Physiological heterogeneity and starvation in mature Pseudomonas aeruginosa biofilms
by Dongxin Karen Xu

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University
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Abstract:
Bacteria growing in biofilms are often found to be less susceptible to antimicrobial agents than bacteria grown planktonically. Slow growth and starvation were hypothesized to contribute to the reduced susceptibility of mature biofilms. In this study, spatial physiological heterogeneity of mature biofilms was visualized by molecular staining coupled with cryoembedding and cryosectioning. Frozen cross sections of biofilms that had been subjected to a period of phosphate starvation then stained for alkaline phosphatase activity with a fluorogenic stain demonstrated that alkaline phosphatase activity was induced only in a distinct band of approximately 30 μm adjacent to the gaseous interface. The localized pattern of alkaline phosphatase activity correlated well with dissolved oxygen penetration profile measured with an oxygen microelectrode. Biofilm sections stained with acridine orange and Fluorescent-In-Situ-Hybridization (FISH) revealed that faster-growing cells were located in the upper 20-25 μm layer of the biofilms, whereas the majority of cells in the lower part of the biofilms were slower growing. These molecular stains gave indications of different activity measurements in the biofilms.

The gene expression and protein level of the starvation sigma factor was studied to address the possible role of starvation in biofilm resistance. A rpoS-lacZ transcriptional fusion was used to compare the level of gene expression of Pseudomonas aeruginosa cells, grown planktonically and in biofilms. lmmunoblots were used to assay the levels of RpoS, under these different cultivation conditions. In 3-day continuously fed biofilms, rpoS gene expression was three fold higher per mg cell protein, than that of average stationary planktonic cells. In addition, the levels of RpoS in 3 and 4-day biofilms were similar to the level found in the stationary phase planktonic culture. These results demonstrated that the levels of RpoS were high in at least some regions of continuously fed mature biofilms. Since RpoS is involved in the regulation of general stress protection, induction of rpoS in biofilms may contribute to the increased resistance of biofilms.

Taken together, these results show that mature P.aeruginosa biofilms are characterized by striking physiological heterogeneity, including evidence of regions of diminished metabolic activity, slow growth, and starvation response.

PHYSIOLOGICAL HETEROGENEITY AND STARVATION IN MATURE *PSEUDOMONAS AERUGINOSA* BIOFILMS

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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This work is dedicated to my beloved father.

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ABSTRACT

Bacteria growing in biofilms are often found to be less susceptible to antimicrobial agents than bacteria grown planktonically. Slow growth and starvation were hypothesized to contribute to the reduced susceptibility of mature biofilms. In this study, spatial physiological heterogeneity of mature biofilms was visualized by molecular staining coupled with cryoembedding and cryosectioning. Frozen cross sections of biofilms that had been subjected to a period of phosphate starvation then stained for alkaline phosphatase activity with a fluorogenic stain demonstrated that alkaline phosphatase activity was induced only in a distinct band of approximately 30 µm adjacent to the gaseous interface. The localized pattern of alkaline phosphatase activity correlated well with dissolved oxygen penetration profile measured with an oxygen microelectrode. Biofilm sections stained with acridine orange and Fluorescent-In-Situ-Hybridization (FISH) revealed that faster-growing cells were located in the upper 20-25 µm layer of the biofilms, whereas the majority of cells in the lower part of the biofilms were slower growing. These molecular stains gave indications of different activity measurements in the biofilms.

The gene expression and protein level of the starvation sigma factor was studied to address the possible role of starvation in biofilm resistance. A *rpoS-lacZ* transcriptional fusion was used to compare the level of gene expression of *Pseudomonas aeruginosa* cells, grown planktonically and in biofilms. Immunoblots were used to assay the levels of RpoS, under these different cultivation conditions. In 3-day continuously fed biofilms, *rpoS* gene expression was three fold higher per mg cell protein, than that of average stationary planktonic cells. In addition, the levels of RpoS in 3 and 4-day biofilms were similar to the level found in the stationary phase planktonic culture. These results demonstrated that the levels of RpoS were high in at least some regions of continuously fed mature biofilms. Since RpoS is involved in the regulation of general stress protection, induction of *rpoS* in biofilms may contribute to the increased resistance of biofilms.

Taken together, these results show that mature *P.aeruginosa* biofilms are characterized by striking physiological heterogeneity, including evidence of regions of diminished metabolic activity, slow growth, and starvation response.

