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Carlos C. Goller

Graduate Division of Biological and Biomedical Sciences

Program in Molecular Microbiology and Genetics

Regulation of β-1,6-*N*-acetyl-D-glucosamine Production and *Escherichia coli* Biofilm Formation

By

Carlos C. Goller

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Program in Microbiology and Molecular Genetics

_____

Tony Romeo
Adviser

_____ _____

Charles P. Moran
Committee Member

June R. Scott
Committee Member

_____ _____

William Shafer
Committee Member

Philip N. Rather
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the Graduate School

_____

Date

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B.S., Worcester Polytechnic Institute, 2002

Advisor: Tony Romeo, Ph.D.

An Abstract of
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ABSTRACT

Regulation of β-1,6-*N*-acetyl-D-glucosamine Production and *Escherichia coli* Biofilm Formation

By

Carlos C. Goller

Bacterial biofilms are important for persistence of microbes in the environment or within a host. Insight into the regulatory elements involved in biofilm formation is essential to understand and combat biofilm-associated diseases. In *Escherichia coli*, the *pgaABCD* locus is required for production of polymeric β-1,6-*N*-acetyl-D-glucosamine (PGA), which promotes biofilm formation. The CsrA (carbon storage regulator) RNA-binding protein represses PGA production. However, at the onset of this project further insights into the regulatory elements that influence PGA accumulation were lacking.

Disruption of *nhaR*, encoding a transcriptional regulator important for survival of *E. coli* under conditions of high salinity and alkaline pH, was found to greatly reduce biofilm formation without impairing growth. NhaR affected *pga* expression in response to increasing monovalent cation concentrations in the medium and to alkaline pH. *pgaA* transcript levels were reduced 200-fold and PGA was undetectable upon disruption of *nhaR*. Purified NhaR-His_6 bound specifically to the *pgaA* promoter region and was necessary for *in vitro* transcription. Recent studies by other groups suggest that induction of *nhaR* occurs during colonization of the gut and PGA may be critical for this process.

A biofilm screen of a panel of single-gene deletions of GGDEF and/or EAL domain proteins, responsible for synthesis and degradation of the secondary messenger c-di-GMP, respectively, indicated that several of these genes affect biofilm formation.

Over-expression of YhjH (EAL domain) decreased biofilm and PGA accumulation, whereas expression of YdeH (GGDEF) from a plasmid increased both. Since these two proteins have been shown to affect intracellular c-di-GMP levels, these results suggested that c-di-GMP modulates PGA synthesis. Nevertheless, over-expression of YdeH or YhjH did not affect a *pgaA'-'lacZ* translational fusion, suggesting that c-di-GMP does not alter transcription-translation of *pgaA*. We developed an *in vitro* PGA synthesis assay using membrane preparations; however addition of c-di-GMP failed to enhance *in vitro* PGA synthesis. Membranes from strains depleted for c-di-GMP (over-expressing *yhjH*) were unable to synthesize PGA even in the presence of added c-di-GMP. These results may suggest the existence of an unidentified c-di-GMP responsive factor or the requirement of c-di-GMP for a functional Pga complex.

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Chapter 1: Introduction

Rationale and specific aims

When microbes are faced with nutrient limitation or environmental stressors such as high pH or salinity, genetic pathways are engaged that aid the organism in surviving these conditions. The formation of surface-attached microbial communities is a mode of growth that offers several advantages to the organism and has important clinical, industrial, and environmental implications. The formation of a biofilm by a specific organism or a microbial community is often the cause of disease and deterioration of materials, but can also lead to a productive symbiotic relationship between bacteria and other organisms.

By studying the genetic regulation of biofilm formation in the genetically-tractable organism *Escherichia coli*, we sought to better understand the underlying molecular mechanisms as well as biological significance of the biofilm mode of growth. The specific aims of this dissertation were:

1. To better understand the genetic mechanisms by which organisms link environmental signals to regulation of biofilm components (Chapter 2).
2. To characterize the role of the NhaR transcriptional activator in *E. coli* biofilm formation and *pgaABCD* operon expression required for synthesis of the PGA polysaccharide adhesin (Chapter 3).
3. To identify GGDEF/EAL domain proteins that are involved in biofilm formation and the molecular mechanism by which they modulate PGA synthesis (Chapter 4).
4. To survey naturally occurring *E. coli* isolates from different environments (e.g., soil, sediment, aquatic environments) to determine if there is an environmental

niche that promotes (or requires) biofilm formation and if these organisms use PGA as a biofilm component (Chapter 5).

Background and significance

Biofilms

A biofilm is an aggregate of bacteria attached to a solid surface or to each other and typically encased in an exopolysaccharide matrix (Costerton *et al.*, 1995). This is distinct from planktonic or free-living bacterial growth because biofilm cells are genotypically and phenotypically different from planktonic cells (Sauer *et al.*, 2002). Biofilms, although often invisible to the human eye, occur throughout nature (reviewed by Hall-Stoodley *et al.*, 2004). A single species of bacteria may be involved, or more than one species may co-aggregate to form a biofilm. Fungi, including yeasts, are capable of forming biofilms. Furthermore, it is becoming increasingly evident that the biofilm lifestyle is quite possibly the preferred mode of bacterial growth in the environment as well as in colonized hosts. However, it is currently difficult to distinguish what is truly a biofilm community from seemingly random bacterial aggregations, and great debate surrounds findings of biofilm-like structures on wound models (Davis Ricotti, Mertz 2008 (Davis *et al.*, 2008), intracellularly (intracellular biofilm-like communities, IBCs, Anderson *et al.*, 2003) or even on remains of dinosaurian soft tissues (Kaye *et al.*, 2008).

Biofilm history

Studies of free-swimming bacteria in pure culture have led to important findings that have advanced our understanding of the complexities of bacterial physiology. Nevertheless, it is increasingly evident that bacteria in nature rarely exist as free-swimming cells (Costerton *et al.*, 1995). Van Leeuwenhoek first observed "animaculi" scraped from his teeth under the first primitive microscope in the 17th century. This was probably the first record of a biofilm (Slavkin, 1997). In the 1940s, Zobell described microbes attaching to surfaces in aquatic systems (Zobell, 1943). Nevertheless, the term biofilm was not coined until 1978 (Costerton *et al.*, 1978). The recognition of the biofilm mode of growth, along with the availability of complete bacterial genome sequences, is considered by some two of the major landmark events in microbiology of this last decade (Lasa, 2006).

Early studies focused on biofilms found in natural environments such as streams and relied heavily on microscopic observations (Costerton *et al.*, 1987). Transmission electron and confocal microscopes allowed for more detailed observations of the structure of biofilms in the mid-1980s (reviewed by Costerton, 2007; Kjelleberg and Givskov, 2007). Next, studies in the 1990s focused mostly on the genes involved in biofilm development. Currently, the pace of biofilm research is faster than ever, with studies often fusing diverse disciplines such as chemistry, physics, engineering, public health, genetics, and medicine.

Biofilms in the environment

In addition to aquatic environments, biofilms also form in soil and sediment, including on the roots of plants (reviewed by Danhorn and Fuqua, 2007; Ramey *et al.*, 2004). These interactions offer the microbes a sheltered habitat from certain environmental stresses, protection from protozoan predation, consortial metabolism, and the opportunity for horizontal gene transfer. The interaction of prokaryotes with plants can range from mutualism and commensalism (*Pseudomonas putida*, *Pseudomonas fluorescens* and related pseudomonads found on leaves and roots) to a pathogenic relationship (*Enterococcus faecalis* colonizing roots of *Arabidopsis thaliana* or the cause of brown spot disease, *Pseudomonas syringae* pv. *syringae*) detrimental to the plant (Danhorn and Fuqua, 2007 and references therein).

Biofilms can also be found in extreme environments such as acid mine drainage, where they contribute to the cycling of sulfur (Edwards *et al.*, 2000) or cyanobacterial biofilm communities in thermal springs (Ramsing *et al.*, 2000). Biofilms seem to be ubiquitous in the environment and conduct a variety of biological processes including photosynthesis and nitrogen fixation (e.g. rhizosphere, roots). Though often overlooked, biofilms in the environment can be useful indicators of the health of the ecosystem and therefore monitoring of the spatial and temporal variations of these communities can provide important data (e.g., Lear *et al.*, 2008).

Biofilms in industry and medicine

In industrial settings, biofilms can be useful in the production and degradation of organic matter, such as the degradation of environmental pollutants and the cycling of nitrogen, sulfur, and heavy metals (Costerton, 2007; Videla and Herrera, 2005). Biofilms have been shown to be involved in the removal of sewage in ground water contamination (Massol-Deya *et al.*, 1995). The presence of biofilms on certain surfaces ("biofouling layer") may promote physico-chemical reactions and changes in the properties of materials referred to as biocorrosion or microbially influenced corrosion (reviewed by Beech *et al.*, 2005).

Biofilms can also cause alterations in flow and even block conduit, causing significant economic and productivity losses (Costerton *et al.*, 1995). Most importantly, biofilms that form in drinking water systems are a serious health concern and studies have emerged suggesting the presence and persistence of pathogens such as *Helicobacter pylori* in these environments (Giao *et al.*, 2008).

Biofilms are often responsible for human infections that are persistent and difficult to treat. The Centers for Disease Control and Prevention estimate that more than 65% of infections are caused by bacteria growing in biofilms (cited by Lewis, 2007). A major concern is that biofilms develop on dead tissues, bone, and medical devices (Lambe *et al.*, 1991). A few examples include *Staphylococcus epidermidis* and *Staphylococcus aureus* infections of central venous catheters, eye infections occurring when biofilms form on contact and intraocular lenses, the formation of dental plaque, and *Pseudomonas aeruginosa* airway infections in cystic fibrosis patients (reviewed by del Pozo and Patel, 2007). Catheters, including central lines, intravenous, and urinary catheters, are surfaces

often colonized by biofilms in hospital settings (e.g. Donlan, 2001, 2008). Strikingly, reports of biofilms found in different sites continue to emerge (e.g., microbial biofilm in intraamniotic infection, Romero *et al.*, 2008; adenotonsillar biofilms in children, Al-Mazrou and Al-Khattaf, 2008; intracellular bacterial communities in human urinary infections, Rosen *et al.*, 2007). However, additional studies and confirmations are needed, as, for instance, there is still debate in the field whether the clinically significant non-typeable *Haemophilus influenzae* forms biofilms (Moxon *et al.*, 2008). Nevertheless, increasing evidence supports the theory that chronic infections are often due to biofilms, often of polymicrobial composition, and emphasizes the need for new methods of detection and treatment (James *et al.*, 2008). With the discovery of new biofilms in different sites comes a substantial amount of information about sources of the diseases, ecological processes, and the diversity of organisms composing these prolific microbial communities (e.g., discovery of a new organism *Costertonia aggregate* in a marine biofilm; Kwon *et al.*, 2006).

Bacteria within the biofilm often show marked resistance to antimicrobials in contrast to the same strain grown free-living in broth, which helps to explain why it is so difficult to treat infections associated with biofilms (reviewed by Mah and O'Toole, 2001). Bacteria in the exopolysaccharide matrix may be protected from the host's immune mechanisms (Costerton *et al.*, 1999). The matrix also presents a diffusion barrier for some antimicrobials, while others may easily diffuse across it (Mah and O'Toole, 2001). There is genetic evidence indicating that periplasmic glucans of *P. aeruginosa* interact with tobramycin and prevent the antibiotic from reaching its site of action (Mah *et al.*,

2003). After treatment of a biofilm with antibiotics, quite often persister cells survive that lead to re-seeding of new biofilms (reviewed by Lewis, 2007, 2008).

Persistence of some opportunistic pathogens in the hospital environment has been attributed to their ability to form biofilms, as is the case for *Acinetobacter baumannii*, a common cause of device-related nosocomial infections and an organism that is intrinsically resistant to numerous antibiotics (e.g., Loehfelm *et al.*, 2008). It is of great concern that two of the most notable pathogens in the healthcare environment, methicillin-resistant *Staphylococcus aureus* (MRSA) and multi-drug resistant isolates of *Pseudomonas aeruginosa* were recently found to be resistant to commonly used hospital biocides when grown as biofilms (Smith and Hunter, 2008). Because of this, knowledge of the factors that regulate biofilm formation and dispersal is important in the development of novel antimicrobial therapies (reviewed by Lewis, 2007). Research into new biomaterials and antimicrobial medical device coatings is an important and promising area of research (Raad *et al.*, 2008).

Biofilm structure and development

In the basic biofilm model, biofilm formation begins with the interaction between bacteria and surfaces (e.g. Costerton *et al.*, 1995; O'Toole *et al.*, 2000a). Nevertheless, it is likely that signaling preparing a bacterium for biofilm formation initiates even before the interaction with a surface.

The initial step in biofilm formation is colonization of the surface. Bacteria may use flagella to move on a surface and initiate colonization (Goller and Romeo, 2008 and references therein). Some bacteria may use pili to pull themselves together into clumps

while others rely on cell division to initiate colony formation. For the most part, Gram negative bacteria are thought to initiate biofilm formation using flagella and/or type IV pili (motility) in order to overcome repulsive forces between the bacterium and the surface (O'Toole and Kolter, 1998; Pratt and Kolter, 1998).

Early studies of the initial events in adherence of marine bacteria to surfaces distinguished two stages of attachment: an instantaneous reversible phase and a time-dependent irreversible phase (Marshall *et al.*, 1971). The *lapA/lapBCE* and *sadB* genes of *P. fluorescens* and *P. aeruginosa*, respectively, are involved in the transition from reversible to irreversible attachment (Caiazza and O'Toole, 2004; Hinsa *et al.*, 2003). There is also evidence in *E. coli* that suggests a system for pattern formation, where cells attach reversibly and seem to seek out proper positioning at defined distances between cells in early microcolonies before forming permanent attachments with the substrate (Agladze *et al.*, 2003; Agladze *et al.*, 2005).

Surface structures involved in biofilm formation

Bacteria use a variety of structures to attach to surfaces or each other (reviewed by Beloin *et al.*, 2008b; Goller and Romeo, 2008). They are typically classified in three broad categories: protein structures including pili, fimbriae, and large adhesins; polysaccharides; and other structural components of the biofilm matrix (extracellular DNA, for instance).

Protein

Once on the surface, bacteria use specific adhesins and surface appendages to attach permanently. The flagellum is an important organelle proposed to be needed for motility along surfaces and sometimes used as an adhesive element during initial attachment (O'Toole *et al.*, 2000a; Pratt and Kolter, 1998). In *Vibrio cholerae* it has also been proposed to be used as a mechanism for sensing surfaces and signaling production of an exopolysaccharide (Lauriano *et al.*, 2004). Nevertheless, motility can be detrimental to later stages of biofilm formation and is consequently inhibited to facilitate structural stability of the biofilm (e.g., Moorthy and Watnick, 2004; Schembri *et al.*, 2003). In *P. aeruginosa*, type IV-mediated twitching motility is important for movement along surfaces and formation of microcolonies (O'Toole and Kolter, 1998).

Upon reaching a surface, other protein organelles are important for attachment. Specific outer-membrane adhesins such as antigen 43 (Ag43), curli fimbriae, type I pili, type IV pili, and mannose sensitive haemagglutinin pilus (MSHA) are used for stable attachment after the bacterium has reached a favorable site (reviewed in Chapter 2). Antigen 43 was found to be important for attachment of *E. coli* and other species (Kjaergaard *et al.*, 2000), but does not seem necessary for biofilm formation in Luria Broth (Danese *et al.*, 2000a). This highlights the importance of considering particular cell surface attachment factors in the context of the growth conditions and strains tested.

Curli are thin aggregative fibers involved in bacterial attachment and biofilm formation. In many *E. coli* strains, expression of curli is best at temperatures below 30 °C (reviewed by Barnhart and Chapman, 2006). Expression of curli is regulated by a

complex genetic pathway with several environmental inputs, as described in more detail in Chapter 2.

P and type 1 fimbriae correspond to the chaperone-usher subclass of adhesins (reviewed by Beloin *et al.*, 2008b; Hatt and Rather, 2008. In uropathogenic *E. coli* (UPEC) P fimbriae are associated with pyelonephritis and bind to the α-D-galactopyranosyl-(1-4)- β-D-galactopyranoside receptor epitope of glycolipids, recognized by the tip adhesin PapG. Type 1 fimbriae are produced by most *E. coli* strains and used for the colonization of the bladder by binding to α -D-mannosylated proteins, such as uroplakins, recognized by FimH. Recently, type III fimbriae have been described in *E. coli* (Ong *et al.*, 2008) and *Klebsiella pneumoniae* (Jagnow and Clegg, 2003) as having a role in biofilm formation.

The three types of pilus *V. cholera* uses to attach to different substrates exemplify the theme that bacteria form biofilms through more than one genetic pathway and using multiple organelles. The Tcp (toxin co-regulated pilus, Type IV pilus) is needed for colonizing the gut of animals; Msh (mannose sensitive hemagglutinin, Type IV) is used for attachment to glass and plastic surfaces; and colonization of chitin surfaces may require another factor (reviewed by O'Toole *et al.*, 2000a).

Large proteins such as the staphylococcal biofilm-associated protein (Bap; Cucarella *et al.*, 2001) and its recently discovered homologue in the emerging opportunistic pathogen *Acinetobacter baumannii* (Loehfelm *et al.*, 2008) are characterized by tandem repeats, their presence on the bacterial surface, and their requirement for biofilm formation in certain strains (reviewed by Lasa, 2006; Lasa and Penades, 2006). These

proteins share structural and functional similarities and have been found in at least 13 pathogenic organisms.

Polysaccharides

Production of exopolysaccharides is thought to stabilize the microcolonies (reviewed by Sutherland, 2001). Colonic acid of *E. coli* (Danese *et al.*, 2000b) and alginate of *P. aeruginosa* during cystic fibrosis infections (Hentzer *et al.*, 2001) are two examples of polysaccharides important for the architecture of the biofilm, though likely not critical for cell adherence.

Exopolysaccharides such as cellulose and alginate have different roles in the formation of biofilms on plants depending on the microbe. They contribute to disease by clogging vessels if produced in the vasculature of the plant or can protect the microbes from desiccation on leaves and roots (reviewed by Danhorn and Fuqua, 2007).

Staphylococci produce a β -1,-6-*N*-acetyl-D-glucosamine polymer (PIA or PNAG) which facilitates cell-cell and cell-surface interactions (Heilmann *et al.*, 1996; Mack *et al.*, 1994; Mack *et al.*, 1996). Factors affecting expression of the genes required for synthesis of this polymer have been extensively studied (reviewed by Goller and Romeo, 2008; Otto, 2008, see Chapter 2). The biofilm adhesin β-1,6-*N*-acetyl-D-glucosamine of *E. coli* (PGA) promotes cell-surface and cell-cell interactions (Wang *et al.*, 2004) and also is important for biofilm formation of other organisms with homologues of this locus, including Bordetella sp. (Itoh *et al.*, 2005; Parise *et al.*, 2007*), Salmonella enteritidis*, Typhimurium, and some *E. coli* strains use cellulose as a component of their biofilm matrix (Solano *et al.*, 2002; Zogaj *et al.*, 2001). The *pel* and *psl* gene clusters encode for

the machinery necessary for production of polysaccharides important for biofilm formation in *P. aeruginosa* (reviewed by Tart and Wozniak, 2008), however definitive information on the composition of these polymers is currently unavailable.

Gram negative organisms produce lipopolysaccharides (LPS) with chemical properties that often alter binding to substrates. Strains producing truncated and/or mutated LPS often exhibit altered binding to hydrophobic surfaces (e.g., *P. aeruginosa*, Makin and Beveridge, 1996). It is believed that LPS affects biofilm formation by altering the hydrophobicity of the cell, which in turn affects binding to hydrophobic surfaces. Furthermore, several groups have noted an important role for extracellular DNA in biofilm structure (reviewed by Spoering and Gilmore, 2006).

Regulation of biofilm formation

Environmental factors

Environmental conditions such as pH, salinity, temperature and stressors including ethanol and antimicrobials affect gene expression profiles and trigger genetic pathways that often lead to the transition between sessile and planktonic lifestyles (reviewed by Goller and Romeo, 2008; Stanley and Lazazzera, 2004). Studies have indicated that contact with surfaces induces vast changes in gene expression profiles in *E. coli* (Dorel *et al.*, 1999; Prigent-Combaret *et al.*, 1999) and other organisms (Welin *et al.*, 2004). Many of the differentially regulated genes encode proteins necessary for attachment, including Type 1 pili and antigen 43 (Schembri *et al.*, 2003). Alginate synthesis in *P. aeruginosa* is dependent on *algC*, which is induced upon surface attachment (Davies and Geesey, 1995).

It is likely that future studies will indicate that other biofilm factors, such as PGA, are also induced upon attachment in preparation for later stages of biofilm development.

Growth of biofilms *in vitro* has highlighted the significant impact of the growth medium on the ability of the organism to form a biofilm. Increasing sodium concentration (as well as potassium and lithium) in the medium or alkaline pH induce expression of the *pgaABCD* genes required for synthesis of the PGA biofilm adhesin of *E. coli* (Goller *et al.*, 2006; see Chapter 3). Glucose and sodium chloride also induce staphylococcal PIA production (reviewed by Goller and Romeo, 2008; Lim *et al.*, 2004; Otto, 2008). In contrast, glucose inhibits biofilm formation in *E. coli* (Jackson *et al.*, 2002a) and *Bacillus subtilis* (Stanley *et al.*, 2003). Non-domesticated environmental isolates of *E. coli* grown in media with glycerol formed more robust biofilms than those grown in LB alone (Goller and Pannuri, unpublished results). *V. cholerae* forms more robust biofilms in rich media (Watnick and Kolter, 1999) whereas *E. coli* 0157:H7 prefers a low-nutrient medium (Dewanti and Wong, 1995). Temperature affects *pga* expression and consequently biofilm formation in *E. coli*: at 26 °C *pga* is expressed better than at 37 °C (Cerca and Jefferson, 2008; Wang, 2005; Wang *et al.*, 2005; Goller unpublished results). Iron, oxygen, antimicrobials, viscosity are among many environmental factors that affect expression of biofilm factors (Goller and Romeo, 2008 and references therein).

Few but intriguing examples suggest the existence of undiscovered genetic mechanisms that allow a biofilm to respond to fluid shear (e.g., *P. aeruginosa* cell signaling under high shear flow; Purevdorj *et al.*, 2002; Stoodley *et al.*, 2002). *Staphylococcus aureus* microcolonies appear to roll in the direction of the flowing fluid

in a controlled manner using viscoelastic tethers (Rupp *et al.*, 2005). Isberg and Barnes have suggested a model based on structural data where some pili attach efficiently only under conditions of shear stress (Isberg and Barnes, 2002). It is likely that more of these scenarios await discovery.

Signal transduction and quorum sensing

To respond to ever-changing environmental conditions, bacteria have developed complex signal transduction systems that enable adaptive responses to external stimuli by means of genetic modulation. Several important signal transduction pathways play critical roles in biofilm formation. The CpxA-CpxR and EnvZ-OmpR two component systems form a regulatory network with the stationary phase sigma factor σ^S (Sigma S) needed for expression of *csgD* and curli in *E. coli* (Prigent-Combaret *et al.*, 2001). Sigma B of staphylococci is needed for expression of the *ica* locus and production of PIA (reviewed by Goller and Romeo, 2008; Gotz, 2002).

Both Gram positive and Gram negative organisms use quorum sensing molecules as a mechanism of correlating cell density information with particular gene expression patterns (reviewed by Irie and Parsek, 2008; Miller and Bassler, 2001; Waters and Bassler, 2005). Quorum sensing molecules have been found in aquatic biofilms on submerged stones, in urethral catheters, and in cystic fibrosis patients (Davies *et al.*, 1998; McLean *et al.*, 1997; Stickler *et al.*, 1998). For instance, *P. aeruginosa* requires quorum sensing for proper biofilm maturation (Davies *et al.*, 1998).

Catabolite repression

Catabolite repression control (CRC) is the mechanism by which utilization of alternative carbon sources is inhibited in the presence of a preferred carbon source (reviewed by Bruckner and Titgemeyer, 2002). This is a system by which environmental carbon sources ultimately regulate biofilm formation and is still poorly understood in many cases. Studies have indicated that CRC plays an important role in biofilm formation in *E. coli, P. aeruginosa,* and *B. subtilis* (Jackson *et al.,* 2002a; O'Toole *et al.,* 2000b; Stanley *et al.,* 2003; Itoh *et al.,* unpublished results). In *E. coli,* glucose in the medium represses biofilm formation (Jackson *et al.,* 2002a; Itoh *et al.* unpublished results). In contrast, glucose-rich medium strongly stimulates biofilm formation of *Serratia marcescens* through regulation of type 1 fimbriae (Kalivoda *et al.*, 2008).

Csr system

The carbon storage regulatory system (Csr) has profound effects on metabolism, physiology, and biofilm formation of *E. coli* and pathogenic relatives (Jackson *et al.*, 2002b; Romeo and Gong, 1993; Romeo *et al.*, 1993; Romeo, 1998). Homologous systems are distributed across eubacteria and play roles in regulation of bacterial virulence factors (Mercante *et al.,* 2006 and references therein). The Csr system in *E. coli* represses stationary phase pathways including glycogen synthesis, catabolism, and biofilm formation (Jackson *et al.*, 2002b; Romeo *et al.*, 1993; Yang *et al.*, 1996), whereas it activates certain exponential processes such as glycolysis, acetate metabolism, biofilm dispersal, and motility (Jackson *et al.*, 2002b; Wei *et al.*, 2000; Wei *et al.*, 2001).

CsrA, a 61 amino acid RNA-binding protein, is the central component of the Csr system. CsrA represses gene expression by binding to untranslated regions of messenger RNAs and altering their stability (Liu *et al.*, 1995; Liu and Romeo, 1997) or competing for binding to the ribosome binding site and therefore preventing translation (Baker *et al.*, 2002; Dubey *et al.*, 2003). Levels of available CsrA are maintained by two untranslated small RNA molecules, CsrB and CsrC, which antagonize CsrA activity by sequestering it (Romeo, 1998; Weilbacher *et al.*, 2003). The BarA/UvrY two component system directly activates CsrB/CsrC transcription (Suzuki *et al.*, 2002). CsrA indirectly activates transcription of its own antagonists, CsrB and CsrC, constituting an autoregulatory loop. Suzuki *et al.* have added another component to the Csr system with the discovery of CsrD, a protein that controls the degradation of the CsrB/C RNAs (Suzuki *et al.*, 2006). Although pH is known to affect BarA signaling, (Mondragon *et al.*, 2006), a major area of interest continues to be the search for the signal(s) that regulate the CsrA system.

CsrA binds to and affects the stability of the *pgaA* message, having profound effects on PGA synthesis (Wang *et al.*, 2005). Therefore, *csrA* mutant strains are derepressed for *pga* expression and are hyper biofilm formers. Global studies have indicated how broad the effects of CsrA extend. Edwards and Romeo (American Society for Microbiology 2008 General Meeting, poster) have presented evidence that the CsrA protein binds to several hundred different transcripts, thus suggesting that CsrA is indeed a global regulator exerting effects on metabolism, physiology, stationary phase processes, and other regulators. Furthermore, CsrA was recently found to affect transcript levels of c-di-GMP related proteins (Jonas *et al.*, in press; see below and Chapter 4), thus connecting the Csr system to maintenance of c-di-GMP levels.

c-di-GMP

The second messenger 3',5'- cyclic diguanylic acid (c-di-GMP), is an important regulator of the bacterial biofilm lifestyle, virulence, and motility (reviewed by Cotter and Stibitz, 2007; Jenal and Malone, 2006; Tamayo *et al.*, 2007; see Chapter 4). c-di-GMP is synthesized by diguanylate cyclases (DGC, proteins with a GGDEF domain) and degraded by phosphodiesterases (PDE, proteins with an EAL or HD-GYP domain). Since evidence first emerged in 2002 for a role of c-di-GMP in controlling biofilm formation in *Vibrio parahaemolyticus* (Boles and McCarter, 2002) and *P. aeruginosa* (D'Argenio *et al.*, 2002), an explosion of studies has followed. One commonality seems to be that over-expression of GGDEF domain proteins leads to increases in intracellular c-di-GMP levels and activation of pathways necessary for biofilm formation (e.g., expression of Adr in *Salmonella* Typhimurium leads to cellulose biosynthesis; Simm *et al.*, 2004). This has led to the idea that c-di-GMP levels control the transition between biofilm and planktonic lifestyles. Unfortunately, very few mechanistic details are available for most systems, and quite possibly the best understood one is still that of how c-di-GMP acts as an allosteric activator of the cellulose synthase enzyme complex of *Acetobacter xylinum*, which was elucidated in the 1990s by Benziman and colleagues (Weinhouse *et al.*, 1997).

CONCLUSION

The availability of hundreds of microbial genome sequences and novel minimally-invasive techniques in combination with the realization that our most feared nosocomial

pathogens are capable of persisting in biofilms will drive the field of biofilm research in new directions. Techniques that allow for non-invasive monitoring of biofilm processes using, for instance, integrated nuclear magnetic resonance (NMR) and confocal laser scanning microscopes (CLSM), will enable measurements of dynamic metabolic processes in the depths of a live biofilm community (McLean *et al.*, 2008a; McLean *et al.*, 2008b). This will greatly deepen our understanding of these processes. The early steps of bacterial adhesion hold keys to unlocking some of the biophysical and genetic secrets of biofilm formation; however, early bacterial adhesion dynamics remain poorly understood. Recently, the groups of Nelly Henry and Jean-Marc Ghigo have introduced a novel approach to studying this process involving flow cytometry and dispersed colloidal surfaces as microbial adhesion substrates (Beloin *et al.*, 2008a). This approach will likely provide new understanding of the physical, chemical, and genetic requirements for initial bacterial adhesion. Other novel approaches include the creation of individual based mathematical models (IBMs) with the resolution necessary to examine parameters affecting microcolony formation (Johnson, 2008), which could eventually aid in the understanding of the patterns seen during initial attachment (Agladze *et al.*, 2003; Agladze *et al.*, 2005).

Biofilms in natural environments are mostly composed of consortia of species, exhibiting both synergistic and antagonistic behaviors among the members of the community. Few studies address these issues and those that do frequently find unexpected results (e.g., Burmolle *et al.*, 2006). It is therefore important that future studies consider multispecies biofilms, such as marine surface associated microbial communities, which hold tremendous biotechnological potential for profitable bioactive

compounds (e.g., Egan *et al.*, 2008). Furthermore, the need for new energy sources has kindled tremendous interest in microbial fuel cells, and the group of Derek Loveley has made great advances with mixed communities containing *Geobacter sulfurreducens* (e.g., Nevin *et al.*, 2008).

