A COMPARATIVE ANALYSIS OF THE LACTOSE SYSTEMS IN DIFFERENT ENTEROBACTERIACEAE

BY

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I hereby declare that this thesis has been composed by myself and that the work described herein was carried out by myself, except where otherwise stated in the acknowledgements.

SUMMARY

The plasmid and chromosomal *lac* systems from *Klebsiella* were compared with *lac* systems from other enteric species and the implications concerning *lac* gene evolution within the family *Enterobacteriaceae* are discussed.

The chromosomal *lac* system from *Klebsiella* M5a1 was cloned into pBR322, and analysed by restriction mapping and Tn5 and Tn1725 mediated mutagenesis, which indicated that the cloned *lac* genes were organised similarly to those in the *E. coli lac* operon. No common restriction sites were shared by the *Klebsiella* M5a1 *lac* region and the *E. coli lac* operon, and only limited common sites existed with *Klebsiella* 17R1 *lac* operon.

F_K*lac* DNA was isolated from *Klebsiella* V9A, and the molecular weight of the plasmid was estimated as approximately 120Md. The *lac* system on F_K*lac* was cloned into pBR322, and underwent restriction analysis.

Gene expression products coded by the cloned *lac* systems were studied. The β-galactosidase subunits coded by both the cloned *lac* systems were found to be very similar to the *E. coli lac Z* protein, with regards to molecular weight, which possibly indicated a relatively close evolutionary relationship between all three *lac* systems. This was further supported by the observation that the growth inhibitory effect on *E. coli* of elevated levels of *Klebsiella* chromosomal *lac Y* gene protein were similar to that previously reported for the *E. coli lac Y* gene protein.

However, in six enteric species representing 5 genera, the *lac A* protein, thiogalactoside transacetylase, was only detected in *E. coli*, which suggested that the *E. coli lac* operon differed significantly from other enteric *lac* systems.

The extent of DNA homology between the cloned chromosomal *lac* region from *Klebsiella* M5a1 and the equivalent regions in 11 enteric species, representing 9 genera, and on F_K*lac*, was analysed and produced a range extending from 0% to 78%.

Overall the results obtained were not totally consistent with the hypothesis that *E. coli* had acquired its *lac* operon by horizontal transfer from outwith the *Enterobacteriaceae*.

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CHAPTER 1

INTRODUCTION

The Family Enterobacteriaceae consists of bacteria which in general possess similar specific properties and to quote Brenner (1981) "may be defined as Gram-negative, oxidase-negative, asporogenous, non-acid-fast, rod-shaped bacteria. They are motile by peritrichous flagellae or are non motile; grow both aerobically and anaerobically; grow well on artificial media; produce acid and often gas fermentatively from glucose, other carbohydrates, and related compounds; are catalase positive except for Shigella dysenteriae 1; and reduce nitrates to nitrites except for some species of Erwinia".

The bacterium Escherichia coli (E. coli), like several other members of the Enterobacteriaceae, possesses the ability to use the sugar lactose [O-β-D-galactopyranosyl-(1→4)-β-D glucopyranoside] as its sole source of carbon. E. coli synthesises two proteins, a galactoside permease and a β-galactosidase which play an essential part in lactose metabolism. The former protein is involved in the transport of the disaccharide lactose into the bacterium, and the latter in the conversion of lactose to its individual monosaccharide components, D-galactose and D-glucose, and also in the conversion of lactose to allolactose [O-β-D-galactopyranosyl-(1→6)-β-D glucopyranoside]. A third protein, a thiogalactoside transacetylase, is also synthesised in conjunction with the galactoside permease and β-galactosidase, but its role in lactose metabolism remains uncertain. The synthesis of these proteins is greatly stimulated, or induced, by the presence of lactose or certain other galactosides in the media. This induction is transient and is dependent upon the continued presence of the specific galactosides.

The genes which code for the three proteins described above and for the protein involved in the regulation of their synthesis (repressor protein) are closely linked in

a small region of the E. coli chromosome. The genes coding for β-galactosidase, galactoside permease and thiogalactoside transacetylase are referred to as Z, Y and A respectively and are separated from the gene for the repressor protein (I gene) by a short section of DNA within which are contained a promoter (P) and operator (O) region. Transcription of the Z, Y and A genes is initiated from the site of the promoter and the product is a single polycistronic messenger RNA species. However, by interacting with the DNA in the operator region, the repressor protein can inhibit transcription of the Z, Y and A genes and thus synthesis of the respective protein products. Because the Z, Y and A genes are transcribed as a single unit from a single site, they constitute an operon.

The lactose (lac) operon is an example of bacterial acquisition, via natural selection, of the ability to use available nutrients as efficiently as possible and it also provides a capacity to respond rapidly to environmental changes. In addition, β-galactoside transport in E. coli has been,and continues to serve,as a model for studies of active solute uptake.

These features, combined with certain convenient aspects of the chemistry, physiology and genetics of lactose metabolism in E. coli have led to the position whereby the E. coli lac operon represents one of the most intensively studied gene clusters in bacterial genetics. A detailed account of the work which has led to the present understanding of the E. coli lac operon and which is relevant to this thesis is presented below.

1.1 Structural Proteins coded by the *E. coli* *lac* Operon

1.1.1 β-D-Galactoside Galactohydrolase [E.C.3.2.1.23](β-Galactosidase)

1.1.1.1 Correlation Between *lac Z* gene and β-Galactosidase

There was convincing evidence which suggested that the *lac Z* gene was the structural locus for β-galactosidase. Recombination studies using Hfr strains had indicated that the total length of the gene which specified the structure of β-galactosidase was approximately 0.7 map units or 3500 nucleotides in size (Jacob and Monod, 1961), and thus allowed for a polypeptide of approximately 1170 amino acid residues. Both the colinearity and orientation of the protein to its structural gene were demonstrated succinctly by Fowler and Zabin (1966). The authors used extracts from four strains each of which contained a different *lac Z* nonsense mutation. The sites of the nonsense mutations had previously been established (Newton, Beckwith, Zipser and Brenner, 1965), and their protein products had been shown to display immunological activity against anti-β-galactosidase (Fowler and Zabin, 1966). The molecular weight of the immunologically active component from each strain was obtained by sucrose density gradient centrifugation of crude extracts. A direct correlation was obtained between the molecular weight of the immunologically active component and the estimated length of the *lac Z* gene prior to the nonsense mutation, demonstrating that the length of a polypeptide was determined by the effective length of its structural gene. Based on the fact that polypeptide chain synthesis is from the amino to carboxyl terminal (Dintzis, 1961) the results also showed an increase in size in the mutant polypeptide from the operator proximal amino to operator distal carboxyl terminal which was in the same orientation as the mapped positions of the nonsense mutations.

1.1.1.2 Physical Properties

β-galactosidase was isolated in a pure state from E. coli K12 and ML strains following ammonium sulphate precipitation and DEAE cellulose or DEAE sephadex column procedures (Hu et al., 1959; Karlson et al., 1964; Craven et al., 1965). Enzyme samples from both sources were identical with respect to their physical properties: e.g. both samples displayed an apparent molecular weight of 540,000 when subjected to sedimentation equilibrium studies (Craven et al., 1965). The tetrameric nature of the enzyme was indicated by Karlson et al. (1964), whose work involved electron microscopy of crystalline β-galactoside. Data which supported the tetrameric structure was provided by Ullmannet al. (1968), who showed that β-galactosidase completely dissociated in 6M guanidine to yield a single subunit with a molecular weight of 135,000. This figure was in good agreement with the molecular weight of 118,000 previously reported by Wallenfels et al. (1963) and Shifrin and Steers (1967). The observation that the gradual removal of urea from a suspension of dissociated β-galactosidase subunits allowed the formation of active enzyme, was the basis for the work by Zipser (1963) who mixed dissociated radioactive enzyme with a large excess of dissociated enzyme labelled with heavy isotopes. The large excess of enzyme with heavy isotope ensured that the most probable hybrid enzyme to be formed would contain only 1 radioactive subunit. The density of the active hybrid-enzyme formed was compared to that of the two parental enzymes by density centrifugation, and was found to be a quarter of the difference between the densities of the two parental enzymes. This indicated that the subunits which reassociated were a quarter of the size of the active enzyme.

The E. coli β-galactosidase subunit amino acid sequence of 1021 residues was established by Fowler and Zabin (1977, 1978), however this has recently been corrected to 1023

residues following the nucleotide sequencing of the E. coli lac Z by Kalnins et al. (1983). The amino acid sequence deduced from the DNA sequence of Kalnins et al. (1983), also differed from that reported by Fowler and Zabin (1977, 1978) in eight other amino acid residues within the polypeptide chain. Based on these findings the active enzyme tetramer would have a molecular weight of 465,412 considerably smaller than that reported by earlier workers. This discrepancy may have been caused by small amounts of heavier material contaminating the enzyme preparations used in the earlier work mentioned above (Wallenfels and Weil, 1972). Contaxis and Reithel (1974) have reported a "260 nm absorbing compound" bound to β-galactosidase which they suggested acted to stabilise the tetrameric form, but no further work appears to have been undertaken in the analysis of the "260 nm absorbing compound" and its possible effect on β-galactosidase molecular weight determination.

Aggregates larger than the tetramer have been reported for β-galactosidase in extracts from wild-type strains of E. coli (Appel et al., 1965), and at least 30% of the enzyme isolated from the hyper producing strain A 324-5 used by Fowler (1972) was found to be in higher aggregates. Some work has suggested that the tetramer and higher aggregates were the only active forms of the enzyme. Contaxis and Reithel (1971) indicated that in 90% glycerol the monomeric form was inactive, whereas removal of the glycerol allowed the tetramer to reform and regain activity. The protein products from a missense mutant, and an M15-deletion strain which contained a deletion in the operator proximal portion of the lac Z, were dimers and inactive (Steers and Shifrin, 1967; Langley et al., 1975b). Following α complementation between an M15-deletion strain extract and a peptide fragment produced by cyanogen bromide digestion of the wild type enzyme, an active enzyme was formed (Lin et al., 1970). The addition of the "CB2" peptide, which was derived from

residues 3-92 (Langley et al., 1975a), caused a conformational change in the M15 protein's dimeric structure, and allowed the formation of an active tetrameric structure (Langley and Zabin, 1976). However, it should be noted that Kaneshiro et al. (1975) have reported an active dimer form in the presence of Ag^{+} and, therefore some uncertainty remains concerning which forms of the enzyme are active or inactive.

1.1.1.3 Reaction Mechanism

Acting in its capacity as a hydrolase, β-galactosidase cleaved the bond between the anomeric carbon (c1) and the glycosyl oxygen (Wallenfels and Malhotra, 1961; Wallenfels and Weil, 1972), as shown in Figure 1.1. The β-D-galactopyranoside configuration was necessary for enzyme function, however the moiety attached to the galactopyranoside could be either a sugar, an aryl group, or an alkyl group without affecting enzyme activity excessively.

β-galactosidase also acted as a transferase in which the galactoside moiety could be transferred to monosaccharides, oligosaccharides, alkyl alcohols or phenols. This function was demonstrated in the conversion of lactose to allolactose, which involved the transfer of the galactosyl moiety from the c4 to c6 position of the glucopyranoside (Figure 1.2). Huber et al. (1976) has demonstrated that at low concentrations of lactose the rearrangement of lactose to allolactose was achieved without release of glucose. Allolactose has a lower Km than lactose, 6.3×10^{-4}M compared with 5.5×10^{-3}M (Huber et al., 1975) and therefore appears to be a better substrate for β-galactosidase.

Figure 1.1. Lactose (O-β-D galactopyranosyl-(1-4)-β-D-glucopyranoside) and the site of action of the enzyme β-galactosidase and products.

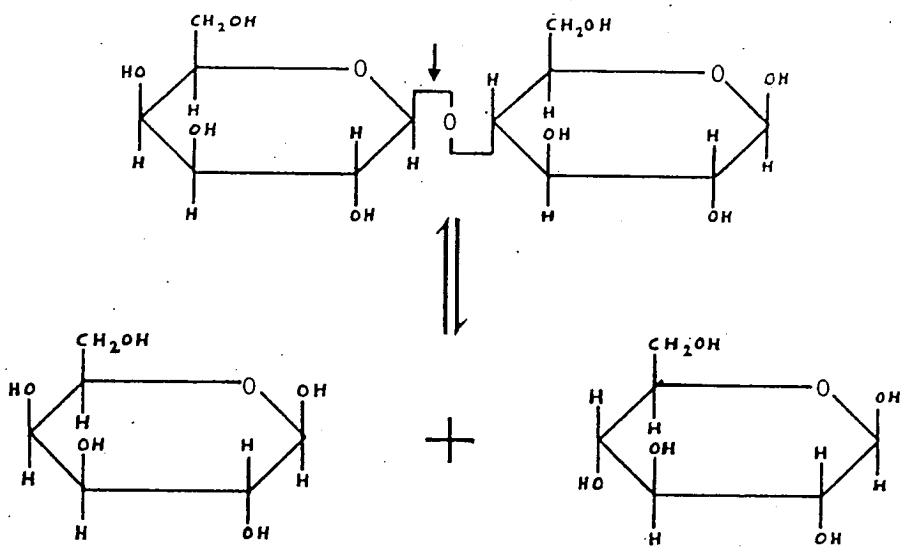
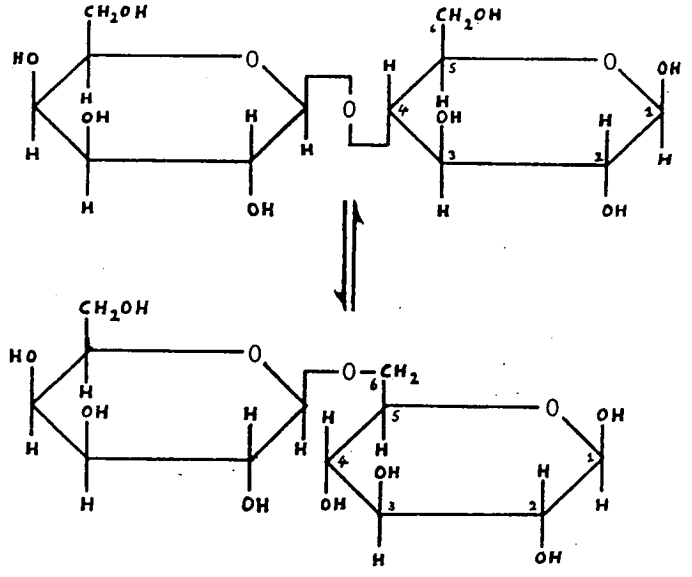


Figure 1.2. The transfer of the galactosyl moiety from C_4 to C_6 on the glucopyranoside molecule catalysed by β-galactosidase.



1.1.1.4 Evolution of the *lac Z* Gene

The evolutionary origin of the *lac Z* gene in *E. coli* has been speculated upon following the publication of the amino acid sequence data of Fowler and Zabin (1977, 1978). The sequence data was analysed using a computer programmed to search for duplications of a given number of amino acids, within the β-galactosidase subunit amino acid sequence, (Hood *et al.*, 1978). The results led the authors to suggest that there was a "highly significant degree of similarity between segments 1-379 and 398-781" from which they concluded that these two segments had evolved by gene duplication. However, the amino acid sequence derived from the nucleotide sequence of the *E. coli lac Z* gene (Kalnins *et al.*, 1983) indicated several discrepancies in the amino acid sequence data of Fowler and Zabin (1977, 1978). When the corrected sequence was analysed by Kalnins and coworkers, five of the internal homologies postulated by Hood *et al.* (1978) were no longer present. This led Kalnins *et al.* (1983) to suggest that the argument of Hood *et al.* (1978) was somewhat weakened.

1.1.1.5 The Second Chromosomal β-Galactosidase Gene in *E. coli*

With respect to the *E. coli lac Z* gene it should be noted that the β-galactosidase coded by this gene was not unique in *E. coli*. The experiments of Campbell *et al.* (1973) and Hall and Hartl (1974) were directed towards the selection, in the laboratory, of a second β-galactosidase gene on the *E. coli* chromosome. Both groups used *lac Z*-deletion *E.coli* strains that produced functional lactose permease at elevated levels and selected for lactose utilisation on indicator plates. The gene which coded for this second chromosomal β-galactosidase was designated *ebg* (evolved β-galactosidase) and following conjugation and transduction

experiments it was mapped on the E. coli chromosome at 65 minutes (Arraj and Campbell, 1975).

Hartl and Hall (1974) showed that the E. coli lac Z-deletion strain used by Hall and Hartl (1974) hydrolysed ONPG, an activity which was inducible by the addition of lactose, but not by IPTG, and displayed virtually no lactase activity. However following the work of Campbell et al. (1973) and Hall and Hartl (1974) described above, E. coli lac Z-deletion strains which possessed lactase activity were isolated.

Hall and Hartl (1975) showed that a regulatory mutation which gave rise to constitutive synthesis of the Ebg^+ enzyme, resided in a linked regulatory gene ebg R.

Recently Hall (1982) has reported Ebg mutants which can synthesise allolactose from lactose.

1.1.2 Lactose Permease

1.1.2.1 General Background

The second gene in the lac operon, lac Y, coded for a protein which was involved in the accumulation of β-galactosides particularly lactose, against a concentration gradient. The presence of such a system in E. coli was indicated by Deere et al. (1939) who described a strain of E. coli which did not ferment lactose, although lactase (β-galactosidase) was present in dried preparations of the cells. It was not until the 1950's that the basis for this observation became clear, when β-galactoside uptake studies were performed on E. coli strains with different lac^- mutations (Cohen and Rickenberg, 1955; Rickenberg et al., 1956). These authors found that unlike wild type E. coli strains which, on induction, gave elevated levels of β-galactosidase activity accompanied by increased β-galactoside transport, the mutants gave a different picture. In some mutants β-galactosidase was produced but no galactoside transport was observed, in

others lactose was transported but accumulated in the cells, due to lac Z mutations. This suggested that β-galactoside transport was an integral part of the lactose utilisation system in E. coli, and that a component of the transport system was coded by a gene (lac Y) at a locus distinct from lac Z.

The specificity of the accumulation process was shown by Pardee (1957) to be partially overlapping in regard to the substrate transported because melibiose, an α-galactoside, could also be accumulated by the lactose transport system described by Rickenberg et al. (1956). Four other transport systems for galactose and α- and β-galactosides have been reported in E. coli, three of which displayed partially overlapping specificities. These included the melibiose permease, whose principal substrate was the α-galactoside melibiose (Prestidge and Pardee, 1965); the methyl-galactoside permease (Rotman, 1959), which was involved with D-galactose and methyl-1-β-D-galactopyranoside transport; the galactose permease, which has been shown to be involved in the transport of D-fucose, D-galactose and L-arabinose (Rotman et al., 1968); and lastly, a second lactose permease (Hobson, 1978), which will be discussed later.

An interesting aspect of the melibiose permease in E. coli K12 was that at temperatures above 37°C, transport of the α-galactoside by this system was inhibited (Buttin, 1968), however melibiose may still enter the bacterium via the lactose permease (Pardee, 1957). Thus growth of E. coli K12 on minimal medium plus melibiose at 40°C provided a convenient indication of the presence or absence of a functional lactose permease, and by inference an intact lac Y gene.

1.1.2.2 Correlation Between *lac Y* gene and Lactose Permease Protein

E. coli strains which contained mutations that affected galactoside uptake and which had invariably also lost acetyl transferase activity but not β-galactosidase activity were isolated. The loci corresponding to this mutant type were mapped by the Hfr mediated recombination studies to a position adjacent to the *lac Z* gene, called the *lac Y* gene (Jacob and Monod, 1961).

The *lac Y* gene product was demonstrated to be a membrane bound protein by Fox and Kennedy (1965). These authors based their work on two sets of observations. Firstly the work of Rickenberg *et al.* (1956) and Herzenberg (1959) had indicated that orthonitrophenyl-β-D-galactoside (ONPG) hydrolysis by β-galactosidase in intact energy-poisoned cells was a measure of lactose permease transport activity, because ONPG was a substrate of the transport system. Secondly, Cohen and Monod (1957) had demonstrated the sensitivity of the lactose permease transport system to sulphydryl poisons. Fox and colleagues therefore showed that under certain conditions ONPG hydrolysis, and thus ONPG transport, could be completely blocked by N-ethylmaleimide (NEM), a sulphydryl poison which bound irreversibly to protein, without affecting β-galactosidase or acetyl transacetylase. The NEM-mediated inhibition of ONPG hydrolysis could be prevented by the addition to the cells of β-D-galactosyl-1-thio-β-D-galactoside (TDG) which also bound to the NEM-sensitive protein, the lactose permease, TDG binding being reversible however. Cells were, therefore, treated with TDG to saturation, thus protecting the NEM-sensitive component of the lactose permease system; NEM was then added and bound to the proteins to which TDG had not bound. The TDG and NEM were washed from the media, during which the TDG was also removed from the cells due to the reversible nature of the TDG binding, and the cells

incubated with radioactive NEM. Induced and uninduced cultures were treated this way and differentially labelled with either ^{3}H or ^{14}C NEM. The results showed that the TDG binding protein necessary for the transport system was localised in the particulate membrane-containing fraction of the cell.

Using cell-free fractions of E. coli, Fox et al. (1967) showed that only lac Y^{+} strains displayed similar binding and inhibition characteristics by NEM and TDG as those described above. The NEM labelling technique was also used with an E. coli mutant which was temperature sensitive for lactose transport. Fox et al. (1967) showed that TDG binding displayed temperature sensitivity which paralleled that displayed by the lactose transport system in the mutant E.coli strain. It was also demonstrated that the results were not due simply to a host strain effect, because similar temperature sensitive TDG binding was observed when the lac genes from the temperature sensitive strain were transferred into a lac-deletion E. coli strain. The above results firmly supported the hypothesis that the membrane bound protein involved in lactose transport was coded by the lac Y gene.

1.1.2.3 Physical Properties

Partial purification of the lactose permease was initially achieved by Jones and Kennedy (1969), who used radioactive N-ethyl [^{14}C] maleimide to specifically label the membrane protein, in a similar manner to that described above. The molecular weight of the partially purified material was established, by SDS polyacrylamide gel electrophoresis, as 31,000. More recently Teather et al. (1978) cloned the E. coli K12 lac Y gene in a multicopy plasmid vector, and established a molecular weight for the protein product (again by SDS-PAGE) of 30,000; in good agreement with that previously reported by Jones and Kennedy (1969).

However, Büchel et al. (1980) established the nucleotide sequence of the E. coli K12 lac Y gene, which was shown to consist of 1251 nucleotides. The predicted primary translation product consisted of 417 amino acid residues resulting in a molecular weight of 46,504, a figure considerably higher than those previously derived by SDS polyacrylamide gel electrophoresis.

Could the difference have been due to post-translational processing? Most secreted proteins, and some integral membrane proteins, in both bacterial and eukaryotic cells, have been shown to be initially synthesised as precursors with hydrophobic amino acid terminal extensions which were then removed by proteolytic cleavage (Sabatini et al., 1982; Blobel, 1980; Kreil, 1981; Wickner, 1979; Inouye and Halegona, 1979; Emr et al., 1980).

On the basis of recent work, it appeared that processing did not take place. Analysis of the in vitro and in vivo products of the E. coli lac permease gene, by Ehring et al. (1980) indicated that the protein products had identical apparent molecular weights when estimated by SDS-PAGE, (30,000 ± 1,000). Also a comparison of the amino acid sequence predicted from the DNA sequence with the partially sequenced N-terminal section of the in vitro and in vivo products of the lac Y gene showed that the initial 15 amino acids were identical. This strongly suggested that there was no processing at the N-terminus.

Ehring et al. (1980) claimed that C-terminus processing was unlikely based on the "good agreement" between the amino acid composition of the lactose permease protein and the predicted figures based on the DNA sequence (Büchel et al., 1980). The amino acid composition of the purified lactose permease protein established by Newman et al. (1981) also supported this claim; however, until the C-terminus amino acid sequence of the in vivo protein has been established the uncertainty regarding processing of the lactose permease protein will remain.

The discrepancy between the predicted molecular weight and apparent molecular weights achieved by SDS-PAGE was shown to be due to an abnormal electrophoretic behaviour on the part of the lactose permease protein (Beyreuther et al., 1980; Padam et al., 1983). The authors showed that the apparent molecular weight of both the in vivo and in vitro products of the lac Y gene could be identically increased by increasing the percentage of acrylamide in the gels and at 20% acrylamide, achieved a molecular weight in good agreement with that based on the DNA sequence. It was suggested that due to the high hydrophobic nature of the protein, it may have bound more SDS than would be expected from the molecular weight, with the consequence of an increased charge to mass ratio displayed by the lac permease-SDS complex. Thus the correct molecular weight may only be established if the molecular sieving component of the gel predominates over the increased charge to mass ratio.

1.1.2.4 Mechanism of Lactose Transport in E. coli

A number of major features demonstrated by the lactose transport system in E. coli led the way to the presently accepted mechanism of lactose transport. Firstly the transport system displayed substrate specificity. In their early studies Rickenberg et al. (1956) compared the affinity of E. coli cells for labelled methyl-β-D-thiogalactoside (TMG) to that displayed by an enzyme for its substrate, and noted that competitive inhibition could take place if unlabelled TMG was added. Later, galactoside binding was shown to be specific for each substrate, each with their individual binding constants (Kennedy et al., 1974). The results suggested that substrate binding, to a limited number of specific sites, was an essential step in the lactose transport system.

Secondly, there appeared to be an energy requirement

for lactose transport. Dinitrophenol, an uncoupler of oxidative phosphorylation, had been shown by Cohen and Rickenberg (1955) to be an effective inhibitor of galactoside accumulation.

Thirdly, although many sugars, including glucose, were transported into the bacterium by the phosphotransferase system (reviewed by Roseman, 1969; Boos, 1974; Cordaro, 1976), Kepes (1960) and Winkler and Wilson (1966) had shown that substrates of the lactose transport system were not phosphorylated. Thus a transport system existed which was specific for the substrate transported, required energy and did not involve chemical modification of the substrate transported. Several theories were put forward to explain the galactoside accumulation. Scarborough et al. (1968) suggested that a high energy compound (e.g. ATP) would interact with the carrier at the outer side of the membrane and increase the affinity of the carrier for the sugar. Schachter and Mindlin (1969), however, proposed that a carrier-sugar complex would interact with the high energy compound on the inside of the membrane and lower the affinity of the carrier for the sugar. According to these hypotheses the action of dinitrophenol would be to decrease levels of the high energy compound within the cell, and consequently any accumulated galactosides would be lost from the cells. However, Pavlasova and Harold (1969) showed that whilst uncoupling agents could abolish TMG accumulation they had little effect on the intracellular levels of ATP, therefore discounting ATP as the high energy compound immediately responsible for lactose accumulation.

An alternative explanation was suggested by Mitchell, based on his hypothesis that the uptake of many solutes would be mediated by specific polypeptide carriers which coupled the uphill translocation of substrate across the cytoplasmic membrane to the simultaneous downhill movement of a cation such as H^+ or Na^+ (Mitchell, 1963; Mitchell,

1970). Thus the concentration gradients of many substrates across the cell membrane would be maintained as a consequence of an electro-chemical ion gradient generated by the action of various cation motive pumps, e.g. membrane bound respiratory chains. Therefore, the primary action of the uncoupling agents such as dinitrophenol would be to promote the passage of H^+ ions through the membrane, which would interfere with the maintenance of the electrochemical potential across the membrane.

A number of experimental observations supported the Mitchell hypothesis in respect of lactose transport. West (1970) and West and Mitchell (1973) reported that in anaerobic, non-metabolising E. coli, lactose influx was correlated with a simultaneous inflow of H^+, the initial rates of which were essentially the same, suggesting a coupled system with 1:1 stoicheiometry. Related to this was the finding that the lactose permease contains a single galactoside binding site per polypeptide chain both in cell membranes, and in detergent micelles (Overath et al., 1979; Teather et al., 1980). Recently purified E. coli lactose permease protein has been reconstituted with phospholipid vesicles from E. coli to form proteoliposomes. Artificial ion gradients were imposed across the "membrane" of these proteoliposomes and lactose uptake was observed (Newman and Wilson, 1980; Newman et al., 1981; Foster et al., 1982). The latter two reports showed that transport was not observed either in the presence of the proton ionophore carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) or in the absence of the purified lactose permease. It was also shown that there was a transient alkalinisation of the medium upon lactose uptake by the proteoliposomes (Foster et al., 1982). Together these results supported the prediction of Mitchell's hypothesis, that the prerequisites necessary for lactose transport were a carrier (the lactose permease) with a specific binding site for the substrate and an intact

membrane structure across which an electro-chemical potential gradient could be formed and maintained.

A diagramatic representation of the presently accepted mechanism of lactose transport into the cell by chemiosmotic coupling is shown in Figure 1.3 (Overath and Wright, 1983). The electro-chemical potential gradient across the membrane is believed to be maintained by electron transport respiratory chains in the cell membrane. In order for this to occur it is necessary for the electron-carrying proteins of the electron transport respiratory chains to be positioned in the membrane in a specific vectorial arrangement, whereby the H^+ absorbing reactions take place on the inner side of the membrane and the H^+ yielding reactions occur on the outer face of the membrane. Thus the electron carriers of the respiratory chain serve as "pumps" which transport H^+ ions across the membrane from the inside to the outside, and hence create the electro-chemical gradient.

The membrane-spanning protein, in this case lactose permease, has two binding sites, one for the galactoside (lactose) and one for the transported proton. The carrier, lactose permease, can exist in two conformational states, either with the binding sites on the exterior of the membrane, or with them on the interior. In phases 1 and 2 the carrier has the conformation state in which the binding sites are on the exterior of the membrane and transport is initiated by the binding of lactose and H^+ to the carrier. A conformational change (phase 3) reorientates the binding sites to the inside where the lactose and proton can dissociate. Finally, the empty binding sites are reorientated from the inside to the outside by a second change in conformation. Lactose may also be transported from inside to outside in this coupled fashion, because the steps in the system are reversible.

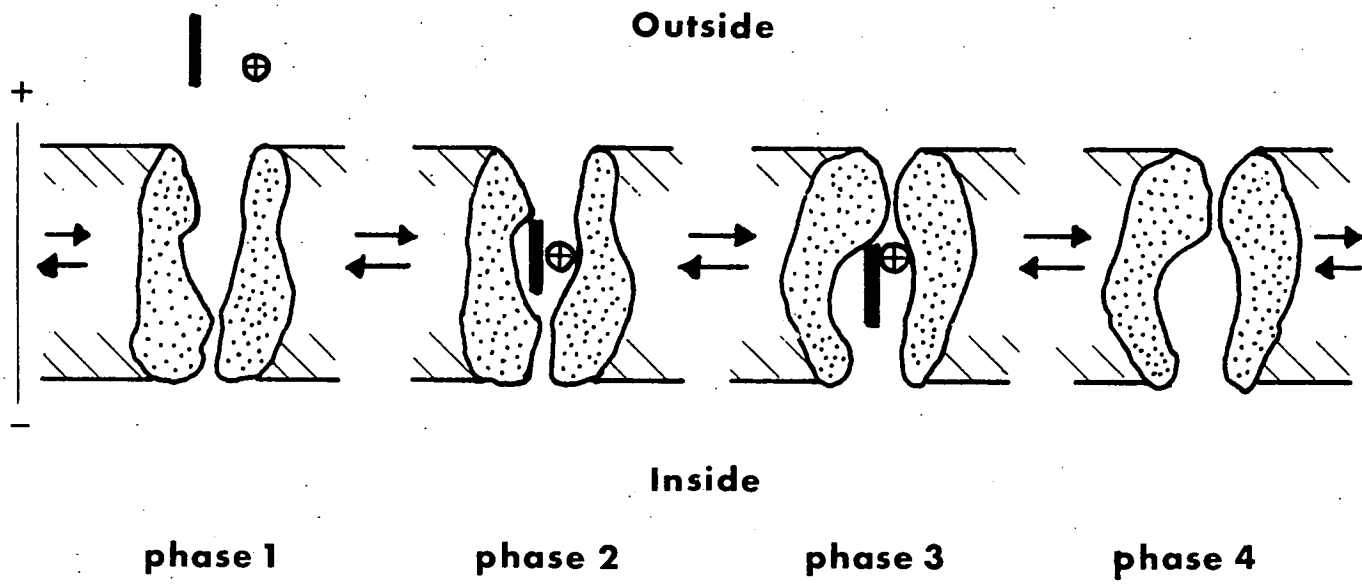


Figure 1·3

Diagramatic representation of lactose:H^+ ion transport by the lactose carrier.

▌: lactose molecule, ⊕: H^+ ion, ▒: lactose carrier, $\overset{+}{|}_{-}$: electrochemical potential gradient across the membrane.

Taken from Overath and Wright, 1983.

1.1.2.5 A Second Chromosomal Lactose Permease Gene in *E. coli*

Hobson (1978) has isolated an *E. coli* mutant which, although initially defective for lactose uptake due to a mutation in the *lac Y* gene, has regained a lactose permease activity. The author showed this was not due to reversion to wild type by the *lac Y* gene, and that it did not represent the selection of an arabinose permease over-producing mutation, shown previously to transport lactose (Messer, 1974), as the new lactose permease mapped at a different position on the *E. coli* chromosome: between 43 and 53 minutes.

Hall (1976) had suggested that the *ebg* and *lac Z* genes may have been derived from a common ancestral gene, perhaps by a duplication of the *E. coli* chromosome followed by divergence, in which case a gene related to *lac Y* might be expected to exist in the *ebg* region, which could mutate to allow lactose uptake. However, the map position of the second lactose permease described by Hobson (1978) did not coincide with that of *ebg*.

1.1.3 Acetyl-CoA: Galactoside-6-O-Acetyl Transferase [E.C.2.3.1.18] (Thiogalactoside Transacetylase)

1.1.3.1 Correlation Between the *lac A* gene and Thiogalactoside Transacetylase

Fox *et al*. (1966) isolated several *E. coli* strains with deletions in the *lac* operon. It was shown by enzyme analysis of β-galactosidase, lactose permease and thiogalactoside transacetylase activity that the Lac^- strains isolated by these authors, produced β-galactosidase, but not lactose permease or thiogalactoside transacetylase. The Lac^+ deletion strains were shown to produce β-galactosidase and lactose permease, but not thiogalactoside transacetylase. The deletions which led only to the loss of thiogalactoside transacetylase were mapped to a region, the *lac A* gene,

which was adjacent to and downstream from the lac Y gene in the lac operon.

1.1.3.2 Physical Properties

Zabin (1963) succeeded in obtaining the purified enzyme by ammonium sulphate fractionation and chromatographic separation and established the amino acid composition as being 268 amino acid residues, corresponding to a minimum molecular weight of 32,000. The molecular weight of the native enzyme was established, using sedimentation equilibrium studies, as 65,300 ± 4,400 (Goldwasser, 1963). Similar studies using 6M guanidine/0.5% mercaptoethanol showed the existence of a single protein subunit species with a molecular weight of 29,700 ± 200 (Brown, Brown and Zabin, 1967). The findings of Brown, Koorajian and Zabin (1967), that heterogeneity existed at the amino terminus of the otherwise identical subunit chains was later attributed to the loss of some of the amino terminal amino acids during the experimental procedures prior to the analysis (Zabin and Fowler, 1980).

More recently, Fried (1980) has established the apparent molecular weights of the thiogalactoside transacetylase subunits from E. coli K12 and ML strains as being 24,800 by SDS polyacrylamide gel electrophoresis, using a range of acrylamide concentrations. The native enzyme molecular weight was estimated to be 48,900 ± 3% using high speed equilibrium centrifugation. The author attributed the variation between these figures and those reported by Zabin (1963) and Brown, Brown and Zabin (1967) to the increased accuracy in the analytical procedures employed in the more recent work.

In conclusion, the results implied that the thiogalactoside transacetylase enzyme consisted of a dimer composed of two identical subunits, each with a molecular weight of 24,800.

1.1.3.3 Reaction Mechanism

The presence and activity of the thiogalactoside transacetylase enzyme in *E. coli* cells was hinted at by Rickenberg *et al.* (1956) who noted that after prolonged incubation of *E. coli* cells with an exogenous carbon source, plus radioactive TMG, a derivative of the radioactive TMG was formed. Zabin *et al.* (1959; 1962) showed that in crude extracts of *E. coli* an enzyme catalysed the acetylation of TMG and certain other galactosides, the product of which was identified by Herzenberg (1961) as a 6-O-acetyl galactoside derivative. A diagrammatic representation of the reaction is shown in Figure 1.4. Musso and Zabin (1973) showed that substrate specificity was not solely confined to thiogalactosides and of those compounds tested, the highest rate of acetylation was observed with p-nitrophenyl-β-D-galactoside.

It had been observed that when β-galactosidase activity in exponentially growing *E. coli* cells was stimulated by the addition of various inducers (e.g. IPTG) a corresponding increase also occurred in thiogalactoside transacetylase activity. This coordinate expression was observed irrespective of the efficiency of the inducer used (Jacob and Monod, 1961). Zabin *et al.* (1962) had shown that thiogalactoside transacetylase was the product of the *lac A* gene, however the work of Herzenberg (1961) had indicated that the 6-O-acetyl derivative produced by the enzyme's activity was neither an inducer of the *lac* operon nor a substrate for the lactose permease and that the derivative diffused passively out of cells. There was, therefore, uncertainty as to the enzyme's role in lactose metabolism.

1.1.3.4 Physiological Role of Thiogalactoside Transacetylase in *E. coli*

Fox *et al.* (1966) constructed mutants of *E. coli* (phenotype $Z^+Y^+A^-$) in which the distal end of the *lac A* gene was deleted and which would also have eliminated any other *lac* genes

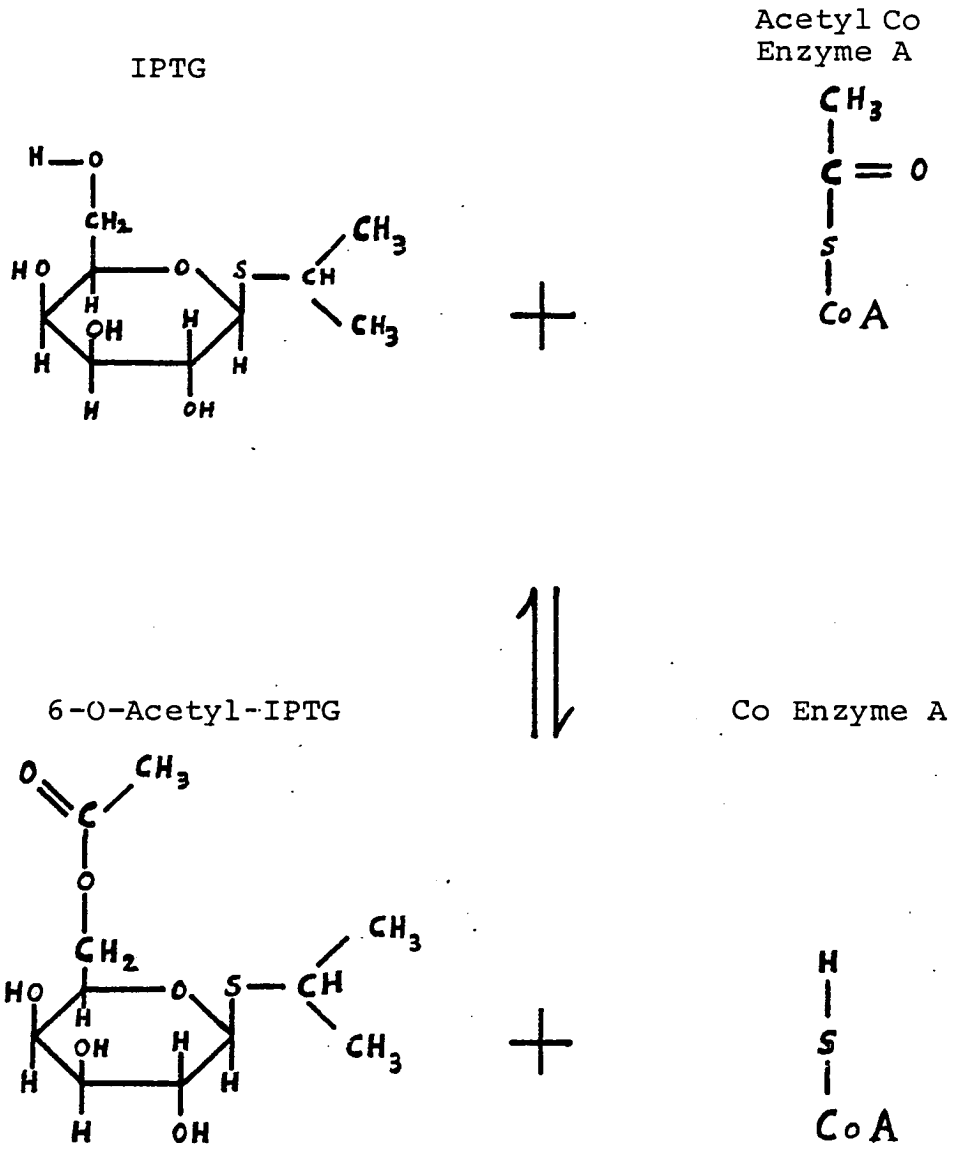


Figure 1·4

The reaction catalysed by the enzyme thiogalactoside transacetylase.

adjacent to and downstream from lac A. These mutants, although lacking a thiogalactoside transacetylase enzyme, grew normally both from the viewpoint of lactose transport and lactose metabolism. This result was later confirmed by Wilson and Kashket (1969). The above results suggested that thiogalactoside transacetylase was not involved in the transport of lactose or its metabolism.

Recently it has been suggested that the role of the E. coli thiogalactoside transacetylase is to assist in the removal from the cell of non-metabolisable analogues that may have gained entry via the lactose permease system during periods of induced lac operon activity (Andrew and Lin, 1976). The authors used a starting population of two isogenic E. coli strains, which differed in the lac A allele, in a ratio of 1:1. After 50 generation there was no observable difference in the ratios of the Lac A^+ to Lac A^- populations; however, after the addition of IPTG and a further 50 generations growth the ratio of Lac A^+ to Lac A^- bacteria had changed to 15:1. The authors suggested that the possession of a thiogalactoside transacetylase conferred upon the E. coli host a selective advantage because the non-metabolisable analogue, IPTG, was detoxified, i.e. acetylated and excreted, as opposed to merely being accumulated. As an example of growth retardation by accumulation of non-metabolisable analogues the authors referred to the work of von Hofsten (1961) who observed growth retardation upon the addition of IPTG or TMG to Lac I^- constitutive mutants of E. coli ML when grown in minimal medium with succinate as a source of carbon and energy. However, the conclusion of von Hofsten (1961), that the accumulation of analogues was the sole cause of growth retardation, may be open to reinterpretation in the light of recent work on the growth inhibitory effects on E. coli cells by elevated levels of lactose permease which are discussed in detail in Chapter 4.

