University of Nevada, Reno

Identification and characterization of novel quorum sensing systems through the scope of interspecies interaction in Streptococcal species

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in
Chemistry

by

Tyler D. Torres

Dr. Yftah Tal-Gan/Thesis Advisor

May, 2022

THE GRADUATE SCHOOL

We recommend that the thesis
prepared under our supervision by

entitled

be accepted in partial fulfillment of the
requirements for the degree of

Advisor

Committee Member

Graduate School Representative

David W. Zeh, Ph.D., Dean
Graduate School

Abstract:

Quorum sensing (QS) is a chemical-based intercellular communication method related to population density used by single-celled organisms to coordinate gene regulation and resultant group behavior. These behaviors include biofilm formation, competence, motility, virulence, pathogenicity, *et al*., and have been associated with human infections causing endocarditis, oral caries, pulmonary disease, orthopedic implant infection, *et al.* The *Streptococcus* genus provides insight as a model organism for studying peptide-based control of QS in Gram positive bacteria. By observing interspecies interactions and investigating their chemical origins, the Streptococcus's own molecular biology aids in the elucidation of crucial elements of the system. For instance, both Competence Stimulating Peptide (CSP) and *sigX*-Inducing Peptide (XIP) promote the transformation of streptococci by inducing competence through distinct and shared biochemical pathways. This allows for the identification of critical structural features of associated enzymes and peptides through the creation of peptide-based probes and use of reporter strains. The ComABCDE pathway, using CSP, and the ComRS pathway, using XIP, present opportunities to develop non-bactericidal treatments for infections dependent on QS systems, avoiding the selective pressure exerted by antibiotics. Bacteriocins, CSPs/XIPs, proteases, transport proteins, histidine kinases, and other transcription factors all present useful targets for academic and therapeutic study. In this work, I set out to identify QS systems in previously unstudied streptococcal strains through the scope of interspecies interactions and begin their characterization. To this end, I used a range of clinically relevant species to observe single-species growth tendencies, as well as combinations of species for growth assays to establish baseline growth expectations. Interspecies interaction patterns assisted in the identification of potential QS systems, and after homology-based searches and gene sequencing, an *ComRS* with an atypical XIP were observed in *S. sobrinus* W1703. Time-dependent protein extraction were unable to confirm native XIP's or CSP's presence in liquid colonies of the strains studied, and

synthesis of the presumed most biochemically active form of XIP from this strain was attempted. The elucidation of the ComRS system in *S. sobrinus* could allow for the development of a model reporter strain for ComRS in the *Streptococcus* genus, including establishing a cross-species interactive view of clinically relevant bacterial communities. Future XIP synthesis, optimization of conditions for native QS signal production, and phenotypic assays (particularly competence, bacteriocin production, and hemolysis) using XIP collectively will contribute to an overarching understanding of QS communication systems and can lead to the advancement of target selection and therapeutic design.

Acknowledgments

This research was funded by the National Institute of General Medical Sciences (Project Number: R35GM128651) and the National Science Foundation (Award Number: CHE-1808370). Acknowledgments are given to the Shared Instrumentation Laboratory of the Department of Chemistry at UNR. MALDI-TOF spectroscopic analysis was performed in the Shared Instrumentation Laboratory. Thank you very much, Dr. Tal-Gan, for your guidance as an academic and researcher, and for your support as a mentor.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

Since the discovery of penicillin in 1929, antibiotic pharmaceuticals have saved countless lives and contributed to the drastically increased life expectancy from 56.4 years in the 1920's to 79.1 years in 2022 by treating and preventing bacterial infections which previously would have proven fatal.[1,2] However, bacteria possess innate means for the development of resistance to adverse physical, chemical, and biological stressors on both an individual and a macroevolutionary level. Through various mechanisms including viral transduction, conjugation, and transformation, genetic material can be transferred between individuals; this coupled with strong selective pressures on antibiotic targets have resulted in the modern spread of resistance.[1,3] This problem has been historically exacerbated from the overuse of broad-spectrum antibacterial compounds. Multiple rounds of non-specific selective sweeps of the microbiome results in species disequilibrium and promotes the success of resistant strains.[2,4] Collectively, this poses substantial risk in global and local public health via the spread of untreatable bacterial infection, resulting in an estimated morbidity of 99,000 annually from antibiotic-resistant pathogen-associated hospital-acquired infections in the US alone.[1] To combat the spread of resistance and to develop effective therapeutic pharmaceuticals capable of treating a narrow range of bacterial targets, model bacterial systems have been constructed, shedding insight on key biochemical features and suggesting molecular targets with research or therapeutic potential.

In 1928, Frederick Griffith demonstrated bacterial transformation demonstrating that cell-free extracts from heat-killed smooth (virulent) strains of *Streptococcus pneumoniae* could become competent—uptake exogenous genetic material—and the cause virulence in living rough (nonvirulent) strains, and in 1944 the Avery-MacLeod-McCarty experiment cemented DNA's formerly uncertain role as the hereditary material.[5] Elemental analysis of active extracts matched the profile for nucleic acids more closely than that of proteins, and DNase, not RNase, degraded the activity of the extracts, ultimately showing that nucleotides, specifically DNA, was responsible

for encoding and storing the biochemical information of cells. In addition to establishing DNA as the carrier of hereditary information, this experiment also demonstrated horizontal gene transfer among bacteria, where genes are passed between living organisms rather than from parent to offspring. Even in that time, Griffith noted the potential for "alteration in the character of the infective organism" resulting from increased selective pressure.[5] The mechanism of action to induce competence was unknown at the time, but further study would reveal that a system known as quorum sensing was what allowed for the transformation of *S. pneumoniae*.

Quorum sensing (QS) is an intercellular communication method used by unicellular organisms whereby the concentrations of excreted chemical signals called autoinducers reflect cell population density and allow for coordinated group behavior.[6] It is a widespread phenomenon among bacteria and archaea, regulating diverse functions including biofilm formation, pathogenicity, virulence, bacteriocin production, motility, competence, conjugation, symbiosis, and more. Group behavior allows for the synchronization of genetic and biochemical changes, which increases the success of the individual via the group. For example, biofilms are a protective cover which prevent physical and chemical damage and provide favorable living conditions. Timed pathogenicity and virulence allow for immune evasion. Bacteriocin and motility impair inter- and intra-species competition and provides a means of actively obtaining prey. Competence and conjugation promote horizontal gene transfer to confer beneficial traits such as antibiotic resistance.[6,7]

QS signals typically regulate gene clusters, such as that in the best-studied QS system, the lux regulon, found in *Vibrio fischeri*, a light-producing Gram-negative bacterium. The *Euprymna scolopes* squid houses a strain of *V. fischeri* in specialized organs rich with nutrients in a symbiotic relationship. At high concentrations, the bacteria produce light to counter-luminate the squid's shadow from moonlight on bright nights to avoid predation. During the day, when light is not necessary, the squid expels most of the *V. fischeri*, conserving resources by preventing the bacteria

from reaching sufficient population density to achieve bioluminescence. Light is produced by the luciferase-catalyzed oxidation of D-luciferin into oxyluciferin, as shown in **Figure 1**.

Acylated homoserine lactones (AHLs) act as autoinducers in Gram-negative bacteria, produced by *luxI* homologues. While signal transport systems remain unelucidated, studies suggest

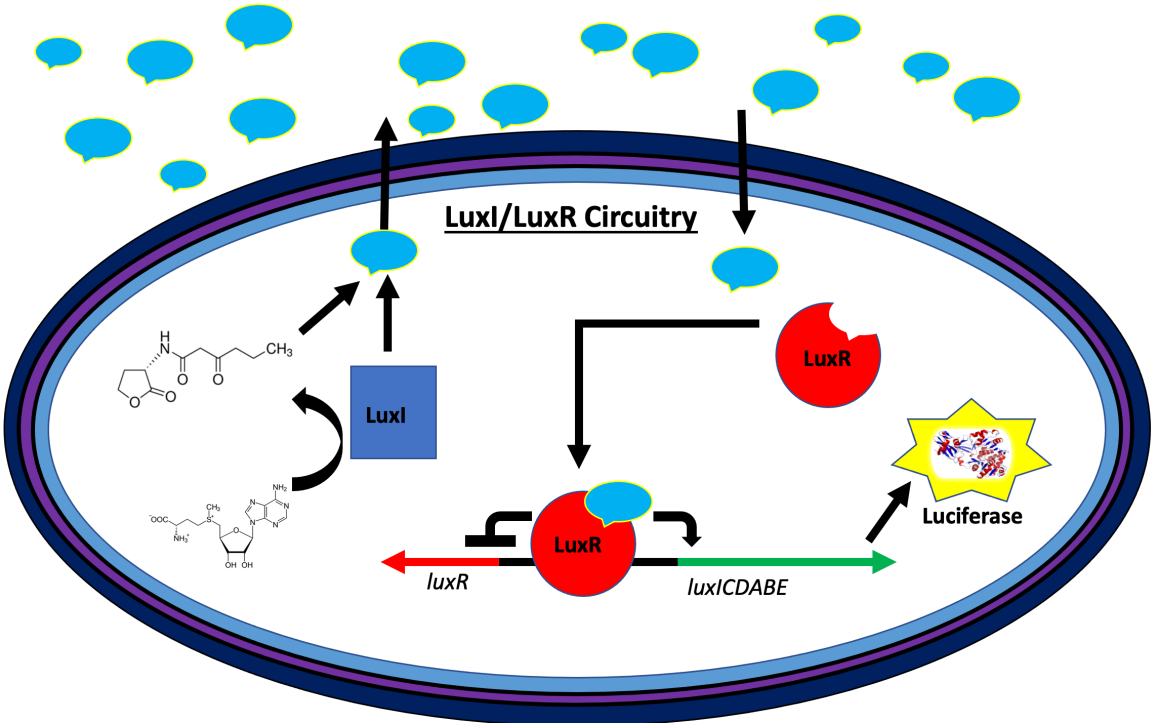

Figure 1. *V. fischeri* LuxI/LuxR circuitry. the rightward transcription of the operon *luxICDABE* consists of *luxCDABE*, encoding the luciferase enzyme, while *luxI* encodes the autoinducer synthase. D-luciferin is encoded as the gene immediately after the *lux* operon. Regulated by the same promotor, the leftward operon *luxR* operon encodes the response regulator, which, upon binding its autoinducer (represented by the blue speech bubbles), exponentially upregulates *luxICDABE* and downregulates *luxR*, in an autoregulatory cycle. Homologues of *luxI* and *luxR* are present in most Gram-negative bacteria using QS, underpinning the shared evolutionary history of this transcriptional regulation system.

that it may be transported by channel proteins, vesicles, or diffusion due to their tough but thin (1.5-10 nm) peptidoglycan cell wall and lipopolysaccharide/protein membrane.[8,9] Similarly, Gram-positive bacteria also use QS to communicate; however, likely due to the thickness of the peptidoglycan cell wall (20-80 nm), the diffusion of small molecule autoinducer signals is unlikely.[9] Therefore, Gram-positive bacteria depend primarily on a different signal transduction system involving autoinducing peptides (AIPs). These circuitries are diverse and include both homologous and analogous systems within and among Gram-positive strains. That said, both Gram-positive and Gram-negative bacteria have been shown to utilize both types of QS machinery to some degree thanks to either convergent evolution or an evolutionary history of horizontal gene transfer.

Impressively, although at the time Frederick Griffith had no idea about the future implications of his 1928 competence research with *S. pneumoniae*, that very species later became a model system for the study of peptide-based QS in Gram-positive bacteria. Considerable progress in genetic research of *Streptococcus*, a clinically relevant genus of human microbe, allowed for the identification and study of QS systems. The rich genetic diversity of *Streptococcal* strains provides for the comparison of cellular machinery and the passive inference of critical structural aspects. However, the innate ability to induce competence as first demonstrated by Griffith has proven invaluable in the control of more genetically explorative approaches.

