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Perspective Series

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Quorum sensing and biofilm formation in Streptococcal infections

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Members of the bacterial genus *Streptococcus* are responsible for causing a wide variety of infections in humans. Many Streptococci use quorum-sensing systems to regulate several physiological properties, including the ability to incorporate foreign DNA, tolerate acid, form biofilms, and become virulent. These quorum-sensing systems are primarily made of small soluble signal peptides that are detected by neighboring cells via a histidine kinase/response regulator pair.

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Streptococcal infections

Members of the genus *Streptococcus* (i.e., streptococci) are ubiquitous parasites of humans. Some are part of the indigenous microflora that are involved in opportunistic infections such as dental caries and others are exogenous pathogens that cause infections ranging from mild respiratory or skin diseases to life-threatening conditions such as pneumonia, septic shock, and necrotizing fasciitis. Contemporary research has found that many species of streptococci and other Gram positive bacteria have evolved similar peptide pheromone quorum-sensing systems that probably help them adapt to and survive host-imposed fluctuations in their local environment and coincidentally regulate the expression of virulence factors that promote their pathogenicity. This review focuses on streptococcal peptide signaling pathways that are population density-dependent and that impact on vital survival and virulence traits.

Biofilms

Biofilms are dense aggregates of surface-adherent microorganisms embedded in an exopolysaccharide matrix. The study of bacteria residing in biofilms as an interactive community rather than free-living planktonic cells has recently gained a great deal of attention. This has arisen, in part, because of the estimate by the Centers for Disease Control and Prevention that 65% of human bacterial infections involve biofilms. Many

species of streptococci are known to form biofilms; however, the relationship between the pathogenic state and the biofilm mode of growth has been most clearly established with the oral streptococci, which are known to initiate dental caries when the bacteria are living in the biofilm environment of dental plaque.

Dental plaque

The human oral cavity is a complex ecosystem that supports an extremely diverse microflora consisting of about 500 species of microorganisms (1). Numerous physical and nutritional interactions between oral bacteria contribute to a complex biofilm community (Figure 1) (2). Streptococci, including *Streptococcus mutans*, are ubiquitous in the oral microbiota of humans. *S. mutans* is considered to be a principal etiological agent of dental caries, where it can cause dissolution of tooth enamel by acid end-products resulting from carbohydrate metabolism. The tooth surface is an indispensable natural habitat for *S. mutans* (3). *S. mutans*' dental biofilm tropism most likely reflects its evolution of glucan synthesis and binding functions as well as its relative aciduricity. Its competitiveness for this ecological niche may also relate to cell density-dependent regulation of its acid tolerance response (ATR), natural genetic competence, and bacteriocin activity. Since *S. mutans* has evolved to depend on a biofilm lifestyle for survival and persistence in the oral cavity combined with its role as an opportunistic pathogen, it has become the best-studied example of a biofilm-forming, disease-causing *Streptococcus* (4). Biofilm-like populations of pathogenic streptococci may also reach higher densities in confined areas like heart valves, prosthetic devices, sinuses, tonsillar crypts, terminal respiratory passages, and in infectious skin lesions.

Quorum sensing in streptococci

Many bacteria, including streptococci, are known to regulate diverse physiological processes through a mecha-

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Nonstandard abbreviations used: acid tolerance response (ATR); two-component signal transduction system (TCSTS); competence-stimulating peptide (CSP); extracellular induction component (EIC).