The question still remained as to the evolutionary advantage of such a system because thiogalactosides are not known in nature (Fried, 1980). It has been demonstrated however, that the substrate specificity of the thiogalactoside transacetylase is not limited to thiogalactosides, but also extends with varying degrees of activity to compounds such as O-nitrophenyl-β-D-galactoside and 4-methyl phenyl sulphone β-D-glucoside (Alpers et al., 1965; Musso and Zabin, 1973). From his study of the thiogalactoside transacetylases from strains of E. coli K12 and ML, Fried (1980) showed that the two enzymes were identical with respect to enzyme kinetics, subunit and native enzyme molecular weights and isoelectric focussing analysis. He argued that a dispensable enzyme would be expected to accumulate mutations which would eventually alter its physical and enzymatic properties. The fact that the thiogalactoside transacetylases from the two E. coli strains K12 and ML (whose evolutionary relationship is unknown) were identical suggested, according to Fried (1980), that they have been structurally and functionally conserved because the enzyme played a vital role in lactose utilisation. To account for the observed normal growth characteristics on lactose reported for Lac A^- mutants (Fox et al., 1966; Wilson and Kashket, 1969), Fried (1980) suggested that the acetylase had retained normal physiological activity while losing the ability to acetylate thiogalactosides, based on the findings that the Km values for the thiogalactosides and other acetyl acceptors tested indicated that they were not the true substrates of the enzyme (Musso and Zabin, 1973).

However, an alternative explanation of the results presented by Fried (1980) was that the E. coli K12 and ML strains used in the work had not undergone sufficient divergence for observable differences to have occurred between the thiogalactoside transacetylases from each strain.

It is interesting to note that milk, which is essentially the sole natural source of lactose (Clamp et al., 1961), contains a number of galactosides other than lactose (Stacey and Barker, 1962). Therefore the possibility remains that the thiogalactoside transacetylase detoxifies some as yet unidentified natural sugar derivative; it is tempting to speculate that a detailed chemical analysis of milk may yield the natural substrate of the enzyme. It should also be noted that, recently, acetylation of maltose was observed in E. coli strains totally deleted for the lac operon which indicated that the lacA gene product was not involved in this process and that a separate system existed for maltose acetylation (Boos et al., 1981; Freundlieb and Boos, 1982). The authors indicated that acetylation of the maltose was unlikely during transport and that acetylmaltose was not a substrate of the mal B dependent transport system, and nor were mal B gene products involved in the acetylation process. The similarity between these findings and those described above for the thiogalactoside transacetylase did not escape the attention of the authors, and it would appear that the acetylation process followed by passive diffusion of the modified substrate, initially demonstrated in E. coli by thiogalactoside transacetylase, may not be as unique as was once thought to be the case.

1.2 Regulation of the *lac* Operon

1.2.1 The *lac I* Gene and Repressor Protein

1.2.1.1 General Background

Early research on the *lac* operon had shown that wild type *E.coli* cells grown in the absence of a galactoside contained between 1 and 10 units of activity of β-galactosidase per mg dry weight, and that when introduced to a suitable inducer the bacterial galactosidase content could reach 10,000 units of activity per mg dry weight. The question as to whether this increase in activity represented the synthesis of "new" enzyme molecules or was simply the conversion or activation of pre-existing protein precursors was answered by the work of Cohn and Torriani (1952), Monod and Cohn (1953) and Rotman and Spiegelman (1954). They showed that the "new" enzyme produced upon induction did not contain any significant fraction of sulphur or carbon from the pre-existing proteins and that they were distinct from all the proteins in the uninduced cells. Thus, new enzyme was synthesised upon induction, which led to the question of how this was regulated.

The measurement of β-galactosidase and transacetylase activity after the addition of a number of individual inducers showed that synthesis of these enzymes was co-ordinated (Jacob and Monod, 1961), which suggested that their induction and hence control was co-ordinated; but was the control at the level of transcription or translation?

Attardi *et al.* (1963) had shown that induced *E.coli* strains carrying the plasmid F'*lac* produced approximately 10 times more *lac* mRNA than uninduced strains. The de-induction experiments of Kepes (1963) also supported this: the removal of induction was accompanied by a decrease in β-galactosidase activity at a rate comparable to the observed instability of the mRNA species. Also the addition of specific inhibitors of RNA synthesis and

analogues known to alter the coding properties of mRNA, to cultures during early induction, resulted in little active β-galactosidase formation, and in the case of analogue addition, the formation of inactive, though serologically cross-reacting protein. Thus these results strongly suggested that the induction response was dependent upon increased synthesis of mRNA.

The genetic studies in E.coli by Pardee et al. (1959) using merozygotes, which were formed following Hfr transfers, had found in addition to mutations in the lac Z and lac Y genes, mutations which produced uncontrolled or constitutive production of the gene products. The mutations were located at a separate locus, the lac I gene, and acted in trans, implying that a cytoplasmic factor coded by the lac I gene was involved in the regulation of β-galactosidase, lactose permease and thiogalactoside transacetylase production. This conclusion was supported by work using F prime factors and Hfr crosses to map other lac I mutations (Jacob and Adelberg, 1959; Jacob and Monod, 1961) which indicated that the lac I gene was located upstream from the lac Z and lac Y gene.

1.2.1.2 Physical Properties

Although the lac I gene had been mapped, the nature and mode of action of the lac I gene product remained unresolved. Pardee and Prestidge (1961) had suggested that the lac I gene functional product was not a protein, and must be RNA, however a considerable body of information was assembled during the 1960's which argued strongly that the functional product of the lac I gene was a protein. Finally Gilbert and Müller-Hill (1966) used the binding affinity between the product of the lac I gene and inducer (radioactive labelled IPTG) to isolate and purify the bound substance, which was not detected in lac I nonsense mutants. The product was a protein (the repressor) with an approximate

molecular weight of 150,000-200,000.

The lac repressor was shown to be a tetrameric protein, consisting of four identical subunits, and which contained four binding sites per tetramer for the inducer IPTG (Riggs and Bourgeois, 1968; Muller-Hill et al., 1971). While monomers and dimers could bind inducers, the tetrameric structure was necessary for binding to the lac operator (Hamada et al., 1973). An amino acid sequence of 347 residues was established by Beyreuther et al. (1973, 1975) for the repressor. However, on the basis of the lac I gene DNA sequence (Farabaugh, 1978), the deduced amino acid sequence was estimated to have 360 residues and a molecular weight of 38,590. Following further work on the lac repressor amino acid sequence, Beyreuther reported a revised amino acid sequence (Beyreuther, 1978) that was in complete agreement with the amino acid sequence predicted from the DNA sequence of the lac I gene (Farabaugh, 1978).

1.2.1.3 Repressor Mode of Action

Gilbert and Müller-Hill (1966; 1967), and Riggs et al. (1968) had shown that the lac repressor bound specifically to λ lac DNA and did not do so to λ DNA alone, and that lac repressor, bound to lac DNA was released from the DNA in the presence of IPTG.

The repressor protein was shown by fluorescence and UV absorption studies and differential sedimentation experiments to have two conformations: an operator-binding form and an inducer binding form (Ląiken et al., 1972; Oshima et al., 1972; Mathews et al., 1973; Wu et al., 1976). The binding sites for the operator and inducer were shown to be physically distinct. The changes observed in the fluorescence of the repressor protein upon binding IPTG were the same in the presence or absence of DNA (von Hippel et al., 1975), indicating that the same conformational change occurred in the repressor whether bound to DNA or

not. It was also shown that inducer would bind to a repressor-DNA complex, or to free repressor in an identical way (Maurizot *et al.*, 1974; von Hippel *et al.*, 1975; Butler *et al.*, 1977). These results, coupled with those of Riggs *et al.* (1970) who showed that the addition of inducer increased the rate of decomposition of the repressor-operator complex, suggested a simple model for the repressor-inducer interaction.

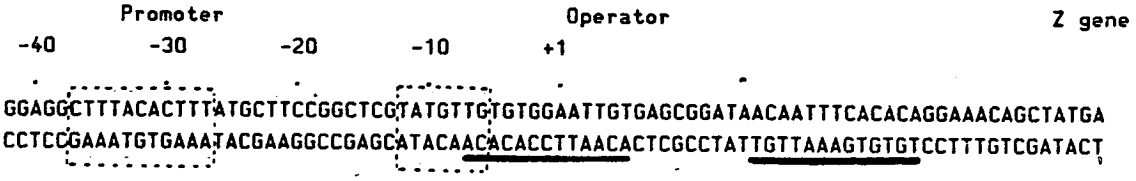
The repressor shows a high affinity for the operator and forms a stable repressor-operator complex. This high affinity is greatly reduced when the repressor moiety of a repressor-operator complex binds to an inducer, resulting in a conformational change of the repressor. As a consequence of this reduced affinity the inducer-repressor moiety is released from the operator.

1.2.2. The *lac* Operator

The existence and position of the *lac* operator was indicated by Jacob *et al.* (1960) who isolated constitutive *lac* mutations (O^c) which were shown not to map in the *lac I* gene; they were later mapped to a position between *lac I* and *lac Z* (Jacob and Monod, 1961). This mapping work was further extended by the isolation of a large number of *lac* operator point mutations(Smith and Sadler, 1971).

Gilbert and Maxam (1973) deduced the DNA nucleotide sequence of the *lac* operator, using RNA transcripts of a DNA fragment which had been protected from nuclease attack by the *lac* repressor. The sequence containing the operator was 27 bp long, and as shown in Figure 1.5 it revealed a high degree of symmetry about the operator region, which from the viewpoint of repressor-operator interaction has been the subject of much experimentation and speculation. As this is outside the scope of this thesis, however, the reader is recommended to Barkley and Bourgeois (1980) for a detailed account of the repressor-operator interaction.

Figure 1.5. The DNA sequence of a section of the lac promoter-operator region (adapted from Reznikoff and Abelson, 1980).



The solid lines indicate those sequences which revealed a high degree of symmetry and which may be involved in the repressor-operator interaction. The broken-line boxes indicate those sequences similar to the equivalent regions in other E.coli promoter regions.

1.2.3 The lac Promoter

The site from which transcription of the lac operon mRNA was initiated, the lac promoter, was initially believed to be in the lac operator region (Jacob and Monod, 1961), based on the isolation of a mutant (O^c) which produced a partially constitutive phenotype, and an operator-negative mutant (O^o) which appeared to be defective in transcription initiation. Following further work the latter was identified as an extremely polar mutation which mapped in the lac Z gene (Beckwith, 1964; Brenner and Beckwith, 1965). Later Jacob et al. (1964) proposed that initiation of mRNA transcription was from a site between lac O and lac Z, which was termed the promoter. However a class of mutations was studied which had a pleiotropic effect on the synthesis of lac operon-coded enzymes, that was cis-dominant and as such was not relieved by the introduction of a second lac region into the cell (Ippen et al., 1968; Miller et al., 1968); these mutations mapped to a position between lac I and lac O. The concept of distinct lac P and lac O regions was supported by the fact that deletions in the lac promoter did not affect repressor binding. In addition to this, a number of lac O-Z deletions were identified (Eron et al. 1970) which displayed constitutive production of thiogalactoside transacetylase.

With the aid of numerous deletion mutations it has been shown that the lac promoter region may be divided into two functional domains. The mutations which mapped in the lac I proximal region appeared to affect the response to the CAP-cAMP complex (discussed under Catabolite Repression) and those which mapped distal to lac I acted by reducing RNA polymerase activity from lac P primarily by lowering the affinity between RNA polymerase and the lac promoter (Beckwith et al., 1972; Hopkins, 1974). The DNA sequences were established for the lac promoter region and several

lac P mutations, which further supported the above conclusion that the lac promoter region was divided into two functional domains (Dickson et al., 1975; Gilbert, 1976).

The sequence data also revealed a region between nucleotide -6 and -12 (Figure 1.5) which was similar to that described by Pribnow (1975a,b) and Schaller et al. (1975) and latterly by numerous other authors (see Reznikoff and Abelson, 1980). Also, the nucleotide sequence extending from position -37 to -27 was similar to the equivalent region in several other E.coli promoter regions. Based on the pancreatic DNase protection experiments of Gilbert and Maxam (1973) and Gilbert (1976) it appeared that the binding of RNA polymerase to the lac promoter and lac repressor to the lac operator were mutually exclusive. Although they may have recognised distinct non-overlapping sequences, the region protected by the repressor was almost completely overlapped by the region protected by RNA polymerase.

1.2.4 Catabolite Repression

Early experiments had shown that when E.coli was grown in media containing both glucose and lactose, glucose was preferentially used as a source of carbon (Monod, 1947; Magasanik, 1961); the term used to describe this observation was catabolite or glucose repression of lactose utilisation. This repression by glucose was not unique to the lactose metabolising enzyme but had been observed for enzymes which catabolised a number of other carbohydrates (Epps and Gale, 1942; Magasanik, 1962; Koch et al., 1964). The repression effect was not only exerted by glucose, gluconic acid had a similar effect and glucose-6-phosphate (G6P) a much stronger effect (Hsie and Rickenberg, 1967).

Neidhardt and Magasanik (1956) proposed that enzymes involved in the catabolism of carbon compounds would be repressed by their end products, i.e. the catabolites

such as ATP, phosphoenolpyruvate etc., or by an "effector compound" whose intracellular concentration was dependent upon the level of these catabolites. Thus, because various catabolic pathways eventually led to a common pool of catabolites, metabolism of one carbon compound could produce catabolites capable of causing repression of enzymes responsible for the degradation of other carbon compounds. Catabolite repression would therefore occur when the rate at which a compound was metabolised, and as a consequence the rate at which catabolites were formed, was in excess of the requirements of biosynthetic reactions.

This was demonstrated by Tyler *et al.* (1969) who showed that it was the metabolism of glucose rather than the glucose molecule *per se* that produced the permanent repression of β-galactosidase in *E.coli* strains. These authors showed that *E.coli* mutants lacking glucose-6-phosphate dehydrogenase and hexosephosphate-isomerase did not produce the permanent repression effect.

An indication as to the identity of the "effector compound" was provided by Makman and Southerland (1965) who observed that the levels of cyclic adenosine monophosphate (cAMP) in *E.coli* cells grown in glucose rose rapidly upon withdrawal of glucose from the media. Furthermore cAMP was shown to overcome glucose repression of β-galactosidase synthesis when added to *E.coli* grown in medium containing glucose (Perlman and Pastan 1968a,b; Ullmann and Monod, 1968; Perlman *et al.*, 1969). Additionally Epstein *et al.* (1975) showed that there was a correlation between intracellular levels of cAMP in *E.coli* and the ability to synthesise β-galactosidase. *E.coli* grown in glucose or glucose-6-phosphate containing medium displayed severe reductions in both β-galactosidase synthesis and cAMP levels, whilst growth in glycerol containing medium allowed increased synthesis of β-galactosidase and an accompanying rise in cAMP levels. The above results led to the conclusion that

cAMP played a role in controlling the synthesis of catabolite sensitive enzymes.

Genetic studies also indicated that cAMP played a central role in catabolite repression. Perlman and Pastan (1969) isolated a class of mutant E.coli, which grew very poorly even in rich medium, and which had a defective adenylate cyclase enzyme. They were thus unable to convert adenosine triphosphate (ATP) to cAMP, however, the addition of cAMP to the cells restored their ability to ferment lactose and other sugars; these were Cya^- mutants. A second type of mutant was obtained, Crp^-, which displayed similar growth characteristics to Cya^- mutants but differed in that the addition of cAMP did not restore the ability to ferment lactose and other sugars (Emmer et al., 1970; Schwartz and Beckwith, 1970). This result suggested that a second factor might be involved with cAMP in effecting enzyme synthesis and this was shown by Emmer et al. (1970) to be a protein to which cAMP bound. The protein called cAMP-receptor-protein (CRP) or catabolite-activator-protein (CAP, Zubay et al., 1970), was absent or greatly reduced in Crp^- strains of E.coli.

Using a DNA-dependent cell free system for the synthesis of β-galactosidase it was shown that cAMP did not stimulate β-galactosidase synthesis in extracts from Crp^- mutants (Emmer et al., 1970; Zubay et al., 1970), but addition of either wild type extract or purified CAP protein to the system restored cAMP-dependent synthesis of β-galactosidase. This strongly suggested that the action of cAMP on catabolic enzyme synthesis was via the CAP protein.

Silverstone et al. (1969) identified the lac P region as the catabolite sensitive site of the lac operon, and following from this a number of point and deletion mutations in this region were isolated which allowed the delimitation of the catabolite-sensitive domain within the lac promoter region (Miller et al., 1970; Beckwith et al.,

1972; Hopkins, 1974; Mitchell *et al.*, 1975). Majors (1975) made use of these deletions to demonstrate that specific cAMP dependent binding of CAP to the *lac* promoter required an intact, repressor-proximal domain, which was confirmed by Mitra *et al.* (1975).

On the basis of the data above and that presented on the *lac* promoter, a possible mechanism for catabolite control of transcription would necessitate the presence both of the RNA polymerase and the CAP-cAMP positive regulator at the promoter, in order that high levels of transcription might occur. The role of the CAP-cAMP complex in the stimulation of the high levels of transcription could be either by a protein-protein interaction with the RNA polymerase (Gilbert 1976) or by the melting of the DNA helix in the promoter region thereby producing an 'open promoter' structure, which would enhance RNA polymerase binding (Burd *et al.*, 1975; Dickson *et al.*, 1975). When *E.coli* is grown in the presence of glucose internal cAMP levels are low and since CAP requires this cyclic nucleotide in order to function, expression of the *lac* operon and other genes under similar control is repressed.

There exists another level of control of bacterial operons coding for carbohydrate catabolic enzymes, including the *lac* operon. This was believed to act upon the transport into the cell of specific inducers of these operons, and was termed "inducer exclusion". Initially it had been observed that when glucose was added to a bacterial cell suspension, the activities of several carbohydrate permeases, including the lactose permease, were inhibited (Magasanik, 1970; Winkler and Wilson, 1967). D-Glucose and several other sugars entered the bacterial cell by a phospho-transferase system in a process termed group translocation, during which they were phosphorylated, and evidence existed to suggest that protein(s) of this system were directly involved in the regulation of the uptake of

certain carbohydrates including lactose (Dills et al., 1980).

A model was proposed by Saier and co-workers to explain the molecular basis of the regulation (Saier and Feucht, 1975; Osumi and Saier, 1982). The authors suggested that lactose permease activity was dependent upon whether or not the glucose specific enzyme III (enzyme III^{glc}) of the phosphotransferase system was phosphorylated.

When the intracellular pool of enzyme III^{glc} was mainly dephosphorylated, the formation of a lactose permease-substrate complex would permit the enzyme III^{glc} to bind to it, which reduced the relative transport activity of the permease. The dephosphorylated state of the enzyme III^{glc} was assumed to predominate when sugar substrates of the phosphotransferase system were being transported into the cell, which would drain the phosphate of the enzyme III^{glc}. When in the phosphorylated form (due to the sequential reactions of the phosphotransferase system) enzyme III^{glc} would dissociate from the lactose permease and allow the transport of lactose to be restored to normal levels.

1.2.5 Relative Rates of Synthesis of lac Operon Structural Proteins

As previously mentioned, the measurement of β-galactosidase activity and thiogalactoside transacetylase activity in E.coli strains after induction demonstrated co-ordinated expression of the two genes (Jacob and Monod, 1961). However, a comparison of the enzyme activities and also of the estimated percentage of total protein each enzyme represented, indicated that the ratio of β-galactosidase to thiogalactoside transacetylase was unexpectedly high: between 12:1 and 20:1 by weight, which, taking the molecular weights of the two species into account gave a

molecular ratio of between 3:1 and 5:1 (Brown *et al.*, 1967a). This was unexpected because the *lac* operon was believed to be transcribed as a polycistronic mRNA species with translation occurring from a single site at the 5' end; and this should have produced identical rates of protein molecule synthesis.

However, de Crombrugghe *et al.* (1973) had shown that *in vitro* transcription of the *lac* DNA gave different size classes of mRNA depending upon the presence or absence of Rho, a factor involved with RNA polymerase transcription that prevented further elongation of RNA polymerase at certain sites and augmented release of RNA at other terminators. Thus the *lac* operon appeared to have within it a number of Rho-dependent transcription-termination signals. On the basis of this and a considerable body of other information (reviewed by Adhya and Gottesman, 1978) the authors proposed a model to explain polarity in gene expression. It was suggested that when an operon was being transcribed, the nascent mRNA was normally covered with ribosomes which block the attachment of Rho to the RNA and prevent movement of Rho along the RNA chain. During transcription RNA polymerase was believed to pause at the Rho-mediated termination signal, which in turn allowed a region of "naked" mRNA devoid of ribosomes to form. With the aid of it s NTPase activity Rho, having bound to the "naked" RNA, translocated itself in the 5' $\longrightarrow$ 3' direction along the transcript towards the RNA polymerase complex. If Rho made contact with the RNA polymerase complex while the complex was close to or at an internal transcription termination sequence premature termination occurred and transcription of the distal genes in the operon was prevented. However, RNA chain termination did not take place if there was reinitiation of translation prior to the interaction of Rho and RNA polymerase. Thus ribosome binding to the mRNA and subsequent polypeptide chain initiation at a neighbouring gene physically

prevented Rho action and allowed distal gene expression.

The natural polarity observed in the lac operon could therefore be explained by occasional transcriptional termination events, probably in the intracistronic regions.

1.3 Bacterial Genome Evolution Within the Enterobacteriaceae and in Particular the Evolution of the lac Operon

In the period up to the end of the 1950's bacterial variation and consequently bacterial evolution was considered to be essentially due to the acquisition of mutations within individual cells, which became bacterial populations following selection (Demerec, 1957).

The evolution of the bacterial genome from a primitive to a contemporary form would also by necessity have involved an increase in the total genetic information in the bacteria. Thus if contemporary genomes of species of the Enterobacteriaceae were descended from a common ancestor, divergence would have occurred by a succession of changes in the genomic information.

These changes may have included inversions of the genome, as demonstrated by a comparison of the positions of the trp genes on the chromosomal maps of E.coli and Salmonella typhimurium, which show an inversion of approximately 10 minutes of chromosomal DNA (Case et al., 1973). An increase in genome size by duplication of a section of pre-existing genomic DNA may also have been involved; Koch (1972) referred to such an occurrence as an "epochal event" which could dramatically increase the evolutionary potential of an organism. Experimentally produced duplications of part of the genome have been reported in E.coli, Salmonella typhimurium and Klebsiella pneumoniae (reviewed by Riley and Anilionis, 1978); however, these were unstable and were lost upon release from the selective pressure. Naturally occurring duplications within the genome of

E.coli have been reported, e.g. the non-tandem duplications of the rRNA genes (Nomura et al., 1977), and the two ornithine transcarbamylase genes argI and arg F (Kikuchi and Gorini, 1975); divergence of duplicated genes is believed to have been a potential major evolutionary route for the acquisition of new functions (Koch, 1972; Ohno, 1970 and Ohta and Kimura, 1971). One group of enzymes found in E.coli believed to be an example of divergence following gene duplication are aspartokinase-I-homoserine-dehydrogenase-I, aspartokinase-II-homoserine-dehydrogenase II, aspartokinase-III, and homoserine-kinase which were shown by Truffa-Bachi et al. (1975) to all share common antigenic determinants.

However, an alternative mechanism by which the bacterial genome may have acquired additional DNA via wide host range plasmids and bacteriophages has been suggested. It was proposed that with the aid of either plasmid or bacteriophage vectors frequent inter- and intra-specific genetic exchange among the bacteria would occur. This has been extensively reviewed by Hedges (1972), Jones and Sneath (1970), Reanney (1976) and Campbell (1981). This would allow the horizontal transfer of genetic information across species boundaries. This hypothesis has considerable support from the evidence of genetic transfer among prokaryotes in nature mediated by broad host range conjugative plasmids (Datta and Hedges, 1972; Falkow, 1975), and of the transposition of certain genetic elements (extensively reviewed in Bukhari et al., 1977). Although much of the data available concerned the transfer of plasmid-mediated drug resistances there also existed what appeared to be examples of the transfer of host chromosomal genes. One R-factor isolate was shown to confer on E.coli the ability to ferment raffinose and production of H_2S (Orskov and Orskov, 1973). Of particular interest were the numerous reports of plasmid borne lac genes, in a variety of enteric bacteria: Salmonella (Falcoa et al., 1975;

Easterling et al., 1969; Gonzalez, 1966), Serratia (Le Minor et al., 1974), Klebsiella (Reeve and Braithwaite, 1970; Brenchley and Magasanik, 1972), Hafnia (Guiso and Ullmann, 1976), Proteus (Tiern and Steinberg, 1975), Yersinia (Cornellis et al., 1976), Erwinia (Chatterjee and Starr, 1973).

The lac plasmids from several different enteric sources were transferred to E.coli hosts that were deleted for the entire lac region, and their β-galactosidases and lactose permeases were compared with the enzymes coded by the E.coli lac operon (Guiso and Ullmann,1976). All of the plasmid coded lac systems were inducible with IPTG and the β-galactosidase Km's for ONPG hydrolysis for each were similar to that of the E.coli lac enzyme. The molecular weights of these β-galactosidases, established by sedimentation studies, were similar to that of the E.coli β-galactosidase; antibodies against the E.coli enzyme recognised all of the plasmid coded β-galactosidases.

TMG uptake studies demonstrated that the plasmid encoded lactose permeases were all very similar to that of E.coli, and measurement of β-galactosidase and permease levels indicated that expression of the plasmid lac genes was co-ordinated. However, unlike the E.coli lac operon the plasmid borne lac systems did not appear to code for a thiogalactoside transacetylase enzyme, the product of the E.coli lac A gene, as judged either by enzyme assays of fully induced cultures or by an immunological test involving antibodies against E.coli thiogalactoside transacetylase (Guiso and Ullmann,1976). The data strongly suggested that the plasmid encoded lac genes and the E.coli lac operon shared a common evolutionary origin, a hypothesis previously proposed by Reeve and Braithwaite (1973).

Recently Cornelis et al. (1978) showed that the lac genes on a plasmid isolated from Yersinia enterocolitica were on a transposon (Tn 951) which could apparently

transpose to the plasmid RP1. The transposon carried lac I, Z and Y genes. Via DNA-DNA hybridisation, Tn 951 was compared with DNA from several independent lac plasmid isolates and with E.coli lac DNA on λd lac (Cornelis, 1981; Cornelis et al., 1978). The results indicated that homology between Tn 951 and lac DNA from λd lac and the lac plasmids studied was essentially confined to the lac region and that the DNA sequences adjacent to the lac DNA on Tn 951 were dissimilar to the equivalent sequences on λd lac and the lac plasmids. The results further supported the suggestion that the lac systems located on the plasmids and the E.coli lac operon shared a common evolutionary origin. However, as there was little apparent homology between the DNA on Tn 951, believed to be concerned with transposition, and the plasmid DNA, the distribution of the lac genes on plasmids could not be attributed to a spread of the transposon.

Nevertheless, plasmids provided a mechanism by which lac genes similar to those on the E.coli chromosome could be disseminated throughout the Enterobacteriaceae, which posed the question as to how similar the chromosomal lac systems in the various enteric species were to that in E.coli and to those located on plasmids? Thus a comparative study of the lac genes and their protein products from the lac plasmids and the chromosomal lac systems within the various enteric species may give an indication as to their evolutionary origins and mechanism of inheritance throughout the Enterobacteriaceae.

1.4 Comparison of the lac Systems in the Enterobacteriaceae with the E.coli Chromosomal and Plasmid Borne lac Systems

1.4.1 General Background

The first question to be asked must be, do all members of the Enterobacteriaceae possess genes for lactose metabolism? To answer this, one may look at two levels. Firstly, can the bacteria grow using lactose as a sole carbon source and for example, give a positive result on MacConkey lactose agar. A positive answer to this would imply the presence in the bacterium of both a functional β-galactosidase and lactose transport system. Should either be inactivated, then a negative result would be recorded after 48 hours incubation in normal growth conditions. However, hydrolysis of ONPG would allow detection of a functional β-galactosidase, even in the absence of lactose transport.

Thus traditional biochemical tests used in taxonomic classification rely on a functional protein product as an indication as to the presence or absence of the gene coding for it. However, if the genes were present but were not expressed or expression produced an inactive protein product then analysis at the second level, the nucleic acid level, would be required to reveal their presence; this is dealt with in detail later.

Thus on the basis of the biochemical tests one answer to the question above would be as described in Table 1.1, from which it can be seen that most genera displayed varying degrees of lactose fermentative ability, depending upon the strains. For example Salmonella subgenus group 1&2 species, which included Salmonella typhimurium were all lactose-negative and ONPG-negative, whereas Salmonella arizona (subgenus III) species were in general both lactose-positive and ONPG-positive. Also, many of the enteric species that were lactose-negative actually produced an enzyme resembling β-galactosidase, as judged by the reaction with ONPG, which

Table 1.1. Published data on lactose fermentation and ONPG hydrolysis by members of the Enterobacteriaceae.

Genus	Species	Lactose Fermentation	ONPG Hydrolysis
Escherichia	coli	+	+
Shigella	dysenteriae	vs	+
	flexneri	-	-
	boydii	-	-
	sonnei	+s	+
Edwardsiella	tarda	-	-
Salmonella	subgenus I		
	cholerae-suis	-	-
	typhi	-	-
	subgenus II		
	typhimurium	-	-
	subgenus III		
	arizoniae	+	+
	subgenus IV		
	hortenae		-
Citrobacter	freundii	v	+
Klebsiella	pneumoniae	+	+
	ozaenae	v	
	rhinoscleromatis	-	
	oxytoca	+	+
Enterobacter	aerogenes	+	+
	cloacae	+	+
	agglomerans	v	+
Hafnia	alvei	-	+
Serratia	marcescens	-	+
	liquifaciens	v	v
	rubidaea	+	+
Proteus	mirabilis	-	-
	vulgaris	-	-
Providencia	rettgeri	-	-
Morganella	morganii	-	-
Erwinia	amylovora	-	
	carotovora	+	+
	chrysanthemi	-	+
	herbicola	v	
Yersinia	ruckeri	-	+

+: 90-100% of strains positive; v: 20-90% of strains positive; -: 0-20% of strains positive; s: ferments lactose slowly. Taken from Cowan et al., 1974; Edwards and Ewing, 1972 and Starr et al., 1981.

would imply that their inability to ferment lactose was due to the absence of a functional lactose transport system. This was clearly demonstrated in Shigella dysenteriae which produced a β-galactosidase, albeit with a reduced activity, but was deleted for the gene(s) involved in lactose transport (Sarkar, 1966).

It can therefore be seen in Table 1.1 that within almost every genus of the Enterobacteriaceae there are species which produce a β-galactosidase enzyme. Ideally one would like to compare the amino acid or nucleotide coding sequences of each of the β-galactosidases from enteric species in order to ascertain the evolutionary relationship between them, as had been achieved for the tryptophan synthetase α-subunit from E.coli, Salmonella typhimurium and K.pneumoniae (reviewed by Crawford 1975). However the sole β-galactosidase to be sequenced to date was that of E.coli (Fowler and Zabin, 1977, 1978; Kalnins et al., 1983). An alternative which may give an indication as to the similarity or otherwise between the various β-galactosidases would be to compare their physical properties e.g. enzyme kinetics, immunological cross-reacting characteristics, subunit and native enzyme molecular weights, and amino acid composition. Unfortunately the amount of data concerning the properties of β-galactosidases from enteric species excluding E.coli is minute by comparison to that known about the E.coli enzyme; that available is presented below.

1.4.2 Physical Properties of the β-Galactosidases from Various Enteric Species

1.4.2.1 Molecular Weights

As previously mentioned, the molecular weight of both the subunit and native enzyme in E.coli had been established as 116,353 and 455,412 respectively (Kalnins et al., 1983; Fowler and Zabin, 1977, 1978). The native enzyme from Shigella dysenteriae had the same sedimentation coefficient

as the E.coli β-galactosidase, and therefore was presumed to have the same molecular weight (Sarkar, 1966). The Ebg enzyme in E.coli had a smaller subunit size than that of the E.coli lac Z encoded enzyme, and was estimated to be 120,000 at a time when the E.coli β-galactosidase was believed to be 135,000, also the native Ebg enzyme apparently predominated in a hexameric form with a molecular weight of 720,000 (Hall, 1976).

Hall and Reeve (1977) examined the properties of a Klebsiella strain that did not express either the plasmid or chromosomal lac Z genes normally present, but which synthesised a β-galactosidase, referred to as β-galactosidase-III. This enzyme was similar to the E.coli Ebg enzyme in that expression of β-galactosidase-III was induced by lactose but not by IPTG, and β-galactosidase-III hydrolysed ONPG but appeared to have virtually no lactose activity. β-galactosidase-III had a subunit molecular weight of 68,000 (Hall and Reeve, 1977), and an apparent native enzyme molecular weight of 226,000 (Hall, 1980) which implied a trimer structure. However to quote the author, "given the errors associated with the sedimentation constant of an enzyme and the molecular weight of the subunit, and in the light of the rarity of trimer enzymes, further studies are required before the trimeric structure of the β-galactosidase-III is taken very seriously".

1.4.2.2 Immunological Relatedness

Immunological cross reactivity has been reported between anti-E.coli β-galactosidase serum and the Shigella dysenteriae and Aerobacter aerogenes enzymes (Monod and Cohn, 1952). Aerobacter aerogenes species were later reclassified as Enterobacter aerogenes (Cowan et al., 1974). No cross-reactivity was observed between anti-Ebg serum and either the E.coli β-galactosidase or the β-galactosidase III from K.pneumoniae (Hall and Reeve, 1977). Guiso and Ullmann

(1976) have reported that E.coli β-galactosidase antiserum did not cross-react with the β-galactosidases from Klebsiella oxytoca, Serratia rubidae and Enterobacter aerogenes. The latter result is in apparent contradiction to that reported by Monod and Cohn (1952). However this may be explained by the fact that the Aerobacter aerogenes strain used by Monod and Cohn (1952) may well have been a Klebsiella species carrying a lac plasmid (Reeve, personal communication). The result observed by Monod and Cohn (1952) could thus be explained because the anti-E.coli β-galactosidase serum would react with the plasmid-encoded β-galactosidase.

1.4.2.3 Enzyme Kinetics

It can be seen from Table 1.2 that the kinetic properties of β-galactosidase III of K.pneumoniae are substantially different to the Shigella β-galactosidase and the enzyme products of the E.coli lac Z and Ebg genes. It is noticeable that neither the Ebg nor β-galactosidase III enzyme can hydrolyse lactose efficiently.

1.4.2.4 Amino Acid Composition

With regard to the amino acid composition of the β-galactosidases from the enteric bacteria no information was available other than that previously described for the E.coli enzyme coded by lac Z.

1.4.2.5 Induction of β-Galactosidases

The E.coli lac operon, the K.pneumoniae lac system and the lac systems of the various F-lac plasmids analysed, have all been shown to be inducible with IPTG (Jacob and Monod, 1961; Guiso and Ullmann, 1976; Reeve and Braithwaite, 1973). Both the E.coli chromosomal and the F-lac plasmid lac genes were also induced with melibiose (Jacob and Monod, 1961; Reeve and Braithwaite, 1974).

Table 1.2. Comparison of enzyme kinetics of different β-galactosidases.

Property	Enzyme			
	β-galactosidase III (*Klebsiella*)	Ebgo (*E.coli*)	β-galactosidase (*E.coli lacZ*)	β-galactosidase (*Shigella*)
Km ONPG (mM)	34	160	0.16	0.18
Km lactose (mM)	29	1200	2	

Data from Hall and Reeve (1977) and Sarkar (1966).

However the K.pneumoniae chromosomal lac system was not induced with melibiose,and IPTG induction of the system was repressed by the presence of melibiose, which was not due to catabolite repression of the lac system by the melibiose (Reeve and Braithwaite, 1974).

1.4.3 Properties of the Lactose Permeases from the Enterobacteriacea.

As with the β-galactosidases from enteric species discussed above the overwhelming majority of available data on lactose permeases from enteric species is primarily concerned with the E.coli lac Y product, and has been described above.

The TMG uptake studies on lactose permeases encoded on lac plasmids isolated from various enteric species showed that their Km's were in good agreement with that of the E.coli lac permease (Guiso and Ullmann, 1976).

The rate at which TMG was accumulated by the K.pneumoniae lactose permease system was shown by Wilson et al. (1979) to be approximately one-third the rate of the E.coli system. It is interesting to note that Shigella dysenteriae, whose β-galactosidase has been shown in the preceding sections to be extremely similar to the E.coli lac Z product, does not possess a lactose permease, apparently as the result of a deletion (Sarkar, 1966).

1.4.4. Properties of the Thiogalactoside Transacetylases from the Enterobacteriaceae

Data concerning the thiogalactoside transacetylase is essentially confined to E.coli, as above. Alpers et al. (1965) devised a colo rimetric assay procedure to measure the amount of thiogalactoside transacetylase in extracts from induced cells. They measured the amount of thiogalactoside transacetylase in E.coli K12 and E.coli B, but failed to detect the presence of a functional thio-

galactoside transacetylase in Shigella dysenteriae, Salmonella typhimurium or Serratia marcescens. Guiso and Ullmann (1976) obtained negative results when extracts from cultures of a total lac deleted E.coli strain, containing Lac$^+$ plasmids from various enteric sources were assayed for the presence of functional thiogalactoside transacetylase. They also reported a negative immunological reaction between E. coli thiogalactoside transacetylase antiserum and extracts from each of the plasmid carrying E.coli lines.

Thus on the basis of the limited information available on physical properties of the lactose utilisation proteins from the enteric bacteria, there would appear to be considerable similarity between the products from the E.coli, Shigella dysenteriae and various plasmid borne lac systems. However, there appeared to be little similarity between the E.coli lac operon encoded proteins and either the Ebg enzyme from E.coli, the β-galactosidase III from K.pneumoniae or those lac proteins analysed from other enteric species.

1.4.5 Genetic Studies of the Enterobacteriaceae which May be Used to Indicate Evolutionary Relationships.

Which of the genetic studies that have involved enteric species could be used, in part, to answer the original question concerning the evolutionary relationship of the lac systems found in the Enterobacteriaceae?

It has been suggested that there may be a tendency within members of bacterial families, such as the Enterobacteriaceae, towards conservation of gene location (Riley and Anilionis, 1978), a suggestion based on linkage map data. Therefore,do the lac genes map in a similar position throughout the enteric bacteria?

In E.coli K12 the lac genes are located at approximately 8 minutes on the chromosomal map (Bachmann, 1983), and

relevant adjacent markers are shown in Figure 1.6. Linkage maps have been produced for many other enteric species including Shigella dysenteriae (Schnieder and Falkow, 1964), Citrobacter freundii (De Graaff et al., 1974), Salmonella typhimurium (Sanderson and Hartman, 1978) Proteus mirabilis (Beck et al., 1982) Yersinia pseudotuberculosis (McMahon, 1973) Erwinia chrysanthemi (Katonjansky et al., 1982) Erwinia amylovora (Pugashetti et al., 1978) Erwinia carotovora (Forbes, 1983) Serratia marcescens (Matsumoto et al., 1974) K.pneumoniae (Matsumoto and Tazaki, 1970, 1971) and Enterobacter aerogenes (Matsumoto, 1973).

Unfortunately several of the chromosomal linkage maps are severely limited in the number of genes mapped and only in the K.pneumoniae linkage map have the lac genes been positioned (Matsumoto et al., 1971). These authors reported that the lac genes in the K.pneumoniae strain studied were congruent with the position of the lac genes in E.coli. However, in recent studies involving the production of R primes containing sections of the K.pneumoniae chromosome, the subsequent analysis of genes "picked up" by this process did not produce the expected high cotransfer of markers found adjacent to the lac genes in E.coli (Reeve unpublished). This strongly suggested that the lac genes were not adjacent to those genes which flanked the lac genes in E.coli, and by inference the K.pneumoniae lac genes were thus positioned differently on the chromosome, at least in the strain studied by Reeve.

Several of the linkage maps were produced using an F' lac episome to mobilise the chromosome (De Graaff et al., 1974; McMahon, 1973; Schneider and Falkow, 1964; Forbes, 1983; Chatterjee and Starr, 1977). Therefore the possibility existed that on the basis of the order and distance of the chromosomal markers mapped, the site of the F'lac integration into the chromosomes of the various

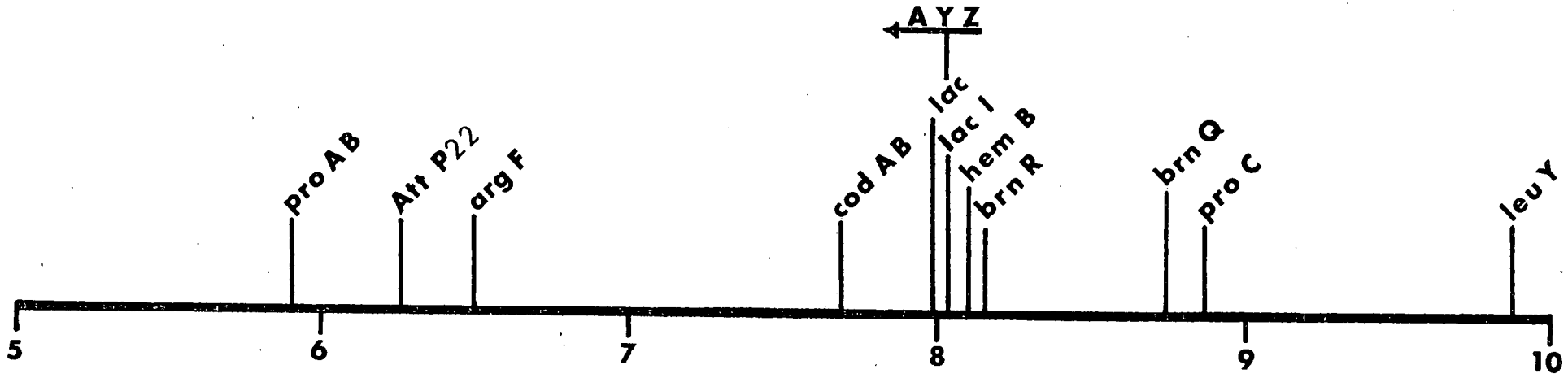


Figure 1·6

The position of the lac genes on the chromosome map of Escherichia coli, showing adjacent loci, (Bachmann, 1983). Arrow denotes direction of transcription.

enteric bacteria could be established and compared with the equivalent position of the lac genes in the E.coli chromosome. However, many of the chromosomal markers used in these studies were not well characterised thereby rendering direct comparison with the E.coli chromosomal map extremely difficult, with regard to establishing the site of F' lac integration.