In *Streptococci*, the ComABCDE system (**Figure 2**) ultimately upregulates *comX*, an alternative sigma factor which controls a host of genes associated with competence, bacteriocin production, biofilm formation, and other physiological behaviors. The AIP in ComABCDE is a 16-21-mer known as competence stimulating peptide, or CSP. Pre-CSP, encoded by *comC*, is transported out of the cell and cleaved by a peptide-specific ATP-binding cassette (ABC) transporter known as ComAB, encoded by *comAB*. In some strains of *S. mutans*, further cleavage by an extracellular protease, SepM, produces the mature CSP signal, which accumulates in the

extracellular environment in relation to cell population density.[10] At high enough concentrations, CSP binds to the CSP-specific extracellular loop of ComD, a transmembrane histidine kinase with 5-8 transmembrane segments, encoded by *comD*.[11,12] Activated ComD prompts the phosphorylation of ComE, a transcriptional regulator encoded by *comE* which upregulates transcription of *comABC*, *comDE*, and *comX*. The phenomenon of CSP production leading to increased CSP production outlines a classic positive feedback loop, which allows for rapid signal amplification and better coordination of group physiological changes upon signal recognition.

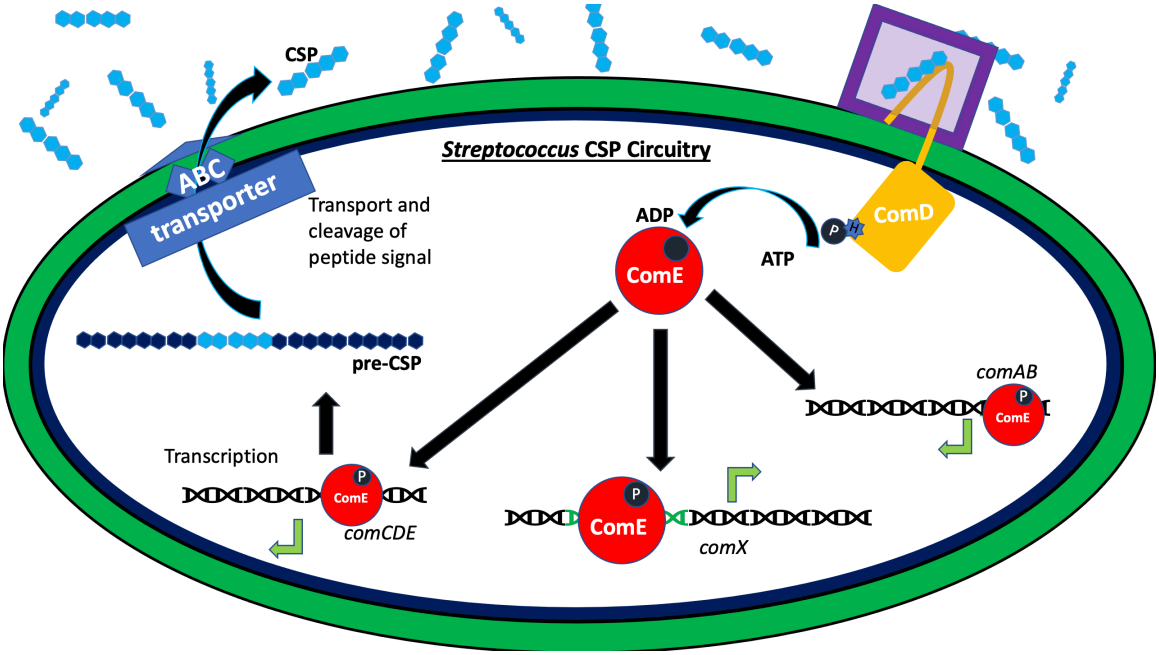

Figure 2. *Streptococcus* ComABCDE circuitry. *comC* is translated to pre-CSP, which is cleaved upon active export by the ComAB transporter into the functional CSP signal. Once the extracellular concentration of CSP is sufficiently high, it binds the transmembrane histidine kinase, activating a phosphorylation cascade, phosphorylating the transcriptional regulator ComE. Upon binding their respective promotors, ComE upregulates expression of *comAB* (encoding the transporter), *comCDE* (encoding CSP, the histidine kinase, and the response regulator), and the alternative sigma factor *comX*.

While not as well-studied as the ComABCDE system, the *Streptococcal* ComRS system (**Figure 3**) still targets *comX* as the ultimate competence regulon control switch, but activation of the alternative sigma factor occurs via another AIP. In this case, *comS* encodes ComS, an unprocessed sigX-inducing pheromone (XIP) analogue to pre-CSP. A ComS transporter exports the ComS, and an unknown protein cleaves the leader sequence, leaving the *C*-terminal end as mature XIP. A key difference here, once XIP builds up in the extracellular environment, an unknown mechanism imports XIP to the cell, and XIP binds ComR, a transcriptional regulator.

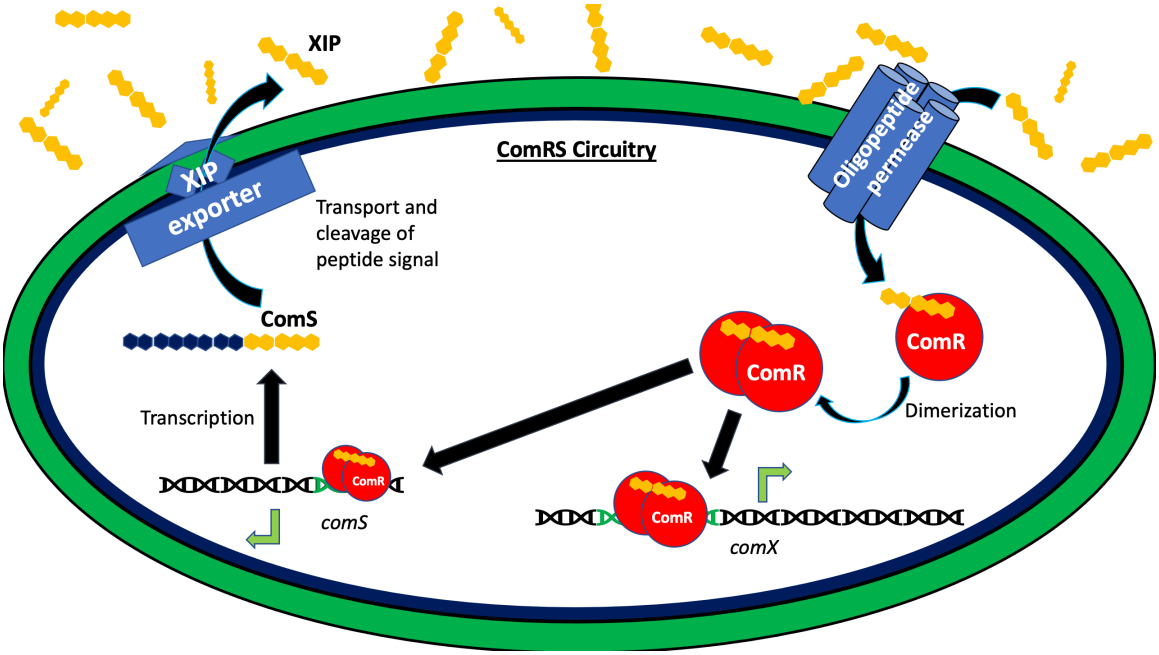

Figure 3. *Streptococcus* ComRS circuitry. *comS* is translated to ComS, which undergoes cleavage to the functional XIP, which is transported outside of the cell by an XIP transporter which is thought to cleave it to the functional peptide. Once the concentration of XIP is high enough, it is transported back into the cell, by either a non-specific oligopeptide transporter, or another mechanism. XIP binds the transcriptional regulator ComR, causing dimerization, and formation of the DNA-binding site. ComR binds the promotors and increases transcription of *comS* and *comX*, but not *comR*.

Studies show that a functional oligopeptide permease can increase cellular uptake of XIP, suggesting a role for the non-specific channel protein in signal transport. Once ComR binds XIP, a conformational change allows for the dimerization of ComR, resulting in the stabilization of a helix-turn-helix DNA-binding domain on ComR.[13,14] The transcriptional regulator then binds and upregulates *comS* and *comX*, but not *comR*, which lacks the binding sequence.[15]

Interestingly, QS's utility extends beyond single-signal intraspecies communication. A marine bacterium, *V. harveyi*, possess two homologous QS circuits which produce and respond to different AHLs. As it stands, one signal is only encoded by one other marine bacterium, *Vibrio parahaemolyticus*, while the other AHL appears to be for intraspecies communication with *V. harveyi* itself.[6] In fact, it is not uncommon to find several QS systems in one organism, often affecting transcription levels in both distinct and overlapping sets of genes to varying degrees in response to variable environmental conditions. *Streptococcus mutans* UA159 uses both ComRS and ComABCDE circuitries to activate *comX* and induce competence, but which is used depends on the environmental growth conditions. When grown in chemically defined media (CDM), exogenous CSP does not upregulate *comX*, while exogenous XIP does; conversely, when grown in rich media, exogenous CSP upregulates *comX*, while exogenous XIP does not.[16] These competence circuitry regulatory mechanisms compliment growth conditions: *comABCDE* translation (more active in nutrient-rich conditions) undergoes a positive feedback loop, while in the ComRS system (more active in nutrient-poor conditions), unchanged translation rates of ComR prevents such a strong amplification of the signal. The strength of expression of competence machinery, then, self-moderates based on the cell's surroundings. Such subtle intricacies allow for fine-tuned responses to external stimuli by bacterial neighbors and environmental factors.

A step further, quorum quenching entails interfering with or co-oping existing QS systems in surrounding bacteria to increase one's own competitive fitness. This process can take a few forms, including degradation of the autoinducer, receptor agonism or antagonism. For instance,

many members of the *Bacillus* bacterial genus produce AiiA, a secreted lactonase which hydrolyzes the AHL, rendering the autoinducer ineffective for other members of its species.[7] Surprisingly, quorum quenching has been observed across species and even kingdom lines. The seaweed *Delisea pulchra* produces HSL analogues, halogenated furanones and enones which act as LuxR antagonists, inhibiting LuxR activation in *V. fischeri* and *Serratia liquefaciens*, and preventing the swarming behavior of *S. liquefaciens*. Antagonism of LuxR in this way allows for non-bacteriocidal management of the microbial community, which reduces selective pressure and microbial pathogenesis. Although the application of this treatment is mostly therapeutically limited based on the sub-species identity of the pathogen (and thus on which, if any, QS circuitries are elucidated/useful/understood for manipulation), insight on model QS systems offers the potential to revolutionize the future of treating bacterial infection.

Considering that selection through the overuse of antibiotics has led to the current crisis of resistance, modern medicine must begin to mitigate bacterial pathogenesis through means such as targeted quorum quenching, without generally exerting the same selective pressure on other members of the human microbiome. As exemplified above, quorum quenching offers options in the fight against the spread of antibiotic resistance through non-bacteriocidal means. While HSL-based QS in Gram-negative bacteria is fairly well-understood, the peptide-based systems of Gram-positive bacteria remain largely understudied. The model genus *Streptococcus*, thus, has proven invaluable to furthering the understanding of the framework and variety within of AIP-based QS systems. Aside from the clinical benefit in the treatment of infections including dental caries, pneumonia, and meningitis from members of the genus itself, the study of *Streptococcal* QS systems allows for the detailed elucidation of this biochemical communication process in a model that can be widely applied throughout Gram-positive species as a whole.[17] Targets of interest become evident by observing interactions between co-cultured strains, and further genetic and phenotypic analysis reveals key features of species of interest. Further studies should involve

bacteriocin and hydrogen peroxide production, as well as critical structural features of QS machinery.

Chapter 2: Identification of species, establishment of growth baseline, and prospective QS indicated by inter-/intra-species interactions

Introduction

The microbial environment is rarely unispecific; that is to say, in the vast majority of cases, microbes encounter neighboring foreign species on a regular basis. On a genetic level, closely related individuals likely possess considerable homology in their prospective intercellular communication machinery, which increases the likelihood of interspecies interactions, either constructively in co-culture, or destructively through inhibition. Several species of *Streptococci* tend to be found together in biofilms such as *S. downei*, *S. sobrinus*, and *S. mutans* co-cultures in dental caries. At one point, all these species were classified as *S. mutans* due to morphological and ecological similarities, but phylogenetic analysis revealed that they were each distinct species, but still members of the Mutans clade.[18] Conversely, interactions like *S. cristatus'* conversion of the lactic acid excreted by *S. mutans* during fermentation into pyruvate to also hydrogen peroxide, which inhibits *S. mutans* growth, garnished further research because of their competitive natures.[19] In either scenario, the observation of interspecies interactions can serve as a useful tool to target promising candidates for further study.