CHAPTER 1

GENERAL INTRODUCTION

Ubiquity of Biofilm Formation

Bacteria in natural aquatic populations have a marked tendency to interact with surfaces and form biofilms. The real significance of bacterial biofilms has gradually emerged since their first description (Zobell & Anderson, 1936), and the first recognition of their ubiquity (Costerton et al., 1978). Biofilms are found in natural aquatic environments (Lock et al., 1984), in industrial aquatic systems and on medical biomaterials. They are involved in biodeterioration of materials, including the digestion of insoluble nutrients by bacterial populations in the digestive tracts of higher animals and protective and pathogenic association with tissue surfaces (Costerton et al., 1987). It has become increasingly clear that the biofilm mode of growth (sessile) predominates in natural ecosystems both in medical and non-medical situations. In an exhaustive survey of the sessile and planktonic bacterial populations of 88 streams and rivers, the sessile populations exceeded the planktonic populations by 3-4 logarithm units in pristine alpine streams and by 200 fold in sewage effluent (Lock et al., 1984). In the investigation of medical-devices-associated infections, extensive bacterial biofilms were found by scanning and transmission electron microscopy on transparent dressings, sutures, wound drainage tubes, intraarterial and intravenous catheters (Peters et al., 1981), cardiac pacemakers (Marrie & Costerton, 1982), Foley urinary catheters (Nickel et al., 1985a) and urine collection systems.

Biofilm Resistance

It is well documented that biofilms are generally less susceptible to antimicrobial agents than their free-living counterparts (Brown and Gilbert, 1993; Costerton, 1984; LeChevallier et al., 1988). Treatment with traditional concentrations of biocides kills planktonic microorganisms but leaves the biofilm populations virtually unaffected (Ruseska et al., 1982; LeChevallier et al., 1988). Millions of dollars each year have been wasted in ineffectual treatments. There are also numerous reports about the biofilm resistance to antibiotics (Nickel et al., 1985a; Nickel et al., 1985b; Evans & Holmes, 1987; Anwar et al., 1989). The resistance of biofilms to antibiotics was demonstrated (Nickel et al., 1985b) by the inability of tobramycin to kill *Pseudomonas aeruginosa* cells embedded in a biofilm at antibiotic levels more than 50 times the MIC for the same strain grown in a liquid culture.

A bacterial cell initiates the process of irreversible adhesion by binding to the surface using exopolysaccharide glycocalyx polymers (Costerton et al., 1987). Cell division then produces sister cells that are bound within the glycocalyx matrix, initiating the development of adherent microcolonies. The eventual production of a continuous biofilm on the surface is a function of cell division within microcolonies and new recruitment of bacteria from the planktonic phase (Malone & Caldwell, 1983). The biofilm finally consists of single cells and microcolonies of sister cells all embedded in a highly hydrated, predominantly anionic matrix (Sutherland, 1977) of bacterial exopolymers and trapped extraneous macromolecules. These so-called extracellular-polymers (EPS) may protect the biofilm cells from the onslaught of antimicrobial agents by serving as a diffusional barrier (Anwar et al., 1992; Hoyle et al., 1992). The reduced penetration of antimicrobial agents results from the binding, absorption or reaction of the antimicrobial agents within the biofilms (de Beer et al., 1994; Nichols et al., 1988). de Beer et al. (1994) measured the chlorine penetration into biofilms during disinfection using chlorine microelectrode and found the limited penetration was caused by neutralization of the chlorine in the biofilm matrix. Monochloramine was also observed to be more effective than free chlorine for inactivation of biofilm bacteria due to its lower reaction rates resulting in greater penetration power (LeChevallier et al., 1988).

Biofilm Physiology

While reduced penetration of antimicrobial agents could explain some cases of biofilm resistance, transport limitation is not sufficient to explain all biofilm recalcitrance. Nichols (1989) mathematically modeled the penetration of two antibiotics into a

Pseudomonas aeruginosa biofilm. The amino-glycoside, tobramycin and a β-lactam, cefsuldin, were used. Based on their model the authors concluded that transport limitation of the antibiotics was not the only factor reducing the susceptibility of this biofilm. Using ATR/FT-IR, Vrany et al. (1997) observed that the transport of the fluoroquinolones, levofloxacin and ciprofloxacin, was rapid (<20min) for a 15-25 μm thick *P.aeruginosa* biofilm. Differences in levels of recalcitrance observed for this biofilm system were suggested to be due to a less susceptible physiological status which bacteria assume during biofilm life (Vrany et al., 1997). Stewart (1996) recently argued that, for most antibiotics, transport limitation was insufficient to explain the reduced susceptibility of biofilms because most antibiotics do not react or sorb sufficiently within the biofilm. Therefore, physiological and genetic modifications of biofilms resulting in biofilm resistance are receiving more and more attention (Anwar et al., 1992; Gilbert et al., 1990).

How solid surfaces may influence bacterial activity is an important question in microbial ecology. For over 50 years researchers have been trying to address the question and it appears to be complex, particularly in natural environments. Most measurements deal directly or indirectly with the efficiency of substrate utilization. It is speculated that biofilm bacteria have a nutritional advantage over the planktonic cells. Surfaces in aquatic environments rapidly adsorb organic molecules. These organic molecules are a source of nutrients for the attached bacteria. Griffith and Fletcher (1991) reported the adsorption of bovine serum albumin (BSA) by particles, derived from diatoms. Attached bacteria degraded 100% of the protein absorbed, while the planktonic cells were unable to utilize the BSA. McFeters et al. (1990) found a shorter lag time and greater specific activity in the degradation of nitrilotriacetate by attached bacteria than by bacteria in the bulk aqueous phase. Other investigations have produced different results. Fowler (1988) found that growth of *Escherichia coli* was improved after surface adsorption, but only at a nutrient (glucose) concentration less than 25 ppm. Jeffery & Paul (1986) reviewed a number of studies and found an increase in metabolic activities for surface-associated bacteria at low or zero nutrient concentrations.

