A Quorum Sensing-Regulated Protein Involved in Biofilm Formation and Lysozyme Resistance in *S. pyogenes*

BY

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THESIS

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“The truth is rarely pure and never simple”

Oscar Wilde

The Importance of Being Earnest

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CONTRIBUTION OF AUTHORS

Chapter I (Introduction) of this dissertation includes parts of a published review of the literature concerning this thesis work (Jimenez JC and Federle MJ, 2014. Quorum sensing in group A Streptococcus. *Front. Cell. Infect. Microbiol*. **4**:127. doi: 10.3389/fcimb.2014.00127). This publication was written primarily by me, and Dr. Michael J. Federle contributed to the writing process as well.

Chapter III contains results of my own unpublished experiments as well as my own results extracted from two published manuscripts (Aggarwal, C., Jimenez, J.C., Nanavati, D., and Federle, M.J. 2014, *JBC*, doi: 10.1074/jbc.M114.583989; and Aggarwal, C., Jimenez, J.C., Lee, H., Chlipala, G.E., Ratia, K., and Federle, M.J. 2015, *mBio*, doi: 10.1128/mBio.00393-15). In both of these publications I was second author, and I performed, analyzed and wrote the results for key experiments that helped us to phenotypically follow transcriptional processes studied by Dr. Aggarwal.

Chapter IV contains data of my own unpublished experiments directed at answering the mechanics of the phenotypes described in Chapter III. This chapter harbors key data for future experimental endeavors in the lab, and this data together with the unpublished results from Chapter III will be soon featured in a first author publication written by me.

Chapter V contains my own results extracted from one published manuscript (Chang JC, Jimenez JC and Federle MJ, 2015. Induction of a quorum sensing pathway by environmental signals enhances group A streptococcal resistance to lysozyme. *Molecular Microbiology* **97**:(6), doi: 10.1111/mmi.13088). I was a second author in this manuscript, and I performed, analyzed and wrote the results required for the understanding of the mechanics of one environmental signaling process discovered by Dr. Chang.

Chapter VI contains an overview of the obtained data, and discussion of the possible molecular targets that could complete the working model proposed based on the results of this work.

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LIST OF ABBREVIATIONS

::	Novel junction (fusion or insertion)
AHL	Acylated homoserine lactones
AI-2	Autoinducer-2
ABC	ATP-binding cassette
AMP	Antimicrobial peptides
bp	Base pair
CCR	Carbon catabolite repression
CDM	Chemically defined medium
CRE	Catabolite responsive element
Cm	Chloramphenicol
C-terminal	Carboxyl-terminal
DMSO	Dimethylsulfoxide
Erm	Erythromycin
GAS	Group A *Streptococcus* (*S. pyogenes*)
gDNA	Genomic DNA
GlcNAc	N-acetylglucosamine
h	Hours
HTH	Helix-Turn-Helix
HGT	Horizontal gene transfer

LIST OF ABBREVIATIONS (continued)

Kan	Kanamycin
luxAB	Luciferase genes A and B
m	Minute(s)
MurNAc	N-acetylmuramic acid
OD_{600}	Optical density at 600nm
PCR	Polymerase chain reaction
PTS	Phosphotransferase system
QS	Quorum sensing
RBS	Ribosome binding sequence
SHP	Short hydrophobic peptide
Sp	Spectinomycin
TCS	Two-component system
THY	Todd-Hewitt broth

SUMMARY

Cell-to-cell communication events in bacteria coordinate collective behaviors in a process known as quorum sensing (QS). QS systems are based on the basic mechanism of production, accumulation, and detection of chemical signals called autoinducing peptides or pheromones. Recently, a novel QS pathway in the human pathogen *Streptococcus pyogenes* has been characterized, the Rgg2/3 pathway. Rgg2 and Rgg3 are two antagonistic transcription factors that regulate expression of target genes in response to SHP pheromone peptides. While the molecular events involved in the signaling through the Rgg2/3 pathway are well understood, the behaviors regulated and their effect over the physiology of *S. pyogenes* are unknown. In this work we show how the activation QS signaling by SHP (Small Hydrophobic Peptide) pheromones triggers the processes of cellular aggregation and biofilm formation in the NZ131 isolate of *S. pyogenes.* The increase in biofilm is dependent on the activation of target gene expression by Rgg2, and can be triggered by the different SHP pheromone variants produced by *S. pyogenes.* Additionally, the increase in biofilm levels be prevented by the use of specific QS inhibitor molecules cyclosporin A and valspodar, which directly compete for binding of Rgg2/3 with the SHP pheromones. In order to understand the molecular mechanisms at work, we generated deletions in several target genes of Rgg2/3 and followed biofilm formation. Results showed that a small secreted protein, 0414c, was required and sufficient to trigger biofilm increase. Additionally, lysozyme resistance, a novel phenotype related with Rgg2/3 activation, was also dependent on 0414c. Bioinformatic analysis has led us to hypothesize that the 0414c protein works as a cysteine protease inhibitor. In order to unveil putative targets of 0414c inhibition, we generated deletions in several cysteine proteases encoded in the NZ131 genome. Deletion of the *isp2* gene partially recapitulated the phenotypes seen by 0414c expression, suggesting the Isp2 protein is a putative target of 0414c. Isp2 encodes a CHAP cysteine peptidase, predicted to be involved in cell wall modification processes. Additionally, the secreted 0186c protease was required to trigger the full biofilm response seen after 0414c expression. These results point towards a role for 0414c in modulating the activity of cysteine proteases that can tailor the components of the cellular envelope of *S. pyogenes.*

Finally, we also uncovered the genes required for the transduction of environmental cues involved in triggering Rgg2/3 signaling. Previously, we have shown that SHP pheromone expression is induced in the presence of the monosaccharide mannose. In this work we show a precise sugar importer complex, PtsABCD, is dispensable for growth but essential for activation of Rgg2/3 QS communication in response to mannose.

I. INTRODUCTION

(Reprinted in part, with permission, from Jimenez JC and Federle MJ (2014) Quorum sensing in group A Streptococcus. *Front. Cell. Infect. Microbiol*. **4**:127. doi: 10.3389/fcimb.2014.00127[a])

1.1 Quorum sensing

For a long time, bacteria were thought of as organisms carrying out self-sufficient and independent unicellular lifestyles. During the last forty years, several studies have demonstrated how, in fact, bacteria interact and establish complex social behaviors with their siblings and with other bacteria in their community to develop beneficial actions for the population, by means of conserved chemical languages. Quorum Sensing (QS) is the communication process in which bacteria produce, secrete and detect chemical signals with the purpose of triggering specific phenotypes. QS regulates genes involved in population-wide responses and behaviors that are beneficial when performed as a synchronous group rather than at the individual level and which include bioluminiscence, sporulation, competence, antibiotic production, biofilm formation and secretion of virulence factors [1]–[3].

While all QS systems are based on the basic mechanism of production, accumulation, and detection of a chemical signal, the nature of these signals differ between different bacterial groups. Three general types of QS signals have been described: acylated homoserine lactones (AHL), utilized by Gram-negative bacteria; peptide signals, used by Gram-positive bacteria; and the cyclic furanone compounds of the autoinducer-2 (AI-2) family, used by both Gram-negative and Gram-positive bacteria. Although AI-2 has been shown to be produced by several bacteria, the involvement of this molecule in precise QS signaling pathways has only been demonstrated in a subgroup of species, and within the Gram-positives only the *Bacillaceae* family has characterized AI-2 receptor proteins. In the other Gram-positive species, the cellular machinery required for sensing and transducing the AI-2 signal, or even the precise effects of AI-2 over gene regulation are unknown [4], [5]. For this reason, peptide based signaling is the major mechanism characterized to date by which Gram-positive bacteria establish QS communication events.

1.2 Peptide-mediated quorum sensing in Gram-positive bacteria

QS signaling in Gram-positive bacteria (Figure 1) operates mainly through the activity of post-translationally modified oligopeptides, named autoinducing peptides or pheromones, which can range from 5 to 34 amino acids in length and can adopt either linear or cyclical conformations [6]–[12]. These pheromones are initially synthesized as inactive pro-peptides by the ribosome, and then exported from the cell by either the general secretion pathway (Sec) or by dedicated ABC transporters. During the export process, pro-peptides undergo proteolytic processing (and in some cases additional covalent modification) to generate the active pheromone, and a variety of enzymes are involved in these maturation processes [8], [11], [13]–[17]. When the pheromones surpass threshold concentrations in the extracellular medium they are efficiently detected by transmembrane receptors of the two-component system (TCS) signal transduction family, leading to differential phosphorylation of a response regulator and consequent change in target gene expression. Alternatively, pheromones can be imported into the cytoplasm via peptide transporter complexes, most commonly the Opp/Ami oligopeptide permease, a promiscuous peptide transporter involved in the import of nutritional peptides, peptidoglycan recycling components as well as pheromone peptides for QS systems. Once inside the cell, peptide pheromones bind and directly modulate the activity of transcriptional regulators. As a result, target genes change their expression pattern and genes encoding for the pheromone pre-peptides are upregulated, increasing the production of mature pheromone and generating a positive-feedback loop (or auto induction process), that helps strengthen the QS signaling at the individual level and increases pheromone levels in the environment to activate signaling at the population level.

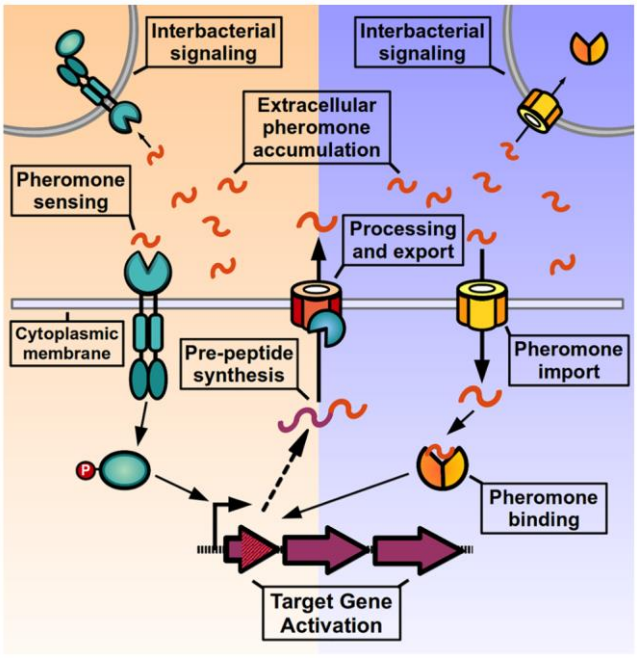


FIGURE 1. Mechanisms of quorum sensing in Gram-positive bacteria. After being translated by the ribosome, pre-peptides are processed, and exported from the cell to generate an active signaling pheromone. Pheromones accumulate in the extracellular medium where they are detected by the producer cell and neighboring bacteria. Pheromone detection can either occur through two-component systems in the bacterial membrane or by direct binding by transcription factors after peptide import. After pheromone detection, the activated response regulator or the pheromone-bound transcription factor induce changes in target gene expression, and genes encoding for pheromone pre-peptides are up-regulated, increasing pheromone production and generating an auto induction process.

1.3 ***Streptococcus pyogenes***

Also known as Group A *Streptococcus* (GAS), *S. pyogenes* is a human-restricted, Gram-positive bacterial pathogen of the Firmicutes phylum. It resides primarily in the oropharynx and the skin of its human host and generates a variety of illnesses that range from uncomplicated superficial infections, like pharyngitis and impetigo, to severe and life threatening invasive infections, like necrotizing fasciitis and toxic shock syndrome [18]. Immune mediated post infection sequelae can also develop in the host, generating pathologies like rheumatic heart disease and glomerulonephritis [19], [20]. Millions of cases of new *S. pyogenes* infections are estimated to occur per year, posing a large health and economic burden [21]. Isolation of *S. pyogenes* from an individual does not necessarily correlate with a disease state, as this bacterium can be isolated from several body sites in asymptomatic individuals, and has been reported to be

carried asymptomatically in approximately 20% of school-aged children. *S. pyogenes* is an organism that develops its complete life cycle inside the human host, passing from non-virulent asymptomatic colonization to symptomatic infection, to transmission and dissemination [22]. Because of this, it is of great interest to understand the ways by which *S. pyogenes* populations regulate their collective behaviors, and how these behaviors may be associated with adaptation to the host environment or with the switch between pathogenic and non-pathogenic states.

A common mechanism by which bacteria change their transcriptional programs is the use of alternative sigma factors of the RNA polymerase, which allow them to redirect transcription initiation to promoters of interest based on the environmental conditions faced by the bacterium [23]. *S. pyogenes* has only one alternative sigma factor, which has a limited role in triggering a cryptic natural competence pathway [24]. Thus, the transcriptome changes in *S. pyogenes* rely on the activity of several transcriptional regulatory proteins [25]. We now know that some of these transcriptional factors form part of novel quorum sensing pathways.

1.4 Quorum sensing in *Streptococcus pyogenes*

Some of the earliest reports describing cell-to-cell communication in bacteria arose from studies of genetic exchange in *Streptococcus pneumoniae*, where it was shown that a "hormone-like cell product" secreted by the bacteria into the culture medium activated their ability to import extracellular DNA and undergo genetic transformation in a process termed competence [26]. Several years later, the signaling molecule was shown to be a secreted peptide that formed part of a quorum-sensing circuit [6]. Since then, several pheromones and their associated QS systems have been discovered and characterized in species of the *Streptococcus* genus [27]. The existence of conserved QS systems in *S. pyogenes* was unknown until recently.

The *S. pyogenes* pan-genome encodes several varieties of QS circuits, harboring the potential to produce an assortment of dedicated signaling molecules that affect expression of several target genes. While there is increasing information regarding the mechanics of these QS networks, our current understanding of these systems is still in its infancy, and despite data regarding regulated genes and observed QS-dependent phenotypes, we do not fully comprehend yet when and where these communication systems are triggered *in vivo*, and how *S. pyogenes* benefits from these processes of population-wide coordination of gene expression.

1.4.1 Sil signaling system

The Sil system was the first QS network characterized in *S. pyogenes*. The *sil* locus was discovered in a tagged-transposon mutagenesis screen designed to identify bacterial mutants that lost the ability to spread systemically on a highly invasive isolate [28]. One of the isolated mutants lost its ability to reach the spleen and had an insertion in a novel locus that was termed streptococcal invasion locus (*sil*), which harbored the machinery to produce, secrete and sense a short oligopeptide. The *sil* QS locus (Figure 2A) is composed of *silAB,* a putative TCS, *silDE,* a putative ABC transporter and *silCR,* the pheromone pro-peptide gene. An additional small ORF encoded in the complementary DNA strand and overlapping with *silCR* is *silC,* which encodes a 39 amino acid peptide disrupted by the original transposon hit during the mutant screen [28]. SilA was shown to be necessary together with SilB, the putative sensor histidine kinase, to trigger expression of genes in response to SilCR [29]. Most of the genes that possess putative SilA binding sites are present in a putative genomic island of 15 - 17 Kbps in size (Figure 2B). These genes increase their expression rapidly after SilCR pheromone addition, and several correspond to predicted bacteriocins and/or bacteriocin maturation components, while the rest of them correspond to transposons or insertion sequences [28]–[30]. The presence of *sil* genes in *S. pyogenes* genomes is not widespread, with only four of the 19 sequenced S. pyogenes genomes containing this system (MGAS8232, MGAS10750, Alab49 and HSC5) [31], [32]. Epidemiological studies have shown that the prevalence of Sil ranges from 12% in non-invasive isolates to 16%-25% in invasive isolates, and that the locus is restricted to a few *emm*-serotypes with M4 being the most common *sil*-harboring isolate [31], [33], [34]. The sequenced strains that do not possess Sil have instead remnants of the genomic island, suggesting that DNA recombination may have been responsible of loss of *sil* genes (Figure 2B) [29].

Although the *sil* genes have been shown to be expressed *in vivo* using infection models, and while Sil signaling has been involved with modulating the degradation of chemokines from the host by bacterial peptidases, the precise mechanism by which this QS system effects virulence remains ununderstood [30], [35]–[37].

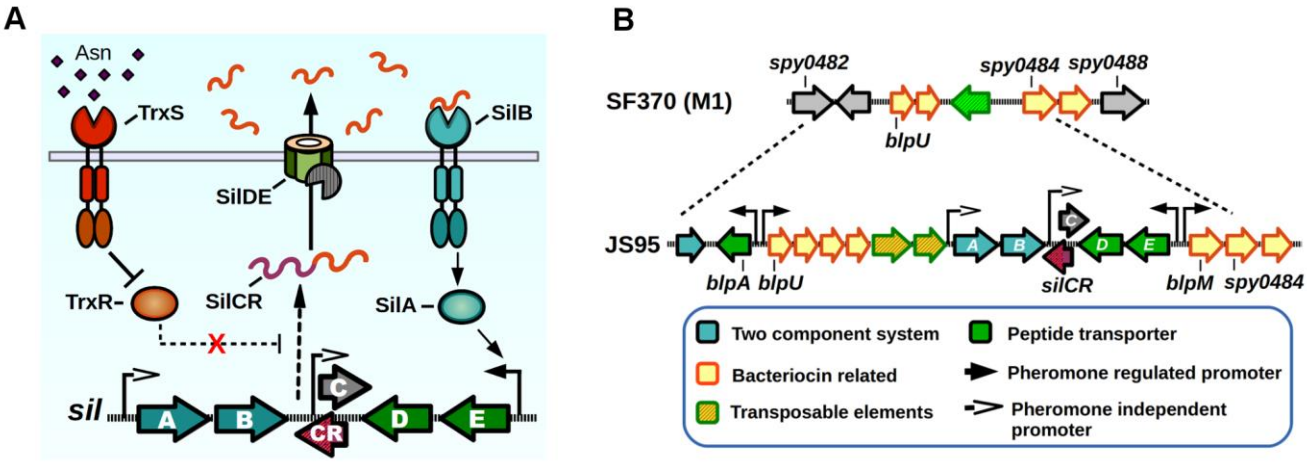


FIGURE 2. Sil signaling system. A. Model of signaling. SilCR pre-peptide is produced, secreted and processed. Mature SilCR is detected by SilB, activating the response regulator SilA which activates expression from select promoters, including the promoter for the expression of *silED-CR* genes. Expression of the *sil* locus is also activated in an unknown manner by a second two-component system, TrxSR. Asparagine sensing by TrxS alleviates repression of target genes by TrxR, generating an increase in expression of SilCR dependent promoters. **B.** *sil* genomic island. The *sil* locus plus neighboring genes are located in a putative genomic island in the strain JS95. Chromosomal location is compared with the SF370 strain that does not possess the *sil* locus.

1.4.2 Lantibiotic regulatory systems

During the process of colonization and establishment of their niche, bacteria face constant competition for nutrients from other individuals. For this reason, several bacteria have devised systems to give them a competitive advantage against other bacterial organisms. One such way is the production of bacteriocins, ribosomally synthesized antimicrobial peptides (AMP) that can target bacteria in the same species or across genera, with producing bacteria expressing specific immunity proteins to protect themselves from their cognate peptide. These antimicrobial molecules can act through a variety of mechanisms including membrane pore formation, cell wall synthesis inhibition, and target enzyme inhibition [38], [39]. Class I bacteriocins, termed lantibiotics, are post-translationally modified peptides that are produced by Gram-positive bacteria, including *Bacillus*, *Staphylococcus*, *Lactococcus* and *Streptococcus* species [40]. These molecules are synthesized as inactive pro-peptides and then modified through amino acid dehydration and/or thioether bridge formation to generate unusual amino acid residues, and are cleaved during the secretion process to generate a mature active lantibiotic [40], [41]. The lantibiotic acts not only as an AMP to neighboring bacteria, but also has QS pheromone

properties, as lantibiotic-producing bacteria can detect their own bacteriocin through a TCS. For these reasons, lantibiotic production in several bacteria exhibits a cell-density dependent pattern and is regulated by quorum-sensing like circuits [41]. The machinery required for lantibiotic production is usually encoded in gene clusters of conserved architecture formed by two or more operons in which genes are grouped by their function. A model of a prototypical lantibiotic producing system and its mechanism is shown in Figure 3A: The inactive product of the pre-peptide gene is post-translationally modified, cleaved and exported to give rise to the active lantibiotic, which can exert is antimicrobial activity towards sensitive individuals. The mature lantibiotic is also sensed by the TCS, which signals to generate an autoinduction process and increase the production of lantibiotic as well as activates the production of lantibiotic in its siblings. In some cases lantibiotic biosynthetic operons are shared amongst different species, meaning that a bacteriocin can act both as an inter- and intraspecies signaling molecule. To remain resistant to the activity of their lantibiotic, bacteria can express membrane bound immunity peptides that bind the bacteriocin and/or ABC transporters that prevent the accumulation of lantibiotics on the bacterial surface [42].

Certain *S. pyogenes* isolates have been reported to produce different lantibiotic molecules that can inhibit growth in other streptococcal species, or in sensitive *S. pyogenes* isolates, including Salivaracin, Streptin and Streptococcin [43]–[49]. The ability to produce mature bacteriocins is not widespread amongst all *S. pyogenes* isolates, and in most cases conserved mutations have occurred in biosynthetic loci (Figure 3B). Nonetheless, strains that cannot produce active lantibiotic still do express the immunity genes during the host infection process [50]. This has led authors to hypothesize that some of these circuits may have lost their lantibiotic and QS ability and instead have adapted them to perform other functions inside the host [51], [52].

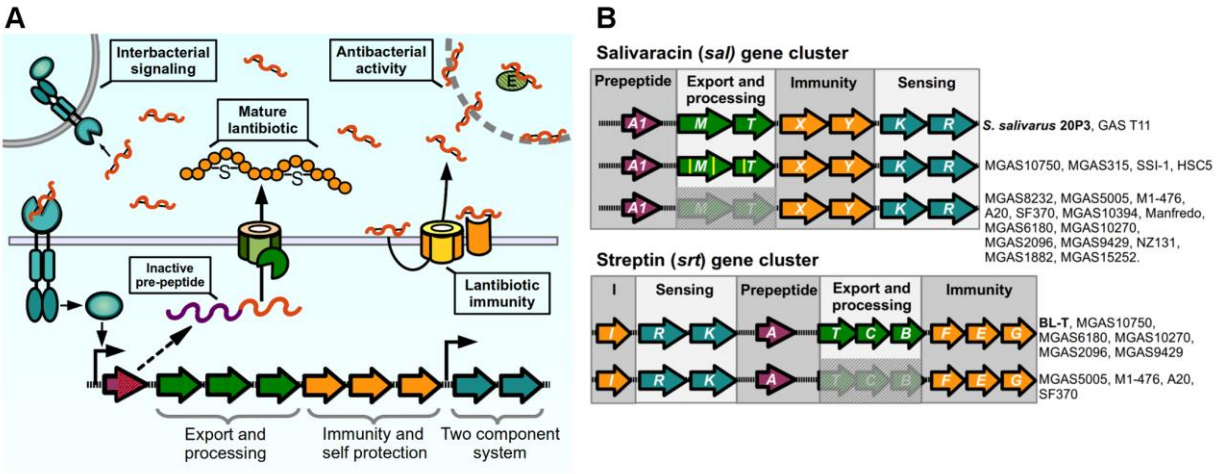


FIGURE 3. Lantibiotic regulatory systems. A. Model of production and sensing. Lantibiotic pre-peptide is synthesized, exported, cleaved, and modified through thiosulfur bridge formation and amino acid dehydration. The mature lantibiotic can exert its antibacterial effect on sensitive bacteria either by targeting the cytoplasmic membrane or inhibiting activity of target enzymes. Lantibiotic-producing bacteria express membrane immunity proteins to bind their cognate lantibiotic, or to re-export via an ABC transporter. Two-component systems sense the lantibiotic and trigger activation of the lantibiotic gene cluster in producing and neighboring bacteria. **B.** Lantibiotic gene clusters present in *Streptococcus pyogenes*. Complete or partial components of lantibiotic genes in sequenced strains of GAS compared with reference strains (in bold) for *sal* cluster (*S. salivarius* 20P3, accession AY005472), and *srt* cluster (*S. pyogenes* BL-T, accession AB030831).

1.4.3 Rgg transcriptional regulators

In Gram-positive bacteria, two families of conserved transcription factors have been reported to interact with imported peptide pheromones: RNPP and Rgg. The RNPP family, named for its prototypical members Rap, NprR, PlcR and PrgX, is characterized by the presence of tetratricopeptide repeat domains (TPRs) in their C-terminal domains, defined by motifs that are involved in protein-protein interactions and are involved in peptidic pheromone binding. Some members of the RNPP family also possess Helix-Turn-Helix (HTH) motifs for DNA binding and direct regulation of gene expression. RNPP Representatives are found in *Bacillus* and *Enterococcus* species and have been shown to regulate processes of sporulation, conjugation, biofilm formation and pathogenic responses [53].

The second conserved group of peptide-binding transcription factors is the Rgg family. Members of this family possess characteristic HTH motifs in their N-terminal domains and a C-terminal region rich in alpha-helical structures [54]. The Rgg family members are widespread in the low-G+C Gram-positive bacteria and only absent in the *Clostridiaceae*. Rgg proteins have

been shown to behave like activators or repressors of DNA expression, while some can exhibit simultaneously both regulatory functions [55]–[57]. The first Rgg family member was identified in the oral bacterium *Streptococcus gordonii,* in which extracellular glucosyltransferase activity required for tooth surface colonization was shown to be promoted by the activity of the Rgg protein (regulator gene of glucosyltransferase) [58]. Since then, several other Rgg members have been characterized in streptococcal species including *S. oralis* [59] , *S. thermophilus* [60]–[62], *S. salivarius* [61], *S. pneumoniae* [63], *S. mutans* [64], *S. agalactiae* [55], and *S. suis* [65]. Some species may even harbor multiple *rgg*-like genes in their genomes, like the case of *Streptococcus thermophilus,* predicted to encode in its genome seven different Rgg paralogs [66]. After the discovery that the deletion of a small pre-peptide gene inhibited the regulatory activity of an Rgg protein in *S. thermophilus,* it was recognized that activity of Rgg regulators was modulated by short peptides, constituting putative QS circuits [62]. Commonly, Rgg genes are located next to a short open reading frame that encodes the pro-peptide of their cognate pheromone, short genes which are usually overlooked in genome annotation processes but have been predicted by *in silico* analysis [66]. Rgg pheromones have been classified in two groups to date, short hydrophobic peptides (SHPs) and peptides involved in competence pathways, termed XIPs [54], [67]. Since some of the Rgg/pre-peptide loci show high conservation among different streptococci, it has also been shown that interspecies cross-talk can occur via SHP-pheromones [68], [69]. In *S. pyogenes,* four Rgg paralogs can be identified based on sequence homology: RopB (Rgg), Rgg2, Rgg3 and ComR (Rgg4).

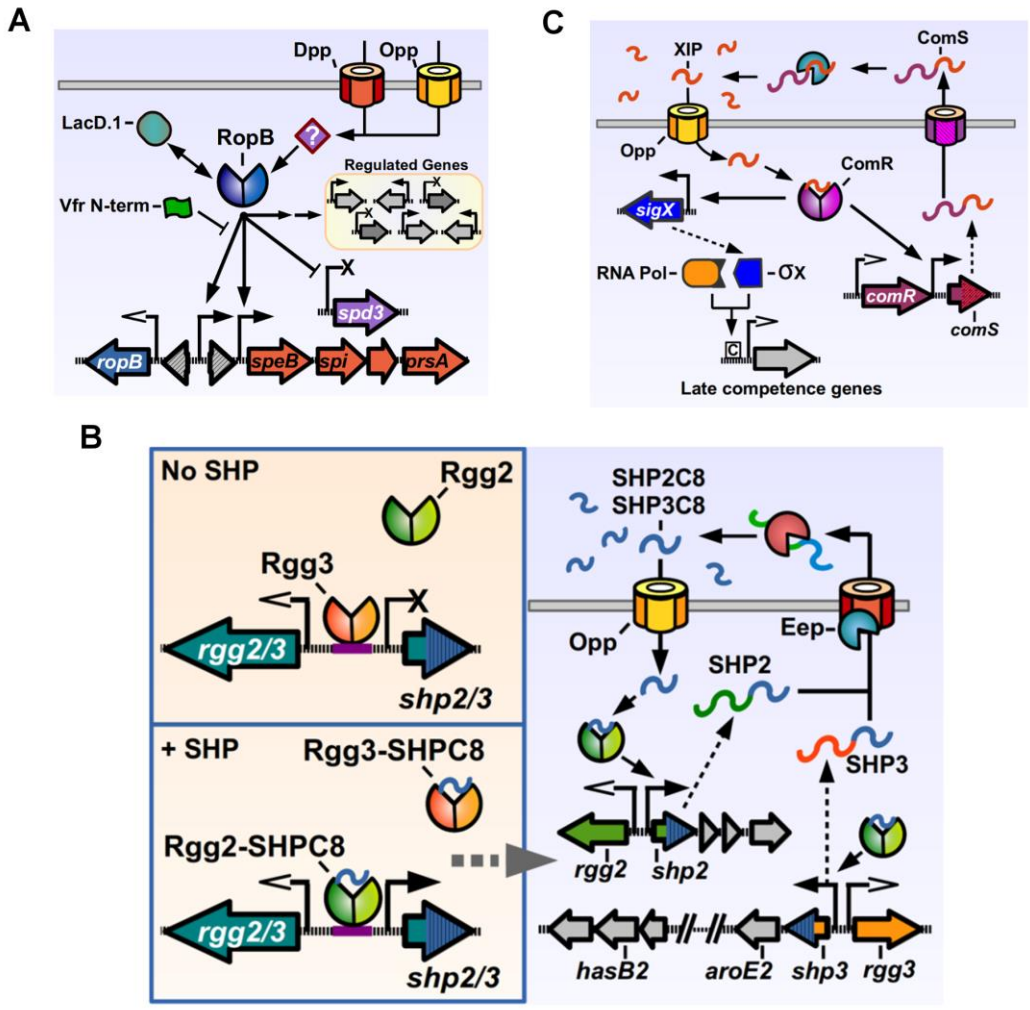


FIGURE 4. Rgg regulators of *Streptococcus pyogenes*. A. RopB directly activates the expression of *speB* and its associated downstream genes, while directly repressing the prophage encoded *spd3* DNase. RopB also affects, by direct and indirect manners, the expression of a varying group of genes in different isolates. Other factors can modulate RopB activity, like LacD.1, the N-terminal peptide of the Vfr protein and unknown factors imported by the Opp and Dpp permeases. **B.** The Rgg2/3 system. Left panel: in the absence of SHPs, Rgg3 is bound to the promoter region of the pheromone genes, inhibiting their expression. Addition of exogenous SHP pheromones that bind Rgg3, cause its release from DNA, while allowing Rgg2 to bind the same promoter region and promote expression of pheromone genes. Right panel: activation of the Rgg2/3 system triggers expression of *shp2*, *shp3* and their downstream genes. Translated SHP2 and SHP3 pro-peptides are secreted through an unknown exporter and processed by the activity of Eep and additional extracellular enzymes. The active SHP2C8 and SHP3C8 pheromones are imported via Opp to complete the autoinduction loop. **C.** Regulation of competence genes by ComR. ComS pro-peptide is produced, secreted and processed to generate the XIP peptide. After being imported through Opp, XIP can bind ComR, which binds to the promoters of *comS* and *sigX* to activate their expression. The alternative sigma factor σX together with RNA polymerase bind to Com box sites in target genes and activate the expression of competence related genes. Promoters with open arrows are Rgg-independent. Promoters with filled arrows are activated by Rgg-proteins. Promoters with X symbol are repressed by Rgg-proteins.

1.4.3.1 RopB (Rgg1)

The most studied Rgg member of *S. pyogenes* is the RopB protein. One of the important 'stand-alone' regulators, transcription factors whose cognate sensor partners are unknown [25], RopB, a predicted dimeric protein, has been shown to both positively and negatively modulate the expression of several genes (Figure 4A) [70]–[77]. RopB was initially discovered in a mutant screen for regulators of the potent cysteine protease SpeB which targets a wide range of proteins from the host, including components of the extracellular matrix and modulators of the immune response [78]. RopB is essential for *speB* transcription, and regulates its expression by directly binding the distal P1 promoter in the intergenic region between the *ropB* and *speB* genes. As with other genes regulated by activation factors, the *speB* P1 promoter lacks a canonical -35 promoter sequence, which may result in poor binding by RNA polymerase and low expression in the absence of the transcriptional activator. Located upstream of the -35 region of P1 are a two inverted repeat sequences, common recruitment motifs for dimeric DNA-binding proteins, which RopB may interact with in order to bind and recruit polymerase and activate *speB* expression, consistent with the activity of a type II activator [79], [80]. How RopB represses the expression of target genes is less understood, but it has been shown to be able to bind two different promoters in the upstream region of the repressed, prophage encoded *spd-3* gene, which possess -35 sequences closer to the consensus, suggesting that in this case RopB binding blocks the promoter region, inhibiting polymerase recruitment.

Even though it is a member of the Rgg family, RopB has not been widely characterized as a pheromone binding protein or as a QS component, as its cognate peptide/pheromone has not been discovered yet. Additionally, several studies on SpeB production and mutagenesis screens performed to understand its regulators in *S. pyogenes* have revealed a variety of factors modulating RopB activity, but so far no small peptide-encoding ORF. Nonetheless, several results have shown that RopB activity is affected by interaction with peptides and proteins: The regulation of *speB* expression by RopB has been shown to be dependent on cell cycle, with the highest degree of expression beginning in stationary phase, suggesting a requirement for high cell density conditions for RopB-mediated gene activation. If *ropB* is expressed from a non-native promoter at high levels during early exponential phase, *speB* transcription is still not activated, implying that additional factors are required for RopB regulation [80]. Mutations in *opp* genes, or in its paralog *dpp* (dipeptide permease) generate a drastic decrease in *speB*

expression, suggesting that peptide import into the cell is also required for *speB* regulation [81]–[83]. Other results have shown the role of proteins that directly interact with RopB to affect its regulatory function: LacD.1 is an aldolase enzyme that has been proposed to link metabolic status of *S. pyogenes* with virulence gene regulation, as it is able to bind intermediates of the glycolytic pathway that are present in high concentrations during exponential growth and consequently influence gene expression. LacD.1 and RopB can be co-precipitated, and in the absence of LacD.1 a constitutive *ropB* allele is able to partly uncouple SpeB activity from cell cycle regulation. Other reports, based on the observation of differential gene regulation by RopB during stages of bacterial growth, have suggested that LacD.1 binds to RopB during exponential phase, changing its DNA binding preferences and affecting the repertoire of genes regulated [84]. Another protein involved in repressing RopB-dependent SpeB activation is Vfr (virulence factor related) [85]. The *vfr* gene was identified by transposon mutagenesis, as a mutant with increased protease activity, and SpeB activity is inversely correlated with *vfr* expression; *vfr* expression is maximal in early logarithmic stages and decreased towards stationary phase. Additional work by Shelburne *et al.* showed that the N-terminal region of the Vfr protein, which forms part of a putative cleavable secretion signal, was able to bind RopB and was sufficient to exert the inhibiting activity [86]. However, a factor accounting for a necessary activating signal of SpeB regulation by RopB has yet to be identified.

1.4.3.2 Rgg2 and Rgg3

The *rgg2* and *rgg3* genes were identified after a search for additional Rgg orthologs in the *S. pyogenes* genome [87]. Rgg2 and Rgg3 proteins share a high degree of similarity (55% identical, 76% similar), and are encoded divergently from the genes for the pro-peptide pheromones, which are termed SHP2 and SHP3, respectively (Figure 4B). Both SHP2 and SHP3 pro-peptides are also highly similar (58% identical, 62% similar), 23 and 22 amino acids long, respectively, and contain almost identical C-terminal regions (Figure 5). Basal expression of SHP2/3 pheromones is repressed under normal laboratory culturing conditions, suggesting that specific environmental signals are required to activate the QS system endogenously. Addition of synthetic SHP2 or SHP3 full length pro-peptides are able to generate a small increase in expression from the P*shp2* and P*shp3* promoters, consistent with an autoinducing signaling system, and addition of the last eight C-terminal amino acids of SHP2 or SHP3 (termed SHP2C8

and SHP3C8) generate a strong inducing activity [87]. Further studies have shown that multiple variants of SHP2 and SHP3 pheromones can be found in *S. pyogenes* culture supernatants, corresponding to the C7, C8, C9 and C10 regions of the pheromone pro-peptides, and that the SHPC8 variants of the pheromones are the most abundant and most biologically active forms [88]. These results also show that both SHP2 and SHP3 signaling peptides have the same function of activating the Rgg2/3 circuit, in comparison with other dual pheromone systems in which one peptide acts as an activator and the other as a repressor of the QS circuit [89]. If a strain that lacks both *shp2* and *shp3* genes is used, the induction of the system by synthetic pheromones is only transient, suggesting that the autoinduction process is required to generate the full extent of the response [90]. Even though they seem redundant in function, both *shp2* and *shp3* genes are required for efficient signaling and autoinduction processes, as mutations in either of them affects the timing and breadth of the signaling response [90]. As reported for other Rgg systems, an intact Opp system is required in order to import the pheromones and trigger activation of the system. Experiments also revealed that a deletion of the *rgg3* gene derepresses expression of the *shp2* and *shp3* genes, while a double mutant Δ*rgg2* Δ*rgg3* lacks nearly all expression of pheromones. A single deletion of *rgg2* also renders *S. pyogenes* grossly unresponsive to synthetic SHPC8 pheromones, illustrating how Rgg3 acts as a repressor of gene expression, while Rgg2 activates gene expression in the absence of the repressive effect of Rgg3 (Figure 4B). Deletion of the *eep* gene, encoding for a metalloprotease involved in pheromone processing in other Gram-positive QS systems, reduces the effectiveness of the autoinduction process, suggesting a role in the processing of SHP2/3 pre-peptides into their mature form. This effect can however be ameliorated if SHP3 is overexpressed, suggesting that additional proteins are involved in SHP2/3 processing [87].

SHP2(C8): MKKVNKAL-LFTLIMDILIIVGG
SHP3(C8): MKKISKFLPILILAMDIIIIVGG

FIGURE 5. SHP peptides of *S. pyogenes*. Sequence of SHP2 and SHP3 peptides. In red, sequence of the C8 section, the most abundant and active form of the secreted processed pheromone.