The Tal-Gan lab group researches the competence regulon of *Streptococci*, with particular focus on identification and characterization. QS activators and inhibitors have been produced, which point to therapeutic potential; additionally, cellular transformation allows for molecular probing using gene knockouts and structure-activity relationship (SAR) assays such as alanine screens.[20,21] In my studies, QS circuitry identification was performed through the scope of interspecies interactions and genome analysis, then baseline independent and co-culture growth patterns were noted for later comparison.

A variety of oral *Streptococci* were selected to observe the widest range of interactions with combinatorial significance. All strains are clinical isolates: *S. cristatus* (strains F0329, ATCC551100, A53, and A54), *S. mutans* (strains UA159 and ATCC), *S. oralis tigurinus*, *S. sobrinus* W1703, and *S. downei* F0415. While *S. cristatus* is generally considered commensal and non-pathogenic, *S. mutans* and *S. downei* are known to be pathogenic. *S. oralis tigurinus* (Mitis group, like *S. cristatus*) and *S. sobrinus* (Mutans group, like *S. mutans* and *S. downei*) have been reported as both beneficial and pathogenic, though this is likely a case of guilt by association, since the bacteria are typically found in co-culture at relatively low concentrations with other *Streptococci*. QS machinery has been reported in strains of S. *cristatus*, *S. mutans*, and *S. oralis tigurinus*, but debate continued on the existence of functional QS in *S. sobrinus* and *S. downei*.

With a mix of phenotypes among isolates in both solid and rich liquid media, as well as clear bacterial contamination in the *S. cristatus* A54—two distinct colony morphologies appeared when plated on sterile solid rich media—strain identification was necessary to move forward. The bacterial *rpoB* gene encodes a 740-AA highly conserved subunit of RNA polymerase, which means that it is ubiquitously present across Gram-positive and Gram-negative bacteria. This allows for the broad application of the DNA barcode *rpoB* as a generic target for species-level identification.[22]

Experimental Section

To begin, one physically distant colony of each morphology was isolated from solid cultures of the contaminated *S. cristatus* A54 isolate and grown individually in THY rich (3% Todd-Hewitt broth, 0.5% yeast extract) liquid media then THY solid media (with 1% agar), resulting in two visibly differentiable types of bacteria. Liquid THY cultures of *S. cristatus* strains F0329, ATCC551100, A53, and A54 (large cells, small cells, and mixed), and *S. oralis tigurinus*, grew overnight in sterile complex THY liquid media at 37ºC, 5% CO_2, to achieve high cell density. A 500 uL aliquot of each liquid culture was pelleted and washed with 500 uL 1X PBS three times

and water once. After resuspension in 250 uL water, cells were lysed using heat shock by incubating for first for 3-5 minutes at 95ºC, then for 10 more minutes at -80ºC, with quantification of the decantate via NanoDrop in preparation for polymerase chain reaction (PCR).

Strepto F ([5'-AARYTIGGMCCTGAAGAAAT-3']) and Strepto R ([5'-TGIARTTTRTCATCAACCATGT G-3']) primers were used to target *rpoB* during PCR. The PCR reaction mixture was comprised of 2X Hot Start Taq Master Mix from VWR, genome template extract, Strepto F/R primers, and water to a final volume of 25 uL. The Eppendorf Mastercycler gradient 5331 PCR used the settings seen on **Table 1**. After amplification, PCR products were quantified and assessed for purity using the A260/A280 reading from the NanoDrop. A 1% agarose gel was prepared using 1X TAE buffer, and GelRed Nucleic Acid Gel Stain, 10,000X from Biotium.

Table 1. Eppendorf Mastercycler Gradient 5331 PCR Settings. The following time and temperature conditions were used to perform polymerase chain reaction amplification.

Number of Cycles	Step	Temperature (ºC)	Time
1	Initial denaturation	94	3 min
30	1. Denaturation	94	30 sec
	2. Annealing	50	45 sec
	3. Extension	72	1 min
1	Final extension	72	5 min

Gel electrophoresis revealed successful amplification of *rpoB*, as evident by the bands at ~740 BP, as shown in **Figure 5**. The Omega Biotek E.Z.N.A Cycle Pure PCR Clean-Up Kit was used to remove impurities and amplification fragments prior to sequencing. Samples were submitted to Nevada Genomics Center (NGS) at UNR for Sanger sequencing with the ABI Prism 3730 DNA Analyzer. Phred was used to analyze sequencing data, returning trimmed reads with 99.9% (Q>20) confidence in base identity along with raw sequencing data. The Basic Local

Alignment Search Tool for Nucleotides (BLAST-N) from the National Center for Biotechnology Information (NCBI) compared trimmed sequences to previously published genomes in search of sequence similarity (**Table 2**).

Having identified the isolates and addressed/removed a contaminant, growth baseline expectations must be established. To study growth patterns, growth curves were generated, and colony-forming units (CFUs) were quantified. Liquid THY colonies of the isolates were prepared, and after incubating overnight at 37ºC and 5% CO_2, 200 uL of a 1:100 THY dilution of resuspended bacteria was added to a 96-well plate. The Biospa Automated Incubator and Plate Reader records optical density absorbance readings at 600 nm (OD600), which is related to cell density. The cells incubated in THY at 37ºC and 5% CO_2 for 16 hours, cell division occurred, and as cell density increased, so too would absorbance, as shown in **Figure 6**. In addition, the total number of CFUs (**Figure 7**) were observed in hopes of perceiving a relationship between number of colony-forming units and OD600. A $1{:}10^6$ dilution prior to plating on solid THY and a small volume of liquid culture provided enough space between individual colonies for an accurate count to be carried out.

Time-based invasion assays were performed to observe native interspecies interactions and the effect of pre-establishment of *Streptococcal* colonies. Isolates were grown in THY at 37ºC and 5% CO_2 overnight, then vortexed to separate *Streptococcal* chains. A lawn of a species was established by evenly spreading 200 uL of liquid culture across a THY plate. After allowing the lawn to incubate independently for a given amount of time (see **Tables 3** and **4**), 7.5 uL aliquots of each invading strain were added. The lawn and invading species were then left to incubate overnight at 37ºC and 5% CO_2.

To identify the source of inhibitory patterns observed in **Tables 3** and **4**, cell-free supernatants were applied to lawns in well-diffusion assays. Supernatants were obtained by incubating 1.5 mL liquid cultures of each strain at 37ºC, 5% CO_2 for 16 hours to the stagnant phase, and after vortexing and pelleting, the liquid cultures were passed through a 0.22 micron filter to

remove all living cells. To prepare the lawns, 25 mL of 1% agar in THY was plated in each Petri dish used for well-diffusion. After spreading 150 uL of liquid culture on each of the THY plates, they were incubated for 30 min at 37ºC, 5% CO_2. The back end of a sterile 200 uL pipette tip was used to form holes for the wells, and 100 uL of each supernatant was added to the wells, and they were left to incubate overnight.

Finally, "sticky" peptides which remain attracted or bound to the membrane after excretion, may have fostered the need for direct cell contact to cause inhibition. Targeting early log-phase, 0.75 mL liquid cultures were inoculated in 14.25 mL THY for 3 hours at 37ºC, 5% CO_2. To release the peptides into the supernatant, cells were pelleted, decanted (saving the supernatant), and washed cells with phosphate-buffered saline (PBS) twice. The cells and supernatant were incubated separately at room temperature for 20 minutes with shaking in varying total concentrations (0.1%-1.0%) of Tween20, a surfactant.

Results and Discussion

After successful amplification (**Figure 4**), species-level results were afforded through the use of *rpoB*, but strain-level identification becomes more convoluted due in part to high levels of both sequence homology and variation, and in part to the impressive number of total strains which remain heretofore unsequenced.[22] Therefore, as long as the species identity fit expectations, the originally established strain labels were used; departures from previous names only took place in the case that BLAST displayed results suggested impurities or misidentification of a species. Contaminant identification was accomplished with *Staphylococcus hominis*; since this genus is known for habitation of human skin, the contamination likely was acquired at some point in the oral collection or lab cultivation of *S. cristatus* A54. Serial dilution allowed for the isolation of individual colonies prior to genome extraction, so the contaminant was effectively removed. Aside from the contaminant, the other sequencing results confirmed previously assigned identities.

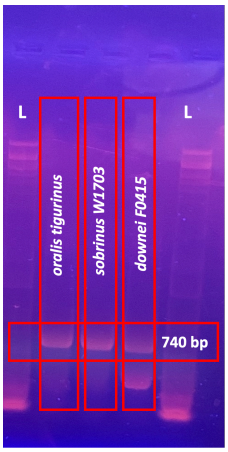

Figure 4. Gel electrophoresis of *rpoB* amplicon for species identification. Gel electrophoresis of PCR amplicons for the *rpoB* gene revealed bands at 740 base pairs, indicating the expected mass for the amplicon, allowing for sequencing to proceed. One note, a lab member separately sequenced the *S. mutans* strains UA159 and ATCC, so they are not present in gels.

The *Streptococcal* QS circuitry functions as the control switch to activate *comX*, which goes on to regulate a host of behaviors, including cooperation (ex. biofilm formation) and inhibitory (ex. bacteriocin/hydrogen peroxide production). Either way, deviations from unperturbed growth patterns indicate potentially promising relationship dynamics for further study. Growth baselines for future comparison were measured using growth curves measuring OD600 Absorption measurements (**Figure 5**) and counting CFUs (**Figure 6**) as well. Unfortunately, with regard to relating OD to CFUs, there seemed to be no discernable relationship between absorbance readings and the number of colony-forming units. Such a result suggests that in addition to cell number, other factors such as cell size likely affect the OD measurement.

Table 2. Species identification: *rpoB* BLAST-N Results. This table delineates the results of the sequencing of the *rpoB* gene barcode for species-level identification. Most species had their identities confirmed, except for one sample, where a contaminant (*Staphylococcus*) was detected.

Presumed Species	Strain	Top BLAST Species Candidate	BLAST Description	Query Coverage	Percent Identity	Accession
S. cristatus	F0329	*S. cristatus*	*Streptococcus cristatus* strain 3965_07 RNA polymerase beta subunit (rpoB) gene, partial cds	96%	95.13%	MK322522.1
S. cristatus	ATCC551100	*S. cristatus*	*Streptococcus cristatus* strain NCTC 12479 genome assembly, chromosome: 1	100%	100.00%	LS483383.1
S. cristatus	A53	*S. cristatus*	*Streptococcus cristatus* strain NCTC 12479 genome assembly, chromosome: 2	98%	97.09%	LS483383.1
contam. *S. cristatus*	contam. A54	*S. cristatus*	*Streptococcus cristatus* strain NCTC 12479 genome assembly, chromosome: 2	99%	97.07%	LS483383.1
S. cristatus	A54 (small colonies)	*S. cristatus*	*Streptococcus cristatus* strain NCTC 12479 genome assembly, chromosome: 3	99%	97.11%	LS483383.1
S. cristatus	A54 (large colonies)	*Staphylococcus hominis*	*Staphylococcus hominis* strain FDAARGOS_136 chromosome, complete genome	99%	100.00%	CP014107.1
S. oralis	*tigurinus*	*S. oralis*	*Streptococcus oralis* strain NCTC 11427 genome assembly, chromosome: 1	94%	98.64%	LR134336.1
S. mutans	UA159	*S. mutans*	*Streptococcus mutans* strain FDAARGOS_685 chromosome	98%	99.11%	AE014133.2
S. mutans	ATCC	*S. mutans*	*Streptococcus mutans* strain FDAARGOS_685 chromosome	97%	98.15%	AE014133.2
S. sobrinus	W1703	*S. sobrinus*	*Streptococcus sobrinus* strain NCTC 12279 genome assembly, chromosome 1	98%	100%	LS483378.1
S. downei	F0415	*S. downei*	*Streptococcus downei* strain CIP 103222 RNA polymerase beta subunit (rpoB) gene, partial cds	86%	98.81%	DQ132984.1

During the growth cycle of *Streptococci*, response to AIPs such as bacteriocin production, biofilm formation, and competence often peak during the early to mid-exponential growth phase, usually about 3-6 hours post-inoculation of a new culture.[23,24] Based on this trend, time-based invasion assays were performed on bacterial mats ("lawns") to detect the effect of preincubation on interspecies interactions. The next day, phenotypic changes were noted as either -1, meaning that the invading species displayed an extra-colonial halo on the lawn; 0, meaning that neither the invading species nor the lawn colony appeared present; 1, meaning that the invading species was

