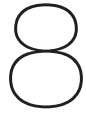

Peptide Signaling



Jeremy M. Yarwood

Abstract

The study of peptide signaling is yielding both fascinating and valuable information regarding bacterial biofilm development and helping to elucidate the disease processes caused by several human pathogens. Peptide signaling potentially impacts all stages of the biofilm “life-cycle” for many bacterial species, from attachment to maturation and detachment. While the particular roles of the signaling systems in biofilm formation varies among species, the implications of several phenomena, from natural transformation of streptococci to quorum sensing variant generation in staphylococci, may only be fully appreciated in the context of biofilms and cell-to-cell signaling. Understanding the mechanisms by which the peptide signaling systems exert their effects on biofilms may yield therapeutic strategies with limited, but important, uses.

Introduction

The role of peptide signaling (also known as quorum sensing) in bacterial biofilm development continues to attract significant attention, in no small part due to the potential of disrupting biofilm formation, preventing expression of pathogenic factors, and understanding certain disease processes. However, as quickly becomes evident through review of relevant literature, the contribution of peptide signaling to pathogenesis and biofilm behavior can vary significantly not only species to species but even within species. The pleotropic regulatory effects exerted by these signaling systems, their interaction with other regulatory elements, and the effects of varied growth conditions can make for somewhat conflicting results from study to study. Still, important insights are being made into the physiological and signaling processes involved in biofilm development, including both the commonalities and differences between species.

While bacteria have historically been studied in planktonic cell cultures, it is clear that biofilm-associated organisms are different by just about any measure. They exhibit markedly different gene and protein expression profiles as compared to planktonic cells and are remarkably resistant to treatment with antimicrobials. Furthermore, the frequently heterogeneous nature of the biofilm environment (gradients in carbon sources, oxygen, metabolic by-products, etc.) makes for a correspondingly heterogeneous population of cells unlike those in fairly uniform planktonic cultures. Yet, elements of the traditional batch culture appear to translate meaningfully to various stages of biofilm formation, perhaps best

illustrated by the staphylococci (Figure 8.1). Expression of staphylococcal cell surface-associated adhesins occurs during early growth at low cell densities in planktonic cell culture when peptide signal concentrations are also low. These adhesins, such as the fibrinogen-binding and fibronectin-binding proteins, can mediate attachment to biological surfaces through ligand–receptor interactions. Large cell-surface proteins, including the adhesins, can also enhance attachment to abiotic substrates through non-specific physiochemical means, such as hydrophobic interactions. Correspondingly, these colonizing factors would be important *in vivo* when initial cell densities are relatively low and nutrient supply non-limiting. In contrast, once staphylococcal planktonic cell cultures reach high densities and nutrient supply is limited, signal concentrations are high and the cultures tend to produce extracellular enzymes (e.g. proteases, lipases and hemolysins), immunostimulatory factors (e.g. superantigens) and even some molecules with surfactant properties (e.g. staphylococcal δ -toxin). *In vivo*, such cell density and limited nutrient supply could be found in biofilms or abscesses, and these extracellular factors may contribute to cell detachment and dissemination though cleavage of cell-to-cell or cell-to-host bonds and degradation of surrounding host tissue. The increased vascular permeation that accompanies expression of extracellular virulence factors may also enhance the spread of organisms from one site to another. Once

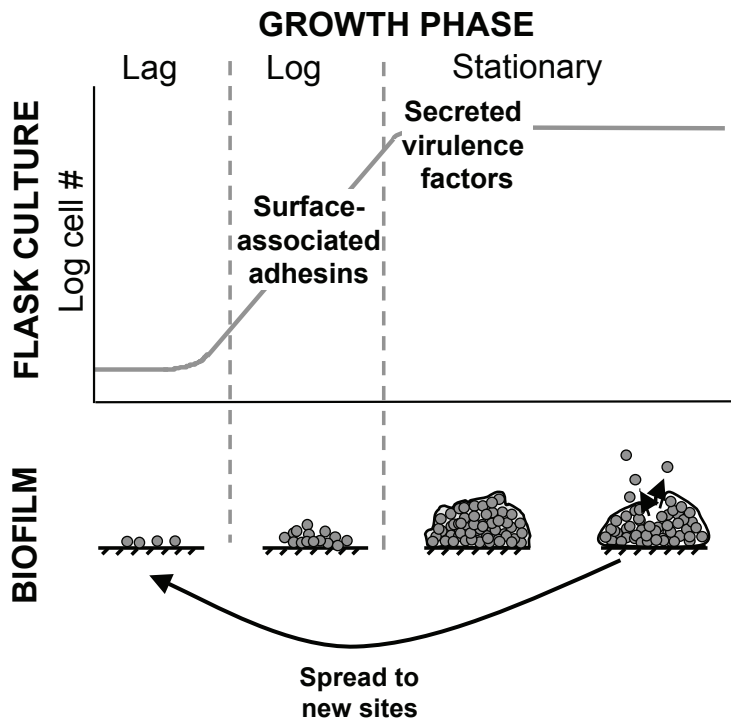


Figure 8.1 Model of staphylococcal virulence gene expression *in vitro* and *in vivo*. Expression of cell surface-associated adhesins enhances colonization and proliferation of staphylococci at the site of infection. Growth of the staphylococcal colony leads to a mature biofilm. Expression of extracellular enzymes, toxins and surfactants facilitates escape of staphylococci from the localized infection and subsequent colonization of secondary sites or hosts.

released from the colony, cells experience low signal concentrations and revert to expression of surface adhesins that mediate attachment, enabling colonization of secondary sites. This model can only be presented in a simplistic fashion in Figure 8.1 as the specific bacterial agents involved in this process, the timing of their induction, and their activity will vary from species to species, and even within species, based on the disease process. Yet, as this chapter will show, evidence of interplay between peptide signaling, cell density, and bacterial products involved in colonization and dissemination is widespread enough among certain bacteria to deserve further investigation into the regulatory systems that control the biofilm “life-cycle” of cell attachment, biofilm growth and maturation, cell detachment, and finally, cell attachment at secondary sites.

Though peptide-based signaling systems have been identified in some Gram-negative bacteria, little is known regarding their role, if any, in biofilm formation. Thus, this chapter will focus on the biology of peptide signaling and biofilm formation by Gram-positive bacteria. Among these, the signaling systems have been best characterized in three genera, *Staphylococcus*, *Streptococcus*, and *Enterococcus*. Unlike the acyl-homoserine lactone signals found in many Gram-negative bacteria (Atkinson *et al.*, this volume), the peptide signals that mediate quorum sensing in these Gram-positive bacteria cannot freely diffuse through the cell membrane. Instead, these signaling systems are generally characterized by two-component regulatory systems that sense and respond to secreted signals. Usually, distinct genetic loci exist that encode the two-component system, the peptide signal precursor, and proteins likely involved in the processing and/or secretion of the signal.

Peptide signaling and *Staphylococcus* biofilms

Staphylococci are remarkably adept at causing a variety of human and animal diseases. These range from relatively benign skin infections, such as impetigo, to much more serious ones, including toxic shock syndrome. Many staphylococcal infections appear to take the form of biofilms, including endocarditis, osteomyelitis, and even some skin infections. In fact, biofilm formation is considered to be the primary “virulence factor” of certain coagulase-negative staphylococci, including *S. epidermidis*, a common cause of implanted medical device-related infections.

The Agr quorum-sensing system

Since the identification of the accessory gene regulator (Agr) quorum sensing system in *Staphylococcus aureus* and subsequently in other staphylococcal species it has been assigned a central role in the regulation of staphylococcal virulence (Kong *et al.*, 2006; Novick, 2003; Novick, 2006; Yarwood, 2006). As such, it has attracted substantial attention as a potential target for controlling staphylococcal disease. While virulence gene regulation by Agr appears to be considerably more complex *in vivo* than initially understood from *in vitro* studies, expression of Agr, or even lack thereof, remains an important determinant in staphylococcal disease development. *agr* mutants have been shown to be attenuated for virulence in some animal models of infection, including a murine arthritis model, an osteomyelitis model, and a skin abscess model (Novick, 2003). It has also been shown that expression of Agr, and Agr-regulated exotoxins, facilitates escape of *S. aureus* internalized by epithelial cells (Shompole *et al.*, 2003). Now evidence is accumulating that the Agr sys-

tem plays a significant role in biofilm formation, development and behavior, with important implications for human disease.

This chapter will focus primarily on the Agr system of *S. aureus*, in which Agr, and virulence in general, has been best studied, and to a lesser extent, *S. epidermidis*. Agr homologs have been identified in many additional staphylococcal species, but little or no investigation has been conducted into regulation of biofilm formation by Agr in these staphylococci.

The *agr* locus consists of two divergent operons (Figure 8.2) (Novick, 2003). The P2 operon (*agrACDB*) encodes the proteins necessary for signal synthesis, processing, secretion, and recognition, while the transcript of the P3 operon, RNAIII, mediates the regulatory effects of Agr expression. The autoinducing peptide (AIP) signal is formed by cleavage and processing of the AgrD protein. A characteristic thiolactone ring is formed between a generally conserved central cysteine and the peptide's C-terminal carboxyl group, and this cyclical structure is generally required for the activity of AIP. Both the cleavage of AgrD and the secretion of AIP are thought to be mediated by the membrane protein AgrB, though other proteins may be involved as well. In comparison of AIP sequence from multiple *Staphylococcus* species, only the central cysteine and the five-membered thiolactone ring are generally conserved (Novick, 2003; Otto, 2001). The length of the N-terminal

