Hydrating the *Pseudomonas aeruginosa* periplasm under desiccating conditions

by

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Dedicated to my wife, Lindsay.

Thank you for always supporting me in this endeavor.

I love you!

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CHAPTER 1. GENERAL INTRODUCTION

For terrestrial bacteria, the world can be a dry place. On a micro-scale, limited water-availability can be one of the defining features of diverse environments from soil to the surface of plants and animals. Yet, bacteria are able to survive and grow in dry conditions, inhospitable to other organisms. Our understanding of how these single celled microbes adapt to such conditions is of great importance considering their fundamental role in many ecosystems including carbon and nutrient cycling and the survival of pathogens and beneficial bacteria involved in plant, animal and human health.

Water availability is a function primarily of two components, solute and matric water potentials, that influence the total water potential of the surrounding environment. Water will diffuse across semi-permeable barriers, such as cell membranes, to conditions of lower water potential. Even if a cell is bathed in water, high concentrations of solutes reduce the solute (osmotic) potential, limiting the propensity of water to enter the cytosol. Similarly, inter-molecular forces (including surface tension) between water and the surrounding matrix reduce the matric potential external to the cell, particularly when the water content is low.

We know some well characterized mechanisms bacteria utilize to counter these reduced water potential stresses. Hygroscopic exopolysaccharides and other polymers produced by a community of cells called a biofilm can absorb and sequester water, creating a more-hydrated micro environment relative to the surrounding dry habitat. Additionally, under both high solute and low-water-content conditions, the accumulation of compatible cytosolic solutes helps reduce the water potential in that compartment, facilitating water uptake and retention.

While both solute and matric components reduce the environmental water potential, they exert physiologically different stresses on bacterial cells, especially in relation to the periplasmic space between the inner and outer membranes of gram negative bacteria. Under high solute conditions, solutes enter the periplasm through porin channels, allowing for hydration of the compartment. However, when water-content is low, the reduced water potential differential lies across the outer membrane; thus cells must hydrate the periplasm to maintain optimal function. Surprisingly, it is unknown how the periplasm, a compartment housing many critical functions, is hydrated under low-water-content conditions. A likely mechanism would be to reduce the water potential of the periplasm with charged solutes. Small solutes, like the compatible solutes found in the cytoplasm would be ineffective as they could diffuse out through porins, though intermediately sized periplasmic glucans may fulfill this role.

Ubiquitously found among proteobacteria, these oligosaccharides are often anionically charged and come in two general types, linear and cyclic. Interestingly, under hypo-osmotic (water-replete) conditions, periplasmic glucans accumulate in the periplasm to balance the water-potential of the fully hydrated periplasm with the cytosol, containing solutes important for normal metabolism, preventing over-swelling of the inner membrane. But no one has examined whether glucans accumulate under low-water-content conditions. If glucans function to reduce the periplasmic water potential, hydrating the compartment during low-water-content conditions, we would expect to see glucans accumulate and or become more anionically charged. We would also expect that the absence of periplasmic glucans under low-water-content conditions would exacerbate the matric stress already experienced by the cells.

Members of the genus *Pseudomonas* make interesting models for studying the roles of periplasmic glucans because most species are able to produce both linear and cyclic glucans.

Recent research with the soil borne gram negative bacterium *Pseudomonas putida* revealed that periplasmic glucan biosynthesis gene expression is elevated under low-water-content conditions. Similarly, there was a significant increase in the amount of glucose-rich low molecular weight carbohydrate in the extracellular matrix of *P. putida* in response to low-water-content conditions, suggesting that excess periplasmic glucans may have leaked out of the cell. These two pieces of evidence led us to develop our hypothesis that glucans accumulate in the periplasm to hydrate the periplasm. The work discussed in this thesis aims to explore the role of periplasmic glucans in the model organism *Pseudomonas aeruginosa* under low-water-content conditions by exploring their abundance and anionic properties under different water availability conditions and to further explore the effect of periplasmic glucan deficiency on physiological properties and fitness under low-water-content conditions.

Thesis Organization

This thesis is organized into five chapters. The first chapter is a general introduction to water availability and associated stresses under various environmental conditions and the potential role glucans play in hydrating the periplasm under low-water-content conditions. The 2nd chapter is a review of relevant literature pertaining to water stress physiology and the biological roles of periplasmic glucans. Chapter 3 investigates the accumulation of periplasmic glucans produced by *P. aeruginosa* under varying water-stress conditions. Changes in the physical properties of the glucans are also discussed as well as the effect glucan deficiency has on matric stress survival. Chapter 4 explores the physiological consequences of glucan deficiency in *P. aeruginosa* cells grown under low-water conditions to better understand the function of each glucan. Among other phenotypes, loss of glucan production exacerbates the

envelope stress response in cells grown in low-water-conditions, suggesting a poorly hydrated periplasm. The final chapter summarizes our overall conclusions as well as looks to future avenues of research on glucan-mediated hydration of the periplasm. Appendix I describes research investigating a putative amyloid fiber-like protein produced by *P. putida* called purli. We investigated purli subunit structure and the interactions between purli and exopolysaccharides. Appendix II explores transcription of glucan biosynthesis genes. We used 5' RACE to identify transcriptional start sites with varying success. Additionally we describe a potential σ^{22} (AlgU/T) alternative sigma-factor binding site upstream of the linear glucan biosynthesis operon.

CHAPTER 2. LITERATURE REVIEW - WATER STRESS PHYSIOLOGY AND THE NEED TO HYDRATE THE PERIPLASM

Water availability is a defining characteristic of microbial habitats as diverse as soil, plant, animal and man-made surfaces. The availability of water to a bacterium is a function of primarily two components, osmotic and matric, influencing the thermodynamic water potential of the external environment. Water diffuses across semipermeable cellular membranes from higher to lower water potentials, a process that cells must regulate to ensure proper cellular hydration under diverse conditions.

When environmental water is plentiful and solute concentrations are low, a hypo-osmotic stress is imposed on a bacterial cell. Because the cytosol contains osmolalities of around 300 mosM required for normal cellular processes (75), the cytosolic water potential is reduced relative to the water-replete environment outside the cytoplasmic membrane. This difference increases the propensity of water to diffuse into the cytosol, causing it to swell (13, 56). Following a sudden wet-up (e.g., rainfall) event of a dry soil, the environment rapidly becomes hypo-osmotic which can be extremely stressful to a cell. However, bacteria can counteract this stress by secreting excess solutes thereby increasing the cytosolic water potential (53). Additionally, gram-negative bacteria have both an inner and outer membrane with the periplasm between them. Within this space, the accumulation under hypo-osmotic conditions of intermediately sized oligosaccharides called glucans has been well studied (6, 35, 56) and will be discussed in detail below. These molecules can have anionic substitutions such as phosphoglycerol or succinyl groups, which increase the fixed osmolyte concentration of the periplasm (6, 13, 35), reducing the periplasmic water potential to balance the differential across the cytoplasmic (inner) membrane.

Under a solute stress, dissolved solutes reduce the water potential of external water bathing the cell, increasing the propensity of water to diffuse out rather than into the cell (13). We can impose this stress in the lab with the addition of dissolved solutes such as NaCl to our growth media. For gram-negative bacteria solute (osmotic) stress creates a situation in which solutes enter the periplasm through outer membrane porins (13). This hydrates the periplasm, albeit with water of reduced activity, creating a water potential differential across the inner membrane. Bacteria counteract this water potential difference by accumulating compatible solutes within the cytosol (13). The transient increase of cytosolic potassium ions is one of the first compatible solutes to accumulate in response to increased osmolarity (19, 64, 71), before being secreted as other molecules such as glutamate, glutamine, trehalose, proline and glycine betaine eventually accumulate either from uptake from the environment or de novo synthesis (19, 24, 40, 79). These osmolytes further reduce the cytosolic water potential, balancing the differential across the inner membrane.

Unlike solute stress, low-water-conditions impose a matric stress on the cell as water associates with the environmental matrix via intermolecular forces, reducing the water-potential external to the entire cell, including the periplasm. In the lab we can simulate a low-water-content condition by amending our media with polyethylene glycol MW 8000 (PEG). Much as dry soil would interact with water, limiting its availability to a bacterium, this large non-permeating hygroscopic solute reduces the external water potential and imposes a matric stress on the cell (28). Depending on the media, PEG amendments can easily achieve reduced water-potential of -2.5 MPa relative to water-replete (hypo-osmotic) conditions. An additional method of imposing a matric stress on the cells is a novel process involving porous ceramic plates wetted with a thin layer of growth media under suction through the plate (17). However, it was only

with the recent modification of added pressurization that a reduce water potential of -1.5 MPa was achieved (27).

We know of some ways that the cell remains hydrated under low-water-content conditions. For a community of cells living as a biofilm on a surface, the secretion of extracellular polymeric substances coat the cells, limiting exposure to environmental stresses such as inhibiting diffusion of antibiotics or access of leukocytes to pathogens (3, 31, 41, 59, 74) but also protecting organisms from water-limitation (10). Extracellular polymeric substances can contain hygroscopic polysaccharides, creating a more hydrated micro-environment relative to the bulk environment. Produced by many Pseudomonads, the well characterized exopolysaccharide alginate fulfills this role (10). This anionic polysaccharide, primarily composed of mannuronic and sometimes guluronic acid, can be hygroscopic (10, 46). While it is typically absent in the biofilm (84), under low-water-content conditions alginate becomes abundant. In *Pseudomonas putida*, *Pseudomonas aeruginosa* and *Pseudomonas syringae*, cells grown on matric stress, but not solute stress or water-replete conditions increase alginate production (10). This was demonstrated in *P. putida* to be the result of a transient increase in expression of the alginate biosynthesis operon (45).

However, the build-up of hygroscopic exopolysaccharides and the more-hydrated micro-environment it provides is not enough to maintain hydration of the cell. Hydration of the cytosolic compartment during low-water-content conditions occurs by accumulating compatible solutes mitigating dehydrating forces similar to the response to solute stress (62). However, for a gram-negative bacterial cell a low-water-content induced matric stress is physiologically very different from a solute stress, especially from the standpoint of the periplasmic space. Under a matric stress, the water potential differential lies across both the inner and outer membranes, not

just the inner membrane as is the case with osmotic stress when solutes and water enter the periplasm. However, we know that many bacterial species are able to remain metabolically active under low-water-content conditions; clearly they must be able to hydrate the periplasm and draw-in the limited environmental water required for survival.

Periplasm must be hydrated

Consequences for the periplasm, a compartment composing 20-40% of the total cell volume, drying out would be catastrophic (75). Membrane fluidity could become compromised due to dehydration. Additionally, the periplasm houses many important functions including structural, transport, metabolic and signaling proteins requiring proper hydration. In many gram-negative bacteria, envelope stability issues are sensed by periplasmic- and membrane-associated sensory proteins and trigger the envelope stress response due to release of an alternative sigma factor that regulates the expression of a suite of genes. In *E. coli*, periplasmic proteins such as DegS, RseB and RseP sense misfolded proteins and trigger the release of the alternative sigma factor σ^E via proteolytic lysis of the anti-sigma factor, RseA (1, 2, 15). There are parallel processes in many other gram-negative bacteria, including *P. aeruginosa*, where AlgW and other periplasmic proteins sense and trigger the release of the alternative sigma factor σ^{22} (AlgU/T) via intramembrane proteolysis of the anti-sigma factor MucA (30, 32, 52, 72). Desequestered AlgU is then free to regulate a diverse set of genes under its regulon, including those involved in adaptation and protection, transcriptional regulators, LPS synthesis and other membrane-associated proteins (18, 32, 82, 83). One of the targets of AlgU is the alginate biosynthetic operon. Pseudomonads with MucA mutations display hyper-mucoid phenotypes due to overproduction of alginate (51, 69). This is a common mutation in *P. aeruginosa* isolated from

Cystic Fibrosis patients, resulting in poor prognosis as overproduction of EPS such as alginate contributes to better tolerance to immune responses (20, 31, 51). Because of its clinical importance, regulation of alginate and other EPS components has been an active area of research (36, 66, 73). Overproduction of alginate, especially in cells grown on a matric stress, often yields a distinctive mucoid phenotype, indicative of an envelope stress response (10).

Without a mechanism for hydrating the periplasm, the compartment would become dehydrated, though we do not currently know how the cell counteracts this. One option would be to modify the water potential of the periplasm, increasing the likelihood of water remaining in the space and even drawing water from the external environment. The accumulation of the same compatible solutes found in the cytosol would not be effective in reducing the periplasmic water potential as these molecules could diffuse out through porins to the external environment. However, the accumulation of intermediately-sized, anionically-charged molecules could be effective. Periplasmic glucans might fit this role under low-water-content conditions; they fulfill a similar role of reducing the water potential of the periplasm under water-replete (hypo-osmotic) conditions. However, no one has looked at whether glucans accumulate or what role glucans play in tolerating water-limited conditions.

Current understanding of periplasmic glucans

Periplasmic glucans are ubiquitous amongst gram-negative proteobacteria and fall into two general classes, linear and cyclic (6, 8, 11, 35, 44, 49, 56, 76-78). They accumulate under hypo-osmotic conditions, constituting up to 7% of the cell dry mass (35). Linear glucans have been studied primarily in enteric bacteria, such as *E. coli* where they were first described (80). Synthesized by the membrane bound glycosyl transferases encoded in the *opgGH* operon

(*mdoGH* and formerly "locus *mdoA*") (39), linear glucan backbones typically range from 5 to 16 β-1→2 linked glucose residues with β-1→6 branches (6, 43, 44, 68). Along with a glycosyl transferase domain, OpgH has several transmembrane domains, leading to speculation that the linear glucan is exported to the periplasm through an OpgH derived channel as it is being synthesized from UDP-glucose (6, 16). Despite being required for linear glucan synthesis, the exact function of OpgG has not yet been fully determined. It is thought that OpgG is involved in the addition of β-1→6 branches (6, 29). Another gene, *mdoD* appears to be involved in regulating glucan size as mutants have increased backbone chain length (43).

Cyclic glucans have been studied primarily in Rhizobiaceae where they are produced by the cytosolic NdvB glycosyl transferase (21, 56, 63). Depending on species, sizes range from 10 to 40 glucose residues arranged in a cyclic chain and linkages can vary from β-1→2, β-1→3, or β-1→6 (8, 49, 55, 63). The cyclic glucan is translocated to the periplasm via NdvA, a possible ATP-binding cassette transporter (9). *Bradyrhizobium japonicum* cyclic glucans have β-1→3 and β-1→6 linkages, with NdvC responsible for the formation of β-1→6 linkages (4, 55).

Both linear and cyclic glucans can have substitutions giving them anionic properties. Depending on species, phosphoglycerol and succinyl substitutions are common, although other substitutions such as phosphocholine and acetyl groups have been identified (35, 54, 80). In *E. coli*, OpgB (MdoB) adds phosphoglycerol head groups from membrane phospholipids to linear glucans in a process influencing phospholipid turnover (34, 42, 70), while MdoC is thought to add succinyl groups from cytoplasmic succinyl-CoA (38). In *Sinorhizobium meliloti*, phosphoglycerol additions are carried out by CgmB, which is also involved in phospholipid turnover (54, 81). Under hypo-osmotic conditions, glucans with anionic substitutions can accumulate to regulate the fixed osmolyte concentration of the periplasm. In fact, there is

evidence that the degree of anionic decoration is regulated by environmental osmolarity, since *S. meliloti* grown in the presence of more solutes accumulate more neutral glucans (14).

In many species, periplasmic glucan abundance is dependent on osmolarity with high glucan concentrations accumulating under hypo-osmotic conditions (35, 56). Conversely, under high osmolarity, glucans decrease in abundance and can become scarce. Regulation of glucan accumulation is thought to occur at several levels. In *E. coli*, linear glucan production is regulated transcriptionally and at the enzymatic activity level. Expression of the *opgGH* operon is reduced with increasing concentrations of NaCl (39). Inhibiting *de novo* OpgG protein synthesis with chloramphenicol prevents accumulation of linear glucans, even when cells are transitioned from high to low osmolarity, suggesting enzymatic activity is not up-regulated by hypo-osmotic conditions and implying that continued expression of *opgG* is required (35, 37). However, in vitro experiments suggest that glucosyl transferase activity of membrane preparations is inhibited by the addition of salt (65). Synthesis of cyclic glucans appears not to be regulated at the transcriptional level (21). Rather, in vitro experiments show that increasing medium ionic strength reduces *S. meliloti* NdvB activity, demonstrating that protein activity is the primary regulator of cyclic glucan synthesis (33). However, in *S. meliloti* export of the cyclic glucan is transcriptionally regulated by the sensor kinase / response regulator FeuQP, which induces *ndvA* expression under hypo-osmotic conditions (26). This suggests that in response to a hypo-osmotic shock, glucan synthesis could begin but export would require expression and assembly of the NdvA secretory apparatus. Additionally it's not known if there are conditions under which cyclic glucans are synthesized but not exported.

Mutants deficient in linear glucan synthesis exhibit numerous pleiotropic phenotypes. *P. aeruginosa* exhibits reduced virulence in *Caenorhabditis elegans*, mice, and Arabidopsis and

does not form fully structured biofilms in a flow cells (44, 50). Linear glucan-deficient mutants of plant pathogen *Dickeya dadantii* exhibit reduced virulence due to lower pectinase, cellulase and protease activities as well as reduced swarming motility and increased exopolysaccharide production (7, 60). Similarly *hrpM* (*opgH*) mutants of *P. syringae* pv. *syringae* are avirulent on host bean plants and pear trees and are unable to induce the hypersensitive reaction on tobacco plants (47, 57). Motility and survival in hypo-osmotic conditions is also reduced while alginate production increases 10-fold (61). Similarly, in *E. coli*, linear glucan mutants exhibit 38-fold increased colonic acid capsular polysaccharide expression in a manner similar to alginate over production by *P. syringae* (23) suggesting that glucan-deficient cells experience envelope stress. In addition to reduced hypo-osmotic survival and reduced virulence in mice, proteomic studies of *Salmonella enterica* showed reductions in flagellar, osmo-sensing and chemotaxis proteins and increases in ABC transporters including those involved in uptake of compatible solutes glycine betaine and choline (5, 12).

It is thought that in addition to their role osmoregulating the periplasm, Rhizobial cyclic glucans may function as signal molecules for establishing nodule formation on leguminous plants, possibly by suppressing plant defenses (48). Cyclic glucans of Rhizobiaceae are essential for proper nodule development (which is the origin of the gene name *ndv*), since cyclic glucan-deficient mutants form non-nitrogen fixing pseudonodules (22). Similar to species producing linear glucans, cyclic glucan mutants of *S. meliloti* also have slower growth and reduced swarming motility in hypo-osmotic media, though addition of solutes restores wild-type growth and swarming (21). Reduced motility by glucan mutants is likely due to reduced stability of the flagellar structure which may be the result of a stressed envelope; this could also explain increased bacteriophage resistance of *S. meliloti* glucan mutants (21). Reduced swarming is also

observed in *Bradyrhizobium japonicum ndvB* mutants where it also becomes apparent that the specific structure of cyclic glucans is important to successful symbiosis though not osmoregulation of the periplasm (4). NdvC-deficient mutants produce glucans composed entirely of β-1→3 linkages, and no β-1→6 linkages, form non-functional pseudonodules with their soybean host, but swarm similarly to wild-type (4). In a similar fashion, cyclic glucans are required for *Agrobacterium tumefaciens* virulence, influencing association with the host (63). Cyclic glucans contribute to antibiotic resistance as *P. aeruginosa* mutants are more sensitive to tobramycin. Co-purification assays suggest that cyclic glucans bind both tobramycin and kanamycin (67), though destabilization of the cell envelope could also play a factor by reducing antibiotic permeability (49, 67).

Pseudomonads are excellent models for studying periplasmic glucans

While most bacterial species appear to make a linear or cyclic glucan, Pseudomonads are able to produce both (44, 49). This makes them interesting models for studying the roles of linear and cyclic glucans as well as exploring overlapping functions. In addition to the identified OpgGH (MdoGH) orthologs, *P. aeruginosa* hypothetical protein PA1689 has 46% similarity to the *E. coli* MdoB phosphoglycerol transferase. Surprisingly, it appears that the *P. aeruginosa* linear glucan is primarily substituted by succinyl groups, rather than phosphoglycerol (44), while the cyclic glucan is exclusively decorated with phosphoglycerol (67). Though an NdvA exporter ortholog has not been identified yet in *P. aeruginosa*, PA2656 and PA2657 share significant homology to the *S. meliloti* FeuQP; the 2-component sensor kinase and response regulator controlling expression of *ndvA*. This suggests they regulate expression of a yet unidentified cyclic glucan exporter. It is not yet known why Pseudomonads are capable of producing both

glucans. Growing in diverse habitats from aquatic to soil to plant and animal surfaces, perhaps a specific glucan may be critical in specific habitats, while the other is required in other conditions. Alternatively, linear and cyclic glucans may perform slightly different functions under the same conditions.

Evidence glucans play a role in hydrating the periplasm under low-water content conditions

While no one has yet directly looked at the role glucans play under low-water-content conditions, there have been some intriguing preliminary findings suggestive of the possible role of glucans in tolerating matric stress-mediated dehydration. Matric stress stimulates *Pseudomonas putida* EPS production and 60% is low-MW components, which appear to be glucose rich (10, 58). This glucose-rich component was found in both wild-type and cellulose-deficient mutants, suggesting it could be a periplasmic glucan (10, 58). The presence of extracellular glucans could be a consequence of secretion, as observed for some Rhizobia, where it is thought to act as an extracellular signal molecule (25, 48), or a consequence of the process of harvesting EPS from unsaturated biofilms. Furthermore, a transcriptome study of *P. putida* found that 15 minutes following a -1.0 MPa ψ matric stress shock there was an almost 4-fold increase in expression of *opgG* (58). This presence of a low-MW carbohydrate accumulating in response to matric stress, and the increased expression of the linear glucan biosynthesis operon, strongly supports the hypothesis that glucans accumulate to hydrate the periplasm under matric stress conditions.

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CHAPTER 3. *PSEUDOMONAS AERUGINOSA* "OSMOREGULATED" PERIPLASMIC GLUCANS ACCUMULATE AND BECOME MORE ANIONIC UNDER LOW-WATER-CONTENT CONDITIONS.

Abstract

For bacteria to survive desiccating conditions they must make adaptations to facilitate water retention in all cellular compartments. In the cytosol, accumulating compatible solutes reduce the cytosolic water potential in a well characterized process. However, we do not know how the periplasm of gram negative bacteria is hydrated. Housing many critical functions, hydration of the periplasm is important. Osmoregulated periplasmic glucans accumulate under water-replete (hypo-osmotic) conditions to reduce the periplasmic water-potential relative to the solute rich cytosol, limiting swelling of the cytoplasmic compartment. Interestingly, compared to cells grown on solute stress or water-replete media, *Pseudomonas aeruginosa* biofilm cells accumulate more linear glucans under matric stress conditions. Furthermore, there are three populations of linear glucans with varying anionic properties, ranging from neutral to highly anionic. Under matric stress there is a shift towards a greater proportion of highly anionic linear glucans. A transient increase in the linear glucan biosynthesis operon expression suggests accumulation is regulated transcriptionally, though regulation of enzyme activity is also likely. While we were unable to purify cyclic glucans from *P. aeruginosa* biofilm cells, we show that cyclic glucan-deficient mutants exhibit lower matric stress tolerance than the wild type. Collectively, our data suggests that to facilitate periplasm hydration under desiccating conditions anionic linear glucans accumulate and cyclic glucans contribute to matric stress tolerance by a presently unknown mechanism.

Introduction

For many terrestrial bacteria, their environment can be quite dry. Even under non-drought conditions bacterial communities living in soil, the phyllosphere or on the skin of animals often experience conditions of limited water availability. The availability of water can be determined by measuring the total water potential of an environment, which is primarily comprised of two components; the solute potential and matric potential.

Dissolved solutes, such as NaCl, reduce the water potential by structuring water. As water moves to areas of lower potential, bacteria must regulate the water potential within the cell relative to their immediate surroundings (6). Porin channels in the outer membrane of gram negative bacteria allow solutes to enter periplasm, hydrating this compartment with water of reduced potential, while imposing a solute (osmotic) stress due to water potential differences across the inner (cytosolic) membrane. To hydrate the cytosol, compatible solutes including potassium ions, glutamate, glutamine, trehalose, proline and glycine betaine accumulate (8, 33, 39, 42), reducing the water potential to retain water. In water-replete (hypo-osmotic) conditions, the environmental water potential is greater than the cytosol, raising the possibility that over swelling of the cell will occur from an influx of water. In response, excess cytosolic solutes are secreted, increasing the water potential of the compartment (24). However, as the cytosol contains at least 300 mOsM of solutes required for normal metabolism (41) it is necessary for there to be an equilibrium between the cytosol and periplasm water activities to avoid over-swelling of the cytosol. Consequently, many gram negative bacteria accumulate oligosaccharides called glucans in the periplasm, which can have anionic substitutions to improve hydrophilic interactions and to increase the osmolarity of the compartment, reducing the water potential differential across the inner membrane (2, 16, 27).

As a habitat dries the matric potential becomes the predominant factor contributing to the total water potential due to the interaction of water with matrix (e.g. soil, leaves). Under these low-water-content conditions, bacteria are desiccated due to the physical removal of water and experience "osmotic" pressure across the outer membrane, periplasm, and cytosol: this is referred to as a matric stress (32). We can simulate low-water-content in the laboratory by amending media with non-outer membrane permeable polyethylene glycol MW8000 (PEG), creating a matric stress, since it tightly sorbs water and reduces the water potential like dry soil (11). We know that similar to solute stress, the cytosolic compartment is hydrated during a matric stress by the accumulation of compatible solutes (6, 30).

However, we do not know how the periplasm is hydrated under dry or low-water-content conditions. This compartment can comprise 20-40% of the cell volume and houses many important structural, metabolic, signaling and transport functions, necessitating proper hydration to function (33, 41). Accumulation of the small compatible solutes found in the cytosol would likely not effectively hydrate the periplasm as they could diffuse out of the periplasm into the environment through outer membrane porins. Intermediately sized anionic molecules, such periplasmic glucans, could fulfill this role. Unfortunately, no studies have directly investigated glucan abundance in response to low-water-content conditions. In a transcriptomic study, confirmed by qRT-PCR, Nielsen observed that expression of the periplasmic glucan biosynthesis gene *opgG* was elevated 3.9 fold in *Pseudomonas putida* after 15 minutes of growth under matric stress (29). Additionally, it was found that the extracellular matrix of *P. putida* contains a significant amount of low molecular weight, glucose-rich polysaccharide when grown under matric stress (3). This was observed even in a cellulose-deficient mutant. These findings suggest that these low molecular weight polysaccharides could be periplasmic glucans accumulating in

response to low-water-content conditions that may have been secreted or released from the periplasm during our extraction procedure.