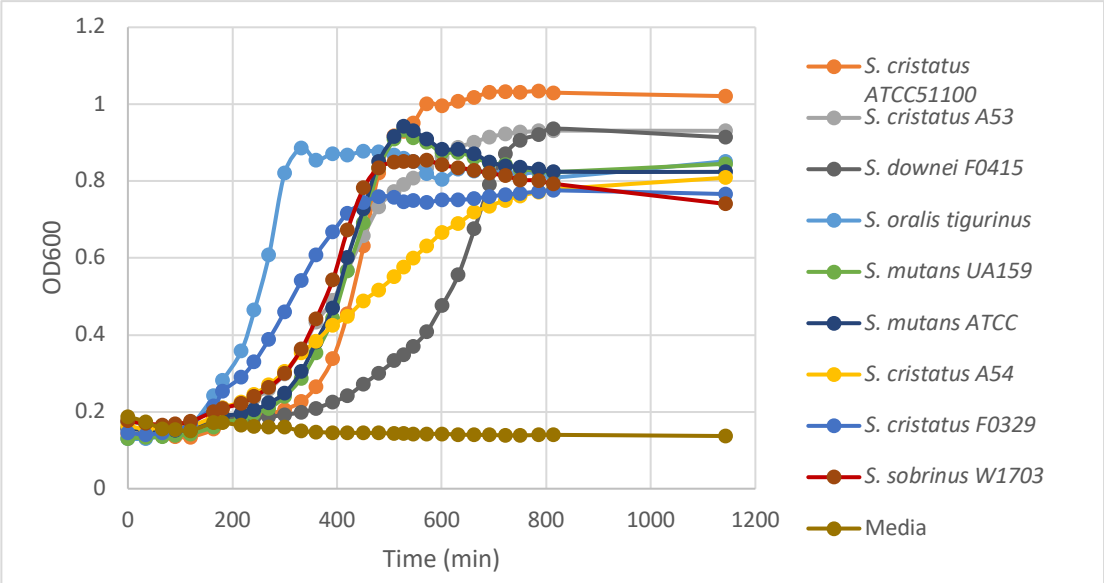

Figure 5. Growth curves monitored using OD600. To establish baseline growth standards for each of the species, the optical density was measured by taking the absorbance at 600 nm every half hour until colonies reached the stationary phase. Error bars have been omitted for clarity.

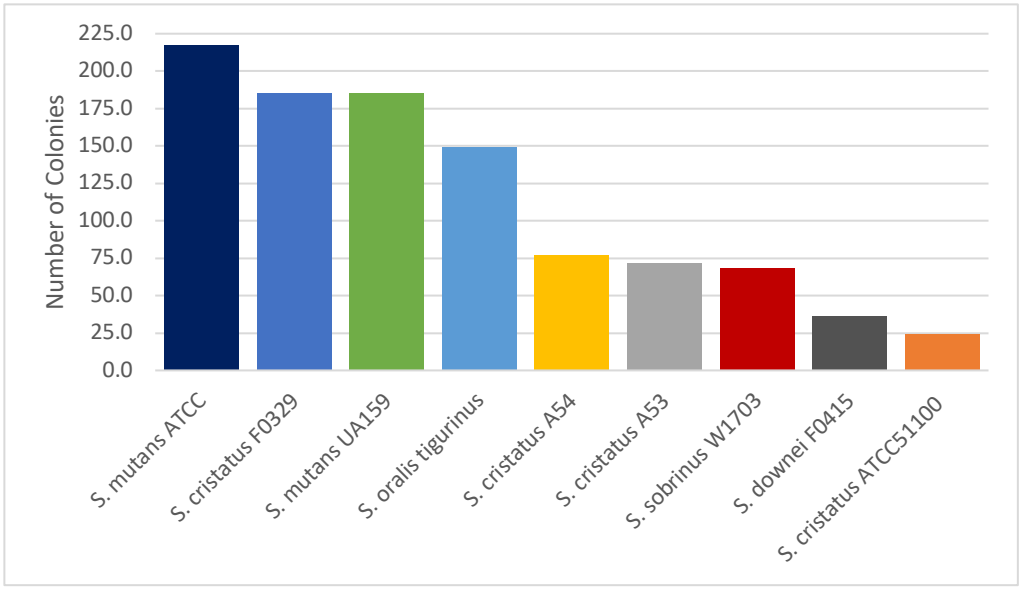

Figure 6. Colony forming units (1:10^6 dilution). Colony counts were carried out following serial dilutions of 1:10^6 to establish a baseline and compare to growth curves. Dilutions were carried out on cultures as they arrived at the stationary phase.

Figure 7. Example of invasion assay results. This figure shows examples of the three main types of interspecies interaction: inhibition of the bacterial lawn producing a halo (-1), blank (0), either through mutual inhibition or unclear coculture, and clear coculture with the lawn (2). Trials were done in triplicate on different days, showing favorable reproducibility.

not visible on the lawn; or 2, meaning that the invading species and the lawn survived in co-culture. **Figure 7** presents examples of each case. Results from invasions of Mitis group lawns (*S. cristatus* and *S. oralis tigurinus*), as well as those from the invasion of Mutans group lawns (*S. mutans, S. sobrinus, and S. downei*) can be seen below (**Tables 3** and **4**). A summary of this data can be seen in **Table 5**. Invading Mutans group strains tend to co-culture for longer with other Mutans group strain lawns, while Mitis lawns would tolerate co-culture, but typically only with less than an hour of lawn preincubation. Mitis group invaders outcompeted Mutans lawns with less than 1-4 hours of lawn preincubation. Mitis group strains frequently did not culture with Mitis lawns, although results varied widely from coculture-ability to clear zones of inhibition.

Invading Mutans group isolates showed a general propensity to co-culture with any of the lawns, but this ability diminished with all lawns sometime between the 1- and 4-hour mark of preincubation. Generally, invading Mitis species inhibited Mutans lawns but co-cultured with other Mitis species when the lawn was incubated less than 4 hours. The exception here is that invading *S. cristatus* ATCC51100 showed mild success being able to co-culture with most lawns regardless of group, (excluding *S. cristatus* A54 and *S. mutans* UA159), which was uncommon for the other

Mitis species tested. Interestingly, invading *S. oralis tigurinus* was able to co-culture with most other fellow Mitis (but not Mutans) group *Streptococci* lawns as well, whereas 3 of the 4 *S. cristatus* isolates mostly lacked this ability. In fact, *S. cristatus* ATCC51100 alone was inhibited by rest of the Mitis group as well as mildly by *S. mutans* strains, which seems odd considering that co-culture

Table 3. Mitis group-lawn invasion assay results. Time-based invasion assays were performed on Mitis group strains as lawns. Invading strains were added to pre-incubated lawns at various timepoints from 0 to 16 hours. Trends are outlined in the main body of the text.

S. cristatus F0329 lawn preincubation duration (hr)	S. cristatus F0329	S. cristatus ATCC51100	S. cristatus A53	S. cristatus A54	S. oralis tigurinus	S. mutans UA159	S. mutans ATCC	S. sobrinus W1703	S. downei F0415
0	0	1	1	1	2	2	2	2	2
0	1	2	1	1	2	2	2	2	2
0	1	2	1	1	2	2	2	2	2
0.5	0	1	1	1	2	1	2	1	1
0.5	0	2	1	1	1	1	2	2	2
0.5	0	2	1	1	1	1	2	2	2
1	2	1	1	1	2	1	2	1	1
1	1	1	1	1	1	1	2	1	1
1	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1
6	1	1	1	1	2	2	2	2	2
6	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1
16	1	1	2	2	2	0	0	2	2
16	1	1	1	1	1	1	1	1	1
16	1	1	1	1	1	1	1	1	1

S. cristatus ATCC51100 lawn preincubation duration (hr)	S. cristatus F0329	S. cristatus ATCC51100	S. cristatus A53	S. cristatus A54	S. oralis tigurinus	S. mutans UA159	S. mutans ATCC	S. sobrinus W1703	S. downei F0415
0	-1	-1	0	-1	-1	-1	-1	2	2
0	-1	2	0	-1	-1	1	2	2	2
0	-1	2	0	-1	-1	1	2	2	2
0.5	-1	1	0	-1	-1	-1	-1	2	2
0.5	1	2	0	0	1	2	2	2	2
0.5	1	2	0	0	1	2	2	2	2
1	-1	2	-1	-1	-1	-1	-1	2	2
1	0	1	1	0	1	1	2	1	2
1	0	1	1	0	1	1	2	1	2
4	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1
6	1	1	1	1	2	1	1	1	1
6	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1
16	1	1	1	1	2	2	1	1	1
16	1	1	1	1	1	1	1	1	1
16	1	1	1	1	1	1	1	1	1

S. cristatus A53 lawn preincubation duration (hr)	S. cristatus F0329	S. cristatus ATCC51100	S. cristatus A53	S. cristatus A54	S. oralis tigurinus	S. mutans UA159	S. mutans ATCC	S. sobrinus W1703	S. downei F0415
0	2	1	1	2	2	2	2	2	2
0	1	2	1	2	2	2	2	2	2
0	1	2	1	1	2	2	2	2	2
0.5	1	1	1	1	2	1	2	2	1
0.5	1	2	1	0	2	2	2	2	2
0.5	1	2	1	0	2	2	2	2	2
1	1	1	1	1	2	1	2	1	1
1	2	2	1	1	2	1	1	2	1
1	2	2	1	1	2	1	1	2	2
4	1	1	1	1	0	1	2	1	1
4	1	1	1	1	0	1	2	1	1
4	1	1	1	1	0	1	2	1	1
6	1	2	2	1	2	0	1	1	1
6	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
12	2	2	2	2	2	2	1	-1	2
12	1	2	1	1	2	1	1	1	1
12	1	2	1	1	2	1	1	1	1
16	2	2	0	0	2	0	0	2	2
16	1	1	1	1	1	1	1	1	1
16	1	1	1	1	1	1	1	1	1

S. cristatus A54 lawn preincubation duration (hr)	S. cristatus F0329	S. cristatus ATCC51100	S. cristatus A53	S. cristatus A54	S. oralis tigurinus	S. mutans UA159	S. mutans ATCC	S. sobrinus W1703	S. downei F0415
0	2	1	1	1	2	2	2	2	2
0	1	0	1	1	2	1	2	1	2
0	1	0	1	1	2	1	2	1	2
0.5	1	1	1	1	2	2	2	2	2
0.5	1	1	1	1	1	1	2	2	1
0.5	1	1	1	1	1	1	2	1	1
1	1	1	1	1	2	2	2	2	2
1	1	1	1	1	1	1	2	1	2
1	1	1	1	1	1	1	2	1	2
4	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
12	1	1	1	1	2	1	1	1	1
12	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1
16	1	1	1	1	2	1	1	1	1
16	1	1	1	1	2	1	1	1	1
16	1	1	1	1	2	1	1	1	1

S. oralis tigurinus lawn preincubation duration (hr)	S. cristatus F0329	S. cristatus ATCC51100	S. cristatus A53	S. cristatus A54	S. oralis tigurinus	S. mutans UA159	S. mutans ATCC	S. sobrinus W1703	S. downei F0415
0	1	1	1	1	1	1	1	1	1
0	1	0	1	1	1	1	2	1	2
0	1	0	1	1	1	1	2	1	2
0.5	-1	1	1	0	2	1	1	2	2
0.5	1	2	1	0	2	1	1	1	1
0.5	1	2	1	0	2	1	1	1	1
1	2	1	1	1	2	1	2	1	1
1	1	1	1	1	1	0	0	1	0
1	1	1	1	1	1	0	0	1	0
4	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1
12	1	1	1	1	2	1	1	1	1
12	1	1	1	1	2	1	1	1	1
16	1	1	1	1	1	1	1	1	1
16	1	1	1	1	2	1	1	1	1
16	1	1	1	1	2	1	1	1	1

Table 4. Mutans group-lawn invasion assay results. Time-based invasion assays were performed on Mutans group strains as lawns. Invading strains were added to pre-incubated lawns at various timepoints from 0 to 16 hours. Trends are outlined in the main body of the text.

