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A Novel Competence Pathway in the Oral Pathogen *Streptococcus sobrinus*

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Abstract

Streptococcus sobrinus is an etiologic cause of dental caries (tooth decay) in humans. Our knowledge of *S. sobrinus* is scant despite the organism's important role in oral health. It is widely believed that *S. sobrinus* lacks the natural competence pathways that are used by other streptococci to regulate growth, virulence, and quorum sensing. The lack of natural competence has also prevented genetic manipulation of *S. sobrinus*, limiting our knowledge of its pathogenicity. We discovered that most strains of *S. sobrinus* contain a new class of the ComRS competence system. Although *S. sobrinus* is typically placed among the mutans group streptococci, the *S. sobrinus* ComRS system is most similar to the competence pathways in the salivarius group. Unlike all other ComRS systems, the *S. sobrinus* pathway contains 2 copies of the transcriptional regulator ComR and has a peptide pheromone (XIP) that lacks any aromatic amino acids. Synthetic XIP enables transformation of *S. sobrinus* with plasmid or linear DNA, and we leverage this newfound genetic tractability to confirm that only 1 of the ComR homologs is required for induced competence while the other appears to suppress competence. Exogenous XIP increases the expression of bacteriocin gene clusters and produces an antimicrobial response that inhibits growth of *S. mutans*. We also identified 2 strains of *S. sobrinus* that appear to be "cheaters" by either not responding to or not producing XIP. We show how a recombination event in the nonresponsive strain could restore function of the ComRS pathway but delete the gene encoding XIP. Thus, the *S. sobrinus* ComRS pathway provides new tools for studying this pathogen and offers a lens into the evolution of ecological cheaters.

Keywords: dental caries, genetics, microbiology, streptococcus, pheromones, quorum sensing

Introduction

Dental caries results from acid fermentation by bacteria, most commonly the oral pathogens *Streptococcus mutans* and *Streptococcus sobrinus*. Although *S. sobrinus* is rarer than *S. mutans*, studies show that *S. sobrinus* is more strongly associated with the development of caries, especially in children (Hirose et al. 1993; Nurelhuda et al. 2010; Gross et al. 2012; Singla et al. 2016). Coinfection with *S. sobrinus* and *S. mutans* is associated with greater incidence or severity of caries (Seki et al. 2006; Kanasi et al. 2010; Okada et al. 2012), suggesting that *S. sobrinus* and *S. mutans* may interact by an unknown mechanism to exacerbate the disease. Such an interaction is consistent with the ecological plaque hypothesis (Takahashi and Nyvad 2008; Philip et al. 2018), a viewpoint that caries results from complex interactions among microbes, the host, and the environment.

Streptococci use peptide pheromones for intra- and intercellular communication. The pheromones regulate competence pathways that innervate metabolism (Underhill et al. 2019), virulence (Koirala et al. 2018; Lin and Lau 2019), quorum sensing (Shanker and Federle 2017), and antibiotic tolerance (Slager et al. 2014). However, no peptide pheromones or functional competence pathways have been discovered in *S. sobrinus*. The apparent lack of competence pathways raises questions about how *S. sobrinus* interacts with *S. mutans* and other microbes during cariogenesis.

Avery et al. first discovered the natural competence of streptococci in 1944. Fifty years later, researchers showed that a peptide pheromone named CSP controlled competence through the ComCDE pathway (Håvarstein et al. 1995; Håvarstein et al. 1996). A second competence pathway, ComRS, was later discovered in many streptococci (Fontaine et al. 2010; Mashburn-Warren et al. 2010). ComRS systems contain a transcriptional regulator (ComR) that is activated by XIP, a small peptide derived from a precursor peptide ComS. The classic XIP pathway functions as an autocrine loop (Fig. 1A; Fontaine et al. 2015). The precursor peptide ComS is exported and cleaved to form active extracellular XIP. The processed XIP is imported by an unknown mechanism and binds to the transcriptional regulator ComR. The ComR/XIP

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A supplemental appendix to this article is available online.