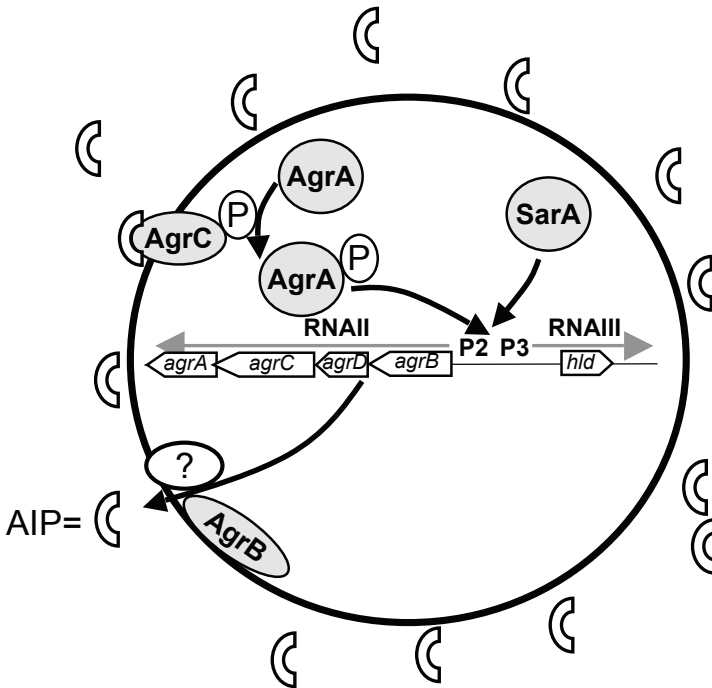


Figure 8.2 Model of the *Staphylococcus aureus* Agr system (see text for additional description). The *agrD* product is processed in part by AgrB into AIP and secreted into the extracellular environment. Recognition of AIP by AgrC triggers transfer of a phosphate group between AgrC and AgrA. AgrA, together with the transcriptional regulator SarA, acts to increase transcription of both the P2 and P3 operons. The transcript of the P3 operon, RNAIII, is the effector molecule of the *agr* locus and encodes δ -toxin (*hld*) as well.

tail varies from two and four amino acids, which results in pheromones with seven to nine residues in all. Lengthening or shortening the tail of an *S. epidermidis* AIP by a single amino acid residue results in a lack of biological activity in this bacterium, suggesting that AIP length is important for biological activity.

Four distinct Agr groups, based on the identity of the AIP produced, have been described in *S. aureus* (Novick, 2003). AIP produced by one group generally inhibits signaling by staphylococci from a different Agr group by competitive binding to the AgrC receptor. In addition, one AIP produced by *S. epidermidis* inhibits signaling by three of the four *S. aureus* Agr groups. Conversely, *S. aureus* Agr group IV is the only one capable of inhibiting *S. epidermidis* signaling.

In planktonic cultures, the amount of AIP in the medium generally increases in correlation with increasing cell density. Upon reaching sufficient AIP concentration (this has been reported to be during the mid-log phase, though the timing may vary from strain to strain) signaling via the AgrA-AgrC system leads to increased transcription of both the P2 and P3 operons. AgrA and AgrC form the response regulator and histidine kinase receptor, respectively, of a two-component regulatory system that responds to the secreted AIP. AgrA is thought to be constitutively phosphorylated, thus its activation may require dephosphorylation. Binding of AgrA to the *agr* promoters has not been demonstrated, and other regulatory proteins such as staphylococcal accessory regulator A (SarA) likely are a critical component of Agr autoinduction and response to the AIP.

The transcript of the P3 operon, RNAIII, is considered to be the effector of the *agr* locus in mediating the repression or induction of quorum-controlled genes. Levels and timing of RNAIII transcription vary from strain to strain, and can be correlated with the relative levels of Agr-regulated secreted or surface factors (Li *et al.*, 1997). δ -toxin (*hld*) is also translated from the RNAIII molecule, though disruption of δ -toxin translation does not appear to impair the regulatory capabilities of RNAIII. Though the RNAIII nucleotide sequence is not well conserved, the secondary structure is, including several stem-loop motifs. When RNAIII from *S. epidermidis*, *S. simulans*, and *S. warneri* were expressed in *S. aureus*, they completely repressed expression of the protein A (*spa*) gene, similar to native *S. aureus* RNAIII, and they stimulated expression of α -toxin (*hla*) and serine protease, suggesting conservation of some important regulatory function among species (Tegmark *et al.*, 1998).

Much remains unknown of how RNAIII exerts its regulatory effects, though RNAIII is capable of regulation both at the transcriptional and translational levels (Novick, 2003). For instance, the transcription termination loop of RNAIII is necessary for repression of *spa* transcription, whereas the 3' end of RNAIII is complementary to the translation initiation site of *spa* mRNA and reportedly blocks its translation. In regulation of *hla* translation, the 5' region of RNAIII is complementary to the *hla* mRNA leader sequence. The *hla* mRNA leader folds into an untranslatable form unless prevented from doing so by RNAIII. Benito *et al.* (2000) proposed that several structurally different populations of RNAIII might coexist *in vivo*, and that RNAIII undergoes conformational changes necessary for specific functions.

In fact, many of the regulatory effects exerted by RNAIII are likely to be indirect. An analysis of Agr regulation of *sed* expression suggests that the late log growth phase

increase in *sed* transcription occurs via the Agr-mediated reduction in Rot (repressor of toxin) activity rather than a direct effect of Agr (Tseng *et al.*, 2004). This was supported by the observation that the *sed* promoter is not regulated by the Agr system in a *rot* mutant background. Agr does not appear to affect *rot* transcription. However, it has been shown to downregulate Rot activity by an as yet undefined mechanism. One explanation is that Rot might be an RNAIII-binding protein, and activation of *agr* results in titration of Rot from its gene targets (McNamara *et al.*, 2000).

Agr-regulated genes

In general, Agr expression in planktonic cultures of *S. aureus* leads to the increased expression of secreted virulence factors and the decreased expression of several surface-associated adhesins and virulence factors. For most strains this occurs during the transition from late-log growth to early stationary phase, also known as the postexponential phase. A model for how this might affect biofilm development *in vivo* was presented in the introduction to this chapter (see Figure 8.1).

With the exception of capsular polysaccharides, secreted factors upregulated by the Agr system with particular significance for virulence can generally be divided into two classes, exoenzymes and exotoxins (Novick, 2006). Exoenzymes, such as lipase and the proteases, contribute to host tissue degradation, perhaps to create a source of nutrients or to facilitate escape from the localized infection. Also, proteases degrade host proteins important for the immune response, such as the neutrophil defensins, platelet microbicidal proteins, and antibodies, thus providing some protection from host immunity. Proteases may also contribute to the degradation of cell-to-cell or cell-to-host bonds formed by staphylococcal cell surface adhesins. Regulation of lipase production itself may be due to the upregulation by Agr of proteolytic activity, enhancing conversion of the pro-form of the lipase to mature lipase, rather than regulation at the transcriptional level. Fatty-acid modifying enzyme (FAME) is also strongly activated by Agr. FAME can inactivate bactericidal lipids often found in staphylococcal abscesses through esterification of the lipids into alcohols. These lipids are frequently released from glycerides in the abscess, perhaps by the action of staphylococcal lipase. Accordingly, most *S. aureus* strains that produce lipase also produce FAME.

Exotoxins upregulated by Agr include the hemolysins (α -, β -, δ -, and γ -toxin), which have general lytic activity against a broad range of host cells, and the pyrogenic toxin superantigens (SAGs), which have broad immunostimulatory activity. α - and δ -toxins are both thought to be pore-forming agents strongly regulated by Agr. α -toxin is positively regulated at both the transcriptional and translational levels by RNAIII, whereas δ -toxin is directly encoded via RNAIII. α -toxin is a highly potent toxin, killing erythrocytes, mononuclear immune cells, epithelial and endothelial cells. δ -toxin is a small, surfactive protein and is active against many types of membranes. β -toxin is likely a sphingomyelinase, causing hydrolysis of sphingomyelin in the membrane outer leaflet patches of erythrocytes and eventual collapse of the lipid bilayer. γ -toxin is a member of a family of bi-component toxins of *S. aureus* in which their pore-forming activity is mediated by two synergistically acting proteins, and is strongly hemolytic but much less leukotoxic.

SAGs generally exert their effects through non-antigen specific binding of professional antigen-presenting cells and T-cells. Typical antigens might stimulate one out of 10 000

T-cells that specifically recognize that particular antigen; superantigens may stimulate and cause polyclonal proliferation of 20% or more of all circulating T-cells. This results in the release of high levels of cytokines, leading to the symptoms of toxic shock such as vascular dilation, loss of blood pressure, and subsequent organ damage and failure. It is possible that the increased permeability of organs and tissues due to this immune response could also facilitate the spread of staphylococci from one site to another by fluid flow.

In general, most of the SAGs are activated by the Agr system, though there are some exceptions. Staphylococcal enterotoxin A (SEA), for instance, is produced throughout growth in an Agr-independent manner. It is not clear what the significance of this Agr-independent expression and the relative contribution of Agr-independent toxins versus Agr-activated toxins in a particular infection type might be. Many of the SAGs are also enterotoxins, exhibiting emetic activity which is separable within the protein from their superantigenic activity, and are responsible for the dominance of *S. aureus* as a leading cause of food poisoning.

There are numerous factors important for colonization and virulence that are down-regulated by Agr as well. These include several surface-associated adhesins such as protein A, fibronectin-binding proteins (*fnbA*, *fnbB*), vitronectin-binding protein and coagulase (Novick, 2006). Many of these proteins can also be found in substantial quantities in the growth medium, suggesting that their release from the cell may have importance as well. This release may be due in part to proteolytic activity, particularly at high cell densities.

Protein A was the first staphylococcal surface protein to be characterized and is noted for its ability to bind the Fc region of mammalian IgG. By binding IgG, Protein A may interfere with phagocytosis of opsonized bacteria. Protein A can also mediate staphylococcal adherence to von Willebrand factor, a host extracellular matrix protein, suggesting that protein A influences several aspects of the colonization and infectious processes. Two structurally similar proteins, FnbpA and FnbpB, have been shown to mediate *S. aureus* binding to fibronectin. Fibronectin is a ubiquitous protein found in the extracellular matrix of most tissues, as well as in soluble form in many body fluids, and is necessary for the adhesion of almost all cell types. Fibronectin is one of the host proteins that rapidly coat foreign objects, such as an intravascular catheter, thus facilitating adherence of staphylococci to this *de facto* biological surface. The fibronectin-binding proteins may also play a role in invasion of host cells by binding soluble fibronectin which is then recognized by integrins on the host cell. This results in phagocytosis of the host protein-coated bacteria. Vitronectin is an adhesive glycoprotein found in circulation at several extracellular matrix sites, particular during tissue or vascular remodeling. Similar to fibronectin- or fibrinogen-binding proteins, vitronectin-binding likely facilitates colonization by staphylococci of host tissues or host-protein coated implanted devices. Coagulase production is the primary criterion used to distinguish *S. aureus* from other staphylococcal species in a clinical microbiology setting. Coagulase binds soluble fibrinogen and also binds human prothrombin to form a complex which converts soluble fibrinogen to insoluble fibrin. Coagulase is cell-wall associated, though does not have a cell-wall anchoring sequence. The role of coagulase in staphylococcal pathogenesis is not well understood. It could be that fibrin clotting around infection foci protects the bacteria from elements of host immunity.

