Quorum Sensing in Streptococci

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Abstract Emerging studies aimed at understanding the molecular underpinnings of streptococcal infections highlight the importance of quorum sensing (QS) for biofilm formation and virulence in many streptococcal species. Among streptococci, the best characterized quorum sensing system belongs to *Streptococcus pneumoniae*. Although initially QS was believed to act mainly to acquire and incorporate foreign DNA into the host chromosome, studies implicating its involvement in biofilm formation, fratricide as well as virulence suggested a broader and more varied role for this system in *S. pneumoniae*. In this chapter, we will discuss the QS system of pneumococci, as well as other streptococci including *Streptococcus mutans* and *Streptococcus pyogenes*. Furthermore, we will also discuss some interesting studies that have been conducted recently to target QS as a tool to combat or modulate infections associated with streptococcal biofilms.

1 Streptococcal Biofilms

In humans, most members of the genus *Streptococcus* belong to the normal commensal flora that inhabit the mouth, skin, intestine, and upper respiratory tract. Infections such as strep throat, dental caries, meningitis, bacterial pneumonia, endocarditis, and necrotizing fasciitis (infamously known as the "flesh-eating" disease) are caused by pathogenic streptococcal strains whose disease etiology has been studied extensively. In contrast to the original view that most of these bacteria act as individual unicellular organisms, the discovery of quorum sensing (QS) and how this

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elegant mechanism can synchronize the production of virulence actors concomitant to cell population density has shed new light on understanding and conquering infectious diseases caused by these bacteria. Moreover, the finding that streptococcal QS plays a major role in maintaining the structural and functional integrity of the biofilm phenotype makes QS an attractive and rather promising target to attenuate virulence caused by these strains. In this chapter we will discuss some of the recent findings pertaining to QS-based virulence modulation strategies that are being developed for use in *Streptococcus pneumoniae*, *Streptococcus mutans*, and *Streptococcus pyogenes* infections as potential chemotherapeutic agents to control or prevent streptococcal biofilm-mediated infections.

2 Peptide-Based Quorum Sensing in Streptococci

There is a growing body of literature strongly implicating the switch from planktonic to sessile growth as a major phenotypic shift required for streptococcal virulence (Cvitkovitch et al. 2003). One of the most well-known and best-studied pathogenic biofilms predominated by streptococci is human dental plaque, which harbors hundreds of species of bacteria (Kroes et al. 1999). This chapter will focus on the QS systems of streptococci that reside permanently or transiently in the human oral cavity and the ways that biofilms are linked to infection and signaling processes. The primary components and mechanisms utilized for QS by *S. mutans*, *Streptococcus gordonii*, and other streptococci are essentially the same as for *S. pneumoniae*. The phenotype originally associated with density-dependent signaling was the induction of genetic competence, the transient physiological state that bacteria enter to facilitate uptake and incorporation of exogenous DNA (Morrison and Lee 2000).

The signal molecule associated with the QS phenomenon is called competence-stimulating peptide, or CSP (Håvarstein et al. 1995a). CSP in *S. pneumoniae* and *S. mutans* and competence factor in *S. gordonii* are derived from their precursor propeptide molecules that contain a typical Gly–Gly cleavage site and an N-terminal leader sequence that is removed via proteolysis by the ComAB transporter during export to produce the biologically active autoinducer peptide (Håvarstein et al. 1995b). The *S. pneumoniae* CSP is a 17-amino-acid (aa) peptide processed from a 41-aa precursor (Håvarstein et al. 1995a), whereas the *S. gordonii* competence factor comprises 19 aa obtained from a 50-aa precursor (Lunsford and London 1996). The leader sequence of the *S. pneumoniae* CSP was found to belong to a family of double-glycine-type leader peptides that contained the consensus LSX_2ELX_2IXGG with hydrophobic residues at positions -4, -7, -12, and -15 relative to the Gly–Gly cleavage site (Håvarstein et al. 1994, 1995b). Sequence analysis of the *S. mutans* genome database allowed deduction of its CSP to consist of a 21-aa CSP derived from a 46-aa precursor, and the addition of synthetic CSP to growing cultures confirmed its identity (Li et al. 2001b).

In pneumococci, the QS system is encoded by two distinct genetic loci, *comCDE* and *comAB* (Håvarstein et al. 1995a). The *comC*, *comD*, and *comE* genes encode the CSP peptide-precursor, the histidine kinase (HK), and response regulator (RR), respectively (Cheng et al. 1997; Håvarstein et al. 1995a, 1996; Pestova et al. 1996). The secretion apparatus necessary for CSP maturation and export is encoded by the *comA* and *comB* genes. Based on the model proposed in *Streptococcus pneumoniae*, when the mature CSP peptide reaches a threshold concentration, it binds to the membrane-bound HK sensor ComD, causing its autophosphorylation. Phosphorylated ComD then transfers the phosphate group to its cognate intracellular RR ComE, which then activates the transcription of *comX* encoding an alternative sigma factor. ComX (Luo et al. 2003) together with the ComW protein (Luo et al. 2004; Sung and Morrison 2005) enable transcription of several late competence-related genes involved in DNA uptake and integration.

