# Carbohydrate Metabolism Regulated by Antisense *vicR* RNA in Cariogenicity

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## Appendix

## Materials and Methods

### *Ethics statement and clinical specimens*

Clinical strains used in this study were isolated from early childhood caries (ECC) patients who originated from the West China Hospital of Stomatology, Sichuan University (Sichuan, China). This study was approved by the Ethical Committee of West China School of Stomatology, Sichuan University (WCHSIRB-D-2015-084). For animal experiments, approval was obtained from the Ethics Committee of West China Hospital of Stomatology (WCHSIRB-D-2018-083). Isolates of strains were kindly provided as described previously (Chen et al, 2017). Children, aged from 3 to 5 years old, were recruited from the pediatric department of West China Hospital of Stomatology for this study. They were divided into two groups: caries-free (CF) children and ECC children. For each participant, dmft scoring was performed by Pediatric Department of West China Hospital of Stomatology. This study was approved by the Institutional Ethical Committee of West China School of Stomatology, Sichuan University (WCHSIRB-D-2015-084). Dental plaque from the buccal surfaces of anterior teeth and the first mandibular molar was obtained using sterile dental probes and pooled. The pooled samples were kept in sterilized tubes containing PBS buffer on ice and transferred to the lab within 2 hours. Samples were diluted and plated onto mitis salivarius (MS) agar (Difco Laboratories, Detroit, USA) supplemented with 0.2 U/ml bacitracin (Sigma Chemical Co., St Louis, USA) and 15%(wt/vol) sucrose (Saravia et al., 2013). Plates were incubated at 37°C in an anaerobic atmosphere for 48 hours. Five colonies were randomly chosen from each sample based on their morphology. *S. mutans* strains were identified by their morphological, biochemical and physiological characteristics, as well as 16S rRNA gene sequencing identification as previously described (Chen et al, 2017). Ten CF clinical isolates and ten ECC strains were involved in present study and used for the comparisons of gene expression.

The details of the participants are listed in the **Appendix Table 1**.

***Bacterial strains and growth conditions***

Bacterial strains and plasmids used in this study are listed in **Appendix Table 2.** The *S. mutans* UA159 strain was provided by the State Key Laboratory of Oral Diseases (Sichuan University, Chengdu, China). *S. mutans* strains were routinely grown in Brain Heart Infusion (BHI) media as described previously (Lei et al, 2015). Spectinomycin (spec, 500 μg/mL) were added to the media when necessary. *Escherichia coli* (*E. coli*) was grown aerobically in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/mL) as needed. *S. mutans* cells were cultured to mid-exponential phase (optical density at 600 nm [$OD_{600}$] 0.5) in BHI medium and *E. coli* strains were grown overnight in 500 mL LB broth, respectively. For mutant and wild-type strains, a total of 50 μL of mid-log-phase cells was inoculated in triplicate into 1mL BHI medium. To generate growth curves, a Spec 2000 reader was programmed to monitor the $OD_{600}$ at 37°C every 60 min for 24 h.

***Construction of recombinant t4rnl1 plasmid***

The shuttle vector pDL278 was used to express the *t4rnl1* sequence under the control of the *S. mutans* endogenous promoter region of the *vicR* gene (**Appendix Figure 1**). First, the *t4rnl1* promoter sequences were obtained by oligonucleotide synthesis (Sangon Biotech, Shanghai, China). Next, the sequences were cloned into vector pDL278 at SmaI and SalI restriction sites, generating recombinant plasmid pDL278_t4rnl1, which was transformed into *S. mutans* UA159 and *rnc* deletion (Smurnc) strains. For the transformation, *S. mutans* cultures were grown to mid-exponential phase, and the competence stimulating peptide (CSP) was added to the culture to achieve a final concentration of 1 μg/mL. Recombinant pDL278 vectors were simultaneously added to the culture and incubated for 60 min (Senadheera et al, 2007). UA159_t4rnl1 cells were isolated using BHI plates, which contained 500 μg/mL spectinomycin for selection. Smurnc_t4rnl1 strains containing the pDL278_t4rnl1 recombinant plasmid were used as a control. Shuttle vectors (pDL278) were constructed to express the *vicR* sequence under the control of the *vicR* gene promoter region. The vicR and promoter sequences were obtained by oligonucleotide and gene synthesis (Sangon Biotech, Shanghai, China). All expression plasmids were verified using PCR

and DNA sequencing analyses.

***Isolation of RNA and cDNA reverse transcription for RT-PCR assays***

For total RNA extraction, *S. mutans* strains were harvested at mid-exponential phase in BHI media, supplemented with 1% sucrose. In brief, overnight *S. mutans* cultures in BHI media supplemented with 10 μg/mL erm and/or 500 μg/mL spec, were diluted into fresh BHI media containing 1% sucrose and grown to mid-exponential phase (optical density at 600 nm [$OD_{600}$] 0.3). The growth of biofilms was established in 12-well plates after 24 hours and cells were harvested by scraping as previously described (Lei et al, 2015; Mao et al, 2016). Total RNA was isolated from *S. mutans* cells using a MasterPureTM RNA purification Kit from Epicentre Technologies (Epicentre, Madison, WI, USA) in accordance with the recommendations of the supplier. Contaminating genomic DNA was removed by digestion with Turbo RNase-free DNase I (Ambion, Austin, TX, USA) according to the manufacturer's instructions. DNA contamination was assessed by PCR amplification using *gryA* gene primers (**Appendix Table 3**), and agarose gel electrophoresis. The purity (A260/A280) and concentration of RNA were determined using a NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA was reverse transcribed to cDNA using random hexamer primers or gene-specific primers (**Appendix Table 3**) using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA).

