Aston University

Some pages of this thesis may have been removed for copyright restrictions.

If you have discovered material in AURA which is unlawful e.g. breaches copyright, (either yours or that of a third party) or any other law, including but not limited to those relating to patent, trademark, confidentiality, data protection, obscenity, defamation, libel, then please read our Takedown Policy and contact the service immediately

STUDIES ON BIOFILM GROWTH OF *Pseudomonas aeruginosa* PAO1

SHONA MARGARET NELSON

Doctor of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

April 1993

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without proper acknowledgement.

THE UNIVERSITY OF ASTON IN BIRMINGHAM

STUDIES ON BIOFILM GROWTH OF *Pseudomonas aeruginosa* PAO1

by

Shona Margaret Nelson

Doctor of Philosophy

1993

SUMMARY

The development of *in vitro* techniques to model the surface-associated mode of growth is a prerequisite to understanding more fully the physiological changes involved in such a growth strategy. Key factors believed to influence bacterial persistence in chronic infections are those of the biofilm mode of growth and slow growth rate. Methods for controlling *Pseudomonas aeruginosa* biofilm population growth rates were investigated in this project. This microorganism was incompatible with the *in vitro* 47mm diameter membrane filter-based biofilm technique developed for the study of *Escherichia coli* and *Staphylococcus epidermidis* by Gilbert *et al* (Appl. Environ. Microbiol. 1989, **55**, 1308-1311). Two alternative methods were designed. The first comprised a 25mm diameter cellulose acetate membrane filter supported in an integral holder. This was found to be limited to the study of low microbial population densities with low flow rates. The second, based on a cylindrical cellulose fibre depth filter, permitted rapid flow rates to be studied and allowed growth rate control of biofilm and eluted cells.

Model biofilms released cells to the perfusing medium as they grew and divided. The viability of released cells was reduced during, and shortly after, inclusion of ciprofloxacin in the perfusate. Outer membrane profiles of biofilm populations exhibited at least two bands not apparent in planktonic cells grown in batch and chemostat culture, and LPS profiles of biofilm populations showed variation with growth rate. Cell surface hydrophobicity of resuspended biofilm cells varied little with growth rate, whilst it decreased markedly for cells released from the biofilms as growth rate increased. Cells released from the biofilm were more hydrophilic than their sessile counterparts. Differing growth rates, LPS profiles and hydrophobicity are proposed to have a bearing on the release of cells from the adherent population.

Key words: *Pseudomonas aeruginosa*, biofilms, growth rate, outer membrane, cell surface hydrophobicity.

To Dad

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Professor M.R.W. Brown, for his guidance, ideas and many suggestions throughout the course of the project work. Sincere thanks are due also to Dr. Peter Gilbert, Department of Pharmacy, University of Manchester, for numerous informative discussions and allowing me to work in his laboratory.

I wish to express my gratitude to many people; Dr. Peter Lambert for his boundless enthusiasm and expert help with outer membrane preparations, Mr. Roy Tilling and Mrs. Dorothy Townley for their generous technical assistance and unfailing encouragement, Drs. Anthony Smith, David Evans and Phil Collier for their considerable help and advice and Dr. A. D'Emanuele at Manchester for the use of the Electroscan microscope.

To my friends in the Aston and Manchester laboratories, many thanks for making the time fly past and providing a life beyond work.

Most of all, thanks to Mum for understanding and putting up with me and David for avoiding the subject.

I acknowledge, gratefully, receipt of financial support from the Science and Engineering Research Council and Aston University.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

CDM	chemically defined media
CDM-Fe	iron-depleted CDM
CDM+Fe	iron-plentiful CDM
CF	cystic fibrosis
cfu(s)	colony-forming unit(s)
℃	degrees centigrade
(d)dH_2O	(double) distilled water
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
h	hour(s)
HIC	hydrophobic interaction chromatography
IRMP(s)	iron-regulated membrane protein(s)
kDa	Kilodaltons
l	litre(s)
LPS	lipopolysaccharide
µg	microgramme(s)
µm	micrometer(s)
mg	milligramme(s)
ml	millilitre(s)
min	minute(s)
M	molar concentration
M_{wt}	molecular weight
MIC	minimum inhibitory concentration
MOPS	4-morpholino-propane sulphonic acid
NA	nutrient agar
NB	nutrient broth
OD	optical density
OD_{470}	optical density at 470nm
OD_{630}	optical density at 630nm
OM	outer membrane
OMP(s)	outer membrane protein(s)
Opr B	outer membrane protein D1
OprC	outer membrane protein C
OprD	outer membrane protein D2
OprE	outer membrane protein E
OprF	outer membrane protein F
OprG	outer membrane protein G
OprH	outer membrane protein H1

OprI	outer membrane protein I
OprL	outer membrane protein H2
OprP	outer membrane protein P
rpm	revolutions per minute
Sarkosyl	N-lauryl sodium sarcosine
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electophoresis
SEM	scanning electron microscopy
sp.	species
μ	specific growth rate
t_d	doubling time
w/v	weight per volume

1. INTRODUCTION

Most microorganisms have an obligate requirement for an aqueous environment, being dependent upon the transport of essential soluble substrates and nutrients across their plasma membranes, yet they exhibit a tendency for adsorbing to and colonising solid surfaces submerged in the liquid phase. The solids may be organic, inorganic, living or dead materials, ranging from plants and rocks to animals and man-made structures, and bacterial adhesion may be of a transient or permanent nature (Costerton *et al*, 1987). The term adhesion can be defined unambiguously only in terms of the energy involved in the formation of the adhesive junction and, consequently, when work is required to separate a bacterium from a solid substratum to its original condition then it can be said truly to have adhered to that surface (Marshall, 1991). The reasons for this preferential growth at surfaces are unclear but it was suggested, originally, to be a survival tactic, in the case of small starved marine bacteria (Dawson *et al*, 1981). Certainly, in their natural environments, microorganisms are subjected to fluxes of nutrients ranging from conditions of nutrient-surfeit to varying degrees of deficiency and recover when nutrients are available once again (Dawes, 1985). Such a feast and famine lifestyle necessitates the ability of a bacterium to adapt to its immediate surroundings and, generally, bacteria can be considered as being in a dynamic state, extremely versatile in their responses to environmental changes (Roszack and Colwell, 1987). Alterations of morphology, cell volume and internal pools of compounds (Kjelleberg and Hermansson, 1984) are but a few examples of adaptive mechanisms in the so-called "starvation-survival" response. Moreover, if small starved cells can reach an interface, additional survival mechanisms become available to them (Kjelleberg *et al*, 1982), such as the rapid triggering of "ultramicrocell" formation which increases their surface/ volume ratio packing density (Humphrey *et al*, 1983).

The pathogenic bacterium in a nutrient-rich environment (such as the human body) relative to habitats such as soil, water or air, can respond to such changes by developing requirements for relatively complex growth factors (Roszack and Colwell, 1987). However, host defence mechanisms are mobilised in response to the presence of invasive bacteria, resulting in the rapid killing of planktonic microorganisms, and it is

often to the distinct advantage of the bacteria to adhere to host tissue as an evasion strategy (Costerton *et al*, 1985). Thus, the adherent biofilm mode of growth can be considered as part of both the offensive and defensive weaponry of microorganisms.

The attached bacteria grow and divide, resulting in the formation of adherent microcolonies (Costerton *et al*, 1990), the production of a highly hydrated polysaccharide glycocalyx (Sutherland, 1980) enhancing adhesion further. In addition to protecting the enclosed cell from harsh fluctuations in the physical conditions of the surrounding environment (Costerton *et al*, 1987) the glycocalyx may also place the cells in optimal positions for nutrient acquisition (Costerton *et al*, 1988). Moreover, it may protect them from host antibodies and phagocytes and, to a certain degree, aid in bacterial recalcitrance to antimicrobial chemotherapy (Costerton *et al*, 1990). An important feature of the enclosed cells is that, physiologically, they are quite distinct from their planktonic counterparts (Gilbert *et al*, 1990). Furthermore, as the thickness of the adherent biofilm increases over time, so the levels of cell heterogeneity within the biofilm increase. Cells deeply embedded within the structure experience a very different physical and chemical environment from that experienced by cells at the surface (Anwar and Costerton, 1992). Resultant physiological differences play major roles in the success or otherwise of therapeutic treatment of chronic bacterial infection *in vivo* (Gilbert et al, 1990).

1.1 THE FORMATION OF BIOFILMS

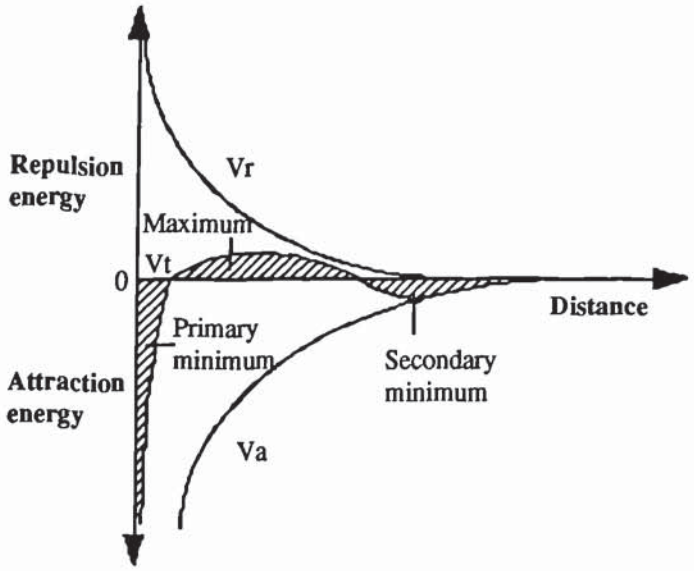
1.1.1. Reversible adhesion

Before bacterial biofilm formation can commence, cells must come into contact with a solid surface. The specific mechanism by which this is achieved is dependent upon the composition both of the organism and of the particular surface (Costerton and Lappin-Scott, 1989). Transport of cells by fluid dynamic forces, to a point where long-range forces may be important in positioning the cells close to a surface, may occur (Van Loosdrecht *et al*, 1989; Marshall, 1991), with van der Waals forces being the only ones

operative at distances greater than 50nm (Busscher and Weerkamp, 1987). This distance is too large for the opposing surfaces to recognise specific surface components. Other transport mechanisms to surfaces include sedimentation (i.e. gravitational forces), chemotaxis and Brownian motion (Marshall, 1985).

Understanding the adhesion of bacteria to solid substrata involves a consideration of various aspects of colloid chemistry since, in physicochemical terms, bacteria can be regarded as colloidal particles. The Derjaguin-Landau and Verwey-Overbeek (DLVO) theory of hydrophobic surfaces has been used to explain bacterial interaction with a surface (Norde and Lyklema, 1989). The DLVO theory states that as rigid bodies of like charge approach each other, they are subject to attractive and repulsive forces that are additive but vary independently with the distance of separation between the bodies. In simple terms, the derivation of the DLVO theory employed in biofilm formation is applicable to the analysis of the weak long-range interactions of initial, reversible adhesion (Weiss and Harlos, 1972). It involves consideration of the net charges of the contributory surfaces and, as such, at separation distances between approximately 10 and 20nm, "secondary minimum" interactions occur as a result of van der Waals and electrical double layer forces (see fig. 1.1.). Most living cells have negative surface charges and therefore repel one another. However, van der Waals forces, which are electromagnetically generated by atomic and molecular vibrations with similar fluctuation frequencies, act over long distances and may attract particles or cells of similar charge (Dickinson and Bisno, 1989a). The most important outcome is that the bacterium is held at some small, but finite, distance from the surface. In the absence of strong shear forces, the bacterium being held at the secondary minimum is in an ideal position to make use of other means to bind irreversibly to surfaces (Marshall, 1985). Marshall *et al* (1971) showed that cells held at this point exhibit Brownian motion and can be removed easily by washing with 2.5% NaCl. In addition to electrostatic forces, hydrophobic interactions have been implicated in the attachment processes (Fletcher and Loeb, 1979; Van Loosdrecht *et al* 1987b), although the relative contribution of these two factors varies depending upon the bacterial species.

Fig. 1.1. Schematic diagram of the total interaction energy Vt (shaded area) between two particles e.g. a bacterium and epithelial cell, as the sum of the electrostatic energy of repulsion (Vr) and the energy of attraction (Va). (Adapted from De Graaf and Mooi, 1986).



As well as physicochemical explanations for adhesion phenomena, the presence of complex organic molecules in the liquid phase, conditioning of the solid surface through binding of proteins (McEldowney and Fletcher, 1986) and temperature (Fletcher, 1977) have all been proposed as contributing to initial adhesion. Moreover, microorganisms often show some physiological response to the presence of substrata in the form of modification of the cell surface (Marshall, 1986). Properties and composition of the bacterial cell envelope are influenced greatly by nutrient status and growth rate (Brown and Williams, 1985a), thus cell physiology will ultimately play a major role in the degree of success in surface colonisation exhibited by a bacterium.

1.1.2. Irreversible adhesion

Irreversible adhesion has been defined as a time-dependent firm adhesion where bacteria no longer exhibit Brownian motion and are not removed by washing (Marshall *et al*,

1971). At cell-solid surface separation distances of less than 1.5nm, where any potential energy barrier has been overcome, several specific or non-specific short range forces may occur which lead to irreversible adhesion (Marshall, 1985; Busscher and Weerkamp, 1987). In the host-bacterium interaction, specific proteins on the bacterial cell surface may interact with receptors in the host tissue. Surface appendages such as fimbriae and pili may span the distance between the cell and solid surface (Isaacson, 1985; Krogfelt, 1991) and, as a result of diminished electrostatic repulsion, irreversible adhesion is facilitated (Van Loosdrecht *et al*, 1989).

The property leading to irreversible adhesion which has been studied most widely, however, is that of exopolysaccharide (EPS) formation. This appears to be involved in the development of microcolonies and ultimately in the evolution of a microbial surface film consisting of single cells and microcolonies of sister cells (Costerton *et al*, 1985; Marshall, 1985; Allison and Sutherland, 1987; Costerton *et al*, 1987).

1.1.2.1. The bacterial glycocalyx

Bacterial glycocalyces are defined as polysaccharide-containing structures of bacterial origin lying outside the integral elements of the outer membrane of Gram-negative and peptidoglycan of Gram-positive cells (Costerton *et al*, 1981a). Slime polysaccharides are loosely associated with the cell surface, electrostatic, hydrophilic or hydrophobic forces predominating as the adherent glue (Beveridge, 1988). In contrast, capsular polysaccharide (CPS) is intimately associated with the cell surface and may be bound covalently (Whitfield, 1988). Differentiation between the two forms can be difficult, since cells producing large amounts of CPS may "release" some material at the periphery, giving the appearance of slime production. Most bacteria show a preference for production of one form over the other, although some strains of *Klebsiella* sp. can produce, simultaneously, chemically identical slime and capsule (Whitfield, 1988).

Exopolysaccharides are composed of a range of monomers, either homopolysaccharides or heteropolysaccharides, both types of which occur widely (Sutherland, 1977).

Heteropolysaccharides (as well as some homopolymers) are composed, generally, of anionic repeating units, with structural diversity arising from a broad range of monosaccharide components and increased by potential noncarbohydrate substituants and linkage types (Kenne and Lindberg, 1983). Some components are unique to EPS, whilst others are also found commonly in other bacterial cell surface polysaccharides such as lipopolysaccharides and teichoic acids.

The generation and maintenance of a glycocalyx requires the expenditure of energy and, in the protected environment of a pure culture, the glycocalyx would confer no selective advantage to a bacterial cell (Costerton *et al*, 1981a). In the cell's natural environment, however, its synthesis is a characteristic feature associated with growth and survival and the matrix accounts for 75 to 95% of the volume of the biofilm (Geesey *et al*, 1992).

1.2. PHYSIOLOGY OF BIOFILM BACTERIA

The subject of metabolic activity of bacteria growing as adherent biofilms in their natural environments has long been a grey area, partly because of inherent difficulties in the measurement of physiological parameters but due, also, to the misconception held generally that experimental work in the laboratory yields results which can be extrapolated directly to the situation in the field. The influence of solid surfaces on bacterial activity is complex (Fletcher, 1985) and the complexity of the situation increases further as biofilm formation proceeds and cells are entrapped within the glycocalyx. It is likely that, in aging biofilms, cells which are embedded deep within the structure will differ quite markedly from those close to the surface and, hence, the external environment (Anwar and Costerton, 1992). The former, many having been involved in the initial biofilm formation, will most probably be experiencing a microenvironment limited for nutrients including oxygen and accumulating waste metabolites. In contrast, cells at the surface of the biofilm will be experiencing a more dynamic environment, with more ready access to nutrients and, as such, will continue to grow and divide, contributing to an increased thickness of biofilm. Some biofilms

do, however, appear to have channels in the polymer matrix that perhaps allow limited diffusion of nutrients to lower levels of the biofilms (Marshall, 1992). With diffusivity through biofilms thought to control the metabolic reaction rates, the use of a microprobe, 15μm in diameter, to measure the dissolved oxygen concentration confirmed that the microbial respiration reaction is indeed diffusion-controlled (Lewandowski *et al*, 1991). Such microelectrode probes are proving useful and non-destructive tools in the study of physiological heterogeneity within *in situ* biofilms. The technique of scanning confocal laser microscopy (SCLM) in conjunction with 3-dimensional computer reconstructions of biofilms, has also provided evidence for the existence of large void spaces within biofilms of natural microbial communities (Lawrence *et al*, 1992).

In a mixed species biofilm, different organisms will benefit from differential positioning throughout the matrix e.g. anaerobic microorganisms will continue cellular activities deep within the structure and products resulting from such metabolism may be utilised by organisms of other species at different levels within the biofilm (Marshall, 1992). SCLM has shown that mixed-species biofilms of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* contain regions where each species occurs independently and areas where they exist in intimate association (Geesey *et al*, 1992). Many biofilms can be viewed, therefore, as functional consortia of microorganisms, with various communities contributing to the localised nutrient gradients and, ultimately, to the overall activity within the biofilm as a whole.

The effect a solid surface has on a microorganism will depend on the bacterial species involved in the association, as well as the environmental conditions. For example, in the marine environment, in response to low nutrient conditions, bacteria which reach a solid surface tend to alter their cell surfaces (Kjelleberg and Hermansson, 1984) and patterns of peptide synthesis (Nyström *et al*, 1990). Kjelleberg *et al* (1985) reported that the initial responses of starved *Vibrio* and *Pseudomonas* sp., at nutrient-enriched and deficient interfaces, were fragmentation and continuous size reduction, accompanied by intense metabolic activity. Hydrophilic bacteria became even smaller at nutrient-deficient surfaces whilst hydrophobic strains exhibited no further change. A different study

compared responses of starved adhesive marine *Pseudomonas* sp. and non-adherent marine *Vibrio* sp. when exposed to surface-bound stearic acid as the only available energy substrate (Power and Marshall, 1988). *Vibrio* exhibited cell growth and began cell division near the surface, with the dividing cells returning to the aqueous phase for completion of division, whilst *Pseudomonas* remained surface-attached and daughter cells migrated across the solid substratum after division.

Microorganisms deep within a biofilm may be non-growing, but should not necessarily be considered as dead. The quiescent (or "dormant") bacterium is a fascinating phenomenon, rather poorly understood, but acknowledged as being a real occurrence and such cells are reported to be retained within biofilms (Lewis and Gattie, 1991). Should a situation where nutrients become available to these microorganisms prevail, they can adapt to take advantage of such a change.

The temporal and spatial heterogeneity of biofilms in nature poses the researcher numerous problems in the quest to understand the physiological consequences of adhesion to surfaces. It is clear, nevertheless, that the species make-up and environmental surroundings will dictate, to a large extent, the levels of microbial activity within these structures.

1.3. IMPORTANCE OF BACTERIAL BIOFILMS IN CLINICAL INFECTION

It is only in the last few decades that consideration has been given to the concept of bacterial biofilms and association with *in vivo* pathogenic medical infections. Previously, pathogenic microorganisms were envisaged as being freely accessible to the antimicrobial action of administered agents and little thought was given to the notion that bacteria inhabiting or infecting the host must somehow hold on to surfaces in order to avoid physical removal. It is now well established, however, that bacteria can exist in the body in two forms (Costerton, 1982): a relatively unprotected mobile "swarmer" cell which seeks out new areas to colonise, and a sessile microcolonial form which adheres

to tissue and persists under adverse conditions, consequently acting as a source of new infective organisms on eradication of the planktonic population.

1.3.1. Infection of implanted medical prosthetic devices

Modern medical practice is dependent upon a wide variety of therapeutic interventions and a huge number of plastic and metal prostheses are implanted into patients in both short and long-term applications (Costerton *et al*, 1985; Dougherty, 1988). When non-living substrata such as biomaterial implants are introduced into mammalian hosts they often become favoured sites for adhesive bacterial colonisation, especially in the immunocompromised patient (Gristina, 1987). The invasive microorganisms employ similar biofilm-formation tactics to those exercised in the external environment. Infection may be due to one species or additional symbionts which join the consortia and, characteristically, such infections can only be treated by the removal of the colonised substratum (Gristina *et al*, 1987; Christensen *et al*, 1989; Dickinson and Bisno, 1989b; Bandyk, 1991). The incidence of infection varies with the particular type of prosthetic device and recent data shows some rates of infection as follows (Finch, 1993): hip prostheses - 0.5%, central venous lines - 10%, ventricular assist systems - 25%, continuous ambulatory peritoneal dialysis (CAPD) - one or two episodes of peritonitis per patient per year.

Microorganisms can cause infection of medical devices via a number of routes. They may be resident pathogens or itinerant organisms which are passing through the implant site, either at the time of graft placement or later, via the bloodstream (Dougherty, 1988). Alternatively, contamination may occur during manufacture of the device, or by operating-room personnel during implantation. If the device communicates directly between the external surface of the skin and internal tissue, as in the case of intravascular catheter systems, the risk of infection is increased. It has been estimated that, with temporary haemodialysis catheter systems, the accumulative risk of colonisation at 50 days post-operation is at least 70%, the pattern of infecting organisms in the initial months most closely reflecting the skin flora (Finch, 1993).

Current management varies according to the nature of the infected system, its site of insertion and the ability to define the contaminating organism. The significant cost of containing and eradicating these infections through conventional antibiotic therapy is necessitating deeper understanding of the mechanisms of bacterial pathogenesis through adhesion and biofilm formation, as well as research into biopolymer surfaces less prone to bacterial attachment. Exacerbating the problem, there is evidence that contact between implant devices and neutrophils reduces the latters' microbiocidal capacity and that there is a decrease in opsonisation, reducing the likelihood of efficient phagocytosis of bacteria (Dougherty, 1988). Further still, the body coats foreign implants with a film containing various proteins (e.g. fibronectin, laminin, collagen) and some serve as receptors for colonising organisms (Dickinson and Bisno, 1989a). Progressive fibrin trapping thereafter may lead to protection of the invasive microorganism from the action of leukocytes. The physical properties of the implant, too, can be problematic since porous material may allow bacteria to invade interstices, soon after implantation, before host tissue is able to do so (Dougherty, 1988).

Bacterial infections not involving artificially-implanted devices are able, also, to persist by virtue of their biofilm mode of growth. Examples of such cases include lung infections of cystic fibrosis (CF) patients (Lam *et al*, 1980), endocarditis (Mills *et al*, 1984), urinary tract infections (Costerton, 1982) and osteomyelitis (Dan *et al*, 1990).

1.4. RECALCITRANCE OF BACTERIAL BIOFILMS

The refractory nature to antimicrobial therapy of bacteria growing as adherent biofilms has, in recent years, become a topic of much debate and research (Gilbert *et al*, 1987; Brown *et al*, 1988; Hoyle *et al*, 1990; Nichols, 1991; Anwar and Costerton, 1992). Several important factors are believed to contribute to the survival and pathogenicity of bacteria *in vivo*, including growth rate of the organisms, nutrient availability and the physical, protective and diffusion barrier afforded by the glycocalyx.

1.4.1. Effect of biofilm cell growth rate and nutrient limitation upon suceptibility to antimicrobial agents

It has been reported widely for many years that bacterial growth rate influences markedly the outcome of antimicrobial sensitivity testing of planktonic cells (Brown and Williams, 1985a; Tuomanen *et al*, 1986a; Gilbert *et al*, 1987; Chen *et al*, 1991). Moreover, attention has focussed increasingly on the physiology of slowly- or non-growing bacteria in acknowledgement of the belief that bacteria in infections, as well as in nature, are growing more slowly than traditional laboratory-grown cultures (Gilbert *et al*, 1987; Brown *et al*, 1990). Phenotypic tolerance, the ability of bacteria to evade the bactericidal activity of antibiotics, is typified by the inadequacy of beta-lactam therapy, for example, for treatment of non-growing cells (Tuomanen, 1986) and research has been ongoing for a number of years in an effort to develop new compounds with bactericidal activity on cells in a quiescent state. The modern fluoroquinolone drugs (section 4.1.1.), in particular, have offered the most effective treatment of such cells (Zeiler, 1985; Eng *et al*, 1991; Widmer *et al*, 1991; Tanaka *et al*, 1992). With physiological heterogeneity an integral factor of biofilms, then a range of cell growth rates is likely to be found within biofilm populations, especially as such a structure thickens. This will have a sigificant bearing upon the effectiveness of antibiotic therapy. Nutrient limitation, too, is a major determinant of the physiology of the adherent cell, given the many reports of bacterial adaptation to altered nutrient status ranging from the extreme starvation-survival response mentioned previously, to the alteration of cell envelope properties exhibited by iron-limited organisms (Brown and Williams, 1985b).

1.4.2. Effect of the glycocalyx upon susceptibility to antimicrobial agents

It has been postulated that the extracellular polymers of a biofilm constitute a diffusion barrier for antibiotics (Costerton *et al*, 1987) as well as excluding host antibodies which can, however, form immune complexes at the biofilm surface (Dasgupta *et al*, 1987). As a consequence of the highly hydrated anionic nature of the glycocalyx (Sutherland,

1977) it might be thought that biofilms could increase the concentration of a soluble antimicrobial agent in the cellular environment by trapping and concentrating its molecules in a manner similar to that by which it traps and concentrates nutrients (Costerton *et al*, 1987). Alternatively, the many charged binding sites on the matrix polymers and outermost cells might afford the organisms embedded more deeply a large degree of protection.

Evidence for the occurrence of each of these situations has been forthcoming in the literature and the whole subject has been a source of some controversy over the past few years. Two possible mechanisms of reduced diffusion leading to antimicrobial agent resistance have been described (Nichols, 1991). The first involves the concept of a detoxifying reaction, such as enzyme-catalysed hydrolysis, occurring within the biofilm along the diffusion pathway by which the antimicrobial agent must reach deeply-embedded cells. An example of this, as shown by calculations (Nichols *et al*, 1989), could be the production of beta-lactamase which acts as a barrier to penetration of beta-lactam antibiotics. Indeed, it has been reported that beta-lactamase production was induced in *Pseudomonas aeruginosa* biofilms by imipenem and piperacillin and was protective to the cells located within the structure (Giwercman *et al*, 1991). The second mechanism of reduced permeability described by Nichols is associated with binding of the toxic compound to cell-synthesised material, and must be analysed in terms of the kinetics of diffusion and the stoichiometry of binding along with the kinetics of synthesis of further binding sites (Nichols *et al*, 1989).

Nichols argues that the work which has supported the hypothesis that the glycocalyx is relatively impermeable to antimicrobial agents (Slack and Nichols, 1981; Taylor *et al*, 1988; Hoyle *et al*, 1990) has not been justifiable in terms of diffusion kinetics or with the kinetics of detoxification or binding. He believes that the glycocalyx material in bacterial biofilms of up to 100μm in thickness is not a significant diffusion barrier (Nichols, 1993).

It has been reported that *P. aeruginosa*-derived alginate protected mucoid and nonmucoid strains of the organism against the action of ciprofloxacin (uncharged at

neutral pH) and various beta-lactams, whilst other neutral or negatively-charged polysaccharides had no such effect (Hodges and Gordon, 1991). However, these workers were investigating the effects of dispersed alginate, not of intact biofilms, and since in real biofilms there are other potential barriers to penetration (Nichols, 1991; 1993) these results cannot be extrapolated to the *in vivo* situation. Previously, Gordon *et al* (1988) found that positively-charged aminoglycosides bound to bacterial alginate even when calcium, known to alter the structure of exopolysaccharide, was present in appreciably high quantities. It was found, though, that the ratio of alginate to antibiotic influenced drug penetration, with a low ratio most likely resulting in antibiotic saturation of all the electronegative binding sites (as suggested by Costerton *et al*, 1987) and, therefore, allowing its passage through the alginate at an increased rate. Recent reports by Hoyle *et al* (1992a,b) inferred that *P. aeruginosa* mucoid exoploysaccharide's (MEP) role as a diffusion barrier might vary according to its soluble state, since treatment of such biofilms with $CaCl_2$ condensed the MEP, thus altering its structure, and protected the glycocalyx-enclosed cells from tobramycin. These authors suggested that the binding of tobramycin was not as important a factor in reduced bacterial kill as was the steric hindrance imposed by the calcium ions, thus the quasi-organisation of biofilms plays an important role in resistance. Nichols (1993) forwarded alternative explanations: perhaps not only the EPS, but also other bacterial extracellular polymers, precipitated with the calcium within the pores of the membrane on which the biofilm was grown, effectively clogging the pores, or the initial membrane colonisation involved cells with virtually complete cell-to-cell contact, unlike more naturally-formed biofilms, thus permeability of intact cells and not of the film as a whole was being measured. Most recently, Hoyle *et al* (1993) monitored MEP transcription and production through biofilm formation, reporting that its production was enhanced, although only transiently, following *P. aeruginosa* adhesion and, once again, implicated the biofilm in the age-related antibiotic resistance of older biofilm cells (Anwar *et al*, 1989a,b; Anwar and Costerton, 1990; Anwar *et al*, 1992a).

There would appear to be a considerable amount of experimental work to be carried out before arrival at a concensus of opinion, if at all, on the permeability properties of the bacterial glycocalyx but it is likely that results between species will always show

variation owing to the different chemical structures of their EPS.

1.4.3. Electrically-enhanced biofilm treatment with antimicrobial agents

With host defences and currently available antimicrobial agents proving largely ineffective in the eradication of bacterial biofilms, such a mode of growth therefore presents a major health and economical problem. Industrial ecosystems, too, are prone to biofilm growth of bacteria with water and fuel pipelines, oil rig structures and ships' hulls but a few examples where biofouling can be a costly and often hazardous phenomenon (Costerton and Lappin-Scott, 1989). However, recent research has demonstrated that electric currents sensitise bacteria quite dramatically to antibacterial agents. This so-called "bioelectric effect" was reported by Blenkinsopp *et al* (1992) who found that within a low strength electric field of low current density, the polarity of which was altered every 64 seconds, the industrial biocides tested exhibited enhanced killing action against *P. aeruginosa* biofilms grown on stainless steel studs. Neither the electric current (direct, not alternating) nor the biocides applied alone inhibited biofilm development, but used in concert, biofilms were eliminated completely within 24h.

The mechanism by which this application works is, as yet, unknown although possibilities may include electrophoretic forces, electroporation or removal of cations from the biofilm material (Fox, 1992). Khoury *et al* (1992), who reported a similar effect of enhancement of bactericidal activity of tobramycin against *Staphylococcus epidermidis* and *P. aeruginosa* biofilms hypothesised that, in these cases, the electric field impels the cationic tobramycin molecule through the biofilm. Nichols (1993), however, has pointed out that this explanation would not hold for the similar case of electrically-enhanced glutaraldehyde- and isothiazolone-killing of *P. aeruginosa* biofilms (Blenkinsopp *et al*, 1992) since these biocides are electrically neutral. This intriguing technique requires further research and development but it could possibly revolutionise treatment of biofilms in both industrial and medical environments.

1.5. *Pseudomonas aeruginosa* : INCIDENCE AND VIRULENCE

Having presented a general overview of biofilm formation and physiology, the following sections will focus on the particular problems encountered on infection with the Gram-negative bacterium, *P. aeruginosa.*

P. aeruginosa is essentially an aquatic microorganism but constitutes a serious threat to patients whose defence mechanisms have been disrupted (Costerton *et al*, 1990). Infection is seen mainly in patients with burns, cancer, CF, diabetes and indwelling medical devices. As a nosocomial pathogen it causes the highest mortality rate of any bacterium (Korvick and Yu, 1991). The spectrum of *P. aeruginosa* infections ranges from progressive chronic disease in the lungs of CF sufferers to fulminating septicaemia in neutropenic patients (Pedersen, 1992). An opportunistic pathogen, rarely causing infection in a healthy host, *P. aeruginosa* possesses a formidable array of virulence factors which contribute to the successful invasion and colonisation of the immunocompromised host (table 1.1.).

Table 1.1.
Virulence factors of *P. aeruginosa* (from Pedersen, 1992).

pili	proteinase
flagella	leucocidin
alginate	rhamnolipid
siderophores	phospholipase C
outer membrane proteins	lipase
pyocyanin and other phenazine	exotoxin A
pigments	exoenzyme S
lipopolysaccharide	slime
elastase	beta-lactamase
alkaline protease	

It must be stressed that the role of many of these virulence factors is the subject of some debate and the reader is referred to Pedersen's review for fuller explanations.

1.5.1. Mechanisms of *P. aeruginosa* adhesion *in vivo*

A major factor in the general inability of antimicrobial therapy to eradicate *P. aeruginosa* successfully, especially in chronic infection, is that of its adoption of the biofilm mode of growth on host tissue. As described in the preceding sections, numerous traits of this growth strategy lead to the increased resistance of enclosed microorganisms. As such, despite often aggressive antimicrobial therapy, the nidus of infection will remain and lead to recurrent infection episodes. *P. aeruginosa* can be considered a prolific surface coloniser, showing a marked lack of specificity for various substrata in its natural aquatic ecosystem by virtue of its production of alginate, regarded as being an extremely sticky polymer (Costerton *et al*, 1990). Indeed, with or without the organism that produced it, alginate has a high affinity for surfaces.

In addition to alginate, several other adhesins of *P. aeruginosa* have been identified or proposed, amongst them pili. Pili on *P. aeruginosa* exist as fine filaments in a polar orientation and have been reported as mediators of adherence to buccal cells stripped of fibronectin by proteases (Woods *et al*, 1981), damaged tracheal epithelium (Ramphal *et al*, 1984) and tracheobronchial mucin (Ramphal *et al*, 1987). Indeed, Wong *et al* (1992) identified the C-terminal region of *P. aeruginosa* PAK pilin as containing an epithelial binding domain. One model proposed for pilus-mediated adhesion suggests that pili do not function directly in binding but, rather, they carry the adhesin molecules (Baker and Svanborg-Edén, 1989). This was prompted by the observations that, although purified pili from strain PAK bound to gangliotriaosylceramide, gangliotetraosylceramide and lactosylceramide, a non-piliated mutant of PAK bound to the same compounds (Johnson and Lory, 1987). It was thus suggested that in a non-piliated mutant the adhesins could still be presented over the bacterial surface and that they may be carried on alginate or pili, respectively. Baker and Svanborg-Edén suggested, also, that pili-mediated adhesion is of most significance in colonisation of the upper respiratory tract of compromised patients and damaged epithelium of the lower respiratory tract, whilst alginate-mediated adhesion could be an early selective process in the colonisation of the lower respiratory tract when mucociliary clearance is impaired but there is no serious damage to the epithelium.

Receptors for pili on host cells were identified as glycolipids and glycoproteins containing lactosyl and sialosyl residues (Baker and Svanborg-Edén, 1989). Injury to epithelial cells of mucus membranes by trypsin or human leukocyte elastase *in vitro* was thought to expose previously-masked receptors for pili and thus increase bacterial adhesion (Plotkowski *et al*, 1989). Damage of this type is expected to follow inflammatory reactions provoked by bacteria or viruses, common in CF, before *P. aeruginosa* infection (Høiby and Koch, 1990). Further, the usual events of wound repair such as cell migration and proliferation, and associated changes of cell surface glycoconjugates, may also enhance *P. aeruginosa* adhesion (Plotkowski *et al*, 1991). However, epithelial respiratory cells from CF patients reportedly have no more affinity for *P. aeruginosa* cells than those of non-CF patients (Plotkowski *et al*, 1992). This has lead to speculation that increased mucin secretion by cells from CF patients may play a crucial role in the interaction between the bacterial cells and the respiratory epithelium.

Exoenzyme S, an enzyme which ADP-ribosylates several membrane-associated eukaryotic proteins has also been implicated as a possible adhesin (Baker *et al*, 1991). Preliminary studies on the binding of this enzyme to cells indicated that it may recognise the same carbohydrate sequence as the whole bacteria and, further, exoenzyme S and antibodies to the enzyme were found to inhibit *P. aeruginosa* attachment to buccal cells.

1.5.2. The mucoid phenotype of *P. aeruginosa*

Respiratory colonisation by *P. aeruginosa*, in particular by variants exhibiting a mucoid phenotype, is regarded widely as the major microbial challenge in CF (Govan and Glass, 1990). Infection often begins with non-mucoid strains which only acquire the mucoid phenotype when infection becomes chronic (Høiby, 1974). Up to 80% of patients have been found to harbour mucoid strains (Pedersen *et al*, 1990). The alginate produced by mucoid strains has long been considered a major virulence factor (Høiby *et al*, 1975), enhancing growth as a biofilm, thus affording the bacteria some protection in a hostile environment where the host immune defence system is still intact (Kharazmi, 1991).

1.5.2.1. Physicochemical properties of *P. aeruginosa* alginate

Alginate is a strongly anionic polysaccharide, composed of linear chains of two types of acidic sugars; ß-D-mannuronic (M) and α-L-guluronic (G) acids (Russell and Gacesca, 1989). Physical properties of alginate include water retention, ion binding and the ability to form gels, these being dependent upon the arrangement of the uronic acids. Unlike alginates from other biological sources (such as seaweeds), the alginates from mucoid *P. aeruginosa* always lack poly-G blocks and, since this is found in non-CF mucoid strains, it is not a specific response to the disease (Russell and Gacesca, 1989).

Alginate solutions will form gels in approximately 3mM Ca^{2+}, a concentration at which mucoid strains of *P. aeruginosa* will form gelatinous microcolonies *in vitro* (Govan and Harris, 1986). It is not known precisely what is the calcium ion concentration in CF lungs, but it is elevated above normal and is approximately at a concentration of 3mM in the extracellular fluid (Russell and Gacesca, 1989). Poly-G blocks bind Ca^{2+} very strongly by chelation, and alginates containing a significant proportion of these acids form brittle gels in the presence of divalent cations. The lack of poly-G blocks in *P. aeruginosa* alginate would ensure that, in the CF lung, the microcolony environment of the bacteria remains elastic, allowing them to grow and divide. It does not explain, however, the prevalence of mucoid strains in CF patients but does, probably, contribute to their persistence over many years.

1.5.2.2. Alginate production and its significance

The mucoid phenotype of *P. aeruginosa* has been reported as being rather unstable *in vitro* (Govan *et al*, 1979), although culture conditions such as varying growth rate and specific nutrient limitation may influence the conversion (Ombaka *et al*, 1983). Also, stable strains are isolated frequently from clinical strains (Buckmire, 1984). In recent years the genetic basis for alginate expression has begun to be elucidated. It is known now that the genes for alginate biosynthesis are clustered at several different loci on the

bacterial chromosome (Martin *et al*, 1993). The genes *algS* and *algT* (the *algST* region often designated *muc*) function as switch genes which control a cluster of regulator/ modulator genes *algP*, *algQ*, *algR* and *algB*. These in turn, positively control the cluster of genes *algA*, *algD* and *algG* which code for the biosynthetic enzymes. It was reported that mutations in the *muc* loci affected transcription of both *algD* and *algR* and was influenced by environmental factors such as NaCl or nitrogen concentration (Deretic *et al*, 1990; May *et al*, 1991). For instance, increased concentration of NaCl, an osmolyte that is elevated in CF lung secretions, resulted in increased *algD* transcription (Deretic *et al*, 1990). Recently, a genetic locus required for *algD* transcription was characterised, this gene termed *algU* (Martin *et al*, 1993). Another alginate regulatory protein described recently, AlgR3, has strong homology to a eukaryotic histone protein (Kato *et al*, 1990). Investigation of environmental factors using prolonged chemostat culture of non-mucoid PAO1 found that slow dilution rate, limitation of particular nutrients, utilisation of growth components found in lecithin and high medium osmolarity all resulted in the appearance of a persistent mucoid subpopulation (Terry *et al*, 1991). Chemostat culture demonstrated 100% conversion of non-mucoid *P. aeruginosa* to the mucoid variant within 48h of treatment with ciprofloxacin, accompanied by an increase in MIC from <0.5μg/ml to >10μg/ml (Piña *et al*, 1992). The results implicate the involvement of DNA gyrase, the bacterial intracellular target of ciprofloxacin, and supercoiling in the regulation of genes involved in alginate synthesis.

It is unlikely that environmental signals alone switch on alginate production, however. The composition of secretions exclusive to the CF lung do not alter from birth, yet the mucoid phenotype does not emerge until the disease has progressed significantly (Pedersen, 1992). Strains that overproduce alginate may enjoy a selective survival advantage in the hostile environment and subsequently outgrow the non-mucoid strains, and the additional advantage of enclosed biofilm growth will increase persistence in the lung. Thus, it has not been established precisely whether the prevalence of mucoid *P. aeruginosa* in the CF lung is a result of the phenotypic plasticity of this organism or the induction of a genotypic switch, but it is probable that the two factors are not mutually exclusive.

Alginate-overproducing strains can be isolated from infection sites other than in CF, such as the urinary tract (McAvoy *et al*, 1989), burned skin (Schultz, 1947) and other pulmonary diseases (McAvoy *et al*, 1989), and it can be assumed that the mucoid phenotype is not induced by some abnormality specific for CF patients, but provides the most successful means of survival for the bacteria in particular disease conditions.

1.5.3. Recalcitrance of *P. aeruginosa in vivo*

P. aeruginosa is notoriously recalcitrant to antimicrobial therapy and successful management of infections of this organism requires prompt, often aggressive, and appropriate antimicrobial administration (Staneck *et al*, 1989). The individual bacterium has been described as being relatively impermeable to many antimicrobial agents and it must be stressed, also, that its extracellular environment will, to a large extent, dictate the growth rate and cell surface characteristics of the organism and, consequently, its level of sensitivity to drug treatment (Brown and Williams, 1985a; Gilleland, 1988). Growth as part of an alginate-enclosed microcolony may, therefore, increase the refractivity of an already resistant microorganism although, as discussed in section 1.4.2., the matter of drug-permeation of through the glycocalyx is the subject of much debate.

1.5.3.1. Evasion of the host immune response by *P. aeruginosa* biofilm cells

Infection of burned tissue, where host defences would be reduced as a consequence of the damage inflicted on wounding, involves a planktonic mode of growth and fast-growing cells are able to amount an overwhelming assault on undamaged tissue and the circulatory system (Costerton *et al*, 1990). However, active and passive immunisation using cell surface and flagellar antigens may be effective against the planktonic cells of such acute infections. Lung infection in CF represents a paradoxical situation where *P. aeruginosa* persists in an environment with a high presence of T cells, neutrophils and monocytes/macrophages which are all able to kill the organism *in vitro* (Pedersen

1992). Antibody levels in serum appear unimpaired, with IgsA, G and M all present. Kharazmi (1991) stated that evasion of the host defence system takes place by two mechanisms, these being the production of a large number of extracellular products, some of which interfere with the host defence system, and the adoption of the defensive microcolony and biofilm modes of growth. Briefly addressing the former mechanism, two of the many extracellular products which play a major role are alkaline protease (AP) and elastase (Ela). AP and Ela have been shown to inhibit phagocytic cell function, the cytotoxicity of natural killer cells, T cell function and to cleave, interfere with and inhibit cytokines (Kharazmi, 1989). AP also cleaves IgA, whilst Ela cleaves both IgG and IgA, and they both inactivate complement (Schultz and Miller, 1974). Thus, they play an important role early in infection by interfering with the local host defence system and providing the environment for bacterial colonisation of the lungs, but their role is not so important in later stages of the infection.

The bacteria within the biofilm are not overtly pathogenic but their persistence and growth results in massive unresolved bacterial masses in the CF lung (Lam *et al*, 1980). With host defences unable to penetrate biofilms, immune complexes (IC) are formed at the biofilm surface (Dasgupta *et al*, 1987). These workers proposed an immunopathogenic sequence where the permanent immunological response stimulated by the biofilm resulted in ICs saturating the local and systemic immunological mechanisms, persistent high levels of antibodies and circulating IC in the serum, and probable lung damage as a result.

Biofilms have been shown to induce only a slow oxidative burst response by polymorphonuclear leukocytes (PMN) (Jensen *et al*, 1990), although Pedersen *et al* (1990) found that alginate enhanced the release of PMN toxic oxygen radicals which, over the course of the disease, would contribute to damage of lung parenchyma. Moreover, mucoid strains are known to be phagocytosed less readily than non-mucoid strains (Baltimore and Mitchell, 1980). Complement activation, an important initial step in the inflammatory reaction often triggered by bacterial lipopolysaccharide (LPS) or peptidoglycan, is not triggered by alginate (Pedersen *et al*, 1990), thus the biofilm cells are protected from lysis and opsonisation, enhancing persistence of infection.

1.5.4. Therapeutic strategies for treatment of *P. aeruginosa in vivo*

Antibiotic therapy of *P. aeruginosa* in the CF lung serves to improve the patient prognosis and prolong life expectancy, but it does not eradicate completely chronic mucoid *P. aeruginosa* infection (Döring, 1991). Therapy with the new quinolone antimicrobial agent, ciprofloxacin, has shown most promise in the containment of *P. aeruginosa* (Rubio and Shapiro, 1986; Wolfson and Hooper, 1989) although the development of resistance to this drug has added to the list of persistence phenomena (Chamberland *et al*, 1990). (The fluoroquinolone drugs are described in more detail in chapter 4). Resistance has been shown to be highly pleiotropic in terms of accompanying changes in sensitivity to non-quinolone antibiotics and changes to outer membrane proteins and LPS (Diver *et al*, 1991). Valerius *et al* (1991) reported that administration of oral ciprofloxacin and aerosolised antibiotic during the early stages of *P. aeruginosa* lung colonisation prevented or delayed the development of chronic lung infection, illustrating the importance of rapid action on first isolation of the organism from CF sputum.

Another strategy being developed and tested for the treatment of the CF patient is the use of human recombinant DNase, administered by nebuliser (Suter, 1992). Adhesion blockers, too, are being researched, focussing on substances which can interfere with *P. aeruginosa* attachment to the CF patient's cells (Döring, 1991). Alginase, an alginate depolymerising enzyme, was found to render glycocalyx-bearing pseudomonads more susceptible to both antibiotic-induced and PMN-mediated killing (Bayer *et al*, 1991) and further *in vivo* studies on animals with endocarditis confirmed the removal of a large amount of the glycocalyx surrounding intravegetation mucoid pseudomonads (Bayer *et al*, 1992). Co-administration of alginase and suitable antimicrobial agents in aerosol form may improve therapy for CF patients. A similar approach is the administration of α1-antitrypsin, the main inhibitor of neutrophil elastase (McElvaney *et al*, 1991). It is hoped this will prevent damage to the normal respiratory tissues by neutrophil elastase and increase host defence efficiency on the respiratory epithelial surface. Other

measures range from the physical prevention of major transmission routes to immunisation against various prevalent bacterial cell characteristics or factors (Pedersen, 1992).

1.6. *IN VITRO* MODELS OF BIOFILM GROWTH

The detrimental consequences of biofilm growth of microorganisms in both industrial and medical environments have prompted increased study of this phenomenon. A number of *in vitro* models have been developed to varying degrees of success. These models vary from simple agar plates to complex biofilm fermenters and choice of which to use is dependent upon the particular needs of the researcher. The aims of such systems must be, however, to replicate the situation *in vivo* or in the environment as closely as possible. An appreciation of the physiological constraints and effects of such surroundings is therefore required.

1.6.1. Agar lawn method

The most elementary method simulating *in vivo* surface growth is that described for testing the antibacterial susceptibility of organisms grown on solid agar (Lorian, 1989). This involves growth of a confluent lawn of bacteria on agar which contains the antimicrobial agent within it. Nichols *et al* (1989) developed this one step further by inoculating bacteria onto semicircular halves of cellulose nitrate filters, placed on the agar surface, in order to ascertain whether the presence of the filter would alter the minimum inhibitory concentration (MIC) of the drugs being investigated. Further, biofilms grown on agar can be cryosectioned and the activity of enzymes within the microcolonies may be mapped (Keevil, 1993). Although more representative of many *in vivo* conditions than liquid culture and having provided some helpful data on MICs and cell ultrastructural changes, this simplistic approach has the major drawback of lacking physiological control.

1.6.2. Inoculated disc method

A method was developed by Prosser *et al* (1987), for studying the effects of antibiotics on established biofilms. This involves resuspending agar-grown bacteria onto sterile discs of silicon latex catheter material. After washing in buffer and transfer to Petri dishes containing appropriate broth medium, with or without antibiotic, the discs are incubated and the attached cells grow and divide, forming an adherent biofilm. Susceptibility to antimicrobial agents is assessed by viable counting, on resuspension of the cells from the discs' surfaces. Similarly, Gristina *et al* (1989) used such a method for investigating biofilms grown on catheter discs and titanium squares whilst antiseptic activity on biofilms grown on silicone discs was investigated by another group (Stickler *et al*, 1989; Stickler and Hewett, 1991). All groups reported increased resistance of biofilm-grown cells. The advantages of this method are its relative ease of handling and the fact that it allows determination of antibacterial efficacy against sessile populations without the drawbacks of dealing with inactivation of the agents by heavy concentrations of planktonic organisms (Prosser *et al*, 1987). The major handicap of the system, however, is that biofilm growth rate is not controlled and so direct comparison with results from rapidly-growing planktonic populations cannot be made (Brown *et al*, 1988).

1.6.3. Immersion of surfaces in chemostat cultures

A chemostat system was developed by Keevil *et al* (1987) to model the attachment of oral bacteria to acrylic tiles immersed in steady state cultures. The advantages of such an approach is that manipulation of the growth environment is possible and attachment processes and interactions of particular communities can be monitored, since plaque bacteria are known to show great diversity in attachment to the walls of continuous culture vessels.

Anwar *et al* (1989a,b), using a similar strategy, suspended tiles in growth-rate-controlled, iron-limited *P. aeruginosa* cultures, allowed biofilms to establish, and on

removal of the tiles, tested the biofilm populations' sensitivity towards tobramycin. The general method is of considerable use in the long-term generation of biofilms in a controlled environment but it is only the planktonically-grown cells which are being controlled to any great extent and, as such, no extrapolation to the growth rate kinetics of the biofilm cells can be made. Further, accurate assertions on the growth rate kinetics of the planktonic cells are not possible either, since some cells which may have otherwise left the vessel via the overflow will be adhering to the tiles, whilst the biofilm population itself will most likely be releasing cells into the liquid culture in unknown quantities. The authors reported a steady state in viable planktonic cells from day one of experimentation, whereas the biofilm population reached stasis on the fifth day, thus the growth kinetics of the system as a whole are more complex than in a traditional-type chemostat. However, provided that such aspects are taken into consideration this method for biofilm study does give an insight into general biofilm formation over long time periods. Factors pertinent to the *in vivo* situation, such as iron-limitation, and the assortment of materials used in the construction of medical devices can be investigated and, indeed, information on the relative susceptibilities of young and old biofilms has been forthcoming (Anwar and Costerton, 1990; Anwar *et al*, 1992a,b). The establishment of aging biofilms appears to be a mechanism of bacterial persistence and this confirms that the strategy used currently by some CF centres, where aggressive antibiotic therapy is initiated immediately on detection of *P. aeruginosa* in sputum (Valerius *et al*, 1991), is beneficial in as much as it will delay, if not prevent, the colonisation and persistence of *P. aeruginosa* populations.

1.6.4. The Robbins Device

The "Robbins Device" (McCoy *et al*, 1981) has been used widely in the study of biofilm formation, this system providing an effective model of biofouling in tubular flow systems such as waste water pipes and heat exchangers. The apparatus consists of a spool tube section from which sample pieces may be withdrawn either in the form of replaceable studs or as sections of the tube itself. The spool section is incorporated into a circulating loop through which growing cultures in appropriate nutrient media are

passed, and an adherent biofilm develops on the inner surface of the tube. The biofilm can be examined on removal of the replaceable studs and direct sampling from their surface. Research work employing this technique includes the study of biocide treatment of corrosion-causing bacteria (Ruseska *et al*, 1982; Costerton and Lashen, 1984), particularly pertinent to the problems encountered in the oil industry.

A modified Robbins Device (MRD) has been used to model infections of soft tissues and implanted medical devices. Nickel *et al* (1985) first reported the development of an artificial multiport sampling catheter in place of the sample section used in the original tubular-flow model. This allowed formation of *P. aeruginosa* biofilms on latex catheter discs, attached to retractable pistons, which were continually exposed to a (non-recirculating) flow of artificial urine and cell culture, followed by sterile medium, with or without antibiotic. (Alternatively, the discs may be seeded with cells flowing from a chemostat). This system has been used for antimicrobial studies of biofilms on various biomaterials (Evans and Holmes, 1984; Gristina *et al*, 1987; Hoyle and Costerton, 1989) as well as the induction of beta-lactamase in *P. aeruginosa biofilms* (Giwercman *et al*, 1991). The innovative investigation into the effect of combining exposure of biofilms to antimicrobial agents with an electrical field (Blenkinsopp *et al*, 1992) and, most recently, the production of mucoid exopolysaccharide by *P. aeruginosa* biofilms (Hoyle *et al*, 1993) also employed MRD-grown biofilms.

Obviously of considerable use and versatility, the Robbins Device and MRD, however, lack effective growth-rate-control of the adherent population and, consequently, do not enable the researcher to differentiate between properties attributable to growth rate and those associated with adherence *per se*.

1.6.5. The annular reactor

The annular reactor (or RotoTorque reactor) consists of two concentric cylinders, a stationary outer and a rotating inner cylinder, and provides a large wetted surface-area-to-volume ratio and a turbulent liquid phase (Charaklis *et al*, 1982). Biofilm

accumulates on the walls of the reactor, any which detaches being washed out in the effluent, and can be sampled via removable slides contained in the inside wall of the outer cylinder. As with the Robbins Device, cells are pumped in from a steady state chemostat before sterile medium is fed into the system. Such continuous-flow fermenters are used to model microbial fouling in industrial pipelines, for example, and are particularly sensitive to changes in fluid frictional resistance.

1.6.6. Constant thickness biofilm fermenter

The thin film fermenter (Wimpenny *et al*, 1989) provides a mechanism whereby a large number of identical films can be obtained at constant depth at any point predetermined within a range of depths of up to 300μm. At the centre of the vessel is a rotating plate and the upper surface is swept by a blade, over which fresh medium is passed. Organisms are continually washed by nutrient medium and shaved off the surface by the rotating blade, but biofilm formation occurs in the recessed areas of known depth. Growth of films can be measured by the time-dependent removal of individual films and determination of their weight and content. This technique has provided much information on spatial gradients within biofilms.

1.6.7. Modified glass fermenter

This method was first described by Gilbert *et al* (1989) and involves perfusion of a cellulose filter membrane, loaded with cells on one side, with nutrient medium at a constant flow rate. After initial detachment of loosely-bound cells a steady state population may be achieved, dependent upon the bacterial species used. (See chapter 3 for full description). The overriding advantage of this system is the growth rate control that can be achieved for organisms such as *Escherichia coli* and *S. epidermidis* (Gilbert *et al*, 1989; Evans *et al*, 1991b). This allows valid comparison of results of antimicrobial-testing, for example, of growth-rate-controlled biofilm cells with the outcome of parallel studies on chemostat-grown populations. Thus, one is able to

distinguish whether a particular property of biofilm cells is attributable to changes in population growth rate or whether some other property of biofilm-growth is prevalent. Additionally, eluate cells, corresponding to the cells dispersed from a biofilm (Gilbert *et al*, 1989) may be studied in the same way as the biofilm and chemostat "control" populations.

The resident biofilm population is perfused with nutrients from its underside, a situation atypical of *in situ* biofilms on inanimate surfaces. It is, however, representative of bacterial surface infections of soft tissue and, as such, provides a good model of *in vivo* infection. Investigations into antimicrobial sensitivity (Evans, 1990; Evans *et al*, 1990a,b; Gander *et al*, 1992; Duguid *et al*, 1992a,b), electrokinetic mobility (Makin *et al*, 1992), cell surface hydrophobicity (Allison *et al*, 1990a,b), EPS production (Evans *et al*, 1992) and adhesion studies (Evans, 1990) have been carried out using cells generated by this technique.

1.7. AIMS OF THE STUDY

P. aeruginosa can adopt a planktonic mode of growth in acute lung and burn tissue infections, whilst in the CF lung chronic infection is in the form of adherent biofilm populations (Costerton, 1990). Furthermore, in both acute and chronic infection *P. aeruginosa* has been shown to proliferate under iron-limited conditions (Ward *et al*, 1988; Brown *et al*, 1984) and, in many cases, is refractory to antimicrobial therapy, a result of numerous factors ranging from an innate resistance to certain antibiotics to the particular growth strategy employed during infection.

