THE CHARACTERIZATION AND OCCURRENCE OF CLINICALLY IMPORTANT GRAM-NEGATIVE ANAEROBIC BACILLI

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Thesis presented for the degree of Doctor of Medicine (MD)

University of Edinburgh 1979

FRONTSPIECE

Bacteroides asaccharolyticus : 72-h culture on human-blood agar.

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Statement regarding the content of Dr Brian Duerden's thesis

This is to certify that I have studied the content of this thesis most carefully, with particular reference to Dr Duerden's personal contribution to some of the joint studies. (See chapters 3 & 4 containing material published in papers by Duerden, Holbrook, Collee and Watt, and Holbrook, Duerden and Deacon; see also chapters 4 & 5 containing material reported to the International Commission for Systematic Bacteriology, Taxonomic Sub-committee.)

With due appreciation of the difficulty involved in separating work that was done by a team, I wish to report categorically that Dr Duerden has incorporated in his thesis areas of work personally conducted and often generated by him. As the ranking of his authorship in many of our papers indicates, he has been a prime mover in these investigations and he has made a major contribution in all of them.

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1 March, 1979.

TO my wife Marjorie, whose tolerance and support throughout has made these studies possible.

ABSTRACT

Awareness of the Bacteroidaceae as important members of the normal human flora and as pathogens has increased dramatically in recent years. Classification, however, has been confused and the identification of isolates difficult. In particular, the role of pigment production in the classification of *Bacteroides* spp. was debated. The aims of this investigation were (i) to study the classification of Bacteroidaceae with specific reference to pigment production by *B. melaninogenicus*; (ii) to examine conventional bacteriological tests for the characterization and identification of clinically - important gram-negative anaerobic bacilli; and (iii) to apply these methods to the study of *Bacteroides* spp. isolated from the normal human flora and from infections.

In studies on pigment production, *B. melaninogenicus* strains produced a characteristic pigment when grown on media containing blood. The pigment was extracted by ultrasonic disintegration of washed cells of strains of *B. melaninogenicus* grown in blood broth and on blood agar. It was intra-cellular or cell-associated, soluble in water and had the spectrophotometric characteristics of a derivative of haemoglobin. No such pigment was extracted from strains of *B. fragilis*, *F. necrophorum* and *Cl. clostridiiforme*. The pigment was unrelated to the dense black colloidal precipitate of ferrous sulphide that resulted from the production of H_2S by *Bacteroides* spp. and facultative species in the presence of ferrous ions. However, the pigment-producing strains were not a homogeneous species and were

divided into three subgroups: B. melaninogenicus ss. melaninogenicus, ss. intermedius and ss. asaccharolyticus.

A scheme for the identification of unknown isolates of Bacteroides spp. was devised following studies in which 165 reference strains and laboratory isolates were subjected to a series of simple laboratory tests that included conventional biochemical and fermentation tests, tests for resistance to antibiotics, and tolerance of dyes and bile salts. These tests allowed a clear separation of strains into three main groups - B. fragilis, B. melaninogenicus and Fusobacterium spp. - and certain tests were useful for identifying the subspecies of B. fragilis and B. melaninogenicus.

The classification of B. melaninogenicus and related species was further studied in a series of tests with 175 strains of B. melaninogenicus, 17 strains of B. oralis and 6 strains of B. ochraceus. The pigmented asaccharolytic strains formed a distinct group and have been assigned a separate species - B. asaccharolyticus. B. melaninogenicus ss. intermedius strains formed a homogeneous group. B. ochraceus was distinguished from other Bacteroides spp. by its ability to grow in air plus 10% CO_2 and its resistance to metronidazole; it is suggested that it should be removed from the genus Bacteroides. B. melaninogenicus ss. melaninogenicus and B. oralis gave similar patterns of results and were often indistinguishable except for the production of pigment by B. melaninogenicus strains.

The following system of classification was derived after studies with additional reference and referred strains. The Bacteroidaceae were divided into 4 main groups - B. fragilis group, B. melaninogenicus/oralis/ruminicola group, asaccharolytic group and Fusobacterium group. The B. fragilis group comprised the 5 subspecies of B. fragilis that have been reinstated to species rank - B. fragilis, B. vulgatus, B. distasonis, B. thetaiotaomicron and B. ovatus - and several related species - B. splanchnicus, B. eggerthii, B. uniformis and B. variabilis. The B. melaninogenicus/oralis/ruminicola group contained the 2 saccharolytic subspecies of B. melaninogenicus, ss. melaninogenicus and ss. intermedius, a weakly fermentative subspecies - ss. levii, and 4 non-pigmented species - B. oralis, B. bivius, B. disiens and B. ruminicola. B. oralis and B. melaninogenicus ss. melaninogenicus strains share many characteristics and it is suggested that pigment production might not be a valid criterion for their separation. The asaccharolytic group included B. asaccharolyticus, B. corrodens and non-pigmented strains that were not further identified, and the Fusobacterium group was represented by reference strains of F. polymorphum, F. varium, F. necrogenes, F. necrophorum and L. buccalis. These species were identified by a combined set of tolerance tests with taurocholate, deoxycholate, Victoria blue 4R and ethyl violet, antibiotic disk resistance tests with neomycin, 1000µg, kanamycin 1000µg, penicillin 2 units and rifampicin 15µg, pigment production and biochemical

tests for indole production, gelatin digestion, aesculin hydrolysis and the fermentation of glucose, lactose, sucrose, rhamnose, trehalose, mannitol and xylose. Strains were allocated to the appropriate group by the results of the tolerance and resistance tests and to species/subspecies level by the results of biochemical and fermentation tests.

The scheme was evaluated satisfactorily in studies with Bacteroides strains isolated from the normal human flora and clinical infections. Specimens of faeces, vaginal secretions and sub-gingival plaque were obtained from 20 normal healthy adults. A heavy growth of Bacteroides spp. was obtained from all specimens of faeces and 10 colonies were selected from each subject for identification. Most(84%) isolates belonged to the B. fragilis group. The commonest species/subspecies were B. fragilis ss. vulgatus and ss. thetaiotaomicron (22% of B. fragilis-group isolates each), ss. distasonis (18%) and the B. eggerthii/variabilis group (14%). B. fragilis ss. fragilis accounted for only 9% of B. fragilis-group isolates. Bacteroides spp. were recovered from 65% of vaginal specimens. Most (78%) isolates belonged to the B. melaninogenicus/oralis/ ruminicola group and the commonest species/subspecies were B. bivius/disiens (42% of the group isolates) B. melaninogenicus ss. melaninogenicus (16%) and ss. intermedius (22%). Only 6 B. fragilis strains were identified and 5 were from a single subject. A heavy growth of Bacteroidaceae was obtained from all specimens

of sub-gingival plaque; 68% of isolates were members of the B. melaninogenicus/oralis/ruminicola group. B. oralis (42% of the group isolates), B. melaninogenicus ss. melaninogenicus (26%) and ss. intermedius (17%) were the commonest species. Fusobacterium spp. and L. buccalis were common isolates from sub-gingival plaque and accounted for 36 isolates.

In studies of the role of Bacteroides spp. in infections, 399 significant isolates were obtained from 356 specimens from 332 patients. A variety of species were identified; the B. fragilis group accounted for 261 isolates and there were 55 isolates of B. asaccharolyticus. Many (68%) were from infections related to the gastro-intestinal tract but others were from gynaecological, soft tissue and a variety of other infections. B. fragilis ss. fragilis accounted for 51% of all isolates and 78% of B. fragilis-group isolates, which indicates that this subspecies has particular pathogenic potential, not only in infections derived from the gastro-intestinal tract. The Bacteroides spp. were isolated in pure culture from only 26% of the infections, 73% were mixed infections with Bacteroides spp. and facultative organisms that may act synergistically.

Bacteroides spp. can be identified by a simple set of conventional bacteriological tests that can be performed in any dia nostic laboratory. These studies have shown that different species are predominant in the normal flora of the mouth, faeces and vagina and that a number of species, particularly B. fragilis ss. fragilis, form only a minor part of the normal flora but are the commonest pathogens.

CHAPTER 1

Awareness of anaerobic bacteria as important members of the normal human flora and as pathogens has increased dramatically amongst clinicians and clinical bacteriologists in recent years, but the history of anaerobic bacteriology extends from the origins of modern microbiology. Pasteur (1861-63) investigated the role of bacteria in putrefaction and was the first to report bacteria that grew only in the absence of oxygen. He introduced the term anaerobe. Loeffler (1884) associated similar organisms with calf diphtheria. The first isolations of gram-negative anaerobic bacilli that we would now recognise as bacteroides were made by Vincent (1896), Veillon and Zuber (1897, 1898) and Hallé (1898). During subsequent years gram-negative anaerobic bacilli were isolated by enthusiastic and careful workers from a wide variety of clinical conditions with the common characteristics of suppuration, tissue necrosis and a foul smell. The generic name Bacteroides was first used for these bacteria by Castellani and Chalmers (1919).

The isolation of many anaerobes and their characterization was beset with technical difficulties; reliable anaerobic conditions were difficult to achieve and many workers were unable to obtain pure cultures. The most significant advance in anaerobic technology was the introduction of the MacIntosh and Fildes anaerobic jar (1916) that enabled the growth of anaerobes on solid

media. However, anaerobic techniques remained essentially inadequate and anaerobes such as bacteroides organisms were infrequently isolated in routine diagnostic laboratories. Dack (1940) quoted Thompson who stated in 1939 that "In clinical bacteriology, anaerobes play a minor role. The finding of anaerobes is analogous to the occurrence of red-letter days on a calendar - when they occur they are usually worthy of consideration".

Techniques improved slowly during the 1950's but the current interest in the role of the non-sporing gram-negative anaerobes of the Bacteroides group in clinical infections developed during the 1960's, stimulated by work done by the teams working with Moore at the Virginia Polytechnic Institute and Finegold at the Wadsworth Hospital Center, Los Angeles, with Prévot in France and Werner and his colleagues in Germany.

Growth and recovery of anaerobes

In diagnostic bacteriology, anaerobes are generally defined as those bacteria that require an anaerobic atmosphere and will not grow in air, air plus 5-10% CO_2, or in the reduced oxygen tension of a candle jar (Finegold 1977). This definition is necessarily imprecise and there is a spectrum of anaerobes from the less demanding Clostridium perfringens, through moderately strict anaerobes such as Bascteroides fragilis to the more demanding Cl. novyi type D and some of the B. melaninogenicus strains (Watt & Collee, 1974). The definition has been strengthened recently by observations

that anaerobes are sensitive to metronidazole whereas all aerobes, micro-aerophiles and CO_2-dependent (but not anaerobic) organisms are resistant (Prince et al., 1969; Whelan & Hale, 1973; Ralph & Kirby, 1975; Watt, 1978). This test is now used to confirm that an isolate is an anaerobe.

The reasons why bacteria are "anaerobes" are debated (Finegold, 1977; Watt, 1972a); some workers have suggested that anaerobes lack significant amounts of catalase and superoxide dismutase (McCord, Keele & Fridovich, 1971; Gregory & Fridovich, 1973; Tally et al., 1975a, b) and are, therefore, susceptible to oxygen toxicity, whereas others relate the effect of oxygen to its influence on the redox potential (Eh) of the environment (Walden & Hentges, 1975). Anaerobes require a low redox potential for the initiation of growth (Smith, 1975) and the function of their electron transport system (Rizza et al., 1968) but it is probable that a combination of both aspects of anaerobiosis is important. In practical terms, anaerobic conditions are achieved by removing oxygen from the environment and thus reducing the Eh. Additional reducing agents such as l-cysteine, iron filings etc. (Watt, Hoare & Collee, 1973; and Watt, 1972b) may also be used to facilitate the surface growth of demanding anaerobes.

The early workers could only use deep broth cultures in sealed tubes to grow anaerobes and liquid culture was the basis of anaerobic bacteriology. The reducing power of the medium was improved by the addition of

reducing agents such as cooked-meat particles (Robertson, 1915-16), thioglycollate or an iron nail, and by excluding air with a layer of oil or paraffin wax. These are still used for culture of anaerobes in fluid media, but better results are obtained by incubation of demanding species in an anaerobic atmosphere.

The introduction of the MacIntosh and Fildes jar (1916) enabled the study of anaerobes in pure culture on solid media. Developments since then have improved anaerobic jar techniques by the introduction of standardised methods (Collee et al., 1972), and controls to monitor anaerobiosis and catalyst activity (Watt, Collee & Brown, 1976). Safety has also been improved by the replacement of heated catalysts by room-temperature catalysts, but the principles of anaerobic culture have remained the same. This method is the mainstay of clinical anaerobic bacteriology but two alternative methods were developed for the study of very strict anaerobes. The roll-tube method (Hungate, 1950) and the anaerobic glove box (Aranki et al., 1969; Aranki & Freter, 1972) enable all manipulations of cultures to be performed under anaerobic conditions, and both use media prepared and sterilised under an anaerobic atmosphere (Pre-Reduced Anaerobically Sterilised - PRAS; Holdeman & Moore, 1972). However, prompt conventional handling at the bench with a well-controlled and standardised jar technique and freshly-prepared or pre-reduced media has given as good

quantitative and qualitative recovery of the anaerobes found in clinical material and human faeces as an anaerobic glove box technique (Watt, Collee & Brown, 1974).

Anaerobic conditions are not the only requirements for the growth of bacteroides; many strains are nutritionally demanding (Varel & Bryant, 1974). In particular, the quantitative and qualitative recovery of many Bacteroides spp. is improved by the addition of 5-10% CO_2 to the anaerobic atmosphere (Watt, 1973; Stalons, Thornsberry & Dowell, 1974). Metal ions are growth factors for certain bacteroides (Caldwell & Arand, 1974) and some species require haemin (Gibbons & Macdonald, 1960; Gilmour & Poole, 1970). Certain B. melaninogenicus strains also require menadione (Vitamin K) (Lev, 1959) or its precursors (Robins, Yee & Bentley, 1973) and succinate (Lev, Keudall & Milford, 1971) as growth factors. A rich medium containing a variety of growth factors is generally necessary for optimal growth of a wide range of bacteroides and several complex media have been developed for this purpose, e.g. BM medium (Williams et al., 1975; see Appendix IV).

Anaerobic technology and media are, however, only the laboratory aspects of the isolation of bacteroides from specimens. The organisms must be viable on arrival at the laboratory and bacteroides fare badly on swabs (Collee et al., 1974) because of a combination of desiccation, oxygen toxicity and adhesion to the swab. Three approaches to this problem have been advocated :

(i) immediate direct plating of material obtained from the patient or subject; (ii) the use of transport media that provide reduced and moist conditions, e.g. thioglycollate transport media (Stuart, 1956; Cary & Blair, 1964), and VMG II medium (Möller, 1966); and (iii) the use of gassed-out tubes with an atmosphere of O_2-free CO_2 for transport, although this does not overcome the effects of desiccation. It is significant that bacteroides survive well in pus held under aerobic conditions (Bartlett et al., 1976).

It is often necessary to separate anaerobes from facultative species that are also present in the normal flora or in clinical specimens. Anaerobes are resistant to high concentrations of aminoglycoside antibiotics. Some workers recommend that a selective medium should contain neomycin 70µg/ml, but kanamycin 75µg/ml is less inhibitory to some bacteroides and fusobacteria (Loesche, Hockett & Syed, 1971) and may be more suitable. Vancomycin 2.5µg/ml can also be added to eliminate both aerobic and anaerobic gram-positive organisms and achieve the selection of gram-negative anaerobes only (Collins & Hood, 1968; Finegold, Sugihara & Sutter, 1971).

The studies described in this thesis employed standard bench methods for the manipulation of bacteria and a standardised jar technique (Collee et al., 1972; Appendix I). Specimens from the normal human flora were

either plated directly on to a selective medium of modified BM medium plus kanamycin and vancomycin or collected in VMG II transport medium and then plated on the selective medium. These methods and media were evaluated for the isolation of bacteroides from dental plaque by Holbrook (1976; Holbrook, Ogston & Ross, 1978) and found to give similar results that were better than those obtained with other methods and media.

Taxonomy and Classification of the Bacteroidaceae

The classification of gram-negative anaerobic bacilli has undergone many changes since Veillon & Zuber (1898) named their isolates Bacillus fragilis and Bacillus fusiformis. Castellani & Chalmers (1919) introduced the generic name Bacteroides for species that they called Bacteroides fragilis, B. brunei, B. pseudo-ramosus, B. anaerobius, B. corutus, B. thetaiotaomicron, B. variabilis, B. variegatus, B.bifidus and B. bullosus, and Knorr (1922) introduced the name Fusobacterium for the small spindle-shaped anaerobic bacilli. The strains that produced black-pigmented colonies were described by Oliver & Wherry (1921) and called Bacterium (later Bacteroides) melaninogenicum. Eggerth & Gagnon (1933) produced a further scheme based upon the classification of Castellani & Chalmers (1919) for the identification of strains isolated from the gastro-intestinal tract; they defined 18 species on the basis of morphology and carbohydrate fermentation tests, but it is clear that all

their strains belonged to the fragilis group (Speirs, 1971; Holdeman & Moore, 1974).

In the sixth editionof Bergey's Manual of Determinative Bacteriology, Murray & Breed (1948) classified the Bacteroideae, comprising the two genera Bacteroides and Fusobacterium, as a tribe of the family Parvobacteriaceae. They described 23 species of Bacteroides; most would now be classified in the fragilis group except for B. melaninogenicus, that was described as saccharolytic, and one non-saccharolytic species, B. caviae. Their genus Fusobacterium (Knorr) comprised four species - F. plauti-vincenti, F. biacutum, F. nucleatum and F. polymorphum. They also devoted an appendix to the classification of Prévot (1938), who studied anaerobes extensively and produced a detailed classification (1938; Prévot & Fredette, 1966). However, Prévot used generic names that differed from those in general use and were taxonomically invalid on the basis of precedence in biological nomenclature. He divided gram-negative anaerobic bacilli into two families : Ristellaceae comprised five genera - Ristella, which included organisms generally recognised as Bacteroides fragilis, B. melaninogenicus and several other species, Pasteurella, which included the species vulgatus, ovatus, convexus and coagulans, Dialister, Capsularis and Zuberella, which were motile; the family Sphaerophoraceae comprised two genera - Sphaerophorus, which corresponded with the accepted genus Fusobacterium, and Sphaerocillus which

comprised motile species.

In the seventh edition of Bergey's Manual of Determinative Bacteriology, Breed, Murray & Smith (1957) re-classified the gram-negative anaerobic bacilli in the family Bacteroidaceae with five genera. The genus Bacteroides contained 30 species, of which two were motile; they were distinguished by gas production, gelatin liquifaction, cell morphology and the production of acid from carbohydrates; there were two non-fermentative species - B. coagulans and B. putredinis. They included six species in the genus Fusobacterium - F. fusiforme (incorrectly regarded as Vincent's organism), F. polymorphum, F. praeacutum, F. nucleatum, F. vescum and F. biacutum - and two of Dialister. Their second largest genus were Sphaerophorus, with 14 species, and they also included the genus Streptobacillus in the Bacteroidaceae, despite the fact that streptobacilli are facultative organisms.

The confusion in the taxonomy of the Bacteroidaceae is reflected in the sixth edition of Topley and Wilson's Principles of Bacteriology, Immunology and Virology in which Wilson & Miles (1974) discussed the problems of classification and defined three genera. Their genus Fusobacterium contained F. necrophorum, F. fusiforme and F. nucleatus; Bacteroides included only B. fragilis, B. oralis, B. melaninogenicus (described as an asaccharolytic species), B. corrodens and B. putredinis; and the

genus Dialister was reserved for the very small gram-negative bacillus D. pneumosintes (Olitsky and Gates, 1921; 1922). This treatment, however, confused the distinction between the genera Fusobacterium and Leptotrichia; they regarded Leptotrichia spp, as gram-positive.

The first general agreement on taxonomy and classification was at the meeting of the International Commission for Systematic Bacteriology Sub-committee for gram-negative anaerobic rods at Lille in 1967, when the major and minor characteristics for classification were defined (Beerens, 1970). These principles were embodied in the classification of Holdeman & Moore (1974) in the eighth edition of Bergey's Manual of Determinative Bacteriology. With their colleagues at the Virginia Polytechnic Institue, these workers had studied many strains in a wide range of tests and their findings had been incorporated in the Anaerobe Laboratory Manual (Holdeman & Moore, 1972) and in a confirmatory paper (Moore & Holdeman, 1973).

They divided the Bacteroidaceae into three genera : Bacteroides, Fusobacterium and Leptotrichia. There were 22 species of Bacteroides in five groups:

(i) B. fragilis divided into five subspecies - ss. fragilis, ss. vulgatus, ss. distasonis, ss. ovatus and ss. thetaiotaomicron. All grew well in 20% bile, were saccharolytic and

produced succinic acid as a major metabolic product. This species included most of the species described by Eggerth and Gagnon (1933) and previous workers.

(ii) Similar strains that were inhibited by bile included B. ruminicola (Bryant, Small, Bouma & Chu, 1958), B. oralis (Loesche, Socransky & Gibbons, 1964), B. ochraceus and B. amylophilus (Hamlin & Hungate, 1956).

(iii) A group of six species that did not produce succinic acid but were otherwise unrelated - B. hypermegas, B. serpens, B. termitidis, B. biacutus, B. clostridiiforme and B. constellatus.

(iv) Non-saccharolytic non-pigmented strains that were divided into nine species - R. putredinis, B. coagulans, B. praeacutus, B. corrodens (Eiken, 1950) B. nodosus, B. furcosus, B. capillosus, B. succinogenes, and B. pneumosintes.

(v) B. melaninogenicus strains that produced black-pigmented colonies on laked-blood agar; they were divided into two saccharolytic subspecies - ss. melaninogenicus and ss. intermedius - and one non-saccharolytic subspecies - ss. asaccharolyticus. A further species, B. niger, was defined as producing black pigment in gelatin culture.

Their genus Fusobacterium contained 16 species that were, in general, small spindle-shaped rods but were defined by the production of butyric acid as a major metabolic product; it included most of the species

that had previously been designated Fusobacterium or Sphaerophorus spp. It did not include Vincent's organism (F. plauti-vincenti) which was now assigned to the third genus, Leptrotrichia (Gilmour, Howell & Bibby, 1961), as L. buccalis because it produced lactic but not butyric acid as a major product.

Approaches to the identification of bacteroides

It is generally accepted that the Bacteroidaceae are strictly-anaerobic gram-negative bacilli, although the inclusion of several facultative species has caused confusion in the past. Early workers relied almost exclusively upon observations of microscopic and colony morphology. Eggerth & Gagnon, (1933) established the first biochemical key for the group, but these tests were carried out under varied and often inadequate conditions so that the results were deceptive and there was little indication of the taxonomic significance of the results. In particular, microscopic morphology was an extremely unreliable criterion that varied considerably with the incubation conditions (Guillaume, Beerens & Osteux, 1956). As a result, different workers gave different names to the same organism and, in some cases, the same name was given to obviously different organisms (Moore & Holdeman, 1973; Holdeman & Moore, 1974). Prévot undertook extensive series of conventional tests that were the basis of his classification scheme (Prévot, 1938; 1966).

These tests are still widely used for the identification of bacteroides (Dowell, 1972) and are an important part of the identification scheme set out in the Anaerobe Laboratory Manual (Holdeman & Moore, 1972); they are also the only method for distinguishing between closely related groups such as the subspecies (or separate species) of the B. fragilis complex. Now, however, the conventional tests are selected to discriminate between strains assigned to accepted species that have been defined on the basis of a wide variety of more complex and refined investigations (Aalbaek, 1973).

The short-chain fatty acids formed as end products of protein or carbohydrate metabolism are of particular importance in current systems of classification (Holdeman & Moore, 1974). The family Bacteroidaceae has been assigned two principal genera, Bacteroides and Fusobacterium, largely on the basis that Fusobacterium spp. produce major amounts of n-butyric acid. A third genus, Leptotrichia, comprises oral fusiform bacteria that do not produce major amounts of any fatty acid other than lactic. Classification of members of the Bacteroidaceae on the basis of their fatty acid end products was first attempted by Guillaume, Beerens & Osteux (1956) by distillation and paper chromatographic separation; the use of gas-liquid chromatography (GLC) was developed by Werner (1969), Moore (1970), Cato et al. (1970) and Carlsson (1973).

Johnson (1973) used the analysis of DNA homologies to confirm the relationships between some members of the Bacteroidaceae and several groups of workers have compared the ratio of the DNA bases guanine and cytosine to confirm the similarities or differences between members of the group (Sébald, 1962; Williams et al., 1974; 1975). Cell surface and capsule analysis have also been used in the classification of bacteroides (Kasper & Seiler, 1975; Kasper, 1976) and Strom et al., (1976) used polyacrylamide gel electophoresis to divide strains into groups on the basis of protein profiles.

Barnes & Goldberg (1968) produced a computer analysis of the results obtained with 77 named strains in a wide range of tests using the principle of numerical taxonomy and identified four phena :
(1) strains of Sphaerophorus, Fusobacterium and B. melaninogenicus; (2) other Bacteroides spp.; and (3, 4) two groups of isolates from poultry caeca.
They found the most useful differentiating tests to be cell morphology, terminal pH in glucose broth, production of formic, acetic, propionic and butyric acids, threonine deamination, growth in 20% bile and the effect of several inhibitors and antibiotics.

The refined and complex methods pursued by research and reference laboratories are too complicated, laborious and expensive for routine use in clinical laboratories and several simpler approaches to the identification of the clinically important Bacteroidaceae have been devised.

Suzuki, Ushijama & Ichinose (1966) proposed that tests for glutamic acid decarboxylation and threonine deamination (Beerens & Tahon-Castel, 1965) could be used to distinguish the major groups, and Funderburk & Kester (1975) used tests for valine, malic and pyruvate dehydrogenases. Werner (1972) used susceptibility to rifampicin to distinguish between Bacteroides, Fusobacterium Leptotrichia and Sphaerophorus strains. Tests for resistance to antibiotic disks that contained kanamycin 1000µg, penicillin 2 units, rifampicin 15µg, colistin 10µg, erythromycin 60µg, and neomycin 1000µg (Finegold, Harada & Miller, 1967; Sutter & Finegold, 1971) and the ability to grow in the presence of 20% bile or the bile salts sodium taurocholate and sodium deoxycholate (Shimada, Sutter & Finegold, 1970; Sutter & Finegold, 1971; Peach, 1975) were used to identify B. fragilis, B. melaninogenicus, B. oralis, and several Fusobacterium and Sphaerophorus species. Suzuki et al. (1966) and Ninomiya et al. (1972) used differential inhibition by specified high dilutions of the dyes ethyl violet, Victoria blue 4R, brilliant green and gentian violet to distinguish between species of Bacteroides, Fusobacterium and Sphaerophorus. These approaches, however, could only assign strains to the appropriate major group or genus; they could not determine the species or subspecies of isolates. Biochemical tests were still needed for specific identification; rapid methods were developed for tests for fermentation

reactions (Fay & Barry, 1974b; Schreckenberger & Blazevic, 1975) and indole production (Fay & Barry, 1974a) and a micromethod was developed by Wilkins & Walker (1975a, b).

Serological studies offered a more specific approach, Sharpe (1971) studied the surface antigens of Bacteroides spp. and Lambe prepared fluorescent antibody conjugates against B. melaninogenicus subspecies; he identified serogroups that corresponded, in general, with the three subspecies of B. melaninogenicus (Lambe, 1974; Lambe & Jerris, 1976). Stauffer et al., (1974) used an indirect fluorescent antibody technique with polyvalent antiserum pools to detect and identify bacteroides in smears prepared directly from clinical specimens. However, none of the serological approaches proved entirely satisfactory for general use.

Several commercial companies have produced simple kits for the identification of anaerobes. The API Anaerobic Multitest microsystem (API Laboratory Products) is a set of biochemical tests carried out in a strip of individual cupules that contain the substrates; this gave satisfactory results when evaluated by Moore, Sutter & Finegold (1975), but was less satisfactory in other hands (Duerden, unpublished observations). An alternative approach by the same manufacturers was the API ZYM system that detects specific enzymic activity against substrates contained in the strip of cupules

(Tharagonnet et al., 1977; see Chapter VII). The Minitek Miniaturised Differentiation System (BBL) was another microsystem for performing a set of biochemical tests that gave satisfactory results when evaluated by Stargel et al. (1975). Mast Laboratories Ltd (38 Queensland Street, Liverpool) adopted the antibiotic-resistance approach to identification and produced a "Mastring" with the six antibiotics used by Sutter & Finegold (1971) which is commercially available.

However, despite the increased awareness of the importance of gram-negative anaerobes and the improved anaerobic technology that enables laboratories to isolate bacteroides reliably from a wide variety of clinical conditions, few clinical laboratories identify their gram-negative anaerobic isolates. Many report "Bacteroides spp. isolated", or, at most, report the non-pigmented penicillin-resistant strains as B. fragilis, the pigmented ones as B. melaninogenicus and the others as Bacteroides spp.

Bacteroidaceae in the normal human flora.

Gram-negative anaerobic bacilli are prevalent at several sites throughout the body as part of the indigenous flora; in particular they are found in relation to the mucous membranes of the mouth, lower gastro-intestinal tract and vagina. Eggerth & Gagnon (1933) recognised their importance in the faecal flora

and Rosebury (1962) stressed their normal occurrence in the three sites. Since then there have been several studies to determine the precise sites of colonisation and identify the species isolated; these studies have been reviewed by Smith (1975) and Finegold (1977).

Bacteroides in the mouth. The mouth consists of several microenvironments with different predominant flora (Hardie & Bowden, 1974). The surface of the tongue is almost devoid of bacteroides and the saliva contains a variable number of fusobacteria ($2.72 \times 10^5 - 1.79 \times 10^6$/ml) derived from the more populous gingival crevice; the significantly higher results were obtained from patients with acute ulcerative gingivitis (Hadi & Russell, 1968; 1969).

The gingival crevice and dental plaque are the principal sites of colonisation by gram-negative anaerobic bacilli (Socransky & Manganiello, 1971; Hardie & Bowden, 1974). B. melaninogenicus constituted 4·8% of the cultivable flora of the gingival crevice examined by Gibbons (1974); other non-pigmented bacteroides were also found here (Loesche et al., 1964) and called B. oralis. Gibbons et al., (1963) found that 16.1% of the cultivable flora from the gingival crevice were gram-negative anaerobic rods and devised a practical scheme for their identification (Loesche &

& Gibbons, 1965). Similarly Gibbons et al., (1964) found that Bacteroides spp. formed c.4% of the cultivable flora of dental plaque in normal subjects and Loesche, Hockett & Syed (1972) found that gram-negative anaerobic rods were 17% (including B. melaninogenicus 5.6% and fusobacteria 6.8%) of the cultivable flora in plaque from institutionalised subjects.

However, there are several technical problems common to all studies of the oral anaerobic flora : many oral anaerobes, particularly the fusiform organisms, are very demanding, both anaerobically and nutritionally, and their quantitative recovery on solid media has never approached the numbers calculated by direct microscopy; moreover, the oral microflora is nutritionally interdependent and some strains of bacteroides will grow on solid media only by satellitism in mixed cultures and have not been isolated in pure culture for identification. The isolates in some of the series have not been identified beyond calling the pigmented strains B. melaninogenicus.

Bacteroides in the gastro-intestinal tract. "B. fragilis is the most numerous bacterial species in the intestine ..... (and) Bacteroides is probably the most important genus of intestinal bacteria" (Draser & Hill, 1974). Obligate anaerobes are seldom found in the normal stomach, duodenum, jejunum or proximal ileum and only in the distal ileum do Bacteroides and

Bifidobacterium spp. appear (Finegold, 1977). In quantitative studies Bacteroides spp. were recovered in counts of 10^{11} organisms/g of faeces (Finegold, Attebury & Sutter, 1974; Finegold, 1977; Drasar, 1967; Hill et al., 1971). A comparison of the caecal flora from subjects in different countries (Drasar, 1974) showed that bacteroides were recovered in greater numbers from specimens from developed countries whereas gram-positive anaerobes were predominant in specimens from developing countries and Japan.

B. fragilis has been the dominant species encountered in all studies and when more detailed identification has been undertaken, the commonest subspecies have been ss. vulgatus and ss. thetaiotaomicron with ss. fragilis present in appreciably smaller numbers (Werner, 1974; Moore & Holdeman, 1975; Finegold et al., 1975). Eggerth & Gagnon (1933) divided their strains into 18 species but all would now be classified as B. fragilis. Many other Bacteroides and Fusobacterium spp. have been found in small numbers and inconsistently in faeces. B. melaninogenicus strains have been isolated but in much smaller numbers than the B. fragilis complex; all were asaccharolytic and this lead Werner, Pulverer & Reichertz (1971) to suggest that B. melaninogenicus is a non-saccharolytic species that produces n-butyric acid as a major metabolic end product, a view adopted by Wilson & Miles (1974).

Bacteroides in the vaginal flora. The vagina is not a single environment; the normal flora of the lower vagina is a mixture of organisms from the true vaginal flora with those from the perineum and introitus that include both skin and faecal organisms such as Staphylococcus epidermidis and Escherichia coli, whereas the flora of the cervix and the vaginal fornices more closely represents the true vaginal flora. Moreover, the vagina is not a constant environment; the physiological state of the mucosa and secretions changes (i) with age, as a woman passes from childhood through puberty to the reproductive age and then the menopause, (ii) with each menstrual cycle, and (iii) with pregnancy (Hurley et al., 1974). The most reliable studies of the vaginal flora have used specimens carefully taken under direct vision from the cervices or fornices of healthy women in the reproductive years.

The presence of bacteroides in the vagina was reported by Burdon (1928) who isolated B. melaninogenicus from 28 out of 35 normal women. Mead & Louria (1969) and Suzuki & Ueno (1971) found that bacteroides were commonly present in the normal vaginal flora. Gorbach et al. (1973) isolated Bacteroides spp. (including B. fragilis and B. oralis) from the normal cervical flora of 57% of 30 healthy women, and Sanders et al. (1975) isolated Bacteroides spp. from endocervical cultures in 65% of 26 healthy women. However, Neary

et al. (1973) recovered Bacteroides spp. from only 8.6% of vaginal swabs from 246 pre-operative gynaecological patients, and Leigh, Kershaw & Simmons (1976) isolated bacteroides from only 4.6% of 500 women attending a family planning clinic and 5% of 200 patients attending a gynaecological out-patient clinic. These differences in isolation rates in different series probably reflect differences in sampling methods and in anaerobic techniques that were more rigorously controlled in the studies of Gorbach et al. (1973) and Sanders et al. (1975). Hurley et al. (1974) collected specimens of vaginal secretion from the posterior fornix of 280 unselected pregnant women and found Bacteroides spp. in only 15 (5.4%), but whether this reflects the anaerobic methods used by these authors or a true alteration in the flora during pregnancy has not been established. The Bacteroides spp. isolated in most studies of the vaginal flora have not been further identified and the relationship of these isolates to those from other sites is not clear.

Infections caused by Bacteroides spp.

Gram-negative anaerobic bacilli have been isolated from a wide variety of infections. The frequencies of their isolation in different studies generally reflect varying standards of care and enthusiasm of different investigators for anaerobic bacteriology. The common factors in almost all bacteroides infections are :
(i) the infections are endogenous and their source

is the normal flora of the patient; (ii) the infections are usually related to damage to, or breach of, the mucous membrane surfaces colonised by bacteroides; and (iii) bacteroides infections are usually characterised by tissue damage, cell necrosis and/or abscess formation and the production of foul-smelling pus. The extensive literature on bacteroides infections has been reviewed recently by Balows et al., (1974), Leigh (1976), Finegold (1977) and Willis (1974; 1977).

Much of the data on the incidence of bacteroides infections is unreliable, and poor anaerobic techniques have led to serious underestimation of their incidence. Dack (1940) recovered non-sporing anaerobes from $< 4\%$ of 5180 surgical unit specimens. Stokes (1958) found that anaerobes were present in 10.5% of 4737 specimens that yielded bacterial growth. Martin (1971), however, recovered anaerobes from 35% of specimens received in the Mayo Clinic Clinical Laboratory and these were 49.3% of all bacteria isolated; in a subsequent study, anaerobes were isolated from 49% of culture-positive specimens (Martin, 1974). Holland, Hill & Altemeier (1977) isolated anaerobes from 58.5% of bacteria-positive cultures from specimens excluding stool, urine, sputum and blood; this represented 48.8% of all specimens cultured and bacteroides were found in 70% of the anaerobe-positive cultures. The influence of techniques on these results was shown by Leigh (1974): in 1970 - 72 he recorded a wound infection rate of 11%

following abdominal surgery and bacteroides were recovered from 17%; in six months in 1973, the incidence of wound infections was 13% and 81% were caused by bacteroides. Even when bacteroides are recovered from specimens it may be difficult to assess the significance of the findings, especially when the specimen is contaminated with normal flora (Finegold, 1977; Bartlett & Finegold, 1970). Moreover, bacteroides are usually isolated from mixed cultures with facultative species that have, in the past, been considered more important. It is possible, however, that part of the reported increase in the isolation of anaerobes may reflect a true increase in anaerobic infections because of more adventurous surgery performed on many compromised patients.

Abdominal and perineal infections. The most common sources of Bacteroides isolates in the routine clinical laboratory are specimens from wound infections, abscesses or peritonitis following surgery or injury to the appendix or large intestine; the source of infection is the faecal flora. Finegold (1977) found that 86% of intra-abdominal infections involved anaerobes, of which most were bacteroides, and the majority of post-operative wound infections following surgery to the appendix or large intestine involve bacteroides (Swenson et al., 1974; Leigh, 1974; 1976; Willis et al., 1975; 1976; 1977). Similarly peritonitis and intra-

abdominal abscesses (e.g. sub-phrenic and pelvic abscesses) following perforation or penetration of the large intestine almost always involve bacteroides (Gorbech & Bartlett, 1974). Bacteroides are important causes of postappendicectomy sepsis (Willis et al. 1976); the incidence of this complication varies from 4% for normal appendices to 77% for gangrenous or perforated appendices and the average frequency is c.30% (Willis et al. 1976; Willis, 1977). Werner et al. (1975) have also suggested that bacteroides are implicated in the pathogenesis of appendicitis. There is evidence (summarised by Finegold, 1977) that bacteroides are a major cause of liver abscess and they have been implicated in cholecystitis and cholangitis.

Diverticulosis is a common non-infective condition, and bacteroides are almost invariably involved in the infections complicating diverticulitis i.e. diverticular abscesses, peritonitis and bacteraemia (Felner & Dowell, 1971). They are also found in most perirectal and perianal abscesses (Finegold, 1977; Mitchell, 1973).

A common feature of many of these infections is that anaerobes are usually found in mixed culture with facultative organisms such as coliforms; evidence is accumulating that the bacteroides are the more significant pathogens. Drainage of the pus and removal of necrotic tissue which removes sites with anaerobic conditions may be effective treatment, and treatment with anti-microbial agents effective only against

anaerobes will usually eliminate the sepsis despite the presence of facultative organisms; moreover, Willis et al. (1976; 1977) have shown that prophyllaxis with metronidazole, an agent active only against anaerobes, will prevent sepsis following surgery to the appendix or lower gastro-intestinal tract.

It is also of interest that bacteroides infections of the abdomen are commonly associated with carcinoma of the bowel and may be the first manifestation of malignancy (Finegold, 1977).

Oral and dental infections. Gram-negative anaerobic bacilli are involved in many types of infection related to the teeth and the oral mucous membranes. They are important causes of infections of the pulp and root canals of teeth and of dentoalveolar abscesses that follow pulp infections (Hardie, 1974; Moore & Russell, 1972; Möller, 1966). These infections may progress to involve the jaw in osteomyelitis or the soft tissues of the face and neck, although prior tooth involvement is not invariable in these infections (Finegold, 1977), and bacteroides have been frequently isolated as co-pathogens in actinomycotic abscesses (Hardie, 1974). The gram-negative anaerobic bacilli that colonise the gingival crevice are also significant pathogens in periodontal disease. Acute ulcerative gingivitis (Vincent's stomatitis) has been generally accepted as a synergistic anaerobic bacterial infection involving

F. nucleatum and Borrelia vincenti (Willis, 1977) but there is evidence that other oral bacteroides, in particular B. melaninogenicus, are an essential component of the infection and may be the more specific cause of the gingival damage in this condition (Kaufman et al., 1972; Hardie, 1974).

Other infections of the head and neck. In addition to the soft tissue infections originating in the mouth, there are two further sites of significant anaerobic infection - the middle ear and mastoid and the paranasal sinuses. Bacteroides have not been implicated in acute otitis media but chronic otitis media and mastoiditis are commonly caused by anaerobes or associated with them (Finegold, 1977). Similarly, chronic infection of the sinuses by bacteroides from the mouth and pharynx are not uncommon (Frederick & Braude, 1974). As in other sites, bacteroides are rarely isolated in pure culture and are usually mixed with anaerobic cocci and/or facultative orgnisms.

Central nervous system infections. Anaerobes are a major cause of brain abscess (Heineman & Braude, 1963). Bacteroides spp. are particularly implicated in abscesses resulting from chronic otitis media and mastoiditis (Ingham, Selkon & Roxby, 1977) and sinusitis, although they may also be found in metastatic brain abscesses complicating lung abscess and

bronchiectasis. Bacteroides are less commonly found in other infections of the central nervous system but they have been reported to cause meningitis, unrelated to brain abscess, and subdural empyema. The isolated case reports were reviewed by Finegold (1977).

Infections of the female genital tract. Bacteroides spp. are frequently involved in infections of the female genital tract and are the predominant pathogens in most serious infections (Ledger, Sweet & Headington, 1971; Gorbach & Bartlett, 1974; Willis, 1977). They are found in vulvovaginal infections (Pearson & Anderson, 1970a; Thadepalli, Gorbach & Keith, 1973) but are of major importance in infections of the uterus and pelvis. Pearson & Anderson (1970b) estimated that 10 - 15% of cases of septic abortion were associated with bacteroides infections, but Thadepalli et al. (1973) and Rotheram (1974) suggested that the figure should be >70%. The majority of peurperal infections are also due to Bacteroides spp., often in association with anaerobic or micro-aerophilic cocci (Thadepalli et al. 1973; Pearson & Anderson, 1970b; Willis, 1977). Similar results have been obtained in studies of pyometra and endometritis in patients with carcinoma of the cervix (Carter et al., 1951; Burdon, 1928). Bacteroides are commonly recovered from other pelvic infections : they are important causes of non-gonococcal tubo-ovarian

abscesses, pelvic abscesses and post-operative gynaecological infections (Thadepalli et al., 1973; Swenson et al., 1973; Swenson, 1974). In all of these infections, Bacteroides spp. from the normal vaginal flora reach the normally-sterile uterus, fallopian tubes and pelvis as a result of surgery, trauma, unskilled interference or dysfunction of those structures (Galask, Larsen & Ohm, 1976).

Infections of the Cardiovascular system. Significant bacteroides bacteraemia is almost invariably a complication of some pre-existing anaerobic sepsis (Felner & Dowell, 1971; Chow & Guze, 1974). The incidence of bacteroides bacteraemia is difficult to determine because of differences in techniques; Washington & Martin (1973) found anaerobes in 27% of positive blood cultures and Medeiros (1972) found that 7.6% of bacteraemias were due to Bacteroides spp. Finegold (1977) estimated that 5 - 15% of bacteraemias involve anaerobes. Several workers have stressed that bacteroides are not uncommonly involved in polymicrobial bacteraemias (Hermans & Washington, 1970; Wilson et al., 1972; Chow et al., 1974) and may only be detected by subculture on to a selective medium (von Graevenitz & Sabella, 1971). Without treatment, the mortality of bacteroides bacteraemia is as high as 60-80% (Felner & Dowell, 1971; Chow & Guze, 1974).

These bacteraemias, however, generally represent one manifestation of underlying anaerobic sepsis.

True cardiovascular infections manifest as bacteroides endocarditis is rare. Felner (1974) reviewed 15 cases due to Bacteroides spp. and 7 due to fusobacteria.

Lower respiratory tract infections. Bacteroides infections of the lungs and pleura are relatively common according to studies by Bartlett & Finegold (1972) and Bartlett, Gorbach & Finegold (1974), but there have been few reports of such infections in Great Britain. This probably reflects differences in investigative techniques : the American workers diagnose these infections by the examination of specimens obtained by percutaneous transtracheal aspiration or lung biopsy whereas British workers rarely examine these specimens and sputum contaminated with saliva is unsuitable for anaerobic culture (Bartlett, Rosenblatt & Finegold, 1973). Three types of infection are recognised - pneumonia, necrotizing pneumonia and lung abscess - with or without empyema. Bacteroides are also frequently found in the mixed bacterial flora from bronchiectatic cavities. Aspiration is the commonest primary event in the development of anaerobic lung infections (Finegold, 1977) and the infecting organisms are usually derived from the mouth and upper respiratory tract.

Bone and joint infections. Bacteroides infections of bone and joint are infrequent and generally occur as complications of bacteroides bacteraemia or by direct

extension of a soft tissue infection such as occurs in the jaw (see above) or foot in a patient with diabetic gangrene (Ziment, Miller & Finegold, 1968; Ziment, Davis & Finegold, 1969).

Superficial and soft tissue infections. Bacteroides are implicated in a variety of infections related to wounds, including some types of Meleney's synergistic gangrene and necrotizing fasciitis (Willis, 1977: Finegold, 1977); in particular, human and animal bites may be infected with oral bacteroides (Linscheid & Dobyns, 1975). Decubitus ulcers are also commonly infected with Bacteroides spp. and despite the very mixed flora of these lesions, the bacteroides appear to be the predominant and significant pathogens (Rissing et al., 1974). Similarly, bacteroides are frequently implicated in infections of pilonidal sinuses.

Bacteroides species as infections

The identification of the Bacteroides spp. isolated from the infections described above has rarely been precise; the classification of gram-negative anaerobic bacilli has been confused and methods have not been available in many laboratories for accurate identification. Most workers, therefore, have reported their isolates as Bacteroides spp., or B. fragilis, B. melaninogenicus and fusobacteria. Even when

more specific identification has been attempted, the results have often been unreliable and the nomenclature variable. Furthermore, comparison of isolation rates for different species must take account of the ease or difficulty of recovering and identifying those species; a common pathogen may be recovered only rarely if it is a demanding anaerobe and the techniques used are inadequate, whereas the less demanding species will be isolated more frequently.

B. fragilis has been by far the commonest species isolated from bacteroides infections in general; a variety of names have been used to describe the isolates in the earlier literature but most would now be included in the B. fragilis group. Few workers, however, have identified the subspecies of their B. fragilis isolates; when this has been done, B. fragilis ss. fragilis has been the commonest subspecies isolated from all types of infection and accounts for > 50% of all isolates in series where precise identification has been made. B. fragilis ss. thetaiotaomicron has been isolated regularly but much less frequently from abdominal infections. Werner & Pulverer (1971) and Werner (1974) identified 208 isolates as B. fragilis ss. fragilis and 47 isolates as ss. thetaiotaomicron, and Holland et al. (1977) identified 44 isolates as B. fragilis ss. fragilis, 27 as ss. thetaiotaomicron, 10 as ss. distasonis,

4 as ss. vulgatus and one as ss. ovatus.

B. melaninogenicus is the only other species that has been identified consistently in bacteroides infections (Burdon 1928; 1932; Heinrich & Pulverer, 1960), but it has generally been recovered much less frequently than B. fragilis and is rarely found in pure culture. However, in studies carried out in the pre-antibiotic era, F. necrophorum was relatively common and was clearly a virulent pathogen; it has been encountered much less frequently in the antibiotic era (Finegold, 1977).

Although B. fragilis is generally the most common species isolated, the frequency of isolation of different species varies with the site of infection and the source of the infecting strain.

Abdominal and perineal infections. B. fragilis (usually ss. fragilis) is the commonest isolate from these infections and is recovered from most abdominal wound infections, abscesses etc. However, most series also show a small but consistent number of infections due to B. melaninogenicus. Spaulding et al. (1971) identified 18 isolates of B. fragilis, 10 of B. melaninogenicus and 6 of F. nucleatum in a study of post-operative abdominal wound infections; in a similar study, Gorbach identified 28 isolates as B. fragilis, 4 as B. melaninogenicus and 6 as fusobacteria (Finegold, 1977). Weiss (1943) isolated 10 strains of B. melaninogenicus

from abdominal infections and Heinrich & Pulverer (1960) isolated B. melaninogenicus from sub-phrenic and intra-abdominal abscesses. B. melaninogenicus has frequently been isolated from perianal abscesses.

Dental and oral infections. B. fragilis is not a common commensal of the mouth (Hardie, 1974) and is only rarely involved in infections related to the mouth. The species most commonly isolated from infections in the mouth and surrounding tissues are B. melaninogenicus and fusobacteria, with a few reports of B. oralis infections (Bartlett & Finegold, 1972).
B. melaninogenicus has been described as a co-pathogen in some actinomycotic abscesses (Hardie, 1974), but a greater variety of Bacteroides spp. is found in chronic infections of the middle ear, mastoid and sinuses and B. fragilis is commonly isolated from these infections (Frederick & Braude, 1963; Finegold, 1977).

Central nervous system. Most of the brain abscesses that involve bacteroides are otogenic, as complications of chronic otitis media and mastoiditis, and B. fragilis is the commonest species isolated, although B. melaninogenicus has also been recovered from some of these infections (Ingham et al., 1977).

Bacteraemia, endocarditis and bone and joint infections. B. fragilis has been the causative species in almost

all cases of bacteroides endocarditis, in most bacteraemias and in most bone and joint infections (Felner & Dowell, 1971).

Female genital tract infections. B. fragilis remains the commonest species isolated from these infections, but in contrast with abdominal infections there is a much greater proportion of infections with B. melaninogenicus and fusobacteria (Gorbach & Bartlett, 1974) and B. oralis has also been isolated (Finegold, 1977)

Lower respiratory tract infections. Most anaerobic pleuropulmonary infections result from aspiration, and most of the species involved are derived from the mouth and pharynx; the predominant species isolated have been B. melaninogenicus, B. oralis and Fusobacterium spp. (Bartlett, Gorbach & Finegold, 1974).

Superficial and soft tissue infections. The isolates from superficial infections reflect the general preponderance of B. fragilis. However, B. melaninogenicus and fusobacteria have been regularly isolated from human and animal bites and B. melaninogenicus appears to be particularly common in decubitus and diabetic ulcers (Peromet et al., 1973; Rissing et al., 1974; Willis, 1977)

Aims of the present study

The studies reported in this thesis form a series of investigations into the taxonomy and classification of the Bacteroidaceae and their occurrence in the normal flora and in clinical infections. The aims of these investigations were;

1. To study classification of the gram-negative anaerobic bacilli (i) with specific reference to pigment production by B. melaninogenicus and its relevance to the taxonomy and classification of B. melaninogenicus and related species, and (ii) with reference to the occurrence of specific biotypes as commensals or pathogens of man (see 4 below)
2. To examine conventional bacteriological methods for the characterization of clinically important gram-negative anaerobic bacilli.
3. To devise a scheme for the identification of these species by conventional tests that could be undertaken in routine clinical laboratories.
4. To apply this scheme to study the Bacteroides spp. isolated from (i) the normal flora of the lower gastro-intestinal tract, the vagina and the mouth, and (ii) specimens of pus and other exudates obtained from infected patients.

CHAPTER 2

PIGMENT PRODUCTION BY BACTEROIDES SPECIES WITH REFERENCE TO SUB-CLASSIFICATION

Oliver & Wherry (1921) first described bacteroides - like organisms that produced black-pigmented colonies when grown on blood agar, and called them Bacterium melaninogenicum. This characteristic appearance was regarded as highly specific and remained the sole basis for differentiation from other organisms now generally grouped as Bacteroides. Oliver & Wherry thought that the pigment was extracellular and identified it as melanin on the basis of its insolubility in organic solvents and its slow solubility in sodium hydroxide. Schwabacher, Lucas & Rimington (1947) found that the pigment was intracellular and was not melanin but a derivative of haemoglobin; it was spectroscopically identical with haematin. However, Tracy (1969) found that clinical isolates of B. fragilis produced a dense black pigment in mixed culture with Escherichia coli or Staphylococcus aureus in cooked-meat-broth containing a rusty nail. She found that standard strains of B. fragilis, B. melaninogenicus and B. necrophorus (F. necrophorum) produced this pigment when grown in pure culture in a medium containing cysteine, ferrous sulphate and vitamin K. The pigment was extracellular and was identified as colloidal ferrous sulphide. The following series of experiments was undertaken to

investigate the nature of the pigment and to study the conditions necessary for its production by Bacteroides.

Materials and Methods

Throughout the investigations described in this thesis, cultures were regularly checked for cell morphology and purity by examination of gram-stained smears and by aerobic and anaerobic sub-culture on blood agar. The standard anaerobic procedure described in Appendix I was followed throughout the experiments.

Organisms. The following strains were studied :

B. melaninogenicus NCTC nos. 9336, 9337 and 9338; B. fragilis NCTC9343; F. neocrophorum NCTC10575 Clostridium clostridiiforme (B. necrophorus) NCTC7155; and 36 strains of the B. fragilis group, originally isolated from clinical specimens or human faeces. Laboratory strains of E. coli, S. aureus (Oxford: NCTC6571), Proteus mirabilis, Salmonella typhimurium, and Cl. welchii were included as control organisms and the strains of E. coli and S. aureus were used in studies of mixed cultures with Bacteroides spp.

Culture inocula. One drop (0.02ml) of 48h cooked-meat-broth culture was used to seed each tube and one loopful (c0.01ml) to seed each plate of medium.

Characterization of strains. All the gram-negative anaerobic bacilli were subjected to the following series of morphological, biochemical, tolerance and antibiotic disk resistance tests (for details of media and methods,

see Chapter 3): microscopic and colonial morphology; haemolytic effect on human- and horse-blood agar; lipase activity; oxidase test; catalase test; H_2S production; indole production; gelatinase test; aesculin hydrolysis; fermentation of glucose, lactose, sucrose, maltose, rhamnose, trehalose and mannitol; growth in the presence of the bile salts sodium taurocholate and sodium deoxycholate, separately and in combination, and the dyes brilliant green, Victoria blue 4R, gentian violet and ethyl violet; resistance to disks containing neomycin 1000µg and 10µg, kanamycin 1000µg and 30µg penicillin 1.5 units, methicillin 10µg, erthromycin 60µg, colistin 10µg, rifampicin 15µg, lincomycin 2µg, clindamycin 2µg, bacitracin 0.1 unit, vancomycin 15µg, chloramphenicol 10µg, and metronidazole 5µg.

Media. The basic liquid medium was Robertsons cooked-meat-broth; menadione (1µg per ml), ferrous sulphate (5mg per ml) and cysteine (2mg per ml) were added singly and in all combinations, i.e. eight media in all including the basic medium. Horse-blood broth, containing 10% Defibrinated Horse Blood (Oxoid) in Oxoid Nutrient Broth no1 2, was used to study pigment production in a liquid medium containing blood. Solid media were : (i) human-blood agar; (ii) lysed-human-blood agar; (iii) horse-blood agar; (iv) heated (chocolate) horse-blood agar; (v) the supernate from Robertsons cooked-meat-broth solidified with 1% agar; and (vi) Robertsons cooked-meat-broth made with homogenised meat and solidified with 1% agar. Menadione (1µg per ml),

ferrous sulphate (5mg per ml) and cysteine (2mg per ml) were added when required (see text).

Isolation and examination of pigment.

Presumptive colloidal ferrous sulphide. The dense black pigment produced by culture of any Bacteroides strain in liquid media containing cysteine and ferrous sulphate was separated from the bacterial cells by centrifugation at 800g for one hour. Most of the pigment remained in the supernate, which retained the dense black appearance of the original culture; some was deposited with the cells. The presence of ferrous ions in the supernate was demonstrated in tests with potassium ferricyanide solution with and without acidification with HCl; the Prussian-blue reaction indicated the presence of ferrous ions.

Lead acetate paper was used to detect the evolution of H_2S after acidification of the supernate with HCl.

The supernate was also examined by spectrophotometry over the wavelength range 200-700nm after 50-fold dilution with distilled water.

Pigment from cultures of B. melaninogenicus in 10% horse-blood-broth with menadione. The cells were sedimented by centrifugation at 800g for one hour and the supernate was retained. The deposit of bacterial cells and red-cell debris was washed thrice with distilled water to lyse any remaining RBC and to remove the haemoglobin; then it was disrupted ultrasonically for 30 min. in distilled water and the cell debris separated from the soluble fraction by ultracentrifugation at 20,000g for one hour. The original culture

supernate was diluted 100-fold in distilled water and the aqueous extract after ultrasonication was diluted 10-fold for spectrophotometry over the wavelength range 200-700nm. These preparations were also tested for the presence of sulphide and ferrous ions as above.

Pigment from colonies of B. melaninogenicus on blood agar. Growth after anaerobic incubation for one week on blood agar was suspended in distilled water and treated in the same way as above. The first supernate and the supernate obtained after ultrasonic treatment were examined by spectrophotometry.

Spectrophotometer. Pye Unicam SP8000 Ultraviolet Recording Spectrophotometer.

Ultrasonic disintegrator: MSE-Mullard Ultrasonic Disintegrator No. 4200.

Results

Growth of Bacteroides spp. on blood agar

B. melaninogenicus NCTC9338 produced visible colonies on blood agar after anaerobic incubation for 48h; the colonies became black after anaerobic incubation for 2-4 days. Haemolysis appeared and increased concurrently with colony pigmentation. The same pattern was observed with NCTC nos. 9336 and 9337 except that pigmentation and haemolysis did not develop until 4-6 days.

There was essentially no difference between degree and speed of pigmentation on horse-blood agar and human-blood agar. Menadione increased the rate of pigmentation particularly on horse-blood agar. Pigment

appeared earlier on heated (chocolate) horse-blood agar than on unheated horse blood. Pigmentation was most rapid on lysed-human-blood agar.

No other gram-negative anaerobic bacilli produced pigmented colonies in pure culture on human- or horse-blood agar.

Effect of cultivation with Escherichia coli or Staphylococcus aureus (Oxford) on pigment production by Bacteroides spp. on blood agar

The effect of cultivation with E. coli or S. aureus on pigment production was tested on separate human-blood- agar plates. E.coli or S. aureus was seeded on to a plate of a diametric streak and a streak inoculum of each strain of gram-negative anaerobic bacilli, including the NCTC reference strains, was made at right angles across the E. coli and S. aureus inoculum so that arears of pure and mixed growth were obtained. After incubation for one week, only B. melaninogenicus NCTC nos. 9336, 9337 and 9338 had produced pigmented colonies. Pigment developed more rapidly in NCTC nos. 9336 and 9337 in areas of mixed culture with E. coli and S. aureus, E. coli stimulating pigment production more than S. aureus. No other strains produced pigment.

Growth of Bacteroides spp. in mixed culture with E. coli or S. aureus in cooked-meat broth with iron filings

Each strain was incubated separately with E. coli and S. aureus in cooked-meat broth to which a knife-point

of iron filings had been added. After three days, one laboratory-isolated strain produced a few small black clumps in mixed culture with S. aureus. No others produced pigment.

Growth of Bacteroides spp. with menadione, cysteine and ferrous sulphate

Each strain was grown for 48h in plain cooked-meat broth and in cooked-meat broth to which menadione, cysteine, and ferrous sulphate, singly and in all combinations, had been added, i.e. eight media in all (see Methods). All produced dense black pigmentation throughout media containing both cysteine and ferrous sulphate; this effect was independent of the presence of menadione. Two strains produced clumps of black pigment in medium with only ferrous sulphate as an additive, and four strains produced black clumps when both ferrous sulphate and menadione were present. Pigment was not produced in media to which ferrous sulphate had not been added.

The same dense black appearance was reproduced by the addition of ferrous sulphate solution to a 48h culture of any strain in the medium that contained only additional cysteine. The black discolouration began to develop as soon as the ferrous sulphate solution was added. A similar result was obtained when copper sulphate solution was added to 48h cultures in the medium with additional cysteine; in this case, the precipitate was blue.

Hydrogen sulphide production in cultures of Bacteroides spp.

All strains were tested for H_2S production in plain cooked-meat broth and in cooked-meat broth with additional cysteine. Weak production of H_2S was generally demonstrable with cultures in the plain medium. All strains grown in cooked-meat broth supplemented with cysteine gave strongly positive results after 48h; the development of the black pigment was correlated with detectable H_2S production and the presence of ferrous sulphate.

Studies with bacteria of other genera in fluid media

Strains of E. coli, P. mirabilis, Salm. typhimurium and Cl. welchii were also inoculated separately into the above test media. They all gave a strongly positive test for H_2S production in media containing added cysteine and they produced the same dense black pigment throughout the medium when ferrous sulphate was also present. The naked-eye appearance of the black broth cultures were indistinguishable from those produced by the Bacteroides strains.

Growth of Bacteroides spp. on solid media containing cysteine, menadione and ferrous suplhate

Ten strains of B. fragilis were incubated on human-blood agar with cysteine and ferrous sulphate incorporated in the medium and with disks of menadione

(0.005 mg) and phytomenadione (o.1mg and 0.005mg) on the surface. One strain failed to grow; the others produced poor growth and colonies were not pigmented.

All test strains of gram-negative anaerobic bacilli were incubated on separate agar media incorporating (i) cooked-meat-broth supernate, (ii) homogenised cooked meat, or (iii) lysed human blood, each containing cysteine, menadione and ferrous sulphate. Fourteen failed to grow on the supernate agar and the remainder produced barely visible growth without pigmentation. All except two strains grew on homogenised cooked-meat-broth agar, but growth was slow and colonies were small and not pigmented. Eight strains grew moderately well on lysed-blood agar; 14 produced barely visible growth and 23 failed to grow. After one week, colonies of the eight moderately good cultures began to develop black centres and after 10 days most colonies had black centres. None of these strains was B. melaninogenicus and one was B. fragilis NCTC9343.

Examination of black pigment from cultures of Bacteroides strains and E. coli in cooked-meat- broth with cysteine and ferrous sulphate

The black pigment did not sediment on centrifugation, and a wet film of the deposit showed normal bacterial cells with clumps of extracellular pigment. The cell-free, pigmented supernate did not react with potassium ferricyanide solution; this indicated the absence of free ferrous ions, whereas the supernate from the medium control gave a strong Prussian-blue reaction attributable

to the ferrous sulphate content. When the culture supernate was acidified with HCl, H_2S was evolved and the pigmentation gradually cleared. The acidified supernate gave a strongly positive Prussian-blue reaction with potassium ferricyanide solution. This is a prima-facie evidence that this pigment was the colloidal ferrous sulphide identified by Tracy (1969) in similar circumstances. Spectrophotometry of a 50-fold dilution of the black supernate, the medium control and plain cooked-meat broth revealed a general increase in turbidity over the whole range for the black supernate as one might expect with a black substance in colloidal suspension.

Examination of pigment produced by B. melaninogenicus in blood- containing media

B. melaninogenicus NCTC nos. 9336, 9337 and 9338, B. fragilis NCTC 9343, F. necrophorum NCTC10575 and Cl. clostridiiforme (B. necrophorus) NCTC7155 were all grown for one week in horse-blood broth with menadione. The centrifuged culture supernate and an aqueous ultrasonic extract of the deposited bacterial cells were examined by spectrophotometry for pigmentation. There was no difference between the spectra of the supernates from the six cultures and from an uninoculated control. There was a small increase in absorption at 525-575 nm and a single large narrow peak at 405-410 nm representing haemoglobin from lysed RBC. When the cells

were washed with distilled water, haemoglobin was again the only pigment detected in the washings and it was equally present in all six cultures and the control. The final washings were clear.

At this stage the cells of B. melaninogenicus strains were considerably darker than those from the test cultures of B. fragilis, F. necrophorum and Cl. clostridiiforme strains. The pigment was clearly cell-associated and not freely extracellular. The cells were disrupted ultrasonically in distilled water and the supernate, after ultracentrifugation to remove cell debris, was dark brown in the case of B. melaninogenicus strains and colourless in the case of B. fragilis, F. necrophorum and Cl. clostridiiforme strains and the medium control. Spectrophotometry of these aqueous extracts from the disrupted cells showed a broad peak in the 370-450 nm band, with a maximum of 410 nm, for B. melaninogenicus strains; this was not so with preparations derived from B. fragilis, F. necrophorum or Cl. clostridiiforme strains or from the medium control (fig. 2. 1).

The Prussian-blue reaction was not produced by the addition of potassiumferricyanide solution to this pigment extract, either with or without acidification, and H_2S was not evolved after acidification.

Colonies of B. melaninogenicus NCTC9336, 9337 and 9338, B. fragilis NCTC9343, F. necrophorum NCTC10575 and Cl. clostridiiforme NCTC7155 after incubation for one week on blood agar were suspended in distilled water,

washed and examined as above. The first washing showed a small amount of haemoglobin carried over with each, but subsequent washings were clear. The B. melaninogenicus cells remained dark and the B. fragilis, F. necrophorum and Cl. clostridiiforme cells were pale. After ultrasonic treatment and ultracentrifugation, the extracts from B. melaninogenicus were all dark brown and showed spectrophotometry peaks very similar to those obtained from cells grown in horse-blood broth. The extracts from B. fragilis, F. necrophorum and Cl. clostridiiforme strains had no such peak (fig 2. 2).

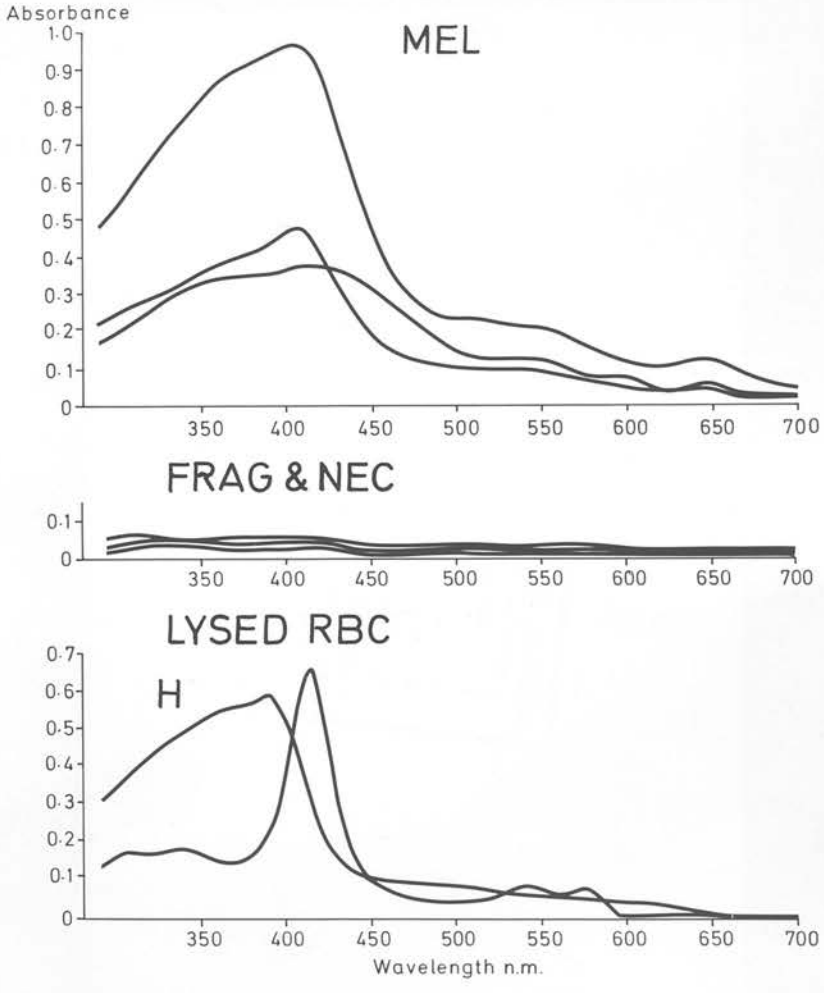
Characterization of Bacteroides strains

The detailed characteristics of the NCTC reference strains are given in Chapter 3. The 36 laboratory isolates were also included in the 165 strains tested in the studies described in Chapter 3; all were identified as belonging to the B. fragilis group.

Discussion

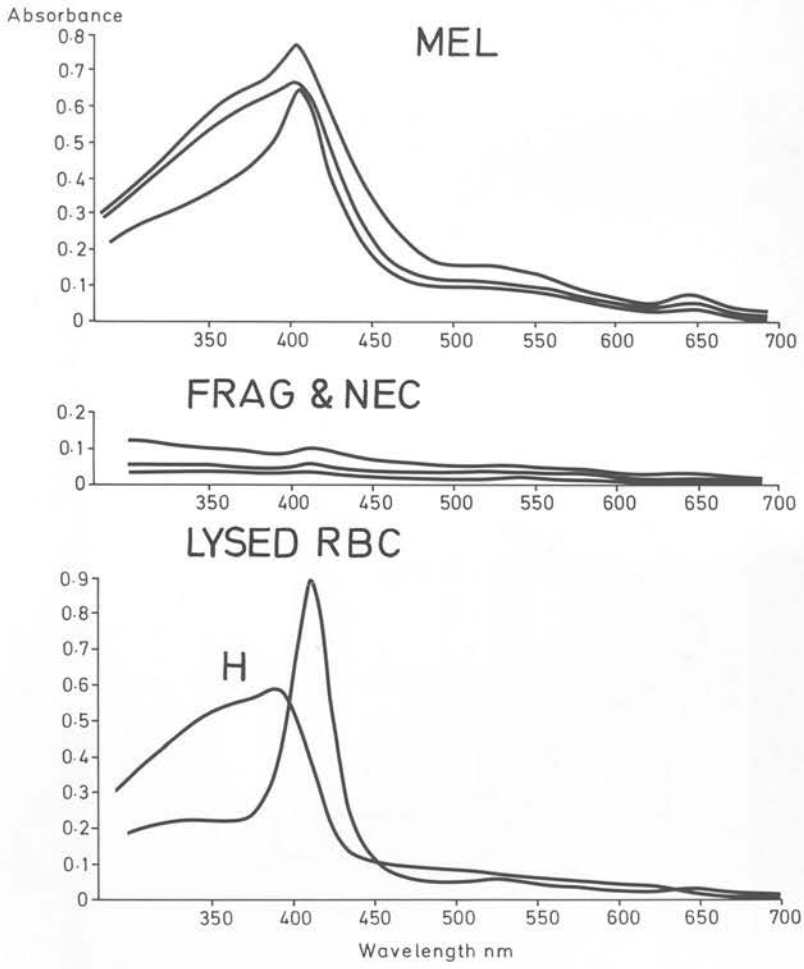
The confusion that has surrounded studies on B. melaninogenicus, because of the difficulties experienced in maintaining pure cultures, the variability of results of biochemical tests, and the specific growth requirements of some strains (Gibbons & Macdonald, 1960), has resulted in the distinctive property of pigmentation remaining the primary basis for the identification of B. melaninogenicus. Bacteroides-like organisms that produce black colonies on blood agar have been grouped together as B. melaninogenicus by some workers solely on the basis of

FIGURE 2.1



Typical patterns of plot obtained in spectrophotometric studied on extracts of disintegrated washed preparations of Bacteroides spp. derived from cultures in horse-blood broth. MEL : B.melaninogenicus NCTC nos. 9336, 9337 & 9338; FRAG & NEC : B.fragilis NCTC 9343, F.necrophorum NCTC 10575 and Cl.clostridiiforme NCTC 7155; LYSED RBC : aqueous extract of lysed horse red cells; H : aqueous solution of haemin.

FIGURE 2.2



Typical patterns of plots obtained in spectrophotometric studies on extracts of disintegrated washed preparations of Bacteroides spp. derived from cultures on human-blood agar. MEL : B.melaninogenicus NCTC nos. 9336, 9337 & 9338 (upper line); FRAG & NEC : B.fragilis NCTC 9343, F.necrophorum NCTC 10575 and Cl.clostridiiforme NCTC 7155; LYSED RBC : aqueous extract of lysed human red cells; H : aqueous solution of haemin.

this property, irrespective of any other similarities or differences (Beerens, 1970). Doubt was cast on the validity of this approach by the claim of Tracy (1969) that other Bacteroides species can produce black pigment under particular cultural conditions, and that the pigment is extracellular colloidal ferrous sulphide. Earlier workers had identified the pigment of B. melaninogenicus as extra-cellular melanin (Oliver & Wherry, 1921), whereas Schwabacher et al. (1947) considered that it was intracellular or cell-associated haematin.

The first group of experiments in the present study with 42 bacteroides-like organisms and one strain of E. coli, P. mirabilis, Salm. typhimurium and Cl. welchii grown in media containing additional cysteine and ferrous sulphate showed that all of these organisms produced H_2S in such a medium and all produced a dense black colloidal precipitate of ferrous sulphide. However, this is not equivalent to the synthesis of a specific pigment; it is an inorganic chemical reaction occurring whenever H_2S is introduced into a solution containing ferrous ions. When a sulphur source such as cysteine was provided, all the bacteroides-like organisms produced H_2S; ferrous sulphate, either incorporated in the original medium or added after incubation, was then reduced to ferrous sulphide and merely acted as an indicator of H_2S production. This indicator system, however, is not responsible for the

development of the typical black colonies when B. melaninogenicus strains are grown on media containing blood.

In the second part of the investigation, three reference strains of B. melaninogenicus were grown in media containing blood, and the washed bacterial cells and the cell-free medium supernate were examined for pigment. There was no extracellular pigment in the supernate, and pigment was not eluted when the cells were washed with distilled water, but the washed cells of the three B. melaninogenicus strains were dark brown in colour. Parallel treatment of cultures of B. fragilis F. necrophorum and Cl. clostridiiforme yielded pale cells. The dark pigment that gives the colonies of B. melaninogenicus their characteristic appearance is either intracellular or cell-associated in such a way that it cannot be removed by washing with water despite the finding that, when released by ultrasonic treatment of the cells, the pigment is water soluble and can then be extracted in aqueous solution.

The extracted pigment has none of the properties of ferrous sulphide; it is in solution and not in suspension; it does not give the Prussian-blue reaction with potassium ferricyanide solution after acidification; and acid-treatment does not liberate H_2S from it. Moreover, ferrous sulphide produced in colonies of other bacteroides-like organisms grown on lysed-blood agar with additional cysteine and ferrous sulphate faded

when left exposed to air and light; the (intact) pigmented colonies of B. melaninogenicus did not fade. When the pigment was examined by spectrophotometry, there was an absorption peak at 410 nm. This is the wavelength at which haemoglobin in the medium supernate gives its peak. The position of the peak is constant for extracts from all three B. melaninogenicus strains grown in blood broth or on blood agar, whereas a comparable peak was not given by extracts from B. fragilis, F. necrophorum or Cl. clostridiiforme. Moreover, the likely source of the pigment is indicated by the observations that pigmentation develops only in colonies on blood agar in association with haemolysis of the surrounding medium and is more rapid when lysed blood is used for the medium. These results support the conclusions of Schwabacher et al. (1947) that the pigment of B. melaninogenicus is derived from haemoglobin and is either intracellular or cell-associated.

The present experiments like those of Schwabacher et al. are open to the criticism that the pigment was extracted from preparations of cells grown in blood-containing media. In both studies, however, great care was taken to wash away all traces of extraneous haemoglobin; and in the present study no trace of haemoglobin was revealed by spectrophotometry in control extracts similarly derived from cultures of B. fragilis, F. necrophorum and Cl. clostridiiforme or in control

extracts of the uninoculated medium.

The results indicate that pigment production during growth in blood-containing media is a stable characteristic of certain strains of Bacteroides, that were all called B. melaninogenicus. It is not a property that can be expressed by any strain of Bacteroides under suitably manipulated cultural conditions. However, it does not follow that all pigment-producing strains form a biochemically homogeneous group: the three pigment-producing strains used in the present study are not identical. The results of the biochemical tests and antibiotic disk resistance tests show that strain NCTC9337 is clearly different from the other two; it is non-saccharolytic, resistant to colistin and does not produce a lipase or phospholipase.

