Control of Biofilm Formation: Bacteriocins, Bacteriophage and Biocides

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DECLARATION

This thesis has been composed by myself and the work, of which it is a record, has been carried out by myself. All sources of information have been specifically acknowledged by means of a reference.

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ABSTRACT

Bacterial biofilms are consistently more resistant to antimicrobial agents when compared to planktonically-growing cells, and this is a cause of concern in many diverse fields in industry and medicine. Several theories have been developed to explain the increased recalcitrance of biofilms. These include 'penetration limitation' which states only surface bacteria are subjected to lethal doses of antimicrobial, and 'physiological limitation', which proposes that biofilm bacteria exist in a more quiescent state. The attachment of bacteria to surfaces also causes derepression in a large number of genes associated with a sessile existence, so that biofilm cells are phenotypically distinct from planktonic cells. This may inadvertently alter resistance.

In natural and industrial environments, biofilms often consist of complex communities of microorganisms. Recent studies have indicated that mixed species biofilms can often be thicker and more stable than monospecies biofilms, and this may further influence their resistance to disinfectants (Skillman *et al.*, 1999). An aim of this work was to compare interactions between bacteria, and to correlate them with increased or decreased biofilm formation. A better understanding of the interactions occurring within biofilms may lead to more effective control strategies. As the strains used in this study were closely related Enteric species, considerable bacteriocin activity occurred. Bacteriocin-producing strains were found to have a competitive advantage over bacteriocin-sensitive strains, both in gaining a foothold into a new community, and discouraging the attachment of potential competitors. Bacteriocins and bacteriocin-producing strains may be used as a novel strategy to control biofilm growth, and discourage the attachment of pathogenic strains.

In general, a decrease in biofilm size and stability, and an increase in sensitivity to disinfectants was exhibited by bacteriocin-producing mixed species biofilms. There were, however, exceptions: certain biofilms of *Enterobacter agglomerans/Ent* when antagonised with a second, competitive strain produced a signal to repress bacteriocin synthesis in the competing strain, leading to a co-operative state. These biofilms were thicker, more stable and demonstrated an increased resistance to disinfectants.

There is also the possibility that bacteriophage can be used to control biofilm formation. Studies indicated that small titres of phage were more successful in the removal of *Enterobacter cloace*/5920 biofilms. However, infection by three phages, ϕ1.15, Winchburgh and Blackburn phage, was required to completely eradicate the biofilms. The triple-combination of phage was also found to selectively remove a single bacterial species from a mixed species biofilm.

The role of EPS in biofilm resistance and the adaptation of biofilms to increasing concentrations of disinfectant were also investigated. While the involvement of EPS was found to be transient, it was thought that repeated exposure to an antimicrobial agent may select for a more resistant phenotype, leading to biofilm resistance. For example, biofilms responded to increasing concentrations of triclosan by producing a triclosan mutant, and it was thought that increasing concentrations of benzalkonium chloride selected for strains utilising increased expression of multi-drug efflux pumps

ABBREVIATIONS

BEC	biofilm eradicating concentration
BC	benzalkonium chloride
CFU	colony forming unit
CPC	cetyl pyridinium chloride
dH_20	distilled H_20
EPS	extracellular polysaccharide
Fuc	fucose
GFP	green fluorescent protein
Gal	galactose
Glc	glucose
HPC	sodium hypochlorite
HSL	N-acyl-homoserine lactone
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride
INT-formazan	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride formazan
Man	mannose
MIC	minimum inhibitory concentration
MDR	multi-drug resistant pump
MSHA	mannose-sensitive haemagglutin
OMP	outer membrane protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
phage	bacteriophage
PI	propidium iodide
QAC	quaternary ammonium compound
Rha	rhamnose
SCV	small colony variants
SDS	sodium dodecyl-sulphate
SE	standard error
TR	triclosan
YE	yeast extract medium
ϕ	bacteriophage

CONTENTS

Chapter 1

INTRODUCTION

1.1 BIOFILMS

A biofilm can be defined as a microbial population, adherent to each other and/or to surfaces or interfaces, frequently embedded in a matrix of microbially produced organic polymers (Costerton *et al.*, 1995). In all natural habitats studied to date, bacteria prefer to reproduce on available surfaces rather in liquid phase. It has been estimated that > 99% of all bacteria live in biofilm communities (Costerton *et al.*, 1995). These findings have led to the proposal that the planktonic phase may only serve as a mechanism for translocation from one surface to another.

Bacteria in biofilms are profoundly different to planktonic cells. Recent research has also indicated that through the use of chemical signalling, bacteria are capable of developing complex, co-operative communities. A number of advantages can be gained through a biofilm-dwelling existence, including a high resistance to biocides, preservatives and antibiotics. Reports have estimated biofilms can be as much as x1000 less susceptible to antimicrobial agents than their planktonic counterparts. This problem of high resistance has generated interested in many diverse fields in medicine and industry.

1.2 BIOFILM FORMATION

Biofilm formation on surfaces has been depicted as a sequence of events. The first step involves absorption of conditioning film components. Once transported to the surface, microorganisms may adhere, depending on the forces involved. This initial interaction with the surface is reversible, followed by an irreversible adherence due to the production of an extracellular polymer matrix by the attached cells. At this point the coadhesion of secondary colonisers with organisms already adhered to the surface can also start to occur. Subsequently, growth of the attached microroganisms takes place, continuing until naturally occurring shear forces detach parts, or all of the biofilm.

1.2.1 *Surface Conditioning Film*

Macromolecules and other low-molecular weight, hydrophobic molecules from the aqueous phase rapidly absorb to surfaces to form the conditioning film. For example, proteins and glycoproteins in saliva, serum and mucus, and in aquatic environments, humic acids and other organic molecules can coat surfaces. This alters the surface electrostatic charge and surface-free energy, and in turn encourages or discourages bacterial colonisation (Marshall, 1992). The conditioning film contains higher concentrations of nutrients than is found in the mass of fluid and cells are attracted to the conditioned surface by complex physiological interactions.

Bacteria are transported to a surface by several mechanisms. This includes random contacts with the surface due to Brownian motion, convective transport by the bulk liquid bringing cells into contact with the surface, sedimentation due to differences in specific gravity between bacteria and the bulk liquid, and by force-generating mechanisms such as flagellar activity (Davies, 2000). Initial microbial adhesion is a result of non-specific van der Waal forces. However, only when bacteria reach a distance of 5 nm from the surface do specific structures on the interacting surfaces become effective enough to facilitate strong adhesion (Bussher *et al.*, 1992).

1.2.2 *Genetic Studies of Initial Biofilm Formation*

The genetic bases of the steps in initial biofilm formation have been investigated for a number of bacterial species such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Vibrio cholerae*. A profile of gene transcription that is distinct from planktonic cells has been demonstrated in each case.

As bacteria approach a surface, motility is slowed. The bacteria may then choose to form a transient association with the surface or other bacteria, allowing the bacteria to search for the ideal conditions before it chooses to adhere irreversibly. Initial interactions with the surface can be accelerated by force-generating organelles such as type IV pili and flagella. In *E. coli*, type I pili are essential for the initial attachment (Pratt and Kolter, 1998). Mutations in the genes for the regulation and biosynthesis of type I pili yielded cells that did not attach to PVC. Type I pili contain the mannose-specific adhesin, FimH and biofilm formation of *E. coli* was inhibited by addition of D-mannose. *E. coli* flagella mutants demonstrated reduced biofilm forming capabilities. Microscopic analysis of the mutant strains suggested that motility is important for both initial surface interactions, and for movement along the surface to form microcolonies. In contrast, in *Ps. aeruginosa* flagella are important only for initial attachment to the surface; flagella mutants could not form biofilms on PVC. Mannose-sensitive haemagglutin (MSHA) type IV pili-mediated twitching motility was found to be important for microcolony formation (O'Toole and Kolter, 1998b). Twitching motility is thought to occur through anchoring of a pilus to a fixed surface, followed by retraction of the pilus against the surface (Wall and Kaiser, 1999). Biofilm formation by *V. cholerae* El Tor also required the synthesis of a MSHA type IV pilus and flagellar motility. As in *E. coli*, the type IV pilus and flagella accelerated attachment and the flagellum mediated spread across the surface. However, both pili and flagella mutants eventually attached to surfaces, and there were no differences between the rates of biofilm formation by mutant strains in which the flagellum and the MSHA type IV pili were either paralysed or absent. This suggests 'accelerated' attachment was due to the force-generating properties of these structures. If flagella or pili were absent, it is probable that bacteria will collide with a surface with enough force to overcome repulsion, and a biofilm may still form at a slower rate (Watnick and Kolter, 1999). Vidal *et al.*, 1998 also demonstrated that the

absence of force-generating motility in non-motile strains of *E. coli* was not inhibitory to biofilm formation. Examination of mutants exhibiting increased adhesion to PVC showed that the 'stickiness' of these strains was due to increased production of Curli synthesis. Many of the surface attachment-defective *Ps. aeruginosa* mutants isolated by O'Toole and Kolter (1998a) could be 'rescued' by addition of iron, or by growing in glutamate or citrate. This suggests that at least 3 overlapping pathways leading to the initiation of biofilm formation exist and multiple strategies for initiating cell-surface interactions in *Ps. aeruginosa* exist. Following on from these studies, O'Toole *et al.* (2000) demonstrated the importance of the catabolite repression control (Crc) protein, which is involved in the regulation of carbon metabolism, for *Ps. aeruginosa* biofilm formation. Mutations to the *crc* gene decreased the expression of *pilA*, the pilin structural gene required for twitching motility.

Interestingly, Kawagishi *et al.* (1996) developed a mechanosensor theory for the attachment of a marine *Vibrio* to surfaces. Surface attachment leads to the conversion of planktonic, rod-shaped swimmer cells with polar flagellum into 30 μm swarmer cells, which possessed many lateral flagella. The polar flagellum of the swimmer cell derives energy for rotation from sodium ion transport, whereas lateral flagella utilise proton transport. Blocking the sodium ion channel resulted in production of lateral flagella. This suggested that as a cell approaches a surface, the rotation of the polar flagellum will be negatively affected, and the consequent decrease in rotation, and sodium ion flux, causes an upregulation of lateral flagella.

1.2.3 *Intercellular Communication*

The term quorum sensing is used to describe the phenomenon whereby the accumulation of a diffusable, low molecular weight signal molecule, or autoinducer, allows individual cells to sense when the minimal population size has been achieved for a concerted population response to be initiated. Quorum sensing is known to control a variety of physiological processes, including bioluminescence, swarming, conjugation, antibiotic biosynthesis and the production of virulence factors. In Gram-negative bacteria, the most intensively investigated group of quorum sensing

molecules are the N-acyl homoserine lactones (HSLs) (Bassler, 1999; Parsek and Greenberg, 2000; Williams *et al.*, 2000).

HSL signalling systems were first recognised in *Vibrio fischeri*; the genes responsible for bioluminescence are expressed in response to an increase in cell numbers. *LuxI* is responsible for generation of the signal: N-(3-oxohexanoyl)-L-homoserine lactone (OHHL) and a second gene, *luxR* activates the expression of the remaining *lux* structural genes (Williams *et al.*, 2000). In *Erwinia cartovora*, the same OHHL molecule activates biosynthesis of the antibiotic carbapenem, and is one of a number of signals required to activate plant-cell-wall degrading enzymes. Regulation of the genes for many *Ps. aeruginosa* virulence determinants, including elastase, alkaline protease, exotoxin A, phenazine antibiotic and siderophore production are under quorum sensing control (Williams *et al.*, 2000). *Ps. aeruginosa* contains two pairs of LuxR homologues, LasR and RhlR, and can produce four HSLs, OHHL, N-3-oxododecanoyl-L-homoserine lactone (OdDHL), N-butanoyl-L-homoserine lactone (BHL) and N-hexanoyl-L-homoserine lactone (HHL). A multilayered regulatory cascade system is employed. LasR and OdDHL activate expression of both RhlR and the stationary-phase sigma factor RpoS (Latifi *et al.*, 1995).

Production of HSLs have been demonstrated in both cultured and natural biofilms (McLean *et al.*, 1997; Stickler *et al.*, 1998). HSL signalling is ideally suited to a diffusion-limited environment such as a biofilm, and the importance of HSLs in *Ps. aeruginosa* biofilms has been clearly demonstrated. For example, HSLs are responsible for creating the 3-D structure of the biofilm. Mutants that were unable to produce LasI formed flat, thinner, undifferentiated biofilms that were more susceptible to sodium dodecyl sulphate (Davies *et al.*, 1998). HSLs were also mediators of surface attachment (Allison *et al.*, 1998), and both *las* and *rhl* were required for type IV pilus twitching motility (Glessnar *et al.*, 1999).

1.2.4 *Role of Exopolysaccharide Production in Biofilm Formation*

Exopolysaccharide Structure

Exopolysaccharides (EPS) form integral components of all biofilms, representing up to 95% of their biomass. The nature and physical properties of the biofilm will be

greatly influenced by the chemical composition and structure of the EPS (Sutherland, 1998). Microbial EPS can be homopolyers composed of a single sugar. Among the homopolysaccharides are bacterial cellulose, levans and dextrans, and some bacterial alginates composed solely of D-mannuronic acid. Alternatively, EPS may be heteropolymers composed of repeating units of several different monosaccharides, commonly 2 to 8 in number. A huge variation of structures and properties are possible due to the vast number of possible linkages and configurations. Each hexose can be α or β linked, in the pyranose or furanose form and be linked through the 2, 3, 4, or 6 position. Heteropolysaccharides can be composed of linear repeat units, or more commonly possess short side chains of 1 - 4 sugars in length. The polysaccharides often carry acyl substituents, commonly ester-linked acetate or ketal-linked pyruvate. Inorganic substituents frequently include phosphate, although certain cyanobacterial polymers can also contain sulphur (Sutherland, 1994).

Exopolysaccharides in Biofilms

Most microbial polysaccharides are relatively water-soluble and easily removed from the bacterial surface. These polysaccharides are probably fairly ineffective in attaching bacterial cells to a solid substratum. However, some EPS are virtually insoluble in water or form rigid gels and are likely to be extremely effective in maintaining the integrity of the biofilm. Amongst these insoluble polysaccharides are several which are commonly found in biofilms. Examples include mutan, the 1,3-α-linked glucan of the oral bacterium *Streptococcus mutans*, and the corresponding 1,3-β-D-linked curdlan synthesised by *Agrobacterium radiobacter*. These macromolecules are particularly effective in bacterial adhesion and exclusion of water soluble molecules (Sutherland, 2000a). Interestingly, studies in our laboratory have shown the polysaccharides of two *Enterobacter agglomerans* strains to be poorly soluble in water, and in alkali and dimethylsulphoxide. These polymers are composed entirely of neutral monosaccharides and contain no acyl substituents. Both these polysaccharides also contain a high proportion of 1,3-linked residues.

The vast majority of EPS macromolecules exist in an ordered or disordered state. At elevated temperatures, and at extremely low ionic concentrations, the disordered state is favoured. In the natural environment EPS can, therefore, be

expected to be present as the ordered form. In this ordered configuration, many bacterial EPS adopt a double helical form. Associations between the double helices are facilitated by ions and by water molecules, and this can influence the physical properties of the polysaccharides, and, consequently, the properties of the biofilm (Sutherland, 2000a). For those polysaccharides that are relatively water soluble, adhesiveness may depend on the conformation adopted. The presence of anionic residues, such as uronic acid, and acyl substituents such as pyruvate ketals or succinyl half-ester groups may enhance the adhesive properties of the polysaccharides. For example, alginates containing contiguous sequences of L-guluronosyl residues tightly bind Ca^{2+} within their adopted 'eggbox' structure to form a strong, thick gel (Sutherland 2000a).

When two or more microbial strains form a biofilm, the presence of polymer material may not only assist in establishing the biofilm, but also promote enhanced biofilm growth than the comparable single species biofilm. Skillman *et al.* (1999b) described a synergistic effect, possibly involving polysaccharide-polysaccharide interactions between two enterobacterial strains; *Klebsiella aerogenes* and *Ent. agglomerans*. Either following pre-treatment with EPS, or during growth as a mixed culture, the biofilm-forming capacity of the two strains was enhanced. Allison and Matthews (1992) also found that mixtures of polymers were more viscous than the pure solutions. Addition of Ca^{2+} also enhanced gelation of a mixture of bacterial alginate, mucin and *Burkholderia cepacia* polysaccharide.

If present in a biofilm, naturally occurring enzymes such as glucanases or esterases could have a marked effect on the physical properties of bacterial EPS. While glucanases can be expected to destroy the integrity of a biofilm, the action of certain enzymes such as esterases may result in enhanced biofilm formation. For example, removal of acetyl groups from the *Enterobacter* XM6 EPS produced a highly microcrystalline structure (Nisbet *et al.*, 1984). Similarly, the weak rubbery gels of the *Sphingomonas* sp. EPS, gellan can be converted to hard brittle gels by removal of ester-linked acyl groups (Chandraeskaran and Rhadha, 1955).

Biofilm-Specific Polysaccharides

The question remains as to whether biofilm exopolysaccharides are biofilm-specific, being only synthesised under biofilm as opposed to planktonic conditions. The ability of bacterial cultures to form several chemotypes of EPS is rare, and it is even less common for a bacteria to simultaneously express more than one type of EPS (Sutherland, 1995). Studies by Hughes *et al.* (1998a) demonstrated both biofilm and planktonic exopolysaccharides could be degraded by the same specific endoglycanase, and the resultant oligosaccharides produced were similar. Costerton *et al.* (1981) found antibodies directed against planktonic EPS also bound to biofilm EPS. This suggests, at least in these cases, biofilm-specific EPS was not being produced. If different biofilm-specific polymers do exist, it is more likely that they are variations of the same structures, differing only in a degree of acetylation, for example, which may affect their physical properties, as discussed above.

However, the production of more than one type of polymer has been recognised in certain strains. For example, a marine *Pseudomonas* species, unusually, synthesised one polymer in exponential phase, and a totally different polymer in stationary phase (Christensen *et al.*, 1985). *Rhizobium meliloti* are capable of producing a heteropolysaccharide succinoglycan or the homopolysaccharide curdlan (Glazebrook and Walker, 1989). Production of the complex colanic acid by the *Enterobacteriaceae* requires specific physiological conditions, such as increased osmotic pressure or reduced incubation times. However, certain strains of *E. coli* K12, or natural isolates of *Ent. cloace* synthesise copious amounts of colanic acid under all conditions tested (Sutherland 1995).

Functions of Biofilm Polysaccharides

Adhesion to a surface has been shown to cause an increase in EPS production. Davies and Geesey (1995) showed alginate production by *Ps. aeruginosa* was induced upon contact with a surface. Prigent-Combaret *et al.* (1999) screened for genes that are differentially expressed during biofilm formation in *E. coli*. The transcription of 38% of genes was affected during biofilm formation, including the decrease of flagellin synthesis and the increase of colanic acid. Studies with *V. cholerae* and *Staphylococcus epidermidis* have shown that EPS was required for the

initial attachment to surfaces (Watnick and Kolter, 1999; McKenney *et al.*, 1998). Examination of biofilm formation of freshwater isolates found that mutants devoid of exopolysaccharide still attached, but failed to form microcolonies (Allison and Sutherland, 1987). The importance of colanic acid production in the construction of the three dimensional structure of *E. coli* biofilms has also been demonstrated (Danese *et al.*, 2000). The rugose colony variant of *V. cholerae* O1 El Tor was shown to produce an EPS that confers biofilm-forming capacity and chlorine resistance (Yildiz and Schoolnik, 1999). Thus, exopolysaccharides have been shown to be important both in the attachment of cells to surfaces, and for the architectural development of the biofilm.

Hughes *et al.* (1998b) demonstrated the involvement of polysaccharides in the protection of biofilms from desiccation. The highly hydrated nature of EPS allows survival under conditions of severe dehydration. The role of EPS in the protection of biofilm cells from the action of antimicrobial agents is discussed in detail in section **1.3.2**. Microbial cells in a biofilm may also be protected from the action of toxic metal cations such as Cd, Zn, Pb, Cu and Sr through the binding of these agents by exopolysaccharide material (Sutherland, 2000a).

The occurrence of several functional groups, such a carbonyl, carboxyl and hydroxyl groups may act as sites of attachment for cations. In oligotrophic environments, this ion trapping may act as a reservoir of essential trace elements.

1.2.5 *Three Dimensional Architectural Development*

At an appropriate time, the adhered microcolonies differentiate into true biofilms. The structure of a mature biofilm will vary with location, the microflora present, and the availability of nutrients. It can range from the thick confluent layers of cells found in oral biofilms, to the thin biofilms formed in oligotrophic environments, with dispersed microcolonies and protruding stacks of cells, to the extravagant pillar and mushroom-shaped structures of *Ps. aeruginosa* biofilms (**Figure 1.1**). In this case, water-filled channels form between the microcolonies, constituting a primitive circulatory system, delivering nutrients to and removing waste products from the communities of cells in the microcolonies (Costerton *et al.*, 1995). The involvement of acyl homoserine lactones and exopolysaccharide production in the process of

differentiation has already been discussed. However, physical shear forces and mass transfer processes can also significantly influence the shaping of a biofilm. In an examination of the responses to shear forces on a community consisting of *K. pneumoniae, Ps. aeruginosa* and *Ps. fluorescens*, Stoodley *et al.* (1999) demonstrated the formation of spherical colonies at low shear, and at high shear, elongated microcolonies and streamers downstream of the direction of flow. Thus, the hydrodynamics of an aqueous environment will determine the shear stresses acting on a biofilm, the rate of erosion of cells from the biofilm and the transport rate of nutrients and planktonic cells to a surface.

For bacteria to detach under unfavourable conditions, and colonise new environments, there must be some mechanism for dispersion. Pieces of biofilm can break off in the flow, or specific enzymes may sever the EPS matrix, liberating the cells. Such glycanases and polysaccharide lyases are often gene products associated with biosynthesis of the polysaccharide itself, and will be released upon lysis of the producing cell (Sutherland 2000b). Interestingly, an acyl homoserine lactone signal is required for the release of individual cells of the photosynthetic bacteria *Rhodobacter sphaeroides* from community structures (Puskas *et al.*, 1997).

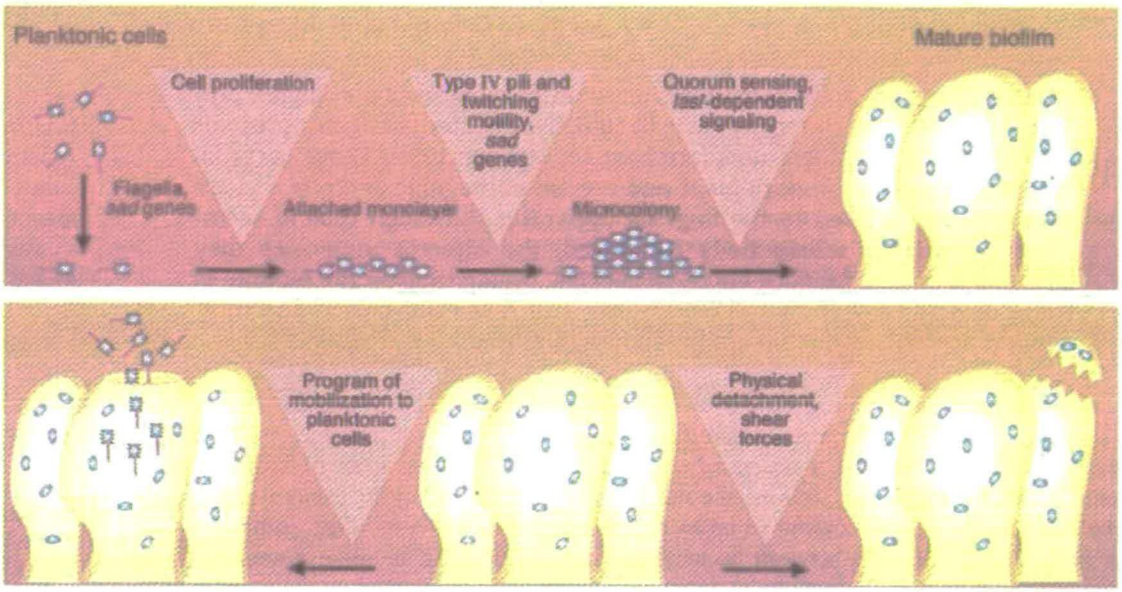


Figure 1.1

The development of *Ps. aeruginosa* biofilms from planktonic cells. Studies of surface attachment deficient mutants (sad) have shown the importance of flagella in initial attachment, type IV pili twitching motility in microcolony formation, and quorum sensing and exopolysaccharide production in the architectural development of the biofilm. Dispersal of bacteria from a biofilm is through a localised hydrolysis of the EPS matrix, leading to release of planktonic cells, and through physical detachment (Costerton *et al.*, 1999).

1.3 BIOFILM RESISTANCE

Although growth as a biofilm can be beneficial in certain areas, for example, billions of pounds are saved annually due to the use of biofilms in the treatment of sewage; the formation of biofilms is generally considered to be problematic. Life within a biofilm confers a number of advantages to the microorganisms that dwell within its layers, including a high resistance to antimicrobial agents. Concentrations of antimicrobials that would ordinarily eradicate planktonic cells are unable to control biofilm cells and estimates suggest that bacteria in biofilms are somewhere between 100 and 1000 times more resistant to antimicrobial agents. The problem of biofilm recalcitrance to antimicrobials and disinfectants are discussed below.

1.3.1 *Problems Caused by Resistance of Biofilms to Antimicrobial Agents*

The problems caused by antimicrobial resistance of biofilms encompass a wide range of disciplines, and so have been studied in a number of different fields. In medicine, for example, *Ps. aeruginosa* respiratory tract infections are common in patients with cystic fibrosis (CF). Isolates from these patients frequently display mucoid colony morphology as a result of the overproduction of the exopolysaccharide alginate. This is regarded as an important virulence factor and has been linked to the antibiotic resistance of the biofilms. Most *Ps. aeruginosa* strains have the genetic capacity to synthesise alginate, but the scale of mucoidy in these respiratory tract infections is rarely seen in environmental isolates. It is thought that mutations amongst the genes for alginate regulation and production are responsible for the overproduction of alginate in CF patients (Deretic *et al*, 1994). Infections associated with medical implants, such as cardiac pacemakers and catheters also cause problems. Biofilms of bacteria such as *S. epidermidis*, a organism not previously recognised as a pathogen, often form on the surface of these devices and continue to provide a source of infection to other parts of the body by bacterial detachment. The cells within the deeper layers of these biofilm can resist the action of antibiotics (Nickel *et al*, 1985). Presently, this problem can only be solved by replacement of the contaminated device. Dental plaque is also an excellent example of biofilm formation in which a wide variety of aerobic and anaerobic bacteria co-exist as relatively stable consortia (Kolenbrander *et al.*, 2000).

Industries also suffer due to the unwanted formation of biofilms. For example, sulphate reducing bacteria are the main culprits in bacterial corrosion. The action of such bacteria can penetrate 5/8 inch-thick steel plates in less than 6 months by formation of a biofilm on the metal surface (Costerton & Lappin-Scott, 1989). Biofilms also accumulate on water pipelines in power station heat exchangers, reducing the efficiency of heat transfer and narrowing the working diameters of pipes, thus reducing their carrying capacity. The fouling of ship hulls by biofilms results in energy loss and reduced performance. Along with the need for regular cleaning, this can be very costly (Marshall, 1992).

In their review of problems caused by biofilms in the food industry, Carpentier and Cerf (1993) stated that unsterilised food equipment will harbour microorganisms, which, between two cleaning and disinfection processes, start a re-colonisation process. Biofilm material can build up within the space of a few hours, and very quickly exhibit increased resistance to antimicrobial agents. For example, it was stated that *Listeria monocytogenes* and *S. aureus* persist on the rubber fingers of poultry pluckers and trolleys that carry carcasses after cleaning. It was also reported that cleaning and disinfection of a conveyor belt with polyurethane bands in a ham slicing plant had no effect on the level of microbial contamination. Chlorine dosing of drinking water pipes was found to kill planktonic bacteria but left biofilm bacteria on pipe surfaces unaffected, ready to recontaminate the water (Costerton & Lappin-Scott, 1989).

1.3.2 *Mechanisms of Resistance*

Three principal approaches have been made in recent years in an attempt to explain the resistance of microbial biofilms to chemical and antibiotic treatments. The first hypothesis is penetration limitation. Following attachment to surfaces, cells initiate the production and accumulation of extracellular polymers that eventually surround and encase the developing microcolony. This exopolysaccharide layer influences the access of molecules and ions to the cell wall and membrane (Costerton *et al.*, 1981), and so it was initially thought that the production of exopolysaccharide provided a physical exclusion barrier to antimicrobial agents. This is now regarded as an unlikely explanation for the recalcitrance of biofilms. Any decrease in diffusion

coefficient of the antimicrobial within a biofilm is insufficient to account for any increased resistance since, at equilibrium, concentrations at the cell surface will be the same as those in the bathing medium (Gristina *et al*, 1989; Gordon *et al*, 1988). However, the penetration of cationic agents through a biofilm may be inhibited due to the binding of cationic agents by negatively charged EPS, thus protecting underlying biofilm cells. For example, direct binding of positively charged aminoglycosides to polyanionic alginate resulted in poor diffusion of the antibiotics through a *Ps. aeruginosa* biofilm (Kumon *et al.*, 1994). The synthesis of antibiotic-degrading enzymes, such as β-lactamases, may give rise to a similar situation. Anderl *et al.* (2000) noted that ampicillin did not penetrate through *K. pneumoniae* biofilms due to the production of β-lactamases; the drug was inactivated faster than it could react. It has also been shown that biofilm cells of *Ps. aeruginosa* produced 32-fold more β-lactamase than cells of the same strain grown planktonically (Giwercman *et al.*, 1991). De Beer *et al* (1994) reported that limited penetration of chlorine into biofilms of *Ps. aeruginosa* and *K. pneumoniae* was due to neutralisation by the surface biofilm cells.

The second hypothesis, physiological limitation, proposes that bacteria in deeper layers of the biofilm exist in a quiescent state. Gradients of oxygen exist across the biofilm and this leads to corresponding gradients in growth rates or other physiological activities within the biofilm. It has been shown that the resistance of *E. coli* and *Ps. aeruginosa* biofilms to ciprofloxacin was due to their slow growth (Evans *et al.*, 1991). McLeod and Spector (1996) reported that starved and stationary-phase *Salmonella typhimurium* cells exhibited increased resistance towards polymixin B. Biofilm age may also be a factor; young biofilms of *Ps. aeruginosa* were less resistant to tobramycin and piperacillin than older biofilms (Anwar *et al.*, 1992). However, growth rate related resistance might only delay cell death and complete eradication of the biofilm (Xu *et al.*, 2000). It is generally agreed that the cells at the outermost layers of the biofilm, will be rapidly growing (Anwar *et al.*, 1992). These cells will succumb quickly to antimicrobials and cease to consume nutrients. Underlying cells will then become bathed in nutrients produced from the lysing bacteria, and begin to grow quickly, thus, in turn, becoming

susceptible to antimicrobial attack. Layer by layer, all biofilm microorganisms will be alternately nourished and killed (Xu *et al.*, 2000).

Nutritional limitations brought on by nutritional gradients not only reduce growth rates, but can also give rise to cells with physiology and cell envelopes unique to each limitation. These effects have been reported to influence susceptibility to a wide range of antibiotics (Brown & Williams, 1985). For example, nutrient limitation may result in the induction of new outer membrane (OM) proteins. The resistance of *Ps. aeruginosa* to gentamicin, polymyxin and EDTA has been correlated with the induction of H1, an OM protein induced under magnesium limitation (Nicas & Hancock, 1980). Thus, biofilm cells may be recalcitrant due to changes to fatty acids, phospholipids, metal cations, proteins, extracellular enzymes and polysaccharides in the cell envelope.

Suci *et al.*, (1998) suggested that the two explanations of biofilm resistance, penetration limitation and physiological limitation are not mutually exclusive. If the structure of a biofilm can moderate the delivery of an antimicrobial to cells within the biofilm, this may give cell time to adopt a set of physiological, protective, changes. Similarly, Hughes *et al.* (1997) noted an increased production of EPS at certain sub-lethal concentrations of antibiotic. It was thought that the action of the antibiotic inhibited bacterial growth, followed by a reallocation of resources into increased EPS production of EPS, and this may further increase the resistance of the biofilm.

The third hypothesis states that the attachment of cells to surfaces causes the depression of a large number of genes associated with sessile existence so that biofilm cells are phenotypically distinct from their planktonic counterparts. This may inadvertently alter antimicrobial susceptibility. Two regulatory genes that may be involved in mediating the biofilm physiology of *Ps. aeruginosa* are *rpoS* and *algU*. The general stress response sigma factor in *E. coli*, RpoS, controls 30 or more genes that are expressed during stress and starvation, and during the transition to stationary phase. To explain the high resistance of biofilms to antimicrobial agents, Brown and Barker (1999) proposed that biofilm growth leads to an early accumulation of density-dependent signals and to an early general stress response, and possibly a more complete expression of the response, relative to that in planktonic culture.

There is evidence to suggest that RpoS is being expressed in sputum from cystic fibrosis isolates with chronic *Ps. aeruginosa* infections (Foley *et al.* 1999). The sigma factor AlgU, involved in alginate biosynthesis, is also activated during the formation of biofilms in *Ps. aeruginosa* (Deretic *et al*, 1994). However, Cochran *et al.*, (2000) demonstrated that *algU* and *rpoS* played only a transient role in protecting thin biofilms from hydrogen peroxide. Interestingly, Hasset *et al.* (1999) stated that *Ps. aeruginosa* mutants devoid of one or both AHLs exhibited decreased expression of superoxide dismutase and catalase, suggesting quorum sensing mediates biofilm susceptibility to hydrogen peroxide.

Other mechanisms that could contribute to biofilm resistance are being investigated. For example, many organisms have multidrug resistant pumps (MDRs) that can extrude chemically unrelated compounds. The involvement of the MDR of *Ps. aeruginosa*, MexAB-OprM, in biofilm resistance was investigated. The resistance of *Ps. aeruginosa* to ofloxacin was dependent on the expression of MexAB-OprM, but only at low concentrations (Brooun *et al.* 2000). Similar results for the MDR of *E. coli*, *marAB*, and ciprofloxacin resistance were published (Maira-Litran *et al.* 2000). Thus, the induction of MDR within biofilms could not explain the elevated levels of antibiotic resistance.

At this moment in time, the phenomenon of biofilm resistance remains a mystery. However, Brooun *et* al., (2000) have suggested the answer lies in a small 'superbacteria' resistant population residing amongst the biofilm. Dose-response killing of *Ps. aeruginosa* biofilms demonstrated the presence of a subpopulation of cells that were resistant to quinolones. When removed form the biofilm, these cells exhibited higher resistance levels to tobramycin when compared to planktonic cultures, but lower levels than that of the intact biofilm. Ashby et al. (1994) and Muli et al. (1998) have made similar observations. Small colony variants (SCV) of *S. aureus* and *S. epidermidis* are frequently isolated from chronic infections. These SCV exhibit slow growth, auxotrophy and antibiotic resistance (Bayston and Wood, 1997). Further investigation of these phenotypes may provide the solution to the problem of antibiotic resistance.

1.3.3 *Alternative Methods of Control*

Treatment with antimicrobial agents that has proven to be effective against suspension cultures often fails to have any effect *in situ* (Bloomfield *et al.*, 1994; Holah and Kearney, 1992). Antibiotics may suppress the symptoms of a biofilm infection by killing planktonic cells, but once chemotherapy ceases, the biofilm quickly regrows and the infection manifests itself repeatedly. Antimicrobial agents need to be tested against biofilm, not planktonic cells. For example, roxithromycin, a derivative of erythromycin, inhibits biofilm formation (Ozeki *et al.*, 1996). When used in combination with other drugs, roxithromycin was successful in eradicating biofilms of *Ps. aeruginosa* and *S. epidermidis* (Kondoh *et al.*, 1996). In the oral cavity, colonisation of *S. mutans* increases following sugar consumption. This has been linked to fall in pH that is known to be a factor in the selective colonisation of *S. mutans*. From this, using sugar substitutes may control *Streptococcus* colonisation, such that *S. mutans* no longer produces acids from sugar metabolism. Alternatively, the addition of fluoride may also interfere with the effects of acidification, and also inhibit *S. mutans* attachment (Suchett-Kaye *et al.*, 1996).

Targeting the HSL regulatory system may prevent biofilm formation. For example, the macroalgae *Delisea pulchra* produces a number of halogentaed furanones that are structurally similar to HSLs. These furanones inhibited bioluminescense by *V. fischeri* and swarming motility in *Serratia liquefaciens*, also an HSL regulated process (Giskov *et al.*, 1996). The ability of various HSL analogues to inhibit the action of the cognate HSL has also been demonstrated (McLean *et al.*, 1997; Passador *et al.*, 1996).

Attempts have been made to control biofilms in other ways, for example, the incorporation of biocides into substrates to make surfaces that are intrinsically resistant to microbial colonisation. For example, incorporation of copper and cobalt phthalocyaine catalysts within polymers generated the break down of peroxides and persulphates to form active oxygen species at the biofilm-surface interface (Wood *et al.*, 1997). Rogers *et al.* (1995) used silver-coated surfaces that slowed downed initial colonisation but did not prevent biofilm formation. Reduction of surface roughness has also been successful in controlling biofilm formation. Holah and Thorpe (1990) suggested the use of hardwearing, ultrapolished stainless steel. Polyurethane is

commonly used on conveyor belts, but this is abraded very easily, and, consequently, difficult to clean. Peterson and Pitt (2000) have reported that low-frequency ultrasound renders bacteria more susceptible to antibiotics. This system has also been tested *in vivo*, and successfully reduced bacterial viability (Rediske *et al.*, 2000). Low electrical currents were also reported to enhance the activity of the antibiotics ciprofloxacin and polymyxin B (Jass *et al.*, 1995). Similarly, the effects of UV radiation on biofilm communities are being investigated (Elasri and Miller, 1999).

1.4 MIXED SPECIES BIOFILMS

Biofilms in most industrial and natural environments consist of complex communities of microorganisms. Interactions between bacteria in biofilms have a profound influence on the formation, structure and physiology of the biofilm. However, the majority of research on bacterial interactions has focused on planktonic cultures. The close association and stability of cells within biofilms and local areas of hindered diffusion are likely to increase the possibility of physiological interactions occurring. This suggests interspecies interactions in biofilms may be more significant than in planktonic cultures (James *et al.*, 1995).

1.4.1 *Bacterial Interactions*

Bacterial interactions begin to influence a biofilm during the initial stages of development. Adhesion of one species can have a positive, negative or neutral influence on the adhesion of another species (McEldowney and Fletcher, 1987). Positive interactions can be indirect due to modification of the conditioning film by bacterial products, or by direct mechanisms involving cell-cell contact. This is exemplified by the coaggregation of oral bacteria (Kolenbrander and London, 1993). Inhibitory actions can also occur due to one cell blocking the attachment site of another or through modification of the conditioning film by the absorption of inhibitory macromolecules. Neutral adhesion interactions are likely to occur when species occupy separate binding sites on the substratum (James *et al.*, 1995). Therefore, species can coexist, compete or co-operate in a biofilm, and all these processes may occur at the same time. Examples of how interactions between species can influence the community structure and stability of biofilms are discussed below. Although the focus here is on bacterial interactions, it must be remembered that interactions between other micro- and macroorganisms often occur. Interactions between algae and bacteria are common in natural aquatic environments. Predation by protozoa is also a common occurrence (Hahn and Hofle, 1999).

1.4.2 *Commensalism, Co-operation and Synergism*

Commensal interactions occur when one population benefits and the other is unaffected. Such an interaction is demonstrated by the role of *Fusobacterium*

nucleatum in oral biofilms. *F. nucleatum* facilitates the survival of obligate anaerobes in aerated environments (Bradshaw *et al.*, 1998).

A commensal relationship between a *Pseudomonas* sp. and a *Burkholderia* sp. was described by Neilson *et al.* (2000). When growing on citrate, the pseudomonad and *Burkhoderia* sp. developed as separate colonies. However, only the *Burkholderia* was capable of utilising the aromatic compound chlorobiphenyl, oxidising this to chlorobenzoate. The *Pseudomonas* could degrade chlorobenzoate, and under these conditions the *Pseudomonas* was seen to move towards the *Burkholderia*, presumably by chemotactic mechanisms, to produce microcolonies consisting of the two species in close association.

Biofilms formed by mixed communities are often thicker and more stable than single species biofilms. One species can often enhance the stability of another in a biofilm. For example, the copious production of an exopolymer can increase the stability of another species within a biofilm. Buorion and Cerf, (1996) demonstrated the importance of *Ps. aeruginosa*, an exopolymer-producing bacteria, for enhancing attachment by *Listeria inocua*. *L. inocua* was protected from disinfectants in the mixed biofilm due to the presence of *Ps. aeruginosa* cells and associated polymers. Production of drug inactivating enzymes can also be useful. Investigating the interactions between a β lactamase producing strain, *Moraxella catarrhalis* and a pathogenic strain, *S. pneumoniae*, Budhani and Struthers, (1998) showed that the production of the β lactamase by *M. catarrhalis* had a protective effect. Concentrations of antibiotic that would have ordinarily killed the pneumococcus were ineffective.

When each species benefits from the other, this process is termed co-operation. For example, the thicker biofilm production, and enhanced resistance to disinfection by a dual species biofilm of *Ent. agglomerans* and *K. pneumoniae* was partially attributed to interactions between the EPS produced, changing the physical properties of the mixed polymers (Skillman *et al.*, 1999b). A similar, stabilising situation was described by Allison and Matthews (1992). An increase in attachment and increased metabolic activity was also described between *Salmonella enteritidis* and *K. pneumoniae* when grown as a dual species biofilm (Jones and Bradshaw, 1997).

Co-operating interactions between microorganisms can often result in the increased metabolism of a food source. When more of a particular compound is produced or consumed by a microbial community than by a single population, this is termed synergism. For example, the degradation of a pesticide by a consortium of nine bacteria was enhanced by the presence of an alga, presumably due to the provision of alternative sources of carbon (Wolfaardt *et al.*, 1994). The degradation of toluene by *Ps. putida* was enhanced by the presence of an *Acinetobacter* sp. Only in the presence of the *Acinetobacter* was the meta-pathway of degradation of *Ps. putida* switched on, allowing more efficient degradation by the two strains (Moller *et al.*, 1998). A classic synergistic relationship exists between cellulolytic microbial communities and methanogenic bacteria in the rumen. The fermentative products of cellulose degradation are formate, H_2 and CO_2. Formate and H_2 are utilised by the methanogens to from CH_4. This reduces the levels of H_2 in the gut, making the fermentation of cellulose more thermodynamically favourable (James *et al.*, 1995).

The development of plaque is the most widely studied example of a complex mixed species biofilm. The oral cavity contains a large and diverse population of microbes, with over 700 species and 37 genera recognised. Most and probably all oral bacteria coaggregate with at least one partner cell type. Coaggregation, or cell-to-cell recognition of genetically distinct cell types, is mediated by ionic, hydrophobic and lectin-receptor mechanisms. A lectin is a sugar binding protein or glycoprotein of non-immune origin that agglutinates cells and/or precipitates glycoconjugates. These adhesins are found intercalated into the outer membrane or cell wall, or may be associated with fimbriae (Behmlander and Dworkin, 1994). Binding sites on lectins are thought to be clefts or grooves into which particular sugars fit, with certain side groups of the sugar making contact with small combining regions on the lectin.

Adherence of oral bacteria occurs by a multistep process. Following cleaning, teeth are covered by a thin film (acquired pellicle) that consists of glycoproteins, mucins and enzymes. Many of the early colonisers recognise components of the acquired pellicle. For example, *Streptococcus gordonii* binds to acidic proline-rich proteins and α-amylase, and *Actinomyces naeslundii* and *F. nucleatum* bind to the

phosphoprotein, statherin. Most of the early-colonising streptococci offer receptor molecules to the indigenous flora, allowing further accretion to occur by intergeneric coaggregation (**Figure 1.2**). Each strain has its own specific partners, but often several isolates of a genus exhibit the same set of partners. The fusobacterium are depicted as a bridge between early and late colonisers. For example, late colonisers, such as *Selenomonas flueggei*, do not coadhere with early colonisers, but colonise exclusively with *F. nucleatum* (Kolenbranger and London, 1993). The ability to aggregate is increased when the bacteria possess multiple adhesins. Six different mechanisms of adhesion for *S. pyogenes* were described (Hasty *et al.*, 1992). Different streptococcal species preferentially colonise different oral sites, and coadhere to a varied range of bacteria. Thus, species are able to cooperate, not compete, with each other for binding sites (Wittaker *et al.*, 1996).

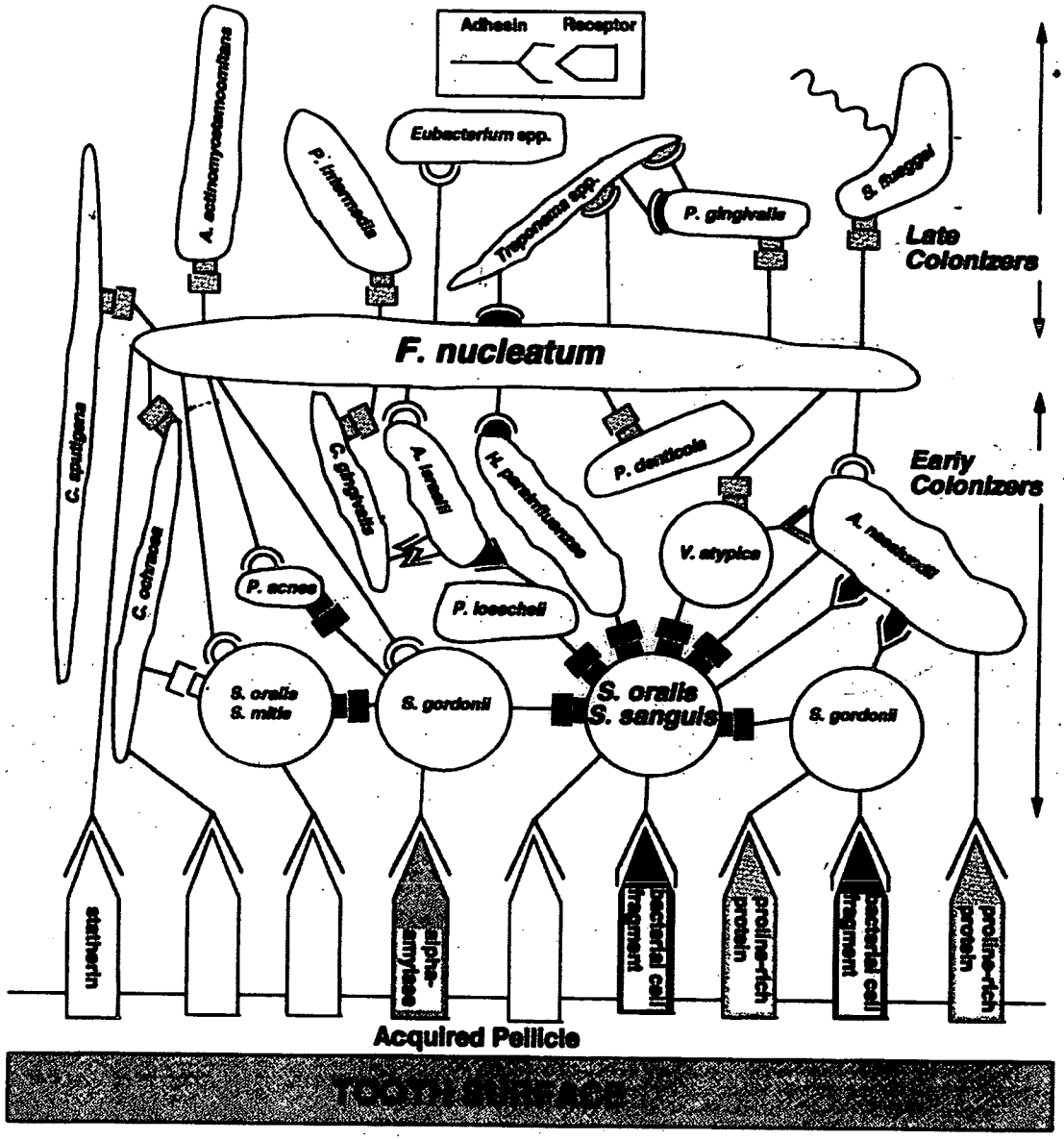


Figure 1.2

Bacterial colonisation in dental plaque. Early colonisers bind to receptors in the acquired pellicle, and each newly adhered cell type becomes, in turn, a surface for additional coadhesion (Kolenbrander & London, 1993).