Thus, at the outset of this project, the aims were defined clearly as focussing around the comparative study of *P. aeruginosa* PAO1 grown by growth-rate-controlled planktonic and biofilm culture methods. Work was to be carried out to gain an insight into the nature of the iron-restricted biofilm-grown cell and how it differs from the ubiquitously-studied planktonic cell.

However, during the course of the project, it became clear that the chosen technique for generation of growth-rate-controlled PAO1 biofilms was beset with technical difficulties and unsuitable for use with this particular microorganism. Consequently, the emphasis of the project switched to the development of a functional and reliable technique for biofilm cultivation. Physiological studies were to be carried out on successful attainment of such a system.

2. MATERIALS AND METHODS

2.1. ORGANISMS AND CULTURE MAINTENANCE

Throughout this study, the organisms used were *Pseudomonas aeruginosa* ATCC 15692 (PAO1) and *P. aeruginosa* PAO1 JD, another PAO1 strain obtained from B. Holloway via Dr. J. Doig, Department of Bacteriology, University of Edinburgh. They were maintained at 4°C on nutrient agar (Lab M Ltd., Bury) plates. Long term storage was either at -70°C in nutrient broth (Lab M) plus 10% glycerol or in liquid nitrogen.

Organisms were grown on nutrient agar at 37°C. Successive subculturing by streaking out on nutrient agar was found to result in slowing the growth rate of 15692 indicating that the organism had changed in some manner. 15692 was used only in nutrient limitation studies and PAO1 JD was used in all further experimental work. Samples from the -70°C store were streaked out once a month in order to avoid any subculture-induced alterations.

2.2. CHEMICALS

Chemicals used in this project were obtained from BDH Chemicals Ltd. (Poole), Sigma Chemical Company (Poole) and Fisons (Loughborough). AnalaR and Aristar grades or equivalent were used. Ciprofloxacin was obtained from Bayer UK Ltd. (Newbury).

2.3. GLASSWARE

All glassware to be used in iron restriction studies was soaked overnight in 0.01% ethylenediaminetetraacetic acid (EDTA) solution, to chelate any contaminating iron, and washed ten times in single- then ten times in double-distilled water.

2.4. PREPARATION OF CHEMICALLY DEFINED MEDIA (CDM)

Organisms were grown in CDM liquid medium, shaken on a rotating incubator (New Brunswick G10) at 37°C. CDM_{10} was first employed, 10 being the theoretical optical density at 470nm which can be obtained by growth of cells in this medium. In later studies CDM_{12} was used, this being isotonic with serum. The composition of CDM was based on that of Noy (1982) and is given in table 2.1.

Table 2.1.

P. aeruginosa chemically defined media.

Component	CDM_{10} mM	CDM_{12} mM
Glucose	40.00	48.00
KCl	0.62	0.74
NaCl	0.50	0.60
$(NH)_4.SO_4$	40.00	48.00
$MgSO_4.7H_2O$	0.40	0.48
MOPS*	50.00	60.00
$K_2HPO_4.3H_2O$	3.20	3.84
ddH_2O to 1l		
$FeSO_4.7H_2O$	0.01	0.01
(acidified with 1ml H_2SO_4 per litre to prevent precipitation of iron)		

* MOPS = 3-(N-Morpholino) propane-sulphonic acid

The pH was adjusted to 7.8 using 1M NaOH and the medium autoclaved at 121°C for

20 min. Both glucose and $FeSO_4.7H_2O$ were autoclaved separately, in the former case to prevent caramelisation of the glucose and in the latter to give the choice of whether or not to iron-restrict the medium.

2.4.1. MICRONUTRIENT SUPPLEMENTATION

The CDM was supplemented with the following trace elements (table 2.2.) in order to ascertain whether growth of PAO1 was enhanced by such additions.

Table 2.2.

Trace elements added to CDM.

Component	M ($x10^{-6}$)
$CaCl_2.6H_2O$	0.50
H_3BO_3	0.50
$CoCl_2.6H_2O$	0.05
$CuSO_4.7H_2O$	0.05
$ZnSO_4.7H_2O$	0.05
$MnSO_4$	0.10
$(NH_4)6Mo_7O_{24}.4H_2O$	0.005

The micronutrient solution was sterilised separately by autoclaving.

2.5. ENUMERATION OF ORGANISMS BY VIABLE PLATE COUNTING

In order to test the reproducibility of the viable counting (spread plate) method, 100µl of a logarithmic phase culture of PAO1 grown in CDM_{12}+Fe was serially diluted 1:10 in CDM_{12} salts solution (CDM without glucose) to give around 100 colony forming units (cfu) per 100µl after overnight incubation at 37°C. Five replicate dilution tubes of the final dilution were made and, from each, 100µl was plated out onto five nutrient agar plates, giving 25 plates in all. The plates were incubated at 37°C for 24h and the resulting colonies counted. The results are shown in table 2.3.

Table 2.3.

Colony counts per plate for five replicate counts.

	1	2	3	4	5
1	89	74	67	95	79
2	103	106	68	84	94
3	102	82	107	98	85
4	101	90	94	114	78
5	109	92	62	82	92
Total(T)	504	444	398	473	428
Mean($\bar{x}$)	100.8	88.8	79.6	94.6	85.6

The results in table 2.3. were subjected to an analysis of variance as shown in table 2.4.

n = number of observations per count = 5

m = number of counts = 5

n.m = total number of observations = 25

(1) $\Sigma x^2 = 206513$

(2) $\Sigma T^2/n = 203293.8$

(3) $(\Sigma x^2)/n.m = 201960.36$

Table 2.4.

Analysis of variance of five replicate counts.

SOURCE OF VARIANCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	VARIANCE RATIO (F)
Between counts	(2)-(3) 1333.44	m-1 4	333.36	2.07
Within counts	(1)-(2) 3219.2	n.m-m 20	160.96	

The tabulated values of "F" for 4/20 degrees of freedom at 5% and 1% levels are 2.87 and 4.43, respectively. Therefore the variation between counts is not significantly greater than the variation within counts.

2.6. OPTICAL DENSITY MEASUREMENTS OF CELL NUMBERS

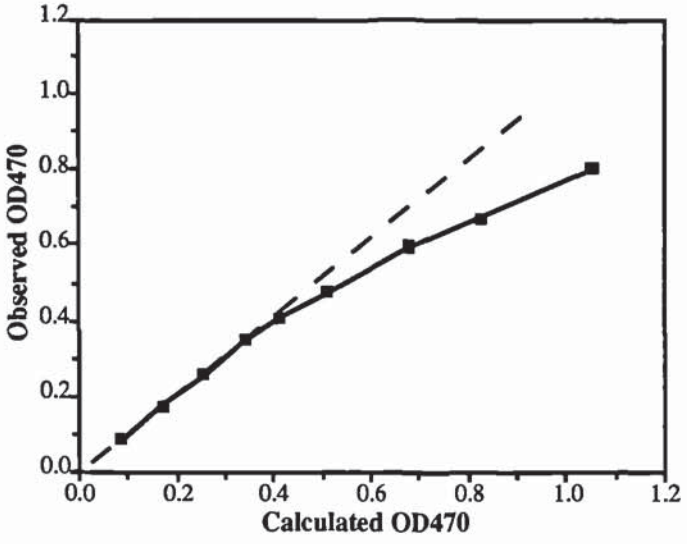
All spectrophotometric observations were carried out on a Unicam SP6-400 U.V.

spectrophotometer (Pye Unicam Instruments Ltd., Loughborough) at 470nm using disposable plastic microcuvettes (1cm light path). At low cell concentrations the light scattered by a bacterial cell suspension is directly proportional to the cell concentration in the suspension.

A dilution series of a suspension of *P. aeruginosa* cells was prepared using sterile CDM salts and the optical densities of each measured at 470nm. (This wavelength was used to minimise absorption by medium constituents and bacterial metabolic products such as pyocyanin). With knowledge of the optical density of the most dilute suspension, and the dilution factor of each, the expected optical densities were calculated. Measured optical density was plotted against calculated (true) optical density (fig. 2.1.) and deviation from the Beer-Lambert law observed when the optical density exceeded 0.4. At values above 0.4, the cell density is such that some cells are in the shadow of others and scatter no light. As a result of this and internal reflection, the measured optical density becomes much lower than the true value. Therefore, in subsequent experiments suitable dilutions of cultures were made when OD_{470} exceeded a value of 0.4.

In order to correlate viable cell numbers with OD_{470} serial 1:10 dilutions into sterile CDM salts were prepared from suspensions of known optical densities and 100µl aliquots spread on nutrient agar plates. After overnight incubation at 37°C, the number of cfus was counted and it was found that ODs of 0.1 and 1.0 at 470nm indicated concentrations of approximately $1x10^8$ $cfu.ml^{-1}$ and $1x10^9$ $cfu.ml^{-1}$, respectively, as previously described by Anwar (1981).

Fig. 2.1. Observed versus calculated OD_{470} of suspensions of *P. aeruginosa* PAO1 in CDM_{10} showing deviation from the Beer-Lambert law for the opacity of bacterial cell suspensions.



2.7. TOTAL CELL COUNTS

Samples to be counted for the total number of cells were stored in formyl saline (4% formaldehyde in 0.85% saline) in order to preserve the cells, whilst inhibiting growth. A drop of appropriately treated suspension was then placed in a 0.1mm haemocytometer slide (Improved Neubauer, Hawksley) and the chamber left in a humid atmosphere for 30min to allow cells to settle into one plane of vision, after which total counts were made in 80 small squares. The total number of cells was calculated, taking the total volume into consideration.

2.8 BATCH CULTURE STUDIES

2.8.1. Theory

Bacteria are traditionally grown in the laboratory in batch culture. By this method, a fixed volume of medium is inoculated with a bacterial suspension and incubated, generally resulting in a rapidly dividing culture, although this depends largely on the medium used. There is no further input or output (apart from sampling) and such a culture is described as a closed system. Growth in such systems follows a characteristic pattern which can be considered as several separate, yet continuous, stages.

2.8.1.1. Lag phase

The lag phase represents an initial adaptive phase, the extent and duration of which is mainly dependent upon the nature of the cells' previous extracellular environment and its similarity to the new environment. Less common causes of lag are the presence of autoinhibitors which require to be broken down, or the state of the actual inoculum i.e. if the cells are in the form of spores, germination precedes the next stage of growth. During lag phase, cell biomass (x) increases but cell number does not.

2.8.1.2. Acceleration phase

This stage occurs as the cells gradually come out of the lag phase and begin to divide and grow faster.

2.8.1.3. Logarithmic growth phase

An exponential increase in cell number with time occurs with the specific growth rate (μ) being defined as the actual increase in cell number per unit time, specific to the cell numbers present at that time. If the basic law of growth is described by the equation $dx/dt = \mu x$, where dx/dt is the growth rate of the cell population, then for exponential cell growth:

$x = x_0 e^{\mu t}$ and $\mu = \ln 2/t_d$ where x_0 is the initial cell population and t_d = doubling time.

In a simple defined chemical salts medium, exponential growth is possible at any value of μ up to μ_{max} (the maximum specific growth rate) provided that the substrate concentration (s) is maintained at a constant value (Monod, 1949). The relationship between μ and s is described in the Monod equation:

$\mu = \mu_{max}(s/k_s+s)$ where k_s is a constant parameter termed the "saturation constant".

The duration of the logarithmic period of growth depends upon several factors such as the complexity of the medium, initial inoculum size and environmental conditions. It should be noted that, whilst no essential nutrient is at a growth-limiting concentration, the rate at which bacterial growth proceeds depends upon the speed at which nutrients can be assimilated by the cells. Metabolism of compounds requires enzymic action, thus the rate of growth is related to enzyme activity.

2.8.1.4. Deceleration phase

Eventually, growth rate declines because specific nutrients run out and inhibitory compounds (waste products of metabolism) build up. Further, the pH of the medium may be altered as a result of these compounds and oxygen may be at a limiting concentration, although these factors can be minimised through adequate buffering and aeration of the culture.

2.8.1.5. Stationary phase

Biomass concentration is now constant and the cells are no longer growing. Cell density at stationary phase can be controlled by providing all but one of the essential nutrients to excess and one critical nutrient at a level that will limit growth. The point of onset of stationary phase is dependent upon the nature of the depleted nutrient. Throughout this phase, there is further accumulation of inhibitors, this also preventing any cell growth.

2.8.1.6. Decline phase

Eventually, cell number and biomass concentration decline as a consequence of nutrient exhaustion and the build up of toxic wastes.

The length and presence of these described phases varies with extracellular conditions. The cells are continually changing throughout growth e.g. cell size is not constant, being greatest in exponential and deceleration phases and, further, subcellular structure varies. Thus, cells sampled at various times during batch growth are likely to be physiologically different and care must be taken to distinguish when cells are in logarithmic phase or in stationary phase.

Closed growth systems are of much use in the laboratory in the study of chemical

composition of cells, but they do not accurately reflect the *in vivo* situation, especially where growth rate is concerned.

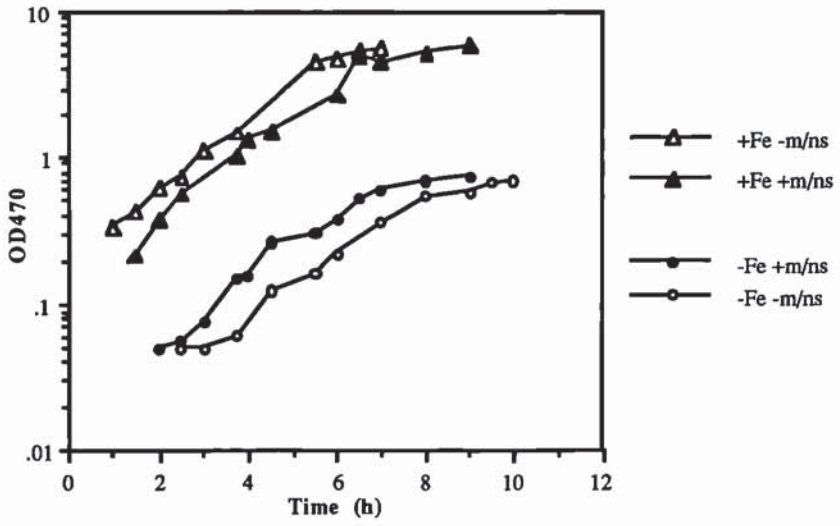
2.8.2. Nutrient depletion studies in batch culture

The final cell density reached by a culture of *P. aeruginosa* has been found to be dependent on the initial concentration of a growth-limiting nutrient in the medium (Brown and Melling, 1969). The object of this study was to investigate the effect of iron or carbon limitation upon growth of PAO1.

2.8.2.1 Method

The growth of *P. aeruginosa* 15692 in CDM_{10} with and without iron supplementation was followed by inoculating 50ml of the medium of interest with 500µl of an overnight culture, grown up in that same medium at 37°C, shaking. Growth was then followed by measuring OD_{470} at regular intervals. The effect upon growth of micronutrient supplementation to CDM_{10} +/-Fe was also investigated as above and the resultant growth curves are illustrated in fig. 2.2. Benefit gained by such supplementation was observed to be minimal and the micronutrient supplement was not used any further during the course of the project.

Fig. 2.2. Effect of micronutrient (m/ns) supplementaion upon batch culture growth of *P. aeruginosa* 15692 in CDM_{10}+/- Fe.



2.8.2.2. Iron limitation

A series of growth curves for *P. aeruginosa* 15692 growth in CDM_{10} containing varying amounts of iron were carried out. Concentrations of iron used were ($Mx10^{-6}$) : 0.0, 0.004, 0.008, 0.016, 0.031, 0.062, 0.125, 0.250, 0.500, 2.0. Several of the resultant growth curves and a plot of final (stationary phase) OD_{470} against time are illustrated in figs. 2.3. and 2.4., respectively. The final OD reached by 15692 was determined by all concentrations of iron shown. It was found, however, that iron concentrations of over 1μm resulted in formation of clumps of cells in liquid culture, thus this level of iron was never exceeded in subsequent culture. To ensure iron-plentiful culture was never limiting for iron, cultures were limited for glucose to stop growth at ODs below which iron would have become a growth-limiting factor.

2.8.2.3. Carbon limitation

A series of growth curves for *P. aeruginosa* 15692 growth in CDM_{10}+Fe containing varying amounts of glucose were carried out. Concentrations of glucose were (mM) : 0.0, 0.031, 0.125, 0.5, 1.0, 2.0, 4.0, 8.0, 12.0, 16.0, 28.0. Several of the resultant growth curves and a plot of maximum (stationary phase) OD_{470} against time are illustrated in figs. 2.5. and 2.6., respectively. At lower glucose concentrations (i.e. 1 and 2mM), the growth rate of cultures appeared to be lower than that displayed by cultures growing in medium containing higher carbon concentrations (8 and 4mM). This is indicative of two different carbon uptake systems, one with high and the other with low affinity for carbon. Plotting the maximum OD_{470} reached by a culture against the initial glucose concentration in that culture showed that glucose concentrations of below 16mM affected the time at which stationary phase was entered.

Fig. 2.3. Effect of iron concentration (μM) in the medium on batch culture growth of *P. aeruginosa* 15692 in CDM_{10} at 37°C. (The curves have been offset for clarity).

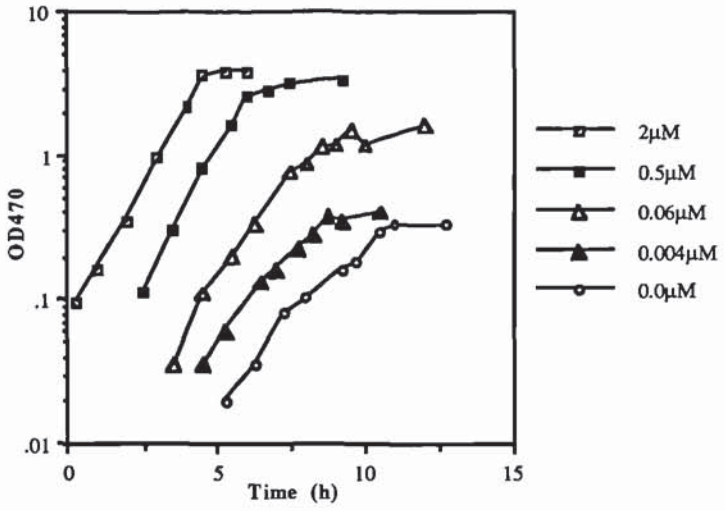


Fig. 2.4. Final OD_{470} of *P. aeruginosa* 15692 grown by batch culture in CDM_{10} with graded concentrations of iron.

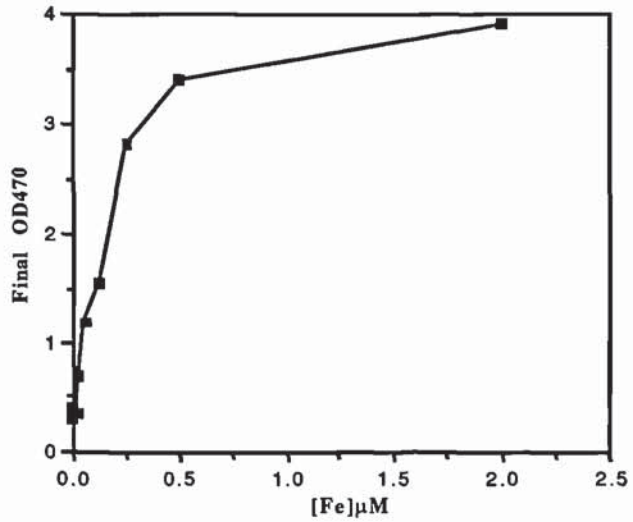


Fig. 2.5. Effect of the concentration of carbon source (mM glucose) in CDM+Fe on batch culture growth of *P. aeruginosa* 15692 at 37°C. (Curves have been offset for clarity).

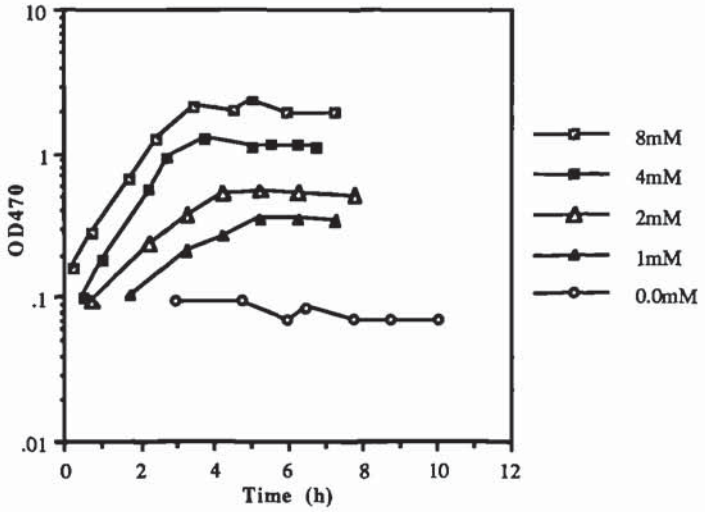
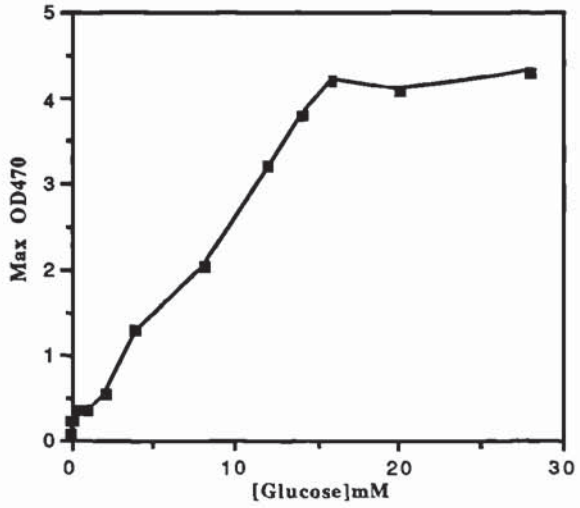


Fig. 2.6. Maximum OD_{470} of *P. aeruginosa* 15692 grown by batch culture in CDM with graded concentrations of glucose.



2.9. CONTINUOUS CULTURE

2.9.1. Theory

The continuous culture of microorganisms in the laboratory overcomes many of the problems of batch culture where the growth medium is eventually altered by the actions of the growing organisms and rendered unsuitable for growth. A continuous culture is essentially a flow system of constant volume to which fresh medium is added continuously and from which spent medium and cells are continually removed via an overflow. Once such an open growth system is in equilibrium, cell number and nutrient status remain constant and the system is said to be in steady state (Herbert *et al*, 1956). The most common type of continuous culture device used is the chemostat, a fermenter which allows control of both growth rate and population density of a culture (fig. 2.7.). Two variables are used in the control of a chemostat: the flow rate and the concentration of a limiting nutrient or growth factor. Dilution rate (D), the proportion of medium replenished in one hour, is an expression of the rate of medium supply, where:

$D = F/V$ where F = rate of fresh medium inflow
V = volume of culture in vessel

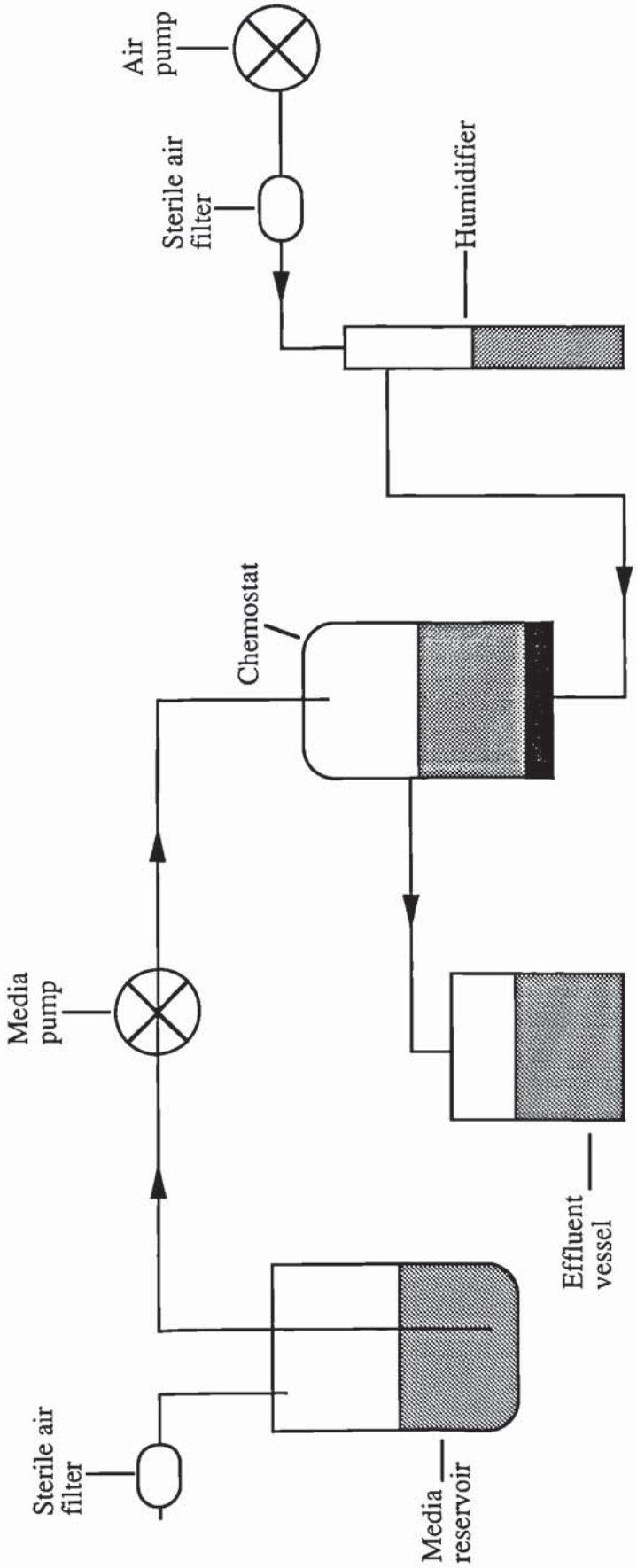
The most commonly used function for μ, the specific growth rate, is derived from the Monod equation (Monod, 1949):

$$\mu = \mu_{max} (s/k_s+s) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

s = substrate concentration
k_s = saturation constant
μ_{max} = maximum specific growth rate

Exponential growth is possible at any value of μ up to μ_{max} provided that substrate concentration is maintained at a constant value and equation (1) assumes that, during bacterial growth, a single enzymic step becomes growth-rate limiting. At steady state, an equilibrium exists between cell density ($\bar{x}$) and concentration of substrate remaining

Fig. 2.7. Schematic representation of a chemostat design.



in the outflow ($\bar{s}$), and μ equals D.

$$\mu = D = \mu_{max}(\bar{s}/k_s+\bar{s}) \quad \text{..... (2)}$$

If s_r is the substrate concentration in fresh media and Y the yield constant (weight of cells formed per weight of substrate used) then:

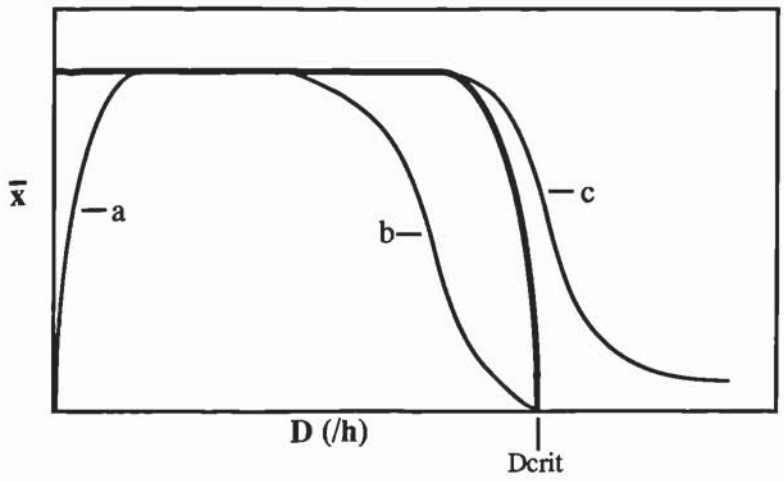
$$\bar{x} = Y(s_r-\bar{s}) \quad \text{..... (3)}$$

Rearranging equation (2) and substituting for $\bar{s}$:

$$\bar{x} = Y(s_r-Dk_s/\mu_{max}-D) \quad \text{..... (4)}$$

As shown by the bold curve in fig. 2.8., dilution rates can be varied from zero through to μ_{max} , or D_{crit}, the point at which washout of the culture occurs and $\bar{x}$=0. At D rates below the critical dilution rate, biomass is, theoretically, constant. However, when there is a maintenance requirement for the growth-limiting substrate (e.g. for turnover of cell materials, maintenance of concentration gradients), the effect on the steady state biomass concentration is as illustrated by (a). Trace element deficiency results in kinetics illustrated by (b) and (c) shows the effect on steady state biomass of wall growth of the organisms in the vessel (Pirt, 1975).

Fig. 2.8. Theoretical plot of steady state biomass concentration ($\bar{x}$) with dilution rate (D) in chemostat culture.



2.9.2. Method

Continuous culture studies were carried out using mini air-lift glass chemostats constructed according to the design described by Gilbert and Stuart (1977). The all-glass construction enables low-iron media to be employed without contamination from metal parts. Furthermore, the absence of moving parts provides a robust and durable function over many days. Vessels of 50ml and 350ml volume were employed (fig. 2.9.). Chemostats were soaked in detergent (Lipsol) and rinsed thoroughly before assembly and sterilisation by autoclaving. For iron-limitation studies, all glassware was EDTA-treated as described in section 2.3. Fresh medium was pumped from a 10l storage vessel by a peristaltic pump (Watson-Marlow MHRE HR Flow Inducer, Falmouth) through silicone tubing and the dropper (F) into the fermentation chamber (A). As the vessel filled with medium to level (D) excess medium flowed through the overflow (E) into a glass vessel which was enclosed except for an air inlet and filter.

Aeration was achieved by the passage of filtered air delivered through the glass sinter (C). The use of the sinter also provided rapid and efficient mixing of the culture to ensure an even and instantaneous distribution of fresh medium as it dropped in. The fermentation chamber was jacketed (B) and linked to a recirculating water bath to allow the accurate control of incubation temperature.

2.9.2.1. Pump calibration

The peristaltic pump was calibrated by measuring the rate of delivery of water through the tubing for a range of speeds and tubing sizes. A linear relationship between pump speed and flow rate was achieved. An approximation of the working volume was ascertained by running water through the system and, at a given pump rate and air flow rate, the volume within the vessel was measured.

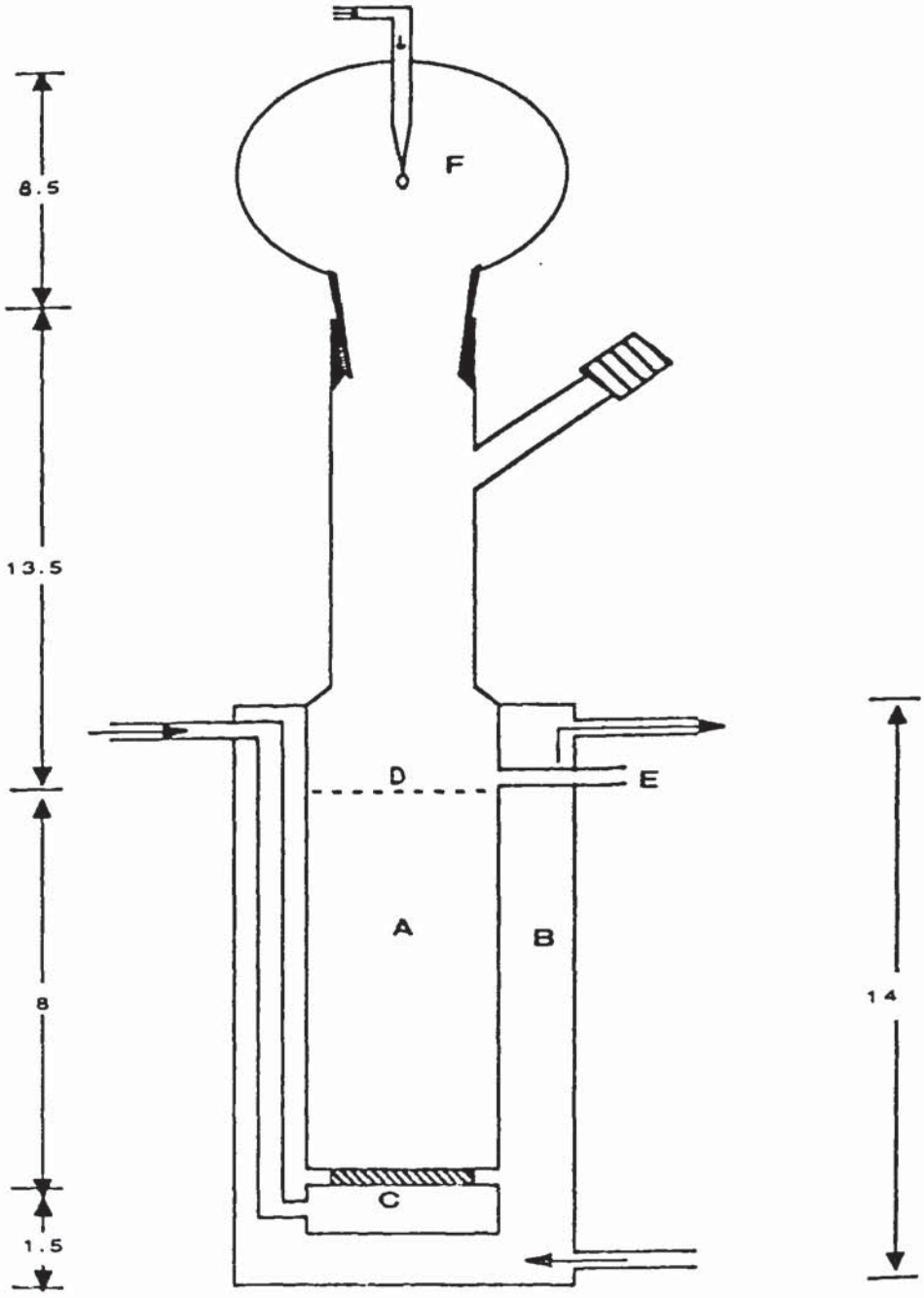
These procedures provided an estimate of dilution rates (D) which could be achieved, but in order to allow for discrepancies due to changing pump tubing length, air flow

rate or frothing of cultures, flow rate was monitored throughout experiments by measuring the volume of spent medium and cells expelled via the overflow in a given time.

When filling the chemostat in preparation for experimentation, media was pumped in with aeration, then stopped just before the working volume was reached. The vessel was then inoculated aseptically with a log-phase culture of PAO1 cells in the same media (1% of working volume) and allowed to grow as a batch culture until stationary phase was achieved. The pump speed was then increased gradually until the required medium flow rate was attained.

Before using the cells, or after changing the growth rate, the chemostat was allowed to reach a steady state. The chemostat was run for sufficient time to give five complete volume changes before the cells were used. This procedure was used for all chemostats running at a single dilution rate. In cases where the dilution rate was altered during a run, five volume changes were also allowed before cells were sampled.

Fig. 2.9. Diagram of mini air-lift, all-glass chemostat.
(50ml volume vessel, dimensions are in cm).



ASTON UNIVERSITY
LIBRARY AND
INFORMATION SERVICES

2.9.3. Nutrient limitation in continuous culture

PAO1 JD was grown by continuous culture under conditions of iron restriction in CDM_{12}-Fe and carbon limitation in CDM+Fe (2mM glucose). Optical densities, viable counts and total cell counts were monitored throughout, at each dilution rate employed. Figures 2.10. and 2.11. illustrate that, for iron-restricted cultures, between dilution rates of $0.06h^{-1}$ and $0.26h^{-1}$ cell density and viability remained relatively constant. Washout, which was never achieved since interest was focussed on slow growth rates, was estimated to occur at a dilution rate between 0.7 and $0.8h^{-1}$. Carbon-limited cultures (figs. 2.12. and 2.13.) displayed relatively constant cell density and viability between dilution rates of $0.09h^{-1}$ and $0.4h^{-1}$. Again, total washout of viable cells was not achieved.

Direct comparison of OD_{470} and viable counts may be slightly misleading since the light-scattering properties of cells will vary according to their sizes at different growth rates (fast-growing cells are larger than their slower growing counterparts (Maaløe and Kjeldgaard, 1966)). Additionally, at faster dilution rates the proportion of viable cells is likely to increase, but this will not necessarily affect OD readings, since these reflect total cell numbers.

Fig. 2.10. Relationship between iron-restricted chemostat optical density at steady state and dilution rate for *P. aeruginosa* PAO1 JD.

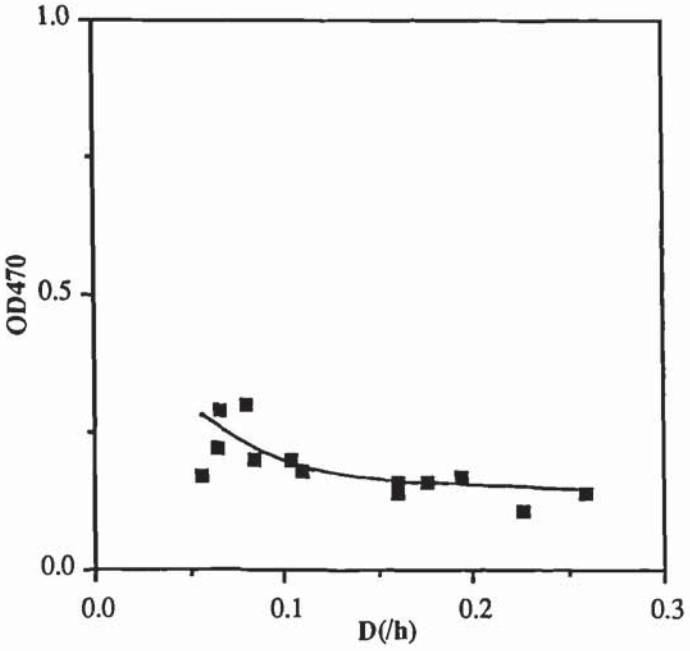


Fig. 2.11. Relationship between iron-restricted chemostat viable count at steady state and dilution rate for *P. aeruginosa* PAO1 JD.

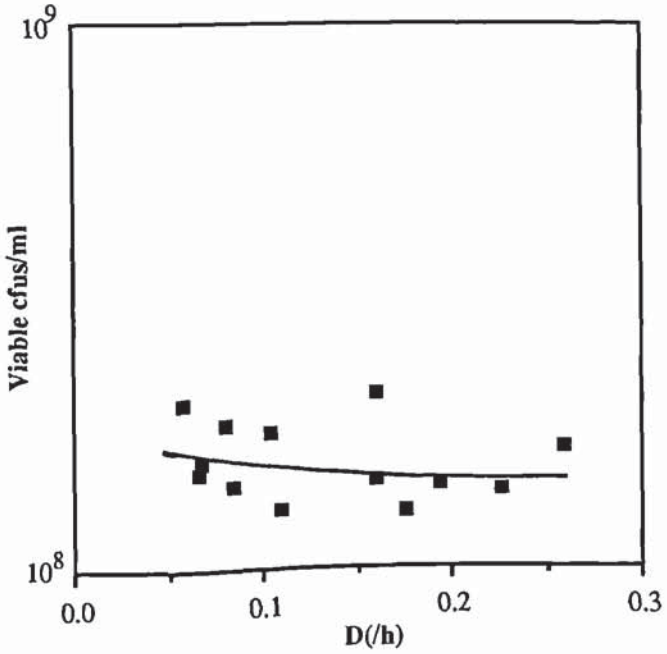


Fig. 2.12. Relationship between carbon-limited chemostat optical density at steady state and dilution rate for *P. aeruginosa* PAO1 JD.

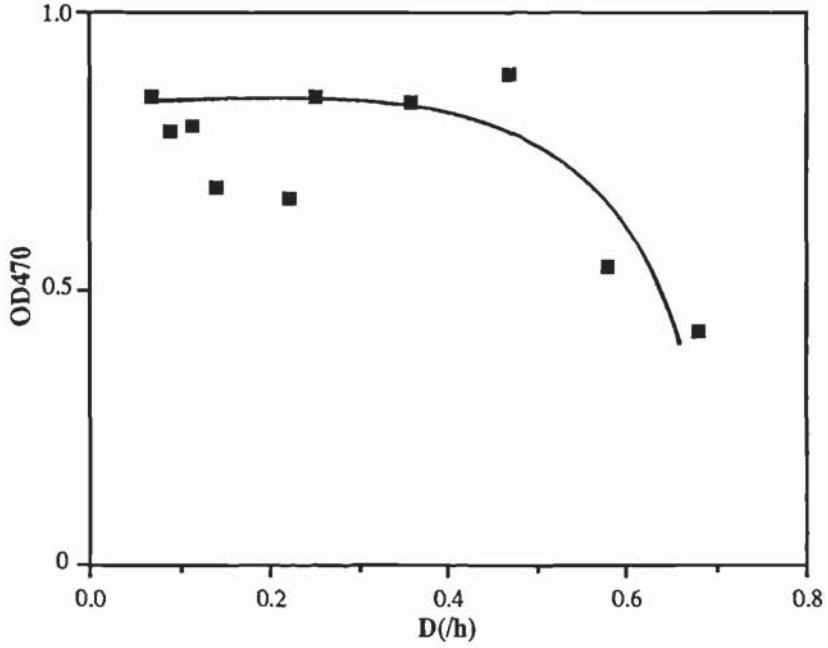
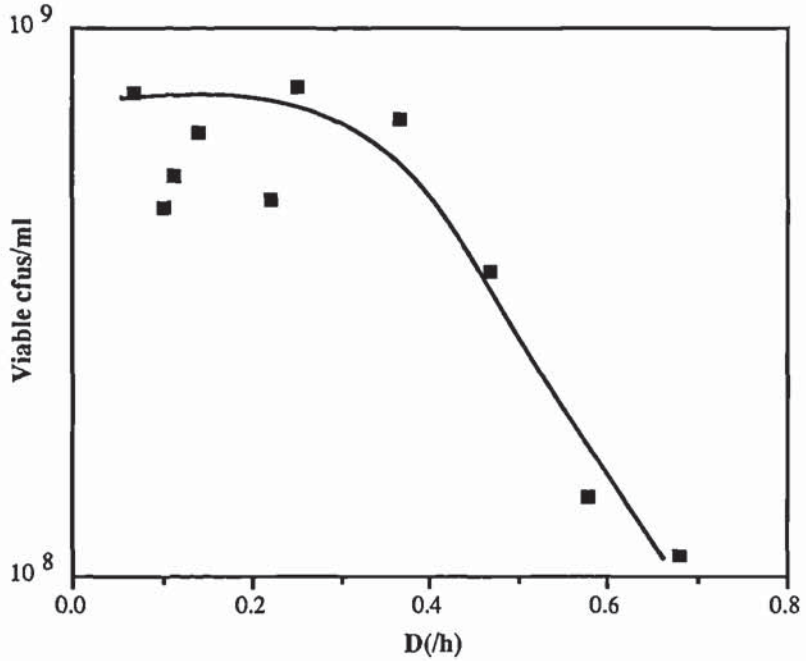


Fig. 2.13. Relationship between carbon-limited chemostat viable count at steady state and dilution rate for *P. aeruginosa* PAO1 JD.



2.10. METHODS FOR THE STUDY OF CELL SURFACE PROPERTIES

2.10.1. Introduction

Alteration of the composition of the microbial cell surface occurs as a result of changes in the cell's environment (Ellwood and Tempest, 1972) and growth rate (Gilbert *et al*, 1990). Physical changes include altered hydrophobicity (Rosenberg and Kjelleberg, 1986), cell surface charge (Van Loosdrecht *et al*, 1987b) and the presence of additional polymers bound to the exterior of the cell envelope (Fletcher and Floodgate, 1973).

In this study, a number of techniques have been used to study several of these surface properties in relation to the method of cell culture and changes in growth rate.

2.10.2. Hydrophobic interaction chromatography

Surface hydrophobicity was determined by hydrophobic interaction chromatography (HIC), the procedure being adapted from that of Smyth *et al* (1978). Octyl sepharose (Sigma) and, in some cases, phenyl sepharose columns were used as the non-polar ligands with sepharose columns to correct for non-specific adsorption. The principle of the technique is that, on passing cells down the HIC columns, their relative hydrophobicity can be found by determination of the proportion of cells being retained on the columns. The use of non-polar sepharose derivatives gives a measure of both specific and non-specific interactions and ascertaining non-specific adsorption subsequently allows calculation of hydrophobic interaction with the resins. The whole technique relies upon the fact that such hydrophobic interactions between cell surface and resin are promoted by the presence of a high ionic strength buffer (NaCl).

2.10.2.1. Preparation of chromatography columns

Columns were prepared using glass Pasteur pipettes (143mm length, 5mm diameter) with the tapered end shortened to 10mm. A small quantity of glass wool was placed at the constriction of the pipette to support the sepharose resin, whilst still allowing the elution of liquid from the column. The pipettes were held vertically in a suitable stand.

To prepare the HIC column packings, the pre-swollen gel slurries were first washed several times in distilled water to ensure the removal of preservative (sodium azide). A thick slurry of packing in 4M sodium chloride was then prepared (with the sepharose constituting 70% of the final volume). Each slurry was degassed for 15 minutes at reduced pressure then used to fill the pipettes to give 3cm of packed bed volume, taking care to ensure that no air bubbles were added into the system. Columns were then washed with at least 5ml 4M NaCl. To prevent the column from drying out, the tip was sealed until such time as when it was required. Fresh columns were prepared each day.

2.10.2.2. Varying NaCl concentration

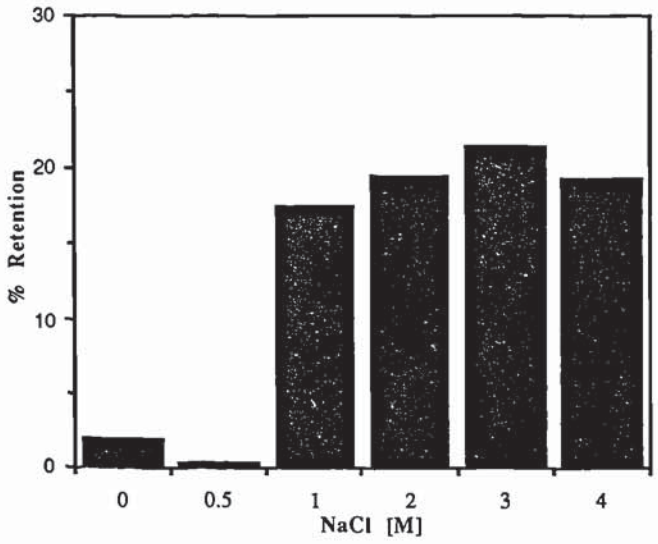
To demonstrate the importance of the concentration of NaCl used, prepared columns were washed thoroughly with various NaCl solutions of strength 0M, 0.5M, 1M, 2M, 3M and 4M in order to give at least five volume changes of eluting NaCl. From the outcome of this trial (fig. 2.14.) it was decided to use 4M NaCl, as shown previously (Smyth *et al*, 1978). This concentration is used generally by other workers (Allison *et al*, 1990a,b; Evans, 1990) and was chosen in this project to justify direct comparison of results with those gained by other groups.

2.10.2.3. Method

Cell suspensions of *P. aeruginosa* were diluted with CDM salts to give ca.10^5 cells.ml^{-1}

and 2ml of such suspensions added to the columns by Pasteur pipette, with a further 2ml of 4M NaCl being added to ensure adequate wash-through of cells. Retention of cells was assessed by viable counts on the initial suspension and on the column eluates. Retention of cells by octyl or phenyl sepharose was expressed as the percentage of cells retained, relative to retention by the sepharose column.

Fig. 2.14. Effect of NaCl concentration on retention of PAO1 JD cells in sepharose columns.



2.10.3. Analysis of outer membrane proteins of *P. aeruginosa* by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

2.10.3.1. Preparation of outer membranes - classical method

Cells were grown overnight in 1l conical flasks containing CDM (250ml) on an orbital shaker at 37°C, harvested from the chemostat onto ice (to prevent further growth and phenotypic variation of the organisms) and, in the case of biofilm-grown organisms, vortexed and scraped from cellulose acetate membranes as described in section 3.2.2.6. Cells were harvested by centrifugation (10,000 rpm, 10min: Beckman J2-21, Beckman Instruments Ltd., High Wycombe), suspended in 0.85% saline, recentrifuged and resuspended in dH_2O to a volume of 9ml. The suspension was then subjected to sonication on ice (MSE Soniprep, 150W: MSE Ltd., Crawley) for 5 minutes (10 x 30sec sonication, 30 sec cooling intervals between each sonication) or until the OD_{470} was reduced by 90%. In order to solubilise the cytoplasmic membrane, whilst leaving the outer membrane intact, 1ml of 20% N-lauroyl-sarcosine (Sarkosyl, BDH) was added to give a final concentration of 2% w/v and the suspension incubated for 1 hour at 37°C (Lambert and Booth, 1982). Mild centrifugation (5,000 rpm, 5min) pelleted any unbroken cells and the supernatant was then centrifuged at 20,000 rpm for 1 hour to obtain a pellet consisting of outer membranes. This was washed and finally resuspended in dH_2O and stored at -20°C.

A Lowry assay (see section 2.10.3.5.) was carried out to determine the amount of protein present and the preparation diluted, if necessary, to give a concentration of 2.4mg.ml^{-1} protein. This was subsequently diluted 1:1 with sample buffer and boiled for 10 minutes. 15µl of sample were applied per lane and the gels run at a constant voltage of 200V until the dye-front had migrated to within 0.5 cm of the bottom of the gel. 12% discontinuous gels were prepared as described by Lutgenberg *et al* (1975)

using Mini-Protean gel apparatus (Bio-Rad, Watford).

Sigma molecular weight markers were included to facilitate the determination of the molecular weight of the outer membrane proteins. The markers used are given in table 2.5.

2.10.3.2. Preparation of outer membranes - modified method for small samples

When only a very small amount of cells was available for outer membrane preparation (e.g. when filter membranes from Swinnex and Sorbarod devices had been sacrificed and the resulting cell suspensions pooled) the classical method for outer membrane preparation could not be used. In such cases, either whole cell preparations could be run on gels or, preferentially, a modified method of outer membrane preparation was carried out.

Cell suspensions were centrifuged (10,000 rpm, 10min) and the pellet resuspended in a small volume of 0.85% saline in an Eppendorf tube. The sample was sonicated, still in the Eppendorf tube, for a few seconds to resuspend and evenly redistribute the pellet. 10% Sarkosyl was added to give a final concentration of 2%w/v, the sample was sonicated briefly, then centrifuged (MSE MicroCentaur) at 13,000 rpm for 1 hour. The pellet was resuspended in a 50µl solution of sample buffer and dH_2O (1:1) and 10µl of sample used to load the gel.

Table 2.5.

Sigma molecular weight markers.

Marker	Approximate molecular weight (KDa)
Albumin, bovine	66
Albumin, egg	45
Glyceraldehyde-3-phosphate dehydrogenase	36
Carbonic anhydrase	29
Trypsinogen	24
Trypsin inhibitor	20.1
α -lactalbumin	14.2

2.10.3.3. Coomassie Brilliant Blue stain for protein gels

Protein gels were stained for 1 hour, with gentle agitation, in a solution containing Brilliant Blue R-250 (1g; Sigma), methanol (500ml) and glacial acetic acid (100ml) in 400ml dH_2O. Background stain was then removed by successive washes in a destain solution consisting of methanol (100ml) and glacial acetic acid (200ml) in 700ml dH_2O.

2.10.3.4. Silver stain for protein gels

When only small amounts of protein were present on SDS-PAGE gels, as was the case with outer membrane preparations from Swinnex biofilm-grown cells, the gels were stained with a silver stain solution. This method (a modification of that of Merril *et al*, 1981) was more sensitive to the presence of protein than the Coomassie Brilliant Blue

stain.

Once electrophoresis had been completed, the gels were gently agitated in fixing solution (50% methanol, 10% acetic acid, 40% dH_2O) for 30 minutes. They were then fixed in destain solution (5% methanol, 7% acetic acid, 88% dH_2O) for at least 1 hour before fixing in 10% gluteraldehyde for 30 minutes. Gels were subsequently given 4 x 30 minute washes with dH_2O and stained with silver nitrate solution (1.75ml 30% NH_4OH, 21ml 0.1M NaOH, 77.25 ml H_2O; 8ml 19.4% silver nitrate added dropwise to solution) for 15 minutes, shaking vigorously. Following staining, gels were given 5 x 1 minute washes with dH_2O then agitated in developing solution (0.5g sodium citrate, 0.5ml 37% formaldehyde, 100ml dH_2O ; 5ml of this solution diluted with 100ml dH_2O and this used for developing) until bands appeared to the required intensity. The gels were transferred immediately to Kodak Rapid Fix (Kodak, UK) for 5 minutes and, finally, washed exhaustively in dH_2O to ensure complete removal of the Rapid Fix solution.

2.10.3.5. Lowry assay for protein

The protein content of OM preparations was determined by the method of Lowry *et al* (1951) and simplified according to Peterson (1977). Bovine serum albumin (BSA) standards (0-300μg) and the samples for analysis were made up to 0.5ml with ddH_2O. An equal volume of 1M NaOH was added to each sample and heated to 100°C for 5min. On cooling, 2.5ml of a solution containing 1ml 0.5%w/v $CuSO_4.5H_2O$ and 1ml 1%w/v Na/K tartrate solution in 50ml of 5%w/v Na_2CO_3 in 0.1M NaOH was added. After leaving for 10min, 0.5ml Folin-Ciocalteau phenol reagent (Sigma), diluted 1:1 with dH_2O, was added to each sample and mixed by vortexing. The OD_{750} was recorded after 30min against a BSA blank and a calibration curve constructed. Standards and samples were assayed in triplicate.

2.10.4. Polyacrylamide gel electrophoresis for analysis of lipopolysaccharide

The method was that of Hitchcock and Brown (1983). One ml of whole cell suspensions (OD_{470} approximately 0.5) was pelleted and resuspended in SDS-PAGE sample buffer (100µl). The preparation was heated to 100°C for 10 minutes to lyse the sample, then 25µl of a 2.5mg.ml^{-1} solution of proteinase K (from *Tritirachium album* type XI; Sigma) was added to digest any protein and the sample was incubated at 60°C for 60 minutes (Hitchcock and Brown, 1983). (Alternatively, OM preparations were used directly and suitable volumes of proteinase K added after boiling). Samples were loaded in the same way as for outer membrane protein preparations and the gels run at a constant voltage of 100V and stopped as the dye-front ran within 0.5mm of the bottom of the gel.

2.10.4.1 Silver stain for lipopolysaccharide

Two methods for visualising LPS preparations separated by PAGE were used. Method 1 was performed as described by Tsai and Frasch (1982) and Method 2 was a modification of this, as reported by Fomsgaard *et al* (1990). Method 2 is believed to avoid the possibility of losing visibility of any bands, especially O-antigen banding, through excessive washing of the gel. In both cases, double distilled water and scrupulously clean gel dishes were used and gels were handled with washed gloves at all times, owing to the sensitivity of the stains.

2.10.4.1.1. LPS silver stain - Method 1

After the gel had run, it was fixed overnight in a solution of ethanol (40%) and glacial acetic acid (5%). The following day, the fixing solution was replaced for 5 minutes with an oxidising solution (1g periodic acid, 4ml fresh fixing solution, 150ml dH_2O). The

oxidase solution was removed from the gel by 3 fast then 3 x 30 minute washes in dH_2O. The staining solution was freshly prepared by adding ammonia (2ml) to 0.1M NaOH (28ml) followed by dropwise addition of 20% $AgNO_3$ (5ml), then addition of dH_2O (115ml). The gel was agitated in the staining solution for 10 minutes then given 4 x 10 minute washes in dH_2O to remove any excess silver stain. It was then developed for as long as necessary in a solution containing citric acid (10mg) in 37% formaldehyde (0.5ml) and 200ml dH_2O. The developing was stopped by immersion in a solution of glacial acetic acid (0.7ml) in dH_2O (200ml) and the gel photographed as soon as possible as the stain deteriorates.

2.10.4.1.2. LPS silver stain - Method 2

After electrophoresis, the gel was immediately put in oxidising solution (0.7% periodic acid, 40% ethanol, 5% glacial acetic acid) for 20 minutes, thus eliminating the fixation step. It was given 3 x 5 minute washes in dH_2O then stained for 10 minutes in fresh staining solution.The silver stain solution was prepared by adding ammonia (0.67ml) to 0.1M NaOH (9.33 ml) followed by addition of 33.33ml dH_2O. A solution of 20% $AgNO_3$ (1.67ml) was added in a dropwise fashion and the volume of the final solution adjusted to 50ml with dH_2O. The gel was then given 3 x 5 minute washes in dH_2O and developed in a solution containing citric acid (10mg), 37% formaldehyde (0.1ml) and 200ml dH_2O. Developing was stopped by immersion in acetic acid (10%) for 1 minute and the gel washed repeatedly in dH_2O.

2.11. SIDEROPHORE ASSAY

The assay used in this study is a procedure described by Schwyn and Neilands (1987) which is designed to determine non-specifically the amount of siderophore produced by the test organism. This colorimetric assay works on the principle that the dye, chrome azurol S (CAS), complexed with iron gives a blue colour but in the presence of higher affinity siderophore, iron dissociates from the CAS and is chelated by the siderophore.