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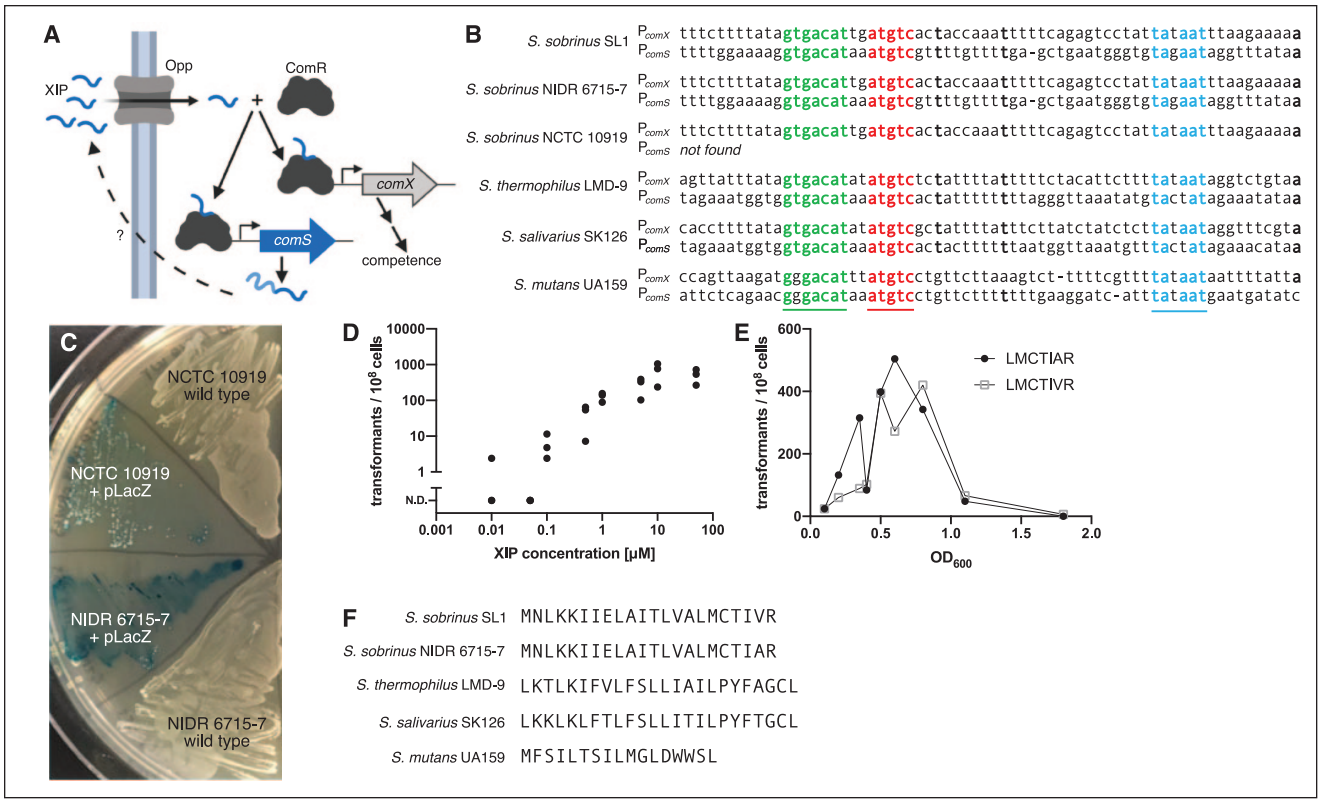

Figure 1. The peptide XIP induces competence in *Streptococcus sobrinus*. (**A**) The ComRS competence pathway in *Streptococcus mutans* forms an autocrine signaling loop. An unknown protein cleaves the leader peptide from ComS and exports the XIP precursor. Activated XIP is imported, where it facilitates dimerization of the transcriptional regulator ComR. The ComR/XIP complex binds a DNA motif to promote transcription of *comX* and *comS*. (**B**) The ComR/XIP binding motif appears upstream of the sigma factor *comX*. Using this sequence, we identified a *comS* gene in 2 strains of *S. sobrinus*. The *comS* gene appears downstream of a homolog of the regulator *comR*. (**C**) *S. sobrinus* strains NIDR 6715-7 and NCTC 10919 can be transformed with exogenous XIP. Both strains were transformed with a plasmid expressing LacZ. When plated with X-gal, the plasmid-carrying strains produce a blue color, but the wild type strains do not. (**D**) The transformation efficiency of *S. sobrinus* strain NCTC 10919 increases with XIP concentration. Transformation assays used linear DNA with homology to regions flanking *comR*. No transformants were observed without XIP. (**E**) Transformation efficiency peaks in the midexponential phase. Transformation assays were performed with strain NCTC 10919 and the pLacZ plasmid (Appendix Fig. 1) by using the predicted XIP for strains SL1 (LMCTIVR) and NIDR 6715-7 (LMCTIAR). No XIP precursor gene (*comS*) is found in the NCTC 10919 genome. (**F**) The *S. sobrinus* ComS peptides differ from sequences in *S. mutans, Streptococcus salivarius*, and *Streptococcus thermophilus*. In particular, all previously known XIP sequences in streptococci contain 2 aromatic amino acids; the XIP in *S. sobrinus* has none.

