Core-Gene-Encoded Peptide Regulating Virulence-Associated Traits in Streptococcus mutans

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Recently, high-coverage genome sequence of 57 isolates of Streptococcus mutans, the primary etiological agent of human dental caries, was completed. The SMU.1147 gene, encoding a 61-amino-acid (61-aa) peptide, was present in all sequenced strains of S. mutans but absent in all bacteria in current databases. Reverse transcription-PCR revealed that SMU.1147 is cotranscribed with scnR, which encode the histidine kinase and response regulator, respectively, of a two-component system (TCS). The C terminus of the SMU.1147 gene product was tagged with a FLAG epitope and shown to be expressed in S. mutans by Western blotting with an anti-FLAG antibody. A nonpolar mutant of SMU.1147 formed less biofilm in glucose-containing medium and grew slower than did the wild-type strain under aerobic and anaerobic conditions, at low pH, or in the presence of H₂O₂. Mutation of SMU.1147 dramatically reduced genetic competence and expression of comX and comY, compared to S. mutans UA159. The competence defect of the SMU.1147 mutant could not be overcome by addition of sigX-inducing peptide (XIP) in defined medium or by competence-stimulating peptide (CSP) in complex medium. Complementation with SMU.1147 on a plasmid restored all phenotypes. Interestingly, mutants lacking either one of the TCS components and a mutant lacking all three genes behaved like the wild-type strain for all phenotypes mentioned above, but all mutant strains grew slower than UA159 in medium supplemented with 0.3 M NaCl. Thus, the SMU.1147-encoded peptide affects virulence-related traits and dominantly controls quorum-sensing pathways for development of genetic competence in S. mutans.

Streptococcus mutans is the primary etiologic agent of dental caries (1–4), and multiple characteristics contribute to its successful colonization, persistence, and virulence in the dynamic environment of the human oral cavity. Pathogenesis by S. mutans requires efficient biofilm formation on the tooth surface, as well as the fermentation of carbohydrates to organic acids that directly cause demineralization of tooth enamel (5, 6). Acid tolerance, or aciduricity, which is the ability to grow and metabolize at low pH, is also a defining characteristic of cariogenic microorganisms. Oral microbial communities are one of the most complex microbiomes of the human body. To date, hundreds of different taxa have been identified using DNA-based methodologies (7, 8), and many species that are abundant in the oral flora display remarkable degrees of genetic and phenotypic heterogeneity (9), which undoubtedly has a profound effect on the pathogenic potential of the oral microbiome.

Environmental conditions in the oral cavity change frequently and rapidly due to the intermittent eating patterns and diurnal rhythms of the host. Bacteria in oral biofilms must cope with these changes, and adaptations to fluctuations in environmental conditions are often accomplished by two-component systems (TCSs), which consist of a histidine kinase (HK) and response regulator (RR) that regulate gene expression as a function of a wide variety of inputs (10). In S. mutans UA159, there are 14 putative TCS pairs plus GcrR (also annotated as CovR or TarC), a so-called orphan response regulator that is not encoded in an operon with a cognate histidine kinase (11–15), and many of these TCSs have been demonstrated to affect the expression of virulence-related traits of this organism. For example, the ComDE TCS consists of the membrane-bound HK ComD and the RR ComE and has a profound impact on the development of competence, biofilm formation, and bacteriocin production (16–19). The ComDE TCS is activated through a quorum-sensing pathway that includes competence-stimulating peptide (CSP), encoded by comC. Although CSP is able to enhance natural genetic competence, the system appears primarily designed to control the expression of a variety of bacteriocins produced by S. mutans. Recently, it was shown that activation of competence appears to require the transcriptional activator ComR and a 7-aminoo-acid (7-aa) coactivating peptide known as XIP, for comX-inducing peptide (20–23). A variety of other TCSs in S. mutans have been shown to impact virulence-related phenotypes, including VicRK, CiaRH, LiaRS, and ScnRK. Of relevance here, ScnRK has been shown to contribute to hydrogen peroxide stress tolerance and to enhance the resistance of S. mutans to killing by macrophages (24). ScnRK, which has also been called HK/RR3 (25), has been reported to have a modest effect on acid tolerance. Finally, an apparent orthologue of this system in Streptococcus pyogenes has been reported to influence the expression of bacteriocins (26).