It appears that pigmentation on blood-containing media is a significant property and merits considerable weight in the taxonomy of the Bacteroides group. It is, however, only one feature and it must be clear that there can be wide variations between pigment-producing strains in their biochemical properties and antibiograms. Some workers have described pigmented Bacteroides strains that varied in their individual growth requirements (Lev, 1959; Gibbons & Macdonald, 1960). Other workers have described pigmented Bacteroides strains that have different biochemical properties (Sawyer et al., 1962; Holdeman & Moore, 1972) and antibiograms (Finegold, Harada & Miller, 1967) and identified three sub-species

of B. melaninogenicus on these grounds.

The ICSB taxonomic sub-committee on gram-negative anaerobic rods has studied this problem and has now recommended (Finegold & Barnes, 1977) that the asaccharolytic strains of B. melaninogenicus should be transferred to a separate species, B. asaccharolyticus, and that the specific epithet melaninogenicus should be retained for the saccharolytic strains that produce black or brown pigmented colonies on media containing blood. The species B. melaninogenicus is at present regarded as having two sub-species - ss. melaninogenicus and ss. intermedius. The production of this pigment by Bacteroides strains therefore remains an important taxonomic criterion but it is not the sole basis for the definition of the species B. melaninogenicus.

CHAPTER 3

CHARACTERIZATION OF CLINICALLY IMPORTANT GRAM-NEGATIVE ANAEROBIC BACILLI BY CONVENTIONAL BACTERIOLOGICAL METHODS

One hundred and sixty five strains of gram-negative anaerobic bacilli were subjected to a series of simple laboratory tests that included morphological and biochemical tests, tests of resistance to antibiotics, and tolerance to dyes and bile salts. On the basis of the results of these tests, a short set of tests that were of particular discriminative value was selected for the identification of unknown laboratory isolates.

Materials and Methods

Organisms

The following strains were studied : Bacteroides fragilis (B. fragilis ss. fragilis) NCTC nos. 9343, 9344, 10581, 10584 and 8560; B. thetaiotaomicron (B. fragilis ss. thetaiotaomicron) NCTC10582 and ATCC8492; B. vulgatus (B. fragilis ss. vulgatus) NCTC10583 and ATCC8482; B. ovatus (B. fragilis ss. ovatus) ATCC8483; B. distasonis (B. fragilis ss. distasonis) ATCC8503; B. melaninogenicus NCTC nos. 9336, 9337 and 9338; B. melaninogenicus ss. melaninogenicus VPI4196; B. oralis NP333 and ATCC15930 (B. melaninogenicus ss. melaninogenicus, see Appendix V); B. melaninogenicus strains 2296, 3502, and 3586;

B. corrodens strains 124, NC-1, NC-2 and NCL20; *F. polymorphum* NCTC10562; *F. necrogenes* NCTC10723; *F. necrophorum* NCTC nos. 10575, 10576 and 10577; *L. buccalis* NCTC10249; *L. dentium* NCTC10206; *B. necrophorus* (*Cl. clostridiiforme*) NCTC7155; *B. ochraceus* VPI2845. In addition, 39 strains were isolated from clinical specimens at the Royal Infirmary, Edinburgh; 50 strains were isolated from clinical specimens at the Central Microbiological Laboratories, Western General Hospital, Edinburgh; 26 strains were isolated from normal human faeces in the Microbial Pathogenicity Research Laboratory, Department of Bacteriology, Edinburgh University Medical School; and 17 strains were isolated from human gingival crevice material and dental plaque sampled at the Dental Hospital, Edinburgh.

Tests for the characterization of strains

Culture inocula. In the following tests, one drop (0.02ml) of a 48-h cooked-meat-broth culture was used to seed each tube and either one drop or one loopful (*c*.0.01ml) to seed each plate of medium.

Media. Details of the media used are given in Appendix II

Colony and cell morphology. In general, observations of colony morphology were recorded after growth for 48h on human-blood agar; colonies of strains that grew

slowly were described as soon as they were visible.

Cell morphology was noted in gram-stained smears from cultures grown for 48h (i) on blood agar and (ii) in cooked-meat broth.

Haemolysis on blood agar was observed after incubation for 48h and one week.

Pigment production. Strains were observed for production of black-pigmented colonies on lysed-blood agar with menadione after incubation for up to two weeks.

Motility. A wet film prepared from a 48h cooked-meat-broth culture was examined by phase-contract microscopy. Strains were also stab inoculated into semi-solid agar (motility test medium) which was then incubated anaerobically until growth was visible.

Lipase activity. This was detected by observing the effect of growth on egg yolk agar (EYA).

Oxidase test. A freshly prepared 1% solution of tetramethyl-p-phenylene-diamine dihydrochloride in distilled water was poured on to a one-week culture on EYA. A blue colour indicated oxidase production.

Catalase test. A one-week culture on EYA was flooded with 10% hydrogen peroxide solution; a stream of bubbles arose from colonies of catalase-producing organisms.

Hydrogen sulphide production. A strip of lead acetate paper was suspended during incubation in the neck of a tube of a cooked-meat-broth culture. Blackening of the paper indicated H_2S production.

Nitrate reduction. This was tested in supplemented

thioglycollate medium with KNO_3(200µg/ml). The presence of nitrite ions was indicated by a deep red colour when 0.5ml of Nitrate Solution A and 0.5ml of Nitrate Solution B (Cruickshank, 1968) were added to 48h cultures.

Indole production. Indole was detected by adding 0.5ml of benzol to the liquid supernate of a 48h cooked-meat-broth culture and then adding a few drops of Ehrlich's Reagent. A pink colour indicated the presence of indole. Positive and negative control strains were included in each batch of tests.

Gelatinase test. A charcoal-gelatin disk in a cooked-meat-broth culture was observed for digestion of the disk during incubation for two weeks. In view of difficulties encountered with commercially available disks, the disks were prepared in our own laboratory (see Appendix II).

Aesculin hydrolysis. The organisms were grown for 48h in cooked-meat-broth containing 1% aesculin. If aesculin was hydrolysed a black discoloration developed when 0.5ml of a 1% aqueous solution of ferric ammonium citrate was added.

Dextran hydrolysis. Strains were grown for one week on blue dextran test medium. Hydrolysis was indicated by the development of a zone of clearing around colonies growing on the blue medium.

Carbohydrate-fermentation tests. Filter-sterilized 20% solutions of glucose, lactose, sucrose, maltose, rhamnose, trehalose and mannitol in distilled water were added separately to tubes of pre-steamed cooked-meat-broth, or supplemented thioglycollate medium to

give a final concentration of 1% of the test sugar. The tubes were seeded with one drop (0.02ml) from a 48h cooked-meat-broth culture and incubated anaerobically for 48h. Slow-growing strains were also incubated for one week. The final pH was measured with a Pye Unicam model 292 pH meter and a Pye Ingold combined Glass and Reference Electrode No. 401-S/160 after aerobic exposure on the bench for at least one hour. Controls included cultures of the test strains in plain cooked-meat-broth or thioglycollate medium and tubes of uninoculated (but incubated) 1% sugar media. The test was regarded as positive if the pH fell > 0.5 unit below that of the uninoculated control and > 0.5pH unit in comparison with the value of the 48h culture in plain cooked-meat-broth (derived from Rutter, 1970).

Tolerance tests. (a) Inhibition of growth by bile salts. Each strain was seeded on a series of four supplemented nutrient agar test media containing (i) no bile salt (control); (ii) 0.5% sodium taurocholate; (iii) o.1% sodium deoxycholate; or (iv) 0.5% sodium taurocholate plus 0.1% sodium deoxycholate. The plates were examined for growth after 48h, or longer for fastidious strains. (b) Sensitivity to dyes. Each strain was seeded on a series of five supplemented nutrient agar media containing (i) no dye (control); (ii) brilliant green (1 in 80 000); (iii) Victoria blue 4R (1 in 80 000); (iv) gentian violet (1 in 100 000); or (v) ethyl violet (1 in 80 000). The plates were examined

for growth after 48h. Each dye was initially prepared in aqueous solution and the concentrations above are those finally achieved in the test medium.

Antibiotic disk resistance tests. The test strains were grown on fresh blood agar and tested for resistance to antibiotics by the disk-diffusion method (modified from Sutter & Finegold, 1971). The plates were seeded by spreading 0.02ml of a 48h cooked-meat-broth culture on the surface with a glass spreader. The diameters of zones of inhibition were measured after either 24 or 48h, as soon as good growth was visible.

Disks containing neomycin sulphate 1000µg, kanamycin sulphate 1000µg, benzyl penicillin 1.5 units, erythromycin ethyl succinate 60µg, colistin sulphate 10µg, rifampicin (Rimactane) 15µg, vancomycin 15µg, and chloramphenicol 10µg were prepared in our laboratory. Disks containing methicillin 10µg, lincomycin 2µg, clindamycin 2µg, bacitracin 0.1 unit, neomycin 10µg and kanamycin 30µg, were obtained from Mast Laboratories.

B. fragilis NCTC9343 was selected as the reference strain for tests. It was tested in parallel experiments with a standard Oxford staphylococcus that was sensitive to neomycin, kanamycin, penicillin, methicillin, erythromycin, rifampicin, lincomycin, clindamycin, vancomycin and chloramphenicol but resistant to colistin and bacitracin. The diameters of zones of inhibition were grouped in four grades: < 15mm, 16-25mm; 26-35mm; > 35mm. The grades obtained with B. fragilis NCTC9343 were classified as resistant (R) or sensitive

(S) by comparison with the grades obtained with the Oxford staphylococcus. The grades obtained with test strains were compared with those from B. fragilis NCTC9343 and classified as 'resistant' or 'sensitive' in relation to that reference strain.

Results

Cell morphology

All strains were gram-negative, non-sporing, non-motile, obligately anaerobic bacteria, except L. dentium NCTC10206 which was a gram-variable facultative aerobe with many gram-positive cells in young cultures. Pleomorphism was common. Cell shape varied from filamentous to cocco-bacillary, often in the same smear; some strains formed chains; some had pointed ends and others rounded ends. Cell morphology varied further with the culture medium and had little discriminatory value. However, many B. melaninogenicus strains were predominantly cocco-bacillary and many Fusobacterium spp. showed long filamentous forms.

Colony morphology

Colonies on blood agar differed in size from pin-point to 3-4mm in diameter. Some but not all Fusobacterium spp. produced rhizoid colonies which were never produced by Bacteroides spp. Black-pigmented colonies with haloes of haemolysis were typical of B. melaninogenicus. Many strains gave a small zone of incomplete haemolysis after prolonged incubation, but a wider zone of complete haemolysis was

characteristic of B. melaninogenicus and some Fusobacterium spp. (Tables 3. 1,2,3).

The test strains were divided into three groups on the basis of the results of biochemical, tolerance and antibiotic resistance tests. Strains that broadly resembled B. fragilis NCTC9343 were designated 'B-strains'; strains that produced black-pigmented colonies on blood agar were regarded as B. melaninogenicus strains and designated 'M-strains'; the remaining strains that included reference strains of Fusobacterium spp. and Leptotrichia spp. and a small heterogeneous group of laboratory isolates that did not conform with the broad characteristics of the other two groups were designated 'F-strains'.

Results of biochemical tests

None of the test strains produced oxidase and only L. dentium NCTC10206 produced catalase.

The 'B-strains'. The 20 patterns of results in a series of biochemical tests on 107 B-strains are listed in Table 3.1 and are coded Bi-Bxx. Almost all results were clearly positive or negative. Haemolysis, however, was variable and at most weak and incomplete with B-strains, and a few variable results were obtained in tests for sucrose fermentation.

The five reference strains of B. fragilis ss. fragilis and 47 strains isolated from clinical specimens gave pattern Bi. B. fragilis ss. vulgatus NCTC10583 and four laboratory isolates gave pattern

TABLE 3.1. Results of biochemical tests with non-pigmented Bacteroides organisms (B-strains)

Test	Pattern of results*																			
	Bi	Bii	Biii	Biv	Bv	Bvi	Bvii	Bviii	Bix	Bx	Bxi	Bxii	Bxiii	Bxiv	Bxv	Bxvi	Bxvii	Bxviii	Bxix	Bxx
H_2S production	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Haemolysis on blood agar	⊥	−	⊥	⊥	−	⊥	⊥	−	⊥	⊥	⊥	⊥	⊥	−	⊥	⊥	⊥	+	⊥	−
Lipase production on EYA	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Digestion of gelatin	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
Indole production	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−
Hydrolysis of aesculin	+	−	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+
Fermentation of:																				
glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sucrose	+	+	+	+	+	−	+	+	+	⊥	+	+	⊥	+	+	+	+	−	+	+
rhamnose	−	−	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	−	+	−
trehalose	−	−	−	−	−	−	+	+	−	−	+	−	−	+	+	+	+	−	−	−
mannitol	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Hydrolysis of dextran	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+	−	−	+	+
Reference strains conforming to the given pattern	NCTC 9343 NCTC 8560 NCTC 9344 NCTC 10581 NCTC 10584	...	NCTC 10583	...	...	...	ATCC 8503	ATCC 8482	...	...	ATCC 8483	...	...	ATCC 8492	NCTC 10582	...	...	...	NP 333	VPI 2845
No. of laboratory isolates conforming	47	1	4	12	1	4	0	2	1	2	0	1	3	2	7	1	5	1	0	0
Total no. of strains conforming	52	1	5	12	1	4	1	3	1	2	1	1	3	3	8	1	5	1	1	1

*+ = positive result; − = negative result; ⊥ = weak reaction.

Biii, distinguished by the ability to digest gelatin and ferment rhamnose. Pattern Biv, obtained with 12 strains from normal human faeces differed from Biii in only one respect (aesculin hydrolysis negative); B. fragilis ss. vulgatus ATCC8482 was significantly different and gave a pattern (Bviii) similar to that obtained with B. fragilis ss. distasonis ATCC8503 (Bvii). B. fragilis ss. ovatus ATCC8483 (pattern Bxi) was the only strain that fermented mannitol. Nine laboratory isolates gave the same pattern (Bxiv and Bxv) as the two reference strains of B. fragilis ss. thetaiotaomicron ATCC8492 and NCTC10582 that differed only in the ability to hydrolyse dextran. B. oralis NP333 and B. ochraceus VPI2845 gave individual patterns (Bxix and Bxx) that differed only in the fermentation of rhamnose. The 20 remaining laboratory isolates gave patterns that were intermediate between the patterns obtained with the reference strains.

The 'M-strains'. Twelve patterns of results (Mi-Mxii) were obtained from the biochemical tests on 41 M-strains (Table 3.2). All strains produced black-pigmented colonies on blood agar surrounded by a zone of clear haemolysis and all except one strain digested gelatin. The patterns of results with most strains fell within two groups clearly differentiated by the results of carbohydrate-fermentation tests.

B. melaninogenicus NCTC nos. 9336 and 9338 and 12 laboratory isolates - 10 from human gingival crevice

TABLE 3.2. Results of biochemical tests with black-pigmented Bacteroides organisms (M-strains)

Test	Pattern of results*											
	Mi	Mii	Miii	Miv	Mv	Mvi	Mvii	Mviii	Mix	Mx	Mxi	Mxii†
H_2S production	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	−	−	−	−	−	−	−	−	−	+	+	−
Haemolysis on blood agar	+	+	+	+	+	+	+	+	+	+	+	+
Lipase production on EYA	+	−	+	+	−	−	−	−	−	−	−	...
Digestion of gelatin	+	+	+	+	+	+	+	+	+	+	−	+
Indole production	−	−	+	+	+	+	−	−	+	+	−	+
Hydrolysis of aesculin	+	−	−	−	−	−	−	−	−	−	−	−
Fermentation of:												
glucose	+	+	+	+	+	+	+	−	−	−	+	−
lactose	+	+	+	−	−	+	−	−	−	−	+	−
maltose	+	+	+	+	+	+	+	−	−	−	−	−
sucrose	+	+	+	+	+	+	−	−	−	−	+	−
rhamnose	−	−	−	−	−	−	+	−	−	−	−	−
trehalose	−	−	−	−	−	−	−	−	−	−	−	−
mannitol	−	−	−	−	−	−	−	−	−	−	−	−
Hydrolysis of dextran	+	+	−	−	−	−	−	−	−	−	−	−
Reference strains conforming to the given pattern	ATCC 15930	VPI 4196	...	NCTC 9338	NCTC 9336	...	...	...	NCTC 9337	...	...	...
No. of laboratory isolates conforming	0	0	1	11	1	4	1	4	7	1	1	5
Total no. of strains conforming	1	1	1	12	2	4	1	4	8	1	1	5

*+ = positive result; − = negative result.

† Five laboratory isolates that did not grow on EYA are shown in the Tables as biochemical group Mxii.

material and dental plaque and two from clinical specimens - gave similar patterns (Miv and Mv) that differed only in the production of lipase; five strains (patterns Miii and Mvi) differed only in the fermentation of lactose.

The second group were asaccharolytic. B. melaninogenicus NCTC9337 and 11 laboratory isolates - 10 from abdominal wounds and high vaginal swabs and one oral isolate - gave similar patterns (Mviii and Mix). Five asaccharolytic strains failed to grow on supplemented EYA and are, therefore, placed in a separate pattern (Mxii). B. melaninogenicus ss. melaninogenicus VPI4196 and B. melaninogenicus (formerly B. oralis) ATCC15930 gave similar patterns but differed in the production of lipase and the hydrolysis of aesculin. The remaining three strains gave individual patterns (Mvii, Mx and Mxi). In particular, the strains that gave patterns Mx and Mxi were the only Bacteroides strains except B. corrodens that reduced nitrate to nitrite.

The 'F-strains'. The results of the biochemical tests with reference strains of Fusobacterium spp. and a small miscellaneous group of laboratory isolates are given in Table 3.3. None of these strains produced black-pigmented colonies on blood agar. The four strains of B. corrodens gave pattern Fi. F. necrophorum NCTC nos. 10575, 10576 and 10577 gave pattern Fii; they gave variable results in fermentation tests with

TABLE 3.3. Results of biochemical tests with miscellaneous organisms of the Bacteroides - Fusobacterium group (F-strains)

Test	Pattern of results*									
	Fi	Fii	Fiii	Fiv	Fv	Fvi	Fvii	Fviii	Fix	Fx
H_2S production	+	+	−	+	−	+	−	+	+	−
Nitrate reduction	+	−	−	−	−	−	−	−	−	+
Haemolysis on blood agar	⊥	+	+	−	−	−	−	−	−	⊥
Lipase production on EYA	−	+	+	−	−	−	−	−	−	−
Digestion of gelatin	+	−	−	−	−	−	−	−	−	−
Indole production	−	+	−	+	−	−	−	−	−	−
Hydrolysis of aesculin	−	−	−	−	+	+	−	−	+	−
Fermentation of										
glucose	−	⊥	+	−	+	−	⊥	−	+	−
lactose	−	−	−	−	+	−	⊥	−	+	−
maltose	−	⊥	+	−	+	−	⊥	−	+	−
sucrose	−	−	−	−	+	−	⊥	−	+	−
rhamnose	−	−	−	−	−	−	−	−	−	−
trehalose	−	−	−	−	+	+	−	−	−	−
mannitol	−	−	−	−	−	−	−	−	−	−
Hydrolysis of dextran	−	−	−	−	−	−	−	−	−	−
Reference strains conforming to the given pattern	...	NCTC 10575 NCTC 10576 NCTC 10577	...	NCTC 10562	NCTC 10249	NCTC 10723	...	...	NCTC 7155	NCTC 10206
No. of laboratory isolates conforming	4	0	1	1	0	0	1	2	0	0
Total no. of strains conforming	4	3	1	2	1	1	1	2	1	1

*+ = positive result; − = negative result; ⊥ = weak reaction.

glucose and maltose but the results were never more than weakly positive (a fall in pH of 0.5 unit). F. polymorphum NCTC10562, F. necrogenes NCTC10723 and L. buccalis NCTC10249 gave individual patterns of results. Two laboratory isolates were unreactive except in H_2S production (pattern Fviii), and one isolate gave positive results only weakly in some fermentation tests (pattern Fvii). Cl. clostridiiforme (B. necrophorus) NCTC7155 gave the pattern (Fix) of B. fragilis ss. fragilis. The pattern (Fx) obtained with L. dentium NCTC10206 is included although the organism is not a true gram-negative anaerobe.

Results of tolerance tests

Sixteen patterns (A-R) of results were obtained with the 165 test strains in tolerance tests with bile salts and dyes (Table 3-4). B. fragilis ss. fragilis NCTC nos. 8560, 9343, 9344, 10581 and 10584, B. fragilis ss. thetaiotaomicron NCTC10582 and ATCC8492, B. fragilis ss. ovatus ATCC8483, and 48 laboratory isolates gave pattern A. Fifteen laboratory isolates gave a similar pattern (B) but inhibition of growth by deoxycholate was not prevented by the presence of taurocholate. B. fragilis ss. vulgatus NCTC10583 and 15 laboratory isolates grew in the presence of Victoria blue 4R and ethyl violet (pattern E). B. fragilis ss. vulgatus ATCC8482 was inhibited by these dyes and gave the same pattern (D) as B. fragilis

TABLE 3.4. Results of tolerance tests with 165 strains of gram-negative anaerobic bacilli

Test	Pattern of results* A	B	C	D	E	F	G	H	J	K	L	M	N	P	Q	(R)†
Growth on basal medium	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	...
Growth on basal medium plus:																
taurocholate (0·5%)	+	+	+	+	+	+	+	+	I	I	I	I	+	I	+	...
deoxycholate (0·1%)	I	I	I	I	I	I	I	+	I	I	I	I	+	I	+	...
taurocholate (0·5%) & deoxycholate (0·1%)	+	I	+	+	+	I	I	+	I	I	I	I	+	I	+	...
Victoria blue 4R (1/80 000)	+	+	+	I	+	+	I	+	+	I	+	+	+	+	+	...
ethyl violet (1/80 000)	I	I	I	I	+	+	I	I	I	I	I	+	+	+	+	...
gentian violet (1/100 000)	I	I	I	I	I	I	I	I	I	I	I	+	+	+	+	...
brilliant green (1/80 000)	I	I	+	I	I	I	I	I	I	I	+	+	I	I	+	...
Reference strains conforming to the given pattern	NCTC 8560, NCTC 9343, NCTC 9344, NCTC 10581, NCTC 10582, NCTC 10584, ATCC 8483, ATCC 8492	...	...	ATCC 8482, ATCC 8503	NCTC 10583	...	...	...	NP 333	NCTC 9336, NCTC 9337, NCTC 9338, VPI 4196, VPI 2845, ATCC 15930, NCTC 10206	...	NCTC 10575, NCTC 10576, NCTC 10577, NCTC 10562	NCTC 10723	NCTC 10249	NCTC 7155	...
No. of laboratory isolates conforming	48	15	2	0	15	1	1	1	7	38	6	2	0	0	0	3
Total no. of strains conforming	56	15	2	2	16	1	1	1	8	45	6	6	1	1	1	3

*+ = growth; I = inhibition of growth.

† Three laboratory isolates of *B. melaninogenicus* that did not grow on tolerance test basal medium are shown in the tables as tolerance group R.

ss. distasonis ATCC8503.

B. melaninogenicus NCTC nos. 9336, 9337 and 9338, ATCC15930 and VPI4196 and 30 laboratory isolates of B. melaninogenicus gave pattern K: B. ochraceus VPI2845, L. dentium NCTC10206, seven laboratory isolates of B. fragilis-like strains, and one F-strain gave the same pattern. B. oralis NP333, two laboratory isolates of B. melaninogenicus, two F-strains, and three B. fragilis-like strains gave pattern J, that differed from pattern K by growth in the presence of Victoria blue 4R.

The four B. corrodens strains, one B. melaninogenicus strain and one B. fragilis-like strain gave pattern L. F. necrophorum NCTC nos. 10575, 19576 and 10577, F. polymorphum NCTC10562, and two laboratory isolates gave tolerance pattern M. F. necrogenes NCTC10723, L. buccalis NCTC10249 and Cl. clostridiiforme (B. necrophorus) NCTC7155 gave unique tolerance patterns (N, P and Q). Strain NCTC7155 was the only test strain that grew in the presence of all the dyes and bile salts used. Three B. melaninogenicus strains did not grow on tolerance test basal medium (pattern (R)).

Results of antibiotic disk resistance tests

The patterns of results obtained with the 165 test strains in antibiotic disk resistance tests are shown in Tables 3. 5 and 6. The 105 B. fragilis-like strains

TABLE 3.5. Results of antibiotic disk resistance tests with the test strains of gram-negative anaerobic bacilli : patterns 1-20

Antibiotic (content per disc)	Patterns of results*																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Neomycin (1000 μg)	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S	R	R
Neomycin (10 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kanamycin (1000 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
Kanamycin (30 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Penicillin (1·5 units)	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	S	S
Methicillin (10 μg)	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	S	S	S
Erythromycin (60 μg)	S	R	S	S	S	S	R	R	R	S	S	R	S	S	S	S	S	S	S	S
Colistin (10 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R
Rifampicin (15 μg)	S	S	S	S	S	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S
Lincomycin (2 μg)	R	R	R	S	S	S	R	R	S	S	R	R	R	S	S	S	S	S	S	S
Clindamycin (2 μg)	S	S	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S
Bacitracin (0·1 unit)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	R	S	S
Vancomycin (15 μg)	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
Chloramphenicol (10 μg)	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	R	S	S	S	S
Reference strains conforming to the pattern	NCTC 9343 NCTC 9344 NCTC 10583 NCTC 10584	NCTC 8560	NCTC 10582	NCTC 10581	...	ATCC 8492	ATCC 8483	...	...	...	...	...	ATCC 8482	...	...	ATCC 8503	...	...	...	...
No. of laboratory isolates conforming	57	7	4	0	5	0	0	1	1	5	1	3	3	2	2	0	1	2	2	1
Total no. of strains conforming	61	8	5	1	5	1	1	1	1	5	1	3	4	2	2	1	1	2	2	1

* S = sensitive; R = resistant

TABLE 3.6. Results of antibiotic disk resistance tests : patterns 21-41

Antibiotic (content per disc)	Patterns of results*																				
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
Neomycin (1000 μg)	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Neomycin (10 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S
Kanamycin (1000 μg)	R	R	R	R	R	R	R	R	S	S	S	R	R	S	S	S	S	S	S	S	S
Kanamycin (30 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	S	S
Penicillin (1·5 units)	S	S	R	S	S	S	S	S	S	R	S	R	R	S	S	S	S	S	S	S	S
Methicillin (10 μg)	S	S	R	S	S	S	S	S	S	R	S	S	R	S	S	S	S	S	R	S	S
Erythromycin (60 μg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	S
Colistin (10 μg)	S	R	S	S	S	R	R	S	S	S	R	R	R	S	S	S	S	S	S	S	R
Rifampicin (15 μg)	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S
Lincomycin (2 μg)	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S
Clindamycin (2 μg)	S	S	S	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S	S	S	S
Bacitracin (0·1 unit)	S	R	S	S	S	S	R	R	S	R	R	R	R	R	R	S	R	R	R	R	S
Vancomycin (15 μg)	S	S	R	R	S	S	S	R	R	S	S	R	S	R	R	S	R	R	S	S	S
Chloramphenicol (10 μg)	R	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S
Reference strains conforming to the pattern	...	...	...	NCTC 9336 NCTC 9338	...	NCTC 9337	ATCC 15930	VPI 4196 NP 333	...	...	VPI 2845	...	...	NCTC 10575 NCTC 10576 NCTC 10577 NCTC 10723	...	NCTC 10249	NCTC 10562	NCTC 7155	...	...	NCTC 10206
No. of laboratory isolates conforming	1	1	2	15	3	4	2	2	2	1	0	1	1	0	1	1	0	0	1	4	0
Total no. of strains conforming	1	1	2	17	3	5	3	4	2	1	1	1	1	4	1	2	1	1	1	4	1

* S = sensitive; R = resistant

gave 18 patterns of resistance (1 - 18); 61 strains gave pattern 1; they were resistant to the test concentrations of neomycin, kanamycin, penicillin, methicillin, colistin, lincomycin, bacitracin and vancomycin. Fifteen strains (patterns 12 - 18) were sensitive to the 1000µg neomycin disk, one strain (pattern17) was sensitive to the 1000µg kanamycin disk, and two strains (patterns 4 and 17) were sensitive to vancomycin. Six strains were sensitive to penicillin and methicillin (patterns 10 and 11) and two strains (pattern 18) to methicillin alone. Fourteen strains were resistant to erythromycin (patterns 2, 7, 8, 9 and 12); three strains were resistant to rifampicin (patterns 6, 7 and 8) and two strains were resistant to chloramphenicol (patterns 7 and 16). Twenty one strains were sensitive to lincomycin (patterns, 4, 5, 6, 9, 10, 14, 15, 16, 17 and 18) but all except six strains (patterns 3 and 8), were sensitive to clindamycin. Two strains were sensitive to colistin (pattern 18) and three strains were sensitive to bacitracin (patterns 15 and 17).

The 41 B. melaninogenicus strains gave 12 patterns of resistance (19-30). Seventeen strains gave pattern 24; they were resistant to the 10µg neomycin disk, 30µg and 1000µg kanamycin disks, and vancomycin, and sensitive to the other antibiotics. Three strains were resistant to penicillin and methicillin (patterns 23 and 30), three to chloramphenicol (patterns 21 and 29), and one strain (pattern 30) was resistant to

lincomycin and clindamycin. All except five strains (patterns 19-22) were sensitive to the 1000µg neomycin disk; 15 strains were sensitive to vancomycin (patterns 20-22, 25-27 and 30), but only three strains were sensitive to the 1000µg kanamycin disk (patterns 29 and 30). Twelve strains were resistant to colistin (patterns 19, 20, 22, 26 and 27) and eight strains were resistant to bacitracin (patterns 22, 27, 28 and 30). B. oralis NP333 gave the same pattern (28) as three B. melaninogenicus strains. B. ochraceus VPI2845, however, was sensitive to the 1000µg kanamycin disk and vancomycin, and resistant to colistin, clindamycin and bacitracin (pattern 24).

The 17 F-strains gave 10 patterns of resistance (32-41). L. dentium NCTC10206 was sensitive to all agents except colistin (pattern 41). All four strains of B. corrodens gave pattern 40; they were sensitive to all agents except rifampicin and bacitracin. F. necrophorum NCTC nos. 10575, 10576 and 10577, and F. necrogenes NCTC10723 (pattern 34) and Cl. clostridiiforme (B. necrophorus) NCTC7155 (pattern 38) were also resistant to rifampicin. The remaining F-strains gave varied patterns.

Comparison of results of biochemical tests with results of tolerance tests

The patterns of tolerance obtained with the 107 B-strains that gave 20 patterns of biochemical results are shown in Table 3.7. Forty nine of the 52 strains

TABLE 3.7. The relationship of biochemical patterns to tolerance patterns of B-strains

Biochemical pattern	Number of strains with the stated tolerance pattern																Key to position of reference strain(s) (indicated by superscript)
	A	B	C	D	E	F	G	H	J	K	L	M	N	P	Q	(R)	
Bi	41[a]	8	2						1								a *B. fragilis* ss. *fragilis* NCTC 9343, 9344, 10581, 10584 and 8560
Bii										1							
Biii					4[b]	1											b *B. fragilis* ss. *vulgatus* NCTC 10583
Biv					12												
Bv										1							
Bvi									1	2	1						
Bvii				1[c]													c *B. fragilis* ss. *distasonis* ATCC 8503
Bviii	2			1[d]													d *B. fragilis* ss. *vulgatus* ATCC 8482
Bix		1															
Bx		2															
Bxi	1[e]																e *B. fragilis* ss. *ovatus* ATCC 8483
Bxii		1															
Bxiii		2							1								
Bxiv	3[f]																f *B. fragilis* ss. *thetaiotaomicron* ATCC 8492
Bxv	7[g]						1										g *B. fragilis* ss. *thetaiotaomicron* NCTC 10582
Bxvi								1									
Bxvii	2	1								2							
Bxviii										1							
Bxix									1[h]								h *B. oralis* NP 333
Bxx										1[j]							j *B. ochraceus* VPI 2845

TABLE 3.8. The relationship of biochemical patterns to tolerance patterns of M-strains

Biochemical pattern	Number of strains with the stated tolerance pattern																Key to position of reference strain(s) (indicated by superscript)
	A	B	C	D	E	F	G	H	J	K	L	M	N	P	Q	(R)	
Mi										1[k]							k *B. melaninogenicus* ATCC 15930
Mii										1[l]							l *B. melaninogenicus* VPI 4196
Miii										1							
Miv										12[m]							m *B. melaninogenicus* NCTC 9338
Mv										2[n]							n *B. melaninogenicus* NCTC 9336
Mvi									1	3							
Mvii										1							
Mviii										4							
Mix									1	7[p]							p *B. melaninogenicus* NCTC 9337
Mx											1						
Mxi										1							
Mxii										2						(3)	

Table 3.9. The relationship of biochemical patterns to tolerance patterns of F-strains

Biochemical pattern	Number of strains with the stated tolerance pattern																Key to position of reference strain(s) (indicated by superscript)
	A	B	C	D	E	F	G	H	J	K	L	M	N	P	Q	(R)	
Fi											4						
Fii												3[q]					q *F. necrophorum* NCTC 10575, 10576 and 10577
Fiii										1							
Fiv												2[r]					r *F. polymorphum* NCTC 10562
Fv														1[s]			s *L. buccalis* NCTC 10249
Fvi													1[t]				t *F. necrogenes* NCTC 10723
Fvii									1								
Fviii									1			1					
Fix															1[u]		u *B. necrophorus* NCTC 7155
Fx										1[v]							v *L. dentium* NCTC 10206

that gave the biochemical pattern Bi (B. fragilis ss. fragilis) and 19 of the 22 strains that gave patterns Bviii-Bxv and contained all B. fragilis ss. ovatus and B. fragilis ss. thetaiotaomicron strains gave tolerance patterns A and B. The 15 strains in tolerance pattern B did not grow in the presence of the deoxycholate and taurocholate mixture.

The 17 strains of B. fragilis ss. vulgatus in biochemical groups Biii and Biv gave tolerance patterns E and F, distinguished by growth in the presence of ethyl violet. Eleven strains that gave biochemical patterns conforming with the B. fragilis-like group (patterns Bi-Bxviii) gave tolerance patterns J, K and L that were the same as patterns obtained with B. melaninogenicus strains, B. oralis NP333, B. ochraceus VPI2845 (biochemical patterns Bxix and Bxx), L. dentium NCTC10206 and three F-strains also gave tolerance patterns J and K.

Thirty five of the 41 strains of B. melaninogenicus (biochemical patterns Mi-Mxii) gave tolerance pattern K (Table 3.8). Three strains grew in the presence of Victoria blue 4R (tolerance patterns J and L) and the remaining three strains failed to grow on the tolerance test basal medium.

The patterns of tolerance obtained with 17 F-strains are shown in Table 3.9. The four strains of B. corrodens that gave biochemical pattern Fi gave tolerance pattern L. F. necrophorum NCTC nos. 10575, 10576 and 10577

(biochemical pattern Fii), F. polymorphum NCTC10562 (biochemical pattern Fiv) and two laboratory isolates (biochemical patterns Fiv and Fviii) gave tolerance pattern M.

Comparison of results of biochemical tests with results of antibiotic disk resistance tests

The patterns of antibiotic resistance and biochemical results are compared in Tables 3.10, 11 and 12. Sixty-one of the 105 B. fragilis-like strains (biochemical patterns Bi-Bxviii) gave resistance pattern 1 and a further eight strains differed only in resistance to erythromycin (pattern 2). B-strains with the antibiotic resistance patterns 3 - 19 were distributed amongst biochemical groups Bi-Bxviii and the recognized subspecies of B. fragilis were not distinguishable by their antibiotic resistance patterns.

Sixteen saccharolytic strains of B. melaninogenicus and one asaccharolytic strain gave resistance pattern 24, but the distribution of the other 24 strains between 11 resistance patterns was unrelated to their biochemical patterns. All four strains of B. corrodens gave unique biochemical (Fi) and resistance (40) patterns. F. necrophorum NCTC nos. 10575, 10576 and 10577 (biochemical pattern Fii) and F. necrogenes NCTC10723 (biochemical pattern Fvi) gave the same resistance pattern (34) and the other F-strains gave

TABLE 3.10. The relationship of biochemical patterns to antibiotic resistance patterns of B-strains

Bio-chemical pattern	Number of strains with the stated pattern of antibiotic resistance																	..		..		..	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		28		31	41
Bi	36[a]	8[b]		1[c]	2			1	1			3											
Bii																		1					
Biii	2[d]				2						1												
Biv	11				1																		
Bv																	1						
Bvi													1	1	2								
Bvii																1[e]							
Bviii	2												1[f]										
Bix	1																						
Bx										2													
Bxi							1[g]																
Bxii			1																				
Bxiii										3													
Bxiv	1		1			1[h]																	
Bxv	4		3[j]										1										
Bxvi	1																						
Bxvii	3												1	1									
Bxviii																		1					
Bxix																				1[k]			
Bxx																						1[l]	

Key to position of reference strain(s) (indicated by superscript)
a *B. fragilis* ss. *fragilis* NCTC 9343, 9344 and 10584
b *B. fragilis* ss. *fragilis* NCTC 8560
c *B. fragilis* ss. *fragilis* NCTC 10581
d *B. fragilis* ss. *vulgatus* NCTC 10583
e *B. fragilis* ss. *distasonis* ATCC 8503
f *B. fragilis* ss. *vulgatus* ATCC 8482
g *B. fragilis* ss. *ovatus* ATCC 8483
h *B. fragilis* ss. *thetaiotaomicron* ATCC 8492
j *B. fragilis* ss. *thetaiotaomicron* NCTC 10582
k *B. oralis* NP 333
l *B. ochraceus* VPI 2845

TABLE 3.11. The relationship of biochemical patterns to antiobiotic resistance patterns of M-strains

Bio-chemical pattern	Number of strains with the stated pattern of antibiotic resistance																					Key to position of reference strain(s) (indicated by superscript)
	1	..	..	18	19	20	21	22	23	24	25	26	27	28	29	30	31	..	..	41		
Mi													1[m]									m *B. melaninogenicus* ATCC 15930
Mii														1[n]								n *B. melaninogenicus* VPI 4196
Miii										1												
Miv										9[p]				1	2							p *B. melaninogenicus* NCTC 9338
Mv										2[q]												q *B. melaninogenicus* NCTC 9336
Mvi										4												
Mvii														1								
Mviii									1	1		1	1									
Mix					1		1				2	3[r]	1									r *B. melaninogenicus* NCTC 9337
Mx								1														
Mxi																1						
Mxii					1	1			1		1	1										

TABLE 3.12. The relationship of biochemical patterns to antibiotic resistance patterns of F-strains

Biochemical patterns	Number of strains with the stated pattern of antibiotic resistance														Key to position of reference strain(s) (indicated by superscript)	
	1	..	..	31	32	33	34	35	36	37	38	39	40	41		
Fi													4		s	*F. necrophorum* NCTC 10575, 10576 and 10577
Fii							3^{s}									
Fiii									1							
Fiv								1		1^{t}					t	*F. polymorphum* NCTC 10562
Fv									1^{u}						u	*L. buccalis* NCTC 10249
Fvi							1^{v}								v	*F. necrogenes* NCTC 10723
Fvii						1										
Fviii					1							1				
Fix											1^{w}				w	*B. necrophorus* NCTC 7155
Fx														1^{x}	x	*L. dentium* NCTC 10206

varied resistance patterns.

There was no overlap between the antibiotic resistance patterns obtained with B. fragilis-like strains (biochemical patterns Bi-Bxviii), B. melaninogenicus strains (Mi-Mxii) and F-strains (Fi-Fx). B. oralis NP333 (Bxix) and B. ochraceus VPI2845 (Bxx) gave similar antibiotic resistance patterns to B. melaninogenicus strains.

Discussion

In this part of the study, conventional bacteriological tests were selected and adapted to meet the special requirements of these anaerobes. The details of the procedures and of the controls included in the tests take account of problems of growth of strains and reproducibility of results. Findings with a few exacting strains among the 165 tested indicated that all of the problems had not been solved but the results in general were encouraging.

The test strains fell readily into three main groups on the basis of the series of biochemical, tolerance and antibiotic-resistance studies; (i) non-pigmented B. fragilis-like strains; (ii) B. melaninogenicus strains, and (iii) Fusobacterium-like strains. The biochemical tests gave the most useful discrimination of strains within the groups. In the B. fragilis-like group (B-strains) there was clear separation of the reference strains representing five recognized sub-species

of B. fragilis. The patterns obtained with the remaining B-strains showed clusters of strains related to the reference strains in a spectrum that included intermediate patterns. B. oralis NP333 and B. ochraceus VPI2845 gave biochemical patterns that were closely related to those obtained with the B. fragilis-like strains.

In the B. melaninogenicus group (F-strains) there was clear separation of the strains into two clusters; (i) saccharolytic, and (ii) asaccharolytic; there were minor differences between strains within these clusters. Three sub-species of B. melaninogenicus were described by Holdeman & Moore (1974) as B. melaninogenicus ss. melaninogenicus, ss. intermedius, and ss. asaccharolyticus. B. melaninogenicus NCTC9337 and the non-fermentative laboratory isolates are clearly B. melaninogenicus ss. asaccharolyticus. B. melaninogenicus NCTC nos. 9336 and 9338 conform to the published reference patterns of B. melaninogenicus ss. intermedius (Holdeman & Moore, 1973) and the remainder of our laboratory isolates of B. melaninogenicus were similar to these two reference strains. B. melaninogenicus ss. melaninogenicus VPI4196, differed in a number of tests from the cluster of strains identified as B. melaninogenicus ss. intermedius. B. melaninogenicus (formerly B. oralis) ATCC15930 was similar to but not identical with strain VPI4196.

The F-strains were a heterogeneous collection of

organisms that were clearly different from the B-strains and M-strains. There were considerable biochemical differences between reference strains of individual species within the group.

The tolerance tests largely confirmed the separation of the test strains into the three main groups. The B. fragilis-like strains gave a number of similar tolerance patterns; small differences were not generally related to the different sub-species, but B. fragilis ss. vulgatus NCTC10583 and the similar laboratory isolates (but not B. fragilis ss. vulgatus ATCC8482) gave a unique pattern. A single tolerance pattern was typical of the B. melaninogenicus strains but complete discrimination between B-strains and M-strains on the basis of tolerance tests was marred by a small number of B-strains; these included B.oralis, B. ochraceus and a few intermediate strains not biochemically conforming with recognized sub-species of B. fragilis, which gave tolerance patterns typical of B. melaninogenicus strains. The reference strains in the F-group were separated from the B-strains and M-strains by their tolerance patterns, although other F-strains gave similar patterns to B. melaninogenicus strains.

The most detailed separation between the test strains was obtained with the antibiotic disk resistance tests. There was no overlap of patterns obtained with

B. fragilis strains, B. melaninogenicus strains and F-strains when the complete series of antibiotic disks were included. However, B. oralis NP333 and B. ochraceus VPI2845, which were classified as B-strains on the basis of biochemical tests and lack of black pigment, gave antibiotic resistance patterns that closely resembled those obtained with B. melaninogenicus strains. Sutter & Finegold (1971) suggested that disks containing specified concentrations of colistin, erythromycin, kanamycin, neomycin, penicillin and rifampicin could be used for preliminaryidentification of gram-negative anaerobic bacilli. The results of the present study show that a significant number of strains of B. fragilis and B. melaninogenicus gave atypical results with one or more of these antibiotics. Resistance tests with neomycin 1000µg, kanamycin 1000µg, penicillin 1.5 units and rifampicin 15µg were, however, useful for group discrimination in a combined approach with tolerance tests and biochemical tests. The separation of B. fragilis strains and B. melaninogenicus strains on the basis of antibiotic resistance patterns did not correlate with the biochemical separation of B. fragilis into sub-species and B. melaninogenicus into saccharolytic and asaccharolytic groups.

In this series of biochemical, tolerance and antibiotic resistance tests, the reference strains gave consistent results that correlated well with other published work, except that the type-strain of

B. fragilis ss. vulgatus ATCC8482 (Holdeman & Moore, 1974), was significantly different from B. fragilis ss. vulgatus NCTC10583 and its related cluster of laboratory isolates. Strain ATCC8482 appeared to be more closely related to B. fragilis ss. distasonis ATCC8503.

Dye tolerance tests (Suzuki et al., 1966), studies of growth in the presence of bile salts (Shimada et al., 1970) and antibiotic disk resistance tests (Finegold et al., 1967) have been used for preliminary identification of gram-negative anaerobic bacilli. If these approaches were used separately, a significant number of strains in the present series would be wrongly classified because of atypical results in individual tests. Most of these problems can be overcome by using a short combined set of tolerance tests and antibiotic disk resistance tests and a small number of biochemical tests. The occurrence of atypical results is then clear.

The set of tests shown in Table 3.13 will identify the major species and the sub-species of B. fragilis and B. melaninogenicus and enable a preliminary allocation of the intermediate strains into the appropriate main group. The tolerance tests and antibiotic disk resistance tests are not used in a sequential manner but as a single combined set of tests for the provisional identification of strains. They are of confirmatory value for typical B. melaninogenicus strains

TABLE 3.13. Scheme for the identification of gram-negative anaerobic bacilli

	Result obtained in test															
Pigment production†	–												+			
Antibiotic disc resistance tests:																
neomycin (1000 μg)	R‡			S				S					S‡			
kanamycin (1000 μg)	R			R				S					R			
penicillin (1·5 units)	R			S				S					S‡			
rifampicin (15 μg)	S			S			R		S				S			
Tolerance tests:																
taurocholate	+			I			I	+	I				I			
deoxycholate	I			I			I	+	I				I			
Victoria blue 4R	+	I	+	+	I		+	+	+	+			I			
ethyl violet	I	I	+	I	I		I	+	+	+			I			
Biochemical tests:																
indole production	–	+	+	–	–	–	–	–	+	–	+	–	–	+	+	
digestion of gelatin	–	–	+	+	+	–	–	+	–	–	–	–	+	+	+	
hydrolysis of aesculin	+	+	+	+	+ or –	+	+	–	–	+	–	+	+ or –	–	–	
Fermentation of:																
glucose	+	+	+	+	+	+	+	…§	…	…	…	…	+	+	–	
rhamnose	–	+	+	+	+	+	–	…	…	…	…	…	…	…	…	
trehalose	–	+	+	+	–	–	–	…	…	…	…	…	…	…	…	
mannitol	–	–	+	–	–	–	–	…	…	…	…	…	…	…	…	
	B. fragilis												*B. melaninogenicus*			
	ss. *fragilis*	ss. *thetaiotaomicron*	ss. *ovatus*	ss. *distasonis*	ss. *vulgatus*	*B. oralis*	*B. ochraceus*	*B. corrodens*	*F. necrophorum*	*F. necrogenes*	*F. polymorphum*	*L. buccalis*	ss. *melaninogenicus*	ss. *intermedius*	ss. *asaccharolyticus*	

† Pigment production (black colonies) observed on lysed blood agar after anaerobic incubation for up to 7 days.
‡ Occasional strains may give anomalous results.
§... Results of these tests are not of primary importance in the identification of these species.
Note: This gives sets of results that distinguish the species. The scheme should not be regarded as a sequential key.

but are important for the early detection of strains that develop pigment only after prolonged incubation. This Table is based principally upon the results that were obtained with reference strains. However, Fusobacterium spp., B. fragilis ss. distasonis and ss. ovatus, and B. melaninogenicus ss. melaninogenicus were not adequately represented in the laboratory isolates. The validity of the patterns given for some of the latter species was the subject of subsequent investigation (Chapters 4 and 5). Holdeman & Moore (1974) stressed the need for further study of the B. fragilis-like organisms that they considered to be a continuum of variants with clusters of strains that had been designated sub-species. The present investigation confirmed that clusters of strains closely resemble reference strains of recognized sub-species with a number of intermediate strains that differ from the presently accepted sub-species.

CHAPTER 4

CHARACTERIZATION AND CLASSIFICATION OF *BACTEROIDES MELANINOGENICUS* AND RELATED SPECIES

The characteristics and interrelationships of the three subspecies of *Bacteroides melaninogenicus* (ss. *melaninogenicus*, ss. *intermedius* and ss. *asaccharolyticus*), *B. oralis* and *B. ochraceus* were investigated in studies with 175 strains of *B. melaninogenicus*, 17 strains of *B. oralis* and six strains of *B. ochraceus*. Results were obtained in a series of biochemical, tolerance and antibiotic disk resistance tests and by the gas-liquid chromatographic analysis of the acid end products of metabolism.

Materials and Methods

Organisms

The following strains were studied:
B. melaninogenicus NCTC nos. 9336, 9337 and 9338; ATCC15930; WAL2721 and WAL 2724; GUI1011 and GUI1034; VPI4196; G11a-d, P11a-k, AB13a-f, UJB13a-c and D13a-f; 2296, 3502 and 3586; T588 and 127/2845. In addition, five strains were isolated from clinical specimens at the Royal Infirmary, Edinburgh; seven strains were isolated from oral infections and 94 strains from subgingival dental plaque in the Microbial Pathogenicity Research Laboratory, Department of Bacteriology, Edinburgh University Medical School; 19 strains were isolated in the same laboratory from specimens of

faeces and 12 from high vaginal swabs sent to the Bacteriology Laboratory, Royal Infirmary, Edinburgh; 19 strains were isolated from high vaginal swabs at the Central Microbiological Laboratories, Western General Hospital, Edinburgh.

B. oralis J1, 7CM and 30; VPI5832 and VPI7570A; NP333 and 11 strains isolated from subgingival dental plaque in the Microbial Pathogenicity Research Laboratory.

B. ochraceus VPI2845; 1956C and 2467B; 10, 79B and 73.

Forty-one strains of B. melaninogenicus and one strain each of B. oralis and B. ochraceus were isolated in the earlier studies with clinically-important gram-negative anaerobic bacilli (Chapter 3). B. melaninogenicus strains WAL2721, WAL2724, GUI1011, GUI1034 and ATCC15930, B. oralis strains VPI5832, VPI7570A, J1, 7CM and 30, and B. ochraceus strains 1956C and 2467B were studied as part of the collaborative study instigated by the International Committee for Systematic Bacteriology (ICSB) Taxonomic Sub-Committee on gram negative anaerobic rods (Appendix VII; Finegold & Barnes, 1977).

Characterization of strains

Ninety-two strains of B. melaninogenicus, 15 strains of B. oralis and the six strains of B. ochraceus were subjected to the following series of morphological, biochemical, tolerance and antibiotic disk resistance tests (for details of methods, see Chapter 3).

Morphological and biochemical tests. Microscopic and colonial morphology; haemolysis on blood agar; pigment

production; motility; lipase activity; oxidase test; catalase test; H_2S production; indole production; gelatinase test; aesculin hydrolysis; dextran hydrolysis; fermentation of glucose, lactose, sucrose, maltose, rhamnose, trehalose and mannitol.

Tolerance tests. Growth in the presence of(i) the bile salts sodium taurocholate, sodium deoxycholate, separately and in combination, and (ii) the dyes brilliant green, Victoria blue 4R, gentian violet and ethyl violet (separately).

Antibiotic disk resistance tests. Resistance to disks containing neomycin 1000µg and 10µg, kanamycin 1000µg and 30µg, penicillin 1.5 units, methicillin 10µg, erythromycin 60µg, colistin 10µg, rifampicin 15µg, lincomycin 2µg, clindamycin 2µg, bacitracin 0.1 unit, vancomycin 15µg, chloramphenicol 10µg, tetracycline 10µg, and metronidazole 5µg.

The remaining 85 strains were identified by the following short, combined scheme derived from the above series of tests as a result of the studies described in Chapter 3; pigment production; antibiotic disk resistance tests with neomycin 1000µg, kanamycin 1000µg, penicillin 1.5 units, and rifampicin 15µg disks; separate tolerance tests with sodium taurocholate, sodium deoxycholate, Victoria blue 4R and ethyl violet; biochemical tests for the production of indole, digestion of gelatin, and hydrolysis of aesculin; and the fermentation of glucose, rhamnose, trehalose and

mannitol.

GLC analysis of short-chain fatty acids

One hundred and two strains of B. melaninogenicus, 13 strains of B. oralis and six strains of B. ochraceus were subjected to gas-liquid chromatographic (GLC) analysis of their short-chain fatty acid products of metabolism. The GLC analyses were performed by A. G. Deacon; for details of the media, equipment and methods see Deacon A.G. (1977) and Holbrook, Duerden & Deacon (1977). The test strains were grown in a proteose peptone medium (PPYS and PPYSG) and the analysis of volatile and non-volatile (methylated) acids were performed using a Pye-Unicam series 104 gas chromatograph fitted with flame-ionization detectors. The columns were packed with Chromosorb 101. The approximate concentration values of acids for test samples were calculated by comparing the corrected peak heights of the test samples with those of the appropriate acid standards. Results were recorded as follows:

Volatile acids. ++, concentration value > 10µmol/ml; +, 1.1-10µmol/ml; tr(trace), 0.2-1.0µmol/ml; -, < 0.2 µmol/ml.

Lactic and succinic acids. ++, concentration value > 20µmol/ml; +, 10-20µmol/ml; tr,1-9µmol/ml; -, < 1µmol/ml.

Results

The test strains were provisionally allocated to one

of three species:

(1) Strictly anaerobic strains that produced black or brown pigmented colonies when grown on lysed-human-blood agar for up to one week were assigned to the species B. melaninogenicus. The three subspecies of B. melaninogenicus were distinguished by tests for the production of indole and the fermentation of glucose:

(a) Strains that did not produce acid from glucose were labelled ss. asaccharolyticus (46 strains). These included strains NCTC9337, AB13a-f, 2296, 3502 and 3586.

(b) Strains that produced acid from glucose and produced indole were labelled ss. intermedius (78 strains). These included strains NCTC nos. 9336 and 9338, G11a-d, P11a-k, UJB13a-c, and D13a-f, 127/2845 And T588.

(c) Strains that produced acid from glucose but did not produce indole were labelled ss. melaninogenicus (53 strains). These included strains ATCC15930, WAL2721 and WAL2724, GUI1011 and GUI1034, VPI4196 and VPI7570A, and 30.

(2) Strictly anaerobic, non-pigmented strains that were inhibited by bile salts were assigned to the species B. oralis (15 strains). Two strains (VPI7570A and 30) were submitted to us as strains of B. oralis but produced black or brown pigmented colonies and were transferred to the B. melaninogenicus ss. melaninogenicus group (above).

(3) Non-pigmented strains that were able to grow in 10% CO_2 in air were assigned to the species B. ochraceus (6 strains).

Cell morphology

All strains were gram-negative bacilli or cocco-bacilli and many were pleomorphic. B. melaninogenicus and B. oralis strains were predominantly cocco-bacilli or short bacilli with rounded ends, often arranged in short chains. A few longer rods were seen and some strains were highly pleomorphic. B. ochraceus strains were long, slender bacilli with rounded or tapered ends and often with a central oval swelling.

Colony morphology

The colonies of B. melaninogenicus ss. asaccharolyticus were 0.5mm diameter, round, convex and opaque. Individual colonies were light grey after incubation for 48h but confluent growth was sometimes brown and appeared moist; colonies were 1mm in diameter, dark brown or black after further incubation on lysed-blood agar. After one week, some strains produced very small variant colonies that were light brown in colour.

Colonies of B. melaninogenicus ss. intermedius were 1-2mm in diameter, round, convex and opaque. After incubation for 48h individual colonies were grey but confluent growth was becoming black; all colonies were black after further incubation

Colonies of B. melaninogenicus ss. melaninogenicus were 1-2mm in diameter, round, convex and opaque.

After incubation for 48h they were typically light grey, becoming brown after further incubation. The pigmentation varied between strains from light brown to almost black. The colonies of many strains had a light brown annulus around a dark brown centre.

All strains of B. melaninogenicus ss. melaninogenicus and ss. intermedius produced zones of complete or incomplete haemolysis on human-blood agar; the development of these zones paralleled the development of pigment. Many strains of B. melaninogenicus ss. asaccharolyticus were similarly haemolytic but some strains of this subspecies were non-haemolytic and, therefore, non-pigmented on human-blood agar although all of these strains produced black pigment when grown on lysed-blood agar.

Colonies of B. oralis were 1-2mm in diameter, round, convex, opaque and grey; they tended to coalesce. After incubation for 7 days the colonies of some strains (including VPI5832 and NP333) became light brown and were difficult to distinguish from the lighter-pigmented strains of B. melaninogenicus ss. melaninogenicus.

B. ochraceus strains typically produced two colony types: (a) 1mm diameter, round or with an irregular edge, smooth, opaque and blue-grey; (b) 1mm in diameter, rhizoid, granular and ochre in colour.

Biochemical tests

None of the strains tested produced oxidase or catalase;

all strains produced H_2S although some strains produced only small amounts. The results of the other biochemical tests are shown in Table 4.1. Strains of B. melaninogenicus ss. asaccharolyticus (46) did not ferment any of the test carbohydrates and did not hydrolyse aesculin or dextran; most strains produced indole and digested gelatin; only a few strains produced lipase. All strains of B. melaninogenicus ss. intermedius (78) fermented glucose, sucrose, and maltose, but not rhamnose; a few strains fermented lactose but only two strains fermented mannitol and one strain fermented trehalose. All strains produced indole and only one strain failed to digest gelatin; only three strains hydrolysed dextran and one strain hydrolysed aesculin. Many strains produced lipase. B. melaninogenicus ss. melaninogenicus strains (53) fermented glucose but not trehalose or mannitol; only one strain failed to ferment maltose, another failed to ferment sucrose, and two strains failed to ferment lactose. Two strains fermented rhamnose. The strains assigned to this subspecies did not produce indole. In tests for the hydrolysis of aesculin and dextran and the digestion of gelatin this subspecies did not give a constant pattern; some strains gave negative results in all three tests whereas other strains gave positive results in one, two or all three tests. There was no correlation between the results obtained in these three separate tests. A few strains produced lipase.

TABLE 4.1. Results of biochemical tests with 198 test strains

Test result	Number of positive strains/number tested in each group				
	B. melaninogenicus				
	ss. *asaccharolyticus*	ss. *intermedius*	ss. *melaninogenicus*	*B. oralis*	*B. ochraceus*
Growth in air + CO_2	0/46	0/78	0/53	0/15	6/6
Pigment production (black/brown)	46/46	78/78	53/53	0/15	0/6
Indole production	42/46	78/78	0/53	0/15	0/6
Aesculin hydrolysis	0/46	1/78	17/53	14/15	5/6
Gelatin digestion	45/46	77/78	30/53	7/15	0/6
Dextran hydrolysis	0/44†	3/75‡	28/53	5/15	6/6
Lipase production	5/22*	31/39	9/26	0/13	0/6
Fermentation of:					
glucose	0/46	78/78	53/53	15/15	6/6
lactose	0/29	8/39	24/26	13/13	6/6
sucrose	0/29	39/39	25/26	13/13	6/6
maltose	0/29	39/39	25/26	13/13	6/6
rhamnose	0/46	0/78	2/53	8/15	0/6
trehalose	0/46	1/78	0/53	0/15	0/6
mannitol	0/46	2/78	0/53	0/15	0/6

* Seven strains failed to grow on test medium.
† Two strains failed to grow on test medium.
‡ Three strains failed to grown on test medium.

B. oralis strains (15) fermented glucose, lactose, sucrose, and maltose, but not trehalose or mannitol; eight strains fermented rhamnose. All except two strains hydrolysed aesculin; some strains hydrolysed dextran and some strains digested gelatin. None of the strains produced indole or lipase.

B. ochraceus strains (6) fermented glucose, lactose, sucrose and maltose, but not rhamnose, trehalose or mannitol. All strains hydrolysed dextran and only one strain failed to hydrolyse aesculin. None of the strains digested gelatin or produced indole or lipase.

Tolerance tests

The results of these tests are shown in Table 4.2. All the test strains of *B. melaninogenicus* and *B. ochraceus* and all except one strain of *B. oralis* (7CM) were inhibited by bile salts. All strains were inhibited by ethyl violet, gentian violet and brilliant green except one strain of *B. oralis* (7CM) that grew in the presence of ethyl violet and one strain each of *B. melaninogenicus* ss. *melaninogenicus* and ss. *asaccharolyticus* that grew in the presence of brilliant green. The atypical strain of *B. oralis* (7CM) gave a pattern of results in biochemical, tolerance and antibiotic disk resistance tests that was more typical of a strain of the *B. fragilis* group.

Antibiotic disk resistance tests

The results of these tests are shown in Table 4.3. All

TABLE 4.2. Results of tolerance tests with 198 test strains

Test result	Number of positive strains/number tested in each group				
	B. melaninogenicus				
	ss. *asaccharolyticus*	ss. *intermedius*	ss. *melaninogenicus*	*B. oralis*	*B. ochraceus*
Growth on basal medium	44/46	75/78	53/53	14/15	6/6
Growth on basal medium plus:					
taurocholate (0·5%)	0/44	0/75	0/53	1/14	0/6
deoxycholate (0·1%)	0/44	0/75	0/53	1/14	0/6
taurocholate (0·5%) and deoxycholate (0·1%)	0/28	0/39	0/26	1/13	0/6
Victoria blue 4R (1/80 000)	7/44	4/75	6/53	7/14	4/6
ethyl violet (1/80 000)	0/44	0/75	0/53	1/14	0/6
gentian violet (1/100 000)	0/28	0/39	0/26	0/13	0/6
brilliant green (1/80 000)	1/28	0/39	1/25	0/13	0/6

strains of the anaerobic species were sensitive to metronidazole and one strain of B. ochraceus was also sensitive to this agent. All except one strain of B. melaninogenicus ss. intermedius and two strains of ss. melaninogenicus were resistant to kanamycin (1000µg disk). Most strains were sensitive to neomycin, but 19 strains of B. melaninogenicus ss. asaccharolyticus, five strains of ss. intermedius, three strains of ss. melaninogenicus and one strain of B. oralis were resistant. Most strains were sensitive to penicillin, but four strains of B. melaninogenicus ss. asaccharolyticus, six strains of ss. intermedius, 14 strains of ss. melaninogenicus, and two strains of B. oralis were resistant. The results obtained with methicillin and penicillin disks were the same for strains tested with both disks, except that two strains of B. ochraceus were sensitive to penicillin but resistant to methicillin. Almost all strains of B. ochraceus, B. oralis, B. melaninogenicus ss. melaninogenicus and ss. intermedius were resistant to the vancomyin disk but most strains of ss. asaccharolyticus were sensitive,

GLC analysis

The results of these tests are shown in Table 4.4. All strains produced acetic acid. Strains of B. melaninogenicus ss. asaccharolyticus produced n-butyric acid and lactic acid as major products; nine strains

TABLE 4.3. Results of antibiotic disk resistance tests with 198 test strains

Test result		Number of positive strains/number tested in each group				
		B. melaninogenicus				
		ss. *asaccharolyticus*	ss. *intermedius*	ss. *melaninogenicus*	*B. oralis*	*B. ochraceus*
Neomycin (1000 μg)	S	27/46	73/78	50/53	14/15	6/6
Kanamycin (1000 μg)	R	46/46	77/78	51/53	15/15	6/6
Penicillin (1·5 unit)	S	42/46	72/78	39/53	13/15	6/6
Methicillin (10 μg)	S	25/29	37/39	20/26	11/13	4/6
Erythromycin (60 μg)	S	29/29	39/39	26/26	13/13	6/6
Tetracycline (10 μg)	S	24/29	35/39	24/26	12/13	6/6
Colistin (10 μg)	S	12/29	38/39	18/26	4/13	0/6
Rifampicin (15 μg)	S	46/46	78/78	51/53	15/15	6/6
Lincomycin (2 μg)	S	29/29	39/39	25/26	13/13	6/6
Clindamycin (2 μg)	S	29/29	39/39	25/26	13/13	6/6
Bacitracin (0·1 unit)	R	4/29	17/39	25/26	12/13	6/6
Vancomycin (15 μg)	R	9/29	38/39	24/26	13/13	5/6
Chloramphenicol (10 μg)	S	28/29	39/39	26/26	13/13	6/6
Metronidazole (5 μg)	S	29/29	39/39	26/26	13/13	1/6

R, resistant; S, sensitive.

TABLE 4.4. Results of GLC analysis of acid end-products from 121 test strains

Acid concentration		Number of strains in each group*				
		B. melaninogenicus				
		ss. *asaccharolyticus* (18†)	ss. *intermedius* (36†)	ss. *melaninogenicus* (48†)	*B. oralis* (13)	*B. ochraceus* (6)
Acetic	++	13	31	44	9	2
	+	5	5	4	4	4
Propionic	++	5	1			
	+	13	16	19	4	2
	tr		11	21	5	1
	−		8	8	4	3
iso-Butyric	+	16	4			
	tr	2	27	16		
	−		5	32	13	6
n-Butyric	++	7				
	+	11	2			
	tr		1			
	−		33	48	13	6
iso-Valeric	++	1				
	+	17	18	11		
	tr		18	27	5	1
	−			10	8	5
n-Valeric	−	18	36	48	13	6
Lactic	++	17	19	11		
	+	1	11	14	3	
	tr		6	21	2	1
	−			2	8	5
Succinic	++	2	32	38	7	3
	+	5	1	10	6	3
	tr	2	1			
	−	9	2			

* Numbers in parentheses indicate number of strains tested in each group.
† Seven strains of *B. melaninogenicus* ss. *intermedius*, five strains of ss. *melaninogenicus* and one strain of ss. *asaccharolyticus* were tested in medium (without added glucose) only.

did not produce succinic acid but seven strains produced significant amounts of this acid. The two strains that produced > 10µmol/ml of succinate had been incubated for 7 days to obtain good growth; they also produced larger quantities of acetic, n-butyric and lactic acids. All strains of ss. asaccharolyticus produced significant amounts of propionic acid and smaller amounts of iso-butyric and iso-valeric acids. In general, the saccharolytic organisms i.e. B. melaninogenicus ss. intermedius and ss. melaninogenicus, B. oralis and B. ochraceus, produced succinic acid as a major product but did not produce n-butyric acid. However, three strains assigned to the B. melaninogenicus ss. intermedius group consistently produced n- butyric acid but not succinic acid in repeated tests. Strains of B. melaninogenicus ss. intermedius and ss. melaninogenicus produced variable amounts of lactic acid; propionic, iso-butyric, and iso-valeric acids were minor products of most strains. None of the test strains of B. oralis produced iso-butyric acid; propionic, iso-valeric and lactic acids were minor products of some strains. In general, the test strains of B. ochraceus produced significant amounts of acetic and succinic acids only, but they produced smaller amounts of acetic acid than the other test strains. None of the test strains of B. ochraceus produced iso-butyric acid but three strains produced propionic acid and one strain produced iso-valeric and lactic acids as minor products.

Identification of strains from different sites

Strains of B. melaninogenicus were isolated from the mouth, the vagina, and from faeces; almost all the oral strains were identified as ss. melaninogenicus or ss. intermedius whereas all three subspecies were regularly isolated from the vagina and from faeces. All the strains of B. oralis and B. ochraceus were isolated from the mouth except for one strain of B. oralis that was isolated from a putrid lung abscess.

Discussion

The results of this part of the study confirm the separation of the pigmented bacteroides strains into three broad groups. Strains of B. melaninogenicus ss. asaccharolyticus are clearly distinguished by the failure to ferment carbohydrates and the production of significant amounts of n-butyric acid; they produce indole and are proteolytic. Werner et al. (1971) and Williams et al. (1975) did not detect the production of succinic acid by any of their test strains of the asaccharolytic group. The production of minor quantities of succinate by seven asaccharolytic strains under the conditions of our tests is consistent with the findings of Holdeman & Moore (1972). The ICSB Taxonomic Sub-committee on gram-negative anaerobic rods has clarified the taxonomic status of the asaccharolytic strains and has suggested that B. melaninogenicus ss. asaccharolyticus is sufficiently different from the other subspecies to be regarded as

a separate species designated B. asaccharolyticus (Finegold & Barnes, 1977).

The saccharolytic strains of B. melaninogenicus could be divided into two clear groups on the basis of the series of tests. The strains that produced indole appeared to be a fairly homogeneous group that corresponds with the subspecies intermedius (Holdeman & Moore, 1974). Strains that failed to produce indole were designated B. melaninogenicus ss. melaninogenicus; many strains were easily separated from strains of ss. intermedius by their distinctive colony morphology (See Results) that has also been observed by Lambe & Jerris (1976). GLC analysis of the acid end-products of metabolism were not helpful in differentiating between individual strains of these two subspecies. There were differences between the median concentration of acid end-products but there was considerable overlapping between the ranges of concentrations obtained with the two subspecies. B. melaninogenicus ss. melaninogenicus did not form a homogeneous group but defined sub-groups could not be detected on the basis of the results of biochemical, tolerance or antibiotic disk resistance tests.

Some of the non-pigmented strains of bacteroides that were identified as B. oralis closely resembled strains of B. melaninogenicus ss. melaninogenicus in the series of tests adopted in this study. They were differentiated only by their failure to produce

pigment; this distinction was even less clear with strains of B. melaninogenicus ss. melaninogenicus that produced brown pigmented colonies only slowly and strains of B. oralis that gave buff-coloured colonies after prolonged incubation. However, some strains identified as B. oralis grew in the presence of Victoria blue 4R and fermented rhamnose; these strains were more clearly distinguished from B. melaninogenicus ss. melaninogenicus. As a result of the similarities between some strains of B. melaninogenicus ss. melaninogenicus and B. oralis, the relationship between these two groups has been the subject of some taxonomic debate that remains unresolved (Finegold & Barnes, 1977). Terada et al. (1976) have suggested that B. oralis and B. ruminicola might be closely related groups that have many similarities with B. melaninogenicus ss. melaninogenicus. In a numerical taxonomic study, Sundqvist (1976) found close similarities between strains of B. melaninogenicus ss. melaninogenicus, B. oralis, and B. ruminicola; he did not regard pigment production as a good basis for differentiation between B. melaninogenicus ss. melaninogenicus and B. oralis. It remains to be decided whether the ability to assimilate haemoglobin and produce brown pigmented colonies when grown on media containing blood is a valid criterion for dividing these very similar strains of B. melaninogenicus ss. melaninogenicus and B. oralis

into two species.

B. ochraceus strains were clearly differentiated from the other test strains; they were able to grow in air plus CO_2 and were resistant to metronidazole (except for one aberrant strain), an antimicrobial agent to which only anaerobic bacteria are susceptible (Prince et al., 1969). The status of these strains within the genus Bacteroides requires further investigation but on the present evidence it would seem that they should be removed from the genus.

Most of the test strains were readily identified from the pattern of results obtained in the short, combined set of biochemical, tolerance and antibiotic disk resistance tests. The results in tolerance and antibiotic disk resistance tests distinguish the groups of bacteroides studied in this investigation from other gram-negative anaerobic bacilli. These tests are used as a combined set to prevent an anomolous result in any single test leading to an incorrect identification. The results of the biochemical tests provide the basis for the differentiation between the species and subspecies within this group of bacteroides.

CHAPTER 5

CHARACTERIZATION OF AN EXTENDED RANGE OF SPECIES OF GRAM-NEGATIVE ANAEROBIC BACILLI

The methods for the characterization of gram-negative anaerobic bacilli by conventional bacteriological tests were initially developed and evaluated in tests with the reference strains available at the beginning of the study, together with a series of faecal isolates from normal healthy adults and isolates from clinical material (Chapter 3). Therefore, the identification scheme based upon a short combined set of conventional tests (Table 3.13) was designed for the identification of the five subspecies of B. fragilis, three subspecies of B. melaninogenicus, B. oralis, B. corrodens and several Fusobacterium spp. The range of species studied was extended by the investigations of strains of B. melaninogenicus and related species and some of the initial results were validated by the study of more strains in this group of species (Chapter 4).

During these studies, however, several reference strains that were classified as additional species of Bacteroidaceae became available. Some were recognised type-strains that had been deposited recently in culture collections (N.C.T.C. and A.T.C.C.) and others were sent by colleagues in other laboratories. Twenty two strains were received for investigation as part of an I.C.S.B. collaborative study of the relationship between B. melaninogenicus ss. melaninogenicus, B. oralis

and B. ruminicola. These included strains that were provisionally designated B. oralis, B. ruminicola, B. disiens and B. bivius. The latter two species were described and named by Holdeman & Johnson (1977). In addition representative strains of the putative species of B. splanchnicus (Werner et al., 1975), B. uniformis, B. variabilis and B. eggerthii (Holdeman & Moore, 1974) that shared similarities with B. fragilis, and the reference strains of F. varium and B. multiacidus (Mitsuoka et al., 1974) were obtained from culture collections or from colleagues.

The strains were studied in the set of conventional tests already developed although the number of carbohydrate fermentation tests was increased to include xylose and arabinose because other studies (L. V. Holdeman, personal communication) had indicated their value in the separation of B. oralis and B. ruminicola (arabinose and xylose fermented).

The aims of this investigation were:
(i) to study the properties of these strains in relation to existing species and assess the validity of their species status, and (ii) to extend the short identification scheme to include these additional species.

Materials and Methods

The following 32 strains were studied:
B. splanchnicus NCTC nos. 10825 and 10826; B. uniformis VPI11227; B. variabilis VPI11368; B. eggerthii NCTC11155;

B. oralis VPI9958*, VPI8906D*, WAL3281*, B1/15, B1.3/54, 1000, 1210* and 1221*; B. ruminicola C12*, B56029*, B38024*, and B38080*; B. disiens VPI8057* and VPI7852*; B. bivius VPI6318*, VPI6822*, VPI5540*, VPI7880*, 07073*, B3/36 and B3/68; B. melaninogenicus ss. melaninogenicus WAL3030*, B56007* and B56020*; B. melaninogenicus ss. levii VPI3300; B. multiacidus NCTC10934; and F. varium NCTC10560.

*These 19 strains were studied as part of the I.C.S.B. collaborative study; strains VPI5540, VPI7880, WAL3030 and 07073 were originally submitted as strains of B. oralis and strains B56007 and B56020 were submitted as strains of B. ruminicola (see Appendix VIII).

Characterization of Strains

The 32 test strains were submitted to the following series of morphological, biochemical, tolerance and antibiotic disk resistance tests (for details of methods, see Chapter 3).

Morphological and biochemical tests. Microscopic and colonial morphology; haemolysis on human-blood agar; pigment production on BM agar; motility; lipase activity; oxidase test; catalase test; H_2S production; indole production; gelatinase test; aesculin hydrolysis; fermentation of glucose, lactose, sucrose, maltose, rhamnose, trehalose, mannitol, arabinose and xylose. The broth medium for these tests was BM broth.

Tolerance tests. Growth in the presence of (i) the

bile salts sodium taurocholate and sodium deoxycholate, separately and in combination, and (ii) the dyes brilliant green, Victoria blue 4R, ethyl violet and gentian violet (separately).

Antibiotic disk resistance tests. Resistance to disks containing neomycin 1000µg, kanamycin 1000µg, penicillin 1.5 or 2 units, and rifampicin 15µg.

In addition reference and representative strains of the five subspecies of B. fragilis, B. melaninogenicus ss. intermedius and ss. melaninogenicus and B. asaccharolyticus were tested in fermentation tests with arabinose and xylose.

Results

The results of the tolerance and antibiotic disk resistance tests are shown in Table 5.1 and the results of the biochemical tests in Table 5.2.

Morphology. The colonial morphology of many of the test strains was essentially similar and of no help in distinguishing between them. After incubation for 2 days on blood agar, most strains produced small (0.5-1.5mm) circular, convex, semi-translucent, grey colonies that were either non-haemolytic or gave a narrow zone of incomplete haemolysis. Many strains showed incomplete haemolysis after incubation for 7 days. The 3 strains of B. melaninogenicus ss. melaninogenicus were indistinguishable from the other test strains after incubation for 2 days but produced dark brown pigmented colonies after incubation for 7 days on BM agar.

B. melaninogenicus ss. levii VPI3300 produced small, translucent, non-pigmented colonies after incubation for 2 days; the colonies became larger (1.5-3mm) and mucoid with prolonged incubation and began to develop black pigmented centres after 6-7 days. Pigmentation developed more quickly (3-4days) on BM agar. F. varium NCTC10560 produced pale colonies 1-2mm diameter, low convex or flat and rhizoid with an irregular edge, and B. multiacidus NCTC10934 produced medium-sized (1-2mm), circular, low convex or flat, smooth, pale colonies after incubation for 2 days, which became spreading, irregular and flat after 5-6 days.