Dynamic in nature, biofilms are ideal situations for gene transfer to occur. Close proximity of cells within a biofilm promotes conjugation (Ghigo, 2001). A study demonstrated that *B. subtilis* could transfer a transposon to streptococcus within a biofilm (Roberts *et al.*, 1999). Most importantly, there is evidence that gene transfer between organisms within a biofilm may have produced multi-drug resistant bacteria (e.g. Weigel *et al.*, 2007). This cannot be overlooked, for its clinical and evolutionary consequences are tremendously important. A biofilm is much more than an aggregation of cells, it is teeming with life and processes vital to it.

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Chapter 2: Environmental Influences on Biofilm Development

Carlos C. Goller[1] and Tony Romeo[1,2]

[1] Department of Microbiology and Immunology, Emory University School of Medicine, 3105 Rollins Research Center, 1500 Clifton Rd., N.E., Atlanta, GA 30322.

[2] Department of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611.

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SUMMARY

Bacteria must coordinate genetic responses to changes in the local environment. In order to determine when formation of a biofilm is appropriate, expression of biofilm factors is carefully regulated in response to defined conditions. This review focuses on drawing a connection between environmental signals or conditions and the activation of genetic pathways leading to biofilm formation, highlighting examples in the published literature. Furthermore, it aims to compare and contrast the distinct mechanisms and triggers of biofilm formation that characterize the process in different organisms. The influence of physical and chemical factors such as hydrodynamics and substrate properties is discussed. Motility plays an important role during the initial approach to a surface and is regulated by numerous factors, such as osmolarity and temperature in *Escherichia coli*. The intriguing possibility that bacteria have genetic mechanisms to sense surfaces and proximity to neighboring cells to form surface patterns is discussed. Surface factors such as pili, fimbriae, and specialized adhesin proteins (e.g., Ag43) are then needed to transition from a reversible to a more permanent attachment and begin microcolony formation. Known conditions affecting the regulation of surface factors are cited exemplifying the complex regulatory networks involved, even in some of the better understood model organisms. Production of polysaccharides is often required for a true biofilm structure and important for the architecture of a mature biofilm. Factors regulating biofilm polysaccharide production are covered as well as triggers for biofilm dispersal, which is an area of great interest for the design of more efficient anti-infective therapies. Finally, one frontier in biofilm research is a better understanding of multispecies biofilms, and this chapter concludes with some fascinating facts from recent studies of microbial communities.

ABSTRACT

Bacterial biofilms are found under diverse environmental conditions, from sheltered and specialized environments found within mammalian hosts to the extremes of biological survival. The process of forming a biofilm and the eventual return of cells to the planktonic state involve the coordination of vast amounts of genetic information. Nevertheless, the prevailing evidence suggests that the overall progression of this cycle within a given species or strain of bacteria responds to environmental conditions via a finite number of key regulatory factors and pathways, which affect enzymatic and structural elements that are needed for biofilm formation and dispersal. Among the conditions that affect biofilm development are temperature, pH, O_2 levels, hydrodynamics, osmolarity, the presence of specific ions, nutrients, and factors derived from the biotic environment. The integration of these influences ultimately determines the pattern of behavior of a given bacterium with respect to biofilm development. This chapter will present examples of how environmental conditions affect biofilm development, most of which come from studies of species that have mammalian hosts.

INTRODUCTION

In the past decade, substantial advances in the understanding of the genetic and physiological bases of biofilm formation have been made. Dramatic differences in gene expression patterns exist between planktonic and sessile cells, and indeed even between different stages of biofilm development (e.g. Sauer ***et al.*** 2003). Nevertheless, the environmental and genetic factors that promote the transition from planktonic to sessile communities are only beginning to be understood in a few model organisms

(reviewed by Stanley and Lazazzera 2004). It is clear that different species and even strains of bacteria can exhibit unique patterns of response to the environment. What environmental conditions predispose various species of bacteria to initiate a given biofilm? How are the molecular genetic, biochemical and structural elements that mediate biofilm development regulated in response to environmental conditions? The following sections describe some of the environmental influences on biofilm development in the context of the molecular genetics and biochemistry of the biofilm development cycle (Figure 2-1).

Surface factors and hydrodynamic effects

Virtually any material that comes into contact with fluids containing bacteria is a substrate for biofilm formation. The roughness, chemistry, and presence of conditioning films affect attachment of bacterial cells to a surface. While rough surfaces are readily colonized because shear forces are diminished and surface area is increased in rougher surfaces (Donlan 2002), studies have indicated that non-domesticated strains of at least some species seem to colonize smooth surfaces equally as well (Donlan and Costerton 2002). Studies have also demonstrated that microorganisms typically attach more rapidly to hydrophobic surfaces such as plastics than to hydrophilic glass or metals (reviewed by Donlan 2002). For instance, hydrophobic substrata promote biofilm formation by most clinical isolates of *S. epidermidis* (Cerca *et al.* 2005). Hydrophobic interactions between the cell surface and the substratum may enable the cell to overcome repulsive forces and attach irreversibly (Donlan 2002). A notable exception is that *Listeria monocytogenes* forms biofilms more rapidly on hydrophilic than on hydrophobic surfaces (Chavant *et al.* 2002).

Submerged surfaces adsorb solutes and small particles, including bacteria (Geesey 2001). Studies dating back to the 1940's showed that glass surfaces adsorb nutrients from sea water, with consequent effects on metabolic activity associated with bacterial attachment (e.g. ZoBell 1943). Furthermore, the metabolic activities of bacteria associated with a surface cause temporal and spatial changes in the three-dimensional chemical gradients at the liquid-solid interface (Geesey 2001; Rani *et al.* 2007). When surfaces exposed to fluid environments adsorb proteins, coatings or conditioning films are formed that alter the surface properties and affect attachment of bacteria (Dunne 2002; Murga *et al.* 2001; Tieszer *et al.* 1998). For example, the proteinaceous conditioning films called acquired pellicles that develop on tooth enamel within the oral cavity are colonized within hours by Gram-positive cocci (Donlan and Costerton 2002; Rickard *et al.* 2003). The surface of a central venous catheter is in direct contact with the bloodstream and becomes coated with platelets, plasma, and tissue proteins including albumin, fibrinogen, fibronectin, and laminin (see the chapter by Donlan). This coating acts as a conditioning film that is colonized by organisms such as *S. aureus*, which adheres to fibronectin, fibrinogen, and laminin via large surface proteins known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (see the chapter by Otto; Mack *et al.* 2007; Patti *et al.* 1994).

Fluid flow or hydrodynamics influences biofilm structure and can have dramatic effects on the type of biofilm that is formed. Physical properties of biofilms such as cell density and strength of attachment can be affected by fluid sheer (reviewed by Stoodley *et al.* 2002 a, b; van Loosdrecht *et al.* 2002). Furthermore, biofilms grown under low flow conditions may form isotropic structures, whereas higher unidirectional flow may produce filamentous cells or groupings of cells with evidence of di-

rectionality (Stoodley *et al.* 1999, 2002a). Furthermore, *Pseudomonas aeruginosa* biofilms grown under high shear were more strongly attached than those grown under lower shear (Stoodley *et al.* 2002b). Others speculate that turbulent flow may enhance bacterial adhesion and biofilm formation by impinging cells on the surface (Donlan and Costerton 2002). In contrast, "rolling" of entire staphylococcal microcolonies over surfaces has been observed in biofilms grown under turbulent flow, perhaps allowing mature biofilms to colonize new surfaces downstream (Hall-Stoodley and Stoodley 2005; Rupp *et al.* 2005). Similarly, *Escherichia coli* attachment to mannose-coated surfaces via the type 1 fimbrial adhesive subunit, FimH, is shear-dependent. At low shear, the cells tended to roll over the surface, however as shear was increased, they became more firmly attached (Anderson *et al.* 2007; Thomas *et al.* 2004). Weak rolling adhesion at low shear force allows for cells to spread out and colonize more surface area than under high shear stress, where cells remain in tight microcolonies. Thus, preferred sites of colonization may be those with the necessary flow to maintain a stable interaction between the bacteria and host proteins (Isberg and Barnes 2002). In a study of *E. coli* biofilm formation under flow, fluid flow altered the spatial organization of cell attachment patterns (Agladze *et al.* 2003). While these and other studies document the important role of hydrodynamics in biofilm development and structure, little is known about the possible molecular genetic responses to fluid flow.

Approach and initial attachment to the surface

Motility and chemotaxis

Although both motile and nonmotile species form biofilms, in motile species, the ability to move using flagella or pili is generally required for efficient cell-to-

surface attachment. Microscopic observations indicate that motility promotes both initial interaction with the surface and movement along it (O'Toole and Kolter 1998; Pratt and Kolter 1998). However, there are reports suggesting that motility may only be important for biofilm formation under certain conditions (McClaine and Ford 2002). Motility may be needed to overcome the repulsive forces generated between cellular and abiotic surfaces and to permit favorable cell-surface interactions required for attachment (Geesey 2001). However, flagellar motility is not essential for initial adhesion and biofilm formation when the cell is equipped with an efficient adhesin (Jackson *et al.* 2002b; Prigent-Combaret *et al.* 2000; Wang *et al.* 2004). Furthermore, steric hindrance and/or movement caused by a flagellum can destabilize cellular attachments. Accordingly, motility genes are repressed after the bacterium attaches to the surface (Prigent-Combaret *et al.* 1999). Another example of the complex influence of environmental conditions on motility and biofilm development is the finding that while twitching motility via type IV pili appears to be needed for *P. aeruginosa* biofilm formation (O'Toole and Kolter 1998), overstimulation of twitching by the chelation of iron with lactoferrin, a component of innate immunity, prevents this bacterium from establishing productive surface contacts and forming biofilm (Singh *et al.* 2002).

Surface motility is widespread among flagellated Gram-negative bacteria. When it involves groups of long, hyperflagellated cells, moving as an organized mass, it is referred to as swarming motility. In *P. aeruginosa*, swarming motility is regulated through Rhl quorum sensing, while swimming is not. In recent studies, Rhl-dependent quorum sensing and nutritional conditions determined whether a flat, uniform biofilm or a structured biofilm was formed (Shrout *et al.* 2006). In contrast to motility, chemotaxis is not required for *E. coli* biofilm development in batch cultures (Pratt and Kolter 1998). However, in topologically-constrained environments, che-

motaxis may be important for assembling a quorum of cells that can initiate biofilm development (Park *et al.* 2003).

Expression of the genes involved in flagellum synthesis, motility, and chemotaxis in *E. coli* occurs in a hierarchical fashion, permitting ordered synthesis and assembly of the flagellum components (e.g. Macnab 2003; Soutourina and Bertin 2003). The master regulator $FlhD_2C_2$ is a DNA-binding protein that is directly or indirectly required for expression of all other motility and chemotaxis genes, over 50 in total. These are expressed from at least 15 operons, clustered at several regions on the chromosome. Expression of the *flhDC* operon serves as a pivotal point for integrating environmental signals (Figure 2-2). Its expression is controlled by numerous regulators including H-NS, Crp, EnvZ-OmpR, CsrA, QseBC, LrhA, and RcsCDB, which sense environmental conditions such as osmolarity (H-NS, EnvZ-OmpR), envelope stress (RcsCDB), nutritional conditions (Crp), or quorum sensing (QseBC).

In *E. coli*, high osmolarity and acetyl-phosphate levels inhibit *flhDC* expression and motility through the phosphorylation and subsequent binding of OmpR to the *flhDC* promoter region (Shin and Park 1995). The synthesis of flagella is also controlled by growth temperature: cells are not flagellated at 42°C, perhaps because of competition for the heat shock chaperones DnaK, DnaJ and GrpE, which are needed for flagellum gene expression (Shi *et al.* 1992). Furthermore, *flhDC* and flagellum biosynthesis are regulated by catabolite repression, i.e. activated by the cyclic AMP-Crp complex, and are repressed by the nucleoid-associated protein H-NS (Silverman and Simon 1974; Soutourina *et al.* 1999). Overall, stressful conditions such as high concentrations of salts, sugars, or alcohols, high temperature, both low and high pH, or conditions of blocked DNA replication inhibit flagellum biosynthesis (Maurer *et al.* 2005; Shin and Park 1995; Soutourina *et al.* 2002).

The Csr (carbon storage regulator) system of *E. coli* also controls motility and flagellum biosynthesis. The RNA binding protein CsrA positively regulates *flhDC* expression by binding to the untranslated leader and stabilizing this mRNA (Wei *et al.* 2001). Although much information has been obtained concerning the regulatory circuitry and mechanisms of this complex system (e.g. Romeo 1998; Suzuki *et al.* 2002, 2006; Weilbacher *et al.* 2003), the environmental signals are still somewhat obscure. At the present time, it is evident that quorum sensing via SdiA and environmental pH affect the expression of noncoding RNA antagonists that sequester CsrA (Babitzke and Romeo 2007; Suzuki *et al.* 2002; Mondragon *et al.* 2006). Importantly, while CsrA activates motility, its dominant role in biofilm formation is to repress expression of the polysaccharide adhesin PGA of *E. coli* K-12 (e.g. Wang *et al.* 2005) and overall it acts as a strong repressor of biofilm formation (Jackson *et al.* 2002a).

The temporal control of flagellum biogenesis also involves the Rcs phosphorelay and acetyl-phosphate (Fredericks *et al.* 2006). The Rcs (Regulator of capsule synthesis) phosphorelay activates genes required for capsular biosynthesis and membrane proteins (Boulanger *et al.* 2005), while repressing genes required for flagellum biogenesis (Francez-Charlot *et al.* 2003). The Rcs regulon is thought to be activated by surface contact and envelope stress; however the exact nature of the signal remains unknown (reviewed by Majdalani and Gottesman 2005).

Recent studies implicate the ubiquitous bacterial secondary messenger c-di-GMP (3', 5'-cyclic diguanylic acid) as a central regulator of motility and biofilm formation in diverse Gram-negative species. In general, this nucleotide, which is synthesized by GGDEF domain-containing proteins and is degraded by EAL or HD-GYP domain proteins, affects the transition from planktonic to sessile communities by promoting the production of adhesins and exopolysaccharides and inhibiting flagel-

lum- and pilus-based motility (reviewed in Jenal and Malone 2006; Ryan *et al.* 2006). While c-di-GMP metabolizing proteins often contain sensory domains (e.g. PAS, GAF, CheY-like and REC), only a few environmental cues are known or suspected to influence c-di-GMP metabolism, and with the exception of cellulose synthase, the mechanism of action of this nucleotide is unknown. As in the case of Csr regulation, c-di-GMP generally has opposite effects on biofilm formation and motility, consistent with the idea that while motility facilitates initiation of biofilm formation, it may be detrimental at later stages.

Surface sensing?

Are bacteria able to sense contact with a surface and respond by expressing adhesins? The Cpx signaling system in *E. coli* has provided some circumstantial evidence for surface sensing. Cpx is a two-component system composed of CpxA, a sensor kinase/phosphatase, and CpxR, a DNA-binding response regulator (Raivio and Silhavy 1997). Studies by Otto and Silhavy (2002) showed that a *cpxR* mutant strain forms altered cell-surface interactions in comparison with the wild-type strain and that Cpx-regulated gene expression is enhanced by surface attachment. The mechanism of "surface sensing" is unknown and may be indirect. Studies indicate that the Cpx system responds to misfolded proteins in the periplasm (Danese and Silhavy 1998). In a microtitre plate assay for biofilm formation, *cpxA* mutants that apparently have lost the phosphatase activity of the CpxA protein formed biofilm with less biomass than wild-type strains (Dorel *et al.* 1999). This was due to decreased transcription of the curlin-encoding gene *csgA* (described below). In uropathogenic *E. coli*, Cpx responds to misfolded pyelonephritis-associated P pilin subunits in the periplasm. In turn, DNA binding by CpxR, in conjunction with other transcription factors, induces transcription

from the *papB* and *papI* promoters (Hung *et al.* 2001). Finally, transcriptome analysis in *E. coli* K-12 showed that *cpxP* is highly expressed in biofilms and affects biofilm structure (Beloin *et al.* 2004). Whether attachment to a surface leads to denaturation of certain envelope proteins and mediates the proposed "surface-sensing" by Cpx remains to be determined.

Environmental effects on surface attachment proteins

Bacteria make extensive use of proteinaceous extracellular fimbriae or pili, which permit them to establish surface contacts that promote biofilm formation. Fimbriae are generally under complex regulatory controls, often involving multiple physiological and/or environmental inputs. The following discussion presents some examples in which the environmental conditions and genetic regulation of fimbriae of *E. coli* and its relatives have been examined, illustrating the complexity of the regulatory networks involved in biofilm formation.

Curli

Proteinaceous extracellular fibers called curli were first observed in *E. coli* (Olsen *et al.* 1989), and have been shown to mediate adhesion, colonization, and biofilm formation in this and other species. In *Salmonella* spp., curli are also known as thin aggregative fimbriae (Romling *et al.* 1998). In *E. coli*, curli promote both initial adhesion and cell-cell interaction (Prigent-Combaret *et al.* 2000). A variety of environmental isolates of *E. coli* form biofilms according to their ability to express curli (Castonguay *et al.* 2006). Curli synthesis in *E. coli* is dependent on at least six genes located in the divergently transcribed *csgBA* and *csgDEFG* operons. CsgD activates transcription of the *csgBA* operon, which encodes CsgA, the structural subunit that is

secreted outside of the cell, where CsgB nucleates it into a fiber (Barnhart and Chapman 2006).

Expression of curli is activated under conditions of low temperature, microaerophilic conditions, low nitrogen, phosphate, and iron, low osmolarity, and slow growth or starvation (Gerstel *et al.* 2003; Maurer *et al.* 1998; Olsen *et al.* 1993a, b; reviewed in Barnhart and Chapman 2006) (Figure 2-3). These features imply that curli are produced in the external environment, as opposed to in the mammalian host. However, in addition to abiotic surfaces, curli mediate bacterial binding to extracellular matrix proteins such as fibronectin and laminin (Barnhart and Chapman 2006), suggesting that they may be produced in anticipation or preparation for host attachment and colonization. Other studies have indicated that within a biofilm, curli fimbriae may be expressed at 37°C (Kikuchi *et al.* 2005). Of note, curli are not expressed in many laboratory strains of *E. coli*, due to silencing of the *csgD* promoter (Hammar *et al.* 1995).

Curli expression responds to environmental conditions through at least three different phosphorelay signaling systems. The EnvZ-OmpR two-component regulatory system activates *csgD* transcription and thereby promotes production of curli fimbriae and stable cell-surface interactions at low osmolarity (Vidal *et al.* 1998; Prigent-Combaret *et al.* 2001). However, in conditions of low osmolarity, there is a reduced level of the active response regulator, phosphorylated OmpR, due to the decreased kinase/phosphatase ratio of EnvZ (Cai and Inouye 2002). This would seem to suggest that an increase in osmolarity should result in higher *csgD* transcription and curli biosynthesis. However, high osmolarity has a negative effect on transcription of the curli genes (Prigent-Combaret *et al.* 2001). This apparent contradiction can be reconciled by the observation that the Cpx pathway, which represses transcription of curli, is in-

duced by high osmolarity and masks OmpR activation (Prigent-Combaret *et al.* 2001). Whereas CpxR represses *csgD* in high salt concentrations, the nucleoid-associated protein H-NS mediates *csgD* repression in high sucrose, independently of CpxR (Jubelin *et al.* 2005). Activation of the Cpx pathway by curlin accumulation also results in the repression of the *csgD* and *csgB* operons (Prigent-Combaret *et al.* 2001). In addition, the RcsCDB phosphorelay system, which controls the synthesis of capsule and flagella, also represses expression of curli (Vianney *et al.* 2005). A comprehensive model in which EnvZ-OmpR, Cpx, and Rcs regulate *csgD* transcription and curli gene expression in response to changes in osmolarity has been proposed (Jubelin *et al.* 2005).

Transcription from the *csgD* promoter is also regulated by other global transcription factors, including *rpoS*, *crl*, and *hns* (Romling *et al.* 1998). The stationary phase sigma factor RpoS (σ^s) directly activates transcription of the *csgBA* promoter in response to slow growth or other stresses (Hengge-Aronis 2002). The small protein Crl, which is preferentially expressed at low temperature and in stationary phase, interacts with the σ^s subunit and apparently promotes curli production by strengthening the association of σ^s with core RNA polymerase to enhance transcription initiation at *csgBA* (Bougdour *et al.* 2004). The protein H-NS has both direct and indirect effects on curli, depending on the environmental conditions (Jubelin *et al*. 2005). Apparently, integration host factor (IHF), H-NS, and OmpR form a nucleoprotein complex with the *csgD* promoter, resulting in elevated expression under microaerophilic growth conditions (Gerstel *et al.* 2003).

The regulatory nucleotide c-di-GMP (which is produced in response to complex regulatory cues) activates the production of both curli and cellulose in certain *E. coli* strains and in *Salmonella enterica* serovar Typhimurium (e.g. Kader *et al.* 2006;

Weber *et al.* 2006). Curli and cellulose together produce a strong biofilm matix facilitating attachment to hydrophilic and hydrophobic surfaces (Zogaj *et al.* 2003).

Type 1 fimbriae

Type 1, or mannose-sensitive fimbriae are rigid 7-nm-wide and ≈1-µm-long, rod-shaped surface structures found on the majority of *E. coli* strains and are widespread among the Enterobacteriaceae (Schembri *et al.* 2001). Type 1 fimbriae are important in the colonization of various host tissues by *E. coli* and in biofilm formation on abiotic surfaces (see the chapter by Hatt and Rather; Pratt and Kolter 1998). The FimH adhesive protein expressed on the tip of type 1 fimbriae binds to glycoproteins including natural ligands such as uroplakins on urinary epithelial cells in urinary bladders, and immunoglobulin A or mucin in intestines and lungs (e.g. Mulvey *et al.* 2000). A typical type 1 fimbriated bacterium has 200-500 peritrichously-arranged fimbriae (Lowe *et al.* 1987).

Production of type 1 fimbriae requires a polycistronic operon comprising the seven structural genes (*fimAICDFGH*) and two monocistronic operons encoding the site-specific recombinases FimB and FimE. Transcription of type 1 fimbriae genes is phase variable due to FimB- and FimE-mediated inversion of a 314 bp DNA fragment that contains the promoter for the polycistronic *fim* operon (Klemm 1986). Within a cell population, type 1 fimbriae expression is activated at body temperature and is repressed by high osmolarity and low pH. These effects are mediated through altered switching frequency of the *fim* operon promoter (e.g. Gally *et al.* 1993; Schwan *et al.* 2002). Although the environmental signals remain to be shown for LrhA, this transcriptional regulator represses motility and chemotaxis genes and represses production of type 1 fimbriae by altering phase variation (Blumer *et al.* 2005). Furthermore,

the alarmone ppGpp (guanosine 3', 5'-bispyrophosphate), which is produced in response to amino acid or carbon starvation, activates expression of type 1 fimbriae and biofilm formation in uropathogenic *E. coli* through its role in expression of the FimB recombinase (Aberg *et al.* 2006). Acetyl-phosphate activates production of type 1 fimbriae, perhaps by serving as a phosphodonor for the FimZ response regulator (discussed in Wolfe *et al.* 2003). Acetyl-phosphate accumulates at the transition to stationary phase in the presence of excess carbon and/or the lack of oxygen (Wolfe 2005). Thus, the production of type 1 fimbriae on cells is complex; it is governed by nutritional status and repressed by stresses such as low pH, low temperature and high osmolarity.

Antigen 43 and related proteins

Antigen 43, encoded by the *flu* locus, is an autoaggregation factor produced by many *E. coli* strains. It was originally discovered for its ability to cause bacterial aggregation (reviewed in Klemm *et al.* 2006). Antigen 43 is a member of the self-associating autotransporter (SAAT) group of proteins consisting of a signal peptide for transfer across the inner membrane, a translocator domain, and a secreted passenger domain. SAATs, including Ag43, TibA, and AIDA (adhesin involved in diffuse adherence), can interact with each other to cause formation of mixed bacterial aggregates. These proteins are anchored directly to the outer membrane and protrude only ~10 nm from the surface, resulting in closer cell-cell interactions than those seen with curli or other fimbriae. Expression of bulky surface structures that protrude beyond this distance in the bacterial envelope (e.g. type 1 pili or capsules) interferes sterically with Ag43-mediated aggregation (Klemm *et al.* 2006).

Ag43 expression undergoes phase variation controlled by OxyR and Dam (deoxyadenosine methylase). The cellular redox sensor OxyR represses Ag43 expression by binding to the *flu* promoter, while Dam activates Ag43 expression by methylating DNA that overlaps the OxyR binding (Wallecha *et al.* 2002; see the chapter by Beloin *et al.*). OxyR plays an important role in sensing peroxides encountered during oxidative stress, although its oxidation state may not influence *flu* regulation (Wallecha *et al.* 2003). It activates protective measures, such as enzymes that detoxify reactive oxygen compounds or repair damage caused by them. Furthermore, Ag43-mediated cell aggregation confers protection from hydrogen peroxide killing (Schembri *et al.* 2003a). Ag43 and other SAAT proteins, including AIDA-I and TibA, also impair bacterial motility (Ulett *et al.* 2006). Several studies have indicated that Ag43 is induced specifically during biofilm growth, and its expression enhances *E. coli* biofilm formation (discussed in Klemm *et al.* 2004; Schembri *et al.* 2003b). In urinary track infections, Ag43 is expressed by *E. coli* cells that form biofilm-like structures within bladder cells (Anderson *et al.* 2003).

Finally, environmental pH affects antigen 43-mediated cellular aggregation, which occurs more rapidly as the pH decreases from10 to 4 (Klemm *et al.* 2004). This strong effect of pH on cellular aggregation has been proposed to facilitate more rapid transit through and thus improved survival in the stomach (Klemm *et al.* 2006).

Conversion from temporary to permanent attachment: A regulated process?

During normal biofilm development, some species of bacteria bind to a surface reversibly or temporarily, followed by irreversible or permanent attachment. This phenomenon was first reported in the 1940s (reviewed in Stoodley *et al.* 2002a). Genes affecting this transition and biofilm development have been studied in *Pseu-*

domonas aeruginosa and *Pseudomonas fluorescens* (Caiazza and O'Toole 2004; Hinsa *et al.* 2003) as well as in *E. coli* (Agladze *et al.* 2005). In these species, temporarily attached cells interact with a surface by a cell pole, whereas permanently attached cells are associated via the lateral cell surface. Mutants of *P. fluorescens* that failed to produce a large adhesive protein (LapA) and *E. coli* mutants that fail to produce a polysaccharide adhesin (PGA, described below) were similarly defective in the conversion from temporary to permanent attachment (Agladze *et al.* 2005; Hinsa *et al.* 2003). In *E. coli*, the kinetics of this transition process was monitored with an assay developed for this purpose (Agladze *et al.* 2005).

Conversion from temporary to permanent attachment has been proposed to be a regulated process, perhaps allowing the cell to sample its local environment before committing to a sessile lifestyle (Caiazza and O'Toole 2004). Furthermore, because cell attachment in both monolayers and more mature biofilms of *E. coli* exhibit distinct, nonrandom spatial organization, it has been suggested that proximity to neighboring cells might govern the conversion to permanent attachment (Agladze *et al.* 2003; 2005). *E. coli* mutants lacking the polysaccharide adhesin PGA exhibited aperiodic cell distribution and no apparent cell-cell adhesion. In theory, formation of such patterns could be guided by a reaction-diffusion or Turing process (e.g. Maini *et al.* 2006), based on the sensing of a bacterially synthesized inhibitor of attachment. Validation of such hypotheses will require an understanding of the putative signals in the local environment that are being recognized, the putative signal transduction pathways through which this information flows, and a better appreciation of the biochemistry of temporary and permanent attachment processes.

Environmental effects on matrix polysaccharides

A hallmark of prototypical biofilms is that they are composed of cells embedded within a complex matrix (reviewed in Branda *et. al* 2005; Sutherland 2001 a, b). While polypeptides, nucleic acids, lipids, and a host of small molecules are often present in biofilm matrices, polysaccharide, which may include multiple different polymers, is often the main component (e.g. Morikawa *et al.* 2006; Schooling and Beveridge 2006; Steinberger and Holden 2005; Whitchurch *et al.* 2002). Due to their roles in cellular interactions with surfaces and their direct exposure to cells of the immune system, matrix polysaccharides have become topics of considerable interest. However, an understanding of these polymers is limited, even for the best studied biofilms. Certain polysaccharides influence biofilm architecture, ion selectivity, resistance to desiccation, and other properties, but probably do not function as biofilm adhesins *per se*. Acidic polysaccharides, such as alginate of *P. aeruginosa*, colanic acid and K antigens of *E. coli*, and capsular polysaccharides of *Pantoea stewartii* and *Xanthomonas campestris* may be considered in this class; they are not essential for biofilm formation and may even be inhibitory under certain conditions (Crossman and Dow 2004; Hanna *et al.* 2003; Schembri *et al.* 2004; Stapper *et al.* 2004; von Bodman *et al.* 2003; Wozniak *et al.* 2003). In contrast, other polysaccharides serve as adhesins that assist cell-surface and/or cell-cell attachment. The conditions and regulatory factors that promote the synthesis of the latter polysaccharides drive biofilm formation. Polymers that fall into the latter category tend to be basic or neutral, and include β-1,6-*N*-acetyl-D-glucosamine polymers of staphylococci, *E. coli, Yersinia pestis, Bordetella* species, *Actinobacilli*, and *P. fluorescens* (Heilmann *et al.* 1996a, b; Itoh *et al.* 2005; Litran *et al.* 2002; Maira- Wang *et al.* 2004; Parise *et al.* 2007; Kaplan *et al.* 2004), Psl and Pel of *Pseudomonas aeruginosa* (Friedman *et al.* 2004; Jackson *et al.* 2004; Vasseur *et al.*

2005), cellulose, which is produced by many eubacteria (reviewed in Lasa 2006), and the extracellular D-glucans of *Streptococcus mutans* (Munro *et al.* 1995). Some examples that illustrate the complex regulation of poly-β-1,6-*N*-acetyl-D-glucosamine polymers in Gram-positive and negative bacteria follow.

Poly-β-1,6-*N*-acetyl-D-glucosamine was discovered in *Staphylococcus epidermidis* (Heilmann *et al.* 1996 a, b; see the chapter by Otto) and later was found to serve as a biofilm adhesin in Gram-negative bacteria (Wang *et al.* 2004). This polymer is referred to as PIA (polysaccharide intercellular adhesin) or PNAG in *S. epidermidis* and *Staphylococus aureus,* respectively. Production of PIA/PNAG is dependent on the *ica* operon (*icaADBC*), which is regulated by a divergently transcribed gene (*icaR*) that encodes a transcriptional repressor, which responds to various environmental conditions (Conlon *et al.* 2002, Figure 2-4).

