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Molecular Interactions in Biofilms

Review

Mark E. Shirtliff,[1] Jon T. Mader,[2] and Anne K. Camper[1,3]
[1]Center for Biofilm Engineering
Montana State University
Bozeman, Montana 59717
[2]Hyperbaric Facility
University of Texas Medical Branch
Galveston, Texas 77555

A biofilm may be defined as a microbially derived, sessile community characterized by cells that are attached to an interface, embedded in a matrix of exopolysaccharide, and demonstrate an altered phenotype. This review covers the current understanding of the nature of biofilms and the impact that molecular interactions may have on biofilm development and phenotype using the motile gram-negative rod *Pseudomonas aeruginosa* and the nonmotile gram-positive cocci *Staphylococcus aureus* as examples.

Introduction

A biofilm may be defined as a microbially derived sessile community characterized by cells that are attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substance, and exhibit an altered phenotype with respect to growth, gene expression, and protein production [1]. Biofilm thickness can vary between a single cell layer to a thick community of cells embedded within a thick polymeric matrix. Recent structural analyses have demonstrated that these thick biofilms possess a sophisticated architecture in which microcolonies can exist in discrete pillar- or mushroom-shaped structures [2]. Between these structures, an intricate channel network provides access to environmental nutrients.

By adopting this sessile mode of life, biofilm-embedded microbes enjoy a number of advantages over their planktonic counterparts. One advantage is the ability of the polymeric matrix to capture and concentrate a number of environmental nutrients, such as carbon, nitrogen, and phosphate [3]. Another advantage to the biofilm mode of growth is it enables resistance to a number of removal strategies, such as antimicrobial and antifouling agent removal, shear stress, host phagocytic clearance, and host oxygen radical and protease defenses. This inherent resistance to antimicrobial factors is mediated through very low metabolic levels and drastically downregulated rates of cell division (e.g., small colony variants) of the deeply embedded microbes [4]. In fact, one researcher went as far as to conclude that *Pseudomonas aeruginosa* stationary phase cells and biofilm cells have similar resistance to killing by antimicrobials [5]. While low metabolic rates may explain a great deal of the antimicrobial resistance properties of biofilms, other factors may play a role. One such factor may be the ability of biofilms to act as a diffusion barrier to slow down the penetration of some antimicrobial agents [6]. For example, reactive chlorine species (such as hypochlorite, chloramines, or chlorine dioxide) in a number of antimicrobial/antifouling agents may be deactivated in the outer layers of the biofilm faster than they can diffuse into the lower layers [7].

The last advantage to the biofilm mode of growth is the potential for dispersion via detachment. As mentioned, microcolonies can exist in discrete mushroom-shaped structures. These microcolonies may detach under the direction of mechanical fluid shear or through a genetically programmed response that mediates the detachment process [8]. Under the direction of fluid flow, this microcolony travels to other regions of the host or water system to attach and promote biofilm formation on virgin areas. In addition, detachment and seeding of virgin surfaces may be accomplished by the evacuation of single, motile cells from attached microcolony cores [9]. Therefore, this advantage allows a persistent bacterial source population that is resistant to antimicrobial agents and host immune clearance, while at the same time enabling continuous shedding to promote bacterial spread.

Understanding the mechanisms involved in initial attachment, development, and maturation of the biofilm phenotype is of extreme interest for its impact on medical, industrial, and public health arenas. In this review we will explore the current understanding of the nature of biofilms and the impact that molecular interactions between the bacteria themselves and bacteria and the surface may have on biofilm development and phenotype using the motile gram-negative rod *P. aeruginosa* and the nonmotile gram-positive coccus *Staphylococcus aureus* as case study bacterial species.

Pseudomonas aeruginosa Biofilms

P. aeruginosa is a gram-negative, ubiquitous, free-living bacterial species that is able to survive in a wide variety of environmental extremes. It has a predilection for moist environments and it can infect plants, insects, lower animals, and humans [10]. This bacterial species has been described as the quintessential opportunist in human infections and is capable of causing fatal systemic disease in certain conditions. Some of these conditions arise when normal cutaneous or mucosal barriers have been breached or bypassed, when immunologic defense mechanisms have been compromised, when the protective function of the normal bacterial flora has been disrupted by broad-spectrum antibiotic therapy, and/or when the patient has been exposed to reservoirs associated with a hospital environment [11]. Infections with this pathogen are particularly common in patients with cystic fibrosis. Virtually all people with this respiratory disease eventually develop a *P. aeruginosa* infection, and it is the cause of death in more than 90% of these patients [12]. It also causes a number of other infections, including skin and soft tissue infections,

[3]Correspondence: anne_c@erc.montana.edu

medically implanted device infections, osteomyelitis, pneumonia, malignant external otitis, endophthalmitis, endocarditis, meningitis, and septicemia, through its ability to form conglomerates and biofilm.

P. aeruginosa biofilms may develop on devitalized tissue and medically implanted devices to produce an infection. In one study by O'Toole and Kolter, biofilm formation was found to be initiated by flagella and twitching motility [13]. In their study, they found that one class of mutants had a greatly reduced ability to attach to polyvinylchloride plastic to form a biofilm monolayer; these mutants were nonmotile strains that were defective in the production of the flagellum. Another class of mutants was able to form a monolayer but was unable to produce small cellular aggregates, termed microcolonies. This class of mutants was defective in the production of type IV pili. Therefore, it has been hypothesized that biofilm formation initially requires flagellar-dependent association and binding with a surface to form a single-cell monolayer. Individual cells on this monolayer then conglomerate into a number of microcolonies through twitching motility via type IV pili [13]. Also, this organism produces neuraminidase that enhances pili binding by removing sialic acid residues from host glycoprotein G_{M1}, making it a better receptor for the pili [14]. Once attached and twitching motility begins, this organism can form fully mature biofilm structures composed of complex channel systems that provide even deeply embedded bacteria access to nutrients in this modular community. High molecular weight alginate polymers and possibly microbial DNA in the early stages of biofilm development [15] are used to efficiently retain *P. aeruginosa* cells within the biofilm matrix. However, once the biofilm gains maturity, detachment processes ensue. This occurs partly through the detachment of large conglomerates of cells that diffuse away from the parental biofilm and reattach to the surface, thereby enabling biofilm spread in the mature biofilm form. This large-scale detachment may be mediated by stress due to hydrodynamic flow and/or by pseudomonal programmed responses such as upregulation of alginate lyase (encoded by *algL*) production. This enzyme is capable of alginate degradation and therefore may induce biofilm sloughing and dispersion [8]. Detachment was also found to occur in *P. aeruginosa* biofilms through the evacuation of single, motile cells from the central portions of large, mushroom-shaped cell clusters, leaving behind void spaces [9]. Prior to evacuation, these motile cells appeared to "swim" within this central cluster region and were surrounded by nonmotile cells in the cluster walls. The observed stages of attachment-mature biofilm formation-dispersion may not be the final story. Another study has potentially identified a cycle of increased biofilm biomass followed by a significant detachment phase and then a return to increasing biomass in *P. aeruginosa* biofilms [16]. Therefore, biofilm development may be cyclical in nature.