***Transcription analysis by quantitative RT-PCR***

Real-time PCR was carried out with a Bio-Rad Applied Biosystems ABI 7500 System (Bio-Rad Laboratories, Hercules, CA, USA) using a Quantitect SYBR-Green PCR kit (QIAGEN, Valencia, CA, USA). For each real-time PCR reaction, 20 μL of a mixture containing 10 μL SYBR Premix Ex TaqII, 2.0 μL template cDNA, 1μL 10 μM PCR Forward Primer, 1μL 10 μM PCR Reverse Primer, 0.4μL ROX Reference Dye, and 5.6μL deionized water was placed in each well. Primers used in real-time PCR reactions are shown in **Appendix Table 3** and were obtained commercially (Sangon Biotech, Shanghai, China). Conditions for Real-time PCR were as follows: 95°C, 3 min (initial denaturation), followed by 35 cycles of 95°C for 30 s (denaturation), 55°C for 30s (primer annealing), and 72°C for 30s (extension). For msRNAs, stem-loop quantitative real-

time PCR (Stem-loop RT-PCR) using SYBR Green was performed (RiboBio, Guangzhou, China) following the manufacturer's protocol. For technical replicates, reactions were carried out in triplicate. Threshold cycle values (CT) were quantified and the expression of each gene was normalized relative to the expression of the *gyrA* gene, which was used as an internal reference. Data were calculated according to the $2^{-\Delta\Delta CT}$ method (Bustin et al, 2009). To confirm that a single PCR product was amplified, melting curve analysis was performed as follows: 95°C for 1 min, 55°C for 1 min, and 55 to 95°C at a rate of 0.5°C per 10 s.

***Northern blotting***

Northern blot assays were performed as previously described with some modifications (Stipp et al, 2013; Biswas et al, 2007). Briefly, equal amounts (10 μg) of total RNA was purified from *S. mutans* cultures. Samples were separated by electrophoresis in 1% formaldehyde-agarose gels and transferred to positively charged Nylon membranes. The probe sequence was obtained by PCR reactions (oligonucleotides was listed in **Appendix Table 3**). The probes were labeled using the DIG Luminescent System (Roche). Blots were incubated with 10 ng/mL probes at 50°C overnight, and signals were detected using the CSPD Star substrate (Roche) according to the manufacturer instructions.

***Protein production analysis by Western blotting***

*S. mutans* cells grown as planktonic cultures were harvested at mid-exponential phase (optical density at 600 nm [$OD_{600}$] 0.3) in 15 mL of BHI media, supplemented with 1% sucrose. For the measurement of GtfB, GtfC, and GtfD produced in culture fluids, the supernatant was filtered through a 0.22-μm pore size filter membrane (Corning Incorporated, New York, NY, USA), followed by dialysis against 0.2 mM sodium phosphate, containing 10 μM phenylmethylsulfonyl fluoride (PMSF) as a proteinase inhibitor. After dialysis, samples were lyophilized to dryness as described (Matto-Graner et al, 2004). To evaluate the VicR production, harvested cells were harvested, washed and re-suspended in 300 μL cold phosphate buffered saline (PBS, pH 7.3). Cells were mechanically disrupted by a FASTPREP Beater apparatus (MP Biomedicals, Irvine, CA, USA) using glass beads (diameter, 0.1 mm) for three cycles of 20 sec. Clear supernatants were

collected by centrifugation (13000 rpm, 2 min, 4°C) and protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (BioRad, Hercules, CA, USA) according to the manufacturer's instructions.

For western blot analysis, equal amounts of protein (30 μg) were mixed with 2X SDS-PAGE Sample Loading Buffer (Beyotime Biotech, Shanghai, China) in boiling water for 10 min and loaded on 10% SDS-PAGE gels (Bio-Rad). Proteins were fractionated and then electrotransferred to polyvinylidene fluoride (PVDF) membranes (Biosharp Biotech, Shanghai, China). Membranes were blocked in 100 mM Tris-HCl, 2.5 mM NaCl (pH 7.5) containing 5% w/v nonfat dry milk at room temperature for 2 h. For measurement of GtfB, GtfC, and GtfD production, membranes were incubated with monoclonal antibodies (MAbs): anti-GtfB, anti-GtfC, and anti-GtfD at dilutions of 1:60, 1:30, and 1:60, respectively as previously described (Mattos-Graner et al, 2004) for 2 h of incubation at room temperature. Then membranes were washed in Tris-buffered saline containing 0.1% Tween 20, pH 7.5. Incubation with goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase (Amersham Biosciences, Piscataway, N.J.) was then performed at room temperature for 2 h. For measurement of VicR production, membranes were incubated with purified VicR-specific antibodies (1:500, AbMax Biotechnology, Beijing, China) for 2 h at room temperature, washed in Tris-buffered saline containing 0.1% Tween 20, and incubated with horse-radish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (1:5,000) for 2 h at room temperature as previously described (Lei et al, 2018). Protein immunoreactive bands were visualized using an Immobilon Western Chemiluminescent kit (Millipore, Billerica, MA, USA). A Quantity One Software Imaging Densitometer (Vision 4.62, Bio-Rad, Hercules, CA, USA) was used to analyze the signal density of the protein bands. To estimate the protein production by *S. mutans* strains, the respective intensity values were divided by the intensity for the UA159 control strain.