Expression of the *icaADBC* operon is increased during growth in nutrient-rich or iron-limiting conditions and is induced by stressful stimuli such as heat, ethanol, and high concentrations of salt increase *ica* expression and PIA production (Vuong *et al.* 2005 and references therein). The latter stressors are known to repress tricarboxylic acid (TCA) cycle activity, and the TCA cycle inhibitor fluorocitrate increases PIA production (Vuong *et al.* 2005). Furthermore, anaerobic conditions induce PIA production (Cramton *et al.* 2001). Sub-inhibitory concentrations of tetracycline and the semisynthetic streptogramin antibiotic quinupristin-dalfopristin enhance *icaADBC* expression 9- to 11-fold (Rachid *et al.* 2000). Ethanol induction of PIA synthesis is *icaR*-dependent (Conlon *et al.* 2002). Interestingly, glucose addition causes repression of *icaADBC*, but enhances PIA production, possibly via its precursor-product relationship with PIA (Dobinsky *et al.* 2003).

SarA is a global regulatory DNA-binding protein involved in expression of a variety of staphylococcal virulence genes. Transcription of *icaADBC,* which is essential for biofilm development in *S. aureus,* is activated by SarA binding (Tormo *et al.* 2005; Valle *et al.* 2003). In turn, *sarA* is activated by the stress response sigma factor, σ^B, which modulates responses to environmental stress and energy depletion. It is important to note that SarA, but not σ^B, is essential for biofilm development by *S. aureus* (Valle *et al.* 2003), suggesting that there are other means of activating *sarA* expression. σ^B also represses, possibly indirectly, *icaR* expression (Tormo *et al.* 2005), indicative of the complex interactions within this regulatory system.

The bacterial LuxS-dependent quorum sensing systems are found in diverse species, and may permit bacteria to assess the overall microbial density of the environment (Schauder and Bassler 2001; Xavier and Bassler 2003). Biofilm formation in a *luxS* mutant strain of *S. epidermidis* was considerably enhanced, suggesting that the reaction product of the LuxS protein, autoinducer 2 (AI-2), represses *icaADBC* (Xu *et al.* 2006). Of note, quorum sensing systems generally promote the expression of factors required for biofilm formation (Kirisits and Parsek 2006; Kong *et al.* 2006, Spoering and Gilmore 2006); although another example of quorum sensing inhibition of biofilm formation is found in *Vibrio cholerae* (Hammer and Bassler 2003). A well-studied quorum sensing system of *S. epidermidis* and *S. aureus*, *agr* (accessory gene regulator), also inhibits biofilm formation, but does not affect PIA levels (Vuong *et al*. 2003).

The IS256 insertion element is able to integrate into and inactivate or excise from *icaADBC* or genes that affect *ica* expression (e.g. *sarA*), thus constituting a phase-variable mode of regulation (Conlon *et al.* 2004; Ziebuhr *et al.* 1999). Transposition of IS256, but not transcription of the transposase, is repressed by σ^B (Valle *et*

al. 2007). Valle and colleagues believe that environmental stress conditions activate σ^B and decrease the generation of biofilm-negative variants, in line with evidence indicating that NaCl and other stressors induce *ica*-dependent biofilm formation. The authors of this study also speculate that the IS256 element may modulate biofilm dispersal by affecting the proportion of biofilm-negative variants in a biofilm.

In *E. coli*, an understanding of biofilm regulation preceded the discovery of the *pgaABCD* structural genes, which in turn led to the identification of novel regulatory genes for biofilm formation (Figure 2-5). An initial observation was that the global regulatory gene *csrA* of *E. coli* dramatically represses biofilm formation (Romeo *et al.* 1993), a phenotype that could not be explained by any previously known adhesin (Jackson *et al.* 2002a). A genetic screen for factors that cause hyper-biofilm formation in the *csrA* mutant led to discovery of the *pgaABCD* locus, which encodes gene products similar to the glycosyltransferase IcaA (PgaC) (Gerke *et al.* 1998) and the *N*-deacetylase IcaB (PgaB) (Vuong *et al.* 2004). The mechanism of CsrA in this regulation is to bind to the *pgaABCD* mRNA leader at six sites, including sites that overlap the Shine-Dalgarno sequence and initiation codon, and thereby prevent ribosome binding (Wang *et al.* 2005). Translational repression likely results in the observed destabilization of this transcript by CsrA. Transcription of the *pgaABCD* operon is activated by the binding of the LysR family protein NhaR to the sole promoter of this operon in response to high pH or high Na^+ (Goller *et al.* 2006). The biosynthesis of PGA is also regulated by c-di-GMP (Suzuki *et al.* 2006; unpublished studies) and is increased at low temperature (Wang *et al.* 2005; unpublished data). The latter findings are reminiscent of regulation in the homologous *hmsHFRS* system of *Yersinia pestis* (Bobrov *et al.* 2005; Perry *et al.* 2004; Simm *et al.* 2005).

The *hmsHFRS* operon and *hmsT* are required for *Yersinia pestis* biofilm formation in the gut of the flea vector and are important in the transmission of plague (see the chapter by Hinnebusch and Erickson). Hms-dependent biofilm formation is optimal at low temperature. The levels of HmsH, HmsR, and HmsT proteins are lower at 37°C than at 26°C, and temperature-dependent degradation of HmsH, HmsR and HmsT proteins seems to be responsible for the Hms^- phenotype at 37°C (Perry *et al.* 2004). Additionally, biofilm formation is stimulated by HmsT, a protein that synthesizes c-di-GMP, and is inhibited by HmsP, which likely degrades c-di-GMP (Bobrov *et al.* 2005).

Conditions and factors mediating biofilm dispersal

No doubt, there are times when it is advantageous for cells to be able to escape from a biofilm. Entrapment within the biofilm environment limits bacterial growth (e.g. Rani *et al.* 2007). Furthermore, the transcriptome of mature biofilm, on average, has been suggested to be more similar to that of stationary phase cells than of exponentially growing cultures, although many changes in gene expression appear to be biofilm specific (Beloin *et al.* 2004; Sauer *et al.* 2002; Waite *et al.* 2005). In addition, the biofilm matrix may prevent or at least deter cells from fleeing deleterious conditions. Dispersal processes are of interest because of their potential to promote spread of bacteria in the environment and because of the possibility to exploit these processes to combat detrimental biofilms. Release of cells or clumps of cells from biofilm can be accomplished by constitutive low level "sloughing" as well as active "dispersion" in which a substantial proportion of the population synchronously exits the biofilm. Several different cellular patterns of biofilm dispersal or escape have been documented under microscopic examination (reviewed by Hall-Stoodley and Stoodley

2005). In addition, the dissolution of cell attachments by surfactant production in *Bacillus subtilis*, *Pseudomonas aeruginosa* and *S. epidermidis* may help to shape biofilm architecture (Boles *et al.* 2005; Branda *et al.* 2001; Davey *et al.* 2003; Vuong *et al.* 2003).

Environmental conditions that influence biofilm dispersal include nutrient availability, oxygen levels, pH, and specific compounds (Gjermansen *et al.* 2005; Jackson *et al.* 2002a; Sauer *et al.* 2004; Thormann *et al.* 2005; Table 2-1). Changes in nutrient availability are a well-recognized determinant of dispersal. This is not surprising, given the importance of nutrient acquisition to bacterial survival. For example, early studies revealed that introduction of a rich medium to a tightly-aggregated *Acinetobacter* biofilm that had been grown under low nutrient conditions led to a more open, widely dispersed cell arrangement (James *et al.* 1995). Although the molecular genetics of biofilm dispersal has lagged behind that of formation, recent breakthroughs have paved the way for understanding the dispersal process, from the detection of environmental cues to signal transduction circuitry to the biochemical activities responsible for dispersal.

Biofilm dispersion in *Pseudomonas aeruginosa* is perhaps the most studied and best understood process. Sauer and coworkers examined the proteome of this bacterium during active dispersion and found expression patterns that more closely resembled those of planktonic cells than biofilm cells (Sauer *et al.* 2002). Specific carbon nutrients, including succinate and glutamate, were found to trigger immediate large-scale release of cells (Sauer *et al.* 2004). Genes for motility, ribosomal proteins and phage Pf1 were induced in the dispersed cells, while cells remaining attached contained elevated transcripts for pilus production and anaerobic nitrogen respiration.

The latter activity indicates that insufficient oxygen was available for complete aerobic metabolism of the added carbon substrate in this biofilm.

The above observations support the recent discovery that trace amounts of nitric oxide (NO) or a metabolite thereof mediates dispersal (Barraud *et al.* 2006). This product of anaerobic respiration facilitated the "seeding dispersal" of cells from mature biofilm. In this phage Pf1-dependent process, mature biofilm structures appear to "liquefy" internally, involving both cell death and release of viable cells, and leaving behind hollow, shell-like structures. In addition, exposure to NO dispersed immature biofilms without causing cell death. The normal resistance of biofilm cells to certain antibacterial agents reverted back to the planktonic, sensitive phenotype during this dispersion process. Sauer and coworkers recently identified a gene encoding an apparent chemotaxis protein, BdlA, which is crucial for nutrient dispersal of *P. aeruginosa* (Morgan *et al.* 2006). A mutant lacking this protein also exhibited increased adherence and increased c-di-GMP levels. The latter observation is consistent with a rapidly expanding role of this nucleotide in stimulating bacterial exopolysaccharide synthesis and enhancing adherence properties of cells (reviewed in Jenal and Malone 2006; Romling and Amikam 2006), and the correlation of degradation of this nucleotide with biofilm dispersal (e.g. Gjermansen *et al.* 2006; Morgan *et al.* 2006 Thormann *et al.* 2006). It is tempting to suggest that BldA might regulate c-di-GMP levels in response to, perhaps even by binding to NO. This is consistent with the observations that (i) nutrient-induced dispersal leads to increased anaerobic respiration and (ii) the BdlA protein structure includes a PAS domain, which is typically involved in signal detection. Another consideration is that lung infections of cystic fibrosis patients by *P. aeruginosa* become anaerobic. How these observations might apply to this host environment is still an open question (discussed in Romeo 2006).

There are parallels in other species that suggest dispersal processes that are related, though not identical, to those of *P. aeruginosa*. *Shewanella oneidensis* biofilm disperses rapidly in under anoxic conditions and is likewise induced by an increase in c-di-GMP levels and possibly mediated via effects on exopolysaccharide production (Thormann *et al.* 2006). Is it possible that the cue for this process might not be the decrease of oxygen, but rather the production of NO or another product of anaerobic respiration? *Pseudomonas putida* responds to carbon starvation by inducing dispersal in a process that might involve c-di-GMP regulation, exopolysaccharide and a large proteinaceous adhesin that has also been studied in *Pseudomonas fluorescens* (Gjermansen *et al.* 2005; 2006; Hinsa *et al.* 2003; 2006).

Studies in *Escherichia coli* suggest that CsrA may facilitate dispersal (Jackson *et al.* 2002a). This RNA-binding protein, alternatively referred to as RsmA (repressor of stationary phase metabolites) in some species, post-transcriptionally represses production of the biofilm polysaccharide adhesin β-1,6-*N*-acetyl-D-glucosamine, or PGA, with dramatic effects on biofilm formation (Wang *et al.* 2005). The mechanism of CsrA in biofilm dispersal remains unknown, but could be based on inhibition of PGA synthesis if this polysaccharide is continuously removed by turnover or sloughing. The relatively slow rate of biofilm release (a few hours) that occurs in response to *csrA* induction suggests that the way in which CsrA affects dispersal may be different than in the preceding examples. CsrA activity is governed to a large extent by noncoding regulatory RNAs that sequester this protein, e.g. CsrB, CsrC in *E. coli* (Gudapaty *et al.* 2001; Liu *et al.* 1997; Suzuki *et al*. 2002; Weilbacher *et al.* 2003). Thus, CsrA activity should increase as CsrB and CsrC synthesis decreases or their turnover increases. The environmental control of Csr RNAs is not well defined in any species. However, it typically involves transcriptional activation via BarA-UvrY or

homologous two component signal transduction systems, such as BarA-SirA, ExpS-ExpA, GacS-GacA, or VarS-VarA, and is connected to quorum sensing pathways (Lenz *et al.* 2005; Suzuki *et al.* 2002; reviewed in Babitzke and Romeo 2007). Furthermore, while quorum sensing systems often promote biofilm formation, they can activate biofilm dispersal in some species (e.g. Dow *et al.* 2003; Hammer and Bassler 2003; Yarwood *et al.* 2004).

Mixed species biofilms

The natural environments that most bacteria inhabit are typically complex and dynamic. Unfortunately, this complexity is not fully appreciated when growing organisms in monocultures under laboratory conditions. Biofilm communities associated with the plant rhizosphere (Ramey *et al.* 2004), intestinal mucosa (Eckburg *et al.* 2005), oral cavity and gingival crevices (Kroes *et al.* 1999; Kolenbrander 2000) and many other natural sites are inhabited by numerous different species in close proximity. Such environments are rich in biological stimuli to be processed by bacterial cells and used to direct biofilm development in response to changing conditions.

Studies using species-specific probes and microscopy have revealed complex spatial organization of species in natural biofilm communities (e.g. Bottari *et al.* 2006). Furthermore, co-culture experiments have demonstrated the importance of competition for nutrients and commensal metabolic networks in the dynamics of mixed-species biofilms (e.g. Christensen *et al.* 2002). Thus, spatial and metabolic interactions between species contribute to the organization of multispecies biofilms, and the production of a dynamic local environment (Battin *et al.* 2007; Tolker-Nielsen and Molin 2000). The distribution of cells and biomass in complex biofilms is influenced by the physiology of the organisms present, which in turn leads to the development of

local nutrient gradients. Furthermore, mixed species biofilms can evolve rapidly and lead to stable interactions between species when driven by selective pressure for co-metabolism (Hansen *et al.* 2007). In the latter model system, a commensalistic relationship was established between *Acinetobacter* sp. strain C6 and *Pseudomonas putida* KT2440 when the latter species evolved the ability to adhere and form biofilm close to *Acinetobacter* microcolonies, and thereby capture the metabolite benzoate.

Mutualistic relationships can also occur in mixed-species biofilms. For example, biofilm formation by *E. coli* PHL565 was synergistically enhanced by growth in mixed culture with *Pseudomonas putida* MT2 (Castonguay *et al.* 2006). Particularly striking mutualistic effects on biofilm formation have been shown for species that inhabit dental plaque (e.g. Palmer *et al.* 2001). Conjugative plasmids have been demonstrated to induce bacterial biofilm development in co-culture experiments (e.g. Ghigo 2001), and biofilm formation increases the chance for lateral gene transfer and thus the risk for interspecies gene transfer and the consequent spread of virulence factors and antibiotic resistance (e.g. Weigel *et al*. 2007). In fact, many examples of synergistic induction of biofilm formation were observed when a large collection of nondomesticated *E. coli* strains were individually co-cultivated with a laboratory strain or with each other. This was most often precipitated by conjugal transfer of natural plasmids carried by the isolates.

Quorum sensing can have somewhat unpredictable effects on biofilm formation (Merritt *et al.* 2003; Schauder and Bassler 2001; Waters and Bassler, 2005; Bassler and Losick 2006). While many quorum sensing systems are relatively species specific, the autoinducer-2 based (or LuxS-dependent) quorum sensing system is widespread among eubacteria and may serve as a universal language for these organ-

isms. Despite its profound implications, the impact of interspecies communication on biofilm development is presently not well understood.

CONCLUSIONS AND OUTLOOK

Recent advances in our understanding of the biofilm development cycle have indicated that in most cases, it is a dynamic process in which common environmental factors such as nutritional conditions, temperature, oxygen tension and osmolarity have strong influences. We have begun to understand the factors and pathways that respond to environmental cues and regulate the surface transformations that drive the biofilm development cycle. The distinctive conditions that govern biofilm development for a given species can provide important clues to its natural ecology and life-cycle and vice versa. Many surprises lay in store, and the rules for biofilm development seem to be made to be broken. For example, the minimalist bacterium *Mycoplasma pulmonis* lacks any two component signal transduction system or recognizable global regulator. Nevertheless, it is able to modulate biofilm formation through slipped-strand mispairing of the gene for an adhesive surface protein (Simmons *et al*. 2007).

At the present time, there are many unanswered or partially answered questions concerning the influence of the bacterial environment on biofilm development. (1) Which steps in development are most important for regulation and how are these steps regulated? The transition from reversible to irreversible attachment would seem to be an important site for regulation to occur, but this remains to be shown. (2) While a variety of environmental influences on biofilm are now known in a few model organisms, information on their relative importance and integration is lacking. (3) Systematic analysis of gene expression by array studies has provided much information

concerning gene expression patterns during biofilm development. How these genes are regulated and which of these genes are critical for the development process? (4) How does the presence of other microorganisms and growth in association with eukaryotic hosts influence biofilm formation by a given species? This is a complex biological question that likely differs for each species of interest. Nevertheless, it is critical for the development of new therapeutic strategies and other applications.

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FIGURE LEGENDS

Fig. 2-1. A model for biofilm development. Planktonic cells (1) use motility to approach to and swim on a surface (2). Upon interacting with the substratum by a pole, cells can become reversibly attached, which may allow for sampling of the environment before committing to a sessile lifestyle (3). Next, cells become laterally-attached to the surface, involving adhesins such as PGA or LapA (4). During this time, the attachment of cells begins to create a two-dimensional biofilm, which in *E. coli*, exhibits distinct periodicity in cellular distribution (5). The biofilm grows in thickness as more cells are incorporated into its structure. Extracellular polysaccharides and other substances are produced, resulting in more firmly attached cells within an extracellular matrix. The architecture of the biofilm may be modified by production of surfactant and release of attached cells (6). In response to environmental or physiological clues, cells may be released from the matrix and return to a planktonic state, thus completing the developmental cycle (7). The entire process of biofilm development is dynamic, and is influenced by numerous environmental factors.

Fig. 2-2. Regulation of *E. coli* motility. The *flhDC* operon encodes a DNA binding protein ($FlhD_2C_2$) that serves as a central regulatory point to initiate the motility and chemotaxis cascade of gene expression, which is needed for optimal biofilm formation. Stressful conditions such as high concentrations of salts, sugars, or alcohols, high temperature, both low and high pH, or conditions of blocked DNA replication inhibit flagellum biosynthesis. The RcsCDB phosphorelay system, which somehow is activated by envelope stress, represses *flhDC*. Acetyl-phosphate and high osmolarity activate the EnvZ-OmpR two component signal tranduction system, which represses *flhDC*. The heat shock chaperones DnaK, DnaJ and GrpE are needed for flagellum gene expression, but may be limiting at high temperatures. In addition, *flhDC* transcription is under catabolite repression, and is activated by cAMP-Crp. The RNA binding protein CsrA activates *flhDC* expression by binding to the untranslated leader and stabilizing this mRNA. However, the main effect of CsrA on biofilm formation is to repress expression of the adhesin PGA (see Figure 2-5). LrhA, a LysR-type transcriptional regulator, represses motility as well as expression of type 1 fimbriae. In various species, c-di-GMP, which is synthesized by GGDEF domain-containing proteins and is degraded by EAL domain proteins, inhibits flagellum-based motility.

Fig. 2-3. Conditions affecting curli fimbriae in *E. coli*. Curli fimbriae aid in biofilm formation in certain *E. coli* strains and related species, and are produced through the expression of the divergent operons *csgDEFG* and *csgBA*. CsgD is a DNA binding protein necessary for transcription of *csgBA*, which encodes the nucleation factor and pilin for curli fimbriae, respectively. Other Csg proteins are involved in pilus biogenesis. Both OmpR-P (activator) and CpxR-P (repressor) can simultaneously occupy the *csgDEFG* promoter. EnvZ-OmpR promotes *csgDEFG* transcription at low osmolarity, while CpxA-CpxR represses this operon under envelope stress and high osmolarity. H-NS has multiple effects on these pathways, one of which is to repress *csgD* in high sucrose, independently of CpxR. The RcsCDB phosphorelay system, which controls synthesis of capsule and flagella, also represses curli in response to membrane perturbations and high osmolarity. c-di-GMP activates production of both curli and cellulose in response to uncharacterized stimuli. Low temperature, nitrogen, phosphorus or iron limitation, slow growth and microaerophilic conditions promote curli production. RpoS, in conjunction with Crl, activates transcription of the *csgBA* promoter in response to several of these conditions.

Fig. 2-4. Environmental influences on staphylococcal polysaccharide intercellular adhesin (PIA). The β-1,6-GlcNAc polymer PIA or PNAG, is required for cell-cell adhesion and biofilm formation in *S. epidermidis* and *S. aureus.* Production of PIA depends on *icaADBC* and is repressed by the divergently transcribed *icaR* gene. *icaABCD* expression is increased by growth in nutrient-replete, iron-limiting, anaerobic, and stress-inducing conditions. Several of these environmental conditions repress tricarboxylic acid (TCA) cycle activity. Sub-inhibitory concentrations of certain antibiotics also enhance *icaADBC* expression. IS256 causes phase variation by integrating into and excising from *icaADBC* or genes that affect its expression. The global regulator SarA activates transcription of *icaA* and is essential for biofilm development in *S. aureus*. In turn, *sarA* is activated by the general stress sigma factor σ^B, which also represses *icaR* and IS256 transposition. Glucose apparently represses *icaADBC* expression, but nevertheless, enhances PIA production via a possible product-precursor relationship. The *agr* quorum sensing system negatively regulates biofilm development.

Fig. 2-5. Regulation of the biofilm adhesin PGA in *E. coli*. Poly-β-1,6-*N*-acetyl-glucosamine (PGA) synthesis is regulated at several levels. NhaR binds to the *pgaABCD* promoter and activates transcription in response to high pH or high sodium ion concentrations. CsrA protein binds to six sites in the leader of the *pgaABCD* transcript, which blocks ribosome binding and accelerates the turnover of this mRNA. Expression of *csrA* is activated as cultures approach stationary phase, by unknown mechanism(s). In addition, CsrA is sequestered by the noncoding RNAs CsrB and CsrC. Transcription of these RNAs requires the BarA-UvrY two component signal transduction system, and CsrA itself. The signal for this system is not known, although BarA-UvrY signaling is blocked at low pH. SdiA activates *uvrY* transcription upon binding to N-acyl-homoserine lactones (HSL). *E. coli* does not produce HSL. Therefore, *csrB* and *csrC* transcription should be enhanced in the presence of Gram-negative species that produce such quorum-sensing compounds. CsrB and CsrC RNAs are degraded by a pathway involving a possible sensory protein, CsrD, and RNase E.

Table 2-1. Molecular genetics of biofilm dispersal processes in Gram-negative bacteria.

Organism	Environmental cue	Signal transduction	Output	Reference
P. aeruginosa	carbon nutrients	BdlA, c-di-GMP	Adhesins?	Morgan *et al.* 2006
P. aeruginosa	nitric oxide	?	Phage induction other?	Barraud *et al.* 2006
P. aeruginosa	quorum sensing (*las/rhl*)	?	Phage induction	Purevdorj-Gage *et al.* 2005
P. putida	carbon starvation	c-di-GMP?	LapA protein? Polysaccharide?	Gjermansen *et al.* 2005; 2006
X. campestris	quorum sensing	Rpf signal pathway	β-1,4-mannanase	Dow *et al.* 2003
S. oneidensis	anaerobic conditions	c-di-GMP?	Polysaccharide?	Thormann *et al.* 2005; 2006
E. coli	quorum sensing, other?	Csr system	PGA, other?	Jackson *et al.* 2002a; Wang *et al.* 2004; 2005
A. actinomycetemcommitans	?	?	PGA hydrolase (dispersin B)	Itoh *et al.* 2005; Kaplan *et al.* 2003; 2004

Fig. 2-1.

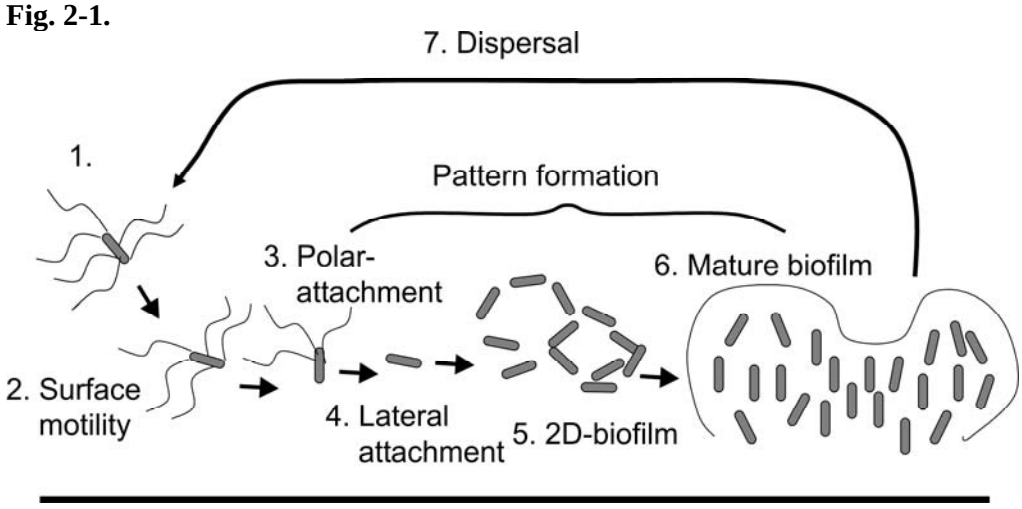

7. Dispersal
1.
Pattern formation
3. Polar-
attachment
6. Mature biofilm
2. Surface
motility
4. Lateral
attachment
5. 2D-biofilm

Fig. 2-2.

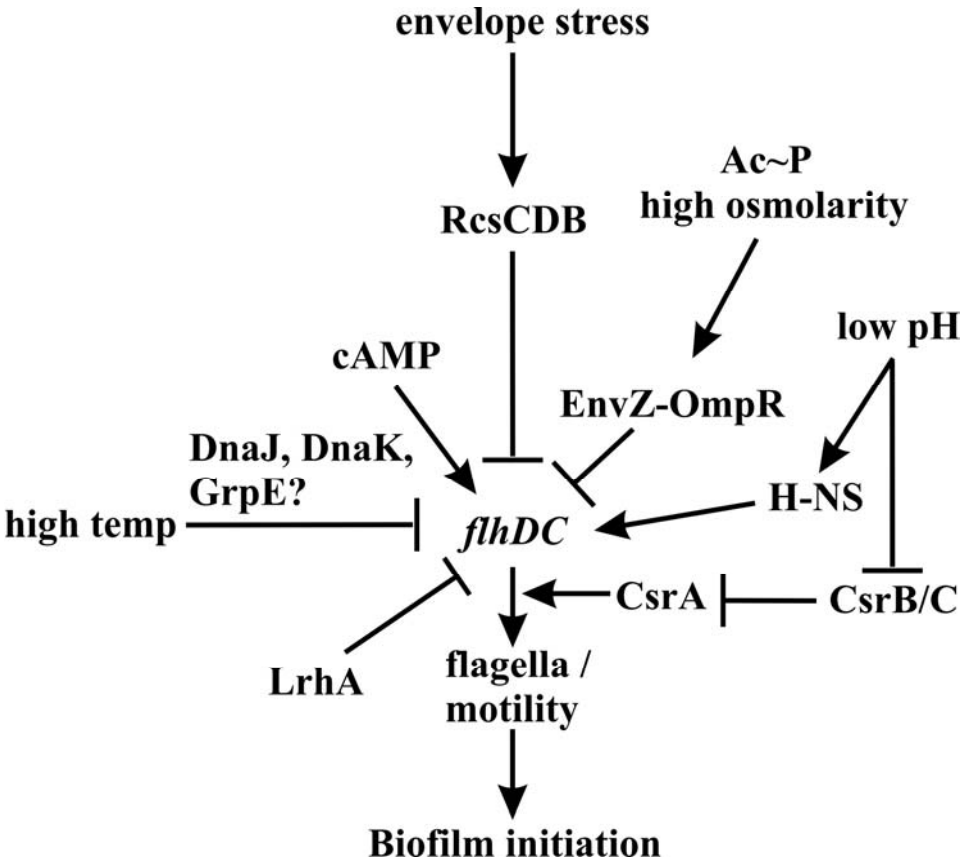

envelope stress
RcsCDB
Ac~P
high osmolarity
low pH
cAMP
EnvZ-OmpR
DnaJ, DnaK,
GrpE?
H-NS
high temp
flhDC
CsrA
CsrB/C
LrhA
flagella /
motility
Biofilm initiation

Fig. 2-3.

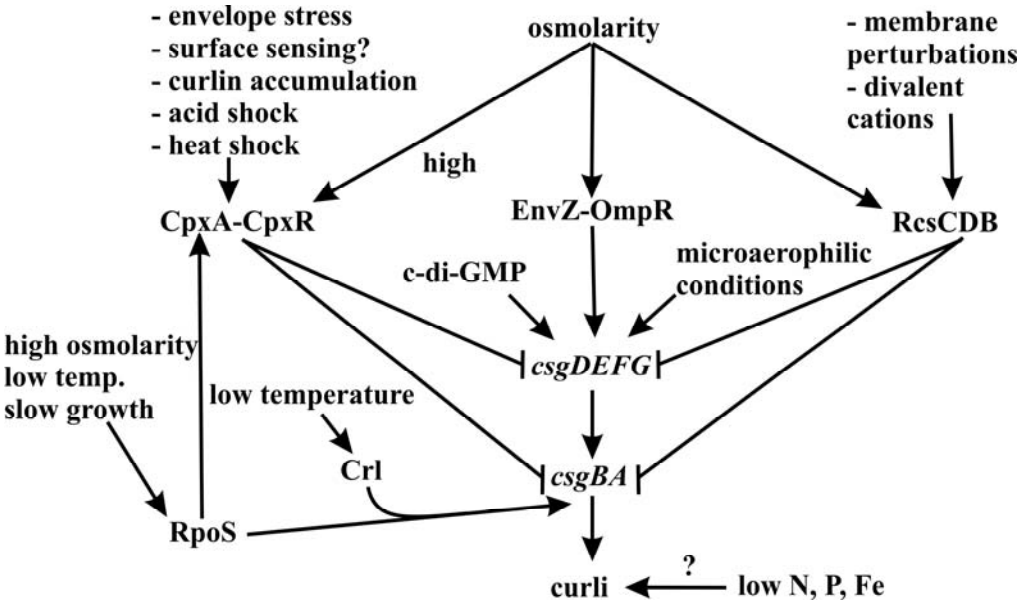

- envelope stress
- surface sensing?
- curlin accumulation
- acid shock
- heat shock
osmolarity
- membrane perturbations
- divalent cations
high
CpxA-CpxR
EnvZ-OmpR
RcsCDB
c-di-GMP
microaerophilic conditions
high osmolarity
low temp.
slow growth
csgDEFG
low temperature
Crl
csgBA
RpoS
curli
?
low N, P, Fe

Fig. 2-4.

