

Two-Component System VicRK Regulates Functions Associated with Establishment of *Streptococcus sanguinis* in Biofilms

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Streptococcus sanguinis is a commensal pioneer colonizer of teeth and an opportunistic pathogen of infectious endocarditis. The establishment of *S. sanguinis* in host sites likely requires dynamic fitting of the cell wall in response to local stimuli. In this study, we investigated the two-component system (TCS) VicRK in *S. sanguinis* (VicRK_{SS}), which regulates genes of cell wall biogenesis, biofilm formation, and virulence in opportunistic pathogens. A *vicK* knockout mutant obtained from strain SK36 (SKvic) showed slight reductions in aerobic growth and resistance to oxidative stress but an impaired ability to form biofilms, a phenotype restored in the complemented mutant. The biofilm-defective phenotype was associated with reduced amounts of extracellular DNA during aerobic growth, with reduced production of H₂O₂, a metabolic product associated with DNA release, and with inhibitory capacity of *S. sanguinis* competitor species. No changes in autolysis or cell surface hydrophobicity were detected in SKvic. Reverse transcription-quantitative PCR (RT-qPCR), electrophoretic mobility shift assays (EMSA), and promoter sequence analyses revealed that VicR directly regulates genes encoding murein hydrolases (*SSA_0094*, *cwdP*, and *gbpB*) and *spxB*, which encodes pyruvate oxidase for H₂O₂ production. Genes previously associated with *spxB* expression (*spxR*, *ccpA*, *ackA*, and *tpK*) were not transcriptionally affected in SKvic. RT-qPCR analyses of *S. sanguinis* biofilm cells further showed upregulation of VicRK targets (*spxB*, *gbpB*, and *SSA_0094*) and other genes for biofilm formation (*gtfP* and *comE*) compared to expression in planktonic cells. This study provides evidence that VicRK_{SS} regulates functions crucial for *S. sanguinis* establishment in biofilms and identifies novel VicRK targets potentially involved in hydrolytic activities of the cell wall required for these functions.

Streptococcal species represent about 80% of the microorganisms during the initial 4 to 8 h of biofilm formation on tooth surfaces (1, 2). These include *Streptococcus sanguinis*, a commensal pioneer colonizer of tooth surfaces, which is also an opportunistic pathogen of bacterial endocarditis (3). Early establishment of *S. sanguinis* during tooth eruption in children is associated with reduced colonization by the opportunistic pathogen of dental caries, *Streptococcus mutans* (4, 5). As a pioneer commensal species of teeth, *S. sanguinis* adhere to molecules of the film (also called pellicle) adsorbed to tooth surfaces, which consists mostly of salivary glycoproteins and microbial components. Adhesion involves an initial step in which the *S. sanguinis* surface adheres to pellicle components by hydrophobic and electrostatic interactions (6). Afterwards, receptor interactions with pellicle ligands, e.g., α -amylase/secretory IgA (SIgA) complexes and proline-rich proteins, take place (7). Pellicle-adherent cells provide new binding sites and promote local changes, which include production of H₂O₂, production of extracellular polysaccharides, and DNA release. These environmental changes may promote or inhibit potential microbial successors of the complex biofilm community, influencing its pathogenicity (8). Hydrogen peroxide is a major product of *S. sanguinis* metabolism which inhibits growth of *S. mutans* and influences its biofilm formation capacity (9, 10). Production of H₂O₂ likely affects cell wall homeostasis of *S. sanguinis* by mechanisms not entirely understood, promoting release of genomic DNA, which in turn seems to function as a structural component of the extracellular matrix of biofilms and as a source of genes conferring competitive advantages (11).

The VicRK two-component system (TCS) is a conserved two-component transcriptional regulatory system that is known to play essential roles in cell wall and surface biogenesis in several streptococcal species of the human microbiota and to regulate critical functions involved in biofilm formation in dental strepto-

cocci, e.g., *S. mutans* (12–14). Additionally, this TCS includes the unique response regulator (VicR) that is known to be essential in several bacterial species (15). In this study, we investigated the role of the TCS VicRK in the ability of *S. sanguinis* to form biofilms and in biofilm-associated physiological functions. The effects of inactivating the gene encoding the membrane sensor kinase of this TCS (*vicK*_{SS}) on biofilm formation and on biofilm-associated functions (cell surface properties, production of hydrogen peroxide, and extracellular amounts of genomic DNA) were analyzed in *S. sanguinis* strain SK36. Transcriptional comparisons between SK36 and the *vicK* mutant revealed genes involved in the altered phenotypes. Promoter sequence analysis and DNA interaction assays with recombinant VicR_{SS} further established direct regulation by VicR_{SS}. Finally, upregulation of VicR_{SS} gene targets during biofilm initiation supported the role of VicR_{SS} in biofilm formation.

MATERIALS AND METHODS

Strains, plasmids, oligonucleotides, and culture conditions. The strains and plasmids used in this study are described in Table 1, and oligonucleotides are described in Table S1 in the supplemental material (16, 17). All reagents were purchased from Sigma-Aldrich unless otherwise specified.

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
<i>Streptococcus</i>		
spp.		
UA159	Erm ^s Spec ^s	ATCC
UA159p	Erm ^r ; carrying pVA838	This study
UAvic	$\Delta vicK::Erm^r$	13
SK36	Erm ^s	ATCC
SK36p	Erm ^r ; carrying pVA838	This study
SKvic	$\Delta vicK::Erm^r$	This study
SKvic+	$\Delta vicK::Erm^r$; pDL278:: <i>vicK</i> ; Spec ^r	This study
<i>E. coli</i>		
DH5 α	General cloning and plasmid amplification	Invitrogen
BL21	Expression of pET22B[27]:: <i>covR</i> and pET22B[27]:: <i>vicR</i>	Novagen
Plasmids		
pVA838	Erm ^r source	16
pDL278	Spec ^r cassette; low-copy-no. vector for construction of complemented strains	17
pET22b+	Amp ^r ; empty vector for construction and expression of His-Tag VicR protein	Novagen

S. mutans UA159 and its respective *vicK* knockout mutant (UAvic) were also used in phenotypic analyses as reference strains (13, 14). *Escherichia coli* strain DH5 α was used for plasmid propagation. Streptococcal strains were grown from the frozen stocks in brain heart infusion (BHI) agar (Difco) and incubated at 37°C under aerobiosis (rotational aeration at 80 to 100 rpm or static incubation at 10% CO₂) or anaerobiosis (10% H₂, 10% CO₂, 80% N₂). Erythromycin (Erm) (10 μ g/ml) and/or spectinomycin (Spec) (300 μ g/ml) were added to medium for selection and maintenance of the respective mutant and complemented strains. The Erm selective pressure was kept in phenotypic analyses of SKvic to avoid mutant revertants, because the VicRK TCS is known to positively regulate essential genes in other streptococcal species (13, 18). The presence of Erm in the culture medium does not affect growth of SKvic under the conditions tested (data not shown).

Construction of *vicRK_s* knockout and complemented mutants. The *vicK* nonpolar knockout mutant (SKvic) was obtained from *S. sanguinis* SK36 by double-crossover recombination with a null allele constructed by PCR ligation (19). In the recombinant allele of 1,893 bp, an internal sequence of 1,142 bp of *vicK* coding region (*SSA_1564*) was replaced by an Erm resistance cassette (Erm^r) obtained from plasmid pVA838. For *vicR* inactivation, a 2,169-bp recombinant allele was constructed by replacing a 509-bp internal sequence of *vicR* (*SSA_1565*) by the Erm^r cassette. Plasmid pVA838 was used to control transformation efficiency of SK36. The complemented *vicK* mutant (SKvic+) was obtained by transforming SKvic with plasmid pDL278 containing the intact copy of *vicK* and a spectinomycin resistance gene.

