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Involvement of Sensor Kinases in the Stress Tolerance Response of *Streptococcus mutans*▽

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The gram-positive bacterium *Streptococcus mutans* is the primary causative agent in the formation of dental caries in humans. The ability of *S. mutans* to adapt and to thrive in the hostile environment of the oral cavity suggests that this cariogenic pathogen is capable of sensing and responding to different environmental stimuli. This prompted us to investigate the role of two-component signal transduction systems (TCS), particularly the sensor kinases, in response to environmental stresses. Analysis of the annotated genome sequence of *S. mutans* indicates the presence of 13 putative TCS. Further bioinformatics analysis in our laboratory has identified an additional TCS in the genome of *S. mutans*. We verified the presence of the 14 sensor kinases by using PCR and Southern hybridization in 13 different *S. mutans* strains and found that not all of the sensor kinases are encoded by each strain. To determine the potential role of each TCS in the stress tolerance of *S. mutans* UA159, insertion mutations were introduced into the genes encoding the individual sensor kinases. We were successful in inactivating all of the sensor kinases, indicating that none of the TCS are essential for the viability of *S. mutans*. The mutant *S. mutans* strains were assessed for their ability to withstand various stresses, including osmotic, thermal, oxidative, and antibiotic stress, as well as the capacity to produce mutacin. We identified three sensor kinases, Smu486, Smu1128, and Smu1516, which play significant roles in stress tolerance of *S. mutans* strain UA159.

Streptococcus mutans, a pathogenic gram-positive bacterium with a low G+C content, is primarily associated with the formation of dental caries, which is one the most common human infectious diseases (39). *S. mutans* adheres to the tooth surface and forms dental plaque, a classical biofilm. The pH of the oral cavity is predominantly acidic; within the dental plaque, *S. mutans* induces an acid tolerance response that facilitates its survival under conditions of low pH (30, 31). In addition, *S. mutans* experiences various environmental stresses, including temperature fluctuation, nutritional limitation, and variation in oxygen tension (14); despite these harsh conditions, *S. mutans* is able to maintain its presence by forming biofilms in the oral cavity. Similar conditions are also encountered in the biofilm formed on the heart valves, where the inhibitory effects of immune response elements in the blood must be overcome. *S. mutans*, along with other oral bacteria, can enter the bloodstream after dental extractions and cause transient bacteremia and infective endocarditis (39). As much as 14% of cases of streptococcus-induced endocarditis are thought to be mediated by *S. mutans* (66). The extraordinary ability of *S. mutans* to adapt and survive in the diverse and hostile environment of the oral cavity demonstrates the fundamental importance of detailed analysis of the molecular mechanisms of stress tolerance response in this organism.

In prokaryotic organisms, adaptive responses to environmental changes, such as nutrient limitation, oxygen deprivation, and osmotic shock, are regulated by the so-called two-component signal transduction systems (TCS) (18, 20, 36, 42). These systems share a common biochemical mechanism involving phosphoryl transfer between two distinct protein components: a sensor kinase and a response regulator. The sensor kinase is generally a transmembrane receptor that detects environmental changes and undergoes autophosphorylation of a specific histidine residue within the cytoplasmic signaling domain, which is relatively well conserved and contains about 200 residues (28). The membrane-integral N-terminal region of the protein can vary significantly between different histidine kinases and may consist of a variable number of transmembrane helices that, along with the cytoplasmic regions, play a crucial role in signal recognition. The phosphoryl group is subsequently transferred from the sensor kinase to a specific aspartic acid residue located on the response regulator, which typically functions as a cytoplasmic transcriptional regulator (for reviews, see references 42 and 58). Phosphorylation activates the response regulator, which then allows it to modulate expression of its target genes necessary for growth under a particular environment.

Sequence analysis of the complete genome of *S. mutans* strain UA159 reveals the presence of 13 putative TCS (3). While several of these systems have been studied and characterized, most of the studies focused on the role of sensor kinases in virulence regulation and/or biofilm formation (2, 7, 37, 38, 54, 57). To date, there has not been any systematic study performed to characterize the involvement of sensor kinases in the stress tolerance response of *S. mutans*. In an effort to gain a more comprehensive view of the role of these sensor kinases, we systematically inactivated each of the genes coding for the 14 putative sensor kinases in *S. mutans* UA159. Each of the sensor kinase mutants was then tested against a variety of environmental stresses, as well as a panel of antibiotics. Several putative sensor kinases were identi-

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TABLE 1. Oligonucleotides used for construction of plasmids containing sensor kinase gene fragments