There was also the possibility that integration by an F' lac into a chromosome could have been by recombination between non-lac chromosomal DNA on the plasmid, and a region of homology on the chromosome of the recipient bacterium. Furthermore it should be borne in mind that F-primes including F' lac, and the chromosomes of several different enteric species have been shown to carry insertion sequences (Nymen et al., 1981; Starlinger and Saedler, 1976). Therefore, the observed integration of the F' lac episomes into the chromosomes of the various enteric species may have involved recombination between the insertion sequences rather than between the lac DNA regions.

Thus, until considerably more detailed information is available concerning the exact linkage map positions of the lac genes within enteric species no conclusion may be drawn concerning conservation of gene location in respect to the lac genes.

An interesting point concerning the Salmonella typhimurium linkage map was the absence from the chromosome of lac DNA (Sanderson and Hartman, 1978). A comparison of the E.coli linkage map with that of Salmonella typhimurium showed that the two chromosomes differed from each other at a number of positions, by the addition or deletion of genetic material. Thus, when common markers were aligned together loops were formed. The lac genes were found on one such loop in the E.coli chromosome and were not present in the Salmonella typhimurium chromosome.

Recombination between the lac genes of E.coli and Shigella dysenteriae was reported by Sarkar (1966), which indicated that there existed a reasonable degree of DNA homology. The author also showed that the lac repressor-protein from E.coli could repress the Shigella dysenteriae β-galactosidase production, and conversely the Shigella repressor-protein could repress the E.coli lac operon. Thus both the E.coli and Shigella dysenteriae repressor proteins and operator-regions must have been very similar, which was not unexpected because it had been suggested that E.coli and Shigella should be classified as a single species (Brenner et al., 1972, 1973). This was not the case with the repressor of the K.pneumoniae chromosomal lac system which did not cross react with E.coli. Reeve and Braithwaite (1974) demonstrated that a consitutive lac I^- E.coli lac operon on an F' was not repressed when transferred into a K.pneumoniae strain. However, in the presence of melibiose the E.coli lac genes were repressed, and it was suggested that the melibiose formed an active complex with the K.pneumoniae chromosomal lac repressor, which could then repress the E.coli lac operon. The authors also showed that the E.coli chromosomal lac genes were repressed by the lac repressor protein coded by the lac I gene on F_K lac, a lac plasmid resident in K.pneumoniae (Reeve and Braithwaite, 1970). Recently a Lac I^- K.pneumoniae was constructed,which was lac constitutive, and which was not repressed by the E.coli repressor protein (Reeve, personal communication).

These results further supported the proposal by Reeve and Braithwaite (1973) that the lac genes on the F_K lac plasmid were derived from an ancestral E.coli chromosome, and that distinct differences existed between the chromosomal lac I gene in K.pneumoniae and that of the F_K lac plasmid and E.coli.

1.4.6 The Use of Nucleic Acid Hybridisation in Comparative Studies of the *Enterobacteriaceae*

It will be clear from the above discussion that the approaches used and data obtained provided little insight concerning the evolutionary relatedness between the *lac* systems within the *Enterobacteriaceae*. What was required was a system which, short of obtaining the complete nucleotide sequences for the genomic *lac* genes from each enteric species, would provide data on the nucleotide sequence homology between the various enteric *lac* systems.

An analytical procedure which appeared to satisfy this requirement was the nucleic acid reassociation technique in which homology between DNA sequences from different strains or species was assessed by the extent to which the complementary DNA or RNA strands cross hybridised. It was assumed that the extent and stability of the duplex formed after annealing would provide a direct-measure of the degree of relatedness between the two strands involved in the hybrid duplex.

Thus it had been shown that DNA duplexes formed between genomic DNA from *Shigella flexneri* and *E.coli* were more thermally stable than DNA duplexes formed between less related enteric species such as *E.coli* and *Proteus mirabilis* (reviewed in Brenner and Falkow, 1971; Brenner, 1973). This technique was applied to the study of the similarity between specific regions of the bacterial genome of several enteric species by using radioactive labelled mRNA, enriched for *lac* mRNA from *E.coli*, which was incubated with bound, denatured genomic DNA from various enteric species (Brenner *et al.*, 1969). The authors showed that the *Shigella* DNA-*E.coli* *lac* mRNA hybrid was more thermally stable than hybrids formed between *E.coli* *lac* mRNA and other enteric species tested, from six different genera. Brenner *et al.* (1969) also showed that the relative binding of the *E.coli* *lac* mRNA to genomic DNA from the various

enteric species studied was lower than the binding between E.coli bulk mRNA and the genomic DNA from the enteric bacteria. When the relative binding of E.coli 23S rRNA to genomic DNA from other enteric species was measured, it was shown to be considerably higher and considerably more uniform throughout the Enterobacteriaceae than had been observed for the lac mRNA relative binding.

Brenner et al. (1969) suggested that the results implied that, assuming a common ancestor, particular regions of the genome of enteric species had diverged at quite different rates. However an alternative possibility not suggested by Brenner et al. (1969) was that particular regions of the genome of enteric organism may have been acquired from different sources.

The basic approach to the question of relatedness of a specific region of genomic DNA between enteric species was taken a step further by the work of Riley and Anilionis (1980). The authors used the Southern transfer and hybridisation techniques (Southern, 1975) in order to bind restriction enzyme digested, genomic DNA, separated by agarose gel electrophoresis to nitrocellulose filters. In this way denatured restriction fragment profiles of several different enteric species were analysed. The tryptophanase (tna) gene, thymidylate synthetase (thy A) gene, the tryptophan operon (trp), and of particular interest the lac Z gene were all analysed using this method. Riley and Anilionis (1980) hybridised these probes to genomic DNA from species of nine genera within the Enterobacteriaceae. They found that the lac Z probe displayed exceptionally low levels of relatedness to all but one of the strains tested. The exception was Shigella dysenteriae which showed a high degree of relatedness and, as shown above, was sufficiently similar to E.coli to be regarded by some as almost inseparable from it. Between each genus the degree of relatedness

observed for the tna, trp and thy A DNA probes was considerably variable with Shigella displaying the highest homology figures. However, within each genus the difference between the degree of relatedness of the tna, trp and thy A DNA was much less variable. The limited conservation of fragment size displayed by the fragments which hybridised to the tna, thy A and trp DNA probes, was absent from the fragments hybridising to the lac Z DNA probe.

Riley and Anilionis (1980) suggested the above results indicated that portions of the genome including tna, trp and thy A appeared to have evolved in concert with the genome as a whole, but that by contrast the lac Z varied considerably from the tna trp and thy A, possibly reflecting a separate evolutionary origin.

Following from the work of Riley and Anilionis (1980) a DNA fragment containing lac genes from K.pneumoniae was cloned into the multicopy plasmid pBR322 (McDonald and Riley, 1983). Restriction enzyme analysis of the fragment and comparison with that of E.coli showed an absence of common restriction sites within the lac region, for those restriction enzymes used. In total, the results described above invited speculation and further investigation into the relationship between the lac systems found in enteric species for the following reason.

1.5 The Next Step

Although this project was started prior to the publication of the work of Riley and Anilionis (1980) other work described above and in particular that of Brenner et al. (1969) had indicated that similarities between the E.coli lac operon and lac systems in other enteric species were confined to the Shigella species and those lac plasmids analysed. This conclusion made untenable a hypothesis that the chromosomal lac systems in the Enterobacteriaceae were of an ancestral E.coli origin and had been acquired via F' lac mediated

transfer. It also begged the question that as the *E. coli* and *Shigella* *lac* systems were in general atypical of other chromosomal *lac* systems observed in the *Enterobacteriaceae*, had *E. coli* and *Shigella* acquired their *lac* genes by lateral transfer? Following directly from this was the question concerning how similar to each other were the *lac* systems observed in enteric species excluding *E. coli* and *Shigella*? It therefore appeared essential to analyse in depth an enteric chromosomal *lac* system which had been shown to be dissimilar to the *E. coli* *lac* operon, and which could be used to answer the following questions.

Could confirmation be obtained for the hypothesis that the *lac* system from an enteric species was demonstrably different from the *E. coli*/*Shigella* *lac* systems?

Were the *lac* systems of the enteric species, excluding those of *Escherichia* and *Shigella*, demonstrably similar to each other and considerably different from the *Escherichia* and *Shigella* *lac* systems as would be expected if only *Escherichia* and *Shigella* species had acquired a *lac* system by lateral transfer? If, on the other hand, other genera had also acquired *lac* by lateral transfer but in separate evolutionary events to *Escherichia* and *Shigella* then the remaining enteric bacteria would have *lac* systems which were evolutionary related, but to varying degrees. Could sufficient differences between the chromosomal *lac* systems in the remaining enteric species be demonstrated in support of this hypothesis?

A subsidiary or tangential question was also asked how similar was the *lac* system on F_K*lac* to the chromosomal *lac* system of an enteric species other than *Escherichia* and *Shigella*, such as *Klebsiella*.

Table 2.1. Bacterial, phage and plasmid strains.

Strain	Description	Source
BACTERIAL STRAINS		
Escherichia coli		
K-12 λ (EGSC5073)	wild *E.coli*	JA
RE254	F^- *lac his trp* str^R nal^R	RE
RE400	F^- *lac his trp* str^R nal^R /T_K	RE
RE412	*lac his trpR gal bio* sup^o rK^- mK^+ str^R nal^S	RE
RE431	F^- *lac his met proAB recA* str^R	RE
RE439	F^- ΔXIII(*proAB argF lac*) *argI thi* rK^- mK^-	RE
RE476	F^- ΔXIII(*proAB argF lac*) *argI thi* rK^- mK^- *recA*	this work
W1485 (EGSC5024)	wild *E.coli* K-12 λ	JA
Klebsiella		
M5a1	wild strain (UNFS)	RE
V9A (RE1401)	wild strain carrying F_k*lac* and T_k	RE
RE1435	F_k*lac* 'free', Tc^S variant of RE1401	RE

Strain	Description	Source
Other *Enterobacteriaceae*		
Citrobacter freundii (ATCC8090)		JA
Enterobacter aerogenes (ATCC13048)		JA
Erwinia carotovora (SCRI193)		FK
E.chrysanthemi (SCRI409)		FK
E.herbicola (SCRI427)		RE
Hafnia alvei (NCTC6578)		JA
Proteus morganii (NCTC5710)		JA
Salmonella typhimurium (NCTC5710)		SJ
Serratia marcescens (NCTC1377)		JA
Shigella sonnei (NCTC8574)		SJ
PHAGE STRAINS		
λ *b221 rex*::Tn5 *cI857* O_{am8} P_{am29}	Km	JA
Mu *cts62*		RE
P1 *clr100* Km	thermoinducible, Km	RE

Strain	Description		Source
PLASMID STRAINS			
F_k lac		Lac^+	RE
F9505	[73.3]	F^+::Tn10, Tc	FK
pATC58	[280]	A. tumefaciens C58 cryptic plasmid	FK
pBR322	[2.9]	Ap Tc	SD
pCM21			BJ
pHE1		Lac^+ Ap	this work
pHE3		Lac^+ Ap	this work
pHE6		Ap	this work
pHE7		Ap	this work
pHE8		Lac^+ Ap Tc	this work
pHE10 (pHE8 lacY::Tn5)		Ap Km	this work
pKF1	[63]	R68::Mu $c^+\Delta$445-7	FK
pMG1	[320]	Gm Hg Sm Su Uv	FK
pTi-C58	[123]	A.tumefaciens C58 Ti plasmid	FK
R6K		Ap Sm	SD
RK2	[37]	Ap Km Tc	SD
RP4	[37]	Ap Km Tc	FK
Rts1::Tn1725		Cm Km	JA
T_k		Tc	RE
pHE11		Ap	this work

Sources of bacterial, phage and plasmid strains were as follows. ATCC: American Type Culture Collection, Rockville, Maryland, USA. BJ: J.Bishop, Edinburgh University, Edinburgh, UK. EGSC:E.Coli Genetics Stock Center. FK: K.Forbes, Edinburgh University, Edinburgh, UK. JA: A.Jenkins, Edinburgh University, Edinburgh, UK. NCTC: National Collection of Type Cultures, Central Public Health Laboratory, London. RE: E.Reeve, Edinburgh University, Edinburgh, UK. SCRI: M.Perombelon, Scottish Crop Research Institute, Dundee, UK. SD: D.Sherratt, Glasgow University, Glasgow, UK. UNFS: Unit of Nitrogen Fixation, Sussex University, Falmer, UK. SJ: J.Stuart, Bacteriology, Edinburgh University, Edinburgh, UK.

Nomenclature was as recommended by Demerec (1966) for bacterial strains, Novick et al. (1976) for plasmids, Campbell et al. (1977) for transposable elements and Howe and Bade (1975) for phage Mu.

Plasmid molecular weights [Md] are from; F95O5: Sharp et al., 1972, Halling et al., 1982; pAtC58: Villaroe and Van Montague pers. comm. in Rosenberg et al., 1982; pBR322: Sutcliffe, 1978; pKF1: Thomas, 1981 and Martuscelli et al., 1971; pMG1: Hansen and Olsen, 1978; pTiC58: Watson et al., 1975; RK2: Thomas, 1981; RP4: Meyer et al., 1977.

CHAPTER 2

MATERIALS AND METHODS

2.1. Introduction

This chapter describes in detail the bacterial strains, plasmids and phage used in this study and also includes the materials employed and methods used in the analysis of DNA and proteins. Where used,modifications to previously published procedures are described, and unless stated to the contrary all procedures mentioned in the text are as described within this chapter.

2.2.1 Bacterial, Phage and Plasmid Strains

The bacteria, phage and plasmids used in this study are listed in Table 2.1.

2.2.2 Abbreviations.

AGE, agarose gel electrophoresis; c-AMP, adenosine 3', 5'-cyclic monophosphate; BSA, bovine serum albumin; EDTA (Na_2), ethylene diamine tetra acetic acid di-sodium salt; IPTG, isopropyl-β-D-thiogalactoside; Kb, kilobase pairs; M.dal, megadalton; ONPG, orthonitrophenyl-β-D-galactoside; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; TONPG, orthonitrophenyl-β-D-thiogalactoside; Tris, Tris (hydroxymethyl) aminomethane; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D- galactoside.

2.2.3 Chemicals

Chemicals and solvents used in this work were analytical grade (A.R.) unless otherwise described. Restriction enzymes and E. coli DNA polymerase 1 were obtained from Boehringer Mannheim and T4 DNA ligase was purchased from Miles Chemicals Ltd.

2.2.4 Solutions

AGE sample buffer consists of:- 10% Ficoll 400,000; 0.5% SDS; Orange G; in either TA or TB buffer.

Denhardt Solution consists of: 0.02% B.S.A.; 0.02% Ficoll 400,000; 0.02% polyvinyl pyrrolidine; in distilled water and stored as a 50 x concentrate at -20°C.

Eckhardt Solution I, 100ml contains:- 1.08g Tris base; 0.42g EDTA (Na_2); 0.55g Boric acid; 20g Ficoll 400,000; 0.37mg Ribonuclease 1 (82u/mg); 0.1mg bromophenol blue; in distilled water.

Eckhardt Solution II, 100ml contains:- 10g Ficoll 400,000; 0.2g SDS; in distilled water.

Eckhardt Solution III, 100ml contains:- 5g Ficoll 400,000; 0.2g SDS in distilled water.

Hybridisation Solution consists of:- 50% formamide (Bethesda Research Laboratories 'Ultrapure grade'); 3 x SSC; 1 x Denhardts Solution: 0.3% SDS; 250µg/ml of sonicated and heat denatured calf thymus DNA; in 50mM sodium phosphate buffer, pH 6.5.

PAGE sample buffer consists of:- 0.0625M Tris pH 6.8; 2% SDS; 20% glycerol; 0.01% bromophenol blue; in distilled water and 1/10 volume of mercaptoethanol was added to the buffer before use.

SSC solution consists of:- 0.15M sodium chloride; 0.015 sodium citrate (pH 7.0); in distilled water.

TA buffer consists of:- 40mM Tris (pH 8.1) (Trizma pH 7-9, Sigma, London); 20mM acetic acid; 2mM EDTA (Na_2); in distilled water.

TB buffer consists of:- 89mM Tris (pH 8.3)(Trizma pH 7-9 Sigma, London); 89mM boric acid; 2.5mM EDTA (Na_2); in distilled water.

TE buffer consists of:- 10mM Tris (pH 8.0); 1mM EDTA (Na_2); in distilled water.

Tris/Glycine buffer consists of:- 0.025M Tris (pH 8.3); 0.192M glycine; 0.1% SDS; in distilled water.

2.2.5 Media

Nutrient broth (NB):- 8g nutrient broth powder (Difco) plus 5g sodium chloride per litre of distilled water. 10ml 20% sugar, as required, added per litre after autoclaving.

Nutrient agar (NA):- as above solidified with 1.5% Difco Bacto agar.

L. broth (LB):- 10g tryptone (Difco), 5g yeast extract (Difco), 5g sodium chloride, 1g glucose, per litre of distilled water.

L. agar (LA):- as above solidified with 1.5% Difco Bacto agar.

Top agar (TA):- 15.5g tryptone (Difco), 8.75g yeast extract (Difco), 0.5g NaCl, 4.25ml 1M sodium hydroxide, 5g Difco Bacto agar per litre of distilled water.

MacConkey (lactose) agar (ML):- 51.5g MacConkey agar No.3 (Oxoid) per litre of distilled water.

MacConkey (sugar free) agar (M):- 20g peptone (Difco), 1.5g bile salts No.3 (Oxoid), 5g sodium hydroxide, 30mg neutral red, 1mg crystal violet, 15g Difco-Bacto agar per litre of distilled water; sugars added to 1%.

Hobson salts (10x):- 105g di-potassium hydrogen orthophosphate, 10g tri-sodium citrate, 45g potassium di-hydrogen orthophosphate and 10g ammonium sulphate per litre of distilled water.

Hobson medium (HM):- 1ml of 0.1M magnesium sulphate, 1ml 20% sugar and 10ml of Hobson salts (10x) added to 87ml of autoclaved distilled water. Magnesium sulphate and sugar solutions were autoclaved separately.

Hobson agar (HA):- As above solidified with 1.5% Difco Bacto agar.

X-Gal agar:- Hobson agar plus X-Gal at a final concentration of 40µg/ml, glucose at 0.2% and iso-propyl-β-D-thiogalactoside (IPTG) at a final concentration of 5 x 10^{-4}M, when added.

TONPG agar:- Hobson agar plus TONPG at a final concentration of 10^{-3}M, IPTG at a final concentration of 10^{-5}M and 0.2% sodium succinate.

Amino acids and vitamins:- Added at a final concentration of 50µg/ml as required. Casamino acids (Difco) used at 0.15% final concentration.

Unless stated otherwise in the text, antibiotics were used in the media described above at the following final concentrations:- ampicillin, 20µg/ml; chloramphenicol, 20µg/ml; kanamycin, 40µg/ml; tetracycline, 10µg/ml.

Tryptophan, vitamins and antibiotics were sterilised by filter sterilisation, all other media were autoclaved at 15p.p.s.i. for 15 minutes.

2.3 Methods

2.3.1. Bacterial Strain Construction

A *rec A* mutant of *E. coli* K12 strain RE 439 (F^- *thi* ΔxIII [*pro AB arg F lac*] *arg* I r^-m^-) was needed as a recipient for the hybrid *lac* plasmids produced in this work. To produce such a mutant use was made of the fact that the genes for sorbitol utilisation (*srl*) and *rec A* are cotransducible

by the phage P1. Thus the temperature sensitive phage Mu cts 62 (Abelson *et al.*, 1973) was used to produce *srl* mutants of RE 439, following the method described by Bukhari and Ljungquist (1977). A phage P1 *clr 100* Km lysate was made on a sorbitol-positive, *rec A* host (RE 431) and was used to transfect the RE 439 *srl* line, as described by Goldberg *et al.* (1974). Following selection for sorbitol utilisation, bacteria found to be positive were screened for cotransduction of *rec A* by the method of Clark and Margulies (1965). A mutant line found to be *rec A* was designated RE 476.

2.3.2 Chromosomal DNA Preparation

A modified version (Forbes, 1983) of the method of Schwinghamer (1980), in which the prewashing of cells in Sarkosyl prior to lysis was omitted, was used to isolate chromosomal DNA from members of the *Enterobacteriaceae*. Bacterial strains were grown to stationary phase in 20ml of LB then harvested and washed twice in TE buffer. With all manipulations performed at between 0-5°C, the cells were resuspended in 0.6ml of sterile 25% sucrose - 0.05M Tris (pH 8.0) solution and then 0.2ml of lysozyme solution (1mg/ml in TE buffer) was added. After 5 minutes, 6.4ml of TE buffer was added, followed by the addition of 0.8ml of 10% sarcosyl in TE buffer. The suspension was mixed gently for 5 seconds and 8.08g of caesium chloride was added, followed by 0.5ml of ethidium bromide (10mg/ml in TE buffer). The samples underwent ultracentrifugation for 40 hours at 45,000 rpm in a R65 rotor (Beckman) at 18°C. DNA in the gradient formed was visualised by UV illumination and was collected by puncturing the side of the centrifuge tube with a No. 18g needle.

Ethidium bromide was removed from the DNA sample by extractions with propan-2-ol saturated with caesium chloride

in TE buffer. Caesium chloride was removed by dialysis against chilled TE buffer containing Na^+ Dowex 50w ion exchange resin.

The DNA solution was made 0.3M for sodium acetate (pH 8.0) and precipitated with 2.5 volumes of chilled 100% ethanol and storage at -70°C for one hour. The DNA precipitate was pelleted, washed twice in 70% ethanol, briefly vacuum desiccated and resuspended in 0.5ml of TE buffer.

DNA concentrations were established by spectrophotometric readings at 260nm (Humphreys et al., 1975), and DNA samples were stored in TE buffer at -20°C.

2.3.3 Plasmid DNA Isolation

The method of Hansen and Olsen (1978) was used to isolate the F'_k lac plasmid from the original Klebsiella host strain (Klebsiella V9A) and from the E. coli strain RE 254 into which the plasmid had been mated.

Two litres of cells in HM supplemented with casamino acids, thiamine, tryptophan and 0.2% lactose were grown shaking at 37°C to an OD_{425} of 2.0 and were treated as per the method of Hansen and Olsen (1978). The resulting suspension was made 7.7M for caesium chloride (Humphreys et al., 1975) which precipitated the polyethylene glycol in the suspension and which was removed by centrifugation at 3000G for 5 minutes. Ethidium bromide was added to the suspension at a final concentration of 100µg/ml. Covalently closed circle (c.c.c.) plasmid DNA was isolated from the suspension by the dye-buoyant-density method of Radloff et al. (1967) with the samples undergoing ultracentrifugation as described in Section 2.3.2. Plasmid c.c.c. DNA (seen as the lower band in the caesium chloride gradient) was visualised, collected, treated and stored as described for chromosomal DNA in Section 2.3.2.

A scaled-up version of the method of Birnboim and Doly (1979) was used for the isolation of pBR322 and all the hybrid plasmids produced in this work. Plasmid-containing strains were grown shaking overnight, at 37°C, in 250ml of LB containing the appropriate antibiotic. The cells were pelleted, washed in TE buffer, pelleted once more and resuspended in a small amount of residual buffer. 10ml of a solution containing 4mg/ml lysozyme, 50mM glucose, 10mM EDTA (Na_2) 25mM Tris (pH 8.0) was added to the cells and the mixture was vortexed briefly, then held at 0°C for 30 minutes. Cell lysis was produced by the addition of 20ml of 1% SDS in 0.2M sodium hydroxide. The addition of 15ml of 3M sodium acetate (pH 4.8) to the suspension followed by storage at 0°C for one hour precipitated the contaminating macromolecules which were removed by centrifugation. The DNA in the supernatant was precipitated by the addition of 2 volumes of ethanol, and storage at -70°C for one hour. The DNA precipitate was pelleted, resuspended in 8ml of TE buffer and c.c.c. plasmid DNA was isolated by the dye-buoyant-density method of Radloff et al. (1967) described in Section 2.3.2. The subsequent treatment and storage of the plasmid DNA was also as described in Section 2.3.2.

2.3.4 Plasmid Molecular Weight Estimation by Agarose Gel Electrophoresis.

A modified version of the method of Eckhardt (1978) was used to estimate the molecular weight of plasmids. A vertical 0.8% agarose gel in TB buffer with sample wells capable of holding 200µl was used. 0.3ml of overnight bacterial culture in LB was harvested and the supernatant poured off. The modification involved the thorough resuspension of the cell pellet in Eckhardt Solution 1 prior to the addition of cells to the gel well, as opposed to the original method in which the cells were resuspended in Eckhardt solution 1 in the gel well. The samples were

therefore treated individually in the following way, 40µl of Eckhardt solution 1 was added to the cell pellet which was vortexed for 15 seconds, and the suspension was then loaded into a dry gel well. This procedure was repeated for each sample. Each gel well was overlayed with 40µl of Eckhardt solution II followed by 100µl of Eckhardt solution III. The gel wells were sealed by the addition of hot 0.8% agarose in TB buffer and the gel was electrophoresed in TB buffer for one hour at 8m amps on constant current. This was then increased to 40m amps and the gel electrophoresed until the dyefront had reached the base of the gel. For maximum resolution of large plasmids, the electrophoresis was carried out at 20m amps overnight. Gels were stained for one hour in TB buffer containing 0.5µg/ml ethidium bromide and were photographed using a model C62 transilluminator (U.V. Products Inc., USA), Ilford FP4 5" x 4" Professional sheet film and an orange filter (Hoya). Using plasmids of known molecular weight as standards on each gel, a graph was plotted of molecular weight (log) versus distance travelled, which allowed the molecular weight of the plasmid under investigation to be estimated (Meyers et al., 1976). Plasmid molecular weight in M.dal may be converted to kilobase pairs of duplex DNA by multiplying by 1.5 (Old and Primrose, 1981).

2.3.5 Plasmid Size Estimation by Electron Microscopy

Plasmid DNA was isolated from bacterial cultures as described in Section 2.3.3, and was prepared for visualisation in the electron microscope by the method of Davis et al. (1971), using the aqueous technique. Following the staining and drying steps, the copper grids bearing plasmid DNA were rotary-shadowed using 6-7cm of platinum/palladium wire on a double tungsten filament at about 8° and 8.5cm to the grids. The sizes of the plasmids were established by

comparing the contour lengths of the "standard molecules" (pCM 21, Bishop, 1979) with those under investigation.

2.3.6 Transformation of *E. coli* Strains with Plasmid DNA

The method of Dagert and Ehrlich (1979) was used to produce competent *E. coli* cells in which 0.2ml of cells were transformed with 1µg of plasmid DNA. The cells were then plated on LA containing the relevant antibiotic(s) and incubated overnight at 37°C. Routinely an aliquot of competent cells, minus DNA, was treated as above to act as a control. Transformants were repurified on HA containing the necessary growth supplements and relevant antibiotic(s).

When a maximum of transformants per µg DNA was not required competent cells prepared by the bulk preparation and long term storage procedure of Morrison (1977) were used.

2.3.7 Restriction Enzyme Digestion and Ligation Conditions

Both the digestion and ligation conditions employed in the production and analysis of hybrid plasmids were those described by Davis *et al*. (1980). When restricting chromosomal DNA samples from enteric species spermidine tri-hydrochloride (pH 7.0) at a final concentration of 20mM was routinely added to enhance full digestion of the DNA. The spermidine was added to the DNA solution and the mixture was left on ice for 5 minutes before the addition of the restriction enzyme (Bouche, 1981).

2.3.8 Agarose Gel Electrophoresis (AGE)

DNA fragments produced by restriction enzyme digestion were separated by electrophoresis through a vertical 1% agarose gel using TA buffer and run at either 70 volts (3.5 volts/cm) for 6 to 7 hours or 30 volts (1.5 volts/cm) overnight. A 0.2 volume of AGE 'sample buffer' was added

to the digested DNA samples before loading onto the gel. Restriction fragments of known size, produced by the digestion of λcI_{857} DNA with either Hind III or Eco R1, or both were used to estimate the size of the DNA fragments being studied. This was achieved by plotting a graph of mobility versus size similar to the plasmid molecular weight estimation by agarose gel electrophoresis described in Section 2.3.4.

The screening of low molecular weight plasmid DNA (<30 x 10^6 daltons) was done by electrophoresis through a vertical 0.8% agarose gel with TB buffer at 40 volts overnight. For rapid screening of these plasmids a "mini-gel" apparatus was employed, which consisted of a horizontal 0.8% agarose gel measuring 10cm x 5cm. Electrophoresis in TB buffer at 80 volts for 1 to 1.5 hours produced sufficient separation for convenient plasmid analysis.

2.3.9 Extraction of DNA Fragments from Agarose Gels

The method of Yang et al. (1979) was used to remove DNA restriction fragments from agarose gels. The method involved initial separation of the fragments by electrophoresis through a horizontal 0.8% agarose gel in TA buffer. The electrophoresis was then stopped and a dialysis membrane was placed in the path of the DNA fragment to be isolated. Electrophoresis was then continued and the DNA fragment was collected against the dialysis membrane then removed in a small volume of electrophoresis buffer and purified as described by Yang et al. (1979).

2.3.10 Transposon Insertion Mutagenesis of lac Genes

2.3.10.1. With Tn5.

Bacterial strains from which fresh λ::Tn5 lysates were to be prepared were grown overnight at 37°C in NB supplemented with maltose. The cells were pelleted and resuspended in

approximately 0.4 x the original volume of sterile 0.01M magnesium sulphate. Plate lysate stocks were prepared and assayed as described by Maniatis et al. (1982) and the λ::Tn5 containing supernatants were stored at 4°C with 0.3% chloroform.

E. coli strain RE 412 (pHE8) was grown at 37°C in NB plus 0.4% maltose and ampicillin to a density of approximately 10^9 cells/ml and then infected with λ::Tn5 at a multiplicity of 0.1 particles per cell (Berg, 1977). Following resuspension in 10ml of LB and incubation for 60 minutes at 30°C, the infected cells were pelleted, resuspended in 2ml of sterile saline and 0.1ml aliquots spread on LA containing ampicillin and kanamycin to select for cells in which Tn5 transposition had occurred.

Colonies from the selection plates were pooled and plasmid DNA was isolated from the cell suspension by the scaled up method of Birnboim and Doly (1979). The DNA was transformed into the E. coli strain RE 476 by the method of Dagert and Ehrlich (1979). Transformants containing pHE8 with Tn5 insertion-inactivated lac genes were selected initially on ML containing ampicillin and kanamycin, with a Lac-negative phenotype indicating insertion into either the lac POZ or lac Y genes. A Lac-negative phenotype on ML accompanied by failure to produce β-galactosidase on X-Gal agar containing ampicillin, kanamycin and IPTG, was taken to indicate transposon insertion into either the lac PO or Z DNA. A Lac-negative phenotype on ML accompanied by β-galactosidase production on X-Gal agar, containing ampicillin, kanamycin and IPTG, inferred transposon insertion into the lac Y gene. This was confirmed by growth tests on TONPG agar plus IPTG and ampicillin and kanamycin.

The Tn5 insertion sites in pHE8 were mapped using the known Bam Hl and Hind III restriction sites in pHE8 (Chapter 3, this work) and Tn5 (Jorgenson et al., 1979) as described in Chapter 3.

2.3.10.2 With Tn 1725.

The plasmid RTS1::Tn1725 (Tn1725 carries chloramphenicol resistance) was transferred by membrane mating on LB to RE 476 (pHE8). The recipient strain was grown on HM plus glucose, ampicillin, chloramphenicol and the necessary amino acid requirements at 40°C to reduce the frequency of the temperature sensitive RTS1::Tn1725 in the cell population, but retain RE 476 (pHE8) in which Tn1725 transposition had occurred.

Plasmid DNA was isolated from this culture by the scaled up version of the method of Birnboim and Doly (1979) described above. The plasmid DNA was transformed into RE 476 by the method of Dagert and Ehrlich (1979) and transformants containing pHE8 with a transposon insertion-inactivated *lac* gene were selected on ML containing ampicillin and chloramphenicol. Identification of the *lac* gene inactivated, was as described above for Tn5 insertions but chloramphenicol instead of kanamycin was used in the selection media.

The Tn1725 insertion sites in pHE8 were mapped using the known *Eco* R1 and *Hind* III restriction sites in pHE8 (Chapter 3, this work) and Tn1725 (Schmitt, pers.comm.; Altenbuchner *et al.*, 1983, and Schöffl *et al.*, 1981) as described in Chapter 3.

2.3.11 DNA-DNA Hybridisation

2.3.11.1 Transfer of DNA to Nitrocellulose Filters

The DNA samples for hybridisation analysis were digested with either *Hind* III or *Eco* R1 as described in Section 2.3.7. The DNA fragments produced were separated by agarose gel electrophoresis, and the gels were stained and photographed as described in Sections 2.3.8 and 2.3.4 respectively.

The method of Southern (1975) was used to transfer DNA

from the agarose gel to the nitrocellulose filter. As suggested by Wahl et al. (1979) a light depurination step which consisted of two 15 minute washes in 250ml of 0.25M hydrochloric acid was included, to increase the efficiency of transfer of large DNA fragments to the nitrocellulose filter.

After drying, and before the ^{32}P labelled probe DNA was added, the filters were incubated in 20ml of hybridisation solution at 37°C for three hours (shaking gently) in heat sealed polyethylene bags.

2.3.11.2 Preparation of ^{32}P labelled DNA

Plasmid or DNA fragments were ^{32}P-labelled by nick-translation and the reaction was terminated after 90 minutes at 14°C, all as described by Davis et al. (1980). Incorporation of labelled nucleotide into the probe DNA, as judged by the method described by Maniatis et al. (1982) was routinely between 45% and 50% of the total labelled nucleotide used. The reaction mixture then underwent one phenol-chloroform extraction, followed by one back extraction of the phenol-chloroform phase with 50µl of TE buffer. The incorporated and non-incorporated labelled nucleotides were separated by passage down a Bio-Gel P60 (100-200 mesh) 'mini column' prepared in a siliconised pasteur pipette containing a siliconised glass wool plug. The passage of the ^{32}P labelled DNA probe through the 'mini column' was followed using a hand monitor, and when collected the labelled probe DNA was denatured by heating at 95°C for 10 minutes.

2.7ml of a 50% dextran sulphate solution (previously dissolved at 65°C) was added to 5ml of hybridisation solution (pre-incubated at 37°C) and thoroughly mixed. The denatured labelled probe DNA was added to this solution gently mixed and the solution immediately added

to the nitrocellulose filters pre-incubating in 20ml of hybridisation solution (described in the previous section). The nitrocellulose filters in their hybridisation mixture were maintained overnight at 37°C shaking gently. They were then washed three times for 5 minutes at room temperature in 250ml aliquots of a solution containing 2 x SSC and 0.1% SDS. This was followed by two 15 minute washes in 250ml aliquots of a solution containing 0.2 x SSC and 0.1% SDS at 45°C.

The nitrocellulose filters were then dried and exposed to Kodak Ortho-G X-ray film and intensified with a Kodak Lanex Regular screen in a Kodak X-omatic cassette at -70°C for 4 to 7 days.

2.3.11.3 Homolog Size Estimation

The sizes of the DNA fragments which formed homologs with labelled DNA were estimated in the following way. DNA fragments of known size, Hind III digested pBR322 (Sutcliffe, 1979) and the 2.5kb Hind III fragment from pHE8 (this work) which would hybridise with the labelled probe DNA used in this work, were added to samples of restricted λcI_{857} DNA, used as size standard markers in the agarose gels. The homologs formed between labelled probe DNA and these DNA fragments of known size allowed the autoradiograms to be aligned on life size photographs of their respective agarose gels. This in turn allowed the λcI_{857} standard fragments seen in the photographs to be used to estimate the size of other homologs produced.

This method was used because Riley and Anilionis (1980) had shown that labelled λDNA formed homologs with the restricted genomic DNA from a number of enteric species. These homologs were similar in size to those formed between E. coli lac DNA and the restricted genomic DNA and had partially obscured the lac DNA homologs. Therefore the

addition of labelled λDNA to hybridisation mixtures was omitted.

2.3.11.4 Estimation of the Relative Amounts of Labelled DNA Involved in Homolog Formation

Autoradiograms were traced with a Joyce-Loebl densitometer using a fixed ratio arm. The density of each band was established by measuring the area under each peak, minus the baseline count, using a Joyce-Loebl automatic integrater linked to the densitometer.

The accurate resolution of all of the bands observed on an autoradiogram was not possible, using a single wedge, because of the large differences in the densities displayed by the various homologs. Therefore the dense bands were traced using a wedge suitable for their resolution, and the wedge was then replaced with one sufficiently sensitive to resolve the less dense bands. A number of bands were separately traced using each wedge. The two sets of figures thus obtained were used to establish a ratio between the two wedges from which all readings could be transferred to the same scale.

2.3.12 Polypeptide Chain Molecular Weight Determination by SDS-PAGE.

Polypeptide molecular weights were estimated by SDS-PAGE using the Tris-glycine buffer system of Laemmli (1970).

2.3.12.1 Preparation of Protein Samples

Parallel samples of RE476 containing either pHE1, pHE8 or pHE10 were grown with and without induction by IPTG (10^{-4}M) in HM with the necessary supplements and antibiotic(s) with 0.4% glycerol as a carbon source. The cells were pelleted, washed once in TE buffer and then resuspended in 40µl of 'Z buffer' (Miller, 1972) plus 5µl of 20% SDS and one drop

of chloroform. The suspension was vortexed for 30 seconds, the cell debris pelleted and 20µl of the supernatant was added to an equal volume of 2 x 'PAGE sample buffer'. This solution was maintained for 2.5 minutes in boiling water to thoroughly denature the sample and the 40µl were then loaded onto the polyacrylamide gel.

2.3.12.2 Preparation of Polyacrylamide Gels and Electrophoresis of Proteins

Polyacrylamide gels contained a 5% acrylamide stacking section and a 7.5% acrylamide separating section, and were prepared from a stock solution of 30% by weight acrylamide and 0.8% N, N'-bis-methylene acrylamide.

The stacking section also contained 0.125M Tris-HCl (pH 6.8) and 0.2% SDS (final concentrations) and the separating section also contained 0.375M Tris-HCl (pH 8.8) and 0.1% SDS. The electrode buffer (pH 8.3) contained 0.025 Tris-HCl, 0.192M glycine and 0.1% SDS.

The samples were electrophoresed through the stacking gel at 100 volts and then run at 180 volts through the separating gel for approximately 5 hours. The gels were stained for 30 minutes at 65°C in a solution of 45% ethanol, 10% glacial acetic acid and 45% distilled water containing Coomassie brilliant blue at a final concentration of 2mg/ml. The gels were diffusion-destained in a similar solution to that described above, minus the Coomassie brilliant blue. The gels were photographed using Ilford FP4 roll film.

2.3.13 β-Galactosidase Assay

β-galactosidase activity was assayed as described by Miller (1972), from bacterial cultures grown in HM on 0.4% citrate as a carbon source for Klebsiella strains and 0.4% glycerol as a carbon source for all other enteric species used. Parallel cultures were grown with and without

induction for 90 minutes by IPTG at 10^{-4}M.

Chloroform and SDS were used to make the bacterial cells permeable to ONPG, following Miller (1972).

β-galactosidase activity was expressed as units of enzyme in 1ml of cells at OD_{600}1.0 which produced 1 n-mole O-nitrophenol/minute at 28°C and pH 7.0 (assuming a molar extinction coefficient of 4500 at 420nm, Miller, 1972).

The formula used was:-

$$\text{Units of } \beta\text{-galactosidase activity} = \frac{OD_{420} - 1.75 \times OD_{550}}{RT \times V \times OD_{600} \times 0.0045}$$

2.3.14 Thiogalactoside Transacetylase Assay

Thiogalactoside transacetylase activity was assayed as described by Miller (1972) from bacterial cultures grown overnight with induction by IPTG (10^{-4}M) in HM on 0.4% citrate as a carbon source for Klebsiella strains and 0.4% glycerol as a carbon source for all other enteric species used. The cell concentrations were increased by centrifugation and resuspension in smaller volumes of TE buffer and the cells were then disrupted by sonication for four periods of 30 seconds with an MSE 150 Watt Ultrasonic Disintegrator (at Low power and amplitude 1).

Cell debris was removed from the samples by centrifugation and aliquots of supernatant were removed for β-galactosidase assay (as described above). The supernatant was then heat treated at 70°C for 5 minutes and assayed for thiogalactoside transacetylase activity as described by Miller (1972). Enzyme activity was calculated as units of enzyme which formed 1nmole of thionitrobenzoic acid per minute at 28°C, assuming a molar extinction coefficient of 13,300 for thionitrobenzoic acid at 412nm (Fried, 1980).

2.3.15 Measurement of the Effect of Cloned *lac* Gene Induction on RE476 Growth Rate

Cultures of RE476, either plasmid free or containing pHE1 pHE8 or pHE10, were grown in HM containing the necessary supplements, antibiotic(s) and 0.4% glucose as a carbon source. Parallel cultures were grown with and without induction by IPTG (10^{-4}M) and the growth rates of these cultures were monitored by measuring their optical densities at 550nm every hour. The above procedure was repeated using 0.4% glycerol instead of glucose as a carbon source.