Two recent studies have evaluated the differential genomic and proteomic expression profiles seen in *P. aeruginosa* grown under planktonic and biofilm conditions. The differential gene expression studies utilized a microarray that enabled the simultaneous evaluation of mRNA levels of 5500 of the 5570 predicted *P. aeruginosa* genes [17]. In this study, only approximately 1% of the total genome displayed differential gene expression between the two modes of growth. Although the quorum-sensing system was not evaluated in this study, since planktonic control cultures and biofilms were both sufficiently dense to activate quorum-dependent genes [18], the small number of differentially regulated genes was surprising. This may be especially true when one notes that approximately 35% (or 525 proteins) had differential production between each stage of biofilm development when proteomics were used as the analytical method [9]. These differences were amplified when researchers compared the protein expression profile of planktonic *P. aeruginosa* to mature biofilms. In this case, over 50% of the proteome showed altered levels of production. These studies also found different proteins to be upregulated in the mature biofilm condition. The differences between the two studies may have been due to a number of factors, including very different growth conditions, different sensitivities of the two analytical approaches, altered rates of turnover between mRNA (for genomic studies) and proteins (for the proteomic studies), or the possibility that transcription is not a true measure of translation.

P. aeruginosa Cell-to-Cell Signaling

A large number of other bacterial species are known to possess quorum-sensing systems. These systems all stimulate an autoinduction response that occurs when the bacteria respond to concentrations of a secreted compound reached only if the cell population collectively represents a critical density. Therefore, the function of this system is to assure an individual cell of a critical population density before it induces the expression of specialized functions [19]. In gram-negative bacteria (and possibly fungi), the signaling molecule is a derivative of an *N*-acylated homoserine lactone (HSL) that is usually synthesized in its active form. The HSL is passively and/or actively secreted from the cell and accumulates in the extracellular milieu [20]. As the HSL accumulates, the intracellular concentration of HSL also increases through equilibration (especially with the freely diffusible short chain HSLs), thereby enabling it to bind to an autoinducer-responding element. This complex is then able to control the expression of the respective quorum-sensing-regulated genes. Therefore, bacterial species are able to incorporate this system into regulatory circuits whenever multicellular control is adaptively useful [19]. The quorum-sensing ability in *P. aeruginosa* is dependent upon two distinct but interrelated systems, *las* and *rhl*. There is a definite hierarchy between these systems, with the *las* system taking precedence. These two systems work in concert to upregulate a number of pseudomonal factors that enable this pathogen to survive in highly diverse environments (see Figure 1).

A. LasR/LasI System

P. aeruginosa regulates virulence gene expression in a cell density-dependent manner, known as quorum sensing. The ability of *P. aeruginosa* to detect cell density and regulate gene expression is accomplished through two separate but interrelated and homologous quorum-

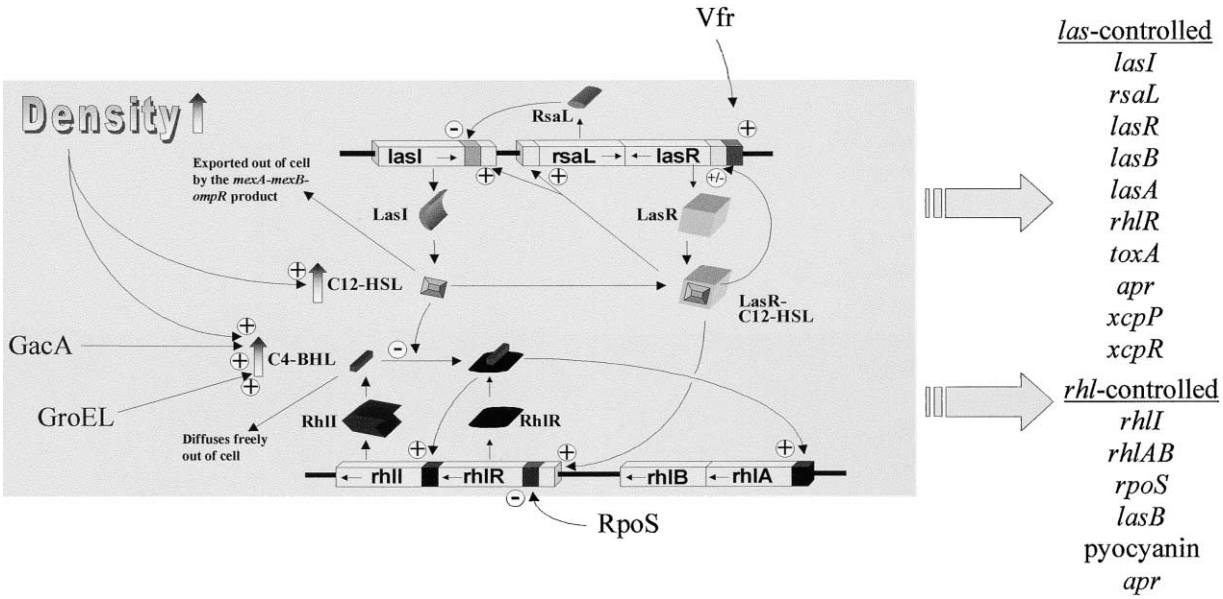


Figure 1. Model of the *las/rhl* Quorum-Sensing System in *P. aeruginosa*
This figure was adapted from de Kievet et al., 1999 (613). See text for gene descriptions.

sensing systems, *las* and *rhl*. These systems are arranged in a hierarchy where *las* is dominant over *rhl* [21]. In the *las* quorum-sensing system, the lasI gene product directs the formation of the extracellular autoinducing signal *N*-(3-oxododecanoyl) homoserine lactone (3-oxo-C_{12}-HSL) (see Figure 1), whose secretion to the extracellular environment is aided by *P. aeruginosa* efflux pumps encoded by the *mexA-mexB-ompR* operon [20]. As 3-oxo-C_{12}-HSL concentrates in the extracellular environment, it is taken up by *P. aeruginosa* cells and interacts with the LasR transcriptional activator. This LasR-3-oxo-C_{12}-HSL complex is then able to activate the expression of a number of genes, including *lasB* (elastase), *lasA* (a serine protease that nicks elastin and works synergistically with elastase), *apr* (alkaline protease), *toxA* (exotoxin A), both *xcp* operons (*xcpPQ* and *xcpR-Z*, encoding the type II secretion apparatus), *rhlR*, and *lasI* itself [21]. The transcription of *lasR* is induced in response to glucose limitation, and this induction is mediated through the vfr-cAMP complex (see below) [22].