Primer	Sequence (5′ to 3′)[a]	Purpose
SMU45-F	CTATATAATGCAAGCAAATCAGTTGAG	pIB12 construction for smu45 inactivation
SMU45-R	CCGTTTCTTAATTTCATGAAATCCGAC	pIB12 construction for smu45 inactivation
SMU486-F	TTCTGCTTAATCAGGCCATCACACAGG	pIB14 construction for smu486 inactivation
SMU486-R	CCTCAATATTACTCTTATCAGTTAATTCTC	pIB14 construction for smu486 inactivation
SMU577-F	CATTCCTATGATGTTGCTTAACAGTCTAGG	pIB15 construction for smu577 inactivation
SMU577-R	CTGCAAGGCCTTGATTTCTGCAATATTAG	pIB15 construction for smu577 inactivation
SMU660-F	CATTATTCAGTTGCCTGGATGAATAAGTATC	pIB16 construction for smu660 inactivation
SMU660-R	GAATCTTTATATCTTTCGTTGCAGCAAG	pIB16 construction for smu660 inactivation
SMU928-F	GTCTTATATGAGAAATATTAATCTCCATG	pIB17 construction for smu928 inactivation
SMU928-R	CTTCTGCTTTGACATAAATATCAGAAGC	pIB17 construction for smu928 inactivation
SMU1009-F	GAACCAAGGAATTTAACTATGTTAACTGCAC	pIB18 construction for smu1009 inactivation
SMU1009-R	CCTTAATCTTAATGGTGATCTGCCCAC	pIB18 construction for smu1009 inactivation
SMU1037-F	GACAAGCCTTATAGCTCTTCAGG	pIB19 construction for smu1037 inactivation
SMU1037-R	TTCTTCAAGATTAACTGTGCTTG	pIB19 construction for smu1037 inactivation
SMU1128-F	GATATTGTTGAACAAGAAGCTAAGAATATTTAC	pIB20 construction for smu1128 inactivation
SMU1128-R	GAATTTCTGTTATTTCTGGTTTGATACCATC	pIB20 construction for smu1128 inactivation
SMU1145-F	GTCCAATCTTATCGTTTACCTCAACAGC	pIB21 construction for smu1145 inactivation
SMU1145-R	CTCCGTTTGCTTTCCATGACCATATTG	pIB21 construction for smu1145 inactivation
SMU1516-F	GATAGTTATACTTACAATGATTTGATTAC	pIB22 construction for smu1516 inactivation
SMU1516-R	GTATCAATTTCAATCCAAACTGATTTGTC	pIB22 construction for smu1516 inactivation
SMU1548-F	GATTAATGGCATTATTTATGGGATTG	pIB23 construction for smu1548 inactivation
SMU1548-R	GATAGACTGATATCAGATAAACCAAAGAG	pIB23 construction for smu1548 inactivation
SMU1814-F	CCAAAAGACAGTATTATCCGATATAGC	pIB24 construction for smu1814 inactivation
SMU1814-R	CTCTAAGTTACCATTTGTCAATCGAGC	pIB24 construction for smu1814 inactivation
SMU1916-F	GCAATCATATTCTTTATCTTGGATGGAAC	pIB25 construction for smu1916 inactivation
SMU1916-R	GATATTATAACGGGTATCCTGCAATTG	pIB25 construction for smu1916 inactivation
SMU1965-F	TTGTCTTAATGATTCTTACCTTTATTTC	pIB13 construction for smu1965 inactivation
SMU1965-R	CATCTTGAATACCTTCTCTAACAACATCTG	pIB13 construction for smu1965 inactivation
Bam-SMU486-F1	cgcggatccTGTTGATGTCTCAGTTAGTTTGG	pIB55 construction for smu486 complementation
Bam-SMU487-R2	cgcggatccAACTCTGTTGGTTTTGAAACAACAGC	pIB55 construction for smu486 complementation
Bam-SMU1127-R2	ggcggatccGGAGGTAATAAAGACATTGGC	pIB302 construction for smu1128 complementation
Bam-SMU1129-F2	ggcggatccGGAAGATACTCCACCTAATGGC	pIB302 construction for smu1128 complementation
Eco-SMU1516-R2	ggcgaatTCCTTTCTTTTATCCTTAGACCTTTC	pIB303 construction for smu1516 complementation
Bam-SMU1516-F2	ggcggatccATTGATTTTGATATAATCGTAGAGGG	pIB303 construction for smu1516 complementation

[a] Sequences homologous to the *S. mutans* genome sequence are in uppercase. Synthetic restriction sites added for cloning purposes are underlined.

fied which contribute to the stress tolerance response of *S. mutans*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* strain DH5α was grown in Luria-Bertani medium supplemented (when necessary) with erythromycin (300 μg/ml). *S. mutans* UA159 and its derivatives were used for all of the genetic experiments in the present study. For identification of TCS in various strains of *S. mutans*, the following strains were used: NG8, V-100, and OMZ175 (A. S. Bleiweis, University of Florida); GS-5 and SP-2 (H. Kuramitsu, SUNY, Buffalo, NY); SJ32, T-8, and 8VS3 (M. Duncan, The Forsyth Institute, Boston, MA); 3209 and UA130 (J. Banas, University of Iowa); 109Sc (Y. Sato, Tokyo Dental College, Tokyo, Japan); and V403 (F. L. Macrina, Virginia Commonwealth University, Richmond). *Streptococcus gordonii* (DL-1) and *Streptococcus sanguinis* (SK36) were kindly provided by M. Gilmore (Harvard Medical School, Boston, MA) and T. Kitten (Virginia Commonwealth University, Richmond), respectively. With the exception of some stress experiments, as described below, *S. mutans* cultures were routinely grown in Todd-Hewitt medium (BBL, Becton Dickson) supplemented with 0.2% yeast extract (THY). *S. mutans* was also grown in chemically defined media (CDM) supplemented with 0.5% glucose, prepared by a modification of methods previously described (38, 40). CDM consisted of 58 mM K_2HPO_4, 15 mM KH_2PO_4, 10 mM $(NH_4)_2SO_4$, 35 mM NaCl, 0.1 mM $MnCl_2$, 2 mM $MgSO_4 \cdot 7H_2O$, and 0.2% (wt/vol) casein hydrolysate. The medium was supplemented with filter sterilized vitamins (Sigma catalog no. R7256), amino acids (1 mM L-arginine HCl, 1.3 mM L-cysteine HCl, 4 mM L-glutamic acid, and 0.1 mM L-tryptophan). When necessary, THY or CDM was supplemented with erythromycin (10 μg/ml).

Inactivation of sensor kinases in *S. mutans*. The genes encoding the various putative sensor kinases of *S. mutans* strain UA159 were inactivated as described here. Internal fragments (~500 bp) corresponding to the open reading frames of 14 putative sensor kinase genes were amplified from chromosomal DNA of UA159 by using high-fidelity thermostable polymerase and the primers listed in Table 1. Throughout the text, sensor kinases are designated by the open reading frame number, according to the nomenclature of the National Center for Biotechnology Information. Each PCR fragment was cloned into pBSKEry (8) that was linearized with EcoRV and verified by DNA sequencing. These plasmids were then used to inactivate the corresponding sensor kinase genes in *S. mutans* by natural transformation. Transformants were selected on THY agar containing erythromycin. To confirm that the mutagenic plasmids had integrated at the expected loci, genomic DNA was isolated from the erythromycin-resistant transformants and analyzed by PCR. For analysis of each of the sensor kinase mutants, one of the primers was specific to the sequence of the integrated plasmid, while the other primer corresponded to the targeted sensor kinase gene sequence found in the genomic DNA sequence but not within the fragment cloned into the plasmid.