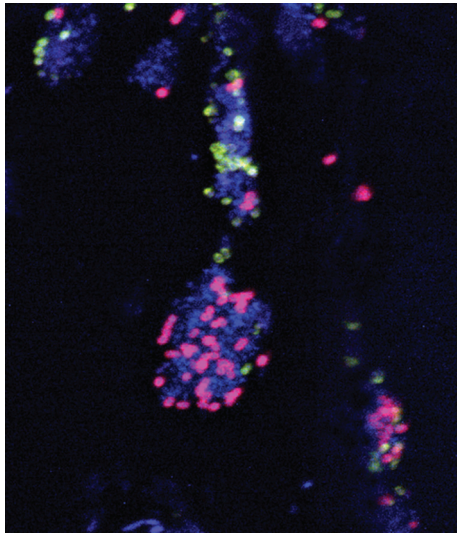


Figure 1

Eight-hour-old dental plaque was visualized using confocal scanning laser microscopy. Antibodies against *Streptococcus gordonii* DL1 and anti-receptor polysaccharides (RPS), commonly found on *Streptococcus oralis*, were used for indirect immunofluorescence along with the stain Syto 59. At least two staining types are seen within the aggregates: antibody-reactive (anti-DL1 [green], anti-RPS [purple]) cells found in direct association with antibody-unreactive (blue Syto 59-stained) cells. Single colonies containing all three staining types (anti-DL1, anti-RPS, and antibody unreactive) were frequently seen. Reproduced with permission from *The Journal of Bacteriology* (2).

nism called quorum sensing. In Gram positive bacteria, quorum-sensing systems generally consist of three components, a signal peptide and a two-component regulatory system (also called two-component signal transduction system or TCSTS) that has a membrane-bound histidine kinase sensor and an intracellular response regulator (5). Quorum sensing in Gram positive bacteria has been found to regulate a number of physiological activities, including competence development in *Streptococcus gordonii*, *S. pneumoniae*, and *S. mutans* (6), sporulation in *Bacillus subtilis* (7), antibiotic biosynthesis in *Lactococcus lactis* (8), and induction of virulence factors in *Staphylococcus aureus* (8).

Many of the genes involved in competence induction and the transformation process of streptococci, which is very similar to that described for the mitis group of *Streptococcus* (including *S. pneumoniae*), have been identified. Induction of genetic competence in these streptococci is mediated by quorum sensing, which depends on a competence stimulating peptide (CSP) signaling system illustrated in Figure 2.

Evidence for the existence of other CSP-modulated pathways

It has been recently recognized that the quorum-sensing signal in *S. pneumoniae* initiates competence through the activity of a global transcription modulator, ComX, which acts as an alternate sigma factor during the development of genetic competence (9). Transcription of the

comX gene is regulated by ComE, the response regulator of the quorum-sensing system (10). The ComX sigma factor then initiates the transcription of competence-specific operons involved in DNA uptake and recombination by recognizing a *com*-box (also referred to as *cin*-box) consensus sequence (TACGAATA) in their promoter regions (9). *S. mutans* and *S. pyogenes* also have *comX* genes present in their genomes (9, 11), and inactivation of one copy of the *S. mutans comX* renders the cell transformation deficient (12). The presence of the conserved *com*-box consensus sequence in the promoter region of *S. mutans* late-competence genes such as *comFA*, *celA*, and *cglA* supports the hypothesis that transcriptional regulation of these genes is also mediated by the ComX sigma factor (13). There is much accumulating evidence that many genes not involved in competence are under the control of the CSP-ComX system. This includes the observations that many “other” *S. pneumoniae* genes contain the *com*-box sequence and are activated by CSP (14, 15). These genes likely encode products that aid in the cell’s adaptation to a high cell density, and it is likely that they (or their homologs in other streptococci) contribute to the biofilm phenotype.

LuxS quorum sensing

Recently, the *luxS* gene has been identified in streptococci (16, 17). The LuxS protein is required for the biosynthesis of the type 2 autoinducer, AI-2, which is involved in quorum sensing in a wide range of bacterial species. Mutation of *luxS* in *S. mutans* caused a defect in biofilm formation, while disruption of this gene in *S. pneumoniae* resulted in reduced virulence in mouse infections. It has been suggested that the AI-2 pathway is a very good target for chemotherapeutic control of bacterial virulence.