Biofilm formation assays. To analyze biofilm formation on saliva-coated glass surfaces, samples of whole stimulated saliva were collected from a healthy adult volunteer under a protocol approved by the Ethical Committee of the Piracicaba Dental School, University of Campinas (proc. 067/2009). Saliva samples were clarified by centrifugation (16,000 \times g, 15 min, 4°C), filter sterilized under vacuum using membrane filters (0.22- μ m pore size) (Nalgene, USA), and stored in sterile glass bottles at -70°C until use. For biofilm formation, glass slides previously treated with saliva (overnight, 4°C) were placed horizontally on 24-well plates

containing 1:10 dilutions of *S. sanguinis* cultures (BHI with 1% sucrose; A₅₅₀ of 0.3) and incubated (37°C) under aerobiosis (rotary aeration at 80 rpm) for 2 or 4 h. Slides were then gently washed with distilled water (dH₂O) and processed for scanning electron microscopy (SEM) analysis. The planktonic growth (A₅₅₀) of strains was monitored in the same culture dilutions used for biofilm formation.

Biofilms grown in the presence or not of DNase I were also analyzed. Briefly, cultures in BHI (1% sucrose) were supplemented or not with 50 μ g/ml of DNase I (DN-25), and volumes of 200 μ l were transferred to saliva-coated wells of 96-well polystyrene plates. Plates were incubated under aerobiosis (37°C) for 2 or 4 h. Biofilms were gently washed by immersion in distilled water to remove nonadherent cells and stained with crystal violet. Stain eluted from biofilms in ethanol was used as an indirect measure of biofilm biomass, as previously described (14). Heat-inactivated DNase I (2 h at 65°C) was used as a control. Three independent assays were performed, with eight replicates for each condition.

Electron microscopy analysis. Biofilm slides and planktonic cells at the mid-log phase of growth (A₅₅₀ of 0.3) were harvested by centrifugation (16,000 \times g, 5 min, 4°C), washed three times with phosphate-buffered saline (PBS), and processed in microcentrifuge tubes for SEM analysis as described elsewhere (13). Digital images of biofilms and planktonic cells were obtained with a scanning electron microscope (JSM 5600LV; JEOL, Japan).

Viability under oxidative stresses. VicK of *S. sanguinis* has a conserved PAS domain involved in oxidative stress responses (12), which might influence the ability of *S. sanguinis* to initiate tooth colonization under high oxygen tension. We thus compared sensitivities of the *vicK* mutants to oxidative stress with those of the respective parent strains as described elsewhere (20), with some modifications. Briefly, strains were grown aerobically or anaerobically until reaching an A₅₅₀ of 0.2. H₂O₂ (10 μ M) was then added to the cultures, and they were incubated at room temperature for 1 h. After that, a lethal dose of H₂O₂ (100 μ M) was added and incubation continued for a further 30 min. Serial dilutions of cells were plated on BHI agar supplemented or not with antibiotics for strain maintenance and incubated (37°C, 48 h, 10% CO₂) for determination of viable cell counts (CFU). Sensitivities to this oxidative stress were expressed as the ratios of the mean cell counts (log₁₀ CFU) of samples before the addition of H₂O₂ to the mean cell counts recovered after H₂O₂ exposure. At least three independent experiments were performed in triplicate.

Quantification of production of hydrogen peroxide. Concentrations of H₂O₂ in planktonic cultures were determined as described elsewhere (21) with minor modifications. *S. sanguinis* strains were grown aerobically until the late log phase of growth (A₅₅₀ of 0.7). Afterwards, cells were harvested from volumes (1 ml) of cultures by two cycles of centrifugation (each 16,000 \times g, 5 min, 4°C), and 40 μ l per well of culture supernatants was transferred to 96-well microplates (CralPlast, Brazil) containing 160 μ l per well of fresh sodium acetate solution (0.1 M, pH 5.0) with 0.1 μ g of horseradish peroxidase and 10 μ l of *o*-dianisidine solution (1 mg/ml in methanol). Plates were then incubated protected from light at room temperature for 10 min, and the absorbance (A₅₇₀) of the reaction mixtures was measured using a microplate reader (VersaMAX; Molecular Devices, USA). The concentrations of H₂O₂ produced were calculated from standard curves prepared with BHI (4.68 to 0.29 mM H₂O₂; Synth) in MilliQ water.

Autolysis assay. The autolytic activities of strains were determined as previously described (13) with modifications. Briefly, strains were incubated at 10% CO₂ until the A₅₅₀ reached 0.3. Cells were then collected by centrifugation (16,000 \times g, 5 min, 4°C) and resuspended to an A₅₅₀ of 0.9 in autolysis buffer (1.36 g 20 mM KH₂PO₄, 37.27 g 1 M KCl, 0.074 g 1 mM CaCl₂, 0.10 g 1 mM MgCl₂, 0.4% sodium azide [pH 6.5]). Suspensions were incubated aerobically at 44°C, and autolysis was monitored spectrophotometrically (A₅₅₀) at 24, 48, and 72 h. Three independent experiments were performed in duplicate.

Quantification of eDNA. Amounts of extracellular DNA (eDNA) during planktonic and biofilm growth were measured by quantitative

PCR (qPCR) as previously described (22), with some modifications. Volumes of 1 ml of cultures grown aerobically to an A_{550} of 0.3 or 0.7 were collected by centrifugation (twice at $16,000 \times g$, 4°C, 10 min) to obtain cell-free supernatants. The same procedure was performed with biofilm fluids collected from biofilms formed on saliva-coated surfaces during 2 and 4 h of incubation (37°C, aerobiosis). For qPCR, volumes of 1 μ l of cell-free culture fluids were mixed with 3.4 μ l molecular-grade water, 5 μ l of Power SYBR green PCR master mix (Life Technologies, USA), and 0.3 μ l of 10 mM stock solution of each primer for the 16S rRNA gene. qPCR cycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 30 s. The DNA concentration was calculated based on average threshold cycle values against a 10-fold dilution series of purified SK36 genomic DNA in the same medium. qPCR negative controls included sterile culture medium and, for biofilm cultures, sterile culture medium exposed to saliva-coated surfaces. Three independent experiments performed in duplicate were used for planktonic cultures, and four independent experiments with six replicates were performed for biofilm fluids.

Competition assay on solid medium. To compare the capacities to inhibit *S. mutans* UA159 of *S. sanguinis* SKvic and parent strain SK36, we used the *Erm*^r strains SK36p and UA159p (Table 1). Thus, all the strains could be grown in the presence of erythromycin. The competition assays were performed as previously described with modifications (23). Briefly, 8 μ l of cultures of *Erm*^r SK36 and SKvic at an A_{550} of 0.3 or 0.7 were inoculated onto agar plates with BHI agar with erythromycin and incubated (37°C, 10% CO₂) for 6 h. After that, 8 μ l of cultures of *S. mutans* UA159 (A_{550} of 0.2) were inoculated next to the *S. sanguinis* inoculum, and incubation was continued for 24 h. Inhibitory capacities were assessed by measuring the proximal zones of inhibition in standard digital images of the culture plates using the Gel Logic 200 Imaging System (Kodak). Three independent experiments were performed in six replicates.

RNA isolation, reverse transcription, and qPCR. RNA was isolated from cells subjected to mechanical disruption using a modified protocol from the RNeasy minikit (Qiagen), and treated with Turbo DNase (Ambion, USA) as described elsewhere (14). The cDNA was obtained from 1 μ g of RNA using random primers (24) and SuperScript III (Life Technologies, USA) according to the manufacturer's instructions. Quantitative PCR was performed in a StepOne real-time PCR system (Life Technologies) with cDNA (1 μ l), 30 μ M each primer (see Table S1 in the supplemental material), and 1 \times Power SYBR green PCR master mix (Lifetech) in a total volume of 10 μ l. Results were normalized against *S. sanguinis* 16S rRNA gene expression, which was invariant under the experimental test conditions. Assays were performed in duplicate with at least three independent RNA samples.