There are several review articles addressing the changes of physiological activity in biofilm bacteria (van Loosdrecht et al., 1990; Costerton et al., 1995; Marshall & Goodman, 1994). However, during the 1984 Dahlem Workshop on Microbial Adhesion and Aggregation, the discussion group on activity on surfaces concluded: "Attachment to a surface can undoubtedly affect the activity of microorganisms, although sometimes in ways that are not readily predictable on our current knowledge" (Breznak, 1984). In the review article by van Loosdrecht et al, this statement was claimed to be still true. Although they examined the current work on microbial activity case by case, due to the great diversity in experimental set-up and parameters involved, the conclusion drawn was: "The presence of surfaces may positively or negatively (or not at all) affect microbial substrate utilization rates and growth yields. The results often depend on the nature of the organisms, the kind and concentration of substrate, and the nature of the solid surface. In interpreting the effect of surfaces on bioconversion processes, all possible physical and chemical interactions (e.g., diffusion ad- and desorption, ion-exchange reactions, conformation changes, etc.) of a given compound and its possible metabolites with a given surface have to be considered before general conclusions can be drawn".

The effects of adhesion on microbial physiology at genetic level have just begun to come under investigation using molecular approaches. The application of reporter gene technology has allowed in situ studies of gene expression directly at surfaces and has provided possibilities of identification of genes "switched on" or "switched off" at surfaces (Davies et al., 1993; Hoyle et al., 1993; Marshall et al., 1994). Using an *algC-lacZ* transcriptional fusion, Davies et al. (1993) found *algC* expression was upregulated in bacterial cells at the time of adhesion and often ceased when cells were surrounded by large amounts of alginate. Dagostino et al. (1991) employed reporter gene technology to demonstrate the "switching on" of genes at polystyrene surfaces in mutants that failed to express the genes in either liquid or semisolid media. Using the plasmid vector pJO100 (Östling et al., 1991) to transfer the transposon mini-*Mu* containing the promoterless *lacZ* into the marine *Pseudomonas* S9, mutants were selected that failed to express β-galactosidase in liquid or on semisolid media but produced the enzyme at a solid-liquid interface.

Hodgson et al. (1995) developed a perfused biofilm fermenter to achieve growth rate control in adherent population cells. Whole cell proteins of *Staphylococcus aureus* were isolated from biofilm cells and chemostat-grown cells and analyzed by SDS polyacrylamide gel electrophoresis (PAGE). SDS-PAGE demonstrated significant differences between the protein profiles of biofilm and chemostat controls cultured at equivalent growth rates. The differences include the repression of a 48 KDa protein and increased expression of a 21 KDa protein in the biofilm.

Physiological Heterogeneity and Starvation in Thick Biofilms

A major factor in biofilm growth that is different from planktonic growth is that biofilms are usually mass transport limited. The three factors that govern concentrations of a particular solute in a biofilm are 1) external mass transport to the biofilm, 2) diffusion within the biofilm, and 3) reaction or consumption of the solute by biofilm cells. Nutrient concentrations at the liquid-biofilm interface can be much lower than bulk liquid concentration due to a diffusion boundary layer (Characklis et al., 1990). Biofilms are predominantly water and diffusion into a biofilm should be relatively rapid, except that the presence of EPS and other cellular materials may impede the diffusion of nutrients. Reaction/ consumption plays an important role that leads to the depletion of a nutrient in the biofilm. Mass transport limitation within the biofilm results in a concentration gradient, where cells embedded deeper may experience starvation for nutrients. Thus in a thick aging biofilm the physiological status of biofilm cells is hypothesized to be heterogeneous and is determined by the location of each individual cell within the multiple layers of cells (Wentland et al., 1996). Cells located in the upper regions of the biofilm may have easy access to nutrients, including oxygen, and have fewer problems with the discharge of metabolic waste products. These cells are speculated to be metabolically active. In contrast, cells of the same species deep within the biofilm are likely to be less metabolically active.

Concentrations of oxygen, hydrogen sulphide, nitrous oxide and hydrogen ions (pH) can be measured within biofilms by microsensors with tip diameters down to a few micrometers. The size of the tip is so small that it provides an accurate measurement of concentration profiles in biofilms without disturbing the system. The use of such sensors has revealed steep concentration gradients, not only in the biofilm itself, but also in the aqueous phase above it (Lewandowski, 1994; Revsbech, 1989). In natural systems, this spatial differentiation generates a range of habitats providing niches for different physiological types of bacteria. A level of organization may develop in which cells of different species form consortia with integrated metabolic processes. Ritz (1969) examined the species composition of the sections of dental plaque by probing with fluorescent antibodies. The aerobic *Neisseria* were found to be most abundant in young plaque and in the upper layers of mature plaque. *Veillonella* (anaerobes) were limited to the inner two-thirds of the plaque.