Both Rgg2 and Rgg3 have been shown to bind directly to the promoter regions of *shp2* and *shp3* [87], [91]. Interestingly, when the precise DNA binding sites of both regulators were mapped by DNase I footprinting, Rgg2 and Rgg3 were shown to bind to the same conserved sequence in the P*shp2* and P*shp3* promoters, hence only one Rgg protein is able to bind per promoter at any given time, indicating that Rgg3 exerts it repressing activity by binding the shared DNA site and generating steric interference that inhibits Rgg2 binding [91]. This is consistent with the fact that the HTH domains of Rgg2 and Rgg3 are distinctively analogous, sharing 71% identity and 94% similarity. A higher degree of variability is seen in the C-terminal regions of the Rgg2 and Rgg3 proteins, which are predicted to be involved in protein oligomerization and interaction with RNA polymerase, and this variability may determine the fundamental differences in the activities of these two regulators [91]. Similar to the RopB interaction with the *speB* promoter, Rgg2 and Rgg3 bind the P*shp2* and P*shp3* promoters in their -35 regions, which have poor resemblance with consensus sequences, suggestive of a class II activator dependent promoter. Using EMSA assays, it was revealed that either Rgg can bind the target DNA sequences in the absence of pheromones. However, when pheromones are added in increasing concentrations, Rgg3 is released from the DNA while Rgg2 remains unaffected. When the experiment is set up with both regulators to mimic the DNA-binding competition event *in vitro*, addition of synthetic pheromones affects the binding ratio, which becomes skewed in favor of Rgg2-DNA interactions over Rgg3-DNA interactions [91].

The function of the Rgg2/3 circuit and its contribution to the *S. pyogenes* lifestyle has not been completely elucidated. Apart from the *shp2* and *shp3* genes, other major regulatory targets of the Rgg2/3 pathway are the genes located downstream of the pheromone genes (Figure 4B) [87]. Downstream of *shp3*, a putative biosyntethic operon composed of nine genes is encoded, which harbors genes with diverse enzymatic functions, like shikimate dehydrogenase, sugar isomerase, glycosyltransferase and oxidoreductase. Components from this operon have been identified in mutagenesis screens for genes affecting mucoid colony morphology, virulence in zebra fish, and have also been involved in capsule formation in *S. pyogenes* [32], [92], [93]. Downstream of *shp2*, Rgg2/3 also controls a highly conserved region in *S. pyogenes*, which encodes a series of hypothetical genes of unknown function.

1.4.3.3 ComR (Rgg4)

ComR is another Rgg member present in *S. pyogenes*, involved in natural genetic transformation processes in other streptococci through the regulation of early genes in the competence cascade (Figure 4C). Competence, the temporal physiological state of DNA receptivity, relies on the coordinated and sequential expression of a series of genes, encoding proteins responsible for pheromone signaling, DNA binding, uptake, processing and recombination into the chromosome (reviewed in [94]). In the streptococci, the master regulator of competence is SigX/ComX, an alternative sigma factor of RNA polymerase that recognizes a conserved DNA sequence termed cinbox or combox and controls the expression of the competence 'late genes' required for DNA binding, uptake and recombination [95]–[97]. All the genes induced before *sigX* in the competence cascade and that are involved in the signaling processes that lead to *sigX* expression are termed 'early genes'. In *S. pneumoniae,* the expression of *sigX* is under the control of a QS system, composed of the ComDE TCS which senses and responds to the competence inducing pheromone CSP. *S. pyogenes* and other streptococci of the Pyogenic, Bovis and Salivarius groups lack ComDE but have an alternative circuit, based on ComR activity, to trigger *sigX* expression and concomitant upregulation of late genes in the bacterial population. The *sigX* promoter, bound by ComR, lacks a canonical -35 sequence and has an inverted repeat element in this region, suggestive with a Class II activator dependent promoter, similar to the P*shp2/3* promoters activated by Rgg2 and the P1 *speB* promoter activated by RopB [67]. *In silico* screening in streptococcal genomes revealed the presence of *comRS* orthologs in the genomes of pyogenic, bovis and mutans streptococci [67]. The predicted ComS pro-peptides from these species had similar features, being composed by a high degree of hydrophobic residues, possessing a net positive charge and a double tryptophan (WW) motif in their C-terminal region. Characterization of the ComRS system in *S. mutans* showed that a synthetic pheromone consisting of the last 8 C-terminal amino acids of the 17 amino-acid long ComS peptide was able to trigger *sigX* expression and transformation in an Opp- and ComR-dependent manner, mimicking the activity of the putative mature pheromone. This active pheromone was named XIP for *sigX*-inducing peptide [67].

The effect of ComRS signaling in *S. pyogenes* and other pyogenic-group streptococci, and its effect over transformation have proven to be more cryptic than in non-pyogenic streptococcal counterparts. Even though genome sequencing data has shown that horizontal transfer and genetic exchange between *S. pyogenes* and other streptococcal species is common, the demonstration of natural transformation of *S. pyogenes* in laboratory conditions has proven elusive. Genomic analysis by Mashburn *et al* found *comRS* genes in all sequenced *S. pyogenes* genomes, and two allelic variants named M1 and M3 were found [24]. In the absence of exogenous XIP, *sigX* expression is low. Addition of synthetic XIP activates ComR and triggers expression of *sigX* in S. pyogenes strains, in an allele-specific manner. Microarray analysis revealed that XIP sensing by ComR generated the upregulation of 30 different genes, 21 of these containing com boxes, and thus regulated directly by SigX [24]. Most of the regulated genes corresponded to predicted competence late genes and all gene functions known to be required for genetic transformation in *S. pneumoniae* and *Bacillus subtilis* were upregulated in response to XIP in *S. pyogenes*, Nonetheless, transformation was not seen *in vitro* and further experiments revealed that competence was blocked at the DNA uptake process, suggesting that unknown post-transcriptional regulation events were inhibiting transformation [24]. Additional environmental signals may therefore be required to unblock the competence machinery, and the first evidence for this notion has been associated with growth in biofilms. As sessile bacterial aggregates, biofilms contain high densities of bacterial cells in close contact and studies have shown that Streptococci that undergo natural transformation do so in higher efficiencies when growing inside biofilms [98], [99]. Recently, the first report of effective *S. pyogenes* natural transformation in the laboratory was seen in biofilm cultures [100]. *S. pyogenes* biofilms grown on a substrate of fixed epithelial cells were shown to internalize and incorporate exogenous DNA at a low frequency, in a ComR-dependent manner. Addition of XIP generated a ten-fold increase in efficiency, but was not required for transformation, suggesting that growth on epithelia provides a signal(s) for Com system activation. The transformation phenotype could also be replicated during an in vivo biofilm growth assay, where intranasally colonized mice were also given donor DNA. Again, the addition of the XIP pheromone generated a ten-fold increase in the number of transformed colonies [100]. These results suggest that *S. pyogenes* are indeed capable of undergoing genetic transformation, but an unidentified regulatory check-point requires an environmental condition that appears to be satisfied through growth on host cells.

1.5 Biofilms

Biofilms are sessile communities of bacterial aggregates surrounded by self-generated matrices. They are thought of as the main mode of growth for several bacteria in their environments, and have been observed in a variety of habitats including river streams, bovine rumen, abiotic surfaces in the hospital and food industry, and in the human body, in places like the surface of the teeth [101]. Biofilms have an important relevance in human health, since its formation has been shown important for colonization of the host, evasion of the immune system and tolerance to the activity of antibiotics. While in some bacteria, the process of biofilm formation can be directly correlated with a pathogenic event (*P. aeruginosa* biofilms in cystic fibrosis patients lungs [102], *S. epidermidis* biofilms in venous catheters [103], *L. monocytogenes* biofilms in food processing surfaces [104]), in other bacteria the role of biofilm formation in their life cycle and its contribution to disease is not yet well understood.

1.5.1 *Streptococcus pyogenes* biofilms

S. pyogenes has shown the ability to form biofilm on several surfaces *in vitro* [105]–[108], as well as over the skin of infected patients [109], and on tonsils of infected individuals as well as on asymptomatic carriers [110]. A variety of genes have been shown to contribute to biofilm formation in *S. pyogenes*, most of them encoding for cell surface components, including the promiscuous adhesin M-protein [111], [112], adhesive pili [113], hyaluronic acid capsule [111], the adhesin AspA [114], and the collagen-like Scl1 adhesin [115]. Contrarily, the potent protease SpeB has been shown to be able to mediate dispersion of formed biofilms when its expression is activated [116].

While biofilm growth increases the tolerance of *S. pyogenes* to some antibiotics [107], [117], the role of biofilm in the pathologies induced by this bacterium is unknown. Since *S. pyogenes* biofilms can be found in asymptomatic patients, and biofilm contributes to tissue colonization in animal models, it has been proposed that biofilm may represent a non-pathogenic mode of growth for *S. pyogenes* in its host, and that the activation of important virulence factors, including proteases shown to degrade biofilm, may tip the balance towards escape from biofilm and virulent behaviors [110], [118], [119]. Nonetheless, the precise role of biofilm growth in *S. pyogenes* life cycle is still unknown.

1.6 Bacterial cell wall

The bacterial cell wall plays an essential role in protecting the cells from osmotic rupture, as well as serving as a scaffold for the attachment of proteins and teichoic acids. The main component of the cell wall is peptidoglycan, a polymer formed by strands of alternating N-acetyl-glucosamine (GlcNAc) and N-acetyl-muramic acid (MurNAc) monosaccharides, crosslinked to other sugar strands by short peptide chains of three to five amino acids [120], [121] (Figure 6). Due to its crucial role in survival, the bacterial cell wall is an attractive target for other organisms that compete for the same biological niches or for higher organisms that defend themselves from bacterial infection; and usual strategies include the use of small molecules like antibiotics that inhibit key enzymes for peptidoglycan biosynthesis, or the activity of peptidoglycan hydrolase enzymes that degrade the cell wall polymer [122], [123].

In Gram-positive bacteria, the cell wall is the outermost layer of the bacterial envelope, hence is also the surface which physically interacts with the host and isolates the bacterial cell from larger host molecules. A dedicated enzyme machinery covalently attaches specific proteins to the peptidoglycan structure and these cell wall anchored proteins, which in *S. pyogenes* include various adhesins, complement inhibiting peptidases, interleukin-targeting proteases and antiphagocytic proteins, usually play important roles in the interaction with the host [124]–[127]. Hence, bacterial-induced processing and/or modification of the peptidoglycan polymer is important not only due to its effects over resistance towards antibacterial compounds, but also because it can alter the dynamics of the surface attached proteins and thus the interaction of the bacterium with the host [128].

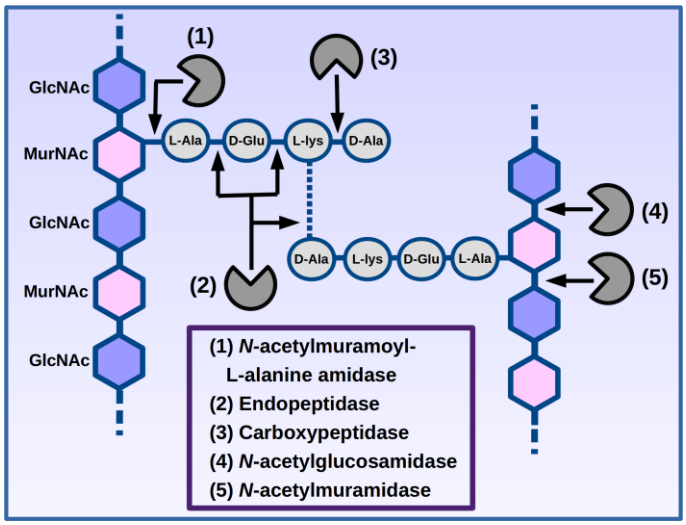


FIGURE 6. Structure of *S. pyogenes* peptidoglycan. The alternating N-acetyl-glucosamine (GlcNAc) and N-acetyl-muramic acid (MurNAc) strands are crosslinked together through interpeptide bridges (dotted line) that bind the stem tetrapeptides. The interpeptide bridge in *S. pyogenes* is composed of two to three residues of L-Ala. Shown are the different enzymatic activities known to cut the cell wall, and their targets.

1.6.1 Peptidoglycan hydrolases

A variety of enzymes of diverse origin have the ability to cleave residues in peptidoglycan. Based on their precise substrate and mechanism, these can be classified in 5 different groups (Figure 6): *N*-acetylmuramoyl-L-alanine amidases that cleave the bond between MurNAc and L-Alanine, carboxypeptidases that cleave short peptides, endopeptidases that cleave inner segments of the peptide chains and/or the cross-bridges in between different peptide chains, and *N*-acetylglucosaminidases and *N*-acetylmuramidases that hydrolyze the bonds between the saccharides [129]. Bacterial genomes can harbor genes for every hydrolase type (either by genes of bacterial origin or present in integrated bacteriophage genomes), while in animals, to date only two activities have been described: peptidoglycan recognition proteins with amidase activity [130], [131] and the well described family of *N*-acetylmuramidases, the lysozymes [132].

1.6.1.1 Lysozyme

Lysozyme-like enzymes can be found in all major taxa of living organisms. In higher organisms lysozyme is found in high concentrations in mucosal tissues and can be produced as well by recruited neutrophils, playing an important role in the clearance of various bacterial pathogens from infection sites [133]–[136].

In order to successfully colonize host mucosal tissues, bacteria have devised different ways to gain resistance towards the effects of lysozyme. One example is the acetylation and deacetylation of different molecules in the glycan strands of peptidoglycan [121]. Alternatively, teichoic acids, glycopolymers that attach to the cell wall, can also be modified by D-alanylation to provide resistance to lysozyme [137]. Finally, some bacteria like *E. coli* and *Salmonella* have been shown to secrete small proteins that have the ability to work as direct lysozyme inhibitors [132]. Interestingly, the 'Streptococcal inhibitor of complement' (Sic), a protein that has been shown to bind components of the membrane attack complex of complement, has also been shown to be able to inhibit lysozyme activity [138], [139]. However, this gene is only present in a small number of highly virulent *S. pyogenes* isolates of the M1 and M57 serotypes.

1.6.1.2 Bacterial peptidoglycan hydrolases

The genomes of Gram-positive bacteria in the Firmicutes phylum encode a variety of peptidoglycan hydrolases. The genetic architecture of these is modular, having at least one catalytic domain and one recognition/cell wall-binding domain; while in some cases some hydrolases can carry more than one catalytic domain and are thus able to cleave more than one type of bond in peptidoglycan [129]. One well studied function of peptidoglycan hydrolases is their key role during the cell division process, as peptidoglycan must be cut in order to cytokinesis and daughter cell separation to occur [140]. Cell lysis events, triggered by either bacteria or infecting bacteriophages, have also been shown to be dependent on peptidoglycan hydrolase lysins [141].

While the hydrolases involved in daughter cell separation and cell lysis events have been thoroughly characterized, a wide range of peptidoglycan hydrolases are still of unknown function. New research is slowly unveiling how these enzymes also play roles in cell shape determination, cell wall modification and turnover, and in the release of peptidoglycan-attached

proteins [128], [140], [142], [143]. Additionally, efficient degradation of exposed peptidoglycan by bacteria has been shown to help avoid detection by the immune system [144].

S. pyogenes has been shown to harbor in its genome 22 hydrolase domains, including two L-alanine amidases, seven N-acetylglucosaminidases, two N-acetylmuramidases, six endopeptidases, five carboxypeptidases [129], a number that may vary depending in the number of integrated prophages. Apart from precise lysins carried in prophages, most of the bacterial enzymes carrying hydrolase domains in *S. pyogenes* have not been yet characterized, hence their role remains mostly unknown.

II. MATERIALS AND METHODS

2.1 Strains, plasmids, and culture media.

All strains used in this work are listed in table I. Construction of deletion and insertional mutants is described in detail below (see Section 2.1.2). All plasmids used in this work are listed in table II. 'JCC' designated strains and 'pJC' designated plasmids were generated by Jennifer C. Chang, 'Sar' designated strains and 'pSar' designated plasmids were generated by Breah LaSarre, and the NZ131 Δ*speB* strain was generated by Chaitanya Aggarwal. All primers used in this work are listed in Table III.

S. pyogenes was routinely grown in THY, Todd-Hewitt medium (BD Biosciences) supplemented with 2% (w/v) yeast extract (AMRESCO). For all quorum-sensing phenotype studies, *S. pyogenes* was grown in chemically defined medium [87], [145] containing 1% (w/v) glucose. When required, antibiotics were added in the following concentrations: chloramphenicol (Cm), 3 μg/ml; erythromycin (Erm), 0.5 μg/ml; spectinomycin (Sp), 100 μg/ml. *E. coli* strain BH10C [146] was used for cloning procedures, and was grown in Luria broth (LB). When required, antibiotics were added in the following concentrations: chloramphenicol (Cm), 10 μg/ml; erythromycin (Erm), 500 μg/ml; spectinomycin (Sp), 100 μg/ml.

TABLE I

BACTERIAL STRAINS USED IN THIS STUDY

Wild-type isolates			
Strain Name	**Description**	**Chapter(s)**	**Reference**
NZ131	*S. pyogenes* M49 serotype	III-V	[147]
GA19681	*S. pyogenes* M6 serotype	III, IV	[148]
HSC5	*S. pyogenes* M14 serotype	III	[149]
MGAS5005	*S. pyogenes* M1 serotype	III	[150]
MGAS10394	*S. pyogenes* M6 serotype; Erm^R	III	[151]
CS101	*S. pyogenes* M49 serotype	III	[152]
Mutant Strains			
Strain Name	**Description**	**Chapter(s)**	**Reference**
JCC131	NZ131 Δ*rgg3*::*cat;* Cm^R	III	[87]
JCC137	NZ131 Δ*rgg2;* unmarked	III, V	[87]
BNL145	NZ131 Δ*rgg2 Δrgg3::cat;* Cm^R	III	[87]
BNL170	NZ131 $shp2_{GGG}$ $shp3_{GGG}$, START → STOP codon mutants; unmarked	III	[90]
JCJ133	NZ131 Δ*0450-0460::cat;* Cm^R	IV	This study
JCJ185	NZ131 Δ*0412-0414c*-IGR*::cat;* Cm^R	IV	This study
JCJ67	NZ131 Δ*0412::aph3A;* Kan^R	IV	This study
JCJ61	NZ131 Δ*0413::aph3A;* Kan^R	IV	This study
JCJ173	NZ131 Δ*0414c;* unmarked	IV	This study
JCJ310	NZ131 Δ*0414c*-IGR::*cat*; Cm^R	IV	This study
JCJ471	NZ131 Δ*0015::cat;* Cm^R	IV	This study
JCJ420	NZ131 Δ*0026;* unmarked	IV	This study
JCJ202	NZ131 Δ*0186c::cat*; Cm^R	IV	This study
JCJ456	NZ131 Δ*0790*-Φ2 (Deletion in prophage NZ131.2*);* unmarked	IV	This study
JCJ484	NZ131 Δ*pepC;* unmarked	IV	This study
JCJ328	NZ131 Δ*isp1;* unmarked	IV	This study

TABLE I (continued)

Strain name	Description	Chapter(s)	Reference
JCJ365	NZ131 Δ*isp2::cat*; Cm^R	IV	This study
JCJ454	NZ131 Δ*1459*-Φ3 (Deletion in prophage NZ131.3*);* unmarked	IV	This study
–	NZ131 Δ*speB;* unmarked	IV	[153]
JCJ413	NZ131 Δ*isp1 Δisp2::cat*; Cm^R	IV	This study
JCJ257	GA19681 Δ*0871*::*cat*; Cm^R (NZ131 *0414c* homolog)	IV	This study
JCJ256	GA19681 Δ*0826*::*cat*; Cm^R (NZ131 *0186c* homolog)	IV	This study
JCJ304	NZ131 *manM*::pJJ216; Kan^R	V	[154]
JCJ343	NZ131 *agaW*::pJJ215; Kan^R	V	[154]
JCJ336	NZ131 *ptsC*::pJJ217; Kan^R	V	[154]
JCJ337	NZ131 *mga*::pJJ219; Kan^R	V	This study

Cm: Chloramphenicol, Kan: Kanamycin, Erm: Erythromycin

TABLE II

PLASMIDS USED IN THIS STUDY

Backbone plasmids			
Plasmid Name	**Description**		**Source**
p7INT	Shuttle-suicide vector that integrates at streptococcal bacteriophage T12 *attB* site; *erm* cassette, Erm^R		[155]
pGh9(Tres)	Temperature resistant variant of pGh9, pWV01 ori; *erm* cassette, Erm^R		[156]
pLZ12-Sp	Shuttle vector; pWV01 ori; *add9* cassette, Sp^R		[157]
pJC162	Shuttle vector pGH9-ISS1 with IS*S1* element deleted; pWV01 ori, temperature sensitive; *erm* cassette, Erm^R (a.k.a. pFED760)		[67]
pUC-Kan	Gram-negative vector; pMB1 ori; *aphA3* cassette, Kan^R		[158]
pJC303	P_{recA} promoter cloned next to MCS in pLZ12-Sp; Sp^R		[154]
pJJ180	Promotorless *luxAB* genes from *V. fisheri* cloned; Erm^R		[154]
Deletion/disruption plasmids			
Plasmid Name	**Description**	**Backbone**	**Source**
pSar64.1	*0412-0414c*-IGR KO plasmid*;* Erm^R Ts	pJC162	This study
pJJ134	pSar64.1 plus *cat* cassette. *0412-0414c*-IGR::*cat* KO plasmid*;* Erm^R Cm^R Ts	pJC162	This study
pJJ144	*0450-0460*::*cat* KO plasmid; Erm^R Cm^R Ts	pJC162	This study
pJJ114	*0412*::*aph3A* KO plasmid*;* Erm^R Kan^R Ts	pJC162	This study
pJJ115	*0413*::*aph3A* KO plasmid*;* Erm^R Kan^R Ts	pJC162	This study
pJJ158	*0414c* KO plasmid; Erm^R Ts	pJC162	This study
pJJ202	*0414c*-IGR::*cat* KO plasmid*;* Erm^R Cm^R Ts	pJC162	This study
pJJ273	*0015*::*cat* KO plasmid*;* Erm^R Cm^R Ts	pJC162	This study
pJJ260	*0026* KO plasmid; Erm^R Ts	pJC162	This study
pJJ165	*0186c*::*cat* KO plasmid*;* Erm^R Cm^R Ts	pJC162	This study
pJJ276	*0790(Φ2)* KO plasmid; Erm^R Ts	pJC162	This study
pJJ290	*pepC* KO plasmid; Erm^R Ts	pJC162	This study
pJJ224	*isp1* KO plasmid; Erm^R Ts	pJC162	This study
pJJ250	*isp2*::*cat* KO plasmid*;* Erm^R Cm^R Ts	pJC162	This study
pJJ277	*1459(Φ3)*::*cat* KO plasmid; Erm^R Cm^R Ts	pJC162	This study

pJJ215	482 bp middle fragment of *agaW* gene, suicide plasmid; Kan^R	pUC-Kan	[154]
pJJ216	517 bp middle fragment of *manM* gene, suicide plasmid; Kan^R	pUC-Kan	[154]
pJJ217	430 bp middle fragment of *ptsC* gene, suicide plasmid; Kan^R	pUC-Kan	[154]
pJJ219	750 bp middle fragment of *mga* gene, suicide plasmid; Kan^R	pUC-Kan	This study
Complementation / overexpression plasmids			
Plasmid Name	**Description**	**Backbone**	**Source**
pJJ109	*0412-0414-IGR* region driven by native P_{shp2}, $shp2_{GGG}$ (START→STOP codon); Erm^R	p7INT	This study
pJJ132	*0414-IGR* region driven by native P_{shp2}, $shp2_{GGG}$ (START→STOP codon); Erm^R. Cloned into pJJ119 (p7INT + PstI SalI sites)	p7INT	This study
pJJ137	pJJ132 with IGR deleted; Erm^R	p7INT	This study
pJJ138	pJJ132 with *0414c* gene deleted; Erm^R	p7INT	This study
pJJ163	0414c fused with P_{recA} promoter; Erm^R	pGh9 (Tres)	This study
pJJ251	*ptsABCD* operon cloned downstream of P_{recA}; Sp^R	pJC303	[154]
Reporter plasmids			
Plasmid Name	**Description**	**Backbone**	**Source**
pBL111	P_{shp2} promoter fused to *luxAB* genes from *V. fisheri*; Erm^R	p7INT	[87]
pJC219	P_{shp3} promoter fused to *luxAB* genes from *V. fisheri*; Erm^R	p7INT	[90]
pJC229	P_{shp3} promoter fused to *luxAB* genes from *V. fisheri*; Sp^R	pLZ12-Sp	[154]
pJJ252	P_{manL} promoter cloned next to *luxAB*; Erm^R	pJJ180	[154]
pJJ244	P_{ptsA} promoter cloned next to *luxAB*; Erm^R	pJJ180	[154]

Cm: Chloramphenicol, Kan: Kanamycin, Erm: Erythromycin, Sp: Spectinomycin

TABLE III

PLASMIDS USED IN THIS STUDY

Purpose	Primer	Sequence	Description
pJJ109	BL279(**BamHI**)	CATG**ggattc**GTGAAATGAATCATATTT	Used to amplify and clone *0412-0413-IGR-0414c* genes
	BL280(**BamHI**)	CATG**ggattc**CAGCTACTTCAGAAGA	
pJJ114	JJ47(PstI)	CATG**ctgcag**AGACAAACGACAGAAGTGCT	Used to amplify and clone *0412* gene plus neighborhood
	JJ48(SalI)	CATG**gtcgac**GCGAGACTTACTAGCACATAATT	
	JJ53(PacI)	CATG**ttaattaa**TAGTTAATAATTTGCCGGTTG	Used to remove *0412* gene by iPCR
	JJ54(PacI)	CATG**ttaattaa**CATCATCATGTTCCTTTCATTA	
pJJ115	JJ49(PstI)	CATG**ctgcag**GAGCCTTACCGGACAATAT	Used to amplify and clone *0413* gene plus neighborhood
	JJ50(SalI)	CATG**gtcgac**AAGGACTGCGATGACCATA	
	JJ55(PacI)	CATG**ttaattaa**AAGAGTGCTTAAGAATTATGTGC	Used to remove *0413* gene by iPCR
	JJ56(PacI)	CATG**ttaattaa**CAATTCTTCCGGCTATGAG	
pJJ132	JJ21(EcoRI)	CATG**gaattc**ATTTTTCCCACTTTCA	Used to amplify and clone IGR-0414c region
	JJ82(SalI)	CATG**gtcgac**GCTTAAGAATTATGTGCTAG	
pJJ134	JJ87(PacI)	CATG**ttaattaa**TGTTCCTTTCATTATTTTTTA	Used to add PacI and NcoI sites to pSar64.1 to add antibiotic cassette
	JJ88(PacI NcoI)	CATG**ttaattaa**A**ccatgg**GGTCGCTAACACTCTTT	
pJJ137	JJ59(PacI)	CATG**ttaattaa**GTTTCCCCAAAGTGGATT	Used to remove IGR by iPCR
	JJ60(PacI)	CATG**ttaattaa**TCAACTAGAAAGGCCTAAACA	
pJJ138	JJ57(PacI)	CATG**ttaattaa**GGTCATTGTTTAGGCCTTTC	Used to remove *0414c* gene by iPCR
	JJ58(PacI)	CATG**ttaattaa**TAGCTCTCCTAAAATCAGTGGA	

TABLE III (continued)

pJJ144	JJ96(PacI)	CATG**ttaattaa**TTAGATGCCAGTTTGGG	To iPCR pJC230, removing 0457 gene and leaving PacI site
	JJ97(PacI)	CATG**ttaattaa**CTTCTAAACACACTGAGTCTACC	
	JJ98(PacI)	CATG**ttaattaa**ATTCGTTAACAGTTAGTACTAA	To amplify and clone 0461 and 0462 genes
	JJ99(SpeI)	CATG**actagt**GAATTCTCAACTGAACGT	
pJJ158	JJ51(EcoRI)	CATG**gaattc**TAATTTGCCGGTTGCTTTA	To amplify and clone 0414c plus neighborhood
	JJ52(SalI)	CATG**gtcgac**GACTAGGTGCAATTGAGGAG	
	JJ57(PacI)	CATG**ttaattaa**GGTCATTGTTTAGGCCTTTC	Used to remove *0414c* gene by iPCR
	JJ58(PacI)	CATG**ttaattaa**TAGCTCTCCTAAAATCAGTGGA	
pJJ163	JJ71(PstI)	CATG**ctgcag**GTCCGTAAAACTTTATTAAAAC	To amplify PrecA with fragment homologous to *0414c* region (underlined)
	JJ123	ttttggtcatTACTTTCTCCTCTTTTTTAACAA	
	JJ78(SalI)	CATG**gtcgac**GAGAGCTAAGATTGTTCATCT	To amplify *0414c* with fragment homologous to PrecA region (underlined)
	JJ122	ggagaaagtaATGACCAAAAAACAGTTTTAT	
pJJ165	JJ124(PacI)	tagttcct**ttaattaa**CTTTTGTTTCATCATAACTCC	To amplify upstream region of *0186c* gene with fragment homologous to downstream region (underlined)
	JJ125(EcoRI)	CATG**gaattc**TATAACGCGATTCCGAAT	
	JJ126(PacI)	aacaaaag**ttaattaa**AGGAACTAGGTTTAGACACATG	To amplify downstream region of *0186c* gene with fragment homologous to upstream region (underlined)
	JJ127(SalI)	CATG**gtcgac**GCGTGAAGAGAGTAACCA	

TABLE III (continued)

pJJ202	JJ61(SalI)	CATG**gtcgac**TTGCTGGTATTGAACGAAT	To amplify downstream region of 0414c-IGR segment with fragment homologous to upstream region (underlined)
	JJ62	ctttcgcttaGCGAGACTTACTAGCACATAAT	
	JJ63	taagtctcgcTAAGCGAAAGGTTATCCAC	To amplify upstream region of 0414c-IGR segment with fragment homologous to downstream region (underlined)
	JJ64(EcoRI)	CATG**gaattc**CCTTACCTCCCAGCACTAT	
	JJ65(PacI)	CATG**ttaattaa**TAAGCGAAAGGTTATCCAC	To add PacI site for *cat* cassette addition
	JJ66(PacI)	CATG**ttaattaa**GCGAGACTTACTAGCACATAAT	
pJJ224	JJ208(SalI)	CATG**gtcgac**GGTCAACCTAGAACCAAAC	To amplify and clone *isp1* gene plus neighborhood (Cloned using HindIII + EcoRI)
	JJ238(EcoRI)	CATG**gaattc**CTCATCAAAATTAGTAATGCTG	
	JJ240(NcoI)	CATG**ccatgg**GCTAACAATTTCCTTTTCTTC	To remove *isp1* gene by iPCR
	JJ241(NcoI)	CATG**ccatgg**ATAACCCCACAACCAAGAA	
pJJ215	JJ226(SalI)	CATG**gtcgac**AGCTATCTCCGAATTAGCTT	To amplify and clone inner region of *agaW* gene
	JJ227(EcoRI)	CATG**gaattc**CCAATCAGTACAAAAGGAAT	
pJJ216	JJ224(SalI)	CATG**gtcgac**GTCATCCTTGGTGGTACA	To amplify and clone inner region of *manM* gene
	JJ225(EcoRI)	CATG**gaattc**ATAGCTGCAAGAGCAAAAC	
pJJ217	JJ222(SalI)	CATG**gtcgac**GCAACAACCTTTACCATTAT	To amplify and clone inner region of *ptsC* gene
	JJ223(EcoRI)	CATG**gaattc**CTGGTAATTTCAAATAAGCTG	
pJJ219	JJ237(SalI)	CATG**gtcgac**TCCTTAGAAGAGTTAGCTGAA	To amplify and clone inner region of *mga* gene
	JJ238(EcoRI)	CATG**gaattc**CTCATCAAAATTAGTAATGCTG	

TABLE III (continued)

pJJ244	JJ254(SalI)	CATG**gtcgac**TTATCGTGATGGCTATGG	To amplify and clone *ptsA* promoter region
	JJ255(NotI)	CATG**gcggccgc**CTATTAACAAAAACCGCTGA	
pJJ250	JJ268(PstI)	CATG**ctgcag**CCTGATGATCTGTTGTTCC	To amplify downstream region of *isp2* gene with fragment homologous to upstream region (underlined)
	JJ265	ttagaaagaagtgacCTAATGACTTTTCTTACGGC	
	JJ267(SalI)	CATG**gtcgac**GGTATTTTTACTCATTTTGCC	To amplify upstream region of *isp2* gene with fragment homologous to downstream region (underlined)
	JJ265	ttagaaagaagtgacCTAATGACTTTTCTTACGGC	
pJJ251	JJ273(NotI)	CATG**gcggccgc**ATGAAACGAAAATTTCTCATT	To amplify and clone *ptsABCD* operon
	JJ274(BglII)	CATG**agatct**CTTACCTTTATTGCTAGGA	
pJJ252	JJ276(SalI)	CATG**gtcgac**TGGAACTGGTTATGCTATGA	To amplify and clone *manL* promoter region
	JJ257(NotI)	CATG**gcggccgc**CTCCTTTAAAATATTTTTCGTTG	
pJJ260	JJ298(EcoRI)	ATCCAA**gaattc**CTCGATAA	To amplify upstream region of *0026* gene with fragment homologous to downstream region (underlined)
	JJ312	agtatttacccaTGAGACCAAAAAACGATGA	
	JJ299(SalI)	CATG**gtcgac**GGTGGAAAACATCGACATC	To amplify downstream region of *0026* gene with fragment homologous to upstream region (underlined)
	JJ311	tttttggtctcaTGGGTAAATACTGATAAAGCTAA	
pJJ273	JJ294(SalI)	CATG**gtcgac**ACCTGTCTGGTATAATGAAAGAG	To amplify upstream region of *0015* gene with fragment homologous to downstream region (underlined)
	JJ309	atgttagtgtggCTACTCCTTTTCGATAAGACA	
	JJ295(SalI)	CATG**gtcgac**CATCTTCACCCACTAAACCAT	To amplify downstream region of *0015* gene with fragment homologous to upstream region (underlined)
	JJ310	gaaaaggagtagCCACACTAACATATTATTTAGGTTA	

TABLE III (continued)

pJJ276	JJ333(PacI)	GTAACTGAT**ttaattaa**GAGGTAAAGCTCCGAGATAAG	To amplify downstream region of *0790* gene
	JJ334	aattatcaagcttatcgatACGCTTCATACCTTTTCC	
	JJ308(EcoRI)	CATGgaattcCTGTTATCCCGCAACTTGT	To amplify upstream region of *0790* gene
	JJ332(PacI)	CTTACCTC**ttaattaa**ATCAGTTACTTCCTTTCTTCAAAAAATG	
pJJ277	JJ327(SalI)	CTCGAG**gtcgac**GGTTGTTTAGCTCAGGGATAATC	To amplify downstream region of *1459* gene
	JJ328(PacI)	GAGGACAG**ttaattaa**ATAAGACAAATGCCCTCG	
	JJ44	CATGCTGCAGTATAAAGGCATGCTTGAGCTA	To amplify upstream region of *1459* gene. Inner SalI site in amplified region will be used for cloning.
	JJ329(PacI)	TTTGTCTTAT**ttaattaa**CTGTCCTCCTTATCTAAAAACGGATAAAAGATAAG	
pJJ290	JJ335(SalI)	ctcgag**gtcgac**ggtCATTACAAGAAGAGATTATCCG	To amplify upstream region of *pepC* gene.
	JJ336(PacI)	AGAAAGGAA**ttaattaa**TATTGTCCTCCTAAAATATTAAGATAAG	
	JJ337(PacI)	AGGACAATA**ttaattaa**TTCCTTTCTTTGCGACTG	To amplify downstream region of *pepC* gene.
	JJ338(ClaI)	AATTATCAAGCTT**atcgat**ACCACGGCCTATATGATG	
cat	BL119(PacI)	GCGTG**ttaattaa**GGTATCGATAAGCTTGATGAA	To amplify *cat* cassette from pEVP3 plasmid, for marked deletion construction
	BL120(PacI)	GCGTG**ttaattaa**GAAAAAGGAGAAGTCG	
aph3A	JC320(PacI)	CATG**ttaattaa**CGATACTATGTTATACGC	To amplify *aph3A* cassette from pOskar plasmid, for marked deletion construction
	JC321(PacI)	CATG**ttaattaa**AGCGAACTTTTAGAAAAG	

2.1.1 Starter culture preparation

Strains of interest were inoculated into THY with appropriate antibiotics and grown overnight at 30° C. Next morning, cultures were diluted 1:100 into CDM and grown at 37° C until mid-exponential phase ($OD_{600} = 0.4$ to 0.7). At this point, glycerol was added to a final concentration of 20% and aliquots stored at −80°C.

2.1.2 Construction of *Streptococcus* mutant strains

The majority of *S. pyogenes* strains in this study were derived from the serotype M49 strain NZ131. When mutations were studied across serotypes, mutations were also generated in a strain of M6 serotype (GA19681).

To generate plasmids for allelic replacement, 750-1000 bp fragments encompassing the upstream and downstream regions flanking the gene of interest were amplified from NZ131 gDNA by PCR and cloned into the pJC162 temperature sensitive plasmid [67], by the use of restriction enzymes. Alternatively, the gene of interest plus is flanking upstream and downstream regions were cloned into pJC162, and the targeted gene removed by inverse PCR reaction (iPCR), restriction cut, and ligation. When necessary, antibiotic resistance cassettes for kanamycin (aph3A, amplified from pOskar [159]) or chloramphenicol (cat, amplified from pEVP3 [160]) were cloned in between the upstream and downstream fragments, to generate plasmids for selective allelic replacement. All deletion vectors were electroporated into competent *S. pyogenes* cells, and a two-step temperature dependent selection process was used to isolate the desired mutants [161]. Briefly, cells containing deletion plasmids were grown at the plasmid-replication permissive temperature (30° C), then shifted to the replication restrictive temperature (37° C) and plated on the appropriate antibiotic to select bacteria harboring the recombined plasmid into one of the regions flanking the gene of interest. Bacteria were then grown at the permissive temperature to allow the plasmid to recombine out of the genomic DNA, a process that was followed by the loss of Erm resistance, and loss of the gene of interest was screened by PCR and sequencing. This process was repeated to generate double mutants.

To generate plasmids for insertional gene disruption, 450-750 bp fragments from the middle region of the ORF of interest were amplified from NZ131 gDNA by PCR and cloned into the pUC-Kan plasmid by the use of restriction enzymes. The pUC-Kan plasmid contains an

origin of replication that is only functional in Gram negative bacteria. In order to generate the gene disruptions, 1 μg of each plasmid was electroporated into *S. pyogenes,* and mutants selected by growth in kanamycin, and checked by PCR.

2.1.3 Construction of luciferase transcriptional reporters

DNA fragments of 250-500 bp located upstream of the RBS of the genes of interest were amplified from NZ131 gDNA by PCR, and cloned by restriction digestion and ligation into the NotI and SalI sites of plasmid pJJ180, located next to an RBS and the *luxAB* ORFs.

2.2 Synthetic peptides

Synthetic peptides of >95% purity were purchased from Neo-Peptide (Cambridge, MA), and reconstituted as 1mM stocks in DMSO and stored at -80° C. Dilutions for working stocks were made in DMSO and stored at -20° C.

