Activity of *Streptococcus mutans* VicR Is Modulated by Antisense RNA

Journal of Dental Research
2018, Vol. 97(13) 1477–1484
© International & American Associations
for Dental Research 2018
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/0022034518781765
journals.sagepub.com/home/jdr

L. Lei^{1,2}, R.N. Stipp^{2,3} , T. Chen², S.Z. Wu⁴, T. Hu¹, and M.J. Duncan²

Abstract

The VicRK 2-component system of *Streptococcus mutans* regulates genes associated with cell wall biogenesis and biofilm formation. A putative RNase III-encoding gene (*rnc*) is located downstream from the *vicRKX* operon. The goals of this study were to investigate the potential role of VicR in the regulation of adjacent downstream genes and evaluate transcription levels of *vicR* during planktonic and biofilm growth. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to investigate whether *vicRKX* and adjacent downstream genes were cotranscribed. Binding of purified recombinant VicR protein to promoter regions of *vicR*, *rnc*, and *syfA* genes was confirmed by electrophoretic mobility shift assay and by chromatin immunoprecipitation analyses. VicR antisense (*ASvicR*) RNA was detected by Northern blotting and qRT-PCR assays. *ASvicR* overexpression mutants were constructed, and the biofilm biomass was determined by crystal violet microtiter assay. Adjacent downstream genes *rnc*, *smc*, *syfA*, *smu.1511*, and *syfB* were cotranscribed with *vicRKX*. The predicted promoter regions of *vicR*, *rnc*, and *syfA* genes were directly regulated by VicR. An *ASvicR* RNA transcript was detected upstream of the *rnc* gene. Expression of the *ASvicR* RNA transcript was elevated in planktonic cultures and repressed during biofilm growth. In addition, Western blot data showed that expression of the VicR protein decreased by 35% in planktonic as compared with biofilm cultures. Furthermore, we show that overexpression of *ASvicR* led to a reduction in biofilm formation. The downstream genes *rnc*, *smc*, *syfA*, *smu.1511*, and *syfB* are cotranscribed with *vicRKX*. VicR is autophosphorylated, and *rnc* and *syfA* are directly regulated by VicR. Expression of VicR protein correlated inversely with different levels of *ASvicR* RNA transcript and growth conditions. The biofilm biomass decreased in the *ASvicR* overexpression mutant. These data suggest a role for the *ASvicR* RNA transcript in posttranscriptional regulation of VicR protein production in *S. mutans*.

Keywords: gene regulation, protein production, biofilm growth, mRNA interference, non-coding RNA characterization, bacterial genetics

Introduction

Streptococcus mutans is the major cariogenic pathogen that resides in a multispecies microbial biofilm on the tooth surface. The ability of *S. mutans* to sense and adapt to environmental conditions during biofilm formation contributes to its pathogenicity (Smith and Spatafora 2012). The VicRK 2-component signal transduction system (TCS), originally identified in *Bacillus subtilis*, is highly conserved in Gram-positive bacteria and regulates genes associated with extracellular polysaccharide synthesis, cell wall homeostasis, and genetic competence (Fukuchi et al. 2000; Senadheera et al. 2005; Stipp et al. 2013). A typical TCS comprises a histidine sensor kinase membrane receptor and a cognate intracellular response regulator that, upon activation, binds to the regulatory regions of target genes. A third gene, *vicX*, annotated as a putative hydrolase, is associated with the *vicRK* operon in *S. mutans* (Senadheera et al. 2007).

Mapping of *vicR* and neighboring genes with BLAST searches demonstrated the presence of a gene that potentially encodes RNase III (*rnc*) and a chromosome segregation protein (*smc*) immediately downstream from the *vicRKX* operon (Senadheera et al. 2005). In *Escherichia coli*, endonuclease RNase III plays a critical role in the specific cleavage of double-stranded RNA structures (Régner and Grunberg-Manago

1990). Recently, an *rnc*-null mutation in *S. mutans* UA159 was constructed that showed a decrease in biofilm formation (Mao et al. 2018). Deep sequencing and bioinformatics analyses suggested that RNase III probably affects expression of *vicRKX* via putative microRNA-size small RNAs that target the *vicRKX* genes (Mao et al. 2016). The 108-bp noncoding intergenic region between the *vicX* and *rnc* genes contains a predicted VicR-binding consensus motif (Dubrac and Msadek 2004; Ayala et al. 2014). However, information regarding potential interaction between the *S. mutans* VicR regulon and RNase III is limited.

¹State Key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, Department of Preventive Dentistry, West China Hospital of Stomatology, Sichuan University, Chengdu, China

²The Forsyth Institute, Cambridge, MA, USA

³Department of Oral Diagnosis, Piracicaba Dental School, University of Campinas, Campinas, Brazil

⁴West China Hospital, West China Medical School, Sichuan University, Chengdu, China

A supplemental appendix to this article is available online.

Corresponding Author:

M.J. Duncan, The Forsyth Institute, 245 First Street, Cambridge, 02142, USA.