Construction of complementing plasmids. Three sensor kinase-encoding genes were chosen for expression in *trans*. For expression of smu486, the region encoding Smu486/487 was amplified from UA159 chromosomal DNA using primers Bam-SMU486-F1 and Bam-SMU487-R2 and cloned into pCRII-TOPO (Invitrogen) to generate pIB54. A BamHI-HindIII-restriction-digested fragment containing smu486/487 was isolated from pIB54 and subcloned into BamHI-HindIII-digested pJRS1315 (52) to construct pIB55. To express Smu1128, a fragment containing smu1128 was amplified from UA159 chromosomal DNA using primers Bam-SUM1127-R2 and Bam-SMU1129-F2. The resulting fragment was restriction digested with BamHI-EcoRI and cloned into BamHI-EcoRI-digested pIB164 plasmid (a derivative of pJRS1315 [I. Biswas, unpublished data]) to create pIB302. Similarly, for expression of smu1516 in *trans*, a fragment containing smu1516 was amplified from UA159 chromosomal DNA using primers Eco-SMU1516-R2 and Bam-SMU1517-F2, restricted by BamHI and EcoRI, and cloned into BamHI-EcoRI-restricted pIB164 plasmid to create pIB303.

Sensitivity to oxidative stress. To evaluate the sensitivity of each of the *S. mutans* sensor kinase mutants to various oxidative stress-promoting chemicals, cultures were exposed to reagents either through disk diffusion assays or growth on THY agar plates containing the chemical of interest. For disk diffusion assays, cultures were grown overnight in THY broth (with or without antibiotics as required) and then swabbed onto THY agar plates, followed by placement of filter paper disks (6 mm in diameter) containing various stressors onto the inoculated agar. After overnight incubation at 37°C, under microaerophilic conditions, the diameters of the zones of bacterial growth inhibition were measured. For growth on THY agar plates containing stress-inducing chemicals, cultures were grown to exponential phase in THY broth with appropriate antibiotics at 37°C. Cultures were pelleted via centrifugation, washed twice with 0.85% NaCl, and resuspended in 0.85% NaCl. The cultures were adjusted to an optical density (A_{600}) of 5.0 and serially diluted 10-fold, and 7.5 μl of each dilution was spotted onto THY agar containing the oxidative-stress-inducing chemicals. The plates were incubated overnight at 37°C, under microaerophilic conditions, and bacterial growth was evaluated as previously described (10). The following chemicals were used as indicated. Methyl viologen (paraquat; Sigma) was added to THY agar medium to a final concentration of 5 or 10 mM, while 10 μl from a 1 M stock was added to each disk for the disk diffusion assay. Hydrogen peroxide (Sigma) was used only for THY plate growth assays, at a concentration of 2 or 4 mM. Cumene hydroperoxide (Sigma) and *t*-butyl-hydroxyperoxide (*t*-BOOH; Sigma) were used only in disk diffusion assays; 10 μl of 5% cumene hydroperoxide or 70% *t*-BOOH was added to each disk.

Antibiotic susceptibility stress. Disk diffusion assays were performed as described above to evaluate antibiotic susceptibility of the *S. mutans* sensor kinase mutants. Antibiotic disks (6 mm in diameter; Becton and Dickinson Laboratories) were placed on THY agar plates inoculated either with wild-type UA159 or with one of the sensor kinase mutant strains. The zones of inhibition were measured after overnight incubation. The antibiotics used for the study included bacitracin (10 U), cefaclor (30 μg), cefazolin (30 μg), cefepime (30 μg), cefixime (5 μg), cefotaxime (30 μg), cefoxitin (30 μg), ceftazidime (30 μg), cefuroxime (30 μg), cephalothin (30 μg), chloramphenicol (5 μg), ciprofloxacin (5 μg), clindamycin (2 μg), colistin (10 μg), ertaperiem (10 μg), fosfomycin (200 μg), imipenem (10 μg), meropenem (10 μg), mezlocilin (75 μg), nalidixic acid (30 μg), penicillin (2 μg), polymyxin B (300 μg), rifampin (5 μg), trimethoprim (5 μg), and vancomycin (5 μg).

Acid stress tolerance. To analyze the growth of *S. mutans* cultures at low pH, the initial pH of the THY agar medium was adjusted, prior to sterilization, to pH 5.5 or 7.0 with HCl. Citrate-phosphate buffer (50 mM) of the desired pH was added to media after sterilization. Different dilutions of *S. mutans* cultures, prepared as described above, were spotted onto the plates and incubated at 37°C under anaerobic conditions.

Sensitivity to osmotic stress. Overnight cultures were diluted 20-fold in fresh THY media containing sorbitol (final concentration, 4 or 5%), NaCl (final concentration, 0.5 M), or ethanol (final concentration, 4 or 5%), and grown at 37°C. Growth was monitored by using a Klett-Summerson colorimeter with a red filter, as previously described (8).

Sensitivity to other chemical reagents. Sensitivity of the *S. mutans* sensor kinase mutant strains to diamide, puromycin, and mitomycin C was evaluated by using the disk diffusion method or by growth on THY agar plates containing these reagents. THY agar plates inoculated with culture were overlaid with disks containing 1 M of diamide, for assessment of diamide sensitivity. Mitomycin C was added to THY agar medium at a final concentration of 6 ng/ml or to disks at a concentration of 12.5 μg/ml. Puromycin was added to a final concentration of 6.0 μg/ml in THY agar plates.

Deferred antagonism assay for mutacin production. *S. mutans* cultures (wild type and mutant) were spotted (at various dilutions) onto a THY plate and grown overnight at 37°C under microaerophilic conditions. Each plate was overlaid with soft agar containing an overnight culture of indicator strain consisting of either *S. gordonii* (DL-1) or *S. sanguinis* (SK36). The zone of inhibition of the indicator strains was evaluated after an overnight incubation.