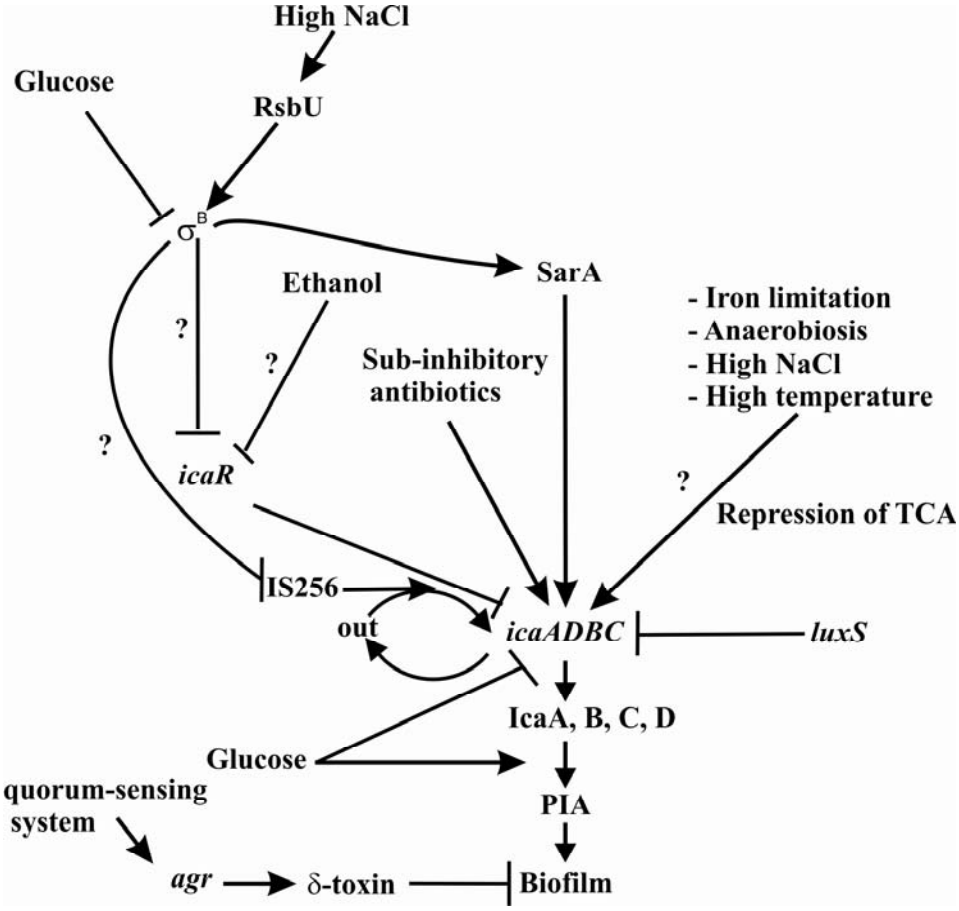
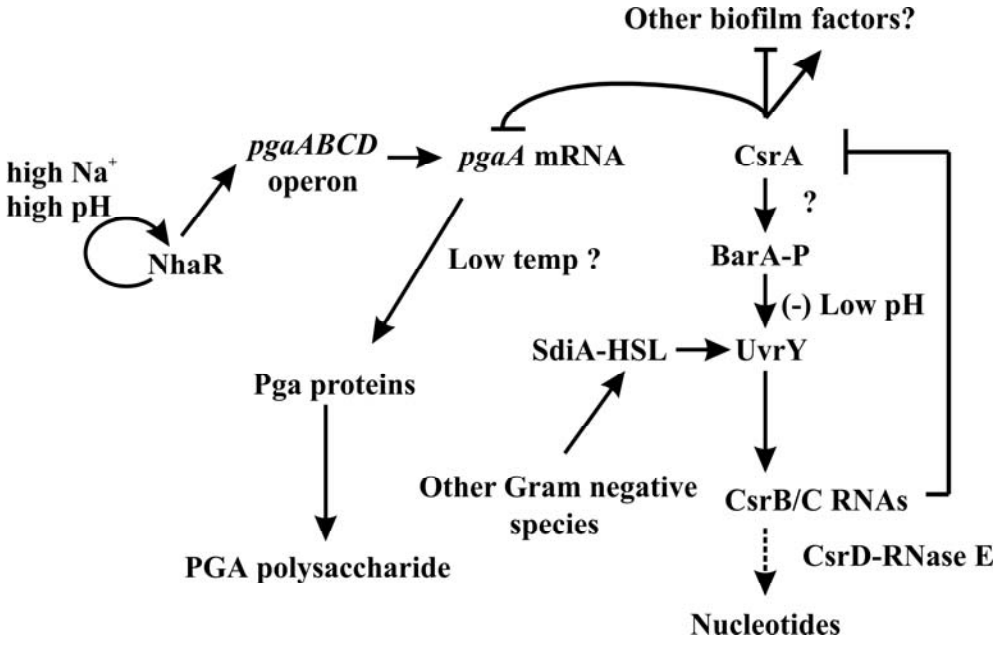

High NaCl
Glucose
RsbU
σB
SarA
Ethanol
- Iron limitation
- Anaerobiosis
- High NaCl
- High temperature
?
?
Sub-inhibitory
antibiotics
?
icaR
?
Repression of TCA
IS256
out
icaADBC
luxS
IcaA, B, C, D
Glucose
quorum-sensing
system
PIA
agr
δ-toxin
Biofilm

Fig. 2-5.


Other biofilm factors?
high Na+
high pH
pgaABCD
operon
pgaA mRNA
CsrA
?
NhaR
Low temp ?
BarA-P
(-) Low pH
SdiA-HSL
UvrY
Pga proteins
Other Gram negative
species
CsrB/C RNAs
CsrD-RNase E
PGA polysaccharide
Nucleotides

Chapter 3: The Cation-responsive Protein NhaR of *Escherichia coli* Activates *pgaABCD* Transcription, Required for Production of the Biofilm Adhesin Poly-β-1,6-*N*-acetyl-D-glucosamine

Carlos Goller[1], Xin Wang[1], Yoshikane Itoh[1] and Tony Romeo[1,2]

[1]Department of Microbiology and Immunology, Emory University School of Medicine, Emory University School of Medicine, 3105 Rollins Research Center, 1510 Clifton Rd. N.E., Atlanta, GA 30322.

[2] Department of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611.

This chapter consists of a manuscript that was published in *J. Bacteriol* **188**: 8022-8032. The manuscript was written by Carlos C. Goller and Tony Romeo. The *pga* deletion strains and over-expression plasmid were constructed by Xin Wang. Yoshikane Itoh conducted the dot plot analysis. All remaining experiments were performed by Carlos C. Goller.

SUMMARY

A transposon mutagenesis screen for novel genes involved in *Escherichia coli* biofilm formation, conducted previously by Dr. Xin Wang and Rebecca DesPlas, yielded numerous mutants with altered biofilm formation, with respect to the parent strain. One promising candidate, NhaR, is a LysR-type transcriptional regulator that upon disruption results in a strain with a severe decrease in biofilm formation without affecting planktonic growth. Characterization of the mechanism by which NhaR affects biofilm formation provided evidence that this protein is responsible for transcriptional activation of the *pgaABCD* operon required for production of the biofilm adhesin poly-β-1,6-*N*-acetyl-D-glucosamine (PGA). PGA production was undetectable in an *nhaR* mutant strain and complemented to wild-type levels upon plasmidic expression of *nhaR*. Expression of a *pgaA'-'lacZ* translational fusion was induced in a *nhaR*-dependent fashion by NaCl and alkaline pH, but not by $CaCl_2$ or sucrose. Furthermore, primer extension and quantitative real-time reverse-transcription PCR (q-RT-PCR) analyses revealed that NhaR affects the steady state level of *pga* mRNA. A purified recombinant NhaR protein bound specifically and with high affinity within the *pgaABCD* promoter region with an apparent binding site overlapping the -35 element and a second site that lies immediately upstream of the first. Recombinant NhaR was necessary and sufficient for activation of *in vitro* transcription from the *pgaA* promoter. These results indicate that NhaR is critical for *pgaABCD* expression and define a novel role for this protein in sensing environmental cation concentrations and pH to trigger biofilm formation and sodium-stress survival mechanisms.

ABSTRACT

The *pgaABCD* operon of *Escherichia coli* is required for production of the biofilm adhesin poly-β-1,6-*N*-acetyl-D-glucosamine (PGA). We establish here that NhaR, a DNA-binding protein of the LysR family of transcriptional regulators, activates transcription of this operon. Disruption of the *nhaR* gene decreased biofilm formation without affecting planktonic growth. PGA production was undetectable in an *nhaR* mutant strain. Expression of a *pgaA'-'lacZ* translational fusion was induced by NaCl and alkaline pH, but not by $CaCl_2$ or sucrose, in a *nhaR*-dependent fashion. Primer extension and quantitative real-time reverse-transcription PCR (q-RT-PCR) analyses further revealed that NhaR affects the steady state level of *pga* mRNA. A purified recombinant NhaR protein bound specifically and with high affinity within the *pgaABCD* promoter region; one apparent binding site overlaps the -35 element and a second site lies immediately upstream of the first. This protein was necessary and sufficient for activation of *in vitro* transcription from the *pgaA* promoter. These results define a novel mechanism for regulation of biofilm formation in response to environmental conditions and suggest an expanded role for NhaR in promoting bacterial survival.

INTRODUCTION

Bacteria in the natural environment often form complex communities of cells associated with a surface or interface and containing a polysaccharide matrix (Branda *et al.*, 2005; Costerton *et al.*, 1995; Donlan and Costerton, 2002; Hall-Stoodley *et al.*, 2004). This physiological state, known as a biofilm, provides an environment that can facilitate horizontal gene transfer and promote survival under hostile conditions (Costerton *et al.*, 1995; Costerton *et al.*, 1999; Sorensen *et al.*, 2005). Biofilm-associated cells are

generally more resistant to antimicrobial treatments than free-living bacteria (Davies, 2003; Fux *et al.*, 2005; Mah and O'Toole, 2001). In addition, pathogenic organisms growing as biofilms are protected from attack by the immune system, and these resilient communities of microbes can lead to chronic infections (Costerton *et al.*, 1999; Fux *et al.*, 2005; Hall-Stoodley *et al.*, 2004; Hall-Stoodley and Stoodley, 2005), resulting in medical complications and substantial economic losses (Costerton and Stewart, 2001; O'Toole *et al.*, 2000; Prigent-Combaret *et al.*, 1999). An understanding of the factors that regulate the colonization process is important for developing approaches to combat and control biofilm formation.

Biofilm development is a complex process, initiated by cell attachment to a surface and formation of microcolonies (Costerton and Stewart, 2001; O'Toole *et al.*, 2000). A temporary attachment step often precedes “permanent” attachment (Agladze *et al.*, 2003; Agladze *et al.*, 2005; Caiazza and O'Toole, 2004; Hinsa *et al.*, 2003) and may be aided by motility (Pratt and Kolter, 1998; Wood *et al.*, 2006). In various strains of *Escherichia coli*, attachment and microcolony formation is facilitated by proteinaceous adhesins and polysaccharides such as cellulose and poly-β-1,6-*N*-acetyl-D-glucosamine (PGA) (Da Re and Ghigo, 2006; Pratt and Kolter, 1998; Prigent-Combaret *et al.*, 2000; Reisner *et al.*, 2003; Vidal *et al.*, 1998; Wang *et al.*, 2004; Zogaj *et al.*, 2001). The latter polymer is involved in both cell-cell adhesion and attachment to certain abiotic surfaces by *E. coli* K-12, and also stabilizes biofilm structure of other gram negative bacteria and staphylococci (Itoh *et al.*, 2005 and references therein). Its production depends upon the *pgaABCD* operon, which encodes a GT-2 family vectoral glycosyltransferase (PgaC) that synthesizes this polymer and other proteins thought to be involved in PGA export and

localization. Quorum sensing molecules and other polysaccharides influence the further maturation of various biofilms (Domka *et al.*, 2006; Gonzalez Barrios *et al.*, 2006; Parsek and Greenberg, 2005). Eventually, planktonic cells are released from the biofilm, completing the developmental cycle and leading to colonization elsewhere (O'Toole *et al.*, 2000). Thus, biofilm formation may be viewed as a flexible or dynamic developmental process involving sequential gene expression patterns that are influenced by environmental cues (Beloin and Ghigo, 2005; Ghigo, 2003; O'Toole *et al.*, 2000).

Several regulatory systems affect *E. coli* biofilm formation (e.g. Adams and McLean, 1999; Corona-Izquierdo and Membrillo-Hernandez, 2002; Dorel *et al.*, 1999; Jackson *et al.*, 2002a; Jackson *et al.*, 2002b; Prigent-Combaret *et al.*, 2001; Vidal *et al.*, 1998). The RNA-binding protein CsrA regulates biofilm formation primarily by binding to the untranslated leader and proximal coding region of *pgaABCD* mRNA, which blocks *pgaA* translation and destabilizes this transcript (Jackson *et al.*, 2002a; Jackson *et al.*, 2002b; Romeo, 1998; Wang *et al.*, 2005). Thus, a *csrA* mutant overproduces PGA and exhibits a dramatic increase in biofilm formation. CsrA activity in the cell is controlled by two noncoding RNAs, CsrB and CsrC, which bind to and sequester multiple copies of CsrA, and thus activate *pga* expression and biofilm formation (e.g. Wang *et al.*, 2005). EnvZ/OmpR, H-NS, and the Cpx and Rcs systems all appear to affect biofilm formation by regulating the production of curli fimbriae in response to osmotic conditions (Da Re and Ghigo, 2006; Jubelin *et al.*, 2005; Prigent-Combaret *et al.*, 2001; Stanley and Lazazzera, 2004). The Cpx system also may mediate surface sensing to activate biofilm formation (Lejeune, 2003; Otto and Silhavy, 2002).

Living cells not only respond to changing osmotic conditions, but must also maintain an externally directed sodium gradient and a relatively constant intracellular pH (Padan *et al.*, 1981). Na^+/H^+ antiporters, membrane proteins that exchange Na^+ (or Li^+) for H^+, play important roles in these processes. In *E. coli*, NhaA is the key antiporter that protects against sodium stress, and is essential for growth in the presence of high sodium concentrations, while NhaB becomes essential only in the absence of NhaA (Padan and Schuldiner, 1994; Padan *et al.*, 2001). The *nhaA* gene is located in a two-gene operon, *nhaAR*, which is induced by the presence of monovalent cations. The gene *nhaR* of this operon encodes an autoregulatory protein that activates *nhaAR* transcription, and is homologous to the LysR-OxyR family of prokaryotic transcriptional regulators (Rahav-Manor *et al.*, 1992; Schell, 1993). NhaR also activates *osmC* transcription, which is required for resistance to organic peroxides and long term survival in stationary phase (Gutierrez and Devedjian, 1991; Lesniak *et al.*, 2003; Sturny *et al.*, 2003; Toesca *et al.*, 2001).

LysR-type transcriptional regulators (LTTRs) respond to low molecular weight co-inducer molecules, although co-inducer binding often has been indirectly inferred by isolating mutants that fail to respond to, or have altered specificity for, the co-inducer (Schell, 1993; Tyrrell *et al.*, 1997; Wang and Winans, 1995). Co-inducers typically do not increase promoter affinity, but instead activate transcription via a conformational change in the LTTR-DNA complex (Chen *et al.*, 2005; Hryniewicz and Kredich, 1991; Muraoka *et al.*, 2003; Schell, 1993). While NhaR activates gene expression *in vivo* in response to Na^+, K^+ or Li^+, this cation response has not been reconstituted *in vitro* (Carmel *et al.*, 1997; Dover *et al.*, 1996; Dover and Padan, 2001; Toesca *et al.*, 2001).

Here we establish that NhaR stimulates *pga* transcription, and thus biofilm formation, in response to monovalent cations and alkaline conditions. We propose that this represents a novel means by which NhaR promotes survival of *E. coli* and perhaps other enteric bacteria in response to environmental conditions. Despite its regulatory role in the cell, NhaR-dependent *in vitro* transcription did not respond to monovalent cations, suggesting that additional factors might be involved in this regulation.

MATERIALS AND METHODS

Bacterial strains, phage, plasmids and growth conditions. All *E. coli* strains, phage and plasmids used in the present study are listed in Table 1. Unless otherwise indicated, bacteria were routinely grown at 37°C in Luria-Bertani (LB) medium pH 7.4 (tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 10 g/L) with shaking at 250 rpm. L-broth was identical to LB, but lacked NaCl. Biofilms were grown in 96-well flat-bottom polystyrene microtiter plates (Corning Inc. Life Sciences, Acton, MA) at 26°C for 24 h under static conditions. Colonization factor antigen (CFA) medium (1% Casamino Acids, 0.15% yeast extract, 0.005% $MgSO_4$ and 0.0005% $MnCl_2$, pH 7.4) was used for initial isolation of biofilm mutants. Media were supplemented with antibiotics as needed at the following final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 100 μg/ml; and tetracycline, 10 μg/ml.

Molecular biology and genetics. Standard procedures were used for isolation of supercoiled plasmids, restriction digests, ligations, transformation and P1*vir* transduction of antibiotic markers, including *nhaR::cam* (Miller, 1972; Sambrook *et al.*, 1989).

Quantitative biofilm assay. Biofilm formation was assayed by crystal violet (CV) staining of adherent cells in microtiter wells, as described previously (Jackson *et al.*, 2002b). Overnight cultures were diluted 1:100 into fresh LB medium without antibiotics or supplemented with ampicillin for strains harboring a plasmid. Bacterial growth was determined by measuring the absorbance at 600 nm using a Synergy-HT plate reader (BioTek, Winooski, VT) prior to CV staining. At least 16 replicates were conducted for each sample, and each experiment was performed at least twice. The results were calculated as averages and standard errors from the mean using the GraphPad Prism software package (San Diego, CA). Tukey's multiple comparison test was used for statistical analysis of data (GraphPad Prism).

Detection of PGA. Overnight cultures were diluted 1:100 into fresh LB medium. Cultures were incubated for 24 h at 26°C with shaking at 250 rpm, and were harvested (10 ml) and resuspended in 400 μl of a solution containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA and 1 μg lysozyme. After incubation at room temperature for 30 min, a solution (300 μl) containing 10 μg DNase I, 40 μg RNase, 200 μg α–amylase and 40 mM $MgCl_2$ was added. The mixture was incubated at room temperature for 1 h with occasional mixing before heating to 37°C for 2 h. The resulting cell lysate was then extracted once with 50 mM Tris (pH 8.0) saturated phenol and once with chloroform. The aqueous phase (1 ml from 10 ml of culture) was collected and residual chloroform was allowed to evaporate overnight at room temperature. The samples were concentrated using a YM-3 membrane (Amicon, Houston, TX; molecular cutoff 3,000 Da).

For cell-bound PGA, 3 μl of sample, corresponding to 1 ml of the culture, was applied onto a nitrocellulose membrane and allowed to air-dry overnight at room

temperature. For PGA from spent medium 3 μl of sample corresponding to 75 μl of culture were used. The membrane was blocked for 1 h in 5% non-fat dry milk in PBS-T(1.47 mM NaH_2PO_4, 8.09 mM Na_2HPO_4, 0.145 mM NaCl, and 0.5% Tween 20). A primary anti-staphylococcal PIA (poly-β-1,6-*N*-acetyl-D-glucosamine) rabbit antiserum was used at a dilution of 1:5,000 in 1% BSA / PBS-T for 1 hr. This antiserum was a generous gift from Dr. Dietrich Mack (Heilmann *et al.*, 1996; Rupp *et al.*, 1999). After washing the membrane twice for 5 min and twice for 10 min with PBS-T, the secondary HRP-conjugated antibody (1:10,000, Sigma-Aldrich) was applied for 1 h. The membrane was then washed, and the signal was detected by chemiluminescence, as recommended (Western Lightning Plus protocol, Perkin Elmer). Membranes were photographed using a Bio-Rad ChemiDoc system. The complete experiment was conducted twice with essentially identical results. PIA positive and negative strains, *Staphylococcus epidermidis* 1457 and 1457-M10, respectively, were used as controls.

Plasmid construction. Enzymes for molecular cloning were purchased from Promega (Madison, WI) or New England Biolabs (Ipswich, MA). Constructs were electroporated into DH5α cells, and recombinant plasmids were isolated using Qiagen (Valencia, CA) reagents. Cloned inserts were determined to be free of mutations by DNA sequencing.

Molecular cloning of the *nhaAR* operon involved PCR amplification of chromosomal DNA using the primers NhaAR FWD and NhaAR REV (Table 2) and Elongase (Invitrogen, Carlsbad, CA) under the reaction conditions described by the manufacturer. Annealing temperatures and extension times were based on primer melting

temperature (T_M) and final product size, respectively. The 2.4 kb product was cloned into pCR 2.1 TOPO (Invitrogen).

The *nhaR* gene was amplified from pCRnhaAR plasmid DNA using Elongase and the primers NhaR FWD and NhaR REV (Table 2). The 940 bp PCR product and pKK223-3 were each digested with *Eco*RI and *Pst*I, gel purified using the Qiagen Gel Extraction kit (Qiagen), and ligated to create pNhaR.

A C-terminal his-tagged *nhaR* gene was amplified from pCRnhaAR using Pfu Turbo DNA polymerase (Stratagene) as indicated by the manufacturer, with primers NhaR FWD and NhaR HIS_6 REV (Table 2). The pNhaR-His_6 plasmid was constructed using the procedures described for pNhaR and confirmed to complement the *nhaR* mutant phenotype *in vivo*.

β-galactosidase and total protein assays. β-Galactosidase activity was assayed as described (Romeo *et al.*, 1990), except that activities of *csrA* wild type and mutant strains were determined with 2 h and 1 h reaction times, respectively, and expressed as activity per h. Cultures were grown at 26°C in LB with shaking, and 500 μl of cells were concentrated and used for each assay. Reactions were performed in triplicate. Total cellular protein was measured by the bicinchoninic acid method (BCA Protein Assay, Pierce, Rockford, IL) using bovine serum albumin as the standard (Smith *et al.*, 1985). Absorbance measurements were conducted in flat bottom 96-well microtiter plates using a Synergy HT plate reader (BioTek).

Isolation of total RNA. Total cellular RNA was prepared using the MasterPure™ RNA Purification Kit (Epicentre, Madison, WI). DNA was removed from the preparations that were to be analyzed by q-RT-PCR, using two DNase I digestion

steps, as recommended (Epicentre). RNA was quantified by its absorbance at 260 and 280 nm, and rRNA integrity was assessed on formaldehyde agarose gels. RNA samples were stored at -80°C in 70% ethanol.

Primer extension analysis. Primer extension analyses were performed according to the protocol of Wang *et al.* (Wang *et al.*, 2005). Cells were grown in LB at 26°C to the transition to stationary phase of growth, harvested and total RNA was prepared. A primer, PEXT3, that anneals between position 134 to 154 relative to the transcript initiation site of *pgaABCD* was 5'end-labeled with [γ-^{32}P]ATP (3,000 Ci/mmol, Perkin Elmer, Boston, MA) using T4 polynucleotide kinase (Promega), as described in the manufacturer's manual. Unincorporated [γ-^{32}P] ATP was removed using MicroSpin™ G-25 Columns (Amersham Biosciences, Piscatawa, NJ). Labeled primer (~6 pmol) was added to 40 μg of total RNA. Reverse transcription was performed for 60 min at 50°C using the ThermoScript™ RT-PCR system (Invitrogen), according to the manufacturer's instructions. The labeled primer and a plasmid template (pPGA372) were used to generate a corresponding DNA sequencing ladder with the SequiTherm EXCEL™ II DNA Sequencing Kit (Epicentre). The primer extension products were analyzed on a 6% polyacrylamide sequencing gel containing 6 M urea, which was dried and subjected to autoradiography using a phosphorimager (Storm® 860, Amersham Bioscience). Two different concentrations of RNA were examined to ensure that the signal was limited by the RNA transcripts and not the primer concentration.

q-RT-PCR analysis of *pgaA* mRNA. For q-RT-PCR, *csrA* wild type (MG1655) and mutant (TRMG) strains containing a wild type or mutant *nhaR* gene were grown at 26°C in LB medium to transition phase. Total RNA was isolated, treated to remove

DNA, and the steady level of *pgaL-A* was determined with the primer pair PGART1/PGART2 (Table 2) using the iScript one-step RT-PCR Kit with SYBR® Green (Bio-Rad, Hercules, CA), according to the manufacturer's guidelines. Reactions were performed in duplicate using 100 ng RNA template. A reaction lacking reverse transcriptase was included for each sample as a control for DNA contamination. Reactions were conducted using the iCycler iQ real-time system (Bio-Rad) under the following conditions: 65°C—5 min, 53°C—60 min, 95°C—10 min, (95°C—30 sec, 57°C —1 min, 68°C— 1 min) for 35 cycles. The difference in cycle threshold (ΔC_t) between samples from wild type and mutant strain pairs was calculated. The PCR product identities were confirmed by agarose gel electrophoresis and product uniformity was determined using melting curves (iCycler Instruction Manual, Bio-Rad). These experiments were conducted three times with similar results, and the mean values of the three experiments were determined. A standard curve for each experiment was constructed using a plasmid template, to ensure linearity of the reactions under our experimental conditions. For normalization of pgaA RNA levels, a primer pair 16S-4 / 16S-5 (Table 2) was used to amplify a 99 bp of 16S rRNA using 1 and 10 ng samples of total RNA and a 20 minute reverse transcription time.

Purification of NhaR-His$_6$. NhaR-His$_6$ was purified as described previously, with some modification (Carmel *et al.*, 1997). An overnight culture of *E. coli* DH5α [pNhaR-His$_6$] was used (20 ml/L) to inoculate 1 L of LB medium, and the culture was grown at 37°C with aeration to an A_{600} of 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG, 2 mM) was added to the culture, and growth was continued for 3 h before cells were harvested by centrifugation and stored at -80°C. Frozen cells were thawed and

resuspended (2 g of cells per mL) in lysis buffer containing 1 mg/ml of lysozyme, 4 mM imidazole, 20 mM Tris-HCl (pH 7.9), 500 mM KCl, 5 mM β–mercaptoethanol (BME). After incubation on ice for 30 min, cells were disrupted by sonication. NhaR-His_6 was then purified by affinity chromatography using a His-Trap column (QIAexpressionist, Qiagen), as recommended by the manufacturer. The binding buffer (BB) consisted of 4 mM imidazole, 500 mM KCl, 20 mM Tris-HCl pH 7.9, and 5 mM β-mercaptoethanol (BME). Wash and elution buffers were identical to BB, but contained 60 and 400 mM imidazole, respectively. This single chromatographic step provided an almost homogeneous preparation of NhaR-His_6 with greater than 98% purity, as assessed by SDS-PAGE with Coomassie blue staining. Fractions containing the peak concentration of NhaR-His_6 were pooled and dialyzed overnight at 4°C against 20 mM Tris-HCl (pH 7.9), 50 mM KCl, 1 mM EDTA (pH 8.0), 15 mM BME and 10% glycerol. Glycerol was added to 10% and aliquots (100 μl) were stored at -80°C. This procedure yielded ~0.54 mg of his-tagged NhaR per liter of culture. For experiments designed to examine cation dependency, the protein was extensively dialyzed against this buffer lacking the KCl.

Matrix-assisted laser desorption-ionization time of flight (MALDI-TOF) mass spectrometry. NhaR-His_6 was confirmed by MALDI-TOF mass spectrometry. Spectra were acquired in a linear mode by using a Voyager STR (Applied Biosystems, Foster City, CA) employing a nitrogen laser (λ = 337 nm). One hundred to 600 laser pulses were used to obtain the spectra. The matrix was a saturated solution of 1,4-hydroxyphenylazobenzoic acid in 50% acetonitrile and 1% trifluoroacetic acid, and was mixed in a 10:1 ratio with sample. One microliter of the mixture was transferred to the sample plate and allowed to dry. The predicted mass of recombinant NhaR-His_6 is

35,110 Da, and the experimental mass was determined to be 35,168 Da. The Voyager MALDI-TOF MS with internal calibration is sensitive to ±0.05% (±20 Da). The larger experimental mass was suggestive of a K^+ (40 Da) ion associated with the protein.

Gel mobility shift assay with purified NhaR-His_6 and digoxigenin (DIG)-labeled *pga* DNA. A 132 bp PCR product containing the promoter region of *pgaABCD* was amplified using primers PgaA GS FWD and PgaA GS REV (-115 to +18; Table 2) and Taq Polymerase (Promega). This product was 3'-end labeled with DIG-11-ddUTP (DIG Gel Shift Kit, 2nd Generation, Roche Diagnostics, Indianapolis, IN) as suggested by the manufacturer. Purified recombinant NhaR (NhaR-His_6, 1.17 μM) was added to DNA binding reactions (10 μl) containing 50 mM KCl, 20 mM Tris–HCl, (pH 7.9), 1 mM DTT, 10% glycerol, 125 μg/ml bovine serum albumin and 1.38 fmol of DIG-labeled PCR product. Reactions were carried out for 20 min at 25°C. Loading dye (1.6 μl) containing bromophenol blue and glycerol, supplied with the labeling kit (Roche Diagnostics), was immediately added, gently mixed and reactions were subjected to native gel electrophoresis (5% acrylamide, 0.5X TBE (50 mM Tris, 40 mM boric acid, 0.5 mM EDTA)) at room temperature. The DNA was electroblotted (400 mA, 12 v, 45 min) onto positively charged nitrocellulose membranes (Roche Diagnostics). Membranes were rinsed for 10 min in 2X SSC and nucleic acids were crosslinked (UV Stratalinker™ 1800, Stratagene, La Jolla, CA). The signal was detected by chemiluminescence as recommended (Wash and Block Buffer Set, Roche Diagnostics) using a Bio-Rad ChemiDoc system.

A nonspecific control DNA (*pgaB-C*) of similar size and G+C content to the promoter DNA was amplified from the region overlapping *pgaB* and *pgaC* using the

primer set PgaB-C FWD/PgaB-C REV (Table 2). Quantity One (Bio-Rad) software was used to analyze the blots and quantify the bound and unbound probe. An apparent equilibrium binding constant (K_d) for the NhaR-*pgaA* DNA complex was calculated using GraphPad Prism (Graph Pad) according to a previously described cooperative binding equation (Yakhnin *et al.*, 2000), adapted as follows:

$$pgaA_b = Y_{max} * ((NhaR_f / K_d)n)/(1+((NhaR_f / K_d)n)$$

Y_{max} is the maximum possible bound fraction (100%) of *pgaA* probe DNA ($pgaA_b$). K_d is the concentration of free protein ($NhaR_f$) at which $pgaA_b$ reaches 50% bound. Isolated NhaR protein was assumed to be 100% active for the calculations. The cooperativity of binding is described by the Hill coefficient (n).

DNase I footprint of NhaR-His$_6$ on a linear DNA fragment of *pgaA*.