Found within diverse gram negative species (2, 16, 26, 27), there are two types of periplasmic glucans: linear and cyclic. Studied primarily in enteric bacteria, linear glucans are oligosaccharides composed of 5-16 glucose residues with a β1→2 linked backbone and β1→6 linked branches (2, 19, 37). They are produced by the membrane-bound glycosyl transferases encoded by the *opgGH* operon (formerly *mdoGH*) from UDP-glucose (7). In *E .coli*, they can be anionically decorated with both succinyl and phosphogycerol groups by OpgC and OpgB, respectively (15, 17). Cyclic glucans have been studied extensively in Rhizobia. Depending on the species, cyclic glucans are 10-40 glucose residues comprised of β1→2, β1→3 or β1→6 linkages synthesized by the glycosyl transferase NdvB and can have phosphoglycerol and succinyl substitutions (2, 19, 27, 36, 43).

Interestingly, most Pseudomonads have the potential to produce both linear and cyclic glucans. As of January 2014, based on gene annotation we identified 43 *opgGH* and 36 *ndvB* orthologs in the 44 genomes available in the Pseudomonas Genome Database (pseudomonas.com; (Table S1)) (45). All genomes harbored at least one *opgGH* or *ndvB* ortholog. Separate BlastP searches confirmed the absence of *opgGH* or *ndvB* orthologs in genomes missing an ortholog. *Pseudomonas aeruginosa* linear and cyclic glucans have both been characterized when produced under water-replete conditions (19, 22, 36), making this genetically-tractable and commonly-used model organism an excellent subject for glucan research.

In the present study, we show that linear glucan abundance increases under low-water-

content conditions, expanding our understanding of what are commonly referred to as "osmoregulated" glucans. The increase of the linear glucan is partially due to transiently elevated expression of the glycosyl transferase genes during growth under low-water-content conditions, although it is likely that glucan production is also regulated at the enzyme activity level (35).We further demonstrate that linear glucans become more anionic under low-water-content conditions, presumably improving their hydrating ability.

Materials & Methods

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1. Cells were routinely maintained on tryptic soy agar and for examining effects of water stress they were grown on either 21C medium (0.5 g NH_4Cl, 1.725 g $Na_2HPO_4 \cdot 7H_2O$, 1.38 g KH_2PO_4 per L deionized water) with 20 mM glucose and Hutner's minimal salts, or CTYE medium (21C amended with 0.63 g tryptone and 0.32 g yeast extract per L) solidified with 10 g phytagel (Sigma-Aldrich) (11, 20, 40). The water potential of media was lowered with NaCl to simulate a solute (osmotic) stress or PEG 8000 to simulate a matric stress as described previously (11). The water potential of solid media was validated with a thermocouple psychrometer (Decagon Devices). Incubations were performed at 25° C.

Generation of mutants

Deletion of the *opgGH* operon was performed using a method developed by Chen, et al. (4). Briefly, a 5,962-bp amplicon comprised of 1,013-bp upstream and 793-bp downstream of the 4,156-bp *opgGH* operon was amplified using primers opgGH-UpF and opgGH-DnR with Phusion Taq polymerase (Thermo Scientific). The blunt-end amplicon was ligated using T4 DNA ligase (Promega) into a *Sma*I digested pTsacB and transformed into *E. coli* Top10. Using

Phusion Taq polymerase, kinase-treated outward facing primers opgGH-F2 and opgGH-R1 amplified a linearized plasmid that included the upstream and downstream regions with 5' phosphate ends. The plasmid was ligated with T4 DNA ligase, creating pTsacBΔ*opgGH*, which was electroporated (5) into both PA01 and Δ*ndvB*, selecting for Tc^R single recombinants. Double recombinant deletion mutants were obtained by screening for Tc^S colonies after 1 round of growth in the presence of 10% sucrose. Deletion of the *opgGH* operon was confirmed using PCR with primers opgGHseq and opgGHseqR targeting sites external to the deletion site (Figure S1).

Table 1. Strains, plasmids and primers used in this study

Strains	Description	Source
Pseudomonas aeruginosa		
PAO1	Wild-type	(13)
Δ*ndvB*	Δ*ndvB* deletion	(22)
Δ*opgGH*	Δ*opgGH* deletion	This study
Δ*ndvB* Δ*opgGH*	Δ*ndvB* Δ*opgGH* deletions	This study
Δ*algD*	Δ*algD* deletion	(44)
Δ*ndvB* Δ*algD*	Δ*ndvB* Δ*algD* deletions	(44)
Δ*opgGH* Δ*algD*	Δ*opgGH* Δ*algD* deletions	(44)
Δ*ndvB* Δ*opgGH* Δ*algD*	Δ*ndvB* Δ*opgGH* Δ*algD* deletions	(44)
E. coli		
Top10	F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *nup*G *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
Plasmids		
pTsacB	Suicide vector with *sacB* gene, Tc^R	(4)
pTsacBΔ*opgGH*	pTsacB with upstream and downstream region of *opgGH*	This study
pProbe-GT	Transcriptional reporter fusion plasmid, Gm^R	(28)
pP_{PA}*opgG-gfp*	Transcriptional fusion of PAO1 *opgG* promoter, Gm^R	This study
pP_{PA}*ndvB-gfp*	Transcriptional fusion of PAO1 *ndvB* promoter, Gm^R	This study
pP_{PA}*algD-gfp-G*	Transcriptional fusion of PAO1 *algD* promoter, Gm^R	(44)
Primers		
rimM-RT1F	5'-TCGAGGAAGGTGAGTACTACTGGCA-3'	This study
rimM-RT1R	5'-ACCGGTTTCCAGCAGATGGTCGAT-3'	This study
opgG-RT1F	5'-CACCAAGTTCGATTTCGGATCCCTG-3'	This study
opgG-RT1R	5'-TGTCGGCCTTGTTGATCGGATAGAG-3'	This study
ndvB-RT2F	5'-ATGATCATCGTGCCCAAGCGCGTC-3'	This study
ndvB-RT2R	5'-TACAGCACCGCCTTGGCAATGG-3'	This study
opgGH-UpF	5'-GAACTGCAGATCATCCGCGACGC-3'	This study
opgGH-DnR	5'-CCTTGCCGTTGACCACGTCCATC-3'	This study
opgGH-F2	5'-GGCATGCGCTCAAGGATGGCTTCC-3'	This study
opgGH-R1	5'-GTTCAGGGGTCTCCGACGGCACC-3'	This study
opgGHseq	5'-AGCGAATGTGGCGAAACCATGTTC-3'	This study
opgGHseqR	5'-AAGTGCTACCGGGTCACTCAACA-3'	This study
PopgG-F	5'-ATTAGGATCCCGAAGATGGCTGCACAAGCTGCTC-3'	This study
PopgG-R	5'-AATTGGTACCAAATCGGTGTTTGAAACGGAACGG-3'	This study
PndvB-F	5'-ATTAGGATCCGAACACGCAGGGCACGCTGTAG-3'	This study
PndvB-R	5'-ACTTGGTACCGGTTGAGCCCGATCTTGCGTGAAGACAT-3'	This study

Growth curves of mutants in CTYE water-replete or amended with NaCl (solute stress) or PEG (matric stress) to lower the water potential 1.5 MPa were conducted at 25° C. Growth was measured using either a BioTek EL340 (water-replete and solute stress) microplate reader measuring a relative OD $405/600_{nm}$ ratio or a BioTek Synergy H1 (matric stress) microplate reader measuring OD 600_{nm}. Doubling times were calculated from the slope of natural log-transformed OD's during exponential phase.

Glucan crude extraction

Linear and cyclic glucans were isolated using previously described methods with modifications due to growth on solid rather than liquid media (19, 22). Briefly, PAO1 and glucan mutant cells were grown overnight on unamended CTYE (water-replete) media and resuspended in 1mM phosphate buffer to an $OD_{600nm} = 0.1$. A 1.2 ml aliquot of the suspension was spread onto 0.45µm pore nylon membranes (Maine Manufacturing) overlaying CTYE media unamended or amended with either NaCl or PEG to lower the water potential by 1.5 MPa in 9 x 13 inch glass baking trays. After 48 h of growth, cells were scraped off the membranes and resuspended in 0.85% saline. An aliquot was removed for protein quantification (Bradford method) prior to centrifugation for 10 min at 5,000 x g. For isolation of linear glucans the cell pellet was resuspended in fresh 0.85% saline. Bovine serum albumin (BSA) and trichloroacetic acid were added to the sample to achieve final concentrations of 8.3 and 50 g L^{-1}, respectively. Samples were vortexed, incubated 5 min at room temperature, and centrifuged 10 min at 5,000 x g. The resulting supernatant was mixed with activated charcoal on a hematology mixer for 10 min at room temperature. Samples were centrifuged and supernatant was removed. The charcoal pellet was washed with water on a mixer, followed by centrifugation and repeated twice. Glucans

were eluted twice from the charcoal with 15% (vol/vol) pyridine. Extractions were pooled and concentrated using a speedvac concentrator (Savant).

Cyclic glucans were extracted from pelleted cells by adding 75% ethanol and incubating at 70° C for 20 minutes prior to centrifugation for 5 minutes at 5000 x g. The pellet was discarded and the supernatant containing the released cyclic glucans were precipitated with the addition of a 10X volume of 100% ethanol and 100mM NaCl prior to incubating overnight at -20° C. Precipitated glucans were pelleted by centrifugation at 16,000 x g for 20 min and resuspended in water. Alternatively, cyclic glucans were isolated using a previously described procedure (22). Briefly, *P. aeruginosa* was grown 24 h in M63 liquid media and pelleted by centrifugation for 10 min at 5,000 x g. Ethanol extractions and precipitations were carried out as described above.

Size exclusion chromatography

Linear glucans were isolated from crude cell extracts by size-exclusion chromatography using a 75 x 1.5 cm column containing G-25 Sephadex (Sigma-Aldrich) with a gravity flow of 0.15 M ammonium acetate buffer in 7% 1-propanol (pH=7.4) at 12 ml h^{-1} at 4° C. Fractions (2 ml) were assayed for total carbohydrate by the phenol/sulfuric acid method (9) with glucose as a standard. Cyclic glucans were isolated from crude cell extracts using the method described above but also by using a 75 x 1.5 column containing G-75 Sephadex (Sigma-Aldrich) with gravity flow of 0.1 M pyridium acetate buffer (pH=5.0) at 10 ml h^{-1} at 4° C. Fractions (2 ml) were assayed for total carbohydrate by the anthrone/sulfuric acid method with glucose as the standard (34).

Anionic exchange chromatography

Following size exclusion chromatography, carbohydrate-positive fractions were pooled, concentrated using a speedvac, and desalted on a G-25 Sephadex column (30 x 1.5 cm) with 7%

1-propanol as the eluent by gravity flow (15 ml/h) at 4° C. Carbohydrate-containing fractions were pooled and concentrated by speedvac. Anionic exchange chromatography was then carried out on a DEAE-Sephacel (Sigma-Aldrich) column (30 x 1.5 cm) with gravity flow of 10 mM Tris-HCl in 7% 1-propanol and a 20 mL step NaCl gradient was applied, beginning with 0 and ending with 0.3 M NaCl. Fractions (2 mL) were collected and assayed for total carbohydrate using the phenol/sulfuric acid method.

Quantitative real-time PCR

P. aeruginosa strain PAO1 was grown overnight on CTYE solid media amended with NaCl. Cells were resuspended in CTYE broth with NaCl for preparation of inoculum. For 0-3 h samples, cells were resuspended to OD_{600} = 0.8, centrifuged 5 minutes at 5,000 x g and resuspended in $1/20^{th}$ the original volume. In preparation for 6 and 9 h samples, cells were resuspended to OD_{600} = 0.4, centrifuged and resuspended in $8/25^{th}$ the original volume. The 9 h inoculum was diluted 1:100 to make the 24 h inoculum. One hundred µl of each inoculum was spread onto CTYE plates either unamended, or amended with NaCl or PEG. At designated time points, biofilm cells were resuspended from plates in a 2:1 solution of RNA protect (Qiagen) and CTYE broth. RNA was isolated from cells using an RNA miniprep kit (Qiagen) following manufacturer's protocol including enzymatic lysis with proteinase K and on-column DNAse treatment. Samples were further treated with a second DNAse treatment (Ambion). An Aligent 2100 Bioanalyzer and RNA 6000 Nano kit (Aligent) was used to check RNA integrity. Expression of *opgG* and *ndvB* were measured relative to the reference gene *rimM* in PAO1 using primers rim-RT1F/ rimMRT1R (*rimM*), opgG-RT1F/opgG-RT1R (*opgG*) and ndvB-RT2F/ndvB-RT2R (*ndvB*) with conditions listed in Table S2. qRT-PCR was performed using

qScript 1-step qRT-PCR kit (Quanta) according to the manufacturer's protocol. Gene expression was calculated using the Livak ($2^{-\Delta\Delta C(t)}$) method (21).

Measurement of glucan biosynthesis promoter activity

To better measure the overall intensity of linear and cyclic glucan biosynthesis gene expression relative to each other, a transcriptional promoter/reporter fusion system was developed. Plasmid pP_{PA}*opgG-gfp* was constructed by inserting a 799 bp PCR amplicon (primers: PopgG-F and PopgG-R) into pProbe-GT. Similarly, we inserted an 836 bp PCR amplicon (primers: PndvB-F and PndvB-R) into pProbe-GT, creating pP_{PA}*ndvB-gfp*. PAO1 cells transformed with Plate cultures were grown 24 h on CTYE unamended or amended with NaCl or PEG to reduce the water potential 1.5 MPa to simulate solute or matric stress. Cells were resuspended in water to OD_{600nm} = 0.05-0.08 and fluorescence intensity was measured with a Fluoromax 2 spectrometer (Jobin-Yvon-Spex Instruments). Excitation and emission wavelengths, bandpass, and integration time were 488 nm, 510 nm, 5 nm, and 0.1s, respectively. A relative fluorescence unit is defined as the fluorescence intensity relative to the optical density of the cell suspension at the time of sampling.

Cell survival assay

To test the effect of glucan deficiency on survival, PAO1 and glucan mutants were grown overnight in 21C media amended with NaCl to lower the water-potential 1.5 MPa. Cells were diluted to an OD_{600} = 0.025 in either water, or water amended with either NaCl or PEG to lower the water potential 1.5 MPa. Cultures were incubated with shaking (200 rpm) at 25 °C. At various times, an aliquot was removed for dilution plating onto TSA to determine survival.

Results

Growth of glucan-deficient mutants

Once glucan mutants were obtained, we monitored growth in water-replete, solute stress and matric stress conditions to see if glucan deficiency impacted fitness. Growth curve plots show similar growth patterns among strains under water-replete and solute stress (Figures 1 A and C), though doubling time calculations show a slight, but significant, increase for Δ*ndvB* (Figures 1 B and D) suggesting somewhat impaired growth. Growth curve results from matric stress conditions show similar growth patterns among strains, though Δ*ndvB* eventually reaches a higher OD than the other strains (Figure 1 E). However, the roughly 4-5 fold increase in doubling time for all strains when cells are grown under matric stress highlights the stressful nature of such conditions on cell physiology. Furthermore, both Δ*opgGH* and Δ*ndvB* Δ*opgGH* strains have significantly increased doubling times under matric stress, implying linear glucans are important for fitness under matric stress (Figure 1 F).

Accumulation of linear glucans under matric stress

In order to directly test our hypothesis that glucans accumulate to facilitate periplasm hydration under water-limiting conditions we obtained crude carbohydrate extracts from PAO1 biofilm cells. These extracts were used to isolate linear and cyclic glucans for assessing whether more glucans accumulate under matric stress than a thermodynamically equivalent solute stress or water-replete conditions. Figure 2 shows the size exclusion chromatography elution profile of the periplasmic carbohydrate extracts of wild-type PAO1 cultivated under water-replete, and solute or matric stress conditions, on a Sephadex G-25 column. The linear glucan peak was identified by the absence of the highlighted peak (arrow in Fig. 1) in a PAO1 Δ*opgGH* mutant

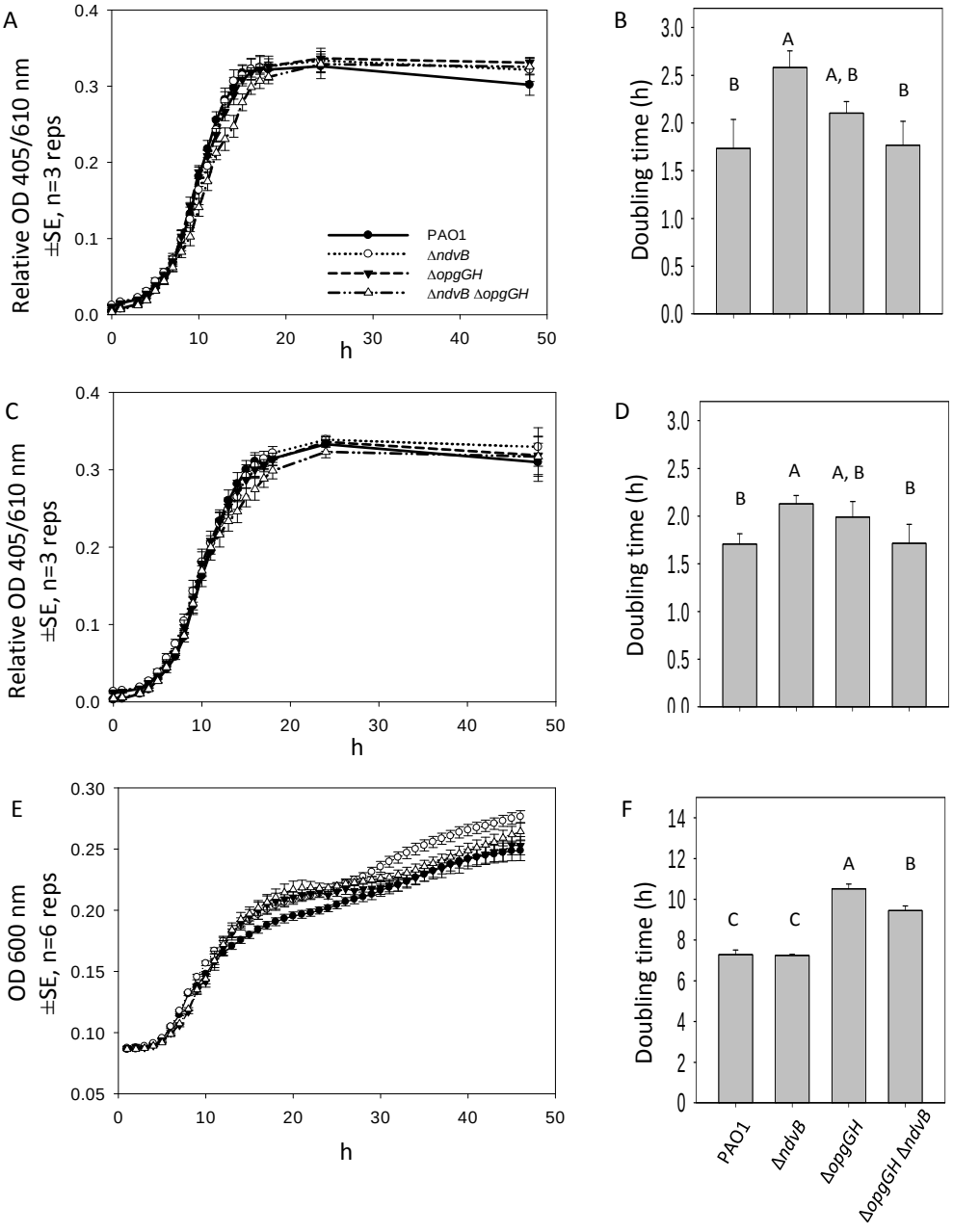

Figure 1. Growth analysis of PA01 and glucan mutant derivatives in CTYE. A) Water-replete growth curve, B) Water-replete doubling times, C) Solute stress growth curve, D) Solute stress doubling time, E) Matric stress growth curve, F) Matric stress doubling time. Doubling time values with the same letters are not statistically different based on an LSD test ($p \leq 0.05$).

cultivated under matric stress conditions. On average, the lowest concentration of linear glucans were for biofilm cells under solute stress (2.5 ± 1.2 μg glucose-equivalents/mg protein) conditions which was roughly 50% of the yield for cells cultivated under water-replete conditions (5.4 ± 1.8 μg glucose-equivalents/mg protein). Strikingly, linear glucans were 8 fold

more abundant under matric stress conditions (19.8 ± 5.4 μg glucose-equivalents/mg protein) than a thermodynamically equivalent solute stress. This result clearly suggests that low-water-content conditions stimulate glucan accumulation.

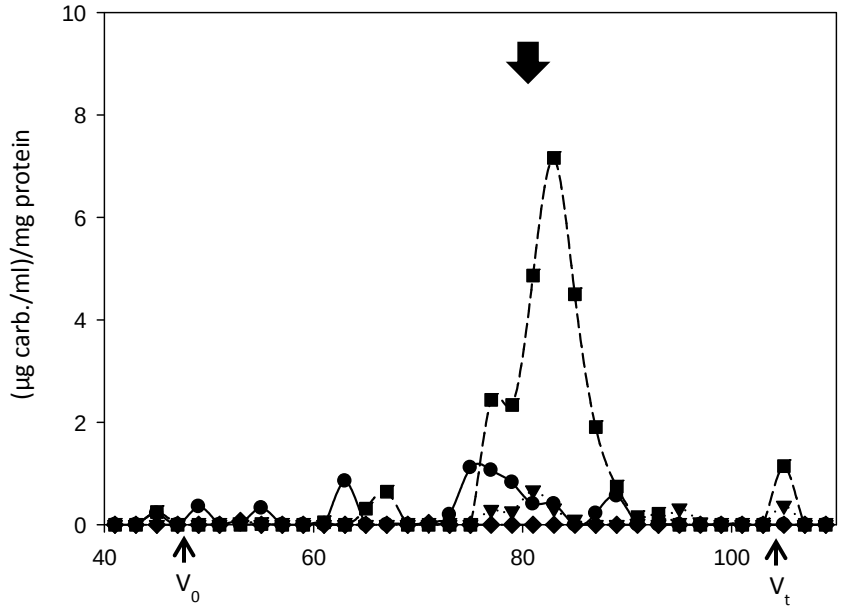

Figure 2. Size exclusion chromatography (Sephadex G-25) of periplasmic carbohydrate extracts from wild-type PAO1 biofilm cells cultivated under water-replete (circles), solute-stress (triangles), and matric-stress (squares) conditions and Δ*opgGH* biofilm cells cultivated under water-replete conditions (diamonds). V_0 denotes void volume, V_t denotes total volume. The downward arrow indicates the linear glucan peak. Data is representative of a single experiment.

Unfortunately, we were unsuccessful in isolating cyclic glucans from biofilm cells using a variety of procedures that have been reported to be successful, including eluting with ammonium acetate buffer (25). For example, we observed a peak in extracts from Δ*ndvB* but not Δ*opgGH* biofilm cells cultivated under water-replete conditions (Figure 3A). However, we successfully isolated cyclic glucans from cell extracts of PAO1 and PA14 but not Δ*ndvB* cultures grown in M63 liquid media (water-replete) that were eluted on Sephadex G-75 with pyridium acetate buffer (Figure 3B), indicating that we had the ability to isolate cyclic glucans. Apparently, the pyridium acetate buffer was a significant factor - which was as ammonium

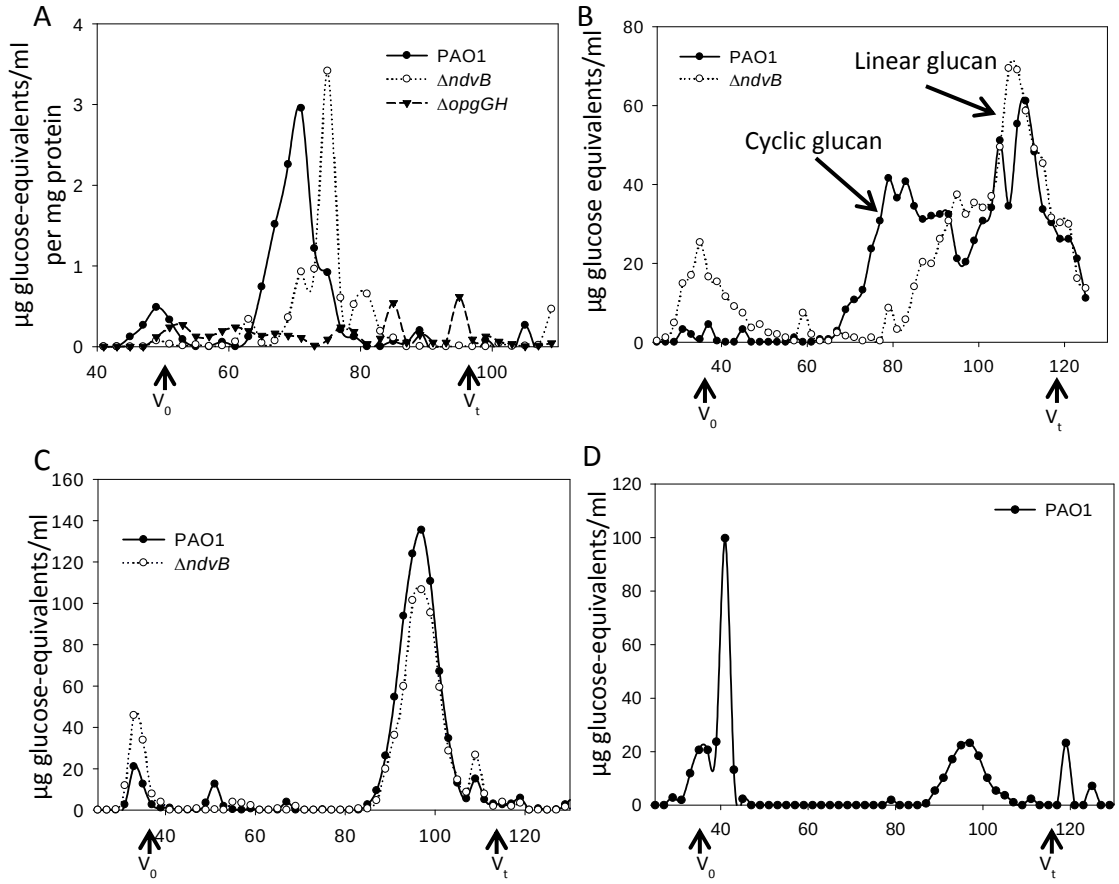

Figure 3. Representative size exclusion chromatography elution profiles of carbohydrate cell-extracts using various cyclic glucan isolation protocols: A) carbohydrate extracts from biofilm cells on Sephadex G-25 columns with ammonium acetate buffer; B) carbohydrate extracts from M63 liquid cultures on Sephadex G-75 columns with pyridium acetate buffer, arrows denote linear and cyclic glucans; C) carbohydrate extracts from biofilm cells on Sephadex G-75 columns with pyridium acetate buffer; D) extracellular carbohydrates isolated from biofilm cells on Sephadex G-75 columns with pyridium acetate buffer. V_0 denotes void volume, V_t denotes total volume.

acetate buffer has been used to isolate cyclic glucans from other species (25). Despite our success with PAO1 liquid cultures we were unsuccessful in identifying a cyclic glucan peak from carbohydrate extracts of PAO1 biofilm cells that were eluted on Sephadex G-75 with a pyridium acetate buffer. This is surprising as ndvB has stronger expression during biofilm rather than planktonic growth (1, 22).We did obtain a peak that is likely the linear glucan in extracts from Δ*ndvB* biofilm cells since it eluted in the same fraction as the presumptive linear glucan peak from PAO1 (compare Figure 3 C with 3 B). Since in some Rhizobia (10) cyclic glucans are

secreted we explored the possibility that the cyclic glucan was retained in the supernatant after biofilm cells were harvested by resuspending them in a saline solution for isolation of cells by centrifugation. We concentrated the supernatant and it was eluted on a Sephadex G-75 column with pyridium acetate buffer. While a peak corresponding to the linear glucan was found, no cyclic glucan peaks were observed (Figure 3 D).