Email: mduncan@forsyth.org

Figure 1. *VicRKX* operon and downstream genes in *Streptococcus mutans* UA159. **(A)** Schematic of gene loci and RT-PCR primers sets with putative intergenic *VicR* binding sites. Downstream from the *vicRKX* operon in *S. mutans* UA159, a cluster of 5 genes (SMU_1514 to SMU_1510) is similarly transcribed in the same orientation. These genes encode RNase III (*rnc*, SMU_1514), a chromosome segregation ATPase (*smc*, SMU_1513), phenylalanine-tRNA synthetase α subunit (*syfA*, SMU_1512), acetyltransferase (*act*, SMU_1511), and phenylalanine-tRNA synthetase β subunit (*syfB*, SMU_1510). **(B)** RT-PCR with lanes numbered 1 to 5 according to primer sets used. **(C)** Total RNA was used as the negative control for all the PCR reactions. Amplicons could not be generated from RNA, indicating that reverse transcription was not contaminated with genomic DNA. **(D)** Genomic DNA was used as the positive control for all the PCR reactions, which did yield amplicons.

Evidence from transcriptome analyses revealed that substantial antisense (AS) as well as intergenic and intragenic transcription occurs in bacteria (Rasmussen et al. 2009; Dornenburg et al. 2010). A noncoding AS RNA sequence is complementary to that of messenger RNA (mRNA), and their interaction to form a duplex blocks translation to a functional protein. In some cases, the double-stranded RNA complex is a substrate for complete degradation by RNase III (Krinke and Wulff 1987, 1990). As expected for regulatory factors, some AS RNAs are expressed in response to environmental conditions. In *B. subtilis*, AS RNA expression levels are associated with sporulation (Silvaggi et al. 2006). In *Helicobacter pylori*, expression of *ureAB* is regulated by AS RNA under neutral-pH conditions (Wen et al. 2011). In streptococci, *VicR* activity is modulated by the cognate sensor kinase *VicK* in response to environmental signals (Dalton and Scott 2004). In this study, the potential role of *VicR* in the regulation of adjacent genes was investigated. The close proximity of the *rnc* gene to the *vicRKX* operon suggested a functional relationship; therefore, we investigated whether potential AS RNA was specifically associated with regulation of *VicR* function.

Materials and Methods

Bacterial Strains and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Appendix Table 1. See Appendix for details of the mutant strains and growth conditions.

RNA Purification for qRT-PCR Assays

Total RNA was extracted with a MasterPure RNA purification Kit (Epicentre). See Appendix for details.

cDNA for qRT-PCR

Aliquots of total RNA were reverse transcribed to cDNA with random hexamer primers or gene-specific primers (Appendix Table 2) with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific; see Appendix for details). qRT-PCR reactions were carried out in triplicate. Threshold cycle values were quantified, and expression of each gene expression was normalized relative to that of *gyrA*. Data were calculated according to the $2^{-\Delta\Delta CT}$ method (Bustin et al. 2009).

Protein Extraction and Western Blotting

The expression of *VicR* protein in *S. mutans* cells after planktonic or biofilm growth was quantified by Western blot. See Appendix for details of protein extraction and Western blotting.

Electrophoretic mobility shift assays (EMSAs) were used to determine whether *rnc* and *syfA* genes in the extended *vicR* operon were directly regulated by *VicR*, as previously described (Stipp et al. 2013) with modifications (see Appendix for details).

Chromatin Immunoprecipitation and PCR Analyses

Chromatin immunoprecipitation (ChIP) assays were performed with anti-*VicR* antibody as previously described (Nishikawa et al. 2004) with minor modifications. Primers were designed to amplify predicted promoter regions for *vicR*, *rnc*, and *syfA* genes (Appendix Table 2).

Detection of *vicR* AS RNA by Northern Blotting and RT-PCR Assays

Detection of AS RNA was carried out by Northern blotting as well as RT-PCR with a first strand-specific primer for synthesis of cDNA transcripts (Ho et al. 2010). Oligonucleotide primers (PCR3) used for first strand synthesis PCR are listed in Appendix Table 2 (see Appendix for details).

5' Rapid Amplification of cDNA Ends

Total RNA (10 μ g) from UA159 was ligated to the 5' rapid amplification of cDNA ends (RACE) outer adapter from the FirstChoice

RLM-RACE Kit (Ambion) as described previously (Nishikawa and Duncan 2010). Primers used for PCR reactions for 5' RLM-RACE were listed in Appendix Table 2. See appendix for subsequent nested PCR conditions and sequencing.

Construction of ASvicR Mutants

The low copy number shuttle vector pDL278 was used to express the ASvicR sequence under the control of the *vicR* promoter region (Appendix Fig. 3) and transformed into *S. mutans* UA159 strains. Biofilm growth and acid production curves were measured in mutant and UA159 parent strains (see Appendix for details).

Crystal Violet Microtiter Assay to Measure Biofilm Biomass

The biomass of UA159, ASvicR, and pDL278 empty vector control strain biofilms was determined by crystal violet assay as previously described (Chen et al. 2017; see Appendix for details).

Data Analysis

The Shapiro-Wilk test was used to determine whether the data were normally distributed, and Bartlett's test was performed to assess the homogeneity of variances. For parametric testing, 1-way analysis of variance was used to compare the data, followed by pairwise multiple comparisons (Tukey test).