complex initiates transcription of the competence-inducing sigma factor ComX (Talagas et al. 2016).

S. mutans has functional ComCDE and ComRS pathways, but genomic studies predicted that both pathways are incomplete in *S. sobrinus* (Song et al. 2013; Conrads et al. 2014). The lack of natural competence has led to a widespread belief that *S. sobrinus* is genetically intractable. Researchers can genetically modify other streptococci using synthetic CSP or XIP (Morrison et al. 2015; Junges et al. 2017; Salvadori et al. 2017), but these methods do not work with *S. sobrinus*. The paucity of genetic tools for *S. sobrinus* creates gaps in our mechanistic understanding of how this pathogen affects oral health.

Here we report that *S. sobrinus* does, in fact, contain a functional ComRS competence pathway. Although *S. sobrinus* is commonly classified among the mutans streptococci, the *S. sobrinus* ComRS system is most similar to pathways in the salivarius group. Most strains of *S. sobrinus* contain 2 homologs of ComR, and we show that only 1 is essential for transformation with foreign DNA. Unlike all known XIP sequences, the XIPs from *S. sobrinus* contain no aromatic amino acids. The ComRS pathway regulates bacteriocin production in *S. sobrinus*, and we discovered that *S. sobrinus* inhibits the growth of *S. mutans* through a ComRS-dependent response. Finally, we report 2 strains of *S. sobrinus* that appear to be "cheating" by not sensing or not producing XIP. Overall, the discovery of a novel competence pathway suggests that *S. sobrinus* can communicate extracellularly within the complex oral microbiome. The ComRS pathway also provides a robust method for genetic manipulation of *S. sobrinus*.

Methods

Strains, Reagents, and Growth Conditions

Appendix Data Set 3 lists the strains and plasmids used in this study. Complete genome sequences are now available for all of

the strains used in this article (Sales et al. 2018), confirming that the strains are *S. sobrinus*.

Liquid cultures were grown anaerobically (5% H_2, 10% CO_2, and 85% N_2) at 37 °C in chemically defined medium (CDM; Chang et al. 2011) containing 1% glucose. Solid agar plates were made with Todd-Hewitt broth plus 0.5% yeast extract, 1.5% agar, and antibiotic selection when needed: 1-mg/mL kanamycin, 400-μg/mL spectinomycin, or 4 μg/ml chloramphenicol. Plates were incubated aerobically under 5% CO_2 at 37 °C.

All chemicals were purchased from Sigma Aldrich unless otherwise stated. Enzymes were purchased from New England Biolabs. Oligonucleotides were synthesized by Integrated DNA Technologies. Synthetic peptides were purchased from GenScript, Inc. at >90% purity.

DNA Manipulation and Strain Construction

Linear DNA fragments were constructed by Golden Gate assembly (Engler et al. 2008) following the procedures outlined in the Appendix Methods. A new Golden Gate–compatible backbone (pRW17) was constructed from the *Escherichia coli*/streptococcal shuttle vector pDL278 (LeBlanc et al. 1992). Appendix Table 4 lists all primers and genomic coordinates.

Transformation Assays

An overnight culture of *S. sobrinus* in CDM with antibiotic selection was diluted 150× into fresh CDM without antibiotics. The culture was grown to the midexponential phase (OD600 between 0.55 and 0.75). XIP dissolved in DMSO was added to a final concentration of 10 μM. The culture was mixed well by flicking the tube, and 200 μL was transferred to a microcentrifuge tube where 400 ng of DNA was added. After another 2 h of anaerobic incubation, 150 μL was plated on solid agar and incubated for 24 h aerobically. Transformation efficiency was calculated by comparing colony counts after 24 h on selective and nonselective plates. A detailed transformation protocol is given in the Appendix Methods.