The second *P. aeruginosa* quorum-sensing system consists of the regulatory protein RhlR and the diffusible autoinducer *N*-butyryl homoserine lactone (C_4-HSL) (see Figure 1) synthesized by the product of *rhlI*. In contrast to 3-oxo-C_{12}-HSL, C_4-HSL is freely permeable. Apparently the length and/or degree of substitution of the *N*-acyl side chain determines whether an autoinducer is freely diffusible or is subject to active efflux by *P. aeruginosa*. Like the homologous *las* system, once the diffusible autoinducer C_4-HSL attains adequate levels, it binds and activates the RhlR transcriptional regulator. RhlR-C_4-HSL has been shown to regulate the rhamnolipid biosynthesis operon *rhlAB*, alkaline protease, pyocyanin, PA-IL and PA-IIL lectins, the *lasB*-encoded elastase, and *rhlI* itself [21]. The hierarchy of the *las/rhl* system is aided by the inhibitory action of the unbound *las* autoinducer 3-oxo-C_{12}-HSL on the binding of C_4-HSL to the RhlR transcriptional activator. The upregulation of *lasR* transcription and the resulting elevated concentrations of the LasR-3-oxo-C_{12}-HSL complex allow the *rhl* system to be subsequently activated. The *rhl* quorum-sensing system has also been recently shown to be inhibited by the alternative sigma factor RpoS [23] and activated by the *gac* two-component regulatory system that responds to growth phase (see Figure 3) [24]. In addition, the formation of the RhlI-C_4-HSL complex is aided by the formation of the LasR-3-oxo-C_{12}-HSL complex [25].

It has also been theorized that at low cell density, RsaL inhibits transcription of *lasI* by binding to the *lasI* operator region, thereby blocking activation by LasR-3-oxo-C_{12}-HSL [26]. As the cell density increases, so does the intracellular concentration of 3-oxo-C_{12}-HSL, which enables sufficient LasR-3-oxo-C_{12}-HSL formation to competitively inhibit RsaL for binding to the *lasI* operator. Thus, it appears that during the early stages of growth, RsaL blocks the quorum-sensing cascade by inhibiting the transcription of *lasI*. Finally, it was found that this organism produces another intercellular signal termed the *Pseudomonas* quinolone signal (PQS) that was identified as a 2-heptyl-3-hydroxy-4-quinolone [27]. PQS is produced maximally at late stationary phase and works by activating transcription of the *rhlI* gene (and to a lesser degree *lasR* and *rhlR*). It is not known what activates the production of PQS, but this molecule is probably not involved in sensing cell density.

While the 3-oxo-C_{12}-HSL and the C_4-HSL of the *las* and *rhl* systems, respectively, have been well studied, up to five additional HSLs have been identified in *P. aeruginosa* and include *N*-hexanoyl-L-homoserine lactone (C_6-HSL), *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C_6-HSL), *N*-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C_8-HSL), *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C_{10}-HSL), and *N*-(3-oxotetradecanoyl)-L-homoserine lactone (3-oxo-C_{14}-HSL) (refer to Figure).