***Immunoprecipitation Analyses***

The chromatin immunoprecipitation (ChIP) assay was performed using a method previously described (Nishikawa et al, 2004) with minor modifications. In brief, *S. mutans* UA159 cultures

in 200 mL BHI media were grown to mid-exponential phase ($OD_{600}$ 0.3), followed by the addition of 1% formaldehyde to form cross-links of DNA-protein complexes *in vivo*. Then, UA159 cells were washed twice with cold PBS buffer and resuspended in 2 mL lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM NaCl, and 10 mg/ml lysozyme). A total of 2 μL of immunoprecipitation (IP) buffer (50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton-100, 0.1% sodium deoxycholate, and 1 mM PMSF) was added to UA159 cell suspensions. Precipitation of DNA-protein complexes was performed as previously described (Scott et al, 2013), using a purified anti-FLAG antibody and anti-IgG antibody as a negative control, and was carried out overnight at 4°C. Then, the soluble suspension was incubated with protein A/G agarose (Millipore, Bedford, MA, USA) on a rotating plate for 2 h at 4°C. Antibody–protein–DNA complexes were eluted twice with 50 μL elution buffer (1% SDS, TE pH 8.0, 10 mM DTT) as the output precipitate. Specific precipitation of DNA-VicR protein complexes in input, output, or negative precipitates was confirmed by western blot analysis as described above. For PCR analyses, 50% output and 3% of the immunoprecipitated input samples were diluted with Tris-HCl (pH 8.0) and reverse cross-linked for 12 h at 65°C in the presence of proteinase K. The FLAG-RIP-ChIP approach was performed as previously described (Chen et al, 2015). The monoclonal antibody against FLAG was purchased (Sangon Biotech, Shanghai, China). Briefly, cell lysates were collected and cleared with preblocked Protein G beads and incubated with Pierce™ Co-Immunoprecipitation Kit (Thermo Scientific). As controls, IP with a nonimmune serum was performed in parallel.

***Production of Recombinant Rnc and RNase III activity assays***

To generate Rnc-His-Tag fusion proteins, the ORF was obtained by oligonucleotide synthesis (Sangon Biotech). Next, the NdeI- and XhoI-digested ORF sequences were cloned into pET-21a (Novagen) with His-Tag to yield pET-rnc. Plasmids were sequenced and then transformed into *E. coli* BL21, and recombinant proteins were isolated from 500 mL of overnight culture after 3 h of induction with 1 mM IPTG ($OD_{600}$ 0.5). After cell lysis, recombinant proteins were purified by affinity chromatography on $Ni^{2+}$ NTA agarose (Qiagen) as previously described (Stipp et al, 2013).

The purity/integrity of the purified protein was visualized by Coomassie staining after SDS-PAGE. Polyclonal antibodies to r-Rnc were produced using the standard 77 Days Rabbit Protocol (AbMax). For RNase activity assays, total RNAs were extracted from the UA159, AS*vicR* and VicR+ strains and purified as previously described. Equal amounts of RNA (2 μg) were incubated with 4 nM of recombinant Rnc in 20 μL interaction buffer (10 mM Tris-HCl, pH=8.0) for 30 min at 37°C. The mixtures were assessed by agarose gel electrophoresis.

***Detection of bacterial growth and biofilm structural formation***

Bacterial strains were cultured at 37℃ in BHI media and growth curves were prepared by measuring the OD values at 600 nm. We used scanning electron microscopy (SEM) of *S. mutans* UA159, ASvicR, and vicR+ 6 h biofilm to observe their morphological and physiological characteristics. For the biofilm establishment, *S. mutans* UA159, AS*vicR*, and vicR+ strains were routinely grown in Brain Heart Infusion (BHI) media as described previously (Lei et al, 2015). Spectinomycin (spec, 500 μg/mL) were added to the media when necessary. Overnight *S. mutans* cultures were grown to mid-exponential phase (optical density at 600 nm [$OD_{600}$] 0.5) in BHI medium. The suspension of S. mutans UA159, ASvicR, and vicR+ strains were followed by diluting 0.1 mL culture into 2 mL of BHI supplemented with 1% sucrose at the same ratio. Biofilms of *S. mutans* UA159, ASvicR, and vicR+ strains were established in 24-well plates for 6 hours and prepared. For observation of biofilm matrix, *S. mutans* biofilms at 24 h on glass plates were washed twice with PBS and fixed with 2.5% glutaraldehyde at room temperature for 4 h. Serial dehydration included preparations with ethanol solutions (30%, 50%, 70%, 95%, and 100%), critical-point drying with liquid $CO_2$, and coating with gold powder. Biofilm specimens were evaluated via scanning electron micrographs we obtained by a scanning electron microscope (Inspect Hillsboro, OR, USA).

For the assessment of biomass structure, the EPS matrix of *S. mutans* biofilms was stained with Alexa 647-labeled dextran conjugate (Invitrogen, Eugene, OR, USA), and bacterial cells in the biofilm were labeled with SYTO9 (Invitrogen, Carlsbad, CA, USA). Next, confocal laser scanning microscopy was performed using a microscope (CLSM, TSP SP2; Leica, Solms,

Germany) at 63× magnification using an oil-immersion objective lens. Three-dimensional reconstruction of the biofilms as well as imaging biomass quantification was analyzed using Imaris 7.0 software (Bitplane, Zurich, Switzerland). Experiments were performed in triplicate for three randomly selected areas of each specimen.