Results

Extended VicR Gene Cluster

Downstream from the *vicRKX* operon in *S. mutans* UA159, a cluster of 5 genes (SMU_1514 to SMU_1510) is transcribed in the same orientation (Fig. 1A). RT-PCR with a series of overlapping primers suggested cotranscription of a polycistronic mRNA comprising at least *vicX* and the 5 downstream genes (Fig. 1B–D), suggesting that the cluster may be transcribed as a single unit. It was previously demonstrated that the *vicRKX* genes were cotranscribed (Senadheera et al. 2005); therefore, there are possibly 8 genes in an extended regulon. In addition, using the FindTerm database (<http://linux1.softberry.com/berry.phtml?topic=findterm&group=programs&subgroup=gfindb>) predicted a putative transcriptional terminator located at the end of *syfB* gene that was scored as a “strong” rho-independent bacterial terminator.

The extended *vicR* gene cluster included 2 noncoding regions of 442 and 106 bp that are potential promoter regions for *rnc* and *syfA*, respectively (Fig. 1A; Appendix Fig. 1A, B).

Figure 2. Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) for *rnc*, *syfA*, and *vicR* promoter regions. (A) EMSA in which promoter regions were obtained by PCR and DIG labeled. Excess cold (unlabeled) promoter DNAs were used to outcompete VicR-specific binding. In each case, binding was reduced in the presence of excess nonlabeled DNA, suggesting specificity of VicR binding. The promoter of the *gtfB* gene, which contains a known VicR binding site, was used as a positive control. As the negative control, a DNA fragment the same size as the *vicR* promoter and with a similar AT:GC mole ratio, though missing the VicR consensus-binding sequence, was used to rule out nonspecific binding. (B) PCR was used to detect promoters of *rnc*, *syfA*, and *vicR* in ChIP input and output fractions after precipitation anti-VicR; IgG-immunoprecipitated fractions were used as controls. As in panel A, the negative control was the *vicR* promoter region without the consensus-binding sequence, which could not be immunoprecipitated by VicR-specific antibody in the output DNA. (C) Western blotting of anti-VicR-immunoprecipitated ChIP fractions was probed with affinity-purified VicR-specific antibody. Western blotting of the immunoprecipitates showed that VicR-DNA complexes were present in the starting DNA fraction (input) and in the VicR-immunoprecipitate (output), while the IgG immunoprecipitate contained only traces of protein.

By EMSA, we showed that VicR protein bound to DNA fragments from *vicR*, *rnc*, and *syfA* promoter regions (Fig. 2A). To confirm the EMSA results, we used ChIP in which *S. mutans* UA159 DNA-VicR complexes that formed in vivo were immunoprecipitated with VicR-specific antibody or with Immunoglobulin G (IgG) (negative control). VicR ChIP input and output fractions contained DNA sequences for the promoter regions of *rnc*, *syfA*, and *vicR* that were identified by PCR with gene-specific primers, while only background levels of these sequences were found in IgG immunoprecipitates (Fig. 2B, C). These data indicate that at least *rnc*, *smc*, *syfA*, *act*, and *syfB* are part of the VicR regulon.

vicR Gene Cluster Contains a vicR AS RNA That Negatively Affects Production of VicR Protein

We investigated whether potential AS RNA was specifically associated with *vicR* with first-strand cDNA synthesis to detect

Figure 3. Detection of *vicR* antisense (AS) RNA. **(A)** Differences in AS RNA abundance in planktonic versus biofilm cells detected by Northern blotting (676-nt size of RNA). Transcript amounts were measured in cultures of UA159 in a representative Northern blot (10 μ g of total RNA applied per lane). Lane 1, planktonic cells grown in brain heart infusion (BHI) without added sugar; lane 2, planktonic cells in BHI with sucrose (final concentration of 1%); lane 3, planktonic cells in BHI with glucose (final concentration of 1%); lane 4, biofilm cells in BHI without added sugar; lane 5, biofilm cells in BHI with sucrose (1%); lane 6, biofilm cells in BHI with glucose (1%); lane 7, sample buffer without RNA was used as the loading control; lane C, positive control (DIG-labeled RNA as a hybridization control). **(B)** Total RNA samples were isolated from *Streptococcus mutans* grown under several conditions and used as templates to detect *ASvicR* RNA transcripts by first cDNA-strand synthesis and RT-PCR with *vicR*-specific antisense- and sense-strand primers. Lane 1, planktonic cells grown in BHI without added sugar; lane 2, planktonic cells in BHI with sucrose (1%); lane 3, planktonic cells in BHI with glucose (1%); lane 4, biofilm cells in BHI without added sugar; lane 5, biofilm cells in BHI with sucrose (1%); lane 6, biofilm cells in BHI with glucose (1%); lane 7, negative control: total RNA from *Porphyromonas gingivalis* as template for cDNA synthesis. The *S. mutans* housekeeping gene *gyrA* was used as the internal reference for sample loading (lower panel). **(C)** *ASvicR* quantified in cells by qRT-PCR. The threshold cycle values were compared with the control groups of planktonic cells of UA159 in BHI medium without added sugar. The *gyrA* gene was used as an internal standard (* $P < 0.05$). Values are presented relative to that of planktonic cells grown in BHI without additional carbon source. Error bars indicate standard deviations. **(D)** *VicR* protein was quantified in the cells grown as previous from Western blots probed with anti-*VicR* antibody (upper panel). The lower panel shows a Coomassie-stained gel supporting equal loading of samples. **(E)** The expression of *VicR* protein in planktonic or biofilm growth was quantified by Western blot assays. The intensity values of Western blot bands were divided by the intensity of the control strain (UA159 planktonic cells grown in BHI without added sugar) to estimate *VicR* production (* $P < 0.05$). Values are presented relative to that of planktonic cells grown in BHI without additional carbon source. Error bars indicate standard deviations.