1.4.3 *Neutral*

It would seem unlikely that neutral, or non-interactions, would occur in a biofilm, due to the close association of the cells, however, Siebel and Characklis (1991), described such a system. The product (cellular and extracellular carbon) formation rates and glucose:oxygen consumption ratios of *Ps. aeruginosa* and *K. pneumoniae* were unaffected when the bacteria were grown as a mixed species biofilm; species interaction was not observed.

1.4.4 *Competition*

Competition refers to an interaction where two populations are competing for a growth-limiting nutrient, and is detrimental to both populations. The outcome for competition in planktonic cultures is thought to be growth rate dependent (Meers, 1973). Differences in growth rates may also be a factor in biofilm competition. Banks and Bryers (1991) studied the invasion of a faster growing strain, *Pseudomonas putida*, into a biofilm of a slower growing strain, *Hyphomicrobium* sp. The faster growing strain rapidly became the dominant species within the biofilm. Similarly, *Ps. aeruginosa* biofilms in a packed-bed reactor were outgrown by invading *K. pneumoniae* cells (Sturman *et al.*, 1994). In each of these studies, when the roles of invader and established population were reversed, the faster growing strain again dominated the biofilm. Interestingly, the subordinate strains were not outcompeted by the dominant strains and also remained established within the biofilms. Siebel and Characklis, (1991) suggested that factors other than specific growth rate, such as colony morphology may influence competitive interactions between strains in biofilms. Stewart *et al.*, (1997) attributed the co-existence of nutrient-competing strains in a dual species biofilm system to microscale structural heterogeneity and differing rates of attachment and detachment of the two species. The enhanced competitiveness of *Fibrobacter succinogenes* when grown on a solid substrate was also attributed to its ability to colonise the surface more effectively (Odenyo *et al.*, 1994).

An ammensalistic interaction can also occur when one microrganism produces a compound that is inhibitory to another. Bacteriocins are antimicrobial agents produced by a wide range of bacteria, and result in the killing of bacterial

competitors. Production is tightly linked with an immunity protein to protect the producer cell. Competition between two *Ruminococcus* sp. was based on the production of a bacteriocin (Odenyo *et al.*, 1994). The production of an extracellular substance by *Staphylococcus sciuri* was also attributed to the decreased adhesion of *Listeria monocytogenes* (Leriche and Carpentier, 2000). Addition of unpastuerised buttermilk to silicone rubber voice prosthesis in an artificial throat removed both yeasts and bacteria. This has been associated with the production of a biosurfactant by *Streptococcus* sp., present in the buttermilk (Busscher and van der Mei, 2000).

Taken to the extreme, negative interactions also include predation, where one organism is consumed by another, and parasitism, where one organism is invaded intracellularly by another. Shi and Zusman (1993) described the release of attractants by *Myxococcus xanthus*, harnessing the chemotactic behaviour of *E. coli*, causing the cells to swim towards the *Myxococcus* colony. *Bdellovibrio bacteriovorus* is a Gram negative bacterium, which uses other Gram negative bacteria as its sole source of nutrition. Koval and Bayer (1997) demonstrated that EPS did not protect planktonic cells from *Bdellovibrio* attack, suggesting biofilm cells would also be susceptible to predation by these parasitic bacteria.

1.5 BACTERIOCINS

The production of substances that inhibit the growth of closely related species is universal among groups of bacteria. Antimicrobials include toxins, bacteriolytic enzymes, bacteriophage, by-products of metabolic pathways, antibiotics and bacteriocins. Bacteriocins are compounds produced by bacteria that inhibit or kill closely related species. Their production is abundant in all the major lineages of Eubacteria and Archaebacteria. These highly specific toxins are usually produced under stressful conditions and result in the killing of bacterial competitors that are not resistant to their effects. Most come equipped with an immunity protein which protects the producer from the effect of the bacteriocins. Bacteriocins produced by the Gram negative bacteria, in particular the *Enterobacteriaceae* and those produced by *Pseudomonas* sp., are discussed below. However, bacteriocins are also produced by a wide range of Gram positive species, such as the lantibiotics (nisin, epidermin and cinnamycin) of species such as *Bacillus, Staphylococcus* and *Streptomyces*, and lysostaphin, produced by *Staphylococcus*.

1.5.1 *Colicins*

Colicins are generally encoded on plasmids; the genes are usually repressed and tightly linked with an immunity gene. Production is amplified several orders of magnitude by induction, such as exposure to UV light and radiomimetic chemicals, agents which elicit the SOS response (Walker, 1984). Colicin-production results in the killing of neighbouring cells that are not immune or resistant to the cell. Colicin synthesis is a rare and lethal event. Only one cell in 10^3 – 10^4 produces colicin in each generation, and this suicidal act poisons the surrounding environment. Cell lysis requires the function of a lytic protein whose gene is part of the colicin operon. Colicins bind to specific cell surface receptors and are transported into the cell. Having gained access, they go on to kill the cell. Nomura (1964) classified the colicins according to their biochemical effects on sensitive bacteria. Colicins of the E2 class degrade DNA, another class, typified by colicin E3, inhibits protein synthesis and class E1 colicins damage the cytoplasmic membrane.

Colicin E2 Class

Colicin E2 acts as a non-specific DNA endonuclease. The bacteriocin is released from the cell as a 1:1 complex of two polypeptides (60 kDa). The larger polypeptide (50 kDa), termed E2*, is the active moiety of the complex and contains the structural information required for receptor selection. The smaller subunit has no bacteriocin activity but is a highly specific inhibitor of the larger polypeptide. Tests with proteolytic digests of colicins have shown that a C-terminal segment carries the nuclease activity and combines with the immunity protein (Ohno-Iwashita and Imahori, 1979). The N-terminal region is thought to be involved in translocation across the cell membrane (Konisky, 1982).

Colicin E3 Class

The two most extensively studied members of the colicin E3 group are E3 and DF13 (isolated from *Enterobacter cloace*). These bacteriocins have RNAse activity. Each causes an endonucleolytic break that splits a 49-nucleotide fragment from the 3• - terminus of the 16S RNA molecule of the 30S ribosomal subunit (Konisky, 1982). Similar to colicin E2, E3 and DF13 colicins have immunity proteins which are tightly bound to the C-terminal segment of the molecule.

Colicin E1 Group

Killing of sensitive bacteria by the colicins of the E1 group (E1, 1a, 1b, A, K) has been correlated with a rapid loss of membrane potential and a consequent decrease of the proton motive force across the cytoplasmic membrane. Measurements of the components of the proton motive force have shown that, although protons continue to be expelled from colicin-treated bacteria, the membrane potential decreases to near zero. K^+ ions flow out of the cells according to the concentration gradient. Each individual colicin molecule is thought to be capable of forming a single channel in the cytoplasmic membrane; this would elicit sufficient ion flow to depolarise the membrane within a few minutes (Konisky, 1982). E1 group colicins are not released from the cell in complex with an immunity protein. The location of immunity protein is the cytoplasmic membrane.

Colicin Release, Binding and Entry into Cell

Colicin release from the cell relies on the expression of the Kil protein. It has been proposed that Kil activates phospholipase A in the outer membrane of *E. coli* cells, altering the permeability of the outer membrane and facilitating colicin release from the periplasm and cell lysis (James *et al.*, 1996).

In order to reach their cellular targets, colicins follow a three-step process. First they bind to bacterial receptors; second, they translocate the outer membrane and third, they act by forming a pore into the inner membrane, or by exerting nuclease activity in the cell cytoplasm. In line with the three steps constituting their mode of action, the colicin polypeptide chains comprise three linearly organised domains, each of which corresponds to one of these steps. The N-terminal domain is involved in translocation, the central terminal domain is responsible for receptor binding and the C-terminal domain carries the lethal activity.

The outer membrane protein BtuB, involved in vitamin B_{12} transport, acts as a receptor for many colicins, including the E colicins and colicin A. However, it is thought that only newly synthesised BtuB molecules can mediate colicin action, possibly while they are associated with areas of contact between the inner and outer membrane. Once colicins have bound to a specific receptor, they are translocated across the outer membrane. Tanslocation is mediated by the Ton or Tol system (**Figure 1.3**). Colicins can be classified into groups on the basis of the translocation system they use. The group A colicins (A, E1 to E9, K, L, N and S4) use the Tol system and require porins such as OmpF, together with TolA, TolB, TolQ, and TolR proteins. The Tol-dependent pathway is also used for the uptake of filamentous phage. The group B colicins (B, D, Ia, Ib, M, Q) require the Ton system and require specific receptors and the TonB, ExbB and ExbD proteins. This system also facilitates the flow of energy from the cytoplasm to the outer membrane for the energy-dependent transport of siderophores and vitamin B_{12} (James *et al.*, 1996).

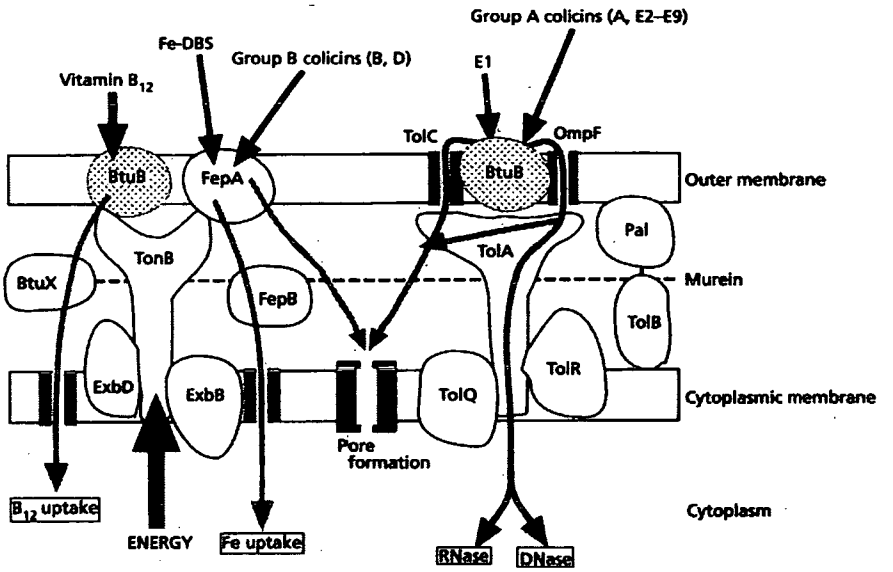


Figure 1.3

Receptor binding and translocation of Group A colicins, two of which are colicin E1 and A, form pores in the cytoplasmic membrane, whilst the colicins, E2-E9, are translocated to the cytoplasm. As a comparison, the group B colicins (B and D) bind to the FepA receptor and also cause pore formation in the cytoplasmic membrane, along with the vitamin B_{12} and Fe-DBS, all of which are translocated via the TonB-dependent uptake system (James *et al.*, 1996).

The Col Plasmids

In colicinogenic bacteria, the genetic determinants for colicin adsorption, transfer and biochemical action are located in plasmids, the Col factors. Production of the toxin requires the simultaneous expression of the colicin structural gene, *cea*, and a second gene, *kil*, which is responsible for cell lysis. In most cells, transcription of *cea*, *kil* and the immunity gene, *imm*, is repressed by the LexA protein but when the SOS response is triggered by DNA damage, the repressor is inactivated by RecA protease activity. All Col factors are DNA replicons, replicating from a unique origin. Replication is independent of the bacterial replication controls, although it requires bacterial transcription products. Two sets of Col factors exist, grouped according to molecular weight, group I (3×10^6 to 6×10^6) and group II (7×10^7 to 12×10^7) (Hardy, 1975). Group I replicons are present in high copy number. Copy

number is maintained through regulation of the formation of the RNA primer required for plasmid replication and by random equipartition during cell division. Group II Col factors are present in only one copy per cell and are distributed at cell division by physical separation of membrane attachment sites. The inability of two plasmids to stably coexist in the same cell, or incompatibility, is thought to be associated with competition for replication regulators. Thus, a cell containing incompatible plasmids A and B will give rise to descendants containing either A or B, but not both. Group I Col factors are generally too small to encode a full set of genes for conjugation, but are mobilisable, achieving transfer by 'borrowing' the gene products of a self-transmissible plasmid. In Col E1, a group I Col factor, the Mob proteins nick DNA at the *nic* site, bind to the nicked strand and lead it through a mating channel into a recipient cell. The conjugative plasmid provides pilus-mediated cell-to-cell contact and formation of the conjugation pore. Group II Col factors are fully conjugative. Many Col factors confer a membrane-associated property of exclusion, preventing the entry of related factors during conjugation (Luria and Suit, 1987).

1.5.2 *Microcins*

The microcins are antibiotic substances secreted by a wide range of the *Enterobacteriaceae* (Baquero and Moreno, 1984). They are distinguished from the majority of colicins by their low molecular weight (less than 20 kDa) and because their synthesis is not induced by conditions that lead to induction of the SOS repair pathway. However, they are synthesised during stationary phase, similar to the larger colicins. The majority of microcins are ribosomally synthesised polypeptides which undergo unusual posttranslational modifications to yield the mature molecule. They often contain amino acid residues not found in proteins e.g. lanthionine and dehydroalanine, and along with unusual crosslinks, this may help the molecule achieve and retain their small confirmation (Gross and Morell, 1971). The unusual side chains found in microcins are also thought to provide a wider repertoire of chemical activity (Gross and Morell, 1967) and this may be very important for the biological activity of these peptides.

A microcin-producing bacterium must be able to synthesise and export the peptide, protect itself from the action of the antibiotic, and go on to interact with a sensitive cell and interfere with its growth or survival. Examples of how these problems can be overcome are discussed with reference to the microcins B17 and colicinV.

Microcin B17

Microcin B17, and immunity to it, is determined by *E. coli* strains harbouring the plasmid pMccB17. Seven genes, *mcbABCDEFG*, are involved in the production of MccB17 and this is under the control of a stationary phase promotor. The structural gene, *mcbA*, codes for the 69-residue MccB17 precursor (Kolter and Moreno, 1992). A complex of MccB-D converts the cysteine and serine side chains of preMccB17 to thiazole and oxazole, respectively. This is then converted to mature MccB17 by proteolytic removal of the first 26 amino acids, possibly by the action of the unlinked gene *pmbA* (Kolter and Monero, 1992). A complex of McbE and McbF constitutes a dedicated exporter for active MccB17, translocating mature MccB17 across the double-membrane envelope of *E. coli* into the surrounding medium (Garrido *et al*, 1989). A cytosolic immunity factor, McbG is synthesised which protects the host cell from MccB17 activity. The membrane transporters McbE and McbF can also confer partial immunity, presumably because they recognise the mature MccB17 as an export substrate (Garrido *et al*, 1989).

MccB17 inhibits DNA replication and induces the SOS repair response system. To reach its target, it first binds to the outer membrane receptor OmpF to reach the periplasm and then binds to SbmA, to translocate the inner membrane (Kolter and Moreno, 1992).

Colicin V

Although it is called a colicin, ColV fits the description of a microcin: it has a molecular weight of 6000 and its synthesis is not SOS-inducible. Four plasmid genes from pColV-K30 are required for ColV synthesis, export and immunity (Gilson *et al*, 1987). These are arranged on two converging operons. The immunity gene, *cvi*, and the structural gene, *cvaC*, constitute one operon and *cvaA* and *cvaB*, the exporter

genes are located on the other operon. The product of the unlinked gene, *tolC*, is also required for immunity. The export signal of ColV, which is recognised by the CvaA/CvaB exporter, is located within the N-terminal 39 residues of the product of *cvaA*. ColV kills by disrupting the membrane potential (Yang and Konisky, 1984) and so only has to cross the outer membrane to reach its target: the cytoplasmic membrane. Mutants lacking the outer membrane protein Cir and the inner membrane protein TonB are immune to ColV. TonB is required for translocation of many substances across the outer membrane (Kolter and Moreno, 1992).

The contribution of ColV towards virulence has been investigated. For example, ColV production was shown to increase the invasiveness of *E. coli* in chickens. Transferring ColV to *E. coli* K-12 also increased the number of deaths by the strain (Hardy, 1975). More recently, production of ColV and the presence of smooth LPS were significantly correlated with chicken embryo lethality (Wooley *et al.*, 1993).

1.5.3 *Pyocins*

Pseudomonas sp. produce various types of bacteriocins or pyocins, namely, R, F and S-type pyocins. The S pyocins are a group of simple proteins, of low molecular weight and are sensitive to proteases. Studies of the four representative members of the S pyocin group suggest they share functional similarity, nuclease activity and some DNA sequence with the nuclease colicins of *E. coli*. The structures of R and F-type pyocins closely resemble contractile bacteriophage tails, each being composed of a contractile sheath, a core and fibres. Of each of the pyocin's over 20 distinct subunit proteins, only that polypeptide that comprises the main component of the fibre differs from pyocin to pyocin. This is thought to be the basis of the host range of each R-pyocin, as these fibres allow the absorption of each bacteriocin to its cognate lipopolysaccharide receptor on the sensitive cell (Ito *et al.*, 1970b). There is evidence to suggest that the R-type pyocins originated from a common temperate bacteriophage, which by mutation became defective in assembly of normal particles. Several *Ps. aeruginosa* strains produce phage that display immunologic cross-reaction with the R-type pyocins (Ito *et al.*, 1970a).

The apparent mode of killing is by pore formation in the membrane and disruption of the membrane potential. Pyocin typing of more than 1400 *Ps. aeruginosa* isolates from environmental and clinical sources has indicated that more than 90% produce one or more pyocins. R and F pyocins are produced by over 90% of clinical isolates, and S pyocins are produced by 70% of those same strains (Riley, 1998).

1.5.3 *Bacteriocin Ecology*

The role of bacteriocins in natural populations is poorly understood. There must be an advantage to be gained by bacteria that are able to kill other strains, but not all investigations have demonstrated a selective advantage conferred by colicinogeny. In fact, there are indications that colicins are inactivated by intestinal contents, presumably by proteases. But as colicins seem so perfectly adapted to killing *E. coli* in the laboratory, an entirely different role for colicins in nature seems improbable. Indeed, mathematical models have shown that colicins play a key role in mediating *E. coli* population dynamics. In general, it seems as if colicinogeny may play a significant part in the succession of strains in the intestine, but this may be frequency dependent. For example, when the initial frequency of the colicin-producer is low, too little colicin is produced to kill enough sensitive cells, and so the sensitive cell predominates. Similarly, a sensitive strain can only invade a colicinogenic population at high frequencies (Riley and Gordon, 1999).

There have been few *in vivo* studies, which back up these mathematical models, however certain research has show that colicinogency is a significant factor in the succession of strains in the intestine. For example, in examining the fate of ingested broth cultures of *E. coli* in healthy persons, Cooke *et al.* (1972) reported eight instances in which colicinogency was apparently responsible for the establishment of a strain, or displacement of a strain. Microcins are thought to be less susceptible to the action of proteases in the intestine. The colonisation process of the intestinal tract in hospitalised, bottle-fed new-borns was investigated, and the ability for colonisation was related to microcin production (Baquero and Moreno, 1984). More recently, Gordon *et al* (1998), studied the temporal changes in the frequency of colicinogency in *E. coli* from house mice, and has shown that there is a constant flux

of sensitive, producer and resistant cell types in natural populations. Bacteriocin production by *Haemophilus influenza* in the nasopharyngeal region of the rat and *Streptococcus mutans* in the human oral cavity (Riley, 1998) have also been investigated. In each case, the action of the bacteriocin increased the competitive advantage of the producing strain in invading or maintaining population levels.

A relationship between the course of bacterial intestinal infections and changes to the colicinogeny of the intestinal flora has also been noted. In several cases, the percentage of Col+ strains amongst the commensal flora accompanying the pathogen increased during the course of dysentery infections. This was thought to be due to the transfer of Col factors from the pathogen to the accompanying enterobacteria (Hardy, 1975).

1.6 BACTERIOPHAGE

Bacteriophage were first identified 80 years ago, and have played a key role in the development of modern biotechnology. Their initial isolation appeared to offer a solution to the control of therapeutic diseases. With the increase in multiply antibiotic-resistant pathogens, bacteriophage are now being re-evaluated as the basis of new therapeutic strategies. Bacteriophage have also been successfully applied to the identification and typing for many groups of pathogenic bacteria. More recently, their host specificity has been applied to the development of bacterial detection and diagnostic strategies. The technologies of molecular cloning were also developed following studies of phage infection.

1.6.1 *Occurrence*

Extensive reservoirs of viral particles occur in natural ecosystems (Ripp and Miller, 1997). Wichels *et al.* (1998) investigated bacteriophage diversity in the North Sea. Using electron microscopy, 10^4 – 10^7 particles per ml were identified. DNA-DNA hybridisation techniques revealed the large genetic diversity of bacteriophage, which far exceeded the morphological diversity. Bergh *et al.* (1989) calculated that as much as one third of the biofilm population in natural waters may experience a phage attack each day, and along with protozoan grazing, bacteriophage may be very important in controlling bacterial populations. Wiggins and Alexander (1985) determined the distribution of bacteria that contained DNA from a particular temperate phage by hybridising isolated colonies to phage DNA. They showed that phage and related particles were widely distributed in Pseudomonad communities from 257 environmental samples. Bacteriophage for three representative strains of Gram negative bacteria were also found to be of widespread occurrence in sewage (Hughes *et al.*, 1998a). Araujo *et al.* (1997) investigated the use of phage as indicators of faecal pollution in fresh water. The levels of somatic and F specific coliphages and phages infecting *Bacteroides fragilis* in freshwater samples were studied. Levels of bacteriophage were estimated to be 2.1 x 10^5 for somatic coliphages, 7.2 x 10^4 for F-specific coliphages and 2.3 x 10^3 for *Bacteroides fragilis* phages. These levels increased 10 fold in sediments. Phage, therefore, can be expected to exert considerable influence on bacterial survival in the biofilm.

1.6.2 *Structure*

Structurally, bacteriophage are all built on the same principles, a nucleic acid core surrounded by a protein coat. The nucleic acid can be DNA or RNA, and may be of a double- or single stranded type, depending on the virus. The protein coat is formed of a number of individual protein molecules, called structural subunits or capsomeres, which are arranged in a highly repetitive pattern around the nucleic acid. The small genome size of most bacteriophage restricts the number of different viral proteins. A few phage have only a single kind of protein in their capsid, but most viruses have several distinct kinds of structural subunits that are themselves associated in specific ways to form larger assemblies called morphological subunits or capsids. The protein coat may also have appendages attached to it or be surrounded by an envelope. Bacteriophage are all built symmetrically and along strict geometrical lines. Bradley (1967) described six basic morpholgical lineages of phage (**Figure 1.4**). The most complex, group A, has a hexagonal head and a tail with a contractile sheath. This is usually rigid and may have various appendages such as fibres or spikes. Group B also have a six-sided head, and a more flexible tail. The third group, C, has a much shorter tail. The fourth group has no tail and each apex of the hexagon has a large capsomere attached. Group E is similar to group D, but no capsomeres are present at the apex of the protein coat. The final group, F, is in the form of a long flexible filament, there are no additional structures or appendages. Groups A to D are unique to bacteriophage, whereas, structures similar to E and F can be found amongst eukaryotic viruses.

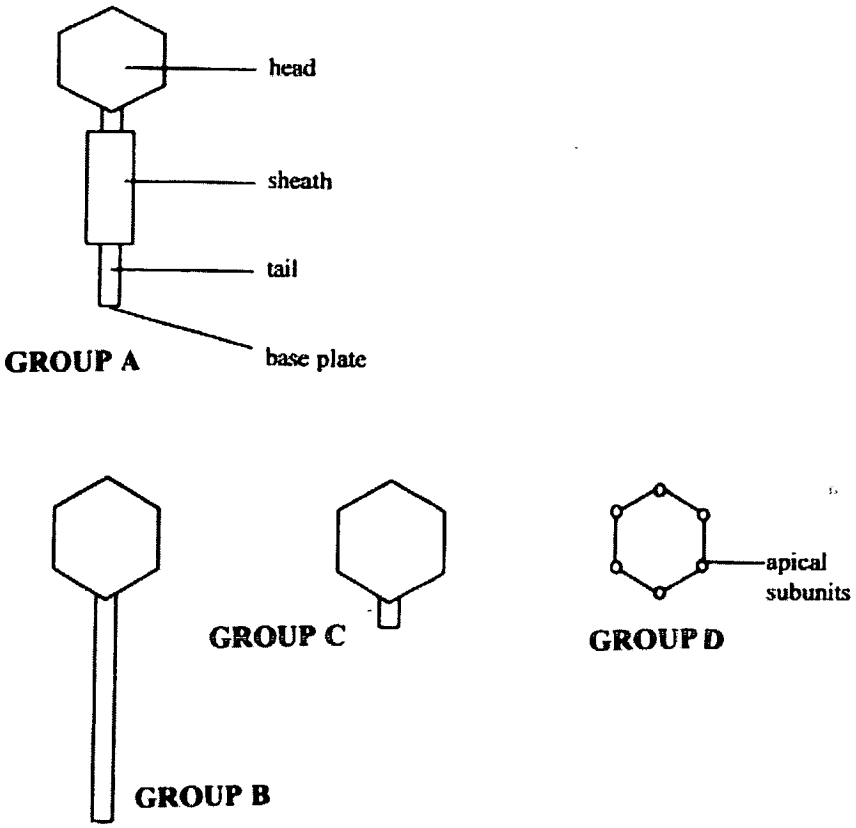


Figure 1.4
Basic morphological types of bacteriophage (Bradley, 1967)

1.6.3 *Replication*

Bacteriophage must induce a bacterial cell to synthesise all the essential components needed to make more virus particles. These components must then be assembled into the proper structure, and the new virions must then escape from the cell to infect other cells. The various phases of this replicative process in a bacteriophage can be categorised by the following steps: attachment, penetration, replication, assembly and release. This process is called the one-step growth curve (**figure 1.5**).

During infection, bacteriophage must adsorb to a receptor on the cell surface, such as flagella, pili, EPS, lipopolysaccharides (LPS) and teichoic acid-peptidoglycan complexes (Lindberg, 1977). There is high specificity in the interaction between the host and virus; a particular phage will only absorb to a limited range of bacterial strains that carry the phage receptor. The term 'adsorption' refers to two different steps, binding and eclipse. Binding is the process by which the receptor is recognised. Eclipse results in loss of infectivity of the phage and is characterised by a conformational change to the phage.

Penetration into the cell then occurs. For example, the group D phage ϕX174, possesses an icosahedral protein coat containing spikes at the twelve vertices. The spikes interact with the core oligosaccharides of LPS and guide the DNA through the membrane. This tends to takes place at points of fusion between wall and cytoplasmic membrane. The penetration of phage T4 of *E. coli*, a Bradley type A phage, is more complex. T4 first attaches to the cell by means of tail fibres. The fibres interact with LPS and contract, allowing the core of the tail to make contact with the cell envelope of the bacteria. A lysozyme-like enzyme makes a hole in the cell envelope. The tail sheath contracts, and DNA passes through into the cell via hole in the tip of the tail. The majority of the coat protein remains outside.

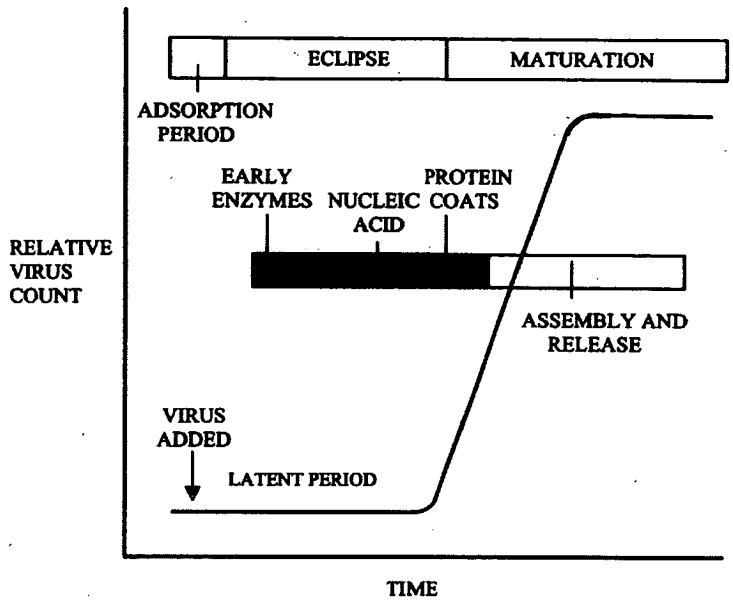


Figure 1.5

One step growth of virus replication. This graph displays the results of a single round of viral multiplication in a population of cells. Following absorption, the infectivity of the viral particles disappears, a phenomenon called *eclipse*. This is due to the uncoating of the viral particles. During the *latent period*, replication of viral nucleic acid and protein occurs. The *maturation period* follows, when virus nucleic acid and proteins are assembled into mature virus particles. Finally, *release* occurs, following cell lysis (adapted from Bradley, 1967).

Bacteriophages can have two different replicative cycles. In the first, virulent cycle, nucleic acid is injected into a sensitive cell that subsequently lyses. In the second, lysogenic or temperate type of cycle, nucleic acid is injected, but no intracellular virions are formed. Instead, the phage genome becomes inserted into the bacterial genome and is replicated as part of the bacterial chromosome. Many phage are capable of both replicative cycles, depending on the bacterial strain they infect.

During the latent period, synthesis of the proteins used as structural subunits of the virus coat, and replication of viral occurs replication occurs. The phage takes over the biosynthetic machinery of the host – the phage may provide a few proteins, but the host provides everything else including energy-generation systems,

ribosomes, amino acid activating enzymes and transfer RNA. The maturation period follows, when nucleic acid and protein are assembled into mature virus particles.

Finally, the mature virions are released from the cell. Filamentous phages can extrude through the envelope layer without fatal consequences for the host. However, others have to deal with the strong, stable peptidoglycan mesh surrounding the cell. Double stranded DNA phages produce a soluble, muralytic enzyme known as an endolysin, and a small membrane factor, 'holin'. The holin permeabilises the membrane, allowing the endolysin to gain access to the murein. Thus, the holin controls the timing of lysis. In some cases, the lytic process is so fine-tuned that a holin inhibitor is also produced. Single strand DNA phages have a single lysis gene. In the case of φX174, the protein transcribed inhibits a specific step in murein biosynthesis (Young *et al.*, 2000).

1.6.4 *Bacteriophage Polysaccharide Depolymerases*

Polysaccharide depolymerases are produced endogenously from polysaccharide synthesising microorganisms, exogenously from a wide range of microorganisms that degrade polymers as a food source, and from bacteriophage of EPS-producing bacteria. Adams and Park (1956) first noted the ability of phage to degrade the EPS of bacteria. Bacteriophage active on EPS-producing bacteria are generally EPS-specific, non-capsulate or non-slime producing mutant strains are generally resistant. Enzyme producing phage tend to belong to Bradley group C, although groups A and B can also have polysaccharide depolymerase activity (Lindberg, 1977).

To reach cell surface receptors, bacteriophage must first remove the polymer material that surrounds the bacterial cell envelope (**figure 1.6**). These enzymes degrade a pathway to the cell surface where the phage can bind to the primary receptor and initiate infection (Lindberg, 1977). Bacteriophage enzymes may act hydrolytically, cleaving specific linkages in the polysaccharide, or as a polysaccharide lyase, acting by eliminative cleavage at a monosaccharide-uronic acid linkage and introduction of an unsaturated bond at the C_4 and C_5 of the non-reducing uronic acid terminal (Sutherland, 2000b).

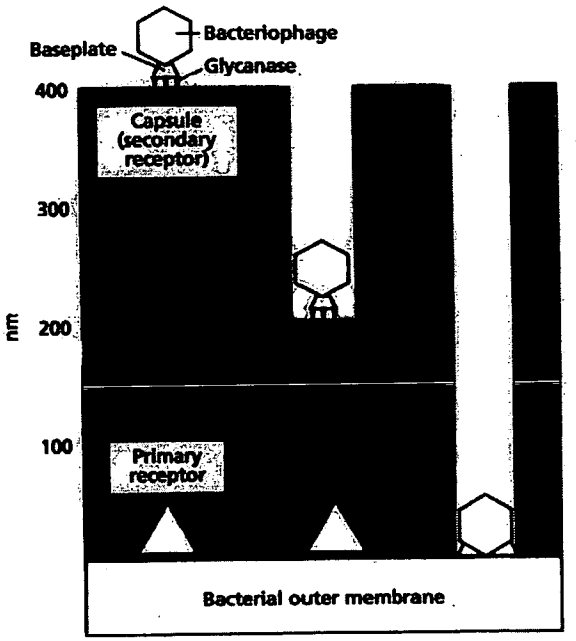


Figure 1.6

Degradation of bacterial capsular polysaccharide by a phage polysaccharide depolymerase occurs in 3 stages. (1) Phage binds to capsular polysaccharide (secondary receptor). (2) Phage polysaccharide depolymerase degrades polymer until phage reaches cell surface. (3) Phage binds to primary receptor and infects cell (Hughes *et al.*, 1998b)

This enzyme activity is associated with small spikes attached to the baseplate. In addition to the phage-associated enzymes, a soluble form of the protein is released following viral maturation and cell lysis.

The attack of bacteriophages on EPS-producing bacteria is often revealed by the fact that the plaques are surrounded by a halo. This characteristic halo production is due to the diffusion of phage induced enzyme which hydrolyses the capsule without killing the bacteria. The release of large quantities of enzyme during lysis is thought to permit egress of the mature phage particles. Decapsulated bacteria often exhibit increased sensitivity to other phage (Adams and Park, 1956). Sutherland and Wilkinson (1965) isolated several phage from sewage which were active on colanic acid producing bacteria of *E. coli*, including *E. coli* K12 and *Enterobacter cloace*. The phage studied often produced plaques only on their respective host strains. The

isolated depolymerases were, however, active on colanic acid preparations isolated from different strains and species.

1.6.5 *Bacteriophage Interactions with Biofilms*

The action of phage on biofilm will depend on whether the phage is temperate or virulent, or capable of producing polysaccharase depolymerase. As much of the biofilm is composed of polymer material excreted from the constituent micro-organisms, the possession of an enzyme capable of degrading these polymers will have a marked affect on the integrity of the biofilm. It may only require infection by one phage to destroy the entire biofilm. Infected cells in the outer layers will provide a focus for the generation of further phage particles on cell lysis, together with the release of soluble enzyme. Similarly, a lysogenic phage that is incorporated into a cell deep within the biofilm, may revert to a lytic cycle, destroying the biofilm from within. Biofilm topography will also influence the rate of infection. Phage may be able to move through the pores and channels to the basal layers where polymer degradation would cause sloughing off of the whole biofilm (Keevil *et al.*, 1995). Wiggins and Alexander (1985) stated that a biofilm may actually be the preferred site for phage reproduction when compared to the less accessible bacteria found in liquid cultures.

There are few studies on the ability of phage to degrade biofilms, and even less on the influence of phage-borne polysaccharase. For example, Doolittle *et al.* (1995) infected biofilms of *E. coli* K12, with T4, a phage with no polysaccharide depolymerase activity. It was shown that the phage successfully infected the biofilm, and the bacteria were not protected against bacteriophage attack by EPS as was previously suggested (Costerton *et al.*, 1987). Doolittle *et al.* (1996) also examined the infection of *E. coli* and *P. aeruginosa* biofilms by phage T4 and E27, respectively. Full infection of the thinner *E. coli* biofilm occurred, but the thicker biofilm of *Ps. aeruginosa* was only infected at the surface layers and access to the deeper layers was restricted. Hughes *et al.* (1998a,b) tested the separate effects of phage and of the soluble phage enzyme from phage lysates. The greatest decline in biofilm size was achieved through the use of a phage containing an associated enzyme capable of degrading the EPS. It was thought that the majority of bacteria

were being removed from the biofilm due to the action of the enzyme before the cells had a chance to lyse. When phage was added to a phage-resistant strain of *Ent. agglomerans*, and again where only soluble enzyme was added to the biofilm, the action of the phage enzyme alone still removed a substantial quantity of biofilm material. However, even when excess polysaccharase was added later in the experiment, some cells remained attached, suggesting factors other than EPS are involved in adhesion. Roy *et al.* (1993) studied the action of listeriaphages and a quaternary ammonium compound on biofilms of *Listeria monocytogenes.* It was noted that phage were as efficient as a quaternary ammonium compound in reducing biofilm size. A synergistic effect was observed when 2/3 different phage were used together, as was a combination of phage and the disinfectant. Similarly, Johansen *et al.* (1997) used a mixture of enzymes and disinfectants to eradicate mixed biofilms.

There may be some potential in use of phage as a tool to study pure and mixed culture biofilms. If the host bacteria produce exopolysaccharide, a phage with polysaccharase activity could be used to selectively remove the polysaccharide from the biofilm. Similarly, in a dual species biofilm, phage could be used to selectively attack a single species. However, given the close confinements of strains in mixed biofilms, and mixing of EPS, a strain-specific phage may not be able to eradicate one strain in a mixed species biofilm. In a synergistic dual species biofilm of *Ent. agglomerans* and *K. pneumoniae*, addition of a polysaccharase specific for the EPS of the *Ent. agglomerans* strain removed both bacteria from the biofilm (Skillman *et al.*, 1999b).

Biofilm bacteria may also be resistant to the effects of bacteriophage. Bacterial cells at the centre of the biofilm have very low growth rates. In low nutrient environments, pseudolysogeny is known to occur (Ripp and Miller, 1997). Pseudolysogeny is a form of host-cell interaction in which the nucleic acid of the phage resides within its starved host in an unstable, inactive state. In such cells, there is insufficient energy to initiate genetic expression for the lytic cycle to occur. The different phenotypic states of bacteria at different levels in a biofilm may lead to changes to the cell surface, and subsequently to the expression of a primary receptor required for bacteriophage attack.

1.8 AIMS & OBJECTIVES

The aim of this project was to study the high resistance of biofilms to antimicrobial agents. Biofilms more commonly consist of complex communities of microorganisms. Recent research has indicated that biofilms formed by mixed microbial communities are often thicker and more stable than monospecies biofilms, and this could further influence their susceptibility to antimicrobial agents (Buorion & Cerf, 1996). The biofilms formed by synergistic communities, communities in which the interactions are mutually beneficial to each of the species involved, can be more resistant to antimicrobial agents than when grown in isolation (Skillman *et al.*, 1999b). It was of importance to determine if different species could exert influences on each other, further altering biofilm resistance. A number of enterobacterial bacteria isolated from industrial surfaces were available for study. An understanding of the interactions between bacteria in a biofilm may lead to the development of novel, preventative treatments.

Hughes *et al.*, (1998a,b) provided evidence to suggext that bacteriophage and their associated polysaccharide depolymerases would be very useful, highly specific tool for the study of mixed species biofilms. For example, bacteriophage polysaccharide depolymerases may be used to selectively remove polysaccharide material from biofilms in an investigation of the role of EPS in the resistance of biofilms to disinfectants. Their high specificity also makes phage particularly useful tools in the study of mixed species biofilms. Selective removal of one species may allow its role in a dual species biofilm to be determined. There is also the possibility that bacteriophage can be used for biofilm control.

Chapter 2

MATERIALS AND METHODS

2.1 STRAINS

2.1.1 *Bacteria*

	Abbreviation in text	Source
Escherichia coli ATCC 11229	*E. coli*	Standard disinfectant-test strain
Escherichia coli	S61	I. W. Sutherland
Escherichia coli	S53	I. W. Sutherland
Escherichia coli	K12	Lab strain
Escherichia coli	Eck5	I. W. Sutherland
Escherichia coli	*E. coli* B	Lab strain
Enterobacter agglomerans	*Ent*	Industrial environment*
Enterobacter agglomerans	53b	Industrial environment*
Enterobacter cloace	5920	I. W. Sutherland
Enterobacter gergoviae	1.15	Local isolate
Klebsiella oxytocei	109c	Industrial environment*
Klebsiella oxytocei	86b	Industrial environment*
Klebsiella pneumoniae	G1	Industrial environment*
Klebsiella pneumoniae	107b	Industrial environment*
Klebsiella sp.	K66	I. W. Sutherland
Klebsiella sp.	D451.1	E. Yurewicz
Klebsiella sp.	XM6	I. W. Sutherland
Serratia liquefaciens	5Xa	Industrial environment*
Serratia marcesens	Pd3a	Industrial environment*
Serratia marcesens	87b	Industrial environment*
Serratia sp.	A1	Local isolate
Serratia sp.	A4	Local isolate
Pseudomonas aeruginosa ATCC 15442	Pseud	Standard disinfectant-test strain

*Given by Dr. M. V. Jones (Unilever Research Laboratory, Port Sunlight, UK).

2.1.2 *Bacteriophage*

Bacteriophage specific for the strains *E. coli, Ent*, 5920, S61 and S53 were isolated from primary effluent sewage during the course of this work. A bacteriophage specific for *Enterobacter gergoviae* 1.15 (ϕ1.15) and phage specific for *Klebsiella* sp. (K34 and K60) had previously been isolated. The phage ϕE. coli and Phil were isolated for the strain *E. coli*. ϕEnt and Armadale phage were isolated for the strain *Ent*. The phage ϕ5920, Blackburn and Winchburgh were isolated for the strain 5920. A phage ϕS61 was isolated for the strain S61, and East Calder phage was isolated for the strain S53.

2.2 MEDIA

Yeast Extract Media (YE Media) (Sutherland and Wilkinson, 1965).

	gL^{-1}
Yeast extract	1
Casein hydrolysate	1
X10 salts solution	100 ml
Distilled water	900 ml

x10 Salt Solution

	gL^{-1}
Na_2HPO_4	100
KH_2PO_4	30
K_2SO_4	10
NaCl	10
$MgSO_47H_2O$	2
$CaCl_22H_2O$	0.01
$FeSO_47H_2O$	0.001

Each salt was dissolved sequentially in distilled H_2O. Each item was dissolved before adding the next. The YE medium was buffered to pH 7.2 due to the ratio of Na_2HPO_4

and KH_2PO_4 in the salt solution. To prepare yeast extract plates and slopes, 15 g l^{-1} of Oxoid Agar No.3 was added before autoclaving. Sloppy agar, used for phage counts, was prepared by adding 5 g l^{-1} agar. Glucose was autoclaved separately, then added to allow a final concentration of 1% (w/v). Yeast extract, casein hydrolysate and Agar No.3 were from Oxoid, Unipath Ltd, Basingstoke, Hampshire, England.

Phosphate Buffered Saline

	g L^{-1}
NaCl	8
KCl	0.2
Na_2HPO_4	1.15
KH_2PO_4	0.2

The ingredients listed above were dissolved in 1l of distilled water and autoclaved.

2.3.GROWTH OF BIOFILM MATERIAL

Flask and Bead Method

The culture system comprised 250 ml Erlenmeyer flasks containing 100 ml Y.E media and 15 g of 4 mm glass beads. Cultures were incubated at 30 °C with shaking at 80 rpm.

Yellow Box Method

The batch culture system comprised a stand capable of supporting glass coverslips and a culture vessel in which the stand was immersed. The stand consisted of two parts, both made of 0.7 mm thick sheet stainless steel (Brown & Glegg Ltd, Edinburgh). The first part consisted of a series of 12 grooves in which the coverslips were supported vertically. The second part was a shallow walled tray, in which the first part sat, preventing the coverslips from moving laterally out of the grooves. The culture vessel was a yellow tip box (Greiner, Labortechnik Ltd., Stonehouse, Gloucestershire - yellow rack with 96 Gilson yellow prepacked tips). A magnetic flea (25 mm) was placed inside the box which, when placed on a magnetic stirrer,

agitated the media and allowed aeration. The tip box held 250 ml of media and was incubated at 30 °C. After sealing of the semi-transparent tip box with autoclave tap the whole system could be autoclaved.

Microtitre Plates

Plastic 96 well microtitre plates (Lifesciences International, UK) were used for biofilm cultivation. Volumes of 100 µl were dispensed into each well and the plates incubated at 30 °C for the required time period.

2.4 BIOFILM ANALYSIS

2.4.1 *Standardisation of Inoculum Size*

A liquid culture was prepared by inoculating into a 250 ml flask containing 100 ml YE, and incubating on an orbital shaker (100rpm) at 30 °C for 16 h. Each sample was then serially diluted with sterile PBS, 0.1 ml aliquots plated out onto YE agar, spread and incubated at 30 °C for 16 h. The colony forming units (CFU) per plate were counted and the CFU ml^{-1} of the initial sample calculated.

2.4.2 *Viable Counts*

Viable counts of the beads were estimated by removal of the bead from the flask and gentle washing in three changes of sterile PBS. The bead was then placed in a sterile eppendorf containing 1 ml sterile distilled water and vortexed for 1 min to remove the adhered cells. A viable count was made of the solution of the mechanically removed cells, and the number of CFU cm^{-2} was estimated

Viable counts of the slides were estimated by removal of the slide from the tip box and gentle washing in three changes of PBS. The slide was then placed in a sterile 10 ml beaker containing 1 ml sterile distilled water and mechanically crushed to remove the adhered cells. A viable count was made of the solution of the mechanically removed cells, and the number of CFU cm^{-2} was estimated.

2.4.3 *Total Carbohydrate Determination*

Total carbohydrate determination was carried out following the method of Dubois *et al.* (1956). Biofilm grown on glass coverslips were rinsed and crushed in 2 ml dH_2O. For biofilms grown on glass beads, 5 beads were removed, rinsed and placed in an eppendorf with 0.5 ml dH_2O and vortexed for 60 sec.

Solution 1 5% aqueous phenol

Solution 2 concentrated sulphuric acid

To 200 µl of test solution, 200 µl of solution 1 was added. After mixing, 1 ml of solution 2 was quickly added and the solution re-mixed. The reaction mixtures were left for 15 min and then read in a glass cuvette at 490 nm. A standard curve was prepared from samples containing 0-10 µg glucose.

2.4.4 *Estimation of Metabolic Activity Using INT*

The metabolic activity of biofilm material was measured using 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT). INT is converted to red (nonfluorescent) crystals of INT-formazan within metabolically active bacteria. Biofilm material was grown on glass slides. One glass slide was crushed into an Eppendorf containing 0.5 ml of 2 mg ml^{-1} INT and incubated with shaking for 4 h. The crushed glass and INT mixture was vortexed thoroughly to resuspend the crushed material, and 100 µl placed in a glass cuvette. N,N-dimethylformamide (900 µl) was added to the cuvette to extract the INT-formazan crystals and immediately measured spectrophotometrically at 435 nm using the extraction solution as a blank. This was compared to INT-formazan standards in N,N-dimethylformamide.

2.4.5 *Microscopy*

Glass coverslips were immersed in 10 mM cetyl pyridinium chloride (CPC) for 5 min, then immersed in 25 µg ml^{-1} propidium iodide (PI) (Sigma, UK). Pre-treatment with the detergent caused cell membrane damage and access of PI to the cell interior. GFP was not masked by the presence of PI in the cell, therefore *EntGFP* cells appeared green (or yellow) and other strain appeared red under UV illumination. The

biofilms were observed on a Polyvar microscope with tungsten bulb attachment (Reichert-jung, Austria) using a violet-blue excitation filter (395-446 nm).

2.5 BACTERIOCINS

2.5.1 *Testing for the Presence of Bacteriocin Activity*

A 250 ml flask containing 100 ml of YE medium was inoculated with the strain to be tested and incubated overnight at 30 °C. The culture was then centrifuged at 10 000g for 20 min to separate the cells from the supernatant. The supernatant was filtered (Millipore 0.22 μm pore size) to sterilise and stored at 4 °C. The activity of the spent media was estimated by the following two methods.

