# STUDIES ON SURVIVAL OF
## *Pseudomonas aeruginosa* 6750

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by

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## SUMMARY

The growth of *Pseudomonas aeruginosa* 6750 as a biofilm was investigated using a novel system based on that of Gilbert *et al* (1989). The aim was to test the effect of controlled growth of the organism on antibiotic susceptibility and examine the survival of the organism as a biofilm. During the investigations it became clear that, because of the increasing growth of *P. aeruginosa* and production of exopolysaccharide, a growth rate controlled monolayer could not be achieved and so the method was not used further. The data, however, showed that there was an increase in the smooth colony type of the organism during growth.

Investigations were focused on the survival of *P. aeruginosa* in batch and chemostat studies. Survival or percentage culturability, as measured by total and colony count ratio, was found to decrease both in extended batch culture and for chemostat cells with decreasing growth rate. Extended batch culture, however, did not exhibit further increases in resistance to ciprofloxacin and polymyxin B. Survival was also measured using other parameters namely the direct viable count, vital staining, effect of temperature downshift and measurement of lag. In batch culture, the most notable change was a decrease in cell size along the growth curve. This was accompanied by an increase in the cellular protein content. Protein per volume was calculated from the data which showed a marked increase in batch culture, which was not demonstrated for chemostat cells with decreasing growth rate.

Outer membrane protein profiles were obtained for batch and chemostat cells. An LPS profile of batch culture cells was also demonstrated. In general, there was little difference in the outer membrane protein profiles of cells from early and late stationary phases. The result of the LPS profile showed that there appeared to be an increase in the B-band of the region of the LPS in the older stationary phase cultures.

Key words : *Pseudomonas aeruginosa*, biofilms, growth rate, survival, size

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## ABBREVIATIONS

| | |
|---|---|
| BCA | Bicinchoninic acid |
| CDM | chemically defined media |
| CDM-Fe | iron-depleted CDM |
| CDM+Fe | iron-plentiful CDM |
| CF | cystic fibrosis |
| CFTR | cystic fibrosis transmembrane regulator |
| cfu(s) | colony-forming units |
| CM | cytoplasmic membrane |
| CSLM | confocal scanning laser microscopy |
| CTC | 5-cyano-2, 3-ditolyl tetrazolium chloride |
| $^{o}C$ | degrees centigrade |
| (d)dH$_2$O | (double) distilled water |
| DAPI | 4', 6-diamidino-2-phenylindole |
| DiBAC$_4$(3) | bis-(1, 3-dibutylbarbituric acid)trimethine oxonol |
| DiOC6(3) | 3, 3' -dihexyloxacarbocyanine |
| DNA | deoxyribonucleic acid |
| DVC | direct viable count |
| EDTA | ethylenediaminetetraacetic acid |
| EPS | extracellular polymeric matrix |
| hr | hour(s) |
| HSL | $N$-acyl homoserine lactone |
| INT | 2-($p$-iodophenyl)-3-($p$-nitrophenyl)-5-phenyltetrazolium chloride |
| IROMP(s) | iron-regulated outer membrane protein(s) |
| kDa | kilodaltons |
| KDO | 2-keto-3 deoxyoctonic acid |
| l | litre(s) |
| LPS | lipopolysaccharide |
| μg | microgramme(s) |
| μl | microlitre(s) |
| μm | micrometre(s) |
| μM | micromolar |
| mg | milligramme(s) |
| ml | millilitre(s) |
| min | minutes(s) |
| M | molar concentration |
| MOPS | 4-morpholino-propane sulphonic acid |
| nm | nanometre |

| | |
|---|---|
| OD | optical density |
| $OD_{470}$ | optical density at 470nm |
| OM | outer membrane |
| OMP(s) | outer membrane protein(s) |
| OprB | outer membrane protein D1 |
| OprC | outer membrane protein C |
| OprD2 | outer membrane protein D2 |
| OprE1 | outer membrane protein E1 |
| OprF | outer membrane protein F |
| OprG | outer membrane protein G |
| OprH | outer membrane protein H1 |
| OprI | outer membrane protein I |
| OprL | outer membrane H2 |
| Oxonol VI | bis-(3-propyl-5oxoisooxazol-4-yl)pentamethine-oxonol |
| PBS | phosphate buffered saline |
| PHA | poly-3-hydroxyalkanoate |
| PHB | poly(3-hydroxybutyrate) |
| PCS | photon correlation spectroscopy |
| RNA | ribonucleic acid |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| Sp. | species |
| UV | ultraviolet |
| $\mu$ | specific growth rate |
| $\sigma^s$ | sigma factor |
| $t_d$ | doubling time |

# 1. INTRODUCTION

## 1.1. Survival of Microorganisms

Microorganisms are capable of surviving for extremely long periods of time. Records suggest that bacteria have survived for centuries and even millennia (Kennedy *et al*, 1994). However, careful evaluation of data is required to establish whether microorganisms have actually been revived after a survival period of dormancy in which they have existed with a low endogenous metabolism, or if an example such as *in situ* reproduction of an isolated organism or contamination of the sample has taken place so disproving the theory of long-term storage.

Survival of microorganisms is an important consideration in the areas of ecology, medicine and the food and water industries. Studies have been completed that have investigated survival of microorganisms especially in the area of marine biology. Microorganisms have been found to exist in oligotrophic environments of the sea in the form of ultramicrobacteria. Some have claimed that formation of ultramicrobacteria is a result of a stress response or survival strategy (Novitsky and Morita, 1978) in which small cells were formed as a result of several reductive divisions so forming an increase in cell number without an increase in cell biomass. A further cell size reduction is caused by the degradation of endogenous material. These cells are thought to survive for long periods of time with an accompanying decrease in metabolic activity. Marine biology studies and work in natural systems have then been related to the food and water industries because of the potential pathogenicity of such microorganisms in an extended survival state.

## 1.2. "Viable" but Non-Culturable Bacteria

Much debate has surrounded the term "viable" non-culturable and whether or not the state actually exists. The term has been used in general to represent those bacterial cells that are unable to replicate in conventional media but have been shown to possess either some form of active metabolic process or the ability to grow after an incubation period in a specific environment. This may be analogous to germination of dormant bacterial spores.

The problem concerning the metabolic state of organisms that are unable to undergo cell division but have been observed in direct counting techniques, was documented in the early 1900s (Knaysi, 1935). In 1953, Heinmets *et al* recognised that metabolic growth processes could continue in the absence of cell division. Studies that were performed in the late 1970s confirmed findings that cells that were thought to be nonculturable were

able to metabolize actively and were therefore considered to be viable (Kogure *et al*, 1979). The limitations of the definition of "viability" has been discussed (Roszak and Colwell, 1987) but an overall accepted definition of the term has not as yet been produced. Other workers have recently proposed that the evidence supporting the viable but non-culturable hypothesis is not conclusive, and so a re-examination of concepts is thought to be necessary (Barer *et al*, 1993).

Selected definitions of terms used in the thesis are given below :

*Viability* : The use of this term will be limited in this thesis. In general, it describes the ability of cells to undergo replication to produce similar viable progeny under certain culture conditions.

*Total count* : The count refers to the total number of cells that can be evaluated microscopically.

*Colony count* : The count relates to the number of culturable cells that can be observed as isolated colonies on solid media.

*Culturability* : The term refers to the ability of cells to form colonies on solid media.

*Vitality* : The term refers to the ability of cells to retain certain assayable activities.


## 1.3. Assays to Assess Culturability or Vitality in Non-Culturable Cells

To assess culturability, bacterial cells are traditionally grown on culture media by a process of plating out serial dilutions and by determination of numbers of colony forming units that appear after incubation. However, where the colony count is less than the total count, it is not known whether there are cells that have not grown because they are either dead or that they cannot be cultured in this way but possess some form of metabolic activity. Because of this problem, various assays have been developed that assess whole populations of cells directly. Many of the assays are in the form of vital stains that utilize a metabolic process of the cell and manifest themselves by means of fluorescence.

## 1.3.1. Nucleic Acid Stain : Acridine Orange

Acridine orange has been used by microbial ecologists to stain bacterial cells and provide an indication of physiological activity in order to distinguish between active and inactive cells (Hobbie *et al*, 1977, Lopez-Torres *et al*, 1988). The rationale for use of acridine orange as an indicator of vitality was based on the fact that the dye intercalates into nucleic acid and fluoresces if excited with UV light. When acridine orange binds to RNA (single-stranded nucleic acids) it fluoresces red-orange, whereas when it binds to DNA (double-stranded nucleic acids) it fluoresces green. Since a living cell contains more RNA than DNA it stains orange, but a cell that has had its RNA degraded should retain its DNA and so will stain green.

There are limitations, however, to this assay. If cell death were to occur without loss of RNA then the stain would not be able to detect the true lack of activity (Siegele *et al,* 1993). Studies have also shown that differentiation between active and inactive cells could not be seen when bacteria were grown in minimal media. These findings suggested that physiological activity could only be determined under defined conditions (McFeters, 1991).

## 1.3.2. Direct Viable Count

Direct count of viable bacterial cells (DVC) was developed by Kogure *et al* (1979). Samples were incubated for a certain period of time with small amounts of yeast extract and nalidixic acid. The yeast extract provided nutrients in order to induce growth whereas the nalidixic acid, which is a specific inhibitor of DNA synthesis (Goss *et al*, 1965), prevented cell division of Gram-negative bacteria. Cell replication is thereby inhibited but at the same time cell growth is promoted. The cell growth manifests itself in filament formation. Further work with the DVC method demonstrated that cells responding to the procedure were in an active metabolic state and that the filament formation was a result of metabolism and growth of viable cells (Roszak and Colwell, 1987).

Several points have been made regarding the technique, which suggest that processes underlying the phenomenon have not been explained (Barer *et al*, 1993). Since many of the cells tested by the DVC method may not be able to undergo cell division anyway using routine culture techniques, addition of nalidixic acid may not in fact prevent DNA replication. This could mean that yeast extract alone may produce the same effect. It is therefore important to examine this by performing control experiments. Additionally, quinolones such as nalidixic acid have been shown to produce an SOS response which

involves formation of filaments. It would be unclear whether filament formation was due to SOS response or to growth of viable cells.

DVC method has mainly been used on Gram-negative bacteria as the procedure is dependent on the bacterium being sensitive to nalidixic acid. Recent developments, however, have modified the procedure by using other suitable DNA gyrase inhibitors (Servis et al, 1995). Experimenters found that the antibiotic and concentration required to give optimal results, varied between different species of Gram-positive bacteria. Other workers have discovered that ciprofloxacin is a suitable antibiotic in DVC method for Gram-positive and Gram-negative bacteria (Barcina et al, 1995). Modifications to the original method involved measurement of biovolume increase to determine percentages of viable cocci, instead of filament formation.

## 1.3.3. Membrane Potential Dyes

Rhodamine 123 is a water-soluble, cationic, lipophilic fluorescent dye that has been used recently to distinguish, using the definitions of Kaprelyants and Kell, between "non-viable", "viable" and "non-viable-but-resuscitable" *Micrococcus luteus* cells (Kaprelyants and Kell, 1992). Trans-membrane potential is a sign of vitality in bacterial cells. It is thought to be generated by metabolic processes and helps the membrane transport of nutrients and ions (Harold, 1972). Rhodamine 123 moves along with the trans-membrane potential in an uncoupler-sensitive manner and is accumulated cytosolically in cells with an inside negative electrochemical potential. Matsuyama (1984) showed that the accumulation of the dye could be reversed by uncouplers. This enabled "viable" and "non-viable" cells to be quantitatively distinguished in a flow cytometer (an instrument by which cells are analysed individually) by the extent to which they accumulated Rhodamine 123 (Kaprelyants and Kell, 1992).

The method was adapted for Gram-negative bacterial cells by pre-treatment with Tris-EDTA that permeabilized the outer membrane and allowed adequate staining with the dye (Kaprelyants and Kell, 1992, Yu and McFeters, 1994).

Another group of dyes are the cyanine dyes such as 3, 3'-dihexyloxacarbocyanine (DiOC6(3)). DiOC6(3) stains a large number of bacteria but its accumulation does not always reflect membrane potential. This is because the dye undergoes non-specific staining in which it binds to cell structures such as lipids. However, the dye has been found useful in the assessment of vitality for *Pseudomonas putida* (Pinder et al, 1993).

A third type of membrane potential sensitive dye belongs to the oxonol family. It is a lipophilic anion unlike Rhodamine 123. It is therefore not accumulated cytosolically but its action is opposite to that of the cationic dyes. This means that as membrane potential increases, the fluorescent response decreases (Mason *et al*, 1995). Two oxonol dyes have been tested : bis-(1, 3-dibutylbarbituric acid) trimethine oxonol (DiBaC$_4$(3)) and bis-(3-propyl-5-oxoisoxazol-4-yl) pentamethine oxonol (Oxonol VI). Some work has suggested that oxonol VI is the more useful for the detection of membrane depolarization during starvation survival processes. This is because oxonol VI has a longer wavelength of emission which is far removed from its autofluorescence emission (López- Amorós *et al*, 1995).

## 1.3.4. Respiratory Chain Activity Stain

The redox dye 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) was used to directly observe active bacteria (Iturriaga, 1979). The stain acts as an artificial electron acceptor. The reducing activity which is produced by the electron transport system of the cells converts INT into insoluble INT-formazan crystals. The crystals accumulate in metabolically active bacteria and can be visualized using a microscope with bright field optics. Recently a new compound has been more widely used. 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) has been found to be more useful (Rodriguez *et al*, 1992). The stain acts in a similar way to INT except that when it is reduced it forms a fluorescent insoluble formazan compound. Because of the fluorescence of the formazan compound it is more easily detected when illuminated with longwave UV light, which increases the sensitivity of the method.

CTC has been used with flow cytometry to determine respiratory activity in individual cells (Kaprelyants and Kell, 1993a). Because of the sensitivity of the technique, CTC has been used for a variety of applications : it has been used to measure dormancy in *Micrococcus luteus cells* (Kaprelyants and Kell, 1993b), to quantify respiring bacteria in drinking water and surface biofilms (Schaule *et al*, 1993) and to assess respiratory activity of indigenous bacteria (Winding *et al*, 1994).

## 1.3.5. Esterase Activity Stains

Fluorogenic esters such as fluorescein diacetate and various substituted derivatives of this compound have been used as vital stains in bacteria. Fluorescein diacetate is a lipophilic non-fluorescent derivative of fluorescein. It diffuses through cell membranes after which it is cleaved by non-specific esterases to release fluorescein, which is then retained intracellularly. A brilliant green fluorescence is emitted when irradiated with blue light (Chrzanowski *et al*, 1984). The accumulation and hydrolysis depends on an intact membrane and metabolism in bacteria and this gives an indication of vitality in a cell (McFeters *et al*, 1995). In dead cells that lack intact membranes, the fluorochrome leaks out of the cell. Additionally, these cells may lack the enzymes required for conjugate cleavage (Pinder *et al*, 1993).

Derivatives of fluorescein diacetate are thought to be better retained by viable cells (Musgrove and Hedley, 1990). They include carboxyfluorescein diacetate and 2', 7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethylester. It has been suggested that carboxyfluorescein is better retained because Gram-negative species tend to cleave fluorescein diacetate in the periplasm so that the fluorescein may not enter the cell. Carboxyfluorescein, on the other hand, may be less susceptible to this periplasmic cleavage and so may enter the cell and undergo cleavage intracellularly (Pinder *et al*, 1993). Another fluorogenic ester that has been used is ChemChrome B which is a commercially available stain (Chemunex UK, Cambridge).

The use of a number of fluorogenic esters was tested using flow cytometry (Diaper and Edwards, 1994). The results showed that no one dye was found to be universally successful in staining. ChemChrome B was found to stain the widest number of Gram-positive and Gram-negative species but the fluorescein diacetate derivatives were better able to stain Gram-positive species.

## 1.3.6. Calcafluor White

Calcafluor White is the disodium salt of 4, 4'-bis(4 anilino-bis-diethyl amino-s-triazin-2-ylamino)-2, 2'-stilbene disulphonic acid). The compound is strongly fluorescent when excited by u.v. light. Studies have been made examining absorption and transport of this type of dye in microorganisms (Darken, 1962). The exact mode of action is unknown at present (Lloyd and Hayes, 1995) but viable cells are able to exclude the dye whereas non-viable cells appear brightly fluorescent (Mason *et al*, 1995). In experiments comparing the ability of membrane potential dyes and calcafluor white to distinguish between viable and non-viable bacteria, calcafluor white appeared an

effective dye (Mason *et al*, 1995). The dye was tested on non-viable cells killed in two ways namely heat treatment and gramicidin treatment. The control viable cells were early logarithmic phase cells ($OD_{600}$ 0.3). The results showed that calcafluor white produced larger changes in fluorescence between viable and non-viable populations of bacteria compared to the carbocyanine and rhodamine 123 dyes tested.

## 1.3.7. Future use of Fluorescent Probes

There has been an increase in the use of fluorescent probes aided by more advanced techniques for analysis (McFeters *et al*, 1995). Technical advances include scanning confocal laser microscopy, digital image analysis, more highly sensitive cameras (Caldwell *et al*, 1992) and flow cytometry.

## 1.3.8. Flow Cytometry and Bacteria

Flow cytometry is a method by which cells can be analysed individually. Cells are carried one by one in a microscopic jet of water and subjected to an intense beam of excitation light. This intense light excites a fluorescent dye which is bound to a cellular constituent of the cell. Each cell then produces a short flash of fluorescence proportional to the content of that constituent (Steen, 1983). The fluorescence produced is collected by appropriate optics and then transformed by a photomultiplier tube into an equivalent electrical pulse. The electrical pulses are sized, sorted into "bins" or "channels" and stored by a multichannel pulse height analyser. A histogram can be produced which accumulates the data and presents it in a two or three dimensional graph.

Additionally, each cell can produce scattering of the excitation light as it passes through the beam. The magnitude of the scattering is a function of the size, shape or structure of the cell. The flash of scattered light is recorded by a separate detector (Steen, 1990).

Flow cytometry is a rapid and sensitive method for the analysis of bacterial cells (Kell *et al*, 1991). The major feature of the instrument is its ability to characterise individual bacterial cells in populations. Since analysis with fluorescence can also be undertaken, the capability to analyse and identify a population of cells that appear dormant can be achieved with suitable resuscitation studies (Kaprelyants *et al*, 1993). It should be noted that the physiological state of the test cells may influence the uptake of the dye. Cells with decreased permeability could wrongly suggest that they were either dead or non-viable because of reduced levels of fluorescence. Results should therefore be carefully interpreted.

No single stain has been found to be universally useful. Studies have shown that Rhodamine 123 works well for most species, but because it undergoes non-specific binding to particulates it is of limited value for environmental preparations, as a high background fluorescence is produced (Pinder *et al*, 1993). Overall, a range of dyes should be tested for a given bacterial species to assess suitability.

## 1.4. Size and Volume Reduction

It has been referred to already that cells become smaller in situations where there has been extended survival. Ultramicrobacteria, referred to previously, are seen in a number of marine bacteria, and have been shown to decrease to sizes as small as $0.03\mu m^3$ (Kjelleberg et al, 1987). The morphological differentiation into an ultramicrocell can be a consequence of adaptation to starvation (Kjelleberg et al, 1993). It has been shown in seawater samples that a fraction of these cells grow back to normal sized bacteria when nutrients become available (Torrella and Morita, 1981).

It has also been shown that cells incubated at low temperature experience a morphological change from rods to cocci (Oliver et al, 1991). Size reduction was obtained using *Vibrio vulnificus* in which it was suggested that the effect of the temperature downshift was a result of the viable nonculturable response. However, since cells exposed to the temperature downshift did not respond with a reductive division, it was said that the nonculturable response was different from the starvation response of marine bacteria.

Other workers have found that a dormant state was produced when the copiotrophic *vibrio* sp. strain DW1 requiring relatively high nutrient concentrations for growth, was subjected to starvation conditions. The cells become smaller and tend to accumulate at the air-water interface. It has been suggested that interfaces lacking a sufficiency of nutrients trigger copiotrophic cells to become smaller as a result of a survival strategy (Kjelleberg et al, 1982). Conversely, the native oligotrophs that have adapted to low nutrient conditions may not respond to high nutrient environments.

It has been suggested that a possible advantage of the increase in cell number experienced by marine bacteria is to improve survival of the population by increasing the possibility that cells may encounter nutrients (Morita, 1986). Another survival advantage would be that a small cell would require less energy for maintenance if adverse conditions were encountered (Kjelleberg et al, 1982). The morphological change in cell shape from rods to cocci also results in an increase in the surface area to volume ratio which may increase cells' chance of encountering nutrients in low nutrient environments

Changes in cell size have been shown to be accompanied by changes in subcellular compartments (Siegele and Kolter, 1992). This occurs in the case of *E. coli* where it is found that the cytoplasm is condensed and the volume of the periplasm increases (Reeve et al, 1984b). Therefore it is concluded that entry into a starvation phase requires the

cell to make both physiological and structural changes to the cell, one of which is manifested as a change in cell size.

## 1.4.1. Direct Measurements of Cell Size

One of the simplest methods in which to measure size is the direct microscopic observation of bacterial cells by eye and measurement using a graticule and ocular micrometer. Fluorescent dyes can also be used to enhance the visualization of the cells. A problem that occurs with the use of fluorescence, however, is the difficulty in seeing the outline of fluorescent images of bacteria due to the so- called "halo" effect (Lee and Fuhrman, 1987). The halo is caused by several factors including the spread of fluorescent light from the cell interior through the cell wall and various optical limitations as a result of the limit of resolution of the light microscope (Fry, 1990). There is no overall consensus about how to judge the true edge of the bacterium. This method has proved popular as there is no need for specialist equipment.

Photographic methods are also relatively straightforward and are often used for measuring sizes of natural bacteria (Fry, 1990). Photographs are taken of the images of the bacteria using high-contrast, high-speed films. The bacteria on the negatives or slides can then be measured directly by projection. The projection should be at a magnification of between 10,000 x (Lee and Fuhrman, 1987) and 14,000 x (Bratbak, 1985). This magnification can be achieved with a slide projector using a final image size of about 70cm x 48cm from a complete 35-mm negative (Fry and Davies, 1985). Measurements of all bacteria can then be made of the projected images with a ruler or micrometer gauge. An internal standard consisting of polystyrene latex particles of known diameter should be photographed in the same manner as the bacteria. After projection, the sizes can be measured and using the actual values for the latex particles, the actual sizes of the bacteria can be calculated. The major disadvantage of the method is that it is time-consuming, since the photographs have to be developed before sizes can be measured.

Electron microscope methods were thought to be unsuitable for measuring sizes of bacteria as they all caused a considerable amount of shrinkage and so did not produce a true estimate of cell size (Fuhrman, 1981).

More recent developments in electron microscopy, however, have shown that the technique can be adapted to examine morphological aspects of bacterial cells and so give an indication of cell size (Kondo et al, 1994) which could be used as a future method of cell sizing. The fixation method that was used to examine the bacteria was

said to be sufficiently mild so as to preserve the fine structures of the bacterial surface which cannot be achieved by use of the conventional chemical fixation method (Amako *et al*, 1988).

## 1.4.2. Indirect Measurements of Cell Size

Light scattering techniques have been used for many years to examine microbial cell size and cell morphology by differential light scattering (Koch, 1968). The dynamic light scattering technique of photon correlation spectroscopy (PCS) or quasi-electric light scattering has also been widely used as a method to size particles (Pecora, 1985).

In the procedure of PCS, a population of particles or bacterial cells scatter coherent light, namely laser light. The principle of PCS involves the analysis of time scales over which the coherent light, fluctuates as a consequence of the particle's Brownian motion. In this way, an accurate determination of the diffusion coefficient is made, which can then be related to cell size (Jepras *et al*, 1991).

The advantages of the procedure are that small volumes can be analysed in a relatively quick time. The technique requires no calibration and is not dependent on sample concentration or incident light intensity (Cummins and Pike, 1977).

Flow cytometry is a technique in which large numbers of single cells (more than $10^3$ cells per second) can be analysed as they move in a fluid stream. Several physical and chemical properties can be measured, one of which is cell size (Boye and Løbner-Olesen, 1991). Bacteria are stained with a fluorescent dye. The amount of scattered light that has emitted from a bacterium as it passes through the excitation focus of a flow cytometer is said to be a good measure of the cell mass (Boye *et al*, 1983). This means that the mass of individual bacterial cells can be analysed so giving an indication of cell size.

Kaprelyants and Kell (1993b) measured bacterial cell size using flow cytometry. Low-angle light scattering data, obtained from the flow cytometer, have been converted to apparent cell diameters by calibrating the instrument with latex beads of known diameters (Davey *et al*, 1993). The bacterial cells and latex beads scatter light differently and so this method uniformly underestimated the diameter of unfixed cells by approximately 26%. Despite the underestimation of cell size, a trend showing a change in mean cell diameter was shown.

## 1.5. Ability of Cells to Resuscitate

Dormancy has been classed as a reversible state in which cells persist for extended periods of time without division, with a low metabolic activity (Kaprelyants *et al*, 1993 review). It is the word "reversible" that indicates that so-called dormant cells should possess the ability to resuscitate and resume "normal" metabolic activity and possible growth when appropriate conditions present themselves.

It is known that only a limited fraction of cells in a population of ultramicrobacteria isolated from soil is capable of forming colonies on agar (Bakken and Olsen, 1987). However, after various resuscitation procedures it has also been shown that the bacteria can be revived and revert to "normal" bacteria (Morita,1988). It is clear that there is a need for special resuscitation procedures for bringing cells out of dormancy : minimal nutrient concentration and a prolonged incubation time appear to be extremely important for recovering ultramicrobacteria (Tabor *et al*, 1981). Other workers have hypothesized that the laboratory concentrations of nutrients used in an experiment were thought to be inappropriately high, as the bacterial isolates would have been conditioned to low nutrient conditions (Torrella and Morita, 1981).

An experiment was carried out with starved *vibrio* sp. marine bacteria (Albertson *et al*, 1990). The recovery media was a glucose-containing liquid media. Various parameters were measured during the recovery procedure including total cell number, average cell volume and respiratory and biosynthetic activities of the cells. Results showed that an increase in average cell volume from 0.5 $\mu m^3$ to 3-4 $\mu m^3$ was achieved with an accompanying constant cell number. These results indicated that the recovery was unlikely to be due to multiplication of a few culturable bacteria in the starved population. It has to be noted that the cells used in the study demonstrated a low but readily detected level of RNA and protein synthesis even after 200 hours of starvation, and so were not classed as dormant.

Cells that are in the viable but nonculturable state do not respond quickly to a reversal of the factor which initially induced the nonculturable state. This is opposite to the behaviour of starved bacteria (Oliver, 1993). The resuscitation of *S. enteritidis* from the viable nonculturable state has been reported (Roszak *et al*, 1984). The results showed that bacterial cells that were made unculturable in sterile river water were resuscitated to almost the original cell density after the addition of nutrients. An incubation period of 25 hours was required before cells appeared culturable.

The potential problem of resuscitation studies that rely on nutrient additions to nonculturable cells has been highlighted by Oliver *et al* (1991). It is often difficult to

prove whether true resuscitation has taken place or whether a few culturable cells that have remained undetected have in fact multiplied because of the addition of nutrient. Re-examination of several studies have found that evidence for dormancy was able to be disproved (Rose *et al*, 1990). In an attempt to overcome this problem, experimenters tried to recover *V. vulnificus* without addition of nutrients (Nilsson *et al*, 1991). The nonculturable bacteria were subjected to a temperature upshift. The original bacterial count was achieved after 3 days without an increase in total cell counts, suggesting that resuscitation took place without growth of a few culturable cells. Microscopic examination of the bacteria revealed no cocci, suggesting resuscitation of cocci to rods, and so confirmed the findings. However, later suggestions stated that a possibility still existed in which regrowth of a few cells could have taken place by the utilization of nutrients released by dead cells present in the main population. To account for the constant number of total counts during resuscitation, it was suggested that the majority of the nonculturable cells may undergo lysis after temperature upshift and may be replaced by the regrowing cells (Weichart *et al*, 1992).

More recent resuscitation studies have used the flow cytometer and various staining techniques to gain insight into the problems (Kaprelyants and Kell, 1993b). *Micrococcus luteus* cells that were stored in spent growth medium for an extended period of time were pretreated with penicillin to remove any culturable cells. The cells were monitored with flow cytometry and measuring total and colony counts. Resuscitation of the cells was accompanied by the appearance of a population of larger cells that could accumulate rhodamine 123 and reduce CTC. A similar fraction of the cells possessed the ability to form colony forming units as well as there being a similar decrease in the fraction of small cells. The results suggested that the initial population of cells was not dead but dormant and that resuscitation and not growth had taken place.

Another study investigated the influence of culturable cells on the resuscitation of dormant cells in *Micrococcus luteus* cultures held in an extended stationary phase (Votyakova *et al*, 1994 ). Under certain conditions, it was found that the use of a 1 : 1 mixture of fresh lactate medium and supernatant from a late-logarithmic phase *M. luteus* cultures resulted in a medium that favoured resuscitation and cell growth (Kaprelyants *et al*, 1994). It was concluded that the presence of culturable cells in a population before resuscitation is a requirement for the recovery of dormant cells. It was also suggested that the resuscitation was due to some factor being excreted which promoted the transition from dormancy to a state capable of colony formation.

Following on from this work, it was discovered recently that during the initial resuscitation stage, the dormant cells divide for 10 to 17 generations in lactate minimal medium containing yeast extract, whilst still remaining nonculturable on agar plates.

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After further incubation there was a decrease in the total cell number in liquid medium. The addition of culturable *M. luteus* cells to either resuscitating cells or supernatant from these cultures resulted in the discovery that an excretion of a "factor" or "factors" had taken place which inhibited the proliferation of the supposedly culturable cells. Supernatant that was taken from late logarithmic phase cultures of the organism removed this inhibitory effect. It was suggested that the effect of late logarithmic culturable cells and supernatant, on resuscitation of dormant cells, may be involved with overcoming the activity of a certain antibacterial factor. It was concluded that this factor may cause a "self-poisoning" effect on the dormant cells during the resuscitation process (Mukamolova *et al*, 1995a).

## 1.6 Increased Resistance to Stress

Bacterial spores are able to survive extreme adverse conditions. However, non-differentiating bacteria can develop a resistant state which enables them to survive for long periods of time under conditions of environmental stress.

Starvation is known to induce the development of a more resistant state in non-sporulating bacteria (Siegele and Kolter, 1992). Experiments have demonstrated that glucose or nitrogen starved cultures of *Escherichia coli* have increased resistance to heat or hydrogen peroxide compared to cultures that are growing exponentially (Jenkins *et al*, 1988). These results were found to be different to well established findings that stationary phase cells are in general more resistant than exponentially growing cells (Elliker *et al*, 1938; Sherman *et al*, 1923). This is because these were the first results to state that the enhanced resistance could be attributed to the synthesis of starvation proteins. The studies investigated the effect of chloramphenicol, a protein synthesis inhibitor. In the presence of chloramphenicol, the starved cultures failed to exhibit either type of enhanced resistance, which suggested that protein synthesis was essential for the development of the observed cross-protection. The resistance produced by starvation was even more protective than that produced by heat or hydrogen peroxide adapted cells.

A similar investigation showed that glucose starvation of stationary phase cultures and osmotic adaptation of exponentially growing cells both produced enhanced osmotic resistance. The osmotolerance that developed during starvation or osmotic adaptation was also shown to require "de novo" protein synthesis (Jenkins *et al*, 1990).

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Experimenters have discovered similar findings in several species of bacteria: Nyström *et al* (1992) reported the onset of the development of resistance in *Vibrio* species, against a variety of stress conditions.

Other workers have investigated the effects of starvation and osmotic stress on heat resistance of *Pseudomonas fluorescens* (Jørgensen *et al*, 1994). The results showed that carbon-starved *P. fluorescens* developed increased heat resistance when compared to logarithmic phase controls. A further experiment tested the ability of osmotic stress to induce cross-protection against heat. However, an increased thermotolerance could not be shown.

Another study analysed the responses to nutrient starvation in *Pseudomonas putida*, including an investigation into general cross-protection (Giskov *et al*, 1994). Studies were undertaken in which growing cells, cells in the transition phase, and carbon-starved cells were challenged with various environmental stresses. It was shown from the results that as cells shifted from exponential growth in glucose-supplemented medium to a medium depleted of carbon, a high degree of resistance was developed to ethanol, heat shock, elevated medium osmolarity, hydrogen peroxide, and the effects of freezing. It was concluded that in general, a development of cross-protection was seen following exhaustion of carbon source and other nutrients such as nitrogen.

## 1.6.1. Increased Resistance to Antibiotics

It is well established that stationary phase bacterial cultures exhibit enhanced resistance to antibiotics (Cozens *et al*, 1986; Tuomanen *et al*, 1986). It has also been widely reported that the effect of the decrease in growth rate of stationary phase cells can contribute to the increased resistance seen in these cells (Brown and Williams, 1985). However, few studies have examined the effect of starvation and increased survival in relation to enhanced resistance to antibiotics. More studies in this area may increase understanding of recalcitrance to antibiotics in established infections.

## 1.7. Outer Membrane Changes

It has already been mentioned that the capability of bacterial cells to survive starvation and to tolerate exposure to various forms of physical stress is thought to be based on the synthesis of a set or proteins known as starvation or stress proteins. However, it is also known that several other changes take place in the outer membrane of cells exposed to such stresses.

1.7.1. Outer Membrane Proteins

Some starvation-induced proteins are found in the outer membrane. One study sought to determine the nature, location and time of expression of starvation antigens using antibodies specific for starved cells (Albertson *et al*, 1987). The results from an unidentified Gram-negative motile rod showed that the antigens detected were proteinaceous and located in the outer membrane and periplasmic space. Additionally, titrations of whole cells indicated that the antigenic change in the cell surface took place in the initial phase of starvation and increased during the first 14 hours of the starvation period.

Studies also examined changes in outer membrane profiles of *Pseudomonas fluorescens* DF57 and *Pseudomonas putida* DF14 during starvation for carbon, nitrogen and phosphorus (Kragelund and Nybroe, 1994). An outer membrane protein of 55kDa appeared specifically during phosphorus starvation in DF57 whereas another protein of 63kDa appeared during all starvation conditions. DF14 induced two outer membrane proteins of 28 and 29kDa during starvation for carbon and nitrogen. It was concluded that starvation-induced outer membrane proteins were not induced in fluorescent pseudomonads alone. It was also proposed that a unique starvation response may be found in individual strains.

The exact function of most of the induced proteins is not known (Kragelund and Nybroe, 1994). However, it has been suggested that the function of the proteins may be to provide the starving cells with the required nutrients (Sterkenberg and Wouters, 1984).

Experiments with the organism *Vibrio fluvialis*, a marine bacterium, revealed four new proteins that were absent from growing cells but were seen in gels of membranes from ultramicrocells formed as a result of starvation conditions (Smigielski *et al*, 1989). The functions of the proteins were not known, but it was proposed that they could be involved in altering membrane structure or function to increase chances of survival. One

suggestion was that the new proteins could reduce proton permeability of the membrane.

The induction of outer membrane proteins as a response to environmental stresses was found not to occur in all species of bacteria. *Vibrio vulnificus* was subjected to changes in osmolarity and investigation was made into the effect on its outer membrane proteins (Simpson *et al*, 1994). It was stated that no differences were seen in outer membrane proteins with varying salt concentrations. Additionally, an upshift in osmolarity during growth had no effect on the outer membrane protein profile. It was concluded that under the osmotic conditions of the studies, *V. vulnificus* may not need to regulate transport through modification of its outer membrane proteins.

It has also been reported, however, that in *E. coli* and *S. typhimurium*, synthesis of the outer membrane porin proteins F and C (OmpF and OmpC) is controlled by osmotic pressure (Hasegawa *et al*, 1976; Smit and Nikaido, 1978). The inclusion of 10% sucrose in the growth medium, as well as raising the growth temperature to 42°C, repressed the production of OmpF porin. It was proposed that bacteria in the bodies of animals "sensed" the high osmotic pressure and temperature and produced only the narrower OmpC porin, which would benefit the bacteria in protecting them from some of the inhibitory substances in the animals (Nikaido and Vaara, 1985).

## 1.7.2. Changes in Peptidoglycan

Experiments investigating changes in cell morphology have been performed with *Vibrio vulnificus*. Ampicillin, a peptidoglycan synthesis inhibitor, was added to cells of *V. vulnificus* incubated in artificial seawater at 5°C. Loss of culturability was seen very quickly, whereas control cells without ampicillin exhibited an expected gradual loss in culturability. The results suggested that the nonculturable response involved active peptidoglycan synthesis (Oliver, 1993).

The morphological aspects of nonculturable *Vibrio cholerae* stored in low nutrient conditions have also been examined (Kondo *et al*, 1994). Results were obtained using a freeze fixation technique of electron microscopy. The most dramatic observation of the cells was that the peptidoglycan layer was thick and more electron dense than that of cells that were not subjected to the starvation medium. It was suggested that the presence of such a thick layer may reduce or prevent the transport of bacterial protein or nutrients across the membrane. This prevention of loss of nutrients could aid the maintenance of the state of dormancy.

## 1.7.3. Changes in Lipid and Fatty Acid Composition

A psychrophilic marine *Vibrio* sp. was studied as it was known to survive prolonged periods of starvation (Oliver and Stringer, 1984). The overall lipid phosphate content was found to decrease with starvation, which would be expected as the molecules would act as a store of potential energy. However, an increase of 57 % was detected in the neutral lipid fraction, the identity of which was not known. A large amount of short chain fatty acids was found to occur in the *Vibrio* organism. Analysis of the fatty acid and phospholipid composition suggested that these changes may have increase membrane fluidity, which in turn may influence various cell transport systems.

Studies were performed to characterize the cell envelopes of *Vibrio vulnificus* and *Escherichia coli*, by examination of their fatty acid composition after incubation of the organisms in artificial-seawater microcosms at 5°C (Linder and Oliver, 1989). At the point at which *V. vulnificus* became unculturable, considerable change in the fatty acid distribution took place. The percentage of saturated fatty acids increased from 49 % to 69.5 %, and unsaturated fatty acids decreased from 45 % to 17 %. The percentage of fatty acid species with chain lengths less than 16 increased when the cells became unculturable. It was found that *E. coli* exhibited a similar but more gradual change in fatty acid composition. It was suggested, as with results of Oliver and Stringer (1984), that the changes in lipid composition resulted in an effect on membrane fluidity. The decrease in chain length had the same effect as addition of double bonds in increasing membrane fluidity at lower temperatures.

Investigations into the biochemical properties of *Micrococcus luteus* cells stored in spent growth medium revealed that the total lipids of the cells declined rapidly within the first 10 days of starvation (Mukamolova *et al*, 1995b). Starvation resulted in changes in phospholipid composition with the almost complete disappearance of phosphatidylglycerol after 10 to 100 days. This change meant that cardiolipin was the predominant phospholipid in the membrane. In contrast to the above results, other measurements showed that membrane fluidity also declined during starvation which were also thought to be attributed to the changes in membrane phospholipid.

A recent study examined the permeability of the outer membrane of *Myxococcus xanthus*, a Gram-negative bacterium, after starvation (Laval-Favre *et al*, 1995). The experiment examined the secretion of a foreign protein by the organism. The results showed that the arrest of protein diffusion that took place after starvation was not due solely to the lack of accumulation of the protein in the periplasm. It was suggested, therefore, that the structure of the outer membrane had been modified and that the

permeability had decreased. It was mentioned, however, that the molecular basis surrounding the permeability of the outer membrane of *M. xanthus* was far from clear.

Various structural changes in the outer membrane occur when cells become unculturable or when they undergo starvation, depending on the stress and the organism. Therefore, a universal structural modification does not appear to exist.

## 1.7.4. Changes in poly (3-hydroxybutyrate) (PHB) content

It is well known that PHB is accumulated by various species of Gram-positive and Gram-negative bacteria (Dawes, 1976). The ability to store PHB is thought to be a survival strategy, since it functions as an endogenous source of carbon during starvation. Recent experiments investigating the survival of wild-type strains and PHB negative mutants of *Bacillus megaterium* and *Alcaligenes eutrophus* have shown that for both organisms the survival was higher in the wild-type vegetative cells compared to the PHB mutant strains (López *et al*, 1995).

Recent studies with a Gram-negative organism *Pseudomonas putida* have shown that cells subjected to phosphate starvation increase in size. This increase is thought to be attributed to the accumulation of an intracellular storage component, namely poly-3-hydroxyalkanoate (PHA) (Eberl *et al*, 1996). Phase contrast and electron microscopy confirmed the findings. Accumulation of the reserve material could be seen in the wild-type organism but not in the mutant organism that could not synthesize the polymer. The results confirm that accumulation of storage polymers occurs in species of non-vegetative cells and affects the survival of the organism.

## 1.7.5. Hydrophobicity and Charge

The surface properties of starved cells are different from those of growing cells (Siegele and Kolter, 1992). It has been shown that the surface of marine bacteria can become increasingly hydrophobic, and as a result the cells can become more adhesive during starvation (Kjelleberg *et al*, 1987). Studies have been performed in which changes in hydrophobicity, charge and degree of irreversible binding to glass surfaces were followed during starvation (Kjelleberg and Hermansson, 1984). The results showed that, in general, significant changes in the degree of irreversible binding took place at various times during starvation-survival for viable cells of several species of marine bacteria. No overall trend in charge effects was seen during starvation, but they were considered to be involved in binding.

## 1.7.6. Survival : Genetic Approach

It has been discovered that adaptation to growth arrest is a highly ordered response that utilizes sets of regulatory networks during both initial and long-term starvation (Kjelleberg *et al*, 1993). The identification of a central regulator of stationary-phase gene expression in some species of bacteria has led to greater understanding of these processes. The central regulator of stationary phase gene expression is the *rpoS* gene product and is known as a specific stationary phase sigma factor. It is responsible for the induction of a specific subset of bacterial genes that are only expressed under stress conditions (Rees *et al*, 1995).

The sigma factor ($\sigma^s$ factor) is the sixth subunit of RNA polymerase in *E. coli*. The enzyme is involved in the transcription of DNA to RNA which precedes protein synthesis. The sixth subunit is one of several different inter-changeable subunits of the core enzyme. The function of the sigma factor is to recognise and bind to promoter sequences in the primary DNA sequence. The binding then promotes the correct alignment of the RNA polymerase and once a gene has been identified, transcription is initiated and the specific gene is expressed (Rees *et al*, 1995).

The exact mechanism of how the sigma factor mediates selective transcription of specific stationary phase genes has not been entirely elucidated. However, it has also been discovered that a histone-like protein H-NS is also involved in the regulatory mechanisms. H-NS is an abundant non-basic DNA-binding protein with a preference for AT-rich curved DNA regions (Lucht *et al*, 1994; Owen-Hughes *et al*, 1992). The protein is encoded by the *hns* gene. It can influence the degree of negative superhelicity of DNA and when present in high concentrations, it can compact DNA. Additionally, it represses the expression of a number of genes with unrelated functions (Barth *et al*, 1995), and prevents RNA polymerase binding correctly to initiate transcription (Higgins *et al*, 1990). Yashimona *et al* (1995) has recently indicated that H-NS is intimately involved with the activity of RpoS. It appears to inhibit the expression of the sigma factor itself by a mechanism that acts at the posttranscriptional level.

*N*-acyl homoserine lactones (HSLs) are molecules that are adapted for intercellular communication. They have been identified in a wide range of Gram-negative bacteria (Bainton *et al*, 1992). It has been proposed recently that homoserinelactone arises as a natural response to starvation/stationary phase in *E. coli* (Huisman and Kolter, 1994). The results suggested that the expression of $\sigma^s$ was induced by homoserinelactone. However, no HSL has been identified in an *E.coli* strain and the exact relationship between starvation, RpoS expression and HSL production has not be discovered in any organism (Rees *et al*, 1995).

## 1.8. Dormancy and Infection

The major aspect of the studies of cells that appear nonculturable but possess numerous active vital processes is the cells' capability of being potentially hazardous. There have been few studies that have proved conclusively that cells that possess vitality are in fact potential pathogens. Difficulties in this area of work are of a similar nature to the problems encountered in resuscitation studies. Much of the research has focused on organisms that are important in the food and water industries which is of importance to public health. However, organisms that exist in an already established recalcitrant infection may also contain unculturable cells that pose as a reservoir of cells capable of producing infection at other sites of the body.

Studies have investigated the potential pathogenicity for humans of *Vibrio cholerae* (Colwell *et al*, 1990). Cells of an attenuated strain were made nonculturable by incubation at 4°C. Samples of the culture were ingested by two human volunteers at a dosage of ca. $10^8$ cells/ml. After a time period of around 48 hours one of the volunteers passed culturable *V. cholerae* in his stool. After 5 days the second volunteer also passed *V. cholerae* cells. *In vitro* studies involving incubation of unculturable *V. cholerae* cells in nutrient medium did not show resuscitation. However, because unculturable cells that were fed to the volunteers induced infection, it was claimed that this provided evidence to prove the pathogenic potential of the organism in humans.