2.3 Biofilm formation assays

S. pyogenes strains were grown overnight in THY at 30° C. Next morning, strains were diluted 1:25 into fresh CDM and 500 μL dispensed in duplicate into cell culture treated 24-well polystyrene plates. 25mM synthetic pheromones were added to each well, and plates incubated statically at 37° C 5% CO_2 for 20h. Medium was aspirated, wells washed once with 300 μL of 0.9% NaCl and biofilms were dry-fixed at 37° C for at least 6h. Biofilms were stained with 0.2% crystal violet solution, washed three times with 0.9% NaCl 10% EtOH, and quantified by measurement of absorbance at 595nm by area scan of the wells in a Synergy 2 BioTek plate reader. A minimum of three experimental replicates was performed for each strain and condition.

2.4 Luciferase reporter assays

Starter culture aliquots of strains of interest were thawed and diluted into fresh CDM into an OD_{600} = 0.025, and grown at 37° C. Every 30m, 80 μL samples were taken from each culture and transferred to a white bottom 96-well plate, samples exposed to decyl aldehyde (Sigma) fumes, and luminescence counts per second (CPS) were measured using a Turner BioSystems microplate luminometer. OD_{600} of the culture was measured at each time point using a Spectronic 20D sepctrophotometer (Thermo), and relative light units (RLU) calculated by normalizing CPS to OD_{600}.

For the analysis of peptide pheromone signaling (chapter III and IV), 25nM of synthetic peptides were added at the beginning of growth. For the analysis of the effect of carbon source over target gene expression (chapter V), starter cultures were diluted into CDM in which glucose was replaced by mannose or fructose as explained in section 2.9.

2.5 Bacterial sedimentation assays

Starter cultures were diluted into THY media and allowed to grow until they reached early exponential phase. Cells were pelleted and resuspended to a final $OD_{600} = 0.05$ in fresh CDM containing 50 nM synthetic pheromones. Cells were then grown at 37° C and briefly vortexed every 15 min until they reached an $OD_{600} = 0.5$. Cultures were then placed at room temperature, duplicate 100 µl samples taken from below the meniscus every 20m and immediately measured for absorbance at 600 nm in a Synergy 2 microplate reader (Biotek).

2.6 Quorum sensing inhibition in biofilm assays

Bacterial strains were grown overnight in THY medium at 30°C and then back-diluted 1:100 into fresh CDM and grown at 37° C until they reached late exponential phase. Bacteria were back-diluted into tubes of fresh CDM containing 10 nM SHP2C8 and 2-fold dilutions of the quorum sensing inhibitors cyclosporine A or valspodar, at concentrations ranging from 5 to 0.156 µM. NZ131 strains were back-diluted to an OD_{600} of 0.02, and GA19681 and MGAS5005 strains were back-diluted to an OD_{600} of 0.005. Tubes containing 0.1% DMSO were used as controls. Bacteria were incubated for 1 hour at 37°C until they reached an OD600 of ~0.1 for NZ131 strains and an OD600 of ~0.02 for GA19681 and MGAS5005 and then plated in duplicate in cell culture-treated 24 well polystyrene plates. Plates were then incubated at 37° C with 5% CO2 for 20 h to promote biofilm growth. Biofilms were quantified as stated above.

2.7 Lysozyme challenge assays

Starter cultures were diluted into CDM to an $OD_{600} = 0.025$ and grown at 37° C until they reached an $OD_{600} = 0.1$. Cultures were then divided into two tubes, and SHPC8 pheromone or reverse SHPC8rev control peptide added to medium. Cells were incubated for one hour at 37° C, backdiluted to an $OD_{600} = 0.1$, and lysozyme from chicken egg white (Sigma) added to a final concentration of 2 mg/mL. Cells were incubated at 37° C and every hour aliquots were taken, serially diluted and plated into THY plates. After incubating plates overnight, colonies were counted and CFU/mL estimated for each time point. Values were expressed as fold change in CFU/mL relative to the viability prior lysozyme addition. Each strain was assayed in duplicate.

2.8 Bioinformatic analysis

The sequenced genomes from *S. pyogenes* NZ131, *S. pneumoniae* R6, *S. sanguinis* SK36, *S. anginosus* C238, *S. themophilus* LMD-9, *S. downei* F0415, *S. mutans* UA159, *S. gallolyticus* ATCC43143, *S. canis* FSL-Z3, *S. dysgalactiae* subspecies *equisimilis* ATCC12394, *S. dysgalactiae* subspecies *dysgalactiae* ATCC27957, *S. equi* subspecies *zooepidemicus* H70, *S. ictaluri* 707-05, *S. porcinus* Jelinkova176, *S. uberis* 0140J, *S. iniae* YSFST01-82, *S. urinalis* FB127-CNA-2, and S. agalactiae 2603V/R were obtained from the NCBI webpage (https://www.ncbi.nlm.nih.gov/) in final assembly or contig form. The DNA segments encompassing the *era-fpg* region were identified and extracted using the CLC Sequence Viewer software (QIAGEN), and the genetic composition of each species was assembled over a reconstructed *Streptococcus* phylogenetic tree derived from a set of 136 core genes, conserved along the genus [162]. For the comparison of DNA sequences of *S. pyogenes* NZ131 and *S. canis* FSL-Z3, alignments were generated to assess DNA identity (blastn, http://blast.ncbi.nlm.nih.gov/Blast) and proteins similarity (EMBOSS matcher, http://mobyle.pasteur.fr). Stop codon analysis and putative ORFs from IGR region of NZ131 were predicted using ClustalO and ORF prediction tools from CLC Sequence Viewer software.

2.9 Growth curves with alternative carbon sources

Carbon source free CDM was prepared by omitting glucose, and bringing the volume up to 90% of the final value. Carbon sources of interest (mannose, fructose, N-acetylglucosamine, glucosamine, sucrose and glucose) were prepared as 10% (w/v) stocks and diluted into carbon source free CDM. Starter cultures were diluted into regular CDM and allowed to grow to an OD_{600} = 0.3, pelleted, washed once to remove residual glucose and diluted into fresh CDM containing the carbon source of interest at a starting OD_{600} = 0.05. Growth was monitored using a Spectronic 20D+ spectophotometer (Thermo Scientific).

2.10 Scanning electron microcopy

S. pyogenes biofilms were grown over polystyrene coverslips, in 6-well polystyrene plates. After 20h of growth, biofilms were prepared as described [163]. Briefly, biofilms were fixed in a 2.5% glutaraldehyde, 0.0075% ruthenium red, 0.1 M sodium cacodylate buffer pH 7.2 solution, for one hour. After three washes in cacodylate buffer, sample was dehydrated with increasing concentrations of EtOH (30% → 50% → 75% → 95%→100%) for 10m each. Samples were then treated with 100% hexamethyldisilazane, and submitted to SEM analysis to RRC imaging service.

III. Activation of the Rgg2/3 quorum sensing pathway leads to increased cellular aggregation and biofilm formation in *Streptococcus pyogenes*

(Some results from this chapter are reprinted in part, with permission, from Aggarwal, C., Jimenez, J.C., Nanavati, D., and Federle, M.J. 2014, *JBC*, doi: 10.1074/jbc.M114.583989 [a]; and from Aggarwal, C., Jimenez, J.C., Lee, H., Chlipala, G.E., Ratia, K., and Federle, M.J. 2015, *mBio*, doi: 10.1128/mBio.00393-15 [b])

3.1 Rationale

The Rgg2/3 signaling pathway is the only characterized QS system conserved across *Streptoccus pyogenes* isolates. Although we possess transcriptional data regarding the Rgg2/3 regulon, the cellular behaviors being regulated by this system are currently unknown. Biofilm formation is a polyfactorial event, in which a variety of enzymatic functions and cellular processes come together to favor the aggregation and attachment of bacterial cells in order to generate a sessile community. In both Gram-negative and Gram-positive bacteria, QS has been shown to modulate biofilm production through the regulation of genes involved in bacterial attachment (*S. aureus* adhesins [164]), cellular aggregation (regulation of *Y. pseudotuberculosis* clumping [165]), matrix production (exopolysaccaride formation in *V. cholera* [166]) , biofilm maturation (promotion of complex biofilm architecture in *P. aeruginosa* [167]), and biofilm dispersal (activation of proteases in *S. aureus* [168]).

Currently, there is little understanding of the events that regulate biofilm formation in *S. pyogenes*, and while genes that contribute or hinder to this process have been discovered, no regulatory system that modulates the switch between planktonic and biofilm modes of growth has been discovered yet. The aim of this chapter is to evaluate the effect of pheromone signaling through the Rgg2/3 QS pathway over the process of biofilm formation in *S. pyogenes.*

3.2 Results

3.2.1 SHP2C8 and SHP3C8 pheromones increase biofilm production in *S. pyogenes* NZ131

To test the effects of Rgg2/3 signaling on biofilm formation, cultures of the NZ131 strain were grown in 24-well polystyrene plates in the presence of active signaling pheromones SHP2C8 or SHP3C8, with their inactive reverse sequence counterparts SHP2revC8 or SHP3revC8, and in a vehicle only (DMSO) control condition. After 20 h of growth, a significant increase in the attached biomass was observed in the wells containing active signaling pheromones, when compared with the control conditions (Figure 7A). Crystal violet staining and absorbance quantification revealed a 4-to-5 fold increase in biofilm production when comparing SHP2C8 and SHP3C8 to the vehicle control (Figure 7B). This increase in biomass was not due to the hydrophobic nature of the added peptides since the reverse sequence peptides SHP2revC8 and SHP3revC8, which share the same chemical composition of SHP2C8 and SHP3C8 while lacking the ability to trigger QS signaling, were unable to increase biofilm production. When viable counts of both the surface-attached (biofilm) and free-floating (planktonic) communities were analyzed, it revealed a 5-fold increase in biofilm cell counts in the presence of SHPC8 pheromones, coupled with a decrease in the planktonic cell count compared with control conditions (Figure 7B). To analyze the dose effect of pheromone concentrations over biofilm production, we used a mutant strain with start codon mutations in both pheromone genes (*shp2*$_{GGG}$ *shp3*$_{GGG}$*)*. This strain lacks the ability to produce its own pheromone peptides and is thus unable to induce a positive feedback loop, and therefore we can define the exact concentration of peptide pheromones in the media. As seen in Figure 8, biofilm formation followed a dose-dependent behavior with increasing SHPC8 concentrations, saturating at around 200nM SHPC8 peptides.

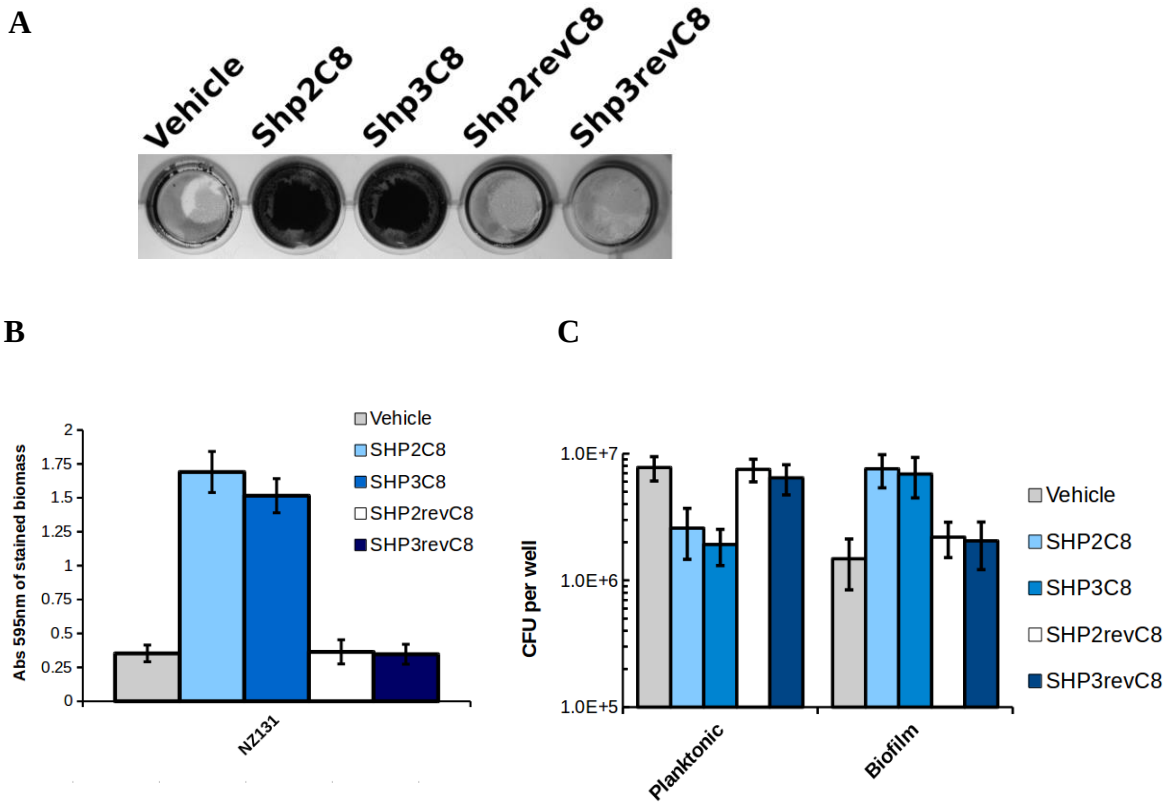


FIGURE 7. SHP pheromones enhance biofilm formation in *S. pyogenes* NZ131. The ability of NZ131 to form biofilms in polystyrene plates was assessed. Strains were grown in CDM for 20h in the presence of 25nM of the designated peptides, or with the same volume of DMSO, the vehicle used to dissolve the peptides. **A.** Picture of a crystal violet stained biofilm plate. **B.** Biofilm formation in several experiments was quantified by measuring the crystal violet absorbance by scanning the stained biofilm area. Bars show standard error between experiments. **C.** Comparison of viable counts in planktonic and biofilm communities in the presence and absence of signaling peptides.

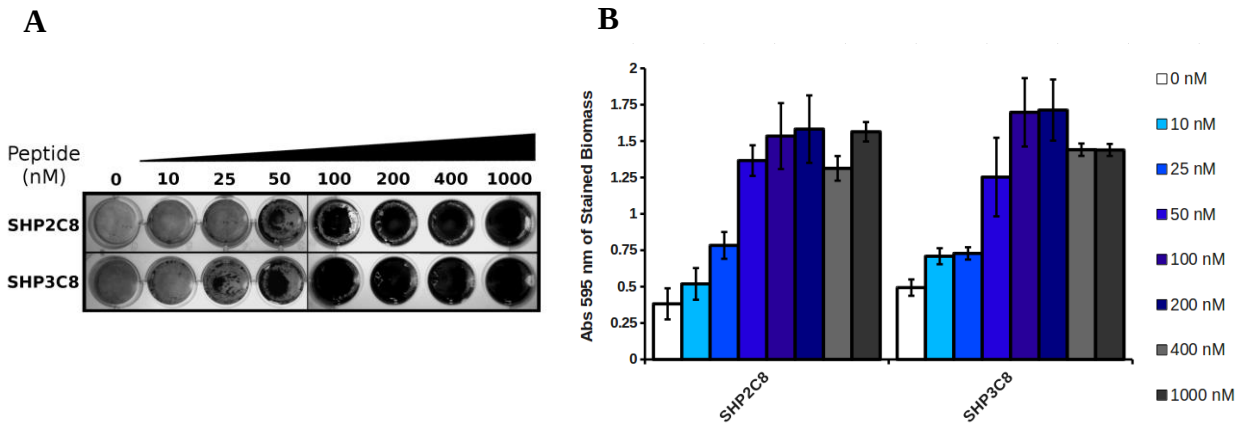


FIGURE 8. Dose dependent effect of SHP pheromones over biofilm formation in *shp2*$_{GGG}$ *shp3*$_{GGG}$ strain. A strain devoid of SHP pheromone gene expression was used to measure the effect of defined pheromone concentrations over biofilm formation. Strains were grown in CDM for 20h in the presence of 25nM of the designated peptides, or with the same volume of DMSO. **A.** Picture of a crystal violet stained biofilm plate. **B.** Biofilm formation in several experiments was quantified by measuring the crystal violet absorbance by scanning the stained biofilm area. Bars show standard error between experiments.

3.2.2 Different peptide pheromone variants promote biofilm formation

Work from our lab has shown that while the eight amino acid long SHPC8 is the preferential length of produced SHP pheromones, nine (SHPC9) and ten (SHPC10) amino acid long fragments are also secreted at lower concentrations. These SHP variants also possess the ability to trigger QS signaling, although they require higher concentrations to do so [88]. When assayed for their ability to induce biofilm production in the NZ131 strain, the C9 and C10 variants of SHP2 and SHP3 peptides are able to induce biofilm formation, although at concentrations that are at least 5-fold higher than those required for the SHPC8 versions (Figure 9), supporting the weaker QS ability of these longer SHP variants.

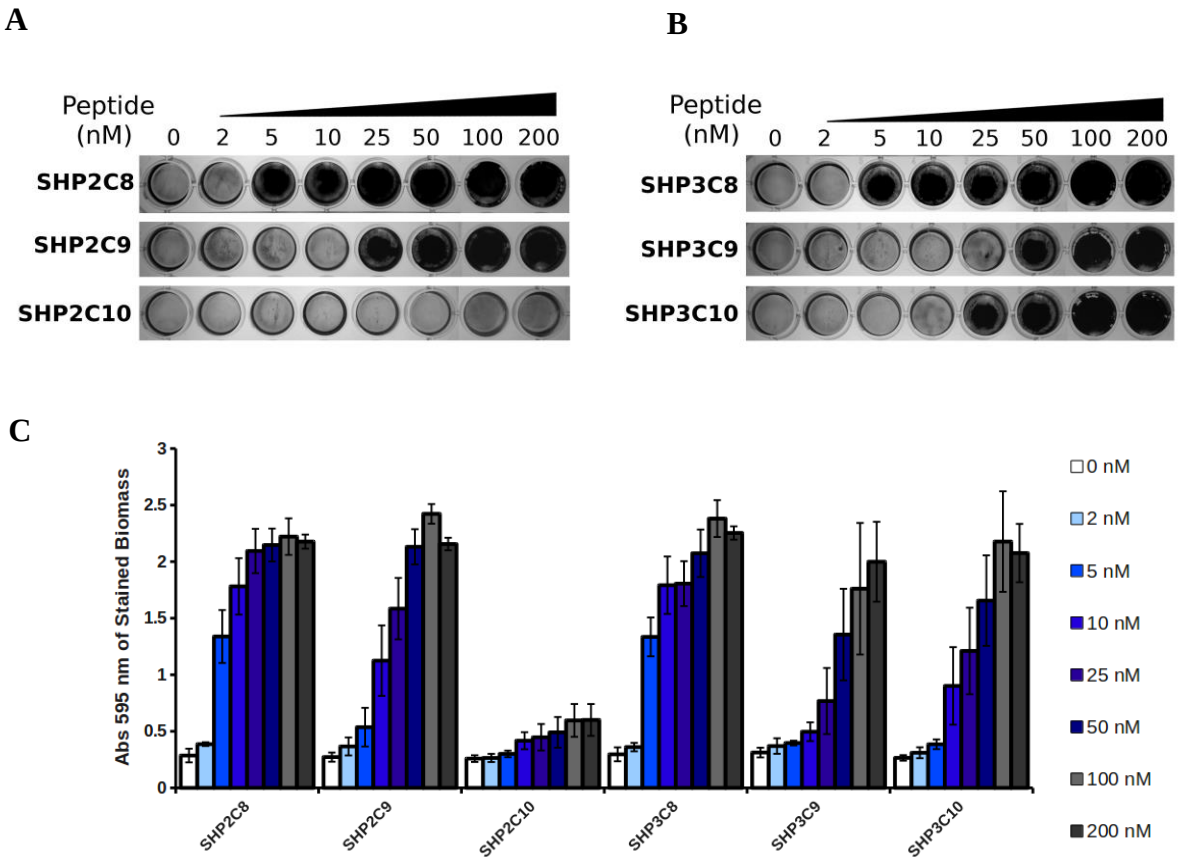


FIGURE 9. Different SHP pheromone variants promote biofilm formation. Wild-type NZ131 strain was incubated with various concentrations of SHP2 (**A**) and SHP3 (**B**) pheromone variants of different lengths, and their biofilm abilities measured. **A.** and **B.** Pictures of a crystal violet stained biofilm plates. **C.** Biofilm formation in several experiments was quantified by measuring the crystal violet absorbance by scanning the stained biofilm area. Bars show standard error between experiments.

3.2.3 Biofilm production is stimulated by the activation of the Rgg2/3 QS pathway

Although only active signaling pheromones (and not control reverse peptides) were able to increase biofilm formation, this does not exclude the possibility that the SHP peptides are working through other mechanisms different from Rgg2/3 QS activation to increase biomass. To demonstrate that the biofilm-enhancing effect of SHPC8 pheromones relies on signaling through the Rgg2/3 pathway, we proceeded to test biofilm production in strains containing diverse gene deletions in this QS circuit. We also used a luciferase reporter construct containing the QS target promoter of the *shp*2 pheromone gene (P_{shp2}-*luxAB*) to follow the activation of QS in planktonic cultures, and we analyzed both the patterns of QS signaling and biofilm formation in mutants of the pathway. In the wild-type strain, addition of either SHPC8 pheromone leads to a 500-fold increase in P_{shp2}-*luxAB* activity (Figure 10A), and this activation is reflected in the increase of biofilm formation with the pheromones (Figure 10B). The deletion of the *rgg*2 gene, encoding for the transcriptional activator of the QS pathway, leads to a significant hindrance in target promoter activation, as well as the inhibition of biofilm increase, when pheromones are added. Deletion of *rgg*3, encoding the transcriptional repressor of QS, leads to a derepression of *shp* pheromone genes, thus the addition of exogenous synthetic pheromones is not required to activate QS and high levels of signaling as well as high biofilm levels become constitutive. The double Δ*rgg*2 Δ*rgg*3 deletion generates a strain that is unable to produce pheromones and unable to respond synthetic pheromones, and thus is also unable to increase biofilm formation in the presence of SHP2C8 or SHP3C8. These results show how the pattern of transcriptional activation of QS is correlated with the increase in biofilm formation in response to SHP pheromones.

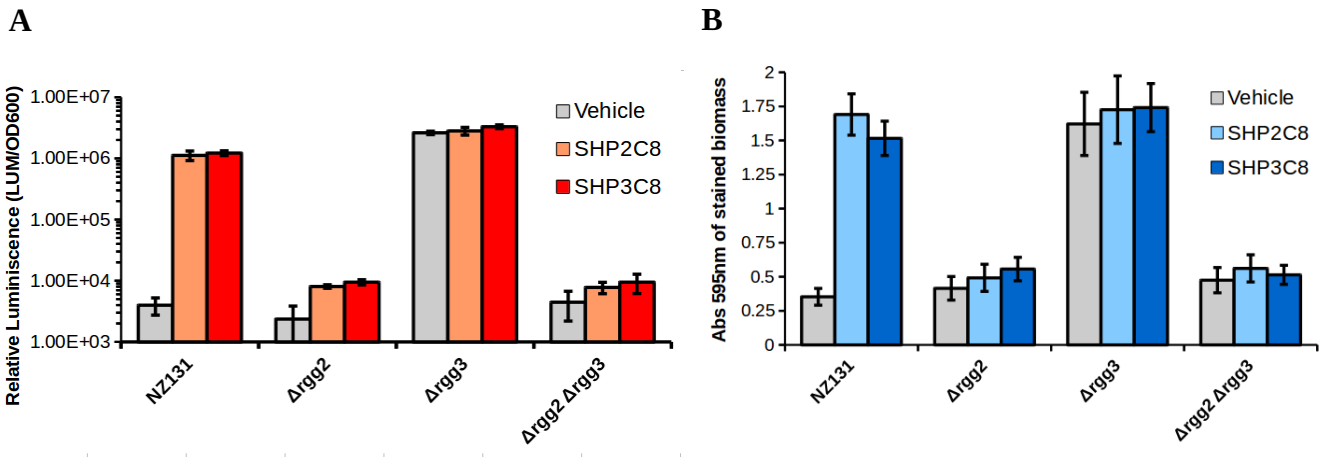


FIGURE 10. Involvement of Rgg2/3 genes in biofilm formation. Several mutants in components of the Rgg2/3 system were assessed for their ability to activate QS, and to form biofilms in response to SHPC8 pheromones. **A.** Activation of QS by SHPC8 pheromones as measured with a P_{shp2}-*luxAB* transcriptional reporter. Bars show standard deviation. **B.** Quantification of biofilm formation in several experiments. Bars show standard error.

We proceeded to analyze the biofilm by scanning electron microscopy (SEM), in order to detect any gross morphological changes in biofilm matrix that could explain the increased biofilm formation seen in presence of SHP pheromones. We analyzed the wild-type strain grown in the absence and presence of SHP3C8, plus a Δ*rgg*3 mutant that exhibits constitutive high levels of biofilm (Figure 11). SEM revealed that no drastic changes occurred in the constitution of the biofilms analyzed.

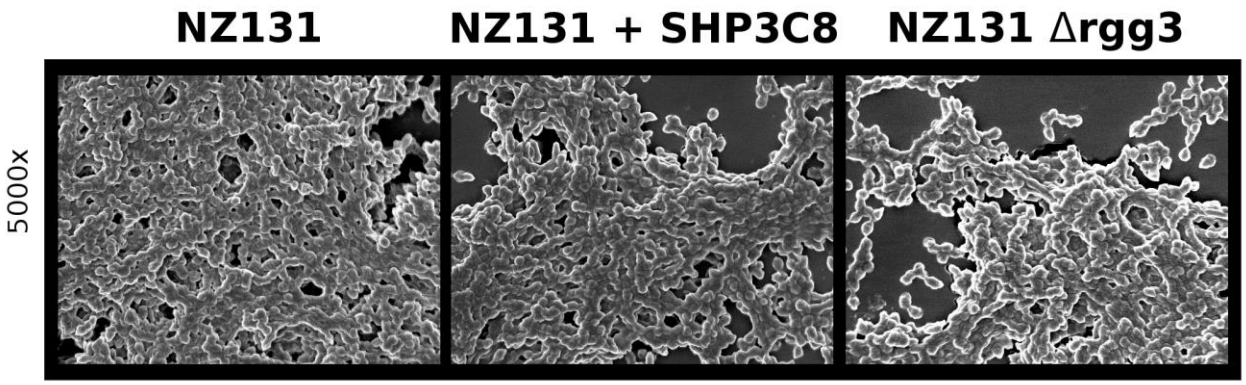


FIGURE 11. Microscopic analysis of *S. pyogenes* biofilm. *S. pyogenes* biofilms were grown over polystyrene coverslips, fixed in a 2.5% glutaraldehyde 0.075% ruthenium red solution and analyzed by scanning electron microscopy. 5000X magnification images shown.

3.2.4 *S. pyogenes* isolates show differential ability to increase biofilm in response to SHP pheromone signaling

Within the species, *S. pyogenes* presents many serotypes (more than 100 M-types) and displays diversity in its pathogenic potential and tissue tropism. A vast amount of genetic variability is found among isolates, in some cases in the form of large mobile elements as prophages, or in other cases as simple point mutations in key transcription factors that can alter transcriptional responses of the organism [169]. For this reason, although the Rgg2/3 pathway is conserved through all sequenced isolates, it is important to prove that the phenotypes being regulated are conserved as well. To this end, we tested the effects of QS on biofilm formation in different isolates of *S. pyogenes* (Figure 12). Our first observation was that the basal levels of biofilm formation in the absence of QS were strikingly different between isolates, with some exhibiting low levels (NZ131, MGAS8232) and others having exceedingly high levels of biofilm in the absence of pheromones (HSC5, MGAS5005, MGAS10394). After adding SHPC8 pheromones, apart from NZ131, only the GA19681 strain exhibited an increased ability to form biofilms, while the HSC5 strain exhibited an opposite phenotype. All other strains showed no difference in their biofilm levels in presence of pheromones. These results show that the ability to increase biofilm production in response to pheromones is only seen in particular isolates.

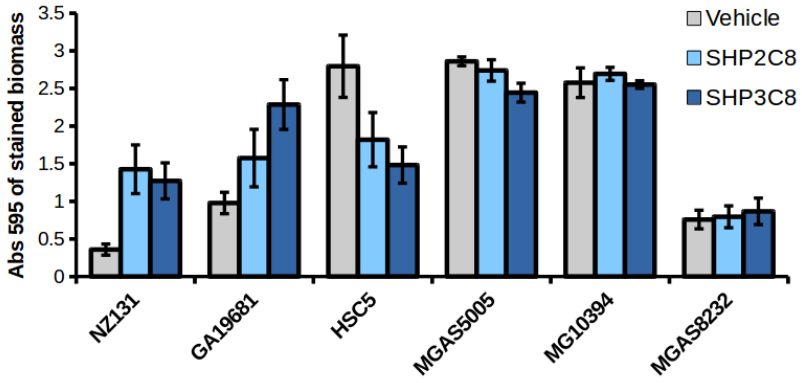


FIGURE 12. Biofilm formation response of diverse *S. pyogenes* isolates. 5 isolates corresponding to 4 different serotypes were assessed in their biofilm forming capabilities and their ability to respond to SHPC8 pheromones, and compared to the NZ131 strain. Biofilm was quantified in several experiments. Bars show standard error.

3.2.5 QS inhibiting molecules prevent increase of biofilm formation by SHP pheromones

The use of purified Rgg2 protein in a high throughput fluorescence polarization platform has led our lab to discover small molecules that inhibit the interaction of SHP pheromones with their target transcription factors [170]. In order to test the effect of QS inhibitors over biofilm formation we used cyclosporin A, the top inhibiting compound, and its non-immunosupressive derivative compound valspodar. Bacteria were grown with 10 nM SHP2C8, or with 10 nM SHP2C8 plus a range of concentrations of the QS inhibitors, and then cultured in 24-well plates to assess the process of biofilm formation. When cyclosporin A was added at concentrations of 1.25 μM or above, the increase in biofilm formation promoted by SHP2C8 was inhibited; while valspodar was able to abolish the process in concentrations of 630 nM and higher (Figure 13).

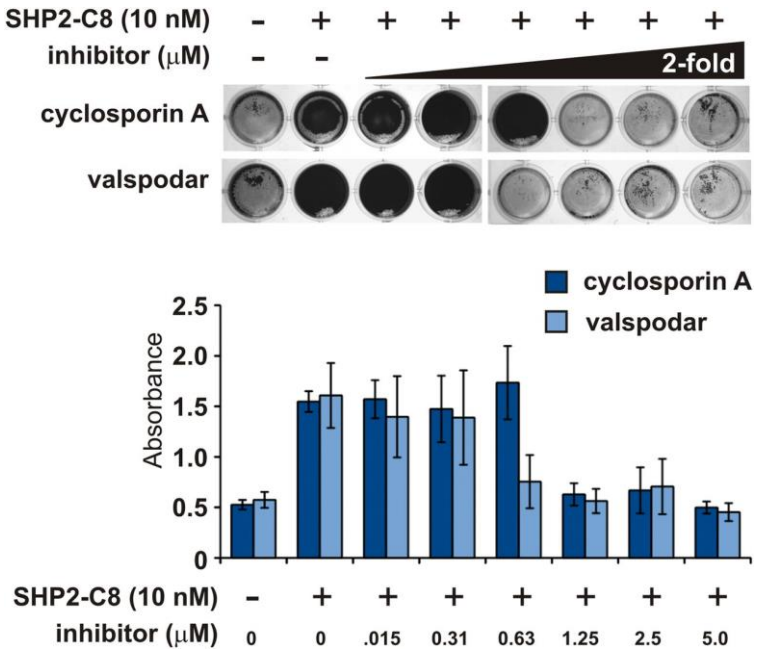


FIGURE 13. Effects of quorum sensing inhibition over biofilm formation. The NZ131 strain was incubated in the prescience of SHP2C8, plus increasing concentrations of known QS inhibitors cyclosporin A and valspodar, and then plated to assess biofilm formation. Top, plate of one representative biofilm and QS inhibition experiment. Bottom, quantification of several experiments. Bars show standard error.

As reported above, the effect of SHP pheromones on biofilm formation in *S. pyogenes* is strain-dependent. For this reason, we tested the effects of cyclosporin A over biofilm in three more strains: one isolate that is able to increase its biofilm levels in response to QS (GA19681), one isolate that shows constitutively high levels of biofilm formation independent of SHP-signaling (MGAS5005) as well as an NZ131 Δ*rgg3* mutant strain that exhibits constitutively high levels of biofilm dependent on QS-signaling (Figure 14). Cyclosporin A was able to repress SHP signaling in all tested isolates (Figure 14A), and was able as well to repress the SHP-induced biofilm increase in wild-type NZ131, NZ131 Δ*rgg3* and GA19681 (Figure 14B). The high biofilm levels of MGAS5005, which are independent of QS, remain unchanged. Additionally, cyclosporin A only inhibits the SHP-dependent contribution to biofilm, and does not have an effect over basal biofilm levels, as better seen for strain GA19681. Thus, QS inhibitors can be used to suppress the SHP-dependent increase in biofilm in select *S. pyogenes* strains, but do not have a general biofilm-inhibiting effect.

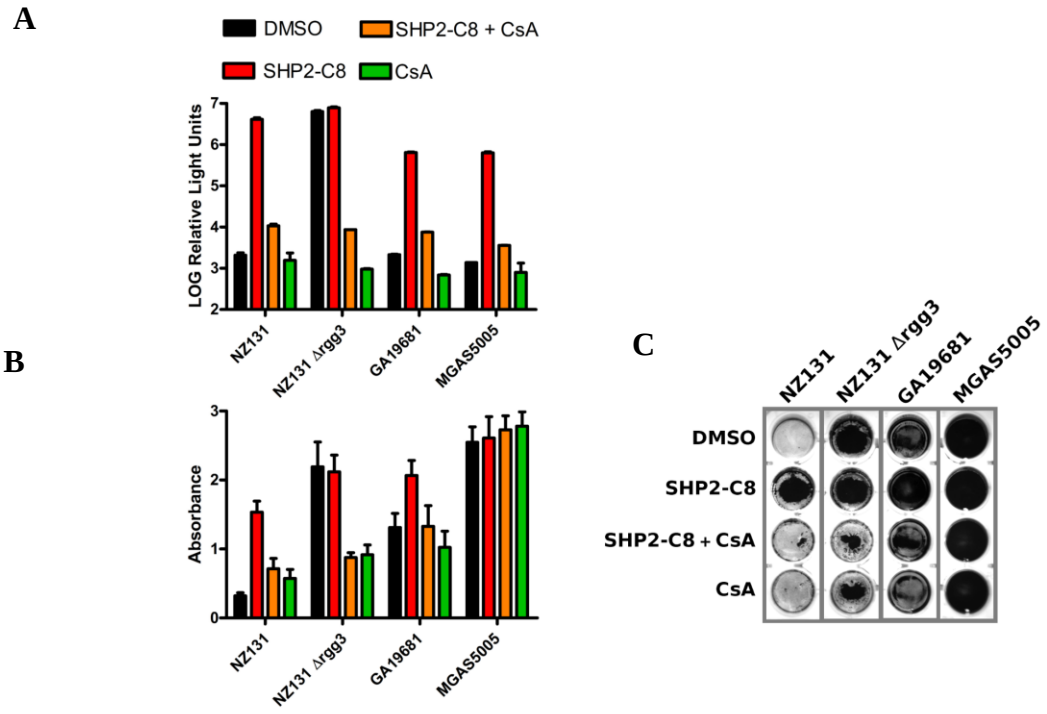


FIGURE 14. Effects of quorum sensing inhibition and biofilm formation in different *S. pyogenes* isolates. *S. pyogenes* strains were tested for QS inhibition and its effect over biofilm formation. **A.** Inhibition of SHP2C8 induced QS by cyclosporin A (CsA) as measured with a P_{shp2}-*luxAB* transcriptional reporter. Bars show standard deviation. **B.** Quantification of biofilm formation in the presence of SHP2C8 and/or cyclosporin A in several experiments. Bars show standard error. **C.** Picture of a biofilm plate from a representative experiment.

3.2.6 Activation of Rgg2/3 QS pathway leads to increased cellular aggregation of NZ131 strain

As with other bacterial organisms, *S. pyogenes* cultures undergo a process of self-aggregation at high cellular densities. A variety of factors can affect this process, including capsule formation, pili expression, and the homophilic interaction between surface attached proteins in neighboring cells; and this process is highly variable between species and isolates [113], [171]. During the process of performing our experiments, we noticed that the rate at which bacterial cultures aggregated differed between those exposed to control peptides and those exposed to SHPC8 pheromones. In order to quantify this process, we performed a simple assay to assess the process of cellular aggregation by measuring the decrease in optical density during sedimentation of bacterial aggregates. Cells grown in the presence of SHP3C8 pheromone showed an increased sedimentation rate when compared with cells grown with the SHP3revC8 control, suggesting that increased aggregation was a result of QS signaling and not an effect of hydrophobic peptides in the medium (Figure 15A and C). Additionally, when the experiment was repeated using a Δ*rgg*2 strain, which is unable to activate QS in response to SHP pheromones, no drastic difference in sedimentation between the SHP3C8 and SHP3revC8 condition was observed (Figure 15B and C). The effects of pheromone on cellular aggregation can also be seen when cultures are analyzed by microscopy (Figure 15D). These results show how activation of the Rgg2/3 QS pathway leads to increased cellular aggregation in *S. pyogenes* NZ131.