1. Lawns of a wide selection of bacteria were spotted with 10 μl of spent media solution. The plates were incubated overnight at 30 °C, and the lawns examined for any effects produced by addition of the spent media.
2. A 250 ml flask containing 90 ml of YE medium was inoculated with bacteria. During mid-logarithmic phase, 10 ml of the filter-sterilised spent media was added to the culture and the O.D. at 600 nm monitored over a period of 24 h. Cultures were incubated at 30 °C. A drop in the O.D. of the culture was indicative of cell lysis, and possible bacteriocin production.

2.5.2 *Assays of Bacteriocin Activity*

Proteinase activity

The solution to be tested (0.3 ml) was added to a test tube, with 1.7ml of distilled water and 1.0 ml azoalbumin solution (5 mg ml^{-1} azoalbumin in 0.1 M Tris buffer [pH7.5]). A blank was prepared containing 2.0 ml distilled water and 1.0 ml azoalbumin solution. The test tubes were incubated for 1h in a water bath at 30 °C. To stop the reaction and precipitate unhydrolysed azoalbumin, 2.0 ml of 8% trichloroacetic acid was added and the mixture centrifuged at 10 000 g to sediment the precipitated protein. 2.0 ml of the clear supernatant fluid was transferred to a clean tube and 2.0 ml of 0.5 M NaOH added to intensify the colour. The absorbency of the solution was then read at 400 nm.

Release of nucleic acid

The action of a bacteriolytic agent on a susceptible cell will release nucleic acid. This was an ideal way of estimating quantities of smaller bacteriocins. Overnight cultures of a susceptible strain were washed in PBS and resuspended in PBS containing 1% glucose. The O.D. of the culture was adjusted to 1.0 at 600 nm. Washed cell suspension (0.9 ml) and 100μ l of the sample to be tested were added to a sterile eppendorf and vortexed. Samples were incubated in a water bath at 30 °C for 1 h, microfuged at 10 000 *g* for 5 min and the absorbency read at 260 nm. The absorbency at 260 nm of a 24 h single species culture of the strain being tested was included as a reference point.

Decrease in culture O.D.

For those strains that produced bacteriostatic agents, the percentage reduction in biofilm size due to bacteriocin activity was estimated. Susceptible strains were grown in microtitre plates for 4 h and 25 μl of the sample to be tested added. After 4 h incubation, the well absorbencies were read at 570 nm. A bacteriocin-free control was included, allowing the percentage reduction in culture O.D. due to bacteriocin activity to be estimated. The activity of a 24 h single species culture of the strain being tested was included as a reference point.

2.6 DISINFECTION

Three commonly used household disinfectants were used to assess the resistance of single and mixed species biofilms: sodium hypochlorite (Fisher Chemicals, UK), benzalkonium chloride (Aldrich, UK) and triclosan (gifted from Ciba Speciality Chemicals PLC, Macclesfield, UK).

2.6.1 *Minimum Inhibitory Concentrations (MIC) of Disinfectants on Liquid Cultures*

Stock solutions of disinfectant were prepared and 200 µl aliquots of the stock solution dispensed into a row of wells in a microtitre plate. 100 µl aliquots of YE medium were dispensed into the adjacent 11 rows. 100 µl of the stock solution was introduced into the wells in the second row, effecting a two-fold dilution. This was repeated until the twelfth row, when 100 µl was removed and discarded, to maintain a constant volume across the wells. An overnight suspension of the bacteria to be tested were standardised by adjusting the $O.D._{570}$ to 0.25, 0.5 µl of which was dispensed into each well. The plates were agitated at 200 rpm for 60 s and the A_{570} read on a Dynatech 5000 plate reader. The plates were incubated for 24 h at 30 °C, agitated for 60 s and the A_{570} read. The MIC concentration was taken as the lowest concentration of disinfectant, which prevented the development of turbidity in the samples.

2.6.2 *Biofilm Eradicating Concentration (BEC)*

The BEC of the disinfectants against single and mixed species biofilms was measured. The appropriate concentration of disinfectant was added to 72 h biofilms in microtitre plates and left at room temperature for 5 min. The wells were rinsed with PBS and replaced with fresh YE medium (100 µl) Any surviving micro-organisms in the biofilm could regrow into the liquid phase. The BEC was taken as the lowest concentration of disinfectant to prevent any regrowth.

2.6.3 *Antibiotic Susceptibility Determination Using MASTRING-S*

Susceptibility of bacteria to a variety of antibiotics was determined by use of MASTRING-S (Mast Lab Ltd, Merseyside, England). The rings contained a range of

antibiotics at concentrations, which are commonly bactericidal. Lawns of each strain were prepared by pouring overnight cultures of each strain onto YE medium agar plates, and the plates drained and allowed to dry. The MASTRING-S was placed onto the plate surface and incubated at 30 °C overnight. On examination, the bacterial lawn had grown except where the antibiotics had diffused out of the MASTRING and produced zones of inhibition. The diameter of these zones was measured and compared to that of other strains. Any increase in EPS production, shown as a thick halo surrounding the zone of inhibition, was also noted.

2.7 ANALYSIS OF EXOPOLYSACCHARIDE (EPS)

2.7.1 *Preparation of Exopolysaccharide*

Bacterial cultures were grown in 2 l flasks containing YE medium with 2% glucose at 30 °C for 96h on a rotary shaker at 150 rpm. The culture was then centrifuged at 10 000g for 20 min to separate the cells from the supernatant. The supernatant fluid, containing the exopolysaccharide (EPS), was poured into two volumes of cold acetone. This precipitated the EPS from the medium. After the EPS had settled to the bottom of the container, the liquid was decanted off and the EPS washed again with acetone. Following re-precipitation and removal of the acetone, a small volume of distilled water was added to the EPS, which either re-dissolved or became suspended. Extensive dialysis against distilled water was performed to remove acetone, glucose, salts and other low molecular weight molecules. The EPS was de-ionised by passage through Amberlite Mixed Bed Resin (MB1), then lyophilised.

2.7.2 *Preparation of Biofilm EPS*

Biofilms material was grown on glass beads (150 g) in 2 l flasks containing YE media with 1% glucose at 30 °C for the appropriate incubation time required. The glass beads were removed, washed in sterile PBS to remove any unadhered cells. The glass beads were agitated vigorously to remove biofilm material and the liquid centrifuged at 10 000 g. This was then dialysed for 48 h before lyophilising.

2.7.3 *Identification of Monosaccharides in the Polymers Using Acid Hydrolysis.*

Polysaccharide (20mg) was dissolved or suspended in 300ml of 0.25M sulphuric acid, sealed in a hard glass tube and placed in an oven at 100°C for 16h. The hydrolysate was neutralised using amberlite IRA-410 (HCO_3^- form) and Amberlite MB1 mixed bed resin. The solution and washings were evaporated to dryness then redissolved in 400 µl ion free water. The hydrolysate was centrifuged to remove particulate material, passed through an HPLC filter and stored at –20 °C.

2.7.4 *EPS Analysis*

Paper Chromatography

The hydrolysate was analysed by descending paper chromatography using butan-1-ol:pyridine:water (6:4:3) as the solvent. The hydrolysate (25 µl) was applied to a sheet of Whatman No.1 chromatography paper along with 1 µl of each 0.1 M standard sugar solution. The chromatograms were run for 24 h then developed using the following reagents (Trevelyn *et al.*, 1950).

Reagent 1

Acetone	200 ml
Std. $AgNO_3$	1.0 ml
DH_2O	0.5 ml

Reagent 2

NaOH	10 g
H_2O	7.5 ml
C_2H_5OH	500 ml

Reagent 3

$Na_2S_2O_35H_2O$	39.2 g
DH_2O	500 ml

The chromatograms were sequentially run through the reagents in a trough. Once dry, a permanent record of the chromatograms was made by photocopying. The

image was also reduced in size and intensified using this method. The distance of the centre of the glucose standard spot from the origin was measured. The other standards' movement could then be measured and compared to glucose.

$$R_{glc} = \frac{\text{distance of spot}}{\text{distance of glucose}}$$

Thus, in the experimental samples, a value close to 1 was likely to be glucose.

High Pressure Liquid Chromatography (HPLC) Analysis

A Dionex HPLC was used consisiting of a CarbPak PA1 anion-exchange column (4 mm diameter, 250 mm length) with a pulsed amperometric detector. A method to separate monosaccharides and uronic acids was used. Between 0 and 1.8 min, the eluent was a linear gradient of 20 mm NaOH, from 1.8 to 30 min, an isocratic gradient of 100% H_2O, between 30 and 52 min, a linear gradient of 0.8 mM, between 52 and 57 min, an isocratic gradient of 0.8 mM NaOH, and from 57 to 60 min, an isocratic gradient of 20 mM NaOH. The flow rate was maintained at 1.0 ml min^{-1} at room tempreture, and 20 µl samples were injected through a 100 µl loop.

Uronic Acid Determination (Blumenkrantz and Adsoe-Hansen, 1973)

Solution 1 12.5 mM sodium tetraborate in concentrated sulphuric acid

Solution 2 0.15 % m-hydroxyphenyl in 0.5 % sodium hydroxide

Glass tubes of similar diameters, containing 200 µl of test sample, were cooled in an ice water bath and 1.2ml of solution 1 added and mixed. The assay mixture was boiled for 5 min and cooled in a cold water bath. Solution 2 (20 µl) was added, mixed and read at 520 nm. Glucuronic acid solutions were used to prepare a standard curve containing 0.5-20 µg 200 μl^{-1}.

2.8 ANALYSIS OF OUTER MEMBRANE PROTEINS

2.8.1 *Isolation of Outer Membrane Using Sodium N-Lauroyl Sarcosinate (Sarkosyl)* (Filip *et al.*, 1973)

Bacteria (100 ml) were harvested by centrifugation, washed in distilled water and resuspended in 10 ml of distilled water. The cell suspension was passed through a French Press at 103 500 kPa (15 000 P.S.I.) to disrupt the cells. Any remaining intact cells were removed by centrifugation at 5 000 *g* for 10 min. Sodium N-lauroyl sarcosinate was added to a final concentration of 0.2 g ml^{-1} and the mixture incubated at room temperature for 30 min. A visible clearing of the envelope suspension indicated solubilisation of the cytoplasmic membrane. The outer membrane-peptidoglycan complex was recovered by centrifugation at 40 000 *g* for 60 min, washed twice in distilled water containing 1mg ml^{-1} PMSF, resuspended in 0.5 ml distilled water and stored frozen at –20°C.

2.8.2 *Polyacrylamide Gel Electrophoresis*

Polyacrylamide gel electrophoresis (PAGE) was carried out following the method of Laemmli (1970). A 1 mm resolving gel of 10 % acrylamide was allowed to polymerise and a 4.5 % stacking gel was poured on top. A comb was placed in the stacking gel. 10 % SDS was added to a final concentration of 0.1 % to make a denaturing gel. The following running buffer was used:

Running buffer

	gL^{-1}
TRIS base	3.0
Glycine	14.4
SDS	1.0

Protein samples (30 µg) were prepared for electrophoresis by mixing in a 1:1 ratio with X2 sample buffer and heated at 100 µC for 3 min and microfuged for 3min to pellet any insoluble material. The gel was pre-electrophoresed at 100 mV constant voltage (10-20 mA) for 1-2 h. The samples were loaded into the wells and a mixture of molecular markers was included for reference. The gel was run at 10 mA (constant

current) for 1 h to allow the proteins to stack in gel, followed by 20 mA until the blue dye front was within 1 cm of the bottom of the gel. After electrophoresis was complete, the gel was stained with PAGE Blue, photographed and dried down on 3 MM chromatography paper.

2.9 BACTERIOPHAGE

2.9.1 *Isolation, Purification and Concentration*

Primary effluent sewage samples were tested to see if they contained a phage capable of infecting the bacteria. Each sewage sample was centrifuged at 10 000 g for 20 min to remove particulate material. It was then passed through a 0.45 μm pore size filter (Millipore), twice, to remove bacteria. Preliminary checks for the presence of phage were carried out by spotting the filtrate onto bacterial lawns. To 50 ml of prewarmed double strength Y.E. medium, an equal volume of filtered sewage and 20 ml of overnight bacterial culture was added. The optical density (600 nm) of the culture was monitored over a 12 h period. A fall in O.D. due to cell lysis indicated the presence of an infectious phage. The culture was centrifuged and the supernatant (containing the phage particles, phage components and the contents of the lysed bacterial cells) was collected. When dialysed, concentrated and filtered (Millipore, 0.45 μm pore size) the supernatant acted as an impure source of bacteriophage and bacteriophage proteins (including any polysaccharide depolymerases). This process was repeated with sewage from each location and *E. coli*, Ent, S53, 5920 and S61.

To purify the bacteriophage, the supernatant was serially diluted in phage buffer and 0.1 ml added to 3 ml sloppy agar to which 0.1 ml of overnight culture had been added. The sloppy agar mixture was poured onto a YE agar plate and incubated overnight at 30 °C. Individual plaques were picked off and inoculated into exponentially growing flasks of bacteria, and again incubated overnight at 30 °C. The above process of centrifugation and filtration was then repeated.

The supernatants were concentrated against polyethylene glycol (PEG 6000) for 16 h, filtered (Millipore, 0.45 μm pore size) and stored at 4 °C.

Phage buffer

	$g\ L^{-1}$
TRIS	6.06
NaCl	5.84
$Mg\ SO_4.7H_2O$	2.46

The ingredients listed above were dissolved in 1 l of distilled water and autoclaved.

2.9.2 *Screening of Bacteriophage Activity on Bacteria*

The phage were screened for lytic and polysaccharide depolymerase enzyme activities on *E. coli*, Ent, 1.15, S53 and 5920. A lawn of each bacterial strain was spotted with 10 µl of each concentrated phage solution, and 10^{-1} and 10^{-2} dilutions. The plates were incubated overnight at 30 °C, and the lawns examined for formation of plaques and haloes of decapsulated bacteria, which is indicative of the presence of phage which possess a polysaccharide degrading enzyme.

2.9.3 *Burst Time of Phage*

A 250ml flask containing 100ml of Y.E. medium was inoculated with bacteria and incubated at 30 °C. During mid-logarithmic phase the culture was infected with bacteriophage and the O.D. at 600nm monitored over an 8h period. A sudden drop in O.D. indicated bacterial cell lysis and the release of the phage particles.

2.9.4 *Approximate Estimation of Phage Burst Size (Degree of Multiplicity)*

A liquid culture of 25ml in logarithmic phase was inoculated with phage at a ratio of 1 phage for every 10 cells to ensure that each cell was infected by only a single phage, since multiple infection might give an incorrect result. Optical density measurements at 600nm and phage counts were taken until a drop in O.D. occurred indicating the onset of cell lysis. At this time the culture was diluted 1000-fold to reduce the possibility of the newly infected phage encountering and infecting any remaining bacterial cells. Phage numbers were determined by serially diluting the phage in phage buffer and plating out in 0.1ml aliquots in 3ml of sloppy agar to

which 0.1ml of susceptible bacteria had been added. Subsequent calculations, involving the number of phage added to the bacterial culture and the number of phage present after the burst, gave information on the burst size.

2.9.5 *Separation of Phage Enzyme from Phage Particles*

The phages were produced in high titre by replication in susceptible host. The phage were separated from most bacteria cell debris by centrifugation at 21 000g (8 x 50 ml tubes) for 21 min. The supernatant was dialysed against phage buffer at 4 °C for 24 h to remove low molecular weight material including glucose, then concentrated against PEG for 16 h. The concentrated supernatant was re-centrifuged at 21 000g for 20 min, then ultracentrifuged at 100 000g for 3 h (Sorvall OTD65B ultracentrifuge and Sorvall A841 rotor, 8 x 36 ml tubes) to separate soluble phage enzyme from phage particles. The supernatant was filtered (Millipore, 0.22 μm pore size) to ensure no phage remained. Drops of filtered supernatant (100 μl) were dispensed onto freshly spread lawns of bacteria. After incubation at 30 °C for 16 h, the phage enzyme solution was tested for the presence of phage. The phage solution was spotted onto lawns of bacteria. The production of no clear plaques was used as confirmation that the supernatant was phage free. However, extensive degradation of the bacterial EPS can be observed. The phage free supernatant containing the soluble enzyme was concentrated against PEG then frozen.

2.9.6 *Bacteriophage Polysaccharide Depolymerase Enzyme Assays*

The enzyme activities of purified phage enzyme extract with EPS as a substrate were assayed. Phage enzyme extract (100 μl) was added to EPS (2 ml of 10 mg ml^{-1} solution), and incubated for 4 h at 37 °C. Azide (0.02%) was added to prevent bacterial growth. Enzyme activity was measured using a reducing sugar assay, with no-substrate and no-enzyme controls. The protein content of the purified phage enzyme and EPS was estimated using the Biorad Microassay Procedure (Bradford, 1976).

Reducing Sugar Determination (Park & Johnson, 1949)

Solution 1 0.05% potassium ferricyanide

Solution 2 0.53% sodium carbonate
0.065% potassium cyanide

Solution 3 0.15% ferric aluminium sulphate
0.1g 100 ml^{-1} Triton x100 in 0.05 N H_2SO_4

To 200 µl of sample, 200 µl of solution 2 was added, followed by 200 µl of solution 1. The assay mixture was boiled for 15 min in tubes of the same diameter. The reaction mixture was cooled in ice water and 1 ml of solution 3 added and allowed to stand at room temperature for 15 min. The samples were read at 690 nm against a water blank. A standard curve, 0-10 µg 200 μl^{-1}, was prepared using 1 mg ml^{-1} glucose.

Protein Determination (Bradford, 1976)

The Biorad Microassay Procedure (Biorad Laboratories GmbH, Munchen) was used to determine protein concentration. The samples were read at 595 nm. Standard curve solutions were prepared containing 0-12 µg glucose.

Chapter 3

BACTERIOCIN PRODUCTION AND COMPETITION IN BIOFILMS

3.1 INTRODUCTION

Previously, biofilm research has focused on single species or undefined mixed biofilms. Although single species biofilms can be found in a few specialised niches, for example, on contaminated catheters, biofilms in natural and industrial environments more commonly consist of complex communities of microorganisms. Diversity in microbial communities leads to a variety of complex relationships involving interspecies and intraspecies interactions. A wide range of potential interactions among populations in microbial communities can occur, and can have a profound influence on the initial stages of biofilm formation and development (James *et al.*, 1995). These interactions can be beneficial to the participating populations, and this includes increased metabolic efficiency and substrate accessibility. The breakdown of many complex organic molecules involves the co-operative action of several groups of microorganisms with complementary enzyme profiles. A broader habitat range for growth may also be achieved. For example, the mouth is an aerobic environment, yet obligate anaerobes survive in high numbers. The anaerobic strains cope with this stress by interacting with oxygen-consuming species. It has been found that the facultative anaerobe *F. nucleatum* can act as a bridging organism between weakly co-aggregating strict aerobes and anaerobes (Bradshaw *et al.*, 1998). Mixed species biofilm can often be thicker and more stable than monospecies biofilms, and this may further influence their resistance to antimicrobial agents (Skillman *et al.*, 1999b).

Alternatively, interactions can have an adverse effect. For example, the production of antagonistic compounds such as bacteriocins may give an organism a selective advantage when interacting with other microbes. In a study of colonisation of teeth by oral streptococci, the degree of colonisation was proportional to their level of bacteriocin activity (Hillman *et al.*, 1987).

The aim of this work was to compare interactions between bacteria, and to correlate them with increased or decreased biofilm formation. A two-species system was used, as this is simple enough to allow quantitative analysis of interactions and *in situ* speciation (Camper *et al.*, 1996; Stewart *et al.*, 1997; Banks and Bryer, 1991; Siebel and Characklis, 1991).

A further aim was to determine if different species could exert influences on each other, further altering biofilm resistance towards biocides. Biocides lack selective toxicity against different classes of microorganisms and exert their effects at multiple sites in the cell. These agents have many advantages over antibiotics, which are typically far more specific in action, making biocides very useful in industry and food processing. A selection of disinfectants were chosen for this study: benzalkonium chloride, sodium hypochlorite and triclosan. From previous studies (Hughes, 1997), it was noted that a range of *Enterobacteriaceae* were particularly sensitive to benzalkonium chloride. This agent is also widely used as a general disinfectant and preservative. Sodium hypochlorite was similarly chosen because of its wide use as a disinfecting agent. Triclosan has received particular attention lately, and is now widely used in food packaging, antiseptic soap, toothpastes and other materials. There is considerable interest in the site of action of action of triclosan, giving rise to concern that its wide-spread use may select for multi-drug resistant bacteria.

The strains selected for use are all Enteric bacteria. Many of these were isolated from biofilm material. For example, *Ent. agglomerans/Ent* and *Ent. agglomerans*/53b were isolated from industrial surfaces, and *Ent. gergoviae*/1.15 was isolated locally from a biofilm developed on a glass slide in a stream. Other strains were selected for their copious polysaccharide production. For example, the *E. coli* strain S53, and the *Enterobacter* strains 1.15 and 5920 produce a negatively charged polysaccharide of the colanic acid type. *Ent*, unusually, produces a neutral, poorly soluble polysaccharide.

3.2 INTERACTIONS IN DUAL SPECIES BIOFILMS

3.2.1 *Initial Discovery of Competitive Interactions*

The adhesion of the strains *Enterobacter agglomerans/Ent* and *Enterobacter gergoviae*/1.15 to glass beads was used to determine the interactions between the strains (**figure 3.1**). *Ent* and 1.15 were first grown as single species biofilms, and then as dual species biofilms. Examination of fig 3.1 shows that growth as a dual species biofilm was detrimental to both strains. The amount of cells colonising the glass beads in the *Ent* single sp. biofilm was 9.89 x 10^5 CFU cm^{-2} (± 4.4 x 10^4) after 72 h growth, and 1.11 x 10^6 CFU cm^{-2} (± 4.7 x 10^4) for the 1.15 single species biofilm. This was reduced to a total cell count of only 4.8 x 10^5 CFU cm^{-2} (± 3.6 x 10^4) in the dual species biofilm.

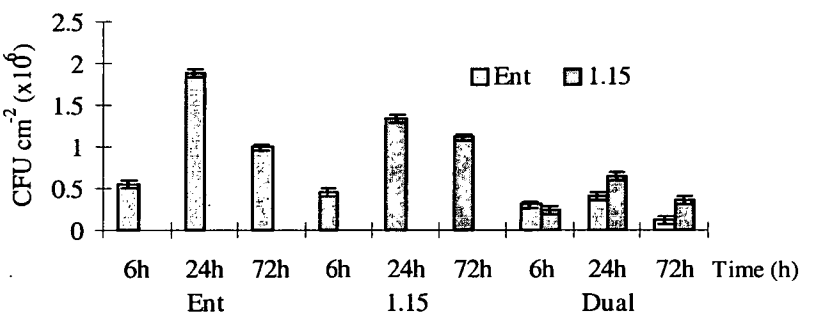


Figure 3.1

The colonisation of glass beads by the strains *Enterobacter agglomerans/Ent and Enterobacter gergoviae/* 1.15 as single and dual species biofilms was investigated to determine the interactions between the strains. Strains in dual species biofilms were inoculated in a 1:1 ratio. Viable counts of biofilm material were estimated at 6, 24 and 72 h. Growth was reduced in the dual species biofilms. Results are the outcome of 4 replicate experiments and bars represent standard error.

3.2.2 *Investigation of Possible Bacteriocin Activity*

The reduced adhesion in the *Ent*/1.15 dual species biofilm suggested antagonism was occurring between the strains, and possible bacteriocin activity. To test this theory, the presence of bacteriocin production was investigated first by spotting filter-

sterilised spent media onto lawns of bacteria. For example, spent media taken from a planktonic culture of *Ent* was filter-sterilised and spotted onto a lawn of 1.15. These results were then confirmed by the addition of *Ent* filter-sterilised spent media (10% v/v) to growing 1.15 planktonic cultures. The OD_{600} of the cultures were monitored and compared to a control containing no additional spent media. Any drop in OD of the culture containing spent media was indicative of bacteriocin activity. These experiments were repeated for the strains *Escherichia coli*/*E. coli*, *Enterobacter agglomerans*/*Ent*, *Ent. gergoviae*/1.15, *Ent. cloace*/5920 and *E. coli*/S53, and filter-sterilised spent media obtained from *E. coli*, *Ent*, 1.15, 5920 and S53 **(table 3.1)**. This showed that all the strains tested could have an antagonistic effect on a number of the strains in use. From these results, it was decided to select three systems for further study: a non-competitive biofilm (*E. coli*/*Ent*), and two competitive systems, *Ent*/1.15 and 5920/1.15. Whereas *Ent* and 1.15 each produce a bacteriocin directed against its competitive partner, the agents produced by 5920 are ineffective against 1.15. It was thought this type of interaction may produce interesting results, and so the pair of 5920/1.15 was selected for further study.

The colonisation of glass beads by the pairs *E. coli*/*Ent* and 1.15/5920 as single and dual species biofilms were also investigated **(figure 3.2)**. Again, a decrease in the number of cells adhering to the glass beads in the dual species biofilm was observed. In the *E. coli*/*Ent* dual species biofilm, cell numbers after 72 h growth only reached 5.65×10^5 CFU cm^{-2} ($\pm 8.21 \times 10^4$) for *E. coli*, and 5.98×10^5 CFU cm^{-2} ($\pm 8.3 \times 10^4$) for *Ent*. Single species controls at 72 h were 2.01×10^6 CFU cm^{-2} (9.9×10^4) for *E. coli*, and 1.13×10^6 CFU cm^{-2} ($\pm 8.3 \times 10^4$) for *Ent*. Similarly in the 1.15/5920 biofilms, CFU cm^{-2} dropped from 2.21×10^6 ($\pm 4.5 \times 10^4$) in the 5920 single species biofilm to 6.61×10^5 ($\pm 3.7 \times 10^4$) in the dual species biofilm. For 1.15, CFU cm^{-2} was 8.79×10^5 ($\pm 4.5 \times 10^4$) in the single species biofilm and 6.62×10^5 ($\pm 3.5 \times 10^4$) in the dual species biofilms.

	SPENT MEDIA				
STRAIN	*E. coli*	*Ent*	1.15	S53	5920
E. coli			+	+	+
Ent			+	+	+
1.15	+	+		+	
S53	+	+	+		
5920	+	+	+	+	

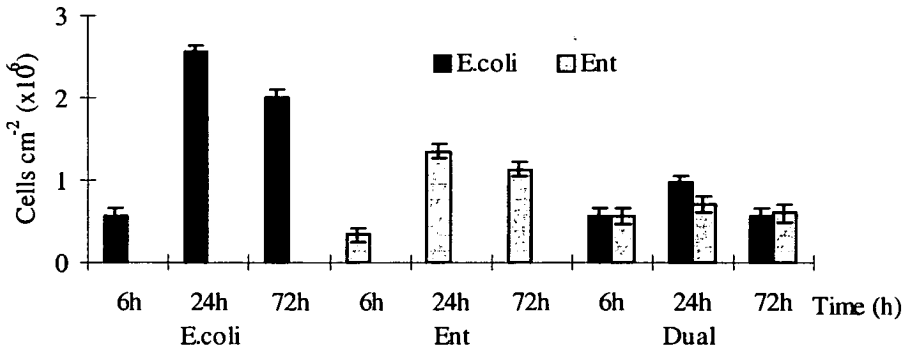
Table 3.1

Overnight cultures of *E. coli*, *Ent*, 1.15, S53 and 5920 were centrifuged and the supernatant filtered (Millipore 0.45 μm pore size) to sterilise. The activity of the spent media was estimated by the following two methods:-

1. **A lawn of each bacterial strain was spotted with 10 μl of each spent medium solution. The plates were incubated overnight at 30 °C, and the lawns examined for any effects produced by addition of the spent media.**
2. **A 250 ml flask containing 90 ml of Y.E. medium was inoculated with either *E. coli*, *Ent*, 1.15, S53 or 5920. During mid-logarithmic phase, 10 ml of the filter-sterilised spent media was added to the culture and the OD at 600 nm monitored over a period of 24 h.**

Any clear zones on the lawns produced by addition of spent media or reduction in growth rates are marked as + in the table.

3.2a) *E. coli* and *Ent* single and dual species colonisation of glass beads.



3.2b) 1.15 and 5920 single and dual species colonisation of glass beads.

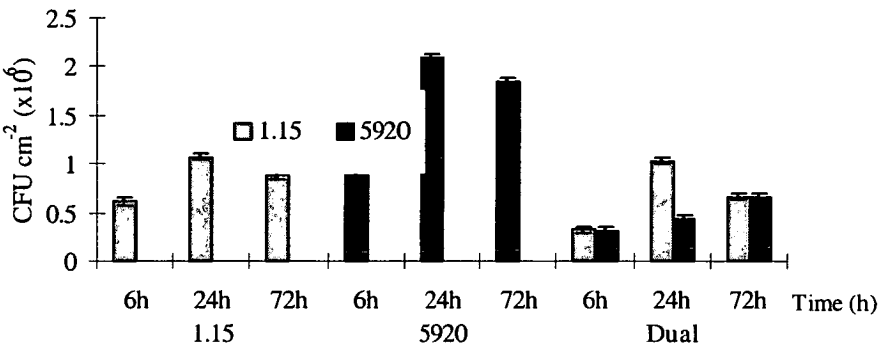


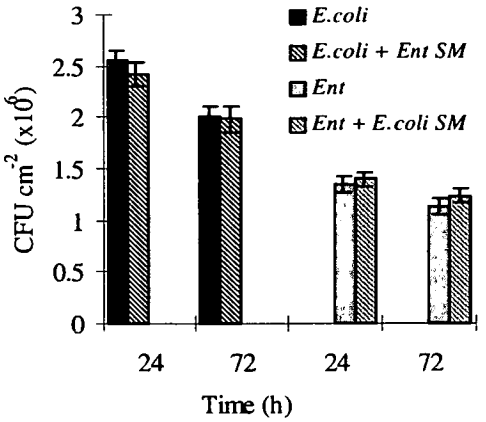
Figure 3.2

The colonisation of glass beads by dual species biofilms were investigated to determine the interactions between the strains. From investigations of the effects of spent media on the strains, it was thought that *E. coli* and *Ent* (fig. 3.2a) produced a non-competitive biofilm and the pairs *Ent*/1.15 (fig. 3.1) and 1.15/5920 (fig. 3.2b) produced competitive biofilms. Dual species biofilms are compared to single species biofilms. Strains in dual species biofilms were inoculated in a 1:1 ratio. Viable counts of biofilm material were estimated at 6, 24 and 72 h. Growth of the strains was reduced in the dual species biofilms. Results are the outcome of 4 replicate experiments.

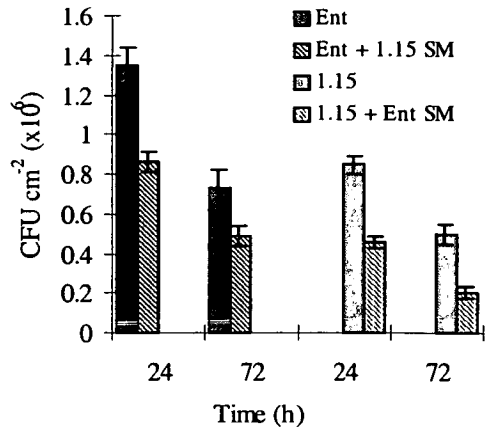
3.2.3 *The Effect of Bacteriocin-Production on Biofilms*

The effect of bacteriocin activity on biofilm bacteria was further investigated by addition of filter-sterilised spent media (10% v/v) to single species biofilms (**figure 3.3**). As expected, *E. coli* and *Ent* spent media had no effect on the growth of their respective biofilms (fig 3.3a). However, addition of 1.15 spent media (SM) to *Ent*, and *Ent* SM to 1.15 reduced the viable counts considerably (fig 3.3b). It was also noted that in the competitive *Ent*/1.15 biofilms, the addition of 1.15 SM to *Ent* biofilms produced a greater effect than addition of *Ent* SM to 1.15. At 24h, the percentage reduction in CFU cm^{-2} due to the addition of *Ent* SM to 1.15 single species biofilms was 36.52% (± 2.2%), and a 46.45% (± 3.2%) reduction was produced due to the addition of 1.15 SM to *Ent* single species biofilms. A similar decrease was seen due to the addition of 1.15 SM to 5920 single species biofilms (fig 3.3c).

3.3a) *E. coli* and *Ent*



3.3b) *Ent* and 1.15



3.3c) 1.15 and 5920

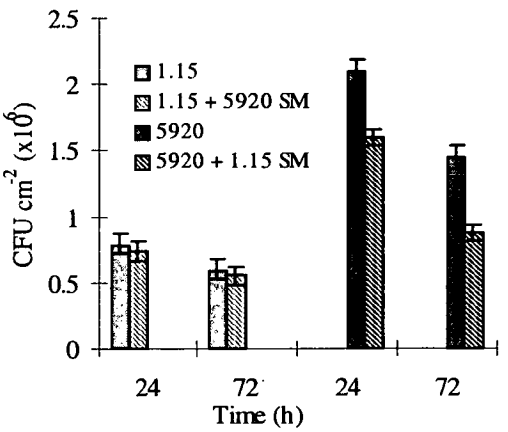
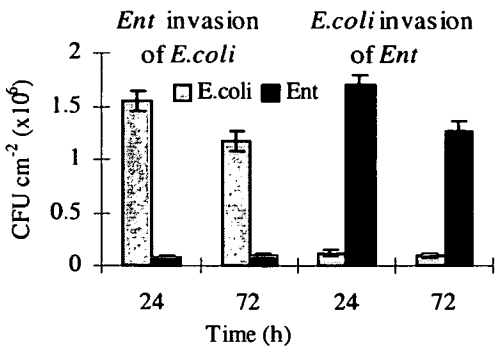


Figure 3.3

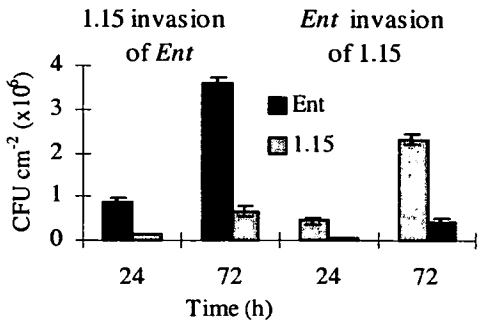
To investigate the effect of bacteriocins on biofilm cultures, single species biofilms growing on glass beads were allowed to reach stationary phase, and filter-sterilised spent medium (10% v/v) was added. Cultures were incubated with shaking at 30 °C, and viable counts were estimated at 24 and 72 h. A reduction in the number of cells that adhered to the glass beads was seen in those strains which were susceptible to the bacteriocins. Results are the outcome of 3 replicate experiments, and bars represent standard error.

Again to demonstrate the competition between the strains and the effect of bacteriocin activity, single species biofilms were grown to stationary phase (8h), inoculated with a second bacterial strain and the integration of the second strain into the biofilm monitored (**figure 3.4**). The production of a bacteriocin active against the pre-established strain was seen to enhance the invasive properties of the secondary colonising strain. For example, *E. coli* and *Ent*, the strains forming a non-competitive biofilm, were not able to colonise pre-established biofilms of *Ent* and *E. coli*, respectively (fig 3.4a). 5920 was also unsuccessful in its attempts to invade a biofilm of 1.15 (fig 3.4c). The bacteriocin-sensitive strains *Ent*, 1.15 and 5920 were, however, successfully invaded by the strains 1.15, *Ent* and 1.15, respectively. It was also noted that 1.15 was more successful in invading a biofilm of *Ent* than *vice versa* (fig 3.4b). Also of interest was the large increase in the number of cells that adhered to the glass beads due to the integration of a bacteriocin-producing strain into a bacteriocin-sensitive biofilm, for example, the invasion of 1.15 into *Ent* biofilms.

3.4a)



3.4b)



3.4c)

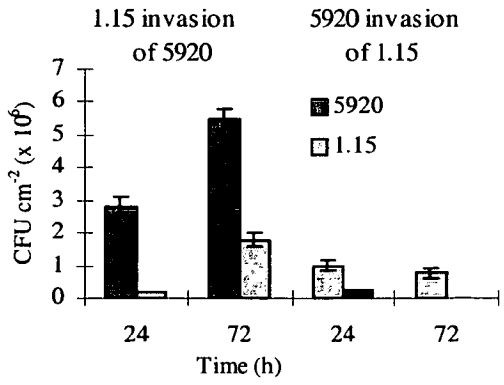


Figure 3.4

To investigate the effectiveness of bacteriocins in a biofilm situation, pre-established single species biofilms were inoculated with a second, competing strain. The inoculum was adjusted to allow a 20:1 ratio of planktonic cells, with the pre-established strain dominating in each case. Biofilms were grown on glass beads, incubated with shaking at 30 °C, and viable counts were estimated at 24 and 72 h. Strains susceptible to bacteriocin were 'invaded' by the bacteriocin-producing strains. Those which were not susceptible did not allow secondary colonisation by the second, invading strain. Results are the outcome of 3 replicate experiments, and bars represent standard error.

3.3 INVESTIGATION OF BACTERIOCINS

3.3.1 *Estimation of Bacteriocin Size*

There are two major groups of bacteriocins produced by the Enteric bacteria: colicins and microcins. Due to the very small molecular mass of the microcins (<20 kDa), these can be easily distinguished. Spent media from the strains *Ent*, 1.15 and 5920 were passed through a 1000 Mr cut-off filter (Millipore, UK), and the activity of the filtrates estimated. To assess the activity of the *Ent* and 5920 'spent media' fractions, cultures of 1.15 were grown in microtitre plates, the filtrates added and the percentage reduction in OD_{570} due to the action of bacteriocin assessed. Similarly 1.15 'spent media' was used to estimate 1.15 bacteriocin activity.

The filtered spent media from all three strains contained bactericidal activity. Cultures of 1.15 were reduced by 53.2% (± 3.1%) by the action of the *Ent* filtrate, and by 44.95% (± 4.7%) due to the addition of the 5920 filtrate. The *Ent* filtrate reduced the OD_{570} of 1.15 by 47.65% (± 5.2%). However, further investigation of the spent media produced by 1.15 indicated the presence of another fraction with bactericidal activity. This fraction was estimated to be between 50 and 100 kDa.

To compare the effects of the bacteriocins on biofilm and planktonic cultures, 1.15, *Ent* and 5920 spent media were split into fractions (<10 KDa and >10<100 KDa) by passing the spent media through Millipore Vivacell 70 membrane filters. These were then filter-sterilised by passage through 0.22 nm cut-off filters (Millipore, UK). These fractions, along with standard spent media, were added to 24 h planktonic cultures and glass-bead biofilm cultures (10% v/v), and incubated for a further 4 h. Viable counts were estimated and the percentage reduction in CFU cm^{-2} or CFU ml^{-1} estimated (**table 3.2**). This table shows that the smaller fractions, >10 KDa, reduced the CFU of biofilm and planktonic cultures by approximately the same amounts. When comparing the effect of the >10<100 KDa 1.15 fraction on biofilm cells than on planktonic cells, it can be seen that this fraction reduced biofilm size by 62.4%, but reduced the number of planktonic cells by only 17. 8%.

1.15 spent media fractions		Control	SM	>10K<100K	<10K
Ent	Biofilm CFU cm^{-2}	5.03×10^6	3.07×10^6	1.89×10^6	2.54×10^6
	% reduction in CFU		38.97	62.43	49.5
	Planktonic CFU ml^{-1}	2.25×10^9	1.85×10^9	2.35×10^9	1.23×10^9
	% reduction in CFU		17.78	22.22	45.33
5920	Biofilm CFU cm^{-2}	2.32×10^6	1.89×10^6	1.65×10^6	1.27×10^6
	% reduction in CFU		18.53	0	45.26
	Planktonic CFU ml^{-1}	4.85×10^9	4.05×10^9	4.86×10^9	2.7×10^9
	% reduction in CFU		16.49	0	44.32
Ent spent media fractions		**Control**	**SM**	**>10K<100K**	**<10K**
1.15	Biofilm CFU cm^{-2}	1.68×10^6	1.33×10^6	1.71×10^6	9.98×10^5
	% reduction in CFU		20.83	0	40.59
	Planktonic CFU ml^{-1}	2.65×10^9	2.01×10^9	2.72×10^9	1.73×10^9
	% reduction in CFU		24.15	0	34.72
5920 spent media fractions		**Control**	**SM**	**>10K<100K**	**<10K**
1.15	Biofilm CFU cm^{-2}	1.74×10^6	1.29×10^6	1.79×10^6	9.87×10^5
	% reduction in CFU		25.86	0	43.28
	Planktonic CFU ml^{-1}	2.72×10^9	2.06×10^9	2.64×10^9	1.68×10^9
	% reduction in CFU		24.26	0	38.23

Table 3.2

1.15, *Ent* and 5920 spent media were split into the fractions >10K<100K and <10K by passing through Millipore Vivicell 70 membrane filters, and then filter-sterilised. 10% (v/v) was added to 24 h planktonic and biofilm cultures and incubated for a further 4 h. Viable counts were made and the percentage reduction of cell numbers calculated. Standard error for the biofilm CFU cm^{-2} was $\pm 2.03 \times 10^5$, and for planktonic CFU ml^{-1} was $\pm 2.59 \times 10^8$. Results are the outcome of 3 replicate experiments.

3.3.2 *Microcin Activities of Ent, 1.15 and 5920*

Due to the small size (>1000 Mr) of the bacteriocins produced by *Ent*, 1.15 and 5920, it was likely that these were microcins. Microcins, unlike colicins are not amplified by agents which elicit the SOS response (Walker, 1984). Therefore, the affect of exposure to UV radiation on bacteriocin production by the strains *Ent*, 1.15 and 5920 was investigated. Planktonic cultures of the three strains were centrifuged,

washed in PBS and placed in a UV cabinet for 5 min. Cells were left to recover for 30 min and bacteriocin activities assayed as usual. No increases in bacteriocin production were measured, indicating that the smaller agents produced by the strains were likely to be microcins.

The lytic activity of the agents was investigated. Cells of *Ent*, 5920 and 1.15 were centrifuged, washed in PBS and resuspended in PBS containing 1 % glucose. Spent media (1% v/v) was added to the cell suspensions and the cultures incubated for 1 h. Release of nucleic acid, due to cell lysis, can be read at an absorbency of 260 nm. The larger bacteriocin and the microcins of 1.15 lysed cells of *Ent* and 5920 whereas the addition of the microcins of to the cells of 5920 and *Ent* did not cause an increase in absorbency at 260 nm, having a bacteriostatic effect.

3.3.3 *Bacteriocin Activity Curves*

Bacteriocins are generally produced during stationary growth phase. Profiles of the activities of the microcins produced by *Ent* and 5920, and the larger bacteriocin produced by 1.15 have shown that production is induced upon entry into stationary growth phase (**figure 3.5**). While investigating the induction of 1.15 microcin production, it was discovered that 1.15 produced two microcins, one at 4 h, and another at 8 h. **Figure 3.6** shows 1.15 microcin production. This was measured by the % reduction in the OD_{570} of microtitre plate cultures of 5920 and of *Enterobacter agglomerans*/53b, and by the lysis of 5920 and 53b, measured at 260 nm. 53b was used instead of *Ent* as the larger bacteriocin produced by 1.15 has no activity against 53b, allowing estimation of microcin activity alone. Only the microcin produced at 8 h (1.15 M-8) has activity against *Ent*, whereas the microcin produced at 4 h (1.15 M-4) has activity against 5920.

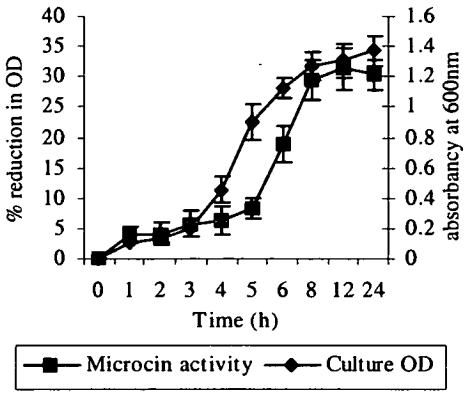


Figure 3.5

Typical activity profile of *Ent* microcin production. Microcin production was measured by the % reduction of a microtitre plate culture of 1.15, and the OD_{600} of a culture of *Ent* is also shown. Microcin production is induced upon entry into stationary phase. Cultures were incubated at 30 °C with shaking. Results are the outcome of 3 experiments, and bars represent standard error.

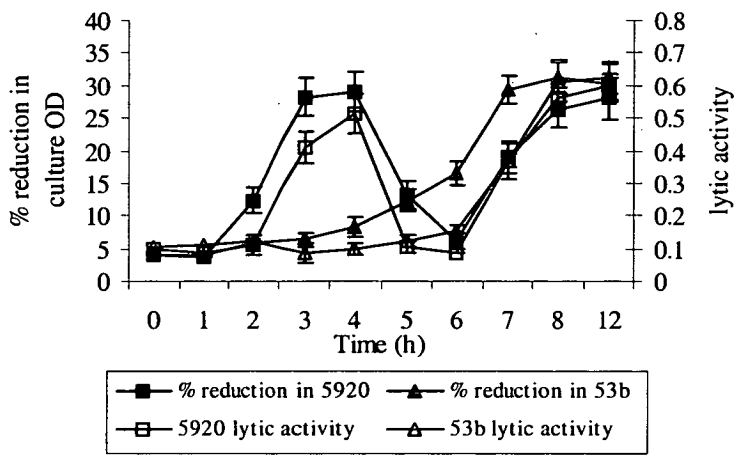


Figure 3.6

1.15 microcin production was measured by the % reduction in the OD_{570} of microtitre plate cultures of 5920 and of *Enterobacter agglomerans*/53b, and by the lysis of 5920 and 53b, measured at 260 nm. This suggested 1.15 produced 2 microcins, one at 4 h, and another at 8 h. Results are the outcome of 4 replicative experiments, and bars represent standard error.

3.3.4 *Protease Activity of the Larger Bacteriocin of 1.15*

Examination of 1.15 Protease

The larger bacteriocin of 1.15 had a greater effect on biofilm than planktonic cells and was, therefore, tested for reducing sugar activity, to test for the presence of a polysaccharide depolymerase, and protease activity. While no reducing sugar activity was found, there was protease activity: 1 ml of a 24 h culture of 1.15 hydrolysed 52 mg of protein in 1 h. Proteases of bacteria can be divided into four groups: the acid proteases, PMSF-sensitive-alkali proteases, metal-chelator sensitive neutral proteases and thiol proteases. PMSF is phenyl methyl sulfonyl flouride (Sigma, UK), an inhibitor of alkaline proteases. The pH optimum of 1.15 protease was elucidated by incubation at various pH. **Figure 3.7** indicated that the protease of 1.15 was active from pH 5 to pH11, and, thus, probably a PMSF-alkali-sensitive protease. To test this further, 1.15 protease was incubated with PMSF (25 μg ml^{-1}) for 1h before protease activity was assayed. Incubation with PMSF inactivated the enzyme. Addition of EDTA at concentrations of 10 μg ml^{-1} had no effect on protease activity. From this, it was concluded that 1.15 protease is a PMSF-sensitive-alkali protease. This group is best represented by the subtilisins, serine proteases similar to trypsin.

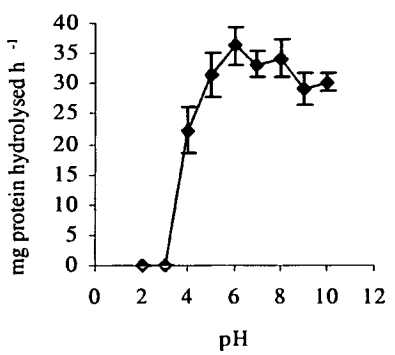


Figure 3.7

The pH optimum of 1.15 protease was determined. 1.15 protease is active from pH 5 to pH 11.

Comparison of Effect of Proteases on Biofilm and Planktonic Cells

It was previously noted that the larger bacteriocin of 1.15 had a greater effect on biofilm than on planktonic cells (**table 3.2**). The effect of 1.15 protease and a selection of commercially available proteases (subtilopeptidase A, *Rhizopus* sp. type XVIII and thermolysin type X, Sigma) on *Ent* biofilm and planktonic cells was, therefore, compared (**table 3.3**). Enzyme activities were adjusted to a constant activity of 50 μg ml^{-1}. Table 3.3 demonstrates that Subtilopeptidase A also had a greater effect on biofilm than on biofilm cells. *Rhizopus* sp. type XVIII had similar effects on biofilm and planktonic cells, and thermolysin type X had a greater effect on planktonic cells.