For atomic force microscopy (AFM) assessment, *S. mutans* biofilms were rinsed twice with PBS and dried for 10 min in air. AFM procedures were performed using an SPM-9500J2 (Shimadzu, Tokyo, Japan) in the contact mode (Lei et al, 2015), and the bacterial adhesion force was calculated (lijima et al, 2012). For each measurement, the probe was positioned over the center of a bacterial surface and ten force cycles were recorded for five randomly-selected bacterial cells in the biofilms (Ivanov et al, 2011).

***Exopolysaccharide measurements***

*S. mutans* biofilm cells cultured in 12-well plates for 24 h in BHI media, supplemented with 1% sucrose, were collected by scraping, and were resuspended in PBS. Water-insoluble glucan (WIG) and water-soluble glucan (WSG) of *S. mutans* biofilms was isolated as previously described (Lei et al, 2015). The supernatant was filtered through a 0.22-μm pore size filter membrane (Corning Incorporated, New York, NY, USA) and separated for WSG measurement using the anthrone method (Lei et al, 2015). Precipitated cells were obtained by centrifugation (12000rpm, 4℃, 10min) and washed twice with 4 mL of 0.4 M NaOH. For WIG assessment, 200 μL of soluble suspension was mixed with 600 μL of anthrone reagent. The mixtures were heated at 95 °C for 10 min and put on ice. The absorbance of each sample at 625 nm was measured using a microplate reader (Gene Co., Hong Kong, China). The corresponding polysaccharide concentration was calculated according to a prepared standard curve with a dextran standard (**Appendix Figure 2**).

Exopolysaccharide extraction and purification from *S. mutans* biofilms were performed according to our previous protocol (Lei et al, 2015). The polysaccharide fractions that were washed with distilled water were pooled and lyophilized to dryness.

The Gel permeation chromatography (GPC) is a type size exclusion chromatography which is used for the analysis of polymers (Lathe et al, 1956). In present study, the average molecular weights of the polysaccharides from UA159, AS*vicR*, and vicR+ biofilms were determined according to our previous report (Lei et al, 2015). An Agilent Technologies 1200 series HPLC was included

and equipped with a gel permeation chromatographic column (7.8 ×300 mm) (TSKgel G5000PW, Tosoh Bioscience, Tokyo, Japan) at 40°C. The corresponding molecular weights of the polysaccharides were calculated using the Agilent GPC software. The monosaccharide composition analysis was conducted using the acetylation method (Fujii et al, 2012). The purified exopolysaccharide samples from *S. mutans* biofilms were treated according to our previous protocols (Lei et al, 2015; Yang et al, 2019). The monosaccharide analysis was proceeded via GC/MS (Gas Chromatography-Mass Spectrometry, Agilent, 19091J-413) which is equipped with a chromatographic column (HP-5, Agilent). Each exopolysaccharide sample was technically replicated three times.

***RNA extraction and RNA-Seq performance***

Samples of UA159, AS*vicR* or Smurnc were grown in fresh BHI media supplemented with 1% sucrose in biofilm or planktonic growth. Total RNA was isolated as described above. RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific), and RNA quality was evaluated using an Agilent 2100 bioanalyzer (Agilent Technologies). All RNAs were determined to have RNA integrity numbers (RIN) of 9.8 and above. Removal of rRNA was performed using a Ribo-Zero$^{TM}$ rRNA Removal Kit for Gram-Positive Bacteria (Epicentre) according to the supplier's specifications. The final quality and purity of enriched bacterial mRNA samples was analyzed using an Agilent Bioanalyzer (Agilent Technologies). From enriched mRNA samples, cDNA libraries were prepared using a Truseq$^{TM}$ RNA sample prep kit from Illumina following instructions from the supplier. Subsequently, RNA-Seq was performed on a HiSeq 4000 (2×150 bp read length) by the NextGen DNA Sequencing Core Laboratory of Majorbio Biotechnology Research (Shanghai, China). Read mapping was performed on a Galaxy server with Bowtie2 for Illumina (He et al, 2017). Reads were mapped to the genome of *S. mutans* UA159 (https://www.ncbi.nlm.nih.gov/genome/?term=AE01413).

***Statistical analysis of RNA-Seq data and data validation***

Fold-changes and significant differences in gene expression between growth conditions were calculated using edgeR (http://www.bioconductor.org/packages/2.12/ bioc/html/edgeR.html) (Moulos et al, 2015). Gene Ontology (GO) terms were assigned to genes using Blast2GO

(http://www.blast2go.com/b2ghome) (Gotz et al, 2008). Relative enrichment (overrepresentation) of GO terms for upregulated genes compared to a background of GO terms for all genes was assessed using Fisher's exact tests. In addition, a false discovery rate (FDR) procedure was used to correct for multiple hypothesis testing (FDR < 0.05). To gain further insight into the effects of AS *vicR* RNA on *S. mutans*, we performed Kyoto encyclopedia of genes and genomes (KEGG) pathway impact analysis using the software package Pathway-Express. By combining all evidence in gene expression level data, pathway impact was calculated, and the FDR procedure was used to correct for multiple hypothesis testing (FDR < 0.05). To validate the RNA-Seq data, quantitative real-time PCR (qRT-PCR) was conducted to measure changes in mRNA expression of selected genes as described above.