Oligonucleotides PgaA GS FWD and PgaA GS REV (100 pmol) were individually end-labeled with [γ-^{32}P]ATP (6,000 Ci/mmol, Perkin Elmer) using T4 polynucleotide kinase (Epicentre) as per the manufacturer's instructions. Each labeled primer (20 pmol) was then used with the corresponding unlabeled primer (20 pmol) to amplify the promoter region of the *pgaABCD* operon from pPGA372 plasmid DNA using Platinum Taq Polymerase (Invitrogen). The final PCR products corresponded to a 132 bp fragment containing the promoter region of *pgaA* (-115 to +18), and were gel purified and cleaned with Qiagen QuickSpin columns.

Footprinting reactions contained 0.67 pmol (≥10,000 CPM) of the end-labeled DNA and were incubated with increasing concentrations of NhaR-His$_6$. The 20-µl reaction mixtures contained 33 mM Tris acetate (pH 8.0), 0.15 mg/ml BSA, 10 mM Mg

acetate, 1 mM dithiothreitol and 10% glycerol. The reactions were incubated at 25°C for 15 min before adding DNase I (2 μl of 0.01 mg/ml, 51.8 Units). After 5 min at 25°C, the digests were terminated by addition of 200 μl of Stop Buffer (570 mM ammonium acetate, 80% ethanol). The DNA in each reaction was precipitated with ethanol using Pellet Paint® co-precipitant (Novagen, San Diego, CA). The same labeled primer and plasmid template pPGA372 were used to generate a DNA sequencing ladder with the SequiTherm EXCEL™ II DNA Sequencing Kit (Epicentre). Products were separated on a 6 M urea-polyacrylamide (8%) sequencing gels. Gels were dried under vacuum and the labeled fragments were detected using a Storm® 860 phosphorimager (Amersham Bioscience). Footprinting reactions were conducted in the presence or absence of added sodium chloride. The experiment was conducted four times.

***In vitro* transcription.** For *in vitro* transcription reactions, supercoiled plasmid pPGA372 (1 μg) and his-tagged NhaR (455 nM) were mixed in a 50 μl reaction containing 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol and incubated at 25°C for 20 min. *E. coli* σ^{70}-RNA polymerase holoenzyme (1.25 U; Sigma-Aldrich Corp., St. Louis, MO) was added and the reactions were incubated for 10 min at 37°C. For transcription, 3.5 μl of a mixture of the four nucleoside triphosphates (2.5 mM each) and 40 U of Superasin RNase inhibitor (Ambion, Austin, TX) was added and the solution was incubated for 30 min at 37°C. Potassium and sodium content of the reactions was estimated to be no greater than 1.1 mM. Transcription reactions were terminated by incubation with DNase I (5 units, Epicentre) for 15 min. The mixtures were extracted twice with phenol-chloroform, three times with chloroform, and RNA was precipitated with ethanol and resuspended in diethylpyrocarbonate-treated water (10 μl).

The *pgaA* transcript was detected by primer extension. PEXT3 labeled primer (3.5 pmol) was added to *in vitro* transcribed RNA (5 μl). For comparison, total RNA (25 μg) from the indicated strains was analyzed as stated above (Primer extension analysis), except that Superasin (Ambion) was added to the reaction instead of the RNase inhibitor supplied with the reverse transcriptase.

Microscopy. Sterile borosilicate coverslips were placed into 15-cm petri dishes containing 25 ml of a freshly inoculated (1:100) overnight culture. The petri dishes were incubated at room temperature, and coverslips were removed at various times and rinsed gently with water. Each coverslip was inverted over a Parafilm gasket on a microscope slide and images were obtained 10 min thereafter, as described (Agladze *et al.*, 2003; Agladze *et al.*, 2005). Adherent cells were viewed by transmitted light with an Olympus 1X71 microscope (40X objective lens with a 1.6X selector; Olympus, Thornwood, NY). The images were captured by using a charge-coupled device camera (COHU-4915, COHU, Inc., Florence, KY) connected to a frame-grabber board installed in a personal computer and processed using Image Pro-Plus 4.5 software (Media Cybernetics, Silver Spring, MD).

Bioinformatics. Phylogenetic distribution of NhaR was assessed using BLAST analyses (Altschul *et al.*, 1990) at the NCBI website using the *E. coli* protein as the query sequence.

RESULTS AND DISCUSSION

***nhaR* is needed for optimal biofilm formation.** In order to identify genes that affect biofilm formation in *E. coli* K-12 (DJ25), Wang *et al.* conducted a random transposon

mutagenesis and screened for altered biofilm phenotypes (Wang *et al.*, 2004). This screen yielded an insertion in the *nhaR* open reading frame (*nhaR::cam*), which was determined by transduction to be linked to the biofilm-deficient phenotype of this strain (96B10). The gene product of *nhaR* is a 301 amino acid DNA-binding protein of the LysR family, which is involved in adaptation to elevated Na^+ and alkaline pH, and activates expression of the *nhaAR* operon (Carmel *et al.*, 1997; Dover *et al.*, 1996; Padan and Schuldiner, 1994; Rahav-Manor *et al.*, 1992). Transduction of the *nhaR::cam* mutation into both MG1655 and its isogenic *csrA::kan* mutant caused growth inhibition under the combination of high sodium (0.6 M NaCl) and high pH (8.5) on solid media, but was normal on LB medium. This growth defect was restored by complementation with pNhaR, a plasmid clone of *nhaR* (data not shown). The *nhaR* mutant exhibited normal motility (data not shown).

Disruption of *nhaR* significantly decreased biofilm formation in both MG1655 (4-fold; Fig.3-1A, compare bars 1, 2) and its isogenic *csrA* mutant (>10-fold; compare bars 5, 6). A plasmid containing *nhaR* (pNhaR) complemented the biofilm defect of *nhaR::cam* mutants (Fig. 3-1A and data not shown) confirming the role of this gene in biofilm formation. In fact, this plasmid enhanced biofilm formation beyond that formed by the wild type strain (Fig. 3-1A, compare bars 1, 4). Disruption of *nhaR* also compromised the ability of these strains to adhere to borosilicate glass coverslips, even after 60 h of incubation (Fig. 3-1B and data not shown), demonstrating that these mutants are not simply delayed in biofilm development. Multilayered biofilms were not formed by *nhaR* mutants, and these strains were defective in making the transition from temporary to permanent attachment (data not shown), as described previously (Agladze *et*

al., 2003). Consistent with the known response of NhaR to sodium levels, biofilm formation in MG1655 was inducible (~5-fold) by the addition of NaCl (100 mM) to L-broth (Fig. 3-1C). The *nhaR* mutant showed more modest effects (~2.5-fold), which were complemented *in trans* by pNhaR or a similar plasmid expressing a hexahistidine-tagged NhaR protein (Fig. 3-1C). Because *nhaR* was expressed from a *tac* promoter in the latter example, it is clear that activation via NaCl does not simply reflect increased *nhaAR* expression by this autoregulatory protein. This *nhaR* mutation caused strains to become defective for biofilm formation at 26°C or 37°C in CFA medium or in unbuffered LB medium or LB buffered to pH 6.4, 7.4 or 8.4 (data not shown). The altered attachment behavior of the *nhaR* mutant was similar to that of *pga* mutants, which do not produce the polysaccharide adhesin PGA (Agladze *et al.*, 2003; Wang *et al.*, 2004).

Regulation of biofim formation by NhaR depends on the *pgaABCD* operon. Next, we conducted experiments to gain insight into the mechanism by which NhaR affects biofilm formation. Plasmidic expression of *nhaR* in an *nhaA* mutant strain increased biofilm, establishing that NhaR effects on biofilm were mediated independently of the Na^+/H^+ antiporter NhaA (data not shown). The biofilm defect of an *nhaR* mutant was restored by ectopic expression of *pgaABCD* from a multicopy plasmid (Fig. 3-1D, compare bars 3 and 7). However, biofilm formation in a strain deleted for the *pgaABCD* operon was not affected by ectopic expression of *nhaR* (data not shown). These results are consistent with the possibility that *nhaR* might be needed for the production of PGA.

An *nhaR* mutant does not accumulate PGA. To examine the effect of NhaR on PGA accumulation, relative levels of cell-bound PGA were compared by immunoblot

analysis of *nhaR* wild type and isogenic mutant strains, as well as a control strain deleted for *pgaABCD*. Parental and complemented *nhaR* mutant strains (Fig. 3-2a, c, respectively) produced anti-PIA-reacting material; whereas *nhaR::cam* and Δ*pgaABCD* mutants failed to react with the antiserum (Fig. 3-2b, d). In addition, disruption of *nhaR* prevented the accumulation of PGA in the culture supernatant (data not shown).

NhaR is required for induction of *pgaA'-'lacZ* expression by monovalent cations and alkaline pH. To test whether NhaR regulates the *pgaABCD* operon itself, expression of a chromosomally-encoded *pgaA'-'lacZ* translational fusion containing the upstream non-coding region through the initiation codon of *pgaA* (Wang *et al.*, 2005) was examined in *nhaR* wild type and mutant strains. This reporter fusion was activated by increasing concentrations of NaCl in the *nhaR* wild type strain, but not in the isogenic mutant (Fig. 3-3A).

Previously, NhaR was found to activate gene expression in response to increasing [NaCl], [KCl], [LiCl], or pH of the medium (Dover and Padan, 2001; Toesca *et al.*, 2001). We found that increasing [NaCl], [LiCl], and alkaline pH also activated expression of *pgaA'-'lacZ* in an *nhaR*-dependent fashion (Fig. 3-3B and C, respectively). Addition of KCl or $MgCl_2$ at the same osmolarity as NaCl weakly induced *pgaA'-'lacZ* expression, whereas sucrose or $CaCl_2$ did not (Fig. 3-3B and data not shown), suggesting that this response is not due to increasing osmolarity or Cl^- concentration.

Previous studies indicated that transcription of *nhaAR* in *E. coli* increases as the pH of the medium is increased from 6.5 to 8.5 (Dover *et al.*, 1996). Furthermore, exposure of *Shewanella oneidensis* to alkaline pH caused upregulation of *nhaA* and *nhaR* transcripts (Leaphart *et al.*, 2006). Thus, we grew cultures at pH 6.4, 7.4, and 8.4 in LB

buffered with 60 mM 1,3-bis[tris(hydroxymethyl)-methylamino]propane-HCl (Dover *et al.*, 1996) and examined the expression of the *pgaA'-'lacZ* fusion. This expression increased from pH 6.4 up to 8.4 in a *csrA* mutant strain and was virtually eliminated by disruption of *nhaR* (Fig. 3-3C). Biofilm formation in the strain increased modestly from pH 6.4 to 7.4, but in contrast to *pgaA'-'lacZ* expression, decreased slightly in medium at pH 8.4 (data not shown). In *csrA* wild type strains, expression of the fusion also increased modestly from pH 6.4 to 7.4 and then decreased modestly at pH 8.4, more closely reflecting the results of biofilm assays in this strain (data not shown). Expression of *csrA'-'lacZ* and *csrB-lacZ* fusions did not vary substantially at these pH levels (data not shown). This suggests that *csrA* and *csrB* genes, which post-transcriptionally control *pgaABCD* expression, were not responsible for the effects of pH on the *pgaABCD* operon.

Examination of other possible influences on *pga* expression. H-NS regulates many stress response genes by acting as a major component of chromatin and by responding to changes in osmolarity (Atlung and Ingmer, 1997; Dorman, 2004). Furthermore, *hns* mutants are de-repressed for *nhaAR* expression (Dover *et al.*, 1996). We observed that *pgaA'-'lacZ* expression was only slightly increased (25-30%) and was still inducible by NaCl in an *hns* mutant strain background (data not shown), suggesting that HN-S is not a major regulatory determinant of *pga* gene expression. Mutations eliminating the stationary phase sigma factor, RpoS, and the response regulator OmpR did not affect *pgaA'-'lacZ* expression (data not shown). However, OmpR modestly activates biofilm formation in these strains (Jackson *et al.*, 2002b and data not shown). Thus, the modest induction of biofilm formation by NaCl in an *nhaR* mutant (Fig. 3-1C)

may be due to the effect of OmpR. A transposon insertion that was isolated during the course of these studies in the *rcsD* (*yojN*) gene of the RcsC-RcsD-RcsB signal transduction system, affected biofilm formation but not *pgaA'-'lacZ* expression (data not shown), indicating that the Rcs regulatory system, which responds to osmolarity and envelope stress, does not affect *pga* expression. In addition, we previously determined that *cpxR* does not affect biofilm formation by MG1655 or its isogenic *csrA* mutant (Agladze *et al.*, 2005).

***nhaR* affects *pga* transcript levels *in vivo*.** Our previous studies revealed that (i) the *pgaABCD* genes are co-transcribed as an operon, (ii) the 5'-end of the *pgaABCD* transcript corresponds to an A residue 234 nucleotides upstream from the *pgaA* initiation codon, and (iii) all four coding regions of this transcript are elevated in a *csrA* mutant (Wang *et al.*, 2005). Primer extension analysis (Fig. 3-4, compare lanes 1 and 5) further demonstrated that this transcript was absent from *nhaR* mutants (lanes 2, 3 and 6, 7) and was restored upon complementation by a multicopy plasmid clone of *nhaR* (lanes 4 and 8). *pga* transcript levels were positively correlated with the amount of biofilm formed by these strains (Figs. 3-4 and 1A, and data not shown).

q-RT-PCR was used to quantitate the effect of *nhaR* on steady-state levels of the *pgaA* transcript (Materials and Methods). Disruption of *nhaR* decreased *pgaA* transcript levels 200-fold in both the *csrA* wild type and mutant backgrounds, respectively.

Purified recombinant NhaR binds specifically to *pgaABCD* promoter DNA. To determine whether NhaR-His_6 binds to *pgaA* promoter DNA, gel mobility shift assays were conducted using a 132 bp labeled DNA fragment containing the promoter region and putative NhaR binding sites of *pgaA*. Binding produced a single shift with an

apparent K_d of 0.21 μM (Fig. 3-5), which is within the range of at least one other LTTR (Blumer *et al.*, 2005). The Hill coefficient was determined to be 1.28 ± 0.17, suggesting that the binding might be weakly cooperative. Binding was not affected by the presence or absence of 50 and 100 mM NaCl, KCl or LiCl in the reactions (data not shown). Competition studies confirmed the specificity of this binding (Fig. 3-5B).

LysR-type transcriptional regulators (LTTRs) generally bind degenerate inverted repeats that have a T-N_{11}-A motif and are found upstream of -35 promoter elements (Schell, 1993). Comparison of the putative *pgaABCD* promoter region with NhaR binding sites of *nhaA* (Carmel *et al.*, 1997) and *osmC* (Sturny *et al.*, 2003) revealed two T-N_{11}-A motifs, one overlapping the -35 promoter element and a second further upstream (Fig. 3-6E and data not shown).

DNase I footprinting of NhaR binding to *pgaA* promoter DNA. To more precisely define the NhaR binding sites on the *pgaA* promoter, DNase I footprint analysis was conducted with NhaR-His_6 and a DNA fragment (from -115 to +18 with respect to the 5' end of the *pga* transcript). NhaR protected a region spanning ~60 bp which overlapped and extended upstream from the putative -35 promoter region (Fig. 3-6). Within this protected region, two apparent binding sites with dyad symmetry were separated by a small region that became hypersensitive to DNase I digestion in the presence of NhaR suggestive of an altered conformation. The extended region of protection was seen on both strands and is characteristic of LysR-type transcriptional regulators (Chen *et al.*, 2005; Schell, 1993; Tropel and van der Meer, 2004). In addition, a hypersensitive region immediately downstream of the -10 element also became evident

in the presence of NhaR. Addition of 50 mM NaCl had no consistent effect on the footprint (compare Fig. 3-6A and C, B and D).

NhaR is required for *in vitro* transcription of *pgaABCD*. To further examine the role of NhaR in *pga* expression, *in vitro* transcription of a *pgaABCD*-containing supercoiled plasmid template by σ^{70}-saturated *E. coli* RNA polymerase was determined in the presence or absence of NhaR-His_6. Recombinant NhaR was necessary and sufficient to activate *in vitro* transcription from the *pga* promoter (Fig. 3-7). The 5'- end of the NhaR-dependent *in vitro* transcript was identical to that observed *in vivo*. The intensity of two transcripts originating from vector DNA (labeled V1 and V2) did not increase in the presence of NhaR (Fig. 3-7, left panel). This indicates that NhaR was not acting as a general activator of transcription. Addition of 25 mM NaCl or KCl did not affect these reactions (Fig. 3-7 and data not shown).

NhaR is conserved in the Enterobacteriaceae. BLAST analyses revealed that homologs of *nhaAR* are present in the genomic sequences of most Enterobacteriaceae (82% of total), including pathogens such as *E. coli* O157:H7 (99% NhaR identity), *Yersinia pestis* (79%), and *Erwinia carotovora* (77%), which also contain loci homologous to the *pgaABCD* operon of *E. coli* (Wang *et al.*, 2004). The genes of the *pgaABCD* operon encode proteins necessary for the synthesis and possibly the subcellular localization of polymeric β-1,6-N-acetyl-D-glucosamine (PGA) to the cell envelope (Wang *et al.*, 2004). This polymer promotes biofilm formation in *E. coli* and several other species (Itoh *et al.*, 2005; Kaplan *et al.*, 2004). Furthermore, PGA-like polysaccharides are important determinants of disease transmission and virulence in *Yersinia pestis* and Staphylococci, respectively (cited in Wang *et al.*, 2004). Additional

investigation will be required to determine whether these species, which inhabit distinctly different environmental niches than *E. coli* K-12, utilize similar strategies for regulating PGA production and biofilm formation in response to environmental cues.

CONCLUSIONS

The present study demonstrates that the LysR-type transcriptional regulator NhaR is necessary and sufficient to activate transcription from the *pgaABCD* promoter of *E. coli* K-12 (Fig. 3-7), and consequently activates PGA production (Fig. 3-2) and biofilm formation (Fig. 3-1). It is evident that NhaR activates expression of its target operons, *nhaAR*, *osmC* and *pgaABCD* as a coordinated response to elevated Na^+ and/or pH. Consequently, NhaR is required for survival under high concentrations of NaCl, high pH, and certain oxidative stresses (Padan and Schuldiner, 1994; Toesca *et al.*, 2001). While the full ramifications of our findings remain to be determined, the regulatory role of NhaR in adhesion and biofilm formation is consistent with the idea that biofilm formation itself provides protection against a variety of biological and chemical stresses (Costerton *et al.*, 1995; Costerton *et al.*, 1999; Costerton and Stewart, 2001; Davies, 2003; Donlan and Costerton, 2002), and further suggests that NhaR is a stress-response regulator of substantial importance. These studies also demonstrate that monovalent cation concentrations, distinct from osmolarity, can be an important regulatory cue for biofilm formation by a gram negative bacterium (Figs. 3-1 and 3-3). Surprisingly, activation of *pga* in vitro transcription by NhaR did not require monovalent cations (Fig. 3-7). As LTTR proteins often bind to their DNA targets in the absence of co-inducer ligands (Chen *et al.*, 2005; Schell, 1993; Toledano *et al.*, 1994), it was not surprising that NhaR

bound to *pga* promoter DNA without the addition of salts. Likewise, it was not unexpected to find that the DNase I footprint pattern of NhaR was not altered by the addition of salts (Fig. 3-6; Carmel *et al.*, 1997; Dover and Padan, 2001). It is tempting to suggest that NhaR might not bind directly to its co-inducer cations, but might function similarly to the LTTR protein GcvA. In this case, the binding of glycine to GcvR prevents this protein from interacting with GcvA to block activation (Ghrist and Stauffer, 1995; Heil *et al.*, 2002). Further studies will be required to assess this or other mechanisms that could explain the discrepancy between *in vitro* and *in vivo* cation requirements of NhaR.

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Table 3-1. Strains, plasmids and bacteriophage used in this study.

Strain, plasmid or phage	Description or genotype	Source or reference
E. coli K-12 strains		
DH5α	*supE44* Δ*lacU169 (ϕ80 lacZ* Δ*M15) hsdR17 relA1 recA1 endA1 gyrA96 thi-1*	
MG1655	$F^- \lambda^-$	Michael Cashel, NICHD, National Institutes of Health, Bethesda, Maryland, USA
TRMG	MG1655 *csrA::kan*	Romeo *et al.*, 1993
CF7789	MG1655 Δ*lacI-Z (MluI)*	Michael Cashel
TRCF7789	CF7789 *csrA::kan*	Suzuki *et al.*, 2002
DJ25	TRMG Δ*motB uvrC-279::Tn10* Δ*fimB-H*	Jackson *et al.*, 2002a
XWMGDABCD	MG1655 Δ*pgaABCD*	Wang *et al.*, 2004
TRXWMGDABCD	TRMG Δ*pgaABCD*	Wang *et al.*, 2004
NM81	*nhaA*::*kan*	Etana Padan, Hebrew University of Jerusalem, Israel
96B10 *	DJ25 *nhaR::cam* at +432 relative to the initiation codon	This study
CGMR	MG *nhaR::cam*	This study
CGTR	TRMG *nhaR::cam*	This study
XWZ4	CF7789 *pgaA*'-'*lacZ*	Wang *et al.*, 2005
TRXWZ4	XWZ4 *csrA::kan*	Wang *et al.*, 2005
CGCFR	XWZ4 *nhaR::cam*	This study
CGTRCFR	TRXWZ4 *nhaR::cam*	This study
Staphylococcus epidermidis		
S. epidermidis 1457	PIA positive	Dietrich Mack, Universitätsklinikum, Hamburg-Eppendorf, Germany
S. epidermidis 1457-M10	PIA negative, Ery^R, $Cipro^R$, Gm^R	Dietrich Mack
Plasmids		
pKK223-3	cloning vector, Ap^R	Pharmacia Corp.
pUC19	cloning vector, Ap^R	
pCR 2.1 TOPO	cloning vector, Ap^R and Kn^R	Invitrogen
pCRnhaAR	*nhaAR* operon in pCR2.1	This study
pNhaR	*nhaR* in pKK223-3	This study
pNhaR-His_6	C-terminus hexahistidine tagged NhaR for protein purification	This study
pPGA372	*pgaABCD* in pUC19	Wang *et al.*, 2004

Bacteriophage P1vir	strictly lytic P1	Carol Gross, University of California San Francisco, USA

* Original transposon mutant.

Table 3-2. Oligonucleotide primers used in this study. [a]

Primer name	Sequence 5'to 3'	Comments
NhaAR FWD	GATTCCTCTATTTATTCGCCCGC	400 bp upstream of *nhaAR*
NhaAR REV	CACTCGTGAGCGCTTACAGCCG	3' end of *nhaR*
NhaR FWD [b]	AAGAATTCAACGGCTCCCTTTTCATTGTTATCAGGG	Contains upstream region of *nhaR* and *Eco*RI site for cloning
NhaR REV [b]	AACTGCAGTTAACGCACCGCTGGACTAAAAAG	Contains stop codon of *nhaR* and *Pst*I site for cloning
NhaR-His_6 REV [b]	AACTGCAGTCAGTGGTGGTGGTGGTGGTG ACGCACCGCTGGACTAAAAAG	Contains six His codons, stop codon, and *Pst*I site.
PgaA GS FWD [c]	CAATTAAATCCGTGAGTGCCG	Anneals -115 of *pgaA*
PgaA GS REV [c]	TCTTCAGGAATACGGCATAAAT	Anneals +18 of *pgaA*
PgaB-C FWD [c]	ATCATTAATCGCATCGTATCG	Intergenic region between *pgaB* and *pgaC*
PgaB-C REV [c]	AGTGAAAGTACGCTACGCATAGGG	Intergenic region between *pgaB* and *pgaC*
PEXT3 [c]	CCTCATAATCCGTTATTAAACGC	Anneals between +134 to +154 nt relative to the initiation of transcription of *pgaA*
PGART1	TTCTCATCATCAACAATTCACGTCTC	*pga* leader – *pgaA* Forward primer
PGART2	GCGGCAGTAAGAAGTTTCAAAGC	*pga* leader – *pgaA* Reverse primer
16S-4 [d]	AGTTATCCCCCTCCATCAGG	16S rRNA distal, forward primer
16S-5 [d]	TGCAAGTCGAACGGTAACAG	16S rRNA distal, reverse primer

[a] All primers were purchased from Integrated DNA Technologies Inc., Coralville, Iowa.

[b] Restriction sites are underlined.

[c] Primers used for gel mobility shift assays and primer extensions were desalted and HPLC purified by the manufacturer.

[d] Sequences were previously published (Gong *et al.*, 2006).

FIGURE LEGENDS

Fig. 3-1. Disruption of *nhaR* of *E. coli* affects biofilm formation. **(A)** Cultures were grown in microtiter plates in LB pH 7.4 for 24 h at 26°C and biofilm formation was assessed by crystal violet staining (Materials and Methods). Bars depict the results obtained with a series of isogenic strains: 1, MG1655 (wild type); 2, *nhaR::cam;* 3, *nhaR::cam* [pKK223-3]; 4, *nhaR::cam* [pNhaR]; 5, *csrA::kan;* 6, *csrA::kan nhaR::cam;* 7, *csrA::kan nhaR::cam* [pKK223-3]; 8, *csrA::kan nhaR::cam* [pNhaR]. **(B)** Time course of adherence to coverslips by isogenic *E. coli* K-12 MG1655 strains (described in Materials and Methods). Representative fields are shown. **(C)** Effect of sodium chloride on biofilm formation. Cultures were grown in L-broth (LB without NaCl) supplemented with the indicated concentrations of NaCl. Symbols represent the results obtained with a series of isogenic strains: ■, MG1655 (wild type); ▲, *nhaR::cam*; ▼, *nhaR::cam* [pKK223-3]; ◆, *nhaR::cam* [pNhaR]; ●, *nhaR::cam* [pNhaR-His_6]. Bars represent the average of 2 wells, and error bars correspond to the SEM. **(D)** Epistasis analyses of *nhaR* and *pgaABCD* on biofilm formation. Biofilm formation was assessed as in Fig. 1A in the following isogenic strains: 1, MG1655; 2, Δ*pgaABCD;* 3, *nhaR::cam*; 4, MG1655 [pUC19]; 5, MG1655 [pPGA372] (contains *pgaABCD)*; 6, *nhaR::cam* [pUC19]*;* 7, *nhaR::cam* [pPGA372]; 8, Δ*pgaABCD* [pUC19]; 9, Δ*pgaABCD* [pPGA372]; 10, Δ*pgaABCD* [pKK223-3]; 11, Δ*pgaABCD* [pNhaR]. Bars represent the average of 16 wells normalized to the biofilm formed by MG1655, and error bars correspond to the

SEM. The asterisks denote significant differences relative to the corresponding parent strain ($P < 0.001$ [Tukey's multiple comparison test]).

Fig. 3-2. Effect of *nhaR* mutation on accumulation of PGA. Cell lysates were prepared from a series of isogenic strains and analyzed by immunoblotting with an anti-PIA antiserum (Materials and Methods). Sample identities are as follows: (a) parent (TRMG), (b) *nhaR::cam*, (c) *nhaR::cam* [pNhaR], (d) Δ*pgaABCD*. This experiment was repeated in entirety three times, with essentially identical results. A representative blot is shown.

Fig. 3-3. Effects of *nhaR::cam*, monovalent cations and pH on expression of a *pgaA'-'lacZ* translational fusion. (**A)** Activity of the *pgaA'-'lacZ* chromosomal fusion in XWZ4 (■) and its isogenic *nhaR* mutant (CGCFR) (□) strains after 14 h of growth at 26°C in L-broth (LB without NaCl) or supplemented with the indicated concentrations of NaCl. **(B)** Activity of the reporter fusion in L-broth containing the indicated mM amounts of NaCl, KCl, LiCl or sucrose. **(C)** Effect of pH on *pgaA'-'lacZ* expression. Strains were grown for 24 h at 26°C in LB buffered with 60 mM 1,3-bis[tris(hydroxymethyl)-methylamino]propane, and the pH was adjusted to the indicated values with HCl. Values represent the means of three separate reactions ± SEM. Error bars smaller than the symbols are not visible. These experiments were repeated at least three times with similar results.

Fig. 3-4. Primer extension analysis of *pgaA* transcript in *nhaR* wild type and mutant (*nhaR::cam*) strains. Cultures were grown at 26°C with shaking and harvested for RNA

isolation in the late exponential phase of growth. Strain identities are as shown in Fig. 1A. The dideoxy-sequencing ladder (lanes G, A, T and C) was generated with PEXT3 using pPGA372 as a template. The 5' end of the *pgaA* transcript is marked with an asterisk.

Fig. 3-5. Purified NhaR-His_6 binds specifically to *pgaABCD* promoter DNA. **(A)** A 132 bp PCR product containing the promoter region and putative NhaR binding site of *pgaA* was 3'-labeled with DIG-11-ddUTP and used for gel mobility shift assays. The apparent equilibrium binding constant *(K_d)* was determined as described in Materials and Methods. **(B)** Five hundred and 1,000-fold molar excess of unlabeled specific DNA (*pgaA)* and nonspecific DNA (*pgaBC*) were used as competitors in the binding reactions.

Fig. 3-6. DNase I protection footprint of NhaR on the *pgaA* promoter. A 132 bp PCR product containing the promoter region of *pgaA* (-115 to +18) was 5'-end labeled with [^{32}P] on the top **(A, C)** or bottom strand **(B, D),** as diagrammed in panel **E**, and subjected to DNase I footprinting in the presence of increasing concentrations of NhaR- His_6 (0, 0.29, 0.57, 1.14 μM). The binding reactions contained no NaCl **(A, B)** or 50 mM NaCl **(C, D)**. Protected regions are marked by vertical lines adjacent to the sequences. Hypersensitive sites are indicated by asterisks. Numbers depict positions relative to the initiating nucleotide (+1). These footprints were repeated four times, with no significant difference in the protection pattern observed in the presence or absence of NaCl. **(E)** Summary of DNase I footprinting. Protected regions are shaded grey; boxed sequences

contain a LysR-type transcriptional regulator binding motif (T-N_{11}-A). The –35, –10 and +1 promoter elements are bolded. Hypersensitive sites are indicated by asterisks.

Fig. 3-7. NhaR-His_6 activates *in vitro* transcription from the *pgaA* promoter. NhaR-His_6 and σ^{70}-saturated RNA polymerase were used for *in vitro* transcription of plasmid DNA containing the *pgaABCD* operon (pPGA372). The resulting transcripts were analyzed by primer extension (Materials and Methods). The full image **(A)** and a close up **(B)** obtained from a single gel are shown. In the full gel image, two transcripts originating from the vector are indicated by solid arrowheads labeled V1 and V2; an asterisk denotes the 5' end of the transcript originating from the *pga* (P_{pga}). Identical results were obtained when 25 mM KCl was used instead of NaCl in the transcription reaction (data not shown). The first two lanes (*in vivo*) depict primer extension analysis of total cellular RNA isolated from the indicated strains. The last four lanes (*in vitro*) correspond to transcription reactions conducted in the presence or absence of NhaR (455 nM) and NaCl (25 mM).

Fig. 3-1.