	PLANKTONIC		BIOFILM	
	CFU ml^{-1}	% decrease in CFU	CFU cm^{-2}	% decrease in CFU
Control	2.25×10^9		5.03×10^6	
1.15 protease	1.96×10^9	12.8	1.89×10^6	62.4
Subtilopeptidase A	1.73×10^9	23.1	2.14×10^6	57.4
Rhizopus sp. Type XVIII	8.91×10^8	60.4	1.78×10^6	64.6
Thermolysin Type X	1.71×10^9	24.12	4.13×10^6	18.2

Table 3.3

The effect of 1.15 protease and commercially available proteases on *Ent* planktonic and biofilm cultures were compared. Enzyme activities were adjusted to an activity of 50 μg protein hydrolysed h^{-1}. Standard error for biofilm CFU cm^{-2} was ± 3.1×10^5, and for planktonic CFU ml^{-1}, ± 1.23×10^8. Results are the outcome of 3 replicate experiments.

3.3.5 *Range of Activities of Bacteriocins*

The range of activities of the bacteriocins produced by 1.15, *Ent*, 5920, and the strains *E. coli* and 53b, used in later experiments, were determined. *E. coli* bacteriocin was found to be a microcin, and 53b a larger bacteriocin (<100 KDa >50 KDa). Spent media fractions were prepared using Millipore Vivacell 70 membrane filters, and then filter-sterilised (0.22 μm filter, Millipore, UK). A wide range of *E. coli*, *Enterobacter*, *Klebsiella*, *Serratia* species, and a *Pseudomonas* test strain were

used. Each strain was inoculated into 96 well microtitre plates, incubated for 24 h and filter-sterilised spent media fractions added to the wells. After further incubation for 4 h, the OD_{570} of the cultures were read in a microtitre plate reader. Any decrease in the OD of the cultures was indicative of bacteriocin activity (**table 3.4**). Interestingly, 1.15 protease was only active against *Ent*. The microcins produced by 1.15, *Ent*, 5920 and *E. coli* were active against strains in many of the *E. coli*, *Enterobacter*, *Klebsiella* and *Serratia* groups. The larger bacteriocin produced by 53b was active against all the strains tested.

STRAINS		BACTERIOCINS						
		1.15 prtase >10K	1.15-M4 <10K	1.15-M8 <10K	*E. coli* <10K	*Ent* <10K	5920 <10K	53b <10K
Escherichia coli	*E. coli*			+			+	+
	S53							+
	K12				+	+		+
	Eck5		+					+
	E.coli B						+	+
Enterobacter	*Ent*	+		+			+	+
	53b			+		+		
	5920		+	+		+		+
	1.15				+			+
Klebsiella	109c			+	+			+
	86b			+				+
	G1			+				+
	107b		+	+	+			+
	K66			+				+
	D451.1			+				+
	XM6		+		+	+		+
Serratia	5Xa							+
	Pd3a							+
	87b				+			+
	A1		+	+				+
	A4		+	+				+
Pseudomonas	Pseud							+

Table 3.4

The bacteriocins produced by *E. coli*, *Ent*, 1.15, 5920 and 53b were tested against a wide range of bacteria. Membrane cut-off filters (sizes 10 and 100 K) were used to produce the fractions tested. 1.15 produces two microcins, one at 4 h, 1.15-M4, and at 24 h, 1.15-M24. Strains to be tested were inoculated into 96 well microtitre plates, incubated for 24 h and filter-sterilised spent media fractions added to the wells. After further incubation for 4 h, the OD of the wells were read in a microtitre plate reader at 570 nm. A decrease in the OD of the culture (<5%) is marked with +. Results are a consensus of 5 replicate experiments.

3.4 COMPETITIVE INTERACTIONS IN DUAL SPECIES BIOFILMS

3.4.1 *Consequences of Inoculum Size*

1.15 is capable of producing two microcins and a protease, whereas *Ent* produces a single microcin. Consequently, competitive interactions between *Ent* and 1.15 have shown that 1.15 often gains the upper hand. Addition of filter-sterilised 1.15 spent media to biofilms of *Ent* reduced the number of cells adhering to the glass beads to a greater degree than the addition of *Ent* spent media to 1.15 biofilms (fig 3.2b). Similarly, 1.15 was more successful in colonising a pre-established biofilm of *Ent* than *vice versa* (fig 3.3b). It was, therefore, decided to investigate the competitive interactions between 1.15 and *Ent* further, specifically the effect of changing the initial inoculum of the two strains. Glass beads were inoculated with 1.15 and *Ent* in the following ratios: 5:95, 25:75, 50:50, 75:25 and 95:5 and viable counts made after 24 h (**table 3.5**). The same cultures were then subjected to a sub-inhibitory disinfection treatment; 5 min in 40 $\mu g\ ml^{-1}$ triclosan. The glass beads were washed to remove the triclosan and left to recover for 4 h before viable counts were again made. The cell counts were also expressed as ratios of *Ent*:1.15. This process was also repeated for the strains 1.15 and 5920 (**table 3.6**).

Table 3.5 showed that 1.15 managed to dominate every *Ent*:1.15 biofilm, except the biofilm with a 95% *Ent* and 5% 1.15 inoculum where a 50:50 mix of the two strains occurred. 1.15 was also successful in dominating 5920/1.15 biofilms (table 3.6). Only those biofilms with a 5% 1.15 and 95% 5920 inoculum resulted in a marginal 5920 dominance. Biofilms with a 25% 1.15 and 75% 5920 inoculum resulted in a 50:50 mix.

Disinfection of the competitive biofilms produced interesting results. In the *Ent*/1.15 biofilms where 1.15 had previously dominated, 1.15 increased in numbers again (table 3.5). However, the disinfection treatment completely eradicated the biofilm with the initial inoculum of 5% *Ent* and 95% 1.15. Table 3.6 shows that disinfection of the 5920/1.15 biofilms resulted in an increase in 5920 numbers in all the conditions tested.

% inoculum		x10^4 CFU cm^{-2} pre-triclosan			x10^4 CFU cm^{-2} post-triclosan		
1.15	*Ent*	1.15	*Ent*	1.15:*Ent*	1.15	*Ent*	1.15:*Ent*
0	100	0	202		0	9	
5	95	26	24	1.1	0	0	
25	75	14	9	1.8	0.6	0.3	2
50	50	101	29	3.5	0.9	0.1	9
75	25	42	5	8.4	4.0	0.1	40
95	5	67	6	11.2	39	0	
100	0	156	0		47	0	

Table 3.5

Consequences of inoculum size on the competitive interactions between *Ent* and 1.15. Cultures were inoculated with the ratios 5:95, 25:75, 50:50, 75:25 or 95:5, and viable counts were made after 24 h growth. Biofilm cultures were also challenged with 50 μg/ml triclosan and incubated for a further 6 h before viable counts were made. Data is also represented as ratios of *Ent*:1.15 cells. Standard error for the mixed species biofilms was ± 9.31 x 10^3, and for the triclosan-treated mixed species biofilms, ± 8.13 x 10^2. Results are the outcome of 3 replicate experiments.

% inoculum		x10^4 CFU cm^{-2} pre-triclosan			x10^4 CFU cm^{-2} post-triclosan		
1.15	5920	1.15	5920	1.15:5920	1.15	5920	1.15:5920
100	0	156	0		47	0	
95	5	139	26	5.3	88	9	9.8
75	25	136	40	3.4	13	7	1.9
50	50	133	77	1.7	154	75	2.1
25	75	104	100	1.0	56	80	0.7
5	95	99	143	0.7	35	79	0.4
0	100	0	428		0	9	

Table 3.6

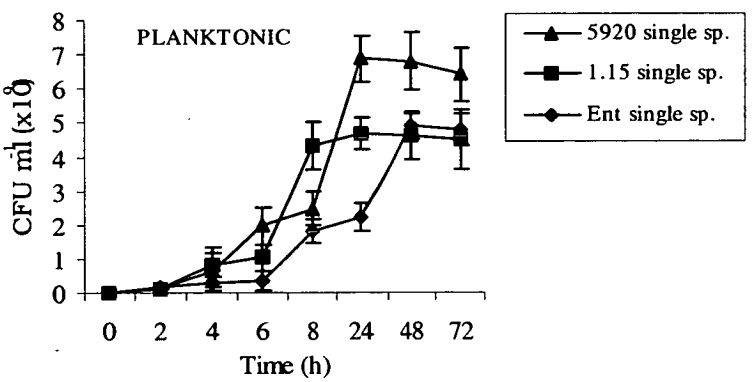
Consequences of inoculum size on the competitive interactions between 5920 and 1.15. Cultures were inoculated with the ratios 5:95, 25:75, 50:50, 75:25 or 95:5, and viable counts were made after 24 h growth. Biofilm cultures were also challenged with 50 μg/ml triclosan and incubated for a further 6 h before viable counts were made. Data is also represented as ratios of 5920:1.15 cells. Standard error for the mixed species biofilms was ± 8.23 x 10^4, and for the triclosan-treated mixed species biofilms, ± 7.11 x 10^3. Results are the outcome of 3 replicate experiments.

3.4.2 *Comparison of Competitive Interactions in Planktonic and Biofilm Cultures*

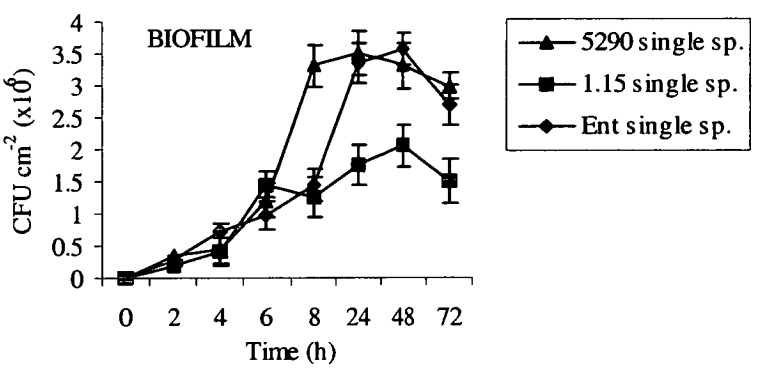
Previous studies have shown that the long-term co-existence of bacteriocin-producing and bacteriocin-sensitive strains in liquid cultures can not be achieved, one strain will always be out-competed (Riley and Gordon, 1998). Results in this chapter suggest the situation may be different for biofilm competition. The dual species competitive biofilms studied of *Ent*/1.15 and 1.15/5920 resulted in a culture containing a mixture of the two strains. The competitive interactions between *Ent* and 1.15, and 5920 and 1.15 in planktonic and biofilm cultures was, therefore compared. Biofilm cultures were grown on glass beads. Tables 3.5 and 3.6 showed that biofilms with a 5:95 inoculum resulted in a more even mixture of *Ent*/1.15 and 5920/1.15 in the 24 h biofilms. A 20:1 ratio of cells was, therefore, used, with *Ent* and 5920 being the dominant strain in each pair. **Figure 3.8** shows the outcome of

the competitive interactions between *Ent* and 1.15, and between 1.15 and 5920. Single sp. controls for planktonic (figure 3.8a) and biofilm (figure 3.8b) cultures were included. Figures 3.8c and 3.8d demonstrate planktonic and biofilm competition between *Ent* and 1.15, and figures 3.8e and 3.9f planktonic competition between 1.15 and 5920. Viable counts were estimated over a 72 h. Results show there was considerable differences between planktonic and biofilm competition. In both the *Ent*/1.15 and 1.15/5920 planktonic cultures, 1.15 was gradually out-competed whereas 1.15 remained established within both the biofilm cultures.

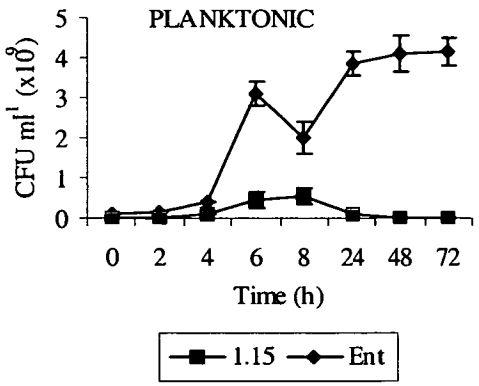
3.8a) planktonic single. sp controls



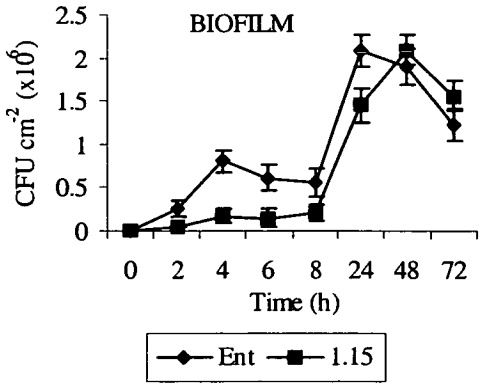
3.8b) biofilm single sp. controls



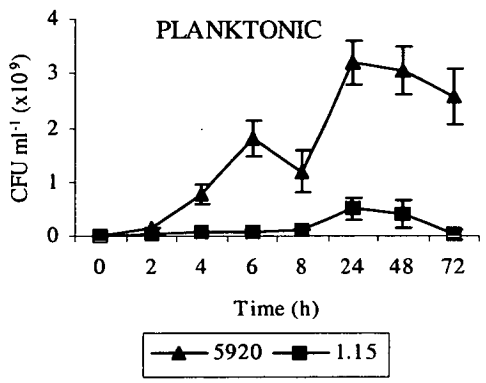
3.8c) *Ent*:1.15 planktonic competition



3.8d) *Ent*:1.15 biofilm competition



3.8e) 5920/1.15 planktonic competition



3.8f) 5920/1.15 biofilm competition

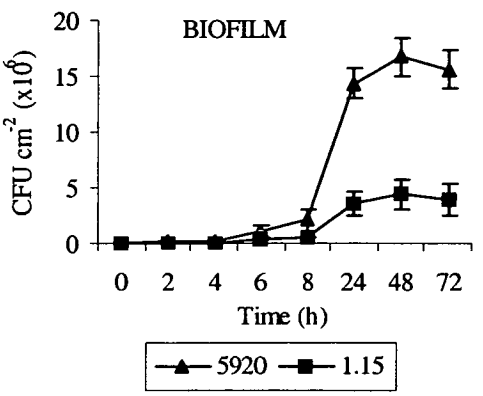


Figure 3.8

A comparison of the competitive interactions between liquid and biofilm cultures was made. Single sp. controls are shown in fig 3.8a (planktonic) and fig 3.8b (biofilm). For the dual species cultures, an inoculum containing a 20:1 ratio of cells was used, with the dominant strains being *Ent* for the *Ent*/1.15 cultures, and 5920 for the 5920/1.15 cultures. Fig 3.8b and c show competition between dual species planktonic and biofilm cultures of *Ent*/1.15. Fig 3.8e and f show competition between dual species planktonic and biofilm cultures of 5920/1.15. Cultures were incubated at 30 °C with shaking and viable counts were estimated over a 72 h period. Whereas the planktonic cultures (fig 3.8c and e) resulted in dominance by one species, the biofilm cultures (fig 3.8d and f) contained a mixture of the two species. Results are the outcome of 3 replicate experiments and bars represent standard error.

Unlike competition between bacteriocin-producing strains in planktonic cultures, where one strain will always be out-competed, results in figure 3.8 suggested bacteriocin-producing strains can co-exist in biofilms. To test this theory further, 3 more competitive pairs of bacteria were selected, *E. coli*/1.15, *Ent*/53b and 1.15/*Ent.* The 1.15/*Ent* differed from the pair used in figure 3.8 as 1.15 was the dominant strain in the inoculum. Previously, *Ent* was the dominant strain in the initial inoculum. **Table 3.7** demonstrates the outcome of competition between these pairs in biofilm and planktonic cultures. Again, the initial inoculum was adjusted to a ratio of 20:1, with the underlined strain being the dominant strain in each pair. Ratios of each strain in each pair were monitored over a 3 day period, and the figures shown in the table are the ratios after 72 h growth. In the biofilm cultures, the subordinate strain became established within the biofilm, sometimes even dominating the biofilm as in the case of *Ent*/1.15. As in figure 3.8, the subordinate strain was gradually being out-competed in the planktonic cultures.

STRAINS	BIOFILM	PLANKTONIC
Ent/1.15	0.6	all *Ent*
1.15/*Ent*	2	125
5920/1.15	5	all 5920
E. coli/1.15	15	all *E. coli*
Ent/53b	3.5	50

Table 3.7

The competitive interactions between biofilm and planktonic cultures were compared. The initial inoculum was adjusted to a 20:1 ratio of cells, with the underlined strain being the dominant strain in each pair. Ratios of each strain in each pair were monitored over a 3 day period, and the figures shown here are the ratios after 72 h growth. The biofilm cultures consisted of a mixture of the two strains, whereas in the planktonic cultures the subordinate strains were gradually being out-competed to leave a single species culture.

Figure 3.9 depicts a competitive biofilm of *Ent* and 1.15. To distinguish microscopically between the two strains, an *Ent* variant containing a GFP plasmid was used. Strain 1.15 was stained using propidium iodide, a red fluorescent pigment. GFP was not masked by the presence of PI in the cell, therefore *EntGFP* cells appeared green (or yellow). Figure 3.9 demonstrates the two strains formed distinct colonies.

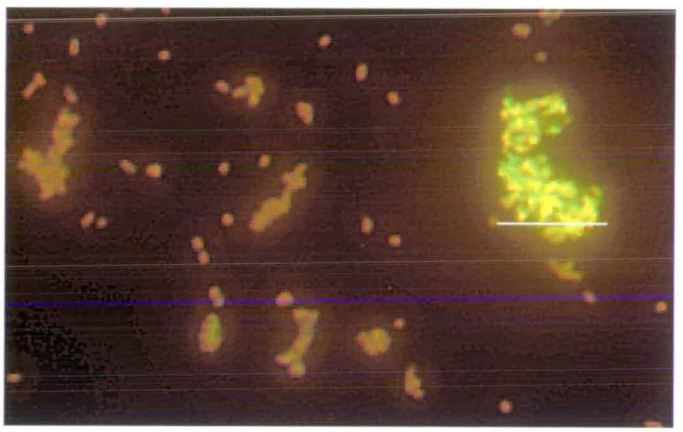


Figure 3.9

Fluorescent microscopy of a competing biofilm of *EntGFP* and 1.15. Propidium iodide was used to stain 1.15 red. Separate microcolonies of the two strains can be distinguised. Bar = 75 μm

3.5 RESISTANCE OF COMPETITIVE BIOFILMS TO DISINFECTANTS

The biofilms formed by co-operating communities, communities in which the interactions are mutually beneficial to each of the species involved, can be more resistant to antimicrobial agents than when grown in isolation (Skillman *et al.*, 1999). Little is known of the resistance of competing biofilms to disinfectants. The resistance of biofilm material to the common household disinfectants sodium hypochlorite (HPC), benzalkonium chloride (BC) and triclosan (TR) were estimated.

Table 3.8 shows the biofilm eradicating concentrations (BEC) of BC, HPC and TR against single and dual species biofilms. The pairs *E. coli*/*Ent*, *Ent*/1.15 and 1.15/5920 were examined. The initial inoculum contained a 1:1 ratio of cells in each case. Biofilms were grown on microtitre plates for 24 h and the appropriate concentration of disinfectant added. This was incubated for 5 min, the disinfectant rinsed out and the plates re-incubated. Any surviving bacteria could grow into the liquid phase. The BEC was taken as the lowest concentration of disinfectant to cause any re-growth. Theoretical results are given based on the concentrations of disinfectant required to eradicate the single species biofilms and the ratio of cells in the dual species biofilms. When comparing the theoretical and actual data, the results show that the competitive biofilms *Ent*/1.15 and 1.15/5920 were actually less resistant to disinfectants in each case. When compared to the competitive biofilms, the non-competitive biofilm of *E. coli*/*Ent* was only slightly less resistant to the disinfectants.

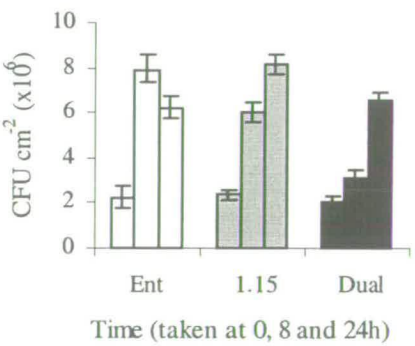
Similarly, **figure 3.10** depicts the effect of addition of 50 μg triclosan to pre-established single and dual species biofilm of *Ent* and 1.15. Biofilms were grown overnight on glass beads, washed 3 times in PBS to remove loosely adhered cells and placed into fresh media with (fig 3.5a) and without (fig 3.5b) triclosan. Viable counts were taken at 0, 8 and 24 h. Growth was reduced in all the triclosan-containing cultures, however, the dual species biofilm was particularly effected. The dual species biofilm grown in triclosan-free media also contained reduced numbers.

STRAINS	BC (mg/ml)		HPC (%)		TR (μg/ml)	
E. coli	0.4		0.018		50	
Ent	0.4		0.018		45	
1.15	0.4		0.03		55	
5920	0.45		0.022		55	
	THEORETICAL	ACTUAL	THEORETICAL	ACTUAL	THEORETICAL	ACTUAL
E. coli/Ent	0.4	0.35	0.018	0.018	47.32	45
Ent/1.15	0.4	0.3	0.027	0.02	52.5	30
1.15/5920	0.425	0.35	0.026	0.022	55	40

Table 3.8

The biofilm erradicating concentrations of sodium benzalkonium chloride (BC), sodium hypochlorite (HPC) and triclosan (TR) against single and dual species biofilms. Biofilm material was grown in microtitre plates and the appropriate concentration of disinfectant added. This was incubated for 5 min, the disinfectant rinsed out and the plates re-incubated with fresh YE media. Any surviving bacteria could re-grow into the liquid phase. The BEC was taken as the lowest concentration of disinfectant to prevent any growth. Theoretical results are given based on the concentrations of disinfectant required to eradicate the single species biofilms and the ratio of cells in the dual species biofilms. The competitive biofilms *Ent*/1.15 and 1.15 /5920 were less resistant to disinfection in each case.

3.10a)



3.10b)

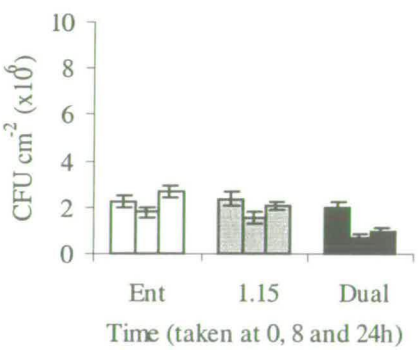


Figure 3.10

The effect of addition of 0.5 μg triclosan to pre-established biofilms of *Ent*, 1.15 and *Ent*/1.15. Single and dual species biofilms were grown overnight on glass slides. The slides were washed 3 times in sterile PBS to remove loosely adhered cells and placed into fresh media with (fig 3.10a) and without (fig 3.10b) triclosan. Viable counts were taken at 0, 8 and 24 h. In both the control and triclosan-containing cultures, the competitive dual species biofilm contained less adhered cells. Results are the outcome of 3 replicate experiments and error bars represent standard error.

3.6 DISCUSSION

3.6.1 *Bacteriocin Production*

Studies have shown that the agents produced by 1.15, *E. coli*, *Ent* and 5920 were of low molecular size (<1000 Mr), and their production unaffected by UV treatment. This suggests these agents were likely to be microcins. Interestingly, 1.15 was shown to produce two microcins. A protease (<100 KDa >50 KDa) was also produced by 1.15. *Ent. Agglomerans*/53b also produced a larger bacteriocin. This agent was found to be <100 KDa >10 KDa in size.

Range of Activities of Bacteriocins

The strains used in these studies are all closely related species. Consequently, it was not surprising to find that considerable bacteriocin activity occurred amongst the strains. Table 3.4 indicated that the microcins produced by 1.15, *E. coli*, *Ent* and 5920 had activities against representative strains of *E. coli*, *Enterobacter*, *Klebsiella* and *Serratia*. A cross over of bacteriocin activity into other Enteric Genus has previously been demonstrated. For example, colicin L is a bacteriocin produced by *Serratia marcescens* JF246 that is active against certain *E. coli* strains, but not those *Serratia* strains tested (Konisky, 1982). Many ColE plasmids of *E. coli* were also found to be stable in *Salmonella* strains (Hardy, 1975). Studies of protein sequence similarities of colicins of *E. coli*, a cloacin of *Ent. cloace*, a klebicin of *K. pneumoniae* and a marcescin of *S. marcescens* reveal shared evolutionary ancestries (Riley, 1998). While the origin of the microcins remains unclear, it can be expected that these too can have activities amongst the closely related groups of *Enterobacter*, *Klebsiella* and *Serratia*.

The bacteriocin produced by *Ent. agglomerans*/53b had activity against all the *E. coli*, *Enterobacter*, *Klebsiella* and *Serratia* strains tested. However, no activity was produced against the *Pseudomonas* test strain. Bacteriocins are classified as compounds produced by bacteria that inhibit or kill closley related species. The broad-spectrum lytic activity of 53b suggests that this compound may be better classified as a broad-spectrum antibiotic.

Methods Used to Estimate Bacteriocin Production

Traditional methods to evaluate bacteriocin production involve the use of agar plates overlaid with sloppy agar. Double dilutions are made of the culture supernatant and this is then applied to the sloppy agar. After incubation, the plates are examined for zones of inhibition. This method can be costly and time-consuming. The microcins produced by *Ent* and 5920 are bacteriostatic, and so addition of these agents to lawns of bacteria inhibit the growth of susceptible strains, but do not produce clear zones of inhibition. Other methods for the estimation of bacteriocin production were, therefore, required. This problem was overcome by the use of microtitre plates. Cultures of a susceptible strain were grown for 4 h, and spent media containing the bacteriocin added to the microtitre plate wells. After further incubation, the inhibition of growth due to the addition of a bacteriostatic agent, and the resultant drop in OD, could be read in a microtitre plate reader.

For bacteriolytic agents, such as 1.15 microcins, an alternative method could be used. Cells of a susceptible strain were centrifuged, washed and resuspended in PBS. Spent media of the sample to be tested was added to the cell suspension and incubated for 1 h. The cells were then spun out and the supernatant examined for the release of nucleic acid due to cell lysis. This can be read at an absorbency of 260nm.

Figure 3.6, an estimation of the production of the two microcins of 1.15, demonstrates the reproducibility of these two techniques.

1.15 Protease

1.15 protease was shown to be a PMSF-sensitive-alkali protease, active only on *Ent* (fig 3.7; table 3.4). Tables 3.2 and 3.3 indicated that 1.15 protease was more active against biofilm than planktonic cultures. PMSF-sensitive proteases are best represented by the subtilins, serine proteases similar to trypsin. These are generally active at neutral and alkaline pH, and have considerable esterolytic activity. Subtilopeptidase A, a PMSF-sensitive-alkali protease, was also more active against biofilm cells. It is likely that these proteases are not lysing the biofilm cells, but removing the entire biofilm by targeting proteins involved in adherence to surfaces. This knowledge may be useful in the control of biofilm formation. However, it must be noted that the pH range of activity of 1.15 protease is pH 5-11. In a heterogenous

environment such as a biofilm, pockets of acidity are likely to occur and protease activity would be restricted to areas of neutral or alkaline pH.

3.6.2 *Competition Amongst Bacteriocin-Producing Strains*

The Effect of Bacteriocins on Biofilm Cultures

It was shown that 1.15 was capable of producing two microcins and a protease. This protease and the 1.15 M-8 had activity against *Ent*, both with bacteriolytic activity. *Ent* was only capable of producing a microcin with bacteriostatic against 1.15. Consequently, it was not surprising to see that competitive interactions between 1.15 and *Ent* resulted in dominance by 1.15. For example, addition of filter-sterilised 1.15 spent media to biofilms of *Ent* reduced the number of cells adhering to the glass beads to a greater degree than the addition of *Ent* spent media to 1.15 biofilms (fig 3.3b). Similarly, 1.15 was more successful in colonising a pre-established biofilm of *Ent*, than *Ent* was in colonising a biofilm of 1.15 (fig 3.4b).

In the second competitive pair studied, 5920 and 1.15, both microcins produced by 1.15, M-4 and M-8, had lytic activity against 5920. Although 5920 has capable of producing a microcin, this had no activity against 1.15. As expected, addition of 5920 SM to biofilms of 1.15 had no effect on the number of cells adhered to the glass beads, whereas addition of 1.15 SM to biofilms of 1.15 had no effect on the number of cells adhered to the glass beads (fig 3.3c). While 1.15 was capable of colonising pre-established biofilms of 5920, 5920 was incapable of colonising biofilms of 1.15. It therefore seems that bacteriocin-producing strains can colonise a biofilm of a pre-established bacteriocin-sensitive strain, and that non-bacteriocin-producing strains are unsuccessful.

A reduction in the number of cells adhering to the glass beads was seen in the dual species competitive biofilms of *Ent*/1.15 and 5920/1.15. This was thought to be due to bacteriocin production amongst the strains. However, the non-competitive dual species biofilm of *E. coli* and *Ent*, also exhibited a reduction in cell numbers when compared to the single species biofilms. The reason for this reduction in biofilm size is unclear. Although *E. coli* and *Ent* are both bacteriocin-producing strains, these bacteriocins are inactive against the respective strains. This could also be seen in figure 3.3a); the addition of filter-sterilised spent media to the strains had

no effect on biofilm size. The strains were also ineffective in colonising pre-established biofilms (fig3.4a). This decrease in biofilm size may have been associated with the spatial distribution of colonies within the biofilm, with each strain forming their own microcolonies.

Consequences of Inoculum Size

1.15 is capable of producing a microcin and a bacteriocin against *Ent*, whereas *Ent* produces only one microcin. Although the amount of bacteriocin being produced and the effectiveness of the bacteriocin must be taken into account, 1.15 appears to have an advantage over *Ent*. To reset any advantage 1.15 had over *Ent* the initial inoculum size was adjusted (table 3.5). This was also repeated for 5920 and 1.15 (table 3.6). 1.15 was capable of dominating every biofilm except the biofilm with an initial inoculum of 5% 1.15 and 95% *Ent*, where the 24 h culture contained a 50:50 mix of the two strains. 1.15 was also able to dominate the dual species biofilms of 5920. Only the biofilm with an initial 95% inoculum of 5920 resulted in 5920 dominance. Figure 3.8b represents the colonisation of glass beads by single species biofilms of 5920, 1.15 and *Ent*. Although *Ent* and 5920 reach higher cell densities, the initial colonisation rates by the three strains is identical. The domination of *Ent* and 5920 by 1.15 is probably due to bacteriocin production in the later stages of biofilm formation alone.

Biofilm *vs.* Planktonic Competition

In their review of the ecological role of bacteriocins in bacterial planktonic cultures, Riley and Gordon (1999) state that the long-term co-existence of bacteriocin-producing and bacteriocin-sensitive strains can not be achieved, one strain will always be out-competed. The same may not be true of biofilms. While *Ent* dominated and eventually out-competed 1.15 in the competitive planktonic culture (fig 3.8c), resulting in a culture solely composed of *Ent*, the competitive biofilm resulted in a 3:2 mix of 1.15/*Ent* cells. Both strains remained established within the biofilm. This was demonstrated for four more competitive dual species biofilms (table 3.7). Mathematical modelling has shown it is possible to achieve a dynamic equilibrium between bacteriocin-producing and sensitive strains when the cells are

growing on a solid surface (Frank, 1994). This is thought to be due to the spatial distribution of cells within the biofilm, each strain will form its own microcolonies, and fig3.9 demonstrates a competitive biofilm of the strains *Ent* and 1.15 formed distinct microcolonies within the biofilm. However, other factors such as the diffusion of the bacteriocin through the biofilm, and the subsequent gradients formed, may also affect the equilibrium between the strains.

Disinfection of Competitive Biofilms

Competition between the strains also caused a decrease in the resistance of the biofilms to sodium hypochlorite, benzalkonium chloride and triclosan (table 3.8 and fig 3.9). The non-competitive dual species biofilms of *E. coli* and *Ent* had BECs similar to those of the single species biofilms. The increased sensitivity of the competitive biofilms to the disinfectants may be because the cells in the biofilm have to deal with a double assault, being affected by both bacteriocin and disinfectant. Tables 3.5 and 3.6 demonstrated how complex competitive interactions can affect the disinfection of dual species biofilms. Biofilms of *Ent*/1.15 and 1.15/5920 were subjected to a sub-inhibitory disinfection treatment of 40 $\mu g\ ml^{-1}$ tricolsan. *Ent* had a triclosan BEC of 45 $\mu g\ ml^{-1}$, whereas 1.15 was 55 $\mu g\ ml^{-1}$. An increase in 1.15 cell numbers would, therefore, be expected. This did occur, but the biofilm size after the disinfection treatment also decreased with increasing *Ent* population size. It seems that those biofilms where most competition would be expected to occur, for example in the biofilm with 1:1 ratio of cells, caused a further decrease in stability, and, consequently, a further decrease in the resistance of the biofilm to disinfection. 5920, like 1.15, has a triclosan BEC of 55 $\mu g\ ml^{-1}$. In this case, 1.15 bacteriocin production was ineffective. 5920 numbers actually increased after the triclosan treatment, resulting in domination over 1.15. While both 1.15 and 5920 produce copious EPS, the EPS of 5920 is more viscous, and perhaps this affected the outcome of 1.15/5920 interactions here.

3.6.3 *Conclusions*

The production of bacteriocins by the strains resulted in competitive biofilm formation. Examination of the dual species biofilms formed by the pairs *Ent*/1.15 and 5920/1.15 indicated that competition in biofilms appears to differ markedly from competition within planktonic cultures. Whereas planktonic competition results in dominance by one strain, bacteriocin-producing and bacteriocin-sensitive strains can co-exist in biofilms. However, the competitive biofilms exhibited decreased biofilm size, decreased stability and an increase in sensitivity to the disinfectants benzalkonium chloride, sodium hypochlorite and triclosan.

Chapter 4

COMPETITION AND CO-OPERATION

4.1 INTRODUCTION

Results in fig 3.4b indicated that addition of 1.15 to a dual species biofilm of *Ent* resulted in a thicker biofilm. This biofilm reached a total cell count of 4.45 x 10^6 CFU cm^{-2} (± 2.05 x 10^5), whereas a competitive biofilm of *Ent*:1.15 contained 2.79 x 10^6 CFU cm^{-2} (± 1.43 x 10^5) (fig 3.8d). Sloughing off of biofilm material was a problem when using glass beads, possible due to the high shear forces involved. This can be seen clearly in figures 3.8b, d and f. The *Ent* biofilm antagonised with 1.15 did not slough off, suggesting that introduction of a competitive strain not only increased adhesion, but also increased the strength of adhesion.

It was decided to investigate this phenomenon further, first by comparing the competition strategies used by 1.15 as it colonises a clean surface with *Ent*, and as it invades a pre-established biofilm of *Ent*. As a control, biofilms of 5920/1.15 will be compared. Particular attention will also be paid to the EPS production by the two strains. This may influence the stability of the biofilm (Skillman *et al.*, 1999b).

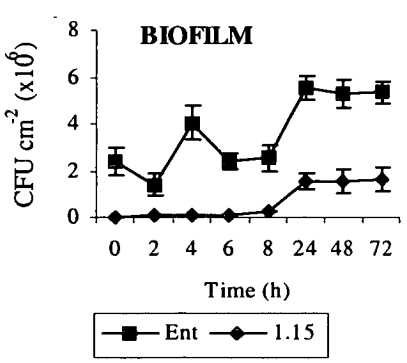
Studies have also suggested that biofilms may produce more bacteriocin than planktonic cells. For example, when compared to planktonic cultures it was found that a biofilm of *Staph. epidermidis* exhibited increased protease production (Evans *et al.*, 1994). The bacteriocin production by biofilm cultures will, therefore, be compared to planktonic cultures.

4.2 INVASION OF A PRE-ESTABLISHED BIOFILM BY A SECOND, BACTERIOCIN-PRODUCING STRAIN

4.2.1 *1.15 Invasion of Pre-Established* Ent *Cultures*

The integration of 1.15 into pre-established cultures of *Ent* was monitored over a 72 h period. Viable counts (**figure 4.1**) and bacteriocin production (**figures 4.2** and **4.3**) were estimated. Planktonic cultures were also compared with biofilm cultures. The inoculum of 1.15 was adjusted to allow for a 20:1 ratio of cells. 1.15 protease was estimated as mg protein hydrolysed h^{-1}. 1.15 microcin was measured by its lytic effect on 53b cells. As the protease is not active against 53b, this gives an estimation of 1.15 microcin only. 1.15 M-4 also has no activity against *Ent* and 53b, and so use of 53b also gave an estimation of 1.15 M-8, and not 1.15 M-4 activity. *Ent* microcin had only bacteriostatic activity against 1.15 cells. This agent was, therefore, measured using the percentage reduction in the OD_{570} of growing cultures of 1.15. Figures 4.2 and 4.3 also show bacteriocin production of single species cultures, and of 'competitive' cultures, where 1.15 and *Ent* were inoculated simultaneously.

4.1a)



4.1b)

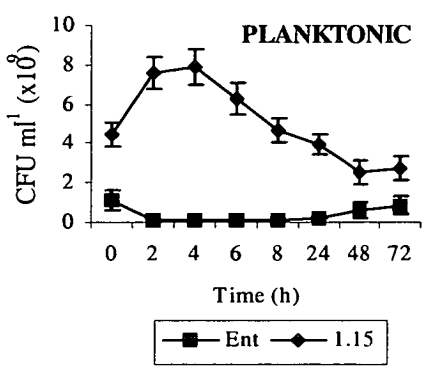
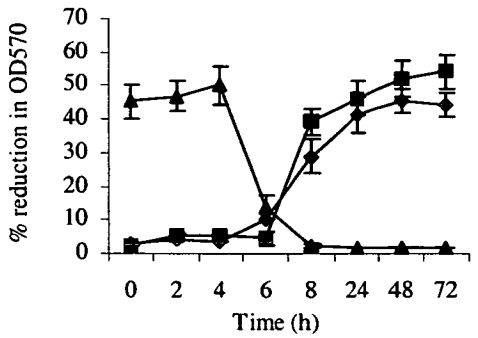


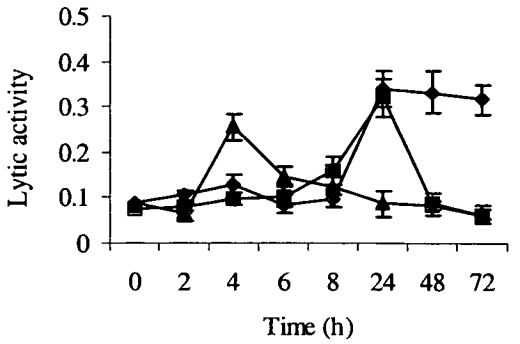
Figure 4.1

The invasion of a pre-established *Ent* biofilm (fig 4.1a) and planktonic (fig 4.1) cultures by the strain 1.15 was examined. The inoculum was adjusted to allow for a 20:1 ratio of 1.15:*Ent* cells. Viable counts were estimated over a 72 h period. Results are the outcome of 4 replicate experiments, and the bars represent standard error.

4.2a) *Ent* microcin



4.2b) 1.15 microcin



4.2c) 1.15 protease

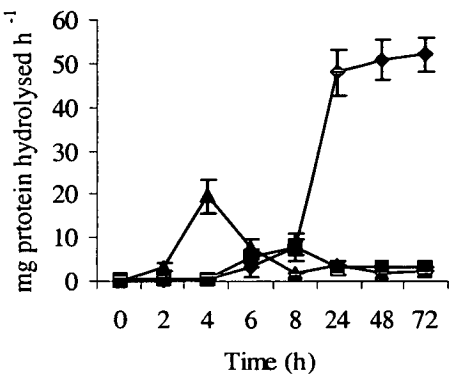
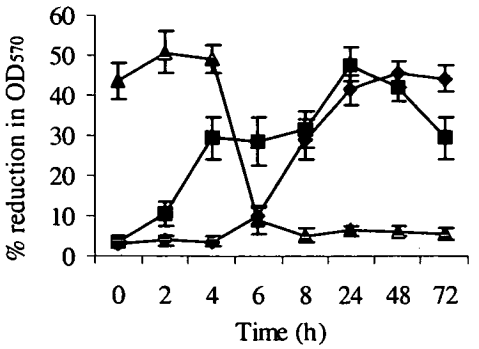


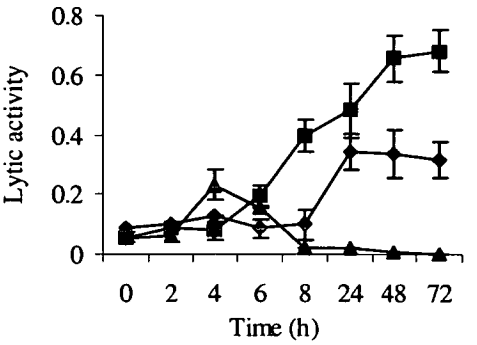
Figure 4.2

The different competition strategies used when *Ent* and 1.15 were inoculated simultaneously (competitive cultures ■), and as 1.15 invaded a stationary phase PLANKTONIC culture of *Ent* (invasive cultures ▲) were compared. The inoculum was adjusted to contain a 20:1 ratio of cells, with *Ent* being the dominant strain in each case. Single sp. planktonic controls (◆) are also shown. *Ent* microcin was estimated by the percentage reduction in OD_{570} of cultures of 1.15 (fig 4.2a). 1.15 microcin was estimated by the lysis of *Enterobacter agglomerans*/53b (fig4.2b). This releases nucleic acid that can be read at 260 nm. Protease activity was measured as mg protein hydrolysed h^{-1} (fig4.2c). Results are the outcome of 4 replicate experiments and bars represent SE.

4.3a) *Ent* microcin



4.3b) 1.15 microcin



4.3c) 1.15 protease

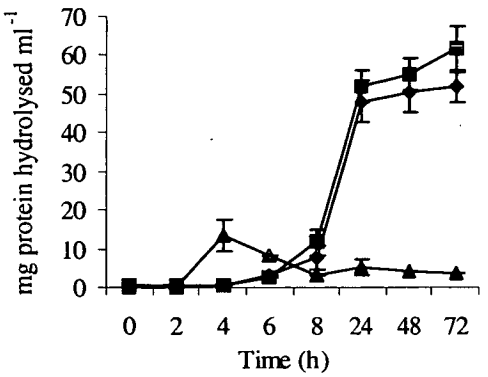


Figure 4.3

The different competition strategies used when *Ent* and 1.15 were inoculated simultaneously (competitive cultures ■), and as 1.15 invaded a stationary phase BIOFILM of *Ent* (invasive cultures ▲) were compared. The inoculum was adjusted to contain a 20:1 ratio of cells, with *Ent* being the dominant strain in each case. Single sp. biofilm controls (◆) are also shown. *Ent* microcin was estimated by the percentage reduction in OD_{570} of cultures of 1.15 (fig 4.3a). 1.15 microcin was estimated by the lysis of *Enterobacter agglomerans*/53b (fig4.3b). This releases nucleic acid that can be read at 260 nm. Protease activity was measured as mg protein hydrolysed h^{-1} (fig 4.3c). Results are the outcome of 4 replicate experiments and bars represent SE.

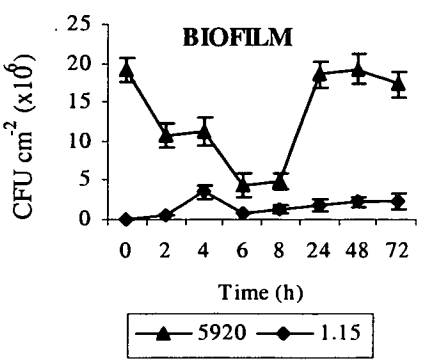
Invasion of an *Ent* biofilm by 1.15 resulted in an increase of cell numbers adhering to the glass beads (fig 3.4b). This large increase of cell numbers can be seen again in figure 4.4a. A competitive culture of *Ent* and 1.15, where the strains are inoculated at the same time, only reach a maximum of 3.98 x 10^6 CFU cm^{-2} (± 2.1 x 10^5) at 48 h. The invasive biofilm culture reached a maximum of 6.9 x 10^6 CFU cm^{-2} (± 5.26 x 10^5) at 72 h. Unlike the competitive cultures, there was also no sloughing off of biofilm material (fig 3.8d). Examination of the viable counts of 1.15 integration into the pre-established cultures of *Ent* show 1.15 gradually increased numbers after 24 h (figure 4.1). This increase in cell numbers after 24 h may be correlated with the bacteriocin production of *Ent*; this is completely switched off at approximately 8 h (figures 42a & 4.3a). By comparing 1.15 microcin and protease activities in single sp. biofilms with the activities in the invasive biofilms, it can be seen that 1.15 bacteriocin production is also switched off. Induction would, ordinarily, begin at 8 h. Instead of competing, this biofilm begins to co-operate. Interestingly, *Ent*, in both the biofilm and planktonic cultures, increased in numbers at 4 h, and this may be associated with the peaks in 1.15 microcin and protease activities at the same time period, particularly in the biofilm cultures.

There were no differences between the bacteriocin production in the invasive planktonic and biofilm cultures (figures 4.2 and 4.3). There were also no differences between planktonic and biofilm bacteriocin production in the single sp. cultures. However, there were differences between bacteriocin production in the competitive planktonic and biofilm cultures. *Ent* and 1.15 were inoculated simultaneously in these cultures, in a 20:1 ratio of cells. Viable counts for these cultures can be seen in figure 3.8. Both *Ent* and 5920 remained established within the biofilm cultures, whereas 1.15 was gradually out-competed in the planktonic cultures. The gradual disappearance of 1.15 in the planktonic cultures can also be seen in the graphs of bacteriocin production (figure 4.3). There is a slight peak in 1.15 microcin activity at 24 h (fig 4.3b), but this quickly disappears and protease production was not induced (fig 4.3c). Interestingly, there was an early induction of *Ent* microcin production in the competitive biofilm culture (fig 4.3a).

4.2.2 *1.15 Invasion of Pre-Established 5920 Cultures*

Figure 3.8f showed the cultures of 5920 antagonised with 1.15 also increased in cells numbers. It was, therefore, decided to repeat the above experiments and examine the invasion of pre-established 5920 cultures with 1.15. Viable counts (**figure 4.4**) and bacteriocin activities (**figure 4.5**) were again estimated over a 72 h period. The inoculum of 1.15 was adjusted to allow a 20: 1 ratio of cells. Because there were no differences in bacteriocin production between the *Ent* planktonic and biofilm cultures antagonised with strain 1.15 (fig 4.2 & 4.3), only the bacteriocin production of biofilm cultures was examined. Again bacteriocin production by the invasive biofilms was compared to the production in competitive, where strains were inoculated simultaneously, and single species biofilms. 1.15 microcin was estimated by the lysis of 5920 cells, and 1.15 protease as mg protein hydrolysed h^{-1}. Although 1.15 protease is not active against 5920, an assay was included to show a more complete picture of bacteriocin production. Similarly, 5920 microcin production was also assayed, as percentage reduction in the OD_{570} of microtitre plate cultures of 1.15.