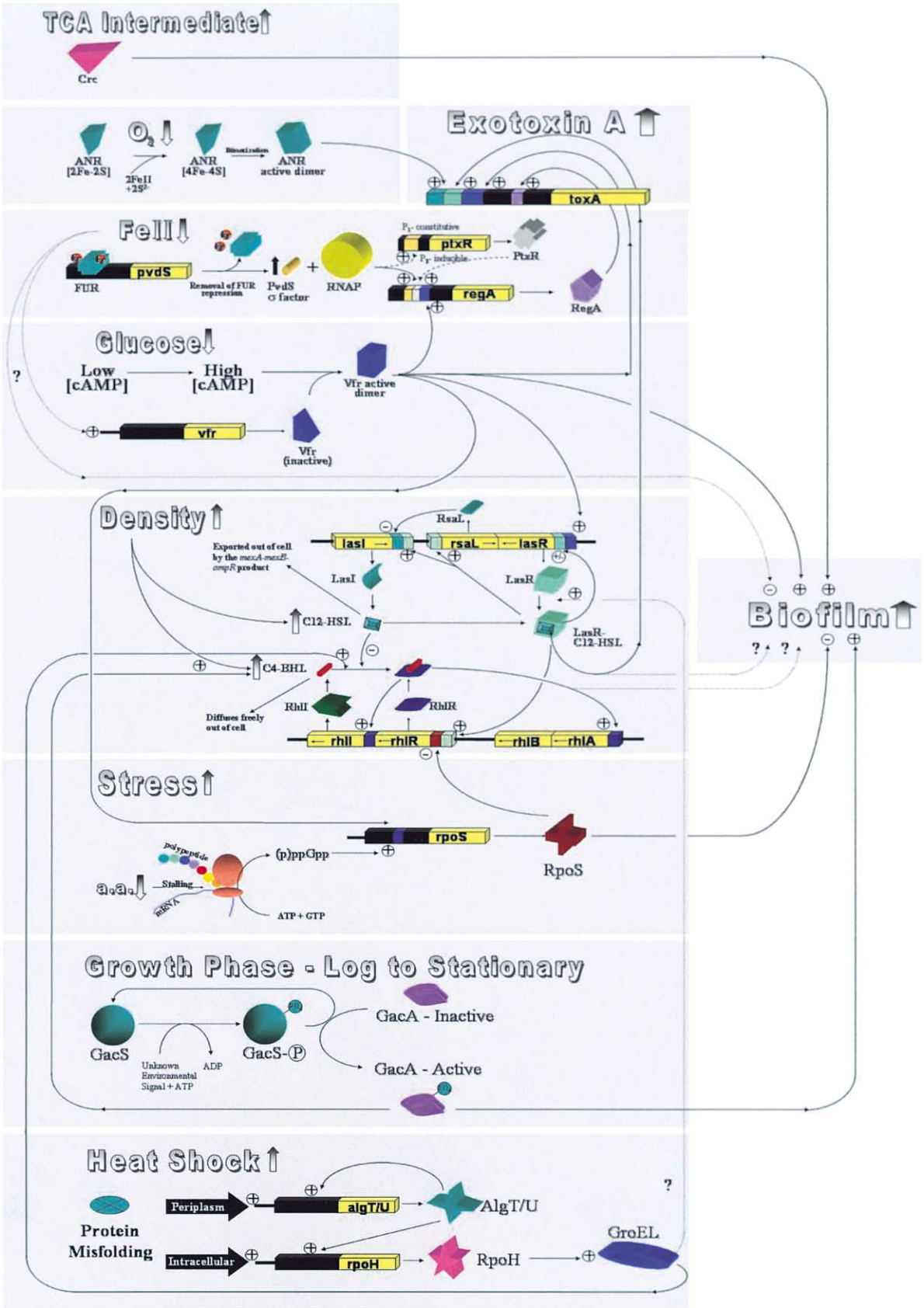


Figure 2. Interaction of the *P. aeruginosa* Quorum-Sensing System and Environmental Stimuli in the Regulation of *toxA* (Exotoxin A) Transcription and Biofilm Formation

See text for gene descriptions.

In a normal, differentiated *P. aeruginosa* biofilm, cells are embedded within a thick polymeric matrix in which an intricate channel network provides lower levels of the structure with access to environmental nutrients. A recent study has suggested that the development and maintenance of this fully mature biofilm phenotype may be under the direction of the *las/rhl* quorum-sensing systems [28]. In this study, a *lasI* mutant that was deficient in the production of the autoinducer 3-oxo-C_{12}-HSL formed biofilm cell clusters that were 20% of the wild-type biofilm thickness and were sensitive to detergent removal. These mutant biofilms were composed of single continuous sheets without the highly developed and differentiated structure of the wild-type strain. When the 3-oxo-C_{12}-HSL was added to the system, the *lasI* mutant was once again able to form normal biofilms. Other researchers have found that inhibiting the quorum-sensing system with a synthetic halogenated furanone compound (derived from the Australian macroalga *Delisea pulchra*) enhanced bacterial detachment [29]. However, recent studies have challenged these findings. Stoodley et al. were unable to find significant structural differences when the biofilms of *lasR lasI* mutants and the wild-type strain were compared, even though biofilms were grown under similar laminar and turbulent flow conditions with minimal glucose medium [30]. These confounding results were also found in other recent studies of *P. aeruginosa* biofilms grown under flow and static conditions [31, 32]. Therefore, while the quorum-sensing HSLs are produced by *P. aeruginosa* biofilms in both the clinical setting [33, 34] and in the environment [35] and seem important for virulence, their exact role in biofilm formation is still a source of debate.

As mentioned previously, biofilms can spread through the detachment of large conglomerates of cells that diffuse away from the parental biofilm and reattach to virgin areas or through the individual escape of planktonic, motile cells. The control of biofilm detachment by the pseudomonal quorum-sensing system was evaluated in a set of preliminary experiments in which the number of cells, dispersal mode, growth rate, and the effect of shear were compared in the biofilms of wild-type *P. aeruginosa* (PAO1) and mutants defective in the production of either C_4-HSL or 3-oxo-C_{12}-HSL [36]. Researchers found that mutants defective in the production of the longer chain signaling molecule produced a biofilm that dispersed through erosion and was relatively thin even though this biofilm had bacterial concentrations equal to wild-type biofilms. Since the wild type and mutant had equal numbers of cells per unit of biofilm volume, the difference in biofilm thickness may be due to a number of factors, including the alignment of the individual alginate polymers, the levels of biofilm hydration, or the amount of exopolysaccharide produced per cell. However, the researchers did not further elucidate this scientific observation. When the properties of the C_4-HSL mutant were evaluated, they found that the biofilms were much thicker, contained 10-fold more cells, detached as large sloughs, and were very sensitive to shear. Therefore, this shorter chain HSL may be responsible for either limiting the size of the biofilm or upregulating detachment.

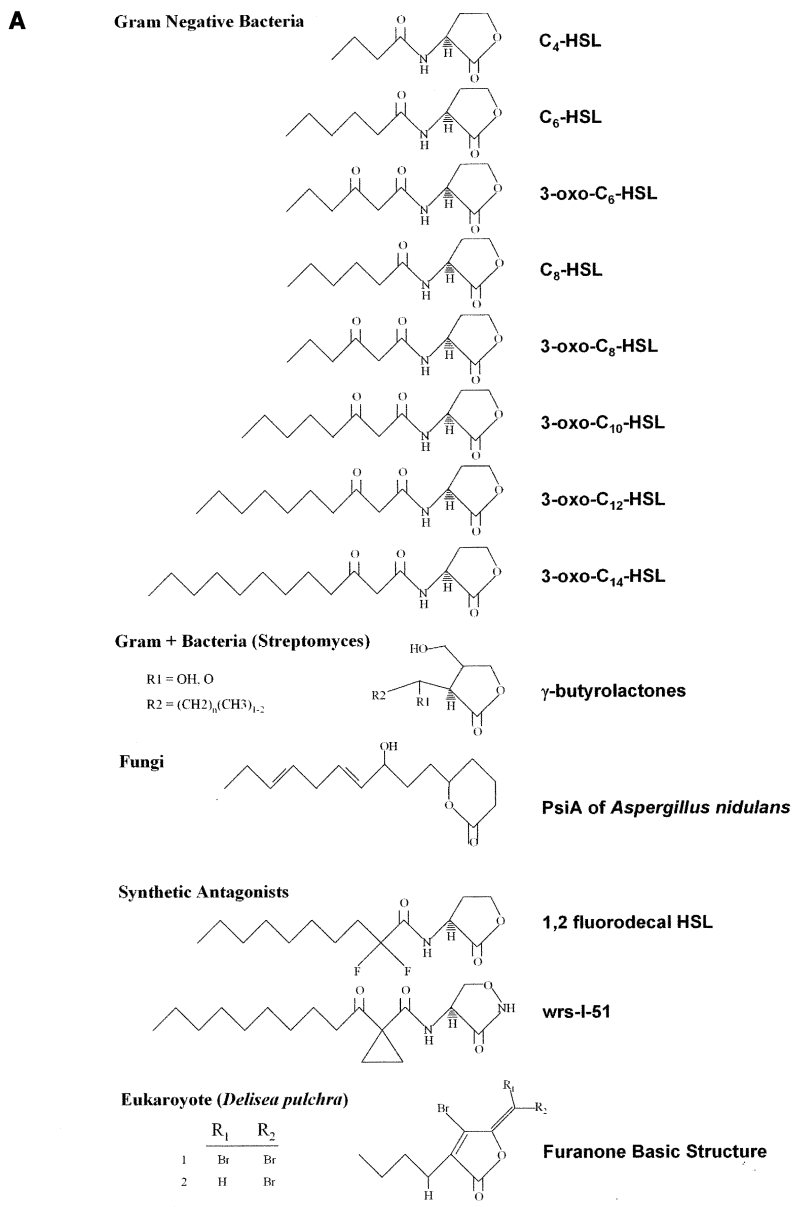
Environmental Cues and *P. aeruginosa* Biofilms