Studies investigating the infectivity of *Vibrio vulnificus* indicated that nonculturable cells of the organism were not virulent (Linder and Oliver, 1989). A 50% lethal dose was administered by intraperitoneal injection into mice. The results obtained suggested that the viable, nonculturable cells lost their virulence with this mouse model. Recent results, using similar procedures to the above, revealed that *V. vulnificus* maintains its ability to initiate infection during entry into the viable but nonculturable state, but its virulence decreases over time (Oliver and Bockian, 1995). This was suggested as a possible explanation for the negative results obtained previously, since the cells used by Linder and Oliver were injected after 3 weeks incubation at 5°C. It was concluded that cells of *V. vulnificus* remain virulent for a certain time period when present in the viable, nonculturable state, and that they are capable of causing fatal infections after *in vivo* resuscitation.

Another study that showed resuscitation and evidence of continued virulence was carried out with both *E. coli* and *V. cholerae* (Colwell *et al*, 1985). Inoculation of the unculturable cells into ligated rabbit ileal loops revealed recovery of culturable cells after passage. The identity of the cells was confirmed using plasmid characterization.

*Legionella pnemophilia* is known to enter a viable but nonculturable state in drinking water. In an experiment, nonculturable cells of the organism were injected into chick embryos (Hussong *et al*, 1987). It was found that the number of culturable cells that were recovered were considered much greater than the initial inoculum and the lethality of the procedure was thought to be attributed to the virulence of these nonculturable cells.

*Campylobacter* spp. is another example of an organism that can survive in natural environments in a viable nonculturable state for extended periods of time after being shed by animal hosts. In addition, *Campylobacter* spp. has been found to survive in water samples and retain infectivity in mice (Rollins and Colwell, 1986). Unsuccessful attempts at colonization of the organism in the intestines of one-day-old chicks took place after viable but nonculturable cells of *Campylobacter jejuni* were fed orally (Medema *et al*, 1992). The results were surprising as fresh cultures were infective at 26 and 260 colony forming units per inoculum, but no sign of caecal colonization could be shown 7 days after the ingestion of the nonculturable inoculum, which had a direct viable count of $1.8 \times 10^5$ cells.

Another group examined the effect of nonculturable *C. jejuni* and its potential infectivity (Jones *et al*, 1991). Experimenters were able to demonstrate that infections were established in suckling mice after a dose was given of an inoculum of less than 0.2 colony forming units/ml. The results showed that an inoculum that was not culturable using routine methods could act as a potential pathogen. However, it was not proved that it was the viable but nonculturable cells that were responsible for the infection (Barer *et al*, 1993).

The prevalence and lack of recovery of *Helicobacter pylori* from environmental sources makes the organism a possible example of one that produces a viable nonculturable state. The organism has been isolated from relatively few places, most notably from the gastric-type mucosa, and its high rates of transmission in some communities (Sullivan *et al*, 1990) are in keeping with a viable nonculturable form (Barer *et al*, 1993).

*Shigella* spp. has been shown to enter a viable nonculturable state under specific experimental conditions. More recent studies have examined the virulent strain of *S. dysenteriae* type 1 after its induction into the viable nonculturable state (Rahman *et al*, 1996). The results demonstrated the maintenance of the Shiga toxin gene, involved in the production of a potent toxin. The toxin was also found to remain biologically active and also continued to adhere to the intestinal epithelial cells used in the studies. The experimenters concluded that the viable nonculturable cells of *S. dysenteriae* type 1 retained several virulence factors and so remained potentially pathogenic.

In general, some organisms have been examined to establish the infectivity or potential pathogenicity of the viable nonculturable cells but other species should be investigated to evaluate if the occurrence is more widespread. Additionally, more direct evidence is required (Barer *et al*, 1993) for the viable nonculturable state and its role in the transmission of infection.

Another area of work that is worth investigating would be the capability of bacteria in chronic infections to act as a reservoir of potentially pathogenic bacteria and to increase the persistence of infection. Many chronic infections that grow as adherent biofilms contain multiple layers of cells in a heterogeneous formation. Cells that are embedded deep in the lower regions of the biofilm may be at a stage in dormancy (Lewis and Gattie, 1991). Upper layers of bacteria are metabolically active and larger in size. This means that antibiotic treatment would be able to reduce populations of planktonic and surface biofilm cells but embedded biofilm cells would not be eradicated. It has been proposed that the establishment of ageing biofilms is a possible mechanism for the persistence of the bacteria and resistance to further antibiotic treatment, in medical device-associated infection as well as chronic *Pseudomonas* bronchopulmonary infection (Anwar *et al*, 1992).

## 1.9. Cystic Fibrosis and *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a Gram-negative, non-spore forming bacillus. The organism can be found in natural habitats such as water and soil. It is an opportunistic pathogen that generally does not cause infection. However, in burn wounds, immunocompromised patients and those suffering from cystic fibrosis (CF) the organism has the capability to establish infection.

CF was thought to be a genetically based disease, when Anderson first described the syndrome in 1938 (Anderson, 1938). The gene responsible is located on chromosome 7. The product of the cystic fibrosis gene is the cystic fibrosis transmembrane regulator (CFTR), which is thought to function as a chloride channel (Anderson, 1991). It has also been suggested that this chloride channel contributes to chloride secretion through the apical cell membrane of epithelial cells such as those found in the airways (Lindsay and Bosso, 1993). Patients suffering with CF have an absence of chloride secretion in affected epithelial cells together with an accompanying increase in sodium reabsorption, leading to dehydration of pulmonary secretions. It is these thick dried pulmonary secretions which act as an appropriate medium for the growth of the common lung pathogens of CF.

The majority of patients suffering from CF eventually contract chronic *Pseudomonas aeruginosa* lung infection (Pederson, 1992, Koch and Høiby, 1993). CF patients have no detectable immune deficiency and, except in the case of respiratory infection, are no more likely to become susceptible to infections. It is thought, therefore, that CF patients suffer from recurrent and chronic respiratory tract infections because of the altered secretions of the respiratory tract which leads to thick dehydrated mucous (Høiby *et al*, 1995). It is believed that this dehydration also plays an important role in the defective mucociliary clearance seen in these patients (Gilligan, 1991).

Overall, the main characteristics of the condition are malabsorption due to exocrine pancreatic insufficiency, recurrent bacterial infections in the lower respiratory tract, increased loss of sweat, and male infertility (Koch and Høiby, 1993). However, it is the chronic lung infection that determines the outcome of patients. Pulmonary dysfunction is responsible for approximately 90% of deaths in CF patients and is said to be largely a result of chronic airway infection (Gilligan, 1991).

It has been shown that many chronic infections involve bacterial consortia which grow as adherent biofilms within extended polysaccharide glycocalices (Brown *et al*, 1985; Costerton *et al*, 1987). The chronic *Pseudomonas aeruginosa* bronchopulmonary infection is an example of such growth as this organism grows as a biofilm/biomass in the lungs. Often the infection produced is recalcitrant to antibiotic treatment. However, antibiotic treatment plays a central role in the management of CF (Lindsay and Bosso, 1993), and can have a beneficial effect on both mortality and morbidity.

## 1.10. Formation of Biofilms

Biofilms grow extensively in nature and disease. Their formation is important in very many situations including metal corrosion in the shipping and oil industries, colonization of pipe surfaces in the food and water industries, and in medicine associated with infections of tissues of the body, prosthetic devices and implants, dental plaque and bronchopulmonary infections.

Microorganisms are able to adhere to surfaces that are either living tissues in the *in vivo* situation or to inert surfaces. Bacteria adhere to these surfaces initially in a reversible association and eventually produce irreversible adhesion (Marshall *et al*, 1971), so forming the adherent biofilm. The process of adhesion is initiated by the binding of a bacterial cell to the surface by the use of exopolysaccharide glycocalyx polymers (Costerton *et al*, 1987). Cell division then occurs which produce sister cells within the glycocalyx matrix to form adherent microcolonies. Development of the biofilm is carried on through cell division within the microcolonies (Malone *et al*, 1983) and through the addition of planktonic bacteria from the bulk phase. Biofilms have therefore been defined as a matrix-enclosed bacterial population, adherent to each other and/or to surfaces of interfaces (Costerton *et al*, 1995).

## 1.11. Structure of Biofilms

Throughout the 1980s biofilms were thought to be homogeneous in structure (Costerton *et al*, 1994). They were considered to be composed of bacteria distributed relatively evenly and randomly throughout an exopolysaccharide matrix. Similarly, mathematical models of biofilm activity, treated the biofilm as a planar aggregate of uniform thickness (Stewart *et al*, 1994). However, through the application of confocal scanning laser microscopy (CSLM), it was discovered that biofilms contained many heterogeneous features in its structure. It has also been discovered that the area of highest cell density varies among different species.

CSLM allowed nondestructive, *in situ* analysis of a living fully hydrated biofilm structure. The technique was in contrast to other electron microscopy procedures, in that it did not require dehydration and harsh chemical fixation which could collapse and destroy the extra-cellular polymeric substance (EPS) matrix, and so present an inaccurate picture of the structure as well as artefacts (Handley, 1995) The collapse of the biofilm had previously given researchers the false impression that mature biofilms were confluent and had also greatly underestimated the thickness and topography (Keevil, 1995).

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The overall structure which recent studies have confirmed, show that living biofilms are often comprised of a variable distribution of cells or cellular aggregates, extracellular polymers, and void spaces or water channels (Keevil and Walker, 1992; Korber et al, 1994; Costerton et al, 1995). It has been shown that many native biofilms when fully mature consist of a low background of microorganisms on the surface out of which arise tall finger-like stacks or fronds of microorganisms (Keevil et al, 1993).

CSLM allowed the observation of the positioning of cells within the biofilm matrix as well as whether the cells were in chains of small groups (Lawrence et al, 1991). Recently, experiments using a combination of CSLM and epifluorescence revealed that physico-chemical properties of the substratum influenced cell morphology of the biofilm (Dalton et al, 1994). The results showed that biofilms grown on hydrophobic surfaces had tightly packed biofilms which consisted of single or paired cells whereas in contrast, biofilms on hydrophilic surfaces exhibited sparse colonization and the formation of long chains of cells.

It is also important to note that despite the fact that biofilms are known now to be heterogeneous, they are said to demonstrate homogeneity of function (Hamilton, 1987). The individual microorganisms do not function in isolation but show significant interaction and even interdependency.

Recent studies have been useful in investigating the phenotypic changes that bacteria undergo during their adherence to surfaces (Davies et al, 1993). The results have shown that two genes algC and algD are upregulated during adhesion. These genes are known to control the production of various enzymes in the alginate synthesis pathway, and hence biofilm formation. The results show that the process of adhesion triggers the expression of several enzymes that are involved in biofilm formation and so gives a greater understanding of the development of the biofilm in nature and disease (Costerton et al, 1995).

## 1.12. *In Vitro* Models of Biofilms

Because of the ubiquitous nature of bacterial biofilms, their enhanced resistance to antimicrobials and resultant difficulty in their eradication, it is both of importance and interest to investigate this highly specialized form of growth. In an attempt to mimic the *in vivo* situation various models have been devised. Much of the resistance to antibiotics shown by biofilm-associated cells relates to their slow growth rate, rather to any innate properties of the glycocalyx (Gilbert and Brown, 1995). For this reason, models that are designed to understand biofilm physiology should have an element of growth rate control, so that the effect of growth rate can be assessed.

Overall, in the evaluation of the mechanisms of resistance, *in vitro* models of biofilm growth are required which differentiate between adherence and the associated influences of the glycocalyx, nutrient status and growth rate (Brown and Gilbert, 1993).

## 1.12.1. Closed Growth Models

Growth in these models is akin to growth in batch culture, as it takes place in a closed environment with a limited source of nutrients.

One of the simplest methods of biofilm production is the formation of confluent growth of an organism on the surface of medium solidified with agar. A disadvantage of this method is the necessity of removal of the biofilm to carry out any antibiotic resistance studies. The spatial arrangement of the microorganisms would therefore be disrupted, so lessening the influence of the exopolysaccharide on the flux of solutes and macromolecules into the biofilm (Williamson and McCarty, 1976), and so would make the antibiotic studies difficult to interpret. An additional problem occurs if antimicrobial testing is performed on the agar itself, as it is polyanionic in nature and so may affect the availability of the antimicrobial (Brown and Gilbert, 1993).

The problem can be resolved by the use of intact biofilms for susceptibility testing. Cultures were inoculated onto cellulose acetate membranes which were then placed onto agar (Millward and Wilson, 1989). The intact membrane could then be used to test antimicrobial sensitivity.

Another simple means of generating a biofilm *in vitro* involved agitation of an aerobic organism in liquid culture (Govan, 1975). During agitation, a vortex is formed at the air-medium interface (Chan *et al*, 1984) and a bacterial biofilm is formed on the available

glass surfaces. Once again, however, in removing the biofilms for antimicrobial testing the biofilms are disrupted.

More reproducible biofilms can be formed by placing sample discs into liquid culture (Gristina *et al*, 1987). Prosser *et al* (1987) initially exposed the discs to bacterial suspensions after which they were transferred to a sterile broth medium where the attached cells were able to grow and divide. In both cases the sample discs could be subjected to antibiotics for susceptibility testing.

The above methods are useful for preliminary tests on antimicrobial sensitivity, but more controlled systems are needed to understand biofilm growth more fully.

## 1.12.2. Continuous Growth Models

One of the most widely used models is the Robbins device. The system involves passage of culture medium through a tubular section, which is fitted with removable circular test pieces within its inner wall (McCoy *et al*, 1981). A modified version of this device has also been used. It is composed of retractable pistons which form the inside of a pipe of rectangular section (Nickel *et al*, 1985). As the culture medium flows through the pipe, biofilms develop on the inside of the pipe and can be examined more closely by removing the retractable pistons. Another continuous method involves test pieces submerged in a chemostat (Keevil *et al*, 1987).

These continuous methods are more useful than closed systems, since the constituents of the medium in contact with the biofilm remain constant. However, the systems are not ideal, since the biofilms are continuously increasing and so the growth rate alters over time. They are not, therefore, classed as steady state models.

Other models have been devised that do reach a steady state condition. The constant depth fermentor (Wimpenny *et al*, 1989), produces biofilms of a known thickness. The upper surface of the biofilm is swept by a blade over which fresh medium is passed.

The other main steady state model consists of a perfused biofilm (Gilbert *et al*, 1989). This system was adapted from a model which was used to investigate cell synchronization (Helmsetter and Cummings, 1963; Helmsetter *et al*, 1992). A filter membrane is inoculated with bacterial cells, inverted and placed in a modified continuous fermentation apparatus. Fresh medium is perfused into the fermentation chamber via a peristaltic pump. The biofilm produced is of constant thickness, since the newly formed daughter cells disperse from the underside of the membrane and are

collected in the eluate. Steady state conditions were reached after approximately 100 minutes using the organism *Escherichia coli*. After this point, the biofilm was under growth rate control which was brought about by the rate of perfusion of the filter. This biofilm method was also used with the organism *Staphylococcus epidermidis* (Evans *et al*, 1992).

Experiments performed with two clinical isolates of *P. aeruginosa* produced biofilms on cellulose acetate membranes using the procedures of Gilbert *et al* (Evans *et al*, 1991). However, the technique was found to be unsuccessful with *P. aeruginosa* PAO1 (Nelson, 1993). A growth rate controlled biofilm could not be achieved mainly because the number of cells in the eluate began to increase within 24h of running the fermenter. A steady state could not therefore be maintained for any significant length of time. It was also concluded that any EPS produced caused blockage of the filter pores and so prevented further perfusion of the media.

## 1.13. Resistance of Biofilms

Growth of microorganisms in the form of biofilms provides many advantages to the organisms within the structure (Costerton *et al*, 1985). Growth of biofilms *in vivo* provides protection from host immune defences and antibiotic treatment (Kharazmi *et al*, 1986, Marrie *et al*, 1990), as well as from desiccation. It also enables the micoorganisms within the structure to experience an element of homeostasis, in which they are protected from the external environment to a certain degree in a more stable situation. This is aided by the ability of the biofilm structure to bind nutrients for growth, so creating a nutrient-rich environment for the bacteria (Costerton *et al*, 1987). It is a well established fact that bacterial biofilms are often difficult to treat *in vivo*. It has also been demonstrated that growth as an adherent biofilm, as opposed to growth of planktonic cells, confers some degree of resistance on the bacterial cells (Nickel *et al*, 1985, Gristina *et al*, 1987).

There are many explanations of the increased resistance of bacteria grown as a biofilm. One possibility is that the structure of the bacterial glycocalyx could form a protective barrier against antibacterial agents.

Other explanations revolve around the concept of slow growth rate (Evans and Holmes, 1987) in which the reduced growth rate may affect antibiotic action. This work is based on the findings that stationary phase cells are recalcitrant to many antibiotics (Brown *et al*, 1988) and so it is conceivable that the resistance of biofilm grown cells could also be due to the decreased growth rate.

Another area of work is focused on the fact that changes in growth rate and nutrient limitation are often accompanied by changes in cell components. These alterations in cell components could in turn alter sensitivity to antimicrobial agents (Gilbert and Brown, 1978).


## 1.13.1. Role of Glycocalyx and Lipopolysaccharide (LPS) in Resistance

In general the glycocalyx consists of 50 to 90 % of the total biofilm (Characklis and Cooksey, 1983) and so has the potential to affect the activity of the biofilm to a great extent. It consists of an extensive anionic matrix surrounding bacterial cells. In the area of resistance, it is thought to act as an ionic barrier which binds charged cationic antibiotic molecules, thereby reducing the free concentration of antibiotic and so protecting the innermost cells (Nichols *et al*, 1988).

It has been shown in the CF lung that the bacterial glycocalyx increases the biofilm resistance to antibacterial surfactants (Costerton, 1981). In addition, it has been shown that glycocalyx-enclosed cells are less efficiently phagocytosed (Schwarzman and Boring, 1971), thereby allowing cells to possess an increased chance of establishing an infection in the host environment.

The direct role of the glycocalyx in resistance, as mentioned earlier, is one which suggests that it inhibits the movement of animicrobials to the cell surface (Costerton 1977; Slack and Nichols, 1981; 1982). However, experiments have provided evidence which challenged this exclusion theory. It was proved that in the case of the antibiotic tobramycin, penetration through the alginate of *Pseudomonal* exopolysaccharide was relatively unimpaired. It was also suggested that unless much greater binding occurred, this may be true for other antibiotics (Nichols *et al*, 1988). Other experimenters have found no difference in sensitivity to antibiotics of *Staphylococcus epidermidis* biofilms composed of slime producing and non-slime producing bacteria (Gristina *et al*, 1989). As a result of the observations, it was concluded that the slime had little effect in the hindrance of antibiotic to the bacterial cell surface.

Additionally, a similar finding was made in the studies of susceptibility to tobramycin of intact and resuspended biofilms of *E. coli* (Evans *et al*, 1990). The results showed no difference between the two types of sensitivity testing. However, other investigations have shown that *P. aeruginosa* biofilms of a mucoid phenotype were less susceptible to ciprofloxacin compared to the non-mucoid strain (Evans *et al*, 1991).

An indirect role of the glycocalyx in resistance can be attributed to a certain extent to its thickness. Because of differences in the thickness, gradients can be created across the structure to different degrees and so cells at different locations within the biofilm will experience different nutrient and physicochemical environments (Gilbert and Brown, 1995). It has also been shown that gradients arise within the biofilm as a result of consumption of nutrients and oxygen by the cells and the generation of secondary metabolites and waste products (Wimpenny et al, 1989). In this way, cells that are embedded deep within the biofilm are subjected to limitations in nutrient that would give rise to a reduced growth rate compared with cells that are on the surface of the biofilm or in the planktonic culture itself (Brown et al, 1988).

Overall, it is likely that resistance to a particular antibiotic can be attributed to a number of factors namely the type of antibiotic, its ability to bind to the glycocalyx, the concentration of antibiotic used and the thickness of the glycocalyx.

## 1.13.2. Nutrient Limitation and its Role in Resistance

Rapid growth of bacteria takes place in an environment for a relatively short period of time. Toxic metabolites will limit growth or nutrients will be exhausted at some point and the cells will encounter a situation in which there is a depletion of one of the essential nutrients. As a result of this shortage, bacteria adapt to the new situation to enable them to acquire the limited nutrient.

It has been stated that when a dividing bacterium encounters a depletion of a nutrient, it will produce an envelope which is characteristic of the particular depletion (Brown, 1977). This has been shown to occur during phosphate limitation of Gram-positive cells, in which the teichoic acids of the cell walls are found to be replaced by teichuronic acids (Ellwood and Tempest, 1972). It is the cell envelope that allows a bacterium to interact with its environment. Additionally, it is the plasticity of the cell envelope which enables the bacterium to adjust to a change in environment. Eventually the effect of the changes is a decrease in the growth rate.

Nutrient limitation can result in the induction of new outer membrane proteins (OMPS). These OMPS can play a role in antimicrobial resistance. The resistance of P.aeruginosa to gentamicin, polymyxin and ethylenediaminetetraacetic acid (EDTA) has been correlated with the induction of a specific protein H1 (Nicas and Hancock, 1980). In a similar investigation, protein H2 was found to be induced under conditions of $Mg^{2+}$-depletion (Anwar et al, 1983). It was proposed that this protein also could be implicated in the resistance of P.aeruginosa.

Changes in envelope lipid composition, brought about by nutrient depletion, have also been linked to alterations in susceptibility to antimicrobial agents and antibiotics (Klemperer et al 1980; Finch and Brown, 1978).

1.13.2.1. Effect of Iron deprivation

Iron is an important nutrient as it is required in essential biochemical reactions and in oxygen transport. During many infections, the body withholds iron from microorganisms by transferrin and lactoferrin, the body's natural iron-binding proteins, which constitute a major component of non-specific mammalian defence mechanisms. Consequently, the ability of bacteria to obtain iron is a significant virulence factor, and in response to iron deprivation, many microorganisms produce low-molecular-weight, iron-chelating agents (siderophores) (Weinberg, 1978).

Siderophores are powerful iron chelators, which have stronger affinity for iron than the host proteins. The iron-siderophore complexes are then taken up by the cells via iron-regulated outer membrane proteins (IROMPS), which act as receptors on the outer surface of the membrane (Lambert, 1988).

In studies using mucoid *P. aeruginosa*, recovered without subculture from the sputum of a CF patient, several high molecular mass IROMPS were expressed indicating that they were present *in vivo* (Brown *et al*, 1984).

As mentioned previously in the context of biofilms, nutrient gradients exist in the glycocalyx, so subjecting different bacterial cells to possible nutrient limitations. As a result, cells that are embedded deep within the biofilm are exposed to concentrations of nutrients which may be different from other cells in the biofilm or those experienced by planktonic cultures growing in the same medium (Brown and Gilbert, 1993). It is not known which nutrients will be limited, but is thought that cation availability and also carbon and nitrogen utilization may produce nutrient gradients (Gilbert and Brown, 1995). As a consequence of the nutrient limitations, phenotypic changes as described above may occur to bacteria, thus altering resistance to antimicrobial agents.

## 1.13.3. The Effect of Slow Growth Rate

It is difficult to isolate the effects of nutrient limitation and slow growth rate, since slow growth can be a consequence of nutrient limitation. However, it is important to mention the effect of slow growth *per se*, as this effect has been studied extensively.

Traditional *in vitro* methods of culture can produce good yields of bacteria. However growth rates bear little resemblance to growth of a chronic bacterial infection within a host (Gilbert *et al*, 1987). Therefore, in order to assess the effects of antibiotics in the laboratory, growth rate of the organism should be controlled. Continuous culture is often used as a means to maintain cells growing under steady conditions at a constant growth rate (Herbert, 1956; Tempest, 1970). In general, experiments have shown that slow growing cells are more resistant to antimicrobial agents compared to faster growing bacteria (Brown, 1975; Gilbert and Brown 1978; Tuomanen *et al*, 1986; Eng *et al*, 1991).

Growth rate has an effect on cell envelope composition, as with nutrient limitation. Investigations have reported changes in fatty acid and phospholipid (Gilbert and Brown, 1978), metal cation (Boggis *et al*, 1979) and protein composition (Brown and Williams, 1985). Other changes that have been documented include those of extracellular enzyme and exopolysaccharide production (Sutherland, 1982; Ombaka *et al*, 1983). These alterations in cell envelope constituents affect susceptibility of the cells to antimicrobial agents.

The production of penicillin binding proteins (PBP) has been found to be influenced by growth rate and also affected by the availability of cation (Turnowsky *et al*, 1983). It has been shown that the antibiotics cefotoxidine and ceftriaxone have little activity against slow-growing *E. coli*, as it is known that the specific PBP is poorly expressed (Cozens *et al*, 1986; Tuomanen, 1987). However, in other studies, another β lactam antibiotic CGP 17520 was found to possess good bactericidal activity against slow growing cultures (Tuomanen and Schwartz, 1987). This indicates that the suggestion that slow growth rate of bacteria produces cultures with increased antibiotic resistance is not universal.

It has been proposed that slow growth may be the factor that influences the resistance of bacterial biofilms (Brown *et al*, 1988). The use of models that have been able to distinguish between the effects of the biofilm as a whole and those relating to a reduced growth rate, has established that some of the resistance shown by biofilm cells is related to a reduced growth rate rather than to the barrier properties of the glycocalyx (Evans *et al*, 1990a; Evans *et al*, 1991). Findings from the experiments were that susceptibility to

antibiotics increased with growth rate for planktonic chemostat cultures and also for the biofilms. When the sensitivity of intact biofilms was compared to planktonic chemostat cells and cells resuspended from the biofilm, it was found that in most instances, cells that were in the biofilm formation possessed increased resistance. These results show that even though the increased resistance can account to some extent to the reduced growth rate, other factors such as organization of cells in the biofilm structure also have some effect.

As mentioned previously (see section 1.1), cell populations can exhibit very low growth rates or even growth rates of zero (Moyer and Morita, 1989), under extreme adverse conditions. These cells may be analogous to cells in chronic infections, and so the studies on dormancy may help to elucidate the problems of recalcitrance in such infections (Gilbert *et al*, 1990).

### 1.13.4. Cell Cycle

Studies have shown that the different stages of the cell cycle can affect susceptibility to antibiotics. The results of Evans *et al*, (1990b), using synchronous populations indicate that the sensitivity of *Escherichia coli* to tobramycin changes during the division cycle, and is at its greatest immediately after cell division.

In summary, it is probable that the resistance of biofilms can be attributed to several of the factors described above, each of which affects resistance to varying extents.

## 1.14. Antibiotics

### 1.14.1. Ciprofloxacin

Ciprofloxacin is a 4-quinolone antibacterial drug. It is a highly active bacterial agent that is effective against a broad spectrum of Gram-positive and Gram-negative bacteria (King *et al*, 1984), including *P. aeruginosa*. It is therefore, an antibiotic that has been found to be useful in the treatment of *P. aeruginosa* infection in CF.

1.14.1.1. Mode of Action

Studies have indicated that two or more mechanisms are involved in the lethal action of ciprofloxacin (Smith, 1984, Diver and Wise, 1986). One of its mechanisms of action is believed to involve inhibition of bacterial DNA gyrase, an essential enzyme which negatively supercoils the DNA (Smith, 1984). It is not known exactly how the 4-quinolones interfere with DNA gyrase function, but it has been suggested that they prevent the A subunits of DNA gyrase from finally sealing the staggered nicks introduced into chromosomal DNA (Gellert *et al*, 1977). These interruptions could then induce the synthesis of exonucleases which could enlarge the gaps until they become permanent double strand excisions (Fenwick and Curtiss, 1973), thus preventing supercoiling. Supercoiling of bacterial DNA by DNA gyrase influences all metabolic processes involving DNA, and is essential for replication (Cozzarelli, 1980). Hence, if supercoiling is prevented, the replication of DNA is also prevented and possible death of the organism could ensue.

Experiments with nalidixic acid, another quinolone, have shown a second mechanism of action in that it induces the SOS response in *E. coli*, which is a complex cascade of gene activations accompanied by many effects on cell metabolism. The many effects include inhibition of DNA synthesis, enhanced capacity for DNA repair and mutagenesis, and inhibition of cell division so producing filamentation (Little and Mount, 1982). Sustained induction of the SOS response is thought to be a lethal event (Gottesman, Halpern and Trisler, 1981).

## 1.14.1.2. Morphological and Biochemical Changes

Investigations into the morphological changes that occur as a result of exposure to ciprofloxacin, can be related to the biochemical changes of the SOS response that happen at the same time (Diver and Wise, 1986). At concentrations close to the MIC, extensive filamentation of the *E. coli* was observed, whereas at the bactericidal concentration, cells were elongated but not filamented. This is probably due to ciprofloxacin inhibiting RNA and therefore protein synthesis at the higher concentration, and so producing less filamentation. At even higher concentrations, ovoid cells were seen which could be attributed to the ciprofloxacin totally blocking the RNA and protein synthesis and so preventing any filamentation.

The data obtained in the study indicated that the major mechanism of induction of cell death in *E. coli* K1-16 did not require protein synthesis and accounted for > 90% of cell death. It was proposed it was caused by the inhibition of bacterial DNA gyrase leading to irreversible DNA damage. The secondary mechanism, which was induced via the SOS-response pathway, did require protein synthesis and was found to involve cell filamentation.

## 1.14.1.3. Uptake of Antibiotic

Because quinolone antibiotics interact with the intracellular enzyme, DNA gyrase, their antimicrobial activities depend greatly on rapid intracellular accumulation. It has been suggested that quinolones can penetrate the cell envelope of Gram-negative bacteria by three routes (Asuquo and Piddock, 1993), the hydrophilic pathway through porin channels (Nikaido and Vaara, 1985), the hydrophobic pathway by means of penetration of the phospholipid bilayer (Nikaido and Hancock, 1986) and the self-promoted uptake pathway (Chapman and Georgopapadakou, 1988). The first two methods of penetration are thought to be influenced by the hydrophobicity of the antibiotic, whereas the self-promoted uptake pathway involves the displacement of divalent cations from the lipopolysaccharide of the outer membrane.

Recent studies however, investigating the mechanism by which antibacterial activities of the quinolones are reduced in the presence of divalent cations, have shown that the self-promoted uptake pathway did not appear to be involved in the penetration of quinolones (Marshall and Piddock, 1994). It was found that none of the quinolones studied permeabilized Gram-negative bacteria to lysozyme or increased leakage of periplasmic β-lactamase into the culture medium. Further studies were recommended in this area in order to elucidate the problem.

## 1.14.2. Polymyxin

Polymyxin B is a polycationic decapeptide antibiotic. It is active against Gram-negative bacteria including *Pseudomonas aeruginosa*, but is not used very much clinically in the treatment of infection in CF. It has been found useful, however in laboratory studies as an antibiotic used to study the structure and function of biological membranes. Polymyxin E (colistin), a related compound, has been used clinically in the management of CF. It can be administered by inhalation as an adjunct to other antibiotic treatment.

The mechanism of uptake of polymyxin and other polycationic antibiotics, such as aminoglycosides, was the first identified example of the self-promoted uptake pathway (Hancock *et al*, 1981a).

### 1.14.2.1. Mode of Action

Polymyxin B acts on Gram-negative bacteria by electrostatic and hydrophobic interactions which constitute the primary action of the compound. Electrostatic interactions are thought to occur between the peptides of the antibiotic and lipid phosphates of the outer membrane as well as there being interactions between the molecules' fatty acid side chains and lipids of the outer and cytoplasmic membranes (Noy, 1982).

Electron microscopic examination revealed interaction of polymyxin B and LPS extracted from *E. coli* (Lopes and Innis, 1969). Stable noncovalent complex formation between LPS of *Salmonella typhimurium* and polymyxin B was also shown (Bader and Teuber, 1973). The main interaction was thought to be between the antibiotic molecule and the negatively charged 2-keto-3 deoxyoctonic acid (KDO)-lipid A portion of the LPS. These findings were confirmed by other workers (Morrison and Jacobs, 1976; Schindler and Osborn, 1979). The interactions were thought to be both ionic and hydrophobic in nature.

Studies have also been performed that reveal that polymyxin B acts not only on the cell wall but also on the cytoplasmic membrane. Electron microscopic investigations showed that polymyxin produced projections that originated from the outermost layers of the Gram-negative cell wall and partially disorganized the cytoplasmic membrane (Koike, Iiida, & Matsuo, 1969). Other studies involving a technique of freeze etching of polymyxin treated *S. typhimurium* demonstrated blebs and projections which originated from the outer monolayer of the OM (Schindler and Teuber, 1975). The inner part of

this layer has also been shown to be involved in bleb formation (Lounatmaa and Nanninga, 1976).

These interactions would subsequently cause disruption to the cell envelope consisting of the cell wall and cytoplasmic membrane so leading to leakage of the cell contents (Newton, 1956), resulting in cell death.

There is an involvement of divalent cations in the activity of polymyxin, with competition between the antibiotic molecule and $Mg^{2+}$ or $Ca^{2+}$ ions for sites on the outer membrane. It had been suggested that polymyxins acted at a common site on the outer membrane which was thought to be a phosphate site on the lipopolysaccharide (Brown, 1975; Nicas and Hancock, 1980).

This suggestion was confirmed by the induction of a protein in the OM termed H1, which was produced by growth of *P. aeruginosa* under conditions of $Mg^{2+}$ depletion or using mutants of the organism which overproduced the protein H1. The results demonstrated that the susceptibility of *P. aeruginosa* to polymyxin B, EDTA, gentamicin and streptomycin decreased (Nicas and Hancock, 1980, Hancock *et al*, 1981a), which supported the idea of a specific site for action. It was suggested that the protein H1 acted by replacing $Mg^{2+}$ at a specific site on the lipopolysaccharide which in other situations could be attacked by cationic antibiotics such as polymyxin. The discovery of protein H2 produced similar findings (Anwar *et al*, 1983).

## 1.15. Unculturability in Biofilms and *In vitro* Models

### 1.15.1. Biofilms

It has already been established that plate counts are not a complete indication of the vitality of the bacteria within a culture. In the same respect, studies have been performed that have investigated biofilms using fluorescent probes to establish the vitality of individual cells. In this section, investigations into activity in biofilms as opposed to planktonic cultures are analysed.

Respiratory activity and culturability of biofilms during monochloramine disinfection could be examined easily by scraping biofilms of *P. aeruginosa* and *Klebsiella pneumoniae* and analysing the homogenized cell suspensions (Stewart *et al*, 1994). However, novel methods in which biofilms did not undergo any removal procedures allowed the investigations of biofilms *in situ*.

As previously mentioned (section 1.3.4), CTC was found to be a useful compound in order to assess vitality (Rodriguez *et al*, 1992). One of the main advantages of the method was related to the investigation of biofilms. The procedure allowed the examination of actively respiring cultures on cell membrane filters, as well as bacteria in biofilms on surfaces that were non-transparent or optically opaque. Counterstaining of the CTC-treated samples was performed using a DNA-specific fluorochrome 4', 6-diamidino-2-phenylindole (DAPI), that allowed the enumeration of total numbers of bacteria. In general, the results obtained from samples of wastewater, groundwater, and seawater, supplemented with exogenous nutrients, showed that the CTC counts were lower than DAPI counts but equal or higher than standard plate counts.

Adaptations of the above procedure were made by other workers (Schaule *et al*, 1993). In this instance, direct microscopic quantification of respiring bacteria was made for drinking water samples and biofilms grown on different opaque substrata. By the use of computerized digital image analysis, the colonized surface was scanned and the respiratory activity of the CTC-treated cells could be rapidly quantified. It was thought, however, that the procedure would not be useful in the examination of thick biofilms.

In another study the physiological activities of *Klebsiella pneumoniae* biofilms on stainless steel was investigated (Yu and McFeters, 1994). The DVC method as well as CTC procedures were employed in these experiments. Counts were found to be approximately 2-fold higher using both the DVC and CTC methods, compared to the conventional plate counts. An added advantage of CTC procedures was that the results were obtained in 2 hr compared to the 4 hr required to incubate the cells with the DVC

method. The *in situ* approach to the analysis of biofilms was able to be undertaken because the cells were arranged in bacterial monolayers. Once again it was stressed that thicker biofilms may respond differently.

An assessment of biofilm disinfection could also be made by the above procedures. In one report, the DVC method was used to enumerate attached bacteria and assess the disinfection ability of chlorine and monochloramine (Yu *et al*, 1993). An environmental isolate of *Klebsiella pneumoniae* was used to form biofilms on stainless steel coupons which were grown to monolayers so that enumeration could be undertaken. The DVC method was used both *in situ* and in the conventional way. The results for the chlorine treated cells showed that the *in situ* DVC method produced higher counts than the plate counts and the conventional DVC method.

Overall, the results show that *in situ* applications of the techniques are useful and may produce a more accurate picture of the vitality of the bacterial cells within the biofilm structure. From the findings it can be said that many of the conventional plate count results obtained from dispersed biofilms have underestimated the total number of actively respiring bacteria.

## 1.15.2. Chemostat Cultures

Since it is has been suggested that in oligotrophic environments non-sporulating bacteria can adopt a possibly dormant state in which they possess a decreased metabolic activity, it has been proposed that chemostat cultures at low dilution rates could also possess similar properties (Kaprelyants *et al*, 1993).

It has been established that the steady state percentage viability, as judged by the conventional plate counts, of the organism *Aerobacter aerogenes*, decreases as the dilution rate of the continuous culture is lowered (Tempest *et al*, 1967).

Long-term studies were performed with a bacterium *Cytophaga johnsonae* in a glucose limited chemostat at high and low dilution rates (Höfle, 1983). One of the points of investigation was the examination of whether the reduction of viability observed at low growth rates reflected dead cells in the chemostat or was an artifact. During prolonged culture, a downward drop in colony counts took place for slow and fast growing cultures. However, the trend was more pronounced in slowly growing cultures compared with faster growing ones. The results suggested that if the unculturable cells in the chemostat at low dilution rates were in fact dead then some of the dividing population would have to grow at a very fast growth rate in order to maintain the

biomass. As this was thought unlikely, it was proposed that a proportion of the chemostat culture had lost their culturability but had still retained metabolic activity.

Morphological changes have also been reported that support the idea that bacterial cells adopt a dormant state. At dilution rates of less than 0.05 hr$^{-1}$, the mean cell size of *Aerobacter aerogenes* decreased. Additionally, the culture became more heterogeneous in nature as it contained both long cells and small cocci (Tempest *et al*, 1967). This decrease in mean cell size with the lowering of dilution rate has also been observed for other bacteria as well (Gottschal, 1992). Studies using *Micrococcus luteus* cells also investigated the effect of dilution rate on size. Experiments were performed in lactate-limited chemostat cultures. As the dilution rate was decreased from 0.1 hr$^{-1}$ to 0.01 hr$^{-1}$ the mean cell size increased. There was however, heterogeneity within this population, in which a fraction of very small cells was also present (Kaprelyants and Kell, 1992). The viability as represented by colony counts decreased to around 40% of the original culture. Rhodamine 123 was used so that its accumulation within the cells could discriminate between viable, dead and possible dormant cells. Flow cytometric measurements confirmed that resuscitation had taken place. The small cells increased in size which was accompanied by membrane energization as well as a comparable increase in colony counts.

More investigations are required that evaluate the metabolic state and activity of the bacteria in chemostat cultures. However, in order to evaluate fully the behaviour of the chemostat cultures at low dilution rates, it has been suggested that a fraction of the cells are in a dormant state (Pirt, 1987). Experiments performed using carbon-limited chemostat cultures of *Klebsiella pneumoniae* have led other researchers to classify bacterial cells as being either alive, dead or non-culturable cells but still metabolically active (Mason *et al*, 1986).

## 1.16. Aims of Project

The initial aim of the project was to develop a novel biofilm system for the growth of *Pseudomonas aeruginosa*. The system was to be an *in vitro* model of the growth of the organism in the CF lung. The intention was to produce growth rate control, so that the properties of the biofilm attributed to adhesion and slow growth rate could be separated. Once this was achieved the aim was to investigate the antibiotic susceptibilities of the organism growing as a biofilm. Further studies were to be performed to investigate the survival of the organism in the growth of a biofilm as well as the role of dormancy.

Unfortunately, the technique used to establish growth of a biofilm with *Pseudomonas aeruginosa* was found to be unsuccessful, and incapable of producing a growth rate controlled biofilm. Consequently, studies of biofilm growth were terminated and investigations were focused on the survival of *Pseudomonas aeruginosa* firstly in batch culture and then in continuous culture with the use of the chemostat.

Various parameters were used to measure the survival of *P. aeruginosa* and to establish dormancy-like properties in extended batch cultures. Subsequently, the effect of growth rate was investigated with respect to survival of the organism.

# 2.     MATERIALS AND METHODS

## 2.1.     Organisms and Culture Maintenance

The organism used in the nutrient depletion studies was *Pseudomonas aeruginosa* PAO1 JD obtained from Professor B. Holloway via Dr. J. Doig, Department of Bacteriology University of Edinburgh. It was maintained at 4°C on nutrient agar (Lab M Ltd., Bury) plates. Long term storage was at -70°C in nutrient broth (Lab M) plus 10% glycerol.

During the chemostat studies, extensive wall growth was obtained with *P.aeruginosa* PAO1 JD, and so *P. aeruginosa* NCTC 6750 was used instead, with the same storage conditions as for the previous organism except that the organism was stored at -70°C in chemically defined media (CDM$_{12}$).

Two colonial types were identifiable with 6750, a rough and a smooth colony type. Cultures were inoculated so that approximately 50% rough and 50% smooth colony types were produced initially. During chemostat studies, after steady state was achieved, the colonies were found to have reverted to the smooth colony type. Additionally, during prolonged incubation of cultures at 37°C, the rough type had decreased in numbers (not quantified), whereas the smooth type was maintained (see section 6.3).

## 2.2.     Chemicals

The chemicals used in this project were obtained from BDH Chemicals (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 of the glassware that was used for iron restriction studies was machine-washed, with an acid rinse (Lancer 910E), soaked overnight in 0.01% EDTA solution, and then washed ten times in single- then ten times in double-distilled water.

## 2.4. Preparation and Composition of Chemically Defined Media (CDM)

Cells were grown in CDM liquid medium and shaken on a rotating incubator (New Brunswick G10) or a shaking incubator (The Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey) at 37°C. $CDM_{12}$ was used throughout the project, which is isotonic with serum, the composition of which was based on that of Noy (1982) and is given in table 2.1. The value 12 is the theoretical optical density at 470nm which can be obtained by batch culture growth of the cells in this medium.(i.e. major nutrients all would become depleted at this cell density).

**Table 2.1.** *P.aeruginosa* chemically defined media

| Component | $CDM_{12}$(mM) |
|---|---|
| Glucose | 48.00 |
| KCl | 0.74 |
| NaCl | 0.60 |
| $(NH)_4SO_4$ | 48.00 |
| $MgSO_4.7H_2O$ | 0.48 |
| MOPS* | 60.00 |
| $K_2HPO_4.3H_2O$ | 3.84 |
| ddH$_2$O to 1 litre | |
| $FeSO_4.7H_2O$ (acidified with 1ml $H_2SO_4$ per litre to prevent precipitation of iron). | 0.01 |

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

The pH was adjusted to 7.8 using 1M NaOH and the medium was autoclaved at 121°C for 20 minutes. Glucose, $FeSO_4.7H_2O$ and $K_2HPO_4.3H_2O$ were all autoclaved separately. The glucose was autoclaved separately in order to prevent caramelisation, $FeSO_4.7H_2O$ so that the choice of whether or not to iron-restrict could be made, and $K_2HPO_4.3H_2O$ to prevent any precipitation.

## 2.5. Enumeration of Organisms by Colony Counting

### 2.5.1. Colony Counting Method : Spread Plate Method

A procedure was used to test the reproducibility of the colony counting method (spread plate method). One hundred microlitres of a logarithmic phase culture of 6750 grown in $CDM_{12}+Fe$ was serially diluted 1:10 and 1:100 in $CDM_{12}$ salts solution (CDM without glucose) to give around 80 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 total. The plates were incubated at 37°C for 24 hours and the resulting colonies were counted (table 2.2). The results were subjected to an analysis of variance (table 2.3).

**Table 2.2.** Colony counts per plate for five replicate counts (spread plate method)

| count plate | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 78 | 63 | 77 | 57 | 64 |
| 2 | 76 | 64 | 57 | 69 | 62 |
| 3 | 67 | 75 | 77 | 76 | 69 |
| 4 | 59 | 66 | 69 | 74 | 81 |
| 5 | 73 | 65 | 80 | 62 | 51 |
| Totals T | 353 | 333 | 360 | 338 | 327 |

n = number of observations per count = 5

m = number of counts = 5

n.m.= total number of observations = 25

1. $\sum x^2 = 121667.00$

2. $\dfrac{\sum T^2}{n} = 117100.84$

3. $\dfrac{(\sum x)^2}{n.m.} = 117254.2$

**Table 2.3.**   Analysis of variance of five replicate counts

| Source of Variance | Sum of Squares | Degrees of freedom | Mean Squares |
|---|---|---|---|
| Between counts | (2) - (3)<br>153.36 | m -1<br>4 | 38.34 |
| Within Counts | (1) - (2)<br>4566.16 | nm - m<br>20 | 228.308 |

Variance Ratio ( F ) = $\dfrac{38.34}{228.308}$       = 0.168

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.