CHAPTER 3

THE CLONING AND PHYSICAL CHARACTERISATION OF THE LACTOSE UTILISATION SYSTEMS IN KLEBSIELLA

3.1 Introduction

As described in Chapter 2 certain Klebsiella strains possessed both a chromosomal and plasmid borne lac system, the former of which had been shown to be dissimilar to the E. coli lac operon, whereas the latter shared common characteristics with the E. coli system. Cloning of these different lac systems found in Klebsiella would allow their DNA and protein products to be compared with their counterparts in other members of the Enterobacteriaceae. The results obtained could provide an indication as to the general relatedness to each other of the lac systems present in Klebsiella, and their relatedness to the chromosomal lac systems in other enteric species.

Klebsiella pneumoniae M5al (Hamilton and Wilson, 1958) was chosen as the source of Klebsiella chromosomal lac genes, rather than Klebsiella aerogenes V9A (Reeve and Braithwaite, 1970) whose chromosomal lac system had been extensively studied by Reeve and coworkers, because when the work was undertaken only Klebsiella M5al complied with the Genetic Manipulation Advisory Group guidelines for cloning work in our laboratory.

Klebsiella M5al was analysed for the presence or absence of a lac plasmid and the chromosomal lac genes were then cloned using a direct in vitro approach whereby restriction fragments of chromosomal DNA were ligated into the plasmid cloning vector pBR322 (Bolivar et al., 1977a,b). Fragments of chromosomal DNA of a suitable size were produced by digesting the DNA with Hind III which has a hexanucleotide recognition sequence, and the possibility of

cutting the DNA within the lac genes was reduced by using digestion conditions which favoured partial digestion of the DNA.

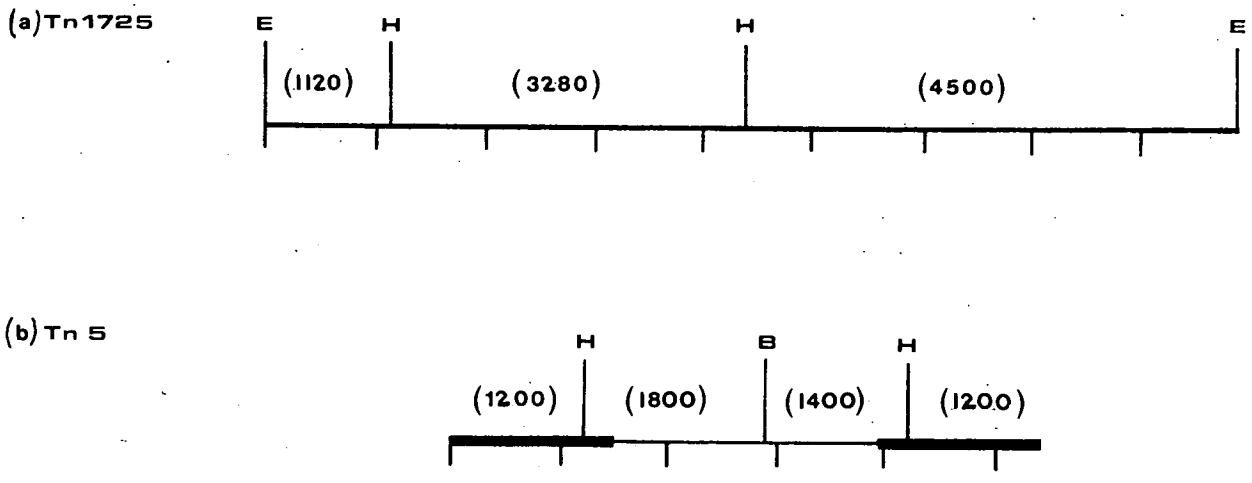
A restriction map was constructed of the hybrid plasmid containing the Klebsiella M5al lac genes, and the lac genes were subsequently sub-cloned. This produced a smaller hybrid lac plasmid, which was subjected to further restriction mapping.

The positions of the lac genes on the latter hybrid lac plasmid were established by transposon mediated "site directed mutagenesis" (Ruvkun and Ausubel, 1981). Transposon insertion into the coding sequence of a gene normally inactivates that gene, and Berg (1977) showed that in the majority of cases insertions into the E. coli lac Z gene were polar to the expression of distal genes.

The transposon Tn5 (Berg et al., 1975) was chosen for use in these experiments for the following reasons:- it had a relatively high transposition frequency (Kleckner, 1977); it carried the gene for kanamycin resistance, which provided the means for selecting transposon insertion into the hybrid lac plasmids (which already carried the gene for ampicillin resistance); Tn5 contained suitable restriction sites (Jorgenson et al., 1979) which allowed the position of transposon insertion to be mapped (see Figure 3.1).

Tn1725 (Wiebauer et al., 1981) was used in these experiments because it had a relatively high transposition frequency, carried the gene for chloramphenicol resistance and also contained suitable restriction sites which enabled the transposon insertions to be mapped conveniently (see Figure 3.1). Tn1725 also had the attraction of Eco R1 restriction sites at its extreme ends. Thus Tn1725 could be used to introduce 'new' Eco R1 restriction sites into the hybrid lac plasmid. This would in turn allow restriction fragments containing various discrete sections of the

Figure 3.1. Restriction sites on transposons Tn1725 and Tn5 used to establish the locations of the transposon insertions on pHE8.



Scale in 1kb units. Figures in parenthesis are fragment sizes in base pairs of DNA. a: R.Schmitt (pers. comm.) based on Altenbuchner et al. (1983) and Schoffl et al. (1981), and Wiebauer et al. (1981). b: Auerswald et al. (1980) modified from Jorgensen et al. (1979).

cloned *lac* genes to be isolated, and used as *lac* DNA hybridisation probes (see Chapter 5).

A preliminary analysis of Tn5 insertion into the sub-cloned *Klebsiella* M5al *lac* genes was undertaken to investigate whether Tn5 displayed preferential insertion into regions of the *lac* system, because Miller *et al*. (1980) had shown that Tn5 displayed such a characteristic when integrating into the *E. coli lac* operon. The restriction maps of the *lac* DNA from *Klebsiella* M5al,T17R1 (MacDonald and Riley, 1983) and *E. coli* (Chacones *et al*., 1981) were compared.

The plasmid F_K *lac* (Reeve and Braithwaite, 1970) was chosen as a source of plasmid borne *lac* genes because it had been extensively investigated by Reeve and coworkers and had been shown to be similar to the *E. coli lac* operon and to those *lac* systems reported on *lac* plasmids in other *Enterobacteriaceae* (see Chapter 1). The plasmid was thus an established and available source of *lac* plasmid DNA.

F_K *lac* plasmid DNA was isolated and the size and molecular weight were estimated by electron microscopy and agarose-gel-electrophoresis. The lactose genes from F_K *lac* were cloned using a similar strategy to that employed in the cloning of the *Klebsiella* M5al chromosomal *lac* genes, described above. A restriction map was constructed of a hybrid plasmid which contained the *lac* genes from F_K *lac*. The approximate position of the *lac* genes on this hybrid *lac* plasmid was established by DNA-DNA hybridisation between the *Klebsiella* M5al *lac* gene containing hybrid plasmid, and restriction fragments of the hybrid *lac* plasmid containing F_K *lac* DNA.

3.2 Results and Discussion

3.2.1 Cloning and Analysis of the *Klebsiella* Chromosomal *lac* genes.

3.2.1.1 Analysis of *Klebsiella* M5al for the Presence of Lac^+ Plasmids.

Klebsiella M5al produced pale red colonies on ML compared with the deep red colonies and precipitation of bile salts associated with *Klebsiella* strains containing Lac^+ plasmids (Reeve and Braithwaite, 1973). This result indicated the absence of a Lac^+ plasmid bearing a functional *lac* system. The presence of plasmid DNA in *Klebsiella* M5al was tested for by the modified DNA preparation method of Schwinghamer (1980) and the modified plasmid analysis method of Eckhardt (described in Chapter 2). No plasmid DNA was detected in *Klebsiella* M5al by either method.

The plasmid analysis procedure of Eckhardt (1978) was shown to be capable of detecting plasmid DNA over a wide molecular weight range from many members of the *Enterobacteriaceae*, including *Klebsiella* (see Figure 3.11). The absence of detectable plasmid DNA in *Klebsiella* M5al, in conjunction with the phenotype displayed by this strain on ML strongly suggested that *Klebsiella* M5al did not contain a Lac^+ plasmid and was plasmid-free.

3.2.1.2 Cloning the *Klebsiella* M5al Chromosomal Lactose Utilisation Genes

7.5µg of chromosomal DNA isolated from *Klebsiella* M5al by the modified method of Schwinghamer (1980) was partially digested with *Hind* III and then ligated with a sample of 3µg of pBR322 DNA which had been completely digested with *Hind* III. The plasmid DNA was then used to transform the *E. coli* strain RE476 by the method of Dagert and Ehrlich (1979). The transformants were grown on X-Gal agar containing ampicillin and IPTG, with selection for blue, β-

galactosidase producing, ampicillin resistant colonies.

Approximately 10,000 ampicillin resistant colonies were screened in this manner and 1 colony (designated RE476 Lac$^+$1) gave a dark blue-green β-galactosidase positive result. When grown on X-Gal agar without IPTG paler blue colonies were obtained, which implied that β-galactosidase production was inducible with IPTG. This was clearly demonstrated when β-galactosidase assays were performed on RE476 Lac$^+$1 in the presence and absence of IPTG (Table 3.1). Thus RE476 Lac$^+$1 contained a functional lac I gene whose protein product repressed lac Z gene activity, and the represson of which could be released by the addition of IPTG.

When grown on ML containing ampicillin RE476 Lac$^+$1 produced a strong Lac positive phenotype, i.e. deep red colonies, due to acids produced early in lactose utilisation (Eddy, 1961). Lactose may only enter E. coli in sufficient amounts necessary to produce such an effect, by the lactose permease system (Rotman et al., 1968). RE476 Lac$^+$1 therefore also contained a functional lac Y gene and its associated protein lactose permease, whose synthesis was apparently co-inducible with β-galactosidase. It was inferred that the lac PO regions were also present from the expression of the lac Z gene in the presence and absence of IPTG.

The presence in RE476 Lac$^+$1 of an approximately 15Mdal. plasmid, designated pHE3 was established by the plasmid isolation procedure of Birnboim and Doly (1979) and agarose gel electrophoresis as described by Meyers et al. (1976) (not shown).

It was therefore concluded that RE476 Lac$^+$1 had acquired a plasmid, pHE3, the presence of which conferred on the E. coli total lac deletion strain the ability to utilise lactose and in conjunction with the results described above indicated that pHE3 contained the chromosomal lac I PO Z and Y genes from Klebsiella M5al. The plasmid was further analysed, as described below.

Table 3.1. β-galactosidase activity expresed by wild strains of Klebsiella and E.coli strains carrying cloned lac genes.

Strain	Uninduced	Induced	Source
Klebsiella			
M5a1	1.75	660	
A1	1.0	71	RE
D8	2.0	81	RE
K122	1.5	81	RE
7242	2.0	146	RE
8017	3.0	156	RE
8595	1.5	81	RE
E.coli			
RE476	0	0	
RE476 Lac^+1 (pHE3)	57	5800	
RE476 Lac^+2 (pHE8)	88	5500	
RE476 Lac^+3 (pHE1)	72	3990	
RE476 Lac^+4 (pHE2)	99	4675	

β-galactosidase assays were performed as described by Miller (1972). Enzyme activity is expressed as units of enzyme per ml of culture at OD_{600} 1.0 which produced 1nmole o-nitrophenol per minute at 28°C and pH7.0 (Miller, 1972).

Growth was in HM plus citrate in the case of Klebsiella strains, and HM plus glycerol and growth supplements in the case of E.coli strains. 100μg ampicillin per ml of culture was used with E.coli strains carrying plasmids. Induction with 10^{-4}M IPTG was for 1.5 hours.

RE: Reeve and Braithwaite, 1973.

3.2.1.3 Restriction Analysis of pHE3

A restriction map of pHE3 was constructed using Hind III, Eco Rl, Bam Hl and Pst 1. The restriction enzymes were used either singly, or in combinations of two of the four enzymes mentioned above. In the case of Eco Rl, partial digestion of pHE3 was also used to establish the positions of the restriction sites. A typical experiment is shown in Figure 3.2 in which it can be seen that complete digestion of pHE3 with Hind III produced three fragments (Figure 3.2, lane 5). This clearly showed that the chromosomal DNA fragment from Klebsiella M5al cloned in pHE3 was the product of a partial Hind III digestion.

In order to determine the positions of the restriction sites on pHE3 it was necessary to first establish the orientation of the pBR322 region of the plasmid. Following this the known restriction sites in pBR322 could be used as fixed points from which the position of other restriction sites could be measured.

pBR322 contains only one Bam Hl restriction site (Sutcliffe, 1979), however digestion of pHE3 with Bam Hl revealed the fragments of 20.5kb and 1.9kb (Figure 3.2, lane 3). Therefore the cloned chromosomal DNA in pHE3 contained a Bam Hl restriction site 1.9kb from the Bam Hl restriction site in the pBR322 region of the plasmid. Figure 3.3 shows the alternative orientations of the pBR322 region in pHE3and the associated alternative positions of the Bam Hl site in the cloned chromosomal DNA in pHE3. A comparison of the expected restriction digest fragment profiles for alternatives A and B in Figure 3.3 with that actually obtained when pHE3 was digested with Bam Hl (Figure 3.2, lane 3) showed that alternative A was correct. Having thus established the orientation of the pBR322 region in pHE3, the known restriction sites in that region were used in conjunction with the restriction

Figure 3.2. pHE3 restriction digest patterns.

The restriction enzymes used were HindIII (H), EcoRI (E), BamHI (B), PstI (P).

The sizes of the restriction fragments of λ DNA digested with EcoRI are 21.8kb, 7.6kb, 5.9kb, 5.5kb, 4.9kb and 3.4kb and digested with HinDIII are 23.7kb, 9.5kb, 6.7kb, 4.3kb, 2.3kb, 2.0kb and 0.6kb.

The λ DNA restriction fragments in Lanes 1 and 6 were used to establish restriction fragment sizes in Lanes 2, 3, 4 and 5; Lanes 7 and 12 were used for restriction fragments in Lanes 8, 9, 10 and 11; Lane 15 was used for Lanes 13 and 14.

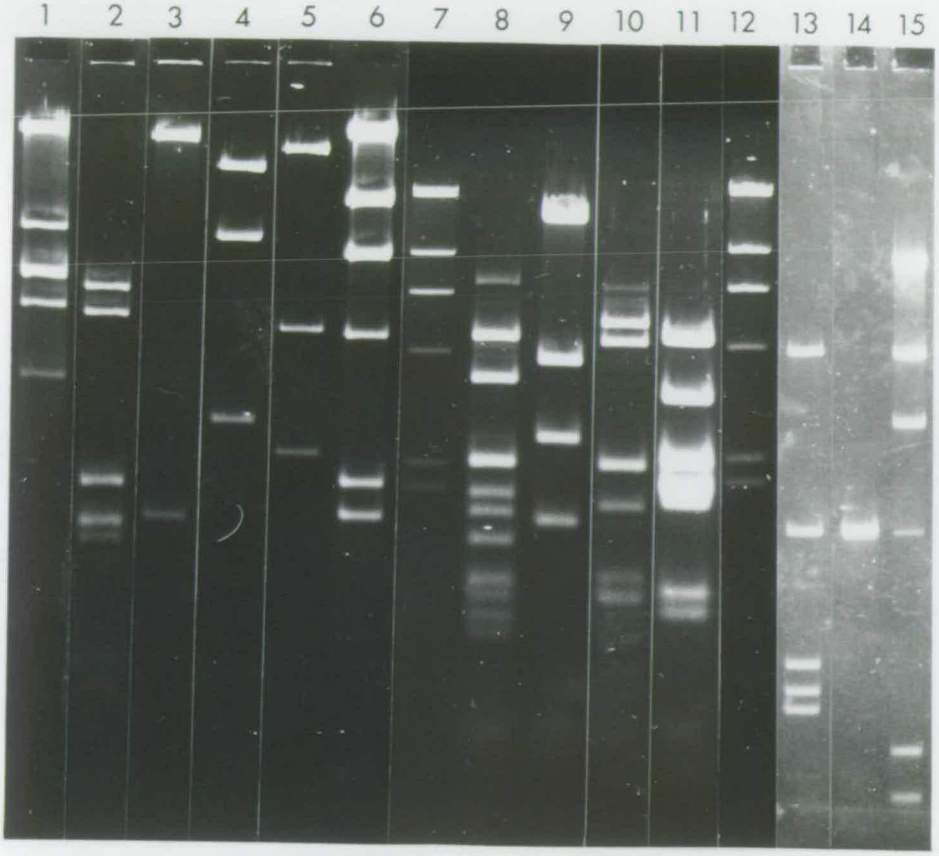


FIGURE 3.2

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
DNA Digested	λ	pHE3	pHE3	pHE3	pHE3	λ	λ	pHE3	pHE3	pHE3	pHE3	λ	pHE3	pBR322	λ
Restriction Enzyme Used	E	P	B	E	H	H	H	H+P	H+B	E+P	B+P	H	H+E	H+E	H
		5.4	20.5	12.6	15.6			4.8	14.0	5.2	4.7		10.0	4.2	
		4.8	1.9	7.0	4.4			3.7	4.0	4.5	3.3		4.2		
		2.4		3.2	2.5			2.4	2.5	2.3	2.25		2.9		
		2.0						2.0	1.6	1.9	2.1		2.7		
		1.9						1.9		1.3	2.0		2.5		
		1.1						1.6		1.2	1.9				
		1.0						1.3			1.8				
								1.2			1.2				
								1.0			1.0				
								0.9							
								0.5							

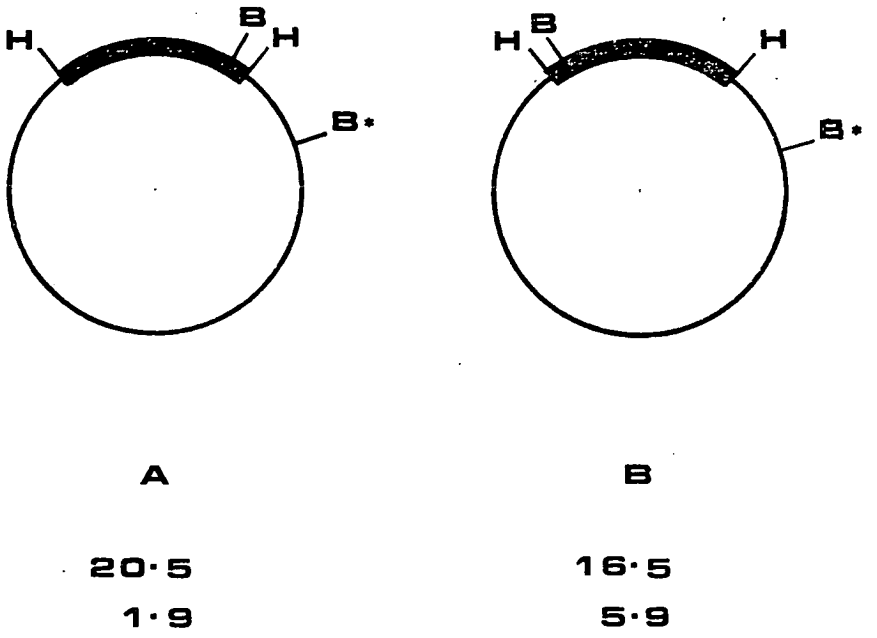


Figure 3·3

The alternative orientations of the pBR322 region in pHE3.

Alternatives A and B show the two possible orientations of the pBR322 region (thick line) in pHE3, and the corresponding relationship of the BamHl restriction site in the pBR322 DNA to the BamHl restriction site (designated B*) in the cloned Klebsiella M5al DNA (thin line).

The boundaries of the pBR322 and cloned DNA are indicated by the HindIII (H) restriction sites.

Below each alternative is shown the expected restriction fragment profile (in Kb) which would result from digestion with BamHl.

digest patterns shown in Figure 3.2 and partial digestion of pHE3 with Eco Rl (not shown) to produce a restriction map of pHE3 (Figure 3.4).

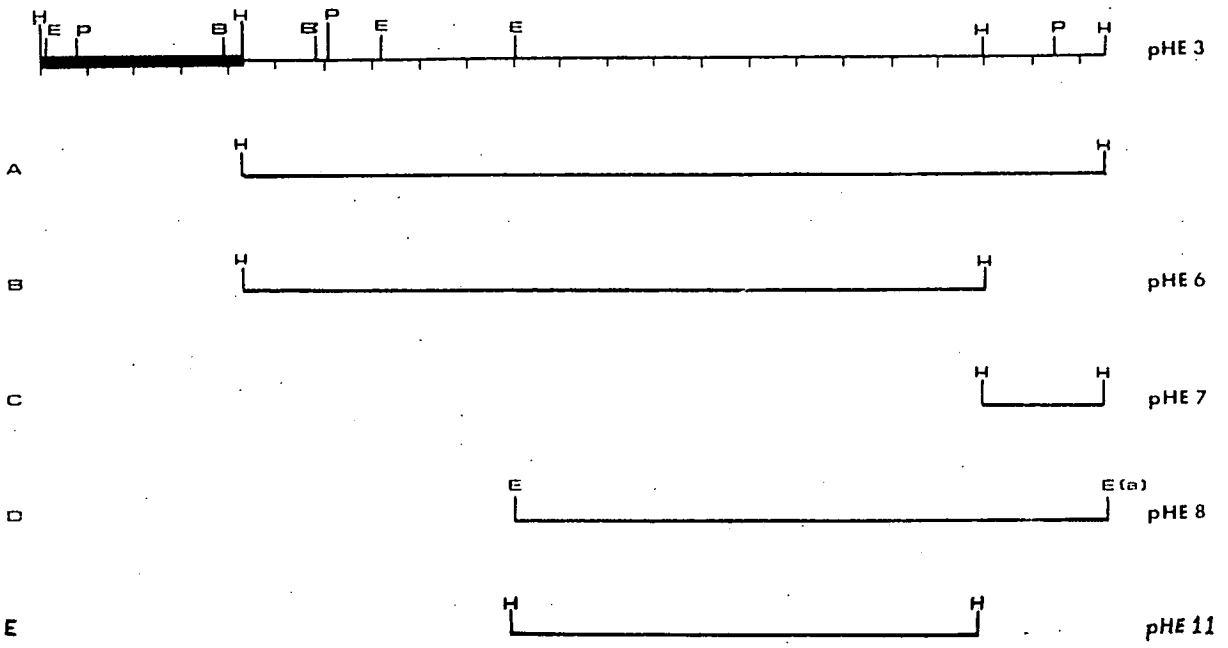
3.2.1.4 The Sub-cloning of DNA Fragments from pHE3

The two Hind III DNA fragments (referred to as B and C in Figure 3.4) which made up the cloned chromosomal DNA in pHE3 were separately isolated by the method of Yang et al. (1979) and were ligated into Hind III digested pBR322 DNA as described in Chapter 2. The plasmids produced from the cloning of fragments B and C (designated pHE6 and pHE7 respectively) were used to transform cultures of RE476 by the method of Dagert and Ehrlich (1979).

Neither RE476/(pHE6) nor RE476/(pHE7) formed blue β-galactosidase positive colonies on X-Gal agar containing IPTG and ampicillin, which suggested that the Hind III restriction site within the cloned chromosomal DNA in pHE3 was in either lac PO or Z DNA. Fragment D (Figure 3.4) extended from the pBR322 Eco Rl restriction site, overlapped in part both fragments B and C and thus contained the DNA flanking the internal Hind III restriction site referred to above, and by inference, the lac PO and Z DNA.

Fragment D DNA was therefore isolated, ligated into the Eco Rl restriction site of pBR322 and transformed into RE476 as described in Chapter 2. The transformed RE476, designated RE476 $lac^{+}2$ gave a strong Lac^{+} phenotype on ML containing ampicillin and was shown to be inducible for β-galactosidase by enzyme assay (Table 3.1). It was inferred from these results that a functional lac Y and lac PO region were also present for reasons described previously. The presence of a functional lac Y gene was confirmed by failure of the RE476 $Lac^{+}2$ to grow on TONPG agar containing amp icillin. The accumulation of TONPG in E. coli via the lactose permease transport system is known to be lethal (Müller-Hill et al., 1968; Smith and Sadler, 1971).

Figure 3.4. Restriction map of pHE3 and subclones produced therefrom.



The thick line indicates pBR322 DNA (from Sutcliffe, 1978) and the thin line DNA from K.pneumoniae M5a1. Scale calibrated in kb. Restriction sites are; H: HindIII, E: EcoRI, P: PstI, B: BamHI. Fragments A, B and C were isolated and separately ligated into pBR322; the resultant subclone pHE numbers are given. Fragment D was produced by EcoRI digestion of pHE3 and has EcoRI restriction sites from the K.pneumoniae chromosomal DNA and from the pBR322 DNA at either end, thus fragment D contains not only K.pneumoniae chromosomal DNA but also 31bp of pBR322 DNA. Fragment E was produced by Hind III digestion of pHE8 and has Hind III restriction sites from the K.pneumoniae chromosomal DNA and from the pBR322 DNA. Not all Pst I sites are shown.

3.2.1.5 Restriction Analysis of pHE8

Plasmid DNA (designated pHE8) was isolated from the RE476 Lac$^+$2 strain and underwent further restriction mapping. The results of a typical experiment are shown in Figure 3.5. The orientation of the pBR322 vector region of pHE8 was established using Hind III digestion and restriction fragment profiles. Figure 3.6 shows the alternative orientations and a comparison of the expected DNA fragment sizes following Hind III digestion of pHE8, with those actually produced (Figure 3.5, lane 2) revealed that alternative B was correct.

With the orientation of the pBR322 region in pHE8 established the known restriction sites in pBR322 were used in conjunction with the restriction digest patterns shown in Figure 3.5 and partial digestion with Pst 1 to produce a restriction map of pHE8 (Figure 3.7). Further characterisation of pHE8 was undertaken and is described below.

3.2.1.6 Mapping of lac Genes on pHE8

pHE8 was subjected to transposon mediated mutagenesis, using Tn5 and Tn1725, insertions into the lac genes were selected and plasmid DNA was isolated from those lines displaying lac gene inactivation as described in Chapter 2. DNA from those lines was digested separately with Bam Hl and Hind III in the case of Tn5 insertions and Eco Rl and Hind III in the case of Tn1725 insertions, in order to estimate the position of transposon insertion and thus the inactivated gene.

It can be seen in Figure 3.1 that Tn5 contains a single Bam Hl restriction site, as does pHE8 (Figure 3.7) Bam Hl digestion of pHE8 containing a Tn5 insertion would therefore yield two fragments. The sizes of these two fragments allowed the Tn5 to be mapped to one of two sites on pHE8. A typical experiment is shown in Figure 3.8(A) in which it can be seen that Bam Hl digestion of pHE8 carrying a Tn5

Figure 3.5. pHE8 restriction digest patterns.

The restriction enzymes used were HindIII (H), EcoRI (E), SalI (S), PstI (P). Fragment D* was isolated by the method of Yang et al. (1979) and digested with PstI. The numerals refer to restriction fragment sizes in kb.

The sizes of the restriction fragments of λ DNA digested with EcoRI are 21.8kb, 7.6kb, 5.9kb, 5.5kb, 4.9kb and 3.4kb and digested with HinDIII are 23.7kb, 9.5kb, 6.7kb, 4.3kb, 2.3kb, 2.0kb and 0.6kb.

The λ DNA restriction fragments in Lane 1 were used to establish restriction fragment sizes in Lane 2; Lane 3 for Lane 4; Lane 5 for Lanes 6, 7 and 8; Lane 9 for Lanes 10, 11 and 12; Lane 13 for Lane 14.

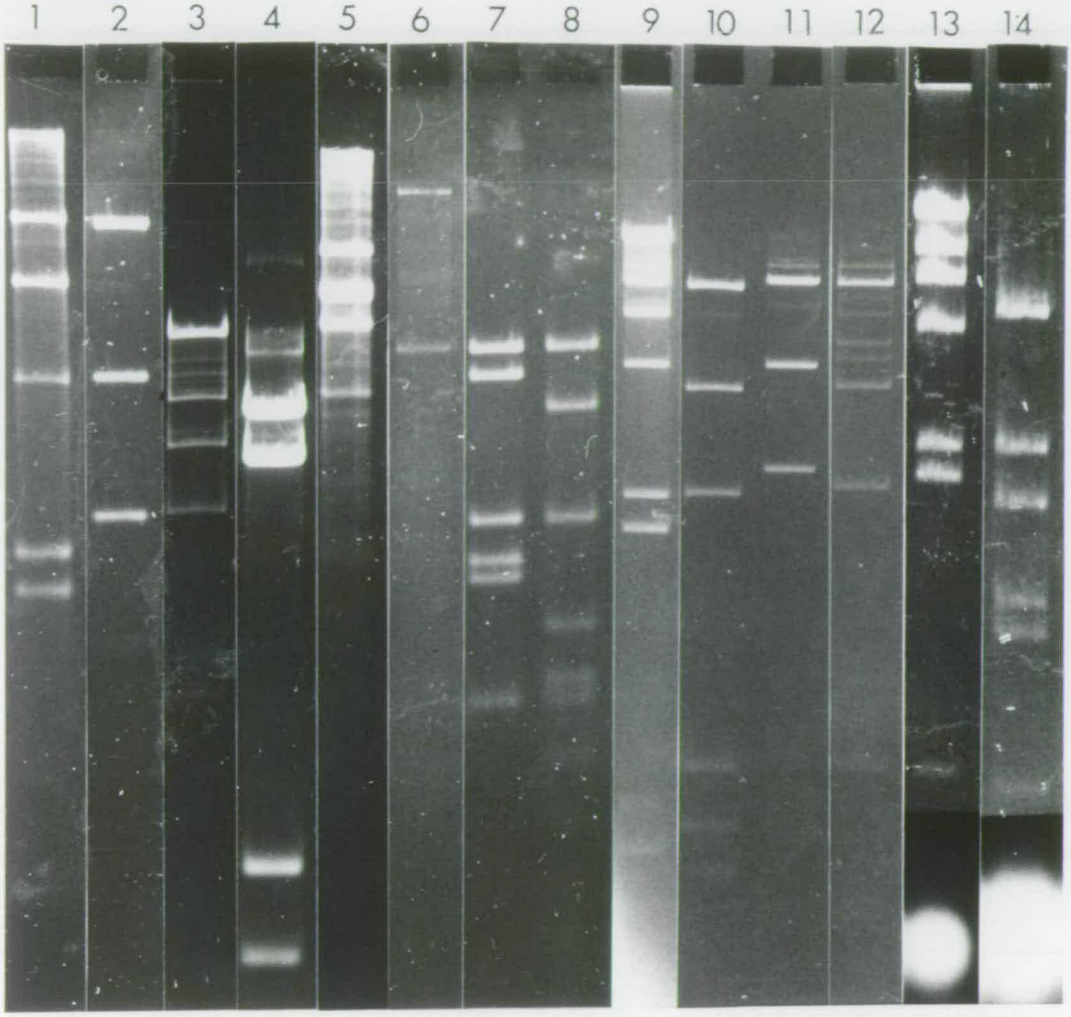
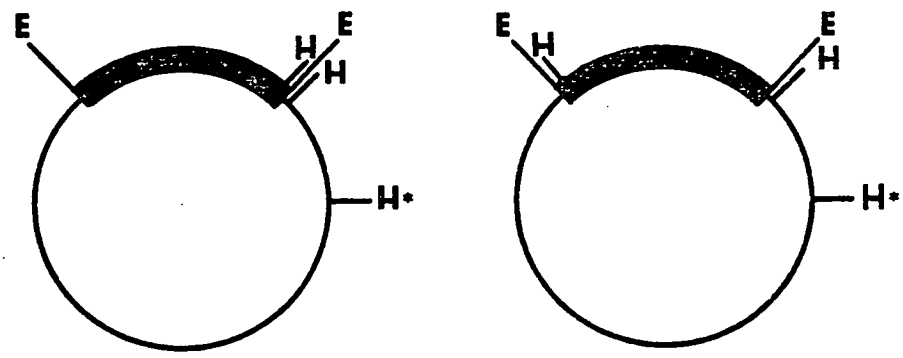


FIGURE 3.5.

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
DNA Digested	λ	pHE8	λ	pHE8	λ	pHE8	pHE8	pHE8	λ	pHE8	pHE8	pHE8	λ	Fragment D*
Restriction Enzyme Used	H	H	H	S	E	E	P	P+H	H	H+S	H+E	E+S	H	P
		9.8		9.0		12.6	4.3	4.3		8.5	9.8	9.0		4.6
		4.3		6.1		4.3	3.7	3.3		3.7	4.3	3.7		2.3
		2.5		1.0			2.4	2.4		2.1	2.5	2.1		1.8
				0.8			2.0	1.6		0.8		0.8		1.2
							1.8	1.2		0.7		0.6		1.0
							1.1	1.1		0.6				
								0.8						
								0.5						



A	B
14·2	9·8
2·5	4·4
	2·5

Figure 3·6

The alternative orientations of the pBR322 region in pHE8.

Alternatives A and B show the two possible orientations of the pBR322 region (thick line) in pHE8 and the corresponding relationship of the HindIII restriction site in pBR322 to the HindIII restriction site (designated H*) in the sub-cloned Klebsiella M5a1 DNA (thin line).

The boundaries of the pBR322 and sub-cloned DNA are indicated by the EcoR1 (E) restriction sites.

Below each alternative is shown the expected restriction fragment profile (in Kb) which would result from digestion with HindIII.

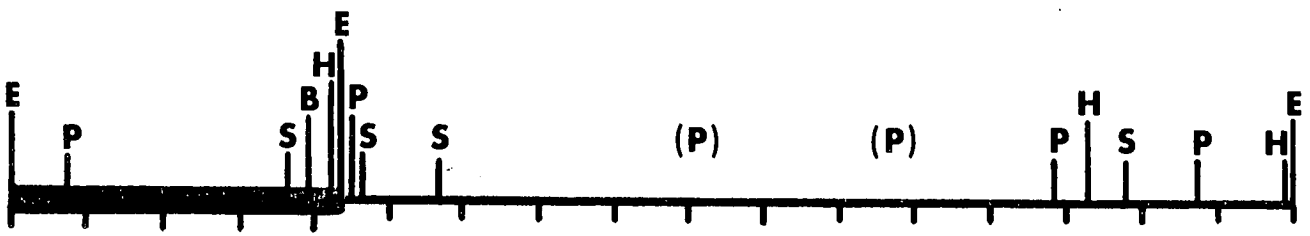


Figure 3·7

Restriction map of pHE8.

The restriction sites were based on several experiments, an example of which is shown in Figure 3.5. The thick line represents pBR322 DNA and the restriction sites therein are from Sutcliffe (1978). The thin line represents Klebsiella M5a1 chromosomal DNA subcloned from pHE3.

The restriction enzymes used were EcoRl, (E); Pstl, (P); Sal1, (S); BamHl, (B); HindIII (H). The parentheses indicate Pstl restriction sites whose precise locations are unknown, but which were approximated from Pstl partial digestion fragment sizes. Calibration is in Kb.

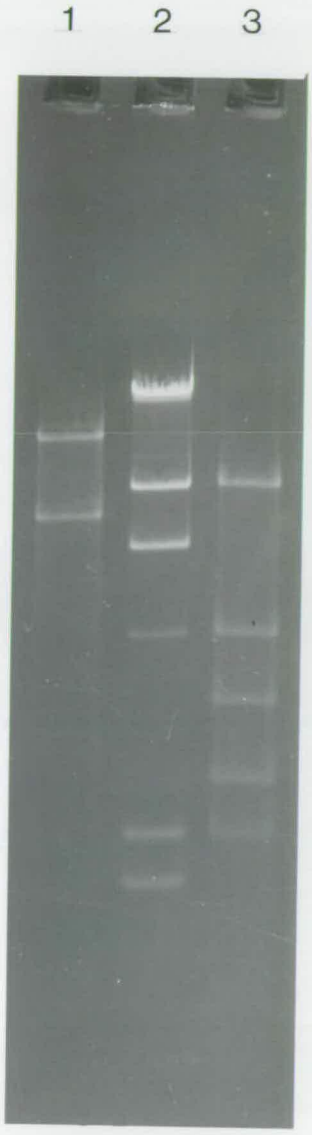


FIGURE 3.8A

Restriction analysis of pHE8 lac Y::Tn5 (designated Tn10)

Plasmid DNA was digested with either BamHl (Lane 1) or HindIII (Lane 3) and subjected to electrophoresis through 1% agarose.

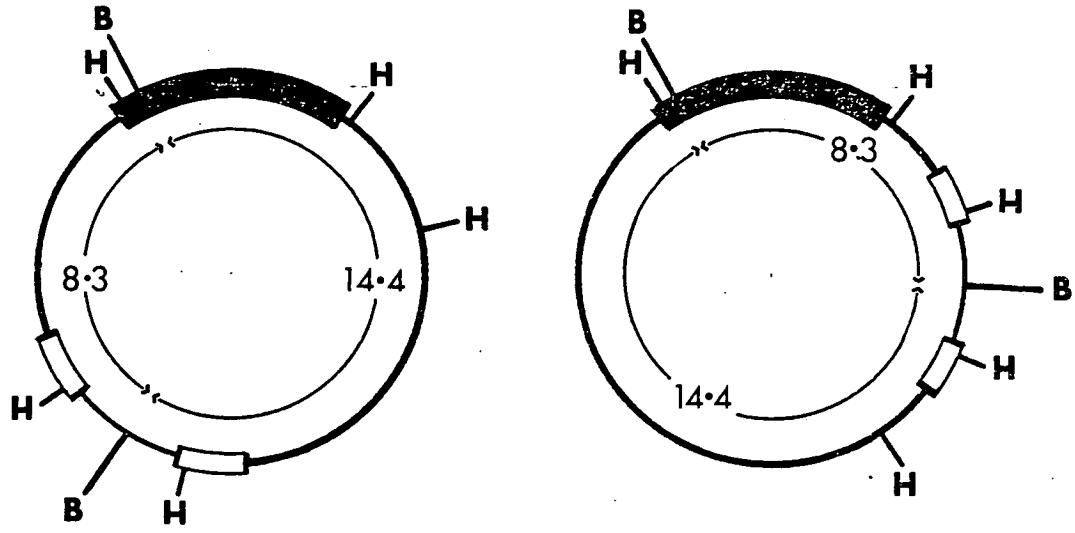
λ DNA digested with HindIII (Lane 2) gave fragments of 23.7kb, 9.5kb, 6.7kb, 4.3kb, 2.3kb, 2.0kb, 0.6kb.

insertion yielded two fragments of 14.4kb and 8.3kb. The established Bam Hl restriction site in pHE8 was therefore separated from the Bam Hl restriction site in the inserted Tn5 by a distance equivalent to the lengths of the two fragments, and it followed that two alternative sites of Tn5 insertion could be estimated, as shown in Figure 3.8B.

The distances from the ends of the Tn5 DNA to the Hind III and Bam Hl restriction sites in the transposon and the distances between those restriction sites themselves (Figure 3.1) plus the pHE8 restriction map (Figure 3.7) were used to produce an expected Hind III restriction profile for alternative insertion positions A and B (Figure 3.8B). When compared with the actual Hind III restriction profile obtained (Figure 3.8A. lane 3) alternative B was shown to be correct.

The above rationale was used to map the position of seven different Tn5 insertsions, three of which mapped to the lac PO Z region and four to lac Y (Figure 3.9).

Tn1725 insertions in pHE8 were mapped using the Eco Rl restriction sites at both ends of the transposon and the two internal Hind III sites (Figure 3.1). The rationale employed to map the insertion positions was similar to that described above, with Eco Rl digestion of pHE8 carrying a Tn1725 insertion indicating two possible sites of insertion. However, because of the position of the Hind III restriction sites in Tn1725 (Figure 3.1) the orientation of the transposon in pHE8 made a considerable difference to the expected Hind III digestion profiles that could be produced. Thus for each of the two alternative sites of Tn1725 insertion indicated by the Eco Rl digestion profiles, there existed two different expected Hind III digestion profiles depending upon the orientation of the transposon in pHE8. Comparison of the actual Hind III digestion profile of pHE8 containing a Tn1725 insertion with the four



A	B
6·4	10·0
6·2	4·4
4·4	3·3
3·3	2·7
2·5	2·2

Figure 3·8 B

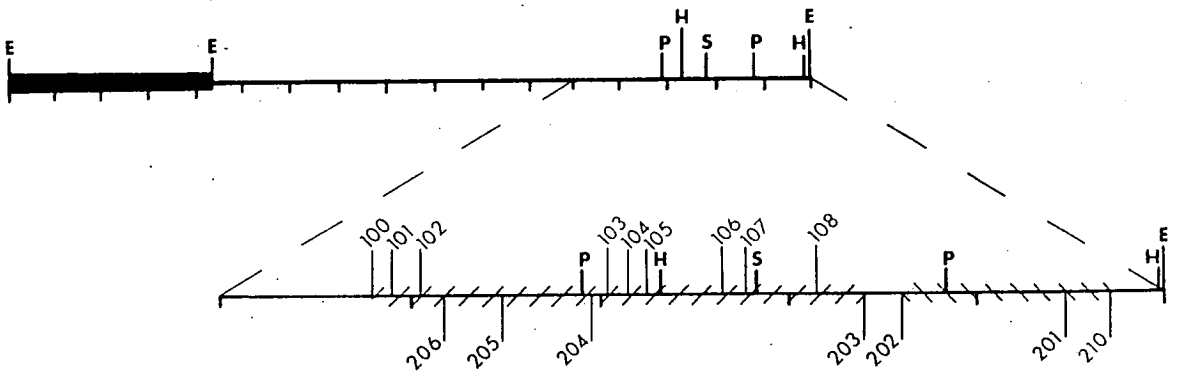
Tn5 insertion in pHE8.

Alternatives A and B show the two possible sites of Tn5 insertion in pHE8 based on the results shown in Figure 3.8A (lane 1), also shown as numerals within the circles. The thick line represents the pBR322 region of pHE8; the thin line represents the sub-cloned Klebsiella M5al DNA; Tn5 is represented by the flanking inverted repeats (open boxes) and the short region containing a BamHl restriction site; HindIII restriction sites are represented by H.