Through whole-genome shotgun sequencing of 57 genomes of clinical isolates of S. mutans, we recently identified a so-called “unique core genome” (UCG), which consists of genes that are present in all isolates of S. mutans but are absent in closely related mutants streptococci (27). Among the UCGs, we found that SMU.1147, which encodes a 61-aa peptide and is part of a three-gene operon containing the scnRK genes for a two-component...
system, is apparently unique to \textit{S. mutans}, as apparent homologues are found in all sequenced \textit{S. mutans} strains but not in any other organism in the database (28–30). Notably, the SMU.1147-encoded peptide was identified as a potential bacteriocin based on its genetic linkage to genes for the TCS and an ABC transporter (letfEG) (31). However, the authors concluded that the peptide did not have characteristics of known bacteriocins. In this study, we explored whether the SMU.1147 product could be produced in \textit{S. mutans}, evaluated the influence of the 61-aa peptide on traits that are associated with the persistence and virulence of \textit{S. mutans}, and probed whether the effects of this peptide could be exerted through the ScnRK system. The findings indicate that this apparently unique peptide plays critical roles in the expression of virulence-related traits and has a dominant effect over the primary quorum-sensing and competence development pathways of \textit{S. mutans}.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** All strains and plasmids used in this study are listed in Table S1 in the supplemental material. \textit{S. mutans} UA159 and its derivatives were grown in brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI) at 37°C and a 5% CO\textsubscript{2} atmosphere. Media were supplemented with spectinomycin (1 mg ml\textsuperscript{-1}), kanamycin (1 mg ml\textsuperscript{-1}), or erythromycin (10 μg ml\textsuperscript{-1}) (Sigma-Aldrich, St. Louis, MO), as needed. For growth measurements, overnight cultures grown in BHI were diluted 1:50 in the defined medium FMC (32) containing 10 mM glucose and grown to early exponential phase (optical density at 600 nm (OD\textsubscript{600}) \approx 0.2) at 37°C in a 5% CO\textsubscript{2} atmosphere. These exponential-phase cultures were diluted 1:100 into fresh FMC medium and overlaid with sterile mineral oil to reduce exposure to air. The optical density of cells growing at 37°C was measured at 600 nm (OD\textsubscript{600}) every 30 min using a Bioscreen C lab system (Helsinki, Finland). To test the sensitivity to stress conditions, the growth medium was supplemented with HCl to lower the pH of the medium to 5.5, with 0.003% H\textsubscript{2}O\textsubscript{2}, or with 0.3 M sodium chloride (Sigma-Aldrich, St. Louis, MO) and overlaid by filter-sterilized mineral oil to create anaerobic conditions.

**Construction of mutant strains.** Deletion mutants of \textit{S. mutans} were constructed as previously described (33, 34). Briefly, using primers flanking the gene of interest, two flanking sequences that incorporated restriction enzyme sites for BamHI for the upstream and downstream fragments were amplified. The PCR products were digested with BamHI and ligated with T4 DNA ligase (Invitrogen) to a promoterless nonpolar kanamycin resistance (NPKm) (35) gene that had been digested with the same restriction enzymes. The resulting ligation mixtures were transformed into competent \textit{S. mutans} to generate the deletion mutant strains. Mutant construction was confirmed through PCR and DNA sequencing, including confirmation that the sequences flanking the gene of interest were intact. To generate a FLAG-tagged fusion protein (see Fig. S1 in the supplemental material), two fragments, including 565 bp upstream of the coding regions of the SMU.1147 gene and 372 bp downstream of the SMU.1147 gene, were amplified with gene-specific primers carrying the restriction enzyme sites listed in Table S1 and then doubly digested with restriction enzymes (EcoRI-KpnI and XbaI-HindIII, respectively). A pFLAG-NpEm plasmid (unpublished data) was digested with KpnI and XbaI restriction enzymes to release a 1.2-kbp fragment encoding the FLAG tag and an erythromycin resistance marker (Em\textsuperscript{r}), which was then ligated with the above fragments. The resulting construct was transformed into a wild-type strain, and the FLAG tag fusion was established in single copy in the chromosome by double crossover homologous recombination, with the wild-type SMU.1147 gene serving as the integration site and the expression of the fusion peptide driven from the cognate promoter and ribosome binding site (RBS) for SMU.1147.