Inhibition Assays

An inhibition assay protocol was adapted from Mignolet et al. (2018) and Van de Rijn and Kessler (1980). Assay plates (60 mm petri dish) were made with 2 layers: a 3-mL bottom layer of 1% agarose and CDM, supplemented with XIP, and a 0.6-mL top layer of 0.4% agarose and CDM mixed with 12 μL of overnight culture of *S. mutans* UA159. A 3-μL spot of *S. sobrinus* (OD 0.6 to 0.85, grown in CDM) was added to the top layer. Plates were imaged after 24 h of incubation on an Axygen Gel Documentation System.

Growth Assays

Overnight CDM cultures of *S. sobrinus* were diluted 100× into fresh CDM and grown to near OD 0.8. A 96-well plate was prepared with 200 μL of fresh CDM and either 10μM XIP (dissolved in DMSO) or an equal volume of DMSO and prewarmed in the incubator. A 50-μL inoculum was added to the prewarmed 96-well plate, and growth was monitored every 30 min with a BioTek Epoch2 spectrophotometer.

Gene Expression Profiling

Changes in gene expression were measured by quantitative reverse transcription polymerase chain reaction via biological triplicates at each time point. Protocols (including RNA isolation) and primer information are available in the Appendix Methods and Appendix Table 5.

Statistical Analysis

We used a linear statistical model to quantify the overall effects of deleting or complementing *comR1* and *comR2*. Deleting *comR2* reduces transformation efficiency below our limit of detection ($P < 1 \times 10^{-16}$). On average, strains of NIDR 6715-7 with extra copies of *comR2* in *trans* are transformable with 10.5-fold higher efficiency ($P < 8 \times 10^{-15}$). Deleting *comR1* increases transformation efficiency by 2.7-fold ($P < 2 \times 10^{-4}$), and strains carrying extra copies of *comR1* have a transformation efficiency that is barely detectable (110-fold decrease, $P < 2 \times 10^{-16}$).

Bioinformatic Searches

Targeted queries for ComRS homologs were performed via BLAST with blastp or tblastn through the NCBI web interface. Searches for homologs of ComR in all *S. sobrinus* strains was performed offline through custom R scripts and the blastp toolkit (Camacho et al. 2009).

Results

Previous studies have searched unsuccessfully for ComCDE and ComRS systems in *S. sobrinus* (Song et al. 2013; Conrads et al. 2014). Searches for ComS (XIP) and ComC (CSP) have also failed, although these small genes are difficult to find. We used a hybrid strategy to search the recently completed genomes of 3 *S. sobrinus* strains (Sales et al. 2018) for both ComR homologs and the promoter sequence recognized by the ComR/XIP complex. The ComR/XIP motif is usually located upstream of the sigma factor *comX* and the *comS* gene (Mashburn-Warren et al. 2010). The ComR/XIP motif from *S. mutans* UA159 (GGGACATNNATGTC) was not present in *S. sobrinus*; however, the ComR/XIP motif from *Streptococcus thermophilus* LMD-9 and *Streptococcus salivarius* SK126 (GTGACAT NNATGTC) was found upstream of *comX* in *S. sobrinus* (Fig. 1B, Appendix Data Set 1). In 2 *S. sobrinus* strains (SL1 and NIDR 6715-7), the ComR/XIP motif appeared between a homolog of the *S. thermophilus comR* gene and a short ORF that we believed to be *comS* (Fig. 1B). The third strain (NCTC 10919) contained a homolog of *comR* but no *comS* gene.