**Electrophoretic mobility shift assays (EMSA)**

EMSA were used to determine whether the *lacA* gene was directly regulated by VicR as previously described (Stipp et al, 2013) with modifications. PCR amplicons of the *lacA* gene promoter region were generated from *S. mutans* UA159 genomic DNA using primers listed in **Appendix Table 3**. The DNA fragments were purified and labeled with the DIG Gel Shift Kit (Roche). Recombinant VicR protein was incubated (30 min, 25°C) with 50 mM acetyl phosphate for phosphorylation prior to binding (Stipp et al, 2013). Increasing amounts of phosphorylated recombinant VicR (0, 20, 30, 40, and 60 pmols) with labeled DNA probes (0.02 pmol), and 100-fold excess of unlabeled DNA fragments (cold DNA) as competitor (Stipp et al, 2013) were used for EMSA. Binding reactions were carried out in 20 μL volumes containing reaction buffer as previously described (Lei et al, 2018). After incubation for 20 min at 25°C, the reactions were mixed with (5X) Hi-Density TBE Sample Buffer (Invitrogen), and loaded onto the 6% polyacrylamide precast gels (Stipp *et al*, 2013) in 0.5×TBE buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA, pH 8.0). Protein-DNA complexes were separated at 4°C (100 V, 3 h), transferred to positively charged nylon membranes (GE Health Care). The membranes containing protein-DNA complexes were washed, incubated with anti-digoxigenin antibody (Roche), and

detected using the DIG wash and block buffer set (Roche) according to the manufacturer's instructions.

***Animal study***

Animal experiments were reviewed and approved by the Ethics Committee of West China Hospital of Stomatology, Chengdu, China (Number of permit: WCHSIRB-D-2018-083). To investigate the effects of UA159, AS*vicR* and VicR+ mutants on the formation of dental plaque and caries *in vivo*, a rat model was used under specified pathogen-free (SPF) conditions as previously described with some modifications (Bowen et al, 1988). Forty caries-susceptible, Wistar rats (male, 3 weeks of age) were included (IVC Experimental Animal Center of Public Health, Sichuan University, Chengdu, China). Before the infection, rats were randomized into four experimental groups: UA159 as a positive control, AS*vicR* group, VicR+ group, and a blank group, which served as the negative control ($n$=10 rats per group). On days 1 to 7, each rat was infected orally with 200 μL of a bacterial suspension that contained either the UA159 strain, AS*vicR* strain, or the VicR+ strain and 5% sucrose water ad libitum. Rats were weighed twice a week and then their physical appearance was routinely recorded. On day 21, animals were sacrificed by $CO_2$ asphyxiation. The lower jaws were aseptically removed and divided into two pieces. After being sonicated in PBS twice, the lower jaws were dissected and immersed in 10% buffered formalin phosphate for 48 h. The teeth were observed under a stereomicroscope and caries were scored according to a modified Keyes score (Keyes, 1958; Koo et al, 1999; Su et al, 2014). The dental plaque on the tooth surface was stained with Alexa 647-labeled dextran conjugate (Invitrogen, Eugene, OR, USA), and bacterial cells in the biofilm were labeled with SYTO9 (Invitrogen, Carlsbad, CA, USA). Three-dimensional reconstruction of the biofilms as well as imaging biomass quantification was analyzed using Imaris 7.0 software (Bitplane, Zurich, Switzerland). The experimental method has been mentioned and used in our previous publications (Mao et al,2016; Yang et al, 2019).

***Data analysis***

Statistical analyses of the data were performed using SPSS 16.0 (SPSS Inc., Chicago, IL,

USA). The Shapiro–Wilk test demonstrated whether the data were normally distributed, whereas Bartlett's test was used to assess the homogeneity of variances. For parametric testing, one-way ANOVA was used to detect the significant effects of variables followed by the Student-Newman-Keuls test to compare the means of each group. For nonparametric testing, the Kruskal-Wallis test and least significant difference (LSD) multiple comparisons were used. A value of $p < 0.05$ was considered significant.

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**Appendix Table 1. Detailed information on each participant in this study**

| Patient Number | Gender | Age (Year/Month) | dmft Score | Isolation of *S.mutuans(Yes* or No) |
|---|---|---|---|---|
| 1 | Female | 3Y6M | 0 | Yes |
| 2 | Female | 3Y10M | 0 | Yes |
| 3 | Female | 4Y8M | 0 | Yes |
| 4 | Male | 4Y6M | 0 | Yes |
| 5 | Female | 3Y1M | 6 | Yes |
| 6 | Male | 3Y11M | 0 | Yes |
| 7 | Male | 4Y6M | 8 | Yes |
| 8 | Male | 4Y3M | 6 | Yes |
| 9 | Female | 4Y6M | 0 | Yes |
| 10 | Male | 3Y5M | 7 | Yes |
| 11 | Male | 4Y3M | 8 | Yes |
| 12 | Female | 3Y6M | 5 | Yes |
| 13 | Female | 3Y7M | 7 | Yes |
| 14 | Male | 4Y8M | 0 | Yes |
| 15 | Male | 5Y | 0 | Yes |
| 16 | Female | 3Y3M | 0 | Yes |
| 17 | Male | 3Y5M | 8 | Yes |
| 18 | Female | 3Y8M | 6 | Yes |
| 19 | Female | 4Y4M | 7 | Yes |
| 20 | Female | 4Y6M | 0 | Yes |