**RNA isolation, RT-PCR, and real-time PCR.** Cells were grown to mid-exponential phase in BHI or FMC, supplemented if desired with 2 μM synthetic sigX-inducing peptide (XIP) (GLDWWSL) (21) synthesized by NeoBioSci, at 37°C with 5% CO\textsubscript{2}. The cells were stabilized with bacterial RNAprotect reagent (Qiagen), resuspended in 10 mM TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5), and then subjected to mechanical disruption in a Bead Beater-16 (Biospec Products, Inc., Bartlesville, OK). Total RNA was isolated using the RNeasy minikit (Qiagen) and treated with RNase-free DNase (Qiagen) in order to remove genomic DNA. The concentration of RNA in the samples was determined using a spectrophotometer. By use of the SuperScriptIII first-strand synthesis system (Invitrogen), cDNA was synthesized from 2 μg of total RNA by target-specific primers (see Table S1 in the supplemental material). Then, reverse transcription-PCRs (RT-PCRs) were performed as follows: one cycle of 95°C for 10 min, followed by 30 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 2 min. For quantitative real-time RT-PCR (qRT-PCR), cDNAs were generated using random oligonucleotides (Invitrogen) and qRT-PCR was conducted using iQ SYBR green Supermix (Bio-Rad) in an iCycler qRT real-time PCR detection system (Bio-Rad) as follows: one cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Target-specific primers used for real-time RT-PCRs were designed with Beacon Designer 4.0 software (Premier Biosoft International, Palo Alto, CA). All expression data were normalized to the copy number of 16S rRNA in each sample.

**Protein fractionation of \textit{S. mutans} cells.** Fractionation of \textit{S. mutans} strains carrying a FLAG tag on the C terminus of the SMU.1147-encoded peptide was performed as previously described (36, 37), with minor modifications. Briefly, cells were grown in 5 ml of BHI broth to an OD\textsubscript{600} of 0.4. Cultures were centrifuged at 3,200 × g for 10 min at 4°C to obtain culture supernatants (S) and cell pellets. Supernatants were filtered through a 0.22-μm syringe filter (Millipore), precipitated with 100 μl of 100% (wt/vol) trichloroacetic acid (TCA; Fisher Scientific), collected by centrifugation, washed twice with 300 μl of acetone (Fisher Scientific), and resuspended in 50 μl buffer A (0.5 M sucrose, 10 mM Tris-HCl [pH 6.8], 10 mM MgSO\textsubscript{4}) containing 17 μg ml\textsuperscript{-1} of phenylmethanesulfonyl fluoride (PMSF) (ICN Biomedicals Inc.). Cell pellets were washed once with 1 ml buffer A, collected by centrifugation, and resuspended in 1 ml buffer A. To obtain cell wall (CW) fractions and protoplasts, the cell pellets were incubated at 37°C overnight following the addition of 25 μl of 10 mg ml\textsuperscript{-1} lysozyme (Sigma) and 2 μl of 10,000 U ml\textsuperscript{-1} proteasinulin (Sigma). Protoplasts and digested cell wall (CW) fractions were separated by centrifugation at 3,200 × g at 4°C, and the supernatants (CW) were filtered through a 0.22-μm syringe filter and precipitated with TCA as described above. The protoplasts were washed twice with 1 ml of buffer A, resuspended in 1 ml of osmotic lysis buffer (50 mM Tris, pH 7.5, 10 mM MgSO\textsubscript{4}, 0.8 M NaCl), and broken by 3 cycles of sonication for 10 s each on a setting of 5 using an Ultra-Sonicator W-370 (Heat Systems-Ultrasonics Inc., USA). After adding 10 μl of 1 mg ml\textsuperscript{-1} DNase (Promega) and 1 mg ml\textsuperscript{-1} RNase (Sigma), the protoplasts were incubated for 30 min at room temperature. Unlysed protoplasts and cell debris were removed by centrifugation, and the protoplasts were centrifuged twice at 3,200 × g for 5 min at 4°C. To separate cytoplasmic (C) and membrane (M) fractions, supernatants were transferred into 1.5-ml ultracentrifuge tubes (Beckman Coulter) and centrifuged at 100,000 × g for 1 h at 4°C in an Optima ultracentrifuge (Beckman Coulter). After centrifugation, the supernatant fluids were transferred to a new 1.5-ml tube as the cytoplasmic (C) fraction and the pellets were resuspended in 50 μl of buffer A as the membrane (M) fraction. For the preparation of whole-cell lysates (WL), cell pellets grown and collected as described above were resuspended in 1 ml of buffer A supplemented with 25 μl of 10 mg ml\textsuperscript{-1} lysozyme and 2 μl of 10,000 U ml\textsuperscript{-1} mutanolysin and incubated at 37°C overnight. After incubation, the cells were treated with 160 μl of 5 M NaCl and then disrupted by sonication. The lysed cells were then treated with 10 μl of 1 mg ml\textsuperscript{-1} DNase and 1 mg ml\textsuperscript{-1} RNase and centrifuged at 3,200 × g for 5 min at 4°C. The supernatants (WL) were transferred into a new tube and TCA precipitated as described above. Protein concentration was determined using the bicinchoninic acid assay (BCA) (Thermo Scientific).