the transcript in *S. mutans* UA159. The sense and AS sequences of the *vicR* gene (SMU_1517) are shown in Appendix Figure 1C. Total RNA was prepared from strain UA159 grown as planktonic or biofilm cultures in brain heart infusion (BHI) without and with added sugars (to final concentrations of 1% glucose or sucrose). These RNA samples were used as templates for first-strand DNA synthesis with *vicR* AS- (PCR3) and sense-specific primers (AS11; Appendix Table 2). A *vicR* AS transcript of the predicted size (approximately 587 bases) was detected in each culture except for biofilm cells grown in BHI without additional carbon source (Fig. 3C, lane 4). The *ASvicR* RT-PCR product was subsequently sequenced. Expression of *ASvicR* quantified by qRT-PCR was highest in planktonic

cells grown without additional carbon source (Fig. 3B); expression decreased by approximately 60% in planktonic cells grown with glucose and by almost by 70% in the presence of sucrose. The level of *ASvicR* in biofilm cultures was lower than that in planktonic cells, especially in the absence of an additional carbon source (Figs. 3B, lane 4; 3C, column 4). In addition, the differences in *ASvicR* abundance in planktonic cells versus biofilms were confirmed with Northern blotting (Fig. 3A). Western blots probed with anti-*VicR* antibody clearly showed that the level of *VicR* protein was highest in biofilm cells cultured without additional carbon source (Fig. 3D, lane 4 in the upper panel and column 4 in the lower) and that *ASvicR* was significantly reduced in the same culture (Fig. 3B, lane 4; 3C, column 4). A similar trend was observed in planktonic cultures in BHI with 1% sucrose (Figs. 3C, D, lane 2).

We used 5' RACE for additional confirmation of *ASvicR* and to determine its 5' terminus; the primers used in the PCRs are listed in Appendix Table 2. The position of primers used for 5' RACE assays are indicated in Figure 4A. Gel electrophoresis of the 5' RACE PCR amplicon indicated that the size was approximately 289 bp (Fig. 4B), and sequencing showed that it contained 253 bp of *ASvicR* RNA sequence from 1,446,139 to 1,446,392 bp in the *S. mutans* UA159 genome (Fig. 4C) and 36 bp of 5' RACE inner primer. The sequence predicts that the 5' terminus of *ASvicR* begins within the 5' terminus of the *vicK* sensor kinase and covers most of the *vicR* coding sequence, including the DNA binding and dimerization domains, and extends into the receiver domain.

Overexpression of *ASvicR* Leads to a Reduction in Biofilm Formation

An *ASvicR* overexpression mutant was constructed, and expression levels of *ASvicR* and *vicR* mRNA were quantified. *S. mutans* cells were incubated to midexponential phase in BHI media, supplemented with sucrose (1%). The transcription of *ASvicR* in the *ASvicR*-overproducing strain was 2.5-fold higher than in UA159 parent cells (Fig. 5C), which was confirmed by Northern blotting (Fig. 5B). Correspondingly, the level of *vicR* mRNA in the *ASvicR* strain decreased by 74% when compared with the UA159 strain, while the addition of a pDL278 empty plasmid did not affect the expression levels of *ASvicR* RNA and *vicR* mRNA (Fig. 5C). Next, we evaluated the specific effects of *ASvicR* overexpression on the ability of the strains to form biofilms. Bacteria were allowed to form

biofilms in BHI with 1% sucrose for 24 h. The biomass was quantified by crystal violet microtiter assay, which showed that overexpression of AS*vicR* resulted in a 50% decrease in biofilm growth as compared with the UA159 parent strain (Fig. 5D). The effects of AS*vicR* overexpression on acid production by the strains were also investigated. A progressive decrease of pH values was observed after 9 h of incubation in the AS*vicR* overexpression strain, starting close to 7.5 and ending <5.5, which was a slower decrease versus the UA159 strain (Fig. 5E). However, additional studies on the linkage of AS*vicR* to cariogenic phenotypes of *S. mutans* are justified.

In the present study we showed that VicR binds to the promoter region of 3 downstream genes: *syfA*, SMU.1511c, and *syfB*. How this regulator affects their expression and whether AS*vicR* and RNase III are involved also warrant further investigation. In summary, we detected and confirmed 676-bp noncoding AS RNA with the potential to attenuate the functions of the essential response regulator VicR. Furthermore, we show that overexpression of AS*vicR* leads to a reduction in biofilm formation.