Matric stress alters the anionic properties of linear glucans

We hypothesized that increasing anionic substitutions of the glucans would better facilitate periplasm hydration under water-limiting conditions. The linear glucan fraction was pooled following size exclusion chromatography, concentrated and then subjected to anion exchange chromatography (Figure 4). Under water-replete conditions linear glucans are comprised of three distinct populations (Figure 4, Table 2); a small neutral or nominally anionic, a moderately anionic, and a small highly anionic population that elute with increasing NaCl

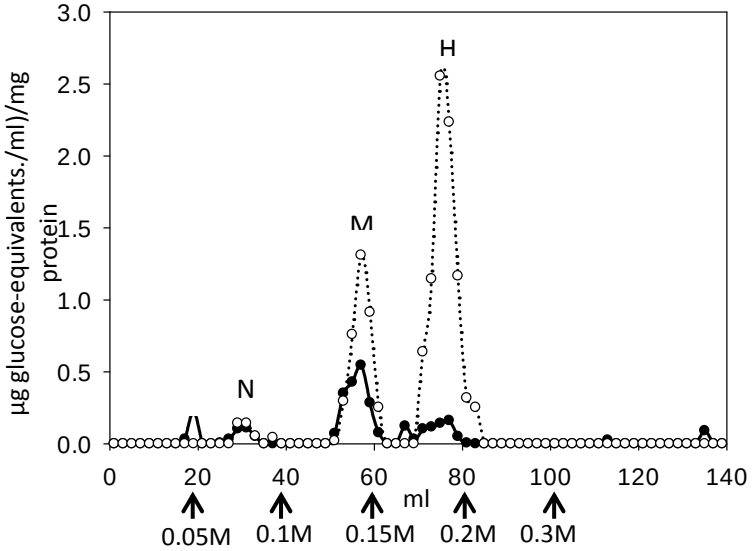

Figure 4. Anionic exchange chromatography elution profile of the linear glucan fraction. Arrows indicate step gradient concentration of NaCl. Closed circles: water-replete; open circles: matric stress. N: neutral, M: moderately anionic, H: highly anionic.

concentration. Notably under matric stress, there is a shift towards a greater proportion of highly anionic linear glucans (Figure 4, Table 2).

Table 2. Effect of water stress on anionic properties of linear glucans

	% of Total linear glucans	
	Water-replete conditions	**-1.5 MPa ψ Matric stress conditions**
Neutral	11	3
Moderately anionic	63	29
Highly anionic	26	68

γ-cyclodextrin (MW 1297.12) eluted in the same fractions as "Neutral" glucans indicating this population is neutral to slightly anionic.

Linear and cyclic glucan biosynthesis gene expression

To explore the mechanisms contributing to linear and possibly cyclic glucan accumulation under matric stress, we assessed the effect of osmotic and matric stress on cyclic (*ndv*B) and linear (*opg*G) glucan glycosyl transferase gene expression using qRT-PCR. Matric stress highly and transiently induced *opgG* expression with peak transcription occurring within 15 min after exposure to a matric stress. After 1 h, it was decreased substantially and reached water-replete levels by 6 h (Figure 5). In contrast, *opgG* expression was unaffected by a -1.5 MPa ψ solute stress (data not shown). Expression of *ndvB* was unaffected by matric (Figure 5) and solute stress (data not shown). Interestingly, *opgG* expression was rather high under all conditions with c(t) values consistently similar to the constitutively expressed *rimM* reference gene (data not shown). In Chapter 4 we show with using promoter-*gfp* transcriptional fusions at 24 h of growth in the presence or absence of water stress that *opgG* promoter activity was significantly greater than the *ndvB* promoter as well as the *algD* promoter. Under matric stress *opg*G promoter activity is highly induced (44).

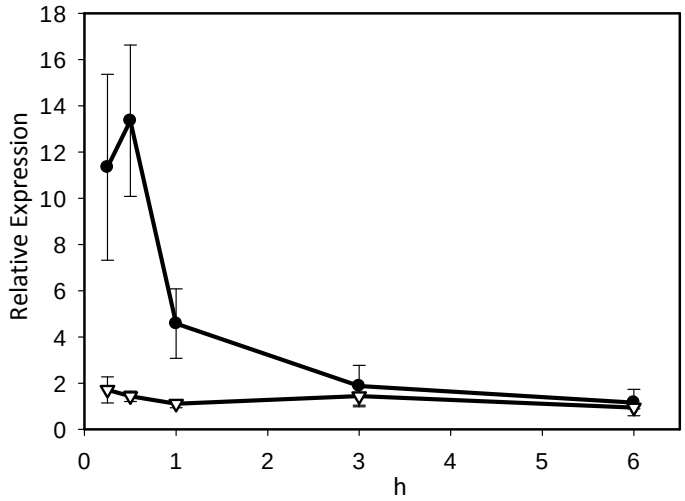

Figure 5. Effect of -1.5 MPa Ψ matric stress on ● *opgG* and ∇ *ndvB* expression. Values expressed relative to water replete condition using the method of Livak et al. (21). Values are mean ± SE, n = 2-4.

Cyclic glucan deficiency reduces matric stress tolerance

We assessed the effect of cyclic and linear glucan deficiency on matric stress tolerance. Bacterial cultures were cultivated under -1.5 MPa ψ solute stress prior to their exposure to water-replete or matric stress conditions. We reasoned that growth under high osmolarity would decrease the cellular pool sizes of glucans whose presence could facilitate tolerance to a hypo-osmotic or matric stress. Cell culturability of the wild type and glucan mutants did not decrease over time in the water-replete and solute stress treatments, rather PAO1 continued with cellular division under water replete conditions (Figure S3 A) and the cyclic glucan mutant had higher culturability at day 2 in the -1.5 MPa ψ NaCl treatment (Figure S3 B). However, in the -1.5 MPa ψ PEG treatments there was a rapid decrease in culturability over time with survival of the *ΔndvB* mutant the poorest (Figure 6). Notably, culturability of the *ΔndvB* mutant was significantly reduced compared to the wild-type PAO1 while *ΔopgGH* survival was similar to PAO1 throughout the experiment. Interestingly, survival of the *ΔndvB ΔopgGH* mutant was intermediate to *ΔndvB* and *ΔopgGH* mutants (Figure 6), suggesting that linear glucan deficiency

facilitated survival under these conditions. One possibility is that linear glucan deficiency activates a matric stress tolerance mechanism. In the following chapter, we demonstrate that both cyclic and especially linear glucan-deficient mutants exhibit a hyper-active envelope stress response, which can lead to global changes in gene expression that have been implicated in stress tolerance (46). Importantly, the envelope stress response has been shown to induce expression of the exopolysaccharide alginate, a known contributor to matric stress tolerance (3, 23, 38), and in Chapter 4 we show that linear glucan deficiency leads to alginate overproduction. However, deletion of the alginate biosynthesis glycosyl transferase gene *algD* in the wild type and glucan mutants did not affect their survival (Figure S4), suggesting that linear glucan deficiency is likely influencing some other matric stress tolerance mechanism.

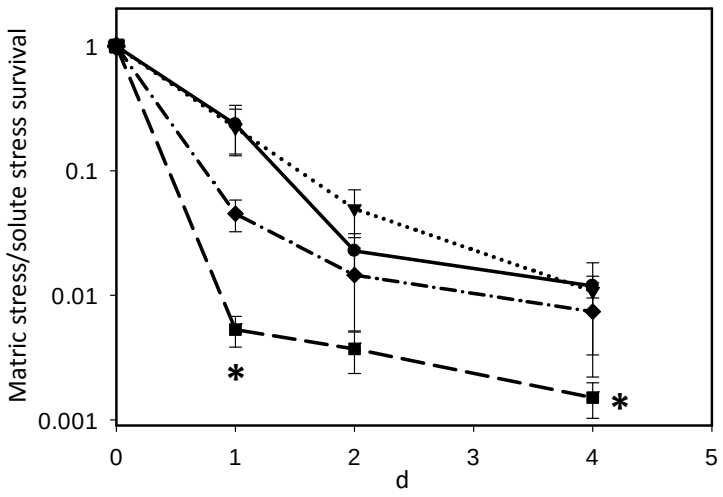

Figure 6. Survival in matric stress relative to solute stress for strains PA01 (solid line), *ΔndvB* (dashed line), *ΔopgGH* (dotted line), and *ΔndvB ΔopgGH* (dash/dot line). * denotes significantly different from PAO1 using an LSD test ($p \leq 0.05$). Values are mean ± SE, n=6.

Discussion

In the present study we show that *P. aeruginosa* biofilm cells accumulate linear periplasmic glucans in response to water-limitation (Figure 1) compared to water-replete or

hypo-osmotic conditions. These results strongly support our hypothesis that linear periplasmic glucans contribute to hydrating the periplasm under low-water-content conditions. This is a novel finding, substantially changing our understanding of the regulation and role of what are traditionally referred to as "osmoregulated" periplasmic glucans (2), adding a new aspect to their function and contributing to our understanding of desiccation tolerance. The slower growth rate observed in linear glucan-deficient cells (Figure 1 F) highlights the importance of linear glucan accumulation in the periplasm during low-water-content conditions. This is possibly a consequence of decreased fitness of glucan-deficient cells and/or greater resource allocation to production of the exopolysaccharide alginate, which is shown in Chapter 4.

The possible role of cyclic glucans in contributing to periplasm hydration is less clear. Although we were able to identify the cyclic glucan in carbohydrate extracts of liquid cultures of PAO1 (Figure 3 B) following size exclusion chromatography using an established protocol (22) we were unable to isolate cyclic glucans from PAO1 biofilm cells using the same procedure (Figure 3A and C). However, our data (Figures 6 and S3 C) suggests that cyclic glucans have an important role in matric stress tolerance. This is consistent with data that will be shown in Chapter 4: cyclic glucan deficiency dramatically stimulates the envelope stress response under matric stress conditions. There are several potential reasons why we were unable to detect cyclic glucans in biofilm cells. Although it's possible that during harvesting of biofilm cells, the cyclic glucan was released from the cell, size exclusion chromatography of the concentrated saline supernatant did not contain a cyclic glucan peak, though there appears to be some linear glucan (Figure 3 D). It's possible that biofilm cells require the accumulation of small amounts of cyclic glucans in the periplasm and the amounts were below our detection limits. Alternatively, the cyclic glucan size or chemical properties change during the biofilm lifestyle, precluding our

ability to isolate it from the periplasm with our extraction procedures or it co-elutes in the void volume in our size exclusion chromatography. Lastly, it could be sequestered due to an interaction with a protein or other molecule thereby preventing their extraction using standard procedures. Since we can purify cyclic glucans from liquid-grown cultures, it's conceivable that cyclic glucan-specific antibodies could be used to detect cyclic glucans in situ or in various cellular fractions independent of size exclusion chromatography. Unfortunately, developing antibodies to specific carbohydrates can prove difficult, but may be the best option for quantifying cyclic glucans during unsaturated biofilm growth.

At present we don't know if matric stress stimulation of linear glucan accumulation is due to the transient increase in linear glucan biosynthesis *opgGH* operon expression (Figure 5). Matric stress induction of *opgG* expression supports our hypothesis that glucans accumulate in response to low-water-content. The rapid induction of *opgG* expression following exposure to a matric stress suggests that linear glucans may contribute to early adaptation responses to cellular desiccation. Other studies that explored the effect of chemical disruptions of the cell wall also trigger an envelope stress response, as revealed by a rapid increase in *opgH* expression within 15 min to 2 h (31, 46). Contrasting with our observations, previous studies exploring *opgH* expression in *Pseudomonas syringae* observed an increase in expression after transfer to hypo-osmotic conditions (31). Additionally, *opgG* expression in *E. coli* is inhibited by solute stress (18), whereas we still observed high *opgG* expression under both hypo-osmotic water-replete and solute stress conditions. It's possible that *P. aeruginosa* regulatory mechanisms are different from *E. coli* and even *P. syringae*. We observed changes in glucan abundance between water-replete, solute-stress and matric-stress that were likely regulated beyond gene transcription, including a decrease in linear glucans isolated after growth on solute stress, while we did not

record any changes in gene expression. Regulation of glucan production at the enzyme activity level has been reported for cyclic and linear glucan synthesis in *Sinorhizobium meliloti* and *E. coli*, respectively (14, 35). Future work exploring regulation of glucan production by *P. aeruginosa* at the level of enzyme activity under varying water availability conditions would help us better understand glucan accumulation mechanisms.

We propose that increasing anionic charges under matric stress conditions make the glucans more hydrophilic, thereby improving their water retention abilities. Previous research suggests linear glucans from *P. aeruginosa* grown under water-replete conditions can be decorated with succinyl groups while cyclic glucans are decorated with phosphoglycerol (19, 36). It is possible that under water-limited conditions the degree of substitution increases or mixing of both substitutions occur to increase anionic properties. Future experiments with the different anionically charged populations could utilize GC-MS to determine the identity of the substitutions. Additionally, confirming that linear (and cyclic) glucans have water-retention capabilities that are improved with anionic substitutions would directly support our hypothesis of glucans hydrating the periplasm under desiccating conditions.

Despite our inability to isolate cyclic glucans from biofilm cells, cyclic glucans clearly contribute to matric stress tolerance (Figure 6 S3 C). However it is less clear how loss of the linear glucan affects survival. That deficiency of both glucans improved survival relative to cells deficient in just the cyclic glucan suggests one of two scenarios. Either linear glucan deficiency improves survival by virtue of its absence because cellular resources devoted to linear glucan synthesis are diverted to stress tolerance or it induces another stress tolerance mechanism that masks the effect of glucan deficiency on survival. In the next chapter, we show that linear glucan mutants exhibit elevated envelope stress response under water-limited conditions, evidence that

loss of the linear glucan is not in of itself beneficial as well as suggesting a potential mechanism for activating other stress tolerance mechanisms (46). We hypothesized that alginate over production, a well understood consequence of the envelope stress response (12, 46), may play a role in improving survival. However, loss of alginate production did not affect survival under matric stress (Figure S4). It is possible that one of the other targets of the envelope stress response or some tolerance mechanism independent of the envelope stress response is responsible for the improved survival of double glucan-deficient cells.

Taken together our findings support our model that linear glucans accumulate under water-limited conditions to maintain hydration of the periplasm. While this is a new understanding of glucan function, it is perhaps not surprising. Already periplasmic glucans were known to accumulate in the periplasm under hypo-osmotic conditions in order to balance the water potential differential across the inner membrane (2, 16). There is no reason to think that under conditions where the water potential differential lies across the outer membrane - as is the case with low environmental water availability - that accumulating glucans would not act to balance the water status, as well. The question remains as to how the cell differentiates hypo-osmotic from desiccating conditions to modulate glucan accumulation to optimize periplasm function.

Supplemental

Table S1. Pseudomonads lacking *opgGH* or *ndvB* orthologs

Species	Strain	Missing glycosyl transferase
P. resinovorans	NBRC 105553	*opgGH*
P. aeruginosa	PA7	*ndvB*
P. brassicacearum	subsp. *Brassicacearum* NFM421	*ndvB*
P. fluorescens	F113	*ndvB*
P. mendocina	NK-01	*ndvB*
P. mendocina	Ymp	*ndvB*
P. stutzeri	DSM 10701	*ndvB*
P. stutzeri	RCH2	*ndvB*
P. syringae	pv. *phaseolicola* 1448A	*ndvB*

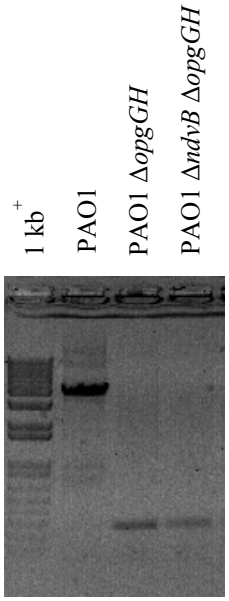

Figure S1. Confirmation of *opgGH* gene deletions in *P. aeruginosa*. We observed a dramatic decrease in amplicon size from 4,108-bp in the wild-type PAO1 to 265-bp in the *ΔopgGH* and *ΔndvBΔopgGH* mutants.

Table S2. Thermocycler program for qRT-PCR.

Step	Temperature	Time	Note
1	50° C	10:00	
2	95° C	5:00	
3	95° C	0:15	
4	60° C	0:30	
5	-	-	Read, go to step 3 x 39 times
6	95° C	1:00	
7	55° C	1:00	
8	55-95° C	-	Melt curve: read every 0.5 C, hold 0:10

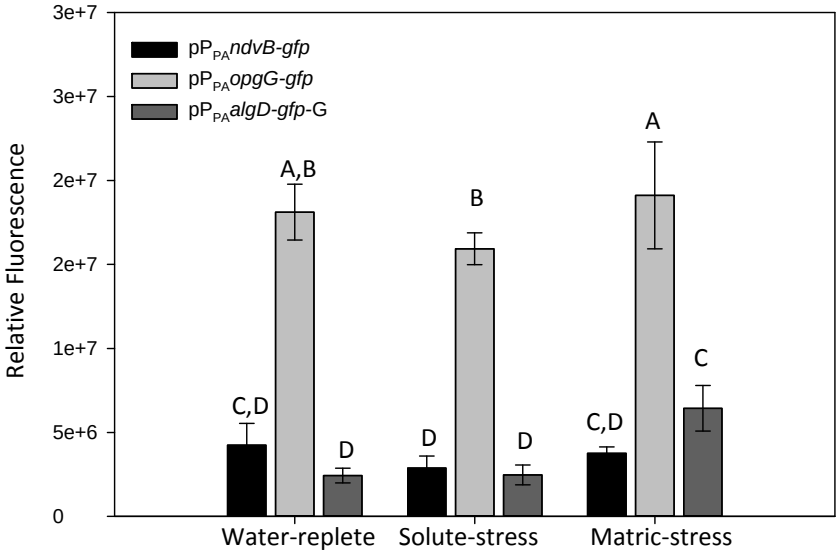

Figure S2. Promoter activity of glucan biosynthesis genes after in cells grown 24 h on CTYE unamended or amended with NaCl (solute-stress) or PEG (matric-stress) to reduce the water-potential 1.5 MPa. Promoter activity of matric-stress-responsive alginate biosynthesis operon used to compare expression levels. Values are mean relative fluorescence ± SE, n =4. Values not linked with the same letter are statistically different using LSD ($\alpha \leq 0.05$).

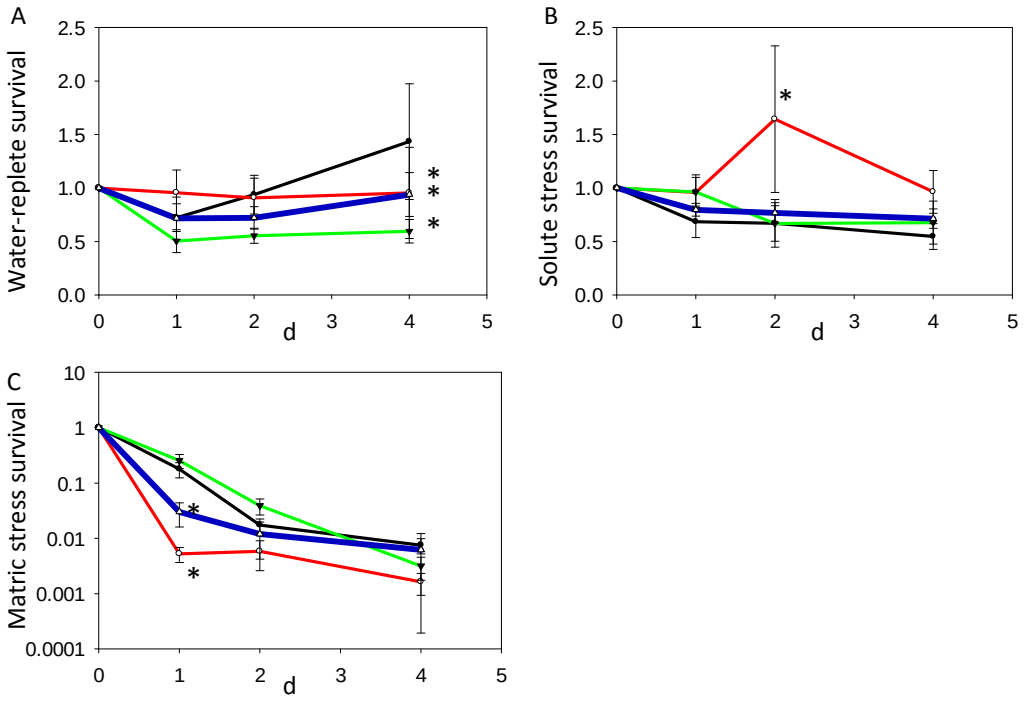

Figure S3. Survival in A) water replete, B) solute stress and C) matric stress relative to initial inoculum for strains PA01 (black line), *ΔndvB* (red line), *ΔopgGH* (green line), and *ΔndvB ΔopgGH* (blue line). * denotes significantly different from PAO1 using an LSD test ($p \leq 0.05$). Values are mean ± standard error of mean, n=6.

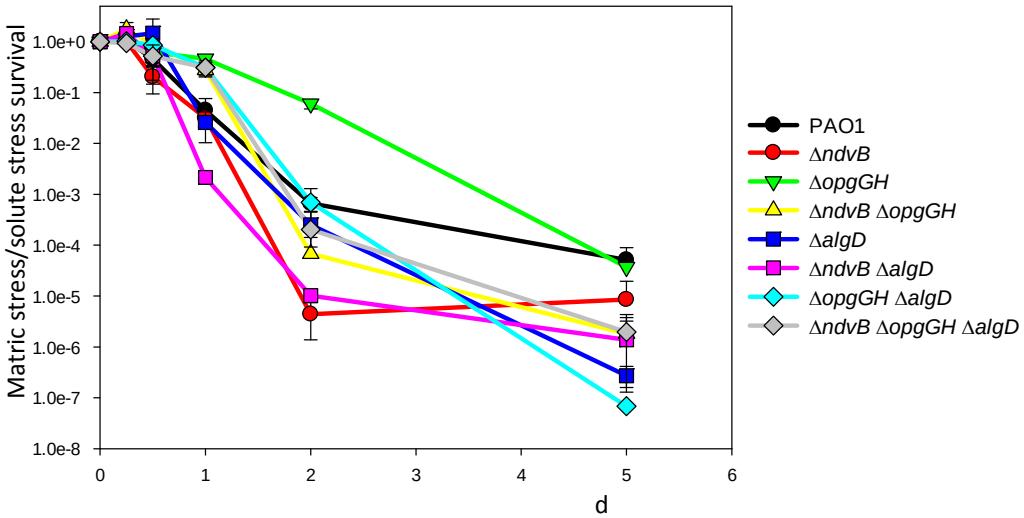

Figure S4. Survival in matric stress relative to solute stress conditions for strains with and without functional alginate biosynthesis. Values are mean of 3 replicates ± SE.

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CHAPTER 4. *PSEUDOMONAS AERUGINIOSA* CYCLIC AND LINEAR PERIPLASMIC GLUCANS ATTENUATE MATRIC STRESS-MEDIATED ENVELOPE STRESS

Abstract

Periplasmic glucans accumulate in *Pseudomonas aeruginosa* during matric stress, suggesting glucans play a role hydrating the periplasm under low-water-content conditions. In this study, we show that accumulation of linear and cyclic glucans attenuate matric stress-mediated envelope stress. Both linear (Δ*opgGH*) and cyclic (Δ*ndvB*) glucan mutants exhibit a hyper-mucoid phenotype due to over production of the exopolysaccharide alginate under matric stress conditions, which is indicative of an envelope stress response. Using an alginate (*algD*) promoter-*gfp* transcriptional fusion, we measured envelope stress experienced by glucan-deficient mutants under water stress. Our data suggest that each glucan has a distinct role in attenuating matric stress-mediated envelope stress. Linear glucan deficiency inherently activates an envelope stress response but the extent of the response peaks at moderate matric water potentials. Envelope stress in linear glucan mutants was relieved by low to high osmolarity. In contrast, the extent of the envelope stress response in cyclic glucan-deficient mutants increased with increasing matric stress severity. Interestingly, overexpression of the linear glucan biosynthesis operon rescues the cyclic glucan deficiency-mediated alginate over-production, though not vice versa, suggesting some functional redundancy between the glucans. However, other pleotropic differences between Δ*ndvB* and Δ*opgGH* strains including increased motility and changes in fatty acid profiles, suggest linear and cyclic glucans are not completely functionally redundant. Collectively our data suggests that both linear and cyclic glucans may contribute to

periplasm hydration under low-water content conditions and their absence triggers σ^{22} (AlgU/T) release and activation of the envelope stress response manifested by alginate overproduction.