RESULTS

The genome of *S. mutans* UA159 encodes 14 putative TCS. Analysis of the annotated genome of *S. mutans* strain UA159 (GenBank accession no. AE014133) suggests that UA159 encodes 13 putative TCS, many of which share significant homology to TCS characterized in other bacteria (Fig. 1). *S. pyogenes*, which is closely related to *S. mutans*, encodes an additional orphan sensor kinase, which was not annotated in its genome (8). BLAST-P analysis was performed to determine whether *S. mutans* also encoded an orphan sensor kinase. The sequences encoding the C-terminal kinase domain from each of the 13 sensor kinases was used as a query and compared against the complete *S. mutans* UA159 genome. The BLAST-P results also indicated the presence of one additional open reading frame, smu45, which was originally designated as a gene coding for a "hypothetical protein." Smu45 consists of 533 residues with significant similarity to the BaeS family of sensor kinases from *Microscilla marina* (e^{-38}) and *Haemophilus influenzae* (e^{-25}). The C-terminal region of Smu45 also contains the conserved HATPase_c (Pfam02518) domain. The gene imme-

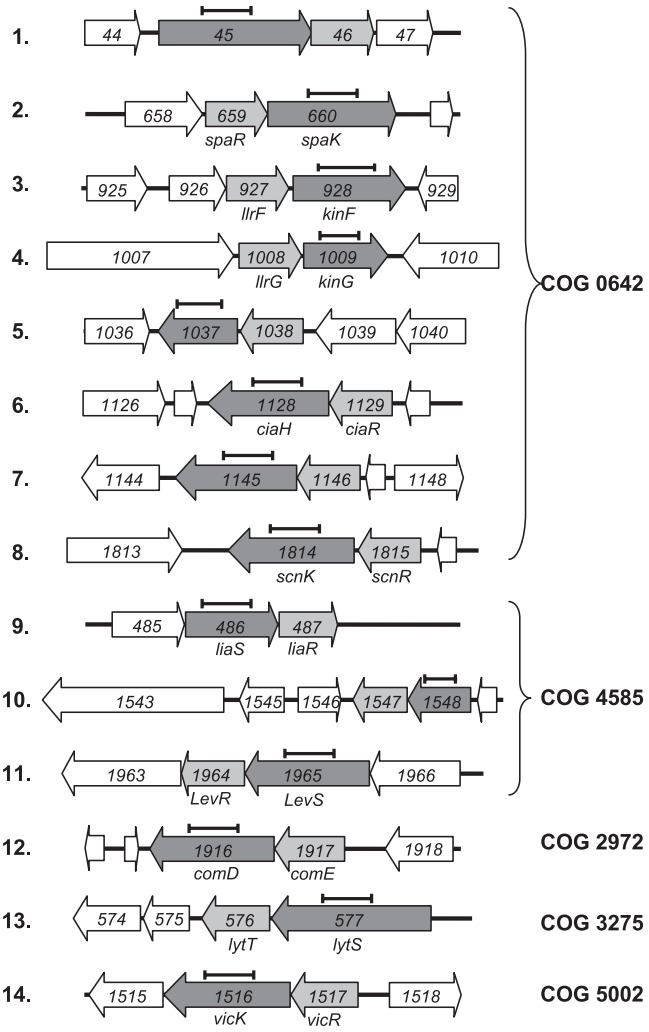

FIG. 1. Schematic diagram of the loci encoding TCS in the *S. mutans* UA159 genome. TCS are depicted by light gray (response regulator) and dark gray (sensor kinase) arrows, and the direction of transcription is indicated by the orientation of the arrows. Open reading frames are designated according to National Center for Biotechnology Information nomenclature. TCS are organized according to their COG (cluster of orthologs) groups (60). TCS from *S. mutans* showing significant homology with previously characterized TCS are as noted. The location of the fragments that were amplified and used for insertion mutagenesis and probes are represented by horizontal lines above the block arrows.

TABLE 2. *S. mutans* strains used for this study

Strain	Genotype	Source or reference
UA159	Wild type, serotype c	R. A. Burne, University of Florida
IBS10	UA159 (*covR*::*aad9*)	9
IBS340	UA159 with smu45 inactivated by pIB12	This study
IBS341	UA159 with smu486 inactivated by pIB14	This study
IBS342	UA159 with smu577 inactivated by pIB15	This study
IBS343	UA159 with smu660 inactivated by pIB16	This study
IBS344	UA159 with smu928 inactivated by pIB17	This study
IBS345	UA159 with smu1009 inactivated by pIB18	This study
IBS346	UA159 with smu1037 inactivated by pIB19	This study
IBS347	UA159 with smu1128 inactivated by pIB20	This study
IBS348	UA159 with smu1145 inactivated by pIB21	This study
IBS349	UA159 with smu1516 inactivated by pIB22	This study
IBS350	UA159 with smu1548 inactivated by pIB23	This study
IBS351	UA159 with smu1814 inactivated by pIB24	This study
IBS352	UA159 with smu1916 inactivated by pIB25	This study
IBS353	UA159 with smu1965 inactivated by pIB13	This study

diately downstream of smu45, smu46, encodes a protein of 211 residues with significant similarity to the LuxR family of response regulators. Therefore, the smu45/smu46 locus constitutes a new TCS in *S. mutans*, bringing the total number of TCS in this organism to 14. Apart from this new putative TCS, BLAST-P analysis did not reveal the presence of any other sensor kinase in *S. mutans*.

Not all sensor kinase are encoded in each strain. The number of TCS present in a given bacterial species may vary among different strains. For example, there are several TCS that are present in one strain of the gram-positive pathogen *Clostridium perfringens* that are absent in other strains (49). Similarly, only 18% of clinical isolates of *S. pyogenes* contain the SilA/B TCS, which is involved streptococcal invasion (22).