Quorum sensing in *S. mutans* biofilms

Horizontal gene transfer through genetic transformation has been observed in many natural ecosystems, and recent studies suggest that growth of bacteria in biofilms may facilitate horizontal gene transfer among bacterial species via either transformation or conjugation (18, 19). Natural genetic transformation has been extensively studied in streptococci, but these studies relied exclusively on bacteria grown in fluid cultures, where they would become transiently competent after reaching a critical cell density. Since biofilms are more representative of bacterial growth in natural environments and *S. mutans* is an organism that relies on a biofilm lifestyle, we set forth to investigate the ability of this bacterium to transport and integrate exogenous DNA when living in its biofilm state. To facilitate assays for genetic transformation of biofilm-grown cells, we utilized a chemostat-based continuous flow biofilm system, which allows observation of physiological activities of a bacterial population under controlled growth conditions (20). Using this system, we have demonstrated that growth rates, culture pH, and biofilm age are several important factors that influence competence development of *S. mutans* growing in biofilms.

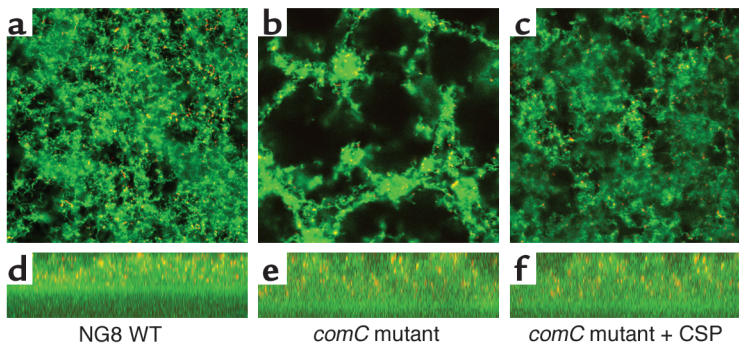


Figure 3
Scanning confocal laser microscope images of 16 h *S. mutans* biofilms. Panels **a-c** show the X-Y planes (top view), while the panels **d-f** show the X-Z planes (side view). The panels illustrate (**a, d**) a normal biofilm formed by the parent strain NG8 and (**b, e**) an aberrant biofilm formed by the *comC*⁻ mutant defective in making a CSP. The wild-type appearance of the biofilm is restored by addition of synthetic CSP (**c, f**). Reproduced with permission from *The Journal of Bacteriology* (12).

S. mutans ComCDE quorum-sensing system was involved in biofilm formation, we examined the ability of *S. mutans* mutants defective in various components of the system to form biofilms. We found that inactivation of any one of the genes encoding the components of the quorum-sensing signaling system results in the formation of an abnormal biofilm (Figure 3) (12). Particularly, the *comC* mutant (unable to produce or secrete the signal peptide) formed a biofilm that lacked the wild-type architecture, whereas the *comD* and *comE* mutants defective in sensing and responding to the signal peptide formed biofilms with a reduced biomass. The architectural change in the *comC* mutant biofilms may be associated with a defect in cell separation with mutations in this gene resulting in the formation of large aggregates or “weblike” biofilms that were easily removed from the surface. The observation that the mutants unable to produce or secrete the CSP formed biofilms that differed from those formed by the mutant defective in the *comD* or *comE* genes suggested that *S. mutans* has multiple CSP receptors. Although we have clearly demonstrated that the ComCDE quorum-sensing system is directly connected to the ability of *S. mutans* to form biofilms, the molecular and biochemical mechanisms involved in expression of the “wild-type biofilm phenotype” remain to be investigated.