CAS in the absence of iron is orange coloured, thus the resulting drop in optical density at 630nm gives a measure of iron chelation by siderophore in the test sample. The OD_{630} falls with increasing siderophore concentration.

2.11.1. Method

Culture supernatants (1ml), after centrifugation (10,000 rpm, 10 minutes), were mixed with siderophore reagent (1ml; see below) in a 3ml, 1cm path length cuvette. The solutions were left for 1 hour at room temperature for the iron to equilibrate between the siderophore and the CAS. Absorbance was read at 630nm with CDM salts as a blank and reagent plus CDM (1ml + 1ml) as a negative control (i.e. zero siderophore). The decrease in optical density was used as an indication of the amount of siderophore present in the sample, and compared with the values for the standard desferrioxamine (Desferal: Ciba Geigy, Horsham) calibration curve.

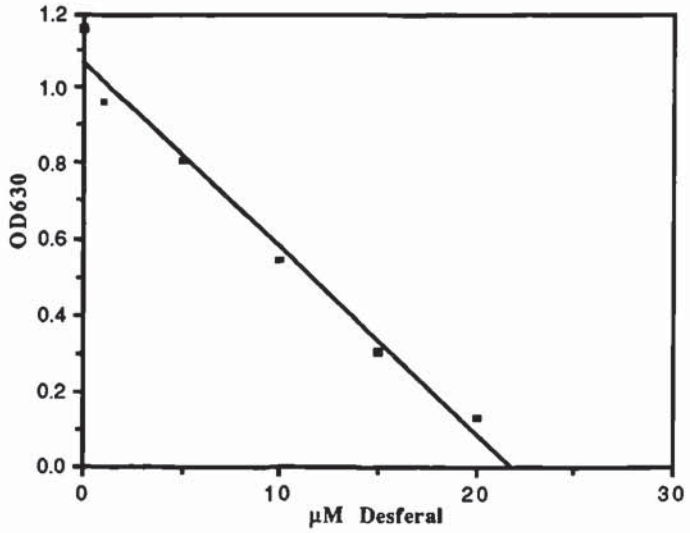
Stock reagents:
- 10mM cetrimide
- 1mM $FeCl_3.6H_2O$ in 10mM HCl
- 2mM CAS (Chrome azurol S)

To prepare the siderophore reagent, 6ml of the cetrimide solution was added to a 100ml glass bottle and diluted with 30 ml dH_2O. 1.5ml of the ferric chloride solution and 7.5ml of the CAS solution were then added. Anhydrous piperazine (4.307g) was added to a universal bottle, two thirds filled with dH_2O, and 6.25ml of conc. HCl added. This solution was then added to the 100ml bottle and the volume made up to 100ml with dH_2O. Finally, 101.6mg of 5-sulphosalicylic acid was added to act as a "shuttle" which accelerates the reaction. After shaking, the reagent was left at 37°C for several hours to allow the shuttle to dissolve.

Since the assay is sensitive to siderophore production in general, some type of comparison values for a known iron-chelator were required in order to give "units of siderophore" more meaning. The iron-chelator desferrioxamine methanesulphonate

(Desferal) was chosen and a calibration curve was plotted to illustrate how changes in optical density were related to known amounts of iron-chelator present (fig. 2.15).

Fig. 2.15. Calibration curve for Desferal iron chelation.



2.12. Antimicrobial bactericidal assay

The aim of this assay was to give an indication of the variation with growth rate of the susceptibility of *P. aeruginosa* PAO1 JD to ciprofloxacin. It was necessary that such an assay was relatively rapid to perform in order to assess the properties of the cells after harvesting from the chemostat or biofilmer device, before any alteration in physiological properties could occur. A further reason for the use of this assay lies in the fact that the fluoroquinolones cause filamentation of bacteria which increases the turbidity of liquid cultures. Consequently, bacterial killing by fluoroquinolones antibacterials cannot be judged by measurements such as light scattering or optical density that merely reflect a change in bacterial mass. Hence, the levels of kill of bacteria by the 4-quinolones are studied by viable counts (Smith, 1984).

Preliminary experiments were conducted to select concentrations of antimicrobial agents which gave appropriate levels of killing, relative to control suspensions, within a 1h contact time period at 37°C. It was noted that the killing curve for ciprofloxacin (fig. 2.16.) did not display the paradoxical dose-dependent response described by Crumplin and Smith (1975) and Smith (1986), with levels of survival instead decreasing with increasing drug concentration. However, it may be the case that the biphasic response would become apparent if an increased range of drug concentrations was used.

In iron-restricted batch culture (mid-logarithmic phase), a concentration of 0.3 $\mu g.ml^{-1}$ ciprofloxacin was found to give 40% kill, and this concentration was used in subsequent experiments on chemostat and biofilm-generated cells.

2.12.1. Method

Tubes containing the appropriate concentration of ciprofloxacin were prepared (see table 2.6.) and pre-warmed at 37°C for 10 minutes. The samples to be exposed to the drug were standardised by dilution with CDM salts to give ca.10^5 $cells.ml^{-1}$ and aliquots (100µl) of such suspensions added to the antimicrobial solutions and incubated at 37°C for 1 hour. The treated cells and controls were then diluted (1 in 100) in CDM salts (minus glucose) in order to inactivate the ciprofloxacin, and 100µl samples plated out on nutrient agar. Plates were incubated at 37°C overnight and relative reductions in colony forming units (cfu) assessed.

Fig. 2.16. Survival of PAO1 JD cells after incubation with ciprofloxacin at 37°C for 1h.

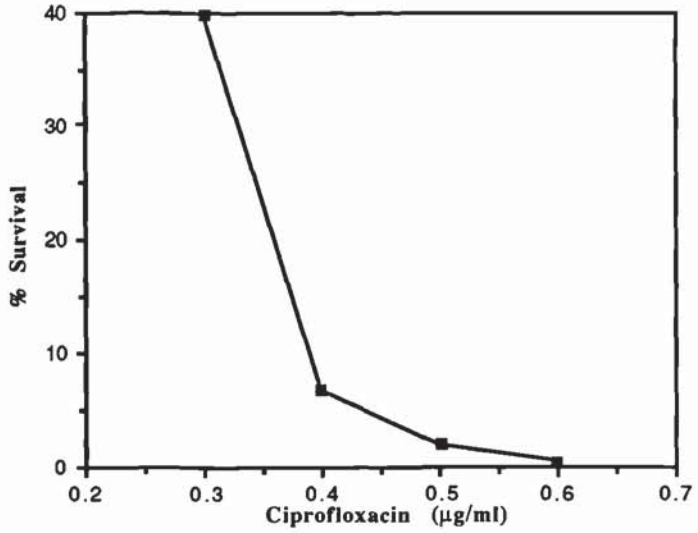


Table 2.6.

Antibiotic solutions for use in antimicrobial bactericidal assay

Antimicrobial stock solution	Vol. to use (µl)	Simple salts soln. (ml)	Inoculum (µl)	Final conc. ($\mu l.ml^{-1}$)
Ciprofloxacin				
30 µg/ml	100	9.8	100	0.3
100 µg/ml	100	9.8	100	1.0

3. DEVELOPMENT OF TECHNIQUES FOR THE STUDY OF GROWTH RATE-CONTROLLED BIOFILMS OF *Pseudomonas aeruginosa* PAO1 JD

3.1 THE 47MM BIOFILM FERMENTER

3.1.1. Introduction

The importance of bacterial biofilm growth in the environment, industrial pipelines and medical devices has gained increasing recognition in recent years (Costerton and Lashen, 1984; Costerton *et al*, 1987; Costerton *et al*, 1990; Christensen *et al*, 1989) and numerous studies have attempted to model such populations in the laboratory. (For an overview of methods used to study the biofilm mode of growth, see section 1.6.).

Despite producing much valuable information on biofilm growth in general, these models lack effective growth rate control of the biofilm population, a factor required if distinction between the effects of attachment and slow growth rate is sought (Gilbert *et al*, 1989). To date, the vast majority of studies have focussed on the physiological effects of cells actually adhering to a surface without separating these from those effects of the growth rate of the adherent cells. Mounting evidence suggests that many of the properties of biofilm cells are related to slow growth (Brown *et al*, 1988; Holmes and Evans, 1989; Gilbert *et al*, 1990).

Gilbert *et al* (1989) first described the use of a novel biofilm fermenter enabling growth rate control of adherent *E. coli* cells and have since studied several aspects of the biofilm and dispersed cells produced by this method. These include investigations on the effect of growth rate of biofilms to treatment with various antimicrobial agents (Evans *et al*, 1990a,b; Evans *et al*, 1991a; Gander *et al*, 1992) and how cell surface hydrophobicity affects dispersal of cells from biofilms (Allison *et al*, 1990a).

In this project great difficulty was encountered in the application of this technique with

P. aeruginosa PAO1 JD, and this chapter will deal with the progression of events which led to the abandonment of this method, followed by the design and use of new techniques.

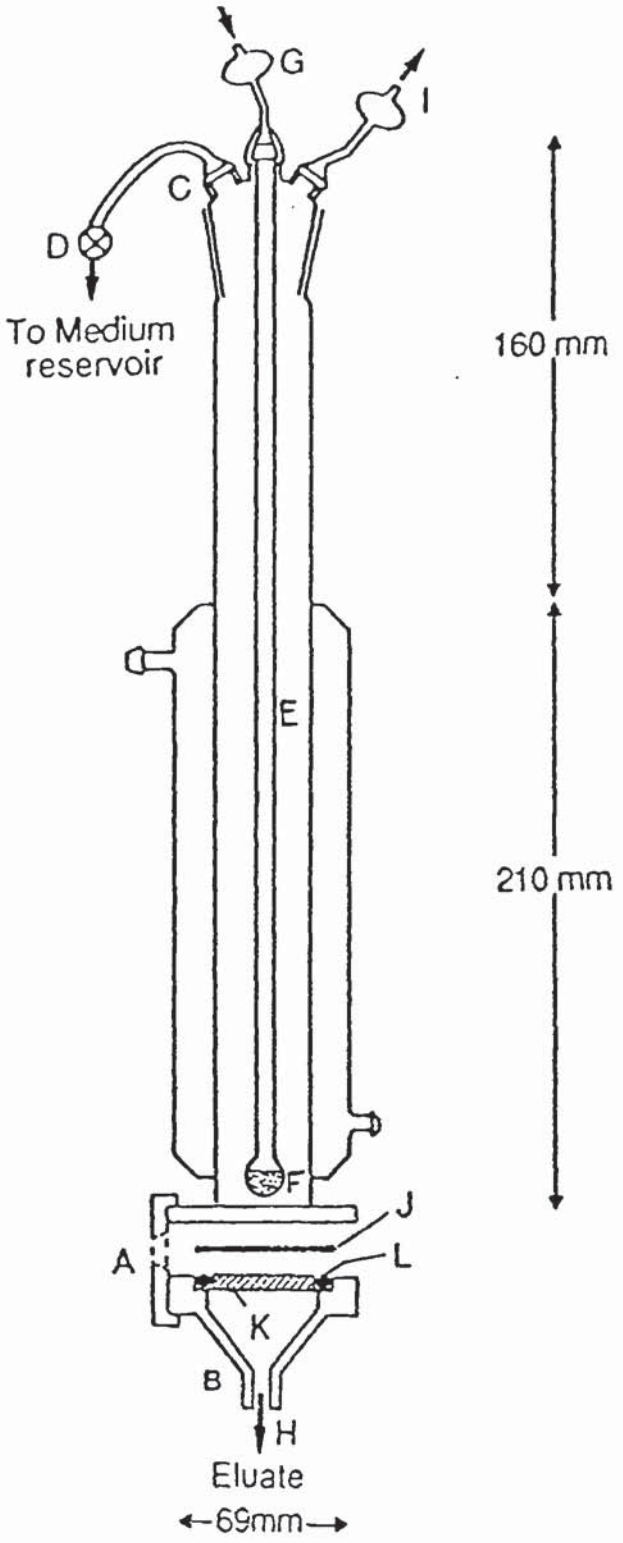
3.1.2. Basic Method

3.1.2.1. Preparation and initial running of 47mm biofilm fermenter

PAO1 cells were grown in shake flasks at 37°C in CDM_{12}-Fe until an OD_{470} of 0.2 was reached. After removing 100µl for viable counting, a known volume of this culture was passed through a 47mm diameter cellulose acetate/nitrate mix membrane filter (Oxoid, 0.2µm pore size) using a Millipore pressure filtration unit ($35kN/m^2$). (The filter had been prewashed by boiling in ddH_2O for 5min, then sterilised by autoclaving). The cell-impregnated filter membrane was removed, using sterile forceps, inverted, then placed and clamped (A) above the Teflon base (B) of the continuous fermentation apparatus as shown in fig. 3.1., supported by a Teflon-coated stainless steel sinter (K) and bounded by an O-ring seal (L). Temperature control was achieved by placing the whole apparatus in a room continuously maintained at 37°C (see section 3.1.3.1.). Fresh medium was pumped in (C) at a known rate into the vessel by way of a peristaltic pump (D) and the eluate passing through the filter membrane (J) collected at outlet H at various time intervals, for viable counting. Aeration was achieved by passing air through a sterile filter (G), then via a glass tube (E) and sinter (F) just above the membrane. The air outlet is labelled I. The apparatus was cleaned and sterilised as described for the glass chemostats in section 2.9.2.

The system was run at various flow rates in order to investigate if the resident membrane population size, the number of cells being eluted and, consequently the specific growth rate (µ) of the biofilm cells, were under the control of the rate at which incoming medium was being fed into the system.

Fig. 3.1. Diagram of 47mm biofilm apparatus.



3.1.2.2. Enumeration of cells adhering to membrane

After the required period of medium perfusing the filter membrane had elapsed, the membrane was removed aseptically then placed, "biofilm" side down, into a beaker containing 10ml sterile CDM salts. This was vortexed for 5min, the filter transferred to a further 10ml CDM and the vortexing repeated. To ensure that this procedure removed the largest quantity possible of the adherent population, this washing was carried out a total of four times and viable counts made using 100µl aliquots from each of the four beakers.

After this initial experiment, the four washes were thought to be sufficient in removing a maximum number (i.e. over 95%) of cells from the membrane.

3.1.2.3. Calculation of specific growth rate (µ) of membrane population cells: method used for *E. coli* model

According to the method of Gilbert *et al* (1989), for *E. coli*, if the number of cells residing on the filter membrane, the flow rate of the medium (ml/h) and the number of cells being eluted from the membrane (cfus/ml) are known and constant, then the rate at which cells are eluted can be calculated (cfus/h) and the expected doubling time (t_d) of the biofilm population ascertained (i.e. how long a population of x amount of cells on the membrane would take to shed x cells into the perfusing medium, assuming the biofilm itself is not growing in size).

Given the equation relating specific growth rate to t_d,

$$\mu = \ln 2 / t_d$$

then the specific growth rate of the biofilm cells may be calculated.

3.1.2.4. Attainment of a synchronously-dividing culture of *P. aeruginosa*

According to the technique of Helmstetter and Cummings (1964) the cells eluted from the *E. coli* B/r-impregnated filter membrane, after 30 minutes of perfusion with medium, corresponded to newly formed daughter cells. In the method described by Gilbert *et al* (1989) the system was allowed to run until such time as a constant viable cell count in the eluate was achieved (the "steady state"). A method of evaluating whether only newly formed daughter cells were being eluted was to collect a sample of eluate cells over a short period of time (no more than 2min worth in order to minimise the age range) and transfer it directly to pre-warmed medium of known volume. This medium was eluate which had been collected over ice and filtered through a 0.2μm filter membrane before warming to 37°C.

Viable counts made over a period of around 3h should indicate whether the eluate cells grow in synchrony in the fresh medium.

3.1.3. Results

3.1.3.1. Temperature control of the 47mm biofilmer

According to the method of Gilbert *et al* (1989), temperature control of the biofilm population of cells is achieved by way of a water jacket surrounding the main body of the vessel (see fig. 3.1.) filled with recirculating water heated to 37°C. However, this jacket is 2cm above the membrane and it was envisaged that, if there was no substantial hydrostatic head of medium above the membrane to be warmed by the jacket, then there would be some discrepancy between the jacket temperature and the temperature of the medium perfusing the membrane. This was investigated using a thermocouple to record the temperature actually on the underside (i.e. the biofilm side) of the membrane under normal experimental conditions.

3.1.3.1.1. Method

A non-inoculated biofilmer was set up in the laboratory and perfused with dH_2O instead of CDM, temperature control being provided by the water jacket. Room temperature (the temperature of the inflowing water) and the recirculating heated water temperature were monitored with a thermometer whilst the temperature at the membrane surface was ascertained by inserting the thermocouple via H (fig. 3.1.) and touching the membrane gently with the wire.

Temperature was followed over time, as if a normal experiment was taking place, and a hydrostatic head allowed to build up.

3.1.3.1.2. Results

As the first drops of "medium" flowed through the membrane, the temperature at the surface was only 20°C as opposed to the 37°C of the water jacket. Over time, as the head built up, the temperature at the membrane was still unacceptably low, thus after 300min, the flow rate was increased to allow as large a head, for heat transfer, as possible to build up. Finally, after 375min, the water jacket temperature itself was raised to 40°C and the hydrostatic head allowed to reduce in height. (See table 3.1.).

Table 3.1.

Temperature control of the 47mm biofilmer.

Time (min)	Water jacket temp (°C)	Room temp (°C)	Membrane temp (°C)	Liquid head height (cm)
0	37	14	20	0.0
15	"	15	24	1.8
30	"	"	28	2.5
40	"	"	29	3.0
50	"	"	30	3.5
60	"	16	31	3.6
90	"	"	31	3.8
120	"	"	"	4.0
180	"	17	32	4.3
240	"	"	32	4.4
300	"	18	32.5	6.7
360	"	"	33	7.2
375	40	"	36	7.5
420	"	"	"	4.6
450	"	19	"	4.4

3.1.3.1.3. Conclusion

Only when the water jacket temperature was raised to 40°C did the membrane surface approach an acceptable temperature. It was clear, also, that the hydrostatic head must build up sufficiently to expose it to the water jacket for efficient heat transfer to take place. Evidently, the whole system should be run in a 37°C warm room where the incoming medium is at 37°C and the need for a water jacket could be dispensed with.

It could be argued that, unless all previous experiments with this system had been carried out with the water jacket several degrees above the desired temperature of the

membrane, then optimum growth conditions, to date, have never been achieved.

3.1.3.2. Elution of cells from the 47mm biofilmer

3.1.3.2.1. Results and modifications of technique

As reported by Gilbert *et al* (1989), perfusion of the cell-impregnated filter membrane with medium flowing at a constant rate resulted in the number of eluted cells rapidly decreasing (fig. 3.2.) and reaching a steady state after a period of time (in this case, after 2.25h). Unlike the findings of Evans (1990), such a steady state could not be maintained for as substantial an amount of time as 14 days, as reported for *E. coli.* Indeed, within 24h of commencement of perfusion, the number of cells in the eluate began to increase. Furthermore, and to the detriment of the system, the hydrostatic head increased in height until it approached the top of the fermenter and the experimental run had to be abandoned. Another occasional problem was that of the apparatus leaking where the base was clamped to the main body of the fermenter.

3.1.3.2.2. Attempts to achieve synchronously-dividing batch cultures of PAO1 from 47mm biofilmer eluate

Using the method described in section 3.1.2.4., synchronous cultures of *P. aeruginosa* could not be achieved reproducibly. An example of a typical result is shown in fig. 3.3. Reasons for this could be;

1) the eluate cells were not all of the same age (i.e. stage of cell division cycle)
2) the eluate cells were not all growing at the same rate
3) the strain of bacterium used was not suitable for use in synchronisation studies of this type (Helmstetter *et al*, 1992).

This third possibility was discounted after Swinnex biofilm culture studies (section 3.2.2.2.) were able to produce synchronously-dividing cells, albeit irregularly.

Fig. 3.2. Elution of iron-restricted PAO1 JD from a 47mm, 0.2μm pore-size membrane at a constant flow rate of 0.27ml/min.

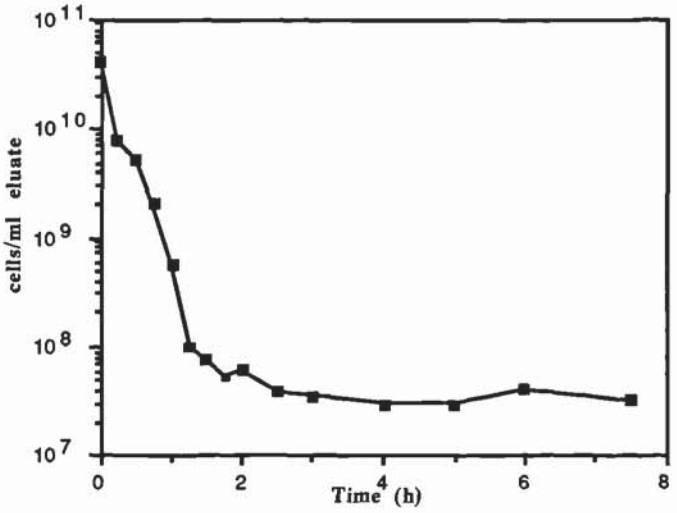
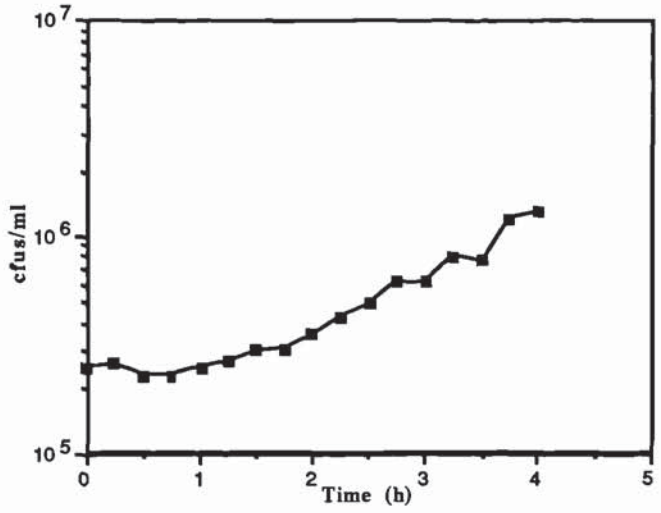


Fig. 3.3. Example of asynchronous growth of iron-lmited PAO1 JD cells eluted from a 47mm biofilmer and inoculated into pre-warmed CDM_{12}-Fe.



3.1.3.2.3. Hydrostatic head

The build-up of the hydrostatic head of medium above the membrane was a matter for concern, namely because it resulted ultimately in the premature termination of experimental runs. Furthermore, moving the apparatus to a 37°C warm room brought to light a previously hitherto unnoticed problem. As the system was run and the head increased in size, it was noticed that it became colonised with *P. aeruginosa* within 24h. This suggested that, either the upper side of the filter membrane was being contaminated during the process of handling the filter with forceps and positioning it on the fermenter base, or the organism was able to grow through the membrane pores from the underside. The former possibility could be very likely since the actual area of the filter which was not cell-impregnated, and therefore suitable to grasp, was limited to a 1mm wide rim around the perimeter of the filter membrane.

To investigate the first possibility, after loading the membrane with *Pseudomonas* and placing it on the fermenter base as normal, it was exposed to UV_{254nm} radiation from a germicidal UV lamp for 30secs in order to kill any bacteria which may have been on the upper surface of the filter. The base was then clamped in place and the system run in the usual manner. Despite this procedure, the hydrostatic head showed signs of contamination the following day.

A final modification to the apparatus was to attempt to improve the seal on the fermenter by placing a silicone rubber gasket over the filter membrane before clamping the pieces of apparatus together. This dispensed with the need to lubricate the base surface with silicone grease and, indeed, appeared to improve the overall seal on the system. However, the build-up of the hydrostatic head continued to be a problem as did its contamination, although the onset of growth of *Pseudomonas* here appeared to be delayed.

3.1.4. Discussion of 47mm biofilmer technique

Utilisation of the 47mm biofilm fermenter to give growth-rate-controlled biofilms of *P. aeruginosa* PAO1 was obviously beset with problems not apparent when growing *E. coli* (Gilbert *et al*, 1989) or *S. epidermidis* (Evans *et al*, 1992) biofilms by this method. The increasing hydrostatic head and its subsequent contamination could not be avoided by the methods used and it could be surmised that PAO1 and any EPS produced were leading to blockage of the filter pores. Further, the organism was somehow contaminating the upper surface of the membrane, perhaps through the manipulation step with forceps, or by growing through the membrane or leaking around the edges of the filter.

It was decided to develop an alternative biofilm fermenter where the possibility of contamination through handling would be avoided as would the development of a large hydrostatic head. This is described in the following section.

3.2. THE SWINNEX BIOFILM MODE OF GROWTH

3.2.1. Introduction

As a consequence of the problems encountered in the running of the 47mm biofilm apparatus (section 3.1.) it was decided to construct a smaller, more "user-friendly", biofilm device, but one which still adhered to the main growth rate control principle. The major criteria involved in the choice of apparatus were that it was small (for ease of handling), reusable, and that several could feasibly be run at the same time.

The "Swinnex" filter unit (Millipore Corporation, USA) appeared to be an ideal choice for use as the alternative biofilmer, being a ready-made filtration device which could be reused simply by replacing the membrane inside. The major advantage this unit had over the 47mm biofilmer was that, on loading the membrane with cells, there was no need to touch the membrane with forceps in order to invert it, ready for perfusion with

medium. Instead, the whole apparatus itself was simply inverted, without opening or readjustments being made, thus reducing the risk of contaminating the filter surfaces and reducing the time during which the cells on the membrane were not being perfused with medium. Additional favourable aspects of the Swinnex filter unit were that, being constructed of polypropylene, liquid and cells were less likely to adhere to the inner surfaces, allowing rapid flow-through of eluate, its small size (see fig.3.4.) allowed several to be set up in a relatively small space, it was readily autoclavable and a rapid turnover of these units in experimental work might be expected due to their ease of handling and smaller requirement for medium.

3.2.2. Method

As with the 47mm biofilmer, an initial set of calibration experiments were required in order to establish optimum conditions for running the devices and the limits within which it was most suitable to run them.

What follows is an account of the sequence of events which took place whilst attempting to standardise the method for use of the Swinnexes. It became obvious that this was not going to be a straightforward process of development, but it was necessary to define clearly the working limits of the system and to explain why this method of biofilm growth of *P. aeruginosa* PAO1 JD was not as successful as had been hoped at the outset.

3.2.2.1. Preparation and basic method of operation of the Swinnex

As can be seen from fig. 3.4., the Swinnex consists of two pieces which, once the filter membrane (B) and silicone O-ring (D) are in place, are screwed together to give the complete, sealed unit. For use simply as a filter, the whole unit is inverted from the position seen in fig. 3.4., a syringe containing the sample to be filtered inserted at port F and filtrate passes out at E. One major modification of the Swinnex, made before the

apparatus was put into use as a biofilmer, was to bore a hole in the side of the conical section i.e. what would be the lower half of the unit whilst operational and, into this, insert a needle (A) to serve as an air inlet. The needle was kept in position using Silastic Multifunctional Sealant (Dow-Corning GMBH, Munich). The whole unit was autoclaved (121°C, 20 min) with the membrane (0.2μm pore, 25mm diameter, Millipore mixed esters) (B) *in situ*.

Before loading the membrane with cells, the air inlet was blocked off and the membrane wetted with 10ml pre-warmed, filtered CDM salts by the normal method of inoculating the Swinnex. Once loaded with an appropriate amount of cells (initially based on the number of cells per mm^2 of membrane loaded onto the 47mm biofilmer) the unit was inverted and held in a clamp. The medium inlet tube was attached at E, the air inlet tube attached to the needle (A) and the system run either in a warm room or incubator at 37°C. Eluate containing cells and partially-spent medium could be collected at F, as with the 47mm biofilmer.

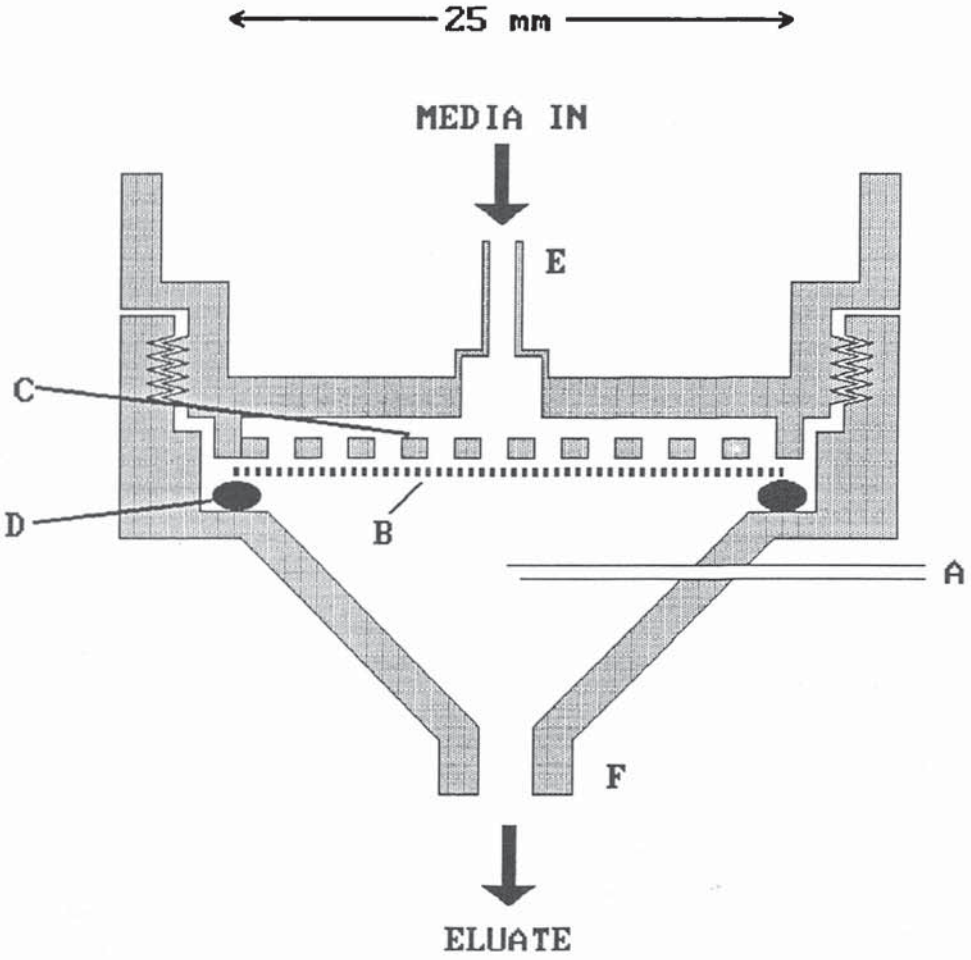
3.2.2.2. Initial calibration experiments

The principle aims of the first Swinnex experimental runs were to establish the numbers of cells in the eluate and on the membranes at different flow rates and to use these to calculate the specific growth rate (μ) of the cells on the membrane at each particular flow rate. At the same time, eluate cells were collected and used to inoculate warmed medium in order to establish if a synchronously-dividing culture could be achieved (see section 3.1.2.4. for method).

These initial experiments demonstrated that eluate viable cell numbers (cfus/ml) decreased as flow rate increased, as expected, but calculated μ values were low and, furthermore, no synchrony was achieved.

The inner design of the Swinnexes was altered in order to remove an horizontal

Fig. 3.4. Diagram of the Swinnex biofilmer.



ledge above the outlet (not seen in fig. 3.4.) which appeared to be allowing a small amount of eluate to lie on its surface, thus giving ineffective eluate outflow and the possibility of bacterial colonisation. Additionally, the inner surface was sprayed with Teflon (PTFE Aerosol Spray, Fisons Scientific Apparatus, Leics. or Dry Film Lubricant, TBA Industrial Products Ltd., Rochdale) to give improved liquid repellency.

Following the eluate viable count with time, a type of plot similar, at first, to fig. 3.2. for the 47mm biofilmer could be obtained, with an initial washing-off of loosely-attached cells but, thereafter, the number of viable cells in the eluate began to increase. An example of this phenomenon is illustrated in fig. 3.5.

It was possible to achieve a synchronously-dividing population of cells from the eluate cells, although this was not a satisfactorily reproducible occurrence. Fig. 3.6. illustrates one of the rare examples of synchrony, with a cell division time in the region of 60 mins. More commonly, however, a plot such as fig. 3.7. was obtained, indicative of batch growth of the cells. It would appear, then, that in most instances it is not just cells of one age which were being eluted from the membrane. It could be the case that there is a very short critical period when only newly-formed cells are being eluted, perhaps just at the point when all loosely-associated cells have been washed off. Alternatively, cells were not all growing at the same growth rate when the samples, which resulted in asynchronous growth, were collected. Wall-growth of cells would certainly contribute to the problem in this latter case.

As more Swinnexes at different flow rates were run, it was seen clearly that flow rate had a bearing on cell numbers eluted and cell numbers remaining on the membrane. In general, eluate viable counts would increase over time, as would the number of cells resuspended from the membrane. However, cell counts being achieved initially could not be taken as accurate since, when retrieving the membranes from the units, they were found repeatedly to be either split completely, especially when used at fast flow rates, or punctured slightly, if using slow rates of flow. Furthermore, the membranes were no longer flat but appeared to be sagging, creating a domed shape (fig. 3.8.). It seemed that, on sagging, the membranes were being holed by the air inlet needles.

Fig. 3.5. Elution of iron-restricted PAO1 JD from a Swinnex filter membrane at a constant flow rate of 1.3 ml/min (initial loading ca. 3.0×10^9 cells).

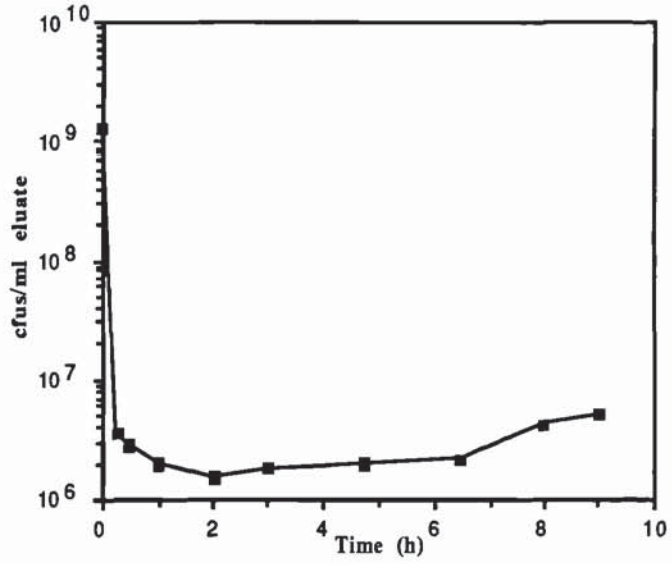


Fig. 3.6. Example of synchronous growth of PAO1 JD in CDM-Fe using Swinnex eluate cells as inoculum.

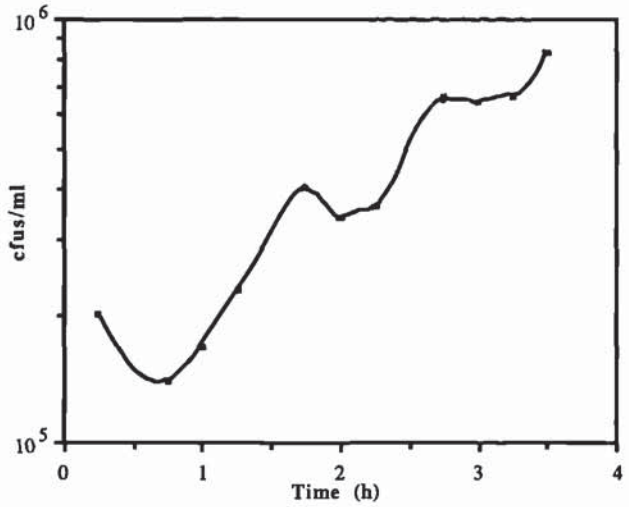
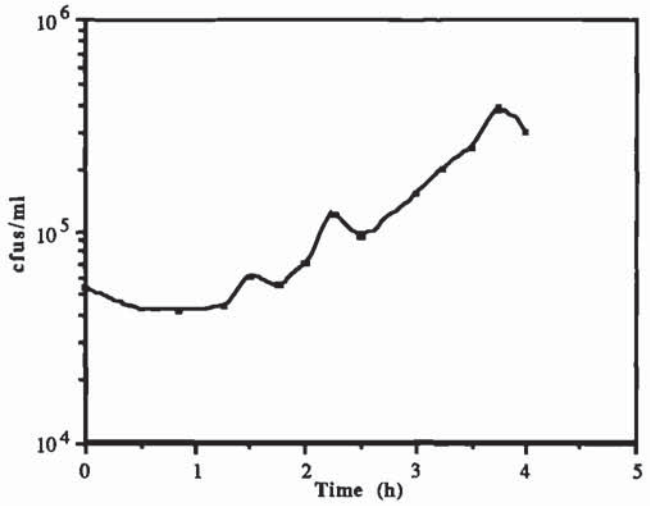


Fig. 3.7. Example of asynchronous growth of PAO1 JD in CDM-Fe using Swinnex eluate cells as inoculum.



Clearly, the doming problem needed to be remedied for, apart from the holing, the shape of the membrane would also affect the path of flow of medium through the system. It was envisaged that media would take a more central route with perhaps the periphery experiencing a slightly altered through-put of medium. It was noted that the central part of the membrane often appeared a green colour, indicative of either more profuse growth of PAO1 or the preferential flow of pigment produced in this area.

3.2.2.3. Use of grid support

To combat the sagging of the membrane, a Teflon-covered metal grid was placed over the washer in the Swinnex thus, when inverted, the membrane would be supported from below instead of simply at its edges.

At the end of the trial run, the membrane was sacrificed and found to be intact. However, putting the grid through an identical washing and vortexing procedure to that of the membrane resulted in ca. $5.5x10^8$ cfus being washed from the grid, whilst ca. $6.8x10^8$ cfus were removed from the membrane. Therefore, there were effectively two separate biofilm populations within the one flow system and the grid support could not be used further.

3.2.2.4. Use of two O-ring seals

The distortion of the membrane resulted, presumably, from the pressure of inflowing medium pushing the membrane down, causing it to slip from the grip of the rubber washer. In an attempt to prevent this, a second washer was introduced into the system and placed on the Swinnex support base (C) before the membrane was positioned on top. Finally, the other O-ring was placed on top of the filter as normal. It was hoped that, consequently, the membrane would be held in place more firmly.

A further modification was to lower the air inlet to ensure that, if sagging did occur, the membrane would not be pierced by the needle. The outcome of these modifications was that, although doming of the filter continued to occur, the membrane remained intact. It was decided to proceed with quantifying the system with the improved design as it appeared that the doming could not be circumvented.

3.2.2.5. Quantification of the Swinnex biofilm system

In order to determine specific growth rates for various flow rates, duplicate units at each flow rate were set up, with one unit being sacrificed after 8h of perfusion with medium, and the second broken down after 24h had elapsed. Figs. 3.9. and 3.10. illustrate the effect of increasing the flow rates on the number of cells eluted in the medium whilst fig. 3.13. shows the biomass of cells resuspended from the filter membranes. As can be seen from fig. 3.11., a crude level of growth rate control was being achieved, although by no means accurately reproducible and certainly, the calculated values for μ were much lower than would have been expected for the faster flow rates.

It was apparent that, at the slow end of the flow rate spectrum, there was more obvious control of growth rate.

Fig. 3.8. Illustration of doming of the Swinnex filter membrane.

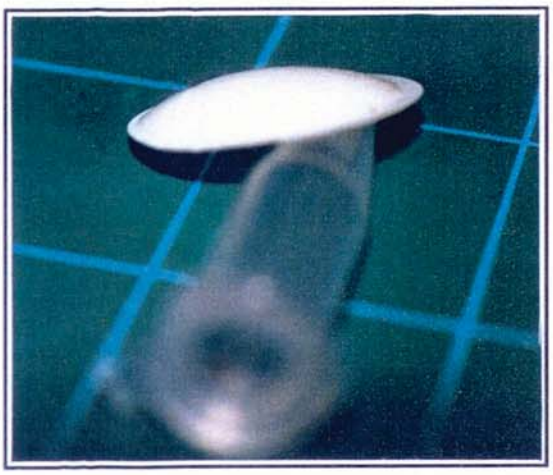


Fig. 3.9. Relationship between the Swinnex medium (CDM_{12}-Fe) flow rate and viable count of PAO1 JD in the eluate, at time 8h.

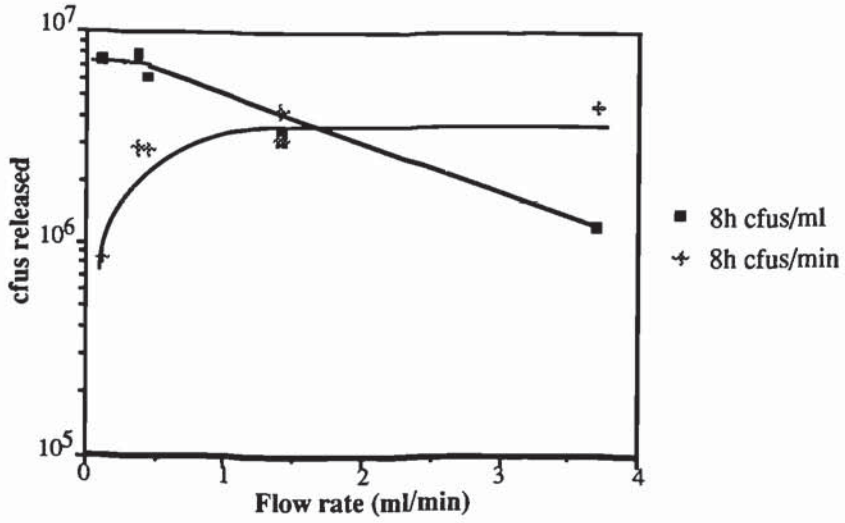


Fig. 3.10. Relationship between the Swinnex medium (CDM_{12}-Fe) flow rate and viable count of PAO1 JD in the eluate, at time 24h.

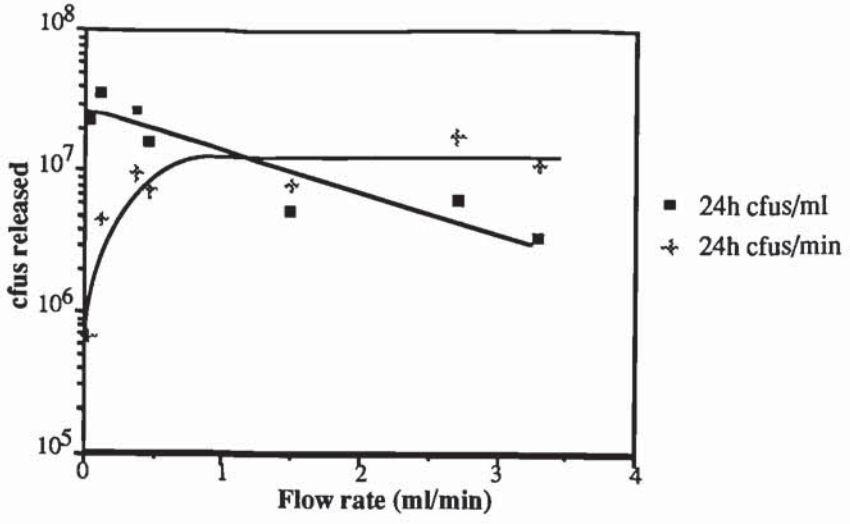
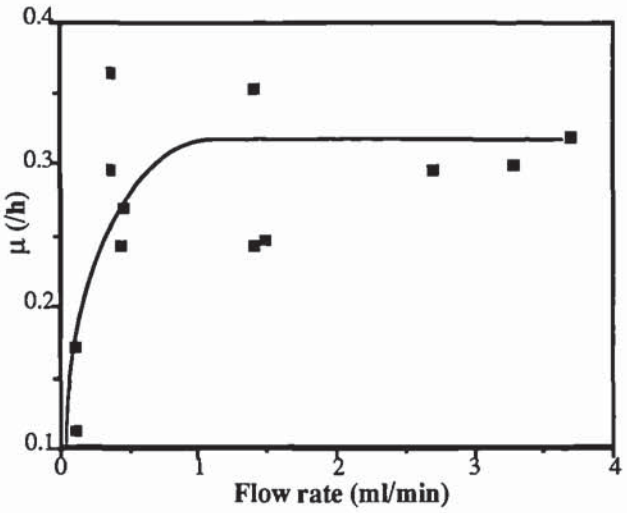


Fig. 3.11. Growth rate control of PAO1 JD in CDM_{12}-Fe achieved using the Swinnex biofilm system.



3.2.2.6. Membrane washing procedure

For the accurate calculation of specific growth rate of the biofilm cells it is essential that all surface-grown cells are removed from the filter for viable counting. For *E.coli*, shaking in 10ml saline for 10min would appear sufficient to remove biofilm organisms (Evans, 1990) whereas with *P. aeruginosa* it has been reported that scraping, vortexing and use of a low output ultrasonic bath are required to dislodge successfully all biofilm cells from a surface (Costerton *et al*, 1986).

Initially in this project, membranes were treated by a series of 6 x 2min vortex-washes (Fisons Whirlimix, maximum speed) in known volumes of CDM salts (5ml for 25mm membranes, 10ml for 47mm membranes) with the washes being pooled for viable counting. This number of washes had been chosen after repeatedly washing and vortexing a Swinnex membrane and viable counting each set of washings (fig. 3.12.). However, it appeared that there was a proportion of cells still adhering to the membrane since the washing procedure still generated organisms on the tenth wash. It was decided to introduce a scraping step into the procedure. It was also noted from sacrificing 8h and 24h biofilms over a range of flow rates, that the membrane bioburden increased over time (fig. 3.13.).

3.2.2.6.1. Method and results

An 8h biofilm was vortex-washed 5 x 2min, each time in fresh CDM salts. After the fifth wash, it was cut in half with a sterile scalpel and one half washed as normal, the other being scraped using a sterile glass rod before a vortex wash. It was found that the non-scraped half yielded $4.3x10^6$ cfus on the sixth wash, whilst the scraped portion yielded $1.6x10^7$ cfus.

A 24h biofilm was similarly vortex-washed five times and halved, then both halves subjected to scraping before washing, one by sterile glass rod, the other by sterile

cotton swab. It was found that both methods yielded $2.6x10^8$ cfus from the sixth wash and it was decided to continue using the (more convenient) glass rod method. One final alteration was to introduce the scraping step on the second wash, which was found to dispense with the requirement for 6 x 2min washes and allowed for only 4 x 2min vortex-washes to dislodge the cells from the filter membrane.

3.2.3. Visualisation of the membrane

In order to verify that the scraping and washing procedure indeed removed the vast majority of cells from the membrane, several microscopy techniques were employed.

3.2.3.1. Light microscopy

Viewing the membranes by light microscopy necessitated optical clearing of the filter membranes. Using the method of Dutton *et al* (1983), air-dried membranes were floated on immersion oil until they became transparent. Light microscopic examination revealed little difference between a sterile, cleared filter and one which had undergone the scraping and washing steps to remove a 24h biofilm.

3.2.3.2. Scanning electron microscopy (1)

Scanning electron microscopy (SEM) work was carried out on dried membranes which had undergone the washing procedures. Portions of filters were stuck onto metal discs, gold-plated then viewed by SEM (Cambridge Instruments Stereoscan 90). The membrane surface appeared to be, on the whole, bacteria-free, although it was clear that, for better visualisation, modification of the preparatory technique was required.

Fig. 3.12. Multiple vortex-washes of a Swinnex filter membrane.
(Cells removed on each 2 min wash in 5ml of CDM salts).

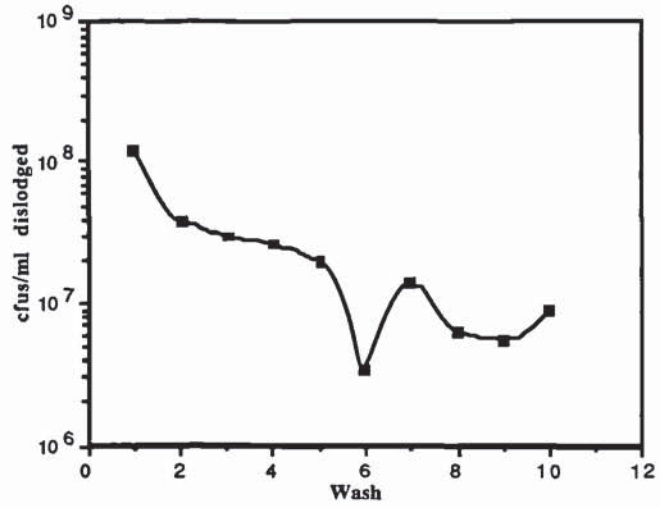
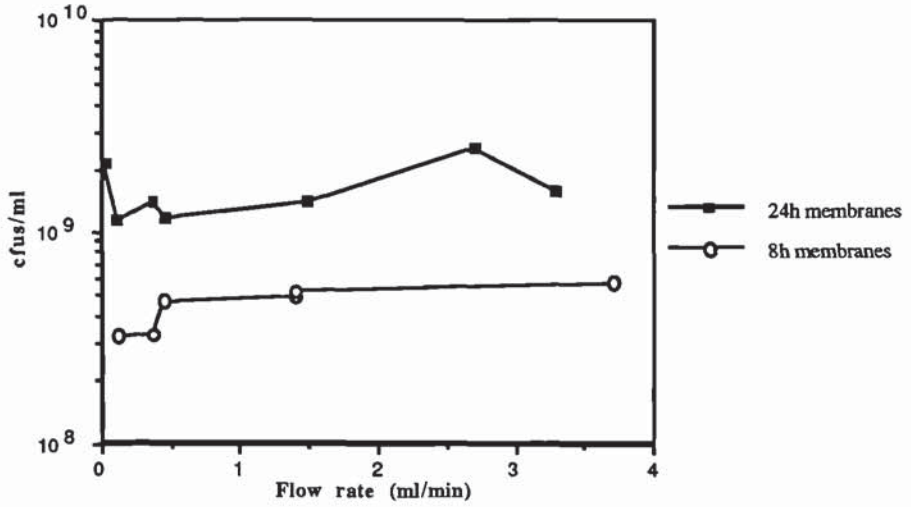


Fig. 3.13. Numbers of PAO1 JD cells resuspended in CDM salts from 8h and 24h Swinnex biofilms over a range of flow rates.



3.2.3.3. Scanning electron microscopy (2)

A SEM technique was utilised where no prior treatment of the membrane was required. Samples were placed directly on the viewing stage of the microscope (Electroscan, Electroscan Corporation, Mass., U.S.A.) and the amount of wetting and drying altered as required. This relatively new type of electron microscopy has several advantages over classical scanning electron microscopy, namely that a high vacuum is not required in the sample chamber, no preparatory techniques need be employed, thus reducing the incidence of artefact occurrence, and the potentially harmful results of over-drying the sample are also avoided more easily. Instead, the sample is placed directly in the sample chamber under low vacuum, atmospheric conditions (with the electron gun maintained at high vacuum conditions quite separately from the sample) and the level of hydration of the sample can be altered as required. A high resolution image is obtained from secondary electron detection, as with traditional SEM methods. Resolution is enhanced further by the production of more electrons due to the ionisation of gas in the chamber by the secondary electrons from the sample.

A "Swiss-cheese" effect was seen clearly (fig. 3.14.), presumably this being created by holes in the carbohydrate layer of the biofilm. However, as this was the first occasion that the microscope had been used for this type of work, the possibility of electron beam damage or over-drying of the sample exacerbating the effect cannot be wholly ruled out. The holes were 1-2μm wide.

Unfortunately, the presence of individual *Pseudomonas* cells could not be detected owing to the copious amounts of EPS present. Future work using this method of microscopy may benefit from prior incubation of the biofilm cells with a metal compound which would be clearly visible on the micrograph, or perhaps labelled antibodies could improve visualisation of individual cells. It should be noted that other workers have employed electron microscopy techniques for visualisation of bacterial biofilm growth (Costerton *et al*, 1981a,b; Costerton *et al*, 1985) with transmission electron microscopy and ruthenium red-staining being an example of a successful method for observing bacteria and EPS material (Costerton, 1981a; Fletcher, 1990).

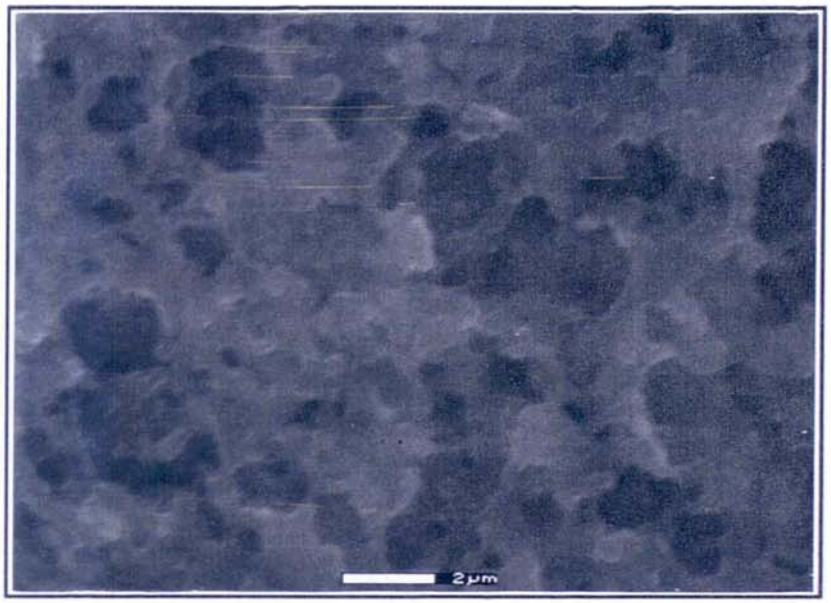
3.2.4. Leakage from the system

Having solved the problems of piercing and splitting of the membranes, a difficulty of a different nature arose. Liquid was seen to be leaking from the sides of the Swinnex units, in the region of the thread for screwing the two separate pieces of the unit together. The leaking would commence at varying times, normally 4 to 8 hours after the system had been set running, but occasionally it was not observed until the following day.

When this problem first occurred, biofilms perfused with iron-plentiful medium (CDM[2mM glucose]+Fe) were being grown, as opposed to the more usual iron-limited culture. Suspecting iron precipitation on the membrane to be blocking the filters, eluate samples were taken for siderophore assays and membrane washes pelleted and prepared for SDS-PAGE of outer membrane proteins to establish whether the biofilm cells actually had iron available to them. (See fig. 5.8. for OM profiles, illustrating the absence of IRMPs). However, even when using CDM-Fe, the units continued to leak at the sides, discounting any possibility of iron precipitation blocking the membrane pores. To confirm that no other constituents of the medium were causing a blockage, a 0.2μm filter was placed in-line with the medium. Despite its presence, the Swinnexes continued to show signs of leaking.

Brand new Swinnex units were used to investigate the possibility that continual autoclaving had distorted the units reducing the efficiency of the seal, but little improvement was seen. Other steps taken were to place the filter membranes in the units *after* autoclaving, to counteract any shrinkage of the cellulose which has been known to occur during sterilisation, wrapping PTFE tape around the thread of the Swinnex to tighten the seal and to use 0.45μm pore membranes. Nevertheless, leaking continued unabated. Thus, the leaking problem with these units could not be solved during the course of the thesis.

Fig. 3.14. Scanning electron micrograph of intact 20h *P. aeruginosa* biofilm using Electroscan environmental microscope. (20 kV, x5900 magnification).



It was noted also that, during the course of experimental runs, there was a visible build-up of polysaccharide material on the inner, Teflon-coated surfaces of the units, especially directly above the outlet. As a result, outflow of eluate was not instantaneous and *Pseudomonas* was colonising the Swinnex surface.

3.2.5. Colonisation of the Swinnex surface

To evaluate the extent of such colonisation, several units were run for 24h, the filter membrane biofilms subjected to the usual washing procedure and the Swinnex surfaces swabbed, washed with CDM salts and the resulting samples viable counted.

In some cases, the difference between cell numbers on the membrane and those from the sides was less than tenfold. The Teflon spray was, clearly, not preventing surface colonisation and may even have been contributing to the problem since, if the spray coating was not absolutely uniform, this may have created more sites for colonisation. Thereafter, units were no longer Teflon-coated, but were cleaned scrupulously after each use.

3.2.6. Further appraisal of the leakage problem

The combined problems of the doming of the membranes and leakage of liquid from the Swinnexes' sides suggested a large build-up of pressure within the system. This could have occurred as a result of either an increase of organisms and/or polysaccharide on the membrane. Inoculation of the Swinnexes with *E. coli* ATCC 8739 and the subsequent running of the system provided no such difficulties. Thus, clogging of the system was a problem inextricably linked with the use of *Pseudomonas aeruginosa* PAO1 JD.

Three different routes were considered appropriate at this stage:

1) to determine how much the leakage actually disrupted the system i.e. could it be tolerated?
2) to modify the design further in an attempt to prevent leakage
3) to reduce the initial inoculum to such a level where a biofilm was still formed, but the system was able to run for a sufficient length of time for experimental work to be carried out on samples before leakage began to occur.