Discussion

The VicRK TCS is highly conserved in Gram-positive bacteria and is annotated as YycG/YyF in some genera. It was recently proposed to rename the system WalKR because of its larger role in the regulation of genes associated with cell wall synthesis (Dubrac et al. 2008). Transcriptome data indicated that 128 *S. mutans* genes (approximately 6.5% of the genome) were differentially regulated by VicR (Dmitriev et al. 2011). The inability to construct *vicR* mutants in *S. mutans* predicted that the regulator gene was also essential for growth in this species (Senadheera et al. 2005). Subsequently, it was shown that the essentiality of VicR was related to its role as a regulator of the expression of glucan-binding protein B (*gbpB*), the *S. mutans* orthologue of *pcsB*, an essential gene in *Streptococcus pneumoniae* (Mattos-Graner et al. 2006; Stipp et al. 2013) that appears to play a role in cell division (Bartual et al. 2014). Although *vicK* mutants were viable, they showed altered expression of genes associated with the synthesis of extracellular matrix and decreased genetic competence (Senadheera et al. 2005). In addition to activating its cognate response regulator VicR, the sensor kinase VicK facilitates in vitro cross talk by phosphorylating the orphan response regulator GcrR in the presence of manganese (Downey et al. 2014).

Several new genes in the region of *vicR* locus were assigned to the VicR regulon because EMSA showed that VicR bound to

Figure 4. Structure of the AS*vicR* transcript and correlation with *vicR* mRNA. **(A)** Locations of primers used for 5' rapid amplification of cDNA ends (RACE): a, gene-specific outer primer; b, gene-specific inner primer; c and d, 5' RACE outer and inner primers, respectively. **(B)** Detection of the 5' terminus of the AS*vicR* transcript by 5' RACE: lane 1, the PCR product was approximately 289 bp; lane 2, negative control PCR without template; lane 3, size markers. **(C)** Schematic of AS*vicR* showing that transcription starts within the 5' terminus of the *vicK* open reading frame (ORF) and extends through most of the *vicR* ORF. The sequence of the first-strand synthesis AS*vicR* RT-PCR amplicon was 587 bp from 1,446,228 to 1,446,815 bp in the genome. The overlapping sequence from 5' RACE and first strand-specific AS*vicR* RT-PCR was 164 bp from 1,446,228 to 1,446,392 bp in the genome. The full length for AS*vicR* RNA is 676 bp (1,446,139 to 1,446,815). **(D)** Sequence of AS*vicR* (676 nucleotides). Candidate -35 and -10 transcription motifs are underlined. Bioinformatic searches for promoter motifs for the transcript started within the *vicK* ORF (BPROM at <http://linux1.softberry.com/berry.phtml>). A putative -35 box, TTAAGT, started at nucleotide 78 of the sequence, and a -10 box, TTTTAACT, at nucleotide 92 as underlined. Sequence in italics and bold is the starting site of the AS*vicR* transcript determined by 5' RACE. A predicted transcription start (Neural network at http://www.fruitfly.org/seq_tools/pro-moter.html) coincided with that found experimentally by 5' RACE at nucleotide 141 depicted in italic. Sequence in bold depicts the overlap between first-strand synthesis RT-PCR and 5' RACE as well as the starting site of first-strand synthesis RT-PCR product.

their promoters. However, the function annotated for the *smc* gene, an ATPase associated with chromosome segregation (Minnen et al. 2011; Attaiech et al. 2015), aligns more closely with the function of other genes in the extended VicR regulon (i.e., cell wall biogenesis and cell division). The most distal genes in the *vicR* cluster encoded phenylalanine tRNA synthetase subunits α and β and an acetyltransferase that may play a role in modulating protein function by acetylation; links between these and other genes regulated by VicR are, as yet, unclear. The close proximity of *rnc* to *vicRKX* suggested a potential interaction and role in regulation of VicRK TCS. Evidence from transcriptome analyses revealed that substantial AS transcription, as well as intergenic and intragenic transcription, occurs in bacteria (Dornenburg et al. 2010). *Rnc*-encoded RNase III endonuclease activity was shown to cleave RNA-RNA duplexes in *Escherichia coli*, such as AS RNA complexed with its complementary sense strand (Thomason and Storz 2010). AS RNA has long been used as a strategy of mRNA interference to inhibit the translation of specific proteins in bacterial pathogens (Ji et al. 2001). In *S. mutans*, an inducible AS RNA approach was used to identify and characterize several novel essential genes (Wang and Kuramitsu 2005). Induction of *gbpB* AS RNA led to reduced production