There is also some evidence showing the spatial physiological heterogeneity and growth rate limitation within the biofilm as a result of restriction for a particular nutrient that fails to fully penetrate the biofilm. Tresse et al. (1995) entrapped viable cells of

Escherichia coli in agar gel layers to form artificial biofilm-like structures. Killing assays of immobilized bacteria by latamoxef and tobramycin were performed under different oxygenation conditions of the culture medium and compared with suspended cells. Under moderate aeration, agar-entrapped bacteria displayed higher resistance to the two antibiotics than suspended cells. In anaerobic conditions, suspended bacteria were highly resistant to the two antibiotics. Sustained oxygenation enhanced tobramycin efficacy against suspended and immobilized cells. These results show that oxygen deficiency in the gel layer contributes to the enhanced antibiotic resistance of biofilm-like cells. Oxygen concentration gradients in biofilms have been experimentally demonstrated many times using dissolved oxygen microelectrodes (de Beer et al., 1994).

Acridine orange (AO) stains double stranded nucleic acids green and single stranded nucleic acid orange. An actively-growing cell will have a higher RNA/DNA ratio than a less active cell, and therefore will have higher orange/green fluorescence intensity ratio after staining with AO. Wentland (1995) sampled colony-biofilms at different growth phases and stained the cryosections with AO. He found that mid-exponential phase colonies had high overall growth rates ($\mu > 1 hr^{-1}$) and were bright orange; stationary phase colonies had cells at the colony edges that fluoresced orange thus indicating high growth rates while cells in the interior of the colony fluoresced green indicating slower growth.

Kinniment and Wimpenny (1992) measured the distribution of adenylate concentrations and adenylate energy charge across *Pseudomononas aeruginosa* biofilms. The method involved freezing and sectioning of the intact biofilm, followed by extraction and assay of the adenylates in the sectioned material. Results indicated an increase in adenylate energy charge of about 0.2 units from the bottom to the surface of the biofilm and total adenylates formed a peak just below the interface. Energy charge values were generally low throughout the biofilm, reaching a maximum of only 0.6 units. Of the adenylates measured, AMP was the predominant nucleotide, especially in the deeper parts of the biofilm.

The same researchers along with Scourfield (Wimpenny et al., 1993) also used transmission electron microscopy (TEM) to examine biofilm growth. Scourfield (1990) grew the Bowden dental plaque community in the Cardiff constant-depth film fermenter (CDFF) as a model biofilm and investigated the structure with TEM. Examination by TEM of the dental biofilm and the above-mentioned *Pseudomonas aeruginosa* biofilm showed that healthy cells are present in the upper two-thirds of the biofilm. Below this the majority of cells appeared to be lysed. It was remarkable that there was a sharp division between the two zones. They suggested that in steady-state biofilm, nutrients diffuse downwards to a reproducible position below which cells were starved and/or anaerobic.

In Situ Indicators of Physiological Activities

The ability of microorganisms to grow and form colonies on solid culture media has been used as the traditional approach to study bacterial viability. Conventional microbiological methods for assessing the viability of bacteria within biofilms are based on the mechanical removal of cells from substrata followed by enumeration by colony formation. However, these methods not only require at least 24 hours incubation but also often underestimate bacterial activity (Morita, 1985; Brock, 1987). Most importantly, spatial relationships that are inherently complex and important in studying biofilm ecology will be lost by culture-dependent approaches.

To reveal spatial physiological heterogeneity in situ, fluorogenic probes can be utilized to stain biofilm before or after cryoembedding and cryosectioning depending upon the probes and targeting activities. Cryoembedding and cryosectioning is a simple technique developed by Yu et al. (1994) to mechanically remove biofilms from the substratum with minimal disruption of the structure and enable the imaging of sections of thick biofilms under light and epifluorescent microscopes.

There are extensive fluorescent reagents originally used by cellular biologists to observe the activities of cells (Mason, 1993; Haugland, 1992) and some have been exploited in microbiological applications. The compounds, 4,6-diamidino-2-phenylindole (DAPI), propidium iodide, ethidium bromide and Hoechst 33342 are all fluorescent nucleic acid stains that have been applied by microbiologists to determine a ‘total bacterial count' in a range of circumstances (Porter and Feig, 1980; Swannell and Williamson, 1988).