3.2.6.1. Analysis of leakage from the system

Duplicate units were run overnight at a flow rate of 0.2 ml/min and both were found to be leaking the following morning. The amount of leakage was, very crudely, estimated through soaking up liquid visible at the sides of the units with a pre-weighed piece of tissue, then re-weighing the wetted tissue and relating weight gained to volume of liquid. The rate of leakage was found to worsen gradually over a 66h period.

Streaking a sample of liquid from the side of the unit on nutrient agar revealed that PAO1 cells were leaking out. To find if the flow of cells was bidirectional (i.e. cells could re-enter the system), the sides were inoculated with 100µl of a culture of *E. coli* (ca. $1.2x10^8$ cfus) at 22h. This Swinnex had originally received an inoculum of $1.2x10^9$ *P. aeruginosa* cells. By time 28h, the eluate viable count was $9.4x10^6$ cfus/ml, all being *Pseudomonas*, and at 48h there were $1.5x10^7$ cfus/ml, again all PAO1. A viable count of the leaking liquid at 48h showed there to be $8.5x10^7$ PAO1 cfus/ml and $1.85x10^7$ *E. coli* cfus/ml present.

The eluate viable count at 66h resulted in $5.2x10^6$ PAO1 cfus/ml and $6.5x10^5$ *E. coli* cfus/ml i.e. eight times more pseudomonads. In contrast, there were $7.55x10^8$ *E. coli*

cfus/ml sampled from the leak and only $6.5x10^7$ PAO1 cfus/ml i.e. just over tenfold more *E. coli*. Examination of the membrane by washing and scraping showed $2.2x10^9$ *P. aeruginosa* and $1.2x10^8$ *E. coli* present in the biofilm.

Quite clearly, then, *E. coli* was able to establish itself on the membrane after being inoculated on the outside of the Swinnex and, indeed, appeared to flourish in the medium which formed a pool around the thread areas of the Swinnex unit. The fact that cells could leave the apparatus by a route other than that which was intended in the design, demonstrated a major fault in the apparatus, one which would have to be remedied if valid and meaningful claims were to be made on the usefulness of the system for controlling the growth rate of microorganisms.

3.2.6.2. Sealing the filter membrane and O-ring

In an attempt to remedy the inefficient sealing of the system, the filter membranes were glued to the washers before placing them in the Swinnex. Filter membranes were glued to the washers using Unibond frame sealant (Unibond Ltd., Surrey), allowed to set for 24h, then used as normal in experimental runs. Repeat experiments, at various flow rates, showed that this procedure resolved the leakage problem but introduced another problem. The membranes fractured in the region where they were attached to the washers, normally within 10h, due to the pressure build-up within the system. The problem occurred with 0.45 and 0.2μm pore size membranes and was most apparent at flow rates over 0.25ml/min, although slower flow rates still exhibited membrane breakage, albeit after a longer time period had elapsed. This approach was therefore not pursued further.

3.2.6.3. Reduction of loading inoculum

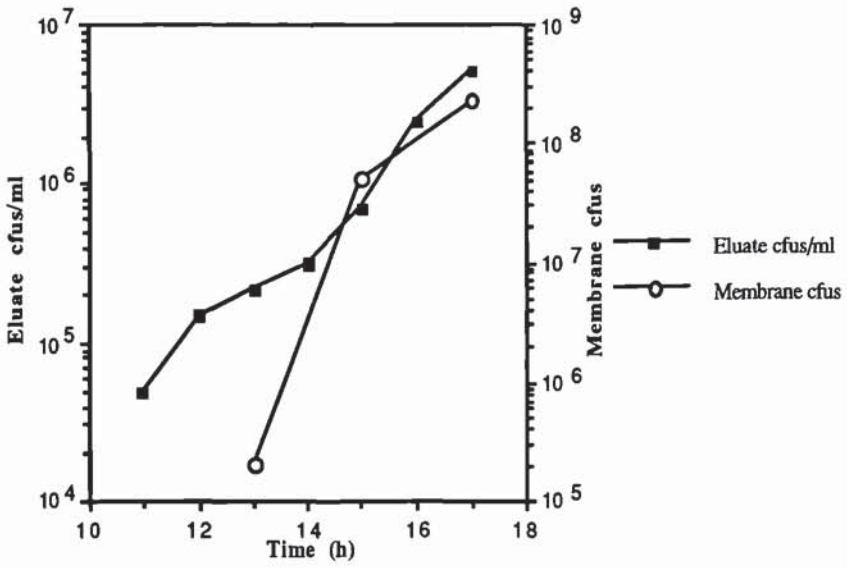
The loading inoculum was reduced to one tenth of the original amount, ca. $2.8x10^8$

cells now being inoculated onto the surface. This entailed diluting the cells in pre-warmed CDM first, to ensure the even distribution of cells on the membrane which would not have been achieved if inoculating only a few hundred microlitres of culture. This procedure was used in all subsequent experiments with the Swinnex system. As mentioned previously, it is envisaged that, in the growth-rate-controlled biofilm system, *E. coli* grows as a monolayer, shedding newly-formed cells into the eluate at a constant rate (Gilbert *et al*, 1989). With PAO1 apparently behaving in quite a different manner, it was necessary to establish the proportion of newly-formed cells remaining on the membrane, or within the biofilm, and the proportion which was being eluted with the medium.

3.2.6.3.1. Method

Five Swinnexes were prepared, each loaded with ca. $2.4x10^8$ cells and all perfused with CDM-Fe at a rate of 0.5ml/min. After running for 11h, perfusion continued and eluate viable cell counts were carried out on an hourly basis. Every two hours, one of the units was stopped and a viable count performed on the resuspended biofilm population. The experiment was brought to a premature conclusion after 17h, owing to the breakage of membranes, but a sufficient amount of data could be gleaned from the work to confirm the hypothesis that PAO1 was increasing its population on the membrane (fig. 3.15.).

Fig. 3.15. Increase in number of PAO1 JD cells being dispersed in the eluate and resuspended from the biofilms after reduction of initial loading volume of cells to ca. $2.4x10^8$. (Perfusion of CDM_{12}-Fe at a constant rate of 0.5ml/min).



3.2.7. Discussion of attempts to improve the Swinnex system

The results for three Swinnexes clearly demonstrate a steady increase in the number of cells being eluted with the eluate and a concomitant increase in the membrane bioburden over time. It is not entirely clear, but the membrane numbers appeared to be increasing at a slightly faster rate than that of the increase in eluate cell numbers.

Power and Marshall (1988) demonstrated that *Pseudomonas* JD8 cells adhering to a membrane were able to grow and divide, remaining attached to the surface, with daughter cells migrating across the surface after division. Subsequent detachment of cells from the surface was observed, probably as a result of changes in the substratum free energy. The results of this report are comparable to those of the present study in so much as they support the finding that this strain of *P. aeruginosa* is able to divide and remain on the membrane, unlike *E. coli* which sheds all newly formed cells into the eluate, as demonstrated by the "steady state" of eluate cell counts and the static biofilm bioburden assessed over time (Evans, 1990).

Further experiments, of similar design to that described in this section, confirmed the rapid increase in cell numbers shed into the eluate, but no concrete statements on the actual proportion of cells leaving the membrane could be made. The difficulties encountered with the breakage of the membranes could not be resolved, even with the use of a more pliable sealant (Dow-Corning 3145 RTV adhesive/sealant, Dow-Corning Corp., USA) to fix the O-rings and membranes together, and a further modification of the Swinnex base which allowed the membrane to lie flush on the base whilst the glued washer could fit around the base structure. This final modification involved sanding 1mm of the base periphery away and tapering the central segment (C on fig. 3.4.) at the edges, thus enabling the membrane and washer to lie flat over the base, as the basic Swinnex design intends, but providing an extra seal on the apparatus. It transpired, however, that this alteration was actually detrimental to the overall seal of the unit.

Dispensing with the procedure of sealing the membrane and washer, it was decided that the only modification in methodology which was of actual merit was that of reducing

the initial loading inoculum. Even this in practice was of limited application since, eventually, the biofilm on the filter would increase to such a density where pressure in the system forced medium and cells out of the Swinnex sides. Several experimental runs indicated that, generally, when the cell bioburden on the membrane was somewhere in the region of 10^8 cells, then leakage would begin to occur, depending on both the flow rate being used and the length of time the system had been running.

A fast flow rate generally resulted in leakage occurring more rapidly than in a slowly flowing system. The reasons for this could be twofold. Firstly, the greater pressure exerted by a fast-flow system could result in more rapid onset of leaking. Secondly, the fast flow rate could be allowing more rapid growth on the membrane, thus the "critical" amount of cells and extracellular polysaccharide material on the membrane, which results in liquid being forced out of the sides, is reached sooner. In contrast, a slow flow rate is less stressful on the mechanics of the unit, whilst it also limits the division rate of the biofilm cell population.

In some cases, it was found that the "critical" bioburden in slowly-growing systems was less than the amount for the fast systems. This may be explained by the possibility that, because these slow systems were taking longer to reach their own particular "critical" level, EPS production could have been playing a more important role in blocking the membrane.

It should be noted that this is merely conjecture, however, and the concept of the critical density of cells has not been investigated further.

3.2.8. Conclusions

Growing *P. aeruginosa* PAO1 in the Swinnex biofilm system appears to have only limited applications. Media flow rates must be extremely slow, ideally no faster than 0.1ml/min, the loading inoculum reduced to one tenth of the amount conveniently used

per mm^2 for *E. coli*, and the operating time severely shortened as a result of the eventual leakage problem. This last situation seems to be unavoidable as a result of PAO1's ability to grow, divide and remain on the membrane, whether it be actually adhering to the filter itself or attached to other cells and EPS.

A level of growth rate control can be exerted, particularly noticeable at slow flow rates, but accurate calculation of specific growth rate using the method described in section 3.1.2.3. is not possible. It is not known what proportion of newly divided cells remains on the membrane or within the biofilm, and consequently, the rate of release of cells into the eluate does not necessarily reflect the rate at which biofilm cells are replicating. Indeed, it is not clear what factors contribute to this phenomenon - whether cells are physically entrapped within the polysaccharide matrix and cannot drop off as the medium flows through, whether it is determined genetically that they will remain as part of the biofilm, or whether nutritional status and physicochemical factors play a major role.

With the increasing density of cells and EPS on the filter, blockage of the filter membrane inevitably occurs, along with a build-up of pressure within the system. As a result of this growth behaviour of PAO1, it is not feasible to control accurately μ and, as such, the method must be adjudged to have limited applications.

3.3. THE SORBAROD BIOFILM MODE OF GROWTH

3.3.1. Introduction

With the lack of applicability of the Swinnex biofilm system one final method of biofilm culture was devised. This involved the use of Sorbarod filters (Sorbarod Systems, Kent) which differ markedly from all previous filters employed in this project. The filters used are constructed of a paper cylindrical sleeve encasing a cellulose filling and are 10mm in diameter and 20mm in length (fig. 3.16.). Principally, they are known to

be used for plant cell culture. The main reason for using these filters was that the packed cellulose filling would provide a large surface area for bacterial adhesion and growth, resulting in a large quantity of dispersed and biofilm cells with which to work. Further, it was hoped that the system would not have such a tendency to block up, as did the 47mm and Swinnex biofilmers and, again, their ease of handling and small size would allow for several units to be run at the same time.

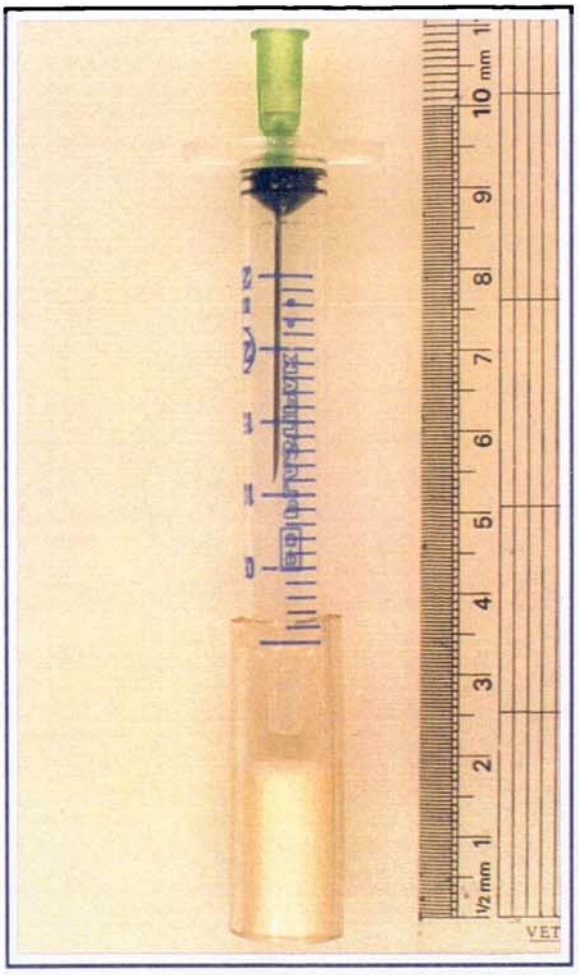
Quantification of these systems was a prerequisite to the proposed biofilm and dispersed cell analyses as it was of importance to assess the extent to which growth rate-control of cells was possible. With a relatively thick (20mm) depth of filter to perfuse, it was envisaged that there was a greater chance for heterogeneity to occur within the system. Nevertheless, it was hoped that control of the nutrient input rate would ultimately control growth rate of adherent bacteria to a useful extent.

3.3.2. Method

To prepare a Sorbarod filter for experimental work, it was placed within a piece of clear PVC tubing, 40mm in length, internal diameter of 10mm (Oakes Eddon, Liverpool), then autoclaved as normal. To inoculate with cells, a 2ml syringe with a central outlet was used, as this could fit snugly within the PVC tubing and ensure as even a spread of liquid as possible. The filter was pre-wet with warmed CDM salts (5ml) then inoculated with a predetermined quantity of cells.

A fresh sterile syringe replaced the first, with its plunger withdrawn to leave only the rubber bung (originally attached to the piston) within the main body of the syringe. Through this, a sterile needle could be inserted and this attached to the medium inlet tubing. Thus, the syringe served as a medium reservoir above the Sorbarod filter and medium could drip, at a controlled rate, onto the filter. This reduced any possibility of grow-back and subsequent contamination of the medium to a minimum. The medium was delivered to the unit through silicone tubing from a 10l storage vessel via a peristaltic pump (Watson-Marlow MHRE HR Flow Inducer). The Sorbarod unit was

Fig. 3.16. Photograph of basic Sorbarod biofilm apparatus.



held upright by a clamp and placed in a 37°C incubator for experimental runs.

3.3.3. Calibration of Sorbarods

In a similar fashion to the previous biofilm systems, quantification of cells released from the filter and those adhering to the fibres, with different flow rates, was necessary. Preliminary experiments demonstrated that the system could not withstand extremely fast flow rates, such as 5ml/min, for any great length of time, 20h being the maximum time achieved for the running of the system at such a rate. All experimental runs were carried out using mid-logarithmic phase *P. aeruginosa* PAO1 JD as inocula and CDM_{12} (iron-restricted) growth medium.

3.3.3.1. Quantification of adherent cells

As a consequence of the tubular construction of the Sorbarod, the removal of attached cells required a different approach from that used for the 47mm and 25mm diameter flat membranes. On completion of perfusion of the filter, excess liquid was allowed to drip through and then the Sorbarod was removed from its PVC housing. Using a sterile scalpel, the paper sleeve surrounding the matrix of filter fibres was cut open, then both fibres and sleeve were dropped into 10ml sterile CDM salts in a wide- bore glass test tube for vortex-washing. At the end of each 2min vortex-wash (with four being carried out in total) the diffused fibres were separated from the liquid by way of a plunger mechanism which compressed the fibres to the base of the tube allowing the liquid and resuspended cells to be poured off. This plunger was constructed from a stainless steel rod with a silicone disc attached at one end which pushed the fibres down whilst allowing liquid to pass between it and the sides of the boiling tube. On completion of this process, the 40ml of CDM and resuspended cells were pooled and used for viable counting. Direct light microscopic examination of the washed fibres revealed complete removal of the adherent cells from the Sorbarod fibres (see fig. 3.21.).

3.3.3.2. Elution of cells from the Sorbarod

Sorbarods were inoculated with ca. $1x10^9$ cells in the mid-logarithmic phase of growth and eluate viable counts made at various time intervals throughout separate runs at different flow rates. An example of the elution kinetics of cells on commencement of perfusion with medium is shown in fig. 3.17.

3.3.4. Results

On completion of a number of trial runs with the Sorbarod system, it was decided that calculation of the growth rate of the surface-associated cells by the method intended originally for the 47mm biofilmer (Gilbert *et al*, 1989) would not give an accurate value for μ. As with the Swinnex system, the number of cells in the eluate was clearly increasing after the initial washing off of loosely-associated cells, this being indicative of an increasing adherent population on the filter fibres. Therefore, it was determined to run multiple Sorbarods at different flow rates, sacrificing each unit at specified time intervals to give a measure of how both the eluate and biofilm cell numbers increased over the course of 48h.

Owing to time constraints, three media flow rates only were selected, these being 0.02ml/min, 0.5ml/min and 1.15ml/min and designated as "slow", "medium" and "fast", respectively. On each experimental run, four units were set up and sacrificed progressively after 10, 24, 34 and 48h. Fig. 3.18. illustrates, for each flow rate, the cell numbers collected in the eluate and from the corresponding resuspended biofilm populations.

Fig. 3.17. Numbers of iron-restricted PAO1 JD cells released from a Sorbarod biofilm unit, on commencement of perfusion with medium, during set (half hour) time periods. (Flow rate = 5ml/min).

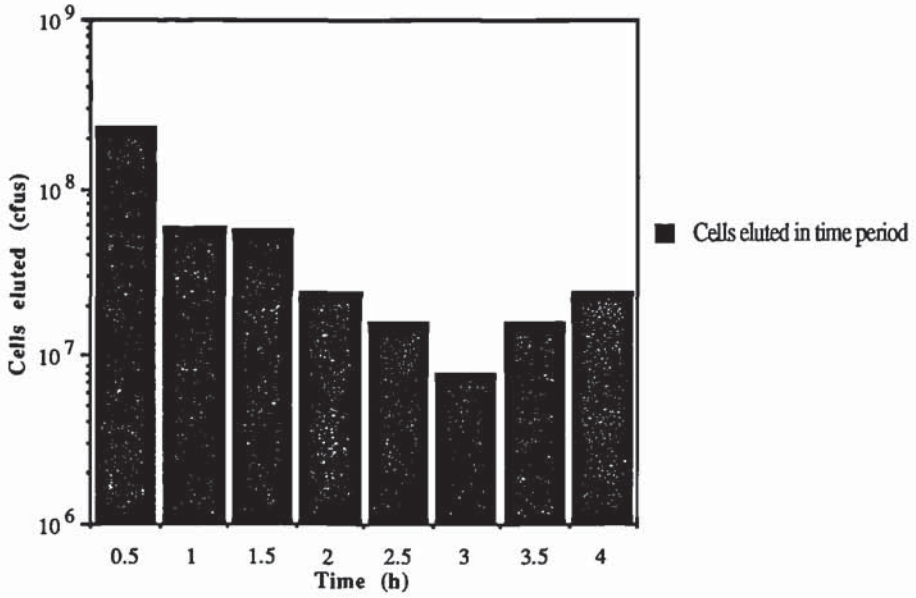
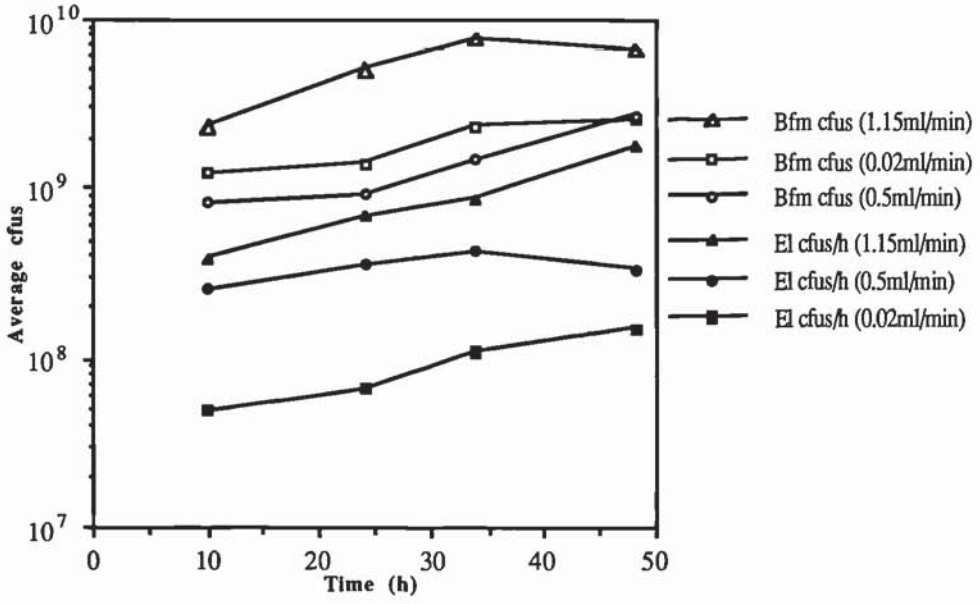


Fig. 3.18. Numbers of resuspended PAO1 JD iron-restricted biofilm cells (Bfm cfus) and eluate cells (El cfus/h) obtained from Sorbarod units monitored over 48h.



3.3.4.1. Method for calculation of population growth rate

It can be seen in fig. 3.18., that over 38h at least, increasing numbers of cells were obtained at the designated time points. Beyond 38h, however, the situation was less easy to interpret. Eluate cell numbers for the system run at a flow rate of 0.5ml/min no longer appeared to increase by 48h, and repeat experiments also followed this pattern. Similarly, the 48h biofilm population for the system run at the flow rate of 1.15ml/min was never seen to exceed the value found at 38h.

Thus, these seemingly unexpected end points could not be treated as mere anomalies and had to be taken into consideration when devising a method for growth rate calculation.

The simplest method of approaching the question of how to calculate the growth rate of each population would have been to draw lines of best fit through each set of points. It was appreciated, however, that the aforementioned low values for 0.5 and 1.15ml/min systems would obviously bias the fit of the lines through the respective sets of points.

3.3.4.1.1. Calculation of proportion of cells released from biofilm

For each set of flow rates, at each sample time, it was possible to calculate the total biomass present at that time by simply totalling the number of resuspended biofilm cells with the number of cells in the eluate. Hence, the actual proportion of cells being shed into the eluate/h relative to the total biomass could also be calculated (see table 3.2.). Since at the middle flow rate (0.5ml/min) a larger proportion of cells relative to the overall biomass was being released into the eluate, it seemed appropriate to ascertain for each flow rate the patterns of cell release from, or retention within, the biofilm populations.

Table 3.2.

Proportion of PAO1 cells shed from a Sorbarod-grown biofilm with regard to the total biomass (biofilm plus eluate cells) present.

Sample time (h)	Eluate as % of biomass at flow rates:- 0.02	0.5	1.15ml/min
10	3.73	23.89	14.32
24	4.46	28.27	12.04
34	4.43	22.03	10.16
48	5.54	13.13	21.23

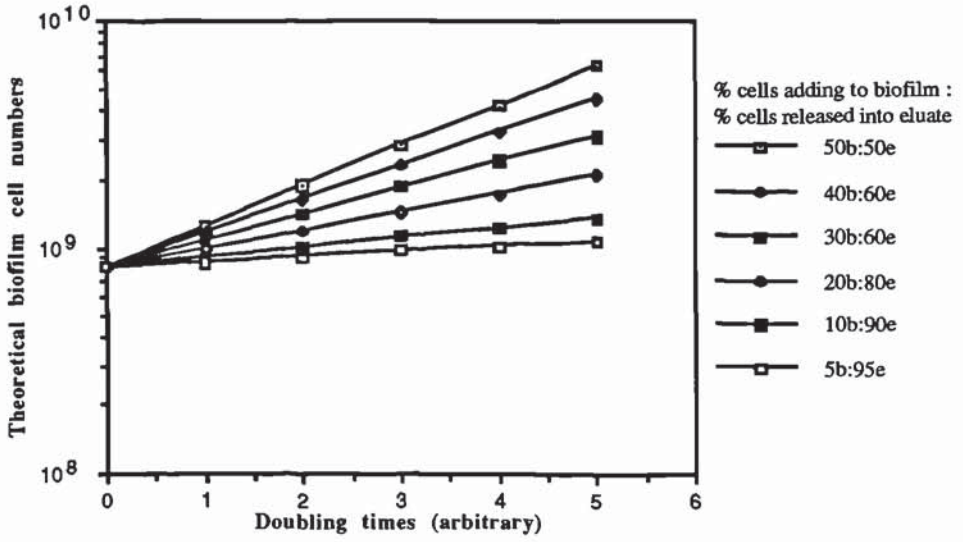
It was necessary to establish the productivity of the biofilm population and it was envisaged that the most suitable method for doing so was to calculate how many new-born cells were sticking to the biofilm in one hour and how many were being released in that same hour. The increase in biofilm population size per hour was calculated over several time periods e.g. from 10-24h, 24-34h and 34-48h for each flow rate. This eliminated the potential for averaging out the overall experimental run. However, it could also be argued that this approach interprets the results too literally and that there is bound to be variation between different Sorbarod units and the exact numbers of cells which they yield on sacrificing the units. This is discussed in section 3.3.5. The increase in the number of cells/h released in the eluate was found similarly for the corresponding time period (i.e. increase/h of eluate cells/h collected). Thus, addition of the values for the increase in biofilm cells/h and eluate cells/h collected plus the increase in eluate cells/h over that one hour time period gave a value for the number of cells being "born" in that particular hour i.e. cell production/h.

Next, a series of calculations was carried out to give data which charted the potential retention of cells within the Sorbarod and subsequent increase in biofilm size over a number of unspecified doubling times. In each data set, different ratios of retention to

release were calculated e.g. 60% retention:40% release; 70% retention:30% release etc. and the data plotted (fig. 3.19.). The starting value for the membrane population used was that which had been found experimentally at the 10h time point of each experimental run. These plots were merely theoretical patterns of biofilm increase, but actual cell numbers found experimentally could be compared to these plots and a good idea of the kinetics of biofilm growth and cell-shedding obtained.

A worked example for calculation of growth rates in the experiment run at the "medium" flow rate is given in the following section .

Fig. 3.19. Theoretical increases in biofilm population when cells produced during each doubling period incorporate into and disperse from the Sorbarod in various ratios.



3.3.4.1.2. Example: calculation of growth rate for flow rate of 0.5ml/min.

10-24h: Increase in biofilm population/h = 6.28×10^6cfus

Rate of release of cells at 10h = 2.58×10^8cfus/h

Incr. in eluate cells/h in 1 hour = 7.2×10^6cfus

Total cfu produced over 1 hour = 2.72×10^8cfus (i.e. from 10-11h)

Thus, time to reproduce the number of biofilm cells (population of 8.26×10^8 cfus) = 3h 2min, and $\mu = 0.228 h^{-1}$ at 10h.

Thus, if t_d = 3h 2min, then from 10h-24h theoretically constitutes 4.6 doubling times and supposed values of cell numbers after such a number of population doublings can be calculated. Using the theoretical plots (fig. 3.19.) it could be discovered which distribution pattern the cells actually followed. If they did not follow a pattern over a period of time then, over the piece, either the t_d did not remain at the calculated value or the proportion of cells being released changed. Having already calculated the proportion of cells being released (section 3.3.4.1.1.) it was therefore known which was the variable factor.

Thus, in 14h (4.6 doublings), a population of 8.26×10^8cfus should theoretically increase to 2.12×10^{10}cfus. In fact, the Sorbarod sacrificed after 14h yielded only 9.14×10^8cfus which suggests a shortfall of 95.7%, cells which presumably were eluted from the system. Following the plot for "5% retention: 95% release", the predicted value for the biofilm population after 4.6 doublings is 1.04×10^9 cfus, slightly more than were actually recovered. This might suggest that either less cells than were estimated to stick to the biofilm actually did so, or the population was not in fact doubling as quickly as proposed.

We know further that 2.72×10^8cfus were "born" in 1h and if 95.7% of these were

eluted, $2.6x10^8$cfus/h would have been collected. In fact, at 10h, the rate of elution of cells was $2.58x10^8$cfus/h. Therefore, it could be assumed that, allowing for a degree of variation between the Sorbarod units used to generate the data, the calculations for t_d and amounts of cells being shed from the system are acceptable. What is clear is that, at this point in the experiment, almost all of the biomass production was being shed into the eluate.

Studying fig. 3.18. at this flow rate (0.5ml/min) for the time period 24-34h, it is immediately noticeable that the rate at which the biofilm increased in size increased significantly, whilst the rate of cell release into the eluate reduced very slightly. Calculating in the same way as described above, the population t_d was found to be 2h 9min with a greater percentage of the newly-produced cells being retained within the Sorbarod. During the time period 34-48h, eluate cell production was found to decrease and the rate at which the biofilm was increasing in size decreased slightly, suggesting a reduced growth rate. Indeed, the calculated t_d was, at the very least, 3h at 34h and estimated to be over 5h by 48h.

In summary, as the experiment progressed, the total biomass of the system increased and the rate of shedding of cells into the eluate decreased quite significantly by 48h. By 34h, a larger proportion of newly produced biomass was being incorporated into the biofilm than at the beginning of the experiment, and the growth rate of the population began to decrease.

3.3.4.1.3. Flow rate of 0.02ml/min

The plots of increase in biofilm and cell numbers for the Sorbarods run at a flow rate of 0.02ml/min exhibit reasonably steady rates of increase, as well as the proportions of biofilm to released cells remaining constant throughout. At this slow flow rate, eluate cells accounted for only a small percentage of the total biomass in the system (see table 3.1.). The rate of production of new cells appeared to increase slightly during the

middle time period of the run then decreased to a level still greater than its initial level, and calculated values for t_d correspondingly increased then decreased. The actual values found for the t_ds, however, were 20h 41min, 8h 43min and 18h 29min for the time periods 10-24h, 24-34h and 34-48h respectively, showing far greater fluctuation in length of time than the plots of the data would suggest. Thus, the suitability for these plots to be calculated in this way must be called into question. Variability between Sorbarod units generating the data will have a bearing on the final outcome of the calculations and in this case in particular, it would seem to be more beneficial to draw a line of best fit through both data sets. Obviously, repetition of the experiment is required.

3.3.4.1.4. Flow rate of 1.15ml/min

Interestingly, at this fastest flow rate, eluate cells did not, on average, account for a higher percentage of the total biomass present than they did at the medium flow rate. The biofilm populations in this experimental run were substantially greater than those achieved at the lower flow rates, although there was no increase noted after the 34h sample. The rate of cell release into the eluate during the corresponding time period increased dramatically, however. Over the first 34h, between 60 and 70% of newly-produced cells were shed from the Sorbarod, but thereafter, an estimated 90% were released.

Actual values for t_d were computed as being 3h 50min and 5h 14min for the first and second time periods, respectively and estimated, for the 34-48h period, to be a doubling time similar to that of the 10-24h period.

3.3.5. Discussion of method and results

Obviously, this system is still in its infancy and many further calibration experiments are required in order to verify both the general trend of these results and the method used to

compute the growth rates and dispersion kinetics. As discussed later, (section 3.3.7.) a thorough study of the actual cells being dispersed from the biofilm is also required). However, it seemed clear that there was a definite effect of flow rate on the outcome of each experimental run. The flow rate which was originally expected to generate the fastest growth rates, hence shortest doubling times, (i.e. 1.15ml/min) in fact, resulted in longer doubling times over the first 34h than the intermediate flow rate, 0.5ml/min, although they were significantly shorter than those obtained for the 0.02ml/min system. This indicates, then, that within the range of experiments carried out, optimal conditions for population growth were produced at the flow rate of 0.5ml/min during the first 34h of operation at this rate.

Calculating growth rates as exactly as possible during specified time periods during the course of the experiment does have drawbacks, however. There will almost certainly be variation between Sorbarods and, consequently, deviation from a line of best fit through the data points may be more exaggerated than is the actual case i.e. one is attempting to model the increase in size of a biofilm population over time but, to do so, one has to use several "models" of that one biofilm since, to calculate the progress of the biofilm over time, a Sorbarod has to be sacrificed to recover and count the cells. Therefore, if the initial loadings varied or if there was unexpected variation in flow rates between individual units, then these may yield biofilms of differing population density. Further, the actual process of biofilm recovery with multiple vortex-washes could be another potential, albeit unavoidable, source of variation.

It would probably be appropriate to ignore minor deviations from a line of best fit through data points, but when data such as those for flow rates of 0.5 and 1.15ml/min are obtained, with major deviation from steady increases in cell numbers, then closer analysis of the contributory data is required.

3.3.6. Alternative method for calculating population growth rates

Growth rates for cell populations at each flow rate were calculated by drawing lines of best fit through each set of points, calculating biomass production over an arbitrary hour period, taking into account the cells which both dispersed from the membrane in that hour plus the amount by which the biofilm increased in the said hour. Thus, knowing how many cells were produced in one hour, the length of time that would be required to double the biofilm population, and hence the specific growth rate, could be calculated.

Using this method it was found, as in section 3.3.4., that the fastest flow rate did not correspond to the fastest growth rate. Values of μ are given in table 3.3.

Table 3.3.

Population doubling times and growth rates at different flow rates calculated using averaged data for change in population densities.

Flow rate (ml/min)	Doubling time	μ (h^{-1})
0.02	17h 23min	0.04
0.5	3h 22min	0.21
1.15	5h 24min	0.13

As described in section 3.3.5. (for the first 34h), it was found that the flow rate of 0.5ml/min resulted in the shortest t_d, thus fastest growth rate, for Sorbarod-grown biofilm populations. Further experimental runs would be required in order to pinpoint more closely μ_{max} for the Sorbarod biofilm system run under conditions of iron limitation. It is clear, however, that there is a substantial measure of growth rate control in this system.

Examining the data from a different standpoint, however, it could be argued that what is being observed is a batch-growth-type of behaviour for this system of cell culture. That is to say that there is an increase in cell numbers at a maximum rate for each particular flow rate, followed by a decline in the rate of cellular reproduction. Patently, there is no entry into stationary phase due to the continued through-put of fresh medium as well as the removal of potential cell-growth-inhibitors which would remain in a closed culture system.

3.3.7. Visualisation of Sorbarod-grown biofilms

Growth of PAO1 on individual Sorbarod fibres was visualised by light microscopy (Zeiss Axioskop 20, Zeiss, Germany). Aggregates of bacteria in the matrix of fibres could be seen clearly after 24h of perfusion of the filters with growth medium. Two methods of staining the samples were attempted; specimens were stained using either a dilute solution of methylene blue or the congo red method of Allison and Sutherland (1984) which was specific for staining extracellular carbohydrate material. Actual biofilms were best viewed without staining, however.

Examples of Sorbarod fibres on which *Pseudomonas* has been growing for 48h and fibres which have been subjected to the cell-removing washing procedure (section 3.3.3.1.) are illustrated in figs. 3.20. and 3.21., respectively.

Fig. 3.22. is a photograph of dispersed cells from a Sorbarod which has been run for 40h. As can be seen, the eluate consists mainly of single cells, but there appears also to be a number of doublets present . This suggests that it is not just newly-formed cells which are being eluted but both potential daughter cells from a division are dispersed in some instances. This, obviously, has great significance as far as accurate calculation of cell production in the system is concerned since the so-called "baby" cells are not the only ones likely to disperse from the biofilm. (The whole question of how to term the

newly-produced cells must be treated with caution here). It is not known, however, what the level of statistical significance is in the occurrence of this phenomenon and it would seem to be important that this matter is investigated further in the future.

Certainly, the release of cells of different ages into the eluate would result in any experiment attempting to grow a synchronously-dividing culture from an initial inoculum of eluate cells being unsuccessful. Owing to time constraints, this was attempted once only, the outcome being a culture growing and dividing as a batch culture. Obvious comparisons to the results of similar experiments using the 47mm and Swinnex biofilm devices can be made but since, on occasion, sychronously-dividing cultures could be obtained using Swinnex dispersed cells (section 3.2.2.2.), the possibility of success using the Sorbarod system cannot be discounted completely. The likelihood is, however, that if it *is* to be achieved at all using a system which has greater potential for heterogeneity within it (as a consequence of the depth of the filter), then the eluate sample to be used must be collected as soon as any loosely-attached cells have all been removed. Any further delay would merely increase the liability for recently-attached, but not newly-formed in relation to the age of instantly-eluted "baby cells", cells to be dislodged into the constantly-flowing perfusate.

Fig. 3.20. 48h biofilm of iron-restricted PAO1 JD on Sorbarod fibre, unstained. (Light microscopy, x400 magnification).

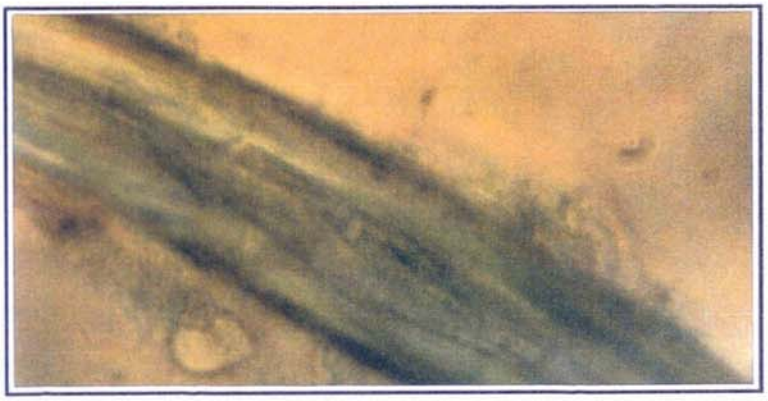


Fig. 3.21. Washed Sorbarod fibres. Cells removed by vortex-washing, 4x2min, in fresh 10ml CDM salts for each wash (light microscopy, x400 magnification).

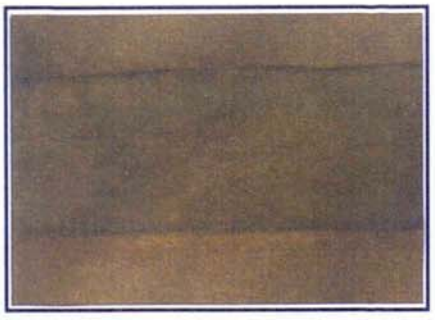
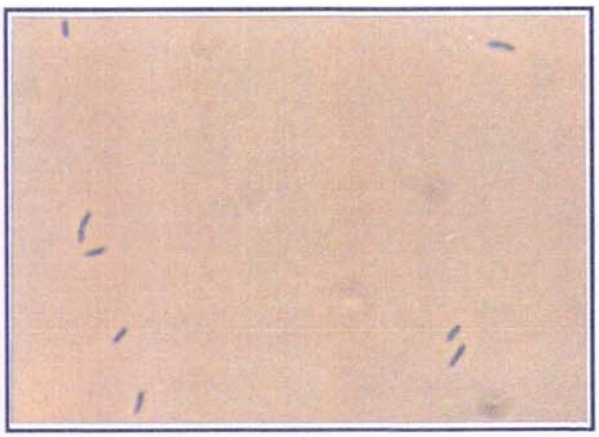


Fig. 3.22. PAO1 JD cells dispersed from a 40h Sorbarod biofilm, stained with dilute methylene blue (x 400 magnification).



4. SENSITIVITY OF PLANKTONICALLY AND BIOFILM-GROWN *Pseudomonas aeruginosa* PAO1 ID TO CIPROFLOXACIN

4.1. INTRODUCTION

Pseudomonas aeruginosa is often found as a normal inhabitant of the skin and intestinal tract of man and animals but infection usually occurs only when the defence mechanisms of the host are suppressed or compromised or when anatomical barriers have been breached. *P. aeruginosa* infections range from acute bacteraemia of very compromised patients to chronic non-toxaemic infections of CF patients. Its growth strategies differ in that it is essentially planktonic in the former instance whilst adopting a biofilm mode of large alginate-enclosed microcolonies in the latter case (Lam *et al*, 1980). Thus, several important factors must be taken into consideration during the design and implementation of antimicrobial testing. Along with careful general preparative procedures (Gilbert and Brown, 1991) an appreciation of the adaptive physiology of microorganisms (Brown *et al*, 1991), such as growth on solid surfaces and alterations in growth rate, is a prerequisite for more meaningful studies to be carried out than have previously been the case.

In the present study the susceptibility to the 4-quinolone drug, ciprofloxacin, of both planktonically- and biofilm-grown *P. aeruginosa* cells was assessed. Comparison between the results of the two methods of growth could be made by virtue of the level of growth rate control imposed on the systems. Thus, independent assessment of growth-rate effects and biofilm-growth effects was possible.

Bacterial resistance to the action of antimicrobial agents can occur as a result of several different factors (Williams, 1982). Microorganisms may be naturally resistant to a particular agent or they may acquire resistance. Resistance may be a determinant on the bacterial chromosome or be extra-chromosomal (i.e. on a plasmid). Mechanisms of acquisition of drug-resistance include selection of resistant clones, mutation of the

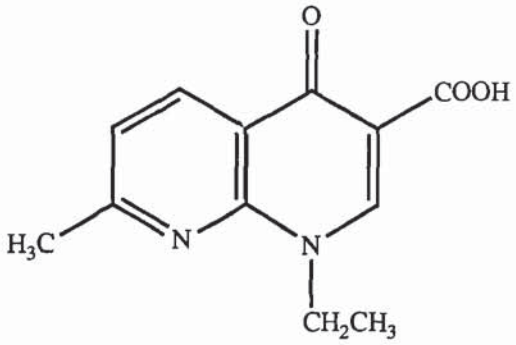
chromosome, bacteriophage transduction or via R-factors. Biochemical mechanisms of resistance include: production of destructive enzymes, physical barriers to penetration or altered enzyme specificity. A resistance mechanism fundamentally different from all other resistance mechanisms is that of tolerance, where bacteria evade only the killing action of the antibiotic (Tuomanen *et al*, 1986b). Both genetic and physiological factors are known which can selectively suppress susceptibility to the bactericidal action of antibiotics without involving a change in the MIC of the drug. The universal example of phenotypic tolerance, a property of virtually all strains of bacteria only apparent under particular growth conditions, is that of the non-growing dormant bacterium (Tuomanen, 1986).

4.1.1. The 4-quinolones

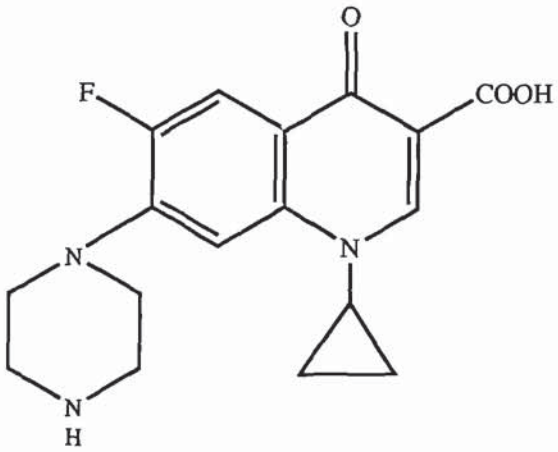
The 4-quinolones are unlike most agents of antimicrobial chemotherapy in that they are not naturally-occurring compounds but products of laboratory chemical syntheses. The earliest quinolone agents, nalidixic acid and oxolonic acid, were developed in the 1960s but their use was restricted to the management of uncomplicated lower urinary tract infections. This was a consequence of their poor absorption and tissue penetration, only moderate activity against a limited range of Gram-negative bacilli and comparatively high incidence of adverse reactions. In the 1970s further research resulted in the development of chemically-substituted 4-quinolones, such as cinoxacin and flumequine, which demonstrated improved pharmacokinetics, increased spectra of activity and much enhanced potency. The structural formulae of nalidixic acid and two of its chemical analogues are shown in fig. 4.1.

The two chemical modifications of the basic quinolone structure, which resulted in the production of newer compounds with improved biologic and toxicologic properties were the addition of a fluorine atom at position 6 of the molecule and the addition of a piperazine or similar ring structure at position 7. These modifications are common to all of the new agents (Wolfson and Hooper, 1989). Their enhanced spectrum of activity includes a wide variety of Gram-positive microorganisms, most common enterobacteria

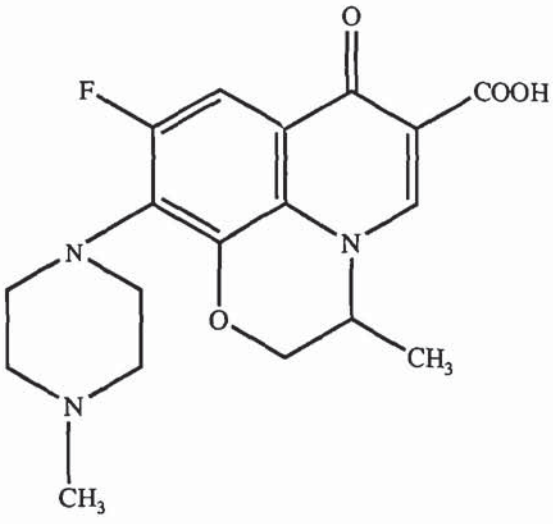
Fig. 4.1. Structural formulae of three quinolone antimicrobial agents.



Nalidixic acid



Ciprofloxacin



Ofloxacin

and opportunist pathogens such as *P. aeruginosa*, but not anaerobic organisms (Eliopoulos and Eliopoulos, 1989). With the possible exception of *P. aeruginosa* (Cullman *et al*, 1985; Fernandes *et al*, 1987), bacteria tend to be 100 to 1,000 times less likely to develop high-level resistance to the newer quinolones than to their older predecessors (Wolfson *et al*, 1987). Since they are novel synthetic molecules it was originally claimed that they were highly unlikely to become susceptible to the resistance mechanisms which currently compromise therapy with beta-lactam and aminoglycoside antibiotics. Thus, it was hoped that the emergence of highly-resistant mutants during therapy would be less likely to occur. However, selection of resistant strains is possible in the laboratory by serially passing bacteria in the presence of increasing drug concentrations (Barry and Jones, 1984; Hooper and Wolfson, 1988; Yates, 1992) and, as described later, resistance does appear to be an increasing problem.

4.1.1.1. Mode of action

The bactericidal action of 4-quinolones results from their ability to inhibit the action of an enzyme which manipulates DNA. Although the precise mechanism of their action has not yet been fully elucidated, it is known that the primary target is DNA gyrase (bacterial topoisomerase II), the only bacterial enzyme capable of introducing negative supercoils into DNA (Wang, 1974; Gellert *et al*, 1976.). DNA supercoiling plays an important role in bacterial metabolism in three ways: by compacting the chromosome, its involvement in the regulation of gene transcription (Fisher, 1984; Hulton *et al*, 1990) and its role in the response of a bacterium to its environment (Dorman *et al*, 1990).

DNA gyrase consists of four subunits, two A monomers (products of the *gyrA* gene) and two B monomers (products of the *gyrB* gene) (Higgins *et al*, 1978; Mizuuchi *et al*, 1984; Lewin, 1992). It is thought that the A subunits first introduce a nick into each strand of the double-stranded chromosomal DNA, the DNA is supercoiled by the B subunits in an ATP-dependent process, then the resultant negative supercoils are locked into the chromosome by the resealing activity of the A subunits (Gellert *et al*, 1977). Since these discoveries were made with DNA gyrase of *E. coli*, analogous gyrases have

been found in *Pseudomonas* and *Micrococcus* species (Miller and Scurlock, 1983; Liu and Wang, 1978).

Initial study on the effects of nalidixic acid on *E. coli* chromosome replication concluded that the agent caused the abnormal accumulation of single-stranded DNA precursors (Crumplin and Smith, 1976) which led to the understanding that the 4-quinolones, in some way, prevent the A subunit of DNA from finally resealing the staggered nicks already made in the DNA. Precisely how quinolones exert their effect on gyrase is still an issue of some controversy. One recent model suggested that gyrase could stimulate binding of quinolones to relaxed DNA in the presence of ATP, with interaction with gyrase via the C-7 group (Shen *et al*, 1989). An opposing school of thought found that norfloxacin bound to the gyrase-DNA complex but not to the protein or to the DNA alone (Willmott and Maxwell, 1993). These workers thus believe that both the enzyme and DNA are required for stable interaction with quinolones.

Mutations in both the *gyrA* and *gyrB* genes have been shown to confer quinolone resistance to intact bacteria (Inoue *et al*, 1978; Smith, 1984) although the majority of quinolone-resistant mutants have *gyrA* mutations. In the *gyrA* gene, quinolone-resistance is caused by a point mutation in a narrow region of amino acids, termed the quinolone resistance-determining region (Yoshida, 1990). In the *gyrB* gene, two quinolone resistance-determining sites have been identified (Yoshida *et al*, 1991).

Differences have been observed amongst the quinolones in the conditions required for their lethal action. Protein and RNA synthesis are essential for the bactericidal activity of the older quinolones e.g. nalidixic acid (Dietz *et al*, 1966; Smith, 1984). Ciprofloxacin and ofloxacin, by contrast, were shown to be also capable of killing *E. coli* cells in the absence of synthesis of protein or RNA (Smith, 1984). This suggests that these drugs have two bactericidal mechanisms of action: mechanism A (common to all 4-quinolones) which requires protein and RNA synthesis and cell division, and mechanism B which does not require these and allows killing of non-dividing bacteria. It is known that the *nalA* mutation in subunit A abolishes mechanism B (ciprofloxacin was unable to kill such a mutant in the absence of protein synthesis (Lewin *et al*,

1991)). Conversely, alterations in the B subunit or outer membrane proteins had no effect on mechanism B. Therefore, both mechanisms involve DNA gyrase subunit A. However, mutations in *gyrB* do cause resistance to quinolones, possibly by altering the binding of the B subunit to the A subunit (Cozzarelli, 1980). Whether the gyrase-DNA contacts are just with the A protein or with the B protein as well is not yet clear.

After the formation of the quinolone-gyrase-DNA complex an event must take place which initiates the process of cell death. (DNA degradation occurs in bacteria exposed to 4-quinolones without appearing to contribute to the lethal action, but may play a role in induction of the SOS response (Lewin and Smith, 1990)). It may have been supposed that killing is due to inhibition of supercoiling by gyrase, but two observations suggest not (Maxwell, 1992). Firstly, wild-type (nalidixic acid-sensitive) *gyrA* and *gyrB* genes are known to be dominant over their corresponding nalidixic acid-resistant alleles (Hane and Wood, 1969). Transformation of resistant *E. coli* strains with a plasmid carrying the wild-type *gyrA* or *gyrB* gene resulted in a sensitive phenotype (Nakamura *et al*, 1989). The second evidence stems from the unusual biphasic response (MIC paradox) exhibited by all 4-quinolones which have been tested, where these agents can be at concentrations that are both too low and too high for optimal killing to occur (Crumplin and Smith, 1975). The concentration of quinolone required to inhibit DNA supercoiling by gyrase exceeds, by a factor of 10-100, that needed to inhibit bacterial growth (Gellert *et al*, 1977; Zweerink and Edison, 1986). This is surprising as it would have been expected that the isolated target (gyrase) would be more, not less, sensitive than the intact cell. Since the relaxation and decatenation reactions of gyrase were found to be no more sensitive to drug-inhibition than was DNA supercoiling (Hallett and Maxwell, 1991), some other explanation other than inhibition of the enzymic reactions of gyrase is sought to explain quinolone-induced inhibition of bacterial growth. Speculation, for the moment, is focussing on the importance of the DNA-gyrase-quinolone ternary complex which may form a barrier to the passage of DNA and RNA polymerase molecules in the cell (Maxwell, 1992).

4.1.1.2. Quinolone uptake and resistance

Quinolones penetrate bacteria by diffusion through porins in the outer membrane of Gram-negatives (Bedard *et al,* 1989; Bryan and Bedard, 1991). The hydrophobic quinolones e.g. nalidixic acid, are also able to penetrate through the phospholipid bilayer. Active processes may play a role in the bacterial accumulation (Diver *et al,* 1990) since it was found to be dependent, partly, on cell metabolism. Previously, resistant *E. coli* strains were found to show decreased diffusion through the OM porin channels coupled to an energy-dependent drug efflux system in the cytoplasmic membrane (Hooper *et al*, 1989). Other factors reported to have a bearing on 4-quinolone uptake and activity include temperature and pH (Bedard *et al*, 1987; Diver *et al*, 1990), concentration of magnesium ions (Ratcliffe and Smith, 1983), elevated inoculum size (Eliopoulos and Eliopoulos, 1989) and anaerobic conditions (Morrisey *et al*, 1990; Hooper and Wolfson, 1991).

Plasmids often carry several genes conferring resistance to several antimicrobial agents, therefore, in clinically important bacteria they tend to be the main reservoir of resistance to most antimicrobial agents. This is not the case with the 4-quinolones as, unlike other commonly used drugs, plasmid-mediated resistance has yet to be identified in clinical isolates. However, one potential mechanism of resistance reported recently involves drug accumulation. *NorA* in *S. aureus* codes for a cytoplasmic membrane-associated 50kDa protein and results in high resistance to hydrophilic quinolones (Neyfakh *et al.* 1993). Transformation of a plasmid containing the cloned gene for this protein into a quinolone-sensitive strain of *E. coli* resulted in conferral of resistance (Ubukata *et al*, 1989). As mentioned previously, though, this type of plasmid-conferred resistance has not yet been found in a clinical isolate.

In most species investigated to date, high levels of resistance to all 4-quinolones seems to be conferred by mutations in the *gyrA* gene, but mutations in the *gyrB* gene also cause resistance in *E. coli* and *P. aeruginosa* (Lewin, 1992). *GyrA* mutations confer high-level cross-resistance to all 4-quinolones but do not appear to be associated with resistance to other, unrelated, antibacterial agents. *GyrB* genes, by contrast, do not

always cause cross-resistance to all 4-quinolones. Unlike alterations in DNA gyrase, however, resistance caused by reduction in quinolone accumulation can be associated with decreased sensitivity to unrelated antibacterial agents (Diver, 1989).

A resistance mechanism identified in PAO was found to be associated with reduced drug permeability (Fukuda *et al*, 1990). The *nfxC* mutation was associated with cross-resistance to imipenem and chloramphenicol but rendered bacteria highly susceptible to cephalosporins and to aminoglycosides. Clinical significance arises from the fact that quinolone-resistant *P. aeruginosa*, which are resistant to imipenem but susceptible to other beta-lactams, have been isolated from patients. Another mutation, the *nfxB* mutation, resulted in increased resistance to the newer quinolones and hypersensitivity to beta-lactams and aminoglycosides (Hirai *et al*, 1987) and has subsequently been proposed as having a role in regulating the expression of genes associated with cell permeability of drugs (Okazaki and Hirai, 1992). Aubert *et al* (1992) recently reported the emergence of ciprofloxacin-resistant *P. aeruginosa* variants after quinolone treatment of patients, one of which showed ciprofloxacin-imipenem cross-resistance despite the patient not having received imipenem. Thus, fluoroquinolone monotherapy in *P. aeruginosa* runs the potential risk of development of resistance not only to quinolones but also to other agents.

4.1.1.3. Clinical applications

The newer quinolones have demonstrated remarkable potency against many common bacterial pathogens as well as a number of less common but therapeutically troublesome microorganisms such as mycobacteria and *Legionella* species (Eliopoulos and Eliopoulos, 1989). With such potency, the use of these drugs in the treatment of *P. aeruginosa* infection of CF patients has been discussed widely in the literature and controversy still persists over what approach to take in containment and treatment of infection in these patients. A recent review of quinolone-treatment of CF patients (LeBel, 1991) concludes that ciprofloxacin is particularly effective in the treatment of mild and moderate infection of *P. aeruginosa* already resistant to beta-lactams and

aminoglycosides. However, the potential for resistance emerging still exists and treatment should be limited in time. The isolation of the mucoid form from the lung is an ominous portent for the future clinical well-being of the patient since this colonising strain is invariably never permanently eradicated (Govan and Glass, 1990). Even with the advent of ciprofloxacin as the first oral anti-pseudomonal agent, other therapeutic approaches are still required and, as with all antimicrobial agents, careful and vigilant administration of the drug will be required to ensure maximum effectiveness (LeBel, 1991).

4.2. METHODS

It has been established that the growth environment of bacterial cells, as well as their growth rate, have a major bearing on the outcome of challenging microorganisms with antimicrobial agents (Gilbert *et al*, 1990; Brown *et al*, 1990). Consequently, a number of studies were undertaken during the course of this project to examine the effects of different methods of cell culture on the survival of PAO1 JD when exposed to ciprofloxacin, a quinolone used frequently in the treatment of *P. aeruginosa* infections (Wolfson and Hooper, 1989; Maiche, 1991).

4.2.1. Antimicrobial bactericidal assays

Bactericidal assays using ciprofloxacin were carried out on a number of differently cultured samples of PAO1 JD according to the method described in section 2.12.1. Preliminary experiments had established that a concentration of 0.3µg/ml gave 40% kill and 1.0µg/ml was known to be the minimum inhibitory concentration of ciprofloxacin for growth of PAO1 JD. The sources of the challenged organisms are listed below, along with the concentrations of ciprofloxacin (Bayer, UK) used.