S. mutans UA159 lawn preincubation duration (hr)	S. cristatus F0329	S. cristatus ATCC51100	S. cristatus A53	S. cristatus A54	S. oralis tigurinus	S. mutans UA159	S. mutans ATCC	S. sobrinus W1703	S. downei F0415
0	-1	0	-1	-1	-1	1	1	2	2
0	-1	-1	-1	-1	-1	2	2	2	2
0	-1	-1	-1	-1	-1	2	2	2	2
0.5	-1	1	-1	-1	-1	2	2	2	2
0.5	-1	-1	-1	-1	-1	2	1	2	2
0.5	-1	-1	-1	-1	-1	2	1	2	2
1	-1	0	-1	-1	-1	2	2	2	2
1	-1	-1	-1	-1	-1	-1	1	1	2
1	-1	-1	-1	-1	-1	-1	1	1	2
4	1	1	1	-1	-1	1	1	1	1
4	1	1	1	-1	-1	1	1	1	1
4	1	1	1	-1	-1	1	1	1	1
6	1	1	1	1	1	1	1	2	1
6	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1
12	1	1	1	1	2	1	1	1	1
12	1	1	1	1	2	1	1	1	1
16	1	1	1	1	1	1	1	1	1
16	1	1	1	1	2	1	1	1	1
16	1	1	1	1	2	1	1	1	1

S. mutans ATCC lawn preincubation duration (hr)	S. cristatus F0329	S. cristatus ATCC51100	S. cristatus A53	S. cristatus A54	S. oralis tigurinus	S. mutans UA159	S. mutans ATCC	S. sobrinus W1703	S. downei F0415
0	-1	1	-1	-1	-1	2	2	2	2
0	-1	-1	-1	-1	-1	2	2	2	2
0	-1	-1	-1	-1	-1	2	2	2	2
0.5	-1	1	-1	-1	-1	2	2	2	2
0.5	-1	2	-1	-1	-1	2	2	2	2
0.5	-1	2	-1	-1	-1	2	2	2	2
1	-1	1	-1	-1	-1	2	2	2	1
1	-1	1	-1	-1	-1	2	2	2	2
1	-1	1	-1	-1	-1	2	2	2	2
4	1	1	1	1	1	1	1	1	2
4	1	1	1	1	1	1	1	1	2
4	1	1	1	1	1	1	1	1	2
6	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1
12	1	1	1	1	2	1	1	1	1
12	1	1	1	1	2	1	1	1	1
16	1	1	1	1	1	1	1	1	1
16	1	1	1	1	2	1	1	1	1
16	1	1	1	1	2	1	1	1	1

S. sobrinus W1703 lawn preincubation duration (hr)	S. cristatus F0329	S. cristatus ATCC51100	S. cristatus A53	S. cristatus A54	S. oralis tigurinus	S. mutans UA159	S. mutans ATCC	S. sobrinus W1703	S. downei F0415
0	-1	2	-1	-1	-1	2	2	1	1
0	-1	2	-1	-1	-1	2	2	2	2
0	-1	2	-1	-1	-1	2	2	2	2
0.5	-1	1	-1	-1	-1	2	2	2	2
0.5	0	-1	-1	-1	-1	2	2	2	2
0.5	0	-1	-1	-1	-1	2	2	2	2
1	-1	2	-1	-1	-1	2	2	2	2
1	-1	2	-1	-1	-1	2	2	2	2
1	-1	2	-1	-1	-1	2	2	2	2
4	-1	-1	1	-1	-1	2	2	1	2
4	-1	-1	1	-1	-1	2	2	1	2
4	-1	-1	1	-1	-1	2	2	1	2
6	1	1	1	1	1	2	2	1	2
6	1	1	0	1	1	2	1	1	2
6	1	1	0	1	1	2	1	1	1
12	1	1	1	1	1	2	1	1	1
12	1	1	1	1	2	1	1	1	1
12	1	1	1	1	2	1	1	1	1
16	1	1	1	1	1	2	2	1	1
16	1	1	1	1	2	1	1	1	1
16	1	1	1	1	2	1	1	1	1

S. downei F0415 lawn preincubation duration (hr)	S. cristatus F0329	S. cristatus ATCC51100	S. cristatus A53	S. cristatus A54	S. oralis tigurinus	S. mutans UA159	S. mutans ATCC	S. sobrinus W1703	S. downei F0415
0	-1	2	-1	-1	-1	2	2	2	2
0	0	-1	0	0	-1	2	2	2	2
0	0	-1	0	0	-1	2	2	2	2
0.5	-1	-1	-1	-1	-1	2	2	2	2
0.5	-1	-1	-1	-1	-1	2	2	1	1
0.5	-1	-1	-1	-1	-1	2	2	2	2
1	0	2	0	-1	-1	2	2	2	2
1	-1	2	-1	-1	-1	2	2	1	2
1	-1	2	-1	-1	-1	2	2	1	2
4	1	1	1	-1	-1	1	2	1	1
4	1	1	1	-1	-1	1	2	1	1
4	1	1	1	-1	-1	1	2	1	1
6	1	1	1	1	1	2	2	1	1
6	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1
12	1	1	1	1	2	1	1	1	1
12	1	1	1	1	2	1	1	1	1
16	1	1	1	1	1	2	2	1	2
16	1	1	1	1	2	1	1	1	1
16	1	1	1	1	2	1	1	1	1

occurred frequently with Mitis *Streptococci* when *S. cristatus* ATCC51100 was the invader. An *S. oralis tigurinus* lawn, on the other hand, sparingly co-cultured both with members of the Mutans group, and *S. cristatus* ATCC51100.

Whether species tended not to co-culture by preventing the invading strain from establishing itself in the first place or through biochemical defenses, each of these interactions speaks towards a potential for QS-based mediation of cohabitation or competition. Those mentioned above which defied expectations (*S. cristatus* ATCC51100 and *S. oralis tigurinus*) draw particular interest in future experimentation. The importance of lawn preincubation time on invasion success also signals toward a time-based communication system, reminiscent of QS.

The halo seen in **Figure 7** indicates that some substance diffused beyond the area of direct cell-cell contact to cause inhibition. As such, liquid cultures—which are often used as bulk growth media—may allow for the increased production of inhibitory substances and present the chance to observe more dramatic interactions. Liquid cultures plated on solid media displayed nonreciprocal phenotypic change; specifically, invading (liquid) colonies of *S. cristatus* ATCC51100 could co-culture with lawns of other Mitis group members, whereas when *S. cristatus* ATCC51100 lawns were invaded by Mitis *Streptococci*, they inhibited *S. cristatus* ATCC51100's growth.

Table 5. Summary of invasion assay results. This table summarizes the competition assay results from **Tables 3 and 4**. The numbers represent the number of hours that the interaction (see figure legend) was visible before returning to 1: aliquot not visible. "N/A" represents crosses which maintained the "1" interaction regardless of the lawn's preincubation time.

	Invading strain									
Lawn strain	*S. cristatus* F0329	*S. cristatus* ATCC51100	*S. cristatus* A53	*S. cristatus* A54	*S. oralis tigurinus*	*S. mutans* UA159	*S. mutans* ATCC	*S. sobrinus* W1703	*S. downei* F4103	
S. cristatus F0329	0.5	0.5	N/A	N/A	0	0	1	0.5	0.5	Mitis group
S. cristatus ATCC51100	0.5	0.5	0.5	1	1	0.5	1	0.5	1	Mitis group
S. cristatus A53	N/A	12	N/A	0.5	12	1	4	1	0.5	Mitis group
S. cristatus A54	N/A	0	N/A	N/A	0.5	0.5	1	0.5	1	Mitis group
S. oralis tigurinus	N/A	0.5	N/A	0.5	0.5, 12-16	N/A	1	N/A	0	Mitis group
S. mutans UA159	1	1	1	4	4	1	0.5	0.5	1	Mutans group
S. mutans ATCC	1	0.5	1	1	1	1	1	1	4	Mutans group
S. sobrinus W1703	4	4	1	4	4	6	4	1	6	Mutans group
S. downei F4103	1	1	1	4	4	1	4	0.5	1	Mutans group
	Mitis group					Mutans group				

-1	Aliquot inhibits lawn
0	0: Neither aliquot nor lawn visible
1	Aliquot not visible
2	Both cultures grew
v	Variable patterns over time

This prompted the question of whether phenotypic changes could be observed in crosses of liquid cultures. The texture of the bacterial mat in liquid cultures is moderately differentiable; however, upon double-inoculation of liquid THY with competitive strains, visual differentiation was impossible for every cross, as expected. To circumvent that obstacle, well-diffusion assays

were performed with sterile-spent liquid media on lawns of the previously tested *Streptococci*—essentially undertaking a cell-free version of the invasion assay. None of the 9 tested lawns displayed any signs of inhibition, so variations to the procedure were necessary.

First, increasing the initial volume of the liquid culture to 5 mL was proposed to aid in the total amount of inhibitory substance produced; this yielded no change to the lawns. Next, incubating the 5 mL liquid cultures to early log phase (3 hours) could allow for the collection of inhibitory substance or QS signal prior to cellular reuptake; this yielded no change to the lawns. After filter sterilization of the Tween20 cell-wash, and of the initial supernatant, each was used in well-diffusion assays; neither yielded a change to the lawns. Depending on the concentration of Tween20 used, either no change or ubiquitous inhibition for each isolate's supernatant—including that of the THY control—was noted, meaning that inhibition was owed to the surfactant. Given such results, it seems likely that the export of inhibitory molecules to the supernatant either happens at such a small level that the concentration in the supernatant is too low to take effect, or the source of these inhibitory patterns lied elsewhere. Additional types of analysis such as insight into the genome could provide useful information.

Chapter 3: Genome analysis to aid in QS circuitry detection, and progress towards characterizing the ComRS in *S. sobrinus* W1703

Introduction

Having exhausted most likely/accessible tests of direct inhibition by the supernatant, I decided to turn to genetic analysis to identify quorum sensing genes to direct further work. Since it appeared that direct cell-cell contact was necessary to observe inhibitory patterns in these isolates, which was not conducive to manipulation using the supernatant, more advanced means of analysis became necessary. Unfortunately, the myriad species and vast genetic diversity between members of the same species of the *Streptococcus* genus translates to a low likelihood of finding a particular strain's genome, or one that's "close enough" on NCBI. This problem came up once before in the identification process beginning Chapter 2. To invest more time and attention to fewer systems, I narrowed my focus to three strains' genomes: *S. sobrinus* W1703, *S. downei* F0415, and *S. oralis tigurinus*. These species and strains stand on the bleeding edge of *Streptococcal* study, in that published genomes were either in fragments, unannotated, both, or wholly missing from NCBI. Accordingly, related model genomes were used to find prospective genes for amplification, and with any luck, primer homology between the model and the strain of interest should allow for Sanger sequencing to spell out sequences from the strain of interest.

In May 2021, after I had identified primer candidates for *comC* from the genome of *S. sobrinus* NIDR6715-7, Li, et al. published an insightful paper Department of Bioengineering, University of Illinois at Urbana-Champaign concerning several *S. sobrinus* strains and their unique ComRS qualities (**Figure 8**). Fortunately, the work they published facilitated the identification of XIP and other genes in the cluster for *S. sobrinus* W1703. One distinctly encouraging feature in the search for *comRS* genes in *S. sobrinus* W1703, *comRS* seems to be extremely diverse among species, and even within strains of the same species.

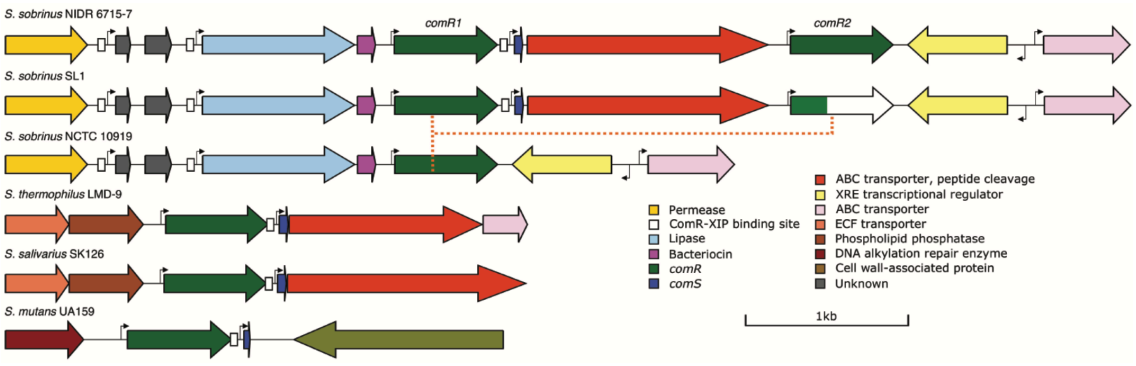

Figure 8. *S. sobrinus* ComRS cluster compared to other *Streptococci*.[23] This figure from Li, *et al*. compares the ComRS gene cluster in several *Streptococcal* species. The gene cluster in *S. sobrinus* appears more complex than in related species, possibly due to the apparent lack of ComABCDE-based activation of *comX*.