PCR analysis was used to determine whether each of the genes coding for the 14 sensor kinases present in *S. mutans* strain UA159 were present in various *S. mutans* strains. Based on analysis of the UA159 genome sequence, two internal primers were designed for each of the sensor kinases encoded by the UA159 genome and used for PCR amplification to generate a 500-bp fragment, using chromosomal DNA isolated from 13 different *S. mutans* strains as a template. Of the 13 strains chosen for the analysis, 11 belong to serotype c, including three commonly used lab strains (UA159, NG-8, and GS-5), and the remainder taken from serotype e (V100) and serotype f (OMZ175). Genes encoding 11 sensor kinases were present in all 13 strains, but genes coding for two sensor kinases, smu45 and smu1814, were only present in UA159 and 8VS3 strains. In addition, smu1037 was not present in strain V-100 but was present in each of the other 12 strains tested.

Southern hybridization was performed to verify the results of the PCR analysis, using HindIII-restricted genomic DNA isolated from each of the 13 strains. Probes were generated by PCR amplification using UA159 chromosomal DNA, with the same primers used for the initial PCR analysis (Table 2). As shown in Fig. 2, smu45 and smu1814 were present in only two strains (UA159 and 8VS3), while smu1037 was present in all strains, except for strain V-100, confirming the results of the PCR analysis. The genes coding for each of the other sensor kinases were present in all of the strains tested. Taken together, the results indicate that there is some variability with respect to the number of sensor kinases encoded by various strains of *S. mutans*.

Inactivation of the sensor kinases in *S. mutans* UA159 and phenotypic analysis. An internal DNA fragment from each sensor kinase-encoding gene was cloned into the suicide vector pBSKEry (8), which confers erythromycin resistance. The resulting plasmid constructs (Table 1) were transformed into *S. mutans* UA159, and transformants were selected on the basis of erythromycin resistance on THY agar plates. Insertion mutants were obtained for each of the sensor kinases and verified by PCR as described in Materials and Methods. Since insertions were obtained in each of the sensor kinase genes, the results indicate that none of the TCS are required for cell viability.

The growth rates of UA159 and of each of the 14 sensor kinase mutants were examined during incubation in THY broth at 37°C. The growth assays showed that all of the mutants grew with similar generation times, with all of the TCS mutant cultures having similar cell densities at stationary phase. However, while the majority of mutant strains grew as homogeneous cell suspensions, one mutant strain, IBS349

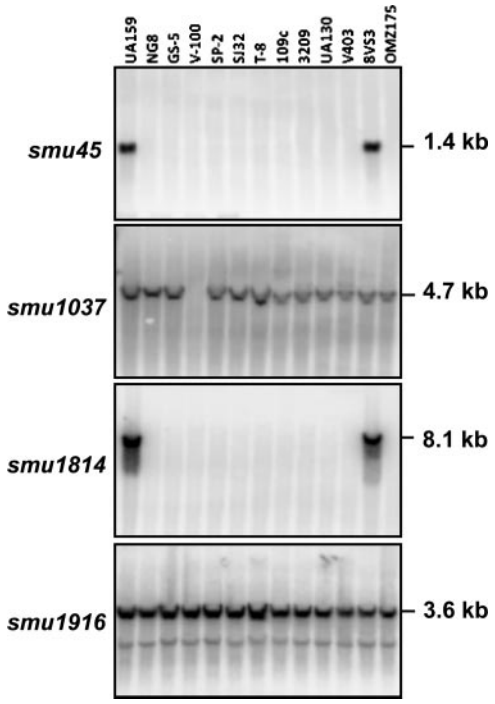

FIG. 2. Southern analysis for the presence of various sensor kinases in *S. mutans*. Chromosomal DNA from different strains was restricted with HindIII and separated in a 0.7% agarose gel by electrophoresis. The presence of various sensor kinases was detected through hybridization with sensor kinase probes as shown in Fig. 1. The strains studied included UA159 (lane 1), NG8 (lane 2), GS-5 (lane 3), V-100 (lane 4), SP-2 (lane 5), SJ32 (lane 6), T-8 (lane 7), 109Sc (lane 8), 3209 (lane 9), UA130 (lane 10), V403 (lane 11), 8VS3 (lane 12), and OMZ175 (lane 13). Strains V100 and OMZ175 belong to serotypes e and f, respectively. The values on the right are the expected molecular sizes (in kilobases) of the genes encoding the putative sensor kinase obtained from the UA159 genome sequence.

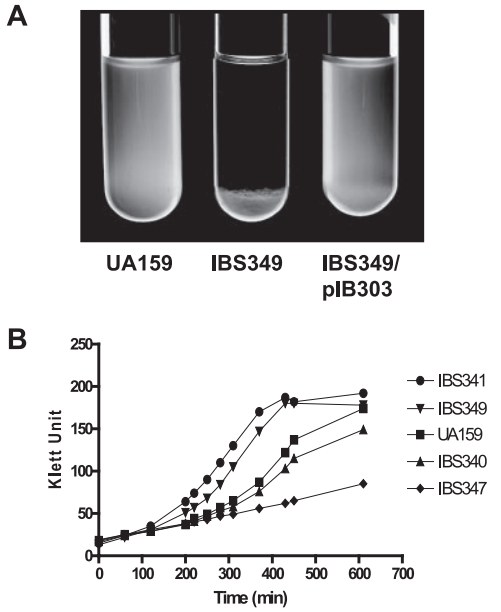

FIG. 3. Characterization of the sensor kinase mutants. (A) Autoaggregation of IBS349 (Smu1516). *S. mutans* cultures were grown in THY broth medium, and aggregation of IBS349 into cell clumps was observed when growth of the cultures entered the late exponential phase. Aggregation was not observed after complementation of IBS349 with wild-type smu1516 strain in *trans*. (B) Growth defects of the sensor kinase mutants. The growth of *S. mutans* cultures in CDM with 0.5% glucose at 37°C was monitored by using a Klett-Summerson colorimeter. The experiments were repeated twice, and a representative growth curve is shown. The strains used for the study include: UA159 (wild type), IBS340 (Smu45), IBS341 (Smu486), IBS347 (Smu1128), and IBS349 (Smu1516). All of the other mutant strains displayed profiles similar to those of UA159 and/or IBS340. Experiments were repeated multiple times, and a representative growth curve is presented here.