4.4a)



4.4b)

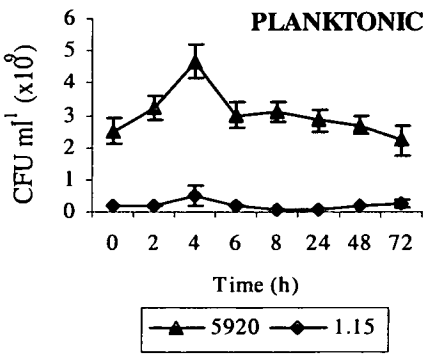
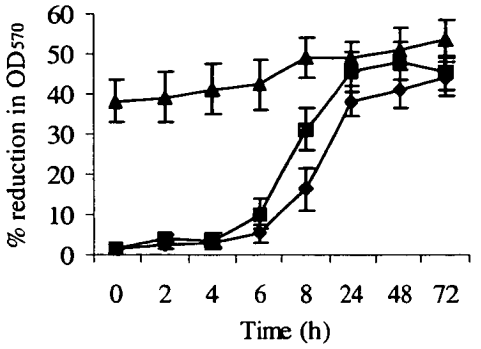


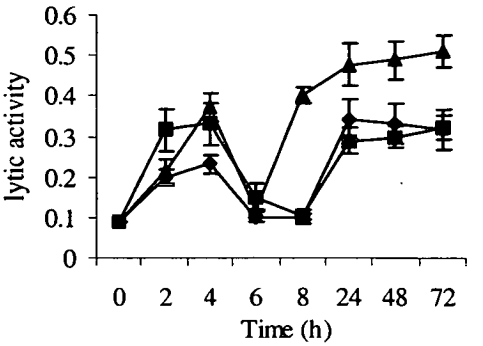
Figure 4.4

The invasion of a pre-established 5920 biofilm (fig 4.4a) and planktonic (fig 4.4b) cultures by the strain 1.15 was examined. The inoculum was adjusted to allow for a 20:1 ratio of cells. Viable counts were estimated over a 72 h period. Results are the outcome of 4 replicate experiments, and the bars represent standard error.

4.5a) 5920 microcin



4.5b) 1.15 microcins



4.5c) 1.15 protease

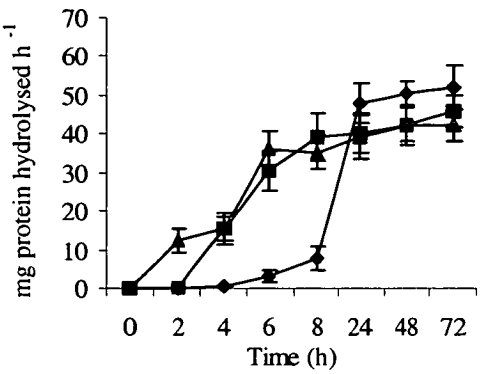


Figure 4.5

The different competition strategies used when 5920 and 1.15 were inoculated simultaneously (competitive cultures ■), and as 1.15 invaded a stationary phase biofilm of 5920 (invasive cultures ▲) were compared. The inoculum was adjusted to contain a 20:1 ratio of cells, with 5920 being the dominant strain in each case. Bacteriocin production by single. sp controls ◆ were included. 5920 microcin was estimated by the percentage reduction in microtitre plate cultures of strain *Ent* (fig 4.5a). 1.15 microcin was estimated by the lysis of 5920. This releases nucleic acid that can be read at 260 nm (fig 4.5b). Protease activity was measured as protein hydrolysed h^{-1} (fig 4.5b). Results are the outcome of 4 replicate experiments and bars represent SE.

There was only a marginal increase in the number of cells adhering to the glass beads in the invasive 5920/1.15 biofilm. Cells reached a maximum of 2.14 x 10^7 CFU cm^{-2} (± 1.97 x10^5) in the invasive biofilm (fig 4.4a), compared to 19.98 x 10^6 CFU cm^{-2} (± 1.54 x 10^5) in the competitive biofilms (fig 3.8f). Also unlike the *Ent*/1.15 invasive biofilms, there was no drop in bacteriocin production (fig 4.5). 5920 microcin was actually increased at 8 h (fig 4.5). The biofilms of *Ent* when antagonised with 1.15 formed a co-operative biofilm. The biofilms of 5920/1.15 were competing, not co-operating.

An early induction of bacteriocin production occurred in many of the competitive and invasive biofilms. This was particularly apparent with 1.15 protease production (fig 4.5c).

4.3 BACTERIOCIN REPRESSION IN *Ent*/1.15 INVASIVE CULTURES

4.3.1. *Addition of Extra Nutrients*

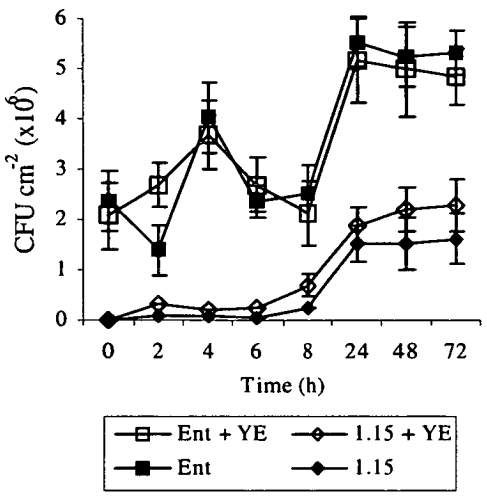
It was thought that the switching off of bacteriocin production as 1.15 integrated a pre-established culture of *Ent* may be due to nutrient depletion; there is no point in competing if there is nothing to compete for. This was investigated by adding additional glucose (1% w/v), yeast extract (0.1% w/v) and casein hydrolysate (0.1% w/v) to pre-established biofilms of *Ent*, along with the usual 1.15 inoculum. As a comparison, this experiment was repeated with 5920/1.15 biofilms.

Viable counts (**figure 4.6a**) and bacteriocin production (**figure 4.7**) of biofilms of *Ent* antagonised with 1.15 were monitored over a 72 h period. The experiment was repeated for 5920 biofilms antagonised with 1.15 (figures 4.6b and 4.8). Slight increases in bacteriocin production were seen for the cultures with additional nutrients (fig 4.7a + 4.7b). However, figure 4.6 shows there were no differences between the biofilms with and without additional nutrients for both *Ent*/1.15 and 5920/1.15.

4.3.2 *Investigation of Bacteriocin Inhibitors*

There was the possibility that an inhibitor of bacteriocin activity was being produced in the *Ent*/1.15 invasive cultures. 1.15 spent media was separated into two fractions, <10 KDa and <100 >10 KDa, using Millipore Vivacell 70 membrane filters. This separated the protease from the smaller 1.15M-8. A fraction containing 1.15 M-4 was also prepared. *Ent* microcin was filtered through a 10 KDa cut-off filter. Spent media from the *Ent*/1.15 (72 h) was filter-sterilised and added to the bacteriocin fractions to give a 50:50 mix of solutions. A control with no *Ent*:1.15 spent media was included. Each solution was assayed for the activity of bacteriocins in the usual fashion. Addition of spent media from an invasive culture of *Ent*:1.15 caused no reduction in the activity of the bacteriocins.

4.6a) *Ent*/1.15 biofilms



4.6b) 1.15/5920 biofilms

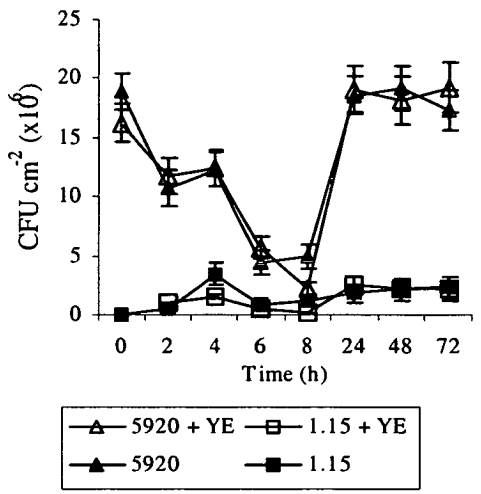
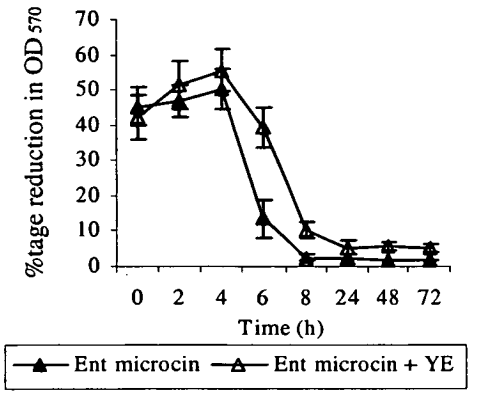


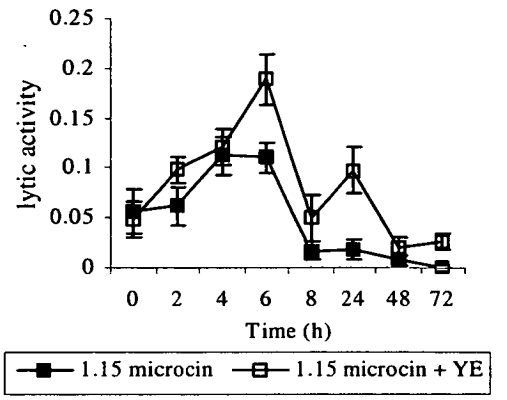
Figure 4.6

To investigate the possibility that the co-operative relationship between *Ent*/1.15 (fig 4.6a) was due to nutrient depletion, extra nutrients were added to the cultures. This was compared to the non-co-operative culture of 1.15/5920. Cultures without the addition of extra nutrients are also shown as a comparison. Cultures were incubated at 30 °C with shaking, and viable counts were taken over a 3 day period. Addition of extra nutrients did not affect the secondary colonisation of the strain 1.15. Results are the outcome of 3 replicate experiments and bars represent standard error.

4.7a) *Ent* microcin



4.7b) 1.15 microcin



4.7c) 1.15 protease

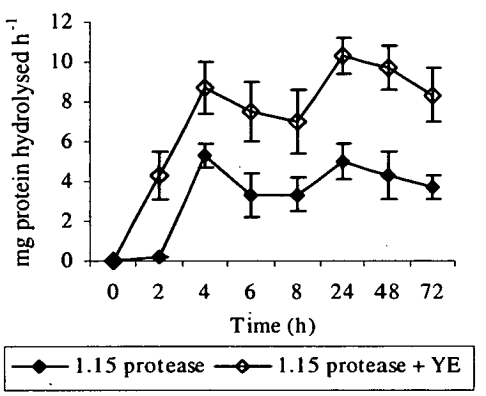
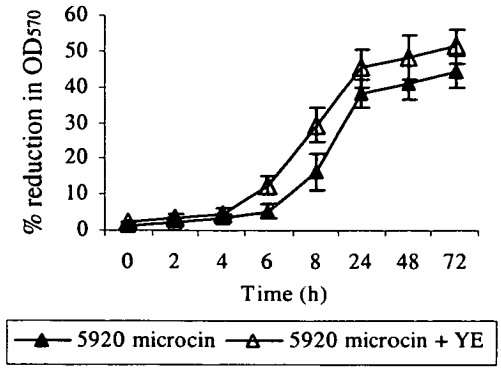


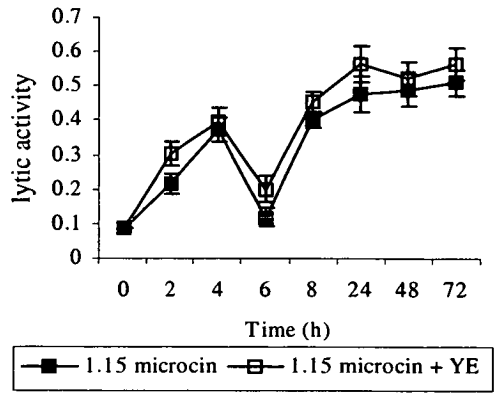
Figure 4.7

The effect of addition of extra nutrients on bacteriocin production in pre-established biofilms of *Ent* antagonised with 1.15 were examined. Cultures that did not receive additional nutrients are included for comparison. *Ent* microcin was estimated by the percentage reduction in OD_{570} of cultures of 1.15 (fig 4.7a). 1.15 microcin was estimated by the lysis of *Enterobacter agglomerans*/53b (fig4.7b). This releases nucleic acid that can be read at 260 nm. Protease activity was measured as protein hydrolysed h^{-1} (fig4.7c). Biofilm with additional nutrients showed a slight increase in bacteriocin production. Results are the outcome of 4 replicate experiments and bars represent SE.

4.8a) 5920 microcin



4.8b) 1.15 microcins



4.8c) 1.15 protease

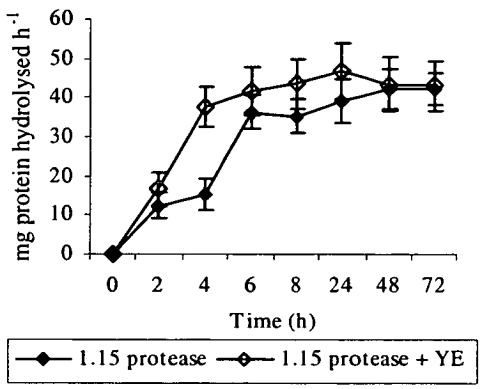


Figure 4.8

The effect of addition of extra nutrients on bacteriocin production in pre-established biofilms 5920 antagonised with 1.15 was examined. Cultures that did not receive additional nutrients were included for comparison. 5920 microcin was estimated by the percentage reduction in OD_{570} of cultures of *Ent* (fig 4.8a). 1.15 microcin was estimated by the lysis of 5920 (fig4.8b). This releases nucleic acid that can be read at 260 nm. Protease activity was measured as protein hydrolysed h^{-1} (fig4.8c). Biofilm with additional nutrients showed a slight increase in bacteriocin production. Results are the outcome of 4 replicate experiments and bars represent SE.

4.3.3 *Changes to Cell Surface*

The strain *Ent* was involved in co-operative biofilm formation with a *K. pneumoniae* strain (Skillman *et al.*, 1999). In this case, the EPS produced by *Ent* played an important role in the dual species biofilm formation, acting both as an adhesin and as the EPS of both strains interacted, changing their physical properties. EPS production was increased in the co-operating *Ent*/G1 biofilm. The production of EPS by the *Ent* and 1.15 in single, competitive and invasive biofilms was investigated. An assay of the total cell carbohydrate of 24 h biofilms revealed *Ent* produced 3.58 μg carbohydrate cm^{-2} (± 0.2 μg cm^{-2}), 1.15 produced 5.32 μg carbohydrate cm^{-2} (± 0.32 μg cm^{-2}), the competitive biofilm 3.98 μg cm^{-2} (± 0.26 μg cm^{-2}) and the invasive biofilms 4.32 μg cm^{-2} (± 0.31 μg cm^{-2}). This indicated that the co-operating 'invasive' biofilms were not producing more EPS than the single species biofilms.

The monosaccharide components of the single and dual species biofilms were also analysed by HPLC. **Table 4.1** is a representation of the molar ratios of monsaccharide constituents of *Ent* and 1.15 single species biofilm EPS, the competitive biofilm EPS and EPS from the *Ent* biofilm antagonised with 1.15. The *Ent*:1.15 ratio of cells in the competitive biofilm was 3:5, and in the invasive biofilm, 10:3 (table 4.2). Examination of table 4.1 indicates that in the dual species biofilms, *Ent* was contributing more EPS to the biofilm than 1.15. This is particularly clear in the invasive biofilm. The molar ratio of fucose drops from 1.0 in a 1.15 single species biofilm to 0.1 in the dual species biofilm, indicating 1.15 contributed only 10% of the EPS, and approximately 23% of the cells in the dual species invasive biofilm. This was probably due to the poorly soluble nature of *Ent* EPS.

	Fuc	Rha	Gal	Glc	Man
Ent		0.15	0.75	1	0.2
1.15	1		1.3	1	
Ent/1.15 Comp.	0.5	0.1	0.5	1	
Ent/1.15 Inv.	0.1	0.1	0.85	1	0.1

Table 4.1

Molar ratios of the monosaccharide components of *Ent* and 1.15 single species biofilms, and the competitive and invasive dual species biofilms.

4.3.4 *Investigation of Activities of* Ent *and 1.15 with Other Strains*

To understand why *Ent* and 1.15 form a co-operating biofilm, the interaction of 1.15 and *Ent* with other strains was investigated. The pairs *E. coli*/1.15 and *Ent*/53b were chosen, with *E. coli* and *Ent* being the pre-established strain within the pair. Table 3.4 showed that *E. coli* and *Ent* each produce a microcin active against the respective strain, and 53b produced a larger, broad-spectrum bacteriocin with activity against *Ent*. The combination of *Ent* and 1.15 where 1.15 is the dominant, pre-established strain within the pair was also included to see if this biofilm could also co-operate. Data for the pairs *Ent*/1.15 and 5920/1.15, obtained from figures 4.3 and 4.5, were also included as a comparison. Amounts of *E. coli* bacteriocin were estimated by the lysis of 1.15 cells, and 53b were calculated by lysis of *Ent* cells.

Single species biofilms were grown to stationary phase and the second, competitive strain added. The biofilms were incubated for 72 h, and then the ratios of each pair, in each strain calculated by viable counts. The bacteriocin production by each strain was also investigated. This process was also repeated for the competitive biofilms, where each strain was inoculated simultaneously. In all cases, the inoculum was adjusted to allow a 20:1 ratio of cells. The results of these experiments are shown in **table 4.2**. The dominant strain in each case is underlined. The amount of bacteriocin produced by a 72 h single species biofilm was calculated for each of the strains, and this was assigned a value of 1. The figures given for bacteriocin production in the dual species biofilms are relative to this figure, taking into account both bacteriocin production and the ratio of cells within the dual species biofilm producing that amount of bacteriocin.

A biofilm of *Ent* antagonised with another *Enterobacter agglomerans* strain, 53b also co-operates. Bacteriocin production again seemed to have been switched off. The dual species biofilms of 1.15/*Ent*, 5920/1.15 and *E. coli*/1.15 biofilms are competing. Competing biofilms produce more bacteriocin. Often, the smaller the size of the subordinate population within the biofilm, the more bacteriocin was produced. This can be seem clearly with the *E. coli*/1.15 biofilms. A ratio of 25 *E. coli* cells to 1 1.15 cell was reached in the invasive biofilm, and a ratio of 15:1 in the competitive biofilm. 1.15 produced more microcin and protease in the invasive '25:1' biofilm than in the competitive '15:1' biofilm. The same scenario occurred with the biofilms

of 5920/1.15. More bacteriocin was being produced in the invasive biofilm, where ratios of 10:1 were reached than in the competitive biofilm, where the strains reached a status quo of 5:1. As each type of biofilm always received a 20:1 inoculum, this also demonstrated that it is harder to establish a population amongst a pre-established biofilm than when a clean surface is being colonised along with a competing partner.

		INVASIVE BIOFILM			COMPETITIVE BIOFILM			
STRAINS	Ratio of cells at 72 h	Bacteriocin Production		Protease	Ratio of cells at 72 h	Bacteriocin Production		Protease
Ent/1.15	10:3	*Ent* **0.15**	1.15 **0.26**	**0.09**	3:5	*Ent* **3.27**	1.15 **2.45**	**1.9**
1.15/Ent	9:2	1.15 **5.35**	Ent **2.17**	**2.43**	2:1	1.15 **3.32**	Ent **1.48**	**1.43**
Ent/53b	10:1	*Ent* **0.38**	53b **0.27**	_	7:2	*Ent* **1.47**	53b **4.5**	_
5920/1.15	10:1	5920 -	1.15 **10.4**	**5.01**	5:1	5920 -	1.15 **4.83**	**4.89**
E.coli/1.15	25:1	*E.coli* **1.29**	1.15 **7.21**	**10.87**	15:1	*E.coli* **1.23**	1.15 **5.61**	**6.02**

Table 4.2

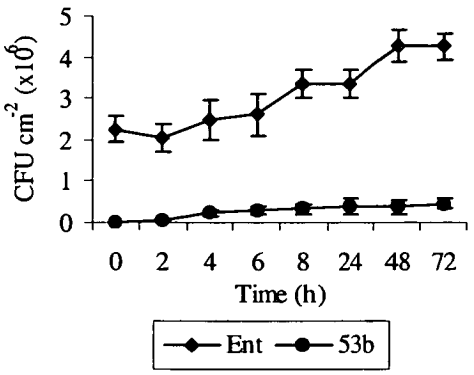
The interaction of 1.15 and *Ent* with other strains, 53b and *E. coli* was investigated. Competitive biofilms are where the strains were inoculated simultaneously, and the invasive biofilms where a second, competitive strain was added to a stationary phase biofilm. The initial inoculum was adjusted to a 20:1 ratio of cells. Strains underlined were the dominant strain in each pair. The amount of bacteriocin produced in a 72 h single species culture was assigned the value of 1, and the amounts produced in the competitive and invasive biofilms are relative to this figure. The ability to co-operate in a competitive biofilm may be an *Ent*-associated effect as addition of 53b to *Ent* also produces a co-operating biofilm. Competing biofilms demonstrated increased bacteriocin production.

4.3.5 *Invasion of* Ent *Pre-Established Biofilms*

Results in table 4.1 indicated that 53b integration into a pre-established *Ent* biofilm also produced a co-operating biofilm with reduced bacteriocin production. This was further investigated by examination of the invasion of 53b into pre-established *Ent* biofilms over a 72 h period using viable counts (**figure 4.9**) and assays of bacteriocin production (**figure 4.10**). *Ent* microcin production was calculated using the percentage reduction in OD_{570} microtitre plate cultures of 1.15, and 53b bacteriocin activity by the lysis of *Ent* cells (measured at A_{260}). Viable counts and assays of bacteriocin activity of *Ent* and 53b single sp. biofilms were included as controls.

A competitive biofilm where *Ent* and 53b were inoculated simultaneously, in a 20:1 ratio, contained 2.66×10^6 CFU cm^{-2} ($\pm$ 1.41×10^5). Figure 4.9 shows an invasive biofilm of *Ent*/53b reached 4.69×10^6 ($\pm$ 3.2×10^5). Unlike the single sp. biofilms, the competitive biofilm did not slough off at 72 h. Although not as marked as the *Ent*/1.15 invasive biofilm (fig 4.5), there was also a reduction in both *Ent* and 53b bacteriocin activity (fig 4.10). The *Ent*/1.15 invasive biofilm switched off bacteriocin production at 8 h, and the *Ent*/53b invasive biofilm switched off bacteriocin production after 24 h.

4.9a) Invasive biofilm



4.9b) Single sp. biofilms

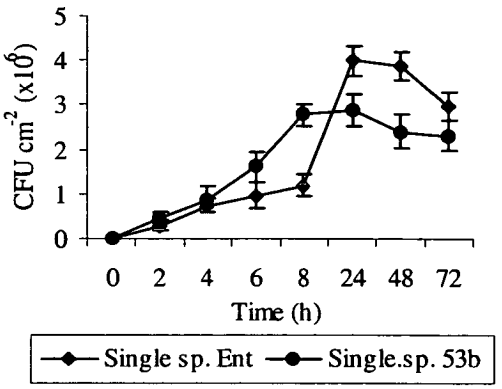


Figure 4.9

The invasion of an *Ent* pre-established biofilm by 53b was examined (fig 4.9a). The inoculum was adjusted to allow for a 20:1 ratio of cells. Single sp. *Ent* and 53b biofilms are shown as a control (fig 4.9b). Viable counts were estimated over a 72 h period. Results are the outcome of 4 replicate experiments, and the bars represent standard error.

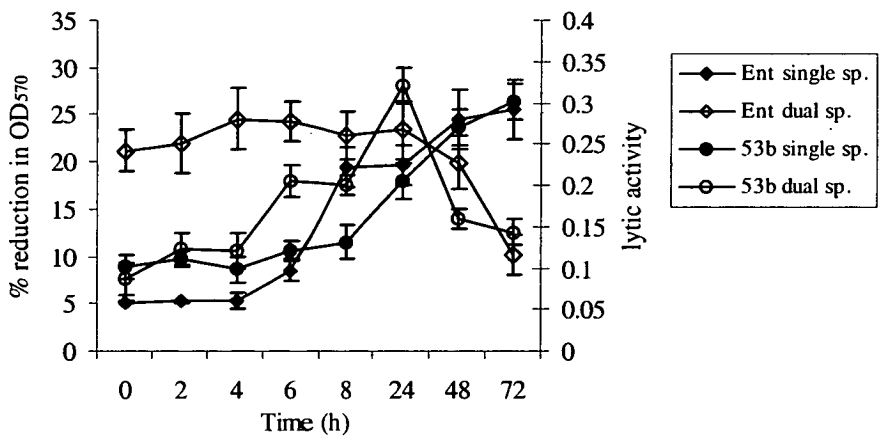


Figure 4.10

Bacteriocin production by single sp. and invasive biofilms of *Ent* and 53b was examined. The 53b inoculum in the dual species biofilm was adjusted to allow a 20:1 ratio of cells. *Ent* microcin was estimated by the percentage reduction in OD_{570} microtitre plate cultures of 53b. 53b bacteriocin was estimated by the lysis of *Ent*. This releases nucleic acid that can be read at 260 nm. In the *Ent*/53b dual species biofilm bacteriocin activities were switched off at 24 h. Results are the outcome of 4 replicate experiments and bars represent SE.

4.3.6 *Addition of* Ent/*1.15 Spent Media to* E.coli *biofilms*

Spent media (25% v/v) from a co-operating culture of *Ent*/1.15 was added to pre-established *E. coli* biofilms. Again viable counts and *E. coli* microcin production was monitored over 72 h (**figure 4.11**). As a control, samples of the co-operating spent media were sterilised by autoclaving and also added to biofilms of *E. coli*. The *E. coli* biofilms were expected to increase in size due to the addition of the co-operating media. Figure 4.11 indicated that there was an increase in the number of cells adhering to the glass beads, and also a slight decrease in microcin production. *E. coli* and 1.15 are capable of producing microcins with activity against each other. 1.15 was added along with the co-operating spent media to biofilms of *E. coli*, and viable counts examined (**figure 4.12**). This showed that while there was an increase in biofilm size 6 h after the addition of the co-operating spent media and 1.15, the end-products of each biofilm were identical: each contained identical CFU cm^{-2} for *E. coli* and 1.15.

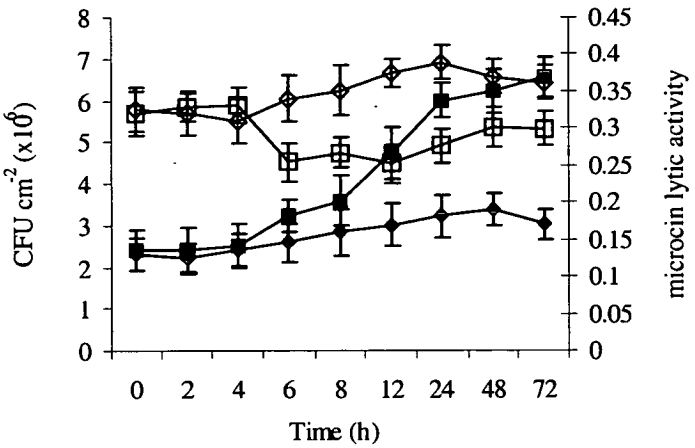


Figure 4.11

Filter-sterilised spent media from co-operating cultures of *Ent*1.15 was added to pre-established biofilms of *E. coli* and viable counts ■ and microcin production ◆ monitored. *E. coli* microcin production was estimated by lytic activity on 1.15 cells. As a control, heat sterilised spent media was added to *E. coli* biofilms and viable counts • and microcin production ◇ again monitored. An increase in biofilm size and decrease in microcin production was seen in the cultures with filter-sterilised spent media. Results are the outcome of 3 replicate experiments, and results represent standard error.

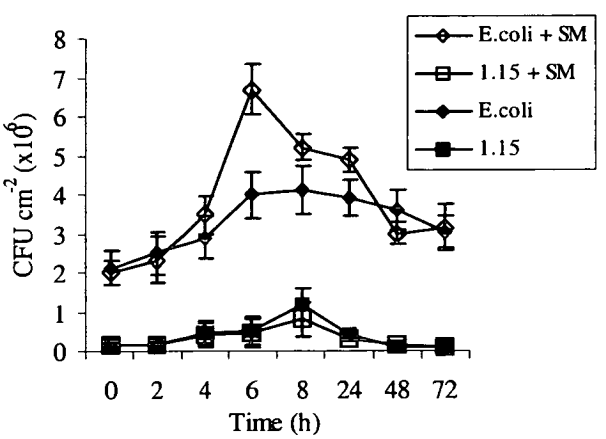


Figure 4.12

1.15 cells were added to pre-established biofilms of *E. coli* along with filter-sterilised spent media from a co-operating *Ent*1.15 biofilm. Viable counts were estimated over a 72 h period. Results are the outcome of 3 replicate experiments, and bars represent standard error.

4.3.7 *Fluorescent Microscopy of Co-operating Biofilms*

The GFP-variant of *Ent* was grown overnight on glass slides. The strains 1.15 or 53b were added to the biofilms, and incubated for a further 24 h, before examining microscopically. The red-fluorescent pigment propidium iodide was used to highlight the strains 1.15 and 53b (**figure 4.13**). This demonstrates that unlike the competitive biofilms, where each strain formed distinct, separate microcolonies, the microcolonies in these biofilms contained a mixture of the two strains.

Fig 4.13a) Co-operating *EntGFP* and 1.15 biofilm (bar = 100 μm)

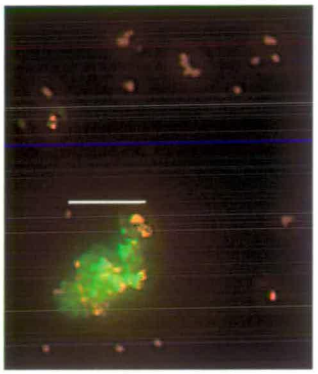


Fig 4.13b) Co-operating *EntGFP* and 53b biofilm (bar = 150 μm)

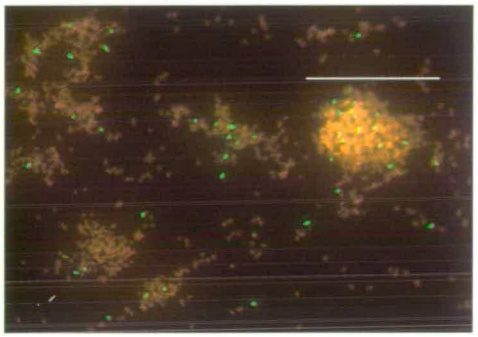


Figure 4.13

Fluorescent microscopy of co-operating biofilms of *EntGFP*/1.15 (fig4.12a) and *EntGFP*/53b (fig4.12b). Propidium iodide was used to stain 1.15 and 53b red.

4.3.8 *Disinfection of Invaded Biofilms*

The biofilm eradicating concentrations (BEC) of HPC, BC and TR against single and invasive dual species biofilms was investigated. The dual species pairs *Ent*/1.15, *Ent*/53b, 1.15/*Ent*, 5920/1.15 and *E. coli*/1.15 were selected. The BEC was taken as the lowest concentration of disinfectant to prevent any re-growth. Theoretical results are given based on the concentration required to eradicate the single species biofilms, and the ratio of cells in the dual species biofilms (**table 4.3**).

The competing biofilms 1.15/*Ent* and *E. coli*/1.15 were less resistant to disinfection in each case, whereas the co-operating biofilms *Ent*/1.15 and *Ent*/53b are more resistant. Interestingly, the dual sp. biofilms of 5920/1.15, although competing, were only marginally less resistant to disinfection than expected.

STRAINS	BC (mg/ml)		HPC (%)		TR (μg/ml)	
Ent	0.4		0.018		45	
1.15	0.4		0.03		55	
5920	0.45		0.023		55	
53b	0.4		0.018		45	
E. coli	0.4		0.018		50	
	THEORETICAL	ACTUAL	THEORETICAL	ACTUAL	THEORETICAL	ACTUAL
Ent/1.15	0.4	0.47	0.02	0.025	47.31	50
Ent/53b	0.4	0.45	0.018	0.02	45	50
1.15/*Ent*	0.4	0.35	0.028	0.02	53.18	35
5920/1.15	0.45	0.45	0.024	0.025	55	50
E.coli/1.15	0.4	0.35	0.019	0.015	50.31	30

Table 4.3

The biofilm eradicating concentrations of sodium hypochlorite (HPC), benzalkonium chloride (BC) and triclosan (TR) against single and invasive biofilms of *Ent*/1.15, 1.15/*Ent*, *Ent*/53b, 5920/1.15 and *E. coli*/1.15. The BEC was taken as the lowest concentration of disinfectant to prevent any re-growth. Theoretical results are given based on the concentrations required to eradicate the single species biofilms and the ratio of in the dual species biofilms. The competitive biofilms (1.15/*Ent*, 5920/1.15 and *E. coli*/1.15) were less resistant to disinfection in each case, while the co-operating biofilms (*Ent*/1.15 and *Ent*/53b) were more resistant. Results are a consensus of 5 replicate experiments.

4.4 DISCUSSION

Examination of the biofilm produced by 1.15 as it colonised a clean surface along with *Ent*, and as the strain invaded a stationary phase biofilm of *Ent* showed considerable differences in the competition strategies used by 1.15 (fig 4.3). The *Ent* biofilm antagonised with 1.15 did not produce any bacteriocin after 8 h: the strains began to co-operate. (fig 4.3). In comparison, the simultaneous colonisation of a surface by *Ent* and 1.15 lead to a competitive state. This also caused an early induction of *Ent* microcin synthesis (fig 4.3a). Conversion to the co-operating state is advantageous to the bacteria that dwell within the biofilm: the resultant biofilm was thicker, and as no sloughing off of biofilm material occurred, also more stable. Competition between the strains caused a decrease in the resistance of the biofilms to sodium hypochlorite, benzalkonium chloride and triclosan (table 3.7). However, examination of the *Ent* biofilms antagonised with 1.15 demonstrated an increase in resistance. It must be noted, however, that this phenomenon was not unique to the biofilm cultures, the same switching off of bacteriocin production also occurred within the invasive planktonic cultures (fig3.2).

The addition of 1.15 to pre-established 5920 biofilms did not produce a co-operating biofilm: this biofilm continued to compete. Again an early induction of bacteriocin-production was seen, demonstrating that the close proximity of a competitive strain can cause increased bacteriocin production. Table 4.1 exemplifies this fact. In some cases, competition between two bacteriocin-producing strains caused a 10-fold increase in bacteriocin production, for example, protease production by 1.15 in a dual species *E. coli*/1.15 biofilm.

It was thought that the lack of bacteriocin production was due to nutrient depletion: there was no point in competing in a nutrient depleted environment. However, addition of extra glucose, yeast extract and casein hydrolysate when 1.15 was inoculated into the *Ent* cultures did not cause bacteriocin production to continue. There was also the possibility that the bacteriocins could have an inhibitory effect on the other bacteriocins. However, further investigation of this showed no inhibition was occurring. The decrease in bacteriocin at 8 h was a true switch off of production. Investigation of the interaction of *Ent* and 1.15 with other strains indicated that an *Ent* biofilm antagonised with another strain, 53b, also co-operates. There was a

similar decrease in bacteriocin production after 24 h, and this biofilm was, like the co-operating *Ent*/1.15 biofilm, more resistant to disinfection (table 4.3). It, therefore, seems to be an *Ent*-associated phenomenon, and there was perhaps some signalling on *Ent*s part that caused this co-operating state. Certainly, addition of spent media from a co-operating *Ent*:1.15 biofilm to *E. coli* biofilms also caused an increase in adhesion. A slight decrease in microcin production was also detected (fig 4.11). This signal was ignored, however, upon the inoculation of 1.15 into the *E. coli* biofilms (fig 4.12). Competing strains in biofilms can, therefore, sometimes co-operate to produce a thicker, more stable biofilm that is also less resistant to disinfectants.

In the addition of 1.15 or 53b to *Ent* pre-established biofilms, *Ent* is the first to switch off bacteriocin production. The fact that 1.15 cannot form a co-operative biofilm with the strains 5920 or *E. coli* also suggests that *Ent* initiates co-operation. Likewise, the invasion of a pre-established biofilm of 1.15 by strain *Ent* continued to produce bacteriocin, and did not produce a co-operative state. Addition of *E. coli* to *Ent* pre-established biofilms (fig 3.3a) does not result in thicker biofilm formation, suggesting these strains also do not form a co-operating dual species biofilm. Only when a pre-established *Ent* biofilm is sensitive to the bacteriocin produced by the antagonistic, invading strain produces an inhibition of bacteriocin production in a secondary colonising strain. The signal produced only in these co-operating biofilms was also capable of increasing the adhesion of *E. coli* (fig 4.11).

The exact nature of this signal is unknown. However, this alloinhibitory action of *Ent* on the strains 1.15 and 53b is not uncommon. A similar situation was described for a pathogen-suppressive strain of *S. diastatochromogenes* (Becker *et al.*, 1997). Addition of filter-sterilised spent media from a range of *Streptomyces* strains isolated from soil to cultures of *S. diastatochromogenes* stimulated, suppressed or had no affect on antibiotic production by the strain. The quorum sensing signalling molecules, acyl-homoserine lactones, allow population density-dependent gene regulation within a species, as well interspecies communication amongst different bacteria (Parsek and Greenberg, 2000). In *S. aureus*, the autoinducer Accessory Gene Regulator (*agr*) regulates the expression of a number of virulence factors. These include agents to allow attachment to host cells, defence mechanisms to avoid elimination by the host, and promotes bacterial internalisation. The autoinducers

produced by various *S. aureus* strains are known to vary, and can specifically induce Agr-mediated quorum sensing in the strains that produce the factor, or inhibit Agr-mediated quorum sening in the strains producing a different autoinducer (Bassler, 1999). The macroalgae *Delisea pulchra* is capable of controlling bacterial colonisation by interference with the HSL regulatory system. *D. pulchra* produces a number of halogenated furanones that are structurally similar to HSLs. These furanones were shown to inhibit bioluminescense by *V. fischeri* and swarming motility in *Serratia liquefaciens*, also an AHL regulated process (Giskov *et al.*, 1996). It has speculated that bacteriocins also mediate intraspecific or population-level interactions (Riley, 1998). Lack of such as signal, such as through the inhibition of bacteriocin production, may lead to an increase in cell densities, and thicker biofilm formation, as seen in the co-operating biofilms.

The signal produced by *Ent* is only triggered under high population densities, such as in a biofilm, or a stationary phase planktonic culture. Perhaps this has evolved as a means of protecting biofilms of *Ent* from competing strains.

4.4.1 *Conclusion*

A biofilm of *Ent* antagonised with a bacteriocin-producing competitor was shown to produce a co-operating state. These biofilms were thicker, more stable and demonstrated an increased resistance to disinfectants. This was correlated with a cease of bacteriocin production. A signal produced by *Ent* was thought to cause this co-operative effect.

Chapter 5

INTERACTIONS OF BACTERIOPHAGE WITH DUAL SPECIES BIOFILMS

5.1 INTRODUCTION

Hughes *et al.* (1998a,b) provided evidence to suggest that bacteriophage and their associated polysaccharide depolymerases would be very useful, highly specific tools for the study of biofilms. The polysaccharide material surrounding bacteria in a biofilm was initially thought to provide protection from bacteriophage attack. However, studies by Hughes *et al.* (1998a,b) have shown that phage possessing specific polysaccharide depolymerases were able to degrade a pathway through the biofilm EPS to gain access to the bacterial surface. It was shown that the majority of bacteria were being removed from the biofilm due to the action of the enzyme before the cells had a chance to lyse. Adding phage to biofilms containing a phage-resistant strain of *Ent. agglomerans*, and again where only soluble enzyme was added to the biofilm, the action of the phage enzyme alone still removed a substantial quantity of biofilm material. It was hypothesised that if the host bacteria produced exopolysaccharide, phages with polysaccharide depolymerase activity could be used to selectively remove the polysaccharide from the biofilm.

The high specificity of phage also makes them particularly useful tools to study mixed species biofilms. In a dual species biofilm, phage could be used to selectively attack a single species. However, in a synergistic dual species biofilm of *Ent. agglomerans* and *K. pneumoniae*, addition of a phage enzyme specific for the EPS of the *Ent. agglomerans* strain removed both the bacteria and the biofilm (Skillman *et al.*, 1999b). The inability of a strain-specific phage to eradicate one strain in this mixed species biofilm was thought to be due to the close confinements of the strains within the biofilm. Further studies in our laboratory have indicated that this removal of both species in a dual species biofilm by a strain-specific phage may also have been due to the high titres of phage used. It was indicated that smaller titres of phage might be more successful in selectively removing one species (Napier & Sutherland, unpublished results).

Smaller titres of bacteriophage will, therefore, be used as a tool to study the structure of dual species biofilms. The bacteriophage selected will also contain polysaccharide depolymerase activity to enable the effects of intact phage and phage-free enzyme extracts to be compared. This will allow the role of a single bacterial species in a mixed species biofilm to be determined.

5.2 ISOLATION OF BACTERIOPHAGE

5.2.1 *Isolation of Bacteriophage from Primary Effluent Sewage*

Bacteriophage specific for the strains *Ent*, 5920, S53 and the standard disinfectant strain *E. coli* were isolated, purified and concentrated from primary effluent sewage. A phage for 1.15 (ϕ1.15) and phage specific for *Klebsiella* sp. (K34 and K60) had previously been isolated. The phage ϕE.coli and Phil were isolated for the strain *E. coli.* ϕEnt and Armadale phage were isolated for strain *Ent.* The phage ϕ5920, Blackburn and Winchburgh were isolated for strain 5920. Phage ϕS61 was isolated for strain S61 and East Calder (E.Calder) were isolated for the strain S53.

5.1.2 *Selection of Bacteriophage for Dual Species Biofilms*

The aim of this work was to investigate the effect of bacteriophage on dual species biofilms. The strains used in the dual species biofilm studies depended on the range of activities of the bacteriophage isolated. For experimental purposes, a phage that would selectively lyse only one species of bacteria in a dual species biofilm was required. The isolated phages were, therefore, tested for activity against a wide range of *E. coli*, *Enterobacter*, *Klebsiella* and *Serratia* species.

Screening for Bacteriophage and Depolymerase Activity

The action of each bacteriophage was tested by spotting 10μl of phage solution onto freshly poured lawns of bacteria. The presence of polysaccharide depolymerase activity, seen as an EPS-free halo of bacteria surrounding the plaque, was also investigated. Table 5.1a) show the activities against a range of *E. coli* and *Enterobacter* species, and 5.1b) the activities against *Klebsiella* and *Serratia* species. The phage ϕ*E. coli*, ϕ*Ent*, ϕ1.15, ϕ5920, ϕS53, Armadale, Blackburn, E.Calder, Phil and Winchburgh had no activity against the *Klebsiella* and *Serratia* strains, and so were not included in the tables. Examination of the activities of the phages shows that a combination of an *Enterobacter* strain with either a *Klebsiella* or *Serratia* strain was possible. For example, *Ent. agglomerans*/53b and *K. oxytocei*/109c or *Ent. cloace*/5920 and *S.liquefaciens*/5Xa.

5.1a) *E. coli* and *Enterobacter* strains

PHAGE NAME	PHAGE ACTIVITY	*Escherichia coli* strains					*Enterobacter* strains			
		E.coli	K12	S53	*E.coliB*	Eck5	*Ent*	1.15	53b	5920
ϕE.coli	Phage	+	+	+	−	+	−	+	+	+
	Enzyme			+			−	+	+	+
ϕEnt	Phage	−	−	+	−	−	+	+	+	+
	Enzyme			+			+	+	+	+
ϕ1.15	Phage	+	−	+	−	+	−	+	+	+
	Enzyme			+			−	+	+	+
ϕ5920	Phage	−	+	+	−	−	+	+	+	+
	Enzyme			+			+	+	+	+
ϕS61	Phage	−	−	−	−	−	+	+	+	+
	Enzyme			−			+	+	+	+
Armadale	Phage	+	−	+	−	−	+	+	+	+
	Enzyme			+			+	+	+	+
Blackburn	Phage	+	−	+	−	−	−	+	+	+
	Enzyme			+			−	+	+	+
E.Calder	Phage	−	−	+	−	+	+	+	+	+
	Enzyme			+			+	+	+	+
Phil	Phage	+	+	+	−	+	+	+	+	−
	Enzyme			+			−	+	+	−
Winchburgh	Phage	−	+	+	+	+	−	+	−	+
	Enzyme			+			−	+	−	−
K34	Phage	−	+	+	+	−	+	−	−	−
	Enzyme			+			+	−	−	−
K60	Phage	+	+	+	+	−	+	−	−	−
	Enzyme			+			+	−	−	−

5.1b) *Klebsiella* and *Serratia* strains

PHAGE NAME	PHAGE ACTIVITY	*Klebsiella* strains						*Serratia* strains					
		109c	86b	G1	107b	K66	D451.1	XM6	5Xa	Pd3a	87b	A1	A4
K34	Phage	+	+	–	+	–	–	–	+	–	+	–	–
	Enzyme	–	–	–	–	–	–	–	–	–	–	–	–
K60	Phage	+	+	–	+	–	–	–	+	–	+	–	–
	Enzyme	–	–	–	–	–	–	–	–	–	–	–	–

The action of each bacteriophage and polysaccharide depolymerase activity on a range of *E.coli* (5.1a), *Enterobacter* (5.1b), *Klebsiella* (5.1b) and *Serratia* (5.1b) species was tested by spotting 10µl of phage solution onto freshly poured lawns of bacteria. Cultures were incubated at 30 °C for 24 h. The production of a plaque was indicative of phage activity (+). The presence of an EPS-free halo surrounding the plaque was used as an indication of polysaccharide depolymerase activity (+). No activity (–). The phage ϕ*E.coli*, ϕ*Ent*, ϕ1.15, ϕ5920, ϕS53, Armadale, Blackburn, E.Calder, Phil and Winchburgh had no activity against the *Klebsiella* and *Serratia* strains, and so were not included in the tables. Results are a consensus of 5 replicate experiments.

Concentration of Phage K34 and K60

It was attempted to prepare concentrated solutions of the phage K34 and K60. However, a solution concentrated 100-fold still produced very low titres: 3.12×10^3 phage ml^{-1} ($\pm$ 1.12×10^2). The phage burst times of the two phages were also investigated. Planktonic cultures of *Klebsiella oxytocei*/109c was grown to mid-log phase and phage solutions added to the cultures. The OD_{600} of the cultures were monitored over a 24 h period. A sudden drop in OD_{600} indicated bacterial cell lysis and the release of the phage particles. However, there was no clear drop in the culture OD (**figure 5.1**). It was, therefore, thought that K34 and K60 phage were temperate, not lytic phage, and were unsuitable for these studies.

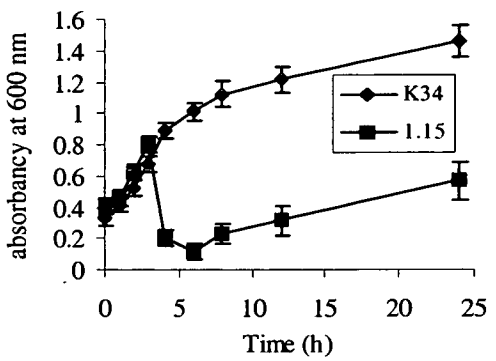


Figure 5.1

Estimation of phage burst times of K34 phage using the strain 109c as a host. The addition of phage ϕ1.15 to the strain 1.15 is shown as a comparison. During mid-log phase, the cultures were infected with bacteriophage and the absorbency read at 600 nm. A sudden drop in OD indicated bacterial cell lysis and the release of phage particles. A clear drop in OD was seen for ϕ1.15, but not for K34. Results are the outcome of 3 replicate experiments and bars represent standard error.

Selection of a Pair of Bacteria for Dual Species Biofilm Studies

Due to the unsuitability of phage K34 and K60, the pair *Ent*/5920 and the phage Phil, ϕ1.15 and Winchburgh were selected for further study. ϕ1.15 has phage and enzyme activity against 5920, and Winchburgh has phage activity. Phil was active against *Ent*, again with no enzyme activity.