Although the export and import mechanisms are unknown, the presence of three ABC transporters under the control of the same modulator system (**Figure 8**) suggests that they may still play a role in ComRS, since transporter proteins comprise part of the *comRS* gene cluster. Alternatively, the production of bacteriocin also brings up the idea of predation and/or defensive behavior. Following a similar narrative as that which explains *S. pneumoniae*'s bacteriocin gene in the competence regulon, competence paired with bacteriocin production kills surrounding bacteria and enables the aggressor to undergo horizontal gene transfer. Li, et al. also point out the two variations of ComR, one of which, ComR1, inhibits ComRS signal transmission, and one of which, ComR2, activates ComRS signal transmission.[23] The presence of the same pair of ComR genes in *S. sobrinus* W1703 prompts the question of functionality for this strain.

Ultimately genome analysis should hopefully reveal potential QS circuitry and/or bacteriocins. Normally, that would settle the matter, but the problem is that native XIP has never been isolated from *S. sobrinus*, so the exact structure and mass are unknown.[23] Previous studies

have shown that the most activity is generated using XIP made of the last 6-9 *C*-terminus residues, but that in *S. sobrinus*, 7 AAs tends to work well, so this length will be a synthetic target and the basis for isolating the native peptide. Even with these complications, genome analysis, peptide synthesis, and native protein extraction offer progress on the characterization of the system, and (through competence) can even lead to the development of more informative models such as reporter strains.

Experimental Section

Unipro's UGENE program assisted in the initial visualization and analysis of genomes. Annotated genomes were compared to the published, unannotated shotgun sequences for *S. sobrinus* W1703, *S. downei* F0415, and *S. oralis tigurinus*. Workflows created on UGENE combined genome and annotation files from NCBI to allow for browsing annotations or gene segments as necessary to find similar sequences in related species. After searching based on full-genome similarity, I found that searching for individual genes was more helpful in finding higher sequence-similarity.

After I had identified *S. sobrinus* NIDR6715-7 as a model genome for *S. sobrinus* W1703, Li, et al. published their paper on ComRS in *S. sobrinus*. Their published ComR-XIP promotor binding site sequence of *comX* and *comS* in *S. sobrinus* SL1 helped to locate homologous sites in *S. sobrinus* W1703. Next, the ComRS gene cluster in *S. sobrinus* NIDR6715-7 acted as a scaffold for the identification of the remaining components of *S. sobrinus* W1703's ComRS. This allowed for the determination of primer targets, particularly the genes for ComS and bacteriocin.

Having finally identified key sequences for primer targets in each strain, primers were designed and ordered from Integrated DNA Technologies (IDT). See **Table 6** for more information on primer specifications. Samples were prepared for sequencing (same as *rpoB* procedure) and sent to the NGS at UNR for Sanger sequencing with the ABI Prism 3730 DNA Analyzer. Once

sequences had been identified (**Table 7** on page 32), ChemDraw version 20.1 was used to calculate the exact masses (**Table 8** on page 33) of peptide targets to monitor during matrix-assisted laser desorption/ionization coupled with time-of-flight (MALDI/TOF) spectrometry. These include XIP (6, 7, 8, and 9 AAs), ComS, and bacteriocin from *S. sobrinus*, and the potential CSP from *S. downei*.

Table 6. Primer information for amplification of potential QS circuitry. The following primers were designed to target QS genes, including *comC*, *comS*, and bacteriocins using homologous annotated segments identified in model strains, either by referring back to the published shotgun sequence (*S. downei* F0415 and *S. sobrinus* W1703), or by using segments directly from the model genome (*S. oralis tigurinus* osk-001 and *S. sobrinus* NIDR6715-7).

Species	Gene	Sense	Amplicon Name	Primer Length (bp)	Amplicon length	Primer Sequence
S. downei F0415	*comC*	5'	SDF-C-5	27	529	TGGTATAAATGCTT TGATTTCTTCTCG
S. downei F0415	*comC*	3'	SDF-C-3	26	529	CCCAGACTAACAC TCAACCATACTGG
S. oralis tigurinus osk-001	*comC*	5'	SOT-C-5	25	679	CGCCTTCTAAGCG AACGGTCGCAGG
S. oralis tigurinus osk-001	*comC*	3'	SOT-C-3	24	679	CATCACCTGTTATC GAGGATAGGG
S. sobrinus W1703	*comS*	5'	SSW-S-5	26	617	GCTCAACTCCCCAC CTACAAGGTAGG
S. sobrinus W1703	*comS*	3'	SSW-S-3	25	617	GGGCCGCCCTTATC AAGCCCAAGGC
S. sobrinus W1703	*bacteriocin*	5'	SSW-B-5	26	820	GCCATCAAATAGG GAAGAGGCGAAGC
S. sobrinus W1703	*bacteriocin*	3'	SSW-B-3	26	820	CCGGTAATTGGAT ATCCCTACCTTCC
S. sobrinus NIDR6715-7	*comS*	5'	SSN-S-5	25	535	GGGAGATATTCTA GATGATCCCACC
S. sobrinus NIDR6715-7	*comS*	3'	SSN-S-3	23	535	CGGATAGCCTTGG TTTTAAGTCC

Solid phase peptide synthesis was carried out using a Wang resin base to make *S. sobrinus* W1703's 7-AA XIP using the last 7 residues from ComS. Since solid phase peptide synthesis occurs in the *C*-to-*N* direction, contrary to biological peptide synthesis, the Wang resin was selected based on the last residue in the peptide. Amino acids were prepared in 2.5 mL dimethylformamide (DMF), and a solution of 1 mL HBTU in 10 mL DMF was added to each. After swelling the resin

for 30-45 minutes in DMF, the first amino acid (already attached to the resin) was deprotected, meaning the Fmoc protecting group which shielded the reactive amine end of the growing peptide was removed, freeing the *N*-terminus up for chain elongation. The mechanism for Fmoc deprotection using 20% piperdine as a strong base and DMF as an electron-donor solvent is shown in **Figure 9**. Initial deprotection was repeated to maximize yield, and the resin was washed 3-4 times with DMF after each deprotection.

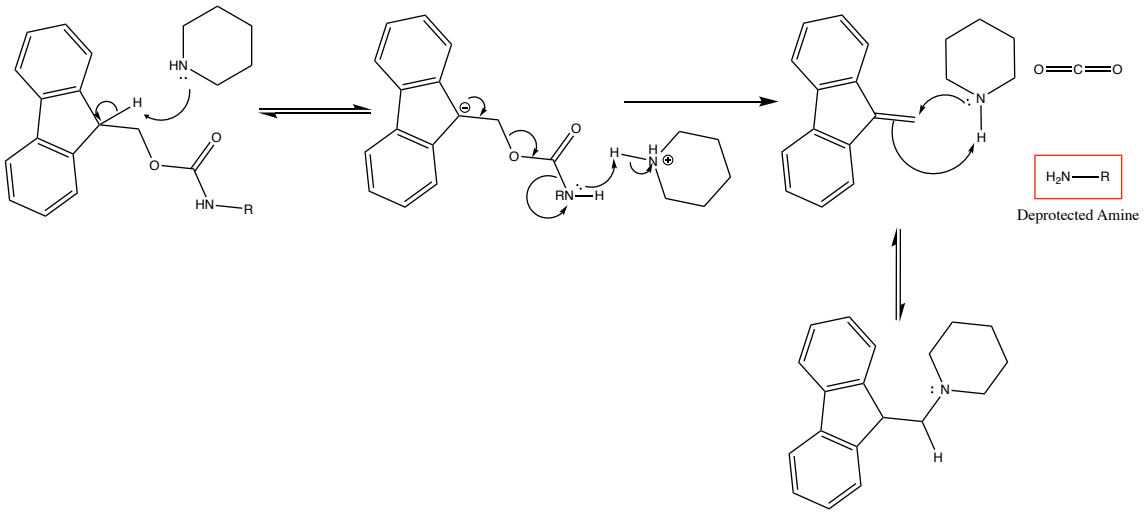

Figure 9. Solid phase synthesis: Fmoc deprotection mechanism. During solid phase peptide synthesis, Fmoc deprotection of the oncoming amino acid's *C*-terminus is accomplished using 20% piperdine in DMF.

As previously mentioned, solid phase peptide synthesis occurs in the *C*-to-*N* direction, meaning that the order amino acid addition is the reverse sequence of the peptide being synthesized. The next AA in the sequence was mixed with 0.5 mL *N,N*-diisopropylethylamine (DiPEA) and added to the resin, and coupling took place in a microwave synthesizer. The mechanism for coupling using hexafluorophosphate benzotriazole tetramethyl uronium (HBTU) as a coupling agent and *N,N*-diisopropylethylamine as a base can be seen in **Figure 10**. Deprotection and coupling were repeated until the final residue was added, then after the final deprotection, cleavage

from the resin was accomplished by incubating the resin in 3 mL of a solution containing 2.5% triisopropylsilane (cation scavenger), 2.5% water, and 95% TFA for 3-4 hours with shaking. A filter made of a syringe and cotton plug separated the solvated product from the solid resin. A 50:50 ether:hexane mixture at -20ºC was used to precipitate the peptide, and after 10-15 minutes of centrifugation, the precipitate was dissolved in 50:50 ACN:H_2O, frozen in the -80ºC freezer, and lyophilized for 24 hours. After resuspension in 5% ACN and filtration through a 0.22 micron filter, the sample was run through the High pressure liquid chromatography (HPLC), and fractions were analyzed on MALDI/TOF. No peaks at the expected mass were detected, so repeating the synthesis will be the next step in the process.

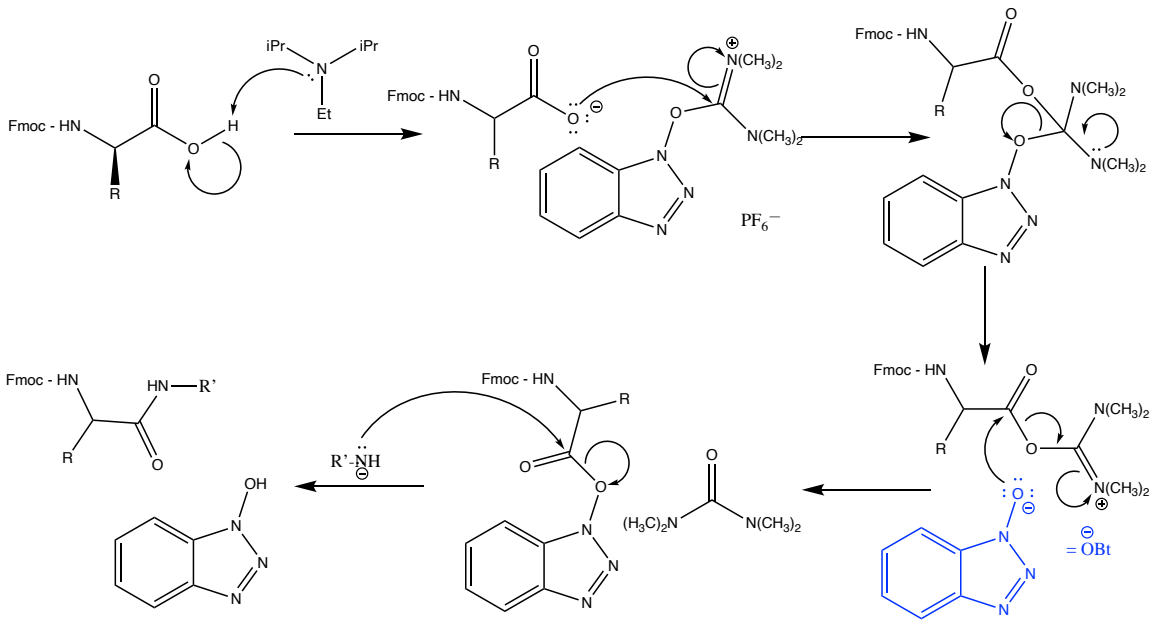

Figure 10. Solid phase synthesis: DiPEA/HBTU coupling mechanism. During solid phase peptide synthesis, addition of the oncoming amino acid is accomplished using DiPEA and HBTU.