There were few significant differences in microscopic morphology between the strains. Most were small pleomorphic gram-negative bacilli; smears prepared from growth on blood agar showed many cocco-bacilli, often in pairs or short chains, and short filamentous forms. This appearance was most marked with strains of B. oralis, B. ruminicola, B. bivius, B. disiens, B. melaninogenicus ss. melaninogenicus and ss. levii; the other species were more pleomorphic but predominantly bacillary. Films prepared from broth cultures were similar but less pleomorphic. Gram-stained smears prepared from B. oralis strain 1221 differed from other similar strains; they showed large gram-negative bacilli with many filaments and few cocco-bacilli. B. multiacidus NCTC10934 was a large regular gram-negative bacillus and F. varium

TABLE 5.1. Results obtained with 32 strains in tolerance and antibiotic disk resistance tests

Test	Patterns of results* obtained with the given strains											
Tolerance tests:												
taurocholate (T)	I	I	I	I	I	I	I	I	I	+	I	+
deoxycholate (D)	I	I	I	I	I	I	I	I	I	I	I	+
T + D	I	I	I	I	I	I	I	I	I	+	I	+
Victoria blue 4R	I	+	+	I	+	+	I	+	+	+	+	+
ethyl violet	I	I	I	I	I	I	I	I	I	+	+	+
gentian violet	I	I	I	I	I	I	I	I	I	I	+	+
brilliant green	I	I	I	I	I	I	I	I	I	I	+	+
Antibiotic disk resistance tests:												
neomycin 1000µg	S	S	R/S	R/S	S	R/S	S	R	R	R	S	S
kanamycin 1000µg	R	R	R	R	R	R	R	R	R	R	S	S
penicillin 2 units	S	S	S	S	R	R	R	R	S	R	S	S
rifampicin 15µg	S	S	S	S	S	S	S	S	S	S	R	R
Strains conforming to the given pattern	B38024 B56007 B56020 1000 WAL3030 VPI3300	VPI9958 VPI8906D WAL3281 VPI8057 B3/68	VPI6318	VPI7880	07073 C12 B56029 NCTC 10825 1210 1221	VPI6822	VPI7852 VPI5540 B1/15 B1.3/54	VPI11227 VPI11368 NCTC 10826 NCTC 11155	B3/36	B38080	NCTC 10934	NCTC 10560

* I = inhibited; + = growth; S = sensitive; R = resistant; R/S = reduced sensitivity.

NCTC10560 was a typical fusiform organism.

Tolerance tests. All the test strains except B. ruminicola B38080, B. multiacidus NCTC10934 and F. varium NCTC10560 were inhibited by the bile salts, both separately and combined, and by the dyes ethyl violet, gentian violet and brilliant green. The 3 strains of B. melaninogenicus ss. melaninogenicus, B. melaninogenicus ss. levii VPI3300, 3/8 strains of B. oralis, 2/7 strains of B. bivius, 1/3 strains of B. ruminicola and 1/2 strains of B. disiens were also inhibited by Victoria blue 4R but the remaining strains were not inhibited by this dye. Strain B38080 was tolerant of taurocholate, taurocholate plus deoxycholate, Victoria blue 4R and ethyl violet. F. varium NCTC10560 was tolerant of all the dyes and bile salts and B. multiacidus NCTC10934 was tolerant of four dyes but inhibited by the bile salts.

Antibiotic disk resistance tests. F. varium NCTC10560 and B. multiacidus NCTC10934 gave patterns of results that were typical of some members of the genus Fusobacterium. They were resistant to rifampicin but sensitive to the neomycin, kanamycin and penicillin disks. All of the other test strains were resistant to the kanamycin disk and sensitive to the rifampicin disk. The test strains of B. eggerthii, B. uniformis, and B. variabilis, B. splanchnicus NCTC10826, B. bivius B3/36 and strain B38080 were resistant to the neomycin disk but the remaining strains of B. oralis, B. ruminicola, B. melaninogenicus ss. melaninogenicus

TABLE 5.2. Results obtained with 32 strains in biochemical tests

Test	Patterns of results* obtained with the given strain																		
Pigment production	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-
Haemolysis	±	±	±	-	±	-	±	+/-	+/-	±	+	+	±	±	±	±	±	-	-
Indole production	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	+
Gelatin digestion	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Aescluin hydrolysis	+	+	±	+	+	+	+	+	-	-	-	+	+	+	+	+	±	-	-
Fermentation of:																			
glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-
sucrose	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	-
maltose	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	-
rhamnose	+	+	-	+	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-
trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
mannitol	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-
arabinose	+	-	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-
xylose	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-
Strains conforming to the given pattern	C12	B 56029	B 38024	VPI 9958	VPI 8906D WAL 3281 B1/5 B1.3/54 1000 1210 1221	WAL 3030	B 56007 B 56020	B 3/68 B 3/36	VPI 5540 VPI 7880 07073 VPI 6318 VPI 6822	VPI 8057 VPI 7852	VPI 3300	NCTC 10825	NCTC 10826	NCTC 11155	VPI 11368	VPI 11227	B 38080	NCTC 10934	NCTC 10560

* + = positive result; - = negative result; ± = weak result.

and ss. levii, B. bivius and B. disiens, and B. splanchnicus NCTC10825 were sensitive; 3 strains of B. bivius (VPI6318, VPI7880 and VPI6822) showed reduced sensitivity to the neomycin disk (R/S; zone size 14-16mm). The test strains of B. eggerthii, B. uniformis, B. variabilis and B. splanchnicus, 5 strains of B. oralis, 2 strains of B. ruminicola, 2 strains of B. bivius, one strain of B. disiens and strain B38080 were resistant to the penicillin disk; the remaining strains were sensitive.

Biochemical tests. None of the test strains produced lipase, oxidase or catalase and all produced small to moderate amounts of H_2S. All of the test strains except F. varium NCTC10560 digested gelatin. The strains of B. eggerthii, B. uniformis, B. variabilis, and B. splanchnicus produced indole and hydrolysed aesculin; the strains of B. bivius, B. disiens, B. melaninogenicus ss. levii and B. multiacidus gave negative results in both of these tests whereas the strains of B. ruminicola, B. oralis, B. melaninogenicus ss. melaninogenicus and strain B38080 hydrolysed aesculin but did not produce indole.

The test strains gave distinctive patterns of results in the carbohydrate fermentation tests. Strains of B. ruminicola fermented glucose, lactose, sucrose, maltose, rhamnose, arabinose and xylose except that strain B38024 did not ferment rhamnose and strain B56029 fermented xylose but not arabinose. B. oralis strains fermented glucose, lactose, sucrose and maltose

but not xylose or arabinose and only strain VPI19958 fermented rhamnose. The 3 strains of B. melaninogenicus ss. melaninogenicus fermented glucose, lactose, sucrose and maltose, and strains B56007 and B56020 also fermented mannitol, but B. melaninogenicus ss. levii VPI13300 only fermented glucose and lactose, weakly, after 5-7 days incubation. B. bivius strains fermented glucose, lactose and maltose and B. disiens strains only fermented glucose and maltose. The test strains B. eggerthii, B. uniformis, B. variabilis and B. splanchnicus fermented glucose, lactose, arabinose and xylose, except for B. splanchnicus NCTC10825 that failed to ferment xylose and arabinose. B. eggerthii NCTC11155 also fermented maltose and rhamnose, B. uniformis VPI11227 fermented sucrose, maltose and trehalose and B. variabilis VPI11368 fermented sucrose, maltose and rhamnose. Strain B38080 fermented all the carbohydrates except mannitol and trehalose. B. multiacidus NCTC10934 fermented all the carbohydrates except trehalose and F. varium NCTC10560 fermented only glucose.

Arabinose and xylose fermentation. Most strains of the B. fragilis subspecies tested fermented both arabinose and xylose, except B. fragilis ss. fragilis strains fermented xylose but not arabinose. Strains of B. melaninogenicus ss. melaninogenicus, ss. intermedius and B. asaccharolyticus fermented neither pentose.

B. ruminicola B38080. This strain was referred as a strain of B. ruminicola, but it is clear from the results obtained in this investigation that the strain

recovered from the lyophilised ampoule was a strain of B. fragilis ss. vulgatus. The strain received by Professor J. G. Collee and Mr. R. Brown in Edinburgh was different from the strain described in this Chapter and the results given in Appendix VIII may represent the true characteristics of this strain.

Discussion

The results obtained with the reference strains of B. splanchnicus, B. eggerthii, B. variabilis and B. uniformis are similar, in general, to the results obtained with the B. fragilis subspecies and they share many common properties. The results of antibiotic disk resistance tests conform with those of B. fragilis but all the representative strains were inhibited by taurocholate and taurocholate-plus-deoxycholate, whereas most strains of B. fragilis were tolerant of these bile salts. They are also distinguished from the B. fragilis subspecies and from each other by different patterns of results in fermentation tests. These differences are only small and may indicate a close relationship between some of the species.

From the results reported in this Chapter and the previous one (Chapter 4), it is clear that B. ruminicola, B. oralis, B. melaninogenicus ss. melaninogenicus, B. bivius and B. disiens are closely related species that differ in only a small number of tests. The relationships between the strains allocated to these species is discussed in Chapter 10 and Appendix VIII. Three strains submitted as B. oralis have been reported

here as B. bivius because their patterns of results more closely resemble those of the reference strains of B. bivius than other B. oralis strains.

Classically, B. melaninogenicus is distinguished by pigment production (Chapter 2) but it is now debatable whether pigment production has major taxonomic significance. In this Chapter, three strains that were submitted as strains of B. ruminicola (2) and B. oralis have been designated B. melaninogenicus ss. melaninogenicus because they produce pigment. The validity of this approach will be discussed in Chapter 10.

It is evident from the results reported in this Chapter that the different test species give patterns of results in the series of tests used that are readily recognisable and may be used as the basis for an expanded identification scheme for gram-negative anaerobic bacilli. This scheme is presented in the next Chapter (6).

CHAPTER 6

SCHEME FOR THE IDENTIFICATION OF GRAM-NEGATIVE ANAEROBIC BACILLI BY CONVENTIONAL BACTERIOLOGICAL METHODS

An important aim of the studies on the characterization of gram-negative anaerobic bacilli was to produce a scheme for the identification of these organisms by a relatively simple set of conventional bacteriological tests. Such a scheme could then be used to study the epidemiology of strains isolated from the normal flora and from infections, and would also be useful for the clinical diagnostic bacteriology laboratory.

The prototype scheme was presented in Chapter 3 (Table 3.13) based upon studies with many strains of B. fragilis but only small numbers of some other species. The extended characterization studies reported in Chapters 4 and 5 (i) validated some of the patterns that were based upon small numbers of strains, (ii) shown that the results obtained in certain tests with different strains of a single species may differ, but that this variation is not of taxonomic significance, and (iii) shown that some species that were not included in the original studies give characteristic patterns of results in the established tests but additional tests may be necessary to identify other species.

The following species have been studied: B. fragilis ss. fragilis, ss. vulgatus, ss. distasonis, ss. ovatus, ss. thetaiotaomicron, B. uniformis, B. variabilis, B. eggerthii, B. splanchnicus, B. ruminicola,

B. oralis, B. bivius, B. disiens, B. melaninogenicus ss. melaninogenicus, ss. intermedius, ss. levii, B. asaccharolyticus, B. corrodens, F. necrophorum, F. necrogenes, F. varium, F. polymorphum, L. buccalis and B. multiacidus. B. ochraceus and L. dentium were also studied, but they were not strict anaerobes and have been excluded from further consideration.

Representative and reference strains of these species have been subjected to the following tests (for details of methods see Chapters 3 - 5):

Morphological and biochemical tests. Microscopic and colonial morphology; haemolysis on human-blood agar; pigment production on lysed-human-blood agar or BM agar; motility; lipase activity; oxidase test; catalase test; H_2S production; indole production; gelatinase test; aesculin hydrolysis; nitrate reduction; fermentation of glucose, lactose, sucrose, maltose, rhamnose, trehalose, mannitol, arabinose and xylose.

Tolerance tests. Growth in the presence of sodium taurocholate and sodium deoxycholate (separately and combined), Victoria blue 4R, ethyl violet, brilliant green and gentian violet.

Antibiotic disk resistance tests. Resistance to disks containing metronidazole 5µg, neomycin 1000µg, kanamycin 1000µg, penicillin 1.5 or 2 units and rifampicin 15µg.

The results obtained with the different species are shown in Tables 6.1 and 6.2; the results of tolerance

TABLE 6.1. Patterns of results obtained in tolerance and antibiotic disk resistance tests with organisms of the Bacteroides - Fusobacterium group

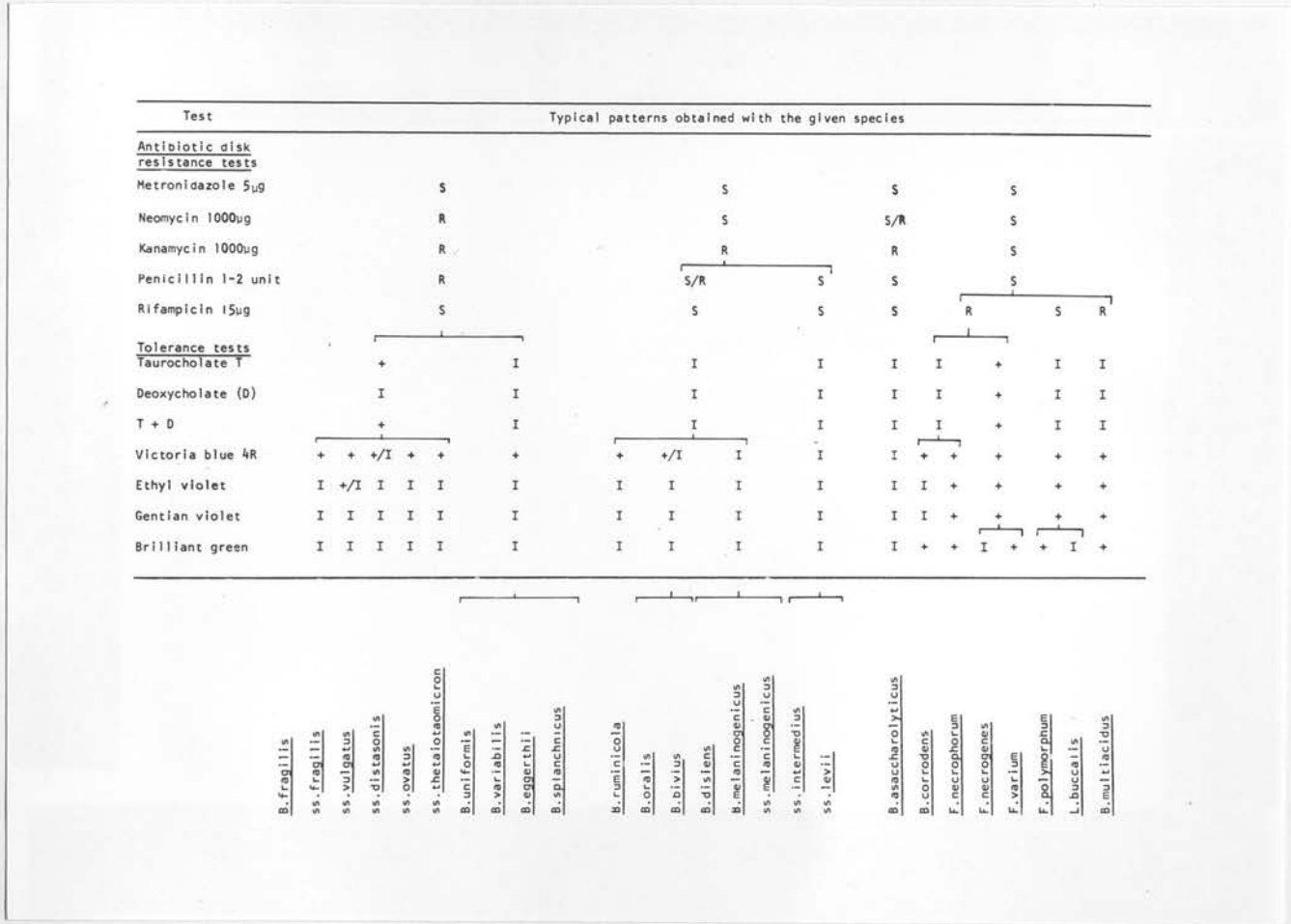


TABLE 6.2. Patterns of results obtained in biochemical tests with organisms of the Bacteroides - Fusobacterium group

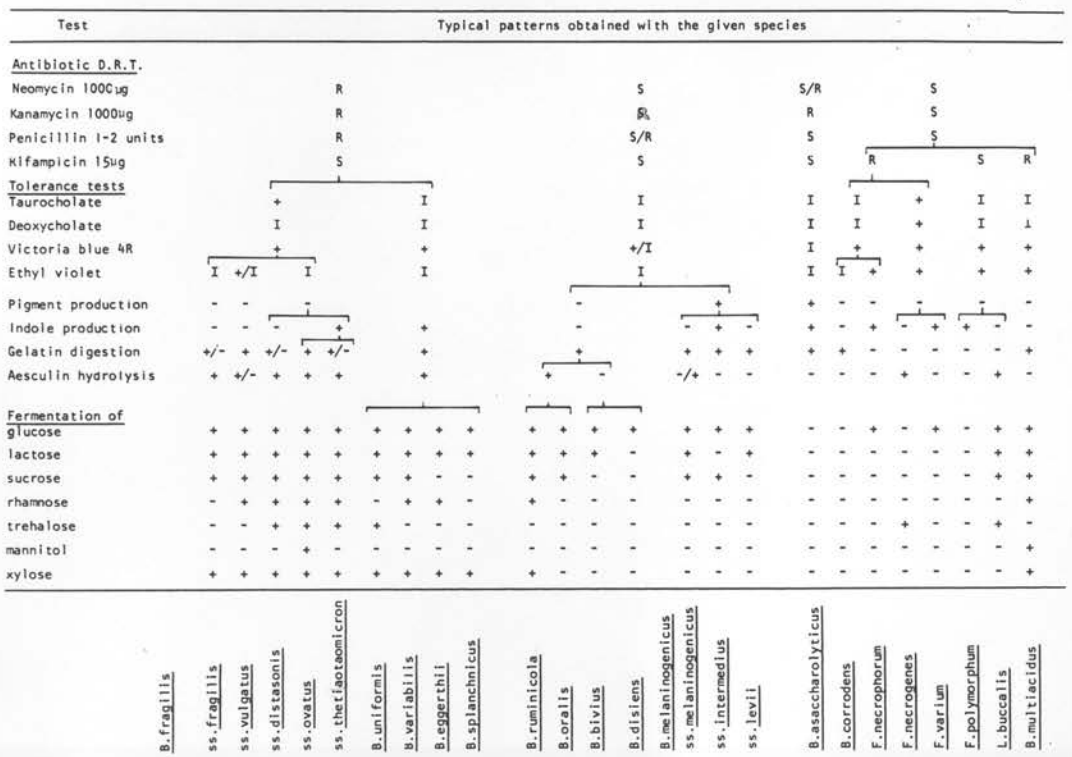
Test	B.fragilis	ss.fragilis	ss.vulgatus	ss.distasonis	ss.ovatus	ss.thetaiotaomicron	B.uniformis	B.variabilis	B.eggerthii	B.splanchnicus	B.ruminicola	B.oralis	B.bivius	B.disiens	B.melaninogenicus	ss.melaninogenicus	ss.intermedius	ss.levii	B.asaccharolyticus	B.corrodens	F.necrophorum	F.necrogenes	F.varium	F.polymorphum	L.buccalis	B.multiacidus
Pigment production						-						-				+			+	-	-			-		
Haemolysis						±						±				+			+	±	+			-		
Motility						-						-				-			-	-	-			-		
Lipase activity						-						-				-			+/-	-	+			-		
Oxidase test						-						-				-			-	-	-			-		
Catalase test						-						-				-			-	-	-			-		
H_2S production						+						+				+			+	+	+	-	+	-	-	+
Indole production									+			-				-			+	-	+	-	+	-	-	-
Gelatin digestion	+/-	+	+/-	+	+/-				+			+				+			+	+	-	-	-	-	-	+
Aesculin hydrolysis	+	-/+	+	+	+				+		+		-		-/+	-	-		-	-	-	+	-	+	-	-
Nitrate reduction	-	-	-	-	-				-		-		-		-	-	-		-	+	-	-	-	-	+	-
Fermentation of																										
glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	-	+	-	+	-	+	+	+
lactose	+	+	+	+	+	+	+	+	-	+	+	+	+	-		+	-	+	-	-	-	-	-	+	+	+
sucrose	+	+	+	+	+	+	+	+	-	-	+	+	-	-		+	+	-	-	-	-	-	-	+	+	+
maltose	+	+	+	+	+	+	+	+	-	-	+	+	+	+		+	+	-	-	-	+	-	-	+	+	+
rhamnose	-	+	+	+	+	+	-	+	+	-	+	-	-	-		-	-	-	-	-	-	-	-	-	-	+
trehalose	-	-	+	+	+	+	+	-	-	-	-	-	-	-		-	-	-	-	-	-	+	-	+	-	-
mannitol	-	-	-	+	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	+
arabinose	+	+	+	+	+	+	+	+	+	+	+	-	-	-		-	-	-	-	-	-	-	-	-	-	+
xylose	+	+	+	+	+	+	+	+	+	+	+	-	-	-		-	-	-	-	-	-	-	-	-	-	+

and antibiotic resistance tests are shown in Table 6.1 and the results of biochemical tests in Table 6.2. The patterns of results are derived principally from the study of reference strains but where many strains of the species have been studied, a typical composite pattern of results is given, and any results that are commonly variable between strains in a species are shown.

From the tables it can be seen that some of the tests have little or no discriminatory value. When these are excluded, a condensed set of tests can be selected that has particular discriminatory value and from which all the species studied can, in general, be identified. The set of tests comprises: pigment production; biochemical tests for indole production, aesculin hydrolysis, and gelatin digestion, and the fermentation of glucose, lactose, sucrose, rhamnose, trehalose, mannitol and xylose; antibiotic disk resistance tests with neomycin, kanamycin, penicillin and rifampicin; and tolerance tests with taurocholate, deoxycholate, Victoria blue 4R and ethyl violet.

The species can be divided into four broad groups - fragilis group, melaninogenicus-oralis group, asaccharolytic group and fusobacteria - on the basis of the results of the antibiotic resistance and tolerance tests, and these groups are subdivided into

TABLE 6.3. Scheme for the identification of clinically-important species in the Bacteroides - Fusobacterium group



species and subspecies by the results of the biochemical and fermentation tests (Table 6.3).

It is important to stress, as with the prototype scheme, that the tests in this more advanced identification scheme should be used as a complete set and that individual test results, especially in tolerance and antibiotic resistance tests, should not be viewed in isolation. Some strains that clearly belong to a particular species give atypical results in individual tests but these results are obvious when the set is analysed as a whole.

The remainder of the investigations reported in this thesis were the application of the prototype scheme, initially, and, later, the more advanced scheme described in this Chapter, to the identification of gram-negative anaerobic bacilli isolated from the normal flora and from cases of clinical infection.

CHAPTER 7

STUDIES WITH THE API ZYM SYSTEM FOR THE IDENTIFICATION OF GRAM-NEGATIVE ANAEROBIC BACILLI

The API ZYM system is a semi-quantitative micromethod for the assay of enzyme activity. It allows the rapid study of 19 enzyme reactions using very small samples of complex, unpurified material. The test strip is composed of 20 microtubes, the bottom of which forms a support that contains the substrate and buffer and allows contact between enzyme and substrate; enzyme activity is revealed by the addition of indicators to detect released products.

The system was designed to detect enzyme activity in specimens of tissue, blood, biological fluids, etc. and also in bacteria. An assay of particular "key" enzyme in bacteria may more closely reflect the genotypic profile of a micro-organism and lead to a more precise characterization than is obtained with conventional biochemical tests that detect the presence of complete, and often complex or multiple, pathways. This type of system has been used with some success in the identification of staphylococci (Joubert and Boussière, 1968), and Erwinia spp. (Peny, 1970), and the API ZYM system has been applied to the identification of non-haemolytic streptococci (Waitkins et al., 1977). Tharagonnet et al. (1977)

described their favourable experience with the API ZYM system in studies with gram-negative anaerobes. A small investigation was undertaken, therefore, to assess the results obtained by the API ZYM system with a set of strains of gram-negative anaerobic bacilli previously identified by the combined set of morphological, tolerance, antibiotic disk resistance and biochemical tests described in Chapters 3 - 6.

Materials and Methods

Organisms

Thirty six reference and referred strains, nine clinical isolates (C - strains) and 5 strains isolated from normal human faeces (S - strains) were studied. The 50 test strains are listed in Table 7 - 2, and the source of the referred and reference strains are given in Appendix IV: they included representative strains of the following species: B. fragilis ss. fragilis, ss. vulgatus, ss. distasonis, ss. ovatus and ss. thetaiotaomicron, B. splanchnicus, B. uniformis, B. variabilis, B. eggerthii, B. oralis, B. ruminicola, B. disiens, B. bivius, B. melaninogenicus ss. intermedius, ss. melaninogenicus and ss. levii, B. asaccharolyticus (B. melaninogenicus ss. asaccharolyticus), L. buccalis, F. necrophorum, F. necrogenes, F. varium, F. polymorphum and B. multiacidus. All the reference and referred strains were identified by the complete set of conventional tests (Chapters 4, 5 and 6) and the C - strains and S - strains were identified by the short set

TABLE 7.1. Enzymes detected by the API ZYM test

Test No.	Enzyme assayed	Substrate	pH
1	Control	None	
2	Alkaline phosphatase	2-naphthyl phosphate	8.5
3	Esterase (C4)	2-naphthyl butyrate	7.1
4	Esterase-lipase (C8)	2-naphthyl caprylate	7.1
5	Lipase (C14)	2-naphthyl myristate	7.1
6	Leucine aminopeptidase	L-leucyl-2-naphthylamide	7.5
7	Valine aminopeptidase	L-valyl-2-naphthylamide	7.5
8	Cystine aminopeptidase	L-cystyl-2-naphthylamide	7.5
9	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5
10	Chymotrypsin	N-benzoyl-DL-phenylalanine-2-naphthylamide	7.1
11	Acid phosphatase	2-naphthyl phosphate	5.4
12	Phosphoamidase	Naphthol-AS-Bl-phosphodiamide	5.4
13	α-Galactosidase	6-Br-2-naphthyl-αD-galactopyranoside	5.4
14	β-Galactosidase	2-naphthyl-βD-galactopyranoside	5.4
15	β-Glucuronidase	Naphthol-AS-βl-βD-glucuronate	5.4
16	α-Glucosidase	2-naphthyl-αD-glucopyranoside	5.4
17	β-Glucosidase	6-Br-2-naphthyl-βD-glucopyranoside	5.4
18	β-Glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide	5.4
19	α-Mannosidase	6-Br-2-naphthyl-αD-mannopyranoside	5.4
20	α-Fucosidase	2-naphthyl-αL-fucopyranoside	5.4

of tests (Chapters 8 and 9).

API ZYM Test

The enzymes detected by the API ZYM system are shown in Table 7.1. The naphthyl, naphthylamide and naphthol derivatives released by enzyme activity are detected by the addition of a single indicator system comprising two reagents:

Reagent A	Tris (hydroxymethyl) methylamine	250g
	Hydrochloric acid 37%	110ml
	Laurylsulphate	100g
	Distilled water to	1000ml
	pH = 7.6 - 7.8	
Reagent B	Fast Blue BB (Sigma No. F0250)	3.5g
	2-methoxyethanol to	1000ml

Both reagents were prepared in the laboratory and held at 4°C.

Each test strain was grown for 48h on blood agar in an anaerobic jar. The growth was removed from the surface of the plate and suspended in 2ml sterile distilled water to produce a very dense suspension. Two drops (0.06ml) of the suspension were added to each microtube in the API ZYM strip placed in a plastic chamber moistened with 5ml water. The chamber was covered and incubated at 37°C for 4h. After incubation one drop each of reagents A and B was added to each microtube. The intensity of the colour reaction after 5 min. was graded from 0 - 5 by comparison with the API ZYM colour chart (fig 7.1). The small differences

FIGURE 7.1

api
API ZYM
5 4 3 2 1 0
1 2 3 4 5 6 7 8 9 10
2 naphtyl phosphate
2 naphtyl butyrate
2 naphtyl caprylate
2 naphtyl myristate
L leucyl 2 naphtylamide
L valyl 2 naphtylamide
L cystyl 2 naphtylamide
N benzoyl DL arginine 2 naphtylamide
N benzoyl DL phenyl-alanine 2 naphtylamide
11 12 13 14 15 16 17 18 19 20
2 naphtyl phosphate
Naphtol AS BI phospho-diamide
6 Br 2 naphtyl αD galactopyranoside
2 naphtyl βD galacto-pyranoside
Naphtol AS BI βD gluco-ronic acid
2 naphtyl αD glucopyra-noside
6 Br 2 naphtyl βD gluco-pyranoside
1 naphtyl N acetyl βD glu-cosaminide
6 Br 2 naphtyl αD mannopy-ranoside
2 naphtyl αL fucopyranoside

in colour between, e.g., grades 4 and 5, did not appear to be significant; therefore, the analysis was simplified by grouping the results into only four grades: 0, 1 (weak reaction; grades 1 and 2 on the chart), 2 (moderate reaction; grade 3 on the chart) and 3 (strong reaction; grades 4 and 5 on the chart).

Results

The simplified API ZYM profiles obtained with the 50 test strains are shown in Table 7.2. Test no. 1 was a negative control for all tests, Substrates 5, 7 and 9 were not degraded by any test strain and only weakly-positive results were given by occasional strains with substrates 8, 10 and 19, except that *B. fragilis* ss. *distasonis* S14/3 and *B. ruminicola* B38024 gave strong reactions with substrate 10. With the remaining 14 substrates a wide variety of profiles was obtained and 45 different profiles were obtained with the 50 test strains, although some differences were only minor. The profiles were allocated to 12 groups (Table 7.2, groups a-l) in which the profiles appeared to share many close similarities; however, some groups contained only a single strain that gave a unique profile.

B. fragilis ss. *distasonis* S14/3 gave a unique profile and differed from all other strains by giving a strong reaction with substrate 10; it differed from other strains in the fragilis group by giving only weak

TABLE 7.2. Results obtained with 50 test strains in the API ZYM test

Test Strain	Grade of result obtained with substrate no.						Profile group
	1 2 3	4 5 6	7 8 9 10	11 12 13	14 15 16	17 18 19 20	
ss. distasonis (S14/3)	031	203	0003	323	311	0300	a
ss. thetaiotaomicron NCTC10582	031	203	0100	333	333	1302	b
ss. fragilis (C288)	031	203	0000	333	333	1303	
ss. fragilis NCTC9344	031	303	0000	333	333	1303	
ss. thetaiotaomicron (C291)	031	303	0000	333	333	1303	
ss. fragilis (C284)	031	201	0100	333	333	1303	
ss. fragilis (C286)	031	201	0100	333	333	1303	
ss. distasonis (S5/2)	030	202	0000	323	333	1303	
ss. vulgatus NCTC10583	031	200	0000	333	333	0303	c
B. ruminicola B38080S	031	100	0000	333	333	0301	
ss. vulgatus (S8/1)	030	100	0000	333	333	0301	
ss. thetaiotaomicron (C285)	031	200	0000	313	333	0302	
ss. ovatus ATCC8483	031	200	0000	333	323	1311	
ss. thetaiotaomicron (S10/2)	030	300	0000	333	313	1301	
B.splanchnicus NCTC10826	031	203	0001	113	301	0303	d
B.eggerthii NCTC11155	031	300	0000	321	303	0100	e
B.mel. ss. mel. B56020	032	300	0000	323	303	0101	

TABLE 7.2. (Cont'd)

Test Strain	Grade of result obtained with substrate no. 1 2 3	4 5 6	7 8 9 10	11 12 13	14 15 16	17 18 19 20	Profile group
B.disiens VPI8057	031	100	0000	333	303	0303	
B.mel. ss. mel. WAL3030	031	100	0000	333	303	0302	
B.mel. ss. mel. ATCC15930	020	100	0001	333	203	0302	
B.bivius 07073	030	200	0000	332	303	0303	
B.bivius VPI7880	030	100	0000	331	303	0303	
B.oralis VPI8906D	030	100	0000	331	303	0303	f
B.bivius VPI6822	031	100	0000	331	303	0303	
B.bivius VPI6318	031	100	0000	332	303	0303	
B.oralis VPI9958	031	100	0000	332	303	0303	
B.mel. ss. mel. B56007	031	200	0000	322	303	0302	
ss. vulgatus (S9/3)	031	200	0001	333	313	3301	
B.ruminicola B38024	031	200	0003	333	313	3302	
B.oralis WAL3281	031	100	0000	333	303	3303	
B.variabilis VPI11368	031	200	0000	333	303	2302	
B.oralis WPH179	030	100	0000	332	303	2302	g
B.uniformis VPI11227	031	200	0001	313	303	3301	
ss. distasonis ATCC8503	031	200	0000	312	203	3200	
B.ruminicola NP333	031	100	0000	332	303	3000	

TABLE 7.2. (Cont'd)

Test Strain	Grade of result obtained with substrate no.						Profile group
	1 2 3	4 5 6	7 8 9 10	11 12 13	14 15 16	17 18 19 20	
B.mel. ss. levii VPI3300	030	200	0002	331	200	0300	h
B.mel. ss. inter. NCTC9338	031	100	0000	330	003	0001	
B.disiens VPI7852	031	100	0001	330	003	0000	i
B.eggerthii (S13/10)	031	300	0000	221	003	1000	
B.asaccharolyticus (C272)	031	100	0000	330	000	0001	
B.asaccharolyticus (C276)	031	100	0000	320	000	0001	
B.asaccharolyticus (C287)	030	100	0000	320	000	0001	
L.buccalis NCTC10249	031	101	0000	311	001	0000	j
F.necrophorum NCTC10575	031	300	0000	310	000	0000	
B.asaccharolyticus NCTC9337	031	200	0000	110	000	0000	
F.necrogenes NCTC10723	030	100	0000	311	001	0000	
F.varium NCTC10560	011	100	0001	210	000	0000	
F.polymorphum NCTC10562	011	100	0000	110	000	0000	k
Fusobacterium spp. (C292)	011	100	0000	110	000	0000	
B.multiacidus NCTC10934	010	100	0000	323	303	2000	l

results with substrates 15 and 16 and a negative result with substrate 20. The seven strains allocated to profile-group b were all from the fragilis group; all gave strong reactions with substrates 2, 11, 12, 13, 14, 15, 16, 18 and 20, a moderate reaction with substrate 4 and some reaction with substrates 6 and 17. The strains in profile-group c also belonged to the fragilis group but they gave a negative result with substrate 6 and, mostly, with substrate 17.

B. splanchnicus NCTC10826, profile-group d, gave a unique profile that appeared to be unrelated to any other profile in the test series. The two strains in profile-group e, B. eggerthii NCTC11155 and B. melaninogenicus ss. melaninogenicus B56020, were typified by strong reactions with substrates 2, 4, 11, 14 and 16, but negative, or very weak, reactions with substrates 6, 15, 17, 18, 19 and 20. The 10 strains in profile-group f belong to the melaninogenicus/ oralis/ ruminicola group and share similar profiles. They gave strong reactions with substrates 2, 11, 12, 14, 16, 18 and 20 and negative reactions with substrates 6, 15, 17 and 19. The strains in profile-group g differed from those in profile-group f by giving a strong reaction with substrate 17; 4 strains in profile-group g belong to the melaninogenicus/oralis/ruminicola group but strains of B. fragilis ss. vulgatus (S9/3), B. fragilis ss. distasonis (ATCC8503), B. variabilis

VPI11368) and B. uniformis (VPI11227) were also included. B. melaninogenicus ss. levii VPI3300 gave a unique profile (h) and B. multiacidus NCTC10934 was also unique (l). The strains allocated to profile-group i gave strong reactions with substrates 2, 11, 12 and 16, but those in profile-group j gave negative or very weak reactions with substrate 16. Profile group i contained strains identified as B. melaninogenicus ss. intermedius, B. disiens and B. eggerthii whereas profile-group j contained strains of B. asaccharolyticus and Fusobacterium spp. that were relatively unreactive. Profile group k contains the three least reactive strains in the test series; they failed to give any strong reaction and were restricted to only five or six very weak reactions.

Discussion

Tharagonnet et al. (1977) found that the API ZYM patterns were quite distinct for all the species that they tested and in their opinion the system was a reliable and simple method for the identification of gram-negative anaerobes. However, the results of the present study did not show such clear delineation of species.

The analysis of the profiles of the 50 strains gave seven major groupings (b, c, f, g, i, j and k) with several unique profiles that were distinct from

these groups of very similar profiles. The profile - groups corresponded, on the whole, to the species groups identified by the set of conventional tests; profile - groups b and c contained strains that all belonged to the fragilis group; profile-groups f and g contained most of the test strains drawn from the melaninogenicus/ oralis/ruminicola group; group i contained three dissimilar organisms that gave very similar profiles, whereas group j contained all the B. asaccharolyticus strains, together with some Fusobacterium and Leptotrichia strains, and the three strains in group k were all fusobacteria. The groups obtained with the API ZYM system, however, identified strains to only a broad taxonomic level and the system did not appear suitable for the more precise identification to species or sub-species level that was achieved by the set of conventional tests.

Perhaps the most interesting finding was that certain strains that gave unique API ZYM profiles had been allocated to one or other of the major groups by conventional methods and also that some strains that were allocated to one group (e.g. fragilis group) by conventional methods gave an API ZYM profile typical of another major group (e.g. melaninogenicus/oralis/ ruminicola group). This would indicate either that there were errors made in the identification of strains by the conventional methods because undue weight was placed on one or more key tests, or that the API ZYM system detected apparently significant differences

between related strains that were not important in their specific identification. Only sophisticated investigation of the genetic and biochemical make-up of the strains and the relationship of conventional tests and the API ZYM tests to these most basic parameters can determine which approach is more valid. It is, however, worth noting that the API ZYM system was not designed for the identification of bacteria and the present experiments were an attempt to adapt a system designed for the detection of enzymes in human tissue for such purposes; as a result, many of the tests were redundant for the identification of gram-negative anaerobic bacilli and only 10 out of the 19 tests showed any useful differences between strains. A more logical approach might be to study the enzyme complement of the bacteria in question and select a set of tests to detect enzymes of taxonomic significance. The present system, however, has no advantage over a simple set of conventional tests for the separation of the gram-negative anaerobic bacilli into their broad taxonomic groups and can only provide information beyond that for strains that give atypical or aberrant results and require further investigation.

CHAPTER 8

THE ISOLATION AND IDENTIFICATION OF *BACTEROIDES* SPP. FROM THE NORMAL HUMAN FLORA

In man, gram-negative anaerobic bacilli are a major component of the normal commensal flora of the mouth, lower gastro-intestinal tract and the female genital tract. In order to assess the contribution of the different species of bacteroides to the normal flora, strains of *Bacteroides* spp. were isolated from the faeces, upper vagina and gingival crevice of normal healthy adults and identified according to the scheme set out in Chapter 7.

Materials and Methods

Faecal strains

Two hundred strains isolated from 20 specimens of normal human faeces were studied.

Specimens. Fresh specimens (*c.* 5mg) of faeces from 20 normal healthy adults were collected in 6ml of VMGII transport medium (See Appendix II) in small screw-capped vials. The subjects were students, professional and technical colleagues, and nurses undergoing routine screening for faecal pathogens. None had any known gastro-intestinal disorder and none were undergoing antibiotic therapy. The specimens were processed on the same day that they were collected.

Isolation of bacteroides. The specimens were dispersed in the transport medium by vortex mixing (Rotamixer, Hook & Tucker Ltd) for 3 min. One loopful (0.01ml) of the suspension was plated on pre-reduced BM agar with

kanamycin and vancomycin by a standard plating method (Gillies & Dodds, 1976). One ml of the suspension was diluted in 9ml pre-steamed nutrient broth and one loopful of the dilution was similarly plated on a second plate of the same medium. The plates were examined after anaerobic incubation for 48h and all colony-types were noted; five representative colonies were sub-cultured from each specimen on to plain BM agar for further study. After incubation for a further 48h, any additional colony-types were noted and another five representive colonies were subcultured. A total of 10 colonies was studied from each specimen; the colony-types were selected in approximate proportion to their comparative numbers on the primary isolation plates.

Vaginal strains

One hundred and twenty strains isolated from 20 vaginal specimens were studied.

Specimens. High vaginal swabs were collected from 20 normal healthy women and the swabs were immediately broken off into a vial of VMGII transport medium. The swabs were collected from the vaginal vault under direct vision during examination with a Sim's speculun and prior to manual examination. The subjects were women aged between 20 and 40 years attending a family planning clinic for routine vaginal examination and cervical cytology. Samples were not accepted from women who had any vaginal pathology or who were undergoing

antibiotic therapy. The specimens were processed on the same day that they were collected.

Isolation of bacteroides. Each swab was used to seed a sector of a plate of pre-reduced BM agar with kanamycin and vancomycin and this inoculum was streaked over the remainder of the plate in the standard manner. The plates were examined after anaerobic incubation for 48h and all colony types were noted; five representative colonies were sub-cultured from each specimen on to plain BM agar for further study. After incubation for a further 48h, any additional colony types were noted and another five representative colonies were sub-cultured. The colony types were selected in approximate proportions to their comparative numbers on the primary isolation plate. Where possible, 10 colonies were selected from each specimen; 10 colonies were obtained from 11 specimens that yielded a moderate or heavy growth of Bacteroides spp., eight colonies were obtained from one specimen and only 2 colonies from another. There was no growth of Bacteroides spp. from seven of the specimens.

Oral strains

Two hundred strains isolated from 20 specimens of subgingival plaque from normal subjects were studied.

Specimens. Sterile toothpicks were used to collect samples of subgingival plaque from 20 normal healthy adults. The subjects were medical students aged 19 - 20 years; none had any gross oral or dental pathology

and none was undergoing antibiotic therapy.

Isolation of bacteroides. The methods and media were derived from those of Baird-Parker (1957), Loesche et al. (1971), Syed & Loesche (1973) and Williams et al. (1975); they were evaluated by Holbrook (1976; Holbrook et al., 1978). The samples of plaque were seeded directly on to a sector of a plate of pre-reduced BM agar with kanamycin and vancomycin so that losses in transit would be avoided and a transport medium was unnecessary. The inoculum was streaked over the remainder of the plate in the standard manner. The plates were examined after anaerobic incubation for 48h; all colony types were noted and representative colonies were sub-cultured from each specimen on to plain BM agar. After incubation for a further 48h, any additional colony types were noted and further representative colonies were sub-cultured to a total of 10 from each specimen. The colony types were selected in approximate proportion to their comparative numbers on the primary isolation plate. Ten colonies were selected from the heavy growth of gram-negative anaerobes obtained from each specimen.

Preservation of test strains

The pure strains were grown in BM broth for 48h to provide a heavy suspension for storage at -70°C. The liquid phase of the BM broth-culture was centrifuged at 600g for 1h and the supernate discarded. The pellet of cells was resuspended in 1ml freezing medium (see Appendix II) and transferred to a screw-capped

plastic vial. The suspension was rapidly frozen by immersion of the vial in either (i) a solid CO_2-alcohol mixture or (ii) liquid nitrogen. The vials were stored at -70°C in the vapour phase of a liquid N_2 container. When required for study, the frozen suspensions were thawed at room temperature; the purity of each strain was checked by aerobic and anaerobic culture on blood agar and the remainder of the suspension was used as the inoculum for a fresh tube of BM broth. After incubation for 24-48h, this culture was used to seed the following tests.

Tests for the characterization of strains

The strains were subjected to the following set of cultural, biochemical, tolerance and antibiotic disk resistance tests (for details of materials and methods, see Chapter 3): colony morphology after incubation for 48h on blood agar and cell morphology in gram-stained smears from 48h-cultures on blood agar and in BM broth; pigment production on BM agar; haemolysis on blood agar; motility in BM broth; antibiotic disk resistance tests with neomycin 1000µg, kanamycin 1000µg, penicillin 2 units, and rifampicin 15µg disks; tolerance tests with taurocholate, deoxycholate, Victoria blue 4R and ethyl violet; biochemical tests for the production of indole, digestion of gelatin, and hydrolysis of aesculin; fermentation tests with glucose, rhamnose, trehalose, mannitol and xylose; in the later stages of the study additional fermentation tests with sucrose and lactose

were included for some strains. The basic broth medium used for these tests was BM broth.

Results

Faecal isolates

Complete sets of results were obtained with 197 isolates of gram-negative, non-sporing, non-motile, obligately anaerobic bacilli from samples of faeces; 3 isolates proved to be gram-positive bacilli and were discarded. One hundred and eighty strains representing 11 recognised species and subspecies of bacteroides were identified by the scheme described in Chapter 6 and the numbers of strains allocated to each species are shown in Table 8.1; 165 strains were allocated to the B. fragilis group; 8 asaccharolytic isolates produced black or brown pigmented colonies on BM agar and were identified as B. asaccharolyticus; one of the remaining pigmented strains was identified as B. melaninogenicus ss. intermedius and the other two were ss. melaninogenicus; 4 isolates were B. ruminicola; 13 isolates were non-pigmented and non-saccharolytic; and 4 were fermentative bacteroides strains that could not be allocated to a recognised species.

B. fragilis group. The 165 isolates were non-pigmented, resistant to kanamycin, sensitive to rifampicin and fermented glucose. Most isolates were also resistant to neomycin and penicillin, and tolerant of taurocholate and Victoria blue 4R. They were allocated to one of the following seven sub-groups:

TABLE 8.1. The identification of faecal isolates

Species/sub-species	Number of isolates
B.fragilis group	(165)
B.fragilis ss. *fragilis*	15
ss. *vulgatus*	37
ss. *distasonis*	30
ss. *ovatus*	0
ss. *thetaiotaomicron*	36
B.uniformis	8
B.variabilis/eggerthii	24
B.splanchnicus	15
B.melaninogenicus/oralis/ruminicola group	(7)
B.melaninogenicus ss. *melaninogenicus*	2
ss. *intermedius*	1
B.ruminicola	4
asaccharolytic group	(21)
B.asaccharolyticus	8
non-pigmented non-saccharolytic spp.	13
Bacteroides spp (unidentified)	4
TOTAL	197

B. fragilis ss. fragilis (15 isolates). Most isolates gave the typical pattern of results of this sub-species; they fermented glucose and, usually, xylose but did not produce indole. The results of the gelatinase test divided the sub-group into two sets: 9 strains digested the gelatin disk after incubation for 10-14 days and 6 did not. Four isolates gave atypical results: 2 identical isolates from the same specimen did not ferment xylose, one isolate was inhibited by taurocholate and one was inhibited by taurocholate and Victoria blue 4R and also did not ferment xylose.

B. fragilis ss. vulgatus (37 isolates). All isolates gave patterns of results typical of this subspecies: they digested gelatin, fermented glucose, rhamnose and xylose, and did not produce indole; 23 isolates were tolerant of ethyl violet and 13 isolates failed to hydrolyse aesculin. Three isolates from the same specimen were sensitive to penicillin.

B. fragilis ss. distasonis (30 isolates). The patterns of results obtained with these isolates were generally typical of the sub-species; they hydrolysed aesculin, did not produce indole and fermented glucose, rhamnose, trehalose and xylose but 20 strains gave isolated atypical results in some of the tests. Four strains, including 2 isolates from one specimen, were tolerant of ethyl violet; 7 strains from 2 specimens were sensitive to neomycin and 3 strains, including 2 isolates from the same specimen, were also inhibited

by Victoria blue 4R,; one strain was inhibited by taurocholate and Victoria blue 4R and another also failed to ferment xylose; one strain did not ferment rhamnose and 2 strains did not digest gelatin.

B. fragilis ss. thetaiotaomicron (36 isolates). Most isolates gave patterns of results typical of the subspecies: they produced indole, hydrolysed aesculin and fermented glucose, rhamnose, trehalose and xylose; all except 3 isolates digested gelatin. Twelve isolates gave atypical results in individual tests: 4 strains, including 3 from one specimen, were inhibited by taurocholate and another 4 strains, including 3 from another specimen, were tolerant of ethyl violet; 2 strains were sensitive to neomycin and one strain was sensitive to penicillin; one strain was inhibited by Victoria blue 4R and also failed to ferment xylose.

B. uniformis (8 isolates). None of these isolates was identical with the reference strain but the differences were generally minor. All the strains produced indole, hydrolysed aesculin, digested gelatin and fermented glucose and trehalose but not rhamnose. They differed from the reference strain as follows: only one isolate was inhibited by taurocholate, 3 were inhibited by Victoria blue 4R and 4 isolates failed to ferment xylose.

B. variabilis/B.eggerthii group (24 isolates). These two species could not be separated because the only significant difference between them in our tests

is the failure of B. eggerthii to ferment sucrose and this test was not performed on the isolates in this part of the study. The isolates gave patterns of results generally similar to those obtained with the reference strains: they produced indole and fermented glucose, rhamnose and xylose but 15 isolates differed from the reference strains and were tolerant of taurocholate. In addition, 3 isolates did not digest gelatin, 4 isolates, including 3 from the same specimen, were sensitive to penicillin, one was sensitive to neomycin and one did not hydrolyse aesculin.

B. splanchnicus (15 isolates). Five isolates gave patterns of results identical with those of the reference strains and the other 10 isolates showed only minor differences. All strains produced indole, hydrolysed aesculin and fermented glucose. Five isolates were tolerant of taurocholate and 2 were inhibited by Victoria blue 4R; one isolate did not digest gelatin, 2 did not ferment xylose and one of them was also sensitive to neomycin.

B. melaninogenicus/oralis/ruminicola group. The 7 isolates comprised 3 pigmented strains of B. melaninogenicus - one ss. intermedius and 2 ss. melaninogenicus (identical isolates from the same specimen) - and 4 strains of B. ruminicola. The 3 isolates of B. melaninogenicus gave typical patterns of results and were sensitive to penicillin. The 4 isolates of B. ruminicola, including 2 identical isolates from the

TABLE 8.2. Results obtained with the unidentified faecal isolates

Test	Result* obtained with strain no.					
	7/10	12/1	12/7	19/3	6/4	16/5
Antibiotic disk resistance tests						
neomycin	R	R	R	S	S	S
kanamycin	R	R	R	R	R	S
penicillin	R	R	R	R	R	S
rifampicin	S	S	S	S	S	S
Tolerance tests						
taurocholate	+	I	I	I	+	I
deoxycholate	I	I	I	I	I	I
Victoria blue 4R	+	I	I	+	+	+
ethyl violet	I	I	I	I	+	I
Pigment production	-	-	-	-	-	-
Indole production	-	-	-	+	-	-
Gelatin digestion	-	+	+	+	+	+
Aesculin hydrolysis	+	-	-	-	-	-
Fermentation of						
glucose	+	+	+	+	-	-
rhamnose	-	-	-	+	-	-
trehalose	+	-	-	+	-	-
mannitol	-	-	-	-	-	-
xylose	+	-	-	+	-	-

* R = resistant; S = sensitive; I = inhibited;

+ = positive result (growth in tolerance tests);

- = negative result

TABLE 8.3. The isolation of species of bacteroides from the faeces of 20 subjects

Species/sub-species	Isolation of the given species/sub-species from subject no.																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
B.fragilis																				
ss. fragilis	+		+					+			+	+			+	+	+		+	
ss. vulgatus	+	+	+	+	+	+	+	+	+	+		+	+	+		+			+	+
ss. distasonis				+	+	+	+	+	+	+	+		+	+			+	+	+	+
ss. ovatus																				
ss. thetaiotaomicron		+		+	+	+			+	+	+	+		+		+	+	+	+	+
B.uniformis	+	+				+	+					+		+						
B.variabilis/eggerthii		+	+	+		+		+	+	+			+	+		+	+	+	+	
B.splanchnicus	+									+	+		+		+				+	
B.melaninogenicus ss. melaninogenicus			+																	
ss. intermedius	+																			
B.ruminicola													+				+	+		
B.asaccharolyticus		+			+			+		+	+	+	+							+
non-pigmented non-saccharolytic	+		+	+		+		+				+			+	+				+
Bacteroides spp.							+					+							+	

same specimen, were sensitive to neomycin and rifampicin, resistant to kanamycin and penicillin, inhibited by taurocholate, deoxycholate and ethyl violet, digested gelatin and fermented glucose and xylose; 3 isolates, however, did not hydrolyse aesculin.

B. asaccharolyticus. The 8 isolates gave patterns of results typical of the species; they were sensitive to penicillin and rifampicin and resistant to kanamycin, inhibited in all four tolerance tests, produced indole, digested gelatin, but did not ferment glucose or hydrolyse aesculin. They differed only in resistance tests with neomycin - 4 isolates were resistant to the neomycin disk.

Non-pigmented non-saccharolytic strains. Thirteen isolates did not ferment glucose but did not produce pigment. Ten isolates produced indole and were inhibited in the four tolerance tests; all except one isolate digested gelatin. These 10 isolates were provisionally allocated to the species B. putredinis. They gave similar patterns of results to B. asaccharolyticus strains in tests other than pigment production. All were resistant to kanamycin, one was resistant to neomycin and two were resistant to penicillin; however, a single isolate was resistant to all four test antibiotics. The remaining 2 asaccharolytic isolates could not be allocated to a recognised group. The patterns of results obtained with the 2 unidentified asaccharolytic strains and the 4 fermentative bacteroides that could

not be identified are shown in Table 8.2.

Identification of isolates from individual subjects. The distribution of species and subspecies amongst the 20 subjects is shown in Table 8.3. The mean number of patterns of results obtained from subjects was 6.1 (range 4 - 9). A variety of strains was isolated from most subjects and the overall distribution of isolates between the sub-groups was not distorted by the presence of large blocks of identical isolates from single subjects.

Colony morphology. The mean number of colony-types isolated from each subject was 4.2 (range 3 - 5). The results of the characterization tests on each isolate were compared with the original description of the colony that was picked from the isolation plate. Five colony-types were recognised. Three types were similar; they were circular, convex, smooth, shiny and grey, many appeared moist and some were frankly mucoid. They differed only in size and were allocated to one of three size-ranges: 0.5-1mm, 1-2m and >2mm. The fourth type were pinpoint colonies that could not be further described and the fifth type were black or brown pigmented colonies. Except for pigmented strains, the subgroups could not be distinguished on the basis of colony morphology on primary isolation. In individual subjects, isolates from different sizes of colony gave the same pattern of results and isolates

from apparently identical colonies gave different patterns of results. Similarly, the different subgroups could not be distinguished on the basis of colony morphology after subculture. The colony-type of a subculture was notinvariably identical with the primary isolate in size; in particular, isolates that were selected as different colony-type on primary isolation but gave the same pattern of results usually produced similar colonies on subculture. It was noticeable that many of the isolates that gave atypical results in the tests produced smaller colonies on blood agar. Many isolates that produced small colonies on blood agar, moreover, produced larger colonies on BM agar. Isolates allocated to the B. putredinis, B. asaccharolyticus and B. melaninogenicus/oralis/ruminicola groups produced very small colonies on blood agar; B. asaccharolyticus and B. melaninogenicus/oralis/ruminicola isolates produced larger colonies and more rapid pigmentation on BM agar.

Cell morphology. Many isolates were highly pleomorphic in gram-stained smears; cell shape varied from filamentous to cocco-bacillary, often in the same smear. The appearance of isolates allocated to the B. fragilis group varied from fairly regular bacilli with rounded ends to highly pleomorphic smears comprising mixtures of cocco-bacilli, bacilli and some bizarre, swollen forms; some isolates formed chains. Cell morphology varied further with the culture medium and had no discriminatory value withinthe B. fragilis group. However,

the B. melaninogenicus ss. intermedius and ss. melaninogenicus isolates and 4 B. asaccharolyticus strains were predominantly cocco-bacillary. The non-pigmented asaccharolytic isolates were generally regular, slender, pale-staining gram-negative bacilli, often with slightly pointed ends; 2 isolates, however, were cocco-bacilli and one was highly pleomorphic.

Vaginal isolates

Gram-negative anaerobic bacilli of the bacteroides group were isolated from 13 (65%) of the women examined; no growth was obtained on the selective medium from the swabs from the remaining seven subjects. Where possible, 10 colonies from each subject yielding a growth of bacteroides were selected for identification; 10 colonies were picked from each of the 11 plates that gave a heavy growth but only eight colonies were grown from one subject and only two colonies from another. Therefore a total of 120 isolates were selected from the 13 subjects.

Complete sets of results were obtained with 113 isolates; 7 isolates failed to grow on subculture from the preserved cultures. The identity of these 113 isolates, according to the scheme given in Chapter 6, is shown in Table 8.4; 6 isolates were allocated to the B. fragilis group, 88 to the B. melaninogenicus/oralis/ruminicola group, 16 were non-saccharolytic, including 9 B. asaccharolyticus strains, and the 3 isolates labelled Bacteroides spp. could not be allocated to a currently recognised species.

TABLE 8.4. The identification of 113 vaginal isolates

Species/sub-species	Number of isolates
B.fragilis group	
B.fragilis ss. *distasonis*	1
ss. *vulgatus*	3
ss. *thetaiotaomicron*	1
B.splanchnicus	1
B.melaninogenicus/oralis/ruminicola group	
B.melaninogenicus ss. *melaninogenicus*	14
ss. *intermedius*	19
B.bivius	16
B.bivius/disiens	21
B.oralis/bivius/disiens	1
B.oralis	10
B.ruminicola	7
asaccharolytic group	
B.asaccharolyticus	9
non-pigmented non-saccharolytic	7
Bacteroides spp.	3

TABLE 8.5 Patterns of results obtained with 6 vaginal isolates allocated to the B.fragilis group

Test	Results* obtained with the test strains				
Tolerance tests					
taurocholate	+	+	+	+	I
deoxycholate	+	I	I	I	I
Victoria blue 4R	+	+	+	+	+
ethyl violet	+	+	I	I	+
Antibiotic disk resistance tests					
neomycin	R	R	R	R	R
kanamycin	R	R	R	R	R
penicillin	R	R	R	R	R
rifampicin	S	S	S	S	S
Indole production	-	-	-	+	+
Gelatin digestion	+	+	+	-	+
Aesculin hydrolysis	+	-	+	+	+
Fermentation of					
glucose	+	+	+	+	+
rhamnose	+	+	+	+	-
trehalose	-	-	+	+	-
mannitol	-	-	-	-	-
xylose	+	+	+	+	+
No. of strains conforming	2	1	1	1	1
Species/sub-species	B.fragilis ss.vulgatus		ss.distasonis	ss.thetaiotaomicron	B.splanchnicus

*R= resistant; S =sensitive; I = inhibited;
+= positive result (growth in tolerance tests); - = negative result.

TABLE 8.6. Patterns of results obtained with 88 vaginal isolates allocated to the melaninogenicus/oralis/ruminicola group

Test	Patterns of results* obtained with the test isolates																							
Tolerance tests																								
taurocholate	I		I	I		I	I	I	I	I	I	I	I		I	I	I	I		I				
deoxycholate	I		I	I		I	I	I	I	I	I	I	I		I	I	I	I		I				
Victoria blue 4R	I		I	+		I	+	I	+	I	+	I	+	I		I	I	I	+		I			
ethyl violet	I		I	I		I	I	I	I	I	I	I	I		I	I	I	I		I				
Antibiotic disk resistance tests																								
neomycin	R	S	S	R	S		S		S		S		S	S	S	S	S	S	S		S			
kanamycin	R	R	R	R	R		R		R		R		R	R	R	R	R	R	R		R			
penicillin	S	S	S	S	R		R		S		S		R	R	S	S	R	S	R		S			
rifampicin	S	S	S	S	S		S		S		S		S	S	S	S	S	S	S		S			
Pigment production	+		+			-		-		-			-	-	-	-	-	-	-	-				
Indole production	+		-			-		-		-			-	-	-	-	-	-	-	-				
Gelatin digestion	+		+			+		+		+			+	+	+	+	+	+	+	+				
Aesculin hydrolysis	-		-			-		-		-			-	+	+	+	+	+	+	+				
Fermentation of																								
glucose	+		+			+		+		+			+	+	+	+	+	+	+	+				
lactose	...		...			+		...		+			...	...	...	...	...	...	...	...				
sucrose	...		...			-		...		+			...	...	...	...	...	...	...	...				
rhamnose	-		-			-		-		-			-	-	-	-	+	+	+					
trehalose	-		-			-		-		-			-	-	-	-	-	-	+					
mannitol	-		-			-		-		-			-	-	+	-	-	-	+					
xylose	-		-			-		-		-			-	+	+	+	+	+	+					
No. of isolates conforming	11	8	7	1	6	7	3	4	2	7	1	9	4	1	7	1	2	2	1	1	1	2		
Species/subspecies	B.melaninogenicus ss.intermedius		B.melaninogenicus ss. melaninogenicus			B.bivius				B.bivius/ disiens					B.oralis			B.ruminicola					

* R = resistant; S = sensitive; I = inhibited; + = positive result (growth in tolerance tests); - = negative result.

B. fragilis group. All except the strain of B. fragilis ss. distasonis were isolated from the same subject. The patterns of results obtained with the six isolates are shown in Table 8.5; most were typical of the designated species or subspecies except that the B. splanchnicus isolate was not inhibited by ethyl violet and two identical isolates of B. fragilis ss. vulgatus from the same subject were not inhibited by deoxycholate.

B. melaninogenicus/oralis/ruminicola group. The majority (78%) of the vaginal isolates were allocated to this group and the patterns of results obtained with the 88 isolates are shown in Table 8.6. All the isolates were inhibited by taurocholate, deoxycholate and ethyl violet, resistant to kanamycin and sensitive to rifampicin.

B. melaninogenicus ss. intermedius. All 19 isolates produced black or dark-brown pigmented colonies readily; they produced indole and fermented glucose but 11 isolates (including 6 identical isolates from one subject and 3 from another) were resistant to neomycin and 10 of these fermented glucose only weakly after 4 - 7 days.

B. melaninogenicus ss. melaninogenicus. Most of the 14 isolates from four subjects gave patterns of results typical of the subspecies. One isolate was resistant to neomycin and 6 identical isolates from one subject were tolerant of Victoria blue 4R, resistant to penicillin and also produced brown pigmented colonies only slowly.

B. bivius/disiens (37 isolates). This was the commonest group of isolates from the vagina. The two species are distinguished by tests for the fermentation of lactose but the significance of this test in the speciation of the B. melaninogenicus/oralis/ruminicola group was not appreciated until the study was in progress and it was not included in the set of tests until the later stages of the investigation. All the isolates gave patterns of results typical of B. bivius and B. disiens; 10 isolates were tolerant of Victoria blue 4R and 18 were resistant to penicillin.

Fermentation tests with lactose and sucrose were performed on 16 isolates tested in the later stages of the investigation; all fermented lactose but not sucrose and were, therefore, identified as B. bivius.

B. oralis. The 10 isolates identified as B. oralis were inhibited in the tolerance tests, sensitive to neomycin and rifampicin, hydrolysed aesculin and fermented glucose but not xylose. Seven isolates were resistant to penicillin and two identical isolates from the same subject fermented mannitol. One isolate gave a pattern of results intermediate between those of B. bivius/disiens and B. oralis; it fermented glucose, lactose and sucrose, but not xylose - typical of B. oralis - but did not hydrolyse aesculin.

B. ruminicola. The 7 isolates identified as B. ruminicola hydrolysed aesculin and fermented glucose and xylose. In addition 2 isolates fermented rhamnose and 2 identical isolates from one subject

TABLE 8.7. Patterns of results obtained with 19 vaginal isolates allocated to the asaccharolytic and "unknown" groups

Test	Results* obtained with the test isolates							
Tolerance tests								
taurocholate	I			I	I	I	I	+
deoxycholate	I			I	I	I	I	I
Victoria blue 4R	I			I	I	I	I	I
ethyl violet	I			I	I	I	I	I
Antibiotic disk resistance tests								
neomycin	S	R	S	R	S	S	S	R
kanamycin	R	R	R	R	R	S	R	R
penicillin	S	S	S	S	S	S	S	R
rifampicin	S	S	S	S	S	S	S	S
Pigment production	+	+	-	-	-	-	-	
Indole production	+	-	+	-	-	+	-	
Gelatin digestion	+	+	+	+	+	+	+	
Aesculin hydrolysis	-	-	-	+	-	+	-	
Fermentation of								
glucose	-	-	-	-	-	+	+	
rhamnose	-	-	-	-	-	-	-	
trehalose	-	-	-	-	-	-	-	
mannitol	-	-	-	-	-	+	-	
xylose	-	-	-	-	-	-	-	
No. of strains conforming	2	6	1	3	2	2	1	2
Identification	B.asaccharolyticus			non-pigmented non-saccharolytic			unknown	

*R = resistant; S = sensitive; I = inhibited;
+ = positive result (growth in tolerance tests); - = negative result

fermented rhamnose, trehalose and mannitol. Only one isolate was tolerant of Victoria blue 4R and 3 isolates were resistant to penicillin.

Asaccharolytic group. (16 isolates). The patterns of results obtained with the asaccharolytic isolates and also those of the unidentified Bacteroides strains are shown in Table 8.7.

B. asaccharolyticus. Nine asaccharolytic isolates produced black colonies on BM agar; 8 isolates gave patterns of results typical of the species, although 6 isolates (including 4 from the same subject) were resistant to neomycin. The remaining isolate, however, did not produce indole.

Non-pigmented non saccharolytic isolates. This group comprises 7 isolates with three distinct patterns of results. Three isolates gave patterns of results similar to B. asaccharolyticus except that they did not produce pigment; they were inhibited in the tolerance tests, resistant to neomycin and kanamycin, produced indole and digested gelatin. A pair of isolates from one subject were inhibited in the tolerance tests; resistant only to kanamycin and digested gelatin, but they also hydrolysed aesculin and did not produce indole. The remaining two isolates, from different subjects, were inhibited in the tolerance tests, sensitive to the four antibiotics and unreactive in the other tests except that they digested the gelatin disk.

Bacteroides spp. (unidentified). Two identical isolates from one subject gave the tolerance and

TABLE 8.8. The identification of vaginal strains isolated from 13 subjects

Species/sub-species	Number of strains isolated
B.fragilis group	
B.fragilis ss. *distasonis*	1
ss. *vulgatus*	2
ss. *thetaiotaomicron*	1
B.splanchnicus	1
B.melaninogenicus/oralis/ruminicola group	
B.melaninogenicus ss. *melaninogenicus*	5
ss. *intermedius*	7
B.bivius	4
B.bivius/disiens	11
B.oralis/bivius/disiens	1
B.oralis	5
B.ruminicola	5
asaccharolytic group	
B.asaccharolyticus	6
non-pigmented non-saccharolytic	5
Bacteroides spp.	2
TOTAL	56

antibiotic disk resistance patterns of the B. fragilis group, but they did not produce indole or hydrolyse aesculin and fermented only glucose. The third unidentified isolate gave the tolerance and antibiotic disk resistance patterns of the B. melaninogenicus/oralis/ruminicola group but it produced indole, hydrolysed aesculin and fermented glucose and mannitol.

The isolation of different strains from individual subjects. When identical isolates from one subject are treated as a single strain, 56 strains were isolated from the 13 subjects and the identity of these is shown in Table 8.8. The average number of strains isolated from subjects where full sets of results were obtained for >6 isolates was 4.6 (range 2 - 8). The subject that yielded 8 strains was unusual because the 8 strains included all except one of the B. fragilis group isolates and only one isolate from the B. melaninogenicus/oralis/ruminicola group. The distribution of the species identified amongst the 13 subjects is shown in Table 8.9. At least one strain of the B. melaninogenicus/oralis/ruminicola group was isolated from each subject and one member of the group was often predominant; strains of the B. fragilis and asaccharolytic groups were detected only in occasional subjects.

Colony morphology. Colony size and morphology was of little value in the identification of the vaginal isolates except for the characteristic pigmentation of

TABLE 8.9. The isolation of species of bacteroides from vaginal swabs from 13 subjects

	Isolation of the given species/sub-species from subject no.												
	1	2	3	4	5	6	7	8	9	10	11	12	13
B.fragilis ss. distasonis								+					
ss. vulgatus							+						
ss. thetaiotaomicron							+						
B.splanchnicus							+						
B.melaninogenicus ss. melaninogenicus		+	+						+			+	
ss. intermedius	+	+	+								+		+
B.bivius					+	+							
B.bivius/disiens	+			+			+	+	+	+	+		+
B.oralis/bivius/disiens				+									
B.oralis						+			+		+		
B.ruminicola			+	+								+	+
B.asaccharolyticus				+			+				+		+
non-pigmented non-saccharolytic		+					+	+		+			
Bacteroides spp.		+									+		

B. melaninogenicus and B. asaccharolyticus strains. After incubation for 48h most strains of B. melaninogenicus could be seen only as pin-point colonies or a haze of growth in the inoculum well on blood agar; on BM agar the colonies were larger (0.5 - 1mm diam.) and pigmentation was present in the well. After incubation for 5 - 7 days, colonies on blood agar remained small but were pigmented whereas growth of these strains on BM agar was more luxuriant, colonies were larger (1 - 2mm diam.), and pigmentation was more advanced. The colonies of B. asaccharolyticus strains were similar to those of B. melaninogenicus strains but were always deeply pigmented whereas some strains of B. melaninogenicus ss. melaninogenicus, in particular, produced brown rather than black colonies. The colonies of the non-pigmented species in the B. melaninogenicus/oralis/ruminicola groups were initially indistinguishable from the early, small, non-pigmented colonies of B. melaninogenicus strains and after incubation for 5-7 days they were generally small (c.1mm diam.), circular colonies with an entire edge, smooth and shiny but not mucoid and either pale grey or translucent; there were no consistent differences between the species. The non-pigmented non-saccharolytic strains only produced very small colonies (< 0.5mm, diam.) even after prolonged incubation.

Cell morphology. The microscopic appearance of the B. fragilis isolates in gram-stained smears was typical of their group: the cultures consisted of small pleomorphic

gram-negative bacilli. Many strains from the B. melaninogenicus/oralis/ruminicola group showed a similar microscopic morphology, although a little more than half of them were predominantly cocco-bacillary and a few strains were highly pleomorphic with many distorted and bizarre forms. The asaccharolytic strains, pigmented andnon-pigmented, were also predominantly cocco-bacillary.

Oral isolates

A heavy growth of gram-negative non-sporing non-motile anaerobic bacilli was obtained on the selective medium from the 20 specimens of sub-gingival plaque. Ten colonies were selected from each subject for identification and results were obtained with 187 isolates; 13 isolates failed to grow on subculture from the preserved cultures. The 187 isolates were identified according to the scheme given in Chapter 6 and the numbers of isolates allocated to each species are shown in Table 8.10; 9 isolates were identified as members of the B. fragilis group, 127 were members of the B. melaninogenicus/oralis/ruminicola group, 10 were non-saccharolytic Bacteroides spp., 29 were fusobacteria, 10 were probably L. buccalis and 2 isolates labelled Bacteroides spp. could not be allocated to a recognised species.

B. fragilis group. The 7 strains of B. fragilis ss. fragilis and 2 strains of B. fragilis ss. thetaiotaomicron were isolated from 8 subjects and were present in very small numbers in comparison with other species. All gave patterns of results typical of the species except

TABLE 8.10. The identification of 187 oral isolates

Species/subspecies	Number of isolates
B.fragilis group	(9)
B.fragilis ss. *fragilis*	7
ss. *thetaiotaomicron*	2
B.melaninogenicus/oralis/ruminicola group	(127)
B.melaninogenicus ss. *melaninogenicus*	33
ss. *intermedius*	22
B.bivius	3
B.disiens	7
B.oralis	53
B.oralis group	4
B.ruminicola	6
Asaccharolytic group	(10)
B.asaccharolyticus	1
non-pigmented non-saccharolytic	9
Fusobacterium group	(39)
F.polymorphum	12
Fusobacterium spp.	17
L.buccalis	10
Bacteroides spp.	2
TOTAL	187

that one isolate of B. fragilis ss. fragilis was inhibited by Victoria blue 4R.

B. melaninogenicus/oralis/ruminicola group. The majority (68%) of the oral isolates were allocated to this group. All were inhibited by taurocholate, deoxycholate and ethyl violet in tolerance tests and were sensitive to rifampicin in antibiotic disk resistance tests, and all fermented glucose. Most isolates were sensitive to neomycin and penicillin and resistant to kanamycin.

B. melaninogenicus ss. melaninogenicus. The 33 isolates produced pigmented colonies on BM agar; some produced black colonies but many produced colonies with a brown or dark brown centre only after incubation for 5-7 days, and a few strains produced pigmented colonies only in the presence of a contaminant organism (e.g. a staphylococcus). Most of the isolates gave patterns of results typical of the subspecies. Four isolates were resistant to penicillin and one was resistant to neomycin. Eight isolates, including 2 identical isolates from one subject, were tolerant of Victoria blue 4R; 3 of these isolates hydrolysed aesculin and one was resistant to penicillin. A total of 8 isolates hydrolysed aesculin including 2 identical isolates from one subject which were also resistant to penicillin.

B. melaninogenicus ss. intermedius. All 22 isolates produced black or dark-brown pigmented colonies readily on blood agar and on BM agar. They produced indole and fermented glucose and most gave the pattern of results shown in Table 6.3 as typical of the

subspecies. The exceptions were 2 isolates from one subject which were sensitive to the kanamycin 1000µg-disk and 4 isolates that hydrolysed aesculin, including the 2 isolates that were sensitive to kanamycin.

B. bivius. The 3 isolates identified as B. bivius were inhibited by Victoria blue 4R, resistant to kanamycin and sensitive to neomycin and penicillin; they fermented glucose and lactose but not sucrose, and 2 isolates hydrolysed aesculin.

B. disiens. The 7 isolates identified as B. disiens were sensitive to neomycin and resistant to kanamycin; they fermented only glucose in the set of fermentation tests and did not hydrolyse aesculin. Two isolates were resistant to penicillin and one was also tolerant of Victoria blue 4R.

B. oralis (53 isolates). This was the commonest species isolated from subgingival plaque. All the isolates gave patterns of results typical of the species. They fermented glucose, lactose and sucrose but did not produce indole; one isolate fermented rhamnose. All except one isolate were resistant to kanamycin and only 3 isolates, including 2 identical isolates from one subject, were resistant to penicillin. Eighteen isolates, including pairs of identical isolates from 4 subjects, did not hydrolyse aesculin, and 20 isolates, including pairs of identical isolates from 6 subjects, were tolerant of Victoria blue 4R.

B. oralis group. Three isolates were identified as non-pigmented members of the B. melaninogenicus/oralis/

ruminicola group but could not be allocated to a recognised species on the basis of the set of tests performed. They did not ferment xylose but their patterns of results were not typical of B. oralis, B. bivius or B. disiens. Two identical isolates from one subject were inhibited by both bile salts and ethyl violet, tolerant of Victoria blue 4R, resistant to kanamycin and sensitive to neomycin, penicillin and rifampicin. They hydrolysed aesculin but did not produce indole and they fermented glucose and sucrose but not lactose. The other isolate fermented glucose and sucrose but not lactose and did not produce indole, but was inhibited by Victoria blue 4R, resistant to neomycin and did not hydrolyse aesculin.

B. ruminicola. The 6 isolates identified as B. ruminicola hydrolysed aesculin and fermented glucose and xylose. All were sensitive to neomycin and penicillin and 3 isolates were inhibited by Victoria blue 4R. Five isolates fermented rhamnose and one also fermented trehalose.

Asaccharolytic group (10 isolates). One isolate that produced black pigmented colonies but did not ferment glucose was identified as B. asaccharolyticus and 9 isolates were non-pigmented non-saccharolytic strains. The patterns of results obtained with the non-pigmented non-saccharolytic strains, the fusobacteria and the unidentified Bacteroides spp. are shown in Table 8.11.