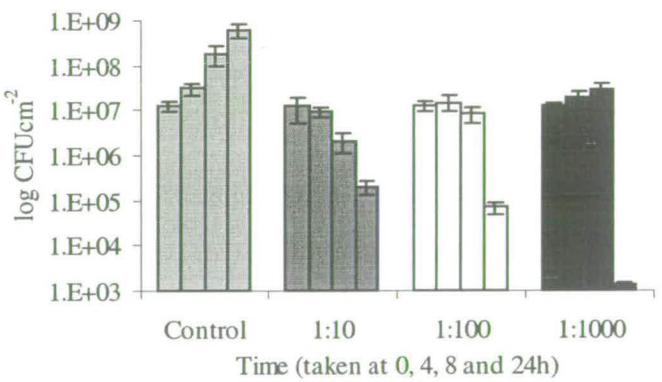
5.3 5920/Ent DUAL SPECIES BIOFILM STUDIES

5.3.1 *Addition of ϕ1.15 to Single and Dual Species Biofilms of* Ent and *5920*

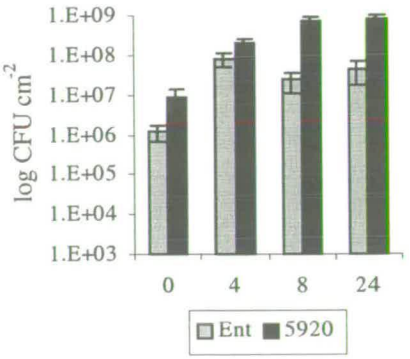
Single and dual species biofilms of *Ent* and 5920 were grown on glass slides overnight. The slides were removed from the cultures, washed in PBS to remove any unadhered cells and placed into fresh YE media. The cultures were inoculated with bacteriophage ϕ1.15 to allow a 1:10 ratio of phage and cells, and also 1:100 and 1:1000 ratios. Single and dual species controls without phage were also included. Viable counts were estimated at 0, 4, 8 and 24 h.

Figure 5.2a) demonstrates the effect of different concentrations of phage ϕ1.15 on single species biofilms of 5920. While the higher concentrations of phage (1:10) initially had a greater effect on the single species biofilms of 5920, it was the lowest concentrations of phage (1:1000) that eradicated most 5920 cells after 24h. No effect was seen on single species biofilms of *Ent*, and so these results are not represented graphically.

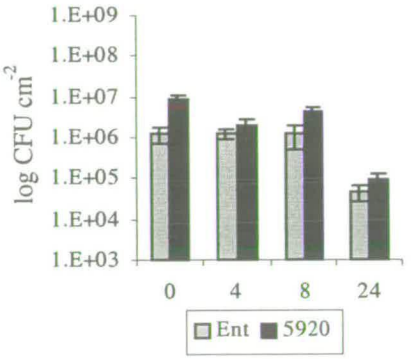
5.2a) Addition of ϕ1.15 to single species biofilms of 5920



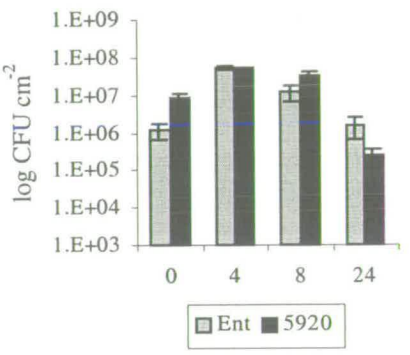
5.2b) Dual species biofilm controls



5.2c) 1:10



5.2d) 1:100



5.2e) 1:1000

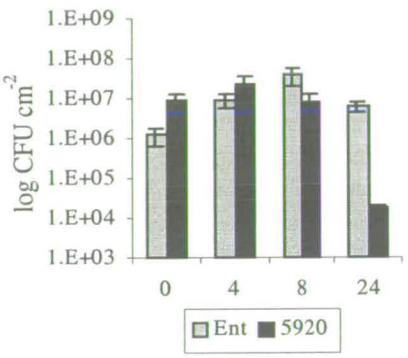


Figure 5.2

The action of ϕ1.15 on dual species biofilms of *Ent*/5920 was investigated. ϕ1.15 has phage and polysaccharide depolymerase activity against 5920. Single species controls of the effect of ϕ1.15 on 5920 are shown in 5.2a). In the dual species biofilms, phages were inoculated to give a ratio of 1 phage: 10cells (5.2c), 1:100 (5.2d) and 1:1000 (5.2e). Figure 5.2b) demonstrates dual species biofilm controls without phage. Biofilms were grown on glass slides, and viable counts were estimated at 0, 4, 8 and 24 h. Results are the outcome of 3 replicate experiments and bars represent standard error.

It was hoped that lower concentrations of phage ϕ1.15 would be more selective in removing 5920 from dual species biofilms of *Ent*/5920. The results obtained form the single species biofilms were promising in that the lowest concentration of phage did eradicate most biofilm cells after 24 h. Dual species biofilm data are represented in graphs 5.2b) phage-free controls, 5.2c) 1:10, 5.2d) 1:100 and 5.2e) 1:1000. While the highest concentration of phage ϕ1.15 eradicated most cells, both *Ent* and 5920 cells were present in equal amounts (5.2d). This indicated that the action of the higher concentrations of phage were too destructive, removing both *Ent* and 5920 cells from the biofilm. Again the lower concentration of ϕ1.15 was most successful (5.2e). This reduced the numbers of 5920 in the dual species biofilm from 8.71×10^6 CFU cm^{-2} ($\pm 2.98 \times 10^5$) to 2.73×10^4 CFU cm^{-2} ($\pm 1.8 \times 10^3$) over a period of 24 h. However, complete eradication of 5920 cells did not occur.

5.3.2 *Addition of Winchburgh to Single and Dual Species Biofilms of* Ent + *5920*

Winchburgh bacteriophage has phage but no polysaccharide depolymerase activity against 5920. It has been suggested that due to the production of polysaccharide material, a phage with no enzyme activity would be unable to infect biofilm cells (Hughes, 1997). It was, therefore, hoped to compare the effects of a phage with enzyme activity (ϕ1.15) with a phage with no enzyme activity (Winchburgh) on single and dual species biofilms of *Ent* and 5920.

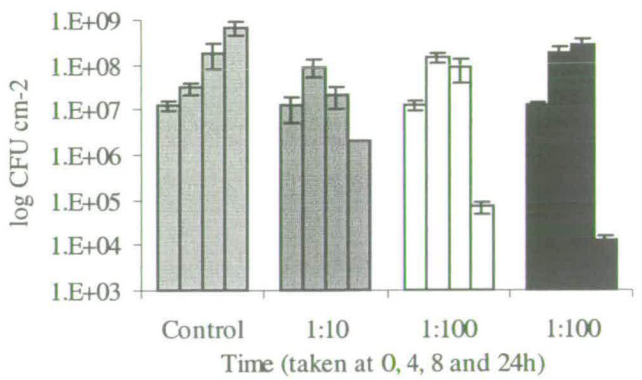
Again single and dual species biofilms of *Ent* and 5920 were grown overnight on glass slides, the slides removed, washed in PBS and placed into fresh media. The cultures were inoculated with Winchburgh to allow a 1:10 ratio of phage and cells, and also 1:100 and 1:100 ratios. Single and dual species controls without phage were also included. Viable counts were estimated at 0, 4, 8 and 24 h.

Figure 5.3a) indicates the effect of different concentrations of Winchburgh on single species biofilms of 5920. Again, the lowest initial concentration of phage was the most successful in selectively eradicating 5920 from the dual species biofilm. A comparison of the action of Winchburgh with ϕ1.15, however, indicated that ϕ1.15 removed more cells from the biofilm than Winchburgh. The Winchburgh phage also needed longer incubation before the effect of the phage could be detected.

A drop in viable counts was seen 4 h after the addition of ϕ1.15 (fig5.2a). However, a similar drop in cell numbers was seen 8 h after the addition of Winchburgh. No effect was seen on the single species biofilms of *Ent*.

The effect of lower concentrations of phage on dual species biofilms of *Ent* and 5920 was also tested. Phage were inoculated to give a ratio of 1:10 (5.3c), 1:100 (5.3d) and 1:1000 (5.3e). A comparison of the action of ϕ1.15 and Winchburgh on 5920 and *Ent* dual species biofilm material indicated that ϕ1.15 selectively removed slightly more 5920 cells from the biofilm. The lowest inoculum of Winchburgh phage (1:1000) reduced 5920 cell numbers to 5.03 x 10^4 CFU cm^{-2} (± 3.83 x 10^3), compared with 2.73 x 10^4 CFU cm^{-2} (± 1.8 x 10^3) due to the action of ϕ1.15. Thus, a bacteriophage with both lytic and enzyme activity was marginally more successful in the selective removal of one strain from a dual species biofilm.

5.3a) Addition of Winchburgh phage to single species biofilms of 5920



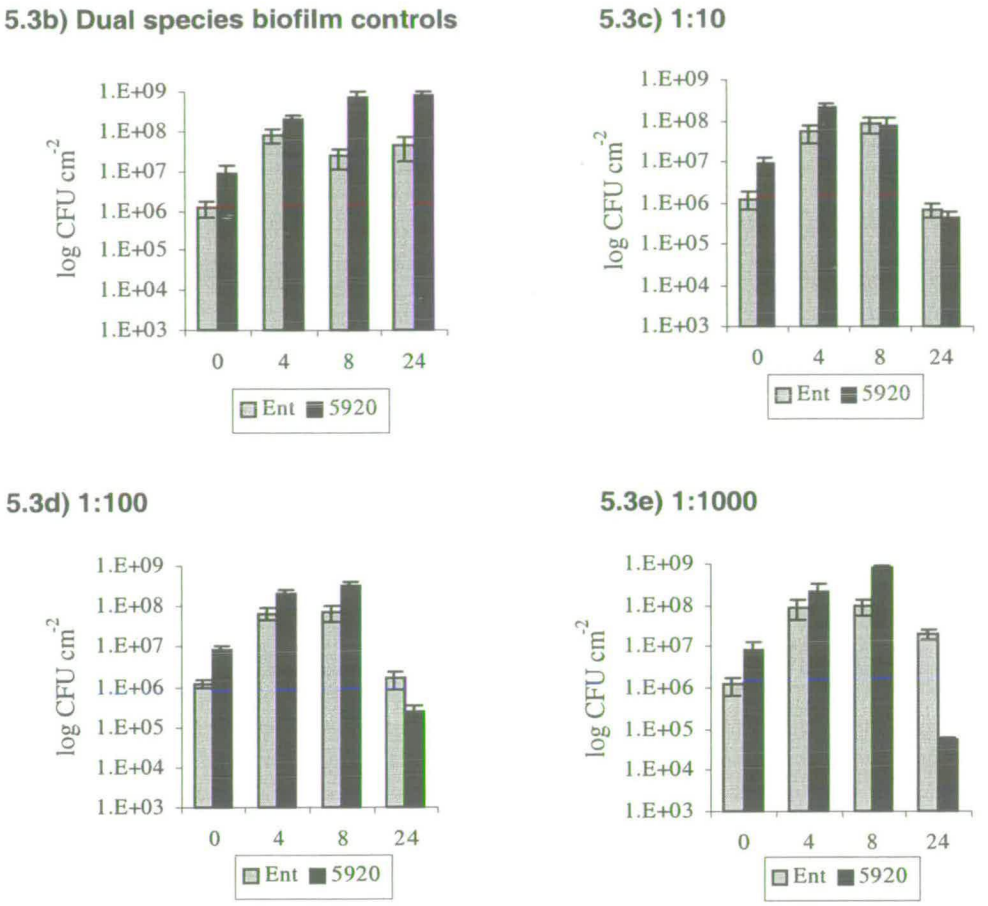


Figure 5.3

The action of Winchburgh phage on dual species biofilms of *Ent*/5920 was investigated. Winchburgh phage has phage but no polysaccharide depolymerase activity against 5920. Single species controls of the effect of Winchburgh phage on 5920 are shown in 5.3a). In the dual species biofilms, phages were inoculated to give a ratio of 1 phage: 10cells (5.3c), 1:100 (5.3d) and 1:1000 (5.3e). Figure 5.3b) demonstrates dual species biofilm controls without phage. Biofilms were grown on glass slides, and viable counts were estimated at 0, 4, 8 and 24 h. Results are the outcome of 3 replicate experiments and bars represent standard error.

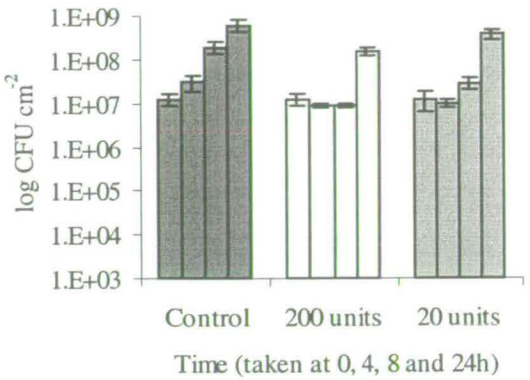
5.3.3 *Addition of Enzyme to Single and Dual Species Biofilms of* Ent *and 5920.*

Concentrated solutions of ϕ1.15 and ϕE.coli polysaccharide depolymerase enzymes were separated from the phage particles, and the action of soluble phage enzyme on biofilm material investigated. Again, single and dual species biofilms of *Ent* and 5920 were grown overnight on glass slides, the slides removed, washed in sterile PBS and placed in fresh media. The specific activities of the enzymes were assayed, and adjusted to allow final enzyme activities of 200 and 20 μg reducing sugar released g protein^{-1} h^{-1} ml^{-1}. This specific enzyme activity was similar to that of the ϕ1.15 phage inoculum used in figure 5.2c). Viable counts were estimated at 0, 4, 8 and 24 h. **Figure 5.4** depicts the effect of ϕ1.15 enzyme, and **figure 5.5**, the effect of ϕE.coli phage enzyme on the single and dual species biofilms.

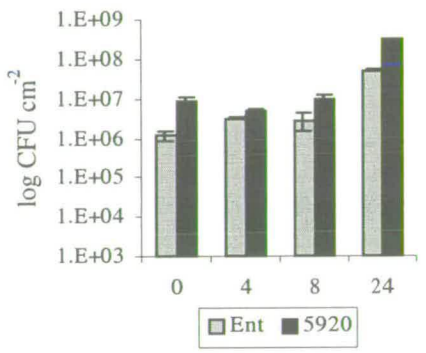
Similar results were obtained for both ϕ1.15 and ϕE.coli phage enzymes. Examination of the action of the larger dose of phage enzyme, 200 units, on the single species biofilms (fig5.4a & fig5.5a) indicated that at 4 h there were large reductions in biofilm size. Comparing biofilm cell numbers of the single species control at 4 h, it can be seen that ϕ1.15 enzyme reduced biofilm size by 77.6% (± 4.2), and ϕE.coli enzyme by 77.4% (± 3.8%). However, adhesion increased again at 8 and 24 h. A smaller reduction in biofilm size was seen with the lower dose of phage enzyme, 20 units: 69.2% (± 4.2) for ϕ1.15 and 71.6% (± 4.1) for ϕE.coli.

In the dual species biofilms, both *Ent* and 5920 cells were removed from the biofilm at 4 h due to the addition of 200 units of page enzyme. However, when the biofilms that received 20 units of phage enzyme biofilms were compared with an *Ent*/5920 dual species biofilm control (fig 5.3b), it can be seen that *Ent* numbers increased slightly more than expected during recolonisaton of the glass slides. A 24 h dual species biofilm control culture contained 8.21 x 10^8 (± 4.4 x 10^7) 5920 and 4.32 x 10^7 (± 3.1 x 10^6) CFU cm^{-2} *Ent*. After addition of 20 units of ϕ1.15 phage enzyme, a 24 h dual species biofilm contained 5.12 x 10^8 (± 3.4 x 10^7) 5920 and 7.39 x 10^7 (± 3.8 x 10^6) CFU cm^{-2} *Ent*.

5.4a) Addition of ϕ1.15 phage enzyme to single species biofilms of 5920



5.4b) Addition of 200 units of enzyme



5.4c) Addition of 20 units of enzyme

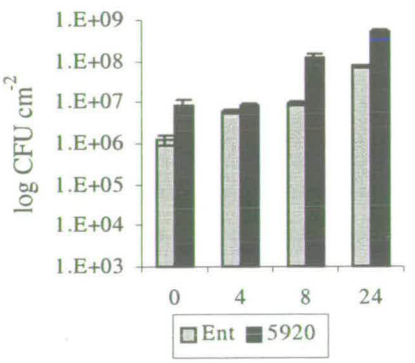
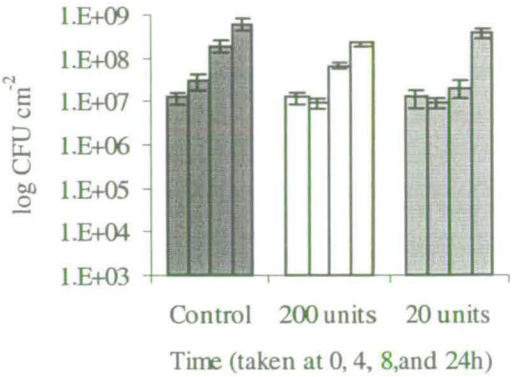


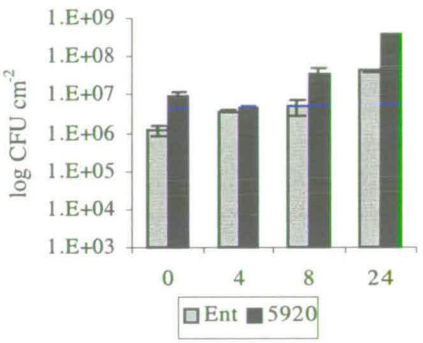
Figure 5.4

Addition of ϕ1.15 phage enzyme to dual species biofilms of *Ent*/5920. Figure 5.4a) shows addition of phage enzyme to single species biofilms of 5920; figures 5.4b) and c) show the addition of phage enzyme to dual species biofilms of *Ent* and 5920. Units of phage enzyme are mg reducing sugar released g protein h^{-1} ml^{-1}. Biofilm material was grown on glass slides, and viable counts were taken at 0, 4, 8 and 24 h. Results are the outcome of 3 replicate experiments, and bars represent standard error.

5.5a) Addition of ϕ*E. coli* phage enzyme to single species biofilms of 5920



5.5b) Addition of 200 units of enzyme



5.5c) Addition of 20 units of enzyme

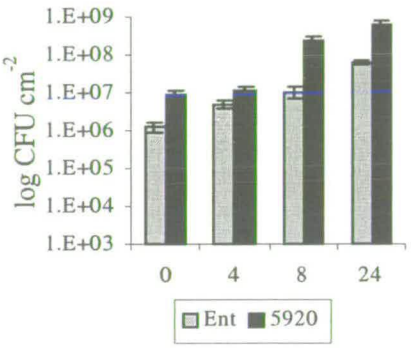


Figure 5.5

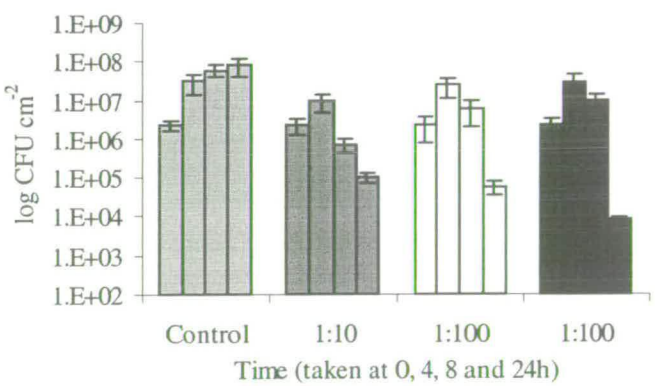
Addition of ϕE.coli phage enzyme to dual species biofilms of *Ent*/5920. Figure 5.5a) shows addition of phage enzyme to single species biofilms of 5920. Figures 5.5b) and c) show the addition of phage enzyme to dual species biofilms of *Ent*/5920. Units of phage enzyme are mg reducing sugar released g protein h^{-1}. Biofilm material was grown on glass slides, and viable counts were taken at 0, 4, 8 and 24 h. Results are the outcome of 3 replicate experiments, and bars represent standard error.

5.3.4 *Addition of Phil Phage to Dual Species Biofilms of* Ent/*5920*

Phil phage preparations had phage, but no enzyme activity against *Ent*. Unfortunately, due to the susceptibility of 5920 to many of the phage isolated, no other combinations of bacteria and phage were possible. Single and dual species biofilms of *Ent* and 5920 were grown on glass slides overnight. The slides were removed from the cultures, washed in PBS to remove any unadhered cells and placed into fresh YE media. The cultures were inoculated with bacteriophage Phil to allow a 1:10 ratio of phage and cells, and also ratios of 1:100 and 1:100. Single and dual species controls without phage were also included. Viable counts were estimated at 0, 4, 8 and 24 h.

The results were similar to those produced by the action of Winchburgh phage on 5920 in *Ent*/5920 dual species biofilms. Again, the higher concentrations of Phil phage removed both *Ent* and 5920 cells from the biofilm (5.6b), whereas lower concentrations of phage were more selective in removing *Ent* from the biofilm (5.6e). Complete eradication of *Ent* from the dual species biofilms was not achieved: a small number of *Ent* cells remained within the biofilm: 2.04×10^4 CFU cm^{-2} ($\pm$ 3.12×10^3).

5.6a) Addition of Phil phage to single species biofilms of *Ent*



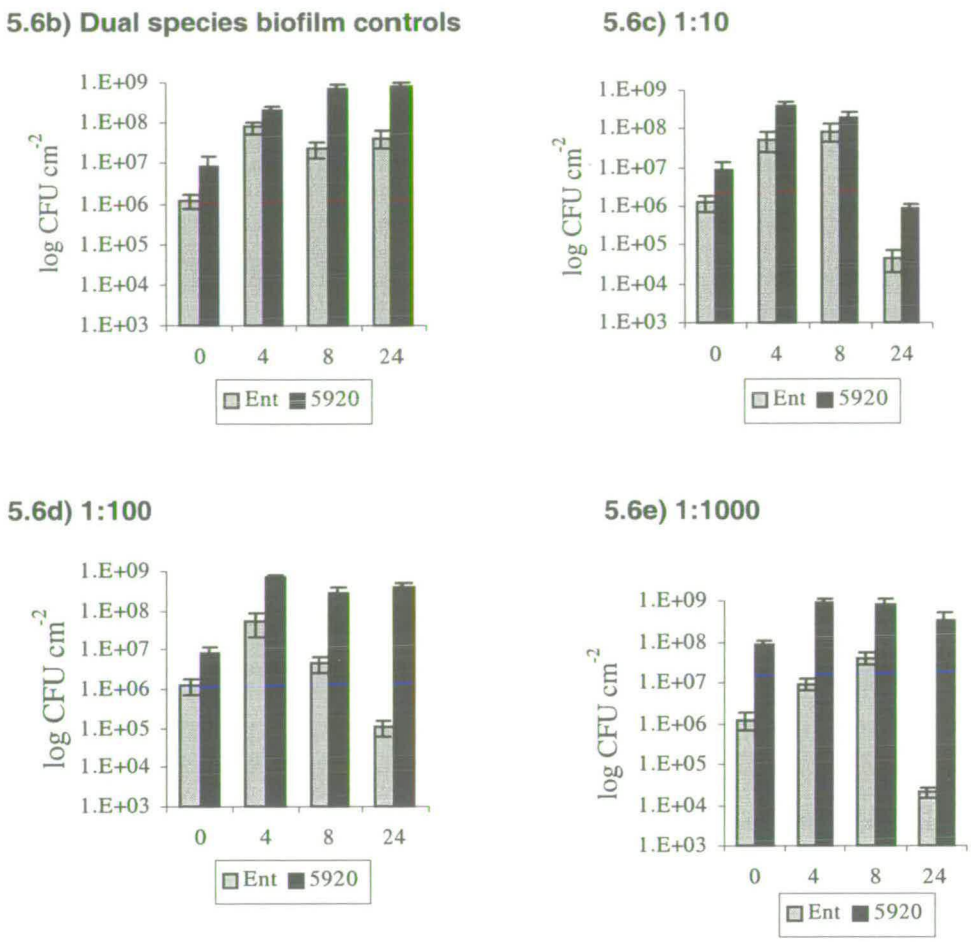


Figure 5.6

The action of Phil phage on dual species biofilms of *Ent*/5920 was investigated. Phil phage has phage activity, but no polysaccharide depolymerase activity against *Ent*. In the dual species biofilms, phages were inoculated to give a ratio of 1 phage: 10cells (5.6c), 1:100 (5.6d) and 1:1000 (5.6e). Figure 5.6b) demonstrates dual species biofilm controls without phage. Single species controls of the effect of Phil phage on 5920 are shown in 5.6a). Biofilms were grown on glass slides, and viable counts were estimated at 0, 4, 8 and 24 h. Results are the outcome of 3 replicate experiments and bars represent standard error.

5.4 ϕ1.15 BACTERIOPHAGE INTERACTIONS WITH 5920 BIOFILMS

Over a period of 24 h, lower concentrations of phage were more successful in eradicating one species from a dual species biofilm. The reason why this occurred was unclear, and so the quantities of phage within phage-infected biofilms were also investigated. It was hoped that an examination of the numbers of phage within the biofilm would give a clearer indication of the interactions between the biofilm bacteria and bacteriophage.

Biofilms of 5920 were grown overnight on glass slides, and then inoculated with different concentrations of ϕ1.15 to allow phage: bacteria ratios of 1:10, 1:100 and 1:1000. Bacteria and phage counts were estimated at 24 and 48 h. To estimate the numbers of phage, the glass slides were washed in PBS and crushed in 1 ml PBS to remove the biofilm from the slides. This gave an estimation of the numbers of both free-living phage, and of phage contained within bacteria.

	24 h			48 h		
	5920 CFU cm^{-2}	ϕ1.15 phage cm^{-2}	Ratio	5920 CFU cm^{-2}	ϕ1.15 phage cm^{-2}	Ratio
1:10	6.23×10^5 ($\pm 3.58 \times 10^4$)	7.53×10^7 ($\pm 4.23 \times 10^6$)	1:120	2.65×10^5 ($\pm 1.13 \times 10^4$)	1.99×10^7 ($\pm 8.89 \times 10^5$)	1:75
1:100	2.11×10^5 ($\pm 2.11 \times 10^4$)	2.28×10^7 ($\pm 1.02 \times 10^6$)	1:108	1.93×10^6 ($\pm 9.95 \times 10^5$)	5.79×10^7 ($\pm 2.55 \times 10^6$)	1:30
1:1000	5.56×10^4 ($\pm 4.19 \times 10^3$)	6.4×10^6 ($\pm 2.13 \times 10^5$)	1:115	4.11×10^5 ($\pm 1.97 \times 10^4$)	3.32×10^7 ($\pm 2.08 \times 10^6$)	1:81

Table 5.2

Single species biofilms of 5920 were infected with different concentrations of ϕ1.15 to give phage: biofilm cell ratios of 1:10, 1:100 and 1:1000. Biofilms were grown on glass slides. Cell and phage counts were estimated after 24 and 48 h. Results are the outcome of 4 replicate experiments.

Bacteria and phage counts are shown in **table 5.2**. Examination of the data from 24 h again demonstrated that lower concentrations of phage remove the largest quantity of cells from the biofilm. Interestingly, in each case biofilm and phage numbers reach a status quo, with bacteria: phage ratios of 1:120, 1:108 and 1:115 occurring. After 48 h incubation, cell numbers increased slightly. This was most evident in the biofilm with a 1:100 phage inoculum, where cell numbers increased to 1.93×10^6, giving a bacteria: phage ratio of 1:30. To test if these bacteria were less susceptible to the action of ϕ1.15, 5920 cells were removed from the 48 h biofilms, and phage-free cells isolated. These cells were then spread onto agar to form bacterial lawns, and spotted with 10 µl of ϕ1.15 solution (**figure 5.7**). Both neat solutions, and 10^{-1}, 10^{-2} and 10^{-3} dilutions of phage were examined. All four solutions produced plaques on the original 5920 culture, whereas the most dilute solutions (10^{-3}) did not produce a plaque on the 5920 cells isolated from the 1:100 phage inoculum biofilm. Although not resistant to the action of ϕ1.15, the cells isolated from the 48 h biofilm with the 1:100 phage inoculum were less susceptible to the action of ϕ1.15.

5.7a) 5920 control **5.7b)5920 cells from phage-infected biofilm**

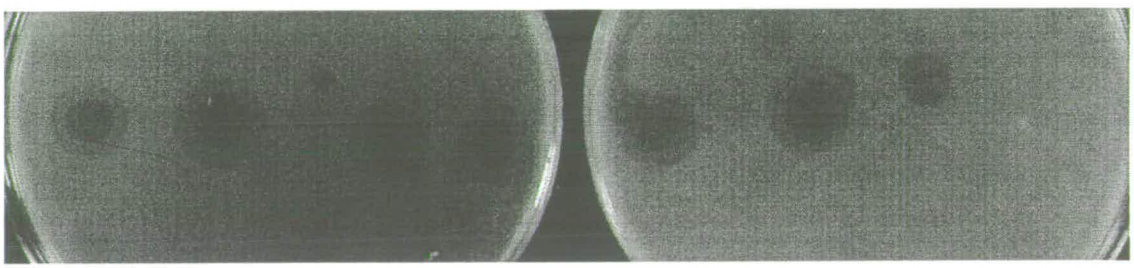


Figure 5.7

5920 cells from the ϕ1.15 phage-infected biofilm (1:100 inoculum) were tested for their phage-susceptibility. Lawns of bacteria were prepared, and spotted with both neat solutions, and 10^{-1}, 10^{-2} and 10^{-3} dilutions of phage. After 24 h incubation, it can be seen that the cells from the phage-infected biofilm were less susceptible to ϕ1.15, as the lowest dilutions did not produce a plaque.

5.5 ADDITION OF TWO AND THREE PHAGE TO 5920 BIOFILMS

The addition of one type of bacteriophage was unsuccessful in the eradication of one species within a dual species biofilm. It was hoped that the addition of 2, or 3 phage to a dual species biofilm of *Ent* and 5920 would be capable of selectively removing 5920 from the biofilm. From the phage isolated from primary effluent sewage, ϕE.coli and Blackburn bacteriophage had both phage and enzyme activity against 5920, but no activity against *Ent*. The double effect of phage ϕ1.15 and ϕE.coli, and triple effect of ϕ1.15, ϕE.coli and Blackburn was, therefore, examined.

Table 5.3 shows the addition of single, double and triple doses of phage to single species biofilms of 5920. This was repeated for dual species biofilms of 5920 and *Ent* (**table 5.4**). Biofilms were grown overnight and then inoculated with different concentrations of phage to allow phage: bacteria ratios of 1:10, 1:100 and 1:1000. Each bacteriophage inoculum contained equal amounts of ϕ1.15 and ϕE.coli, in the case of the double phage inoculum, or equal amounts of ϕ1.15, ϕE.coli and Blackburn in the case of the triple inoculum. Cultures were incubated for 24 h, and viable counts estimated. To allow more accurate counts of the two strains, biofilm material was also plated out onto agar containing 1.0 • g ml^{-1} chloramphenicol. This inhibited the growth of much of the 5920 cells, but allowed *Ent* to grow.

Examination of tables 5.3 and 5.4 show that a low inoculum of several types of phage was most successful in eradicating single species biofilms of 5920, and in selectively removing 5920 from dual species biofilms of *Ent* and 5920. Complete eradication of 5920 from the dual species biofilms was not achieved.

	1 phage	2 phage	3 phage
1:10	2.05×10^5 ($\pm 8.89 \times 10^3$)	7.67×10^4 ($\pm 2.8 \times 10^3$)	2.55×10^3 (± 189.3)
1:100	6.88×10^4 ($\pm 3.03 \times 10^3$)	3.48×10^3 (± 139.3)	0
1:1000	1.31×10^3 (± 716)	121.2 (± 72.6)	0

Table 5.3

Combinations of ϕ1.15 and ϕE.coli ('2 phage'), and ϕ1.15, ϕE.coli and Blackburn ('3 phage') were compared to the action of a single dose of ϕ1.15. Single species biofilms of 5920 were grown overnight and infected with different concentrations of phage to allow bacteria: phage ratios of 1:10, 1:100 and 1:1000. Viable counts were taken at 24 h. Results are the outcome of 3 replicate experiments.

	1 phage	2 phage	3 phage
1:10	9.5×10^4 **5920**	1.11×10^5 **5920**	7.32×10^4 **5920**
	($\pm 5.76 \times 10^3$)	($\pm 5.31 \times 10^4$)	($\pm 4.5 \times 10^3$)
	4.57×10^4 ***Ent***	5.53×10^5 ***Ent***	8.57×10^5 ***Ent***
	($\pm 2.56 \times 10^3$)	($\pm 2.23 \times 10^4$)	($\pm 4.58 \times 10^4$)
1:100	2.32×10^5 **5920**	1.02×10^4 **5920**	8.61×10^3 **5920**
	($\pm 8.98 \times 10^3$)	($\pm 4.21 \times 10^3$)	(± 541.2)
	1.6×10^6 ***Ent***	3.19×10^6 ***Ent***	2.91×10^6 ***Ent***
	($\pm 6.23 \times 10^5$)	($\pm 3.11 \times 10^5$)	($\pm 2.13 \times 10^5$)
1:1000	1.73×10^4 **5920**	428.3 **5920**	103.2 **5920**
	($\pm 9.68 \times 10^3$)	(± 33.2)	(± 18.3)
	4.04×10^6 ***Ent***	5.35×10^6 ***Ent***	6.67×10^6 ***Ent***
	($\pm 1.75 \times 10^5$)	($\pm 3.68 \times 10^5$)	($\pm 4.39 \times 10^5$)

Table 5.4

Combinations of ϕ1.15 and ϕE.coli ('2 phage'), and ϕ1.15, ϕE.coli and Blackburn ('3 phage') were compared to the action of a single dose of ϕ1.15. Dual species biofilms of *Ent* and 5920 were grown overnight and infected with different concentrations of phage to allow bacteria: phage ratios of 1:10, 1:100 and 1:1000. Viable counts were taken at 24 h. Results are the outcome of 3 replicate experiments.

5.6 DISCUSSION

5.6.1 *Bacteriophage Host Range*

Bacteriophage for representative strains of the *Enterobacteriaceae* were found to be of widespread occurrence in sewage. Results also indicated that certain strains, for example, S53, *Ent*, 1.15, 53b and 5920 were all extremely susceptible to many of the bacteriophage isolated. This suggests that these strains may have common cell surface properties. For example, S53, 1.15 and 5920 all produce a negatively charged polysaccharide of the colanic acid type. *Ent* and 53b are also known to produce similar, neutral polysaccharides. Examples of outer membrane protein receptors for bacteriophage and colicins for *E. coli* are demonstrated in **table 5.6**. As can be seen, the outer membrane proteins can act as receptors for a wide range of bacteriophage and colicins.

Bradley (1967) noted that the exceptions to the specificity observed in bacteriophages of most bacterial genera are those of the Enterobacteriaceae family. Many can infect more than one host genus. For example, phages for *E. coli* often have the ability to infect *S. typhimurium* strains. Similarly, phages isolated for *Serratia* species often grow on *E. coli*. This was thought to be due to the classification of the Enterobacteriaceae, which describes as separate genera organisms that are often extremely closely related.

Name	Gene	Mol wt	Receptor for phages and colicins	Solutes transported
Phage λ receptor	lamB	47 393	λ, K10, TP1, TP5, SS1	Maltose
Phage T6 receptor	tsx	26 000	T6, colicin K	Nucleosides
BtuB	btuB	66 400	BF23, E colicins	Vitamin B_{12}
Cir	cir	74 000	Colicins I and V	Ferric Iron?
Iut	iut	74 000	Cloacin DF13	Fe^{3+} -aerobactin
FhuE	fhuE	76 000		Fe^{3+} -copragen
FhuA (TonA)	fhuA	78 000	T1, T5, ϕ80, colicin M	Ferrichrome
FecA	fecA	80 500		Ferric citrate
FepA	fepA	81 000	Colicins B and D	Fe^{3+}-enterobactin
Fiu	fiu	83 000		Ferric iron?

Table 5.6

Outer membrane protein receptors for phage anf colicins in *E. coli* (adapted from Nikaido and Vaara, 1987).

5.6.2 *Comparison of ϕ1.15, Winchburgh and Phage Enzyme on 5920 Biofilms*

While the addition of bacteriophage with enzyme activity is capable of disrupting a biofilm, it was not known if a phage without a polysaccharide depolymerase would be capable of infecting a biofilm, and so the action of phage with and without polysaccharide depolymerase activity was compared. Phage ϕ1.15 was capable of producing an enzyme with activity against 5920, whereas Winchburgh had only phage activity. Addition of ϕ1.15 was initially more successful, a large reduction in cell numbers was achieved within 4 h incubation. In comparison, reductions in cell numbers were not detected until 8 h after the addition of Winchburgh. However, over a period of 24 h, the end results produced by addition of ϕ1.15 and Winchburgh were similar. Both had reduced cell numbers in 5920 single species biofilms to a similar degree, and ϕ1.15 was only marginally more successful in the selective removal of 5920 cells from dual species biofilms of *Ent* and 5920. The dual species biofilm infected with Winchburgh contained 2.04×10^7 ($\pm$ 2.6×10^6) *Ent* cells, and the biofilm infected with ϕ1.15 contained 6.04×10^6 ($\pm$ 3.2×10^5) *Ent* cells. Therefore, given longer periods of incubation, phage without enzyme activity are also capable of disrupting biofilm formation.

Examination of the effect of phage-free polysaccharide depolymerase on the single and dual species biofilms indicated that the initial large reduction in biofilm size in both the single and dual species cultures due to the addition of ϕ1.15 was through the action of polysaccharide depolymerase. This removed vast quantities of cells from the biofilm through enzymatic action on the polysaccharide material, without any cell lysis occurring. Hughes et al. (1998b) produced similar results: the addition of phage-free polysaccharide depolymerase enzyme produced a dramatic drop in bacterial numbers within biofilms of *Ent. agglomerans*/53b. The addition of large quantities of phage enzyme was also unselective, both *Ent* and 5920 cells were pulled from the biofilm. However, during the recolonisation of the glass slides, when compared to a control culture, *Ent* numbers were seen to increase slightly. This suggested that the decapsulated 5920 cells had more difficulty in adhering to the glass slides.

5.6.3 *Bacteria and Bacteriophage Population Dynamics*

The complete eradication of susceptible bacteria by a bacteriophage would be expected. However, communities of bacteriophage and bacteria have been shown to be remarkably stable. Horne (1970) reported the coexistence of phage T4 and *E. coli* for periods as long as 52 weeks. Several theories have been suggested to explain this observation. Working with the phage T5 and lambda, Schrag and Mittler (1996) provided evidence to suggest that the occurrence of spatial 'refuges', brought about through growth on the walls of culture vessels, might be important in stabilising bacteria-phage interactions. Nutritional limitation is also known to influence stability. Bohannan and Lenski (1997) observed that increasing the input of nutrients led to a large increase in phage numbers, a small increase in bacteria and a reduction in the dynamic stability of both populations. Taking this into consideration, within a biofilm, both spatial heterogeneity and nutritional limitations are common occurrences. Theoretically, a biofilm environment would, therefore, increase the stability of bacteria-phage interactions.

Interestingly, in a study of bacteriophage replication in cultures of *Staphylococcus epidermidis, Bacillus subtilus* and *E. coli*, cell densities of over 10^4 CFU ml^{-1} were required before bacteriophage multiplication occurred (Wiggins and Alexander, 1985). Again, this suggests bacteriophage are able to detect the number of cells within a population, and control multiplication in order to achieve a stable phage:bacteria co-existance.

In many cases, introduction of a phage into a susceptible population results in an initial epidemic of phage, followed by a stable equilibrium that can last from weeks to months. At equilibrium, both temperate and virulent phages are much less abundant than the bacteria (Pantasticocaldas *et al.*, 1992). Biofilms of bacteria and phage were incubated for a maximum of 48 h in these experiments. It was expected that within the time-scale examined, the phages were reaching epidemic proportions within the biofilms and any stable interactions between the phage and bacteria had not yet been achieved. However, table 5.2 indicated that single species biofilms of 5920 infected with different concentrations of ϕ1.15 all contained similar proportions of phage and bacteria at 24 h, indicating stabilising interactions were already evident at this point.

Under certain conditions, 5920 biofilms were seen to have reduced susceptibility to the action of ϕ1.15 (fig 5.7). Most resistance to bacteriophage is achieved through the loss or modification of the receptor molecule to which the phage initially binds. The bacteriophage receptor molecules are also involved in bacterial metabolism, and so mutations often simultaneously reduce the cell's competitiveness. Despite this cost of resistance, phage-resistant mutants have been found to quickly evolve and invade most laboratory communities (Lenski, 1998a). In a study of T phage and *E. coli*, Lenski (1998b) thought that this trade-off between resistance and competitiveness allowed the stable coexistence of sensitive and resistant *E. coli* when phage were present. This coevolutionary 'arms race' is also thought to be involved in stabilising interactions between phage and bacteria. In their review of interactions between bacteria and bacteriophage, Bohannan and Lenski, (2000) stated that different resistance mutations produce distinct resistance phenotypes. These differ in whether resistance is partial or complete, the cost in competitiveness associated with the mutation, and whether the mutation can be countered by a host-range mutation in the bacteriophage. These factors will determine if the mutant can overtake the sensitive population, and the resultant structure of the community. For example, T4-resistant bacteria were found to be inferior competitors relative to the sensitive population, and so a community of both sensitive and resistant phenotypes, and bacteriophage developed (Bohannan and Lenski, 1997). Lenski and Leven (1985) produced evidence to suggest that *E. coli* B produced a mutation that phage T7 could not overcome with a corresponding host-range mutation. However, T7 can persist within the population because the phage-sensitive bacteria are competitively superior to the resistant mutant. In comparison, mutations in *E. coli* B that conferred resistance to phage T5 did not restrict the competitive fitness of the strain, and so the phage became extinct.

These studies of resistance mutations within populations of bacteria infected with phage can be applied to a dual species biofilm. The biofilms used in these studies contained a sensitive strain, and a resistant strain. Both are also capable of producing a microcin with activity against the other strain, and so a competitive biofilm is formed. It would be interesting to allow the infected competitive biofilms to develop for longer periods of time and monitor the outcome. If a community

contains resistant bacteria, an increase in nutrients will cause an increase in the resistant population, no change in the average density of phage or sensitive bacteria, and reduced stability of the sensitive bacteria and phage populations, but increased stability of the resistant population (Bohannan and Lenski, 1999). Given longer periods of incubation, the resistant strain within the biofilm may have outcompeted the sensitive strain. Alternatively, development of a phage-resistant mutation may alter the dynamics of the system, leading to a completely different outcome.

5.6.4 *Interactions of Biofilm Bacteria with Bacteriophage*

Smaller Doses of Phage are More Successful

Smaller initial doses of phage were more successful in both reducing cell numbers in a single species biofilm, and also eradication of a single species from a dual species biofilm. A possible mechanism of how this occurs is shown in **figure 5.8**, a representation of 5920 biofilm infection over a period of 24 h, with high and low titres of a bacteriophage containing a polysaccharide depolymerase enzyme. The 5920 biofilms are represented as containing stacks and pillars of cells, with water-filled channels circulating around the microcolonies. It is thought that the large phage inoculum would contain high quantities of enzyme (fig 5.8a). The action of the polysaccharide depolymerase would quickly carve through the EPS, resulting in disintegration of the biofilm before the phages are able to infect the cells. Hughes *et al.* (1998b) estimated that removal of vast quantities of biofilm material through the action of enzyme activity alone could occur within 30 min. This may remove the stacks and pillars resulting in a flatter, undifferentiated biofilm. Infection of the exposed surfaces would then occur, allowing the phages to work slowly through the biofilm to reach bacteria beneath the flattened surface while cell growth occurred from the uninfected areas. Figure 5.8b represents an infection process with a smaller phage inoculum. This would be expected to contain lower quantities of enzyme, and a substantial removal of biofilm material would not occur. Phage will be able to move through the biofilm, including through the pores and channels to the basal layers of the biofilm, infecting all available surfaces. The combined efforts of phage and enzyme will slowly carve through the biofilm, decreasing stability and gradually sloughing off the entire biofilm. This may result in a thinner layer of cells.

A similar scenario to the situation described in fig 5.8 may also occur with the smaller doses of Winchburgh and Phil, phage with no enzyme activity against 5920 and *Ent*. The lytic action of a large phage inoculum may again be too destructive, removing vast quantities of cells from the biofilm to leave a flatter, undifferentiated biofilm. It is imagined that smaller quantities of phages would work gently through a biofilm, gradually decreasing stability until the whole biofilm sloughs off.

Eradication of Biofilms through Addition of Multiple Phage-Types

The fact that bacteria and phage have been shown to coexist in stable communities explains why addition of a single phage type to a biofilm failed to eradicate the bacteria. However, the infection of a single species biofilm of 5920 by three different phages, ϕ1.15, ϕE.coli and Blackburn, resulted in complete eradication of the biofilm. It seems likely that the addition of the three different types of phages put a stop to any stabilising interactions that could potentially occur between a single phage and sensitive bacteria. The phages would, ultimately, have to compete with one another to survive, and this would cause a decrease in the stability of the community, and complete eradication of the host bacteria. It would also be unlikely for a mutant resistant to all three bacteriophage to develop, providing another advantage to using multiple phage-types.

The infection of a dual species biofilm of *Ent* and 5920 by the triple-dose of phages failed to completely remove 5920 from the biofilm. Even though *Ent* and 5920 are competitive, there is the possibility that *Ent* protected 5920 from attack by creating 'spatial refuges' in the depths of the biofilm.

Fig5.8a) Addition of high titres of phage

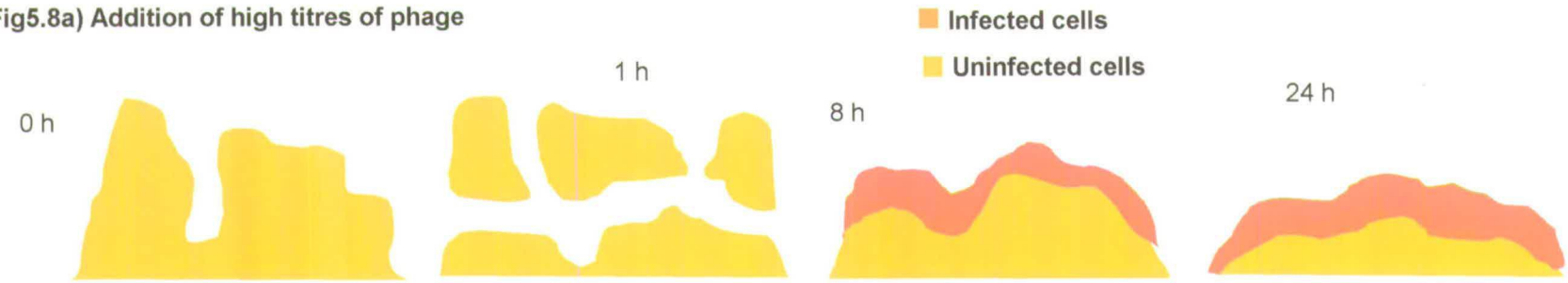


Fig5.8b) Addition of low titres of phage

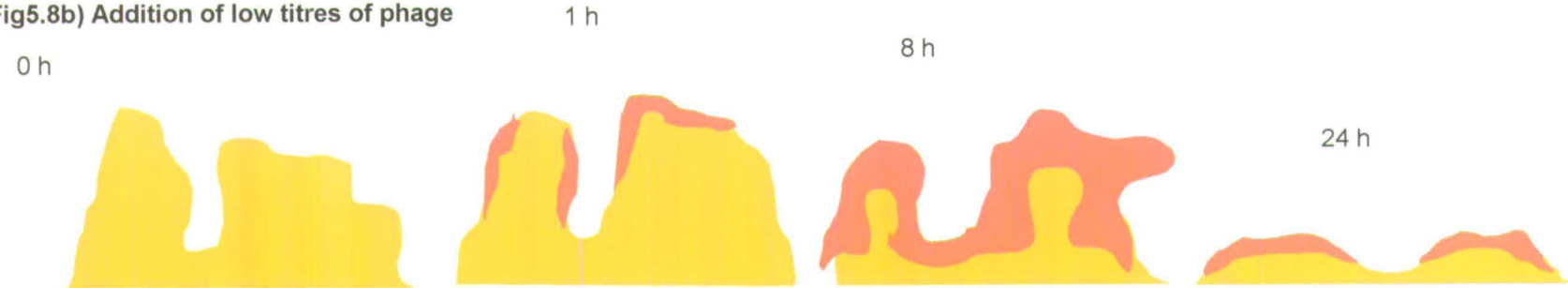


Figure 5.8

Theory of phage infection. During addition of high titres of phage (fig5.8a), the large quantity of soluble polysaccharide depolymerase would quickly disintegrate polymer material, pulling the biofilm apart. This may result in a flatter, undifferentiated biofilm. During recolonisation, only the surface layers would be re-infected by newly released phage. The phage would then have to slowly carve through the biofilm to reach bacteria beneath the flattened surface. A smaller phage inoculum would not contain a large quantity of soluble enzyme (fig5.8b). Phage would infect all available surfaces, including the pores and channels at basal layers of the biofilm. The combined efforts of phage and polymer degradation would slowly eat into the biofilm, decreasing stability. Channels would become larger, stacks of cells thinner, resulting in removal of the entire biofilm to leave a thinner layer of cells.