The BamHl restriction site in Tn5 is positioned off-centre (see Figure 3.1), however for the initial mapping of the site of Tn5 insertion in pHE8 the BamHl restriction site was taken as being equidistant from either end of Tn5.

Below each alternative is shown the expected restriction fragment profile (in Kb) which would result from digestion with HindIII.

Figure 3.9.Diagramatic representation of the transposon insertions in pHE8 and the lac genes inactivated by these insertions.



The heavy line indicates pBR322 DNA with restriction sites taken from Sutcliffe (1978), and the thin line K.pneumoniae chromosomal DNA. Insertions by Tn1725 (⊥) and Tn5 (⊤) are identified by their allele number (e.g. 206 is mutation lacZ206::Tn5). The hatched boxes represent the regions in which transposon insertions inactivate the lacPOZ region (////), or the lacY gene (\\\\). Scales are calibrated in kb.

The restriction fragment sizes, and consequently the insertion positions, are the average of two agarose gel electrophoresis experiments.

expected Hind III digestion profiles allowed the position of insertion and the orientation of the transposon to be established. Nine different Tn1725 insertions were mapped in pHE8 and are shown in Figure 3.9.

The identities of the lac genes inactivated by transposon insertion were established using initially ML and then X-Gal agar containing IPTG and TONPG agar with all of the media containing either kanamycin or chloramphenicol depending upon whether Tn5 or Tn1725 respectively had been used. The following results were obtained.

RE476 lines containing separately pHE8 with the Tn1725 insertions lac-100 through lac-108 (Figure 3.9) and the Tn5 insertions lac-203 through lac-206 did not produce either β-galactosidase or lactose permease protein. However, RE476 lines containing either lac-201 202 or 210 produced β-galactosidase, but not lactose permease. Thus the loss of β-galactosidase activity was accompanied by the loss of lactose permease activity, whereas the loss of lactose permease activity did not necessarily lead to the loss of β-galactosidase activity.

This implied that the insertions lac-201, 202 and 210 were within the lac Y gene, and that insertions lac-100 to 108 inclusive, and lac-203 to 206 inclusive were within the lac PO or lac Z sequences.

These results indicated that transcription of the lac genes in Figure 3.9 was not from the lac Y to the lac Z gene, in a right to left direction, because inactivation of the lac Y gene did not also inactivate the adjacent lac Z gene. An alternative hypothesis that transcription was initiated from a site between lac Z and lac Y and proceeded in both directions was also untenable as insertion into the lac Z also inactivated lac Y, but insertions into lac Y did not inactivate lac Z.

The results did, however, suggest that transcription of

the lac genes was from the lac Z to lac Y direction, with a lac promoter region upstream of lac Z.

As judged by the ability to grow on TONPG agar those RE476 lines which contained pHE8 with a transposon insertion in the lac PO Z region did not produce lactose permease. This implied that lac Y did not have a separate promoter from that for lac Z.

An indication of the sizes of the lac Z and lac Y genes cloned in pHE8 were obtained from the transposon insertion mapping data.

The insertions lac-202 and lac-210 both inactivated lac Y only, and were 1.1kb apart, thus providing a minimum length for the lac Y gene. The downstream boundary of lac Y was provided by the pBR322 region of pHE8 (Figure 3.9). The upstream boundary of lac Y was provided by insertion lac-203 which inactivated both lac Z and lac Y and therefore was positioned in lac PO or Z and was the closest insertion to lac Y to do so. The distance between these two boundaries or limits was 1.6kb, which represented the maximum possible length of lac Y. Similarly the minimum possible length of lac PO Z was estimated by measuring the distance between the two lac PO Z insertions which were furthest apart. The distance between these insertions, lac-100 and lac-203 was 2.6kb.

It should be noted that insertions into lac Z and lac Y were separated at their closest point by only 0.2 kb which is compatible with there being a small intergenic region between lac Z and lac Y as is the case with the E. coli lac operon (Büchel et al., 1980).

The results of the transposon insertion mutagenesis of pHE8 also inferred that there was insufficient available DNA (approximately 0.3 kb) between the mapped downstream limit of lac Y (Figure 3.9, Lac-210) and the pBR322 boundary of the cloned DNA in pHE8, to code for a repressor protein

unless it was considerably smaller than the 1.081kb *E. coli lac I* gene (Farabangh, 1978). The most probable position of the *lac I* gene on pHE8 was therefore upstream of *lac Z*. Unfortunately it was not possible to isolate *E. coli* carrying pHE8 with a transposon insertion in the *lac I* gene for the following reasons.

Firstly, *E. coli* containing pHE8 produced deep-blue colonies on X-Gal agar, due to the high basal level of β-galactosidase synthesis (see Table 3.1) and induction with IPTG did not change the colony colour. Convenient selection of *E. coli* containing pHE8 with transposon insertion in the *lac I* was dependent upon colony differentiation between basal and constitutive β-galactosidase synthesis. This was clearly not possible with this system.

Secondly, there were reports that elevated levels of lactose permease in *E. coli* were lethal (discussed in detail in Chapter 4). As the vector region of pHE8 was the multi-copy plasmid pBR322 (Bolivar *et al.*, 1977a & b) it was highly probable that pHE8 would exist in a multicopy state. Constitutive expression of the *lac* genes on pHE8 could therefore lead to cell death. As both of these problems were related to the multicopy state of pHE8, and the *lac* genes thereon, they could have been circumvented by transferring the cloned *lac* genes to a plasmid vector with a much lower copy number, for example RK2 (Ingram *et al.*, 1973).

Although RK2 is a relatively large plasmid by plasmid vector standards (RK2 is 56kb in size, Meyer *et al.*, 1977 cf. with the 4.362kb of pBR322, Sutcliffe, 1979) hybrid RK2 plasmids containing "extra" DNA ligated into the *Eco* R1 restriction site have been transformed into *E. coli* (Meyer *et al.*, 1975). Therefore a hybrid RK2-Lac$^+$ plasmid containing the *lac* gene DNA from pHE8 could have been produced by cloning the 12.6kb *Eco* R1 fragment from pHE8 into the unique *Eco* R1 site in RK2 and then transforming the hybrid

plasmid into the lac deletion strain RE476 with selection for Lac$^+$, kanamycin and tetracycline resistant transformants.

Transposon mutagenesis of the lac genes cloned in RK2 could then have been undertaken, by mating RTS1::1725 into strains carrying the RK2 Lac$^+$ hybrid plasmid. Overnight growth of the mating mixture at 42°C on ML containing tetracycline and chloramphenicol would have eliminated RTS1::Tn1725 from the bacterial population and allowed the identification of colonies containing RK2 Lac$^+$ hybrid plasmids and Tn1725 insertions. Replica plating of these colonies on X-Gal agar containing tetracycline and chloramphenicol, with and without IPTG would then have allowed differentiation of transposon insertions into the lac I, lac PO Z or lac Y genes.

Plasmid DNA could then have been isolated from lines containing Tn1725 insertions in lac I and the site of insertion estimated by Eco RI and Hind III restriction analysis using essentially a similar rationale to that employed in the mapping of Tn1725 insertions in pHE8.

Unfortunately there was insufficient time to undertake this experiment.

3.2.1.7 Provisional Analysis of Tn5 Insertion into pHE8 lac Genes

A measure of the ratio of lac PO Z to lac Y insertions in pHE8 was undertaken to estimate whether Tn5 displayed preferential integration in the lac DNA in pHE8.

pHE8 DNA was isolated from E. coli RE412 (pHE8), which had been infected with λTn5, and plasmid DNA was transformed into RE476, as described in Chapter 2. Initial selection for plasmid lac gene insertional inactivation was on ML containing ampicillin and kanamycin. These lines were then differentiated into lac PO Z or lac Y transposon insertions by plating on X-Gal agar containing ampicillin and kanamycin as previously described.

Of 40 Lac$^-$ colonies detected on ML containing ampicillin and kanamycin, 36 were lac Y insertions and 4 were lac PO Z insertions, giving a ratio of 9 to 1. On the basis of the transposon mutagenesis mapping data described above, and the β-galactosidase sub-unit molecular weight estimation (described in Chapter 4) the pHE8 lac PO Z region is at least twice the length of lac Y. Allowing for this difference in size gives a preference for lac Y to lac PO Z insertion of 18 fold per unit distance, compared with the 5 to 7 fold preference displayed by Tn5 for the E. coli lac Y compared with lac Z (Miller et al., 1980; Berg et al., 1980).

However, more work would be necessary in order to verify the above result for preferential Tn5 insertion into the lac DNA of pHE8.

3.2.1.8 Comparison of the lac Region Restriction Maps from E. coli and the Klebsiella M5a1 and T17R1

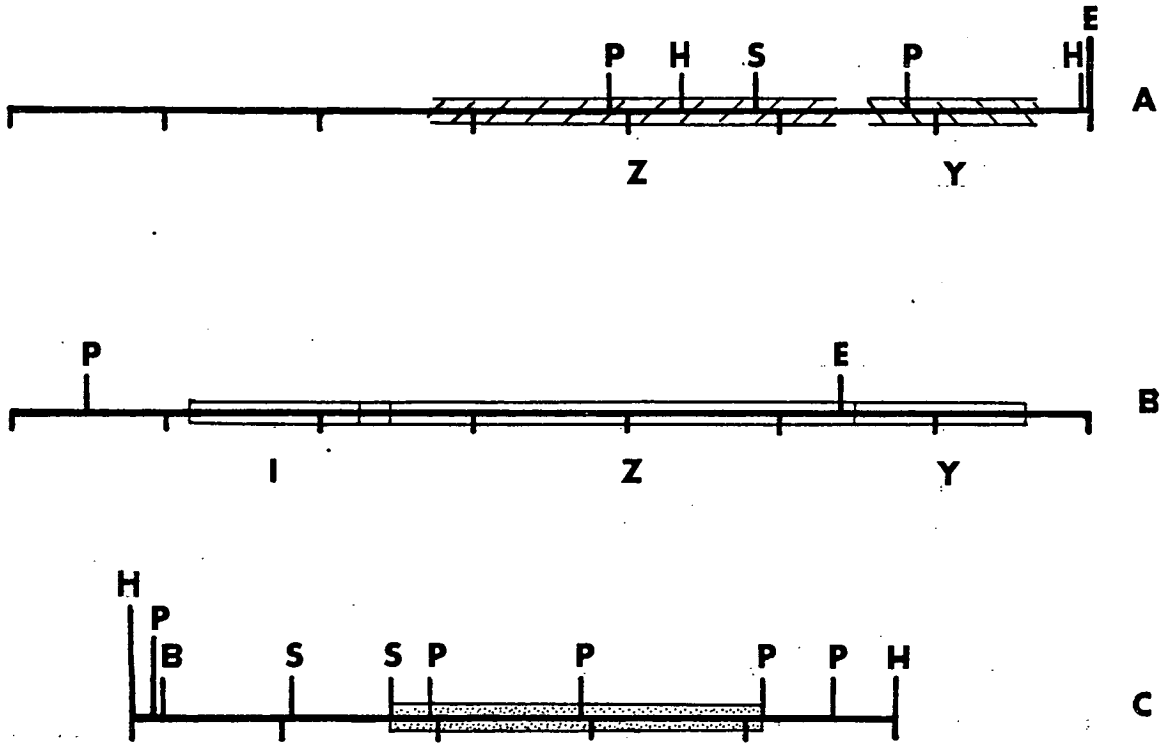
Figure 3.10 shows that the E. coli lac operon does not share any of the restriction sites demonstrated in Klebsiella M5a1 (this work) or Klebsiella T17R1 (MacDonald and Riley, 1983). However it can be seen in Figure 3.10 that both M5a1 and T17R1 have restriction sites for Pst 1 and Sal 1 in their lac regions and that the Pst 1 sites may be common to both.

3.2.2 The Cloning and Analysis of the Klebsiella Lac$^+$ Plasmid lac Genes

3.2.2.1 The Isolation of $F_K$lac.

To facilitate the molecular analysis of the plasmid borne lac genes on $F_K$lac it was first necessary to isolate the Lac$^+$ plasmid.

Figure 3.10. Restriction maps of three different lac regions.



Restriction sites in and immediately adjacent to lac DNA are shown for: (A) K.pneumoniae M5a1 (pHE8, this work), (B) E.coli (pFB140, Chaconas et al., 1981; only restriction sites for those enzymes used in the analysis of pHE8 are shown), (C) K.pneumoniae T17R1 (pCR100, MacDonald and Riley, 1983).

The scale is calibrated in kb. The hatched boxes in A show the DNA regions occupied by either lacZ or lacY (this work). The open boxes in B show the lac genes in E.coli. The dotted boxes in C show the fragments with homology to lacZ DNA from E.coli (MacDonald and Riley, 1983).

Restriction enzyme recognition sites are represented by H, (HindIII); E, (EcoR1); B, (BamH1); P, (Pst1); S, (Sal1).

Attempts to isolate F_K*lac* ccc DNA from either *Klebsiella* or *E. coli* host strains using the methods of Bazaral and Helinski (1968) and Clewell and Helinski (1969) proved unsuccessful. These methods involved the use of the non-ionic detergents Triton-X-100 or Sarkosyl, and following a centrifugation step, produced a "cleared lysate". Plasmids such as pBR322, R6K and RK2 which have molecular weights of 2.9Mdal (Sutcliffe, 1978) 25Mdal (Kontomichalou *et al.*, 1970) and 40Mdal (Burkhardt *et al.*, 1979) respectively were successfully isolated by these methods, therefore these methods were working, but were not applicable to the isolation of F_K*lac* ccc DNA.

Several methods of plasmid isolation which used ionic detergents were then used in attempts to isolate F_K*lac* from either *E. coli* or *Klebsiella* host strains. They included the methods of Guerry *et al.*, (1973), Willshaw *et al.* (1978), Duggleby *et al.* (1977) and Hansen and Olsen (1978), but only the latter method gave positive results. The ccc plasmid DNA yield from a 2 litre starting culture was approximately 40µg.

F plasmid DNA is thought to be complexed with the bacterial chromosome and involves an RNA moiety (Miller and Kline, 1979) in a similar manner to the stabilised folded state of the *E. coli* chromosome (Worcel and Burgi, 1972). Miller and Kline (1979) demonstrated that the stabilised F plasmid-chromosome complex was disrupted by the addition of SDS which released 70% of the ccc F plasmid DNA from the complex, whereas the addition of the non-ionic detergent Sarkosyl released less than 10% of ccc F plasmid DNA.

The method of Hansen and Olsen (1978) incorporated SDS lysis of the cells, a brief alkali denaturation step and exceptionally gentle manipulation of the samples throughout the isolation procedure, all of which presumably maximised the release of intact ccc plasmid DNA.

3.2.2.2 Size and Molecular Weight Estimation of F_K*lac*

The molecular weight of the F_K*lac* plasmid was estimated using a modified version (Forbes, 1983) of the gel electrophoresis method of Eckhardt (1978) described in Chapter 2. The results from fourteen such agarose gel electrophoresis experiments (an example of which is shown in Figure 3.11) indicated that *Klebsiella* V9A contained plasmids of two distinct size classes, one of 113Mdal +/- 10 (170kb) and the other of 39.6Mdal +/- 3.0 (59.4kb)(Figure 3.11, lane 6). The transfer of the strong Lac^+ phenotype from *Klebsiella* V9A to the *E. coli* *lac* deletion strain RE254 was accompanied by the acquisition of the *E. coli* strain of a plasmid similar in size to the 113Mdal plasmid seen in *Klebsiella* V9A (Figure 3.11, lanes 5 and 6 respectively).

Similarly the separate transfer of a tetracycline resistance phenotype from *Klebsiella* V9A to RE254 (designated RE400) was accompanied by the appearance in RE400 of a plasmid similar in size to the 39.6Mdal plasmid observed in *Klebsiella* V9A (Figure 3.11, lanes 9 and 6 respectively).

Electron microscope studies of plasmid DNA isolated from *Klebsiella* V9A and RE254, following transfer of the Lac^+ phenotype to the *E. coli* strain were undertaken. Figure 3.12(A and B) shows electron micrographs of the two plasmid species isolated from *Klebsiella* V9A. The mean length of four of the molecules representing the larger of the two plasmid species in *Klebsiella* V9A (Figure 3.12A) was estimated to be 185kb +/- 12.5 and that of the smaller plasmid species (Figure 3.12B) to be 62kb +/- 6.5 based on the measurement of 10 molecules. These results were in close agreement with the mean lengths of 174kb and 61kb respectively which had been calculated from the molecular weights of the two plasmids (Figure 3.11) estimated by agarose gel electrophoresis.

Figure 3.13 shows an electron micrograph of plasmid

FIGURE 3.11

Molecular weight estimation of F_K*lac* and T_K plasmids in *Klebsiella* V9A by agarose gel electrophoresis.

The position of chromosomal DNA is indicated by X, and where present the plasmid DNA may be seen as thin bands above the thicker diffuse chromosomal bands.

Lane 1: *P. putida* S1239 (pMG1, 320Md), Lane 2: *E. coli* KF 47 (FTn10, 73Md), Lane 3: *A. tumafaciens* C58 (pAT-C58, 280Md, and pTi-C58, 123Md), Lane 4: *E. coli* RE254, "plasmid free" strain, Lane 5: *E. coli* RE254 (F_K*lac*), Lane 6: *Klebsiella* V9A, Lane 7: RE254 (RP4, 37Md), Lane 8: *E. coli* KF57 (R68::Mu, 63Md), Lane 9: *E. coli* RE400 (T_K).

The plasmid DNA in *Klebsiella* V9A is indicated by arrows.

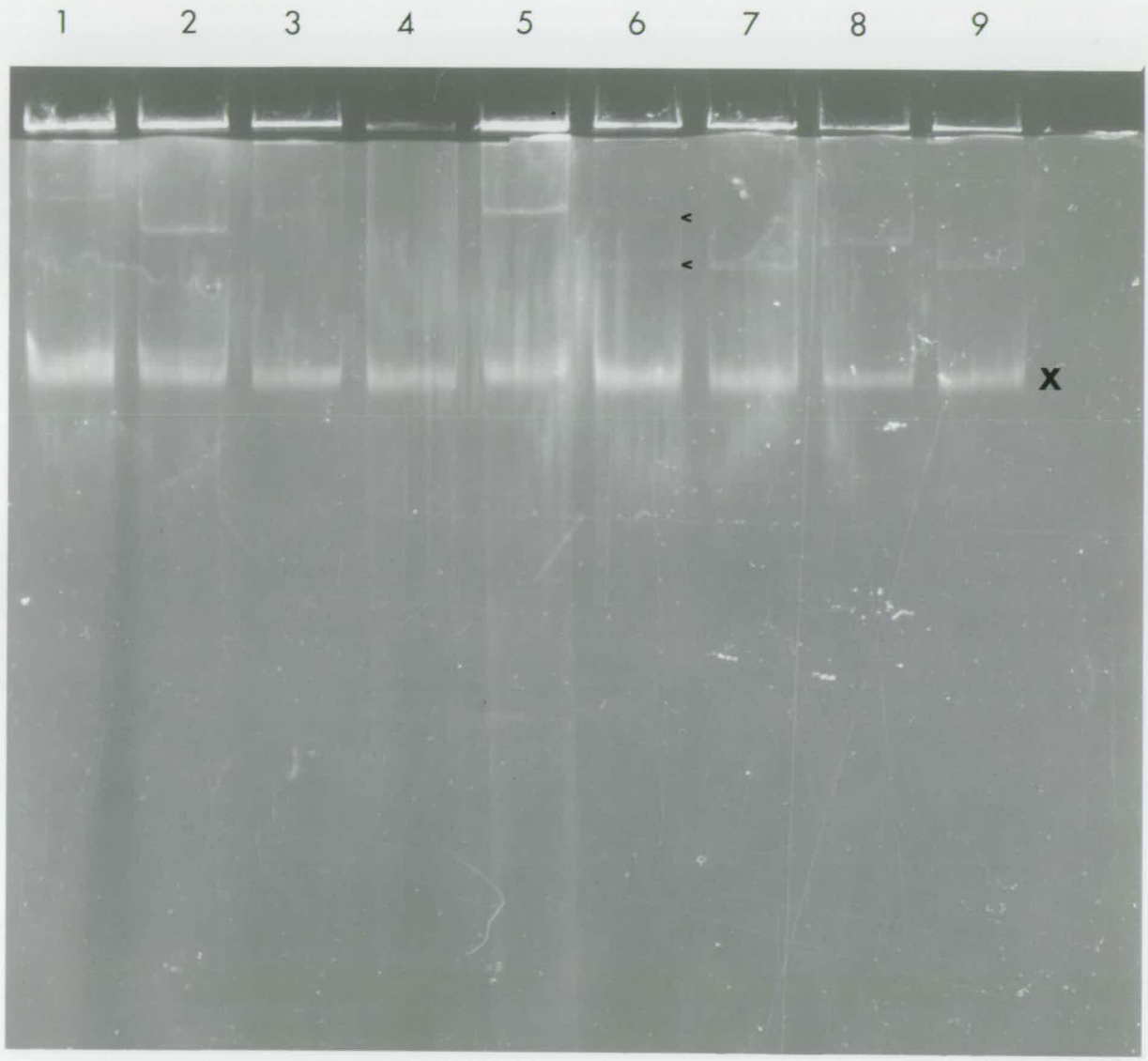

1
2
3
4
5
6
7
8
9
X

FIGURE 3.12A

Electron micrograph of F_K*lac* plasmid DNA isolated from *Klebsiella* V9A.

The arrows indicate some of the plasmid pCM21 molecules (5.4kb in size, Bishop 1979) used in the preparation as standard size markers.

FIGURE 3.12B

Electron micrograph showing the second size class of plasmid (T_K) isolated from Klebsiella V9A.

The arrows indicate some of the plasmid pCM21 molecules (5.4kb in size, Bishop 1979) used in the preparation as standard size markers. Magnification is approximately 2.5 x that of Figure 3.12A.

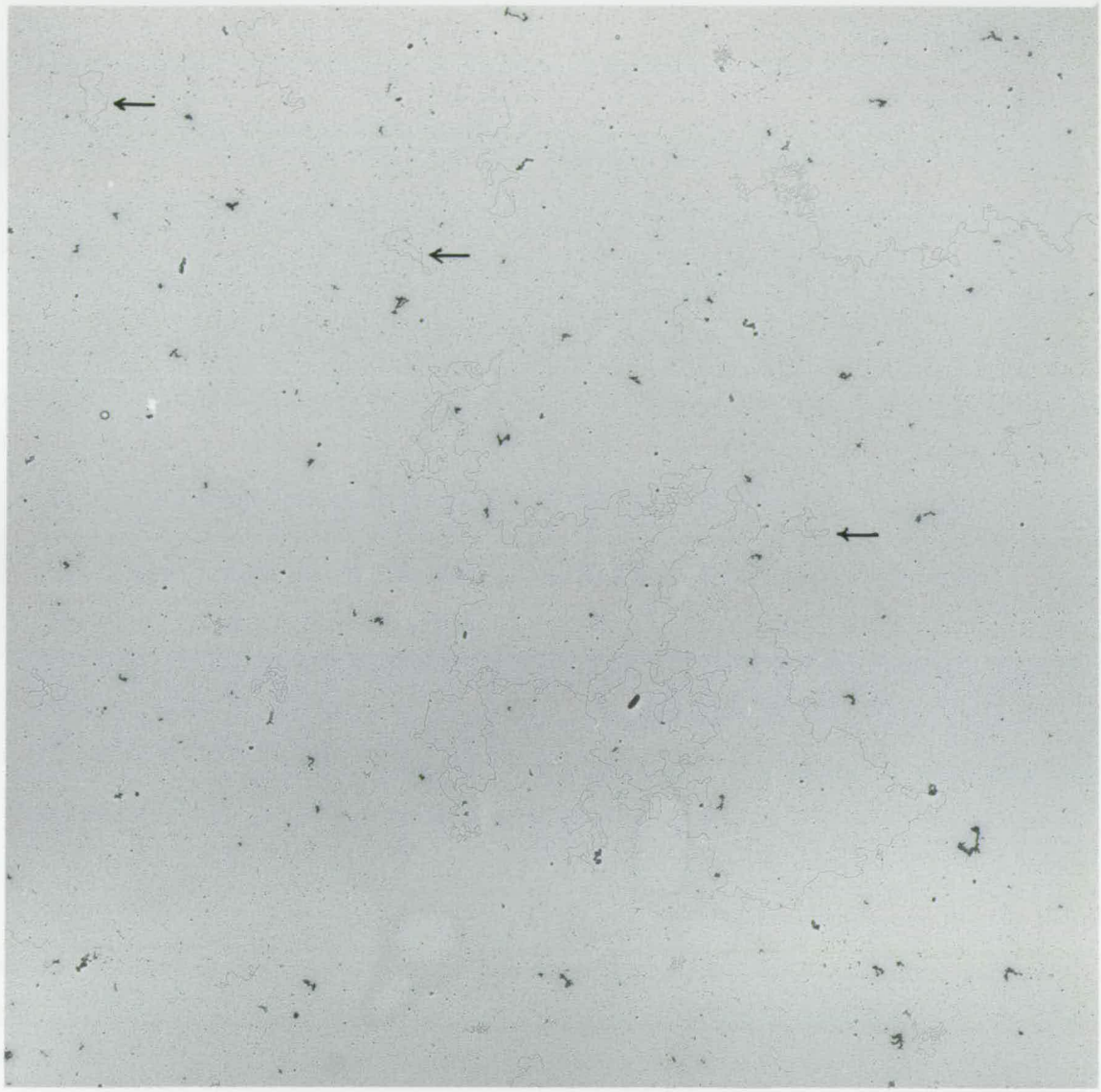


FIGURE 3.13

Electron micrograph of the F_K*lac* plasmid DNA isolated from RE254 F_K*lac*

The arrows indicate some of the plasmid pCM21 molecules (5.4kb in size, Bishop 1979) used in the preparation as standard size markers.

DNA isolated from RE254 F_K*lac* following the acquisition by the *E. coli* strain of a strong Lac^+ phenotype after mating with *Klebsiella* V9A. The mean length of three molecules was estimated to be 180kb +/- 11 which was in good agreement with the 185kb plasmid observed in *Klebsiella* V9A.

Thus the upper and lower plasmid bands seen in Figure 3.9 (track 6) were clearly shown to represent two distinct size classes of plasmid ccc DNA and were not simply the ccc and open circle form of one plasmid species. The 61kb plasmid in *Klebsiella* V9A was shown to be responsible for the tetracycline resistance phenotype displayed by *Klebsiella* V9A and acquired by RE254 following a mating with *Klebsiella* V9A, and is the tetracycline resistance plasmid T_K referred to by Reeve and Braithwaite (1970) and Reeve (1970). The larger plasmid in *Klebsiella* V9A was shown to be responsible for the strong Lac^+ phenotype displayed by *Klebsiella* V9A, and RE254 F_K*lac* following mating with *Klebsiella* V9A. F_K*lac* DNA isolated from the latter strain was used in the experiments described below.

3.2.2.3 Cloning the Lactose Utilisation Genes of F_K*lac*

It has been demonstrated in the previous section that the F_K*lac* plasmid was of a relatively large size and therefore if an analysis of the plasmid *lac* genes was to be undertaken they would first have to be transferred to a more manageable vector. Thus the *lac* genes from F_K*lac* were cloned into pBR322.

Direct and indirect evidence, discussed in Chapter 1, indicated that the F_K*lac* plasmid could be expected to carry a *lac* operon similar in size and organisation to that of the *E. coli* chromosome though lacking the thiogalactoside transacetylase gene (discussed in detail in Chapter 4). The *lac* genes on F_K*lac* could therefore be contained on a DNA fragment with a minimum length of between 5 and 6kb,

and therefore a similar cloning strategy to that adopted for the cloning of the Klebsiella M5al lac genes was used because of the similar problems involved.

1.5µg of $F_K$lac DNA isolated from RE254 $F_K$lac was partially digested with Hind III and ligated with 2.5µg of fully Hind III digested pBR322 DNA, then transformed into RE476. Initial selection was for ampicillin resistant Lac^+ colonies on ML containing ampicillin, and two colonies from the approximately 5,000 screened displayed both these characteristics and were designated RE476 Lac^+3 and RE476 Lac^+4.

When grown on X-Gal agar containing ampicillin, RE476 Lac^+3 and RE476 Lac^+4 both produced two colony types, white colonies and dark blue colonies; the intensity of the latter did not increase when IPTG was present in the medium. However what was observed was a dramatic difference in colony size, from approximately 2mm diameter colonies observed on X-Gal agar to approximately pin-head sized colonies when RE476 Lac^+3 and RE476 Lac^+4 were grown on X-Gal agar with IPTG. The fact that there was no observable difference in colony colour on X-Gal agar with or without IPTG implied that the lac Z in RE476 Lac^+3 and RE476 Lac^+4 were constitutively expressed. However both of these strains were shown to contain an active lac I gene, whose repression upon lac Z activity was released by the addition of IPTG, when β-galactosidase assays were performed on them (Table 3.1).

The high basal (non-induced) level of β-galactosidase production by RE476 Lac^+3 and RE476 Lac^+4 (Table 3.1) presumably accounted for the fact that no difference in colony colour was observed on X-Gal agar with or without IPTG. The presence of a lac P and lac O region was inferred from the above result because lac Z transcription was presumably initiated from a promoter site and the lac I gene protein product appeared to have a site at which repression of lac Z

expression was exerted. Neither RE476 Lac$^+$3 or RE476 Lac$^+$4 grew on TONPG agar containing ampicillin, confirming the presence of a functional lac Y gene in both clones which had previously been implied by the strong Lac$^+$ phenotype displayed by them on ML containing ampicillin.

However, 10% of the colonies formed on this medium were Lac$^-$ with a further 5% of the Lac$^+$ colonies displaying Lac$^-$ segments. This instability of the Lac$^+$ phenotype was also observed when IPTG induced and uninduced cultures of RE476 Lac$^+$3 and RE476 Lac$^+$4 growing in HM containing glycerol and ampicillin, were spread onto ML containing ampicillin. Approximately 50% of the colonies from the induced cultures were Lac$^-$ on ML, with the non-induced cultures showing approximately 10% Lac$^-$ colonies.

Plasmid DNA was isolated from RE476 Lac$^+$3 and RE476 Lac$^+$4, by the scaled up method of Birnboim and Doly (1979) and the molecular weights of the plasmids were estimated by the method of Meyers et al. (1976). The plasmid isolated from RE476 Lac$^+$3 (designated pHE1) had a mobility which indicated a molecular weight of 26Mdal. The plasmid isolated from RE476 Lac$^+$4 (designated pHE2) had a mobility indicating a molecular weight of 28Mdal.

Thus evidence has been presented to show that the lac IPO Z and Y genes from the $F_K$lac plasmid have been cloned into pBR322, to produce two hybrid Lac$^+$ plasmids pHE1 and pHE2. The presence of pHE1 and pHE2 conferred upon an E. coli strain deleted for lac pro a strong Lac$^+$ phenotype which was observed to be unstable when the plasmid-containing E. coli strain was grown on ML. This instability was observed to be at its greatest when the lac pro deletion E. coli strain containing either pHE1 or pHE2 was induced with IPTG whilst growing in HM with a poor carbon source (such as glycerol). The basis for the observed instability is discussed in Chapter 4.

As both pHE1 and pHE2 appeared to contain the lac genes from $F_K$lac, all further work was confined to the smaller of the two plasmids, pHE1, whose physical characterisation is described in the following section.

3.2.2.4 Restriction Map of pHE1

A restriction map of pHE1 was produced using the enzymes Hind III, Eco R1, Bam H1 and Pst 1 either singly or in combinations involving two of the restriction enzymes, and partial digestions were employed in the case of Eco R1 and Bam H1. The DNA restriction fragments produced were digested and separated by agarose gel electrophoresis and a typical result is shown in Figure 3.14.

It can be seen in Figure 3.14 (lane 2) that Hind III digestion of pHE1 yielded 2 fragments, the 4.3kb pBR322 vector region and the approximately 36kb cloned DNA fragment from $F_K$lac. The pBR322 region of pHE1 contains one Bam H1 restriction site, approximately 4.0kb and 0.36kb from the Hind III restriction sites at either end of the pBR322 DNA in pHE1. The pHE1 Bam H1 digestion profile (Figure 3.14, lane 6) was therefore compared with the Bam H1/Hind III digestion profile to see which two Bam H1 restriction fragments had been reduced by 4.0kb and 0.36kb. It can be seen in Figure 3.14, lane 7 that a 10.5kb Bam H1 fragment is restricted by Hind III to give two bands of 6.2kb and 4.0kb. Similarly a 4.8kb Bam H1 restriction fragment was reduced in size to give a 4.5kb fragment following Bam H1/Hind III digestion.

Therefore a 4.8kb Bam H1 fragment overlaps the Hind III restriction site that is 0.36kb from the Bam H1 restriction site in the pBR322 region of pHE1. Similarly a Bam H1 restriction fragment of 10.5kb overlaps the Hind III at the opposite end of the pBR322 region of pHE1. A similar rationale was employed to produce the restriction map shown in Figure 3.15.

FIGURE 3.14

pHE1 restriction digest patterns

The restriction enzymes used were HindIII (H), EcoR1 (E), BamH1 (B) and Pst1 (P). The numerals refer to restriction fragments sizes in kb. The λ DNA EcoR1 restriction fragments are 21.8kb, 7.6kb, 5.9kb, 5.5kb, 4.9kb and 3.4kb. The λ DNA EcoR1 restriction fragments are 23.7kb, 9.5kb, 6.7kb, 4.3kb, 2.3kb, 2.0kb and 0.6kb. The λ DNA restriction fragments in Lanes 1 and 3 were used to establish the sizes of the restriction fragments in Lanes 2, 4, 5, 6 and 7; Lane 10 was used for the restriction fragments in lanes 8 and 9; Lane 12 was used for the restriction fragments in Lane 11.

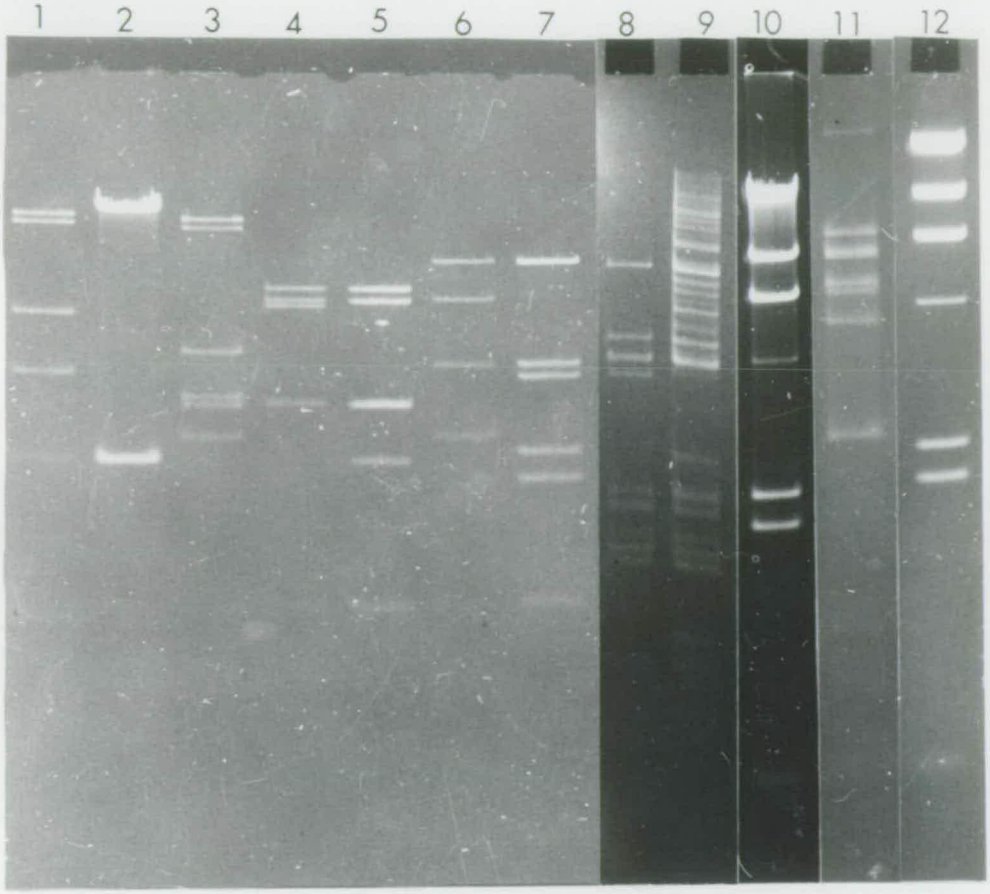


FIGURE 3.14

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12
DNA Digested	λ	pHE1	λ	pHE1	pHE1	pHE1	pHE1	pHE1	pHE1	λ	pHE1	λ
Restriction Enzyme used	H	H	E	E	H+E	B	H+B	P+H	P	H	B/E	H
		36.0		11.5	11.5	14.5	14.5	8.7	8.7		6.8	
		4.4		10.5	10.5	10.5	6.8	5.1	6.0		5.9	
				10.0	5.7	6.8	6.2	4.5	5.1		5.7	
				5.7	5.7	4.8	4.5	4.5	4.5		4.9	
				2.4	4.4	2.4	4.0	2.4	2.7		4.6	
					2.4		2.4	2.2	2.4		4.0	
								1.9	2.2		2.3	
								1.8	1.9		2.3	
								1.7	1.8		1.1	
								0.9	1.7		0.9	
								0.8	0.9			
								0.6	0.8			
									0.6			

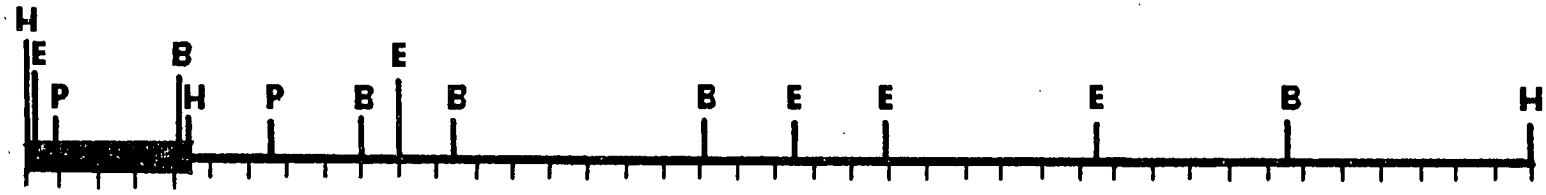


Figure 3·15

Restriction map of pHE1.

The thick line indicates the pBR322 region, and the thin line DNA cloned from $F_K$lac. The restriction enzymes used were HindIII (H), EcoR1 (E), BamH1 (B), and Pst1 (P), and the data from single, double, and partial digestions using these enzymes were used in the construction of the map.

The restriction sites in pBR322 are from Sutcliffe (1978); the scale is calibrated in Kb.

3.2.2.5 Estimation of lac DNA Position on pHE1 by DNA-DNA Hybridisation

DNA homology between the lac region of pHE8 and pHE1 was used to estimate the approximate position of the lac genes on pHE1.

Restriction fragments produced by separate Hind III and Eco R1 digestions of pHE1 DNA were separated by agarose gel electrophoresis, transferred to nitrocellulose filters and hybridised with radioactively labelled pHE8 DNA, as described in Chapter 2.

The labelled pHE8 DNA probe hybridised to both of the Hind III restriction fragments that made up pHE1, and therefore displayed homology with both the pBR322 DNA and the DNA cloned from F_K lac. However the result obtained when pHE8 was hybridised to Eco R1 digested pHE1 DNA was of much more interest, because only one homolog was formed (Figure 3.16, lane 2). One explanation of this result was that the pBR322 DNA and the region of cloned F_K lac DNA on pHE1 which hybridised to pHE8, were both contained on the same Eco R1 restriction fragment. An alternative explanation was that more than one Eco R1 restriction fragment was hybridising to the pHE8 probe, but the differently sized fragments were not being individually resolved on the autoradiogram. This was indeed a possibility because pHE1 contained three Eco R1 restriction fragments of similar sizes (10.0kb, 10.5kb and 11.5kb).

The presence of the lac genes on the 10.0kb Eco R1 restriction fragment was tested for in the following way. pHE1 DNA was fully digested with Eco R1, ligated overnight, and the DNA was transformed into RE476 and transformants were selected on LA containing ampicillin. Only plasmid molecules made up of pBR322 DNA can replicate and confer on the transformants resistance to ampicillin.

The ampicillin resistant colonies were screened for plasmids, and ability to produce β-galactosidase on X-Gal

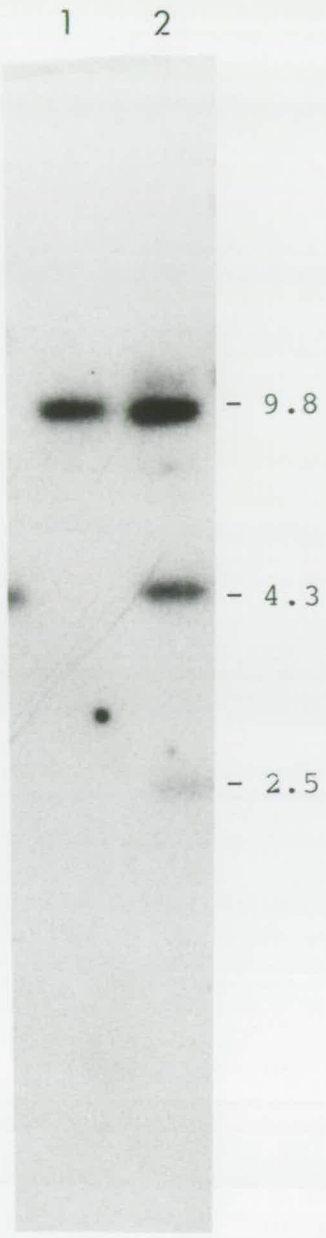


FIGURE 3.16

Analysis of pHE1 EcoR1 restriction fragments by hybridisation with ^{32}P labelled pHE8 DNA.