A transcriptome analysis of Agr function in *S. aureus* identified 104 genes induced and 34 genes repressed in an Agr-dependent manner (Dunman *et al.*, 2001). This study supports in general the idea that extracellular virulence factors are activated by Agr and surface adhesins repressed. However, the majority of genes identified as being Agr-regulated were in fact not known virulence factors, but were instead involved in such cellular processes as amino acid metabolism and nutrient transport. Considering this evidence, as well as the identification of Agr homologs in other, less virulent staphylococci, one can speculate that the Agr system may not have evolved originally to facilitate virulence, but perhaps for the coordination of more basic biological functions. Some of these biological functions could well include colonization and dissemination at appropriate cell densities—functions essential to biofilm development.

Several aspects of the *in vitro* model of the Agr regulatory circuit were confirmed in an experimental endocarditis model, although with an intriguing exception (Xiong *et al.*, 2002). As might be expected, maximal RNAII activation in vegetations occurred early, followed by increasing RNAIII expression. This correlated with increased bacterial densities within the vegetations (as compared to lower densities in kidney and spleen tissues), supporting the idea that RNAIII activation *in vivo* is time and cell-density dependent, and perhaps also tissue-specific. Surprisingly, RNAIII activation was also observed in vegetations formed using Agr signaling mutants (though to a lesser extent than the wild type), suggesting that an RNAII-independent mechanism of RNAIII activation may exist *in vivo*. Also, there was no correlation between RNAII promoter activity and vegetation densities.

The plasma protein fibrinogen is an important component of the acute inflammatory response. It helps to promote neutrophil migration and adhesion, induction of cytokine synthesis, coating of foreign bodies, walling off of infection sites, and initiation of wound healing. As discussed earlier, *S. aureus* possesses several cell-associated and secreted factors that directly interact with fibrinogen or its soluble precursor, fibrin. In a murine abscess model, transient depletion of the animal of fibrinogen significantly reduced the bacterial burden and overall morbidity and mortality in the animals (Rothfork *et al.*, 2003). This was not observed in infection by an *agr* mutant. Fibrinogen depletion also inhibited *in vivo* activation of RNAIII transcription, as well as expression of the quorum-activated virulence factors α -toxin and capsule. The data suggest that fibrinogen-mediated clumping is sufficient to concentrate the autoinducer and promote quorum sensing. The same effects could also be mediated by fibronectin. This study provides an important mechanistic link between the innate immune response and pathogenesis of *S. aureus*, as well as insight into regulation of *agr* expression *in vivo*.

Peptide signaling in *Staphylococcus epidermidis*

In general, the Agr system in *S. epidermidis* appears to be highly similar to that in *S. aureus* (Van Wamel *et al.*, 1998; Vuong *et al.*, 2000a). Agr expression is growth-phase dependent, and with a few exceptions, upregulates exoprotein production while downregulating several surface-associated proteins. In particular, both lipase and protease activity are greatly downregulated in a *S. epidermidis agr* mutant. Overall, homology of the *agr* loci between *S. aureus* and *S. epidermidis* is 68%. The δ -toxin presumably encoded by the *S. epidermidis* RNAIII molecule differs in three amino acids from that produced by *S. aureus*, and is upregulated in

post-exponential phase, as is RNIII. δ -toxin activity was found in 21 of 23 *S. epidermidis* strains tested. Agr was also shown to be indirectly involved in production of the lantibiotic epidermin by *S. epidermidis* via regulation of EpiP, a protease involved in the formation of mature epidermin (Kies *et al.*, 2003).

Agr and staphylococcal biofilms

There are at least three important stages in staphylococcal biofilm development and behavior, similar to those for many other bacterial species. The first is the initial attachment of cells to a biotic or abiotic surface, usually mediated by surface adhesins. The second, or maturation, stage involves the accumulation of cells into multi-layered clusters enclosed in an at least partly self-produced matrix, or glycocalyx. The third stage of biofilm development involves detachment of cells from the biofilm which may facilitate the colonization of distant sites from the original infection site. The factors contributing to detachment are both external and internal to the biofilm. Physical factors such as shear and physical disruption of the biofilm induce large-scale detachment, while emerging evidence suggests that biofilm-associated bacteria may also actively promote their own detachment (see also Webb, this volume).

There are mechanisms whereby Agr expression might impact each of these stages of biofilm development, based on a limited number of *in vitro* studies.

Initial attachment

There appear to be two general mechanisms by which staphylococci attach to a surface as illustrated by colonization of an intravascular catheter. During insertion of the catheter, attachment to the naked polymer surface occurs through non-specific, physiochemical interactions, such as hydrophobic interactions. Subsequent to implantation, the catheter surface becomes coated with components of the host matrix, such as fibrinogen, fibronectin, and collagen. This facilitates more specific interactions between the staphylococci and what is now a biological surface mediated by specific receptors on the staphylococci, such as the fibrinogen- and fibronectin-binding proteins. Several of these specific staphylococcal receptors are negatively regulated by Agr. In some staphylococcal species large proteins that might mediate non-specific, hydrophobic interactions with the uncoated polymer surface are also regulated by Agr (e.g. the autolysin AtlE in *S. epidermidis*).

Maturation

Little evidence exists for or against a contribution of the Agr system to the maturation of biofilms. The matrix is thought to usually consist of the polysaccharide intercellular adhesion (PIA), and the expression of PIA (encoded by the *ica* locus) is not regulated by Agr (Vuong *et al.*, 2003). In the host milieu, however, it is not entirely clear whether PIA is in fact required to form a biofilm-like community. Through intracellular binding mediated by host cell matrix components [e.g. fibrinogen (Rothfork *et al.*, 2003)], a biofilm-like structure could be achieved together with the important characteristics of a biofilm (nutritional gradients, protection from host immune factors and predation, etc.) without the presence of PIA. Thus, continued expression of surface adhesins through downregulation of Agr might enhance accumulation in the host of staphylococcal cells in a biofilm.

Detachment

Expression of δ -toxin, a protein with surfactant properties and encoded by the *agr* locus is thought to contribute to detachment of cells from a biofilm (Vuong *et al.*, 2000b). Thus, in combination with the downregulation of surface adhesins, Agr may well play an important role in facilitating release of staphylococcal cells from the biofilm. Indeed, we have observed enhanced detachment of Agr-expressing cells from a biofilm (Yarwood *et al.*, 2004), but have not been able to confirm the contribution of Agr to this phenomenon. Large-scale detachment events would also be expected to influence mature biofilm structure, at least temporarily, thus potentially influencing the maturation stage of biofilm development as well.

At first glance, studies of the role of the Agr system in staphylococcal biofilm formation and behavior appear somewhat inconsistent in their conclusions. A survey of *S. aureus* strains found a strong correlation between lack of Agr activity (as measured by δ -toxin production) and ability to adhere to polystyrene (Vuong *et al.*, 2000b). This was attributed, at least in part, to the surfactant properties of δ -toxin, as addition of increasing concentrations of δ -toxin decreased attachment of *S. aureus* to polystyrene. In two studies with somewhat conflicting results *agr* mutants were first found to demonstrate increased adherence to immobilized fibrinogen, increased induction of platelet aggregation, and had little impact on adherence to immobilized fibronectin, von Willebrand factors, bovine corneal extracellular matrix and endothelial cells (Shenkman *et al.*, 2001). The difference in adherence properties developed primarily under flow conditions, suggesting different adhesion mechanisms under static and flow conditions. In the second study, it was concluded that RNAIII downregulated *S. aureus* adherence to fibrinogen under static conditions while upregulating *S. aureus* adherence to fibronectin and endothelial cells under both static and flow conditions (Shenkman *et al.*, 2002). In addition, the contribution of activated platelets to *S. aureus* adherence to endothelial cells was downregulated by RNAIII, likely due to decreased adherence to fibrinogen, a plasma protein thought to bridge *S. aureus*, platelets, and endothelial cells. Finally, pleiotropic effects were shown of both the *agr* and *sar* operons on expression of surface molecules responsible for binding to substrata (Pratten *et al.*, 2001).

To address whether the variable results found in the literature were the result of different strains or different growth conditions, biofilms of an isogenic pair (wild type versus *agr* mutant) were grown under several conditions (Yarwood *et al.*, 2004). In this study, the contribution of Agr to biofilm development was found to be dependent on growth conditions. In some cases, Agr expression decreased bacterial attachment and biofilm formation. Under other conditions, it enhanced biofilm formation or, in the case of flow-cell biofilms, appeared to have little effect on biofilm structure at discrete time points even when clearly expressed. However, in time-course studies Agr expression did often precede cell detachment. Given these results and those of other studies, it is clear that the Agr contribution to biofilm formation is heavily dependent on growth conditions (medium, shear, temperature, etc.), surface character (e.g. biological or abiotic), and strains used. Going forward, the most useful results will likely come from either *in vitro* studies that closely mimic the *in vivo* environment, or from animal models of staphylococcal biofilm infection.

In the first study of its kind to address directly the biofilm-forming capabilities of *agr* mutants *in vivo*, Vuong *et al.* (2004) found that a *S. epidermidis agr* mutant shows increased

binding to epithelial cells and a higher colonization rate in a rabbit model of an indwelling medical device-related infection. They also confirmed that deletion of *agr* or inhibition of Agr activity leads to thicker biofilms *in vitro*. These results were consistent with a study conducted earlier by the same laboratory group in which a *S. epidermidis agr* mutant showed increased primary attachment and biofilm formation, as well as expression of the cell surface-associated autolysin AtlE (Vuong *et al.*, 2003). (Repetitive sequences in AtlE are thought to interact hydrophobically with abiotic surfaces.) Like *S. aureus*, production of PIA by the *S. epidermidis agr* mutant was similar to the wild type. As expected, the *agr* mutant lacked δ -hemolysin production. Addition of increasing concentrations of δ -toxin resulted in decreased attachment of *S. epidermidis* cells to polystyrene, where 10 $\mu\text{g/ml}$ δ -toxin was sufficient to reduce biofilm formation of the *agr* mutant strain to the same levels found using the *agr* wild-type strain.