Introduction

Bacteria colonizing terrestrial environments routinely or periodically experience conditions of reduced water availability. The thermodynamic availability of water to a cell can be assessed by measuring water activity or the mathematically related water potential of the environment. Water has a propensity to move to areas of lower water potential, for example out of a cell if the external water potential is lower than inside the cell. In unsaturated environments, water potential is influenced by the osmotic and matric components. Increasing dissolved solute concentrations impose an osmotic stress by reducing the potential of the water bathing the cells. Interactions between limited water content and the surrounding environmental matrix by inter-molecular forces also reduce external water potential, imposing a matric stress on a cell. For bacteria, osmotic and matric stresses are physiologically very different especially when considering the periplasmic space between the inner and outer membrane of gram negative bacteria. Under high osmolarity conditions, solutes enter the periplasm through outer membrane porins, hydrating that compartment, but reducing the periplasmic water potential, creating a differential across the inner membrane (13). The accumulation of charged compatible solutes reduces the cytosolic water potential, balancing with the solutes in the external environment and periplasm (13, 50). However, under low-water-content imposed matric stress, bacteria become dehydrated by the removal of water from the entire cell, including the cytosplasm *and* periplasm. Non-outer-membrane-permeating high MW polyethylene glycols (PEG) can simulate (in both liquid and solid media) low-water-content conditions on account of their exertion of a matric stress across the outer rather than inner membrane (25).

Hydration of the periplasm is important as the compartment comprises 20-40 % of the cell volume, housing many important sensing, structural and metabolic functions (54, 62). Yet little is known as to how the periplasm is hydrated under low-water-content matric stress conditions. The accumulation of compatible solutes found in the cytosol would likely be ineffective in the periplasm as they could diffuse out of the periplasm through outer membrane porins. However, the accumulation of intermediately sized anionic molecules could help hydrate the periplasm. In the previous chapter, we showed that under matric stress linear periplasmic glucans accumulated in the periplasm of *Pseudomonas aeruginosa* (67). These intermediate-sized oligosaccharides are more anionic under low-water-content conditions than under water-replete or high osmolarity conditions. Together, this suggests that glucan (linear and possibly cyclic) accumulation likely contributes to hydrating the periplasm under low-water-content conditions. This complements their role of accumulating under hypo-osmotic conditions to increase the fixed osmolyte concentration in the periplasm, balancing the water potential across the inner membrane with the cytosolic solutes and preventing over-swelling of the cytosolic compartment (33).

Periplasmic glucans influence many aspects of bacterial biology, with glucan-deficient mutants exhibiting pleotropic phenotypes. Both plant and animal pathogens show reduced virulence when unable to produce periplasmic glucans (7, 39). Reduced virulence in the plant pathogen *Dickeya dadantii* is due to lower pectinase, cellulase and protease activities (5, 49). In *D. dadantii*, this is likely due to inhibition of de-repression by the PecS transcriptional regulator in glucan-deficient cells (4). Additionally, altered motility is a common phenotype associated with glucan deficiency, as it is thought that under hypo-osmotic conditions, envelope stress destabilizes flagellar proteins (16). Similar to reduced virulence, Rhizobia deficient in glucan

production do not develop fully functional root nodules (17). Linear glucan-deficient *P. aeruginosa* mutants form altered biofilms in hypo-osmotic media, failing to form the same large mushroom-like structures as the wild-type (35). In linear glucan-deficient *Pseudomonas syringae* mutants, hypo-osmotic conditions triggers increased production of the exopolysaccharide alginate, indicative of elevated envelope stress (51). Similar results suggesting envelope stress were observed in *E. coli* glucan mutants, leading to increased production of the capsular polysaccharide colonic acid (18).

P. aeruginosa, like most Pseudomonads, synthesizes two periplasmic glucans (35, 38, 67). The linear glucan is formed by the glycosyl transferases OpgGH (MdoGH) and has been studied primarily in the Enterobacteriaceae (2, 65). The glycosyl transferase NdvB produces a cyclic glucan which has been characterized mainly in Rhizobia (2, 45). To our knowledge, no studies have concurrently investigated the functional role of both linear and cyclic glucans in the same species.

In this study, we aim to investigate the potential role both linear and cyclic glucans play in hydrating the *P. aeruginosa* periplasm under low-water-content conditions. Sensing and regulating the water status of the periplasm may be a singularly important feat dictating the outcome of various regulatory networks important for both growth and survival. A parched periplasm could disrupt envelope integrity to induce an envelope stress response, manifested in pseudomonads by liberating the alternative sigma factor σ^{22} (AlgU/T) by regulated intramembrane proteolysis from the anti-sigma factor, MucA (21, 22, 26, 41, 59, 68, 70). In this chapter we show that both linear and cyclic glucan deficiency under matric stress simulating low-water-content conditions results in hyper-activation of the envelope stress response, indicating that hydration via periplasmic glucans strongly influence envelope integrity.

Materials & Methods

Strains and growth conditions

All strains and plasmids used in this study are listed in Table 1. Routine culturing of cells was done with tryptic soy agar or broth. Unless otherwise noted, for experiments cells were cultured on CTYE media (36) with Hutners minimal salts (60, 67) and 20 mM glucose, either unamended (water-replete) or amended with NaCl (solute stress) or polyethylene glycol (PEG) with an average molecular weight of 8000 (matric stress) to lower the water potential of the media. All incubations were at 25° C unless otherwise noted. Antibiotic concentrations were as follows, *E. coli*: gentamycin (Gm) – 15 μg ml^{-1}, tetracycline (Tc) – 20 μg ml^{-1}, ampicillin (Ap) – 100 μg ml^{-1}; *P. aeruginosa*: Gm – 50 μg ml^{-1}, Tc – 50 μg ml^{-1}, Carbenicillin – (Cb) 400 μg ml^{-1}.

Table 1. Strains, plasmids and primers

Strains	Description	Source
P. aeruginosa		
PAO1	Wild-type	(31)
ΔndvB	*ndvB* deletion	(38)
ΔopgGH	*opgGH* deletion	(67)
ΔndvB ΔopgGH	*ndvB* and *opgGH* deletions	(67)
ΔalgD	*algD* deletion	(67)
ΔndvB ΔalgD	*ndvB* and *algD* deletions	(67)
ΔopgGH ΔalgD	*opgGH* and *algD* deletions	(67)
ΔndvB ΔopgGH ΔalgD	*ndvB*, *opgGH* and *algD* deletions	(67)
PDO300	Truncated MucA due to frame-shift mutation	(42)
PA14	Wild-type	(53)
PA14 *ΔndvB*	*ndvB* deletion	(38)
PA14 *ΔopgGH*	*opgGH* deletion	This Study
PA14 *ΔndvB ΔopgGH*	*ndvB* and *opgGH* deletions	This Study
Pseudomonas putida		
mt-2	Wild-type, Rf^R	(64)
mt-2 *ΔndvB*	*ndvB* deletion, Rf^R	This Study
mt- 2 *ΔopgGH*	*opgGH* deletion, Rf^R	Lucas Linz
mt-2 *ΔndvB ΔopgGH*	*ndvB* and *opgGH* deletions, Rf^R	This Study
E. coli		
Top10	F^- *mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL* (Str^R) *endA1 nupG*	Invitrogen
DH5α	F^- *Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-, mK+) phoA supE44 λ– thi-1 gyrA96 relA1*	Invitrogen

Table 1. Continued

Plasmids	Description	Source
pProbe-GT	Transcriptional reporter fusion plasmid, Gm^R	(46)
pProbe-AT	Transcriptional reporter fusion plasmid, Ap^R/Cb^R	(46)
pP_{PA}*algD-gfp* -G	Transcriptional fusion of PAO1 *algD* promoter, Gm^R	This Study
pP_{PA}*algD-gfp*-A	Transcriptional fusion of PAO1 *algD* promoter, Ap^R/Cb^R	This Study
pP_{PA}*algU-gfp*	Transcriptional fusion of PAO1 *algU* promoter, Gm^R	This Study
pHERD-30T	AraC-P_{BAD} expression system, Gm^R	(52)
pHERD-*ndvB*	Arabinose inducible *ndvB* expression, Gm^R	This Study
pHERD-*opgGH*	Arabinose inducible *opgGH* expression, Gm^R	This Study
pTsacB	Suicide vector containing *sacB* counter selection, Tc^R	(11)
pTsacBΔ*PPndvB*::Km	pTsacB with Km^R and arms of homology for mt-2 *ndvB*, Tc^R, Km^R	This Study
pTsacBΔ*algD*::Gm	pTsacB with Gm^R and arms of homology for PAO1 *algD*, Tc^R, Gm^R	This Study
pCR2.1	TA cloning vector Ap^R/Km^R	Invitrogen
pRK2073	Tra^+, Mob^+ ColE1 replicon helper plasmid	(20)
pFlp2Tc	Source of Flp recombinase, Cb^R/Tc^R	(67)
p*algD-gfp*	Transcriptional fusion of mt-2 *algD* promoter, Km^R	(36)
Primers		
AlgDF	5’-ATTAGGATCCTCCTCTTTCGGCACGCCGA-3’	This Study
AlgDR	5’-AATTGGTACCCGCATTCACCTCGATTGTTTGT CGC-3’	This Study
PalgUfw	5’-ATTAGGATCCATTCCACGGTGTCGGTATCGTCCA-3’	This Study
PalgURe	5’-AATTGGTACCGAAAGCTCCTCTTCGAACCTGGA-3’	This Study
ndvBcompF	5’-CATCGAATTCCATGTCTTCACGCAAGATCGGGC-3’	This Study
ndvBcompR	5’-ATCTAAGCTTACGCCTCGCCCGACCTCAACC-3’	This Study
opgCompFN	5’-TATAGGTACCCGTGATTTTCCGTTCCGTTTCAAAC ACC-3’	This Study
opgCompRN	5’-TATAAAGCTTCATGGGATCAGGACTGGGCGG-3’	This Study
PPndvBuF	5’-GGTGATGTACTCGGCCATGGTCTC-3’	This Study
PPndvBuRK	5’-GAAGCAGCTCCAGCCTACACCGAACTCGACCGAAT CCTTCATCG-3’	This Study
PPndvBdFK	5’-GGAACTAAGGAGGATATTCATATGGACCGATGATC ATCGTGCCGCATC-3’	This Study
PPndvBdR	5’-CGTCGCTGGTGATCAGAAACG-3’	This Study
PKD4F	5’-CCATGGTCCATATGAATATCCTCC-3’	(48)
PKD4R	5’-ATTGTGTAGGCTGGAGCTGCTTC-3’	(48)
algDupF2	5’-CAATGCCCACGGCTATTACTTCAGC-3’	This Study
algDupRG	5’-TCAGAGCGCTTTTGAAGCTAATTCGTGATTCGCATC GCATTCACCTCG-3’	This Study
algDdnFG	5’-AGGAACTTCAAGATCCCCAATTCGTCGTCGACCTGG TGAACAAGACC-3’	This Study
algDdnR2	5’-TCGTTCTCGACGTCGGTGATG -3’	This Study
FRTGmF	5’-CGAATTAGCTTCAAAAGCGCTCTGA-3’	This Study
FRTGmR	5’-CGAATTGGGGATCTTGAAGTTCCT-3’	This Study
algDinSF	5’-CTTCCCTCGCAGAGAAAACATCC-3’	This Study
algDinSR	5’-GCAGGATGAAATCCTTGGAGTCG-3’	This Study
HERDgfpF	5’-AGGTACCCATGAGTAAAGGAGAAGAAC-3’	This Study
HERDgfpR	5’-CAAGCTTCTATTTGTATAGTTCATCC-3’	This Study
algD-RT3F	5’-CTGATCAACCAGGGCAAGTCG-3’	This Study
algD-RT1R	5’-TCGAGGAAGGTGAGTACTACTGGCA-3’	This Study
rimM-RT1F	5’-CAAGCTTCTATTTGTATAGTTCATCC-3’	(67)
rimM-RT1R	5’-ACCGGTTTCCAGCAGATGGTCGAT-3’	(67)

Plasmid construction

Transcriptional-reporters pP_{PA}*algD-gfp* -G and pP_{PA}*algD-gfp* -A were created by inserting an 871-bp fragment (PCR amplified using primers algDF and algDR) upstream of the *algD* translational start into pProbe-GT and pProbe-AT. Plasmid pP_{PA}*algU-gfp* was constructed by inserting a 595-bp PCR amplicon (primers: PalgUfw and PalgURe) into pProbe-GT. Arabinose inducible pHERD-*ndvB* and pHERD-*opgGH* were created by inserting a promoterless amplicon of *ndvB* (2625-bp using primers ndvBcompF and ndvBcompR), and *opgGH* (4163-bp using primers opgCompFN and opgCompRN) into the pHERD-30T vector. Plasmid pHERD-gfp was constructed by cloning a 717-bp promoter-less amplicon of the *gfp* gene using pProbe-GT as a template (primers: HERDgfpF and HERDgfpR) into pHERD-30T. Plasmid pFlp2Tc was created by ligating a PCR amplified Tc^R marker from pTsacB (1319-bp, primers TetKpnF and TetKpnR) into pFlp2.

Quantitative real-time PCR

Cell cultures were grown, RNA isolated and thermocycler program run as described previously (67). The Livak ($2^{-\Delta\Delta C(t)}$) method (37) was used to measure *algD* (Primers: algD-RT3F and algD-RT1R) expression relative to *rimM* (Primers: rim-RT1F and rimMRT1R) over time during growth on CTYE media, unamended media, as well as media amended with PEG to reduce the water potential by 1.5 MPa.

Deletion of glucan biosynthesis genes

Deletions of PA14 *ΔopgGH* were created using the PAO1 derived deletion system pTSacB*ΔopgGH* described in the previous chapter (67). To create gene deletions of *ndvB* in *P. putida* mt-2, upstream (1086-bp, primers PPndvBuF & PPndvBuRK) and downstream (1095-bp, primers PPndvBdFK & PPndvBdR) regions were PCR amplified as was a Flp recombinase

target (FRT) flanked kanamycin cassette from pKD4 (1490-bp, primers: PKD4F and PKD4R). A second round of phusion Taq PCR amplification using splice overlap extension procedures (14) created an amplicon with the upstream, FRT-Km^R-FRT and downstream regions. The product was blunt-end ligated into the SmaI site of pTsacB creating pTsacBΔ*PPndvB*::Km. Tri-parental mating using donor *E. coli* strain Top10 with pTsacB*ΔPPndvB*::Km, helper *E. coli* strain DH5α with pRK2073 and recipient's mt-2 or mt-2 *ΔopgGH* produced several Km^R and Tc^S double-recombinant transconjugants. The Km^R gene was removed with pFlp2Tc Flp recombinase as described previously (29). The *ndvB* deletion was confirmed by PCR with primers PPndvBupF and PPndvBdnR (Figure S1A).

Deletion of alginate biosynthesis gene in PAO1 and glucan mutant derivatives

Site specific gene deletions of the PAO1 alginate biosynthesis *algD* gene were created by PCR amplifying upstream (933-bp, primers algDupF2 and algDupRG) and downstream (1079-bp, primers algDdnFG and algDupR2) regions around *algD* as well a FRT-Gm^R-FRT cassette (1053-bp, primers FRTGmF and FRTGmR). Splice overlap PCR was used to amplify an amplicon with the upstream region, FRT-Gm^R-FRT, and downstream region. This amplicon was ligated into the TA cloning vector pCR2.1 (Invitrogen). The plasmid was transformed into *E. coli* Top10, before being purified and restricted with SacI and XhoI. The excised insert was then ligated into SacI/XhoI restricted pTsacB, creating pTsacBΔ*algD*::Gm. Triparental mating using donor strain *E. coli* Top10 with pTsacB*algD*::Gm, helper strain DH5α with pRK2073 and recipient PAO1 and glucan mutant derivatives produced several Tc^R transconjugants when grown on M9 media with succinate as the carbon source (to limit *E. coli* growth). The Gm^R gene was removed using pFlp2Tc Flp recombinase (29). Deletion of *algD* was confirmed by PCR with primers algDinSF and algDinSR (Figure S1B)

Mucoid phenotype screen

Cells were grown overnight on CTYE solid media, resuspended in 1 mM phosphate buffer (OD_{600nm} = 0.1) and diluted 100-fold prior to spotting 5 µl aliquots on CTYE solid media either unamended or amended with NaCl or PEG to reduce the water potential by 1.5 or 2.5 MPa. Congo red (40 µg/ml) and Coomassie blue (15 µg/ml) were added to the media to facilitate visualization of the mucoid phenotype. The mucoid phenotype was scored relative to PAO1 under water-limiting conditions after 48 h of growth at 25° C or 37° C. Non-mucoid was scored as zero, mucoid as 1, and hyper-mucoid as 2-3.

Quantification of total extracellular carbohydrates and uronic acids

High molecular weight extracellular polysaccharides were isolated as described previously (10). Briefly, cells were grown overnight on ½-strength TYE (5 g tryptone, 2.5 g yeast extract, 15 g agar L^{-1}) solid media, and resuspended in 1 mM phosphate buffer (OD_{600nm} = 0.1) prior to spreading 1.2 ml onto a 0.45 µm nitrocellulose membrane overlaying solid ½-strength TYE media that was unamended or amended with either NaCl (solute stress) or PEG (matric stress) to lower the water potential by 1.5 MPa. After 48 h of growth, cells were scraped off the membranes and resuspended in 0.85 % saline solution. After removal of an aliquot for protein quantification (Bradford assay), the cell suspension was centrifuged for 20 minutes at 16,000 x g. The resulting supernatant was then filtered (0.45 µm) prior to DNAse (100 µg/ml, 4 h at 37 °C) and Proteinase K (1 mg/ml, overnight at 37 °C) treatment. The solution was desalted by dialysis (1000 MW cut-off). High MW-exopolysaccharides were precipitated with the addition of 3 volumes 100% ethanol overnight at -20° C prior to centrifugation for 20 min at 16,000 x g. The pellet was air dried and resuspended in water. Total carbohydrates were

quantified using the phenol-sulfuric acid assay with glucose as a standard (15). Uronic acids were quantified using the meta-hydroxyl phenol method with glucoronic acid as the standard (1).

Effect of glucan deficiency on alginate operon promoter activity

To measure *algD* operon promoter activity, cells were transformed by electroporation (12) with pP_{PA}*algD-gfp*-G or pP_{PA}*algD-gfp*-A. Plate cultures were grown for 48 h on CTYE without and with various concentrations of NaCl or PEG amendments to reduce the water potential to simulate solute or matric stress. Cells were resuspended in water to OD_{600nm} = 0.05-0.08 and fluorescence intensity was measured with a Fluoromax 2 spectrometer (Jobin-Yvon-Spex Instruments). Excitation and emission wavelengths, bandpass, and integration time were 488 nm, 510 nm, 5 nm, and 0.1s, respectively. A relative fluorescence unit is defined as the fluorescence intensity relative to the optical density of the cell suspension at the time of sampling. For *P. putida* mt-2 alginate promoter activity was measured with pP*algD-gfp*. Activity of the *algU* operon promoter was measured similarly using cells containing pP_{PA}*algU-gfp*.

Expression of plasmid-based glucan biosynthesis genes

Wild-type or glucan-deficient mutants were transformed with pHERD-30T, pHERD-*ndvB* or pHERD-*opgGH* for complementation studies where expression of the glucan biosynthesis genes was controlled by arabinose-mediated induction. To assess the effect of complementation or glucan biosynthesis gene overexpression on alginate promoter activity strains also harbored pP_{PA}*algD-gfp*-A, which was stably maintained in the presence of the pHERD-derived vectors. We did not use the pP_{PA}*algD-gfp*-G system for these studies since its antibiotic resistance marker was the same as the pHERD-derived vectors. Plate cultures of PAO1 and glucan-deficient mutants transformed with pHERD-30T, pHERD-*ndvB* or pHERD-*opgGH* grown on CTYE water-replete media were resuspended in 1 mM phosphate buffer. Aliquots (5

µl) were placed onto CTYE plates amended with 0 to 0.5% L-arabinose and either unamended or amended with PEG (matric stress) or NaCl (solute stress) to lower the water potential by 1.5 MPa. Relative fluorescence was measured as described above after 48 h of growth.

Swarming motility assay

Overnight TYE broth cultures were diluted to OD_{600nm}=0.1 and 2 µl aliquots were spotted onto 0.5% BM2 media (40). Plates were incubated for 24 h at 37° C prior to visually assessing swarming motility.

Fatty acid methyl ester analysis

Fatty acids were extracted from cells grown on 0.45 µm membranes overlaying CTYE plates that were either unamended or amended with NaCl (solute stress) or PEG (matric stress) to reduce the water potential by 1.5 MPa. After 48 h, cells were scraped off membranes for methylation, saponification, extraction and flame-ionization detection-gas chromatography using the Sherlock-Microbial Identification System (MIDI Inc.) according to manufacturers recommended protocols.

Results

Periplasmic glucan mutants appear mucoid when grown under matric stress conditions.

Under matric stress conditions wild-type PAO1 becomes slightly mucoid (Figure 1 and Table S1). Visually, Δ*opg*GH and Δ*ndv*B mutants exhibited a hyper-mucoid colony morphology phenotype on matric stress (Figure 1 top, Table S1), but not solute stress or water-replete solid media (Tables S1, S2, Figure S2). Interestingly Δ*ndv*B mutants exhibit reduced Congo red binding on water-replete media (Figure S2); whether this is due to Congo red interactions with the cyclic glucan itself or if Δ*ndv*B mutations alter the production of some other polysaccharide

is not clear. The extent of the hyper-mucoid phenotype of the glucan mutants is comparable to that exhibited by PDO300, a PAO1-derivative with a *mucA* mutation (Figure 1 and Table S1). When the cell experiences envelope instability, the anti-sigma factor MucA releases σ^{22} (AlgU/T) to activate the envelope stress response, including expression of the alginate biosynthesis operon (26). The observed hyper-mucoid phenotype suggests that under matric stress, glucan-deficient cells hyper-activate the envelope stress response leading to overproduction of the exopolysaccharide alginate. The extent of mucoidy increased with greater matric stress severity (Table S1 and S2) and at lower (28 °C) than higher (37 °C) temperature (Table S2). There was a slight but detectable increase in mucoidy with colony age (Table S2). The hyper-mucoidy phenotype was not restricted to PAO1 since we also observed a hyper-mucoid phenotype by linear glucan-deficient strains of *P. aeruginosa* PA14 (Table S2) and *P. putida* mt-2 (data not shown). Mucoidy of the Δ*ndv*B mutants was comparable to wild-type when grown on media amended with PEG to lower the water potential 1.5 MPa, but appeared to have increased mucoidy when grown on media amended with PEG to lower the water potential 2.5 MPa (Table S2).

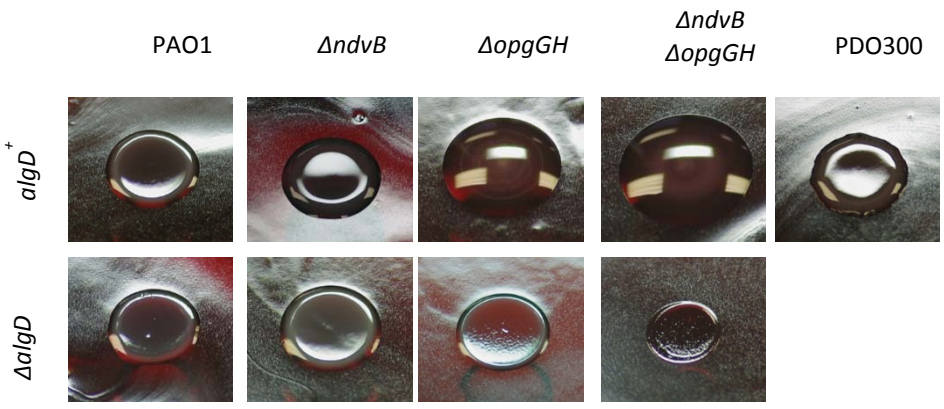

Figure 1. Effect of matric stress on the mucoid phenotype of PAO1, PDO300, and glucan-deficient mutants. Strains were grown for 48 h on CTYE media amended with PEG to lower the water potential by 1.5MPa. Top row are strains capable of producing alginate (*algD*$^+$) and the bottom row are alginate-deficient (*ΔalgD*) strains.

Quantitative analysis of high-MW EPS isolated from unsaturated PAO1 biofilms indicates that the mucoid and hyper-mucoid phenotype of wild-type and Δ*opgGH* and Δ*ndvB* mutants is due primarily to the presence of uronic acids as measured by colorimetric assays (Figure 2). Similar results were observed for PA14 and its Δ*opgGH* and Δ*ndvB* mutants (Figure S3). The high amount of uronic-acids associated with the mucoid phenotype is likely due to alginate (19) production rather than Psl or Pel exopolysaccharides that can be made by *P. aeruginosa*, which are thought to be neutral; though unlike Psl, Pel has not been fully characterized (8, 23). To demonstrate that glucan deficiency-mediated hyper-mucoidy is due to alginate production, deletions of the alginate glycosyl transferase *algD* were made in PAO1 and the glucan-deficient strains. Under matric stress, the hyper-mucoid phenotype disappeared for all strains; conclusively showing that alginate over production causes the mucoid phenotype under matric stress (Figure 1, bottom).

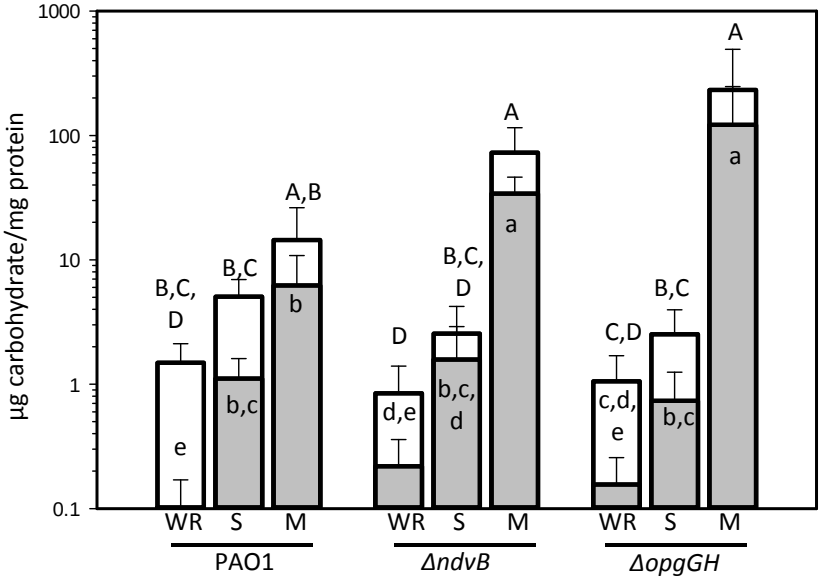

Figure 2. Effect of glucan deficiency on extracellular polysaccharide production. Water stress was imposed by amending media with NaCl or PEG to lower the water potential by 1.5 MPa. Open bars: total carbohydrates; grey bars: uronic acids. WR=water-replete, S= solute stress; M=matric stress. Values are mean ± SE of 3 replicates. Bars with the same letter (capital for total and lower-case for uronic acids) are not statistically different based on a LSD test ($p \leq 0.05$).