## 2.5.2. Colony Counting Method : Drop Method

A procedure was used to test the reproducibility of the drop method as a means to assess colony counts. A culture of 6750 was grown overnight in $CDM_{12}$ + Fe. The Miles and Misra method was used to enumerate the numbers of colonies. Drops of 0.05ml of diluted culture were deposited on over-dried agar plates. Each plate had six drops consisting of three drops of each of the dilutions. At least three dilutions were made, to plate for each of three levels. After incubation for 24 hours at 37°C, counts were made in drop areas showing the largest number of colonies without confluence (30 to 70). The mean count per drop gave the colony count per 0.05ml of that dilution (Ombaka, 1980). All the counts were performed in triplicate. The reproducibility of the counting procedure was tested by performing five replicate counts (table 2.4) and the results subjected to an analysis of variance (table 2.5).

**Table 2.4.**     Colony counts per drop for five replicate count (drop method)

| count drop | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 56 | 60 | 59 | 52 | 42 |
| 2 | 42 | 58 | 64 | 62 | 64 |
| 3 | 59 | 48 | 66 | 62 | 52 |
| 4 | 45 | 47 | 50 | 57 | 45 |
| 5 | 62 | 50 | 65 | 45 | 39 |
| 6 | 69 | 65 | 69 | 57 | 47 |
| 7 | 56 | 47 | 56 | 53 | 54 |
| 8 | 44 | 52 | 68 | 47 | 44 |
| 9 | 40 | 39 | 63 | 52 | 53 |
| Totals T | 473 | 466 | 560 | 487 | 440 |

n = number of counts          = 9

m = number of plates          = 5

n.m. = total number of observations   = 45

1. $\sum x^2 = 134084.00$

2. $\dfrac{\sum T^2}{n} = 131694.89$

3. $\dfrac{(\sum x)^2}{n.m.} = 130788.36$

**Table 2.5.**     Analysis of variance of five replicate counts

| Source of Variance | Sum of Squares | Degrees of freedom | Mean Squares |
|---|---|---|---|
| Between counts | (2) - (3)<br>906.53 | m -1<br>4 | 226.6325 |
| Within Counts | (1) - (2)<br>2389.11 | nm - m<br>40 | 59.73 |

Variance Ratio ( F ) = $\dfrac{226.6325}{59.73}$     = 3.79

The tabulated values of "F" for 4/40 degrees of freedom at 5% and 1% levels are 2.61 and 3.83 respectively. Therefore, the variation between counts is not significantly greater than the variation within counts at the 1% level.


## 2.6. Enumeration of Organisms by Total Cell Counts

### 2.6.1 Chamber Counts

Total counts were performed on exponential and stationary phase cultures grown in $CDM_{12}$ and diluted with $0.2\mu$ filtered saline. Formaldehyde solution was found to increase the clumping of the cells and so it was not used. Samples were analysed as soon as they were harvested. Counts were made using 0.1mm haemocytometer slides (Improved Neubauer, Hawksley). The coverslip was placed evenly over the chamber so that "Newton rings" could be seen over the areas of contact. Samples were diluted so that counts could be made of between 2 and 10 cells per small square (Herbert, 1990). Before counts were made, slides were placed in a humid atmosphere so that cells could settle in one plane. At least 400 cells were counted for each sample using a phase contrast microscope with x 40 magnification.

A stationary phase culture of *P. aeuginosa* 6750 was diluted appropriately with filtered saline. Five replicate counts were made in five counting chambers and the results recorded in table 2.6. The results were subjected to an analysis of variance (table 2.7).


**Table 2.6.**    Total counts per slide for five replicate counts

| Slide Replicates | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 477 | 504 | 461 | 551 | 462 |
| 2 | 534 | 531 | 485 | 344 | 500 |
| 3 | 499 | 517 | 478 | 340 | 485 |
| 4 | 495 | 449 | 462 | 368 | 444 |
| 5 | 505 | 495 | 452 | 403 | 536 |
| Totals T | 2510 | 2496 | 2338 | 2006 | 2427 |


n = number of counts per slide            = 5

nm = number of slides                     = 5

n.m. = total number of observations       = 25

65

1. $\sum x^2 = 5624077.00$

2. $\sum \dfrac{T^2}{n} = 5582145.00$

3. $\dfrac{(\sum x)^2}{n.m.} = 5547909.2$

**Table 2.7.**    Analysis of variance of five replicate counts

| Source of Variance | Sum of Squares | Degrees of Freedom | Mean Squares |
|---|---|---|---|
| Between Slides | (2) -(3) 34235.8 | m -1 4 | 8558.95 |
| Within Slides | (1) -(2) 41932.0 | nm - m 20 | 2096.6 |

Variance ratio ( F ) = $\dfrac{8558.95}{2096.6}$ = 4

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

In general, experiments were performed at least twice unless otherwise stated.

## 2.6.2. Direct Counting by Epifluorescence Microscopy

Culture cells were harvested and then diluted with filtered PBS to an OD of approximately 0.0018. The cells were passed repeatedly through a 0.4-mm gauge needle to eliminate clumps. Vacuum filtration apparatus was assembled with a $0.2\mu m$ pore-size black polycarbonate membrane filter (Poretics Corp., USA). One ml of the diluted culture was filtered and 0.6ml of $0.2\mu$ filtered 0.02% acridine orange(AO) was placed on the filter and left to stain (without vacuum filtration) for 3 min. The membrane was washed twice with 10ml filtered PBS. The filter was allowed to dry before its removal from the filter apparatus. It was placed on a microscope slide with a coverslip and a drop of paraffin oil above and below the filter, and observed under oil immersion x 100 magnification using a fluorescence microscope (Zeiss, Axioscope, Germany). At least 200 bacteria were counted from at least 10 randomly chosen microscope fields (Hobbie *et al*, 1977). The procedure was performed at least twice for each sample analysed and the average number of cells per ml was calculated.

## 2.7. Optical Density Measurements of Cell Numbers

Spectrophotometric measurements 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 cell suspension.

A dilution series of a suspension of *P. aeruginosa* 6750 cells was prepared using sterile CDM salts solution and the optical densities of each were measured at 470nm (This wavelength was used to minimise absorption by medium constituents and bacterial metabolic products such as pyocyanin). Since the optical density of the most dilute suspension and the dilution factor of each suspension were known, the expected optical densities were calculated. Deviation from the Beer-Lambert law was discovered when the optical density exceeded about 0.3.

At values above 0.3, the cell density is such that some cells are in the shadow of others and so scatter no light. Because of this and internal reflection, the measured optical density becomes much lower than the true value. Therefore in subsequent experiments suitable dilutions were made when the optical density exceeded approximately 0.3 to ensure that the true value was recorded.

## 2.8. Correlation of Colony Counts with Optical Density

An overnight culture of 6750 grown in $CDM_{12}$-Fe was diluted with sterile $CDM_{12}$ salts to create a series of suspensions of varying optical densities. These suspensions were serially diluted 1:10 and 1:100 in CDM salts (without glucose) and 100µl aliquots plated out onto nutrient agar. After overnight incubation at 37°C, the number of colony forming units was counted and the colony counts were determined. Optical density was plotted against the number of colony forming units per ml to establish the relationship between colony forming units and optical density for *P. aeruginosa* 6750 (figure 2.1).

**Fig. 2.1.** Relationship between colony forming units and optical density for *P. aeruginosa* 6750.
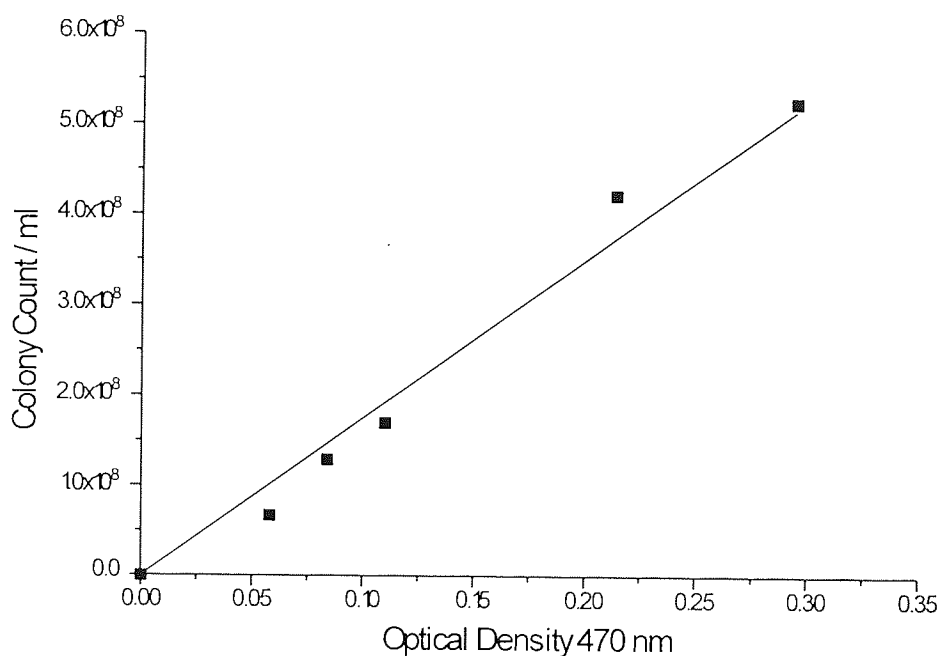
## 2.9. Batch Culture Studies : Nutrient Depletion

### 2.9.1. Iron Depletion Studies

An overnight culture of *P. aeruginosa* PAO1 JD was grown without iron supplementation. Two hundred microlitres were added to several flasks containing 20ml of $CDM_{12}$ with graded concentrations of iron. Growth was then followed by measuring $OD_{470}$ at regular intervals.

### 2.9.2. Carbon Depletion Studies

An overnight culture of *P. aeruginosa* PAO1 JD was grown in $CDM_{12}$ but with a concentration of glucose which was 10% (4.8mM) of the value for $CDM_{12}$. The method as for iron studies was then followed.

### 2.9.3. Phosphate Depletion Studies

An overnight culture of *P. aeruginosa* PAO1 JD was grown in $CDM_{12}$ but with a concentration of phosphate which was 10% (0.38mM) of the value for $CDM_{12}$. The method as for iron studies was then followed.

## 2.10. Continuous Culture

Continuous culture studies were carried out using mini air-lift glass chemostats constructed according to the design described by Gilbert and Stuart (1977). The apparatus is of an all-glass construction which enables low-iron media to be used without contamination from metal parts. Fifty millilitres vessels were used throughout the studies. 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 10 litre glass vessel by a peristaltic pump (Watson-Marlow MHRE HR Flow Inducer, Falmouth) through the 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 a filter.

Aeration was achieved by the passage of filtered, humidified air through the glass sinter (C). The air was humidified by the passage through sterile water. 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 into the vessel. The fermentation chamber was jacketed (B) and linked to a recirculating water bath to allow the accurate control of incubation temperature.

## 2.10.1. 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 set pump rate and air flow rate, the volume within the vessel was measured.

These procedures provided an estimate of the flow rates and ultimately the dilution rates (D) which could be achieved. However, in order to allow for discrepancies due to changing pump tubing length, air flow rate or frothing of cultures, flowrate was monitored throughout experiments by measuring the volume of spent medium and cells expelled via the overflow in a given time.

**Fig. 2.2.**    Diagram of the mini air-lift, all glass chemostat.
(50ml volume vessel, dimensions are in cm).
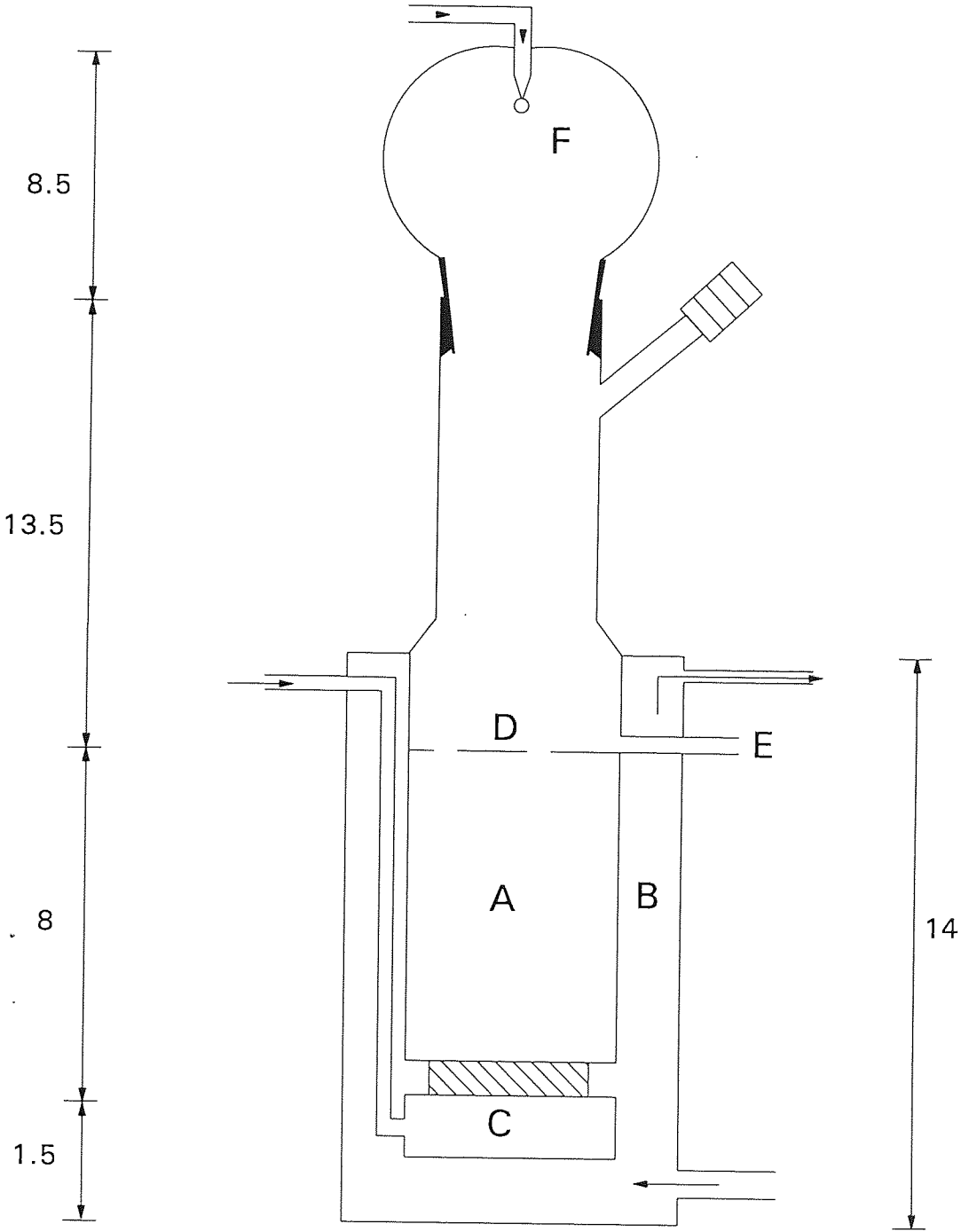
At the start of the chemostat studies, media was pumped in with aeration and then stopped when the vessel was approximately a quarter full. The vessel was then inoculated with a log phase culture of PAO1 or 6750 cells in the same medium (1% of working volume) and allowed to grow as a batch culture until stationary phase was achieved. The pump speed was then increased gradually until the required medium flowrate was attained.

The chemostat was then run for sufficient time to give five complete volume changes before the cells were used. This allowed a steady state to be achieved, and the procedure was followed each time the flowrate was changed.

## 2.10.2. Iron Limitation in Continuous Culture

*P. aeruginosa* 6750 was grown by continuous culture under iron limitation in $CDM_{12}$-Fe. Optical densities and colony counts were monitored throughout the studies at each dilution rate employed. Samples of the eluate cells were collected over ice for subsequent outer membrane preparations. Samples from the body of the chemostat were removed aseptically and subjected to various tests. However, after this procedure five additional volume changes were allowed to elapse before additional removal of samples. Iron added to an iron restricted chemostat resulted in an increase in the optical density, thereby demonstrating the initial iron restriction.

## 2.10.2.1 Evaporation in the Chemostat at Low Dilution Rates

Initial experiments with water flowing at low dilution rates were used to check that evaporation did not take place. The level of the cultures in the chemostat were monitored throughout the experiments. After completion of the chemostat runs, the volume of the cultures was measured to check if the volumes remained constant.

## 2.11. Parameters to Investigate Survival and Possible Dormancy

### 2.11.1. Longevity

Cultures were kept at 37°C in conical flasks of 20ml, 40ml or 200ml depending on the length of time for storage. The flasks were shaken for approximately 30 days after which time they were sealed more firmly with Parafilm and allowed to stand at 37°C.

Survival of cultures was monitored using total and colony counts (cfus) throughout the period of storage.

### 2.11.1.1. Survival of *P.aeruginosa* 6750 Stored at 5°C under Glucose, Phosphate and Iron Depletion.

To investigate the survival of *P.aeruginosa* 6750 after a temperature downshift, batch cultures of *P. aeruginosa* 6750 were grown to stationary phase at 37°C depleted for the nutrient under investigation, and then transferred to a cold room maintained between 3°C and 7°C. Cultures were grown in shake flasks containing CDM. To investigate the influence of growth stage, a set of cultures were grown at 37°C to an $OD_{470}$ of 0.2 and transferred to the cold room. Total cell counts and colony counts were performed over a period of 30 to 35 days.

To evaluate the effects of starvation and temperature downshift on exponential cells, a further set of flasks were grown as before to an $OD_{470}$ of 0.2 and put directly into the cold room as a control. The test set of flasks were grown to the same OD, centrifuged (1,000g, 50 mins), supernatant was removed and the pellets were washed (5,000g, 20 mins) in CDM minus the limiting nutrient. Supernatant was again removed and the pellets were resuspended to the original volume with CDM minus the limiting nutrient. Cultures were placed in flasks and transferred to the cold room. Total cell counts and colony counts were performed throughout the period of storage.

## 2.11.1.2. Resuscitation of Iron -depleted *P.aeruginosa* 6750

*P.aeruginosa* 6750 grown to stationary phase, and stored in a cold room at 5°C for approximately 80 days was subjected to a temperature upshift to 37°C. Total and colony counts were performed over a period of 24 hours to investigate any change.

## 2.11.1.1.1. Stacking of Plates: Colony Counts

When plates were initially stacked in high piles or in trays, small colonies appeared but may have been due to the lower temperature of enclosed plates rather than the suspected lag of these colonies. Plates were stacked therefore in piles of no more than three, so that an even incubation temperature was maintained.

## 2.11.1.1.2. Cell Aggregation and Total Counts

Cultures kept at 37°C for extended storage times suffered from an increased amount of cell aggregation or clumping. In order to quantify the problem during the procedure of total counting, the size of the cells were classed roughly into three catagories :

1) single cell
2) Clump size > 2 but < 10
3) Clump size > 10

The procedure was adopted whenever total counts were made, but unless stated otherwise the counts were calculated by classing one clump as a single cell whatever the clump size.

Different strains of *P. aeruginosa* were cultured at 37°C in $CDM_{12}$ for varying lengths of time and compared to *P. aeruginosa* 6750 to investigate if aggregation was peculiar to the strain.

## 2.11.1.1.3. Evaporation of Culture in Shake Flasks

Weights of flasks containing cultures stored up to 30 days were monitored at intervals to investigate the extent of evaporation.

## 2.11.2 Measurement of Size

### 2.11.2.1. Size: R.T.G. Correlator

Size was measured using a commercially-available goniometer (R.T.G. Correlator, SEMAtech) based on the principle of photon correlation spectroscopy (PCS) or quasi-elastic light scattering. PCS analyses the time scales over which a laser light source, scattered from a population of particles fluctuates as a consequence of the particles' Brownian motion. From this, the diffusion coefficient can be established which can then be related to particle size (Jepras *et al*, 1991).

Samples of bacteria were diluted with filtered PBS to avoid dust particles. Samples were analysed at working temperatures of 25°C in 1cm diameter round glass cuvettes immersed in an index-matching, temperature-stabilised ethanol bath. Polystyrene spheres suspended in double distilled water were used as a control. Measurements were carried out with 90° angle recommended for bacteria (Jepras, 1991). In the optimal configuration, a series of concentrations of bacteria were analysed to establish the mean diameter and working range of the instrument (fig. 2.3).

The graph shows that the instrument could be used between optical densities of 0.05 and 1.3. In general cultures were standardised to an $OD_{470}$ of 0.1.

**Fig. 2.3.**    Calibration curve to evaluate working range of RTG Correlator
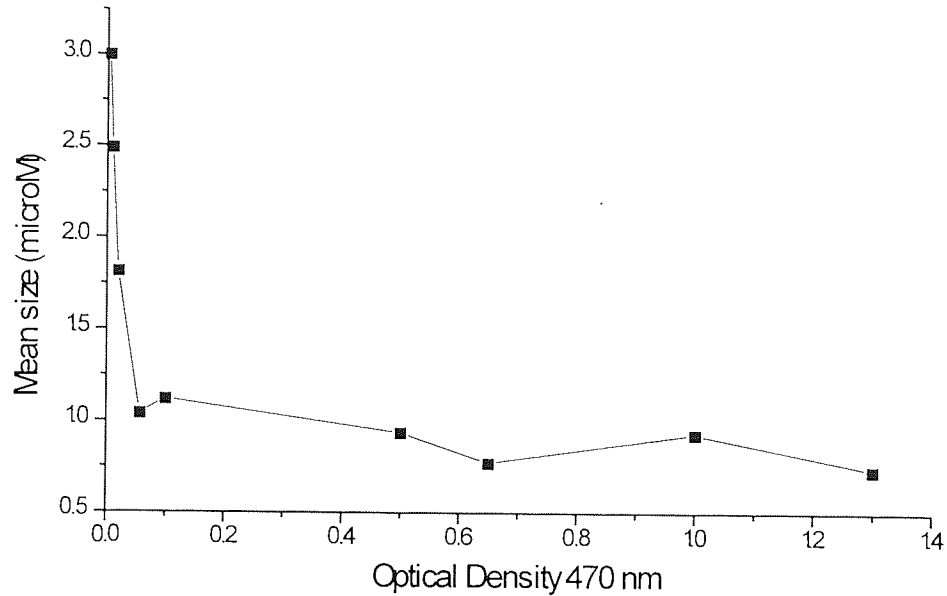


## 2.11.2.2. Photographic Method of Size Measurement

Samples of 10μl bacterial cultures were prepared on glass microscope slides with a glass coverslip. Samples were left for 30 minutes so that the majority of bacteria settled in one plane. Slides were examined under phase contrast using a microscope (Zeiss, Axiotrop, Germany) with x 100 magnification and oil immersion. Images were carefully focussed to ensure sharp images with minimal movement. Photographs were taken using a 35mm black and white 100 ASA film (Ilford) using 1/8th second exposure time. Pictures were taken. from at least 10 fields of view for each sample. Latex particles of known sizes suspended in double-distilled water were also photographed as reference sizes. After development of the film, the negatives were mounted in slide mounts and projected with a slide projector so that the final image size of the whole 35mm negative was approximately 70cm x 48cm (Fry and Davies, 1985). Measurements of at least 100 bacteria were made directly from the projected image with a ruler. Using the projected images of the latex particles as reference points, the size of the bacteria could be calculated.

## 2.11.3. Estimation of Bacterial Cell Volume

Estimation of bacterial cell volume was made by using the most common model : a straight sided rod with hemispherical ends. The volume of a single cell (v) is therefore calculated by

$$v = ( d^2 \pi /4 ) ( 1 - d) + \pi d^3 / 6$$

where d = width and l = central length (Fry, 1990)

## 2.11.4. Bicinchoninic Acid Protein Assay

The method used was an adaptation of that of Lowry *et al*, (1951). Proteins react with copper(II) to produce a protein-copper(I) complex. The BCA Protein Assay Reagent reacts with copper(I) to form an intense purple colour. Bicinchoninic acid is the main component of the assay (Smith *et al*, 1985) and forms alkali metal salts which are soluble in water. As the purple reaction product is also soluble in water, it allows the spectrophotometric measurement of an aqueous protein solution.

The BCA protein reagent was freshly prepared by mixing 50 volumes of reagent A (aqueous solution of 1% bicinchoninic acid, 2% $Na_2CO_3.H_2O$, 0.16% disodium tararate, 0.4% NaOH and 0.95% $NaHCO_3$) with one volume of reagent B (4% $CuSO_4.5H_2O$ in double distilled water. Ten µl of sample or standard was mixed with 200µl of the protein reagent on a microtire plate. The plate was incubated for 30 min. at 60°C so that the colour developed. The plates were allowed to cool at room temperature after which the absorbance was measured ar 550nm (Anthos Reader 2001, Anthos Labtec Instruments, Austria). Standard curves were constructed using Bovine Serum Albumin (BSA) stock solution as protein samples.

## 2.11.4.1. Preparation of Standards and Protein Samples for BCA Method

One ml of bacteria was harvested and centrifuged in a microcentrifuge (MSE MicroCentaur) at 13,000rpm for 5 minutes. The procedure was performed in triplicate. The supernatant was removed and sterile saline was added to a volume of 1ml in order to wash the samples free of $CDM_{12}$ which was found to affect the reaction. The samples were vortexed to resuspend the pellet and respun as before. The saline was carefully removed and the pellets were resuspended in a 50:50 mixture of 1M NaOH and sterile water to dissolve completely the pellet up to volumes of 1ml, 0.5ml or 0.1ml depending on the optical density of the bacterial culture. Stock solution of BSA was also made up

in the NaOH solution. Control solutions of saline were tested to investigate its affect on the assay.

## 2.11.5. Methods for the Study of Cell Surface Properties

### 2.11.5.1 Preparation of Outer Membranes : Method for Small Samples

At fast dilution rates in the chemostat, only a small impractical number of cells was obtained for outer membrane preparation. A "mini-method" for the preparation of samples was used (Nelson, 1993).

Cell suspensions were centrifuged (10,000 rpm, 10 min), and the pellet suspended in a small volume of 0.85% saline in a microcentrifuge tube (Eppendorf). The sample was sonicated, whilst still in the same tube, for a few seconds to resuspend and evenly redistribute the pellet. N-lauryl sarcosinate (Sarcosyl) 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 hr. The pellet was resuspended in approximately 100 μl solution of sample buffer and double distilled water ddH$_2$O (1:1) and was boiled for 10 minutes.

### 2.11.5.2. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Separation of OM proteins was carried out by 12% discontinuous gels as described by Lutgenberg et al (1975) using the Mini-Protean system. Ten to fifteen μl were applied to each lane and the gels run at a constant voltage of 200V until the dye-front had migrated to within 0.5cm of the bottom of the gel. The gels were then stained.

### 2.11.5.3 Coomassie Brilliant Blue Stain for Protein Gels

Protein gels were stained for one hour, with gentle agitation, in a solution containing Brilliant Blue R-250 (1g; Sigma), methanol (500ml) and glacial acetic acid (100ml) in 400ml dH$_2$O. Background stain was removed by successive washes in a destain solution of methanol (100ml) and glacial acetic acid (200ml) in 700ml dH$_2$O.

## 2.11.5.4 Polyacrylamide Gel Electrophoresis for the Analysis of LPS

The amount of bacteria from which the LPS was to be extracted was standardised. Different volumes of bacteria were centrifuged at 13,000 rpm for 5 min. and resuspended to produce 1ml of culture with $OD_{470}$ of 8 in microcentrifuge tubes. Cultures were washed with saline (13,000 rpm, 5 min.), the supernatants were discarded and pellets were resuspended in 40$\mu$l lysis buffer. Samples were steamed at 100$^o$C for 15 min. using a waterbath. After the samples had cooled, 10$\mu$l Proteinase K (2.5mg/ml in ddH$_2$O) was added and samples were incubated at 58$^o$C $\pm$ 2$^o$C for 1 hr. Samples were centrifuged at 13,000 rpm for 5 min. and supernatants were transferred to clean microcentrifuge tubes.

Samples were loaded in the same way as for outer membrane protein preparations except that the gels were 15% for the separating gel and 5% for the stacking one. Gels were pre-run at 15V for 1 hr, 20V for 4 hr and then 35V for 2 hr so that the dye-front ran within 0.5mm of the bottom of the gel. The gels were then stained.

## 2.11.5.5. Silver Stain for LPS

The gel was fixed overnight in a solution of methanol (60%), ddH$_2$O (30%) and glacial acetic acid (10%). The following day, the fixing solution was replaced for 30 min. with a solution of glacial acetic acid (7.5% in ddH$_2$O). The solution was removed and an oxidising solution was added (0.4% periodic acid in ddH$_2$O). The oxidising solution was removed from the gel by using at least 5 changes of ddH$_2$O to wash it over a period of 2 hr. Staining solution was freshly prepared by adding concentrated ammonium hydroxide (718$\mu$l) to 10M NaOH (450$\mu$l) followed by dropwise addition of 10% AgNO$_3$ until precipitate just started to form. A further drop of the ammonia was then added so that the precipitate dissolved. To this solution was added ddH$_2$O to make the volume up to 50ml. The gel was agitated in the staining solution for 10 to 15 min., and then given at least 3 washes in ddH$_2$O over a period of 1 hr, to remove any excess stain. It was developed within 10 to 15 min. in a freshly prepared solution containing citric acid (5mg) in 37% formaldehyde (52$\mu$l), methanol (10ml) and ddH$_2$O to 100ml. Developing was stopped by removal of the solution and addition of ddH$_2$O. the gel was photographed immediately as the stain deteriorates quickly. It was washed in at least 5 changes of ddH$_2$O before drying.

## 2.11.6 Vital Stains

### 2.11.6.1. 5-Cyano-2,3-Ditolyltetrazolium Chloride (CTC)

CTC is a monotetrazolium redox dye which produces a fluorescent insoluble formazan compound when it is chemically or biologically reduced. It has been found that CTC can be used to visualize microscopically actively respiring bacteria (Rodriguez $et$ $al$, 1992 ).

The method of Rodriguez $et$ $al$ (1992) was used with some modifications for $P.$ $aeruginosa$. The CTC (Polysciences, Inc., Warrington, Pa.) was dissolved in water to a concentration of 5mM. Cell cultures were harvested and mixed directly with CTC stain in duplicate to obtain a final CTC concentration of 4.5mM. Standardised cell cultures with $CDM_{12}$ were also made (ca. $10^8$ cells/ml) and mixed with the CTC stain to examine the affect of cell number on the efficacy of staining. Cells were incubated with CTC for 2 hours at room temperature protected from light. Cultures were mixed throughout incubation to minimize cell aggregation.

Samples were observed directly on microscope slides to ensure that the intracellular CTC-formazan deposits corresponded directly with actual cells, and so total counts could be performed at the same time. Bacterial cells were examined under 100 x oil immersion fluorescence objective using a Zeiss Axioskop microscope and appropriate filter blocks. The respiring cells were identified as the cells with the deposits and percentages of these were calculated using total numbers. Colony and total counts were made to check the procedures.

A late exponentially grown culture was used to constuct a calibration curve. A portion of the culture was killed by incubation at 60°C in a water bath for 1 hr. Varying amounts of the live culture and dead culture were mixed to produce different percentages of live and dead. Total and colony vounts were performed to verify the percentages. Cultures were subjected to the CTC treatment and the respiring cells were counted to produce a calibration curve.

**Fig. 2.4.**     Relationship between percentage CTC stained cells and percentage
live cells of exponential phase *P. aeruginosa* 6750 grown in iron
- depleted $CDM_{12}$ at 37°C.
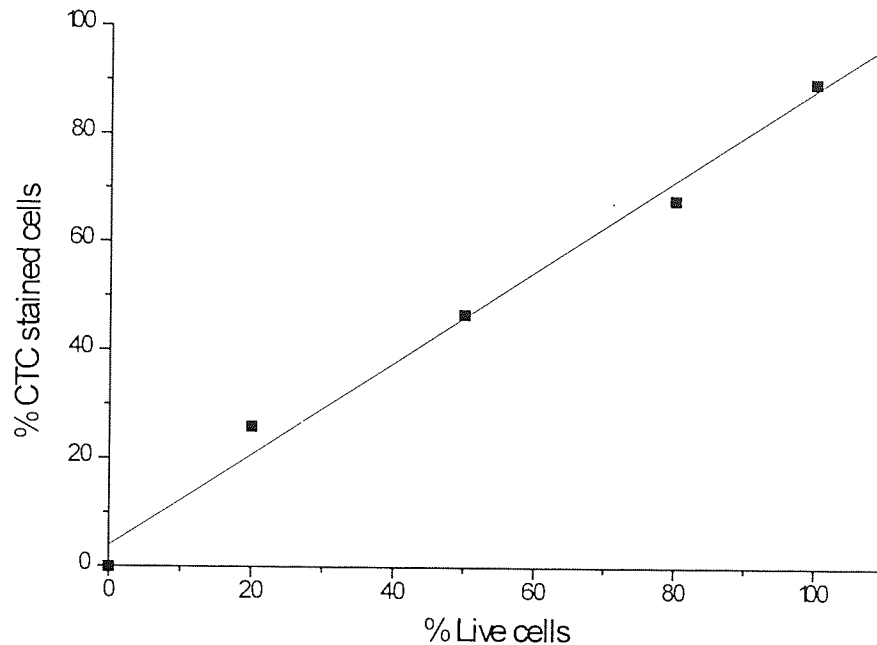


## 2.11.6.2. Direct Viable Count (DVC)

DVC method was carried out according to Singh *et al* (1990). Preliminary experiments
were performed to evaluate the concentration of nalidixic acid needed to inhibit
bacterial division. It was found that an overnight culture of cells incubated in 300μg/ml
nalidixic acid for 5 hours was sufficient to inhibit cell division and this protocol was
used for future experiments.

Cell cultures were incubated at 37°C in PBS containing 0.3% Casamino Acids (Difco)
and 0.03% yeast extract and concentration of nalidixic acid 300μg/ml. Control cultures
were examined without nalidixic acid at time zero. Cultures were removed after 5 hr
incubation and subjected to the treatment used to directly count cells using
epifluorescence microscopy (section 2.6.2.). Measurements of colony and total counts
and percentage of elongated cells were made using the method of Hobbie *et al* (1977)
with minor modifications. Counts were made within 5 min. of cell preparation on the
membrane. Cells longer than three times the normal control cell length were classed as
elongated and made up the counts of the DVC method. Control cultures were set up
containing PBS and nalidixic acid without the yeast extract and Casamino acids in order
to examine the effect.

## 2.11.7. Measurement of Lag

Cultures of different types of cell were used as inoculum for flasks of 20ml of fresh $CDM_{12}$. Growth was followed by measuring $OD_{470}$ at regular intervals. The time taken for each of the cultures to reach an $OD_{470}$ of 0.05 was recorded and interpreted as a measure of lag.


## 2.11.8. Antimicrobial Bactericidal Assay

### 2.11.8.1. Ciprofloxacin

The assay was performed to give an indication of the susceptibility of *P. aeruginosa* 6750 to ciprofloxacin. Fluoroquinolones cause abnormal elongation of bacteria which increases the scattering of light when measuring optical density of liquid cultures. Consequently, bacterial killing by fluoroquinolone antibacterials cannot be judged by measurements such as light scattering or optical density that only reflect a change in bacterial mass. Hence, the levels of kill of bacteria by the 4-quinolones are studied by colony counts (Smith, 1984).

Iron-restricted chemostat cultures or batch cultures were used. Tubes were prepared in triplicate to give appropriate concentrations of the drug ciprofloxacin (table 2.8), and pre-warmed at 37°C for 10 min. Culture samples were standardised by dilution with $CDM_{12}$ salts solution to give approximately $10^5$ cells $ml^{-1}$. One hundred microlitres of these suspensions were added to the antimicrobial solutions and incubated for 1 hr. The treated cells and controls were diluted 1:100 in CDM salts (minus glucose), and 100µl samples were plated onto nutrient agar. Plates were incubated at 37°C overnight and relative reductions in cfu(s) were assessed. Concentrations of ciprofloxacin which gave appropriate levels of killing (1-2 log cycles), relative to control suspensions were chosen for future experiments.

**Table 2.8.**    Antibiotic solutions for use in antimicrobial bactericidal assay

| Antimicrobial stock solution ($\mu$g/ml ) | Volume of antibiotic to use($\mu$l) | CDM salts solution (ml) | Inoculum ($\mu$l) | Final concentration ($\mu$l./ml) |
|---|---|---|---|---|
| 0 | 0 | 9.90 | 100 | 0 |
| 20 | 50 | 9.85 | 100 | 0.1 |
| 20 | 100 | 9.80 | 100 | 0.2 |
| 30 | 100 | 9.8 | 100 | 0.3 |
| 50 | 100 | 9.8 | 100 | 0.5 |
| 100 | 100 | 9.8 | 100 | 1.0 |

## 2.11.8.2. Polymyxin B

The assay was performed to give an indication of the susceptibility of *P. aeruginosa* 6750 to polymyxin B. The assay was performed in the same way as for ciprofloxacin, except for the treated cells and controls were diluted in letheen broth (Difco) as it contained lecithin glycerol (Kohn *et al*, 1963). Preliminary experiments assessed the neutraliser efficiency (data not shown). The test ensured that the antimicrobial agent was neutralised by the inactivator and that the inactivator was not inhibitory to the growth of the test organism (Noy, 1982).

## 2.11.8.3. Susceptibility of "True" Exponential Cells

Experiments were carried out involving "true" exponential phase cells. These cells were allowed to undergo at least four generations of growth from initial inoculum and were subjected to polymyxin B and ciprofloxacin antibiotic assay when they were at least four generations from onset of stationary phase. True exponential phase cells were compared to another set of exponential phase cells (further along the growth curve) that were allowed to grow so that they were not four generations from stationary phase. It was ensured that, the latter set of exponential cells were tested before they entered stationary phase.

## 2.12. Study of Biofilm Growth

### 2.12.1. Apparatus

Biofilm growth was studied using a 47mm inline filter unit (Gelman Sciences Inc.), fitted with a rifle connector and sealed with silicone sealant (Silastic, Dow Corning). Filter units were autoclaved and then 47mm sterile cellulose acetate membrane filters of 0.22µm pore size (Millipore Corporation, USA.) were introduced aseptically into the units. The method was adapted from that of Helmsetter $et$ $al$, (1992).

An early stationary phase culture of $P.$ $aeruginosa$ 6750 was adjusted to an optical density of 0.1 using sterile $CDM_{12}$ salts media and placed on a rotating incubator at 37ºC for approximately 1 hr. Media were prepared in the same way as for the chemostat studies without the addition of iron and delivered to the units by means of a peristaltic pump (see section 2.9), through silicone tubing and into the entry port of the filter units which were supported in clamps. Temperature control was achieved by placing the whole apparatus in a room maintained continuously at 37ºC. Aeration was achieved by passing air through a sterile filter and then via a humidifier (as for the chemostat), which was then attached to the air inlet on the underside of the filter unit. The pump was turned on and the medium allowed to flow through the silicone tubing until it reached the ends of the tubing. The pump was then turned off and the filter units were prepared, so that a minimum of time delay occurred between inoculation and the assembly of the apparatus.

### 2.12.2. Inoculation of the Membranes

Filter units were perfused with 10ml of sterile water in order to wash off the surfactant which was incorporated in the manufacture of the membranes. 10ml of culture ($OD_{470}$ 0.1) was perfused through the units, and care taken to avoid pushing air through the device. The units were then inverted and 50ml of sterile saline 0.85% was perfused through them at approximately 1ml/sec. The saline eluate was collected and a colony count was carried out to check the number of cells eluting each membrane. The units were then assembled in the warm room, the air pump and the peristaltic pump were turned on and samples of eluate were collected intermittently in sterile bijou bottles.

## 2.12.3. Enumeration of Cells Adhering to the Membrane

When a time-point was reached to sample a membrane, the appropriate media line was closed off and the filter unit was removed from the apparatus. The membrane was removed using sterile forceps and placed in a sterile petri dish. Ten millilitres of sterile saline were added to the petri dish and cells were scraped off the membrane using a sterile spreader and sterile forceps. The saline and cells were placed in a sterile universal tube, and the membrane was subjected to a second round of washing with an additional 5ml of sterile saline. After the addition of the second saline washing to the universal tube, the membrane was placed in the tube. A final 5 ml of sterile saline were added to the petri dish to remove any excess cells and it was also placed in the universal tube. The membrane underwent a 30 second vortex treatment (Fisons Whirlimix, maximum speed), after which time a colony count was carried out. The procedure was repeated twice and a colony count was performed after each treatment. The membrane was frozen at -20°C after the final colony count was made.

## 2.13. Verification of *P. aeruginosa* 6750

### 2.13.1. Oxidase Test

The test was used to prove the presence of the organism *P. aeruginosa* (Cowan and Steel).

### 2.13.2. Growth on *Pseudomonas* Isolation Agar (PIA)

The test was used to verify that the organism present was *P. aeruginosa* and to eliminate the possibility of contamination. The cetrimide in the PIA agar inhibits many organisms but allows the growth of *P. aeruginosa*.

## 2.14. Investigation of Slow Growth

During the course of some of the experiments it was discovered that cultures experienced slow growth : the growth rate of the cultures decreased which manifested itself with an increase in doubling times. Temperatures and pH of cultures were monitored throughout growth and were found to be consistent with previous results. However, it was noted that upon addition of iron to cultures the problem did not arise. Several flasks of *P.aeruginosa* 6750 were set up in CDM with varying amounts of iron. Growth was followed by measurement of $OD_{470}$. It was found that the higher the amount of iron added to the culture the faster the growth rate. Therefore, it was concluded that the water being used contained less contaminating iron than previously which resulted in the observed slow growth.

# 3.    RESULTS AND DISCUSSIONS

## 3.1    Studies on Biofilm Growth

### 3.1.1.    Introduction

The principles of the biofilm system of growth for the *P. aeruginosa* organism were based on the work of Gilbert *et al* (1989) in which growth rate control of adherent bacterial populations was achieved. A steady state was developed in which the size of the biofilm population remained constant, in the form of a monolayer, and dispersed cells were collected in the spent medium. At steady state, the rate of perfusion of the fresh medium controlled the growth rate of the adherent biofilm.

In work with *P. aeruginosa* 6750, difficulties were encountered in the application of the above techniques and so these studies were terminated. Since studies relating to survival were to be made with respect to changes in growth rate, the chemostat system was used instead in order to pursue this line of work.

### 3.1.2.    Results and Discussion

Figure 3.1 represents the numbers of cells eluted from a biofilm during the first 6 hours of perfusion. The perfusion of medium through the membrane resulted in a decreasing number of eluted cells. After approximately 3 hrs a steady state was achieved. In contrast to the findings of Gilbert *et al* (1989) and Evans (1990) in which a steady state biofilm was maintained for 14 days with *E. coli*, the steady state in these experiments could not be maintained for a longer period of time than 6 hrs. Figure 3.3 shows that when the biofilm was run for a longer time, the numbers of cells eluted tended to increase with respect to time, this being indicative of an increasing adherent biofilm population (Nelson, 1993). At each time point on fig. 3.2, one biofilm was sacrificed.