While the quorum-sensing system is an extremely important determinant of virulence factor expression in *P. aeruginosa*, this bacterial species must also be able to adaptively respond to environmental stimuli, and this is accomplished by the complex interaction between a number of regulatory cascades. For example, the production of both biofilms and Exotoxin A, the diptheria-like toxin responsible for protein synthesis disruption in eukaryotic cells, are regulated by a number of environmental and quorum-sensing signals (see Figure 2). Some of the environmental signals that *P. aeruginosa* responds to include the presence of glucose, iron and nitrogen availability, oxygen levels, temperature variations, pH, osmolarity, amino acid starvation, and ultraviolet damage.

P. aeruginosa response to glucose limitation is mediated through the virulence factor regulator (Vfr) that demonstrates significant homology to the cAMP receptor protein (CRP) of *E. coli* [22]. When glucose is in short supply, the intracellular concentration of cAMP is upregulated in most microbes. In *P. aeruginosa*, two cAMP molecules bind the inactive Vfr dimer. This cAMP-Vfr complex is then able to bind a consensus dyad symmetrical sequence in the promoter region of a number of operons, including the quorum-sensing regulator (*lasR)* and genes required for the utilization of various carbon sources, through a helix-turn-helix binding motif [22]. Upon binding, the complex promotes the localization of the RNA polymerase holoenzyme (RNAP) to the promoter region through interaction between the β subunit of the RNAP and the cAMP-Vfr complex. This complex is able to increase the expression of type IV pili, resulting in increased twitching motility and biofilm formation [37, 38]. In addition, this complex is able to activate the transcription of *lasR*, thereby activating the quorum-sensing cascade when the autoinducer 3-oxo-C_{12}-HSL is present at significant levels, which may indirectly affect biofilm development.

Another carbon metabolite regulator, termed the catabolite repression control (Crc) protein, has been recently found. This protein is able to sense carbon source availability and repress carbohydrate catabolism enzymes, possibly through BkdR, the transcriptional activator of the inducible multienzyme complex branched-chain keto acid dehydrogenase [39]. This protein also affects expression of the type IV pili structural subunit PilA to promote microcolony formation on biofilms. As demonstrated in mutation studies, *P. aeruginosa* Crc mutants are only capable of forming thin biofilm monolayers instead of conglomerating into a number of microcolonies through type IV-dependent twitching motility [40]. Therefore, Crc may represent a link between carbon availability and the decision of whether or not to enter into a biofilm mode of growth.

Since *P. aeruginosa* prefers aerobic metabolism that utilizes a number of iron-containing enzymes, this microbial species has evolved a number of strategies to obtain iron from its environment [41]. In order to conserve energy and resources, *P. aeruginosa* tightly regulates the expression of its iron acquisition systems to limit their activity in iron-rich environments. This iron-dependent regulation centers upon the activity of a recently isolated



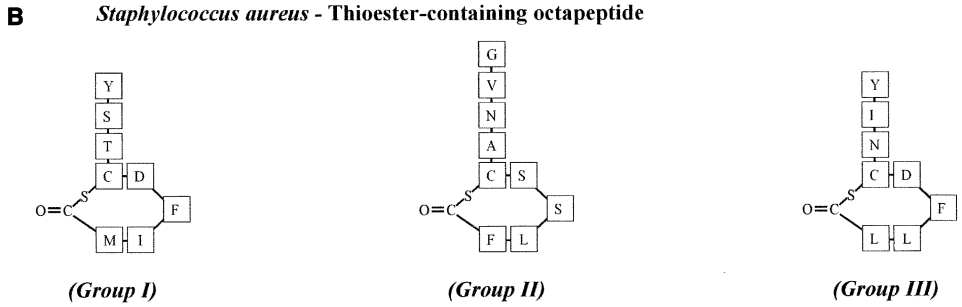


Figure 3. ■■■

Some common acylated homoserine lactones and derivatives (A) and thioester-containing octapeptides (B) for *S. aureus* groups I–III. See text for gene descriptions.

homolog of the ferric uptake regulator (Fur) in *E. coli* [41, 42]. When iron is in ample supply, Fur binds Fe^{2+} and is able to attach to a palindromic consensus sequence (termed the "Fur box") in the promoter regions of iron-regulated genes, thereby repressing their expression. When *P. aeruginosa* is grown in iron-limiting conditions, Fe^{2+} dissociates from the complex, causing Fur release and repression removal. Some of the genes controlled either directly or indirectly by the *fur* system include those that code for other regulatory proteins (e.g., the sigma factor PvdS); iron-scavenging proteins (e.g., pyochelin and pyoverdin siderophores); proteases that degrade the iron binding host proteins; the cytotoxin exotoxin A, which enables iron release from susceptible host cells; proteins involved in basic metabolic processes (e.g., Krebs cycle); and proteins responsible for oxidative stress survival (e.g., superoxide dismutase) [43-45]. While alterations in iron concentrations have been shown to affect the quorum-sensing system, this interaction may be mediated indirectly through the *vfr* regulation system as seen in *V. fischeri* [46]. The effect of iron limitation on biofilm growth was recently studied by Singh et al. [47]. It was found that iron chelation resulted in the formation of thin monolayer biofilms that did not display the intricate three-dimensional biofilm architecture seen in other studies. The authors concluded that twitching motility was induced by iron limitation, thereby preventing microcolony formation. This conclusion seems to contradict previous findings by O'Toole and Kolter that twitching motility was required for microcolony and biofilm development [13]. However, the disparity between these two studies can be resolved when one takes into account that the O'Toole and Kolter study was performed on static biofilms for a short duration (8 hr), while the Singh et al. study was performed over 13 days in a dynamic laminar flow system. Therefore, twitching motility may be only required in young biofilms and may be used as an effective spreading mechanism in order to allow *P. aeruginosa* to colonize the entire substrate [31].