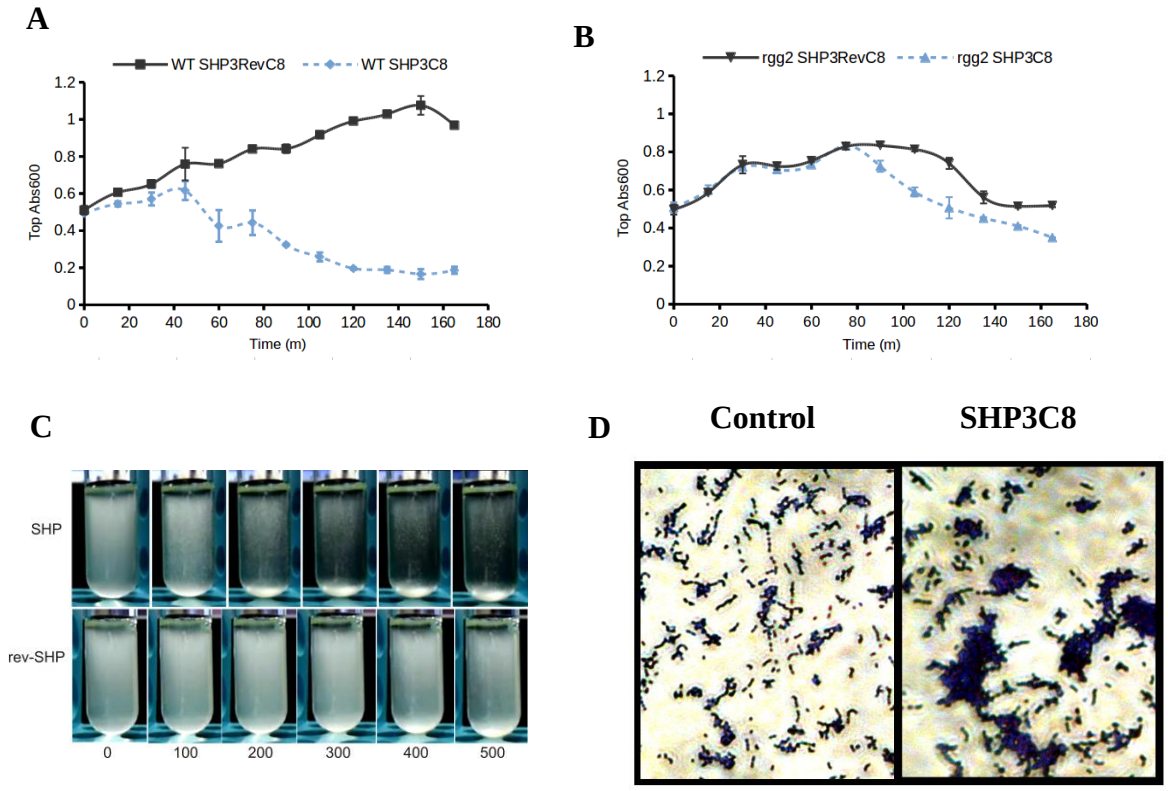


FIGURE 15. Aggregation and sedimentation of *S. pyogenes* NZ131 cultures after SHPC8 signaling. A. wild-type and **B.** Δ*rgg2* strains were tested for their aggregation by measuring the decrease in apparent absorbance in the upper region of culture tubes. **C.** Pictures of a time-course analysis of the sedimentation of the wild-type strain. **D.** Light microscope picture, appearance of *S. pyogenes* grown over glass coverslips in the presence or absence of SHP3C8.

3.3 **Discussion**

Recent works in the area of biofilm formation in *S. pyogenes* are slowly unveiling the contribution of this growth phenotype to the lifestyle and pathogenesis of this organism. While precise genes, including proteases, DNases, capsule synthesis proteins and cell wall-attached adhesins have been shown to modulate the biofilm formation process; their contribution and effect seem to fluctuate depending the isolate and the biofilm model tested [172].

The results in these chapter are summarized in Figure 16. The internalization and detection of SHP pheromones, and the consequent activation of the Rgg2/3 pathway lead to increased cellular aggregation and the promotion of enhanced biofilm formation in *S. pyogenes*. Interaction between pheromones and the Rgg2/3 receptors can be prevented if QS inhibitor molecules are used, preventing as well an increase in biofilm production. Even though the process of pheromone signaling can be observed among different isolates, the ability of Rgg2/3 signaling to effect biofilm formation is only restricted to a few of these. Thus, this pathway is not a global regulator of biofilm formation in this species, but rather, it may be regulating other processes that indirectly affect biofilm and cellular aggregation in a subset of strains. We will elaborate on this process further in the next chapter, in which we will use the biofilm phenotype as a proxy to uncover the possible molecular changes that are being triggered by activation of Rgg2/3 QS in *S. pyogenes.*

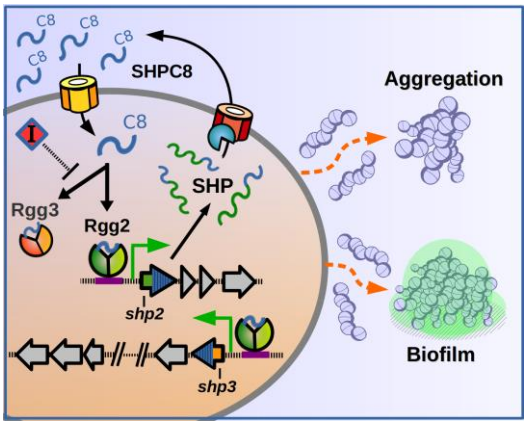


FIGURE 16. Chapter III working model. Activation of the Rgg2/3 quorum sensing pathway leads to increased cellular aggregation and biofilm formation.

IV. A small secreted protein is required for quorum sensing-dependent increase in biofilm formation and lysozyme resistance in *Streptococcus pyogenes*.

4.1 Rationale

As discussed in the previous chapter, activation of a conserved QS pathway in *S. pyogenes* leads to increased biofilm formation and cellular aggregation in *S. pyogenes* NZ131. Additionally, recent results from our lab show that activation of Rgg2/3 QS also lead to increased resistance to the bactericidal effects of lysozyme, an enzyme that targets the bacterial cell wall [154]. We propose that these phenotypes are indirect consequences reflecting changes occurring at the molecular level of the surface envelope of *S. pyogenes* in response to QS signaling.

This chapter reveals the genes regulated by Rgg2/3 that lead to an increase in biofilm formation. The experiments described here provide a better understanding of the gene functions regulated by QS in *S. pyogenes* and their effect over this pathogen's biology. We hypothesize that the genes modulated by this QS pathway play a role in the modification of the cellular envelope, the cell wall and/or the proteins that decorate this structure.

4.2 Results

4.2.1 The *shp2 downstream region encodes a gene involved in biofilm formation*

To understand the molecular mechanism responsible for biofilm increase in the NZ131 strain, we generated mutations in target genes controlled by the Rgg2/3 pathway, and asked which mutants affected biofilm formation. We targeted the genetic regions showing the highest increase in expression upon exposure to pheromones, as determined by microarray experiments [87]. Downstream of the *shp3* gene, a nine gene operon is encoded (genes *0450* to *0460*, Figure 17), whose expression we predict is driven by the P_{shp3} promoter. This operon harbors genes of diverse enzymatic functions, including a shikimate dehydrogenase, a sugar isomerase, a sugar dehydrogenase and an efflux pump protein, suggesting the involvement of this region in the production and secretion of a hypothetical secondary metabolite. Downstream of the *shp2* gene, three genes encoding hypothetical proteins of unpredicted function plus a 913bp intergenic

region (referred in this work as IGR) are encoded. In the same coding strand as *shp2*, and predicted to be driven by the P_{shp2} promoter, lies the *0414c* gene encoding a hypothetical secreted protein, and on the opposite strand lie two additional genes (*0413* and *0412)*. The deletion of the *0450 – 0460* operon, as well as the deletion of the *0412-IGR-0414c* region, do not affect the ability of the Rgg2/3 circuit to signal, as addition of SHP2C8 and SHP3C8 leads to activation of the QS regulated P_{shp2} promoter in these mutants in the same degree as in the wild-type strain, as assessed by a luciferase reporter (Figure 18A). However, when biofilm production was assessed, deletion of the *0412-IGR-0414c* region disrupted the increase in biofilm stimulated by the pheromones (Figure 18B and C). Biofilm formation was restored when the *0412-IGR-0414c* region was complemented in single copy and stimulated by pheromones, indicating that the biofilm-promoting gene is encoded within this genetic neighborhood (Figure 18C).

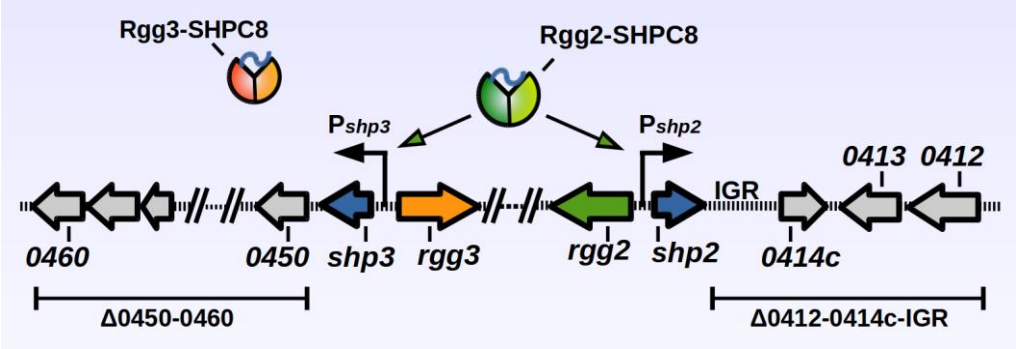


FIGURE 17. Targets of Rgg2/3 regulation. Previous transcriptional analysis and DNA-binding assays have revealed that Rgg2 and Rgg3 antagonistically compete to regulate two target promoters, P_{shp3} and P_{shp2}, which promote transcription of their corresponding pheromone genes. Additionally, the downstream genes after each *shp* gene also are upregulated.

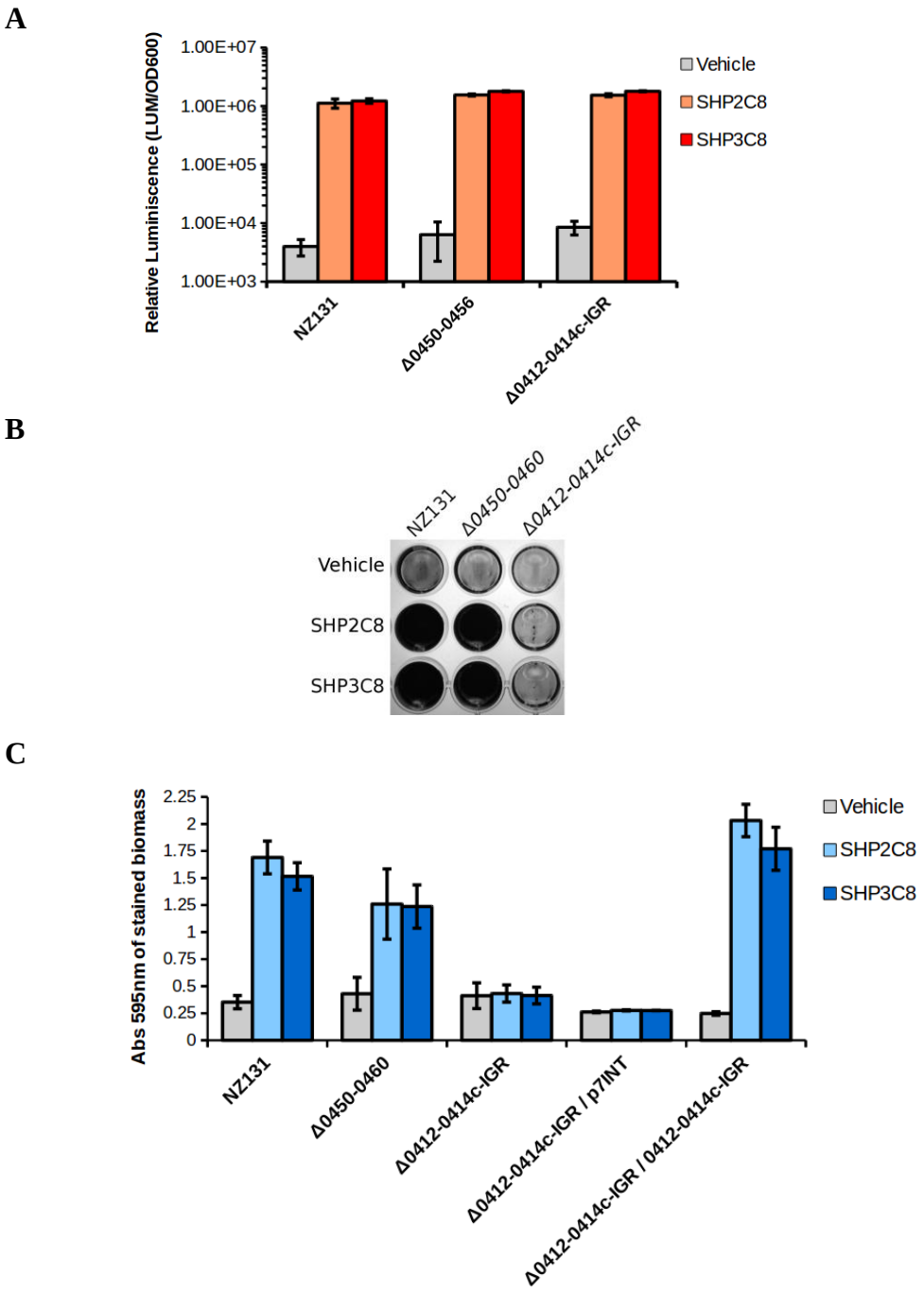


FIGURE 18. Involvement of Rgg2/3 regulated genes in biofilm formation. Large deletions were generated in the regions encoded downstream of the *shp2* and *shp3*, and the effects over QS signaling and biofilm formation measured. **A.** Activation of QS by SHPC8 pheromones as measured with a P_{shp2}-*luxAB* transcriptional reporter. Bars show standard deviation. **B.** Picture of a crystal violet stained biofilm plate form a representative experiment **C**. Quantification of biofilm formation in several experiments. Bars show standard error.

4.2.2 The 0414c protein is necessary and sufficient to promote biofilm formation

To identify the gene(s) required for biofilm formation in the *0412-IGR-0414c* region, we generated individual defined deletions in the *0412, 0413,* and *0414c* genes. While deletions of *0412* and *0413* did not affect biofilm formation, the deletion of *0414c* was able to disrupt the biofilm-increase phenotype, and the phenotype was restored when *0414c* was provided back in single copy (Figure 19A and B). The role of 0414c is not restricted to the NZ131 isolate, as when the homolog of 0414c was deleted in the GA19681 *S. pyogenes* strain, the ability to develop enhanced biofilms in response to pheromones was lost in this isolate also (Figure 19C). While these experiments show the requirement of the *0414c* gene, they do not assess the role the preceding IGR may play in the biofilm phenotype. Our attempts to generate non-polar deletions of the IGR were unsuccessful. For this reason, in order to assess the role of the IGR for the biofilm phenotype, we generated an antibiotic cassette-marked mutant of the IGR-*0414c* region, and attempted to restore the wild-type phenotype with different complementation constructs (Figure 20). The ΔIGR-*0414c* mutant, which as expected was unable to increase biofilm in response to SHP3C8 pheromone, could be complemented with a plasmid carrying the whole IGR-0414c region, and could also be complemented with a construct lacking the IGR region, ruling out the requirement of this region to increase biofilm formation (Figure 20). To show that *0414c* was not only required but also sufficient to trigger the increase in biofilm production, we fused the *0414c* ORF to the constitutive promoter of the r*ecA* gene and cloned the construct in a multicopy plasmid. When compared with the empty plasmid control, constitutive expression of *0414c* generated a large increase in biofilm production even in the absence of exogenous SHP addition, and achieved similar biomass level as the ones obtained after SHPC8 pheromone addition (Figure 21A and B). When the 0414c overexpression construct was transformed in a Δ*rgg2* strain, which is unable to generate QS signaling, a similar increase in biofilm formation is observed. These results demonstrate that *0414c* was both required and sufficient to promote the increase in biofilm, and that constitutive expression of *0414c* bypasses the requirement of QS signaling through the Rgg2/3 pathway to increase biofilm. Finally, filtered supernatants of the *0414c* overexpressing strain, but not of the control strain containing an empty vector, were able to increase biofilm formation without the need to add SHPC8 pheromones, supporting the notion that this protein is secreted into the surrounding medium (Figure 21C).

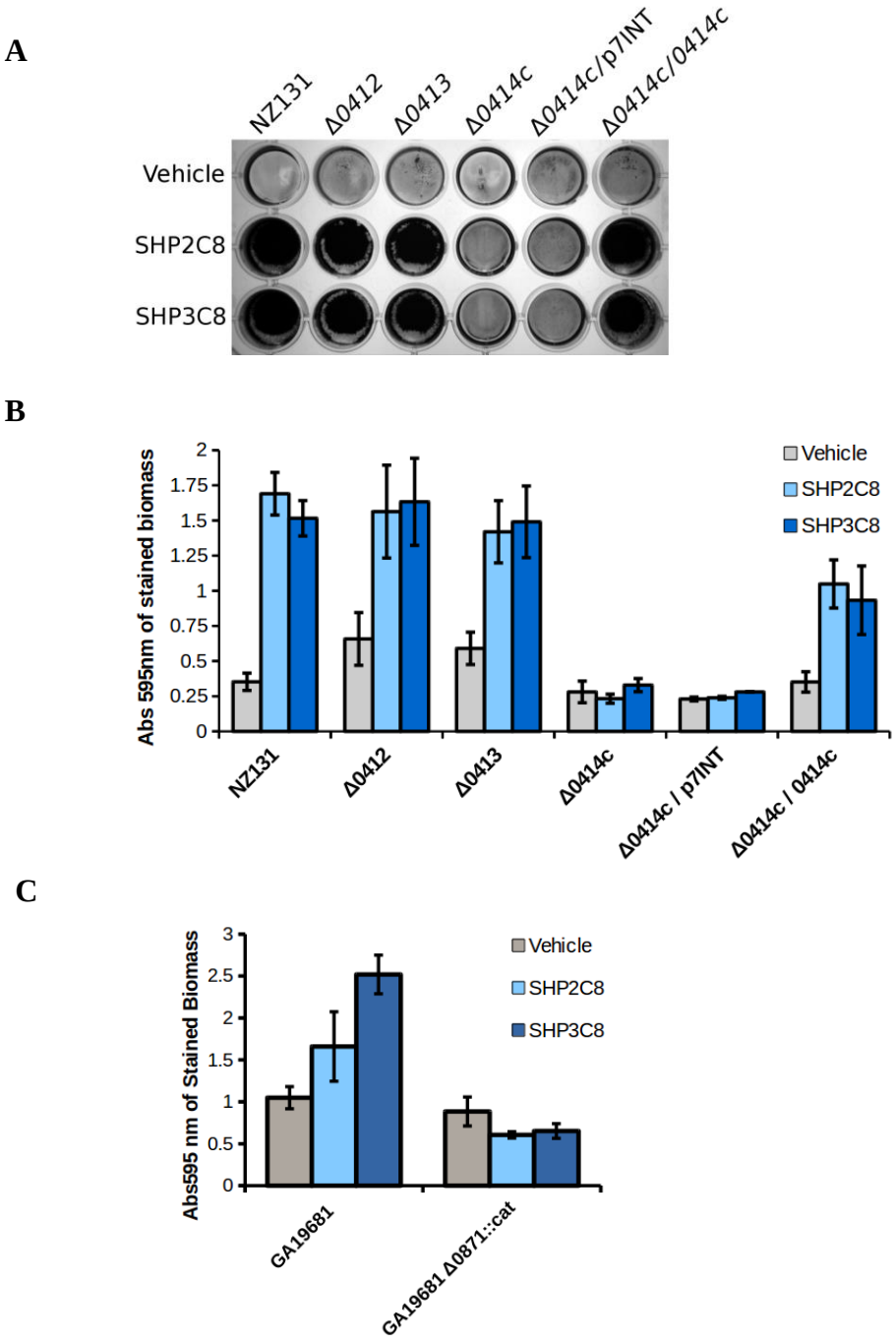


FIGURE 19. The *0414c* gene is necessary for SHP-dependent biofilm increase. Deletions in open reading frames encoded downstream of the *shp2* gene were generated, and the effects over biofilm formation assessed **A.** Biofilm formation in several experiments was quantified by measuring the crystal violet absorbance by scanning the stained biofilm area. Bars show standard error between experiments. **B.** Picture of a crystal violet stained biofilm plate. **C.** Biofilm formation in the *S. pyogenes* GA19681 isolate, and the mutant of the NZ131 *0414c* homolog gene, gene *0871*.

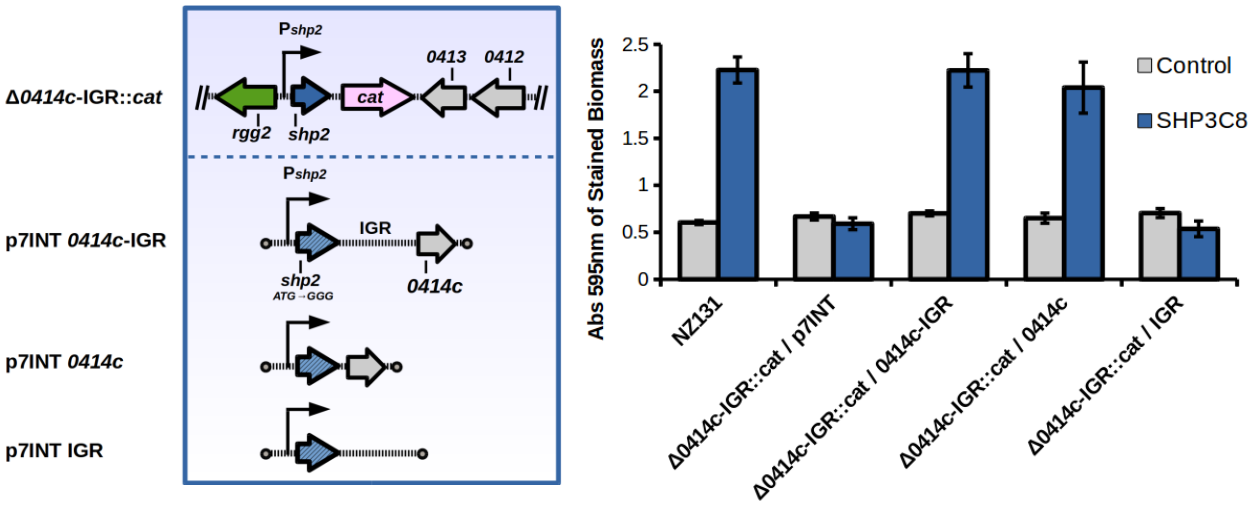


FIGURE 20. The IGR is not required for the biofilm phenotype. A IGR-0414c::*cat* mutant in strain NZ131 was generated and transformed with different complementation constructs. Left, diagram showing *shp2* downstream region in mutant, and different constructs. Right, biofilm formation of mutant and complemented strain. Bars show standard error between experiments.

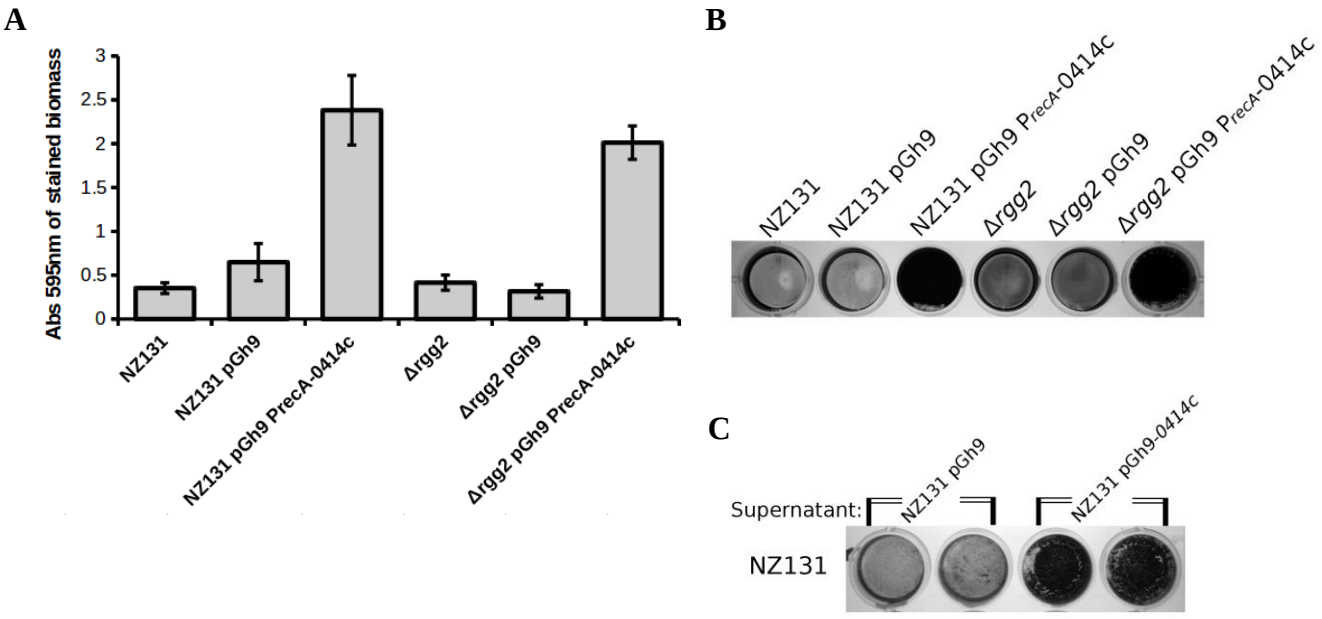


FIGURE 21. 0414C is sufficient to promote biofilm increase. Expression of the *0414c* gene from a constitutive P_{recA} promoter generates an increase in biofilm formation in the absence of QS signaling. **A.** Quantification of biofilm formation in several experiments. Bars show standard error **B.** Picture of a crystal violet stained biofilm plate form a representative experiment. **C.** Effect of transfer of supernatants from control and 0414c expressing strains over biofilm formation.

4.2.3 0414c promotes lysozyme resistance in *S. pyogenes*

Work from our lab has shown that SHPC8 pheromones increase the survival of *S. pyogenes* when challenged with lysozyme. In these experiments, bacteria that are preincubated with SHP3C8 pheromone are able to withstand the bactericidal effects of lysozyme, when compared with a strain treated with a control peptide (Figure 22A). This phenotype is dependent on signaling through the Rgg2/3 pathway, and was induced in every *S. pyogenes* isolate tested when incubated with SHPC8 pheromones, including those strains whose biofilm phenotype was not responsive to pheromones [154]. We therefore tested the involvement of 0414c in the process of QS-induced lysozyme resistance. When compared to the wild-type strain, the Δ*0414c* mutant remained sensitive to lysozyme, even after the preincubation with SHP pheromones (Figure 22A). Moreover, constitutive expression from P_{recA}-*0414c* generates a strain that is able to survive the effects of lysozyme without the need to induce QS signaling (Figure 22B). These results demonstrate that *0414c* is also necessary and sufficient to promote lysozyme resistance in *S. pyogenes* NZ131.

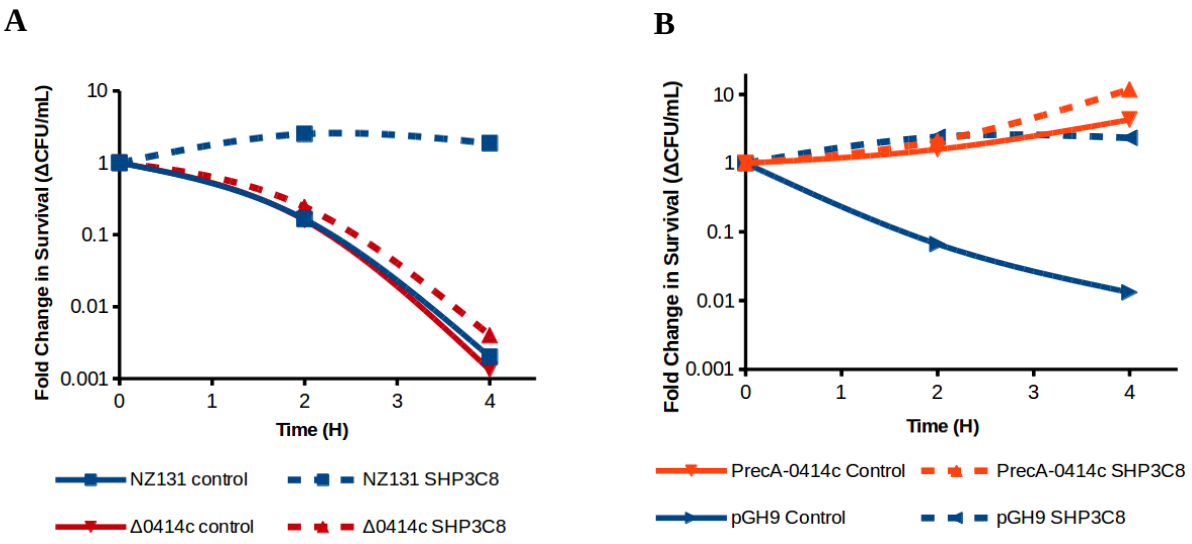


FIGURE 22. The *0414c* gene promotes lysozyme resistance in *S. pyogenes* NZ131. Wild-type and mutant strains were subjected to a lysozyme challenge experiment after preincubation with control peptide (SHP3revC8), or with active SHP3C8 peptide. **A.** wild-type NZ131 and Δ*0414c* strains were assessed for their survival in a lysozyme challenge experiment. **B.** NZ131 was transformed with empty plasmid (pGh9) or the plasmid carrying the constitutive PrecA-*0414c* construct, and the ability to survive 2 mg/mL lysozyme challenge assessed.

4.2.4 *0414c* encodes a putative Cysteine Proteinase inhibitor protein

We next used bioinformatic tools to gain a better understanding of the mechanism by which *0414c* promotes the different behaviors described. Basic domain analysis in the MicrobesOnline.org website revealed that the *0414c* gene does not encode any predicted conserved protein domains, and protein similarity searches only revealed similar proteins in two closely-related species, *Streptococcus canis* and *Streptococcus dysgalactiae subsp. dysgalactiae*. Based on the knowledge that other streptococcal species have *rgg2-shp2* paralog genes [68], and based on the fact that Rgg-regulated genes are commonly encoded in the same genetic neighborhood as the *rgg* genes [27], [173], we examined other *rgg2-shp2* loci with their adjoining regulated genes in other species. Genome analysis revealed that the *rgg2-shp2* locus in *Streptococcus pyogenes* lays in between two highly conserved genes across the Firmicutes phylum: the *era* gene, encoding for an essential GTP-binding protein involved in cell cycle control in bacteria [174]; and the fpg/mutM gene, encoding for a DNA-glycosylase involved in DNA repair [175]. To date, the *Streptoccus* genus includes 72 species that are subclassified into 8 groups depending on the genetic relatedness of core genes [162]. We first analyzed the genetic composition of the *era-fpg* region in one representative species of each *Streptococcus* group, and assembled the data on a tree of the predicted phylogenetic relations between the groups [162] (Figure 23). In the representative species of the Mitis, Salivarius and Bovis groups, the *era* and *fpg* genes are adjacent. Mutans, Anginosus and Sanguinis representative species show a few additional genes between *era* and *fpg*, including the conserved *mutT* gene that like *fpg/mutM*, is also involved in DNA repair processes [176]. The Downei group species also have additional genes encoded in the *era-fpg* region, but none of these encode for Rgg-type transcriptional regulators. Thus, initial analysis reveals that only the Pyogenic group harbors an *rgg2-shp2* locus in the genetic region analyzed. We proceeded to analyze in further detail the species that comprise the Pyogenic group, and inspected the *era-fpg* regions in eleven species (Figure 24). Our first observation was that 7 of the 11 species analyzed (*S. pyogenes*, *S. canis*, *S. dysgalactiae* subsp. *equisimilis*, *S. dysgalactiae* subsp. *dysgalactiae*, *S. ictaluri*, *S. iniae* and *S. agalactiae*) had an *rgg2-shp2* locus, while the remaining four species, although having several genes in this region, did not possess any encoded Rgg transcription factors. In all species carrying a *shp2* homolog, the translated, eight C-terminal amino acids of the gene have the same sequence (DILIIVGG), suggesting that these species have the potential to develop interspecies

communication via these peptides. Additionally, *S. dysgalactiae* subspecies *dysgalactiae* has also a second QS system encoded in this region, the two-component based *sil* system, which is also present in a minority of *S. pyogenes* isolates, and which in the case of the isolate analyzed (NZ131), there only remains a few genes of this system (shown in yellow arrows). Finally, when analyzing the arrangement of the genes downstream of each *shp2* homolog, we noticed that *S. pyogenes* was the only one in which a large IGR exists. In *S. canis*, *S. dysgalactiae* subsp. *dysgalactiae*, *S. iniae* and *S. agalactiae*, the *shp2* gene is immediately followed by a ORF in place of the IGR found in *S. pyogenes*, and further downstream a small ORF that resembles in length that of the *0414c* gene. In *S. ictaluri* and *S. dysgalactiae* subsp. *equisimilis*, the larger ORF can also be found, but in the first one has been displaced further downstream by the insertion of two genes in the opposing strand, while in the later the large and small ORFs have been fused to encode one single gene downstream of the *shp2* homolog. In all cases, the large ORF encoded downstream of the *shp2* gene is predicted to encode a transglutaminase-like protease (TG-like protease), a novel subfamily of the cysteine proteinases [177]. We hypothesized that mutations may have ocurred to disrupt the TG-like protease gene in *S. pyogenes*. To test this, we performed DNA alignments of the *rgg2-shp2* locus between *S. pyogenes* and its phylogenetically closest counterpart *S. canis*, a pathogen capable of generating similar pathologies than *S. pyogenes* causes in humans, in cattle and dogs [178], [179]. While there is a high degree of sequence conservation, starting from *rgg2* and decreasing towards the *shp2* downstream region, several mutations show how the original protease gene encoded was lost in *S. pyogene*s (Figure 25). These mutations include a start codon mutation (ATG to ATA), several non-sense mutations in the coding frame, plus a 747 bp deletion in the coding region. Most of these mutations are well conserved in other *S. pyogenes* isolates, as the IGR region has a 98% DNA identity amongst 45 different strains, suggesting that the events generating the loss of the protease gene occurred early during the speciation event that defined the *S. pyogenes* and *S. canis* species. When analyzing the DNA sequence for possible expression of remnants of the pseudogene, three putative ORFs are present that are preceded by RBS-like sequences. Only one is encoded in the same reading frame as the original protease gene. Although we cannot be sure these peptides are actually being expressed, it remains interesting that the IGR is well conserved in *S. pyogenes*, suggesting a possible function for the components encoded in this region.

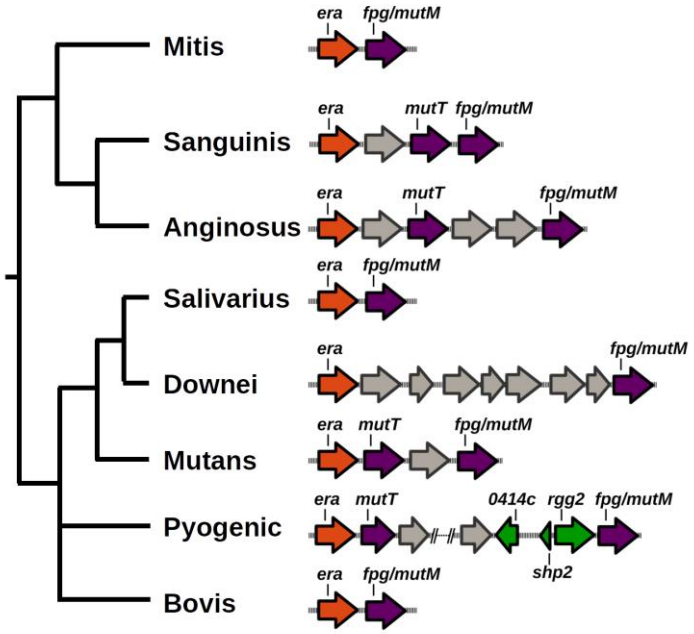


FIGURE 23. Genetic composition of the *era-fpg* region in representative species of the different *Streptoccus* genus groups. The genes encoded in between the conserved *era* and *fpg/mutM* genes was analyzed in species of the Mitis (*S. pneumoniae* R6*)*, Sanguinis (*S. sanguinis* SK36), Anginosus (*S. anginosus* C238), Salivarius (*S. themophilus* LMD-9*)*, Downei (*S. downei* F0415), Mutans (*S. mutans* UA159), Pyogenic (*S. pyogenes* NZ131*)* and Bovis (*S. gallolyticus* ATCC43143*)*.

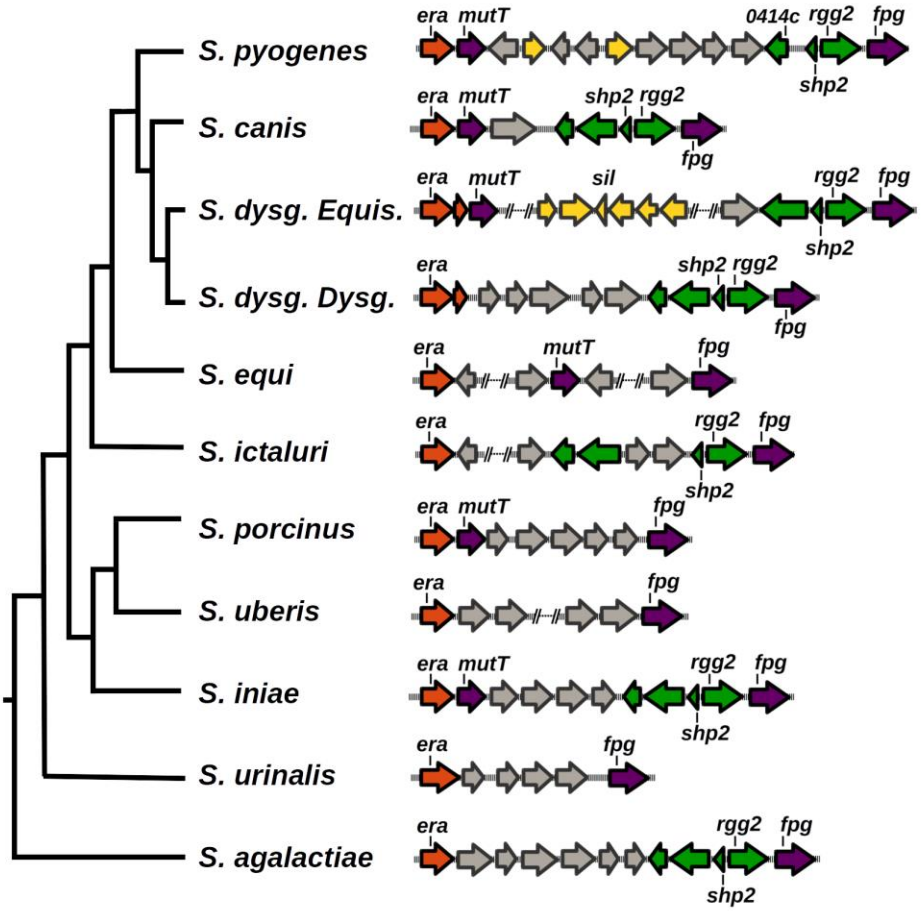


FIGURE 24. Genetic composition of the *era-fpg* region in species of the Pyogenic group. The genes encoded in between the conserved *era* and *fpg/mutM* genes was analyzed in *S. pyogenes* NZ131, *S. canis* FSL-Z3, *S. dysgalactiae* subspecies *equisimilis* ATCC12394, *S. dysgalactiae* subspecies *dysgalactiae* ATCC27957, *S. equi* subspecies *zooepidemicus* H70, *S. ictaluri* 707-05, *S. porcinus* Jelinkova176, *S. uberis* 0140J, *S. iniae* YSFST01-82, *S. urinalis* FB127-CNA-2 and S. *agalactiae* 2603V/R. In green, *rgg2-shp2* QS related genes. In yellow, *sil* QS related genes.