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Detection of FLAG-tagged proteins by Western blotting. FLAG-tagged SMU.1147-encoded protein was detected by Western blotting as described previously (36) with modifications. Briefly, 10 μg of protein was mixed with 5× SDS sample buffer (200 mM Tris–HCl, pH 6.8, 10% [vol/vol] SDS, 20% [vol/vol] glycerol, 10% [vol/vol] β-mercaptoethanol, 0.02% [vol/vol] bromophenol blue). The protein samples were denatured in boiling water for 5 min and then resolved by 12% SDS-PAGE with a Mini-Protein 3 cell system (Bio-Rad Laboratories). Following electrophoresis, the proteins were transferred to Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore) using a TE series Transphor electrophoresis unit (Hoefer Scientific Instruments, USA) in transfer buffer (25 mM Tris, 192 mM glycine, 10% [vol/vol] methanol). FLAG-tagged proteins were detected using a primary monoclonal anti-FLAG M2 antibody (Sigma) and a secondary peroxidase-labeled goat anti-mouse IgG antibody (1:10,000 dilution; Kirkegaard & Perry Laboratories, USA). The detection of FLAG-tagged proteins was performed using a SuperSignal West Pico chemiluminescent substrate kit (Thermo Scientific) and visualized with a FluorChem 8900 imaging system (Alpha Innotech, USA). Whole-cell lysates prepared as described above from wild-type S. mutans UA159 with no FLAG epitope expressed were used as a negative control.

Quantification of biofilm formation. Biofilm assays were performed as previously described (38). Cell cultures were grown in a 96-well polyvinylidene difluoride (PVDF) membrane (Millipore) using a TE series Transphor electrophoresis unit (Hoefer Scientific Instruments, USA) in transfer buffer (25 mM Tris, 192 mM glycine, 10% [vol/vol] methanol). FLAG-tagged proteins were detected using a primary monoclonal anti-FLAG M2 antibody (Sigma) and a secondary peroxidase-labeled goat anti-mouse IgG antibody (1:10,000 dilution; Kirkegaard & Perry Laboratories, USA). The detection of FLAG-tagged proteins was performed using a SuperSignal West Pico chemiluminescent substrate kit (Thermo Scientific) and visualized with a FluorChem 8900 imaging system (Alpha Innotech, USA). Whole-cell lysates prepared as described above from wild-type S. mutans UA159 with no FLAG epitope expressed were used as a negative control.