In reference to respiration, *P. aeruginosa* can utilize inorganic electron acceptors (other than oxygen) for growth. However, this species is incapable of fermentative metabolism and generally grows more fastidiously in oxygenated environments since it prefers aerobic metabolism. Therefore, it is not surprising that this organism alters its gene expression in response to oxygen levels. This control is mediated through the oxygen-sensing transcriptional regulator protein ANR (anaerobic nitrate respiration) that is homologous to the FNR in *E. coli* [48]. This protein forms a $[4Fe\text{-}4S]^{2+}$ cluster under conditions of low O_2. This cluster formation has been shown to promote dimerization and binding to promoter regions of genes in whose functions facilitate adaptation to growth under anaerobic conditions (e.g., denitrification enzymes and/or their regulators) [49]. There is no data to support the direct role of ANR on the regulation of the quorum-sensing system. However, O_2 levels must be taken into account when evaluating biofilm experimental data, since ANR has been shown to activate the transcription of a large number of enzymes associated with anaerobic metabolism while repressing the expression of those enzymes responsible for aerobic metabolism. By taking into account the effect of oxygen-dependent regulation, one may avoid the incorrect assumption of causal relationships between gene expression and biofilm formation.

This organism has been shown to adapt to amino acid starvation through a complex series of regulatory events, termed the stringent response, which has been described as a global regulation mechanism. Briefly, this response (well elucidated in *E. coli*) is mediated through the accumulation of uncharged cognate tRNA. When the ratio of aminoacyl-charged to -uncharged tRNA falls below a critical threshold, occupation of the vacant mRNA codon at the ribosomal A site by uncharged cognate tRNA leads to stalling of peptide chain elongation. Also, the synthesis of the pseudomonal nucleotide (p)ppGpp from GTP and ATP is induced in a ribosomal-dependent idling reaction [50, 51]. It has been demonstrated in *E. coli* that the (p)ppGpp inhibits RNA polymerase, causing the downregulation of a wide range of energetically demanding cellular processes (e.g., the synthesis of stable RNA), stimulation of certain amino acid synthesis pathways (e.g., isoleucine), and the induction of stationary phase-specific genes through the effects of the stationary-phase/stress-specific sigma factor (a.k.a. σ^s or RpoS) [50, 52]. While the σ^{70} (a.k.a. σ^D) factor is responsible for the transcription of constitutive-expressed and housekeeping genes, RpoS has been implicated in the transcription regulation of over 50 genes in *E. coli* in response to not only amino acid starvation, but also osmotic stress, acid shock, heat shock, oxidative DNA damage, and transition to stationary phase. Transcriptional regulation of *rpoS* expression has been demonstrated to be under positive control by (p)ppGpp and negatively by the cAMP receptor protein. Also, translational control has been ascribed to a number of other factors including an RNA binding protein (Hfq), a nucleoid histone-like protein (H-NS), and a small regulatory RNA (*dsrA* RNA) that destabilizes the secondary structure in *rpoS* mRNA to allow translational initiation. However, proteolysis of RpoS by the ClpPX protease (due to the removal of protection of RpoS by the chaperone protein DnaK) seems to be the main regulation mechanism in *E. coli* [53]. The role of RpoS in *P. aeruginosa* biofilm formation was recently evaluated using an *rpoS* mutant. The biofilm architecture developed by this mutant was significantly thicker than the structures of the corresponding wild type. Therefore, RpoS may act to limit the size of the biofilm under stationary conditions of growth (such as those seen in deeper biofilm layers) in order to maintain access to the limited nutrients.

P. aeruginosa also mediates changes in gene expression through a complex array of other alternative RNA polymerase sigma factors in response to a number of environmental stressors. Heat shock is one example of an environmental stress, and the pseudomonal response is mediated through the combined effect of the extracytoplasmic stress factor, σ^E (a.k.a. AlgT or AlgU) and σ^H (a.k.a. RpoH or σ^{32}) [54]. AlgT/U and RpoH respond to the accumulation of misfolded proteins in the periplasmic and cytosolic bacterial compartments, respectively. Specifically, AlgT/U is able to upregulate its own expression as well as the expression of genes cod-

ing for RpoH and the enzymes of the alginate biosynthetic pathway [54]. The antisigma factor products of *mucA* and *mucB* normally inhibit the activity of AlgT/U [55, 56]. However, in patients suffering from cystic fibrosis, these antisigma factor coding regions are often mutated resulting in the conversion of nonmucoid strains into the mucoid variety by allowing for the constitutive overproduction of alginate [56, 57]. This excess of alginate production in *P. aeruginosa* results in the formation of biofilm microcolonies consisting of exopolysaccharide-embedded cells. These biofilm microcolonies demonstrate high-level resistance to host or antimicrobial clearance strategies. The enzymes of the alginate biosynthetic pathway are also under the negative regulation of a sigma factor that responds to nitrogen depletion (σ^{54} or RpoN) [58, 59]. In reference to RpoH, many of its regulatory effects can be linked to its activation of GroESL proteins [25]. These proteins act as chaperonins to sequentially promote correct folding of a number of proteins and aid in the formation of protein complexes, possibly including the LasR quorum-sensing regulator with the LasI product 3-oxo-C_{12}-HSL and the formation of the RhlR-C_4-HSL complex [21, 25]. Another environmental stressor that regulates gene expression through an alternative sigma factor includes the need for flagellin (mediated by σ^F, a.k.a. σ^{28} or RpoF coded by *fliA*) [60]. All of these sigma factors discussed above have been well studied in *E. coli*, but their role in pseudomonal stress response or biofilm formation remains to be fully elucidated.