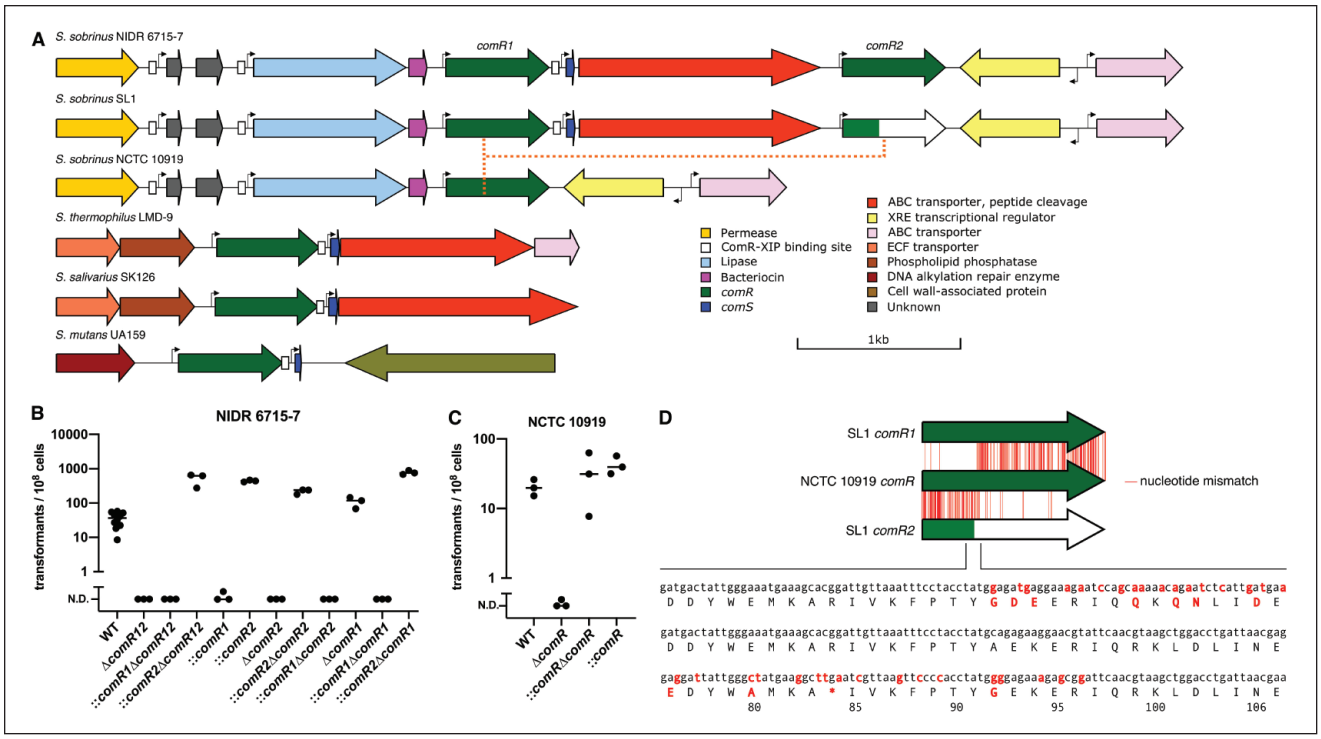

Figure 2. The *Streptococcus sobrinus* ComRS gene cluster is distinct from other streptococci. (**A**) The ComRS gene cluster in strains NIDR 6715-7 and SL1 contain 2 homologs of *comR* that we call *comR1* and *comR2*. The type strain SL1 contains a truncated *comR2* gene (green/white) and cannot be transformed. Strain NCTC 10919 has a single homolog of *comR* and no *comS* gene or a ComS export/cleavage gene. (**B**) Strain NIDR 6715-7 cannot be transformed if *comR2* or the region from *comR1* to *comR2* is deleted. An additional copy of *comR1* on a plasmid does not rescue transformation, but strains complemented with extra *comR2* can be transformed. The horizontal bars represent the mean of the biological replicates (black dots). (**C**) The single *comR* homolog in strain NCTC 10919 is required for transformation. (**D**) The *comR* gene in strain NCTC 10919 appears to be a fusion of *comR1* and *comR2*. A recombination event that produced *comR* would have removed the premature stop codon found in the *comR2* gene of strain SL1.

Based on ComS cleavage patterns in *S. thermophilus* and *S. mutans*, we purchased peptides containing the last 7 amino acids of ComS for strains NIDR 6715-7 and SL1. Both synthetic peptides induced competence in strain NIDR 6715-7, allowing us to transform the strain with either linear or plasmid DNA (Fig. 1C). Both peptides also induced competence in NCTC 10919 (the strain lacking a *comS* gene), but neither peptide induced competence in strain SL1. Synthetic peptides with only the last 9 amino acids of ComS also induced competence in NIDR 6715-7 and NCTC 10919, but peptides with only the last 5 amino acids did not. We used the 7–amino acid peptide for all subsequent experiments and refer to this peptide as XIP; however, we do not know the exact location of the ComS cleavage site that produces active XIP in vivo.