EMSA and promoter analysis. Electrophoretic mobility shift assays (EMSAs) were performed as previously described (14) with modifications. Briefly, amplicons of the promoter regions of candidate and control genes were generated with specific primers (see Table S1 in the supplemental material), purified, and labeled with digoxigenin (DIG) using the DIG Gel Shift kit (Roche). Binding reactions of labeled DNA (~3 fmol) with recombinant VicR_{ss} (rVicR_{ss}) (0, 9, 22.5, and 45 pmol) were carried out in volumes of 25 μ l containing 1 \times DIG Gel Shift buffer [20 mM HEPES, 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM dithiothreitol (DTT), 0.2% Tween 20, 30 mM KCl, pH 7.6], poly-L-lysine (5 ng/ μ l), and unspecific competitor poly[d(I-C)] or salmon sperm DNA (50 ng/ μ l). The range of rVicR_{ss} concentrations used in these assays was determined in preliminary experiments. Samples were incubated (25°C, 60 min), and DNA-protein complexes were separated in nondenaturant 6% acrylamide gels (120 V, 1.5 h) in 0.5 \times Tris-borate-EDTA (TBE) buffer (pH 8.0). Protein-DNA complexes were electrotransferred to positively charged nylon membranes (Amersham, GE) and detected using anti-DIG antibodies conjugated with alkaline phosphatase and the CDP Star system (Roche) according to the manufacturer's protocol. To assess the specificity of binding, a 200-fold excess of unlabeled test fragment (cold DNA) was incubated with rVicR in each reaction mixture.

Promoter sequences of potential VicR_{ss}-regulated genes were retrieved from the GenBank (NCBI) and screened to identify VicR binding motifs (15). Alignment of the identified nucleotide sequences was performed using a computational program (<http://weblogo.berkeley.edu/>) for WebLogo graphical representation.

Data analysis. Biofilm growth was analyzed from SEM digital images, at a magnification of $\times 1,300$, obtained from 32 predetermined areas (97 to 63 μ m) equally distributed on each glass slide sample. ImageJ Image Processing and Analysis software in Java (NIH, <http://rsbweb.nih.gov/ij/index.html>) was used to assess mean coverage areas (μ m²) in 64 predetermined areas per strain at each time point. The chain length of planktonic cells was also determined by the counts of cocci in a total of 200 randomly selected isolated chains per strain. Phenotypic comparisons were performed using the nonparametric Kruskal-Wallis test with *post hoc* Dunn's multiple comparisons or Mann-Whitney tests. Parametric analysis of variance (ANOVA) with *post hoc* Dunnett's multiple comparisons was used to compare transcriptional changes. Differences were considered significant when a *P* value of <0.05 was obtained.

RESULTS

Analysis of the gene locus encoding the TCS VicRK_{ss} and vicRK_{ss} inactivation. The genes encoding the TCS VicRK of *S. sanguinis* are organized in an operon-like fashion, composed of *vicX* (SSA_1563, 801 bp), *vicK* (SSA_1564, 1,350 bp), and *vicR* (SSA_1565, 702 bp), which are located in the minor strand of the SK36 chromosome. BLASTP analyses revealed that, as in other streptococci (12), *vicX* encodes a metal-dependent hydrolase of the beta-lactamase superfamily I. To investigate whether *vicRK* are transcribed as an operon, reverse transcription-PCR (RT-PCR) analysis was performed using primer sets to amplify sequences spanning *vicK* and *vicR* sequences (see Table S1 in the supplemental material). Amplicons yielded from SK36 cDNA using primer pairs VicKP1/VicKR (1,100 bp), VicKP1/VicKP2 (564 bp), VicKF/VicKP4 (1,106 bp), VicKP3/VicKP4 (349 bp), and VicKP1/VicKP4 (2,037 bp) are compatible with polycistronic transcription of *vicRKX*. Negative and positive controls were the same used in reverse transcription-quantitative PCR (RT-qPCR) analysis.

In SK36, it was possible to generate stable nonpolar *vicK* knockout mutants via double-crossover recombination with the *vicK* mutant allele. However, no transformants could be recovered by transforming SK36 with the *vicR* mutant recombinant allele. These data indicate that *vicR* is essential for viability of SK36, which is consistent with a previous study in which *vicR* was identified as an essential gene of SK36 (25).

VicK inactivation affects *S. sanguinis* morphogenesis, planktonic growth, and sensitivity to oxidative stress. Given the general role of the VicRK TCS in cell division and morphogenesis (12, 14, 15, 18), the effects of *vicK* inactivation on SK36 morphology and growth were assessed. Inactivation of *vicK* in SK36 promoted formation of extremely long chains, which were not observed in SK36 or in complemented mutant SKvic+ (Fig. 1A). At the mid-log phase of growth (A_{550} of 0.3), the SKvic chains were formed by a mean of $33.9 (\pm 8.6)$ cocci, which was significantly longer than the chains for SK36 (mean of 10.1 ± 8.6 cocci per chain) or complemented mutant SKvic+ (21.2 ± 7.4) (Kruskal-Wallis test with *post hoc* Dunnett test, *P* < 0.05). Of note, because the long chains of SKvic tended to aggregate, only the most isolated SKvic chains could be quantitatively analyzed.

SKvic showed slower growth than SK36 in BHI_{Erm} under aerobiosis, but this trait was not completely restored in the complemented mutant grown in BHI_{Erm/Spec} (Fig. 1B). Fluctuation in the

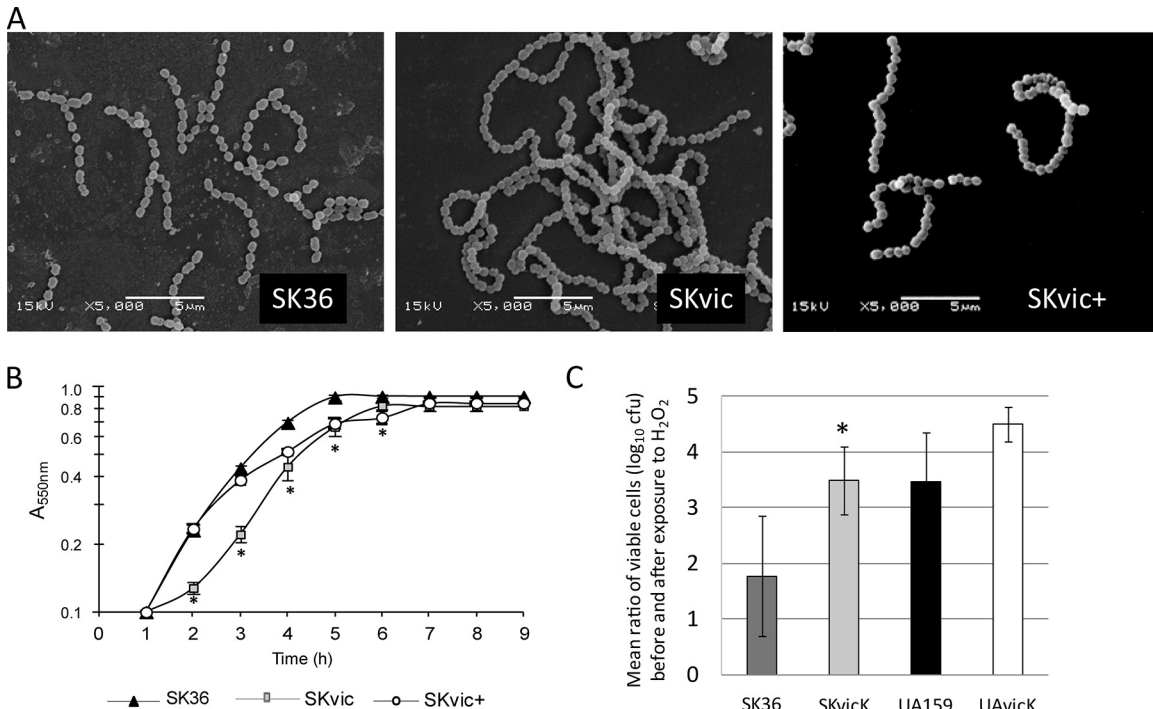


FIG 1 Effects of *vicK* inactivation on chain formation (A), aerobic growth (B), and sensitivity to oxidative stress (C) in *S. sanguinis* and *S. mutans* strains. Symbols represent means from triplicates of one representative experiment. Bars represent means from three independent experiments. Error bars indicate standard deviations. Significant differences between a mutant and the respective parent strain are indicated by asterisks (Kruskal-Wallis test; *, $P < 0.05$).

