Influence of reduced water availability on Pseudomonas putida unsaturated biofilms and the role of alginate in desiccation tolerance

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Influence of reduced water availability on *Pseudomonas putida* unsaturated biofilms and the role of alginate in desiccation tolerance

by

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For the Major Program
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Abstract

Biofilms are aggregates of cells adhering to surfaces embedded in a matrix of extracellular polymeric substances of their own making. Microbial water availability in many terrestrial habitats is one of the most important factors influencing unsaturated biofilm development and biofilm cell survival and death. In general, Pseudomonas putida strain mt-2 unsaturated biofilm formation proceeds through three distinct developmental phases, culminating in the formation of a microcolony. The form and severity of reduced water availability alters cell morphology. The dehydration treatments resulted in biofilms comprised of smaller cells but they were taller and more porous, and had a thicker exopolysaccharide (EPS) layer at the air interface. In the osmotic stress treatments, cell filamentation occurred more frequently in the presence of high concentrations of ionic, but not non-ionic, solutes and these filamented cells drastically altered biofilm architecture.

P. putida produced more EPS in response to dehydration stress, but not thermodynamically equivalent osmotic stress. Carbohydrate composition analysis showed that alginate was a component of unsaturated biofilm EPS only in the presence of dehydration stress. The absence of alginate altered biofilm architecture in that biofilms were shorter, covered more surface area, and were less porous than when alginate was present. By measuring intracellular water potential changes and monitoring fatty acid alterations during dehydration stress, we demonstrated that alginate slows the rate of cellular dehydration. Alginate production also contributed to the ability of cells to survive a severe desiccation stress. These results suggest that an important consequence of cellular dehydration is alginate production, which contributes to the fitness of P. putida in water-limited environments.
Reduced water availability influenced the temporal and spatial localization of dead cells within unsaturated biofilms. Dead cells were organized in arrays of various lengths one cell width. In general, more dead cells were localized in the lower layers, while active, growing cells were localized primarily in the upper layers.

Taken together, reduction of water availability influences biofilm architecture, EPS production, and cell death patterns, which all reflect a status of adaptation of *P. putida* to dehydration stress. This study provides new insights into microbial dehydration physiology.
Chapter 1. General Introduction

INTRODUCTION

Bacteria in soils are periodically or routinely exposed to a variety of changing environmental conditions, such as extreme fluctuations in temperature, availability of nutrients and water, and the presence of toxic molecules/substances produced naturally or from anthropogenic sources (pollutants). To cope with these stresses, bacteria by necessity need to utilize a broad range of colonization and survival strategies.

Given the complexity of the soil habitat, questions arise as to how soil bacteria are able to successfully colonize a particular habitat and what spectrum of strategies they employ for growth and survival. This is particularly important for bacteria residing in soils contaminated with organic pollutants (e.g., toluene), since they will consistently be exposed simultaneously to multiple stresses that potentially impact their ability to degrade those pollutants. It is conceivable, if not likely, that regulatory hierarchies and the adaptive responses they direct for challenging physical environmental stressors overlap with those employed for responding to organic pollutants.

Among environmental factors including temperature, pH, and water and nutrient availability implicated as critical determinants of pollutant degradation, water availability is likely the most important, and probably least understood. Because water is essential for microbial survival and metabolic activity, knowledge of the physiological and molecular mechanism(s) bacteria employ to adapt to water-limited conditions will provide insight into microbial ecology in general, but in particular their ability to degrade pollutants under these conditions.
DISSEMINATION ORGANIZATION

This dissertation is organized into five chapters. The first chapter provides a review of literature relevant to understanding and appreciating the research presented in the subsequent chapters. The second chapter describes how reduced water availability influences the dynamics, development, and ultrastructural properties of *Pseudomonas putida* biofilms. The material present in this chapter was published in the Journal of Bacteriology in a slightly condensed version. The following chapter then presents the role of exopolysaccharide (EPS) alginate produced by *P. putida* in desiccation tolerance and biofilm ultrastructure in water-limited environments and how the presence of alginate slows the rate of cellular dehydration the bacteria actually experience. In the fourth chapter, we describe the influence of reduced water availability on the spatial and temporal patterns of cell death within *P. putida* biofilms. In the fifth chapter, conclusions and future directions of this research are presented. There are also six appendices that follow the main chapters: Appendix A, the effect of reduced water availability on fractionation and visualization of EPS by gel electrophoresis; Appendix B, the effect of reduced water availability on *P. aeruginosa* PAO1 EPS production; Appendix C, a more detailed description of the strategy I used to generate the alginate deficient mutant used in these studies; Appendix D, the effect of reduced water availability during steady-state growth on fatty acid profiles; Appendix E, role of flagella motility in colonization of the substratum; and Appendix F, normalization of ice nuclei activity (INA) values relative to colony forming units at time zero before membrane transfer and recalculation of estimated water potential.
Microbial water availability in microbial habitats

Water is arguably the most important resource for living things including microorganisms. Water is the universal solvent of life and dissolves many substances necessary to microbial growth and survival. It is also integral to most, if not all, biological processes in the microbial world; it contributes to the stabilities of proteins, DNA, and lipids and confers structural order upon cells (124). Moreover, water is a critical component of the recycling of many elements, such as nitrogen or carbon, within ecosystems. It is also a limiting factor in the biodegradation of organic pollutants in soils, since microbial activity of these processes is significantly influenced by water availability.

Water potential in microbial habitats. Water potential is a measure of the free energy of the water and its fundamental concept is now widely, although not entirely, accepted among microbiologists (2, 18, 65, 115). In natural environments, the water potential can be used to predict the direction of water flow and therefore the energy status of water. Since the water potential is always negative in most environments compared to pure, free water whose potential energy (per unit mass or volume) is zero, water flows spontaneously from high to low potentials (115). As the water potential decreases, the availability of water for cellular activities decreases and in consequence water stress on microorganisms increases.

There are two major types of microbial habitats where microbes could suffer from water stress (18). One is aquatic habitats, such as rivers and oceans, in which water availability is almost exclusively determined by the concentration of dissolved solutes. Increasing the concentration of solutes decreases water availability and increases the osmotic pressure imposed on microorganisms. The other is terrestrial habitats, such as soil matrices and plant
surfaces, which are frequently unsaturated (i.e., water limited). Particularly in soils, drainage can induce physical discontinuities of water films by capillary and surface effects (18). The total soil water potential is a quantitative term comprised primarily of the sum of the solute potential (osmotic stress), which is due to the interaction of water with solutes, and the matric potential (dehydration stress), which is due to the interaction of water with soil matrices. From a bacterial perspective, the major difference between these two stresses is that with an osmotic stress microbes are bathed in water of diminished activity, whereas with a matric stress microbes become dehydrated by the physical removal of water from their environment and the availability of the remaining water is reduced through its sorptive interaction with soil constituents (66). In fact, as soils dry the matric potential becomes the predominant component contributing to the total water potential (67, 115).

**Experimental approaches to study reduced water availability on bacteria.** In order to explore how water availability controls microbial activity, many microbiologists lower water activity by adding solutes such as NaCl into a growth medium, and then monitor how reductions in osmotic (solute) potential influence a biological process or property (11, 121, 123, 157). In unsaturated habitats, however, this method may not be applicable because matric potential is the primary component of the total water potential influencing microbial activity in these habitats (67, 115). Therefore, studying the effect of matric stress on bacteria requires different approaches. One method for controlling matric potential is an isopiestic system in which cells are immobilized on an inert support, such as a filter, in an enclosure and they are placed in close proximity to agar containing a solute with an appropriate concentration to lower the relative humidity of the chamber (125). In this system, cells are subjected to matric potential controlled by isopiestic equilibrium through the vapor pressures
by solutes, especially when saturated, and humidities at different temperatures. More
realistically, soil samples can be saturated, and then directly dried to reduce water content.
However, subpopulations of bacteria located in different positions in a soil may experience
different matric potential, due to soil water heterogeneity, and this inherent heterogeneity can
hinder the establishment of cause and effect relationships (145).

Alternatively, dehydrating agents can be used directly in both liquid and solid culture
media. Ethanol has been a widely used dehydrating agent that affects the physicochemical
state and biological functions of cell membranes (37). However, its toxicity and permeability
of membranes results in a perturbed cytoplasmic membrane structure and function (174),
which may not reflect authentic dehydrating situations. Alternatively, high molecular weight
(MW) (i.e., a MW of >3,000) polyethylene glycols (PEGs) have been used to study microbial
and plant responses to reduced water content, because they are inert, non-ionic, and too large
to penetrate cell walls and can cause reduction in water content available to microbes by
binding water molecules tightly (21, 66, 67, 104). Thus, these high MW PEG amended media
could better reflect in situ dry soil habitats at the given water potential than those amended
with ethanol.

**Bacterial adaptive mechanisms to reduced water availability**

Bacteria residing in soil frequently suffer from reduced water availability derived from
unsaturated environments, although they are surrounded by water films, which vary in
thickness, depending on the water content of the habitat and properties of the surfaces. When
a soil dries, resident microbes can be exposed to matric (dehydration) stress and solute
(osmotic) stress caused by increased concentrations of dissolved solutes in the soil water. Bacterial growth and survival in these unfavorable environments requires a wide range of adaptive responses. In most cases, the bacterial response involves transcriptional activation of genes whose products help the cells cope with a given environmental stress. Recently, van de Mortel and Halverson (168) identified water deprivation-controlled (wdc) genes in Pseudomonas putida encoding cell envelope proteins using the mini-Tn5-'phoA system. In this study, many wdc loci identified were differentially regulated by solute and matric stress, although some genes were induced by both stresses. This result suggests that when the stresses are thermodynamically equivalent, cells are differently affected by each form of reduced water availability (66, 67, 124), and hence they employ different adaptive responses to matric and solute stresses.

In this review, I will primarily focus on the strategies bacteria may employ to overcome dehydration-mediated damage and membrane shrinkage with alteration of the physical properties of membranes (124), since matric stress is the major part of the total water potential in the soil water, specifically when soils dry (67, 115).

**Compatible solutes.** The drying of cells requires adaptations to maintain an intracellular water potential that is in equilibrium with the external environment in a fashion similar to that observed for responding to high osmotic conditions (67, 87, 105, 134). This phenomenon can occur by i) reducing intracellular water content, which can cause crowding of macromolecules in the cytoplasm (124) and therefore could influence bacterial growth and survival, and/or ii) accumulating intracellular organic and inorganic compatible solutes, such as K⁺, amino acid (e.g., glutamate), and carbohydrate (e.g., trehalose), which are non-toxic cytoplasmic co-solvents whose level can be modulated over a broad range by de novo
synthesis or by transport from the environment without disrupting cellular functions (36, 124, 184, 186). One of the best characterized compatible solutes that accumulate when cells are desiccated is the disaccharide sucrose or trehalose. In particular, trehalose has been known to act as an osmoprotectant in many organisms and provide cells with increased resistance to desiccation (132, 177). Interestingly, the synthesis and accumulation of trehalose is also induced in stationary phase and by osmotic shock, indicating an overlapped adaptive response among different environmental stressors (132).

**Lipid membranes.** As cell membranes become dehydrated, they undergo a series of phase transitions from liquid crystalline to gel-like phases; the membranes become less fluid as they are dehydrated and decreasing membrane fluidity can restrict the diffusion of nutrient and waste products (33, 124). Recently, it has been shown that cis-to-trans isomerization of membrane fatty acids by a constitutively expressed cis-trans isomerase (Cti) (77, 78, 83, 119) is a rapid means by which *P. putida* can mitigate the dehydration-mediated disruption of membrane integrity (66). In addition to altering membrane fatty acids, bacteria may employ an adaptive strategy by exporting trehalose or sucrose to stabilize dehydrated membranes (53, 124). Trehalose can replace water molecules around the polar head groups of the phospholipids in the dry membranes (60), and this proposed mechanism is called the “water replacement hypothesis” (29, 32-35). Moreover, maintenance of proper membrane integrity or fluidity has been implicated as a central mechanism of desiccation tolerance in anhydrobiotic organisms (22, 32).

**Oxidative stress.** In general reactive oxygen species (ROS), including superoxide anion \((O_2^-)\), hydrogen peroxide \((H_2O_2)\), and hydroxyl radicals \((HO-)\), are generated during aerobic metabolism (166). ROS will also significantly accumulate during drying, especially when
cells are subjected to solar radiation (124). In plants, membrane perturbations induced by dehydration stress can cause aberrant electron flow in an electron transport chain, leading to increased formation of ROS (75). Unfortunately, ROS can cause damage to proteins, lipids, carbohydrates, and DNA (17, 80, 124, 166). Defense against ROS is primarily provided by the induction of antioxidant enzymes such as superoxide dismutase (SOD) which converts $O_2^-$ to $H_2O_2$ and $O_2$, catalase which converts $H_2O_2$ to $H_2O$ and $HO^-$, and peroxidase. For example, *P. aeruginosa* possesses two Fe- and Mn-SODs (68, 69), three catalases (KatA, KatB, and KatC) (19, 101), and four alkyl hydroperoxide reductases (AhpA, AhpB, AhpCF, and Ohr) (110). Recently, it has been shown that when desiccated for 13 years, cyanobacterium *Nostoc commune* accumulates active Fe-SOD, which was the third most abundant protein (148) following water stress proteins (Wsp) and phycobiliproteins (142). In fact, high levels of *sodF* mRNA also accumulate after long-term desiccation (148).

In addition to these enzymatic defense systems, the synthesis of non-enzymatic antioxidants such as glutathione, vitamins C and E, and ubiquinol could function to scavenge free radicals (81). In fact, in the study by van de Mortel and Halverson there was increased expression of the $\gamma$-glutamyl transpeptidase (GGT) gene upon dehydration (168), and this gene product catalyzes transfer of the $\gamma$-glutamyl moiety of glutathione to amino acids, dipeptides, and to glutathione itself (164), suggesting that increased GTT levels might be required for recycling glutathione and its related compounds to counteract dehydration-mediated oxidative stress (168).

**Protein damage.** One of the ultimate targets for desiccation-induced damage is proteins whose surface topology is strongly associated within water molecules (100). As cellular water moves out of the cell, dry microenvironments will influence folding pathways for each
protein, resulting in unfolded and/or misfolded proteins. To cope with protein damage at the molecular level, cells may induce heat shock proteins (HSPs), many of which are chaperones and proteases that can facilitate protein folding or degrade misfolded proteins (132). In *E. coli*, expression of genes that encode HSPs are transcriptionally regulated via the heat-shock sigma factor $\sigma^{32}$ (20). Although HSPs were first discovered in response to heat shock, several forms of stress including pH, osmotic stress, UV radiation, desiccation, and the presence of toxic substrates (e.g., ethanol, oxygen peroxide, and heavy metals) are known to increase the rate of synthesis of HSPs (20, 84, 85, 91, 99, 169, 188). These results indicate that stress responses overlap and $\sigma^{32}$ is a key element in the regulatory hierarchy.

In particular, cell envelope proteins could be more vulnerable to dehydration stress due to their direct exposure to the environment. The so-called envelope stress response consists of two distinct regulatory pathways that respond to bacterial envelope stress imposed on the inner membrane, periplasm and outer membrane (127, 128); i) the $\sigma^E$ pathway which responds to misfolded outer membrane proteins that accumulate in the periplasm (129), and ii) the two-component signal transduction system where the membrane-bound sensor histidine kinase perceives a variety of envelope perturbations (i.e., misfolded proteins) (38, 82, 153) and this signal is transduced to the cytoplasmic response regulator through conserved phosphotransfer reactions (47, 130, 175).

**Exopolysaccharides (EPSs).** There is a vast body of literature suggesting that EPS production occurs in response to desiccation and its presence is important for desiccation tolerance (112, 134). EPSs can be hygroscopic and presumably their enhanced water holding capabilities sustain the moisture level in the microenvironment surrounding cells upon dehydration. In this study, we hypothesize that EPS creates a more hydrated
microenvironment, thereby contributing to bacterial desiccation tolerance (27, 134). A more detailed review on bacterial EPS will be discussed below.

**Bacterial exopolysaccharides**

Bacteria are capable of producing capsule polysaccharide (CPS) that is intimately associated with the cell, and/or EPS that is loosely associated and can be easily separated from the cell by rinsing (43, 151). However, CPS can be considered part of EPS, since they are both exported outside cells. The presence of EPS is likely a factor in the ecology of the organisms, since these polysaccharides define the immediate environment surrounding the bacteria. Their physicochemical properties such as hydrophilicity, charge, and viscosity are thought to provide a favorable microenvironment. Increased EPS production by soil bacteria improves soil aggregation and aggregation stability and water retention properties (4, 5, 134).

**Properties of bacterial EPS.** Bacterial EPS vary dramatically in not only their composition but also their physicochemical properties, conferring unique features to each EPS. Some are neutral and a few are cationic, but the majority are polyanionic due to the presence of uronic acids, or acetyl-, succinyl-, or pyruvyl-decoration of the carbohydrates (162). Moreover, different monomer units and linkages between monomers alter their physicochemical properties, such as their water solubility (161). For example, bacterial cellulose (β-1, 4 glucan) is hydrophobic (44, 103, 116, 139), whereas α-1, 6 glucan is slightly hydrophilic and bacterial alginate is highly hydrophilic (61). Computer-based models of many EPSs revealed that charged groups are located on the exterior of the molecular chains, suggesting that it facilitates the interaction between these groups and ions or other molecules
(24). For instance, alginate can form gels by interacting with divalent cations such as Ca$^{2+}$, Cu$^{2+}$, Mg$^{2+}$, Pb$^{2+}$, or Ni$^{2+}$ (135). Alginate comprised of D-mannuronic acids (M) and its C-5 epimer L-guluronic acids (G) is produced by many plant-associated fluorescent pseudomonads including *P. fluorescens*, *P. syringae*, and *P. aeruginosa* (57, 58). Like algal alginate, bacterial alginate has an irregular sequence of MM or MG residues which determine its viscosity; the more L-guluronic acids, the greater the viscosity (135, 160). But, bacterial alginates differ from algal alginate in that the mannuronic acid residues are frequently O acetylated at the C-2 and/or C-3 positions. O-acetyl groups are known to affect physicochemical properties, including viscosity, interactions with Ca$^{2+}$, and the mannuronan epimerase and lyase (59, 144, 150), and to influence biofilm architecture and properties (109). This is a clear example of how decorations of a carbohydrate backbone structure can substantially alter its physicochemical properties. Since modification of the polysaccharide residues is a microbially mediated response, it is conceivable that different function can be attributed to each type of EPS that would enhance bacterial fitness.

*Environmental regulation of EPS biosynthesis.* Various environmental signals regulate EPS biosynthesis. An increased carbon-to-nitrogen ratio and a lower incubation temperature than that optimal for bacterial growth induce EPS synthesis (163). EPSI (succinoglycan) and EPSII (galactoglucan) production by *Sinorhizobium meliloti* is regulated by phosphate concentration (10, 106, 189). Copper, oxygen, and calcium with low pH are also known to induce EPS biosynthesis in various organisms (46, 86, 136).

Of particular interest is alginate production in several *Pseudomonas* species that can be stimulated by dehydration and/or osmotic stress (45, 51, 149). There is also genetic evidence that transcription of algD gene, the first gene in the alginate biosynthesis operon, in *P.*
*P. aeruginosa* is activated by ethanol (i.e., dehydrating agent) and high osmolarity (12, 45). In fact, the stimulatory effect of environmental factors on alginate production is species and strain specific; for example, while the optimal concentration of NaCl for activation of the *algD* promoter in *P. aeruginosa* has been reported to be 0.35 M (approximately -1.5 MPa water potential) (12), this concentration does not induce alginate production by some *P. fluorescens* strains (149). More importantly, the environmental signals leading to EPS production are likely to overlap, since it is almost, if not always, induced under stressful conditions. One of the key elements that contribute to bacterial survival under these conditions is the alternative sigma factors (e.g., $\sigma^E$, AlgU/T). Sigma factor $\sigma^E$ was first characterized in *E. coli*, and played a role in the bacterial envelope stress response (See *Protein damage*). Moreover, the $\sigma^E$-homolog in many *Pseudomonas* species, AlgU (AlgT), is a positive regulator for EPS alginate production and contributes to tolerance toward desiccation and osmotic stress (147). This suggests that EPS production is an active process and that modulation of the amounts and/or types of EPS synthesis can contribute to protection against harsh environmental conditions.

**The roles of bacterial EPS.** There are many studies (50, 95, 180) suggesting that bacterial EPSs play roles in pathogenicity, survival, and interaction with other molecules (e.g., proteins). In this review, I will primarily focus on the protective roles by which bacteria can survive challenging habitats.