(Δsmu1516), formed visible clumps at the onset of exponential growth. As shown in Fig. 3A, the wild-type phenotype was restored after complementation with plasmid pIB303, which contains the wild-type smu1516 in *trans*. This particular mutant was also distinguished from the other mutants when grown on THY agar, forming darker yellow colonies. By comparison, *S. mutans* colonies are typically white in color.

The growth characteristics of several sensor kinase mutants cultured in CDM-glucose were different compared to growth in THY medium. Two mutants, IBS341 (Δsmu486) and IBS349 (Δsmu1516), grew faster in CDM-glucose medium compared to both wild-type and other mutant strains (Fig. 3B). In contrast, IBS347 (Δsmu1128) grew significantly more slowly compared to the wild type (Fig. 3B). Differential growth characteristics of these TCS mutants strongly suggest that sensor kinases in *S. mutans* indeed play a vital role in cell growth and development.

Response to environmental and antibiotic stress, and mutacin production. *S. mutans* is a catalase-negative, facultative anaerobe, such that its response to oxidative stress is unique compared to other bacteria that produce catalase. To study the role of sensor kinases in response to oxidative stress, each mutant was streaked onto THY agar plates and incubated at 37°C under aerobic conditions. As shown in Table 3, the

TABLE 3. Growth of sensor kinase mutants under aerobic conditions[a]

Strain	Growth on:	
	THY	THY + O_2
UA159	++	+
IBS341 (Δsmu486)	++	–
IBS347 (Δsmu1128)	++	–
IBS341/pIB55 (+smu486)	++	+
IBS347/pIB302 (+smu1128)	++	+

[a] THY agar plates containing streaked bacterial cultures were incubated under either anaerobic conditions (THY) or aerobic conditions (THY + O_2) at 37°C. Growth of wild-type and complemented bacteria under anaerobic conditions was twofold greater than growth under aerobic conditions.

growth of two mutants IBS341 (Δsmu486) and IBS347 (Δsmu1128) was significantly reduced. Growth under aerobic conditions was restored for IBS341 and IBS347 after complementation with plasmids pIB55 and pIB302, which contain wild-type smu486 and smu1128, respectively (Table 3). These two mutant strains also showed reduced growth on THY agar medium supplemented with hydrogen peroxide, confirming that Smu486 and Smu1128 are essential for growth during conditions of oxidative stress.

The sensor kinase mutants were then tested for their ability to withstand superoxide stress generated by methyl viologen. In contrast to the results presented above, while IBS347 displayed a growth defect when grown with methyl viologen, there were no changes to the growth characteristics of IBS341 (Fig. 4A). Interestingly, there were no significant differences observed when the mutants were subjected to superoxide stress generated by cumene hydroperoxide and *t*-BOOH (Table 4).

Disulfide stress is a subcategory of oxidative stress and is defined as the accumulation of non-native disulfide bonds in the cytoplasm (4). Disulfide stress was induced in *S. mutans*

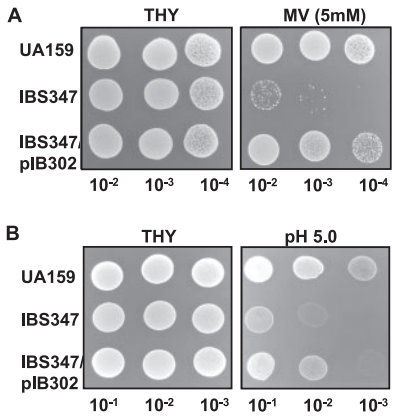

FIG. 4. Stress-sensitive phenotypes of Smu1128 (CiaH). (A) Superoxide stress. Dilutions of fresh overnight cultures were spotted on THY agar plates with or without methyl viologen (10 mM; MV). Plates were incubated at 37°C under aerobic conditions. Experiments were repeated no fewer than three times, and relevant areas of representative plates are shown. (B) Acid stress. Cultures were prepared and spotted as described above. Cultures were grown on THY agar plate in the presence or absence of sodium citrate buffer to generate a pH of 5.0 to mimic acid stress. Plates were incubated at 37°C for 48 h under aerobic conditions. Experiments were repeated no fewer than three times, and the relevant areas of representative plates are shown.

TABLE 4. Growth of sensor kinase mutants under various stress conditions

Strain	Growth under various stress conditions[a]								
	Oxidative stress	Superoxide stress	Disulfide stress	Acid stress	Thermal stress	Osmotic stress	DNA damage	Antibiotic stress	Mutacin production
UA159 (wild type)	+	+	+	+	+	+	+	+	+
IBS340 (Δsmu45)	+	+	+	+	+	+	–	+	+
IBS341 (Δsmu486)	–*[b]	+	+	+	–*	+	+	+	–*[c]
IBS342 (Δsmu577)	+	+	+	+	+	+	+	+[d]	+
IBS343 (Δsmu660)	+	+	+	+	+	+	+	+[d]	+
IBS344 (Δsmu928)	+	+	+	+	+	+	+	+	+
IBS345 (Δsmu1009)	+	+	+	+	+	+	+	–[e]	+
IBS346 (Δsmu1037)	+	+	+	+	+	+	+	+	+
IBS347 (Δsmu1128)	–*[b]	–[f]	+	–*	–*	+	–*	+	–*[c]
IBS348 (Δsmu1145)	+	+	+	+	+	+	+	+	+
IBS349 (Δsmu1516)	+	+	+	+	+[g]	+	+	–*[h]	+
IBS350 (Δsmu1548)	+	+	+	+	+	+	+	+	+
IBS351 (Δsmu1814)	+	+	+	+	+	+	+	+	+
IBS352 (Δsmu1516)	+	+	+	+	+	+	+	–[e]	–[i]
IBS353 (Δsmu1965)	+	+	+	+	+	+	+	+	+