The first evidence that QS was involved in streptococcal biofilm formation came from a study of *S. gordonii*, a commensal bacterium in human dental plaque (Loo et al. 2000). These investigators recovered a biofilm-defective mutant following transposon mutagenesis that had an inactivated *comD* gene encoding the HK receptor that detects the QS autoinducer peptide. More recently, Gilmore et al. used real-time polymerase chain reaction to study the expression of *S. gordonii* genes known or assumed to be involved in biofilm formation. They demonstrated that both *comD* and *comE* – which comprise the two-component signal transduction system in its QS – were both upregulated in the biofilm phase (Gilmore et al. 2003).

Following the pioneering work in *S. gordonii*, a link between biofilm formation and QS was solidly established in *Streptococcus mutans* (Li et al. 2001b, 2002). This bacterium, which resides in the oral cavity, is strongly regarded as a principal etiologic agent of dental caries, which is one of the most prevalent chronic childhood ailments (more common than asthma). In the mouth, *S. mutans* relies on a biofilm mode of growth for its survival by anchoring itself to hard surfaces and evading the hostility of the salivary flow.

The linkage between QS and biofilm formation in *S. mutans* was established by demonstrating that mutants defective in any of the *comC*, *comD*, *comE*, or *comX* genes formed abnormal biofilms (Li et al. 2001b, 2002), and the addition of exogenous CSP to a *comC* mutant restored the normal biofilm architecture (Li et al. 2002). Other phenotypes activated by CSP were an increased resistance to acid (Li et al. 2001a) and modulation of bacteriocin production (Kreth et al. 2006).

In a recent study, Oggionni et al. (2004, 2006) established a link between the CSP QS system and biofilm formation in *S. pneumoniae*. CSP was able to induce biofilm formation *in vitro*, and a mutant defective in the ComD receptor, which did not form biofilms, also showed reduced virulence. These results were in contrast to those found in a bacteremic sepsis model of infection, in which the competence system was downregulated. These researchers also found that when the infective bacteria from different physiological states were used to infect mice, biofilm-grown cells were more effective in inducing meningitis and pneumonia whereas liquid-grown planktonic cells were more effective at inducing sepsis.

One can now argue that the simple transient phase of competence was merely the first discovery of the multiple roles of CSP-mediated QS in most streptococci, which we propose is the "master switch" between planktonic and biofilm lifestyles.

3 Population-Density-Dependent Cell Death

Although natural genetic transformation of pneumococci was extensively investigated for over eight decades following its initial discovery by Frederick Griffith in the 1920s, the source of donor DNA for transformation was traditionally regarded as DNA that originated from dead cells that fell apart from natural causes. Despite this belief, accumulating evidence from recent experiments shows that in *S. pneumoniae*, recipient or competent cells are capable of ensuring the availability of donor DNA by coordinating competence with the lysis of noncompetent cells of the same strain in the population (Steinmoen et al. 2002, 2003). Moreover, in *S. mutans* it was observed that the addition of exogenous CSP in excess of the amount necessary for competence inhibited cell growth and that a further increase of CSP led to cell death (Qi et al. 2005). Clearly, during a time in which there is a great need to develop novel strategies to combat virulence, these studies come as positive news in getting us one step closer to developing successful drugs against biofilm-mediated infections. In the following sections we will discuss competence-induced cell death in *S. pneumoniae* and *S. mutans*, as well as how we can possibly exploit QS mechanisms of these bacteria to design novel therapies against bacterial infections.

3.1 QS-Induced Allolysis in S. Pneumoniae

Steinmoen et al. (2002) recently demonstrated that during cocultivation, competent streptococci grown in liquid culture were able to actively acquire transforming DNA by killing 5–20% of their noncompetent siblings when exposed to CSP. These researchers showed that lysis and DNA release were initiated with the induction of the competence state and that the efficiency of this process was influenced by cell density (Steinmoen et al. 2003). The discovery of this fratricide phenomenon has enormous significance from an evolutionary perspective in enhancing the genetic plasticity of *S. pneumoniae*. Although identification of the molecular underpinnings of this cell-lysis mechanism is still in the early stages, a two-peptide bacteriocin called CibAB is believed to be required for the allolysis. Moreover, competence-induced allolysis also requires the production of the major autolysin LytA and the lysozyme LytC, which can be supplied by the competent cells or the targeted cells (Guiral et al. 2005; Knutsen et al. 2004; Moscoso and Claverys 2004; Steinmoen et al. 2002, 2003). In addition, it was also observed that a QS-mutant deficient in the *comE* gene was incapable of cell lysis, thereby linking the ComDE signal transduction system with DNA release and uptake (Steinmoen et al. 2002).