1) Batch-grown iron-restricted and iron-replete/ carbon-limited cultures (0.3 and 1.0 µg/ml ciprofloxacin),

2) chemostat-grown iron-restricted and iron-replete/ carbon limited cultures at different growth rates (0.3 and 1.0μg/ml ciprofloxacin), and
3) iron-restricted, Sorbarod-grown eluate cells and their resuspended surface-grown counterparts at different growth rates (1.0μg/ml ciprofloxacin).

All samples were diluted to a theoretical OD_{470} of 0.005 (equivalent to ca. $5x10^6$ cfu/ml) using appropriate volumes of pre-warmed CDM salts solution. This was necessitated by the fact that the Sorbarod eluate samples contained low concentrations of cells and direct comparison of such samples with undiluted samples obtained from the other methods of culture would not be valid.

4.2.2. Perfusion of intact PAO1 JD biofilms with ciprofloxacin

As described in section 3.3., the Swinnex biofilm mode of growth of PAO1 JD had limited applications. However, the technique was believed to be of use when the biofilm population size was below a critical density level. Thus, it was reasoned that if perfused with medium containing an antimicrobial agent, the cell numbers on the filter may be reduced and the possible effects of this perfusion could be studied by following any change in cell number in the eluate. Consequently, for a short period at least, experimental work could be carried out using the Swinnex system, with the build-up of microorganisms on the membrane possibly being delayed by exposure to the particular agent being used.

Thus, intact iron-restricted biofilms were perfused *in situ* with ciprofloxacin at a concentration of 1.0μg/ml in order to investigate the susceptibility of surface-associated cells without disrupting the organisation of the biofilms. Initial experimentation followed the effects of a single exposure to the antimicrobial agent, and this was followed up by investigating the outcome of two successive exposures.

Duplicate Swinnexes (0.2μm pore membrane, single non-glued washer) were loaded with ca. 8.0 x 10^8 PAO1 JD cells and perfused with CDM_{12}-Fe at a rate of 0.5ml/min. After 4h, one unit was perfused with medium containing 1.0μg/ml ciprofloxacin for 90 min, followed by continued perfusion with drug-free medium until 24h in total had elapsed. Samples of eluate for viable and total counting were taken over the course of the experiment. It was noted, however, that the total count values were rendered inaccurate since filamentation of cells resulted in difficulty in distinguishing one filament from another. The expansion and filamentation of bacteria, whilst they are dying, during exposure to quinolones has been reported previously (Smith, 1984; Diver and Wise, 1986; Nishino *et al*, 1989). It is for this reason that only viable counting of treated cells can be used to follow the bactericidal effects of these drugs since light-scattering or optical density measurements would be affected by changes in cell size and shape.

In the second set of experiments, duplicate Swinnexes were set up in a similar fashion, but loaded this time with ca. 1.8x10^8 PAO1 JD cells and perfused with CDM_{12}-Fe at a rate of 0.2ml/min. After 4h, one unit was perfused with medium containing 1.0μg/ml ciprofloxacin. Following 90min of exposure to the drug, perfusion with normal CDM_{12}-Fe resumed until a total of 21.5h had passed, when the Swinnex was once again perfused for 90min with drug-containing medium followed by drug-free CDM until the experiment was terminated after 30h. Samples of eluate for viable counting were taken throughout.

4.3. RESULTS

4.3.1. Bactericidal assays on PAO1 grown in batch culture

The results for survival of PAO1 JD cells harvested at different stages of batch growth in iron-restricted and iron-replete media are illustrated in figs. 4.2. & 4.5. and 4.3. & 4.6., respectively. Iron-restricted cultures were more resistant to the action of the drug than were iron-replete cells. The former displayed increasing levels of survival in the

presence of 1.0μg/ml ciprofloxacin as growth rate gradually decreased and survived the presence of 0.3μg/ml ciprofloxacin throughout growth, increasing to 100% survival in deceleration phase. Stationary phase cells survived to levels of 74% and 85% for 1.0 and 0.3μg/ml drug-treated cells, respectively. In contrast, iron-replete cells survived incubation with 1.0μg/ml of drug poorly, although the lower concentration of drug resulted in survival to a maximum level of 44% during cell growth. It is important to point out here that the results for iron-replete cultures would be very much influenced by the fact that such batch-grown cultures had a tendency to clump in the flask. Clearly, the erratic nature of the results seen especially in fig. 4.3. has been a consequence of aggregates of cells being exposed to the antimicrobial agent on some occasions, these most probably being afforded a certain degree of protection from the drug and resulting in higher levels of survival. These experiments were repeated on four occasions, each time with similar results.

4.3.2. Bactericidal assays on PAO1 grown in continuous culture

Changes in sensitivity towards ciprofloxacin (at concentrations of 0.3 and 1.0μg/ml) with specific growth rate of iron-restricted and iron-replete chemostat cells are shown in figs. 4.6. and 4.7., respectively. As with batch culture, iron-restricted cells appear to be more resistant to the action of the drug. Clearly, both sets of chemostat populations were significantly more resistant to ciprofloxacin, at a concentration of 0.3μg/ml, at slow growth rates, with a marked increase in susceptibility as the dilution rate was increased. At the higher drug concentration, however, even at relatively slow dilution rates, iron-replete cells were highly sensitive to the drug whereas the iron-restricted cells demonstrated over 20% survival at D rates below $0.05h^{-1}$.

Fig. 4.2. Survival of PAO1 JD, throughout batch culture in CDM_{12}-Fe, after incubation with ciprofloxacin (0.3µg/ml, 1h).

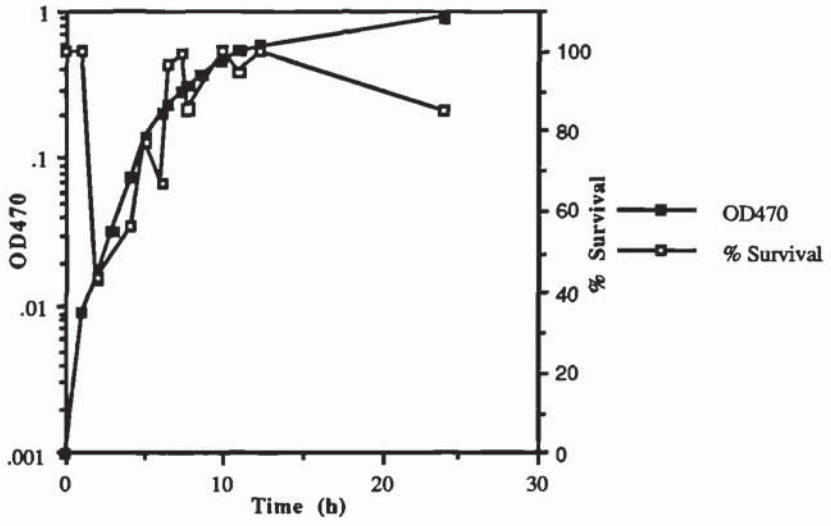


Fig. 4.3. Survival of PAO1 JD, throughout batch culture in carbon-limited CDM+Fe, after incubation with ciprofloxacin (0.3µg/ml, 1h).

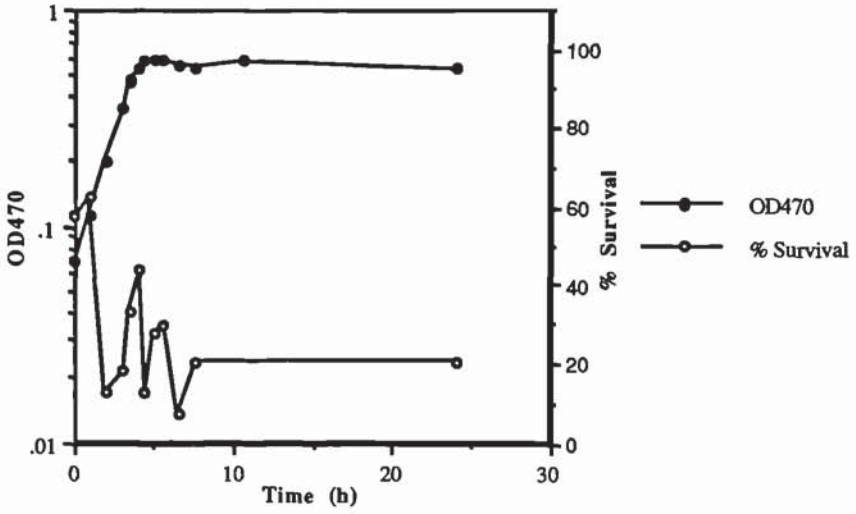


Fig. 4.4. Survival of PAO1 JD, throughout batch culture in CDM_{12}-Fe, after incubation with ciprofloxacin (1.0μg/ml, 1h).

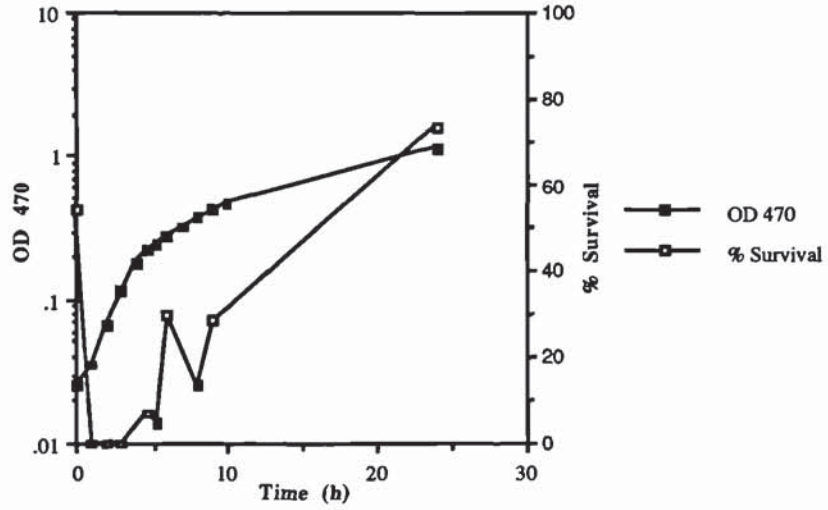


Fig. 4.5. Survival of PAO1 JD, throughout batch culture in carbon-limited CDM+Fe, after incubation with ciprofloxacin (1.0μg/ml, 1h).

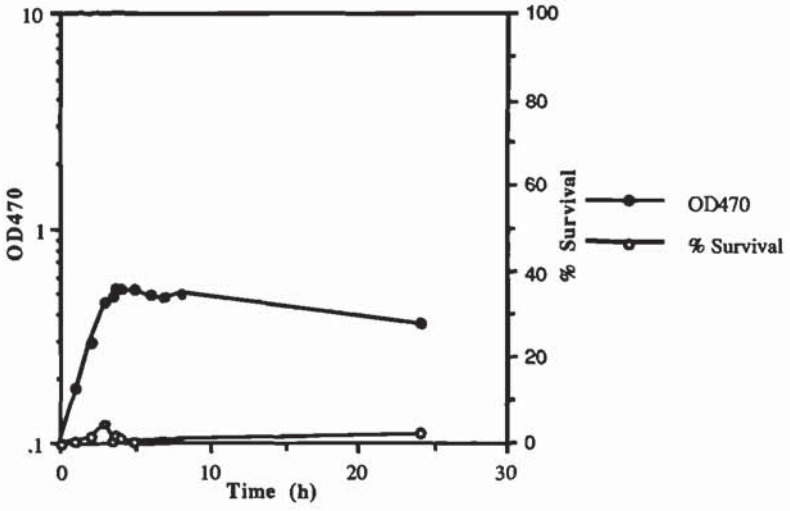


Fig. 4.6. Effect of growth rate of iron-restricted and iron-replete PAO1 chemostat cells on survival after incubation with 0.3µg/ml ciprofloxacin for 1h.

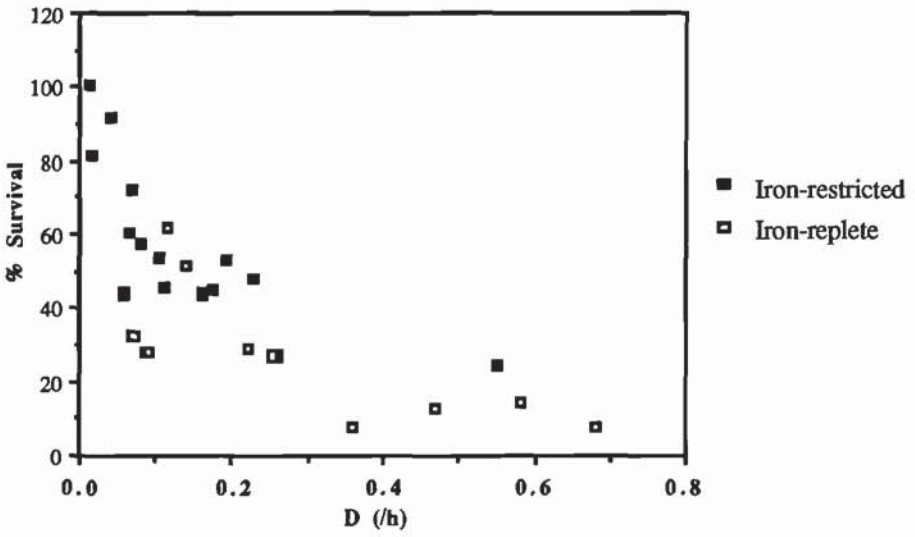
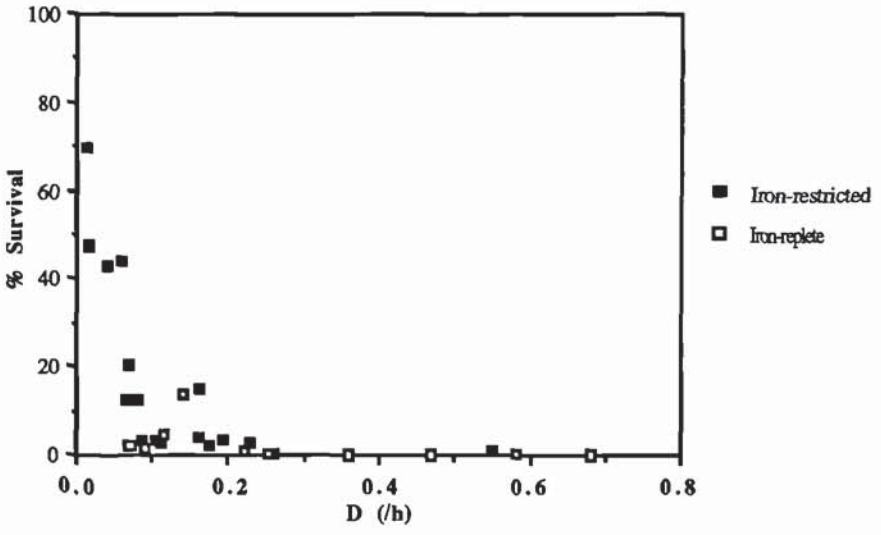


Fig. 4.7. Effect of growth rate of iron-restricted and iron-replete PAO1 chemostat cells on survival after incubation with 1.0µg/ml ciprofloxacin for 1h.

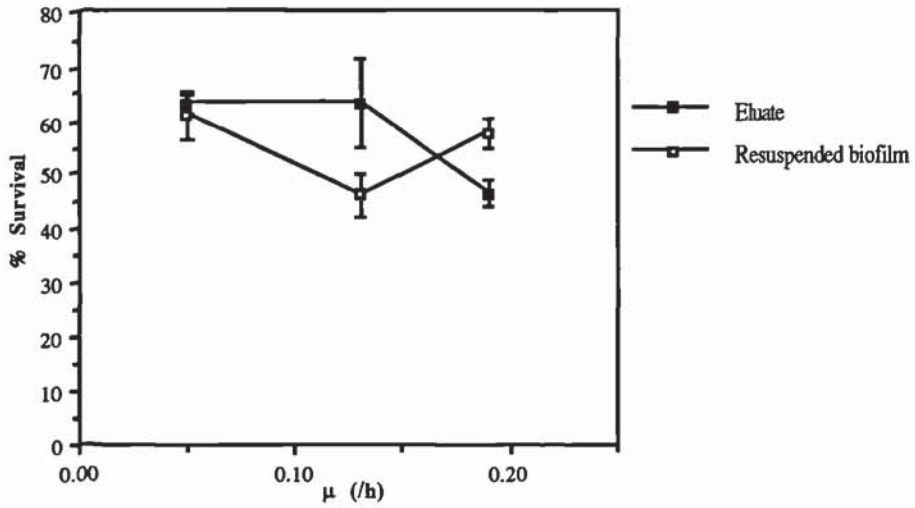


4.3.3. Bactericidal assays on Sorbarod-grown PAO1 JD cells

The results for the survival of PAO1 JD eluate and resuspended biofilm cells on incubation with 0.3μg/ml ciprofloxacin are presented in fig. 4.8. The samples used in the assays were taken from Sorbarod units which had been running for 48h, thus values for μ are taken from the calculations in section 3.3.4.1. as being 0.05, 0.13 and 0.19h^{-1}. The range of growth rates was not particularly wide and the effect of growth rate upon survival was not at all pronounced. Eluate cell survival decreased by almost 20% from the slowest to the fastest growth rates whilst resuspended biofilm cell survival was at its lowest value for the middle growth rate.

Obviously, more samples would be required in order to discern if some sort of pattern of survival was emerging. However, survival levels of eluate and resuspended biofilm cells did not differ to a great extent.

Fig. 4.8. Survival of Sorbarod-grown, iron-restricted PAO1 JD eluate and resuspended biofilm cells after incubation with ciprofloxacin (0.3µg/ml, 1h).



4.3.4. Survival of ciprofloxacin-perfused Swinnex-grown PAO1 JD cells

Fig. 4.9. illustrates the effects on viable eluate cell numbers of a single perfusion of a PAO1 JD biofilm with 1.0μg/ml ciprofloxacin. There was a dramatic decrease in viable cell release from the biofilm within one hour of commencement of exposure to the drug, with eluate viable counts remaining low for several hours after drug-perfusion had ceased. Study of samples of the eluate by light microscopy revealed that cells were elongated, with no apparent division of newly-formed cells from their parents. By 24h, however, the number of viable cells in the eluate had increased to pre-exposure levels. (The specific growth rate of the biofilm was estimated as being $0.3h^{-1}$).

The effects of double exposure to ciprofloxacin on eluate viable cell numbers are presented in fig. 4.10. It can be seen that, on perfusion with ciprofloxacin, there was a rapid decrease in viable cells being eluted from the biofilm which continued for several hours after exposure to the drug had ceased. Thereafter, recovery was swift, especially after the second perfusion where the number of cells shed into the eluate more than doubled every hour.

Fig. 4.9. Effect of exposure of intact, iron-restricted PAO1 JD biofilm to ciprofloxacin (1.0μg/ml, 90min) on numbers of viable cells present in the eluate. The arrows indicate commencement (a) then cessation (b) of exposure to ciprofloxacin.

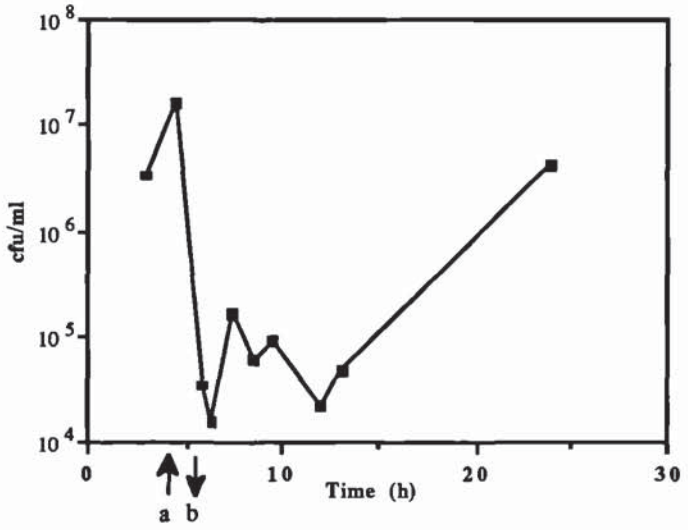
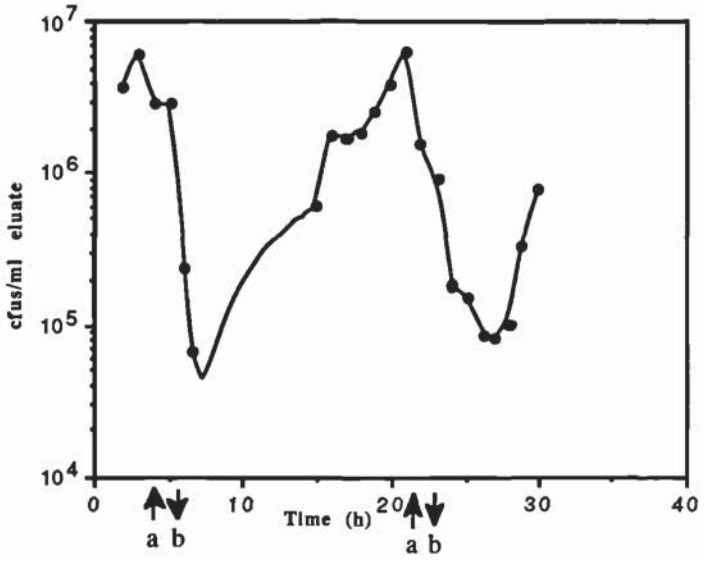


Fig. 4.10. Effect of 2 x 90min exposures of intact, iron-restricted PAO1 JD biofilm to ciprofloxacin (1.0μg/ml) on numbers of viable cells present in the eluate. The arrows indicate commencement (a) then cessation (b) of exposure to ciprofloxacin.



4.3. DISCUSSION

The antimicrobial susceptibility studies of batch culture cells, although without the benefit of growth rate control, were included to lay the foundations for subsequent experimental work on cultures which would yield more meaningful data. What was clear from batch culture work was that iron-restricted cultures were less susceptible to ciprofloxacin than were iron-plentiful cells and sensitivity varied with growth phase. A problem noted frequently with batch culture of PAO1 JD in iron-plentiful medium however, was that of the tendency for aggregates of cells to develop in the flasks, despite being shaken vigorously during incubation. No reasonable explanation can be given for this other than to speculate that the iron-plentiful cells have altered cell envelope properties enhancing stickiness. Other workers in this laboratory have also encountered the clumping in shake-flask culture and, as yet, the problem has not been circumvented.

Chemostat populations demonstrated growth-rate-dependency of antibiotic sensitivity, both in iron-deficient and iron-replete conditions. As with batch-grown populations, iron-restricted cells showed higher levels of survival after exposure to both drug concentrations, especially at slow growth rates. The growth-rate-dependence of antibiotic susceptibility for chemostat cells has been reported widely in the literature, for various antimicrobial agents on a range of microorganisms under different nutrient limitations (Finch and Brown, 1975; Dean *et al*, 1976; Tuomanen, 1986a; Wright and Gilbert, 1987; Wu and Livermore 1990; Evans *et al*, 1990b; Chen *et al*, 1991; Duguid *et al*, 1992a,b).

The results of the bactericidal assays on eluted and resuspended Sorbarod-grown cells were unexpected in as far as the eluate cells were concerned. There is much less difference between the sensitivity of eluted cells and resuspended biofilm cells than might have been expected from results previously obtained in a similar study of two different strains of *P. aeruginosa* grown using the 47mm biofilmer system (Evans *et al*, 1991a). In that instance, eluate cells from both mucoid and non-mucoid biofilm populations displayed much greater sensitivity to ciprofloxacin (0.5mg/ml) in

comparison to their resuspended biofilm counterparts, although the difference between the two types of populations lessened as growth rate increased. In the present study over the range of growth rates used, survival of the Sorbarod-cultured cells was similar to that of iron-restricted planktonic (chemostat) cells. Obviously, it would be necessary to study the effects of the use of higher concentrations of the quinolone, over a wider range of growth rates, for more significance to be given to the experiment. Further, work on the sensitivity of intact biofilms would help ascertain whether the actual structural integrity of the biofilm affords yet higher survival of cells. Hoyle *et al* (1992b) reported that the quasi-organisation of *P. aeruginosa* biofilms is important in resistance to the aminoglycoside tobramycin. Similarly, the ciprofloxacin-susceptibility of intact *P. aeruginosa* biofilms was significantly less than that for resuspended cells (Evans *et al*, 1991a), a finding particularly obvious for the mucoid strain. In contrast, no such differences were observed for intact and resuspended *E. coli* biofilm populations. The authors suggested that, for the particular *E. coli* strain used (rough LPS phenotype), the presence of the glycocalyx was of reduced significance.

A recent report of similar antimicrobial sensitivity testing of chemostat- and biofilm-derived *S. epidermidis* cells demonstrated further the differences between microorganisms used (Duguid *et al*, 1992a). Newly-formed daughter cells in the eluate were extremely tolerant towards ciprofloxacin whilst resuspended and intact biofilm cells displayed increasing sensitivity as growth rate increased. Interestingly, planktonic (chemostat) cells were less sensitive than were biofilm cells growing at the same rate.

The outcome of using a different antimicrobial agent was markedly altered, illustrated by the result of exposing *S. epidermidis* biofilms to tobramycin (Duguid *et al*, 1992b). In comparison to the results obtained from ciprofloxacin exposure, the sensitivity of eluate cells was drastically increased and there was a marked difference in survival of intact, as opposed to resuspended, biofilm cells. Whereas eluate cell ciprofloxacin-survival did not appear to be growth-rate-dependent, growth rate did have a bearing on all biofilm- and chemostat-derived cell tobramycin-survival. The glycocalyx played a role in enhancement of resistance to tobramycin, as resuspended cells were found to be more sensitive to this agent, whereas little difference existed in the survival rates

between these two populations on exposure to ciprofloxacin, a result comparable with that obtained for *E. coli* (Evans *et al*, 1991a).

In the present study, the use of 48h-old cells meant that cells were possibly growing more slowly than they had previously been doing (see section 3.3.5.), especially in the cases of the faster flow rates. It would be beneficial, instead, to carry out similar studies using Sorbarod units which had been set up for shorter periods of time, but long enough for sufficient numbers of cells to adhere. By this method, a wider range of growth-rate effects could be investigated.

For the Swinnex-grown biofilms, the observed recovery of dispersed cells to pre-drug exposure levels could suggest there was little reduction in viability of the biofilm population. One could speculate further that there had been no reduction in viability at all, merely a decrease in cell reproduction, and/or the newly-formed cells were sensitive to the ciprofloxacin. However, it is not possible assuredly to state as such, since it is known that a proportion of PAO1 cells remain on the filter surface, or within the biofilm, after cell division and, therefore, the increase in viable eluate cells may merely be reflecting the growth of the biofilm population which survived drug perfusion. That is to say, many of the biofilm cells could have been killed but, on cessation of drug treatment, those recalcitrant to the agent began to grow and divide once more, thus building up the biofilm population and, consequently, increasing the number of cells which could disperse progeny, or themselves be dispersed, into the eluate. Alternatively, filamentation of biofilm cells without cell division could mean that less cells were being released from the biofilm, thus the number of cells in the eluate decreased. The observation of elongated cells in the eluate during, and for several hours after, ciprofloxacin exposure could be demonstrating that it is not just newly-formed daughter cells which disperse from the biofilm, but also their "parent" cells. Also the colonial morphology on nutrient agar of cells collected from the eluate for viable counting was noted to change for samples plated during, and for a short period after, ciprofloxacin perfusion. These colonies tended to be smaller than normal and have been found previously by other workers in this laboratory (Langford *et al*, 1989; Yates, 1992).

Under similar experimental conditions (Gander *et al*, 1992), *E. coli* biofilms showed little or no reduction in viability of biofilm cells (since the biofilm is known not to increase over time, grown in this system) with eluate cell numbers recovering to their pre-drug exposure level. In contrast, perfusion with ciprofloxacin of *Staphylococcus epidermidis* biofilms, grown using the 47mm biofilm system, resulted in a permanent reduction in viable eluate cell numbers even after drug-perfusion had ceased (Duguid *et al*, 1992a). This reflected bactericidal or bacteriostatic effects on the parent biofilm since the eluate cells were known to be highly resistant to the drug, as described previously. The results of tobramycin-perfusion differed once again (Duguid *et al*, 1992b), with eluate cell numbers recovering to levels approaching those obtained pre-drug-exposure. Simultaneous tobramycin and ciprofloxacin perfusion resulted in a large decrease in viable eluate cells, but recovery to original levels was achieved rapidly post-drug-exposure. This suggested the growth-inhibitory effects of tobramycin on the biofilm population afforded the cells protection from the bactericidal activity of the quinolone. Ciprofloxacin has been shown to remain bactericidal to non-dividing Gram-negative bacteria (Smith, 1984) by virtue of the so-called "mechanism B" mode of action (section 4.1.1.1.) but this is not the case with Gram-positive microorganisms.

In the CF patient, as the number of antipseudomonal treatment courses increases, the temporary eradication rate falls gradually and, once established firmly in the CF lung, mucoid *P. aeruginosa* cannot be eradicated permanently (Høiby and Koch, 1990). There is much debate as to what the best form of management should be. For instance, the Danish CF Centre in Copenhagen advocates early and aggressive therapy with colistin, a polymyxin antibiotic, and ciprofloxacin as soon as *P. aeruginosa* is isolated (Pedersen *et al*, 1992). *In vitro* work studying exposure of *P. aeruginosa* grown on tiles suspended in chemostats, although essentially lacking growth rate control does demonstrate the importance of early exposure of biofilms to antimicrobial agents (Anwar *et al*, 1989a,b; Anwar and Costerton, 1990; Anwar *et al*, 1992a,b). Resistance of biofilms increased with age, although relatively young biofilms could still be eradicated by a combination of tobramycin and piperacillin treatment. However, the problem of how to combat effectively well-established bacterial biofilms still remains.

5. SURFACE COMPOSITION AND RELATED PROPERTIES OF PLANKTONIC AND BIOFILM CELLS

5.1. ANALYSES OF OUTER MEMBRANES OF PLANKTONICALLY- AND SURFACE-GROWN PAO1 JD POPULATIONS

5.1.1. Introduction

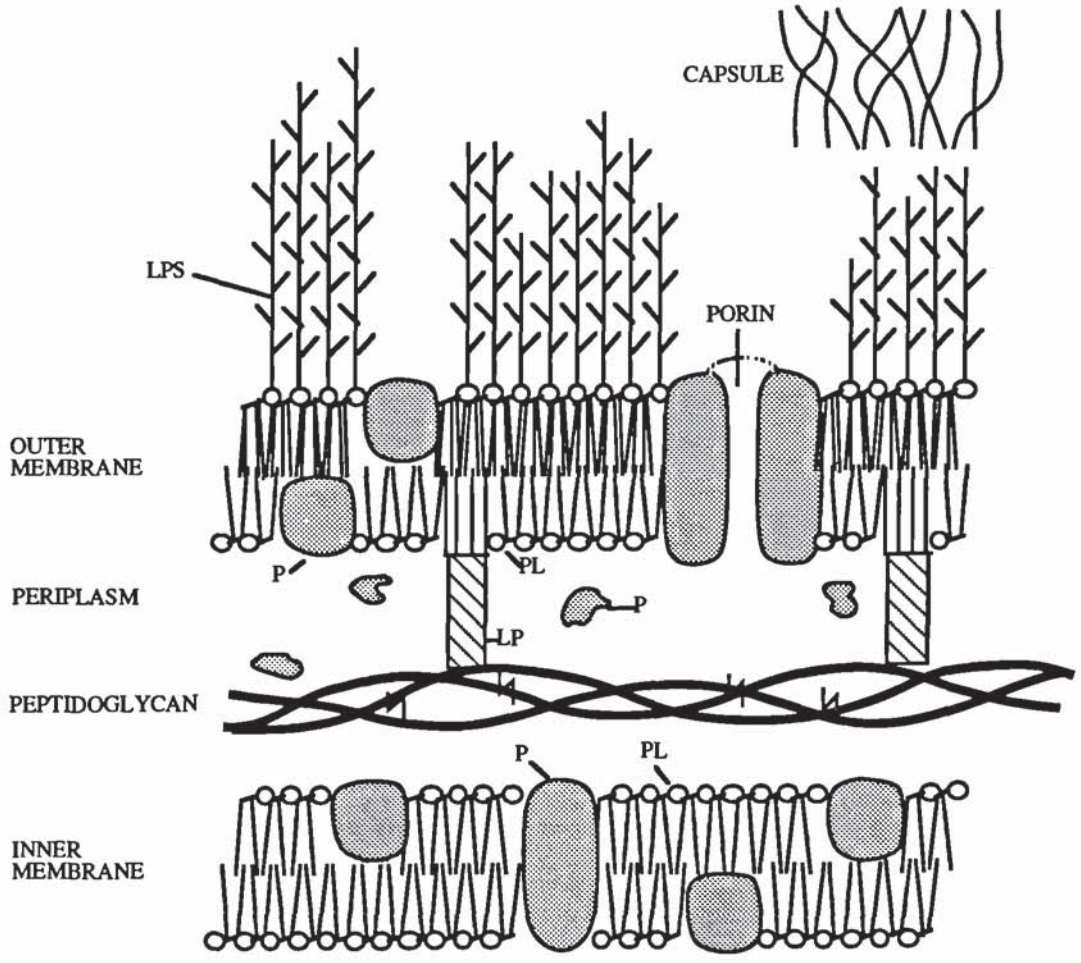
The cell envelope is the structure through which a bacterium reacts with its environment and it is the remarkable plasticity of this structure which confers a major survival advantage upon a cell in a changing environment (Costerton *et al*, 1979). The bacterial cell has an innate ability to adapt its physiology to specific nutrient limitations and environmental conditions favouring, for example, faster or slower growth rates or growth on a surface as opposed to planktonic growth (Brown and Williams, 1985b) and the envelope is the prime structure through which such adaptations are mediated.

5.1.1.1. Composition, structure and function of the Gram-negative cell envelope

The Gram-negative envelope is known to consist of three principle layers: the cytoplasmic membrane (CM), the peptidoglycan (PG) and the outer membrane (OM). In addition, other surface components outside of the OM can be present and include exopolysaccharide and surface appendages such as flagella, fimbriae and pili. A schematic representation of the Gram-negative cell envelope is shown (fig. 5.1).

The CM is a double leaflet membrane composed primarily of phospholipid (up to seven kinds) and approximately two hundred different proteins, in amounts characteristic of the specific growth conditions (Ingraham *et al*, 1983). It is the site of many important biochemical and biophysical activities, containing many enzymes, permeases and components of the respiratory chain.

Fig. 5.1. Schematic diagram of the Gram-negative cell envelope. (Adapted from Sleytr *et al*, 1988).



LPS Lipopolysaccharide P Protein
PL Phospholipid LP Lipoprotein

The PG layer accounts for 10-20% of the weight of the envelope and provides the skeleton around which the other components are assembled (Lambert, 1988). An important stress-bearing polymer, it comprises a backbone of N-acetylmuramic acid and N-acetylglucosamine residues cross-linked by ß-1,4 glycosidic bonds. It is not entirely clear how the PG is situated in the envelope, one school of thought having suggested the formation of an extensive gel-like structure occupying the entire "periplasmic space" (the region between the CM and the OM) (Hobot, 1984). More recent conflicting evidence suggested that the PG is basically a monolayered structure, although a partially multilayered structure cannot be ruled out (Wientjes *et al*, 1991). It should be noted that the concept of terminology for the "periplasm" must be treated warily. "Periplasm" implies substance and functionality and is dynamic whilst "periplasmic space" is the physical region that contains most, but not necessarily all, of the periplasm (Graham *et al*, 1981). The size of the periplasmic space very possibly varies according to bacterial species and growth conditions, and one estimate puts the range of thickness to be from 10.6 to 25.3nm (Graham *et al*, 1981). Many nutrient-binding proteins and enzymes, as well as oligosaccharides involved in osmotic regulation and proteins destined for export from the cell, are contained in the periplasm (Lambert, 1988).

The OM consists of phospholipids, LPS and an array of different proteins which are present, as with CM proteins, in amounts characteristic of the growth environment. Unique to Gram-negative cells, the OM forms a physical and functional barrier to the cell and its surrounding environment and is unusual in its asymmetrical structure, a consequence of the replacement by LPS of some of the standard phospholipid molecules (Ingraham *et al*, 1983). LPS consists of three covalently-linked regions: the hydrophobic lipid A moiety, a highly ordered (Labischinki *et al*, 1985) and conserved region in all Gram-negative bacteria; the hydrophilic core polysaccharide which has similar composition in many species; and the hydrophilic O-polysaccharide side chains which are extremely variable in comparison (Hitchcock and Brown, 1983) and are used for serotyping species (Orskov *et al*, 1977). LPS forms a hydrophilic layer covering the cell, presenting a permeability barrier to hydrophobic entities with far-reaching consequences as far as antimicrobial agent permeation and host defence systems, including complement, are concerned (Nikaido abd Vaara, 1985). As well as being the

major antigenic determinant of the wall surface, the lipopolysaccharides serve as receptors for the adsorption of many bacteriophages. In *P. aeruginosa*, LPS is heterogeneous in size because of variations in the amount of O-side-chain polymer substitution on the core and in the types of side-chain sugars present. Most *P. aeruginosa* strains can synthesise simultaneously an A-band form and a B-band form of LPS (Rivera and McGroarty, 1989). A-band LPS, a common antigen, is composed of uncharged sugars, whereas B-band LPS is a highly charged O-antigen-containing LPS which determines the serotype of a particular strain (Lam *et al*, 1989).

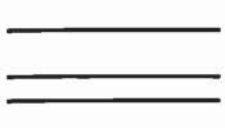
The focus of this section of work was on *P. aeruginosa* OMPs, thus the following section provides an overview of the organism's OMPs characterised to date.

5.1.1.2. Outer membrane proteins of *P. aeruginosa*

It is reported widely that the OM of *P. aeruginosa* acts as an unusually effective permeability barrier (Brown, 1975; Bryan *et al*, 1984; Nikaido and Hancock, 1986). As with enteric bacteria, its OM contains a large number of proteins, some of which have been isolated and characterised by a number of workers. Their nomenclature has arisen mainly according to the migration pattern achieved by SDS-PAGE (fig. 5.2.) and for some proteins migration is determined further by the temperature of denaturation (Hancock and Carey, 1979).

Under conditions of iron deprivation, *P. aeruginosa* produces a number of high molecular mass (75-90KDa) OMPs which function as receptors for the binding of complexes of iron with siderophores (see section 5.3) and *P. aeruginosa* expresses these IRMPs *in vivo* (Anwar *et al*, 1984; Cochrane *et al*, 1987; Shand *et al*, 1991) as well as *in vitro*. Recent reports in the literature are providing formal proof that at least some of the IRMPs of *P. aeruginosa* have a role in siderophore-mediated iron transport (Poole *et al*, 1991; Gensberg *et al*, 1992; Smith *et al*, 1992).

Fig. 5.2. Pattern of OMPs of *P. aeruginosa* following separation by SDS-PAGE on 12% acrylamide gels after denaturation at 100°C in SDS and mercaptoethanol. (Adapted from Hancock *et al*, 1990).



75-90 kDa IROMPs

70 kDa porin protein (OprC)

48 kDa phosphate pore protein P (OprP)

46 kDa glucose pore protein D1 (OprB)

45.5 kDa imipenem/basic amino acid selective porin D2 (OprD)

44 kDa porin protein E (oprE)

38 kDa major porin/structural protein F (OprF)

25 kDa protein G (OprG)

21 kDa protein H1- stabilisation of Mg^{2+} deprived cells (OprH)

20.5 kDa lipoprotein/structural protein H2 (OprL)

9-14 kDa FBP- ferripyochelin binding and transport (fbp)

9 kDa lipoprotein/structural protein I (OprI)

rough LPS

Proteins C (OprC) and E (OprE) are general porins (Yoshihara and Nakae, 1989) although their actual exclusion limits and functions are subject to some debate as yet (Hancock *et al*, 1990). Protein C has been found to allow the diffusion of antipseudomonal anionic beta-lactams (Satake *et al*, 1990).

The presence of protein P (OprP) is an indicator of low-phosphate (≤0.15mM) (Hancock *et al*, 1982). Proteins D1 (OprB) and D2 (OprD) have very similar molecular weights (Hancock and Carey, 1979). OprB is co-regulated with the glucose-uptake system and a periplasmic glucose-binding protein (Hancock and Carey, 1980) and has also been shown to function as a porin. OprD functions as a specific channel for the diffusion of lysine, arginine, ornithine, histidine and some peptides containing these basic amino acids (Trias and Nikaido, 1990a), facilitates diffusion of imipenem and its analogues (Trias and Nikaido, 1990b) and has recently been proposed as catalysing facilitated diffusion for sparfloxacin (Michéa-Hamzehpour *et al*, 1991).

Its function the subject of some controversy, protein F (OprF) had been assigned status as the major porin species in the *P. aeruginosa* OM (Hancock *et al*, 1979; Yoshimura *et al*, 1983). However, later work has contested its porin-forming ability (Yoshihara and Nakae, 1989) and it was found that mutants lacking protein F were unaltered in their antibiotic susceptibility (Gotoh *et al*, 1989). However, Nikaido *et al* (1991) attributed the findings of this latter work to the techniques used having inadvertently inactivated the protein. OprF is proposed as a representative of a class of porins which exist as monomers, producing large channels, but having low permeability due to drag resistance. It plays an important function in OM stability and cell shape determination (Hancock *et al*, 1990) and may be functionally related to OmpA of *E. coli* (Woodruff and Hancock, 1989). Recently, Meyer (1992) suggested that, besides specific routes for iron uptake, *P. aeruginosa* is also able to take up siderophore-liganded iron through the porin-OprF channels. This was prompted by the observation that inability to synthesise pyoverdin results, in response to iron-starvation, in increased efficiency of some of the other possible iron-acquisition routes.

Expression of protein G (OprG) is very much dependent upon growth conditions,

including iron-status, growth temperature and growth phase (Hancock and Carey, 1979; Ohkawa *et al*, 1980; Kropinski *et al*, 1987). Suggested roles encompass fluoroquinolone uptake (Chamberland *et al*, 1989) and low affinity iron uptake (Yates *et al*, 1989). Recently, a reduction of OprG was implicated as a possible mechanism of OM permeability modulation in the expression of quinolone resistance when PAO1 trained to resist ciprofloxacin showed a dose-dependent loss of OprG (Yates, 1992).

Proteins H1 (OprH) and H2 (OprL) have similar molecular weights but their respective functions are unclear. H1 replaces divalent cations in the OM, is over-expressed under conditions of particular cation-deficiency and blocks the self-promoted uptake of certain polycationic antibiotics (Nikaido and Hancock, 1986; Bell *et al*, 1991). H2 is strongly associated with PG, belongs to the class of OMPs termed as PG-associated lipoproteins (Mizuno, 1981) and is not thought to be surface-exposed (Lambert and Booth, 1982). A lower molecular weight lipoprotein, protein I (OprI) is broadly analogous to the Braun lipoprotein of *E. coli* (Mizuno and Kageyama, 1979). OprI has, as yet, not been assigned a function.

Continuing research into the isolation and identification of *P. aeruginosa* OMPs is resulting in the appearance in the literature of new information on a regular basis. Newly-described proteins include a 54KDa protein in the so-called *nfxB* mutant (Okazaki, 1992; section 4.1.1.2.) and a new 49KDa protein, designated OprM, the overproduction of which is associated with cross-resistance to meropenem, cephems and quinolones in *P. aeruginosa* (Masuda and Ohya, 1992). An 84KDa OMP has been identified in the resistance of *P. aeruginosa* to a catecholic cephem (Hazumi *et al*, 1992) and the *nfxC* mutation associated with an increase in a 50KDa protein and decrease in a 46KDa protein (Fukuda *et al*, 1990).

Thus, study of organisms resistant to antimicrobial agents is leading to expression of hitherto uncharacterised OMPs, although it must be stressed that the composition of the OM as a whole must be examined. Since many porin molecules isolated from the OM are tightly associated with LPS, it was originally suggested that such an association is necessary for porin function (Lugtenberg and Van Alphen, 1983). Findings vary with

the protein in question: Parr *et al* (1986) found that LPS was not required for porin function of OprP in *P. aeruginosa* and *E. coli* OmpF, whilst evidence for interplay between LPS and porin F was provided by Hancock and Woodruff (1988), with the state of the LPS being able to influence the number of open functional OprF pores in the OM (Kropinski *et al*, 1982; Nicas and Hancock, 1983). Moreover, changes only in the core fraction of LPS resulted in discrimination of antibiotics based on their relative hydrophobicities (Godfrey *et al*, 1986). More recent work has demonstrated that LPS alterations may contribute largely to the development of resistance to various classes of chemically unrelated antibiotics such as quinolones and beta-lactams (Leying *et al*, 1992). LPS side chain presentation could well affect permeability more than a change in the number of active porins.

The plasticity of the cell envelope probably plays a major role in the success of the biofilm mode of growth. It would seem logical to assume that free-floating bacteria have quite different cell surface properties from those adhering to a surface and numerous studies have investigated properties such as cell surface hydrophobicity and charge (see section 5.2.) which must determine, to a large extent, the initial attraction and adhesion to a surface. However, the factors contributing to such properties i.e. the actual composition of the cell surface, also merit investigation.

Increased EPS production is well documented as being a characteristic of biofilm growth (section 1.1.2.1.) but alterations in the OM *per se* have not been studied as widely, at least by methods which yield information pertinent to both the surface mode of growth and the growth rate of the organism. Many workers have studied the properties of *in vivo* surface-grown cells through the use of chamber implants, these having the advantage of localising the infection but the disadvantage of excluding some cellular antimicrobial defences (Brown and Williams, 1985a). Further, such methods, although of considerable value, possibly present the researcher with too much information i.e. many proteins will be seen in the OM but the reason for their expression cannot be attributed to any particular aspect of their growth. As such, adaptations to single factors (eg. surface growth alone, slow growth rates, nutrient limitations, host defence mechanisms) cannot be distinguished from one another. By

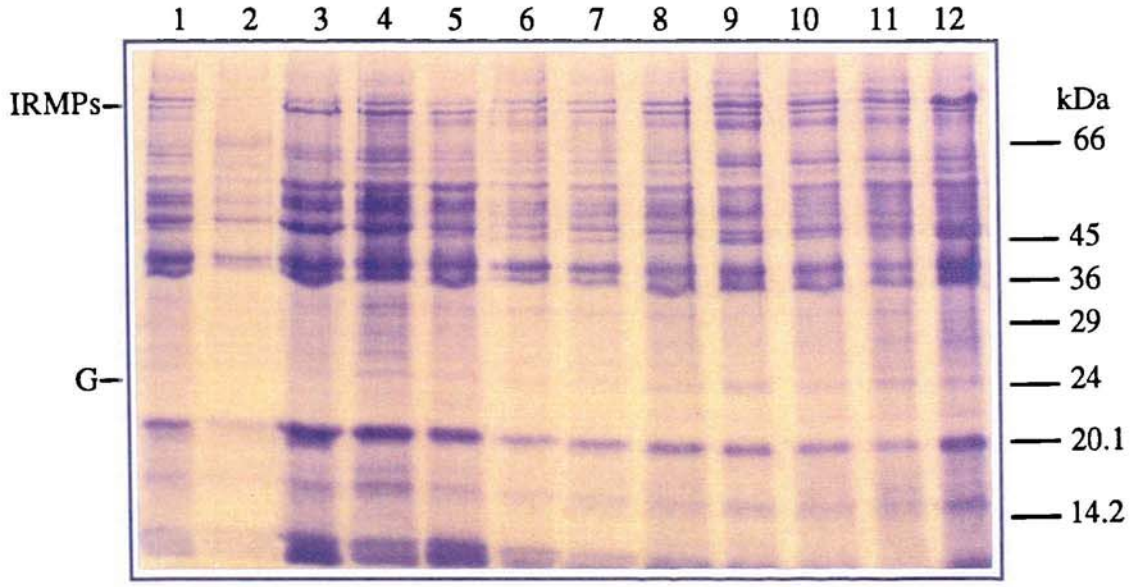
use of a growth-rate-controlled system *in vitro*, however, one can study effects of controlled variables and hopefully accumulate information on cell envelope modifications resulting from growth, at different rates, as an adherent cell. With this in mind, the cell surface properties of Swinnex- and Sorbarod-grown PAO1 JD populations, alongside their eluted counterparts, were investigated and compared to cells harvested from batch and growth-rate-controlled chemostat populations.

5.1.2. Results

The techniques used in the analysis OMPs and LPS have been described in section 2.10.3. The Coomassie blue-stained mini-prep. OMP profiles of PAO1 JD sampled progressively through batch culture growth in CDM_{12}-Fe (from OD_{470} 0.04 to 1.1) are shown in fig. 5.3. Three changes to note are; 1) the progressively stronger expression of protein G, 2) the alterations in levels of expression of two of the IRMPs, with the higher molecular weight (M_{wt}) protein, by late stationary phase, becoming the more strongly expressed, and 3) the increasing expression of a band in the region of 60KDa. LPS analysis by SDS-PAGE (not shown) did not show any discernible differences through batch growth. The profiles were essentially the same as those shown in fig. 5.10. i.e. with O-antigen laddering and the lipidA/ core region at the gel front.

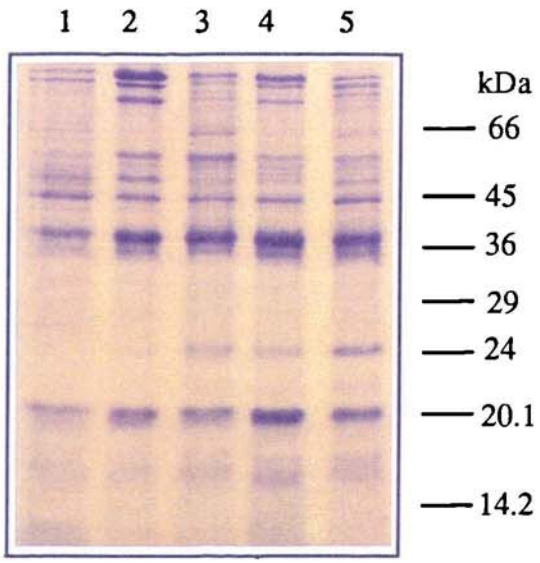
Direct comparison of OMs of cells harvested from iron-restricted batch and chemostat culture can be seen in fig. 5.4. The change in IRMP expression from mid to late logarithmic phase cultures is clearly seen, with high level expression of the 85KDa band and switching on of the 75KDa protein. Protein G is present only in the chemostat samples and may be increasing with growth rate. However, analysis of cells from a wider range of chemostat growth rates (fig. 5.5) (along with some OM preparations of cells scraped from the sides of chemostats) does not confirm the increase in OprG incorporation into the OM as growth rate increases. These samples, again, show alterations in relative expression of IRMPs, and whereas four IRMPs are present at slow growth rates, only two are expressed at the faster dilution rates. No M_{wt} markers are shown on fig 5.5. as this was for a general view only of relative banding patterns.

Fig. 5.3. Coomassie blue-stained SDS-PAGE OMP profiles of PAO1 JD sampled throughout batch growth in iron-limited culture. (All samples prepared by mini-method).



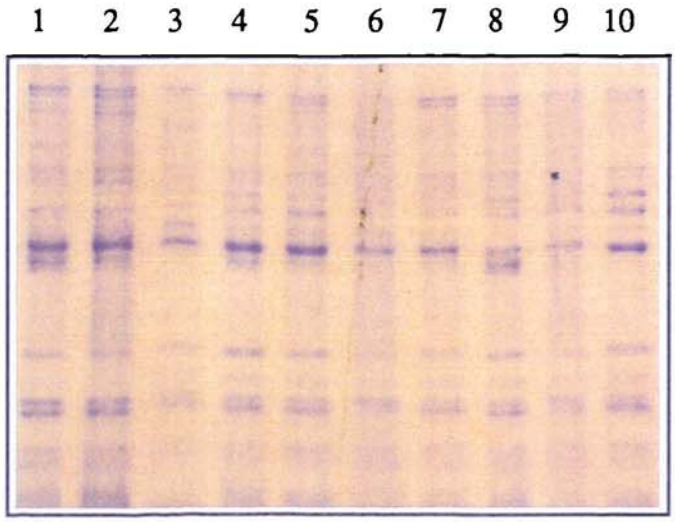
Lane	OD_{470}	Lane	OD_{470}
1	0.024	7	0.270
2	0.075	8	0.394
3	0.090	9	0.592
4	0.156	10	0.764
5	0.202	11	0.925
6	0.226	12	1.025

Fig. 5.4. Coomassie blue-stained SDS-PAGE OMP profiles of PAO1 JD grown by batch and chemostat iron-limited culture. (All samples prepared by mini-method).



Lane	Sample
1	mid-logarithmic phase batch
2	late logarithmic phase batch
3	chemostat ($D=0.069h^{-1}$)
4	chemostat ($D=0.09h^{-1}$)
5	chemostat ($D=0.228h^{-1}$)

Fig. 5.5. Coomassie blue-stained SDS-PAGE OMP profiles of PAO1 JD grown by chemostat iron-limited culture. (All samples prepared by classical method; w = sample from wall of vessel).



Lane	Dilution rate (h^{-1})	Lane	Dilution rate (h^{-1})
1	0.069	6	0.228w
2	0.076	7	0.241
3	0.185	8	0.329
4	0.213	9	0.329w
5	0.228	10	0.508

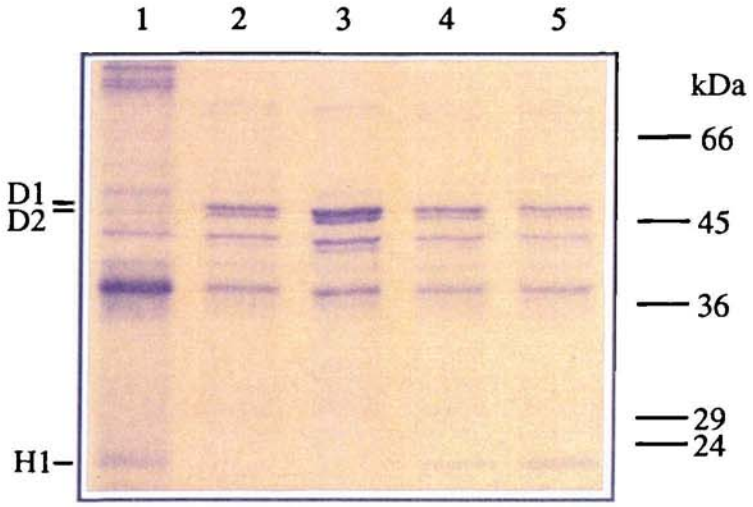
SDS-PAGE analysis of iron-plentiful (carbon-limited) chemostat cells of increasing growth rates (fig. 5.6) reveals complete suppression of IRMPs and strong expression of proteins D1 and D2. Just visible, levels of protein H1 appear to be gradually increasing with growth rate.

Coomassie blue-stained OM profiles of resuspended Swinnex-grown whole cell, sarkosyl-treated whole cell (pellet and supernatant) and "standard" chemostat preparations are shown in fig. 5.7. All were grown under iron restriction. Bands in the region of 61 and 58KDa in size can be seen in the resuspended Swinnex biofilm OM profiles but not in the full OM preparation of chemostat cells.

Owing to the relatively small quantities of cells recovered from the Swinnex biofilm system, Coomassie blue-staining was thought not to be adequately sensitive to the reduced levels of protein present in these samples. Consequently, a silver stain for protein was used in preference. Initial studies utilising this method of staining indicated strong expression of a novel band at around 58KDa. In order to verify if this was indeed expression of a new protein, or whether its appearance was a result of the mini-method of OM preparation, NB and CDM+Fe batch culture populations were both subjected to the classical and modified methods of OM preparation. Fig. 5.8., showing the profiles of all of these samples, illustrates that the new band was present only in the surface-grown samples, thus was not arising as a consequence of the preparatory technique. The presence of proteins 54K and 43KDa in size was also noted in the OMs of Swinnex-grown cells.

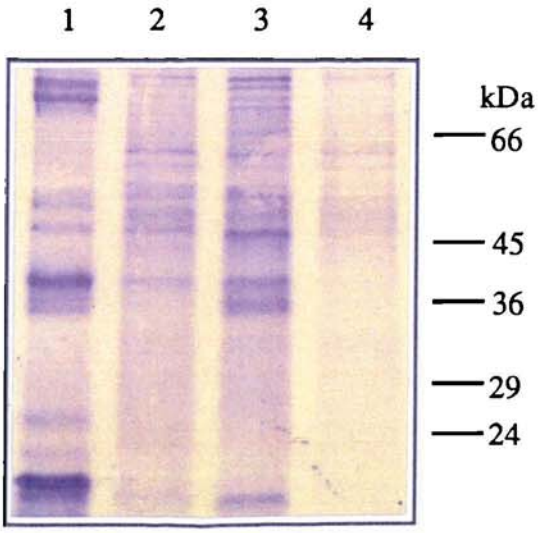
The OM profiles of cells harvested from 48h Sorbarod filter fibres alongside their respective eluate cells at each particular flow rate are shown (fig. 5.9.). As "standards", batch- and chemostat-grown cell preparations were also run on the gel. The particular chemostat samples were chosen in order to give a range of population doubling times which were comparable to the rates calculated for the 48h Sorbarod samples. All samples were prepared by the modified method. Close inspection of the profiles of the Sorbarod-grown cells shows that there were no major differences in protein expression between the eluate cells and their surface-grown counterparts. All show the presence

Fig. 5.6. Coomassie blue-stained SDS-PAGE OMP profiles of PAO1 JD grown by chemostat iron-restricted and carbon-limited/ iron-plentiful culture. (All samples prepared by classical method).