As a virulence factor, CPS is widely produced by many human pathogens, including *Streptococcus pneumoniae, Streptococcus pyogenes*, and *E. coli* (7, 70, 89, 111, 118, 152, 158, 159, 185). It has been postulated that a possible physiological mechanism by which pathogens confer their tolerance to phagocytosis is repulsion either between the hydrophilic
CPS and the possibly hydrophobic phagocyte, or between different charges on the two surfaces (49, 181). Likewise, the EPS produced by plant-associated pathogens alters the physicochemical properties of the cell-immediate environment (95, 131) that could improve epiphytic fitness (187), or possibly attenuate plant defense. For example, Ramirez et al. found positive correlations among lesion area, the viscosity of the culture, and the degree of EPS xanthan gum acetylation (131). Yu et al. demonstrated that alginate contributes to \textit{P. syringae} virulence, perhaps by facilitating their epiphytic fitness (187). In addition to pathogenesis, EPS functions as a specific signaling molecule recognized by leguminous host plants in the \textit{Rhizobium}-legume symbiosis. For example, i) bacterial mutants that fail to produce certain EPS are substantially impaired in their ability to invade root nodules (63, 96, 108, 133), and ii) there are significant differences in efficiencies of root nodule invasion depending on the nature of the EPS synthesized, even in the same bacterium (120).

A variety of physical stresses, such as UV radiation, ROS, or drought conditions can also influence the ability of pathogenic and saprophytic bacteria to produce EPS. It has been suggested that EPS production provides protection to the bacterial cells against UV radiation and oxidative stress (8, 9, 26), and it also confers desiccation tolerance (112), possibly by absorbing large amounts of water due to the hygroscopic nature of many EPS constituents (27). An EPS matrix may create a buffering system surrounding microenvironments and therefore prevent such a rapid loss of water. The greater water content in the immediate vicinity of cell membranes may reduce dehydration-mediated damage of membranes. Furthermore, slowing the rate of drying could increase bacterial survival by increasing the time available for making metabolic adjustments (134) necessary for tolerating or preventing desiccation-mediated cellular damage.
In addition to its protective roles, EPS can also stabilize extracellular enzymes. Kuwabara and Lloyd (90) demonstrated that the enzyme β-lactamase II produced by *Bacillus cereus* is associated with a polysaccharide that enhances its activity by as much as 50%. This would suggest that EPS can influence enzyme activity by increasing protein stability, although it has no enzymatic activity. In addition, enzyme secretion is dependent on the cosecretion of at least one of the *Micrococcus sodonensis* EPSs (16), which suggests that young polypeptide chains vulnerable to extracellular protease are protected by EPS (50).

**Biofilms in terrestrial habitats**

*Definition of biofilm.* Bacteria residing in terrestrial habitats (e.g., soils) often form aggregates of cells adhering to surfaces embedded in a matrix of extracellular polymeric substances including EPS, proteins, and nucleic acids, of their own making, and can be defined as biofilms (25, 30, 31, 62, 113). The biofilm concept of bacterial growth has been widely accepted in a variety of fields including civil and environmental engineering, medical and environmental microbiology, and the food industry (117).

*Experimental approaches to study biofilms.* Scanning confocal laser microscopy (SCLM) is the major tool to study biofilm ultrastructure. With SCLM, biofilm ultrastructure and development can be directly visualized without modification (e.g., electron microscopy requires dehydration and fixation steps of matrices that could be highly hydrated). Biofilm properties are quantitatively analyzed using various image analysis programs, such as COMSTAT (13, 72-74). There are a variety of methods available to generate biofilms, including drip flow reactors, tube reactors, rotating disk reactors, and flow cell systems. In
particular, the flow cell system has been used extensively to generate biofilms to explore biofilm developmental processes and biofilm architecture by SCLM.

Drip flow reactors are comprised of inert slides positioned at angle (~10°) and are bathed continuously with the medium that drips onto the slides (1, 79). These biofilms are fully hydrated and nutrients for biofilm growth are provided by bathing the cells periodically with a medium that is gently flowing down the slide, and consequently there is great heterogeneity in nutrient supply and flow rates. Tube reactors consist of clear plastic tubes where biofilms form on the tubing walls and the medium flows at a consistent rate through the system (140, 146). Rotating disk reactor systems are used for quantitative analysis of biofilm cell susceptibility to antibiotics and are comprised of a reactor vessel containing a rotor, several removable inert disks (e.g., stainless steel) for sampling, and medium exchange (71, 94, 122, 165). The rotor is embedded with a magnetic stir bar on the bottom and driven by a stirrer (ca. 60 rpm). In flow cell systems, nutrients are provided through an inlet and waste products are removed via an outlet (72, 74, 93, 154, 167), since it is often accessible to combine the use of SCLM to investigate biofilm development and architecture.

However, all of the systems described so far are aquatic-based systems. In other words, biofilms formed in these systems are basically fully hydrated and hence will be different from biofilms formed in unsaturated habitats (See Biofilm ultrastructure and properties). Therefore, it would be an advantage to develop methods for characterizing unsaturated biofilm development, properties, and ultrastructure in real time.

**Biofilm ultrastructure and properties.** In a flow cell system, biofilm architecture (three-dimensional (3-D) structure) is very heterogeneous and constantly changes depending upon external and internal processes; i) fluid flow regulates the concentrations of available
substrates and waste products, ii) fluid flow shears the biofilm surface and may determine dispersal processes, and iii) the diffusion and convection of chemical species affect ion exchange and nutrient utilization. Moreover, different bacterial species reveal different biofilm architecture, even when using the same flow rate or substrates (74). Despite this heterogeneity two conceptual models of mature biofilm ultrastructure have been presented. One is the water-channel model in which biofilms have interconnected mushroom-like shapes (183) and channels (void spaces) that act as conduits for waste removal and nutrient re-supply (42, 155). The other is the heterogeneous mosaic model in which tower-like structures are separated from their neighbors and hence towers are not interconnected and surrounded by the water phase (183).

Unlike those in the aquatic habitats, biofilms in terrestrial habitats are commonly unsaturated, although they are surrounded by water films varying in thickness, depending on environmental conditions. Hence, these biofilms are likely to experience periods of reduced water availability that may influence cell physiology and biofilm development and architecture. The lack of significant fluid flow will also dramatically influence nutrient availability patterns, metabolic waste accumulation, and disposal. Biofilms cultured under confined, unsaturated conditions at a range of matric water potentials as low as -1.5 MPa (6, 76) exhibit high cell densities and more uniformly shrunken polymers associated with the surface than those found in the water-saturated habitats. Moreover, studies of biofilm formation in the phyllosphere where air drying frequently occurs have provided some evidence that bacterial populations are highly aggregated even at small scales (88). By quantitatively analyzing the frequency, size, and spatial distribution of bacterial aggregates on bean leaf surfaces, Monier and Lindow indicate that bacteria exist on the leaf surface in a
wide range of cluster sizes, ranging from single cells to over $10^4$ cells per aggregate (107). In unsaturated soil habitats, however, relatively few studies have been attempted to visualize intact biofilms, although several studies have examined biofilms that form along roots (14, 56).

In addition to natural unsaturated habitats, *in vitro* colony biofilms (e.g., colonies grown on the agar surface), which can be considered a form of biofilm growth, have been cultivated on filters overlaying agar (6), since they may mimic, in part, natural unsaturated habitats due to the lack of bulk fluid surrounding them. However, colony biofilms on media are generally examined only after the biofilms have matured (55, 178), and therefore few studies allow scientists to get knowledge in biofilm development. Clearly, the paucity of information on the influence of reduced water content on biofilm development, properties, and architecture is especially great. In order to fill these gaps to better understanding microbial biofilm biology and related physiological traits, there is a strong need for developing new techniques for studying unsaturated biofilms, since most experimental systems only reflect fully saturated conditions (See Experimental approaches to study biofilms).

**Physiological traits contributing biofilm developmental processes.** A variety of bacterial traits have been implicated as being important in biofilm development. One of the most notable traits is EPS production, which can be considered a hallmark of bacterial biofilms (156, 171), since the biofilm typically contains polysaccharides as its major components (160, 171). Moreover, increased EPS production results in enhanced biofilms comprised of taller and rougher architecture (39, 71). Quorum sensing may also be involved in the properties of biofilm architecture. Bollinger et al. (15) and Davies et al. (40) have shown that cells located in different regions of a biofilm exhibit different expression patterns
of quorum-regulated genes and the alginate biosynthesis genes. In particular, quorum sensing may be required for the differentiation of biofilms into complex mushroom- or tower-like structures (41), although this has not been consistently observed (72). Flagellar motility and twitching motility have also been shown to influence biofilm developmental processes and the resulting biofilm architecture. Based on the mutant studies by Pratt and Kolter, one-half of mutants defective in biofilm formation displayed a decrease in normal flagellar function (126), indicating that flagellar motility is necessary for biofilm formation. In contrast, lack of twitching motility results in taller and rougher biofilms, indicating that twitching motility affects the development of biofilm architecture (28, 72), although O'Toole and Kolter showed that a twitching mutant was unable to develop 3-D architecture after forming a monolayer of cells on the PVC plastic (114).

**Tolerance of biofilm cells to chemical and physical stressors.** One of the striking features of microbial biofilms is their increased resistance to adverse environmental conditions including antimicrobial agents, heavy metals, low pH, UV radiation, and desiccation compared to planktonic cells (23, 52, 92, 97, 165, 168, 170, 176, 182). This increased tolerance to chemical and physical stressors could be that biofilm cells are protected by i) an extracellular polymeric matrix that functions as a diffusional barrier to toxic chemicals (31) and a physical shield to physical stressors, ii) slower growth rates, therefore lowering metabolic activities and making cells less susceptible to toxic compounds (31, 48), and iii) a distinct and protective biofilm phenotype that confers enhanced stress tolerance (31). Recently, DNA microarray technology of global gene expression profiles of biofilm cells compared to planktonic cells revealed that only 1% of the genes were differentially expressed in the two growth modes of *Pseudomonas aeruginosa*, while 5%
were differentially expressed in *Escherichia coli* (141, 179). The majority of genes whose expression is altered are associated with motility and attachment, metabolism, transport/membrane proteins, and hypothetical proteins, while some are associated with enhanced autoaggregation and heavy metal resistance. These results suggest that these genes may be important elements that trigger the enhanced environmental stress tolerance of biofilm cells.

**Bacterial death**

Failure of successful adaptation to stressful conditions could lead to death of residents within a biofilm community. In fact, death is one of the least understood aspects of bacterial life in soil. Reduced water and nutrient availability and exposure to other stresses clearly influence the death of individual cells, populations, or entire communities. However, cell death may not always be, in part, bad for the community. From a population perspective, one of the possible mechanisms by which cells within a community survive in water- or nutrient-limited habitats may be to reduce competition for limiting resources by eliminating defective or damaged cells, possibly by killing them, which may also provide a pool of substrates available for the survivors.

Cannibalism by the sporulating bacterium *Bacillus subtilis* in which cells produce a killing factor triggered by nutrient limitation (64) and toxic protein production by the marine bacterium *Pseudoalteromonas tunicata* (102) are examples where cells are killed by their neighbors or siblings. A number of death mechanisms are thought to be a genetically sophisticated bacterial programmed cell death (PCD) under starvation or in the presence of
antibiotics (3, 98, 137, 138). Cannibalism could be considered a form of PCD, as well (54). Recently, Webb et al. proposed that cell death in biofilms is a normal, but important, process by which surviving cells benefit from the death of a subpopulation of community members (173). They have shown a possible mechanism contributing to death of cells in *P. aeruginosa* biofilms by which a prophage is activated to produce superinfective phages that, when released, lead to the killing of susceptible cells (173). Activation of the prophage apparently requires physiological or genetical changes in the biofilm cells and possibly the activities of a toxin-antitoxin (TA) system present in the phage genome (172). TA modules are a form of bacterial PCD in which stable (long half-life) toxins kill cells unless unstable (short half-life) antitoxins antagonize the toxic effect by binding to the stable toxins. These TA protein complexes prevent initiating processes leading to cell death (3, 98, 137, 138). Normally cells actively produce the antitoxin that is essential for cell survival. However, expression of the chromosomally located TA module is inhibited by starvation stress which could be a condition that cells routinely experience in mature unsaturated biofilms. Due to the lack of continuous expression of the TA loci, the unstable antitoxins are degraded by protease faster than the stable toxins, which leads to accumulation of the toxin and cell death.

By doing what can be considered as altruistic behaviors, subpopulations within biofilms may i) promote survival of the community by releasing nutrients for the remaining cells to utilize, ii) reduce competition for remaining resources, and iii) provide physical support and/or space for their offspring. For example, autolysis of *Myxococcus xanthus* cells presumably releases nutrients that feed cells that ultimately form the fruiting body and spores (98), whereas the *Saccharomyces cerevisiae* corpses autolysed presumably provide physical support for the stalk-like structures that form when growing on solid surfaces (143). This
leads us to ask the following questions. What are the rates of cell death in unsaturated biofilms and how do reductions in water availability alter those rates? Is cell death a random process or is it possibly organized? Are there distinct spatial and/or temporal patterns of cell death within unsaturated biofilms? By answering these and other questions we will improve our knowledge of biofilm cell death physiology in water-limited habitats.

REFERENCES


Chapter 2. Reduced Water Availability Influences the Dynamics, Development, and Ultrastructural Properties of *Pseudomonas putida* Biofilms

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**ABSTRACT**

We examined the effect of reduced water availability on unsaturated biofilm formation by *Pseudomonas putida* strain mt-2. Cells were grown on a solid medium in which the water potential was lowered with the permeating solutes NaCl, sucrose, or polyethylene glycol (PEG) with a molecular weight of 200 (PEG-200) or with the non-permeating solute PEG-8000. A biofilm chamber system was developed for visualizing the dynamics and cell organization of unsaturated biofilms by confocal scanning laser or epifluorescence microscopy. We demonstrate that unsaturated biofilm formation proceeds through three distinct developmental phases. These growth phases transform individual cells on a surface into communities encased in a polysaccharide matrix to produce a microcolony, the defining feature of an unsaturated biofilm. The form and severity of reduced water availability alters cell size, which influences microcolony size, and microcolony morphology reflects cell
behavior during growth. The more desiccating PEG 8000 treatments resulted in biofilms that were comprised of smaller cells but were taller, more porous, and had a thicker extracellular polysaccharides (EPS) layer at the air-interface. The greater the reduction in water availability with the ionic solute NaCl, the greater the proportion of the cell population exhibiting a filamented phenotype: filamented cells drastically alter the architecture of mature biofilms. Reductions in water availability with the non-ionic solute PEG 200 resulted in biofilms that were similar to those in the absence of a water deprivation treatment. The studies described here form the basis for investigations into the molecular mechanisms of unsaturated biofilm biology, which is necessary to better understand bacterial ecology in terrestrial habitats.

INTRODUCTION

In terrestrial habitats, bacteria reside on surfaces (soil matrices or plant surfaces) as aggregates of cells or microcolonies that are frequently enmeshed in exopolymeric substances of their own making and can be described as biofilms (1, 7, 23, 27). These biofilms are commonly unsaturated, although they are surrounded by water films, which vary in thickness, depending on the environmental conditions. When a soil dries, these biofilms can be exposed to significant periods of water deprivation due to desiccation and osmotic stress caused by increased concentrations of dissolved solutes in the soil water. The lack of significant fluid flow in terrestrial habitats will likely result in vastly different biofilm architecture from that in fully hydrated (aquatic) systems.

In aquatic systems, channels in the biofilm act as conduits for waste removal and nutrient supply (44). Under low-shear laminar flow Pseudomonas aeruginosa PAO1 biofilms consist
of a monolayer of cells with mound-shaped circular microcolonies, but under high-shear, turbulent flow PAO1 biofilms consist of filamentous streamers (10, 16, 30). Quorum sensing may be required for the differentiation of biofilms into complex mushroom- and tower-like structures (10), although this has not been consistently observed (16). Quorum sensing results from sensing high signal concentrations, and these could be achieved at lower population densities in unsaturated than saturated habitats. Consequently, in unsaturated habitats the lack of laminar fluid flow will dramatically influence nutrient availability patterns, metabolic waste accumulation and disposal, and the accumulation of quorum sensing signal molecules. All of these may influence biofilm properties.

In unsaturated habitats, biofilm cells are likely to experience periods of reduced water availability. Water availability may influence cell physiology and biofilm development and architecture. In a saturated system the water potential is comprised almost exclusively of the solute potential (28). But as a soil or leaf surface dries the matric potential becomes the predominant factor contributing to the total water potential. The difference between these two stresses is that with a solute (osmotic) stress bacteria are bathed in water of diminished activity, whereas with a matric stress bacteria become desiccated by the removal of water from its environment and the availability of the remaining water is reduced through its interaction with the matrix (soil or plant surface). Bacterial production of extracellular polysaccharides (EPS) can confer desiccation tolerance in some bacteria presumably because the hydroscopic properties of EPS create a more hydrated microenvironment surrounding the cells (5, 35). EPS production increases in response to increases in medium osmolarity as well.
The purpose of this work is to further our understanding of bacterial growth and biofilm development in unsaturated habitats. Confocal scanning laser microscopy (CSLM) has become an important tool for characterizing the development and dynamics of biofilms in saturated flow-through experimental systems. CSLM has provided the physical description of the mushroom- or columnar-shaped structures and the suggestion that the microcolony is the basic structural unit (45, 46). Detailed descriptions of the development, dynamics, and ultrastructural properties of unsaturated biofilms have not been as extensively explored. In many ways, growth of bacteria on agar surfaces better approximates the conditions bacteria experience in many unsaturated habitats: the acquisition of nutrients from the underlying matrix and the presence of a relatively thin water film covering the biofilm. Examination of the organization of bacterial cells within colonies grown on agar surfaces has revealed that cells within a colony assume particular organizational patterns and that there are differential gene expression patterns within a colony (11, 25, 32, 37-39). Time-lapse microscopy of the early periods of colony development has implicated the importance of cell-cell interactions in morphogenetic control during early microcolony development (39). Recently, atomic force microscopy of fresh and desiccated *Pseudomonas putida* biofilms revealed that drying had little effect on physical morphology and surface properties (1). However, these studies focused on fully developed, mature colonies (biofilms) that were desiccated. The effects of reduced water availability on the development and ultrastructural properties of unsaturated biofilms were not explored.

The purposes of the study reported here were to characterize the development and ultrastructural properties of unsaturated *P. putida* mt-2 biofilms and to assess the effect of reduced water availability on these biofilms properties. We used permeating (NaCl and
polyethylene glycol (PEG 200) [i.e., a PEG with a MW of 200]) and non-permeating (PEG 8000) solutes to simulate the solute and matric components of soil water potential, respectively (3, 14, 20). To monitor unsaturated biofilm growth we developed an experimental system for non-destructively visualizing microcolony development on gellan gum surfaces using CSLM or epifluorescence microscopy.

**MATERIALS AND METHODS**

**Bacterial strains, media, and chemicals.** *P. putida* strain mt-2 (33) and derivatives thereof were used in the experiments. The *gfp* gene was fused to the constitutive neomycin-phosphotransferase promoter, P_{npth}, on the broad-host-range promoter probe vector pPROBE-KT (22). The *dsRed* gene fused to the constitutive synthetic, lac repressible promoter P_{A1/O4/O3}, on the P_{A1/O4/O3}-RBSII-*dsRed*-T0-T1 cassette was randomly inserted into the chromosome of *P. putida* mt-2 using the pUTKan delivery plasmid (46). Biofilms were cultivated on tryptone yeast extract (TYE) medium, which contained 1 g of MgSO_4·7H_2O, 1.38 g of KH_2PO_4, 5 g of tryptone, 2.5 g of yeast extract, and 40 mL of Hutner’s mineral solution (41) per liter of deionized water and was solidified with 8 g L^{-1} of phytagel gellan gum (Sigma Chemical Co., St. Louis, MO). The water potential of the basal medium was lowered with various concentrations of PEG 8000 to simulate a matric water stress or NaCl, KCl, sucrose, or PEG 200 to simulate an osmotic water stress, as described previously (14). For enumeration of bacteria we used a spiral plater (Spiral Biotech, Bethesda, MD) and Luria Bertani medium supplemented with 15 g L^{-1} agar (Bacto).