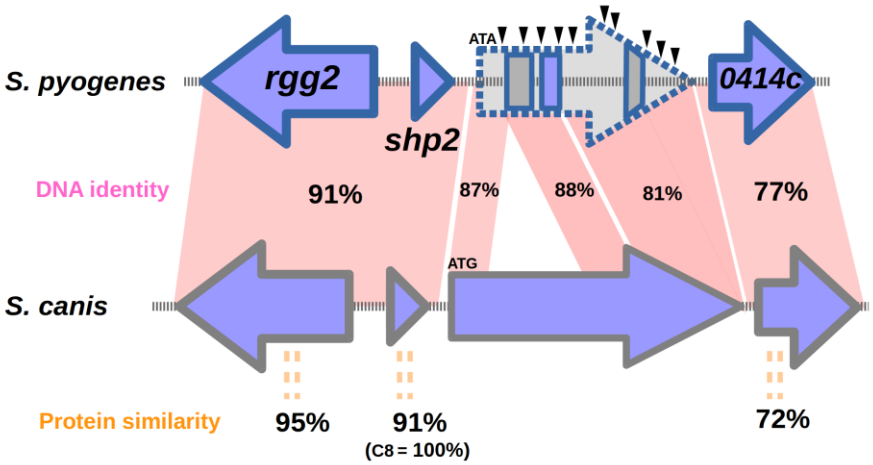


FIGURE 25. Comparison of *rgg2-shp2* region between *S. pyogenes* and *S. canis.* DNA sequences of *S. pyogenes* NZ131 and *S. canis* FSL-Z3 were aligned, and DNA identity and protein similarity of different fragments calculated using BLAST alignments (http://blast.ncbi.nlm.nih.gov/Blast) and EMBOSS matcher (http://mobyle.pasteur.fr). Black triangles indicate stop codons in the pseudogene's (dotted arrow) original reading frame. Blocks inside the pseudogene indicate ORFs that possess RBS sequences close to canonical. Blue block indicates ORF in the same reading frame as original gene.

Organisms of the varied kingdoms of life have devised the use of small proteins in order to inhibit the activity of cysteine proteases [180]. It has been documented in Gram positive bacteria that cysteine protease genes are coexpressed with small ORFs encoding for small chaperone proteins that play the role of enzymatic inhibitors for their cognate proteinases. The most thoroughly studied example of these are the staphostatins, inhibitors of the *S. aureus* papain-like cysteine proteases (Staphopains). Staphostatins show high specifity towards their partner coexpressed protease [181], [182]. In *S. pyogenes,* the potent papain-like protease SpeB has been shown to be inhibited by its coexpressed downstream peptide Spi [183]. These data support the idea that the small ORF encoded downstream of *shp2* in the Pyogenic group streptococci encodes for an inhibitor for its coexpressed protease. In *S. pyogenes* on the other hand, mutations lead to the loss of the protease gene, leaving an orphan protease inhibitor. We hypothesize that the *0414c* gene has evolved to interact with other partner proteases to modify their behavior. The fact that Rgg2 proteins are highly similar between *S. pyogenes* and *S. canis* (95%, Figure 25), while the 0414c protein is only 72% similar to its *S. canis* paralog, supports the notion that 0414c may have evolved to interact with other partners in *S. pyogenes.*

4.2.5 Cysteine Proteinase mutations affect phenotypes promoted by 0414c

We hypothetize that 0414c acts as a cysteine protease inhibitor. If this were the case, deleting the target proteinase(s) of 0414c should recapitulate the phenotypes seen by activating 0414c expression through SHP-signaling or by its constitutive expression from the P_{recA} promoter construct. The *S. pyogenes* NZ131 strain has 10 genes encoding members of the cysteine proteinase superfamily (SSF54001), including the well-studied papain-like protease SpeB, the IgG protease IdeS (encoded in the *mac* gene), and 6 genes harboring CHAP (Cysteine, histidine-dependent amidohydrolases/peptidase) domains, involved in the processing of peptide crosslinkings in cell wall peptidoglycan (Table IV). Four of these protease genes are followed by a short ORF, which could have regulatory functions towards its preceding protease (as already shown for the Spi protein encoded downstream of SpeB [183]). We proceeded to generate mutations in all members of the cysteine protease superfamily of NZ131, except the *mac* gene, since it has been shown that IdeS protease (encoded in *mac*) shows strict specificity towards IgG molecules, and thus is unlikely to cleave proteins on the surface of *S. pyogenes*

[184]. Since some CHAP peptidases have been shown to be required for cytokinesis [185], we tested the growth rate of the CHAP gene mutants. While most exhibited a normal pattern of growth, the mutant in the *0026* gene was strongly impaired in growth (Figure 26A), and hence was not used in further experiments.

Gene number	Gene name	Gene arrangement	*Predicted / Described function*
0015	-		CHAP-domain, predicted to be involved in cytokinesis
0026	-		CHAP-domain, predicted to be involved in cytokinesis
0186c	-		Transglutaminase-like cysteine protease, lipoprotein
0679c	***mac***		IdeS IgG endopeptidase [186]
(Φ2) 0790	-		CHAP-domain, prophage encoded lysin
1280c	***pepC***		Aminopeptidase
1407c	***isp2***		CHAP-domain, endo-beta-N-acetylglucosamidase
1675c	***isp1***		CHAP-domain, endo-beta-N-acetylglucosamidase
(Φ3) 1459	-		CHAP-domain, prophage encoded lysin
1690c	***speB***		Papain-like, broad specificity [187]

TABLE IV. Cysteine proteinases of *S. pyogenes* NZ131. In bold, the name by which each protease is referred to in the text. Gene arrangement diagram shows the presence or absence of downstream short ORF. In the case of *mac* gene, downstream small ORF is predicted to be pseudogene (hatched arrow).

We proceeded to test the mutants for their ability to form biofilms, in the presence or absence of SHP pheromones (Figure 26B). While certain deletions generated a moderate increase in the basal level of biofilm formation, no single deletion was able to increase biofilm to the same levels of the SHP-induced wild-type strain, the condition in which 0414c is produced and secreted. Of the mutations that generated an increase in biofilm, the deletion of the *isp2* gene had the most significant effect, increasing biofilm formation 3-fold in absence of SHP pheromones, while biofilm formation in the presence of SHP3C8 in the Δ*isp2* strain was similar to the SHP-induced wild-type strain. The *isp2* gene is the second allele to *isp1* or "immunogenic secreted protein", a protein of unknown function that has been shown to generate a serological response in infected individuals [188]. Since the absence of one *isp* allele could be compensated by presence of the other, we generated a double Δ*isp1* Δ*isp2* mutant, which nonetheless showed a similar phenotype to the Δ*isp2* strain. These results show that the Isp2 cysteine protease represses biofilm formation in *S. pyogenes,* and suggest that the possible inhibition of Isp2 by 0414c may in turn enhance the ability to form biofilms in this bacteria.

While most mutations did not affect biofilm increases seen in the presence of SHP3C8, deletion of the *0186c* gene greatly reduced this response, with biofilm levels increasing 1.8-fold with SHP3C8 in the Δ0*186c* mutant compared to the almost 6-fold increase seen in the wild-type strain (Figure 26B). To assess for any potential effects that the *0186c* mutation may have over *0414c* expression, we transferred a plasmid providing constitutive expression of the *0414c* gene into the Δ0*186c* mutant. As seen in figure 27A, constitutive production of the 0414c protein in Δ0*186c* mutant has a greatly reduced effect over biofilm when compared with the wild-type strain. Finally, to test the involvement of the 0186c TG-like protease in biofilm production in other *S. pyogenes* isolates, we proceeded to generate a mutation in the *0186c* homolog in the GA19681 isolate. The deletion of the *0826* TG-like protease gene generated a decrease in the basal level of biofilm, as well as reduced the biofilm response to SHP3C8 pheromone addition to growth medium (Figure 27B). Together, these data show that the 0186 protease contributes to biofilm formation, and is also required for the full capacity of biofilm response seen after Rgg2/3 activation and 0414c production.

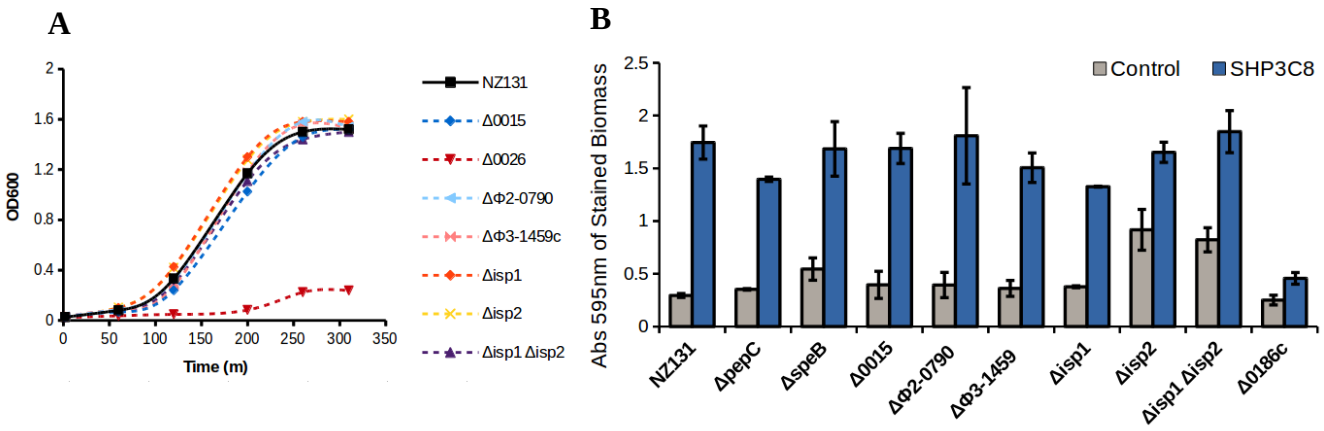


FIGURE 26. Cysteine proteinase mutants and their effect over biofilm formation. A. Growth curve of CHAP domain mutants. **B.** Biofilm formation of wild-type and nine cysteine proteinase mutants, in the presence or absence of SHP3C8 pheromone. Quantification of biofilm formation in several experiments. Bars show standard error

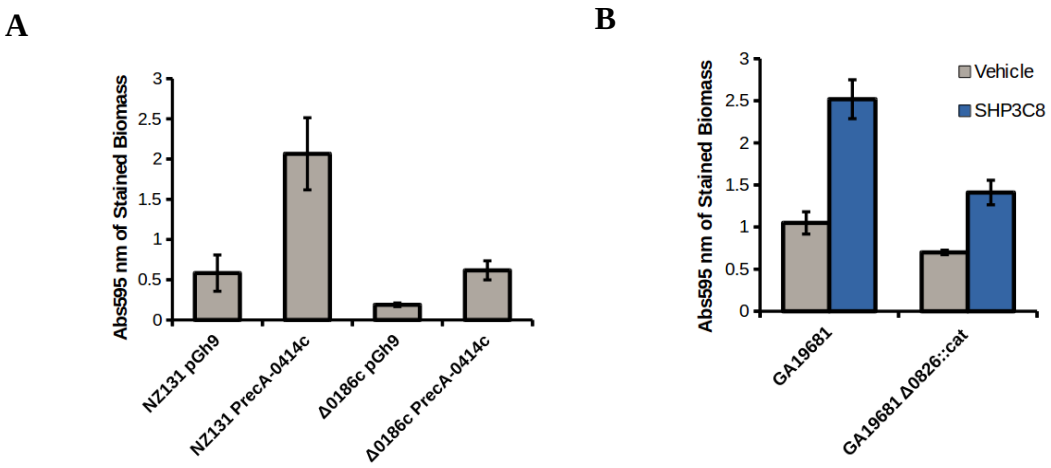


FIGURE 27. Effects of Δ186c deletion over biofilm formation. A. Effect of expression of the *0414c* gene from a constitutive P_{recA} promoter over biofilm formation in wild-type and Δ*0186c* strains. Quantification of biofilm formation in several experiments. Bars show standard error.. Empty pGh9 plasmid used as control. **B.** The *0186c* homolog in the GA19681 strain (gene *0826)* was deleted and biofilm formation assessed *i*n the presence or absence of SHP3C8 pheromone. Quantification of biofilm formation in several experiments. Bars show standard error.

4.2.6 Isp2 plays a role in lysozyme resistance

We proceeded to test the involvement of the Isp1, Isp2 and 0186c proteins on the phenotype of lysozyme resistance promoted by 0414c. Deletion of the *0186c* gene did not greatly affect the response to lysozyme, as this mutant behaved in a similar way to the wild-type strain, only exhibiting a slight difference after 4h of cellular incubation in lysozyme (Figure 28A). In comparison, the deletion of *isp2* generated a strain that is able to survive the bactericidal effects of lysozyme, while an *isp1* mutation also had a slight effect on viability at later time points (Figure 28B). While these mutations enhance bacterial survival, is noteworthy that neither is as effective as the activation of Rgg2/3 signaling by SHP pheromone, as the kinetics of survival of the SHP-induced strains starkly differ from the uninduced Δ*isp2* strain behavior. This suggests that while Isp2 may have a role in lysozyme sensitivity, Rgg2/3 signaling and 0414c may act on additional targets to promote lysozyme resistance. To further analyze this, we proceeded to test the ability of wild-type and Δ*isp2* strains to grown in a range of lysozyme concentrations (Figure 28C). When the overnight growth in a range of lysozyme concentrations was tested, deletion of *isp2* provided protection against lower concentrations of lysozyme, but this phenotype disappears when lysozyme concentrations are increased. On the other hand, incubating cells with SHP3C8 provides protection to a higher level of lysozyme concentrations. Taken together, these results show that deletions of *186c* and *isp1* have little effect on lysozyme resistance, while the Δ*isp2* mutation renders cells resistant to intermediate concentrations of lysozyme. These results suggest that the activity of Isp2 increases the sensitivity of *S. pyogenes* to lysozyme and puts forward the possibility that this protein could be a target for inhibition by 0414c in order to trigger increased resistance to lysozyme when the Rgg2/3 pathway is activated.

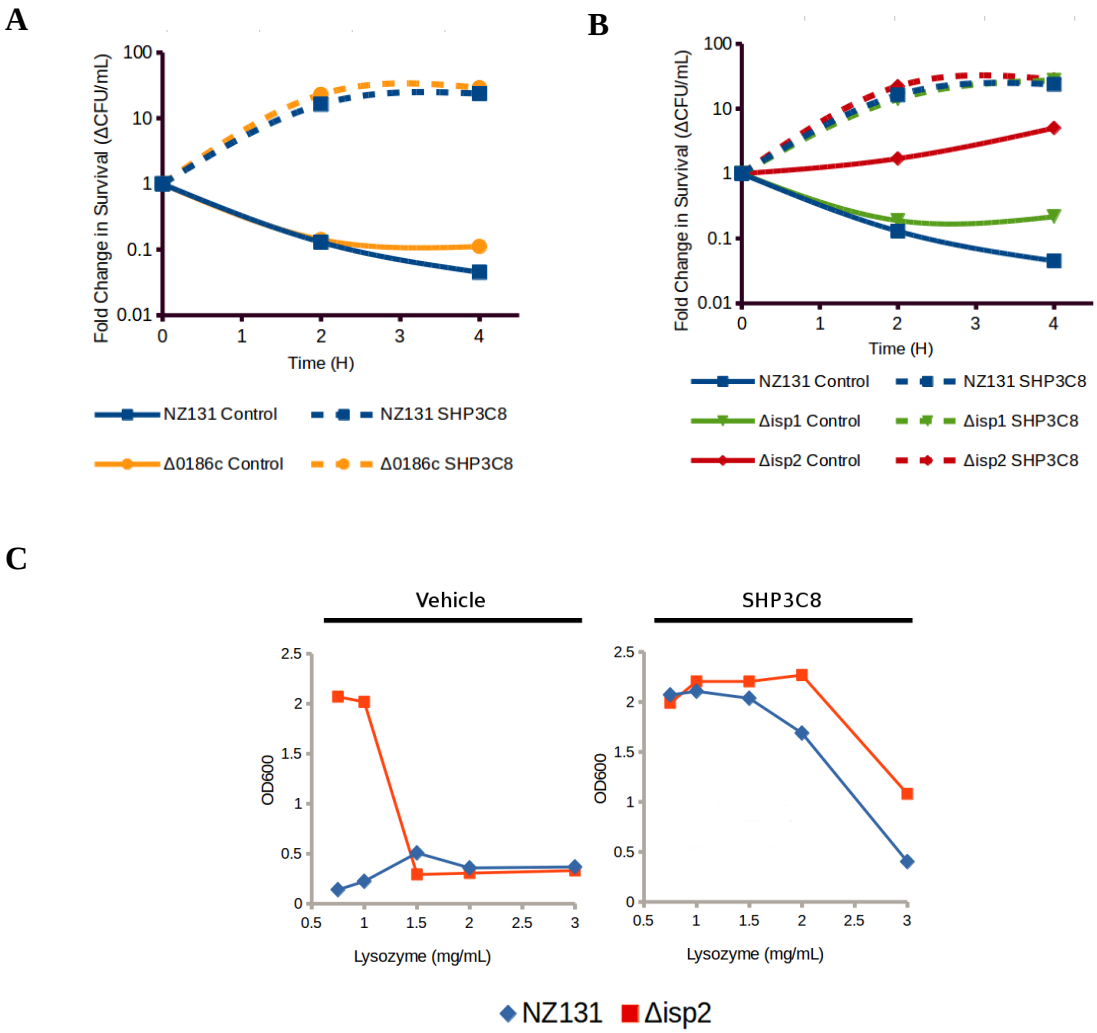


FIGURE 28. Effects of Cysteine proteinase mutations over lysozyme resistance. Wild-type and mutant strains were subjected to a lysozyme challenge experiment after preincubation with control peptide (SHP3revC8), or with active SHP3C8 peptide. **A.** wild-type NZ131 and Δ*186c* strains were assessed for their survival in a lysozyme challenge experiments. Samples were taken at each time point to estimate CFU of surviving bacteria. **B.** wild-type NZ131, Δ*isp1* and Δ*isp2* strains were assessed for their survival in a lysozyme challenge experiment, samples were taken in each time point to estimate CFU of surviving bacteria. **C.** Wild-type NZ131 and Δ*isp2* were assessed on their ability to grow on different concentrations of lysozyme in an end point assay. Growth was assessed by OD600 after 20h of incubation.

4.3 Discussion

The results in this chapter provide the first indications to the mechanism by which activation of the Rgg2/3 QS pathway triggers its diverse phenotypes (Figure 29). Production of the small secreted protein 0414c in *S. pyogenes* NZ131 drives the processes of cellular aggregation, biofilm formation and lysozyme resistance. Based on analogous *rgg2-shp2* loci in other related streptococci, we predict that this small protein is acting as a cysteine protease inhibitor, whose neighboring cognate protease gene has been lost due to several mutational events. Thus, we think 0414c acts by inhibiting one or more cysteine proteases encoded in a different location in the genome. The *S. pyogenes* genome encodes for several proteases, including 10 predicted cysteine proteases. Mutational analysis linked two of these proteases to the phenotypes studied: Isp2, which affected the processes of biofilm formation and lysozyme resistance; and the 0186c protease, which was required to increase biofilm production in response to QS signaling. In the general discussion section I will elaborate on the possible activities these two proteases may be exerting on the cellular envelope of *S. pyogenes.* Additionally, since 0414c appears to be an 'orphan' protease inhibitor, lacking its original cognate protease, there is also the possibility that 0414c has evolved to interact with other enzymes apart from cysteine proteases, or alternatively, it could also target proteins in the host's environment. Further proteomics approaches could be used to discover additional bacterial and human targets of 0414c interaction and their effects on the lifestyle of *S. pyogenes*.

Our bioinformatic analysis revealed that the *rgg2-shp2* locus is well distributed among *Streptococcus* species of the Pyogenic group, and the predicted SHP2C8 pheromone sequence is identical in all strains carrying this QS locus. Our lab has shown that *S. pyogenes* is able to participate in interspecies QS events when co-cultured with *S. agalactiae*, a species that also colonizes the human host [68]. However, some of the species that possess the *rgg2-shp2* locus are not common colonizers of the human host, but rather specialized colonizers of fish (*S. iniae, S. ictaluri*), dogs (*S. canis),* and cows (*S. dygalactiae* subsp. *dysgalactiae*), although in rare cases they can infect humans [179], [189]–[191]. Thus, in most cases, we suggest that conservation of the SHP2C8 pheromone sequence across Pyogenic group species is the result of the maintenance of a well-calibrated interaction between peptide and receptor, rather than the result of a beneficious interspecies signaling event.

It remains intriguing that the IGR segment downstream of the *shp2* gene, in which the TG-like protease was previously encoded, has a high degree of sequence conservation in *S. pyogenes* isolates, up to a 98% identity amongst 45 different strains. This result suggest that while the original protease gene was lost, there is still some function associated with the components present in this region. One possibility may be the presence of small regulatory RNAs (sRNA). One well studied case of QS and sRNA regulation is the *agr* QS system of *S. aureus.* The *agr* locus consists of two divergent transcriptional units that are activated in response to an autoinducting octapeptide pheromone [12], [192]. One of these transcripts, the 514bp RNAIII, has been shown to encode a translated ORF and in the same segment, to harbor a regulatory sRNA that plays an important role in regulating target virulence genes [192]–[194]. Additionally, we predict that at least three short ORFs could be expressed from the pseudogene region, peptides that could as well exert a regulatory activity. Finally, there is the possibility that the region upstream of the *0414c* gene has effects on translation. We have recently generated translational reporter fusions of the *0414c* gene with the *gus* gene (encoding D-β-glucuronidase), and preliminary results show that deletion of the IGR reduces the amount of β-glucuronidase activity when SHPC8 pheromones are added to media, supporting the idea of the requirement of IGR for efficient translation of 0414c.

Taken together, the data presented in this chapter point towards a role for the Rgg2/3 QS of *S. pyogenes* in the modification of the cellular envelope through the activity of a small secreted protein. While biofilm formation and lysozyme resistance are the phenotypes we have been able to observe in our *in vitro* conditions, these phenotypes may be the result of precise molecular changes related to the tailoring of the cell wall composition, and which may play an even bigger role in how *S. pyogenes* interacts with the human host and survives the demanding conditions faced during host tissue colonization.

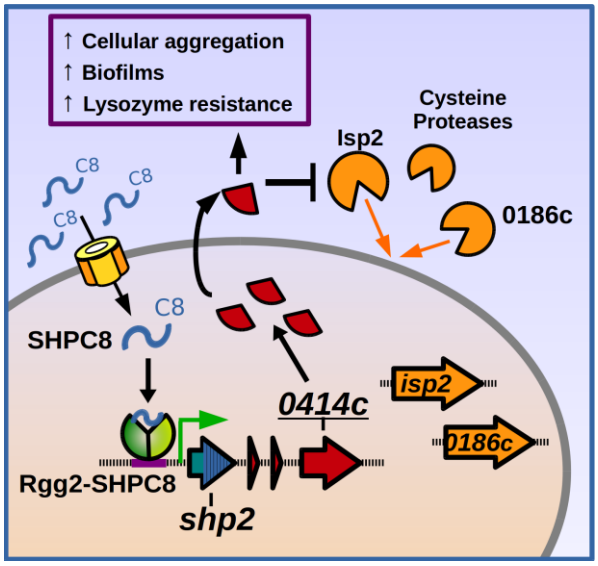


FIGURE 29. Model of 0414c activity. QS signaling by SHPC8 pheromones activates expression and secretion of 0414c, which promotes cellular aggregation, biofilm formation and lysozyme resistance. In the outer media, this enzyme works to inhibit cysteine proteases. The Isp2 and 0186c are putative targets of 0414c inhibition.

V. Identification of mannose transport proteins required for activation of the Rgg2/3 quorum sensing pathway of *Streptococcus pyogenes*.

(Reprinted in part, with permission, from Chang JC, Jimenez JC and Federle MJ (2015) Induction of a quorum sensing pathway by environmental signals enhances group A streptococcal resistance to lysozyme. *Molecular Microbiology* **97**:(6), doi: 10.1111/mmi.13088)

5.1 Rationale

Successful cell-to-cell signaling in bacteria relies on the accumulation of secreted chemical signals in the environment up to threshold concentrations ideal for signal detection. While this process is usually characterized to be only dependent on cellular density, the ability of QS circuits to turn on (and off) also depends on the environmental conditions bacteria encounter, in some cases with certain nutrients being able to activate or repress QS genes [195]–[197].

A screen performed in our lab using a panel of various carbon source nutrients unveiled that the monosaccharide mannose is able to induce a strong activation of the *shp* QS genes in *S. pyogenes* NZ131, triggering endogenous production of SHP pheromones and leading to the activation of the Rgg2/3 QS pathway [154]. A slight activation of *shp* expression was also observed when fructose was present, while 58 other carbon source nutrients that supported growth were unable to promote QS signaling, highlighting the specificity of mannose as a QS promoting signal.

As with other monosaccharides, mannose and fructose are imported into bacteria by the activity of phosphotransferase systems (PTS), multiprotein complexes comprised of two core proteins (Enzyme I (EI) and HPr) and a variety of enzyme II (EII) permease complexes, each one specializing in the import of a subset of mono- or disaccharides. PTS not only catalyze the transport and phosphorylation of sugars, but can also phosphorylate and/or directly interact with other proteins to trigger downstream changes in gene expression, (Figure 30A) and are involved in regulating genes related with metabolism, chemotaxis and virulence in several bacterial pathogens (reviewed in [198]). In streptococcal species, PTS of the mannose family (PTS-Man) have been shown to import several saccharides (referred in this text as PTS-Man sugars) including mannose, fructose, galactose, glucosamine, N-acetyl-glucosamine (GlcNAc), and even glucose; but the substrate specificity of each PTS-Man complex varies depending on the precise

allele and species studied [199]–[202]. The *S. pyogenes* genome encodes three putative PTS-Man systems (Figure 30B): *manLMN*, widely distributed in the *Firmicutes* phylum and identified in other streptococci as the main importer of mannose [200], [201], [203], [204]; *ptsABCD*, present only in some species of pyogenic streptococci and in enterococci; and a third system, *agaFVWD*, annotated as a N-acetyl-galactosamine importer but shown to be required for growth on hyaluronate in *S. pneumoniae* [201].

We hypothesize that the import of mannose by PTS complexes triggers downstream signaling that activates the expression of SHP pheromones, leading to QS communication. The aim of this chapter is to understand the role of PTS-Man systems and their contribution to the activation of Rgg2/3 QS in *S. pyogenes* by mannose.

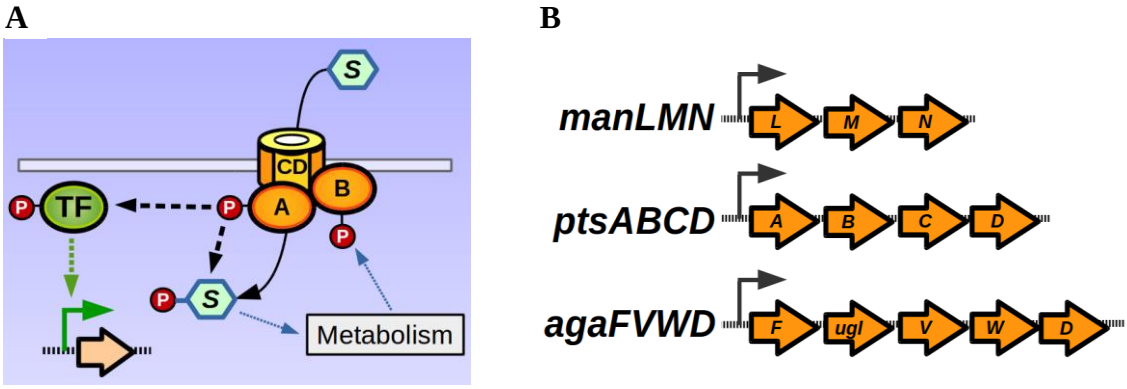


FIGURE 30. PTS systems and their role in signaling. A. The EII permease complex of the PTS has subunits that import the target sugar (C and D), and subunits that are part of a phospho-relay circuit (A and B) that take phosphate groups from the active metabolism to phosphorylate the incoming sugar, targeting it to its corresponding metabolic pathways. In some cases, the phosphorylating subunits can also activate transcription factors by phosphorylation or direct interaction, triggering downstream signaling events. **B.** The *S pyogenes* NZ131 genome encodes for three putative PTS systems of the mannose family (PTS-Man), whose genes are encoded in three distinct operons.

5.2 Results

5.2.1 Role of *S. pyogenes* PTS-Man complexes in growth in alternative carbon sources

To investigate whether a specific PTS system was required to import mannose and mediate SHP signaling, mutants of the membrane-bound permease component (EIIC) of each PTS were generated by insertional disruption using a suicide plasmid approach, as described in materials and methods section.

We first tested the ability of the different mutants to grow on media using PTS-Man substrate sugars as the sole carbon source (Figure 31). We also tested a control condition in the presence of sucrose, a sugar not imported by the PTS-Man family, and a no sugar added condition. The wild-type NZ131 strain was able to grow robustly in all carbon sources tested, although growth on mannose was significantly slower than in other sugars. This observation can be explained by the necessity to isomerize mannose to fructose-6-phosphate before this sugar can enter glycolysis, the main energy production pathway of *S. pyogenes* (www.genome.jp/kegg). The *manM*$^-$ mutant was unable to grow in the presence of mannose, fructose, glucosamine or N-acetyl-glucosamine as carbon sources, but was able to grow on glucose, a sugar that is imported by multiple routes into the cell. The total yield of growth in this mutant in glucose is nonetheless reduced, suggesting the ManLMN complex can as well import glucose, a phenomenon seen with other PTS-Man transporters in Streptococci [203]. As expected, the growth of the *manM*$^-$ mutant on sucrose was not affected. The *ptsC*$^-$ mutant was able to grow on all sugars, with only slightly slower rates when using mannose, fructose and N-acetyl-glucosamine. Apart from a slightly lower growth yield in mannose, growth of the *agaW*$^-$ mutant on all sugars was similar to that observed for wild-type. These results show that ManLMN is the primary PTS for import of mannose, fructose, glucosamine and GlcNAc and that neither PtsABCD nor AgaFVWD are sufficient to support robust growth on these substrates when ManLMN is absent.

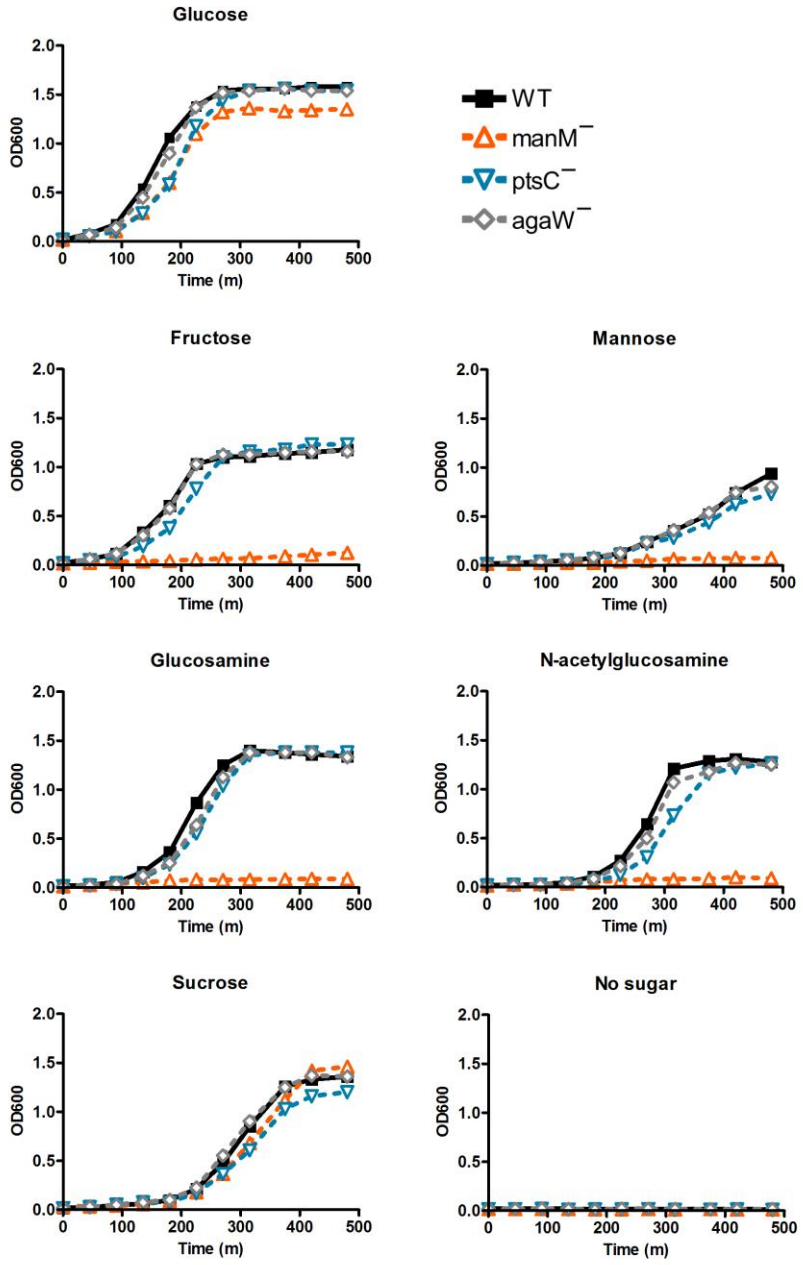


FIGURE 31. Effect of PTS insertional disruptions on growth in varied carbon sources. Three target PTS-Man systems were disrupted using a suicide-plasmid approach, and the ability of the respective mutants to grow using different saccharides as the sole carbon source tested and compared to a wild-type strain. Strains were grown in CDM with 1% of the indicated sugar added. Sucrose was used as a control sugar which is not imported by the PTS-Man family. A condition with CDM with no sugars added was used as a control to show the requirement of saccharides for growth.

5.2.2 Mannose and fructose activate expression of SHP pheromone

As shown in Figure 32, when a wild-type strain harboring a P_{shp3}-*luxAB* luciferase reporter was grown on glucose, very low levels of promoter activity are seen, even when high cell densities are reached. When the same strain is grown in the presence of mannose as the sole carbon source, the activation of P_{shp3}-*luxAB* follows a growth associated pattern, following the classic density-dependent induction of QS, and generating a 5000-fold increase in pheromone promoter activity. Fructose, the other sugar imported by the PTS-Man family of transporters, has a moderate effect over P_{shp3} activity, increasing promoter activity 5-fold after the mid-exponential phase of growth is reached.

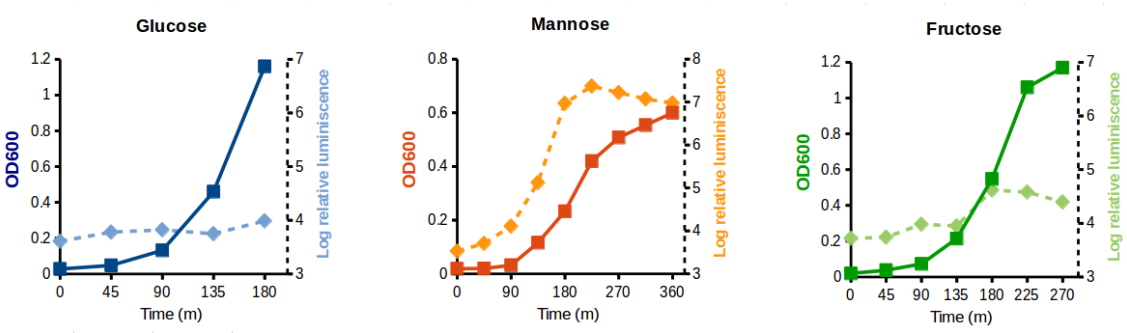


FIGURE 32. Activation of P_{shp3} pheromone promoter expression in response to different carbon sources in a wild-type NZ131 strain. A wild-type strain harboring an integrated plasmid with a P_{shp3}-*luxAB* reporter construct was grown on CDM containing 1% (w/v) of the indicated sugars, and growth and light production monitored. Solid lines represent bacterial growth as measured by OD_{600} while dotted lines represent log of relative light production.

5.2.3 Disruption of *ptsABCD* system inhibits mannose-induced Rgg2/3 QS

We tested the effects of PTS-Man disruption on P_{shp3} induction in the presence of mannose or fructose (Figure 33). While the *agaW*$^-$ mutant followed a similar induction pattern compared to the wild-type strain, *manM*$^-$ and *ptsC*$^-$ mutants exhibited minimal reporter induction when grown on mannose. However, interpretation of the *manM*$^-$ mutant's was hindered by the severe growth defect of this strain on these sugars; it is possible this strain was not able to grow sufficiently to trigger signaling. In contrast, disruption of *ptsC* had minimal effect on growth yet induction of P_{shp3} was dramatically reduced. The slight inducing effect of fructose over P_{shp3} could be seen in both *agaW*$^-$ and *manM*$^-$ strains, but was lost in the *ptsC*$^-$ strain. When the PTS mutant strains were exposed to synthetic SHP-C8 peptide, a condition that bypasses the environmental cues needed to trigger Rgg-SHP signaling, all mutants responded similarly to wild-type, demonstrating that mutations in PTS encoding genes do not disrupt the pheromone detection machinery and that the capacity of these mutants to respond to pheromone is intact. In contrast a Δ*rgg2* strain, which is unable to activate P_{shp3}, shows an impaired response to pheromone addition (Figure 34).