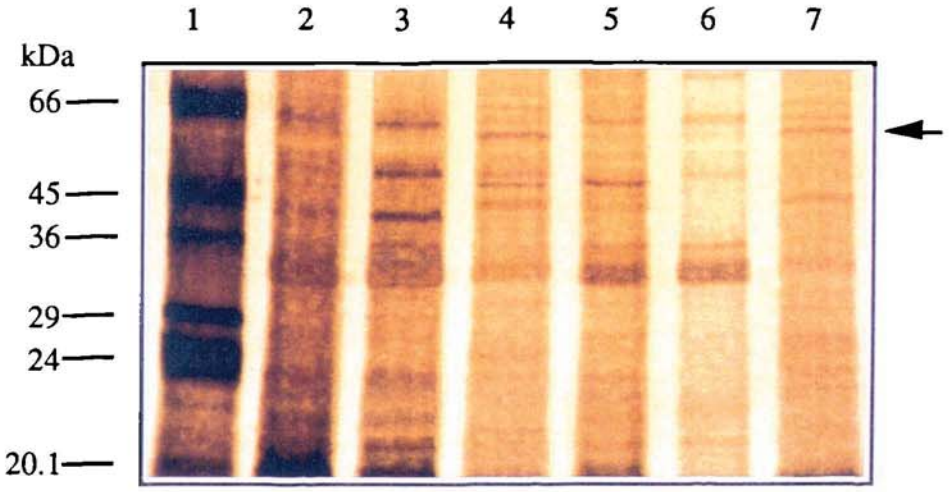
Lane	Dilution rate (h^{-1})	
1	0.014	(Fe-restricted)
2	0.14	(C-limited)
3	0.129	"
4	0.58	"
5	0.68	"

Fig. 5.7. Comparison of Coomassie blue-stained SDS-PAGE OMP profile of PAO1 JD grown by chemostat culture ($D=0.013h^{-1}$) with cells resuspended from Swinnex biofilm units (flow rate=0.9ml/min). (All grown under iron-restricted conditions).



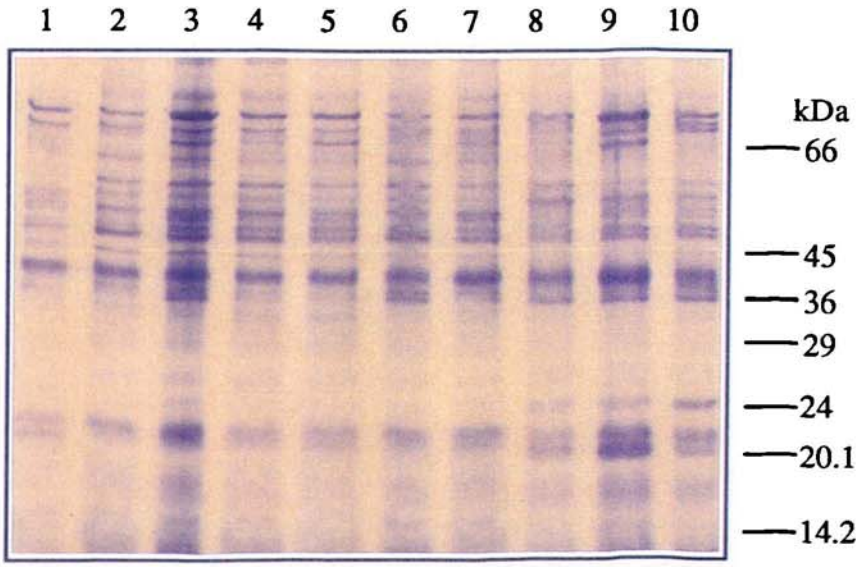
Lane	Sample
1	Fe-restricted chemostat (classical OM preparation)
2	Resuspended biofilm whole cell preparation
3	Resuspended biofilm, sarkosyl-treated: pellet
4	Resuspended biofilm, sarkosyl-treated: supernatant

Fig. 5.8. Comparison of silver-stained SDS-PAGE OMP profile of PAO1 JD grown by chemostat culture ($D=0.013h^{-1}$) with cells resuspended from Swinnex biofilm units (flow rate=0.9ml/min). The presence of a novel band is indicated by an arrow. (NB=nutrient broth-grown).



Lane	Sample
1	Molecular weight markers
2	NB batch, classical OM preparation
3	NB batch, mini OM preparation
4	C-limited/Fe-plentiful biofilm, mini OM preparation
5	C-limited/Fe-plentiful batch, classical OM preparation
6	C-limited/Fe-plentiful batch, mini OM preparation
7	C-limited/Fe-plentiful biofilm, mini OM preparation

Fig. 5.9. Coomassie blue-stained SDS-PAGE OMP profiles of PAO1 JD iron-restricted cultures: comparison of batch, chemostat and Sorbarod-grown (S/rod) cells. (All OMs prepared by mini-method).



Lane	Sample	Lane	Sample
1	Batch, stationary phase	6	S/rod eluate (0.02ml/min)
2	S/rod, eluate (1.15ml/min)	7	S/rod biofilm (")
3	S/rod, biofilm (")	8	Chemostat ($D=0.069h^{-1}$)
4	S/rod, eluate (0.5ml/min)	9	Chemostat ($D=0.09h^{-1}$)
5	S/rod, biofilm (")	10	Chemostat ($D=0.228h^{-1}$)

of the 85kDa IRMP and all, bar the eluted cells from the fastest flow rate, express an IRMP of about 75kDa. In comparison to the chemostat standards, more proteins were expressed by the Sorbarod samples as a whole. Chemostat samples are deficient in bands corresponding to 58 and 54KDa in size, as are the batch-grown cells. Chemostat populations are the only cells to show expression of protein G.

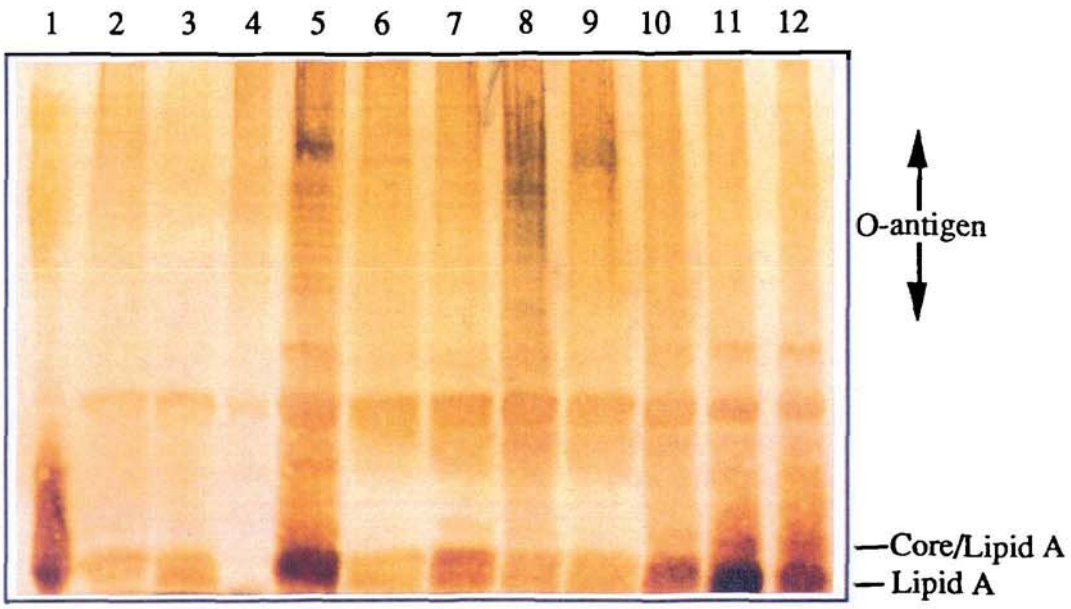
LPS from the Sorbarod-, batch- and chemostat-harvested cells was examined. The silver-stained LPS profiles of proteinase K-digests of mini-OM preparations run alongside a PAO1 LPS standard are shown in fig. 5.10. As mentioned previously (section 2.10.4.1.), the method of Fomsgaard *et al* (1990) for LPS silver staining results in relatively short-lived visualisation of banding patterns. However, O-antigen banding was observed in all lanes, albeit faintly in some. The amount of lipid A from sample to sample appeared to vary and, among the Sorbarod samples, biofilm cells from the fastest perfusion rate displayed the greatest amount.

5.1.3. Discussion

The major problem faced in the analysis of OMPs and LPS of PAO1 JD biofilm and eluate cells in this project was that of the relatively low numbers of cells which were available for the production of outer membranes. e.g. In the region of 10^9 cfus could be recovered from a Sorbarod filter whilst as few as 10^7 cfus/h were, in some instances, released in the eluate from such a unit. In normal laboratory practice, at least 10^{10} cells are considered an appropriate amount for efficient OM preparation. Thus, developing and utilising the mini-preparation method was crucial to the success of examining OM profiles of adherent and dispersed cells.

The OM profiles of traditional batch-grown populations, from very early log through to late stationary phase at 24h, exhibited three progressive differences worth noting. OprG expression became more obvious by early stationary phase, although expression was still relatively weak. It has been shown that OprG expression is dependent upon both

Fig. 5.10. Silver-stained SDS-PAGE profiles of proteinase K digests of OM preparations (all by mini-method) of PAO1 JD iron-restricted cultures: comparison of batch, chemostat and Sorbarod-grown (S/rod) cells.



Lane	Sample	Lane	Sample
1	PAO1 LPS standard	7	S/rod biofilm (0.5ml/min)
2	Mid-log. phase batch	8	S/rod eluate (0.02ml/min)
3	Stationary phase batch	9	S/rod biofilm (")
4	S/rod eluate (1.15ml/min)	10	Chemostat ($D=0.069h^{-1}$)
5	S/rod biofilm (")	11	Chemostat ($D=0.09h^{-1}$)
6	S/rod eluate (0.5ml/min)	12	Chemostat ($D=0.228h^{-1}$)

the level of available iron and growth rate/ phase (Yates, 1992). Previous results suggested suppression of OprG under iron-limitation (Ohkawa *et al*, 1980; Ward, 1987) with the induction of expression in the presence of high iron concentrations in the media. Yates (1992) also found that OprG began to be expressed only as cells in iron-restricted medium entered late stationary phase (24h). A second aspect for discussion is that of the alteration in levels of expression of the IRMPs, with the highest levels of expression seen for the 85KDa band by late stationary phase. The 85KDa IRMP has been correlated with ferri-pyoverdin uptake and it was confirmed recently that *P. aeruginosa* responds specifically to the presence of pyoverdin in the growth medium and not simply to the iron-deficiency imposed by that siderophore (Gensberg *et al*, 1992). Indeed, there are a growing number of reports in the literature which provide evidence that various IRMPS do indeed act as receptors for siderophore-iron complexes (Poole *et al*, 1991; Smith *et al*, 1992), a process having been long believed to occur in *P. aeruginosa* without formal demonstration. A final point was that of the increasing expression of a 60KDa protein as the culture entered stationary phase. The identity of this protein is unknown.

Iron-restricted chemostat populations of PAO1 JD exhibited marginally increasing levels of protein G expression with growth rates between 0.069 and 0.228h^{-1}. However, the results for a wider range of dilution rates did not give any evidence for this trend being continued. Overall, OprG expression in iron-restricted chemostat cell populations was weak, but the protein was nevertheless present. Yates (1992), in contrast, found it expressed only at a relatively slow growth rate. Growth phase certainly affects expression but the effect of growth rate is unclear. Both growth rate and phase would appear to influence the differential expression of IRMPs, however. Growth of PAO1 JD under iron-replete, low carbon conditions in the chemostat abolished the presence of IRMPs and surprisingly, in the light of the aforementioned findings of OprG expression correlating with high iron concentration, there was no expression of OprG whatsoever.

Thus, further evidence of the possible role of OprG as a low-affinity iron-uptake system

(Yates, 1992) has not been forthcoming from this study. Even with full repression of IRMP synthesis, OprG was not present in the OM and, therefore, iron must have been entering the cells by some other means.

OprB is known to be a glucose-inducible pore (Hancock and Carey, 1980) and its presence was clearly visible under low carbon conditions in the chemostat. OprH appeared to be expressed, albeit weakly, only as growth rate increased. OprH is believed to replace OM-stabilising divalent cations and can be over-expressed when the growth medium is limiting for such ions. Bell *et al*, (1991) noted, however, that glucose minimal medium was associated with the expression of OprH gene. Also seen to be expressed was OprC (70KDa), this having been reported as being visualised by SDS-PAGE only when OM samples were heated before electrophoresis (Yamano *et al*, 1990). This group also resolved OprE into two distinct bands, E1 and E2.

SDS-PAGE of Swinnex-grown iron-restricted cells consistently revealed the existence of several proteins not observed in slowly-growing chemostat (planktonic) OM preparations i.e. in the regions of 60KDa, 58KDa and 52-54KDa. Silver-staining of iron-replete surface-grown cells confirmed the presence of a novel protein in the region of 58KDa as well as a lesser-expressed 45KDa band. Not an artifact of the preparatory method, the 58KDa band, at least, was possibly a consequence of growth of PAO1 on a surface.

The expression of new bands on bacterial contact with surfaces is not a novel phenomenon. Previous workers reported the induction of a 76KDa band, an iron-transport protein, on filtration of an *E. coli* strain onto a filter membrane (Boyd and Holland, 1977), and Dagostino *et al* (1991) noted that certain genes can be switched on at a solid surface. A 55KDa protein in the OM of *P. aeruginosa* (Ohkawa *et al*, 1979) is known to be induced by growth on acyl esters. The possibility of its induction due to growth on the Millipore mixed ester filter membranes used in the Swinnex biofilm systems must, therefore, be taken into consideration. SDS-PAGE of OM proteins of *P. aeruginosa* PAO579 isolated directly from the lungs of infected rats showed the expression of IRMPs, weak expression of OprG and on immunoblotting with serum

from infected rats, an antigen with an approximate M_{wt} of 54KDa was recognised by the serum (Cochrane *et al*, 1987). This protein was believed to be flagellin and was not visible by Coomassie blue staining. (Interestingly, there were antibodies to protein G present in the serum suggesting differences in the antigens expressed by the organism growing in the serum and in the lung environments). As far as the role of flagella is concerned, it was shown that, once chronic infection was established in the CF lung a high number of, originally invasive, *P. aeruginosa* strains lacked flagella (Luzar and Montie, 1985) and these were described as reverting to an avirulent state, presumably as a result of selective pressure in an environment where host defence factors would be abundant.

Direct comparison of OM profiles of Sorbarod-associated cells and their released "progeny" did not reveal any significant differences between the two types of sample source, at any of the three flow rates. One difference occurred at the fastest flow rate, with the resuspended biofilm cells expressing three IRMPs as opposed to the expression of only two by the cells in the eluate. Another difference, tentatively suggested, is that of expression of two bands, in the region where D1 and D2 would be expected, by each resuspended biofilm population whereas only the higher M_{wt} band of the two is expressed by eluate cells. Variation in profiles in comparison with the batch grown and chemostat cell OMPs was more obvious, with Sorbarod populations (both types) expressing 54 and 58KDa bands, not seen in the planktonic samples.

Thus, in summary, within the limited range of growth rates studied there were no major differences in OM profiles between sets of Sorbarod-grown cells, but there were differences between cells growing in suspension and those grown, and eluted from, the biofilm systems. A reason for the similarity of OM profiles of eluate and parent biofilm populations could be that the eluate contained not just newly-formed "babies", but a heterogeneous array of older biofilm cells alongside newly divided cells. This could therefore provide more evidence in agreement with the general findings of the calibration experiments (chapter 3) which could rarely achieve synchrony with eluate cells from any of the biofilm systems. Similar OM profiles could also partly provide an explanation for the similar antimicrobial susceptibility exhibited by resuspended biofilm

and eluate cells (section 4.3.3.).

There are many examples in the literature of bacterial cell surface alterations, the occurrence of which are dependent upon the circumstances of the cells. An interesting illustration of necessary membrane changes is that of the induction of several proteins, in *Salmonella* species, by receptors on host epithelial cell surfaces (Finlay *et al*, 1989: 1992). These new proteins are required for adherence to and subsequent invasion of epithelial cells, two properties inextricably linked since most non-invasive *Salmonella* mutants are also non-adherent species. Similarly, *Haemophilus influenzae* initiates *de novo* protein synthesis to produce several new OM proteins probably required for adhesion to host cells (St. Geme and Falkow, 1990). Further, loss of capsule expression of this organism allows function of a nonpilus adhesin-invasin which promotes persistence within the human respiratory tract (St. Geme and Falkow, 1991). Clearly, specific OMP expression, not required for planktonic survival, is required for successful invasion of cells and can , therefore, be considered a crucial virulence factor.

It is, as yet, unknown whether the new proteins observed in the OM of PAO1 JD are synthesised in response to the presence of a surface or as a consequence of adhering by some other means. One could speculate that a cell reversibly binding to a surface may not express such proteins but they may contribute to or be a result of irreversible binding. Within the parameters studied in this project, however, the OMP induction was neither a growth rate- nor nutrient-controlled factor.

Considering the virulence properties of *P. aeruginosa* in the CF lung, it is the switch from the non-mucoid to the alginate-producing mucoid phenotype which is a major virulence determinant of the organism. Grabert *et al* (1990) reported the presence of a novel protein of about 54KDa in the OMs of mucoid, but not non-mucoid, strains. Other workers, investigating the effect of the porosity and chemical nature of a surface on slime formation by *P. aeruginosa* found that growth on a semi-permeable, as opposed to a fully permeable, surface resulted in expression OM proteins of 32-37KDa and 50-56KDa (Ross *et al*, 1991). Comparison of OMPs of mucoid and non-mucoid *P. aeruginosa* showed that a prominent band of approximately 55KDa, as well as a

25KDa band, in mucoid CF isolates was absent or underexpressed in the laboratory-reverted non-mucoid strains (Kelly *et al*, 1990). In a long-term study of CF patients, several (but not all) mucoid *P. aeruginosa* isolates expressed an OMP of about 52KDa (Shand *et al*, 1991). This group also made the point that, owing to difficulties in comparing results from different systems, this protein could be the 58.5KDa protein reported previously as causing an antibody response in CF serum (Fernandes *et al*, 1981). A point to note, but perhaps unrelated, is that a 54KDa OMP of *P. aeruginosa* which has been characterised, cloned and sequenced recently is the product of the *nfxB* gene and is associated with quinolone resistance (Okazaki and Hirai, 1992).

Clearly, *P. aeruginosa* can produce several novel proteins when or after colonising *in vivo*, in CF patients for example, some of which may be linked to the switching of non-mucoid to the mucoid phenotype. Certainly, proteins commonly appear in the 54-58KDa range and whether or not it is coincidence that biofilm-derived cells in this project consistently expressed OMPs in this size range, whilst planktonically-grown cells estimated to be growing at similar rates did not, is certainly an intriguing matter for future investigation.

LPS, a characteristic component of the OM of *P. aeruginosa* and all Gram-negative bacteria, is known to exhibit altered properties in response to the cell's environment. In this project, the study of LPS from Sorbarod- and planktonically-grown cells by SDS-PAGE did not show any major differences between samples. However, only very small sample sizes were available for analysis and it would have been preferable for more work to have been carried out in this area. Generally, O-antigen laddering was observed for all samples, although in varying degrees of strength. Amounts of lipid A varied with growth rate of both Sorbarod eluate and resuspended biofilm samples, the fastest-growing cells appearing to have more lipid A present in their OMs. It could be possible that the samples from the two fastest-growing biofilms showed at least one low M_{wt} core/ lipid A fraction, although fading of the gel has made this rather unclear. It has already been reported that smooth-form *P. aeruginosa* biofilms, from the modified Robbins device, had higher amounts of such bands without O-sugars as compared with

their planktonic counterparts (Giwercman *et al*, 1992). In lipid A, the number and position of fatty acids influence the permeability barrier as well as the toxicity of the LPS, and more appear to be present in the low M_{wt} LPS fractions (Fomsgaard *et al*, 1988). Giwercman *et al* (1992) thus assumed that the biofilms of S-form *P. aeruginosa* contained increased amounts of low M_{wt} core/ lipid A fractions. The significance of this is perhaps reflecting OM structural changes which contribute to the altered physico-chemical properties of biofilm bacteria in CF patients and medical implant-associated infections. Another group found that *P. aeruginosa* cells grown *in vivo* in a chamber implant device lacked a series of high M_{wt} O-antigen LPS bands and gained a new series of lower M_{wt} bands (Kelly *et al*, 1989). Also, comparison of the LPS of mucoid and parent non-mucoid PAO1 showed that the mucoid phenotype had lost a high M_{wt} LPS (Kelly *et al*, 1990).

Alterations in LPS may contribute significantly to development of resistance to various classes of chemically-unrelated antibiotics (Leying *et al*, 1992). Subtle changes within the O-side chain composition of *P. aeruginosa* LPS were correlated with increased MICs of beta-lactam antibiotics (Godfrey *et al*, 1984) with , further, no noted change in porin number to affect the permeability of the drugs (Godfrey *et al*, 1986). In this project, the eluate cell LPS profiles showed weak O-side chain banding in comparison to their parent biofilms. Loadings for the gel had been standardised by calculating volumes which gave equal amounts of protein in each lane, using the SDS-PAGE OMP gels as a basis for rationalising the amounts. It can only thus be assumed that the eluate cells had less O-antigen, relative to amounts of protein, than did their biofilm counterparts. Growth rate of the samples again appeared to affect this portion of LPS, with less O-antigen present in resuspended biofilm samples as growth rate decreased. It was not possible to distinguish relative amounts in the eluate samples. These points will be discussed further later in this chapter, in the light of the results for cell surface hydrophobicity. A change in KDO:protein ratio correlated with resistance to ciprofloxacin (Yates, 1992) and KDO content has also been found to vary with growth rate, being reduced as growth rate increases (Gilbert and Brown, 1978). Thus alterations in LPS structure occur as a result of the growth environment and play a significant role in bacterial resistance and survival.

5.2. STUDIES ON CELL SURFACE HYDROPHOBICITY OF PAO1 JD

5.2.1. Introduction

Bacterial attachment to surfaces, a property integral to the ability of cells to form adhesive biofilms, is dependent upon many contributory factors. Initial adherence is considered to depend mainly upon surface properties such as hydrophobicity (Van Loosdrecht *et al*, 1987a,b) and net surface charge (Fletcher and Loeb, 1979) as well as the presence of surface appendages such as fimbriae and flagella (Rosenberg and Kjelleberg, 1986). Synthesis of exopolysaccharide materials appears to be the crucial factor for irreversible adherence (Martinez-Martinez *et al*, 1991). Specific factors influencing cell surface hydrophobicity in Gram-negative bacteria include components of the OM (OMPs, LPS type, O-antigen production, exopolysaccharide) and associated divalent cations. Variations in the cell's environment known to affect the cell envelope will also be determinants of cell surface hydrophobicity. In addition to adhesion to surfaces, cell surface hydrophobicity has a bearing on the susceptibility of particular organisms to certain antimicrobial agents (Nikaido and Vaara, 1985), phagocytosis and killing by PMNs and macrophages. The more hydrophilic cells are eradicated by host defence systems with greatest difficulty (Absolom, 1988). Moreover, a number of antibiotics, in sub-inhibitory doses, have been shown to alter bacterial hydrophobicity (Absolom, 1988; Domingue *et al*, 1989) probably due to antibiotic-induced changes in the cell membrane biochemistry and/ or structural alterations.

In this project, preliminary studies under specific nutrient limitation on the effects of growth phase, rate and mode on cell surface hydrophobicity were undertaken, using the method of hydrophobic interaction chromatography (HIC; section 2.10.2). Principle objectives were to gain an insight into the differences in this cell surface property of biofilm and eluate cells and, along with knowledge of OMPs and LPS (section 5.1.), to correlate, possibly, findings with the patterns of cell release/ retention observed at each flow rate imposed upon the Sorbarod biofilm system.

5.2.1.1. The measurement of cell surface hydrophobicity

The significance of bacterial adhesion in the environment and in the clinical situation has been outlined in chapter 1. Microbial adhesion at liquid-liquid (e.g. oil and water) and liquid-solid (e.g. medical implant and body fluid) interfaces is of major environmental, health and economic concern and, as such, interest in hydrophobicity and adhesion has increased over recent years. A variety of techniques for measuring hydrophobicity have been proposed but there is a lack of consensus on a definitive measurement method, with results varying between different laboratories and also with those obtained on different occasions within the same laboratory (Mackowiak and Marling-Cason, 1984). Such contradictions stem from both the non-uniformity of the bacterial cell surface and the variation in definition of "hydrophobicity" by different sets of researchers. The subject of measurement has been extensively reviewed, most recently by Rosenberg and Doyle (1990).

Not only is the hydrophobicity of the bacterial cell surface of interest, due consideration must also be given to that of the solid substratum (Loeb, 1985). Wherever the properties of a substratum and the surface properties of the bacterium "match", in terms of surface free energy, adhesion will occur (Busscher and Van der Mei, 1983). Thus, combinations of different organisms and surfaces lead to different results. For example, glass, with a higher surface energy than Teflon, is adhered to less by hydrophilic bacteria, whilst a hydrophobic bacterial strain (having lower surface energy) prefers to adhere to Teflon (Busscher and Van der Mei, 1983).

Further relevance for the study of hydrophobicity lies with the fact that a prerequisite for proliferation of growth as adherent biofilms is that cells must be able to detach from existing biofilm populations before colonising new surfaces. To date, relatively little is known about the processes involved in detachment aside from physical disturbance, such as sloughing. A further point to note is that so-called "hydrophobic" microorganisms mostly disperse in the bulk liquid phase and very few microbial strains are so hydrophobic that they can be wetted by oil and not by water (Rosenberg and Doyle, 1990).

5.2.1.2. Surface components influencing hydrophobicity

Rosenberg and Kjelleberg (1986) introduced the words "hydrophobins" and "hydrophilins" to denote cell surface components which contribute to or reduce cell surface hydrophobicity, respectively. Fimbriae are often implicated as hydrophobins, although not all fimbriae *are* necessarily, and their number and topographic distribution will ultimately contribute to the degree of hydrophobicity which they confer (Rosenberg and Kjelleberg, 1986). Although fimbriated *E. coli* strains, for example, are generally more hydrophobic than their nonfimbriate counterparts, the degree of hydrophobicity conferred by the fimbriae is low. Further, environmental conditions such as temperature, pH, the presence/ absence of alanine and growth rate are known to affect the production of K99 fimbriae in *E. coli* (Van der Woude *et al*, 1990). The ability to express organelles such as pili is known to be important for the spread of *E. coli* from one host to another (Bloch *et al*, 1992). Indeed, many investigations into adhesion to host tissue have been directed towards the study of colonisation of the gastrointestinal tract. Since, during normal transmission of *E. coli* among hosts, it is likely that the bacteria spend time in an extra-intestinal environment, they must phenotypically (and genotypically) adapt to these vastly different environmental surroundings. A considerable proportion of *E. coli* isolates produce thin, coiled, highly aggregative surface fibres called curli, when grown under certain conditions, and their expression is highly correlated to the organisms' ability to auto-aggregate and bind to soluble fibronectin and laminin (Olsén *et al*, 1993). It is thought that, as with fimbriae, different growth conditions may select for curli-expressing or non-expressing variants (Olsén *et al*, 1993).

The determinants for adhesion of *P. aeruginosa* to animal cells have not been established clearly. The successful colonisation of the respiratory system by this organism is initiated upon attachment to the respiratory epithelia (Woods *et al*, 1980) and several studies have indicated that the pilus adhesin provides the initial adhesion (Woods *et al*, 1980; Doig *et al*, 1987; Doig *et al*, 1988; Zoutman *et al*, 1991). Some workers have suggested that hydrophobic interaction does not appear to play a major role in the binding capability of *P. aeruginosa* (Elsheikh *et al*, 1985; Marcus and Baker, 1985;

Garber *et al*, 1985) but it has been noted that the procedures used for measuring the surface hydrophobicity may not be sufficiently sensitive to detect hydrophobic areas of the pilus adhesin (Lachica, 1990). Recently, Ramphal *et al* (1991) showed that the ability of a pilin structural gene mutant to adhere to mucin, in comparison to its wild-type parent, was not impaired. This suggested that *P. aeruginosa* also binds to mucin via adhesins distinct from pilin. Unexpectedly, however, pili were found to block the adhesion of the piliated strain and the authors speculated that binding of *P. aeruginosa* pili to mucins could be a long-range non-specific hydrophobic interaction via receptors for the nonpilus adhesin. Another group (Saiman *et al*, 1990) reported previously that several characteristics act in concert for the binding of *P. aeruginosa* to epithelial cells, with most efficient binding achieved by piliated, motile (flagellated) organisms in the presence of exoproducts that accumulate during growth under low phosphate conditions. It was suggested that the expression of environmentally-regulated genes may be important for determining the ability of the bacterium to bind efficiently. Hydrophobicity of non-mucoid strains of *P. aeruginosa* was found to be important in adhesion to catheters during the first five minutes of interaction, thereafter appearing to be of no further significance (Martinez-Martinez *et al*, 1991). The initial adherence of mucoid strains was lower than non-mucoid with similar hydrophobicities, but the adherent population increased slowly until a steady state was achieved whilst, having attained a maximum level of adhesion, non-mucoid populations decreased in size.

The presence of bacterial capsule or slime, hydrophilic in nature, has been shown to decrease surface hydrophobicity in the vast majority of studies (Rosenberg and Kjelleberg, 1986) and would thus suggest an antiadhesive role of EPS. However, it is more likely that the polysaccharide acts to bridge and cement layers of adhering bacteria without involvement in the initial adhesion processes (Marshall, 1988).

5.2.1.3. Detachment of surface-associated cells

It appears that cell surface hydrophobicity has a degree of importance in the initial binding of certain cells to inert surfaces. The effect of hydrophobicity on detachment

processes, however, is less well defined. One hypothesis for the mechanism of bacterial desorption from an interface suggests changes in cell surface hydrophobicity or, alternatively, localised bacterial production of amphipathic materials that remove bound cells to be important (Rosenberg and Doyle, 1986). Dahlbäck (1983) suggested a life cycle in which planktonic, starved hydrophobic bacteria adhere readily to available interfaces, efficiently scavenge surface-localised materials, increase in biomass and size and then leave the interface, usually in the form of a daughter cell following division (Kjelleberg *et al*, 1982), to face the nutrient-deprived conditions in the planktonic phase. Marshall (1988) reported that a starved *Pseudomonas* strain bound to hydrophobic surfaces but detached once the adsorbed fatty acid layer was completely utilised. An explanation put forward suggested that utilisation of the fatty acids resulted in localised changes in the substratum free energy, thereby creating conditions favouring reversible rather than irreversible adhesion.

In vivo, once established in human tissue *P. aeruginosa* uses the same survival strategy favoured in its natural aquatic environment in its establishment of an alginate-protected biofilm base, from which it attempts to colonise new areas through the release of mobile (planktonic) "scout" cells (Costerton *et al*, 1990). It is only very recently that research has been carried out with a view to establishing how cells dispersed from *in vitro* biofilms (analogous to the "scout cells") may differ from their adherent parent cells in terms of hydrophobicity (Allison *et al*, 1990a,b; Evans, 1990; Gilbert *et al*, 1991) and the results of these studies will be discussed in conjunction with the findings of work carried out on batch and Sorbarod-grown populations in this project.

5.2.2. Results

Hydrophobicity of PAO1 JD throughout growth in batch culture was investigated by HIC for both iron-restricted and iron-replete populations (figs. 5.11. and 5.12.). Iron-restricted organisms showed a sharp decline in hydrophobicity as growth commenced, remaining extremely hydrophilic, relative to the limits of this assay, through growth, and became more hydrophobic by late stationary phase. Carbon-limited/ iron-replete

cultures also demonstrated a decrease in hydrophobicity during cell growth and early stationary phase, although not to the extent observed for iron-restricted cells. Again, cells were seen to have become more hydrophobic by late stationary phase. Use of phenyl-sepharose, as a further measure of specific and non-specific cell retention, resulted in very similar retention to that obtained for octyl-sepharose (results not shown) and it was decided that no further use of this extra source of measurement was required.

The results of HIC assays on 48h Sorbarod eluate and resuspended biofilm cells, shown in fig. 5.13., demonstrate that, even over the narrow range of growth rates investigated, there was a considerable growth-rate-effect upon the cell surface hydrophobicity of cells released into the eluate. Clearly, hydrophobicity decreased markedly as growth rate increased. No such radical changes in hydrophobicity were apparent for the resuspended biofilm cells, there appearing to be only a marginal increase in hydrophobicity with growth rate.

Fig. 5.11. Specific retention on octyl-sepharose columns (% hydrophobicity) of PAO1 JD during batch culture in CDM_{12}-Fe.

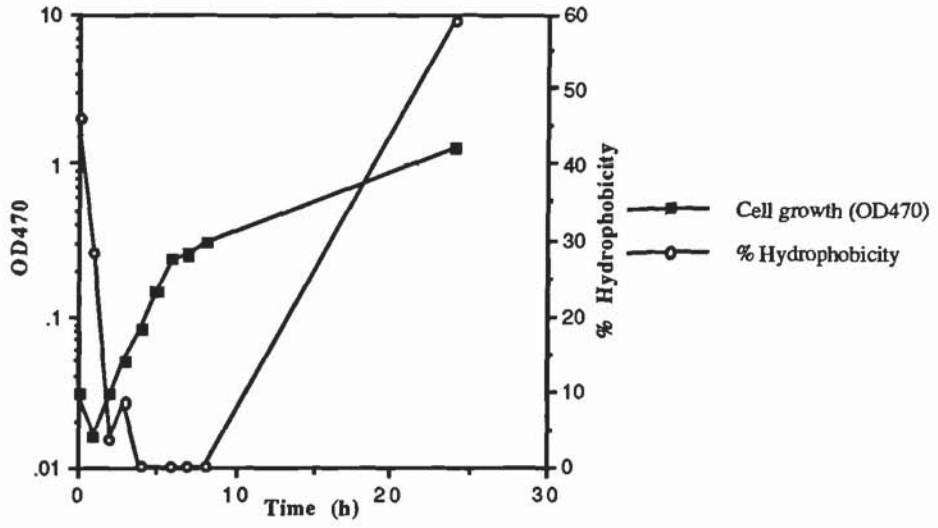


Fig. 5.12. Specific retention on octyl-sepharose columns (% hydrophobicity) of PAO1 JD during batch culture in CDM+Fe.

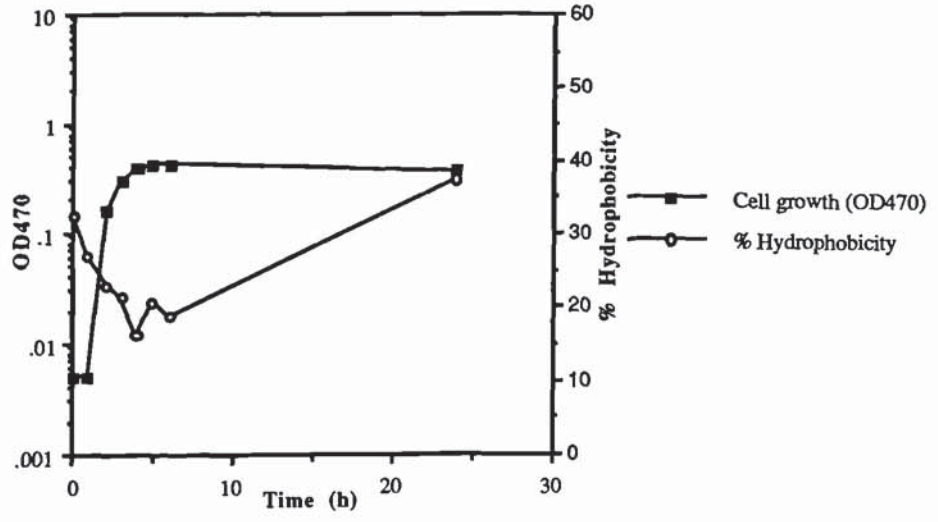
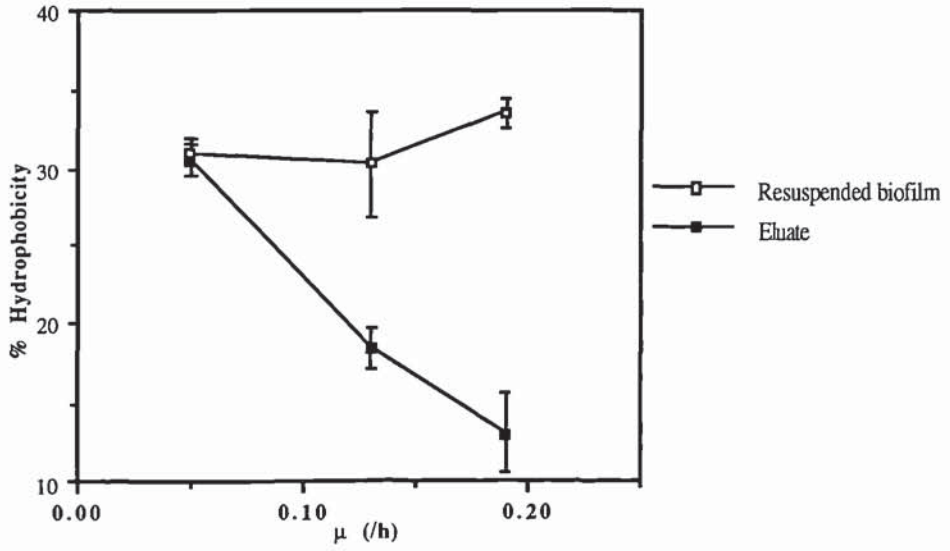


Fig. 5.13. Hydrophobicity (measured by HIC) of iron-restricted Sorbarod eluate and resuspended biofilm cells of PAO1 JD, at different growth rates.



5.2.3. Discussion

Growth phase of *P. aeruginosa* PAO1 JD had a noticeable effect on cell surface hydrophobicity, with batch-grown, iron-restricted cells becoming markedly more hydrophilic through logarithmic phase and remaining as such as growth rate slowed. Iron-plentiful cells followed this trend also, although the drop in hydrophobicity was less pronounced. Both sets of populations showed maximal hydrophobicity in late stationary phase. A previous study of laboratory and clinical strains of *P. aeruginosa* also reported a decrease in hydrophobicity during exponential growth phase (for up to six hours), hydrophobicity increasing slightly thereafter (Elsheikh *et al*, 1985). Both *E. coli* and *S. epidermidis* displayed similar patterns of change in hydrophobicity, measured by HIC, to *P. aeruginosa* (Evans, 1990) and a later study found that adhesiveness of these organisms decreased in early to mid exponential phase (Gilbert *et al*, 1991). Study of the cell surface hydrophobicity of growth-rate-controlled (chemostat) iron-limited cells of mucoid *P. aeruginosa* indicated an increase with growth rate (Allison *et al*, 1990b), as did *S. aureus* (Domingue *et al*, 1989). Comparison of this cell surface characteristic of phosphate-plentiful and limited PAO1, grown in continuous culture, demonstrated remarkably different growth rate effects for the two limitations (Wood, 1990). Phosphate-limited cells' surface hydrophobicity decreased with growth rate whilst the opposite trend was observed for phosphate-plentiful populations. The wide range of results achievable, of which but a few examples have been outlined above, only serve to underline the variable nature of bacterial cell surface hydrophobicity. The outcome of such studies is not only dictated by the rate and phase of growth of the organism, but also by the species, strain and nature of the cell envelope.

Marked differences in cell surface hydrophobicity were observed for resuspended Sorbarod biofilm and eluate cells, measurements for biofilm cells remaining relatively constant over the range of growth rates whilst, in contrast, eluate cells displayed decreasing hydrophobicity. A further result from a Sorbarod unit operated at an extremely rapid flow rate (5ml/min), confirmed this trend, with resuspended biofilm cells showing 36%, and eluate cells 0%, retention. (Unfortunately, only an estimated

specific growth rate, of $0.22h^{-1}$, could be calculated for this unit since the flow rate proved to be beyond the maximal rate which the system could physically withstand. Thus, the biofilm bioburden was not ascertained at the originally appointed time for breaking the system down, since backflow of medium had been occurring for some time). It was of interest to note that, at the slowest growth rate, the biofilm and eluate cells possessed almost identical hydrophobicity. At this flow rate eluate cell number, as a percentage of the overall biomass present, was extremely low (see table 3.2.) and the biofilm population appeared to stop increasing (fig. 3.18). It may be possible that a good proportion of cell release at this stage was a consequence of the shear forces of the flowing medium, hence the similarity in hydrophobicity values of "released" cells and biofilm cells. Otherwise, some factor(s) other than hydrophobicity or shear forces must account for the preferential release of cells of seemingly similar hydrophobicity. Referring again to table 3.2., by 48h, eluate cell number as a percentage of total biomass had decreased from previously high levels for the intermediate growth rate cells, and increased for the fastest growing cell population.

There appears to be a degree of correlation between hydrophilicity and increasing cell release from the biofilm, although this will be intimately tied in with other factors such as shear forces as well as the general physiological consequences of increasing numbers in the biofilm as a whole. For example, rapidly-growing populations initially releasing, as well as retaining, large quantities of cells may then decrease their overall growth rate due to cell density and oxygen limitation, and concomitantly release less cells as the population adapts to this less favourable environment. The population subjected to the intermediate flow rate (0.5ml/min) was initially calculated to be reproducing most rapidly, but this rate subsequently declined, as did the proportion of divided cells being eluted with the medium. Clearly, some major physiological alterations were taking place within the population and heterogeneity within the system is very likely.

Referring to the information available from the study of OM profiles of the Sorbarod-grown cells (fig. 5.9.), no major differences were seen between biofilm and eluate samples at any flow rate. However, LPS profiles (fig. 5.10) showed differences

between samples in the intensity of O-antigen laddering and relative amounts of LPS:total protein present. Lipid A alterations with growth rate were noticed, with increasing levels of lipid A as growth rate of biofilm cells decreased, which suggests increasing cell surface hydrophobicity. The fastest-growing cells appeared also to have a higher proportion of core region along with lipid A and this might negate any large increase in hydrophobicity, tying in with the observed marginal increase with growth rate for hydrophobicity values determined for resuspended biofilm cells. Correlation between LPS and hydrophilicity of eluate cells was rather difficult to ascertain since the profiles for these cells were less visible. Levels of lipid A (with no extra low molecular weight core region) could be seen to decrease as growth rate increased, suggesting a decrease in cell surface hydrophobicity, which agrees with the findings of this section. However, relative amounts O-antigen banding for the eluate cells were not obvious and so little comment can be made on the contribution of these hydrophilic side chains to the overall cell hydrophilicity. Indeed, with the biofilm cells exhibiting more obvious O-antigen banding, it would have been thought that these cells were more hydrophilic than their released counterparts. Interestingly, though, comparison of the LPS profiles of biofilm and eluate cells from the slowest flow rate (which were of similar hydrophobicity) shows that they appeared to contain similar amounts of lipid A, and neither showed extra high molecular weight core regions. In contrast, profiles for the fastest-growing biofilm and eluate cells differed to the greatest extent. Several explanations for the whole scenario may be relevant:

1) the eluate cells were hydrophilic as a consequence of A-band LPS, not obvious from LPS profiles, as has been found for cells isolated from the CF lung (Lam *et al*, 1989),
2) newly-produced eluate cells are fundamentally different from their parents (although some are not released as a consequence of physical entrapment within the biofilm), or
3) factors other than relative amounts of hydrophobic lipid A and hydrophilic core and O-antigen regions play a predominant role in the outcome of cell release from the system.

Similar studies on cell surface hydrophobicity of a clinical mucoid isolate from CF

sputum showed a different trend in levels of hydrophobicity (Allison *et al*, 1990a). Eluate cell hydrophobicity was only slightly affected by growth rate and, at all growth rates, such cells were more hydrophilic than their adherent counterparts. Biofilm and chemostat cells demonstrated increasing hydrophobicity with growth rate. In contrast, cell surface hydrophobicity of resuspended *E. coli* biofilm cells decreased with growth rate whilst, as for mucoid *P. aeruginosa*, the hydrophobicity of the eluate cells remained relatively unaffected by growth rate (Allison *et al*, 1990b). The authors concluded that, since the daughter cells were uniformly hydrophilic, cell surface hydrophobicity must change at some point during the cell division cycle. Thus, not only are differences seen between different bacterial species, exemplified by the opposing trends in surface hydrophobicities with growth rate of the biofilm cells described above, but so too are there fundamental differences between different strains of the same species. Mucoid *P. aeruginosa* eluate cells showed little change with increasing growth rate whilst, in this project, significant growth rate effects were observed on cell surface hydrophobicity of PAO1 eluate cells.

Nevertheless, released cells appear generally to be more hydrophilic than their surface-associated counterparts. It has been suggested that this may be a strategy employed by biofilm populations for the dispersal of new cells, where avoidance of phagocytosis *in vivo* or digestion by amoebae in natural environments would be enhanced by the relative hydrophilicity of the "scout" cells (Evans, 1990).

Van Loosdrecht *et al* (1987) found that, for strains of *Alcaligenes*, *Arthrobacter* and *Pseudomonas*, cell surface hydrophobicity (measured by contact angles) tended to increase as cells grew more quickly in chemostat culture. In agreement, it has been reported that increased adhesion of *Pseudomonas* species occurred in exponential phase cultures (Fletcher, 1977) whereas contradictory evidence showed that extremely slowly growing starved cells had an increased surface hydrophobicity and tendency to attach to surfaces (Kjelleberg and Hermansson, 1984). Complicating matters further, observations on the effect of arresting organisms' activity on their attachment resulted in increased, decreased or unchanged attachment, depending upon the species of bacterium being investigated (Fletcher, 1980). The surface of many marine bacteria

becomes increasingly hydrophobic and the cells become more adhesive during starvation (Kjelleberg *et al*, 1987) and it has been noted that starved *Legionella* cells fed into a Robbins device adhered more efficiently than growing cells (H. Lappin-Scott, personal communication). The phenomenon of the starved cell, then, provides one of the most interesting examples of bacterial adaptation to its environment. However, it is a multifaceted response to stressed surroundings and hydrophobicity is only one of many responses to/ consequences of such environments.

Hydrophobicity, itself, is a multifaceted process and this area of study remains somewhat confusing, with a seemingly inexhaustible number of contradictions contributing to the overall body of knowledge of the subject. Clearly, there is considerable species variation in the dependence of surface hydrophobicity upon growth phase and rate and, further still, variation between different strains of the same species. Although most research has focussed on the role of hydrophobicity during the process of bacterial attachment to surfaces, it probably also has a bearing on detachment processes, as alluded to by the findings of the Sorbarod work. However, as with attachment, many other factors are likely to be involved.

5.3. SIDEROPHORE PRODUCTION OF PAO1 JD

5.3.1. Introduction

Iron is indispensable as a micronutrient to almost all living organisms, yet is present only in low amounts as a freely soluble, ferric ion in nature. Most bacterial growth depends on iron's involvement in a large number of enzymatic reactions, both as a cofactor and a prosthetic group (Martinez *et al*, 1990). In man, ferric iron is bound and transported in plasma and secretions by transferrin and lactoferrin whilst non-functional iron is stored in the cells as ferric oxohydroxy crystals within ferritin molecules (Hershko *et al*, 1988). This results in an extremely low concentration of free ionic iron in body fluids (10^{-18}M Fe^{3+}; Bullen *et al*, 1981) yet pathogenic bacteria can multiply successfully *in vivo* to establish an infection as a consequence of possessing specialised iron uptake and transport systems (Crosa, 1989). One of the most commonly found strategies of iron entrapment evolved by microorganisms is the production of siderophores, low molecular weight iron chelators with an extreme affinity for the trivalent oxidation state of iron (Bagg and Neilands, 1987).

A number of siderophores have been described, the majority of which are either phenolic (catechols) or hydroxamic acid compounds (Martinez, 1990). *E. coli*, for example, produces enterobactin, a phenolate-type siderophore and the plasmid-encoded aerobactin, a hydroxamate-type siderophore (Crosa, 1989). Aerobactin is a known virulence factor in *E. coli* whilst the role of enterobactin as a virulence factor in Enterobacteriaceae has been questioned, (Benjamin, 1985; De Lorenzo and Martinez, 1988; Brock *et al*, 1991) despite it having the higher affinity constant of the two siderophores for iron. A possible explanation for this seemingly illogical situation, however, lies in the fact that during the invasive process, bacteria tend to become intimately associated with host tissues in preference to remaining free in the bloodstream. If other siderophores can, like desferrioxamine for instance, scavenge iron from cells, then cell-associated iron may present a more readily available source than transferrin (Brock *et al*, 1991). Since many mammalian cells, macrophages and

hepatocytes in particular, continually release iron as part of the normal pattern of iron recirculation and there is little evidence that it is attached to a carrier molecule (Brock *et al*, 1984), then any siderophores present in iron-releasing tissues may be able readily to intercept before it is bound to transferrin. Results of the work of Brock *et al* (1991) suggested strongly that, in some cases, cell-delivered iron may actually be more readily available than iron bound to transferrin, thus aerobactin and enterobactin, with their different affinities, may acquire iron from different sources.

Other mechanisms of bacterial iron uptake exist, such as the OM protein receptors evolved by *Neisseria* species which actually recognise the complex of lactoferrin or transferrin with iron, thus dispensing with the need for siderophores to aid the internalisation of iron (Mickelsen and Sparling, 1981), and the ferric-citrate uptake mechanisms of *E. coli* (Frost and Rosenberg, 1973) and *P. aeruginosa* (Cox, 1980). However, in this project, the focus of attention for iron-restriction and uptake was on the siderophore-mediated mechanism only and the following section gives a brief overview of this method of iron-sequestration.

5.3.1.1. Siderophore-mediated iron uptake in *P. aeruginosa*

A schematic diagram of bacterial siderophore-mediated iron acquisition is shown (fig. 5.14.). Under iron-deficient conditions, bacteria such as *P. aeruginosa* synthesise siderophores concomitantly with a number of proteins involved in the ferrisiderophore uptake complex. This iron-siderophore complex binds to the OM via a specific receptor located on the OM, then is transferred from the OM receptor to a periplasmic carrier protein. The ferrisiderophore complex is then released from its complex with the carrier to a CM protein complex and it is thought that the ferrisiderophore may be translocated across the CM, in an energy-dependent process, where subsequent release of the ferrisiderophore complex leads to the eventual release of the iron in the cell (Braun *et al*, 1991).

P. aeruginosa synthesises two known siderophores under iron-limiting conditions,

pyochelin and pyoverdin (Poole *et al*, 1991), the structures of which are given in fig. 5.15. Pyochelin appears to be produced by all strains and the IRMP uptake systems for this siderophore have been identified as a 14KDa ferripyochelin binding protein (Sokol and Woods, 1983) and a 75KDa IRMP (Heinrichs *et al*, 1991). Pyochelin has been shown also to bind other transition metals such as Cu(II), Co(II), Mo(VI) and Ni(II) (Visca *et al*, 1992) and may play a role in their delivery to *P. aeruginosa*. Strains of *P. aeruginosa* produce several different pyoverdins, yellow-green chromopeptides which are structurally related to pseudobactins synthesised by fluorescent pseudomonads (Demange *et al*, 1987). Recent data have identified the 85KDa IRMP of *P. aeruginosa* PAO1 as the specific receptor for its own ferripyoverdin, as well as different ferripyoverdins from other strains, and also indicated the existence of a second ferripyoverdin uptake system of lower affinity and broader specificity (Gensberg *et al*, 1992; Smith *et al*, 1992). A 90KDa ferripyoverdin transporter was reported in a pyoverdin-deficient strain by Poole *et al* (1991), this group also providing evidence for the probable existence of a second transport system.

5.3.1.2. Iron restriction *in vivo*

Bacteria have been shown to grow under iron-restricted conditions in human infections (Anwar *et al*, 1984; Brown *et al*, 1984; Lam *et al*, 1984; Shand *et al*, 1985; Ward *et al*, 1988) and in experimental animal models (Griffiths *et al*, 1983; Sciortino and Finkelstein, 1983). More recent work reported that antibodies to IRMPs only appeared in the serum of infected CF patients in the advanced stages of the disease (Shand *et al*, 1991), perhaps due to the microcolony mode of growth, a masking effect of mucoid EPS or altered dynamics for iron uptake. The finding that elastase secreted by *P. aeruginosa* can completely cleave transferrin, but not lactoferrin, and make iron available to the bacteria (Döring *et al*, 1988) may be of great relevance to the situation in the CF lung. Although lactoferrin is the major iron-binding protein in this environment, transferrin is present in CF lung fluid (Fick *et al*, 1984) and the production of protease is stimulated by iron-limiting conditions (Ombaka *et al*, 1983). Thus, secretion of proteases early in infection may act as a virulence mechanism, increasing the availability

Fig. 5.14. Schematic representation of siderophore-mediated iron uptake in Gram-negative bacteria.

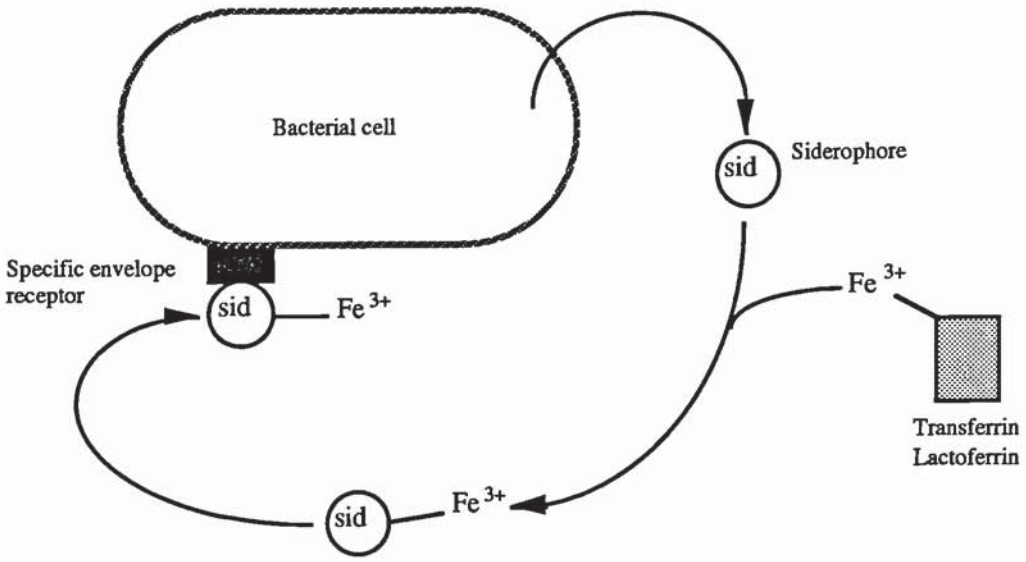
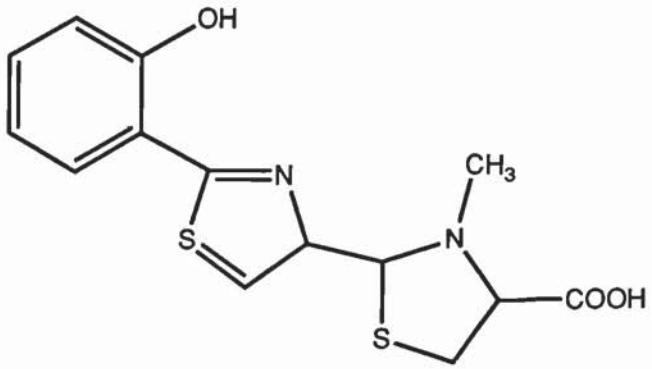
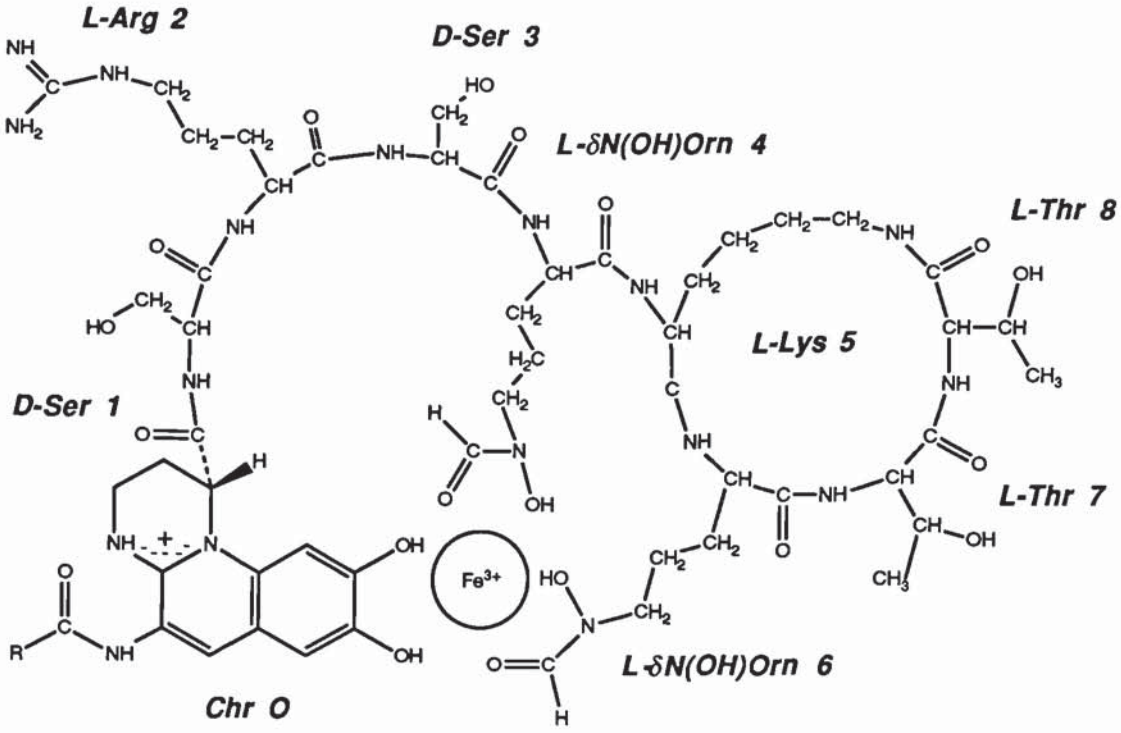


Fig. 5.15. The structures of pyochelin (a) and pyoverdin (b). (Adapted from Demange *et al*, (1987) and Morel *et al*, (1992), respectively).