Interestingly, there may also be some role for Agr expression in the resistance of staphylococcal biofilms to antibiotic exposure. Under conditions where an *agr* mutant formed a smaller biofilm than its wild-type parent, the mutant was also more sensitive to rifampicin treatment, but not oxacillin (Yarwood *et al.*, 2004). The basis for this variation in sensitivity is unknown, though there is precedent for the regulation of other antibiotic resistance mechanisms by Agr. Regulation of NorA, a multi-drug efflux pump involved in resistance to quinolones, by the DNA-binding protein NorR was found to require an intact Agr system (Truong-Bolduc *et al.*, 2003).

Agr variants

Agr variants (cells either in which expression of Agr is significantly higher or lower as compared to the parental strain) have been frequently isolated from cultures *in vitro*, suggesting that staphylococci maintain some capacity to alter their Agr phenotype or maintain Agr-negative subpopulations. Somerville *et al.* (2002) found that repeated passage of *S. aureus in vitro* resulted in the loss of Agr function in a large percentage of the population, along with corresponding hemolytic and aconitase activity. The authors hypothesized that frequent mutations of *agr* create a mixed population of bacteria, with some cells expressing colonization factors, while others would tend to express secreted exotoxins. Under a particular environment with specific ecological and/or immunological selection, the Agr variant best able to adapt would emerge.

Agr mutants are frequently found among clinical isolates. One study (Vuong *et al.*, 2004) showed that the percentage of strains with defective quorum-sensing systems was significantly higher among isolates from patients with infections of joint prostheses than among isolates from the skin of healthy controls (36% versus 5%, respectively). Another study found that 26% of *S. aureus* isolates failed to produce δ -toxin, indicating that they were deficient in quorum-sensing-mediated regulation (Vuong *et al.*, 2000b). When staphylococci were isolated from the lungs of cystic fibrosis patients, not only did the strains generally express low levels of RNAlII, but several isolates were also found to be Agr-negative (Goerke *et al.*, 2000).

Fowler *et al.* (2004) showed that the percentage of *S. aureus* isolates recovered from patients with persistent bacteremia with defective δ -toxin production (a consistent indicator of Agr activity) was higher than in isolates from patients with resolving bacteremia

(71% versus 39%, respectively). The authors postulated that lack of Agr expression might contribute to persistent bacteremia through the increased expression of the *S. aureus* surface adhesion gene, *fnbA*, in these mutants. The fibronectin-binding protein encoded by *fnbA* has been shown to enhance *S. aureus* adhesion to, invasion of, and persistence within endothelial cells. Intracellular invasion may contribute to resistance to antibiotics, as vancomycin penetrates poorly into endothelial cells. Thus, lack of Agr expression may facilitate a protected intracellular reservoir for *S. aureus*. Indeed, an agr mutant is incapable of escape from the endosome (Shompole *et al.*, 2003) or inducing apoptosis (Wesson *et al.*, 1998), suggesting a prominent role for Agr in invasion of and persistence in host cells.

One area of particular concern in staphylococcal pathogenesis is the emergence of staphylococci with intermediate resistance to glycopeptide antibiotics (GISA). Interestingly, GISA are frequently isolated from biomedical device-related infection, which are also likely to be biofilm-associated, and these same GISA have been shown to be predominantly Agr-negative (Sakoulas *et al.*, 2002). The same study also suggested that loss of Agr function might in fact contribute to the development of vancomycin tolerance, an intriguing idea yet to be confirmed.

Behavior of mixed populations of hyper-hemolytic, hemolytic, and non-hemolytic variants was examined in a murine abscess model of infection (Schwan *et al.*, 2003). The percentage of non-hemolytic variants, likely representing Agr-negative bacteria, recovered from the wound increased over time, whereas the number of hyper-hemolytic variants (Agr overexpressors) decreased dramatically over the same time period. A wound infection model demonstrated the same trend, though to a lesser degree. In contrast, hemolytic variants seemed to be favored in isolates recovered from murine livers and spleens in a model of systemic infection. Thus, Agr activity likely facilitates survival and pathogenesis in some host environments, but not others. Additional studies will be of great importance in monitoring Agr phenotypes of clinical isolates from various infection types (preferably multiple isolates from each patient) and determining any correlation to disease progression and outcome.

It can be hypothesized that Agr-negative variants are better suited to biofilm formation and long-term, chronic infection as they tend to (1) express the surface adhesins that mediate cell-to-cell and cell-to-surface interactions, while downregulating factors that may facilitate detachment, such as δ -toxin, and (2) express more immuno-evasive factors, such as protein A, than immuno-stimulatory ones (such as the superantigens). In addressing the first idea, our laboratory has found that Agr-negative variants become the predominant form in biofilms grown in a serum-based medium (Yarwood, 2004; Yarwood and Greenberg, 2006). It is not yet clear whether this is due to a selective pressure against Agr-positive cells, increased generation of Agr variants in the biofilm, active detachment of cells expressing Agr, or some combination of all three. Also, the results indicate that the Agr-positive population is not completely lost from the biofilm (J.M. Yarwood, unpublished data), suggesting a mechanism to retain the capability to express invasive factors at an appropriate stage of infection. Indeed, we have detected the frequent detachment of cells expressing Agr from the biofilm (Yarwood *et al.*, 2004). This may have important clinical implications, as detaching cells expressing Agr are also likely to be expressing extracellular virulence factors important in causing acute infection. The frequency at which variants arise also appears

to vary from strain to strain, and with cell density (J.M. Yarwood, unpublished data), and much work remains to be done to understand the mechanisms of variant generation and the importance of these functional Agr variants in the disease process.

One potential model of *S. aureus* Agr evolution in the context of a chronically infected host was presented previously (Yarwood, 2006) and is summarized here. Upon establishment of infection, mutations (often point mutations) accumulate in the *agr* loci of *S. aureus* cells. These mutations result in the conversion of a significant part of the population to a quorum-sensing negative phenotype. The Agr-negative phenotype confers some protection to the staphylococcal population as a whole due to increased expression of immuno-evasive factors and facilitates attachment and accumulation through increased expression of host protein-binding factors. This protected environment is conducive to the continued growth of staphylococci and additional accumulation of mutations in the *agr* locus. On very rare occasions, appropriate mutations are acquired by the Agr-negative variant to return functionality to the *agr* locus, such as alteration of the AgrC receptor to recognize an AIP variant. These new Agr specificity group cells detach from the biofilm through expression of invasive virulence factors or production of δ -toxin and establish infection elsewhere in the host or, alternatively, colonize a secondary host. In some cases, appropriate ecological pressures are present to allow emergence of this new Agr specificity group from among the established groups. The combined rarity of these events—accumulation of several, eventually positive mutations, and selection for any emergent Agr specificity group would only give rise to a major new *S. aureus* Agr group very infrequently. This would be consistent with the identification of only four distinct *S. aureus* Agr groups thus far, despite frequent mutation of the *agr* locus. The driving force behind this cycle is, in part, the advantage conferred by maintaining a mixed population of cells, where Agr-negative variants prevent recognition by immune surveillance, and cells expressing Agr provide additional nutrient sources through host tissue degradation or facilitate escape from the localized infection at appropriate times. Thus, the emergence of distinct Agr groups may be a byproduct of this mode of Agr evolution in which the generation of variants is itself important. However, it is noteworthy that this Agr-negative phenotype is often generated through non-reversible mutation of the *agr* locus, rather than a reversible, conditional switching of Agr expression on and off. This is consistent with some evolutionary or pathological advantage for generation of distinct Agr specificity groups.

RIP/RAP

With some degree of controversy, a second quorum-sensing system has been described in *S. aureus* that is proposed to regulate Agr activity (see references Balaban *et al.*, 1998; Dell'Acqua *et al.*, 2004; Novick, 2003 and other studies by N. Balaban and colleagues). This system consists of the auto-inducer RNAIII activating protein (RAP) and its target molecule TRAP. RAP is described as an ortholog of the ribosomal protein L2 that is synthesized early in growth. Reportedly, when RAP reaches a threshold concentration, it induces the histidine phosphorylation of the membrane protein TRAP. This event leads to the upregulation of *agr* transcription through an undescribed mechanism. Once AIP is made, it has been reported to lead to the downregulation of TRAP phosphorylation. Immunization against RAP was shown to mitigate pathology in a murine cutaneous *S.*

aureus infection model. A protein produced by *S. xyloso* and resembling in the N-terminal sequence of RAP, RNAIII inhibiting peptide (RIP), has been reported act as an agonist of TRAP, inhibiting its phosphorylation and, consequently, *agr* expression. Treatment with synthetic RIP inhibits several types of *S. aureus* and *S. epidermidis* infections, including those that are biofilm-related or caused by multiple drug-resistance staphylococci. Apparently, these therapeutic effects can be observed when RIP is applied locally or systemically.

The RAP/TRAP/RIP story thus far is somewhat unsatisfying. Little has been described regarding the properties of RIP, and even its amino acid sequence and structure remain questionable. It is also conceivable that when used at high concentrations, RIP has sufficient amphipathic, or surfactant, properties to prevent bacterial attachment, not unlike many other proteins, including staphylococcal δ -toxin. Most of the *in vivo* experiments have administered RIP before or coincident with bacterial challenge and it is clear that, in these cases, RIP inhibits bacterial adherence to surfaces. However, there is no evidence that RIP has any effect against established biofilm, and little data is available as to the overall effect of RIP on bacterial physiology or virulence. It is also not clear whether a RIP-impregnated intravascular catheter would continue to inhibit staphylococcal adhesion against the more or less continual “challenge” that likely occurs *in vivo*—either from the epithelium or transient bacteremias. Implanted devices are soon coated with host matrix proteins, a fact that limits the efficacy of many device surface treatments as the now biotic surface provides several protein receptor specific targets for staphylococci to bind to. Furthermore, very little is known as to the regulatory targets of this system. Besides being reported to inhibit Agr activation, no other gene targets have been conclusively identified. This is particularly unsatisfying, as inhibition of Agr activity would be expected to increase bacterial adhesion, yet, in fact, the RIP-treated cells are less likely to adhere, and suggests that RIP may in fact simply be acting as a surfactant-like molecule. Finally, other laboratory groups have been unable to detect the RNAIII-activating activity in supernatants of *agr*-null strains, despite the presumed presence of RAP, contributing to the controversy as to the true nature of this molecule (Novick, 2003; Novick *et al.*, 2000).