Fluorescent dyes also exist for different cellular functions. 5-cyano-2,3-ditolyl tetrazolium (CTC) has recently been applied with flow cytometry to determine respiratory activity (Kaprelyants and Kell, 1993). This compound is related to 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) which has been extensively used to microscopically discriminate actively respiring bacterial in a wide range of ecological and environmental studies (Rodriguez et al., 1992; Zimmermann et al, 1978). Both CTC and INT act as artificial electron acceptors. INT is converted to insoluble red (nonfluorescent) crystals of INT-formazan within metabolically active bacteria. Results obtained with INT correlated well with cellular ATP content in a study of pure and mixed microbial cultures (Stubberfield and Shaw, 1990). However, when cells are on opaque surfaces the microscopic examination of INT-formazan crystals is virtually impossible since the transmission of visible light through the specimen is required. On the other hand CTC can be utilized to observe respiring bacteria on an opaque surface since CTC is reduced to its fluorescent formazan crystals by succinate dehydrogenase of the respiratory pathway in *E. coli* (Smith and McFeters, 1996). CTC has been used to visualize respiring autochthonous bacteria in drinking water and biofilms (Schaule et al., 1993). Recently CTC was coupled with an immunomagnetic method to observe respiring *E. coli* O137 from hamburger (Pyle et al., 1999).

Cell biologists have extensively used indicators of membrane potential ($\Delta\Psi$) for nearly 20 years (Wu and Cohen, 1993; Loew, 1993). $\Delta\Psi$ is linked to the energy status of the cell (Diaper et al., 1992; Kaprelyants and Kell, 1992). Some studies have shown that $\Delta\Psi$ changes in eukaryotic cells during cellular proliferation with respect to their phase in the cell cycle (Darzynkiewicz et al., 1981). $\Delta\Psi$ can be evaluated in bacteria by using fluorescent probes developed in mammalian cells. Commonly used fluorescent probes include rhodamine 123 (Rh123) and 3,3'–dihexyloxacarbocyanine iodide [$DiOC_6(3)$]. Rh123 is a cationic fluorescent dye that is concentrated in mitochondria by the relatively high negative potential across the energized mitochondrial membrane (Johnson et al., 1981). In bacterial cells Rh123 is accumulated in an uncoupler-sensitive fashion via transmembrane potential (Haugland, 1992). Different studies have shown that Rh123 is a sensitive $\Delta\Psi$ probe for $Gram^+$ bacteria. However, the uptake of this fluorochrome dye by $Gram^-$ bacteria is low because their outer membrane is less permeable to it (Diaper et al., 1992; Kaprelyants, 1992; Matsuyama, 1984). Some recent methods described a pretreatment for staining $Gram^-$ bacteria with Rh123 (Kaprelyants, 1992; Yu and McFeters, 1994; Yu and McFeters, 1994). To eliminate the pretreatment problem, some other dyes have been exploited. For example, $DiOC_6(3)$ is a lipophilic cationic dye that has been shown to be satisfactory to evaluate the $\Delta\Psi$ of *E. coli* cells and to follow $\Delta\Psi$ changes during cells growth and throughout the cell cycle (Monfort and Baleux, 1996).

Acridine orange (AO) has been available for over 100 years and is one of the most commonly used fluorogenic dyes in microbial ecology and environmental microbiology as part of the acridine orange direct count (AODC). Some suggested that the reaction of bacteria with AO will allow discrimination of faster and slower growing cells, as mentioned above. McFeters et al. (1991) confirmed this assumption using purified DNA, ribosomes, bacteriophage-infected cells and *Escherichia coli* under a range of defined physiological circumstances. However, he also suggested that when applying the AO staining reactions as an indicator of physiological activity, the relevant variables including drying, fixation and chlorination should be understood.

Another more direct molecular method for measuring ribosomal RNA (rRNA) is oligonucleotide probes. The use of rRNA sequence divergence to infer phylogenetic relationships and as the basis for developing determative hybridization probes is now well established (Amann et al., 1990a; Amann et al., 1990b; Olsen et al., 1986; Woese, 1987). A popular method is the use of fluorescent-dye-labeled oligonucleotides complementary to rRNAs for the visualization of single cells with fluorescent microscopy (Stahl et al., 1989). This technology has been largely devoted to the detection of microorganisms. Coupled with image analysis, fluorescent-in-situ-hybridization (FISH) has been developed to infer cellular ribosomal (rRNA) content. In some bacteria cellular rRNA content shows good correlation with growth rate (Schaechter et al., 1958). Delong et al. (1989) first demonstrated the application of FISH for the estimation of growth rate of *Escherichia coli* in pure culture. Poulsen et al. (1993) observed good correlation between growth rate and FISH signal intensity of a sulfate-reducing bacterium isolated from an anaerobic fixed-bed bioreactor. He then used this quantitative FISH method to estimate the growth rate of this specific population of sulfate-reducing bacteria in multispecies biofilms. There was also a study to estimate the growth rate of *Escherichia coli* colonizing the large intestine of streptomycin-treated mice (Poulsen et al., 1995).

Other approaches to study the activity of attached bacteria in situ include microautoradiography (Ellis, 1999; Stewart et al., 1991), microcalorimetry and microelectrodes. Radiolabeling and microautoradiography were applied by Fletcher (1979) and Karel (1989) to investigate the activity of attached bacteria. Microcalorimetry has been used to measure heat output by surface-associated microorganisms and provide an estimate of total metabolic activity. Microelectrodes, which have been developed primarily for determining variations in pH and oxygen concentration in structural microbial mats, are useful tools for dissecting the different physiological activities of surface-associated microbial populations (Revsbech et al. 1983; Revsbech and Ward ,1984).