[a] Multiple conditions/reagents were used to generate these stresses. *, Wild-type phenotypes were restored upon complementation of the mutant strain with the wild-type gene in *trans*.
[b] Refer to Table 3.
[c] Mutacin production was reduced more than 100-fold with respect to the wild type.
[d] Higher resistance to mezlocillin was observed compared to the wild type.
[e] Sensitive to bacitracin only.
[f] Sensitive to paraquat but not to cumene hydroperoxide or *t*-BOOH.
[g] ~10-fold-higher resistance was observed compared to the wild type.
[h] Sensitive to cefotaxime, cefuroxime, ceftazidime, meropenem, mezlocillin, penicillin, and trimethoprim.
[i] No mutacin production was observed.

cultures with diamide, using the disk diffusion assay, to determine whether the sensor kinase mutants were more sensitive to disulfide stress. All of the mutant strains were able to tolerate disulfide stress, comparable to the wild-type UA159, suggesting that sensor kinases are not involved in the recognition of the disulfide stresses, at least under the conditions tested.

S. mutans rapidly adapts to an acidic environment by eliciting a strong acid tolerance response (6, 30). Previous studies have shown that TCS play an important role in the acid tolerance response in many bacteria, including *S. mutans* (5, 17, 38). Therefore, the sensor kinase mutants were tested for growth at low pH in an agar plate assay as described in Materials and Methods. Only one mutant, IBS347 (Δsmu1128), showed a significant reduction of growth at pH 5.0 (Fig. 4B). This result suggests that only Smu1128 is involved in the acid tolerance response in *S. mutans*.

Sensor kinases recognize changes in temperature and modulate gene expression accordingly (1, 59). Puromycin, which causes premature chain termination during protein synthesis, was used to mimic thermal stress conditions. Of the 14 sensor kinase mutants tested, growth defects were only observed with strains IBS341 (Δsmu486) and IBS347 (Δsmu1128) (Table 4). Sensitivity to puromycin was eliminated in IBS341 and IBS347 after complementation with plasmids pIB55 and pIB302, respectively. None of the other mutants showed any detectable growth defects in the presence of puromycin. Interestingly, strain IBS349 (Δsmu1516) showed an increased tolerance (~5-fold) to puromycin relative to wild-type UA159. Thus, the results show that, as in many other bacteria, sensor kinases also play an important role in thermotolerance in *S. mutans*.

Sensor kinases are also important for eliciting osmotic stress tolerance in many bacteria (47, 53). Therefore, the sensor kinase mutants were tested for their ability to grow in THY broth containing various osmotic stress-inducing agents, such as ethanol, NaCl, or sorbitol, at 37°C. There were no observable changes to the growth of the sensor kinase mutants, suggesting that sensor kinases are not involved in osmotic stress tolerance in *S. mutans*.

Mutant strains were evaluated for their ability to withstand DNA damage from mitomycin C, which alkylates double-stranded DNA and blocks DNA replication to cause double-stranded DNA breaks. IBS340 (Δsmu45) and IBS347 (Δsmu1128) were more sensitive to the treatments of mitomycin C (Table 4), suggesting that these two sensor kinases may play a role in DNA damage recognition.

Various antibiotic stresses are recognized by bacterial TCS (32, 45, 48). A panel of 25 antibiotics, with different cellular targets, was tested using disk diffusion assays as described in Materials and Methods. While the majority of the sensor kinase mutants did not demonstrate increased sensitivity, some mutant strains were sensitive to antibiotics that specifically target cell wall biosynthesis. IBS349 (Δsmu1516) showed an increased sensitivity (at least greater than a 5-mm increase in diameter compared to the wild type) to the antibiotics cefotaxime, cefuroxime, ceftazidime, meropenem, mezlocillin, penicillin, and trimethoprim (data not shown). However, IBS349 did not show an increased sensitivity to all of the antibiotics belonging to the same group. Two other sensor kinase mutants, strains IBS345 (Δsmu1009) and IBS352 (Δsmu1965), showed an increased sensitivity to bacitracin (20 mm in the mutant compared to 10 mm in the wild type) and fosfomycin (20 mm in the mutant compared to 15 mm in the wild type), respectively. On the other hand, strains IBS342 (Δsmu577) and IBS343 (Δsmu660) were less sensitive (35 mm in the mutants compared to 40 mm in the wild type) to mezlocillin. Taken together, the results suggest that the sensor kinases of *S. mu-*

tans are involved in both positive and negative regulation of antibiotic-induced stress response.

Production of bacteriocins, such as nisin, subtilin, and related compounds, are often regulated by TCS (21, 34). Deferred antagonism assays were used to study the production of mutacins by each of the 14 sensor kinase mutants (15), using *S. gordonii* (DL-1) and *S. sanguinis* (SK36) as indicator strains. Of the 14 sensor kinase mutants, only the inactivation of smu1916 (*comE*, IBS352) resulted in the total loss of mutacin production (Table 4). Inactivation of two other sensor kinases, Smu486 (*liaS*, IBS341) and Smu1128 (*ciaH*, IBS347), resulted in an ~100-fold reduction in mutacin production (Table 4). The production of mutacin was restored when the mutant strains were complemented with plasmids expressing the gene encoding the corresponding sensor kinase of interest. Taken together, the results indicate that at least three sensor kinases, encoded by smu486, smu1128, and smu1916, are required for mutacin production in *S. mutans* UA159.

DISCUSSION

S. mutans is extremely adaptable to a number of adverse environmental conditions. This pathogen can survive over a wide range of temperature, flourish under acidic conditions, grow under high oxygen tension, and persist under nutrient-limited conditions. While the molecular mechanisms that allow *S. mutans* to tolerate these environmental pressures are thought to be important virulence traits (12, 13, 35), these mechanisms are very poorly understood. One such mechanism would be TCS, a primary signal sensing system by which bacteria sense environmental cues and mount an appropriate stress response through the expression of genes that allow for adaptation to a changing environment.