Peptide signaling and *Streptococcus* biofilms

Streptococci are the causative agents of numerous diseases, from indigenous microflora that cause dental caries to exogenous pathogens that are the etiologic agents of both relatively benign infections, such as impetigo, and potentially fatal diseases, including necrotizing fasciitis. First described in *S. pneumoniae* in its relationship to natural transformation, peptide signaling has also been shown to be involved in streptococcal virulence, biofilm formation, acid tolerance and bacteriocin production (Cvitkovitch *et al.*, 2003; Suntharalingam and Cvitkovitch, 2005). Highly homologous competence-stimulating peptide (CSP) quorum sensing systems have been identified in several streptococcal species, including strains in the mitis (including *S. pneumoniae*), anginosus (including *S. intermedius*), and mutans (including *S. mutans*) groups.

Streptococcal quorum sensing, particularly in its relationship to competence induction and transformation, has been most extensively studied in *S. pneumoniae* (Figure 8.3). The CSP precursor is encoded by *comC*. Two secretory proteins, ComA, an ATP-binding cassette transporter, and ComB, described as an accessory protein to ComA, are involved in

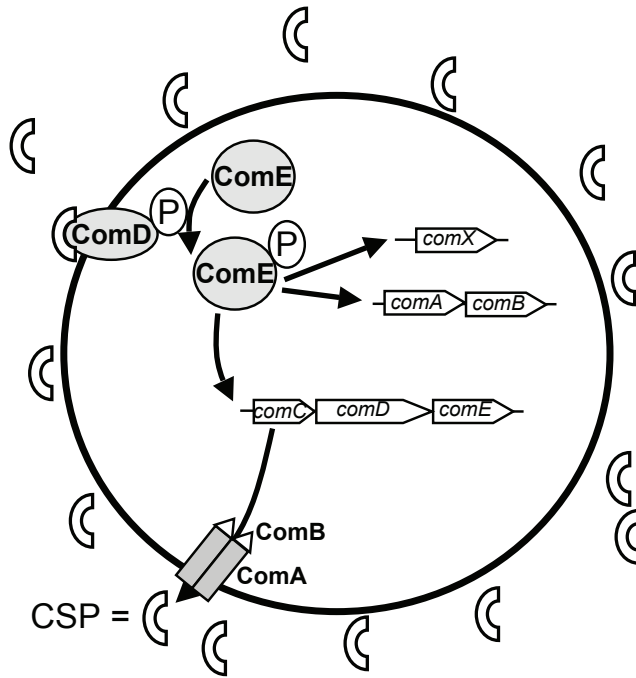


Figure 8.3 Model of the *Streptococcus pneumoniae* competence-stimulating peptide (CSP) system (see text for additional description). The *comC* product is processed by the ComA/ComB complex and secreted as CSP into the extracellular environment. Recognition of CSP by ComD triggers transfer of a phosphate group between ComD and ComE. ComE acts to increase transcription of both the operons encoding the CSP system itself (*comAB* and *comCDE*) as well as *comX*, encoding the alternative sigma factor ComX. ComX activates the transcription of late competence genes, including those involved in DNA uptake and integration into the host cell genome.

the processing and export of a 17-amino acid CSP. Interaction of the CSP with its histidine kinase receptor, ComD, initiates a series of temporally distinct transcription profiles via the response regulator ComE. Among the earliest operons to be induced are those encoding the cell-to-cell signaling system itself, *comAB* and *comCDE*, and an alternative sigma factor, ComX. ComX is integral to later stages of competence development, inducing genes involved in DNA uptake and integration. Many of the *S. pneumoniae* operons regulated by ComX have a *com*-box consensus sequence (5'-TACGAATA-3') in their promoter regions recognized by the sigma factor. The presence of *com*-boxes in *S. mutans* late-competence genes suggest ComX behaves similarly in this organism. ComX also regulates a mechanism whereby a small fraction of the bacterial population initiates lysis and release of donor DNA.

In vitro, accumulation of the CSP in the growth medium triggers a short (20–40 minutes) window of competence throughout the culture. *In vivo*, the ability to take up DNA through natural transformation may enable the streptococci to acquire novel genes, such as antibiotic-resistance cassettes and virulence factors, that provide a selective advantage over

neighboring bacteria competing for the same resources. Also of note, recent transcriptional analyses during *S. pneumoniae* competence development showed that only 23 of 124 CSP-inducible genes were required for transformation, at least in laboratory conditions. It remains to be seen what role many of the remaining gene products might have in cell-density dependent responses, such as nutrient adaptation, biofilm formation, or stress responses.

The mechanics and effects of the CSP quorum sensing systems vary somewhat among the streptococci. While some streptococcal species (particularly the anginosus group) encode and respond to identical CSPs, CSPs are often species, and sometimes strain, specific (streptococci responding to the same CSP signal are identified as a “pherotype”). In many species the genes encoding the CSP, histidine kinase receptor and the cognate response regulator are organized as a single operon. In *S. mutans*, however, *comC* is divergently transcribed from the *comDE* operon. Competence can be completely abolished in *S. pneumoniae* by inactivation of the ComDE two-component system; in *S. mutans*, inactivation of this system only reduces competence. In *S. pneumoniae*, competence development affects most of the cell culture; in *S. mutans*, competence development affects only a small number of cells.

While many species of streptococci have been found to form biofilms, the relationship between biofilm formation, peptide signaling, and pathogenesis has been best studied in the oral streptococci (Cvitkovitch *et al.*, 2003; Suntharalingam and Cvitkovitch, 2005). The oral cavity is one of the most microbiologically diverse environments on earth, with as many as 500 species residing in the human mouth. Dental plaque is also one of the more complex representations of bacterial biofilms, with multiple species colonizing the tooth surface in multiple stages. Furthermore, the oral cavity is a particularly stressful environment for bacteria, with wide swings in nutrient availability, low pH, high osmolarity, and presence of host-produced enzymes and antimicrobials. Thus, biofilm formation likely provides oral bacteria a protected environment, as well as facilitating genetic exchange.

The first indication that a CSP quorum sensing system was involved in streptococcal biofilm formation came when transposon mutants of *comD* in the oral bacterium *S. gordonii* were shown to be defective in biofilm formation (Loo *et al.*, 2000). A subsequent study in *S. mutans* found that inactivation of any component of the ComCDE pathway in results in abnormal biofilms (Li *et al.*, 2002). Biofilms of the *comC* mutant lack the wild-type architecture (which can be restored by addition of synthetic CSP), whereas biofilms of the *comD* and *comE* mutants have reduced biomass. In *S. intermedius*, biofilm formation is enhanced in the presence of CSP without affecting the organism’s growth rate (Petersen *et al.*, 2004). While little is known as to the mechanisms by which the CSP systems contribute to streptococcal biofilm development, initial evidence indicates that CSP influences the early stages of biofilm formation rather than later maturation steps, at least for *S. mutans* and *S. intermedius*. *S. mutans comD* or *comE* mutants adhere less to surfaces, while *S. intermedius* CSP appears to enhance the early buildup of cells in a biofilm.

Of particular interest is the observation that *S. mutans* and *S. intermedius* biofilm cells are much more efficient in incorporating foreign DNA than corresponding planktonic cells (Li *et al.*, 2001b). Furthermore, the CSP quorum sensing system appears to be transcriptionally upregulated in biofilm-associated *S. gordonii* and *S. mutans*. It seems likely that the high cell density of the biofilm likely enhances transformation efficiency through both

increased CSP cell-to-cell signaling and the presence of relatively high concentrations of extracellular genetic material. It may well be that the heterogeneous biofilm environment also provides gradients of CSP signal and other growth conditions that sustain localized clusters of cells with a competence window significantly longer than the 20–40 minutes typically seen in planktonic cultures (Suntharalingam and Cvitkovitch, 2005). Indeed, observations via confocal microscopy of a *comX-gfp* reporter in a *S. mutans* biofilm demonstrated spatial heterogeneity; it appeared that cells in denser areas of the biofilm had increased *comX* activity and likely were genetically competent (Aspiras *et al.*, 2004).

Peptide signaling and the acid-tolerance response

One of the hallmarks of tooth decay (dental caries) associated with colonization by *S. mutans* is the production of acid from fermentable dietary carbohydrates that leads to demineralization of the tooth surface. In the dental biofilm, *S. mutans* encounters pH shifts from above 7 to nearly 3 during ingestion of these carbohydrates. Thus, *S. mutans*' tolerance to low pH is critical to its survival and pathogenicity. The acid tolerance response (ATR) by *S. mutans* requires de novo synthesis of proteins apparently required for adaptation to an acidic environment and is pH-inducible—exposure of *S. mutans* to mildly acidic pH (5–6) results in enhanced survival at lower pH values (3.0–3.5). In many bacteria, the ATR is also growth phase- and time-dependent, leading to speculation that it might be quorum-regulated in *S. mutans*. Indeed, mutations in the *comC*, *comD*, or *comE* genes result in a diminished log-phase ATR in *S. mutans*, whereas addition of synthetic CSP to a *comC* mutant restores the ATR (Li *et al.*, 2001a). Correspondingly, cell density enhances the ATR—planktonic *S. mutans* taken from a high cell density culture or from a biofilm are more resistant to a killing pH (3.5) than planktonic cells taken from a lower cell density culture. Thus, both low pH induction and cell-to-cell communication (including, but not limited to, CSP) appear important for optimal development of acid adaptation.