The autoradiogram shows fragments bearing homology to the pHE8 DNA. Lane **2** contained HindIII digested pHE8 DNA, and Lane **1** contained EcoR1 digested pHE1 DNA. Hybridisation was carried out as described in Chapter 2. Numerals on margin refer to the sizes of the DNA fragments which have hybridised with pHE8.

agar containing ampicillin. Although containing a 10.0kb plasmid none of the ampicillin resistant colonies produced β-galactosidase and therefore did not contain an intact lac PO Z region.

The characterization of pHE1 was not taken further due to the lack of available time and resources, and as a consequence of this the restriction map of pHE1 could not be compared with those of the E. coli and Klebsiella lac regions previously described in this Chapter.

However the positions of the individual lac genes on pHE1 could be rapidly established using radioactively labelled DNA probes containing specific regions of the E. coli lac operon which are now available.

3.3 Conclusion

The following may be concluded from the results presented in this chapter. The chromosomal lac genes from Klebsiella M5a1 have been successfully cloned, resulting finally in the plasmid pHE8. A restriction map has been established for pHE8 and the position of the lac Z and Y genes on the plasmid have been established; by transposon mediated mutagenesis. The positions of the lac I and lac PO regions have also been inferred. It is interesting to note that the Klebsiella M5a1 chromosomal lac genes cloned on pHE8 appeared to be organised in a similar way to the E. coli lac genes, with lac Y transcriptionally downstream of lac Z and lac PO and lac I upstream of lac Z.

Tn5 has been shown to undergo apparent preferential integration into the pHE8 lac Y region. Miller et al. (1980) found a strong correlation between A + T richness and regions of preferential Tn5 insertion in the E. coli lac operon. The apparent preferential Tn5 insertion in the lac Y on pHE8 may therefore also reflect a region of

A + T richness.

A comparison of the restriction map of the pHE8 *lac* region with that of the *E. coli lac* operon revealed no shared restriction sites for those enzymes used. However a comparison of the restriction map of the *lac* region on pHE8 with that of the cloned *Klebsiella pneumoniae* T17R1 *lac* genes (MacDonald and Riley, 1983) revealed the likelihood of common *Pst 1* restriction sites in these regions.

It has also been shown that a plasmid of approximately 120 M.dal was responsible for the "strong" lactose positive phenotype displayed by *Klebsiella* V9A F_k lac. This plasmid was isolated and the *lac* genes on it were successfully cloned into pBR322 resulting in the hybrid plasmid pHE1. A restriction map of pHE1 was established, but due to the instability of pHE1 the location of the *lac* genes on the plasmid could not be achieved using transposon mediated mutagenesis. However the *lac* genes were shown by hybridisation experiments to reside in part or in total on an approximately 10.0kb *Eco* R1 fragment.

E. coli containing either pHE1, or pHE8 were used to study the expression products of the cloned *lac* genes, and the results are described in Chapter 4.

CHAPTER 4

Proteins Coded by the *lac* Genes from *Klebsiella* and other Enteric Species

4.1 Introduction

It has been shown in Chapter 1 that the majority of the available information concerning the protein products of the lac systems in enteric species is based upon the *E. coli*, *Shigella dysenteriae* and Lac^+ plasmid systems. However the cloning of the F_k *lac* and *Klebsiella* M5a1 *lac* genes into pHE1 and pHE8 respectively, described in Chapter 3, provided for convenient comparisons to be made of some of the *lac* encoded proteins from each of the clones. The comparisons undertaken with the cloned *lac* systems fell into two distinct sections:

(a) Estimation and Comparison of β-Galactosidase subunit Molecular Weights.

The molecular weights of the β-galactosidase subunits coded on pHE1 and pHE8 were estimated by SDS-PAGE and compared with that previously reported for the *E. coli* enzyme subunit, which was based upon DNA sequencing data (Kalnins *et al.*, 1983). The comparison allowed for speculation concerning the relatedness of the various β-galactosidases.

(b) Effect on *E. coli* of Elevated Cellular Levels of Lactose Permease coded on pHE1 and pHE8

The pHE1 and pHE8 plasmids provided an opportunity to compare the effect on the bacterium of elevated levels of the cloned *lac Y* proteins with that previously reported for the *E. coli lac Y* protein (see below). This was because pHE1 and pHE8 are believed to exist in a multicopy state and would thus provide elevated cellular levels of lactose permease.

The phenomenon of the inhibition of E. coli cell growth by lactose was reported by von Hofsten (1961) who found that lactose constitutive mutants when grown on succinate were sensitive to lactose, whereas no sensitivity to lactose was observed when the mutant strains were grown on lactate.

The phenomenon was further investigated by Horiuchi et al. (1962) who grew E. coli on a limited supply of lactose in a chemostat, and thereby selected for strains which produced elevated levels of lac operon coded proteins. These hyper-producing strains made between three and four times as much β-galactosidase as the induced wild type E. coli K12, and on transfer to a medium containing excess lactose the hyper-producing strains rapidly stopped growing, with survival ranging between 0.01% to 10% of the original culture. The survivors were composed of cells which either produced no β-galactosidase or made it at a rate characteristic of normal E. coli wild type strains.

The addition of IPTG to the hyper-producing E. coli strains did not appear to affect their growth rate which implied that the lethality effect in these strains was not inducible. Horiuchi et al. (1962) also estimated the E. coli cellular levels of lactose permease by TMG uptake studies and concluded that the level of lactose permease in the hyper-producing strains was similar to that found in wild type E. coli. The authors therefore concluded that the β-galactosidase level, and not the level of lactose permease was in some way stopping cell growth.

Dykhuizèn and Hartl (1978) undertook similar studies to those above, but also used an E. coli strain which carried a deletion in the lac Z gene which did not alter lac Y gene expression. An E. coli strain identical to the lac Z deletion strain, but which possessed an intact lac operon was used as a control. The two strains were grown separately on limited melibiose in a chemostat, in order to

select for lactose permease constitutive mutants. High levels of lactose induced cell death were observed for both strains, when after 8 days of chemostat culturing, samples were plated on minimal media containing either lactose or other energy sources. This result strongly suggested that the β-galactosidase was not the cause of lactose killing. The authors also showed that catabolic products of lactose were not involved in lactose cell death, and suggested that the lac A enzyme, thiogalactoside transacetylase was not involved, as it would not accept lactose as a substrate.

Finally, Dykhuizen and Hartl (1978) suggested that the mechanism by which lactose is transported into the cell (described in Chapter 1) was directly involved in lactose induced cell death, because lactose uptake was driven by a proton gradient and rapid uptake of sugars would also be accompanied by a rapid influx of associated protons. They suggested that an expected consequence of this would be the collapse of the electro-chemical potential across the membrane and cessation of any transport into the cell by this mechanism.

If rapid sugar and associated proton uptake were the cause of lactose inhibition of growth the effect should be duplicated by other substances which are co-transported with protons. Ramos and Kaback (1977) demonstrated that growth of an E. coli strain constitutive for the hexose phosphate transport system (HP6R mutation), was inhibited by the addition of glucose-6-phosphate, which also produced a partial collapse of the membrane potential.

Further support for the concept that substrate transport was responsible for the decrease in membrane potential, and in turn the observed growth inhibition of E. coli was provided by Wilson et al. (1981). They demonstrated that several substrates of the lac permease transport system for example IPTG, TMG, TONPG and raffinose all produced growth

inhibitory effects, but to varying degrees, on an E. coli strain constitutive for lac and grown on glycerol. When sodium succinate was used as the sole energy source the inhibition was considerably more severe, however cells grown in rich medium were far less susceptible to the inhibition.

As the E. coli strain used by Wilson et al. (1981) was constitutive for lac expression, the substrates IPTG, TMG and lactose (following its conversion to allolactose) all of which are inducers, should not have increased the level of constitutive lac expression. Prior to the addition of the substrates, the lac constitutive E. coli strain grew normally on glycerol with no obvious growth inhibition. The results therefore suggested that it was the active transport of the substrates by the lactose permease which was responsible for the observed growth inhibition in E. coli rather than induced levels of lactose permease per se. This conclusion was further supported by the growth inhibition also observed when raffinose was used as a substrate in the experiments of Wilson et al. (1981). Raffinose, an α-galactoside is a substrate of the lactose permease transport system, but is not an inducer of the E. coli lac operon (Beckwith, 1970). Thus transport of the raffinose by the elevated levels of lactose permease in the E. coli lac constitutive strain was apparently sufficient to inhibit cell growth.

More recently the growth inhibitory affects of lactose permease on E. coli have been studied using the E. coli lac Y gene coding for the lactose permease, cloned into a multicopy plasmid (Padam et al., 1983). The plasmid borne lac DNA also contained the lac PO region but not the lac I gene, however constitutive expression of the multicopy lac Y genes was presumed to be partly repressed, because the E. coli host strain contained the lac I PO and Z genes on the chromosome. Padam et al. (1983) used the E. coli lac Y gene cloned into the multicopy plasmid pACYC184

which during normal bacterial growth exists at approximately 20 copies per chromosome (Chang and Cohen, 1978). E. coli synthesises approximately 10 molecules of lac repressor protein per cell (Gilbert and Müller-Hill, 1970), therefore many of the copies of the lac Y gene will be repressed. Padam et al. (1983) added IPTG or TMG to cultures of E. coli containing the lac Y plasmid and growing in Minimal medium plus glucose and tetracycline for plasmid selection. They observed cessation of cell growth within one hour of the addition, and a rapid decrease in colony forming ability. These results were to be expected on the basis that the IPTG or TMG not only induced expression of all of the copies of the lac Y gene per cell but also served as transport substates for the elevated levels of lactose permease.

However, Padam et al. (1983), using similar growth conditions to those described above observed that the addition of cAMP to E. coli carrying the multicopy lac Y genes produced severe cell growth inhibition, which was greater than that produced by either IPTG or TMG. The growth inhibition associated with the addition of cAMP was not observed in the E. coli host strains minus the lac Y multicopy plasmid, nor in the E. coli host strain containing only pACYC184, the multicopy vector minus the lac DNA. The addition of cAMP to cultures of E. coli growing on glucose as an energy source had previously been shown to release gene expression from catabolite repression (discussed in Chapter 1). It appeared from the above results that the addition of cAMP to a culture of E. coli containing the multicopy lac Y plasmid and growing in Minimal medium plus glucose and tetracycline released plasmid lac Y gene expression from the catabolite repression exerted upon the lac DNA by glucose. Thus elevated synthesis of lactose permease would occur, but most importantly it would occur in the absence of a substrate of the lactose permease active transport

system. This result implied that growth inhibition was not due to the elevated levels of lactose permease being involved in the active transport of substrates, but was due to elevated levels of lactose permease alone. These results contradicted the findings of Wilson et al. (1981) and the conclusions of those authors and Dykhuizen and Hartl (1978).

There have been several reports that the expression of amplified genes hindered cell growth and survival (Little, 1979; Fiil et al., 1979 and Shimatake and Rosenberg, 1981) however the molecular basis for these effects and that associated with elevated levels of lactose permease are not clear. Postgate and Hunter (1962) found that when K. pneumoniae, grown on a limited carbon source, was supplied with an excess of that carbon source increased amounts of uracil, guanine and hypoxanthine were released into the medium. The authors also provided evidence which suggested that the osmotic barrier of the bacterium was intact, however results presented later in this section suggest that this may not be the case with E. coli containing many copies of expressed lac genes.

It is obvious that further work is necessary to unravel the exact basis for the phenomenon of lactose induced cell death and pHE1 and pHE8 provided an opportunity to compare the effect on E. coli cells of amplified lactose permease from the F_k lac and Klebsiella M5a1 chromosomal encoded lac Y genes with that described above for the E. coli lac Y gene product.

Finally the question as to whether or not enteric species other than E. coli synthesised active thiogalactoside transacetylase, the product of the lac A gene, was asked.

Early work by Alpers et al. (1965) using a spectrophotometric assay procedure showed that of the enteric species tested only E. coli K12 and E. coli B, both with intact

lac Z and lac Y genes, synthesised the thiogalactoside transacetylase enzyme upon induction of the lac system. The authors obtained negative results for E. coli K12 strains which were permeaseless, Shigella dysenteriae (Sh 60), Salmonella typhimurium (LT2), and Serratia marcescens.

The results of Alpers et al. (1965) were interesting because all of the negative results were obtained from enteric species which lacked an intact lac system. Shigella dysenteriae is virtually indistinguishable from E. coli (see Chapter 1) and might therefore have been expected to also synthesise thiogalactoside transacetylase. However the Shigella dysenteriae strain used by Alpers et al. (1965) (Sh 60) has been shown by Sarkar (1966) to be deleted for lac Y. This deletion may have overlapped the thiogalactoside transacetylase gene, if present, or may have adversely effected expression of the lac A gene. Salmonella typhimurium LT2 has been shown to lack the equivalent chromosomal region to the lac region in E. coli (Sanderson and Hartman, 1978). Alpers et al. (1965) gave no indication as to the identity or lactose permease status, of the Serratia marcescens strain used by them. However <7% of Serratia marcescens strains tested (Table 1.1, Chapter 1) were indicated to produce a functional lactose permease, therefore there was a high probability that the strain used by Alpers et al. (1965) did not possess a functional lac Y gene.

The obvious question which arose from the negative results described by Alpers et al. (1965) was that if the permeaseless enteric species tested lack thiogalactoside transacetylase activity, did other enteric species, which possessed intact lac Z and lac Y genes and fermented lactose, produce inducible thiogalactoside transacetylase? Therefore several enteric species, including Klebsiella M5a1 and V9A, which had been shown to ferment lactose and thus to synthesise functional lac Z and Y protein products were assayed for the presence of thiogalactoside transacetylase.

4.2 Results and Discussion

4.2.1 β-Galactosidase Polypeptide Molecular Weight Estimation

The β-galactosidase polypeptide molecular weights were estimated by SDS-PAGE as described in Chapter 2. IPTG (10^{-4}M) induced, and uninduced cultures of RE476 containing either pHE1, pHE8 or pHE10 were included in the analysis. The plasmid pHE10 is pHE8 containing a Tn5 insertion in the lac Y gene (lac-202 Figure 3.9) which inactivated the lac Y gene and inhibited lactose permease synthesis (Chapter 3, Section 3.2.1.5). The inclusion of pHE10 in the analysis therefore enabled the β-galactosidase polypeptide to be positively identified because the only IPTG inducible polypeptide common to both RE476 (pHE8) and RE476 (pHE10) was the lac Z gene protein.

Figure 4.1A shows that a prominent band, whose mobility was similar to the E. coli β-galactosidase subunit (lane 4, marker B) was observed in the induced samples of RE476 containing either pHE10 or pHE8 (lanes 2 and 3, respectively) which was not present in the uninduced samples of RE476 (pHE8) (lane 1).

The Tn5 insertion in pHE10 was mapped to a position in the lac Y gene adjacent to lac Z (Figure 3.9). Therefore if a lac Y protein product was produced at all in RE476 (pHE10) it would be expected to have a reduced size and hence differ in its mobility to the functional lac Y protein, when subjected to SDS-PAGE. However it can be seen in Figure 4.1A that the prominant bands in lanes 2 and 3, which have similar mobilities to that of the E. coli β-galactosidase subunit (size standard marker B, lane 4) do not differ significantly in their mobilities. This was clearly demonstrated in Figure 4.1B (lanes 1 and 3).

These results strongly suggested that the prominent

Figure 4.1A, B. β-Galactosidase subunit molecular weight estimation by SDS-polyacrylamide gel electrophoresis.

The protein samples and standards were prepared and subjected to electrophoresis through SDS-polyacrylamide gel (7.5%) containing 0.2% bis-acrylamide as described in Chapter 2.

Figure 4.1A. Lane 1: RE476 (pHE8), uninduced; Lane 2: RE476 (pHE10), induced with IPTG; Lane 3: RE476 (pHE8), induced with IPTG; Lane 4: protein size standards (described below); Lane 5: RE476 (pHE1), induced with IPTG; Lane 6: RE476 (pHE1), uninduced; Lane 7: protein size standards.

Figure 4.1B. Lane 1: RE476 (pHE10), induced with IPTG; Lane 2; protein size standards; Lane 3: RE476 (pHE8), induced with IPTG.

The protein size standards are, A: myosin (rabbit muscle) 205,000; B: β-galactosidase (E.coli) 116,000; C: phosphorylase B (rabbit muscle) 97,400; D: albumin (bovine) 66,000; E: albumin (chicken) 45,000.

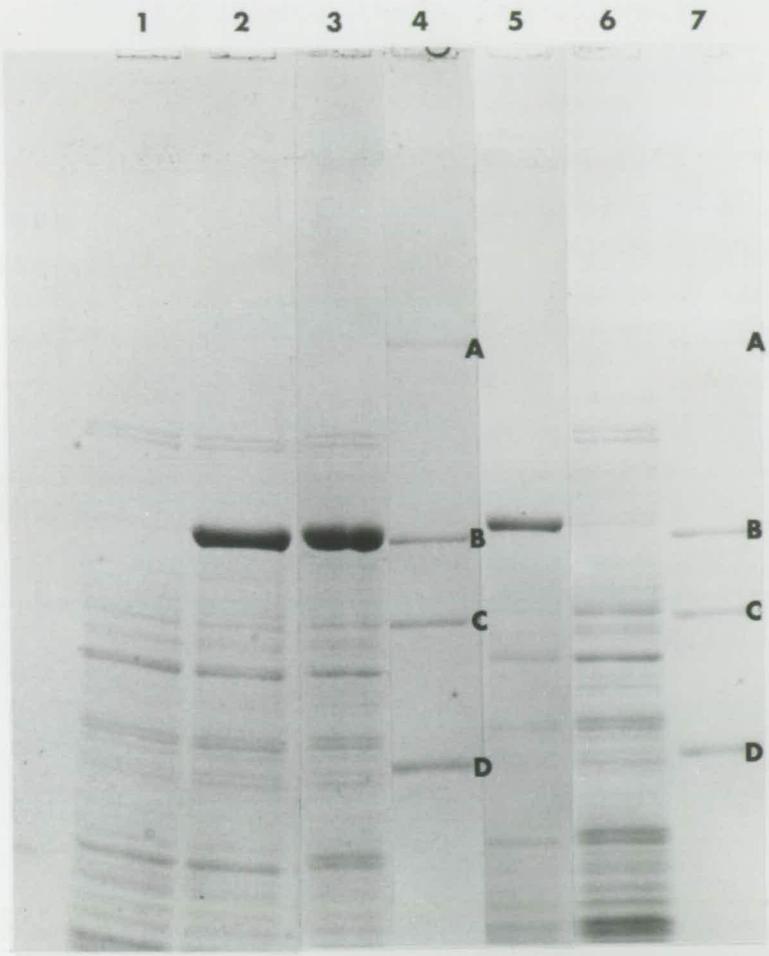


Figure 4·1A

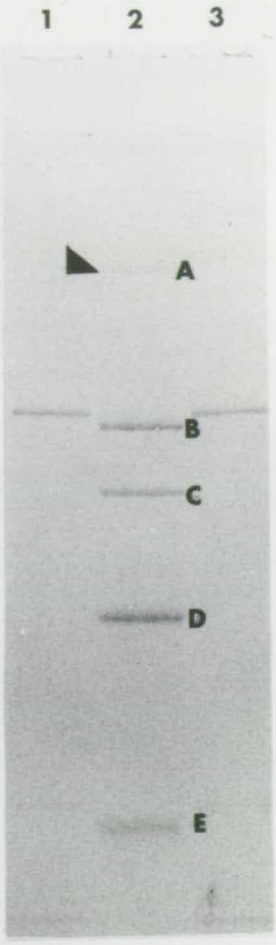


Figure 4·1B

bands in lanes 2 and 3 (Figure 4.1A) and lanes 1 and 3 (Figure 4.1B) were not the protein product of the *lac Y* gene. Further support for this conclusion was based on the observation that the prominent band represented a polypeptide chain with a molecular weight of approximately 126,000. This was considerably larger than would be expected for the intact *lac Y* gene polypeptide product, based on the size estimate of the pHE8 *lac Y* gene of a maximum possible length of 1.6kb (established in Chapter 3).

The transposon mutagenesis mapping work described in Chapter 3 also demonstrated that there would be insufficient DNA down-stream of *lac Y* to code for a gene co-ordinately induced with *lac Z* and *lac Y*.

The results above therefore indicated that the prominent bands seen in Figure 4.1A (lanes 2 and 3) and Figure 4.1B (lanes 1 and 3) represented the β-galactosidase polypeptide with a molecular weight of 126,000, coded on pHE8 by the *lac Z* gene which had been originally cloned from the chromosome of *Klebsiella* M5a1.

The analysis of RE476 (pHE1) is also shown in Figure 4.1A in which a prominent band can be seen in the induced sample (lane 5) which was not present in the uninduced sample (lane 6). Unfortunately transposon mutagenesis was not undertaken on pHE1 for reasons outlined in Chapter 3 and thus the prominent band in Figure 4.1A, lane 5 could not definitely be shown to be the polypeptide product of the *lac Z* gene on pHE1. The possibility therefore remains that the prominent band seen in Figure 4.1A lane 5 represents a polypeptide other than the *lac Z* gene product, and whose synthesis is induced by IPTG. However the work of Guiso and Ullmann (1976) who studied the *lac Z* and *Y* protein products encoded on Lac^+ plasmids from several enteric species, indicated that the plasmid encoded β-galactosidases were practically identical to that of the *E. coli lac Z* when

judged by several criteria (see Chapter 1). The prominent band seen in Figure 4.1A, lane 5 indicated a polypeptide with a molecular weight of approximately 125,000, which is similar to that of the E. coli β-galactosidase subunit (protein size standard marker B, lane 7). Taken in conjunction with the results of Guiso and Ullmann (1976) the SDS-PAGE results implied that the 125,000 polypeptide was the product of the pHE1 lac Z gene, originally cloned from $F_k$lac, but they cannot be said to definitely show this.

What was the evolutionary significance of the observation that the lac Z protein products from E. coli, $F_k$lac and Klebsiella M5a1 were very similar in size? Rohlfing and Crawford (1966a,b) observed that the molecular weight of the β-galactosidase native enzyme from Aeromonas formicans, a Gram-negative bacterium resembling the enteric species in their fermenting ability, "did not differ significantly" from that of the E. coli β-galactosidase enzyme, (molecular weight 465,412, based upon the subunit molecular weight of 116,353, Kalnins et al., 1983). However the authors also observed that Bacillus megaterium, a Gram-positive bacterium, contained a β-galactosidase enzyme which had a molecular weight of 150,000, a figure much smaller than the molecular weights of the β-galactosidase enzymes of E. coli or Aeromonas formicans.

Based on the observations of Rohlfing and Crawford (1966a,b) and Distler and Jourdain (1973), Hall (1976) argued that there were no apparent physical constraints on enzymes capable of hydrolysing β-galactoside bonds, which required the enzyme to be the size of the E. coli β-galactosidase or the Ebg^+ enzyme, because as demonstrated by the β-galactosidase from bovine testes (native enzyme molecular weight of 68,000, Distler and Jourdain, 1973) other β-galactosidases were considerably smaller. Hall (1976) suggested that this was evidence of ancestral homology between the ebgA gene and the E. coli lac Z.

This argument equally applies to the Klebsiella M5a1 chromosomal lac Z product, encoded on pHE8, with the implication of ancestral homology between the Klebsiella M5a1 lac Z gene and that in the E. coli lac operon and on F_K lac.

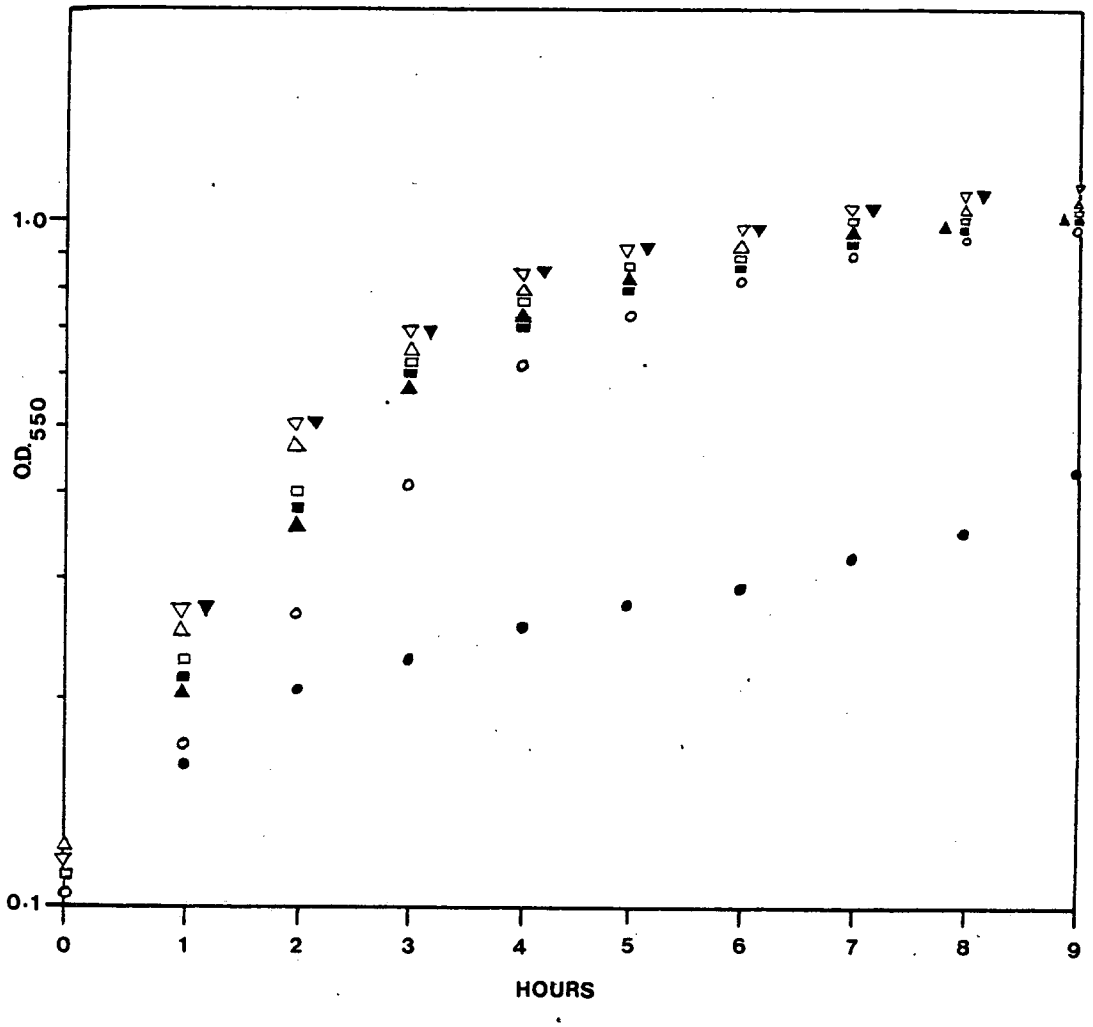
4.2.2 Effect on E. coli of Elevated Cellular Levels of Lactose Permease, Coded on pHE1 or pHE8

Cultures of RE476 either plasmid free or containing pHE1, pHE8 or pHE10 were grown in HM containing the necessary supplements, ampicillin and either glucose or glycerol as a carbon source. The effect on host cell growth, of IPTG induction of the plasmid borne lac genes, was examined as described in Chapter 2.

IPTG induction of RE476 (pHE8) grown on glucose rapidly inhibited cell growth (Figure 4.2, filled circles). The inhibition appeared to be transient, because 6 hours after the addition of IPTG to the medium the bacteria appeared to be capable of growth once more, as judged by the increase in the optical density at 550nm.

The IPTG mediated effect was not displayed by RE476 containing pHE10, which as described previously was Z^+Y^- and thus synthesised β-galactosidase, but not lactose permease. The results therefore indicated that the IPTG mediated inhibition of cell growth shown in Figure 4.2 required IPTG induced synthesis of the pHE8 lac Y gene protein. In those cases where gene expresseion is catabolite repressible (such as the E. coli lac operon) glucose exerts a greater degree of repression than does glycerol (Mandelstam, 1962). It might therefore be expected that growth inhibition of RE476 (pHE8) by IPTG induction of the plasmid borne lac genes, would be more severe for strains grown on glycerol than those grown on glucose. This would be due to the increased level of gene expression possible on the former carbon source.

Figure 4.2. Effect of induced expression of the lac genes on plasmids pHE1, pHE8 and pHE10 on growth of the host strain RE476 in HB glucose medium.

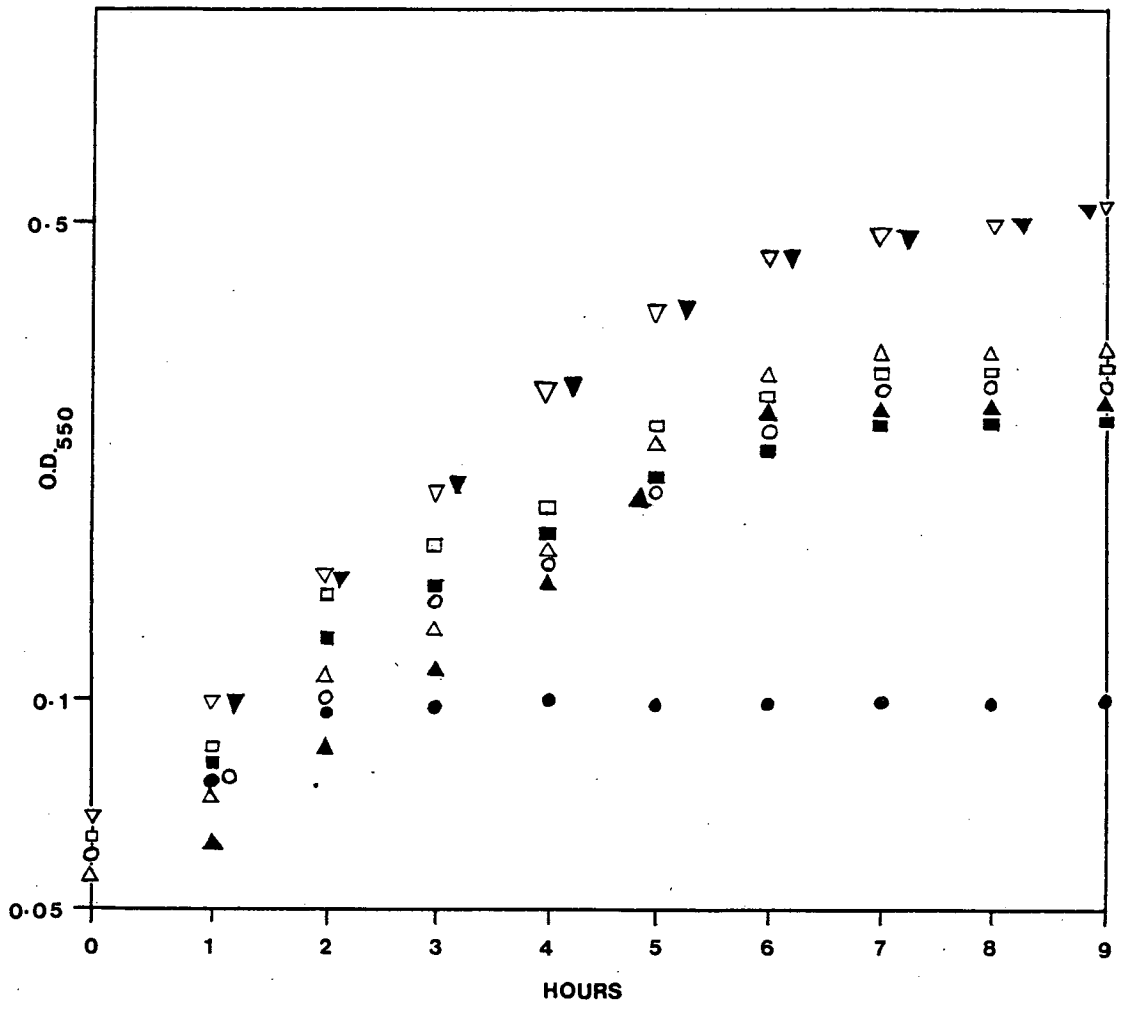


Cells of the plasmid free strain (▽) and of the strain transformed with pHE1 (△), pHE8 (○) or pHE10 (□) were grown in minimal medium plus 0.5% glucose, required supplements as described in Chapter 2, and ampicillin for those strains carrying plasmids. Growth was monitored by optical density (at 550nm) of aliquots of culture; after one doubling in fresh medium (0hours) IPTG (10^{-4}M) was added and growth monitored for a further 9hours as indicated by the solid symbols.

Figure 4.3 (filled circles) shows that this was the case, as the addition of IPTG to RE476 (pHE8) grown on glycerol, produced a greater inhibition of cell growth than that observed in Figure 4.2. The inhibition continued throughout the period in which cell growth was monitored. As with RE476 (pHE10) grown on glucose, IPTG induction of the strain grown on glycerol did not produce growth inhibition, although the growth rate of the induced culture appeared to be reduced slightly, presumably due to the metabolic burden of induced β-galactosidase synthesis.

The results obtained when RE476 (pHE1), growing on glucose or glycerol as a carbon source, was induced with IPTG were surprising because no growth inhibition was observed (Figures 4.2 and 4.3, filled triangles). In view of the unstable Lac^+ phenotype displayed by RE476 (pHE1) (previously described) aliquots of the induced and non-induced cultures were spread on ML containing ampicillin to see what proportion of the cell population were Lac^+. Approximately 40% of the colonies were Lac^- and 60% were Lac^+, of which 25% displayed Lac^- segregants within the colonies. The Lac^- colonies and the Lac^- segregants from within Lac^+ colonies were repurified on ML containing ampicillin, which implied that the ampicillin resistance gene on pHE1 was intact and retained, but that the *lac* genes on pHE1 had been lost or inactivated, or that the host bacterium was no longer capable of expressing the *lac* genes. The latter alternative was tested by growing the Lac^- lines of RE476 (pHE1) on X-Gal agar plus ampicillin with and without IPTG. Many of the Lac^- ampicillin resistant lines produced blue colonies on the above medium, indicating that they had synthesised β-galactosidase and that the host bacterium was capable of *lac* gene expression. This further suggested that the Lac^- phenotype displayed by these lines was due to either loss or inactivation of the *lac Y* gene on pHE1.

Figure 4.3. Effect of induced expression of the lac genes on plasmids pHE1, pHE8 and pHE10 on growth of the host strain RE476 in HB glycerol medium.



Cells of the plasmid free strain (▽) and of the strain transformed with pHE1 (△), pHE8 (○) or pHE10 (□) were grown in minimal medium plus 0.5% glycerol, required supplements as described in Chapter 2, and ampicillin for those strains carrying plasmids. Growth was monitored by optical density (at 550nm) of aliquots of culture; after one doubling in fresh medium (0hours) IPTG (10^{-4}M) was added and growth monitored for a further 9hours as indicated by the solid symbols.

the intact *E. coli lac Y* gene and some adjacent *lac* DNA into pBR322, observed similar Lac phenotype instability to that described above for RE476 (pHE1). Taken together these observations implied that cloned *E. coli*-like *lac* DNA has an inherent instability produced by an unknown mechanism which is not obviously dependent upon the size of the DNA fragment containing the *lac* DNA and which is *recA* independent. The cloned *lac* genes from *Klebsiella* M5a1 did not however display this instability. The stability of the pHE8 plasmid was further demonstrated by the addition of 0.9M cAMP to RE476 (pHE8) growing on ML containing ampicillin, the result of which is shown in Figure 4.4, where it can be seen that clear zones of growth inhibition were produced in the 'bacterial lawn' at the positions where cAMP was added. The clear (white) zones were surrounded by Lac^+ colony growth (dark areas). No zones of growth inhibition were observed when either 0.56M or 0.1M cAMP were added. Similarly no growth inhibition was observed when 0.9M cAMP was added to RE476 (pHE8) growing on MacConkey "lactose free" agar containing ampicillin and either glucose, maltose, arabinose or glycerol or when no carbon source was added. No zones of growth inhibition were observed when the above experiments were repeated using RE476 (pHE10) or RE476 (pHE1). These results may be explained as follows. The lactose in the ML, after conversion to allolactose would act as an inducer of the *lac* genes on pHE8. However the 'rich nature' of the ML would reduce the extent of induction due to catabolite repression. This repression would be released by the addition of cAMP to the system (see Chapter 1) which in turn would allow elevated levels of lactose permease to be produced by RE476 (pHE8) in those areas of cAMP addition, which led to growth inhibition. Growth inhibition was not observed for RE476 (pHE1) or RE476 (pHE10) due to plasmid instability, and plasmid *lac Y* gene inactivation respectively, both of which have been discussed previously.

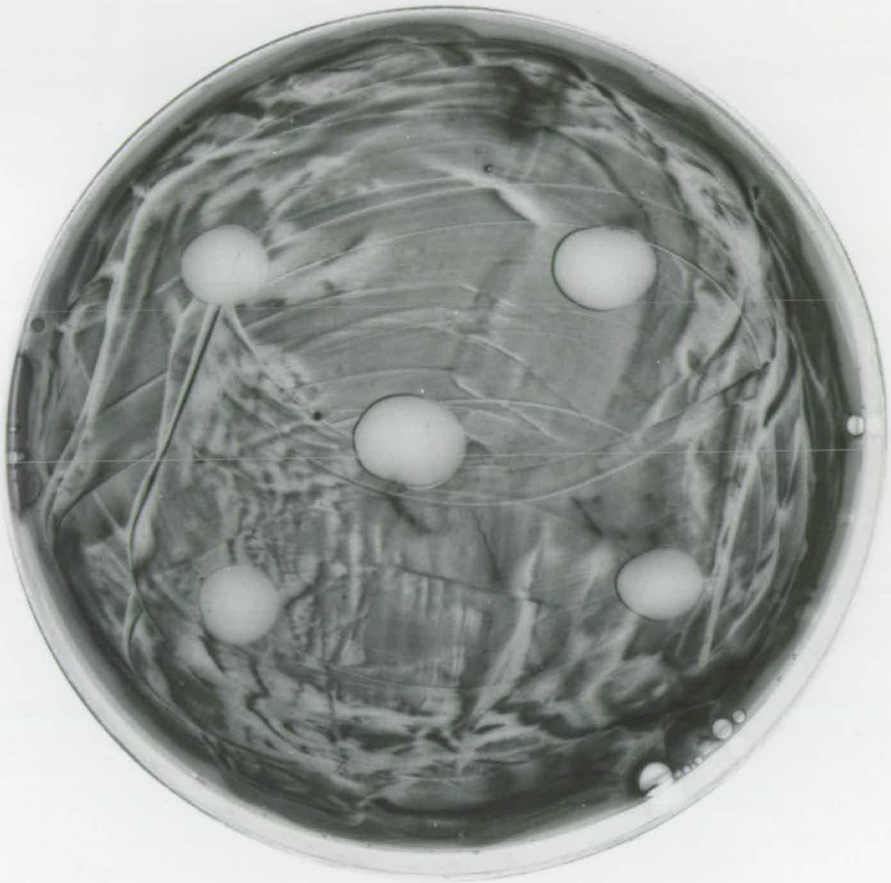


FIGURE 4.4

Growth inhibition of RE476 (pHE8) by the addition of cAMP.

A 0.1ml aliquot of an overnight culture of RE476 (pHE8) in LB plus ampicillin was spread onto ML containing ampicillin. Drops of 0.9M cAMP were added and allowed to dry and the bacteria were incubated overnight at 37°C. White circles indicate regions of inhibited bacterial growth, dark areas indicate regions of Lac^+ bacterial growth.

4.2.3. Investigation into the Mechanism of IPTG Induced Inhibition of Cell Growth

When RE476 (pHE8) was grown on X-Gal agar containing glucose, ampicillin and IPTG the blue colour, produced by the hydrolysis of the X-Gal, was seen to diffuse a distance of approximately 3mm through the medium around the colonies. RE476 (pHE10) did not display this diffusion when grown on similar medium, and nor did uninduced samples of RE476 (pHE8) or RE476 (pHE10). The observed diffusion appeared to be related to the IPTG induced expression of the lac Y gene on pHE8. X-Gal produces a non-diffusible dye when hydrolysed, and for the X-Gal to be hydrolysed contact with β-galactosidase would be required; two alternative explanations existed to account for the diffusion of blue dye beyond colonies of E. coli containing pHE8. Either the X-Gal was hydrolysed in the cell and diffused out through the membrane, which would imply a change in membrane permeability associated with the elevated levels of lactose permease. Or β-galactosidase was released from the cell into the surrounding medium where the enzyme hydrolysed the X-Gal. However because of the large size of the enzyme this possibility would probably necessitate a very severe breakdown in the integrity of the cell if not total lysis. Accompanying the release of the enzyme would also be the release of hydrolysed X-Gal which had been hydrolysed within the cell.

In an attempt to differentiate between the two hypotheses outlined above, the following experiment was undertaken. Parallel cultures of RE476 (pHE8) were uninduced or induced with IPTG, and then centrifuged to produce 'cell-free' supernatants. 2ml aliquots of these supernatants were freeze dried, then resuspended in 40μl of SDS-PAGE sample buffer and subjected to SDS-PAGE as described in Chapter 2. No protein with a mobility equivalent to a molecular weight of approximately 126,000 was detected in

either the induced or uninduced samples. This suggested that β-galactosidase was not released into the medium after induction of the RE476 (pHE8) culture, and that the ring of diffused hydrolysed X-Gal was most probably due to the blue dye passing through the effected membrane. How an increase in the cellular levels of lactose permease could bring about such a change in the membrane permeability is as yet unknown. However it may well be associated with the breakdown of membrane potential which is brought about by elevated levels of active transport across the membrane (see Introduction to this Chapter). Such a breakdown in membrane potential might be produced by an increase in the lactose permease mediated membrane transport of IPTG.

An interesting aspect of the growth inhibition results obtained with E. coli containing pHE8 was the potential for a cloning vector based on the pBR322 vector and the Klebsiella M5al lac I PO Y DNA. A problem encountered in molecular cloning, results from the fact that during ligation reactions intramolecular ligation usually occurs more rapidly than intermolecular ligation. The majority product of an in vitro ligation reaction thus tends to be vector DNA which has simply recircularised covalently. Following transformation the majority of transformants will contain vector DNA only. The advantage of a cloning vector based on the lac Y gene system would be that positive selection could be applied for a fragment cloned into the lac Y gene. This would be because all of the transformants which simply received vector DNA would not be capable of growth on HA plus glycerol IPTG and ampicillin. Only those cells with a cloned fragment in the lac Y gene which would inactivate the lac Y gene and thus inhibit lactose permease synthesis could grow under these conditions.