(a)



(b)



R = CH_2-CH_2-$CONH_2$ in *P. aeruginosa* pyoverdin *Pa*. HPLC analysis of pyoverdin has revealed the presence of other minor compounds, *Pa A, B and C* whose structures differ only in the chromophore (Demange *et al*, 1987).

of free iron to the colonised bacteria, and this may explain the seemingly delayed production of antibodies to IRMPs in serum. It has not been reported for *P. aeruginosa* that iron may be scavenged directly from cells, as was mentioned possible previously for *E. coli* (Brock *et al*, 1991), but this would also be of relevance in the CF lung, where *P. aeruginosa* is growing in the form of a tissue-associated biofilm. Haas *et al* (1991a, b) reported that *P. aeruginosa* isolated from CF patients synthesised pyoverdin and pyochelin, indicating that the CF lung is indeed an iron-stressed environment. Since the CF patient's immune system has been reported widely to be fully functional (Parmley and Hovart, 1986; Berger *et al*, 1989; Kharazmi, 1991) then the presence, in copious amounts, of macrophages and hepatocytes which are releasing free iron will ultimately provide a source of iron, some of which which may be entrapped by the siderophores before it can be bound by lactoferrin or transferrin.

Siderophore production, in response to iron deprivation, is obviously an important virulence mechanism playing a role in the success of *P. aeruginosa* pathogenesis. The purpose of this section of study was to carry out some preliminary work to determine whether production of siderophores by PAO1 JD was affected by the growth phase, rate or mode of the organism.

5.3.2. Results

Siderophore assays were carried out as described in section 2.11.1. "Siderophore units" describes the drop in OD_{630} of the chrome azurol-siderophore-iron culture supernatant mixture as a result of iron-chelation by any siderophore present in the sample.

Through batch growth of PAO1 JD in CDM_{12}-Fe, as can be seen from fig. 5.16., the amount of siderophore present in the culture medium increased with cell density, after an initial lag period of 4h. Expressed as units of siderophore produced per 10^6 cfus, however (fig. 5.17.), it can be seen that after the initial presence of a high amount of siderophore (probably due to carry-over from the initial inoculum), actual siderophore

production remained at a fairly constant level, increasing only slightly as cell growth slowed. Relating the pattern of siderophore production to the OM profiles of PAO1 JD grown in batch culture (fig. 5.3.), siderophore production for the particular culture from which the OMs were prepared was detectable by OD_{470} 0.202, and it was noticeable that at this point, the 83KDa IRMP was expressed less strongly than previously. By stationary phase, the 85KDa IRMP was, of all the IRMPs, expressed to the greatest extent and siderophore production per cell quantity remained fairly static. Fig. 5.18. illustrates the relationship between viable cell number in the chemostat and siderophore production with increasing dilution rate. Over the range of growth rates shown, siderophore production increased with growth rate.

Rates of cell release from an iron-restricted Swinnex biofilmer system and siderophore production (fig. 5.19.) both increased over time. Direct comparison of siderophore production and total cell biomass present was not possible since the actual numbers of biofilm cells present at each time point were not known. However, such a study was undertaken using the Sorbarod biofilmer system, with siderophore assays carried out on eluate from the three different flow rates looked at (0.02, 0.5 and 1.15ml/min). Only in the slowest system was there any detection of siderophore, though, with amounts increasing over the 38h time period. Presence of siderophore in the eluate, in relation to the overall cell biomass present, increased with time (fig. 5.20.). Putting this in perspective, total biomass size approximately doubled over the 38h time period, whilst siderophore production/h increased 50-fold.

Fig. 5.16. Siderophore production, measured by the Schwyn-Neilands method, through batch culture growth of PAO1 JD in CDM_{12}-Fe.

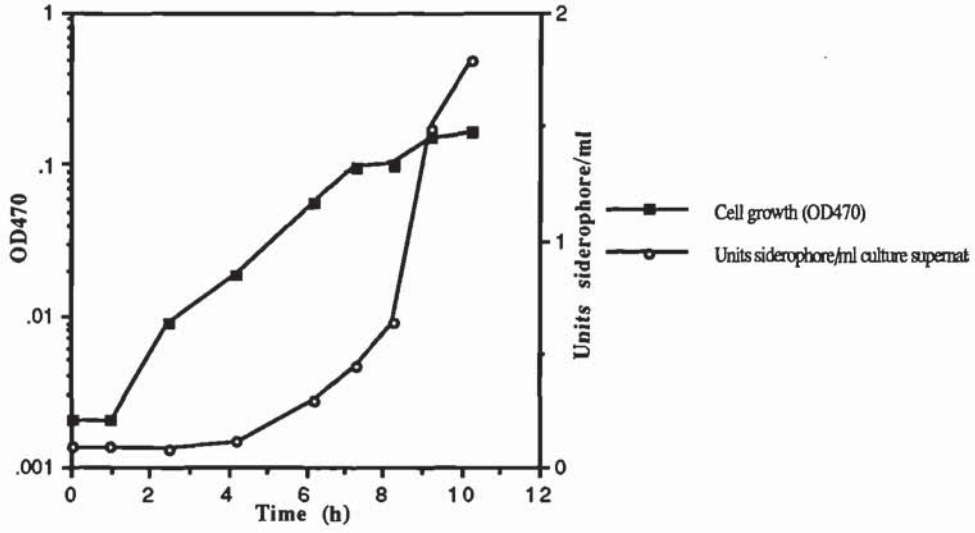


Fig. 5.17. Siderophore production/10^6 cfus, through batch culture growth of PAO1 JD in CDM_{12}-Fe.

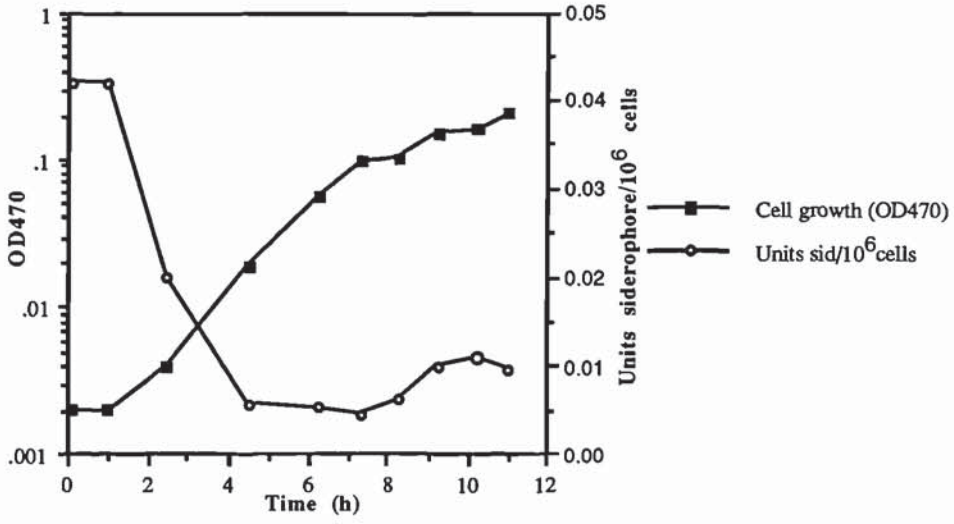


Fig. 5.18. Siderophore production/10^8 viable cfus of PAO1 JD in iron-restricted chemostat culture.

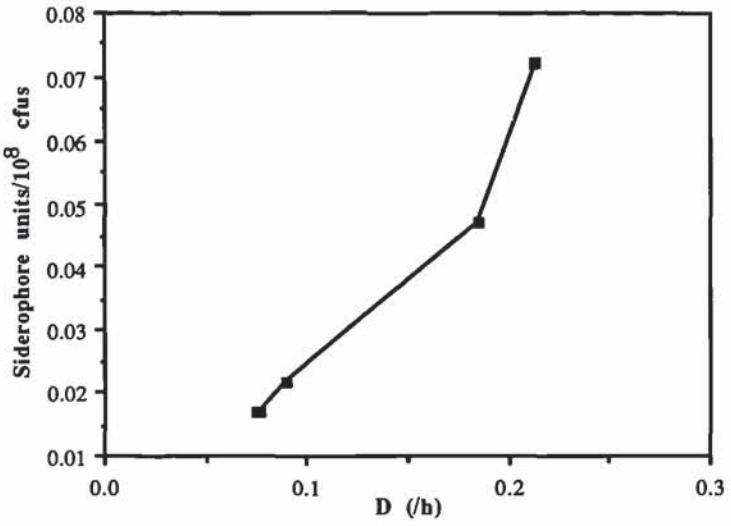


Fig. 5.19. Rates of siderophore production and cell release per hour from Swinnex biofilmer system run under iron-restriction (flow rate = 30ml/h) for 22h.

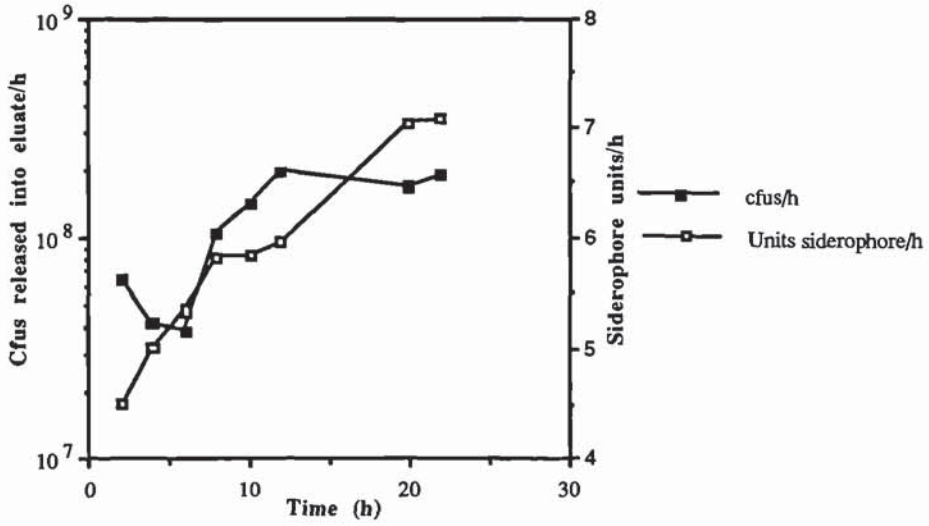
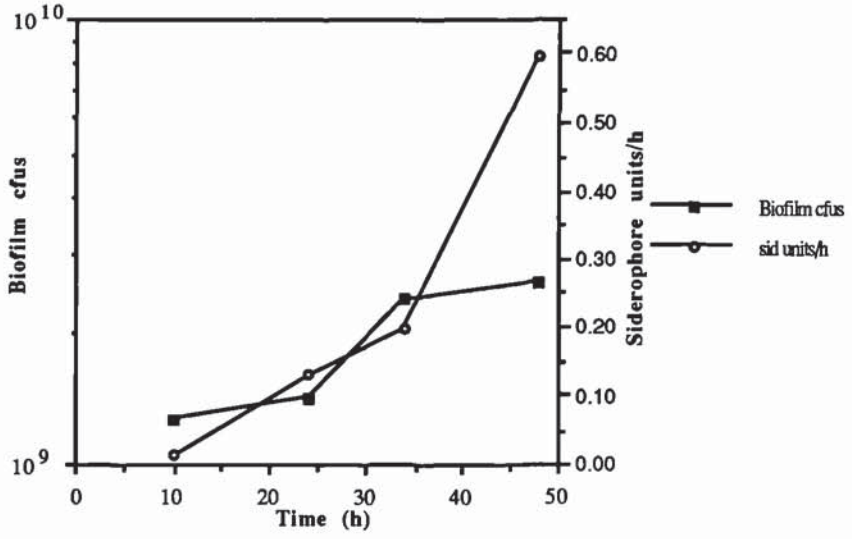


Fig. 5.20. Relationship between rate of siderophore production by iron-restricted PAO1 JD, detected in eluate, and total cell biomass present in Sorbarod biofilmer system run for 48h (flow rate = 1.2ml/h).



5.3.3. Discussion

The Schwyn and Neilands (1987) method for the detection of siderophores in culture fluid supernatant, although of use in the indication of siderophore production in general, does not distinguish between the type of siderophores produced by the bacterial culture. It works only on the principle that siderophores have a higher affinity for Fe(III) than the chrome azurol dye present in the experimental reagent, and is thus independent of their structure. Initially, such a crude level of detection was all that was adjudged to be necessary for the needs of this section of project work. However, in light of the results obtained, precise identification of specific siderophore production (i.e. pyochelin and/ or pyoverdin) would have been more informative. This could possibly have been achieved by an HPLC method (M. Abdallah, personal communication) but would have required much larger volumes of culture than could be obtained from the experimental systems.

As cell density increased through shake flask batch culture of PAO1 JD in iron-restricted medium, there was an increase in the amount of siderophore detected, corresponding to over 30μM of Desferal equivalents. However, expressed as siderophore production relative to cell numbers present, during logarithmic phase the rate of siderophore production did not increase, although it appeared to increase as the cells moved into deceleration phase. Study of the OM profiles of PAO1 through batch culture growth showed that the expression of the two dominant IRMPs switched over the course of the experiment. The presence of a 75KDa IRMP reported by Heinrichs *et al* (1991) was not obvious although a band in the 9-14KDa range, where the ferripyochelin binding protein (fbp) has been described as being resolved (Sokol and Woods, 1983), was present. Possible explanations for these observations could be that, during exponential phase, either there was less requirement for iron or there was sufficient to be scavenged from the medium and an OMP with only low iron-binding affinity was required, and as iron became scarce, more siderophore was produced and higher affinity iron-uptake systems were required. Heinrichs *et al* (1991) noted that induction of the 75KDa protein required growth to the late log or early stationary phase.

In chemostat culture, siderophore production per cell quantity increased over the range of dilution rates looked at, this observation in agreement with those of Wood (1990) for the growth of PAO1 in iron-limited/ phosphate plentiful medium, and Lodge (1987) for growth of *Klebsiella aerogenes* in iron-limited culture. Wood (1990) noted also that phosphate-limited cells expressed approximately ten-fold less siderophore than phosphate-plentiful cells, suggesting that a phosphate-limited environment reduced the requirement for iron. In iron-limited culture, however, it appears that, over the range of growth rates shown, as the cell grows faster its requirement for iron increases. It is well documented that iron plays a role in many cellular processes such as oxygen and electron transport and enzyme function (Hershko *et al*, 1988). Even in iron-replete/ carbon-limited culture as growth rate of *K. aerogenes* increased, it produced increasing amounts of enterochelin (Lodge, 1987) reflecting the greater need for iron, in general, of cells with high metabolic activity.

Direct comparison of siderophore production in the Swinnex and Sorbarod biofilm systems could lead to rather misleading conclusions as a result of the different type of biofilm systems which they represent. Most, if not all, siderophore produced by cells growing on the flat membrane filter of the Swinnex system will be eluted with the constant through-put of medium. By contrast, the Sorbarod is of considerable depth and there is more opportunity for ferrisiderophore complexes to be taken up by the cells at different depths within the Sorbarod, particularly if the flow rate is extremely slow (e.g. 0.02ml/min). In the Swinnex system, as eluate cell numbers increased, siderophore detection increased also. Knowledge of the total biomass present in the system would have given some indication of levels of siderophore production per cell. With this in mind, siderophore production per hour in relation to the biofilm biomass was calculated using the Sorbarod biofilm system and was found to increase to a much greater extent than did the actual cell biomass. Thus the surface-associated cells within the Sorbarod became increasingly iron-stressed over the course of the experiment, and despite the fact that the population of cells was calculated as growing extremely slowly, they obviously required a higher level of iron uptake. The slow growth rate could, therefore, be partly a consequence of the lack of iron availability.

It is not entirely clear, then, how siderophore synthesis is affected by growth rate. Certainly, the argument that as a cell grows faster it will require more iron for metabolic processes (Lodge, 1987) appears to be logical. However, it could equally be argued that in a chemostat operating below a particular critical slow dilution rate, it might be expected that the cells are extremely stressed for iron. Hansford and Humphrey (1966) warned that obtaining uniform distribution of the growth rate limiting nutrient among cells in the chemostat approached mechanical impossibility as D decreased below $0.05h^{-1}$. Thus, if iron is available only in very small amounts and with relatively long periods of time passing between each drop of medium into the system, it would seem probable that the cells would produce more siderophore to entrap any iron that may suddenly become available. This may have been the case in the Sorbarod system, where slowly growing cells appeared to elevate their mechanism for iron entrapment, whilst no siderophore was detectable at the faster rates of media input.

It is known that, under iron-limitation, as well as siderophores, *P. aeruginosa* produces toxin A, haemagglutinin, proteinase, lipase and biosurfactant (Bjorn *et al*, 1979; Guerra-Santos *et al*, 1984). In this project, noticeably more frothing of chemostat cultures occurred at low dilution rates, in agreement with the findings of Persson *et al* (1990) for *P. fluorescens*. These workers noticed also that both nutritional and morphological features of the organism changed with time to a larger extent at a low dilution rate than at a faster rate, especially when limited for phosphate or iron, even suggesting that, since in a chemostat it is an advantage that the steady state culture changes as little as possible with time, it would be advisable not to run such chemostats at very low dilution rates.

Although the results of this section do not clearly define how rate of growth has a bearing on siderophore production, the observation of differential expression of the high molecular weight IRMPs during batch culture growth might suggest preferential production of one or another siderophore, depending upon the growth environment of the cell. Indeed, Gensberg *et al* (1992) recently suggested that *P. aeruginosa* may produce low amounts of each siderophore in iron-deficient growth environments and

monitor which chelator is most effective at complexing iron before becoming committed to, and inducing, iron uptake via that route. The roles of each one of the IRMPs have not been elucidated as yet, however, thus it is not possible to speculate further on this.

The methods used to study siderophore production in this project, it must be stressed, are not indicative of the situation in the iron-restricted lung, for example. In the present scenario, it is envisaged that most iron-chelators synthesised by the cell will be washed from the system, perhaps rendering the bacteria more iron-stressed as a result of their inability to recapture the lost siderophores. This may explain the increase in siderophore production which far outstripped the rate of biomass production within the Sorbarod (the biofilm population merely doubling, whilst siderophore production/h increased almost fifty-fold). In the lung, though, siderophore recapture will be possible, aiding the overall success of colonisation of and survival within the host.

6. CONCLUDING REMARKS

Throughout the preceding chapters, the significance and consequences of bacterial growth associated with surfaces were emphasised continually. The requirement to understand fully such a growth strategy, in order to prevent, control or eradicate bacterial populations where necessary, has led to many *in vitro* models of growth on a solid substratum. Each has its own particular merits, but the technique described by Gilbert *et al* (1989) was the first to attempt to control the growth rate of the biofilm population and, for this reason, it was determined to apply this method to study biofilm growth of *P. aeruginosa* PAO1.

The modified glass fermenter (47mm biofilm device) had been used previously to study physiological growth characteristics of *E. coli* and *S. epidermidis*, both species forming steady-state biofilm populations on the filter membrane surface (Evans, 1990; Evans *et al*, 1991b). *P. aeruginosa* PAO1 grown by this method, however, behaved in a manner quite different to these organisms. A prolonged steady-state biofilm was never achieved and the design of the system was such that, with the increasing biofilm size, the hydrostatic head of perfusing medium would build up as a consequence of the blocking effect of cells and associated EPS. This liquid would build up to exceed the physical limits of the glass fermenter, often accompanied by leakage of cells and medium from around the sides of the membrane, an area which could be considered as the weak-point of the apparatus. Inevitably, this led to the abandonment of each experimental run. It has since been noted that *S. aureus* behaves in a fashion similar to that of PAO1 (A. Hodgson, personal communication).

Thus, the problems encountered with this apparatus necessitated the use of a modified technique which, nevertheless, still operated on the principle of perfusion of medium to control the biofilm population growth rate. Again, however, build-up of cells on the membrane was the root of the difficulties faced with the new "Swinnex" biofilmer. With hydrostatic head build-up precluded by virtue of the apparatus design, increased pressure within the system became the major complication, causing cells and medium to

be forced from the sides of the device. This would occur in a time- and biomass-dependent manner, with experiments involving reduced inoculum loadings able to run for longer periods of time before leakage began.

The behaviour of *P. aeruginosa* should, perhaps, not have been so surprising as there are reports in the literature which suggest that *Pseudomonas* sp., on solid surfaces, display a tendency to divide and remain associated with the substratum, often migrating across the surface to other areas (Marshall, 1988; Power and Marshall, 1988). Other work has provided evidence for bacterial migration across surfaces in streams by way of "rolling" manoeuvres (Lawrence and Caldwell, 1987) and *P. fluorescens* daughter cells detached from a surface only after several rounds of division had occurred (Lawrence *et al*, 1987).

Within the constraints of the Swinnex system it was possible, nevertheless, to obtain sufficient surface-grown cells to prepare OMs by a novel mini-method, developed during the project especially for small-scale applications. This method was pivotal to the accomplishment of SDS-PAGE analysis of Swinnex- and Sorbarod-derived samples since, often, only low numbers of cells were retrieved from these units, particularly in the case of eluate cell collection.

One facet of biofilm growth studied using the Swinnex biofilmer was that of antibiotic sensitivity. The ciprofloxacin-perfusion experiments demonstrated that PAO1 biofilm cells were affected by the presence of the quinolone, reflected by the reduced numbers of cells in the eluate during, and for some time after, drug exposure. Subsequent recovery to pre-exposure levels of cells in the eluate indicated that a good proportion of biofilm cells survived drug perfusion and were able to resume growth and division.

The limited applications of the Swinnex biofilmer prompted the development of another biofilm system, one which would not tend to block in the manner of the preceding models. The resultant "Sorbarod" system achieved a much greater degree of success, allowing initial studies on biofilm accumulation, cell envelope biochemistry, antibiotic susceptibility and cell surface hydrophobicity to be carried out. It was demonstrated

clearly that the biofilm population increased over time, at a rate dependent upon the media flow rate, and although further work is required to elucidate the kinetics of the system further, there was certainly a level of growth rate control over the adherent cells. The calculated growth rates never reached the maximal rates of reproduction obtained in batch culture, perhaps due as much to oxygen-limitation or severe iron-restriction as the actual biofilm mode of growth itself. No appreciable differences in OM profiles were observed for eluate and resuspended biofilm cells, although both population sets, as well as Swinnex-grown cells, expressed bands not seen in planktonically-cultured populations. It might be assumed, then, that growth of PAO1 on a surface induces specific protein production. LPS profiles of eluted and resuspended biofilm cells differed mainly in their relative quantities of lipid A/ core region present, fastest growing cells showing largest amounts of this portion of LPS and biofilm cells appearing to contain more of this region than their eluted counterparts. LPS is known to affect cell hydrophobicity and, interestingly, eluate cells displayed a marked decrease in hydrophobicity with growth rate, in contrast to biofilm cells which remained relatively hydrophobic. At the slowest growth rate, eluate and biofilm cells were of similar hydrophobicity and displayed similar LPS profiles, suggesting that a shear effect may instead account for much of the cell release from the system. Thus, the differences in LPS profiles and corresponding HIC results may go some way to explaining the different patterns of cell release and retention within the system, hydrophilic cells more likely to be released than relatively hydrophobic cells. Study of susceptibility to ciprofloxacin were inconclusive, eluate and biofilm cells appearing to be of similar sensitivity over the range of growth rates analysed.

In summary, this project resulted in the design and implementation of a novel method for generation of growth-rate-controlled biofilm populations as well as the adaptation of the classical method of OM preparation for small-scale use. Results suggest that PAO1 biofilm cells differ from their planktonic counterparts in aspects of their cell envelope and surface biochemistry and that cell surface hydrophobicity of cells released from the biofilm are affected by the population growth rate.

6.1. SUGGESTIONS FOR FURTHER WORK

The Sorbarod biofilm method appears, potentially, to be a useful means for generating *Pseudomonas aeruginosa* biofilms. In the limited time available during this project, it was not possible to investigate a wide range of growth rates using this technique. Therefore, at the outset of any future work, it would be necessary to investigate the outcome of growth of the organism employing a wider range of flow rates through the system. This would, hopefully, improve upon the present method used for the calculation of growth rates by providing a wider range of data from which to draw conclusions on growth kinetics. Inoculation with varying amounts of cells, too, would be of interest in the analysis of optimising overall population growth rates since it is probable that increased cell density within the Sorbarod leads to reduced growth rates.

Once fully calibrated, the studies on cell envelope and surface biochemistry could be extended, investigation of hydrophobicity and LPS being of particular interest. If the cells released from a biofilm are relatively hydrophilic and therefore engulfed less easily by phagocytes, then the actual growth rate of the biofilm may play a role in spread of infection *in vivo* since the results of the work completed to date suggest that more slowly-growing PAO1 biofilms release more hydrophobic cells. Novel OM protein synthesis on solid surfaces and positive identification of such proteins is another area worth deeper investigation, since protein bands separated by SDS-PAGE in the region of 54-60KDa have been reported as being associated with factors ranging from the appearance of the mucoid phenotype to resistance to quinolone drugs.

7. REFERENCES

ABSOLOM, D.R. (1988). The role of bacterial hydrophobicity in infection: bacterial adhesion and phagocytic ingestion. *Canadian Journal of Microbiology* **34**, 287-298.

ALLISON, D.G. & Sutherland, I.W. (1984). A staining technique for attached bacteria and its correlation to extracellular carbohydrate production. *Journal of Microbiological Methods* **2**, 93-99.

ALLISON, D.G. & Sutherland, I.W. (1987). The role of exopolysaccharides in adhesion of freshwater bacteria. *Journal of General Microbiology* **133**, 1319-1327.

ALLISON, D.G., Brown, M.R.W., Evans, D.J. & Gilbert, P. (1990a). Surface hydrophobicity and dispersal of *Pseudomonas aeruginosa* from biofilms. *FEMS Microbiology Letters* **71**, 101-104.

ALLISON, D.G., Evans, D.J., Brown, M.R.W. & Gilbert, P. (1990b). Possible involvement of the division cycle in dispersal of *Escherichia coli* from biofilms. *Journal of Bacteriology* **172**, 1667-2669.

ANWAR, H. (1981). Fluorescence studies on the structure of bacterial envelopes. Ph.D. Thesis. University of Aston, Birmingham.

ANWAR, H. & Costerton, J.W. (1990). Enhanced activity of combination of tobramycin and piperacillin for eradication of sessile biofilm cells of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **34**, 1666-1671.

ANWAR, H. & Costerton, J.W. (1992). Effective use of antibiotics in the treatment of biofilm-associated infections. *ASM News* **58**, 665-668.

ANWAR, H., Brown, M.R.W., Day, A. & Weller, P.H. (1984). Outer membrane antigens of mucoid *Pseudomonas aeruginosa* isolated directly from the sputum of a cystic fibrosis patient. *FEMS Microbiology Letters* **29**, 225-230.

ANWAR, H., Dasgupta, M., Lam, K. & Costerton, J.W. (1989a). Tobramycin resistance of mucoid *Pseudomonas aeruginosa* biofilm grown under iron limitation. *Journal of Antimicrobial Chemotherapy* **24**, 647-655.

ANWAR, H., Van Biesen, T., Dasgupta, M., Lam, K. & Costerton, J.W. (1989b). Interaction of biofilm bacteria with antibiotics in a novel *in vitro* chemostat system. *Antimicrobial Agents and Chemotherapy* **33**, 1824-1826.

ANWAR, H., Strap, J.L. & Costerton, J.W. (1992a). Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial chemotherapy. *Antimicrobial Agents and Chemotherapy* **36**, 1347-1351.

ANWAR, H., Strap, J.L., Chen, K. & Costerton, J.W. (1992b). Dynamic interactions of biofilms of mucoid *Pseudomonas aeruginosa* with tobramycin and piperacillin. *Antimicrobial Agents and Chemotherapy* **36**, 1208-1214.

AUBERT, G., Pozzetto, B. & Dorche, G. (1992). Emergence of quinolone-imipenem cross-resistance in *Pseudomonas aeruginosa* after fluoroquinolone therapy. *Journal of Antimicrobial Chemotherapy* **29**, 307-312.

BAGG, A. & Neilands, J.B. (1987). Molecular mechanism of regulation of siderophore-mediated iron assimilation. *Microbiological Reviews* **51**, 509-518.

BAKER, N.R. & Svanborg-Edén, C. (1989). Role of alginate in the adhesion of *Pseudomonas aeruginosa*. In *Pseudomonas aeruginosa Infection*, pp. 72-79. Eds. N.Høiby, S.S. Pedersen, G.H. Shand, G. Döring, G. & Holder, I.A. Basel: Karger.

BAKER, N.R., Minor, V. Deal, C., Shahrabadi, M.S. Simpson, D.A. & Woods, D.E. (1991). *Pseudomonas aeruginosa* exoenzyme S is an adhesin. *Infection and Immunity* **59**, 2859-2863.

BALTIMORE, R.S. & Mitchell, M. (1980). Immunologic investigations of mucoid strains of *Pseudomonas aeruginosa*: comparison of susceptibility to opsonic antibody in mucoid and nonmucoid strains. *Journal of Infectious Diseases* **141**, 238-247.

BANDYK, D.F. (1991). Prosthetic infections due to bacterial biofilms: clinical and experimental observations. *Journal of Vascular Surgery* **13**, 757-758.

BARRY, A.L. & Jones, R.N. (1984). Cross-resistance among cinoxacin, DJ-6783, enoxacin, nalidixic acid, norfloxacin, and oxolinic acid after the *in vitro* selection of resistant populations. *Antimicrobial Agents and Chemotherapy* **25**, 775-777.

BAYER, A.S., Speert, D.P., Park, S., Tu, J., Witt, M., Nast, C.C. & Norman, D.C. (1991). Functional role of mucoid exopolysaccharide (alginate) in antibiotic-induced and polymorphonuclear leukocyte-mediated killing of *Pseudomonas aeruginosa*. *Infection and Immunity* **59**, 302-308.

BAYER, A.S., Park, S., Ramos, M.C., Nast, C.C., Eftkhar, F. & Schiller, N.L. (1992). Effects of alginase on the natural history and antibiotic therapy of experimental endocarditis caused by mucoid *Pseudomonas aeruginosa*. *Infection and Immunity* **60**, 3979-3985.

BEDARD, J., Wong, S. & Bryan, L.E. (1987). Accumulation of enoxacin by *Escherichia coli* and *Bacillus subtilis*. *Antimicrobial Agents and Chemotherapy* **31**, 1384-1345.

BEDARD, J., Chamberland, S., Wong, S., Schollaardt, T. & Bryan, L.E. (1989). Contribution of permeability and sensitivity to inhibition of DNA synthesis in determining susceptibilities of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Alcaligenes faecalis* to ciprofloxacin. *Antimicrobial Agents and Chemotherapy* **33**, 1457-1464.

BELL, A., Bains, M. & Hancock, R.E.W. (1991). *Pseudomonas aeruginosa* outer membrane protein OprH: expression from the cloned gene and function in EDTA and gentamycin resistance. *Journal of Bacteriology* **173**, 6657-6664.

BENJAMIN, W.H., Turnbough Jr., C.L., Posey, B.S. & Briles, D.E. (1985). The ability of *Salmonella typhimurium* to produce the siderophore enterobactin is not a virulence factor in mouse typhoid. *Infection and Immunity* **50**, 392-297.

BERGER, M., Sorensen, R.U., Tosi, M.F., Dearborn, D.G. & Döring, G. (1989). Complement receptor expression on neutrophils at an inflammatory site, the *Pseudomonas*-infected lung in cystic fibrosis. *Journal of Clinical Investigation* **84**, 1302-1313.

BEVERIDGE, T.J. (1988). The bacterial surface: general considerations towards design and function. *Canadian Journal of Microbiology* **34**, 363-372.

BJORN, M.J. B., Sokol, P.A. & Iglewski, B.H. (1979). Influence of iron on yields of extracellular products in *Pseudomonas aeruginosa* cultures. *Journal of Bacteriology* **138**, 193-200.

BLENKINSOPP, S.A., Khoury, A.E. & Costerton, J.W. (1992). Electrical enhancement of biocide efficacy against *Pseudomonas aeruginosa* biofilms. *Applied and Environmental Microbiology* **58**, 3770-3773.

BLOCH, C.A., Stocker, B.A.D., & Orndorff, P.E. (1992). A key role for type 1 pili in enterobacterial communicability. *Molecular Microbiolgy* **6**, 697-701.

BODEY, G.P., Bolivar, R., Fainstein, V. & Jadeja, L. (1983). Infections caused by *Pseudomonas aeruginosa. Reviews of Infectious Diseases* **5**, 279-313.

BOYD, A. & Holland, I.B. (1977). Protein *d*, an iron-transport protein induced by filtration of cultures of *Escherichia coli. FEBS Letters* **76**, 20-24.

BRAUN, V., Günter, K. & Hantke, K. (1991). Transport of iron across the outer membrane. *Biology of Metals* **4**, 14-22.

BROCK, J.H., Esparza, I. & Logie, A.C. (1984). The nature of iron released by resident and stimulated mouse peritoneal macrophages. *Biochimica et Biophysica Acta* **797**, 105-111.

BROCK, J.H., Williams, P.H., Licéaga, J. & Wooldridge, K.G. (1991). Relative availability of transferrin-bound iron and cell-derived iron to aerobactin-producing and enterochelin-producing strains of *Escherichia coli* and to other microorganisms. *Infection and Immunity* **59**, 3185-3190.

BROWN, M.R.W. (1975). The role of the cell envelope in resistance. In *Resistance of Pseudomonas aeruginosa*, pp. 71-107. Edited by M.R.W. Brown. London: J. Wiley & Sons.

BROWN, M.R.W. & Melling, J. (1969). Loss of sensitivity to EDTA by *Pseudomonas aeruginosa* grown under conditions of Mg-limitation. *Journal of General Microbiology* **54**, 439-444.

BROWN, M.R.W. & Williams, P. (1985a). Influence of substrate limitation and growth phase on sensitivity to antimicrobial agents. *Journal of Antimicrobial Chemotherapy* **15**, suppl. A, 7-14.

BROWN, M.R.W. & Williams, P. (1985b). The influence of environment on envelope properties affecting survival of bacteria in infections. *Annual Review of Microbiology* **39**, 527-556.

BROWN, M.R.W., Anwar, H. & Lambert, P.A. (1984). Evidence that mucoid *Pseudomonas aeruginosa* in the cystic fibrosis lung grows under iron-restricted conditions. *FEMS Microbiology Letters* **21**, 113-117.

BROWN, M.R.W., Allison, D.G. & Gilbert, P. (1988). Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? *Journal of Antimicrobial Chemotherapy* **22**, 777-783.

BROWN, M.R.W., Collier, P.J. & Gilbert, P. (1990). Influence of growth rate on susceptibility to antimicrobial agents: modification of the cell envelope and batch and continuous culture studies. *Journal of Antimicrobial Chemotherapy* **34**, 1623-1628.

BROWN, M.R.W., Costerton, J.W. & Gilbert, P. (1991). Extrapolating to bacterial life outside the test tube. *Journal of Antimicrobial Chemotherapy* **27**, 565-567.

BRYAN, L. E. & Bedard, J. (1991). Impermeability to quinolones in Gram-positive and Gram-negative bacteria. *European Journal of Clinical Microbiology and Infectious Diseases* **10**, 232-239.

BRYAN, L.E., O'Hara, K. & Wong, S. (1984). Lipopolysaccharide changes in the impermeability-type aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimcrobial Agents and Chemotherapy* **26**, 250-255.

BUCKMIRE, F.L.A. (1984). Influence of nutrient media on the characteristics of the exopolysaccharide produced by three mucoid *Pseudomonas aeruginosa* strains. *Microbios* **41**, 49-63.

BULLEN, J.J. (1981). The significance of iron in infection. *Reviews of Infectious Diseases* **3**, 1127-1138.

BUSSCHER, H.J. & van der Mei, H.C. (1993). Microbial adhesion and the physico-chemistry of implant surfaces. Oral presentation at *BSAC/SGM Meeting*, 12th February. Birmingham.

BUSSCHER, H.J. & Weerkamp, A.H. (1897). Specific and non-specific interactions in bacterial adhesion to solid substrata. *FEMS Microbiology Reviews* **46**, 165-173.

CHAMBERLAND, S., Bayer, A.S., Schollaardt, T., Wong, S.A. & Bryan, L.E. (1989). Characterisation of mechanisms of quinolone resistance in *Pseudomonas aeruginosa* strains isolated *in vitro* and *in vivo* during experimental endocarditis. *Antimicrobial Agents and Chemotherapy* **33**, 624-634.

CHAMBERLAND, S., Malouin, F., Rabin, H.R., Schollaardt, T., Parr, T.R. & Bryan, L.E. (1990). Persistence of *Pseudomonas aeruginosa* during ciprofloxacin therapy of a cystic fibrosis patient: transient resistance to quinolones and protein F-deficiency. *Journal of Antimicrobial Chemotherapy* **25**, 995-1010.

CHARACKLIS, W.G., Trulear, M.G., Bryers, J.D. & Zelver, N. (1982). Dynamics of biofilm processes: methods. *Water Research* **16**, 1207-1216.

CHEN, H.Y. Jackson, G.G. & Livermore, D.M. (1991). Dependence of the interaction of ceftazidime and gentamycin against *Pseudomonas aeruginosa* on the bacterial growth rate. *Journal of Antimicrobial Chemotherapy* **27**, 610-612.

CHRISTENSEN, G.D., Baddour, L.M., Hasty, D.L., Lowrance, J.H. & Simpson, W.A. (1989). Microbial and foreign body factors in the pathogenesis of medical device infections. In *Infections Associated with Indwelling Medical Devices*, pp. 27-59. Edited by A.L. Bisno & F.A. Waldvogel. Washington, D.C.: American Society for Microbiology.

COCHRANE, D.M.G., Anwar, H., Brown, M.R.W., Lam, K. & Costerton, J.W. (1987). Iron deprivation *in vivo*: surface protein antigens of *Pseudomonas aeruginosa* in infection. *Antibiotics and Chemotherapy* **39**, 125-135.

COSTERTON, J.W. (1982). Effects of antibiotics on adherent bacteria. In *Action of Antibiotics in Patients*, pp. 160-176. Ed. L.D. Sabath. Vienna: Huber.

COSTERTON, J.W. (1988). Structure and plasticity at various organisation levels in the bacterial cell. *Canadian Journal of Microbiology* **34**, 513-521.

COSTERTON, J.W., & Lappin-Scott, H.M. (1989). Behaviour of bacteria in biofilms. *ASM News* **55**, 650-654.

COSTERTON, J.W., & Lashen, E.S. (1984). Influence of biofilm on efficacy of biocides on corrosion-causing bacteria. *Materials Performance* **9**, 13-17.

COSTERTON, J.W., Brown, M.R.W. & Sturgess, J.M. (1979). The cell envelope: its role in infection.. In *Pseudomonas aeruginosa: Clinical Manifestations of Infection and Current Therapy*, pp.41-62. Edited by R.G. Doggett. New York: Academic Press.

COSTERTON, J.W., Irvin, R.T. & Cheng, K-J. (1981a). The bacterial glycocalyx in nature and disease. *Annual Review of Microbiology* **35**, 299-324.

COSTERTON, J.W., Irvin, R.T. & Cheng, K-J. (1981b). The role of bacterial surface structures in pathogenesis. *CRC Critical Reviews in Microbiology* **8**, 303-308.

COSTERTON, J.W., Marrie, T.J. & Cheng, K.-J. (1985). Phenomena of bacterial adhesion. In *Bacterial Adhesion. Mechanisms and Physiological Significance*, pp. 3-43. Edited by D.C. Savage & M. Fletcher. New York: Plenum.

COSTERTON, J.W., Nickel, J.C. & Ladd, T.I. (1986). Suitable methods for the comparative study of free-living and surface-associated bacterial populations. In *Bacteria in Nature, Vol. 2*, pp. 49-84. Eds. J.S. Poindexter & E.R. Leadbetter. New York: Plenum.

COSTERTON, J.W., Cheng, K-J., Geesey, G.G., Ladd, T.J., Nickel, J.C., Dasgupta, M. & Marrie, T.J. (1987). Bacterial biofilms in nature and disease. *Annual Review of Microbiology* **41**, 435-464.

COSTERTON, J.W., Brown, M.R.W., Lam, K. & Cochrane, D.M.G. (1990). The microcolony mode of growth *in vivo* - an ecological perspective. In *Pseudomonas Infection and Alginates: Biochemistry, Genetics and Pathology*, pp. 76-94. Eds. P. Gacesca & N.J. Russell. London: Chapman & Hall.

COX, C.D. (1980). Iron uptake with ferripyochelin and ferric citrate by *Pseudomonas aeruginosa*. *Journal of Bacteriology* **142**, 581-587.

COZZARELLI, N.R. (1980). DNA gyrase and the supercoiling of DNA. *Science* **207**, 953-960.

CROSA, J.H. (1989). Genetics and molecular biology of siderophore-mediated iron transport in bacteria. *Microbiological Reviews* **53**, 517-530.

CRUMPLIN, G.C. & Smith, J.T. (1975). Nalidixic acid: an antibacterial paradox. *Antimicrobial Agents and Chemotherapy* **8**, 251-256.

CRUMPLIN, G.C. & Smith, J.T. (1976). Nalidixic acid and bacterial chromosome replication. *Nature* **260**, 643-645.

CULLMAN, W., Stieglitz, M., Baars, B. & Opferkuch, W. (1985). Comparative evaluation of recently developed quinolone compounds with a note on the frequency of resistant mutants. *Chemotherapy* **31**, 19-28.

DAGOSTINO, L., Goodman, A.E. & Marshall, K.C. (1991). Physiological responses induced in bacteria adhering to surfaces. *Biofouling* **4**, 113-119.

DAHLBÄCK, B. (1983). Marine microorganisms and interfaces. Ph.D. Thesis, University of Göteborg, Sweden.

DAN, M., Siegman-Igra, Y., Pitlik, S. & Raz, R. (1990). Oral ciprofloxacin treatment of *Pseudomonas aeruginosa* osteomyelitis. *Antimicrobial Agents and Chemotherapy* **34**, 849-852.

DASGUPTA, M.K., Zuberbuhler, P., Abbi, A., Harley, F.L., Brown, N.E., Lam, K., Dossetor, J.B. & Costerton, J.W. (1987). Combined evaluation of circulating immune complexes and antibodies to *Pseudomonas aeruginosa* as an immunologic profile in relation to pulmonary function in cystic fibrosis. *Journal of Clinical Immunology* **7**, 51-58.

DAWES, E.A. (1985). Starvation, survival and energy reserves. In *Bacteria in Their Natural Environments*, pp. 43-78. Eds. M. Fletcher and G.D. Floodgate. London: Academic Press.

DAWSON, M.P., Humphrey, B.A. & Marshall, K.C. (1981). Adhesion: a tactic in the survival strategy of a marine vibrio during starvation. *Current Microbiology* **6**, 195-1991.

DEAN, A.C.R., Ellwood, D.C., Melling, J. & Robinson, A. (1976). The action of antibacterial agents on bacteria grown in continuous culture. In *Continuous Culture 6: Applications and New Fields*, pp. 251-261. Eds. A.C.R. Dean, D.C. Ellwood, C.G.T. Evans & J. Melling. U.K.: Ellis Horwood.

DE GRAAF, F.K. & Mooi, F.R. (1986). The fimbrial adhesins of *Escherichia coli*. *Advances in Microbial Physiology* **28**, 65-143.

DE LORENZO, V. & Martinez, J.L. (1988). Aerobactin production as a virulence factor: a reevaluation. *European Journal of Clinical Microbiology and Infectious Diseases* **7**, 621-629.

DEMANGE, P., Wendenbaum, S., Bateman, A., Dell, A. & Abdallah, M.A. (1987). Bacterial siderophores: Structure and physicochemical properties of pyoverdins and related compounds. In *Iron Transport in Microbes, Plants and Animals*, pp. 167-187. Eds. G. Winkelmann, D. van der Helm & J.B. Neilands. Weinheim: Verlaggesellschaft.

DERETIC, V., Govan, J.R.W., Konyecsni, W.M. & Martin, D.W. (1990). Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: mutations in the *muc* loci affect transcription of the *algR* and *algD* genes in response to environmental stimuli. *Molecular Microbiology* **4**, 189-196.

DICKINSON, G.M. & Bisno, A.L. (1989a). Infections associated with indwelling devices: concepts of pathogenesis; infections associated with intravascular devices. *Antimicrobial Agents and Chemotherapy* **33**, 597-601.

DICKINSON, G.M. & Bisno, A.L. (1989b). Infections associated with indwelling devices: infections related to extravascular devices. *Antimicrobial Agents and Chemotherapy* **33**, 602-607.

DIETZ, W. H., Cook, T.M. & Goss, W.A. (1966). Mechanism of action of nalidixic acid on *Escherichia coli*. *Journal of Bacteriology* **91**, 768-773.

DIVER, J.M. (1989). Quinolone uptake by bacteria and bacterial killing. *Reviews of Infectious Diseases* **11**, suppl. 5., S941-S946.

DIVER, J.M. & Wise, R. (1986). Morphological and biochemical changes in *Escherichia coli* after exposure to ciprofloxacin. *Journal of Antimicrobial Chemotherapy* **18**, suppl.D, 31-34.

DIVER, J.M., Piddock, L.J.V. & Wise, R. (1990). The accumulation of five quinolone antibacterial agents by *Escherichia coli*. *Journal of Antimicrobial Chemotherapy* **25**, 319-333.

DIVER, J.M., Schollaardt, T. Rabin, H.R., Thorson, C. & Bryan, L.E. (1991). Persistence mechanisms in *Pseudomonas aeruginosa* from cystic fibrosis patients undergoing ciprofloxacin therapy. *Antimicrobial Agents and Chemotherapy* **35**, 1538-1546.

DOIG, P., Smith, N.R., Todd, T. & Irvin, R.T. (1987). Characterisation of the binding of *Pseudomonas aeruginosa* alginate to human epithelial cells. *Infection and Immunity* **55**, 1517-1522.

DOIG, P., Todd, T., Sastry, P.A., Lee, K.K., Hodges, R.S., Paranchych, W. & Irvin, R.T. (1988). Role of pili in adhesion of *Pseudomonas aeruginosa* to human respiratory epithelial cells. *Infection and Immunity* **56**, 1641-1646.

DOMINGUE, P.A.G., Schwarzinger, E. & Brown, M.R.W. (1989). Growth rate, iron depletion, and a sub-minimal inhibitory concentration of penicillin G affect the surface hydrophobicity of *Staphylococcus aureus*. In *The Influence of Antibiotics on the Host-Parasite Relationship III*, pp. 50-62. Eds. G. Gillisen, W. Opferkuck & G. Pulverer. Berlin/Heidelberg: Springer-Verlag.

DÖRING, G., Pfestorf, M., Botzenhart, K. & Abdallah, M.A. (1988). Impact of proteases on iron uptake of *Pseudomonas aeruginosa* pyoverdin from transferrin and lactoferrin. *Infection and Immunity* **56**, 291-293.

DÖRING, G. (1991). Microbial lung infections: new therapeutic strategies. *Pathologie Biologie* **39**, 587-590.

DORMAN, C.J., Bhriain, N.N. & Higgins, C. F. (1990). DNA supercoiling and environmental regulation of virulence gene expression in *Shigella flexneri*. *Nature* **344**, 789-792.

DOUGHERTY, S.H. (1988). Pathobiology of infection in prosthetic devices. *Reviews of Infectious Diseases* **10**, 1102-1117.

DUGUID, I.G., Evans, E., Brown, M.R.W., & Gilbert, P. (1992a). Growth-rate-independent killing by ciprofloxacin of biofilm-derived *Staphylococcus epidermidis;* evidence for cell-cycle dependency. *Journal of Antimicrobial Chemotherapy* **30**, 791-802.

DUGUID, I.G., Evans, E., Brown, M.R.W., & Gilbert, P. (1992b). Effect of biofilm culture upon the susceptibility of *Staphylococcus epidermidis* to tobramycin. *Journal of Antimicrobial Chemotherapy* **30**, 803-810.

DUTTON, R.J., Bitton, G. & Koopman, B. (1983). Malachite green-INT (MINT) method for determining active bacteria in sewage. *Applied and Environmental Microbiology* **46**, 1236-1267.

ELIOPOULOS, G.M. & Eliopoulos, C.T. (1989). Quinolone antimicrobial agents: activity *in vitro*. In *Quinolone Antimicrobial Agents*, pp. 35-70. Eds. J.S. Wolfson & D.C. Hooper. Washington, D.C.: American Society for Microbiology.

ELLWOOD, D. C. & Tempest, D.W. (1972). Effects of environment on bacterial wall content and composition. *Advances in Microbial Physiology* **7**, 83-117.

ELSHEIKH, E.L., Abaas, S. & Wretlind, B. (1985). Adherence of *Pseudomonas aeruginosa* to tracheal epithelial cells of mink. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **93**, section B, 417-422.

ENG, R.H.K., Padberg, F.T., Smith, S.M., Tan, E.N. & Cherubin, C.E. (1991). Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. *Antimicrobial Agents and Chemotherapy* **35**, 1824-1828.

EVANS, D.J. (1990). Physiology and antimicrobial susceptibility of Gram-negative bacterial biofilms. Ph.D. Thesis. University of Manchester, Manchester.

EVANS, D.J., Brown, M.R.W., Allison, D.G. & Gilbert, P. (1990a). Susceptibility of bacterial biofilms to tobramycin: role of specific growth rate and phase in the division cycle. *Journal of Antimicrobial Chemotherapy* **25**, 585-591.

EVANS, D.J., Allison, D.G., Brown, M.R.W., & Gilbert, P. (1990b). Effect of growth rate on resistance of Gram-negative biofilms to cetrimide. *Journal of Antimicrobial Chemotherapy* **26**, 473-478.

EVANS, D.J., Allison, D.G., Brown, M.R.W., & Gilbert, P. (1991a). Susceptibility of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms towards ciprofloxacin: effect of specific growth rate. *Journal of Antimicrobial Chemotherapy* **27**, 177-184.

EVANS, E., Brown, M.R.W. & Gilbert, P. (1992). Effect of specific growth rate upon the surface properties of *Staphylococcus epidermidis* biofilms and their production of extracellular virulence factors. Poster presentation, *92nd General Meeting of the American Society for Microbiology*, 26th - 23rd May. New Orleans.

EVANS, E., Duguid, I.G., Brown, M.R.W. & Gilbert, P. (1991b). Surface properties and adhesion of *Staphylococcus epidermidis* in batch and continuous culture. Poster presentation, *91st General Meeting of the American Society for Microbiology*, 5th - 9th May. Dallas.

EVANS, R.C. & Holmes, C.J. (1987). Effect of vancomycin hydrochloride on *Staphylococcus epidermidis* biofilm associated with silicone elastomer. *Antimicrobial Agents and Chemotherapy* **31**, 889-894.

FERNANDES, P.B., Hanson, C.W., Stamm, J.M., Vojtko, C., Shipkowitz, N.L. & St. Martin, E. (1987). The frequency of *in vitro* resistance development to fluoroquinolones and the use of a murine pyelonephritis model to demonstrate selection of resistance *in vivo*. *Journal of Antimicrobial Chemotherapy* **19**, 449-465.

FERNANDES, P.B., Kim, C., Cundy, K.R. & Huang, N.N. (1981). Antibodies to cell envelope proteins of *Pseudomonas aeruginosa* in cystic fibrosis patients. *Infection and Immunity* **33**, 527-532.

FICK Jr, R.B., Naegel, G.P., Squier, S.U., Wood, R.E., Gee, J.B.L. & Reynolds, H.Y. (1984). Proteins of the cystic fibrosis respiratory tract. *Journal of Clinical Investigation* **74**, 236-248.

FINCH, J.E. & Brown, M.R.W. (1975). The influence of nutrient limitation in a chemostat on the sensitivity of *Pseudomonas aeruginosa* to polymyxin and EDTA. *Journal of Antimicrobial Chemotherapy* **1**, 379-386.

FINCH, R.G. (1993). Medical problems associated with biofilms. Oral presentation at *British Biofilm Club Meeting*, 31st March - 2nd April. Gregynog, Wales.

FINLAY, B.B., Heffron, F. & Falkow, S. (1989). Epithelial cell surfaces induce *Salmonella* proteins required for bacterial adherence and invasion. *Science* **243**, 940-943.

FINLAY, B.B., Leung, K.Y., Rosenshine, I. & Garcia-Del Portillo, F. (1992). *Salmonella* interactions with the epithelial cells. *ASM News* **58**, 486-489.

FISHER, L.M. (1984). DNA supercoiling and gene expression. *Nature* **307**, 686-687.

FLETCHER, M. (1977). The effects of culture concentration and age, time and temperature on bacterial attachment to polystyrene. *Canadian Journal of Microbiology* **23**, 1-6.

FLETCHER, M. (1980). The question of passive versus active attachment mechanisms in non-specific bacterial adhesion. In *Microbial Adhesion to Surfaces*, pp. 197-210. Eds. R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter & B. Vincent. Chichester: Ellis Horwood Ltd.

FLETCHER, M. (1985). Effect of solid surfaces on the activity of attached bacteria. In *Bacterial Adhesion. Mechanisms and Physiological Significance* pp. 339-362. Eds. D.C. Savage and M. Fletcher. New York: Plenum Press.

FLETCHER, M. (1990). Methods for studying adhesion and attachment to surfaces. In *Methods in Microbiology* vol. 22, pp. 251-283. Eds. R. Grigorova & J.R. Norris. London: Academic Press Ltd.

FLETCHER, M. & Floodgate, G.D. (1973). An electron-microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces. *Journal of General Microbiology* **74**, 325-334.

FLETCHER, M. & Loeb, G.I. (1979). Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. *Applied and Environmental Microbiology* **37**, 67-72.

FOMSGAARD, A., Conrad, R.S., Galanos, C., Shand, G.H. and Høiby, N. (1988). Comparative immunochemistry of lipopolysaccharides from typable and polyagglutinable *Pseudomonas aeruginosa* strains isolated from patients with cystic fibrosis. *Journal of Clinical Microbiology* **26**, 821-826.

FOMSGAARD, A., Freundenberg, M.A. & Galanos, C. (1990). Modification of the silver staining technique to detect lipopolysaccharide in polyacrylamide gels. *Journal of Clinical Microbiology* **28**, 2627-2631.

FOX, J.L. (1992). Electric fields augment cell killing in biofilms. *ASM News* **12**, 667.

FROST, G.E. & Rosenberg, H. (1973). The inducible citrate-dependent iron transport system in *Escherichia coli* K12. *Biochimica et Biophysica Acta* **330**, 90-101.

FUKUDA, H., Hosaka, M., Hirai, K. & Iyobe, S. (1990). New norfloxacin resistance gene in *Pseudomonas aeruginosa* PAO. *Antimicrobial Agents and Chemotherapy* **34**, 1757-1761.

GANDER, S., Duguid, I.G., Nelson, S.M., Brown, M.R.W. and Gilbert, P. (1992). Effects of growth rate upon the susceptibility of intact *Staphylococcus epidermidis, Pseudomonas aeruginosa* and *Escherichia coli* biofilms towards ciprofloxacin. Poster presentation, *92nd General Meeting of the American Society for Microbiology*, 26th - 23rd May. New Orleans.

GARBER, N., Sharon, N., Shohet, D., Lam. J.S. & Doyle, R.J. (1985). Contribution of hydrophobicity to hemagglutinin reactions of *Pseudomonas aeruginosa*. *Infection and Immunity* **50**, 336-337.

GEESEY, G.G., Characklis, W.G. & Costerton, J.W. (1992). Centers, new technologies focus on biofilm heterogeneity. *ASM News*, **10**, 546-547.

GELLERT, M., Mizuuchi, K., O'Dea, M.H. & Nash, H.A. (1976). DNA gyrase: an enzyme that introduces superhelicle turns into DNA. *Proceedings of the National Academy of Sciences of the USA* **73**, 3872-3876.

GELLERT, M., Mizuuchi, K., O'Dea, M.H., Itoh, T. & Tomizawa, J.I. (1977). Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. *Proceedings of the National Academy of Sciences of the USA* **74**, 4772-4776.