levels of *vicK* expression from pDL278::*vicK* in SKvic+ could explain differences in growth curves between SKvic+ and the parent SK36 (Fig. 1B). SKvic and SK36 showed means of 89.9% ($\pm 2.4\%$) and 94.6% ($\pm 2.1\%$) of hydrophobic cells, respectively. Although this 3% increase in cell surface hydrophobicity achieved statistical significance (means of Kruskal-Wallis test, $P < 0.05$), differences between strains were modest. However, SKvic formed tight clumps of cells, which were more intensely observed during aerobic growth (data not shown). Deletion of *vicK* also promoted increased sensitivity to oxidative stress when strains were grown anaerobically before exposure to H₂O₂ (Fig. 1C). Colonies of SKvic on BHI_{erm} agar plates incubated aerobically (37°C, 48 h)

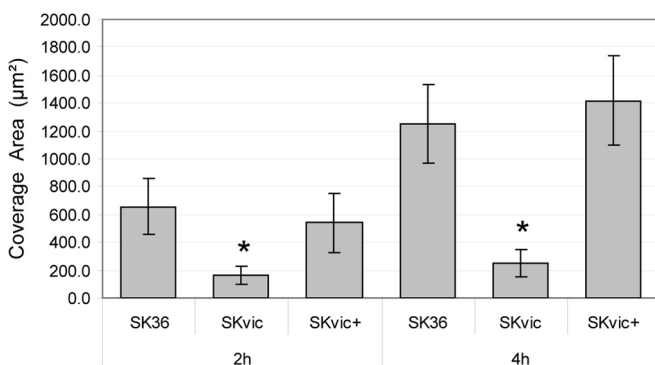


FIG 2 Quantitative comparisons of biofilms formed on saliva-coated glass surfaces. Bars represent mean coverage areas (μm²) per strain at each time point. Error bars indicate standard deviations. Asterisks indicate statistically significant differences compared to the parent SK36 (Kruskal-Wallis test with post hoc Dunn's multiple comparison; *, $P < 0.05$).

were typically irregular and slightly more opaque than those of SK36 or SKvic+ (data not shown).

VicK inactivation impairs biofilm formation in *S. sanguinis* SK36. To assess the role of the TCS VicRK_{S_s} in biofilm formation, initial phases of biofilm growth on saliva-coated surfaces were investigated, because *S. sanguinis* is a major pioneer colonizer of teeth. Biofilm initiation by SK36 was strongly dependent on saliva, since small amounts of biofilms were formed on uncoated glass slides during 2 to 4 h of aerobic growth in BHI with 1% sucrose (data not shown). However, SK36 formed consistent biofilms under the same conditions on saliva-coated glass surfaces (Fig. 2). Biofilm formation capacity was impaired in the SKvic mutant, while the biofilm phenotype was completely restored in the complemented mutant (Fig. 2). Planktonic growth (A₅₅₀) determined in the same bath cultures used in biofilm assays was quite similar for all tested strains at 2 h (A₅₅₀ range, 0.11 to 0.13) and 4 h (range, 0.36 to 0.42) of growth, revealing that differences in biofilm biomass were not a result of altered growth yields.

VicK inactivation in SK36 promotes significant reductions in amounts of eDNA during planktonic and biofilm growth. *S. sanguinis* does not efficiently produce a stable extracellular matrix of glucan for biofilm formation (26, 27). On the other hand, release of genomic DNA appears to be important for *S. sanguinis* biofilm formation (11, 21). To investigate the mechanism by which VicRK affects biofilm formation, we compared amounts of eDNA among *S. sanguinis* strains during planktonic growth (A₅₅₀ of 0.3) and during biofilm initiation. As shown in Fig. 3, the SKvic mutant showed a 2.8-fold reduction in amounts of eDNA during planktonic growth, and this phenotype was rescued in the complemented mutant SKvic+ (Fig. 3A). Additionally, a clear reduc-

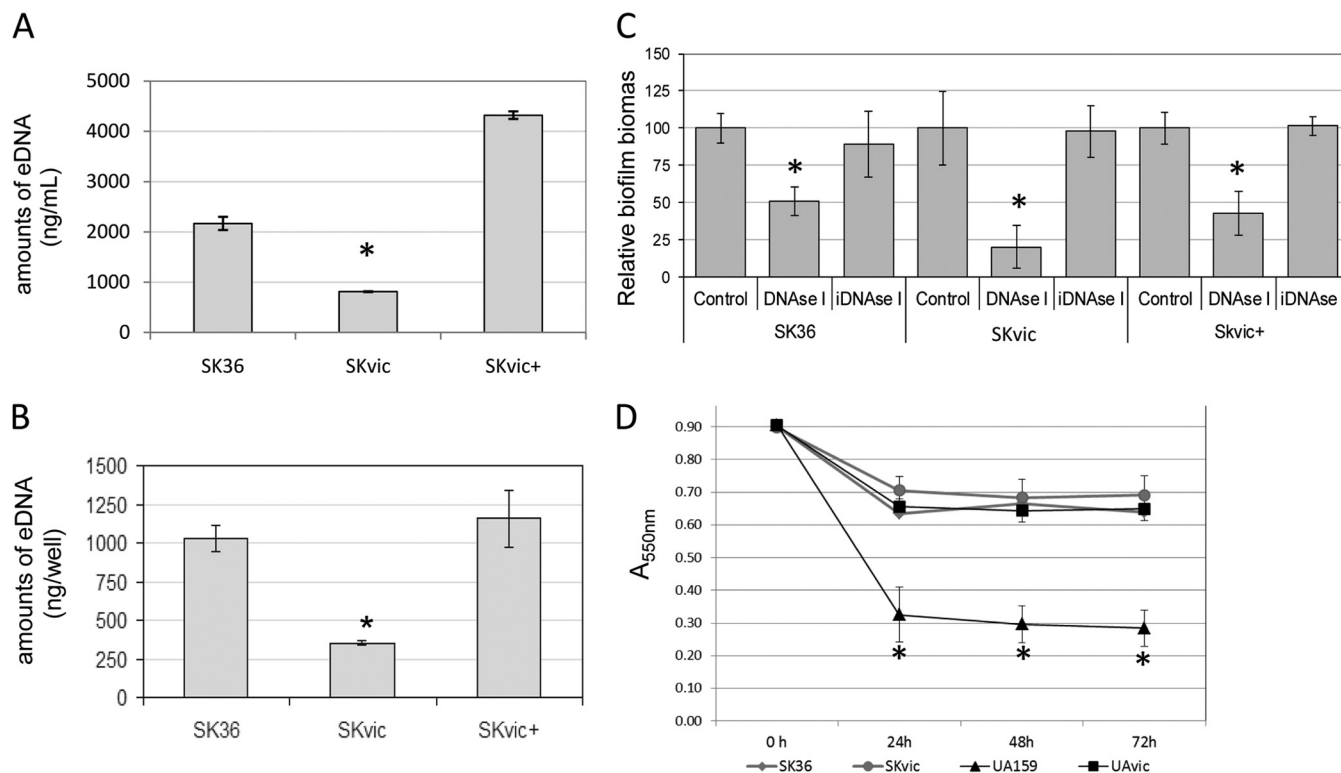


FIG 3 Amounts of extracellular DNA (eDNA) during planktonic or biofilm growth and autolysis. Amounts of eDNA in culture fluids were determined by qPCR. (A) Fluids of planktonic cultures (A_{550} of 0.3). (B) Fluids of biofilms formed during 4 h. (C) Mean biomass of biofilms formed during 4 h in the presence of active or heat-inactivated (i) DNase I. Non-DNase control bars are relative to each respective strain. (D) Induced autolysis at 44°C. Bars or symbols represent means from three independent experiments performed in two (A), three (B), or six (C) replicates. Error bars indicate standard deviations. Asterisks indicate significant differences in comparison to the respective parent strain SK36 or UA159 (Kruskal-Wallis test with *post hoc* Dunn multiple comparisons; *, $P < 0.01$).