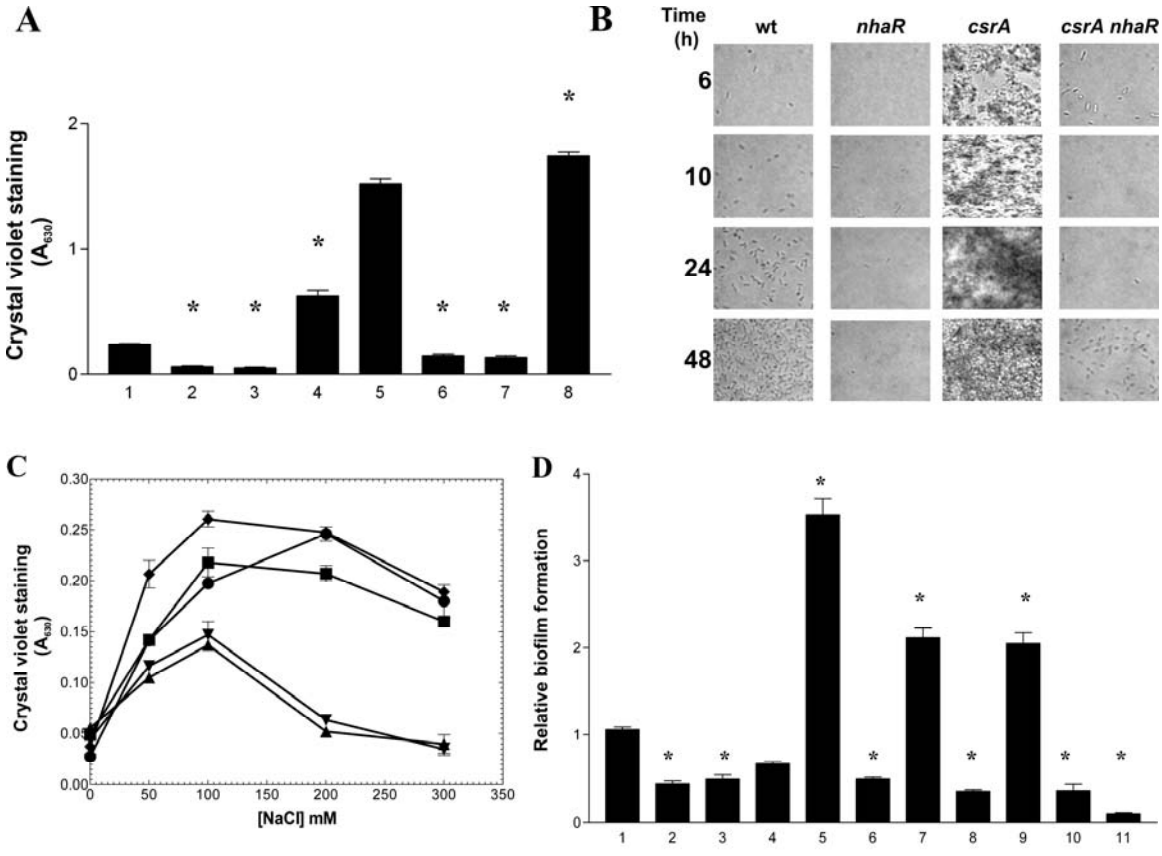

A
Crystal violet staining (A630)
B
Time (h)
wt
nhaR
csrA
csrA nhaR
6
10
24
48
C
Crystal violet staining (A630)
[NaCl] mM
D
Relative biofilm formation

Fig. 3-2.

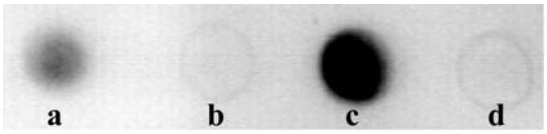


Fig. 3-3.

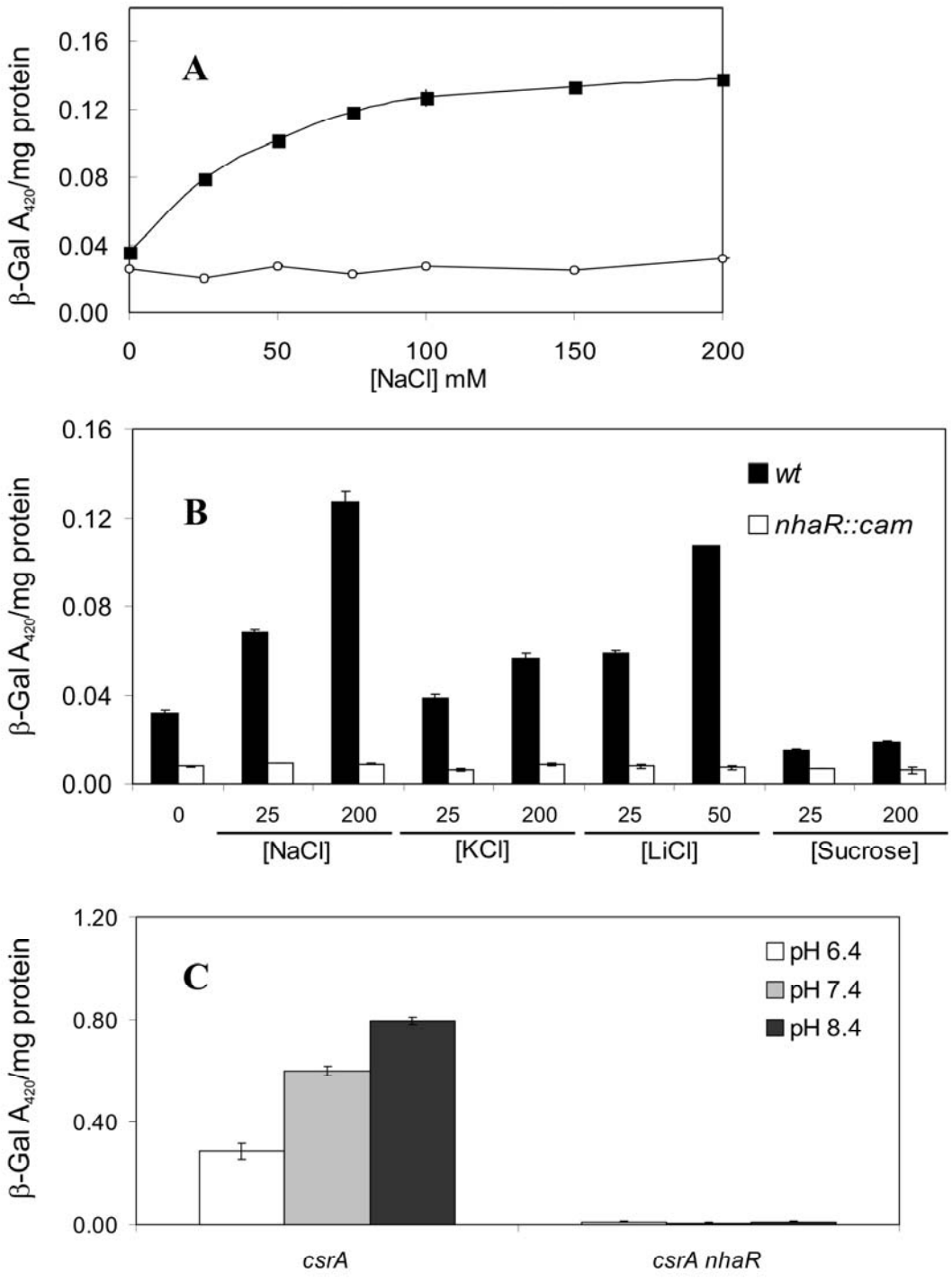

A
β-Gal A_{420}/mg protein
0.16
0.12
0.08
0.04
0.00
0
50
100
150
200
[NaCl] mM
B
wt
nhaR::cam
0
25
200
25
200
25
50
25
200
[NaCl]
[KCl]
[LiCl]
[Sucrose]
C
pH 6.4
pH 7.4
pH 8.4
1.20
0.80
0.40
0.00
csrA
csrA nhaR

Fig. 3-4.

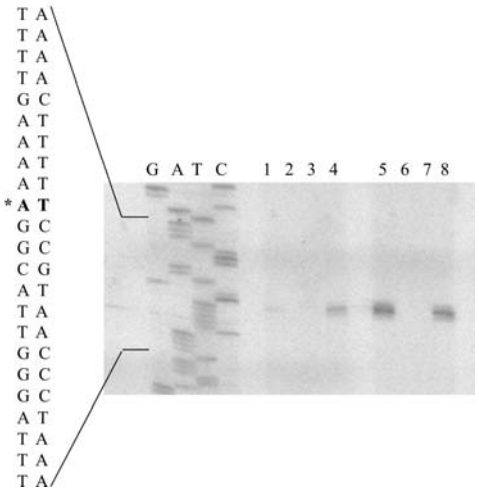

G A T C 1 2 3 4 5 6 7 8
T A
T A
T A
T A
G C
A T
A T
A T
A T
*A T
G C
G C
C G
A T
T A
T A
G C
G C
G C
A T
T A
T A
T A

Fig. 3-5.

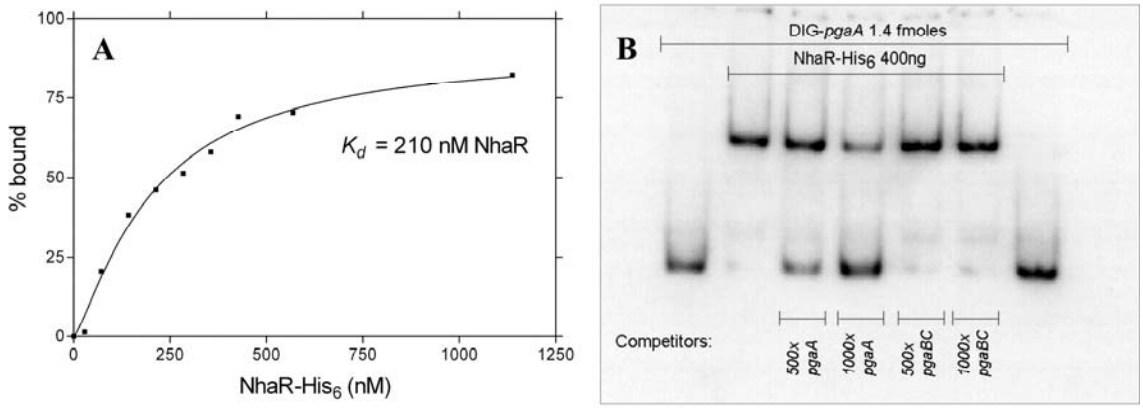

A
% bound
100
75
50
25
0
0
250
500
750
1000
1250
NhaR-His6 (nM)
Kd = 210 nM NhaR
B
DIG-pgaA 1.4 fmoles
NhaR-His6 400ng
Competitors:
500x pgaA
1000x pgaA
500x pgaBC
1000x pgaBC

Fig. 3-6.

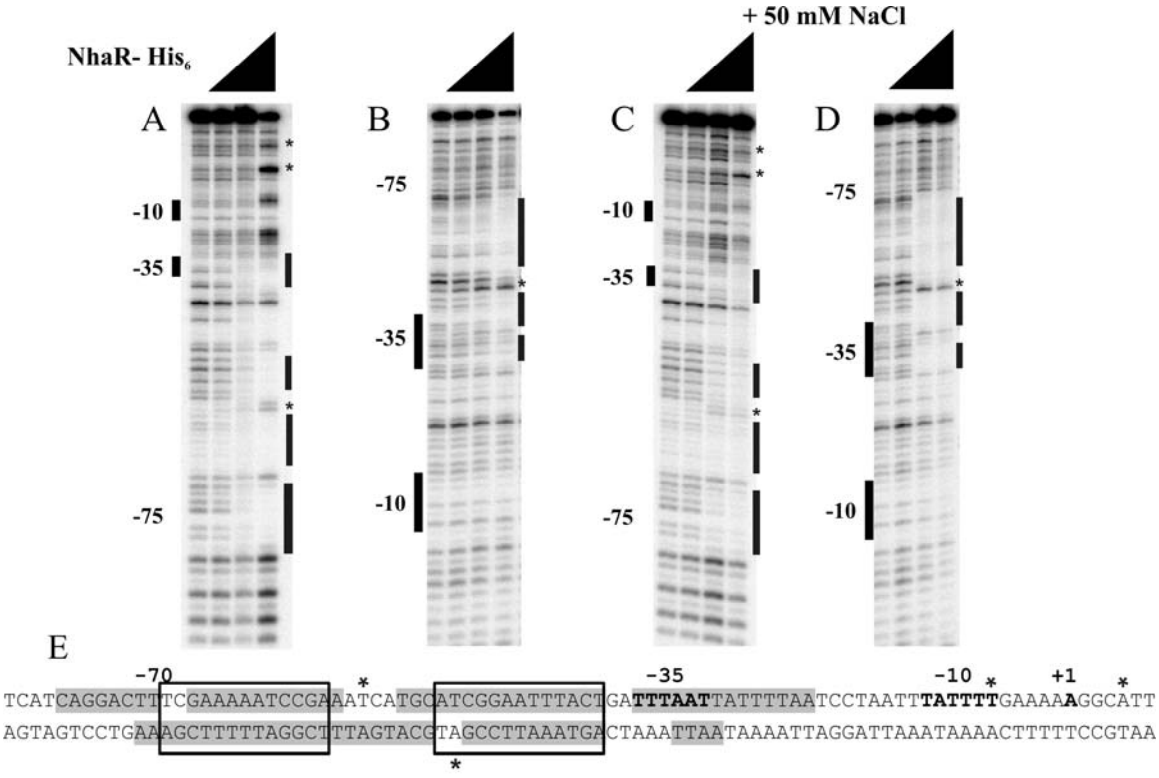
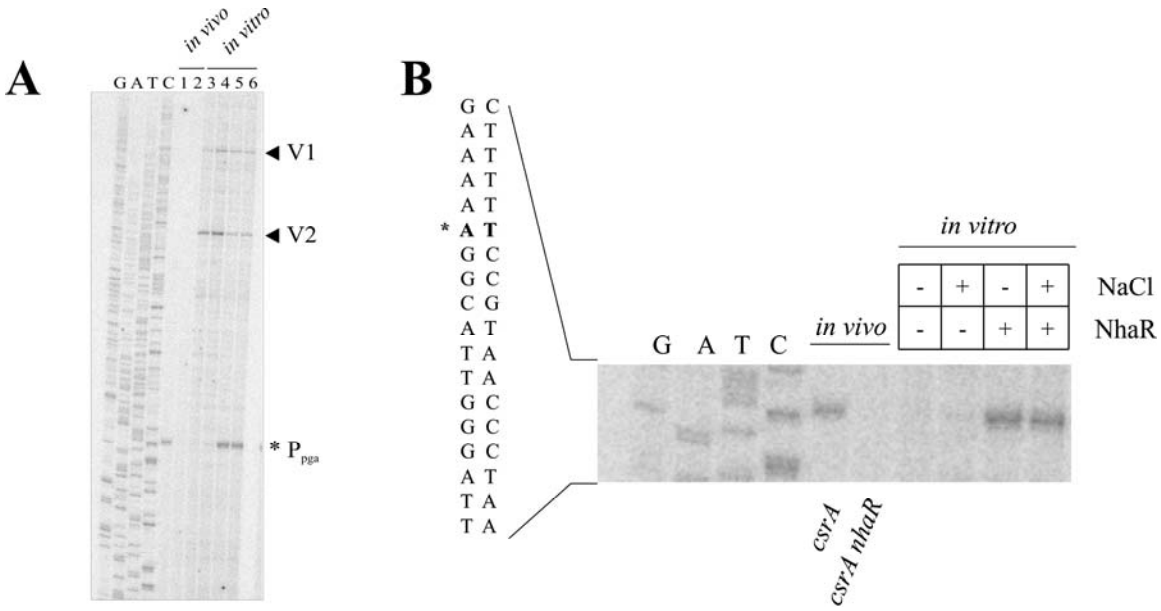

+ 50 mM NaCl
NhaR- His6
A
B
C
D
-10
-35
-75
E
-70
-35
-10
+1
TCATCAGGACTTTCGAAAAATCCGAAATCATGCATCGGAATTTACTGATTTTAATTATTTTAATCCTAATTTATTTTGAAAAAGGCATT
AGTAGTCCTGAAAGCTTTTTAGGCTTTAGTACGTAGCCTTAAATGACTAAATTAATAAAATTAGGATTAAATAAAACTTTTTCCGTAA

Fig. 3-7.


A
in vivo
in vitro
G A T C 1 2 3 4 5 6
V1
V2
* P pga
B
G C
A T
A T
A T
A T
* A T
G C
G C
C G
A T
T A
T A
G C
G C
G C
A T
T A
T A
in vitro
- + - + NaCl
in vivo - - + + NhaR
G A T C
csrA
csrA nhaR

Chapter 4: Regulation of PGA accumulation and biofilm formation in *Escherichia coli* by GGDEF and EAL domain proteins

Carlos C. Goller[1], Archana Pannuri[1,4], Yoshikane Itoh[2], Kazushi Suzuki[3], and Tony Romeo[1,4]

[1] Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322.

[2] Hokkaido University, Sapporo, JAPAN.

[3] Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, Niiagata, Japan 950-2181.

[4] Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611.

This chapter consists of a manuscript in preparation. The manuscript was written by Carlos C. Goller and Tony Romeo. The *pga* dot blot procedure was optimized by Yoshikane Itoh. Dr. Kazushi Suzuki performed the first experiment over-expressing the EAL domain protein YhjH and noticed it did not affect *pgaA*'-'lacZ expression. Dr. Archana Pannuri and Carlos Goller screened the KEIO collection of GGDEF/EAL

domain protein gene disruptions for biofilm and PGA accumulation and constructed the *ydeH* clone pBADydeH-6. All remaining experiments were performed by Carlos C. Goller, including characterization of the effect of *ydeH* and *yhjH* over-expression on PGA accumulation and development of an *in vitro* PGA labeling system using crude membrane preparations.

SUMMARY

The secondary messenger c-di-GMP is involved in regulation of a large number of bacterial processes including motility, expression of virulence factors, and biofilm formation. Genes encoding enzymes that produce (GGDEF domain) or degrade (EAL domain) this molecule are often found in large numbers in Gram negative organisms. Furthermore, in several organisms, c-di-GMP accumulation leads to expression of factors required for a sessile bacterial lifestyle and depletion of this molecule enhances motility and dispersal of biofilms. We were interested in examining the effect of c-di-GMP on expression of the polymer β-1,6-*N*-acetyl-D-glucosamine (PGA, PIA, PNAG), which functions as an adhesin for the formation and structural stability of biofilms of diverse eubacteria. To do so, we examined a panel of (27) single gene deletions of GGDEF and/or EAL domain proteins in *E. coli* for their effects on biofilm formation. Several affected biofilm formation and a few mutants substantially increased or reduced biofilm levels with respect to the parental strain. One gene encoding an EAL domain protein, *yhjH*, and one encoding a GGDEF protein, *ydeH*, were chosen for further study and cloned. Expression from a plasmid complemented the biofilm phenotype in all backgrounds tested. Over-expression of *yhjH* reduced biofilm and PGA accumulation to

levels barely detectable by our biofilm and immuno assays. Over-expression of *ydeH* drastically increased biofilm and PGA accumulation, and was completely dependent on the presence of an intact copy of the *pgaC* gene, encoding the glycosyl-transferase required for PGA synthesis. Nevertheless, over-expression of *ydeH* or *yhjH* did not alter activity from a *pgaA'-'lacZ* translational fusion, suggesting that the product of these genes was not affecting transcription-translation of *pgaA*. In addition, deletion of the *ycgR* gene encoding a protein with a PilZ c-di-GMP binding domain had no significant effect on biofilm formation. Since both YdeH and YhjH have been shown to alter c-di-GMP levels in previous studies, we next focused on determining the mechanism underlying c-di-GMP modulation of PGA accumulation. To do so, we developed an *in vitro* assay which allowed us to measure incorporation of UDP-[^{14}C]GlcNAc into PGA using crude membrane preparations. However, purified c-di-GMP failed to stimulate incorporation *in vitro*. Surprisingly, membranes from *yhjH*-over-expressing cells (depleted for c-di-GMP) were defective in UDP-[^{14}C]GlcNAc incorporation in the presence or absence of exogenous c-di-GMP. These findings imply that c-di-GMP does not allosterically activate PGA glycosyltransferase activity, as seen in other systems (e.g., cellulose synthase in *Gluconacetobacter xylinus*), but is apparently necessary for the production and/or maintenance of a functional PGA biosynthetic membrane complex.

ABSTRACT

The polymer β-1,6-*N*-acetyl-D-glucosamine (PGA, PIA, PNAG) serves as an adhesin for the formation and structural stability of biofilms of diverse eubacteria. In *Escherichia coli,* PGA biosynthesis and secretion require structural genes of the *pgaABCD* operon. Biofilm formation in *E. coli* and a variety of other species is stimulated or inhibited, respectively, by genes encoding enzymes that produce (GGDEF domain) or degrade (EAL domain) the widespread bacterial secondary messenger, c-di-GMP. Analysis of a panel of (27) single gene deletions of GGDEF and/or EAL domain proteins in *E. coli* indicated that only a few of these genes substantially (>2-fold) affected biofilm formation under our experimental conditions. Furthermore expression of *ydeH* (GGDEF) from a plasmid increased, while *yhjH* (EAL) decreased, biofilm formation and PGA levels, respectively. These genes did not affect the expression of a chromosomal *pgaA'-'lacZ* translational fusion. To further investigate the effect of c-di-GMP on PGA synthesis, we measured incorporation of UDP-[^{14}C]GlcNAc into PGA by crude membrane preparations. Conserved c-di-GMP-binding domains are not apparent in the PGA synthesis proteins PgaC and PgaD. Addition of c-di-GMP to this assay failed to stimulate incorporation. Furthermore, membranes from *yhjH*-overexpressing cells were defective in UDP-[^{14}C]GlcNAc incorporation in the presence or absence of exogenous c-di-GMP. Our findings imply that c-di-GMP does not allosterically activate PGA glycosyltransferase, but is apparently required for the production and/or maintenance of a functional PGA biosynthetic membrane complex.

Because PgaC and the other proteins of the PGA system do not appear to contain a classical c-di-GMP binding domain (e.g. PilZ domain), and the known PilZ domain protein YcgR of *E. coli* does not account for effects of c-di-GMP on biofilm formation, these studies suggest that there is a novel response system for c-di-GMP in *E. coli* responsible for modulation of PGA levels.

INTRODUCTION

Cyclic nucleotides are widespread secondary messengers used by bacteria to convey information about nutritional and metabolic conditions as well as environmental signals. Cyclic diguanylate (3', 5'-cyclic, diguanylic acid, c-di-GMP) is a second messenger in bacteria, which was initially described as an allosteric activator of cellulose synthase in *Gluconacetobacter xylinus* (originally *Acetobacter xylinum*, Ross *et al.*, 1987). This molecule is known to regulate numerous functions in diverse bacteria, including expression of virulence factors, motility, and biofilm formation (reviewed by Jenal and Malone, 2006; Tamayo *et al.*, 2007). In several species, a rise in intracellular c-di-GMP results in expression of factors required for biofilm formation, while a decrease in c-di-GMP pools leads to increased motility (e.g., Simm *et al.*, 2004).

The protein domains GGDEF and EAL have been implicated in synthesis and degradation, respectively, of c-di-GMP (Ryjenkov *et al.*, 2005; Schmidt *et al.*, 2005), contributing to the regulation of virulence factors in many bacteria (reviewed by Tamayo *et al.*, 2007). More recently, the HD-GYP domain was shown to act in c-di-GMP degradation (Ryan *et al.*, 2006). Surprisingly, these three domains are typically either abundant or non-existant in bacterial genomes (Galperin, 2005; Galperin and Nikolskaya,

2007). For example, *Escherichia coli* encodes 27 GGDEF and/or EAL domain-containing proteins (identified by bioinformatics), while to date the sequenced genomes of some representative low-GC-content Gram-positive bacteria, such as Staphylococcus, encode only one GGDEF protein and a second with a modified non functional domain (Holland *et al.*, 2008). Since many of the GGDEF/EAL domain proteins also contain sensory and signal transduction domains, it is believed that their activity is regulated by uncharacterized environmental signals. Furthermore, very few molecular details are known about how c-di-GMP affects complex processes such as biofilm formation. Thus, a better understanding of the signals and c-di-GMP molecular targets is required.

Studies have shown that proteins with the PilZ domain function as c-di-GMP receptors (e.g., Weinhouse *et al.*, 1997; Merighi *et al.*, 2007). Recently, a c-di-GMP receptor required for expolysaccharide production has been discovered in *Pseudomonas aeruginosa* (Lee *et al.*, 2007). The PelD protein binds c-di-GMP and mediates Pel polysaccharide biosynthesis. The mechanism of action of PelD remains unknown. It has been proposed to involve an allosteric activation similar to the one found in *G. xylinus* for activation of cellulose synthase. Furthermore, it was found that c-di-GMP levels in eubacteria can be sensed by a riboswitch class in messenger RNA able to control gene expression (Sudarsan *et al.*, 2008), emphasizing the importance of c-di-GMP levels in control of a wide variety of bacterial processes. Currently there is only one example in the literature of a c-di-GMP-responsive transcription factor in *P. aeruginosa* (Hickman and Harwood, 2008).

The *pgaABCD* operon in *E. coli* (and *ica* locus in Staphylococci) is necessary for biosynthesis of β-1,6-*N*-acetyl-D-glucosamine (PGA, Wang *et al.*, 2004), a polymer that

promotes biofilm formation by *E. coli* K-12 and various other eubacteria, including *Staphylococcus epidermidis*, Bordetella sp., *Aggregatibacter actinomycetemcomitans*, *Actinobacillus pleuropneumoniae* and *Yersinia pestis* (Heilmann *et al.*, 1996a; Heilmann *et al.*, 1996b; Hinnebusch *et al.*, 1996; Hinnebusch and Erickson, 2008; Itoh *et al.*, 2005; Izano *et al.*, 2007a; Izano *et al.*, 2008; Parise *et al.*, 2007). In *E. coli* K-12, PGA is a critical factor for biofilm formation and its synthesis is regulated transcriptionally by NhaR in response to increases in sodium and pH. The RNA-binding protein and global regulator CsrA postranscriptionally represses PGA synthesis by decreasing the half-life of the *pga* message (Goller *et al.*, 2006; Wang *et al.*, 2005a).

The biological significance of PGA in biofilm formation is gradually being uncovered. We now know that this polymer likely has several different roles depending on the organism producing it. In addition to being necessary for binding to abiotic surfaces and intercellular adhesion in *E. coli* K-12 (Wang *et al.*, 2004), PGA has been shown to be critical for *E. coli* 0157:H7 attachment to alfalfa sprouts (Matthysse *et al.*, 2008). This suggests a role for this polysaccharide in persistence of *E. coli* in the environment and contamination of produce. Such a role is consistent with enhanced expression of PGA at low temperature (26°C vs. 37°C; Cerca and Jefferson, 2008; Wang *et al.*, 2005a). In addition, NhaR was required for sodium induction of *pga* expression in a uropathogenic strain of *E. coli* (Cerca and Jefferson, 2008), suggesting that sodium and pH in the human host may be triggers of biofilm formation. In *Y. pestis*, the *hms* locus is responsible for blockage of the foregut of the flea and efficient transmission of plague (Hinnebusch *et al.*, 1996; Pendrak and Perry, 1991). Moreover, the components and regulation of the *ica* operons of *Staphyloccoccus epidermidis* and *Staphylococcus aureus*,

which were initially described in 1996, have been extensively studied, resulting in a better understanding of how environmental conditions affect *ica* expression and modulate biofilm formation (reviewed by Gotz, 2002; O'Gara, 2007; Otto, 2008). PGA has recently been found to be a component of biofilms formed by additional organisms of agricultural and clinical significance, such as *Aggregatibacter actinomycetemcomitans* and *Actinobacillus pleuropneumoniae* (Izano *et al.*, 2008*;* Izano *et al.*, 2007a,). Currently, the efficacy of the enzyme Dispersin B (DspB), which leads to hydrolysis of β-1,6-*N*-acetyl-D-glucosamine polymers and aids in removal of biofilms, has sparked the interest of several groups (e.g. Itoh *et al.*, 2005; Izano *et al.*, 2007b; Lu and Collins, 2007; Ramasubbu *et al.*, 2005).

The differential roles of the individual genes and proteins involved in PGA biosynthesis have been characterized in *E. coli*, *Yersinia pestis*, and *Staphylococcus epidermidis* (e.g., Bobrov *et al.*, 2008; Forman *et al.*, 2006; Itoh *et al.*, 2008; Lillard *et al.*, 1997; Vuong *et al.*, 2004; Wang *et al.*, 2004). In *E. coli*, PgaA and PgaB are necessary for export of the polysaccharide, while PgaC, a glycosyltransferase, and PgaD are essential for its polymerization (Itoh *et al.*, 2008). PgaB is also required for deacetylation of the polymer. Although also required for biofilm formation, the deacetylase IcaB of *S. epidermidis* is needed to introduce positive charges into the polymer that are important for its surface localization (Vuong *et al.*, 2004), a role different from that of PgaB in *E. coli*.

The membrane topology of the HmsR and HmsS proteins of *Yersinia pestis*, homologues to PgaC and PgaD of *E. coli,* has been examined and their interactions recently described (Bobrov *et al.*, 2008; Forman *et al.*, 2006). Over-expression of HmsT

(a GGDEF domain protein) results in robust biofilm formation (Kirillina *et al.*, 2004). In *E. coli* the *ycdT* gene (GGDEF domain) is divergently transcribed from the *pga* operon, but deletion of this gene had no apparent biofilm phenotype under the conditions tested (Wang *et al.*, 2005a).

Intrigued by the role of c-di-GMP-related proteins in biofilm formation by several different organisms and our goal of better understanding the regulatory players involved in PGA production, we asked whether c-di-GMP plays a part in PGA accumulation. Our studies suggest that c-di-GMP levels modulate PGA accumulation and identify specific GGDEF/EAL proteins responsible for activating or inhibiting PGA production. Using a panel of mutants and an *in vitro* assay, we aimed to discover which GGDEF/EAL proteins affect PGA accumulation and biofilm formation. Next, we focused on the molecular mechanism of action of c-di-GMP.

MATERIALS AND METHODS

Bacterial strains, phage, plasmids, and growth conditions. All *E. coli* strains, phage, and plasmids used in the present study are listed in **Table 1**. Unless otherwise indicated, bacteria were routinely grown at 37°C in Luria-Bertani medium (LB) (pH 7.4) (tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 10 g/liter) with shaking at 250 rpm. Biofilms were grown in 96-well flat-bottom polystyrene microtiter plates (#3595, Corning Inc., Life Sciences, Acton, MA) at 26°C for 24 hrs under static conditions. Media were supplemented with antibiotics as needed at the following final concentrations: ampicillin, 200 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 100 μg/ml; and tetracycline, 10 μg/ml. For induction of genes cloned into the pBAD24

expression vector, LB was supplemented with 0.2% (final) filter sterilized L-arabinose in water.