CSP modulation of the ATR

The pH levels in dental biofilms are highly variable and frequently shift from above pH 7.0 in the resting pH state to as low as pH 3.0 during the ingestion of dietary carbohydrates by the host. Thus, pH exerts a significant ecological pressure on *S. mutans*, and its ability to tolerate and grow in low pH environments is crucial to its survival and eventual dominance in dental plaque, leading to caries (27). Considerable evidence has shown that *S. mutans* has evolved a number of sophisticated mechanisms to survive these pH changes including induction of an ATR in which exposure of *S. mutans* cells to a mild

or moderately acidic pH (5.0–6.0) results in enhanced survival of a significant proportion of the cell population in a lower pH of 3.0–3.5 (28). This ATR involves a number of de novo proteins that appear to be important for adaptation to an acidic environment (29). Although many of the molecular mechanisms of the ATR in *S. mutans* remain unclear, this “signal pH” that results in synthesis of protective proteins appears to be important for induction of the ATR.

It is widely accepted that bacteria living in biofilms are more resistant to mechanical, physical, and chemical stresses. Since *S. mutans* normally resides in high cell density biofilms, the ability to withstand acid in this physiological state is likely an important adaptive response. We therefore addressed the question of whether acid adaptation involved cell density-dependent events or cell-cell signaling in biofilms. Changes in external pH can significantly influence many physiological parameters, such as energy coupling, ion transport, proton movement, and export of metabolic products, thereby triggering numerous secondary signals. During growth at pH 5.0, *Escherichia coli* can signal stress tolerance to other unadapted cells by secreting a proteinlike molecule, termed extracellular induction component (EIC) (30). Although the signal molecule remains unidentified, induction of stress (including acid) adaptation in *E. coli* presumably involves cell-cell communication. Since *S. mutans* normally encounters acid while living in dense biofilm communities, we proposed that quorum sensing at high cell densities of *S. mutans* might facilitate its survival against low pH challenges. Testing this hypothesis has led us to find that the ATR interfaces with the density-dependent signaling pathway that also initiates genetic competence. We have demonstrated that mutants defective in the *comC*, *D*, or *E* genes have a diminished log-phase ATR and the neutralized culture filtrates prepared from acid-adapted wild-type cells also induce a partial log-phase ATR in cells that have never encountered the signal pH (31). *S. mutans* grown at high cell density established adaptation to the signal pH more rapidly than cells grown at low density. Similarly, *S. mutans* cells grown in a high cell-density biofilm were more resistant to the killing pH than planktonic-phase cells. In fact, *S. mutans* cells grown in biofilms not only survived better than the planktonic cells but also were capable of growth at the lower pH following a glucose pulse. Based on the evidence obtained from this work, we propose that *S. mutans*, upon exposure to low pH in a growing culture, releases extracellular signal molecules, one of which is the CSP, to enhance induction of acid adaptation in the population. It is likely that optimal induction of acid adaptation in a population of *S. mutans* requires a coordinated activity through mechanisms involving both low pH induction and cell density-dependent intercellular signals.

Biofilms likely provide bacterial cells with a unique environment to fully express their adaptive survival mechanisms. Because of three-dimensional structures, high cell density, and diffusion barriers, bacterial cells at different locations within a biofilm may not sense the same degree of extracellular stress simultaneously. The cells that first sense a pH stress may rapidly process the information and pass their “secondary signal” to the other members of the population through cell-cell signaling systems to initiate a coordinated protective response against potentially lethal forces, like acid. Unlike some planktonic cells that need to reach a critical concentration of signal molecules and cell density, biofilms can allow signal molecules to accumulate rapidly in the local environment to initiate coordinated activities far more quickly (25). In addition, physiological states of bacterial cells living in a biofilm, in terms of growth rate, growth phase, or metabolic activities, are heterogeneous; this allows the cells to respond to stress in different ways. Apparently, biofilm populations have several advantages over their free-living counterparts since the cells have more time, a sufficient concentration of signal molecules, and high population density to adapt to stress relative to planktonic cells. The high cell density biofilms may provide a unique environment for induction of acid adaptation via quorum sensing in *S. mutans*. It is likely that the *S. mutans* quorum-sensing signaling pathway is significant for the ATR to intersect with the regulatory networks initiating genetic competence as well as a switch to the “biofilm phenotype.”