To isolate XIP and/or bacteriocins from a liquid culture of *S. sobrinus* W1703, as well as CSP from *S. downei* F0415, protein extraction was accomplished using a cold acetone crash. A 1:100 THY dilution of liquid culture was grown to ODs of 0.2, 0.3, and 0.5, and 3-4 volumes of -20ºC acetone were used to precipitate soluble proteins out of solution. Next, the solid was collected using centrifugation, resuspended in 50:50 ACN:H_2O, frozen in the -80ªC freezer, and lyophilized to collect the solid. These extracts were then separated using HPLC and analysis was performed on MALDI/TOF, but neither of the protein extracts indicated the presence of XIP (and related peptides), bacteriocin, or CSP. This result suggests that a different method for protein extraction, perhaps through ammonium sulfate precipitation, may yield better results.

Results/Discussion

To avoid using unannotated shotgun gene sequences, model systems were sought out. Considerable genome homology was found between *S. sobrinus* NIDR6715-7 and W1703; to a lesser extent, *S. downei* F0415 seemed to be related to *S. downei* MFe28 (although several Mutans group bacteria were very similar). *S. sobrinus* NIDR6715-7 possesses the same genetic components in the ComRS gene cluster as *S. sobrinus* W1703, including those for permease, lipase, bacteriocin, two ComR's, ComS, an XRE transcriptional regulator, and three ABC transporters, as seen in **Figure 8**. The absence of published genes for comparison to annotated sequences presents difficulty to even begin assessing the genome's potential as a model for *S. oralis tigurinus*, with no clear close genetic relations, so it was decided that the highest homology "*oralis tigurinus*" strain would be used as a model.

A point of clarification: although "*tigurinus*" is part of a subspecies designation, related subspecies can also be coined "*tigurinus*" with another designation after, but that does not always reflect a high degree of genome similarity. For instance, *S. oralis tigurinus* osk-001 uses ComABCDE, while *S. oralis* NCTC11427 uses ComRS. Given the futility of finding a match for

the unpublished and unknown genome of *S. oralis tigurinus*, and knowing that ComABCDE is the best-studied QS system in *Streptococci*, it seems the most likely to be present and detectable from my position. Hence, I chose *S. oralis tigurinus* osk-001 as a potential model for QS genes in *S. oralis tigurinus*.

Ultimately, the aim was to design primers using model genomes to then amplify with PCR and sequence genes of the organism at hand. For perspective, the genome of *S. sobrinus* NIDR6715-7 contains 2.15 million base pairs, and over 2000 genes—this would be impossible to effectively dig through without annotations, which are typically automatically generated by a computer. Homology relieves some of the burden of genome analysis by using sequence similarity to characterize unannotated segments, but populational genome diversity hinders what otherwise would have been as straightforward process as a computer's "find" function.

Several steps closer to the "find" function, annotation allows for searches of key terms such as "ABC transporter", "ComC", and "transcriptional regulator", although many are labeled vague terms such as "protein coding". Following extensive genome searches on UGENE, annotated models, *S. downei* MFe28 revealed 3 prospective ABC transporter a ComC genes. Typically translated ComC comprises a leader sequence, a double-glycine motif to direct cleavage, and a 15-18-AA CSP, whereas *S. downei* MFe28's CSP is still 24-25-AA post-cleavage. This means that either the gene is not truly *comC*, or the CSP it produces may be dysfunctional in the model; however, if the primer can still bind, the sequence will still translate, although the rest of the translated peptide may be dissimilar in size and/or composition to the model. Options for identifying comC were narrowed to one candidate based on the identities of surrounding genes. Antithetically, the clearly labeled, singular ComC annotation in *S. oralis tigurinus* osk-001's made for an easy primer target.

Upon arrival of the primers, PCR was used (same procedure as before) to amplify *comC* in *S. oralis tigurinus* and *S. downei* F0415, as well as bacteriocin and *comS* in *S. sobrinus* W1703.

Gel electrophoresis (**Figure 11**) revealed successful production of approximately correct-sized amplicons during PCR. The results of Sanger sequencing are displayed on **Table 7**. As expected with primers made from strain stand-ins, *S. oralis tigurinus* osk-001's primer did not work for sequencing in *S. oralis tigurinus*. However, the 5' *comC* primer from *S. downei* MFe28 resulted in a sequence from *S. downei* F0415 partially like that of the model, but which differed at the last 14 (model) to 18 (F0415) amino acids. The 5' and 3' *comS* primers from *S. sobrinus* W1703 both returned the same sequence for *comS*. Slight sequence disparities between the model genome and that of *S. sobrinus* W1703 prompted me to test *comS* primers from *S. sobrinus* NIDR6715-7 with the W1703 genome to probe for parallel ComRS circuitry, but these yielded the same results as the primers from W1703. Finally, although the 5' bacteriocin primer from *S. sobrinus* W1703 yielded incomplete sequence reads, the 3' primer resulted in almost the same sequence, with the first AA

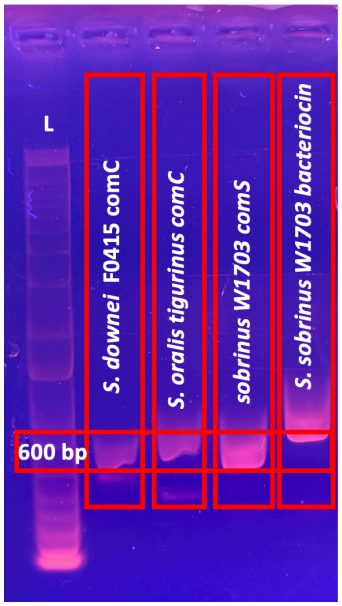

Figure 11. Gel electrophoresis of potential QS genes. Gel electrophoresis of PCR amplicons for various QS gene revealed bands around 600, base pairs, indicating the correct expected mass for the amplicon, allowing for sequencing to proceed.

at the *N*-terminus and the 4th-to-last AA from the *C*-terminus being the only differences. These results, while not encouraging for *S. downei* F0415 or *S. oralis tigurinus*, provide strong support to delve further into the biochemistry of *sobrinus* ComRS circuitry.

Table 7. QS molecule Sanger sequencing results. The following results were sent from NGS at UNR following Sanger sequencing with the ABI Prism 3730 DNA Analyzer. Failed sequencing attempts (labeled "N/A") are likely due to insufficient primer binding affinity to respective target sequences, but even so, the sequence for *S. downei* F0415's CSP, as well as those for *S. sobrinus* W1703's ComS and bacteriocin were revealed. Sequences highlighted in yellow represent differences between model and actual genomes; the sequence highlighted in blue represent the 7-AA XIP found in *S. sobrinus* W1703

Species	Name	Primer Sequence	Amplicon length	Reference Translated Peptide	Translated Sequenced Peptide of Interest
S. downei F0415	*comC* 5'	TGGTATAAATGCT TTGATTTCTTCTCG	497	MKFNFTNYENLSTKELSF IQGGGWRQELLNFLIETS MPIGWNWHQKK-	MKFNFTNYENLSTKELSF IQGGGWRQELLNFLIGD QYANWLELASKKVNQI
S. downei F0415	*comC* 3'	CCCAGACTAACAC TCAACCATACTGG	472	MKFNFTNYENLSTKELSF IQGGGWRQELLNFLIETS MPIGWNWHQKK-	N/A
S. oralis tigurinus	*comC* 5'	CGCCTTCTAAGCG AACGGTCGCAGG	195	MKNTVKLEQFKEVTEAE LQEIRGGEIRKENNFLFY FFKRK-	N/A
S. oralis tigurinus	*comC* 3'	CATCACCTGTTATC GAGGATAGGG	332	MKNTVKLEQFKEVTEAE LQEIRGGEIRKENNFLFY FFKRK-	N/A
S. sobrinus W1703	*comS* 5'	GCTCAACTCCCCA CCTACAAGGTAGG	609	MNLKKIIELAITLVTLVC TIAR	MNLKKIIELAITLVTLVC TIAR
S. sobrinus W1703	*comS* 3'	GGGCCGCCCTTAT CAAGCCCAAGGC	583	MNLKKIIELAITLVTLVC TIAR	MNLKKIIELAITLVTLVC TIAR
S. sobrinus W1703	*bacteriocin* 5'	GCCATCAAATAGG GAAGAGGCGAAGC	199	MKTKELLFSELNEEDLA AIRGGSLWDFKGIIIGDP WWYPRIGIPEHPILLDK-	N/A
S. sobrinus W1703	*bacteriocin* 3'	CCGGTAATTGGAT ATCCCTACCTTCC	794	MKTKELLFSELNEEDLA AIRGGSLWDFKGIIIGDP WWYPRIGIPEHPILLDK-	KKTKELLFSELNEEDLAA IRGGSLWDFKGIIIGDPW WYPRIGIPEHPIFLDK
S. sobrinus NIDR6715-7	*comS* 5'	GGGAGATATTCTA GATGATCCCACC	535	MNLKKIIELAITLVALMC TIAR	MNLKKIIELAITLVTLVC TIAR
S. sobrinus NIDR6715-7	*comS* 3'	CGGATAGCCTTGG TTTTAAGTCC	802	MNLKKIIELAITLVALMC TIAR	MNLKKIIELAITLVTLVC TIAR

From here, testing the phenotypic effects of bacteriocin, XIP, and even CSP would only be possible through one of two means: peptide synthesis, or isolation of native QS peptides from protein extracts. HPLC (size/structure/mass-based reverse-phase chromatography) would assist in the purification of the peptides, and a MALDI/TOF spectrometry would allow for identification on the basis of mass. Exact masses for detection on MALDI/TOF are shown below (**Table 8**).

Table 8. Exact mass and molecular weights of peptide targets in MALDI/TOF. Exact masses were determined using ChemDraw version 20.1. These masses were determined to indicate what to look for in MALDI/TOF analysis of synthetic peptides and native protein extracts (after HPLC). Extended and truncated versions of XIP are also considered, since functional XIP has not been isolated from *S. sobrinus*.

Peptide	1-letter-AA-code	Exact Mass (Da)	Molecular Weight (Da)
W1703 ComS	MNLKKIIELAITLVTLVCTIAR	2455.46	2457.12
W1703 XIP-9 AA	VTLVCTIAR	974.56	975.22
W1703 XIP-8 AA	TLVCTIAR	875.49	876.09
W1703 XIP-7 AA	LVCTIAR	774.44	774.98
W1703 XIP-6 AA	VCTIAR	661.36	661.82
W1703 bacteriocin	MKTKELLFSELNEEDLAAIRGGSLW DFKGIIIGDPWWYPRIGIPEHPILLDK	6019.18	6023.02
downei F0415 CSP	GWRQELLNFLIETSMPIGWNWHQKK	3110.59	3112.61

To probe the ComRS circuitry of *S. sobrinus* W1703, synthetic 7-AA XIP was synthesized (**Figures 9 and 10**) with hopes of preferably inducing competence, an ability that until last year was unheard of in *S. sobrinus*. However, synthesis of the 7-mer was unsuccessful thus far. In addition to obtaining XIP through synthesis, native protein extracts also offer potential to isolate the AIP. However, there exist some hurdles to native protein extraction, including that XIP production is time-dependent in a currently unknown fashion, so time-based extractions are necessary. Also, as previously discussed, although 7 AAs seems to generally yield the best results,

XIP with other lengths (anywhere from 6 to 9 AA) are also possible candidates for the structure of biologically produced XIP. Considering these factors, a wide net can be cast to isolate native XIP.

To further the study and use *S. sobrinus* W1703, HPLC to separate peptides and MALDI/TOF for analysis on a larger starting mass of synthetic XIP lengths could aid in transformations and the construction of reporter strains. Furthermore, alternative extraction methods of natively produced peptides would offer great assistance. The isolation of XIP has potential to unlock study into a previously obscure species of *Streptococcus*. In addition to clinical relevance as a human pathogen, the study of this system lends itself to a broader understanding of QS systems as a whole, and the treatment of other similarly cryptic and muddled bacterial systems.