tion in amounts of eDNA was observed during the initial 2 h (2.2-fold reduction) and 4 h (3-fold reduction) of biofilm formation on saliva-coated slides, suggesting that the *vicK* mutant is defective in releasing genomic DNA during biofilm initiation. To further establish the role of eDNA as a scaffold of *S. sanguinis* biofilms, we compared biofilm growth in the presence or absence of DNase I. As shown in Fig. 3C, SK36 shows an impaired ability to form biofilms in the presence of active DNase I. Moreover, addition of DNase I to SKvic cultures reduced biofilm formation to irrelevant levels (Fig. 3C).

To investigate whether reduced release of DNA of SKvic was associated with changes in autolytic activity, temperature-induced autolytic activities of the strains were compared. The *S. mutans vicK* mutant UAvic and parent UA159 were used in these comparisons because UAvic was previously shown to have impaired autolysis compared to UA159 (14). As shown in Fig. 3D, no clear changes in autolytic activities could be verified in SKvic. Thus, the influence of the TCS VicRK_{SS} on amounts of eDNA during planktonic and biofilm growth of strain SK36 does not involve detectable changes in autolysis.

Inactivation of *vicK* in *S. sanguinis* impairs the production of hydrogen peroxide and affects the ability to inhibit *S. mutans* growth. DNA release in *S. sanguinis* is associated with the production of H₂O₂ under oxygen exposure by mechanisms not entirely understood (11). By comparing *S. sanguinis* strains at the mid-log (A_{550} of 0.3) and late log (A_{550} of 0.7) phases of growth, it was observed that the SKvic mutant has a reduced production of H₂O₂

(Fig. 4A). H₂O₂ production was completely restored in the complemented mutant SKvic+ at an A_{550} of 0.3 and was partially restored at an A_{550} of 0.7, likely as a result of variation in *vicK* expression in the complemented mutant at late log phase. Because production of H₂O₂ is one of the main mechanisms involved in the capacity of *S. sanguinis* to competitively inhibit *S. mutans* (9), we compared *S. sanguinis* strains in interspecies competition assays with *S. mutans* UA159. Consistent with its low production of H₂O₂, SKvic showed a reduced capacity to inhibit UA159, producing inhibitory zones about 2-fold shorter than those of the parent SK36 (Fig. 4B and C).

The VicRK system regulates genes involved in production of hydrogen peroxide and cell wall biogenesis/degradation. The mechanisms of biofilm formation in *S. sanguinis* likely require genes involved in cell surface interactions with salivary components (*sspC* and *sspD*), sensitivity to oxidative stress and production of hydrogen peroxide (*spxB*, *spxR*, *ccpA*, *ackA*, *tpK*, and *sodA*), synthesis of glucan (*gtfP*), competence (*comCDE*), and murein hydrolase activities and/or cell envelope biogenesis (*gbpB*, *SSA_0094*, *cwdP*, and *cgt*). To investigate roles of the TCS VicRK_{SS} in the regulation of these functional sets of genes, transcriptional comparisons between *S. sanguinis* strains were performed by RT-qPCR.

Transcriptional comparisons of SK36 with SKvic did not reveal changes in orthologues of genes of the major adhesin of the AgI/II family (*sspC* and *sspD*) under the experimental conditions tested. This family of cell surface polypeptides has been implicated in cell

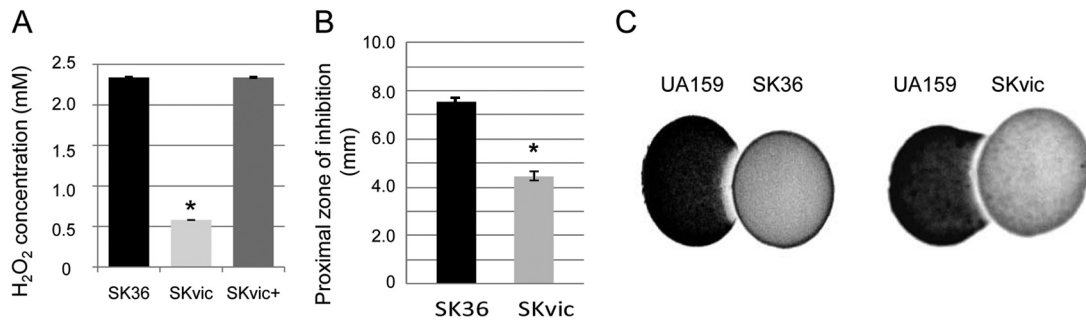


FIG 4 Comparisons of the production of H₂O₂ and the inhibitory activity on *S. mutans* UA159. (A) Amounts of H₂O₂ produced by *S. sanguinis* strains at the mid-log phase of growth (A_{550} of 0.3). (B) Mean proximal zones of inhibition (mm) obtained in three independent experiments performed for six replicates of *S. sanguinis* strains inoculated at an A_{550} of 0.3. Means and standard deviations are shown. Asterisks indicate statistically significant differences between SK36 and SKvic (Kruskal-Wallis test; *, $P < 0.05$). (C) The inhibitory zones on UA159p were assessed on agar plates. After initial incubation for 6 h, plates were inoculated with UA159p, and incubation was continued for 24 h.

surface interactions with tooth-adsorbed salivary components (28). On the other hand, significant downregulation of *spxB*, which is involved in H₂O₂ production, was detected at the mid-log (A_{550} of 0.3) and late log (A_{550} of 0.7) phases of growth (Table 2).

spxB encodes the pyruvate oxidase (SpxB) which converts pyruvate to acetyl phosphate (AcP) in a reaction that requires oxygen, generating H₂O₂ and CO₂ (29). CcpA (catabolite control protein A) is a known repressor of *spxB* in *S. sanguinis* (21), but *ccpA* was

TABLE 2 Transcriptional profiles of the *vicK* mutant (SKvic) and complemented mutant (SKvic+) relative to parental strain SK36