**Chamber for visualizing unsaturated biofilms.** We developed a chamber system in which biofilms developed on a thin agar layer covering a cover slip (Fig. 2-1). In a series of
Figure 2-1. Schematic illustration of the chamber used for cultivating and visualizing unsaturated biofilms. Chamber and reservoir dimensions were 2.5 x 7.5 x 0.5 (width x length x depth) cm and 1.4 x 3.5 x 0.3 (width x length x depth) cm, respectively. The chamber was made of black delrin plastic. A sterile glass cover slip was dipped into molten agar and the agar was allowed to solidify before trimming it with a sterile knife to dimensions smaller than the chamber reservoir. It was then placed medium-side down onto the chamber after inoculating it with 1 μL aliquots of a diluted cell suspension, which spread to create a 3 to 5 mm diameter spot. The chamber reservoir contained 2 to 3 mL of the same solid medium used for preparing the substratum for growth to maintain the desired relative humidity. The cover slip was taped to the surface of the chamber.

Preliminary studies we determined that agar thickness was 0.2 to 0.3 mm and this thickness did not impair visualization of cells with a microscope. By including approximately $1 \times 10^4$ Linear Flow Deep Red fluorescent flow cytometry microspheres (2.5 μm diameter) (Molecular Probes Inc., Eugene, Oreg.) in the inoculum we identified the air-substratum interface and determined that the surface was relatively smooth based on the even distribution of the beads. All chambers were inverted and incubated at 27°C. When using the 100X objective, the cover slip was removed and taped onto a microscope slide with the biofilm away from the slide, and another cover slip was gently placed over the tape to cover
the biofilm. The tape raised the second cover slip above the biofilm and caused minimal, if any, disturbance to the biofilm.

**Growth dynamics of unsaturated biofilms.** For all experiments, the inoculum was prepared by resuspending cells from a 24-h old TYE-agar plate culture in fresh TYE broth and diluting 10- to 100-fold in TYE broth. The initial inoculum size was approximately $10^5$ cells per inoculated spot. The effect of reduced water availability on the growth dynamics of *P. putida* on a solid surface was determined by placing a 1-μl aliquot of a dilute cell suspension onto an agar-covered cover slip and then measuring the number of colony forming units (CFU) recovered from an inoculated area over time. Plates were incubated for 1 to 2 days prior to determining the number of colony forming units (CFU) per spot. Values reflect the mean of 3 to 5 experiments.

**Calcofluor staining.** Calcofluor-white (Sigma Chemical Co., St. Louis, MO), which binds to β-linkages of polysaccharides, was added to the medium at 200 µg mL$^{-1}$ for visualization of EPS. In preliminary studies, we determined that we could visualize by epifluorescence microscopy calcofluor staining of the EPS layer surrounding cells grown on solid surfaces.

**Microscopy and image analysis.** Confocal images were obtained using a Leica TCS-NT scanning confocal laser microscope equipped with UV ($\lambda_{ex}$=365 nm), argon ($\lambda_{ex}$=488 nm), and HeNe ($\lambda_{ex}$=633 nm) lasers for visualization of calcofluor-white ($\lambda_{em}$=425 nm, short-cut filter), green fluorescent protein ($\lambda_{em}$= fluorescein isothiocyanate filter set), and fluorescent micro-beads ($\lambda_{em}$=, CY5 filter set), respectively. All multicolor images were collected simultaneously using a multitrack mode (blue = calcofluor; green = GFP; red = fluorescent microspheres). When calcofluor was included in the medium we adjusted the
sensitivity of the photomultiplier tube of the CSLM until blue autofluorescence of the medium was no longer detectable.

Horizontal (x-y) images were taken at 0.5 μm and 0.05 μm intervals using the 100X objective. Vertical cross section images (x-z) were generated with Image J software (http://rsb.info.nih.gov/nih-image). Biofilms were also viewed with a Nikon EFD-3 epifluorescence microscope (Nikon Instruments Inc., Melville, NY) and images were taken with a charged-couple device camera (SPOT Camera, Diagnostic Instruments, Sterling Heights, MI). GFP and DsRed fluorescence was imaged by using a 465-495 nm excitation and 515-555 nm emission filter set, and 528-553 nm excitation and 600-660 nm emission filter set, respectively. Multiple images were overlaid and cropped using Adobe Photoshop (Adobe Systems, Mountain View, CA).

**Cell size determination.** Sizes of cells growing on agar surfaces were determined by measuring the length of individual cells 3 h after inoculation. Although there was a green fluorescent halo around individual cells, we determined in preliminary experiments that cell sizes determined by epifluorescence and phase-contrast microscopy were similar and consequently we used images obtained by epifluorescence microscopy. Approximately 30-90 cells were analyzed for each treatment from at least 5 to 7 fields of view per growth condition. The experiment was repeated twice.

**RESULTS**

**Reduced water availability alters growth dynamics on solid surfaces.** In order to study the effect of reduced water availability on the properties of unsaturated *P. putida*
biofilms, we tagged *P. putida* mt-2 with the GFP by transferring a stable, broad-host-range plasmid containing the *gfp* gene fused to a strong promoter, to derive mt2-GFP. We first examined the effect of water availability on the survival, acclimation time, and growth dynamics of mt2-GFP following inoculation of the substratum using the biofilm chamber described in Fig. 2-1. Lowering the water potential with either the permeating (NaCl, KCl, PEG 200, and sucrose) or non-permeating (PEG 8000) solutes had no affect on the acclimation time before there was visible growth, except at water potentials of -1.0 MPa or lower (data not shown). The acclimation time for growth was approximately 1, 6, and 12 h for the unamended, and -1.5 MPa ψ NaCl- and PEG 8000-treatments, respectively, yet the final yield of colony forming units were comparable by 48 h (Fig. 2-2).

**Figure 2-2.** Effect of reduced water availability on the growth dynamics of *P. putida* mt2-GFP. ●, unamended; ▼, -1.5 MPa ψ NaCl-treatment; ■, -1.5 MPa ψ PEG8000-treatment. Each inoculated spot was excavated to determine the total number of CFU in the spot. The inset highlights the first 24 h of growth. Data points are means ± SEM of three to five replications.
Reduced water availability alters cell morphology and biofilm development. It is possible that lowering the water potential of the growth medium with the permeating or the non-permeating solutes could affect mt2-GFP cell morphology and biofilm development. To address this, we examined the dynamics of biofilm development over a 96 h-period by epifluorescence microscopy and CSLM, and we show representative images to illustrate our findings. Microscopic examination within 1 h after inoculation indicated the vast majority of cells (>96%) colonized the surface as individuals, which indicates that the microcolonies would be comprised of a clonal population.

On the unamended medium, mt2-GFP cells initially grew parallel to the surface in a monolayer to form elongated or crescent moon-like shaped microcolonies (Fig. 2-3A). Within 12 h, the center of the microcolonies were usually a brighter green than the leading edges (Fig. 2-3B), which consisted of tightly clustered arrays of cells protruding from the microcolony (Fig. 2-3B and C). Frequently, these growth projections extended toward adjacent microcolonies (Fig. 2-3B) or the center of the colony from which it emerged. This could be explained by cell-to-cell communication that may direct microcolony development towards neighboring microcolonies or to areas of higher cell densities. We assume the brighter green fluorescence in some cells was due to greater accumulation of GFP in these cells. Initially, regions of the microcolonies consisted of a single cell layer, and over time multiple layers developed. These multiple layers did not develop at the leading edge of the microcolonies. After 24 h the micocolonies typically consisted of a bright green center with some having growth projections in direct contact with adjacent microcolonies (Fig. 2-3D). After 4 days adjacent microcolonies would frequently merge into each other. We examined the degree of intermixing of cells between microcolonies by inoculating the substratum with
Figure 2-3. Temporal dynamics of mt2-GFP biofilm development on unamended TYE medium. A: 6 h; B: 12 h; C: 16 h; D: 24 h; E: 24 h, intermixing of mt2-GFP (green cells) and mt2-dsRed (red cells) during biofilm development. Images were obtained by epifluorescence (A, B, E) and confocal (C, D) microscopy.
a mixture of mt2-GFP and mt2-dsRed cells at a high density. In all treatments, 24 h post-inoculation, even if microcolonies of red and green cells were adjacent to each other there was relatively little observable intermixing (Fig. 2-3E). Frequently, there were clearly visible cell-free areas between adjacent microcolonies suggesting that the two clonal populations did not make direct contact with each other.

As the water potential of the medium was lowered with the non-permeating solute PEG 8000 amendments lower than -0.25 MPa, there was a reduction in cell length compared to cells in the unamended treatment (Table 2-1, Figs. 2-3A, and 2-4B, D, and E). Not only were cell sizes similar in the unamended- and -0.25 MPa ψ/PEG8000-treatments (Figs. 2-3A and 2-4A, Table 2-1), but also the dynamics of microcolony development and the shape of mature microcolonies were similar (data not shown). As the water potential of the medium

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell length (μm)(^a)</th>
<th>Doubling time (min)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.3 ± 0.2</td>
<td>65.1 ± 8.2</td>
</tr>
<tr>
<td>-0.25 MPa ψ/PEG 8000</td>
<td>4.3 ± 0.1</td>
<td>60.9 ± 5.8</td>
</tr>
<tr>
<td>-0.5 MPa ψ/PEG 8000</td>
<td>1.9 ± 0.1</td>
<td>54.2 ± 4.8</td>
</tr>
<tr>
<td>-1.0 MPa ψ/PEG 8000</td>
<td>1.7 ± 0.1</td>
<td>82.9 ± 4.3</td>
</tr>
<tr>
<td>-1.5 MPa ψ/PEG 8000</td>
<td>2.1 ± 0.1</td>
<td>75.9 ± 7.7(^c)</td>
</tr>
<tr>
<td>-0.5 MPa ψ/PEG 200</td>
<td>3.3 ± 0.1</td>
<td>63.5 ± 4.1</td>
</tr>
<tr>
<td>-0.5 MPa ψ/Sucrose</td>
<td>3.3 ± 0.1</td>
<td>61.7 ± 3.6</td>
</tr>
</tbody>
</table>

\(^a\) Values are the mean ± standard error cell length 3 h after inoculation of the substratum. There was no significant difference (P=0.05) in cell diameter among all treatments. As the biofilm ages (24 h-old or older), there was as much as a 25% reduction in cell length, except in the -0.5 MPa or lower ψ/PEG8000 treatments cell length did not change appreciably.

\(^b\) Values are the mean ± standard error of 3 experiments and were derived by monitoring the number of cells in a microcolony 12 h after inoculation of the substratum.

\(^c\) Doubling time was estimated by the change in CFU between 12 and 24 h after inoculation in the data presented in Fig. 2-2.
was lowered with PEG 8000 amendments below -0.25 MPa, microcolonies were no longer elongated, but instead there was a more uniform radial expansion from the colony center (Fig. 2-4C, F, G, and H). Eventually, some of the bright green fluorescent cells in the colony center were more circular than rod-shaped (Fig. 2-4D and E), which suggests that cells changed their alignment from being parallel to perpendicular relative to the surface or cell morphology changed. In general, a reduction in cell size corresponded with a decrease in microcolony size (data not shown). Doubling times were longer in the -1.0 MPa ψ or lower PEG 8000 treatments (Table 2-1).

We observed that ionic and non-ionic permeating solutes affected cell morphology differently, which ultimately influenced biofilm ultrastructure. With the non-ionic solutes (PEG 200 and sucrose) there was a slight reduction in cell size compared to cells in the unamended medium (Figs. 2-3A and 2-5A, Table 2-1), but the reduction in size did not change with increasing PEG 200 or sucrose concentrations (data not shown). Occasionally, there was a filamentous cell amongst a population of typical rod shaped cells in both the PEG 200 (Fig. 2-5B) and the unamended medium treatments (Fig. 2-3C). In contrast, with increasing ionic (NaCl or KCl) solute concentration there was a corresponding increase in the proportion of the cell population exhibiting a filamented phenotype. For example, at 6 h post-inoculation, 3.4 ± 0.8%, 66.1 ± 3.0%, and 100% (mean ± SEM; n= 16 to 23) of the microcolonies (2 to 82 per experiment) contained filamented cells (cells that were at least 10-times longer than the typical cell length) in the unamended, and -1.0 and -1.5 MPa ψ NaCl-treatments (Fig. 2-5E), respectively. This behavior prevented accurate measurements of cell sizes and doubling times. Examination of these cells at higher magnifications indicated that some had indentations resembling those that form during septation (data not shown).
Figure 2-4. Effect of lowering the water potential with the non-permeating solute PEG 8000 on the temporal dynamics of the early-to-intermediate phase of mt2-GFP biofilm development. A: -0.25 MPa, 6 h; B: -0.5 MPa, 6 h; C: -0.5 MPa, 12 h; D: -0.75 MPa, 9 h; E: -1.0 MPa, 6 h; F: -1.0 MPa, 16 h; G and H: -1.5 MPa, 24 h. Images were obtained by epifluorescence (A-F) and confocal (G-H) microscopy. Arrows point towards circle-shaped cells.
The dynamics of microcolony development in the non-ionic solute and unamended treatments were very similar. The biggest difference was that in the non-ionic solute treatments the projections of growth away from the colony center were fewer and those that did form were shorter, rounder and rarely made contact with neighboring microcolonies unless they were in close physical proximity to each other (Fig. 2-5C and D). Multiple cell layers in the microcolonies were evident in microcolonies in the ionic solute and unamended treatments by 12 h of growth (Fig. 2-5D). In contrast, the dynamics of microcolony development of filameneted cells in the ionic solute and the unamended treatments were very different. First, the microcolonies derived from filameneted cells did not have protrusions (Fig. 2-5F), as did the colonies in the unamended medium (Fig. 2-3B). Second, whereas cells in microcolonies on the unamended medium remained similar in length as the microcolony aged, the proportion of the cells exhibiting a typical rod-shaped morphology increased, and the proportion that was filameneted decreased, as the microcolonies on the ionic-solute amended media aged. Microcolonies derived from typical rod-shaped cells in the NaCl or KCl treatments were similar to those derived in the non-ionic solute treatments (data not shown). In general, microcolony surface area in the ionic and non-ionic solute treatments was comparable to those in the unamended medium treatment after 4 days (Table 2-2). However, microcolony surface area was substantially smaller on the PEG-8000 amended medium than on the unamended or the −1.5 MPa ψ NaCl-amended medium (Table 2-2). In general, the greater the number of microcolonies present in a defined area the smaller the microcolony, which suggests competition for resources can limit microcolony size.
Figure 2-5. Effect of lowering the water potential with the permeating solutes PEG 200 or NaCl on the temporal dynamics of the early-to-intermediate phase of mt2-GFP biofilm development. A-D: -0.5 MPa PEG 200-treatment. E-F: -1.5 MPa NaCl-treatment. A: 6 h; B: 9 h; C: 12 h; D: 12 h; E: 12 h; F: 24 h. Images were obtained by epifluorescence (A-E) and confocal (F) microscopy. Arrow in panel B indicates filamented cell and in panel D multiple cell-layers.
Table 2-2. Influence of reduced water availability on biofilm properties

<table>
<thead>
<tr>
<th>Treatment and day</th>
<th>Microcolony surface area (μm²)(^b)</th>
<th>Density (cells/100 μm²)(^c)</th>
<th>% cell-free area(^c)</th>
<th>Biofilm cell height (μm)(^d)</th>
<th>EPS thickness at air interface (μm)(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>32,622 ± 5,234</td>
<td></td>
<td></td>
<td>6.2 ± 0.3</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>40,801 ± 2,457</td>
<td>114.8 ± 0.8</td>
<td>22.2 ± 0.4</td>
<td>6.3 ± 0.3</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1.5 MPa NaCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>23,899 ± 3,821</td>
<td>72.3 ± 0.9</td>
<td>24.6 ± 0.3</td>
<td>6.7 ± 0.2</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>38,568 ± 2,457</td>
<td></td>
<td></td>
<td>6.8 ± 0.9</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>-1.5 MPa PEG8000</td>
<td></td>
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<td>1</td>
<td>1,568 ± 405</td>
<td>71.8 ± 1.6</td>
<td>49.7 ± 1.1</td>
<td>11.0 ± 0.8</td>
<td>2.7 ± 0.5</td>
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<tr>
<td>4</td>
<td>4,900 ± 1,056</td>
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<td></td>
<td>14.4 ± 0.9</td>
<td>3.9 ± 0.5</td>
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\(^a\) Data are expressed as means ± standard errors of the means.
\(^b\) Microcolony area at the substratum surface. Values were derived from the average microcolony surface area per field of view (number of samples, 3 to 4) containing 1 to 14 microcolonies.
\(^c\) Density as the number of cells/100μm² and the proportion of a 100 μm² area devoid of cells. Values were derived from 29-39 x-y plane CSLM images from 8 to 13 randomly chosen microcolonies obtained in two separate experiments.
\(^d\) The height from the substratum to the top of biofilm. Values were derived from nine random measurements of three to eight x-z projections of a stacked series of x-y plane green fluorescence (cells) CSLM images taken with a 40X objective.
\(^e\) The thickness of the calcofluor-stained layer from the top of the biofilm to the air interface. Values were derived from the images used for the biofilm cell height measurements.
Reduced water availability alters ultrastructural properties. It is possible that due to different growth strategies in the permeating and non-permeating solute treatments (Figs. 2-4 and 2-5), cells within these biofilms are organized differently. We investigated cell organization within 12- to 24-h old microcolonies that formed under reduced water availability conditions. Representative images of single optical sections (x-y plane) from mt2-GFP biofilms grown on the unamended and -1.5 MPa NaCl- and PEG 8000-treatments are shown in Fig. 2-6. It is clear from these images that the form of reduced water availability influences cellular organization within the biofilm. A highly organized pattern of circular cells packed in an orderly array was observed in the x-y sections from the top of the microcolony to the surface of the substratum in the unamended treatment (Fig. 2-6A). This pattern was consistently observed in all sections except at the periphery of the microcolony. Since P. putida is a rod-shaped organism the consistent circular shapes of bacterial cells throughout the depth of the biofilms on the unamended medium suggested that the rod-shaped cells were arranged perpendicular to the surface. In the center of the microcolonies cell density was generally the same at all depths (Table 2-2, and date not shown). Approximately 22% of the biofilm volume did not contain cells (Table 2-2). Cells at the edges were generally arranged parallel to the surface as observed during early periods of microcolony development (Fig. 2-3C).

Cells in microcolonies that formed under a -1.5 MPa NaCl treatment were present as a mixture of circular- and oval-shaped cells (Fig. 2-6B) that were at a much lower density than in the unamended treatment (Table 2-2). We also occasionally observed a filamented cell that snaked its way through several sagital sections (Fig. 2-6B, and data not shown). The presence of a mixture of filamented and typical rod-shaped cells suggests that some of the filamented
Figure 2-6. Overhead (x-y) confocal scanning laser images of 24 h-old mt2-GFP biofilms. A: unamended medium; B: -1.5 MPa NaCl-treatment; C: -1.5 MPa PEG 8000-treatment.

cells were capable of fragmentation by septation during microcolony maturation to give a population of ordinary-sized and filamented cells (Figs. 2-5E and 2-6B).

In the −1.5 MPa ψ PEG8000 treatment heterogeneously distributed aggregates of small, circular-shaped cells were frequently adjacent to large regions devoid of cells (Fig. 2-6C). Biofilms in the −1.5 MPa ψ PEG 8000 treatment had a lower density and were more porous (more cell free areas) than in the unamended and −1.5 MPa ψ NaCl treatments, respectively (Table 2-2).

Examination of cell organization in the x-z plane suggests that mt2-GFP rod-shaped cells were generally arranged perpendicular to the surface in a columnar fashion (Fig. 2-7). In the unamended and −1.5 MPa ψ NaCl treatments there were generally 2 to 3 cells stacked pole-to-pole almost directly on top of each other (Fig. 2-7A and B). In the −1.5 MPa ψ PEG8000 treatment, cells were arranged perpendicular to the surface in a more-or-less columnar fashion (Fig. 2-7C). Because of the substantially reduced cell length in the PEG-8000 treatments below −0.25 MPa ψ there could be as many as 6 to 9 cells in a column. Biofilm
cell height increased over time in the $-1.5$ MPa $\psi$ PEG8000 treatment, but not in the unamended and $-1.5$ MPa $\psi$ NaCl treatments (Table 2-2). In the $-1.5$ MPa $\psi$ PEG8000 treatment, the presence of cell-free regions only one or two cell-diameters in width that could create channels spanning the entire height of the microcolony were visible (Fig. 2-7C). It is unclear whether the large cell-free regions or the area immediately surrounding the cells contain extracellular polymeric substances, which may help support the columnar arrangement of the cells.