TABLE 8.11. Patterns of results obtained with 46 oral isolates of non-saccharolytic Bacteroides and fusobacteria

Test	Patterns of results obtained with 46 isolates																					
Antibiotic D.R.T.																						
Neomycin 1000µg	S	R	S	S	S	S	R	S	S	S	S/R	S	S	R	S	S	S	S	S	S	S	R
Kanamycin 1000µg	R	R	S	S	S	S	R	S	S	S	S/R	S	S	S	S	S	S	S	S	S	S	R
Penicillin 2 units	S	S	S	S	R	R	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	R
Rifampicin 15µg	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	R
Tolerance tests																						
Taurocholate	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Deoxycholate	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Victoria blue 4R	I	I	I	I	I	I	I	+	+	+	+	+	+	+	+	+	+	+	+	I	I	I
Ethyl violet	I	I	I	I	I	I	I	I	I	+	+	+	+	+	+	+	I	I	I	I	I	I
Pigment production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-
Gelatin digestion	+	-	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aesculin hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-
Fermentation of glucose	-	-	-	+	+	-	+	-	+	-	+	+	-	-	+	+	+	-	+	+	+	+
Fusiform morphology	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
No. of isolates conforming	8	1	1	2	1	3	1	1	3	1	1	4	5	1	1	4	1	2	3	1	1	

non-pigmented

non-saccharolytic

F.polymorphum

L.buccalis

Bacteroides spp.

Fusobacterium spp.

Fusobacterium group (39 isolates). Seventeen patterns of results were obtained with 35 isolates that had a fusiform morphology in gram-stained smears (Table 8.11). Twelve isolates were provisionally identified as F. polymorphum. They were inhibited by both bile salts but tolerant of both dyes and most isolates were sensitive to the four antibiotic disks; one isolate was resistant to neomycin and penicillin and another was moderately resistant (zone diam. = 14-15mm) to neomycin and kanamycin. The 12 isolates produced indole and digested gelatin and one also hydrolysed aesculin, but only 6 isolates fermented glucose. Ten isolates were provisionally identified as L. buccalis; the cells were larger than most other fusobacteria in gram-stained smears. They were sensitive to the four antibiotic disks and inhibited by both bile salts; 4 isolates that gave the typical pattern of results of L. buccalis were tolerant of both dyes, hydrolysed aesculin, digested the gelatin disk slowly and fermented glucose. The remaining isolates were less tolerant of the dyes and 2 isolates did not ferment glucose.

Twelve isolates were only identified as Fusobacterium spp. In general, they were sensitive to the four antibiotic disks, except for 4 isolates that were resistant to penicillin and one that was resistant to neomycin and kanamycin; they were inhibited in the tolerance tests, except for 4 isolates that were tolerant of Victoria blue 4R. They did not produce indole or hydrolyse aesculin and 6 isolates did not

ferment glucose. One isolate may have been a strain of F. necrophorum, it was resistant to rifampicin, tolerant of both dyes and produced indole, but it did not ferment glucose. Four fusiform isolates did not survive a second subculture and could not be further characterized.

Unidentified Bacteroides spp. The results obtained with the 2 isolates that could not be allocated to a recognised species or group are shown in Table 8.11. One isolate gave the same pattern of results as 2 isolates of Fusobacterium spp. but its cell morphology was not fusiform.

The isolation of different strains from individual subjects. When identical isolates from one subject are treated as a single strain, 131 strains were isolated from the 20 subjects and the identity of these is shown in Table 8.12. The average number of strains isolated from each subject was 6.5 (range 4-9). A variety of species was isolated from each subject and the distribution of isolates between the subgroups or species was not distorted by the presence of large blocks of identical isolates from single subjects. The distribution of the species and subspecies identified amongst the 20 subjects is shown in Table 8.13. At least one strain of B. oralis was isolated from each subject and pigmented strains (B. melaninogenicus) were isolated from all except 2 subjects; members of the Fusobacterium group were isolated from all except 4 subjects.

Nutritional interdependence of oral species. The gram-negative anaerobic flora of the gingival crevice

TABLE 8.12. The identification of oral strains isolated from 20 subjects

Species/subspecies	Number of strains
B.fragilis group	(8)
B.fragilis ss. *fragilis*	6
ss. *thetaiotaomicron*	2
B.melaninogenicus/oralis/ruminicola group	(89)
B.melaninogenicus ss. *melaninogenicus*	24
ss. *intermedius*	15
B.bivius	3
B.disiens	6
B.oralis	32
B.oralis group	3
B.ruminicola	6
Asaccharolytic group	(7)
B.asaccharolyticus	1
non-pigmented non-saccharolytic	6
Fusobacterium group	(25)
F.polymorphum	7
Fusobacterium spp.	15
L.buccalis	3
Bacteroides spp.	2
TOTAL	131

TABLE 8.13. The isolation of species of bacteroides from gingival plaque from 20 subjects

Species/subspecies	Strain of the given species isolated from subject no.																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
B.fragilis ss. fragilis		+				+	+	+	+	+										
ss. thetaiotaomicron			+															+		
B.melaninogenicus ss. melaninogenicus	+		+	+	+	+		+	+		+	+		+	+	+	+	+	+	+
ss. intermedius		+	+	+	+	+			+	+	+					+	+	+	+	+
B.bivius												+		+				+		
B.disiens						+				+	+	+		+						
B.oralis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B.oralis group		+	+		+															
B.ruminicola				+			+			+	+	+								+
B.asaccharolyticus				+																
non-pigmented non-saccharolytic	+				+						+		+		+		+			
F.polymorphum			+				+	+	+	+			+							
F.necrophorum						+														
Fusobacterium spp.	+				+	+	+		+	+	+	+	+	+					+	+
L.buccalis						+	+									+				
Bacteroides spp.		+																+		

is nutritionally interdependent and this causes difficulties in attempts to study pure isolates. Several attempts to isolate particular strains by subculture of a single colony on to fresh solid medium failed because the strain would only grow in mixed culture with other strains that provided essential nutrients. Eleven isolates of 8 strains from 7 subjects in this study were unable to grow in pure culture on the solid media used but would grow well in liquid culture or on solid media in mixed culture, where they showed marked satellitism around contaminant colonies of e.g. a staphylococcus. Most of the strains were from the B. melaninogenicus/oralis/ruminicola group (B. melaninogenicus ss. melaninogenicus, B. oralis (2), B. disiens (2) and B. bivius) but there was also a non-pigmented non-saccharolytic isolate and an unidentified Bacteroides spp.

Colony morphology. Colony size and morphology was helpful in the initial recognition of only a few species amongst the oral bacteroides. According to the definition of the species, isolates identified as B. melaninogenicus ss. melaninogenicus and ss. intermedius produced black or brown pigmented colonies. After incubation for 48h most strains of B. melaninogenicus were seen as small colonies or a haze of growth in the inoculum well on blood agar; on BM agar the colonies were larger and pigmentation was present in the well with some strains. Generally, deep pigmentation developed earlier in strains of B. melaninogenicus ss.

intermedius and most strains produced shiny black or very dark brown colonies, 1mm in diameter and convex with uniform pigmentation throughout the colony; a few strains, however,produced colonies with a very dark centre and a pale edge which were more typical of ss. melaninogenicus. Most strains of B. melaninogenicus ss. melaninogenicus produced brown colonies that were often larger than those of ss. intermedius; pigmentation usually commenced in the centre of the colony and the margin was pale. Some strains, however, produced deeply and uniformly pigmented colonies. Strains identified as B. oralis produced a variety of colonies on primary isolation; initially all produced grey, semi-translucent, shiny and low-convex colonies that varied from 0.5-2mm diameter. Most strains grew better and produced larger colonies on BM agar than on blood agar, and some failed to grow on blood agar. Colony size was not a useful guide to their identification. Many colonies were indistinguishable from those of B. melaninogenicus ss. melaninogenicus in the early, non-pigmented stages of development. However, many isolates of B. oralis that produced the larger colonies developed a characteristic spreading margin that was low, rough and irregular in outline around the shiny convex centre of the colony. The colonies of B. bivius and B. disiens were indistinguishable from the smaller colonies of strains identified as B. oralis but they did not produce the larger,spreading colonies. B. ruminicola strains produced larger colonies that were grey-ochre in colour.

The non-pigmented non-saccharolytic strains produced very small colonies, even after prolonged incubation, as did similar faecal and vaginal isolates. Some Fusobacterium spp. produced characteristic colonies but others were indistinguishable from non-pigmented Bacteroides spp. Most isolates identified as F. polymorphum produced pale yellow colonies 1-2mm in diameter that were raised, rough, irregular in surface and outline, and opaque. The colonies of other Fusobacterium spp. were not clearly recognisable; most were small (0.5-1mm diam.) smooth, low convex, grey and translucent although some became rough, white or pale grey and opaque. Strains identified as L. buccalis produced colonies similar to the smaller colonies of B. oralis.

Cell morphology. The microscopic appearance of B. melaninogenicus ss. melaninogenicus and ss. intermedius in gram-stained smears was typical of the species: the cultures consisted of small pleomorphic bacilli with many cocco-bacilli and a few short filaments; approximately half of the strains were predominantly cocco-bacillary. The appearance of B. oralis and the other non-pigmented species in the group was similar but there were fewer cocco-bacilli and more pleomorphic bacilli. A few strains of B. oralis that produced the large spreading colonies differed; they were predominantly long bacilli with very few short bacilli or cocco-bacilli. The non-pigmented non-saccharolytic

strains were generally small bacilli and cocco-bacilli. The appearance of the fusobacteria was always characteristic but varied from strain to strain. The isolates identified as F. polymorphum were typical fusiform bacilli i.e. long filamentous organisms with pointed ends. The isolates identified as L. buccalis were similarly filamentous but were not necessarily pointed. The remaining Fusobacterium isolates were filamentous organisms of various sizes with pointed ends or, sometimes, blunt ends.

CHAPTER 9

THE IDENTIFICATION OF *BACTEROIDES* SPP. ISOLATED FROM CLINICAL INFECTIONS

Improvements in anaerobic methodology during the past ten years and an increased interest in anaerobes amongst clinicians and medical microbiologists has led to an increased awareness of the role of non-sporing anaerobes, particularly the bacteroides group, in clinical infections. Bacteroides strains are now regularly isolated from a wide variety of clinical conditions but few attempts are made to identify the isolates; they are usually reported as "*Bacteroides* spp. isolated", or at most the non-pigmented penicillin - resistant strains are reported as *B. fragilis*, the pigmented ones as *B. melaninogenicus* and the others as *Bacteroides* spp.

Where further identification has been undertaken medical workers have concentrated upon strains of the *B. fragilis* group isolated from surgical lesions related to the lower gastro-intestinal tract and evidence has started to accumulate that the *Bacteroides* spp. isolated most commonly from infections are not those found most commonly in the normal flora (Werner & Pulverer, 1971; Werner, 1974; Holland *et al.*, 1977). In particular, *B. fragilis* ss. *fragilis* appeared to be the commonest subspecies isolated from genuine cases of infection.

In order to study the occurrence of the different species and subspecies of Bacteroides in clinical infections, bacteroides strains isolated from specimens

submitted to the diagnostic bacteriology laboratories of the Central Sheffield District were identified by the methods described earlier in this thesis.

Materials and methods

Organisms. Three hundred and ninety nine strains of gram-negative anaerobic bacilli isolated from 356 specimens submitted to the diagnostic bacteriology laboratories of the Central District of the Sheffield Area Health Authority (Teaching) were studied. The specimens were pus, exudates or swabs sent routinely to the laboratories from patients with clinically apparent infections. The clinicians were encouraged to send specimens of pus, where possible, and to avoid delay in transport of specimens; these were routine requests and no special measures were taken to obtain special specimens for the purpose of this investigation or to provide special transport facilities.

All the strains selected for identification were regarded as significant isolates and were reported to the clinicians. The Bacteroides strains were present in pure culture in only a minority of the relevant specimens but they were usually predominant or, at least, present in sufficient numbers relative to any other organisms to justify the view that they were significant isolates.

Isolation methods. All cultures, sub-cultures and other manipulations were carried out on the open bacteriology bench. In the early stages of the

investigation, specimens submitted to the laboratories of the Royal Infirmary, Royal Hospital and the Jessop Hospital for Women were seeded on to horse-blood agar and neomycin-horse-blood agar; later the selective medium was changed to KM-lysed-blood agar and both selective and non-selective media were pre-reduced in an atmosphere of H_2 at room temperature overnight and then held in a large anaerobic jar in an atmosphere of CO_2 until seeded, when they were again held under CO_2 until the jar was sealed and evacuated. The plates were incubated in a BTL anaerobic jar in an atmosphere of 80% N_2/10% H_2/10% CO_2; the jar was filled with the gas mixture and then re-evacuated before filling with the final incubation atmosphere. Anaerobiosis was monitored by including a nutrient agar plate seeded with Ps. aeruginosa in every jar. All plates were examined after overnight incubation then reincubated anaerobically for a further 48h.

Specimens submitted to the Childrens' Hospital laboratory were seeded on to horse-blood agar and neomycin-blood agar and the standard anaerobic procedure (Appendix I) was followed. The plates were examined after overnight incubation and then reincubated for a further 48h.

Patients. The 399 strains were isolated from 356 specimens submitted from 332 patients. Details of the clinical condition of each patient were recorded and the sites from which the specimens were obtained are shown in Table 9.3.

Mixed infections. Only a minority of specimens yielded a pure growth of a bacteroides orgnisms; the other specimens yielded a variety of other organisms, sometimes more than one, and these were also reported to the clinicians. Details of all organisms that were thought to be of possible significance in these mixed infections were recorded and are given in the Results section.

Identification of Bacteroides spp. The clinical isolates were characterized by the short combined set of morphological, biochemical, tolerance and antibiotic disk resistance tests; i.e. tolerance of taurocholate, deoxycholate, Victoria blue 4R and ethyl violet, resistance to disks containing neomycin 1000µg, kanamycin 1000µg, penicillin 2 units and rifampicin 15µg, production of pigment and indole, digestion of gelatin, hydrolysis of aesculin and fermentation of glucose, rhamnose, trehalose, mannitol and xylose, with tests for fermentation of lactose and sucrose added when appropriate. The strains were identified according to the scheme given in Chapter 6.

Results

A total of 399 strains of gram-negative anaerobic bacilli were isolated from 356 specimens that yielded a growth of gram-negative anaerobes; the specimens were obtained from 332 patients. Isolates from more than one specimen from a single patient were included in the series if the isolates were different, or if the same strain was isolated from different sites, or from the

same site during different episodes of infection.

Identification of isolates

The identity of each of the 399 isolates from clinical specimens is shown in Table 9.1. B. fragilis was the commonest species and accounted for >50% of all gram-negative anaerobic bacilli isolated; the next commonest species was B. asaccharolyticus with 55 isolates.

B. fragilis group. Two hundred and sixty one isolates were identified as members of the fragilis group.

B. fragilis ss. fragilis. This was the identity of 204 (51%) of the isolates and 184 of them gave the typical pattern of results for the subspecies (Table 6.3); 138 digested the gelatin disk after 14 days' incubation. The other isolates differed from the typical strains in only minor respects : 5 isolates were sensitive to the neomycin 1000µg disk; 4 isolates were inhibited by sodium taurocholate and 4 by Victoria blue 4R, including 2 isolates that were inhibited by both; 3 isolates were tolerant of ethyl violet; 9 isolates did not ferment xylose, including one isolate that was inhibited by sodium taurocholate and the 2 isolates that were inhibited by taurocholate and Victoria blue 4R.

B. fragilis ss. vulgatus. The 8 isolates gave patterns of results that were typical of the subspecies; 6 isolates were tolerant of ethyl violet and 6 hydrolysed aesculin.

TABLE 9.1. The identification of strains isolated from clinical specimens

Species/sub-species	Number of isolates
B.fragilis group	(261)
B.fragilis ss. fragilis	204
ss. vulgatus	8
ss. distasonis	9
ss. thetaiotaomicron	35
ss. ovatus	1
B.eggerthii/variabilis	2
B.uniformis	1
B.splanchnicus	1
B.melaninogenicus/oralis/ruminicola group	(57)
B.melaninogenicus ss. melaninogenicus	13
ss. intermedius	25
B.oralis	4
B.bivius	1
B.bivius/disiens	7
B.disiens	1
B.ruminicola	6
Asaccharolytic group	(73)
B.asaccharolyticus	55
B.corrodens	12
non-pigmented non-saccharolytic	6
Fusobacterium group	8
TOTAL	399

B. fragilis ss. distasonis. In general, the 9 isolates were typical of the subspecies except that 3 isolates failed to ferment rhamnose and one of them was also sensitive to the neomycin 1000µg disk.

B. fragilis ss. ovatus. The one isolate allocated to this subspecies gave the same pattern of results as the reference strain.

B. fragilis ss. thetaiotaomicron. The 35 isolates allocated to this subspecies gave patterns of results that were typical of the subspecies except that one isolate was sensitive to the neomycin 1000µg disk and 2 were tolerant of ethyl violet; all except one isolate digested the gelatin disk after 14 days' incubation.

B. fragilis ss. fragilis and ss. thetaiotaomicron were the only members of the fragilis group isolated from >10 specimens in this study.

B. eggerthii/variabilis. These species were not distinguished in the early part of the study when the significance of the test for sucrose fermentation was not appreciated and when the 2 strains identified as either B. eggerthii or B. variabilis were isolated. Both isolates gave the pattern of results associated with the fragilis group in antibiotic disk resistance and tolerance tests, except that one isolate was inhibited by sodium taurocholate, as were the reference strains of these species; they produced indole, hydrolysed aesculin, digested gelatin and fermented glucose, rhamnose and xylose.

B. uniformis. The isolate assigned to this species gave the same pattern of results as the reference strain.

B. splanchnicus. The isolate identified as B. splanchnicus gave the pattern of results shown in Table 6.3 as typical of the species except that it was inhibited by Victoria blue 4R.

B. melaninogenicus/oralis/ruminicola group. Fifty seven isolates were allocated to species within this group.

B. melaninogenicus ss. melaninogenicus. Thirteen isolates produced black or brown pigmented colonies on lysed-blood agar, fermented glucose and did not produce indole. In general, they gave patterns of results that were typical of the subspecies; 4 isolates were tolerant of Victoria blue 4R, 4 were resistant to penicillin and one isolate that was both tolerant of Victoria blue 4R and resistant to penicillin also hydrolysed aesculin.

B. melaninogenicus ss. intermedius. The 25 isolates allocated to this subspecies produced black or dark brown pigmented colonies on lysed-blood agar, fermented glucose and produced indole. They gave patterns of results consistent with the pattern established for the subspecies, except that 3 isolates were resistant to neomycin and 3 were resistant to penicillin.

B. oralis. The 4 isolates identified as B. oralis gave patterns of results typical of the species; one

strain was resistant to penicillin.

B. bivius/disiens. In the early part of the study, the discriminatory value of the test for sucrose fermentation in the identification of these species was not appreciated and they were not, therefore, distinguished. The patterns of results obtained with the 7 isolates were identical with those of the reference strains except that 4 isolates were resistant to penicillin and one was resistant to the neomycin 1000µg disk, After tests for the fermentation of sucrose and lactose were included in the investigation of strains, one isolate of each species was identified.

B. ruminicola. Six isolates were identified as B. ruminicola; all gave patterns of results identical with those of the reference strains.

Asaccharolytic group. Seventy-three isolates were asaccharolytic Bacteroides spp. of which 55 were B. asaccharolyticus, 12 were B. corrodens and 6 were non-pigmented non-saccharolytic strains.

B. asaccharolyticus. Most of the asaccharolytic strains that produced black or brown pigmented colonies on lysed-blood agar gave patterns of results typical of the species; 16 isolates were resistant to the neomycin 1000µg disk, one was resistant to penicillin and one was tolerant of Victoria blue 4R. However, 6 isolates differed from the typical pattern by not producing indole.

B. corrodens. The 12 isolates were initially recognised by their characteristic colony appearance

on primary isolation. They gave patterns of results similar to those obtained with the referred strains except that 10 isolates were sensitive to rifampicin, one was resistant to the neomycin and kanamycin 1000µg disks, and one was inhibited by Victoria blue 4R.

Non-pigmented non-saccharolytic strains and fusobacteria.

The 6 non-saccharolytic isolates that did not produce black or brown pigmented colonies could not be allocated to recognised species of Bacteroides on the basis of the tests used. Similarly, 7 of the 8 fusobacteria isolated could not be allocated to a recognised species on the basis of these tests; one isolate was clearly identified as F. necrophorum. The patterns of results obtained with the 6 non-pigmented non-saccharolytic isolates and the 8 fusobacteria are shown in Table 9.2.

Sources of Bacteroides spp. isolated from clinical specimens

The 399 isolates of Bacteroides spp. were isolated from 356 specimens that yielded a significant growth of gram-negative anaerobic bacilli. The sites of the infections are shown in Table 9.3. A large proportion (68%) of the isolates were from infections related to the gastro-intestinal tract including abdominal and perianal abscesses, peritonitis and wound infections following surgery. Small numbers of isolates were from a wide variety of sites; most infections were characterised by tissue necrosis, poor vascularity and the production of foul-smelling pus.

TABLE 9.2. Patterns of results obtained with non-pigmented non-saccharolytic isolates and Fusobacterium isolates

Test	Results obtained with the test isolates									
Antibiotic disk resistance tests										
neomycin	S	S	S	S	S	S	S	S	S	S
kanamycin	R	R	S	S	S	R	R	R	S	S	S
penicillin	R	S	S	S	S	S	S	S	S	S
rifampicin	S	S	S	S/R	S	R	R	R	R	R
Tolerance tests										
taurocholate	I	I	I	I	I	I	I	I	I	+	I
deoxycholate	I	I	I	I	I	I	I	I	I	+	I
Victoria blue 4R	I	I	I	+	+	+	+	I	+	+	+
ethyl violet	I	I	I	I	+	I	I	I	I	+	+
Indole production	-	-	-	-	-	-	-	-	-	+	+
Gelatin digestion	+	+	+	+	-	-	+	+	+	+	+
Aesculin hydrolysis	-	-	-	-	-	-	-	-	+	+	-
Fermentation of										
glucose	-	-	-	-	-	+	+	+	+	+	-
rhamnose	-	-	-	-	-	-	-	-	-	-	-
trehalose	-	-	-	-	-	-	-	-	+	-	-
mannitol	-	-	-	-	-	-	-	-	-	-	-
xylose	-	-	-	-	-	-	-	-	-	-	-
No. of isolates conforming	2	3	1	1	1	1	1	1	1	1	1
	non-pigmented non-saccharolytic			*Fusobacterium* spp.							*F. necrophorum*

TABLE 9.3. The sources of specimens that yielded a significant growth of gram-negative anaerobic bacilli

Site of infection	Number of specimens that yielded *Bacteroides* spp.
Infections related to the gastro-intestinal tract	(242)
Upper G.I. tract	40
Appendix	65
Colon and rectum	87
Perianal infections	49
Gynaecological infections	22
Genito-urinary tract (male)	12
Facial and dental infections	6
Central nervous system	5
Bone and joint infections	8
Soft tissue infections	28
Ear, nose and throat infections	3
Respiratory tract	2
Bacteraemia	3
Unknown	26
TOTAL	356

Infections related to the gastro-intestinal tract.

Upper G.I. tract. The species isolated from the 40 specimens from infections related to the upper gastro-intestinal tract are shown in Table 9.4. These strains were isolated principally from abscesses and wound infections following operations on the oesophagus, stomach, small intestine and biliary tract, including five wound infections following operations for the repair for hernias and two infected pancreatic pseudocysts. B. fragilis ss. fragilis accounted for > 50% of the isolates with a few strains identified as other members of the fragilis group. There were 3 isolates of B. asaccharolyticus but it is significant that 4 strains of B. melaninogenicus ss. melaninogenicus and 2 strains of ss. intermedius, which are essentially oral commensals, were isolated from wound infections following surgery to the oesophagus and stomach.

Appendix. The identity of the 72 isolates from 65 infections related to the appendix is shown in Table 9.5. These strains were isolated from appendix abscesses, peritionitis secondary to gangrenous appendicitis and wound infections following appendicectomy; approximately half of the patients were children. B. fragilis ss. fragilis was isolated from 68% of these infections, either as the only species of gram-negative anaerobic bacilli or in mixed infections with other bacteroides, particularly B. asaccharolyticus

TABLE 9.4. *Bacteroides* spp. isolated from infections related to the upper gastro-intestinal tract

Species/sub-species	Number of isolates
B.fragilis group	(33)
B.fragilis ss. *fragilis*	24
ss. *vulgatus*	1
ss. *distasonis*	1
ss. *thetaiotaomicron*	5
B.eggerthii/variabilis	2
B.melaninogenicus/oralis/ruminicola group	(10)
B.melaninogenicus ss. *melaninogenicus*	4
ss. *intermedius*	2
B.bivius/disiens	2
B.ruminicola	2
Asaccharolytic group	
B.asaccharolyticus	3
TOTAL	46

TABLE 9.5. Bacteroides spp. isolated from infections related to the appendix

Species/sub-species	Number of isolates
B.fragilis group	(55)
B.fragilis ss. fragilis	44
ss. vulgatus	1
ss. distasonis	1
ss. thetaiotaomicron	9
B.melaninogenicus/oralis/ruminicola group	
B.melaninogenicus ss. intermedius	7
Asaccharolytic group	(9)
B.asaccharolyticus	7
B.corrodens	2
Fusobacterium group	1
TOTAL	72

or B. melaninogenicus ss. intermedius (7 strains of each).

Colon and rectum. The identity of each of the 97 isolates from 87 infections related to the colon and rectum is shown in Table 9.6. These strains were isolated from wound infections, peritonitis and intra-abdominal abscesses such as pelvic, sub-phrenic and diverticular abscesses following surgery to or perforation of the large intestine. B. fragilis ss. fragilis was again the commonest isolate and was found in 62% of the specimens that yielded a significant growth of gram-negative anaerobic bacilli. B. asaccharolyticus and B. fragilis ss. thetaiotaomicron were also isolated regularly from these patients although much less frequently than B. fragilis ss. fragilis.

Perianal infections. The 57 isolates from 49 specimens of pus from perianal abscesses that included ischio-rectal, inter-sphincteric and pilonidal abscesses are shown in Table 9.7. B. fragilis ss. fragilis was isolated from 50% of these abscesses; there were 9 isolates of B. asaccharolyticus and 5 of the 12 strains of B. corrodens isolated in the whole study were from these abscesses.

Only specimens from infections that yielded a significant growth of gram-negative anaerobic bacilli have been included in this analysis; the derived data indicating the proportions of isolations of individual species from groups of specimens relate only to these selected specimens. However, there were very few

TABLE 9.6. *Bacteroides* spp. isolated from infections related to the colon and rectum

Species/sub-species	Number of isolates
B.fragilis group	(75)
B.fragilis ss. *fragilis*	54
ss. *vulgatus*	3
ss. *distasonis*	2
ss. *ovatus*	1
ss. *thetaiotaomicron*	15
B.melaninogenicus/oralis/ruminicola group	(3)
B.melaninogenicus ss. *intermedius*	1
B.ruminicola	2
Asaccharolytic group	(18)
B.asaccharolyticus	17
non-pigmented non-saccharolytic	1
Fusobacterium group	1
TOTAL	97

TABLE 9.7. Bacteroides spp. isolated from perianal infections

Species/sub-species	Number of isolates
B.fragilis group	(36)
B.fragilis ss. fragilis	29
ss. vulgatus	2
ss. distasonis	1
ss. thetaiotaomicron	3
B.splanchnicus	1
B.melaninogenicus/oralis/ruminicola group	(4)
B.melaninogenicus ss. melaninogenicus	1
ss. intermedius	2
B.oralis	1
Asaccharolytic group	(15)
B.asaccharolyticus	9
B.corrodens	5
non-pigmented non-saccharolytic	1
Fusobacterium group	2
TOTAL	57

specimens from true infections in the "appendix", "colon and rectum" and "perianal" groups (above) that did not yield a significant growth of one or more Bacteroides spp. and the figures given in those sections, therefore, include almost all true infections related to those sites that occurred during the period of study.

Gynaecological infections. The 25 isolates from 22 specimens from infections related to the female genito-urinary tract are shown in Table 9.8. Despite the finding that the fragilis group are not common commensals in the normal vaginal flora, B. fragilis ss. fragilis was isolated from 16 of these infections; 4 strains of B. melaninogenicus ss. intermedius were also isolated. The 16 infections that yielded a growth of B. fragilis ss. fragilis included two pelvic abscesses following a post-partum infection and an infected intra-uterine contraceptive device, a tubo-ovarian abscess, six wound infections following gynaecological surgery (hysterectomy (3), caesarian section, removal of an ovarian cyst and urethroplasty) and two labial or Bartholins abscesses; 5 strains were isolated as heavy, pure growths from swabs of pus from the cervix, including 2 from cases of uterine infection following a normal delivery and a therapeutic abortion. One B. melaninogenicus ss. intermedius isolate was from a pelvic abscess and the others were from cervical pus.

TABLE 9.8. *Bacteroides* spp. isolated from gynaecological infections

Species/sub-species	Number of isolates
B.fragilis group	(17)
B.fragilis ss. *fragilis*	16
ss. *thetaiotaomicron*	1
B.melaninogenicus/oralis/ruminicola group	(7)
B.melaninogenicus ss. *intermedius*	4
B.oralis	1
B.bivius/disiens	1
B.disiens	1
Asaccharolytic group	
B.asaccharolyticus	1
TOTAL	25

Genito-urinary tract infections (male). Gram-negative anaerobic bacilli were isolated as significant pathogens from 12 infections related to the male genito-urinary tract; most of the infections involved the scrotum and penis. One isolate of B. fragilis ss. fragilis was from a wound infection following the removal of a renal calculus but the remaining strains of B. fragilis ss. fragilis (4), B. corrodens (3), B. melaninogenicus ss. melaninogenicus (2), B. uniformis and B. asaccharolyticus were from abscesses and post-operative wound infections of the scrotum (7), penis (2) or groin (2).

Facial and dental infections. Seven strains of Bacteroides spp. were isolated from 6 infections of the mouth and face. B. melaninogenicus ss. melaninogenicus strains were isolated from two dental abscesses and one infected compound fracture of the mandible; B. fragilis ss. fragilis and B. asaccharolyticus were isolated from a facial abscess and B. corrodens was isolated in large numbers from an actinomycotic abscess.

Central nervous system infections. Eight strains of Bacteroides spp. were isolated from 5 specimens from 4 patients with infections of the CNS. B. fragilis ss. fragilis and B. asaccharolyticus were isolated from a frontal lobe abscess secondary to a penetrating injury to the orbit and frontal sinus and 4 strains (B. melaninogenicus ss. melaninogenicus, ss. intermedius

and 2 separate strains of B. ruminicola) were isolated from the cerebro-spinal fluid and the pus from an abscess of a cervical vertebral body with secondary meningitis. B. fragilis ss. fragilis and B. oralis were also isolated from specimens of pus from separate brain abscesses.

Bone and joint infections. Six isolates of B. fragilis ss. fragilis and one each of B. fragilis ss. distasonis and B. asaccharolyticus were from cases of osteomyelitis or infections following bone and joint surgery. Four isolates of B. fragilis ss. fragilis were from the same leukaemic patient following total hip replacement : 3 of the isolates were from the hip joint area at operation, a subsequent soft tissue abscess of the operation site and then, several months later, a persistent sinus, whereas the fourth isolate was from osteomyelitis of a rib that subsequently developed in the same patient. The other 2 isolates of B. fragilis ss. fragilis were from infected stumps following above-knee and mid-thigh amputations. B. asaccharolyticus was isolated from an infection following lumbar laminectomy and B. distasonis from an infected cranial bone flap.

Soft tissue infections. The 28 isolates from soft tissue infections are shown in Table 9.9. The 9 B. fragilis ss. fragilis strains were isolated from infected varicose ulcers (2), paronychia (2), diabetic gangrene of the

TABLE 9.9. Bacteroides spp. isolated from soft tissue infections

Species/sub-species	Number of isolates
B.fragilis group	
B.fragilis ss. fragilis	9
B.melaninogenicus/oralis/ruminicola group	(7)
B.melaninogenicus ss. melaninogenicus	1
ss. intermedius	3
B.bivius/disiens	3
Asaccharolytic group	(12)
B.asaccharolyticus	8
B.corrodens	1
non-pigmented non-saccharolytic	3
TOTAL	28

foot and buttock, an infected forearm wound, a breast abscess and a decubitus ulcer that also yielded B. asaccharolyticus. B. asaccharolyticus was also isolated from infected varicose ulcers (2), paronychia (2), a tracheostomy wound and a leg wound, an infected abdominal fold in a diabetic which also yielded a non-pigmented non-saccharolytic strain, and a case of diabetic gangrene of the toes from which B. melaninogenicus ss. intermedius was also isolated. The remaining 2 isolates of B. melaninogenicus ss. intermedius were also from cases of diabetic gangrene of the foot. B. corrodens was isolated from an abscess originating from the umbilicus and the B. bivius/disiens strains were isolated from a decubitus ulcer and 2 cases of paronychia. B. melaninogenicus ss. melaninogenicus and a non-pigmented non-saccharolytic strain were isolated from a breast abscess and a second isolate of the same non-pigmented non-saccharolytic strain was from a recurrence of the abscess five months later.

Ear, nose and throat infections. There were 3 isolates of Bacteroides spp. from cases of chronic otitis media; they were identified as B. asaccharolyticus, B. fragilis ss. thetaiotaomicron and B. melaninogenicus ss. intermedius.

Respiratory tract infections. Two strains of fusobacteria were isolated from anaerobic infections of the

lower respiratory tract; one was from a lung abscess sampled at post mortem examination and the other from an empyema.

Bacteraemia. Only three strains of Bacteroides spp. were isolated from blood cultures during the course of the study. B. fragilis ss. fragilis was isolated from one patient following abdomino-perineal resection of the colon and rectum and from one patient following gastro-jejunostomy; B. bivius/disiens was isolated from a patient with a pelvic abscess.

Infections in which a Bacteroides spp. was the sole pathogen isolated.

A Bacteroides spp. was isolated in pure culture, or with only a light admixture of coagulase-negative staphylococci, diphtheroids or coliforms, from 92 infections. The species isolated from these infections are shown in Table 9.10.

The sites of the 60 infections from which B. fragilis ss. fragilis was isolated in pure culture are shown in Table 9.11; 43 infections were related to the gastro-intestinal tract. The 10 infections related to the upper gastro-intestinal tract followed gastrectomy (3), duodenal ulcer repair and gastro-jejunostomy (3), Hartman's operation, repair of an inguinal hernia, exploration of the common bile duct and drainage of a pancreatic pseudocyst; 33 infections were related to the appendix, colon or rectum, or were

TABLE 9.10. *Bacteroides* spp. isolated in pure culture from 92 infections

Species/subspecies	Number of isolates
B.fragilis group	(67)
B.fragilis ss. *fragilis*	60
ss. *thetaiotaomicron*	5
ss. *vulgatus*	2
B.melaninogenicus/oralis/ruminicola group	(15)
B.melaninogenicus ss. *melaninogenicus*	4
ss. *intermedius*	4
B.bivius/disiens	5
B.disiens	1
B.oralis	1
Asaccharolytic group	(8)
B.asaccharolyticus	4
B.corrodens	1
non-pigmented non-saccharolytic	3
Fusobacterium spp.	2
TOTAL	92

TABLE 9.11. Sites of pure infections with B.fragilis ss. fragilis

Site of infection	Number of infections
Infectious related to the gastro-intestinal tract	(43)
Upper G.I. tract	10
Appendix	11
Colon and rectum	11
Perianal infections	11
Gynaecological infections	8
Genito-urinary tract (male)	2
Central nervous system	1
Bone and joint infections	3
Soft tissue infections	3
TOTAL	60

perianal infections. The gynaecological infections were pelvic abscesses (2), wound infections following hysterectomy and removal of an ovarian cyst, and a labial abscess; 3 isolates were from specimens of cervical pus.

The genito-urinary tract infections were a wound infection following surgery for the removal of a renal stone and a scrotal abscess and the central nervous system infection was a cerebellar abscess. The bone and joint infections were a wound infection following total hip replacement, an infected above-knee amputation and osteomyelitis of a rib; the soft tissue infections were a facial abscess, a breast abscess and an infected arm wound.

B. fragilis ss. thetaiotaomicron was isolated in pure culture from 4 wound infections following colon surgery (2), repair of a hernia and hysterectomy, and from a case of chronic otitis media; the B. fragilis ss. vulgatus isolates were from wound infections following colon surgery.

In the B. melaninogenicus/oralis/ruminicola group, B. melaninogenicus ss. melaninogenicus was isolated in pure culture from dental abscesses (2), a wound infection following cholecystectomy and a groin abscess, and the B. melaninogenicus ss. intermedius isolates were from perianal abscesses (2) and cervical pus (2). B. bivius/disiens was isolated in pure culture from wound infections following upper gastro-intestinal tract surgery, repair of a hernia and a tubal ligation,

from a case of paronychia and from the blood of a patient with a pelvic abscess; the B. disiens isolate was from cervical pus and the B. oralis isolate from a post-partum uterine infection.

In the asaccharolytic group of species, B. asaccharolyticus was isolated in pure culture from perianal abscesses (2), paronychia and diabetic gangrene of the toes; the non-pigmented non-saccharolytic strains were from wound infections following colon surgery (2) and a breast abscess; and the fusobacteria were from a lung abscess sampled at post-mortem examination and an empyema.

Infections where a single Bacteroides spp. was not the sole potential pathogen isolated.

A single Bacteroides strain was isolated in pure culture from only a minority of infections in this series. More than one species of potentially pathogenic bacteria were isolated in significant numbers from 246 infections for which full details were obtained. The number of species and their identity varied with the site of infection. In the following analysis of mixed infections, one Bacteroides isolate is regarded as the "index strain" and all other strains of Bacteroides spp. or other bacteria isolated are included as "additional strains".

Infections related to the gastro-intestinal tract.

Upper G.I. tract. The bacteria isolated in addition to

the index Bacteroides isolate from 25 mixed infections are shown in Table 9.12. Coliforms were found in 22 (88%) infections and gram-positive cocci, particularly streptococci, were found in 17 infections. Two strains of Bacteroides spp. were found in 5 infections and 3 strains in 2 infections.

Appendix. The bacteria isolated in addition to the index Bacteroides isolate from 52 mixed infections are shown in Table 9.13. Coliforms were the predominant organisms and were found in 43 (83%) infections. Streptococci were found in 22 (42%) infections; the β - haemolytic streptococci included 2 isolates of Lancefield's group B and 2 isolates of group F. Two strains of Bacteroides spp. were isolated from 11 infections : six yielded a mixture of B. fragilis ss. fragilis and B. asaccharolyticus and 2 contained B. fragilis ss. fragilis plus B. melaninogenicus ss. intermedius; B. asaccharolyticus was also isolated in mixed culture with B. corrodens and with a Fusobacterium strain and one infection yielded a mixed growth of B. fragilis ss. thetaiotaomicron and ss. vulgatus.

Colon and rectum. The bacteria isolated in addition to the index Bacteroides isolate from 71 mixed infections are shown in Table 9.14. Coliform organisms were again the commonest group and were found in 58 (82%) infections. Streptococci were isolated from 30 (42%) infections and Cl. perfringens from 11 (15.5%)

TABLE 9.12. Bacteria isolated from 25 mixed infections related to the upper gastro-intestinal tract

Bacterial species	Number of isolates
Coliforms	22
Proteus spp.	4
Cl.perfringens	2
Cl.sporogenes	1
α-haemolytic streptococcus	7
β-haemolytic streptococcus	1
enterococcus	6
anaerobic coccus	2
S.aureus	2
Bacteroides spp. (2 or more strains)	7

TABLE 9.13. Bacteria isolated from 52 mixed infections related to the appendix

Bacterial species	Number of isolates
Coliforms	43
Proteus spp.	2
Ps.aeruginosa	1
Cl.perfringens	1
non-haemolytic streptococcus	8
β-haemolytic streptococcus	6
α-haemolytic streptococcus	5
enterococcus	3
anaerobic coccus	4
S.aureus	3
Bacteroides spp. (2 or more strains)	11

TABLE 9.14. Bacteria isolated from 71 mixed infections related to the colon and rectum

Bacterial Species	Number of isolates
Coliforms	58
Proteus spp.	4
Klebsiella aerogenes	1
Ps. aeruginosa	3
Cl. perfringens	11
non-haemolytic streptococcus	3
α-haemolytic streptococcus	5
β-haemolytic streptococcus	9
enterococcus	13
anaerobic coccus	6
S.aureus	1
Bacteroides spp. (2 or more strains)	14

infections. Several specimens showed evidence of gross faecal soiling in the isolation of several faecal organisms in mixed culture, although there was always clinical evidence of a true infection. Two strains of Bacteroides spp. were isolated from 12 infections, 3 strains from one infection and 4 strains from another. The combinations of Bacteroides spp. isolated were : B. fragilis ss. fragilis plus B. asaccharolyticus (6), B. melaninogenicus ss. intermedius (2) and B. fragilis ss. vulgatus (1); B. asaccharolyticus plus B. ruminicola (1), B. fragilis ss. thetaiotaomicron (1) and a second distinct strain of B. asaccharolyticus (1); B. fragilis ss. fragilis, B. asaccharolyticus and B. ruminicola were isolated from one infection and B. fragilis ss. fragilis, ss. thetaiotaomicron, ss. ovatus and B. asaccharolyticus from another.

Perianal infections. The bacteria isolated in addition to the index Bacteroides isolate from 36 mixed infections are shown in Table 9.15. In contrast with other infections related to the G.I. tract, significant numbers of coliform organisms were isolated from only 12 (33%) infections and enterococci were not detected. Anaerobic cocci were isolated from 8 infections and β - haemolytic streptococci, including one identified as Lancefield's group A and one as group B, were isolated from a further 8 specimens. Two strains of Bacteroides were isolated from 10 infections and 4 strains from one infection. The combinations of Bacteroides spp. isolated were :

TABLE 9.15. Bacteria isolated from 36 mixed perianal infections

Bacterial Species	Number of isolates
Coliforms	12
Proteus spp.	2
Cl. perfringens	2
Clostridium spp.	1
non-haemolytic streptococcus	4
α-haemolytic streptococcus	3
β-haemolytic streptococcus	8
anaerobic coccus	8
S.aureus	1
Actinomyces israelii	1
Bacteroides spp. (2 or more strains)	11

B. fragilis ss. fragilis plus B. asaccharolyticus (6) and B. corrodens (1); B. asaccharolyticus plus B. corrodens (1) and a Fusobacterium spp. (1); B. fragilis ss. vulgatus plus B. splanchnicus; and B. asaccharolyticus, B. oralis, B. corrodens and a Fusobacterium spp. B. fragilis ss. fragilis and B. corrodens were isolated in large numbers from an actinomycotic abscess.

Gynaecological infections. Additional bacterial species were isolated from 10 mixed infections where a Bacteroides spp. was the index isolate. The species isolated were coliform organisms (4), α - haemolytic streptcocci (4), S. aureus (1), Proteus spp. (1) and Candida albicans (1). Two strains of Bacteroides were isolated from 3 infections : B. fragilis ss. fragilis was isolated in mixed culture with B. asaccharolyticus and with B. melaninogenicus ss. intermedius from specimens of cervical pus and B. fragilis ss. fragilis plus B. melaninogenicus ss. intermedius from a pelvic abscess secondary to an infected intra-uterine contraceptive device.

Genito-urinary tract infections (male). The additional species of bacteria isolated from 9 mixed infections where a Bacteroides spp. was the index isolate were : S. aureus (3), coliform organisms (2), α - haemolytic streptococci (2), an enterococcus and an anaerobic coccus. Two Bacteroides strains were isolated from

2 infections; the combinations were B. fragilis ss. fragilis plus B. asaccharolyticus and B. uniformis plus B. corrodens.

Facial infections. Two bacterial species were isolated from 4 facial infections where a Bacteroides spp. was the index isolate. B. fragilis ss. fragilis and B. asaccharolyticus were isolated from a facial abscess and B. corrodens and Actinomyces israelii from an actinomycotic abscess. The two facultative isolates from facial infections were an ∝ - haemolytic streptococcus and Eikenella corrodens.

Central nervous system infections. Three infections of the CNS included in this series were mixed infections. A case of meningitis secondary to an abscess of a cervical vertebral body yielded 4 strains of Bacteroides (B. melaninogenicus ss. melaninogenicus, ss. intermedius, and 2 separate strains of B. ruminicola); B. fragilis ss. fragilis, B. asaccharolyticus, Proteus spp., Strept. pneumoniae, Actinomyces israelii and Actinobacillus actinomycetecomitans were isolated from a fronted lobe abscess secondary to a penetrating injury of the orbit and frontal sinus; and B. oralis an ∝ - haemolytic streptococcus and Eikenella corrodens were isolated from a brain abscess.

Bone and joint infections. The Bacteroides spp. were isolated in mixed culture with other bacteria from

6 bone and joint infections. A coliform organism was isolated from an infection of an amputation site and an infected cranial bone flap, and S. aureus from a wound infection following lumbar laminectomy. Three wound infections following hip surgery yielded (i) a coliform organism, (ii) an anaerobic coccus and (iii) S. aureus, Proteus spp. and an anaerobic coccus.

Soft tissue infections. The bacteria isolated in addition to the index Bacteroides strain from 18 mixed soft tissue infections are shown in Table 9.16. S. aureus was found in 9 infections and other species in only a few infections each. Two Bacteroides strains were isolated from 3 infections : B. fragilis ss. fragilis and B. asaccharolyticus were isolated from a varicose ulcer; B. asaccharolyticus and B. melaninogenicus ss. intermedius from a case of diabetic gangrene, and B. melaninogenicus ss. melaninogenicus and a non-pigmented non-saccharolytic strain from a breast abscess.

Ear infections. The Bacteroides spp. were isolated in mixed culture from 2 cases of chronic otitis media. A Proteus spp. was isolated from one case and S. aureus and Haemophilus influenzae from the other.

TABLE 9.16. Bacteria isolated from 18 mixed soft tissue infections

Bacterial Species	Number of isolates
Coliforms	4
Proteus spp.	6
Ps. aeruginosa	1
Acinetobacter lwoffii	1
α-haemolytic streptococcus	1
β-haemolytic streptococcus	3
enterococcus	2
anaerobic coccus	2
S. aureus	9
Bacteroides spp. (2 or more strains)	3

CHAPTER 10

DISCUSSION

The results of these investigations show that the gram-negative anaerobic non-sporing bacilli are a varied group of organisms that are common commensals of the mouth, vagina and lower gastro-intestinal tract and are significant pathogens in infections related to these sites. They can be recovered from clinical specimens and from their normal carriage sites by conventional bacteriological methods with a controlled and standardised anaerobic jar technique. The common species can be identified by the short set of conventional bacteriological tests described in Chapters 3-6.

The aims of the investigation were : to study the classification of the gram-negative anaerobic bacilli with special reference to pigment production by B. melaninogenicus and to the occurrence of specific biotypes as commensals and pathogens of man, and to examine conventional bacteriological methods for the characterization of clinically important species. A scheme for the identification of these species by conventional tests was developed and then applied to the study of Bacteroides spp. isolated from the normal flora and from clinical specimens.

Pigment production by Bacteroides spp.

The experiments reported in Chapter 2 confirmed the findings of Schwabacher et al. (1947) that the pigment produced by certain Bacteroides spp. when grown on media

containing blood is derived from the haemoglobin in the medium. The pigment is water-soluble, shares similar spectrophotometric characteristics with haemin and is intra-cellular or cell-associated. All pigmented strains that were studied produced the same pigment and this has been confirmed by studies in other laboratories (H. Shah, personal communication), but the pigmented strains do not comprise a homogeneous species.

Oliver & Wherry (1921) called their pigmented isolates Bacterium (Bacteroides) melaninogenicum and subsequently all pigmented strains were assigned to a single species - B. melaninogenicus. Confusion surrounded studies on B. melaninogenicus because of the specific growth requirements of some strains (Gibbons & Macdonald, 1960) and the difficulties experienced by some workers of maintaining them in pure culture (Senos & Mattman, 1955). These problems require the use of properly prepared complex media and careful attention to anaerobic techniques and nutrition; the findings with a few nutritionally exacting strains, particularly the isolates from the normal flora of the gingival crevice, which would not produce pigment or, in some cases, grow in the absence of another organism, indicate that all the problems have not been solved, but the results, in general, are encouraging.

The pigmented strains appeared to share several characteristics in addition to pigmentation that distinguished them from the other recognised non-pigmented

Bacteroides spp. that were mostly members of the B. fragilis group. Major differences in metabolic and biochemical activity were recognised between groups of pigmented strains and the species was divided into three subspecies - ss. melaninogenicus, ss. intermedius and ss. asaccharolyticus (Sawyer et al., 1962; Moore & Holdeman, 1973). The studies of Williams et al. (1975) on cell wall composition and DNA base ratios supported this classification. Lambe (1974) and Lambe & Jerris (1976) distinguished between the same three groups on the basis of fluorescent antibody staining and they also subdivided the ss. intermedius strains into two sero-groups. Harding et al. (1976) studied a variety of pigmented strains and obtained results similar to those presented in this thesis; they divided the pigmented strains into three groups, corresponding to the three named subspecies, on the basis of the results obtained in a short set of biochemical tests. Sedallion, Kerebel and Rouxel (1973) also recognised distinct sub-groups within the species B. melaninogenicus. Werner et al. (1971), however, found that pigmented strains isolated from faeces and from infections related to the lower gastro-intestinal tract formed a homogeneous group of strictly anaerobic, gram-negative, non-sporing rods that could be identified and differentiated by their biochemical properties without taking pigment production into account. The would not include saccharolytic and asaccharolytic organisms in the same species. They recognised only the asaccharolytic strains as B. melaninogenicus but did

not distinguish the saccharolytic strains from non-pigmented bacteroides.

The differences between the subspecies in a wide range of tests was considerable and cast doubt upon the validity of assigning all pigmented strains to a single species. In particular, the asaccharolytic strains were clearly different from the fermentative strains and produced a different pattern of volatile fatty-acid end-products as detected by G.L.C. analysis; B. melaninogenicus ss. asaccharolyticus produced n - butyric acid as a major product but this acid was never produced by the fermentative subspecies. The ICSB taxonomic sub-committee on gram-negative anaerobic rods reviewed the evidence of a collaborative study and recommended that the asaccharolytic strains should be transferred to a separate species - B. asaccharolyticus (Finegold & Barnes, 1977).

However, pigment production remains a major characteristic for the identification of B. asaccharolyticus and the two remaining subspecies of B. melaninogenicus. The evidence presented in this thesis casts doubt upon the overwhelming importance of pigment production in the classification of these species. Studies with strains isolated from faeces and from clinical specimens (Chapters 8 & 9) have shown that there are asaccharolytic strains of Bacteroides that are similar to strains of B. asaccharolyticus in all respects except that they do not produce pigment. Moreover, some of the non-

pigmented strains isolated from the mouth, vagina and clinical specimens and identified as B. oralis closely resemble strains of B. melaninogenicus ss. melaninogenicus. They were only differentiated by their failure to produce pigment; the distinction was even less clear with strains of B. melaninogenicus ss. melaninogenicus that produced brown-pigmented colonies only slowly and strains of B. oralis that produced buff-coloured colonies after prolonged incubation. Terada et al. (1976) suggested that B. oralis and B. ruminicola might be closely related groups that share many similarities with B. melaninogenicus ss. melaninogenicus. Sundqvist (1976) found close similarities between strains of B. melaninogenicus ss. melaninogenicus, B. oralis and B. ruminicola; he did not regard pigment production as a good basis for identifying similar strains as B. oralis or B. melaninogenicus ss. melaninogenicus. The problem associated with the detection of pigment production and its use as a major criterion in the identification of strains is reflected in the results obtained with six strains that were referred by colleagues in a collaborative study as strains of B. oralis and B. ruminicola; they produced black or brown pigmented colonies and, were, therefore, reclassified as B. melaninogenicus ss. melaninogenicus. In particular, strain ATCC15930 had been placed in the American Type Culture Collection as a reference strain of B. oralis but proved to be pigmented.

The results of the present studies indicate that

strains allocated to the species B. melaninogenicus ss. melaninogenicus, B. oralis, B. bivius, B. disiens and B. ruminicola form a closely related group that share many characteristics and that pigment production may not be a valid criterion for the allocation of otherwise similar strains to different species.

In contrast with B. melaninogenicus ss. melaninogenicus, ss. intermedius appears to be a fairly homogeneous group that is different from the B. melaninogenicus ss. melaninogenicus/B.oralis complex and shares fewer characteristics than B. oralis with B. melaninogenicus ss. melaninogenicus. Similar strains that fail to produce pigment have not been detected. From the results obtained in the present studies it appears possible that B. melaninogenicus ss. intermedius should be recognised as a separate species distinct from the B. melaninogenicus ss. melaninogenicus/B. oralis complex.

It would appear, therefore, that pigment production by Bacteroides spp. has less taxonomic significance than was previously thought and that it should be recognised as one characteristic shown by certain strains from different groups or species of Bacteroides.

A pigmented strain identified by the Virginia Polytechnic Institute Anaerobe Laboratory as a fourth subspecies of B. melaninogenicus, ss. levii, became available for study during the course of these

investigations. It was weakly fermentative and was distinct from B. asaccharolyticus. Other strains have not been detected in these studies and the place of this strain in the classification suggested above is not yet clear.

Classification of Bacteroides spp.

The family Bacteroidaceae are gram-negative, non-sporing, non-motile, obligately anaerobic bacilli. They can be divided into four main groups : the B. fragilis group, the B. melaninogenicus/oralis/ruminicola group, the asaccharolytic group and the Fusobacterium group.

B. fragilis group. This group contains a variety of closely-related organisms that share a common ecology as commensals of the lower gastro-intestinal tract and are important pathogens in infections, especially those related to the gastro-intestinal tract. The group can be divided into several sub-groups. In earlier work, these sub-groups were assigned species status and the classification and nomenclature was confused; many names were given to variants within the group but different names were given to the same organism and the same name to obviously different organisms (Breed, Murray & Smith, 1957; Moore & Holdeman, 1973; Holdeman & Moore, 1974).

In the eighth edition of Bergey's Manual of Determinative Bacteriology, Holdeman & Moore (1974) gathered all the members of the group into a single species, B. fragilis, with five subspecies - ss. fragilis

ss. vulgatus, ss. distasonis, ss. ovatus and ss. thetaiotaomicron. They considered that the species represented a continuum of variants with clusters of strains that were designated subspecies, and smaller numbers of intermediate strains. However, other culsters of strains that fell within the group but were different from the five subspecies have been given species status and reference strains of B. splanchnicus, B. eggerthii, B. variabilis and B. uniformis have been included in the present study.

The strains allocated to the subspecies of B. fragilis and to the other species in the group share many phenotypic characteristics and the results obtained in biochemical reactions, tolerance tests with bile or bile salts and antibiotic resistance or sensitivity tests are similar. The sub-groups are differentiated on the basis of a small number of variable characteristics that include indole production and the fermentation of particular carbohydrates (Holdeman & Moore, 1974). Moreover the guanine plus cytosine (G + C) content of the deoxyribosenucleic acid (DNA) isolated from strains in each cluster were found to be similar (40-44 mol %; Moore & Holdeman, 1973) and the G.L.C. analysis of the short-chain fatty acid end-products of metabolism gave similar results for all members of the group (Deacon, 1977; Deacon, Duerden & Holbrook, 1978).

Cato & Johnson (1976) cast doubt upon this classification; they proposed that B. fragilis, B. vulgatus, B. distasonis, B. ovatus and B. thetaiotaomicron

should be reinstated to species rank on the basis of DNA homology studies that showed a high level of diversity in the arrangement of the DNA nucleotides. This proposal was accepted by the International Journal of Systematic Bacteriology and the sub-groups are now recognised as species within a related group by the International Commission for Systematic Bacteriology. However, the change from sub-species to species rank remains the subject of considerable debate because the concept of a continuum of variants in the B. fragilis group (Holdeman&Moore, 1974) is supported by the results ofawide range of phenotypic tests and by ecological and epidemiological studies.

The results obtained in the present studies with alarge number and variety of strains belonging to the B. fragilis group in the biochemical, tolerance and antibiotic disk resistance tests supports the concept that the related strains form a single species with clusters of similar strains designated subspecies. The most significant differences found between members of the B. fragilis group in these studies was the overwhelming preponderance of B. fragilis ss. fragilis amongst strains isolated from clinical infections whereas this subspecies formed only a small proportion of B. fragilis-group isolates from normal faeces.

B. melaninogenicus/oralis/ruminicola group. The six species and subspecies that comprise this group share many phenotypic characteristics and a similar ecology as commensals of the mouth and vagina; they were also

found in specimens from a significant number of infections related to these sites. The role of pigment production in the classification of this group has been discussed above; according to the currently accepted definition of the species, all pigmented strains that ferment glucose belong to the species B. melaninogenicus and are allocated to one of two subspecies; B. melaninogenicus ss. intermedius appears to be a fairly homogeneous group of well-pigmented strains, whereas B. melaninogenicus ss. melaninogenicus strains are more heterogeneous and are probably more closely related to the non-pigmented strains designated B. oralis. The taxonomy of B. oralis has been the subject of much unresolved debate. The species was described by Loesche et al. (1964) but the only reference strain, ATCC15930, was found to produce pigment and was re-classified as B. melaninogenicus ss. melaninogenicus (Appendix V; Holbrook & Duerden, 1974; Finegold & Barnes, 1977). Non-pigmented strains that conformed with the description of Loesche et al. (1964) and were similar to B. melaninogenicus ss. melaninogenicus in other respects have been designated B. oralis in the present studies and have been the subject of an international collaborative study (Appendices VII & VIII; Finegold & Barnes, 1977; Report, in press), but it remains to be decided whether these similar strains should be allocated to two separate species on the basis of pigment production or should be combined as pigmented and non-pigmented variants of a single species. In contrast,

however, Werner et al. (1977) studied 23 propionate-negative, non-pigmented strains that fermented glucose but did not produce indole; these strains were identified as B. oralis. In serological tests with nine strains they found that the B. oralis strains were a homogeneous group that shared antigens with a strain of B. melaninogenicus ss. intermedius but did not cross-react with a strain of B. melaninogenicus ss. melaninogenicus.

B. bivius and B. disiens share many characteristics with the B. melaninogenicus/oralis complex and can be differentiated only by the results of a small number of fermentation reactions. B. bivius was the commonest Bacteroides spp. isolated from the normal vaginal flora, whereas B. oralis was the commonest species isolated from sub-gingival plaque, but whether the differences between the strains justify segregation into three separate species remains to be decided.

The classification of strains in this study as B. ruminicola is also debatable; strains in the B. melaninogenicus/oralis/ruminicola group that ferment the pentoses xylose and arabinose are identified as B. ruminicola according to the currently-accepted definition of the species (Holdeman & Moore, 1974). Several strains with these characteristics were isolated from the normal flora and from clinical infections. The strains of B. ruminicola isolated from animals have been divided into two subspecies, ss. ruminicola and

ss. brevis, and most of the human isolates conform to the description of B. ruminicola ss. brevis. However, their relationship with the animal isolates which are, in general, fastidious anaerobes (Bryant et al., 1958) and with other non-pigmented strains in the B. melaninogenicus/oralis complex is not clear. On the basis of the work described and discussed in this thesis, a reassessment of the classification of the B. melaninogenicus/oralis/ruminicola group would be appropriate.

Asaccharolytic group. The asaccharolytic bacteroides are a heterogeneous group of species and unallocated strains that have been considered together because they share the single property that their metabolism is non-saccharolytic. The group contains two clearly-recognisable species that were well represented in the present studies and a variety of more or less closely related species and strains. The pigmented strains have been separated from the fermentative species B. melaninogenicus as B. asaccharolyticus. This species was found to be a regular commensal of the lower gastro-intestinal tract, although it was isolated in smaller numbers than members of the B. fragilis group; it was also the second commonest species isolated from clinical infections.

B. corrodens was the other asaccharolytic species that was clearly identified in these studies; it is distinct from the CO_2-dependent species Eikenella corrodens (Robinson & James, 1974; Dorf, Jackson & Rytel,

1974). No strains were isolated from the normal flora but 12 strains were isolated from infections. The remaining non-pigmented non-saccharolytic strains that were isolated from the normal flora and from clinical infections could not be further identified by the set of tests used. Some strains were similar to B. asaccharolyticus except for the absence of pigment production and may represent non-pigmented variants of the same species. Holdeman & Moore (1974) recognised eight non-pigmented non-saccharolytic species in addition to B. corrodens; some isolates in the present series conformed with the pattern of results reported for B. putredinis but most results were negative and there were insufficient positive findings to make a reliable identification.

The relationship between B. asaccharolyticus, the non-pigmented non-saccharolytic strains and B. corrodens and between the asaccharolytic group of bacteroides and the fusobacteria remains to be established. The asaccharolytic bacteroides share some characteristics with the fusobacteria; in particular, B. asaccharolyticus producesn-butyric acid as a major metabolic product; an important characteristic that is used to distinguish the fusobacteria that, by definition, produce n-butyric acid, from Bacteroides spp. that do not. In tolerance and antibiotic disk resistance tests, however, B. asaccharolyticus and some of the non-pigmented non-saccharolytic strains gave patterns of results that were typical of the genus Bacteroides whereas some strains of

B. corrodens were resistant to rifampicin and tolerant of Victoria blue 4R, a pattern of results that was more typical of the genus Fusobacterium.

Fusobacterium group. The genus Fusobacterium was originally named after the typical shape of the fusiform organisms, although the definition now rests upon biochemical properties such as the production of n-butyric acid as a major metabolic product. Holdeman & Moore (1974) recognised 16 species of Fusobacterium and segregated Vincent's organisms as Leptotrichia buccalis. Reference strains of F. polymorphum, F. necrogenes, F. necrophorum, F. varium and L. buccalis were studied and some isolates from the normal flora of the sub-gingival crevice and from clinical specimens were allocated to one of the named species. However, the classification is confused and many of the strains isolated from sub-gingival plaque could not be allocated to a recognised species on the basis of the tests used.

A single reference strain was studied that could not be assigned to one of the four main groups. B. multiacidus was isolated from human and animal faeces in Japan (Mitsuoka et al., 1974) and gave a unique pattern of results. It resembled the fusobacteria in tolerance and antibiotic disk resistance tests but was strongly fermentative and did not have a fusiform morphology.

The present studies have concentrated upon the classification of the B. fragilis and B. melaninogenicus/oralis/ruminicola groups, and the asaccharolytic group

because of the problems relating to the classification of B. asaccharolyticus. As a result, the classification of the first two groups is more readily explained in terms of the results of these investigations. The non-pigmented members of the asaccharolytic group and the fusobacteria were studied in less detail; they were not adequately represented in the laboratory isolates in the early stages of the investigations and the validity of their classification must await further studies.

The identification of Bacteroides spp. by conventional tests.

The development of a scheme for the identification of clinically important gram-negative anaerobic bacilli was one of the principal aims of the present studies. A large series of referred strains and laboratory isolates were studied in a series of conventional bacteriological tests that included biochemical tests, tests of resistance to antibiotics and tolerance of dyes and bile salts. The tests were selected and adapted to meet the special anaerobic and nutritional requirements of these anaerobes; the methods adopted and the controls included in the tests took account of problems of growth of demanding strains and reproducibility of results.

The results obtained in the original series of tests (Chapter 3) enabled the selection of a short set of tests that were of particular discriminatory value. The scheme presented in Chapter 3 was developed from a series of investigations that included many strains of the B. fragilis group and only a few representatives of

the other groups. It was ammended following studies with an extended range of Bacteroides spp. (Chapters 4 & 5) and the identification scheme given in Chapter 6 took account of these studies.

The set of tests used for the identification of Bacteroides spp. included : tolerance tests with taurocholate, deoxycholate, Victoria blue 4R and ethyl violet; antibiotic disk resistance tests with neomycin (1000µg), kanamycin (1000µg), penicillin and rifampicin; and tests for the production of pigment and indole, the hydrolysis of aesculin and the fermentation of glucose, lactose, sucrose, rhamnose, trehalose, mannitol and xylose. This set of tests was designed for use in parallel rather than sequentially and the results should not be used as a dichotomous key. This approach takes account of the small degree of variation between strains within each species and subspecies and prevents a single anomalous result from leading to a serious error of identification.

Microscopic and colony morphology play only a minor role in the identification of Bacteroides spp. The cell morphology of most Bacteroides spp. is pleomorphic, and similar for many species; some strains of B. melaninogenicus and B. asaccharolyticus are predominantly cocco-bacillary but this is not a universal finding. Similarly, many Fusobacterium spp. have the typical fusiform morphology but this is not invariable. Many Bacteroides spp. produce indistinguishable colonies.

B. melaninogenicus and B. asaccharolyticus produce pigmented colonies and some Fusobacterium spp. produce characteristic colonies, but the appearance of the colonies of other species is of little help in their identification.

Most gram-negative anaerobic bacilli can be allocated to the appropriate group on the basis of the results of the tolerance and antibiotic disk resistance tests; the biochemical and fermentation tests are used for the identification of the species and subspecies within the groups.

The B. fragilis group is, in general, tolerant of taurocholate and Victoria blue 4R and inhibited by deoxycholate and ethyl violet, except for some strains of B. fragilis ss. vulgatus that are tolerant of ethyl violet; they are sensitive to rifampicin but resistant to neomycin, kanamycin and penicillin. In biochemical tests they hydrolyse aesculin, digest gelatin and ferment glucose and xylose. The sub-groups within the B. fragilis group are identified in tests for indole production and the fermentation of sucrose, rhamnose, trehalose, and mannitol.

In contrast, members of the B. melaninogenicus/oralis/ruminicola group were inhibited by taurocholate, deoxycholate and ethyl violet, and often by Victoria blue 4R; they were sensitive to neomycin and rifampicin and resistant to kanamycin. Most strains were sensitive to penicillin but a significant proportion were resistant.

All strains fermented glucose. The strains in this group were divided into six sub-groups - B. melaninogenicus ss. melaninogenicus, ss. intermedius, B. oralis, B. bivius B. disiens and B. ruminicola - by the results of tests for the production of indole, hydrolysis of aesculin and the fermentation of sucrose, lactose, rhamnose and xylose.

In the asaccharolytic group, B. asaccharolyticus strains were easily identified on the basis of pigment production and the failure to ferment glucose; they also gave a typical pattern of results in the other tests but this pattern was indistinguishable from that of B. melaninogenicus ss. intermedius without the test for glucose fermentation. The only significantly variable test result amongst B. asaccharolyticus strain was in the test for neomycin resistance; a significant minority of strains were resistant. B. corrodens strains were also identified without difficulty on the basis of their typical results, even when strains had lost their characteristic colony morphology after repeated subculture. The scheme was, however, inadequate for the further identification of non-pigmented non-saccharolytic strains.

Similarly, Fusobacterium spp. were reliably allocated to the Fusobacterium group on the basis of cell and, sometimes, colony morphology and the results of tolerance and antibiotic disk resistance tests, but the allocation of fresh isolates to a recognised species was not achieved in many instances.

The results obtained in the sections of the present study where this identification scheme was applied to a large number of fresh isolates from the normal flora and from clinical specimens show that the scheme is valid and useful for the identification of most clinically important gram-negative anaerobic bacilli. The majority of these isolates belonged to the B. fragilis or B. melaninogenicus/oralis/ruminicola groups or were B. asaccharolyticus and were reliably identified by the set of tests selected. However, the studies that lead to the development of the scheme included insufficient experience with Fusobacterium spp. and non-pigmented non-saccharolytic strains. Further experience with these species in a wider range of tests is required so that additional discriminatory tests can be selected and the scheme adapted to include more precise identification of these groups of Bacteroidaceae. However, an identification scheme based upon the conventional tests described in this thesis provides a useful degree of identification for most clinically important gram-negative anaerobic bacilli, usually within 48h, by a small number of tests available in every bacteriology laboratory.

G.L.C. analysis of metabolic products. The short-chain fatty acid end-products of protein and carbohydrate metabolism have been given particular importance in current systems of classification of Bacteroidaceae (Holdeman & Moore, 1974), but they have not been included

in the scheme described above. In parallel studies (Deacon, 1977; Deacon et al., 1978) the acid end-products of 185 isolates of Bacteroidaceae were analysed by gas-liquid chromatography (G.L.C.). Essentially similar fatty-acid profiles were obtained with strains representing all the members of the B. fragilis group; the major products were succinic and acetic acids with traces of lactic and proionic acids. Moreover all the B. fragilis -group strains tested produced fatty acid profiles similar to those produced by strains of the B. melaninogenicus/ oralis/ruminicola group. B. asaccharolyticus produced n-butyric acid as a major product, but B. corrodens and the Fusobacterium spp. also produced this acid. G.L.C. analysis did not provide information that influenced the identification of strains and was not obtained by conventional tests. If G.L.C. were used for the initial allocation of strains to the appropriate main groups of gram-negative anaerobic bacilli, conventional tests would still be required for specific identification. However, the direct detection of the short-chain fatty acids produced by anaerobes in specimens of pus may enable the rapid diagnosis of anaerobic infections and be a valuable investigation in the diagnostic laboratory (Phillips, Tearle & Willis, 1976).

Evaluation of methods

The present studies have confirmed that the clinically-important gram-negative anaerobic bacilli from clinical specimens and from the normal flora can be isolated and

manipulated on the bench by conventional bacteriological techniques. A wide range of species that included demanding strains of B. melaninogenicus, B. asaccharolyticus and Fusobacterium spp. were grown reliably on solid media in the anaerobic conditions provided by a controlled and standardised anaerobic jar technique provided that freshly-prepared media suitable for the nutritionally demanding strains was used.

The conventional tests used for the identification of gram-negative anaerobic bacilli were adapted for use with these somewhat demanding organisms. The tolerance tests were carried out on a specifically enriched nutrient agar medium that supported the growth of most test strains although the findings with a few exacting strains indicate that all the problems have not been solved. These tests could be adapted for application to large batches of strains by using a multiple-loop inoculator/replicator to apply c.20 strains to each plate. The antibiotic disk resistance tests were carried out in conditions that ensured good growth of the test organism, but were not necessarily suitable for sensitivity tests that would be used to predict therapeutic efficacy; the medium was human-blood agar, or BM agar for more fastidious strains that did not grow well on blood agar, and the atmosphere contained 10% CO_2.

The biochemical and fermentation tests were carried out in a liquid medium. The basal medium was chosen to obtain good growth to ensure reproducible results with even the more fastidious strains. The basal medium

used in the initial studies was cooked-meat broth which was replaced by BM broth when this medium was found to support better growth of a wider range of bacteroides. The results of the carbohydrate fermentation tests were obtained by measuring the pH of the cultures with a pH meter and a combined glass and reference electrode; the test was regarded as positive if the pH fell

> 0.5 units below that of the uninoculated control and

> 0.5 pH unit in comparison with the value for a parallel culture in basal medium without added carbohydrate. This method was adapted from that of Rutter (1970) and avoids the use of chemical indicators to determine the fall in pH. Indicators are unreliable in anaerobic bacteriology because they are affected by Eh as well as pH and can be decolourised by the metabolic activity of the test organisms; moreover, the final pH in basal medium without added carbohydrate varies from speies to species and a single indicator added after incubation (Willis, 1977) could give false results with some species.

The identification scheme, however, is not dependent upon the methods used in the present studies and any test method that gives reliable results in the required tests could be used. These methods could include commercially-produced micro-methods (Moore et al., 1975; Stargel et al., 1975), rapid methods for carbohydrate fermentation tests and indole production (Fay & Barry, 1974a, b; Schreckenberger & Blazevic, 1975) or methods developed

for performing the same biochemical and fermentation tests in solid media.

Bacteroides spp. in the normal human flora

A wide variety of gram-negative anaerobic bacilli was isolated in large numbers from all specimens of faeces and sub-gingival plaque and from 60% of specimens taken from the cervix and vaginal fornices of normal healthy subjects. They constitute a major component of the normal bacterial flora of these sites. The identity of the Bacteroides spp. varied with the site examined and reliable recovery of the varied species that included demanding organisms such as B. melaninogenicus, B. asaccharolyticus and Fusobacterium spp. was obtained with the anaerobic methods and selective media used in these studies.

Bacteroides spp. in faeces. A very heavy growth of Bacteroides spp. was obtained from each specimen of faeces, which correlates with the viable counts of c.10" organisms per g found in quantitative studies (Drasar, 1967; Hill et al., 1971; Finegold et al., 1974). Most (84%) of the isolates belonged to the B. fragilis group that has been recognised generally as the predominant group of Bacteroides in the normal flora of the lower gastro-intestinal tract. The commonest species/subspecies isolated were : B. fragilis ss. vulgatus and ss. thetaiotaomicron (22% of B. fragilis-group isolates each), B. fragilis ss. distasonis (18%) and the B. eggerthii/variabilis group (14%). B. fragilis ss. fragilis accounted for only 9% of the B. fragilis-

group isolates. These results are similar to those of Werner (1974), Moore & Holdeman (1975) and Finegold et al. (1975) who also found that B. fragilis ss. vulgatus and ss. thetaiotaomicron were the commonest subspecies isolated from faeces and that B. fragilis ss. fragilis was found in smaller numbers. The occurrence of the different subgroups in the normal flora was not distorted by large numbers of isolates of the common species/subspecies from a few individual subjects; B. fragilis ss. vulgatus was isolated from 80% of subjects, ss. thetaiotaomicron and ss. distasonis from 70% each and the B. eggerthii/variabilis group from 65%, whereas B. fragilis ss. fragilis was isolated from only 45% of subjects.

All except six isolates were clearly identified by the scheme outlined above and in Chapter 6, but in the B. fragilis-group, 55 isolates (33%) gave atypical results in isolated tests; most atypical results were in tolerance tests with taurocholate, Victoria blue 4R or ethyl violet and in antibiotic disk resistance tests with neomycin or penicillin. These results did not cause any difficulties in the identification of strains but they emphasize that it is important to use the tests as a set so that the aberrant results are recognised and do not lead to errors in identification. A few unusual atypical results were restricted to several different strains from individual subjects; this finding cannot be explained but may reflect the close relationship between the different species/subspecies that shared

these particular characteristics and raises the possibility of genetic exchange between them.

Twenty-one asaccharolytic strains, including eight B. asaccharolyticus isolates, were isolated from the specimens of faeces. B. asaccharolyticus (B. melaninogenicus ss. asaccharolyticus) has been isolated regularly from faeces but in smaller numbers than the B. fragilis group (Werner et al., 1971). It is unlikely that B. asaccharolyticus would have escaped detection in the present study unless present in very small numbers in relation to the B. fragilis group because strains of B. asaccharolyticus grewwell under the conditions used and the characteristic pigmented colonies were easily seen amongst the more numerous B. fragilis-group strains.

Only seven strains of the B. melaninogenicus/oralis/ ruminicola group were isolated from normal faeces, which indicates that the lower gastro-intestinal tract is not the normal habitat of this group. Similarly, no Fusobacterium spp. were detected in the specimens of faeces.

Bacteroides spp. in the vagina. Gram-negative anaerobic bacilli of the Bacteroides group were isolated from swabs from the cervix and vaginal fornices from 65% of the women examined. A heavy growth of Bacteroides spp. was obtained from 11 specimens but only a few colonies of Bacteroides spp. were recovered from two specimens. This is a similar carriage rate to the 65% found by Sanders et al. (1975) and the 57% found by Gorbach et al. (1973) but much greater than the 4.6% detected

by Leigh et al. (1976) in a similar population of women attending a family planning clinic. In a quantitative study of the vaginal microflora, Lindner, Plantema & Hoogkamp-Korstanje (1978) found that the mean $\log_{10}$ number of viable Bacteroides spp. per ml of vaginal secretions was 7-8 but they isolated Bacteroides spp. from only 4% of normal women, 1% of pregnant women and 28% of women with cervicitis. These differences in carriage rates between series may reflect the anaerobic methods of the different investigators or, in the case of the quantitative study (Lindner et al.), may indicate that the viable count of Bacteroides spp. in the normal vaginal flora of many women is below the lower limit of sensitivity of their methods. It has been suggested that vaginal carriage of Bacteroides spp. may vary with the menstrual cycle (Hurley et al., 1964) and could be related to the presence of altered blood. The stage of the cycle in the subjects in the present study was not recorded and this aspect of vaginal colonisation could not be assessed.