GacA and GacS are highly conserved among *Pseudomonas* spp. and demonstrate upregulation of expression upon entry into stationary phase due to an unknown signal [24]. *gacS* encodes the cognate sensor kinase that activates the response regulator coded for by *gacA* by phosphorylation [61]. This GacS/GacA system strictly controls the expression of extracellular products (antibiotics, exoenzymes, and hydrogen cyanide) when cells are in the transition from exponential to stationary phase. This system has also been found to increase the production of the C_4-HSL autoinducer of the pseudomonal *rhl* quorum-sensing system [24]. It was hypothesized that activated GacA, by virtue of its typical C-terminal helix-turn-helix DNA binding motif, regulated the transcription of target genes. However, recent evidence points to posttranscriptional control by interacting with the mRNA ribosomal binding site of GacA-controlled genes [62]. The importance of this regulatory system in the development of biofilms can be demonstrated by a study in which a *gacA* mutant of *P. aeruginosa* had a significantly reduced thickness (one-tenth the size of wild-type biofilms) and reduced virulence [63, 64].

Staphylococcus aureus Biofilms

S. *aureus* is a gram-positive, ubiquitous bacterial species, with the predominant reservoir in nature being humans. The carriage rate of this organism in humans is reported to be between 11% and 32% in healthy adults [65, 66]. In the preantibiotic era, bacteremia with *S. aureus* resulted in a 90% death rate [67]. Due to the increasing involvement of *S. aureus* in foreign body-related infections, the rapid development and exhibition of multiple antibiotic resistance by these organisms, and their great propensity to change from an acute infection to one that is persistent, chronic, and recurrent, this pathogen is once again receiving significant attention. *S. aureus* causes a wide variety of infections, each associated with significant morbidity and mortality. Some of the diseases mediated by this species of bacteria include tropical pyomyositis, lower respiratory infections (pneumonia), superficial skin infections (boils, sties, carbuncles), localized abscesses, endocarditis, osteomyelitis, toxic shock syndrome, serious skin infections (furunculosis), food poisoning, bacteremia, empyema, pyopneumothorax, and exfoliative diseases. It is believed that one of the mechanisms by which *S. aureus* is able to persist is through the formation of a well-developed biofilm.

Staphylococcus spp. can produce a multilayered biofilm embedded into a glycocalyx [68]. The glycocalyx develops on devitalized tissue and bone (such as the involucrum) or medically implanted devices to produce an infection [69]. In addition, dental water supplies and saliva ejectors may also be areas for cross contamination between patients [70]. The presence of implants are a predisposing factor in the development of infection since they are coated in host proteins soon after implantation, and this host protein coating provides an excellent source of attachment for any bacteria remaining after debridement surgery [71]. Once attached, the bacteria can form the glycocalyx, or slime layer, which protects the bacteria from normal host defenses and systemic antibiotics [72].

Early studies reported the solid component of the glycocalyx to be mainly composed of teichoic acids (80%) and staphylococcal and host proteins [73]. Host derived proteins such as fibrin may have been derived from the conversion of fibrinogen by the staphylococcal coagulase-prothrombin complex [74]. Later studies were able to isolate a specific polysaccharide antigen named polysaccharide intercellular antigen (PIA) that is composed of β-1,6-linked *N*-acetylglucosamine residues (80%–85%) and an anionic fraction with a lower content of non-*N*-acetylated D-glucosaminyl residues that contains phosphate and ester-linked succinate (15%–20%) [75]. PIA is a polymer of approximately 130 residues and other sizes of these β-1,6-linked *N*-acetylglucosamine have been identified, termed PNAG-I (the immunogenic 460 kDa compound), -II (100 kDa), and -III (21 kDa) [76]. PIA is synthesized in vitro from UDP-*N*-acetylglucosamine by products of the intercellular adhesion (*ica*) locus [77]. The genes and products of the *ica* locus (*icaR* [regulatory] and *icaADBC* [biosynthetic] genes) have been shown to be required for biofilm formation and virulence and are upregulated in response to anaerobic growth like the conditions seen in the biofilm environment [78]. While the regulation mechanism of this locus has not yet been discerned in *S. aureus*, the homologous *Staphylococcus epidermidis* locus is regulated with the reversible inactivation by insertion sequence (IS*256*) phase variation in 25%–33% variants [79]. Other levels of control in *S. epidermidis* are accomplished through IcaR-mediated transcriptional repression (relieved by ethanol stress) and the *sigB* operon

product σ^B (regulated by operon genes *rsbU* and *rsbV*) [79, 80]. Since a number of insertion sequences (e.g., IS*1181* and IS*431*), *icaR* homologs, and the *sigB* operon have been found in most *S. aureus* strains (e.g., Mu50, N315, MW2, and Newman), these regulatory mechanisms may also hold true.

In addition to PIA, a number of other studies have identified important genes and their products in the formation of staphylococcal biofilms. There is recent evidence that attachment of bacterial cells to a polymer surface, thereby representing the prerequisite for biofilm formation, may be aided by an autolysin of *S. epidermidis* [81]. Therefore, the homolog in *S. aureus* (*atl*) may also function in this manner. A two-component regulatory gene locus that mediates adhesion and affects biofilm formation in *S. aureus* has also been recently studied. This locus is a system encoded by *arlRS*, a member of the OmpR-PhoB family of response regulators, that is regulated by the *agr* and *sarA* loci [82, 83]. When upregulated, the product of *arlS* gene inhibits biofilm formation and may control attachment to polymer surfaces by affecting the secreted peptidoglycan hydrolase activity. Teichoic acid structure is also extremely important in the development of biofilms. Specifically, the addition of D-alanine esters to teichoic acids via *dltA* may be an important factor in imparting the correct charge balance on the gram-positive cell surface to enable initial attachment and subsequent biofilm formation. Transposon mutagenesis was able to identify another *S. aureus* gene biofilm-associated protein (Bap) that was required for biofilm formation on inert surface. However, since only 5% of bovine mastitis isolates and none of the 75 clinical isolates tested possessed the coding sequence for Bap, the in vivo importance of this protein may be questionable. In another study, the differential gene expression between planktonic (shaken) and biofilm (not shaken) cultures in *S. aureus* was evaluated and five biofilm-upregulated genes were identified [84]. These included three oxygen starvation response genes, the gene encoding threonyl-tRNA synthetase (upregulated by amino acid starvation), and a stress response gene that encodes ClpC ATPase. As mentioned above, biofilm formation has been shown to be upregulated by anaerobic, osmotic, and ethanol stress due to the stress-induced alternative sigma factor σ^B [85].