The ComC signal peptide may activate more than one signal transduction pathway

Our previous study revealed that an *S. mutans comC* mutant unable to produce CSP formed a biofilm that differed from that formed by the mutant defective in the *comD* or *comE* genes suggested that there might be a second receptor that also responded to the CSP but activated a different pathway in order to invoke the phenotype (12, 20). Based on the available information, we have proposed a “two-receptor” cell-cell signaling model to illustrate how the quorum-sensing system in *S. mutans* functions to regulate genetic competence, biofilm formation, and the ATR (12). The principle of this model is that the signal peptide (CSP) encoded by *comC* can simultaneously interact with two cognate receptors, one encoded by *comD* and another encoded by an unknown gene. These receptors likely transfer the input signal through two different pathways. Although the genes encoding the components involved in the second transduction pathway remain unknown, the work in our lab has recently characterized another TCSTS, named HK/RR11, which is also demonstrated to involve biofilm formation and acid resistance in *S. mutans* (32).

One of the defects observed in HK/RR11 mutant biofilms was the development of a spongelike architecture composed of cells in very long chains, a feature that we previously observed with the biofilm formed by the *comC* mutant unable to produce CSP. Since there is no

putative substrate, signal or function assigned for the HK/RR11 transduction system, we suspected that the TCSTS encoded by *hk/rr11* may activate a second pathway to respond to the CSP. To test this hypothesis, we added CSP to the HK/RR11 mutant cultures to assess the effect on chain formation by the *hk11* and *rr11* mutant biofilm cells. The results revealed that addition of CSP to the mutant cultures had no observable impact on the length of cell chains comprising the mutant biofilms. This result was consistent with HK11 acting as a CSP receptor but provided no direct evidence to conclusively assign a role to HK11 as a CSP receptor. A study for characterizing the relationship between the *comC*-encoded signal peptide and the HK/RR11 signal transduction pathway is now underway.

Evaluation of CSP analogs to act as inhibitors of biofilm formation

Analogues of quorum-sensing peptides can competitively inhibit the activity of the peptide-mediated phenotype. It has been demonstrated that analogous signal peptides from *Staphylococcus epidermidis* can inhibit the *agr* signaling system of *Staphylococcus aureus*, thereby modulating *S. aureus* virulence (33). Since peptide signaling-sensing systems are very similar among Gram positive bacteria (including the *S. mutans* CSP system) analogs of the *S. mutans* CSP may be able to interfere with the quorum-sensing process, leading to inhibition of induction of the “biofilm phenotype.”

Mucosal pathogens

Quorum sensing among mucosal pathogens may not require high cell density. In contrast to the very dense dental plaques colonized by *S. mutans* and several other oral streptococci, pathogenic streptococci that infect mucosal tissues colonize environments where bacterial microcolonies are usually less dense due to bathing effects of secretions and desquamation of epithelium. Yet, several of these streptococcal and other Gram positive species have evolved peptide pheromone signaling pathways that have autoregulating functions analogous to the quorum-sensing systems that we described above for *S. mutans*. The finding that initiation of genetic competence in *S. pneumoniae* is sensitive to relatively low levels of CSP at about 10⁷ bacteria/ml (34) suggests that pathogenic streptococci of mucosal surfaces may respond to gradients of cell densities that occur frequently during natural infections, raising the question of whether quorum sensing affects pathways by which pathogenic streptococci grow to predominance over normally protective indigenous species. It has been recently established that mutants of *S. pneumoniae* defective in the quorum-sensing competence induction pathway have diminished virulence in a murine model relative to the parent strain (35). Differential fluorescence induction also showed that several *Com* genes were induced during infection in mice (36). Clearly, the role of quorum sensing during infection by *S. pneumoniae* warrants further examination.