**Localization of EPS in unsaturated biofilms.** Calcofluor white, which binds to $\beta$-linkages of carbohydrates and fluoresces blue when exposed to UV-light, was incorporated into the medium to facilitate localization of EPS in the biofilm. Individual cells were surrounded by calcofluor-stained material (Fig. 2-8A and B), but most of the stained material was present at the biofilm-air interface (Fig. 2-8C and D, Table 2-2). Similar results were observed in $-1.5$ MPa $\psi$ NaCl treatments (data not shown). Cell-free regions of the microcolony in the $-1.5$ MPa $\psi$ PEG8000 treatment (Fig. 2-8D) usually contained calcofluor-stained material. Calcofluor-stained material was also detected around cells at the periphery of the colony and in areas adjacent to the colony edges (data not shown). The thickness of the EPS layer at the air-interface was highly variable within a treatment and greatest in the $-1.5$ MPa $\psi$ PEG8000 treatment (Table 2-2), although it generally comprised roughly only 20% of the total biofilm height (biofilm cell height + EPS layer thickness) in any treatment.
Figure 2-7. Sagital (x-z) confocal scanning laser images of 24 h-old mt-2-GFP biofilms. Sagital images were created from a collection of 148 (A), 160 (B), and 234 (C) Z-series scans with a 0.05 μm interval between each Z section using a 100 X objective. The arrows in C indicate contiguous cell-free regions.
**DISCUSSION**

*P. putida* unsaturated biofilm formation proceeds in an organized fashion through early, intermediate, and maturation phases of development. Similar distinct developmental phases have been reported for biofilm formation by many microorganisms in fully hydrated static and flow-through systems (4, 9, 27), although the nature of these phases was distinct in the unsaturated biofilms studied here. Reduced water availability plays a particularly important role in the developmental process. Below we discuss our findings in the context of each developmental phase.
The early phase includes an acclimation period before visible growth, and these periods were, for example, as much as 6- and 12-fold longer at water potentials of -1.0 MPa or lower in the NaCl- and PEG 8000-treated samples, respectively (Fig. 2-2, and data not shown). Reduced water availability caused by the nonpermeating solute PEG 8000 at water potentials lower than -0.25 MPa caused 50 to 60% reductions in cell length that did not change with decreasing water potentials, suggesting that there was a threshold level of dehydration at which cell shrinkage occurs, possibly at an optimal surface-to-volume ratio. Mechanisms that could cause cell size reductions include cell membrane changes or decreased nutrient availability, although the latter is unlikely because cell doubling times were comparable in the -0.5 MPa $\psi$PEG8000- and unamended-treatments (Table 2-1).

The presence of high concentrations of ionic solutes alters cell morphology in the early phase, resulting in a more complex developmental process. The proportion of the population that was filamentous increased as the ion concentration increased (data not shown). Mature microcolonies derived from normal sized cells were comprised of an occasional filamented cell (Fig. 2-6B). If the reduction in number of filamented cells resulted from septation and division of these cells, these changes could cause the less orderly arrangement of cells observed in the -1.5 MPa $\psi$NaCl treatments (compare Fig. 2-6A and B). Bacterial filamentation has been observed in response to nutrient, $\text{Mg}^{2+}$ or oxygen deprivation, low temperature, or high osmolarity (18, 19, 40, 43). Although the mechanism causing filamentation is unknown, NaCl and KCl may interfere with the production or action of cell division proteins involved in septation, while permitting biomass increases. The subsequent septation of filaments into typical cell sizes as the microcolony ages may result from specific adaptations countering the inhibitory effects of NaCl or KCl on cell division processes.
Filament formation has also been observed in mature *P. putida* biofilms in flow-through systems (17).

The early-to-intermediate phase of unsaturated biofilm formation is comprised of rapid growth of the rod-shaped *P. putida* cells parallel to the nutrient-containing surface; this orientation maximizes the surface area for nutrient and waste diffusion into and out of the cell. Except for the occasional presence of filamented cells, cellular morphology was relatively constant during this phase. During the rapid radial expansion of the microcolony, cells remained in close contact to one another following division, and protrusions from the microcolonies grew towards other microcolonies presumably to maximize cell-to-cell contact, although there was little visible intermixing of cells (Fig. 2-3E). There was great heterogeneity in the patterns of cell alignment in the microcolonies and each colony harbors within it a pattern related to the developmental history of the biofilm. Production of an extracellular matrix covering the nascent microcolony was evident by the presence of calcofluor-stained material covering the cells at the air interface.

The maturation phase begins when the microcolonies assume a more complex, three-dimensional organization, which was first visible 9 to 12 h into microcolony formation when cells began to change their orientation from being parallel to perpendicular to the surface. This may occur in response to physical crowding or to cell density-dependent signals, or both, and is likely to involve polar type IV pili, which are involved in twitching motility and were recently identified in the *P. putida* genomic sequence (24). Once oriented vertically, cell growth (biomass increase) and reproduction can proceed, which could explain our observation that cells were stacked pole-to-pole 2 or more cells high. Microcolonies that form on the PEG 8000 amended medium increase in height over time (Table 2-2), but it is
unclear whether this was due to growth at the base or at the top of the microcolony. The lack of a columnar arrangement of cells and the more heterogeneous distribution of cells in microcolonies in the $-1.5 \, \text{MPa}$ $\psi$ PEG8000 treatments could create gradients (e.g., nutrient) due to the greater tortuosity of diffusional paths around the cells, and such gradients could alter biofilm properties.

The images of our unsaturated biofilms showed that cells were generally uniformly upright and perpendicular to the surface throughout the depth of the microcolony on the unamended and the $-1.5 \, \text{MPa} \, \psi$ NaCl amended medium (Fig. 2-7). Similar arrangements of cells have been observed in colonies of *Vibrio parahaemolyticus* (11) and *P. putida* (37) grown on agar as visualized by CSLM and scanning electron microscopy, respectively. The fact that the cell arrangement we observed was similar to that of other bacterial species suggests that this pattern may be a general phenomenon and reflects that it is social in nature. In other studies examining the morphology of cells at the bottom of unsaturated *Pseudomonas* biofilms using atomic force microscopy the cells were observed to be parallel rather than perpendicular to the membrane surface (1, 43). This difference in cellular arrangement could be the result of cultivating the biofilms on a nylon membrane or how samples were prepared for atomic force microscopy.

As the biofilm matures, EPS fills the areas between cells. Most of the calcofluor-stained material was localized at the biofilm-air interface, possibly creating a more hydrated microenvironment around the biofilm. We observed an increase in the amount of EPS produced in response to cellular dehydration imposed by the PEG 8000 treatments but not by increases in osmolarity (See Chapter 3). This increased EPS production resulted in a thicker EPS layer over the microcolony (Table 2-2). It is likely that the EPS viscosity in the biofilm
was greater on the PEG 8000 amended medium than on the unamended medium due to the combined effect of increased EPS production, reduced water content, and reduced microcolony biovolume. Although the *P. putida* EPS bound calcofluor, the chemical composition of this EPS produced by *P. putida* mt-2 is unknown. Other *P. putida* strains are known to synthesize marginalan (13), and the genomic sequence of *P. putida* KT2440 contains a novel EPS biosynthetic operon (possibly for marginalan), a nearly complete alginate biosynthetic pathway, and a cellulose biosynthesis operon (24). Although *P. putida* does not produce alginate under normal culture conditions, we observed the production of an uronic acid-containing EPS, presumably alginate, in the PEG 8000- but not the NaCl-treatments (See Chapter 3). However, the amount of this uronic-acid containing EPS produced does not account for observed EPS increases in response to the PEG 8000 treatments; this suggests that other carbohydrates are synthesized. Alginate-overproducing strains of *P. aeruginosa* in flow-through biofilm systems exhibit enhanced microcolony formation, cover less surface area, and have a more highly heterogeneously structured mature biofilm (15), much like the *P. putida* microcolonies on the PEG 8000 amended medium. For some bacterial species, EPS production is required for surface attachment and for the characteristic biofilm architecture in saturated systems (8, 26, 27, 47). The role, if any, of these EPSs in unsaturated biofilm formation or maintenance needs to be explored fully.

Agar surface wetness (inversely related to agar concentration) can have a profound effect on the development and final shape of bacterial and yeast colonies (21, 34, 36, 42) and whether motile strains produce large radial colony forms or small fractal-shaped colonies. Bacterial motility is very sensitive to drying and ceases entirely when the water potential falls below −0.14 MPa (29). We determined that colony development and shape was similar for
the motile mt2-GFP and a non-motile mutant (See Appendix E) in all experimental conditions we examined, indicating that water film thickness was too thin for flagellar motility or this form of motility is not important for unsaturated biofilm development.

The extent to which bacteria colonize soil or plant surfaces as biofilm communities rather than as individuals is not well understood, but several reports have demonstrated the presence of biofilms in natural habitats (12, 23) and particularly on roots grown in soil containing introduced bacteria (2, 6, 31). Biofilms isolated from leaf surfaces may be tens of micrometers thick, have a copious exopolymeric matrix, form extensive networks several millimeters long and thick, and contain several thousand cells or more (23). With the nutrient rich conditions we used in this study, 24 h-old microcolonies in the unamended treatment were typically 175 to 250 µm across, 6 µm thick, and contained $9.4 \times 10^4$ to $1.5 \times 10^5$ cells per microcolony, assuming a density of 115 cells/100 µm$^2$ and they were 2 to 3 cell layers thick. The conditions that we used may not have been optimal for biofilm formation or may lead to the formation of an exopolymeric matrix that is not copious or complex enough to reflect those found in nature. It should be noted that biofilms found on plant surfaces may be encased in a copious exopolymeric matrix derived from both the microbial community and the plant (6).

Bacterial biofilm formation in unsaturated conditions is a multi-stage process that is influenced by the availability of water. Reduced water availability affects cell growth morphologies, growth rates, and cellular physiology (e.g., increased EPS production), all of which influence biofilm development and properties. We examined the developmental process leading to 96 h-old unsaturated biofilms. Studies on the further development of these biofilms will likely identify maintenance and death phases. Further studies should also
examine unsaturated biofilm development by other organisms under more accurately reflecting *in situ* conditions to reveal the true nature of this growth form in soil or on plant surfaces. It will be interesting to determine if the highly organized biofilm architecture we observed is dependent on specific cell surface components, motility systems, or cell-to-cell interactions. The impact of this information will be widespread ranging from new insights into environmental microbiology to the development of strategies to control or enhance biofilm development or their metabolic activities.

**ACKNOWLEDGMENTS**

We thank Carol Casavant for providing the GFP-tagged *P. putida* mt-2 strain, Martijn van de Mortel for providing the non-motile mutant, Naomi Bremer for providing mt2-dsRed, and Margaret Carter of the ISU Image Analysis and Confocal Microscopy Facility for assistance with confocal microscopy. We also thank Gwyn Beattie for critically reviewing a draft of the manuscript. This research was supported in part by the Agronomy Department Endowment Funds, a grant from the Mary and Raymond Baker Family Trust, and the Iowa Agriculture and Home Economics Experiment Station.

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2. **Bloemberg, G. V., A. H. Wijffjes, G. E. Lamers, N. Stuurman, and B. J. Lugtenberg.** 2000. Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere:


Chapter 3. Alginate Contributes to Biofilm Development and Fitness of 
*Pseudomonas putida* in Low-Water-Content Habitats

A paper to be submitted to Proceedings of the National Academy of Sciences of 
the United States of America

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University, Ames, IA 50011-1010

**ABSTRACT**

Bacteria colonize soil and plant surfaces as aggregates of cells encapsulated in an 
exopolymeric substance of their own making to form biofilms. The hygroscopic nature of 
exopolysaccharides (EPS) may improve bacterial growth and survival in low-water-content 
habitats. We assessed whether alginate is a component of the *Pseudomonas putida* EPS layer 
surrounding biofilms in matric stress (low-water-content) environments, what role(s) it has in 
biofilm developmental processes and ultrastructure, and whether it contributes to desiccation 
tolerance. Carbohydrate composition analysis of biofilm EPS revealed that alginate was not a 
component of the EPS in the absence of a matric stress, but instead it was composed of one 
or more potentially novel polysaccharides. With increasing severity of matric stress, there 
was a corresponding increase in alginate production as well as total amount of EPS produced 
by the wild type, but not in an alginate-deficient mutant. Furthermore, alginate was not 
produced under thermodynamically equivalent osmotic stresses, at low temperatures, or in
the presence of a toxic concentration of toluene. The architecture of biofilms formed by the alginate deficient mutant in water-limited conditions was shorter, covered more surface area, had a thinner EPS layer at the biofilm-air interface and was less porous than those formed by the wild type. To demonstrate that the hygroscopic properties of alginate mitigate a sudden desiccation event in the unsaturated \textit{P. putida} biofilm, we assessed the extent to which alginate slows the rate at which water is lost from the cytosol using a water stress-responsive transcriptional fusion to quantify the water potential sensed by individual biofilm cells following a dehydration shock. We also assessed the ability of alginate to protect cellular membranes from dehydration-mediated changes in fatty acid composition following a dehydration shock. These results suggest that alginate plays an important role in maintaining an appropriate microenvironment for developing microbial community upon rapid reductions in water content. Alginate also contributed to the ability of cells to survive a severe desiccation stress. Taken together, alginate production is an important consequence of cellular dehydration, which contributes to the fitness of \textit{P. putida} in low-water-content habitats.

\section*{INTRODUCTION}

Many terrestrial habitats such as soil and plant surfaces are unsaturated, and a thin layer of water surrounds resident microbes that frequently form aggregates called biofilms. Water film thickness depends on the water content of the habitat and properties of the surfaces. \textit{Pseudomonas putida} biofilm formation in unsaturated conditions is a multi-stage process where reduced water availability affects biofilm development and properties including growth rate, microcolony surface area coverage, cell density, biofilm height, and
exopolysaccharide (EPS) thickness at the air interface (See Chapter 2). Since EPS is hygroscopic, its presence may create a more hydrated microenvironment surrounding the cells; therefore, it can presumably confer desiccation tolerance to some bacteria (6, 40). Roberson and Firestone observed more EPS production by a soil *Pseudomonas* sp. in desiccated than undesiccated sand cultures and lowered drying rates of sand cultures by EPS, suggesting that resources were allocated to EPS production, presumably, to prevent water loss in response to desiccation (40). In addition, although in eukaryotes, there is evidence that polysaccharides buffer plant leaves from drying (34).

Alginate, a linear polymer consisting of O-acetylated, β-1,4-linked D-mannuronic acids and its C-5 epimer L-guluronic acid, is one of the many well-characterized EPSs of pseudomonads (13). Many reports of alginate production by plant-pathogenic or saprophytic pseudomonads have indicated that alginate may contribute to pathogenicity as a virulence factor, adhesion to plant surfaces, or protection of cells from adverse environmental conditions (8, 15, 58). Yu et al. have demonstrated that an alginate-deficient mutant of *P. syringae* pv. *syringae* was significantly impaired in its ability to colonize tomato leaves and caused less severe disease symptoms on bean leaves (58).

In most studies, if not all, the role of alginate in biofilm formation has been based on using alginate-overproducing strains in which inactivation of the anti-sigma factor MucA (AlgS), which inhibits alginate biosynthesis, results in a mucoid phenotype. According to recent studies, alginate is not a component of non-mucoid *P. aeruginosa* biofilm EPS (55), although they have the genetic ability to produce it (47). This genetic ability for alginate biosynthesis was also demonstrated in other rRNA homology group I pseudomonads by DNA-DNA hybridization (17). Despite the fact that the role of alginate has been intensively
studied in several saprophytic and plant- and human-pathogenic *Pseudomonas* species, little is known about whether the physical removal of water (dehydration) stimulates alginate biosynthesis and what role(s) this EPS has in tolerance to this environmental stress. Here we report increased alginate production in response to matric stress and its role in desiccation tolerance of *P. putida*. We have chosen a soil saprophyte and rhizosphere colonist *P. putida* strain mt-2, since it is a model soil organism and has a TOL plasmid for toluene biodegradation. The genomic sequence of *P. putida* strain KT2440 has revealed a complete alginate biosynthetic pathway (35) almost identical to that in *P. aeruginosa* PAO1 except that KT2440 is missing *algM (mucC)*, a negative regulator of alginate production (3).

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and media.** *P. putida* strain mt-2 (39) and derivatives thereof used in the experiments are listed in Table 3-1. Cells were cultivated, unless otherwise stated, on tryptone yeast extract (TYE) medium, which contained 1 g of MgSO$_4$·7H$_2$O, 1.38 g of KH$_2$PO$_4$, 5 g of tryptone, 2.5 g of yeast extract, and 40 mL of Hutner’s mineral solution (45) per liter of deionized water and was solidified with 8 g L$^{-1}$ of phytagel gellan gum (Sigma Chemical Co., St. Louis, MO). The water potential ($\psi$), free energy of water, of the basal medium was lowered with various concentrations of PEG-8000 to simulate a matric water stress and NaCl or PEG-200 to simulate an osmotic stress, as described previously (20). Three point two or 100 g, 6.4 or 150 g, 12.8 or 262 g, 19.2 or 330 g, and 32 or 450 g of either NaCl or PEG-8000 were added per liter to make -0.25, -0.5, -1.0, -1.5, and -2.5 MPa $\psi$, respectively (21, 46). For a PEG200 amended medium, 105 g of PEG
added to reduce $\psi$ by -1.5 MPa. For dehydration shock experiments, we used -1.0 MPa and -2.5 MPa $\psi$ PEG8000 amended ½ 21C medium, which contained 0.5 g of NH$_4$Cl, 1.725 g of Na$_2$HPO$_4$·7H$_2$O, 1.38 g of KH$_2$PO$_4$, 2 g of glucose, and 20 mL of Hutner’s mineral solution (45) per liter of deionized water. The medium was solidified with 10 g L$^{-1}$ of phytage l gellan gum (Sigma Chemical Co., St. Louis, MO).

Table 3-1. Bacterial strains and plasmids used in this study

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<td>This study</td>
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<td>LH22</td>
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<td><em>E. coli</em> strains</td>
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<td>N. C. Casavant</td>
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<td>Suicide vector, Gm$^r$</td>
<td>(53)</td>
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<td>pPNptlce</td>
<td>Plasmid containing a constitutive nptII-inaZ fusion</td>
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$^a$ Rif$^r$, rifampicin resistance; Gm$^r$, gentamycin resistance; Sm$^r$, streptomycin resistance; Km$^r$, kanamycin resistance.

**EPS isolation and quantification.** Magnagraph nylon membranes (MSI, Westboro, Mass.) were placed on TYE plates prior to spreading a 100-μl aliquot of a 24 h-old surface-grown *P. putida* culture resuspended in 1 mM phosphate buffer. To maintain the desired relative humidity, plates were incubated in covered plastic containers for 24 h at 27°C. The nylon membrane was removed from the plates and excess agar scraped off the membrane.
prior to placing it in 20 ml of 0.85% saline, vortexing, and centrifuging at 16,300 \times g for 30 min at 4°C to separate the EPS from the cells. The EPS-containing supernatant was filtered (0.45-\mu m), and treated with proteinase as described previously (38). EPS was precipitated by adding 3 volumes of ethanol (-20°C), incubating overnight at -20°C, and then centrifuging at 16,300 \times g for 30 min at 4°C. Precipitation with ethanol was repeated one time on the ethanolic supernatant as described previously (5). The EPS was dried at room temperature and resuspended in deionized water. Cell protein content was determined by the method of Bradford (4) with bovine serum albumin as the standard (Bio-Rad, Hercules, Calif.). Total carbohydrate content was determined by the phenol-sulfuric acid method (11) with glucose as the standard and uronic acid content was determined by the m-phenylphenol method (2) with D-glucuronic acid as the standard.