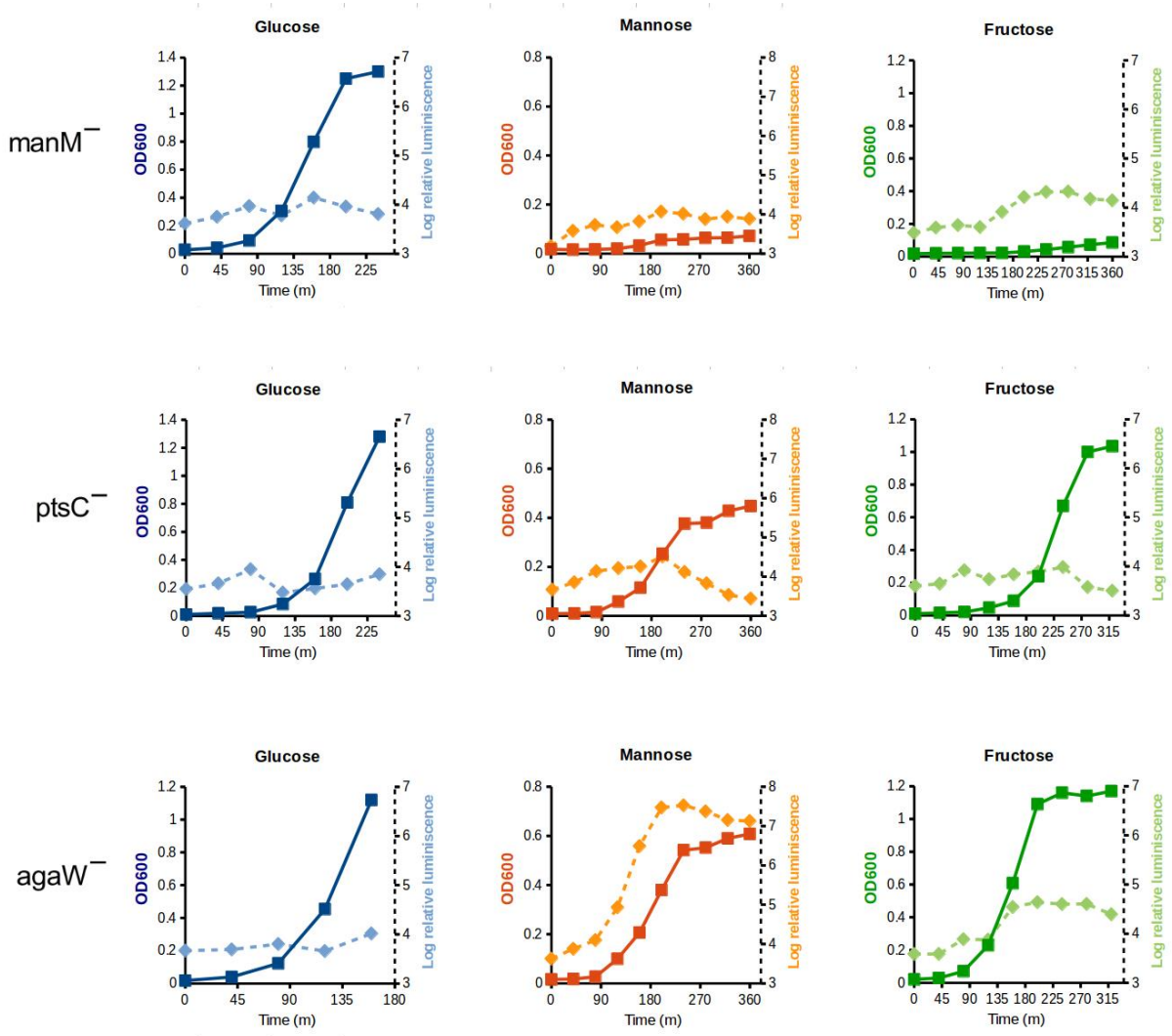


FIGURE 33. Effect of PTS insertional disruptions over expression of QS pheromones. Mutants in three PTS-Man systems of *S. pyogenes* were tested for their ability to activate expression of QS pheromone promoter in response to different carbon sources. The *manM* ⁻, *ptsC* ⁻ *and agaW* ⁻ mutants were transformed with an integrative plasmid carrying a P_{shp3}-*luxAB* reporter construct, and were later grown in CDM containing 1% (w/v) of the indicated sugars. Growth and light production was monitored, solid lines represent bacterial growth as measured by OD_{600} while dotted lines represent log of relative light production.

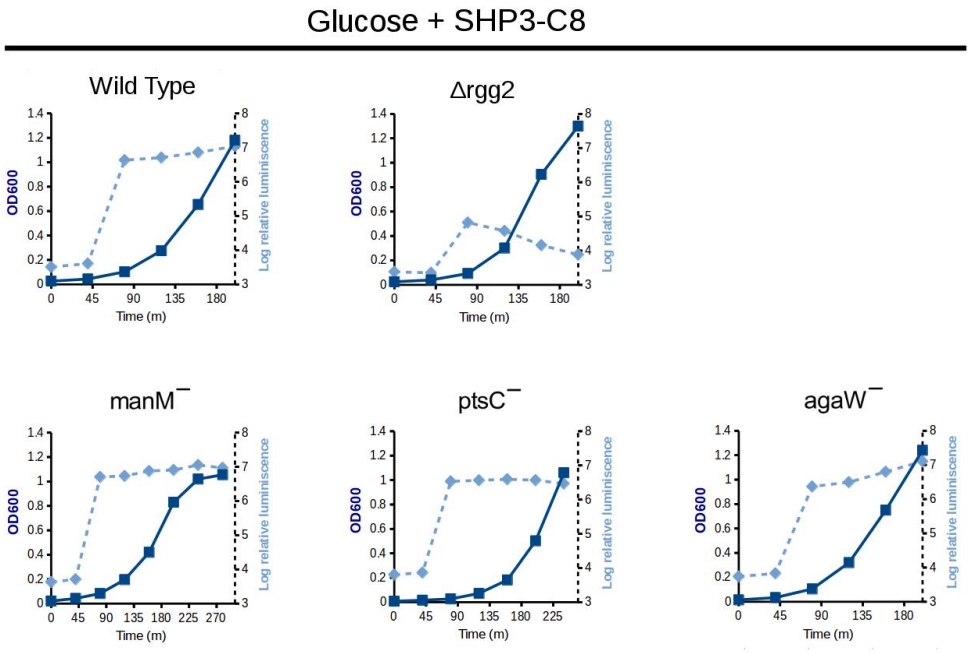


FIGURE 34. Effect of PTS insertional disruptions over QS induction after synthetic SHP3-C8 addition. The ability to respond to synthetic SHP3-C8 pheromone was tested in a wild-type strain, a strain deficient in the *rgg2* gene, and the three mutants in PTS systems. All strains possess an integrated plasmid carrying a P_{shp3}-*luxAB* reporter construct, and were grown in CDM containing 1% (w/v) glucose, plus 25nM SHP3-C8 pheromone. Solid lines represent bacterial growth as measured by OD_{600} while dotted lines represent log of relative light production.

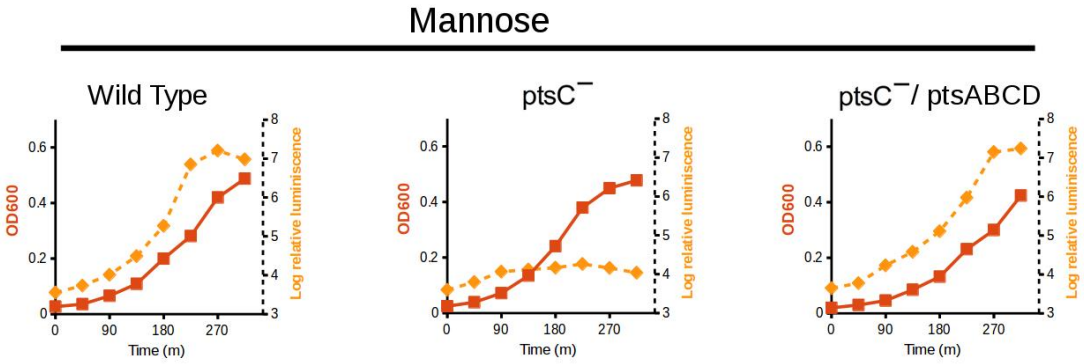


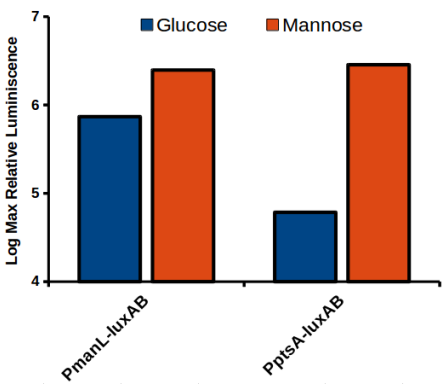
FIGURE 35. Complementation of *ptsC*⁻ mutation restores the ability to induce QS signaling in response to mannose. A *ptsC*⁻ mutant strain was complemented by expressing the *ptsABCD* operon from a constitutive promoter on a multicopy plasmid, and its ability to respond to the QS activating effect of mannose compared to a wild-type and the parental mutant strain. All strains possess an integrated plasmid carrying a P_{shp3}-*luxAB* reporter construct, and were grown on CDM containing 1% (w/v) mannose. Solid lines represent bacterial growth as measured by OD_{600} while dotted lines represent log of relative light production.

Finally, the signaling defect of the *ptsC*⁻ mutant could be fully complemented by expressing the *ptsABCD* operon from a constitutive promoter on a multicopy plasmid (Figure 35). Taken together, these data show that the PtsABCD transporter is essential for SHP signaling in response to activating sugars mannose and fructose.

5.2.4 PTS-Man genes show differential expression patterns

To better understand the role of ManLMN and PtsABCD PTS systems on mannose metabolism and signaling, we studied the expression levels of both systems using luciferase transcriptional reporters. A P_{manL}-*luxAB* reporter exhibited a high level of expression when the strain was grown on glucose, and a further 3-fold higher level in expression when the strain was grown on mannose as the sole carbon source (Figure 36A). In comparison, the P_{ptsA}-*luxAB* reporter showed a low level of expression when grown on glucose, but a 50-fold higher expression level when grown on mannose (Figure 36A). To further assess how the expression of these transporters was activated in the presence of different sugars, reporter strains were briefly incubated in CDM lacking any carbon source, and the signal of luciferase reporters measured. The strains were then transferred to CDM containing 1% (w/v) of each PTS-Man sugar, and the light production estimated immediately after (Figure 36B). Mannose and fructose are able to increase P_{manL} expression more than 10 times, while P_{ptsA} exhibits a 300-fold increase in expression when mannose is added. Glucosamine and N-acetylglucosamine, both of which are unable to effect SHP signaling, have also a poor inducing effect over both PTS-Man transporters. These results suggest that ManLMN is a constitutively expressed PTS, a fact that correlates with its key role in growth when using varied alternate sugars as the sole carbon source (Figure 31). PtsABCD on the other hand is an inducible transporter, highly responsive to addition of mannose. These results remain puzzling, as mutation of the PtsABCD system had little effect on growth rate in the presence of mannose, and this transporter is unable to support robust growth in mannose when ManLMN is absent (Figure 31). One possibility may be that PtsABCD induction provides a growth advantage in the conditions found within the host but that are not replicated in our *in vitro* experiments. Alternatively, one may speculate about the possibility of PtsABCD playing a specialized role focused in the detection of mannose and concomitant signaling events, and playing only a minor role in the further import and metabolism of this sugar.

A



B

Carbon Source	*PmanL-luxAB*	*PptsA-luxAB*
Glucose	6.3	6.5
Mannose	19.0	313.9
Fructose	16.2	26.9
Glucosamine	8.4	15.5
N-acetylglucosamine	7.3	13.6

FIGURE 36. Expression of PTS promoters under different carbon sources. The promoter for the *manLMN* operon (P_{manL}) and the promoter for the *ptsABCD* operon (P_{ptsA}) were studied using luciferase reporter constructs. Strains carrying reporter constructs for either promoter were grown on CDM containing 1% (w/v) of the indicated sugars and light production was monitored. **A.** Maximum promoter activity. **B.** Reporter strains were preincubated briefly in CDM in the absence of any carbon sources, and then transferred to CDM containing diverse carbon sources. The fold increase in luciferase activity in between both conditions was then calculated.

5.2.5 Induction of Rgg2/3 QS by mannose is repressed by glucose

As the preferred energy source, glucose represses the expression of systems involved in the use of secondary carbon sources through a process known as carbon catabolite repression (CCR). CcpA (catabolite control protein A) is the primary transcriptional repressor mediating CCR in *S. pyogenes*. CcpA binds DNA at conserved sequences in target promoters known as CRE or catabolite responsive elements [205], [206]. To test the repressive effects of glucose over mannose-activated QS, increasing amounts of glucose were added to CDM containing 1% mannose, and P_{shp3}-*luxAB* activity monitored. As shown in Figure 37A, we observed a dose-dependent repression of the P_{shp3}-*luxAB* reporter with addition of glucose to the media. Apart from glucose; sucrose, fructose, maltose and lactose are the other main mono- and disaccharides of the human diet [207] and could be carbon sources available to GAS growing on the saliva-bathed surfaces of the oropharynx. Unlike glucose, addition of fructose, sucrose and lactose at

concentrations > 10-fold excess of mannose did not inhibit the expression of the P_{shp3} reporter (Figure 37B). These results show that induction of SHP pheromones is subject to CCR by glucose, and that mannose appears to be a specific signal that can be detected even in the presence of other dietary sugars.

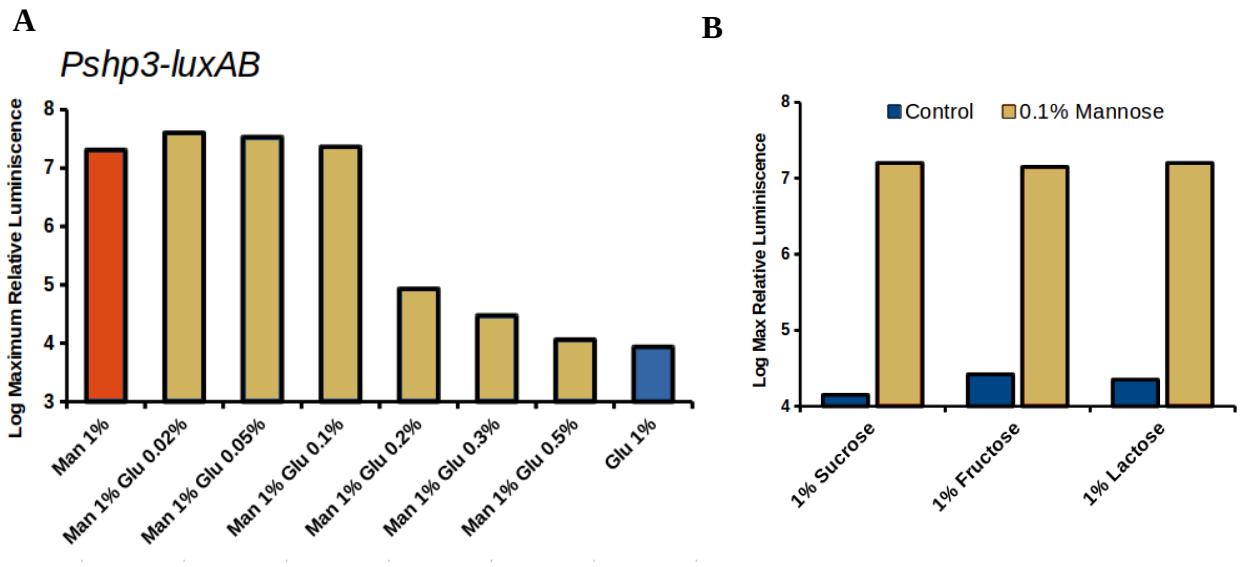


FIGURE 37. Carbon catabolite repression of pheromone activation in response to glucose. **A.** Response of the P_{shp3}-*luxAB* reporter to glucose catabolite repression. Reporter strain was growth in CDM in the presence of 1% mannose alone, or with an increasing concentration of glucose, and the effects over SHP pheromone promoter activation assessed. **B.** Sucrose, fructose and lactose were added to CDM alone, or mixed with mannose in a 10:1 ratio, and activation of P_{shp3}-*luxAB* reporter assessed.

5.2.6 Induction of *ptsABCD* is repressed by glucose

Since PtsABCD expression is highly induced in presence of mannose, we analyzed the effect of glucose on its induction. When we tested the P_{ptsA}-*luxAB* reporter strain, increasing concentrations of glucose were able to repress the induction of expression generated by 1% mannose, in a comparable degree to P_{shp3} repression by glucose (Figure 38A). Furthermore, while the genetic region surrounding both P_{shp2} and P_{shp3} lacks any predicted CRE binding sites for CcpA, the P_{ptsA} region harbors a consensus-fitting site that could be bound by CcpA to exert its

transcriptional repression activities (Figure 38B). These results show that P_{ptsA} is also subject to carbon catabolite repression by glucose, and the presence of a CRE site in this genetic region suggest that this repression is exerted by the CcpA protein. Although we have tried to generate a *ccpA* deletion strain to test this hypothesis, we haven't been able to isolate clones in which the recombination process removes the gene from the chromosome. Additionally, a recent report has shown that CcpA may be essential in certain *S pyogenes* isolates, including the strain used for our transcriptional studies, NZ131 [208].

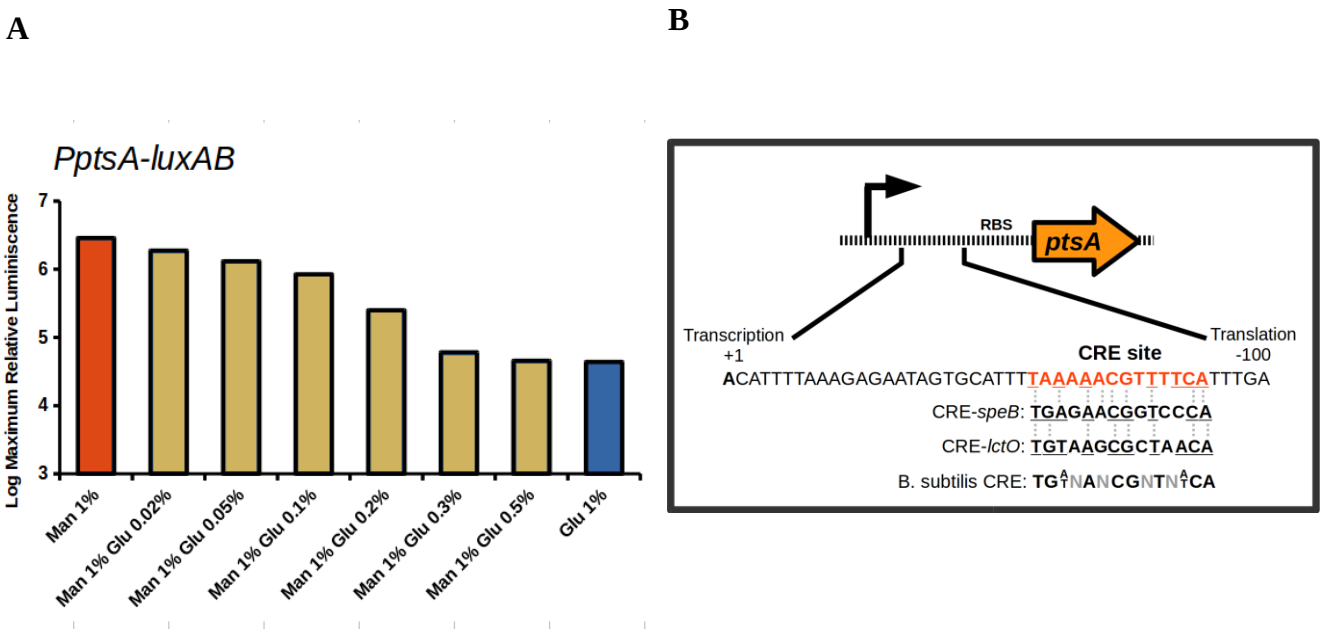


FIGURE 38. Carbon catabolite repression of P_{ptsA} expression. A. Response of the P_{ptsA}-*luxAB* reporter to glucose catabolite repression. Reporter strain was growth in CDM in the presence of 1% mannose alone, or with an increasing concentration of glucose, and the effects over the *ptsABCD* operon promoter activation assessed. **B.** CRE binding site in P_{ptsA}. The CRE (catabolite responsive element) binding site for the CcpA repressor was mapped in the *ptsABCD* operon promoter, and its sequence paired with two characterized CRE sequences of *S pyogenes*, and with the established CRE consensus sequence of *B. subtilis* [209].

5.3 **Discussion**

In bacteria, the processes of nutrient import and metabolism are tightly linked with gene regulation events. It is well understood that changes in the availability of carbon sources, like the depletion of a preferred energy source like glucose and the switch to an alternative secondary carbon source, leads to distinct changes in gene expression [210]. Several transcriptomic studies have shown that *S. pyogenes* triggers the expression of genes involved in binding and importation of different non-glucose carbon sources when in the presence of host tissue, blood, and saliva [211]–[213]; suggesting an important role for saccharides and their signaling effects in the *S. pyogenes* lifestyle. Moreover, the utilization of alternate carbon sources other than glucose has also been linked to the activation of well-known virulence genes of *S. pyogenes*, supporting the important role of carbohydrate sensing and metabolism as a cue to modify cellular behaviors.

When initially characterized, the activity of the Rgg2/3 QS pathway was minimal when bacteria were cultured in presence of glucose, and activation of SHP pheromone expression didn't occur unless small amounts of synthetic peptide pheromones were added to the culture medium [87]. Together with the discovery of mannose as an inducer signal, the results presented in this chapter illustrate how the event of transporting a specific sugar type into the cytosol leads to the activation of a new cellular program, in this case the induction of a QS pathway (Figure 39). Previous works have exemplified how PTS-Man in other Streptococci can affect the process of competence, the development of biofilms, production of secreted hemolysin, and sensitivity to bacteriocins [214]–[216]. Thus, there seems to be a proclivity to tie mannose metabolism with genetic regulation through the activity of PTS-Man importers. Still, the high specificity of mannose and not other related sugars in their ability to activate SHP pheromone expression is intriguing and raises the question of the role of mannose as an environmental cue in the host's environment. Although mannose is not a main dietary sugar [207], it is commonly used for glycosylation of extracellular proteins in eukaryotes [217], [218]. Different streptococcal species have been shown to use a variety of glycosydase enzymes to cleave sugars from human glycoproteins, releasing saccharides that allow bacterial growth and attachment to underlying molecules, a process believed to be key for host-colonization [219]–[222]. Thus, we speculate that release of mannose from host glycoproteins by the activity of *S. pyogenes* glycosydases will

detach mannose molecules that can activate Rgg2/3 QS, and that mannose may then act as an environmental cue to reflect interaction of the bacterium with the host's epithelium.

We do not understand yet how the activity of PtsABCD ties with the activation of Rgg2/3 QS. It is well known that activation of sugar transport by PTS systems can lead to the direct activation of downstream transcription factors [198]. One of the mechanisms for this activation is the phosphorylation of specific domains, termed PTS regulatory domains (PRD), which leads to a change in activity in the target transcription factor. In *S. pyogenes*, six genes encode for proteins with predicted PRD domains. One of these proteins is the extensively characterized 'multiple virulence gene regulator' Mga, a factor that has been shown to regulate up to 10% of the transcriptome in certain *S. pyogenes* isolates [148]. Mga has been shown to be directly phosphorylated by PTS components in two separate PRD domains, and different phosphorylation combinations can lead to the activation or repression of its activity [223]. We think the key role of PtsABCD in activating QS lies in sensing and/or transducing signals specifically related to the presence of mannose, through the phosphorylation and consequent modulation of activity of a target PRD-containing transcription factor. Thus, the study of these PRD proteins may further reveal the mechanistic details on how mannose activates QS pheromone synthesis.

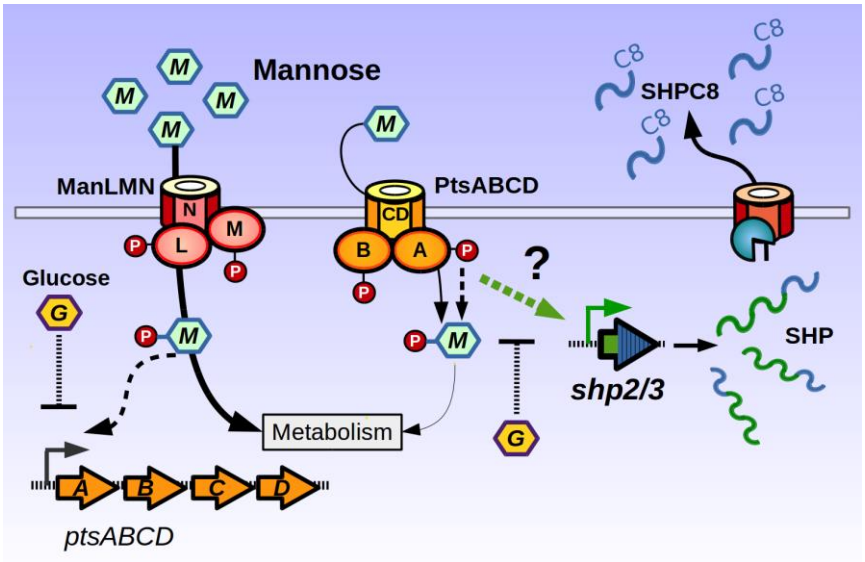


FIGURE 39. Model of PTS-Man role in growth and QS in *S. pyogenes*. Results show that the ManLMN PTS sugar importer is crucial for growth in the presence of Mannose, as well as fructose, glucosamine and N-acetylglucosamine (PTS-Man sugars, not shown in the figure). The detection of mannose by the bacteria leads to the induction in expression of a second transporter, PtsABCD, which has a minor role in supporting bacterial growth in the presence of PTS-Man sugars, but is indispensable to activate expression of SHP pheromones. The mechanism by which this activation occurs is unknown. Additionally, presence of glucose represses activation of SHP expression, while also repressing induction of the *ptsABCD* operon expression.

VI. GENERAL CONCLUSIONS AND DISCUSSION

The work presented herein shows how the activation of a QS pathway leads to several phenotypical changes in *S. pyogenes* by the expression of a single small secreted protein, 0414c. The observed processes of bacterial aggregation, enhanced biofilm formation and lysozyme resistance point towards changes occurring in the bacterial envelope, the cell wall and its attached components. Based on genomic data, we have hypothesized that 0414c acts as a putative cysteine protease inhibitor, and through a genetic approach we have identified two putative targets of 0414c inhibition, 0186c and Isp2. Our current efforts are focused on obtaining biochemical data to support our genomic and genetic findings, in order to establish conclusively the activity exerted by 0414c and its effects over *S. pyogenes*.

6.1 0186c TG-like protease

Interestingly, *0186c* encodes for a putative TG-like cysteine protease, the same subfamily of cysteine protease that is encoded next to *shp2* in other Pyogenic *Streptococcus* species, and that was lost in *S. pyogenes*. Thus, the orphan 0414c protease inhibitor may have evolved to interact with other TG-like cysteine protease in the *S. pyogenes* genome. Proteases are normally thought of as having a repressive effect over biofilm formation, due to their ability to degrade biofilm matrix components and/or bacterial surface adhesins. In this case, the 0186c protease promotes biofilm formation.

TG-like cysteine proteases have been described in bacterial, archeal, viral and eukaryotic genomes [177]. To date, they have only been shown to exhibit protease or peptidase activity, but no transglutaminase activity [224]–[227]. Interestingly, the first studied TG-like protease was isolated from an archeal phage, and was shown to have endopeptidase activity towards the archeal cell wall [224]. Additionally, a quick protein similarity search reveals that the *0186c* gene is differentially annotated through *S. pyogenes* isolates, with some genomes annotations predicting a 'S-layer protein' function, or in some isolates a 'peptidoglycan endo-beta-N-acetylglucosaminidase' activity. S-layer proteins are large proteins that assemble in crystalline-like arrays in bacterial surfaces, and in the case of Gram positive bacteria interact with the peptidoglycan cell wall [228]. The endo-beta-N-acetylglucosaminidase activity is one of the four

different enzymatic mechanisms by which cell wall polymers can be cut [129]. While genome annotation predictions are not always accurate, it is still intriguing that both of these functions link the activity of 0186c to the bacterial envelope.

Recently, we have been able to express the *0186c* gene in *E. coli* and purify the recombinant protein through nickel affinity purification. We have been unable to detect any protease or transglutaminase activities using readily-available proteins casein and BSA as substrates, suggesting either that this enzyme has high specificity for substrates, or its activity is neither a protease nor transglutaminase. Preliminary results also show that while addition of recombinant 0186c protein doesn't enhance biofilm formation by itself, it does enhance biofilm when QS is activated, supporting the notion that 0186c and 0414c work together to generate the biofilm increasing activity.

6.2 The immunogenic secreted proteins, Isp1 and Isp2

The deletion in the *isp2* gene was able to partially recapitulate two phenotypes promoted by 0414c. Isp genes are present in two alleles in *S. pyogenes*, *isp1* and *isp2*, and both genes present similar domain architectures (Figure 40): an N-terminal secretion sequence, a low complexity repetitive sequence region, a predicted N-acetylglucosaminidase domain, and a predicted CHAP (Cysteine-Histidine dependent Amidohydrolases/Peptidases) domain. CHAP domain proteins have been involved in daughter cell separation, cell lysis, and cell-wall processing to promote conjugative transfer of DNA [185], [229], [230]. Interestingly, the *S. pyogenes* genome encodes for multiple CHAP domain proteins, with the NZ131 isolate harboring six genes encoding this domain; two predicted to be involved in cytokinesis (*0015* and *0026* genes), two encoded in prophage genomes (*0790*Φ2 and *1459*Φ3 genes), and two secreted *isp1* and *isp2* hydrolases. Neither the deletion of each single allele, or the double Δ*isp1* Δ*isp2* mutant exhibited deficiencies in growth rate, suggesting that while these enzymes have peptidoglycan hydrolase domains, they don’t play an essential role in cell separation and may play other roles, for example in tailoring the composition of the cell wall. The *isp1* allele was first discovered during the genetic characterization of a downstream gene, *mga*, but no role or function has been assigned to it yet, apart from its immunogenicity in infected individuals [188]. Recently, it has been shown that a purified CHAP domain from a *Bifidobacterium bifidum*

hydrolase is sufficient to trigger an immune response, suggesting that these bacterial domains are themselves highly immunogenic [231].

The *isp* genes of *S. pyogenes* are encoded in two distinct predicted operons, *isp1* encoded downstream of genes that encode the Irr/Ihk two component signaling system and a putative ABC transporter, and *isp2* encoded downstream of genes encoding for an acyl carrier protein and an alanine racemase. Interestingly, these last two proteins are involved in synthesis and modification of the bacterial cell envelope: Acyl carrier proteins are necessary for fatty acid synthesis and are as well required to bind lipid cofactors to enzymes involved in teichoic acid modification [232], [233]. Alanine racemase promotes the interconversion of L-alanine and D-alanine, both substrates for peptidoglycan synthesis and teichoic acid modification [234]. The coexpression of these two enzymes with Isp2 supports the idea of this peptidoglycan hydrolase playing a role in the processing and/or modification of the components present in the cell wall. Recently, we have been able to express the *isp2* gene in *E. coli* and purify the recombinant protein through nickel affinity purification. Preliminary results show that recombinant Isp2 is able to degrade crude cell wall extract of *S. pyogenes*, corroborating the peptidoglycan hydrolase activity of this enzyme.

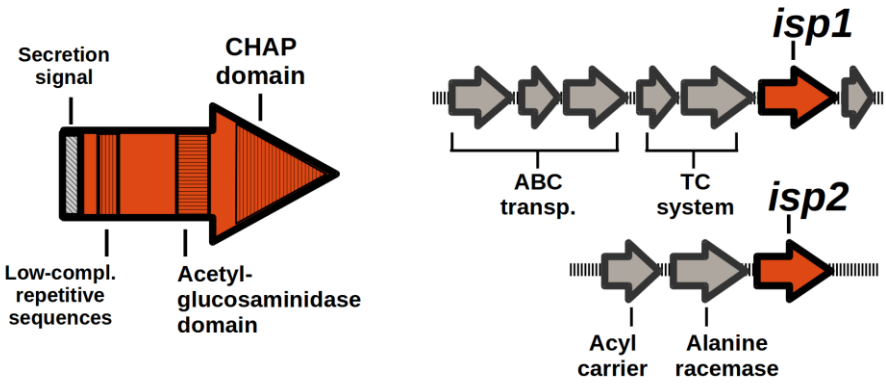


FIGURE 40. Domain architecture and operon distribution of Isp proteins. Left, predicted protein domains of Isp alleles. Right, genetic context of *isp* genes. Predicted operons are shown.

Why does Isp2 affect the processes of biofilm formation and lysozyme resistance?. In the first case, it has been shown that the activity of peptidoglycan hydrolases that cut cell wall bonds also generate the release of cell wall bound proteins [128], and in *S. pyogenes* several peptidoglycan attached proteins can be found in high quantities in the supernatant [127], [235].

Activity of Isp2 could be involved in the release of some of the adhesive proteins that *S. pyogenes* uses in order to promote biofilm formation. In the case of Isp2 and its effect over lysozyme resistance, we hypothesize that processing of the cell wall by Isp2 renders the bacteria susceptible to the effects of lysozyme (maybe through weakening of some of the peptide bonds in between glycan strands) and that either its deletion or its putative inhibition by 0414c results in a more resilient cell wall and enhanced survival towards the action of lysozyme.

6.3 0414c putative cysteine protease inhibitor

The *0414c* gene encodes for a conserved secreted protein of *S. pyogenes,* which expression is regulated in a population-wide manner by QS, suggesting that presence of the 0414c protein in the outer media provides an important function for the community of *S. pyogenes* cells in their environment. Preliminary experiments have shown that expression of the *0414c* gene from a constitutive promoter can generate changes in the composition of *S. pyogenes* supernatants, with new proteins being released to the outer media when 0414c is expressed. Further experiments will assess the identity and source of these proteins, which could be released to the supernatant as a result of a differential processing of cell wall and its attached proteins when 0414c is present.

Research of the cell division machinery of bacteria has shown that peptidoglycan hydrolases are usually regulated at the level of activation by other cell wall binding factors [140]. While bacterial-produced inhibitors of mammalian lysozymes have been described, no known secreted inhibitors of bacterial peptidoglycan endopeptidases have been described to date, hence 0414c could prove to encode for a novel type of inhibitor. Recently, we have been able to successfully express and purify recombinant the 0414c protein from *E. coli,* with the intent of characterizing the effect of 0414c over its putative targets and its effect over changes occurring to the composition of the cell wall.

6.4 Mannose as a QS inducing signal

The PtsABCD PTS transporter proved dispensable for growth, but essential for activation of Rgg2/3 QS in response to mannose. As stated in chapter V, we speculate that *S. pyogenes*-induced release of mannose from glycoproteins in the host's epithelium may act as an environmental cue to reflect interaction of the bacterium with the host, effecting SHP pheromone production and Rgg2/3 signaling. There is also the possibility that mannose serves as substrate for enzymes regulated by QS, for example the enzymes encoded in the putative biosynthetic operon downstream of the *shp3* (Figure 41). As with other bacterial biosynthetic operons [236], the 0450-0460 operon is predicted to encode the enzymes for the step by step synthesis of a secreted secondary metabolite. Some of the enzymes encoded in this operon are predicted to be involved in the isomerization, binding and dehydrogenation of sugars. Thus, mannose could act both as a signal to induce the expression of the biosynthetic genes, and be a building block for the molecule synthetized by these enzymes.

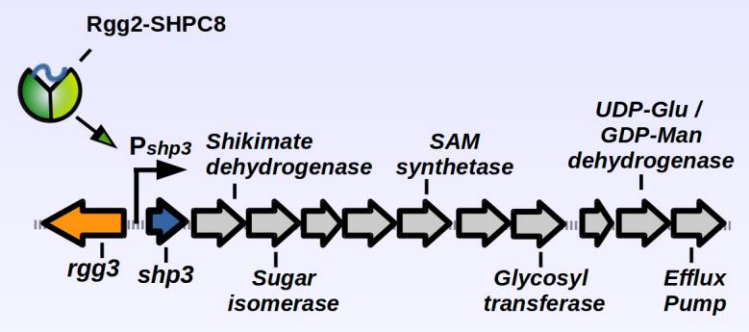


FIGURE 41. Predicted enzymatic functions encoded in the 0450-0460 operon. The predicted enzymatic functions of each gene are annotated.

6.5 Concluding remarks

The *S. pyogenes* NZ131 genome contains 1820 genes in 1.81 Mbp of DNA, and is thus around 2.5 times smaller than the prototypical *E. coli* genome. As with other *Streptococcus* species, *S. pyogenes* lacks the biosynthetic pathways for several nutrients that it obtains directly from its human host, and most of these gene pathways are predicted to be have been lost during the adaptation of a free-living ancestor towards a pathogenic, host-dependent lifestyle [237]. For these reasons, it's assumed that the genes that are conserved along the *S. pyogenes* pan-genome must provide an adaptive advantage towards life inside the human host.

Several QS systems can be found in *S. pyogenes,* but to date, Rgg2/3 represents the only functional QS circuit that is conserved among sequenced strains. We think this is pressing evidence that this signaling system plays an important role in the life cycle of *S. pyogenes* inside the human host. The work presented unveils a possible functionality for the Rgg2/3 system in *S. pyogenes,* and points towards active processing or modification of the bacterial envelope. Future experiments will be geared towards carefully characterizing the possible molecular changes occurring in the cell wall and its associated molecules when SHP signaling and 0414c expression is triggered, and analyzing how these changes can affect the interaction of an important pathogen with its human host.

LITERATURE CITED

[1] S. T. Rutherford and B. L. Bassler, “Bacterial quorum sensing: its role in virulence and possibilities for its control.,” *Cold Spring Harb. Perspect. Med.*, vol. 2, no. 11, Nov. 2012.

[2] S. Atkinson and P. Williams, “Quorum sensing and social networking in the microbial world.,” *J. R. Soc. Interface*, vol. 6, no. 40, pp. 959–78, Nov. 2009.

[3] W.-L. Ng and B. L. Bassler, “Bacterial quorum-sensing network architectures.,” *Annu. Rev. Genet.*, vol. 43, pp. 197–222, Jan. 2009.

[4] C. S. Pereira, A. K. de Regt, P. H. Brito, S. T. Miller, and K. B. Xavier, “Identification of functional LsrB-like autoinducer-2 receptors.,” *J. Bacteriol.*, vol. 191, no. 22, pp. 6975–87, Nov. 2009.

[5] C. S. Pereira, J. a Thompson, and K. B. Xavier, “AI-2-mediated signalling in bacteria.,” *FEMS Microbiol. Rev.*, vol. 37, no. 2, pp. 156–81, Mar. 2013.

[6] L. S. Håvarstein, G. Coomaraswamy, and D. A. Morrison, “An unmodified heptadecapeptide pheromone induces competence for genetic transformation in Streptococcus pneumoniae.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 92, no. 24, pp. 11140–4, Nov. 1995.

[7] J. M. Solomon, B. A. Lazazzera, and A. D. Grossman, “Purification and characterization of an extracellular peptide factor that affects two different developmental pathways in Bacillus subtilis.,” *Genes Dev.*, vol. 10, pp. 2014–2024, 1996.

[8] P. Mayville, G. Ji, R. Beavis, H. Yang, M. Goger, R. P. Novick, and T. W. Muir, “Structure-activity analysis of synthetic autoinducing thiolactone peptides from Staphylococcus aureus responsible for virulence,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 96, no. February, pp. 1218–1223, Feb. 1999.

[9] O. P. Kuipers, M. M. Beerthuyzen, P. G. de Ruyter, E. J. Luesink, W. M. de Vos, E. Luesnik, and W. M. De Vos, “Autoregulation of nisin biosynthesis in Lactococcus lactis by signal transduction,” *J. Biol. Chem.*, vol. 270, no. 45, pp. 27299–304, Nov. 1995.