The Bacteroides spp. isolated in most other studies have not been identified further, except that Lindner et al. (1978), identified their isolates as B. fragilis ss. fragilis, ss. thetaiotaomicron, ss. ovatus and ss. vulgatus. In the present study, all except three isolates were identified by the scheme given in Chapter 6; only six out of 113 isolates (5%) belonged to the B. fragilis group and all except one of these isolates

were from a single subject. The majority (78%) of vaginal isolates belonged to the B. melaninogenicus/oralis/ruminicola group and the commonest sub-group or species was the B. bivius/disiens complex that accounted for 42% of the B. melaninogenicus/oralis/ruminicola-group isolates; B. melaninogenicus ss. melaninogenicus (16%) ss. intermedius (22%) were also common isolates. Fusobacterium spp. were not detected and 12% of vaginal isolates were asaccharolytic strains. The preponderance of the relatively fastidious B. melaninogenicus/oralis/ruminicola group in this study and the absence of these species in the study of Lindner et al. may reflect the differences in the anaerobic methods used. Lindner et al. incubated their cultures in anaerobic jars with an atmosphere of 90%H_2/10%CO_2 but did not give details of their methods, or controls, and their selective medium contained neomycin sulphate 100mg/l; the studies on antibiotic resistance reported in this thesis show that the B. melaninogenicus/oralis/ruminicola group is less resistant to neomycin than the B. fragilis group and this may have affected the recovery of these species.

Pigmented strains of B. melaninogenicus and B. asaccharolyticus were isolated from 9 of the 20 specimens in the present series. This is a lower carriage rate than Burdon (1928) found; he isolated "B. melaninogenicus" from 28 out of 35 specimens from normal women.

Bacteroides spp. in the mouth. A heavy growth of *Bacteroides* spp. was obtained from each specimen of sub-gingival plaque, which reflects the findings of Gibbons *et al*. (1963) and Loesche *et al*. (1972) that gram-negative anaerobic bacilli constitute 15-16% of the cultivable flora of the gingival crevice. The oral isolates were more demanding nutritionally than the faecal and vaginal isolates and some difficulty was experienced in isolating and maintaining some strains in pure culture. One of the problems encountered by earlier workers (Senos & Mattman, 1955) was the difficulty of obtaining pure cultures of *Bacteroides* spp. Several strains of the *B. melaninogenicus/oralis/ruminicola* group would not grow on solid media in the absence of a contaminant organism around which they would show satellitism, and some strains of *Fusobacterium* spp. were difficult to maintain in repeated sub-culture.

The predominant *Bacteroides* spp. isolated from sub-gingival plaque in the present series were members of the *B. melaninogenicus/oralis/ruminicola* group; these species accounted for 68% of the oral isolates. This is the same group that was predominant amongst the vaginal isolates. The similarity in the identity of isolates from the two sites may reflect similarities in the physiological environment of the two sites; a comparison of saliva with cervical secretions shows that the pH, osmolarity, carbohydrate and enzyme content, especially the amylase content are similar (Singer & Jordan, 1976;

Mason & Chisholm, 1975). The proportion of the different sub-groups within the B. melaninogenicus/oralis/ruminicola group amongst oral isolates differed from the proportions amongst vaginal isolates. B. oralis was the commonest species isolated (42% of the B. melaninogenicus/oralis/ruminicola-group isolates) and B. melaninogenicus ss. melaninogenicus (26%) and ss. intermedius (17%) were also common amongst the oral isolates, but only 10 isolates were identified as B. bivius or B. disiens. Gibbons (1974), and Loesche et al. (1972) showed that B. melaninogenicus is a common commensal of the gingival crevice; B. melaninogenicus strains were isolated from all except two of the subjects in the present study. Hardie (1974) stated that B. fragilis had not been shown to be a commensal in the mouth. In the present study, only nine isolates were identified as members of the B. fragilis group; they were present in very small numbers and may represent transient carriage rather than permanent colonisation.

Hadi & Russell (1968; 1969) found Fusobacterium spp. in the gingival crevice and, in smaller numbers, in saliva. In the present study, Fusobacterium spp. were isolated from 65% of the specimens and accounted for 20% of the isolates identified, although only a minority could be assigned to a recognised species on the basis of the tests used. The significance of the fusobacteria as members of the normal flora of the gingival crevice may have been underestimated in the present study as a

result of the difficulty experienced in the isolation of pure cultures of fusobacteria and the maintenance of the isolates in a viable state during repeated subculture.

It can be seen from the present studies that the gram-negative anaerobic bacilli that form a major part of the commensal faecal flora belong to the B. fragilis group but that most of them are not B. fragilis ss. fragilis. In the vagina and the sub-gingival crevice, however, members of the B. fragilis group are uncommon and the B. melaninogenicus/oralis/ruminicola group is predominant. Fusobacterium spp. were not detected in the faecal or vaginal specimens but were a significant proportion of the commensal flora of the gingival crevice.

Bacteroides spp. isolated from clinical infections

A variety of Bacteroides spp. was isolated from infections at many different sites. Infections related to the gastro-intestinal tract have provided the majority of isolates in most studies of Bacteroides infections and a large proportion of the isolates in the present study were from infections related to injury, surgery or some underlying pathology of the gastro-intestinal tract.

B. fragilis ss. fragilis was the commonest species/subspecies isolated from infections overall; 51% of all isolates were B. fragilis ss. fragilis and this subspecies accounted for 78% of all B. fragilis-

group isolates. If isolates from infections related to the appendix, colon and rectum are considered alone, 58% of all isolates and 75% of B. fragilis-group isolates were B. fragilis ss. fragilis. In comparison with the proportion of isolates from the normal faecal flora that were identified as B. fragilis ss. fragilis (9% of the B. fragilis-group isolates) it is clear that this subspecies has a particular pathogenic potential. If all members of the B. fragilis-group were equally virulent and infections were merely related to the opportunity given by faecal soiling, the proportions of the different species/subspecies isolated from infections would be expected to reflect their proportions in the faeces. The preponderance of B. fragilis ss. fragilis in infections must be due to some specific property or properties that make this subspecies particularly virulent. A specific virulence factor has not been identified but several factors may be involved. Most pathogenic strains of B. fragilis ss. fragilis are capsulate; the capsule is not large but serological studies indicate that it may play an important part in pathogenicity (Kasper, 1976; Kasper & Seiler, 1975). Other workers have suggested that the production of exotoxins such as heparinase (Gesner & Jenkins, 1961) and neuraminidase (Müller & Werner, 1970) by B. fragilis ss. fragilis may be important in pathogenicity. The cell wall of B. fragilis ss. fragilis contains endotoxin but there is no detectable difference in relative toxicity between the endotoxin content of

B. fragilis ss. fragilis and other members of the group. Although the role of these virulence factors has not been proved, the epidemiological evidence of the present study and those of Werner & Pulverer (1971), Werner (1974) and Holland et al. (1977) indicates that the identification of clinical isolates to the species/subspecies level would be of practical value in the routine diagnostic laboratory as an indication of the significance of individual isolates of Bacteroides spp. The isolates of B. fragilis ss. fragilis from infections related to the gastro-intestinal tract would probably indicate a true infection whereas the isolation of other members of the B. fragilis-group would suggest gross faecal soiling, particularly if several species/subspecies were isolated from the same specimen. However, the final assessment of the significance of the bacteriological findings must also take account of the clinical condition of the patient.

As in other studies, B. fragilis ss. thetaiotaomicron was the second commonest member of the B. fragilis group isolated from clinical specimens, but the second commonest species isolated overall was B. asaccharolyticus. Some clinical isolates of B. asaccharolyticus were from infections related to the appendix, colon and rectum and from perianal infections. B. fragilis ss. fragilis was also isolated frommany of these infections. However B. asaccharolyticus was isolated from nine soft tissue infections where it appeared to be the most significant pathogen in lesions such as diabetic gangrene and varicose

ulcers where a poor blood supply and a considerable amount of devitalised and necrotic tissue created suitable conditions for anaerobic infection. Peromet et al. (1973), Rissing et al. (1974) and Willis (1977) also reported that B. melaninogenicus was particularly common in decubitis and diabetic ulcers.

The proportion of the various Bacteroides spp. isolated from clinical infections varied with the site of infection. B. fragilis ss. fragilis, ss. thetaiotaomicron and B. asaccharolyticus were the principal species isolated from infections related to the gastro-intestinal tract. B. fragilis ss. fragilis was isolated from 65% of infections related to the appendix, colon and rectum and from 50% of perianal infections and those related to the upper gastro-intestinal tract; most of the other species/subspecies isolated were commensals of the lower gastro-intestinal tract except for six strains of B. melaninogenicus that are commensals in the mouth and were isolated from wound infections following surgery to the oesophagus and stomach.

B. fragilis ss. fragilis was not commonly isolated from the normal vaginal flora but was isolated from 73% of gynaecological infections, emphasising the specific pathogenicity of this subspecies. The identification of Bacteroides strains isolated from the female genital tract as B. fragilis ss. fragilis may help to distinguish true infection from contamination with commensal bacteroides and casts doubt upon the assumption that bacteroides infections of the female genital tract are

caused by those Bacteroides spp. present in the vagina as part of the normal flora. However, some isolates from serious gynaecological infections were identified as species that form part of the normal vaginal flora.

Several Bacteroides spp. were isolated from infections unrelated to the gastro-intestinal or female genital tracts; B. fragilis ss. fragilis was the commonest species/subspecies isolated but was less predominant in these infections and there was a greater variety of species than in infections related to the gastro-intestinal tract. However, the numbers of different infections were small and no species/subspecies appeared to be particularly common in any one type of infection. The isolation of several Bacteroides spp. from cerebral abscesses that followed infections of, or penetrating injury to, the middle ear and mastoid, the paranasal sinuses and the posterior pharyngeal wall supports the findings of Ingham et al. (1977) that Bacteroides spp. are important pathogens in otogenic and similarly derived brain abscesses.

A Bacteroides spp. was isolated in pure culture from only 26% of infections in this study; B. fragilis ss. fragilis was isolated from 65% of these infections. Two or more potentially pathogenic species were isolated from 73% of infections for which full laboratory and clinical details were obtained. Most mixed infections originated in areas with a mixed normal flora, e.g. the gastro-intestinal tract, or where colonisation with a variety of species generally occurs, e.g. varicose

ulcers and diabetic gangrene. The most frequent facultative bacteria isolated were coliform organisms. Evidence from Willis et al. (1975; 1976; 1977) shows that treatment of mixed infections related to the appendix, colon, rectum and female genital tract with metronidazole, which affects only anaerobes, is effective in controlling the infections; the aerobic and facultative orgnisms alone appear to have little virulence under these conditions. Similarly, Jones, Willis & Ferguson (1978) have shown that topical treatment of decubitus ulcers with metronidazole leads to a resolution of the infection and healing of the lesion without any antibacterial treatment effective against the aerobic or facultative species present. It appears, therefore, that the Bacteroides spp. are the most significant pathogens in these mixed infections. However, the facultative and aerobic species should not be disregarded. There may be true synergy between facultative and anaerobic species in the production of serious tissue damage, as a synergistic bacterial gangrene; an anaerobic coccus or a β - haemolytic streptococcus was isolated from 50% of the perianal abscesses in the present study and these infections were associated with considerable cellulitis and tissue necrosis. Alternatively the common occurrence of coliform organisms with Bacteroides spp. in infections may provide suitable conditions for the Bacteroides spp. to cause the tissue damage and necrosis associated with these infections. although the coliform organisms are not an essential

component of the infections.

Two or more Bacteroides strains were isolated from 15% of the bacteroides infections in the present study; most of these infections were related to sites where several Bacteroides spp. are normal commensals. No single combination of two Bacteroides spp. was particularly common, but it was noticeable that B. asaccharolyticus was rarely isolated in pure culture from any infections and the most common combination was B. fragilis ss, fragilis plus B. asaccharolyticus. Burdon (1928; 1932)and Heinrich & Pulverer (1960) also found that B. melaninogenicus was rarely isolated in pure culture from infections and Weiss (1937) found that B. melaninogenicus alone would not cause infection in laboratory animals but was the most important component of various mixed infections. Hite, Locke & Hesseltine (1949) also found that B. melaninogenicus was important in experimental synergistic infections with non-sporing anaerobic bacteria.

The studies reported in this thesis have shown that Bacteroides spp. are important members of the normal flora of the lower gastro-intestinal tract, vagina and gingival crevice and are also significant pathogens in a variety of infections. They can be studied by conventional bacteriological methods adapted to their specific requirements. Gram-negative anaerobic bacilli were divided into four groups containing 24 species/subspecies that could be identified by a simple set of tests. A variety of species was isolated from the normal human flora; the predominant species in the

faeces were members of the B. fragilis group whereas members of the B. melaninogenicus/oralis/ruminicola group were the principal species isolated from the vagina and gingival crevice. There was less variety amongst strains isolated from clinical infections and > 50% of isolates were B. fragilis ss. fragilis.

APPENDIX I

STANDARD ANAEROBIC PROCEDURE

The following anaerobic procedure, based on that of Collee et al., (1972) was used throughout the investigations described in this thesis.

Anaerobic Jars

Two types of anaerobic jars were used:

1. BTL Anaerobic Jar (Baird and Tatlock Ltd). In later experiments the needle valves of some of these jars were replaced by the Whitley Valve System of heavy duty Shrader Valves (Don Whitley Scientific, Shipley, West Yorkshire). Each jar was fitted with either three catalyst sachets (Baird and Tatlock Ltd) or one four-gramme catalyst sachet (Don Whitley Scientific).
2. Whitley 48-plate Stainless Steel Anaerobic Jar (Don Whitley Scientific). Each jar was fitted with two four-gramme catalyst sachets.

Vacuum Pump

An electrically operated vacuum pump that could reliably achieve a vacuum of 660mm Hg below atmospheric pressure was used.

Vacuum Guage

A Speedivac vacuum guage was used to measure the vacuum achieved inside the jar.

Method

1. One inlet (H) was connected to the pump and the other inlet (V) to the guage; the pump was started.

2. The jar was evacuated to -630mm Hg (c.-25 inches Hg), i.e. 630mm below atmospheric pressure.

3. Inlet H was closed and the pump disconnected.

4. A bladder containing a gas mixture of 90% hydrogen and 10% carbon dioxide was connected to inlet H and the gas run in until the guage showed zero.

5. Both inlets were closed and disconnected and the jar was left on the bench.

6. AFTER TEN MINUTES one inlet was connected to a simple manometer to check that a vacuum of at least 20mm Hg had been produced.

7. More of the H_2-CO_2 gas mixture was admitted to equilibrate.

8. The jars were then incubated.

Anaerobic Indicator

A biological indicator of anaerobiosis was used. A nutrient agar plate was seeded with a broth culture of a strain of *Pseudomonas aeruginosa* and incubated in the jar. This organism would not grow within 48h if the atmosphere inside the jar had remained anaerobic. Growth of the *P. aeruginosa* indicated a jar failure.

APPENDIX II

MEDIA

Fluid media

Robertsons cooked-meat-broth

(per tube)

Fresh bullocks' heart (Cruickshank et al., 1975) 2cm depth

Nutrient broth no. 2 (Oxoid) 10 ml.

BM broth (modified from Williams et al., 1975)

Trypticase (BBL)	1%
Proteose peptone (Oxoid)	1%
Yeast extract (Oxoid)	0.5%
Sodium chloride	0.5%
Sodium succinate	0.25%

Dissolve in distilled water, adjust to pH7.4, add to cooked-meat particles as above and autoclave. To cool medium add :

haemin	5µg/ml
menadione	1µg/ml
l-cysteine hydrochloride	0.075%

PPYS medium

Proteose peptone (Oxoid)	2%
Yeast extract (Difco)	1%
Sodium chloride	0.5%

Dissolve in distilled water, adjust to pH7.4 and autoclave. To cool medium add :

haemin	5µg/ml

menadione	1µg/ml
1-cysteine hydrochloride	0.075%
sodium carbonate (anhydrous)	0.04%
Horse serum (filter-sterilised; Wellcome)	2%

PPYSG medium

PPYS medium as above, with glucose 1% added as a 20% filter-sterilised aqueous solution to cooled autoclaved medium.

Supplemented Thioglycollate medium

Thioglycollate medium without dextrose or indicator (BBL) with :

haemin	5µg/ml
menadione	1µg/ml
1-cysteine hydrochloride	0.075%

VMG II transport medium (Möller, 1966)

Salt solution IIc

Phenylmercuric acetate (BDH)	30mg
$CaCl_2 6H_2O$ (BDH, Analar)	2.4g
KCl (BDH, Analar)	1g
NaCl (BDH, Analar)	10g
$MgSO_4 . 7H_2O$ (BDH, Analar)	1g
Sodium glycerophosphate (BDH)	100g
glass distilled water to	1000ml

Solution A

Dissolve 10mg Ionagar (Oxoid no. 2) in 9ml glass distilled water, cool to 50°C and add :

Bacto gelatin (Difco)	1g
Tryptone (Difco)	50mg

Thiotone (BBL)	50mg
l-cysteine hydrochloride	50mg
mercaptoacetic acid (BDH) (5% solution)	1.0ml
bacteriological charcoal (Oxoid)	1g
Salt solution IIc (above)	100ml

Adjust to pH7.5 and dispense in 6ml volumes in bijoux. Autoclave at 121°C for 20 min., tighten caps immediately and store at room temperature aerobically.

Freezing medium

Nutrient Broth no. 2 (Oxoid)	100ml
Inactivated Horse Serum	10ml
20% aqueous solution of glucose	5ml

Solid media

Human-blood agar.

Columbia Agar Base (Oxoid) plus 5% outdated human blood.

Lysed-human-blood agar.

The blood was lysed either by treating with saponin or by freezing and thawing thrice; the medium contained 5% lysed blood in Columbia Agar Base.

Horse-blood agar.

Defibrinated Horse Blood (Oxoid) 10% in Columbia Agar Base (Oxoid).

Heated (chocolate) horse-blood agar.

The blood was lysed by heating at 55-56°C for 1h

before incorporation in the base.

BM agar.

Trypticase (BBL)	1%
Proteose peptone (Oxoid)	1%
Yeast extract (Oxoid)	0.5%
Sodium chloride	0.5%
Sodium succinate	0.25%
Ionagar no. 2 (Oxoid)	1.2%
or Agar no. 1 (Oxoid)	

Dissolve in distilled water, adjust to pH7.4 and autoclave. Cool to 50°C and add :

lysed human blood (see above)	5%
menadione	1µg/ml
L-cysteine hydrochloride	0.075%

BM-kanamycin-vancomycin agar.

The BM agar was prepared as above and filter-sterilised aqueous stock solutions of kanamycin and vancomycin were added to the cool medium to give final concentrations of :

kanamycin	75µg/ml
vancomycin	2.5µg/ml

Supplemented nutrient agar.

Columbia Agar Base (Oxoid) plus	
Sodium succinate	0.25%
Yeast extract (Oxoid)	0.25%

After autoclaving and cooling to 50°C add :

haemin	5ug/ml
menadione	1µg/ml

Egg Yolk Agar.

Supplemented nutrient agar was prepared as above and 5% egg yolk emulsion (Oxoid) added to the cooled medium.

Blue Dextran Test Medium.

Columbia Agar Base (Oxoid) plus	
Dextran T40 (Pharmacia)	0.5%
Blue Dextran 2000 (Pharmacia)	0.5%
Glucose	0.2%
Sodium succinate	0.5%
Yeast extract (Oxoid)	0.25%
haemin	5µg/ml
menadione	1µg/ml

Charcoal-gelatin disks (modified from Kohn, 1953).

Dissolve 12.5g gelatin (Difco) in 100ml nutrient broth no. 2 (Oxoid); add 5g of finely powdered charcoal and pour into metal Petri dishes to solidify at 4°C. Hold the charcoal-gelatin in 10% formalin at room temperature for 5 days and cut into disks 1cm in diameter. Wash the disks in running tap water for 48h at 4°C and pasteurise by heating at 70°C in sterile distilled water for 20 min.

APPENDIX III

SOURCES OF REAGENTS, CHEMICALS AND ANTIBIOTICS

Sources of dehydrated media components (See Appendix II)

OXOID Ltd.,
Basingstoke,
Hants, England

DIFCO LABORATORIES
Detroit, Michigan, U.S.A.

BBL (Baltimore Biological Laboratories)
Div. Becton, Dickinson and Co.,
Cockeysville, MD21030, U.S.A.

Sources of chemicals/reagents.

Aesculin — BDH Chemicals Ltd, Poole, England.

Carbohydrates. — BDH

Filter-sterilised aqueous stock solutions contained 20% carbohydrate.

l-cysteine hydrochloride — BDH

Filter-sterilised aqueous stock solution contained l-cysteine hydrochloride 3.7%

Dextran T40 — Pharmacia Fine Chemicals, Box 604, S - 51 25 Uppsala - 1, Sweden.

Blue Dextran 2000 — Pharmacia

Ferric ammonium citrate — BDH

Ferrous sulphate — BDH

Haemin (haematin hydrochloride). — BDH

Filter-sterilised stock solution contained 500µg/ml in 0.01 N - NaOH.

Hydrochloric acid — BDH

Lauryl sulphate — Sigma Chemical Company P.O. Box 14508, St. Louis, MO.63178, U.S.A.

Menadione — Sigma

Filter-sterilised stock solution contained 100µg/ml in distilled water (see Barnes & Impey, 1971).

2 - methoxyethanol — BDH

Potassium chloride — BDH

Sodium chloride — BDH

Sodium carbonate — BDH

Sodium succinate — BDH

Sodium taurocholate — BDH

Sodium deoxycholate — BDH

Tetramethyl - p - phenylenediamine dihydrochloride. — BDH

Tris (hydroxymethyl) methylamine — BDH

Dyes

Fast Blue BB (No. F0250) — Sigma

The following were prepared as filter-sterilised aqueous stock solutions.

Brilliant green (1/800) — George T. Gurr Ltd, London SW6

Victoria blue 4R (1/800) — Gurr

Gentian violet (1/1000) — Gurr

Ethyl violet (1/800) — Gurr

Antibiotics

The following antibiotic disks were prepared in our own laboratories:

neomycin sulphate 1000µg

kanamycin sulphate 1000µg

benzyl penicillin 1.5 units

erythromycin ethyl succinate 60µg

colistin sulphate 10µg

rifampicin (Rimactane) 15µg

Vancomycin 15µg

The remaining disks were obtained from the manufacturers indicated:

neomycin 10µg kanamycin 30µg methicillin 10µg lincomycin 2µg clindamycin 2µg bacitracin 0.1 unit tetracycline 10µg	Mast Laboratories Ltd., 38 Queensland Street, Liverpool, L7 3JG.
penicillin 2 units	Oxoid
metronidazole 5µg	May & Baker Ltd., Dagenham, Essex, RM10 7XS.

Antibiotics incorporated in media

Kanamycin sulphate	Winthrop Laboratories, Surbiton-upon-Thames, Surrey, England.

The filter-sterilised stock solution contained 15,000µg/ml in distilled water.

Vancomycin	Eli Lilly & Co. Ltd., Kingsclere Road, Basingstoke, Hants.

The filter-sterilised stock solution contained 500µg/ml in distilled water.

The stock solutions were held frozen at -20°C in single-use aliquots.

Human blood.

Outdated human blood was kindly provided by the

Regional Blood Transfusion Service, Edinburgh and by the Blood Transfusion Service, Sheffield; each 500ml volume of the human-blood preparation contained 2g disodium citrate and 1.7g dextrose in 70ml water added to 430ml of whole blood.

Horse serum

Filter-sterilised horse serum - Wellcome Reagents Ltd., Beckenham, England.

APPENDIX IV

SOURCES OF REFERENCE AND REFERRED STRAINS

Several strains were re-named or re-allocated as a result of collaborative studies, some of which are reported in this thesis. These strains are given under their latest designation and their former names are given in parentheses.

Strain	Source
B. fragilis ss. *fragilis*	
NCTC9343	National Collection of Type Cultures (N.C.T.C.) Central Public Health Laboratory, Colindale Avenue, London NW9 5HT.
" 9344	
" 8560	
" 10581	
" 10584	
B. fragilis ss. *vulgatus*	
NCTC10583	N.C.T.C.
ATCC8482	Dr. E. M. Barnes, A.R.C. Food Research Institute, Colney Lane, Norwich
B. fragilis ss. *distasonis*	
ATCC8503	
B. fragilis ss. *ovatus*	
ATCC8483	
B. fragilis ss. *thetaiotaomicron*	
ATCC8492	Dr. E. M. Barnes
NCTC10582	N.C.T.C.
B. splanchnicus	
NCTC10825	N.C.T.C.
" 10826	

B. eggerthii	
NCTC11155	N.C.T.C.
B. uniformis	Dr. E. Cato & Dr. L. Holdeman, Virginia Polytechnic Institute, and State University (V.P.I.), Blacksburg, Va, 24060, U.S.A.
VPI11227	
B. variabilis	
VPI11368	
B. fragilis group	Dr. E. Sharpe & Dr. B. A. Phillips, National Institute for Research in Dairying, University of Reading.
(B. oralis) 7CM	
(B. ruminicola) B38080	Dr. T. Mitsuoka, Animal Physiology Laboratory, The Institute of Physical and Chemical Research, Wako, Saitama, 351, Japan.
B. ruminicola	
B56026	
B38024	
(B. oralis)	
NP333	Dr. J. M. Hardie, London Hospital Dental College
B. ruminicola	
C12	Dr. M. P. Bryant, Dairy Sciences Department, University of Illinois, Urbana, Illinois, U.S.A.
B. oralis	
VPI9958	V.P.I. (via Dr. E. M. Barnes)
VPI8906D	
B1/15	Dr. P. van Assche Laboratorium Voor Industriele, Gistingen, Fakulteit van de Landbouwwetenschappen, Rijksuniversiteit, 9000, Gent, Belgium.
B1.3/54	
1000	

B. oralis

	1220	Dr. Socransky
	1221	
	WAL3281	Dr. S. M. Finegold, Veterans Administration, Wadsworth Medical Centre, Los Angeles, Ca. 90073 U.S.A.

B. bivius

	VPI6318	V.P.I. via Dr. E. M. Barnes
	VPI6822	
(B. oralis)	VPI5540	
(B. oralis)	VPI7880	
	B3/36	Dr. P. van Assche
	B3/68	
	07073	Dr. H. Werner Institut fur Medizinische Mikrobiologie und Immunologie, der Universitat, 53 Bonn-Venusberg, Germany.

B. disiens

	VPI8057	V.P.I. via Dr. E. M. Barnes
	VPI7852	

B. melaninogenicus ss. melaninogenicus

	VPI4196	V.P.I.
(B. oralis)	VPI7570A	
	WAL2721	Dr. S. M. Finegold
	WAL2724	
(B. oralis)	WAL3030	
(B. oralis)	30	Dr. B. A. Phillips
(B. ruminicola)	B56007	Dr. T. Mitsuoka
(B. ruminicola)	B56020	

(B. oralis)	ATCC15930	American Type Culture Collection (A.T.C.C.) 12301 Parklawn Drive, Rockville, Md. 28052, U.S.A.
	GUI 1011	Dr. K. Ueno Department of Bacteriology, Gifu University Medical School, Tsukasa-Machi, Gifu-shi, Gifu-ken, Japan.
	GUI 1034	

B. melaninogenicus ss. intermedius

NCTC9336	N.C.T.C.
NCTC9338	
T588 (VPI9169)	Dr. J. M. Hardie

B. asaccharolyticus (B. melaninogenicus ss. asaccharolyticus

NCTC9337	N.C.T.C.
2296	Dr. R.Wiseman Bangour General Hospital, West Lothian, Scotland.
3502	
3586	

Fusobacterium necrophorum

NCTC10575	N.C.T.C.
" 10576	
" 10577	
F. necrogenes NCTC10723	
F. polymorphum NCTC10562	
F. varium NCTC10560	

Leptotrichia buccalis

NCTC10249	N.C.T.C.

L. dentium (Bacterionema matruchottii)

NCTC10206	N.C.T.C.

Clostridium clostridiiforme

(B. necrophorum) NCTC7155	N.C.T.C.

B. ochraceus

1956C	)	Dr. M. Sebald Institut Pasteur, 25 Rue du Docteur Roux, Paris.
2457B	)	
10	)	Dr. W. H. van Palenstein- Helderman, Preventive Dentistry Department, University of Uttrecht, Netherlands.
79B	)	
73	)	
VPI2845	)	V.P.I.

APPENDIX V

A NOTE ON THE RE-CLASSIFICATION OF *B. ORALIS* ATCC15930 AS *B. MELANINOGENICUS* ss. *MELANINOGENICUS*

As part of the studies on the identification and classification of gram-negative, anaerobic, non-sporing bacteria, the only currently available reference strain of *B. oralis* (ATCC15930) was obtained from the American Type Culture Collection in March 1973. This strain was studied in the series of morphological, biochemical, tolerance and antibiotic disk resistance tests described in Chapter 3. The results of these tests are shown in Chapter 3 and Appendix VII. *B. oralis* ATCC15930 was a non-motile, non-sporing, gram-negative cocco-bacillus with some short chains; occasionally a longer form was seen. Both forms were seen in preparations made from single colonies on human-blood agar. After incubation for 48h on human-blood agar the colonies were small ($<$1mm dia.), circular, convex and translucent with an entire edge. Zones of complete haemolysis were seen around the colonies after 5 days. The colonies initially became grey; 24h after haemolysis appeared, the colonies became black. Pigmentation developed 24 - 48h earlier on lysed-human-blood agar but more slowly on media containing horse blood. The black pigment was extracted from cells grown on 5% human-blood agar for 10 days. The cells were washed thrice in

distilled water and disrupted by ultrasonication; after ultracentrifugation, the pigment in aqueous solution was examined by spectrophotometry (see Chapter 2). There was a broad peak in the 370-450nm band with a maximum at 410nm; this was not found with preparations derived from the medium control. This was the same pattern that was given by pigment extracted from strains of B. melaninogenicus (Chapter 2).

In the currently accepted definition (Loesch & Gibbons, 1965; Holdeman & Moore, 1974), the species B. melaninogenicus is a gram-negative, non-notile, non-sporing, anaerobic bacillus or cocco-bacillus that produces black or brown pigmented colonies when grown on blood agar. The observation that B. oralis ATCC15930, produced black pigment when grown on human- or horse-blood agar indicates that this strain should be re-classified as B. melaninogenicus.

Holdeman & Moore (1974) differentiate between three sub-species of B. melaninogenicus (ss. melaninogenicus, ss. intermedius and ss. asaccharolyticus). The characteristics of the species B. oralis are essentially similar to the characteristics of B. melaninogenicus ss. melaninogenicus except for the property of pigment production that appears to be the sole basis for differentiation between the two species, (Loesche & Gibbons, 1965; Holdeman & Moore, 1974). The results of characterization studies with "B. oralis" ATCC15930

(see Chapter 3 and Appendix VII) show that it should be re-classified as B. melaninogenicus ss. melaninogenicus.

Sharpe (1971) reported a personal communication from Dr. M. P. Bryant that a strain derived from B. oralis ATCC15930 produced black colonies; Sharpe, however, was unable to reproduce the finding. Later observations of pigment production by strain ATCC15930 on lysed-blood agar prompted other workers to consider re-classification of the strain (E. P. Cato, 1974, personal communication). Evidence on the classification of strain ATCC15930 was presented to the ICSB Taxonomic Sub-Committee on Gram-negative anaerobic rods (Appendices VI and VII) and the Sub-committee agreed that the organism listed in the ATCC catalogue as B. oralis ATCC15930 was identical with B. melaninogenicus ss. melaninogenicus (Finegold & Barnes, 1977).

APPENDIX VI

REPORT TO THE I.C.S.B. TAXONOMIC SUB-COMMITTEE ON GRAM-NEGATIVE ANAEROBIC RODS

J. G. Collee

B. I. Duerden

W. P. Holbrook

8th May 1975

Our work has included a study of some 41 strains of black-pigmented Bacteroides-like organisms. Five reference strains, obtained from various culture collections, and thirty-six clinical isolates from various sources have been studied to date.

The sources were broken down as follows:-

Reference strains:

NCTC 9336	
NCTC 9337	
NCTC 9338	
VPI 4196	
ATCC 15930	(see enclosed reprint; Holbrook & Duerden, 1974)

Clinical isolates:

Source -		
	Abdominal wounds/abscesses	4 strains
	Oral wounds/abscesses	3 strains
	Vincent's infection	1 strain
	Gingival crevice scrapings	16 strains
	Root canal of tooth	1 strain
	High vaginal swab	8 strains
	Infected catheter	1 strain
	Routine laboratory specimens from unknown sources	2 strains

We have studied these organisms in (i) a variety of conventional biochemical tests; (ii) tolerance tests with bile salts and dyes incorporated in a solid basal medium; and (iii) antibiotic disk sensitivity tests with fourteen antibiotics. The materials and methods that we used are given in the enclosed reprint (Duerden,

1975). At this stage we are reporting our findings to you for inclusion in the Committee's deliberations. We shall shortly submit a paper recording our observations in tests with Bacteroides melaninogenicus and other gram-negative anaerobic bacilli and we shall give our views on the classification of these organisms. With this in mind, any early comment from you in relation to our findings would be most welcome.

TABLE VI.1. Results of biochemical tests with black-pigmented Bacteroides organisms

Test	Mi	Mii	Miii	Miv	Mv	Mvi	Mvii	Mviii	Mix	Mx	Mxi
Motility	-	-	-	-	-	-	-	-	-	-	-
Aerobic growth	-	-	-	-	-	-	-	-	-	-	-
Oxidase production	-	-	-	-	-	-	-	-	-	-	-
Catalase production	-	-	-	-	-	-	-	-	-	-	-
H_2S production	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	-	-	-	-	-	-	-	-	-	+	+
Haemolysis on blood agar	+	+	+	+	+	+	+	+	+	+	+
Lipase production on EYA	+	-	+	+	-	-	-	-	-	-	-
Digestion of gelatin	+	+	+	+	+	+	+	+	+	-	+
Indole production	-	-	+	+	+	+	-	+	-	-	+
Hydrolysis of aesculin	+	-	-	-	-	-	-	-	-	-	-
Fermentation of:											
glucose	+	+	+	+	+	+	+	-	-	+	-
lactose	+	+	+	-	-	+	+	-	-	+	-
maltose	+	+	+	+	+	+	+	-	-	-	-
sucrose	+	+	+	+	+	+	+	-	-	+	-
rhamnose	-	-	-	-	-	-	+	-	-	-	-
trehalose	-	-	-	-	-	-	-	-	-	-	-
mannitol	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis of dextran	+	+	-	-	-	-	-	-	-	-	-
Black pigmentation on blood agar	+	+	+	+	+	+	+	+	+	+	+
Reference strains	ATCC 15930	VPI 4196		NCTC 9338	NCTC 9336			NCTC 9337			
No. of laboratory isolates	0	0	1	11	1	4	0	7	4	1	1
Total no. of strains in group	1	1	1	12	2	4	1	8	4	1	1

N.B. 5 laboratory isolates do not grow on EYA and are not included in these results.

TABLE VI.2. Results of tolerance tests with black-pigmented Bacteroides species

Test	A	B	C	D
Growth on basal medium	+	+	+	NG
Growth on basal medium plus				
0.5% taurocholate	I	I	I	NG
0.1% deoxycholate	I	I	I	NG
0.5% taurocholate + 0.1% deoxycholate	I	I	I	NG
Victoria Blue 4R (1:80,000)	I	+	+	NG
Ethyl Violet (1:80,000)	I	I	I	NG
Gentian Violet (1:100,000)	I	I	I	NG
Brilliant Green (1:80,000)	I	I	+	NG
Reference Strains	NCTC 9336			
	NCTC 9337			
	NCTC 9338			
	VPI 4196			
	ATCC 15930			
Laboratory isolates	29	1	1	5
Total number of strains	34	1	1	5

+ = growth

I = inhibition of growth

NG = organisms did not grow on the basal medium

TABLE VI.3. Results of antibiotic-disk-sensitivity tests with black-pigmented Bacteroides organisms

Antibiotic (Content per disk)	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Neomycin (1000µg)	S	R	R	R	R	R	S	S	S	S	S	S	S	S
Neomycin (10µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kanamycin (1000µg)	R	R	R	R	R	R	R	R	R	R	R	R	S	S
Kanamycin (30µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Penicillin (1.5 unit)	R	S	S	S	S	S	S	S	S	S	S	S	R	S
Methicillin (10µg)	R	S	S	S	S	S	S	S	S	S	S	S	R	S
Erythromycin (60µg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Colistin (10µg)	S	R	R	R	S	R	S	R	S	R	S	S	S	S
Rifampicin (15µg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Lincomycin (2µg)	S	S	S	S	S	S	S	S	S	S	S	S	R	S
Clindamycin (10µg)	S	S	S	S	S	S	S	S	S	S	S	S	R	S
Bacitracin (0.1 unit)	S	S	S	S	S	R	S	S	R	R	R	S	R	S
Vancomycin (15µg)	R	R	S	R	S	S	R	S	R	S	R	S	S	R
Chloramphenicol (10µg)	S	S	S	S	R	S	S	S	S	S	S	S	S	R
Reference strains							NCTC 9336, NCTC 9338	NCTC 9337		ATCC 15930	VPI 4196			
No. of laboratory isolates	2	1	1	1	1	1	15	4	1	2	1	3	1	2
Total number of strains	2	1	1	1	1	1	17	5	1	3	2	3	1	2

APPENDIX VII

REPORT TO THE I.C.S.B. TAXONOMIC SUB-COMMITTEE ON GRAM-NEGATIVE ANAEROBIC RODS

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11th May 1976

Introduction

As part of the collaborative investigation instigated by the Taxonomic Sub-committee on gram-negative anaerobic rods, 40 strains of Bacteroides melaninogenicus ss. melaninogenicus, B. oralis and B. ochraceus were subjected to a series of tests. These strains included the 12 referred to us by the sub-committee, 6 strains referred by other colleagues and 22 strains isolated from subgingival dental plaque in our laboratory. This work forms part of our continuing studies with B. melaninogenicus and related organisms.

Materials and Methods

Organisms. Bacteroides melaninogenicus ss. melaninogenicus strains WAL 2721* and WAL 2724* (Dr. S. M. Finegold), GUI 1011* and GUI 1034* (Dr. K. Ueno), VPI 4196 (Dr. E. Cato, V.P.I.) and 15 strains isolated from subgingival dental plaque in our laboratory. Bacteroides oralis strains VPI 7570A* and VPI 5832* (V.P.I., Blacksburg, Va.), J1*, 7CM* and 30* (Dr. E.

Sharpe), ATCC15930* (American Type Culture Collection), NP 333 (Dr. J. M. Hardie, London Hospital Medical College), and 7 strains isolated from subgingival dental plaque in our laboratory.

Bacteroides ochraceus (Ristella ochraceus) strains 1956C* and 2467B* (Dr. M. Sebald), VPI 2845 (V.P.I., Blacksburg, Va.), 10, 79B and 73 (Dr. W. H. van Palenstein-Helderman, Preventive Dentistry Dept., University of Utrecht, Netherlands).

Characterization of Strains

The 40 test strains were subjected to the following series of morphological, biochemical, tolerance, and antibiotic disk resistance tests (for details of media and methods, see Duerden et al.,1976) and to gas-liquid chromatographic (G.L.C.) analysis of their short chain fatty acid metabolic products of glucose.

Morphological and biochemical tests: Microscopic and colonial morphology; haemolytic effect on blood agar; pigment production; motility; lipase activity; oxidase test; catalase test; hydrogen sulphide production; indole production; gelatinase test; aesculin hydrolysis; fermentation of glucose, lactose, sucrose, maltose, rhamnose, trehalose, and mannitol.

Tolerance tests: Growth in the presence of (i) the bile salts sodium taurocholate, sodium deoxycholate, and the combination of sodium taurocholate plus sodium deoxycholate, and (ii) the dyes brilliant green, Victoria blue 4R, gentian violet and ethyl violet.

*Strains referred by the I.C.S.B, Taxonomic Sub-Committee.

Antibiotic disk resistance tests: Resistance to disks containing neomycin 1000µg and 10µg, kanamycin 1000µg and 30µg, penicillin 1.5 units, methicillin 10µg, erythromycin 60µg, colistin 10µg, rifampicin 15µg, lincomycin 2µg, clindamycin 2µg, bacitracin 0.1 unit, vancomycin 15µg, chloramphenicol 10µg, tetracycline 10µg and metronidazole 5µg.

G.L.C. analysis of short-chain fatty acids. Medium: The strains were grown for 48h in a glucose-containing medium (PPYG) containing: Proteose peptone (Oxoid) 2%; yeast extract (Difco) 1%; and NaCl 0.5%. Filter-sterilized solutions of the following heat-labile supplements were added aseptically to the (cooled) autoclaved basal medium (pH 7.4) to give final concentrations of: inactivated horse serum, 2%; glucose, 1%; Haemin, 5µg/ml; menadione 1µg/ml; cysteine hydrochloride, 0.75%, and Na_2CO_3, 0.04%. The pH at inoculation was 7.1 ± 0.1. The inoculum was one drop (0.02ml) of a 48h culture in cooked-meat broth.

Chromatograph. A Pye-Unicam series 104 gas chromatograph fitted with heated injection ports and dual flame-ionization detectors was operated isothermally at 190°C with a detector temperature of 250°C. The carrier-gas was oxygen-free nitrogen at a flow-rate of 35ml/min. and the hydrogen flow-rate of both detectors was adjusted for optimum sensitivity. The instrument was fitted with two identical glass columns (1.5m x 4mm) containing Chromosorb 101 (Johns-Manville Corp., U.S.A; supplied by Gas Chromatography Services Ltd., 23 Old Chester Road,

Lower Bebington, Wirral, Merseyside, L63 7LA). Columns were packed in the laboratory. The recorder was a Servoscribe 1S model 541.20 (Belmont Instruments, 6 Belmont Drive, Giffnock, Glasgow, G46 7PA) set at the 10 mV range with a recorder speed of 120mm/h.

Analysis. The procedures used were derived from those of Carlsson (1973). (a) Volatile acids. Cultures were acidified with 50% sulphuric acid to pH 2.0 and a 0.6 µl sample of cell-free supernatant was injected directly on to the analysing column without further pre-treatment or extraction. Contamination of the top 2-3cm of the column occurred during use and required periodic replacement with fresh polymer. The attenuation setting was 2×10^{-2} at the x 1 range setting.

(b) Non-volatile acids. The acidified culture supernates were methylated according to the method of Holdeman & Moore (1972) and a 0.6 µl sample of the chloroform extract was injected on to the column under the same analysis conditions as for the volatile acids but with an attenuation setting of 5×10^{-2}.

Lactic and succinic acids were detected qualitatively in the analysis of volatile acids, but this was confirmed quantitatively by the methylation procedure.

Standards: Single and combined 0.01 M aqueous standards of the volatile acids were used to establish absolute and relative retention times; 0.02 M standards were used in the analysis of the non-volatile acids. Samples of uninoculated (sterile) PPYG medium were included as

controls in every batch of each of the two types of analysis. A PPYG control and a combined acid standard were used to monitor retention times and the sensitivity settings of the instrument as a routine each day.

The approximate concentration values of acids for test samples were calculated by comparing the corrected peak heights of the test samples with those of the appropriate acid standards. Results were recorded as follows:

Volatile acids.

Concentration value	> 10 µM/ml	: ++
" "	1.1 - 10 µM/ml	: +
" "	0.2 - 1.0 µM/ml	: tr (trace)
" "	< 0.2 µM/ml	: -

Lactic and succinic acids.

Concentration value	> 20 µM/ml	: ++
" "	10 - 20 µM/ml	: +
" "	1 - 9 µM/ml	: tr
" "	< 1µM/ml	: -

Results

The results of morphological, biochemical, tolerance and antibiotic disk resistance tests with the 40 test strains are shown in table 1. The proportion of strains that gave each result are shown in table 2, and the results obtained with the 12 strains referred by the I.C.S.B. Taxonomic Sub-committee are shown in table 3.

Preliminary assignment of test strains to groups

The test strains submitted as B. melaninogenicus ss.

TABLE VII.2. Percentage of specified results obtained with the three groups of test strains

Test	Result	% of test strains with the given result				
		B.mel. ss.mel. (23)	B.oralis (11)	B.och-raceus(6)	all non-pigmented strains(17)	all strain (40)
Growth in $O_2 + CO_2$	+	0	0	100	35.3	15
Pigment production	+	100	0	0	0	57.5
Tolerance tests:						
Taurocholate (T)	I	100	90.9	83.3	94.1	95
Deoxycholate (D)	I	100	100	100	100	100
T D	I	100	100	100	100	100
Victoria Blue 4R	I	91.3	54.5	33.3	47.1	72.5
ethyl violet	I	100	90.9	100	94.1	97.5
gentian violet	I	100	100	100	100	100
brilliant green	I	100	100	100	100	100
Antibiotic disk resistance tests:						
neomycin (1000µg)	S	95.7	90.9	100	94.1	95
kanamycin (1000µg)	R	100	100	100	100	100
rifampicin	S	100	100	100	100	100
penicillin	S	78.3	90.9	100	94.1	85
methicillin	S	78.3	90.9	66.6	82.4	80
metronidazole	S	91.3	90.9	100	94.1	92.5
tetracycline	S	69.6	100	100	100	82.5
colistin	S	65.2	36.4	0	23.5	47.5
bacitracin	R	100	100	100	100	100
vancomycin	R	95.7	100	100	100	97.5
Biochemical tests:						
H_2S production	+	91.3	81.8	100	88.2	90
Haemolysis	+	100	81.8	0	52.9	80
Indole production	+	0	0	0	0	0
Aesculin hydrolysis	+	52.2	90.9	83.3	88.2	67.5
Dextran hydrolysis	+	65.2	45.5	100	64.7	65
Gelatin digestion	+	65.2	27.3	0	17.7	45
Lipase production	+	34.8	0	0	0	20

TABLE VII.2. Cont'd

Test	Result	% of test strains with the given result				
		B.mel. ss.mel. (23)	B.oralis (11)	B.och-raceus(6)	all non-pigmented strains(17)	all strains (40)
Fermentation of:						
glucose	+	100	100	100	100	100
lactose	+	100	100	100	100	100
maltose	+	100	100	100	100	100
sucrose	+	100	100	100	100	100
rhamnose	+	4.3	63.6	0	41.2	20
trehalose	+	0	0	0	0	0
mannitol	+	0	0	0	0	0
G.L.C. analysis:						
acetic	+	0	18.2	66.7	35.3	15
"	++	100	81.8	33.3	64.7	85
propionic	-	13	36.4	50	41.2	25
"	tr	34.8	36.4	16.7	29.4	32.5
"	+	52.2	27.2	33.3	29.4	42.5
iso-butyric	-	82.6	100	100	100	90
"	tr	17.4	0	0	0	10
n-butyric	-	100	100	100	100	100
iso-valeric	-	21.7	63.6	83.3	70.6	42.5
"	tr	69.5	36.4	16.7	29.4	52.5
"	+	8.7	0	0	0	5
n-valeric	-	100	100	100	100	100
lactic	-	8.7	63.6	83.3	70.6	35
"	tr	69.6	18.2	16.7	17.6	47.5
"	+	17.4	18.2	0	11.8	15
"	++	4.3	0	0	0	2.5
succinic	+	39.1	45.5	50	47.1	42.5
"	++	60.9	54.5	50	52.9	57.5

melaninogenicus, B. oralis and B. ochraceus were divided into three groups for this analysis:

(i) Strictly anaerobic strains that produced black or brown pigmented colonies when grown on lysed-human-blood agar for up to one week were assigned to one group labelled B. melaninogenicus ss. melaninogenicus (23 strains).

(ii) Strictly anaerobic, non-pigmented strains - B. oralis (11 strains). Three strains (ATCC 15930*, VPI 7570A, and 30) were referred to us as strains of B. oralis but produced black or brown pigmented colonies and are included in our B. melaninogenicus group (above).

(iii) Non-pigmented strains that were able to grow in 10% CO_2 in air - B. (Ristella) ochraceus (6 strains).

Morphology

Microscopic appearance. All strains were gram-negative bacilli and many were pleomorphic. B. melaninogenicus ss. melaninogenicus and B. oralis strains were predominantly cocco-bacilli, occasionally arranged in short chains. B. ochraceus strains were long, slender bacilli with rounded or tapered ends and often with a central oval swelling.

Colony morphology. The colonies of B. melaninogenicus ss. melaninogenicus were 1-2mm diameter, round, convex and opaque. After incubation for 48h, they were typically light grey, becoming brown after further

*See Holbrook & Duerden (1974)

incubation. The pigmentation varied between strains from light brown to almost black.

Colonies of B. oralis were 1-2mm diameter, round, convex, opaque and grey; they tended to coalesce. After incubation for 7 days, the colonies of some strains (including VPI 5823) became light brown and were difficult to distinguish from the lighter-pigmented strains of B. melaninogenicus ss. melaninogenicus.

B. ochraceus strains typically produced two colony types:
(a) 1 mm diameter, round or with an irregular edge, smooth, opaque, and blue-grey: (b) 1 mm diameter, rhizoid, granular and ochre in colour.

Tolerance tests

(a) Bile salts. All strains of B. melaninogenicus ss. melaninogenicus and B. ochraceus were completely inhibited by the test concentrations of sodium taurocholate, sodium deoxycholate, and the combination of the two salts. Ten strains of B. oralis were also inhibited, but the growth of strain 7CM was not inhibited by either of the bile salts alone or by the combination.

(b) Dyes. All test strains were completely inhibited by the test concentrations of gentian violet and brilliant green, and only B. oralis strain 7CM was able to grow in the presence of ethyl violet. Twenty-one strains of B. melaninogenicus ss. melaninogenicus, 6 strains of B. oralis (including VPI 5832) and 2 of B. ochraceus (strains 1956C and

VPI 2845) were completely inhibited by Victoria blue 4R; however, 2 strains of B. melaninogenicus ss. melaninogenicus (WAL 2724 and one laboratory isolate), 5 strains of B. oralis (7CM, J1, NP333 and 2 laboratory isolates), and 4 strains of B. ochraceus (2467B, 10, 79B and 73) were able to grow in the presence of Victoria blue 4R.

Biochemical tests

None of the test strains produced indole, catalase, or oxidase. All test strains produced acid from glucose, lactose, sucrose and maltose, but not from trehalose or mannitol.

Production of H_2S. Most strains produced H_2S, but some produced only small amounts and production by 2 strains of B. melaninogenicus ss. melaninogenicus and 2 strains of B. oralis (all laboratory isolates) was not detected.

Haemolysis. All strains of B. melaninogenicus ss. melaninogenicus produced some degree of haemolysis on human-blood agar. In most cases this was a zone of complete haemolysis around the colonies, but a number of strains, including WAL 2721, WAL 2724, GUI 1034 and VPI 7570A produced only a small zone of incomplete haemolysis. Two strains of B. oralis (J1 and 7CM) produced clear zones of complete haemolysis, 7 strains (including VPI 5832 and NP333) produced small zones of incomplete haemolysis and 2 laboratory isolates were non-haemolytic. None of the B. ochraceus strains were haemolytic.

Rhamnose fermentation. Six strains of B. oralis (including J1, 7CM, VPI 5832 and NP333), but no strains of B. ochraceus and only one laboratory isolate of B. melaninogenicus ss. melaninogenicus produced acid from rhamnose.

Aesculin hydrolysis. Twelve of the 23 strains of B. melaninogenicus ss. melaninogenicus (strains ATCC 15930, GUI 1011, GUI 1034, VPI7570A, 30 and 7 laboratory isolates), all of the test strains of B. oralis except 7CM, and all strains of B. ochraceus except 1956C hydrolysed aesculin.

Dextran hydrolysis. Fifteen strains of B. melaninogenicus ss. melaninogenicus (including VPI 4196, ATCC 15930, WAL 2724 and 30), all strains of B. ochraceus, and 5 strains of B. oralis (including NP333) hydrolysed dextran.

Gelatin digestion. Fifteen strains of B. melaninogenicus ss. melaninogenicus (including VPI 4196, VPI 7570A, ATCC 15930, GUI 1011 and GUI 1034), but only 3 laboratory isolates of B. oralis, digested gelatin disks. The other 8 strains of B. oralis and all 6 strains of B. ochraceus failed to digest the disks.

Lipase production. Strain 30 and seven other strains of B. melaninogenicus ss. melaninogenicus produced a lipase effect. This effect was not produced by any strain of B. oralis or B. ochraceus.

Antibiotic-disk-resistance tests

All test strains were sensitive to erythromycin, rifampicin, lincomycin, clindamycin and

chloramphenicol. They were all resistant to neomycin (10µg), kanamycin (1000µg and 30µg) and bacitracin. Two strains, nos. 30 and 7CM, were resistant to neomycin (1000µg), but all other strains were sensitive. All strains except ATCC 15930 were resistant to vancomycin. Five of the 6 strains of B. ochraceus were resistant to metronidazole; strain VPI 2845 was sensitive to metronidazole, but produced a smaller zone around the disk than the strictly anaerobic bacteroides organisms. All strains of B. oralis and B. ochraceus were sensitive to tetracycline, but 7 strains of B. melaninogenicus ss. melaninogenicus were resistant. Five strains of B. melaninogenicus ss. melaninogenicus (including WAL2721) were resistant to penicillin. Only one strain of B. oralis (7CM) was resistant to penicillin, and all strains of B. ochraceus were sensitive. The five strains of B. melaninogenicus ss. melaninogenicus and one of B. oralis (7CM) were also resistant to methicillin. Two strains of B. ochraceus (10 and 79B) were resistant to methicillin, but sensitive to penicillin. Fifteen strains of B. melaninogenicus ss. melaninogenicus (including VPI 4196, VPI 7570A, WAL 2724, GUI 1011 and GUI 1034) and 4 strains of B. oralis (including NP333) were sensitive to colistin, but all strains of B. ochraceus were resistant.

G. L. C. analysis

All the test strains produced acetic acid. All strains

of B. melaninogenicus ss. melaninogenicus and all except two strains of B. oralis produced moderate amounts (++) but only two strains of B. ochraceus (1956C and 2467B) produced this amount; the remaining two strains of B. oralis (VPI 5832 and 7CM) and 4 strains of B. ochraceus produced minor amounts (+) of acetic acid.

Twelve strains of B. melaninogenicus ss. melaninogenicus, 3 strains of B. oralis and 2 strains of B. ochraceus produced minor amounts of propionic acid; a further 8 strains of B. melaninogenicus ss. melaninogenicus, 4 strains of B. oralis and one strain of B. ochraceus produced trace amounts.

No strains of B. oralis or B. ochraceus produced iso-butyric acid, and only 4 strains of B. melaninogenicus ss. melaninogenicus produced trace amounts of this acid. No strain produced n-butyric acid.

Two strains of B. melaninogenicus ss. melaninogenicus (WAL 2721 and a laboratory isolate) produced minor amounts of iso-valeric acid; a further 16 strains of B. melaninogenicus ss. melaninogenicus and 4 strains of B. oralis but only one strain of B. ochraceus produced trace amounts of this acid. No strain produced n-valeric acid.

One laboratory isolate of B. melaninogenicus ss. melaninogenicus produced a moderate amount of lactic acid; 4 strains produced minor amounts and 16 strains produced trace amounts of this acid. Two strains of

B. oralis produced minor amounts and two produced trace amounts of lactic acid. Strains of B. ochraceus, however, did not produce lactic acid except for a trace amount produced by strain 1956C.

All strains produced succinic acid; 14 strains of B. melaninogenicus ss. melaninogenicus, 6 strains of B. oralis and 3 strains of B. ochraceus produced moderate amounts and the remainder produced minor amounts of this acid.

The patterns of results indicate that differences between "-" and "trace" or "trace" and "+" results are probably less significant than differences between "-", "+" and "++" results.

Comments

As a result of these studies with 40 test strains of B. melaninogenicus ss. melaninogenicus, B. oralis and B. ochraceus, the three groups were distinguished as follows:

B. melaninogenicus ss. melaninogenicus strains were strict anaerobes that produced brown, or occasionally black colonies on media containing blood. They produced acid from glucose (and lactose, sucrose and maltose). They did not produce indole. In general, in disk tests, they were sensitive to neomycin (1000µg disk), rifampicin and metronidazole, and they were resistant to kanamycin (1000µg disk). They were inhibited in tolerance tests with two bile salts (separately and in combination), and they were inhibited by each of the four test dyes. Most strains

were sensitive to penicillin. The results of tests for hydrolysis of aesculin and dextran, gelatin liquefaction, lipase production, and resistance to colistin varied between strains; there was no apparent relationship between the results of these tests.

In G.L.C. analysis, the test strains of this subspecies produced moderate amounts of acetic and succinic acids from glucose; propionic, iso-valeric and lactic acids were variable minor products.

B. oralis strains were strict anaerobes that failed to produce pigment, although the colonies of some strains on lysed-blood agar became pale brown after incubation for 7 days. They were essentially similar to B. melaninogenicus ss. melaninogenicus strains and four laboratory isolates were indistinguishable from B. melaninogenicus ss. melaninogenicus except that they did not produce pigment. The four referred strains of B. oralis and three laboratory isolates were distinguished by the ability to produce acid from rhamnose and five of these strains were able to grow in the presence of Victoria blue 4R. The results of G.L.C. analysis were generally indistinguishable from the results obtained with B. melaninogenicus ss. melaninogenicus strains.

B. ochraceus strains were clearly distinguished by their ability to grow in air plus CO_2. They produced acid from glucose (and lactose, sucrose and maltose) but not from rhamnose, and did not produce indole. They were generally sensitive to disks of neomycin

(1000µg) and rifampicin, but resistant to metronidazole, kanamycin (1000µg), and colistin. They hydrolysed aesculin (except one strain) and dextran. In G.L.C. analysis, they produced smaller amounts of acetic acid than the B. melaninogenicus ss. melaninogenicus and B. oralis groups; propionic acid was a variable minor product; they did not produce lactic, iso-butyric or iso-valeric acids.

The work reported here was financed by the Medical Research Council (MRC Grant No. G974/325B to Professor J. G. Collee). These results are conveyed to the members of the I.C.S.B. Taxonomic Sub-committee on gram-negative anaerobic rods on the understanding that the Edinburgh team will submit a paper incorporating the data for publication in a scientific journal in the near future.

(Tables VII.1 and VII.3 are contained in the pocket inside the back cover of this volume).

APPENDIX VIII

A comparison of strains belonging to the Bacteroides oralis-ruminicola-melaninogenicus Group

Report to the International Commission for Systematic Bacteriology, Taxonomic Sub-committee on Gram-negative anaerobic rods.

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June, 1978

The I.C.S.B. Taxonomic Sub-committee on Gram-negative anaerobic rods instigated a collaborative study of strains belonging to the oralis-ruminicola-melaninogenicus group of bacteroides. The 22 strains examined in our laboratories are shown in table 1.

In addition, we failed to recover two strains - B.oralis 13777/2 (from Dr. Werner) and strain D46 (from Dr. Bryant) - from the cultures sent to us.

METHODS

All strains were tested independently in two laboratories (Edinburgh and Sheffield) except that G.L.C. analysis of the fatty acid end products of metabolism was only carried out in the Edinburgh laboratory. The test strains were studied in a series of morphological, tolerance, antibiotic disk resistance, and conventional biochemical tests, and by G.L.C. analysis of metabolic end products. (For details of media and methods, see our 1976 report; Duerden et al., 1976; Holbrook et al., 1977; and Deacon et al., 1978.)

Morphology. Colonial morphology was observed on human blood agar (Oxoid Columbia Agar Base) and microscopic morphology in Gram-stained smears from growth on blood agar and in cooked-meat broth. Haemolysis was observed after growth on blood agar and pigment production was determined by growth on lysed-human-blood agar or BM-lysed-blood agar.

Tolerance Tests. The test strains were examined for growth in the presence of two bile salts and four dyes. Aqueous stock solutions (10 x final conc. of sod. taurocholate and 100 x final conc. of others) of these substances were added aseptically

to (cooled) autoclaved nutrient agar base.

The preparations used in the two laboratories differed. In the Edinburgh laboratory, stock solutions of Victoria blue 4R and sodium taurocholate were sterilised by autoclaving and the final concentrations were: taurocholate 0.5%; deoxycholate 0.13%; Victoria blue 4R 1/140,000; ethyl violet 1/90,000; brilliant green 1/300,000; gentian violet 1/150,000.

In the Sheffield laboratory, the stock solutions were sterilised by membrane filtration and the final concentrations were: taurocholate 0.5%, deoxycholate 0.1%; Victoria blue 4R 1/80000; ethyl violet 1/80000; brilliant green 1/80000; gentian violet 1/100000.

The effects of different methods of preparation on the results of tolerance tests will be published shortly (Brown, Brown, Collee and Watt).

Antibiotic disk resistance tests. Five antibiotic disks were used and the tests were carried out on blood agar. The disks were: metronidazole 5µg, neomycin 1000µg, kanamycin 1000µg, penicillin 1.5 or 2 units, and rifampicin 15µg.

Biochemical tests. The strains were studied in tests for production of H_2S, oxidase, catalase, dextranase, lipase and indole, hydrolysis of aesculin, digestion of gelatin, reduction of nitrate, stimulation by bile, and fermentation of glucose, lactose, maltose, sucrose, rhamnose, trehalose, mannitol, arabinose and xylose.

Our methods differed from our previous reports as follows:

(i) the fermentation test medium used in one laboratory (Sheffield) was a modified BM broth containing Trypticase 1%, Proteose peptone 1%, yeast extract 0.5%, sodium chloride 0.5%, sodium succinate 0.25%, haemin 5µg/ml, menadione 1µg/ml and l-cysteine hydrochloride 0.075%.

(ii) the medium for bile stimulation test was PPYG medium containing Proteose peptone 2%, yeast extract 1%, sodium chloride 0.5%, haemin 5µg/ml, menadione 1µg/ml, l-cysteine hydrochloride 0.075%, sodium carbonate 0.04%, glucose 1%, ox-bile 2%.

(iii) tests for the reduction of nitrate were carried out in nitrate test medium (BBL).

G.L.C. analysis. Details of the test medium, the methods and the interpretation of results were given in the previous report (1976); see also Deacon et al (1978). The column packing and he operating conditions were changed for the present series. The column packing was 15% SP 1220/1% H_3PO_4 on 100/120 mesh Chromosorb W AW (Supelco) in dual 1.5 m x 4mm glass columns; the operating temperature was 150°C and the N_2 carrier-gas flow rate was 35 ml/min.

RESULTS

The results obtained with 21 of the test strains are shown in table 2a, b and c. The strains within the test group gave broadly similar patterns but there were differences between the strains in a small number of tests. When the

patterns of results were assmbled on the basis of "most similar patterns" they could be separated into five sub-groups, although there was some overlap of results with a few tests and the differences between sub-groups were only small.

sub-group a: strains WPH61, NP333, C12, B56029 and B38024.

sub-group b: strains VPI9958, VPI8906D, WAL3281, 1210, 1221 and WPH179.

sub-group c: strains WAL3030, B56007 and B56020.

sub-group d: strains VPI6318, VPI6822, 5540, 7880 and 07073.

sub-group e: strains VPI8057 and VPI7852.

Strain B38080 has been excluded from this general analysis and the subsequent description of results because different strains were received by the two laboratories, but the results obtained with the strain received in Edinburgh are shown in table 2a, b and c. The results obtained with the strain received in Sheffield are shown in table 3; it does not belong to the melaninogenicus/oralis/ruminicola group and has the characteristics of B.vulgatus.

Morphology. The colonial morphology of the test strains was essentially similar and of no help in distinguishing between them except that three strains (WAL3030, B56007 and B56020) produced dark brown pigmented colonies after incubation for 7 days on lysed-human-blood agar. After 2 days' incubation on blood agar, all strains produced small (0.5-1.5mm) circular (except for strain 5540 that had an irregular colony), convex, semi-opaque, grey colonies that were either non-haemolytic or gave a narrow zone of incomplete haemolysis. Many strains showed incomplete haemolysis after 7 days' incubation.

There were no significant differences in microscopic morphology between the strains except for strain 1221. All other strains were small pleomorphic Gram-negative bacilli; smears prepared from growth on blood agar showed many cocco-bacilli, often in pairs or short chains, and short filamentous forms; films from broth cultures were less pleomorphic. Smears prepared from strain 1221, however, showed larger Gram-negative bacilli with many filaments and few cocco-bacilli.

Tolerance Tests. All the test strains were inhibited by the bile salts (separately and combined) and by the dyes ethyl violet, brilliant green and gentian violet. Eight strains were consistently tolerant of Victoria blue 4R, and a further five strains gave different results in the two laboratories. However, the result of these tolerance tests did not correlate with particular patterns of results in other tests and were not helpful in dividing the test strains into distinct groups.

Antiobiotic disk resistance tests. All test strains were sensitive to the metronidazole and rifampicin disks and resistant to the kanamycin disk. Most strains were sensitive to the neomycin disk but both strains referred as B.bivius (VPI6318 and VPI6822), and strain 7880 (all in sub-group d) were only relatively sensitive (R/S; zone diameter 14-15mm). Thirteen strains were sensitive to the penicillin disk but eight strains were resistant.

Biochemical tests. All except two strains produced H_2S and none of the strains produced oxidase, catalase, lipase or indole; none were stimulated by bile. Most strains digested the gelatin disks readily but five strains (NP333, WPH61, WPH179, 1221 and B38024) did so only weakly after 14 days' incubation and the

results with strains 1210 and B38024 were recorded as negative in one laboratory. Fourteen strains (sub-groups a, b and c) hydrolysed aesculin but the remaining seven strains (sub-groups d and e) gave negative results in this test. None of the strains allocated to sub-groups d and e produced dextranase but seven strains in the other sub-groups gave positive results in this test.

Fermentation Tests. All test strains fermented glucose and maltose and none fermented trehalose. Only the two strains in sub-group e failed to ferment lactose and the seven strains in sub-groups d and e failed to ferment sucrose. Strains allocated to sub-group a fermented rhamnose, except for strain B38024; the only other strain to ferment this carbohydrate was strain VPI9958.

Only two strains fermented mannitol - B56007 and B56020 that were referred as strains of B.ruminicola. Strains allocated to sub-group a fermented xylose and arabinose, except strain B56029 that failed to ferment arabinose. Strain WAL3281 gave a late but consistent positive result in fermentation tests with xylose in one laboratory but a negative result in the other. None of the other test strains fermented these pentoses.

G.L.C. profiles. All test strains gave broadly similar profiles and no distinctive patterns were found. All produced acetic and succinic acids as major products with variable traces of propionic, iso-buytric, iso-valeric and lactic acids. n-Butyric acid was not produced by any strain.

N.B. The results obtained with strains VPI5832 and J1 in the previous study (Report, 1976) are shown in table 4.

NOTES

1. The melaninogenicus/oralis/ruminicola group are a recognisable group of related organisms that can be clearly distinguished from other groups of Gram-negative anaerobic bacilli in the family Bacteroidaceae.
2. All strains within the group tested share many properties. There were differences between the strains in a small number of tests but without data from a wider range of tests, including more sophisticated approaches, the taxonomic significance of differences in some fermentation test results is uncertain. We do not think that the results of a few fermentation tests consistute sufficient evidence to classify the strains within this group as separate species without the support of further evidence from genetic, enzyme and serological studies and cell constituent analyses. There is a spectrum of patterns of results obtained in our tests with the strains examined, and although five sub-groups are tentatively described, differences between them are small and the results of some tests did not conform to these divisions.
3. Classically, B.melaninogenicus is distinguished by pigment production, but it is debatable whether pigment production has major taxonomic significance (see Holbrook et al., 1977). The use of this observation as the prime distinguishing characteristic has already been over-ruled by excluding B.asaccharolyticus from the species. If we were to follow the presently recognised species descriptions, three of the test strains would be designated B.melaninogenicus ss. melaninogenicus (WAL3030, B56007 and B56020); however,

strains in the melaninogenicus/oralis/ruminicola group are very similar in other respects and many strains in the group produce light brown colonies after prolonged incubation on lysed-blood agar, so that it may be difficult to define the presence of pigmentation with certainty.

4. The similarities between strains in this group that have been given different names is emphasised by the results obtained with three of the test strains. Strains 5540, 7780 and 07073 were referred as strains of B.oralis, but gave patterns of results that were almost identical with those of the two strains referred as B.bivius.

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Table 1

Strains		Source
B.oralis	VPI9958	Dr. W.E.C. Moore, Anaerobic Laboratory, Virginia Polytechnic Institute, Blacksburg, Va., U.S.A. (via Dr. Ella M. Barnes)
"	VPI8906D	
"	WAL3030	Dr. S.M. Fingold, Wadsworth Hospital Center, Los Angeles, California, U.S.A.
"	WAL3281	
"	07073	Dr. H. Werner, Institut fur Medizinische Mikrobiologie und Immunologie, der Universitat, 53 Bonn-Venusberg, Germany.
"	7880	
"	5540	
"	WPH179	Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh. EH8 9AG
"	1210	Dr. Socransky
"	1221	(via Dr. Ella M. Barnes)
B.bivius	VPI6318	VPI (via Dr. Ella M. Barnes)
"	VPI6822	
B.disiens	VPI7852	
"	VPI8057	
B.ruminicola	B38024	Dr. T. Mitsuoka, Animal Physiology Laboratory, The Institute of Physical and Chemical Research, Wako, Saitam 351, Japan.
"	B38080	
"	B56007	
"	B56020	
"	B56029	
"	C12	Dr. M.P. Bryant, Dairy Sciences Department, University of Illinois, Urbana, Illinois, U.S.A.
"	NP333	Dr. J.M. Hardie, London Hospita Dental Collegel
"	WPH61	Department of Bacteriology, University of Edinburgh Medical School.

ACKNOWLEDGEMENTS

I am especially grateful to Professor J. G. Collee for his enthusiastic advice and encouragement throughout the studies described in this thesis, particularly for his guidance during the early studies that were carried out in his laboratory, and also for his helpful criticism of the draft chapters of the thesis. I am also grateful to Professor M. G. McEntegart for his continued encouragement and for making excellent laboratory facilities and technical assistance available to me during the second half of these studies.

My thanks are due to all the technical staff who have helped in these investigations: in particular to Mr. R. Brown and his staff in the Microbial Pathogenicity Research Laboratory, Department of Bacteriology, University of Edinburgh, for their help during the early studies on the identification of bacteroides and for continued collaboration in subsequent studies, and to Mrs. Julia Faulkner, Department of Medical Microbiology, University of Sheffield, for her unstinting assistance in carrying out the isolation and identification tests in the studies of bacteroides from the normal flora and from clinical specimens.

I am pleased to acknowledge the invaluable collaboration of Dr. W. P. Holbrook and Dr. A. G. Deacon as colleagues in Professor Collee's research group during the early studies and the many subsequent discussions.

I am also very grateful to the many colleagues (listed in Appendix II) who sent Bacteroides strains for inclusion in these studies: in particular I wish to thank Dr. Ella M. Barnes for two useful discussions, and Dr. J. M. Hardie and Mr. H. Shah, London Hospital Medical School, for cooperation throughout the period of these studies and for several valuable informal discussions.

I am indebted to the colleagues in Sheffield who provided specimens and strains for the studies of bacteroides in the normal flora and clinical infections, in particular to : Dr. J. Colquhoun and his staff in the Bacteriology Laboratory, Royal Infirmary, Sheffield, for collecting all clinically significant isolates of bacteroides; the medical and dental students, and professional and technical colleagues who provided specimens for the study of bacteroides in the normal flora of faeces and sub-gingival plaque; and Dr. M. Griffiths, Family Planning Clinic, Sheffield, for providing vaginal specimens from normal healthy women.

I also thank Mrs. Enid Bailey and Mrs. Hazel Bland for their skill in the preparation of the typescript.

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A COMPARISON OF SOME CHARACTERISTICS OF REFERENCE STRAINS OF *BACTEROIDES ORALIS* WITH *BACTEROIDES MELANINOGENICUS*

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Summary—*Bacteroides oralis* strain ATCC no. 15930 was compared with three reference strains of *B. melaninogenicus* (NCTC nos. 9336, 9337 and 9338) in terms of their microscopic and colonial morphology, their behaviour in a series of simple biochemical tests and disc antibiotic sensitivity tests. The *B. oralis* strain produced black pigmented colonies on blood agar and the extracted pigment appeared identical with that produced by the *B. melaninogenicus* strains. The results of the other tests were also essentially similar. Unless valid reasons can be given for the separation of these reference strains into two species, it is suggested that *B. oralis* strain ATCC no. 15930 be re-classified as *B. melaninogenicus*.

Bacteroides oralis and *B. melaninogenicus* are two species commonly isolated in large numbers from the human mouth. *B. melaninogenicus* was first described by Oliver and Wherry (1921) as a Gram-negative anaerobic bacillus that produces black pigmented colonies on blood agar. This characteristic appearance has been regarded as highly specific and remains the sole basis for the differentiation of *B. melaninogenicus* from other *Bacteroides* species. The species *B. oralis* was proposed by Loesche, Socransky and Gibbons (1964) and is included in three identification schemes for Gram-negative non-sporing anaerobes (Loesche and Gibbons, 1965; Sutter and Finegold, 1971; Holdeman and Moore, 1973). An extensive list of its characteristics is published in the Anaerobe Laboratory Manual (Holdeman and Moore, 1973), and these are similar to *B. melaninogenicus* ss. *melaninogenicus* except that *B. oralis* does not produce pigment.

As part of a larger study of the identification and classification of Gram-negative, anaerobic, non-sporing bacteria, the only currently available reference strain of *B. oralis* (ATCC no. 15930) and three reference strains of *B. melaninogenicus* (NCTC nos. 9336, 9337, 9338) were obtained from the American Type Culture Collection (12301 Parklawn Dr., Rockville, Md. 20852) and the National Collection of Type Cultures (Central Public Health Laboratory, Colindale Avenue, London, NW9 5HT), respectively. These strains were maintained by serial subculture at 10–12 day intervals in pre-steamed Robertson's cooked meat broth (Cruickshank, 1968). Cultures were incubated for 48 hr at 37°C in an atmosphere of 90 per cent H_2 and 10 per cent CO_2 in a Baird & Tatlock Ltd. (BTL) anaerobic jar equipped with three room-temperature catalyst sachets following the method of Collee *et al.* (1972). These stock cultures were then held at room temperature on the bench without further anaerobic measures. Cellular morphology was regularly checked by examination of Gram-stained smears and the purity of cultures checked by aerobic and anaerobic subculture on blood agar. All tests were performed by the methods of Cruickshank (1968) unless otherwise stated.

Growth characteristics, colonial morphology, and the appearance of haemolysis and pigmentation were observed on nutrient agar media (Oxoid Columbia Agar) containing: (i) 5 per cent human blood, (ii) 5 per cent lysed human blood, (iii) 5 per cent horse blood (Wellcome Laboratories) or (iv) 5 per cent lysed horse blood. Lipase and phospholipase activity were detected by growth on egg yolk agar and colonies from this medium were tested for catalase and oxidase production. Indole production in cooked meat broth was tested using Ehrlich's reagent after benzol extraction and H_2S production was detected by lead acetate paper strips. Charcoal–gelatin discs (Oxoid) were added to cooked meat broth cultures to detect proteolysis and these cultures were only discarded as negative after incubation for 2 weeks. Aesculin hydrolysis was determined in cooked meat broth with aesculin added to give a final concentration of 1 per cent. After incubation for 1 week, hydrolysis was indicated by a dense, black colour after adding 1 ml of a 1 per cent solution of ferric ammonium citrate.

Carbohydrate fermentation was determined in thioglycollate medium (BBL) without dextrose or indicator and with added yeast extract (0·2 per cent), haemin (5 μg ml^{-1}), menadione (0·25 μg ml^{-1}), and glucose, lactose, sucrose or maltose added to give a final concentration of 1 per cent. After incubation for 1

week, the pH of the cultures was measured with a Pye Unicam Model 292 pH meter (Pye, Cambridge, England) and a Pye Ingold combined glass and reference electrode No. 401-S/160. Fermentation was defined as a fall of $>0{\cdot}5$ pH unit below the pH value of the inoculated carbohydrate-free control (Rutter, 1970).