Figure 5. Effects of ASvicR overexpression on *Streptococcus mutans*. (A) Extracts were prepared from cells grown in brain heart infusion (BHI) without and with added carbon sources (as described in legend to Fig. 3). VicR production was quantified in Western blots probed with anti-VicR antibody (upper panel). SDS-PAGE gel analysis was conducted (lower panel); pDL278 is the empty vector control. (B) The differences in AS RNA abundance of UA159 and ASvicR cells by Northern blotting (676-bp size of RNA). Transcript amounts were measured in representative Northern blot (10 µg of total RNA applied per lane): lane 1, UA159 cells grown in BHI with sucrose (1%); lane 2, ASvicR cells (ASvicR RNA overexpression) in BHI with sucrose (1%); lane 3, mixture without RNA as the loading control; lane C, positive control (DIG-labeled RNA as a hybridization control). (C) qRT-PCR analysis showed the gene transcripts in UA159, ASvicR, and pDL278. *S. mutans* gene expression was relatively quantified by real-time PCR with *gyrA* as an internal control and calculated per UA159 expression, which was set as 1.0. Experiments were performed in triplicate and are presented as the mean $\pm$ SD (* P < 0.05). (D) Biomass was quantified by crystal violet staining, and optical densities at 550 nm were measured. Data represent 10 biological replicates and are presented as mean $\pm$ SD (* P < 0.05). (E) Effect of overexpression of ASvicR on acid production. UA159, ASvicR, and pDL278 strains were cultured at 37 °C in BHI with sucrose (final concentration of 1%) and acid production curves were determined by measuring the pH values at 1-h intervals. (F) A working model of regulation by ASvicR RNA depends on the formation of ASvicR-vicR mRNA duplexes that prevent the production and function of VicR as an activating response regulator. Included in this model is the ability of RNase III encoded by *rnc* or an as-yet-unknown activity to degrade the ASvicR-mRNA duplexes and restore production of VicR and, potentially, biofilm formation.

of GbpB, indicating the feasibility of an AS RNA strategy to downregulate GbpB protein function (Duque et al. 2011). It is anticipated that, as with 2-component systems, AS RNAs are expressed in response to environmental conditions—for example, regulation of pilin expression in *Neisseria meningitidis* in response to salt stress (Tan et al. 2015).

In *S. mutans*, the VicRK TCS was shown to affect the expression of glucosyltransferases *gtfB/C/D* associated with biofilm formation (Senadheera et al. 2005). Genes regulated by VicR are involved in exopolysaccharide synthesis and cell wall biogenesis (Stipp et al. 2013). As a response regulator, VicR activity is considered to be modulated by the cognate VicK sensor kinase that responds to environmental stimuli (Dalton

and Scott 2004). Expression of *vicR* is not regulated by only VicK and VicX, since loss of *vicK*, *vicX*, or *vicKX* does not alter VicR-LacZ activity (Tremblay et al. 2009). EMSA data indicated that the *vicRKX* operon is potentially autoactivated (Fig. 2A) in response to a specific environmental condition. Additional regulatory mechanisms may also play a role in fine-tuning expression of certain genes.

By searching the AS strand of the *vicRKX* operon, a noncoding cis AS RNA (ASvicR) was identified (Fig. 3A) and detected by northern blotting and confirmed by 5' RACE (Figs. 3C and 4B, respectively). ASvicR was barely detectable by RT-PCR in UA159 cells grown as biofilms in BHI without added sugar (Fig. 3B, C) compared with planktonic cells in the same medium that produced approximately 8-fold more ASvicR. In contrast, VicR production by biofilm cells was highest, as shown in Westerns probed with anti-VicR antibody (Fig. 3D). Planktonic cells in the same medium produced 10 times more ASvicR but 3-fold less VicR protein (Fig. 3D). However, planktonic cells grown in BHI with 1% sucrose showed decreased ASvicR levels and increased production of VicR. Thus, there is an inverse relationship between ASvicR levels and VicR production, which is consistent with the paradigm of the ASvicR-vicR mRNA complex blocking its translation. Our previous study suggested that expression of the *vicR* gene is elevated during biofilm growth in BHI with 0.1% sucrose (Stipp et al. 2013). In this study, ASvicR expression could not be detected in biofilm cells grown without an additional carbon source (Fig. 3C). Possibly, a reduction in carbon source is an environmental signal for decreased expression of ASvicR leading to increased VicR production.

To determine whether ASvicR RNA activity played a role in cariogenic phenotypes of *S. mutans*, we constructed ASvicR overexpression mutant strains. The effects of ASvicR overexpression on VicR production were tested with planktonic cells grown with 1% sucrose, a condition in which ASvicR expression was low and VicR protein production was elevated in *S. mutans* UA159 parent cells (Fig. 3C, D). The ASvicR strains showed a significant increase in ASvicR expression (i.e., 2.5-fold over that of the parent strain; Fig. 5C) and a 2-fold decrease in VicR protein (Fig. 5A), suggesting that ASvicR-mRNA duplexes could interfere with and inhibit *vicR* gene transcription and translation. To provide further confirmation, we evaluated the biomass of the 24h biofilm, and the results showed that the biomass of ASvicR-overexpressing strains was significantly

decreased as compared with that of UA159 (Fig. 5D). Thus, these analyses of biofilm formation again confirm that VicR protein and ASvicR RNA function in biofilm formation by *S. mutans*. However, a limitation of the present study is the absence of direct evidence showing that ASvicR-mRNA duplexes can be disrupted by rnc-encoded RNase III. Whether additional RNase activities are associated with ASvicR-mRNA degradation remains to be explored.