GENSBERG, K., Hughes, K. & Smith, A.W. (1992). Siderophore-specific induction of iron uptake in *Pseudomonas aeruginosa*. *Journal of General Microbiology* **138**, 2381-2387.

GILBERT, P. & Brown, M.R.W. (1978). Influence of growth rate and nutrient limitation on the gross cellular composition of *Pseudomonas aeruginosa* and its resistance to 3- and 4-chlorophenol. *Journal of Bacteriology* **133**, 1066-1072.

GILBERT, P. & Brown, M.R.W. (1991). Out of the test tube into the frying pan: post-growth, pre-test variables. *Journal of Antimicrobial Chemotherapy* **27**, 859-862.

GILBERT, P. & Stuart, A. (1977). Small-scale chemostat for the growth of mesophilic and thermophilic microorganisms. *Laboratory Practice* **26**, 627-628.

GILBERT, P., Brown, M.R.W., & Costerton, J.W. (1987). Inocula for antimicrobial sensitivity testing: a critical review. *Journal of Antimicrobial Chemotherapy* **20**, 147-154.

GILBERT, P., Allison, D.G., Evans, D.J., Handley, P.S. & Brown, M.R.W. (1989). Growth rate control of adherent bacterial populations. *Applied and Environmental Microbiology* **55**, 1308-1311.

GILBERT, P., Collier, P.J. & Brown, M.R.W. (1990). Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy and stringent response. *Antimicrobial Agents and Chemotherapy* **34**, 1865-1868.

GILBERT, P., Evans, D.J., Evans, E., Duguid, I.G. & Brown, M.R.W. (1991). Surface characteristics and adhesion of *Escherichia coli* and *Staphylococcus epidermidis*. *Journal of Applied Bacteriology* **71**, 72-77.

GILLELAND, H.E. (1988). Adaptive alterations in the outer membrane of Gram-negative bacteria during human infection. *Canadian Journal of Microbiology* **34**, 499-502.

GIWERCMAN, B., Jensen, T.E., Høiby, N., Kharazmi, A. & Costerton, J.W. (1991). Induction of ß-lactamase production in *Pseudomonas aeruginosa* biofilm. *Antimicrobial Agents and Chemotherapy* **35**, 1008-1010.

GIWERCMAN, B., Fomsgaard, A., Mansa, B. & Høiby, N. (1992). Polyacrylamide gel electrophoresis analysis of lipolysaccharide from *Pseudomonas aeruginosa* growing planktonically and as a biofilm. *FEMS Microbiology Immunology* **89**, 225-230.

GODFREY, A.J., Hatlelid, L. & Bryan, L.E. (1984). Correlation between lipopolysaccharide structure and permeability resistance in ß-lactam-resistant *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **26**, 181-186.

GODFREY, A.J., Shahrabadi, M.S. & Bryan, L.E. (1986). Distribution of porin and lipopolysaccharide antigens on a *Pseudomonas aeruginosa* permeability mutant. *Antimicrobial Agents and Chemotherapy* **30**, 802-805.

GORDON, C. A., Hodges, N.A. & Marriot, C. (1988). Antibiotic interaction and diffusion through alginate and exopolysaccharide of cystic fibrosis-derived *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy* **22**, 667-674.

GOTOH, N., Wakebe, H., Yoshihara, E., Nakae, T. & Nishino, T. (1989). Role of protein F in maintaining structural integrity of the *Pseudomonas aeruginosa* outer membrane. *Journal of Bacteriology* **171**, 983-990.

GOVAN, J.R.W. & Glass, S. (1990). The microbiology and therapy of cystic fibrosis lung infections. *Reviews in Medical Microbiology* **1**, 19-28.

GOVAN, J.R.W. & Harris, G.S. (1986). *Pseudomonas aeruginosa* and cystic fibrosis: unusual bacterial adaptation and pathogenesis. *Microbiological Sciences* **3**, 302-308.

GOVAN, J.R.W., Fyfe, J.A.M. & McMillan, C. (1979). The instability of mucoid *Pseudomonas aeruginosa*: fluctuation test and improved stability of the mucoid form in a shaken culture. *Journal of General Microbiology* **110**, 229-233.

GRABERT, E., Wingender, J. & Winkler, U.K. (1990). An outer membrane protein characteristic of mucoid strains of *Pseudomonas aeruginosa*. *FEMS Microbiology Letters* **68**, 83-88.

GRAHAM, L.L., Beveridge, T.J. & Nanninga, N. (1981). Periplasmic space and the concept of the periplasm. *TIBS* **16**, 328-329.

GRIFFITHS, E., Stevenson, P. & Joyce, P. (1983). Pathogenic *Escherichia coli* express new outer membrane proteins when growing *in vivo*. *FEMS Microbiology Letters* **16**, 95-99.

GRISTINA, A.G. (1987). Biomaterial-centred infection: microbial adhesion versus tissue integration. *Science* **237**, 1588-1595.

GRISTINA, A.G., Hobgood, C.D. Webb, L.X. & Myrvik, Q.N. (1987). Adhesive colonisation of biomaterials and antibiotic resistance. *Biomaterials* **8**, 423-426.

GRISTINA, A.G., Jennings, R.A., Naylor, P.T., Myrvik, Q.N. & Webb, L.X. (1989). Comparative *in vitro* antibiotic resistance of surface-colonising coagulase-negative staphylococci. *Antimicrobial Agents and Chemotherapy* **33**, 813-816.

GUERRA-SANTOS, L., Käppeli, O. & Fiechter, A. (1984). *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as a carbon source. *Applied and Environmental Microbiology* **48**, 301-305.

HAAS, B., Kraut, J., Marks, J., Zanker, S.C. & Castignetti, D. (1991a). Siderophore presence in sputa of cystic fibrosis patients. *Infection and Immunity* **59**, 3997-4000.

HAAS, B., Murphy, E. & Castignetti, D. (1991b). Siderophore synthesis by mucoid *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients. *Canadian Journal of Microbiology* **37**, 654-657.

HALLETT, P. & Maxwell, A. (1991). Novel quinolone resistance mutations of the *Escherichia coli* DNA gyrase protein: enzymatic analysis of the mutant proteins. *Antimicrobial Agents and Chemotherapy* **35**, 335-340.

HANCOCK, R.E.W. & Carey, A.M. (1979). Outer membrane of *Pseudomonas aeruginosa*: heat- and 2-mercaptoethanol-modifiable proteins. *Journal of Bacteriology* **140**, 902-910.

HANCOCK, R.E.W. & Carey, A.M. (1980). Protein D1- a glucose-inducible pore-forming protein from the outer membrane of *Pseudomonas aeruginosa*. *FEMS Microbiology Letters* **8**, 105-109.

HANCOCK, R.E.W. & Woodruff, W.A. (1988). Roles of porin and ß-lactamase in ß-lactam resistance of *Pseudomonas aeruginosa*. *Reviews of Infectious Diseases* **10**, 770-775.

HANCOCK, R.E.W., Decad, G.M. & Nikaido, H. (1979). Identification of the protein producing transmembrane diffusion pores in the outer membrane of *Pseudomonas aeruginosa* PAO1. *Biochimica et Biophysica Acta* **554**, 323-331.

HANCOCK, R.E.W., Poole, K. & Benz, R. (1982). Outer membrane protein P of *Pseudomonas aeruginosa*: regulation by phosphate deficiency and formation of small anion-specific channels in lipid bilayer membranes. *Journal of Bacteriology* **150**, 730-738.

HANCOCK, R.E.W., Siehnel, R. & Martin, N. (1990). Outer membrane proteins of *Pseudomonas*. *Molecular Microbiology* **4**, 1069-1075.

HANE, M.W. & Wood, T.H. (1969). *Escherichia coli* K-12 mutants resistant to nalidixic acid: genetic mapping and dominance studies. *Journal of Bacteriology* **99**, 238-241.

HANSFORD, G.S. & Humphrey, A.E. (1966). The effect of equipment scale and degree of mixing on continuous fermentation yield at low dilution rates. *Biotechnology and Bioengineering* **8**, 85-96.

HAZUMI, N., Matsuda, K., Sanada, M., Ohtake, N. & Tanaka, N. (1992). Resistance to a new catecholic cephem, BO-1341, in *Pseudomonas aeruginosa* PAO. *Journal of Antimicrobial Chemotherapy* **29**, 287-297.

HEINRICHS, D.E., Young, L. & Poole, K. (1991). Pyochelin-mediated iron transport in *Pseudomonas aeruginosa*: involvement of a high-molecular-mass outer membrane protein. *Infection and Immunity* **59**, 3680-3684.

HELMSTETTER, C.E. & Cummings, D.J. (1964). An improved method for the selection of bacterial cells at division. *Biochimie et Biophysica Acta* **82**, 608-610.

HELMSTETTER, C.E., Eenhuis, C., Theisen, P., Grimwade, J. & Leonard, A.C. (1992). Improved bacterial baby machine: application to *Escherichia coli* K-12. *Journal of Bacteriology* **174**, 3445-3449.

HERBERT, D., Elsworth, R. & Telling, R.C. (1956). The continuous culture of bacteria: a theoretical and experimental study. *Journal of General Microbiology* **14**, 601-622.

HERSHKO, C., Peto, T.E.A. & Weatherall, D.J. (1988). Iron and infection. *British Medical Journal* **296**, 660-664.

HIGGINS, N.P., Peebles, C.L., Suginda, A. & Cozzarelli, N.R. (1978). Purification of subunits of *Escherichia coli* DNA gyrase and reconstitution of enzymatic activity. *Proceedings of the National Academy of Sciences of the USA* **75**, 1773-1777.

HIRAI, K., Suzue, S., Irikura, T., Iyobe, S. & Mitsuhashi, S. (1987). Mutations producing resistance to norfloxacin in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **31**, 582-586.

HITCHCOCK, P.J. & Brown, T.M. (1983). Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *Journal of Bacteriology* **154**, 269-277.

HOBOT, J.A., Carlemalm, E., Villiger, W. & Kellenberger, E. (1984). Periplasmic gel: new concept resulting from the reinvestigation of bacterial cell envelope ultrastructure by new methods. *Journal of Bacteriology* **160**, 143-152.

HODGES, N.A. & Gordon, C.A. (1991). Protection of *Pseudomonas aeruginosa* against ciprofloxacin and ß-lactams by homologous alginate. *Antimicrobial Agents and Chemotherapy* **35**, 2450-2452.

HØIBY, N. (1974). *Pseudomonas aeruginosa* infection in cystic fibosis. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* B **82**, 551-558.

HØIBY, N. & Koch, C. (1990). *Pseudomonas aeruginosa* infection in cystic fibrosis and its management. *Thorax* **45**, 881-884.

HØIBY, N., Adersen, V. & Bendixen, G. (1975). *Pseudomonas aeruginosa* infection in cystic fibosis. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* C **83**, 459-468.

HOLMES, C.J. & Evans, R.C. (1989). Resistance of bacterial biofilms to antibiotics. *Journal of Antimicrobial Chemotherapy* **24**, 84.

HOOPER, D.C. & Wolfson, J.S. (1988). Mode of action of quinolone antimicrobial agents. *Reviews of Infectious Diseases* **10**, suppl.1, S14-S21.

HOOPER, D.C. & Wolfson, J.S. (1991). Mode of action of the new quinolones: new data. *European Journal of Clinical Microbiology and Infectious Diseases* **10**, 223-231.

HOOPER, D.C., Wolfson, J.S., Souza, K.S., Ng., E.Y., McHugh, G.L. & Swartz, M.N. (1989). Mechanisms of quinolone resistance in *Escherichia coli*: characterisation of *nfxB* and *cfxB*, two mutant resistance loci decreasing norfloxacin accumulation. *Antimicrobial Agents and Chemotherapy* **33**, 283-290.

HOYLE, B.D. & Costerton, J.W. (1989). Transient exposure to a physiologically-relevant concentration of calcium confers tobramycin resistance upon sessile cells of *Pseudomonas aeruginosa*. *FEMS Microbiology Letters* **60**, 339-342.

HOYLE, B.D., Jass, J. & Costerton, J.W. (1990). The biofilm glycocalyx as a resistance factor. *Journal of Antimicrobial Chemotherapy* **26**, 1-6.

HOYLE, B.D., Alcantara, J. & Costerton, J.W. (1992a). *Pseudomonas aeruginosa* biofilm as a diffusion barrier to piperacillin. *Antimicrobial Agents and Chemotherapy* **36**, 2054-2056.

HOYLE, B.D., Wong, C.K.W. & Costerton, J.W. (1992b). Disparate efficacy of tobramycin on Ca^{2+}-, Mg^{2+}-, and HEPES-treated *Pseudomonas aeruginosa* biofilms. *Canadian Journal of Microbiology* **38**, 1214-1218.

HOYLE, B.D., Williams, L.J. & Costerton, J.W. (1993). Production of mucoid exoplysaccharide during development of *Pseudomonas aeruginosa* biofilms. *Infection and Immunity* **61**, 777-780.

HULTON, C.S.J., Seirafi, A., Hinton, J.C.D., Sidebotham, J.M., Waddell, L., Pavitt, G.D., Owen-Hughes, T., Spassky, A., Buc, H. & Higgins, C. F. (1990). Histone-like protein H1 (H-NS), DNA supercoilng, and gene expression in bacteria. *Cell* **63**, 631-642.

HUMPHREY, B., Kjelleberg, S. & Marshall, K.C. (1983). Responses of marine bacteria under starvation conditions at a solid-water interface. *Applied and Environmental Microbiology* **45**, 43-47.

INGRAHAM, J.L., Maaløe, O. & Neidhardt, F.C. (1983). *Growth of the Bacterial Cell.* MA, USA: Sinauer Assocs. Inc.

INOUE, Y., Ohue, T., Yamagishi, J., Nakamura, S. & Shimizu, M. (1978). Mode of incomplete resistance among pipemidic, piromidic, and nalidixic acids. *Antimicrobial Agents and Chemotherapy* **14**, 240-245.

ISAACSON, R.E. (1985). Pilus adhesins. In *Bacterial Adhesion. Mechanisms and Physiological Significance*, pp. 307-336. Eds. D.C. Savage & M. Fletcher. New York: Plenum press.

JENSEN, E.T., Kharazmi, A., Lam, K., Costerton, J.W. & Høiby, N. (1990). Human polymorphonuclear leukocyte response to *Pseudomonas aeruginosa* grown in biofilms. *Infection and Immunity* **58**, 2383-2385.

JOHNSON, K. & Lory, S. (1987). Characterisation of *Pseudomonas aeruginosa* mutants with altered piliation. *Journal of Bacteriology* **169**, 5663-5667.

KATO, J., Misra, T.K. & Chakrabarty, A.M. (1990). AlgR3, a protein resembling eukaryotic histone H1, regulates alginate synthesis in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the USA* **87**, 2887-2891.

KEEVIL, C.W. (1993). Methods for addressing the activity of biofilm microorganisms *in situ.* Oral presentation, *British Biofilm Club Meeting*, 31st March - 2nd April. Gregynog, Wales.

KEEVIL, C.W., Bradshaw, D.J., Dowsett, A.B. & Feary, T.W. (1987). Microbial film formation: dental plaque deposition on acrylic tiles using continuous culture techniques. *Journal of Applied Bacteriology* **62**, 129-138.

KELLY, N.M., Bell, A. & Hancock, R.E.W. (1989). Surface characteristics of *Pseudomonas aeruginosa* grown in a chamber implant model in mice and rats. *Infection and Immunity* **57**, 344-350.

KELLY, N.M., MacDonald, M.H., Martin, N., Nicas, T. & Hancock, R.E.W. (1990). Comparison of the outer membrane protein and lipopolysaccharide profiles of mucoid and nonmucoid *Pseudomonas aeruginosa*. *Journal of Clinical Microbiology* **28**, 2017-2021.

KENNE, L. & Lindberg, B. (1983). Bacterial Polysaccharides. In *The Polysaccharides Vol.2*, pp. 287-363. Ed. G.O. Aspinall. New York: Academic Press.

KHARAZMI, A. (1989). Interactions of *Pseudomonas aeruginosa* proteases with cells of the immune system. In *Pseudomonas aeruginosa Infection*, pp. 42-49. Eds. N. Høiby, S.S. Pedersen, G.H. Shand, G. Döring & I.A. Holder. Basel: Karger.

KHARAZMI, A. (1991). Mechanisms involved in the evasion of the host defence by *Pseudomonas aeruginosa. Immnunology Letters* **30**, 201-206.

KHOURY, A.E., Lam, K., Ellis, B. & Costerton, J.W. (1992). Prevention and control of bacterial infections associated with medical devices. *ASAIO Journal* M174-M178.

KJELLEBERG, S. & Hermansson, M. (1984). Starvation-induced effects on bacterial surface characteristics. *Applied and Environmental Microbiology* **48**, 497-503.

KJELLEBERG, S., Humphrey, B.A. & Marshall, K.C. (1982). Effects of interfaces on small, starved marine bacteria. *Applied and Environmental Microbiology* **43**, 1166-1172.

KJELLEBERG, S., Humphrey, B.A. & Marshall, K.C. (1985). Initial phases of starvation and activity of bacteria at surfaces. *Applied and Environmental Microbiology* **46**, 978-984.

KJELLEBERG, S., Hermansson, M. & Mårdén, P. (1987). The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment. *Applied and Environmental Microbiology* **41**, 25-49.

KORVICK, J.A. & Yu, V.L. (1991). Antimicrobial agent therapy for *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **35**, 2167-2172.

KROGFELT, K.A. (1991). Bacterial adhesion: genetics, biogenesis, and role in pathogenesis of fimbrial adhesins of *Escherichia coli*. *Reviews of Infectious Diseases* **13**, 721-731.

KROPINSKI, A.M., Lewis, V. & Berry, D. (1987). Effect of growth temperature on the lipids, outer membrane proteins, and lipolysaccharides of *Pseudomonas aeruginosa* PAO. *Journal of Bacteriology* **169**, 1960-1966.

KROPINSKI, A.M., Kuzio, J., Angus, B.L. & Hancock, R.E.W. (1982). Chemical and chromatographic analysis of lipopolysaccharide from an antibiotic-supersusceptible mutant of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **21**, 310-319.

LABISCHINSKI, H., Barnickel, G., Bradaczek, H., Naumann, D., Rietschel, E.T. & Giesbrecht, P. (1985). High state of order of isolated bacterial lipopolysaccharide and its possible contribution to the permeation barrier property of the outer membrane. *Journal of Bacteriology* **162**, 9-20.

LACHICA, R.V. (1990). Significance of hydrophobicity in the adhesiveness of pathogenic Gram-negative bacteria. In *Microbial Cell Surface Hydrophobicity*, pp. 297-313. Eds. R.J. Doyle & M. Rosenberg. Washington, D.C.: ASM.

LAM, C., Turnowsky, F., Schwarzinger, E. & Neruda, W. (1984). Bacteria recovered without subculture from infected human urines expressed iron-regulated outer membrane proteins. *FEMS Microbiology Letters* **24**, 255-259.

LAM, J., Chan, R. Lam, K. & Costerton, J.W. (1980). Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infection and Immunity* **28**, 546-556.

LAM, M.Y.C., McGroarty, E.J., Kropinski, A.M., MacDonald, L.A., Pedersen, S.S., Høiby, N. & Lam, J.S. (1989). Occurrence of a common lipopolyaccharide antigen in standard and clinical strains of *Pseudomonas aeruginosa*. *Journal of Clinical Microbiology* **27**, 962-967.

LAMBERT, P.A. (1988). Enterobacteriaceae: composition, structure and function of the cell envelope. *Journal of Applied Bacteriology Symposium Supplement*, 21S-34S.

LAMBERT, P.A. & Booth, B.R. (1982). Exposure of outer membrane proteins on the surface of *Pseudomonas aeruginosa* PAO1 revealed by labelling with [^{125}I] lactoperoxidase. *FEMS Microbiology Letters* **14**, 43-45.

LANGFORD, P.R., Anwar, H., Gonda, I. & Brown, M.R.W. (1989). Outer membrane proteins of gentamycin induced small colony variants of *Pseudomonas aerugnosa*. *FEMS Microbiology Letters* **61**, 33-36.

LAWRENCE, J. R. & Caldwell, D.E. (1987). Behaviour of bacterial stream populations within the hydrodynamic boundary layers of surface microenvironments. *Microbial Ecology* **14**, 15-27.

LAWRENCE, J. R., Delaquis, P.J., Korber, D.R. & Caldwell, D.E. (1987). Behaviour of *Pseudomonas fluorescens* within the hydrodynamic boundary layers of surface microenvironments. *Microbial Ecology* **14**, 1-14.

LAWRENCE, J. R., Korber, D.R., Wolfaardt, G.M. & Caldwell, D.E. (1992). Analysis of microbial biofilms using scanning confocal laser microscopy. Oral presentation, *92nd General Meeting of the American Society for Microbiology*, 26th - 23rd May. New Orleans.

LEBEL, M. (1991). Fluoroquinolones in the treatment of cystic fibrosis: a critical appraisal. *European Journal of Clinical Microbiology and Infectious Diseases* **10**, 316-324.

LEWANDOWSKI, Z., Waiser, G. & Characklis, W.G. (1991). Reaction kinetics in biofilms. *Biotechnology and Bioengineering* **38**, 877-882.

LEWIN, C.S. (1992). Resistance to the 4-quinolones. *Journal of Medical Microbiology* **36**, 9-11. Eds. S.G.B. Amyes & C.G. Gemmell.

LEWIN, C.S. & Smith, J.T. (1990). DNA breakdown by the 4-quinolones and its significance. *Journal of Medical Microbiology* **31**, 65-70.

LEWIN, C.S., Howard, M.A. & Smith, J.T. (1991). Protein- and RNA-synthesis independent bacterial activity of ciprofloxacin that involves the A subunit of DNA gyrase. *Journal of Medical Microbiology* **34**, 19-22.

LEWIS, D.L. & Gattie, D.K. (1991). The ecology of quiescent microbes. *ASM News* **57**, 27-32.

LEYING, H.J., Büscher, K.H., Cullmann, W. & Then, R.L. (1992). Lipopolysaccharide alterations responsible for combined quinolone and ß-lactam resistance in *Pseudomonas aeruginosa*. *Chemotherapy* **38**, 82-91.

LIU, L.F. & Wang, J.C. (1978). *Micrococcus luteus* DNA gyrase: active components and a model for its supercoiling DNA. *Proceedings of the National Academy of Sciences of the USA* **75**, 2098-2102.

LODGE, J.M. (1987). Influence of sub-MICs of ß-lactam antibiotics, growth rate, and iron-limitation on the surface structures of *Klebsiella pneumoniae*. Ph.D. Thesis, University of Aston, Birmingham.

LOEB, G.I. (1985). The properties of nonbiological surfaces and their characterisation. In *Bacterial Adhesion. Mechanisms and Physiological Significance*, pp. 111-129. Eds. D.C. Savage & M. Fletcher. London: Plenum Press.

LORIAN, V. (1989). *In vitro* simulation of *in vivo* conditions: physical state of the culture medium. *Journal of Clinical Microbiology* **27**, 2405-2406.

LOWRY, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.

LUGTENBERG, B. & Van Alphen, L. (1983). Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other Gram-negative bacteria. *Biochimica et Biophysica Acta* **737**, 51-115.

LUGTENBERG, B., Meijers, J., Peters, R., Van der Hoek, P. & Van Alphen, L. (1975). Electrophoretic resolution of the major outer membrane protein of *Escherichia coli* K12 into four bands. *FEBS Letters* **58**, 254-258.

LUZAR, M.A. & Montie, T.C. (1985). Avirulence and altered physiological properties of cystic fibrosis strains of *Pseudomonas aeruginosa. Infection and Immunity* **50**, 572-576.

MAALØE, O. & Kjeldgaard, N.O. (1966). *Control of Macromolecular Synthesis.* New York: W.A. Benjamin.

MACKOWIAK, P.A. & Marling-Cason, M. (1984). A comparative analysis of *in vitro* assays of bacterial adherence. *Journal of Microbiological Methods* **2**, 147-158.

MAICHE, A.G. (1991). Use of quinolones in the immunocomprimised host. *European Journal of Clinical Microbiology and Infectious Diseases* **10**, 361-367.

MAKIN, S., Gander, S., Brown, M.R.W. & Gilbert, P. (1992). Influence of growth rate and formation of biofilm upon the cell surface charge of *Escherichia coli*. Poster presentation, *92nd General Meeting of the American Society for Microbiology*, 26th - 23rd May. New Orleans.

MARCUS, H. & Baker, N.R. (1985). Quantitation of adherence of mucoid and nonmucoid *Pseudomonas aeruginosa* to hamster tracheal epithelium. *Infection and Immunity* **47**, 723-729.

MARSHALL, K.C. (1985). Mechanisms of bacterial adhesion at solid-water interfaces. In *Bacterial Adhesion. Mechanisms and Physiological Significance* pp. 133-161. Eds. D.C. Savage and M. Fletcher. New York: Plenum Press.

MARSHALL, K.C. (1986). Adsorption and adhesion processes in microbial growth at interfaces. *Advances in Colloid and Interface Science* **25**, 59-86.

MARSHALL, K.C. (1988). Adhesion and growth of bacteria at surfaces in oligotrophic habitats. *Canadian Journal of Microbiology* **34**, 503-506.

MARSHALL, K.C. (1991). The importance of studying microbial cell suraces. In *Microbial Cell Surface Analysis. Structural and Physicochemical Methods*, pp. 3-20. Eds. N. Mozes, P.S. Handley, H.J. Busscher & P.G. Rouxhet. New York: VCH Publishers Inc.

MARSHALL, K.C. (1992). Biofilms: An overview of bacterial adhesion, activity, and control at surfaces. *ASM News* **58**, 202-207.

MARSHALL, K.C., Stout, R. & Mitchell, R. (1971). Mechanism of the initial events in the sorption of marine bacteria to surfaces. *Journal of General Microbiology* **68**, 337-348.

MARTIN, D.W., Holloway, B.W. & Deretic, V. (1993). Characterisation of a locus determining the mucoid status of *Pseudomonas aeruginosa*: AlgU shows sequence similarities with a *Bacillus* sigma factor. *Journal of Bacteriology* **175**, 1153-1164.

MARTINEZ, J.L., Delgado-Iribarren, A. & Baquero, F. (1990). Mechanisms of iron aquisition and bacterial virulence. *FEMS Microbiology Reviews* **75**, 45-56.

MARTINEZ-MARTINEZ, L., Pascual, A. & Perea, E.J. (1991). Kinetics of adherence of mucoid and non-mucoid *Pseudomonas aeruginosa* to plastic catheters. *Journal of Medical Microbiology* **34**, 7-12.

MASUDA, N., & Ohya, S. (1992). Cross-resistance to meropenems, cephems, and quinolones in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **36**, 1847-1851.

MAXWELL, A. (1992). The molecular basis of quinolone action. *Journal of Antimicrobial Chemotherapy* **30**, 409-416.

MAY, T.B., Shinabarger, D., Maharaj, R., Kato, J., Chu, L., DeVault, J.D., Roychoudhury, S., Zielinski, N.A., Berry, A., Rothmel, R.K., Misra, T.K. & Chakrabarty, A.M. (1991). Alginate synthesis by *Pseudomonas aeruginosa*: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients. *Clinical Microbiology Reviews* **4**, 191-206.

McAVOY, M.J., Newton, V., Paull, A., Morgan, J., Gacesca, P. & Russell, N.J. (1989). Isolation of mucoid strains of *Pseudomonas aeruginosa* from non-cystic-fibrosis patients and characterisation of the structure of their secreted alginate. *Journal of Medical Microbiology* **28**, 183-189.

McCOY, W.F., Bryers, J.D., Robbins, J. & Costerton, J.W. (1981). Observations of fouling biofilm formation. *Canadian Journal of Microbiology* **27**, 910-917.

McELDOWNEY, S. & Fletcher, M. (1986). Effect of growth conditions and surface characteristics of aquatic bacteria on their attachment to solid surfaces. *Journal of General Microbiology* **132**, 513-523.

McELVANEY, N.G., Hubbard, R.C., Birrer, P., Chernick, M.S., Caplan, D.B., Frank, M.M. & Crystal, R.G. (1991). Aerosol α1-antitrypsin treatment for cystic fibrosis. *The Lancet* **337**, 392-394.

MERRIL, C.R., Goldman, D., Sedman, S.A. & Ebert, M.H. (1981). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211**, 1437-1438.

MEYER, J.-M. (1992). Exogenous siderophore-mediated iron uptake in *Pseudomonas aeruginosa*: possible involvement of porin OprF in iron translocation. *Journal of General Microbiology* **138**, 951-958.

MICHÉA-HAMZEHPOUR, M., Furet, Y.X. & Pechere, J.-C. (1991). Role of protein D2 and lipopolysaccharide in diffusion of quinolones through the outer membrane of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **35**, 2091-2097.

MICKELSEN, P.A. & Sparling, P.E. (1981). Ability of *Neisseria gonorrhoeae, Neisseria meningitidis*, and commensal *Neisseria* species to obtain iron from transferrin and iron compounds. *Infection and Immunity* **33**, 555-564.

MILLER, R.V. & Scurlock, T.R. (1983). DNA gyrase (topoisomerase II) from *Pseudomonas aeruginosa*. *Biochemical and Biophysical Research Communications* **110**, 694-700.

MILLS, J., Pulliam, L., Dall, L., Marzouk, J., Wilson, W. & Costerton, J.W. (1984). Exopolysaccharide production by various streptococci in experimental endocarditis. *Infection and Immunity* **43**, 359-367.

MIZUNO, T. (1981). A novel peptidoglycan-associated lipoprotein (PAL) found in the outer membrane of *Proteus mirabilis* and other Gram-negative bacteria. *Journal of Biochemistry* **89**, 1039-1049.

MIZUNO, T. & Kageyama, M. (1979). Isolation and characterisation of a major outer membrane protein of *Pseudomonas aeruginosa*. *Journal of Biochemistry* **85**, 115-122.

MIZUUCHI, K., Mizuuchi, M., O'Dea, M.H. & Gellert, M. (1984). Cloning and simplified purification of *Escherichia coli* DNA gyrase A and B proteins. *Journal of Biological Chemistry* **259**, 9199-9201.

MONOD, J. (1949). The growth of bacterial cultures. *Annual Review of Microbiology* **3**, 371-394.

MOREL, I., Cillard, J., Lescoat, G., Sergent, O., Pasdeloup, N., Ocaktan, A.Z., Abdallah, M.A., Brissot, P. & Cillard, P. (1992). Antioxidant and free radical scavenging activities of the iron chelators pyoverdin and hydroxypyrid-4-ones in iron-loaded hepatocyte cultures: comparison of their mechanism of protection with that of desferrioxamine. *Free Radical Biology & Medicine* **13**, 499-508.

MORRISEY, I., Lewin, C.S. & Smith, J.T. (1990). The influence of oxygen upon bactericidal potency. In *The 4-Quinolones: Antibacterial Agents in vitro*, pp. 23-36. Ed. G.C. Crumplin. London: Springer-Verlag Ltd.

NAKAMURA, S., Nakamura, M., Kojima, T. & Yoshida, H. (1989). *GyrA* and *gyrB* mutations in quinolone-resistant strains of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **33**, 254-255.

NEYFAKH, A.A., Borsch, C.M. & Kaatz, G.W. (1993). Fluoroquinolone resistance protein NorA of *Staphylococcus aureus* is a multidrug efflux transporter. *Antimicrobial Agents and Chemotherapy* **37**, 128-129.

NICAS, T.I. & Hancock, R.E.W. (1983). *Pseudomonas aeruginosa* outer membrane permeability: isolation of a porin protein F-deficient mutant. *Journal of Bacteriology* **153**, 281-285.

NICHOLS, W.W. (1991). Biofilms, antibiotics and penetration. *Reviews in Medical Microbiology* **2**,177-181.

NICHOLS, W.W. (1993). Biofilm permeability to antimicrobial agents. Oral presentation at *British Biofilm Club Meeting*, 31st March - 2nd April. Gregynog, Wales.

NICHOLS, W.W., Evans, M.J., Slack, M.P.E. & Walmsley, H.L. (1989). The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa*. *Journal of General Microbiology* **135**, 1291-1303.

NICKEL, J.C., Ruseska, I., Wright, J.B. & Costerton, J.W. (1985). Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrobial Agents and Chemotherapy* **27**, 619-624.

NIKAIDO, H. & Hancock, R.E.W. (1986). Outer membrane permeability of *Pseudomonas aeruginosa*. In *The Bacteria, Vol. X. A Treatise on Structure and Function*, pp.145-193. Edited by J.R. Sokatch. New York: Academic Press.

NIKAIDO, H. & Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiological Reviews* **49**, 1-32.

NIKAIDO, H., Nikaido, K. & Harayama, S. (1991). Identification and characterisation of porins in *Pseudomonas aeruginosa*. *The Journal of Biological Chemistry* **266**, 770-779.

NISHINO, T., Tanaka, M. & Ohtsuki, M. (1989). Effect of ofloxacin on the ultrastructure of *Escherichia coli* and *Pseudomonas aeruginosa* in the logarithmic and stationary phases of growth. *Reviews of Infectious Diseases* **11**, suppl. 5, 914-915.

NORDE, W. & Lyklema, J. (1989). Protein adsorption and bacterial adhesion to solid surfaces: a colloid-chemical approach. *Colloids and Surfaces* **38**, 1-14.

NOY, M.F. (1982). Effect of phosphate limited growth on drug resistance of *Pseudomonas aeruginosa*. Ph.D. Thesis. University of Aston, Birmingham.

NYSTRÖM, T., Flärdh, K. & Kjelleberg, S. (1990). Responses to multiple-nutrient starvation in marine *Vibrio* sp. strain CCUG 15956. *Journal of Bacteriology* **172**, 7085-7097.

OHKAWA, I., Shiga, S. & Kageyama (1979). An esterase on the outer membrane of *Pseudomonas aeruginosa* for the hydrolysis of long chain acyl esters. *Journal of Biochemistry* **86**, 643-656.

OHKAWA, I., Shiga, S. & Kageyama, M. (1980). Effect of iron concentration in the growth medium on the sensitivity of *Pseudomonas aeruginosa* to pyocin S2. *Journal of Biochemistry* **87**, 323-331.

OKAZAKI, T. & Hirai, K. (1992). Cloning and nucleotide sequence of the *Pseudomonas aeruginosa nfxB* gene, conferring resistance to new quinolones. *FEMS Microbiology Letters* **97**, 197-202.

OLSÉN, A., Arnqvist, A., Hammar, M., Sukupolvi, S. & Normark, S. (1993). The RpoS sigma factor relieves H-NS-mediated transcriptional repression of *csgA*, the subunit gene of fibronectin-binding curli in *Escherichia coli*. *Molecular Microbiology* **7**, 523-536.

OMBAKA, E.A., Cozens, R.M. & Brown, M.R.W. (1983). Influence of nutrient limitation of growth on stability and production of virulence factors of mucoid and nonmucoid strains of *Pseudomonas aeruginosa*. *Reviews of Infectious Diseases* **5**, suppl. 5, S880-S888.

ORSKOV, I., Orskov, F., Jann, B. & Jann, K. (1977). Serology, chemistry and genetics of O and K antigens of *Escherichia coli*. *Bacteriological Reviews* **41**, 667-710.

PARMLEY, M.J. & Horvat, R.T. (1986). Antigenic specificities of *Pseudomonas aeruginosa* alkaline protease and elastase defined by human T cell clones. *Journal of Immunology* **137**, 988-994.

PARR, T.R. JR., Poole, K., Crockford, G.W.K. & Hancock, R.E.W. (1986). Lipopolysaccharide-free *Escherichia coli* OmpF and *Pseudomonas aeruginosa* protein P porins are functionally active in lipid bilayer membranes. *Journal of Bacteriology* **165**, 523-526.

PEDERSEN, S.S. (1992). Lung infection with alginate-producing, mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **100**, suppl. 28.

PEDERSEN, S.S., Kharazmi, A., Espersen, F. & Høiby, N. (1990). *Pseudomonas aeruginosa* alginate in cystic fibrosis sputum and the inflammatory response. *Infection and Immunity* **58**, 3363-3368.

PEDERSEN, S.S., Koch, C. & Høiby, N. (1992). Prevention and early treatment of *Pseudomonas aeruginosa* infection. In *Cystic Fibrosis, Basic and Clinical Research*, pp. 134-144. Eds. N. Høiby & S.S. Pedersen. Amsterdam: Elsevier.

PERSSON, A., Molin, G. & Weibull, C. (1990). Physiological and morphological changes induced by nutrient limitation of *Pseudomonas fluorescens* 378 in continuous culture. *Applied and Environmental Microbiology* **56**, 686-692.

PETERSON, G.L. (1977). A simplification of the protein assay method of Lowry *et al*, which is more generally applicable. *Analytical Biochemistry* **83**, 346-356.

PIÑA, S.E., Terry, J.M. & Mattingly, S.J. (1992). Ciprofloxacin treatment of nonmucoid *Pseudomonas aeruginosa* in a chemostat results in a resistant mucoid population. Poster presentation, *92nd General Meeting of the American Society for Microbiology*, 26th - 23rd May. New Orleans.

PIRT, S.J. (1975). *Principles of Microbe and Cell Cultivation.* Oxford: Blackwell Scientific Publications.

PLOTKOWSKI, M.C., Beck. G., Tournier, J.M., Bernardo-Filho, M., Marques, E.A. & Puchelle, E. (1989). Adherence of *Pseudomonas aeruginosa* to respiratory epithelium and the effect of leucocyte elastase. *Journal of Medical Microbiology* **30**, 285-293.

PLOTKOWSKI, M.C., Chevillard, M., Pierrot, D., Altemayer, D., Zahm, J.M., Colliot, G. & Puchelle, E. (1991). Differential adhesion of *Pseudomonas aeruginosa* to human respiratory epithelial cells in primary culture. *Journal of Clinical Investigation* **87**, 2029-2035.

PLOTKOWSKI, M.C., Chevillard, M., Pierrot, D., Altemayer, D. & Puchelle, E. (1992). Epithelial respiratory cells from cystic fibrosis patients do not possess specific *Pseudomonas aeruginosa*-adhesive properties. *Journal of General Microbiology* **36**, 104-111.

POOLE, K., Neshat, S. & Heinrichs, D. (1991). Pyoverdine-mediated iron transport in *Pseudomonas aeruginosa*: involvement of a high-molecular-mass outer membrane protein. *FEMS Microbiology Letters* **78**, 1-6.

POWER, K. & Marshall, K.C. (1988). Cellular growth and reproduction of marine bacteria on surface-bound substrate. *Biofouling* **1**, 163-174.

PROSSER, B.T., Taylor, D., Dix, B.A. & Cleeland, R. (1987). Method of evaluating effects of antibiotics on bacterial biofilm. *Antimicrobial Agents and Chemotherapy* **31**, 1502-1506.

RAMPHAL, R., Sadoff, J.C., Pyle, M. & Silipigni, J.D. (1984). Role of pili in the adherence of *Pseudomonas aeruginosa* to injured tracheal epithelium. *Infection and Immunity* **44**, 38-40.

RAMPHAL, R., Guay, C. & Pier, G.B. (1987). *Pseudomonas aeruginosa* adhesins for tracheobronchial mucin. *Infection and Immunity* **55**, 600-603.

RAMPHAL, R.B., Koo, L., Ishimoto, K.S., Totten, P.A., Larca, J.C. & Lory, S. (1991). Adhesion of *Pseudomonas aeruginosa* pilin-deficient mutants to mucin. *Infection and Immunity* **59**, 1307-1311.

RATCLIFFE, N.T. & Smith, J.T. (1983). Effects of magnesium on the activity of 4-quinolone antibacterial agents. *Journal of Pharmacy and Pharmacology* **35**, 61P.

RIVERA, M. & McGroarty, E.J. (1989). Analysis of a common-antigen lipopolysaccharide from *Pseudomonas aeruginosa*. *Journal of Bacteriology* **171**, 2244-2248.

ROSENBERG, M. & Doyle, R.J. (1990). Microbial cell surface hydrophobicity: history, measurement, and significance. In *Microbial Cell Surface Hydrophobicity*, pp. 1-37. Eds. R.J. Doyle & M. Rosenberg. Washington, D.C.: ASM.

ROSENBERG, M. & Kjelleberg, S. (1986). Hydrophobic interactions: role in bacterial adhesion. *Advances in Microbial Ecology* **9**, 353-393.

ROSS, N.W., Levitan, R., Labelle, J. & Schneider, H. (1991). Protein and other compositional differences of the extracellular material from slimy and non-slimy colonies of non-mucoid *Pseudomonas aeruginosa*. *FEMS Microbiology Letters* **81**, 257-260.

ROSZAK, D.B. & Colwell, R.R. (1987). Survival strategies of bacteria in the natural environment. *Microbiological Reviews* **51**, 365-379.

RUBIO, T.T. & Shapiro, C. (1986). Ciprofloxacin in the treatment of pseudomonas infection in cystic fibrosis patients. *Journal of Antimicrobial Chemotherapy* **18**, suppl. D., 147-152.

RUSESKA, I., Robbins, J., Lashen, E.S. & Costerton, J.W. (1982). Biocide testing against corrosion causing oilfield bacteria helps control plugging. *Oil and Gas Journal* **80**, 253-264.

RUSSELL, N.J. & Gacesca, P. (1989). Physicochemical properties of alginate from mucoid strains of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients. In *Pseudomonas aeruginosa Infection*, pp. 62-66. Eds. N.Høiby, S.S. Pedersen, G.H. Shand, G. Döring, G. & Holder, I.A. Basel: Karger.

SAIMAN, L., Ishimoto, K., Lory, S. & Prince, A. (1990). The effect of piliation and exoproduct expression on the adherence of *Pseudomonas aeruginosa* to respiratory epithelial monolayers. *The Journal of Infectious Diseases* **161**, 541-548.

SATAKE, S., Yoshihara, E. & Nakae, T. (1990). Diffusion of ß-lactam antibiotics through liposome membranes reconstituted from purified porins of the outer membrane of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **34**, 685-690.

SCHWYN, B. & Neilands, J.B. (1987). Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* **160**, 47-56.

SCHULTZ, E.W. (1947). A gelatinous variant of *Pseudomonas aeruginosa*. *Proceedings of the Society for Experimental Biology and Medicine* **65**, 289-291.

SCHULTZ, D.R. & Miller, K.D. (1974). Elastase of *Pseudomonas aeruginosa*: inactivation of complement components and complement-derived chemotactic and phagocytic factors. *Infection and Immunity* **10**, 128-135.

SCIORTINO, C.V. & Finkelstein, R.A. (1983). *Vibrio cholerae* expresses iron-regulated outer membrane proteins *in vivo*. *Infection and Immunity* **42**, 990-996.

SHAND, G.H., Anwar, H., Kadurugamuwa, J., Brown, M.R.W., Silverman, S.H. & Melling, J. (1985). *In vivo* evidence that bacteria in urinary tract infection grow under iron-restricted conditions. *Infection and Immunity* **48**, 35-39.

SHAND, G.H., Pedersen, S.S., Brown, M.R.W. & Høiby, N. (1991). Serum antibodies to *Pseudomonas aeruginosa* outer membrane proteins and iron-regulated membrane proteins at different stages of chronic cystic fibrosis lung infection. *Journal of Medical Microbiology* **34**, 203-212.

SHEN, L.L., Mitscher, L.A., Sharma, P.N., O'Donnell, T.J., Chu, D.W.T., Cooper, C.S., Rosen, T. & Pernet, A.G. (1989). Mechanism of inhibition of DNA gyrase by quinolone antibacterials: a cooperative drug-DNA binding model. *Biochemistry* **28**, 3886-3894.

SLACK, M.P.E. & Nichols, W.W. (1981). The penetration of antibiotics through sodium alginate and through the exopolysaccharide of a mucoid strain of *Pseudomonas aeruginosa*. *The Lancet* **11**, 502-503.

SLEYTR, U.B., Messner, P., Minnikin, D.E., Heckels, J.E., Virji, M. & Russell, R.B.B. (1988). Structure of bacteria and their envelopes. In *Bacterial Cell Surface Techniques*, pp. 1-31. Eds. I. Hancock & I. Poxton. Chichester: J. Wiley & Sons Ltd.

SMITH, A.W., Hirst, P.H., Hughes, K., Gensberg, K. & Govan, J.R.W. (1992). The pyocin Sa receptor of *Pseudomonas aeuginosa* is associated with ferripyoverdin uptake. *Journal of Bacteriology* **174**, 4847-4849.

SMITH, J.T. (1984). Awakening the slumbering potential of the 4-quinolone antibacterials. *The Pharmaceutical Journal* **233**, 299-305.

SMITH, J.T. (1986). The mode of action of 4-quinolones and possible mechanisms of resistance. *Journal of Antimicrobial Chemotherapy* **18**, suppl. D., 21-29.

SMYTH, C.J., Jonsson, P., Olsson, E., Soderlind, O., Rosengren J., Hjerten, S. & Wadstrom, T. (1978). Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. *Infection and Immunity* **22**, 462-472.

SOKOL, P.A. & Woods, D.E. (1983). Demonstration of an iron-siderophore binding protein in the outer membrane of *Pseudomonas aeruginosa*. *Infection and Immunity* **40**, 665-669.

ST. GEME III, J.W. & Falkow, S. (1990). *Haemophilus influenzae* adheres to and enters cultured human epithelial cells. *Infection and Immunity* **58**, 4036-4044.

ST. GEME III, J.W. & Falkow, S. (1991). Loss of capsule expression by *Haemophilus influenzae* type b results in enhanced adherence to and invasion of human cells. *Infection and Immunity* **59**, 1325-1333.

STANECK, J.L., Glenn, S., DiPersio, J.R. & Leist, P.A. (1989). Wide variability in *Pseudomonas aeruginosa* aminoglycoside results among seven susceptibility testing procedures. *Journal of Clinical Microbiology* **27**, 2277-2285.

STICKLER, D. & Hewett, P. (1991). Activities of antiseptics against biofilms of mixed bacterial species growing on silicone surfaces. *European Journal of Clinical Microbiology and Infectious Diseases* **10**, 157-162.

STICKLER, D., Dolman, J., Rolfe, S. & Chawla, J. (1989). Activity of antiseptics against *Escherichia coli* growing as biofilms on silicone surfaces. *European Journal of Clinical Microbiology and Infectious Diseases* **8**, 974-978.

SUTER, S. (1992). Lung inflammation and anti-inflammatory treatment. In *Cystic Fibrosis, Basic and Clinical Research*, pp. 177-182. Eds. N. Høiby & S.S. Pedersen. Amsterdam: Elsevier.

SUTHERLAND, I.W. (1977). Bacterial exopolysaccharides - their nature and production. In *Surface Carbohydrates of the Prokaryotic Cell*, pp. 27-96. Ed. I.W. Sutherland. London: Academic Press.

SUTHERLAND, I.W. (1980). Polysaccharides in the adhesion of marine and freshwater bacteria. In *Microbial Adhesion to Surfaces*, pp. 329-338. Eds. R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter & B. Vincent. Chichester: Ellis Horwood Ltd.

TANAKA, M., Otsuki, M. & Nishino, T. (1992). Bactericidal activities of ofloxacin and its optically active isomer (DR-3355) on non-growing cells of *Escherichia coli* and *Pseudomonas aeruginosa*. *Chemotherapy* **38**, 21-27.

TAYLOR, D., Prosser, B. & Cleeland, R. (1988). Effect of quinolones on 24 hour biofilms of *S. epidermidis*, *E. coli* and *P. aeruginosa*. Poster presentation at *28th Interscience Conference of Antimicrobial Agents and Chemotherapy*. Los Angeles.

TERRY, J.M., Pina, S.E. & Mattingly, S.J. (1991). Environmental conditions which influence mucoid conversion in *Pseudomonas aeruginosa* PAO1. *Infection and Immunity* **59**, 471-477.

TRIAS, J. & Nikaido, H. (1990a). Protein D2 channel of the *Pseudomonas aeruginosa* outer membrane has a binding site for basic amino acids and peptides. *The Journal of Biological Chemistry* **265**, 15680-15684.

TRIAS, J. & Nikaido, H. (1990b). Outer membrane protein D2 catalyses facilitated diffusion of carbapenems and penems through the outer membrane of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy.* **34**, 52-57.

TSAI, C.M. & Frasch, C.E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Analytical Biochemistry* **119**, 115-119.

TUOMANEN, E. (1986). Phenotypic tolerance: the search for ß-lactam antibiotics that kill nongrowing bacteria. *Reviews of Infectious Diseases* **8** suppl.3, S279-S291.

TUOMANEN, E., Cozens, R., Tosch, W. Zak, O. & Tomasz, A. (1986a). The rate of killing of *Escherichia coli* is strictly proportional to the rate of bacterial growth. *Journal of General Microbiology* **132**, 1297-1304.

TUOMANEN, E., Durack, D.T. & Tomasz, A. (1986b). Antibiotic tolerance among clinical isolates of bacteria. *Antimicrobial Agents and Chemotherapy.* **30**, 521-527.

UBUKATA, K., Itoh-Yamashita, N. & Konno, M. (1989). Cloning and expression of the *norA* gene for fluoroquinolone resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy.* **33**, 1535-1539.

VALERIUS, N.H., Koch, C. & Høiby, N. (1991). Prevention of chronic *Pseudomonas aeruginosa* colonisation in cystic fibrosis by early treatment. *The Lancet* **338**, 725-726.

VAN DER WOUDE, M., Arts, P.A., Bakker, D., Van Verseveld, H.W. & De Graaf, F.K. (1990). Growth-rate-dependent synthesis of K99 fimbrial subunits is regulated at the level of transcription. *Journal of General Microbiology* **136**, 897-903.

VAN LOOSDRECHT, M.C.M., Lyklema, J., Norde, W., Schraa, G. & Zehnder, A.J.B. (1987a). The role of bacterial cell wall hydrophobicity in adhesion. *Applied and Environmental Microbiology* **53**, 1893-1897.

VAN LOOSDRECHT, M.C.M., Lyklema, J., Norde, W., Schraa, G. & Zehnder, A.J.B. (1987b). Electrophoretic mobility and hydrophobicity as a measure to predict the initial steps of bacterial adhesion. *Applied and Environmental Microbiology* **53**, 1898-1901.

VAN LOOSDRECHT, M.C.M., Lyklema, J., Norde, W. & Zehnder, A.J.B. (1989). Bacterial adhesion: a physico-chemical approach. *Microbial Ecology* **17**, 1-15.

VISCA, P., Colotti, G., Serino, L., Verzili, D., Orsi, N. & Chiancone, E. (1992). Metal regulation of siderophore synthesis of *Pseudomonas aeruginosa* and functional effects of siderophore-metal complexes. *Applied and Environmental Microbiology* **58**, 2886-2893.

WANG, J.C. (1974). Interactions between twisted DNAs and enzymes: the effects of superhelicle turns. *Journal of Molecular Biology* **87**, 797-810.

WARD, K.H. (1987). *In-vivo* properties of *Pseudomonas aeruginosa*: outer membrane components and their antigenicity. Ph.D. Thesis. University of Aston, Birmingham.

WARD, K.H., Anwar, H., Brown, M.R.W., Wale, J. & Gowar, J. (1988). Antibody response to outer-membrane antigens of *Pseudomonas aeruginosa* in human burn wound infection. *Journal of Medical Microbiology* **27**, 179-190.

WEISS, L. & Harlos, J.P. (1972). Short term interaction between cell surfaces. *Progress in Surface Science* **1**, 355-405.

WHITFIELD, C. (1988). Bacterial extracellular polysaccharides. *Canadian Journal of Microbiology* **34**, 415-420.

WIDMER, A.F., Wiestner, A., Frei, R. & Zimmerli, W. (1991). Killing of nongrowing and adherent *Escherichia coli* determines drug efficacy in device-related infections. *Antimicrobial Agents and Chemotherapy* **35**, 741-746.

WIENTJES, F.B., Woldringh, C.L. & Nanninga, N. (1991). Amount of peptidoglycan in cell walls of Gram-negative bacteria. *Journal of Bacteriology* **173**, 7684-7691.

WILLIAMS, J.D. (1982). Epidemiological trends in antibiotic resistance and concerns for the future. In *The Control of Antibiotic-Resistant Bacteria*, pp. 35-56. Eds. C.H. Stuart-Harris & D.M. Harris. London: Academic Press.

WILLMOTT, C.J.R. & Maxwell, A. (1993). A single point mutation in the DNA gyrase A protein greatly reduces binding of the fluoroquinolones to the gyrase-DNA complex. *Antimicrobial Agents and Chemotherapy* **37**, 126-127.

WIMPENNY, J.W.T., Peters, A. & Scourfield, M. (1989). Modelling spatial gradients. In *Structure and Function of Biofilms*. Eds. W.G. Characklis & P.A. Wilderer. Chichester: J.Wiley & Sons Ltd.

WOLFSON, J.S. & Hooper, D.C. (1989). Fluoroquinolone antimicrobial agents. *Clinical Micribiology Reviews* **2**, 378-424.

WOLFSON, J.S., Hooper, D.C., Ng, E.Y., Souza, K.S., McHugh, G.L. & Swartz, N.M. (1987). Antagonism of wild-type resistant *Escherichia coli* and its DNA gyrase by the tricyclic 4-quinolone analogues ofloxacin, and S-25930 stereoisomers. *Antimicrobial Agents and Chemotherapy* **31**, 1861-1863.

WONG, W.H., Irvin, R.T., Paranchych. W. & Hodges, R.S. (1992). Antigen-antibody interactions: elucidation of the epitope and strain-specificity of a monoclonal antibody directed against the pilin protein adherence binding domain of *Pseudomonas aeruginosa* strain K. *Protein Science* **1**, 1308-1318.

WOOD, P. (1990). Effects of nutrient limitations and growth rate on physiology and susceptibility to antimicrobial agents of *Pseudomonas aeruginosa* PAO1. M.Sc. Thesis. University of Aston, Birmingham.

WOODRUFF, W.A. & Hancock, R.E.W. (1989). *Pseudomonas aeruginosa* outer membrane protein F: structural role and relationship to the *Escherichia coli* OmpA protein. *Journal of Bacteriology* **171**, 3304-3309.

WOODS, D.E., Straus, D.C., Johanson Jr., W.G., Berry, V.K. & Bass, J.A. (1980). Role of pili in adherence of *Pseudomonas aeruginosa* to mammalian buccal epithelial cells. *Infection and Immunity* **29**, 1146-1151.

WOODS, D.E., Straus, D.C., Johanson, Jr., W.G., Bass, J.A. (1981). Role of fibronectin in the prevention of adherence of *Pseudomonas aeruginosa* to buccal cells. *Journal of Infectious Diseases* **143**, 784-790.

WRIGHT, N.E. & Gilbert, P. (1987). Influence of specific growth rate and nutrient limitation upon the sensitivity of *Escherichia coli* towards polymyxin B. *Journal of Antimicrobial Chemotherapy* **20**, 303-312.

WU, J.P. & Livermore, D.M. (1990). Response of chemostat cultures of *Pseudomonas aeruginosa* to carbapenems and other ß-lactams. *Journal of Antimicrobial Chemotherapy* **25**, 891-902.

YAMANO, Y., Nishikawa, T. & Komatsu, Y. (1990). Outer membrane proteins responsible for the penetration of ß-lactams and quinolones in *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy* **26**, 175-184.

YATES, J.M. (1992). Influence of iron on bacterial infections in leukaemia. Ph.D. Thesis, University of Aston, Birmingham.

YATES, J.M., Morris, G. & Brown, M.R.W. (1989). Effect of iron concentration and growth rate on the expression of protein G in *Pseudomonas aeruginosa*. *FEMS Microbiology Letters* **58**, 259-262.

YOSHIDA, H. (1990). Proportion of DNA gyrase mutants among quinolone-resistant strains of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **34**, 1273-1275.

YOSHIDA, H., Bogaki, M., Nakamura, M., Yamanaka, L.M. & Nakamura, S. (1991). Quinolone resistance-determining region in the DNA gyrase *gyrB* gene of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **35**, 1647-1650.

YOSHIHARA, E. & Nakae, T. (1989). Identification of porins in the outer membrane of *Pseudomonas aeruginosa* that form small diffusion pores. *The Journal of Biological Chemistry* **264**, 6297-6301.

YOSHIMURA, F., Zalman, L.S. & Nikaido, H. (1983). Purification and properties of *Pseudomonas aeruginosa* porin. *The Journal of Biological Chemistry* **258**, 2308-2314.

ZEILER, H.-J. (1985). Evaluation of the *in vitro* bactericidal action of ciprofloxacin on cells of *Escherichia coli* in the logarithmic and stationary phases of growth. *Antimicrobial Agents and Chemotherapy* **28**, 524-527.

ZOUTMAN, D.E., Hulbert, W.C., Pasloske, B.L., Joffe, A.M., Volpel, K., Trebilcock, M.K. & Paranchych, W. (1991). The role of polar pili in the adherence of *Pseudomonas aeruginosa* to injured canine tracheal cells: a semiquantitative morphologic study. *Scanning Microscopy* **5**, 109-126.

ZWEERINK, M.M. & Edison, A. (1986). Inhibition of *Micrococcus luteus* DNA gyrase by norfloxacin and 10 other quinolone carboxylic acids. *Antimicrobial Agents and Chemotherapy* **29**, 598-601.