Peptide signaling and bacteriocin production

Bacteriocins are antimicrobial peptides produced by many bacteria, including streptococci, presumably to enhance their ecological fitness by controlling competing populations of bacteria. In planktonic culture, *S. pneumoniae* releases chromosomal DNA after addition of CSP, a process that is ComDE-dependent (Moscoso and Claverys, 2004). Concentrations of released DNA are highest in stationary phase, coinciding with maximum bacteriocin production. Thus, it has been hypothesized that bacteriocins might aid in release of DNA from surrounding organisms through permeabilization of their cytoplasmic membranes. This liberated DNA might contribute to the biofilm extracellular matrix, serve as a nutrient source, and enhance overall DNA uptake and recombination.

Some evidence is emerging to support these hypotheses. Expression of *nlmAB*, genes which encode the two-peptide non-lantibiotic bacteriocin mutacin IV in *S. mutans*, is highest at high cell density and is abolished with disruption of the *comDE* genes (van der Ploeg, 2005). Kreth *et al.* (2005) also found that CSP induced coordinated expression of competence and mutacin IV in *S. mutans*. Furthermore, in mixed cultures, plasmid transfer from *S. mutans* to *S. gordonii*, which is sensitive to mutacin IV, was CSP and mutacin-dependent. The authors proposed that the coordinated expression of bacteriocins and competence may

exist to effectively acquire DNA from other species living in the same ecological niche. This is consistent with the extensive genomic diversity among *S. mutans* strains, which may well have resulted in part from horizontal gene transfer.

Peptide signaling and *Enterococcus faecalis* biofilms

The Gram-positive commensal bacterium *Enterococcus faecalis*, a normal member of the human intestinal microflora, has emerged as a leading cause of nosocomial infections. Diseases caused by this organism range from endocarditis to urinary tract and dental infections. A majority of clinical isolates form biofilms *in vitro* (Mohamed *et al.*, 2004), though biofilm formation *per se* has yet to be demonstrated as essential for enterococcal virulence. *E. faecalis* biofilms have been observed on dental root canals (Distel *et al.*, 2002), urethral catheters (Tunney and Gorman, 2002), and heart valves (Donlan and Costerton, 2002). It is also worth noting that, while an *E. faecalis* strain may not form a biofilm *in vitro* in the classical sense (i.e. surrounded by a self-produced polymeric matrix), the vegetations that the organism forms *in vivo* may functionally and physiologically mimic a classic biofilm. These vegetations are thought to be an aggregation, in part, of fibrin and platelets and contain very high cell densities (McCormick *et al.*, 2001; McCormick *et al.*, 2002). The vegetation provides an immunologically protected environment for the bacteria similar to what would be expected of an entirely self-produced biofilm (McCormick *et al.*, 2002).

In a survey of the 17 two-component regulatory systems identified in the genome of *E. faecalis*, only one system was identified as affecting biofilm formation (Hancock and Perego, 2004a; Hancock and Perego, 2004b). This system, known as *Enterococcus faecalis* regulator (encoded by the *fsr* locus; Figure 8.4), is similar in many respects to the staphylococcal Agr system. *fsrA*, *fsrB*, and *fsrC* are homologous to the staphylococcal *agrA*, *agrB*, and *agrC* genes, respectively. Furthermore, the 3'-end of *fsrB* is homologous to *agrD*, the gene responsible for encoding the staphylococcal auto-inducing peptide. Indeed, a 11-residue cyclic peptide was identified in culture supernatants of *E. faecalis* that was able to induce early gelatinase production (Nakayama *et al.*, 2001). This pheromone corresponds to a C-terminal portion of FsrB. The Fsr system is autoregulated (expression of *fsrB* and *fsrC* is cell density dependent), and regulates two downstream genes, *gelE* (encoding gelatinase, a secreted thermolysin-like M4 protease) and *sprE* (encoding a secreted serine protease) in a cell-density dependent manner (Qin *et al.*, 2001). The reported percentage of *E. faecalis* isolates carrying the *fsr* locus varies widely depending on patient group from 24% to 100% (Jones and Deshpande, 2003; Pillai *et al.*, 2002; Roberts *et al.*, 2004). Some studies have found that isolates from patients with endocarditis (Pillai *et al.*, 2002) and from ICU patients with urinary tract infections (Jones and Deshpande, 2003) are enriched for the *fsr* locus. While one study contradicted these results by finding similar levels of the *fsr* locus among isolates from diseased and healthy patients (Roberts *et al.*, 2004), it did not address whether presence of the *fsr* locus or gelatinase affects the severity of disease, as they do in animal models of infection (Engelbert *et al.*, 2004; Sifri *et al.*, 2002; Singh *et al.*, 1998).

The Fsr system appears to regulate *E. faecalis* biofilm formation, at least in part, through control of *gelE* expression (Hancock and Perego, 2003; Hancock and Perego, 2004a). Disruption of both the *fsr* and *gelE* genes attenuates *E. faecalis* biofilm development. Expression of the gelatinase gene *in trans* restores a biofilm positive phenotype,

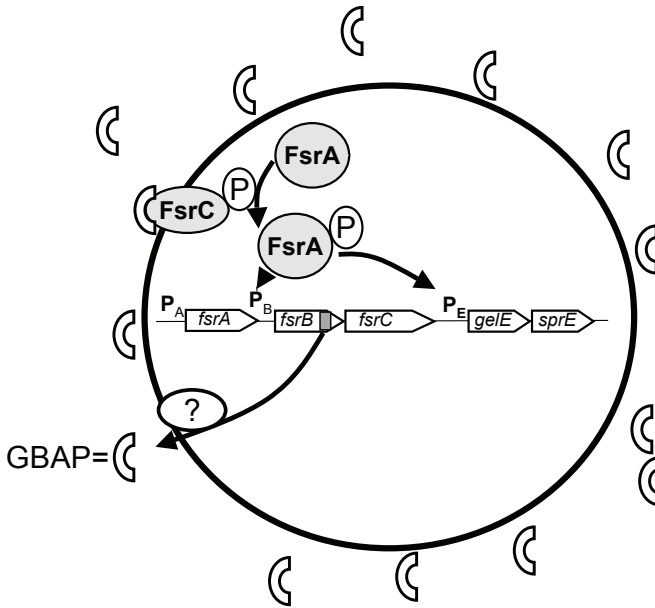


Figure 8.4 Model of the *Enterococcus faecalis* Fsr system (see text for additional description). The GBAP pheromone is liberated from the C-terminal portion of FsrB and secreted by an as yet undefined mechanism into the extracellular environment. Recognition of GBAP by FsrC leads to the phosphorylation of FsrA, which acts to upregulate the *fsrBC* operon as well as the adjacent *gelE* and *sprE* genes. “P” indicates distinct promoter regions.

even in the absence of a functional Fsr system. Observations by Pillai *et al.* (2004) also indicated that Fsr exerted its effects on biofilm control via the downstream protease genes (they did not isolate GelE activity from that of SprE). Interestingly, the same study found that glucose-mediated augmentation of biofilm occurred in wild-type *E. faecalis*, but not in either a *fsrA* mutant or a protease mutant. This indicated that catabolite control of biofilm formation occurs via the Fsr system. Catabolites, such as glucose, have dramatic effects on biofilm production by several bacterial species, yet the mechanisms by which these effects are exerted are poorly understood.

The mechanism by which gelatinase might exert its effect on enterococcal biofilm development is still unclear, nor is it well established what role gelatinase plays in virulence overall (Carniol and Gilmore, 2004). Mohamed *et al.* (2004) found that disruption of the *fsr* locus resulted in less *E. faecalis* biofilm formation, and proposed that the effect was due to decreased primary attachment. Since gelatinase, a secreted protease, tends to cleave its substrates at hydrophobic residues, it is possible that gelatinase activity increases the hydrophobicity of the cell surface, thus promoting non-specific interaction between many surfaces and the cell (Carniol and Gilmore, 2004; Hancock and Perego, 2004a). It has also been proposed that expression of gelatinase, induced in late stages of growth by cell-density dependent mechanisms, enables the dissemination of the organism *in vivo* from vegetations (Waters *et al.*, 2003). Gelatinase appears capable of degrading polymerized fibrin, which likely coats the vegetation, as well as a broad range of other substrates (Carniol and

Gilmore, 2004). Gelatinase could aid in bacterial dissemination by cleaving bacterial attachment proteins and host tissue proteins. The Fsr-controlled serine protease SprE may also be involved in this step, even though it doesn't appear to be involved in biofilm formation (Hancock and Perego, 2004a). This would be consistent with the model presented by Rasmussen and Bjorck (2002) who argued that initial stages of streptococcal infection and colonization are characterized by low levels of protease activity, whereas higher levels of protease activity during later stages of infection, when bacterial density is high, facilitate detachment and spreading of the bacteria.

In the end, many questions remain to be answered regarding the mechanisms by which enterococcal biofilms form, and how peptide signaling contributes to that process *in vivo*. While many endocarditis-derived isolates do not produce gelatinase, a majority of endocarditis-derived isolates do form biofilms *in vitro*, and recent studies have failed to find a correlation between gelatinase activity and biofilm formation in *E. faecalis* (Mohamed and Murray, 2005; Rosa *et al.*, 2006). It may well be that Fsr exerts its effects on biofilm formation both through gelatinase production as well as other mechanisms, particularly *in vivo*, depending on environmental conditions. Indeed, *fsr* locus mutants were found to be more attenuated in a rabbit endophthalmitis than the protease mutants, suggesting additional pleiotropic effects by Fsr disruption (Engelbert *et al.*, 2004). Disruption of *gelE* does attenuate virulence in several animal models of infection (Carniol and Gilmore, 2004).

Inhibition of peptide signaling as a therapeutic tool

Given the role that peptide signaling plays in biofilm control and in virulence factor regulation in several Gram-positive species, the control of peptide signaling by artificial means remains an area of significant interest for inhibiting pathogenesis. However, due to the varied nature of the signals, the complex responses to those signals, and the potential trade-offs for manipulating the quorum response, the practical use of signaling inhibitors remains a challenging proposition. For instance, inhibition of quorum sensing has been proposed as one mechanism for controlling staphylococcal infections (Ji *et al.*, 1997). In a skin abscess model co-administration of the synthetic Agr group II AIP together with the bacterial inoculation significantly attenuated an infection caused by an Agr group I strain (Mayville *et al.*, 1999). However, use of cross-inhibiting pheromones mimics *agr* mutations in both *S. aureus* and *S. epidermidis* and enhances biofilm formation (Vuong *et al.*, 2003; Vuong *et al.*, 2004; Vuong *et al.*, 2000b). This result calls for extreme caution in the use of signaling inhibitors, as it is conceivable that such treatments, while mitigating the acute phase of infections, might facilitate chronic, biofilm-associated infections, at least by staphylococci.