A similar system for positive selection for hybrid plasmids has been proposed by Hagan and Warren (1982) based

on the observation that long palindromic sequences in plasmids are either lethal to the plasmid or to the cell. By removing a restriction fragment from their plasmids a potentially lethal palindromic sequence is produced on the plasmid. If the vector plasmid should simply religate, the palindrome is produced and the plasmid produced is lethal. Only those plasmids with fragments ligated into the palindromic region remain viable upon transformation of E. coli. However, the drawback of this system is that a fragment must be removed from the cloning vector before the potential palindrome may form.

A vector based upon the lac Y gene system negates this step and provides for lethality of religated vector molecules following transformation.

4.2.4. The Determination of the Presence or Absence of Thiogalactoside Transacetylase in Enteric Species

As previously mentioned, in Chapter 1, the measurement of the E. coli β-galactosidase and thiogalactoside transacetylase activity demonstrated coordinated expression of the lac Z and lac A genes, however it was also shown that a polarity of gene expression existed, by which less thiogalactoside transacetylase was synthesised than was β-galactosidase. Therefore if several enteric species were to be analysed for thiogalactoside transacetylase activity, it was necessary to demonstrate that sufficient IPTG induced bacterial material had been used in the assay procedures in order that low thiogalactoside transacetylase activity could be detected.

This was done by harvesting and concentrating IPTG induced cultures of the enteric species, before the cell suspensions were sonicated and then assayed for β-galactoside and thiogalactoside transacetylase activity. By comparing the β-galactosidase activities of the various enteric

species with the β-galactosidase and the thiogalactoside transacetylase activity measured in similarly treated cultures of E. coli K12, an indication was obtained as to whether or not sufficient bacterial material had been used.

Of the two experiments undertaken the first involved the concentration of 50ml cultures by 10X, and the second involved the concentration of 100ml cultures by 40X, before the cultures were sonicated and assayed for β-galactosidase and thiogalactoside transacetylase activity as described by Miller (1972). The combined results of the two experiments are shown in Table 4.1.

The uninduced and induced β-galactosidase activity (standardised to a cell density of 1.0 at 600nm) showed that all of the enteric species tested had been induced by IPTG. The total β-galactosidase units in the sonicates produced in the experiments showed that with the exception of the Enterobacter aerogenes strain, high levels of β-galactosidase were present in the sonicates, due to the concentration procedures outlined above. When the thiogalactoside transacetylase activity in the various enteric species was compared with that in E. coli as described in Table 4.1, it was shown that none of the enteric species tested displayed significant levels of thiogalactoside transacetylase activity.

The analysis was further continued and involved the concentration by 200X of 1 litre of IPTG induced cultures of the Klebsiella strains M5a1 and RE1435. No thiogalactoside transacetylase activity was observed in either sample.

Alpers et al. (1965) had demonstrated that the E. coli thiogalactoside transacetylase enzyme was not sensitive to heat when held at 70°C for 5 minutes, a step which was incorporated in the assay procedure of Miller (1972) in order to eliminate IPTG-independent cleavage of dithiobis (2-nitrobenzoic acid) (DTNB). However the possibility existed that the enteric species tested possessed heat labile

Table 4.1. Thiogalactoside transacetylase presence in various Lac$^+$ Enterobacteriaceae strains induced with IPTG.

Strain	β-galactosidase activity OD_{600} 1.0		Total β-galactosidase units/ml of sonicate (2 assays) $\times 10^{-3}$	% expected thiogalactoside transacetylase activity
	Uninduced	Induced		
E.coli (FRL1)	3100	3400	106	100
Klebsiella V9A	8.0	870	35.5	<2
Klebsiella M5a1	69	570	22	6
Citrobacter freundii	24	355	30	3.3
Enterobacter aerogenes	15	120	8	3.6
Erwinia herbicola	80	1220	59	6.2

The β-galactosidase and transacetylase activity in the sonicates was assayed as described in Chapter 2. β-galactosidase activity was expressed as units of enzyme per ml of sonicate which produced 1nmole o-nitrophenol per minute at 28^{o}C at pH7.0. The ratio of the β-galactosidase activity to thiogalactoside transacetylase activity was used to establish an expected thiogalactoside transacetylase activity for each of the strains based on their individual β-galactosidase activities. The actual thiogalactoside transacetylase activity was then calculated and the average of the two experiments is shown as a percentage of the expected thiogalactoside transacetylase activity. Where OD_{412} per hour for thiogalactoside transacetylase was zero or negative it was taken as <0.01.

E.coli (FRL1) (Reeve and Braithwaite, 1974) is a total lac deletion strain carrying an F'lac which is lacI$^-$ and therefore constitutively expresses the lacZ Y and A genes.

thiogalactoside transacetylases which were inactivated during the assay procedure. Therefore samples of the sonicates of each of the enteric species used in the experiments above were assayed for thiogalactoside transacetylase activity by the method described by Miller (1972), but with the heat inactivation step omitted. No thiogalactoside transacetylase activity was observed in any of the enteric species other than E. coli containing FRL1, therefore those enteric species did not appear to possess heat labile thiogalactoside transacetylase.

It might be argued that the results outlined above were due to the ratio of synthesised β-galactosidase to thiogalactoside transacetylase being greater in the enteric species tested than in E. coli. The concentration steps, in particular that of 200X should have allowed thiogalactoside transacetylase activity, if present, to be detected, however the possibility of very low levels of thiogalactoside transacetylase activity cannot be discounted on the basis of the above results.

In conclusion, assay data described above strongly suggested that with the exception of the E. coli strain containing FRL1 the enteric strains described in Table 4.1 and the Klebsiella strain RE1435 did not produce thiogalactoside transacetylase following IPTG induction.

As all of the enteric species tested above ferment lactose (see Chapter 1) it appeared that thiogalactoside transacetylase activity was not essential for lactose fermentation in these species and it did not "play a vital role in lactose utilisation", as was suggested for the E. coli lac A protein by Fried (1980).

If the absence of thiogalactoside transacetylase activity indicated the related absence of a lac A gene, then presumably E. coli had either acquired its lac operon after divergence from the other enteric species, or E. coli

possessed a common *lac* operon but following divergence had acquired a *lac A* gene which had integrated downstream and directly adjacent to *lac Y*. Alternatively all of the enteric species tested by Alpers *et al*. (1965), and those tested in this work could have lost the ability to synthesise a functional thiogalactoside transacetylase, but due to some unidentified selection pressure on *E. coli*, it had retained a *lac A* gene.

It is interesting to note that the negative results for thiogalactoside transacetylase activity obtained with *Klebsiella* V9A implied that neither the chromosomal nor plasmid *lac* systems encoded a functional thiogalactoside transacetylase. The latter implication supported the findings of Guisso and Ullman (1976) who found that none of the Lac^+ plasmids, from various enteric species, tested by them encoded a functional *lac A* gene.

CHAPTER 5

The Estimation of DNA Homology Between *Klebsiella* M5al *lac* Region DNA and Genomic DNA from Various Enteric Species

5.1. Introduction

The aim of this work was to establish the presence or absence of DNA sequences within the genomes of various members of the *Enterobacteriaceae*, which displayed homology to the *Klebsiella* M5al *lac* region DNA cloned in pHE8. The method of Southern (1975) was employed to achieve this end. Total DNA was isolated from 13 enteric species representing 10 genera by the modified method of Schwinghamer (1980). Each of the enteric species was also screened for the presence of a Lac^+ plasmid, to ensure that any observed hybridisation was only to genomic DNA and not plasmid borne DNA.

The DNA preparations were digested with *Eco*R1 and *Hind* III, subjected to agarose gel electrophoresis, and were then transferred to nitrocellulose filters by the method of Southern (1975). Nitrocellulose filters, bearing the DNA digestion products from the various enteric species were hybridised, under stringent conditions to one of the following radioactively labelled DNA probes, (a) pHE8 which contained an approximately 12kb *Eco*R1 fragment of *Klebsiella* M5al chromosomal DNA shown to contain the Kpn *lac I PO Z Y* DNA (this work) cloned into pBR322 at the *Eco*R1 site; (b) pHE11, which contained a 9.8kb *Hind* III fragment, from pHE8, which has been demonstrated to carry *lac I PO* and 2kb of adjacent *lac Z* (Chapter 3 of this work) cloned into pBR322 at the *Hind* III site; (c) fragment C (Figure 3.4), a 2.5kb *Hind* III fragment from pHE8 shown to contain the *lac Y* and approximately 1kb of adjacent *lac Z* (Chapter 3 of this work) which was isolated by the method of Yang *et al*. (1979); (d) pBR322 vector; (e) fragment D (Figure 3.4) a 12.6kb *Eco*R1 fragment from pHE8. Hybridisation with pHE8

provided an estimate of the apparent relatedness between the lac region of Klebsiella M5a1 and DNA from the various enterics, whilst hybridisation with fragment C and pHE11 enabled detection of any major variation between the two sections of the Klebsiella M5a1 lac region with regards to relatedness to the enteric DNA. Hybridisation with pBR322 DNA enabled identification of the sequences that hybridised with vector DNA.

Autoradiograms were made to locate probe DNA bound to the filters, which permitted identification of the restriction fragments that contained segments of DNA homologous to the probe DNA sequences. The autoradiograms were traced by densitometry and the areas under the peaks were determined.

According to Southern (1975) the blackening of X-ray film by ionizing radiation is proportional to the amount of incident radiation up to the limit where a high proportion of silver grains are exposed. The relative amount of radioactivity in the bands on an autoradiogram could therefore be compared by tracing those bands and comparing peak areas. Assuming that the relative amount of bound radioactive probe reflected the degree of DNA sequence similarity between probe DNA and the genomic DNA to which it was bound, a measure of relatedness between the two could be estimated directly from the densitometry data obtained. When this project was initially undertaken in 1979 it was felt that the DNA-DNA hybridisation studies described above offered a convenient method by which the degree of relatedness between the chromosomal lac region of Klebsiella M5a1 and homologous regions in the genomes of various enteric species could be established. The method appeared to be a viable alternative to the monumental task of sequencing those regions of genomic DNA in the enteric species which displayed homology to the K. pneumoniae M5a1 lac region. The

hybridisation method had been employed for a similar purpose by Riley and Anilionis (1980). They hybridised E. coli DNA probes containing either trp, thy A, tna A or lac Z to restriction enzyme digested genomic DNA from various enteric species. Their results were presented as the percent of homology between the E. coli probes and the genomic restriction fragments from the various enteric species, that displayed homology (see page 48).

More recently however doubts have been raised concerning the applicability of this approach to quantifying DNA-DNA relatedness, and this is discussed in detail later in this Chapter.

As previously described in Chapter 1 there are members of the Enterobacteriaceae that normally display a very weak or zero ability to ferment lactose; however there are numerous reports of these bacteria having acquired Lac^+ plasmids, the presence of which would confuse the interpretation of any hybridisation results. The enteric species used in this study were therefore tested for the presence of Lac^+ plasmids before the hybridisation work was undertaken.

5.2. Results and Discussion

5.2.1. Detection of Lac^+ Plasmids in the Enteric Species Studied

The enteric species used in this study were tested for their ability to ferment lactose on ML, because the presence of a Lac^+ plasmid may be identified, in most cases, by a strong Lac^+ phenotype displayed on such medium (Reeve and Braithwaite, 1973). The presence of plasmid DNA was tested for by the modified methods of Schwinghamer (1980) and Eckhardt (1978). The combined results of these tests are shown in Table 5.1.

Table 5.1. Presence of Lac^+ plasmids in various enteric species.

Strain	Lac phenotype on MacConkey lactose agar	Plasmids detected[a] using method of Schwinghammer, Eckhardt.	
E.coli W1485	strong Lac^+[b]	-	-
E.coli RE439	Lac^-	-	-
S.marcescens NCTC1377	Lac^-[c]	-	-
H.alvei NCTC6578	Lac^-	+	5,40
C.freundii NCTC9750	strong Lac^+	-	-
P.morganii NCTC235	Lac^-	-	-
S.typhimurium NCTC5710	Lac^-	+	40
S.sonnei NCTC8574	Lac^-	+	40
E.carotovora SCRI193	$Lac^{-/+}$	-	-
E.chrysanthemi SCRI409	Lac^-	-	-
Klebsiella M5a1	$Lac^{-/+}$	-	-
Klebsiella RE1435	$Lac^{-/+}$	+	33
E.aerogenes NCTC10006	$Lac^{-/+}$	-	-

a)The presence (+) or absence (-) of plasmids are given and in the case of the Eckhadt (1978) method molecular weights (Md) of plasmids observed. Results are based on two separate gel electrophoresis experiments (Eckhardt,1978), and on one Schwinghammer (1980) experiment.

b)'Strong Lac^+' is indicated by a dark pink to red colony colour with associated precipitation of bile salts in the media, '$Lac^{-/+}$' by a pale pink colony colour and 'Lac^-' by a pale yellow, opaque colony (Reeve and Braithwaite, 1972).

c)Serratia marcescens produced a red pigment, therefore no direct conclusion could be made concerning the lactose phenotype on MacConkey agar, however no precipitation of bile salts was observed in the media, which is associated with rapid lactose utilisation and indicative of a 'strong' Lac^+ phenotype (Eddy, 1961; Reeve and Braithwaite, 1972).

Those enteric species previously reported as weak or negative with regards to lactose fermentation (Edwards and Ewing, 1972; Cowan et al., 1974) gave results consistent with those previously reported and the C. freundii and E. coli species gave the strong Lac^+ phenotype on ML normally associated with their chromosomal lac systems.

The C. freundii, E. coli, E. aerogenes, E. carotovora, E. chrysanthemi, P. morganii, S. marcescens and Klebsiella M5al strains tested were apparently plasmid free. The negative results obtained with C. freundi , E. coli and E. aerogenes were in agreement with the observations of Riley and Anilionis, who used identical strains. Similarly, Forbes (1983) had not detected plasmid DNA in the E. carotovora strain SCRI 193. Plasmids were found in the S. sonnei, S. typhimurium and H. alvei strains and Klebsiella RE1435. RE1435 was a derivative of Klebsiella V9A (Reeve and Braithwaite, 1970) which was thought to have lost $F_k$lac and the tetracycline resistance plasmid T_k, both resident in Klebsiella V9A. No plasmid species similar in size to $F_k$lac was found in RE1435, however a 33M.dal 'cryptic' plasmid was present in the strain. When this plasmid and Tk were separately digested with EcoRl, BamHl or Hind III singly or in combination, it was seen that the plasmids shared restriction fragments of similar sizes. It therefore appeared that the loss of a region(s) of DNA from the 40M.dal T_k plasmid (estimated in Chapter 3) had given rise to the 33M.dal 'cryptic plasmid' and the subsequent loss of tetracycline resistance. The 33M.dal 'cryptic' plasmid did not hybridise with pHE8 probe DNA, as shown in Section 5.2.4, and therefore did not obscure the results obtained for RE1435. Similarly no hybridisation was observed between probe DNA and DNA isolated from S. typhimurium and therefore the presence of plasmid DNA did not confuse data interpretation. The plasmids in H. alvei did not interfere with

interpretation of the hybridisation data, because homology between the probes used and DNA isolated from H. alveii was confined to homology between the pBR322 region of the probe DNA and H. alveii DNA (Discussed in Section 5.2.4). The effect on the hybridisation data of the plasmid in the S. sonnei strain is also discussed in Section 5.2.4.

It should be noted here that failure to detect a plasmid in a bacterial strain does not necessarily mean that the bacterial strain is "plasmid-free", but may merely reflect the limitations of the procedures employed. However, for reasons outlined on page 72 the plasmid analysis results, in conjunction with the lactose fermentation phenotypes obtained on ML, strongly suggested that none of the enteric species used in the hybridisation experiments contained a Lac^+ plasmid.

5.2.2. Bacterial DNA Restriction Digestion Patterns

0.5µg aliquots of DNA from the enteric species were digested with either Hind III or EcoR1 and subjected to gel electrophoresis through 1.0% agarose as shown in Figures 5.1A and 5.1B respectively. It can be seen that each of the patterns of the enteric DNA digestions differed one from another, indicating that the Hind III and EcoR1 restriction target sequences were distributed differently in the genomes of these enteric species.

The denatured DNA restriction fragments were transferred to nitrocellulose by the method of Southern (1975) with the addition of the modification of Wahl et al. (1979) to enhance the transfer of large DNA fragments. The nitrocellulose bound fragments were hybridised with, separately, the radioactively labelled DNA probes previously described in this Chapter.

FIGURE 5.1A

Agarose gel electrophoresis of HindIII digests of DNA from various enteric species.

Digests of 0.5µg of DNA from each enteric species were subjected to electrophoresis through 1% agarose and stained with ethidium bromide and photographed.

Lane 1: λ DNA and pHE7 DNA digested with HindIII, 2: pHE8 DNA (0.2ng) digested with HindIII, 3: pHE8 DNA (0.1ng) digested with HindIII, 4: Klebsiella M5al, 5: P. morganii, 6: E. carotovora, 7: S. typhimurium, 8: E. coli, 9: S. sonnei, 10: H. alvei, 11: λ DNA digested with HindIII, 12: E. chrysanthemi, 13: C. freundii, 14: E. aerogenes, 15: S. marcescens, 16: λ DNA digested with EcoR1.

λ DNA HindIII fragments are 23.7kb, 9.5kb, 6.7kb, 4.3kb, 2.3kb, 2.0kb and 0.6kb.

λ DNA EcoR1 fragments are 21.8kb, 7.6kb, 5.9kb, 5.5kb, 4.9kb and 3.4kb.

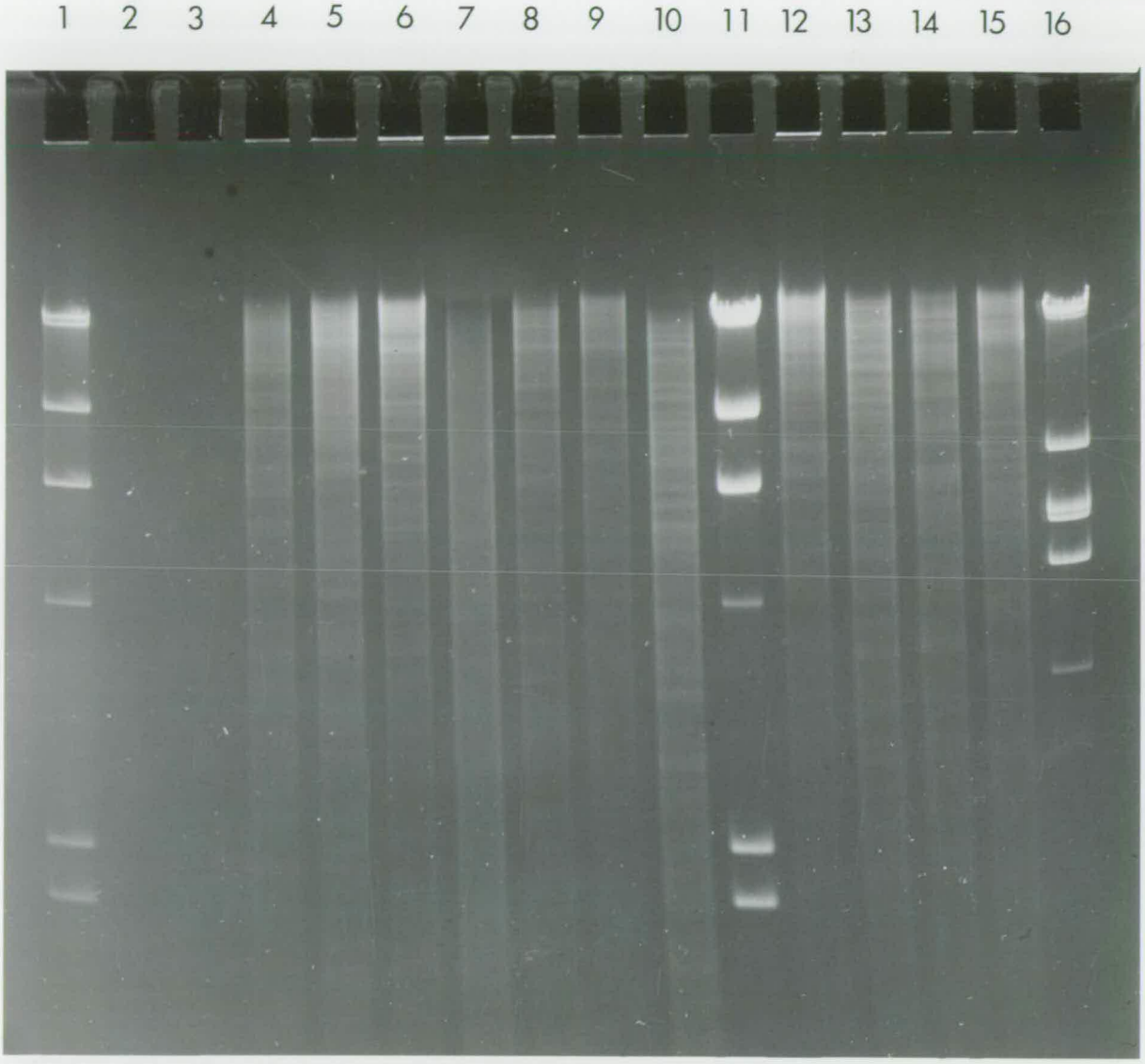

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

FIGURE 5.1B

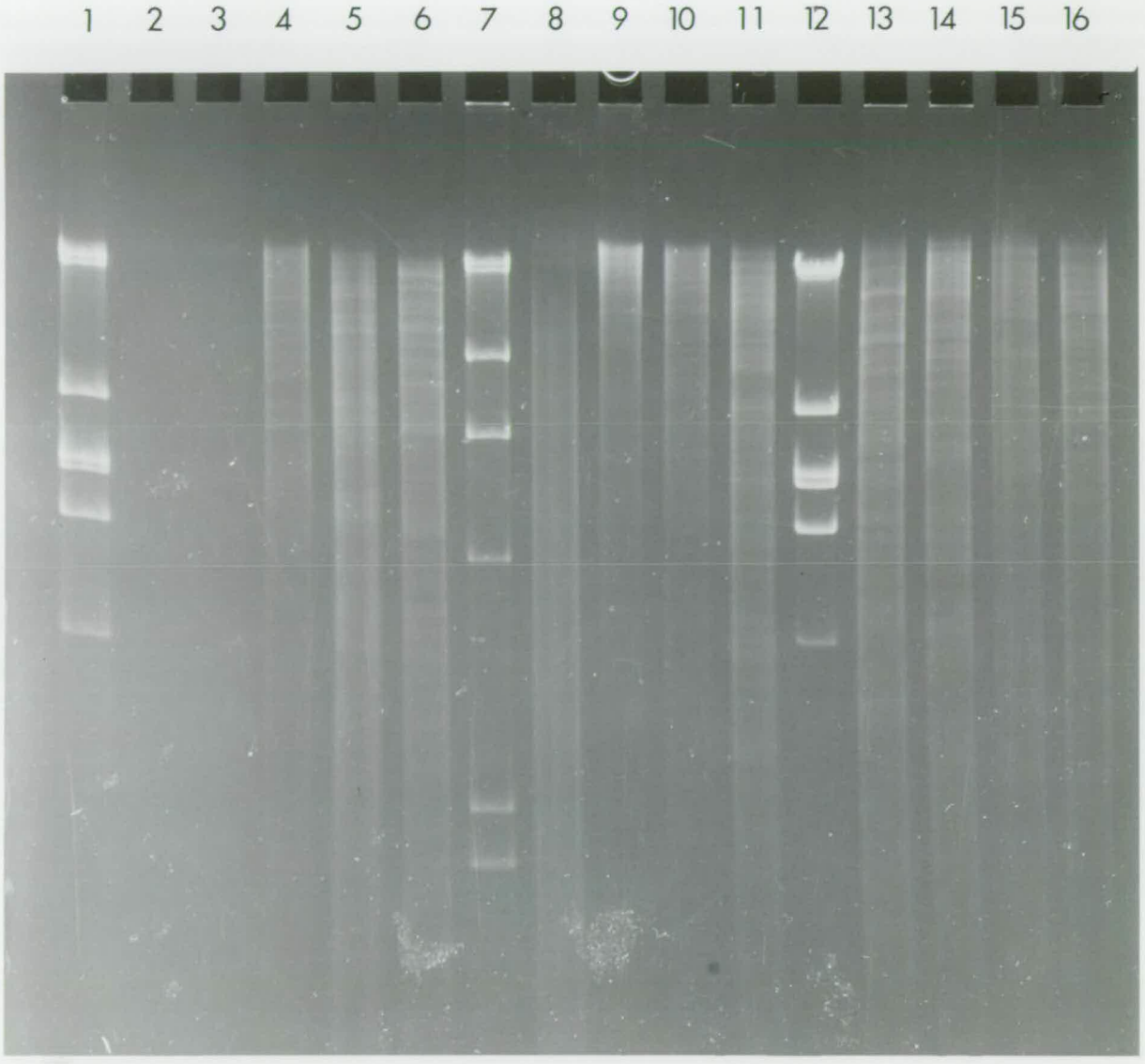
Agarose gel electrophoresis of EcoR1 digests of DNA from various enteric species.

Digests of 0.5µg of DNA from each enteric species were subjected to electrophoresis through 1% agarose and stained with ethidium bromide and photographed.

Lane 1: λ DNA digested with EcoR1, and pHE7 DNA digested with HindIII, 2: pHE8 DNA (0.2ng) digested with EcoR1, 3: pHE8 DNA (0.1ng) digested with EcoR1, 4: Klebsiella M5a1, 5: P. morganii, 6: E. carotovora, 7: λ DNA digested with HindIII, 8: S. typhimurium, 9: E. coli, 10: S. sonnei, 11: H. alvei, 12: λ DNA digested with EcoR1, 13: E. chrysanthemi, 14: C. freundii, 15: E. aerogenes, 16: S. marcescens.

λ DNA HindIII fragments are 23.7kb, 9.5kb, 6.7kb, 4.3kb, 2.3kb, 2.0kb and 0.6kb.

λ DNA EcoR1 fragments are 21.8kb, 7.6kb, 5.9kb, 5.5kb, 4.9kb and 3.4kb.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

5.2.3. Hybridisation of ^{32}P labelled pBR322 DNA to Restricted Enteric DNA.

The results of hybridising labelled pBR322 DNA with EcoR1 digested DNA from enteric species are shown in Figure 5.2. Digested DNA from S. sonnei (lane 7) contained three fragments of 8.5kb, 6.5kb and 3.4kb which shared common sequences with pBR322. DNA from H. alvei also shared common sequences with pBR322 as demonstrated by the formation of three homologs of 6.7kb, 3.2kb and 2.9kb (Figure 5.2, lane 8). Genes coding for the tetracycline and ampicillin resistance expressed by H. alvei may have been responsible for homolog formation with the pBR322 probe. However, the possibility existed that the observed homologs were due to DNA on the plasmids present in the H. alvei strain. The S. sonnei strain did not display resistance to either tetracycline or ampicillin, but did possess a plasmid, which may have been involved in homolog formation with the pBR322 probe.

5.2.4. Hybridisation of ^{32}P labelled pHE8 to Restricted Enteric DNA.

When pHE8was hybridised to nitrocellulose 'blots' produced from the agarose gels seen in Figures 5.1A and B many homologs were formed (Figures 5.3A and B), which were not present when pBR322 was used as the probe (5.2). However the same homolog pattern was obtained when the EcoR1 digested DNA from H. alvei was hybridised with either pHE8 or pBR322. This implied that homology between DNA from H. alvei and pHE8 was confined to the pBR322 vector region of the probe. Similarly three of the four homologs formed between EcoR1 digested S. sonnei DNA and pHE8 were also formed when pBR322 was used as the probe DNA, indicating that those homologs were due to homology with the pBR322 vector region of pHE8. Therefore the remaining homologs observed in Figures 5.3A and B indicated the location of Klebsiella M5a1

Figure 5.2. Hybridisation of ^{32}P labelled pBR322 DNA to EcoRI digests of DNA from various enteric species.

DNA from various enteric species was digested with EcoRI and the restriction fragments separated by electrophoresis through 1% agarose, transferred to a nitrocellulose filter and hybridised to ^{32}P labelledpBR322 DNA.

Lane 1: λ DNA and pHE7 DNA digested with HindIII; Lane 2: 0.2ng pHE8DNA digested with EcoRI; Lane 3: Klebsiella M5a1; Lane 4: Klebsiella RE1435; Lane 5: P.morganii; Lane 6: E.carotovora; Lane 7: S.sonnei; Lane 8: H.alvei; Lane 9: S.typhimurium; Lane 10: E.coli; Lane 11: λ DNA digested with HinDIII; Lane 12: E.chrysanthemi; Lane 13: C.freundii; Lane 14: E.aerogenes; Lane 15: S.marcescens; Lane 16: λ DNA digested with EcoRI.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

FIGURE 5.3A

Hybridisation of ^{32}P labelled pHE8 DNA to HindIII digests of DNA from various enteric species.

The DNA shown in Figure 5.1A was transferred to a nitrocellulose filter and hybridised with ^{32}P labelled pHE8.

Lane 1: λ DNA and pHE7 DNA digested with HindIII, 2: pHE8 DNA (0.2ng) digested with HindIII, 3: pHE8 DNA (0.1ng) digested with HindIII, 4: Klebsiella M5a1, 5: P. morganii, 6: E. carotovora, 7. S. typhimurium, 8: E. coli, 9: S. sonnei, 10: H. alvei, 11: λ DNA digested with HindIII, 12: E. chrysanthemi, 13: C. freundii, 14: E. aerogenes, 15: S. marcescens, 16: λ DNA digested with EcoR1.

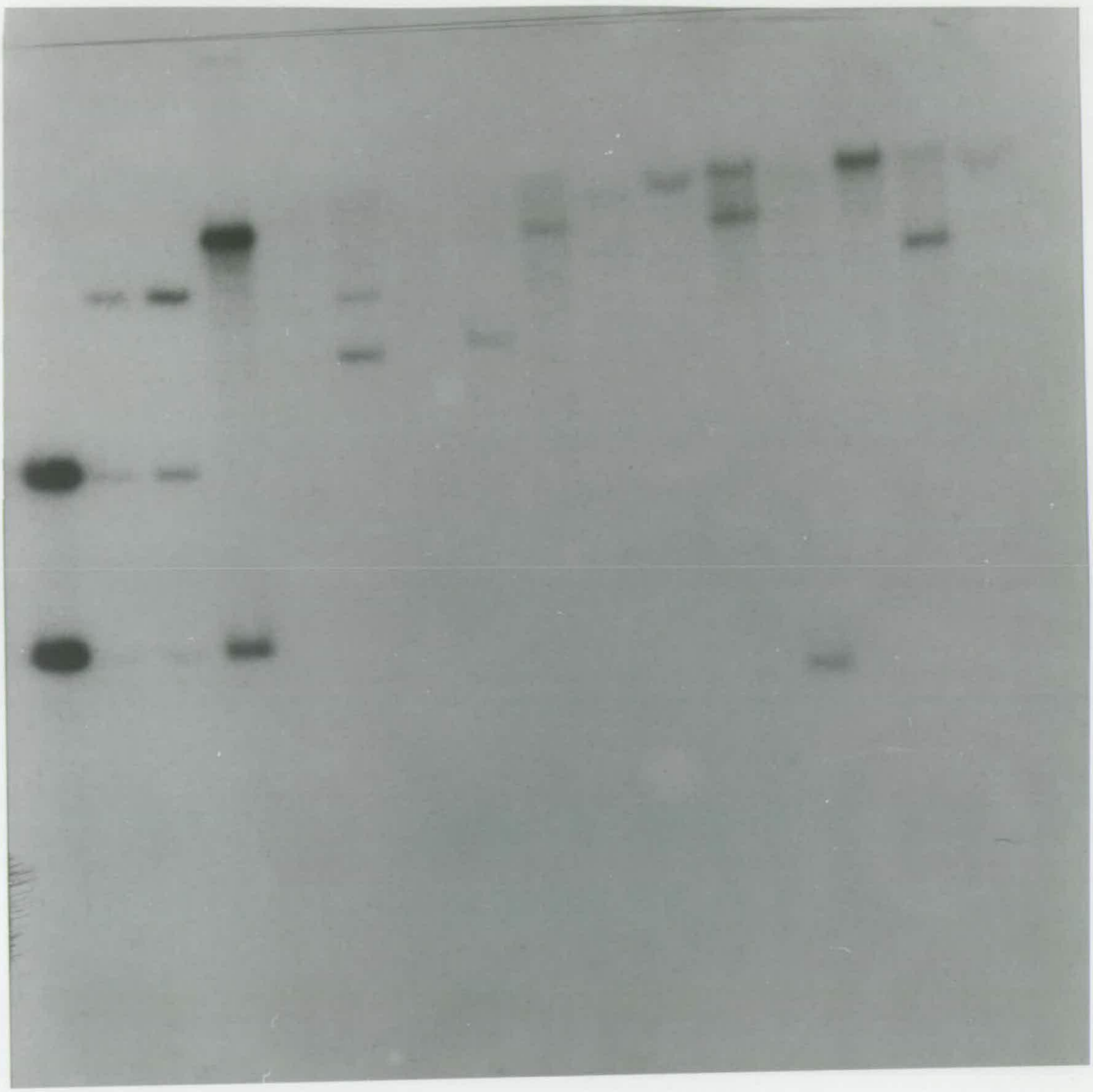

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

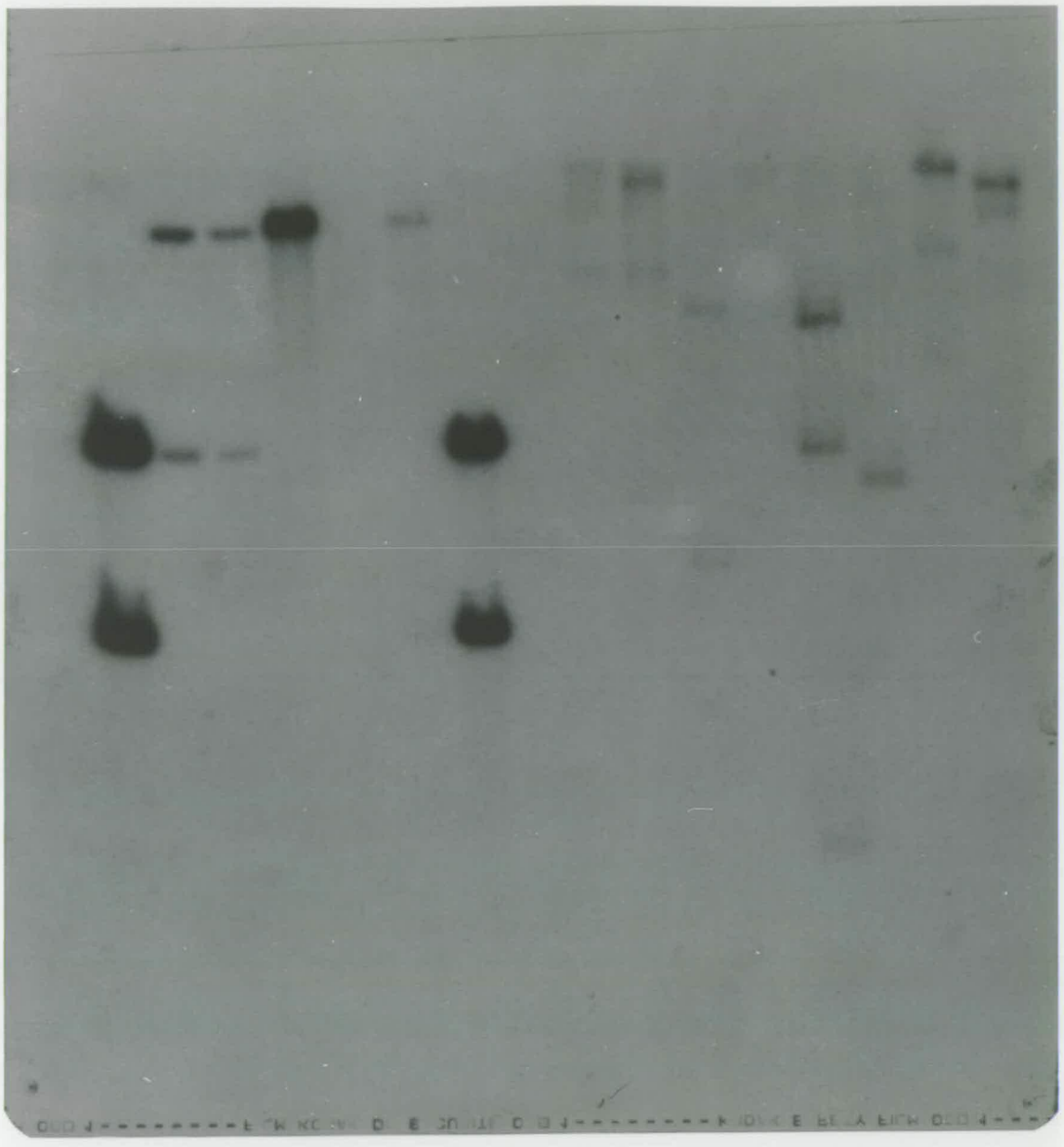
FIGURE 5.3B

Hybridisation of ^{32}P labelled pHE8 DNA to EcoR1 digests of DNA from various enteric species.

The DNA shown in Figure 5.1B was transferred to a nitrocellulose filter and hybridised with ^{32}P labelled pHE8.

Lane 1: λ DNA digested with EcoR1, and pHE7 DNA digested with HindIII, 2: pHE8 DNA (0.2ng) digested with EcoR1, 3: pHE8 DNA (0.1ng) digested with EcoR1, 4: Klebsiella M5a1, 5: P. morganii, 6: E. carotovora, 7: λ DNA digested with HindIII and pHE7 DNA digested with HindIII, 8: S. typhimurium, 9: E. coli, 10: S. sonnei, 11: H. alvei, 12: λ DNA digested with EcoR1, 13: E. chrysanthemi, 14: C. freundii, 15: E. aerogenes, 16: S. marcescens.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16



lac region homologs amongst the DNA fragments of the enteric DNA digestions.

Using similar conditions to those used in the above experiments, pHE1 DNA and DNA isolated from Klebsiella RE1435 were also tested for homology with the Klebsiella M5a1 lac region. pHE1 was digested separately with Hind III and EcoR1 and hybridised with labelled pHE8. DNA isolated from Klebsiella RE1435 was digested with Hind III and hybridised with fragment D,an EcoR1 restriction fragment from pHE8 which contained all of the cloned Klebsiella M5a1 DNA in pHE8 plus approximately 30 base pairs of pBR322 DNA. The results shown in Figure 5.3C indicated that the homology observed between Klebsiella RE1435 and fragment D involved the Klebsiella M5a1 lac region on fragment D and that the 33M.da1 'cryptic' plasmid, previously shown to be in Klebsiella RE1435 was not involved in homolog formation, as no homology was observed between this plasmid and fragment D (Figure 5.3C, lane 1).

The combined results, shown in Table 5.2, indicated that with the exception of P. morgani, S. typhimurium and H. alvei, DNA from all of the enteric species tested and the plasmid pHE1 contained a region(s) of apparent close homology to the lac region of Klebsiella M5a1 cloned on pHE8. The sizes of the restriction fragments involved in lac region homology varied amongst the enteric DNA samples suggesting that there had been little conservation of the EcoR1 or Hind III target sites within the genomes of the enteric species in or near the regions bearing homology to the lac regions of Klebsiella M5a1.

The relative amount of hybridised labelled probe DNA, and thus apparent degree of homology was estimated by tracing the autoradiograms produced by the above experiments, on a densitometer and measuring the area under the peaks of the lac region homologs. The results are shown in Table 5.3.

FIGURE 5.3C

An analysis of the homolog forming ability between ^{32}P labelled fragment D and *HindIII* digests of DNA from *Klebsiella* RE1435 and the 33Md "cryptic" plasmid DNA isolated from *Klebsiella* RE1435.

Lane 1: 0.1µg of 33Md "cryptic" plasmid DNA digested with *HindIII*, which contains no discernible homologs, 2:0.5µg of *Klebsiella* RE1435 DNA digested with *HindIII*, 3 and 4: 0.1ng and 0.2ng respectively of pHE8 DNA (representing approximately 1/10 and 1/5th of the estimated *lac* region DNA in 0.5µg of *Klebsiella* M5a1 DNA), digested with *HindIII*. The densities of the two homologs (indicated by arrows) seen in both lanes 3 and 4 were compared with that of the homolog in Lane 2, to establish the degree of relatedness between the *Klebsiella* RE1435 and *Klebsiella* M5a1 *lac* regions.

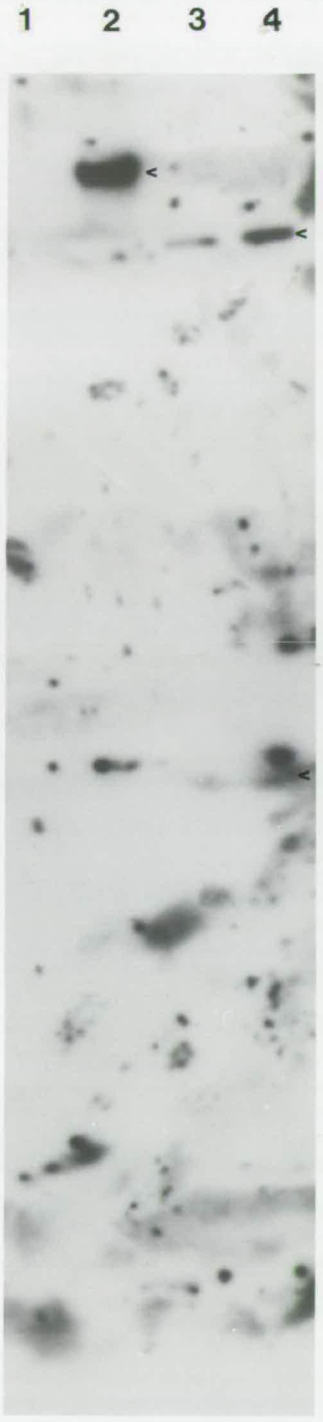

1
2
3
4

Table 5.2. Sizes of pHE8-homologous fragments in EcoRI and HindIII digested DNA from various Enteric species.