In summary, the downstream genes *rnc*, *smc*, *syfA*, *smu.1511*, and *syfB* are cotranscribed with the *vicRKX* operon in *S. mutans* UA159. VicR is autoactivated, and *rnc* and *syfA* are directly regulated by VicR. The expression of VicR protein correlated inversely with the levels of a *vicR* AS RNA transcript and growth conditions. Furthermore, we show that overexpression of ASvicR leads to a reduction in biofilm formation. This model of ASvicR-*vicR* mRNA regulation is depicted in Figure 5E. Collectively, the data suggest a role for ASvicR as a posttranscriptional modulator of the VicR regulator in *S. mutans*.

Author Contributions

L. Lei, M.J. Duncan, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; R.N. Stipp, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; T. Chen, S.Z. Wu, contributed to data acquisition and analysis, critically revised the manuscript; T. Hu, contributed to conception, design, data acquisition, analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

L.L. was supported by grants 81670980 and 81771068 from the National Natural Science Foundation of China and grant 2017SCU12054 from the Fundamental Research Funds for the Central Universities. R.N.S. was supported by grant 2017/03263-3 from FAPESP (São Paulo Research Foundation) and grant 4005-CAPES (Coordination of Improvement of Higher Level Personnel) from PROAP (Postgraduate Support Program). M.J.D. was supported by grants R01-DE24308 and R01-DE024468 from the National Institutes of Health / National Institute of Dental and Craniofacial Research. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

ORCID iD

R.N. Stipp <https://orcid.org/0000-0001-5305-1299>

References

- Attaiech L, Minnen A, Kjos M, Gruber S, Veening JW. 2015. The ParB-parS chromosome segregation system modulates competence development in *Streptococcus pneumoniae*. *Mbio*. 6(4):e00662.
- Ayala E, Downey JS, Mashburn-Warren L, Senadheera DB, Cvitkovitch DG, Goodman SD. 2014. A biochemical characterization of the DNA binding activity of the response regulator VicR from *Streptococcus mutans*. *PLoS One*. 9(9):e108027.
- Bartual SG, Straume D, Stamsås GA, Muñoz IG, Alfonso C, Martínez-Ripoll M, Håvarstein LS, Hermoso JA. 2014. Structural basis of PcsB-mediated cell separation in *Streptococcus pneumoniae*. *Nat Commun*. 5:3842.
- Bustin SA, Benes V, Garson JA, Hellems J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, et al. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 55(4):611–622.
- Chen J, Li T, Zhou X, Cheng L, Huo Y, Zou J, Li Y. 2017. Characterization of the clustered regularly interspaced short palindromic repeats sites in *Streptococcus mutans* isolated from early childhood caries patients. *Arch Oral Biol*. 83:174–180.
- Dalton TL, Scott JR. 2004. CovS inactivates CovR and is required for growth under conditions of general stress in *Streptococcus pyogenes*. *J Bacteriol*. 186(12):3928–3937.
- Dmitriev A, Mohapatra SS, Chong P, Neely M, Biswas S, Biswas I. 2011. CovR-controlled global regulation of gene expression in *Streptococcus mutans*. *PLoS One*. 6(5):e20127.
- Dornenburg JE, Devita AM, Palumbo MJ, Wade JT. 2010. Widespread antisense transcription in *Escherichia coli*. *MBio*. 1(1):e00024-10.
- Downey JS, Mashburn-Warren L, Ayala EA, Senadheera DB, Hendrickson WK, McCall LW, Sweet JG, Cvitkovitch DG, Spatafora GA, Goodman SD. 2014. In vitro manganese-dependent cross-talk between *Streptococcus mutans* VicK and GcrR: implications for overlapping stress response pathways. *PLoS One*. 9(12):e115975.
- Dubrac S, Bisicchia P, Devine KM, Msadek T. 2008. A matter of life and death: cell wall homeostasis and the WalKR (YycGF) essential signal transduction pathway. *Mol Microbiol*. 70(6):1307–1322.
- Dubrac S, Msadek T. 2004. Identification of genes controlled by the essential YycG/YycF two-component system of *Staphylococcus aureus*. *J Bacteriol*. 186(4):1175–1181.
- Duque C, Stipp RN, Wang B, Smith DJ, Höfling JF, Kuramitsu HK, Duncan MJ, Mattos-Graner RO. 2011. Downregulation of GbpB, a component of the VicRK regulon, affects biofilm formation and cell surface characteristics of *Streptococcus mutans*. *Infect Immun*. 79(2):786–796.
- Fukuchi K, Kasahara Y, Asai K, Kobayashi K, Moriya S, Ogasawara N. 2000. The essential two-component regulatory system encoded by *yycF* and *yycG* modulates expression of the *ftsAZ* operon in *Bacillus subtilis*. *Microbiology*. 146(Pt 7):1573–1583.
- Ho EC, Donaldson ME, Saville BJ. 2010. Detection of antisense RNA transcripts by strand specific RT-PCR. *Methods Mol Biol*. 630:125–138.
- Ji Y, Zhang B, Van SF, Horn, Warren P, Woodnutt G, Burnham MK, Rosenberg M. 2001. Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA. *Science*. 293(5538):2266–2269.
- Krinke L, Wulff DL. 1987. OOP RNA, produced from multicopy plasmids, inhibits lambda cII gene expression through an RNase III-dependent mechanism. *Genes Dev*. 1(9):1005–1013.
- Krinke L, Wulff DL. 1990. RNase III-dependent hydrolysis of lambda cII-O gene mRNA mediated by lambda OOP antisense RNA. *Genes Dev*. 4(12A):2223–2233.
- Mao MY, Li M, Lei L, Yin JX, Yang YM, Hu T. 2018. The regulator gene *rnc* is closely involved in biofilm formation in *Streptococcus mutans*. *Caries Res*. 52(5):347–358.
- Mao MY, Yang YM, Li KZ, Lei L, Li M, Yang Y, Tao X, Yin JX, Zhang R, Ma XR, et al. 2016. The *rnc* gene regulates exopolysaccharides and affects the VicRK signal transduction system via microRNA- size small RNAs in *Streptococcus mutans*. *Front Microbiol*. 7:687.
- Mattos-Graner RO, Porter KA, Smith DJ, Hosogi Y, Duncan MJ. 2006. Functional analysis of glucan binding protein B from *Streptococcus mutans*. *J Bacteriol*. 188(11):3813–3825.
- Minnen A, Attaiech L, Thon M, Gruber S, Veening JW. 2011. SMC is recruited to oriC by ParB and promotes chromosome segregation in *Streptococcus pneumoniae*. *Mol Microbiol*. 81(3):676–688.
- Nishikawa K, Duncan MJ. 2010. Histidine kinase-mediated production and autoassembly of *Porphyromonas gingivalis* fimbriae. *J Bacteriol*. 192(7):1975–1987.
- Nishikawa K, Yoshimura F, Duncan MJ. 2004. A regulation cascade controls expression of *Porphyromonas gingivalis* fimbriae via the FimR response regulator. *Mol Microbiol*. 54(2):546–560.
- Rasmussen S, Nielsen HB, Jarmer H. 2009. The transcriptionally active regions in the genome of *Bacillus subtilis*. *Mol Microbiol*. 73(6):1043–1057.
- Régnier P, Grunberg-Manago M. 1990. RNase III cleavages in non-coding leaders of *Escherichia coli* transcripts control mRNA stability and genetic expression. *Biochimie*. 72(11):825–834.
- Senadheera MD, Guggenheim B, Spatafora GA, Huang YC, Choi J, Hung DC, Treglown JS, Goodman SD, Ellen RP, Cvitkovitch DG. 2005. A VicRK signal transduction system in *Streptococcus mutans* affects *gtfBCD*, *gbpB*, and *fff* expression, biofilm formation, and genetic competence development. *J Bacteriol*. 187(12):4064–4076.