Use of quorum sensing inhibitors has also been proposed to as a method for preventing CSP-mediated biofilm formation in streptococci (Suntharalingam and Cvitkovitch, 2005). Since early evidence suggests quorum sensing appears to positively regulate attachment by streptococci, downregulating CSP activity might inhibit biofilm formation without the additional risk of upregulating other virulence factors, particularly in the oral streptococci. Indeed, synthetic CSPs have been used to study and manipulate streptococcal quorum sensing already. Interestingly, addition of CSP beyond levels required for induction of competence inhibits the growth of *S. mutans*, and at higher levels, even lead to cell death (Qi *et al.*, 2005).

Whether these peptide signaling systems can be effectively manipulated for therapeutic purposes is an open question. Regardless of the answer, the role of peptide signaling in biofilm formation remains an intriguing area of study, and promises to greatly enhance our understanding of the physiology and pathogenesis of many important human pathogens.

References

- Aspiras, M.B., Ellen, R.P., and Cvitkovitch, D.G. (2004). ComX activity of *Streptococcus mutans* growing in biofilms. *FEMS Microbiol. Lett.* 238, 167–174.
- Balaban, N., Goldkorn, T., Nhan, R.T., Dang, L.B., Scott, S., Ridgley, R.M., Rasooly, A., Wright, S.C., Larrick, J.W., Rasooly, R., and Carlson, J.R. (1998). Autoinducer of virulence as a target for vaccine and therapy against *Staphylococcus aureus*. *Science* 280, 438–440.
- Benito, Y., Kolb, F.A., Romby, P., Lina, G., Etienne, J., and Vandenesch, F. (2000). Probing the structure of RNAIII, the *Staphylococcus aureus agr* regulatory RNA, and identification of the RNA domain involved in repression of protein A expression. *RNA* 6, 668–679.
- Carniol, K., and Gilmore, M.S. (2004). Signal transduction, quorum-sensing, and extracellular protease activity in *Enterococcus faecalis* biofilm formation. *J. Bacteriol.* 186, 8161–8163.
- Cvitkovitch, D.G., Li, Y.H., and Ellen, R.P. (2003). Quorum sensing and biofilm formation in streptococcal infections. *J. Clin. Invest.* 112, 1626–1632.
- Dell'Acqua, G., Giacometti, A., Cirioni, O., Ghiselli, R., Saba, V., Scalise, G., Gov, Y., and Balaban, N. (2004). Suppression of drug-resistant staphylococcal infections by the quorum-sensing inhibitor RNAIII-inhibiting peptide. *J. Infect. Dis.* 190, 318–320.
- Distel, J.W., Hatton, J.F., and Gillespie, M.J. (2002). Biofilm formation in medicated root canals. *J. Endod.* 28, 689–693.
- Donlan, R.M., and Costerton, J.W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15, 167–193.
- Dunman, P.M., Murphy, E., Haney, S., Palacios, D., Tucker-Kellogg, G., Wu, S., Brown, E.L., Zagursky, R.J., Shlaes, D., and Projan, S.J. (2001). Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. *J. Bacteriol.* 183, 7341–7353.
- Engelbert, M., Mylonakis, E., Ausubel, F.M., Calderwood, S.B., and Gilmore, M.S. (2004). Contribution of gelatinase, serine protease, and *fsr* to the pathogenesis of *Enterococcus faecalis* endophthalmitis. *Infect. Immun.* 72, 3628–3633.
- Fowler, V.G., Jr., Sakoulas, G., McIntyre, L.M., Meka, V.G., Arbeit, R.D., Cabell, C.H., Stryjewski, M.E., Eliopoulos, G.M., Reller, L.B., Corey, G.R., et al. (2004). Persistent bacteremia due to methicillin-resistant *Staphylococcus aureus* infection is associated with *agr* dysfunction and low-level *in vitro* resistance to thrombin-induced platelet microbicidal protein. *J. Infect. Dis.* 190, 1140–1149.
- Goerke, C., Campana, S., Bayer, M.G., Doring, G., Botzenhart, K., and Wolz, C. (2000). Direct quantitative transcript analysis of the *agr* regulon of *Staphylococcus aureus* during human infection in comparison to the expression profile *in vitro*. *Infect. Immun.* 68, 1304–1311.
- Hancock, L., and Perego, M. (2003). The *fsr* signal transduction system of *Enterococcus faecalis* controls biofilm development through the production of gelatinase. Paper presented at: Functional Genomics of Gram-Positive Microorganisms, 12th International Conference on Bacilli (Baveno, Italy).
- Hancock, L.E., and Perego, M. (2004a). The *Enterococcus faecalis fsr* two-component system controls biofilm development through production of gelatinase. *J. Bacteriol.* 186, 5629–5639.
- Hancock, L.E., and Perego, M. (2004b). Systematic inactivation and phenotypic characterization of two-component signal transduction systems of *Enterococcus faecalis* V583. *J. Bacteriol.* 186, 7951–7958.
- Ji, G., Beavis, R., and Novick, R.P. (1997). Bacterial interference caused by autoinducing peptide variants. *Science* 276, 2027–2030.
- Jones, R.N., and Deshpande, L.M. (2003). Distribution of *fsr* among *Enterococcus faecalis* isolates from the SENTRY antimicrobial surveillance program. *J. Clin. Microbiol.* 41, 4004–4005.
- Kies, S., Vuong, C., Hille, M., Peschel, A., Meyer, C., Gotz, F., and Otto, M. (2003). Control of antimicrobial peptide synthesis by the *agr* quorum sensing system in *Staphylococcus epidermidis*: activity of the lantibiotic epidermin is regulated at the level of precursor peptide processing. *Peptides* 24, 329–338.
- Kong, K.-F., Vuong, C., and Otto, M. (2006). *Staphylococcus* quorum sensing in biofilm formation and infection. *Int. J. Med. Microbiol.* 296, 133–139.

- Kreth, J., Merritt, J., Shi, W., and Qi, F. (2005). Co-ordinated bacteriocin production and competence development: a possible mechanism for taking up DNA from neighbouring species. *Mol. Microbiol.* 57, 392–404.
- Li, S., Arvidson, S., and Mollby, R. (1997). Variation in the *agr*-dependent expression of alpha-toxin and protein A among clinical isolates of *Staphylococcus aureus* from patients with septicaemia. *FEMS Microbiol. Lett.* 152, 155–161.
- Li, Y.H., Hanna, M.N., Svensater, G., Ellen, R.P., and Cvitkovitch, D.G. (2001a). Cell density modulates acid adaptation in *Streptococcus mutans*: implications for survival in biofilms. *J. Bacteriol.* 183, 6875–6884.
- Li, Y.H., Lau, P.C., Lee, J.H., Ellen, R.P., and Cvitkovitch, D.G. (2001b). Natural genetic transformation of *Streptococcus mutans* growing in biofilms. *J. Bacteriol.* 183, 897–908.
- Li, Y.H., Tang, N., Aspiras, M.B., Lau, P.C.Y., Lee, J.H., Ellen, R.P., and Cvitkovitch, D.G. (2002). A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. *J. Bacteriol.* 184, 2699–2708.
- Loo, C.Y., Corliss, D.A., and Ganeshkumar, N. (2000). *Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes. *J. Bacteriol.* 182, 1374–1382.
- Mayville, P., Ji, G., Beavis, R., Yang, H., Goger, M., Novick, R.P., and Muir, T.W. (1999). Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. *Proc. Natl. Acad. Sci. USA* 96, 1218–1223.
- McCormick, J.K., Hirt, H., Waters, C.M., Tripp, T.J., Dunny, G.M., and Schlievert, P.M. (2001). Antibodies to a surface-exposed, N-terminal domain of aggregation substance are not protective in the rabbit model of *Enterococcus faecalis* infective endocarditis. *Infect. Immun.* 69, 3305–3314.
- McCormick, J.K., Tripp, T.J., Dunny, G.M., and Schlievert, P.M. (2002). Formation of vegetations during infective endocarditis excludes binding of bacterial-specific host antibodies to *Enterococcus faecalis*. *J. Infect. Dis.* 185, 994–997.
- McNamara, P.J., Milligan-Monroe, K.C., Khalili, S., and Proctor, R.A. (2000). Identification, cloning, and initial characterization of *rot*, a locus encoding a regulator of virulence factor expression in *Staphylococcus aureus*. *J. Bacteriol.* 182, 3197–3203.
- Mohamed, J.A., Huang, W., Nallapareddy, S.R., Teng, F., and Murray, B.E. (2004). Influence of origin of isolates, especially endocarditis isolates, and various genes on biofilm formation by *Enterococcus faecalis*. *Infect. Immun.* 72, 3658–3663.
- Mohamed, J.A., and Murray, B.E. (2005). Lack of correlation of gelatinase production and biofilm formation in a large collection of *Enterococcus faecalis* isolates. *J. Clin. Microbiol.* 43, 5405–5407.
- Moscato, M., and Claverys, J.P. (2004). Release of DNA into the medium by competent *Streptococcus pneumoniae*: kinetics, mechanism and stability of the liberated DNA. *Mol. Microbiol.* 54, 783–794.
- Nakayama, J., Cao, Y., Horii, T., Sakuda, S., Akkermans, A.D., de Vos, W.M., and Nagasawa, H. (2001). Gelatinase biosynthesis-activating pheromone: a peptide lactone that mediates a quorum sensing in *Enterococcus faecalis*. *Mol. Microbiol.* 41, 145–154.
- Novick, R.P. (2003). Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol. Microbiol.* 48, 1429–1449.
- Novick, R.P. (2006). Staphylococcal pathogenesis and pathogenicity factors: genetics and regulation. In: Gram-positive Pathogens, V.A. Fischetti, R.P. Novick, J.J. Ferretti, D.A. Portnoy, and J. I. Rood, eds. (Washington, D.C., ASM Press), pp. 496–516.
- Novick, R.P., Ross, H.F., Figueiredo, A.M.S., Abramochkin, G., Muir, T., Balaban, N., Singh, B., Goldkorn, T., Rasooly, A., Torres, J.V., and Uziel, O. (2000). Activation and inhibition of the staphylococcal AGR system. *Science* 287, 391a.
- Otto, M. (2001). *Staphylococcus aureus* and *Staphylococcus epidermidis* peptide pheromones produced by the accessory gene regulator system. *Peptides* 22, 1603–1608.
- Petersen, F.C., Pecharki, D., and Scheie, A.A. (2004). Biofilm mode of growth of *Streptococcus intermedius* favored by a competence-stimulating signaling peptide. *J. Bacteriol.* 186, 6327–6331.
- Pillai, S.K., Sakoulas, G., Eliopoulos, G.M., Moellering, R.C., Jr., Murray, B.E., and Inouye, R.T. (2004). Effects of glucose on *fsr*-mediated biofilm formation in *Enterococcus faecalis*. *J. Infect. Dis.* 190, 967–970.
- Pillai, S.K., Sakoulas, G., Gold, H.S., Wennersten, C., Eliopoulos, G.M., Moellering, R.C., Jr., and Inouye, R.T. (2002). Prevalence of the *fsr* locus in *Enterococcus faecalis* infections. *J. Clin. Microbiol.* 40, 2651–2652.