Molecular biology and genetics. Standard procedures were used for isolation of plasmids, restriction digests, ligations, transformation, and P1*vir* transduction of antibiotic markers (Miller, 1972; Sambrook *et al.*, 1989).

Plasmid construction. Enzymes for molecular cloning were purchased from New England Biolabs (Ipswich, MA). Constructs were electroporated into DH5α or MG1655 cells, and recombinant plasmids were isolated using QIAGEN (Valencia, CA) reagents.

Molecular cloning of the *yhjH* gene involved PCR amplification of chromosomal DNA with primers yhjH FWD and yhjH REV (**Table 4-2**) with Pfu Ultra™ II Fusion HS DNA polymerase (Stratagene), under the reaction conditions described by the manufacturer. Annealing temperatures and extension times were based on primer melting temperature and final product size, respectively. The 768 kb product was treated with polynucleotide kinase (PNK) and cloned into the filled-in and antartic phosphatase-treated *Nco*I site of pBAD24. The resulting pBADyhjH construct contained the Shine-Dalgarno sequence from pBAD24 as described by Guzman *et al.*, 1995.

Cloning of the *ydeH* gene and creation of pBADydeH involved an identical approach to that employed for *yhjH*, using primers ydeH FWD and ydeH REV (**Table 4-2**). All cloned inserts were determined to be free of mutations by DNA sequencing.

Quantitative biofilm assay. Biofilm formation was assayed by crystal violet staining of adherent cells in microtiter wells, as described previously (Jackson *et al.*, 2002). Overnight cultures were diluted 1:100 into fresh LB without antibiotics or supplemented with 200 μg/ml ampicillin for strains harboring a plasmid. Bacterial growth was determined by measuring the absorbance at 600 nm using a Synergy-HT plate reader (BioTek, Winooski, VT) prior to crystal violet staining. At least four replicates were conducted for each sample, and each experiment was performed a minimum of three times with high reproducibility. The results were calculated as averages and standard errors from the means for a representative experiment using the GraphPad Prism software package (San Diego, CA). Tukey's multiple-comparison test was used for statistical analysis of data (GraphPad Prism).

β-Galactosidase and total protein assays. ß-Galactosidase activity was assayed as described previously (Goller *et al.*, 2006). The activities of *csrA* wild-type and mutant strains were determined with 2-h and 1-h reaction times, respectively, and expressed as activity per hour (A_{420}/mg protein). Cultures were grown at 26°C in LB with shaking, and 500 μl of cells were concentrated and used for each assay. Reactions were performed in triplicate. Total cellular protein was measured by the bicinchoninic acid method (BCA protein assay; Pierce, Rockford, IL) using bovine serum albumin as the standard. Absorbance measurements were conducted in flat-bottom 96-well microtiter plates with a Synergy HT plate reader (BioTek).

Immunoblotting and detection of PGA. For dot blot analysis, cell-associated PGA was prepared as described previously with minor modifications (Goller *et al.*, 2006). Briefly, overnight cultures were diluted 1:100 into fresh LB. Cultures were incubated for 24 hrs at 26°C without shaking and were harvested (10 ml) and resuspended in 400 µl of a solution containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1 µg lysozyme. After incubation at room temperature for 30 min, a solution (300 µl) containing 10 µg DNase I, 40 µg RNaseA, 200 µg α-amylase, and 40 mM $MgCl_2$ was added. The mixture was incubated at room temperature for 1 h with occasional mixing before being heated to 37°C for 2 hrs. The resulting cell lysate was then extracted once with 50 mM Tris (pH 8.0)-saturated phenol and once with chloroform. The aqueous phase (1 ml from 10 ml of culture) was collected, and residual chloroform was allowed to evaporate overnight at room temperature. The samples were concentrated using a YM-3 membrane (Amicon, Houston, TX; molecular mass cutoff, 3,000 Da).

For immunodetection of PGA, the procedure of Itoh *et al.*, 2008 was followed. Sample (3 µl) was applied directly onto a nitrocellulose membrane and the membrane was allowed to air dry overnight at room temperature. It was then blocked for 1 h in PBS-T (10 mM sodium phosphate buffer, 0.15 mM NaCl, pH 7.4 plus 0.2% Tween 20; filtered sterilized) containing 5% dry skim milk and treated for 1 h at room temperature with murine IgM MAb 2F3.1D4 (diluted 1:10,000 in PBS-T + 0.1% BSA) that was raised against *E. coli* PGA (described in Itoh *et al.*, 2008). The membrane was washed twice for 5 min and twice for 10 min with PBS-T, and treated with horseradish peroxidase-conjugated anti-murine IgM antibody (1:10,000; Sigma-Aldrich, St. Louis, MO) for 1 h at room temperature. The membrane was then rinsed with PBS-T, and the signal was

detected by chemiluminescence (Western Lightning Chemiluminescence Plus protocol; PerkinElmer). Membranes were photographed using a Bio-Rad ChemiDoc system.

Preparation of S-100 extracts and crude membranes. *E. coli* cultures (2 L) were grown at 26°C shaking for 24 hrs, harvested by centrifugation, and the cell pellets were resuspended in filtered TEM buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, and 4 mM dithiothreitol; ~ 1 mg dry weight cells per ml of TME buffer). Cells were disrupted by 6 × 1-min sonication (10 seconds on, 10 seconds off) on ice using a Fisher Scientific Dismembrator Model 500. Unbroken cells were pelleted (10 min, 7000 × *g*) twice. Membranes were sedimented by ultracentrifugation (30 min, 354,000 × *g;* 4°C). Crude membranes were then resuspended in 0.75-1.0 ml of TEM buffer using a 7 ml glass homogenizer to obtain protein concentrations between 20-40 mg/ml, as determined by TCA precipitation and BCA protein assay. The supernatants (S-100 extracts) were also collected. Membranes, S-100 extracts, and buffers were all stored at -80 °C in small aliquots. For experiments, the extracts were subjected to a maximum of three freeze-thaw cycles before disposing. All preparative procedures were carried out on ice.

***In vitro* enzymatic assay.** An *in vitro* assay to measure incorporation of UDP-[^{14}C] GlcNAc into PGA using membrane extracts was developed based on the technique used for *in vitro* synthesis of *S. epidermidis* PIA (Gerke *et al.*, 1998). Reactions were performed by incubating crude membranes in TEM buffer containing 2.5 mM $CaCl_2$ and 6.3-7.6 μM UDP-*N*-acetyl-D-[U-^{14}C]glucosamine (specific activity 317 mCi/mmol,

batches 74 and 76, CFB.150; Amersham). Reactions were carried out in a total volume of 50 µl incubated for 8-16 hrs at 26°C. *In vitro* synthesized products were extracted once with alkaline phenol (Ambion Catalog # AM9730 with the supplied Tris Alkaline Buffer added to increase the pH to 7.9) followed by an extraction with chloroform. The aqueous phase was collected and residual chloroform was allowed to evaporate at 26°C for ~8 hrs. Samples (3 ul) were spotted onto nitrocellulose membranes. Membranes were allowed to air dry overnight, washed three times with PBS-T, and dried at RT for 3 hrs. Phosphoimager screens were exposed directly to the membranes for 16-22 hrs, and ^{14}C incorporation was detected using a Storm 860 or Typhoon phosphorimager (Amersham).

Bioinformatics. The amino acid sequences of the PgaC and PgaD proteins were submitted to the HHpred server (http://toolkit.tuebingen.mpg.de/hhpred; Soding *et al.*, 2005) for protein homology detection and structure prediction with the objective of searching for putative c-di-GMP binding targets, as described (Itoh *et al.*, 2008). To gain further insight into the structural features of YhjH and YdeH, the Ecogene, Pfam, RegulonDB, and NCBI databases were consulted.

RESULTS

Assessment of the effects of c-di-GMP-related genes on biofilm formation.

To assess the effect of c-di-GMP-related genes on biofilm formation, we obtained strains with single gene disruptions from the Keio collection of *E. coli* K-12 in-frame knockout mutants (Baba *et al.*, 2006) for 27 GGDEF and/or EAL domain-containing proteins. We also investigated a mutant of the PilZ domain protein YcgR involved in c-

di-GMP binding. The ability of each of these mutants to form biofilm was assessed by crystal violet staining. Others have recently screened the entire Keio collection to identify genes required for biofilm formation (Niba *et al.*, 2007), but we chose to focus on c-di-GMP-related proteins and how they affect biofilm formation specifically through modulation of PGA accumulation.

Our results indicated that few mutations in genes encoding GGDEF/EAL domain proteins substantially affected biofilm formation (>2-fold; **Figure 4-1**). Disruption of the *yddV, yneF, ydeH,* and *yfiN* genes encoding proteins containing GGDEF domains decreased biofilm formation compared to the parental strain.

Deletion of the genes *yjcC, yhjH, ycgF,* and *yahA* (which encode EAL domain-containing proteins) resulted in an increase in biofilm formation to levels at least twice that of the parental strain. Importantly, purified YahA has been shown previously to be a catalytically active phosphodiesterase that cleaves c-di-GMP (Schmidt *et al.*, 2005), suggesting that the biofilm phenotype observed is likely due to alteration of the intracellular c-di-GMP pools. Disruption of the *ylaB* gene (EAL) decreased biofilm formation by ~50%. Disruption of the genes encoding YfgF, YfeA, YciR, and YegE proteins containing both GGDEF and EAL domains, resulted in modest increases in biofilm. A deletion of *dos*, which encodes a protein containing both GGDEF and EAL domains, resulted in 3-4 times more biofilm formation than the parental strain.

Loss of the PilZ domain protein YcgR did not alter biofilm formation. Note that the YcdT and YhdA, also called CsrD (Suzuki *et al.*, 2006), GGDEF domain proteins were not included in these analyses since their effects on biofilm formation and the PGA

system have been previously characterized by our group (Wang *et. al* 2005; Suzuki *et al.* 2006).

Each mutation was transduced into MG1655 and BW25113 *csrA::kan* strain backgrounds and re-tested for biofilm formation (data not shown). MG1655 forms relatively weak biofilms. Introduction of the *csrA* mutation into the BW background results in de-repression of the *pgaABCD* operon and increased PGA production (Wang *et al.*, 2005a; data not shown). The results of these analyses supported the initial studies in the BW25113 background and allowed us to identify mutations likely to result in altered PGA-dependent biofilm formation.

In *Pseudomonas aeruginosa, Vibrio cholera, Salmonella,* and *Shewanella onedensis*, it has been shown that increases in c-di-GMP tend to lead to a sessile lifestyle (e.g., Gjermansen *et al.*, 2006; Simm *et al.*, 2004; Tamayo *et al.*, 2005; Thormann *et al.*, 2006). In line with the theme that increases in intracellular c-di-GMP concentrations (e.g. disruption of a catalytically active EAL domain protein; Simm *et al.*, 2004) result in enhanced biofilm formation, deletion of *yhjH* or *yjcC* (EAL domain) led to a substantial increase in biofilm formation while disruption of *ydeH* or *yneF* (GGDEF domain) decreased biofilm. Because disruption of *ydeH* and *yhjH* consistently showed strong effects on biofilm formation in all three backgrounds tested, we chose to study *ydeH* and *yhjH* more closely to further dissect the mechanism by which these proteins alter biofilm formation. In addition, both proteins lack putative transmembrane segments, suggesting that they are most likely cytoplasmic and more readily purified for other applications.

Over-expression of *ydeH* enhances biofilm and PGA accumulation.

YdeH is a 296 amino acid protein conserved in *E. coli* and its close relatives. It has no predicted transmembrane domains and a single C-terminal putative diguanylate cyclase GGDEF domain (Ecogene, Pfam) consisting of the amino acid sequence "GGEEF".

ydeH was cloned under the control of an arabinose inducible promoter (of pBAD24) and contained the Shine-Dalgarno sequence from the vector. The *ydeH* clone on a multicopy vector complemented the biofilm defect of the *ydeH::kan* gene replacement from the Keio collection in BW25113, MG1655, and TRMG backgrounds, leading to greater than wild-type levels of biofilm in all strain backgrounds tested (**Figure 4-2A** and data not shown). Nonetheless, over-expression of *ydeH* was unable to overcome the biofilm defect of a *pgaC* mutant, which is deleted for the glycosyltransferase required for biosynthesis of β-1,6-*N*-acetyl-D-glucosamine (PGA), a polysaccharide important for biofilm formation in *E. coli* K-12 (Itoh *et al.*, 2005; Wang *et al.*, 2004). This suggested that the effect of *ydeH* on biofilm formation was likely mediated through PGA production.

We then probed cell lysates from BW25113 and isogenic BW *ydeH::kan* strains harboring a vector control (pBAD24) or pBAD24 containing the *ydeH* clone for PGA accumulation using an anti-PGA monoclonal antibody (**Figure 4-2B**). Over-expression of *ydeH* led to increased cell-bound PGA in this assay. Analysis of PGA in the culture supernatants revealed comparable levels among all strains (data not shown). These results indicate that expression of *ydeH* results in increased PGA accumulation.

Ectopic expression of *yhjH* (EAL domain protein) represses biofilm formation and PGA accumulation.

YhjH is a 255 amino acid protein and contains a cyclic-di-GMP phosphodiesterase EAL domain. When it was over-expressed in *Salmonella* Typhimurium, *yhjH* enhanced motility, inhibited cellulose biosynthesis and the "rdar phenotype", and resulting in decreased intracellular c-di-GMP levels (Simm *et al.*, 2004). Introduction of *E. coli* YhjH into *P. putida* cells or YhjH from *Salmonella* into *Shewanella oneidensis* led to rapid dispersal of preformed biofilms (Gjermansen *et al.*, 2006; Thormann *et al.*, 2006).

In agreement with previous studies, over-expression of *yhjH* in TRMG (*csrA::kan*) strains led to a dramatic decrease in biofilm formation (**Figure 4-3A**). Furthermore, induction of *yhjH* in preformed biofilms resulted in a gradual decrease in biofilm over the course of ~4 hrs, which was not as rapid as the dispersal seen in *P. putida* or *Shewanella oneidensis* (data not shown). Immunodetection of PGA produced by strains harboring a *yhjH* clone indicated that elevated synthesis of this phosphodiesterase resulted in a decrease in PGA to levels barely detectable upon overexposure of the membrane (**Figure 4-3B**).

The results of over-expression of *ydeH* and *yhjH* support the emerging idea that both GGDEF- and EAL-domain proteins are involved in regulating the transition of bacteria between a planktonic and a sessile lifestyle by regulating the amount of PGA. Furthermore, the intracellular levels of c-di-GMP are likely responsible for this shift.

Most importantly, our immunoassays indicated that both *ydeH* and *yhjH* influence biofilm formation by affecting PGA accumulation.

***ydeH* or *yhjH* induction does not affect *pgaA'-'lacZ* expression**.

To dissect the mechanism by which these proteins alter PGA levels, we focused on whether over-expression of these two genes altered expression of the *pgaABCD* operon. Expression of *ydeH* or *yhjH* was induced in a strain containing a chromosomal *pgaA'-'lacZ* translational fusion. Compared to the vector control (pBAD24), expression of *ydeH* or *yhjH* did not alter activity of the fusion (**Figure 4-4)**, suggesting that *ydeH* and *yhjH* do not regulate transcription-translation of *pgaA*. Addition of arabinose decreased activity levels of the fusion, possibly through catabolite repression (e.g. Jackson *et al.* 2002). In addition, over-expression of *yhjH* or the GGDEF protein AdrA (YaiC) cloned into an IPTG-inducible vector did not alter activity of the *pgaA'-'lacZ* fusion, but had a strong effect on biofilm formation and PGA accumulation (data not shown; Suzuki and Romeo unpublished results). AdrA has been shown to affect cellulose accumulation in *Salmonella* (Simm *et al.* 2004), but deletion of *bcsA* or *bcsB* required for cellulose synthesis did not alter biofilm formation in a strain over-expressing of AdrA or YhjH (Suzuki and Romeo, unpublished results). Thus, it appears that c-di-GMP is not affecting *pgaA* transcription or translation.

c-di-GMP does not stimulate *in vitro* PGA synthesis

Since over-expression of *ydeH* or *yhjH* did not alter expression of the *pgaA'-'lacZ* fusion, we hypothesized that c-di-GMP might be an allosteric activator of the PGA

synthesis machinery, similar to its role in the cellulose biosynthesis system (Haim *et al.*, 1997; Weinhouse *et al.*, 1997). In order to test this hypothesis, we developed an *in vitro* assay to measure incorporation of a radioactive precursor into PGA. Crude membranes from strains with known biofilm phenotypes were prepared and used in reactions containing UDP-[^{14}C]GlcNAc as a substrate. Membranes from TRMG (*csrA::kan*) incorporated the nucleotide into a form that is trapped in the nicrocellulose membranes, but an isogenic Δ*pgaC* strain, deleted for the glycosyltransferase required for PGA synthesis, showed no detectable incorporation (**Figure 4-5A**). A time course of *in vitro* PGA synthesis at 26°C indicated that incorporation increases over time (**Figure 4-5B**). Furthermore, membranes isolated from strains with single non-polar deletions of the *pga* genes indicated that *in vitro* incorporation results largely mirrored those obtained by immunodot blots of cell lysates of these strains probed for PGA (Itoh *et al.*, 2008; **Figure 4-5A**). Incorporation of label in reactions containing membranes from *pgaA* or *pgaB* deletions, however, was lower than that obtained for the parental strain. The reason for this is currently unclear. These results validate our assay as a tool to test the effect of added extracts or factors (such as purified c-di-GMP) in a cell-free *in vitro* system. However, purified c-di-GMP did not consistently enhance incorporation of UDP-[^{14}C]GlcNAc (**Figure 4-6**). While S-100 extracts from TRMG enhanced apparent PGA synthesis, addition of these extracts also failed to alter labeling in response to c-di-GMP. Label incorporation by membranes from a strain that over expressed *ydeH* failed to respond to c-di-GMP (data not shown).

Membranes over-expressing *yhjH* (EAL) fail to synthesize PGA *in vitro*

YhjH was previously shown to reduce intracellular concentrations of c-di-GMP and to have phosphodiesterase activity (Simm *et al.* 2004). We hypothesized that by depleting intracellular levels of c-di-GMP, the effect of addition of purified c-di-GMP on PGA synthesis, if any, would be enhanced. We then tested the ability of membranes over-expressing *yhjH* to incorporate label into PGA *in vitro*. Surprisingly, these membranes were unable to synthesize PGA either in the presence or the absence of 50 μM c-di-GMP (**Figure 4-7**). These results suggest that c-di-GMP may be required for proper assembly or stability of the PGA biosynthesis machinery within the inner membrane. Whether this involved expression or activity of an effector of Pga synthesis is not known.

Effect of c-di-GMP on PgaC and PgaD accumulation.

Based on the results of the aforementioned *in vitro* experiments, it remained a formal possibility that c-di-GMP could be altering the stability of the Pga components involved in enzymatic synthesis of the polysaccharide. In order to test the effect of varying c-di-GMP levels on PgaC and PgaD protein levels, we created chromosomal C-terminus 1X FLAG fusions of these genes using the method of Uzzau *et al.*, 2001. These fusions proteins retained functionality in biofilm assays (biofilm levels were virtually identical to those of the parental strain) and cells containing them were able to respond to c-di-GMP levels *in vivo* (data not shown). Western blots were then used to detect the fusion proteins and assess relative levels in strains over-expressing the pBAD24 vector control, *yhjH,* or *ydeH*. Unfortunately, we were unable to detect the PgaC-FLAG fusion protein in our assays. This leaves open the possibility that over-expression of the

GGDEF domain protein YdeH or YhjH (EAL) may affect the stability of the critical proteins for PGA production.

DISCUSSION

Our results indicate that production of the polysaccharide adhesin β-1,6-*N*-acetyl-D-glucosamine (PGA) and biofilm development are modulated by levels of the intracellular second messenger c-di-GMP. A panel of single gene disruptions in all GGDEF and EAL domain containing proteins of *E. coli* was tested to determine which genes affected biofilm formation. These surveys indicated that several GGDEF and EAL proteins affect biofilm formation to varying degrees. By focusing on two genes with strong effects on biofilm, we determined that expression of *ydeH* or *yhjH* affects PGA accumulation. These data provide evidence that expression of the GGDEF domain protein YdeH activates biofilm formation by enhancing levels of PGA, while expression of the EAL domain protein YhjH decreases PGA accumulation. Because activity from a translational *pgaA*'-'*lacZ* fusion was not affected by expression of *ydeH* or *yhjH* (**Figure 4-4**) and previous studies have indicated that both YhjH and YdeH affect intracellular levels of c-di-GMP (Jonas *et al.*, in press; Simm *et al.*, 2004) we hypothesized that c-di-GMP was acting allosterically to promote PGA synthesis. However, none of the four Pga proteins appears to contain a classical c-di-GMP binding domain (e.g. PilZ domain; data not shown), and the known PilZ domain protein YcgR of *E. coli* does not account for effects of c-di-GMP on biofilm formation (**Figure 4-1**).

To provide more mechanistic details of how c-di-GMP modulates PGA levels and to test our hypothesis, an *in vitro* enzymatic assay using membrane preparations was

adapted from Gerke *et al.*, 1998 for *E. coli* and validated by testing the PGA synthesis abilities of membranes with defined mutations and biofilm forming capacities (**Figure 4-5**). Exogenously added purified c-di-GMP failed to enhance PGA accumulation *in vitro*. Furthermore, membranes depleted for c-di-GMP were unable to promote synthesis of PGA, suggesting that c-di-GMP may be necessary for assembly and/or activity of membranes competent for PGA synthesis. These results imply that there is a novel response system for c-di-GMP in *E. coli* responsible for modulation of PGA levels.

Currently, factors known to affect PGA biosynthesis include the LysR-type transcriptional regulator NhaR, which activates *pga* transcription in response to elevated monovalent cations and pH (Goller *et al.* 2006) and the RNA-binding protein CsrA, which binds to the *pgaABCD* mRNA leader, blocking translation and destabilizing this mRNA (Wang *et al.* 2005). Catabolite repression control also influences PGA accumulation (Itoh, Pannuri, and Romeo unpublished results). The basis for this response is not yet known. Furthermore, there is now evidence that CsrA binds to and affects levels of the *ydeH* and *ycdT* mRNA transcripts and intracellular c-di-GMP levels (Jonas *et al.*, 2008), thus adding a new and exciting level of complexity to the PGA system.

Although the exact mechanism by which c-di-GMP affects PGA accumulation remains unknown, the findings of this study provide insight into what is likely to be a novel mechanism of action of this molecule. Riboswitches are invariably located at the 5' end of mRNAs that they regulate, and our *pgaA'-'lacZ* fusion contained the full 5'-leader of the *pga* transcript (Wang *et al.*, 2004). Thus, it seems unlikely that the *pgaABCD* transcript can act as a riboswitch (e.g., Sudarsan *et al.*, 2008) in response to c-di-GMP because our assays (translational *pgaA'-'lacZ* fusions under conditions of

varying c-di-GMP) showed no significant effect on transcription/translation. Real time PCR analyses of the intergenic regions between *pgaB/pgaC* and *pgaC/pgaD* showed negligible effects of over-expression of *yhjH* on transcript levels, thus suggesting that the existence of an internal promoter that responds to c-di-GMP is unlikely (data not shown). In addition, results of the *in vitro* assays suggest that allosteric activation of the Pga system may not be the mechanism. Nevertheless, the intriguing results of the *in vitro* PGA synthesis reactions carried out with membranes depleted for c-di-GMP (over-expressing *yhjH*) offer a glimpse into the mechanism of action of this molecule. It is therefore possible that c-di-GMP affects stability of the functional Pga complex or expression/activity of a yet-unidentified c-di-GMP responsive element of the Pga system. Future studies should aim to elucidate the details.

Factors affecting the expression of the GGDEF and EAL domain proteins that influence biofilm formation will offer insight into the conditions that modulate biofilm formation and, most importantly, dispersal. The predicted structures of *yhjH* and *ydeH* do not reveal any apparent additional signaling domains. Therefore, by focusing on the regulatory elements that modulate expression of *yhjH* or *ydeH*, we may be better able to assemble a snapshot of the conditions that are likely responsible for activation of the Pga system. For instance, a putative CpxR binding site is located upstream of the *ydeH* promoter (RegulonDB database; Yamamoto and Ishihama, 2006) and may implicate expression of *ydeH,* and consequently activation of PGA production, under conditions of membrane stress. The Cpx system has been proposed to be responsible for surface sensing in bacteria (Lejeune, 2003; Otto and Silhavy, 2002). It is thought provoking to consider that the *ydeH*-PGA connection may be wired in such a way that allows the

bacterium to sense surfaces for the timely production of the PGA polysaccharide required for attachment and entrance into a sessile lifestyle. Likewise, *yhjH* is known to positively affect motility and its expression may be part of a coordinated response that involves turning off PGA accumulation and activating motility to consequently exit the biofilm mode of growth and swim to other areas.

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FIGURE LEGENDS

Figure 4-1. Effects of c-di-GMP-related genes on biofilm formation. Strains with single gene disruptions were obtained from the Keio collection for 27 GGDEF and/or EAL domain containing proteins, as well as the PilZ domain protein YcgR involved in c-di-GMP binding. Values represent the average of 4 wells and were normalized with respect to the parent strain BW25113. The standard error from the mean (SEM) is shown for the biofilm formed by two independent colonies of each strain. Arrows highlight two strains with substantially altered biofilm phenotypes that were further analyzed (the GGDEF domain protein YdeH, and the EAL protein YhjH). An asterisk denotes that the *rtn* deletion strain displayed substantial heterogeneity in biofilm formation depending on the stock culture used. Note that the YcdT and YhdA GGDEF domain proteins were not included in these analyses since their effects on biofilm formation and the PGA system have been previously characterized by our group (Wang *et. al* 2005; Suzuki *et al.* 2006).

Figure 4-2. Over-expression of *ydeH* enhances biofilm and PGA accumulation. A) Biofilm assay for strains bearing the *ydeH* ORF in pBAD24 under the control of arabinose induction. Biofilms were grown as indicated in the legend of Figure 1. A representative experiment is shown where bars represent the average of 4 wells ± SEM. The *pgaC* gene encodes the glycosyltransferase required for biosynthesis of β-1,6-*N*-acetyl-D-glucosamine (PGA).

B) PGA dot blots of cell lysates from 24 h cultures using anti-PGA monoclonal antibody (see Materials and Methods).

Figure 4-3. Over-expression of *yhjH* (EAL protein) represses biofilm formation and PGA accumulation. A) Biofilm assay for TRMG (*csrA::kan*) strains harboring the arabinose-inducible *yhjH* plasmid clone. B) Immunodetection of PGA produced by these strains.

Figure 4-4. Induction of *ydeH* or *yhjH* does not affect *pgaA'-'lacZ* expression. Expression of *ydeH* or *yhjH* was induced by addition of 0.2% arabinose in a strain containing a chromosomal *pgaA'-'lacZ* translational fusion. Compared to the vector control (pBAD24), expression of *ydeH* or *yhjH* did not alter activity of the fusion. Bars correspond to the average β-galactosidase activity per milligram of protein for reactions conducted in triplicate from a representative experiment. Error bars depict SEM. No significant difference (at $\alpha = 0.95$) in activity levels between any two of these strains was found [Tukey's multiple comparison analyses].

Figure 4-5. *In vitro* biosynthesis of PGA. PGA synthesis by crude membranes from strains with known biofilm phenotypes was monitored *in vitro* using UDP-[^{14}C]GlcNAc as a substrate. Reactions (0.4 mg membrane protein) were incubated at 26°C. A) *In vitro* synthesized PGA from membranes from TRMG (*csrA::kan*) and TRMG Δ*pgaC* strains, deleted for the glycosyltransferase required for PGA synthesis. B) Time course of *in vitro* PGA synthesis. The lower panel depicts the densitometry analyses using ImageQuant software (Molecular Dynamics) of the corresponding dot blot (average of duplicate reactions ± SEM).

Figure 4-6. c-di-GMP does not stimulate the *in vitro* synthesis of PGA. Incorporation of UDP-[^{14}C]GlcNAc label into PGA was examined in the presence of increasing concentrations of purified c-di-GMP (generous gift from M. Gomelsky) and S-100 extracts from TRMG cultures.

Figure 4-7. Membranes from strains over-expressing *yhjH* (EAL) fail to synthesize PGA *in vitro*. Incorporation of UDP-[^{14}C]GlcNAc label into PGA was examined in membranes from strains over-expressing *yhjH* (depleted for c-di-GMP) in both the absence and presence of 50 μM c-di-GMP.

Table 4-1. List of strains, plasmids, and bacteriophage used in this study.

Strain, plasmid of phage	Description or genotype	Source or reference
E. coli K-12 strains		
DH5α	*supE44* Δ*lacU169* *(ϕ80lacZΔM15)* *hsdR17 relA1 recA1 endA1 gyrA96 thi-1*	
MG1655	$F^-\lambda^-$	Michael Cashel
TRMG1655	MG1655 *csrA::kan*	Romeo *et al.*, 1993
CF7789	MG1655 Δ*lacI-Z (MluI)*	Michael Cashel
TRCF7789	CF7789 *csrA::kan*	Suzuki *et al.*, 2002
TRXWMGΔABCD	TRMG1655 Δ*pgaABCD*	Wang *et al.*, 2004
TRXWMGΔA	TRMG1655 Δ*pgaA*	Wang *et al.*, 2004
TRXWMGΔB	TRMG1655 Δ*pgaB*	Wang *et al.*, 2004
TRXWMGΔC	TRMG1655 Δ*pgaC*	Wang *et al.*, 2004
TRXWD146	TRMG1655 *pgaD146*::*cam*	Wang *et al.*, 2004
XWZ4	CF7789 *pgaA*'-'*lacZ*	Wang *et al.*, 2005b
TRXWZ4	XWZ4 *csrA::kan*	Wang *et al.*, 2005b
CFPGACAT2321	CF7789 Δ(att-lom)::*bla::cam* Φ(*pgaA*'-'*lacZ*)4(Hyb) Cam^R	Mercante *et al.*, 2006
TRC-FLAG	TRMG1655*pgaC*-1X FLAG	This study
TRD-FLAG	TRMG1655*pgaD*-1X FLAG	This study
GGDEF/EAL strains from Keio collection		
BW25113	Parent; *rrnB3* Δ*lacZ4787* *hsdR514* Δ*araBAD 567* Δ*rhaBAD 568 rph-1*	Baba *et al.*, 2006
JW2585	*yfiN::kan*; GGDEF	
JW1697	*ydiV::kan*; EAL	
JW2052	*yegE::kan*	
JW2488	*yfgF::kan*; GGDEF & EAL	
JW1278	*yciR::kan*; GGDEF & EAL	
JW1774	*yeaI::kan*; GGDEF	
JW1150	*ycgF::kan*; EAL	
JW0376	*yaiC::kan*; GGDEF	
JW0818	*yliF::kan*; GGDEF	
JW0307	*yahA::kan*; EAL	
JW0817	*yliE::kan*; EAL	
JW2164	*rtn::kan*; EAL	
JW4022	*yjcC::kan*; EAL	
JW3493	*yhjH::kan*; EAL	
JW5206	*ydaM::kan*; GGDEF	
JW5174	*ycgG::kan*; EAL	

JW5062	*ylaB::kan*; EAL	
JW5391	*yfeA::kan*; GGDEF & EAL	
JW5241	*yddV::kan*; GGDEF	
JW5291	*yeaJ::kan*; GGDEF	
JW5292	*yeaP::kan*; GGDEF	
JW1528	*ydeH::kan;* GGDEF	
JW1804	*yoaD::kan*; EAL	
JW5832	*yedQ::kan*; GGDEF	
JW5825	*yneF::kan*; GGDEF	
JW5863	*yhjK::kan*; GGDEF & EAL	
JW1183	*ycgR::kan*; PilZ domain	
JWdos	*dos::kan,* GGDEF & EAL	Graciela Lorca, University of Florida
BWAP	BW25113 *csrA::kan*	This study
BWTRYDEH	BW25113 *csrA::kan* *ydeH::kan*	This study

Plasmids

pKK223-3	cloning vector	Pharmacia
pBAD24	Arabinose-inducible expression vector	Guzman *et al.*, 1995
pPGA372	*pgaABCD* in pUC19	Wang *et al.*, 2004
pBADydeH	*ydeH* in pBAD24	This study
pBADyhjH	*yhjH* in pBAD24	This study

Bacteriophage

P1*vir*	strictly lytic P1	Carol Gross

Table 4-2. List of primers used in this study.