Determination of genetic competence. Competence assays were performed as previously described (39). Briefly, overnight cultures of the wild-type strain and its derivatives were diluted (1:20) into 475 μl of either fresh BHI or defined medium FMC and grown to an OD600 of 0.15 at 37°C, with 0.1% crystal violet for 15 min. After the stained bacteria were washed twice with 200 μl of water, the retained dye was extracted from cells using ethanol-acetone (8:2, vol/vol), and biofilms were quantified by measuring absorbance at a 575-nm wavelength.

Statistical analysis. All graphical data display the means and standard deviations for a minimum of three biological replicates (n = 3). All data were analyzed using the Student t test method with P values provided in the figure legends.

RESULTS AND DISCUSSION

Transcriptional organization of the SMU.1147 operon. Cornejo et al. (27) recently completed whole-genome shotgun sequencing of 57 strains of Streptococcus mutans along with representatives of some of the most closely related mutants streptococci. The report included a population and evolutionary genomic analysis of S. mutans, but the authors also identified what they termed the “unique core genome” (UCG) of S. mutans. The UCG is a subset of the core genome of S. mutans consisting of genes that are in all S. mutans isolates but absent in other sequenced members of the mutans streptococci. Interestingly, the UCG of S. mutans is significantly enriched for genes that encode small proteins or peptides, possibly involved in regulation or signaling, as well as for genes that have helped to contribute to the adaptation of S. mutans to the human oral cavity after the adoption of agriculture and the associated increase in carbohydrates in the human diet. In particular, the UCG contains genes that have been shown to be activated by low pH, sucrose, or starch and genes involved in oxidative stress tolerance. We have been conducting a mutational analysis of a group of UCG genes from S. mutans and have found that the SMU.1147 gene product has properties that make it a potential therapeutic target to control the establishment, persistence, or virulence of this caries pathogen.

The SMU.1147 gene is 186 bp and encodes a hypothetical protein of 61 amino acids. BLASTP and BLASTN searches indicate that it exists uniquely in S. mutans. SMU.1147 is located immediately upstream of the scnRK genes (Fig. 1), carrying GenBank designations SMU.1145 and SMU.1146, respectively, and encoding an apparent two-component system (TCS). Note that some annotations designate ScnK as CovS, but annotations of ScnR as CovR appear to be incorrect, as the apparent true CovR of S. mutans UA159 is an orphan response regulator (40).

To determine whether SMU.1147 could be cotranscribed with scnRK, reverse transcriptase PCRs were performed using cDNAs as a template. As shown in Fig. S2 in the supplemental material, the result indicated that these genes are organized in an operon. It is also notable that inactivation of SMU.1147 with a nonpolar marker did not result in a statistically significant change in the mRNA levels of scnRK (data not shown). Using bioinformatic analysis (BPROM), a promoter region was predicted to be position 38 to 66 bp upstream of the SMU.1147 start codon. A second promoter was predicted to be located just upstream of the SMU.1146 gene, but we could find no evidence to support the idea that this predicted promoter was functional when we fused the 3′ end of SMU.1147 to a reporter gene (data not shown). Therefore, the results support the idea that transcription of the SMU.1147 operon is governed by a promoter 5′ to SMU.1147. It should also be noted that all three genes in this operon were consistently expressed under the growth conditions tested, with roughly 3.5 × 10⁵ copies of mRNA detected per μg of input RNA for each gene.

The SMU.1147-encoded peptide is expressed in S. mutans. The topology of the SMU.1147 gene product was predicted using a web-based TopPred algorithm (http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::toppred) (41). According to the predicted model, the 61-aa peptide contains a single alpha-helical transmembrane segment (SMU.1147,33–53) and an N-terminal protru-
Deletion of SMU.1147 affects growth and biofilm formation.