5.6.5 *Conclusions*

In a comparison of the effect of phages with and without polysaccharide depolymerase activity on biofilm material, over a period of 24 h, near identical results were achieved.

Communities of bacteriophage and bacteria have been shown to be remarkably stable (Horne, 1970). Infection by three phages, ϕ1.15, ϕE.coli and Blackburn, was, therefore, required to eradicate single species biofilms of 5920. This combination of phages was also the most successful in selectively removing a single species form a dual species biofilm. Complete eradication, however, was not achieved.

Chapter 6

STIMULATION OF EPS PRODUCTION BY SUB-INHIBITORY CONCENTRATIONS OF DISINFECTANT

6.1 INTRODUCTION

Biofilms are consistently more resistant to antimicrobial agents when compared to planktonically growing cells (Costerton *et al.*, 1987). Several approaches have been made in recent years in an attempt to explain the resistance of bacterial biofilms to chemical and antibiotic treatments. Three principal hypotheses have been formulated. Physiological limitation proposes that bacteria within the biofilm exist in a more recalcitrant phenotypic state. Penetration limitation states that only surface bacteria are subjected to lethal doses of antimicrobial agents. The third hypothesis states that the attachment of cells to surfaces causes the derepression of a large number of genes associated with sessile existence so that biofilm cells are phenotypically distinct from their planktonic counterparts.

Research into the role of EPS in the resistance of biofilms has produced mixed results. For example, Giwercman *et al.* (1991) found that the accumulation of drug-inactivating enzymes such as β-lactamases within the biofilm created marked concentration gradients of the β-lactam antibiotics and protected, to some extent, the underlying cells. Similarily, Anderl *et al.* (2000) noted that ampicillin did not penetrate through *K. pneumoniae* biofilms due to the production of β-lactamases; the drug was inactivated faster than it could react. The penetration of chlorine into biofilms containing *Ps. aeruginosa* and *K. pneumoniae* was linked to neutralisation of chlorine by surface bacteria (deBeer *et al.*, 1994). The sorption of an antimicrobial to biofilm components may give rise to a similar situation. For example, direct binding of positively charged aminoglycosides to polyanionic alginate resulted in poor diffusion of the antibiotics through a *Ps. aeruginosa* biofilm (Kumon *et al.*, 1994). However, Nichols *et al.* (1988) suggested that for antibiotics such as tobramycin, although the EPS produced by *Ps. aeruginosa* bound the antibiotic, the resulting decrease in diffusion coefficients within the biofilms were not enough to define this as a significant barrier.

Bacteriophage with polysaccharide depolymerase activity specific for the strains used in this study have been isolated. Phage enzymes may be used to selectively remove the polysaccharide material from a biofilm. In this way, the role of EPS in the resistance of bacterial biofilms to disinfectants can be investigated.

6.2 STIMULATION OF 5920 TO FORM THICKER BIOFILMS IN THE PRESENCE OF DISINFECTANTS

Ent. cloace/5920 was selected for study as this strain produces copious amounts of polysaccharide material. Glass beads were inoculated with strain 5920 and viable counts of biofilm material estimated at 6, 24 and 72 h. The same cultures were also grown in disinfectant containing media (**figure 6.1**). Concentrations of disinfectants were 0.5 mg l^{-1} triclosan (TR), 0.01% sodium hypochlorite (HPC) and 6 mg ml^{-1} benzalkonium chloride (BC).

Sloughing off of biofilm material was a major problem when using glass beads, possibly due to high shear forces. Compared to the disinfectant-free control biofilm, the 5920 cultures growing in disinfectant did not slough off: large increases in biofilm size were seen for all three disinfectants tested.

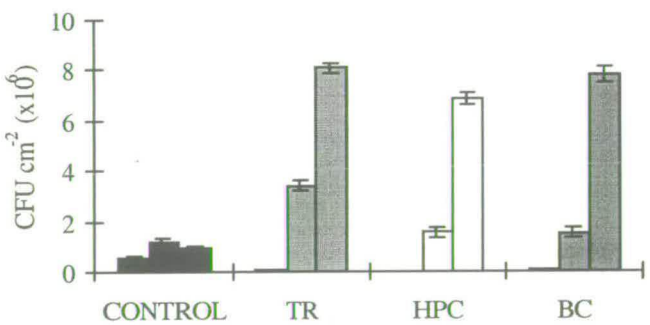


Figure 6.1

Glass bead cultures were inoculated with 5920 (5 ml of o/n culture) and either TR (0.5mg l^{-1}), HPC (0.01 %) or BC (6 mg l^{-1}). Viable counts were estimated at 6, 24 and 48 h. Disinfectant-free controls were also included. Addition of sub-inhibitory concentrations of disinfectant to cultures of 5920 enhanced biofilm production. Results are the outcome of 3 replicate experiments and bars represent standard error.

6.2.1 *Addition of Bacteriophage Polysaccharide Depolymerase*

The enhanced biofilm formation in disinfectant-containing cultures was thought to be associated with the high viscosity of 5920 EPS, and so a bacteriophage polysaccharide depolymerase capable of degrading 5920 EPS was added. Glass bead biofilms of 5920 were grown overnight and ϕ1.15 phage enzyme added to allow a final enzyme activity of 25 μg glucose released g protein^{-1} h^{-1} ml^{-1}. This was incubated for 4 h and disinfectant added to the biofilm cultures. Disinfectant was added to give final sub-inhibitory concentrations of either 0.15% HPC, 40 μg l^{-1} triclosan and 0.40 mg l^{-1} BC. Viable counts were estimated after a further 4 h incubation, and EPS production was measured using the total cell carbohydrate assay (Dubois *et al.* 1956), and by EPS dry weight. Disinfectant and phage-free controls were also included. Addition of ϕ1.15 enzyme to the disinfectant-free control caused a decrease in biofilm EPS (28.1% decrease in total cell carbohydrate and 30.4 % decrease in dry weight), but also a slight decrease in cell numbers (19.6 %) (**table 6.1**). Again, addition of sub-inhibitory concentrations of disinfectant to 5920 caused a large increase in biofilm cell numbers, but an increase in biofilm EPS was also seen. Addition of both phage enzyme and disinfectant reduced cell numbers and EPS, indicating that biofilm EPS may play a role in the enhanced biofilm production in disinfectant-containing cultures of 5920.

Disinfectant	± 1.15 phage enzyme	EPS production		CFU (cells cm^{-2})
		total cell cardohydrate (mg cm^{-2})	EPS dry weight (mg cm^{-2})	
Control	+	0.82	0.64	2.95×10^6
Control	–	1.14	0.92	3.67×10^6
HPC	+	1.64	1.48	1.92×10^6
HPC	–	2.66	2.46	1.33×10^7
TR	+	2.06	1.68	1.04×10^6
TR	–	3.14	2.34	8.22×10^6
BC	+	2.04	1.81	1.32×10^6
BC	–	3.26	3.08	7.47×10^6

Table 6.1

Glass bead biofilms of 5920 were grown overnight and inoculated with ϕ1.15 polysaccharide depolymerase to give a final activity of 25 μg glucose released g protein^{-1} h^{-1} ml^{-1}. The cultures were incubated for a further 4 h at 30 °C with shaking, and disinfectant added to the cultures. HPC concentrations were 0.15%, triclosan 40 mg l^{-1} and BC 0.40 mg l^{-1}. Viable counts were estimated after a further 24 h incubation. Enzyme-free and disinfectant-free controls were also included. Addition of enzyme to remove biofilm EPS rendered 5920 disinfectant-sensitive. Standard error for CFU cm^{-2} was ± 2.35 x 10^5. Results are the outcome of 3 replicate experiments.

6.3 SUB-INHIBITORY CONCENTRATIONS OF ANTIBIOTIC

Studies by Hughes (1997) also indicated that sub-inhibitory concentrations of a wide range of antimicrobial agents caused an increase in EPS production, and hypothesised that this increase was a general stress response. The antimicrobial agents added were at such concentrations to reduce growth rate but allowed EPS synthesis to continue. **Figure 6.2** shows similar results. This demonstrates the increased polysaccharide production by the strains 5920, 1.15 and S53 surrounding Mastring-s® antibiotic discs. Mastring-s® discs allow a rapid method of assessing sensitivity to a large range of antimicrobial agents. The discs were placed onto lawns of bacteria, and the antimicrobial diffused into the agar, creating a concentration gradient. Growth of the cells occurred when the antimicrobial had reached a sub-inhibitory concentration. Surrounding the clear zones of many of the antibiotics, a pronounced build up of polysaccharide material was seen. It was assumed that at this point, the antibiotic concentration was high enough to halt cell division, but not inhibit polysaccharide production. A culture of 5920 showing no increased EPS production is shown in fig6.2a), whereas fig6.2b) demonstrates 5920 increased EPS production produced by sub-inhibitory concentrations of streptomycin. As seen in **table 6.2** , this effect was widespread in all 5 strains tested, and for the antibiotics tested.

6.2a) no increased EPS production

6.2b) increased EPS production

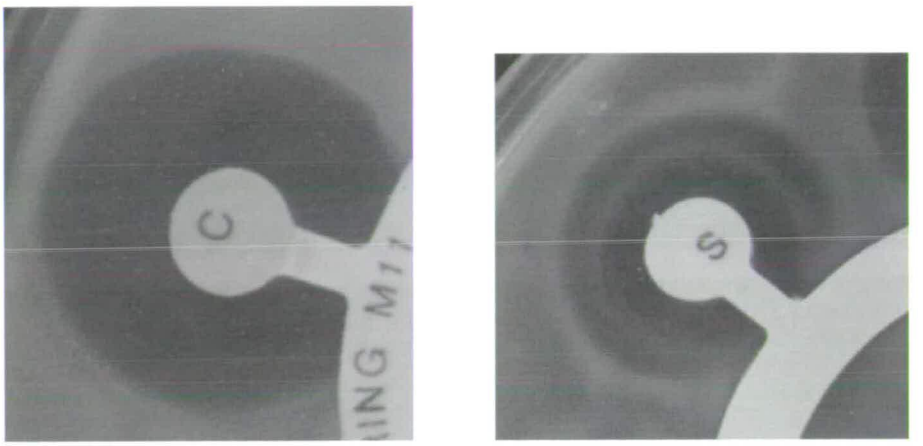


Figure 6.2

Mastring discs were applied to lawns of 1.15, 5920 and S53. Areas of increased EPS production due to the addition of sub-inhibitory concentrations of antibiotic can be seen. 6.2a) no increased EPS production. 6.2b) 5920 increased EPS production produced by subinhibitory concentrations of streptomycin.

Mastring-s® SPECIFICATIONS				*E.coli*	*Ent*	1.15	5920	S53
M26	AP	AMPICILLIN	25 µg	0	0	12 +	27 +	18
	C	CHLORAMPHENICOL	50 µg	18	26	26	22	20
	CO	COLISITN SULPHATE	100 µg	18	20 +	20 +	23 +	28
	K	KANAMYCIN	30 µg	17	18	10	15	18 +
	NA	NALIDIXIC ACID	30 µg	17	20 +	26 +	23 +	23
	NI	NITROFURANTOIN	50 µg	18	14	18	15 +	7
	S	STREPTOMYCIN	25 µg	12	25 +	10	13 +	23 +
	T	TETRACYCLINE	100 µg	20	23	28	23 +	18
M27	AP	AMPICILLIN	25 µg	0	0	12 +	25 +	19
	GM	GENTAMYCIN	10 µg	20	19	18	16	13
	PY	CARBENICILLIN	100 µg	0	0	22	26	23 +
	NA	NALIDIXIC ACID	30 µg	18	20	24	20	23
	NI	NITROFURANTOIN	50 µg	15	20 +	22	14 +	22
	SM	SULPHANMETHIZOLE	200 µg	0	20	7	6 +	26
	T	TETRACYCLINE	100 µg	18	20	26	23 +	20
	TS	COTRIMOXAZOLE	25 µg	18	19 +	20	18 +	24

Table 6.2

Mastring-s® testing of the strains *E. coli*, *Ent*, 1.15, 5920 and S53. Figures are the diameter of the clear zone surrounding the antibiotic disc (mm) and '+' indicates an increase in polysaccharide production by the strain. This was seen as a halo of thick polysaccharide around the edge of the clear zone.

This increase in EPS production due to sub-inhibitory concentrations of antibiotic was investigated further. Streptomycin and nalidixic acid were chosen for further study as these antimicrobial agents produced a marked effect on all the strains tested, and were also capable of increasing polysaccharide production (table 6.2). Streptomycin is an inhibitor of protein synthesis, and nalidixic acid is a DNA Gyrase inhibitor. Four concentrations of antimicrobial agent were selected, each below the BEC. The highest concentration utilised was only marginally below the BEC. The strains 1.15 and 5920 were used. For each situation, a cell count was made and the total cell carbohydrate assayed. This allowed an estimation of the total cell carbohydrate $cell^{-1}$ to be estimated. The results of these investigations are shown in **table 6.3**. For the three lowest sub-inhibitory concentrations of antibiotics, an increase in total carbohydrate with increasing antibiotic concentration was shown. This increase was undoubtedly due to an increase in EPS production by the cell. This increase in EPS production was not seen at the highest concentration of antibiotic.

strain	antibiotic	antibiotic conc^{n} (μg/ml)	CFU cm^{-2}	total carbohydrate cell^{-1} (pg)
1.15		0	**1.07 x 10^{6}** ± 3.07 x 10^{4}	**4.80** ± 0.88
	Streptomycin	6.0	**5.56 x 10^{4}** ± 2.01 x 10^{3}	**11.98** ± 0.91
		4.0	**1.9 x 10^{5}** ± 2.03 x 10^{4}	**18.32** ± 1.98
		3.0	**1.12 x 10^{6}** ± 2.89 x 10^{4}	**5.06** ± 0.32
		2.0	**1.13 x10^{6}** ± 6.23 x 10^{4}	**4.09** ± 0.47
	Nalidixic Acid	1.5	**3.21 x 10^{4}** ± 2.22 x 10^{3}	**16.76** ± 2.03
		1.125	**1.49 x 10^{5}** ± 1.23 x 10^{3}	**37.38** ± 0.31
		0.75	**4.99 x 10^{5}** ± 4.67 x 10^{3}	**11.56** ± 0.18
		0.375	**1.26 x 10^{6}** ± 5.32 x 10^{4}	**5.54** ± 0.57
5920		0	**2.97 x 10^{6}** ± 5.98 x 10^{4}	**6.34** ± 0.078
	Streptomycin	6.0	**2.21 x 10^{4}** ± 1.01 x 10^{3}	**12.26** ± 0.99
		4.0	**4.91 x 10^{5}** ± 9.98 x 10^{3}	**31.57** ± 1.95
		3.0	**1.03 x 10^{6}** ± 2.77 x 10^{4}	**8.49** ± 1.27
		2.0	**2.19 x 10^{6}** ± 4.72 x 10^{4}	**6.1** ± 0.84
	Nalidixic Acid	4.0	**6.12 x 10^{4}** ± 5.01 x 10^{3}	**17.23** ± 2.45
		3.0	**8.23 x 10^{5}** ± 6.82 x 10^{4}	**51.29** ± 4.26
		1.5	**2.31 x 10^{6}** ± 5.98 x 10^{4}	**9.36** ± 1.23
		0.75	**2.68 x 10^{6}** ± 1.73 x 10^{4}	**7.14** ± 1.08

Table 6.3

The effect of antibmicrobial concentration on the production of EPS by 1.15 and 5920 was assessed. Cultures were inoculated, the appropriate amount of antibiotic added and incubated o/n at 30 °C. The absorbency of the culture was read at 600 nm. Viable cell counts were made of each slide, and the total cell carbohydrate of each slide assayed. Results were the outcome of 4 replicate experiments.

6.4 GROWTH RATE EFFECTS

6.4.1 *Artificially Stopping Growth*

The possibility that certain sub-inhibitory concentrations of antimicrobial agents can stop cell growth without killing the cell, and allow EPS production to continue was investigated. Cultures of 5920 were centrifuged to separate the cells. The cells were then washed thoroughly in PBS, and resuspended in PBS to concentrate the cells x5. Glucose was added to allow a final concentration of 1.0 % (w/v). This artificially stopped cell growth. The minimum inhibitory concentrations (MIC) of HPC and triclosan over a 4 h period against the concentrated cell suspension were estimated, and concentrations of HPC (0.3, 0.2 and 0.1%) and triclosan (45, 40 and 30 $\mu g\ ml^{-1}$) were selected. Again, the highest concentration was only marginally below the MIC. Disinfectant was added to the cell suspensions, and the production of EPS monitored over a 3 h period. This was estimated by total cell carbohydrate assay (Dubois *et al.*, 1956) and as EPS dry weight.

The results are shown in **table 6.3**. Addition of 0.2% HPC and 40 $\mu g\ ml^{-1}$ triclosan resulted in an approximate 3-fold increase in EPS production. No reduction in cell viability due to the addition of the disinfectants occurred. The lower (0.1% HPC and 40 $\mu g\ ml^{-1}$ triclosan) and higher (0.3% HPC and 30 $\mu g\ ml^{-1}$ triclosan) concentrations produced no increase in EPS production. Addition of the highest concentrations also reduced the viability of the cells.

Disinfectant	conc^n	time (h)	CFU ml^{-1}	EPS production	
				dry weight (mg ml^{-1})	total cell carbohydrate (mg ml^{-1})
Control	0	0	8.75×10^9	36	49.7
		1	1.13×10^{10}	30	43.9
		2	1.2×10^{10}	38	45.2
		3	1.55×10^{10}	34	56.6
HPC (%)	0.3	0	9.42×10^9	54	52.3
		1	9.11×10^9	56	57.7
		2	6.45×10^9	60	56.5
		3	8.85×10^8	77	67.7
	0.2	0	1.16×10^{10}	81	60.44
		1	1.15×10^{10}	92	153.7
		2	1.43×10^{10}	129	129.8
		3	1.24×10^{10}	209	171.6
	0.1	0	9.56×10^9	56	66.6
		1	9.89×10^9	58	69.9
		2	1.08×10^{10}	62	74.2
		3	1.11×10^{10}	67	81.2
TR (μg ml^{-1})	45	0	2.01×10^8	52	60.2
		1	1.21×10^8	59	62.3
		2	4.23×10^8	58	55.5
		3	4.46×10^7	65	49.5
	40	0	1.15×10^8	43	48.6
		1	1.55×10^8	48	48.5
		2	1.03×10^8	89	86.7
		3	1.21×10^8	151	167.6
	30	0	1.45×10^8	45	56.2
		1	1.25×10^8	49	59.5
		2	1.39×10^8	52	62.1
		3	1.26×10^8	55	71.9

Table 6.3

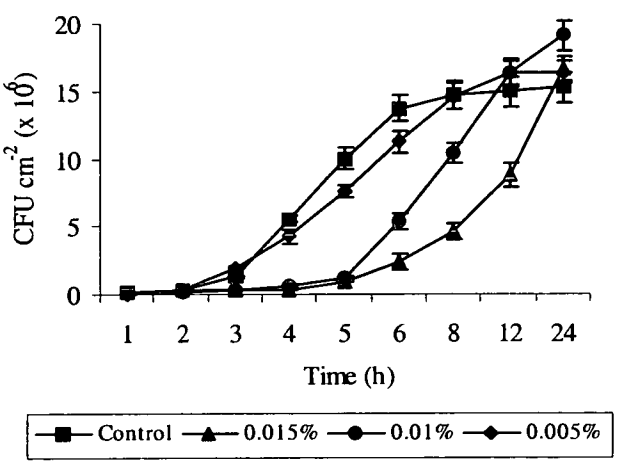
Cultures of 5920 were grown overnight, centrifuged and the cells concentrated x5 by washing and resuspending in PBS. This artificially stopped the growth of the cells. Glucose (1.0 %), and HPC (0.3, 0.2 and 0.1%) and triclosan (45, 40 and 30 μg ml^{-1}) were added to the cell suspension, and the production of EPS monitored over a 4 h period. EPS production was estimated as dry weight and as total cell carbohydrate. Certain concentrations of disinfectant caused an increase in EPS production by 5920. Standard error for HPC treated cultures and controls were 8.89×10^8 CFU ml^{-1}, and 5.56×10^7 CFU ml^{-1} for triclosan treated cultures and controls. Results are the outcome of 3 replicate experiments.

6.4.2 *Metabolic Assays*

INT was used to assay the metabolic activity of biofilm material. INT is converted to red (nonfluorescent) crystals of INT formazan within actively respiring bacteria. Glass slides were inoculated with 5 ml of o/n culture of 5920, and either 0.015, 0.01 or 0.005% HPC. Viable counts and metabolic activity were estimated over a 24 h period.

Examination of the control cultures of 5920 indicated that the INT assay was able to discriminate between actively growing and dormant cells (**figure 6.2**). The INT formazan profile of the culture (fig6.2b) showed the cells going through a typical growth cycle of lag phase, exponential phase and stationary phase. 5920 was able to grow in all concentrations of HPC. The cells were more metabolically active and growth was slowest in 0.015% HPC, presumably due to the high stress the growing cells were subjected to. At concentrations of 0.005% HPC, profiles of metabolic activity and cell growth were very similar to the control. However, at concentrations of 0.01% HPC, the cells were less metabolically active for longer periods of time. Cells were stationary for the first 6 h of growth.

6.2a) Growth of 5920 in HPC: CFU cm^{-2}



6.2b) Growth of 5920 in HPC: metabolic activity

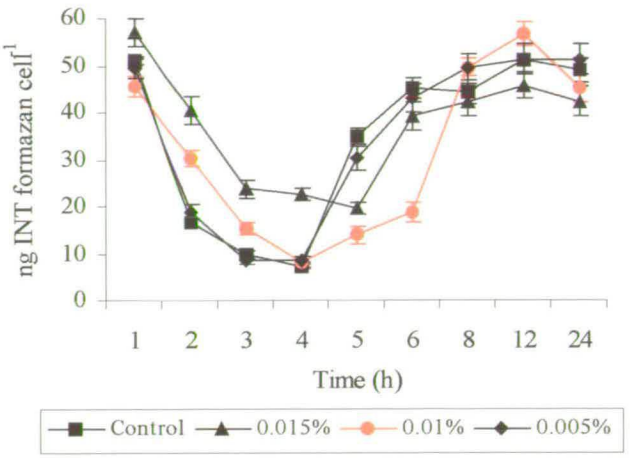


Figure 6.2

Glass slides were inoculated with 5920, and either 0.015, 0.01 or 0.005% sodium hypochlorite. Viable counts and metabolic activity were estimated over a 24 h period. INT was used to assess metabolic activity. This is reduced to a red precipitate of INT formazan in metabolically active cells. Cultures inoculated with 0.01% HPC were less metabolically active. Results are the outcome of 4 replicate experiments and bars represent standard error.

To confirm the hypothesis that certain sub-inhibitory concentrations of hypochlorite caused a reduction in growth rates and a reallocation of resources into polysaccharide synthesis, EPS production by the HPC-containing cultures was also examined. This was calculated as total cell carbohydrate $cell^{-1}$. When compared to the control cultures, it can be seen that the biofilm cultures of 5920 containing 0.01% HPC produced more polysaccharide material during the first 6 h of growth (figure **6.3**). This indicated that the reduction in metabolic activity was accompanied by an increase in EPS synthesis.

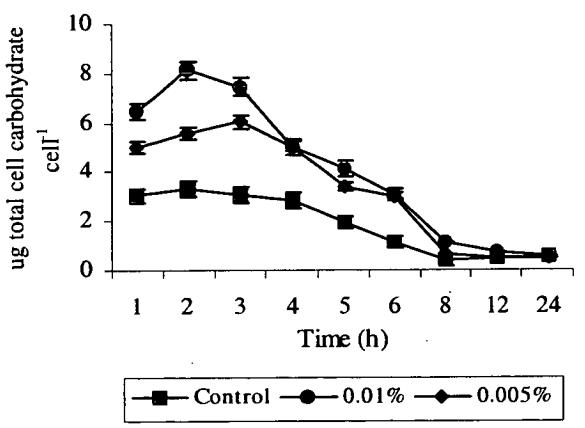


Figure 6.3

The EPS production by biofilms of 5920 was estimated. Glass slides were inoculated with 5920, and either 0.01% or 0.005% sodium hypochlorite. The total cell carbohydrate of biofilm material was assayed over a period of 24 h, and calculated as μg total cell carbohydrate $cell^{-1}$. Disinfectant-free controls were also included. Cultures containing 0.01% HPC produced more polysaccharide material during the first 6 h growth. Results are the outcome of 3 replicate experiments, and bars represent standard error.

6.5 CHANGES TO THE CELL SURFACE

Changes in growth rate can often be accompanied by changes in cell envelope components. The phospholipid and fatty acid (Gilbert and Brown, 1978), metal cation (Kenward *et al.*, 1978) and extracellular enzymes and polysaccharide content (Sutherland, 1982) can all vary with growth rate. This can further influence susceptibility to antimicrobial agents.

The changes to the outer membrane fraction brought about by sub-inhibitory concentrations of HPC were investigated. Biofilms of 5920 were grown overnight on glass beads (500 g), the beads washed to remove any unadhered cells, and placed into fresh media. HPC was added at such a concentration to allow increased EPS production and slowed cell growth under the conditions tested (0.05%). Cultures were incubated for 2, 4, 8 and 24 h and the outer membrane fractions isolated using sodium sarkosyl (Filip *et al.*, 1973). The outer membrane fractions of disinfectant-free controls were also isolated. **Figure 6.3** indicated that no changes in outer membrane composition occurred due to the addition of HPC. There were also no changes made over the 24 h period examined.

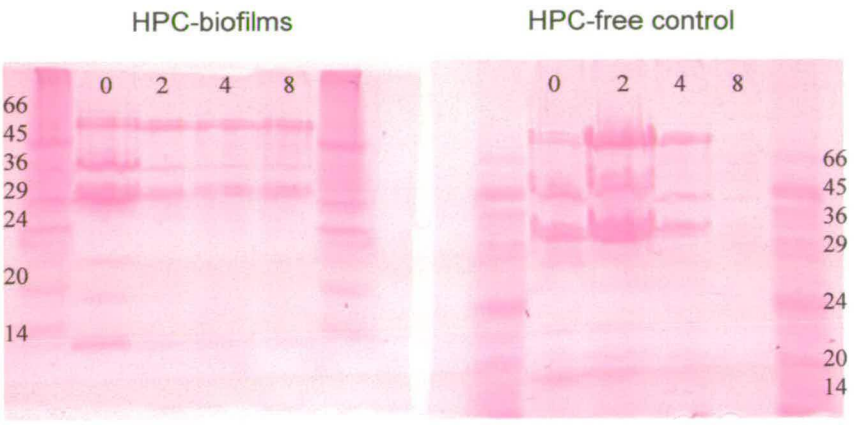


Figure 6.3

SDS-Page of outer membrane fractions of 5920. Carried out on 10% denaturing gels and stained with coomassie blue. Biofilms of 5920 were inoculated with 0.02% HPC, incubated for 2, 4, 8 and 24 h and the outer membrane fraction isolated using sodium sarkosyl (Filip *et al.*, 1973). The following protein markers were used: bovine albumin 66 kDa; ovalbumin 45 kDa; glyceraldehyde-3-phosphate dehydrogenase 36kDa; carbonic anhydrase 29 kDa; trypsinogen 24 kDa; trypsin inhibitor 20.1 kDa; lactalbumin 14.2 kDaThe outer membrane fractions of disinfectant-free controls were also isolated. No changes in outer membrane composition occurred due to the additon of sub-inhibitory concentrations of disinfectant.

6.6 RESISTANCE TO DISINFECTION OF EPS STIMULATED CULTURES

6.6.1 *BEC of Antimicrobial Agents*

The biofilm eradicating concentrations a range of disinfectants against microtitre plate biofilms of 5920 were compared to those of 5920 biofilms grown in EPS-stimulating concentrations of HPC (0.01%). **Table 6.4** indicated that 5920 cultures with increased EPS production were marginally more resistant to the antimicrobial agents.

	BEC	
Disinfectant	**5920**	**5920 ^{EPS}**
HPC	0.022 %	0.025 %
BC	0.45 mg ml^{-1}	0.55 mg ml^{-1}
TR	55 μg ml^{-1}	60 μg ml^{-1}
Dettox	2.5 %	4.0 %
Mr Muscle	3%	3.5%
Ethanol	15 %	20 %
Sainsbury's Microban	1.5%	1.5%
Sainsbury's Multipurpose	2.5%	3.0%
Streptomycin	15 μg ml^{-1}	17.5 μg ml^{-1}
Nalidixic Acid	0.75 μg ml^{-1}	0.9 μg ml^{-1}

Table 6.4

The biofilm eradicating concentrations of disinfectants against microtitre plate biofilms of 5920. These were compared to biofilms that had been grown in subinhibitory concentrations of HPC to stimulate EPS production. Disinfectants were: HPC, BC, TR, Dettox (that contains a cationic compound as its active ingredient), EDTA-containing Mr Muscle, Sainsbury's Microban washing-up liquid (contains triclosan) and Sainsbury's Multipurpose cleaner (contains BC) and ethanol. The antimicrobial agents streptomycin and nalidixic acid were also used. Biofilm material was grown on in microtitre plates and the appropriate concentration of disinfectant added. This was incubated for 5 min, the disinfectant rinsed out and the plates re-incubated with fresh YE media. Any surviving bacteria could re-grow into the liquid phase. The BEC was taken as the lowest concentration of disinfectant to prevent any growth. The biofilms with stimulated EPS production were marginally more resistant.

6.6.1 *Time Taken to Eradicate Biofilm*

It is thought that any increase in resistance due to EPS production is transient. Given enough time, complete eradication of the biofilm can be achieved. Table 6.4 demonstrates the BECs of a variety of agents against microtitre plate biofilms of 5920 where the antimicrobial agents were allowed to react for a period of 5 min. The time taken to eradicate the biofilms of 5920 with enhanced EPS production were estimated. The concentrations of antimicrobials needed to remove the control biofilms were used. Antimicrobial agents were left to react for periods of 6, 7, 8, 9, 10, 12 and 15 min, removed from the microtitre plate wells and replaced with fresh media. After a further 24 h incubation, the time taken to eradicate the biofilms was calculated.

Disinfectant	Time (min)
HPC	7
BC	10
TR	9
Dettox	10
Mr Muscle	7
Ethanol	6
Sainsbury's Microban	6
Sainsbury's Multipurpose	10
Streptomycin	15
Nalidixic Acid	9

Table 6.5

The time taken to eradicate biofilms of 5920 with enhanced EPS production was investigated. The concentrations of antimicrobials needed to remove the control biofilms were used.

6.7 DISCUSSION

Certain sub-inhibitory concentrations of antimicrobial were shown to reduce metabolic activity and increase EPS production in the first 6 h of growth. This increased polysaccharide production protected the cells from further disinfectant stress (table 6.4). The biocides used in these studies have unrelated modes of action: all have different chemistries, charges, bactericidal concentrations, target sites and distinct modes of entry into the cell. It was, therefore, likely that this response was not an antimicrobial-specific response, but a general stress response. The antimicrobial agents caused a reduction in growth rate accompanied by a reallocation of resources into polysaccharide synthesis.

Changes to the outer membrane protein profiles of stressed and unstressed biofilms of 5920 was investigated. Outer membrane protein changes are often responsible for increases in resistance in Gram negative bacteria. Antimicrobial agents cross the outer membrane via a hydrophilic pathway, utilising porins, or via a hydrophobic pathway involving diffusion across the outer membrane bilayer. As the majority of antibiotics used in the treatment of Gram negative infections are small hydrophilic molecules, their effectiveness depends largely in their permeability through the porin channels (Nikaido *et al.*, 1983). Phenotypic or genotypic changes affecting the porins may give rise to altered antibiotic sensitivities. For example, the resistance of *Ps. aeruginosa* to gentamycin, polymyxin and EDTA has been correlated with an induction of H1, an outer membrane protein induced during magnesium limitation (Nicas and Hancock, 1980). However, no changes in the outer membrane content of 5920 biofilms occurred (fig6.3). The apparent increase in resistance of 5920 was, therefore, not associated with changes to the outer membrane, again verifying that this was a general stress response.

The increase in EPS synthesis under stressful conditions has been demonstrated for a variety of situations. For example, Looijesteijn *et al.* (1998) stated that the efficiency of EPS production was highest under glucose limitation. Under acid-stress, *Xanthomonas campestris* was also shown to increase polysaccharide production (Esgalhado and Roseiro, 1998). The production of the capsular polysaccharide colanic acid in *E. coli* K12 is known to be partially regulated by

environmental parameters. Sledjeski and Gottesman (1996) demonstrated that osmotic shock by sucrose or NaCl increased colanic acid production. Similar to the results found within this chapter, Held *et al.* (1995) also demonstrated the stimulation of polysaccharide production in *K. pneumoniae* under sub-inhibitory concentrations of ciprofloxacin and ceftazidime. Thus, a wide variety of environmental stresses can cause an increase in polysaccharide production. In a study of EPS and storage polymer production in an *Enterobacter aerogenes* strain under limiting nutrient conditions, de Souza and Sutherland (1994) revealed that the available substrate was preferentially used for EPS synthesis, rather than for intracellular glycogen production, indicating the importance of this polysaccharide layer to the bacterium. The external EPS layer is thought to concentrate essential trace elements present at low concentrations in oligotrophic environments. The occurrence of several functional groups (carbonyl, carboxyl, hydroxyl and sulphate) would provide various attachment sites for cations, and thereby facilitate nutrient uptake into the cell. Along with ion binding and nutrient scavenging, EPS is also thought to act as a physical barrier to protect against bacteriophage, toxins, protozoa, host defences and desiccation (Sutherland, 2000a).

Brown *et al.* (1990) stated that during extremes of environmental change, bacteria reduce their growth rate. In an analysis of the effect of growth rate on EPS production in biofilm and planktonic *S. epidermidis*, Evans *et al.* (1994) concluded that attachment induced polysaccharide production at rates that were inversely proportional to growth rate. At slower growth rates, EPS production by the attached population was greater. Thus, the reduced growth rates in a biofilm may itself stimulate increased EPS production.

In a heterogeneous biofilm environment, bacteria in natural and industrial communities are likely to experience many of these environmental stresses, including exposure to antimicrobial agents. However, it is unknown if an increase in polysaccharide production would enhance biofilm survival by protecting the underlying cells. Results in this chapter indicate that the increase in resistance brought about by enhanced EPS production was only transient, disinfection for longer periods of time succeeded in eradicating the biofilm. Interestingly, the cationic

agents benzalkonium chloride, streptomycin and Dettox required 15 min incubation in order to eradicate the EPS-stimulated biofilms of 5920. This was probably due to binding of these agents to the negatively charged EPS. However, reduced permeability may give bacteria a chance to develop resistance. For example, the majority of resistance to third generation cephalosporins have originated in *Klebsiella* sp. Much of this was thought to be associated with the large capsule produced by *Klebsiella*. The antibiotics diffuse more slowly, giving sufficient time for point mutations in β-lactamase genes to occur before the bacteria are killed (Philippon *et al.*, 1994).

6.7.1 *Conclusions*

Sub-inhibitory concentrations of disinfectants were seen to increase the EPS production in strain *Ent. cloace*/5920. This was associated with a reduction in cell metabolism. It was thought this was a general stress response: the inhibitory concentrations of hypochlorite caused a reduction in growth rate and a reallocation of resources into polysaccharide synthesis. Biofilm bacteria in industrial and medical environments experience many environmental stresses, including exposure to antimicrobial agents. It was thought this may enhance the survival of the biofilm, however, the increased EPS production only marginally increased resistance towards a range of antimicrobial agents.

Chapter 7

ADAPTATION TO DISINFECTANTS

7.1 INTRODUCTION

The antimicrobial agent triclosan (2,4,4'-trichloro-2'hydroxydiphenyl ether) is widely used in hand soaps, plastics, fabrics and oral hygiene products. Originally thought to be a non-specific biocide, evidence now suggests that triclosan may have a specific target in the cell. Detailed analysis of the effect of triclosan in *E. coli* revealed that nanomolar concentrations inhibited FabI, an enoyl reductase involved in bacterial lipid synthesis, by mimicking its natural substrate (McMurray *et al.*, 1998a). This has lead to concern that wide use of triclosan may lead to cross-resistance to other antimicrobial agents. McMurray *et al.* (1999) provided evidence to suggest that *Mycobacterium smegmatis* mutants selected for resistance to triclosan had mutations to InhA, also an enoyl reductase involved in fatty acid synthesis. Some of these mutants also expressed resistance to the antituberculosis drug isoniazid. Also of concern was the report that a small increase in resistance of *E. coli* to triclosan could select for antibiotic resistance, with the mechanism involving drug efflux (McMurray *et al.* (1998b). The implications these studies may have for the clinical use of triclosan is not yet known. The concentrations of triclosan used in these studies were low ($\mu g\ ml^{-1}$), and do not approach those used in practise: antiseptic products generally contain 2 – 20 $mg\ ml^{-1}$ triclosan. Also in dispute of the results produced by McMurray *et al.*, McDonnell and Pretzer (1998) proposed that mutations to FabI, the enoyl reductase in *E. coli*, were only partially responsible for resistance in their triclosan mutants, overproduction of a second protein also occurred within the mutants isolated. They concluded that triclosan does have multiple targets in bacteria.

Although the question mark still hangs over triclosan, cross-resistance towards other biocides and antibiotics has already been demonstrated. For example, plasmid-mediated resistance to cationic biocides such as chlorhexidine, quaternary ammonium compounds, diamidines and acridines have been found in staphylococci (Russell, 2000). The plasmids encode proton-dependent export proteins. It has been proposed that the presence of such a plasmid in staphylococci may result in selection for antibiotic-resistant bacteria (Paulsen *et al.*, 1996). Similar efflux mechanisms are thought to be responsible for low-level resistance to cationic compounds, dyes, detergents and heavy metals in Gram negative bacteria. For example, strains of *Ps.*

aeruginosa, *Provadencia stuartii* and *Proteus* spp. isolated from urinary tract infections in paraplegic patients were resistant to quaternary ammonium compounds, chlorhexidine, and several chemically-unrelated antibiotics (Stickler *et al.*, 1983).

The aim of this study was to adapt the strains *Ent* and 1.15 to increasing concentrations of triclosan, the quaternary ammonium compound benzalkonium chloride and sodium hypochlorite. The adapted strains would then be tested against a wide range of disinfectants and antibiotics for evidence of cross-resistance. A dual species culture of *Ent* and 1.15 will also be used to determine the effects of competition on the survival fitness of the two strains.

7.2 ADAPTATION TO TRICLOSAN, BENZALKONIUM CHLORIDE AND SODIUM HYPOCHLORITE

Glass bead biofilm cultures were inoculated with 50 µl of overnight culture of *Ent*, 1.15 or an equal mixture of the two strains. Small vials containing 10 ml YE media and 2.5 g beads (diameter 4mm) were used. Disinfectant was also added to give final concentrations of 0.05 µg ml^{-1} triclosan (TR), 0.6 µg ml^{-1} benzalkonium chloride (BC) or 0.01% sodium hypochlorite (HPC). Cultures were incubated for a period of 24 h. The glass beads were then removed from the culture, washed to remove any unadhered cells and placed into fresh vials containing an increased concentration of disinfectant. Again, the cultures were incubated for a further 24 h. Extra vials were also incubated to allow viable counts of biofilm material to be estimated throughout the experiment. Biofilm material was subjected to increasing concentrations of disinfectant until no more growth occurred. The concentrations of disinfectant used throughout the experiment are shown in **table 7.1**. The single and dual species biofilms of *Ent* and 1.15 were unable to adapt to increasing concentrations of Domestos, and so this disinfectant was omitted from further experimentation.

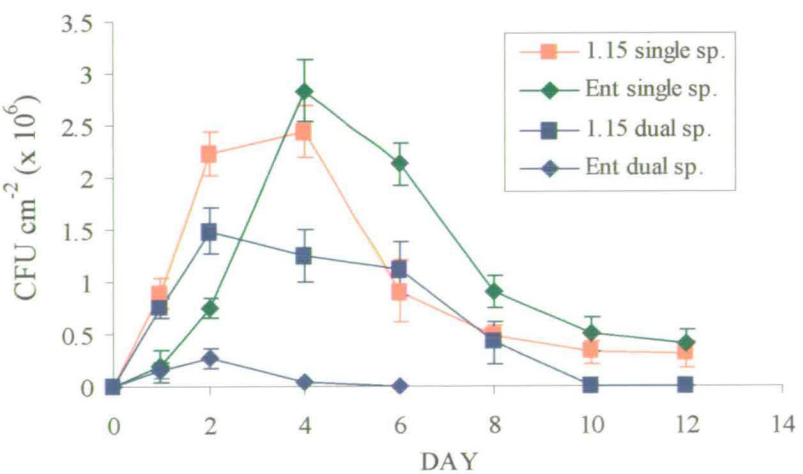
Figure 7.1 demonstrates the adaptation of single and dual species cultures of *Ent* and 1.15 to increasing concentrations of triclosan (7.1a) and benzalkonium chloride (7.1b). Triclosan is only soluble in water up to concentrations of 1.5 µg ml^{-1}. Biofilm cultures of both *Ent* and 1.15 were capable of adapting to this concentration. Growth occurred in biofilm cultures containing higher concentrations of triclosan, up to concentrations1000-fold. However, it was thought that only the soluble fraction of triclosan was able to react with the cells within the biofilms. Studies were, therefore, conducted no further than 1.5 µg ml^{-1}. Strain 1.15 was capable of adapting to an average of 25 µg ml^{-1} BC, and *Ent* adapted to 30 µg ml^{-1} BC. In both cases, the mixed species biofilm was unable to adapt as far as the single sp. biofilms, reaching an average only of 0.4 µg ml^{-1} triclosan and 7.5 µg ml^{-1} BC. Interestingly, *Ent* was out-competed from the biofilm by day 4.

Day	TR ($\mu g\ ml^{-1}$)	BC ($mg\ ml^{-1}$)
1	0.05	0.6
2	0.075	0.8
3	0.1	0.1
4	0.15	0.15
5	0.2	0.2
6	0.25	2.5
7	0.3	3.0
8	0.4	4.0
9	0.5	5.0
10	0.75	7.5
11	1.0	10.0
12	1.5	15.0
13		20.0
14		25.0
15		30.0
16		40.0

Table 7.1

Concentrations of triclosan (TR) and benzalkonium chloride (BC) used in the adaptation of *Ent* and 1.15 to disinfectants.

7.1a) Adaptation to increasing concentrations of triclosan



7.1b) Adaptation to increasing concentrations of benzalkonium chloride

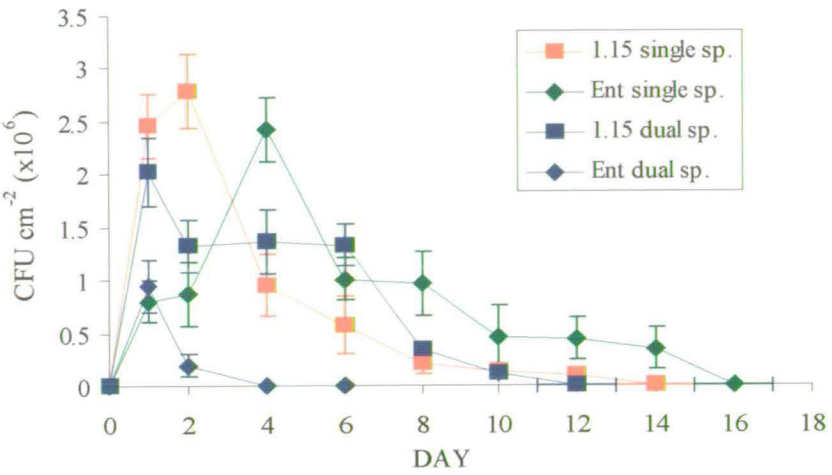


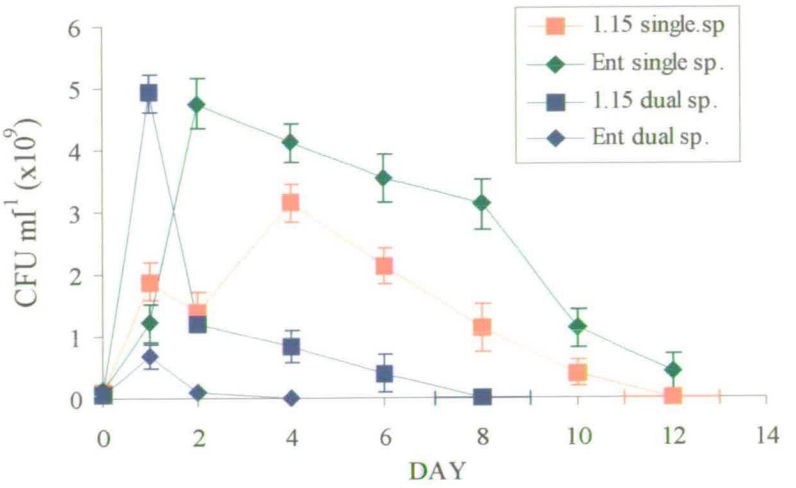
Figure 7.1

The adaptation of single and dual species biofilms of *Ent* and 1.15 to increasing concentrations of triclosan (7.1a) and benzalkonium chloride (7.1b). The concentrations of disinfectants used are shown in table 7.1. Small vials containing 10 ml YE media and 2.5 g beads (diameter 4mm) were used. Cultures were incubated for a period of 24 h, the glass beads removed from the culture, washed to remove any unadhered cells and placed into fresh vials containing an increased concentration of disinfectant. Biofilm material was subjected to increasing concentrations of disinfectant until no more growth occurred. Viable counts were estimated throughout the experiment. Results are the outcome of 3 replicate experiments, and X and Y bars represent standard error.

The above experiment was also repeated for single and dual species planktonic cultures of *Ent* and 1.15. Starter cultures were again inoculated with 50 µl of overnight culture of *Ent*, 1.15 or a mixture of the two strains, and the disinfectant concentrations 0.05 µg ml^{-1} triclosan (TR) and 0.6 µg ml^{-1} benzalkonium chloride (BC). Again after 24 h incubation, the culture was placed into fresh media containing a higher concentration of disinfectant. The inoculum size was adjusted to give a number of cells similar to those received throughout the biofilm experiments (fig7.1). This process was again repeated until no more growth occurred.

Figure 7.2 demonstrates that the planktonic culture of 1.15 adapted to 0.75 µg ml^{-1} triclosan and 7.5 µg ml^{-1} BC. *Ent* also adapted to 7.5 µg ml^{-1} BC, and survived to concentrations of 1.5 µg ml^{-1} triclosan. The dual species cultures adapted to a maximum concentration of 2.5 µg ml^{-1} BC and 0.25 µg ml^{-1} triclosan. Again, *Ent* was very quickly out-competed from the dual species planktonic culture. When compared to the biofilm data, it can be seen that with the exception of the *Ent* culture adapting to triclosan, which reached the optimum concentration within the confines of this experiment, none of the planktonic cultures adapted as far as the biofilm cultures.