The antibiotic disc sensitivity test was modified from Sutter and Finegold (1971). One drop (0·02 ml) of a 48-hr cooked meat broth culture was spread over the surface of a 5 per cent human blood agar plate. Four discs were placed on each plate and the diameters of inhibition zones were measured as soon as good growth was visible, usually after 48 hr. The discs contained neomycin 1000 μg and 10 μg, kanamycin 1000 μg, erythromycin 60 μg, penicillin 1·5 units, tetracycline 10 μg, colistin 10 μg, rifampicin 10 μg, lincomycin 2 μg, bacitracin 0·1 unit, vancomycin 15 μg and chloramphenicol 10 μg.

The black pigment was extracted from cells grown on 5 per cent human blood agar for 10 days. The cells were washed thrice in distilled water and disrupted by ultrasonication. After ultracentrifugation, the pigment in aqueous solution was examined by spectrophotometry (Duerden, 1974).

B. oralis and *B. melaninogenicus* strains were all non-motile, non-sporing, Gram-negative, cocco-bacilli with some short chains. Occasionally a longer form of *B. oralis* with pointed ends was seen. Both forms were seen in preparations made from single colonies on human blood agar. After incubation for 48 hr on human blood agar, the colonies of each strain were small (<1 mm, dia.), circular, convex and translucent, with an entire edge. The *B. oralis* strain grew more slowly than the *B. melaninogenicus* strains. Zones of complete haemolysis were seen around colonies of *B. melaninogenicus* strains after 2–5 days and around colonies of *B. oralis* after 5–7 days. The colonies initially became grey; 24 hr after haemolysis appeared, the colonies became black. Pigmentation developed 24–48 hr earlier on lysed human blood agar but more slowly on media containing horse blood. All of the strains produced pigment within 7 days on media containing either human or horse blood. Spectrophotometry of aqueous solutions of pigment extracted by ultrasonication from black colonies of *B. oralis* and *B. melaninogenicus* strains showed a similar pattern for each strain. There was a broad peak in the 370–450 nm band with a maximum at 410 nm; this was not so with preparations derived from the medium control.

The cultural and biochemical characteristics of the strains are shown in Table 1. All of the strains produced H_2S and digested gelatin and none of them produced oxidase or catalase. *B. oralis* strain 15930 and *B. melaninogenicus* strain 9338 produced a lipase and a phospholipase. Indole was produced by all of the *B. melaninogenicus* strains but not by *B. oralis* strain 15930. Aesculin was hydrolysed by *B. oralis* strain 15930 but not by any of the *B. melaninogenicus* strains. *B. oralis* strain 15930 fermented glucose, lactose, maltose, and sucrose, whereas *B. melaninogenicus* strains 9336 and 9338 fermented glucose, sucrose and maltose, but not lactose. *B. melaninogenicus* strain 9337 was non-saccharolytic.

The results of antibiotic disc sensitivity tests obtained with the four strains were essentially similar (Table 2). The only differences between *B. oralis* strain 15930 and the three strains of *B. melaninogenicus* were that strain 15930 was resistant to bacitracin and sensitive to vancomycin. *B. melaninogenicus* strain 9337 and *B. oralis* strain 15930 were resistant to colistin, whereas *B. melaninogenicus* strains 9336 and 9338 were sensitive.

In the currently accepted definition (Loesche and Gibbons, 1965; Holdeman and Moore, 1973), the species *Bacteroides melaninogenicus* is a Gram-negative, non-motile, non-sporing, anaerobic bacillus or coccobacillus that produces black pigmented colonies when grown on blood agar. All strains that produce pigment in this way are classified as *B. melaninogenicus*. This has been challenged by Tracy (1969) who showed that *Bacteroides*-like organisms generally can produce a black colloidal precipitate in certain fluid media. Duerden (1974) has recently attributed this to the production of H_2S under cultural conditions that provide ferrous ions leading to a precipitate of ferrous sulphide. The true pigment produced in colonies grown on blood agar is a different and a specific product of *B. melaninogenicus* strains. The species comprises a heterogeneous group of strains with wide variations in biochemical properties between strains and some workers have identified sub-species on the basis of these properties (Holdeman and Moore, 1973). Our observation that the reference strain of *B. oralis* ATCC 15930, produced black pigment when grown on human or horse blood agar indicates that this strain should be classified as *B. melaninogenicus* according to the above definition. Sharpe (1971) reported a personal communication from Dr. M. P. Bryant that a strain derived from *B. oralis* ATCC strain 15930 produced black colonies; Sharpe was unable to reproduce that finding. However, recent observations of pigment production by *B. oralis* ATCC strain 15930 on lysed blood agar have prompted other workers to consider re-classification of the strain (E. P. Cato, 1974, personal communication). Moreover, other major characteristics of all of the sub-species of *B. melaninogenicus* are sensitivity to penicillin and to high concentrations of neomycin (Sutter and Finegold, 1971), and the ability to digest gelatin; these characteristics are shared by strain 15930. Considerable differences remain between the four strains in terms of their carbohydrate fermentation reactions and lipase production, but these differences between strain 15930 and the reference strains of *B. melaninogenicus* are no greater than the differences observed between strains accepted as *B. melaninogenicus*.

Holdeman and Moore (1973) differentiate between three sub-species of *B. melaninogenicus* (ss. *melaninogenicus*, ss. *intermedius* and ss. *asaccharolyticus*). The reference strains 9336 and 9338 accordingly correspond with *B. melaninogenicus* ss. *intermedius*, and

Table 1. Results of cultural and biochemical tests with strains of *B. oralis* and *B. melaninogenicus*

Test or observation	Results obtained with strain*			
	B. oralis ATCC 15930	*B. melaninogenicus* NCTC 9336	*B. melaninogenicus* NCTC 9337	*B. melaninogenicus* NCTC 9338
Black pigment on blood agar	+	+	+	+
Haemolysis on blood agar at 48 hr	−	−	−	+
Haemolysis on blood agar at 7 days	+	+	+	+
Phospholipase production	+	−	−	+
Lipase production	+	−	−	+
Oxidase production	−	−	−	−
Catalase production	−	−	−	−
H_2S production	+	+	+	+
Digestion of Gelatin	+	+	+	+
Indole production	−	+	+	+
Aesculin hydrolysis	+	−	−	−
Glucose fermentation	+	+	−	+
Lactose fermentation	+	−	−	−
Maltose fermentation	+	+	−	+
Sucrose fermentation	+	+	−	+
Motility	−	−	−	−
Aerobic growth	−	−	−	−

* + = positive result; − = negative result.

Table 2. Results of antibiotic disc sensitivity tests with strains of *B. oralis* and *B. melaninogenicus*

Antibiotic used*	Diameter of inhibition zone obtained with strain†			
	B. oralis ATCC 15930	*B. melaninogenicus* NCTC 9336	*B. melaninogenicus* NCTC 9337	*B. melaninogenicus* NCTC 9338
Neomycin (1000 μg)	++	++	++	++
Neomycin (10 μg)	R	R	R	R
Kanamycin (1000 μg)	R	R	R	R
Penicillin (1·5 units)	S	S	S	S
Erythromycin (60 μg)	S	S	S	S
Tetracycline (10 μg)	S	S	S	S
Colistin (10 μg)	R	++	R	++
Rifampicin (10 μg)	S	S	S	S
Lincomycin (2 μg)	S	S	S	S
Bacitracin (0·1 units)	R	+	+	++
Vancomycin (15 μg)	++	R	R	R
Chloramphenicol (10 μg)	S	S	++	S

* The amount of antibiotic contained in a disc is given in brackets.

† R = diameter of inhibition zone <15 mm; + = diameter of inhibition zone 15 × 25 mm; ++ = diameter of inhibition zone 25 × 35 mm; S = diameter of inhibition zone >35 mm.

strain 9337 with *B. melaninogenicus* ss. *asaccharolyticus*; strain 15930 corresponds with *B. melaninogenicus* ss. *melaninogenicus*. The characteristics of *B. melaninogenicus* ss. *melaninogenicus* are essentially similar to the reported characteristics of *B. oralis* (Loesche and Gibbons, 1965; Holdeman and Moore, 1973) except for the property of pigment production that appears to be the sole basis for differentiation between the two species. As we have shown that the *B. oralis* strain can produce pigment, we suggest that the reference strain "*B. oralis* ATCC no. 15930" should be re-classified as a strain of *B. melaninogenicus*.

Acknowledgements—We are indebted to Dr. J. G. Collee and Dr. P. W. Ross for their helpful advice and encouragement and to Mr. R. Brown for his skilled technical assistance. The financial support of the Medical Research Council (Grant G 971/113/B) is gratefully acknowledged.

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PIGMENT PRODUCTION BY *BACTEROIDES* SPECIES WITH REFERENCE TO SUB-CLASSIFICATION

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OLIVER and Wherry (1921) first described bacteroides-like organisms that produced black pigmented colonies when grown on blood agar, and called them *Bacterium melaninogenicum*. This characteristic appearance was regarded as highly specific and was the sole basis for differentiation from other organisms now generally grouped as *Bacteroides*. The pigment-producing strains have been studied intermittently since then and much confusion has arisen, partly because of the difficulty experienced by some workers in maintaining them in pure culture (Senos and Mattman, 1955). Some workers were able to study these organisms only in mixed culture and this may be related to a requirement of some strains for vitamin K-like compounds (Lev, 1959). The biochemistry and serology of strains of *Bacteroides melaninogenicus* was found to vary considerably (Weiss, 1937; Sawyer, MacDonald and Gibbons, 1962), and sub-species have been defined on this basis (Holdeman and Moore, 1972).

Study of the pigment has created more confusion. Oliver and Wherry (1921) thought that it was extracellular and identified it as melanin on the basis of its insolubility in organic solvents and its slow solubility in sodium hydroxide. Schwabacher, Lucas and Rimington (1947) found that the pigment was intracellular and was not melanin but a derivative of haemoglobin; it was spectroscopically identical with haematin.

Tracy (1969) found that clinical isolates of *B. fragilis* produced a dense black pigment in mixed culture with *Escherichia coli* or *Staphylococcus aureus* in cooked-meat broth containing a rusty nail. She found that standard strains of *B. fragilis*, *B. melaninogenicus* and *B. necrophorus* produced this pigment when grown in pure culture in a medium containing cysteine, ferrous sulphate and vitamin K. The pigment was extracellular and was identified as colloidal ferrous sulphide.

The present series of experiments was undertaken to investigate the nature of the pigment and to study the conditions necessary for its production by *Bacteroides*, with special reference to its taxonomic significance.

MATERIALS AND METHODS

Throughout these experiments cellular morphology was regularly checked by examination of Gram-stained smears; the purity of cultures was checked by aerobic and anaerobic subculture on human-blood agar.

Received 3 May 1974; *accepted* 25 June 1974.

Culture inocula. One drop (0·02 ml) of a 48-h cooked-meat broth culture was used to seed each tube and one loopful (*c.* 0·01 ml) to seed each plate of medium.

Anaerobic incubation. Anaerobic jars supplied by Baird and Tatlock Ltd (BTL) were each fitted with three catalyst sachets and the anaerobic procedure of Collee *et al.* (1972) was essentially followed. All incubation was at 37°C in an atmosphere of 90% H_2 and 10% CO_2.

Organisms. The following strains were obtained from the National Collection of Type Cultures (NCTC): *Bacteroides melaninogenicus* nos. 9336, 9337 and 9338; *B. fragilis* no. 9343; and *B. necrophorus* nos. 7155 and 10575. In addition, we studied 36 strains of Gram-negative anaerobic non-sporing bacilli, originally isolated from clinical specimens or human faeces. All these anaerobic bacilli were maintained by fortnightly serial subculture in tubes of cooked-meat broth incubated anaerobically at 37°C for 24 h and thereafter kept on the bench.

Laboratory strains of *E. coli*, *S. aureus* (Oxford; no. NCTC6571), *Proteus mirabilis* and *Salmonella typhimurium* had been maintained by serial subculture in nutrient broth and on nutrient-agar slopes for many years; a laboratory strain of *Clostridium welchii* was maintained by serial subculture in cooked-meat broth.

Chemicals. Aqueous solutions of menadione (Sigma, London; 100 μg per ml; see Barnes and Impey, 1971); ferrous sulphate (Analar grade; 250 mg per ml); and L-cysteine (Koch-Light, pure; 100 mg per ml), were prepared immediately before use. All were sterilised by membrane filtration and added aseptically to (cooled) autoclaved media to give final concentrations of menadione 1 μg per ml, ferrous sulphate 5 mg per ml, and cysteine 2 mg per ml.

Media were either freshly prepared or steamed for 30 min. just before use. Media containing menadione or cysteine could not be steamed and were always freshly prepared.

The basic *liquid medium* was Robertson's cooked-meat broth (Cruickshank, 1969); various combinations of chemicals were added (see text). Horse-blood broth, containing 10% Defibrinated Horse Blood (Oxoid) in Oxoid Nutrient Broth no. 2, was used to study pigment production in a liquid medium containing blood. *Solid media* were: (i) human blood-agar containing 5% human blood in Oxoid Columbia Agar Base; human blood was provided by the Regional Blood Transfusion Service, Edinburgh, and contained 2 g disodium citrate and 1·7 g dextrose in 70 ml of water added to 430 ml of whole blood; (ii) lysed human-blood agar prepared by freezing and thawing the blood before incorporating it in the medium; (iii) horse-blood agar (10% Defibrinated Horse Blood, Oxoid, and Oxoid Columbia Agar Base). Special solid media derived from cooked-meat broth were prepared as follows: (i) the supernate from Robertson's cooked-meat broth solidified with 1% agar (Oxoid no. 1); and (ii) Robertson's cooked-meat broth made with homogenised meat and solidified with 1% agar.

Characterisation of strains

All the NCTC strains were subjected to the following tests. *Haemolysis* on blood agar was observed after incubation for 48 h and 1 week. *Lipase* and *phospholipase-C* activity were detected by observation of the effects of growth on egg-yolk agar (Cruickshank, 1969). *Oxidase* production was indicated by a blue colour after a solution of tetramethyl-*p*-phenylene-diamine dihydrochloride had been poured on to cultures on blood agar. *Catalase* was detected by flooding 1 week's growth on egg yolk agar with 10% hydrogen peroxide; a stream of bubbles arose from catalase-producing colonies. *Gelatinase* test: a charcoal-gelatin disk (Oxoid) in a cooked-meat broth culture was observed for digestion of the disk during incubation for 2 weeks. *Indole* production was detected by adding ether followed by a few drops of Ehrlich's Reagent to the liquid phase of a 48-h cooked-meat broth culture; the reagent turned pink in the presence of indole. *Aesculin hydrolysis* was detected by the development of black discolouration when 0·5 ml of a 1% aqueous solution of ferric ammonium citrate was added to a 48-h culture in cooked-meat broth with 1% aesculin. Production of H_2S was detected by blackening of a strip of lead-acetate paper suspended during incubation in the neck of a tube of cooked-meat broth containing cysteine.

Inhibition of growth by bile salts. Each strain was seeded on to a series of four nutrient-agar test media (Oxoid Columbia Agar) supplemented with 1% sodium succinate and 1% glucose and containing (i) no bile salt (control); (ii) 0·5% sodium taurocholate (BDH Chemicals Ltd): (iii) 0·1% sodium deoxycholate (BDH); or (iv) 0·5% sodium taurocholate plus 0·1% sodium deoxycholate. The plates were examined for growth after 24h and 48 h.

Sugar-fermentation tests. Seitz-filtered aqueous solutions of glucose, lactose, sucrose, and maltose were separately added to cooked-meat broth to give a final concentration of 1% of the test sugar. These were seeded with 0·02 ml of a 48-h cooked-meat broth culture. Cultures were incubated for 48 hours and the final *p*H was measured with a *p*H meter after exposure on the bench for at least 1 h. Controls included cultures of the test strains in plain cook-edmeat broth and tubes of the uninoculated 1% sugar media. The test was regarded as positive if the *p*H fell $>0{\cdot}5$ unit below that of the uninoculated control and $>0{\cdot}5$ *p*H unit in comparison with the value for the 48-h culture in plain cooked-meat broth (see Rutter, 1970). *p*H values were measured by a Pye Ingold combined Glass and Reference Electrode, no. 401(E2), with a Pye Ingold Resistance Thermometer no. 622 as temperature compensator.

Antibiotic-sensitivity tests. All NCTC strains were grown on fresh 5% human-blood agar and tested for sensitivity to antibiotics by the disk-diffusion method (modified from Sutter and Finegold, 1971). The plates were seeded by spreading 0·02 ml of a 48-h cooked-meat broth culture on the surface with a standard glass spreader. The sizes of inhibition zones were measured after either 24 or 48 h, as soon as good growth was visible.

Disks containing neomycin sulphate 1000 μg, kanamycin sulphate 1000 μg, rifampicin (Rimactane) 10 μg, and erythromycin ethyl succinate 60 μg were prepared in our laboratory. Disks containing lincomycin 2 μg, and bacitracin 0·1 unit, were supplied by Mast Laboratories Ltd; and disks with colistin sulphate 10 μg, tetracycline 10 μg and penicillin 1·5 units were from Oxoid Ltd.

A standard Oxford staphylococcus was tested in parallel with the *Bacteroides* spp. as a control. This control organism was incubated anaerobically and the validity of such a control continues to be debated. The control strain was sensitive to neomycin, kanamycin, penicillin, erythromycin, rifampicin, lincomycin, and tetracycline, and resistant to colistin and bacitracin. The tests were performed at least four times and the inhibition-zone diameters were grouped into four grades: <15 mm; 16–25 mm; 26–35 mm; >35 mm. The grades obtained with the *Bacteroides* strains were then classified as resistant (R) or sensitive (S) by comparison with the grades obtained with the Oxford staphylococcus.

Isolation and examination of pigment

Presumptive colloidal ferrous sulphide. The dense black pigment produced by culture of any *Bacteroides* strain in liquid media containing cysteine and ferrous sulphate was separated from the bacterial cells by centrifugation at 800 *g* for 1 h. Most of the pigment remained in the supernate, which retained the dense black appearance of the original culture; some was deposited with the cells. The presence of ferrous ions in the supernate was demonstrated in tests with potassium ferricyanide solution with and without acidification with HCl; the Prussian-blue reaction indicated the presence of ferrous ions.

Lead acetate paper was used to detect the evolution of H_2S after acidification of the supernate with HCl. The supernate was also examined by spectrophotometry over the wavelength range 200–700 nm after 50-fold dilution with distilled water.

Pigment from cultures of B. melaninogenicus in 10% horse blood broth with menadione. The cells were sedimented by centrifugation at 800 *g* for 1 h and the supernate was retained. The deposit of bacterial cells and red-cell debris was washed thrice with distilled water to lyse any remaining RBC and to remove the haemoglobin; then it was disrupted ultrasonically for 30 min. in distilled water and the cell debris separated from the soluble fraction by ultracentrifugation at 20,000 *g* for 1 h. The original culture supernate was diluted 100-fold in distilled water and the aqueous extract after ultrasonication was diluted 10-fold for spectrophotometry over the wavelength range 200–700 nm. These prepartions were also tested for the presence of sulphide and ferrous ions as above.

Pigment from colonies of B. melaninogenicus on blood agar. Growth after anaerobic

incubation for 1 week on blood agar was suspended in distilled water and treated in the same way as above. The first supernate and the supernate obtained after ultrasonic treatment were examined by spectrophotometry.

Spectrophotometry. A Pye Unicam SP8000 Ultraviolet Recording Spectrophotometer was used.

Ultrasonic disintegration. An MSE-Mullard Ultrasonic Disintegrator no. 4200 was used.

Results

Growth of Bacteroides spp. on blood agar

B. melaninogenicus strain no. 9338 produced visible colonies on blood agar after anaerobic incubation for 48 h; the colonies became black after anaerobic incubation for 2–4 days. Haemolysis appeared and increased concurrenly with colony pigmentation. The same pattern was observed with strains nos. 9336 and 9337 except that pigmentation and haemolysis did not develop until 4–6 days.

There was essentially no difference between degree and speed of pigmentation on horse-blood agar and human-blood agar. Menadione increased the rate of pigmentation, particularly on horse-blood agar. Pigment appeared earlier on heated (chocolate) horse-blood agar than on unheated horse blood. Pigmentation was most rapid on lysed human-blood agar.

No other Gram-negative anaerobic bacilli produced pigmented colonies in pure culture on human- or horse-blood agar.

Effect of cultivation with Escherichia coli or Staphylococcus aureus (Oxford) on pigment production by Bacteroides spp. on blood agar

The effect of cultivation with *E. coli* or *S. aureus* on pigment production was tested on separate human-blood-agar plates. *E. coli* or *S. aureus* was seeded on to a plate as a diametric streak and a streak inoculum of each strain of Gram-negative anaerobic bacilli, including the NCTC reference strains, was made at right angles across the *E. coli* or *S. aureus* inoculum so that areas of pure and mixed growth were obtained. After incubation for 1 week, only NCTC strains of *B. melaninogenicus*, nos. 9336, 9337 and 9338, had produced pigmented colonies. Pigment developed more rapidly in strains nos. 9336 and 9337 in areas of mixed culture with *E. coli* or *S. aureus*, *E. coli* stimulating pigment production more than *S. aureus*. No other strains produced pigment.

Growth of Bacteroides spp. in mixed culture with E. coli or S. aureus in cooked meat broth with iron filings

Each strain was incubated separately with *E. coli* and *S. aureus* in cooked-meat broth to which a knife-point of iron filings had been added. After 3 days, one laboratory-isolated strain produced a few small black clumps in mixed culture with *S. aureus*. No others produced pigment.

Growth of Bacteroides spp. with menadione, cysteine and ferrous sulphate

Each strain was grown for 48 h in plain cooked-meat broth and in cooked-meat broth to which menadione, cysteine, and ferrous sulphate, singly and

in all combinations, had been added, i.e., eight media in all (see *Methods*). All produced dense black pigmentation throughout media containing both cysteine and ferrous sulphate; this effect was independent of the presence of menadione. Two strains produced clumps of black pigment in medium with only ferrous sulphate as an additive, and four strains produced black clumps when both ferrous sulphate and menadione were present. Pigment was not produced in media to which ferrous sulphate had not been added.

The same dense black appearance was reproduced by the addition of ferrous sulphate solution to a 48-h culture of any strain in the medium that contained only additional cysteine. The black discolouration began to develop as soon as the ferrous sulphate solution was added. A similar result was obtained when copper sulphate solution was added to 48-h cultures in the medium with additional cysteine; in this case, the precipitate was blue.

Hydrogen-sulphide production in cultures of Bacteroides spp.

All strains were tested for H_2S production in plain cooked-meat broth and in cooked-meat broth with additional cysteine. Weak production of H_2S was generally demonstrable with cultures in the plain medium. All strains grown in cooked-meat broth supplemented with cysteine gave strongly positive results after 48 h; the development of the black pigment was correlated with detectable H_2S production and the presence of ferrous sulphate.

Studies with bacteria of other genera in fluid media

Strains of *E. coli*, *P. mirabilis*, *S. typhimurium* and *C. welchii* were also inoculated separately into the above test media. They all gave a strongly positive test for H_2S production in media containing added cysteine and they produced the same dense black pigment throughout the medium when ferrous sulphate was also present. The naked-eye appearances of the black broth cultures were indistinguishable from those produced by the *Bacteroides* strains.

Growth of Bacteroides spp. on solid media containing cysteine, menadione and ferrous sulphate

Ten strains were incubated on human-blood agar with cysteine and ferrous sulphate incorporated in the medium and with disks of menadione (0·005 mg) and phytomenadione (0·1 mg and 0·005 mg) on the surface. One strain failed to grow; the others produced poor growth and colonies were not pigmented.

All strains were incubated on separate agar media incorporating (i) cooked-meat-broth supernate, (ii) homogenised cooked meat, or (iii) lysed human blood, each containing cysteine, menadione and ferrous sulphate. Fourteen failed to grow on the supernate agar and the remainder produced barely visible growth without pigmentation. All except two strains grew on homogenised cooked-meat-broth agar, but growth was slow and colonies were small and not pigmented. Eight strains grew moderately well on lysed-blood agar; 14 produced barely visible growth and 23 failed to grow. After 1 week,

colonies of the eight moderately good cultures began to develop black centres and after 10 days most colonies had black centres. None of these strains was *B. melaninogenicus* and one was a known *B. fragilis* strain no. 9343.

Examination of black pigment from cultures of Bacteroides strains and E. coli in cooked-meat broth with cysteine and ferrous sulphate

The black pigment did not sediment on centrifugation, and a wet film of the deposit showed normal bacterial cells with clumps of extracellular pigment. The cell-free, pigmented supernate did not react with potassium ferricyanide solution; this indicated the absence of free ferrous ions, whereas the supernate from the medium control gave a strong Prussian-blue reaction attributable to the ferrous sulphate content. When the culture supernate was acidified with HCl, H_2S was evolved and the pigmentation gradually cleared. The acidified supernate gave a strongly positive Prussian-blue reaction with potassium ferricyanide solution. This is prima-facie evidence that this pigment was the colloidal ferrous sulphide identified by Tracy (1969) in similar circumstances. Spectrophotometry of a 50-fold dilution of the black supernate, the medium control and plain cooked-meat broth revealed a general increase in turbidity over the whole range for the black supernate as one might expect with a black substance in colloidal suspension.

Examination of pigment produced by B. melaninogenicus in blood-containing media

B. melaninogenicus strains nos. 9336, 9337 and 9338, *B. fragilis* strain no. 9343 and *B. necrophorus* strains nos. 7155 and 10575 were grown for 1 week in horse-blood broth with menadione. The centrifuged culture supernate and an aqueous ultrasonic extract of the deposited bacterial cells were examined by spectrophotometry for pigmentation. There was no difference between the spectra of the supernates from the six cultures and from an uninoculated control. There was a small increase in absorption at 525–575 nm and a single large narrow peak at 405–410 nm representing haemoglobin from lysed RBC. When the cells were washed with distilled water, haemoglobin was again the only pigment detected in the washings and it was equally present in all six cultures and the control. The final washings were clear.

At this stage the cells of *B. melaninogenicus* strains were considerably darker than those from the test cultures of *B. fragilis* and *B. necrophorus* strains. The pigment was clearly cell-associated and not freely extracellular. The cells were disrupted ultrasonically in distilled water and the supernate, after ultracentrifugation to remove cell debris, was dark brown in the case of *B. melaninogenicus* strains and colourless in the case of *B. fragilis* and *B. necrophorus* strains and the medium control. Spectrophotometry of these aqueous extracts from the disrupted cells showed a broad peak in the 370–450 nm band, with a maximum at 410 nm, for *B. melaninogenicus* strains; this was not so with preparations derived from *B. fragilis* or *B. necrophorus* strains or from the medium control (fig. 1).

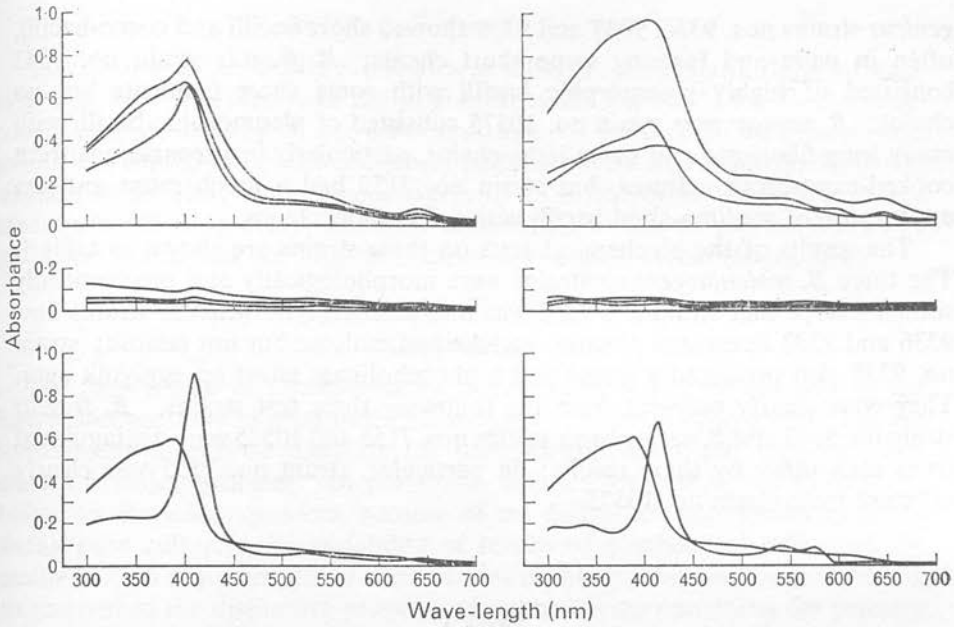

FIG. 1.—Typical patterns of plot obtained in spectrophotometric studies on extracts of disintegrated washed preparations of *Bacteroides* species derived from cultures in horse-blood broth: *top graph*: *B. melaninogenicus* strains nos. 9336, 9337 and 9338; *middle graph: B. fragilis* strain no. 9343 and *B. necrophorus* strains nos. 7155 and 10575; *bottom graph*: aqueous solution of haemin (broader peak) and aqueous extract of lysed horse red cells (narrow peak).

FIG. 2.—Typical patterns of plots obtained in spectrophotometric studies on extracts of disintegrated washed preparations of *Bacteroides* species derived from cultures on human-blood agar: *top graph: B. melaninogenicus* strains nos. 9336, 9337 and 9338 (upper line); *middle graph: B. fragilis* strain no. 9343 and *B. necrophorus* strains nos. 7155 and 10575; *bottom graph*: aqueous solution of haemin (broader peak) and aqueous extract of lysed human red cells (narrow peak.)

The Prussian-blue reaction was not produced by the addition of potassium ferricyanide solution to this pigment extract, either with or without acidification, and H_2S was not evolved after acidification.

Colonies of *B. melaninogenicus* strains nos. 9336, 9337 and 9338, *B. fragilis* strain no. 9343 and *B. necrophorus* strains nos. 7155 and 10575 after incubation for 1 week on blood agar were suspended in distilled water, washed and examined as above. The first washing showed a small amount of haemoglobin carried over with each, but subsequent washings were clear. The *B. melaninogenicus* cells remained dark and the *B. fragilis* and *B. necrophorus* cells were pale. After ultrasonic treatment and ultracentrifugation, the extracts from *B. melaninogenicus* strains were all dark brown and showed spectrophotometry peaks very similar to those obtained from cells grown in horse-blood broth. The extracts from *B. fragilis* and *B. necrophorus* strains had no such peak (fig. 2).

Characterisation of NCTC strains

All of the test strains were non-motile, non-sporing, pleomorphic, Gram-negative and obligately anaerobic bacteria. Gram-stained films of *B. melanino-*

genicus strains nos. 9336, 9337 and 9338 showed short bacilli and cocco-bacilli, often in pairs and forming some short chains; *B. fragilis* strain no. 9343 consisted of highly pleomorphic bacilli with some short filaments but no chains; *B. necrophorus* strain no. 10575 consisted of pleomorphic bacilli with many long filaments and some long chains, particularly in preparations from cooked-meat-broth cultures, but strain no. 7155 had a much more uniform appearance of medium-sized bacilli with a few short chains.

The results of the biochemical tests on these strains are shown in table I. The three *B. melaninogenicus* strains were morphologically and biochemically similar except that strain no. 9337 was non-saccharolytic whereas strains nos. 9336 and 9338 fermented glucose, sucrose and maltose but not lactose; strain no. 9338 also produced a lipase and a phospholipase effect on egg-yolk agar. They were clearly different from the following three test strains. *B. fragilis* strain no. 9343 and *B. necrophorus* strains nos. 7155 and 10575 were distinguished from each other by these results; in particular, strain no. 7155 was clearly different from strain no. 10575.

TABLE I

Results of biochemical tests with reference strains of Bacteroides spp

Test	Results obtained with strain no.*					
	9336	9337	9338	9343	7155	10575
Aerobic growth†	−	−	−	−	−	−
Motility	−	−	−	−	−	−
Black pigment on blood agar	+	+	+	−	−	−
Haemolysis on blood agar	+	+	+	−	−	+
Pearly layer on EYA (lipase)	−	−	+	−	−	+
Phospholipase production	−	−	+	−	−	+
Oxidase production	−	−	−	−	−	−
Catalase production	−	−	−	−	−	−
H_2S production	+	+	+	+	+	+
Indole production	+	+	+	−	−	+
Digestion of gelatin	+	+	+	−	−	−
Hydrolysis of aesculin	−	−	−	+	+	−
Inhibition of growth by:						
0·5% sodium taurocholate	I	I	I	−	−	I
0·1% sodium deoxycholate	I	I	I	I	−	I
0·5% sodium taurocholate +0·1% sodium deoxycholate	I	I	I	−	−	I
Fermentation of:						
glucose	+	−	+	+	+	−
lactose	−	−	−	+	+	−
sucrose	+	−	+	+	+	−
maltose	+	−	+	+	+	−

* *B. melaninogenicus* NCTC nos. 9336, 9337, and 9338; *B. fragilis* NCTC no. 9343; *B. necrophorus* NCTC nos. 7155 and 10575; + = positive result; − = negative result; I = inhibition of growth by the bile salts.

† Aerobic incubation was at 37°C (i) in air and (ii) in air with 10% CO_2 added.

Antibiotic-disk sensitivity tests

The results of these tests are shown in table II. In general, different antibiograms were obtained with the *B. fragilis* strain no. 9343, the *B. melaninogenicus* strains, and the *B. necrophorus* strains. The three *B. melaninogenicus* strains had similar antibiotic-sensitivity patterns except that strain no. 9337 was resistant to colistin; they were sensitive to all of the other antibiotics except kanamycin (1000-μg disk). The only difference between strains no. 7155 and 10575 was that strain no. 7155 was resistant to erythromycin (60-μg disk).

Discussion

The importance of Gram-negative anaerobic non-sporing bacteria of the *Bacteroides* group in clinical medicine is being increasingly recognised, but the development of precise methods for their identification and differentiation remains a major problem. In particular, the confusion that has surrounded studies on *B. melaninogenicus*, because of the difficulties experienced in maintaining pure cultures, the variability of results of biochemical tests, and the specific growth requirements of some strains (Gibbons and MacDonald, 1960), has resulted in the distinctive property of pigmentation remaining the primary basis for the identification of *B. melaninogenicus*. *Bacteroides*-like organisms that produce black colonies on blood agar have been grouped together as *B. melaninogenicus* by some workers solely on the basis of this property, irrespective of any other similarities or differences (Beerens, 1970). Doubt was cast on the validity of this approach by the claim of Tracy (1969) that other *Bacteroides* species can produce black pigment under particular cultural conditions, and that the pigment is extracellular colloidal ferrous sulphide. Earlier workers had identified the pigment of *B. melaninogenicus* as extra-

Table II

Results of antibiotic-disk-sensitivity tests with reference strains of Bacteroides spp.

Antibiotic (content per disk)	Sensitivity or resistance of strain no.*						
	9336	9337	9338	9343	7155	10575	*S. aureus* (Oxford)
Neomycin (1000 μg)	S	S	S	R	S	S	S
Kanamycin (1000 μg)	R	R	R	R	S	S	S
Penicillin (1·5 unit)	S	S	S	R	S	S	S
Erythromycin (60 μg)	S	S	S	S	R	S	S
Colistin (10 μg)	S	R	S	R	S	S	R
Rifampicin (10 μg)	S	S	S	S	R	R	S
Lincomycin (2 μg)	S	S	S	R	S	S	S
Bacitracin (0·1 unit)	S	S	S	R	R	R	R
Tetracycline (10 μg)	S	S	S	S	S	S	S

* *B. melaninogenicus* NCTC nos. 9336, 9337 and 9338; *B. fragilis* NCTC no. 9343; *B. necrophorus* NCTC nos. 7155 and 10575. S = Sensitive; R = resistant (see *Methods*).

cellular melanin (Oliver and Wherry, 1921), whereas Schwabacher *et al.* (1947) considered that it was intracellular or cell-associated haematin.

The first group of experiments in the present study with 42 bacteroides-like organisms and one strain of *E. coli*, *P. mirabilis*, *S. typhimurium* and *C. welchii* grown in media containing additional cysteine and ferrous sulphate showed that all of these organisms produce H_2S in such a medium and all produce a dense black colloidal precipitate of ferrous sulphide. Tracy (1969) regarded this as a true bacterial extracellular pigment synthesised by all species of *Bacteroides* under appropriate conditions; she argued that all could then be called "*B. melaninogenicus*" and hence sought to explain the variability in biochemical reactions of different strains of "*B. melaninogenicus*". However, the present results show that the production of ferrous sulphide in this way is not equivalent to the synthesis of a specific pigment; it is an inorganic chemical reaction occurring whenever H_2S is introduced into a solution containing ferrous ions. When a sulphur source such as cysteine was provided, all the *Bacteroides*-like organisms produced H_2S; ferrous sulphate, either incorporated in the original medium or added after incubation, was then reduced to ferrous sulphide and merely acted as an indicator of H_2S production. This indicator system, however, is not responsible for the development of the typical black colonies that form when *Bacteroides* strains called *B. melaninogenicus* are grown on media containing blood.

In the second part of the present investigation, three reference strains of *B. melaninogenicus* were grown in media containing blood, and the washed bacterial cells and the cell-free medium supernate were examined for pigment. There was no extracellular pigment in the supernate, and pigment was not eluted when the cells were washed with distilled water, but the washed cells of the three *B. melaninogenicus* strains were dark brown in colour. Parallel treatment of cultures of *B. fragilis* and *B. necrophorus* yielded pale cells. The dark pigment that gives the colonies of *B. melaninogenicus* their characteristic appearance is either intracellular or cell-associated in such a way that it cannot be removed by washing with water despite the finding that, when released by ultrasonic treatment of the cells, the pigment is water soluble and can then be extracted in aqueous solution.

The extracted pigment has none of the properties of ferrous sulphide; it is in solution and not in suspension; it does not give the Prussian-blue reaction with potassium ferricyanide solution after acidification; and acid-treatment does not liberate H_2S from it. Moreover, ferrous sulphide produced in colonies of other bacteroides-like organisms grown on lysed-blood agar with additional cysteine and ferrous sulphate faded when left exposed to air and light; the (intact) pigmented colonies of *B. melaninogenicus* did not fade. When the pigment was examined by spectrophotometry, there was an absorption peak at 410 nm. This is the wavelength at which haemoglobin in the medium supernate gives its peak. The position of the peak is constant for extracts from all three *B. melaninogenicus* strains grown in blood broth or on blood agar, whereas a comparable peak was not given by extracts from *B. fragilis* or *B. necrophorus*. Moreover, the likely source of the pigment is indicated by the

observations that pigmentation develops only in colonies on blood agar in association with haemolysis of the surrounding medium and is more rapid when lysed blood is used for the medium. These results support the conclusions of Schwabacher *et al.* (1947) that the pigment of *B. melaninogenicus* is derived from haemoglobin and is either intracellular or cell-associated.

The present experiments and those of Schwabacher *et al.* are open to the criticism that the pigment was extracted from preparations of cells grown in blood-containing media. In both studies, however, great care was taken to wash away all traces of extraneous haemoglobin; and in the present study no trace of haemoglobin was revealed by spectrophotometry in control extracts similarly derived from cultures of *B. fragilis* and *B. necrophorus* or in control extracts of the uninoculated medium.

These results indicate that pigment production during growth in blood-containing media is a stable characteristic of certain strains of *Bacteroides*, at present all called *B. melaninogenicus*. It is not a property that can be expressed by any strain of *Bacteroides* under suitably manipulated cultural conditions. However, it does not follow that all pigment-producing strains form a biochemically homogeneous group: the three pigment-producing strains used in the present study are not identical. The results of our biochemical tests and antibiotic-disk-sensitivity tests show that strain no. 9337 is clearly different from the other two (see tables I and II); it is non-saccharolytic, resistant to colistin and does not produce a lipase or phospholipase. However, the three pigment-producing strains have more properties in common with each other than any one of them has with a strain of *B. fragilis* or *B. necrophorus*. The three species were differentiated on the basis of pigmentation, digestion of gelatin, indole production, aesculin hydrolysis, bile-salt sensitivity and antibiogram. Furthermore, *B. melaninogenicus* strains were culturally more demanding than *B. fragilis* and *B. necrophorus* strains. Cooked-meat-broth cultures of *B. melaninogenicus* held on the bench died more rapidly; subculture at 10–12-day intervals was needed to ensure survival of *B. melaninogenicus*, whereas *B. fragilis* and *B. necrophorus* strains survived for at least 14 days. It is also significant that the morphological and biochemical differences between *B. necrophorus* strains nos. 7155 and 10575 are so considerable that the two strains cannot reasonably be regarded as belonging to the same species (Collee and Watt, personal communication).

From the present study it appears that pigmentation on blood-containing media is a significant property and merits considerable weight in the taxonomy of the *Bacteroides* group. It is, however, only one feature and it must be clear that there can be wide variations between pigment-producing strains in their biochemical properties and antibiograms. Some workers have described pigmented *Bacteroides* strains that varied in their individual growth requirements (Lev, 1959; Gibbons and MacDonald, 1960). Other workers have described pigmented *Bacteroides* strains that have different biochemical properties (Sawyer *et al.*, 1962; Holdeman and Moore, 1972) and antibiograms (Finegold, Harada and Miller, 1967) and have identified sub-species of *B. melaninogenicus* on these grounds. They retain the species name *B. melanino-*

genicus for all pigment-producing strains, even when pigmentation may be the only significant shared characteristic. Provided that such widely different sub-species of a single species are acceptable, it is reasonable to regard all pigmented strains of *Bacteroides* as belonging to the species *B. melaninogenicus* and then divide that into sub-species. The problem then arises that it could be expected that strains of *Bacteroides* would be described that were identical with a sub-species of *B. melaninogenicus* in biochemical properties and antibiogram but did not produce pigment. The identification of such strains would depend upon future decisions based upon the biochemical investigations and antibiogram studies of *Bacteroides* spp. being undertaken in several centres. Finegold *et al.* (1967) advocated a scheme of identification based upon antibiograms and this appears to differentiate well between the species when high-concentration disks of neomycin (1000 μg), kanamycin (1000 μg), and erythromycin (60 μg) (Sutter and Finegold, 1971) are used in addition to the commonly used disks of pencillin (1·5 units), rifampicin (10 μg), colistin (10 μg) and bacitracin (0·1 unit). As a long-term approach to identification this method carries the risk of invalidation by acquired drug resistance and more experience is needed for its evaluation.

Other groups (Werner, Pulverer and Reichertz, 1971; Holdeman and Moore, 1972) have concentrated upon gas-liquid chromatography to detect the acid end-products of glucose fermentation and used these results to differentiate between the *Bacteroides* spp. It remains to be decided whether pigmentation remains the significant basis of identification of *B. melaninogenicus* or whether antibiograms, gas-liquid chromatography of the acid end-products of glucose fermentation, or some other group of biochemical tests, will provide more valid criteria for the taxonomy of the *Bacteroides* group.

Summary

All of six reference strains of *Bacteroides* species, 36 laboratory isolates conforming to this group, and individual strains of *Escherichia coli*, *Proteus mirabilis*, *Salmonella typhimurium* and *Clostridium welchii* produced a dense black pigment, identified as ferrous sulphide, when grown in cooked-meat media containing cysteine and ferrous sulphate. This was an indicator effect resulting from the production of H_2S by the bacteria in the presence of ferrous ions and was unrelated to the characteristic pigment produced by strains of *B. melaninogenicus* when grown on blood agar. A pigment was extracted by ultrasonic disintegration of washed cells of three reference strains of *B. melaninogenicus* grown for 1 week in horse-blood broth and on human-blood agar. It was intracellular or cell-associated, soluble in water and had the spectrophotometric characteristics of a derivative of haemoglobin. No such pigment was extracted from strains of *B. fragilis* or *B. necrophorus* by similar procedures. Pigment production is a stable characteristic of those strains of *Bacteroides* called *B. melaninogenicus* and it is a significant property in the classification of the *Bacteroides* group. However, the pigment-producing strains are not a homogeneous species, and there were considerable differences between the

results of biochemical tests and antibiograms obtained with the three strains of *B. melaninogenicus*.

I wish to thank Dr J. G. Collee for much helpful advice and encouragement, and Mr R. Brown for his skilled technical assistance. Dr Collee acknowledges a valuable discussion with Dr Ella Barnes. The financial support of the Medical Research Council (Grant G971/115/B) is gratefully acknowledged.

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PROCEEDINGS OF THE PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

The 130th meeting of the Society was held at the Middlesex Hospital Medical School, London, on the 9th, 10th and 11th January 1975

26. Pigment production by *Bacteroides* species

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The taxonomic significance of pigment production by members of the *Bacteroides* group of organisms was investigated in studies with six reference strains and 36 laboratory isolates of *Bacteroides* species and with various control organisms. All the *Bacteroides* strains and individual control strains of *Escherichia coli*, *Proteus mirabilis*, *Salmonella typhimurium* and *Clostridium welchii* produced a dense black precipitate of ferrous sulphide in cooked-meat medium containing cysteine and ferrous sulphate. This was found to be an indicator effect resulting from the production of H_2S in the presence of ferrous ions and was unrelated to the characteristic pigment produced by *B. melaninogenicus* when grown on blood agar. A true pigment was extracted by ultrasonic disintegration of washed cells of three reference strains of *B. melaninogenicus* grown for 1 week in horse-blood broth or on human-blood agar. It was intracellular or cell-associated, soluble in water, and had the spectrophotometric characteristics of a derivative of haemoglobin. No such pigment was extracted from strains of *B. fragilis* or *B. necrophorus*. Although the pigment-producing strains show more biochemical heterogeneity than is generally accepted within a single species, pigment production is a stable characteristic of those *Bacteroides*-like organisms currently called *B. melaninogenicus* and is a significant factor in the classification of the *Bacteroides* group.

J. appl. Bact. 1976, **40**, 163–188

The Characterization of Clinically Important Gram Negative Anaerobic Bacilli by Conventional Bacteriological Tests

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Received 18 August 1975 and accepted 10 November 1975

One hundred and sixty-five reference strains and laboratory isolates of Gram negative, non-sporing, anaerobic bacilli were subjected to a series of simple laboratory tests that were initially selected for their discriminatory value. Conventional biochemical tests, tests of resistance to antibiotics, and tolerance to dyes and bile salts were included. These tests allowed a clear separation of strains into three main groups: *Bacteroides fragilis*, *B. melaninogenicus* and *Fusobacterium* spp. Certain tests were found useful for identifying recognized subspecies of *B. fragilis* and *B. melaninogenicus*. A scheme for the identification of unknown laboratory isolates of Gram negative anaerobic bacilli is presented.

BACTERIA OF THE *Bacteroides-Fusobacterium* group are significant anaerobic pathogens in many clinical conditions, notably periodontal disease (MacDonald, Gibbons & Socransky, 1960; Socransky, 1970), bronchiectasis and lung abscess (Bartlett & Finegold, 1972), wound infections following abdominal and pelvic surgery (Finegold, 1974), chronic pelvic infections in women (Thadepalli, Gorbach & Keith, 1973) and post-operative bacteraemia (Chow & Guze, 1974). There are, however, technical problems relating to their culture and identification. The present classification of the bacteroides-like bacteria is confused and most routine diagnostic laboratories are unable to identify the organisms beyond the level of '*Bacteroides* spp'.

Recent advances in techniques of handling and anaerobic culture of these bacteria—e.g. the adoption of a standard anaerobic procedure (Collee, *et al.*, 1972), the addition of 10% carbon dioxide to the anaerobic atmosphere (Watt, 1973), the use of lysed blood agar (Finegold, Sugihara & Sutter, 1971), and the addition of haemin and vitamin K as growth factors for *Bacteroides melaninogenicus* (Gibbons & MacDonald, 1960)—have overcome many of the difficulties previously encountered in isolating them in pure culture. It is now possible to recover them quantitatively on solid media (Watt, 1972) and to obtain good growth in liquid media by conventional bacteriological techniques at the bench.

Our objective in the present study was to produce a scheme for the identification of isolates of the bacteroides group from which a simple approach might be derived for

use in the routine diagnostic laboratory. We have used conventional bacteriological methods to characterize a series of 165 bacteroides-like organisms including standard reference strains, referred strains and new isolates from clinical specimens and from healthy subjects.

Materials and Methods

Cultures were regularly checked for cell morphology and purity by examination of Gram-stained smears and by aerobic and anaerobic subculture on human-blood agar.

Culture inocula

One drop (0·02 ml) of a 48-h cooked meat broth culture was used to seed each tube and either one drop or one loopful (*c.* 0·01 ml) to seed each plate of medium.

Anaerobic incubation

The standard anaerobic procedure of Collee *et al.* (1972) was followed with anaerobic jars supplied by Baird and Tatlock Ltd (BTL), each fitted with three catalyst sachets. All incubation was at 37° in an atmosphere of 90% H_2 and 10% CO_2.

Incubation under low oxygen tension

An anaerobic jar without catalyst was evacuated until the internal atmospheric pressure was reduced to 180 mmHg. The pressure was then equilibrated with CO_2, i.e. *c.* 5% O_2 and 80% CO_2. The jars were incubated at 37° for 48 h.

Organisms

The following strains were obtained from the National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale Avenue, London, NW9 5HT: *Bacteroides fragilis* (*B. fragilis* ss. *fragilis*) 9343, 9344, 10581, 10584 and 8560; *B. thetaiotaomicron* (*B. fragilis* ss. *thetaiotaomicron*) 10582; *B. vulgatus* (*B. fragilis* ss. *vulgatus*) 10583; *B. melaninogenicus* 9336, 9337 and 9338; *Fusobacterium polymorphum* 10562; *F. necrogenes* 10723; *F.* (*Bacteroides*; *Sphaerophorus*) *necrophorum* 10575, 10576 and 10577; *Leptotrichia buccalis* 10249; *L. dentium* 10206; *B. necrophorus* 7155 (see Barnes & Goldberg, 1968; Lapage, 1972). *B. oralis* 15930 (*B. melaninogenicus*, see Holbrook & Duerden, 1974) was from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 20852, U.S.A. *B. fragilis* ss. *vulgatus* ATCC 8482, *B. fragilis* ss. *ovatus* ATCC 8483, *B. fragilis* ss. *thetaiotaomicron* ATCC 8492, and *B. fragilis* ss. *distasonis* ATCC 8503 were from Dr Ella M. Barnes, Agricultural Research Council, Food Research Institute, Colney Lane, Norwich, NOR 70F. *B. ochraceus* VPI 2845 and *B. melaninogenicus* ss. *melaninogenicus* VPI 4196 were from Dr Elizabeth Cato, Virginia Polytechnic Institute and State University, Blacksburg, Va. 24060, U.S.A. *B. oralis* NP 333 was from Mr G. H. Bowden, London Hospital Medical College, Dental School, Turner St., London, E1 2AD. Three strains of *B. melaninogenicus* (strains 2296, 3502 and 3586) were from Dr R. Wiseman, Bangour General Hospital, W. Lothian. Four strains of *B. corrodens*

(strains 124, NC-1, NC-2, and NCL 20) were from Dr A. L. James, Newcastle Polytechnic, Newcastle upon Tyne, England. Thirty-nine strains were isolated from clinical specimens at the Royal Infirmary, Edinburgh; 50 strains were from clinical specimens at the Western General Hospital, Edinburgh; 26 strains were isolated from normal human faeces in this laboratory; and 17 strains were isolated from human gingival crevice material and dental plaque sampled at the Dental Hospital, Edinburgh. A total of 165 strains was examined.

Media

These were either freshly prepared or steamed for 30 min and promptly cooled just before use. Media containing menadione could not be steamed and were always freshly prepared.

The basic liquid media were Robertson's cooked meat broth (Cruickshank, 1968, modified by Watt, 1973) and thioglycollate medium without dextrose or indicator (Baltimore Biological Laboratories); various combinations of chemicals were added (see below). All solid media were prepared with Columbia agar base (Oxoid). Blood agar contained 5% outdated human blood provided by the Regional Blood Transfusion Service, Edinburgh; each 500 ml volume of the human blood preparation contained 2 g disodium citrate and 1·7 g dextrose in 70 ml of water added to 430 ml of whole blood; lysed blood agar was prepared by treating the blood with saponin before incorporating it in the medium (Cruickshank, 1968).

Growth factors. The following chemicals were added as indicated for the growth of the more fastidious organisms. Stock solution of haemin (haematin hydrochloride, BDH) contained 500 μg/ml in 0·01 N-NaOH and stock solution of menadione (Sigma, London) contained 100 μg/ml in distilled water (see Barnes & Impey, 1971); these filter-sterilized solutions were added aseptically to (cooled) autoclaved media to give final concentrations of haemin 5 μg/ml and menadione 1 μg/ml. A filter-sterilized solution of glucose in distilled water was added to (cooled) autoclaved media to give a final concentration of 1%. When required 0·25% yeast extract (Oxoid) and 0·25% sodium succinate (BDH) were added to media before autoclaving.

Characterization of strains

Morphology. In general, observations were recorded after growth for 48 h on blood agar; colonies of strains that grew slowly were described as soon as they were visible. Cell morphology was noted in Gram-stained smears from cultures grown for 48 h (i) on blood agar and (ii) in cooked meat broth.

Haemolysis on blood agar was observed after incubation for 48 h and 1 week.

Pigment production. Strains were observed for production of black-pigmented colonies on lysed blood agar with menadione after incubation for up to 2 weeks.

Motility. A wet film prepared from a 48 h cooked meat broth culture was examined by phase-contrast microscopy. Strains were also stab inoculated into semi-solid agar

(motility test medium) which was then incubated anaerobically until growth was visible. The medium contained 0·2% agar (Oxoid no. 2) in thioglycollate medium with yeast extract, haemin and menadione.

Lipase activity. This was detected by observing the effect of growth on egg yolk agar (EYA) (Cruickshank, 1968), supplemented with sodium succinate, yeast extract, haemin and menadione.

Oxidase test. A freshly prepared 1% solution of tetramethyl-*p*-phenylene-diamine dihydrochloride in distilled water was poured on to a 1-week culture on supplemented egg yolk agar. A blue colour indicated oxidase production.

Catalase test. A 1-week culture on supplemented egg yolk agar was flooded with 10% hydrogen peroxide solution; a stream of bubbles arose from colonies of catalase-producing organisms.

Hydrogen sulphide production. A strip of lead acetate paper was suspended during incubation in the neck of a tube of a cooked meat broth culture. Blackening of the paper indicated H_2S production.

Nitrate reduction. This was tested in thioglycollate medium with yeast extract, haemin, menadione and KNO_3 (200 μg/ml). The presence of nitrite ions was indicated by a deep red colour when 0·5 ml of Nitrate Solution A and 0·5 ml of Nitrate Solution B (Cruickshank, 1968) were added to 48-h cultures.

Indole production. Indole was detected by adding 0·5 ml of benzol to the liquid supernate of a 48-h cooked meat broth culture and then adding a few drops of Ehrlich's Reagent. A pink colour indicated the presence of indole. Positive and negative control strains were included in each batch of tests.

Gelatinase test. A charcoal–gelatin disc in a cooked meat broth culture was observed for digestion of the disc during incubation for 2 weeks. In view of difficulties encountered with commercially available discs, the discs were prepared in our own laboratory by a modification of Kohn's method (Kohn, 1953): 12·5 g gelatin (Difco) was dissolved in 100 ml of nutrient broth (Oxoid no. 2); 5 g of finely powdered charcoal was added and the mixture was poured into metal Petri dishes and allowed to solidify at 4°. The charcoal–gelatin was held in 10% formalin at room temperature for 5 days and then cut into discs 1 cm in diameter. The discs were washed in running tap water for 48 h at 4° and pasteurized by heating at 70° in sterile distilled water for 20 min.

Aesculin hydrolysis. The organisms were grown for 48 h in cooked meat broth containing 1% aesculin. If aesculin was hydrolysed a black discoloration developed when 0·5 ml of a 1% aqueous solution of ferric ammonium citrate was added.

Dextran hydrolysis. Strains were grown for one week on a medium containing 0·5% dextran T40 (Pharmacia, Uppsala, Sweden), 0·5% blue dextran 2000 (Pharmacia), 0·2% glucose, 0·25% sodium succinate, 0·25% yeast extract, 5 μg/ml haemin and

1 μg/ml menadione in Columbia agar base (Oxoid) (modified from Staat, Gawronski & Schachtele, 1973). Hydrolysis was indicated by the development of a zone of clearing around colonies growing on the blue medium.

Carbohydrate-fermentation tests. Filter-sterilized 20% solutions of glucose, lactose, sucrose, maltose, rhamnose, trehalose and mannitol in distilled water were added separately to tubes of pre-steamed cooked meat broth, or thioglycollate medium with haemin, menadione and yeast extract, to give a final concentration of 1% of the test sugar. The tubes were seeded with 1 drop (0·02 ml) from a 48-h cooked meat broth culture and incubated anaerobically for 48 h. Slow-growing strains were also incubated for 1 week. The final pH was measured with a Pye Unicam model 292 pH meter and a Pye Ingold combined Glass and Reference Electrode no. 401-S/160 after aerobic exposure on the bench for at least 1 h. Controls included cultures of the test strains in plain cooked meat broth or thioglycollate medium and tubes of uninoculated (but incubated) 1% sugar media. The test was regarded as positive if the pH fell >0·5 unit below that of the uninoculated control and >0·5 pH unit in comparison with the value for the 48-h culture in plain cooked meat broth (see Rutter, 1970).

Tolerance tests

(a) *Inhibition of growth by bile salts.* Each strain was seeded on a series of four nutrient agar test media supplemented with sodium succinate, haemin and menadione, and containing (i) no bile salt (control); (ii) 0·5% sodium taurocholate (BDH); (iii) 0·1% sodium deoxycholate (BDH); or (iv) 0·5% sodium taurocholate plus 0·1% sodium deoxycholate. The plates were examined for growth after 48 h, or longer for fastidious strains.

(b) *Sensitivity to dyes.* Each strain was seeded on a series of five nutrient agar media supplemented with sodium succinate, yeast extract, haemin and menadione, and containing (i) no dye (control); (ii) brilliant green (1 in 80 000); (iii) Victoria blue 4R (1 in 80 000); (iv) gentian violet (1 in 100 000); or (v) ethyl violet (1 in 80 000). The plates were examined for growth after 48 h. Each dye was initially prepared in aqueous solution and the concentrations above are those finally achieved in the test medium.

(c) *Antibiotic resistance tests.* The test strains were grown on fresh blood agar and tested for resistance to antibiotics by the disc-diffusion method (modified from Sutter & Finegold, 1971). The plates were seeded by spreading 0·02 ml of a 48-h cooked meat broth culture on the surface with a glass spreader. The diameters of zones of inhibition were measured after either 24 or 48 h, as soon as good growth was visible.

Discs containing neomycin sulphate 1000 μg, kanamycin sulphate 1000 μg, benzyl penicillin 1·5 units, erythromycin ethyl succinate 60 μg, colistin sulphate 10 μg, rifampicin (Rimactane) 15 μg, vancomycin 15 μg, and chloramphenicol 10 μg were prepared in our laboratory. Discs containing methicillin 10 μg, lincomycin 2 μg, clindamycin 2 μg, bacitracin 0·1 unit, neomycin 10 μg and kanamycin 30 μg were obtained from Mast Laboratories. *B. fragilis* NCTC 9343 was selected as the reference strain for tests. It was tested in parallel experiments with a standard Oxford staphylococcus that was sensitive to neomycin, kanamycin, penicillin, methicillin,

erythromycin, rifampicin, lincomycin, clindamycin, vancomycin and chloramphenicol but resistant to colistin and bacitracin. The diameters of zones of inhibition were grouped in four grades: <15 mm; 16–25 mm; 26–35 mm; >35 mm. The grades obtained with *B. fragilis* NCTC 9343 were classified as resistant (R) or sensitive (S) by comparison with the grades obtained with the Oxford staphylococcus. The grades obtained with test strains were compared with those from *B. fragilis* NCTC 9343 and classified as 'resistant' or 'sensitive' in relation to that reference strain.

Results

Cell morphology

All strains were Gram negative, non-sporing, non-motile, obligately anaerobic bacteria, except *L. dentium* NCTC 10206 which was a Gram-variable facultative aerobe with many Gram positive cells in young cultures. Pleomorphism was common. Cell shape varied from filamentous to cocco-bacillary, often in the same smear; some strains formed chains; some had pointed ends and others rounded ends. Cell morphology varied further with the culture medium and had little discriminatory value. However, many *B. melaninogenicus* strains were predominantly cocco-bacillary and many *Fusobacterium* spp. showed long filamentous forms.

Colony morphology

Colonies on blood agar differed in size from pin-point to 3–4 mm in diameter. Some but not all *Fusobacterium* spp. produced rhizoid colonies which were never produced by *Bacteroides* spp. Black-pigmented colonies with haloes of haemolysis were typical of *B. melaninogenicus*. Many strains gave a small zone of incomplete haemolysis after prolonged incubation, but a wider zone of complete haemolysis was characteristic of *B. melaninogenicus* and some *Fusobacterium* spp. (Tables 1a, b, c).

The test strains were divided into three groups on the basis of the results of biochemical, tolerance and antibiotic resistance tests. Strains that broadly resembled *B. fragilis* NCTC 9343 were designated 'B-strains'; strains that produced black pigmented colonies on blood agar were regarded as *B. melaninogenicus* strains (Duerden, 1975) and designated 'M-strains'; the remaining strains that included reference strains of *Fusobacterium* spp. and *Leptotrichia* spp. and a small heterogeneous group of laboratory isolates that did not conform with the broad characteristics of the other two groups were designated 'F-strains'.

Results of biochemical tests

None of the test strains produced oxidase and only *L. dentium* NCTC 10206 produced catalase.

The 'B-strains'. The 20 patterns of results in a series of biochemical tests on 107 B-strains are listed in Table 1a and are coded Bi-Bxx. Almost all results were clearly positive or negative. Haemolysis, however, was variable and at most weak and incomplete with B-strains, and a few variable results were obtained in tests for sucrose fermentation.

TABLE 1A

Results of biochemical tests with non-pigmented Bacteroides *organisms (B-strains)*

Test	Pattern of results*																			
	Bi	Bii	Biii	Biv	Bv	Bvi	Bvii	Bviii	Bix	Bx	Bxi	Bxii	Bxiii	Bxiv	Bxv	Bxvi	Bxvii	Bxviii	Bxix	Bxx
H_2S production	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Haemolysis on blood agar	⊥	−	⊥	⊥	−	⊥	⊥	−	⊥	⊥	⊥	⊥	⊥	−	⊥	⊥	⊥	+	⊥	−
Lipase production on EYA	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Digestion of gelatin	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
Indole production	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−
Hydrolysis of aesculin	+	−	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+
Fermentation of:																				
glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sucrose	+	+	+	+	+	−	+	+	+	⊥	+	+	⊥	+	+	+	+	−	+	+
rhamnose	−	−	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	−	+	−
trehalose	−	−	−	−	−	−	+	+	−	−	+	−	−	+	+	+	+	−	−	−
mannitol	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Hydrolysis of dextran	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+	−	−	+	+
Reference strains conforming to the given pattern	NCTC 9343 NCTC 8560 NCTC 9344 NCTC 10581 NCTC 10584	...	NCTC 10583	...	...	...	ATCC 8503	ATCC 8482	...	...	ATCC 8483	...	...	ATCC 8492	NCTC 10582	...	...	...	NP 333	VPI 2845
No. of laboratory isolates conforming	47	1	4	12	1	4	0	2	1	2	0	1	3	2	7	1	5	1	0	0
Total no. of strains conforming	52	1	5	12	1	4	1	3	1	2	1	1	3	3	8	1	5	1	1	1

*+ = positive result; − = negative result; ⊥ = weak reaction.

TABLE 1B

Results of biochemical tests with black-pigmented Bacteroides *organisms (M-strains)*

Test	Pattern of results*											
	Mi	Mii	Miii	Miv	Mv	Mvi	Mvii	Mviii	Mix	Mx	Mxi	Mxii†
H_2S production	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	−	−	−	−	−	−	−	−	−	+	+	−
Haemolysis on blood agar	+	+	+	+	+	+	+	+	+	+	+	+
Lipase production on EYA	+	−	+	+	−	−	−	−	−	−	−	...
Digestion of gelatin	+	+	+	+	+	+	+	+	+	+	−	+
Indole production	−	−	+	+	+	+	−	−	+	+	−	+
Hydrolysis of aesculin	+	−	−	−	−	−	−	−	−	−	−	−
Fermentation of:												
glucose	+	+	+	+	+	+	+	−	−	−	+	−
lactose	+	+	+	−	−	+	−	−	−	−	+	−
maltose	+	+	+	+	+	+	+	−	−	−	−	−
sucrose	+	+	+	+	+	+	−	−	−	−	+	−
rhamnose	−	−	−	−	−	−	+	−	−	−	−	−
trehalose	−	−	−	−	−	−	−	−	−	−	−	−
mannitol	−	−	−	−	−	−	−	−	−	−	−	−
Hydrolysis of dextran	+	+	−	−	−	−	−	−	−	−	−	−
Reference strains conforming to the given pattern	ATCC 15930	VPI 4196	...	NCTC 9338	NCTC 9336	...	...	...	NCTC 9337	...	...	...
No. of laboratory isolates conforming	0	0	1	11	1	4	1	4	7	1	1	5
Total no. of strains conforming	1	1	1	12	2	4	1	4	8	1	1	5

*+ = positive result; − = negative result.

† Five laboratory isolates that did not grow on EYA are shown in the Tables as biochemical group Mxii.

TABLE 1C

Results of biochemical tests with miscellaneous organisms of the Bacteroides–Fusobacterium *group (F-strains)*

Test	Pattern of results*									
	Fi	Fii	Fiii	Fiv	Fv	Fvi	Fvii	Fviii	Fix	Fx
H_2S production	+	+	−	+	−	+	−	+	+	−
Nitrate reduction	+	−	−	−	−	−	−	−	−	+
Haemolysis on blood agar	⊥	+	+	−	−	−	−	−	−	⊥
Lipase production on EYA	−	+	+	−	−	−	−	−	−	−
Digestion of gelatin	+	−	−	−	−	−	−	−	−	−
Indole production	−	+	−	+	−	−	−	−	−	−
Hydrolysis of aesculin	−	−	−	−	+	+	−	−	+	−
Fermentation of										
glucose	−	⊥	+	−	+	−	⊥	−	+	−
lactose	−	−	−	−	+	−	⊥	−	+	−
maltose	−	⊥	+	−	+	−	⊥	−	+	−
sucrose	−	−	−	−	+	−	⊥	−	+	−
rhamnose	−	−	−	−	−	−	−	−	−	−
trehalose	−	−	−	−	+	+	−	−	−	−
mannitol	−	−	−	−	−	−	−	−	−	−
Hydrolysis of dextran	−	−	−	−	−	−	−	−	−	−
Reference strains conforming to the given pattern	...	NCTC 10575 NCTC 10576 NCTC 10577	...	NCTC 10562	NCTC 10249	NCTC 10723	...	...	NCTC 7155	NCTC 10206
No. of laboratory isolates conforming	4	0	1	1	0	0	1	2	0	0
Total no. of strains conforming	4	3	1	2	1	1	1	2	1	1

*+ = positive result; − = negative result; ⊥ = weak reaction.

The five reference strains of *B. fragilis* ss. *fragilis* and 47 strains isolated from clinical specimens gave pattern Bi. *B. fragilis* ss. *vulgatus* NCTC 10583 and 4 laboratory isolates gave pattern Biii, distinguished by the ability to digest gelatin and ferment rhamnose. Pattern Biv, obtained with 12 strains from normal human faeces differed from Biii in only one respect (aesculin hydrolysis negative); *B. fragilis* ss. *vulgatus* ATCC 8482 was significantly different and gave a pattern (Bviii) similar to that obtained with *B. fragilis* ss. *distasonis* ATCC 8503 (Bvii). *B. fragilis* ss. *ovatus* ATCC 8483 (pattern Bxi) was the only strain that fermented mannitol. Nine laboratory isolates gave the same patterns (Bxiv and Bxv) as the two reference strains of *B. fragilis* ss. *thetaiotaomicron* ATCC 8492 and NCTC 10582 that differed only in the ability to hydrolyse dextran. *B. oralis* NP 333 and *B. ochraceus* VPI 2845 gave individual patterns (Bxix and Bxx) that differed only in the fermentation of rhamnose. The 20 remaining laboratory isolates gave patterns that were intermediate between the patterns obtained with the reference strains.

The 'M-strains'. Twelve patterns of results (Mi-Mxii) were obtained from the biochemical tests on 41 M-strains (Table 1b). All strains produced black-pigmented colonies on blood agar surrounded by a zone of clear haemolysis and all except one strain digested gelatin. The patterns of results with most strains fell within two groups clearly differentiated by the results of carbohydrate-fermentation tests.

B. melaninogenicus NCTC 9336 and 9338 and 12 laboratory isolates—10 from human gingival crevice material and dental plaque and 2 from clinical specimens—gave similar patterns (Miv and Mv) that differed only in the production of lipase; five strains (patterns Miii and Mvi) differed only in the fermentation of lactose.

The second group were asaccharolytic. *B. melaninogenicus* NCTC 9337 and 11 laboratory isolates—10 from abdominal wounds and high vaginal swabs and one oral isolate—gave similar patterns (Mviii and Mix). Five asaccharolytic strains failed to grow on supplemented egg yolk agar and are, therefore, placed in a separate pattern (Mxii). *B. melaninogenicus* ss. *melaninogenicus* VPI 4196 and *B. melaninogenicus* (formerly *B. oralis*) ATCC 15930 gave similar patterns but differed in the production of lipase and the hydrolysis of aesculin. The remaining three strains gave individual patterns (Mvii, Mx and Mxi). In particular, the strains that gave patterns Mx and Mxi were the only *Bacteroides* strains except *B. corrodens* that reduced nitrate to nitrite.

The 'F-strains'. The results of the biochemical tests with reference strains of *Fusobacterium* spp. and a small miscellaneous group of laboratory isolates are given in Table 1c. None of these strains produced black-pigmented colonies on blood agar. The four strains of *B. corrodens* gave pattern Fi. *F. necrophorum* NCTC 10575, 10576 and 10577 gave pattern Fii; they gave variable results in fermentation tests with glucose and maltose but the results were never more than weakly positive (a fall in pH of 0·5 unit). *F. polymorphum* NCTC 10562, *F. necrogenes* NCTC 10723 and *L. buccalis* NCTC 10249 gave individual patterns of results. Two laboratory isolates were unreactive except in H_2S production (pattern Fviii), and one isolate gave positive results only weakly in some fermentation tests (pattern Fvii). *B. necrophorus* NCTC 7155 gave the pattern (Fix) of *B. fragilis* ss. *fragilis*. The pattern (Fx) obtained with *L. dentium* NCTC 10206 is included although the organism is not a true Gram negative anaerobe.

TABLE 2
Results of tolerance tests with 165 strains of Gram negative anaerobic bacilli

Test	Pattern of results* A	B	C	D	E	F	G	H	J	K	L	M	N	P	Q	(R)†
Growth on basal medium	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	...
Growth on basal medium plus:																
taurocholate (0·5%)	+	+	+	+	+	+	+	+	I	I	I	I	+	I	+	...
deoxycholate (0·1%)	I	I	I	I	I	I	I	+	I	I	I	I	+	I	+	...
taurocholate (0·5%) & deoxycholate (0·1%)	+	I	+	+	+	I	I	+	I	I	I	I	+	I	+	...
Victoria blue 4R (1/80 000)	+	+	+	I	+	+	I	+	+	I	+	+	+	+	+	...
ethyl violet (1/80 000)	I	I	I	I	+	+	I	I	I	I	I	+	+	+	+	...
gentian violet (1/100 000)	I	I	I	I	I	I	I	I	I	I	I	+	+	+	+	...
brilliant green (1/80 000)	I	I	+	I	I	I	I	I	I	I	+	+	I	I	+	...
Reference strains conforming to the given pattern	NCTC 8560, NCTC 9343, NCTC 9344, NCTC 10581, NCTC 10582, NCTC 10584, ATCC 8483, ATCC 8492	...	...	ATCC 8482, ATCC 8503	NCTC 10583	...	...	...	NP 333	NCTC 9336, NCTC 9337, NCTC 9338, VPI 4196, VPI 2845, ATCC 15930, NCTC 10206	...	NCTC 10575, NCTC 10576, NCTC 10577, NCTC 10562	NCTC 10723	NCTC 10249	NCTC 7155	...
No. of laboratory isolates conforming	48	15	2	0	15	1	1	1	7	38	6	2	0	0	0	3
Total no. of strains conforming	56	15	2	2	16	1	1	1	8	45	6	6	1	1	1	3

*+ = growth; I = inhibition of growth.

† Three laboratory isolates of *B. melaninogenicus* that did not grow on tolerance test basal medium are shown in the tables as tolerance group R.

Results of tolerance tests

Sixteen patterns (A-R) of results were obtained with the 165 test strains in tolerance tests with bile salts and dyes (Table 2). *B. fragilis* ss. *fragilis* NCTC 8560, 9343, 9344, 10581 and 10584, *B. fragilis* ss. *thetaiotaomicron* NCTC 10582 and ATCC 8492, *B. fragilis* ss. *ovatus* ATCC 8483, and 48 laboratory isolates gave pattern A. Fifteen laboratory isolates gave a similar pattern (B) but inhibition of growth by deoxycholate was not prevented by the presence of taurocholate. *B. fragilis* ss. *vulgatus* NCTC 10583 and 15 laboratory isolates grew in the presence of Victoria blue 4R and ethyl violet (pattern E). *B. fragilis* ss. *vulgatus* ATCC 8482 was inhibited by these dyes and gave the same pattern (D) as *B. fragilis* ss. *distasonis* ATCC 8503.

B. melaninogenicus NCTC 9336, 9337, 9338, ATCC 15930 and VPI 4196 and 30 laboratory isolates of *B. melaninogenicus* gave pattern K; *B. ochraceus* VPI 2845, *L. dentium* NCTC 10206, 7 laboratory isolates of *B. fragilis*-like strains, and 1 F-strain gave the same pattern. *B. oralis* NP 333, 2 laboratory isolates of *B. melaninogenicus*, 2 F-strains, and 3 *B. fragilis*-like strains gave pattern J that differed from pattern K by growth in the presence of Victoria blue 4R.

The 4 *B. corrodens* strains, 1 *B. melaninogenicus* strain and 1 *B. fragilis*-like strain gave pattern L. *F. necrophorum* NCTC 10575, 10576 and 10577, *F. polymorphum* NCTC 10562, and 2 laboratory isolates gave tolerance pattern M. *F. necrogenes* NCTC 10723, *L. buccalis* NCTC 10249, and *B. necrophorus* NCTC 7155 gave unique tolerance patterns (N, P and Q). Strain NCTC 7155 was the only test strain that grew in the presence of all the dyes and bile salts used. Three *B. melaninogenicus* strains did not grow on tolerance test basal medium (pattern (R)).

Results of antibiotic disc resistance tests

The patterns of results obtained with the 165 test strains in antibiotic disc resistance tests are shown in Table 3a and b. The 105 *B. fragilis*-like strains gave 18 patterns of resistance (1–18); 61 strains gave pattern 1; they were resistant to the test concentrations of neomycin, kanamycin, penicillin, methicillin, colistin, lincomycin, bacitracin and vancomycin. Fifteen strains (patterns 12–18) were sensitive to the 1000 μg neomycin disc, 1 strain (pattern 17) was sensitive to the 1000 μg kanamycin disc, and 2 strains (patterns 4 and 17) were sensitive to vancomycin. Six strains were sensitive to penicillin and methicillin (patterns 10 and 11) and 2 strains (pattern 18) to methicillin alone. Fourteen strains were resistant to erythromycin (patterns 2, 7, 8, 9 and 12); 3 strains were resistant to rifampicin (patterns 6, 7 and 8) and 2 strains were resistant to chloramphenicol (patterns 7 and 16). Twenty-one strains were sensitive to lincomycin (patterns 4, 5, 6, 9, 10, 14, 15, 16, 17 and 18) but all except 6 strains (patterns 3 and 8), were sensitive to clindamycin. Two strains were sensitive to colistin (pattern 18) and 3 strains were sensitive to bacitracin (patterns 15 and 17).