We also examined the mucoidy of mutants of strains with transposon insertions in genes predicted to be involved in glucan production. PA1689 shares 46.6% similarity to the *E. coli* phosphoglycerol transferase MdoB (OpgB) involved in decorating the linear glucan with phosphoglycerol substitutions, contributing to its anionic properties. In the previous chapter, we found that glucans become more anionic under matric stress, suggesting that the anionic properties contribute to glucan function. Interestingly, *PA1689* mutants generally did not display more mucoidy than wild-type PAO1 under matric stress (Table S1), except when inoculated with a more dilute ($OD_{600nm} = 0.01$) cell suspension, yielding a hyper-mucoid phenotype (data not shown). This may suggest that lower cell numbers and shared resources expose cells to greater need of anionically charged glucans. In *Sinorhizobium meliloti*, the sensor-kinase and response regulator FeuQP regulate the transcription of *ndvA*, the cyclic glucan exporter. While no NdvA ortholog has yet been identified in *P. aeruginosa*, PA2656 and PA2657 share significant homology to *S. meliloti* FeuQ and FeuP based on BLASTP. Under matric stress, *feuP* mutants showed increased mucoidy (Table S1), suggesting that this response regulator could be involved in glucan secretion. The mucoidy of other transcriptional regulator and quorum sensing mutants was also examined, and many exhibited mucoidy greater that PAO1, though increases in mucoidy may be unrelated to glucan production (Table S1).

Cyclic and linear glucan deficiency hyper-activates the envelope stress response

These findings suggest that glucan deficiency under matric stress conditions stimulates an envelope stress response since alginate expression is one of the best characterized components of the σ^{22} (AlgU) regulon, an extracytoplasmic-responsive sigma factor responding to periplasm and outer or inner membrane stress. To validate this, we constructed a transcriptional fusion

between the alginate (*algD)* biosynthesis operon promoter and *gfp*. With the pP_{PA}*algD-gfp* -G bioreporter we observed that σ^{22}-dependent *algD* operon promoter activity of the wild-type PAO1 increased over two-fold under matric stress (Figure 3). This result was corroborated with qRT-PCR expression analysis of *algD* in PAO1 (Figure S4). Cells deficient in either the linear or cyclic glucan exhibited statistically greater *algD* operon promoter activity under matric stress than wild-type (Figure 3 and Tables S3-4). Importantly, Δ*opgGH* cells exhibited greater *algD* operon promoter activity than the wild-type PAO1 under water-replete and solute stress conditions, suggesting that absence of the linear glucan itself stimulates an envelope stress response in the absence of matric stress. As a separate measure of activation of the envelope stress response, we constructed a σ^{22} (AlgU) promoter transcriptional fusion, pP_{PA}*algU-gfp*. Liberation of σ^{22} (AlgU) by envelope stress leads to induction of the *algU* operon itself (28). As

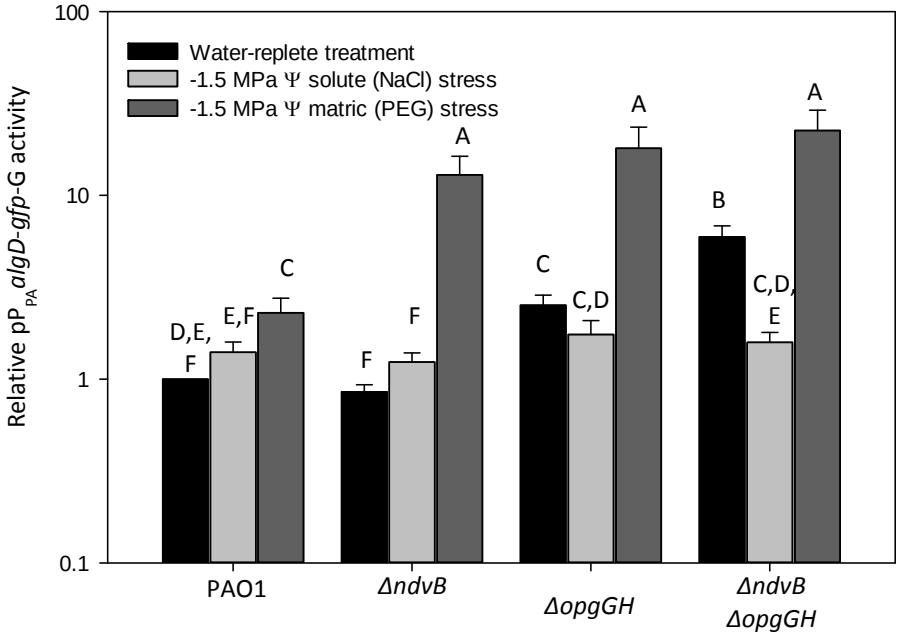

Figure 3. Envelope stress response in *ΔopgGH* & *ΔndvB* mutants after 48 h growth as determined with σ^{22}-dependent bioreporter pP_{PA}*algD-gfp*-G. Values are mean ± SE (n=3) and are expressed relative to PAO1 under water-replete conditions. Bars with the same letter are not statistically different based on a LSD test ($p \leq 0.05$).

with the alginate (*algD*) bioreporter, we observed increased *algU* operon promoter activity (Figure 4), although the level of promoter activity was lower and high promoter activity was only

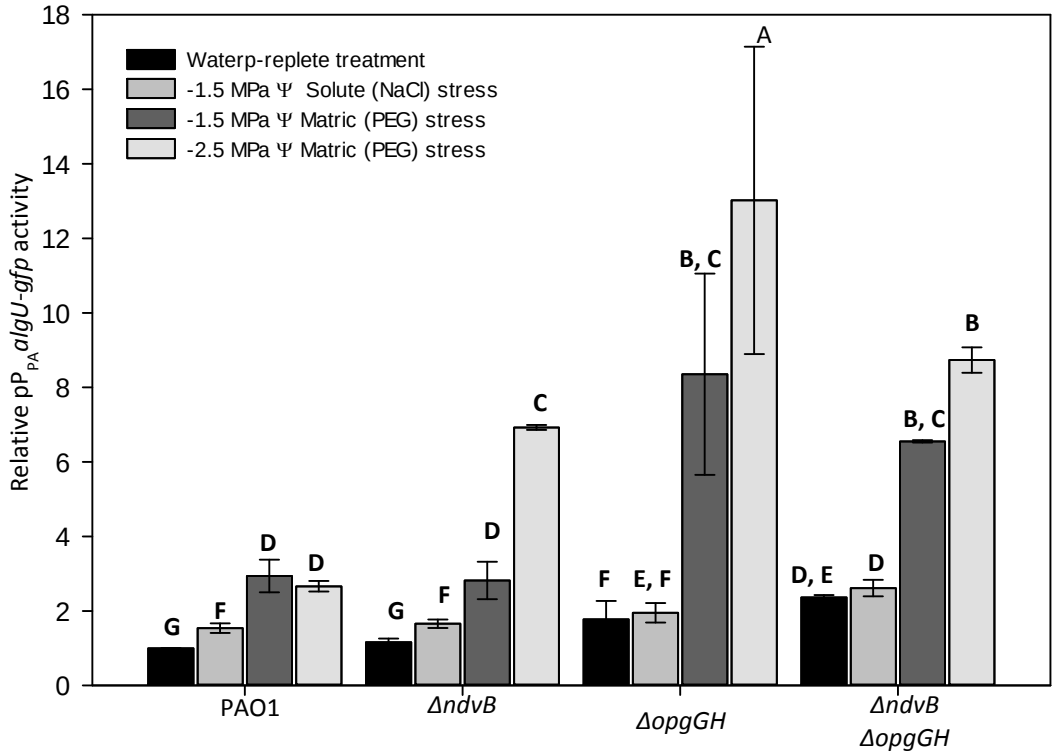

Figure 4. Envelope stress response in *ΔopgGH* & *ΔndvB* mutants after 48 h of growth as determined by σ^{22}-dependent bioreporter pP$_{PA}$*algU-gfp*. Values are mean ± SE (n=3) relative to PAO1 under water-replete conditions. Bars with the same letter are not statistically different based on a LSD test ($p \leq 0.05$).

observed under more stressful (-2.5 MPa Ψ) matric stress conditions. Similar *algD* promoter activity results were observed in *P. aeruginosa* PA14 and *P. putida* mt-2 in that linear glucan-deficient strains had elevated *algD* promoter activity, though cyclic glucan deficiency (Δ*ndvB*) did not stimulate *algD* promoter activity (Figure S5); this is consistent with exopolysaccharide production by the PA14 mutants (Figure S3).

Effect of stress severity on envelope stress response.

The preceding suggests that the presence of periplasmic glucans is necessary for modulating the envelope stress response under water-limiting conditions. However, we used a severe matric stress (-1.5 MPa Ψ) comparable to the permanent wilting point of many agronomic

plants. Consequently, we were interested in understanding the role linear and cyclic glucans have in modulating the envelope stress response under higher matric potentials using the σ^{22}-dependent bioreporter pP_{PA}*algD-gfp-G*. Relative to water-replete conditions alginate promoter activity in PAO1 under solute stress was significantly lower at -0.25MPa and then started to increase with increasing solute stress severity (Figure 5A and Table S3). Similar results were observed for Δ*ndvB* (Fig 5A). Though alginate promoter activity in Δ*opgGH* and Δ*ndvB* Δ*opgGH* under water-replete conditions was elevated compared to PAO1 (Figure 5A and Table S4), increasing solute stress decreased promoter activity dramatically, illustrating that linear glucans function to alleviate envelope stress under hypo-osmotic conditions, and that solutes mitigate the need for glucan accumulation.

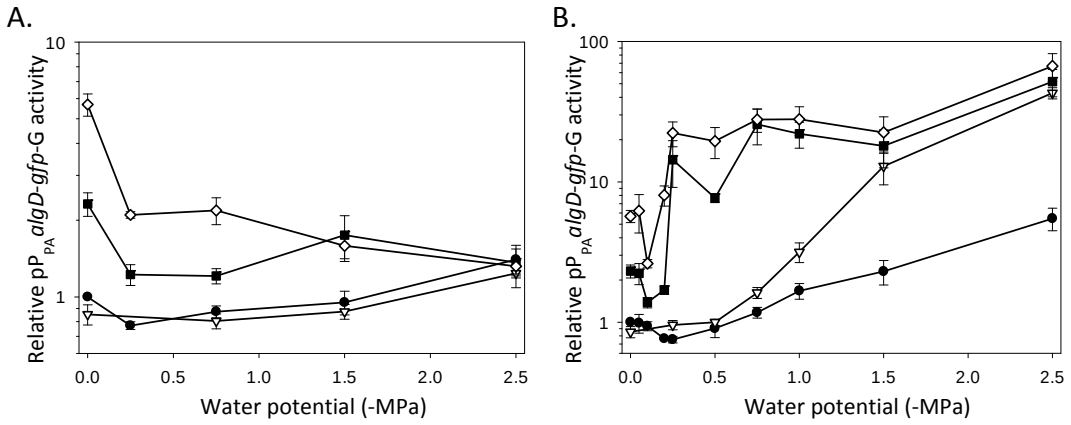

Figure 5. Envelope stress in *ΔopgGH* & *ΔndvB* mutants as determined with the σ^{22}-dependent bioreporter pP_{PA}*algD-gfp*-G. A: Solute (NaCl) stress; B: Matric (PEG) stress. Values are mean ± SE; n=3-9. ● PAO1, ∇ *ΔndvB,* ■ *ΔopgGH* and ◊ *ΔndvB ΔopgGH*. Values are relative to PAO1 under water-replete conditions. Statistical differences listed in Table S3 & S4.

Relative to water-replete conditions alginate promoter activity in PAO1 under matric stress was significantly lower at -0.25MPa and then started to increase with increasing matric stress severity (Figure 5B and Table S3). Likewise, alginate promoter activity in the Δ*ndvB*

mutant increased with increasing matric stress severity and at -0.75 MPa and lower water potentials alginate promoter activity was significantly greater than the wild-type (Fig 5B and Table S4). Absence of the linear glucan resulted in hyper-activation of alginate promoter activity, which decreased with a slight-to-modest matric stress though increased further to a maximal level at water potentials below -0.25MPa (Figure 5B). This response by *ΔopgGH* and *ΔndvB ΔopgGH* to increasing matric stress severity is distinct from the response to solute stress (Compare Figure 5A and B).

Overexpression of linear glucan functionally complements NdvB deficiency

We validated the consequences of linear and cyclic glucan deficiency on the envelope stress response with complementation studies. Our approach was to construct a system where cells harbored promoter-less copies of either *ndvB* or *opgGH* controlled by the arabinose-inducible P_{BAD} promoter and a σ^{22}-dependent bioreporter pP_{PA}*algD-gfp-A* for measuring alginate promoter activity. Evidence for complementation is based on reduced alginate promoter activity under matric stress compared to the activity measured in control strains harboring an empty vector (pHERD-30T). Figure 6 shows alginate promoter activity of the *ΔopgGH* and *ΔndvB* mutants returned to wild-type levels when complemented with *opgGH* and *ndvB*, respectively. Interestingly, alginate promoter activity of Δ*ndvB* and Δ*ndvB* Δ*opgGH* strains harboring pHERD-*opgGH* returned to wild-type levels (Figure 6), suggesting that overexpression of *opgGH* can functionally complement NdvB deficiency-mediated envelope stress response. In contrast, over-expression of *ndvB* does not functionally complement OpgGH deficiency-mediated envelope stress response (Figure 6).

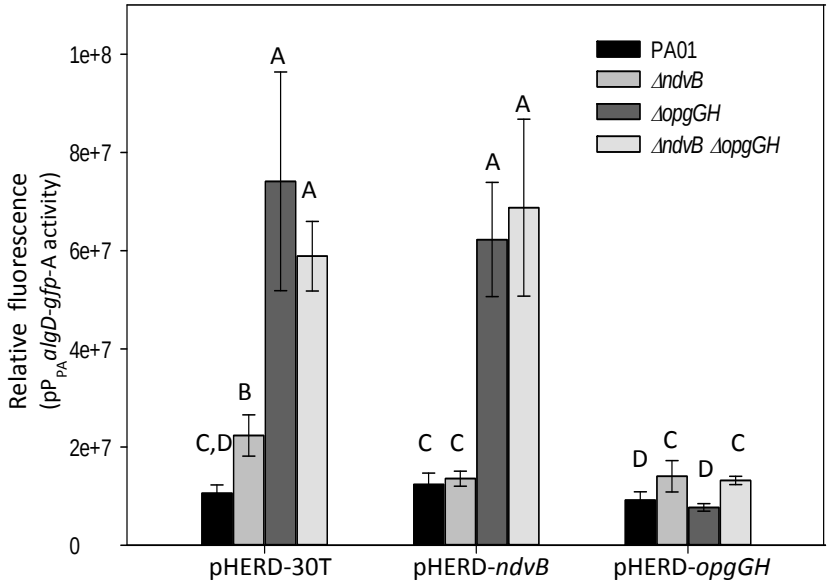

Figure 6. *ndv*B & *opgGH* complementation or overexpression abolishes NdvB and OpgGH deficiency-mediated stimulation of the envelope stress response. Bacteria harbor both the pP$_{PA}$*algD-gfp*-G bioreporter & arabinose-inducible P$_{BAD}$ promoter (pHERD) driving *ndvB* or *opgGH* expression plasmids. Bars with the same letter are not statistically different (P≤0.05). Values are the mean ±SE, n=6. Bars with the same letter are not statistically different based on an LSD test ($p \leq 0.05$).

Interestingly complementation was observed on media without exogenous arabinose, suggesting that the P$_{BAD}$ promoter is leaky and the level of expression was sufficient for complementation. To validate this possibility we constructed pHERD-*gfp* and showed that in the absence of exogenous arabinose there was at least 2-fold more fluorescence than cultures with an empty pHERD-30T (Figure S6). Furthermore, we also showed that arabinose induction increased GFP production for pHERD-*gfp* (Figure S6), demonstrating functionality of the leaky arabinose-inducible system. Additionally, we noted that while linear glucan-deficient cells lost their mucoidy without arabinose amendments the hyper-mucoid phenotype re-appeared with arabinose amendments (Table S5). Experiments with pHERD-opgGH and pP$_{PA}$*algD-gfp*-A may show that increasing arabinose concentration elevates the envelope stress of linear glucan-deficient cells (Figure S7). There was no visual change in mucoidy of *ΔndvB* pHERD-*ndvB* or

ΔndvB pHERD-*opgGH* (Table S3), though *algD* promoter activity confirmed complementation in the absence of arabinose-mediated induction (Figure 5).

Loss of cyclic glucan increases swarming motility

A common pleiotropic effect of cyclic or linear glucan deficiency under hypo-osmotic conditions is altered swimming or swarming motility properties and that wild-type phenotypes are restored under hyper-osmotic conditions (5, 49). There was no apparent defect in swimming motility of the *ΔopgGH*, *ΔndvB*, *ΔndvB ΔopgGH* mutants under water-replete conditions (data not shown), which is consistent with previous reports (35). However, we did observe that the *ΔndvB* and *ΔndvB ΔopgGH* mutants exhibited increased swarming motility under hypo-osmotic conditions (Figure 7). Because *ΔopgGH* swarming was comparable to PAO1 it is likely that *ΔndvB* mediated hyper-motility is due to a mechanism independent of increased envelope stress. Strains harboring either pHERD-*ndvB* or pHERD-*opgGH* failed to return motility of both *ΔndvB* and *ΔndvB ΔopgGH* back to wild-type levels when grown in the absence of arabinose (data not shown).

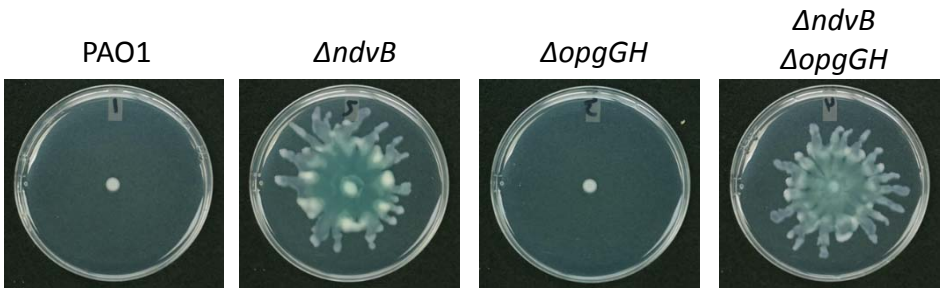

Figure 7. Swarming motility phenotype on BM2 media 24-h post inoculation.

Fatty acid analysis

Previous work had shown that matric stress modulates membrane fatty acid composition (25) and thus we assessed the effect of linear and cyclic glucan deficiency on membrane integrity by performing FAME analysis on whole-cell fatty acid extracts of stationary phase cultures

grown on media without or with NaCl or PEG-amendments to lower the water potential by 1.5 MPa. In general, the fatty acid profiles of PAO1and *ΔopgGH* were similar to each other under water replete and solute stress conditions, though *ΔndvB* and *ΔndvB ΔopgGH* strains have different profiles from PAO1 with a greater proportion of cyclopropyl fatty acids (17:0 cyclopropyl and 19:0 cyclopropyl w8c) and fewer 16:1 w7c and 18:1 w7c fatty acids (Table S6). Conversely, under matric stress the fatty acid profiles of PAO1 and *ΔndvB* were similar, but the *ΔopgGH* and *ΔndvB ΔopgGH* mutants exhibited an increase in short and hydroxyl fatty acids with a decrease in 16:0 and 18:1 w7c fatty acids (Table 6). Glucan synthesis is known to be involved in the turnover of phospholipids as glucans use the phosphoglycerol head groups for anionic decoration (32, 57, 66). It could be that fatty acid composition is affected by disruption of this process. Alternatively, it could be that envelope perturbations due to glucan deficiency cause changes in the fatty acid profiles.

Discussion

In this study, we propose that the previously observed accumulation of periplasmic glucans under low-water-content conditions (67) act to hydrate the periplasm. We found strong evidence that under matric stress simulating low-water-content, both linear and cyclic glucans moderate envelope stress, likely by hydrating the periplasm. We observed that there is likely some one-way functional redundancy between the glucans as over-expressed linear glucans were able to modulate the envelope stress response in cyclic glucan-deficient mutants in a non-reciprocal fashion. However, phenotypic differences between linear and cyclic glucan-deficient cells highlight that both glucans likely have unique functions and importance to the cell.

If hydration was inadequate, periplasmic and membrane associated proteins would become misfolded and trigger the well characterized σ^{22} (AlgU/T)-dependent envelope stress response, activating the AlgU stimulon (26, 28, 43, 58, 69), including the alginate biosynthesis operon. While we observed elevated alginate production in wild-type PAO1 cells grown under low-water-content conditions - with increased alginate-dependent colony mucoidy (Figure 1, Table S1), extracellular uronic acids (Figure 2) and alginate biosynthesis gene expression (Figure 3) - these phenotypes were greatly exacerbated in glucan-deficient mutants, strongly suggesting they were experiencing greater envelope stress.

The fact that glucan-deficient mutants had hyper-mucoidy comparable to PDO300 (Figure 1, Table S1), a truncated MucA (σ^{22} anti-sigma factor) mutant (42), is very significant. This phenotype suggests that glucans play such an important role in facilitating periplasm hydration under low-water-content conditions that glucan absence causes an extensive envelope stress in which MucA sequestering of σ^{22} (AlgU/T) is minimal and comparable to the complete inability to sequester the sigma factor. Highlighting the known understanding of periplasmic glucans under water-replete (hypo-osmotic) conditions, the linear glucan-deficient mutants had elevated envelope stress under these conditions, which was attenuated with increasing environmental solutes (Figure 3 and 5), functionally replacing the absent linear glucan in reducing the water-potential of the periplasm.

It is important to consider alternatives to the σ^{22} (AlgU/T) dependent envelope stress response causing the over-production of alginate. It is plausible that glucan deficiency elevates concentrations of the intra cellular signal molecule cyclic-di-guanosine monophosphate (c-di-GMP). High concentrations of c-di-GMP have been shown to increase exopolysaccharide production in many bacterial species (55). The activity of Alg44, a component of the alginate

polymerization/secretion complex, contains a PilZ c-di-GMP binding domain shown to influence alginate production (44). However this regulation occurs at the enzymatic activity level, and would not explain the observed increase in *algD* or *algU* expression (Figure 3 and 4), both of which do not appear to be transcriptionally regulated by c-di-GMP concentrations (61). Alternatively, there have also been mucoid *P. aeruginosa* strains that do not depend on σ^{22} (AlgU/T), but rather expression of the alginate biosynthesis operon is initiated by σ^{54} (RpoN) which binds the *algD* promoter at a site overlapping the σ^{22} binding site (6). However, it is likely that the mucoid phenotype we observed in our glucan mutants is actually dependent on σ^{22} (AlgU/T)-driven expression as we also observed elevated expression of *algU* (Figure 4), which has a σ^{22} binding site, but no σ^{54} (RpoN) binding site (6).

The fact that linear glucan overexpression reduces the envelope stress response in the cyclic glucan mutant in a non-reciprocal manner (Figure 6) supports the model in which the linear glucan is more versatile and there is at least partial functional redundancy between the linear and cyclic glucans. In our study, "leaky" expression (Figure S6) of the arabinose-inducible plasmid based-glucan biosynthesis genes was sufficient to rescue the envelope stress response in glucan-deficient mutants (Figure 6 and Table S5). This is contrary to the observations of *Dickeya dadantii*, using a similar arabinose-inducible expression plasmid, where no detectable glucans were found without the addition of arabinose and that restoration of swimming motility and virulence were positively correlated with arabinose concentration (3). However, since the source of the P_{BAD} arabinose-inducible promoter is *E. coli* (52) - an enteric like *D. dadantii* - it is possible that the regulation is leakier in the Pseudomonads. Promoters from enterics have been found to be leaky in Pseudomonads before, including the λ *E. coli* phage P_L promoter (9). Additionally, differences affecting transcriptional regulation and posttranscriptional regulation of

glucan production cannot be ruled out. In the previous chapter, we did not observe any changes in *ndvB* expression under water-replete, high solute or low-water-content conditions and only observed a brief increase in *opgG* expression during low-water-content conditions (67). Perhaps glucan production is primarily regulated post transcriptionally in *P. aeruginosa* and that minimal transcription can yield large quantities of glucans depending on post-transcriptional regulation.

Since there appears to be some functional redundancy between the glucans, an intriguing question that arises is what evolutionary advantages there are for Pseudomonads to produce both the linear and cyclic periplasmic glucans while most other gram negative bacteria appear to produce only one. As a group, Pseudomonads are known to live in diverse habitats including soil, on and within plants and animals, even in the atmosphere (24, 34, 47, 63). It is possible that the linear and cyclic glucans differ in function under varying conditions, for which there are several pieces of supporting evidence. The Δ*opgGH* mutant but not the Δ*ndvB* mutant has elevated envelope stress under water-replete conditions, minor to moderate solute stress and minor low-water-content stress (Figure 5), suggesting that linear glucans are preferentially used under non-severe water-stress conditions. The double glucan-deficient mutant has further increased envelope stress relative to the Δ*opgGH* mutant implying the cyclic glucans play a role during these conditions in the absence of linear glucans. Alternatively, linear glucans could fulfill a role additional to reducing the water potential of the periplasm as a whole and hydrating the compartment. One possibility is that linear glucans are closely associated with envelope proteins to ensure proper hydration in their immediate vicinity (from which the larger cyclic glucans are sterically hindered), or provide some other direct structural support. Previous studies have shown that in addition to low-water-content (67), linear glucan biosynthesis gene expression becomes elevated in Pseudomonads after envelope disruption from chemicals

including hydrogen peroxide, sodium dodecyl sulfate, cupric sulfate or d-cycloserine or transfer from osmotic to hypo-osmotic conditions (51, 69). Whether this means that linear glucan biosynthesis helps to stabilize these disruptions or is just part of the envelope stress response is not clear.

An additional role for both glucans may be assisting in phospholipid turnover which results in the addition of lipid-derived phosphoglycerol to glucans. It appears that cyclic glucan deficiency affects fatty acid profiles of cells under water-replete and solute stress, while linear glucan deficiency changes the fatty acid profile of cells under low-water-conditions (Table S5). The discrepancy between the mutants may be explained by preferential phosphoglycerol decoration of one or the other glucan depending on water-stress, in turn affecting turnover of specific phospholipids. However, it is not clear that the observed shifts in the fatty acid profile are due to phospholipid turnover, or alternatively are more directly related to envelope perturbations caused by glucan deficiency.