To investigate the physiological roles of the SMU.1147 operon in S. mutans, the coding regions of the scnK, scnR, and SMU.1147 genes were replaced by a nonpolar kanamycin resistance cassette (see Table S1 in the supplemental material). The integrity and lack of polarity of the deletion-replacements were confirmed using a combination of sequencing and RT-PCR analysis (data not shown). Growth of the wild-type and mutant strains was monitored in a Bioscreen C in FMC broth without a mineral oil overlay to create comparatively aerobic conditions (Fig. 3A) or with a mineral oil overlay for more anaerobic conditions (Fig. 3B). Under aerobic conditions, the wild-type and mutant strains carrying scnK or scnR deletions did not differ in their growth. However, the strain lacking SMU.1147 displayed a significantly increased ($P < 0.05$) doubling time ($T_d = 103.3 \pm 3.9$ min), compared to the wild-type strain ($T_d = 80.8 \pm 1.4$ min) (Fig. 3A). Under anaerobic conditions, the ΔscnK or ΔscnR strain grew slightly faster and achieved a higher final optical density than did the wild-type strain, whereas the SMU.1147 deletion mutant grew significantly slower ($T_d = 104.5 \pm 3.3$ min) than the wild-type strain ($T_d = 76 \pm 3.4$ min) ($P < 0.05$) (Fig. 3B). One unusual observation was that the final OD achieved by the SMU.1147 mutant growing under aerobic conditions was higher than that for growth under anaerobic conditions. Importantly, when the scnRK mutations were introduced into the strain carrying an inactivated SMU.1147 gene, the ΔscnRK SMU.1147 triple mutant grew comparably to the wild-type strain or to strains carrying deletions in only the TCS genes (Fig. 3A and B).

The abilities of the ΔscnK, ΔscnR, ΔSMU.1147, and triple deletion mutant strains to form stable biofilms were compared to that of the wild-type strain (Fig. 4A and B). Inactivation of SMU.1147 resulted in a smaller amount of biofilm formation when grown in BM broth supplemented with 20 mM glucose as the primary carbohydrate source, compared to the parental strain ($P < 0.001$), whereas the inactivation of scnK or scnR elicited no change in biofilm formation (Fig. 4A). As is often observed with strains of S. mutans that display a biofilm defect in glucose-containing medium, biofilm formation in BM-sucrose did not differ among the strains, likely because the production of glucan polymers can mask defects in biofilm formation that are evident in glucose-cultured cells. Similar to what was observed for planktonic growth, the strain carry-
ing deletions of all three genes displayed biofilm formation on par with that of the wild-type strain (Fig. 4A). Thus, an intact ScnRK TCS is required for manifestation of growth and biofilm defects in strains lacking the SMU.1147-encoded peptide.

SMU.1147 has major impacts on stress tolerance. Chen et al. (24) reported that an scnRK-null mutant of S. mutans had diminished resistance to phagocytic killing and oxidative stress tolerance. We examined the growth of the wild-type and mutant strains when exposed to acid, oxidative, and osmotic stressors as detailed in Materials and Methods. To determine whether the mutant strains had altered resistance to low pH, we monitored growth under anaerobic conditions in FMC that had been acidified to pH 5.5 (Fig. 5A). Growth of the SMU.1147 mutant was significantly slower than that of other strains (*, difference from the wild-type genetic background at $P < 0.001$ (Student’s $t$ test)).

![Graph A](image1.png)  
**FIG 4** Biofilm formation. S. mutans wild-type and mutant strains were grown to an OD$_{600}$ of 0.5 in BHI broth, diluted 1:50 in BM semidefinite medium supplemented with 20 mM glucose (A) or with 10 mM sucrose (B) in a 96-well microtiter plate, and incubated at 37°C with 5% CO$_2$ for 48 h. To quantify biofilm formation, the plates were washed twice, stained with 0.1% crystal violet, and resuspended with an ethanol-acetone (8:2 [vol/vol]) mixture. The OD$_{575}$ of the stained biofilm was measured. Results shown are the mean and standard deviation (error bars) of three separate isolates assayed in triplicate. *, difference from the wild-type genetic background at $P < 0.001$ (Student’s $t$ test).