The transformation efficiency of *S. sobrinus* increases with XIP concentration, peaking at 10^{-5} transformants per cell with 10μM XIP for strain NIDR 6715-7 (Fig. 1D). The transformation efficiency also depends on the growth phase of the cells. The efficiency is highest in the midexponential phase (Fig. 1E), similar to other streptococci transformed with XIP (Junges et al. 2017; Salvadori et al. 2017).

The *S. sobrinus* XIP sequence is unlike those of other streptococci (Fig. 1F). ComRS systems are classified by the identity and location of 2 aromatic amino acids in the XIP peptide (Fontaine et al. 2010; Talagas et al. 2016). These aromatic residues are believed to be essential for binding between XIP and ComR (Talagas et al. 2016). We were surprised that the *S. sobrinus* ComS sequence contains no aromatic amino acids and therefore cannot be grouped into any of the 3 established classes of ComRS systems.

To our knowledge, all ComRS gene clusters contain a single copy of the transcriptional regulator gene *comR*. Two *S. sobrinus* strains (SL1 and NIDR 6715-7) contain 2 homologs of *comR* in the same gene cluster (Fig. 2A, Appendix Data Set 2). These genes (*comR1* and *comR2*) flank the *comS* gene and the gene encoding the putative ComS exporter. We used strain NIDR 6715-7 to test if *comR1, comR2*, or both genes are required for competence (Fig. 2B). Deleting *comR1, comR2*, and the 2 genes in between (*comS* and its putative exporter) abolished transformation. Adding a second copy of *comR2* in *trans* restored transformability, but adding back *comR1* did not. In fact, adding extra copies of *comR1* to the wild type strain reduced the transformation efficiency by 111-fold. We verified our results by deleting and complementing *comR2* or *comR1* alone. In all cases, transformation required at least 1 copy of *comR2*, and the transformation efficiency was inversely correlated with the number of copies of *comR1*.

Strain SL1 is unresponsive to XIP, which we believe is explained by a premature stop codon in its *comR2* gene. We predict that the truncated ComR2 in SL1 is nonfunctional on