We observed that loss of the cyclic glucan induced a hyper-swarming phenotype, indicating that the absence of the cyclic glucan is affecting the cell, enabling motility. This is an interesting finding because while modifications to swarming motility are common among glucan-deficient mutants, typically they demonstrate reduced motility, likely due to reduced stability of flagellum (16, 49, 51). Perhaps in *P. aeruginosa*, environmental stresses exacerbated by cyclic glucan deficiency cause the cells to attempt moving out of such conditions.

Findings in several other studies also highlight the importance of the cyclic glucan in *P. aeruginosa*. The ability of cyclic glucans to bind certain antibiotics and improve drug-resistance (38, 56) is very interesting as this could improve survival to both pathogenic and environmental Pseudomonads encountering antibiotics. Perhaps the most compelling argument for maintaining

the cyclic glucan was found in the previous chapter where it was observed that loss of the cyclic (but not linear) glucan resulted in significantly reduced survival under low-water-content conditions (67). Clearly, while there is some functional redundancy between the glucans, both play critical roles and are important to Pseudomonads.

Understanding how glucan-modulated envelope stress influences polysaccharide production would be important to our understanding of infection models as exopolysaccharide production during infection has been an active area of research in recent years. This is especially true in *P. aeruginosa,* where increased polysaccharide production has been found to contribute to better resistance to antibiotic treatments and therefore to poor prognosis (27, 30, 61). In this study we developed a suitable proxy for rapidly and reliably estimating the envelope stress response in *P. aeruginosa* by measuring the *algD* promoter activity with a GFP reporter, which proved useful in exploring the effect of glucan deficiency, but others may find useful in other situations. As periplasmic glucan are found in diverse proteobacteria, living in habitats including soil, plant, animal and man-made surfaces, understanding how periplasmic glucans contribute to fitness in environments of low-water-content presents an exciting area of further research. It is conceivable that characterizations of the type, size and substitutions of the glucans produced by different bacterial species and comparing them to the environmental water-availability conditions experienced by those bacteria will suggest certain trends highlighting the importance of particular glucans. As an example, maybe short linear glucans are more effective for bacteria experiencing shorter duration periods of low-water-content, whereas cyclic glucans provide better hydration during longer-duration dry conditions, or vice versa. Additionally, heterologous production of linear and cyclic glucans in species that would otherwise produce the other glucan

could highlight in what ways the glucans have redundant functions and which are unique, though getting the glucan biosynthesis machinery to work in other strains could prove very difficult.

Supplemental

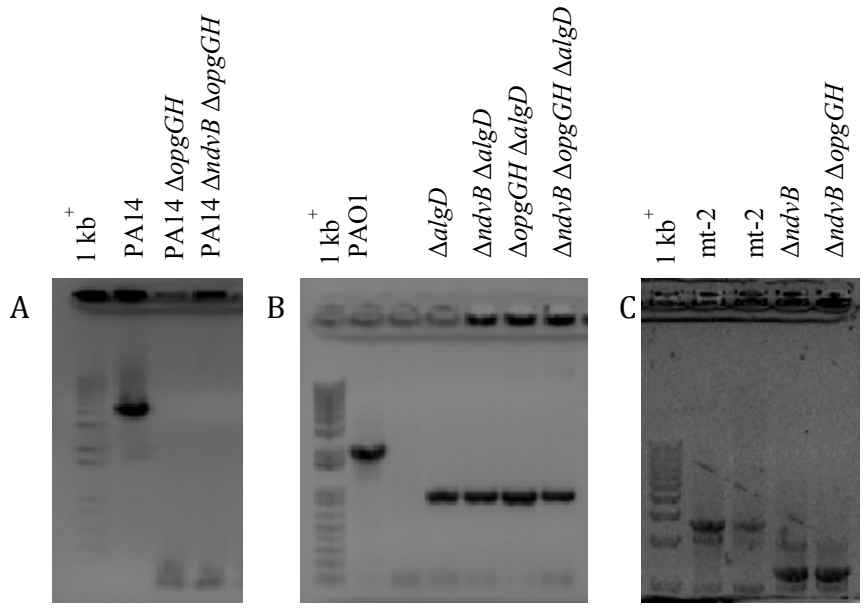

Figure S1. Confirmation of gene deletions. We observed a dramatic decrease in amplicon for A) *opgGH* from *P. aeruginosa* PA14 strains (4,108 bps in wild-type to 265 bps in mutants), B) *algD* from PAO1 strains (1,977 bps in wild-type to 868 bps in mutants), C) *ndvB* from *P. putida* mt-2 strains (4,402 bps in wild-type to 1561 bps in mutants).

Table S1. Mucoid phenotypes of wild type and mutants after 48 h incubation. Values represent relative mucoidy, 0 = non-mucoid, 1 = mucoid, 2- 3 = hyper-mucoid.

Strain	Description	Water-replete	PEG-amended media	
			-1.5 MPa Ψ	-2.5 MPa Ψ
PAO1		0	0	1
ΔalgD		0	0	0
ΔndvB		0	1	3
PW9520	*opgG*	0	2	3
PW9517	*opgH*	0	2	3
PW4001	*PA1689 (mdoB)*	0	0	1
PW4000	*PA1689 (mdoB)*	0	0	1
PW5447	*PA2657 (feuP)*	0	0	2
PW5446	*PA2657 (feuP)*	0	0	2
PW5445	*PA2656 (feuQ)*	0	0	1
PD0300	*mucA*	0	2	3
ΔrpoS		0	0	2
ΔrhlI		0	1	0.5
ΔlasI ΔrhlI		0	0	0
PW3054	*PA1136 (phzR)*	0	0.5	2
ΔrhlR		0	0	1
ΔlasR ΔrhlR		0	0	1
ΔlasR		0	0	1
PW4325	*qscR*	0	0	2
phoQ qscR		0	0	3
PW2548	*trxB2*	0	0	2
PW5783	*ohr*	0	0	2
PW5794	*tesA*	0	0	2
PW5793	*tesA*	0	1	2

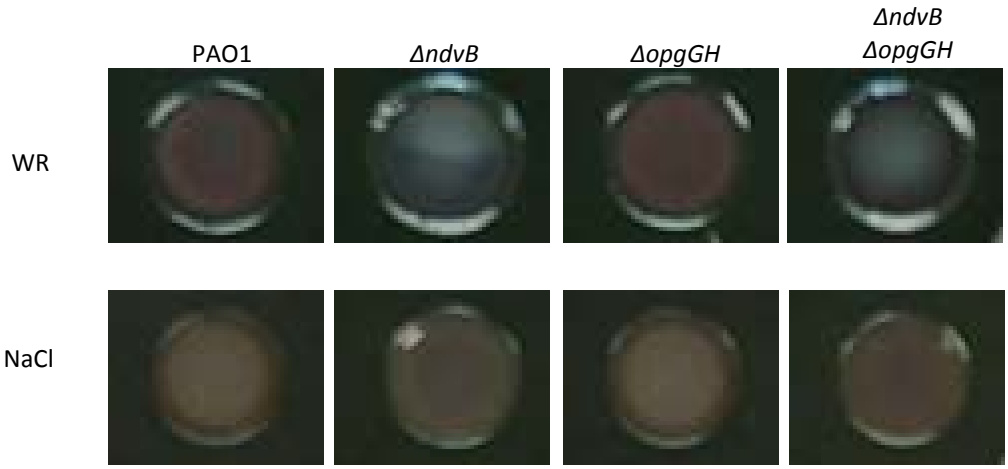

Figure S2. Congo red and Coomassie blue staining of 48 hour-old colonies on CTYE at 37° C. WR = Water-replete. S = -1.5MPa Ψ (Solute stress).

Table S2. Effect of temperature and water stress on mucoid phenotype. Values represent relative mucoidy, 0 = non-mucoid, 1 = mucoid, 2-3 = hyper-mucoid.

	28° C				37° C			
		NaCl	PEG			NaCl	PEG	
24 h	**Water Replete**	**-2.5 MPa Ψ**	**-1.5 MPa Ψ**	**-2.5 MPa Ψ**	**Water Replete**	**-2.5 MPa Ψ**	**-1.5 MPa Ψ**	**-2.5 MPa Ψ**
PAO1	0	0	0.5	1	0	0	0	1
Δ*ndvB*	0	0	1	1	0	0	0.5	1
Δ*opgGH*	0	0	2	2	0	0	1	2
Δ*ndvB* Δ*opgGH*	0	0	2	2	0	0	1	2
PA14	0	0	0	1	0	0	0	0
PA14 Δ*ndvB*	0	0	0	1	0	0	0	0
PA14 Δ*opgGH*	0	0	0	2	0	0	0	0
48 h								
PAO1	0	0	1	1	0	0	1	1
Δ*ndvB*	0	0	2	2	0	0	1	1
Δ*opgGH*	0	0	3	3	0	0	2	3
Δ*ndvB* Δ*opgGH*	0	0	3	3	0	0	2	3
PA14	0	0	0	1	0	0	0	1
PA14 Δ*ndvB*	0	0	0	2	0	0	0	1
PA14 Δ*opgGH*	0	0	0	2	0	0	0	2
72 h								
PAO1	0	0	1	1	0	0	1	1
Δ*ndvB*	0	0	2	3	0	0	1	1
Δ*opgGH*	0	0	3	3	0	0	3	3
Δ*ndvB* Δ*opgGH*	0	0	3	3	0	0	3	3
PA14	0	0	1	1	0	0	0	1
PA14 Δ*ndvB*	0	0	1	2	0	0	0	1
PA14 Δ*opgGH*	0	0	1	2	0	0	0	2

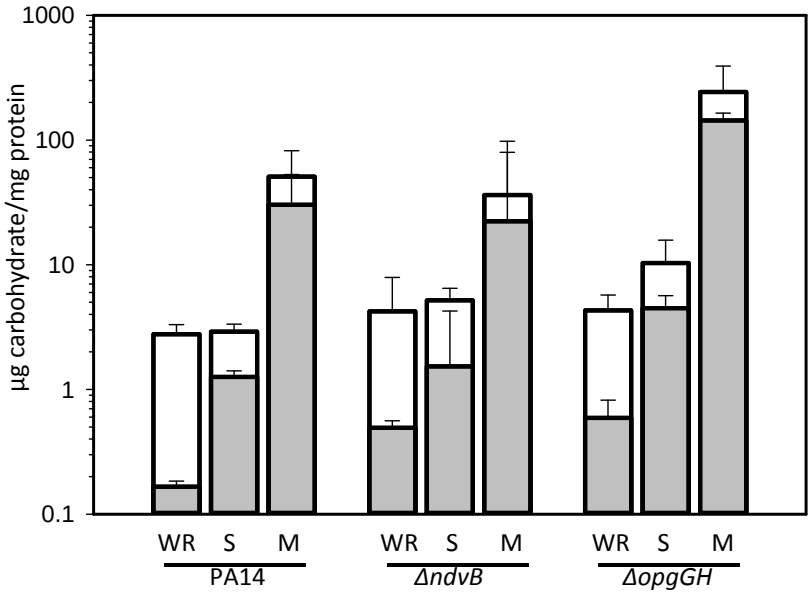

Figure S3. Effect of glucan deficiency on extracellular polysaccharide production in *P. aeruginosa* PA14. Water stress was imposed by amending media with NaCl or PEG to lower the water potential by 1.5 MPa. Open bars: total carbohydrates; grey bars: uronic acids. WR=water-replete, S= solute stress; M=matric stress. Values are mean ± SE of 2-3 replicates.

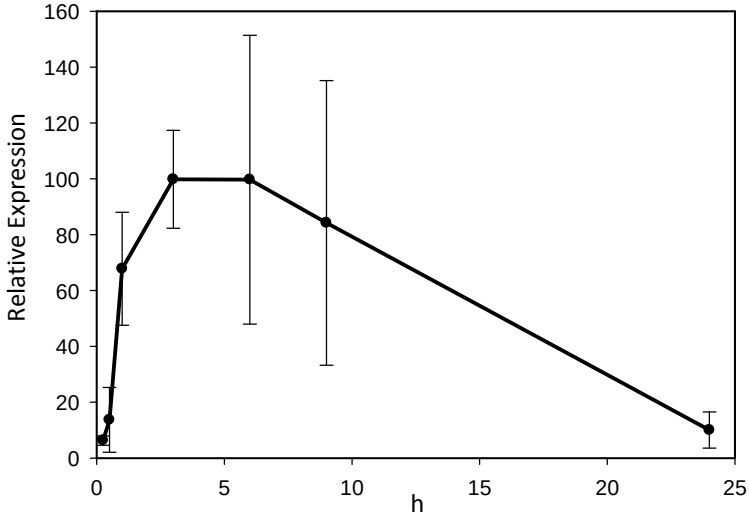

Figure S4. Relative expression of *algD* during growth under matric stress (-1.5 MPa Ψ) relative to water-replete conditions as measured by qRT-PCR. Values are mean of 2-4 replicates ± SE.

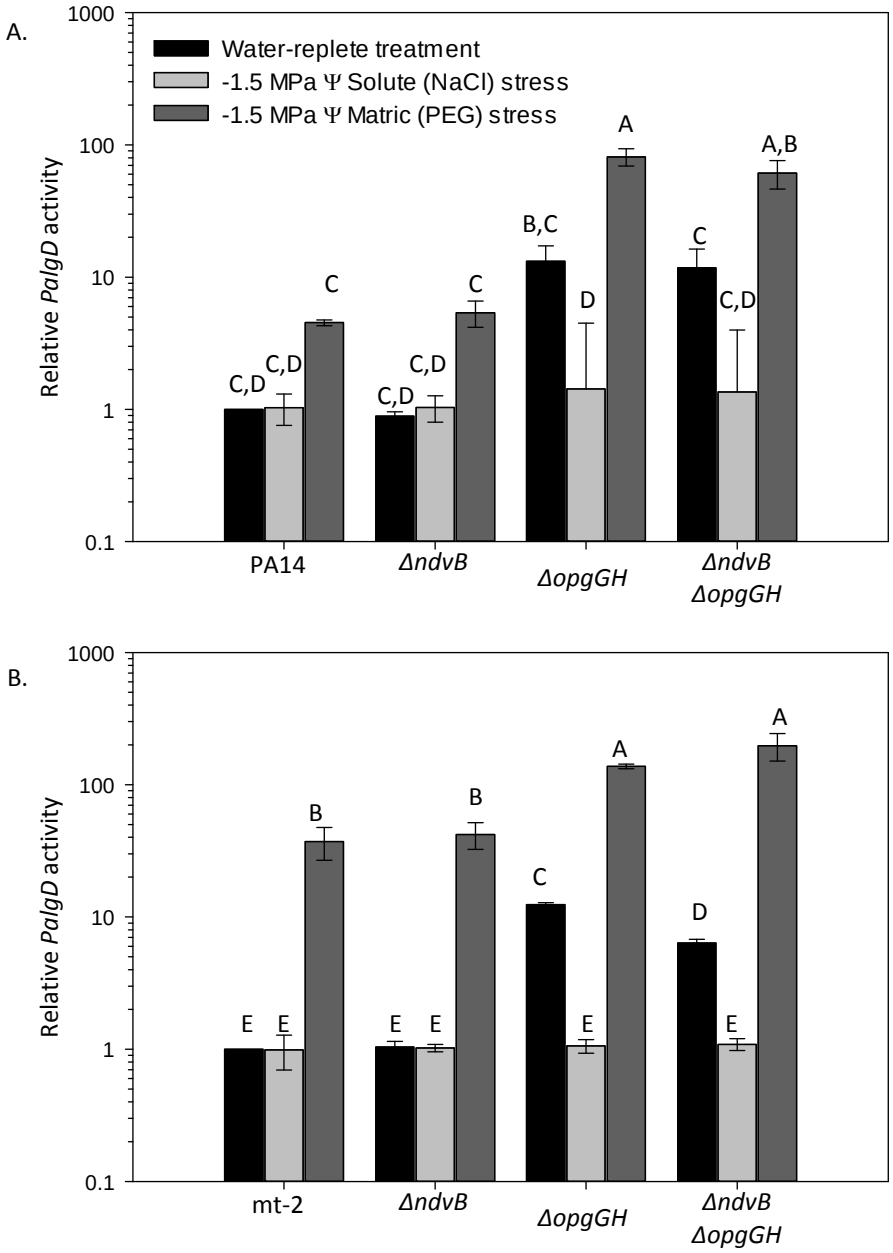

Figure S5. Envelope stress in A) *P. aeruginosa* PA14 and B) *P. putida* mt-2 and their *Δopg* & *Δndv* mutants as determined with a σ^{22}-dependent bioreporter. Values are mean relative to wild-type under water-replete conditions ± SE, n=3. Values not linked by same letter are statistically different using LSD ($\alpha \leq 0.05$).

Table S3. Statistical differences of pP_{PA}*algD-gfp*-G activity shown in Figure 5 within strain, between water-replete and reduced water potential due to solute (NaCl) or matric (PEG) stresses using a t-test, *p≤0.05, **p≤.01. NA denotes not measured.

Treatment	PAO1	*ΔndvB*	*ΔopgGH*	*ΔndvB ΔopgGH*
NaCl -0.25 MPa Ψ	**	NA	**	**
NaCl -0.75 MPa Ψ	*	NA	**	**
NaCl -1.5 MPa Ψ	-	NA	-	**
NaCl -2.5 MPa Ψ	-	*	**	**
PEG -0.05 MPa Ψ	-	NA	-	-
PEG -0.1 MPa Ψ	**	NA	**	**
PEG -0.2 MPa Ψ	**	NA	*	-
PEG -0.25 MPa Ψ	-	-	*	**
PEG -0.5 MPa Ψ	-	-	**	*
PEG -0.75 MPa Ψ	-	**	*	**
PEG -1.0 MPa Ψ	*	**	*	*
PEG -1.5 MPa Ψ	*	*	*	*
PEG -2.5 MPa Ψ	**	**	*	*

Table S4. Statistical differences of pP_{PA}*algD-gfp*-G activity shown in Figure 5 between PAO1 and glucan-deficient mutants within the same treatment under varying water availability conditions using a t-test, *p≤0.05, **p≤.01. NA denotes not measured.

Treatment	*ΔndvB*	*ΔopgGH*	*ΔndvB ΔopgGH*
Water-replete	-	**	**
NaCl -0.25 MPa Ψ	NA	**	**
NaCl -0.75 MPa Ψ	-	*	*
NaCl -1.5 MPa Ψ	-	*	*
NaCl -2.5 MPa Ψ	-	-	-
PEG -0.05 MPa Ψ	NA	-	-
PEG -0.1 MPa Ψ	NA	-	**
PEG -0.2 MPa Ψ	NA	*	*
PEG -0.25 MPa Ψ	-	**	**
PEG -0.5 MPa Ψ	-	**	**
PEG -0.75 MPa Ψ	*	**	**
PEG -1.0 MPa Ψ	*	**	**
PEG -1.5 MPa Ψ	**	**	**
PEG -2.5 MPa Ψ	**	**	**

Table S5 Analysis of relative mucoidy of 48 h colonies grown on PEG amended CTYE (-1.5 MPa Ψ) with various concentrations of arabinose to assess complementation of glucan biosynthesis genes. Values represent relative mucoidy, 0 = non-mucoid, 1 = mucoid, 2- 4 = hyper-mucoid.

	pHERD-30T			
Arabinose conc. (%)	PAO1	Δ*ndvB*	Δ*opgGH*	Δ*ndvB* Δ*opgGH*
0	1	2	3	4
0.1	1	2	3	3
0.5	1	2	3	3
	pHERD-*ndvB*			
Arabinose conc. (%)	PAO1	Δ*ndvB*	Δ*opgGH*	Δ*ndvB* Δ*opgGH*
0	1	2	3	4
0.1	1	2	3	4
0.5	1	2	3	3
	pHERD-*opgGH*			
Arabinose conc. (%)	PAO1	Δ*ndvB*	Δ*opgGH*	Δ*ndvB* Δ*opgGH*
0	1	2	1	2
0.1	1	2	2.5	3
0.5	1	2	2.5	3

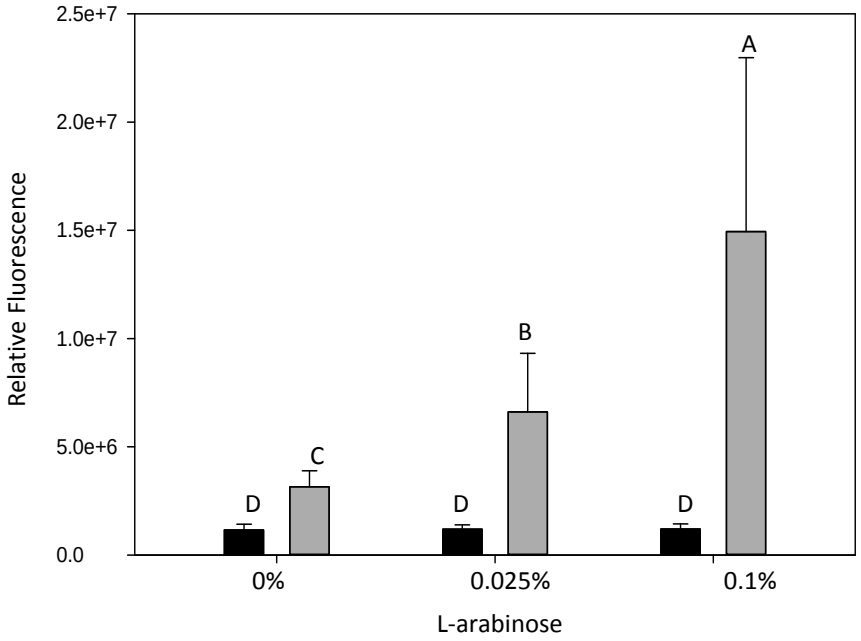

Figure S6. Demonstration of leaky, but arabinose-responsive P_{BAD} promoter expression in PAO1. Values are mean fluorescence. Black bars represent cells with empty pHERD-30T, gray bars represent cells with pHERD-*gfp*. Values are mean ± SE, n=3, those with the same letter are not statistically different based on a LSD test ($p \leq 0.05$).

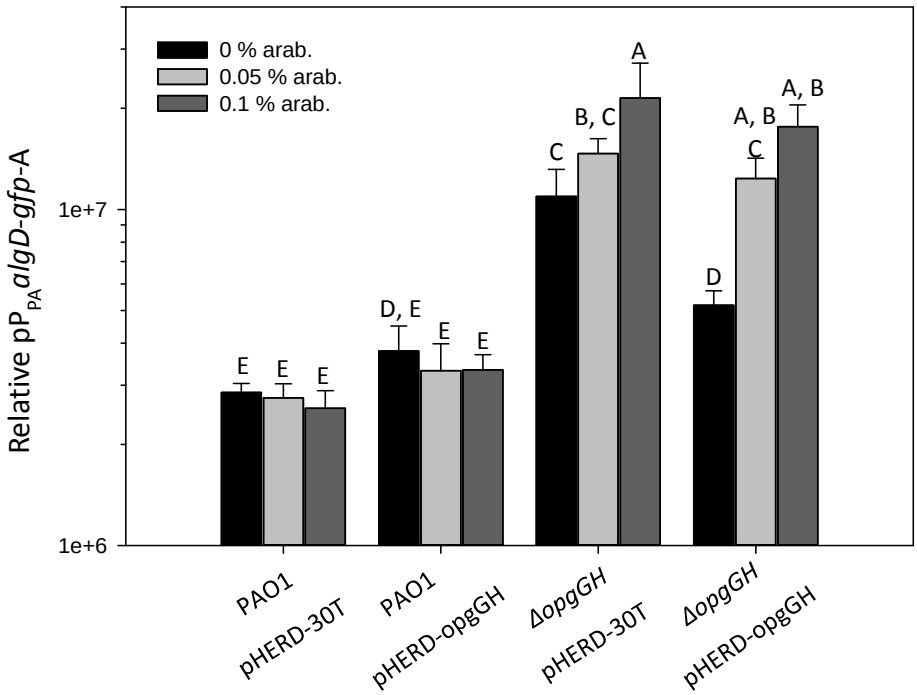

Figure S7. Influence of arabinose conc. on complementation of *ΔopgGH* with pHERD-*opgGH* measured by pP$_{PA}$*algD-gfp*-A activity. Values are mean ± SE, n=3. Bars with the same letter are not statistically different based on an LSD test ($p \leq 0.05$).

Table S6. Whole-cell fatty acid profiles of PAO1 and glucan-deficient mutants grown 48 h on unamended media (water-replete), or media amended with NaCl (solute stress) and PEG (matric stress) to reduce the water potential 1.5 MPa. Values are mean ± SE n=6. Bold values are statistically different from wild-type PAO1 within the same treatment based on an LSD test ($p \leq 0.05$).