Chapter 4: Discussion/Conclusion

In the modern age of antibiotic resistance, medical science fields continue to develop novel methods of identification, classification, experimentation, and treatment of a quickly (relatively speaking) evolving microbial threat. Untargeted, improper, and unfollowed treatments in the past have increased selective pressure both in our human microbiomes, but also in the environment, resulting in an increase in the number of individuals suffering infections that are difficult or impossible to pull through. Various means of horizontal gene transfer accelerate and accentuate the effects of evolutionary pressure, but some bacteria such as many members of the *Streptococcus* genus take this a step further through the use of QS-based group behavior to hunt neighboring microbes and uptake their genetic material using the same QS signal.

Gram-negative bacteria generally use *lux* homologues, depending on a small molecule AHL autoinducer. Likely due to structural differences in cell wall and membrane, Gram-positive bacteria typically use AIPs and transmembrane histone kinases for signal transduction. One exception to this is the ComRS system, which uses aspects of both LuxI/LuxR (import of the signal molecule) and ComABCDE (peptide signals and *comX* activation). Even with a growing body of study, components of most QS circuitry remain unknown, with the most modern understanding of QS originating in AHL-based systems, followed by ComABCDE, and finally ComRS. Thankfully, the molecular biology of QS bacteria often itself is an extremely useful tool in exploring the functionality of cellular communication, for example, by inducing competence and allowing for the use of molecular (generally DNA or protein) probes and the development of reporter strains for further study.

Some organisms depend on multiple lines of QS running in tandem or separately, dictating biochemical changes based on microbial and physical conditions. Furthermore, interspecies and even interkingdom quorum quenching has come to light, hinting at therapeutic potential to commandeer these defensive strategies. Diverging, previously homologous cellular machinery

increases the chance of competitive interactions such as receptor agonism or antagonism due to structural similarity.

Accordingly, interspecies interactions among *Streptococci* can reveal interesting relationships which have a higher likelihood (considering the system being studied) of involving QS. Impressive genus diversity—especially considering the frequently high degree of homology between seemingly distantly related individuals—and a lack of available quality publicly available genomes for interpretation impedes this process. Two such species that have slipped through the cracks until observing their interactions are *S. oralis tigurinus* and *S. cristatus* ATCC51100, which flagged interest in the invasion assays. However, sequence homology also aids in the characterization of related strains' circuitry. Beyond sequence homology, the observation of undisturbed growth trends establishes a baseline for assessing the effects of experimental conditions and can be recorded in the form of CFU counts and growth curves.

Well-diffusion assays using cellular supernatants at various points in their growth pointed away from the hypothesis that inhibitory molecules are being freely secreted to the supernatant media. Well-diffusion assays of surfactant-treated supernatant and cells separately (to release cell-bound molecules) also indicated a lack of excreted inhibitory molecules, which led to the need to employ genetic analysis to uncover more potential explanations for the observed interactions during invasion assays.

Species and sequence homology assisted in the characterization of the *S. sobrinus* ComRS circuitry. This species recently came into the spotlight for its atypical XIP, possessing no aromatic residues which are standard in other species, and recent demonstration of induced competence by XIP stimulation. Further study into the structural aspects of *sobrinus*' can shed more light as to the key chemical features involved in signal transmission. Both total native protein extraction and *de-novo* peptide synthesis allow for the isolation of XIP for phenotypic assays to probe for behaviors such as biofilm formation, hemolysis, bacteriocin or hydrogen peroxide production, and especially

competence. Since XIP has never been isolated from *S. sobrinus*, the exact structure is uncertain, and identification may be difficult due to the obscure nature of cellular export and import and the potential for post-translational modifications of the AIP.

As is typically the case in science, this work has resulted in both questions and answers. The curious existence of both ComR1 (inhibitor) and the ComR2 (activator) in *S. sobrinus* warrants investigation as auto-regulatory mechanisms; although hypothetical mechanisms of function have been proposed, the action and purpose of ComR1 remains elusive. Additionally, the transport system of AIPs in ComRS represents a considerable gap in understanding. Future work should involve the elucidation of the structures and functions of cellular machinery involving ComRS. Phenotypic assays can use baseline growth patterns to note biochemical triggers. Eventually, SAR assays such as alanine screens and truncation assays using ComS, XIP, ComR1, and ComR2 should assist in identifying key structural features.

Unconventional routes of combatting microbial pathogens such as this offer the benefit of being highly targeted and frequently non-bacteriocidal, reducing the potential for selective sweeps and reducing the contribution to antibiotic resistance. Ultimately, a deeper knowledge of strain-specific quorum sensing systems and familiarity with the topic as a whole will lead to the production of probiotic (maybe in the form of a bacteriocin) treatments which can be therapeutically employed to treat tenacious infections and promote better populational health overall.

Conflicts of Interest

There are no conflicts of interest to declare.

Notes and References

1. Aslam, B.; Wang, W.; Arshad, M. I.; Khurshid, M.; Muzammil, S.; Rasool, M. H.; Nisar, M. A.; Alvi, R. F.; Aslam, M. A.; Qamar, M. U.; et al. Antibiotic resistance: a rundown of a global crisis. *Infection and Drug Resistance* **2018**, *11*, 1645-1658, Review. DOI: 10.2147/idr.s173867.

2. Brown, E. D.; Wright, G. D. Antibacterial drug discovery in the resistance era. *Nature* **2016**, *529* (7586), 336-343, Review. DOI: 10.1038/nature17042.

3. Jubeh, B.; Breijyeh, Z.; Karaman, R. Resistance of Gram-Positive Bacteria to Current Antibacterial Agents and Overcoming Approaches. *Molecules* **2020**, *25* (12). DOI: 10.3390/molecules25122888.

4. Blaser, M. J. Antibiotic use and its consequences for the normal microbiome. *Science* **2016**, *352* (6285), 544-545, Editorial Material. DOI: 10.1126/science.aad9358.

5. Griffith, F. THE SIGNIFICANCE OF PNEUMOCOCCAL TYPES. *Reviews of Infectious Diseases* **1981**, *3* (2), 372-395.

6. Miller, M. B.; Bassler, B. L. Quorum sensing in bacteria. *Annual Review of Microbiology* **2001**, *55*, 165-199, Review. DOI: 10.1146/annurev.micro.55.1.165.

7. Waters, C. M.; Bassler, B. L. Quorum sensing: Cell-to-cell communication in bacteria. In *Annual Review of Cell and Developmental Biology*, Annual Review of Cell and Developmental Biology, Vol. 21; Annual Reviews, 2005; pp 319-346.

8. Bose, S.; Aggarwal, S.; Singh, D. V.; Acharya, N. Extracellular vesicles: An emerging platform in gram-positive bacteria. *Microbial Cell* **2020**, *7* (12), 312-322, Review. DOI: 10.15698/mic2020.12.737.

9. Mai-Prochnow, A.; Clauson, M.; Hong, J. M.; Murphy, A. B. Gram positive and Gram negative bacteria differ in their sensitivity to cold plasma. *Scientific Reports* **2016**, *6*, 11, Article. DOI: 10.1038/srep38610.

10. Hossain, M. S.; Biswas, I. An Extracelluar Protease, SepM, Generates Functional Competence-Stimulating Peptide in Streptococcus mutans UA159. *Journal of Bacteriology* **2012**, *194* (21), 5886-5896, Article. DOI: 10.1128/jb.01381-12.

11. Dong, G. F.; Tian, X. L.; Cyr, K.; Liu, T. L.; Lin, W.; Tziolas, G.; Li, Y. H. Membrane Topology and Structural Insights into the Peptide Pheromone Receptor ComD, A Quorum-

Sensing Histidine Protein Kinase of Streptococcus mutans. *Scientific Reports* **2016**, *6*, 12, Article. DOI: 10.1038/srep26502.

12. Iannelli, F.; Oggioni, M. R.; Pozzi, G. Sensor domain of histidine kinase ComD confers competence pherotype specificity in Streptoccoccus pneumoniae. *Fems Microbiology Letters* **2005**, *252* (2), 321-326, Article. DOI: 10.1016/j.femsle.2005.09.008.

13. Talagas, A.; Fontaine, L.; Ledesma-Garca, L.; Mignolet, J.; de la Sierra-Gallay, I. L.; Lazar, N.; Aumont-Nicaise, M.; Federle, M. J.; Prehna, G.; Hols, P.; et al. Structural Insights into Streptococcal Competence Regulation by the Cell-to-Cell Communication System ComRS. *Plos Pathogens* **2016**, *12* (12), 26, Article. DOI: 10.1371/journal.ppat.1005980.

14. Underhill, S. A. M.; Shields, R. C.; Kaspar, J. R.; Haider, M.; Burne, R. A.; Hagen, S. J. Intracellular Signaling by the comRS System in Streptococcus mutans Genetic Competence (vol 3, e00444-18, 2018). *Msphere* **2019**, *4* (1), 1, Correction. DOI: 10.1128/mSphere.00042-19.

15. Fontaine, L.; Wahl, A.; Flechard, M.; Mignolet, J.; Hols, P. Regulation of competence for natural transformation in streptococci. *Infection Genetics and Evolution* **2015**, *33*, 343-360, Article. DOI: 10.1016/j.meegid.2014.09.010.

16. Son, M. J.; Ahn, S. J.; Guo, Q.; Burne, R. A.; Hagen, S. J. Microfluidic study of competence regulation in Streptococcus mutans: environmental inputs modulate bimodal and unimodal expression of comX. *Molecular Microbiology* **2012**, *86* (2), 258-272. DOI: 10.1111/j.1365-2958.2012.08187.x.

17. Weiser, J. N.; Ferreira, D. M.; Paton, J. C. Streptococcus pneumoniae: transmission, colonization and invasion. *Nature Reviews Microbiology* **2018**, *16* (6), 354-367, Review. DOI: 10.1038/s41579-018-0001-8.
18. Yoo, S. Y.; Kim, K. J.; Lim, S. H.; Kim, K. W.; Hwang, H. K.; Min, B. M.; Choe, S. J.; Kook, J. K. First isolation of Streptococcus downei from human dental plaques. *Fems Microbiology Letters* **2005**, *249* (2), 323-326, Article. DOI: 10.1016/j.femsle.2005.06.020.

19. Abranches, J.; Zeng, L.; Kajfasz, J. K.; Palmer, S.; Chakraborty, B.; Wen, Z. Z.; Richards, V. P.; Brady, L. J.; Lemos, J. A. Biology of Oral Streptococci. *Microbiology Spectrum* **2018**, *6* (5), 12, Article. DOI: 10.1128/microbiolspec.GPP3-0042-2018.

20. Lella, M.; Tal-Gan, Y. Strategies to attenuate the competence regulon in Streptococcus pneumoniae. *Peptide Science* **2021**, *113* (4), 11, Review. DOI: 10.1002/pep2.24222.

21. Mull, R. W.; Tal-Gan, Y. Elucidating the Role and Structure-Activity Relationships of the Streptococcus oligofermentans Competence-Stimulating Peptide. *Acs Chemical Biology* **2021**, *16* (12), 2834-2844, Article. DOI: 10.1021/acschembio.1c00746.

22. Drancourt, M.; Roux, V.; Fournier, P. E.; Raoult, D. rpoB gene sequence-based identification of aerobic gram-positive cocci of the genera Streptococcus, Enterococcus, Gemella, Abiotrophia, and Granulicatella. *Journal of Clinical Microbiology* **2004**, *42* (2), 497-504, Article. DOI: 10.1128/jcm.42.2.497-504.2004.

23. Li, J. W.; Wyllie, R. M.; Jensen, P. A. A Novel Competence Pathway in the Oral Pathogen Streptococcus sobrinus. *Journal of Dental Research* **2021**, *100* (5), 542-548, Article. DOI: 10.1177/0022034520979150.

24. Mashima, I.; Nakazawa, F. Role of an autoinducer-2-like molecule from Veillonella tobetsuensis in Streptococcus gordonii biofilm formation. *Journal of Oral Biosciences* **2017**, *59* (3), 152-156, Article. DOI: 10.1016/j.job.2017.06.002.