There is the problem of finding which parameter to measure in order to provide a valid indication of metabolic activity. For example, Lisle et al. (1999) found that using several fluorescent stains and probes could permit a more comprehensive determination of the site and extent of injury in bacterial cells following sublethal disinfection with chlorine.

Starvation, Stringent Response and Antimicrobial susceptibility

Most antibiotics target specific machinery that maintains a viable cell such as cell wall synthesis, protein synthesis, nucleic acid synthesis and cell membrane function. The synthetic functions, which some of these antibiotics target such as cell wall synthesis and protein synthesis are directly related to growth. Bacteria are known for their ability to alter their sensitivity to certain antibiotics and disinfectants with changes in growth rate (Brown et al., 1988; Eng et al., 1991; Gilbert et al., 1990). In some instances the coupling between growth and susceptibility is absolute (Tuomanen et al., 1986); this is the basis for the classical method of counterselection for auxotropic mutants. In studies performed by Harakeh et al. (1985) *Yersinia enterocolitica* and *Klebsiella pneumoniae* were shown to be less susceptible to chlorine dioxide when they were grown at submaximal rates. *Pseudomonas aeruginosa* cells grown to stationary phase were found to be less susceptible to either 0.25% (v/v) acetic acid or 31 mg/L glutaraldehyde treatment than cells growing in the exponential phase (Carson et al.,1972). Matin et al. (1989) also suggested that nutrient-deprived *Escherichia coli* and *Salmonella typhimurium* cells were more resistant to disinfectants' and to osmotic stress. The *E.coli* MAR (Multiple-Antibiotic-Resistance) operon is upregulated in inverse proportion to growth rate (Maira et al., 1998).

The growth of heterotrophic bacteria in natural environments is inhibited by periods of insufficient levels of energy and nutrients (Stenstrom et al., 1989). The survival strategies of bacteria in their natural environments under starvation conditions have been identified (Roszak & Colwell, 1987a) and suggest that the bacterial cultures undergo a series of physiological changes which enable the survival of some of the cells. Rapid multiple divisions of starved cells, which lead to the formation of ultramicrobacteria (<0.3 μm in diameter) have been observed (Novitsky & Morita, 1976). Roszak and Colwell (1987) suggested that ultrabacteria are exogenously dormant forms, responding to unfavorable environmental conditions, are sporelike bacteria. The similarity in responses to nutrient limitation of both sporeforming and nonsporeforming bacterial species may be due to the possession by both groups of the stringent response (SR) gene, *relA*. The SR is a phenotypic adaptation to conditions of amino acid limitation (Cashel, 1987). The gene product of *relA* is (p)ppGpp synthetase I, which phosphorylates GDP and GTP to ppGpp and pppGpp. It is apparent that *relA*-competent cells are unusual because they appear to have an enhanced resistance to many antibiotics. This resistance may be due solely to the reduced rates of metabolism, which could explain the lack of susceptibility to cell wall- and DNA- active antibiotics observed by Stenstrom et al. (1989). Alternatively, some products of the SR may serve to protect intracellular targets from action of antibiotics. In particular, the known binding affinity of (p)ppGpp for ribosomes may protect the cell against the action of aminoglycoside antibiotics.

Regulation of Starvation Sigma Factor RpoS and Role of RpoS in Stress Response

Bacteria are subject to an array of stresses within their natural environment, and it has been demonstrated previously that stationary-phase and starved cells survive these insults better than their exponential-phase counter parts (Jenkins et al., 1988). In *E.coli*, this is due in part to the alternative sigma factor, RpoS (σ^S), which accumulates in stationary-phase cells (Lange and Hengge-Aronis, 1991). Regulation of RpoS levels in *E. coli* cells was believed to occur at three levels (Takayanagi et al., 1994; Lange and Hengge-Aronis, 1994): transcription, translation, and protein stability. Using *rpoS-lacZ* reporter fusions (Mulvey et al., 1990), *rpoS* transcriptional expression in cells was shown to be low in early exponential phase and to increase gradually two-to threefold during exponential phase. The most substantial increase to 20-fold above basal levels occurred during and after the transition to stationary phase (Mulvey et al., 1990; Lange and Hengge-Aronis, 1991). In minimal medium, unexplained strain-specific differences have arisen. One report indicated that *rpoS* expression did not occur in minimal medium (Lange and Hengge-Aronis, 1994). Starvation also elicits an increase in *rpoS* expression depending on the missing component. Starvation for carbon resulted in limited expression, whereas starvation for nitrogen (Mulvey et al., 1990) or phosphate (Lange and Hengge-Aronis, 1991) resulted in maximal expression of *rpoS*. Anaerobiosis reduced the growth rate and stimulated *rpoS* expression during exponential growth in Luria-Bertani (LB) medium (Mulvey et al., 1990). *rpoS* transcription is inversely correlated with growth rate and is negatively controlled by cAMP-CRP. *rpoS* expression begins to increase during slow growth and will stop immediately before growth ceases.