	Water-replete							
	PAO1		*ΔndvB*		*ΔopgGH*		*ΔndvB ΔopgGH*	
	Avg	SE	Avg	SE	Avg	SE	Avg	SE
10:0 3OH	3.45	0.24	3.91	0.26	3.70	0.12	**4.77**	0.68
12:0	2.48	0.06	2.30	0.08	2.74	0.09	2.82	0.22
12:0 2OH	4.68	0.68	4.71	0.48	5.03	0.22	**5.44**	0.59
12:0 3OH	3.93	0.54	3.90	0.37	4.39	0.21	4.46	0.26
16:1 w7c	10.86	0.28	**8.65**	0.30	10.50	0.61	**7.63**	0.24
16:0	24.11	0.51	24.70	0.30	22.80	0.41	23.38	0.66
17:0 cyclo	3.47	0.33	**5.15**	0.41	3.91	0.46	**5.87**	0.58
18:1 w7c	39.53	0.53	**33.41**	0.89	38.23	1.16	**28.62**	1.30
18:0	0.68	0.03	0.67	0.03	0.74	0.02	1.60	1.03
19:0 cyclo w8c	5.84	0.58	**11.39**	1.17	6.93	1.42	**12.82**	1.72
	Solute stress							
	PAO1		*ΔndvB*		*ΔopgGH*		*ΔndvB ΔopgGH*	
	Avg	SE	Avg	SE	Avg	SE	Avg	SE
10:0 3OH	3.77	0.28	4.01	0.20	3.72	.09	3.86	0.13
12:0	3.34	0.16	3.02	0.13	3.25	0.10	2.84	0.11
12:0 2OH	5.15	0.25	4.87	0.21	4.91	0.15	4.71	0.13
12:0 3OH	4.65	0.16	4.26	0.23	**2.32**	0.94	4.24	0.10
16:1 w7c	11.20	2.60	**7.34**	0.38	9.43	0.57	**8.55**	0.74
16:0	21.55	3.69	**25.63**	0.62	24.17	0.66	**24.96**	0.86
17:0 cyclo	4.66	0.34	**5.52**	0.43	4.82	0.46	**5.05**	0.43
18:1 w7c	34.00	0.29	**29.86**	0.45	33.77	1.18	**30.75**	0.62
18:0	0.69	0.05	0.97	0.22	0.76	0.07	0.75	0.03
19:0 cyclo w8c	8.89	0.41	**12.79**	1.04	9.52	0.99	**12.94**	1.12
	Matric stress							
	PAO1		*ΔndvB*		*ΔopgGH*		*ΔndvB ΔopgGH*	
	Avg	SE	Avg	SE	Avg	SE	Avg	SE
10:0 3OH	4.09	0.17	4.69	0.37	**6.83**	0.23	**7.57**	0.38
12:0	3.31	0.11	3.31	0.16	**7.26**	0.37	**7.33**	0.33
12:0 2OH	5.64	0.14	5.45	0.16	**8.15**	0.37	**7.79**	0.30
12:0 3OH	5.10	0.17	3.84	0.42	**7.62**	0.42	7.34	0.70
16:1 w7c	10.66	0.58	11.31	1.17	11.33	0.43	10.98	0.22
16:0	25.56	0.66	25.20	0.83	**22.31**	0.21	**22.55**	0.75
17:0 cyclo	3.98	0.35	3.15	0.55	**2.35**	0.15	3.78	0.35
18:1 w7c	33.26	0.24	**35.28**	1.62	**28.51**	0.65	**26.06**	0.75
18:0	0.50	0.01	0.48	0.02	1.00	0.35	0.66	0.25
19:0 cyclo w8c	6.41	0.65	5.94	1.46	4.49	0.35	6.57	0.47

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CHAPTER 5. GENERAL DISCUSSION

Water-availability is one of the most critical characteristics of bacterial habitats. The availability of water to the cell is a function of the external water potential, with low environmental water potential reducing the propensity of water to enter the cell. The total water potential of a given environment is determined primarily by solute and matric potential. Reduced water potential and availability by these two components can cause two very different stresses on cells, particularly when concerning the periplasm of gram negative bacteria. Under high solute conditions, osmolytes diffuse through outer membrane porins, bathing the periplasm with water of reduced potential, imposing a stress due the need to balance the water potential across the inner membrane with compatible solutes. Under a low-water-content matric stress, limited environmental water associates with the surrounding matrix via inter-molecular forces, limiting the water potential external to the entire cell. We know that the cytosol is hydrated by compatible solutes, but until these studies, it was unknown how the periplasm was hydrated.

We now have strong evidence that matric stress triggers the accumulation of periplasmic glucans to hydrate the periplasm. “Osmoregulated” periplasmic glucans have a well-understood role in reducing the periplasmic water potential during hypo-osmotic (low-solute) conditions. Glucan accumulation prevents over-swelling of the cytoplasm by increasing the osmolyte concentration fixed in the periplasm, reducing the water potential of the compartment which will retain water in the periplasm to push back against the expanding inner membrane as well as limit the water potential differential across that membrane. During conditions of low-water-content, the reduced external matric potential requires the periplasm to somehow reduce its own water potential. The accumulation of linear periplasmic glucans under matric stress does that (Chapter 3, Figure 1). It may seem rather intuitive that periplasmic glucans would fulfill this role under

desiccating conditions, but prior to these studies, no one had looked at glucan accumulation under these conditions. This discovery adds a new facet of understanding to the role of periplasmic glucans. The shift to more anionic populations of linear glucans (Chapter 3, Figure 3) would both further reduce the periplasmic water potential and likely improve the glucans' hydrophilic properties, improving hydration. Previous studies of *P. aeruginosa* linear glucans isolated from low solute conditions found only succinyl group decorations. However the presence of a potential *E. coli mdoB* (*opgB*) ortholog suggests that the *P. aeruginosa* linear glucan can be substituted with phosphoglycerol. Perhaps only under low-water-conditions the glucan is decorated with phosphoglycerol groups.

Unfortunately, our inability to isolate and quantify the cyclic glucan from biofilm cells (Chapter 3, Figure 2) prevents us knowing if cyclic glucans similarly accumulate or become more anionic under matric stress conditions. We do not know why we were unable to isolate cyclic glucans from biofilm cells, though we have isolated it from liquid cultures. We can be reasonably assured that the cyclic glucan is present in wild-type PAO1 biofilm cells, as the inability to produce the cyclic glucan has significant consequences on the envelope stress experienced by the cells (Chapter 4, Figure 3). Prior to our studies, no one appears to have tried isolating either linear or cyclic glucans from unsaturated biofilms. It is possible that during biofilm growth, low glucan abundance, changes in glucan size or interactions with other molecules prevent cyclic glucan isolation.

We know that deficiency of either glucan causes many pleotropic phenotypic changes. Perhaps most striking and relevant to glucan-mediated hydration of the periplasm under low-water-content conditions, there is an increase in envelope stress experienced by glucan deficient mutants under matric stress (Chapter 4 Figures 1-3), suggesting that glucans are moderating the

effect of matric stress on the cell envelope, presumably by hydrating the periplasm. Interestingly, there are distinct differences in these phenotypes. Cyclic glucan deficient mutants appeared to have similar envelope stress as PAO1 under all conditions except once a threshold of moderate to high matric stress is encountered, whereas linear glucans experienced an elevated envelope stress under all conditions except for high solute (Chapter 4, Figure 5). These results seem to suggest that the cyclic glucan only plays an important role in moderating envelope stress during moderate to high matric stress. Linear glucans seem to play an important role in the periplasm under almost all conditions. It is not surprising that linear glucan deficiency is stressful on the cells at low solute concentrations; certainly periplasmic glucans are known to accumulate under these conditions to regulate periplasm osmolarity - their absence would be expected to be stressful. It is perhaps a little surprising that linear glucan deficiency increases the envelope stress at low to moderate matric stress conditions. This may suggest that linear glucan are involved in precise, but critical, adjusting of the periplasmic osmolarity under all but high solute conditions, or rather linear glucans have a role beyond hydrating the periplasm, perhaps directly stabilizing envelope proteins. The ability of over-expressed linear glucans to rescue the cyclic glucan mutant from an elevated envelope stress response in a non-reciprocal (Chapter 4, Figure 6) fashion suggests that there is some functional redundancy between the glucans: linear glucan may be more versatile, possibly due to differences in their respective mode of action, or smaller size. These differences are highlighted further by differences in survival under matric stress (Chapter 3, Figure 5), motility of cyclic glucan-deficient mutants (Chapter 4, Figure 7), and dissimilar changes in fatty acid profiles during different water-stress conditions between linear and cyclic glucan-deficient mutants (Chapter 4, Table S5). These potential differences in modes

of action or conditions of glucan importance may shed light on why Pseudomonads maintain both glucans, while other species produce one or the other.

Future Directions

While the model that accumulation of linear periplasmic glucans under low-water-content conditions facilitates hydrating the periplasm greatly expands our understanding of the function of periplasmic glucans and matric stress tolerance, many questions remain to be answered. Perhaps most pressing would be determining whether cyclic glucans accumulate under matric stress in biofilm cells. If cyclic glucans are present, but with low abundance, increasing the biomass from which we isolate glucans may help us identify cyclic glucans. If there is some other molecule that is binding and interfering with our isolation method, we may have to use a different method to quantify the cyclic glucan. Although size-exclusion chromatography is very effective in isolating glucans perhaps antibodies to the cyclic glucan could be produced allowing for quantification of cyclic glucans from cell extracts using an ELISA assay. Unfortunately, producing good antibodies for carbohydrates can be a challenge. Alternatively, using scanning microprobe matrix-assisted laser desorption/ionization (SMALDI) mass spectrometry may allow us to identify and quantify the cyclic glucan without isolating it. However, the non-uniform populations of glucans (different sizes and charges) may make differentiating cyclic glucan from other components quite difficult.

Another topic of further exploration would be enzymatic regulation of glucan biosynthesis. We observed that linear glucan biosynthesis is transcriptionally regulated, but there is likely further regulation at the enzymatic activity level of glucan biosynthesis. By measuring

periplasmic glucan production under different water stress conditions shortly after disruption of protein synthesis with chloramphenicol we could observe changes in enzymatic activity independent of new protein synthesis. Of course this would only work for the cyclic glucan if we are able to identify and quantify it.

If we could purify enough cyclic glucan, we could determine if they become more anionic under matric stress, which would be beneficial to enhancing understanding of the role of linear and cyclic glucans in regulating periplasm water potential. Continuing with characterizing anionic properties of the glucans, it would be interesting to understand specifically how the compositions of the anionic decorations differ among the populations of glucans. We observed three distinct populations of linear glucans, present in both water-replete and matric stress conditions; it's conceivable that they are all decorated similarly, possibly with increasing numbers of succinyl groups, or that they are substituted with phosphoglycerol as well. Measuring organic phosphate concentrations in the anionic glucan populations with a colorimetric assay or analyzing glucans with GC-MS would help us understand how the glucans are substituted.

An important part of our model is that one function of periplasmic glucans is to hydrate the periplasm. Whether they do this solely by increasing the periplasmic fixed osmolyte concentration or whether they have hygroscopic, water-retention properties is unknown. Showing that the glucans have water-retention properties would provide direct support for our model of glucan mediated periplasmic hydration. It is likely that increased anionic decorations would improve the glucans hydrophilic characteristics, improving their hydrating abilities. Measuring how the water-retention properties of the glucans change with increasing anionic substitutions would further help our understanding of glucan-mediated hydration.

Lastly, understanding how glucan deficiency might affect *P. aeruginosa* fitness in dry natural environments, such as soil, would highlight glucan importance. Changes in survival, motility or attachment to surfaces (biotic and abiotic) could occur due to disruptions of the cell envelope and associated proteins without glucan-mediated periplasm hydration. Such experiments could help us understand the evolutionary forces acting to maintain two glucans in the majority of the Pseudomonads.

APPENDIX I. INTERACTIONS BETWEEN *PSEUDOMONAS PUTIDA* PURLI AMYLOID-LIKE FIBER AND EXOPOLYSACCHARIDES

This appendix covers research I contributed towards understanding the role of a putative, amyloid-like fiber protein produced by *Pseudomonas putida* in biofilm formation and its interaction with exopolysaccharides. Amyloid like-fibers are extracellular fimbre/appendages produced by various species (1, 4) that have been implicated in both cell-cell and cell-surface interactions (4, 5). Amyloid fibers are comprised of repeat subunits that self-assemble with the aid of an outer membrane nucleator protein.

An ortholog to the *E. coli* curli amyloid like fiber called curli was identified in *P. putida*, and was tentatively named purli. Sequences for PP3399 (PsgA; subunit) and PP3398 (PsgB; nucleator) repeat regions were identified using RADAR (www.ebi.ac.uk/Tools/Radar) and manual comparison between *P. putida* KT2440 sequences and *E. coli* CsgA and CsgB sequences. For PsgA, two distinct repeat patterns were identified: there were three repeat R1 (A-C) regions and nine repeat R2 (A-I) regions separated by a linker (Figure 1A). PsgB contained five repeat regions (R1-5) (Figure 2A). Orthologs to *P. putida* KT2440 PsgA and PsgB were identified in five other Pseudomonas strains (*P. putida* strains F1, GB1, and w619, *P. entomophila* L48 (PSEEN), and *P. fluorescens* Pf0-1) using www.pseudomonas.com database in May, 2011. Alignments were constructed manually and using ClustalW2 for repeat regions within a strain and across different strains (Figure 1B & C and Figure 2B & C).

Alignments were visualized using weblogo (www.weblogo.berkeley.edu) to identify and highlight conserved residues. For PsgA, repeat R2 showed more similarity among a specific repeat region across strains (for example, all R2 A's) than when comparing all the R2 regions

within a strain. The PsgB repeats have some similarity to the PsgA R2 regions sharing a similar consensus backbone. Heterologous expression of PsgA was attempted using an *E. coli* expression system. The *psgA* gene with and without the signal peptide sequence were ligated into

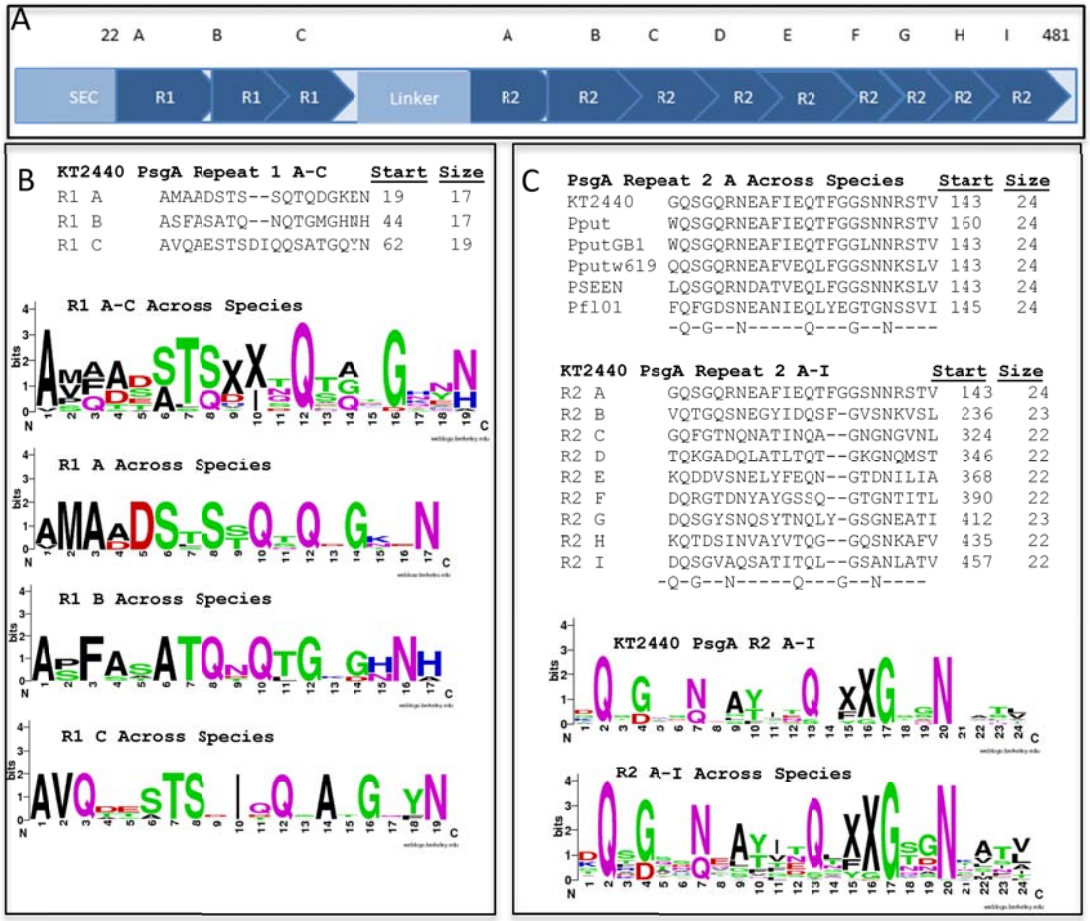

Figure 1. A) Schematic diagram of the primary PsgA structure illustrating the predicted SEC, linker, and R1 and R2 repeat domains. Not to scale B) Alignment of KT2440 PsgA R1 repeats and WebLogo representations of R1 A-C alignments across *P. putida* strains KT2440, F1, GB1, and W619, *P. entomophila* L48 (PSEEN), and *P. fluorescens* Pf0-1 to identify conserved residues. C) Alignments of PsgA repeat 2 A across species, KT2440 PsgA repeat 2 A-I and WebLogo representation of repeat 2 A-I within KT2440 and across species.

pET28a at NcoI and XhoI restriction sites. A positive control pET21 BphP1 was obtained from Liang Wu. Plasmids were transformed into *E. coli* BL21λ De3 RILP cells and grown in Lysogeny broth until an OD_{600nm} =0.6 was reached, then IPTG was added (1mM final concentration) to induce *psg*A expression. Samples were incubated for 18 h at 18° C, pelleted by centrifugation and lysed by sonication (100 V, 12 cycles of 30 s on, 30 s off). The lysate was

poured through a nickel affinity column and then the His-tagged proteins were eluted from the column with imidazole.

The success of the isolation was unclear. BphP1 was clearly produced and isolated (Figure 3). However, while there appears to be a product at 50.7 and 48 kDa (the predicted sizes

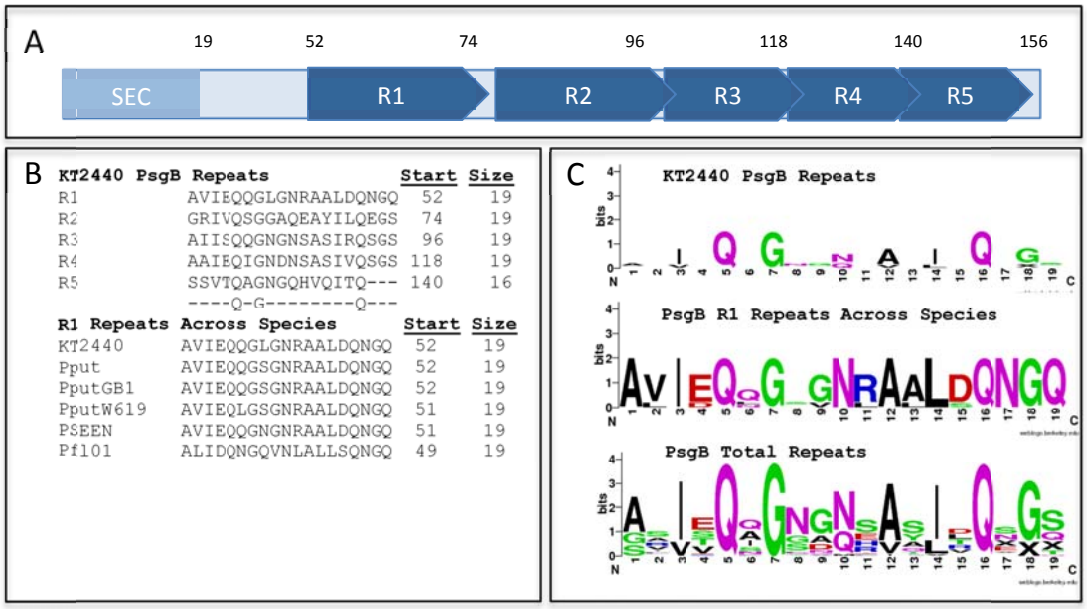

Figure 2. A) Schematic diagram of the primary PsgB structure illustrating the repeat R1 through R5 domains. Not to scale. B) Alignment of KT2440 PsgB repeats (R1-R5) and PsgB R1 repeats across *P. putida* strains GB1, KT2440, F1, and W619, *P. entomophila* L48 (PSEEN), and *P. fluorescens* Pf0-1. C) WebLogo representations of conserved residues of PsgB R1 repeats of KT2440, across species, and all repeats across species.

for the total and no signal peptide sequence PsgA, respectively) the product makes a smear rather than a discreet band. While this smear appears to be approximately the correct size, it is also present in the empty pET28a sample, making it unclear if the observed product is PsgA.

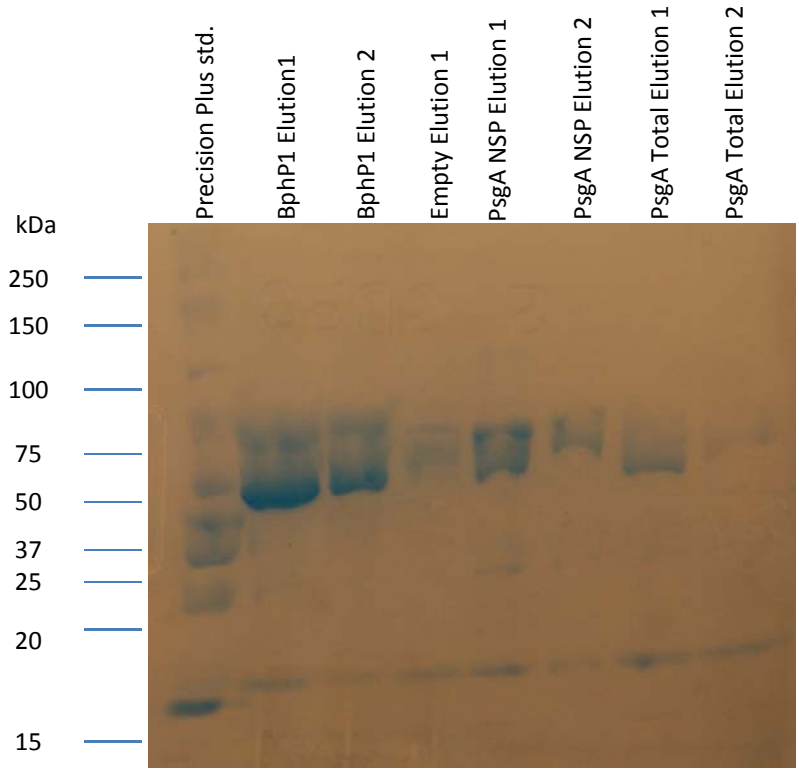

Figure 3. Heterologous isolation of His-tagged PsgA in *E. coli* BL21λ De3 RILP following nickel affinity purification. IPTG induced expression of PsgA with no PsgA signal peptide sequence (NSP) or total PsgA protein using the pET28a expression system. Negative empty pET28a control and positive pET21 BphP1 control.

Previous analyses suggested there was an increase in *psgA* expression after 6 and 24 h of growth on media amended with PEG compared to NaCl to reduce the water potential by 1.5 MPa (8). However, these previous studies had poor amplification efficiencies and when repeated with acceptable efficiencies, no increase in expression was observed (Figure 4A), nor was there any increase in expression for other purli genes (Figure 4 B & C) that we included in these analyses. Interestingly, all of the purli genes do have reduced expression with both NaCl and PEG treatment at 24 compared to 6 h. As a positive control, we included the alginate biosynthesis operon gene *alg*8 since previous work (6) had shown that its expression was highly elevated under matric compared to solute stress.

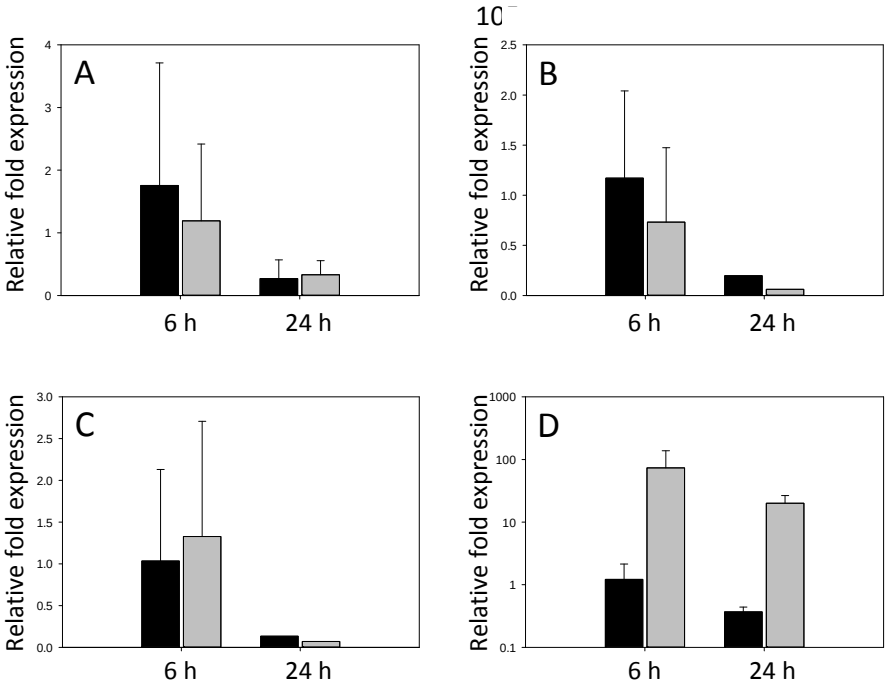

Figure 4. Relative fold expression of A) *psgA*, B) *psgB*, C) *psgX*, D) *alg8* during growth on ½ 21C glucose-succinate media amended with either NaCl (black bars) or PEG (grey bars) to reduce the water potential by 1.5 MPa. Values are the mean ± SD expressed relative to water-replete condition using the method of Livak et al. (7).

We were interested in exploring the possibility that the putative amyloid fiber-like protein Purli interacted with one or more exopolysaccharides, contributing to cell-cell and cell-surface interactions. Our lab had previously constructed null mutants in all *P. putida* exopolysaccharide loci (3, 9) with the exception of the recently described (10) putida exopolysaccharide B (peb) locus. Consequently, I constructed deletions of the *peb* biosynthesis operon that were then were pyramided on top of mutants containing deletions of the other exopolysaccharides (Bcs-bacterial cellulose; Alg-alginate; Pea-putida exopolysaccharide A) and purli. Briefly, the *peb* operon (PP1795-PP1788) was deleted by amplifying the Gm resistance cassette and arms of homology for *peb* from pEX18Ap-ΔPP1795-1788 (10) using PCR with Phusion High fidelity Taq Polymerase (Thermo), producing blunt ends, ligated into pTok2SacB digested with SmaI (Invitrogen), creating pTok2SacB-*Δpeb* and transformed into *E. coli* α-select cells (Bioline). Strains were transformed by tri-parental mating with donor strain α-select with pTok2SacB-*Δpeb* and helper strain DH5α with pRK2073 on LA and subsequent selection for transformants on TSA with Gm and Rf. After growth on TSA containing 10% sucrose, colonies were patched onto

TSA + Tc and TSA + Gm, to identify double recombinants Δ*peb* mutants which were Tc^S, Gm^R. Gm markers were removed with pFLP2Tc flp recombinase.

The binding of Congo red and Coomassie blue to bacterial colonies is a common visual phenotypic screen. Congo red binds β linkages of polysaccharides while Coomassie blue binds proteins. To increase exopolysaccharide production (and therefore Congo red-binding), strains were transformed with pWspR19, which harbors a constitutive diguanylate cyclase producing elevated levels of the intracellular signal molecule c-di-GMP (13). High concentrations of c-di-GMP induce exopolysaccharide production, often through allosteric activation of biosynthesis enzymes or increased exopolysaccharide gene expression (12, 14, 15). Aliquots (5 µl) of cell suspensions (OD_{600nm} = 0.1) were spotted onto Lysogeny broth agar plates amended with 40 µg/ml Congo red and 15 µg/ml Coomassie blue and incubated at 25° C.