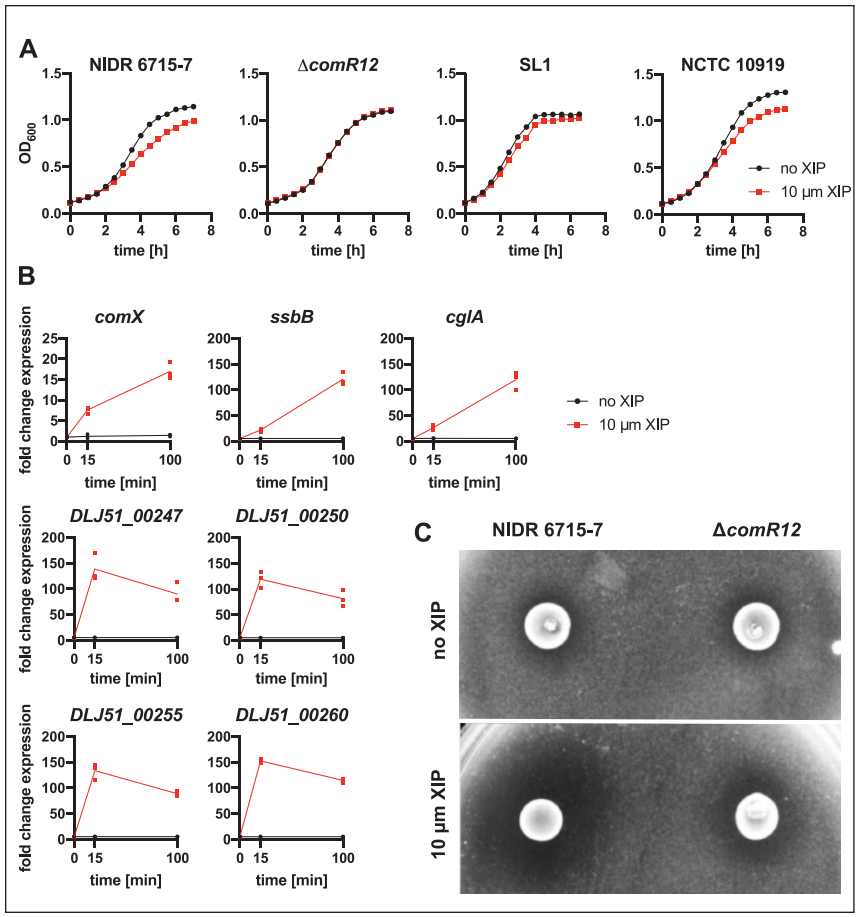

Figure 3. The ComRS pathway controls phenotypic changes in *Streptococcus sobrinus*. (**A**) Adding XIP to cultures of strain NIDR 6715-7 causes a growth defect. An NIDR 6715-7 deletion strain lacking the ComRS pathway (*comR1, comS*, the ComS exporter, and *comR2*) does not show a growth defect after XIP is added. The SL1 wild type strain cannot be transformed with XIP and does not show a growth defect, but the NCTC 10919 strain is transformable and grows slower in the presence of XIP. (**B**) XIP increases the expression of the *comX, ssbB*, and *cglA* genes in strain NIDR 6715-7. The ComR/XIP binding motif appears upstream of 4 genes in a bacteriocin gene cluster (*DLJ51_00247, DLJ51_00250, DLJ51_00255*, and *DLJ51_00260*). All 4 genes are upregulated after XIP is added. Each point is a biological replicate, and fold change is relative to the expression at time zero. (**C**) *S. sobrinus* inhibits the growth of *S. mutans* when XIP is added to cultures on solid agarose. A base layer of agarose containing XIP is topped with a second layer of agarose with embedded *S. mutans* UA159. Spotting *S. sobrinus* 6715-7 on top of the agarose inhibits the growth of *Streptococcus mutans* on plates containing XIP. The inhibition is reduced when the ComRS pathway is deleted from *S. sobrinus*.

the basis of structural studies in other streptococci (Talagas et al. 2016). The strain NCTC 10919 contains only 1 *comR* gene, and this gene is essential for induced competence (Fig. 2C). It appears that the single copy of *comR* is the result of a recombination event between *comR1* and *comR2*, since the 5′ end of the gene is more similar to *comR1* and the 3′ end is more similar to *comR2* (Fig. 2D). Such a recombination event would have deleted the *comS* gene and removed the premature stop codon in the *comR2* of strain SL1. It is possible that strain NCTC 10919 lost its ability to produce XIP in exchange for repairing the broken ComRS pathway in a common ancestor with SL1.

XIP can decrease the growth rate of some streptococci (Desai et al. 2012). Adding exogenous XIP slows the growth of *S. sobrinus* strain NIDR 6715-7, but no defect is observed in a ComRS deletion strain (Fig. 3A). The growth of strain SL1, which is not transformable with XIP, is unaffected by XIP. A XIP-induced growth defect is observed in the competent strain NCTC 10919.

It has been hypothesized that bacteria with a ComRS system could "cheat" by either not producing or not responding to XIP (Fontaine et al. 2010). Instead, the cheating strains would rely on other bacteria to perform the community-level processes controlled by the competence pathway. Two of our *S. sobrinus* strains appear to cheat. Strain SL1 does not respond to XIP due to the loss of a functional *comR2* gene. Strains with this mutation would enjoy a selective advantage by avoiding the growth defect in the presence of XIP. Strain NCTC 10919 does not appear to produce XIP, although it can still respond to XIP produced by other strains.

XIP increases the expression of the competence-related genes *comX, ssbB*, and *cglA*. Transcript levels of these genes are increased by 15 min and continue to increase for up to 100 min (Fig. 3B). The sustained activation of competence genes is consistent with the ComRS system in *S. mutans* (Morrison et al. 2015).

We identified additional ComR/XIP binding sites in *S. sobrinus* that are upstream of putative bacteriocins (Fig. 2A). These genes are also upregulated upon stimulation by XIP (Fig. 3B). Surprisingly, we discovered that *S. sobrinus* inhibits the growth of *S. mutans* when the former is stimulated with XIP (Fig. 3C). The inhibition is attenuated when the ComRS pathway is deleted from *S. sobrinus*.

The ComRS pathway is widespread among strains of *S. sobrinus*. A BLAST search revealed that all 54 publicly available genomes for *S. sobrinus* contain a homolog of the *comR* gene. In 83% of these genomes, the ComR amino acid sequence has >97% amino acid identity with ComR2 in *S. sobrinus* NIDR 6715-7. The conservation of ComR suggests that XIP may be useful for transforming a wide range of *S. sobrinus* strains. While some strains of *S. sobrinus*, like SL1, remain genetically intractable, strains with a functional ComRS system can be manipulated to reveal differences between *S. sobrinus* and other cariogenic species. Thus, the discovery of this novel competence pathway will begin a new phase of *S. sobrinus* research where the mechanisms of cariogenicity can be unraveled with forward genetics.