![Graph B](image2.png)  

![Graph C](image3.png)  
**FIG 5** Growth curves under various stress conditions. The wild-type (●), ΔscnK (■), ΔscnR (▲), ΔSMU.1147 (×), and ΔscnRK SMU.1147 triple mutant (●) strains were grown at 37°C in triplicate to mid-exponential phase in FMC medium and diluted 1:100 into fresh FMC medium supplemented with HCl to lower the pH to 5.5 (A), with 0.003% H$_2$O$_2$ for oxidative stress (B), or with 0.3 M NaCl for osmotic stress (C). The OD$_{600}$ measurements are the mean values of three independent experiments.
growth of the strains carrying the \textit{scnK} and \textit{scnR} mutations was similar to that of the wild-type strain. When cells were grown in the presence of hydrogen peroxide, the SMU.1147-deficient strain displayed a greatly extended lag phase and again did not achieve as high a final optical density as did the wild-type cells ($P < 0.05$) (Fig. 5B), but the strains lacking the TCS components displayed slightly faster growth than did the parental strain ($P < 0.05$). Interestingly, the strain carrying the triple deletion had growth rates and final yields comparable to those of the wild-type strain when exposed to acid or oxidative stress (Fig. 5A and B), demonstrating that an intact ScnRK TCS was needed for the defects in acid and oxidative stress to be manifested in the strains lacking the SMU.1147 gene.

We next determined that wild-type \textit{S. mutans} was unable to grow in FMC broth at NaCl concentrations higher than 0.3 M. When we evaluated the ability of the strains to tolerate osmotic stress, as assessed by monitoring growth in FMC medium supplemented with 0.3 M NaCl, the growth rate of all the single mutants was decreased compared to that of the parental strain ($P < 0.05$) (Fig. 5C). However, unlike for other growth conditions and stresses, the triple mutant displayed the same slow-growth phenotype in 0.3 M NaCl as did the other mutant strains ($P < 0.05$) (Fig. 5C). Taken together, the results support the idea that SMU.1147 plays an essential role in expression of traits that can affect the virulence of \textit{S. mutans} and that the 1147 peptide elicits its effects through the ScnRK TCS. It should also be noted that we determined that nonpolar insertions in the \textit{scnK} or \textit{scnR} gene did not impact the level of expression of SMU.1147 transcripts (data not shown).

Inactivation of SMU.1147 affects genetic competence of \textit{S. mutans}. Natural genetic competence in \textit{S. mutans} is linked to stress tolerance and biofilm formation (42, 43). Since inactivation of SMU.1147 reduced tolerance to low-pH and oxidative stresses and decreased biofilm formation, we evaluated the transformation efficiency of the SMU.1147 mutant strain. The transformation efficiency of the complemented SMU.1147 mutant was tested in the presence of CSP. All strains were grown to mid-exponential phase in BHI medium and diluted 1:20 into fresh BHI medium. Plasmid pBGE (200 ng), which carries an erythromycin resistance (Emr) gene, was added to the culture. Following 2 h of incubation, cells were serially diluted and plated onto BHI plates supplemented with 1 mg ml$^{-1}$ spectinomycin or 10 µg ml$^{-1}$ erythromycin. Transformation efficiency was determined by counting CFU. $^\ast$, difference from the wild-type genetic background at $P < 0.0001$ (Student’s $t$ test).
of \( \text{scn}K \) mutations into the SMU.1147 mutant background restored the ability of the strain to take up exogenous DNA (Fig. 6A and B).

To determine if the results were due solely to the loss of the SMU.1147 gene, we introduced a wild-type copy of SMU.1147 expressed under the control of its own promoter into the wild-type and \( \Delta \text{SMU.1147} \) mutant strains on the multicopy shuttle plasmid pDL278, resulting in overexpression of SMU.1147 (Fig. 6C). Transformation efficiency of these strains was determined using the integration vector pBGE (46) that carries an erythromycin resistance marker, since the pDL278 vector confers spectinomycin resistance. Complementation of the \( \Delta \text{SMU.1147} \) mutant (strain SMU.1147/\( \Delta \text{SMU.1147} \)) resulted in high-level expression of SMU.1147 (Fig. 6C) compared with the wild-type strain, and transformation efficiency was restored to levels that were significantly higher than the wild-type strain (Fig. 6D). Likewise, the complemented strain restored growth under stress conditions and the ability to form biofilms nearly to the level of the wild-type strain (data not shown). When the SMU.1147 gene was overexpressed in wild-type \( S. \text{mutans} \), we again observed higher transformation efficiency compared to the parental strain (Fig. 6D). Thus, SMU.1147 is required for optimal development of genetic competence and stress responses by \( S. \text{mutans} \). It should also be noted that introduction of SMU.1147 on pDL278 into the wild-type genetic background did not alter growth or stress tolerance (data not shown).