Function and gene (NCBI)	Encoded protein	Fold change in expression ^a			
		Mid-log phase (A_{550} , 0.3)		Late log phase (A_{550} , 0.7)	
		SKvic	SKvic+	SKvic	SKvic+
Synthesis of or interaction with extracellular matrix of biofilms					
SSA_0613	GtfP	+1.71 ^c (0.14)	+1.28 ^d (0.19)	+3.38 ^b (0.30)	+1.03 ^d (1.01)
SSA_0019	GbpB	-3.94 ^b (0.34)	-3.31 ^b (0.43)	-4.20 ^b (0.56)	+1.10 ^d (0.21)
Interactions with tooth pellicle or other biofilm components					
SSA_0303	SspC	+1.08 ^d (0.16)	+1.08 ^d (0.08)	+1.12 ^d (0.13)	+1.05 ^d (0.12)
SSA_0956	SspD	-1.08 ^d (0.26)	+1.04 ^d (0.33)	-1.76 (0.26)	+1.04 ^d (0.23)
Synthesis of hydrogen peroxide and tolerance to oxidative stress					
SSA_0391	SpxB	-3.03 ^b (0.20)	+1.09 ^d (0.05)	-3.67 ^b (1.12)	+1.42 ^d (0.34)
SSA_0721	SodA	+1.12 ^d (0.13)	+1.08 ^d (0.04)	-1.33 ^d (0.31)	+1.11 ^d (0.30)
Cell wall biogenesis or cell envelope integrity					
Murein hydrolases					
SSA_0094	Unnamed	-10.62 ^b (0.34)	-2.29 ^b (0.14)	-19.02 ^b (5.73)	-4.81 ^b (1.27)
SSA_0304	CwdP	+1.06 ^d (0.13)	+1.03 ^d (0.51)	+1.41 ^d (0.47)	+1.17 ^d (0.50)
Membrane biogenesis					
SSA_1324	Cgt	+1.02 ^d (0.20)	+1.15 ^d (0.06)	+4.07 ^b (0.12)	+1.22 ^d (0.21)
SSA_1543	Unnamed	-1.14 ^d (0.33)	-1.35 ^d (0.35)	-5.22 ^b (1.82)	-2.24 ^b (0.29)
Regulation of hydrogen peroxide production, metabolism and/or biofilm formation					
SSA_0192	AckA	+1.08 ^d (0.17)	+1.02 ^d (0.25)	+2.15 ^b (0.47)	+1.00 ^d (0.17)
SSA_1492	SpxR	+1.13 ^d (0.20)	+1.11 ^d (0.17)	+1.03 ^d (0.21)	+1.04 ^d (0.06)
SSA_1576	CcpA	-1.05 ^d (0.07)	-1.29 ^d (0.24)	+1.13 ^d (0.29)	+1.47 ^d (0.22)
SSA_2118	TpK	+1.00 ^d (0.07)	+1.05 ^d (0.07)	+1.07 ^d (0.23)	+1.11 ^d (0.12)
SSA_2378	ComE	-1.29 ^d (0.11)	-1.09 ^d (0.35)	-2.36 ^b (0.34)	-1.59 ^d (0.95)
Components of the VicRKX TCS					
SSA_1563	VicX	-1.10 ^d (0.20)	-1.03 ^d (0.18)	-1.13 ^d (0.18)	-1.08 ^d (0.22)
SSA_1564	VicK		+1.07 ^d (0.16)		-1.11 ^d (0.15)
SSA_1565	VicR	-1.07 ^d (0.17)	+1.10 ^d (0.21)	-1.08 ^d (0.16)	+1.06 ^d (0.20)

^a Values represent means (standard deviations) from three independent experiments performed in duplicate.

^b $P < 0.01$ by ANOVA with *post hoc* Dunnett's test.

^c $P < 0.05$ by ANOVA with *post hoc* Dunnett's test.

^d Not significant.

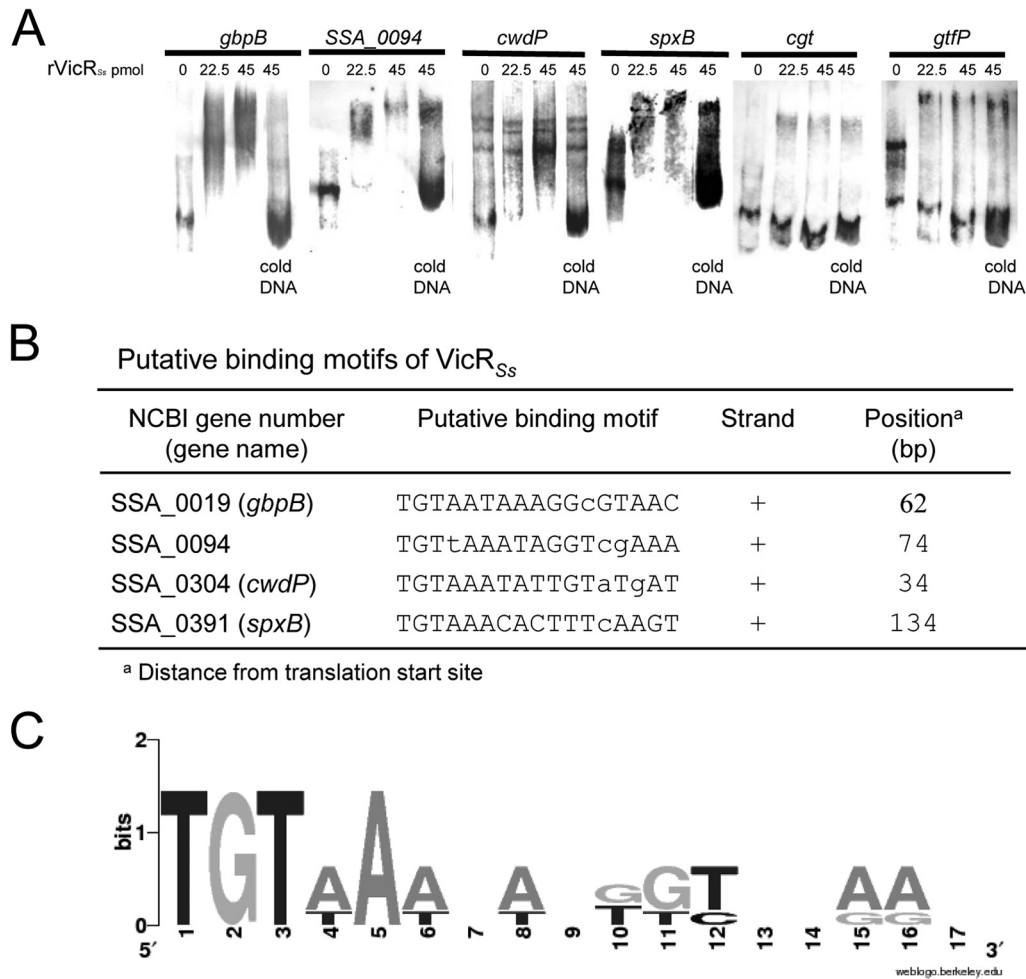


FIG 5 (A) EMSA analysis with promoter region gene fragments labeled with digoxigenin (DIG). DNA fragments were probed with anti-DIG antibodies. Recombinant VicR protein (rVicR) binds DNA fragments of genes with consensus VicR-binding sequences (*gbpB*, *SSA_0094*, *SSA_0304*, and *SSA_0391*). (B) Sequences and positions of the VicR binding sites in VicR-regulated promoters. The *cgt* and *gtfP* promoter regions do not contain a VicR consensus sequence. Consensus for VicR, TGTWAHNNNNNTGTWAH, where W is A or T and H is A, T, or C. Lowercase indicates a mismatch. (C) Sequence logo from the putative VicR-binding sites generated using WebLogo (<http://weblogo.berkeley.edu/>).

not transcriptionally affected in SKvic (Table 2). In addition, no significant changes were detected in *spxR*, which encodes a putative transcriptional regulator of *spxB* (30). Other genes previously shown to affect expression of *spxB* in SK36, including *ackA* (encoding acetate kinase A) and *tpK* (encoding a thiamine pyrophosphokinase) (30), were not significantly affected in SKvic (Table 2). Transcriptional changes were also not detected in *sodA*, which encodes an Mn/Fe-dependent superoxide dismutase (SodA) involved in protection against oxidative stress (31).