[10] M. H. J. Sturme, J. Nakayama, D. Molenaar, Y. Murakami, R. Kunugi, T. Fujii, E. Elaine, M. Kleerebezem, W. M. De Vos, E. E. Vaughan, and W. M. de Vos, “An agr-Like Two-Component Regulatory System in Lactobacillus plantarum Is Involved in Production of a Novel Cyclic Peptide and Regulation of Adherence,” *J. Bacteriol.*, vol. 187, no. 15, 2005.

[11] M. Otto, R. Süßmuth, G. Jung, and F. Götz, “Structure of the pheromone peptide of the Staphylococcus epidermidis agr system,” *FEBS Lett.*, vol. 424, pp. 89–94, 1998.

[12] G. Ji, R. C. Beavis, and R. P. Novick, “Cell density control of staphylococcal virulence mediated by an octapeptide pheromone.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 92, no. 26, pp. 12055–9, Dec. 1995.

[13] L. Zhang, L. Gray, R. Novick, and G. Ji, “Transmembrane topology of AgrB, the protein involved in the post-translational modification of AgrD in Staphylococcus aureus,” *J. Biol. Chem.*, vol. 277, no. 38, 2002.

[14] F. Y. An, M. C. Sulavik, and D. B. Clewell, “Identification and Characterization of a Determinant (eep) on the Enterococcus faecalis Chromosome That Is Involved in Production of the Peptide Sex Pheromone,” *J. Bacteriol.*, vol. 181, no. 19, pp. 5915–21, Oct. 1999.

[15] S. Lanigan-Gerdes, A. N. Dooley, K. F. Faull, B. A. Lazazzera, and S. Lanigan- Gerdes, “Identification of subtilisin, Epr and Vpr as enzymes that produce CSF, an extracellular signalling peptide of Bacillus subtilis,” *Mol. Microbiol.*, vol. 65, no. 5, pp. 1321–1333, Sep. 2007.

[16] R. Magnuson, J. Solomon, and a D. Grossman, “Biochemical and genetic characterization of a competence pheromone from B. subtilis.,” *Cell*, vol. 77, no. 2, pp. 207–16, Apr. 1994.

[17] M. Thoendel and A. R. Horswill, “Identification of Staphylococcus aureus AgrD residues required for autoinducing peptide biosynthesis,” *J. Biol. Chem.*, vol. 284, 2009.

[18] M. W. Cunningham, “Pathogenesis of group A streptococcal infections,” *Clin. Microbiol. Rev.*, vol. 13, no. 3, pp. 470–511, Jul. 2000.

[19] G. P. DeMuri and E. R. Wald, “The Group A Streptococcal Carrier State Reviewed: Still an Enigma.,” *J. Pediatric Infect. Dis. Soc.*, vol. 3, no. 4, pp. 336–42, Dec. 2014.

[20] M. W. Cunningham, “Streptococcus and rheumatic fever.,” *Curr. Opin. Rheumatol.*, vol. 24, no. 4, pp. 408–16, Jul. 2012.

[21] J. R. Carapetis, A. C. Steer, E. K. Mulholland, and M. Weber, “The global burden of group A streptococcal diseases.,” *Lancet Infect. Dis.*, vol. 5, no. 11, pp. 685–94, Nov. 2005.

[22] K. Wollein Waldetoft, L. Råberg, K. W. Waldetoft, and L. Ra, “To harm or not to harm? On the evolution and expression of virulence in group A streptococci.,” *Trends Microbiol.*, vol. 22, no. 1, pp. 7–13, Jan. 2014.

[23] M. J. Kazmierczak, M. Wiedmann, and K. J. Boor, “Alternative Sigma Factors and Their Roles in Bacterial Virulence Alternative Sigma Factors and Their Roles in Bacterial Virulence,” *Microbiol. Mol. Biol. Rev.*, vol. 69, no. 4, pp. 527–543, 2005.

[24] L. Mashburn-Warren, D. A. Morrison, and M. J. Federle, “The cryptic competence pathway in Streptococcus pyogenes is controlled by a peptide pheromone.,” *J. Bacteriol.*, vol. 194, no. 17, pp. 4589–600, Sep. 2012.

[25] B. Kreikemeyer, K. S. McIver, and A. Podbielski, “Virulence factor regulation and regulatory networks in Streptococcus pyogenes and their impact on pathogen–host interactions,” *Trends Microbiol.*, vol. 11, no. 5, pp. 224–232, Apr. 2003.

[26] A. Tomasz, “Control of the competent state in Pneumococcus by a hormone-like cell product: an example for a new type of regulatory mechanism in bacteria,” *Nature*, vol. 208, 1965.

[27] L. C. Cook and M. J. Federle, “Peptide pheromone signaling in Streptococcus and Enterococcus.,” *FEMS Microbiol. Rev.*, vol. 38, no. 3, pp. 473–92, May 2014.

[28] C. Hidalgo-Grass, M. Ravins, M. Dan-Goor, J. Jaffe, A. E. Moses, and E. Hanski, “A locus of group A Streptococcus involved in invasive disease and DNA transfer.,” *Mol. Microbiol.*, vol. 46, no. 1, pp. 87–99, Oct. 2002.

[29] I. Belotserkovsky, M. Baruch, A. Peer, E. Dov, M. Ravins, I. Mishalian, M. Persky, Y. Smith, and E. Hanski, “Functional analysis of the quorum-sensing streptococcal invasion locus (sil),” *PLoS Pathog.*, vol. 5, no. 11, p. e1000651, 2009.

[30] Y. Eran, Y. Getter, M. Baruch, I. Belotserkovsky, G. Padalon, I. Mishalian, A. Podbielski, B. Kreikemeyer, and E. Hanski, “Transcriptional regulation of the sil locus by the SilCR signalling peptide and its implications on group A streptococcus virulence.,” *Mol. Microbiol.*, vol. 63, no. 4, pp. 1209–22, Feb. 2007.

[31] A. Michael-Gayego, M. Dan-Goor, J. Jaffe, C. Hidalgo-Grass, and A. E. Moses, “Characterization of sil in invasive group A and G streptococci: antibodies against bacterial pheromone peptide SilCR result in severe infection.,” *Infect. Immun.*, vol. 81, no. 11, pp. 4121–7, Nov. 2013.

[32] A. A. E. Kizy and M. N. M. Neely, “First Streptococcus pyogenes signature-tagged mutagenesis screen identifies novel virulence determinants.,” *Infect. Immun.*, vol. 77, no. 5, pp. 1854–65, May 2009.

[33] P. Bidet, C. Courroux, C. Salgueiro, A. Carol, P. Mariani-Kurkdjian, S. Bonacorsi, and E. Bingen, “Molecular epidemiology of the sil streptococcal invasive locus in group A streptococci causing invasive infections in French children.,” *J. Clin. Microbiol.*, vol. 45, no. 6, pp. 2002–4, Jun. 2007.

[34] D. S. Billal, M. Hotomi, J. Shimada, K. Fujihara, K. Ubukata, R. Sugita, and N. Yamanaka, “Prevalence of Streptococcus invasive locus (sil) and its relationship with macrolide resistance among group A Streptococcus strains.,” *J. Clin. Microbiol.*, vol. 46, no. 4, pp. 1563–4, Apr. 2008.

[35] M. Baruch, I. Belotserkovsky, B. B. Hertzog, M. Ravins, E. Dov, K. S. McIver, Y. S. Le Breton, Y. Zhou, C. Y. Cheng, C. Y. Chen, and E. Hanski, “An extracellular bacterial pathogen modulates host metabolism to regulate its own sensing and proliferation.,” *Cell*, vol. 156, no. 1–2, pp. 97–108, Jan. 2014.

[36] C. Hidalgo-Grass, M. Dan-Goor, A. Maly, Y. Eran, L. A. Kwinn, V. Nizet, M. Ravins, J. Jaffe, A. Peyser, A. E. Moses, and others, “Effect of a bacterial pheromone peptide on host chemokine degradation in group A streptococcal necrotising soft-tissue infections,” *Lancet*, vol. 363, no. 9410, pp. 696–703, 2004.

[37] C. Hidalgo-Grass, I. Mishalian, M. Dan-Goor, I. Belotserkovsky, Y. Eran, V. Nizet, A. Peled, and E. Hanski, “A streptococcal protease that degrades CXC chemokines and impairs bacterial clearance from infected tissues.,” *EMBO J.*, vol. 25, no. 19, pp. 4628–37, Oct. 2006.

[38] P. Cotter, C. Hill, and R. Ross, “Bacteriocins: developing innate immunity for food,” *Nat. Rev. Microbiol.*, vol. 3, pp. 777–788, 2005.

[39] L. E. N. Quadri, “Regulation of antimicrobial peptide production by autoinducer-mediated quorum sensing in lactic acid bacteria.,” *Antonie Van Leeuwenhoek*, vol. 82, no. 1–4, pp. 133–45, Aug. 2002.

[40] C. Chatterjee, M. Paul, L. Xie, and W. A. van der Donk, “Biosynthesis and mode of action of lantibiotics.,” *Chem. Rev.*, vol. 105, no. 2, pp. 633–84, Feb. 2005.

[41] M. Kleerebezem, “Quorum sensing control of lantibiotic production; nisin and subtilin autoregulate their own biosynthesis.,” *Peptides*, vol. 25, no. 9, pp. 1405–14, Sep. 2004.

[42] S. Gebhard, “ABC transporters of antimicrobial peptides in Firmicutes bacteria - phylogeny, function and regulation.,” *Mol. Microbiol.*, vol. 86, no. 6, pp. 1295–317, Dec.

2012.

[43] W. L. Hynes and J. R. Tagg, “Production of broad-spectrum bacteriocin-like activity by group A streptococci of particular M-types.,” *Zentralblatt für Bakteriologie, Mikrobiologie, und Hygiene*, vol. 259, no. 2. pp. 155–64, Apr-1985.

[44] M. Upton, J. Tagg, P. Wescombe, and H. F. Jenkinson, “Intra- and interspecies signaling between Streptococcus salivarius and Streptococcus pyogenes mediated by SalA and SalA1 Lantibiotic peptides,” *J. Bacteriol.*, vol. 183, no. 13, pp. 3931–8, Jul. 2001.

[45] K. Karaya, T. Shimizu, and a Taketo, “New gene cluster for lantibiotic streptin possibly involved in streptolysin S formation.,” *J. Biochem.*, vol. 129, no. 5, pp. 769–75, May 2001.

[46] J. R. Tagg and S. A. Skjold, “A bacteriocin produced by certain M-type 49 Streptococcus pyogenes strains when incubated anaerobically.,” *J. Hyg. (Lond).*, vol. 93, no. 2, pp. 339–44, Oct. 1984.

[47] W. J. Simpson and J. R. Tagg, “M-type 57 group A streptococcus bacteriocin.,” *Can. J. Microbiol.*, vol. 29, no. 10, pp. 1445–51, Oct. 1983.

[48] J. R. Tagg, R. S. Read, and A. R. McGiven, “Bacteriocin of a group A streptococcus: partial purification and properties.,” *Antimicrob. Agents Chemother.*, vol. 4, no. 3, pp. 214–21, Sep. 1973.

[49] P. A. Wescombe and J. R. Tagg, “Purification and characterization of streptin, a type A1 lantibiotic produced by Streptococcus pyogenes,” *Appl. Environ. Microbiol.*, vol. 69, no. 5, pp. 2737–47, May 2003.

[50] J. Livezey, L. Perez, D. Suciu, X. Yu, B. Robinson, D. Bush, and G. Merrill, “Analysis of group A Streptococcus gene expression in humans with pharyngitis using a microarray.,” *J. Med. Microbiol.*, vol. 60, no. Pt 12, pp. 1725–33, Dec. 2011.

[51] H. a Phelps and M. N. Neely, “SalY of the Streptococcus pyogenes lantibiotic locus is required for full virulence and intracellular survival in macrophages.,” *Infect. Immun.*, vol. 75, no. 9, pp. 4541–51, Sep. 2007.

[52] P. Namprachan-Frantz, H. M. Rowe, D. L. Runft, and M. N. Neely, “Transcriptional analysis of the Streptococcus pyogenes salivaricin locus.,” *J. Bacteriol.*, vol. 196, no. 3, pp. 604–13, Feb. 2014.

[53] J. Rocha-Estrada, A. E. Aceves-Diez, G. Guarneros, and M. de la Torre, “The RNPP

family of quorum-sensing proteins in Gram-positive bacteria.," *Appl. Microbiol. Biotechnol.*, vol. 87, no. 3, pp. 913–23, Jul. 2010.

[54] B. Fleuchot, C. Gitton, a Guillot, J. Vidic, P. Nicolas, C. Besset, L. Fontaine, P. Hols, N. Leblond-Bourget, V. Monnet, and R. Gardan, "Rgg proteins associated with internalized small hydrophobic peptides: a new quorum-sensing mechanism in streptococci.," *Mol. Microbiol.*, vol. 80, no. 4, pp. 1102–19, May 2011.

[55] U. M. Samen, B. J. Eikmanns, and D. J. Reinscheid, "The transcriptional regulator RovS controls the attachment of Streptococcus agalactiae to human epithelial cells and the expression of virulence genes.," *Infect. Immun.*, vol. 74, no. 10, pp. 5625–35, Oct. 2006.

[56] S. Anbalagan, W. M. McShan, P. M. Dunman, and M. S. Chaussee, "Identification of Rgg binding sites in the Streptococcus pyogenes chromosome.," *J. Bacteriol.*, vol. 193, no. 18, pp. 4933–42, Sep. 2011.

[57] E. L. A. Rawlinson, I. F. Nes, and M. Skaugen, "LasX, a transcriptional regulator of the lactocin S biosynthetic genes in Lactobacillus sakei L45, acts both as an activator and a repressor," *Biochimie*, vol. 84, no. 5–6, pp. 559–567, May 2002.

[58] M. C. Sulavik, G. Tardif, and D. B. Clewell, "Identification of a gene, rgg, which regulates expression of glucosyltransferase and influences the Spp phenotype of Streptococcus gordonii Challis.," *J. Bacteriol.*, vol. 174, no. 11, pp. 3577–86, Jun. 1992.

[59] T. Fujiwara, T. Hoshino, T. Ooshima, S. Sobue, and S. Hamada, "Purification, Characterization, and Molecular Analysis of the Gene Encoding Glucosyltransferase from Streptococcus oralis," *Infect. Immun.*, vol. 68, no. 5, pp. 1–10, 2000.

[60] A. Fernandez, F. Borges, B. Gintz, B. Decaris, and N. Leblond-Bourget, "The rggC locus, with a frameshift mutation, is involved in oxidative stress response by Streptococcus thermophilus.," *Arch. Microbiol.*, vol. 186, no. 3, pp. 161–9, Sep. 2006.

[61] L. Fontaine, C. Boutry, M. H. de Frahan, B. Delplace, C. Fremaux, P. Horvath, P. Boyaval, and P. Hols, "A novel pheromone quorum-sensing system controls the development of natural competence in Streptococcus thermophilus and Streptococcus salivarius.," *J. Bacteriol.*, vol. 192, no. 5, pp. 1444–54, Mar. 2010.

[62] M. Ibrahim, A. Guillot, F. Wessner, F. Algaron, C. Besset, P. Courtin, R. Gardan, and V. Monnet, "Control of the transcription of a short gene encoding a cyclic peptide in Streptococcus thermophilus: a new quorum-sensing system?," *J. Bacteriol.*, vol. 189, no.

24, pp. 8844–54, Dec. 2007.

[63] M. E. Bortoni, V. S. Terra, J. Hinds, P. W. Andrew, and H. Yesilkaya, “The pneumococcal response to oxidative stress includes a role for Rgg.,” *Microbiology*, vol. 155, no. Pt 12, pp. 4123–34, Dec. 2009.

[64] F. Qi, P. Chen, and P. W. Caufield, “Functional analyses of the promoters in the lantibiotic mutacin II biosynthetic locus in Streptococcus mutans,” *Appl. Environ. Microbiol.*, vol. 65, no. 2, pp. 652–8, Feb. 1999.

[65] F. Zheng, H. Ji, M. Cao, C. Wang, Y. Feng, M. Li, X. Pan, J. Wang, Y. Qin, F. Hu, and J. Tang, “Contribution of the Rgg transcription regulator to metabolism and virulence of Streptococcus suis serotype 2.,” *Infect. Immun.*, vol. 79, no. 3, pp. 1319–28, Mar. 2011.

[66] M. Ibrahim, P. Nicolas, P. Bessières, A. Bolotin, V. Monnet, and R. Gardan, “A genome-wide survey of short coding sequences in streptococci.,” *Microbiology*, vol. 153, no. Pt 11, pp. 3631–44, Nov. 2007.

[67] L. Mashburn-Warren, D. A. Morrison, and M. J. Federle, “A novel double-tryptophan peptide pheromone controls competence in Streptococcus spp. via an Rgg regulator.,” *Mol. Microbiol.*, vol. 78, no. 3, pp. 589–606, Nov. 2010.

[68] L. C. Cook, B. LaSarre, and M. J. Federle, “Interspecies communication among commensal and pathogenic streptococci,” *MBio*, vol. 4, no. 4, 2013.

[69] B. Fleuchot, A. Guillot, C. Mézange, C. Besset, E. Chambellon, V. Monnet, R. Gardan, and C. Me, “Rgg-associated SHP signaling peptides mediate cross-talk in Streptococci.,” *PLoS One*, vol. 8, no. 6, p. e66042, Jan. 2013.

[70] R. K. Carroll and J. M. Musser, “From transcription to activation: how group A streptococcus, the flesh-eating pathogen, regulates SpeB cysteine protease production.,” *Mol. Microbiol.*, vol. 81, no. 3, pp. 588–601, Aug. 2011.

[71] A. V Dmitriev, E. J. McDowell, K. V Kappeler, M. A. Chaussee, L. D. Rieck, and M. S. Chaussee, “The Rgg regulator of Streptococcus pyogenes influences utilization of nonglucose carbohydrates, prophage induction, and expression of the NAD-glycohydrolase virulence operon.,” *J. Bacteriol.*, vol. 188, no. 20, pp. 7230–41, Oct. 2006.

[72] A. V Dmitriev, E. J. McDowell, and M. S. Chaussee, “Inter- and intraserotypic variation in the Streptococcus pyogenes Rgg regulon.,” *FEMS Microbiol. Lett.*, vol. 284, no. 1, pp. 43–51, Jul. 2008.

[73] A. Hollands, R. K. Aziz, R. Kansal, M. Kotb, V. Nizet, and M. J. Walker, “A naturally occurring mutation in ropB suppresses SpeB expression and reduces M1T1 group A streptococcal systemic virulence.,” *PLoS One*, vol. 3, no. 12, p. e4102, Jan. 2008.

[74] M. S. Chaussee, G. L. Sylva, D. E. Sturdevant, L. M. Smoot, M. R. Graham, R. O. Watson, and J. M. Musser, “Rgg Influences the Expression of Multiple Regulatory Loci To Coregulate Virulence Factor Expression in Streptococcus pyogenes Rgg Influences the Expression of Multiple Regulatory Loci To Coregulate Virulence Factor Expression in Streptococcus pyogenes,” 2002.

[75] M. S. Chaussee, G. A. Somerville, L. Reitzer, and J. M. Musser, “Rgg Coordinates Virulence Factor Synthesis and Metabolism in Streptococcus pyogenes,” *J. Bacteriol.*, vol. 185, no. 20, 2003.

[76] M. A. Chaussee, E. A. Callegari, and M. S. Chaussee, “Rgg regulates growth phase-dependent expression of proteins associated with secondary metabolism and stress in Streptococcus pyogenes,” *J. Bacteriol.*, vol. 186, no. 21, p. 7091, 2004.

[77] M. A. Chaussee, A. V Dmitriev, E. A. Callegari, and M. S. Chaussee, “Growth phase-associated changes in the transcriptome and proteome of Streptococcus pyogenes.,” *Arch. Microbiol.*, vol. 189, no. 1, pp. 27–41, Jan. 2008.

[78] J. Hytönen, S. Haataja, D. Gerlach, a Podbielski, and J. Finne, “The SpeB virulence factor of Streptococcus pyogenes, a multifunctional secreted and cell surface molecule with strepadhesin, laminin-binding and cysteine protease activity.,” *Mol. Microbiol.*, vol. 39, no. 2, pp. 512–9, Jan. 2001.

[79] D. F. Browning and S. J. Busby, “The regulation of bacterial transcription initiation.,” *Nat. Rev. Microbiol.*, vol. 2, no. 1, pp. 57–65, Jan. 2004.

[80] M. N. Neely, W. R. Lyon, D. L. Runft, and M. Caparon, “Role of RopB in Growth Phase Expression of the SpeB Cysteine Protease of Streptococcus pyogenes,” *J. Bacteriol.*, vol. 185, no. 17, 2003.

[81] A. Podbielski, B. Pohl, M. Woischnik, C. Körner, K. H. Schmidt, E. Rozdzinski, and B. A. Leonard, “Molecular characterization of group A streptococcal (GAS) oligopeptide permease (opp) and its effect on cysteine protease production.,” *Mol. Microbiol.*, vol. 21, no. 5, pp. 1087–99, Sep. 1996.

[82] C. Wang, C.-Y. C. Lin, Y. Y.-H. Luo, P.-J. P. Tsai, Y.-S. Lin, M. T. Lin, W.-J. Chuang, C.-

C. Liu, and J.-J. Wu, “Effects of oligopeptide permease in group A streptococcal infection,” *Infect. Immun.*, vol. 73, no. 5, pp. 2881–90, May 2005.

[83] A. Podbielski and B. A. Leonard, “The group A streptococcal dipeptide permease (Dpp) is involved in the uptake of essential amino acids and affects the expression of cysteine protease.,” *Mol. Microbiol.*, vol. 28, no. 6, pp. 1323–34, Jun. 1998.

[84] S. Anbalagan, A. Dmitriev, W. M. McShan, P. M. Dunman, and M. S. Chaussee, “Growth phase-dependent modulation of Rgg binding specificity in Streptococcus pyogenes.,” *J. Bacteriol.*, vol. 194, no. 15, pp. 3961–71, Aug. 2012.

[85] Y. Ma, A. E. Bryant, D. B. Salmi, E. McIndoo, and D. L. Stevens, “vfr, a novel locus affecting cysteine protease production in Streptococcus pyogenes.,” *J. Bacteriol.*, vol. 191, no. 9, pp. 3189–94, May 2009.

[86] S. A. Shelburne, R. J. Olsen, N. Makthal, N. G. Brown, P. Sahasrabhojane, E. M. Watkins, T. Palzkill, J. M. Musser, M. Kumaraswami, and S. A. S. Iii, “An amino-terminal signal peptide of Vfr protein negatively influences RopB-dependent SpeB expression and attenuates virulence in Streptococcus pyogenes.,” *Mol. Microbiol.*, vol. 82, no. 6, pp. 1481–95, Dec. 2011.

[87] J. C. Chang, B. LaSarre, J. C. Jimenez, C. Aggarwal, and M. J. Federle, “Two group A streptococcal peptide pheromones act through opposing Rgg regulators to control biofilm development.,” *PLoS Pathog.*, vol. 7, no. 8, Aug. 2011.

[88] C. Aggarwal, J. C. Jimenez, D. Nanavati, and M. J. Federle, “Multiple Length Peptide-Pheromone Variants Produced by Streptococcus pyogenes Directly Bind Rgg Proteins to Confer Transcriptional Regulation.,” *J. Biol. Chem.*, Jun. 2014.

[89] J. Nakayama, R. E. Ruhfel, G. M. Dunny, a Isogai, and a Suzuki, “The prgQ gene of the Enterococcus faecalis tetracycline resistance plasmid pCF10 encodes a peptide inhibitor, iCF10.,” *J. Bacteriol.*, vol. 176, no. 23, pp. 7405–8, Dec. 1994.

[90] B. LaSarre, J. C. Chang, and M. J. Federle, “Redundant group a streptococcus signaling peptides exhibit unique activation potentials.,” *J. Bacteriol.*, vol. 195, no. 18, pp. 4310–8, Sep. 2013.

[91] B. Lasarre, C. Aggarwal, and M. J. Federle, “Antagonistic Rgg regulators mediate quorum sensing via competitive DNA binding in Streptococcus pyogenes,” *MBio*, vol. 3, no. 6, 2012.

[92] I. Biswas and J. R. J. Scott, “Identification of rocA, a positive regulator of covR expression in the group A streptococcus,” *J. Bacteriol.*, vol. 185, no. 10, pp. 3081–90, May 2003.

[93] J. N. Cole, R. K. Aziz, K. Kuipers, A. M. Timmer, V. Nizet, N. M. van Sorge, M. Anjuli, and N. M. Van Sorge, “A conserved UDP-glucose dehydrogenase encoded outside the hasABC operon contributes to capsule biogenesis in group A Streptococcus.,” *J. Bacteriol.*, vol. 194, no. 22, pp. 6154–61, Nov. 2012.

[94] O. Johnsborg, V. Eldholm, and L. S. Håvarstein, “Natural genetic transformation: prevalence, mechanisms and function.,” *Res. Microbiol.*, vol. 158, no. 10, pp. 767–78, Dec. 2007.

[95] M. Lee and D. Morrison, “Identification of a New Regulator in Streptococcus pneumoniae Linking Quorum Sensing to Competence for Genetic Transformation,” *J. Bacteriol.*, vol. 181, no. 16, 1999.

[96] P. Luo and D. Morrison, “Transient association of an alternative sigma factor, ComX, with RNA polymerase during the period of competence for genetic transformation in Streptococcus pneumoniae,” *J. Bacteriol.*, vol. 185, no. 1, 2003.

[97] S. Peterson, R. T. Cline, H. Tettelin, V. Sharov, and D. A. Morrison, “Gene expression analysis of the Streptococcus pneumoniae competence regulons by use of DNA microarrays,” *J. Bacteriol.*, vol. 182, no. 21, pp. 6192–202, Nov. 2000.

[98] L. R. Marks, R. Reddinger, and A. Hakansson, “High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in Streptococcus pneumoniae,” *MBio*, vol. 3, no. 5, 2012.

[99] H. Wei and L. S. Håvarstein, “Fratricide Is Essential for Efficient Gene Transfer between Pneumococci in Biofilms.,” *Appl. Environ. Microbiol.*, vol. 78, no. 16, pp. 5897–905, Aug. 2012.

[100] L. R. Marks, L. Mashburn-Warren, M. J. Federle, and A. P. Hakansson, “Streptococcus pyogenes Biofilm Growth in vitro and in vivo and its Role in Colonization, Virulence and Genetic Exchange,” *J. Infect. Dis.*, vol. [Epub ahea, pp. 1–32, Mar. 2014.

[101] J. W. Costerton, “Development of the Biofilm concept,” in *Microbial Biofilms - ASM Press*, M. Ghannoum and G. A. O’Toole, Eds. ASM Press, 2004, pp. 4 – 19.

[102] P. K. Singh, A. L. Schaefer, M. R. Parsek, T. O. Moninger, M. J. Welsh, and E. P.

Greenberg, “Quorum-sensing signals indicate that cystic fibrosis lungs are infectedwith bacterial biofilms,” *Nature*, vol. 407, no. 6805, pp. 762–764, Oct. 2000.

[103] M. A. Ishak, D. H. Gröschel, G. L. Mandell, and R. P. Wenzel, “Association of slime with pathogenicity of coagulase-negative staphylococci causing nosocomial septicemia.,” *J. Clin. Microbiol.*, vol. 22, no. 6, pp. 1025–9, Dec. 1985.

[104] M. L. Kalmokoff, J. W. Austin, X.-D. Wan, G. Sanders, S. Banerjee, and J. M. Farber, “Adsorption, attachment and biofilm formation among isolates of Listeria monocytogenes using model conditions,” *J. Appl. Microbiol.*, vol. 91, no. 4, pp. 725–734, Oct. 2001.

[105] K. Hirota, K. Murakami, K. Nemoto, T. Ono, T. Matsuo, H. Kumon, and Y. Miyake, “Fosfomycin reduces CD15s-related antigen expression of Streptococcus pyogenes.,” *Antimicrob. Agents Chemother.*, vol. 42, no. 5, pp. 1083–7, May 1998.

[106] N. Takemura, Y. Noiri, A. Ehara, T. Kawahara, N. Noguchi, and S. Ebisu, “Single species biofilm-forming ability of root canal isolates on gutta-percha points.,” *Eur. J. Oral Sci.*, vol. 112, no. 6, pp. 523–9, Dec. 2004.

[107] J. Conley, M. E. Olson, L. S. Cook, H. Ceri, V. Phan, H. D. Davies, and J. C. L. I. N. M. Icrobiol, “Biofilm Formation by Group A Streptococci : Is There a Relationship with Treatment Failure ?,” vol. 41, no. 9, pp. 4043–4048, 2003.

[108] C. Lembke, A. Podbielski, C. Hidalgo-grass, L. Jonas, E. Hanski, and B. Kreikemeyer, “Characterization of biofilm formation by clinically relevant serotypes of group A streptococci,” *Appl. Environ. Microbiol.*, vol. 72, no. 4, p. 2864, 2006.

[109] H. Akiyama, S. Morizane, O. Yamasaki, T. Oono, and K. Iwatsuki, “Assessment of Streptococcus pyogenes microcolony formation in infected skin by confocal laser scanning microscopy,” *J. Dermatol. Sci.*, vol. 32, no. 3, pp. 193–199, Sep. 2003.

[110] A. L. Roberts, K. L. Connolly, D. J. Kirse, A. K. Evans, K. A. Poehling, T. R. Peters, and S. D. Reid, “Detection of group A Streptococcus in tonsils from pediatric patients reveals high rate of asymptomatic streptococcal carriage.,” *BMC Pediatr.*, vol. 12, p. 3, 2012.

[111] K. H. Cho and M. G. Caparon, “Patterns of virulence gene expression differ between biofilm and tissue communities of Streptococcus pyogenes.,” *Mol. Microbiol.*, vol. 57, no. 6, pp. 1545–56, Sep. 2005.

[112] H. S. Courtney, I. Ofek, T. Penfound, V. Nizet, M. a Pence, B. Kreikemeyer, A. Podbielski, A. Podbielbski, D. L. Hasty, and J. B. Dale, “Relationship between expression of the

family of M proteins and lipoteichoic acid to hydrophobicity and biofilm formation in Streptococcus pyogenes.," *PLoS One*, vol. 4, no. 1, p. e4166, Jan. 2009.

[113] A. G. O. Manetti, C. Zingaretti, F. Falugi, S. Capo, M. Bombaci, F. Bagnoli, G. Gambellini, G. Bensi, M. Mora, A. M. Edwards, J. M. Musser, E. a Graviss, J. L. Telford, G. Grandi, and I. Margarit, "Streptococcus pyogenes pili promote pharyngeal cell adhesion and biofilm formation.," *Mol. Microbiol.*, vol. 64, no. 4, pp. 968–83, May 2007.

[114] S. E. Maddocks, C. J. Wright, A. H. Nobbs, J. L. Brittan, L. Franklin, N. Strömberg, A. Kadioglu, M. a Jepson, and H. F. Jenkinson, "Streptococcus pyogenes antigen I/II-family polypeptide AspA shows differential ligand-binding properties and mediates biofilm formation.," *Mol. Microbiol.*, vol. 81, no. July, pp. 1034–1049, Jul. 2011.

[115] H. A. Oliver-Kozup, M. Elliott, B. A. Bachert, K. H. Martin, S. D. Reid, D. E. Schwegler-Berry, B. J. Green, and S. Lukomski, "The streptococcal collagen-like protein-1 (Scl1) is a significant determinant for biofilm formation by group A Streptococcus.," *BMC Microbiol.*, vol. 11, no. 1, p. 262, 2011.

[116] C. D. Doern, A. L. Roberts, W. Hong, J. Nelson, W. E. Swords, and S. D. Reid, "Biofilm formation by group A Streptococcus: a role for the streptococcal regulator of virulence (Srv) and streptococcal cysteine protease (SpeB)," *Microbiology*, vol. 155, no. Pt 1, pp. 46–52, 2009.

[117] L. Baldassarri, R. Creti, S. Recchia, M. Imperi, B. Facinelli, E. Giovanetti, M. Pataracchia, G. Alfarone, and G. Orefici, "Therapeutic failures of antibiotics used to treat macrolide-susceptible Streptococcus pyogenes infections may be due to biofilm formation.," *J. Clin. Microbiol.*, vol. 44, no. 8, pp. 2721–7, Aug. 2006.

[118] A. L. Roberts, R. C. Holder, and S. D. Reid, "Allelic replacement of the streptococcal cysteine protease SpeB in a Δsrv mutant background restores biofilm formation.," *BMC Res. Notes*, vol. 3, no. 1, p. 281, Jan. 2010.

[119] A. L. Roberts, K. L. Connolly, C. D. Doern, R. C. Holder, and S. D. Reid, "Loss of the group A Streptococcus regulator Srv decreases biofilm formation in vivo in an otitis media model of infection.," *Infect. Immun.*, vol. 78, no. 11, pp. 4800–8, Nov. 2010.

[120] K. H. Schleifer and O. Kandler, "Peptidoglycan types of bacterial cell walls and their taxonomic implications.," *Bacteriol. Rev.*, vol. 36, no. 4, pp. 407–477, 1972.

[121] W. Vollmer, D. Blanot, and M. A. De Pedro, "Peptidoglycan structure and architecture,"

FEMS Microbiol. Rev., vol. 32, no. 2, pp. 149–167, 2008.

[122] L. Maliničová, M. Piknová, P. Pristaš, and P. Javorský, "Peptidoglycan hydrolases as novel tool for anti-enterococcal therapy," *Curr. Res. Technol. Educ. Top. Appl. Microbiol. Microb. Biotechnol.*, pp. 463–472, 2010.

[123] T. J. Wyckoff, J. A. Taylor, and N. R. Salama, "Beyond growth: Novel functions for bacterial cell wall hydrolases," *Trends Microbiol.*, vol. 20, no. 11, pp. 540–547, 2012.

[124] W. W. Navarre and O. Schneewind, "Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope.," *Microbiol. Mol. Biol. Rev.*, vol. 63, no. 1, pp. 174–229, 1999.

[125] S. K. Mazmanian, "Staphylococcus aureus Sortase, an Enzyme that Anchors Surface Proteins to the Cell Wall," *Science (80-.).*, vol. 285, no. 5428, pp. 760–763, 1999.

[126] T. C. Barnett and J. R. Scott, "Differential recognition of surface proteins in Streptococcus pyogenes by two sortase gene homologs," *J. Bacteriol.*, vol. 184, no. 8, pp. 2181–2191, 2002.

[127] N. Chiappini, A. Seubert, J. L. Telford, G. Grandi, D. Serruto, I. Margarit, and R. Janulczyk, "Streptococcus pyogenes SpyCEP Influences Host-Pathogen Interactions during Infection in a Murine Air Pouch Model.," *PLoS One*, vol. 7, no. 7, p. e40411, Jan. 2012.

[128] S. Becker, M. B. Frankel, O. Schneewind, and D. Missiakas, "Release of protein A from the cell wall of Staphylococcus aureus.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 4, pp. 1574–9, 2014.

[129] S. Layec, B. Decaris, and N. Leblond-Bourget, "Diversity of Firmicutes peptidoglycan hydrolases and specificities of those involved in daughter cell separation," *Res. Microbiol.*, vol. 159, no. 7–8, pp. 507–515, 2008.

[130] Z. M. Wang, X. Li, R. R. Cocklin, M. Wang, M. Wang, K. Fukase, S. Inamura, S. Kusumoto, D. Gupta, and R. Dziarski, "Human peptidoglycan recognition protein-L is an N-acetylmuramoyl-L-alanine amidase.," *J. Biol. Chem.*, vol. 278, no. 49, pp. 49044–49052, 2003.

[131] J. Royet, D. Gupta, and R. Dziarski, "Peptidoglycan recognition proteins: modulators of the microbiome and inflammation.," *Nat. Rev. Immunol.*, vol. 11, no. 12, pp. 837–51, 2011.

[132] L. Callewaert, J. M. Van Herreweghe, L. Vanderkelen, S. Leysen, A. Voet, and C. W. Michiels, “Guards of the great wall: bacterial lysozyme inhibitors,” *Trends Microbiol.*, vol. 20, no. 10, pp. 501–510, 2012.

[133] A. M. Cole, H.-I. Liao, O. Stuchlik, J. Tilan, J. Pohl, and T. Ganz, “Cationic Polypeptides Are Required for Antibacterial Activity of Human Airway Fluid,” *J. Immunol.*, vol. 169, no. 12, pp. 6985–6991, 2002.

[134] P. Markart, T. R. Korfhagen, T. E. Weaver, and H. T. Akinbi, “Mouse lysozyme M is important in pulmonary host defense against Klebsiella pneumoniae infection.,” *Am. J. Respir. Crit. Care Med.*, vol. 169, no. 4, pp. 454–458, 2004.

[135] H. T. Akinbi, R. Epaud, H. Bhatt, and T. E. Weaver, “Bacterial killing is enhanced by expression of lysozyme in the lungs of transgenic mice.,” *J. Immunol.*, vol. 165, no. 10, pp. 5760–6, 2000.

[136] I. R. Welsh and J. K. Spitznagel, “Distribution of lysosomal enzymes, cationic proteins, and bactericidal substances in subcellular fractions of human polymorphonuclear leukocytes.,” *Infect. Immun.*, vol. 4, no. 2, pp. 97–102, Aug. 1971.

[137] C. Weidenmaier and A. Peschel, “Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions.,” *Nat. Rev. Microbiol.*, vol. 6, no. 4, pp. 276–87, Apr. 2008.

[138] P. Åkessont, A. G. Sjöholm, and L. Björck, “Protein SIC, a novel extracellular protein of Streptococcus pyogenes interfering with complement function,” *J. Biol. Chem.*, vol. 271, no. 2, pp. 1081–1088, 1996.

[139] B. A. Fernie-King, D. J. Seilly, A. Davies, and P. J. Lachmann, “Streptococcal Inhibitor of Complement Inhibits Two Additional Components of the Mucosal Innate Immune System: Secretory Leukocyte Proteinase Inhibitor and Lysozyme,” *Infect. Immun.*, vol. 70, no. 9, pp. 4908–4916, Sep. 2002.

[140] T. Uehara and T. G. Bernhardt, “More than just lysins: Peptidoglycan hydrolases tailor the cell wall,” *Curr. Opin. Microbiol.*, vol. 14, no. 6, pp. 698–703, 2011.

[141] D. J. Rigden, M. J. Jedrzejas, and M. Y. Galperin, “Amidase domains from bacterial and phage autolysins define a family of ??-D,L-glutamate-specific amidohydrolases,” *Trends Biochem. Sci.*, vol. 28, no. 5, pp. 230–234, 2003.

[142] E. Frirdich and E. C. Gaynor, “Peptidoglycan hydrolases, bacterial shape, and

pathogenesis," *Curr. Opin. Microbiol.*, vol. 16, no. 6, pp. 767–778, 2013.