- Pratten, J., Foster, S.J., Chan, P.F., Wilson, M., and Nair, S.P. (2001). *Staphylococcus aureus* accessory regulators: expression within biofilms and effect on adhesion. *Microbes Infect.* 3, 633–637.
- Qi, F., Kreth, J., Levesque, C.M., Kay, O., Mair, R.W., Shi, W., Cvitkovitch, D.G., and Goodman, S.D. (2005). Peptide pheromone induced cell death of *Streptococcus mutans*. *FEMS Microbiol. Lett.* 251, 321–326.
- Qin, X., Singh, K.V., Weinstock, G.M., and Murray, B.E. (2001). Characterization of *fsr*, a regulator controlling expression of gelatinase and serine protease in *Enterococcus faecalis* OG1RF. *J. Bacteriol.* 183, 3372–3382.
- Rasmussen, M., and Bjorck, L. (2002). Proteolysis and its regulation at the surface of *Streptococcus pyogenes*. *Mol. Microbiol.* 43, 537–544.
- Roberts, J.C., Singh, K.V., Okhuysen, P.C., and Murray, B.E. (2004). Molecular epidemiology of the *fsr* locus and of gelatinase production among different subsets of *Enterococcus faecalis* isolates. *J. Clin. Microbiol.* 42, 2317–2320.
- Rosa, R., Creti, R., Venditti, M., D'Amelio, R., Arciola, C.R., Montanaro, L., and Baldassarri, L. (2006). Relationship between biofilm formation, the enterococcal surface protein (Esp) and gelatinase in clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium*. *FEMS Microbiol. Lett.* 256, 145–150.
- Rothfork, J.M., Dessus-Babus, S., Van Wamel, W.J., Cheung, A.L., and Gresham, H.D. (2003). Fibrinogen depletion attenuates *Staphylococcus aureus* infection by preventing density-dependent virulence gene up-regulation. *J. Immunol.* 171, 5389–5395.
- Sakoulas, G., Eliopoulos, G.M., Moellering, R.C., Jr., Wennersten, C., Venkataraman, L., Novick, R.P., and Gold, H.S. (2002). Accessory gene regulator (*agr*) locus in geographically diverse *Staphylococcus aureus* isolates with reduced susceptibility to vancomycin. *Antimicrob. Agents Chemother.* 46, 1492–1502.
- Schwan, W.R., Langhorne, M.H., Ritchie, H.D., and Stover, C.K. (2003). Loss of hemolysin expression in *Staphylococcus aureus agr* mutants correlates with selective survival during mixed infections in murine abscesses and wounds. *FEMS Immunol. Med. Microbiol.* 38, 23–28.
- Shenkman, B., Rubinstein, E., Cheung, A.L., Brill, G.E., Dardik, R., Tamarin, I., Savion, N., and Varon, D. (2001). Adherence properties of *Staphylococcus aureus* under static and flow conditions: roles of *agr* and *sar* loci, platelets, and plasma ligands. *Infect. Immun.* 69, 4473–4478.
- Shenkman, B., Varon, D., Tamarin, I., Dardik, R., Peisachov, M., Savion, N., and Rubinstein, E. (2002). Role of *agr* (RNAIII) in *Staphylococcus aureus* adherence to fibrinogen, fibronectin, platelets and endothelial cells under static and flow conditions. *J. Med. Microbiol.* 51, 747–754.
- Shompole, S., Henon, K.T., Liou, L.E., Dziewanowska, K., Bohach, G.A., and Bayles, K.W. (2003). Biphasic intracellular expression of *Staphylococcus aureus* virulence factors and evidence for Agr-mediated diffusion sensing. *Mol. Microbiol.* 49, 919–927.
- Sifri, C.D., Mylonakis, E., Singh, K.V., Qin, X., Garsin, D.A., Murray, B.E., Ausubel, F.M., and Calderwood, S.B. (2002). Virulence effect of *Enterococcus faecalis* protease genes and the quorum-sensing locus *fsr* in *Caenorhabditis elegans* and mice. *Infect. Immun.* 70, 5647–5650.
- Singh, K.V., Qin, X., Weinstock, G.M., and Murray, B.E. (1998). Generation and testing of mutants of *Enterococcus faecalis* in a mouse peritonitis model. *J. Infect. Dis.* 178, 1416–1420.
- Somerville, G.A., Beres, S.B., Fitzgerald, J.R., DeLeo, F.R., Cole, R.L., Hoff, J.S., and Musser, J.M. (2002). In vitro serial passage of *Staphylococcus aureus*: changes in physiology, virulence factor production, and *agr* nucleotide sequence. *J. Bacteriol.* 184, 1430–1437.
- Suntharalingam, P., and Cvitkovitch, D.G. (2005). Quorum sensing in streptococcal biofilm formation. *Trends Microbiol.* 13, 3–6.
- Tegmark, K., Morfeldt, E., and Arvidson, S. (1998). Regulation of *agr*-dependent virulence genes in *Staphylococcus aureus* by RNAIII from coagulase-negative staphylococci. *J. Bacteriol.* 180, 3181–3186.
- Truong-Bolduc, Q.C., Zhang, X., and Hooper, D.C. (2003). Characterization of NorR protein, a multi-functional regulator of *norA* expression in *Staphylococcus aureus*. *J. Bacteriol.* 185, 3127–3138.
- Tseng, C.W., Zhang, S., and Stewart, G.C. (2004). Accessory gene regulator control of staphylococcal enterotoxin D gene expression. *J. Bacteriol.* 186, 1793–1801.
- Tunney, M.M., and Gorman, S.P. (2002). Evaluation of a poly (vinyl pyrrolidone)-coated biomaterial for urological use. *Biomaterials* 23, 4601–4608.
- van der Ploeg, J.R. (2005). Regulation of bacteriocin production in *Streptococcus mutans* by the quorum-sensing system required for development of genetic competence. *J. Bacteriol.* 187, 3980–3989.

- Van Wamel, W.J., van Rossum, G., Verhoef, J., Vandenbroucke-Grauls, C.M., and Fluit, A.C. (1998). Cloning and characterization of an accessory gene regulator (*agr*)-like locus from *Staphylococcus epidermidis*. *FEMS Microbiol. Lett.* 163, 1–9.
- Vuong, C., Gerke, C., Somerville, G.A., Fischer, E.R., and Otto, M. (2003). Quorum-sensing control of biofilm factors in *Staphylococcus epidermidis*. *J. Infect. Dis.* 188, 706–718.
- Vuong, C., Gotz, F., and Otto, M. (2000a). Construction and characterization of an *agr* deletion mutant of *Staphylococcus epidermidis*. *Infect. Immun.* 68, 1048–1053.
- Vuong, C., Kocianova, S., Yao, Y., Carmody, A.B., and Otto, M. (2004). Increased colonization of indwelling medical devices by quorum-sensing mutants of *Staphylococcus epidermidis* *in vivo*. *J. Infect. Dis.* 190.
- Vuong, C., Saenz, H.L., Gotz, F., and Otto, M. (2000b). Impact of the *agr* quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. *J. Infect. Dis.* 182, 1688–1693.
- Waters, C.M., Antiporta, M.H., Murray, B.E., and Dunny, G.M. (2003). Role of the *Enterococcus faecalis* GelE protease in determination of cellular chain length, supernatant pheromone levels, and degradation of fibrin and misfolded surface proteins. *J. Bacteriol.* 185, 3613–3623.
- Wesson, C.A., Liou, L.E., Todd, K.M., Bohach, G.A., Trumble, W.R., and Bayles, K.W. (1998). *Staphylococcus aureus* Agr and Sar global regulators influence internalization and induction of apoptosis. *Infect. Immun.* 66, 5238–5243.
- Xiong, Y.Q., Van Wamel, W., Nast, C.C., Yeaman, M.R., Cheung, A.L., and Bayer, A.S. (2002). Activation and transcriptional interaction between *agr* RNAII and RNAPIII in *Staphylococcus aureus* *in vitro* and in an experimental endocarditis model. *J. Infect. Dis.* 186, 668–677.
- Yarwood, J.M. (2004). Quorum sensing in staphylococcal biofilms. Paper presented at: 11th International Symposium on Staphylococci and Staphylococcal Infections (Charleston, SC).
- Yarwood, J.M. (2006). Quorum-sensing-dependent regulation of staphylococcal virulence and biofilm development. In: *Bacterial Cell-to-Cell Communication: Role in Virulence and Pathogenesis*, D.R. Demuth, and R. J. Lamont, eds. (Cambridge, Cambridge University Press), pp. 199–231.
- Yarwood, J.M., Bartels, D.J., Volper, E.M., and Greenberg, E.P. (2004). Quorum sensing in *Staphylococcus aureus* biofilms. *J. Bacteriol.* 186, 1838–1850.
- Yarwood, J.M., and Greenberg, E.P. (2006). Generation of accessory gene regulator variants in *Staphylococcus aureus* biofilms. Paper presented at: American Society for Microbiology General Meeting (Orlando FL).