S. sanguinis express *gtfP*, the unique gene involved in the extracellular synthesis soluble glucan (26, 31, 32). *gtfP* was upregulated in SKvic only at the late log growth phase (Table 2). Because the regulons of VicRK typically include genes for cell wall biogenesis (15) and for cell-surface interactions with glucan (12–14), transcriptional analysis included an orthologue of *S. mutans gbpB* (*SSA_0019*; also called *pcsB*), *SSA_0094*, and *SSA_0304* (encoding a putative murein hydrolase annotated as CwdP [cell wall degradation protein]). *SSA_0094* and *CwdP* contain a lysin motif (LysM domain) for binding to peptidoglycan. *GbpB* and *CwdP* contain a cysteine/histidine-dependent amidopeptidase/amido-

hydrolase (CHAP) domain typical of murein hydrolases with amidase activity (33). *gbpB* and *SSA_0094* were about 4-fold and 10-fold downregulated in SKvic at the mid-log phase of growth (Table 2). In addition, stronger downregulation of *SSA_0094* (19-fold) was observed in SKvic at the late log phase of growth (Table 2). Transcript amounts of these genes were restored to parental levels in the complemented mutant under most of the conditions tested (Table 2). Other genes likely involved in cell envelope integrity (*cgt* [*SSA_1324*] and *SSA_1543*) were affected only at the late log growth phase of SKvic (Table 2). Finally, the gene encoding the response regulator ComE of the TCS ComDE, which is involved in quorum-sensing regulation of competence, showed downregulation (2.36-fold) in SKvic at the late log growth phase (Table 2).

Transcriptional analyses were compatible with EMSA and promoter analyses (Fig. 5). *gtfP* and *cgt* were used as negative controls, because the promoter regions of these genes did not interact with rVicR_{Ss}. No VicR-binding sites were identified in the promoter regions of these genes. rVicR_{Ss} could bind to *gbpB* and *SSA_0094* promoter sequences, which include conserved VicR-binding sites

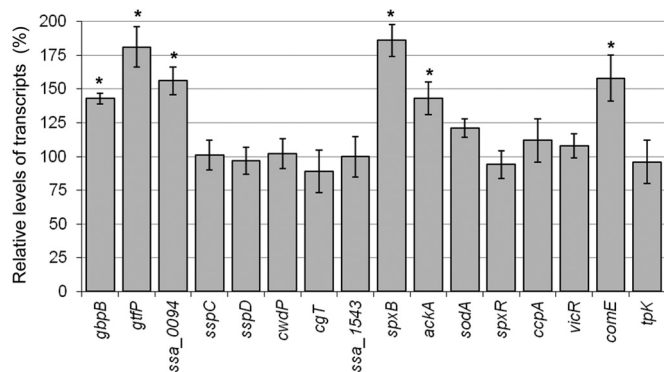


FIG 6 RT-qPCR analysis of gene expression in biofilm and planktonic phases at 4 h of SK36 growth (BHI with 1% sucrose, aerobiosis). Biofilm and planktonic cells were derived from the same cultures. Transcript levels in planktonic phase cells were set to 100% in order to calculate relative amounts of transcripts in the attached cells. Bars represent means from four independent experiments performed in duplicate. Error bars represent standard deviations. Statistically significant changes in gene expression in biofilm versus planktonic cells are indicated (*, $P < 0.01$ by ANOVA with *post hoc* Dunnett's test).

(Fig. 5). Interestingly, although no significant changes in *cwdP* transcript levels were observed in SKvic (Table 2), rVicR_{ss} interacted with the promoter region of *cwdP*, which also encloses a VicR box (Fig. 5). In addition, rVicR_{ss} could bind to the promoter region of *spxB*, and a VicR consensus sequence was identified in this region (Fig. 5).

VicR target genes are upregulated during the initial phases of biofilm formation. To strengthen the role of VicRK in biofilm regulation, we investigated transcriptional activities of VicR_{ss}-interacting genes during initial phases of biofilm formation in SK36. To this end, SK36 cells derived from biofilm or planktonic phases of 4-h biofilms (grown in BHI with 1% sucrose) were collected and subjected to RT-qPCR analyses. Amounts of transcripts obtained in planktonic cells were set to 100% to calculate relative levels of transcripts in biofilm cells. Increases of 43 to 81% in the transcript levels of *gbpB*, *SSA_0094*, and *spxB* were found in SK36 biofilm cells relative to planktonic-phase cells (Fig. 6). Genes expectedly involved in biofilm formation but not directly regulated by VicR (e.g., *comE* and *gtfP*) were also upregulated in biofilm cells (Fig. 6). Thus, genes of the VicR_{ss} regulon are upregulated during initial phases of biofilm growth in SK36.

DISCUSSION

The high ability of *S. sanguinis* to colonize tooth surfaces might be associated with particular cell wall/surface traits. The genome of *S. sanguinis* strain SK36 contains genes encoding a large number of cell wall-anchored proteins and lipoproteins compared to other streptococcal species of the human microbiota, including *S. mutans* and *Streptococcus pneumoniae* (31, 32). During colonization of tooth surfaces, *S. sanguinis* interactions with dental pellicle, biofilm extracellular matrix, and/or coadherent microorganisms likely require dynamic cell surface fitting in response to host and microbial stimuli, a process that involves two-component regulatory systems. The TCS VicRK_{sm} of *S. mutans*, a competitor of *S. sanguinis* which promotes cariogenic biofilms, regulates biofilm formation and virulence (12), which raises interest in this TCS (also called WalRK) as a therapeutic target (34). In this study, we

showed that VicRK_{ss} regulates critical functions necessary for *S. sanguinis* to initiate biofilms, including chain formation, tolerance to oxidative stress, production of hydrogen peroxide, and amounts of eDNA. Comparative analyses of the effects of *vicK* inactivation in *S. sanguinis*, *S. mutans* (14), and *Streptococcus gordonii* (35) further indicate species-specific functions of VicRK in regulating biofilm formation. For example, *S. mutans vicK_{sm}*-deficient strains show significant increases in amounts of eDNA during biofilm formation but reduced autolytic activity (14). In contrast, an *S. sanguinis vicK_{ss}* mutant does not show changes in autolysis but shows reduced amounts of eDNA in biofilm cultures (Fig. 3).

In contrast to the case for *S. sanguinis*, the establishment and virulence of *S. mutans* in dental biofilms rely in part on its ability to effectively enrich biofilm biomass to potentiate biofilm acidic properties that select acid-tolerant organisms (36, 37). This process involves the VicRK_{sm} positive regulation of *gtfB* and *gtfC*, which are, respectively, involved in the synthesis of water-insoluble glucan (rich in α 1-3 linkages) and of a mixture of insoluble and soluble (rich in α 1-6 linkages) glucan (12). Additionally, the VicRK_{sm} TCS regulates genes likely involved in fitting cell surface interactions with the extracellular polymers, including *gbpB* (13), and genes encoding murein hydrolases (*smaA*, *Smu2146c*, and *lysM*) (14). Roles of the TCS VicRK in regulating synthesis of and interaction with extracellular polysaccharides were not previously investigated in *S. sanguinis* or *S. gordonii*. *S. sanguinis* expresses *gtfP* for the synthesis of soluble glucan, which likely has different biological functions in biofilm ecology (26, 27). Although the presence of sucrose, the unique substrate for the synthesis of glucan, is necessary for *S. sanguinis* biofilm formation *in vitro* (21, 32), the mechanisms of *S. sanguinis* interactions with the extracellular glucan are unknown, but they might involve proteins with affinity to glucan. Encoded in the SK36 genome, putative glucan-binding proteins included GbpB (SSA_0019), SspC, and SspD; SspC/D surface proteins enclose glucan binding protein C domains (31, 38). In this study, we established that *gbpB_{ss}*, but not *gtfP* or *sspCD*, is directly regulated by the VicRK_{ss} TCS (Table 2; Fig. 5). Although the 3.4-fold increase in *gtfP* transcripts detected in SKvic at the late log growth phase could imply negative regulation by VicRK_{ss} (Table 2), the *gtfP* promoter region does not include a VicR-binding consensus sequence and does not interact with rVicR_{ss} *in vitro* (Fig. 5). Therefore, the *gtfP* transcriptional change is possibly an indirect effect of *vicK_{ss}* inactivation. Genetic screening of SK36 biofilm-defective mutants in the presence of sucrose did not reveal *gtfP*, *gbpB*, or *sspCD* but did reveal genes for nucleotide and amino acid biosynthesis (32). Thus, the mechanisms involved in *S. sanguinis* biofilm formation remain to be more deeply investigated.