**Fig. 3.1.** Elution of iron-depleted *P. aeruginosa* 6750 from a cellulose acetate membrane at a constant flow rate of 1.1ml/min.
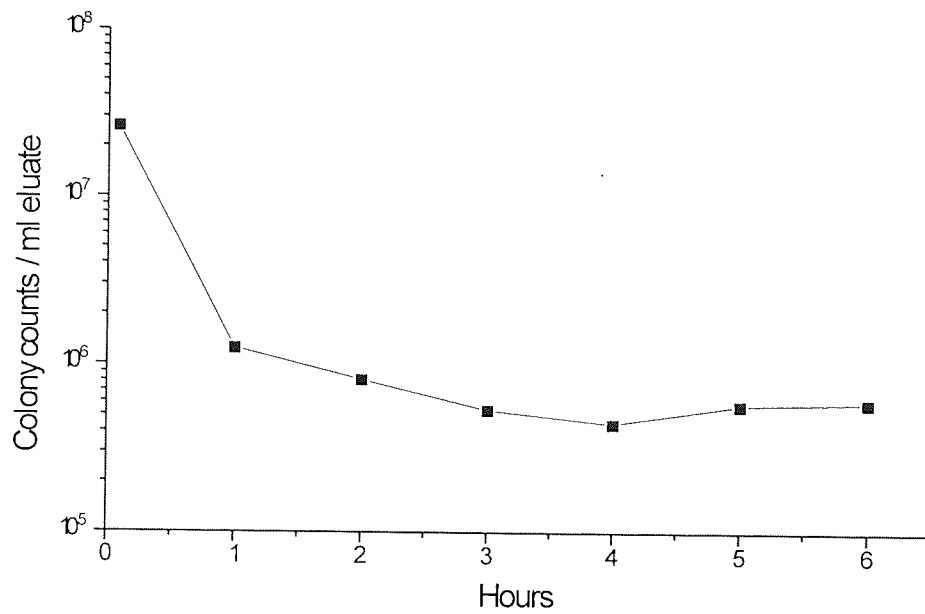
**Fig. 3.2.** Numbers of *P. aeruginosa* 6750 iron-depleted biofilm cells recovered from cellulose acetate membranes, monitored at different flow rates.
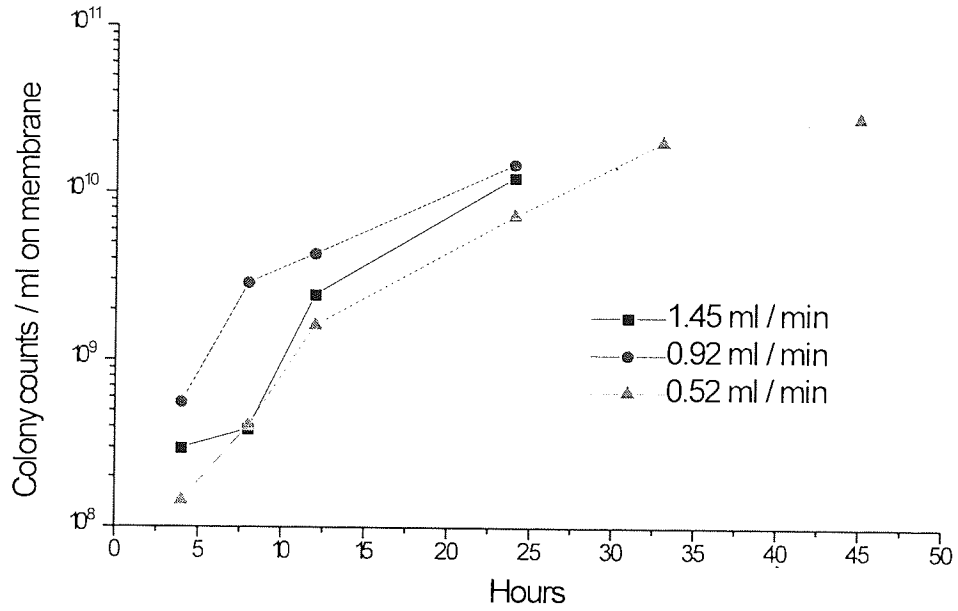


**Fig. 3.3.** Numbers of *P. aeruginosa* 6750 iron-depleted eluate cells from cellulose acetate membranes monitored at different flow rates.
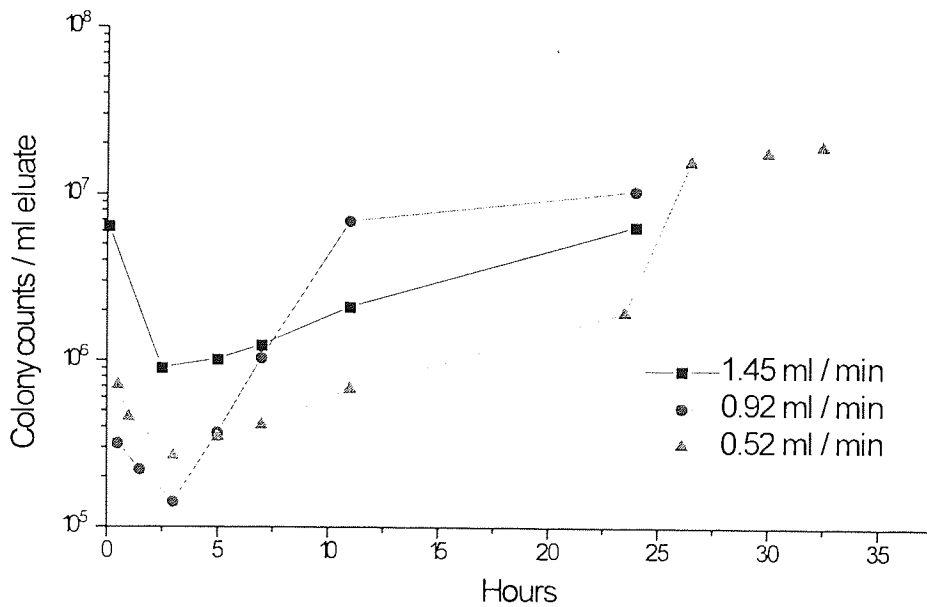
The biofilms were run at three different flow rates. For two flow rates, the biofilms were run over a period of 24 hours, whilst for the third flow rate the biofilm was run for 45 hours. The number of cells released from the biofilms varied according to the flow rate (fig.3.3). This could also be said for the number of cells on the membrane (fig.3.2). An unexpected finding was that for both figure 3.2 and 3.3, the highest flow rate did not correspond to either the greatest numbers of cells recovered from the membrane or the greatest number of cells eluted from the membrane, respectively. Further experiments would have to be performed using other flow rates in order to confirm if this were universally true.

The fact that the highest flow rate did not produce the largest numbers of cells may be a result of the flow rate being too fast. Additionally, the three flow rates were not dissimilar in the number of colony counts on the membrane. The fast flow rate may be washing a large proportion of the cells off the membrane in a manner similar to the "washout" of the chemostat. Indeed, the medium flow rate may also have been too high for differences in growth to be manifested. In both cases the biofilms may be growing at $\mu_{max}$. Consequently, growth on the biofilm should be examined at even lower flow rates in order to achieve a marked differences between the plots so that the true effects of slow-growing biofilms can be analysed. Flow rates, however, should not be too low as problems of perfusion may ensue.

Steady state biofilms could not be produced because of the increasing growth but an element of growth rate control could be achieved if there were definite differences between varying flow rates that were reproducible.

The results of Power and Marshall (1988) confirm the finding that *Pseudomonas* is able to divide and remain on a membrane. Their studies were performed with a different strain, *Pseudomonas* JD8. It was found that the cells that were attached to the membrane were capable of dividing and continued to stay attached to the surface. Detachment of some cells took place as well, but the fact that some dividing cells remained attached to the surface showed that the *Pseudomonas* system for biofilm development was not exactly the same as that of *E. coli* (Evans, 1990).

## 3.1.2.1. "Rough" and "Smooth" Investigation

The results of the colony counts of *P. aeruginosa* 6750 recovered from the cellulose acetate membranes and from the eluate cells, showed that two types of colony were able to grow on the surface. On visual examination of the colony counts, one type had a smooth and round outer appearance on nutrient agar, and the other had a less distinct, rough outer edge and was larger in size. They were termed "smooth" and "rough" respectively.

A differential count of the rough and smooth colonies was performed. The inoculating culture consisted of approximately 50% rough colonies. Figure 3.4 shows the percentage of rough colonies recovered from the membranes with time at the three different flow rates. Figure 3.5 shows the percentage of rough colonies that were released from the membranes as eluate cells with time for the three flow rates. In general, both plots showed that the percentage of rough colonies decreased with time for all three flow rates.

The oxidase test was performed to check for contaminants, and the result was positive for both colony types. The test, which is used to detect both *Neisseria* and *Pseudomonadaceae* genus, detects the presence of cytochrome c oxidase that reduces oxygen. The two types of colony were also found to grow on *Pseudomonas* Isolation agar indicating that they were both likely to be of the genus *Pseudomonadaceae*.

**Fig. 3.4.** Percentage of rough colonies of *P. aeruginosa* 6750 iron-depleted biofilm cells, recovered from cellulose acetate membranes at different flow rates.
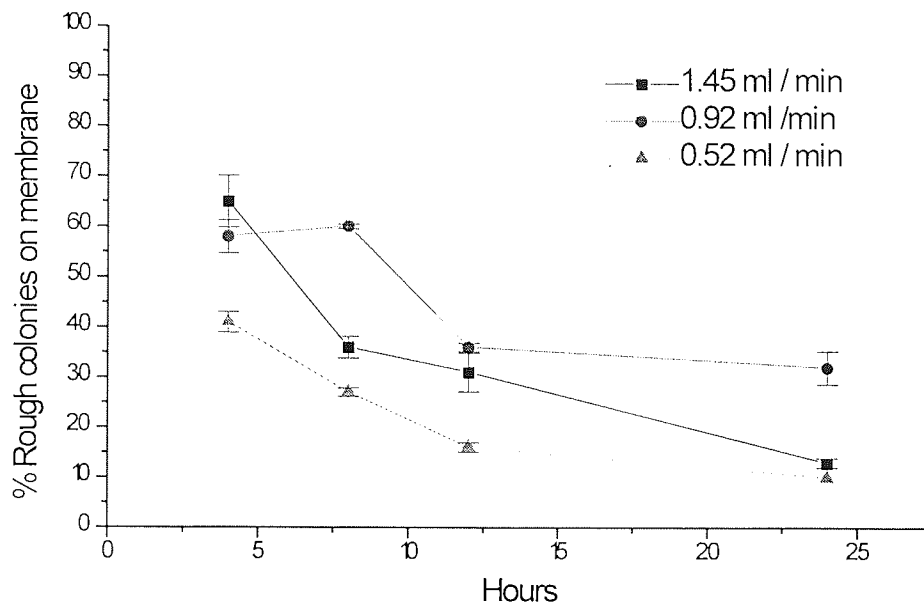


**Fig. 3.5.** Percentage of rough colonies of *P. aeruginosa* 6750 iron-depleted biofilm eluate cells, monitored at different flow rates.
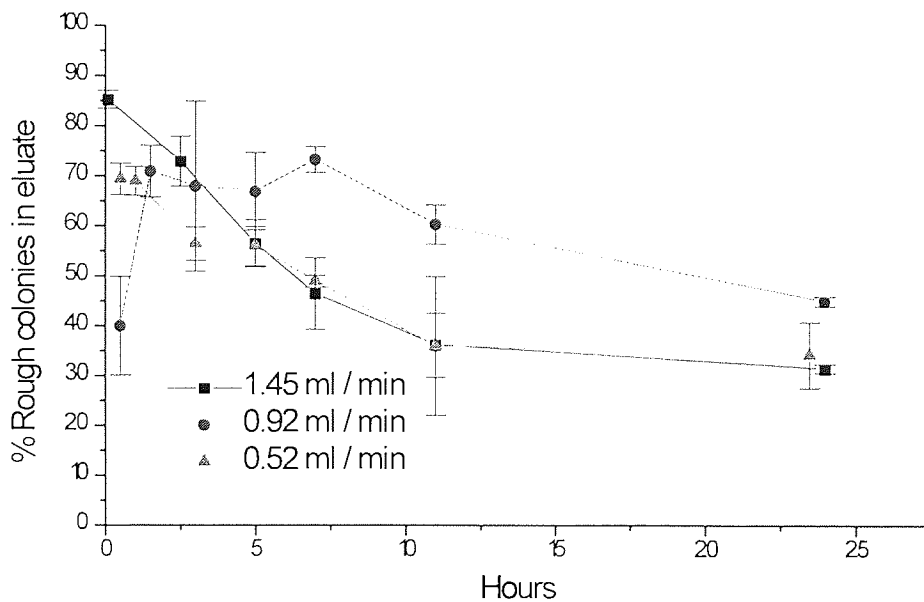
Analysis of LPS of *P. aeruginosa* 6750 has been performed with batch culture cells to investigate the properties of the bacteria important in survival (section 6.3). However, an investigation of LPS structures for the smooth and rough cell types individually was unsuccessful, attributable in part to small cell samples. An alternative method, used especially to detect O-antigen banding could be more sensitive (Fomsgarrd *et al*, 1990).

The LPS structure of *P. aeruginosa* is known to exist in two forms (section 6.2). The R-form LPS (rough form) contains lipid A substituted with core polysaccharide and the S-form LPS (smooth form) represents the R-form LPS capped with side chains (Kropinski *et al*, 1985).

Observations have also been made based on colony morphology that smooth and rough organisms exist. *P. aeruginosa* cultured under humid conditions on nutrient agar plates produced functional fimbriae with the formation of colonies which are large, rough and flat (Hobbs *et al*, 1993), whereas other colonies have been described as small, smooth and domed.

It has been reported in an investigation in which LPS was extracted from biofilm and planktonically grown strains of *P.aeruginosa* isolated from CF patients, that there was an increase in the core/lipid A R-LPS fraction in biofilm-LPS compared to planktonic-LPS extracted from the S-form bacteria (Giwercman *et al*, 1992). It was suggested that these changes in the LPS reflect changes in OM structure when bacteria grow as a biofilm and may contribute to altered physico-chemical surface properties of biofilm bacteria.

# 4. RESULTS AND DISCUSSIONS

## 4.1. Batch Culture Studies

### 4.1.1. Introduction

Organisms grown in batch culture are in a closed system in which there is a finite amount of nutrients to support growth. Cells grow exponentially, possibly after an initial lag phase and then enter stationary phase. The duration of the exponential phase depends partly on the initial concentration of the growth limiting substrate (Pirt, 1975).

Bacteria can be grown in this system using varying concentrations of the growth-limiting substrate, in order to ascertain, for a particular organism, the concentrations of nutrients which will eventually limit growth of the organism. Growth ceases normally through the depletion of one or more essential nutrients or through the production of toxic metabolites. After this stage, cells either survive or experience cell death. Studies in this chapter focus on stationary phase and the outcome of cells after this state.

### 4.1.2. Results

#### 4.1.2.1. Population Growth Curves

It has been found that the final density reached by a culture of *P. aeruginosa* is dependent on the initial concentration of a growth-limiting nutrient in the medium (Brown and Melling, 1969). This was found to be the case where glucose (see figure 4.1), iron and phosphate (data not shown) were the growth limiting nutrients in a culture of *P. aeruginosa* PAO1 JD. The organism was grown in 100 ml flasks to a volume of 20 ml (section 2.9). The curves that were plotted were a representative sample of the graphs obtained using different concentrations of the growth-limiting nutrient.

A graph of $OD_{470}$ at onset of non-linear growth against initial concentration of the potentially growth-limiting nutrient were plotted for all three nutrients. It was found that glucose concentrations below 14.5mM, iron concentrations below 2.44µM and phosphate concentrations below 2mM determined the $OD_{470}$ at which stationary phase was entered (see figures 4.2., 4.3., and 4.4.). Iron is a nutrient which is restricted in its availability in the lung, and so it is important to investigate the concentration below which iron is the limiting nutrient.

**Fig. 4.1.**     Effect of glucose concentration (mM) in $CDM_{12}+$ Fe on batch culture growth of *P. aeruginosa* PAO1 at 37°C. (Curves are representative of ones completed and have been offset for clarity).
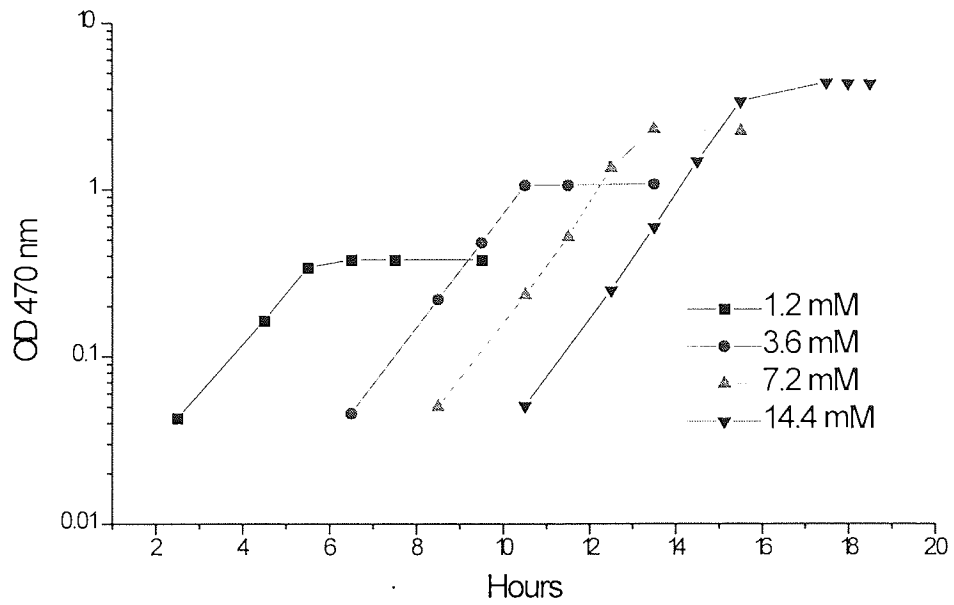


**Fig. 4.2**     Relationship between onset of nonlinear growth of *P. aeruginosa* PAO1, grown in batch culture in $CDM_{12}$, and initial glucose concentration.
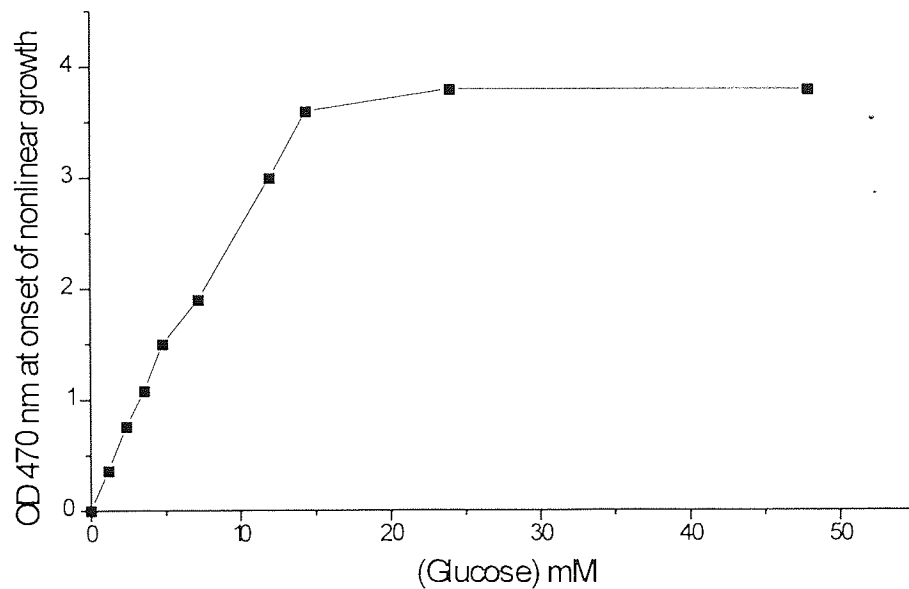
**Fig. 4.3.**     Relationship between onset of nonlinear growth of *P. aeruginosa* PAO1 grown in batch culture in CDM and initial added phosphate concentration.
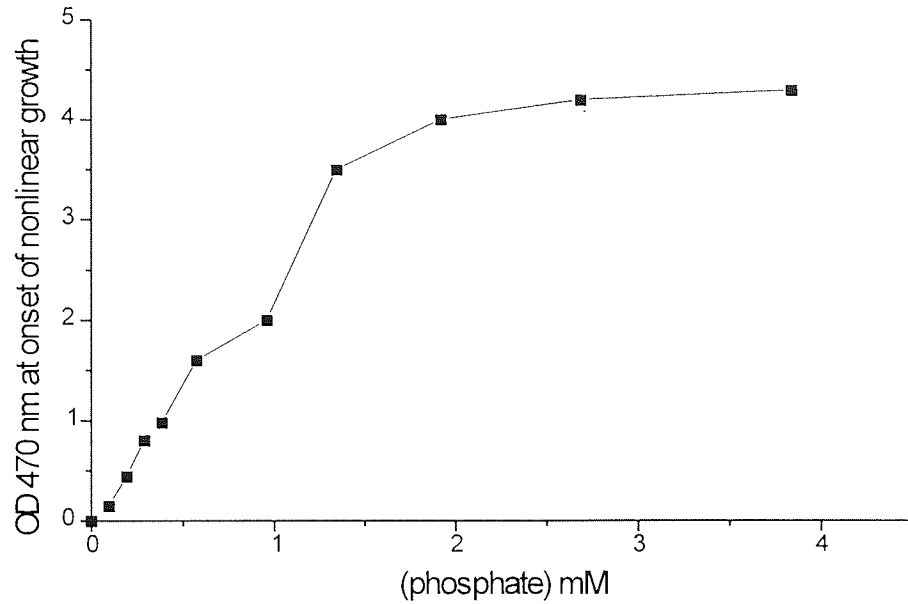


**Fig. 4.4.**     Relationship between onset of nonlinear growth, of *P. aeruginosa* PAO1 grown in batch culture in $CDM_{12}$, and initial added iron concentration.
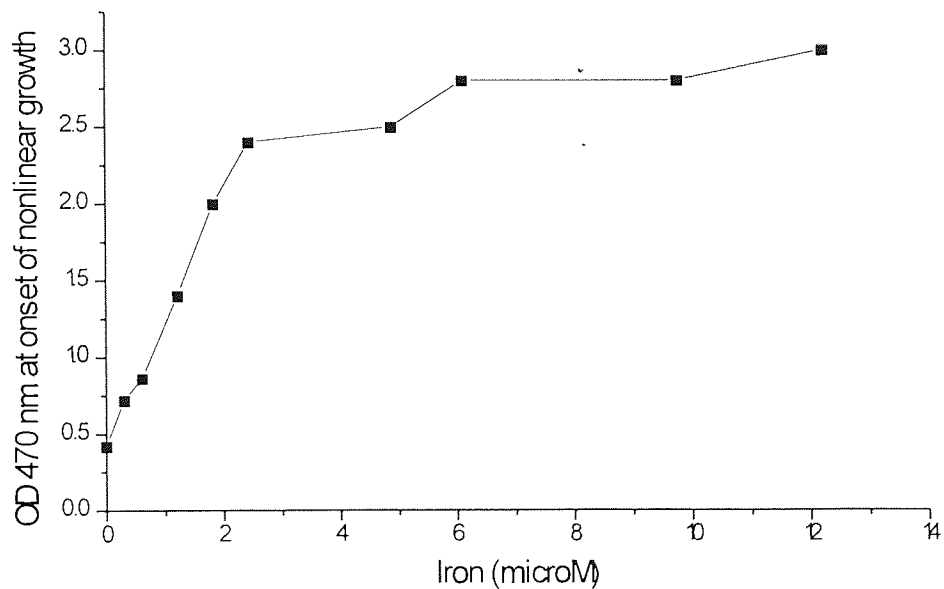
It was known that glassware, chemicals and water contained contaminating iron. However, during the experimental work, it was suspected that the water especially contained varying amounts of iron which resulted in changes in the growth rate of *P. aeruginosa* 6750 and differences in the final $OD_{470}$ of the culture (figure 4.5). When iron was added to the cultures the growth rate was restored and a high final OD was obtained indicating that low amounts of iron in the water did indeed alter the final OD.

**Fig. 4.5.** Effect of contaminating iron in the water and added iron 12.2μM, on the growth of *P. aeruginosa* 6750 in $CDM_{12}$ at 37°C. (Curves have been offset for clarity).
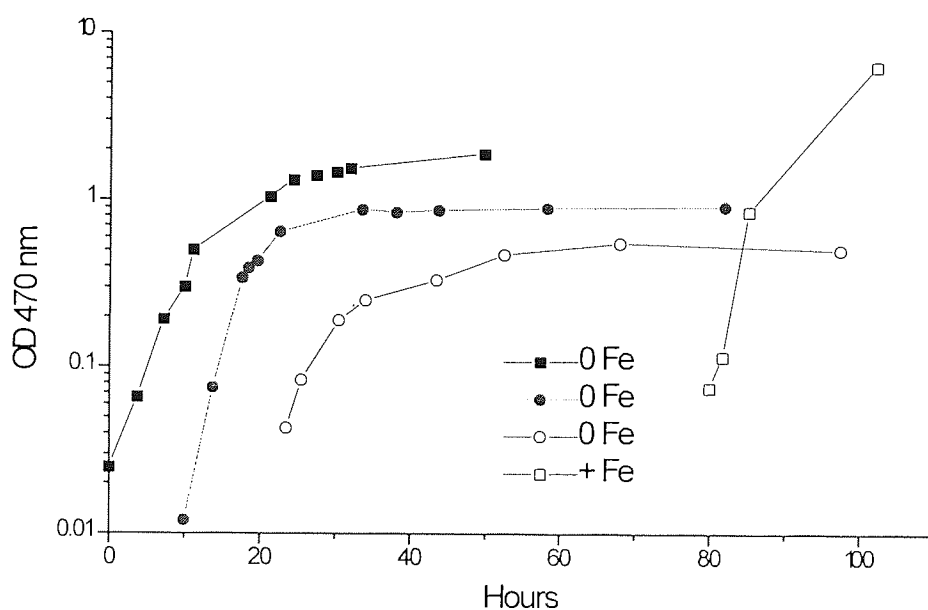
Colony and total counts were made along the growth curve of *P. aeruginosa* 6750 (fig 4.6). The graph shows that there is little difference between the total counts and colony counts during the initial growth of the organism.

**Fig. 4.6.**     Relationship between total counts, colony counts and optical density of *P. aeruginosa* 6750 in iron-depleted $CDM_{12}$ at 37°C, during growth.
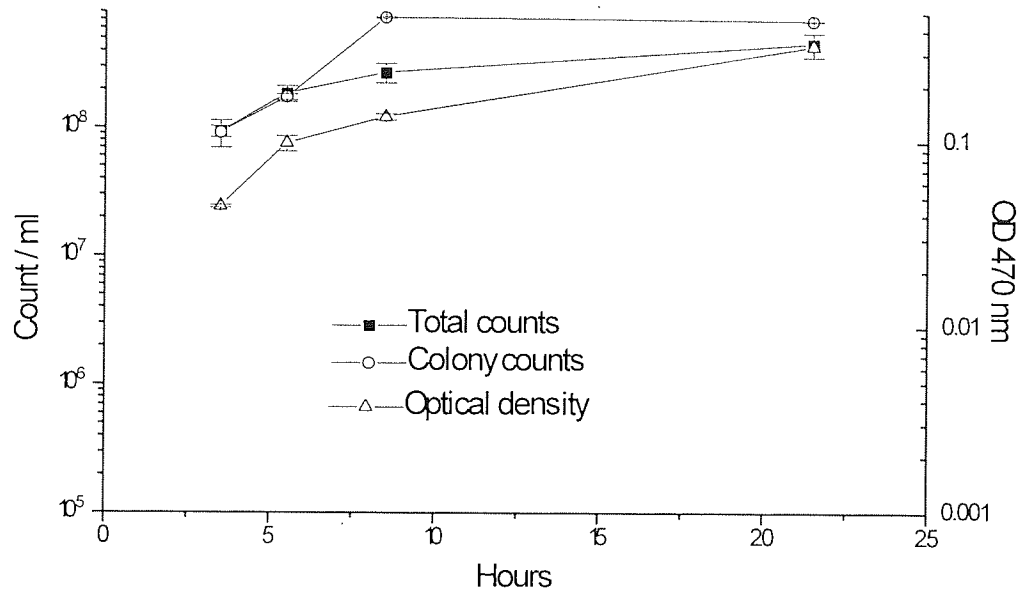
## 4.1.3. Parameters to Measure Survival of *P. aeruginosa* 6750

### 4.1.3.1. Evaluation of Unculturability using Total/Colony Count Ratio

The work investigated the survival of *P. aeruginosa* 6750 under iron, glucose and phosphate depletions maintained at 37ºC (fig 4.7, 4.9, 4.10). Total and colony counts were made over a period of 35 days for the three cultures and a further reading was made after 80 days for the iron-depleted culture.

The results showed that for all three cultures stored at 37ºC culturability decreased, whereas the total counts remained relatively constant. After 35 days for the iron-depleted culture there was a 1.5 log cycle decrease in culturability which progressed to a 2 log cycle decrease after 80 days. For the phosphate and glucose-depleted media, the cultures experienced approximately 1 log cycle decrease in culturability after 35 days.

Further iron depleted cultures were set up and the readings were repeated and expressed on one graph (fig 4.11). The graph showed once again a 1.5 log cycle decrease in culturability after 25 days. This was comparable to figure 4.7 which showed a similar decrease in culturability after 25 days.

Overall, the results show that for the three nutrient depletions, the survival was comparable with each other. It is interesting to note that there was a greater decrease in culturability for the iron-depleted culture compared to the other two nutrient-depleted cultures. Culture with added iron was also analysed in the same way as a control (fig 4.12). The results show that a 1 log cycle decrease in culturability was obtained after 15 days.

When percentage culturability is expressed graphically for the iron depleted culture (fig 4.8), it can be seen that a large decrease occurred initially followed by a tailing off in the curve indicating that a possible steady unculturable state existed for a proportion of the cells which could be a dormant state. The percentage culturability curve of the culture with added iron, on the same graph, shows that there was a slightly larger rate of decrease in culturability during the initial 5 days. After which, the rate of decline was slower for the iron-replete culture compared to the iron-depleted culture. However, readings were only taken up to 15 days. It is not known if more readings were taken in the culture with added iron whether a steadying of the curve would have also been achieved.

### 4.1.3.1.1. Cell Aggregation and Counts

From the categorization of clump sizes, (section 2.11.1.1.2), it was discovered that there was an increase in the number and size of clumps with time. An increase in cell aggregation was seen after the initial 5 days of incubation at 37°C. This may have affected the total counts, however, it was not known to what extent cell aggregation affected the colony counts i.e. Did a colony arise from a single cell or a clump of cells? If the colony arose from a clump of cells, the question of how many culturable cells were present in a clump would have to be asked. Thus it was assumed that the aggregation affected the total and colony counts to the same extent. It was also assumed that each colony arose from either one cell or one group of cells and the total counts were calculated on the basis of classing one clump as a single cell whatever the clump size.

**Fig. 4.7.**   Relationship between total counts, colony counts and optical density of *P. aeruginosa* 6750 with time, grown and maintained in iron-depleted $CDM_{12}$ at 37°C.
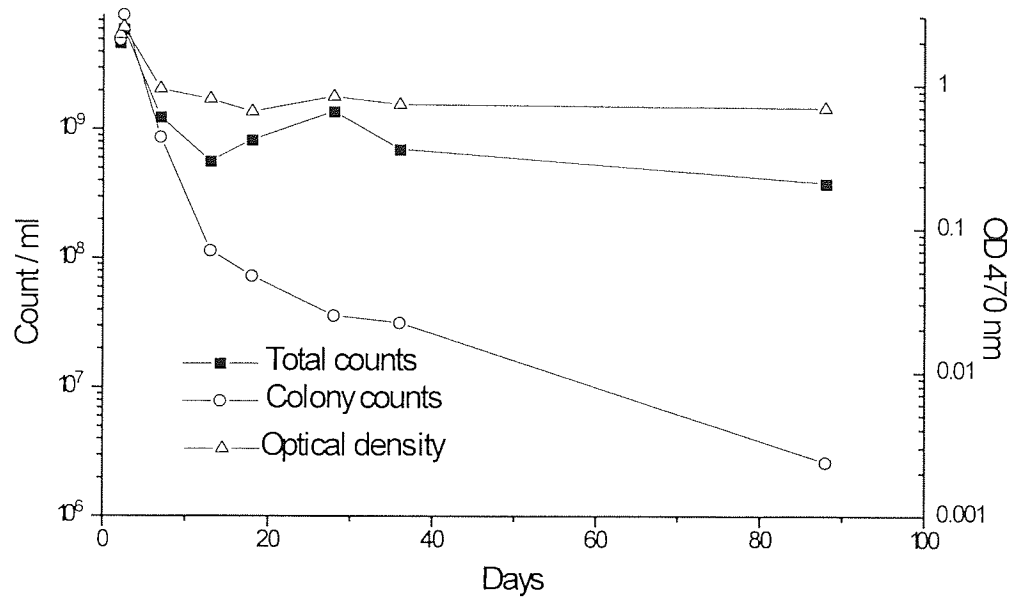


**Fig. 4.8**   Percentage culturability of iron-depleted culture (fig. 4.7) at 37°C and iron-replete culture (fig. 4.12) with time.
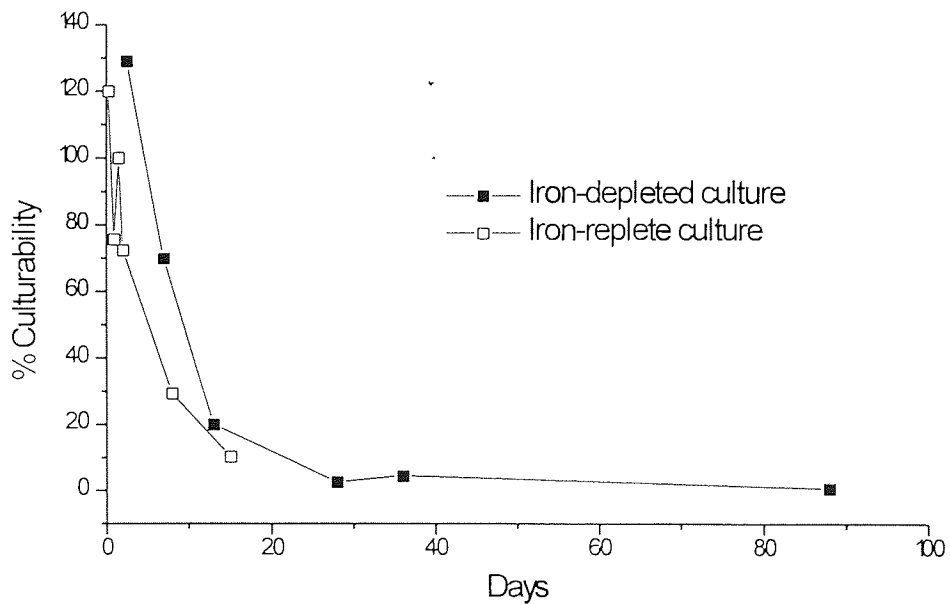
**Fig. 4.9.**      Relationship between total counts, colony counts and optical density of *P. aeruginosa* 6750 with time, grown and maintained in glucose-depleted batch culture at 37°C.
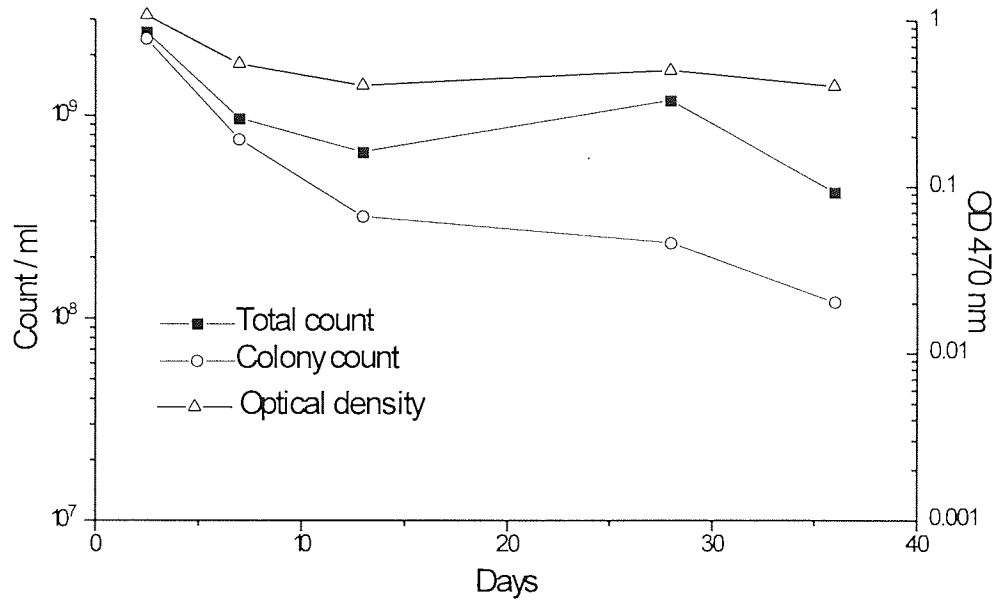


**Fig. 4.10**      Relationship between total counts, colony counts and optical density of *P. aeruginosa* 6750 with time, grown and maintained in phosphate-depleted $CDM_{12}$ at 37°C.
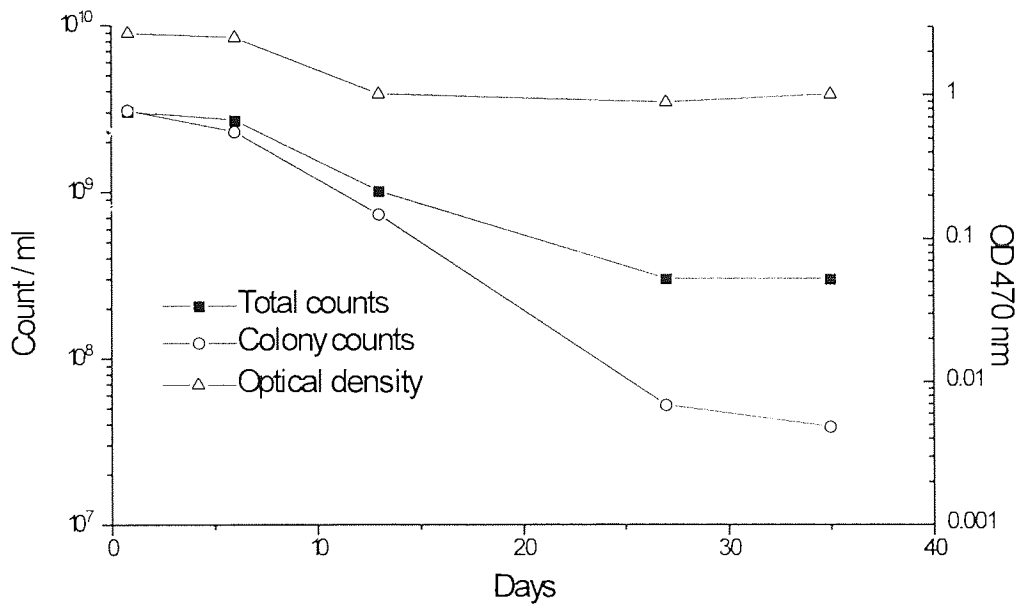
**Fig. 4.11.** Relationship between total counts, colony counts and optical density of several *P. aeruginosa* 6750 batch cultures with time, grown and maintained in iron-depleted $CDM_{12}$ at 37°C.
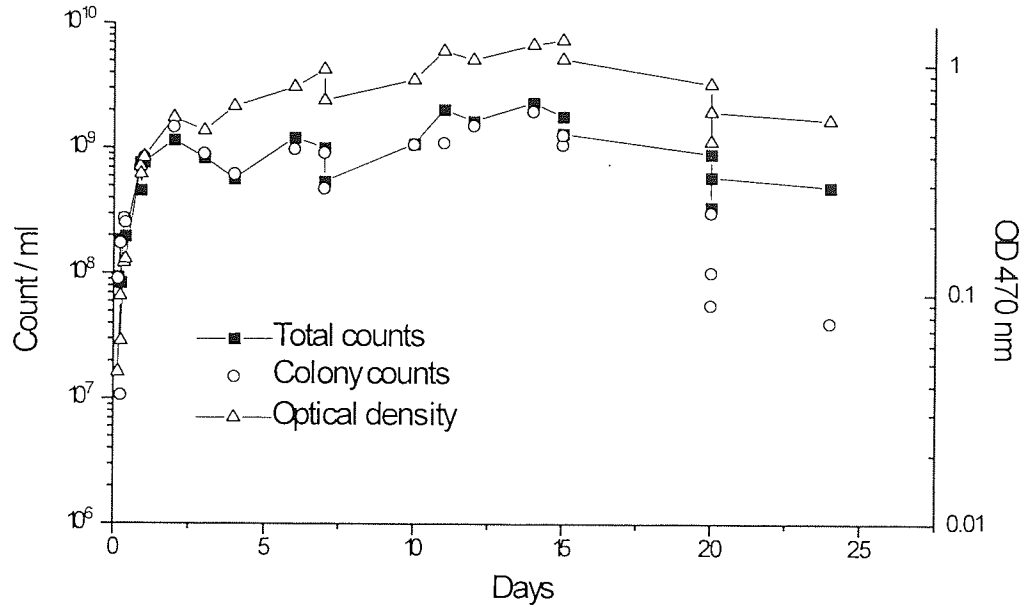


**Fig. 4.12.** Relationship between total counts, colony counts and optical density of *P. aeruginosa* 6750 with time grown and maintained in $CDM_{12}+Fe$ at 37°C.
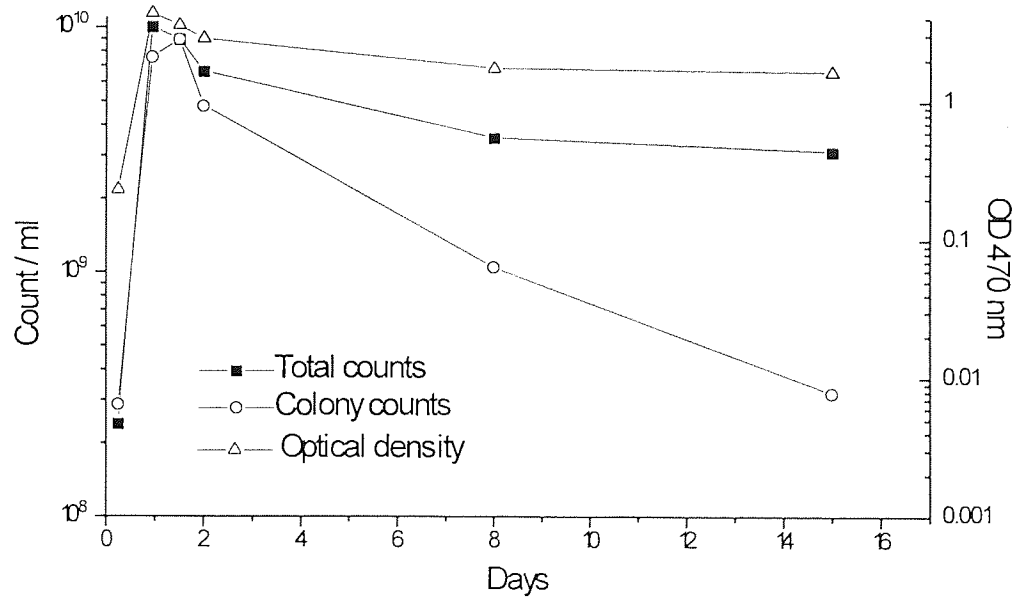
## 4.1.3.2. Discussion

Survival, as measured by the total/colony count ratio, has been measured for several species of bacteria. Bacterial cells lost the ability to form colonies without a significant reduction in total counts. In the above experiments, it has been found that *P. aeruginosa* 6750 did exhibit a loss in culturability but not completely, in the time periods investigated, and so was capable of replication for the duration of the experiments. Additionally, the actual rate of loss of culturability in iron-depleted media decreased with time thereby indicating a stabilisation of the culture (fig. 4.8). The iron-replete culture seemed to follow a similar trend but readings were not taken over the same length of time.

Most other cases have also investigated the loss of culturability induced by exhaustion of glucose or other essential nutrients. In general, studies have discovered that bacteria lose culturability to varying degrees and more recent work has focused on the examination of vitality of these cells and has found a state in which cells are vital but non culturable.

In 1991, Roberto Kolter arrived at the conclusion that many of the cells in starved *E. coli* cultures remained culturable. He described a biphasic death curve in which colony counts dropped by one or two log units in the initial 4 to 5 days after which the remaining viable cells died much slower. In fact it was stated that even after 1 year of incubation, $10^7$ cfus per ml still remained. These findings are similar to those obtained with *P. aeruginosa* 6750 as there was a relatively quick loss of culturability initially followed by a much slower loss.

Experiments with a "wild type" *E. coli* strain, ZK126 have found that the fraction of the population that survives depends on the particular starvation conditions employed (Siegele *et al*, 1993). The results have shown that glucose exhaustion and phosphate exhaustion in MOPS medium did not lead to a significant decrease in the culturability over 10 days. However, ammonium exhaustion did result in a slow decrease in cfus which led to only 14% of the original culture still remaining culturable after 10 days.

In contrast, other workers have found that the *E.coli* strain D10 did not survive phosphate exhaustion (Davis *et al*, 1986) in a medium different to that used by Siegele *et al*. It was concluded that the differences were caused by differences in the medium rather than by strain variations. This was found to be the case for *P. aeruginosa* 6750, as there were differences in the extent of survival depending on the particular nutrient that was depleted.