Discussion

The *S. sobrinus* ComRS system is unique on the basis of its 2 *comR* genes and a XIP sequence that lacks any aromatic amino acids; however, the arrangement and sequence of the ORFs in the ComRS gene cluster is most similar to ComRS clusters in the salivarius group (*S. salivarius* and *S. thermophilus*; Fig. 2A). *S. sobrinus* was originally a subspecies of *S. mutans* and is frequently grouped with the mutans streptococci based on genomic phylogenies (Song et al. 2013). Recent phylogenetic trees place *S. sobrinus* closer to *S. salivarius* and *S. thermophilus* in a separate sobrinus clade (Patel and Gupta 2018). Our functional data on ComRS would agree with *S. sobrinus*'s placement in or near the salivarius group. However, neither *S. salivarius* nor *S. thermophilus* is cariogenic, raising questions about why *S. sobrinus* is associated with aggressive dental caries.

S. sobrinus, like the noncariogenic species *S. salivarius*, has direct links between competence and bacteriocin production. Such a direct link has not been observed in cariogenic streptococci (Mignolet et al. 2018). The ComRS-activated bacteriocins in *S. sobrinus* can kill *S. mutans*, a surprising finding given that coinfection by these 2 species leads to worse oral health outcomes (Seki et al. 2006; Kanasi et al. 2010; Okada et al. 2012). Our scant knowledge of *S. sobrinus* and its behavior has been drawn through the presumed similarity of *S. sobrinus* to *S. mutans*. However, the structural, functional, and phylogenetic differences between *S. sobrinus* and *S. mutans* suggest that the association between *S. sobrinus* and the mutans streptococci needs to be reconsidered.

Streptococci use the ComRS system for intercellular communication, and we now know that *S. sobrinus* can participate in these community-level conversations. Competence affects individual bacteria and the community as a whole, and it has been hypothesized that bacteria with a ComRS system could "cheat" by either not producing or not responding to XIP (Fontaine et al. 2010). Two of our *S. sobrinus* strains appear to cheat. The type strain SL1 does not respond to XIP, most likely because its *comR2* gene is truncated. Strains of *S. sobrinus* that do not respond to XIP would avoid the XIP-induced growth defect (Fig. 3A) and the metabolic costs of producing and exporting bacteriocins; however, they can still benefit from the bacteriocin defenses produced by neighboring cells (Matsumoto-Nakano and Kuramitsu 2006; Shanker and Federle 2017; Mignolet et al. 2018). Even worse, SL1 appears to have functional genes for producing and exporting XIP, so it could activate the ComRS systems in neighboring cells without taking part in the community response.

Strain NCTC 10919 has the potential to cheat by not producing or exporting XIP. NCTC 10919 can sense XIP produced by neighboring cells and participate in the community-wide competence state. This maximizes NCTC 10919's chances of taking up beneficial foreign DNA while avoiding the metabolic costs of producing and exporting XIP. While strain SL1 and NCTC 10919 have the potential to cheat, the mechanisms and benefits of cheating are different. It is interesting that a single recombination event could have switched an SL1-type cheater to an NCTC 10919-type cheater, thereby swapping one type of cheating for another.

The ability to transform *S. sobrinus* clears the way for forward genetic studies into the bacterium's pathogenicity. As compared with *S. mutans, S. sobrinus* produces more acid (de Soet et al. 1989) and better tolerates acid stress (Nascimento et al. 2004). The *S. sobrinus* acid tolerance response is poorly characterized, but we know that it is mechanistically distinct from the acid tolerance response of *S. mutans* (Nascimento et al. 2004; Conrads et al. 2014). Other studies have observed that *S. sobrinus* is capable of immune suppression (Veiga-Malta et al. 2004), hydrogen peroxide production (García-Mendoza et al. 1993; Conrads et al. 2014), and enhanced glucan synthesis (Conrads et al. 2014). Our new genetic system for *S. sobrinus* will allow us to study the molecular details of these phenotypes and identify new targets for reducing cariogenesis by *S. sobrinus*.

Author Contributions

J.W. Li, R.M. Wyllie, P.A. Jensen, contributed to conception, design, and data analysis, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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