7.2a) Adaptation to increasing concentrations of triclosan



7.2b) Adaptation to increasing concentrations of benzalkonium chloride

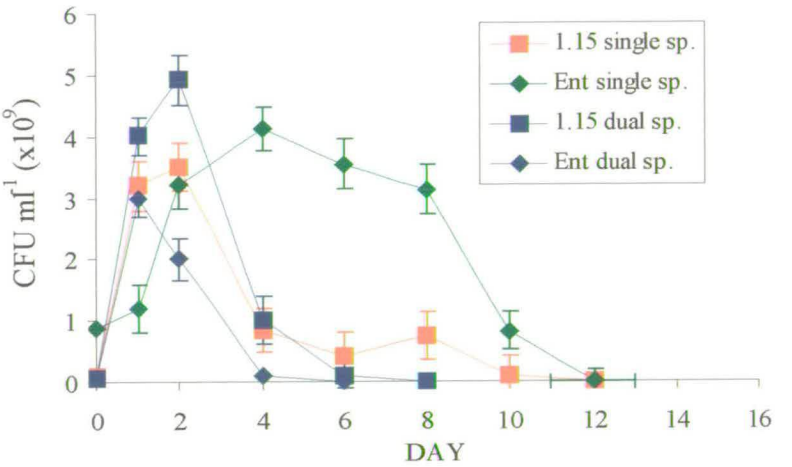


Figure 7.2

The adaptation of single and dual species planktonic cultures of *Ent* and 1.15 to increasing concentrations of triclosan (7.2a) and benzalkonium chloride (7.2b). The concentrations of disinfectants used are shown in table 7.1. Small vials containing 10 ml YE media and 2.5 g beads (diameter 4mm) were used. Cultures were incubated for a period of 24 h, and reinoculated into fresh media containing an increased concentration of disinfectant. The inoculum size used was adjusted to give a number of cells similar to those received throughout the biofilm experiments. Cultures were subjected to increasing concentrations of disinfectant until no more growth occurred. Viable counts were estimated throughout the experiment. Results are the outcome of 3 replicate experiments, and X and Y bars represent standard error.

7.2.1 *Time Taken to Lose Acquired Resistance*

The time taken for the *Ent* and 1.15 adapted biofilm cultures to lose this acquired resistance was estimated. Paper chromatography discs (5 mm) (Whatman No.1) were soaked in either 10 mg ml^{-1} BC or 1 mg ml^{-1} triclosan and placed onto lawns (YE agar) of the resistant cultures. These were incubated for 24 h, and the zones of inhibition measured. The cultures were also subcultured into disinfectant-free media and incubated for a further 24 h. Again, the cultures were tested for their resistance level using the paper chromatography discs soaked in disinfectant.

The cultures adapted to benzalkonium cultures lost their acquired resistance within 24 h incubation in disinfectant-free media. However, the triclosan-adapted cultures did not lose their resistance after 8 days of repeated re-subbing into fresh disinfectant-free media. The strain was still able to grow within media containing 1.5 $\mu g\ ml^{-1}$ triclosan. This occurred for both *Ent* and 1.15 biofilms. It was thought that a mutation towards triclosan had occurred. Samples of biofilm cells from the triclosan-adapted cultures were taken at 2, 4, 8, 10 and 12 days. Mutated cells of both *Ent* and 1.15 were present within 4 days incubation in increasing concentrations of triclosan. Five replicate experiments were conducted, and each case only 4 days incubation was required to produce triclosan mutation-resistant strains of *Ent* and 1.15.

7.3 CROSS-ADAPTATION

The benzalkonium chloride-adapted and triclosan resistant strains of *Ent* and 1.15 were tested against a wide range of commercially available disinfectants. The MICs of the disinfectants against single and dual species cultures of *Ent* and 1.15 are represented in **table 7.2**. The triclosan-resistant cultures were also more resistant to the triclosan-containing Sainsbury's Microban washing-up liquid. The cultures that had adapted to increasing concentrations of BC were more resistant to the BC-containing Sainsbury's multipurpose cleaner, and both triclosan and the Microban washing-up liquid. There was also a slight increase in resistance towards Dettox, which contains an undesignated cationic compound as its active ingredient. This acquired cross-resistance was also lost upon incubation in disinfectant-free media.

	1.15 single sp.			Ent single sp.			Dual sp.		
Disinfectant	Control	BC	TR	Control	BC	TR	Control	BC	TR
TR (μg ml^{-1})	**0.1**	**R**	**R**	**0.1**	**R**	**R**	**0.1**	**R**	**R**
BC (μg ml^{-1})	**1.0**	**300**	**1.0**	**1.0**	**350**	**1.0**	**1.0**	**250**	**1.0**
HPC (%)	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Domestos (%)	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Dettox (%)	**1.25**	**5**	**1.25**	**1.25**	**5**	**1.25**	**1.25**	**2.5**	**1.25**
Mr Muscle (%)	5	5	5	5	5	5	5	5	5
Ethanol (%)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sainsbury's Microban (%)	**0.01**	**0.5**	**0.5**	**0.01**	**0.5**	**0.5**	**0.01**	**0.5**	**0.5**
Sainsbury's Multipurpose (%)	**0.12**	**1.0**	**0.12**	**0.12**	**0.12**	**1.0**	**0.12**	**0.8**	**0.12**

Table 7.2

The MICs of various antimicrobial agents against single and dual species TR-resistant and BC-adapted cultures of *Ent* and 1.15. Unadapted cultures were included as controls. Domestos is sodium hypochlorite-containing disinfectant. Dettox contains a cationic compound as it's active ingredient. Mr Muscle contains EDTA, Sainsbury's Microban washing-up liquid contains triclosan, and Sainsbury's multipurpose disinfectant contains benzalkonium chloride.

Mastring-s® antibiotic discs were applied to lawns of the TR-resistant and BC-adapted cultures, and compared to control cultures of *Ent* and 1.15. **Table 7.3** represents the diameters of the clear zones surrounding the antibiotic discs (mm). Changes in susceptibility are represented in bold. The *Ent* and 1.15 triclosan-resistant cultures had sensitivity patterns identical to the controls. The BC-adapted cultures, however, contained several differences. 1.15 was more resistant to the β-lactam antibiotics ampicillin and carbenicillin, and more sensitive to streptomycin, kanamycin and sulphanmethizole. *Ent* demonstrated increased susceptibility towards the disinfectants streptomycin and sulphanmethizole.

Mastring-s® SPECIFICATIONS				**1.15**	**1.15 TR**	**1.15 BC**	***Ent***	***Ent* TR**	***Ent* BC**
M11	C	CHLORAMPHENICOL	25 μg	20	23	20	18	18	20
	E	ERYTHROMYCIN	5 μg	0	0	0	0	0	0
	FC	FUSIDIC ACID	10 μg	0	0	0	0	0	0
	MT	METHICILLIN	10 μg	0	0	0	0	0	0
	NO	NOVOBIOCIN	5 μg	0	0	0	0	0	0
	PG	PENICILLIN G	1 unit	0	0	0	0	0	0
	S	STREPTOMYCIN	10 μg	9	9	**18**	10	9	**18**
	T	TETRACYCLINE	25 μg	22	20	22	20	18	20
M26	AP	AMPICILLIN	25 μg	12	10	**0**	0	0	0
	C	CHLORAMPHENICOL	50 μg	26	24	25	26	25	25
	CO	COLISITN SULPHATE	100 μg	20	18	21	20	20	20
	K	KANAMYCIN	30 μg	10	10	**19**	18	16	18
	NA	NALIDIXIC ACID	30 μg	26	24	25	20	20	23
	NI	NITROFURANTOIN	50 μg	0	0	0	0	0	0
	S	STREPTOMYCIN	25 μg	8	9	**16**	10	9	**18**
	T	TETRACYCLINE	100 μg	28	26	26	18	20	20
M27	AP	AMPICILLIN	25 μg	12	12	**0**	0	0	0
	GM	GENTAMYCIN	10 μg	18	16	16	19	17	19
	PY	CARBENICILLIN	100 μg	22	20	**0**	0	0	0
	NA	NALIDIXIC ACID	30 μg	24	22	23	20	19	19
	NI	NITROFURANTOIN	50 μg	0	0	0	0	0	0
	SM	SULPHANMETHIZOLE	200 μg	7	6	**25**	20	22	**30**
	T	TETRACYCLINE	100 μg	26	24	25	20	22	22
	TS	COTRIMOXAZOLE	25 μg	20	19	21	19	29	28

Table 7.3

Mastring-s® testing of the triclosan-resistant and BC-adapted cultures of *Ent* and 1.15. Figures are the diameter of the clear zone surrounding the antibiotic disc (mm). Differences in susceptibility when compared to the control cultures are highlighted in bold.

The changes in susceptibility towards the β-lactam antibiotics penicillin G, ampicillin and carbenicillin, the aminoglycosides kanamycin and streptomycin and the folate synthesis inhibitors sulphanmethizole and trimethoprim were further examined. The changes in MICs of the antibiotics are demonstrated in **table 7.4**. Both 1.15 and *Ent* BC-adapted cultures were more resistant to the β-lactam antibiotics. 1.15 was more sensitive to sulphanmethizole, trimethoprim, kanamycin and streptomycin. *Ent* was more sensitive to sulphanmethizole and streptomycin.

ANTIBIOTIC ($mg\ ml^{-1}$)	1.15	
	Control	BC-adapted
Sulphanmethizole	100	25
Trimethoprim	100	6
Kanamycin	200	100
Streptomycin	100	25
Penicillin G	25	200
Ampicillin	12	200
Carbenicillin	25	200
	Ent	
	Control	BC-adapted
Sulphanmethizole	100	12
Streptomycin	25	12
Penicillin G	50	200
Ampicillin	25	200
Carbenicillin	25	200

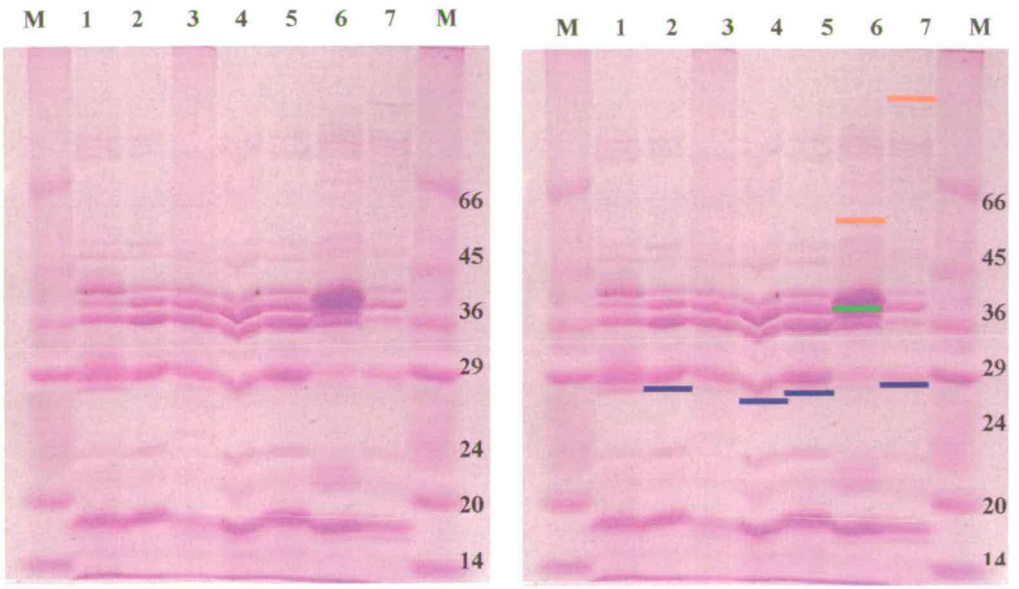
Table 7.5

The change in MIC of antibiotics in BC-adapted cultures.

Acquired resistance to antibiotics and disinfectants often involves changes to the outer cell surface, and so the outer membrane proteins of the adapted cultures were isolated (Filip *et al.*, 1973). **Figure 7.3** represents changes in the outer membrane proteins of triclosan-resistant and BC-adapted cultures of 1.15, and **figure 7.4**, the outer membrane proteins of *Ent*.

The 1.15 triclosan-resistant mutant (lane 3) was identical to the control culture (fig7.3). These cells also contained disinfection-sensitivity profiles identical to the control (lane1). The cultures obtained after one days incubation in triclosan (lane 2) and the BC cultures (lanes 4 & 5) were missing the 28 kDa protein. The BC-adapted culture that had been placed into triclosan-containing medium (lane 6) contained a new protein at approximately 55 kDa, and over expression of proteins at 38 kDa. The BC-adapted culture was also placed into Dettox-containing media (lane 7). This outer membrane fraction was also missing the 28 kDa protein, and contained a very high molecular weight 'new' protein.

The *Ent* tricosan-mutant expressed a new protein at 45 kDa (lane 3), however, like 1.15, these profiles were very similar to the *Ent* control. There were several changes to the biofilms adapted to increasing concentrations of benzalkonium chloride. The one-day BC biofilms contained increased expression of proteins at 44, 40, 22, 19 and 13 kDa, and a 'new' protein at 32 kDa (lane 4). This was identical to the day 12 BC-adapted biofilm that had been incubated in triclosan (lane 6). The day 12 BC-adapted biofilm also contained several changes in protein content, including increased expression of proteins at 44, 40 and 19 kDa, a 'new' protein at 24 kDa, and a missing protein at 13 kDa (lane 5). This was similar to the profile produced by the day 12 BC-adapted biofilm incubated in Dettox-containg media (lane 7).



'missing' protein
'new' protein
overexpression

Figure 7.3

SDS-Page analysis of outer membrane proteins of 1.15. Carried out on 10% denaturing gels and stained with coomassie blue. Lane 1 is an *Ent* control; lane 2, day 1 in triclosan; lane 3, triclosan mutant; lane 4, day 1 in BC; lane 5, day 12 in BC; lane 6, BC-adapted culture in triclosan; lane 7, BC-adapted culture in Dettox. The following protein markers were used: bovine albumin 66 kDa; ovalbumin 45 kDa; glyceraldehyde-3-phosphate dehydrogenase 36kDa; carbonic anhydrase 29 kDa; trypsinogen 24 kDa; trypsin inhibitor 20.1 kDa; lactalbumin 14.2 kDa.

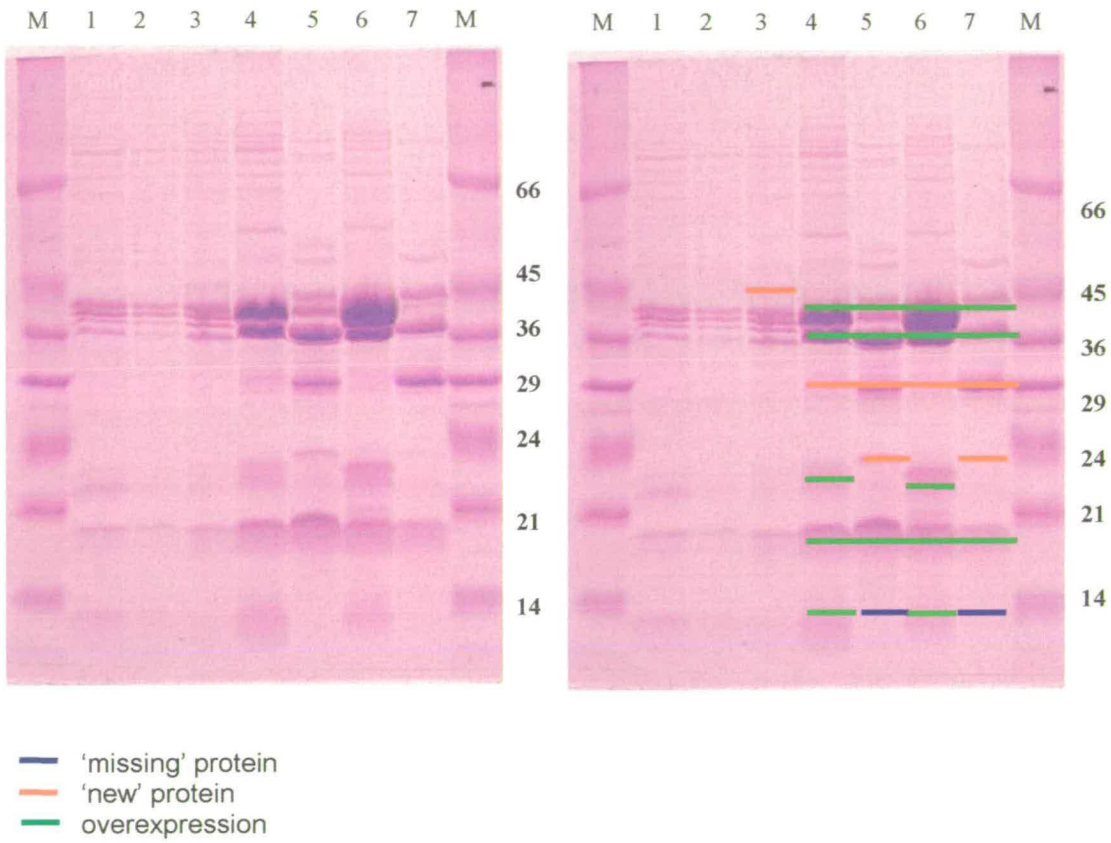


Figure 7.4

SDS-Page analysis of outer membrane proteins of *Ent.* Carried out on 10% denaturing gels and stained with coomassie blue. Lane 1 is an 1.15 control; lane 2, day 1 in triclosan; lane 3, triclosan mutant; lane 4, day 1 in BC; lane 5, day 12 in BC; lane 6, BC-adapted culture in triclosan; lane 7, BC-adapted culture in Dettox. The following protein markers were used: bovine albumin 66 kDa; ovalbumin 45 kDa; glyceraldehyde-3-phosphate dehydrogenase 36kDa; carbonic anhydrase 29 kDa; trypsinogen 24 kDa; trypsin inhibitor 20.1 kDa; lactalbumin 14.2 kDa.

7.4 DISCUSSION

7.4.1 *Adaptation to Disinfectants*

Ent and 1.15 were capable of adapting to increasing concentrations of triclosan and benzalkonium chloride, but not sodium hypochlorite. It is thought that this strong oxidising agent had such a broad effect within the cells that the bacteria were incapable of adapting. Interestingly, biofilm cultures were capable of adapting to higher concentrations of BC and triclosan than the planktonic cultures. This was most evident with the cultures adapting to BC. Biofilms of 1.15 reached concentrations of 25 $\mu g\ ml^{-1}$, x40 the original concentrations used. The planktonic cultures reached only 7.5 $\mu g\ ml^{-1}$. *Ent* biofilms reached concentrations of 30 $\mu g\ ml^{-1}$, whereas the planktonic cultures only reached 7.5 $\mu g\ ml^{-1}$. This is probably due to the innate resistance of biofilms when compared to planktonic cells. The biofilm structure provided some protection for the cells, allowing them to adapt before they were killed. The planktonic cultures were in direct contact with the disinfectants, allowing no time for adaptation to take place.

The bacteriocin-production in the dual species cultures of *Ent* and 1.15 also affected the ability of the culture to adapt to disinfectants. It seems that the competitive interactions between the strains affected the stability of the biofilm. After *Ent* was out-competed from the cultures, the resultant 1.15 biofilm was thinner, and unable to cope with disinfectant stress to the same capacity as the thicker, single species biofilms. Complete eradication of the dual species cultures occurred several days before the single species cultures.

7.4.2 *Resistance to Triclosan*

No changes were detectable in the outer membrane proteins of the triclosan mutants. It is likely that a mutation to the triclosan target within the cell had occurred, such as a change to FabI, the enoyl reductase targeted by low concentrations of triclosan. While no cross-resistance to other antimicrobial agents occurred, the ease of development of a triclosan-resistant mutant was disconcerting. Only four days incubation in increasing concentrations of disinfectant were required to develop resistance in both *Ent* and 1.15. However, the concentrations used within these experiments were much lower that the concentrations used in practise. These

'mutants' may not survive those higher concentrations. It would be interesting to mimic a food preparation or industrial environment, to perceive if triclosan mutants would also develop as easily. If residual concentrations of triclosan are left on a surface, perhaps this could occur.

7.4.3 *Adaptation to Benzalkonium Chloride*

Many changes were made to the outer membrane proteins of *Ent* during adaptation to increasing concentrations of BC. The nature of these changes in relation to the increasing resistance to BC is not known. Until recently, much of the resistance of Gram negative bacteria towards antimicrobial agents was attributed to the efficient permeability barrier provided by the outer membrane. However, recent studies have shown that multiple drug efflux pumps, sometimes with unusually broad specificity, are responsible for the intrinsic resistance of Gram negative bacteria.

The simple efflux pumps of Gram positive bacteria, and some Gram negative bacteria excrete drugs across a single cytoplasmic membrane layer. Examples include the Bmr (*Bacillus* multidrug resistance)-NorA (norfloxacin) group which pump out cationic dyes, fluoroquinolones and quaternary ammonium compounds, and the QacA group, which pump out quaternary ammonium disinfectants and cationic dyes (Paulsen, *et al.*, 1996). For Gram positive bacteria, these are extremely inefficient devices. The offending agent is pumped from the cytoplasm into the periplasmic space. However, lipophilic molecules would be expected to spontaneously diffuse back into the cytoplasm. A high turnover rate is, therefore, required in order to achieve significant levels of resistance (Nikaido, *et al.*, 1998b). Other Gram negative multidrug efflux pumps traverse both the cytoplasmic and outer membranes by utilising three component systems. These systems include the RND (resistance-nodulation-division) family of transporters, which are unique to Gram negative species. Key examples are the AcrAB system of *E. coli*, and the MexAB-OprM system of *Ps. aeruginosa*. Homologs of AcrAB and MexAB-OprM have been found in *Neisseria gonorrhoeae* and *Haemophilus influenzae*, suggesting multidrug efflux pumps are widespread amongst Gram negative bacteria (Nikaido, 1998). Some transporters, such as the *E. coli* AcrAB system, pump out a wide range of substrates including lipophilic and amphiphilic antibiotics, chemotherapeutic agents, metabolic

inhibitors, dyes, detergents, bile salts and solvents (**table 7.4**). The substrates for AcrAB and MexAB-OprM, therefore, must contain hydrophobic domains. A hypothetical mechanism for AcrAB and MexAB-OprM pumps is shown in **figure 7.5**.

Transporter name	Organisms	Class	Thought to function with	Substrates
Gram-positive pumps				
LmrA	*Streptococcus lactis*	ABC	-	EB, R6G, daunomycin, TPP+
QacA	*Staphylococcus aureus*	MFS	-	EB, QA, chlorhexidine, PI
LfrA	*Mycobacterium smegmatis*	MFS	-	EB, AC, QA, FQ
NorA	*Staphylococcus aureus*	MFS	-	EB, AC, QA, R6G, TPP+, puromycin, CP
Bmr	*Bacillus subtilus*	MFS	-	EB, AC, QA, R6G, TPP+, puromycin, CP
Smr	*Staphylococcus aureus*	SMS	-	EB, CV, QA, methyl viologen, TPP+
Gram-negative pumps				
EmrB	*E. coli*	MFS	EmrA, TolC	CCCP, NA, thiolactomycin
AcrB	*E. coli*	RND	AcrA, TolC	EB, AC, CV, SDS, TX100, bile salts, β-lactams, Nov, Ery, Fus, Tet, CP
MexB	*Pseudomonas aeruginosa*	RND	MexA, OprM	β-lactams, Nov, Ery, Fus, Tet, CP, FQ,
MexD	*Pseudomonas aeruginosa*	RND	MexC, OprJ	Fourth generation cephems, Tet, CP, FQ
MexF	*Pseudomonas aeruginosa*	RND	MexE, OprN	CP, FQ

Table 7.5

Examples of bacterial multidrug efflux transporters. Ac, acriflavin; CP, chloramphenicol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CV, crystal violet; EB, ethidium bromide; Ery, erythromycin; FQ, fluoroquinolones; Fus, fusidic acid; NA, nalidixic acid; Nov, novobiocin; PI, pentamidine isothionate; QA, quaternary ammonium compounds, including benzalkonium chloride and cetyltrimethylammonium bromide; R6G, rhodamine 6G; SDS, sodium dodecylsulphate; Tet, tetracycline; TPP+, tetraphenylphosphonium; Tx100, Triton X-100 (adapted from Nikaido, 1998b).

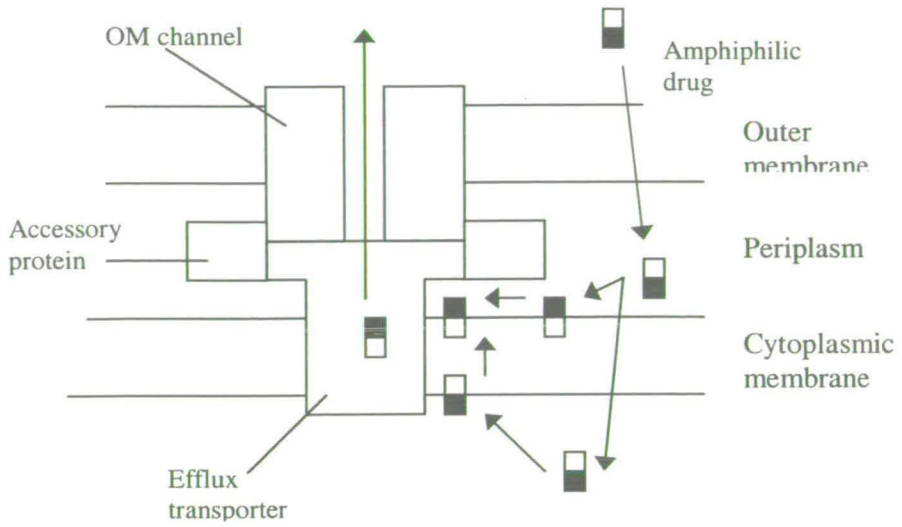


Figure 7.6

Hypothetical model of the multicomponent multidrug efflux pumps. Amphiphilic drugs (with the lipophilic portion shown as the empty rectangle) diffuse into the periplasm and are then captured while inserted in the outer leaflet, or they diffuse into the cytoplasm and are captured, in this case when it partitions into the inner bilayer (adapted from Nikaido, 1998b).

The BC-adapted cultures developed cross resistance to triclosan, the disinfectant Dettox, and the β-lactam antibiotics, penicillin, ampicillin and carbenicillin. It is probable that this was an efflux-mediated response. Quaternary ammonium compounds, β-lactam antibiotics and triclosan are all known to be exported by multidrug efflux pumps (Russell, 2000; Nikaido, 1998; McMurray *et al.*, 1998b). The nature of the cationic compound in Dettox is not known, however, this may also be pumped out of the cell by an efflux pump.

An interesting effect was the increased susceptibility to the aminoglycosides, streptomycin and kanamycin, and the folate synthesis inhibitors trimethoprim and sulphanmethizole. Recent research has provided evidence for an aminoglycoside efflux pump in *Ps. aeruginosa* (Westbrock-Wadman *et al.*, 1999) and *E. coli* (Rosenberg *et al.*, 2000). As it would be more beneficial for the bacterium to concentrate its efforts on excluding BC from the cell, the adaptation may have

induced the increased expression of the efflux pump to extrude BC, but a depression in genes for other resistance mechanisms, such as an aminoglycoside efflux pump. Alternatively, the action of BC may have affected membrane permeability, allowing the aminoglycosides and folate synthesis inhibitors easier access through the outer membrane. Aminoglycosides cross the outer membrane by passive diffusion through porins. The molecule is thought to interact with divalent cation binding sites on surface LPS molecules. The aminoglycosides have a higher affinity for these sites, and so competitively displace them. The bulkiness of the displacing aminoglycoide is though to create a distortion to the membrane structure, and cause an increase in permeabilisation of the outer membrane to a variety of compounds, along with enhanced uptake of the polycationic aminoglycosides (Hancock, 1997).

7.4.4 *Conclusions*

The results in this chapter indicate that biocides can select for antibiotic resistance. Biofilms of *Ent* and 1.15 were able to adapt to increasing concentrations of benzalkonium chloride, and develop cross-resistance to triclosan, Dettox and the β-lactam antibiotics, penicillin, ampicillin and carbenicillin. However, there was a cost to this acquired resistance: the cultures also developed increased susceptibility to some aminoglycosides and folate synthesis inhibitors. This acquired resistance was also temporary: incubation in disinfectant-free media rendered the cultures BC-sensitive.

Chapter 8

GENERAL DISCUSSION

8.1 ROLE OF BACTERIOCINS IN BIOFILMS

The majority of research into interspecies interactions within biofilms has focused on the beneficial aspects of these relationships. Many studies have focused on the increased metabolic efficiency and substrate accessibility in co-operating communities (Wolfaardt *et al.*, 1994; Moller *et al.*, 1998; Neilson *et al.*, 2000). Enhanced protection from environmental stresses has also been demonstrated (Buorion and Cerf, 1996; Skillman *et al.*, 1999). However, as described in chapter 3 of this thesis, not all interactions will be beneficial: antagonistic interactions may also play an important role in the development of microbial communities.

The production of antimicrobial compounds seems to be a generic phenomenon for most bacteria. Antimicrobial agents include toxins, bacteriolytic enzymes, bacteriophages, antibiotics and bacteriocins. Bacteriocins are compounds produced by bacteria that inhibit or kill closely related species. Their production occurs across all major groups of Eubacteria and the Archaebacteria. It has been estimated that 90% of *Ps. aeruginosa* from environmental and clinical sources produce R and F-type pyocins. Of these, 70% also produced an S pyocin (Riley, 1998). In their review of colicin production by *E. coli*, Smarda and Smajs (1998) reported that 35% of *E.coli* strains appearing in human intestinal tract are colicinogenic. Taking this into consideration, it is likely that many of the interactions occurring between bacteria in biofilms will be antagonistic in nature. It has been hypothesised that production of a bacteriocin would give an organism competitive advantage when interacting with other microbes. The production of antagonistic compounds may be beneficial in gaining a foothold in a new environment. Bacteriocins may also prevent the colonisation of a pre-established microbial community by a competitive species, a phenomenon known as 'colonisation resistance' (Marsh and Bowden, 2000).

These studies revealed bacteriocin-producing strains do have a competitive advantage over bacteriocin-sensitive strains. This was exemplified by figure 3.4, the addition of a second strain to a pre-established biofilm. Fig3.4c) demonstrated the addition of strains 1.15 and 5920 to pre-established biofilms of 5920 and 1.15, respectively. 1.15 was capable of producing two microcins, 1.15M-4 and 1.15M-8, with activity against 5920, whereas although 5920 was capable of producing a

microcin, this had no activity against 1.15. Strain 1.15 was seen to successfully colonise a pre-established biofilm of the microcin-sensitive 5920. In comparison, 5920 was unsuccessful in its attempts to colonise biofilms of 1.15. The production of microcin, therefore, allowed 1.15 to invade a pre-established 5920 biofilm, and also protected the 1.15 biofilm population from a secondary colonising population of 5920. However, the inoculum used in these studies was adjusted to contain a 20:1 ratio of cells, with the pre-established strain dominating in each case. In a natural environment, an invading population would contain a much lower quantity of cells, and as bacteriocins are generally produced during stationary phase, it is likely that the incoming population would contain only low expressions of bacteriocin. In comparison, a population-dense biofilm environment would contain high quantities of bacteriocin. It would seem likely that the production of bacteriocin would be more beneficial in protecting the biofilm from incoming competitors and discouraging attachment of incoming species. However, if an invading strain managed to establish itself within the biofilm and the population began to increase, bacteriocin production would also be increased. Table 4.1, the bacteriocin and protease production in competing cultures, demonstrates that the close proximity of a competitive strain can cause increased antimicrobial production. At this stage, the competitive advantage of bacteriocin-production during colonisation of a pre-established biofilm would become more apparent.

Bacteriocins are under density dependent control: they are only induced upon entry into stationary growth phase. This raises the possibility that bacteriocins are under quorum sensing control. Quorum sensing is known to control a variety of physiological processes, including the production of virulence factors involved in pathogenesis. In order to protect the population from bacterial competitors, it seems likely that these regulatory processes also control the production of antagonistic substances such as bacteriocins. This has been shown to be true for certain antimicrobial agents. In *Erwinia cartovera*, the signal OHHL acts as a command for the activation of the β-lactam antibiotic carbapenem (Swift *et al.*, 1993). Carbapenem synthesis was also found to be under quorum sensing control in *Serratia* (Thompson *et al.*, 2000). *Pseudomonas aeruginosa* protease and phenazine production is also known to be controlled by the transcriptional activators LasR and

RhlR. In *Streptomyces griseus*, production of secondary metabolites such as streptomycin is enhanced by a γ-butyrolactone signal, a molecule that closely resembles the HSL sensory molecules used by Gram negative bacteria. This molecule is also produced constitutively at low concentrations and when a critical concentration is reached, antibiotic production is switched on (Duny and Leonard, 1997). Interestingly, a bacteriocin termed '*small*' previously isolated from a *Rhizobium leguminosarum* was also found to be a HSL molecule (Schrimpsema *et al.*, 1996). This agent inhibited the growth of potential competitors for root-nodule occupancy, and also controlled cell-cell interactions involved in plant-microbe interactions. Similarly, the lantibiotic nisin produced by *Lactobacillus lactis* strains acts as a bacteriocin against other strains and also as a quorum sensing signal for the positive regulation of expression of biosynthetic and immunity genes in the nisin-producing population (De Ruyter *et al.*, 1996). Interestingly, the strain 1.15 is capable of producing two microcins: one at 4 h, and another as the population reaches stationary phase, at 8 h. This suggests that, at least for 1.15, two pathways of bacteriocin production with two separate activation signals are in operation. Surette and Bassler (1998) provided evidence to suggest that *E.coli* and *S. typhimurium* also secrete a small, heat labile organic molecule involved in quorum sensing. Unlike the HSL molecules used by a wide range of Gram negative bacteria, this molecule, like 1.15M-4, is produced during midexponential phase.

In these studies, there was no increased bacteriocin production in my biofilms when compared to planktonic cultures; this may be associated with the methodologies used. A batch culture method was used to grow cultures to very high cell densities, densities probably similar to those found in biofilms. However, in natural, low nutrient environment, an increase in bacteriocin production in biofilm cultures would probably occur. In a comparison of protease production in planktonic and biofilm cells, Evans *et al.* (1994) reported production of protease was higher in biofilm populations. A report of upregulation of 'bacteriophage-like' proteins, or pyocin during adhesion in a *Pseudomonas* biofilm was made at the recent ASM conference in Big Sky, Montana, again suggesting bacteriocin production may be very important for biofilm cultures.

Mathematical models have predicted that the production of bacteriocins would be most advantageous in a spatially structured environment such as a biofilm (Frank, 1984). The findings in this thesis also indicate bacteriocins are well adapted for use within a biofilm, both in gaining a foothold into a new community and discouraging the attachment of potential competitors. It has been suggested that these bacteriocins may have evolved specifically for this purpose (Watnick and Kolter, 2000).

8.2 COMMUNITY DEVELOPMENT

Until recently, bacteria were seen as aggressive single-celled planktonic entities, designed to compete with one another. This has now given way to the view that bacteria are capable of developing complex, co-operative communities through the use of chemical signalling. Results in chapter 4 have shown that the strain *Ent. agglomerans/Ent*, when challenged with a bacteriocin-producing, antagonistic strain demonstrated the ability to signal the repression of bacteriocin-synthesis, to produce a co-operative biofilm. This has demonstrated another remarkable aspect of biofilm-forming bacteria, and the use of interspecies signalling. *Ent*, through the use of chemical signalling, has the ability to **choose** to co-operate under adverse conditions. *Ent. agglomerans/Ent* has shown itself to be a phenomenal strain. The same strain was involved in co-operative biofilm formation with a *K. pneumoniae* strain (Skillman *et al.*, 1999). In this case, the EPS produced by *Ent* played an important role in the dual species biofilm formation, acting both as an adhesin and as the EPS of both strains interacted, changing their physical properties. This strain has many attributes to make it an extremely efficient biofilm-former.

These studies have examined biofilm formation under adverse, competitive conditions. Biofilms were challenged with both bacteriocin-producing strains and bacteriophage. However, the biofilms formed were seen to consist of both bacteriocin-producing and bacteriocin-sensitive strains, and bacteriophage infected and uninfected cells. It was thought that the biofilm architecture provided a safe haven for both bacteriocin and bacteriophage-sensitive strains.

8.3 CONTROL OF BIOFILM FORMATION

8.3.1 *Interspecies Interactions in Biofilms*

The close association and stability of cells within biofilms and local areas of hindered diffusion are likely to increase the possibility of physiological interactions occurring between bacterial species (James *et al*, 1995). The interspecies interactions occurring within biofilm can enhance the resistance of biofilms to antimicrobial agents and other environmental stresses. For example, through the production of copious polysaccharide material, *Ps. aeruginosa* protected cells of *Listeria inocua* from disinfection (Buorion and Cerf, 1996). Similarly, the enhanced resistance to disinfection by a dual species biofilm of *Ent. agglomerans* and *K. pneumoniae* was partially attributed to interactions between the EPS produced (Skillman *et al.*, 1999). Cells can also be protected by the inactivation of an antimicrobial agent by enzyme activity. For example, production of a β-lactamase by *M. catarrhalis* was shown to protect a pathogenic *S. pneumoniae* strain (Budhani and Struthers, 1998). By being physically close to neighbours producing neutralising enzymes, such as sIgA proteases (Kilian *et al.*, 1996), cells may also be protected from components of the host defences.

However, antagonistic interactions, such as the production of bacteriocins, decreased the resistance of dual species biofilms to disinfectants (tables 3.7 & 4.2). This was thought to be associated with the increased stress the cells were subjected to. Cells had to deal with both disinfectant and bacteriocin. This again indicates the importance of the nature of the interspecies interactions occurring within biofilms and how this can affect the resistance to disinfectants.

8.3.2 *Bacteriocins*

With the resistance of pathogenic strains to traditional antibiotics becoming increasingly problematic, there has been interest in the development of alternative methods of control, including the possibility that bacteriocins may be used as a replacement for antibiotics. Such technologies have already been developed in Russia. For example, the colicinogenic *E.coli* strain M-17 was used for the treatment of bacillary dysentry. Gelatinous capsules were used to protect the bacteria from the action of intestinal enzymes (Hardy, 1975). The bacteriocins of lactic acid bacteria,

such as nisin, are active against food-borne pathogenic bacteria such as *Bacillus cereus*, *Clostridium botulinum*, *C. perfringens*, *L. monocytogenes* and *Staphylococcus aureus*, and are now widely used in food preservation (Chung, K *et al.*, 1997). The listericidal class IIa bacteriocins of lactic acid bacteria are thought to be next in line if the use of more bacteriocins is approved in the future (Ennahar *et al.*, 2000). Nisin has also been used to inhibit plaque-producing bacteria (Howell *et al.*, 1993). The colonisation of bacteriocin-producing strains of *Haemophilus influenza* in the rat nasopharyngeal region and *Streptococcus mutans* in the human oral cavity has also been investigated (Lipuma *et al.*, 1990; Hillman *et al.*, 1997). These studies indicated that bacteriocin-producing strains may play a role in the control of bacterial infections. The bacteriocin-producing strains used within these studies also demonstrated the ability to prevent the colonisation of bacteriocin-sensitive strains.

6.3.3 *Bacteriophage*

It has been suggested bacteriophage or their associated enzymes could be used as an alternative to chemical treatments. Again, this technology was developed, and is still of wide spread use within the former Soviet Union. Bacteriophage are used to treat both primary and nosocomial infections, alone or in conjunction with traditional antibiotics. Multidrug-resistant pathogenic bacterial strains are routinely sent to the Eliavia Institute of Bacteriophage, Microbiology, and Virology in Tblisi, Georgia, where a bacteriophage specific for that strain is isolated to combat those strains. The institute contains the largest collection of phage for therapeutic use. However, the Russian publications tend to be extremely confusing, throwing doubt over the effectiveness of bacteriophage treatment (Holzman, 1998).

Studies were conducted in Britain to evaluate the effectiveness of bacteriophage in eradicating an *E. coli* infection in mice. A single dose of phage was more effective than a multiple dose of antibiotic (Smith and Huggins, 1982). If bacteriophage are effective, they may have several advantages over conventional antibiotics. For example, as phage multiply, a single dose may be all that is needed to overcome a bacterial infection. Their high specificity would mean that only the pathogenic bacteria are killed while 'good' bacteria would remain unaffeced.

However, this may also be a disadvantage, their specificity means diagnosis must be precise, and treatment must wait for laboratory results. Bacteriophage can also be antigenic, leading to undesired effects involving the immune system. However, it is thought that a one-time exposure to a bacteriophage being used to control a life-threatening infection would represent a minor risk to a patient (Holzman, 1998).

Chapter 5 investigated the use of phage and their polysaccharide depolymerases, both as a tool for the investigation of biofilm structure and as a method of biofilm eradication. Studies demonstrated that infection of a biofilm with a single phage type was unsuccessful in completely eradicating the biofilm: a mixture of infected and uninfected cells remained attached to the surface. These uninfected cells would continue to provide a source of contamination to other areas by bacterial detachment. To completely eradicate single species biofilms of *Ent. cloace*/5920, infection by three different phages was required.

Biofilms in natural or industrial environments more commonly consist of complex communities of microorganisms. The removal of mixed species biofilms using bacteriophage would become increasingly complex, and it would seen unlikely that bacteriophage are suitable for removing contaminating mixed species biofilms from industrial surfaces. As with treating pathogenic infections, knowledge of the bacterial composition of the contaminating biofilm would be required before bacteriophage treatment was attempted. Bacteriophage specific for the key species within the biofilm could them be selected. There may also be difficulty in obtaining large enough quantities for treatment of large surface areas. However, there is the possibility that bacteriophage could be used to selectively remove pathogenic or problematic species from biofilm material. For example, Kudva *et al.* (1999) described the isolation of *E. coli* O157 antigen-specific bacteriophage. These phages could play a role in biocontrol of *E. coli* in fresh foods, without compromising the viability of other normal flora or food quality. Similarly, the use of bacteriophage to control growth of food-poisoning *Salmonella* species in the chicken-processing industry was investigated (Gould, unpublished results).

Other nonmedical uses of phage include the control of various plant pathogens. For example, the bacterial spot pathogen of tomato plants, *Xanthomonas campestris* pv. *vesicatoria* was successfully controlled with bacteriophage (Flaherty

et al., 2000). A viral polysaccharide depolymerase was also evaluated as a potential tool to control fire blight (Kim and Geider, 2000).

Hughes *et al.*, (1998b) demonstrated that treatment to remove EPS using phage polysaccharide depolymerases was effective in removing large quantities of biofilm material from surfaces. Skillman (1999a) provided evidence to suggest that the use of a phage enzyme in conjunction with disinfectants led to a more effective removal of biofilm material than either treatment alone. Table 6.1 also demonstrated the increased susceptibility of enzyme-treated biofilms. It was thought that the polysaccharide depolymerase activity afforded better access for disinfectants and, consequently, better removal and eradication. As in the control of fire-blight (Kim and Geider, 2000), this may be used as a semi-specific treatment, for example, in the removal of pathogenic species from mixed species biofilms. Again, this may not be very effective against complex biofilm communities.

8.3.4 *Biocides*

It has been hypothesised that the high resistance of bacterial biofilms to chemical and antibiotic treatments is associated with both the limited penetration of the agent through the biofilm, and also the recalcitrant phenotypic state of the bacteria dwelling within the biofilm. The general stress response produced by sub-inhibitory concentrations of disinfectant resulted in a reduction in growth rate, and a reallocation of resources into polysaccharide synthesis. However, biofilms of *Ent.* cloace/5920 exhibiting increased polysaccharide production were only marginally more resistant to a range of antimicrobial agents, verifying that, at least in this case, EPS did not play a significant role in biofilm resistance. Although there was a slight delay in the killing of cells with the positively charged antibiotics, complete eradication of the biofilm did occur. Similar results were obtained by Nichols *et al.* (1988). For the antibiotic tobramycin, although the EPS produced by *Ps. aeruginosa* bound the antibiotic, the resulting decrease in diffusion coefficients within the biofilm was not enough to define this a significant barrier. Thus, the EPS produced by *Ent. cloace*/5920 only delayed killing.

Unfortunately, this work has not shed any more light on the problem of biofilm resistance. However, the acquisition of resistance by previously susceptible

strains seen in chapter 7 has shown to be intriguing. Biofilms of *Ent* and 1.15 were gradually exposed to increasing concentrations of triclosan and benzalkonium chloride, each time selecting for the least susceptible phenotype. In clinical practise, if repeated exposure to an antimicrobial agent occurred without compete killing, this may select for antibiotic resistance within a biofilm. Reduced permeability, for example, through increased polysaccharide production, may delay eradication of the biofilm, allowing sufficient time for the selection of a more resistant phenotype to occur. For example, the cultures adapting to increasing concentrations of triclosan responded by producing a triclosan mutant, and it was thought that the biofilm cells responded to benzalkonium chloride stress through selection of strains utilising increased expression of multi-drug efflux pumps.

Antibiotic resistance can also quickly be disseminated through bacterial populations via acquisition of new genetic material. Few studies exist on horizontal gene transfer in biofilms, and these have mainly concentrated on conjugation (Dahlberg, 1998; Hausner and Wicht, 1999; Licht *et al.*, 1999). Biofilms are thought to be more favourable for conjugation than aqueous systems due to stabilisation of the cells on the substratum, and an increased contact time. Factors such as the nutrient conditions, shear stress, cell concentration and temperature can control the extent of conjugation within a biofilm (Ehlers, 2000).

The results in chapter 7 also indicated that biocides could select for antibiotic resistance. Biofilms of *Ent* and 1.15 with resistance to BC had also acquired resistance to triclosan, Dettox, and the β-lactam antibiotics penicillin, ampicillin and carbenicillin. However, there was a cost to this resistance: the cultures had developed increased susceptibility to aminoglycosides and folate synthesis inhibitors. As this acquired resistance was not a permanent effect (incubation in disinfectant-free media rendered the cultures BC-sensitive), it is unlikely these strains would pose much of a threat. In a review of the role of biocides in the selection of antibiotic resistance, Russell (2000) also stated that no relationship could be found between antibiotic and biocide resistance. Several laboratories have shown that antibiotic resistant staphylococci, enterococci, and other hospital pathogens were not any more resistant to biocides than the antibiotic-susceptible strains. At present, no special strategies exist for biocides being used against antibiotic-resistant bacteria. However, the

mechanisms for cross-resistance to occur do exist (Paulsen *et al.*, 1996; Stickler *et al.*, 1993). This area obviously needs more research. Possible cross-resistance should be considered when choosing disinfectants for alternation with quaternary ammonium compound-based disinfectants. This should be taken into account when designing improved disinfection procedures.

Resistance to triclosan can develop as a result of a single point mutation (McMurray *et al.*, 1998a). These studies have also indicated that a mutation to triclosan could develop quickly under low concentrations of triclosan. In practise, a mutation could occur if residual sub-lethal concentrations of triclosan were left on surfaces after treatment. These studies indicate that the wide-spread use of triclosan should be cautioned.

8.4 FUTURE WORK

These studies have involved the use of a high-nutrient batch culture system. If a more natural biofilm system was mimicked, such as a low-nutrient, continually flowing environment, this may result in a completely different outcome, altering both the interactions between bacteriocin-producing and sensitive strains, and between bacteria and phage.

The study of the interactions between bacteriocin-producing strains in biofilms has touched on an area we know little about. The production of antimicrobial agents is a generic phenomenon amongst bacteria, and antagonistic interactions brought about by bacteriocin production may be a common occurrence in biofilms. Further studies into the involvement of bacteriocin production in quorum sensing would be interesting. The nature of the bacteriocin-repressive signal produced by *Ent* could also be investigated. There is also the possibility that bacteriocins and bacteriocin-producing strains could be used to control biofilm formation, or as a novel strategy to control the adhesion of potential pathogens.

The study of bacteriophage infection of bacterial biofilms has provided many possibilities for future research. For example, it was not clear why a smaller inoculum of phage was most successful in the removal of biofilm material from a surface. Microscopy of the biofilm as it is infected with phage may provide some insight into the infection process, and the pattern of biofilm disintegration. It would also be interesting to allow the biofilm communities of bacteria and phage to develop for longer periods of time. Introduction of a phage into a susceptible population results in an initial epidemic of phage, followed by a stable equilibrium that can last from weeks to months. If the bacteriophage were to be considered for the control of biofilm formation, further knowledge of the ecology of the nature of phage:bacteria interactions is required.

Chapter 9

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