**Carbohydrate composition analysis.** Carbohydrate composition analysis was performed by the Complex Carbohydrate Research Center at University of Georgia (Athens, Georgia). In brief, methyl glycosides were first prepared by methanolysis in 1 M HCl in methanol at 80°C (18-22 h), followed by re-N-acetylation with pyridine and acetic anhydride in methanol for detection of amino sugars. The samples were then per-O-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80°C (0.5 h), separated by gas chromatography (GC) by using an All Tech EC-1 fused silica capillary column (30m \times 0.25 mm ID), and analyzed by mass spectrometry (MS). Twenty \mu g of inositol was added to the sample before derivatization as an internal standard. These procedures were carried out as previously described (30, 57). The amount of each sugar was expressed as mole percentage of total carbohydrate.
Construction of an alginate-deficient mutant. We designed PCR primers based on an alginate biosynthesis operon identified in the genomic sequence of *P. putida* KT2440 (www.tigr.org) to amplify a 622-bp internal region from *P. putida* LH1, corresponding to nucleotide position 17-644 of the 1.3 kb *algD* gene of *P. putida* KT2440. Primers for the PCR reaction were 5'-TTGGTTTGGGTTATGTGGG-3' (forward) and 5'-CAGGTGTACTTGATCATTTCGG-3' (reverse). After colony PCR, the PCR product was inserted into the *XcmI* site of the suicide vector pKnockout-G (53) to create the hybrid plasmid pKAD. The insertion was confirmed by sequence analysis (data not shown). The pKAD was transferred from the donor stain *E. coli* S17-1 to *P. putida* LH1 by conjugation with the assistance of the helper functions of *E. coli* pRK2073. By homologous recombination, pKAD was integrated into the *algD* gene of *P. putida* such that it generated a polar mutation. Insertion and orientation of pKAD into the genome was confirmed by PCR using primers for the *algD* gene and the suicide vector pKnockout-G (See Appendix C). The pKnockout-G vector primers were 5'-CCCAGTCACGACGTTGTAAAACG-3' (forward) and 5'-AGCGGATAACAATTTCACACAGG-3' (reverse). To verify alginate deficiency in the putative alginate deficient mutants, we quantified the uronic acid content of the EPS of biofilms cultured on the -1.5 MPa y/PEG8000 amended medium (See Appendix C). One of the alginate deficient mutants, designated LH22, was used for further investigation.

Cultivation of biofilms. Unsaturated biofilms were developed using the previously described experimental system (Chapter 2). In order to visualize unsaturated biofilms, we tagged cells with the green fluorescent protein (GFP) by transferring a stable, broad-host-range plasmid pPROBE-KT (32) containing the *gfp* gene fused to the constitutive neomycin-phosphotransferase (*Pnants*) promoter. The inoculum was prepared by resuspending cells from
a 24-h old plate culture in 1/8-strength Tryptic Soy Broth (TSB) prior to inoculating the solid medium on a coverslip with a 1-µl aliquot containing 10 to 20 cells. The coverslip was placed medium side down onto the chamber, taped to the chamber surface, inverted, and incubated at 27°C. Calcofluor-white (Sigma Chemical Co., St. Louis, MO), which binds to β-linkages of polysaccharides, was added to the medium at 200 µg mL⁻¹ for visualization of EPS.

**Microscopy and image analysis.** Confocal images were obtained using a Leica TCS-NT scanning confocal laser microscope (SCLM) equipped with UV (λ<sub>ex</sub>=365 nm) and argon (λ<sub>ex</sub>=488 nm) lasers for visualization of calcofluor-white (λ<sub>em</sub>=425 nm, short-cut filter) and green fluorescent protein (λ<sub>em</sub>= fluorescein isothiocyanate (FITC) filter set), respectively. All multicolor images were collected simultaneously using a multitrack mode (blue = calcofluor; green = GFP). When calcofluor was included in the medium we adjusted the sensitivity of the photomultiplier tube of the CSLM until blue autofluorescence of the medium was no longer detectable. Horizontal (x-y) images were taken at 0.5 µm intervals using the 40X objective. Vertical cross section images (x-z) were generated with Image J software (http://rsb.info.nih.gov/nih-image). Biofilms were also viewed with a Nikon EFD-3 epifluorescence microscope (Nikon Instruments Inc., Melville, NY) with a FITC filter set and images were taken with a charged-couple device camera (SPOT Camera, Diagnostic Instruments, Sterling Heights, MI). Multiple images were overlaid and cropped using Adobe Photoshop (Adobe Systems, Mountain View, CA).

**Dehydration shock experiment.** A 100-µl aliquot of a 24-h-old surface-grown *P. putida* culture (O.D.=0.001 at 660 nm) was inoculated on a quadrant of an 80 mm diameter nylon membrane (MSI, Westboro, Mass.), prior to overlaying the membrane onto a ½-
strength 21C solid medium in which the water potential was lowered to -1.0 MPa with PEG-8000. This condition was chosen because it was conducive to alginate biosynthesis. Biofilms were incubated for 24 to 36 h at 24°C or 27°C for ice nucleation activity (INA) reporter gene measurements or fatty acid methyl ester (FAME) composition analysis, respectively. The membrane containing the biofilms was then transferred to solid media in which the water potential was lowered to -2.5 MPa ψ with PEG-8000 or NaCl to create a dehydration or osmotic shock, respectively. As a control, membranes were also transferred to -1.0 MPa ψ PEG8000 treatments to ascertain the effect of a fresh supply of nutrients on physiological responses. After membrane transfer, biofilms were incubated at 24°C or 27°C until cells were harvested by resuspending cells from the filters in ½-strength 21C broth for INA assays or by scraping the cells from the filters for FAME analysis, respectively. The actual water potential of the solid media was measured using WP4 Dewpoint PotentiaMeter (Decagon Devices, Pullman, WA), following cell harvest.

**INA assay.** pPProIce or pPNptIce transcriptional fusion plasmid containing the proU promoter inducible by low water potential or the constitutive nptII promoter in front of the reporter inaZ gene, respectively (56), was introduced into both LH1 and LH22 by conjugation. Total ice nuclei were measured at -7.5°C by a droplet freezing assay (27) and the INA of cells was expressed as the number of ice nuclei per cell. Culturable counts were determined with a Model D spiral plater (Spiral Systems Instruments, Bethesda, MD) by plating onto Luria Bertani (LB) medium supplemented with 15 g L⁻¹ agar (Bacto).

**FAME analysis.** We used whole-cell FAME analysis to assess the effect of a dehydration shock on fatty acid composition as described previously (20). Briefly, total cellular fatty acids were extracted from cells harvested from the membranes by mixing them
with a 15% NaOH solution made in methanol and dionized water (1:1). The fatty acids were then methylated using a 6 N HCl-methanol solution (3.25:2.75). Finally, FAMEs were extracted with a 1:1 mixture of hexane and methyl-tert-butyl ether (MTBE) before the base wash. Flame ionization detection gas chromatography was performed using the MIDI system (Newark, Del.) according to the manufacturer's recommended protocols. Peaks were compared to known standards with the Sherlock-MIDI identification system and 16:1 ω7t and ω7c FAME standards (Sigma Chemical Co.), since the Sherlock-MIDI system lumps these two fatty acids into one group (designated sum4). PEG-8000 was previously shown not to produce FAME peaks that could be mistaken for fatty acids (20).

Filter disk desiccation assay. The effect of alginate production on desiccation tolerance was assessed using a filter disk desiccation assay that was performed as described previously (50). In brief, outgrowths were prepared prior to inoculation by diluting overnight cultures in fresh TYE broth to an optical density at 660 nm of 0.2. Four 5 μl-aliquots of outgrowths were then spotted onto each quartered section of a sterile MF™ membrane filter disk (0.45 μm HA, 47 mm diameter; Millipore, Bedford, Mass.) preconditioned with 200 μl TYE broth. Two filter sections were prepared for each strain, and one was transferred to a desiccator where the reservoir was filled with water to generate 100% relative humidity (RH), while the other was transferred to a desiccator where the reservoir was filled with a saturated NaCl solution to generate 75% RH (54). Desiccators were kept at room temperature. Colony forming units (CFU) were determined for undesiccated and desiccated samples at various times by resuspending cells from the filter disk into a phosphate buffer, and then sonicating for 2 min prior to plating dilutions with a Model D spiral plater (Spiral Systems Instruments, Bethesda, MD). Desiccation tolerance (% survival) was calculated as the proportion of
cultrable bacteria surviving the 75% RH treatment relative to those recovered in the 100% RH treatment.

**Statistical analyses.** Statistical analyses were performed using JMP (version 5; SAS institutes, Cary, NC). For comparing INA activity, ANOVA was performed to determine significance of difference. For comparing desiccation survival, we performed a separate ANOVA of the CFU in the 100% and 75% RH treatments and for the proportion of cells that survived the desiccation treatment. Comparisons between LH1 and LH22 (e.g., INA activity at each time point and biofilm properties) were made by Student's t-tests. In all analyses, $P < 0.05$ was used as a criterion for statistical significance.

**RESULTS**

**Increased EPS production in response to matric stress.** There was a dramatic increase in the total polysaccharide and uronic acid content of EPS obtained from *P. putida* biofilms cultivated at water potentials below -0.5 MPa in the PEG-8000, but not NaCl treatments, and further increases in EPS production occurred as the water potential decreased (Fig. 3-1). The amount of non-ethanol precipitable low molecular weight (MW) carbohydrates present in the ethanolic supernatant also increased with increasing matric stress severity (Appendix A). These results clearly show that *P. putida* preferentially produces more EPS in response to matric stress, but not thermodynamically equivalent solute stress.

**Mannuronic acids produced under matric stress.** Composition analysis of ethanol-precipitated EPSs revealed that mannuronic acid, indicative of alginate, was a component of the EPS when LH1 was grown on the -1.5 MPa ψ PEG8000 amended medium (Table 3-2).
In contrast, no mannuronic acids were present in the EPS of strain LH22, a derivative of LH1 with the alginate biosynthesis operon disrupted by site-specific mutagenesis. In fact, in the absence of a matric stress, the compositions of the EPS of LH1 and LH22 were nearly identical, except for the presence of xylose in LH22 (Table 3-2). Interestingly, in both strains the carbohydrate composition of EPS varies between the treatments. For instance, the EPS of

Figure 3-1. Effect of NaCl and PEG-8000 treatments on total carbohydrate (A) and uronic acid (B) of EPS obtained from *P. putida* mt-2 biofilms. Total carbohydrates and uronic acids are expressed as glucose and glucuronic acid equivalents, respectively. Open symbols, NaCl treatment; closed symbols, PEG-8000 treatment. Values represent the mean ± SE of three experiments.
LH1 was comprised of more glucose and less rhamnose in the matric compared to the no stress treatment (Table 3-2), suggesting that other unknown EPSs are made in response to matric stress. This pattern was also observed in LH22 samples, although the proportion of glucose and rhamnose was different (Table 3-2).

**Table 3-2.** Glycosyl composition of the EPS obtained from LH1 and LH22 biofilm cells<sup>a</sup>

<table>
<thead>
<tr>
<th>Glycosyl residues (Mole %)</th>
<th>LH1</th>
<th>LH22</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unamended medium</td>
<td>-1.5 MPa PEG-8000</td>
</tr>
<tr>
<td>Glucose</td>
<td>39.6</td>
<td>62.4</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>42.4</td>
<td>5.8</td>
</tr>
<tr>
<td>Mannose</td>
<td>9.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>8.6</td>
<td>0.0</td>
</tr>
<tr>
<td>Mannuronic acid</td>
<td>0.0</td>
<td>27.4</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> EPS obtained in two experiments were pooled for carbohydrate composition analysis. Values are expressed as mole % of total carbohydrate. Data are representative of results obtained in two separate analyses.

**Environmental stressors influencing alginate production.** Since PEG8000-mediated matric stress also influences membrane fluidity (20), it is possible that alginate production is a consequence of matric stress-mediated alterations in membrane integrity, and not in response to cellular dehydration per se. To test this possibility, we examined the effect of toluene on alginate biosynthesis, since toluene alters fatty acid composition in a fashion similar to PEG-8000 (20, 51). We also examined low temperature that can alter membrane phase transitions similar to what occurs when membranes are dried (9, 29, 37). As shown in Table 3-3, there was no significant difference ($P > 0.05$) in uronic acid contents of the EPS obtained from LH1 and LH22 biofilms exposed to various environmental stressors except for
the -1.5 MPa \( \psi \) PEG-8000 treatment, suggesting that alginate is produced in response to matric stress-mediated dehydration, rather than in response to factors influencing membrane physical properties. We also investigated the effect of the permeating non-ionic solute PEG-200, since alginate production could be occurring in response to the PEG molecule itself.

Uronic acid contents of EPS of LH1 and LH22 in the -1.5 MPa \( \psi \) PEG200 treatment were nearly identical to the unamended treatment (Table 3-3).

**Table 3-3. Alginate production in response to various environmental stressors**

<table>
<thead>
<tr>
<th>Strains</th>
<th>0 MPa ( \psi ) treatments</th>
<th>-1.5 MPa ( \psi ) treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>27°C</td>
<td>15°C</td>
</tr>
<tr>
<td>LH1</td>
<td>2.7 ± 0.8</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>LH22</td>
<td>2.6 ± 0.6</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

\(^a\) Strains were cultivated on solid ½ TYE plates prior to isolation of the EPS as described in the materials and methods. Data are expressed as μg uronic acids / mg protein (n=3, Mean ± SEM).

\(^b\) Toluene was provided in the gas-phase in a desiccator to achieve an aqueous-phase concentration of 100 μg/mL.

**Alginate influences the developmental dynamics and ultrastructure of unsaturated biofilms.** We assessed the role of alginate production on biofilm developmental processes and architecture in the unamended and -1.5 MPa \( \psi \) PEG-8000 treatments by SCLM and epifluorescence microscopy using the previously described unsaturated biofilm system (See Chapter 2). Lag-periods before visible growth and growth rates were comparable for both LH1 and LH22 in the unamended and -1.5 MPa \( \psi \) PEG-8000 treatments (data not shown). In the unamended treatment, the dynamics of microcolony formation by both strains were indistinguishable from each other during the first 24-h of biofilm development (data not...
Table 3-4. Biofilm properties of LH1 and LH22

<table>
<thead>
<tr>
<th>Properties</th>
<th>Unamended treatment</th>
<th>-1.5 MPa ψ PEG8000 treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LH1</td>
<td>LH22</td>
</tr>
<tr>
<td>Microcolony surface area (μm²)</td>
<td>61,427 ± 6,300</td>
<td>65,765 ± 1,413</td>
</tr>
<tr>
<td>Biofilm cell height (μm)</td>
<td>9.3 ± 0.6</td>
<td>9.2 ± 0.3</td>
</tr>
<tr>
<td>EPS thickness at air interface (μm)</td>
<td>2.5 ± 0.3</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>Density (cells/100 μm²)</td>
<td>117.3 ± 1.1</td>
<td>115.6 ± 1.6</td>
</tr>
<tr>
<td>% cell-free area</td>
<td>23.1 ± 0.5</td>
<td>23.7 ± 0.3</td>
</tr>
</tbody>
</table>

*a* One-day old biofilms were used in the measurements of microcolony surface area (μm²), biofilm cell height (μm), and EPS thickness at air interface (μm), while 5-day old biofilms were used in those for density (cells/100 μm²) and % cell-free area. Data are expressed as mean ± standard errors of the means.

*b* Microcolony surface area at the substratum surface. Values were derived from the average microcolony surface area per field of view (n = 3 to 4) containing 5 to 33 microcolonies in two separate experiments.

*c* Height from the substratum to the top of biofilm. Values were derived from 9 random height measurements of 10 to 13 x-z projections of a stacked series of x-y plane CSLM images obtained in three independent experiments.

*d* Thickness of the calcofluor-stained layer between the top of the biofilm to the air interface. Values were derived from the images used for the biofilm cell height measurements.

*e* Number of cells in a 100 μm² area. Values were derived from 13 to 21 x-y plane CSLM images obtained in three experiments.

*f* The proportion of a 100 μm² area devoid of cells. Values were derived from the images used for the density measurements.
shown), leading to biofilms with identical ultrastructural properties (Table 3-4). At 12 h, however, the edges of LH22 microcolonies in the -1.5 MPa ψPEG-8000 treatments were not as smooth as those of LH1 (Fig. 3-2B, F). By 24 h biofilms formed by LH22 covered more surface area, were shorter in height, had a higher cell density, and were less porous than those by LH1 (Fig. 3-2C, D, G, H and Table 3-4). Interestingly, in the matric stress treatments LH1 biofilms had a thicker calcofluor-stained EPS layer at the air interface than LH22 biofilms, although the proportion of EPS thickness to total biofilm height (cell height + EPS thickness at the air interface) was the same for both (Fig. 3-3A, B, and Table 3-4).

**Alginate slows the rate of cellular dehydration.** We used two different approaches to assess whether alginate creates a more hydrated microenvironment that protects cells from dehydration stress by slowing the rate of cellular drying. We generated unsaturated biofilms on membranes overlaying a solid medium in which the water potential was lowered to -1.0 MPa with PEG-8000 amendments, since this condition favored alginate production while at the same time not restricting growth. After generating biofilms comprised of ca. 5,000 cells per microcolony, we transferred intact biofilms to solid media of equivalent water potentials or to plates where the water potential was lowered to -2.5 MPa with PEG-8000 or NaCl to create a dehydration or osmotic shock, respectively. We then assayed the intracellular water potential of biofilm cells using a proU-inaZ transcriptional fusion (56) and the extent of membrane dehydration by monitoring the abundance of membrane fatty acids indicative of dehydration stress (20).

To measure the intracellular water potential sensed by bacteria in biofilms a plasmid, pPProIce, containing a proU-inaZ transcriptional fusion (56) was introduced into LH1 and LH22. The Log10 INA of these strains was directly related to the water potential to which the
Figure 3-2. Temporal dynamics of biofilm development by gfp-tagged LH1 and LH22 in the -1.5 MPa ψ PEG-8000 treatment. Images were obtained by epifluorescence (A, B, C, E, F, G) and confocal (D, H) microscopy. Time points indicate when pictures were taken after inoculation of the substratum with cells.
cells were exposed during growth (Fig. 3-4). Within 2.5 hpt (hours post transfer), LH22(pPProIce) cells exhibited significantly higher \((P < 0.05)\) INA than LH1(pPProIce) cells when exposed to a dehydration (Fig. 3-5A), but not an osmotic shock (Fig. 3-5D). There was no significant difference \((P > 0.05)\) in the INA of LH1(pPProIce) and LH22(pPProIce) cells following transfer to a fresh medium of the same -1.0 MPa water potential (Fig. 3-5A). Within 4 hpt there were fewer culturable LH22(pPProIce) than LH1(pPProIce) cells recovered from biofilms exposed to the -2.5 MPa \(\psi\) PEG8000 treatment (Fig. 3-5B), indicating that alginate provides some protection from dehydration stress. However, there was a similar loss of culturability of LH1(pPProIce) and LH22(pPProIce) biofilm cells following an osmotic shock (Fig. 3-5E) and the decrease in culturability was greater than that observed following a dehydration shock, suggesting that acclimation to a matric stress does not confer protection to a thermodynamically equivalent osmotic stress. By using relations between INA and water potential on solid media (Fig. 3-4), we estimated intracellular water
potentials of LH1(pPProIce) and LH22(pPProIce) cells following the dehydration shock.

After 4 hpt, intracellular water potentials were $-2.28 \pm 0.03$ MPa and $-2.50 \pm 0.03$ MPa for LH1(pPProIce) and LH22(pPProIce), respectively (Fig. 3-5C). There was no significant difference ($P > 0.05$) in the intracellular water potential in the -1.0 MPa $\psi$ PEG8000 or -2.5 MPa $\psi$ NaCl treatments (Fig. 3-5 C and F).

![Figure 3-4](image)

**Figure 3-4.** Correlation between INA of cells and water potential of PEG8000-amended $\frac{1}{2}$ 21C media. ●, LH1(pPProIce); △, LH22(pPProIce).

To determine whether dehydration or osmotic shocks caused physiological changes influencing the activity of the InaZ protein, we used a control plasmid pPNptIce containing a constitutive *nptII* promoter fused to the *inaZ* gene (56). The INA of LH1(pPNptIce) and
Figure 3-5. Temporal dynamics of INA (A, D, and G), survival (B, E, and H), or estimated intracellular water potentials (C and F) of LH1(pPProIce) and LH22(pPProIce) (A - F) and LH1(pPNptIce) and LH22(pPNptIce) (G and H) following a dehydration or osmotic shock. Cells were cultivated on -1.0 MPa ψ PEG-8000 amended medium for 32-36 h prior to transferring to -1.0 MPa ψ PEG-8000 (control), -2.5 MPa ψ PEG-8000, or -2.5 MPa ψ NaCl amended medium. The intracellular water potential (C and F) was derived based on the equation in Fig. 3-4. Closed symbols, LH1; opened symbols, LH22; ▲ or △, -1.0 MPa ψ PEG-8000 treatment; ● or ○, -2.5 MPa ψ PEG-8000 treatment; ■ or □, -2.5 MPa ψ NaCl treatment. Values represent the mean ± SE of two or three experiments, each comprised of three replications.
LH22(pPNptIce) did not change significantly ($P > 0.05$) over time following the dehydration and osmotic shocks (Fig. 3-5G) and the culturabilities of both stains were not statistically different ($P > 0.05$) upon an osmotic shock, whereas they were different upon a dehydration shock (Fig. 3-5H).