**Appendix Table 2. Bacterial strains and plasmids used in this study**

| Strains and plasmids | Relevant characteristics or purpose | Source or reference |
|---|---|---|
| **Strains** | | |
| *S. mutans* | | |
| UA159 | $Erm^s$, $spec^s$ | This study |
| UA159asvicR | pDL278 ($spec^r$): antisense vicR | This study |
| UA159vicR+ | pDL278 ($spec^r$): vicR | This study |
| UA159rnc- | Δrnc:$Erm^r$ | Mao *et al*., 2016 |
| UA159rnc+ | pDL278 ($spec^r$):rnc | Mao *et al*., 2016 |
| UA159 _t4rnl1 | pDL278 ($spec^r$): t4rnl | This study |
| Smurnc _t4rnl1 | Δrnc:Ermr with pDL278 (specr): t4rnl | This study |
| *E. coli* | | |
| DH5-α | General cloning and plasmid propagation | Novagen |
| BL21 | Expression of pET22B (+)::VicR | Novagen |
| **Plasmids** | | |
| pDL278 | ($spec^r$) Empty vector | Novagen |
| pDL278asvicR | ($spec^r$) recombinant and expression asvicR | This study |
| pDL278vicR | (specr) recombinant and expression vicR | This study |
| pDL278_t4rnl1 | (specr) recombinant and expression t4rnl | This study |
| pDL278asvicR-FLAG | (specr) expression as*vicR*+FLAG | This study |

**Appendix Table 3. Sequences of primers used for RT-PCR analysis**

| Primers | sequence 5'-3' (Forward/Reverse) | Amplification size (bp) |
|---|---|---|
| **RT-qPCR** | | |
| *gyrA* | 5'-ATTGTTGCTCGGGCTCTTCCAG-3'/ 5'-ATGCGGCTTGTCAGGAGTAACC-3' | 105 |
| *vicR* | 5'-CGCAGTGGCTGAGGAAAATG-3'/ 5'-ACCTGTGTGTGTCGCTAAGTGATG-3' | 157 |
| *vicK* | 5'-CACTTTACGCATTCGTTTTGCC-3'/ 5'-CGTTCTTCTTTTTCCTGTTCGGTC-3' | 102 |
| *vicX* | 5'-TGCTCAACCACAGTTTTACCG-3'/ 5'-GGACTCAATCAGATAACCATCAGC-3' | 127 |
| *rnc* | 5' -CAGCCTCTTGCTCTGCTAATTTT-3'/ 5' AAGTTGACGGGGATGTTTTGAT 3' | 150 |
| *gtfB* | 5'-ACACTTTCGGGTGGCTTG-3'/ 5'-GCTTAGATGTCACTTCGGTTG-3' | 127 |
| *gtfC* | 5'-CCAAAATGGTATTATGGCTGTCG-3'/ 5'-TGAGTCTCTATCAAAGTAACGCAG-3' | 136 |
| *gtfD* | 5'-AATGAAATTCGCAGCGGACTTGAG-3'/ 5'-TTAGCCTGACGCATGTCTTCATTGTA-3' | 245 |
| *ftf* | 5'-ATTGGCGAACGGCGACTTACTC-3'/ 5'-CCTGCGACTTCATTACGATTGGTC-3' | 103 |
| *gbpB* | 5'-AGCAACAGAAGCACAACCATCAG -3'/ 5'-CCACCATTACCCCAGTAGTTTCC- 3' | 150 |
| *dexA* | 5'-AGGGCTGACTGCTTCTGGAGT-3'/5'-AGTGCCAAGACTGACGCTTTG-3' | 142 |
| *lacA* | 5'-GGTATGATTGCAGCAGAAGTGT-3'/5'-CCGCCGTCATATTTAGCCTC-3' | 155 |

| | | |
|---|---|---|
| *lacB* | 5’-ACACGTACGCACTATCCCAT-3’/5’-TATAATAGCGCCGCCAAAGC-3’ | 192 |
| *lacC* | 5’-ACCAACTGTCATCAAGCCCA-3’/5’-AGCTACCGTTGAATCCCCTG-3’ | 202 |
| *lacD* | 5’-TGCTTCATCCATGCTGCTTG-3’/5’-GCTGACCATTCAACCAGACA-3’ | 150 |
| *lacE* | 5’-ACGCCTTGTTCTGGTTTGTC-3’/5’-GCACCAGTACCACCCATAGT-3’ | 178 |
| *lacG* | 5’-GCGCCAGTAAATGTTCCCAA-3’/5’-TTTGACATTGGCACCGTCTG-3’ | 191 |
| *lacR* | 5’-GTATTCACGGTGGGGCTAGA-3’/5’-CTACAGTTGTTCCCGGTCCT-3’ | 165 |
| *t4rnl1* | 5’- CTCAGATGATGTAAGTGCATCTGGAAG-3’/ 5’- CATAATTCCACGACATTCTAGTGCATC-3’ | 108 |