Although the high cell surface hydrophobicity of *S. sanguinis* could play a role in initial adhesion to salivary pellicle (6, 39), no substantial changes in surface hydrophobicity was observed in SKvic. In addition, expression of genes likely implicated in specific interactions with salivary pellicle, e.g., *sspC* and *sspD*, was not affected in this mutant (Table 2). Downstream from *sspC*, *cwdP* was found to have a promoter region which interacts with VicR (Fig. 5). However, *cwdP* was not transcriptionally affected in SKvic (Table 2). A possible explanation for these data is that *cwdP* could be controlled by additional regulatory mechanisms that might have restored *cwdP* activity in SKvic. Alternatively, *cwdP* could be transcriptionally altered in SKvic under different experimental

conditions, since the VicRK transcriptomes are significantly affected by nutritional and growth conditions in other streptococcal species (14).

Extracellular genomic DNA is recognized as playing important roles as a structural component of the extracellular matrix of bacterial biofilms (40), including those of the oral streptococcal species *S. sanguinis*, *S. gordonii*, and *S. mutans* (11, 41, 42). Extracellular DNA was shown to promote *S. sanguinis* cell aggregation (11). SKvic showed reduced amounts of eDNA in fluids of planktonic and biofilm cultures, which was restored in the complemented mutant (Fig. 3A and B). The requirement for eDNA for biofilm formation under the conditions tested was supported by the reduced amounts of biofilms formed by *S. sanguinis* strains in the presence of active DNase (Fig. 3C). In *S. mutans*, VicRK_{Sm} regulates murein hydrolases that influence amounts of eDNA during biofilm growth (including SMu.2146c, LysM, and SmaA) in a way not entirely dependent on autolysis (14). The SK36 genome does not include orthologues of *smaA*, *lysM*, and *smu.2146c* but has genes encoding putative murein hydrolases analyzed in this study, as well as an orthologue of the major autolysin of *S. gordonii* (AtlS; SGO2013) (49% identity and 66% similarity in amino acid sequences) (41). AtlS of *S. gordonii* is regulated by the TCS LytST and is involved in oxygen-dependent autolysis and DNA release (41). In contrast to *S. gordonii* (41), *S. sanguinis* SK36 has low autolytic activity under aerobiosis (Fig. 3D), suggesting differences in cell wall functions associated with production of eDNA among these species.

Autolysin-independent mechanisms of DNA release seems to occur in *S. gordonii* and *S. sanguinis*, because substantial amounts of eDNA are detected in cultures of these species in the absence of detectable autolysis (11), but the roles of the VicRK_{Ss/Sg} TCS in DNA release and/or turnover remains to be understood. Additionally, murein hydrolases not implicated in autolysis but affecting DNA release during biofilm growth remain to be identified. In this study, we identified a VicRK_{Ss} target gene (Fig. 5) strongly downregulated in SKvic (SSA_0094) at the mid- and late log phases of growth (Table 2). SSA_0094 encodes a protein containing a LysM domain, which is implicated in binding to murein and is typically found in murein hydrolases of *Firmicutes* (44). SSA_0094 is highly conserved among *S. sanguinis* strains with available genomes, and orthologues are present in *Streptococcus mitis*, *Streptococcus oralis*, and *S. pneumoniae*. Upregulation of SSA_0094 during biofilm initiation (Fig. 6) strengthens its involvement in biofilm formation. Thus, this novel gene seems to be an important candidate to elucidate mechanisms of DNA release in *S. sanguinis*.

Amounts of eDNA in *S. sanguinis* cultures are associated with the aerobic production of H₂O₂ (11, 21). Pyruvate oxidase encoded by *spxB* (also called *pox*) is responsible for most of the H₂O₂ aerobically produced by *S. sanguinis* (11); *spxB* deletion impairs DNA release (11). *spxB* orthologues are found in most commensal streptococcal species of the oral cavity, but not in *S. mutans*, and are expressed with relative abundance in dental biofilms *in vivo*, highlighting its importance in biofilm ecology (45). Deletion of *spxB* in *S. sanguinis* further impairs its inhibitory capacity with *S. mutans* (10). Roles of the VicRK TCS in regulating *spxB* or H₂O₂ production in oral streptococci were not previously investigated. In the present study, we established that VicRK_{Ss} positively regulates *spxB* (Table 2; Fig. 5). Inactivation of *vicK*_{Ss} further reduces the production of H₂O₂ at extents sufficient to compromise SK36

capacity to inhibit *S. mutans* growth *in vitro* (Fig. 4). Screening of single-gene deletion mutants defective in H₂O₂ production did not detect *vicRK* deletions (30), which could be explained by growth defects of *vicRK* mutants. VicR is apparently the unique essential response regulator identified in several species of *Firmicutes* (15), including *S. mutans* (12) and *S. sanguinis* (25), likely due to its role in controlling cell division processes (13–15, 46). Consistently, we could not obtain a *vicR*_{Ss} nonpolar deletion mutant of SK36. Although SKvic formed atypical long chains (Fig. 1A), *vicRK* mutants were not recovered from screenings of long-chain-forming gene deletion mutants obtained from SK36 (47). On the other hand, *vicR* is not essential in *S. gordonii* (35), and inactivation of *vicRK*, but not *vicK*, in this species affected aerobic growth and sensitivity to oxidative stress (35), further highlighting species-specific functions of the VicRK systems. SKvic showed increased sensitivity to high doses of H₂O₂ (Fig. 1C) but only when grown under anaerobiosis before the oxidative challenge, a condition under which the *S. mutans vicK* mutant was not significantly affected (Fig. 1C). Therefore, VicRK_{Ss}-independent mechanisms regulating tolerance to oxidative stress might be activated under aerobiosis in SK36. Multiple TCSs are associated with oxidative stress response in *S. gordonii*, including CiaRH and ComDE (35).

In *S. sanguinis*, *spxB* is also directly repressed by the transcription factor CcpA, a component of the carbon catabolite repression (CCR) system (21) which is activated in the presence of glucose (48). However, repression of *spxB* in the presence of glucose is observed only in *S. gordonii* and not in *S. sanguinis* (21). No significant transcriptional changes in *ccpA* or in *spxR*, which encodes another putative regulator of *spxB* (30), were observed in SKvic (Table 2), strengthening direct regulation of *spxB* by VicR_{Ss}. Among other genes indirectly affecting *spxB* activity (*ackA* and *tpK*), only a 2.15-fold upregulation of *ackA* was observed in the late log growth phase of SKvic (Table 2). The acetate kinase (AckA) encoded by *ackA* synthesizes ATP from acetyl phosphate (AcP), the product of pyruvate oxidase, which is important for ATP synthesis in bacterial species that, like *S. sanguinis*, lack the complete respiratory metabolic pathway (31, 49). Thus, upregulation of *ackA* in 4-h biofilms (Fig. 6) might reflect an *ackA* role in metabolic fitness of *S. sanguinis* SK36 during biofilm initiation.

In summary, in this study we identified pathways by which the TCS VicRK regulates biofilm formation in *S. sanguinis* and identified novel genes potentially involved in the release of DNA, a major extracellular component of *S. sanguinis* biofilms. Functional analyses of these genes might help to elucidate the molecular mechanisms by which *S. sanguinis* produces eDNA during biofilm formation. This study further highlights species-specific functions of the TCS VicRK in controlling biofilm growth, which could be explored to design therapeutic strategies to restore the homeostasis of dental biofilms.

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