Primer name	Sequence 5'→ 3'	Comments
yhjH FWD	ata agg cag gtt atc cag cga	*yhjH*, no ATG
yhjH REV	tta tag cgc cag aac cgc cgt att	Contains stop codon of *yhjH*
ydeH FWD	atc aag aag aca acg gaa att gat gcc	*ydeH*, no ATG
ydeH REV	tta aac tcg gtt aat cac att ttg	Contains stop codon of *ydeH*
pgaC FWD 1X FLAG	cgc ccg ttg ggt aag tcc cga tcg cgg gat tct gag agg tga cta caa aga tga cga cga	40 nt homologous to end of *pgaC* in 5', no stop, 1X FLAG tag
pgaC REV FLAG	aac gta ctg gtg att gtc ggg tcg taa taa tta aat tgt tca tag ctg tta cct cct tac ata tga ata tcc tcc tta g	40 nt homologous to region downstream of *pgaC* with optimized SD (from *lacZ*) for *pgaD* to prevent polarity
pgaD FWD 1X FLAG	aat aaa aat ggt tgt ttc aga aaa agc gct agt ccg ggc aga cta caa aga tga cga cga	40 nt homologous to end of *pgaD* in 5', no stop, 1X FLAG tag
pgaD REV FLAG	cgg tgc aga gcc cgg gcg aac cgg gct ttg ttt tgg gtg tca tat gaa tat cct cct tag	40 nt homologous to region downstream of *pgaD*

* Restriction sites are underlined

Fig. 4-1.

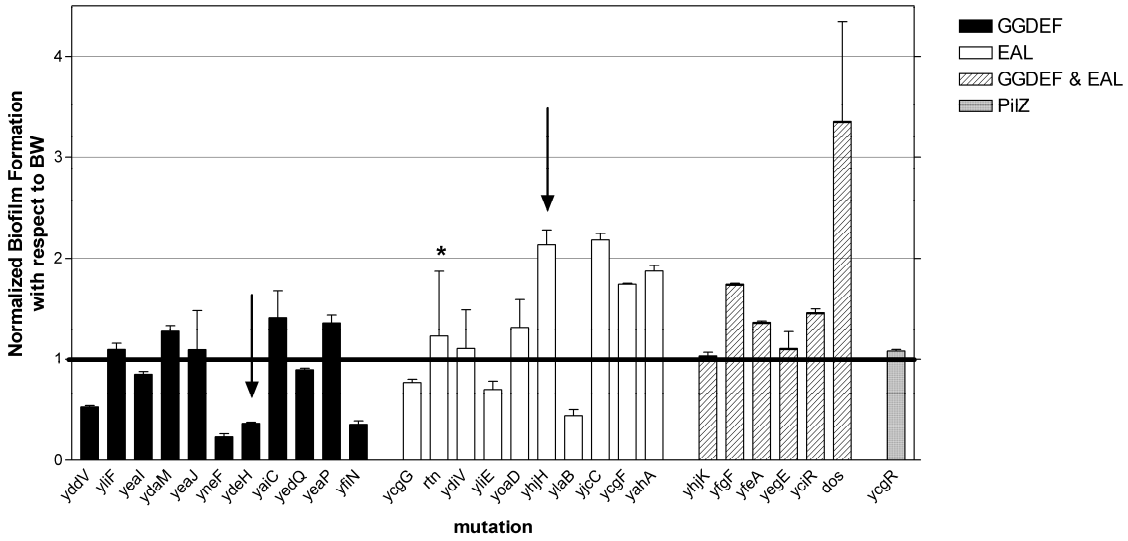

GGDEF
EAL
GGDEF & EAL
PilZ
Normalized Biofilm Formation with respect to BW
0
1
2
3
4
*
ydaV
yliF
yeaI
ydaM
yeaJ
yneF
ydeH
yaiC
yedQ
yeaP
yfiN
ycgG
rtn
ydiV
yliE
yoaD
yhjH
ylaB
yjcC
ycgF
yahA
yhjK
yfgF
yfeA
yegE
yciR
dos
ycgR
mutation

Fig. 4-2.

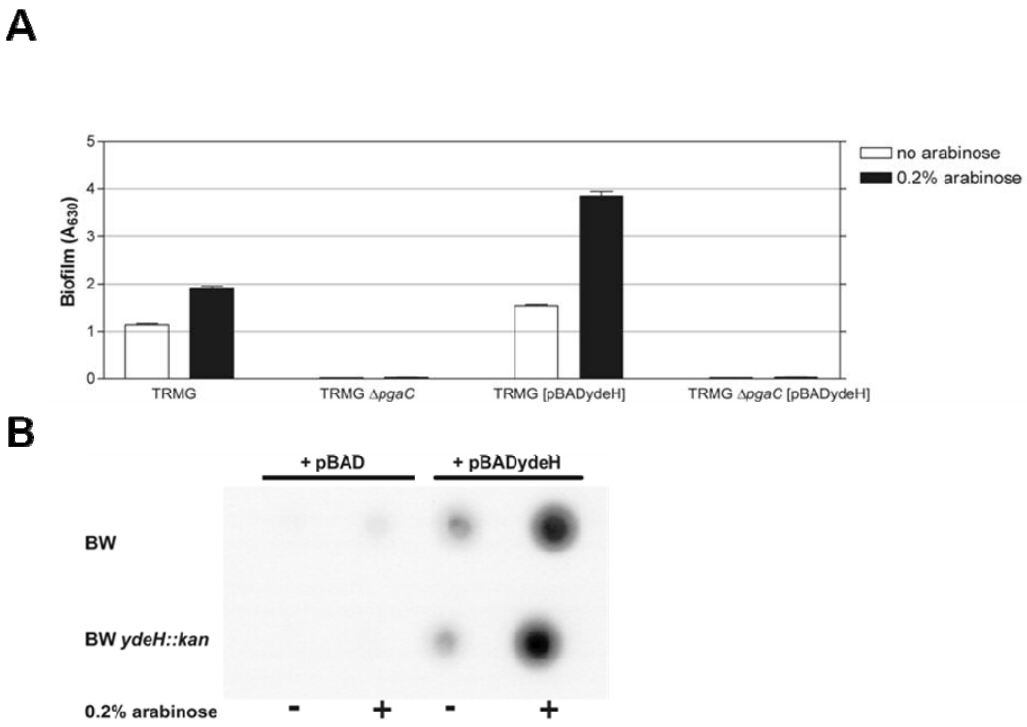

A
Biofilm (A_{630})
5
4
3
2
1
0
no arabinose
0.2% arabinose
TRMG
TRMG ΔpgaC
TRMG [pBADydeH]
TRMG ΔpgaC [pBADydeH]
B
+ pBAD
+ pBADydeH
BW
BW ydeH::kan
0.2% arabinose
-
+
-
+

Fig. 4-3.

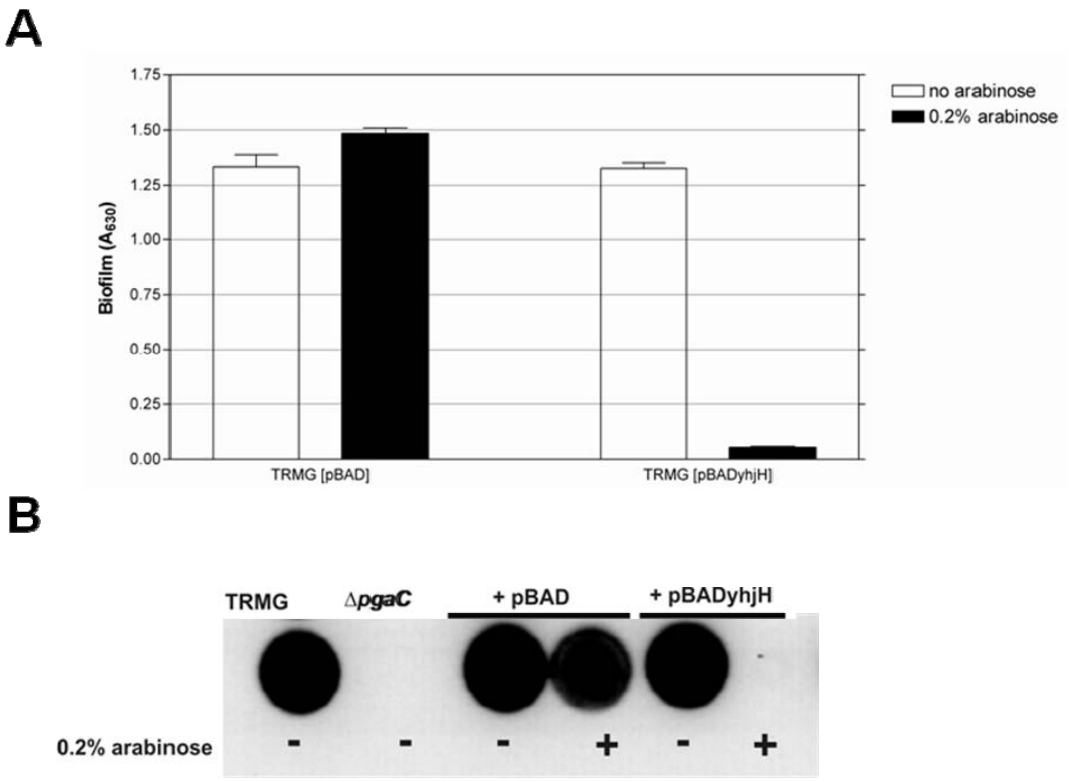

A
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Biofilm (A_{630})
no arabinose
0.2% arabinose
TRMG [pBAD]
TRMG [pBADyhjH]
B
TRMG
ΔpgaC
+ pBAD
+ pBADyhjH
0.2% arabinose
-
-
-
+
-
+

Fig. 4-4.

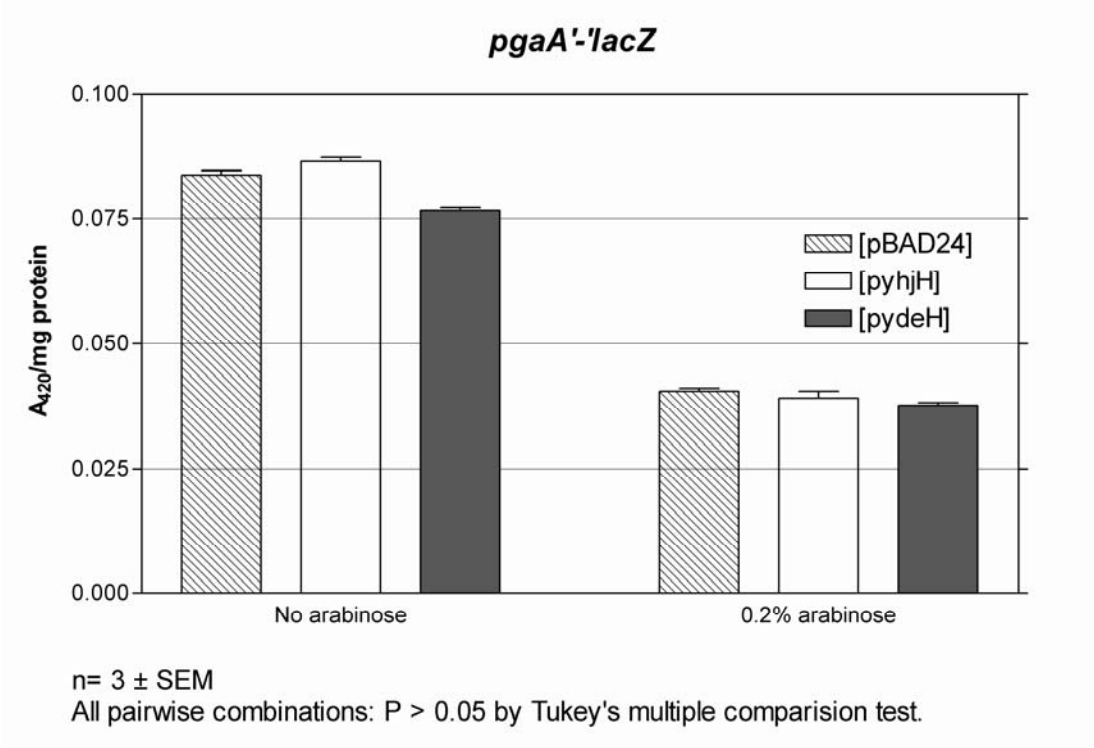

pgaA'-'lacZ
0.100
0.075
0.050
0.025
0.000
A420/mg protein
[pBAD24]
[pyhjH]
[pydeH]
No arabinose
0.2% arabinose
n= 3 ± SEM
All pairwise combinations: P > 0.05 by Tukey's multiple comparision test.

Fig. 4-5.

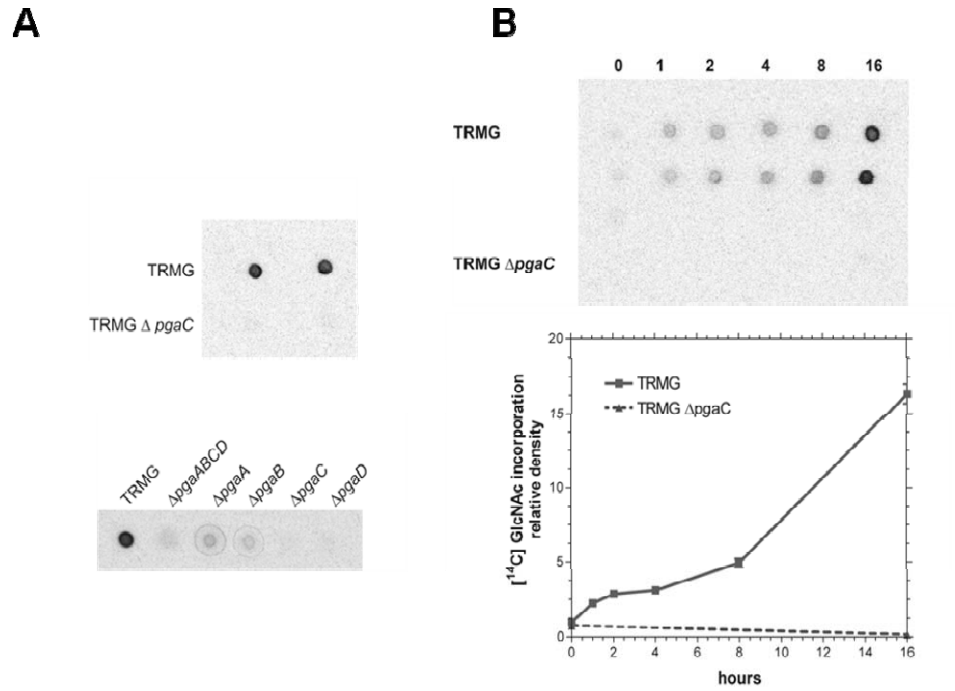

A
TRMG
TRMG Δ pgaC
TRMG
ΔpgaABCD
ΔpgaA
ΔpgaB
ΔpgaC
ΔpgaD
B
0
1
2
4
8
16
TRMG
TRMG ΔpgaC
TRMG
TRMG ΔpgaC
[14C] GlcNAc incorporation relative density
hours
0
5
10
15
20
0
2
4
6
8
10
12
14
16

Fig. 4-6.

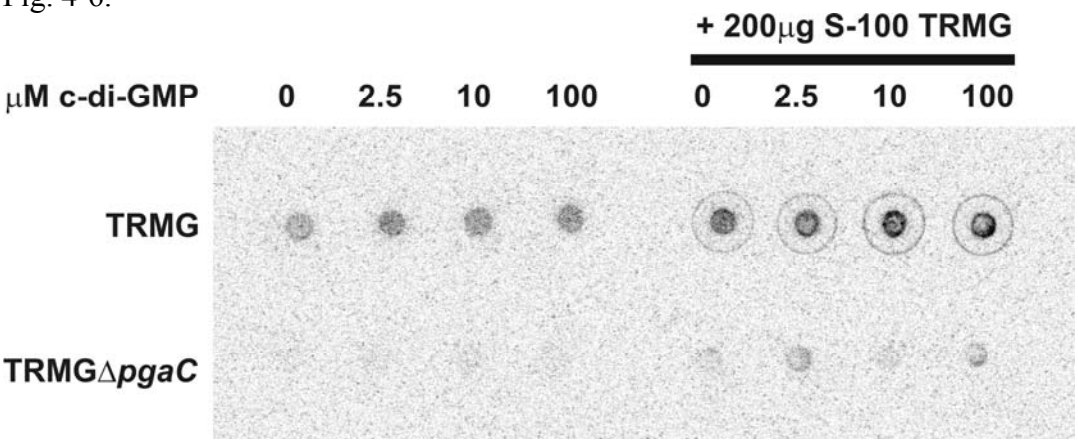
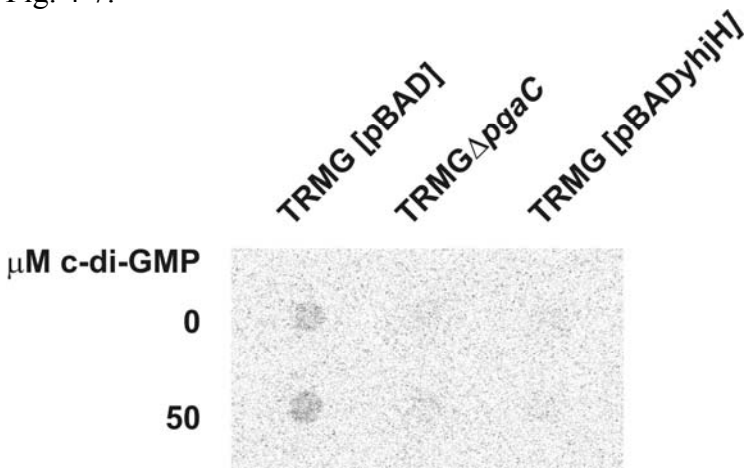

+ 200μg S-100 TRMG
μM c-di-GMP
0
2.5
10
100
0
2.5
10
100
TRMG
TRMGΔpgaC

Fig. 4-7.



Chapter 5: Dissertation Discussion

Role of PGA in biofilm formation

Wang *et al.* clearly demonstrated the importance of the β-1,6-*N*-acetyl-D-glucosamine (PGA) polysaccharide in *Escherichia coli* biofilm formation, paving the road for future studies to discern the regulation of the *pga* genes (Wang *et al.*, 2004; Wang, 2005; Wang *et al.*, 2005). Operons homologous to *pgaABCD* (and the *ica* locus in Staphylococci) are necessary for biosynthesis of polymers that promote biofilm formation by *E. coli* K-12 and other eubacteria, including *Staphylococcus epidermidis*, *Bordetella* sp., *Aggregatibacter actinomycetemcomitans*, *Actinobacillus pleuropneumoniae* and *Yersinia pestis* (Heilmann *et al.*, 1996a; Heilmann *et al.*, 1996b; Itoh *et al.*, 2005; Izano *et al.*, 2007; Izano *et al.*, 2008; Parise *et al.*, 2007). Currently, several groups are studying the roles of these genes on genetic and structural levels. The work presented here defines two additional regulatory elements that have profound effects on PGA production: NhaR and the secondary messenger c-di-GMP.

Elucidating the NhaR regulon

At a transcriptional level, the LysR-type transcriptional regulator NhaR is critical for expression of the *pga* genes (See Chapter 3, Goller *et al.*, 2006). Moreover, it highlights the importance of growth conditions (salinity and pH) on expression of these genes, therefore providing an environmental connection. NhaR is required by cells to maintain internal sodium homeostasis, for it positively activates transcription of the *nhaAR* operon,

where NhaA is a Na^+/H^+ antiporter critical for survival of *E. coli* at high pH and salinity (Carmel *et al.*, 1997; Dover *et al.*, 1996; Dover and Padan, 2001; Goller *et al.*, 2006; Padan and Schuldiner, 1993, 1994; Padan *et al.*, 1999; Rahav-Manor *et al.*, 1992). In addition, NhaR positively affects transcription of the *osmC* gene, encoding an enzyme important for resistance to hydroperoxide stress (Gutierrez and Devedjian, 1991; Lesniak *et al.*, 2003; Sturny *et al.*, 2003; Toesca *et al.*, 2001). Both NhaR and the RcsC-D-B phosphorelay system, which is involved in capsular synthesis, independently regulate *osmC* expression (Sturny *et al.*, 2003). Interestingly, the Rcs regulatory system, which responds to osmolarity and envelope stress, affects biofilm formation but does not alter *pga* expression (Goller and Romeo, unpublished results). Are osmolarity and contact with a surface both triggers for biofilm formation in *E. coli* and other organisms?

Studies to elucidate the NhaR regulon are likely to be very informative. It is tempting to speculate that NhaR is responding to environmental conditions (salinity and pH) that require a coordinated response, including but likely not limited to expression of *nhaA* (extrusion of sodium), *osmC* (peroxide stress), and biofilm formation (*pga*). Discovery of the situations in *E. coli's* lifecycle that require such coordinated response will hold clues as to the biological context surrounding PGA production. The recent studies of Kimberly Jefferson's group on factors affecting PGA expression in a uropathogenic *E. coli* strain support our findings (Cerca and Jefferson, 2008). Furthermore, a signature-tagged mutagenesis in *Proteus mirabilis*, which produces urease and creates an alkaline environment in the urinary tract, indicated that disruption of *nhaR* results in significant attenuation of virulence in competition challenges (Himpsl *et al.*, 2008). The authors

suggest that this is evidence that *P. mirabilis* responds to an alkaline environment. What is the target of NhaR in *P. mirabilis*?

Recently, studies by Edwards and Romeo (unpublished results) suggesting a direct interaction between CsrA and the *nhaR* transcript have supported our finding of a putative CsrA consensus binding sequence between *nhaA* and *nhaR* (Goller and Romeo, unpublished results). If CsrA affects *nhaR* transcript levels or translation, this would provide a novel example of CsrA regulating an internal gene in an operon. Moreover, CsrA would then be regulating PGA biosynthesis indirectly by affecting expression of the activator, NhaR, in addition to its well established direct effects on *pga* message stability (Wang *et al.*, 2005).

Temperature has strong effects on *pga* expression, and this has been noticed in several studies (Cerca and Jefferson, 2008; Wang, 2005; Goller and Romeo, unpublished results). Nevertheless, possible explanations have been lacking until recently. The work by White-Ziegler *et al.* using microarrays to determine gene expression patterns at low temperature indicated that the expression of *nhaR* (and several other biofilm factors) is RpoS-dependent at 23 °C (White-Ziegler *et al.*, 2008). While the early studies on *nhaAR* expression did not indicate that *nhaA* transcription was RpoS-dependent (Dover *et al.*, 1996), later ones relying on an *in vitro* assay did show that a second promoter indeed was RpoS-dependent (Dover and Padan, 2001). In addition, H-NS affects *nhaAR* transcription and an *hns* mutant strain showed increased *pgaA'-'lacZ* activity, presumably through higher levels of NhaR available to activate *pga*, although direct effects of H-NS on the *pga* promoter are still a formal possibility (Goller *et al.*, 2006; Goller and Romeo, unpublished results).

Several questions remain as to the true range of functions served by NhaR and the effect of monovalent cations on its activity. What else is NhaR regulating that could be important for biofilm formation? Is NhaR induced upon contact with a surface? Most importantly, in the human gut, is NhaR being induced by high sodium and alkaline pH (e.g., by bile)? Our *in vitro* transcription assays and DNaseI footprints failed to show an effect on *pga* upon addition of sodium even though stimulation is evident *in vivo* (using *lacZ* fusions). LysR-type transcriptional regulators frequently bind a co-inducer to affect expression patterns (reviewed by Schell, 1993). Is lack of *in vitro* stimulation of transcription by sodium an artifact of the assay? Is there an additional factor or co-inducer required for NhaR-dependent enhancement of *pgaABCD* expression by sodium?

Biofilm forming capacities of *E. coli* isolates from diverse environments

The vast majority of studies of *E. coli* biofilm formation have focused on laboratory strains such as K-12. The ECOR reference collection of *E. coli* isolates has proven to be very useful in studies comparing the genetic differences and abilities of a diverse set of *E. coli* strains from greatly different origins (e.g., Johnson *et al.*, 2001). Reisner *et al.* surveyed 331 non domesticated *E. coli* strains isolated from healthy people and patients with diarrhea, bacteremia, and UTIs (Reisner *et al.*, 2006). Results revealed “remarkable variation” among the (*in vitro*) biofilm forming capacities of the isolates. However, there was a strong dependence on the growth medium composition, and the authors suggest that the diverse *E. coli* isolates respond very differently to changing environmental conditions.

With the knowledge that in our strains and conditions, expression of the polymer β-1,6-*N*-acetyl-D-glucosamine (PGA) is critical for the formation and structural stability of *E. coli* biofilms, we aimed to survey a broad collection of *Escherichia* isolates from diverse environments to determine if the capacity for biofilm formation varies in strains from different niches, and, if so, the factors that are used for adhesion and biofilm development.

We hypothesized that certain strains of *E. coli* use PGA as their main adhesin, and Dr. Pannuri and I initiated a screen of a large panel of environmental isolates obtained from Dr. Mike Sadowsky (University of Minnesota). The panel was divided into isolates obtained from one of four environments: 91 peryphyton (a mixture of cyanobacteria, algae, and microbes attached to a submerged surface), 91 cladophora (isolated from or with green algae), 91 sediment (particles deposited on a bed of a body of liquid), and 16 soil isolates for the initial screen. We also found a strong influence of the growth medium on biofilm formation of these non domesticated strains (addition of glycerol to the growth medium enhanced biofilm formation of several isolates). Most importantly, we found a striking difference in the biofilm forming abilities of these strains: whereas less than 2% of the peryphyton and cladophora isolates formed greater or equal biofilm to an *E. coli* K-12 MG1655 laboratory wild-type strain, 14% of sediment strains formed biofilms ranging from 2 to 6 times that of MG1655 (Goller, Pannuri, Sadowsky, and Romeo, unpublished results). These sediment biofilm formers frequently have more intense calcofluor and congo red binding abilities when compared to the MG1655, suggestive of the presence of polysaccharides, curli, or cellulose. Determination of the component(s) used by these isolates to attach to polystyrene plates is pending. For this, the Dispersin B (DspB)

enzyme, which releases PGA-dependent biofilms of several organisms (Itoh *et al.*, 2005), should be a useful tool in the determination of the nature of these attachments. For factors such as Type I and curli, available antibodies can be used to probe these isolates. This may lead us to isolates and consequently environments where PGA-dependent biofilm formation is required for survival and persistence.

Investigations into the Pga proteins and PGA synthesis machinery

The studies by Itoh *et al*. demonstrated that the PgaC and PgaD proteins are necessary for synthesis of the PGA polysaccharide, while PgaA and PgaB are involved in export and deacetylation, of the polymer (Itoh *et al.*, 2008). These studies also provided insight into the interactions among the Pga proteins and kindled interest by several groups in the study of the distinct structural properties of the Pga proteins. There is also some evidence that PGA is secreted preferentially from the poles of the cell, which is very fascinating considering the observation that cells initiate biofilm formation by polar attachment (Agladze *et al.*, 2003; Agladze *et al.*, 2005). Moreover, this suggests that the Pga protein complex might be localized at the cell poles. Although unsuccessful for the c-di-GMP studies detailed in Chapter 4, I believe that a more sensitive epitope-tagging system using a similar approach might be used to determine the localization of the Pga proteins.

The assembly of the Pga proteins may be important in solving the intriguing mechanism of c-di-GMP enhancement of PGA accumulation. Studies in *Yersinia pestis* with the homologous Hms proteins have provided insight into the protein-protein interactions (Bobrov *et al.*, 2008). It may be worthwhile to use a biochemical approach with the aforementioned tagged Pga proteins to determine which proteins interact. It may

be the case that there is an additional c-di-GMP responsive factor needed for activation of PGA biosynthesis and/or protein complex stability. This would explain the results obtained in Fig. 4-6 and Fig. 4-7, and lead to the discovery of another component of the Pga system.

Outlook

The effect CsrA has on biofilm formation was what led Xin Wang, based on the studies of Debra Jackson-White, to set out on a search for the factor(s) responsible for the striking biofilm phenotype of a *csrA* mutant strain (Jackson *et al.*, 2002; Wang, 2005; Wang *et al.*, 2005). The payoff was a treasure trove of biofilm mutants (including *nhaR* and *yhjH*) crowned by the discovery of the *pga* operon. Two novel pathways that affect PGA accumulation have been characterized in this dissertation and begin to identify the conditions that trigger PGA-dependent biofilm formation. Not surprisingly, CsrA continues to be revealed as a central player in regulating PGA production and the biofilm lifestyle of *E. coli*: emerging evidence suggests that CsrA might affect *nhaR* expression as well as c-di-GMP levels (Jonas *et al.*, 2008; Edwards and Romeo, unpublished results). It remains a challenge to unravel these regulatory networks that feed into and determine PGA levels.

In addition to better understanding the environmental conditions and genetic mechanisms that promote biofilm formation, it remains a priority to elucidate mechanisms to counter biofilm formation, especially in hospital settings. Future studies are needed to address dispersal of *E. coli* biofilms, by addition of DspB or through the discovery of bioactive compounds such as c-di-GMP inhibitors.

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