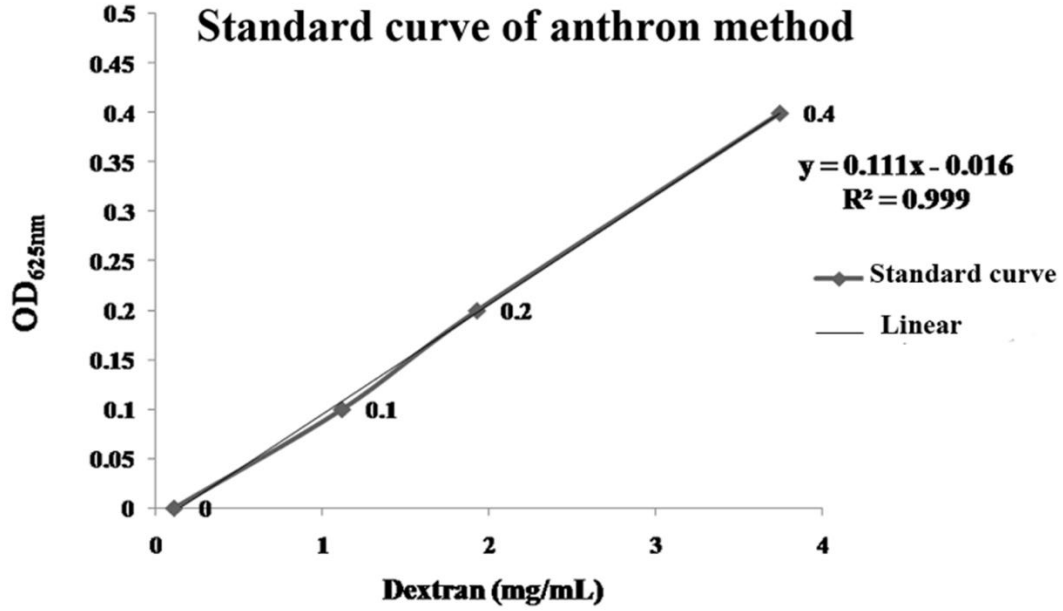
**Appendix Table 3 (continued).**

| Primers | sequence 5’-3’ (Forward/Reverse) | Relevant purpose |
|---|---|---|
| **Antisense RNA detection** | | |
| AS1 | 5’-GCCTTTGATGGGCGTG-3’ | First strand cDNA synthesis |
| PCR1 | 5’-CCAACACCGCGTCGCGTC-3’ | RT-PCR analysis |
| **Reverse transcription for noncoding RNA** | | |
| Antisense vicR | 5’-CCGCAGTGGCTGAGG-3’ | First strand cDNA synthesis |
| msRNA 1657 | 5’ - CCTGGCAGTGATGTTGCGGTCTGCCAGGTGTAAAG -3’ | First strand cDNA synthesis |
| Antisense vicR | 5’- CACGCAGACGACGAACAG -3’ | qRT-PCR analysis |
| msRNA 1657 | 5’- TATCCGAATGACCGGCGGCA -3’/ 5’- CAGTGATGTTGCGGTCT -3’ | msRNA stem-loop qRT-PCR |

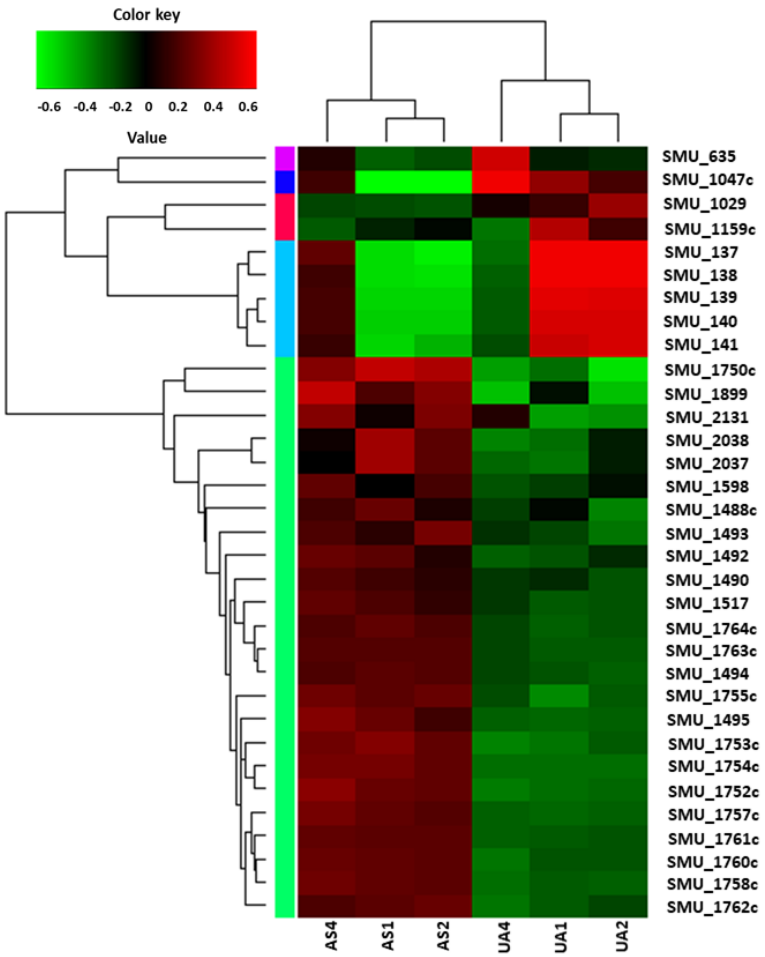
| | | |
|---|---|---|
| **mRNA-msRNA specific chimaera** | | |
| F_tRNA_Gly | 5’ –CTACAGCCTTCCAAGCTGTTGTC- 3’ | This study |
| R_tRNA_Ser | 5’-GGGATTCGAACCCACGCACG-3’ | This study |
| F_vicR-33 | 5’-CTTCTATTTTAAGACCATAAGCGAGG-3’ | This study |
| R_msRNA1657 | 5’-GCCGCCGGTCATTCGGATA-3’ | This study |
| **Primers for Northern blotting probe** | | |
| AS*vicR*_PF | 5'-CACGCAGACGACGAACA-3' | This study |
| AS*vicR*_PR | 5'-CGATTACAGCCTTTGATGG-3' | This study |
| **EMSA** | | |
| *lacA* promoter | 5'-CGTTAGATAACAAAAAGTGATC-3’ | This study |
| | 5'- AGTCATTCTCCTTGAAATGA -3’ | |