Another level of RpoS regulation is posttranscriptional, at the levels of translation and protein stability. The induction of RpoS upon a shift to high osmolarity was shown to result from stimulation of translation and a change in the half-life of RpoS from 3 to 50 minutes (Muffler et al., 1996). RpoS was found to be a highly unstable protein in exponentially growing cells (with a half-life of 1.4 minutes), that was stabilized (with a half-life of 10.5 minutes) in stationary phase (Zgurskaya et al., 1997). By quantifying the relative *rpoS* mRNA levels by RNA_{ase} protection assay, Zgurskaya and Matin (1997) recently found the increase in RpoS level during stationary phase was solely due to a large increase in its stability. In *Pseudomonas aeruginosa*, there is not much known about RpoS regulation. Latifi et al. (1996) reported a hierarchical quorum-sensing cascade in *P. aeruginosa* involved in the activated expression of *rpoS*. They showed that expression of *rpoS* was abolished in a *P. aeruginosa lasR* mutant and in the pleiotropic *N-(3-oxododecanoyl)*-L-homoserine lactone (BHL)-negative mutant. In a heterologous (*E. coli*) background, a *rpoS-lacZ* fusion was regulated directly by RhlR/BHL. A density-dependent phenotype in *P. aeruginosa* biofilms was shown recently by Davies et al (1998). In biofilm, due to the likelihood of slow growth and density-dependent signaling, it is hypothesized that there will be RpoS upregulation.

In *E. coli* RpoS is a master regulator responsible for the gene expression in stationary phase and starvation conditions (Loewen and Hengge-Aronis, 1994). It controls a large group of genes. Analysis of two-dimensional gels has revealed that 18 (Lange and Hengge-Aronis, 1991) to 32 (McCann et al., 1991) proteins are missing or are present in smaller amounts, as well as the presence of some new proteins in an *rpoS* mutant. The identified RpoS-controlled proteins encompass a diverse group of functions, including prevention and repair of DNA damage (*katE*, *katG*, *xthA*, *dps*, *aidB*), cell morphology (*bolA*, *ficA*), modulation of virulence genes in *Salmonella*, *Shigella*, and *E.coli*, osmoprotection and thermotolerance (*otsBA*, *treA*, *csiD*, *htrE*), glycogen synthesis (*glgS*), anaerobically induced genes (*appY*, *appCBA*, *hyaABCDEF*) and membrane and cell envelope functions (*osmB*, *osmY*, *cfa*) (Loewen and Hengge-Aronis, 1994). Several of the genes listed above are involved in stress protection. Protection against H_2O_2 involves *katE* and *katG*, encoding the catalases HPII and HPI, respectively, which destroy H_2O_2 before it can cause damage. Dps forms nuclease-resistant complexes with DNA that also presumably protect the cells from killing by H_2O_2 (Almiron et al., 1992). The RpoS-dependent *otsBA* operon encodes trehalose-6-phosphate synthase (OtsA) and trehalose-6-phosphate phosphatase (OtsB), which together produce large amounts of trehalose in osmotically stressed cells (Giaever et al., 1988). Trehalose acts as an osmoprotectant, and consequently, *otsBA* and *rpoS* mutants exhibit an osmosensitive growth phenotype. Trehalose also acts as a thermoprotectant in a wide variety of species (Van Laere, 1989), presumably through its membrane and protein-protecting properties (Crowe et al., 1984; Crowe et al., 1988). A mutation in the RpoS-dependent *csiD* gene (Weichart et al., 1993) causes a similar heat-sensitive phenotype in addition to causing pleiotropic changes in protein patterns on 2-D gels. In addition, RpoS was reported to protect *E. coli* cells from the electrophile *N*- ethylmaleimide (Ferguson et al., 1998).

Objectives, Rationales and Experimental Design

The main goal of the study is to achieve a comprehensive understanding of spatial physiological heterogeneity and nutrient limitation in thick biofilms, thereby providing a basis for continued investigation of physiological mechanisms of the biofilm recalcitrance to antimicrobial agents.

The specific objectives are presented below:

Objective 1. To demonstrate spatial physiological heterogeneity in a model biofilm with different fluorescent probes which indicate RNA content, protein synthesis and respiratory activity and determine if this spatial physiological heterogeneity is caused by an oxygen gradient in the biofilm (Chapter 2, 3, 4).

Rationale. There is limited work demonstrating the spatial physiological heterogeneity in thick biofilms. Systematic studies of spatial physiological heterogeneity will provide a comprehensive understanding of biofilm physiological ecology and provide an explanation for recalcitrance of thick biofilms to antimicrobial agents.

Objective 2. To investigate the gene expression and protein levels of the starvation sigma factor RpoS in the biofilms.

Rationale. The speculation of starvation and the evidence of quorum-sensing phenomena in the biofilms lead us to hypothesize that the starvation sigma factor is upregulated in the aging biofilms. This would provide a mechanism of recalcitrance of aging biofilms at a genetic and molecular level.