Experiments with *Pseudomonas* isolates have found that the organism has a large capacity to cope with long-term carbon or multiple nutrient starvation (Givskov *et al*, 1994), which would explain the survival of *P. aeruginosa* 6750 for long periods of time. The studies showed that the cultures of *Pseudomonas putida* were almost fully viable during 1 month of carbon, nitrogen and multiple-nutrient starvation. However, *P. putida* KT2442 did not survive well under conditions of sulphate and phosphate starvation. In the case of *Pseudomonas fluorescens*, within the first 10 to 12 days of nitrogen starvation, the culturability of the organism decreased to 5 to 10% of the total cell number (Evdokimova *et al*, 1994). It was suggested that the cells that were unculturable were not dead, however, but under starvation stress.

Investigations with *Micrococcus luteus* stored in lactate minimal medium for approximately 80 days showed that culturability decreased during this time to less than 0.05% (Kaprelyants and Kell, 1993b). Other organisms, the most notable being V*ibrio vulnificus*, have also produced an unculturable state after a period of time and in response to a temperature downshift (Oliver, 1993). In these instances also, although the culturability and survival of the organisms have decreased in terms of colony counts, the survival was in fact found to be maintained in the form of a dormant state that was unculturable but nevertheless capable of metabolic activity.

#### 4.1.3.3. Evaporation of Cultures

During prolonged storage of cultures at 37°C, an investigation was carried out to examine the extent of evaporation of cultures. Evaporation was monitored by measuring the weight of the cultures on a top pan balance at intervals of two to three days over a period of thirty days. It was found that 40ml of culture lost 2.8% of its weight and 20ml of culture lost 4.1% of its weight over a period of thirty days.

Since $OD_{470}$ did not increase significantly over the test period, the effect of evaporation was thought to have minimal effect on the experiments.

## 4.1.3.4. Direct Viable Count (DVC)

Preliminary experiments, adapted from the method of Singh *et al* (1990, section 2.11.6.2.) were carried out to ensure that a specific concentration of nalidixic acid would inhibit cell division such that colony counts would decrease after an appropriate incubation time and that total counts would remain the same. Figure 4.13 shows that the concentration of nalidixic acid that caused a decrease in colony counts was 300µg/ml.

A further experiment was carried out to investigate if the total counts remained the same (fig. 4.14). The graph shows that after an incubation of 5 hours with 300µg/ml of nalidixic acid, the colony counts of early stationary phase *P. aeruginosa* 6750 decreased but the total counts remained the same. DVC made at the same time shows that number of elongated cells correlated with the total count number in these circumstances. Subsequent experiments were made using 300µg/ml nalidixic acid with a 5 hour incubation time.

*P. aeruginosa* 6750 grown at 37°C in iron-limited $CDM_{12}$ in triplicate and maintained at that temperature for approximately 23 days was subjected to the DVC process (fig. 4.15). Results were expressed as percentage of elongated cells because absolute numbers for total counts and DVC counts (obtained using DVC method) were found to be less accurate as the cells became older. The results show that as with the graph of percentage culturability using colony counts (fig 4.8) the percentage of elongated cells decreased with time. However, the culturability of the cells after the DVC process did not decrease at the same rate as the previous graph : there was no initial decrease followed by a "tailing off", as in the previous curve. The decrease seen using the DVC method seemed to follow a steady decrease. It is not known if this meant that it was unlikely that a dormant population existed or that the DVC method was not accurate enough to allow any definite conclusions.

**Fig. 4.13.** Changes in colony counts which show growth of *P. aeruginosa* 6750 in PBS with yeast extract with various concentrations of nalidixic acid at 37°C.
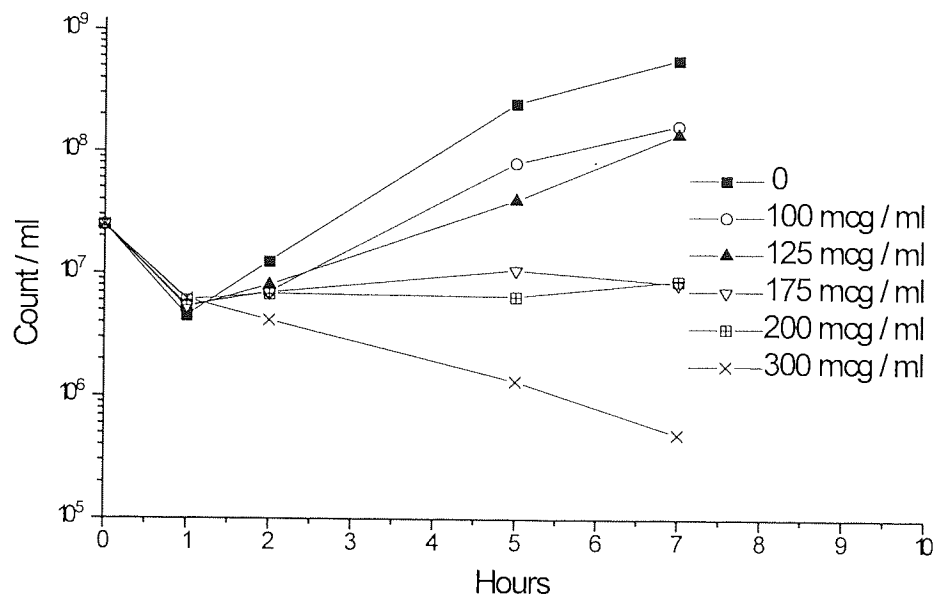


**Fig. 4.14.** Changes in total counts, colony counts and direct viable counts of *P. aeruginosa* 6750 in PBS and yeast extract after a 5 hr incubation with 300mcg/ml nalidixic acid at 37°C.
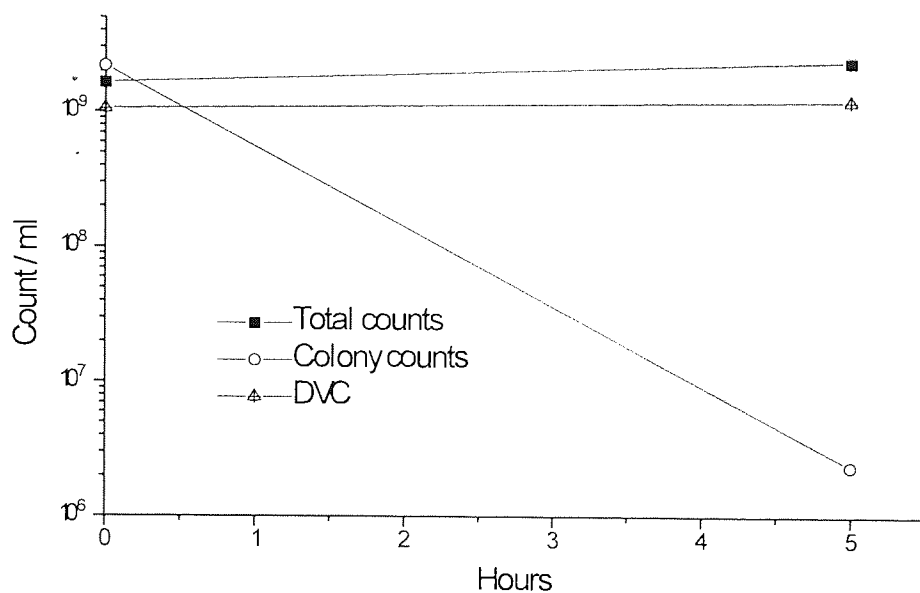
**Fig. 4.15.** DVC changes during the course of incubation of *P. aeruginosa* 6750 in iron-depleted $CDM_{12}$ at 37°C, expressed as percentage of elongated cells.
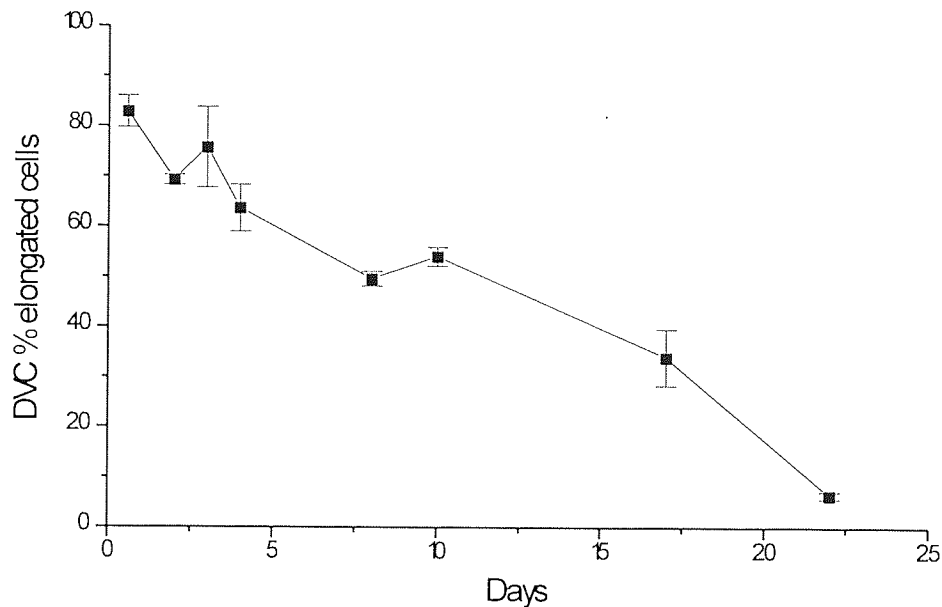


#### 4.1.3.4.1. Discussion

The basis of the DVC method was the finding that although high concentrations of nalidixic acid interfere with several metabolic activities of Gram-negative bacteria, a moderate concentration specifically inhibited bacterial DNA synthesis so that other metabolic activities of the cell would not be affected (Goss *et al*, 1964, Kantor and Deering, 1968). The DVC method used in these studies was adapted from that of Kogure *et al* (1978), so that a higher concentration of nalidixic acid could inhibit DNA synthesis of *P. aeruginosa* 6750 without interfering with other metabolic activities. In this way, extensive filamentation would be seen at appropriate concentrations and ovoid cells which are produced at high concentrations (Diver and Wise, 1986) would be avoided.

Kogure *et al* (1984) proposed the use of piromidic acid, pipemidic acid and nalidixic acid simultaneously in order to address the problem of bacteria resistant to nalidixic acid, but this approach has not been used widely. Additionally, it is known that growth requirements and substrate preferences of bacteria may affect direct viable counts of substrate responsive cells, so it is important to use appropriate concentrations of substrate (Peele and Colwell, 1981).

In these experiments, percentage of elongated cells which represented the cells with metabolic activity, were expressed graphically and compared with the graph of percentage culturability. In general, the percentage elongated cells was higher than percentage culturable cells over a period of 25 days. These results are comparable to those obtained with *E. coli* and *Salmonella enteritidis* (Roszak and Colwell, 1987). Colony counts were found to decrease rapidly but direct viable counts remained relatively stable. In the experiments with *P. aeruginosa* 6750 the percentage elongated cells did not remain stable. However, the fact that the DVC values were higher than the culturable cells at some time-points, could be an indication that a proportion of the unculturable cells possessed some metabolic activity.

Recent studies have adapted this procedure by the use of other antibiotics with Gram-positive organisms and similar results were also found (Servis *et al*, 1995). Direct viable counts of the Gram-positive bacteria were larger than the colony counts for all the bacteria tested. It was therefore concluded that the direct viable count may give more realistic estimate of vitality than colony counts.

## 4.1.3.5. Effect of Temperature Downshift

*P. aeruginosa* 6750 grown to stationary phase at 37°C under different nutrient depletions, was then placed in a cold room for different time periods. Figure 4.16 shows that phosphate-depleted cultures seemed to survive better at 4°C compared to that at 37°C (fig 4.10). Glucose-depleted cultures (fig. 4.17) had a comparable survival to the culture stored at 37°C (fig. 4.9). After 80 days storage, the iron depleted culture (fig. 4.18) experienced a decrease in survival of about 5 log cycles, whereas the culture stored at 37°C (fig. 4.7) exhibited a decrease in survival of 3.5 log cycles after approximately 88 days storage. However, the culture was stored at 4°C for an extended period of time (up to 95 days), and it was found that colony ·counts decreased dramatically until it became less than 1 cfu/ml.

A control culture initially containing all the nutrients was allowed to reach stationary phase, and was then subjected to a temperature downshift (fig. 4.19). The graph shows that after approximately 5 days, there was a decrease in colony counts of nearly 1 log cycle. The result suggests that the relatively quick appearance of unculturable cells could be due to the fact that this culture was allowed to be depleted totally of glucose, whereas the phosphate and iron depleted cultures were not depleted in this way. However, the glucose depleted culture was depleted of the nutrient before it was transferred to the cold room and it did not show the quick appearance of unculturable cells.

**Fig. 4.16.** Changes in total counts, colony counts and optical density of *P. aeruginosa* 6750 grown to stationary phase in phosphate-depleted batch culture at 37°C, and then maintained thereafter at 4°C.
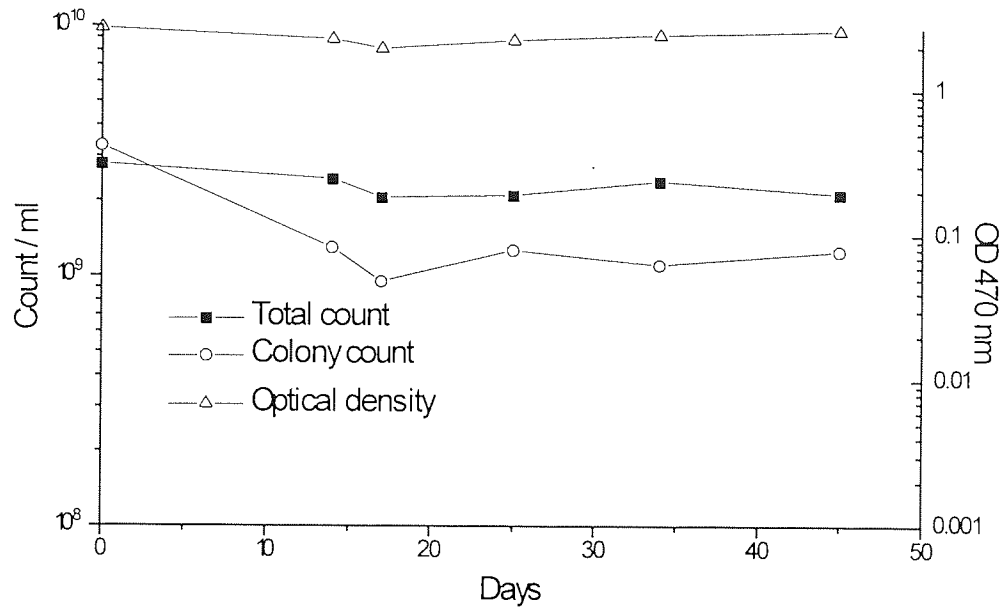


**Fig. 4.17.** Changes in total counts, colony counts and optical density of *P. aeruginosa* 6750 grown to stationary phase in glucose-depleted batch culture at 37°C, and then maintained thereafter at 4°C.
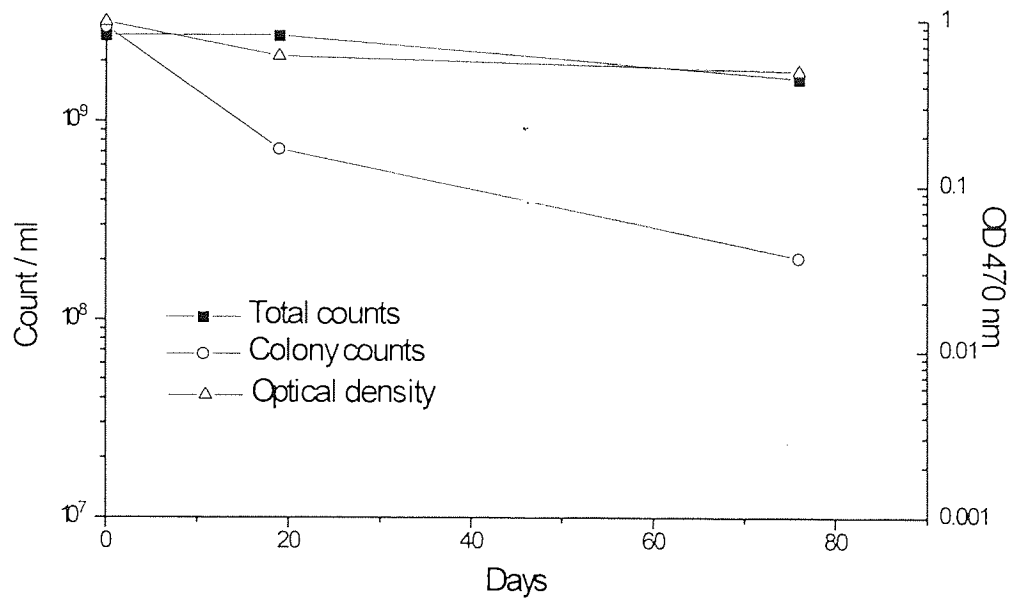
**Fig. 4.18.** Changes in total counts, colony counts and optical density of *P. aeruginosa* 6750 grown to stationary phase in iron-depleted batch culture at 37°C, and then maintained thereafter at 4°C.
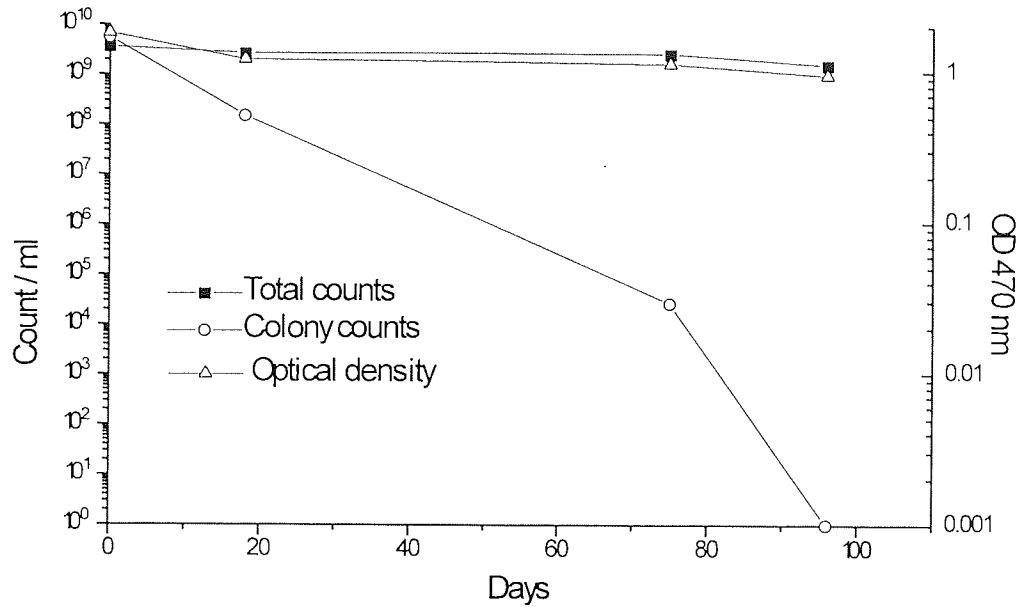


**Fig. 4.19.** Changes in total counts, colony counts and optical density of *P. aeruginosa* 6750 grown to stationary phase in $CDM_{12}$+Fe batch culture at 37°C, and then maintained thereafter at 4°C.
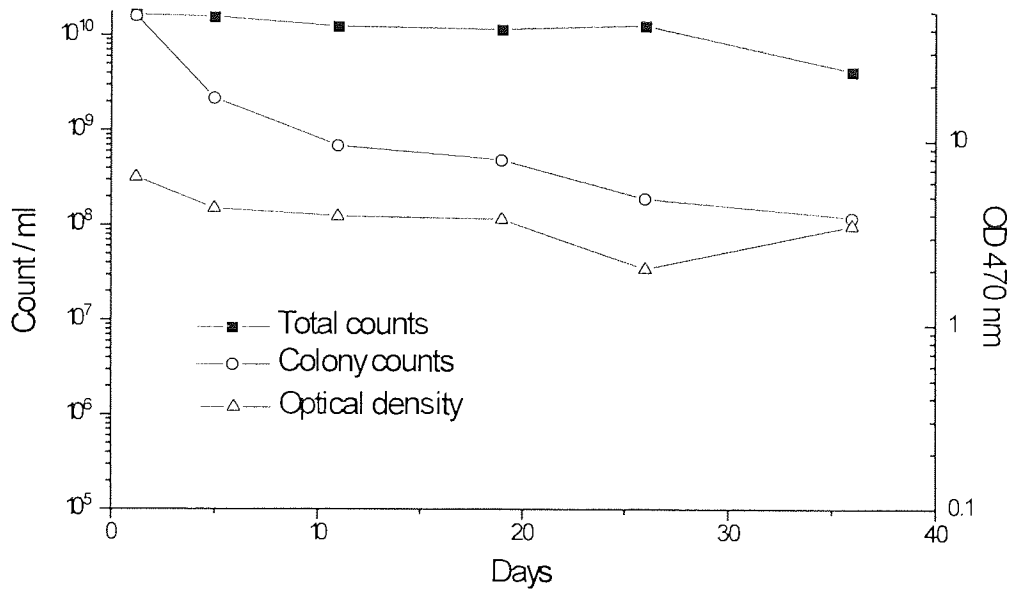
## 4.1.3.5.1. Discussion

The experiments were carried out to investigate the effect of temperature downshift on culturability of *P. aeruginosa* 6750 as was performed on *Vibrio vulnificus* (Oliver *et al*, 1991). According to Oliver *et al*, *V. vulnificus* was found to enter a viable but nonculturable state when it was placed at 5°C, but not at room temperature.

In the case of the phosphate-depleted cultures, and glucose-depleted cultures of *P. aeruginosa* 6750, they were found to survive better at 4°C compared with at 37°C. However, the iron-depleted culturable became unculturable after approximately 88 days which was similar to the *V. vulnificus*. From an initial inoculum of $10^7$ cfu/ml, stationary phase cells of *V. vulnificus* became nonculturable within 40 days at 5°C (Wolf and Oliver, 1992).

Other experimenters who have demonstrated that temperature downshift reduced culturability included Xu *et al* (1982). Studies were performed on *V. cholerae*. The organism was incubated in nutrient free microcosms at 10 or 25°C which resulted in little decrease in culturability. However, when cells were incubated at 4 to 6°C, a rapid decrease in culturability was seen.

## 4.1.3.6. Resuscitation

Iron depleted cultures stored at 4°C after reaching stationary phase were subjected to a resuscitation experiment. When the colony count was ca. $10^4$ cells/ml, after 75 days, the culture was removed from the cold room and subjected to a temperature upshift to 37°C. It was found that after an initial lag period, colony counts approached the original counts with the total count remaining relatively stable (fig 4.20). This suggested a possible dormant state of a proportion of cells kept at 4°C. When the experiment was repeated, after 95 days when the cells were unculturable, resuscitation did not take place. It was considered that perhaps the original increase in colony counts was not resuscitation, but in fact growth of the $10^4$/ml cells.

A control experiment investigated the supernatant of the culture to investigate if it could support growth. A sample of the supernatant from the culture kept at 4°C was filter sterilised with a sterile 0.2μm filter (Nalgene), pre-washed with sterile $CDM_{12}$. The supernatant was inoculated with a colony of *P. aeruginosa* 6750 grown overnight on a nutrient agar plate. A second culture of iron-depleted $CDM_{12}$ was also inoculated with the organism in the same way. Growth was monitored at 37°C in shake flasks using $OD_{470}$. It was found that both cultures supported growth to ca. $10^9$ cells/ml. This implied that the original increase in viable counts could have been due to growth of the few culturable cells as the supernatant supported growth to the same $OD_{470}$ as iron-depleted $CDM_{12}$.

**Fig. 4.20.**    Resuscitation of *P. aeruginosa* 6750 in iron-depleted $CDM_{12}$ at 37°C.
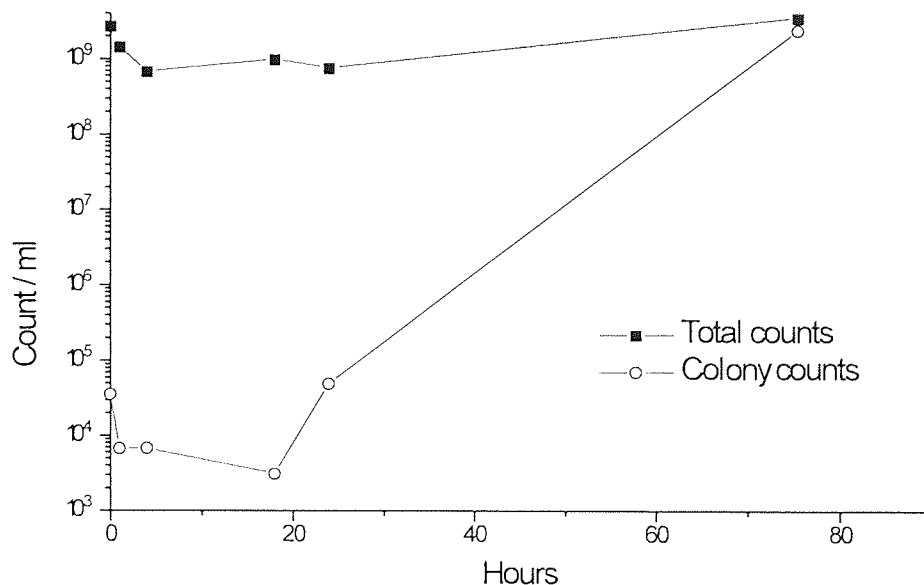
## 4.1.3.6.1. Discussion

Nilsson *et al* (1991) were the first experimenters to report the resuscitation of bacteria from a nonculturable state without the addition of nutrients. *V. vulnificus* became nonculturable after incubation for 27 days at 5ºC. The nonculturable cells were then subjected to a temperature upshift by placing the cells at room temperature. It was found that the original bacterial cell numbers were detectable by colony counts after 3 days. No increase was observed in the total counts. Recent findings have also confirmed that *V. vulnificus* can enter the nonculturable state when placed in natural estuarine waters during the winter months. Resuscitation was also shown when cells in the unculturable state were placed in the same waters in the warmer months (Oliver *et al*, 1995). In this example, the cells took around 24 hours to reach the full culturable state.

The results are similar to those obtained with *P. aeruginosa* 6750 as no nutrients were added but the cells that had a reduced culturability were subjected to a temperature upshift by placing the cells at 37ºC. The resuscitation process in this experiment took place over a period of 2.5 days, and there was little change in the total counts. However, as the cells of *P. aeruginosa* were not completely unculturable there was always a possibility that growth of the $10^4$ cells/ml had taken place. It is suggested that the culture could have been diluted sufficiently so that the number of culturable cells/ml was well below the $10^4$ cells/ml. If the diluted culture were then subjected to a temperature upshift, and if an increase in colony counts had taken place, then it could be assumed that the growth had come from the original non-growing cells.

Other researchers too have encountered problems regarding temperature-induced recovery of bacteria (Ravel *et al*, 1995). *Vibrio cholerae* cells were incubated at 4ºC in nutrient-limited artificial seawater microcosm after which colony counts decreased rapidly to less than 2 cfu/ml in about 23 days. When samples of the microcosms were shifted to 30ºC the colony counts increased to around the original count. Experiments were then performed to determine whether the increase in colony counts was due to resuscitation of the unculturable cells or if it was a result of growth of the original remaining culturable cells. The unculturable cells were diluted 10 and 100 fold and were subjected to the temperature upshift. Since the undiluted unculturable cells recovered to about 2.2 x $10^5$ cfu/ml, it would have been expected that if resuscitation of nonculturable cells had taken place the resultant number of culturable cells would have been 1/10 and 1/100 th of the undiluted samples respectively. In the cultures that were diluted 100 fold, in which there was less than 1 cfu/ml, there was no increase in culturability after the resuscitation process. It was concluded that recovery of culturable cells after temperature upshifts resulted from growth and not resuscitation. Experiments

of this kind would be useful in order to prove or disprove that *P. aeruginosa* 6750 had undergone resuscitation.

## 4.1.3.7. Influence of Growth Phase on Culturability

Experiments to investigate the effect of growth phase on subsequent longevity were performed in two ways. Cells were grown at 37°C to early exponential phase in CDM depleted in one nutrient under investigation and were subjected to a temperature downshift. Secondly, cells were grown as before but this time they were centrifuged and resuspended in media minus the nutrient under investigation. In order to examine the centrifugation effect, cultures were grown in $CDM_{12}+Fe$ and one set were placed directly at 4°C and the other set were centrifuged and resuspended in $CDM_{12}+Fe$ (fig. 4.21, 4.22). The results indicate that the centrifugation effect itself did not induce the production of unculturable cells.

Experiments were performed for cultures low in glucose, phosphate (data not shown) and iron (fig. 4.23, 4.24). In general, there was little difference between the graphs of the cultures centrifuged and resuspended (fig. 4.24) and those that were placed directly at 4°C (fig. 4.23). Results show that overall, exponentially growing cells seemed to survive better over a short period of time (20 days) than the cultures that were allowed to grow to stationary phase before they were subjected to the temperature downshift. This may be due to the fact that the cultures were not completely starved before the change in the temperature conditions. However, the centrifuged and resuspended cells were lacking in the specific nutrients.

It should be pointed out that reduced culturability was in fact observed after the cultures were stored for longer periods (up to 65 days). Therefore, it could be inferred that exponentially growing cultures did not necessarily survive better at 4°C than cultures in the stationary phase but that they took a longer time to form unculturable cells.

**Fig. 4.21.**   Changes in total counts, colony counts and optical density of exponential *P. aeruginosa* 6750 in $CDM_{12}$+Fe and maintained at 4°C without centrifugation.
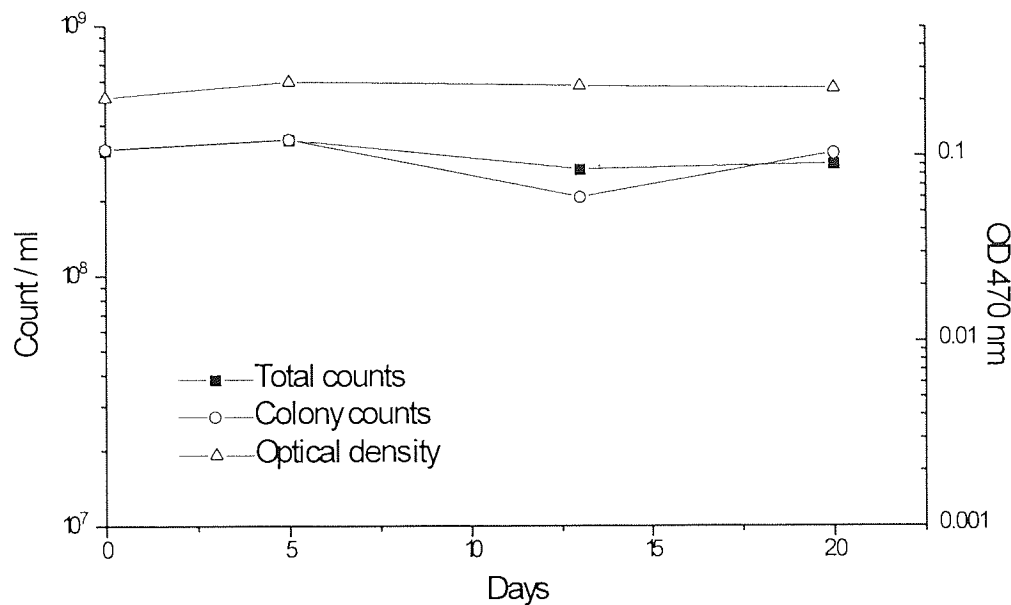


**Fig. 4.22.**   Changes in total counts, colony counts and optical density of exponential *P. aeruginosa* 6750 in $CDM_{12}$+Fe, subjected to centrifugation and maintained at 4°C.
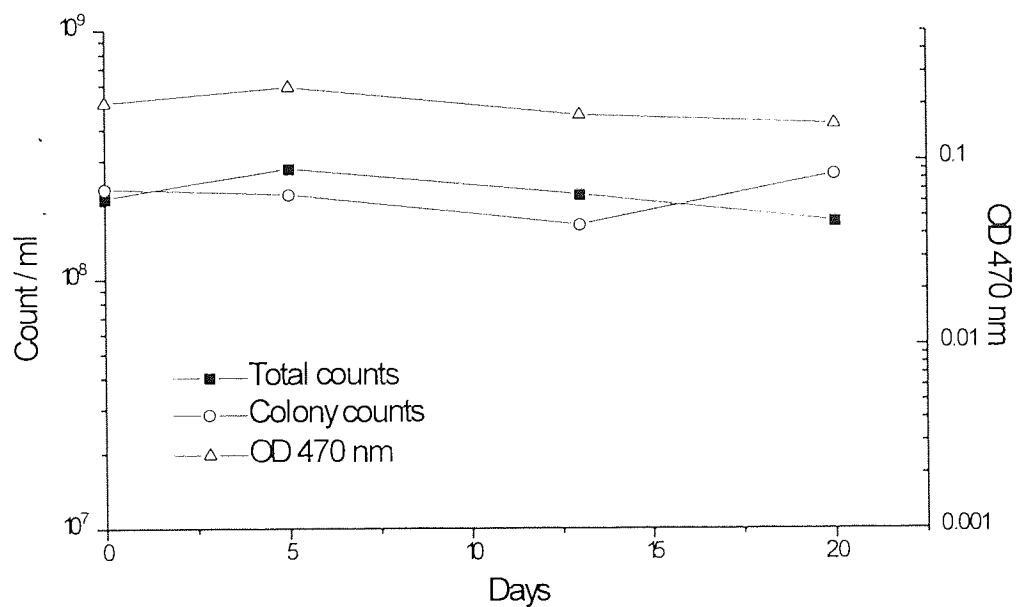
**Fig. 4.23.**     Changes in total counts, colony counts and optical density of exponential *P. aeruginosa* 6750 in iron-depleted $CDM_{12}$ and maintained at 4°C without centrifugation.
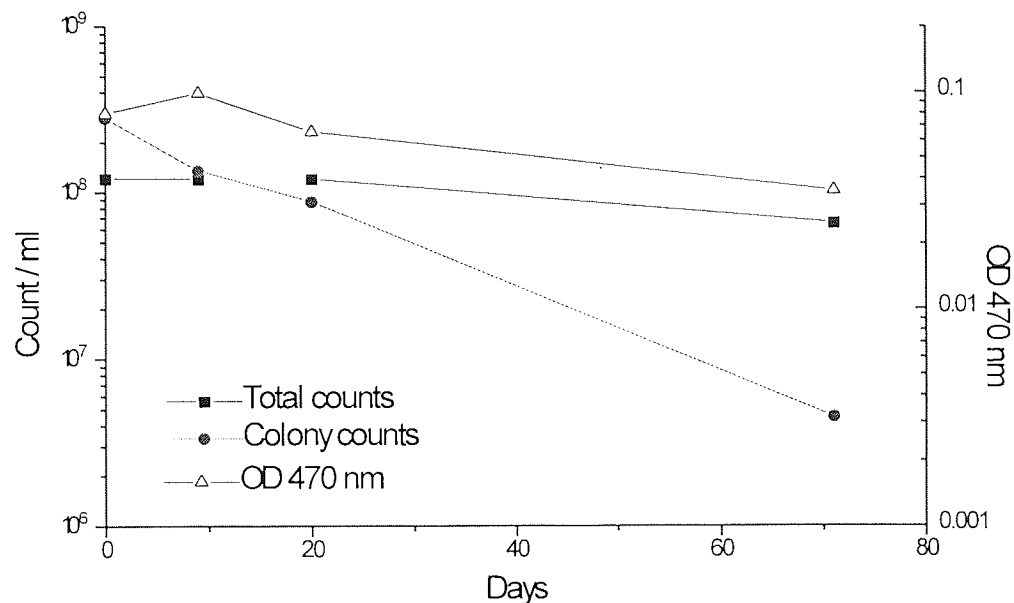


**Fig. 4.24.**     Changes in total counts, colony counts and optical density of exponential *P. aeruginosa* 6750 in iron-depleted $CDM_{12}$, subjected to centrifugation and maintained at 4°C.



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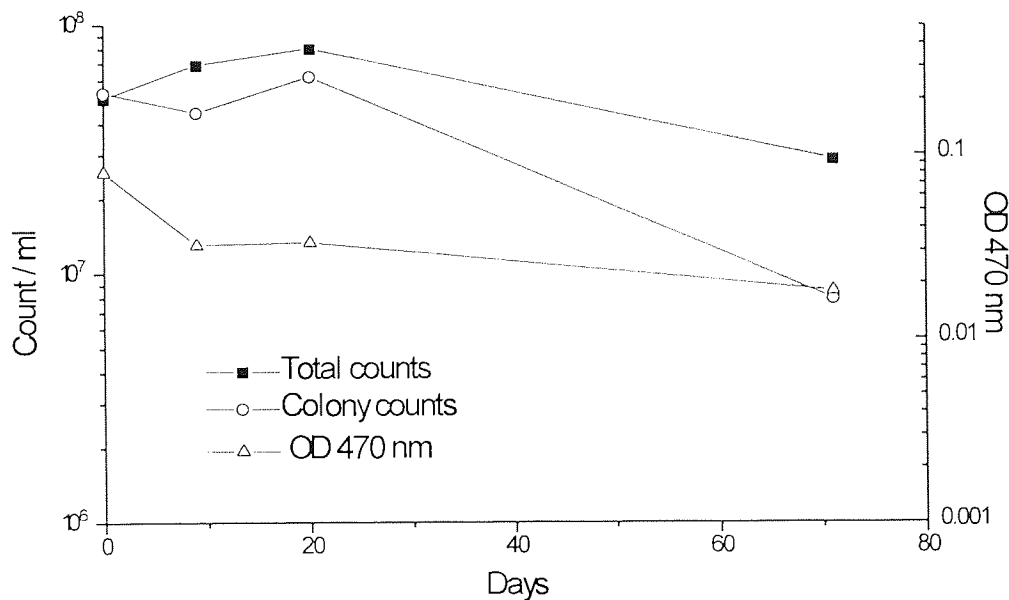
## 4.1.3.7.1. Discussion

The experiments that investigated the influence of growth phase on the culturability of *P. aeruginosa* 6750 at 4ºC were based on those performed on *V. vulnificus* (Oliver *et al*, 1991). In the experiments with *V. vulnificus*, several factors were examined which might influence the nonculturable response of the organism, including incubation temperature, physiological age of the inoculum, whether the cells were washed prior to incubation, the salt content of the plating medium and whether a nitrogen and phosphorus source was present or absent. Of all the factors tested, it was only the physiological age of the inoculum cells (exponential or stationary phase cells) that was found to have a significant effect on the time needed for the cells to enter the nonculturable state.

The results showed that *V. vulnificus* cells taken from the stationary phase required twice as many days to become nonculturable at 5ºC than did exponential phase cells. It was suggested that the effect was related to the production of stationary phase-induced stress proteins.

The results with *P. aeruginosa* 6750 did not show that the stationary phase cells required a longer time to become unculturable than the exponential phase cells. In fact the opposite result was seen with this organism. Unculturable cells were seen in the exponential phase cells later compared to the stationary phase cells.

## 4.1.3.8. Measurement of Size

### 4.1.3.8.1. R.T.G. Correlator

Readings were taken of a culture of *P. aeruginosa* 6750 incubated at 37°C in iron-depleted $CDM_{12}$ over a period of 25 days. One sample was taken each time a reading was to be made and it was divided into two subsamples. Each sample was analysed in triplicate and the mean value of the readings was recorded graphically (fig 4.25). The graph shows the mean value to be initially approximately 0.9μm. This value increased to 1μm between the times of 5 and 17 days, after which a larger increase was observed up to a final value of 1.6μm.

It was discovered that large clumps analysed by the machine displaced the mean value to a much larger value. Fewer clumps were observed earlier in the incubation (section 4.1.3.1.1.). The apparent mean size reflected this as the increase in cell aggregation caused a marked increase in size after 17 days. This method was therefore considered to be unsuitable to measure *P.aeruginosa* 6750 during prolonged storage in stationary phase at 37°C.

**Fig. 4.25.**    Apparent mean size, as measured by the RTG correlator, of
*P. aeruginosa* 6750 grown in iron-depleted $CDM_{12}$ at 37°C.
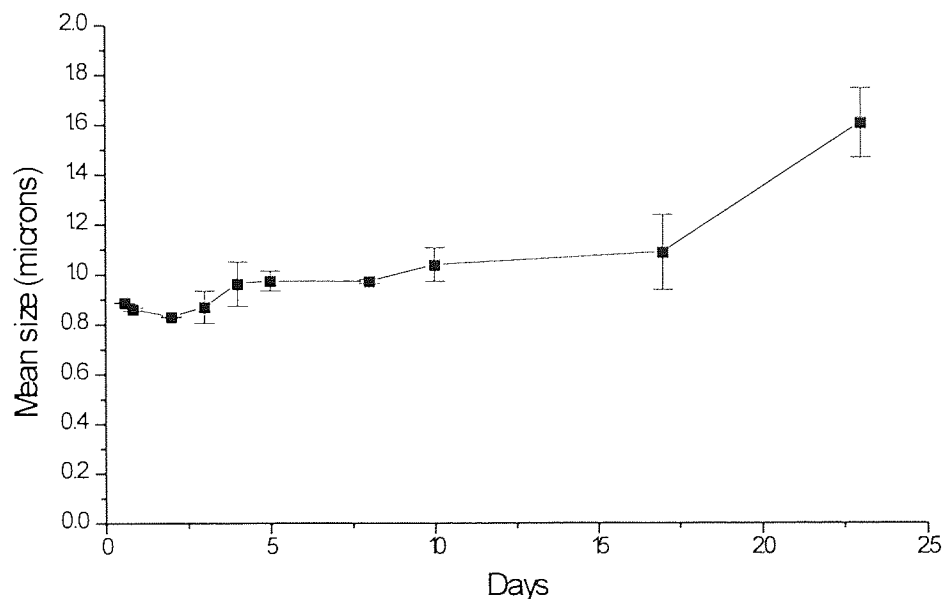
## 4.1.3.8.2. Photographic Method

Figure 4.26 is a graph of size along the growth curve combining measurements of two sets of data compiled on two occasions. The graph shows that exponentially growing cells of *P. aeruginosa* 6750 grown in iron-depleted $CDM_{12}$ at 37°C are approximately twice as large as cells in the stationary phase. Bacterial cells became smaller over a period of two days after which time they remained relatively the same size of approximately 0.75μm, which is a value similar to that obtained with the R.T.G. correlator (fig.4.25). This meant that once cells entered the stationary phase, after approximately one day, the cells became smaller.

It should be noted, however, that there was also an increase in the amount of cell aggregation with time. Cells smaller than 0.7μm were also observed microscopically with the aid of a graticule. However, cells in clumps could not be measured accurately using this method and so their sizes were not recorded.

After about one day, the width was approximately 0.75μm and was subsequently found not to alter significantly with time.

**Fig. 4.26.** Mean size of *P. aeruginosa* 6750 cells during growth in iron-depleted $CDM_{12}$ at 37°C using the photographic method.
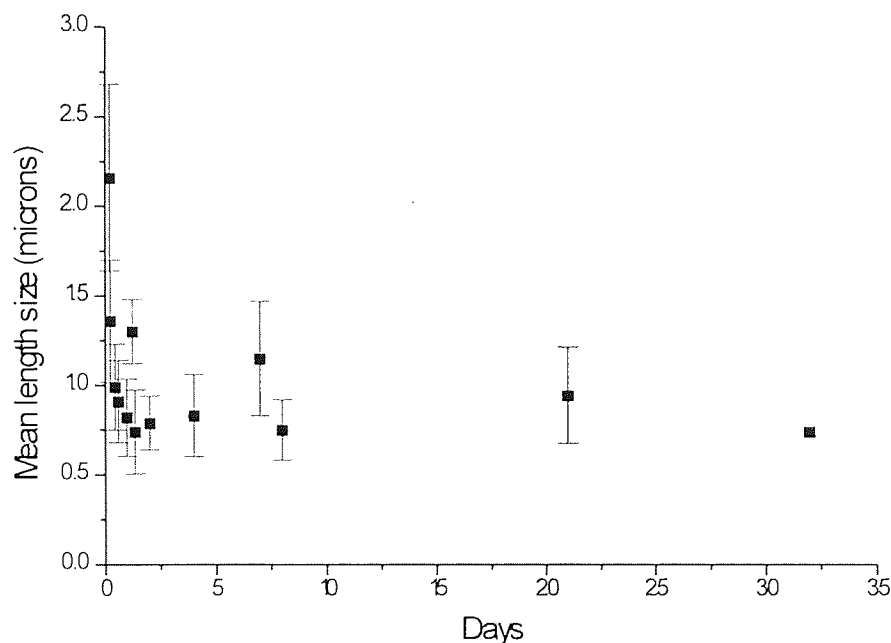
## 4.1.3.8.3. Discussion

The results obtained using the RTG correlator were considered not to reflect the true changes in cell size. However, the values obtained with the photographic method were thought to be more accurate as they were obtained by direct microscopic observation.