**CCCGGG**GGATCCAGTCTTCTCCTTATTTGACATAAATTTATTGTTAGCTTTTCTAACATTATA
TTGTTATACTATCATACCTTTTCTAAGATGTCAAATATTATCAGAAAATTCAGTAAGATAACTT
TCTCTAATAGTTTGAAATAAACTTGTTGGTTTTCATTTGCCATTGATTTTGATATAATCGTAGA
GGGTTACTTTAAGCAGTAAAGCTTTAAGTAAATAAAAATGGTTCTAACATAAAGTTTACTCTAA
GTGGGCTTGATATAAAAATCAGATAAAAATTAATAAAATAGCATGATTTTTAATCTTTCTTACT
TATTTCATAAACTTCTATTTTAAGACCATAAGCGAGGTATTCATA**ATG**CAAGAACTTTTTAACA
ATTTAATGGAACTATGTAAGGATTCGCAGCGTAAGTTTTTTTACTCAGATGATGTAAGTGCATC
TGGAAGAACTTACAGAATTTTCTCATATAATTATGCATCTTATTCTGATTGGTTACTTCCAGAT
GCACTAGAATGTCGTGGAATTATGTTTGAAATGGATGGAGAAAAACCAGTAAGAATTGCTTCTC
GTCCTATGGAAAAGTTTTTTAACTTGAATGAAAATCCGTTCACGATGAATATCGATTTAAACGA
TGTTGATTATATTCTAACAAAAGAAGACGGGTCTTTGGTATCAACTTATTTAGACGGTGATGAA
ATTCTGTTCAAATCAAAGGGTTCAATCAAATCTGAGCAGGCTTTAATGGCTAATGGAATTTTGA
TGAATATTAATCACCATCGGTTGCGTGATAGACTTAAAGAATTAGCTGAAGATGGATTTACTGC
TAACTTCGAATTCGTTGCCCCGACGAATAGAATCGTTCTTGCTTATCAAGAGATGAAAATTATT
TTACTGAATGTTCGTGAAAACGAAACGGGTGAATACATTTCATACGATGATATTTATAAAGATG
CTACTCTTCGTCCGTATCTAGTTGAACGATACGAAATCGATAGCCCTAAATGGATAGAAGAAGC
TAAAAATGCAGAAAACATCGAAGGCTATGTTGCTGTGATGAAAGATGGTTCTCATTTTAAAATT
AAGTCTGACTGGTACGTGTCTCTTCATAGTACAAAAAGTTCATTAGATAATCCAGAAAAATTGT
TTAAGACTATTATTGATGGTGCATCAGATGATCTTAAAGCAATGTATGCTGACGATGAATATTC
ATACAGAAAAATTGAAGCATTTGAAACGACTTATCTGAAGTACTTAGACCGAGCTCTGTTTTTA
GTTCTTGACTGTCATAATAAGCATTGCGGTAAGGATAGAAAGACTTATGCAATGGAAGCACAAG
GTGTTGCTAAAGGTGCTGGAATGGATCACCTGTTCGGTATCATCATGAGCTTATACCAGGGGTA
CGATAGTCAAGAAAAGGTCATGTGTGAAATCGAACAGAATTTTTTGAAAAATTATAAAAAATTT
ATCCCAGAAGGATAC**TAA**GTCGAC

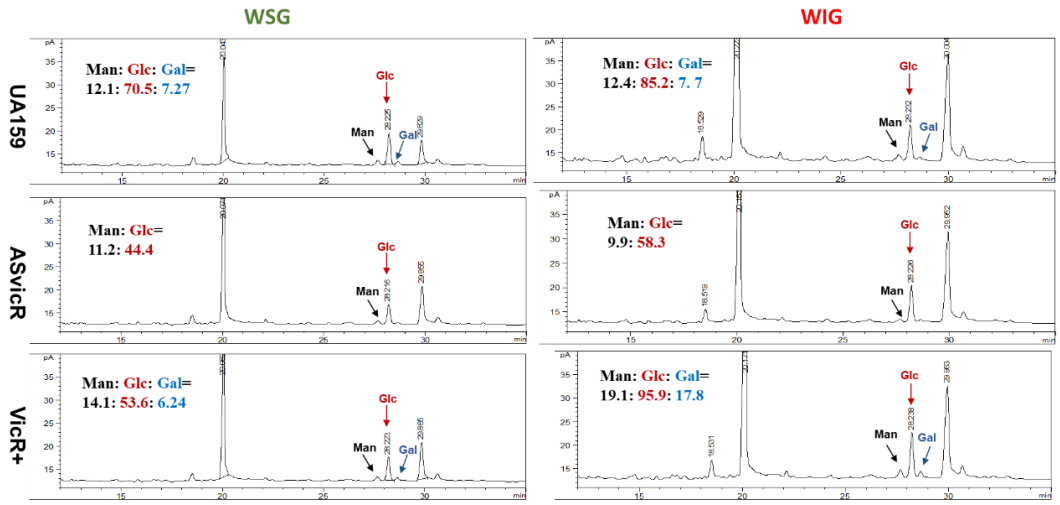
**Appendix Figure 1 The sequences for recombinant t4rnl1 and promoter region.** The start sequence of t4rnl1 is in bold and yellow. Sequence in red and bold is the starting site of the promoter region. The nucleotides in red indicate the SmaI restriction site and nucleotides in blue indicate SalI restriction site.




**Appendix Figure 2.** Standard curve for the anthrone method generated based on known concentrations of dextran.




**Appendix Figure 3. Heatmap for transcriptome analysis.** Transcriptome analysis showed that AS*vicR* RNA affected carbohydrate utilization by *S. mutans*.




**Appendix Figure 4. GC-MS (gas chromatography-mass spectrometry) for monosaccharide composition analysis** in UA159, ASvicR, VicR+ biofilms.