**Transcription of competence genes is regulated by SMU.1147.**

Recently, our group and others (20–23) demonstrated that XIP, a 7-aa peptide derived from processing of the 17-aa ComS peptide, is required for activation of \( \text{comX} \) expression by the ComR transcriptional regulator. ComX can then activate late competence genes, e.g., \( \text{comY} \), that are required for DNA uptake (20, 21). In \( S. \text{mutans} \), competence development does not occur in the absence of XIP in chemically defined medium and peptides present in BHI or tryptone can effectively block activation of \( \text{comX} \) by XIP, apparently by competing for uptake of XIP (20) by the oligopeptide permease Opp (43, 47). We examined whether XIP-dependent signaling was affected in the mutant and whether SMU.1147 affects expression of early (\( \text{comX} \)) or late (\( \text{comY} \)) competence genes. The results revealed that the \( \Delta \text{SMU.1147} \) strain had significantly decreased transformation efficiency in the presence of XIP (\( P < 0.0001 \)), whereas the mutants lacking \( \text{scn}K \) or \( \text{scn}R \) displayed no significant changes compared with their parental strain (Fig. 7A). Interestingly, concurrent inactivation of the TCS genes and SMU.1147 consistently reversed the effects that the SMU.1147 mutant alone displayed (Fig. 7A). Also, consistent with the transformation efficiency shown above, loss of the SMU.1147 gene resulted in significantly reduced mRNA levels of both \( \text{comX} \) and \( \text{comY} \), and neither gene could be induced by exogenously supplied XIP (\( P < 0.001 \)) (Fig. 7B). Thus, the SMU.1147 peptide plays a dominant role in the development of competence, and loss of this peptide somehow interferes with the ability of cells to activate \( \text{comX} \) through the ComRS pathway. Further, as for growth phenotypes, the \( \text{scn}K \) TCS was needed for manifestation of the competence defect associated with the loss of SMU.1147.

**Concluding remarks.** We evaluated a variety of phenotypes of the SMU.1147-deficient strain that are associated with the capacity of \( S. \text{mutans} \) to persist in the oral cavity and to initiate dental caries. Our results clearly show that the SMU.1147 peptide dominantly controls key virulence-related traits of \( S. \text{mutans} \) in a manner that is independent from, and often in contrast to, the behavior of mutants lacking the associated TCS components. Interestingly, when all three genes were deleted, the strain behaved indistinguishably from the wild-type strain for all phenotypes as assessed in this study except osmotic stress. Thus, we propose that the SMU.1147 peptide does indeed work in conjunction with the \( \text{scn}K \) TCS. The simplest interpretation of the results is that the SMU.1147-encoded peptide, which we here designate ScnC, interferes with \( \text{scn}K \) TCS activity, perhaps by complexing with ScnK and holding it in an inactive conformation or blocking access to its cognate signal. When SMU.1147 is deleted, the TCS becomes active, or overly active, and alters gene expression in a manner that elicits the observed phenotypes. While many TCSs interact with additional peptides or proteins that can modulate
their activity, it is unusual for a peptide to block activity in the manner proposed for ScnC. One must also consider that ScnC could have the ability to function as a “TCS connector” (48–50) to modulate the phosphorylation status of ScnR, and possibly even ComDE. Also, since both comX and comY levels were lower in the strain lacking ScnC, ScnCRK may somehow modulate ComRS cascade, and whether interfering with ScnC can diminish ComDE. Also, since both

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