- Senadheera MD, Lee AW, Hung DC, Spatafora GA, Goodman SD, Cvitkovitch DG. 2007. The *Streptococcus mutans* *vicX* gene product modulates *gffB/C* expression, biofilm formation, genetic competence, and oxidative stress tolerance. *J Bacteriol.* 189(4):1451–1458.
- Silvaggi JM, Perkins JB, Losick R. 2006. Genes for small, noncoding RNAs under sporulation control in *Bacillus subtilis*. *J Bacteriol.* 188(2):532–541.
- Smith EG, Spatafora GA. 2012. Gene regulation in *S. mutans*: complex control in a complex environment. *J Dent Res.* 91(2):133–141.
- Stipp RN, Boisvert H, Smith DJ, Höfling JF, Duncan MJ, Mattos-Graner RO. 2013. CovR and VicRK regulate cell surface biogenesis genes required for biofilm formation in *Streptococcus mutans*. *PLoS One.* 8(3):e58271.
- Tan FY, Wörmann ME, Loh E, Tang CM, Exley RM. 2015. Characterization of a novel antisense RNA in the major pilin locus of *Neisseria meningitidis* influencing antigenic variation. *J Bacteriol.* 197(10):1757–1768.
- Thomason MK, Storz G. 2010. Bacterial antisense RNAs: how many are there, and what are they doing? *Annu Rev Genet.* 44:167–188.
- Tremblay YD, Lo H, Li YH, Halperin SA, Lee SF. 2009. Expression of the *Streptococcus mutans* essential two-component regulatory system VicRK is pH and growth-phase dependent and controlled by the LiaFSR three-component regulatory system. *Microbiology.* 155(Pt 9):2856–2865.
- Wang B, Kuramitsu HK. 2005. Inducible antisense RNA expression in the characterization of gene functions in *Streptococcus mutans*. *Infect Immun.* 73(6):3568–3576.
- Wen Y, Feng J, Scott DR, Marcus EA, Sachs G. 2011. A *cis*-encoded antisense small RNA regulated by the HP0165-HP0166 two component system controls expression of *ureB* in *Helicobacter pylori*. *J Bacteriol.* 193(1):40–51.