Our second approach for assessing whether alginate slows the rate of cellular dehydration was to monitor cis-trans isomerase (Cti) activity following a dehydration shock. Cti is a constitutive enzyme that converts cis unsaturated fatty acids to the trans isomer when *P. putida* is dehydrated (20). Within 30 min of the -1.0 to -2.5 MPa $\psi$ PEG8000 treatment, there was a substantial increase in the proportion of 16:1 $\omega 7$ trans relative to its cis isomer in LH22, but not in LH1 (Fig. 3-6A, C). By 120 min, there was an increase in the saturated fatty acid content of LH22, but not LH1, presumably due to de novo synthesis of saturated fatty acids (Fig. 3-6B, D). Interestingly, in the -1.0 to -1.0 MPa $\psi$ PEG8000 control treatments, 16:1 trans/cis ratios and % 16:1 trans fatty acids of both strains dramatically decreased within 30 min (Fig. 3-6A, C), suggesting that fresh nutrients allow for the growth of new cells with lower trans fatty acid contents or the conversion of pre-existing 16:1 trans fatty acids to the cis isomer. These results suggest that alginate slows the rate of cellular dehydration, since fatty acid alterations occur sooner in LH22 than LH1. Unlike in the dehydration shock experiments, during steady state growth in the absence or presence of PEG8000 or NaCl treatments there were no statistically significant differences ($P > 0.05$) in the 16:1 trans/cis and saturated/unsaturated fatty acid ratios of both strains (Appendix D), which suggests that they are not inherently different in their cellular fatty acid composition.
Figure 3-6. Effect of alginate on % 16:1 trans (A) and % saturated fatty acids (B) and 16:1 trans/cis (C) and saturated/unsaturated fatty acid ratio (D) following a dehydration shock. Cells were cultivated on membranes overlaying −1.0 MPa υPEG-8000 amended medium for 24 h prior to transfer of membranes onto either −1.0 or −2.5 MPa υPEG-8000 amended medium. Closed symbols, LH1; open symbols, LH22; ▲ or △, −1.0 MPa υPEG-8000 treatment; ● or ○, −2.5 MPa υPEG-8000 treatment. Values represent the mean ± SE (n=3).
Alginate production is required for desiccation tolerance. To determine whether alginate contributes to desiccation tolerance, we conducted a filter disc desiccation assay as described previously (50). Desiccation stress was imposed by exposing planktonic cells to a 100% or 75% relative humidity (RH) environment, and desiccation tolerance was assessed by monitoring cell culturability (CFU) over time. At 6 h, there was no significant difference \((P > 0.05)\) in the survival of LH1 and LH22 (Fig. 3-7 A, B). By 24 h, the time maximum population sizes are achieved, planktonic cells could form aggregates comprised on average of 23 to 32 cells, assuming that all cells originally deposited in the filter were present as individuals and not aggregates of cells (Fig. 3-7). After 24 h, the population size of LH1 and LH22 gradually decreased in desiccated samples (Fig. 3-7 A, B). By the end of the assay, the number of CFU obtained from desiccated LH1 samples was 13% of the undesiccated LH1 samples (Fig. 3-7A), whereas less than 1% of the LH22 population survived the desiccation stress (Fig. 3-7B). These results show that LH1 is more desiccation tolerant than LH22, indicating that alginate contributes to the ability of cells to survive a desiccation stress.

DISCUSSION

In this study, we demonstrate that alginate is produced by \(P.\ putida\) in response to matric stress, but not thermodynamically equivalent solute stress, and that it has a vital role in biofilm development and survival in water-limited environments. We also address the hydrating nature of alginate in microenvironments surrounding biofilm cells that may slow the rate of cellular drying, and therefore stabilize membrane integrity upon dehydration. We provide two lines of evidence that alginate functions to help create a more hydrated
microenvironment that buffers biofilm cells from drying. First, cells encapsulated with alginate perceive less water stress than those without alginate following a dehydration shock (Fig. 3-5). Second, alginate slows the rate at which membranes become dehydrated as evidenced by the increase in trans unsaturated fatty acids and the de novo synthesis of saturated fatty acids following a dehydration shock (Fig. 3-6). Taken together, alginate

Figure 3-7. Desiccation tolerance of LH1 (A) and LH22 (B). Left y-axis: ●, 100% RH; ■, 75% RH. Right y-axis: △, % Survival. Values represent the mean ± SE (n=3).
production is an important consequence of cellular dehydration and alginate plays an important role in maintaining an appropriate microenvironment for cell survival and developing microbial community in low-water-content habitats.

Carbohydrate composition analysis of the EPS shows other glycosyl residue changes in addition to alginate production under water limiting conditions (Table 3-2). For instance, increased glucose content indicates that a polymer containing glucose is also up-regulated by matric stress. Indeed, the genomic sequence of *P. putida* KT2440 has revealed the presence of cellulose biosynthesis operon (35), and it is reasonable to postulate that cellulose production increases in response to a matric stress. Interestingly, there is possibly a unique EPS comprised of rhamnose, mannose, glucose, and/or glucuronic acids whose production may be directed by a putative EPS cluster on the KT2440 chromosome. It should be noted that the presence of rhamnose is not likely due to lipopolysaccharide (LPS) contamination, since we did not detect KDO (2-keto-3-deoxyoctonate) in our EPS samples.

To the best of our knowledge, this is the first report of alginate production by a wild type *P. putida* strain. Although many *P. putida* strains, including mt-2, possess genes for alginate biosynthesis (17, 35), alginate was not a component of EPS obtained from numerous *P. putida* strains associated with mushrooms (16), a freshwater isolate (38), or an aromatic hydrocarbon degrader in soil (24). Fett et al. reported that EPS produced by mushroom-associated *P. putida* strains is marginalan, an acidic galactogluconan (16), whereas we have no evidence for galactogluconan production by mt-2. Environmental isolate *P. putida* BR7 produces an EPS composed of glucose, galactose, and pyruvate in a ratio of 1:1:1 (38). Carbohydrate content of *P. putida* G7 capsular EPS is comprised of glucose, rhamnose, ribose, N-acetylgalactosamine, and glucuronic acid (24). These results strongly suggest that
there is great heterogeneity in composition of EPS produced by *P. putida* strains that may reflect the habitats from which they were obtained. The only previous evidence, as far as we know, indicating that *P. putida* could synthesize alginate was the isolation of mutant *P. putida* strains (19) that constitutively synthesized alginate, possibly due to a spontaneous mutation that uncoupled the regulatory network controlling alginate biosynthesis.

Characterization of the EPS synthesized by this strain indicated that it produced a low-molecular-weight, highly acetylated alginate comprised of both mannuronic and guluronic acids (7), which differs from the alginate produced by *P. putida* when exposed to a matric stress, since we did not detect guluronic acid residues in this study (Table 3-2).

Similar to the study presented here, it has been reported that ethanol-mediated dehydration stimulates alginate production by several *Pseudomonas* species (10, 12, 44). In particular, transcription of *algD* gene in *P. aeruginosa* is activated by ethanol (10). However, other environmental stressors examined in this study did not stimulate alginate production, although other studies show that the *algD* promoter is up-regulated by high osmolarity (1) and high concentration of glucose (28). Copper and high oxygen are also known to regulate alginate synthesis in *P. syringae* (25) and *Azotobacter vinelandii* (42). In fact, the stimulatory effect of environmental factors on alginate production is species and strain specific. For example, while the optimal concentration of NaCl for activation of the *algD* promoter in *P. aeruginosa* was reported to be 0.35 M (approximately -1.5 MPa water potential) (1), this concentration did not induce alginate production by some *P. fluorescens* strains (44) or *P. putida* mt-2 (Fig. 1 and Table 3). In addition, gene expression of *algD* in response to environmental stimuli does not always result in alginate production. For example, the *algD* promoter is transcriptionally activated by ethanol and NaCl in non-mucoid *P. aeruginosa*
strains, but there is no detectable alginate observed (1, 10), which suggests that some form of post-transcriptional control may also be involved in alginate biosynthesis.

Alginate production contributed to the fitness of *P. putida* under water-limiting conditions (Fig. 3-7). Similarly, colanic acid, a highly viscous EPS (31), protects several enteric bacterial species from desiccation imposed by vacuum (36). It has also been shown that the presence of bacterial EPS slows the rate of water potential decrease in sand that is air dried (40). Furthermore, algal alginate, although different from bacterial alginate in being not acetylated (48), protects seaweed cells from desiccation (14) at low tide (41). Given that alginate is hygroscopic and can hold several times its weight in water (41, 48, 49), it may lose water more slowly and hence cells stay hydrated longer, which could increase time available for making metabolic adjustments that enhance survival during desiccation stress. Alternatively, desiccation tolerance may be attributed to architectural features of biofilms, since alginate production resulted in microcolonies that were taller and less spread-out, thereby creating a smaller biofilm surface area for evaporative water loss. However, this is unlikely due to the fact that aggregates formed by individual planktonic cells in the filter disk desiccation assay are comprised on average of 23 to 32 cells (Fig. 3-7) and do not possess a highly structured three-dimensional (3-D) architecture like that of mature microcolonies. This result further suggests that even small aggregates of cells may produce sufficient amounts of alginate to protect cells within the microcolony from desiccation stress. However, tolerance is likely improved by larger aggregates with a more complex 3-D ultrastructure. Monier and Lindow have suggested that such aggregates might contribute to the survival of bacteria periodically exposed to desiccation stress (33). Large aggregates containing $10^3$ cells
or more account for the majority of cells present on leaves (33) and may contribute to better survival of cells when exposed to subsequent desiccation stress.

The taller and more compact architecture of *P. putida* LH1 biofilms under alginate producing conditions (Table 3-4) is very similar to the architecture of biofilms generated by the *P. aeruginosa* PAO1 isogenic mucA22 mutant (PDO300), which constitutively overproduces alginate in flow-through biofilm systems (22). This similarity suggests that alginate contributes to a highly structured 3-D architecture in biofilm development, which could facilitate increased resistance to physical and/or chemical stressors (22). Twitching motility might also contribute to this taller biofilm architecture, since the biofilm ultrastructure of a twitching motility deficient mutant was similar to that observed for an alginate overproducer (23, 26), although it has been shown that alginate production and twitching motility are positively linked by the alginate regulator AlgR (52).

In summary, this study demonstrates that alginate protects cells from desiccation stress-mediated cellular damage by slowing the rate of cellular drying, possibly by creating a more hydrated microenvironment. It will be interesting to see whether the production of other EPSs, including cellulose and/or the novel EPS, occurs in response to dehydration and whether they also contribute to the fitness of *P. putida* in water-limited environments. A better understanding of the strategies that bacteria employ for their adaptation in low-water-content habitats is important, since this knowledge will allow us not only to assess the effects of the reduced water availability on the microbial community in frequently unsaturated terrestrial habitats, but also to better predict and model their behavior in the environment.
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Chapter 4. Reduced Water Availability Influences Cell Death within Unsaturated *Pseudomonas putida* Biofilms

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**ABSTRACT**

Cell death has been recently described as a factor contributing to biofilm development in a continuous flow cell system. Presumably cell death is an important mechanism providing physical support for biofilm structures and releasing nutrients to feed remaining cells. In unsaturated habitats, the highly structured environment without continuous supply of nutrients and removal of wastes could strongly influence cell death in biofilm communities. We examined temporal dynamics of cell death and spatial localization of dead cells within biofilms when water availability was reduced by solute or matric components of the water potential ($\psi$). There was an arrangement of dead cells organized in arrays of various lengths one cell width in diameter, defined as a “string” of dead cells, in the unamended and -1.5 MPa $\psi$ NaCl treatments. In the -1.5 MPa $\psi$ PEG8000 treatment, however, there was no visible string longer than 6 $\mu$m, and there was an increase in the proportion and in the stratification of dead cells over time. In the unamended and -1.5 MPa $\psi$ NaCl treatments, dead cells were primarily localized in a region about 3 to 4 $\mu$m from the substratum surface,
regardless of biofilm age, whereas most of the dead cells were localized further from the substratum in the -1.5 MPa \(\psi\) PEG8000 treatment. The studies described here provide evidence that reduced water availability dramatically influences cell death patterns within unsaturated biofilms as they age.

**INTRODUCTION**

Bacteria in terrestrial habitats such as soil and plant surfaces, which are frequently unsaturated, can form aggregates, and this microbial cluster is often called an unsaturated biofilm (3). Unlike saturated biofilms (e.g., those in aquatic environments), unsaturated biofilms are surrounded by a very thin layer of water, which varies in thickness, depending on the water content of the habitat. In unsaturated habitats, biofilm bacteria can experience osmotic stress due to the presence of high concentration of dissolved solutes and/or matric (dehydration) stress imposed by physical removal of water from the environment.

The lack of significant fluid flow in terrestrial habitats dramatically influences nutrient availability, metabolic waste accumulation, and disposal patterns. In porous media such as soils, through its relationship to water film thickness (9, 16), matric stress can affect the diffusion of gas and soluble nutrients to microorganisms, which can influence biofilm development and their properties compared to when water is not limiting (3). Hence, as soils dry there can be regions within the soil in which the nutrient supply can be cut off and microbes will experience water deprivation, nutrient deprivation, or both.

Nutrient limitation along with water deprivation could be a crucial factor influencing cell death within biofilm communities. How and why cells die is poorly understood, but it is
becoming clear that many cells die via complex programmed mechanisms (i.e., programmed cell death) (1, 11, 19). Cannibalism of siblings has also been implicated as a means by which a subpopulation of *Bacillus subtilis* can survive nutrient limitation (4, 7). Recently, the distribution of dead cells within biofilms generated in a flow-through system has revealed that they are usually localized in the center of *Pseudomonas aeruginosa* biofilms (23). In addition, the study of oral biofilms indicates that the lower regions of biofilms contain proportionally more dead cells than the upper regions (2, 10), which indicates that nutrients are provided in the bulk fluid flow over the biofilms. In unsaturated biofilms, however, cells are spatially confined without the bulk fluid flow (3) and consequently nutrients or waste products are primarily provided and removed via diffusion through the substratum or through the water layer covering the biofilm. Different paths of nutrients or waste products will likely result in different cell death patterns within unsaturated biofilms from those in fully hydrated systems.

More interestingly, Webb et al. has proposed that cell death in biofilms is a normal process by which surviving cells benefit from the death of their neighbors (23). These findings suggest, at least in part, that cell death in biofilms is not just an end for the community, but a factor contributing to the developmental processes to maintain the community alive and/or active. Despite the importance of understanding cell death in biofilm development, little is known about how reduced water availability influences the dynamics of cell death in unsaturated biofilms. In this study, we explore how osmotic and matric stresses influence cell death of the soil saprophyte and rhizosphere colonist *P. putida* strain mt-2 in unsaturated biofilms. Our findings reveal that death within unsaturated biofilms occurs in a non-random pattern and that the death rate is the greatest when biofilm communities
experience matric stress. Our findings also indicate that nutrients released by dead cells
become a resource that promotes continued bacterial growth.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. *P. putida* strain mt-2 (17) and derivatives
thereof were used in the experiments. The gfp gene was fused to the constitutive neomycin-
phosphotransferase promoter, P<sub>np</sub>, on the broad-host-range vector pPROBE-KT (14), then
this hybrid plasmid was introduced into mt-2 for visualizing unsaturated biofilms. Cells were
cultivated on 1/2 TYE medium, which contained 1 g of MgSO<sub>4</sub>-7H<sub>2</sub>O, 1.38 g of KH<sub>2</sub>PO<sub>4</sub>, 5 g
of tryptone, 2.5 g of yeast extract, per liter of deionized water. Twenty mL of Hutner’s
mineral solution (21) was added into all media prior to solidification with 10 g of phytagel
gellan gum (Sigma Chemical Co., St. Louis, MO) per liter. The water potential of the basal
medium was lowered with PEG8000 or NaCl to simulate a matric (dehydration) stress or a
solute (osmotic) stress, respectively, as described previously (8). Propidium iodide (PI) was
incorporated into the medium (0.5 µg/ml) to differentiate dead from live cells (Molecular
Probes, Inc., Eugene, OR).

Cultivation of unsaturated biofilms. The unsaturated biofilm chamber system
described in Chapter 2 was used for assessing the effect of reduced water availability on the
death of *P. putida* on a solid surface (See Fig. 2-1). In order to visualize dead cells, PI was
incorporated into solid media. In preliminary studies, we determined this concentration of PI
was not toxic, since the total number of cells at 6 dpi (days post inoculation) in the presence
of PI was identical to that in the absence of PI (data not shown). Inoculum of gfp-tagged *P.
*P. putida* mt-2 was prepared by resuspending and diluting cells from a 24- to 36-h old ½ TYE plate culture in fresh ½ TYE broth. A 1-µl aliquot of a dilute cell suspension was placed onto a phytogel-coated cover slip. The initial inoculum size was 15 to 35 cells per inoculated spot. We also included approximately $1 \times 10^4$ Linear Flow Deep Red fluorescent flow cytometry microspheres (2.5 µm diameter) (Molecular Probes Inc., Eugene, Oreg.) in the inoculum to identify the air-substratum interface and define the bottom of the biofilms. We monitored biofilm development along with dead cells within biofilms under static conditions in real time using confocal scanning laser microscopy (CSLM).

**Microscopy and image analysis.** Confocal images were obtained using a Leica TCS-NT confocal scanning laser microscope equipped with argon ($\lambda_{ex}=488$ nm) and HeNe ($\lambda_{ex}=633$ nm) lasers for visualization of green fluorescent protein ($\lambda_{em}=\text{fluorescein isothiocyanate filter set}$) and DNA-bound PI ($\lambda_{em}=\text{CY5 filter set}$), respectively. All multicolor images were collected simultaneously using a multitrack mode (green = GFP; red = PI). When PI was included in the medium we adjusted the sensitivity of the photomultiplier tube of the CSLM until red autofluorescence of the medium was no longer detectable.

Horizontal (x-y) images were taken at 0.5 µm intervals using the 40X objective. Vertical cross section images (x-z) were generated with Image J software (http://rsb.info.nih.gov/nih-image). Multiple images were overlaid and cropped using Adobe Photoshop (Adobe Systems, Mountain View, CA).

**Definition and length determination of strings of dead cells.** We defined a string of dead cells as an array of dead cells adjacent to each other. These arrays were typically one cell width in diameter with various lengths. These strings were quantified in terms of their
lengths and numbers and how many dead cells were present per string. For the analysis of
frequency distribution of strings of dead cells over time, we used the criteria we had
established for defining a string equal to or longer than 6 μm in length, which is equivalent to
2 -3 cells growing pole-to-pole parallel to the substratum or 5-6 cells oriented perpendicular
to the substratum, which is the orientation of most cells within a mature biofilm (Chapter 2).
However, for the analysis of the cumulative proportion of dead cells as a function of the
estimated number of dead cells per string, we included any PI-stained areas equal or greater
than 0.95 μm, which was the average diameter of one cell.

Data transformation and statistical analysis. Calculations of the proportion of dead
cells within biofilms, total biofilm area, and the number of strings of dead cells were
performed using the morphometry tool function of MetaMorph software (Universal Imaging
Corp. Downingtown, PA). Estimations of the total number of dead cells per string and
frequency distributions were obtained by using dynamic data exchange in MetaMorph to
Microsoft Excel software (Microsoft Co., Redmond, WA). All pixel values were converted
into μm units by using an appropriate formula (512 pixels = 250 μm) on the Excel spread
sheet. All statistical analyses were performed with JMP (version 5; SAS institutes, Cary, NC)
and ANOVA was performed to compare the quantitative values of dead cells among different
biofilms. In all analyses, $P < 0.05$ taken by using Student's $t$-tests was considered to be
statistically significant.
RESULTS AND DISCUSSION

Temporal dynamics of dead cells within unsaturated *P. putida* biofilms. We first assessed the temporal dynamics of dead cells within unsaturated biofilms by incorporating propidium iodide (PI) in the medium. We assume that the brightly red fluorescent cells can be considered dead, because the membrane integrity of these cells was compromised to permit passage of PI into the cell (Fig. 4-1). In addition, a recent study has shown that, at least with *Escherichia coli*, a reduction in cell membrane integrity, determined by PI staining, correlated with lost reproductive ability upon subsequent exposure to fresh nutrients and these cells could be considered dead (5). Less than 3% of the inoculum was stained with PI and all dead cells were found as individuals 3 hpi (hours post inoculation), regardless of whether they were or were not exposed to a water stress (Fig. 4-1A and data not shown). In the unamended treatment, by 9 hpi small microcolonies comprised on average of 96 cells were visible, and contained relatively few, if any, dead cells (Fig. 4-1B). By 24 hpi there was a substantial increase in the number of dead cells within a microcolony and frequently they were arranged in arrays of various lengths one cell width in diameter. These strings of dead cells were randomly distributed in a microcolony (Fig. 4-1C, D) and the proportion of dead cells within biofilms was relatively low compared to that observed in biofilms that developed under reduced water availability conditions (Table 4-1 and compare Fig. 4-1C, D and Fig. 4-2A-D). By 96 hpi, in the unamended treatment the frequency and length of strings of dead cells increased, and consequently the proportion of dead cells increased (Fig. 4-1C-F and Table 4-1). In contrast, in the -1.5 MPa ψNaCl treatment the proportion of dead cells did not increase over time (Fig. 4-2A, B, E, F and Table 4-1), although the number of dead cells increased (data not shown). In the -1.5 MPa ψPEG8000 treatment, we observed the greatest
Figure 4-1. Temporal dynamics and spatial localization of dead cells in gfp-tagged P. putida unsaturated biofilms grown on the unamended medium. Time points indicate hours post inoculation (hpi): 3 hpi (A), 9 hpi (B), 24 hpi (C and D), and 96 hpi (E and F). Green cells are expressing GFP and considered viable. Red cells are stained with PI and considered dead. (C and E) Overlay of green and red images; (D and F) corresponding red images only. The arrows in (A) and (B) indicate an individual dead cell. The arrow in (D) indicates the inset magnification of a PI-stained string of dead cells.

proportion of dead cells 96 hpi, and these cells occupied more than a quarter of total biofilm areas chosen at random depths (Fig. 4-2G, H and Table 4-1).