The 41 *B. melaninogenicus* strains gave 12 patterns of resistance (19–30). Seventeen strains gave pattern 24; they were resistant to the 10 μg neomycin disc, 30 μg and 1000 μg kanamycin discs, and vancomycin, and sensitive to the other antibiotics. Three strains were resistant to penicillin and methicillin (patterns 23 and 30), 3 to chloramphenicol (patterns 21 and 29), and 1 strain (pattern 30) was resistant to lincomycin and clindamycin. All except 5 strains (patterns 19–22) were sensitive to the 1000 μg neomycin disc; 15 strains were sensitive to vancomycin (patterns 20–22,

TABLE 3A

Results of antibiotic disc resistance tests with the test strains of Gram negative anaerobic bacilli: patterns 1–20

Antibiotic (content per disc)	Patterns of results*																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Neomycin (1000 μg)	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S	R	R
Neomycin (10 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kanamycin (1000 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
Kanamycin (30 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Penicillin (1·5 units)	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	S	S
Methicillin (10 μg)	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	S	S	S
Erythromycin (60 μg)	S	R	S	S	S	S	R	R	R	S	S	R	S	S	S	S	S	S	S	S
Colistin (10 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R
Rifampicin (15 μg)	S	S	S	S	S	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S
Lincomycin (2 μg)	R	R	R	S	S	S	R	R	S	S	R	R	R	S	S	S	S	S	S	S
Clindamycin (2 μg)	S	S	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S
Bacitracin (0·1 unit)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	R	S	S
Vancomycin (15 μg)	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
Chloramphenicol (10 μg)	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	R	S	S	S	S
Reference strains conforming to the pattern	NCTC 9343 NCTC 9344 NCTC 10583 NCTC 10584	NCTC 8560	NCTC 10582	NCTC 10581	...	ATCC 8492	ATCC 8483	...	...	...	...	...	ATCC 8482	...	...	ATCC 8503	...	...	...	...
No. of laboratory isolates conforming	57	7	4	0	5	0	0	1	1	5	1	3	3	2	2	0	1	2	2	1
Total no. of strains conforming	61	8	5	1	5	1	1	1	1	5	1	3	4	2	2	1	1	2	2	1

* S = sensitive; R = resistant

TABLE 3B

Results of antibiotic disc resistance tests: patterns 21–41

Antibiotic (content per disc)	Patterns of results*																				
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
Neomycin (1000 μg)	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Neomycin (10 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S
Kanamycin (1000 μg)	R	R	R	R	R	R	R	R	S	S	S	R	R	S	S	S	S	S	S	S	S
Kanamycin (30 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	S	S
Penicillin (1·5 units)	S	S	R	S	S	S	S	S	S	R	S	R	R	S	S	S	S	S	S	S	S
Methicillin (10 μg)	S	S	R	S	S	S	S	S	S	R	S	S	R	S	S	S	S	S	R	S	S
Erythromycin (60 μg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	S
Colistin (10 μg)	S	R	S	S	S	R	R	S	S	S	R	R	R	S	S	S	S	S	S	S	R
Rifampicin (15 μg)	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S
Lincomycin (2 μg)	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S
Clindamycin (2 μg)	S	S	S	S	S	S	S	S	S	R	R	R	R	R	R	S	R	R	R	R	S
Bacitracin (0·1 unit)	S	R	S	S	S	S	R	R	S	R	R	R	R	R	R	S	R	R	R	R	S
Vancomycin (15 μg)	S	S	R	R	S	S	S	R	R	S	S	R	S	R	R	S	R	R	S	S	S
Chloramphenicol (10 μg)	R	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S
Reference strains conforming to the pattern	...	...	...	NCTC 9336 NCTC 9338	...	NCTC 9337	ATCC 15930	VPI 4196 NP 333	...	...	VPI 2845	...	...	NCTC 10575 NCTC 10576 NCTC 10577 NCTC 10723	...	NCTC 10249	NCTC 10562	NCTC 7155	...	...	NCTC 10206
No. of laboratory isolates conforming	1	1	2	15	3	4	2	2	2	1	0	1	1	0	1	1	0	0	1	4	0
Total no. of strains conforming	1	1	2	17	3	5	3	4	2	1	1	1	1	4	1	2	1	1	1	4	1

* S = sensitive; R = resistant

25–27 and 30), but only 3 strains were sensitive to the 1000 μg kanamycin disc (patterns 29 and 30). Twelve strains were resistant to colistin (patterns 19, 20, 22, 26 and 27) and 8 strains were resistant to bacitracin (patterns 22, 27, 28 and 30). *B. oralis* NP 333 gave the same pattern (28) as 3 *B. melaninogenicus* strains. *B. ochraceus* VPI 2845, however, was sensitive to the 1000 μg kanamycin disc and vancomycin, and resistant to colistin, clindamycin and bacitracin (pattern 24).

The 17 F-strains gave 10 patterns of resistance (32–41). *L. dentium* NCTC 10206 was sensitive to all agents except colistin (pattern 41). All 4 strains of *B. corrodens* gave pattern 40; they were sensitive to all agents except rifampicin and bacitracin. *F. necrophorum* NCTC 10575, 10576 and 10577, and *F. necrogenes* NCTC 10723 (pattern 34) and *B. necrophorus* NCTC 7155 (pattern 38) were also resistant to rifampicin. The remaining F-strains gave varied patterns.

Comparison of results of biochemical tests with results of tolerance tests

The patterns of tolerance obtained with the 107 B-strains that gave 20 patterns of biochemical results are shown in Table 4a. Forty-nine of the 52 strains that gave the biochemical pattern Bi (*B. fragilis* ss. *fragilis*) and 19 of the 22 strains that gave patterns Bviii-Bxv and contained all *B. fragilis* ss. *ovatus* and *B. fragilis* ss. *thetaiotaomicron* strains gave tolerance patterns A and B. The 15 strains in tolerance pattern B did not grow in the presence of the deoxycholate and taurocholate mixture.

The 17 strains of *B. fragilis* ss. *vulgatus* in biochemical groups Biii and Biv gave tolerance patterns E and F, distinguished by growth in the presence of ethyl violet. Eleven strains that gave biochemical patterns conforming with the *B. fragilis*-like group (patterns Bi-Bxviii) gave tolerance patterns J, K and L that were the same as patterns obtained with *B. melaninogenicus* strains. *B. oralis* NP 333, *B. ochraceus* VPI 2845 (biochemical patterns Bxix and Bxx), *L. dentium* NCTC 10206 and three F-strains also gave tolerance patterns J and K.

Thirty-five of the 41 strains of *B. melaninogenicus* (biochemical patterns Mi-Mxii) gave tolerance pattern K (Table 4b). Three strains grew in the presence of Victoria blue 4R (tolerance patterns J and L) and the remaining 3 strains failed to grow on the tolerance test basal medium.

The patterns of tolerance obtained with 17 F-strains are shown in Table 4c. The 4 strains of *B. corrodens* that gave biochemical pattern Fi gave tolerance pattern L. *F. necrophorum* NCTC 10575, 10576 and 10577 (biochemical pattern Fii), *F. polymorphum* NCTC 10562 (biochemical pattern Fiv) and two laboratory isolates (biochemical patterns Fiv and Fviii) gave tolerance pattern M.

Comparison of results of biochemical tests with results of antibiotic disc resistance tests

The patterns of antibiotic resistance and biochemical results are compared in Table 5a, b and c. Sixty-one of the 105 *B. fragilis*-like strains (biochemical patterns Bi-Bxviii) gave resistance pattern 1 and a further 8 strains differed only in resistance to erythromycin (pattern 2). B-strains with the antibiotic resistance patterns 3–18 were distributed amongst biochemical groups Bi-Bxviii and the recognized subspecies of *B. fragilis* were not distinguishable by their antibiotic resistance patterns.

TABLE 4A

The relationship of biochemical patterns to tolerance patterns of B-strains

Biochemical pattern*	Number of strains with the stated tolerance pattern†																Key to position of reference strain(s) (indicated by superscript)
	A	B	C	D	E	F	G	H	J	K	L	M	N	P	Q	(R)	
Bi	41[a]	8	2						1								a *B. fragilis* ss. *fragilis* NCTC 9343, 9344, 10581, 10584 and 8560
Bii										1							
Biii					4[b]	1											b *B. fragilis* ss. *vulgatus* NCTC 10583
Biv					12												
Bv										1							
Bvi									1	2	1						
Bvii				1[c]													c *B. fragilis* ss. *distasonis* ATCC 8503
Bviii	2			1[d]													d *B. fragilis* ss. *vulgatus* ATCC 8482
Bix		1															
Bx		2															
Bxi	1[e]																e *B. fragilis* ss. *ovatus* ATCC 8483
Bxii		1															
Bxiii		2							1								
Bxiv	3[f]																f *B. fragilis* ss. *thetaiotaomicron* ATCC 8492
Bxv	7[g]						1										g *B. fragilis* ss. *thetaiotaomicron* NCTC 10582
Bxvi								1									
Bxvii	2	1								2							
Bxviii										1							
Bxix									1[h]								h *B. oralis* NP 333
Bxx										1[j]							j *B. ochraceus* VPI 2845

* see Table 1a; † see Table 2.

TABLE 4B

The relationship of biochemical patterns to tolerance patterns of M-strains

Biochemical pattern*	Number of strains with the stated tolerance pattern†															Key to position of reference strain(s) (indicated by superscript)
	A	B	C	D	E	F	G	H	J	K	L	M	N	P	Q	(R)	
Mi										1[k]							k *B. melaninogenicus* ATCC 15930
Mii										1[l]							l *B. melaninogenicus* VPI 4196
Miii										1							
Miv										12[m]							m *B. melaninogenicus* NCTC 9338
Mv										2[n]							n *B. melaninogenicus* NCTC 9336
Mvi									1	3							
Mvii										1							
Mviii										4							
Mix									1	7[p]							p *B. melaninogenicus* NCTC 9337
Mx											1						
Mxi										1							
Mxii										2						(3)	

* see Table 1b; † see Table 2.

TABLE 4C

The relationship of biochemical patterns to tolerance patterns of F-strains

Biochemical pattern*	Number of strains with the stated tolerance pattern†															Key to position of reference strain(s) (indicated by superscript)
	A	B	C	D	E	F	G	H	J	K	L	M	N	P	Q	(R)	
Fi											4						
Fii												3[q]					q *F. necrophorum* NCTC 10575, 10576 and 10577
Fiii										1							
Fiv												2[r]					r *F. polymorphum* NCTC 10562
Fv														1[s]			s *L. buccalis* NCTC 10249
Fvi													1[t]				t *F. necrogenes* NCTC 10723
Fvii									1								
Fviii									1			1					
Fix															1[u]		u *B. necrophorus* NCTC 7155
Fx										1[v]							v *L. dentium* NCTC 10206

* see Table 1c; † see Table 2.

TABLE 5A

The relationship of biochemical patterns to antibiotic resistance patterns of B-strains

Bio-chemical pattern*	Number of strains with the stated pattern of antibiotic resistance†																					Key to position of reference strain(s) (indicated by superscript)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18 .. 28	.. 31	.. 41		
Bi	36[a]	8[b]		1[c]	2			1	1			3										a *B. fragilis* ss. *fragilis* NCTC 9343, 9344 and 10584
Bii																	1					b *B. fragilis* ss. *fragilis* NCTC 8560
Biii	2[d]				2						1											c *B. fragilis* ss. *fragilis* NCTC 10581
Biv	11				1																	d *B. fragilis* ss. *vulgatus* NCTC 10583
Bv																	1					
Bvi													1	1	2							
Bvii																1[e]						e *B. fragilis* ss. *distasonis* ATCC 8503
Bviii	2												1[f]									f *B. fragilis* ss. *vulgatus* ATCC 8482
Bix	1																					
Bx										2												
Bxi							1[g]															g *B. fragilis* ss. *ovatus* ATCC 8483
Bxii			1																			
Bxiii										3												
Bxiv	1		1			1[h]																h *B. fragilis* ss. *thetaiotaomicron* ATCC 8492
Bxv	4		3[j]										1									j *B. fragilis* ss. *thetaiotaomicron* NCTC 10582
Bxvi	1																					
Bxvii	3												1	1								
Bxviii																	1					
Bxix																		1[k]				k *B. oralis* NP 333
Bxx																			1[l]			l *B. ochraceus* VPI 2845

* See Table 1a; † See Table 3.

TABLE 5B

The relationship of biochemical patterns to antibiotic resistance patterns of M-strains

Biochemical pattern*	Number of strains with the stated pattern of antibiotic resistance†																			Key to position of reference strain(s) (indicated by superscript)
	1	..	..	18	19	20	21	22	23	24	25	26	27	28	29	30	31	..	..	41	
Mi													1[m]								m *B. melaninogenicus* ATCC 15930
Mii														1[n]							n *B. melaninogenicus* VPI 4196
Miii										1											
Miv										9[p]				1	2						p *B. melaninogenicus* NCTC 9338
Mv										2[q]											q *B. melaninogenicus* NCTC 9336
Mvi										4											
Mvii														1							
Mviii									1	1		1	1								
Mix					1		1				2	3[r]	1								r *B. melaninogenicus* NCTC 9337
Mx								1													
Mxi																1					
Mxii					1	1			1		1	1									

* See Table 1b; † See Table 3.

TABLE 5C

The relationship of biochemical patterns to antibiotic resistance patterns of F-strains

Biochemical patterns*	Number of strains with the stated pattern of antibiotic resistance†														Key to position of reference strain(s) (indicated by superscript)
	1	..	..	31	32	33	34	35	36	37	38	39	40	41	
Fi													4		s *F. necrophorum* NCTC 10575, 10576 and 10577
Fii							3[s]								
Fiii									1						
Fiv								1		1[t]					t *F. polymorphum* NCTC 10562
Fv									1[u]						u *L. buccalis* NCTC 10249
Fvi							1[v]								v *F. necrogenes* NCTC 10723
Fvii						1									
Fviii					1							1			
Fix											1[w]				w *B. necrophorus* NCTC 7155
Fx														1[x]	x *L. dentium* NCTC 10206

* See Table 1c; † see Table 3.

Sixteen saccharolytic strains of *B. melaninogenicus* and 1 asaccharolytic strain gave resistance pattern 24, but the distribution of the other 24 strains between 11 resistance patterns was unrelated to their biochemical patterns. All 4 strains of *B. corrodens* gave unique biochemical (Fi) and resistance (40) patterns. *F. necrophorum* NCTC 10575, 10576 and 10577 (biochemical pattern Fii) and *F. necrogenes* NCTC 10723 (biochemical pattern Fvi) gave the same resistance pattern (34) and the other F-strains gave varied resistance patterns.

There was no overlap between the antibiotic resistance patterns obtained with *B. fragilis*-like strains (biochemical patterns Bi-Bxviii), *B. melaninogenicus* strains (Mi-Mxii) and F-strains (Fi-Fx). *B. oralis* NP 333 (Bxix) and *B. ochraceus* VPI 2845 (Bxx) gave similar antibiotic resistance patterns to *B. melaninogenicus* strains.

Discussion

The classification of the Gram negative, non-sporing anaerobes is confused. Groups of workers have given different names to the same organism and the same name has been given to obviously different organisms (Moore & Holdeman, 1973; Holdeman & Moore, 1974). The identification of the sub-species of *B. fragilis* and *B. melaninogenicus* has presented particular problems. The difficulties in this field are partly attributable to the variety of methods used by different workers to classify their strains. In addition to conventional bacteriological approaches that include observations of microscopic and colony morphology and biochemical tests (Prévot, 1966; James & Robinson, 1975), other workers have considered numerical taxonomy (Barnes & Goldberg, 1968), gas–liquid chromatographic analysis of the acid end-products of glucose metabolism (Werner, 1969; Moore, 1970), the analysis of DNA homologies (Johnson, 1973), the ratio of the DNA bases guanine and cytosine (Sebald, cited by Barnes & Goldberg, 1968; Williams, *et al.*, 1974), serological studies of surface antigens (Sharpe, 1971), and tolerance tests that demonstrate group differences in (i) antibiotic resistance (Finegold, Harada & Miller, 1967), (ii) sensitivity to bile salts (Shimada, Sutter & Finegold, 1970), and (iii) the effects of dyes on growth (Suzuki, Ushijima & Ichinose, 1966). More recently, bacteriocin-like effects of possible use in characterization have been described in *Bacteroides* spp. (Collee, Watt & Dewhurst, 1974; Watt & Collee, 1974). It is difficult to correlate the results of these different approaches and to establish the taxonomic level at which each operates.

In the present study, conventional bacteriological tests were selected and adapted to meet the special requirements of these anaerobes. The details of the procedures given in the methods section and of the controls included in the tests are important and take account of problems of growth of strains and reproducibility of results. Findings with a few exacting strains among the 165 tested indicate that we have not solved all of the problems, but the results in general are encouraging.

The test strains fell readily into 3 main groups on the basis of our series of biochemical, tolerance and antibiotic-resistance studies: (i) non-pigmented *B. fragilis*-like strains; (ii) pigmented *B. melaninogenicus* strains; and (iii) *Fusobacterium*-like strains. The biochemical tests gave the most useful discrimination of strains within the groups. In the *B. fragilis*-like group (B-strains) there was clear separation of the reference strains representing 5 recognized sub-species of *B. fragilis*. The patterns obtained with the remaining B-strains showed clusters of strains related to the reference strains

in a spectrum that included intermediate patterns. *B. oralis* NP 333 and *B. ochraceus* VPI 2845 gave biochemical patterns that were closely related to those obtained with the *B. fragilis*-like strains.

In the *B. melaninogenicus* group (M-strains) there was clear separation of the strains into two clusters: (i) saccharolytic, and (ii) asaccharolytic; there were minor differences between strains within these clusters. Three sub-species of *B. melaninogenicus* have been described by Holdeman & Moore (1974) as *B. melaninogenicus* ss. *melaninogenicus*, ss. *intermedius*, and ss. *asaccharolyticus*. *B. melaninogenicus* NCTC 9337 and the non-fermentative laboratory isolates are clearly *B. melaninogenicus* ss. *asaccharolyticus*. *B. melaninogenicus* NCTC 9336 and 9338 conform to the published reference patterns of *B. melaninogenicus* ss. *intermedius* (Holdeman & Moore, 1973) and the remainder of our laboratory isolates of *B. melaninogenicus* were similar to these two reference strains. *B. melaninogenicus* ss. *melaninogenicus* VPI 4196, differed in a number of tests from the cluster of strains identified as *B. melaninogenicus* ss. *intermedius*. *B. melaninogenicus* (formerly *B. oralis*) ATCC 15930 was similar to but not identical with strain VPI 4196; as no laboratory isolates gave a similar pattern, it is not clear from these results whether the saccharolytic strains of *B. melaninogenicus* should be divided into two sub-species.

The F-strains were a heterogeneous collection of organisms that were clearly different from the B-strains and M-strains. There were considerable biochemical differences between reference strains of individual species within the group.

The tolerance tests largely confirmed the separation of the test strains into the three main groups. The *B. fragilis*-like strains gave a number of similar tolerance patterns; small differences were not generally related to the different sub-species, but *B. fragilis* ss. *vulgatus* NCTC 10583 and the similar laboratory isolates (but not *B. fragilis* ss. *vulgatus* ATCC 8482) gave a unique pattern. A single tolerance pattern was typical of the *B. melaninogenicus* strains but complete discrimination between B-strains and M-strains on the basis of tolerance tests was marred by a small number of B-strains; these included *B. oralis*, *B. ochraceus* and a few intermediate strains not biochemically conforming with recognized sub-species of *B. fragilis*, which gave tolerance patterns typical of *B. melaninogenicus* strains. The reference strains in the F-group were separated from the B-strains and M-strains by their tolerance patterns, although other F-strains gave similar patterns to *B. melaninogenicus* strains.

The most detailed separation between the test strains was obtained with the antibiotic disc resistance tests. There was no overlap of patterns obtained with *B. fragilis* strains, *B. melaninogenicus* strains and F-strains when the complete series of antibiotic discs was included. However, *B. oralis* NP 333 and *B. ochraceus* VPI 2845, which were classified as B-strains on the basis of biochemical tests and lack of black pigment, gave antibiotic resistance patterns that closely resembled those obtained with *B. melaninogenicus* strains. Sutter & Finegold (1971) suggested that discs containing specified concentrations of colistin, erythromycin, kanamycin, neomycin, penicillin and rifampicin could be used for preliminary identification of Gram negative anaerobic bacilli. Our results show that a significant number of strains of *B. fragilis* and *B. melaninogenicus* gave atypical results with one or more of these antibiotics. Resistance tests with neomycin 1000 μg, kanamycin 1000 μg, penicillin 1·5 units and rifampicin 15 μg were, however, useful for group discrimination in a combined approach with tolerance tests and biochemical tests. The separation of *B. fragilis* strains and *B. melan-*

inogenicus strains on the basis of antibiotic resistance patterns did not correlate with the biochemical separation of *B. fragilis* into sub-species and *B. melaninogenicus* into saccharolytic and asaccharolytic groups.

It is important that the antibiotic disc resistance tests used for identification of strains should not be confused with the sensitivity tests used to determine the susceptibility of an organism to an antibiotic for clinical use. The results of our resistance tests are not a guide to therapeutic efficacy. The method that we used was modified from that of Sutter & Finegold (1971) and the tests were done under conditions that gave good growth of the test strains. The anaerobic atmosphere accordingly contained 10% CO_2 (Watt, 1973) although its presence is known to reduce the zones of inhibition around discs containing pH sensitive antibiotics such as erythromycin and lincomycin (Ingham *et al.*, 1970). We take account of the known effect of ingredients in culture media on acid production during growth in addition to the contributory effect of CO_2 in the anaerobic atmosphere (Watt & Brown, 1975); this is of particular importance in the clinical application of sensitivity test results although the patterns obtained in resistance tests under defined conditions may still be of taxonomic value. The initial control strain for our disc resistance tests was the Oxford staphylococcus, but this was incubated anaerobically and the value of such a control continues to be debated; we are convinced of the need for a standard anaerobic organism analogous to the Oxford staphylococcus for antibiotic sensitivity testing of anaerobic organisms. Our subsequent use of *B. fragilis* NCTC 9343 as the control strain was an attempt to avoid this problem. We were unable to maintain a standard incubation period for all test strains because of the wide variation in the rates of growth of different strains. Results were therefore read as soon as growth was readily visible.

In this series of biochemical, tolerance and antibiotic resistance tests, the reference strains gave consistent results that correlated well with other published work, except that the type-strain of *B. fragilis* ss. *vulgatus*, ATCC 8482 (Holdeman & Moore, 1974), was significantly different from *B. fragilis* ss. *vulgatus* NCTC 10583 and its related cluster of laboratory isolates. Strain 8482 appeared to be more closely related to *B. fragilis* ss. *distasonis* ATCC 8503.

Dye tolerance tests (Suzuki *et al.*, 1966), studies of growth in the presence of bile salts (Shimada *et al.*, 1970) and antibiotic disc resistance tests (Finegold *et al.*, 1967) have been used for preliminary identification of Gram negative anaerobic bacilli. If these approaches were used separately, a significant number of strains in the present series would be wrongly classified because of atypical results in individual tests. Most of these problems can be overcome by using a short combined set of tolerance tests and antibiotic disc resistance tests and a small number of biochemical tests. The occurrence of atypical results would then be clear.

The set of tests shown in Table 6 would identify the major species and the subspecies of *B. fragilis* and *B. melaninogenicus* and enable a preliminary allocation of the intermediate strains into the appropriate main group. The tolerance tests and antibiotic disc resistance tests are not used in a sequential manner but as a single combined set of tests for the provisional identification of strains. They are of confirmatory value for typical *B. melaninogenicus* strains but are important for the early detection of strains that develop pigment only after prolonged incubation. This Table is based principally upon the results that we obtained with reference strains. It has proved useful for the identification of the unknown laboratory isolates although

Table 4

Scheme for the identification of Gram negative anaerobic bacilli

	Result obtained in test*																
Pigment production†									−						+		
Antibiotic disc resistance tests:																	
neomycin (1000 μg)			R‡			S						S			S‡		
kanamycin (1000 μg)			R			R						S			R		
penicillin (1·5 units)			R			S						S			S‡		
rifampicin (15 μg)			S			S			R			S			S		
Tolerance tests:																	
taurocholate			+			I			I		+		I			I	
deoxycholate			I			I			I		+		I			I	
Victoria blue 4R		+		I	+	+	I		+	+	+		+			I	
ethyl violet		I		I	+	I	I		I	+	+		+			I	
Biochemical tests:																	
indole production	−	+	+	−	−	−	−		−	+	−	+	−		−	+	+
digestion of gelatin	−	−	+	+	+	−	−		+	−	−	−	−		+	+	+
hydrolysis of aesculin	+	+	+	+	+ or −	+	+		−	+	+	−	+		+ or −	−	−
Fermentation of:																	
glucose	+	+	+	+	+	+	+		…§	…	…	…	…		+	+	−
rhamnose	−	+	+	+	+	+	−		…	…	…	…	…		…	…	…
trehalose	−	+	+	+	−	−	−		…	…	…	…	…		…	…	…
mannitol	−	−	+	−	−	−	−		…	…	…	…	…		…	…	…
	B. fragilis														*B. melaninogenicus*		
	ss. *fragilis*	ss. *thetaiotaomicron*	ss. *ovatus*	ss. *distasonis*	ss. *vulgatus*	*B. oralis*	*B. ochraceus*		*B. corrodens*	*F. necrophorum*	*F. necrogenes*	*F. polymorphum*	*L. buccalis*		ss. *melaninogenicus*	ss. *intermedius*	ss. *asaccharolyticus*

* For details including time of recording results see *Methods*; the keys to the symbols used here are given in Tables 1, 2 and 3.
† Pigment production (black colonies) observed on lysed blood agar after anaerobic incubation for up to 7 days.
‡ Occasional strains may give anomalous results.
§… Results of these tests are not of primary importance in the identification of these species.
Note: This gives sets of results that distinguish the species. The scheme should not be regarded as a sequential key.

Fusobacterium spp., *B. fragilis* ss. *distasonis* and ss. *ovatus*, and *B. melaninogenicus* ss. *melaninogenicus* are not adequately represented in our laboratory isolates. The validity of the patterns given for the latter species must therefore await confirmation. Holdeman & Moore (1974) stress the need for further study of the *B. fragilis*-like organisms that they consider to be a continuum of variants with clusters of strains that have been designated sub-species. The present investigation confirms that clusters of strains closely resemble reference strains of recognized sub-species with a number of intermediate strains that differ from the presently accepted sub-species.

This study has shown that it is possible to use conventional procedures to obtain good characterization of strains of Gram negative anaerobic bacilli. A combination of tolerance tests, antibiotic disc resistance tests and biochemical tests has allowed us to obtain good differentiation between species of *Bacteroides* and also to identify the major sub-species. We believe this approach will be of value in the clinical microbiological laboratory.

We thank the many colleagues who donated strains for these studies and we are indebted to Professor J. Boyes of the Edinburgh Dental School for facilities for the taking of gingival samples. We are most grateful for the skilled technical assistance of Mr R. Brown and his technical colleagues in the University of Edinburgh and Mrs Fiona Brown at the Central Microbiological Laboratories. Financial support to J.G.C. from the Medical Research Council (MRC Grant no. G974/325B), to B.W. from Abbott Laboratories Ltd, Queensborough, Kent, and to W.P.H. from the University of Edinburgh Faculty of Medicine Scholarship Fund is gratefully acknowledged.

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PROCEEDINGS OF THE PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

The 132nd meeting of the Society was held at the London Hospital, London, on the 7th, 8th and 9th January 1976

49. A SCHEME FOR THE IDENTIFICATION OF CLINICALLY IMPORTANT GRAM-NEGATIVE ANAEROBIC BACILLI BY SIMPLE BACTERIOLOGICAL TESTS

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We have subjected 220 reference strains and laboratory isolates of gram-negative non-sporing non-motile anaerobic bacilli to a series of simple laboratory tests that included conventional biochemical tests, tests of resistance to antibiotics, and tolerance to dyes and bile salts. These tests were poised for use with gram-negative anaerobic bacilli and allowed clear separation of strains into three main groups: *Bacteroides fragilis*, *B. melaninogenicus* and *Fusobacterium* species. Certain tests were found useful for identifying the recognised subspecies of *B. fragilis* and *B. melaninogenicus*. We have selected a short set of simple tests that have particular discriminative value and we present a scheme for the identification of unknown isolates of gram-negative anaerobic bacilli. The tests are: pigment production; antibiotic disk resistance tests with neomycin (1000 μg), kanamycin (1000 μg), penicillin (1·5 units) and rifampicin (15 μg); tolerance tests with sodium taurocholate (0·5%), sodium deoxycholate (0·1%), Victoria blue 4R (1 in 80 000), and ethyl violet (1 in 80 000); and conventional biochemical tests for the production of indole, hydrolysis of aesculin, digestion of gelatin, and fermentation of glucose, rhamnose, trehalose, and mannitol. The determination of these species and certain sub-species is of clinical significance and our procedures could be routinely applied in a conventionally equipped service laboratory.

Journal of Applied Bacteriology 1977, **42**, 259–273

The Classification of *Bacteroides melaninogenicus* and Related Species

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Received 1 December 1976 and accepted 14 December 1976

One hundred and seventy-five strains of *Bacteroides melaninogenicus*, 17 strains of *B. oralis* and six strains of *B. ochraceus* were studied in a series of biochemical, chemical tolerance and antibiotic disc resistance tests and by the gas-liquid chromatographic analysis of the acid end products of metabolism. Strains of *B. melaninogenicus* ss. *asaccharolyticus* formed a distinct group with clear differences from other *B. melaninogenicus* strains. *B. melaninogenicus* ss. *intermedius* strains formed a homogeneous group that could be readily identified. *B. ochraceus* was distinguished from other *Bacteroides* spp. by its ability to grow in air enriched with CO_2. *Bacteriodes melaninogenicus* ss. *melaninogenicus* and *B. oralis* gave very similar patterns of results with the tests used and invariably were indistinguishable; the capacity to produce black-pigmented colonies on blood-containing media may not be a valid criterion for dividing these similar strains into two species.

THE SPECIES *Bacteroides melaninogenicus* contains all strictly anaerobic non-motile non-sporing Gram negative bacilli that produce black or brown pigmented colonies when grown on media containing blood. The pigment is an intracellular or cell-associated derivative of haemoglobin assimilated from the medium (Duerden 1975). This species was first described by Oliver & Wherry (1921) and the characteristic pigmentation remains the basic criterion for differentiation from other *Bacteroides* species (Holdeman & Moore 1974).

Bacteroides melaninogenicus strains are common commensals of the mouth (Socransky *et al.* 1963), lower gastro-intestinal tract (Drasar *et al.* 1969) and vagina (Gorbach *et al.* 1973) and have been implicated as significant pathogens in periodontal disease (MacDonald *et al.* 1960; Socransky 1970), bronchiectasis and lung abscess (Bartlett & Finegold 1972), post-operative wound infections (Finegold 1974) and uterine infections (Thadepalli, Gorbach & Keith 1973). The species, however, is a heterogeneous group. Moore & Holdeman (1973) recognize three subspecies of *B. melaninogenicus*: ss. *melaninogenicus*, ss. *intermedius* and ss. *asaccharolyticus*. These subspecies have a number of distinguishing characteristics but can be simply identified on the basis of glucose fermentation and the production of indole (Duerden *et al.* 1976).

Non-pigmented strains of Gram negative anaerobic bacilli were isolated from the human mouth and designated *B. oralis* by Loesche *et al.* (1964); in other respects, these strains are very similar to some strains of *B. melaninogenicus* ss. *melaninogenicus*. The

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similarities are such that one reference strain described as *B. oralis* was later found to produce pigment and has to be regarded as *B. melaninogenicus* (Holbrook & Duerden 1974). Other seemingly related strains were initially designated *B. oralis* var. *elongatus* (Loesche *et al.* 1964); they require carbon dioxide for growth but are not strict anaerobes and are now called *B. ochraceus* (Holdeman & Moore 1972). The present study was undertaken to investigate the characteristics and interrelationships of the three subspecies of *B. melaninogenicus*, and of *B. oralis* and *B. ochraceus*.

Materials and Methods

Organisms

Bacteroides melaninogenicus

A total of 175 strains was studied. Strains 9336, 9337 and 9338 were obtained from the National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale Avenue, London NW9 5HT. Strain 15930 (formerly *B. oralis*; see Holbrook & Duerden 1974) was from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 28052, U.S.A. Strains WAL 2721 and WAL 2724 were from Dr S. M. Finegold, Wadsworth General Hospital, Veterans Administration, Los Angeles, California, 90024, U.S.A. Strains GUI 1011 and GUI 1034 were from Dr K. Ueno, Department of Bacteriology, Gifu University Medical School, Tsukasa-Machi, Gifu-shi, Gifu-ken, Japan. Strain VPI 4196 was from Dr E. Cato, Virginia Polytechnic Institute and State University, Blacksburg, Va. 24060, U.S.A. Five strains were from Professor G. Sundqvist, Department of Endodontics, University of Umeå, Sweden. Three strains were isolated from abdominal wound infections by Dr R. Wiseman, Bangour General Hospital, W. Lothian, Scotland. Two strains were from Dr J. M. Hardie, London Hospital Medical College, Dental School, Turner Street, London E1 2AD. Four strains were isolated from wound swabs and one from an intravenous catheter in the Bacteriology Laboratory, Royal Infirmary, Edinburgh. Seven strains were isolated in our laboratory from oral infections (two from dental abscesses, two from wound swabs following oral surgery, and one each from cases of Vincent's infection, pericoronitis and a root canal infection). Ninety-four strains were isolated from subgingival dental plaque in our laboratory. Thirty-one strains were isolated from high vaginal swabs (19 by Dr B. Watt, Central Microbiological Laboratories, Western General Hospital, Edinburgh, and 12 in the Bacteriology Laboratory, Royal Infirmary, Edinburgh). Nineteen strains were isolated in our laboratory from specimens of faeces sent to the Bacteriology Laboratory, Royal Infirmary, Edinburgh.

Bacteroides oralis

Seventeen strains were studied. Strains J1, 7CM and 30 were from Dr B. A. Phillips, National Institute for Research in Dairying, University of Reading, England. Strains VPI 5832 and VPI 7570A were from Dr W. E. C. Moore, Virginia Polytechnic Institute. Strain NP 333 was from Mr G. H. Bowden, London Hospital Medical College. Eleven strains were isolated in our laboratory from subgingival dental plaque.

Bacteroides ochraceus

Six strains were studied. Strain VPI 2845 was from Dr E. Cato. Strains 1956C and 2467B were from Dr M. Sebald, Institut Pasteur, 25 Rue du Docteur Roux, Paris. Strains 10, 79B and 73 were from Dr W. H. van Palenstein-Helderman, Preventive Dentistry Department, University of Utrecht, Netherlands.

Forty-one strains of *B. melaninogenicus* and one strain each of *B. oralis* and *B. ochraceus* were included in previous studies on the characterization of clinically-important Gram negative anaerobic bacilli (Duerden *et al.* 1976). *Bacteroides melaninogenicus* strains WAL 2721, WAL 2724, GUI 1011, GUI 1034 and ATCC 15930, *B. oralis* strains VPI 5832, VPI 7570A, J1, 7CM and 30, and *B. ochraceus* strains 1956C and 2467B were studied as part of the collaborative study instigated by the International Committee for Systematic Bacteriology (ICSB) Taxonomic Sub-Committee on Gram negative anaerobic rods.

Characterization of strains

Ninety-two strains of *B. melaninogenicus*, 15 strains of *B. oralis* and the six strains of *B. ochraceus* were subjected to the following series of morphological, biochemical, chemical tolerance and antibiotic disc resistance tests (for details of media and methods, see Duerden *et al.* 1976).

Morphological and biochemical tests

Microscopic and colonial morphology; haemolytic effect on blood agar; pigment production; motility; lipase activity; oxidase test; catalase test; hydrogen sulphide production; indole production; gelatinase test; aesculin hydrolysis; fermentation of glucose, lactose, sucrose, maltose, rhamnose, trehalose and mannitol.

Chemical tolerance tests

Growth in the presence of (1) the bile salts sodium taurocholate, sodium deoxycholate, separately and in combination, and (2) the dyes brilliant green, Victoria blue 4R, gentian violet and ethyl violet (separately).

Antibiotic disc resistance tests

Resistance to discs containing neomycin 1000 μg and 10 μg, kanamycin 1000 μg and 30 μg, penicillin 1·5 units, methicillin 10 μg, erythromycin 60 μg, colistin 10 μg, rifampicin 15 μg, lincomycin 2 μg, clindamycin 2 μg, bacitracin 0·1 unit, vancomycin 15 μg, chloramphenicol 10 μg, tetracycline 10 μg, and metronidazole 5 μg.

The remaining 85 strains were identified by the following short, combined scheme derived from the above series of tests: pigment production; antibiotic disc resistance tests with neomycin 1000 μg, kanamycin 1000 μg, penicillin 1·5 units, and rifampicin 15 μg discs; separate tolerance tests with sodium taurocholate, sodium deoxycholate, Victoria blue 4R and ethyl violet; biochemical tests for the production of indole,

digestion of gelatin, and hydrolysis of aesculin; and the fermentation of glucose, rhamnose, trehalose and mannitol.

GLC analysis of short-chain fatty acids

One hundred and two strains of *B. melaninogenicus,* 13 strains of *B. oralis* and six strains of *B. ochraceus* were subjected to gas-liquid chromatographic (GLC) analysis of their short-chain fatty acid products of metabolism.

Medium

The strains were grown in two tubes of a proteose peptone medium (PPYS) containing (w/v): proteose peptone (Oxoid) 2%; yeast extract (Difco) 1%; and NaCl 0·5%. Filter-sterilized solutions of the following heat-labile supplements were added aseptically to the (cooled) autoclaved basal medium (pH 7·4) to give final concentrations of: inactivated horse serum, 2%; haemin, 5 μg/ml; menadione, 1 μg/ml; cysteine hydrochloride, 0·075%; and Na_2CO_3, 0·04%. Glucose, 1%, was added to one tube (PPYSG medium). The pH value at inoculation was $7·1 \pm 0·1$.

The inoculum was one drop (0·02 ml) of a 48 h culture in cooked-meat broth. After anaerobic incubation (see Collee *et al.* 1972) for 48 h, or longer for slow-growing strains, the cultures were examined microscopically for growth and the pH value of each tube was measured for comparison with the results of the glucose fermentation tests (above). Thirteen strains were tested after growth in PPYS medium without added glucose.

Chromatograph

A Pye-Unicam series 104 gas chromatograph fitted with heated injection ports and dual flame-ionization detectors was operated isothermally at 190 °C with a detector temperature of 250 °C. The carrier-gas was oxygen-free nitrogen at a flow-rate of 35 ml/min and the hydrogen flow-rate of both detectors was adjusted for optimum sensitivity. The instrument was fitted with two identical glass columns (1·5 m × 4 mm) containing Chromosorb 101 (Johns-Manville Corp., U.S.A.; supplied by Gas Chromatography Services Ltd., 23 Old Chester Road, Lower Bebington, Wirral, Merseyside L63 7LA). Columns were packed in the laboratory. The recorder was a Servoscribe 1S model 541.20 (Belmont Instruments, 6 Belmont Drive, Giffnock, Glasgow G46 7PA) set at the 10 mV range with a recorder speed of 120 mm/h.

Analysis

The procedures used were derived from those of Carlsson (1973).

(1) *Volatile acids.* Cultures were acidified with 50% sulphuric acid to pH 2·0 and a 0·6 μl sample of cell-free supernatant was injected directly on to the analysing column without further pre-treatment or extraction. Contamination of the top 2–3 cm of the column occurred during use and required periodic replacement with fresh polymer. The attenuation setting was 2×10^{-2} at the ×1 range setting.

(2) *Non-volatile acids.* The acidified culture supernates were methylated according to the method of Holdeman & Moore (1972) and a 0·6 μl sample of the chloroform ex-

tract was injected on to the column under the same conditions as those used for the volatile acids but with an attenuation setting of 5×10^{-2}. Lactic and succinic acids were detected qualitatively in the analysis of volatile acids, but this was confirmed quantitatively by the methylation procedure.

Standards

Single and combined 0·01 M aqueous standards of the volatile acids were used to establish absolute and relative retention times; 0·02 M standards were used in the analysis of the non-volatile acids. Samples of uninoculated (sterile) PPYSG medium were included as controls in every batch of each of the two types of analysis. A PPYSG control and a combined acid standard were used to monitor retention times and the sensitivity settings of the instrument as a routine each day. The approximate concentration values of acids for test samples were calculated by comparing the corrected peak heights of the test samples with those of the appropriate acid standards. Results were recorded as follows:

Volatile acids. + +, Concentration value >10 μmol/ml; +, 1·1–10 μmol/ml; tr (trace), 0·2–1·0 μmol/ml; –, <0·2 μmol/ml.

Lactic and succinic acids. + +, Concentration value >20 μmol/ml; +, 10–20 μmol/ml; tr, 1–9 μmol/ml; –, <1 μmol/ml.

Results

The test strains were provisionally allocated to one of three species:

(1) Strictly anaerobic strains that produced black or brown pigmented colonies when grown on lysed-human-blood agar for up to one week were assigned to the species *B. melaninogenicus*. The three subspecies of *B. melaninogenicus* were distinguished by tests for the production of indole and the fermentation of glucose:

(a) Strains that did not produce acid from glucose were labelled ss. *asaccharolyticus* (46 strains). These included strain NCTC 9337.

(b) Strains that produced acid from glucose and produced indole were labelled ss. *intermedius* (78 strains). These included strains NCTC 9336 and 9338.

(c) Strains that produced acid from glucose but did not produce indole were labelled ss. *melaninogenicus* (53 strains). These included strains ATCC 15930, WAL 2721 and 2724, GUI 1011 and 1034, VPI 4196 and 7570A and 30.

(2) Strictly anaerobic, non-pigmented strains that were inhibited by bile salts were assigned to the species *B. oralis* (15 strains). Two strains (VPI 7570A and 30) were submitted to us as strains of *B. oralis* but produced black or brown pigmented colonies and were transferred to our *B. melaninogenicus* ss. *melaninogenicus* group (above).

(3) Non-pigmented strains that were able to grow in 10% CO_2 in air were assigned to the species *B. ochraceus* (6 strains).

Cell morphology

All strains were Gram negative bacilli or cocco-bacilli and many were pleomorphic. *B. melaninogenicus* and *B. oralis* strains were predominantly cocco-bacilli or short bacilli with rounded ends, often arranged in short chains. A few longer rods were seen and some strains were highly pleomorphic. *B. ochraceus* strains were long, slender bacilli with rounded or tapered ends and often with a central oval swelling.

Colony morphology

The colonies of *B. melaninogenicus* ss. *asaccharolyticus* were 0·5 mm diameter, round, convex and opaque. Individual colonies were light grey after incubation for 48 h but confluent growth was sometimes brown and appeared moist; colonies were 1 mm in diameter, dark brown or black after further incubation on lysed-blood agar. After one week, some strains produced very small variant colonies that were light brown in colour.

Colonies of *B. melaninogenicus* ss. *intermedius* were 1–2 mm in diameter, round, convex and opaque. After incubation for 48 h individual colonies were grey but confluent growth was becoming black; all colonies were black after further incubation.

Colonies of *B. melaninogenicus* ss. *melaninogenicus* were 1–2 mm in diameter, round, convex and opaque. After incubation for 48 h they were typically light grey, becoming brown after further incubation. The pigmentation varied between strains from light brown to almost black. The colonies of many strains had a light brown annulus around a dark brown centre.

All strains of *B. melaninogenicus* ss. *melaninogenicus* and ss. *intermedius* produced zones of complete or incomplete haemolysis on human-blood agar; the development of these zones paralleled the development of pigment. Many strains of *B. melaninogenicus* ss. *asaccharolyticus* were similarly haemolytic but some strains of this subspecies were non-haemolytic and, therefore, non-pigmented on human-blood agar although all of these strains produced black pigment when grown on lysed-blood agar.

Colonies of *B. oralis* were 1–2 mm in diameter, round, convex, opaque and grey; they tended to coalesce. After incubation for 7 d, the colonies of some strains (including VPI 5832 and NP 333) became light brown and were difficult to distinguish from the lighter-pigmented strains of *B. melaninogenicus* ss. *melaninogenicus*.

Bacteroides ochraceus strains typically produced two colony types: (a) 1 mm diameter, round or with an irregular edge, smooth, opaque and blue-grey; (b) 1 mm in diameter, rhizoid, granular and ochre in colour.

Biochemical tests

None of the strains tested produced oxidase or catalase; all strains produced hydrogen sulphide although some strains produced only small amounts. The results of the other biochemical tests are shown in Table 1. Strains of *B. melaninogenicus* ss. *asaccharolyticus* (46) did not ferment any of the test carbohydrates and did not hydrolyse aesculin or dextran; most strains produced indole and digested gelatin; only a few strains produced lipase. All strains of *B. melaninogenicus* ss. *intermedius* (78) fermented glucose, sucrose and maltose, but not rhamnose; a few strains fermented lactose but only two strains fermented mannitol and one strain fermented trehalose. All strains pro-

duced indole and only one strain failed to digest gelatin; only three strains hydrolysed dextran and one strain hydrolysed aesculin. Many strains produced lipase. *Bacteroides melaninogenicus* ss. *melaninogenicus* strains (53) fermented glucose but not trehalose or mannitol; only one strain failed to ferment maltose, another failed to ferment sucrose, and two strains failed to ferment lactose. Two strains fermented rhamnose. The strains assigned to this subspecies did not produce indole. In tests for the hydrolysis of aesculin and dextran and the digestion of gelatin this subspecies did not give a constant pattern; some strains gave negative results in all three tests whereas other strains gave positive results in one, two or all three tests. There was no correlation between the results obtained in these three separate tests. A few strains produced lipase.

Bacteroides oralis strains (15) fermented glucose, lactose, sucrose and maltose, but not trehalose or mannitol; eight strains fermented rhamnose. All except two strains hydrolysed aesculin; some strains hydrolysed dextran and some strains digested gelatin. None of the strains produced indole or lipase.

Bacteroides ochraceus strains (6) fermented glucose, lactose, sucrose and maltose, but not rhamnose, trehalose or mannitol. All strains hydrolysed dextran and only one strain failed to hydrolyse aesculin. None of the strains digested gelatin or produced indole or lipase.

Chemical tolerance tests

The results of these tests are shown in Table 2. All the test strains of *B. melaninogenicus* and *B. ochraceus* and all except one strain of *B. oralis* (7CM) were inhibited by bile salts. All strains were inhibited by ethyl violet, gentian violet and brilliant green except one strain of *B. oralis* (7CM) that grew in the presence of ethyl violet and one strain each of *B. melaninogenicus* ss. *melaninogenicus* and ss. *asaccharolyticus* that grew in the presence of brilliant green. The atypical strain of *B. oralis* (7CM) gave a pattern of results in biochemical, chemical tolerance and antibiotic disc resistance tests that was more typical of a strain of the *B. fragilis* group.

Antibiotic disc resistance tests

The results of these tests are shown in Table 3. All strains of the anaerobic species were sensitive to metronidazole and one strain of *B. ochraceus* was also sensitive to this agent. All except one strain of *B. melaninogenicus* ss. *intermedius* and two strains of ss. *melaninogenicus* were resistant to kanamycin (1000 μg disc). Most strains were sensitive to neomycin, but 19 strains of *B. melaninogenicus* ss. *asaccharolyticus*, five strains of ss. *intermedius*, three strains of ss. *melaninogenicus* and one strain of *B. oralis* were resistant. Most strains were sensitive to penicillin, but four strains of *B. melaninogenicus* ss. *asaccharolyticus*, six strains of ss. *intermedius*, 14 strains of ss. *melaninogenicus*, and two strains of *B. oralis* were resistant. The results obtained with methicillin and penicillin discs were the same for strains tested with both discs, except that two strains of *B. ochraceus* were sensitive to penicillin but resistant to methicillin. Almost all strains of *B. ochraceus*, *B. oralis*, *B. melaninogenicus* ss. *melaninogenicus* and ss. *intermedius* were resistant to the vancomycin disc but most strains of ss. *asaccharolyticus* were sensitive.

TABLE 1
Results of biochemical tests with 198 test strains

Test result	Number of positive strains/number tested in each group				
	B. melaninogenicus				
	ss. *asaccharolyticus*	ss. *intermedius*	ss. *melaninogenicus*	*B. oralis*	*B. ochraceus*
Growth in air + CO_2	0/46	0/78	0/53	0/15	6/6
Pigment production (black/brown)	46/46	78/78	53/53	0/15	0/6
Indole production	42/46	78/78	0/53	0/15	0/6
Aesculin hydrolysis	0/46	1/78	17/53	14/15	5/6
Gelatin digestion	45/46	77/78	30/53	7/15	0/6
Dextran hydrolysis	0/44†	3/75‡	28/53	5/15	6/6
Lipase production	5/22*	31/39	9/26	0/13	0/6
Fermentation of:					
glucose	0/46	78/78	53/53	15/15	6/6
lactose	0/29	8/39	24/26	13/13	6/6
sucrose	0/29	39/39	25/26	13/13	6/6
maltose	0/29	39/39	25/26	13/13	6/6
rhamnose	0/46	0/78	2/53	8/15	0/6
trehalose	0/46	1/78	0/53	0/15	0/6
mannitol	0/46	2/78	0/53	0/15	0/6

* Seven strains failed to grow on test medium.
† Two strains failed to grow on test medium.
‡ Three strains failed to grown on test medium.

TABLE 2

Results of tolerance tests with 198 test strains

Test result	Number of positive strains/number tested in each group				
	B. melaninogenicus				
	ss. *asaccharolyticus*	ss. *intermedius*	ss. *melaninogenicus*	*B. oralis*	*B. ochraceus*
Growth on basal medium	44/46	75/78	53/53	14/15	6/6
Growth on basal medium plus:					
taurocholate (0·5%)	0/44	0/75	0/53	1/14	0/6
deoxycholate (0·1%)	0/44	0/75	0/53	1/14	0/6
taurocholate (0·5%) and deoxycholate (0·1%)	0/28	0/39	0/26	1/13	0/6
Victoria blue 4R (1/80 000)	7/44	4/75	6/53	7/14	4/6
ethyl violet (1/80 000)	0/44	0/75	0/53	1/14	0/6
gentian violet (1/100 000)	0/28	0/39	0/26	0/13	0/6
brilliant green (1/80 000)	1/28	0/39	1/25	0/13	0/6

TABLE 3

Results of antibiotic disc resistance tests with 198 test strains

Test result		Number of positive strains/number tested in each group				
		B. melaninogenicus				
		ss. *asaccharolyticus*	ss. *intermedius*	ss. *melaninogenicus*	*B. oralis*	*B. ochraceus*
Neomycin (1000 μg)	S	27/46	73/78	50/53	14/15	6/6
Kanamycin (1000 μg)	R	46/46	77/78	51/53	15/15	6/6
Penicillin (1·5 unit)	S	42/46	72/78	39/53	13/15	6/6
Methicillin (10 μg)	S	25/29	37/39	20/26	11/13	4/6
Erythromycin (60 μg)	S	29/29	39/39	26/26	13/13	6/6
Tetracycline (10 μg)	S	24/29	35/39	24/26	12/13	6/6
Colistin (10 μg)	S	12/29	38/39	18/26	4/13	0/6
Rifampicin (15 μg)	S	46/46	78/78	51/53	15/15	6/6
Lincomycin (2 μg)	S	29/29	39/39	25/26	13/13	6/6
Clindamycin (2 μg)	S	29/29	39/39	25/26	13/13	6/6
Bacitracin (0·1 unit)	R	4/29	17/39	25/26	12/13	6/6
Vancomycin (15 μg)	R	9/29	38/39	24/26	13/13	5/6
Chloramphenicol (10 μg)	S	28/29	39/39	26/26	13/13	6/6
Metronidazole (5 μg)	S	29/29	39/39	26/26	13/13	1/6

R, resistant; S, sensitive.

GLC analysis

The results of these tests are shown in Table 4. All strains produced acetic acid. Strains of *B. melaninogenicus* ss. *asaccharolyticus* produced *n*-butyric acid and lactic acid as major products; nine strains did not produce succinic acid but seven strains produced significant amounts of this acid. The two strains that produced >10 μmol/ml of succinate had been incubated for 7 d to obtain good growth; they also produced larger quantities of acetic, *n*-butyric and lactic acids. All strains of ss. *asaccharolyticus* produced significant amounts of propionic acid and smaller amounts of iso-butyric and iso-valeric acids. In general, the saccharolytic organisms i.e. *B. melaninogenicus* ss. *intermedius* and ss. *melaninogenicus, B. oralis* and *B. ochraceus*, produced succinic acid as a major product but did not produce *n*-butyric acid. However, three strains assigned to the *B. melaninogenicus* ss. *intermedius* group consistently produced *n*-butyric acid but not succinic acid in repeated tests. Strains of *B. melaninogenicus* ss. *intermedius* and ss. *melaninogenicus* produced variable amounts of lactic acid; propionic, iso-butyric, and iso-valeric acids were minor products of most strains. None of the test strains of *B. oralis* produced iso-butyric acid; propionic, iso-valeric and lactic acids were minor products of some strains. In general, the test strains of *B. ochraceus* produced significant amounts of acetic and succinic acids only, but they produced smaller amounts of acetic acid than the other test strains. None of the test strains of *B. ochraceus* produced iso-butyric acid but three strains produced propionic acid and one strain produced iso-valeric and lactic acids as minor products.

Identification of strains from different sites

Strains of *B. melaninogenicus* were isolated from the mouth, the vagina, and from faeces; almost all the oral strains were identified as ss. *melaninogenicus* or ss. *intermedius* whereas all three subspecies were regularly isolated from the vagina and from faeces. All the strains of *B. oralis* and *B. ochraceus* were isolated from the mouth except for one strain of *B. oralis* that was isolated from a putrid lung abscess.

Discussion

Moore & Holdeman (1973) divided the pigmented bacteroides into three subspecies: *B. melaninogenicus* ss. *melaninogenicus,* ss. *intermedius* and ss. *asaccharolyticus*. The studies of Williams *et al.* (1975) on cell wall composition and DNA base ratios supported this classification. Lambe (1974) and Lambe & Jerris (1976) have distinguished between the same three groups of fluorescent antibody staining and have also subdivided the ss. *intermedius* strains into two sero-groups. Werner *et al.* (1971), however, would not include saccharolytic and asaccharolytic organisms in the same species. They recognized only the asaccharolytic strains as *B. melaninogenicus* but they did not distinguish the saccharolytic strains from non-pigmented bacteroides. The results of the present study confirm the separation of the pigmented bacteroides strains into three broad groups.

Strains of *B. melaninogenicus* ss. *asaccharolyticus* are clearly distinguished by the failure to ferment carbohydrates and the production of significant amounts of *n*-butyric

TABLE 4

Results of GLC analysis of acid end-products from 121 test strains

Acid concentration		Number of strains in each group*				
		B. melaninogenicus				
		ss. *asaccharolyticus* (18†)	ss. *intermedius* (36†)	ss. *melaninogenicus* (48†)	*B. oralis* (13)	*B. ochraceus* (6)
Acetic	++	13	31	44	9	2
	+	5	5	4	4	4
Propionic	++	5	1			
	+	13	16	19	4	2
	tr		11	21	5	1
	–		8	8	4	3
iso-Butyric	+	16	4			
	tr	2	27	16		
	–		5	32	13	6
n-Butyric	++	7				
	+	11	2			
	tr		1			
	–		33	48	13	6
iso-Valeric	++	1				
	+	17	18	11		
	tr		18	27	5	1
	–			10	8	5
n-Valeric	–	18	36	48	13	6
Lactic	++	17	19	11		
	+	1	11	14	3	
	tr		6	21	2	1
	–			2	8	5
Succinic	++	2	32	38	7	3
	+	5	1	10	6	3
	tr	2	1			
	–	9	2			

* Numbers in parentheses indicate number of strains tested in each group.

† Seven strains of *B. melaninogenicus* ss. *intermedius*, five strains of ss. *melaninogenicus* and one strain of ss. *asaccharolyticus* were tested in medium (without added glucose) only.

acid; they produce indole and are proteolytic. Werner *et al.* (1971) and Williams *et al.* (1975) did not detect the production of succinic acid by any of their test strains of the asaccharolytic group. The production of minor quantities of succinate by seven asaccharolytic strains under the conditions of our tests is consistent with the findings of Holdeman & Moore (1972). The ICSB Taxonomic Sub-committee on Gram negative anaerobic rods has recently clarified the taxonomic status of the asaccharolytic strains and has suggested that *B. melaninogenicus* ss. *asaccharolyticus* is sufficiently different from the other subspecies to be regarded as a separate species designated *B. asaccharolyticus* (Anon. 1977, in press).

The saccharolytic strains of *B. melaninogenicus* could be divided into two clear groups on the basis of our series of tests. The strains that produced indole appeared to be a fairly homogeneous group that corresponds with the subspecies *intermedius* (Holdeman & Moore 1974). Strains that failed to produce indole were designated *B. melaninogenicus* ss. *melaninogenicus*; many strains were easily separated from strains of ss. *intermedius* by their distinctive colony morphology (see Results) that has also been observed by Lambe & Jerris (1976). GLC analysis of the acid end-products of metabolism was not helpful in differentiating between individual strains of these two subspecies. There were differences between the median concentrations of acid end-products but there was considerable overlapping between the ranges of concentrations obtained with the two subspecies. *B. melaninogenicus* ss. *melaninogenicus* did not form a homogeneous group but defined sub-groups could not be detected on the basis of the results of biochemical, chemical tolerance or antibiotic disc resistance tests.

Some of the non-pigmented strains of bacteroides that were identified as *B. oralis* closely resembled strains of *B. melaninogenicus* ss. *melaninogenicus* in the series of tests adopted in this study. They were differentiated only by their failure to produce pigment; this distinction was even less clear with strains of *B. melaninogenicus* ss. *melaninogenicus* that produced brown pigmented colonies only slowly and strains of *B. oralis* that gave buff-coloured colonies after prolonged incubation. However, some strains identified as *B. oralis* grew in the presence of Victoria blue 4R and fermented rhamnose; these strains were more clearly distinguished from *B. melaninogenicus* ss. *melaninogenicus*. As a result of the similarities between some strains of *B. melaninogenicus* ss. *melaninogenicus* and *B. oralis*, the relationship between these two groups has been the subject of some taxonomic debate that remains unresolved (Anon. 1977, in press). Terada *et al.* (1976) have recently suggested that *B. oralis* and *B. ruminicola* might be closely related groups that have many similarities with *B. melaninogenicus* ss. *melaninogenicus*. In a numerical taxonomic study, Sundqvist (1976) found close similarities between strains of *B. melaninogenicus* ss. *melaninogenicus*, *B. oralis* and *B. ruminicola*; he did not regard pigment production as a good basis for differentiation between *B. melaninogenicus* ss. *melaninogenicus* and *B. oralis*. It remains to be decided whether the ability to assimilate haemoglobin and produce brown pigmented colonies when grown on media containing blood is a valid criterion for dividing these very similar strains of *B. melaninogenicus* ss. *melaninogenicus* and *B. oralis* into two species.

B. ochraceus strains were clearly differentiated from the other test strains; they were able to grow in air plus CO_2 and were resistant to metronidazole (except for one aberrant strain), an antimicrobial agent to which only anaerobic bacteria are susceptible (Prince *et al.* 1969). The status of these strains within the genus *Bacteroides* requires

further investigation but on the present evidence it would seem that they should be removed from the genus.

Holdeman & Moore (1974) stressed the value of GLC analysis in the identification of Gram negative anaerobic bacilli. In our studies GLC analysis distinguished between asaccharolytic and saccharolytic strains although three strains that gave positive results in fermentation tests with glucose, and were therefore identified as *B. melaninogenicus* ss. *intermedius*, gave GLC patterns typical of ss. *asaccharolyticus*. These strains produced *iso*-butyric and *iso*-valeric acids and are, therefore, not fusobacteria. They require further investigation to determine whether they constitute a new sub-group or species. We were unable to distinguish between individual strains of *B. melaninogenicus* ss. *intermedius* and strains of the *B. melaninogenicus* ss. *melaninogenicus/B. oralis* group.

Most of our test strains were readily identified from the pattern of results obtained in the short, combined set of biochemical, chemical tolerance and antibiotic disc resistance tests. The results in chemical tolerance and antibiotic disc resistance tests distinguish the groups of bacteroides studied in this investigation from other Gram negative anaerobic bacilli. These tests are used as a combined set to prevent an anomolous result in any single test leading to an incorrect identification. The results of the biochemical tests provide the basis for the differentiation between the species and subspecies within this group of bacteroides.

We thank Professor J. G. Collee for much helpful advice and encouragement and Mr R. Brown and his colleagues for their skilled technical assistance. We thank the many colleagues who donated strains for this study and we are indebted to Professor J. Boyes of the Edinburgh Dental School for facilities for taking gingival samples. W. P. Holbrook thanks the University of Edinburgh for a Faculty of Medicine Scholarship. Financial support from the Medical Research Council (MRC Grant No. G 974/325 B) is gratefully acknowledged.

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Reprinted from the Journal of Clinical Pathology
Volume 30, page 609, 1977.

Recovery of anaerobic bacteria from small inocula: a model for blood culture studies

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J. clin. Path., 1977, **30**, 609-614

Recovery of anaerobic bacteria from small inocula: a model for blood culture studies

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SUMMARY The recovery of anaerobic, facultative anaerobic, and aerobic pathogens from very small inocula was studied under conditions that could be related to routine blood culture procedures. Results with Brain Heart Infusion broth were unsatisfactory. Freshly prepared Brewer thioglycollate medium gave apparently good results, but there are disadvantages when this medium is used for blood culture. Results with Difco Thiol broth were disappointing. A modification of Robertson's cooked-meat broth supplemented with Brain Heart Infusion gave good recovery and sustained viability with a wide range of test organisms including exacting strains. The findings raise points of practical importance.

Although the recovery of certain blood-borne pathogens demands special laboratory procedures, there is a continuing requirement for a general blood culture procedure for use as a routine that might effectively detect a wide range of likely pathogens. This obliges laboratory workers to review their blood culture procedures periodically, to be aware of new technical developments (Brooks and Sodeman, 1974; Smith and Little, 1974; Thiemke and Wicher, 1975; Wood, 1976), and to consider the media used (Washington, 1971; Chow and Guze, 1974; Forgan-Smith and Darrell, 1974; Gantz *et al.*, 1974; Shanson, 1974; Blazevic *et al.*, 1975; Shanson and Barnicoat, 1975; Szawatowski, 1976). In view of developments in clinical anaerobic bacteriology, we studied the recovery of anaerobic pathogens from very small inocula under conditions that could be related to routine blood culture procedures. The study was then extended to the recovery of aerobic or facultative anaerobic pathogens associated with bacteraemic conditions in man.

Material and methods

ORGANISMS

The following strains were used: *Bacteroides fragilis* ss. *fragilis* NCTC nos. 9343 and 9344; *B. melaninogenicus* ss. *intermedius* NCTC no. 9336; *B. melaninogenicus* ss. *asaccharolyticus* NCTC no. 9337; *Fusobacterium polymorphum* NCTC no. 10562; anaerobic coccus group I NCTC no. 9801; anaerobic coccus group III NCTC no. 9803; and laboratory stock strains of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Clostridium welchii*, *Streptococcus pneumoniae*, *Str. viridans*, *Haemophilus influenzae*, and *Neisseria meningitidis*.

MEDIA

These were: (i) Brain Heart Infusion broth (Oxoid) prepared according to the manufacturer's instructions (BHI); (ii) Brain Heart Infusion broth (Oxoid) with 1 g/litre (0·1%) sodium thioglycollate (BHI-Thio); (iii) Brewer Thioglycollate Medium (Oxoid) containing dextrose 5 g/l prepared according to the manufacturer's instructions (Brewer); (iv) Robertson's cooked-meat broth (Cruickshank *et al.*, 1975) made in this department with nutrient broth (Oxoid no. 2) as the liquid phase of the medium (CMB); (v) a cooked-meat medium comprising fresh cooked-meat particles prepared as above with Brain Heart Infusion broth (Oxoid) as the liquid phase (BHI/CMP); (vi) commercially prepared blood-culture bottles containing 50 ml Bacto-Thiol broth with CO_2 under vacuum were purchased from Difco Laboratories (Difco Thiol broth); (vii) blood agar plates made by adding 5% outdated human blood to Oxoid Columbia agar base; each 500 ml of the human blood preparation contained 2 g disodium citrate and 1·7 g dextrose in 70 ml water added to 430 ml whole blood; (viii) plates of lysed blood agar supplemented with filter-sterilised menadione (Sigma,

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Received for publication 17 January 1977

London) 1 μg/ml were used for subculture of *B. melaninogenicus* (see Duerden *et al.*, 1976).

In the initial experiments 50-ml medical flat bottles containing 17 ml of BHI, BHI-Thio, Brewer medium, or CMB (1 cm-depth of cooked-meat particles with 17 ml of nutrient broth) were used. This maintained the same surface:depth and media: bottle volume ratios as with the 150-ml medical flat bottles containing 50 ml of medium that are routinely used for blood culture in the Royal Infirmary, Edinburgh. The latter bottles were used in subsequent experiments. All bottles had perforated metal caps with rubber liners; the perforation was sealed with either a Viskap or autoclave tape.

Unless otherwise stated, bottles were autoclaved with the caps loose; they were tightened as soon as the bottles were cool enough to handle, and further cooling created a partial vacuum in the bottles ('normal' headspace). In the first series of experiments an atmosphere of 10% hydrogen, 10% carbon dioxide, and 80% nitrogen was introduced into one set of bottles of each medium (headspace 'H_2:CO_2:N_2') as follows: a cylinder containing the gas mixture was connected through a reducing valve to the exhaust valve of the autoclave; the bottles were autoclaved with their caps loose; when the autoclave pressure had fallen to atmospheric pressure, the exhaust valve was opened and the gas mixture flowed into the autoclave as it cooled; after 15 minutes the autoclave was opened and the bottle caps were quickly tightened.

BLOOD FOR THE BLOOD CULTURE MODEL

Defibrinated horse blood (Wellcome) was added to each bottle; 5 ml was added to 50 ml of medium in 150-ml bottles and 1·5 ml to 17 ml of medium in 50-ml bottles.

INOCULUM

Stationary phase cultures of test strains (48-hour cultures of *B. melaninogenicus* strains and 24-hour cultures of other species) in cooked-meat broth or a suitable nutrient broth were diluted in pre-steamed nutrient broth to provide an inoculum of approximately 10 cfu per bottle. The dilutions were made on the basis of a total cell count with a 0·1 mm Thoma counting chamber (Hawksley, England) and phase-contrast microscopy (see Collee *et al.*, 1972). A surface viable count was performed by a spread-plate method (see Watt *et al.*, 1973) to determine the actual inoculum size. For the initial series of experiments with 50-ml bottles the inoculum was prepared by serial dilution in pre-steamed nutrient broth with the final dilution in defibrinated horse blood so that 1·5 ml was calculated to contain 10 cfu; this was injected from a syringe through the cap of the bottle. In the subsequent studies with the 150-ml bottles, horse blood (5 ml) was injected into each bottle and then a 0·1 ml inoculum of a suitable serial dilution in pre-steamed nutrient broth. The following procedure (P) was adopted to check the variation in inoculum size between bottles: 0·1 ml inocula of the final dilution, estimated to contain approximately 10 cfu, were injected alternately into the bottles of test media and on to fresh plates of blood agar or lysed blood agar (bottle-plate-bottle-plate) throughout the series of tests; colonies were counted on the blood agar plates after anaerobic incubation for 48 hours.

INCUBATION

All incubation was at 37°C. The atmosphere inside the bottle depended upon the method of preparation until the bottles were opened for the first subculture; this admitted room air to the bottles. Unless otherwise stated, the bottles were thereafter simply incubated in a laboratory incubator. For 'anaerobic + CO_2' incubation the bottles were vented by the insertion through the rubber seal of a sterile needle (25G 5/8 in) plugged with sterile cotton wool and incubated in a BTL anaerobic jar. A standard anaerobic procedure based on that of Collee *et al.* (1972) was followed for these bottles and for all anaerobically incubated plates. The bottles were examined daily for haemolysis and turbidity.

SUBCULTURES

In general, the seeded bottles were subcultured on to fresh plates of blood agar or lysed blood agar by sampling (*c* 0·03 ml) with a sterile wire loop after mixing the contents by gentle rotation (but not inversion). In some studies, e.g. when we compared the effects of different headspace gases, and when we compared recovery in bottles autoclaved with the caps either tight or loose, we did not subculture from the bottles until they had been incubated for 48 hours. In the other studies, Gram smears and subcultures were generally made after overnight incubation (18-20 h) and thereafter daily for up to seven days, with one subculture missed usually at day 5 or 6. Subculture plates were examined after they had been incubated for 48 hours and again at four days. Aerobic plates were prepared with the test anaerobe series as a check for aerobic contaminants.

Results

COMPARISON OF MEDIA AND CONDITIONS

In the first part of the investigation we used small bottles and studied the ability of BHI, BHI-Thio, Brewer medium, and CMB to support the growth of

Table 1 *Results of replicate studies with small inocula of two strains in four media with different headspace atmospheres*

Medium	*Headspace atmosphere*	*Recovery (no. of bottles positive out of 3) with test strain**					
		B. fragilis *ss.* fragilis				B. melaninogenicus *ss.* intermedius	
BHI	Normal	2	0	2	1	0	0
	H_2:CO_2:N_2	2	3	—	0	0	0
	Anaerobic + CO_2	—	—	0	1	0	0
BHI + thioglycollate	Normal	1	3	1	3	0	0
	H_2:CO_2:N_2	3	3	—	3	2	0
	Anaerobic + CO_2	—	—	2	3	1	0
Brewer	Normal	3	3	3	3	3	2
	H_2:CO_2:N_2	3	3	—	3	3	3
	Anaerobic + CO_2	—	—	1	3	3	3
CMB	Normal	—	—	—	3	—	3
	H_2:CO_2:N_2	—	—	—	3	—	3
	Anaerobic + CO_2	—	—	—	3	—	3
Inoculum size†		500	7·4	13	17	8	10·5

*Each column shows results for one set of experiments, — = not done for that set
†Mean no. of bacteria (cfu) inoculated per bottle

small inocula of *B. fragilis* ss. *fragilis* NCTC no. 9343 and *B. melaninogenicus* ss. *intermedius* NCTC no. 9336. Table 1 is a composite table summarising the results of a series of six experiments in which the variables were the four media, three different headspace atmospheres ('normal', 'H_2:CO_2:N_2', and 'anaerobic + CO_2') and the two test organisms. A set of three replicate bottles was seeded for each variable in each experiment. In general, there was no recovery of *B. melaninogenicus* ss. *intermedius* and there was unsatisfactory recovery of *B. fragilis* ss. *fragilis* in BHI medium. Recoveries were marginally improved but still unsatisfactory when the BHI medium was supplemented with thioglycollate (BHI-Thio). Recoveries were much better with Brewer thioglycollate medium, but there was a clear disadvantage in the rapid loss of viability that we observed when cultures were held for more than a few days in this medium. For example, Brewer cultures yielding good growths of the test organism on subculture plates at days 2-4 often gave relatively scanty growths on days 4-6 and no growth on days 6-7. With the occasional exception of an exacting *B. melaninogenicus* strain, which sometimes showed diminished viability at seven days, cultures of the test organisms in cooked-meat media generally maintained their viability and gave good growths on subculture plates up to seven days. There was good recovery and maintained viability in our tests with cooked-meat broth; we already knew that this medium was favourable for the growth of a wide range of anaerobic and aerobic organisms as a result of our experience in this laboratory over many years.

In most cases a positive culture declared itself within 48 hours except with BHI broth, with which incubation was sometimes necessary for seven days before a positive recovery was obtained. As these studies indicated the superior value of cooked-meat particles compared with thioglycollate for the recovery and maintained viability of these test strains from small inocula, we sought to combine the reducing value of cooked-meat particles with the known nutritive value of BHI broth, particularly with a view to extending the study to a wider range of organisms.

The recoveries of two strains of *B. fragilis* ss. *fragilis*, NCTC nos. 9343 and 9344, and one strain of *B. melaninogenicus* ss. *asaccharolyticus*, NCTC no. 9337, were compared in full-size (150-ml) bottles of BHI and BHI/CMP, incubated for up to seven days; BHI gave clearly unsatisfactory results (three successes and six failures) whereas BHI/CMP gave successful recoveries in all nine tests at three days.

VARIATION IN INOCULUM SIZE

At this stage we studied variation in inoculum size in detail and the procedure P (see Methods) was instituted to check the possibility that some bottles might obtain no cells in the inoculum. Plates inoculated with reputedly minimal inocula alternately in series with bottles showed a range of counts as follows: with *B. fragilis* ss. *fragilis* NCTC no. 9344, the estimated inoculum was 4·6 cfu and the plate counts were 9, 6, 5, 4, 6, 8, 6, 5, 1, 10, 1, 5, 6, 1, 5, 2, 7, 4; mean 5·05; SD 2·62. With *B. melaninogenicus* ss. *intermedius* NCTC no. 9336, the estimated inoculum was 12·3 cfu and the plate counts were: 8, 17, 15, 14, 13, 20, 12, 13, 10, 9, 19, 18, 8, 14, 9, 17, 13, 10, 15, 12; mean 13·35, SD 4·67. One strain, *B. melaninogenicus* ss. *asaccharolyticus* NCTC no. 9337, was sometimes unable to grow as colonies when the inoculum fell below a critical level of about 100 cfu

on a plate. In this case we sometimes had to rely upon dilutions estimated from total cell counts to achieve minimal inocula for fluid media.

AUTOCLAVING WITH CAPS LOOSE OR TIGHT

The reducing effect of cooked-meat particles, and the maintenance of this effect during subsequent storage of bottles, depends partly upon the driving off of dissolved oxygen during autoclaving and the subsequent exclusion of air from the bottle headspace after autoclaving. We therefore compared the recoveries of *B. fragilis* ss. *fragilis* NCTC no. 9344 and *B. melaninogenicus* ss. *asaccharolyticus* NCTC no. 9337 in BHI and BHI/CMP in parallel series: one autoclaved with the caps loose and thereafter immediately tightened while the bottles were still hot; the other autoclaved with the caps tight throughout. The results given in Table 2 were obtained. It is clear that it is essential to autoclave these bottles with the caps loose and to tighten them promptly after autoclaving. This is true even with BHI/CMP broth.

BHI/CMP COMPARED WITH DIFCO THIOL BROTH

At this point the value of Difco Thiol broth as a general purpose blood-culture medium was suggested by Szawatowski (1976). Accordingly, we compared Difco Thiol broth with our BHI/CMP medium and we used an extended range of five anaerobes for this study. The results are given in Table 3. The superiority of the BHI/CMP medium is clear; there was only one failure in 25 tests. Contamination was a major problem with the Difco Thiol bottle. We are in no doubt that we introduced the contaminants, most frequently at the first subculture stage when the aseptic manipulation of the rubber stopper proved difficult, but the main point is that one of the test strains was not recovered in the Difco Thiol series, and recovery of another test strain was poor. The experiment with *Fusobacterium polymorphum* was repeated with special aseptic precautions that might be difficult to maintain as a routine. Contamination was avoided, but the Difco Thiol broth scored only one success out of five as compared with four successes out of five tests for the BHI/CMP broth.

Table 2 *Effect on subsequent recoveries if media are autoclaved in bottles with caps loose or tight*

Medium	*Bottle caps tight or loose in autoclave*	*Recovery (no. of bottles positive out of 5) with test strain*	
		B. fragilis *ss.* fragilis	B. melaninogenicus *ss.* asaccharolyticus
BHI	Tight	0	0
	Loose	2	0
BHI/CMP	Tight	3	3
	Loose	5	4
Inoculum size*		23·5	*c.* 50†

*Mean no. of bacteria (cfu) inoculated per bottle
†Estimated from total cell count.