Bacteriocins

Bacteriocins are antimicrobial peptides that are generated by some bacteria and target others that are sensitive. The signaling networks that regulate bacteriocin production and transport as well as immunity to bacteriocins involve peptide pheromone sensing pathways that are very similar to those involved in genetic competence (8). Operons encoding bacteriocin peptide precursors, cognate peptide processing transporters, and two-component regulatory sensors and response regulators are known to be under the control of cell density. For example, the virulence-related *blp* and *com* regulons of *S. pneumoniae* have many analogous features including highly similar signal peptides and peptide secretion systems (15). Moreover, activation of the competence and bacteriocin peptide pheromone response regulators may, in turn, activate downstream pathways that intersect in a common regulatory gene. One such example is the previously mentioned *comX*, a transcriptional regulator of so-called “late” competence genes (9). The genome of M1 *S. pyogenes* strain SF370 contains *comX* and a complete bacteriocin *salA1* locus; yet, it lacks the genes *comABC* that encode the CSP and its secretion apparatus (11). This implies that *S. pyogenes comX* (and downstream pathways) may be activated in response to environmental signals other than CSPs. *S. pyogenes* is a pathogen with a host range limited to humans and a unique set of virulence properties not shared by other streptococci. It is possible that it has evolved a sophisticated bacteriocin peptide regulatory system to help its population competitively emerge on mucosal surfaces, yet protect its genome from contamination with foreign DNA by deleting (or never evolving) genes for the cell density-dependent CSP. Recently, Jenkinson’s lab reported that the sensory and immunity pathways for the pheromone antibiotics SalA1 of *S. pyogenes* and SalA of the indigenous oral species *Streptococcus salivarius* are conserved, closely related, and cross-sensitive (37). Such novel findings suggest that determinants of population dynamics on bathed mucosal surfaces probably encompass subtle combinations of complex environmental sensing systems that are not limited to cell density, bacteriocin production, and competence stimulation. It is also possible that *S. pyogenes* utilizes a bacteriocin-like hemolysin as a quorum-sensing molecule. Recently, *Enterococcus faecalis* was shown to regulate gene expression by such a mechanism involving its cytolysin, a molecule with both antimicrobial and hemolytic activity (38). *S. pyogenes* has a hemolysin that is genetically very similar to quorum-sensing autoinduction operons found in streptococci (39).

Fluctuations in total bacterial burden and population density also determine the pathogenicity of bacterial microcolonies and biofilms. For example, the ability to suppress competing streptococci through bacteriocin activity may be considered a virulence property if it allows the pathogenic species to emerge sufficiently to damage the host. Evidence is growing that cell density-dependent gene regulation also affects the expression of many other virulence-related proteins of pathogenic streptococci. For

example, virulence factor expression and regulation are, in part, determined by growth phase and activation of two-component regulatory sensors of environmental signals, which are characteristics of quorum sensing in Gram positive bacteria (38). Thus, the extracellular concentration of autoregulating peptides appears to serve as one of several environmental conditions that regulate virulence genes through signal transduction pathways that are initiated via two-component regulatory systems. In *S. pyogenes*, in addition to *sagA*, there appear to be several coordinated regulatory loci such as *mga*, *csrRS*, *fasBCA*, and *rgg* that affect the expression of genes for its numerous and diverse virulence factors.

Such a pattern of global regulation is reminiscent of the induction of virulence genes via quorum-sensing pathways in biofilms of *Pseudomonas aeruginosa*. Therefore, it is likely that the genomes of streptococci have evolved density-dependent regulons to control expression of downstream genes that affect bacterial survival in response to changing environmental conditions on mucosal and tooth surfaces, and the selective survival of the pathogenic streptococcal species leads to clinical infection. Although the key molecules of the quorum-sensing pathways of pathogenic streptococci such as *S. pneumoniae*, *S. pyogenes*, and *S. mutans* are distinct from those first reported for Gram negative bacteria, they seem to serve an analogous function, to modulate physiologic homeostasis and adaptation to environmental conditions in response to fluctuations in population density. The use of this information to exploit these pathways to control streptococcal infections is now being implemented and in the near future we may see a more selective and targeted approach to controlling persistent biofilm-dwelling bacteria.

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