These results indicate that cell death occurs in relatively young microcolonies (i.e., 24-h old) and cells continue to die as the biofilm ages. It is possible that many cells were injured during earlier phases of microcolony development but membrane lesions, as detected by
Table 4-1. Influence of reduced water availability on cell death and culturability within unsaturated biofilms

<table>
<thead>
<tr>
<th>Treatment and day</th>
<th>% PI-stained area (b)</th>
<th>String length ((\mu m)) (c)</th>
<th>(\log_{10}(CFU)/spot) (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.21 ± 0.16</td>
<td>12.56 ± 0.66</td>
<td>7.89 ± 0.02 A</td>
</tr>
<tr>
<td>4</td>
<td>7.02 ± 0.73</td>
<td>16.32 ± 0.39</td>
<td>7.68 ± 0.05 A</td>
</tr>
<tr>
<td>-1.5 MPa (\psi) NaCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.62 ± 0.95</td>
<td>19.47 ± 0.53</td>
<td>7.88 ± 0.05 A</td>
</tr>
<tr>
<td>4</td>
<td>6.51 ± 0.62</td>
<td>11.78 ± 0.58</td>
<td>7.72 ± 0.03 A</td>
</tr>
<tr>
<td>-1.5 MPa (\psi) PEG800</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8.62 ± 1.92</td>
<td>NA (^e)</td>
<td>7.14 ± 0.13 B</td>
</tr>
<tr>
<td>4</td>
<td>27.18 ± 3.58</td>
<td>NA (^e)</td>
<td>7.83 ± 0.07 A</td>
</tr>
</tbody>
</table>

\(^a\) The images used were taken at random depths (\(n = 6\) to 11) and values are representative of three replicate experiments. Data are expressed as mean ± standard errors of the mean.

\(^b\) \% PI-stained area = (PI-stained area/total biofilm area) × 100.

\(^c\) Average length (\(\mu m\)) of PI-stained string per field of view. For string length measurement, the string was defined as an array of PI-stained cells in a single file of various lengths greater than 6 \(\mu m\) in length (equivalent to 2 to 3 cell lengths).

\(^d\) Data also shown in Fig. 2-2. Values within a column followed by the same letter indicate no statistical difference at \(P = 0.05\).

\(^e\) NA, Not applicable due to the absence of visible strings longer than 6 \(\mu m\) at a 40X magnification.

permeability to PI, were only evident later. Since the number of dead cells increased over time it is possible that the cells died because of nutrient deprivation, toxic metabolic product accumulation, or limited oxygen penetration in specific, although confined, sites within the microcolony. However, it is unlikely that oxygen deprivation is an issue, since oxygen can penetrate to a depth of 50 \(\mu m\) in mature \(P. \text{aeruginosa}\) PAO1 colony biofilms (24), and \(P. \text{putida}\) biofilms in this study were at most 20 \(\mu m\) in height (Fig. 4-7). Alternatively, the strings of dead cells could reflect the developmental history of the microcolony since a mutation or a physiological defect (non-genetic) in a cell conferring increased susceptibility
Figure 4-2. Effect of reduced water availability on the temporal dynamics and spatial localization of dead cells in gfp-tagged *P. putida* unsaturated biofilms. (A, B, E, and F) -1.5 MPa ψNaCl treatment; (C, D, G, and H) -1.5 MPa ψPEG8000 treatment. (A-D) 24-h old microcolonies; (E-H) 96-h old microcolonies. Green cells are expressing GFP and considered viable. Red cells are stained with PI and considered dead. (A, C, E, and G) Overlay of green and red images; (B, D, F, and H) corresponding red images only.
to environmental conditions favoring death could be passed to daughter cells adjacent to each other in the biofilm or transferred to daughter cells following division. Since all members of this clonal subpopulation would be susceptible to the death-inducing condition(s) they would all die at relatively the same time, assuming they all experience the signal(s) at the same time.

Interestingly, by 4 dpi the number of culturable cells was similar among the treatments (Table 4-1 and See Fig. 2-2), suggesting that the increased proportion of dead cells is not correlated with a reduction in the population of culturable cells. This result further suggests that unsaturated biofilms tend to maintain a certain level of culturable cells over time, presumably, because death rates are close to growth rates in mature biofilms.

**Frequency and relative percentage of strings of dead cells.** We further quantitatively analyzed strings of dead cells to assess how frequently their length is distributed and what relative percentage or number of dead cells account for a typical string length. In the unamended treatment, the average length of strings increased as the microcolony aged, whereas it decreased in the -1.5 MPa $\psi$ NaCl treatment (Table 4-1). In both treatments PI-stained arrays exhibited a right-hand-skewed distribution, and the majority of strings of dead cells were less than 50 µm long (Fig. 4-3). However, the plots of cumulative proportion of dead cells as a function of the number of cells per string show that the proportion of dead cells that were found in longer strings increased with incubation time in the unamended treatment, while it decreased in the -1.5 MPa $\psi$ NaCl treatment (Fig. 4-4). For example, in the unamended treatments approximately 50% of dead cells in 1- and 4-day-old biofilms were present in the strings comprised of fewer than 3 and 7 cells, respectively (Fig. 4-4A), while in the -1.5 MPa $\psi$ NaCl treatments 50% of dead cells were present in strings comprised
Figure 4-3. Frequency distribution of strings of dead cells longer than 6 μm in biofilms generated in the unamended (A) and -1.5 MPa υ NaCl (B) treatments. The same images as those used to generate values for Table 4-1 were used to generate these plots. String lengths were measured in pixels by selecting fiber length measurement in the morphometry tool function of MetaMorph software, and then these pixel values were converted into μm unit using an appropriate formula (512 pixels = 250 μm) on the Excel spread sheet. Values are representative of three replicate experiments.
Figure 4-4. Cumulative proportion of dead cells within *P. putida* biofilms as a function of the estimated number of dead cells per string. (A) Unamended treatment; (B) -1.5 MPa \( \psi \) NaCl treatment. In these analyses, any red-stained areas equal to or greater than 0.95 \( \mu m \) (equivalent to the diameter of one cell) were included. Given the fact that *gfp*-tagged cells were perpendicular to the substratum, we assumed that dead cells within a microcolony in the images used in these analyses were also perpendicular to the substratum. Each curve represents the combined date of three replicate experiments.
of fewer than 19 and 3 cells, respectively (Fig. 4-4B). In the -1.5 MPa $\psi$ PEG8000 treatments we were unable to clearly visualize strings of dead cells, possibly, due to the small size of the cells and the ruggedness of the microcolony (Fig. 4-2C, D, G, and H). However, confocal images taken at a higher magnification indicate that in general dead cells were present as aggregates of several cells rather than as strings of dead cells (Fig. 4-5C, D).

One of the questions that led us to initiate this study was to assess whether void (cell-free) regions within unsaturated biofilms described in Chapter 2 (See Fig. 2-6C) were actually cell free or contained dead cells. Horizontal (x-y) confocal images of 1-day-old biofilms showed that there were no dead cells in some void regions (Fig. 4-5C, D), although we cannot rule out the presence of a cell corpse that has lost its intracellular DNA and thus not stained with PI. Interestingly, overlay images of $gfp$-tagged and PI-stained cells often revealed yellow colored regions, presumably, indicating that some cells accumulate PI while retaining GFP fluorescence and hence are presumably dying. In general, this phenomenon occurred more frequently in the -1.5 MPa $\psi$ PEG8000 treatments (Fig. 4-5). To rule out that this was not a visual artifact, we subjected biofilm cells to flow cytometry and determined that there was a small proportion of cells that were green fluorescent and PI-stained (data not shown).

**Spatial distribution of dead cells within unsaturated *P. putida* biofilms.** Examination of the organization of PI-stained cells in the x-z plane indicates that like $gfp$-tagged cells described in Chapter 2, dead cells were occasionally stacked pole to pole on top of each other and some of these stacks extended from the bottom to the top of the biofilm in a columnar fashion (Fig. 4-6). Quantitative analysis of the ratio of PI-stained area to total biofilm area through the entire depth of the biofilm showed that the greatest proportion of dead cells in the
Figure 4-5. Horizontal (x-y) images of 1-day-old gfp-tagged *P. putida* unsaturated biofilms. Confocal images were taken at the middle of the microcolonies using a 100X objective. Green, cells expressing gfp. Red, cells stained with PI. (A and B) Unamended treatment; (C and D) -1.5 MPa ψ/PEG8000 treatment. (A and C) Overlay of green and red images; (B and D) corresponding red images only.

Unamended treatment occurred primarily in a region 3 to 4 μm from the substratum regardless of age of the microcolony (Fig. 4-7A). Given that the average length of a cell is 2 to 3 μm and that the cells are arranged perpendicular to the substratum, this result suggests that there is vertical stratification of dead cells with more live cells in the upper layers. In the -1.5 MPa ψ/PEG8000 treatments, a stratification of dead cells in the middle of the biofilm was also observed, although the distance from the substratum that the greatest proportion of the dead cells were localized and the maximum proportion of dead cells were greater than
Figure 4-6. Sagital (x-z) images of 1-day-old gfp-tagged P. putida unsaturated biofilms illustrating segregation of dead cells within a microcolony. Green, cells expressing gfp. Red, cells stained with PI. Images were created from a collection of 24 (A) or 58 (B) Z-series scans with a 0.5 μm interval between each Z section using a 40X objective. (A) Unamended treatment; (B) -1.5 MPa ψPEG8000 treatment.

those in the unamended treatment (Fig. 4-7B). Since cell length (ca. 1.5 μm) is shorter in the
-1.5 MPa ψPEG8000 than unamended treatments, most of dead cells were localized about 5
cell layers from the substratum in 1-day-old biofilms, although as the microcolony aged,
most were localized at or near the substratum surface (Fig. 4-7B). Moreover, dead cells
within -1.5 MPa ψPEG8000 treated biofilms were occasionally adjacent to each other to
create large regions of dead cells surrounded by living cells (Fig. 4-6B). The localized pattern
of PI-stained regions within biofilms grown on the -1.5 MPa ψNaCl amended medium
appeared to be similar to those in the unamended treatment (data not shown).

Since it is reasonable to predict that dead cells would be primarily localized in areas of
nutrient depletion or toxic metabolic product accumulation, stratification and segregation of
dead cells in distinct regions of the biofilm suggests that these places are unfavorable to
bacterial survival. More interestingly, regardless of how water availability was reduced, more
than 90% of the dead cells were localized in the lower 2/3 of the biofilm (Fig. 4-7). Similarly,
Figure 4-7. Stratification of dead cells within gfp-tagged *P. putida* unsaturated biofilms. (A) unamended treatment. (B) -1.5 MPa υ PEG8000 treatment. ●, 1-day-old biofilms; ▲, 4-day-old biofilms. Y-axis: % PI-stained pixel area = (PI-stained pixel area/total (GFP- and PI-positive) pixel area) × 100. X-axis: biofilm height (the distance from the substratum). The substratum was defined based on the location of microspheres (2.5 μm diameter) that identified the air-surface interface of the medium.

A recent study of stratified growth within *P. aeruginosa* colony biofilms showed that most dead cells were present at the bottom of the colony, while active, growing cells were localized primarily at or near the biofilm-air interface (i.e., the top of the biofilm) (24).
Taken together, cell death generally occurs in the interior of the microcolony and more live cells are present at the top of the biofilm, suggesting i) that cells within biofilms may rely on turnover of cellular constituent released from dead cells to continue life support of the remaining cells, ii) that dead cells located in the lower layers may provide a sort of physical support, and iii) that metabolically active cells on the top are more likely to be able to disperse and colonize new habitats than cells at the bottom. For example, a rain event could saturate the system and bacteria on the top and/or outer layers are more easily able to swim away than those in the center of a biofilm.

Identification of pemIK-like module on *P. putida* chromosome. In aquatic systems cell death has been recently observed during the normal course of *P. aeruginosa* biofilm development, and it was postulated that surviving cells in the biofilm benefit from the death of their siblings by feeding on the released nutrients (23). One proposed mechanism contributing to death of cells in *P. aeruginosa* biofilms in flow-through systems is the activation of prophage to produce super infective phages that, when released, lead to the killing of susceptible cells (23). Activation of the prophage apparently requires physiological or genetical changes in the biofilm cells and possibly the activities of a toxin-antitoxin (TA) system present in the phage genome (22). TA modules are thought to be a bacterial programmed cell death (PCD) process in which stable (long-half life) toxins kill cells unless unstable (short-half life) antitoxins bind to the stable toxins and inactivate them to prevent initiating processes leading to cell death (1, 11, 18, 19). Cells can live only if they actively produce the antidote antitoxin. In *E. coli*, expression of a chromosomally located TA module is regulated by starvation (1, 19), which could be a condition bacteria experience in mature unsaturated biofilms. In addition to prophage-mediated cell death, a novel autotoxic protein
produced by the marine bacterium *Pseudoalteromonas tunicata* has also been found to be involved in killing cells and in stimulating detachment of cells as biofilms age in flow-through systems (12). By *in silico* analysis of the genome sequence of *P. putida* KT2440 (www.tigr.org), which was derived from strain mt-2, we determined that mt-2 did not have a homologue of the autotoxic protein (AlpP) or the prophage Pf4 or its homologues integrated in the genome. However, we did identify a putative TA pemIK-like module, although it was not obviously associated with other prophages like what was observed in *P. aeruginosa*. Further investigation will be necessary to assess whether this locus is involved in cell death processes within unsaturated biofilms.

**Ecological implication of cell death within unsaturated biofilms.** Like those in saturated biofilms, dead cells within unsaturated biofilms could have a functional role, much like autolysis of *Myxococcus xanthus* cells presumably occurs to release nutrients to feed cells that form the fruiting body and spores (11) or dead cells could provide physical support for the stalk-like structures formed by *Saccharomyces cerevisiae* cells on solid surfaces (20). Various PCD mechanisms have been described in bacteria (6, 11, 13, 15), and these processes have been suggested to be altruistic for the population. Clearly, cell death of injured cells or sub-population could release a pool of readily utilizable resources that could provide life support for other cells better suited for survival or for those best positioned within the biofilm to survive or be dispersed to colonize a new niche.

One of the interesting ecological questions in this study is whether other common soil or plant-associated bacteria exhibit cell death patterns in space and time similar to those observed for *P. putida*. To ascertain this, we examined the distribution and abundance of dead cells within biofilms formed by *E. coli* DH5α, *Enterobacter cloacae* JL1157, *P.
Figure 4-8. Spatial localization of dead cells in 1-day-old gfp-tagged *E. coli* DH5α (A, B), *P. syringae* B728a (C, D), *Enterobacter cloacae* JL1157 (E, F), and *P. fluorescens* A506 (G, H) unsaturated biofilms grown on the unamended medium. (A, C, E, and G) Overlay of green and red images; (B, D, F, and H) corresponding red images only.

Each species was tagged with green fluorescent protein and each exhibited a unique spatial arrangement of dead cells within the biofilm (Fig. 4-8). Individual dead cells were observed more often in DH5α biofilms than the others in which aggregates of dead cells were surrounded by living cells (Fig. 4-8). Interestingly, *P. syringae* and *P. fluorescens* biofilms revealed more aggregates and fewer strings of dead cells than *P. putida* (Compare Figs. 4-1 and 4-8), suggesting that despite evolutionary close relatedness, presumably each species has its own pathway to control cell death within the community. In all four biofilms, more individuals or aggregates of dead cells were located in the center of the microcolony than in the periphery, although degree of centralization depends on the species. In all organisms, the frequency and number of individuals or aggregates of dead cells increased over time (data not shown).
Summary. We demonstrated that reduced water availability influences patterns of cell death within unsaturated biofilms and the spatial and temporal localization and stratification of dead cells. First, unlike other organisms examined in this study, strings of dead cells were only observed in unsaturated *P. putida* biofilms, although we were unable to detect visible strings in the -1.5 MPa ψ PEG8000 treatment. This finding strongly suggests that cell death patterns within biofilms may be species-specific. Second, there was a greater proportion of dead cells in the -1.5 MPa ψ PEG8000 treatment than unamended or -1.5 MPa ψ NaCl treatment, which suggests that PEG8000-mediated matric stress (dehydration) causes more stressful conditions for biofilm cell growth and survival than a thermodynamically equivalent NaCl-mediated osmotic stress. Lastly, quantitative analysis of dead cells through the entire depth of the biofilm showed that regardless of whether biofilms were cultivated in the presence or absence of reduced water availability treatments, more than 90% of the dead cells were localized in the lower 2/3 of the biofilm, which may reflect that these sites are unfavorable to cell growth and survival. This result also suggests that dead cells may provide physical support for live cells localized in the upper layers (e.g., the top of the biofilm) that are more likely to be dispersed to new habitats than cells at the bottom.

Our finding that there was an increase in the number of dead cells (not the proportion of dead cells) over time, possibly due to depletion of nutrient resources, may involve a bacterial PCD process induced by starvation (1, 11, 19). Given that PCD within a subpopulation may be altruistic, we propose that cell death in unsaturated biofilms may reduce competition for remaining resources, promote community survival by releasing nutrients for the remaining cells to utilize, and provide physical support and/or space for their offspring. We conclude that cell death is a normal, yet dynamic, process contributing to the survival of community.
Previous studies demonstrated that the prophage Pf4 and the autotoxic protein (AlpP) are a key element to control cell death in flow-through systems and suggested that cell death is a process by which surviving cells benefit from the death of their neighbors. Similarly, studies on the molecular mechanism(s) by which cells inside multicellular aggregates die on their own or through the activities of their neighbors will provide novel insights into ecological fitness traits in water-limited environments. Furthermore, it will be interesting to ascertain whether cell death of a subpopulation contributes to the fitness of the remaining population of cells under stressful conditions. This may aid the design of strategies to control or enhance biofilm development or the metabolic activities of community members to stimulate pollutant degradation by *P. putida* in soil or the rhizosphere.

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**REFERENCES**


Chapter 5. General Conclusions and Future Directions

The purpose of this study was to understand and elucidate the impact of reduced water availability on unsaturated biofilm biology from the broad perspective of biofilm development, EPS production, and cell death. Through the course of this study, we were able to answer or prove the questions or hypotheses raised, although much is still remaining to be revealed and many more questions will continue to arise.

First, how do soil bacteria develop into aggregates to form biofilm communities in water-limited habitats? To answer this question, we developed a novel experimental system to visualize unsaturated biofilm development and architecture in a non-destructive manner. As a model, we used the common soil saprophyte *P. putida* for these studies. *P. putida* unsaturated biofilm formation proceeded in an organized fashion through early, intermediate, and maturation phases of development. The different type and severity of reduced water availability treatments influenced ultrastructural properties of biofilms in different ways. These results lead to the conclusion that soil bacteria develop biofilms in a coordinated fashion, much like in those that develop in aquatic habitats, and that biofilm cells recognize different forms of water stress and therefore respond differently, and consequently their response to reductions in water availability alters biofilm development and properties.