After 2 d (Figure 5A, Table 1), many pWspR19-containing strains exhibited a strong Congo red-binding phenotype and by 7 d (Figure 5B, Table 1) they had a wrinkled phenotype while those with the empty pVSP61 vector appeared white and non-wrinkly after 7 days. As reported previously (9), *Δpea* mutants have reduced Congo red-binding and do not become wrinkled. This is a dominant feature of strains with *pea* mutations pyramided with other exopolysaccharide mutations. Interestingly, some *ΔbcsΔpsgA* strains showed a distinctive phenotype in which the majority of the colony was white, except for a scattered population of strong Congo red-binding cells in the center of the colony. This phenotype is not consistent among all *ΔbcsΔpsgA* strains, but rather appears to be due to secondary mutations that can develop when purli and a polysaccharide such as cellulose are not produced. Recent observations suggest that light (a variable we did not control) plays a major role in the development of the wrinkly phenotype (data not shown) and may mask some Congo red-binding visualization if not

properly controlled for.

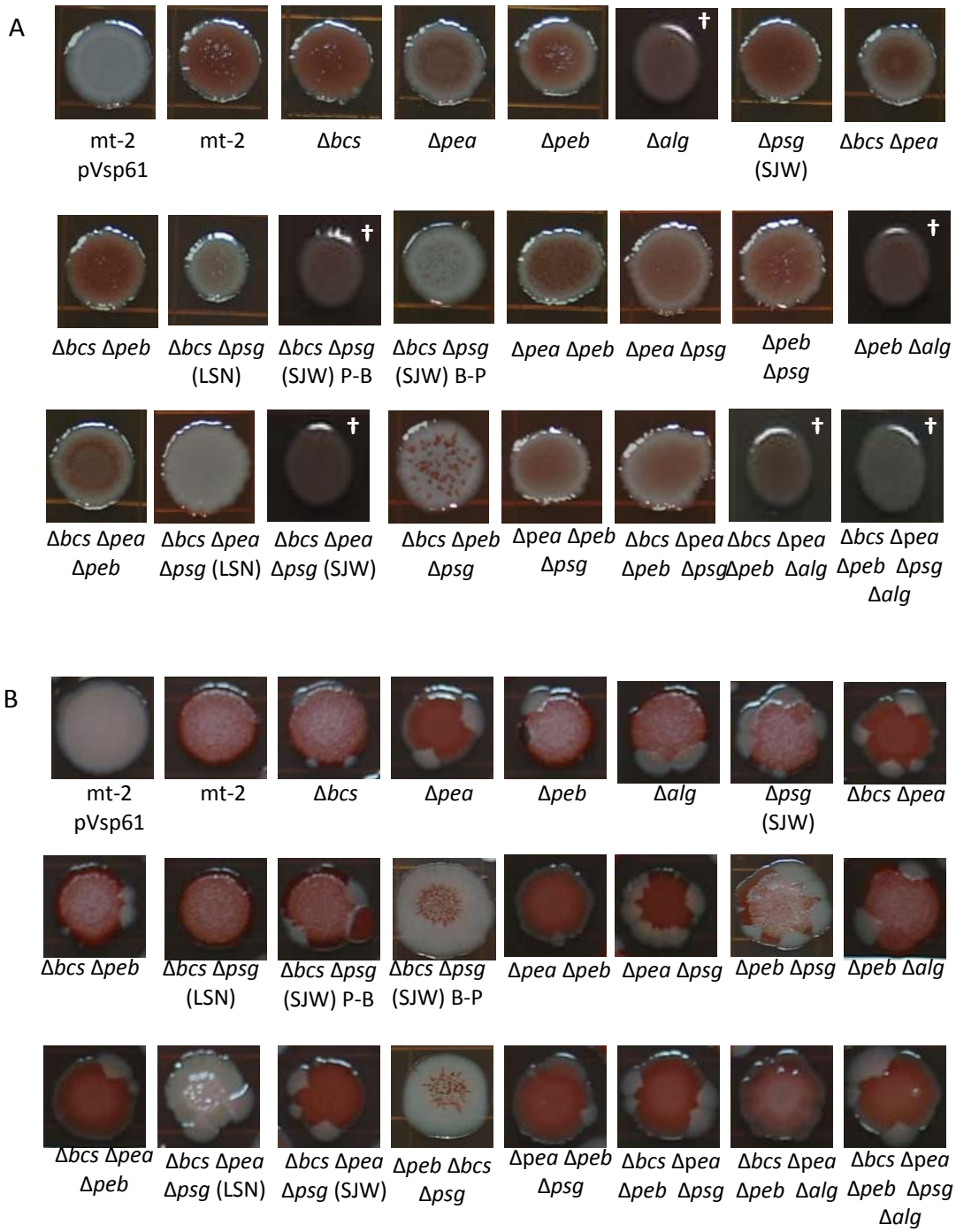

Figure 5. Colony phenotype of mutants grown on CTYE solid medium amended with Congo red and Coomassie blue A) at 2d and B) 7 d. Strains with pVsp61 have wild-type levels of c-di-GMP; all other strains contain pWspR19, elevating c-di-GMP concentrations. Strains names followed by LSN and SJW indicate source of duplicate strains. P-B and B-P indicate order of gene deletion in duplicate strains. † denotes image from alternate experiment, may not be comparable to other strains.

Table 1. Summary of typical phenotypes associated with Congo red binding, pellicle formation and biofilm attachment assays.

Strain	**Day 2 Congo red binding**	**Day 7 Congo red binding**	**Day 7 Colony texture**	**Pellicle phenotype**	**Biofilm Attachment**
mt-2	Wild-type	Wild-type	Wrinkled	Robust	Wild-type
Δbcs	Wild-type	Wild-type	Smooth	Robust	Wild-type
Δpea	Reduced	Reduced	Wrinkled	Disintegrated	Wild-type
Δpeb	Slightly reduced	Wild-type	Wrinkled	Fractured	Wild-type
Δalg	Wild-type	Wild-type	Wrinkled	Robust	Wild-type
Δpsg (LSN)	Not tested	Not tested	Not tested	Not tested	Elevated
Δpsg (SJW)	Wild-type	Wild-type	Usually wrinkled	Robust	Wild-type
ΔbcsΔpea	Reduced	Reduced	Smooth	Not tested	Wild-type
ΔbcsΔpeb	Wild-type	Wild-type	Usually wrinkled	Fractured	Wild-type
ΔbcsΔpsg (LSN)	Reduced	Wild-type	Wrinkled	Not tested	Not tested
ΔbcsΔpsg (SJW) P-B	Wild-type	Wild-type	Wrinkled	Fractured	Elevated
ΔbcsΔpsg (SJW) B-P	Reduced with scattered sub-pop.	Reduced with scattered sub-pop.	Smooth	Disintegrated	Wild-type
ΔpeaΔpeb	Reduced	Reduced	Smooth	Disintegrated	Wild-type
ΔpeaΔpsg	Reduced	Slightly reduced	Smooth	Fractured	Wild-type
ΔpebΔpsg	Slightly reduced	Slightly reduced	Usually wrinkled	Robust	Elevated
ΔpebΔalg	Wild-type	Wild-type	Wrinkled	Robust	Wild-type
ΔbcsΔpeaΔpeb	Reduced	Reduced	Smooth	Disintegrated	Wild-type
ΔbcsΔpeaΔpsg (LSN)	Very little	Very little	Smooth	Not tested	Not tested
ΔbcsΔpeaΔpsg (SJW)	Reduced	Slightly reduced	Smooth	Fractured	Wild-type
ΔbcsΔpebΔpsg	Reduced with scattered sub-population	Reduced with scattered sub-population	Smooth	Disintegrated	Wild-type
ΔpeaΔpebΔpsg	Reduced	Reduced	Smooth	Disintegrated	Wild-type
ΔbcsΔpeaΔpebΔpsg	Reduced	Reduced	Smooth	Disintegrated	Wild-type
ΔbcsΔpeaΔpebΔalg	Reduced	Reduced	Smooth	No pellicle	Reduced
ΔbcsΔpeaΔpebΔpsgΔalg	Reduced	Reduced	Smooth	Disintegrated	Reduced

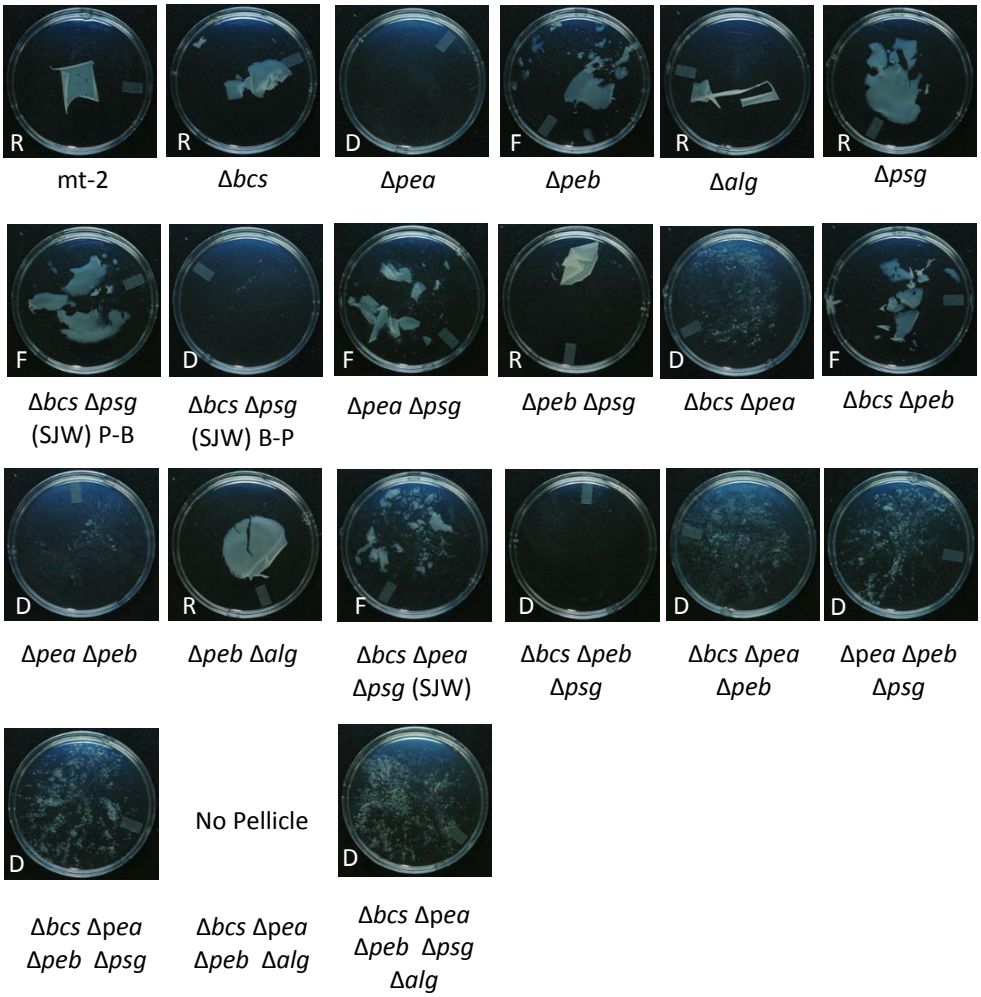

Figure 6. Representative analysis of pellicle strength of wild type and various mutants with pWspR19 after 7 d growth in LB and transfer to water. R = robust (strong), F = fractured (intermediate), D = disintegrated (weak). Strains are as described in Figure 5.

Biofilm formation at the air-liquid interface called pellicles is used to assess the role of exopolymeric matrix components in cell-to-cell interactions. The strength of the pellicle is often used to gauge cell to cell interactions. We grew cells in Lysogeny broth statically for 7 d in 6 well plates at 25° C, then gently transferred the pellicle to a petri dish with water and noted pellicle strength based on whether the film stayed together, fractured into intermediately sized pieces or disintegrated. Again, we used strains transformed with pWspR19 to elevate c-di-GMP levels and consequently exopolysaccharide and possibly other adhesion production (2).

Consistent with the Congo red-binding results, strains with a *Δpea* mutation had weak pellicles, typically disintegrating or fracturing upon transfer (Figure 6, Table 1). This indicates that Pea contributes to robust Congo red-binding and firm pellicle phenotype. Also consistent with the Congo red results, there were inconsistencies between *ΔbcsΔpsg* strains, with one mutant where *bcs* was pyramided on top of a *psgA* deletion (P→B) forming an intermediately strong pellicle while another mutant where *psg* deletion was pyramided on top of a *bcsA* deletion (B→P) forming a very weak pellicle. The same mutant (P→B) exhibited a robust Congo red-binding and firm pellicle phenotype. Mutants deficient in Peb generally had moderately reduced pellicle strength, often fracturing upon transfer, though some *Δpeb* mutants formed very robust pellicles (*ΔpebΔpsg* and *ΔpebΔalg*). Again, recent observations highlighting the importance of light to pellicle formation (a variable we did not control) should be taken into account as without light, pellicle formation was severely hindered (data not shown).

The attachment of bacterial cells to abiotic surfaces and the formation of a biofilm are influenced by exopolysaccharides and adhesions. We looked at how deficiencies in certain exopolysaccharides and purli affected biofilm formation on glass tubes using a crystal violet assay. Overnight cultures were resuspended in CTYE medium to an OD600 of 0.1 and 1 mL was transferred to borosilicate glass tubes and incubated at 25 °C with gentle shaking (50 rpms). At designated times, cell suspensions were decanted and the biofilm attached to the glass surface was air-dried prior to and staining with crystal violet (0.1%). The crystal violet was decanted and the tubes were rinsed three times prior to solubilizing the crystal violet in 70% ethanol as described previously (11). Crystal violet absorbance was read at 540nm.

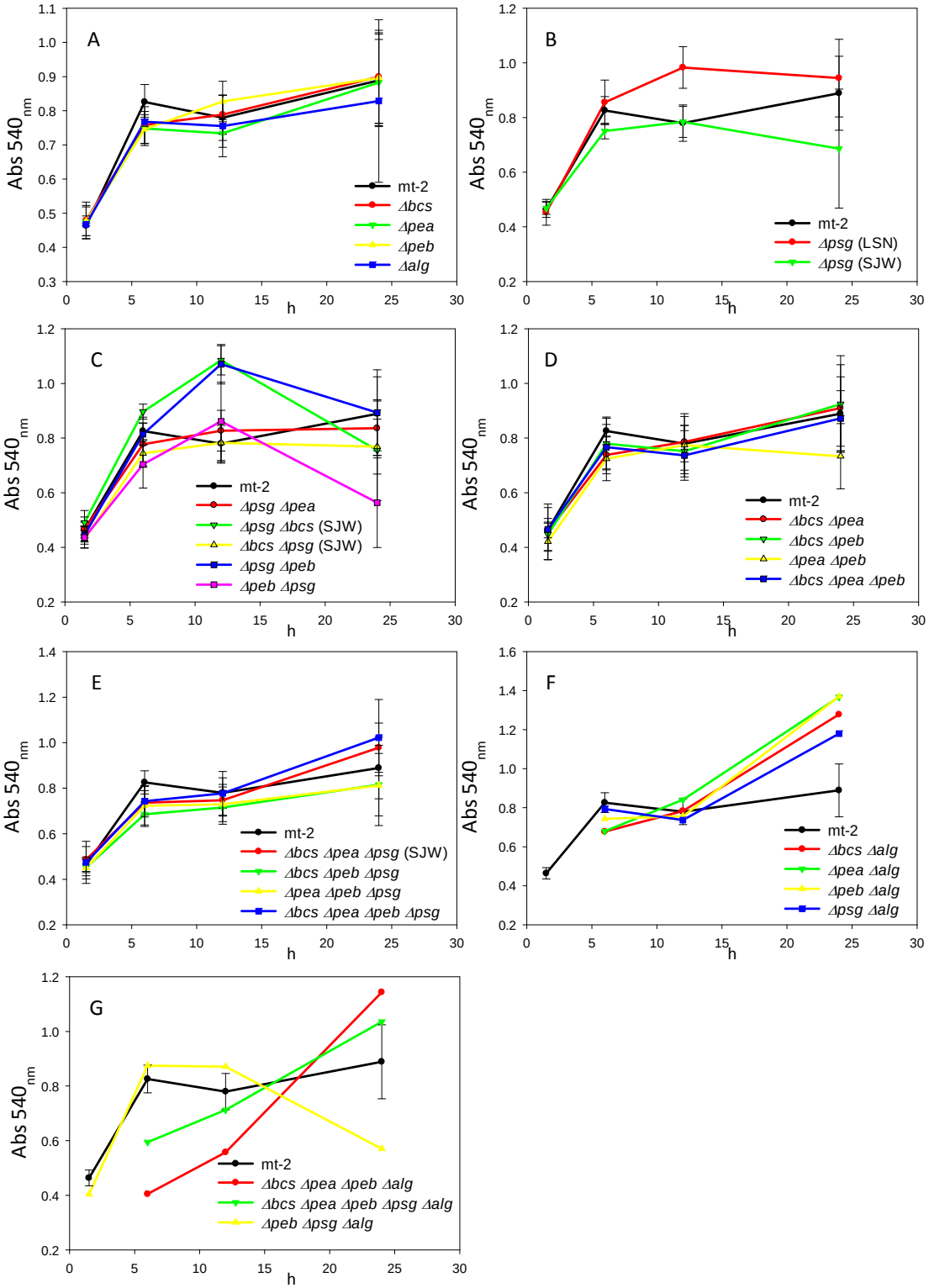

Figure 7. Analysis of attachment/biofilm formation on glass surface over time. Values are mean crystal violet absorbance, n=1-7experimental reps, ± SE if applicable.

In general, most strains behaved like wild-type mt-2 (Figure 7, Table 1). Biofilms at 24 h

were extremely variable between the 7 experiments. In some experiments, biofilm formation peaked at 6-12 h and then dissipated by 24 h; while in other experiments biofilms continued to develop up to 24 h. While we have not yet looked at the effect of light on attachment and biofilm development, it is likely that it would play a significant role (as was observed with Congo red-binding and pellicle formation) and may explain some of the variation at 24 h. Discrepancies between strains with the same combination of genes knocked out highlight potential secondary mutations to *Δpsg* strains (Figure 7B). There is likely a secondary mutation in *Δpsg* (LSN) that persists with subsequent mutations to polysaccharides, except for *ΔpsgΔpea* (Figure 7C). This suggests that the secondary mutation in *Δpsg* (LSN) may cause elevated production of pea. High biofilm formation at 24 h for alginate-deficient mutants was consistent with wild-type in that one experiment (Figure 7F), though poor biofilm formation at 6 h for *ΔbcsΔpeaΔpebΔalg* and *ΔbcsΔpeaΔpebΔalgΔpsg* was unlike wild-type.

In summary, we were able to show the repeats of the purli sub-units PsgA and PsgB, highlighting their conserved nature. Furthermore, we attempted to express the PsgA sub-unit in *E. coli*, but our results were inconclusive. It is possible that interactions between the similar *E. coli* curli machinery interfered with purification. We also looked at interactions between purli and the exopolysaccharides produced by *P. putida*. We recorded Congo red and Coomassie blue binding, as well as pellicle formation and biofilm attachment to glass surfaces (Table 1). There is a clear effect with deficiency of pea generally giving poor Congo red binding and pellicle strength, though phenotypes associated with purli deficiency were inconsistent. Perhaps the most consistent phenotype associated with purli-deficient mutants is an increase in apparent secondary mutations. Purli mutants, but especially those with a pyramided polysaccharide mutation often

exhibited a distinct mutant phenotype. This may highlight an increased stress experienced by the cell when purli is not produced, encouraging mutations to occur.

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APPENDIX II. GLUCAN BIOSYNTHESIS GENE TRANSCRIPTION

This appendix covers research attempting to identify promoter elements, including regulatory DNA binding sites, involved in controlling glucan biosynthesis gene expression. To identify the transcriptional start site for both *ndvB* and the *opgGH* operon, we used 5' Rapid Amplification of cDNA Ends (RACE). In this method, the 5' end of an mRNA transcript is reverse-transcribed into cDNA, amplified and sequenced. One hundred µl of a 1:1000 dilution from an OD_{600} = 0.1 suspension of cells were plated on CTYE media either unamended or amended with PEG to reduce the water-potential by 1.5 MPa and incubated 24 h at 28° C. RNA was extracted using Qiagen RNA isolation Miniprep kit using lysozyme and proteinase K digestion and on-column DNAse treatment. A second Ambion DNAse treatment was also used to ensure no DNA contamination. We used the Takara 5' RACE Core kit according to manufacturer's protocol and the primers listed in Table 1 to generate circular cDNA from the mRNA transcripts. Sequencing of cDNA was done at the ISU DNA Facility.

Table 1. Primers used for 5' RACE

Name	Sequence	Source
ndvB-RT	5'-CGGATGCTGTGGGTGTGC -3'	This Study
ndvB-A1	5'-TGACCGGACGGTTGTACAGG-3'	This Study
ndvB-A2	5'-CGATGACCACCAGGTTGAGC-3'	This Study
ndvB-S1	5'-GAACGCATCTCCGGCTTCTC -3'	This Study
ndvB-S2	5'-TCAACCAGAACCCGCAGAGC -3'	This Study
opgGH-RT	5'-CGGATCTGCTGGTAATCGG -3'	This Study
opgGH-A1	5'-CAAGCAGCAGGGTGCGCACG -3'	This Study
opgGH-A2	5'-CGGTGTTTGAAACGGAACGG -3'	This Study
opgGH-S1	5'-GTTCAGCATCGACGACGTGG -3'	This Study
opgGH-S2	5'-CTCGACCGCGCTGGCATTCG -3'	This Study

We were unable to amplify *ndvB* cDNA from mRNA isolated from cells grown on unamended CTYE, although we were successful using mRNA from cells grown on PEG amended CTYE. We identified an *ndvB* transcriptional start site 95-bp upstream of the translational start site (Figure 1 A). This predicted transcriptional start site was only 9 bases downstream of a Virtual footprint predicted σ^{70} -10 binding site. While we were able to get cDNA amplified from *opgGH* mRNA isolated from cells grown on unamended and PEG amended CTYE, in both cases the predicted transcriptional start site was the same as the

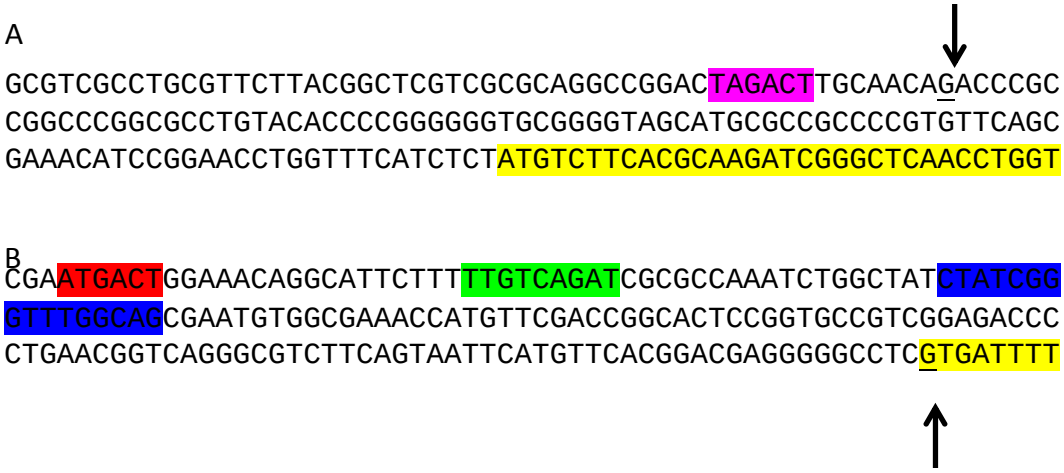

Figure 1. 5' RACE results for identifying transcriptional start sites for A) *ndvB* and B) *opgGH*. Underlined base with arrow indicates 5' end of mRNA , yellow highlights indicate translated region, pink indicates σ^{70} -10 binding site according to Virtual footprint, red and green predict σ^{70} -35 and -10 binding sites according to BPROM, blue indicates LasR and/or RhlR binding site according to Virtual footprint.

translational start site (Figure 1 B). Presumably, there would be some leader sequence for ribosome binding, yet none was identified. It is possible that the 5' end of the *opgGH* mRNA is degraded or that the leader sequence secondary structure prevents complete cDNA synthesis. The presence of an RNA folding ribo-switch could be involved in regulation of linear glucan expression. We know the *P. aeruginosa* exopolysaccharide Psl is regulated post-transcriptionally by the formation of a stem-loop structure stabilized by RsmA, which blocks translation (2). Perhaps one could use computational software to predict RNA folding in the region upstream of

opgGH to identify 2nd degree structure. Perhaps point mutations could be introduced to disrupt folding of a potential ribo-switch, highlighting its role in expression.

While inspecting the region upstream of *opgGH*, we visually identified a potential σ^{22} (AlgU/T) binding site located 620 bps upstream of the translational start site (Figure 2) which was not predicted by BPROM or Virtual footprint (Figure 1). While not a perfect match to the σ^{22} (AlgU/T) consensus sequence (1, 4), it is close, differing primarily in the gap between the -35 and -10 regions (15 bps rather than the typical 16/17). Additionally, transcriptional studies of the σ^{22} (AlgU/T) regulon demonstrate that *opgGH* is likely regulated by the alternative sigma-factor (3). The potential σ^{22} (AlgU/T) binding site is rather far upstream from the translational start site. It would be interesting to test if point mutations to the binding site affect *opgGH* expression, especially during and envelope stress response to confirm involvement in linear glucan regulation.

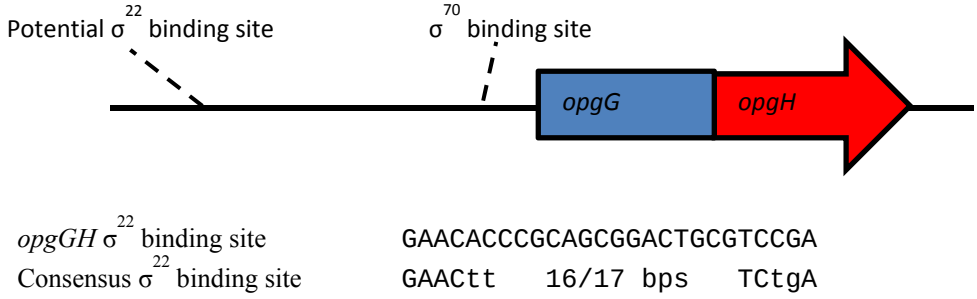

Figure 2. Potential binding site for σ^{22} 620 bps upstream of opgGH. Sequence is similar to consensus σ^{22} binding site sequence, but with a 15 bp gap between -35 and -10 region rather than typical 16 to 17 bp gap.

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