Decreases in cell size and the formation of ultramicrobacteria is a phenomena that has been widely reported in marine studies. One of the first studies using laboratory culture that produced small cells as a result of starvation was performed using a marine psychrophilic bacterium, strain ANT-300 (Novitsky and Morita, 1976, Novitsky and Morita, 1977). It was stated that the appearance of these small cells was a stress response (Novitsky and Morita, 1978). Others have also documented the importance of the "rounding up" phenomena in which cells decrease in size with an accompanying decrease in cell volume (Guelin *et al*, 1979). In the marine environment, cells were found to undergo reductive divisions in response to carbon and nitrogen starvation. In this way, it was emphasised that as well as a decrease in cell size, there was also a concomitant increase in the surface to volume ratio (Novitsky and Morita, 1976).

It was stressed that there was a need for minimal nutrient concentration and a prolonged incubation time in order to recover ultramicrobacteria (Tabor and Colwell, 1981). These factors were found to be important in the studies with *P. aeruginosa* 6750 as the cells were grown in minimal media. Additionally, cells became smaller once they had entered the stationary phase. However, a further decrease in cell size was not seen after prolonged incubation, with this method, as it was not found to be sensitive enough. Cells smaller than 0.75μm were observed microscopically but often they were associated with other cells in aggregates.

Studies with *Vibrio* spp. have demonstrated the changes of cell size and the phenomena of "rounding up" (Felter *et al*, 1969, Kennedy *et al*, 1970). More recent investigations have described a process by which a rapid reduction of cell volume is observed initially as a result of reductive divisions, followed by a slower decrease in size which could be caused by degradation of endogenous cell constituents (Nyström *et al*, 1990).

Similar findings have been shown with *Pseudomonas fluorescens* (Jørgensen *et al*, 1994). Morphological changes in starved cultures were examined microscopically and volumes of cells were measured from enlarged photographs projected on a screen. The results showed a 50-70% reduction in average size during the first day of starvation which was accompanied by a change in shape from rods to cocci. Size continued to decrease at a reduced rate during prolonged starvation.

In the studies performed with *P. aeruginosa* 6750, cultures were iron-depleted which meant that carbon starvation would occur later, however, similar results to those of Jørgensen *et al* (1994) were obtained. An initial decrease of approximately 70 % reduction took place in the first 2.5 days. As mentioned previously, a further decrease was not observed using this method.

Other investigators have used *Pseudomonas putida* and have observed changes in cell shape as well as reductions in cell size during carbon starvation (Givskov *et al*, 1994). In these experiments, cell size was measured using the forward light scatter of a flow cytometer and microscopic observations. The results showed that the change in cell size took place after 1 hr of starvation and after 5 hr all the cells in the culture were small. The results obtained with *P. aeruginosa* 6750 did not appear as quickly but some changes did take place well within the onset of stationary phase.

## 4.1.3.9. Estimation of Cell Volume

An estimation of cell volume was made using formula:

$$v = ( d^2 \pi /4 ) ( l - d) + \pi d^3 / 6$$

where d = width and l = central length (Fry, 1990) (section 2.11.3)

## 4.1.3.10. Protein Content per Cell

Three separate cultures of *P. aeruginosa* 6750 were grown in iron-depleted $CDM_{12}$ at $37^\circ C$ (section 2.11.4) and were analysed over a period of 20 days. Protein content was assessed using the BCA protein assay. Mean values were calculated and represented graphically (fig. 4.27).

Results show that the total amount of protein increased along the growth curve until it reached a relatively steady value. Total and colony counts were examined at the same time as the protein assay (fig. 4.28), so that the protein content per $10^8$ cells could be calculated (fig. 4.29).

The graph shows that there was an increase in the protein content after 2.5 days. The increase was rapid initially, after which it remained relatively stable for the subsequent days. Protein content was initially around 10μg per $10^8$ cells, and later increased to approximately 20μg per $10^8$ cells. The time point at which the increase occurred

corresponded to the cessation of exponential growth and the onset of the extended stationary phase (fig. 4.28).


## 4.1.3.11. Estimation of Protein per Volume

Protein per volume was calculated using the values for the protein per $10^8$ cells divided by the values for the estimation of cell volumes (section 4.1.3.4.3). The results for cell width were found not to vary significantly with time and so a mean value 0.66μm was calculated in the estimation of volume formula.

The results showed that during the first few hours a relatively small increase was observed as the protein/volume increased to around twice the initial value. This would have corresponded to the initial decrease in cell size (fig. 4.26) which would result in a greater decrease in cell volume and so would make the quotient larger. At the onset of stationary phase (after 2 days) there was at least a further doubling in the protein/volume. It is thought that this increase occurred because of the almost doubling in protein per $10^8$ cells that took place at the same time.

**Fig. 4.27.**     Total protein content of *P. aeruginosa* 6750 during growth in iron-depleted $CDM_{12}$ batch culture at 37ºC.
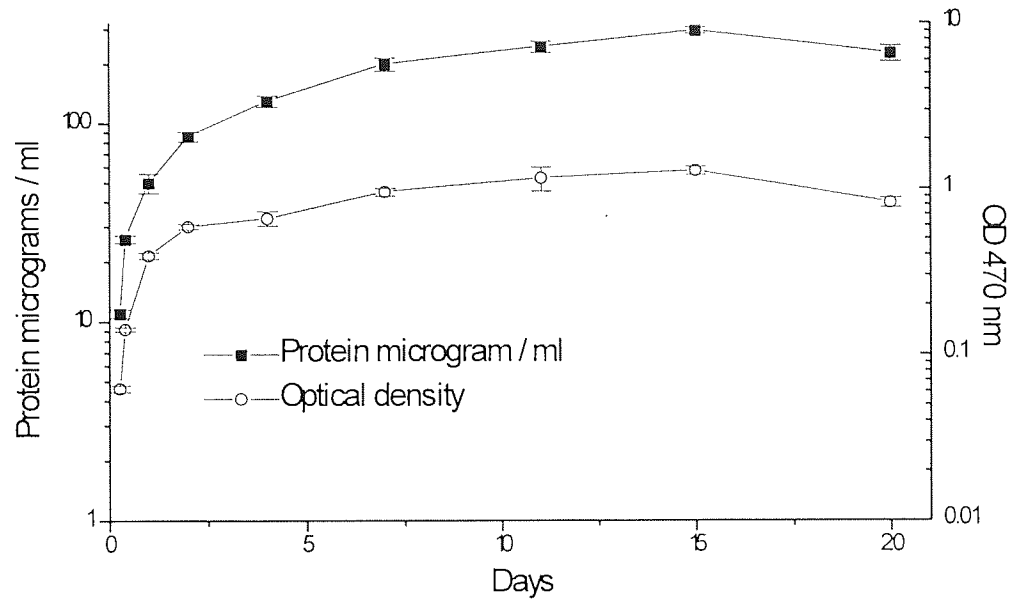


**Fig. 4.28.**     Changes in total counts, colony counts and optical density of *P. aeruginosa* 6750 grown in iron-depleted $CDM_{12}$ at 37ºC.
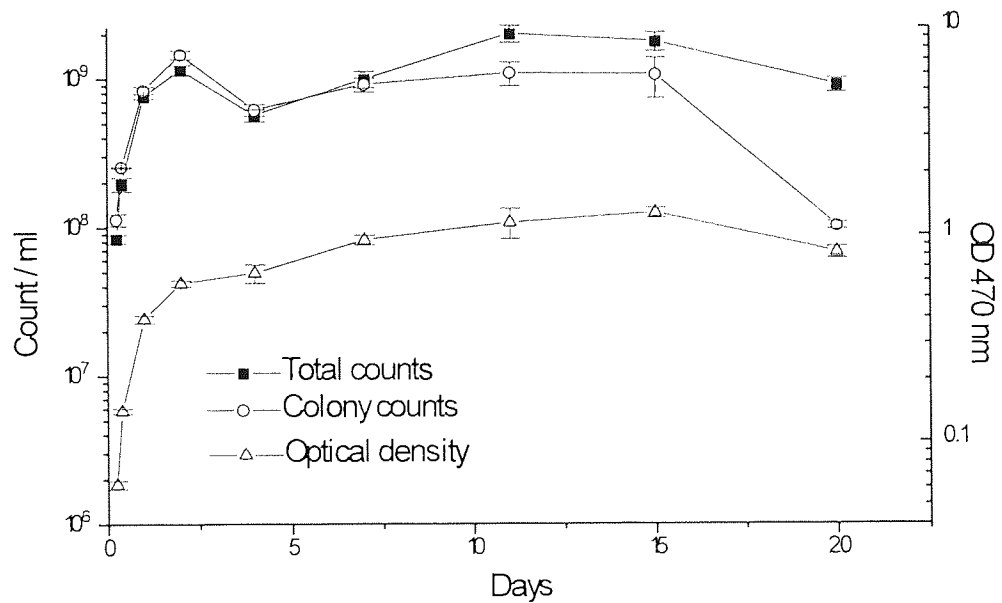
**Fig. 4.29.**  Protein content per $10^8$ cells of *P. aeruginosa* 6750 during growth in iron -depleted $CDM_{12}$ at 37°C.
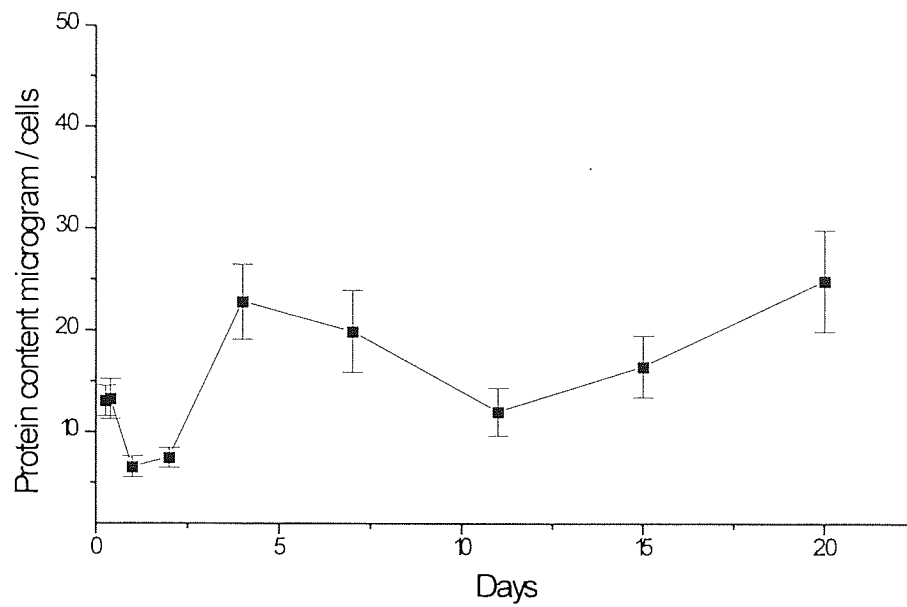


**Fig. 4.30.**  Protein content per $10^8$ cells per volume of *P. aeruginosa* 6750 during growth in iron-depleted $CDM_{12}$ at 37°C.
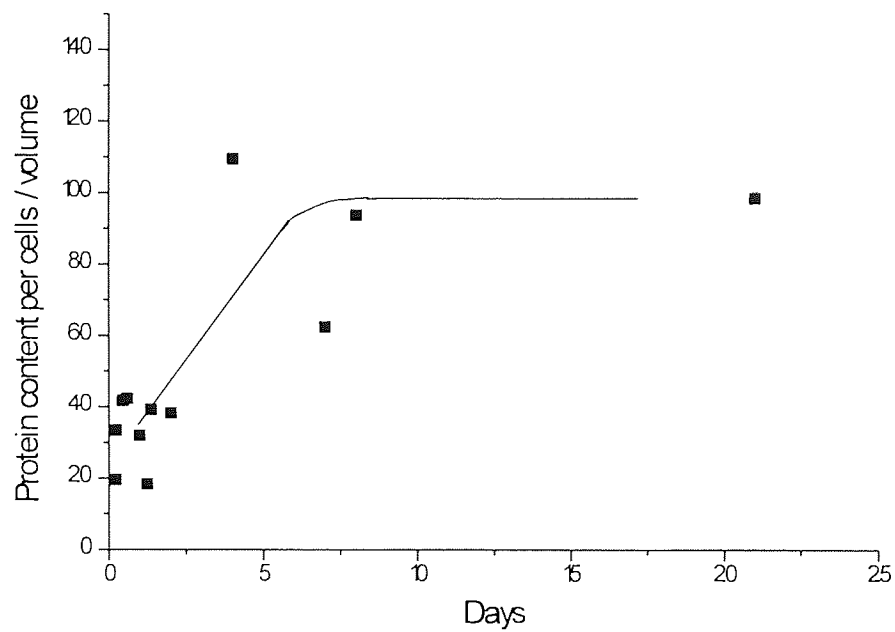
## 4.1.3.11.1. Discussion

As mentioned earlier the size of the cells in an extended culture was shown to decrease (4.1.3.8.3.), and so the volume of the cells would also decrease.

Reeve *et al* (1984a) demonstrated with peptidase-deficient mutants of *Escherichia coli* and *Salmonella typimurium* that protein degradation was important for survival of the cells during carbon starvation. Additionally, protein synthesis was also shown to occur during carbon starvation (Reeve *et al*, 1984b).

Some researchers have investigated specific proteins that have been induced during the stationary phase. The analysis of changes in protein composition for three bacterial isolates from marine waters, at the onset and after 24 hr carbon deprivation, was made with two-dimensional electrophoresis (Jaan *et al*, 1986). The results were expressed as protein per cell and per biovolume. *Enterobacter agglomerans* exhibited no changes in protein content during the starvation period. The organism also showed only a small decrease in size during this time period. A decrease in protein content per cell was seen for the starving *Vibrio* sp. strain DW1. Pronounced specific changes in the protein pattern for the unidentified Gram-negative rod-shaped organism S14 were reflected by both a large decrease in cell volume and an increase in cellular protein concentration during starvation. The results demonstrated that different catabolic-synthetic pathways were exhibited by different bacterial strains.

Multiple-nutrient starvation (i.e. starvation for glucose, amino acids, ammonium and phosphate simultaneously) of a marine *Vibrio* sp. strain S14 was described as a three-phase process (Nyström *et al*, 1990). The first phase demonstrated decreases in RNA and protein synthesis during the first 40min. In the second phase a temporary increase in the rates of RNA and protein synthesis was seen between 1 and 3hr, after which a third phase exhibited a gradual decline in synthesis. The results suggested that most starvation proteins were induced early during starvation and that they appeared to be the most important for survival, since inhibitors of protein synthesis added for a short period at the onset of starvation greatly compromised long-term survival.

Studies have recently investigated the total protein content of *Micrococcus luteus* cells during long term starvation in spent medium (Mukamolova *et al*, 1995b). The results have demonstrated that there was a gradual loss of total protein during starvation. It was also shown that the decrease in protein content was due mainly to the degradation of cytoplasmic protein whereas the membrane protein content was found to be more stable. However, the results did not show the protein content during the exponential phase as a

comparison to the results obtained after starvation. In addition, cell size was not measured and so the quotient of protein per volume could not be analysed.

From our results, it can be seen that an increase in the total protein content occurred which was to be expected in a growing culture as well as more importantly an increase in the protein content per $10^8$ cells. This increase could be explained in terms of changes that may have arisen during the onset of stationary phase. The protein/volume of the cells also increased with time which may be a result of the protein being compacted into the smaller volume of the cell.

It is not known if these changes could have a significance on the survival of the cells. It is proposed that perhaps the increase in the concentration of protein per cell may help the cells to withstand many of the adverse conditions that present themselves during starvation survival.

## 4.1.3.12. Measurement of Cell Vitality using CTC

Duplicate cultures were analysed to examine the vitality of a population of *P. aeruginosa* 6750 in iron-depleted $CDM_{12}$ during growth. Vitality was expressed by the percentage of cells that reduced the CTC stain to the red insoluble CTC-formazan compound. Total and colony counts were made at the same time, so that percentage culturability could be expressed on the same graph (fig. 4.31).

The results show that in general the percentage culturability curve followed a similar pattern to percentage vitality curve. After an initial high level of culturability (up to 15 days), a decrease in culturability was seen. Similarly, in the case of vitality, there was a high level of staining (up to 10 days), followed by a decrease.

However, some differences between the two curves can also be seen : after 2.5 days there was a drop in the vitality without a corresponding drop in culturability, and also the general decrease in vitality appeared sooner than the decrease in culturability. It is not known if the differences that have occurred are due to the fact that CTC stain was found to be unpredictable when used on old cells compared with exponentially growing cells. It is also interesting to note that the cultures exhibited an increase in OD, indicating growth or change in shape, during the extended stationary phase which would account for the high culturability and vitality during the initial 10 days.

Overall, it should be noted that the percentage culturability was never lower than percentage vitality at any time point which indicates that there were no cells in the cultures that were vital but nonculturable. From these results it can not be said that a vital nonculturable population of *P. aeruginosa* 6750 existed.

**Fig. 4.31.** Percentage of culturable and vital cells of *P. aeruginosa* 6750 in iron-depleted $CDM_{12}$ during growth.
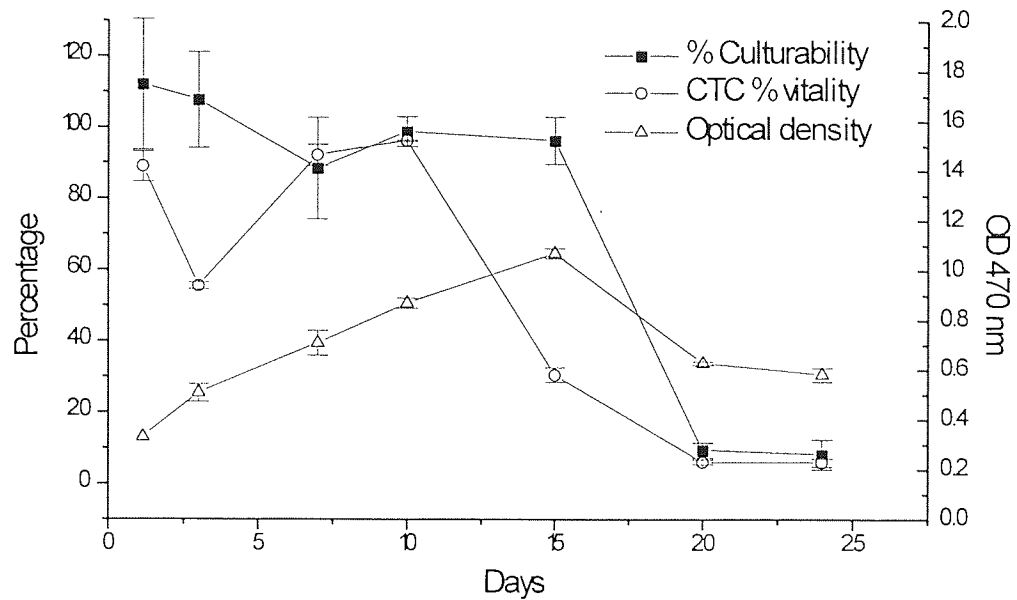


## 4.1.3.12.1. Discussion

CTC has been widely used to evaluate the vitality of unculturable cells. Some have used the method in an effort to prove the existence of a dormant state of bacteria.

As mentioned previously one of the first users of this vital stain was Rodriguez *et al* (1992). They were aware of the potential of the stain to evaluate the numbers of metabolically active bacteria compared to the numbers of culturable bacteria from colony counts. Their results showed that in environmental samples that were supplemented with exogenous nutrients, CTC counts were found to be higher than colony counts. However, in unsupplemented samples the CTC counts were actually lower than the colony counts. This suggested that the lack of oxidizable carbon substrates in the samples limited the *in situ* respiratory of the bacteria under observation.

Our preliminary experiments without supplementation, using *P. aeruginosa* 6750, (fig. 2.4) showed that there was a good correlation between the culturability of exponential phase cells and the staining the of cells with CTC. However, the experiments using older cells did not show higher levels of staining compared to the levels of culturability. Therefore experiments with older cells may necessitate the use of additional supplementation as in the experiments of Rodriguez *et al* (1992).

Other workers have used CTC in staining procedures without supplementation of nutrients so as to avoid preferential stimulation of inactive bacteria (Yu *et al*, 1995). In these studies, an 8 hr incubation time was used which was longer than in other experiments. The experimenters concluded that it was a useful technique to enumerate active bacteria in soil and groundwater sediments.

Studies with *P. fluorescens* in response to starvation and osmotic stress have also highlighted the usefulness of the stain (Jørgensen *et al*, 1994). During 5 days carbon starvation, total counts remained constant, colony counts decreased by one log unit whereas the CTC stained cells decreased gradually by around 80%. However, investigations with multiple starved cells demonstrated that the culturability was greater than the number of CTC-reducing cells. The experiments were performed without the addition of extra nutrients. It was suggested that the use of nutrient additions was required under low nutrient conditions so as to provide an estimation of potentially respiring cells (Thom *et al*, 1993).

The analysis of the effect of CTC with *Micrococcus luteus* suggested the presence of dormant cells (Kaprelyants and Kell, 1993b). Cells that were found to be unculturable after a 75 day incubation were then resuscitated. A certain number were shown to be able reduce CTC and a similar fraction were converted to being culturable again.

Investigations have also been performed that have used CTC both in planktonic cultures and biofilms (Schaule *et al*, 1993). The results showed that the number of CTC-reducing bacteria were consistently greater than the number of colony forming units. A 1 hr incubation time was used in these experiments which showed that it was a rapid and sensitive method for quantification and visualisation of respiring bacteria. The method used in the studies with *P. aeruginosa* 6750 also was short in comparison to the overnight incubation to obtain colony forming units.

## 4.1.3.13. Measurement of Lag

Two flasks of $CDM_{12}$ were set up and inoculum from either of two different *P. aeruginosa* 6750 cultures was added. One inoculum originated from an overnight culture and the other was from a 41 day old culture (fig. 4.32). The graph shows that the time taken for the overnight inoculum culture to reach an OD of 0.1 was 7 hours, whereas the time taken for the older inoculum culture to reach an OD of 0.1 was 12.5 hours. This is an indication of the possible lag of the cells of the older culture compared with the overnight culture. However, it is not known if the growth of the cells from the old culture was brought about from a small number of culturable cells surviving in the old culture.

**Fig. 4.32.**     Growth of *P. aeruginosa* 6750 in iron-depleted $CDM_{12}$ at 37°C using inoculums of 41 day old culture and an overnight culture to measure lag.
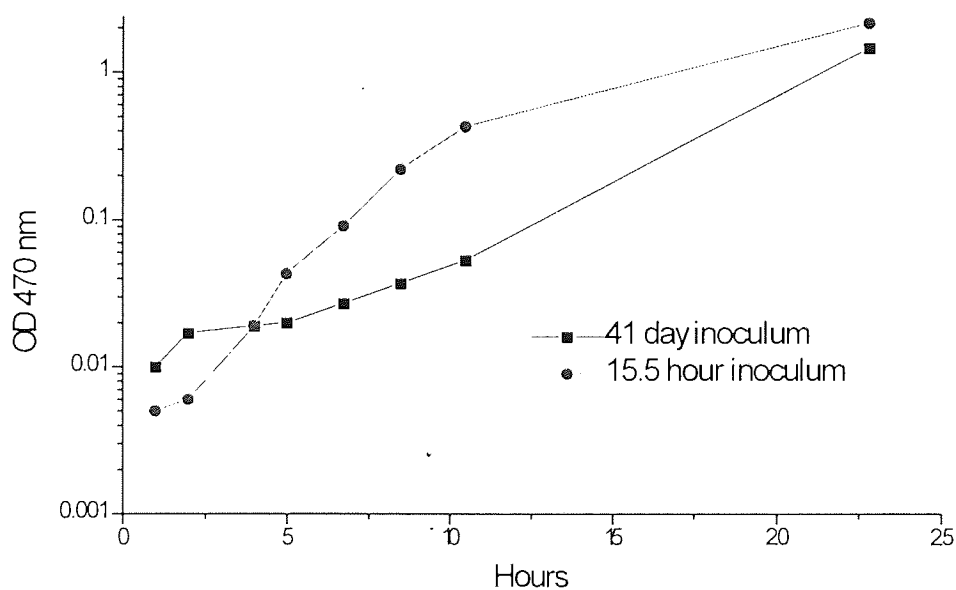
#### 4.1.3.13.1. Discussion

The measurement of lag, as an indication of a cell's slowness to divide, and hence a possible measure of dormancy, has not been quantified in many studies even though some experimenters have commented on this phenomena. Difficulty in interpretation arises, as mentioned above, when it is not known which cells were actually growing.

It has been documented that small bacteria are generally the first colonizers of solid surfaces in marine environments. They are known to arrive at a surface in a dormant state, and are only able to replicate after a lag period (Marshall, 1979).

In an experiment, a dialysis chamber was used to analyse small starved cells. A nutrient was provided at the solid surface of the chamber and the starved cells were found firstly to regain their motility followed by an increase in the cell size. Cell division only took place after the initial events. The observed long lag, before replication occurred, was thought to be a requirement of the time needed to achieve proper size before division (Kjelleberg et al, 1982).

Others have realised that slow-growing bacteria form visible colonies on solid media only after extended periods of incubation and so have suggested that other methods to measure activity of cells require adaptation (Singh et al, 1990). It was proposed that the DVC method may need a prolonged period of incubation in order to obtain adequate cell elongation.

## 4.1.3.14. Antibiotic Susceptibility

## 4.1.3.14.1. Ciprofloxacin

Preliminary results with early stationary phase *P. aeruginosa* 6750 cells showed that 0.5 μg/ml ciprofloxacin gave an approximate one log cycle reduction after 1 hr incubation (fig. 4.33). This concentration of ciprofloxacin was used on subsequent experiments as well as 1μg/ml when higher concentrations were needed to provide suitable killing.

**Fig. 4.33.**     Survival of iron-depleted batch culture of *P. aeruginosa* 6750 after incubation with ciprofloxacin at 37°C for 1 hour.
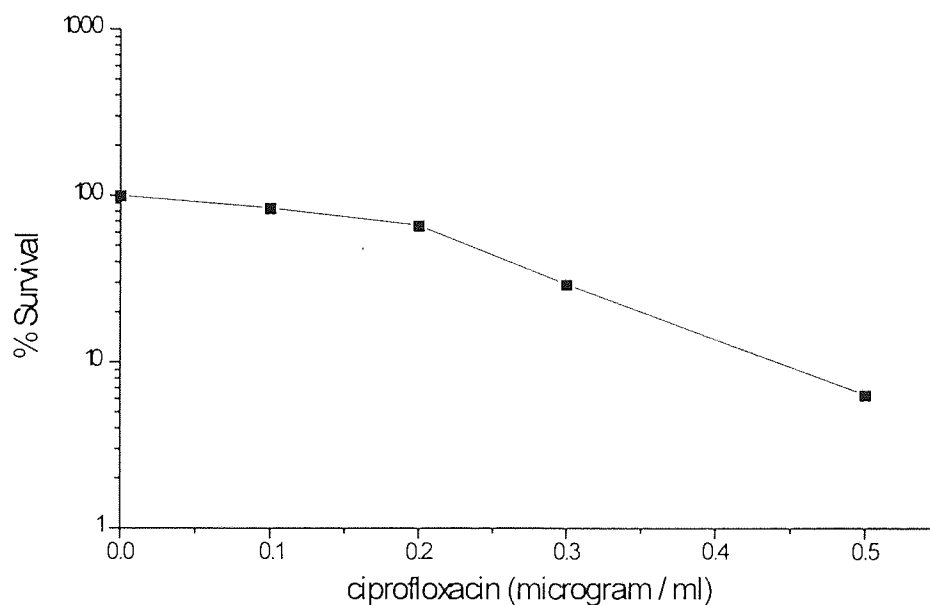
Survival of *P. aeruginosa* 6750 in batch culture was investigated throughout stationary phase starting with a point at early stationary phase. Concentrations of 0.5μg/ml and 1μ g/ml were used (fig. 4.34, 4.35) over a period of 15 days. Optical density of the untreated suspensions was measured at the same time.

Figure 4.34 shows survival of the organism to be 30% at early stationary phase. An increase in survival was observed after 2 days. However between 3 and 4 days, fluctuations in survival were seen. These fluctuations may be attributed to growth of *P. aeruginosa* 6750 during the extended stationary phase. After 4 days, survival reached a maximum value of 95%. This maximum corresponded to a decrease in growth accompanied by $OD_{470}$ reaching a steady value. A decrease in survival to a value of 25 to 30% was seen thereafter.

Results obtained with 1μg/ml ciprofloxacin (fig. 4.35) followed a similar pattern, except that there was a steady increase over a longer period of time (6.25 days). As with 0.5μ g/ml ciprofloxacin, a maximum value was observed (60%) which corresponded once again to a cessation of growth and a steady $OD_{470}$ having been obtained. A slight fluctuation in growth was also seen which may be attributed, as before, to growth in the stationary phase. Overall, after a period of 6.5 days survival of *P. aeruginosa* 6750 failed to increase.

**Fig. 4.34** Survival of *P. aeruginosa* 6750 in iron-depleted $CDM_{12}$, throughout stationary phase, after incubation with ciprofloxacin 0.5μg/ml for 1 hour at 37°C.



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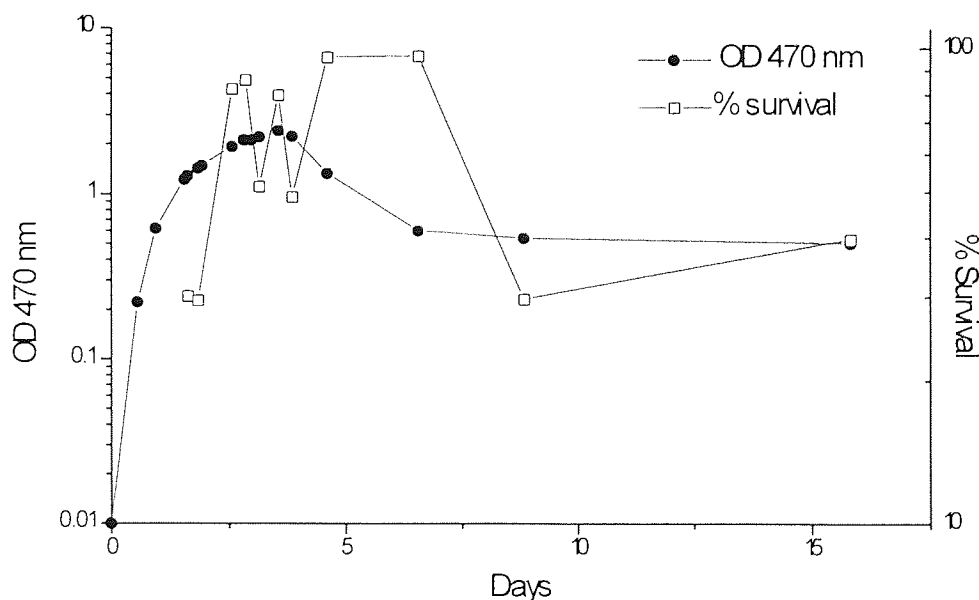
**Fig. 4.35.** Survival of *P. aeruginosa* 6750 in iron-depleted CDM$_{12}$, throughout stationary phase, after incubation with ciprofloxacin 1μg/ml for 1 hour.
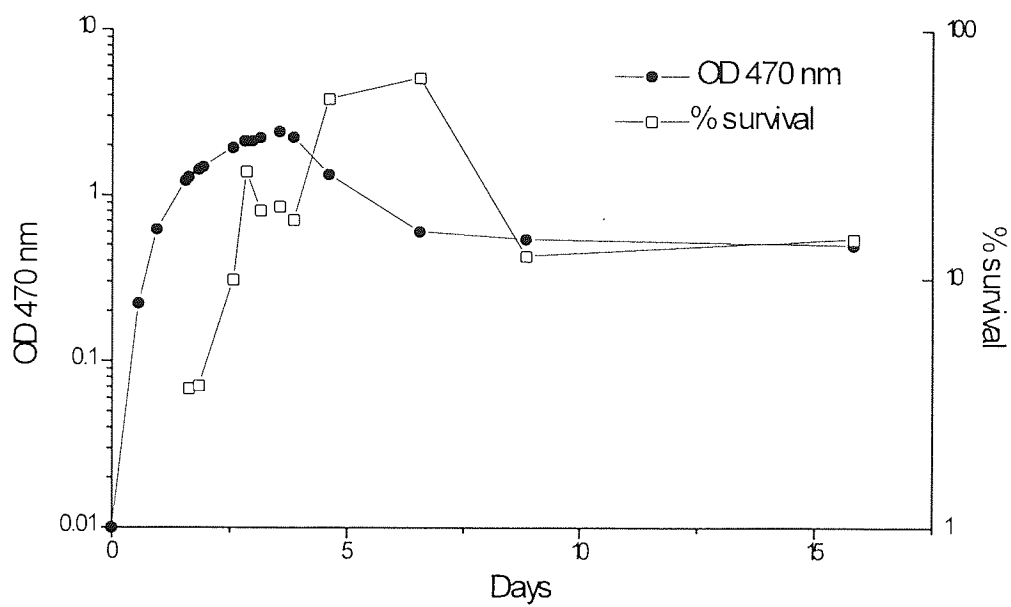
#### 4.1.3.14.2. Polymyxin B

Figure 4.36 shows that early stationary phase *P. aeruginosa* 6750 subjected to 0.5µg/ml polymyxin B after 1 hr incubation produced an approximate 2 log cycle reduction.

Susceptibility of *P. aeruginosa* 6750 to 0.5 and 1.0µg/ml polymyxin B was evaluated throughout stationary phase (fig. 4.37, 4.38).

**Fig. 4.36**      Survival of iron-depleted batch culture of *P. aeruginosa* 6750 after incubation with polymyxin B at 37°C for 1 hour.
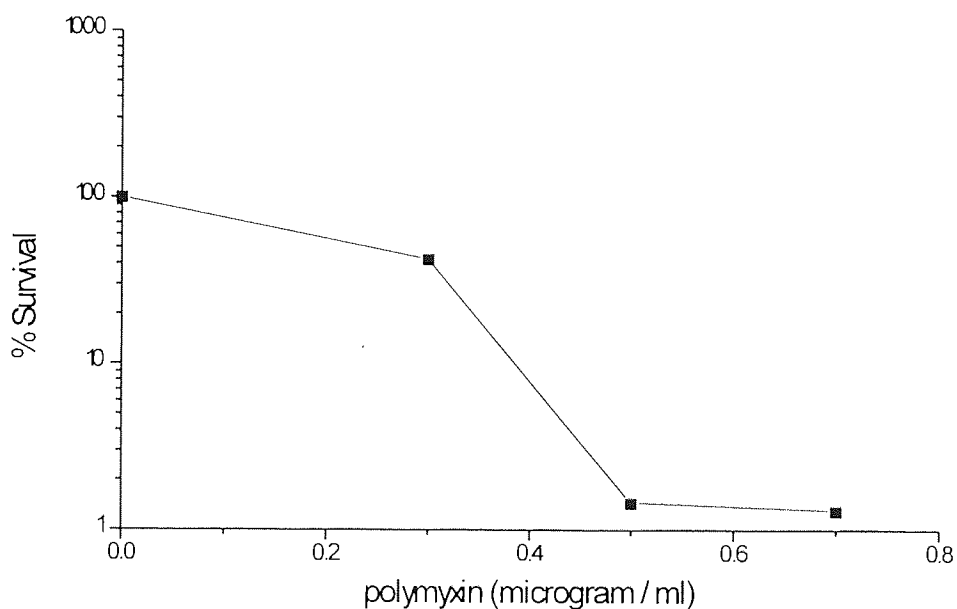
**Fig. 4.37** Survival of *P. aeruginosa* 6750 in iron-depleted $CDM_{12}$, throughout stationary phase, after incubation with polymyxin B 0.5μg/ml for 1 hour.
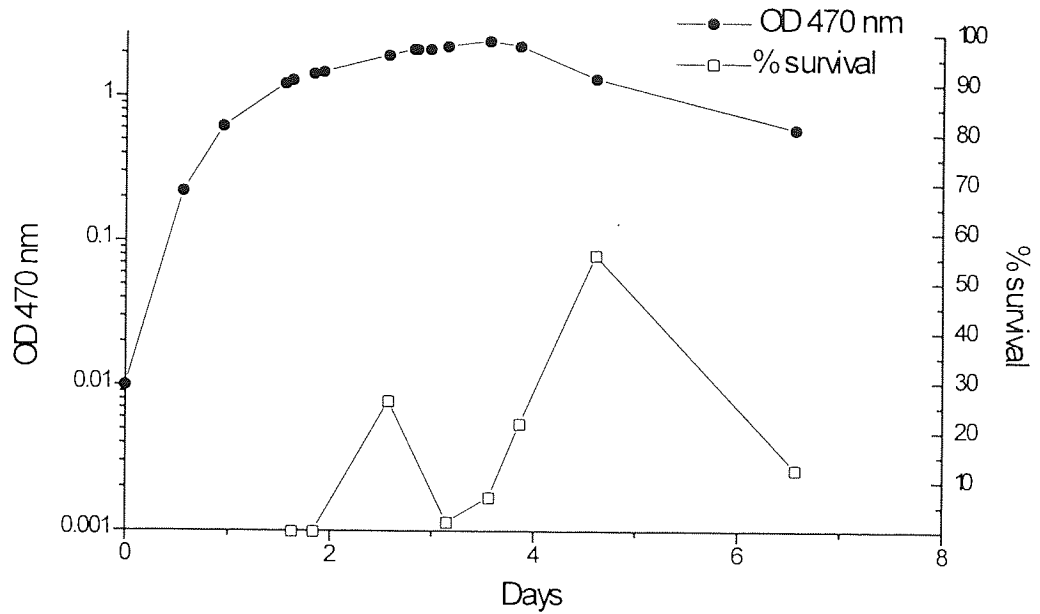


**Fig. 4.38** Survival of *P. aeruginosa* 6750 in iron-depleted $CDM_{12}$, throughout stationary phase, after incubation with polymyxin B 1μg/ml for 1 hour.
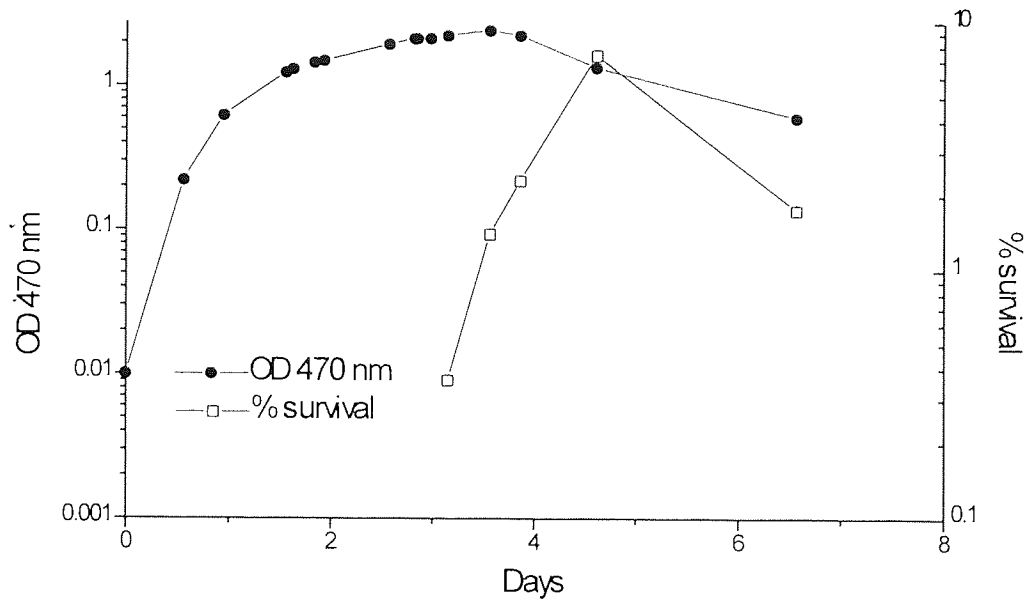
Figure 4.37 shows that an increase in survival occurred after 2 days. A decrease ensued which may be attributed to growth in stationary phase as with ciprofloxacin. After 3 days a steady increase was observed which reached a maximum corresponding to a maximum value reached in the growth curve. After the maximum, a decrease in survival was seen.

Similar results were obtained with $1 \mu g/ml$ polymyxin B. No survival was seen before 5 days, after which time there was a steady increase reaching a maximum value at 4.5 days. Once again, the maximum corresponded to onset of cessation of growth in stationary phase. No further increase was observed thereafter as with ciprofloxacin.

## 4.1.3.14.3. Susceptibility of "True" Exponential phase Cells

Experiments were performed with polymyxin B and ciprofloxacin on "true" exponential phase cells and mid-exponential phase cells. The "true" exponential phase cells were harvested four generations from the inoculum and four generations from the mid-exponential phase cells. The mid-exponential phase cells were harvested four generations from the "true" exponential phase cells and four generations from the onset of stationary phase (section 2.11.8.3). Concentrations of antibiotic used had to be lower than those used for stationary phase cells as these cells were found to be more sensitive. Figure 4.39 shows that concentration of $0.1 \mu g/ml$ of polymyxin B produced nearly one log cycle decrease in survival. However, there was found to be no significant difference between survival of the two types of exponential phase cells.

Experiments with ciprofloxacin 0.05 and $0.1 \mu g/ml$ (fig.4.40) show that a difference did exist between the two types of exponential phase cells. Results show that $0.1 \mu g/ml$ produced a log cycle decrease in survival with "true" exponential cells, but that survival for mid-exponential cells was approximately 4 times greater.

It is not known why a difference occurred with ciprofloxacin and not polymyxin B. It could be that because the antibiotics have very different mechanisms of action the time point on the growth curve at which they exert their maximum effect will be different as well.

The results showed that approximately 1/10th of the concentrations of polymyxin B and ciprofloxacin were needed to produce a similar magnitude of killing of "true" exponential phase cells than was needed for stationary phase cells.

**Fig. 4.39** Survival of exponential phase *P. aeruginosa* 6750 in iron-depleted $CDM_{12}$, after incubation with polymyxin B 0.05µg/ml and 0.1µg/ml for 1 hour at 37°C.
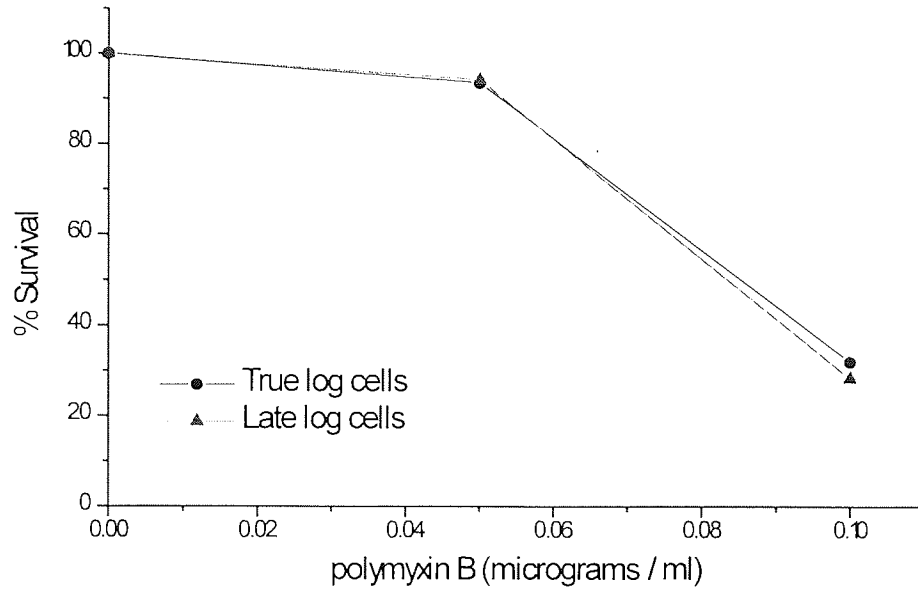


**Fig. 4.40** Survival of exponential phase *P. aeruginosa* 6750 in iron-depleted $CDM_{12}$, after incubation with ciprofloxacin 0.05µg/ml and 0.1µg/ml for 1 hour at 37°C.
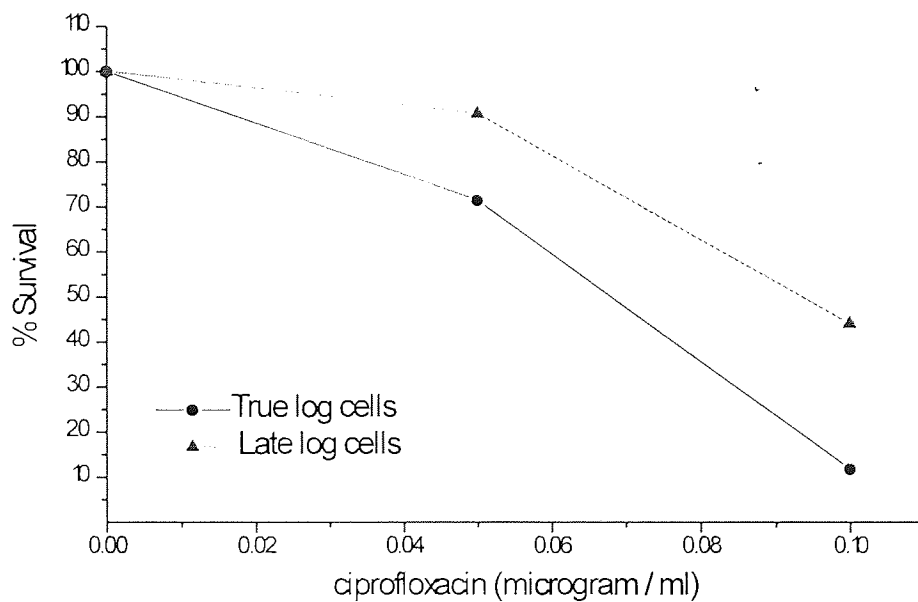
## 4.1.3.14.4. Discussion of Antibiotic Studies

It is widely recognised that stationary phase cells tend to be more resistant to antibiotics than exponentially growing cells. This is shown in the above results with *P. aeruginosa* 6750 as 1/10th of the concentration of antibiotic was used with exponential phase cells to provide the same level of killing as for stationary phase cells. It has also been established that slow-growing cells possess a decreased susceptibility compared to faster growing ones. This too was observed in the previous graphs as in all cases the maximum percentage survival was observed at the slowest growth rate i.e. when the cells ceased to grow and reached a plateau in the extended stationary phase curves. Additionally, it was seen that the exponentially growing cells that would possess the fastest growth rates were the most susceptible.