However, the conditions that we used may not have been optimal for reflecting unsaturated biofilms in nature. Soil is a remarkably diverse, complex, heterogeneous habitat comprised of solid, liquid, and gaseous phases that vary in both space and time. Of particular interest is the rhizosphere whose complexity is influenced by the development, movement, and metabolic activity of plant roots. It would be fascinating to investigate unsaturated biofilm development
under conditions that more accurately reflect those found in soil (e.g., in clay and/or on root surfaces).

Second, is EPS produced by soil bacteria and, if so, where is it localized in biofilms? What is the composition of EPS and how does the composition change with reductions in water availability? To answer these questions we used calcofluor, which binds to β-linkage of carbohydrates, to visualize EPS and carbohydrate composition analysis to explore individual glycosyl residues. Most of the calcofluor-bound EPS was localized at the biofilm-air interface, and EPS production increased in response to cellular dehydration. Carbohydrate composition analysis revealed that there were differences in the composition of the EPS of biofilms that developed in water-limited and water-unlimited conditions. In particular, alginate was only produced under water limiting conditions and was only a minor component of the EPS matrix. These results provide an insight into a key aspect of EPS; bacteria are capable for producing EPS and regulating its production, possibly to create a more favorable microenvironment surrounding them. In other words, one or more EPS biosynthesis is dormant, but it will be activated by an environmental trigger such as dehydration. In addition to alginate production, our result suggests that cellulose production may increase in response to dehydration. To test this hypothesis, it will be necessary to generate a cellulose-deficient mutant to assess what role(s) bacterial cellulose plays in biofilm formation and bacterial survival in water-limited environments.

Third, we hypothesized that alginate provides a more hydrated microenvironment that protects cells from dehydration stress by slowing the rate of cellular drying. To evaluate this hypothesis we conducted two different approaches following a dehydration shock; i) a biosensor to measure the intracellular water potential sensed by bacteria, and ii) FAME
analysis to monitor conversion of cis unsaturated fatty acids to the trans isomer, which is indicative of dehydration. As a result we found that i) cells encapsulated with alginate perceived less water stress than those without alginate, and that ii) alginate slowed the rate at which membranes become dehydrated. Our findings support that our hypothesis is correct and also demonstrate that alginate is a fitness trait necessary for survival in water-limited environments. These results support an earlier finding that colanic acid, a highly viscous EPS, protected several enteric bacteria from desiccation imposed by a vacuum. Hydrophilic polysaccharides like alginate and colanic acids retain water near the cell boundary, which presumably protects cells from drying. Retention of water by these polysaccharides might be related to biofilm architecture in water-limited environments. The taller and more compact biofilm architecture reduces the surface area available for evaporative water loss. Furthermore, our findings led us to raise more questions as to i) whether all cells within a biofilm experience reduced water availability equally when pre-formed biofilms are exposed to low relative humidity? ii) where and when in a biofilm is alginate biosynthesis occurring? Reporter gene technology will allow us to investigate the questions without disrupting biofilm architecture, since the reporter gene products, such as green, red, yellow, or cyan fluorescent proteins, can be visualized in real time by scanning confocal laser microscopy (SCLM). In addition, SCLM and fluorescence reporter gene technology would be useful to determine other properties like whether there are preferential regions of pollutant diffusion within a biofilm, or even in a soil matrix.

Finally, the presence of cell-free regions within unsaturated biofilms led us to assess whether these regions were truly cell-free or contained dead cells. Our results showed that many of these apparent cell-free regions contained dead cells. More intriguingly, they were
frequently non-randomly distributed and stratified within the biofilm. They were arranged in arrays of various lengths one cell width in diameter, which were defined as strings of dead cells. Furthermore, the proportion and localization of dead cells within biofilms were affected by reduced water availability. These results lead to the conclusion that cell death is a normal, yet dynamic, process in unsaturated biofilms. We propose that cell death may be altruistic by facilitating cell survival or maintaining metabolic activity. Two lines of evidence support the notion that cell death is a part of the maintenance process: i) unsaturated biofilms tended to maintain a certain level of culturable, possibly metabolically active cells even when reductions in water availability appeared to increase death rates, and ii) more live cells were localized in the upper layers (e.g., the top of the biofilm), suggesting not only that dead cells located in the lower layers could provide a sort of physical support of biofilm architecture, but also that metabolically active cells on the top are more likely to be dispersed to new habitats than cells at the bottom. For example, rain percolation could saturate the system and bacteria on the top and/or outer layers are predicted to be more easily able to swim away than those in the center of a biofilm. In the future, it would be interesting to elucidate the molecular mechanism(s) determining how, when, and where cells die within a biofilm. One possibility is the chromosomally located putative toxin-antitoxin (TA) pemIK-like module identified in the genome of *P. putida* KT2440 (www.tigr.org). Because TA modules are thought to be a bacterial programmed cell death (PCD) mechanism and the expression of a chromosomally located TA module in *E. coli* is regulated by starvation which could be a condition in mature biofilms in the confined system. It is possible that nutrient limitation within the biofilm is a trigger of a PCD mechanism resulting in the observed spatial patterns of distribution of dead cells. Alternatively, prophage(s) may be activated to produce super
infective phages that lead to the killing of susceptible cells within a biofilm, since many phage related genes have been found in *P. putida* KT2440 genome. It is important to see if there are infective phages released in the microenvironment surrounding biofilm cells and, if so, to determine whether reduced water availability influences the activation of prophage(s). Consequently, identification and characterization of those phages, if any, will provide insight into bacterial cell death process within biofilms.
Appendix A. Fractionation and Characterization of \textit{P. putida} Exopolysaccharides

In Chapter 3, we quantified ethanol precipitable exopolysaccharide (EPS) and concluded that there was increased EPS production in response to matric stress. This result suggests that \textit{Pseudomonas putida} exports more EPS when they are exposed to water-limited environments. Similarly, it is possible that they produce low molecular weight (MW) carbohydrates, such as mono-, di-, or oligo-saccharides in a fashion similar to size fractionation of EPS produced by \textit{Sinorhizobium meliloti}. To ascertain this possibility, we quantified non-ethanol precipitable low MW carbohydrates present in the ethanolic supernatant after the second ethanol precipitation as described in Chapter 3. As expected, the amount of non-ethanol precipitable carbohydrates increased with increasing matric stress severity, although we were unable to detect uronic acids in this sample (Fig. A-1). This result suggests that \textit{P. putida} produces both high and low MW carbohydrates when they suffer from dehydration stress.

We also examined the size distribution of the first ethanol precipitated (high MW) EPSs produced under no, solute, and matric stresses. Alcian blue/silver stained PAGE of the high MW EPS fractions showed significant differences in electrophoretic mobility pattern of the EPS produced under osmotic and matric stresses compared to the no stress control (Fig. A-2). Under matric stress conditions there is a distinct higher mobility ladder pattern corresponding to lower MW EPS. There were no detectable alcian blue/silver stained bands in PAGE analysis of the low MW fractions, suggesting that the increase in the amount of this fraction under matric stress conditions (See Fig. A-1) is not due to contamination from the first ethanol precipitation.
Figure A-1. Total carbohydrate (A) and uronic acid (B) content of EPSs produced by *P. putida* mt-2 under various solute and matric stress conditions. S, solute stress; M, matric stress. Bars are the means of 3 independent replications.
Figure A-2. PAGE analysis of the first ethanol precipitated (high MW) EPSs produced under no (lane 1), solute (lane 2), and matric (lane 3) stresses. Each lane on the gel contained 2 μg of carbohydrate.
Appendix B. Quantification of P. aeruginosa PAO1 Exopolysaccharides

Increased alginate production by P. putida in response to PEG8000 mediated dehydration led us to hypothesize that this phenomenon also occurs in other close related microorganisms (i.e., P. aeruginosa, P. syringae, or P. fluorescens) that have the genetic ability to produce alginate. To test this hypothesis, we chose the opportunistic pathogen P. aeruginosa PAO1, whose alginate biosynthetic pathway is almost identical to that in P. putida KT2440, and quantitatively analyzed EPS isolated from unsaturated PAO1 biofilms as described in Chapter 2.

There was more uronic acid content of EPS obtained from PAO1 biofilms in the -1.5 MPa ψPEG8000 treatments compared to the unamended and -1.5 MPa ψNaCl treatments in both complex (½ TYE) and minimal (½ 21C) media (Fig. B-1). This result suggests that dehydration is a key environmental stimulus to induce alginate production by several Pseudomonas species. In addition, we quantified the total polysaccharide content of PAO1 EPS. As shown in Fig. B-2, basically we observed the same result as that of uronic acids; more total polysaccharide in dehydration (matric stress) than no or solute stress. Our findings clearly show that like P. putida, P. aeruginosa preferentially produces more EPS in response to matric stress, but not to a thermodynamically equivalent solute stress.
Figure B-1. Uronic acid content of EPS isolated from *P. aeruginosa* PAO1 on unamended and -1.5 MPa NaCl and PEG-8000 amended media. Uronic acid contents are expressed as glucuronic acid equivalents. Values represent the mean ± SE (n=3).
Figure B-2. Total carbohydrate content of EPS isolated from *P. aeruginosa* PAO1 on unamended and -1.5 MPa NaCl and PEG-8000 amended media. Total carbohydrate contents are expressed as glucose equivalents. Values represent the mean ± SE (n=3).
Appendix C. Strategy for Generating an *algD* Knockout Mutant

This section will provide more detailed information on how we constructed an alginate-deficient mutant that was used to investigate the role(s) of alginate in biofilm formation and desiccation tolerance.

**Step 1: Amplification of an internal region of *algD* gene.** We designed the primer set to PCR-amplify a 622-bp internal region corresponding to nucleotides 17-644 of the 1.3-kb *algD* gene from genomic DNA of *P. putida* mt-2. Colony PCR was performed in 20 µl volumes containing 20 pmol of forward and reverse primers, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 2 units *Taq* polymerase, 1X *Taq* polymerase buffer, and genomic DNA from a *P. putida* mt-2 colony. In the PCR cycle, annealing temperature and time were 50.5°C and 45 sec. The primers used are as follows:

AF primer: 5' - TTGGTTTGGGTTATGTGGG - 3' (forward)

AR primer: 5' - CAGGTGTACTTGATCATTTCGG - 3' (reverse)

**Step 2: Insertion of a PCR product into the suicide vector pKnockout-G.** The PCR product was inserted into the *Xcm* site of the suicide vector pKnockout-G to create the hybrid plasmid pKAD. The insertion was confirmed by sequence analysis (data not shown). The plasmid pKAD was kept in *E. coli* S17-1 strain for transfer to *P. putida*.

**Step 3: Conjugation.** The plasmid pKAD was transferred from the donor strain *E. coli* S17-1 to the recipient *P. putida* mt-2 by conjugation with the helper strain *E. coli* pRK2073. By homologous recombination the pKAD was inserted into *P. putida* chromosome, resulting in disruption of the *algD* gene. Since the pKAD confers gentamycin resistance (Gm⁰) and *P. putida*, but not *E. coli* S17-1, is resistant to rifampicin (Rif⁰), transconjugants were selected by plating on gentamycin- and rifampicin-containing media.
Step 4: Confirmation of transconjugants in genotype. Insertion and orientation of pKAD was confirmed using PCR amplification from genomic DNA of transconjugants as template with 4 combinations of the internal \textit{algD} primers and the universal primers designed from the original suicide vector (Fig. C-1). The universal primers are as follows:

UF primer: 5' - CCCAGTCACGACGTTGTAAAACG-3' (forward)

UR primer: 5' - AGCGGATACCAATTTCACACAGG-3' (reverse)

Step 5: Confirmation of a polar or non-polar mutation. We examined whether insertion of the pKAD caused a polar effect, since the fate of polar mutation in transconjugants depends on the orientation of a truncated \textit{algD} gene. All of the 16 transconjugants we tested had polar mutation (Figs. C-1 and C-2), indicating that whole genes located on the 16 kb alginate biosynthesis operon were not properly expressed.

Step 6. Confirmation of alginate deficiency in phenotype. To further verify alginate deficiency in the mutants, we conducted an uronic acid assay. All of the tested mutants were not able to produce the same level of uronic acids as the wild type did in -1.5 MPa PEG-8000 treatment, although they produced the basal level of uronic acids (Fig. C-3A, and data not shown), which is presumably glucuronic acids (See Table 3-2 in Chapter 3). We also checked total carbohydrate content, since it is possible that alginate deficiency may affect the biosynthesis of other types of EPS. As shown in Fig. C-3B, the reduced amount of total carbohydrate was due to the alginate deficiency. However, the carbohydrate composition analysis indicates that the composition was changed by knocking out the \textit{algD} gene (See Table 3-2 in Chapter 3).
Figure C-1. Schematic diagram of generating an algD mutant. Orientation 1 and 2 depends on orientation of an inserted algD fragment. Orientation 1 represents disruption of algD without polar mutation due to the presence of the lac promoter to derive expression of downstream alginate biosynthesis genes, whereas orientation 2 results in the generation of polar mutation due to the absence of a promoter to drive expression of downstream alginate biosynthesis genes. Sets 1-4 are primer pairs used to verify which orientation event occurred in the pKAD plasmid integration into the genome.
Figure C-2. Gel electrophoresis of PCR products amplified from the *algD* mutant chromosome with primer set 1 (lane 1), 2 (lane 2), 3 (lane 3), and 4 (lane 4). M represents marker.
Figure C-3. Comparison of uronic acid (A) and total carbohydrate (B) content produced by the wild type strain *P. putida* mt-2 and its isogenic *algD* mutant. We were unable to detect uronic acids in the ethanolic supernatant after the second ethanol precipitation in both strains. Bars are the means of three independent replications.
Appendix D. Comparison of Fatty Acid Composition between the Wild Type and algD Mutant

We compared overall fatty acid composition between the wild type and algD mutant prior to the dehydration shock experiments as described in Chapter 2, since it is possible that both strains are inherently different in their cellular fatty acid composition. As shown in Table D-1, during steady state growth in the absence or presence of PEG8000 or NaCl treatments there were no statistically significant differences ($P > 0.05$) in the fatty acid composition of both strains, specifically in the 16:1 $trans/cis$ and saturated/unsaturated fatty acid ratios. This result suggests that they are not inherently different in their cellular fatty acid composition.
Table D-1. Fatty acid composition of the wild type (wt) and $algD$ mutant in the unamended, -1.5 MPa $\psi$ NaCl, and -1.5 MPa $\psi$ PEG 8000 treatments, respectively$^a$

<table>
<thead>
<tr>
<th></th>
<th>Unamended</th>
<th>-1.5 MPa NaCl</th>
<th>-1.5 MPa PEG 8000</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt</td>
<td>$algD$</td>
<td>wt</td>
</tr>
<tr>
<td>Fatty acid (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>34.2 ± 0.4</td>
<td>33.4 ± 0.6</td>
<td>33.9 ± 0.7</td>
</tr>
<tr>
<td>16:1 $trans$</td>
<td>6.0 ± 0.3</td>
<td>5.7 ± 0.5</td>
<td>8.0 ± 0.7</td>
</tr>
<tr>
<td>16:1 $cis$</td>
<td>24.6 ± 0.6</td>
<td>27.3 ± 0.5</td>
<td>21.2 ± 1.1</td>
</tr>
<tr>
<td>17:0 cyclo</td>
<td>6.7 ± 1.0</td>
<td>4.2 ± 0.4</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>18:0</td>
<td>&lt; 1.0</td>
<td>&lt; 1.0</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>18:1 $trans$</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18:1 $cis$</td>
<td>12.5 ± 0.3</td>
<td>12.8 ± 0.4</td>
<td>14.7 ± 1.0</td>
</tr>
<tr>
<td>19:0 cyclo</td>
<td>0.2 ± 0.1</td>
<td>&lt; 0.1</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Total $trans^b$</td>
<td>6.0 ± 0.3</td>
<td>5.7 ± 0.5</td>
<td>8.0 ± 0.7</td>
</tr>
<tr>
<td>Total $cis^b$</td>
<td>37.1 ± 0.4</td>
<td>40.1 ± 0.7</td>
<td>35.4 ± 1.6</td>
</tr>
<tr>
<td>Total hydroxyl</td>
<td>14.3 ± 0.5</td>
<td>14.4 ± 0.3</td>
<td>15.8 ± 0.4</td>
</tr>
<tr>
<td>Ratio$^c$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:1 $trans/cis$</td>
<td>0.24 ± 0.01</td>
<td>0.21 ± 0.02</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>18:1 $trans/cis$</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total $trans/cis$</td>
<td>0.16 ± 0.01</td>
<td>0.14 ± 0.02</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>Saturated /unsaturated</td>
<td>0.76 ± 0.02</td>
<td>0.85 ± 0.02</td>
<td>0.79 ± 0.05</td>
</tr>
</tbody>
</table>

$^a$ Cells were cultivated on nylon membranes overlaid onto 1/2 21C medium with and without lowering water potentials by NaCl or PEG 8000 for 24 h at 27 °C, and then subject to FAME analysis. Values are expressed as means ± standard errors of the means of three to six replications.

$^b$ Total $trans$ or $cis$ were sum of 16:1 and 18:1 $trans$ or $cis$ unsaturated fatty acids.

$^c$ Ratio was calculated based on % value of fatty acid composition. There was no significant difference between the wild type and $algD$ mutant in each treatment at $P < 0.05$ (Student's paired $t$-test).
Appendix E. Comparison of Early Biofilm Formation between the Wild Type and \textit{flaC} (Flagellar Deficient) Mutant

Taller and less spread-out biofilm architecture in PEG8000-mediated matric stress (dehydration) compared to that in no stress or NaCl-mediated solute stress led us to hypothesize that loss of flagellar motility might be involved in this structure under dehydration conditions. Because, loss of motility could derive cells get clouded. In fact, \textit{P. putida} has one polar flagellum and uses this machinery to swim. To test this possibility, we used a flagellar-deficient (\textit{flaC}) mutant identified using the mini-Tn5-'\textit{phoA} system (provided by Martijn van de Mortel) to compare the surface area of young biofilms of the wild type and mutant in the absence and presence of very mild matric stress. There was no significant difference ($P > 0.05$) in the surface area coverage of the biofilms of the wild type and \textit{flaC} mutant in the 0 MPa and -0.25 MPa \textit{PEG8000} treatments (Table E-1). This result suggests that less spread-out aspect of biofilm formed in matric stress is not due to the loss of flagellar motility, and therefore flagellar motility is not as much as important in unsaturated biofilm formation.

| Table E-1. Effect of flagellar mutation on microcolony surface area coverage$^a$ |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Time (h)        | 0 MPa \textit{PEG8000} treatment | -0.25 MPa \textit{PEG8000} treatment |
|                 | Wild type | \textit{flaC} mutant | Wild type | \textit{flaC} mutant |
| 6               | 6,303 ± 357 | 6,414 ± 578 | 17,576 ± 822 | 19,158 ± 1,147 |
| 12              | 62,537 ± 6,167 | 65,035 ± 9,204 | 34,247 ± 1,169 | 40,858 ± 6,787 |

$^a$ Microcolony surface area (\text{\textmu m}^2) at the substratum surface. Values were derived from the average microcolony surface area per field of view ($n= 3$ to 12) containing 3 to 18 microcolonies.
Appendix F. Normalized INA Values in Fig. 3-5 by CFU at Time Zero and Recalculated Estimated Water Potentials

In the INA assay, theoretically, the maximum INA that can be detected is 1 nucleus per cell. However, we detected more than that in the \textit{algD} mutant after 3 h following a dehydration shock (Fig. 3-5). This result might be explained by the fact either that unculturable bacteria expressed INA, or that cells retained INA for a limited time after they lost viability. Unfortunately, either explanation may open up the question whether alginate really slows the drying rate. To answer this question, we conducted a different method for analyzing the INA data. Because of the decreased population size of both strains upon dehydration and osmotic shock, all INA values were normalized to the CFU at time zero instead of that at a given time point. As a result, the \textit{algD} mutant still showed higher INA than the wild type after 3 h following a dehydration shock (Fig. F-1), which indicates higher water potential (lower water stress) was indeed sensed by the wild type compared to the \textit{algD} mutant.
Figure F-1. Temporal dynamics of the INA (A, C, and E), and estimated water potentials (B and D) by the wild type and algD mutant containing an inducible pPProIce plasmid (A - D) and a constitutive pPNptIce plasmid (E). This is the same experiment as in Fig. 3-5, but all values were re-calculated based on time zero CFU, since a dehydration shock causes decreased population. Closed symbols, wild type; opened symbols, algD mutant; ▲ or △, -1.0 MPa PEG-8000 treatment; ● or ○, -2.5 MPa PEG-8000 treatment; ■ or □, -2.5 MPa NaCl treatment. Values represent the mean ± SE of two or three experiments, each comprised of three replications.
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