Strain	Size of homologous fragments (kb) digested with EcoRI					HindIII	
E.coli W1485	c23.0[a]	14.0	8.5	(6.4)[b]			7.0
S.marcescens NCTC1377			15.5	11.5		26.5	14.0
H.alvei NCTC6578		6.8	3.0	2.8		(19.5)	(10.8)
C.freundii NCTC9750			(13.0)	3.6		(5.0)	2.3
P.morganii NCTC235				ND[c]			ND
S.typhimurium NCTC5710				ND			ND
S.sonnei NCTC8574	21.0	8.5	(6.0)	3.3			13.3
E.carotovora SCRI193		13.5	(8.5)	2.5		9.0	6.6
E.chrysanthemi SCRI409		6.4	4.0	1.3		26.5	14.0
Klebsiella M5a1				13.0		16.0	2.5
Klebsiella RE1435				c18.0			c36.0
E.aerogenes NCTC10006		**21.0**	9.0	(5.5)			**26.5**
pHE1				10.5			4.4

a)Fragment sizes in bold type indicate those homologues which were common to this work and to that of Anilionis and Riley (1980) and Riley and Anilionis (1980) and which were observed in identical strains.

b)Fragment sizes in parenthesis indicate those derived from faint bands on the autoradiogram.

c)No homologues detected in the sample.

Fragment sizes averaged from two experiments.

Table 5.3. Degree of relatedness of *Klebsiella* M5a1 chromosomal *lac* region with *Eco*RI and *Hin*dIII digests of DNA from various Enteric species.

Strain	%-relatedness	
	*Eco*RI	*Hin*dIII
E.coli W1485	3	4
E.coli RE476	0	0
S.marcescens NCTC1377	14	14
H.alvei NCTC6578	0	0
C.freundii NCTC9750	6	7
P.morganii NCTC235	0	0
S.typhimurium NCTC5710	0	0
S.sonnei NCTC8574	7	6
E.carotovora SCRI193	5	10
E.chrysanthemi SCRI409	22	26
Klebsiella M5a1	100	100
Klebsiella RE1435		78[a]
E.aerogenes NCTC10006	20	26
pHE1		9

The relative amounts of hybridisation specific to the *Klebsiella* M5a1 *lac* region probe was determined as described in the text. The %-values are relative to that obtained for the pHE8 probe hybridised to *Klebsiella* M5a1 DNA which was taken as 100% relatedness. %-values are averaged from two experiments except in one instance (a) where patches of dense background prevented densitometric screening.

At this point it is worth considering in detail the nature of the pHE8 probe used in the above experiments, because as previously described in Chapter 3, pHE8 contained not only Klebsiella M5al lac DNA, but also approximately 6kb of non-lac chromosomal DNA from this strain. Therefore what evidence was there to support the hypothesis that the homology observed above involved the lac DNA on pHE8? What genes are adjacent to the lac genes on the Klebsiella chromosome which might be included in the 6kb non-lac region of pHE8 and thus possibly involved in homolog formation? As described in Chapter 1 there is uncertainty as to the nature of the DNA adjacent to the Klebsiella lac, however if the Klebsiella M5al lac genes were similarly positioned and orientated in the chromosome as the E. coli lac operon, the hemB (McConville and Charles, 1979) and brnR (Guardiola et al., 1974) genes would be adjacent to lac I within a distance of 6kb (Bachman and Low, 1980).

The involvement of hemB and brnR in homolog formation between pHE8 and E. coli K12 DNA was tested by hybridising the plasmid with Hind III and EcoR1 digested DNA from RE476. RE476 is deleted for proAB, argF and lac but appeared to have intact hemB and brnR genes (Appendix 1). No homologs were observed therefore the non-lac DNA on pHE8 was not hybridising to hemB or brnR in E. coli K12 DNA.

A comparison of the sizes of the homologs obtained by Anilionis and Riley (1980) and Riley and Anilionis (1980) and in this work (Figure 5.3A & B) showed that where identical strains were used common sized homologs were obtained. As the probe used by Anilionis and Riley (1980) and Riley and Anilionis (1980) consisted of E. coli lac DNA only, this strongly suggested that in the case of those common homologs, the lac DNA on pHE8 was involved in homolog formation.

In order to test whether lac DNA on pHE8 was involved in homolog formation with DNA from those enteric strains

which were not common to both this work and that of Anilionis and Riley (1980) and Riley and Anilionis (1980) fragment C (Figure 3.4) was hybridised to EcoR1 and Hind III digested enteric DNA.

5.2.5. Hybridisation of ^{32}P Labelled Fragment C to Restricted Enteric DNA

^{32}P labelled fragment C DNA was hybridised to the nitrocellulose 'blots' using identical conditions to those used in the previously described hybridisation experiments. The autoradiograms produced, (Figures 5.4A and B) show that a number of homologs which were present in Figures 5.3A and B were absent from Figures 5.4A and B. Fragment C formed homologs with DNA from the E. aerogenes, E. chrysanthemi, E. carotovora and S. marcescens strains, although the homologs formed with the latter three enteric species were barely perceptable. These results indicated that the homologs common to Figures 5.3A and B and Figures 5.4A and B involved lac DNA homology.

The results described above supported, overall, the hypothesis that homolog formation, between the cloned Klebsiella M5a1 DNA on pHE8 and DNA from enteric species, involved the lac DNA on pHE8. However the possibility that non-lac chromosomal DNA on pHE8 was also involved in homolog formation could not be excluded, on the basis of these experiments. Therefore the degree of relatedness between the cloned Klebsiella M5a1 chromosomal DNA, and DNA from the various enteric species (Table 5.3) could only be viewed as relatedness to the lac region of Klebsiella M5a1.

5.2.6. Hybridisation of ^{32}P Labelled pHE11 to Restricted Enteric DNA

To detect any major variation in the ability of two different sections of the cloned Klebsiella M5a1 DNA on pHE8 to form homologs with enteric DNA, pHE11 was hybridised with

FIGURE 5.4A

Hybridisation of ^{32}P labelled fragment C to HindIII digests of DNA from various enteric species.

The DNA shown in Figure 5.1A was transferred to a nitrocellulose filter and hybridised with ^{32}P labelled fragment C.

Lane 1: λ DNA and pHE7 DNA digested with HindIII, 2: pHE8 DNA (0.2ng) digested with HindIII, 3: pHE8 DNA (0.1ng) digested with HindIII, 4: Klebsiella M5a1, 5: P. morganii, 6: E. carotovora, 7: S. typhimurium, 8: E. coli, 9: S. sonnei, 10: H. alvei, 11: λ DNA digested with HindIII, 12: E. chrysanthemi, 13: C. freundii, 14: E. aerogenes, 15: S. marcescens, 16: λ DNA digested with EcoR1.

Arrows indicate the positions of homologs which were visible on the original autoradiogram but which have not been successfully reproduced.

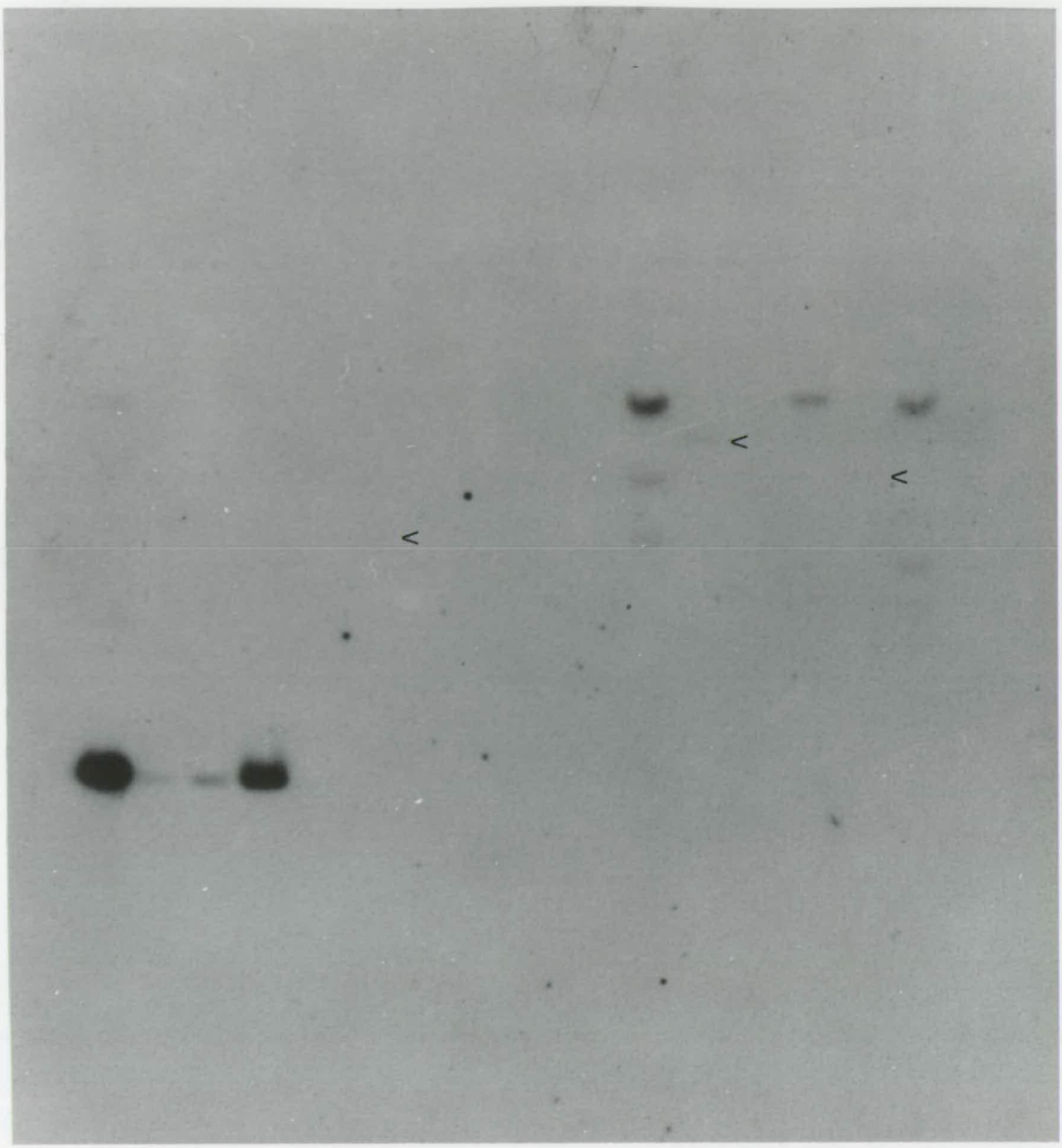

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

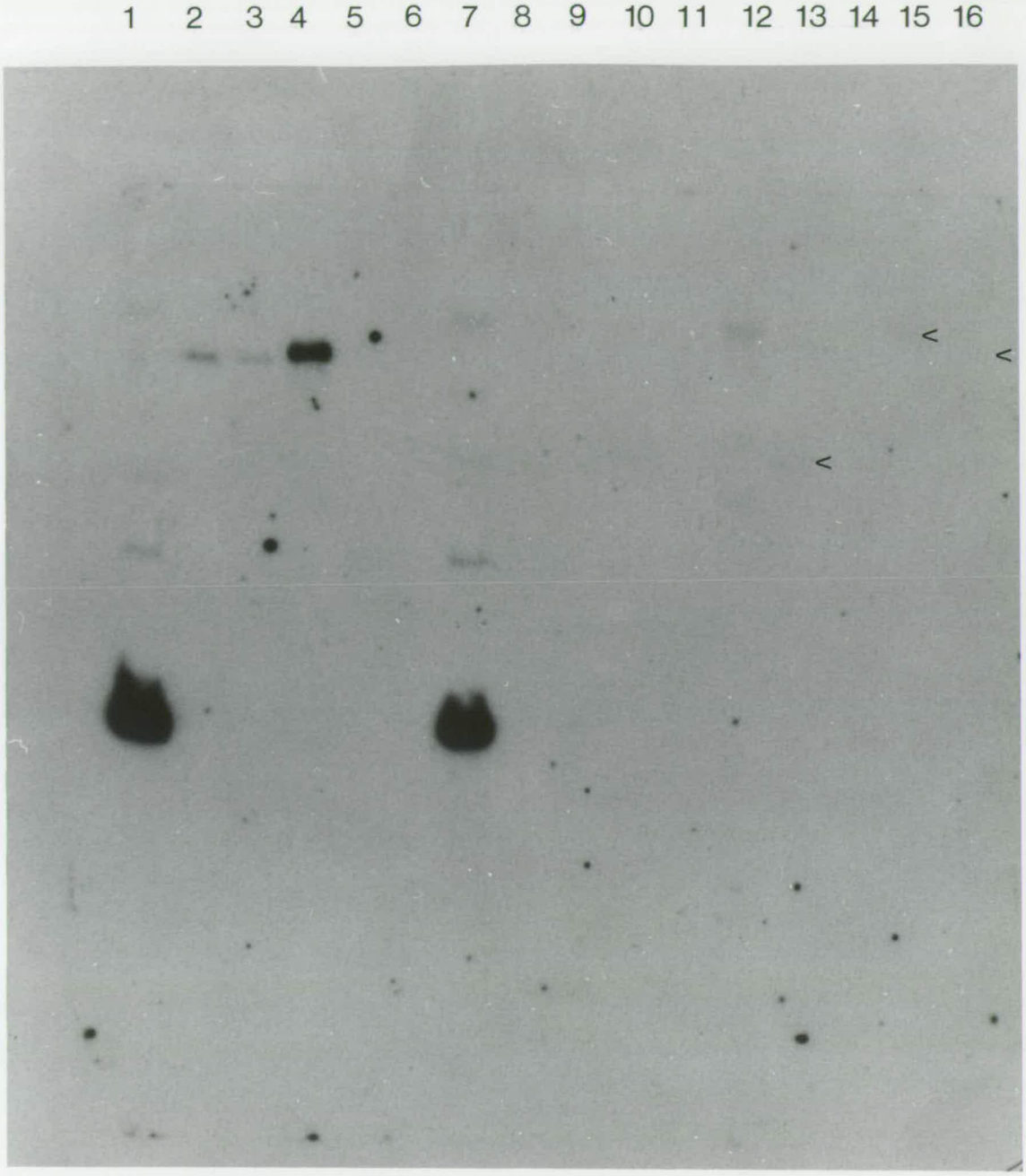
FIGURE 5.4B

Hybridisation of ^{32}P labelled fragment C to EcoR1 digests of DNA from various enteric species.

The DNA shown in Figure 5.1B was transferred to a nitrocellulose filter and hybridised with ^{32} labelled fragment C.

Lane 1: λ DNA digested with EcoR1, and pHE7 DNA with HindIII, 2: pHE8 DNA (0.2ng) digested with EcoR1, 3: pHE8 DNA (0.1ng) digested with EcoR1, 4: Klebsiella M5a1, 5: P. morganii, 6. E. carotovora, 7: λ DNA digested with HindIII and pHE7 DNA digested with HindIII, 8: S. typhimurium, 9: E. coli, 10: S. sonnei, 11: H. alvei, 12: λ DNA digested with EcoR1, 13: E. chrysanthemi, 14: C. freundii, 15: E. aerogenes, 16: S. marcescens.

Arrows indicate the positions of homologs which were visible on the original autoradiograms but which have not been successfully reproduced.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Hind III digested enteric DNA, and the results (shown in Figure 5.5) were compared with those homologs obtained when pHE8 and fragment C were hybridised with the Hind III digested enteric DNA (Figures 5.3A and 5.4A respectively).

An identical pattern of homologs was obtained when either pHE8 or pHE11 were hybridised to the digested enteric DNA. However certain homologs differed in their intensity. This was clearly demonstrated by the homologs formed between DNA from E. aerogenes and pHE11 and pHE8. When hybridised with pHE8 a dense homolog was formed (Figure 5.3A, lane 14) whereas the product of pHE11 hybridisation was barely visible (Figure 5.5, lane 14). This result implied that a proportion of the DNA on pHE8 involved in homolog formation with E. aerogenes DNA was absent in pHE11, i.e. fragment C.

A comparison of the intensities of the homologs formed in Figures 5.4A and 5.5 revealed that in general pHE11 produced denser homologs. The different DNA probes used in the hybridisation experiments were routinely radioactively labelled to approximately the same specific activity and the same experimental conditions were used throughout the work. Therefore the difference in the intensity of identical homologs formed with different probes should reflect a difference in the amount of bound probe DNA, and an apparent difference in sequence homology.

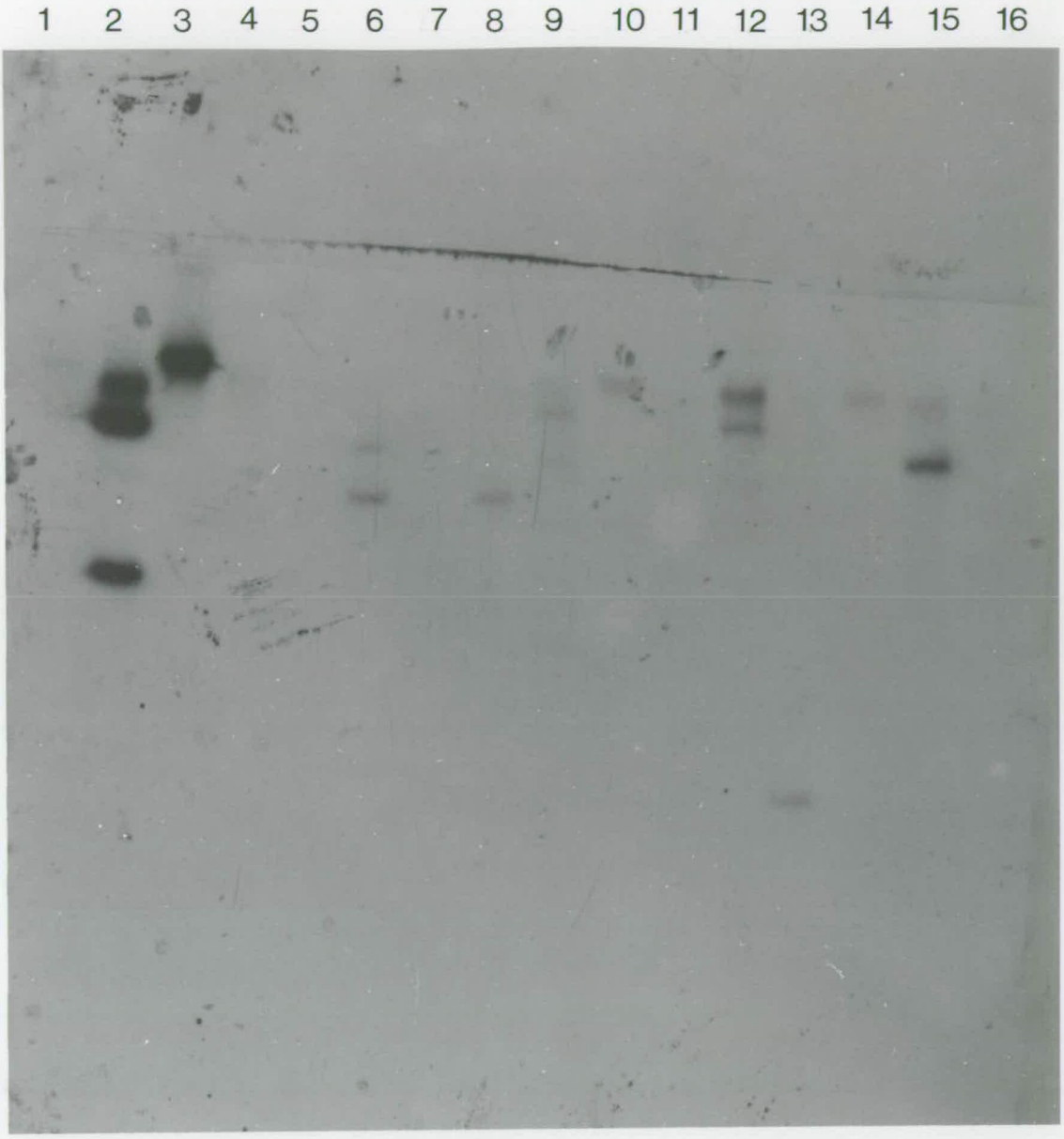
The results shown in Figures 5.4A and 5.5 therefore suggested that the Klebsiella M5a1 DNA on pHE11 had more sequence homology with DNA from E. coli, S. sonnei and C. freundii than did the DNA on fragment C. If the observed homolog formation was confined to the lac DNA on pHE11 and the enteric DNA, it would follow that the Klebsiella M5a1 lac I P O and major portion of the adjacent lac Z DNA had undergone less evolutionary divergence from their equivalent regions in E. coli, S. sonnei and C. freundii than the lac

FIGURE 5.5

Hybridisation of ^{32}P labelled pHE11 to HindIII digests of DNA from various enteric species.

DNA from various enteric species was digested with HindIII and the restriction fragments were separated by electrophoresis through 1% agarose, transferred to a nitrocellulose filter and hybridised with ^{32}P labelled pHE11.

Lane 1: λ DNA digested with HindIII, 2: pHE8 (partially digested) with HindIII, 3: Klebsiella M5a1 (which failed to digest), 4: P. morganii, 5: λ DNA digested with EcoR1, 6: E. carotovora, 7: S. typhimurium, 8: E. coli, 9: S. sonnei, 10:λ DNA digested with HindIII, 11: H. alvei, 12: E. chrysanthemi, 13: C. freundii, 14: E. aerogenes, 15: S. marcescens, 16: λ DNA digested with EcoR1.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Y gene and adjacent 1kb region of lac Z had. This may well have been the case, for as Fried (1980) suggested, although mutations in lac Z and lac Y may accumulate without being necessarily detrimental to a bacterium, it was necessary that a functional lac I gene was maintained "since its function of repressing expression of the lac operon is still necessary for the economy of cellular biosynthesis". On that basis there would be strong selective pressure to reduce mutational change in a lac I gene of common evolutionary origin.

The observation that homologs formed between fragment C DNA and DNA from the E. aerogenes, S. marcescens, E. chrysanthemi and E. carotovora strains, but not with DNA from the other enteric species, suggested that the Klebsiella M5al lac Y and adjacent 1kb lac Z DNA was more homologous to, and less diverged from, the DNA from those strains than to DNA from E. coli, S. sonnei and C. freundii.

A further interesting point which arose from these results concerned the homology displayed between the Klebsiella M5al lac region DNA and DNA from E. carotovora and E. chrysanthemi. The former species gave a weak Lac^+ phenotype on ML whereas the latter species was Lac^- on similar medium. Both species hydrolysed ONPG and therefore produced a β-galactosidase, however due to the failure to ferment lactose E. chrysanthemi appeared not to possess a functional lactose permease transport system. It can be seen, however, that fragment C DNA hybridised with E. chrysanthemi DNA (Figures 5.4A and B). If the lac Y DNA on fragment C was involved in homolog formation with E. chrysanthemi DNA the result would suggest that an equivalent lac Y region existed in that strain. Presumably a non-functional lactose permease in E. chrysanthemi could have arisen by the accumulation of a number of mutational changes in a lac Y gene. This suggestion is supported by the observations of Hugouvieux-Cotte-Pattal et al. (1984)

who isolated spontaneous lactose fermenting derivatives of wild type strains of E. chrysanthemi.

On the basis of the percent relatedness recorded between the Klebsiella M5a1 lac region and DNA from various enteric species it appeared that the Klebsiella M5a1 lac region did not show a high degree of relatedness to DNA from other enteric species other than Klebsiella RE1435. The percent relatedness recorded for the other enteric species did not allow the definite separation of the enteric species into E. coli-like and non-E. coli-like species, although slightly higher degrees of relatedness were recorded for those species which, on the basis of taxonomic/biochemical classification, were believed to be more closely related to Klebsiella.

However as previously mentioned the results described in this Chapter must be interpreted in a very guarded manner for the following reasons.

When double-stranded DNA molecules are subjected to extremes of temperature or pH the hydrogen bonds in the double helix are ruptured and the DNA collapses into two single stranded molecules. In the case of increasing temperature the denaturation is usually characterised by the mid-point transition between the two forms (i.e. double-stranded and single-stranded), which is referred to as the T_m (Marmur, Round and Schildkraut, 1963). Previously, measurement of the degree of hybridisation has been achieved by either the nitrocellulose filter technique (Denhardt, 1966), the hydroxyapatite method (Bernardi, 1965; Britten and Kohne, 1966; Miyazawa and Thomas, 1965) or spectrophotometrically (Seidler and Mandel, 1971). All the procedures involved the measurement of the T_m's of a heterologous DNA sample and its comparison with T_m's produced by the homologous sample. A 1°C difference between the T_m's was taken to indicate a 1% mismatch in base pairs (Bonner et al., 1973).

The procedure based upon the Southern transfer and hybridisation method (Southern, 1975) differs from the methods described above in that it does not measure the individual T_ms between each sample and probe DNA because the conditions in which the hybridisation occurs are fixed. These conditions are therefore a compromise, because DNA reassociates optimally at temperatures 25°C to 30°C below the T_m (Marmur and Doty, 1961; Marmur et al., 1963) and the use of incubation conditions significantly below this optimum would allow hybridisation between distantly related or unrelated sequences. Conversely decreasing the T_m by, for example, decreasing the ionic strength of the hybridisation mixture (Dove and Davidson, 1962) and/or by increasing the percentage of formamide present (McConaughty et al., 1969; Cassey and Davidson, 1977) may produce conditions significantly above the optimum temperature, which would then allow only highly complementary sequences to hybridise (Brenner and Cowie, 1968; Johnson and Ordal, 1968). The T_m's of the homologs formed between probe DNA and DNA from various enteric species will vary genera to genera depending upon the degree of relatedness. Therefore the actual hybridisation conditions chosen are not ideal for each enteric sample, but represent a compromise, one which is weighted towards selection of highly complementary sequences, i.e. stringent conditions.

Recently it has been shown that use of stringent hybridisation conditions can produce misleading results, as was clearly described in a short review by Griffin (1982). The author pointed out that hybridisation conditions previously regarded as "medium stringency" gave negative results with regards to homology between specific probe and sample DNA. However comparison of the DNA sequences of the specific probe and sample DNA revealed approximately 70% homology overall. The reason for the

negative result appeared to be due to the homologous base pairs being well spread out, with the longest stretch of homologous nucleotides being only 17 residues long. Therefore the hybridisation observed between pHE8 and the DNA from the various enteric species may in fact represent only a part of the actual degree of relatedness, simply due to the nature of the conditions chosen. Ideally therefore the hybridisation experiments described in this Chapter should have represented only part of a series, in which the hybridisation conditions and thus the stringency was varied. In this way optimal conditions for the homologs formed may have been achieved. It should also be noted that third base changes, which can significantly contribute to non-homologies may still allow coding for identical amino acids, thus the degree of DNA-DNA relatedness as measured by the system described above may not truly reflect the extent of the similarity between the amino acid sequences coded by related DNA regions from separate sources.

The hybridisation approach does not take into account any major variation in the genome sizes of the various enteric species, which could affect the results if the genome sizes differed significantly.

5.3. Conclusion

In conclusion, the results presented in Chapter 5 indicated that the lac region of Klebsiella M5a1 did not display a high degree of relatedness to any enteric species tested other than Klebsiella RE1435. However because of recent doubts raised about the approach adopted in this work the results obtained may be viewed as underestimates, due to the stringency of the hybridisation conditions used, and the limited number of experiments performed.

However, it is interesting to note that the degree of similarity between the Klebsiella lac region and DNA from

the various enteric species does in general reflect the presently accepted taxonomic relationship between Klebsiella and those other species (Edwards and Ewing, 1972; and discussed in Chapter 1 of this work). The hybridisation method as applied in this Chapter may therefore show general trends of similarity without necessarily providing quantitative estimates for the degree of relationship, as these will invariably change as the hybridisation conditions change.

CHAPTER 6

DISCUSSION

To what extent do the results presented in this thesis answer the questions posed in Chapter One?

Do the results support the hypothesis that the chromosomal *lac* systems from enteric species are demonstrably different from the chromosomal *lac* systems of *Escherichia* and *Shigella* species?

The results obtained suggest that the *Klebsiella* M5al *lac* region differs considerably from the equivalent region in the *E. coli* K12 strain used in this study. A comparison of the restriction maps of these regions show the absence of common restriction enzyme recognition sequences, for the enzymes used, and the data from the DNA-DNA hybridisation experiments suggest that the DNA sequences in the *lac* region of *E. coli* K12 and *Klebsiella* M5al are considerably different.

These results support the findings of Reeve and Braithwaite (1974) who reported that the *K. aerogenes lac* repressor protein did not repress the *E. coli* K12 *lac* operon or the *lac* system on F_k*lac*. The *E. coli* and F_k*lac* repressor proteins were also inactive towards the *K. aerogenes lac* system (Reeve, pers.comm.). These results were presumably due to a variation in the recognition sequences of the *lac* operator regions in *E. coli*, F_k*lac* and *K. aerogenes*.

These results were significant, because in the *Enterobacteriaceae*, other operons, such as the tryptophan operon (*trp*) did not differ to the same extent. The DNA sequences of the *E. coli* and *S. typhimurium trp* promoter-operator region showed that 81 of 105 nucleotide pairs were identical (Bennet *et al.*, 1978a; Bennet *et al.*, 1978b), which explained why the *S. typhimurium trp* repressor protein regulated the *E. coli trp* operon and vice-versa (Somerville, 1966; Manson and Yanofsky, 1976). The *E. coli trp* repressor

protein was also shown to regulate the trp operons from S. dysenteriae, K. pneumoniae, S. marcescens and P. mirabilis (Manson and Yanofsky, 1976), suggesting that a high degree of conservation of the trp promoter-operator region was wide-spread throughout the Enterobacteriaceae.

Another difference between E. coli and the other enteric species studied was the absence of detectable thiogalactoside transacetylase activity in all but E. coli, which is discussed in detail later in this Chapter.

Thus differences between the E. coli lac system and the chromosomal lac system in another enteric species had been shown. However it should be borne in mind that the molecular weights of the β-galactosidase subunit from Klebsiella M5a1 was very similar to the $F_k$lac encoded protein and that of the E. coli lac operon. It has been argued that there is no apparent inherent reason for the enzyme subunits to be so large (Hall, 1976), and therefore the similarity in molecular weight might reflect ancestral homology between the genes coding for the proteins. If the argument of Hall (1976) is accepted then the results discussed above suggest a common, if distant, origin for the lac systems of E. coli and Klebsiella M5a1.

The second question posed in Chapter 1 was "were all of the enteric chromosomal lac regions excluding those of Escherichia and Shigella species, demonstrably similar to each other"? If amongst the enteric species, only the Escherichia and Shigella species had acquired their lac system by lateral transfer, one might expect the homology displayed by the Klebsiella lac region to DNA from the various enteric species tested, to show a discrete boundary separating the Escherichia/Shigella species from the remaining enteric species. However the pattern seen in Chapter 5 (Table 5.3) was more of a gradual variation in the amount of homology between the Klebsiella lac region

and the equivalent region in the various enteric species tested, rather than the levels of homology showing a distinct cut-off, dividing the Escherichia and Shigella species from the other enteric species. This suggests that either the chromosomal lac systems in the enteric species tested were of the same immediate pre-Enterobacteriaceae origin, and had later diverged from each other,or perhaps less likely that lateral transfer of differing lac genes had been commonplace amongst the enteric species, and this was responsible for the observed differences in homology in Table 5.3. A characteristic apparently shared by all of the enteric species, with the exception of E. coli, is the absence of thiogalactoside transacetylase activity (Alpers et al., 1965, and this work). If the absence of thiogalactoside transacetylase activity in the enteric species tested reflects the absence of a lac A gene this result could be interpreted in a number of ways.

Firstly, the absence of a lac A gene in the enteric species other than E. coli could be a clear indication that the E. coli lac operon is not of the same immediate pre-Enterobacteriaceae origin as the chromosomal lac systems in other enteric species. However the hybridisation data discussed above would appear to argue against this.

An alternative was that the E. coli lac system could have acquired the thiogalactoside transacetylase gene after having diverged from the other enteric species. However the work of Guiso and Ullmann (1976) on the expression and regulation of lactose genes carried on plasmids from various sources casts some doubt on this hypothesis because although the plasmid encoded lac genes were shown to be very similar to those of the E. coli lac operon none of the Lac^+ plasmids encoded a functional thiogalactoside transacetylase. Presumably Lac^+ plasmids could have been formed from an Escherichia species before the acquisition by that species of a thiogalactoside

transacetylase gene, however this explanation would necessitate the unlikely situation that all of the Lac^+ plasmids tested by Guiso and Ullmann (1976), and in this work, were formed from a pre-thiogalactoside transacetylase E. coli stock. Another explanation is that the mechanism of Lac^+ plasmid formation always ensures that an intact lac A gene is excluded from the Lac^+ plasmid formed, a hypothesis which is testable and is discussed in the latter section of this Chapter.

A second alternative to explain the absence of thiogalactoside transacetylase activity in enteric species other than E. coli is that all of the enteric species may have, at one time, possessed a functional lac A gene, but only E. coli now retains the ability to produce a functional enzyme, due to some as yet unknown selection pressure on E. coli alone to maintain the presence of the intact lac A gene in the population. This alternative explains in part the variation in the ability to ferment lactose demonstrated by members of the Enterobacteriaceae, as this would be due to lac gene redundancy amongst the enteric species other than E. coli.

However if there was lac gene redundancy amongst the enteric species other than E. coli one might expect this to be confined to the structural genes, and not to the lac I and P/O region, as there would be strong selection pressure on bacteria to maintain control of lac gene expression. In this case, if the E. coli and Klebsiella lac systems were related, even distantly, why were their lac PO regions not as conserved as the trp PO region?

The results obtained in this thesis do not allow a firm conclusion to be drawn concerning whether or not the E. coli lac operon was acquired by lateral transfer, however the hybridisation data, which as previously stated must be viewed with caution, makes this hypothesis less than convincing. Similarly the hypothesis which implies that one

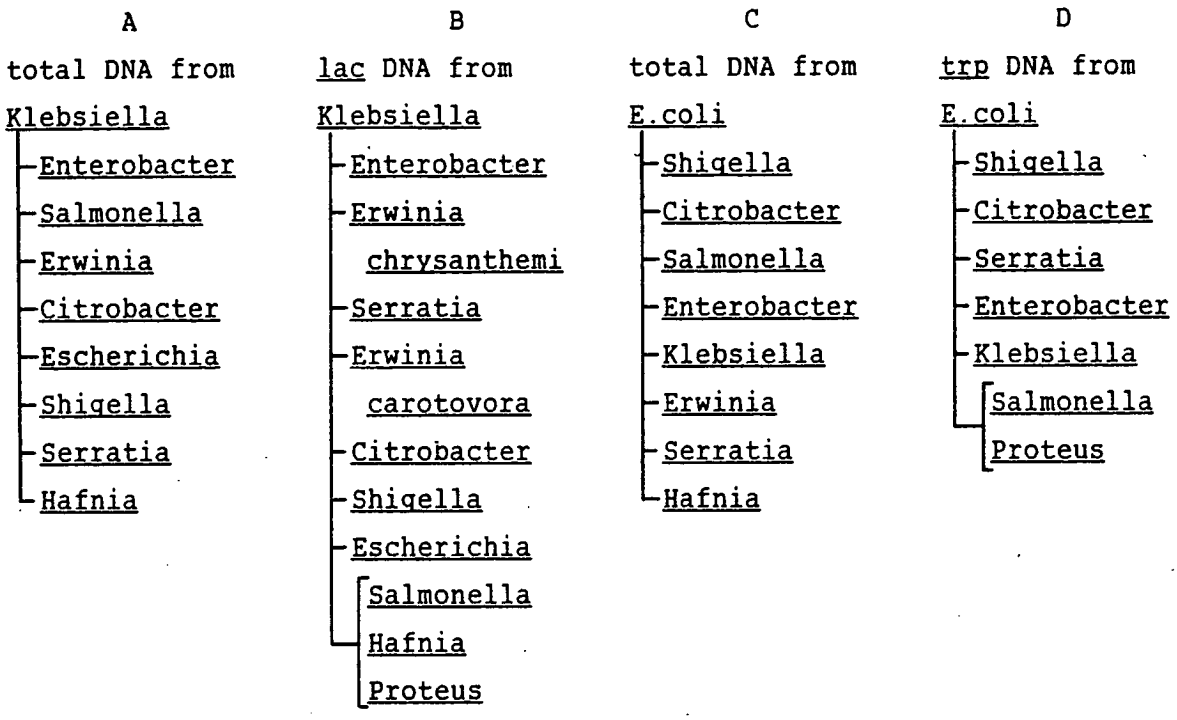
has simply captured a frame in time of the loss of the lac genes from the majority of the enteric species appears also to be unsubstantiated.

Were there sufficient differences between the chromosomal lac systems in the enteric species, other than E. coli, to support the hypothesis that lac systems had been acquired by some enteric species by lateral transfer?

Again the data obtained does not allow a positive statement to be made concerning this question. The pattern of species relatedness based on the lac region hybridisation data in Chapter 5 differed from a pattern based on total DNA-DNA hybridisation data (Figure 6.1A,B). It might be argued that these differences represented lac systems which had not evolved in concert with their genomes and had been acquired by lateral transfer. However a pattern of relatedness based on the E. coli trp operon, which appears to be highly conserved throughout the Enterobacteriaceae, also differs from that based on total DNA-DNA hybridisation (Figure 6.1C,D). Therefore the differences between the patterns of relatedness based on total DNA-DNA hybridisation and lac region DNA hybridisation may not be meaningful.

Finally, how similar is the lac system on $F_k$lac to the chromosomal lac systems of the various enteric species? There is evidence to support the hypothesis that the plasmid borne lac genes are closely related to the E. coli lac operon, and differ from the Klebsiella lac system (discussed on page 46). However the hybridisation data in Chapter 5 suggests that the Klebsiella M5al lac region is more closely related to DNA from $F_k$lac than to E. coli lac DNA, but because of the doubts expressed previously about the quantitative approach undertaken this result may not reflect a significant difference. The $F_k$lac encoded β-galactosidase subunit molecular weight is very similar to those of the Klebsiella M5al and E. coli proteins, which if the argument

Figure 6.1. DNA homology relationships between various enteric species.



A and C: Patterns of relatedness of total DNA from Klebsiella and from E.coli respectively, to total DNA from other enteric species based on %-homology following reassociation at 60°C and hydroxyapatite chromatography (Sanderson, 1976; Steigerwalt et al., 1975; Brenner, 1973; Gardener and Kado, 1972).

B: Pattern of relatedness of Klebsiella lac region DNA to other enteric species using the nitrocellulose filter hybridisation data from Chapter 5.

D: Pattern of relatedness of E.coli trp operon DNA to other enteric species using nitrocellulose filter hybridisation (Riley and Anilionis, 1980).

of Hall (1976) is accepted, suggests ancestral homology between all three lac systems.

What further work may be undertaken to clarify the situation concerning the evolution of the lac systems in the Enterobacteriaceae?

Firstly, it would be worthwhile establishing the absence or presence of a thiogalactoside transacetylase gene in the enteric species other than E. coli, and on Lac$^+$ plasmids. This could be undertaken by hybridising an E. coli lac A probe with digested enteric and Lac$^+$ plasmid DNA. Providing a series of hybridisation conditions were used, ranging from non-stringent to stringent, an indication as to whether or not 'silent' lac A genes existed in the enteric species could be obtained.

Secondly, with the advances which have occurred in DNA sequencing techniques it should be feasible to sequence the entire Klebsiella M5al chromosomal lac genes cloned on pHE8, and the lac genes from $F_k$lac cloned on pHE1. This would provide for a direct comparison of the sequences of these two lac regions with the previously established sequences of the E. coli lac genes and would also allow confirmation as to whether there are lac A gene sequences in the lac systems of Klebsiella M5al and $F_k$lac. It would also be of interest to screen a variety of enteric species, isolated from their natural habitats, for the presence of Lac$^+$ plasmids which unlike those studied to date, are not closely related to the E. coli lac operon. These non-E. coli-like Lac$^+$ plasmids may well have avoided detection simply because they would not confer upon a host bacterium the strong lactose positive phenotype normally associated with Lac$^+$ plasmids. Their detection could be undertaken by transferring plasmids from various enteric species to a suitable lac deletion strain of E. coli, which would then allow the detection of plasmid borne lac genes. If detected they would at least provide a means by which lac genes other than those of E. coli, could have undergone lateral transfer.

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APPENDIX 1

Test for the Presence of hemB and brnR in RE476

RE476 contains the deletion XIII which includes the genes proAB, argF and lac. However the full extension of the deletion is unknown. The genes immediately adjacent to lac in the E.coli chromosome are hemB and brnR. Haemin requiring (hemB) mutants were shown by McConville and Charles (1979) to grow very poorly in HM plus glucose in the absence of haemin, and when grown on similar solid medium hemB mutants produced exceptionally small colonies. RE476 did not display these poor growth characteristics and was therefore presumed not to be deleted for hemB.

The brnR gene product is involved in the high affinity transport of the amino acids isoleucine, leucine and valine into the bacterium. In brnR mutants however, these amino acids may still be transported into the bacterium by another route, a very high affinity (VHA) transport process, but this system is repressed and inhibited by methionine (Guardida et al., 1974). The presence of a functional brnR gene product, and consequent transport into the bacterium of isoleucine, leucine and valine could therefore be demonstrated by studying resistance to valine. This amino acid inhibits growth in E. coli K12 because when valine reaches a critical intracellular concentration it inhibits isoleucine biosynthesis and therefore stops growth on HA. Thus the resistance of RE476 to valine was tested by growing the bacteria on HA containing growth supplements, glucose, valine and methionine (to repress the VHA transport process). RE476 did not grow in the presence of valine, whereas the absence of this amino acid allowed normal growth. Therefore RE476 was presumed to possess the necessary transport systems for valine accumulation and to thus produce a functional brnR gene product.

The XIII deletion in RE476 did not appear to include either brnR or hemB and was therefore presumed not to extend much further than lac I in a direction distal to the lac Z Y A genes.

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