In their reports, Steinmoen et al. (2002, 2003) claimed that the fratricide phenomenon was present in *S. pneumoniae* to ensure the presence of sufficient homologous DNA during genetic transformation. However, a more recent investigation by Moscoso and Claverys (2004) showed that although competence decreased after 20 min following the addition of CSP, the amount of liberated DNA continued to increase and reached a maximum in the stationary phase, when cells were no longer capable of DNA uptake (Moscoso and Claverys 2004). Hence, they argued against the role of fratricide as a means to acquire DNA for maximized genetic exchange and suggested a different role for this observation, including a possible role in nutrient acquisition, biofilm formation, or the release of toxins (such as pneumolysin, teichoic, and lipoteichoic acids).

3.2 CSP-Induced Cell Death in S. Mutans

In *S. mutans*, the ComCDE QS system regulates several physiological properties, including competence development, biofilm formation, acid tolerance, and bacteriocin production. The induction of the latter three phenotypes especially suggests that the CSP-induced QS system likely responds to environmental stress; therefore, it is also likely that an overreaction to stress may cause a detrimental effect on the cells. Recently this idea was tested by Qi et al. by overdosing *S. mutans* with CSP at a concentration higher than that normally used to induce competence (Qi et al. 2005). It was demonstrated that at a slightly higher than normal (0.65 versus 0.5 μM) concentration of CSP, growth of *S. mutans* was inhibited in planktonic and biofilm cells. Further analyses revealed that CSP exerted this effect by inhibiting cell division, ultimately leading to cell death, while mutational analyses suggested that the ComDE QS pathway mediated CSP-induced cell death in a ComX-independent manner. A preliminary examination of the inhibitory effect of CSP on other *S. mutans* strains suggested that the effect was highly variable among strains. Even within the sensitive strain, only about 20% of the cell population progressed to cell death after CSP treatment. Whether this partial inhibition in a cell population would give commensal streptococci a head start to become dominant awaits further experimentation. It remains to be determined whether the higher-than-normal doses of CSP used in the various studies have relevance in the context of a biofilm in which the local concentration of peptide is likely to be high.

3.3 Competence-Induced Bacteriocin Production by S. Mutans

The involvement of CSP in multiple cellular functions has prompted scientists to search for its ecological functions in a multispecies environment such as dental biofilm. In a recent study (Kreth et al. 2005), it was demonstrated that CSP was required to activate a group of bacteriocin (mutacin) genes in *S. mutans*. One of the

mutacins, mutacin IV, was shown to have activities specifically against a group of oral streptococci, the "mitis" group streptococci, which comprise the pioneer colonizers during dental biofilm development and which are known to have antagonistic activities toward *S. mutans*. Further studies demonstrated that mutacin production was required to cause DNA release from neighboring streptococcal species when *S. mutans* became competent (Kreth et al. 2006). More interestingly, there was a programmed time delay for competence development after the addition of CSP. Although mutacin production responds to CSP within 30 min after CSP addition, competence does not develop until 2–2.5 h after the addition of CSP. This time delay was suggested as a mechanism to ensure DNA availability when the cells become competent. Recently, a lantibiotic mutacin, Smb, was also found to be regulated by CSP in strain GS-5. Whether this regulation is also related to DNA release from other streptococcal species has yet to be determined.

4 Targeting Streptococcal Quorum Sensing

4.1 CSP as a Potential Therapeutic Agent

An important study (Oggioni et al. 2004) used an *in vivo* sepsis mouse model to assay the effect of exogenous CSP addition on virulence. This group found that CSP-treated infected mice had a significant increase in survival rates, reduced pneumococcal blood counts, and increased length of survival relative to control mice. *In vitro* CSP addition at the same concentration used in the mice elicited a transient growth inhibition. A receptor HK mutant did not have a bacteriostatic phenotype *in vitro* and was not responsive to CSP *in vivo*. This work shows that CSP induces a temporary growth arrest that alters the outcome of the infection. Interestingly, this therapeutic effect was somewhat unexpected because the positive effect was obtained by activating the target molecule rather than inhibiting it.