Table 3 *Comparison of recoveries obtained with 5 test strains in BHI/CMP and Thiol Broth*

Test organism	*Inoculum* size*	*Recovery (no. of bottles positive out of 5) with the test medium*	
		BHI/CMP	*Difco Thiol broth*
B. fragilis ss. *fragilis*	3·8	4†	5[a](3)
B. melaninogenicus ss. *asaccharolyticus*	6·6	5‡	2[b](5)
F. polymorphum	6·6	5†	0 (5)
Anaerobic coccus group I	4·3	5†	5†(2)
Anaerobic coccus group III	29·7	5†(1)	5†(2)

*Mean no. of bacteria (cfu) inoculated per bottle
[a]4 at 2 days, but one bottle gave recovery only after 4 days' incubation
[b]Both bottles gave recovery only after 7 days' incubation
†These positives were recorded at 2 days
‡These positives were recorded at 4-5 days.
Numbers in parentheses show number of bottles contaminated between first subculture and end of 7 days' incubation.

Table 4 *Recovery of eight test species in BHI/CMP medium*

Test organism	*Inoculum* size*	*Recovery (no. of bottles positive out of 3) with the test strain*
Staph. aureus	5·2	3
E. coli	8·4	3
Ps. aeruginosa	16·6	3
Cl. welchii	4·4	3
Str. pneumoniae	11·9	3
Str. viridans	14·5	3
H. influenzae	1·6†	3
N. meningitidis	6·5	3[a]

*Mean no. of bacteria (cfu) inoculated per bottle
†This is an underestimate of bacterial cells because of clumping of bacterial cells.
[a]One bottle gave recovery only after 7 days' incubation.

BHI/CMP AS A GENERAL PURPOSE MEDIUM

In further studies we tested the ability of BHI/CMP broth to support growth from a small inoculum of a wide range of likely pathogens. Table 4 gives the results. We achieved good recovery in all the tests within 48 hours, except in one bottle in the *N. meningitidis* set that required further incubation.

Discussion

The results obtained with Brain Heart Infusion broth (BHI medium) were unsatisfactory. Results with freshly prepared Brewer medium were apparently good, but cultures of test anaerobes in this medium tended to die within a few days. Cooked-meat broth supplemented with Brain Heart Infusion

(BHI/CMP) gave good recovery and sustained viability with all test strains. Results with Difco Thiol broth were disappointing.

Forgan-Smith and Darrell (1974) ascribed the inactivation of organisms during growth in USP thioglycollate medium to the antibacterial action of acid produced and concluded that a routine blood culture procedure should also include a good cooked-meat medium. Dye (1975) found that toxic properties developed in sterile thioglycollate media during storage. Shanson (1974) did not favour USP thioglycollate broth; he obtained satisfactory results with a Southern Group Brewer's thioglycollate medium without liquoid; a cooked-meat digest broth with glucose also gave good results, but the S. G. Brewer's medium was slightly better. Shanson and Barnicoat (1975) regarded Difco Thiol broth as a suitable alternative to S. G. Brewer's medium. Szawatowski (1976) found that Difco Thiol medium was far superior to thioglycollate broth and cooked-meat broth with glucose for rapid isolation and quantitative growth of test strains of anaerobes that included *B. fragilis* (5 subspecies), *B. melaninogenicus* and various fusobacteria.

The above analysis of the recent literature indicates the confusion confronting the hospital bacteriologist. There are conflicting arguments for and against thioglycollate media, strong support for Difco Thiol broth (containing an unspecified Thiol compound), and conflicting claims for cooked-meat media. Our findings with a cooked-meat medium prepared from fresh bullock heart (see Cruickshank *et al.*, 1975) supplemented with BHI medium and tested with a representative range of aerobes and anaerobes suggest that it is superior to thioglycollate media and to Difco Thiol medium. We found that the rubber bung of the Difco Thiol bottle is difficult to remove and to handle for routine subcultures without a significant risk of contamination; the use of a sterile syringe and needle for each subculture is a possible solution.

The supernate of sterile cooked-meat broth is usually slightly turbid. The common preference for a clear blood culture medium is understandable, but it should be borne in mind that turbidity may be a misleading indication of bacterial growth; we regularly confirmed that an early positive subculture can be obtained from a clear broth and that some turbid blood broths are sterile. Regular subcultures from all inoculated bottles to solid media are essential, and assessments of turbidity of the broth should not be allowed to influence the frequency of this check as a routine blood culture procedure.

In the preparation of blood culture media it is normal laboratory practice to autoclave bottles with their caps loose and to tighten the caps immediately after autoclaving while the bottles and their contents are still hot. Nevertheless, some laboratories autoclave blood culture bottles with the caps tight and our results indicate that this is unwise.

Some limitations of our model are clear. We used a laboratory-prepared inoculum of a stationary phase culture of stock organisms adapted to laboratory media, especially to cooked-meat broth, which we use regularly in this laboratory. We used defibrinated horse blood in place of human blood; outdated human transfusion blood was not used because it contains dextrose-citrate which would not normally be present in a blood culture. By inoculating a plate alternately with each test bottle we sought to demonstrate the range of the actual number of cells delivered and to estimate the probability of delivering no cells in the inoculum, which is a hazard of studies with intentionally small inocula. Results of intensive studies (to be reported elsewhere) with small inocula of strains of *Bacteroides* species from fluid cultures in stationary phase indicate that recoveries on solid media may vary from about 60-95% of the numbers predicted by careful microscopy. We must therefore assume that there is a possible underestimate of up to 40% in the cell counts for the minimal inocula of some of our exacting anaerobes. There may have been larger errors in the estimated inocula of the strain of *B. melaninogenicus* ss. *asaccharolyticus* that could not be grown consistently from small inocula on solid media.

The relative costs (per 100 bottles) of the systems tested are as follows (September 1976): BHI medium £11·36, Brewer thioglycollate medium £9·11, and BHI/CMP medium £12·36; these costs include £7·61 for 100 bottles (re-usable) plus 100 Viskaps and wires, but no allowance is made for technician-time or laboratory overheads. Difco Thiol broth costs approximately £35 + VAT + carriage for 100 bottles; the exact cost depends upon the number ordered.

In our experiments we attempted to simulate clinical microbiological problems in a carefully controlled laboratory model. Blazevic *et al.* (1975) undertook comparative studies in a clinical setting with 'real' specimens. They were limited by the demands of a continuing diagnostic commitment, and their conclusion that both an aerobic and anaerobic bottle are needed for blood culture was essentially based on their experience of the inadequacy of one system alone. We have attempted to assess our blood culture media in clinical practice; there are so many uncontrollable variables that a comparative trial is unlikely to give meaningful results and we favour an assessment based on a controlled laboratory model with a periodic review

of performance in practice.

An ideal medium for a wide variety of potential pathogens might reasonably be rich in nutrients and would incorporate an effective non-toxic reducing system that would not exclude aerobes. These requirements were so well fulfilled by a combination of BHI and properly prepared cooked-meat particles in our studies that such a system perhaps merits wider recognition and clinical application.

We thank Professor B. P. Marmion for identifying the problem in a routine service area and for constructive discussions that led to this study, and we are grateful to Mrs S. Campbell for much technical help. This work was financed by the Medical Research Council (grant no. G 974/325B).

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Printed in England by Eyre & Spottiswoode Ltd, Thanet Press, Margate

PROCEEDINGS OF THE PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

The 134th meeting of the Society was held at Charing Cross Medical School, London, on the 5th, 6th and 7th January 1977

24. Blood culture and the growth of bacteria from small inocula

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The recovery of anaerobic pathogens from very small inocula was studied under conditions that could be related to routine blood culture procedures. The study was then extended to the recovery of aerobic and facultative anaerobic pathogens associated with bacteriaemic conditions in man. Results obtained with Brain Heart Infusion Broth (Oxoid) were unsatisfactory. Results with freshly prepared Brewer Thioglycollate Medium (Oxoid) were apparently good, but loss of viability is a disadvantage with this medium. Results with Difco Thiol Broth were disappointing. A modification of Robertson's cooked-meat broth supplemented with Brain Heart Infusion Broth gave good recovery and sustained viability with a wide range of test organisms including some exacting strains. The findings raise points that may be of practical importance in clinical bacteriology.

GAS-LIQUID CHROMATOGRAPHIC ANALYSIS OF METABOLIC PRODUCTS IN THE IDENTIFICATION OF BACTEROIDACEAE OF CLINICAL INTEREST

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GRAM-NEGATIVE non-sporing anaerobic bacilli of the *Bacteroides-Fusobacterium* group are commonly isolated from clinical specimens (Moore, Cato and Holdeman, 1969; Finegold, 1974). Accurate and prompt generic identification is essential for effective treatment; complete identification may provide information on the pathogenic potential of certain biotypes for man. Significant advances in methods for the identification of clinical isolates have resulted from recent improvements in anaerobic culture procedures (Collee *et al.*, 1972; Holdeman and Moore, 1972; Watt, 1973), culture media (Gibbons and McDonald, 1960; Finegold, Sugihara and Sutter, 1971), and characterisation schemes (Duerden *et al.*, 1976). Conventional bacteriological procedures have been supplemented by a wide variety of more complex and often time-consuming approaches (see Aalbaek, 1973).

The short-chain fatty acids formed as end products of protein or carbohydrate metabolism are of particular importance in current systems of classification (Holdeman and Moore, 1974). The family Bacteroidaceae has been assigned two principal genera, *Bacteroides* and *Fusobacterium*, largely on the basis that *Fusobacterium* spp. produce major amounts of *n*-butyric acid. A third genus, *Leptotrichia*, comprises oral fusiform bacteria that do not produce major amounts of any fatty acid other than lactic. Classification of members of the Bacteroidaceae on the basis of their fatty-acid end products was first attempted by Guillaume, Beerens and Osteux (1956) by means of distillation and paper chromatographic separation; the use of gas-liquid chromatography (GLC) was developed by Werner (1969) and Cato *et al.* (1970).

Received 9 Mar. 1977; *accepted* 24 July 1977.

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The aims of the present study were (1) to determine the fermentation products of members of the *Bacteroides-Fusobacterium* group in various media, (2) to evaluate the use of these media for semi-quantitative GLC analysis of the products, and (3) to assess the value of GLC in identifying a wide range of strains from various clinical sources by comparing the results with those obtained by morphological, biochemical, tolerance and antibiotic-resistance tests.

Materials and methods

Organisms

The following strains were obtained from the National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale Avenue, London NW9 5HT: *Bacteroides fragilis* (*B. fragilis* ss. *fragilis*) nos. NCTC9344 and 8560; *B. thetaiotaomicron* (*B. fragilis* ss. *thetaiotaomicron*) no. NCTC10582; *B. vulgatus* (*B. fragilis* ss. *vulgatus*) no. NCTC10583; *B. melaninogenicus* nos. NCTC9336 and 9337; *Fusobacterium polymorphum* no. NCTC10562; *F. necrogenes* no. NCTC10723; *F.* (*Bacteroides*; *Sphaerophorus*) *necrophorum* no. NCTC10575; *Leptotrichia buccalis* no. NCTC10249.

B. oralis no. ATCC15930 (*B. melaninogenicus*, see Holbrook and Duerden, 1974) was from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md 20852, USA. *B. fragilis* ss. *vulgatus* no. ATCC8482, *B. fragilis* ss. *ovatus* no. ATCC8483, *B. fragilis* ss. *thetaiotaomicron* no. ATCC8492 and *B. fragilis* ss. *distasonis* no. ATCC8503 were from Dr Ella M. Barnes, Agricultural Research Council, Food Research Institute, Colney Lane, Norwich NOR 7OF.

The following strains were referred by the International Committee on Systematic Bacteriology (ICSB) Taxonomic Sub-Committee for Gram-negative anaerobic rods: *B. melaninogenicus* ss. *melaninogenicus* nos. WAL2721 and WAL2724 from Dr S. M. Finegold, UCLA School of Medicine, Los Angeles, Ca 90024, USA; nos. GUI1011 and GUI1034 from Dr K. Ueno, Department of Bacteriology, Gifu University School of Medicine, Tsukasa-Machi, Gifu-shi, Gifu-ken, Japan; no. VPI4196 from Dr Elizabeth Cato, Virginia Polytechnic Institute and State University (VPI), Blacksburg Va. 24060, USA; *B. oralis* nos. VPI7570A and VPI5832 from VPI; nos. J1, 7CM and 30 from Dr B. A. Phillips, National Institute for Research in Dairying, University of Reading, England; no. NP333 from Mr G. H. Bowden, London Hospital Medical College, Dental School, Turner Street, London; *B. ochraceus* (*Ristella ochraceus*) nos. 1956C and 2467B from Dr M. Sebald, Anaerobic Laboratory, Institut Pasteur, 25 Rue du Docteur Roux, Paris XV^{e}, France; no. VPI2845 from VPI; and nos. 10, 79B and 73 from Dr W. H. van Palenstein-Helderman, Department of Preventive Dentistry, University of Utrecht, Netherlands.

Additional strains of Gram-negative anaerobic bacilli were isolated in our laboratory; they consisted of 113 from subgingival dental plaque and 40 from clinical specimens (19 from faeces, 19 from high vaginal swabs, 1 from a rectal abscess and 1 from an abdominal wound swab). In all, 185 isolates were examined.

Bacteriological procedures

Isolation of strains from clinical specimens was as detailed by Holbrook (1976) and Holbrook, Duerden and Deacon (1977). All strains were characterised by the procedures of Duerden *et al.* (1976), and all were independently identified by GLC analysis. Cultures were regularly checked for purity by examination of Gram-stained smears and by aerobic and anaerobic subculture on blood agar (BA) containing 5% human blood in Columbia Agar Base (Oxoid). The standard inocula and anaerobic procedures of Collee *et al.* (1972) were used.

Turbidity readings were performed on a Pye Unicam (Cambridge, England) SP600 spectrophotometer and the absorbance was read at 600 nm in 1-cm cuvettes.

GLC media

Basal media were sterilised by autoclaving at 103·5 kilo-pascals (15 lb per in^2) for 15 min. Stored media were steamed for 30 min. before use to drive off dissolved oxygen. Filter-sterilised stock solutions of the following growth factors were prepared: haemin (haematin hydrochloride; BDH Chemicals Ltd, Poole, England) 500 μg per ml in 0·01N NaOH; menadione (vitamin K_3; Sigma Chemicals, London) 100 μg per ml in distilled water (see Barnes and Impey, 1971); cysteine hydrochloride (BDH) 3·75% (w/v) in distilled water; and Na_2CO_3 4% (w/v) in distilled water. These were added aseptically to all steamed (cooled) basal media to give final concentrations of haemin 5 μg per ml, menadione 1 μg per ml, cysteine hydrochloride 0·075% (w/v), and Na_2CO_3 0·04% (w/v). As required, filter-sterilised aqueous stock solutions of glucose (20% w/v) and vitamin B_{12} (cyanocobalamin; BDH) 10 μg per ml were added to give final concentrations of glucose 1% (w/v) and vitamin B_{12} 0·1 μg per ml. The vitamin B_{12} solution, protected from light, was stored at 4°C.

BM medium was a modification of the medium described by Williams *et al.* (1975); it contained 1% Trypticase (Baltimore Biological Laboratories, BBL), 1% Proteose Peptone (Oxoid), 0·5% Yeast Extract (Difco) and 0·5% sodium chloride. The *p*H was adjusted to 7·4 and the medium was autoclaved. Filter-sterilised horse serum (2% v/v) (Wellcome Reagents Ltd, Beckenham, England) and growth factors were added to the steamed (cooled) medium immediately before inoculation.

The other media were modifications of BM medium without trypticase. Full strength PPY medium contained 2% Proteose Peptone (Oxoid), 1% Yeast Extract (Difco), 0·5% sodium chloride and growth factors; variations of PPY medium were supplemented with glucose (PPYG), with glucose and 2% horse serum (PPYSG), and with serum alone (PPYS). Half-strength PPY ($\frac{1}{2}$PPY) contained 1% proteose peptone, 0·5% yeast extract, 0·5% sodium chloride and growth factors; variations of this medium were supplemented with vitamin B_{12} ($\frac{1}{2}$PPY12) and vitamin B_{12} and serum ($\frac{1}{2}$PPY12S).

Gas liquid chromatography

Apparatus. A dual flame-ionisation gas chromatograph, Series 104 (Pye Unicam, Cambridge, England), fitted with heated injection ports, was used with a Servoscribe 1S recorder, model 541.20 (Belmont Instruments, 6 Belmont Drive, Giffnock, Glasgow G46 7PA), set at the 10-mV range with a chart speed of 120 mm per h. The oxygen-free nitrogen carrier gas, hydrogen and air were supplied by British Oxygen Co. Ltd, London.

Two identical coiled-glass columns (Pye Unicam), length 1·5 m and internal diameter 4 mm, were packed in the laboratory with Chromosorb 101, 80/100 mesh (Johns-Manville, Denver, Colo., available from Gas Chromatography Services Ltd, 23 Old Chester Road, Lower Bebington, Wirral, Merseyside L63 7LA). The columns were conditioned for 24 h at 250°C before gentle repacking; fresh polymer was added to leave a 2·5-cm gap at the top of the column. After further overnight conditioning at 250°C, the columns were purged at the operating temperature of 190°C for 16 h before use.

Operating conditions. The chromatograph was operated isothermally at 190°C for both volatile and non-volatile (methylated) acid analyses. The injection ports and column oven were at 190°C and the detector oven temperature was 250°C. The carrier-gas flow rate in the analysing column was *c.* 35 ml per min. The carrier gas and hydrogen flow rates of the non-analysing column were adjusted for optimal acetic-acid response. The columns were kept in condition by maintaining the flow of carrier gas overnight at the operating temperature. There were no problems of column bleed with the porous polymer. Flow rates were checked each day before analyses commenced.

Sample preparation and analysis. The procedures were derived from those of Carlsson (1973). In general, GLC analyses were performed on 2-day cultures; 4- or 7-day cultures were used for a few slow-growing strains. Cultures were acidified with four drops of 50% H_2SO_4 to approximately *p*H 2 and centrifuged at 800 *g* for 1 h; the cell-free supernates were stored at 4°C in screw-capped 7-ml bijou bottles until analysed. For analysis of volatile acids, a 0·6 μl sample of supernate was injected directly on to the analysing column with a

5-μl syringe (SGE, Melbourne, Australia, available from Phase Separations Ltd, Deeside Industrial Estate, Clywd, North Wales CH5 2LR). No pretreatment or extraction procedures were used. Contamination of the top 2–3 cm of the column occurred during volatile acid analyses and necessitated periodic replacement with fresh polymer. Overnight conditioning followed by 8-h purging restored normal operation.

For the non-volatile lactic and succinic acids the acidified culture supernates were methylated according to the method of Holdeman and Moore (1972) and a 0·6-μl sample of the chloroform extract was injected on to the column operated under the same conditions as those used for volatile analyses. Electrometer attenuation was 2×10^{-2} for volatile analyses and 5×10^{-2} for non-volatile analyses.

The peaks were routinely identified by direct measurement of the relative retention times with the acetic-acid peak as a reference. Accurate identification was made by comparing retention times of test samples with those obtained with a series of chromatographically-pure aqueous standards.

Standards. Aqueous 0·01M standards of acetic, propionic, *iso*-butyric, *n*-butyric, *iso*-valeric and *n*-valeric acids (BDH Analar) were used in the volatile analyses to establish absolute retention times and quantitation values. A combined acid standard containing a 0·01M concentration of each acid was used to establish relative retention times; it was used each day to monitor column performance and sensitivity settings of the instrument. Aqueous 0·02M lactic and succinic acid standards were included with each batch of non-volatile (methylated) acid analyses. Samples of uninoculated sterile medium were included as controls in every batch of each type of analysis.

The approximate concentration values of acids (μ.moles per ml) in the test samples were calculated by comparing the corrected peak heights of the test samples with those of the appropriate acid standards.

RESULTS

Comparison of media for GLC analysis

Preliminary trials showed that PYG medium (Holdeman and Moore, 1972) was unsatisfactory for the culture of some strains of *Bacteroides* spp. whereas good growth of a wide range of species within the genus was obtained in media based on proteose peptone (BM, ½PPY12, PPYG, PPYSG, see *Methods*). There were no growth failures, and even asaccharolytic strains grew well from small inocula, although some required 3–4 days to do so. Some cultures of asaccharolytic strains produced little turbidity and good growth was confirmed by microscopy.

Initial studies with wild strains. BM medium was used for the GLC analyses on 30 of 72 subgingival isolates, and the remaining 42 strains were tested in ½PPY12 medium; 32 of the 72 strains were independently identified as *B. melaninogenicus* ss. *melaninogenicus*, and 40 strains were ss. *intermedius*. There were no differences in the ranges of relative amounts of fatty acids produced by the two subspecies in the two media. All 72 strains produced succinic and acetic acids in significant quantities ($>$ 10 μ.moles per ml) and all produced minor amounts of *iso*-valeric acid with trace amounts of *iso*-butyric acid. Most strains produced traces of propionic acid but none produced *n*-butyric acid. The two subspecies were generally distinguished by the amount of lactic acid produced: 38 of 40 ss. *intermedius* strains produced 60–150 (median 95) μ.moles lactic acid per ml, whereas 28 of 32 ss. *melaninogenicus* strains produced 4–60 (median 38) μ.moles per ml in single analyses on 3-day cultures. The

large amount of acetic acid in sterile BM medium led us to exclude it from our further quantitative GLC studies.

Studies with reference strains grown in a range of derived media. The changes in fatty acid production by five reference strains of the *B. melaninogenicus-B. oralis* group in the different media are shown in table I. No valid type strain of *B. oralis* is currently available, but strain NP333 shares many of the characteristics originally ascribed to this species (Loesche, Socransky and Gibbons, 1964). The three pigmented saccharolytic strains produced characteristic fatty-acid profiles that were consistent in all the media; succinic and acetic acids were the major products. *n*-Butyric acid was not produced by any saccharolytic strain, but it was a significant product of the asaccharolytic strain NCTC9337. This strain also differed from the four saccharolytic strains by not producing succinic acid in any medium.

The incorporation of glucose in the culture medium induced only minor changes in the range of acids produced. Strain NP333 produced *iso*-valeric and lactic acids only in glucose-free media. Glucose stimulated acetic acid production by the strains labelled VPI4196, ATCC15930 and NP333. Strain NCTC9336 produced less acetic, lactic, *iso*-butyric and *iso*-valeric acids in

TABLE I

*Production of volatile fatty acids by five reference strains of Bacteroides spp. in six different peptone media**

Test strain	Test medium*	Approximate concentration of the stated fatty acid produced†						
		Acetic	Propionic	*iso*-Butyric	Butyric	*iso*-Valeric	Lactic	Succinic
B. melaninogenicus ss. *intermedius* NCTC9336	PPY	21	<1	2	0	3	110	26
	PPYS	25	<1	2	0	5	171	46
	½PPY12	15	<1	1	0	3	90	25
	½PPY12S	15	<1	2	0	3	131	26
	PPYG	16	<1	<1	0	1	62	35
	PPYSG	22	<1	<1	0	2	64	37
B. melaninogenicus ss. *melaninogenicus* VPI4196	PPY	11	<1	1	0	2	13	35
	PPYS	11	<1	1	0	2	14	37
	½PPY12	6	<1	1	0	1	14	22
	½PPY12S	6	<1	<1	0	1	13	25
	PPYG	20	<1	1	0	1	8	4
	PPYSG	20	1	1	0	1	6	39
B. melaninogenicus ss. *melaninogenicus* ATCC15930	PPY	10	<1	0	0	0	0	21
	PPYS	9	1	0	0	0	0	17
	½PPY12	5	0	0	0	0	0	14
	½PPY12S	10	<1	0	0	0	11	11
	PPYG	18	1	0	0	0	0	26
	PPYSG	14	0	0	0	0	0	27
B. oralis NP333	PPY	6	<1	0	0	1	32	17
	PPYS	4	<1	0	0	<1	11	17
	½PPY12	4	<1	0	0	<1	9	8
	½PPY12S	3	<1	0	0	<1	9	10
	PPYG	6	1	0	0	0	0	18
	PPYSG	10	1	0	0	0	0	22
B. melaninogenicus ss. *asaccharolyticus* NCTC9337	PPY	21	5	3	12	6	144	0
	PPYS	25	4	2	11	5	121	0
	½PPY12	12	9	1	2	2	64	0
	½PPY12S	19	12	2	4	3	145	0
	PPYG	25	6	3	13	6	148	0
	PPYSG	17	1	1	6	3	148	0

* See *Methods* section for details of the media.
† Fatty acid concentration expressed in μ.moles per ml estimated by measurement of peak height with reference to aqueous standards corrected for values obtained with medium only. Results are those of single analyses for each sample.

glucose-enriched media; this was not attributable to less growth. Glucose did not affect acid production by strain NCTC9337.

The addition of serum had little or no effect on growth or fatty acid production by the four saccharolytic strains. The effect of serum on strain NCTC9337 was inconsistent: production of acetic acid was slightly enhanced by serum in the absence of glucose; growth and production of propionic acid were enhanced when serum and vitamin B_{12} were added; production of other acids was not affected.

The effect of vitamin B_{12} supplement. Strain NCTC9 337 was grown for 23 h in ½PPY, ½PPY12, ½PPY12S, PPY and PPYS medium. The figure shows the volatile fatty-acid profiles obtained from the combined acid standard and the cultures, and table II shows the concentrations of the individual volatile fatty acids achieved in the various media. The production of acetic, *iso*-butyric and *iso*-valeric acids was directly proportional to turbidity. The production of propionic acid was significantly higher in the ½PPY12 and ½PPY12S media.

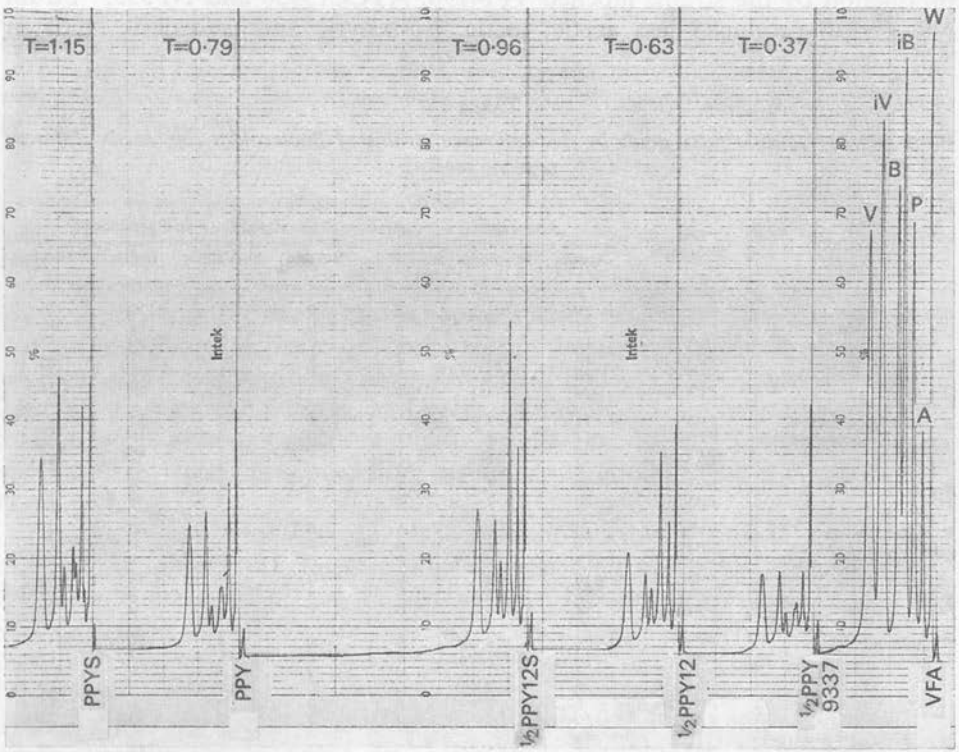

FIGURE.—The volatile fatty-acid profiles of *B. melaninogenicus* ss. *asaccharolyticus* strain NCTC9337, grown for 23 h in five different media, compared with the plot of a combined acid standard (VFA) to show the effect of vitamin B_{12} (cyanocobalamin) and horse serum on growth and acid production. The various media are indicated at the bottom of the chart and the turbidity (T) of each test culture is given at the top of the chart. Each ml of the combined acid standard contained 10 μ.moles of the following acids: acetic (A), propionic (P), *iso*-butyric (*i*B), *n*-butyric (*n*B), *iso*-valeric (*i*V) and *n*-valeric (*n*V). W = " Water " peak. Details of the media used and the chromatographic conditions are given in the *Methods* section. Chart speed = 120 mm per h.

TABLE II

The volatile fatty acids produced by strain NCTC9337 in 23-h cultures of proteose peptone media

Medium	Turbidity at 600 nm	Approximate concentration (μ.moles per ml) of the stated acid				
		Acetic	Propionic	*iso*-Butyric	Butyric	*iso*-Valeric
½PPY	0·365	0·87	<0·2	0·44	1·52	1·44
½PPY12	0·63	4·2	3·04	0·68	1·44	1·8
PPY	0·79	6·1	<0·2	0·56	2·88	2·24
½PPY12S	0·96	7·3	6·0	1·06	2·48	2·6
PPYS	1·15	9·6	0·7	1·06	5·76	3·37

Footnotes as in table I.

Serum and vitamin B_{12} independently stimulated growth; together they produced an additive effect. The production of *n*-butyric acid was slightly inhibited by vitamin B_{12}.

Definitive studies of fatty acid production

Fatty acid profiles of reference strains grown in PPYSG. Table III shows the fatty acids produced by 12 reference strains of Bacteroidaceae in single analyses on 2-day cultures in PPYSG medium. The *Bacteroides* strains all produced similar fatty-acid profiles that were clearly distinct from the profiles of *Fusobacterium* and *Leptotrichia* strains. All *Bacteroides* strains produced succinic, acetic, lactic and propionic acids. The *Fusobacterium* strains produced moderate amounts of *n*-butyric acid and small quantities of lactic and propionic acids but not *iso*-butyric or *iso*-valeric acids; one strain produced a trace of succinic acid. The *Leptotrichia* isolate produced traces of acetic and succinic acids and a moderate amount of lactic acid only.

TABLE III

Approximate concentrations of fatty acids produced by 12 reference strains of the genera Bacteroides, Fusobacterium and Leptotrichia in 2-day cultures in PPYSG medium

Strain identity	Strain no.	Approximate concentration (μ.moles per ml) of the stated acid*						
		Acetic	Propionic	*iso*-Butyric	Butyric	*iso*-Valeric	Lactic	Succinic
Bacteroides fragilis	NCTC9344	22	1	0	0	<1	4	35
Bacteroides fragilis	NCTC8560	21	1	0	0	<1	4	33
Bacteroides thetaiotaomicron	NCTC10582	21	<1	0	0	0	3	26
Bacteroides thetaiotaomicron	ATCC8492	12	<1	0	0	0	2	19
Bacteroides ovatus	ATCC8483	21	1	0	0	<1	3	33
Bacteroides distasonis	ATCC8503	4	4	<1	0	<1	7	22
Bacteroides vulgatus	ATCC8482	5	5	<1	0	<1	17	18
Bacteroides vulgatus	NCTC10583	20	1	0	0	0	0	35
Fusobacterium necrogenes	NCTC10723	6	2	0	13	0	2	0
Fusobacterium polymorphum	NCTC10562	18	2	0	14	0	3	2
Fusobacterium necrophorum	NCTC10575	7	5	0	12	0	3	0
Leptotrichia buccalis	NCTC10249	1	0	0	0	0	22	2

* See footnote to table I.

Studies with isolates grown in glucose-enriched and glucose-free media. GLC was performed on glucose-enriched and glucose-free cultures of 55 saccharolytic subgingival-crevice isolates that included representative strains of *B. melaninogenicus*, *B. oralis*, *Fusobacterium* spp. and *L. buccalis*. Table IV shows the median values and the range of results obtained with 49 isolates tested in glucose-enriched media (PPYG; PPYSG) and 38 isolates tested in glucose-free media (PPY; ½PPY12). Thirty-two isolates were tested in both glucose-enriched and glucose-free media. The volatile fatty-acid profiles of the 49 saccharolytic strains in the *B. melaninogenicus-B. oralis* group were similar to the profiles of the four saccharolytic reference strains. *B. melaninogenicus* ss. *intermedius* strains tended to produce slightly more acetic acid than the ss. *melaninogenicus* strains, but there was considerable overlapping in the ranges of results. The ranges for the other acids produced by these two subspecies also overlapped; the different median values may reflect population differences, but individual strains could not be reliably distinguished. Similarly, *B. oralis* strains could not be distinguished from strains of *B. melaninogenicus* ss. *melaninogenicus* on the basis of the volatile fatty acids produced in the media used. Propionic acid was a minor product of most strains. The reduction in *iso*-valeric and *iso*-butyric acid production in glucose-enriched media correlated with increased growth in these media. *n*-Butyric acid was not produced by any of these strains. The two fusobacterium and four leptotrichia isolates gave volatile fatty-acid profiles that were distinct from each other and consistent with the profiles of the respective reference strains. The leptotrichia strains produced more acetic and succinic acids than the reference strain; two of the isolates produced traces of propionic acid in PPYG medium.

TABLE IV

Concentrations of fatty acids produced by 55 subgingival isolates in glucose-free and glucose-enriched media

Test species or subspecies	Number of strains	Medium*	Number of analyses	Median concentration (and range) of the stated acid† (μ.moles per ml)				
				Acetic	Propionic	*iso*-Butyric	Butyric	*iso*-Valeric
B. melaninogenicus ss. *intermedius*	20	Glu−	17	18 (5-28)	<1 (0-1)	<1 (0-2)	0	3 (1-9)
		Glu+	16	20 (5-25)	1 (0-5)	<1 (0-<1)	0	<1 (0-3)
B. melaninogenicus ss. *melaninogenicus*	22	Glu−	22	9 (5-15)	<1 (0-2)	<1 (0-1)	0	2 (0-4)
		Glu+	25	15 (5-27)	<1 (0-4)	0 (0-<1)	0	<1 (0-2)
B. oralis	7	Glu−	3	14 (9-23)	<1 (0-1)	0 (0-<1)	0	1 (0-4)
		Glu+	12	14 (8-26)	0 (0-2)	0	0	0 (0-<1)
Fusobacterium sp.	2	Glu−	2	14	2	0	14	0
		Glu+	2	11	2	0	14	0
Leptotrichia buccalis	4	Glu−	1	9	0	0	0	0
		Glu+	4	10 (4-20)	0 (0-<1)	0	0	0

* Glu− = Glucose-free medium; Glu+ = glucose-enriched medium.
† See footnote in table I.

Quantitative lactic- and succinic-acid estimations were performed for 29 of the *B. melaninogenicus* and *B. oralis* strains (table V). The lactic acid concentrations were consistently reduced four-fold in glucose-enriched media with 21 strains tested in both glucose-enriched and glucose-free media. *B. melaninogenicus* ss. *intermedius* isolates produced less lactic acid ($\leq$25 μ.moles per ml) than was produced by the reference strain NCTC9336. This finding was clarified by later studies reported below. *B. oralis* strains produced less lactic acid than *B. melaninogenicus* strains, but the ranges obtained in both glucose-enriched and glucose-free media overlapped. Five *B. oralis* strains failed to produce lactic acid in glucose-enriched media, but four strains produced 10 μ.moles or more per ml.

Clinical isolates grown in fully supplemented medium. As a result of the initial studies, PPYSG medium was selected for GLC analysis of the fatty acids produced by 42 clinical isolates of *Bacteroides* spp. Twelve asaccharolytic strains were tested after incubation for 7 days; all other isolates were tested after 2 days. The only *B. fragilis* isolate tested produced major amounts of succinic and acetic acids and a trace of propionic acid; two *B. ovatus* isolates produced similar profiles but one isolate also produced lactic acid 5 μ.moles per ml. One strain identified as *B. corrodens* produced a range of acids similar to that of asaccharolytic *B. melaninogenicus* strains; acetic and lactic acids were the major products with propionic, *iso*-butyric, *n*-butyric and *iso*-valeric acids as minor products; succinic acid was not produced. Seventeen strains of *B. melaninogenicus* ss. *asaccharolyticus* produced typical profiles; lactic (27–150 μ.moles per ml) and acetic (5–27 μ.moles per ml) acids were the major products and *n*-butyric acid was produced in moderate amounts (5–13

TABLE V

Production of lactic and succinic acids by 29 subgingival isolates of B. melaninogenicus and B. oralis in glucose-enriched and glucose-free media

Test species or subspecies	Medium*	Number of analyses	Median concentration (and range) of the stated acid (μ.moles per ml)	
			Lactic	Succinic
B. melaninogenicus ss. *intermedius*	Glu$^-$	10	75 (30-100)	26 (6-52)
	Glu$^+$	5	12 (5-25)	19 (10-38)
B. melaninogenicus ss. *melaninogenicus*	Glu$^-$	13	57 (23-91)	38 (10-74)
	Glu$^+$	15	8 (2-33)	21 (12-40)
B. oralis	Glu$^-$	2	20 & 33	16 & 17
	Glu$^+$	11	5 (0-15)	24 (10-40)

* Glu$^-$ = Glucose-free medium; Glu$^+$ = glucose-enriched medium.

μ.moles per ml); seven strains did not produce succinic acid but five strains produced >10 μ.moles per ml (10–19 μ.moles per ml); propionic and *iso*-valeric acids were minor products.

The saccharolytic strains of *B. melaninogenicus* studied in this section included eight faecal, two subgingival and eight high-vaginal-swab (HVS) isolates. All 18 strains (10 ss. *intermedius;* 8 ss. *melaninogenicus*) produced similar volatile fatty-acid profiles. The median values and the ranges of concentrations of the acids produced were virtually identical to those reported for the same subspecies in glucose-enriched media in table IV. Lactic and succinic acid results differed between the two subspecies on this occasion and also differed from the results reported in table V.

The ranges of values and the median and mean values obtained from faecal, subgingival and HVS isolates are compared in table VI. The values reported for the subgingival isolates include the results for strains included in table V and for nine strains of *B. melaninogenicus* ss. *melaninogenicus* from the collaborative survey (see below).

Subgingival strains of *B. melaninogenicus* ss. *intermedius* produced lactic and succinic acids in quantities similar to those produced by faecal strains of the same subspecies. Eight of 11 subgingival and faecal strains consistently produced lactic acid <30 μ.moles per ml and seven of 11 strains produced succinic acid <40 μ.moles per ml. Two faecal and one subgingival strain produced lactic acid >45 μ.moles per ml. HVS strains of *B. melaninogenicus* ss. *intermedius* produced more lactic and succinic acids than the subgingival or faecal strains. Three of four HVS strains consistently produced lactic acid >60 μ.moles per ml and the other strain produced lactic acid >60 μ.moles

TABLE VI

*Comparison of lactic and succinic acid production by saccharolytic strains of B. melaninogenicus from different clinical sites**

Test subspecies	Source	Number of strains	Number of analyses	Range of concentrations of the stated acid (μ.moles per ml)	Median values	Mean values
B. melaninogenicus ss. *intermedius*	Subgingival	6	10	Lactic 6–46	20	20·5
				Succinic 10–81	38	39·5
	Faecal	5	7	Lactic 10–52	25	28
				Succinic 9–71	40	37
	HVS†	4	7	Lactic 34–110	65	74
				Succinic 24–85	68	65
B. melaninogenicus ss. *melaninogenicus*	Subgingival	17‡	17	Lactic 2–33	8	9
				Succinic 12–40	19	23
	Faecal	3	3	Lactic 4–38	20	20·6
				Succinic 11–69	46	42
	HVS	4	4	Lactic 10–55	33	33·5
				Succinic 35–150	83	95

* All analyses on 2-day cultures in glucose-enriched medium.
† HVS = High vaginal swab.
‡ Includes our strains analysed in collaborative survey.

per ml in one of two cultures tested. All four HVS strains produced succinic acid >66 μ.moles per ml although two of four strains did so in only one of two cultures tested. All analyses were performed on 2-day cultures in PPYSG medium and reasons for the culture-to-culture variation shown by a few strains were not elucidated.

Subgingival and faecal strains of *B. melaninogenicus* ss. *melaninogenicus* produced similar quantities of lactic and succinic acids. Seventeen of 20 strains produced lactic acid <13 μ.moles per ml and 18 of 20 strains produced succinic acid <40 μ.moles per ml. The high median value of 46 μ.moles per ml for succinic acid production by faecal strains in table VI is not significant where only three strains were studied. Three of four HVS strains of *B. melaninogenicus* ss. *melaninogenicus* produced lactic acid >30 μ.moles per ml and succinic acid >80 μ.moles per ml.

Three additional strains were provisionally identified as *B. melaninogenicus* ss. *intermedius* on the basis of indole production, gelatin digestion and the slow, weak fermentation of glucose. These isolates, however, produced *n*-butyric acid on two occasions in PPYSG medium; two also failed to produce succinic acid and were therefore more typical of asaccharolytic strains, but the third isolate produced succinic acid 18 μ.moles per ml.

Collaborative studies with B. melaninogenicus ss. melaninogenicus, B. oralis and B. ochraceus. Forty strains were tested as part of a collaborative investigation instigated by the ICSB Taxonomic Sub-Committee on Gram-negative anaerobic rods. Twelve strains were referred to us by the sub-committee, six strains were referred by other colleagues and 22 strains were isolated from subgingival plaque. Table VII shows the range of fatty acids produced by the strains. Loesche's strain 7CM designated *B. oralis* has been excluded from the *B. oralis* group in the table as it is more typical of a *B. fragilis* strain (see Holbrook, Duerden and Deacon, 1977); similarly, the referred strains ATCC15930, VPI7570A and no. 30 were found to produce black-pigmented colonies on lysed-blood agar and were included in the *B. melaninogenicus* ss. *melaninogenicus* group.

Table VIII shows population differences between the groups represented in the collaborative study. The individual results were assigned to four grades

TABLE VII

Fatty acids produced in PPYSG medium by strains included in the collaborative study

Test species or subspecies	Number of strains	Median concentration (and range) of the stated acid† (μ.moles per ml)						
		Acetic	Propionic	*iso*-Butyric	Butyric	*iso*-Valeric	Lactic	Succinic
Bacteroides melaninogenicus ss. *melaninogenicus*	23*	20 (10-46)	1 (0-4)	0 (0-<1)	0	<1 (0-2)	7 (0-33)	22 (14-40)
Bacteroides oralis	10	15 (4-26)	<1 (0-2)	0	0	0 (0-<1)	0 (0-10)	21 (11-36)
Bacteroides ochraceus	6	10 (3-14)	0 (0-2)	0	0	0 (0-<1)	0 (0-5)	19 (11-31)
Loesche's strain 7CM	1‡	22	<1	0	0	0	0	45

* Includes ATCC15930, VPI7570A and 30 (see text and Holbrook and Duerden, 1974).
† See footnote to table I.
‡ Not included in *B. oralis* (see text).

TABLE VIII

The production of fatty acids by the 40 strains examined in the collaborative study

Fatty acid	Concentration of acid produced*	Percentage of strains producing the stated concentration of acid		
		B. melaninogenicus ss. *melaninogenicus* (23 strains)	*B. oralis* (11 strains)	*B. ochraceus* (6 strains)
Acetic	+	0	18·2	66·7
	++	100	81·8	33·3
Propionic	−	13	36·4	50
	tr	34·8	36·4	16·7
	+	52·2	27·2	33·3
iso-Butyric	−	82·6	100	100
	tr	17·4	0	0
n-Butyric	−	100	100	100
iso-Valeric	−	21·7	63·6	83·3
	tr	69·5	36·4	16·7
	+	8·7	0	0
n-Valeric	−	100	100	100
Lactic	−	8·7	63·6	83·3
	tr	69·6	18·2	16·7
	+	17·4	18·2	0
	++	4·3	0	0
Succinic	+	39·1	45·5	50
	++	60·9	54·5	50

* The system used to grade the concentration values (μ.moles per ml) was as follows. (a) Volatile acids: ++ = >10; + = 1·1−10; tr = 0·2−1·0; − = <0·2. (b) Lactic and succinic acids: ++ = >20; + = 10−20; tr = 2−10; − = <2·0.

that appear to represent significant differences between strains. Individual strains of *B. melaninogenicus* ss. *melaninogenicus* could not be differentiated from strains of *B. oralis* on the basis of GLC results alone. With one exception, the median values and ranges of values in respect of the acids produced by *B. melaninogenicus* ss. *melaninogenicus* and *B. oralis* strains were similar to the values obtained previously (see table IV) and overlapped those obtained with the *B. ochraceus* strains. However, *B. ochraceus* strains generally produced smaller quantities of acetic and lactic acids. It is of interest to note that three referred strains of *B. melaninogenicus* ss. *melaninogenicus* (WAL2724, WAL2721 and VPI7570A) produced acetic acid >28 μ.moles per ml.

A study of culture-to-culture variation. Despite careful attention to detail, particularly with regard to size and condition of inocula, incubation time, sample size at injection and the daily use of appropiate standards and controls, variation occurred in the quantitative results obtained from different cultures of the same strains. The results of analyses on two separate 2-day cultures of each of three reference strains in the same medium are shown in table IX. In all analyses the major products of each strain showed small variations but were always present in significant amounts. Minor products varied in concentration but were consistently detected; trace products were occasionally absent.

TABLE IX

The quantitative variation in fatty acid production by three reference strains of Bacteroides species or subspecies, analysed in PPYSG medium on two separate occasions

Organism	Study*	Approximate concentration of the stated acid†						
		Acetic	Propionic	*iso*-Butyric	Butyric	*iso*-Valeric	Lactic	Succinic
B. oralis strain NP333	I	19	2	0	0	1	3	36
	II	10	1	0	0	0	0	22
B. melaninogenicus ss. *melaninogenicus* strain ATCC15 930	I	20	1	0	0	0	0	25
	II	14	0	0	0	0	0	27
B. melaninogenicus ss. *melaninogenicus* strain VPI4196	I	26	2	1	0	1	12	38
	II	20	1	1	0	1	6	39

* I = Collaborative study; II = media comparison study.
† See footnote to table I.
All analyses were performed on 2-day cultures.

DISCUSSION

All of the 185 Gram-negative, non-sporing, obligate anaerobes studied were identified to species or subspecies level by simple morphological, cultural, biochemical, tolerance and antibiotic-resistance tests (see Duerden *et al.*, 1976). All isolates were correctly identified to genus level by independently-performed GLC analyses of the short-chain fatty acids produced in glucose-free or glucose-enriched broth cultures. This study confirms that presumptive identification of genus can be made on the basis of Gram stain, colonial morphology and fermentation products (see Holdeman and Moore, 1974), and that GLC data must be supplemented with other test data in order to sub-classify the *Bacteroides* and *Fusobacterium* genera.

Essentially similar fatty-acid profiles were obtained with test strains of *B. ovatus* and *B. fragilis* and with reference strains of *B. fragilis* representing all of the *B. fragilis* subspecies now accorded species rank (see Cato and Johnson, 1976). The major products were succinic and acetic acids with traces of lactic and propionic acids; occasional strains produced traces of *iso*-valeric and *iso*-butyric acids. Moreover, all the *B. fragilis*-like strains tested produced fatty acids similar to those produced by the saccharolytic *B. melaninogenicus* strains; quantitative differences and minor qualitative differences existed, but none was of clear discriminatory value at the species or subspecies level. Furthermore, it was impossible to differentiate *B. oralis* from *B. melaninogenicus* ss. *melaninogenicus* and *B. corrodens* from *B. melaninogenicus* ss. *asaccharolyticus*. The status of *B. oralis* (Loesche *et al.*, 1964) as a separate species is currently debated (ICSB Minute, in press); the strains may be non-pigmented variants of *B. melaninogenicus* ss. *melaninogenicus* (Harding *et al.*, 1976; Holbrook *et al.*, 1977). The similarity of *B. oralis* strains to *B. ruminicola* has also been noted (Sundqvist, 1976; Terada, Uchida and Mitsuoka, 1976). It appears that the strains may only be differentiated by arabinose, xylose and salicin fermentation. The six *B. ochraceus* strains were not distinguished with certainty from the *B. melaninogenicus-B. oralis* group. Strains of the two

saccharolytic subspecies of *B. melaninogenicus* produced succinic and acetic acids as major products in all of the test media; production of lactic acid was variable, and there were frequently minor amounts of propionic, *iso*-valeric and *iso*-butyric acids, but *n*-butyric acid was not produced. The strains were clearly distinct from the asaccharolytic subspecies which, in glucose-enriched or glucose-free media, typically produced moderate to large quantities of acetic, lactic and *n*-butyric acids and minor amounts of propionic, *iso*-butyric and *iso*-valeric acids; some produced succinic acid. Isolates belonging to the genus *Fusobacterium* typically produced acetic and *n*-butyric acids with traces of lactic and propionic acids, but did not produce *iso*-butyric or *iso*-valeric acid. The only significant products of isolates belonging to the genus *Leptotrichia* were moderate amounts of lactic and acetic acids. These conclusions are summarised in table X.

The use of aqueous, semi-quantitative GLC analyses of the fermentation products of a wide range of reference and clinical strains of bacteroides organisms in this study revealed the considerable biological variation as reflected in the end products of fermentation that exists within recognised groups of related organisms. In the *B. melaninogenicus-B. oralis* group, strain-to-strain and culture-to-culture variation occurred despite careful standardisation of methods; this obliged us to follow the example of Holdeman and Moore (1972) and we developed a scoring system that took account of these variations but allowed recognition of meaningful differences between strains that might be useful in identification.

Shah *et al.* (1976) recently compared the biochemical properties of 45 *B. melaninogenicus* strains and reported differences in the fatty acid profiles of the two saccharolytic subspecies. Lactic and propionic acids were not detected in BM broth cultures of either subspecies. No *B. melaninogenicus* ss. *melaninogenicus* strain was found to produce *iso*-butyric acid but most ss. *intermedius* strains produced traces of it. Our results differ from those of Shah *et al.* (1976) who also noted discrepancies between their findings and those of Holdeman and Moore (1972) and Lambe (1974). In our experience the detection of lactic or succinic acid in concentrations of <5 μ.moles per ml in the aqueous volatile analyses was unreliable and methylation was necessary. Other small differences in the cultural or chromatographic procedures may account for other variations between our results and those of Shah *et al.* (1976). We consider that the occurrence of such discrepancies in the determination of minor products precludes the use of such small differences for subspecies identification or taxonomic differentiation of strains without support from other tests. We agree with Moore (1970) who stated that the *major* fatty acid products are stable characteristics and it is apparent that these products are consistently and reliably detected by different groups of workers.

The observation that vaginal isolates of saccharolytic *B. melaninogenicus* strains produce more lactic and succinic acids than oral or faecal strains of the same subspecies is interesting. Insufficient numbers were examined to draw definite conclusions; a population difference may exist between the intestinal and vaginal strains and biotyping might be possible on this basis.

TABLE X

Typical fermentation patterns in the family Bacteroidaceae

Species and subspecies	Concentration* of the stated acid produced						
	Acetic	Propionic	*iso*-Butyric	Butyric	*iso*-Valeric	Lactic	Succinic
B. fragilis-like strains	++†	tr	(tr)	**0**	(tr)	tr	++
B. melaninogenicus ss. *intermedius*	+–++	0–tr(+)	0–+	**0**	0–+	tr–++	++
ss. *melaninogenicus*	+–++	0–tr(+)	0–tr	**0**	0–+	0–++	+–++
B. oralis	+–++	0–tr(+)	0–(tr)	**0**	0–tr	0–++	+–++
B. ochraceus	+–++	0–+	**0**	**0**	0–(tr)	0–(tr)	+–++
B. melaninogenicus ss. *asaccharolyticus*	+–++	+	(tr)– +	+–++	+	++	0–+
Fusobacterium spp.	+–++	+	**0**	++	**0**	tr	0–tr
Leptotrichia buccalis	tr–++	**0**–(tr)	**0**	**0**	**0**	++	tr

* See footnote to table VIII for the system used to grade the concentration values. The fatty acid concentrations were estimated as described in the footnote to table I.

† One reference strain each of *B. distasonis* and *B. vulgatus* produced + value only.

( ) = Occasional strains only.

The symbols in bold type indicate results likely to be of discriminatory value.

The BM medium of Williams *et al.* (1975), similar to the isolation medium of Schaedler, Dubos and Costello (1965), proved to be an excellent culture medium and, with meat particles added, it is a useful maintenance medium for a wide range of anaerobic bacteria. We found that the high acetic-acid content of BM medium, contributed by trypticase, limited its value for our GLC studies and it was used only briefly in our preliminary series with freshly-isolated oral strains.

The effect of restricted changes in the growth medium on fatty acid production was evaluated with five reference strains of the *B. melaninogenicus-B. oralis* group in six proteose peptone media that did not contain trypticase (BBL). Each strain produced a characteristic range of acids that was consistent in all the media except for the putative *B. oralis* strain NP333 that produced traces of *iso*-valeric and lactic acids in glucose-free media only. Glucose in general affected the amounts but not the range of acids produced by saccharolytic strains. It did not affect acid production by the single asaccharolytic strain NCTC9337. Similar observations have been reported previously (Werner, Pulverer and Reichertz, 1971; Werner, 1974; Mayhew, Onderdonk and Gorbach, 1975) and support the general conclusion of Holdeman and Moore (1975) that chromatographic analyses could be run on any medium.

The choice of fully-supplemented PPYSG medium, containing proteose peptone, 2% serum and 1% glucose, for definitive GLC studies was based on the following considerations. (1) The full-strength PPY media generally gave better growth rates of a wide range of strains and the addition of serum enhanced the growth of some strains without affecting the fatty-acid profiles. We were concerned to achieve rapid growth wherever possible, to minimise incubation times and to facilitate rapid identification of our isolates. (2) Many clinical isolates are likely to be saccharolytic strains that utilise glucose preferentially and grow more rapidly on this substrate. Asaccharolytic strains may show a slightly-increased lag phase in glucose-enriched media (Lev and Milford, 1975) but in our experience their fatty-acid profiles were not affected. Where glucose is the preferred substrate for energy production, its metabolism will generally cause catabolite repression of amino-acid utilisation, but Loesche and Gibbons (1968) and Wahren and Gibbons (1970) have shown that fermentation of amino acids by some strains of *F. nucleatum* and *B. melaninogenicus* is not repressed by glucose. It therefore seemed reasonable to include glucose in the medium.

The growth and fatty-acid production of the *B. melaninogenicus* ss. *asaccharolyticus* strain NCTC9337 was significantly affected by vitamin B_{12}. Growth and propionic-acid production were stimulated by cyanocobalamin 0·1 μg per ml, but production of *n*-butyric acid was partially inhibited. The initial observation of growth stimulation was made in the course of other studies. The concentration used in the present study was previously found to provide maximum growth stimulation (Deacon, unpublished data). Varel and Bryant (1974) reported that *B. fragilis* strain NCTC9343 required vitamin B_{12} 0·1 ng per ml for good growth in a defined medium. To our knowledge, the minimal vitamin-B_{12} growth requirements of *B. melaninogenicus* ss.

asaccharolyticus strains have not been determined and we are not aware of any previous reports of the pronounced effects that high concentrations of the vitamin can have on the growth and metabolism of these strains. Further studies are in progress.

The GLC approach used in this study provided quantitative data useful in identification of our isolates. There is a high degree of correlation with the other independently-performed tests. GLC may expedite the identification of clinical isolates to generic level without reliance on many other observations and tests, but it is clear that some basic data are necessary, even at this level, and additional information is required for species or subspecies identification. The appeal of GLC techniques for clinical laboratories is likely to be increased with recent reports suggesting that it is possible to detect the presence of anaerobes in clinical specimens by direct analyses on pus or other material (Bricknell, Sugihara and Brook, 1976; Gorbach *et al.*, 1976; Phillips, Tearle and Willis, 1976). However, this calls for skilled and experienced staff, able to monitor and interpret daily analyses. There must also be a sufficient number of specimens yielding anaerobes to justify the capital outlay for the equipment. At present, we consider that if clinical microbiologists are encouraged to use existing GLC facilities available in reference laboratories, they will contribute to the further refinement and development of the procedures and to clarification of the remaining areas of confusion in the taxonomy of the anaerobic bacteria.

Summary

The acid end-products of 185 isolates from the family Bacteroidaceae were separated and analysed by gas-liquid chromatography on broth cultures. Different media were evaluated and definitive studies were performed in a fully supplemented complex medium. The limitations of this approach to the identification of a wide range of strains from various clinical sources were determined and the results were compared with those of a series of morphological, biochemical, tolerance and antibiotic-resistance tests.

All test strains were identified to generic level by simple microscopic and colonial observations and GLC analysis; additional tests were required to allow species or subspecies identification of most strains. Population differences were detected between some species or subspecies isolated from different clinical sites by quantitative analyses of fatty acids, but individual strains could not always be separated because of overlapping ranges of distribution of acids that were common products of more than one species or subspecies. Small differences in minor products between different species or subspecies were variable and are not considered adequate for discrimination at these taxonomic levels without support from other observations.

The potential application of the GLC technique to the rapid and accurate identification of these organisms in hospital laboratories is considered.

We wish to thank Professor J. G. Collee for his helpful advice throughout these investigations and for assistance in the preparation of this paper. We also thank Mr R. Brown and his technical colleagues for skilled assistance.

A. G. Deacon gratefully acknowledges the financial support of the Medical Research Council (MRC Grant no. G974/325B).

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PROCEEDINGS OF THE PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

The 136th meeting of the Society was held at the Preclinical Medical College, St Bartholomew's Hospital, London, on the 4th, 5th and 6th January 1978

40. The characterisation of gram-negative anaerobic bacilli

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Both the classification of the Bacteroidaceae and our understanding of the relationship between the various species are undergoing considerable changes. Gram-negative anaerobic bacilli can be divided into four groups: the "fragilis" group, the "melaninogenicus-oralis" group, the "asaccharolytic" group and the "fusobacteria". The fragilis group contains the species previously designated as subspecies of *Bacteroides fragilis* and several related species; all are commensals of the large intestine. They are saccharolytic non-pigmented organisms that produce succinate as a major end-product of metabolism; the group is generally tolerant of bile or taurocholate and resistant to disks containing penicillin

(1·5 units), neomycin (1000 μg) and kanamycin (1000 μg). The melaninogenicus-oralis group contains the fermentative subspecies of *B. melaninogenicus* and non-pigmented species that share similar characteristics (*B. oralis*, *B. ruminicola* etc.); they are inhibited by bile or taurocholate and are sensitive to penicillin and neomycin disks. Pigment production is no longer the sole basis for the identification of *B. melaninogenicus*. The asaccharolytic group includes *B. asaccharolyticus* (formerly *B. melaninogenicus* ss *asaccharolyticus*), *B. corrodens* and other non-pigmented non-fermentative species. The fusobacteria are generally non- or weakly-fermentative strains that produce major amounts of *n*-butyric acid. This classification is based upon the results of complex biochemical investigations. However, most isolates may be identified by routine bacteriological methods.

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47. The identification of strains of *Bacteroides* spp. from normal human faeces and from clinical infections

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Strains (342) of *Bacteroides* spp. were identified by a short combined set of biochemical, antibiotic-disk resistance and tolerance tests; 197 were faecal isolates from 20 normal human subjects and 145 were isolated from 129 routine clinical specimens, sent to the diagnostic laboratory. The results of these tests enabled the isolates to be allocated to one of four groups—"fragilis", "melaninogenicus-oralis", "asaccharolytic" and "fusobacteria"—and most isolates were identified as belonging to recognised species within these groups. One hundred and seventy-four faecal isolates were allocated to the fragilis group and were divided between several species within that group; only 20 strains were *B. fragilis* (*B. fragilis* ss *fragilis*). However, 75 out of 89 clinical isolates allocated to the fragilis group were *B. fragilis*; 66 out of 92 clinical isolates from infections related to the appendix or large intestine were allocated to the fragilis group and 54 of these were *B. fragilis*. The remaining faecal isolates were *B. melaninogenicus* (3), pigmented *B. asaccharolyticus* (8) and non-pigmented asaccharolytic strains (12). The other clinical isolates were identified as melaninogenicus-oralis group (17), pigmented *B. asaccharolyticus* (30), *B. corrodens* (3), non-pigmented asaccharolytic strains (3) and fusobacteria (3).

PROCEEDINGS OF THE PATHOLOGICAL SOCIETY

OF GREAT BRITAIN AND IRELAND

The 138th meeting of the Society was held at Charing Cross Medical School, London, on the 3rd, 4th and 5th January 1979

31. AN EVALUATION OF THE "MASTRING-S M/ID" IN THE IDENTIFICATION OF CLINICAL ISOLATES OF *BACTEROIDES* SPP. AND THE RESULTS OF ROUTINE SENSITIVITY TESTS

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Clinical isolates of *Bacteriodes* spp. were provisionally identified by the "Mastring – S M/ID" method and definitively identified by conventional bacteriological tests. In general the isolates were allocated to the appropriate groups of species by the results of the "Mastring" test. Isolates of all members of the *B.fragilis* group gave the same result; they were sensitive to the erythromycin 60μg and rifampicin 15μg disks only. Isolates of the *B.melaninogenicus/oralis* group were identified when they were sensitive to the penicillin 2 unit and/or colistin 10μg disks, but sensitivity to colistin and penicillin is variable in this group and resistant strains gave the same pattern of results as the *B.fragilis*-group isolates. Moreover, *B.asaccharolyticus* isolates gave the same pattern of results as the *B.melaninogenicus/oralis*-group isolates. *B.corrodens* isolates were sensitive to all the disks except vancomycim 10μg. In routine sensitivity tests, all isolates were sensitive to metronidazole; four were resistant to clindamycin and five were resistant to chloramphenicol. Amongst *B.fragilis*-group isolates, 79·6% were resistant to erythromycin (2μg disk) and 42·1% to tetracycline (5μg disk). *B.asaccharolyticus, B.corrodens* and *B.melaninogenicus/oralis*-group isolates were sensitive to most agents tested but one *B.asaccharolyticus* isolate and five *B.melaninogenicus/oralis*-group isolates were resistant to penicillin.

APPENDIX VII

TABLE I

Results obtained with the 40 test strains

	B. melaninogenicus ss. *melaninogenicus*															*B. oralis*															*B. ochraceus*					
growth in air + CO_2 *													−															−					+			
Pigment production *													+															−					−			
Antibiotic test †																																				
neomycin 1000µg													S															S					S			
Tolerance tests ø																																				
bile salts													I			I												I					I			
Victoria blue 4R													I			+												I					+			
ethyl violet													I			I												I					I			
Indole production *													−			−												−					−			
Fermentation of *																																				
G L S M													+			+												+					+			
Man. T													−			−												−					−			
rhamnose													−			−												−					−			
Aesculin hydrolysis *			−					+						+	−	+				+			+			+			−			+	−		+	
Dextran hydrolysis *	−	+	+	+	+	+	+	−	−	−	+	+	+				+	+	−	−	+		−	+		+		−			+	+		+	
gelatin digestion *	−	−	+	+	+	+	−	+	+	−	−	−	−	−	+	+	−	−	−	−	−		−	−		−		−			−	−		−	
lipase production *	−	−	−	+	+	−	−	+	−	+	−	+	−	−	−	−	−	−	−	−	−		−	−		−		−			−	−		−	
Haemolysis *	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+	+	+	−		+	+		+		−			−	−		−	
Antibiotic tests †																																				
metronidazole	S		S	S	S			R	S			S	S	S	R	S	S	S	S	S	S	S	S	S		S					S	R		R	
penicillin	S	R	R	S	S	R	S	R	S	S	R	S	S	S	R	S	S	S	S	S	S	S	S	S		R					S	S		S	
methicillin	S	R	R	S	S	R	S	R	S	S	R	S	S	S	R	S	S	S	S	S	S	S	S	S		R					S	S	R	S	
colistin	R	R	R	S	S	S	R	S	R	S	S	S	S	S	R	S	R	R	R	R	S	R	R	S		R					R	R	R	R	
G.L.C. analysis §																																				
acetic	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#		#		#			+	+	#	+	+	
propionic	+	+	⊔	+	+	−	⊔	⊔	⊔	+	−	⊔	+	+	+	⊔	+	−	+	⊔	⊔	+	⊔	−		⊔		−			⊔	+	⊔	−	−	+
iso-butyric	⊔	⊔	−	⊔	−	−	−	−	−	−	⊔	−	−	−	−	−	−	−	−	−	−	−	−	−		−		−			−	−	−	−	−	−
n-butyric	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−		−		−			−	−	−	−	−	−
iso-valeric	+	+	⊔	⊔	⊔	⊔	⊔	−	−	⊔	⊔	−	⊔	⊔	⊔	⊔	⊔	−	⊔	⊔	−	−	−	⊔	⊔	−	−	⊔	−		−	⊔	−	−	−	−
n-valeric	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−		−		−			−	−	−	−	−	−
lactic	#	+	⊔	+	⊔	⊔	⊔	⊔	−	⊔	+	⊔	⊔	⊔	⊔	⊔	⊔	⊔	⊔	⊔	−	−	⊔	−		−		⊔	−		−	−	−	−	−	−
succinic	+	#	+	#	#	+	+	+	#	#	#	#	+	+	#	#	#	+	+	#	#	+	#	#		+		#			+	#	+	#	#	
Reported strain conforming to the pattern	...	WAL 2721	...	VPI 4196	...	...	...	...	ATCC 15930	...	...	...	...	...	...	...	GUI 1011	GUI 1034	VPI 7570A	...	WAL 2724	...	30	...	...	...	...	VPI 5832	...	NP 333	...	...	J1	7CM	VPI 2845	1956C	10 79B	73	2467B
No. of laboratory strains conforming	1 (125)	...	1 (120)	...	2 (63/186)	1 (67)	1 (88)	1 (152)	...	1 (63)	1 (164)	1 (167)	1 (188)	1 (62)	1 (70)	...	...	...	1 (181)	...	1 (140)	...	1 (179)	1 (157)	1 (156)	1 (163)	...	1 (172)	...	1 (61)	1 (144)	...	...	...	...	...	...	...	...
Total no of strains conforming	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1

* + = positive result; − = negative result.

† S = sensitive; R = resistant.

ø I = inhibition; + = growth.

§ see methods.

APPENDIX VII

TABLE 3

Results obtained with I.C.S.B referred strains

Test	WAL 2721	WAL 2724	GUI 1011	GUI 1034	ATCC 15930	VPI 30	VPI 7570A	VPI 5832	J1	7CM	1956C	2467B
growth in air + CO_2	−	−	−	−	−	−	−	−	−	−	+	+
Pigment production	+	+	+	+	+	+	+	±[†]	−	−	−	−
Tolerance tests:												
taurocholate (T)	I	I	I	I	I	I	I	I	I	+	I	I
deoxycholate (D)	I	I	I	I	I	I	I	I	I	+	I	I
T + D	I	I	I	I	I	I	I	I	I	+	I	I
Victoria blue 4R	I	+	I	I	I	I	I	I	+	+	I	+
ethyl violet	I	I	I	I	I	I	I	I	I	+	I	I
gentian violet	I	I	I	I	I	I	I	I	I	I	I	I
brilliant green	I	I	I	I	I	I	I	I	I	I	I	I
Indole production	−	−	−	−	−	−	−	−	−	−	−	−
Fermentation of:												
glucose	+	+	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	+	+	+
sucrose	+	+	+	+	+	+	+	+	+	+	+	+
maltose	+	+	+	+	+	+	+	+	+	+	+	+
trehalose	−	−	−	−	−	−	−	−	−	−	−	−
mannitol	−	−	−	−	−	−	−	−	−	−	−	−
rhamnose	−	−	−	−	−	−	−	+	+	+	−	−
Aesculin hydrolysis	−	−	+	+	+	+	+	+	+	−	−	+
Dextran hydrolysis	−	+	−	−	+	+	−	−	−	−	+	+
gelatin digestion	−	−	+	+	+	−	+	−	−	−	−	−
Lipase production	−	−	−	−	+	+	−	−	−	−	−	−
Haemolysis	+	+	+	+	+	+	+	+	+	+	−	−
H_2S production	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase production	−	−	−	−	−	−	−	−	−	−	−	−
Catalase production	−	−	−	−	−	−	−	−	−	−	−	−
Antibiotic tests:												
neomycin 1000 µg	S	S	S	S	S	R	S	S	S	R	S	S
neomycin 10 µg	R	R	R	R	R	R	R	R	R	R	R	R
kanamycin 1000 µg	R	R	R	R	R	R	R	R	R	R	R	R
kanamycin 30 µg	R	R	R	R	R	R	R	R	R	R	R	R
penicillin 1·5 units	R	S	S	S	S	S	S	S	S	R	S	S
methicillin 10 µg	R	S	S	S	S	S	S	S	S	R	S	S
erythromycin 60 µg	S	S	S	S	S	S	S	S	S	S	S	S
tetracycline 10 µg	R	S	S	S	S	S	S	S	S	S	S	S
colistin 10 µg	R	S	S	S	R	R	S	R	R	R	R	R
rifampicin 15 µg	S	S	S	S	S	S	S	S	S	S	S	S
lincomycin 2 µg	S	S	S	S	S	S	S	S	S	S	S	S
clindamycin 2 µg	S	S	S	S	S	S	S	S	S	S	S	S
bacitracin 0·1 unit	R	R	R	R	R	R	R	R	R	R	R	R
vancomycin 15 µg	R	R	R	R	S	R	R	R	R	R	S	R
chloramphenicol 10 µg	S	S	S	S	S	S	S	S	S	S	S	S
metronidazole 5 µg	S	S	S	S	R	R	S	S	S	S	R	R
G.L.C. analysis												
acetic	++	++	++	++	++	++	++	+	++	+	++	++
propionic	+	+	+	±	±	±	+	−	±	±	±	+
iso-butyric	±	−	−	−	−	−	−	−	−	−	−	−
n-butyric	−	−	−	−	−	−	−	−	−	−	−	−
iso-valeric	+	±	±	±	−	−	±	−	−	−	±	−
n-valeric	−	−	−	−	−	−	−	−	−	−	−	−
lactic	+	±	+	±	−	−	±	−	−	−	±	−
succinic	++	+	++	++	++	++	++	+	++	++	++	++

[†] pale brown after incubation for 7 days.

APPENDIX VIII

TABLE 2a

Results obtained in tolerance tests and antibiotic disk resistance tests

Test	Results obtained with the given strain																							
	WPH 61	NP 333	C12	B 56029	B 38024	WAL 3281	VPI 9958	VPI 8906	WPH 179	1210	1221	WAL 3030	B 56007	B 56020	5540	7880	07073	VPI 6318	VPI 6522	VPI 8057	VPI 7852	B 38080
Tolerance tests:																						
taurocholate (T)	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
deoxycholate (D)	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
T + D	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Victoria blue 4R	+	+	+	I	I	+	+	+/I	I	+/I	+/I	+/I	I	I	I	I	+	+	+	+/I	I	I
ethyl violet	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
gentian violet	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
brilliant green	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Antibiotic disk resistance tests:																						
metronidazole 5 µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
neomycin 1000 µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	RS	S	RS	RS	S	S	R
kanamycin 1000 µg	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
penicillin 1·5–2 units	S	S	R	R	R	S	S	S	S	R	R	S	S	S	R	S	S	S	R	S	R	S
rifampicin 15 µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

+ = growth;
I = inhibition (no growth);
+/I = different results obtained in the two laboratories;
S = sensitive;
R = resistant;
RS = moderately sensitive.

APPENDIX VIII

TABLE 2b

Results obtained in morphological and biochemical tests

Test	Results obtained with the given strain																						
	WPH 61	NP 333	C12	B 56029	B 38024	WAL 3281	VPI 9958	VPI 8906D	WPH 179	1210	1221	WAL 3030	B 56007	B 56020	5540	7880	07073	VPI 6318	VPI 6822	VPI 8057	VPI 7852	B 38080
Pigment production	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−
Haemolysis	+	+	+	−	⊥	−	−	⊥	⊥	⊥	⊥	−	⊥	⊥	+	⊥	⊥	−	−	⊥	⊥	−
H_2S production	+	+	+	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase test	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Catalase test	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Aesculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+
Gelatin digestion	+	+	+	+	+/−	+	+	+	+	+/−	+/−	+	+	+	+	+	+	+	+	+	+	−
Lipase production	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Nitrate reduction	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Indole production	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Bile stimulation	−	−	...	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Dextranase production	+	+	...	+	−	+	−	−	−	−	...	−	+	+	−	−	−	−	−	−	−	−
Fermentation of																						
glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+
maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+
rhamnose	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
trehalose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
mannitol	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
arabinose	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+L
xylose	+	+	+	+	+	−/+L	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+L

\+ = positive result; − = negative result;
+/− = different results obtained by the two laboratories,
⊥ = incomplete haemolysis; or, very poor growth in the bile stimulation test.
+L = late and/or weak positive result

APPENDIX VIII

TABLE 2c

Results of G.L.C. analysis

Product	Results obtained with the given strain																								
	WPH 61	NP 333	C12	B 56029	B 38024	WAL 3281	VPI 9958	VPI 8906D	WPH 179	1210	1221	WAL 3030	B 56007	B 56020	5540	7880	07073	VPI 6318	VPI 6822	VPI 8057	VPI 7852	B 38080
acetate	++	++		+	++	+	+	++	++	+	++	++	+	+	+	++	+	+	+	++	++	+
propionate	tr	+		−	−	tr	tr	+	+	−	−	tr	−	−	−	+	−	−	−	+	tr	tr
iso-butyrate	−	−		−	−	−	−	−	−	−	−	−	−	−	tr	tr	tr	−	−	−	−	−
n-butyrate	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
iso-valerate	−	tr		−	−	−	−	tr	−	tr	tr	−	tr	−	tr	tr	+	tr	tr	tr	tr	−
lactate	−	tr		−	−	tr	−	tr	tr	tr	tr	tr	−	−	−	−	−	−	−	−	−	−
succinate	++	++		++	+	++	+	++	+	++	++	++	++	++	++	++	++	++	++	++	++	++

APPENDIX VIII

TABLE 3

Results obtained with strain B38080 (Sheffield)

Test	Result	Test	Result
Tolerance tests.		Pigment production	−
taurocholate (T)	+	Haemolysis	−
deoxycholate (D)	I	H_2S production	+
T + D	+	Oxidase test	−
Victoria blue 4R	+	Catalase test	−
ethyl violet	+	Aesculin hydrolysis	+w
gentian violet	I	Gelatin digestion	+
brilliant-green	I	Lipase production	−
		Nitrate reduction	...
Antibiotic disk		Indole production	−
resistance tests		Bile stimulation	...
metronidazole 5 µg	S	Dextranase production	...
neomycin 1000 µg	R		
kanamycin 1000 µg	R	Fermentation of:	
penicillin 1·5 iu	R	glucose	+
rifampicin 15 µg	S	lactose	+
		maltose	+
		sucrose	+
		rhamnose	+
		trehalose	−
		mannitol	−
		arabinose	+
		xylose	+

APPENDIX VIII

TABLE 4

Results obtained with strains VPI 5832 and J1 in previous study (see Report, 1976)

Test	Results with VPI 5832	Results with J1
Tolerance tests:		
taurocholate (T)	I	I
deoxycholate (d)	I	I
T + D	I	I
Victoria blue 4R	I	+
ethyl violet	I	I
gentian violet	I	I
brilliant green	I	I
Antibiotic disk resistance tests:		
metronidazole 5 µg	S	S
neomycin 1000 µg	S	S
kanamycin 1000 µg	R	R
penicillin 1.5 units	S	S
rifampicin 15 µg	S	S
G.L.C. profiles:		
acetate	+	++
propionate	−	tr
iso-butyrate	−	−
n-butyrate	−	−
iso-valerate	−	−
n-valerate	−	−
lactate	−	−
succinate	+	++

Test	Results with VPI 5832	Results with J1
Pigment production	−†	−
Haemolysis	+	+
H_2S production	+	+
Oxidase test	−	−
Catalase test	−	−
Aesculin hydrolysis	+	+
Gelatin digestion	−	−
Lipase production	−	−
Nitrate reduction	−	−
Indole production	−	−
Bile stimulation	...	...
Dextranase production	−	−
Fermentation of:		
glucose	+	+
lactose	+	+
maltose	+	+
sucrose	+	+
rhamnose	+	+
trehalose	−	−
mannitol	−	−
arabinose	−	+
xylose	−	+

† pale brown after 7 days