The Genetics of Signaling in *S. aureus*

The effect of the quorum-sensing system in *S. aureus* on biofilm formation has been recently studied in a preliminary sense. Although *S. aureus* has a quorum-sensing system that satisfies the same principal of autoinduction in response to a secreted compound whose concentration is based on cell density, the system does demonstrate some reasonable differences. While gram-negative bacteria passively and actively secrete HSL derivatives that are synthesized in their active form, *S. aureus* utilizes peptide signaling molecules that are originally translated in an inactive form and then secreted. During this secretion process, it has been hypothesized that the polypeptide is converted into a thiolactone-containing peptide that is then able to accumulate in the local environment and act as an external signaling molecule [86]. This signaling peptide interacts with a two component regulatory system in which a cell membrane receptor, usually a histidine kinase, transmits the signal to an intracellular activator, which activates the quorum-sensing-dependent phenotype [87, 88].

S. aureus produce a large number of extracellular and cell-associated products that may contribute to virulence and development of persistent infections. During early exponential growth when cell density is low, proteins that promote adherence and colonization are expressed. When cell growth reaches high densities, the production of the adherence and colonization factors is suppressed, while secreted toxins and enzymes are expressed. Many of these postexponential phase proteins are involved in damaging the host, obtaining nutrients from the host for pathogen growth, and dissemination after the staphylococci have adequately colonized and increased in number to promote an active infection.

The expression of most of these staphylococcal products is under partial or complete control of the staphylococcal accessory regulator (*sar*) and the accessory gene regulator (*agr*) system. During early logarithmic growth, a protein encoded by *rot* (repressor of toxins) inhibits the expression of agr-activated virulence factors [89]. Once activation of the *agr* and *sar* regulatory loci occurs during late exponential phase, there is an increased transcription of a regulatory RNA molecule known as RNAIII (which is a product of *agr*) [90]. RNAIII immediately blocks transcription of surface protein genes and, with a hypothesized timing signal, upregulates transcription of extracellular pathogenicity factors (such as exotoxins). The primary regulatory function of RNAIII is at the level of transcription by an undetermined mechanism but may involve one or more regulatory proteins [87]. This regulatory RNA molecule is also capable of controlling production of at least two virulence factors, α-hemolysin (*hla*) and Protein A (*spa*), at the level of translation. At the beginning of exponential-phase growth, the expression of α-hemolysin is normally inhibited through intramolecular base pairing that blocks the ribosomal binding site [87, 91]. Later in exponential-phase growth, RNAIII is expressed and folds into a stable but inactive regulatory molecule. After a significant lag, the secondary structure of RNAIII changes through an unknown agent, and the 5′ region of RNAIII is then able to hybridize with a complementary 5′ untranslated region of α-hemolysin mRNA, thereby making the transcripts accessible for translation initiation [91]. Conversely, the 3′ region of RNAIII contains sequences complementary to the leader sequence of *spa*, and hybridization is believed to inhibit translation of Protein A.

The activity of RNAIII is regulated through a population-sensing autocrine system (quorum sensing) encoded by the accessory gene regulator locus (*agr*) [92-94]. This locus consists of two divergent transcription units, driven by promoters P2 and P3. The P2 operon contains four genes, *agrB*, *agrD*, *agrC*, and *agrA*, and the P3 operon codes for RNAIII (see above) [92]. An octapeptide with a unique thioester ring structure (referred to as the *agr* auto inducing peptide [AIP]) is generated from its precursor, AgrD, and secreted out of the cell through the action of the AgrB membrane protein [86]. As the concentration of AIP increases in the extra-

cellular microenvironment, the interaction between AIP and the histidine kinase receptor protein, AgrC, also increases. This interaction enables AgrC to phosphorylate and thereby activate an intracellular *agr*-encoded protein (AgrA) [87, 88]. AgrA$\sim$P then increases transcription at the P2 promoter. With SarA (the major transcript of the *sar* operon), AgrA$\sim$P also increases the transcription at the P3 promoter, resulting in elevated intracellular levels of RNAIII [95]. Therefore, as AIP concentrates in the extracellular environment, the level of RNAIII increases, enabling the growth phase-dependent reduction in adherence factor production and increase in extracellular pathogenicity factor production. AIP is not only capable of activating the *agr* regulon in self strains, but can also inhibit the *agr* activation of other *S. aureus* strains.

One study evaluated the ability of *S. aureus* to adhere to polystyrene in 105 isolates of *agr*-positive and negative isolates [96]. Researchers found that 78% of the *agr*-negative mutants were able to attach and form biofilms while only 6% of the *agr*-positive isolates were able demonstrate this growth phenotype. Therefore, the quorum-sensing system in *S. aureus* seemed to have inhibited the formation of biofilms. This inhibition was confirmed by using quorum-sensing blockers and was shown to be independent of changes in autolysin (Atl) and PIA. While this group experimentally demonstrated that the reduction in biofilm formation can be mediated by the well-known surfactant abilities of delta hemolysin [97], more study is needed. It is interesting to note that there is a high frequency of naturally occurring agr mutants (26%). Therefore, a defective quorum-sensing system may enable the microbe to have a low pathogenic and immunogenic activity, but simultaneously enable biofilm formation and persistence.

In addition to the *agr* system, virulence factor expression is also mediated by the sar loci in which SarA (the primary product of sar that is acted upon by σ^B) been shown to directly interact with the promoter regions of a number of genes, including the coding regions for the P2 and P3 promoter of *agr*, collagen receptor, Protein A, fibronectin binding protein, staphylococcal enterotoxin A, α-hemolysin, and the sar loci itself [98, 99]. However, the biofilm-specific interactions of this complex system are still being determined.

Future Research

The research linking biofilm formation with environmental signal response systems and quorum-sensing systems in bacteria is still in its beginning stages. Therefore, a greater understanding of the specific genes and products whose expression and production demonstrate altered regulation in a single species (and multispecies) biofilm system is needed. Also, the relative gene expression, the regulators, and the target genes of these systems and their role during initial attachment, development, and maturation of the biofilm phenotype must also be determined. Studies utilizing microarray technology, 2-D gel electrophoresis, specific reporters, and knockouts may eventually allow researchers to assemble a biofilm development model. However, these studies must be performed in growth conditions that take into account the altered gene expression and production that necessarily results from bacterial cell-cell communication and environmental stimuli in order to draw correct conclusions and discern causal relationships.

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