Many studies have been performed that have supported these findings (Brown, 1975; Finch and Brown, 1978; Gilbert and Brown, 1978; 1980; Tuomanen *et al*, 1986). Researchers have also conducted many investigations in the search to find antibiotics that are effective on slow-growing and non-growing cells (Cozens *et al*, 1986; Tuomanen, 1986; 1987; Eng *et al*, 1991). The results of these studies demonstrated that only select antibiotics were effective against slow growing cells. It has already been mentioned that changes in cell envelope composition as a result of changes in growth rate also influence susceptibility of cells to antimicrobial agents.

The experiments that measured the survival of *P. aeruginosa* 6750 after exposure to antibiotics were performed over a period of 6 days. The results showed that resistance did not increase further after it reached a maximum that corresponded to the cessation of growth at stationary phase. Experiments conducted over greater time intervals, up to 30 days (data not shown) also confirmed the finding that resistance did not increase further but also that it did not decrease greatly.

It has been reported that changes in cell envelope take place at least three generations before the onset of stationary phase due to depletion of a specific nutrient (Brown and Williams, 1985). This was demonstrated in experiments with *Klebsiella pnemoniae*. The organism derepresed its high affinity uptake systems about three generations before the onset of stationary phase (Williams *et al*, 1984). Other studies with magnesium-depleted *P. aeruginosa* have shown that sensitivity to polymyxin B was lost depending on the metal cations in the medium. The sensitivity was only regained after three generations of growth in magnesium plentiful medium (Brown and Melling, 1969b). It was therefore suggested that exponentially growing cells should be harvested at least three generations before the onset of stationary phase and additionally three generations after inoculation. In the present study, although differences in susceptibility of the two types of

exponential phase cells were not seen with polymyxin B, a difference did exist between the two types with ciprofloxacin. This demonstrated that the use of exponential phase cells was no guarantee that envelope and associated properties were constant and reproducible (Brown and Williams, 1985).

## 4.1.3.15. Overall Discussion of Batch Culture Studies

Overall, the results in batch culture suggested culturability increased during the growth curve. After a maximum value was reached, unculturability of a population of cells occurred. This was shown through results obtained with colony and total counts, DVC method and CTC method. The CTC and DVC methods did not indicate that a vital but non-culturable state existed.

The size of the organism was shown to decrease along the growth curve until it reached a steady value. Protein was found to increase until it reached a steady value shortly after onset of stationary phase. From these results, protein/volume ratio was calculated which showed that an increase occurred in which protein was compacted into a smaller volume.

Survival of *P. aeruginosa* 6750 to antibiotics was found to increase during the growth curve and throughout stationary phase until a maximum value was obtained. It is not known if the appearance of unculturable cells played a part in affecting survival after antibiotic treatment. Despite the fact that appearance of unculturable cells occurred at the same time as the increase in survival, no further increase in resistance to antibiotics was observed even though more unculturable cells were produced with time. Therefore the increase in resistance may be because of a change in growth rate due to cessation in growth. Unfortunately, the resistance of unculturable cells cannot be tested by this method as it in itself, involves the culturability of cells using colony counts.

## 5. RESULTS AND DISCUSSIONS

### 5.1. Continuous Culture

### 5.1.1. Introduction

Continuous culture employing a chemostat enables effect of growth rate, nutrient levels and other factors associated with the growth process to be investigated. The essential feature of continuous culture in a chemostat is that microbial growth can take place under steady state conditions; that is growth may occur at a constant rate and in a constant environment (Herbert *et al*, 1956). The chemostat is a device by which a culture medium is kept at a constant volume by the addition of fresh medium at a fixed rate, and the removal of the spent culture medium at the same fixed rate. In this way, a specific growth rate can be maintained.

After the initial inoculation of the chemostat, the bacterial population is allowed to grow as a batch culture without the flow of medium. The medium has an excess of nutrients except for one nutrient which is the potentially limiting substrate $S_R$. The pump is then switched on. If the volume is V, and the medium is added at a rate of f, and removed at this same rate, then the organism (concentration) x grows at a rate $\mu$. The organism is then diluted out at a rate of f/V, which is described by the dilution rate D (volume changes per hour). From this information, an equation can be constructed in which the net rate of increase of concentration of organisms can be given:

Increase = Growth - Dilution (Output)

Or in mathematical terms:

$$dx/dt = \mu x - Dx$$

The outcome of the culture will then be one of three possibilities:

1. If $\mu$ is greater than D, dx/dt is positive and the concentration of organisms will increase.
2. If D is greater than $\mu$, dx/dt is negative and the concentration of organisms will decrease eventually to zero. This means that the culture will experience "washout".
3. When $\mu$ is equal to D, then dx/dt = 0 and x, the concentration of the biomass is constant and a steady state is achieved.

If these conditions can be maintained, the planktonic cells in the culture vessel are grown in a controlled manner at a specific growth rate. Changes can then be made to the

flow rate and time should be allowed until the system reaches steady state again, so that cells will grow at a different specific growth rate.

Dilution rates can be varied from zero up to the maximum dilution rate $D_{crit}$, after which "washout" occurs. At dilution rates below $D_{crit}$, the biomass is theoretically constant.

## 5.1.2. Results

Extensive wall growth was produced using *P. aeruginosa* PAO1 JD and so *P. aeruginosa* 6750 was used instead.

Figure 5.1 and 5.2 show the relationship between optical density and colony count respectively and dilution rate. Each point represented one chemostat run at a particular dilution rate. Results show that between dilution rates of approximately 0.05 $h^{-1}$ and 0.3 $h^{-1}$, cell density and culturability remain relatively constant. "Washout" did not occur at a specific dilution rate as would be expected from a theoretical plot. This can be attributed to wall growth, which tends to prevent a complete "washout". Wall growth or adhesion of biomass can be temporarily prevented by silicone treating the surface of the vessel (Pirt, 1975).

A second variation from ideal behaviour occurs at low dilution rates, where it can be observed that a higher biomass than expected is produced. At low growth rates, and especially where carbon is in excess, it may be stored as an energy reserve which constitutes a high proportion of the biomass (Pirt, 1975), which could influence optical density.

**Fig. 5.1.**    Relationship between optical density and dilution rate of steady state iron
-limited chemostat culture of *P. aeruginosa* 6750 at 37ºC.
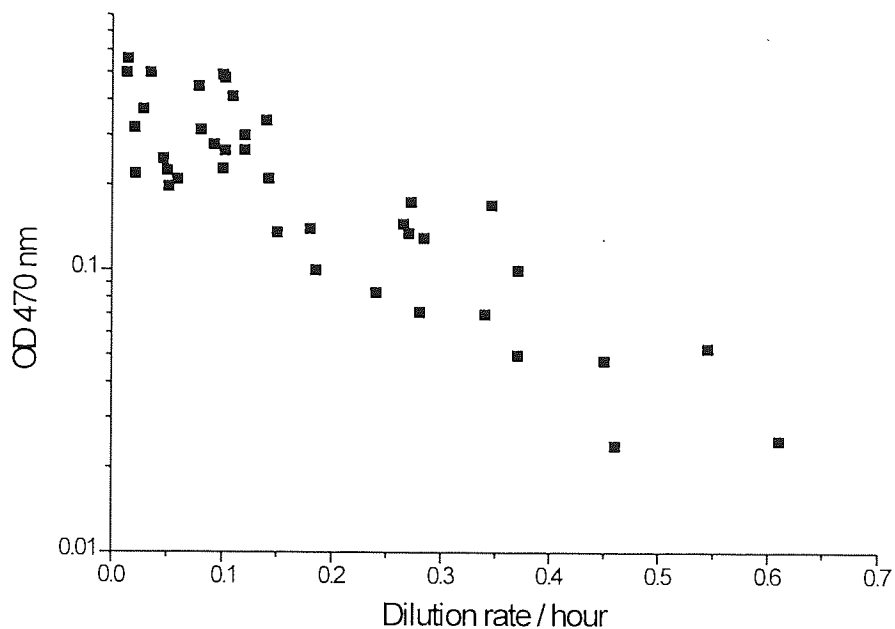


**Fig. 5.2.**    Relationship between colony count and dilution rate of steady state iron-
limited chemostat culture of *P. aeruginosa* 6750 at 37ºC.
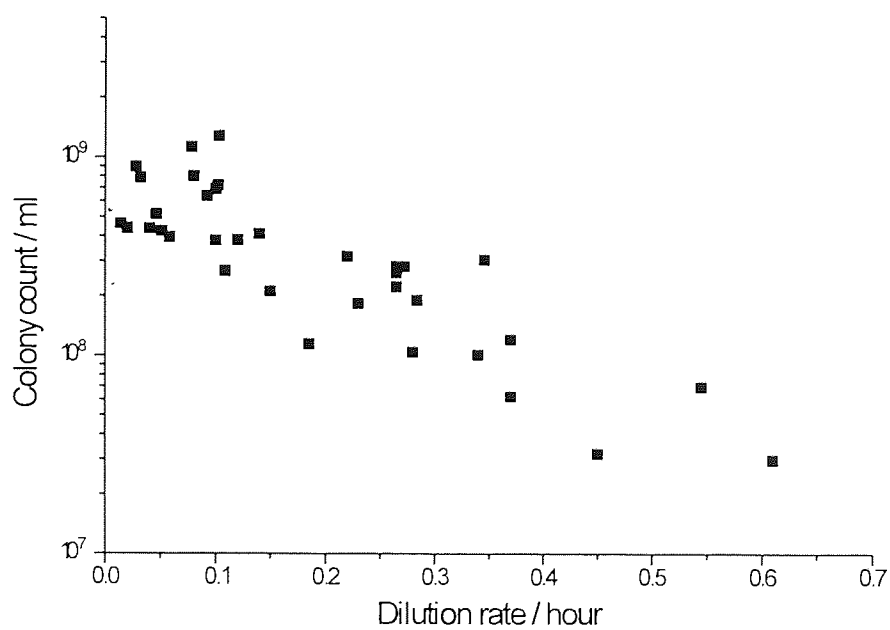
## 5.1.3. Parameters to Measure Survival of *P. aeruginosa* 6750 in Continuous Culture

### 5.1.3.1. Evaluation of Unculturability using Total/Colony Count Ratio

Experiments were carried out to investigate the survival of *P. aeruginosa* 6750 under iron limitation using continuous culture by means of chemostats. Total and colony counts were made at varying dilution rates and results were represented graphically (fig. 5.3). Colony counts were expressed as a percentage of total counts (fig. 5.4).

**Fig. 5.3**    Relationship between total and colony counts and dilution rate of steady state iron-limited chemostat culture of *P. aeruginosa* 6750 at 37°C.
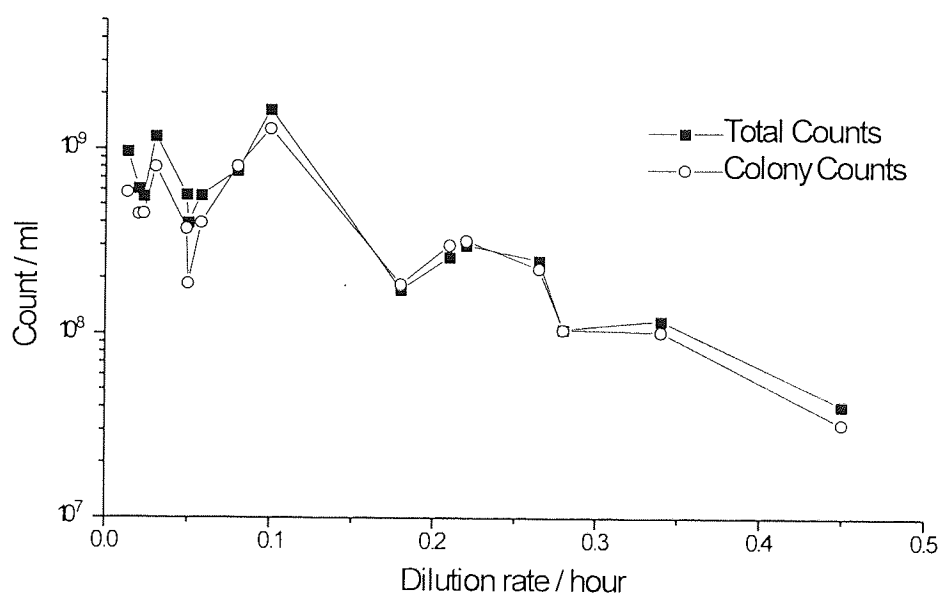


Figure 5.3 shows that overall differences between total and colony counts start to appear around 0.1 hr$^{-1}$ dilution rate. Each point on fig. 5.3 represented one chemostat run at a particular dilution rate. After 0.35 hr$^{-1}$ dilution rate differences between the two counts are also seen. However, this could be attributed to inaccuracy of counting, as counts were low. The graph is similar to that depicting the relationship between optical density and dilution rate (fig. 5.1). As in the previous graph "washout" was seen not to occur at a specific dilution rate and there existed a relatively stable total count between dilution rates of 0.02 hr$^{-1}$ and 0.15 hr$^{-1}$.

Percentage culturability was calculated and expressed graphically in figure 5.4. Results indicate that between dilution rates of 0.45 hr$^{-1}$ and 0.1 hr$^{-1}$ percentage culturability was around 100%. Some values were either slightly above or below 100% which may be a

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that colony counts decreased to a greater extent than above this dilution rate. The decrease in culturability was therefore an indication of unculturability of cells at low dilution rates.

Fig. 5.4    Relationship between percentage culturability and dilution rate of steady state iron-limited chemostat culture of *P. aeruginosa* 6750 at 37°C.
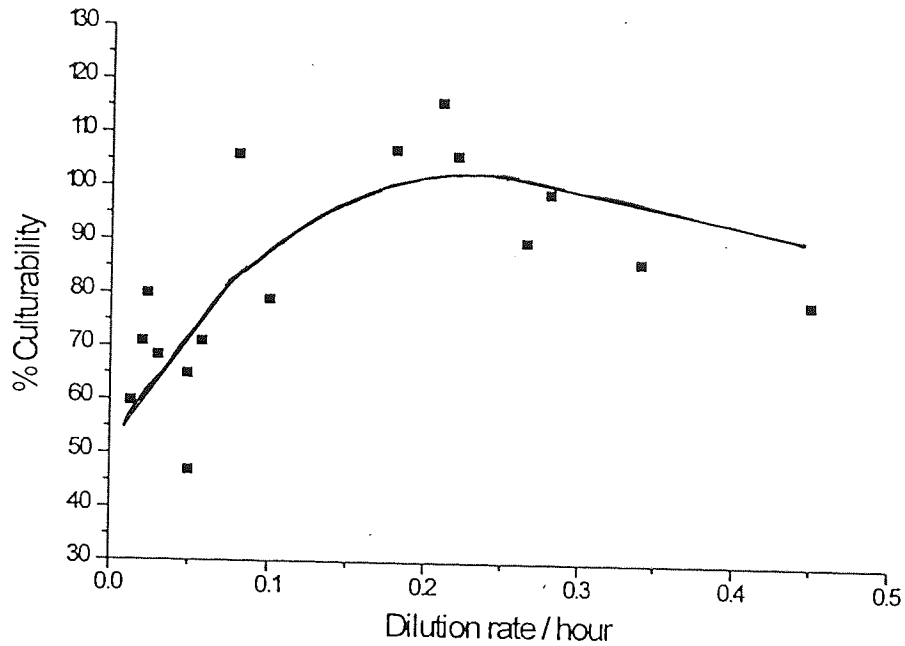


Cells from wall growth were scraped off chemostats and analysed microscopically. It was found that cells from wall growth were clearly seen to be smaller in size than cells in the main body of the chemostat (not quantified). The unculturable cells may have been formed because of low growth rates or perhaps because cells from wall growth detached themselves and became part of the main chemostat culture manifesting themselves as unculturable cells. However, if the latter explanation were true, it would not account for the increased amount of unculturability seen at dilution rates lower than 0.1 hr$^{-1}$ as wall growth appeared even at fast growth rates and the same level of unculturability was not seen.

## 5.1.3.1.1. Discussion

It is generally accepted that at steady state the culturability of bacteria as judged by colony counts is lowered at decreased dilution rates under both carbon and nitrogen limitations (Tempest *et al*, 1967; Gottschal, 1990). An experiment was performed to investigate the prolonged cultivation of *Cytophaga johnsonae* in a glucose-limited chemostat (Höfle, 1983). A decrease in culturability was seen after prolonged cultivation at both high and low dilution rates. It was concluded that the unculturability was caused by the cells inability to grow on agar plates rather than their lack of vitality in the chemostat.

Pirt (1987) described a model to explain the behaviour of cultures at low dilution rates. It had been discovered that at slow growth rates, in continuous cultures, bacterial growth yields from the carbon and energy source were higher than those expected. In order to account for the discrepancy it was proposed that dormant or so-called non viable cells with a zero maintenance energy were generated at slow growth rates. The data that was available at the time were in agreement with the hypothesis.

It was suggested by Mason *et al* (1986) that the proportion of active cells still remains greater than 85% at dilution rates as low as 0.002 $hr^{-1}$. It was also remarked that this suggestion contradicted the findings of Tempest *et al* (1967) who reported culturabilities of 40% at dilution rates of 0.004 $hr^{-1}$. It was considered doubtful for slow-growing, nutrient starved cells actually to be able to replicate on solid medium in a short time period. However, regardless of the cells' capability to replicate on solid medium, further studies should be performed that analyse activity in other ways to assess vitality.

One possible explanation that could account for the loss of culturability that occurs under conditions of prolonged cultivation in chemostats, could be related to the shock that cells may experience in the presence of an abundance of nutrients in the plating medium. This phenomenon is known as substrate-accelerated death. It can occur when the substrate that was limited during growth of the cells is added, and results in accelerated death of the starved cells (Poindexter, 1987; Postgate, 1967). In the present study with *P. aeruginosa* 6750 it is not known if this phenomenon could explain the decrease in culturability since iron was limited in the chemostat. It would be interesting to check the culturability of the cells on agar plates that were depleted in iron, so as to reduce the possibility of substrate-accelerated death.

## 5.1.3.1.2. Chemostat Samples Left to Stand at Room Temperature and 37° C

Samples taken from chemostats at varying dilution rates were allowed to stand at room temperature (20°C to 22°C) and at 37°C. At low dilution rates, (0.013 hr$^{-1}$) results suggested that on standing, unculturability did not increase for samples at room temperature and 37°C (fig. 5.5, 5.6). These results suggest that unculturability occurred as a result of slow growth rate and not starvation which could have been implicated as a result of large time intervals between drops during flow of CDM. However, after 20 days a decrease in colony counts was seen in fig. 5.5. In this case it can be assumed that long term starvation of samples had been attained and as a consequence cells became unculturable.

**Fig. 5.5**     Changes in total counts, colony counts and optical density of samples of *P. aeruginosa* 6750 removed from an iron-limited chemostat (0.013 hr$^{-1}$) culture and allowed to stand at 37°C.
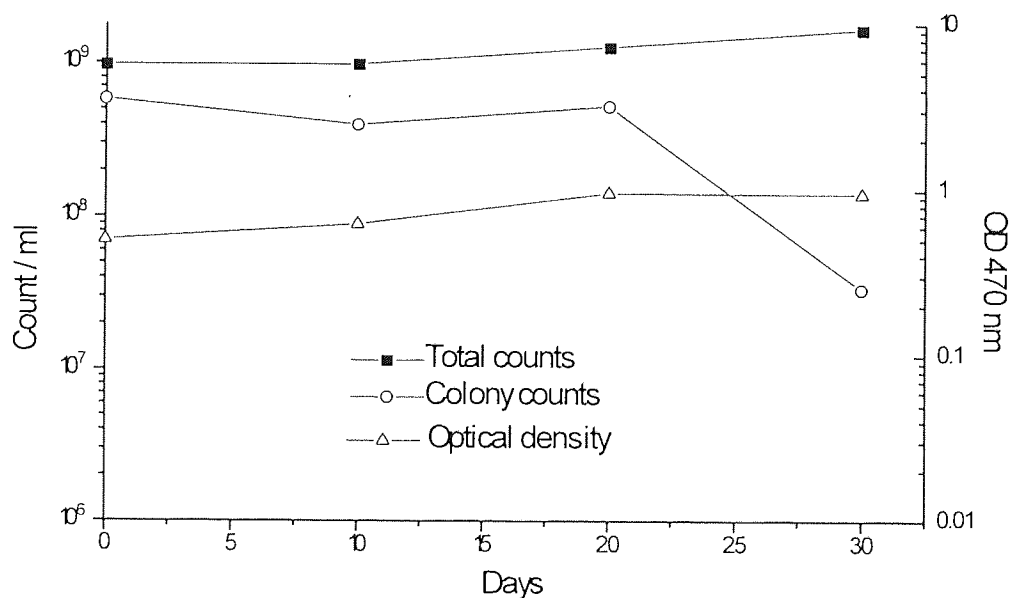
**Fig. 5.6**   Changes in total counts, colony counts and optical density of samples of *P. aeruginosa* 6750 removed from an iron-limited chemostat (0.013 hr$^{-1}$) culture and allowed to stand at room temperature.
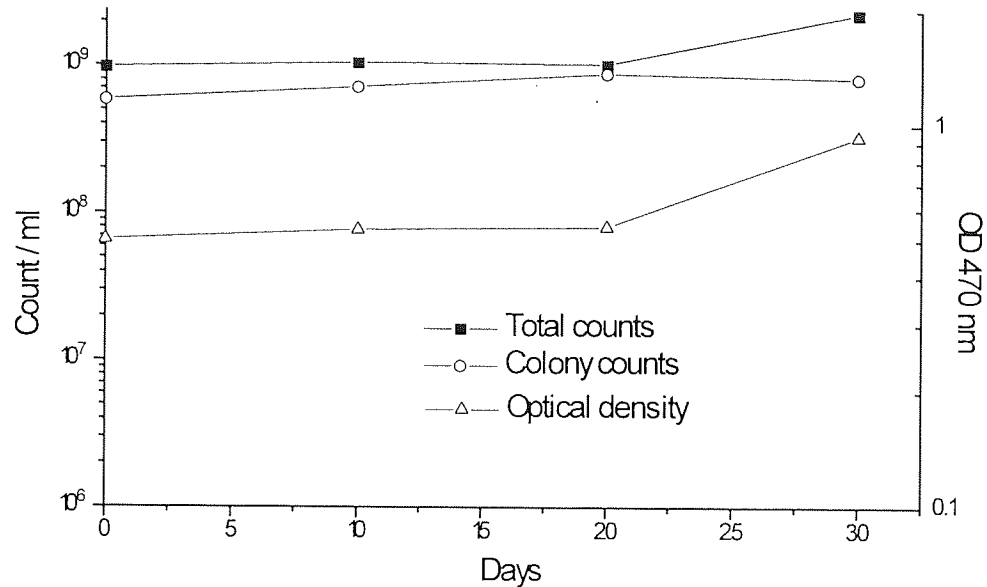


## 5.1.3.1.3. Discussion

Studies were carried out to examine the effect of growth rate and starvation survival on the culturability of a marine bacterium ANT-300 (Moyer and Morita, 1989). The experiment involved the harvesting of samples from the chemostat at several dilution rates and at a temperature of 5°C followed by centrifugation. Batch cell cultures were also analysed at the same time. Total and colony counts were made on the samples over a period of 98 days at 5°C. In general the data showed that three separate stages of physiological changes had occurred : little decrease initially, followed by a large decrease and finally a stabilization of the colony counts. The results demonstrated that at a dilution rate of 0.015 hr$^{-1}$ the culturability of the cells decreased only after 56 days of starvation, when it dropped nearly two log cycles within 1 week. After 70 days of starvation the culturability stabilized at approximately 10$^5$ cfu/ml. In contrast, the batch culture cell began to lose culturability much earlier.

Because of the limitations of time, the experiments with *P. aeruginosa* 6750 were not performed for as long as the above experiment, and were carried out at two different temperatures. However, a similar pattern seemed to be starting to emerge at the temperature of 37°C (which was the temperature of the initial chemostat culture). Little

decrease was observed initially (during the first 20 days). Later, however, nearly one log cycle decrease was demonstrated during the subsequent 10 days. It is not known if there was finally a stabilization of the colony counts. As in the case of the ANT-300 organism, our results from batch culture showed that unculturable cells appeared much earlier i.e. within the first 10 days (fig. 4.7).

It was concluded from the studies of Moyer and Morita (1989) that survival was dependent on the growth rate of the cell source population. This was because cells grown at a dilution rate of 0.015 hr$^{-1}$ showed greater survival than the cell populations from faster dilution rates. This was found not to be the case with *P. aeruginosa* 6750 (data not shown).

The existence of the three stages has also been demonstrated for a marine *Pseudomonas* sp. (Kurath and Morita, 1983). The organism had an initial increase in culturability lasting for 5 days which was considered to be the first stage. Culturability then decreased to 0.1% of the total population after 25 days, after which time the culturability stabilized.

## 5.1.3.2. Direct Viable Count (DVC)

*P. aeruginosa* 6750 grown under continuous culture using a chemostat was subjected to the DVC process, as for batch culture samples (section 4.1.3.4). Results were expressed as percentage of elongated cells compared to total numbers (fig. 5.7). Experiments were performed in duplicate using subsamples obtained at a particular dilution rate. The graph shows that there does not seem to be any variation of percentage elongated cells with dilution rate. A decrease in percentage elongated cells was not seen at lower dilution rates unlike the graph of percentage culturability (fig. 5.4). It was thought that possible inaccuracies found in DVC method using *P. aeruginosa* 6750 could be a reason why no variation in percentage elongated cells with dilution rate was seen. A different concentration of nalidixic acid may be required for cells grown at slow growth rates compared to faster growing cells.

It is also surprising that the graph did not show 100% viability or anything approaching this value for faster growth rates. This is a strong indication that the DVC method using these conditions did not produce expected results with *P. aeruginosa* 6750 grown under iron limitation in a chemostat.

**Fig. 5.7**      Relationship between DVC percentage elongated cells and dilution rate of steady state iron-limited chemostat culture of *P. aeruginosa* 6750 at 37°C.
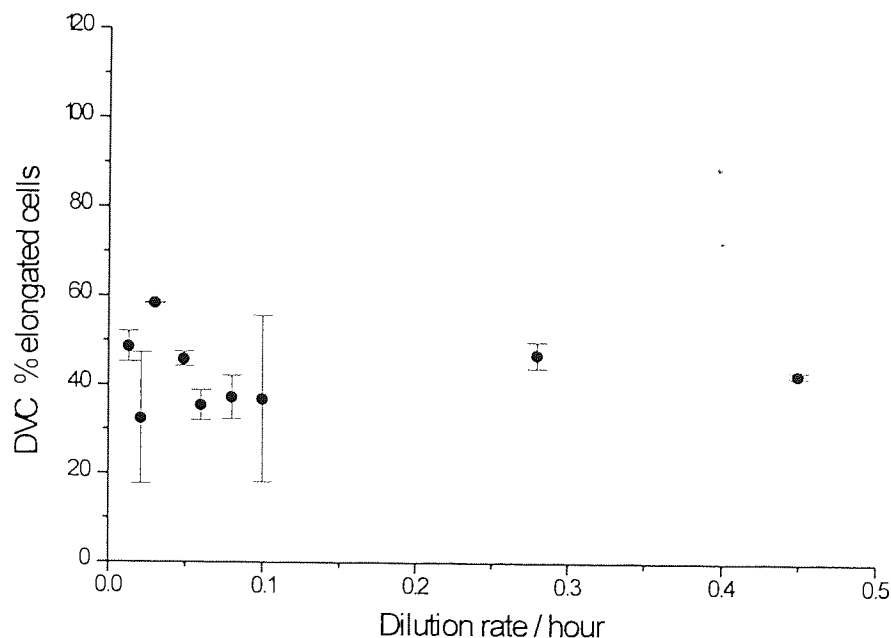
## 5.1.3.3. Measurement of Cell Vitality using CTC

Chemostat cells grown at varying dilution rates were treated with CTC in the same way as batch culture cells. Total and colony counts were made at the same time so that percentage culturability could be expressed on the same graph as percentage vitality (fig. 5.8). Experiments were performed in duplicate using subsamples obtained at a particular dilution rate.

Samples from four dilution rates were analysed. Percentage vitality results did not show any overall trend. Culturability of cells increased from a dilution rate of 0.02 to 0.1 $hr^{-1}$ where a maximum value of 100% was reached. However in contrast, an overall increase in vitality was not seen. It is interesting to note that percentage vitality at 0.02 $hr^{-1}$ was higher than percentage culturability. It is not known if this difference depicts cells that were vital but non culturable at low dilution rates, since there was no such difference at 0.05 $hr^{-1}$ and the difference does not appear to be significant.

**Fig. 5.8**  Relationship between percentage of culturable and vital cells and dilution rate of steady state iron-limited chemostat culture of *P. aeruginosa* 6750 at 37°C.



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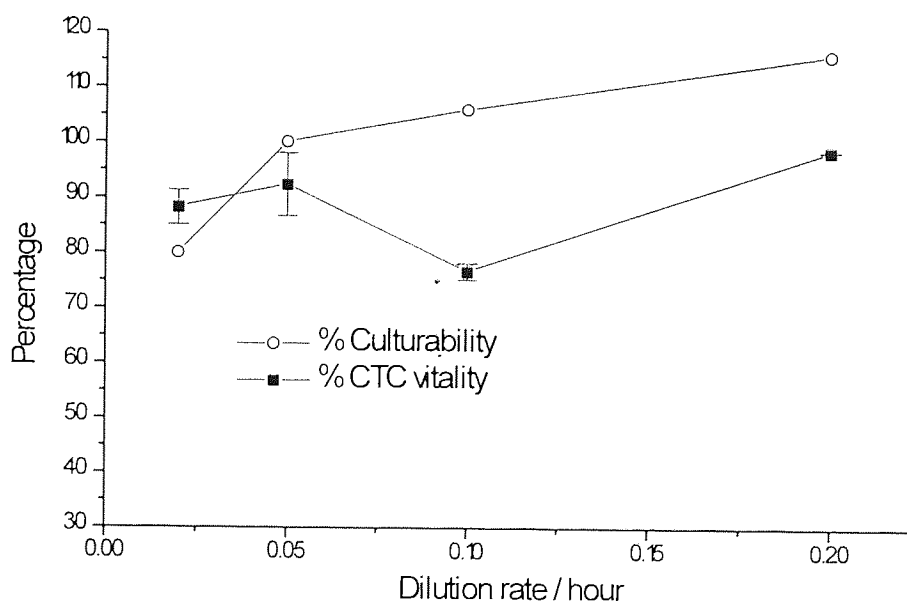
## 5.1.3.3.1. Discussion

Experimental work involving staining to test vitality of chemostat cultures of low dilution rate, was carried out by Kaprelyants and Kell (1992). The culturability of a slowly growing chemostat culture (0.01 hr$^{-1}$) of *Micrococcus luteus* was found to be only around 40-50%. The apparently nonculturable bacteria were able to be resuscitated. It was demonstrated by the use of flow cytometric light scattering and rhodamine 123 accumulation, that the small cells within the heterogeneous population were not dead but increased in size and were accompanied by an increase of membrane energization (associated with the use of rhodamine 123), and an increase of the mean cell size. Additionally, there was an accompanying increase in culturability under conditions of constant cell number. It was suggested that the cells were not dead but dormant capable of being resuscitated into a fully culturable state.

Other workers have studied the decrease in ATP, adenylate energy charge and the ability to accumulate lipophilic cations both in starving cells or in cells that have been cultured at low dilution rates (Horan *et al*, 1981; Jones and Rhodes-Roberts, 1981; Poolman *et al*, 1987). In general, the results of these experiments were unable to show that the bioenergetic parameters could be correlated with the loss of culturability. The disadvantage of these methods was that the measurements were taken from the population as a whole. There was an inability to distinguish if a decrease in ATP level, for instance, was due to the irreversible death of a proportion of the cells' population or caused by a decrease in ATP of all the cells without the accompanying death. The advantage of the studies of Kaprelyants and Kell (1992) was that cells were analysed individually, and as a result their culturability and resuscitation could be quite well correlated with their ability to accumulate rhodamine 123.

Gram-negative bacteria do not accumulate the dye because the dyes do not cross the outer membrane. However, after treatment with Tris and EDTA the dye is able to penetrate the bacteria. Work carried out in our laboratory (Ballestero, 1995 unpublished data) revealed that rhodamine 123 did stain *P.aeruginosa* 6750 after the EDTA treatment, but it was concluded that quantitative data should be obtained by means of a flow cytometer.

## 5.1.3.4. Measurement of Size

### 5.1.3.4.1. R.T.G. Correlator

Measurements of size were made of samples taken from chemostats at different dilution rates. Each sample from a chemostat at a particular dilution rate, was divided into two sub-samples and each was analysed in triplicate. The mean value of the readings was recorded graphically (fig. 5.9).

**Fig. 5.9**    Relationship between apparent mean size and dilution rate of steady state iron-limited chemostat culture of *P. aeruginosa* 6750 at 37°C.
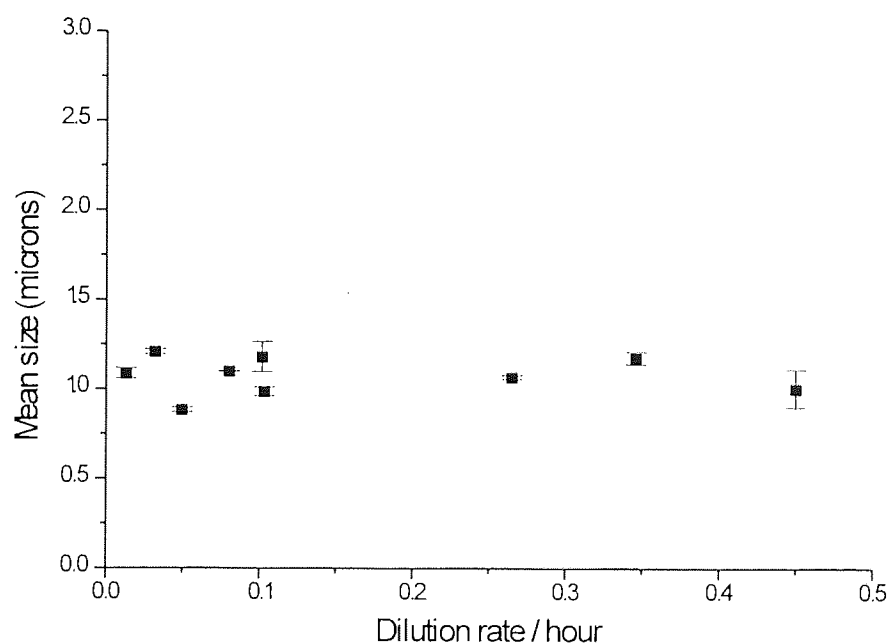


The graph shows that there was little variation in average length of cell with dilution rate. The average length was found to be approximately 1.0μm. Sizes of cells were observed microscopically with the aid of a graticule in order to confirm the readings. There was also less clumping in chemostat samples compared to batch culture. However, since size readings of batch culture were not found to be as accurate with the R.T.G. correlator as with the photographic method (section 4.1.3.8.1) the second method to measure size was also used.

Sizes of cells were measured as for batch culture (section 4.1.3.8.2). Figure 5.10 shows that the average size of cells did not vary significantly with dilution rate. Average size of cells was found to be approximately $2.0\mu m$ in length which was twice the value using the R.T.G. Correlator. The results of size obtained from the chemostat cells were similar in size to the fast-growing exponential cells grown at $\mu_{max}$ after 6 hours incubation, which were around $2.1\mu m$ in length (fig. 4.26). The values at each dilution rate had large standard deviations compared to the results with the R.T.G. correlator. This was thought to be because of the presence of a proportion of small as well as large cells (section 5.1.3.6.1.), especially at low dilution rates.

**Fig. 5.10**      Relationship between mean size and dilution rate of steady state iron-limited chemostat culture of *P. aeruginosa* 6750 at 37°C.
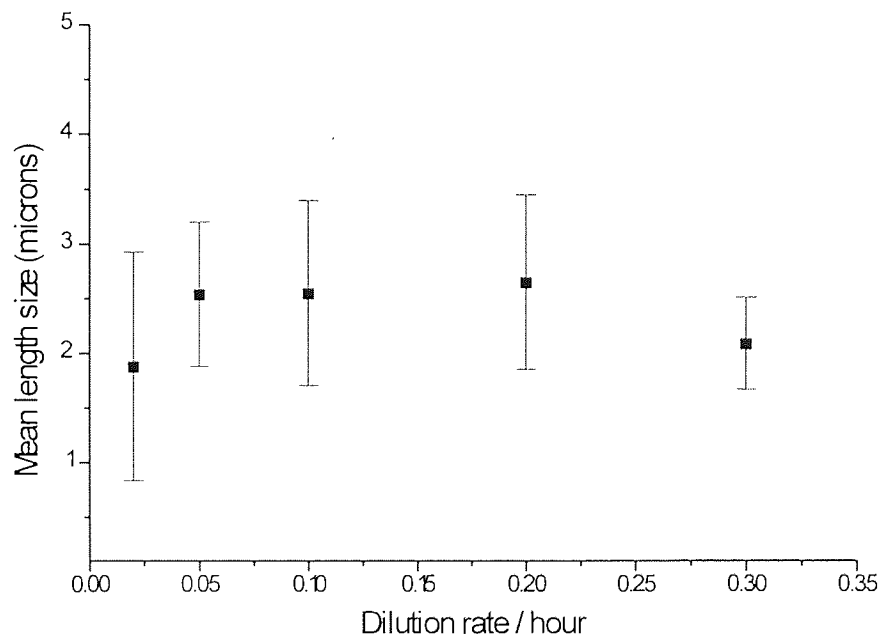
## 5.1.3.4.3. Estimation of Cell Volume

An estimation of cell volume was made using the formula used for batch culture samples (section 3.1.3.4.3) :

$$v = ( d^2 \pi / 4 ) ( l - d ) + \pi d^3 / 6$$

where d = width and l = central length

Width was found not to alter significantly and so an average value of of $0.8\mu m$ was used in the formula.

## 5.1.3.5. Protein Content

Protein content was assessed using the BCA protein assay. Samples were removed at a particular dilution rate and analysed. Figure 5.11 represents the results graphically. The graph shows that between dilution rates of 0.1 $hr^{-1}$ and 0.3 $hr^{-1}$, there was little variation in total protein per ml. At lower dilution rates however, it was seen that there was a slight increase in the amount of protein. This increase can be accounted for by the accompanying increase in optical density which suggested that total numbers had also increased.

Total cell numbers and values for total protein content were combined (fig. 5.12). Results represented graphically prove that there was little variation in protein per $10^8$ cells with change in dilution rate. These results show that chemostat cells possessed different characteristics to old batch culture cells (fig. 4.29). This lack of variation could be due to chemostat cells growing at slow growth rates but still in environments containing high levels of nutrients. This was in contrast to batch culture cells that were actually starved with time.

**Fig. 5.11**     Relationship between protein content, optical density and dilution rate of steady state iron-limited chemostat culture of *P. aeruginosa* 6750 at 37°C.
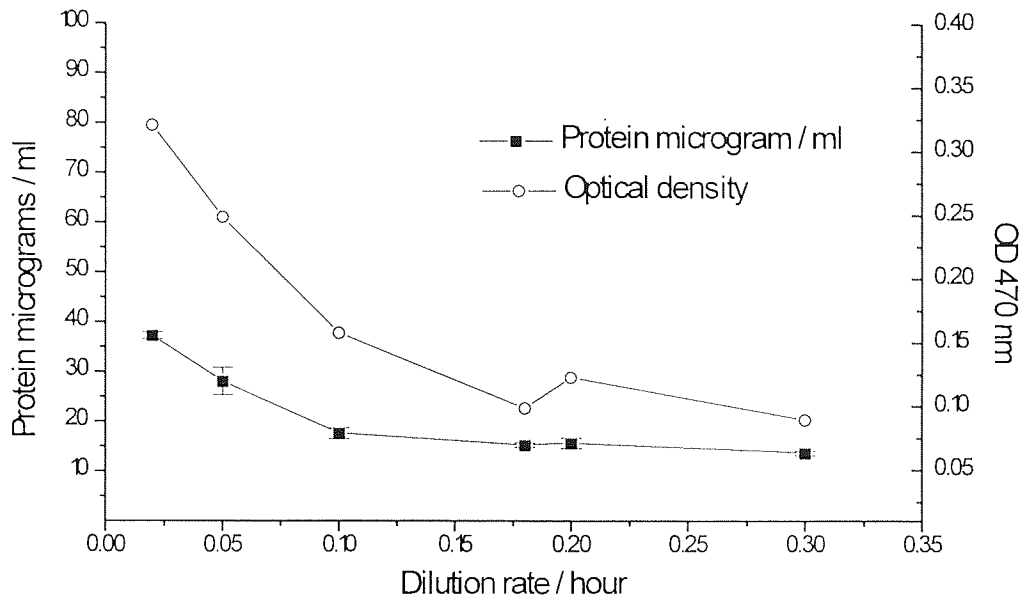


**Fig. 5.12**     Relationship between protein content per $10^8$ cells and dilution rate of steady state iron-limited chemostat culture of *P. aeruginosa* 6750 at 37°C
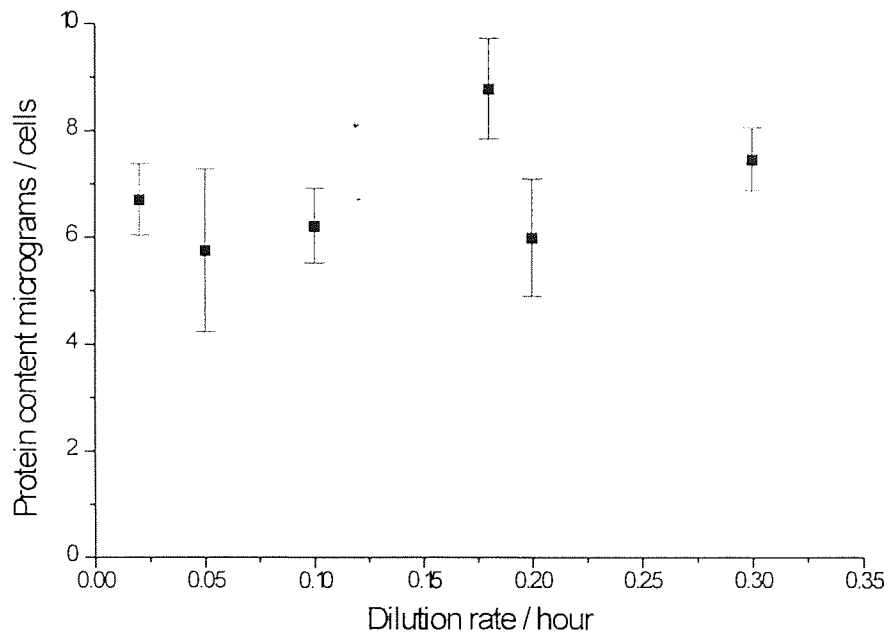
## 5.1.3.6. Protein per Cell Volume

An estimation of the amount of protein per cell volume was made at each dilution rate and results were depicted in figure 5.13. Results show that between dilution rates of 0.05 $hr^{-1}$ and 0.3 $hr^{-1}$ there seemed to be an increase in protein per volume with increase in dilution rate. However, the lowest dilution rate (0.025 $hr^{-1}$) also showed an increase in the quotient compared to the higher dilution rate (0.05 $hr^{-1}$). Therefore, the results are contradictory. These results are different to batch culture results in which a definite increase was shown with time (fig. 4.30).