Experimental Design.

A continuous flow reactor that has been shown to be suitable for growing biofilms and is convenient for physiological analysis was used to generate thick biofilms. To study spatial physiological heterogeneity in the biofilms, biofilms were stained with a fluorogenic alkaline phosphatase substrate, acridine orange, fluorescent-in-situ-hybridization for ribosomal RNA and CTC. These techniques gave an indication of active protein synthesis, RNA/DNA ratio, RNA content and respiratory activity, respectively. The stained biofilm sections were observed microscopically and image analysis was performed to quantify patterns of heterogeneity.

To investigate gene expression of *rpoS*, a plasmid carry *rpoS-lacZ* was transformed into the *P. aeruginosa* ERC strain. Biofilms bearing the plasmid were grown and analyzed for β-galactosidase activity. Western blots were performed to measure RpoS levels.

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CHAPTER 2

SPATIAL PHYSIOLOGICAL HETEROGENEITY OF ALKALINE PHOSPHATASE IN *PSEUDOMONAS AERUGINOSA* BIOFILM IS DETERMINED BY OXYGEN AVAILABILITY

Introduction

It has long been observed that biofilms are much less susceptible to antimicrobial agents than are their planktonic counterparts (Brown and Gilbert 1993; Costerton 1984; LeChevallier et al. 1988; Nickel et al. 1985), but the underlying basis for this recalcitrance is not well established. As introduced in Chapter 1, physiological and genetic modification of biofilms are hypothesized to be a mechanism of biofilm resistance. In a thick aging biofilm, the deep-internal portions of biofilms are hypothesized to experience starvation and slow growth due to nutrient limitation. The biofilm cell growth is hypothesized to be spatially heterogeneous. The purpose of the work in this chapter was to test the dual hypotheses that physiological status varies spatially within the biofilm and that, in the case of a *P. aeruginosa* model biofilm, physiological activity is controlled by oxygen availability. As a physiological indicator, we have used the expression of alkaline phosphatase upon exposure to phosphate starvation, which reflects the capacity for de-novo protein synthesis. Oxygen delivery was controlled by varying the composition of the gaseous environment and measured directly using an oxygen microelectrode.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions.

A pure culture of *Pseudomonas aeruginosa* ERC1 was used thoroughout. It was isolated from an industrial water system, identified with API NFT (bioMérieux Vitek, Inc., MO, USA) and retained in the culture collection of the Center for Biofilm Engineering. The 16S rDNA of the isolate was PCR amplified with 27F (sequence: 5' – AGA GTT TGA TCC TGG CTC AG – 3', corresponds to the *Escherichia coli* 16S rRNA position 8 to 27) as a forward primer and 1392R (sequence: 5' – ACG GGC GGT GTG TAC – 3', corresponds to the *Escherichia coli* 16S rRNA position 1392 to1406) as a reverse primer and commercially sequenced at University of Montana (Missoula, MT). When the sequence was aligned with the most similar Ribosomal Database Project (Maidak et al. 1994) sequence using Genetic Data Environment 2.3 Software, the percent similarity between *P.aeruginosa* strain NIH18 and ERC1 was 99.0%. MOPS (morpholinopropanesulfonic acid) minimal medium prepared as described by Neidhardt et al. (1974) was used in both planktonic and biofilm experiments. High phosphate medium contained 1g/L Na_2HPO_4 while low phosphate medium contained 0.01g/L Na_2HPO_4. Glucose (1.0 and 0.1g/L) was used as sole carbon source in the planktonic and biofilm culture medium, respectively. All the experiments were carried out at room temperature, 22.2 ± 3.0°C.

Planktonic Culture Procedure.

P.aeruginosa overnight cultures were harvested by centrifugation at 7,500 rpm for 10 minutes, washed twice with low phosphate medium, and resuspended in 100ml low phosphate medium to induce phosphate starvation. The low phosphate culture was stirred for 24 hours. Two-milliliter aliquots were withdrawn every hour for alkaline phosphatase and total protein assays. To test the effect of anoxic conditions on alkaline phosphatase production, a low phosphate culture was left in ambient air for 2.5 hours, then connected to pure nitrogen through a bacterial air vent for 3 hours, then changed back to ambient air for another 2.5 hours. Two-milliliter aliquots were withdrawn every 30 minutes.

Biofilm Culture Procedure.

A drip-flow plate reactor was designed to cultivate biofilms (Figure 2.1). It was then modified to a chamber reactor (Figure 2.2). Stainless steel slides in petri dishes were continuously bathed with medium dripping onto the biofilm at a constant flow rate of 50ml/hour. After inoculation with an overnight culture (3×10^8 cells/ml in 0.1g/L glucose MOPS medium) and incubation for 24 hours, the reactor was fed with high phosphate medium for another 72 hour, then replaced with low phosphate medium. The bacterial air vent of the reactor was either connected to pure nitrogen (120~130 ml/min), pure oxygen (120~130 ml/min), or exposed to ambient air to create different gaseous environments. All the biofilm experiments were duplicated.