In addition to being used as a pheromone to disrupt a cell's normal metabolic activity, the target specificity of CSP was also used recently as a homing device to deliver antimicrobial peptide to the target bacterium (Eckert et al. 2006). Eckert et al. demonstrated that by fusing the C-terminal 16 aa of the *S. mutans* CSP (the full-length CSP is 21 aa) to a wide spectrum, defensin-derived antimicrobial peptide G2, they could specifically eliminate *S. mutans* from mixed cell cultures with other closely related streptococcal species. Furthermore, the species specificity of the C16 peptide could be further narrowed down to 8 aa (C8). Interestingly, the target specificity of C16 and C8 is independent of ComD, suggesting that CSP may have a secondary binding site on the cell surface. It is worth noting that a similar process was also attempted in *S. aureus*, where the CIP pheromone fused to colicin was shown to be able to kill *S. aureus*.

Two studies have recently revealed structure-function relationships between *S. mutans* CSP and its competence- and bacteriocin-inducing abilities. The first of these studies showed that a peptide similar to CSP but lacking the three

C-terminal residues was more potent than CSP at inducing competence, biofilm formation, and bacteriocin production (Petersen et al. 2006). A subsequent study examined the ability of synthetic CSP analogs to activate or inhibit competence and modulate expression from the *comC* promotor (Syvitski et al. 2007). The use of truncated CSP peptides in conjunction with circular dichroism and nuclear magnetic resonance revealed that the *S. mutans* CSP has at least two functional domains. The C-terminal end is required for activating the signal transduction pathway, while the alpha-helical core is required for binding to the ComD receptor. If three or more peptides were deleted from the C-terminus, genetic competence was not induced, but QS could be competitively inhibited in the presence of wild-type CSP. These data suggest that at least in vitro, modulation of the CSP regulon can be manipulated by CSP analogs. Further research into their effectiveness in animal and human models may demonstrate chemotherapeutic potential of these or other QS analogs.

4.2 QS Modulation of Infection in Group A Streptococcus

S. pyogenes, or group A streptococcus (GAS), is responsible for causing a number of infections in humans, from the most common form of bacterial pharyngitis (strep throat) to the highly invasive and often fatal necrotizing fasciitis (Cunningham 2000). It has been recently suggested that a functional QS peptide modulates the pathogenic potential of this bacterium (Hidalgo-Grass et al. 2004). The SilCR peptide, or SilC, has features typical of QS signal peptides in gram-positive bacteria (Hidalgo-Grass et al. 2004; Miller and Bassler 2001). The *sil* locus was originally described in an M14 serotype strain of *S. pyogene*s demonstrated to be involved in invasive disease and DNA transfer (Hidalgo-Grass et al. 2002). This five-gene locus encodes a putative two-component system (*silA* and *silB*), a region similar to an ATP-binding cassette transporter (*silD* and *silE*), and a small open reading frame preceded by a combox-like promoter (*silC*). The SilCR region encodes a predicted 41-aa propeptide with a typical glycine-glycine sequence motif that facilitates cleavage, producing a 17-aa mature peptide. SilCR is proposed to be a QS signal peptide that modulates the expression of several unidentified genes in *S. pyogenes* (Hidalgo-Grass et al. 2004).

The exogenous application of SilCR to infection wound sites was shown to affect the outcome of infection in mice inoculated with invasive isolates of *S. pyogenes*. In this study, decreased lesion formation and size were observed in mice infected with *S. pyogenes* in the presence of exogenously added SilCR (Hidalgo-Grass et al. 2004).

Subsequent genomic analyses of several M serotypes of *S. pyogenes* indicated that either the *silCR* gene or other genes in the operon were mutated or absent in the genomes of invasive isolates. It was suggested that SilCR has a regulatory effect on the invasive potential of *S. pyogenes*, somehow reducing invasiveness when present since strains with an intact expressed operon were considered noninvasive.

The therapeutic effect of SilCR was dose dependent and was shown to reduce lesion size with the addition of as little as 3.0 μg of peptide.

The mechanism of SilCR is believed to involve the downregulation of GAS chemokine trypsin-like protease activity responsible for an increase in the migration of neutrophils to the infection site, thus limiting bacterial spread (Hidalgo-Grass et al. 2006). Three serine proteases of *S. pyogenes* were believed to be modulated: X-propyl-dipeptidyl aminopeptidase (PepXP), HtrA/DegP, and PrtS/CspA, enzymes capable of degrading interleukin 8, thus inhibiting neutrophil recruitment to the site of infection.

Further study of SilCR will likely focus on PepXP and PrtS/CspA and other GAS genes and will determine whether SilCR directly affects the expression of host factors.

5 Conclusions and Future Perspectives

It is becoming apparent that QS is nearly always involved in streptococcal biofilm infections. It is therefore logical to study these processes and manipulate these signals to alter disease outcome. The three modes of action of signal perturbation discussed in this article involve application of native signal to alter the colonization stage of infection or addition of CSP analogs to either overstimulate or inhibit CSP action. These works are in their infancy, and further testing of these concepts in animal and human subjects will ultimately determine whether they are feasible therapeutic agents.

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