**Fig. 5.13**    Relationship between protein content per $10^8$ cells per volume and dilution rate of steady state iron-limited chemostat culture of *P. aeruginosa* 6750 at 37°C.
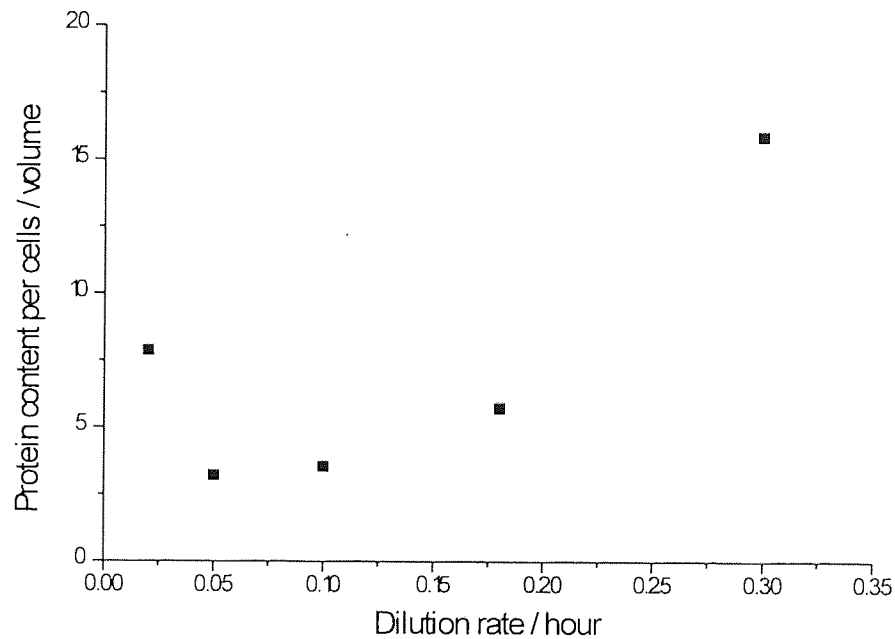
## 5.1.3.6.1. Discussion

The results suggested that there was no compaction of protein in a smaller cell volume with slow-growing iron-limited chemostat cells, in comparison to the results obtained with batch culture (section 4.1.3.11.1).

From the results of cell size, it seemed that even at the slowest growth rates the chemostat cells were still growing at $\mu_{max}$ and so it would not be expected that they would be metabolically inactive and manifest the properties associated with extended survival or dormancy. Different results may be obtained if the chemostat cells were carbon-limited, but in this study the fact that the cells were iron-limited may mean that the cells were not stressed in the same way. This is in contrast to the batch culture cells that were iron-depleted initially but would be starved of carbon as extended batch culture continued. Additionally, in some instances cells have not shown a decrease in size during the survival process. The cell size of *Vibrio* sp. strain S14 increased during the first 6hr of phosphorus starvation, as a result of accumulation of poly-β-hydroxybutyrate (Nyström *et al*, 1992). Therefore, size reduction does not appear to be a universal phenomenon.

Cell size, of chemostat samples at different dilution rates, has been measured by other workers. In general, many bacteria demonstrate a decrease in cell diameter with decreasing dilution rate. It has also been documented for a long time that cell volume is approximately linear with growth rate in chemostat cultures (Maaløe and Kjeldgaard, 1966).

In an experiment that investigated growth of *Aerobacter aerogenes* at low dilution rates, it was demonstrated that at dilution rates of less than 0.05 hr$^{-1}$, the mean size decreased (Tempest *et al*, 1967). In addition it was found that the culture became morphologically more heterogeneous, comprising of long cells and small *cocci*. It has been suggested that this situation is consistent with the idea that the larger cells are growing more rapidly than the smaller cells but if unbalanced growth occurred, the larger cells may even become larger than cells at a higher dilution rate (Kaprelyants *et al*, 1993). This could be a possible explanation for the large cells seen at low dilution rates in the present study, with *P. aeruginosa* 6750. In this study, the smaller cells may not have been seen very easily using the photographic method. The smallest cell size that was reported with the batch culture cells, was indeed only 0.7μm, using the photographic method. Further to this, the large standard errors of the points at low dilution rates may in fact encompass small cells as well as large ones. It can also be seen that, in general, the standard errors do become smaller with increasing dilution rate.

In another experiment, *Saccharomyces cerevisia* was grown in a glucose-limited chemostat at slow growth rates (Bugeja *et al*, 1982). Two types of cell were produced under these conditions, namely phase-bright and phase-dark cells. Both forms of the cell were small in size relative to those seen at high growth rates. The phase-bright cells were unable to multiply whereas the phase-dark ones underwent replication normally.

Cell length distributions of *P. aeruginosa* 6750 have also been measured, using a similar method to the photographic method used here (Gilbert *et al*, 1981). The organism was grown in a magnesium-limited chemostat at growth rates of between $0.037$ hr$^{-1}$ and $0.621$ hr$^{-1}$. In general, mean cell length decreased with decreasing growth rate.

Studies investigating cell volumes were performed with *P. aeruginosa*, by measuring the lengths and widths of cells using an ocular micrometer (Robinson *et al*, 1984). Cells were analysed from chemostats that were carbon-limited, at dilution rates of between $0.05$ and $0.4$ hr$^{-1}$. Once again, the cells of *P. aeruginosa* were found to increase in length with increasing dilution rate. The range of cellular lengths observed was 1.5 to 2.75 $\mu$m, which showed that it was not a wide range. In the present study, the range of mean cellular lengths was 1.88 to 2.65$\mu$m from dilution rates of 0.02 and 0.2 hr$^{-1}$ respectively. The two sets of data are comparable but the dilution rates examined in this experiment were lower than the ones of Robinson *et al* (1984). Another point to mention with the *P. aeruginosa* 6750 results, concerns the value obtained at 0.3 hr$^{-1}$ dilution rate, which did not follow the trend and produced a mean cell length that is 2.09$\mu$m. In general, from the data shown the results were not conclusive.

## 5.1.3.7. Antibiotic Susceptibility

Chemostat cells grown at specific growth rates were subjected to antibiotic treatment as for batch culture cells.

## 5.1.3.7.1. Ciprofloxacin

Percentage survival of cells was represented graphically (fig. 5.14). Results showed that there was an approximate 2 log cycle difference between the fastest dilution rate (0.45 $hr^{-1}$) and the slowest one (0.013 $hr^{-1}$). There was a progressive increase in percentage survival as dilution rate decreased. A maximum value for percentage surviving fraction was attained at a dilution rate of 0.043 $hr^{-1}$. Beyond this point, as dilution rate decreased further, little increase in surviving fraction occurred.

**Fig. 5.14**    Relationship between survival of iron-limited chemostat culture of *P. aeruginosa* 6750 and dilution rate, after incubation with 0.5µg/ml ciprofloxacin.
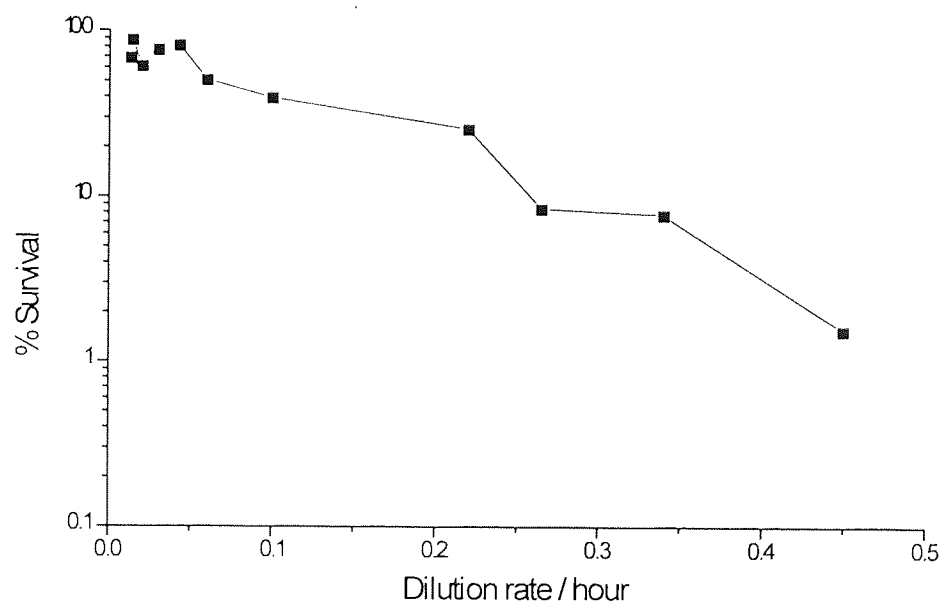
## 5.1.3.7.2. Polymyxin B

The graph representing percentage survival of chemostat cells with polymyxin B (fig.5.15) is similar to that of ciprofloxacin (fig. 5.14). There was a two log cycle difference between the fastest dilution rate (0.45 $hr^{-1}$) and the slowest (0.014 $hr^{-1}$). An apparently linear relationship existed between percentage survival and dilution rate. The results showed that as dilution rate decreased so there was an accompanying increase in the survival of the organism.

**Fig. 5.15**    Relationship between survival of iron-limited chemostat culture of *P. aeruginosa* 6750 and dilution rate, after incubation with 0.5µg/ml polymyxin B.
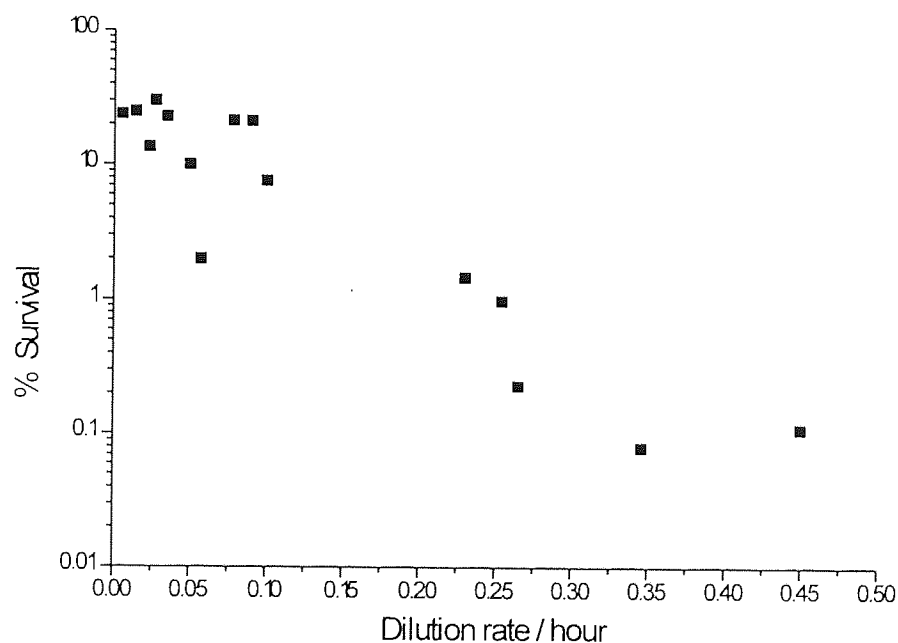
## 5.1.3.7.3. Discussion

It has been reported widely that the antibiotic susceptibility of chemostat cells is dependent on the growth rate. Various species of bacteria have been examined with different antimicrobial agents using different nutrient limitations.

One study investigated the influence of nutrient limitation and growth rate in a chemostat, on the sensitivity of *P. aeruginosa* to polymyxin and EDTA (Finch and Brown, 1975). It was shown that the specific nutrient limitation influenced the antibiotic susceptibility : carbon-limited bacteria showed no change in its high sensitivity to polymyxin with differing growth rates. Polymyxin susceptibility of magnesium-limited cultures was also found to be independent of growth rate although the sensitivity of the bacteria was six times greater than that of the carbon-limited cells. These results are in contrast to the ones obtained in the current study, which show that the sensitivity of iron-limited cells is dependent on growth rate. Differences may be due to the alterations in nutrient limitation. Other workers have found that sensitivity to polymyxin was greater at high dilution rates compared to low ones for *P. aeruginosa* grown under magnesium, carbon and phosphorus limitation (Melling, Robinson and Ellwood, 1974). However, in this study, the experimental conditions were not the same as those employed by Finch and Brown.

The sensitivity of *E. coli* to polymyxin B was examined at a variety of growth rates under conditions of carbon, nitrogen, phosphorus and magnesium limitation (Wright and Gilbert, 1987). A divergent pattern of sensitivity was reported with the different nutrient limitations. It was demonstrated that magnesium and phosphorus-limited cells possessed increased resistance with increasing growth rate whereas carbon and nitrogen-limited cells possessed increased resistance with decreasing growth rates. This provided further evidence to suggest that sensitivity of polymyxin is dependent on nutrient limitation as well as growth rate.

Experiments have been performed to test the susceptibility of chemostat grown *P. aeruginosa* with ciprofloxacin, primarily as a control in order to compare the findings of those results obtained with *P. aeruginosa* biofilms (Evans *et al*, 1991). However, the results are useful to compare with our chemostat data. They show that the susceptibility of the two *P. aeruginosa* isolates, that were used in the study, were dependent directly on the specific growth rate.

It has also been demonstrated that populations of chemostat cells, grown in both iron-limited and iron-deficient conditions, possess sensitivity to ciprofloxacin which was growth rate dependent (Nelson, 1993).

Other investigations with different antibiotics to those used in this study confirm the data obtained with *P. aeruginosa* 6750. The finding of Tuomanen *et al* (1986) stated that the killing rates of *E. coli* by β-lactam antibiotics were a constant function of the bacterial generation time which meant that the killing was proportional to growth rate. Experiments with *E. coli* and *P. aeruginosa* respectively have studied the effect of β-lactam antibiotics and have arrived at the same conclusion (Cozens *et al*, 1986; Wu and Livermore, 1990). Another study established the dependence of the interaction of ceftazidime and gentamicin on bacterial growth rate against *P. aeruginosa* (Chen *et al*, 1991).

Despite the fact that many experiments prove the growth rate dependence of the killing by antibiotics, it is not clear if the increased resistance of cells seen at slow growth rates can be correlated to the unculturable cells and possible dormancy. From the data, it seems that there is no marked increase in resistance occurs at the point at which unculturable cells appear. It would seem that other tests of vitality are better able to examine the hypothesis.

## 5.1.4. Overall Discussion of Chemostat Studies

Overall results with chemostat cells seem to imply that increase in resistance of *P. aeruginosa* 6750 occurred at low dilution rates, indicating that it is slow growth rate of the organism which causes this increase. However the appearance of unculturability was also seen at low dilution rates. Percentage culturability decreased as dilution rate decreased. It is not known if the two phenomena can be related in some way. It could be suggested that the apparent appearance of unculturable cells is an indication of possible dormant cells which may possess properties of enhanced resistance. This was not seen in the results of the present study but additionally would be difficult to show as mentioned in the discussion for batch culture (section 4.1.3.15), because the assay for resistance involved culturing cells using colony counts.

Studies that attempted to elucidate if unculturable cells possessed some degree of vitality were performed but were not conclusive. Both DVC and CTC assays did not show that there existed vital but non culturable cells.

In contrast to the batch culture results, no significant decrease in size was seen as dilution rate was lowered. The mean size of the chemostat cells was around 2$\mu$m, which was the approximate size of the batch culture cells during exponential phase growing at $\mu_{max}$. This indicates that the iron-limited chemostat cells were not equivalent to the starved batch culture cells. There was also no increase in protein content per cell, thereby indicating that chemostat cells were not starved and so perhaps did not enter a phase in which vital processes were reduced.

# 6.   COMPOSITION OF THE GRAM-NEGATIVE CELL ENVELOPE

## 6.1   Introduction

The cell envelope is in contact with the external environment. Its ability to adapt to a changing environment during growth is essential to the cells' survival. These changes are brought about through the remarkable plasticity of this cell envelope, which enables the cell to vary its composition according to the growth conditions. Additionally, it is also vital for the cell to adapt to adverse conditions encountered during the extended stationary phase particularly during starvation.

## 6.1.1. Composition, Structure and Function of the Cell Envelope

This section provides a basic overview of the composition, structure and function of the cell envelope of Gram-negative bacteria. Many reviews have covered this subject comprehensively (Hammond *et al*, 1984; Lugtenberg and Van Alphen, 1983: Lambert, 1988).

The cell envelope of the Gram-negative bacterium is essentially composed of three layers : the inner cytoplasmic membrane (CM), the peptidoglycan layer and the outer membrane (OM). A number of other cell components occupy the outer region of the cell envelope : capsules, which consist mainly of polysaccharides, flagella and fimbriae.

The cytoplasmic membrane is an osmotic barrier. There are thought to be 200 to 300 adhesion sites in which the cytoplasmic membrane and outer membrane are fused (Bayer, 1979). These regions are possibly areas at which export and assembly of outer membrane components take place. The cytoplasmic membrane is mainly composed of phospholipids and protein. The membrane also possesses a considerable amount of enzymatic activity. It contains many enzymes, permeases and various components of the respiratory chain. This membrane is also involved in the synthesis and transport of molecules found outside the membrane. The enzymes involved in the synthesis of the O-antigen layer and core region of the LPS have also been found in the CM (Osborne *et al*, 1972). These components are synthesised in the membrane and translocated through it to the outside membrane.

The peptidoglycan layer lies between the CM and the OM. It accounts for 10 to 20% of the weight of the envelope. It consists of glycan strands that are cross-linked by peptide subunits. The importance of this layer lies in the fact that it provides the basic skeleton of the envelope around which the other components of the envelope are assembled (Lambert, 1988). Evidence obtained from  electron microscopy has suggested that the

peptidoglycan layer forms an extensive gel-like structure which occupies the entire region between the CM and OM, known as the "periplasmic space" or "periplasm" (Hobot *et al*, 1984). However, other findings have reported that this layer is actually a monolayered structure (Wientjes *et al*, 1991). A low molecular weight protein, Braun lipoprotein, is the only component that is covalently attached to the peptidoglycan (Braun, 1975).

It is thought that this layer is vital to the survival of the cell. It is responsible for the size and shape of the cells and it has been reported that its assembly during growth must be carefully controlled in order to maintain the rod shape of the bacterium (Lambert, 1988). It is known that antibiotics that interfere with the assembly of peptidoglycan cause alterations in cell morphology : filamentation, round forms or lysis can be produced (Tipper, 1985). It is also established that dramatic changes to cell morphology occur as a survival process during for example, prolonged starvation, which implies that there could be an involvement of the processes of peptidoglycan synthesis. Experiments with *V. vulnificus* and *V. cholerae* have shown that there does seem to be such an involvement (section 1.7.2).

The OM has a higher density than the CM and has a different composition. The OM is composed of three main components phospholipids, proteins and LPS which are in roughly equal proportions (Lambert, 1988). A unique feature of the OM is the asymmetric nature of the distribution of phospholipid and LPS in the bilayer. The phospholipid is situated at the inner face of the layer whereas the LPS occupies the outer face. In this way, the polysaccharide O-antigen chains are exposed on the outer surface of the OM (Muhlradt and Golecki, 1975). The OM has little enzymatic activity compared to the CM, and contains a range of proteins.

The LPS of the Gram-negative OM is unique feature of the OM. In *P. aeruginosa* the LPS is one of the organisms major virulence factors (Cryz *et al*, 1984). In general it comprises three covalently linked segments : the lipid A region, which is hydrophobic, embedded in the OM and highly conserved in all Gram-negative bacteria; the core polysaccharide region which is hydrophilic and similar in composition for many species; and the O-specific polysaccharide side chain which is also hydrophilic, variable in composition (Hitchcock and Brown, 1983) and used for serotyping within a species. Strains that are devoid of the O-specific side chain result in cells that produce a rough colony morphology whereas those that contain this antigen present a smooth appearance. The length of the O-chains has an important influence on host defences including serum killing and phagocytosis (Taylor, 1983; Joiner *et al*, 1986) as well as an affect on the permeability of the OM to antibiotics (Nikaido and Vaara, 1985).

Recent studies have shown that *P. aeruginosa* produces two antigenically and chemically distinct LPS molecules (Rivera *et al*, 1984; Rivera and McGroaty, 1989). These have been identified as A-band LPS which is a mainly neutral polysaccharide consisting of D-rhamnan and is antigenically conserved, whereas the B-band is the O-antigen containing LPS and is highly charged (Lam *et al*, 1989). Differences in the structure of the B-band have formed the basis of an O-serotyping classification of *P. aeruginosa*. Seventeen standard serotypes (Liu *et al*, 1983) have been discovered as well as an additional 3 that have been recently added to the International Antigenic Typing Scheme (Liu and Wang, 1990).

In contrast to this classification, the core oligosaccharide structure has not been fully documented (DeKievit and Lam, 1994). Additionally, as the LPS lipid A region is considered more conserved among Gram-negative organisms than the O-side chain region, it has been used as a major target for the generation of antibodies and therapeutic agents aimed to antagonize sepsis. In a recent study, four groups of monoclonal antibodies have been isolated which recognize four distinct regions within *P. aeruginosa* : core-plus-one O-repeat unit, outer core, inner core and lipid A (DeKievit and Lam, 1994).

Experimental evidence has demonstrated a rapid and significant decline in lipid in general during starvation for different organisms. This has been reported during the starvation of *Escherichia coli* (Lappin-Scott and Costerton, 1990; Oliver, 1993), *Vibrio vulnificus* (Linder and Oliver, 1989), *Vibrio cholerae* (Hood *et al*, 1986) and *Micrococcus luteus* (Mukamolova *et al*, 1995b).

### 6.1.1.1. Outer Membrane Proteins

The separation of the OM proteins of *P. aeuginosa* into major bands using sodium dodecyl sulphate-polyacrylamide gel electrophoresis was established by Hancock and Carey (1979). The proteins that are isolated are individually named after the genes that encode them, as Omp proteins.

*P. aeruginosa* produces a range of high molecular weight (75-90KDa) Omps as a result of iron deprivation. The organism synthesizes low molecular weight iron chelating agents (siderophores), namely pyochelin and pyoverdin, that are used with which to complex iron. The iron-regulated outer membrane proteins (IROMP) act as receptor proteins and are involved in the uptake and transport of the siderophore-iron complex. The IROMPs have been shown to be produced both *in vitro* and *in vivo* situations (Anwar *et al*, 1984; Shand *et al*, 1991). It has now been proved that some of these

IROMPs of *P. aeruginosa* are directly involved in the transport of iron (Poole *et al*, 1991; Smith *et al*, 1992).

Another major group of proteins are the porin proteins. These proteins are major outer membrane proteins which form transmembrane pores in the OM. The main function of these water-filled pores or channels is to allow the diffusion of relatively small hydrophilic molecules of up to 600D to cross the OM (Nikaido and Nakae, 1979).

OprB (formerly known as D1) and protein D2 (OprD2) both function as porin proteins and are of a similar order of molecular weight (Hancock and Carey, 1979). OprB is thought to form a channel which crosses the OM and facilitates the diffusion of glucose into the periplasm (Hancock and Carey, 1980). A periplasmic glucose binding protein is also thought to be involved and it is suggested that it transports the glucose across the periplasm to as yet unidentified inner membrane transport proteins (Wylie *et al*, 1993). OprD2 functions as a specific porin channel for the diffusion of basic amino acids and peptides (Trias and Nikaido, 1990a). It has also been discovered that this protein facilitates the passage of the carbapenem antibiotic, imipenem, and its analogues across the OM (Trias and Nikaido, 1990b, Fukuoka *et al*, 1991).

Proteins C and E1 (OprC and OprE1) are considered to be more general porins (Yoshihara and Nakae, 1989). OprC has also been involved in the passage of anionic β-lactams through the OM of *P. aeruginosa* (Satake *et al*, 1990). Protein P (OprP) is another porin protein which is expressed under conditions of low phosphate (Hancock *et al*, 1982).

There has been much debate surrounding the function of protein F (OprF). The protein was thought to be a major porin protein of *P. aeruginosa* (Hancock *et al*, 1979; Yoshimura *et al*, 1983). Later work, performed by other workers, disputed this protein's porin-forming ability (Yoshihara and Nakae, 1989). More recent work, however, has maintained that OprF is a major porin protein in *P. aeruginosa* and forms channels through the outer membrane that are large enough to allow the passage of di- and tri-saccharides (Bellido *et al*, 1992). Other studies have discovered an additional role for this protein. Experiments performed with OprF-deficient mutants have found that the cells possessed rounded morphology and grew well only in high osmolarity media (Gotoh *et al*, 1989; Woodruff and Hancock, 1986). This work suggested that OprF was involved in both OM stability and cell structure.

The induction of Protein G (OprG) is influenced by various growth conditions. Iron availability, temperature and growth phase are known to affect the expression of this protein (Hancock and Carey, 1979; Ohkawa *et al*, 1980; Kropinski *et al*, 1987). It has

been suggested that the protein is involved in fluoroquinolone uptake (Chamberland *et al*, 1989) and low affinity iron uptake (Yates *et al*, 1989).

Proteins H1 (OprH) and H2 (OprL) possess similar molecular weights. OprH is expressed in *P. aeruginosa* under specific cationic deficiencies. It has been shown that OprH replaces the divalent cations at the OM binding sites. In this way, the presence of the protein inhibits the self-promoted uptake pathway of some polycationic antibiotics (section 1.14.2.1; Nikaido and Hancock, 1986; Bell *et al*, 1991). OprL is considered to be a lipoprotein, which contains covalently linked fatty acids, and is non-covalently associated with the peptidoglycan (Mizuno, 1981; Hancock *et al*, 1981b). It is thought that this protein, which is not known to be surface-exposed (Lambert and Booth, 1982), is considered to be involved in the maintenance of stability of the OM (Anwar *et al*, 1983). Another lipoprotein of lower molecular weight, lipoprotein I (OprI) is thought to be similar to Braun's lipoprotein (Mizuno and Kageyama, 1979).

The investigations performed in this study focused on the Omp and LPS profiles of *P. aeruginosa* in iron-limited media during extended culture. There has been research in this area of work on different organisms, that has been focussed on specific starvation proteins that are now known to be produced and including their regulation (section 1.7 of introduction).

## 6.2. Results

Figure 6.1 depicts a Coomassie blue stained mini preparation of OMP profiles of *P. aeruginosa* 6750 at exponential phase and after prolonged incubation in iron-depleted $CDM_{12}$. More specifically, the three lanes represented batch culture samples from early exponential phase, after 6.5 hrs incubation, early stationary phase cells after 28.5 hrs incubation and late stationary phase cells after 7 days incubation respectively.

In general, there was no significant difference between the 28.5 hr and 7 day samples. Two main differences could be seen between the exponential phase sample and the stationary phase samples : the two high molecular weight IROMPs were more strongly expressed in the stationary phase samples and there appeared to be a progressive stronger expression of OprH. Other bands that also appeared stronger in the stationary phase samples could be seen at the 46KDa mark and could correspond to OprB as well as there being a band at the 60KDa mark. There appeared to be little expression of OprG despite the growth of *P. aeruginosa* in iron-limited medium.

**Fig. 6.1.**     Coomassie blue-stained SDS-PAGE OMP profiles of *P. aeruginosa* 6750 sampled through growth in iron-limited batch culture
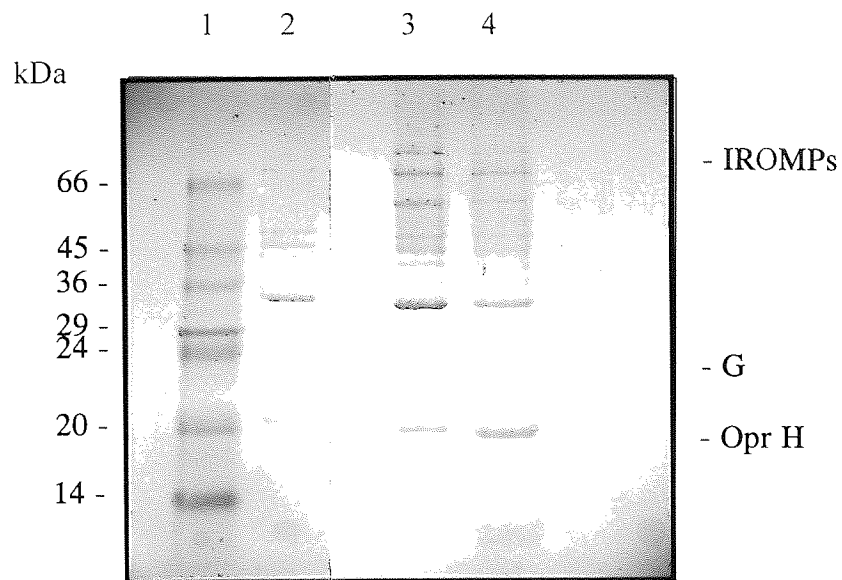


| Lane | Sample |
| --- | --- |
| 1 | marker |
| 2 | early exponential batch culture (6.5hr) |
| 3 | early stationary phase culture (28.5hr) |
| 4 | late stationary phase culture (7 days) |

Figure 6.2 shows a Coomassie stained mini preparation of OMP profiles of *P. aeruginosa* grown in chemostat culture at different growth rates. In the chemostat samples, in contrast to the batch culture results, it could be seen, albeit weakly, that protein G was present. Additionally it seemed to appear to be increasing in its expression with decreasing growth rate. The expression of the IROMPs also seemed to alter with growth rate. The slower growth rates expressed larger numbers of IROMPs than the faster growth rates.

The results of LPS profiles are depicted in figure 6.3. LPS from batch culture cells that were incubated for different time periods were analysed. The LPS profile was run at least three times with different samples in order to evaluate LPS during stationary phase. The samples were harvested from cultures that were at early stationary phase, after 24.5 hr, and late stationary phase after 4 and 17 days respectively. The results demonstrated that there were similar amounts of lipid A present in each sample so that samples were comparable. The most striking feature of the profile was that, despite the absence of banding which depicted the presence of O-antigen, it was clearly seen that there was a marked difference in the amount of high molecular weight bands between the three samples. These bands suggested the presence of differing lengths of B-band in the LPS.

**Fig. 6.2.** Coomassie blue-stained SDS-PAGE OMP profiles of *P. aeruginosa* 6750 grown in chemostat iron-limited culture
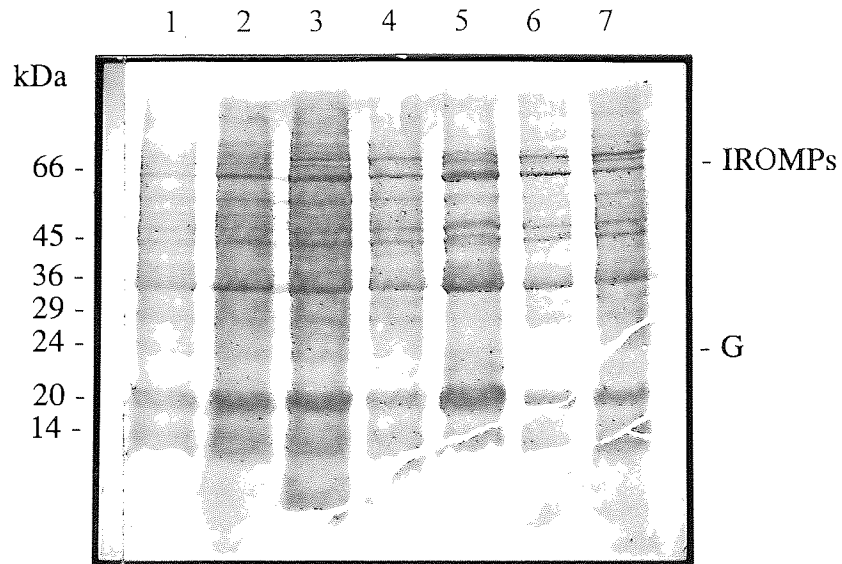


| Lane | Sample |
|------|--------|
| 1 | chemostat sample D= 0.005hr$^{-1}$ |
| 2 | chemostat sample D= 0.014hr$^{-1}$ |
| 3 | chemostat sample D= 0.027hr$^{-1}$ |
| 4 | chemostat sample D= 0.046hr$^{-1}$ |
| 5 | chemostat sample D= 0.078hr$^{-1}$ |
| 6 | chemostat sample D= 0.11hr$^{-1}$ |
| 7 | chemostat sample D= 0.226hr$^{-1}$ |

**Fig. 6.3.**    Silver-stained SDS-PAGE LPS profiles of *P. aeruginosa* 6750 grown in iron-limited batch culture.
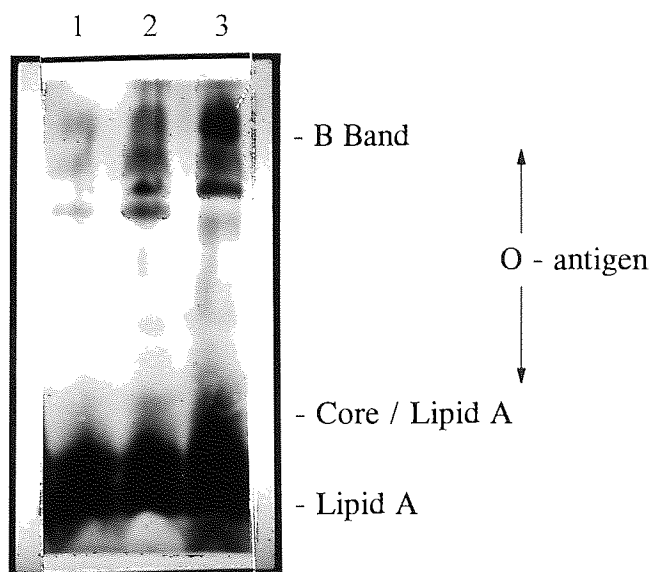


| Lane | Sample |
|------|--------|
| 1 | early stationary phase batch culture (24.5hr) |
| 2 | early stationary phase batch culture (4 days) |
| 3 | late stationary phase batch culture (17 days) |

## 6.3. Discussion

The OMP profiles of the batch culture cells did not exhibit significant differences during prolonged incubation. These findings were in contrast to those obtained with *Pseudomonas fluorescens* and *Pseudomonas putida* in which starvation-induced proteins were found in the OM profiles (Kragelund and Nybroe, 1994; section 1.7.1). However, in a recent study that investigated the starvation-survival processes of the bacterial fish pathogen *Yersinia ruckeri*, similar results in protein profiles were obtained. Nonculturable cells were found not to exhibit changes in their outer membrane proteins (Romalde *et al*, 1994).

The increased expression of IROMPs was to be expected as these proteins would be produced during late exponential phase or early stationary phase. However, there was little difference between the early and late stationary phase profiles as far as IROMPs were concerned.

There appeared to be an increased expression in OprH in the older stationary phase profile. OprH is thought to replace divalent cations that are present in the OM conferring stability. The protein is thought to be over-expressed when the growth medium is limited for these ions. The appearance of this protein after extended stationary phase could be because of the limitation of the ions or perhaps because of the reduced amount of the carbon source after prolonged incubation. Bell *et al* (1991) described the association of the expression of the OprH gene with glucose minimal media and so this may have been the reason for its production in this study.

The band that was also found to be strong in the stationary phase cultures appeared at around 46KDa and was thought to be OprB. This glucose-inducible pore (Hancock and Carey, 1980) was thought to be present because of reduced amounts of glucose in cultures undergoing prolonged incubation. The expression of the band at the 60KDa mark was thought to be a protein of unknown identity (Nelson, 1993).

The results obtained from the chemostat samples also showed that growth rate appeared to influence the expression of IROMPs. Therefore, the results of batch culture and chemostat culture show that both growth phase and growth rate influence the expression of IROMPs. The expression of OprG seemed to be influenced by growth rate. The results were similar to those obtained by Yates (1992), who demonstrated that OprG was expressed only at relatively slow growth rates.

The results of the LPS profile showed that there appeared to be an increase in the length of the B-band, part of the O-antigen of the LPS molecule, as the cells became older. It is

not known if this demonstrated an actual increase in length or if this corresponded to the O-antigen of the LPS which had become more easily oxidizable with time, and so manifested itself by an increased amount of material. Further studies would have to be performed in order to investigate if this change was part of the survival process of *P. aeruginosa* 6750.

Another possibility could be that the increase in B-band was attributed to the increase in the smooth colony type (see section 3.1.2.1) compared to the rough type (not quantified). It is not known if the reduction in rough colonies was a survival strategy or that the cells produced the B-band, thereby producing cells with a survival advantage. Further experiments using perhaps 100 % rough or 100% smooth colony types at the start of the prolonged incubations could clarify the situation.

Similar results were obtained with the investigations of the LPS profiles of *Yersinia ruckeri* (Romalde *et al*, 1994). The results demonstrated that slight differences could be seen : an increase was seen in the high molecular mass O-antigen region.

Experiments have also analysed the concentrations of total lipids and carbohydrates of *Vibrio cholerae* over a 30 day starvation period (Hood *et al*, 1986). Both total lipids and carbohydrates were found to decline rapidly within the first 7 days. However, carbohydrate profiles revealed the relative loss of the five-carbon sugar ribose and *N*-acetylglucosamine and a concomitant relative increase in the total six-carbon sugars. Similar studies on *P. aeruginosa* 6750 would help to examine specific changes in lipid and carbohydrate profiles.

# 7.    CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

The initial aim of the study was to develop a system with which *P. aeruginosa* 6750 could be grown as a biofilm. The proposed model was to produce a growth controlled system in which to study the effects of growth rate, to test antimicrobials and to analyse the survival of the organism. The work was based on that of Gilbert *et al* (1989), in which a growth controlled biofilm was produced with *E. coli*. The main problem with the use of *P. aeruginosa* in this system was the increasing production of the organism and associated exopolysaccharide which resulted in the blockage of the membrane and termination of perfusion of the fresh media.

The limited experimental data that was obtained with this biofilm system provided some information with regards to the rough and smooth colony types of the organism. It was discovered that, in general, with the three flow rates tested, there was an increase in the percentage of the smooth colony types when grown as a biofilm. The significance of this finding was not known, particularly as other workers found an increase in the rough type LPS when using clinical isolates (Giwercman *et al*, 1992).

The development of a growth-controlled *P. aeruginosa* 6750 biofilm model was therefore abandoned, in favour of investigating the survival of the organism in batch culture and a chemostat, to continue the pursuit of the effects of changes in growth rate.

The survival of *P. aeruginosa* 6750 was carried out with the use of various parameters, the total count/colony count ratio being the first parameter investigated. In batch culture, *P. aeruginosa* 6750 was found to lose culturability during the time periods investigated but not completely. Experiments were focused on growth of the organism in iron-depleted media because of the application of the survival of *P. aeruginosa* to situations *in vivo* e.g. the CF lung in which the body withholds iron from microorganisms. A relatively quick loss of culturability of *P. aeruginosa* 6750 in iron-depleted media was observed initially followed by a much slower loss.

The DVC test and CTC staining were used to provide an indication of the vitality of the cells during prolonged incubation. The results of the DVC and CTC tests did not shown that there was a population of cells that was vital but nonculturable.

Investigations were also performed to examine the effect of temperature downshift on the culturability of *P. aeruginosa* 6750. This work was based on that of Oliver *et al* (1991). Phosphate and glucose-depleted cultures of the organism were found to have a reduced loss in culturability at 4°C compared with at 37°C. The iron-depleted culture, however, became unculturable after approximately 88 days. A resuscitation experiment

performed when the culture possessed colony counts of $10^4$/ml showed that the colony count increased to the original count after a temperature upshift. The results were not absolutely conclusive as the culture was not completely unculturable when the resuscitation experiment was performed. It was suggested later, however, that if the $10^4$/ml culture had been diluted sufficiently before the resuscitation experiment had taken place, the result would be more definite as the probability of the increase in colony counts arising from regrowth as opposed to unculturable cells becoming culturable again would be small.

The influence of growth phase on culturability was also investigated. Overall, it was concluded that the exponential phase cells stored at 4°C became unculturable at a later time point compared to the stationary phase cells. This was in contrast to the results of Oliver *et al* (1991) in which it was found that exponential phase cells became unculturable earlier than stationary phase cells.

Size was another parameter that was measured. The photographic method was found to be more useful than the RTG correlator. The results showed that there was a decrease in cell size when cells entered the stationary phase. An estimation of cell volume was made using the cell size data. Protein content of the cells during incubation was also performed which demonstrated an increase in protein content per $10^8$ cells. After analysis of the cell volume data and protein content data, the protein content per $10^8$ cells/volume was represented graphically. It was suggested that the initial increase that was observed, was a result of changes that occurred as cells entered the stationary phase and the larger later increase occurred as a result of the protein being compacted into a smaller volume.

The lag of cells was measured as an indication of cells' slowness to divide and possible measure of dormancy. The results demonstrated that the older culture inoculum took a longer time to reach a certain OD compared to an overnight culture inoculum.

Finally, antibiotic studies demonstrated that early exponential phase cells were more sensitive to ciprofloxacin than late exponential phase cells. Susceptibility tests that were performed along the growth curves of *P. aeruginosa* 6750 showed that the resistance of the organism to both ciprofloxacin and polymyxin B increased in general, until the cessation of growth. After this time no further increase in resistance was demonstrated.

The parameters that were used on batch culture cells were then applied to chemostat cells grown at different growth rates to demonstrate the effect of growth rate on survival.

The percentage culturability of the organism obtained from the total and colony counts showed a decrease below dilution rates of approximately 0.1 hr$^{-1}$. Vitality of cells grown at different growth rates was analysed with the DVC and CTC methods. In general, there was no variation of cell elongation with growth rate, and there was little variation in CTC stained cells with growth rate. It was concluded that vital nonculturable cells did not seem to be present at low growth rates despite the fact that there was a decrease in overall culturability at these growth rates.

Size of chemostat cells grown at different growth rates was also measured using the two methods used for batch culture cells. The two methods showed little variation in mean size with growth rate. However, the standard deviations of the graph of size using the photographic method were large compared to the RTG correlator. An estimation of cell volume was made as well as an analysis of protein content. The results showed that there was little variation in the protein content per $10^8$ cells with dilution rate. This was in contrast to the results obtained with batch culture cells. The findings of protein per $10^8$ cells/volume appeared to show an increase in the quotient with increasing dilution rate. This increase could not be explained. Additionally, an odd point at the very slow growth rate did not follow this trend.

Antibiotic susceptibility studies demonstrated that in general an increase in resistance was seen with decreasing growth rate for both polymyxin B and ciprofloxacin. This could not be correlated with the decrease in culturability of the organism with decreasing growth rate.

Investigations were made into the surface properties of chemostat cells and batch culture cells. The main finding when outer membrane proteins were examined was that there was little difference in the protein profile of the older cells compared to those harvested at early stationary phase. Differences were observed in the amount of the proposed B-band of the LPS of cells of varying ages. It was suggested that the increase in this region of the LPS could be part of a survival process, could be more oxidizable LPS in which case it would manifest itself as an increase or thirdly could be due to the fact that there appeared to be an increase in the smooth colony types with time. It is not known why there was an increase in the smooth type of bacteria.

Further studies would have to be performed to examine this problem. LPS profiles should be made of 100% rough and 100% smooth *P. aeruginosa* 6750 at early stationary phase and after prolonged incubation in order to investigate if the rough colonies reverted to the smooth colony types.

The biofilm work could be re-examined with a different limiting nutrient. Since it was thought in the present study that the iron-depleted biofilm cells were growing at $\mu_{max}$, lower concentrations of carbon should be used, so that there would be less of the limiting nutrient per cell. In this way, the cells may grow more slowly and by varying the concentrations of the nutrient, an element of growth rate control could be achieved. Additionally, it was suuspected that the biofilms may have been oxygen rather than carbon limited and so this too should be further investigated.

During the survival studies of the iron-depleted batch culture of *P. aeruginosa* at 37°C, it was not known if or when the cultures became carbon-depleted. It was thought that when the cells reached the required point on the growth curve, the cells could be harvested, gently centrifuged, washed and resuspended in media minus the limiting nutrients. Therefore, it could be ensured that the cells in prolonged storage were subjected to starvation conditions. The centrifugation process, however, would have to be sufficiently gentle so as to prevent any damage to the cells.

Further work could be performed with the chemostat experiments. *P.aeruginosa* 6750 grown at additional dilution rates should be subjected to the various parameters associated with extended survival. This could possibly confirm the above results. it would also be of interest to examine if the findings were obtained with other Gram-negative organisms.

The nature of this work involved extended periods of time and in a time-limited project this was found to be a major disadvantage. It is not known if tests were performed on even older or more slowly growing cells (chemostat cells) whether the results would be similar to those obtained in these investigations. Further work should therefore examine the effect of the survival parameters on these cells.

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