[143] J. Humann and L. L. Lenz, "Bacterial peptidoglycan-degrading enzymes and their impact on host muropeptide detection," *J. Innate Immun.*, vol. 1, no. 2, pp. 88–97, 2009.

[144] M. L. Atilano, P. M. Pereira, F. Vaz, M. J. Catalão, P. Reed, I. R. Grilo, R. G. Sobral, P. Ligoxygakis, M. G. Pinho, and S. R. Filipe, "Bacterial autolysins trim cell surface peptidoglycan to prevent detection by the drosophila innate immune system," *Elife*, vol. 2014, no. 3, pp. 1–23, 2014.

[145] I. van de Rijn and R. E. Kessler, "Growth characteristics of group A streptococci in a new chemically defined medium.," *Infect. Immun.*, vol. 27, no. 2, pp. 444–8, Feb. 1980.

[146] B. Howell-Adams and H. S. Seifert, "Molecular models accounting for the gene conversion reactions mediating gonococcal pilin antigenic variation," *Mol. Microbiol.*, vol. 37, no. 5, pp. 1146–1158, Sep. 2000.

[147] W. M. McShan, J. J. Ferretti, T. Karasawa, A. N. Suvorov, S. Lin, B. Qin, H. Jia, S. Kenton, F. Najar, H. Wu, J. Scott, B. a Roe, and D. J. Savic, "Genome sequence of a nephritogenic and highly transformable M49 strain of Streptococcus pyogenes.," *J. Bacteriol.*, vol. 190, no. 23, pp. 7773–85, Dec. 2008.

[148] D. a Ribardo and K. S. McIver, "Defining the Mga regulon: Comparative transcriptome analysis reveals both direct and indirect regulation by Mga in the group A streptococcus.," *Mol. Microbiol.*, vol. 62, no. 2, pp. 491–508, Oct. 2006.

[149] G. Port, E. Paluscio, and M. G. Caparon, "Complete Genome Sequence of emm Type 14 Streptococcus pyogenes Strain HSC5," *Genome Announc.*, vol. 86, no. 11, pp. 6379–6380, 2012.

[150] P. Sumby, S. F. Porcella, A. G. Madrigal, K. D. Barbian, K. Virtaneva, S. M. Ricklefs, D. E. Sturdevant, M. R. Graham, J. Vuopio-Varkila, N. P. Hoe, and J. M. Musser, "Evolutionary origin and emergence of a highly successful clone of serotype M1 group a Streptococcus involved multiple horizontal gene transfer events.," *J. Infect. Dis.*, vol. 192, pp. 771–782, 2005.

[151] D. J. Banks, S. F. Porcella, K. D. Barbian, S. B. Beres, L. E. Philips, J. M. Voyich, F. R. DeLeo, J. M. Martin, G. A. Somerville, and J. M. Musser, "Progress toward characterization of the group A Streptococcus metagenome: complete genome sequence of a macrolide-resistant serotype M6 strain," *J Infect Dis*, vol. 190, no. 4, pp. 727–738, 2004.

[152] J. Jacks-Weis, Y. Kim, and P. P. Cleary, “Restricted deposition of C3 on M+ group A streptococci: correlation with resistance to phagocytosis.,” *J. Immunol.*, vol. 128, no. 4, pp. 1897–902, Apr. 1982.

[153] C. Aggarwal, “Elucidating SHP Pheromones, Rgg Receptors and Inhibitors of Gram-Positive Pathogen Quorum Sensing,” University of Illinois at Chicago, 2015.

[154] J. C. Chang, J. C. Jimenez, and M. J. Federle, “Induction of a quorum sensing pathway by environmental signals enhances group A streptococcal resistance to lysozyme,” *Mol. Microbiol.*, vol. 97, no. 6, pp. 1097–1113, Sep. 2015.

[155] W. M. Mcshan, R. E. Mclaughlin, A. Nordstrand, and J. J. Ferretti, “Vectors containing streptococcal bacteriophage integrases for site-specific gene insertion,” vol. 57, pp. 51–57, 1998.

[156] E. Maguin, P. Duwat, T. Hege, D. Ehrlich, and A. Gruss, “New thermosensitive plasmid for gram-positive bacteria,” *J. Bacteriol.*, vol. 174, no. 17, pp. 5633–5638, 1992.

[157] L. K. Husmann, J. R. Scott, G. Lindahl, and L. Stenberg, “Expression of the Arp protein, a member of the M protein family, is not sufficient to inhibit phagocytosis of Streptococcus pyogenes,” *Infect. Immun.*, vol. 63, no. 1, pp. 345–348, 1995.

[158] R. Menard, P. J. Sansonetti, and C. Parsot, “Nonpolar mutagenesis of the ipa genes defines IpaB, IpaC, and IpaD as effectors of Shigella flexneri entry into epithelial cells,” *J. Bacteriol.*, vol. 175, no. 18, pp. 5899–5906, 1993.

[159] Y. Le Breton, P. Mistry, K. M. Valdes, J. Quigley, N. Kumar, H. Tettelin, and K. S. McIver, “Genome-wide identification of genes required for fitness of group A Streptococcus in human blood.,” *Infect. Immun.*, vol. 81, no. 3, pp. 862–75, Mar. 2013.

[160] J.-P. Claverys, A. Dintilhac, E. V. Pestova, B. Martin, and D. A. Morrison, “Construction and evaluation of new drug-resistance cassettes for gene disruption mutagenesis in Streptococcus pneumoniae, using an ami test platform,” 1995.

[161] B. a Degnan, M. C. Fontaine, a H. Doebereiner, J. J. Lee, P. Mastroeni, G. Dougan, J. a Goodacre, and M. a Kehoe, “Characterization of an isogenic mutant of Streptococcus pyogenes Manfredo lacking the ability to make streptococcal acid glycoprotein.,” *Infect. Immun.*, vol. 68, no. 5, pp. 2441–8, May 2000.

[162] V. P. Richards, S. R. Palmer, P. D. P. Bitar, X. Qin, G. M. Weinstock, S. K. Highlander, C. D. Town, R. A. Burne, and M. J. Stanhope, “Phylogenomics and the dynamic genome

evolution f the genus streptococcus," *Genome Biol. Evol.*, vol. 6, no. 4, pp. 741–753, 2014.

[163] S. Hammerschmidt, S. Wolff, A. Hocke, S. Rosseau, E. Müller, and M. Rohde, "Illustration of Pneumococcal Polysaccharide Capsule during Adherence and Invasion of Epithelial Cells Illustration of Pneumococcal Polysaccharide Capsule during Adherence and Invasion of Epithelial Cells," 2005.

[164] J. Pratten, S. J. Foster, P. F. Chan, M. Wilson, and S. P. Nair, "Staphylococcus aureus accessory regulators: expression within biofilms and effect on adhesion," *Microbes Infect.*, vol. 3, no. 8, pp. 633–637, 2001.

[165] S. Atkinson, J. P. Throup, G. S. A. B. Stewart, and P. Williams, "A hierarchical quorum-sensing system in Yersinia pseudotuberculosis is involved in the regulation of motility and clumping," *Mol. Microbiol.*, vol. 33, no. 6, pp. 1267–1277, Mar. 2002.

[166] B. K. Hammer and B. L. Bassler, "Quorum sensing controls biofilm formation in Vibrio cholerae," *Mol. Microbiol.*, vol. 50, no. 1, pp. 101–104, Aug. 2003.

[167] D. G. Davies, "The involvement of cell-to-cell signals in the development of a bacterial biofilm.," *Science*, vol. 280, no. 5361, pp. 295–298, Apr. 1998.

[168] B. R. Boles and A. R. Horswill, "Agr-mediated dispersal of Staphylococcus aureus biofilms.," *PLoS Pathog.*, vol. 4, no. 4, p. e1000052, Apr. 2008.

[169] D. E. Bessen, W. M. McShan, S. V. Nguyen, A. Shetty, S. Agrawal, and H. Tettelin, "Molecular epidemiology and genomics of group A Streptococcus," *Infect. Genet. Evol.*, vol. 33, pp. 393–418, 2015.

[170] C. Aggarwal, J. C. Jimenez, H. Lee, G. E. Chlipala, K. Ratia, and M. J. Federle, "Identification of Quorum-Sensing Inhibitors Disrupting Signaling between Rgg and Short Hydrophobic Peptides in Streptococci," *MBio*, vol. 6, no. 3, pp. e00393–15, Jul. 2015.

[171] I. M. Frick, M. Mörgelin, and L. Björck, "Virulent aggregates of Streptococcus pyogenes are generated by homophilic protein-protein interactions.," *Mol. Microbiol.*, vol. 37, no. 5, pp. 1232–47, Sep. 2000.

[172] T. Fiedler, T. Köller, and B. Kreikemeyer, "Streptococcus pyogenes biofilms-formation, biology, and clinical relevance.," *Front. Cell. Infect. Microbiol.*, vol. 5, no. February, p. 15, 2015.

[173] J. C. Jimenez and M. J. Federle, “Quorum sensing in group A Streptococcus,” *Front. Cell. Infect. Microbiol.*, vol. 4, p. 127, Sep. 2014.

[174] N. Gollop and P. E. March, “A GTP-binding protein (Era) has an essential role in growth rate and cell cycle control in Escherichia coli,” *J. Bacteriol.*, vol. 173, no. 7, pp. 2265–2270, 1991.

[175] S. Boiteux, T. O’Connor, F. Lederer, A. Gouyette, and J. Laval, “Homogeneous Escherichia coli FPG Protein,” *J. Biol. Chem.*, vol. 265, no. 7, pp. 3916–3922, 1990.

[176] T. Tajiri, H. Maki, and M. Sekiguchi, “Functional cooperation of MutT, MutM and MutY proteins in preventing mutations caused by spontaneous oxidation of guanine nucleotide in Escherichia coli,” *Mutat. Res. Repair*, vol. 336, no. 3, pp. 257–267, 1995.

[177] K. S. Makarova, L. Aravind, and E. V Koonin, “A superfamily of archaeal, bacterial, and eukaryotic proteins homologous to animal transglutaminases.,” *Protein Sci.*, vol. 8, no. 8, pp. 1714–1719, 1999.

[178] L. A. Devriese, J. Hommez, R. Kilpper-Balz, and K.-H. Schleifer, “Streptococcus canis sp. nov.: A Species of Group G Streptococci from Animals,” *Int. J. Syst. Bacteriol.*, vol. 36, no. 3, pp. 422–425, 1986.

[179] T. Norihiko, K. Ken, A. Ryuta, H. Tomohiro, T. Kyouichi, S. Tetsuya, U. Takehiro, and S. Hosoda, “Recurrent Septicaemia Caused by Streptococcus canis after a Dog bite.,” *Scand. J. Infect. Dis.*, vol. 33, no. March, pp. 927–928, 2001.

[180] G. Dubin, “Proteinaceous cysteine protease inhibitors,” *Cell. Mol. Life Sci.*, vol. 62, no. 6, pp. 653–669, 2005.

[181] M. Rzychon, A. Sabat, K. Kosowska, J. Potempa, and A. Dubin, “Staphostatins: An expanding new group of proteinase inhibitors with a unique specificity for the regulation of staphopains, Staphylococcus spp. cysteine proteinases,” *Mol. Microbiol.*, vol. 49, no. 4, pp. 1051–1066, 2003.

[182] G. Dubin, M. Krajewski, G. Popowicz, J. Stec-Niemczyk, M. Bochtler, J. Potempa, a Dubin, and T. a Holak, “A novel class of cysteine protease inhibitors: solution structure of staphostatin A from Staphylococcus aureus,” *Biochemistry*, vol. 42, no. 46, pp. 13449–13456, 2003.

[183] T. F. Kagawa, P. W. O’toole, and J. C. Cooney, “SpeB-Spi: a novel protease-inhibitor pair from Streptococcus pyogenes.,” *Mol. Microbiol.*, vol. 57, no. 3, pp. 650–66, Aug. 2005.

[184] P. Akesson, L. Moritz, M. Truedsson, B. Christensson, and U. von Pawel-Rammingen, "IdeS, a Highly Specific Immunoglobulin G (IgG)-Cleaving Enzyme from Streptococcus pyogenes, Is Inhibited by Specific IgG Antibodies Generated during Infection," *Infect. Immun.*, vol. 74, no. 1, pp. 497–503, Jan. 2006.

[185] S. Layec, J. Gérard, V. Legué, M. P. Chapot-Chartier, P. Courtin, F. Borges, B. Decaris, and N. Leblond-Bourget, "The CHAP domain of Cse functions as an endopeptidase that acts at mature septa to promote Streptococcus thermophilus cell separation," *Mol. Microbiol.*, vol. 71, no. 5, pp. 1205–1217, 2009.

[186] U. Von Pawel-Rammingen and L. Björck, "IdeS and SpeB: Immunoglobulin-degrading cysteine proteinases of Streptococcus pyogenes," *Curr. Opin. Microbiol.*, vol. 6, no. 1, pp. 50–55, 2003.

[187] D. C. Nelson, J. Garbe, and M. Collin, "Cysteine proteinase SpeB from Streptococcus pyogenes-A potent modifier of immunologically important host and bacterial proteins," *Biol. Chem.*, vol. 392, no. 12, pp. 1077–1088, 2011.

[188] K. S. McIver, S. Subbarao, E. M. Kellner, A. S. Heath, and J. R. Scott, "Identification of isp, a locus encoding an immunogenic secreted protein conserved among group a Streptococci," *Infect. Immun.*, vol. 64, no. 7, pp. 2548–2555, 1996.

[189] P. Vandamme, B. Pot, E. Falsen, K. Kersters, and L. A. Devriese, "Taxonomic Study of Lancefield Streptococcal Groups C, G, and L," *Int. J. Syst. Bacteriol.*, vol. 46, no. 3, pp. 774–781, 1996.

[190] M. R. Weinstein, M. Litt, D. A. Kertesz, P. Wyper, D. Rose, M. Coulter, A. McGeer, R. Facklam, C. Ostach, B. M. Willey, A. Borczyk, and D. E. Low, "Invasive infections due to a fish pathogen, Streptococcus Iniae," *N. Engl. J. Med.*, vol. 337, no. 9, pp. 589–594, 1997.

[191] P. L. Shewmaker, A. C. Camus, T. Bailiff, A. G. Steigerwalt, R. E. Morey, and M. da G. S. Carvalho, "Streptococcus ictaluri sp. nov., isolated from Channel Catfish Ictalurus punctatus broodstock," *Int. J. Syst. Evol. Microbiol.*, vol. 57, no. 7, pp. 1603–1606, 2007.

[192] R. P. Novick, H. F. Ross, S. J. Projan, J. Kornblum, B. Kreiswirth, and S. Moghazeh, "Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule," *EMBO J.*, vol. 12, no. 10, pp. 3967–3975, 1993.

[193] E. Morfeldt, D. Taylor, a von Gabain, and S. Arvidson, "Activation of alpha-toxin

translation in Staphylococcus aureus by the trans-encoded antisense RNA, RNAIII.," *EMBO J.*, vol. 14, no. 18, pp. 4569–4577, 1995.

[194] E. Huntzinger, S. Boisset, C. Saveanu, Y. Benito, T. Geissmann, A. Namane, G. Lina, J. Etienne, B. Ehresmann, C. Ehresmann, A. Jacquier, F. Vandenesch, and P. Romby, "Staphylococcus aureus RNAIII and the endoribonuclease III coordinately regulate spa gene expression," *Embo J.*, vol. 24, no. 0261–4189 (Print), pp. 824–835, 2005.

[195] L. B. Regassa, R. P. Novick, and M. J. Betley, "expression of the accessory gene regulator Glucose and Nonmaintained pH Decrease Expression of the Accessory Gene Regulator (agr) in Staphylococcus aureus," *Infect. Immun.*, vol. 60, no. 8, pp. 3381–3388, 1992.

[196] K. Duan and M. G. Surette, "Environmental regulation of Pseudomonas aeruginosa PAO1 las and Rhl quorum-sensing systems," *J. Bacteriol.*, vol. 189, no. 13, pp. 4827–4836, 2007.

[197] M. Boyer and F. Wisniewski-Dyé, "Cell-cell signalling in bacteria: Not simply a matter of quorum," *FEMS Microbiol. Ecol.*, vol. 70, no. 1, pp. 1–19, 2009.

[198] J. Deutscher, F. M. D. Aké, M. Derkaoui, A. C. Zébré, T. N. Cao, H. Bouraoui, T. Kentache, A. Mokhtari, E. Milohanic, and P. Joyet, "The bacterial phosphoenolpyruvate:carbohydrate phosphotransferase system: regulation by protein phosphorylation and phosphorylation-dependent protein-protein interactions.," *Microbiol. Mol. Biol. Rev.*, vol. 78, no. 2, pp. 231–56, Jun. 2014.

[199] L. Gauthier, S. Bourassa, D. Brochu, and C. Vadeboncoeur, "Control of sugar utilization in oral streptococci. Properties of phenotypically distinct 2-deoxyglucose-resistant mutants of Streptococcus salivarius," *Oral Microbiol. Immunol.*, vol. 5, no. 6, pp. 352–359, Dec. 1990.

[200] J. Abranches, Y.-Y. M. Chen, and R. A. Burne, "Characterization of Streptococcus mutans Strains Deficient in EIIABMan of the Sugar Phosphotransferase System," *Appl. Environ. Microbiol.*, vol. 69, no. 8, pp. 4760–4769, Aug. 2003.

[201] A. Bidossi, L. Mulas, F. Decorosi, L. Colomba, S. Ricci, G. Pozzi, J. Deutscher, C. Viti, and M. R. Oggioni, "A Functional Genomics Approach to Establish the Complement of Carbohydrate Transporters in Streptococcus pneumoniae," *PLoS One*, vol. 7, no. 3, p. e33320, Mar. 2012.

[202] Z. D. Moye, R. A. Burne, and L. Zeng, "Uptake and Metabolism of N-Acetylglucosamine

and Glucosamine by Streptococcus mutans," *Appl. Environ. Microbiol.*, vol. 80, no. 16, pp. 5053–5067, Aug. 2014.

[203] A. Cochu, C. Vadeboncoeur, S. Moineau, and M. Frenette, "Genetic and Biochemical Characterization of the Phosphoenolpyruvate:Glucose/Mannose Phosphotransferase System of Streptococcus thermophilus," *Appl. Environ. Microbiol.*, vol. 69, no. 9, pp. 5423–5432, Sep. 2003.

[204] H. Tong, L. Zeng, and R. A. Burne, "The EIIABMan Phosphotransferase System Permease Regulates Carbohydrate Catabolite Repression in Streptococcus gordonii," *Appl. Environ. Microbiol.*, vol. 77, no. 6, pp. 1957–1965, Mar. 2011.

[205] A. C. Almengor, T. L. Kinkel, S. J. Day, and K. S. McIver, "The Catabolite Control Protein CcpA Binds to Pmga and Influences Expression of the Virulence Regulator Mga in the Group A Streptococcus," *J. Bacteriol.*, vol. 189, no. 23, pp. 8405–8416, Dec. 2007.

[206] S. A. Shelburne III, D. Keith, N. Horstmann, P. Sumby, M. T. M. T. Davenport, E. a E. A. Graviss, R. G. R. G. Brennan, and J. M. Musser, "A direct link between carbohydrate utilization and virulence in the major human pathogen group A Streptococcus.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 105, no. 5, p. 1698, Feb. 2008.

[207] N. L. Keim, R. J. Levin, and P. . Havel, "Carbohydrates," in *Modern Nutrition in Health and Disease*, M. E. Shils, A. C. Ross, B. Caballero, and R. . Cousins, Eds. Lippincott, Williams, and Wilkins, 2005, pp. 62–82.

[208] Y. Le Breton, A. T. Belew, K. M. Valdes, E. Islam, P. Curry, H. Tettelin, M. E. Shirtliff, N. M. El-Sayed, and K. S. McIver, "Essential genes in the core genome of the human pathogen Streptococcus pyogenes," *Sci. Rep.*, vol. 5, p. 9838, May 2015.

[209] M. J. Weickert and G. H. Chambliss, "Site-directed mutagenesis of a catabolite repression operator sequence in Bacillus subtilis.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 87, no. 16, pp. 6238–6242, 1990.

[210] B. Görke and J. Stülke, "Carbon catabolite repression in bacteria: many ways to make the most out of nutrients.," *Nat. Rev. Microbiol.*, vol. 6, no. 8, pp. 613–24, Aug. 2008.

[211] M. R. Graham, K. Virtaneva, S. F. Porcella, W. T. Barry, B. B. Gowen, C. R. Johnson, F. a Wright, and J. M. Musser, "Group A Streptococcus transcriptome dynamics during growth in human blood reveals bacterial adaptive and survival strategies.," *Am. J. Pathol.*, vol. 166, no. 2, pp. 455–65, Feb. 2005.

[212] K. Virtaneva, S. F. Porcella, M. R. Graham, R. M. Ireland, C. a Johnson, S. M. Ricklefs, I. Babar, L. D. Parkins, R. a Romero, G. J. Corn, D. J. Gardner, J. R. Bailey, M. J. Parnell, and J. M. Musser, "Longitudinal analysis of the group A Streptococcus transcriptome in experimental pharyngitis in cynomolgus macaques.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 25, pp. 9014–9, Jun. 2005.

[213] S. A. Shelburne III, C. Granville, M. Tokuyama, I. Sitkiewicz, P. Patel, and J. M. Musser, "Growth characteristics of and virulence factor production by group A Streptococcus during cultivation in human saliva," *Infect. Immun.*, vol. 73, no. 8, p. 4723, 2005.

[214] J. Abranches, M. M. Candella, Z. T. Wen, H. V Baker, and R. a Burne, "Different roles of EIIABMan and EIIGlc in regulation of energy metabolism, biofilm development, and competence in Streptococcus mutans.," *J. Bacteriol.*, vol. 188, no. 11, pp. 3748–56, Jun. 2006.

[215] S. Lun and P. J. Willson, "Putative mannose-specific phosphotransferase system component IID represses expression of suilysin in serotype 2 Streptococcus suis," *Vet. Microbiol.*, vol. 105, no. 3, pp. 169–180, 2005.

[216] G. G. Nicolas, M. Frenette, and M. C. Lavoie, "*Streptococcus salivarius* mutants defective in mannose phosphotransferase systems show reduced sensitivity to mutacins I-T9 and R-3B," *Can. J. Microbiol.*, vol. 56, no. 8, pp. 692–696, Aug. 2010.

[217] R. G. Spiro, "Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds.," *Glycobiology*, vol. 12, no. 4, p. 43R–56R, 2002.

[218] a Herscovics and P. Orlean, "Glycoprotein biosynthesis in yeast.," *FASEB J.*, vol. 7, no. 6, pp. 540–550, 1993.

[219] H. L. Byers, E. Tarelli, K. A. Homer, and D. Beighton, "Sequential deglycosylation and utilization of the N-linked, complex-type glycans of human ??1-acid glycoprotein mediates growth of Streptococcus oralis," *Glycobiology*, vol. 9, no. 5, pp. 469–479, 1999.

[220] A. M. Burnaugh, L. J. Frantz, and S. J. King, "Growth of Streptococcus pneumoniae on human glycoconjugates is dependent upon the sequential activity of bacterial exoglycosidases," *J. Bacteriol.*, vol. 190, no. 1, pp. 221–230, 2008.

[221] S. J. King, K. R. Hippe, and J. N. Weiser, "Deglycosylation of human glycoconjugates by the sequential activities of exoglycosidases expressed by Streptococcus pneumoniae," *Mol. Microbiol.*, vol. 59, no. 3, pp. 961–974, 2006.

[222] J. Sjögren and M. Collin, “Bacterial glycosidases in pathogenesis and glycoengineering.,” *Future Microbiol.*, vol. 9, no. 9, pp. 1039–51, 2014.

[223] E. R. Hondorp and K. S. McIver, “The Mga virulence regulon: infection where the grass is greener.,” *Mol. Microbiol.*, vol. 66, no. 5, pp. 1056–65, Dec. 2007.

[224] P. Pfister, A. Wasserfallen, R. Stettler, and T. Leisinger, “Molecular analysis of Methanobacterium phage,” *Mol. Microbiol.*, vol. 30, no. 2, pp. 233–244, 1998.

[225] D. Chatterjee, C. D. Boyd, G. a O’Toole, and H. Sondermann, “Structural characterization of a conserved, calcium-dependent periplasmic protease from Legionella pneumophila.,” *J. Bacteriol.*, vol. 194, no. 16, pp. 4415–25, Aug. 2012.

[226] S. Theunissen, L. De Smet, A. Dansercoer, B. Motte, T. Coenye, J. J. Van Beeumen, B. Devreese, S. N. Savvides, and B. Vergauwen, “The 285 kDa Bap/RTX hybrid cell surface protein (SO4317) of Shewanella oneidensis MR-1 is a key mediator of biofilm formation.,” *Res. Microbiol.*, vol. 161, no. 2, pp. 144–52, Mar. 2010.

[227] C. D. Boyd, D. Chatterjee, H. Sondermann, and G. a O’Toole, “LapG, Required for Modulating Biofilm Formation by Pseudomonas fluorescens Pf0-1, is a Calcium-Dependent Protease.,” *J. Bacteriol.*, no. June, Jun. 2012.

[228] M. Sara and U. B. Sleytr, “S-Layer Proteins,” *J. Bacteriol.*, vol. 182, no. 4, pp. 859–868, 2000.

[229] K. Arends, E. K. Celik, I. Probst, N. Goessweiner-Mohr, C. Fercher, L. Grumet, C. Soellue, M. Y. Abajy, T. Sakinc, M. Broszat, K. Schiwon, G. Koraimann, W. Keller, and E. Grohmann, “TraG encoded by the pip501 type IV Secretion system is a Two-Domain Peptidoglycan-Degrading enzyme essential for conjugative transfer,” *J. Bacteriol.*, vol. 195, no. 19, pp. 4436–4444, 2013.

[230] S. C. Becker, S. Dong, J. R. Baker, J. Foster-Frey, D. G. Pritchard, and D. M. Donovan, “LysK CHAP endopeptidase domain is required for lysis of live staphylococcal cells,” *FEMS Microbiol. Lett.*, vol. 294, no. 1, pp. 52–60, 2009.

[231] S. Guglielmetti, I. Zanoni, S. Balzaretti, M. Miriani, V. Taverniti, I. de Noni, I. Presti, M. Stuknyte, A. Scarafoni, S. Arioli, S. Iametti, F. Bonomi, D. Mora, M. Karp, and F. Granucci, “Murein lytic enzyme TgaA of Bifidobacterium bifidum MIMBb75 modulates dendritic cell maturation through its cysteine- and histidine-dependent amidohydrolase/peptidase (CHAP) amidase domain,” *Appl. Environ. Microbiol.*, vol. 80,

no. 17, pp. 5170–5177, 2014.

[232] D. M. Byers and H. Gong, “Acyl carrier protein: structure-function relationships in a conserved multifunctional protein family.,” *Biochem. Cell Biol.*, vol. 85, no. 6, pp. 649–662, 2007.

[233] F. C. Neuhaus and J. Baddiley, “A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria,” *Microbiol. Mol. Biol. Rev.*, vol. 67, no. 4, p. 686, 2003.

[234] A. Watanabe, T. Yoshimura, B. Mikami, and N. Esaki, “Tyrosine 265 of Alanine Racemase Serves as a Base Abstracting α-Hydrogen from L-Alanine: The Counterpart Residue to Lysine 39 Specific to D-Alanine,” *J Biochem*, vol. 126, no. 4, pp. 781–786, 1999.

[235] B. Lei, S. Mackie, S. Lukomski, and M. Musser, “Identification and Immunogenicity of Group A Streptococcus Culture Supernatant Proteins Identification and Immunogenicity of Group A Streptococcus Culture Supernatant Proteins,” 2000.

[236] E. Zazopoulos, K. Huang, A. Staffa, W. Liu, B. O. Bachmann, K. Nonaka, J. Ahlert, J. S. Thorson, B. Shen, and C. M. Farnet, “A genomics-guided approach for discovering and expressing cryptic metabolic pathways.,” *Nat. Biotechnol.*, vol. 21, no. 2, pp. 187–190, 2003.

[237] N. A. Moran, “Microbial minimalism: Genome reduction in bacterial pathogens,” *Cell*, vol. 108, no. 5, pp. 583–586, 2002.

APPENDIX A

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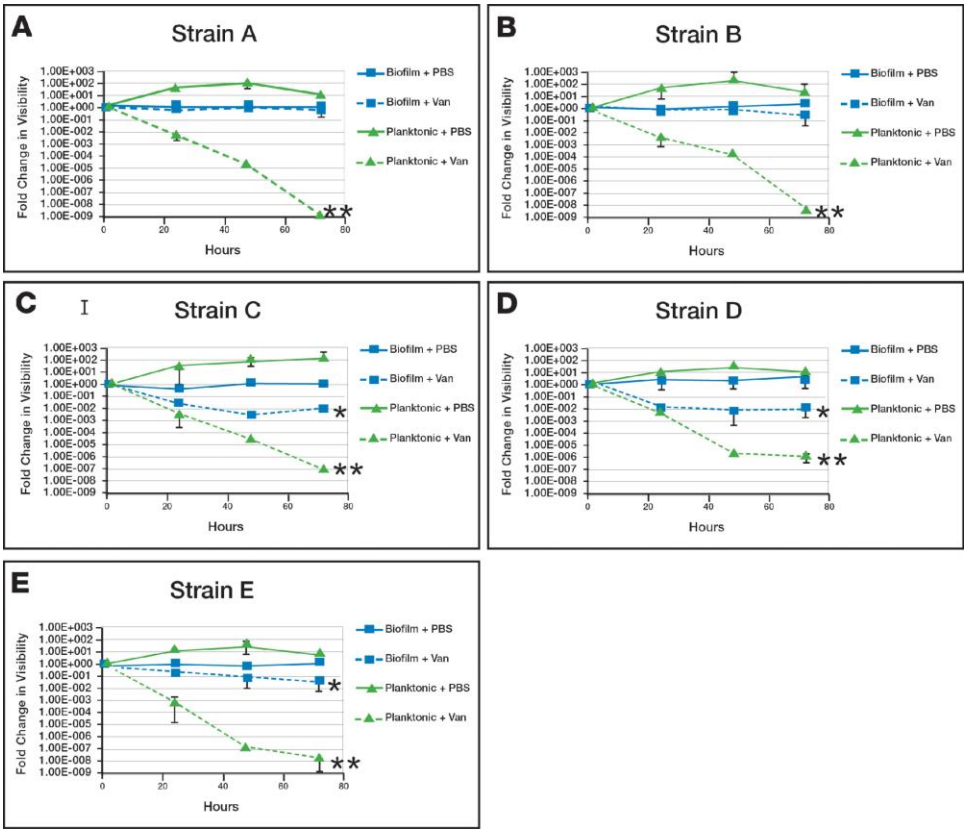
Five selected isolates of coagulase negative *Staphylococcus* (CNS) isolated from ocular surface of patients with Boston type 1 keratoprostheses (K-Pros) were tested for antibacterial resistance patterns when exposed to vancomycin in both planktonic and biofilm forms. For all strains, the rate of bacterial death was significantly higher for planktonic bacterial cells in comparison to the corresponding biofilm state regardless of the specific strain ($P < 0.001$). Eradication of CNS by vancomycin in its biofilm form was statistically significant only in three of the five biofilm-forming strains studied ($P < 0.05$). In the two other strain, vancomycin was ineffective in eradicating or killing CNS in its biofilm form cells at a statistically significant rate.



APPENDIX B

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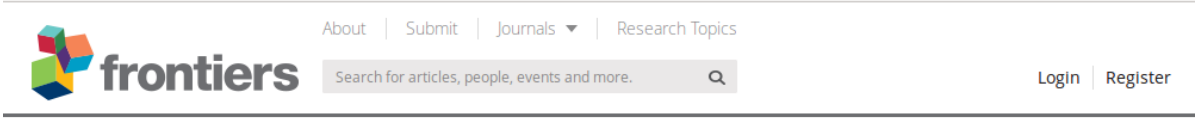

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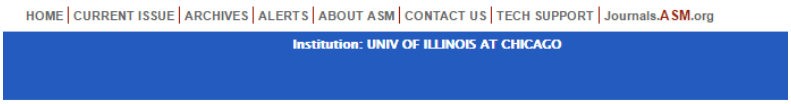

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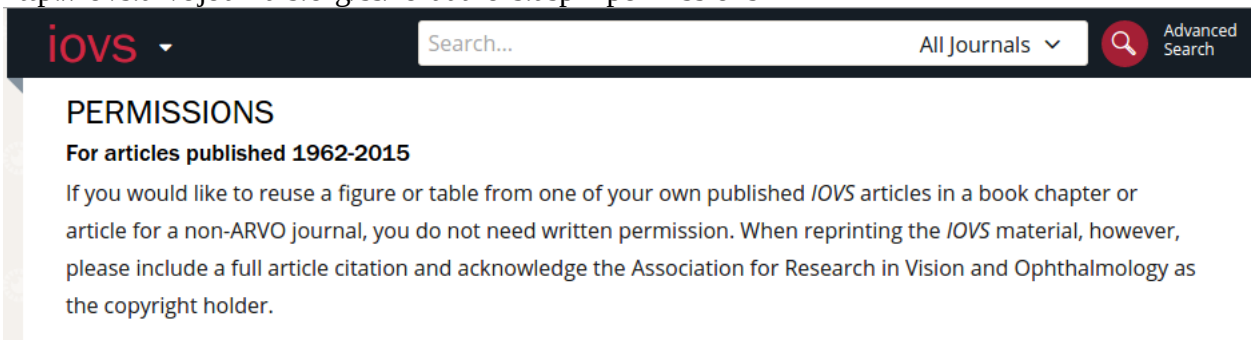

VITA

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EDUCATION

2009 – **B.S. in Biochemistry**
University of Chile - Chile

2010 – **Professional Title in Biochemistry (Post-baccalaureate)**
University of Chile – Chile

2010 – Currently – **PhD Candidate in Microbiology and Immunology**
University of Illinois at Chicago
Mentor: Dr. Michael J. Federle

HONORS / AWARDS

2010 – 2014 **CONICYT *Becas Chile* Doctoral Scholarship**
National Committee for Science and Technology – Chile

2012 – **Registration waiver award**
Midwestern Microbial Pathogenesis Meeting (Milwaukee, USA)

2014 – **Poster Award**
Molecular Genetics of Bacteria and Phages Meeting (Madison, USA)

2014 – **Best oral presentation award**
XXXVI Meeting of the Chilean Society of Microbiology SOMICH (La Serena, Chile)

2015 – **Poster award**
Nexos Chile-USA Meeting (Boston, USA)

PUBLICATIONS

2009 – **The cellular level of O-antigen polymerase Wzy determines chain length regulation by WzzB and WzzpHS-2 in Shigella flexneri 2a.** JA Carter, **JC Jiménez**, M Zaldívar, SA Álvarez, CL Marolda, MA Valvano, I Contreras. *Microbiology 155*(10), 3260-3269.

2009 – **Comparative genomic analysis uncovers 3 novel loci encoding type six secretion systems differentially distributed in Salmonella serotypes.** CJ Blondel, **JC Jiménez**, I Contreras, CA Santiviago. *BMC Genomics 10*(1), 1.

2011 – **Two group A streptococcal peptide pheromones act through opposing Rgg regulators to control biofilm development.** JC Chang, B LaSarre, **JC Jimenez**, C Aggarwal, MJ Federle. *PLOS Pathogens. 7(8), e1002190.*

2013 – **The Type VI Secretion System encoded in SPI-19 is required for Salmonella Gallinarum survival within infected macrophages.** CJ Blondel, **JC Jiménez**, LE Leiva, SA Álvarez, BI Pinto, F Contreras, D Pezoa, CA Santiviago, I Contreras. *Infection and Immunity. IAI-01165.*

2014 - **Multiple Length Peptide-Pheromone Variants Produced by Streptococcus pyogenes Directly Bind Rgg Proteins to Confer Transcriptional Regulation.** C Aggarwal, **JC Jimenez**, D Nanavati, MJ Federle. *Journal of Biological Chemistry. 289(32), 22427-22436.*

2014 - **Quorum Sensing Systems of Streptococcus pyogenes. JC Jimenez** and MJ Federle. *Frontiers in Cellular and Infection Microbiology*. *4*, 127-127.

2015 - **Identification of quorum-sensing inhibitors disrupting Rgg-SHP signaling in streptococci.** C Aggarwal, **JC Jimenez**, H Lee, GE Chlipala, K Ratia and MJ Federle. *mBIO. 6(3), e00393-15.*

2015 - **Induction of a quorum sensing pathway by environmental signals enhances group A streptococcal resistance to lysozyme.** JC Chang, **JC Jimenez** and MJ Federle. *Molecular Microbiology. 97(6), 1097-1113.*

2015 - **Bacteria colonizing the ocular surface in eyes with Boston type 1 keratoprosthesis: Analysis of biofilm-forming capability and vancomycin tolerance.** SH Jassim, KR Sivaraman, **JC Jimenez**, AHJ Jaboori, MJ Federle, J de la Cruz, MS Cortina. *Investigative Ophtalmology and Visual Science. 56(8), 4689-4696.*

POSTER PRESENTATIONS

2008 – XXX Meeting of the Chilean Society of Microbiology SOMICH (Concepcion, Chile)

2009 - XXXI Meeting of the Chilean Society of Microbiology SOMICH (Santa Cruz, Chile)

2012 – Midwestern Microbial Pathogenesis Meeting (Milwaukee, USA)

2012 – 6Th American Society of Microbiology Biofilms Conference (Miami, USA)

2013 – College of Pharmacy Research Day, University of Illinois at Chicago (Chicago, USA)

2013 – Midwestern Microbial Pathogenesis Meeting (Columbus, USA)

2014 – College of Pharmacy Research Day, University of Illinois at Chicago (Chicago, USA)

2014 – Molecular Biology Research Building Retreat, UIC (Chicago, USA)

2014 – Molecular Genetics of Bacteria and Phages Meeting (Madison, USA)

2014 – Midwestern Microbial Pathogenesis Meeting (Chicago, USA)

2015 – American Society for Microbiology General Meeting (New Orleans, USA)

2015 - Midwestern Microbial Pathogenesis Meeting (Indianapolis, USA)

2015 - Nexos Chile-USA Meeting (Boston, USA)

ORAL PRESENTATIONS

2012 – 2016 Gram Positive Seminar, University of Illinois Chicago (USA)

2014 – Nexos Chile-USA Meeting (Philadelphia, USA)

2014 – XXXVI Meeting of the Chilean Society of Microbiology SOMICH (La Serena, Chile)

2014 – Center for Pharmaceutical Biotechnology Seminar, University of Illinois Chicago (USA)

2015 – Seminar, Genomics and Bioinformatics Center, Universidad Mayor (Chile)