The number of TCS varies greatly among bacteria and depends on the genome size, as well as the complexity of the lifestyle of a particular organism (23). In streptococcal spp., the number of TCS varies from fewer than ten, as with *S. thermophilus* (11), to more than 20, as with *S. agalactiae* (27). The annotated genome of *S. mutans* indicates the presence of 13 putative TCS in this organism (3), many of which show significant homology to other well-characterized TCS encoded by closely related bacteria (Fig. 1). In addition to the previously identified 13 TCS, an additional putative TCS, encoded by smu45/46, was identified in *S. mutans* UA159, bringing the total number of TCS to 14 in this cariogenic pathogen. However, smu45/46 was only present in two strains belonging to serotype c (UA159 and 8VS3) and not in the strains belonging to serotype e or f. Similarly, smu1814/1815 was also only present in these two strains UA159 and 8VS3; it is possible that these two putative TCS are required for some linked functions characteristic for these two strains. Further studies are required to determine whether these two TCS are present in other serotypes. On the other hand, smu1037/1038 was identified in every strain, except for V100 (serotype e), which may indicate that this TCS is not physiologically necessary for members of serotype e; other strains belonging to this serotype will need to be studied to confirm this speculation. Thus, our study suggests that the number of TCS greatly varies in different strains of *S. mutans*; the requirement for a particular TCS may be indicative of survival in a particular environmental niche specific for a given host.

The primary aim of the present study was to analyze the contribution of each putative sensor kinase to the stress tolerance response of *S. mutans*. To do so, mutants of strain UA159 were generated by systematically knocking out the genes that encode for each individual sensor kinase, such that each mutant would have one putative TCS inactivated. This was followed by the observation of the phenotype in response to various environmental stresses. Fourteen sensor kinases were tested in the present study, but only three (Smu486, Smu1128, and Smu1516) appear to be involved in the stress tolerance of *S. mutans*. Other sensor kinases, such as Smu45, Smu1009 (MbrD), and Smu1916 (ComD), also contribute to stress tolerance, but their roles were not as prominent as the above-mentioned sensor kinases. Therefore, our discussion is focused on Smu486, Smu1128, and Smu1516, which are homologous to the LiaS, CiaH, and VicK (respectively) sensor kinases characterized from various bacteria (Fig. 1).

Proteins belonging to the LiaS family are generally smaller in length (~300 residues) compared to other sensor kinases and are characterized by the presence of a remarkably shorter transmembrane domain. LiaS belongs to a family of intramembrane-sensing sensor kinase; it has been proposed that LiaS, as well as other related systems, recognizes perturbations of peptidoglycan synthesis through the membrane lipid bilayer (41). In general, gram-positive bacteria, with low G+C content, possess one or more such systems. In *Bacillus subtilis*, LiaS and other related proteins detect antibiotics that target cell wall biosynthesis (43); not surprisingly, these systems are induced by the presence of antibiotics that inhibit cell wall synthesis (45). Unexpectedly, deletion of smu486, which encodes LiaS in *S. mutans*, did not result in the reduction in growth of this bacterium when antibiotics, including bacitracin and vancomycin, were included in the growth medium. However, another putative intramembrane sensor kinase, MbrD, is encoded in the genome of *S. mutans* that is encoded by smu1009; we discovered that this sensor kinase was involved with bacitracin resistance, a finding consistent with previous observations (65). Nevertheless, MbrD was not involved with any other antibiotics specific for cell wall synthesis, including vancomycin. Thus, the mechanism for cell wall stress defense appears to be different in different bacteria.

Our results indicate that LiaS is involved in the production of mutacin in strain UA159 (Table 4); LiaS is also associated with mutacin production in strain UA140 (64). However, the mutacins produced by strains UA140 and UA159 are different; the former predominantly produces mutacin I, a lantibiotic, while the latter only produces mutacin IV, a nonlantibiotic (55). Thus, LiaS appears to play a central role in mutacin production in *S. mutans*, since it influences the production of mutacins in different strains. Therefore, it is possible that LiaS is involved in the secretion of these (non)lantibiotic peptides, perhaps to restrict the growth of other competing bacteria in the biofilm.

LiaS also appears to be involved in oxidative stress tolerance (Table 3). However, LiaS was not required for resistance to superoxide radicals generated by methyl viologen (Table 4). In streptococcal spp., the enzymes superoxide dismutase (SOD) and NADH oxidase (NOXase) are responsible for the reduc-

tion of reactive oxygen species and superoxide radicals (25, 26). SOD converts superoxide anion ($O_2^{\cdot -}$) to O_2 and H_2O_2, with the latter removed by peroxidases. On the other hand, NOXase reduces O_2 directly to H_2O without the formation of harmful reactive O_2 intermediates. As expected, LiaS did not interfere with SOD expression, as determined by reverse transcription-PCR analysis (Biswas, unpublished). While the exact role of LiaS in oxidative stress response awaits further characterization, we speculate that LiaS may regulate the expression of NOXase, since LiaS is not involved in superoxide stress.

The other major player in the stress tolerance response of *S. mutans* was CiaH, encoded by smu1128. This sensor kinase has been extensively studied in many streptococcal spp., and its role in streptococcal physiology is complex. CiaH influences virulence, competence, mutacin production, resistance to antibiotics, and biofilm formation (2, 29, 33, 44, 54, 63). In *S. pneumoniae*, the Cia system is involved with the induction of competence, altered cefotaxime susceptibility (29), and is also thought to be associated with the regulation of cell wall biosynthesis (24). In this organism, CiaH negatively influences induction of competence by controlling the expression of the *comCDE* operon (19); this system also activates the transcription of *htrA*, which encodes a serine protease involved in stress response (56).

The role of CiaH in *S. mutans* is quite different from what was observed in *S. pneumoniae*. In *S. mutans*, CiaH positively regulates the development of competence, since Δ*ciaH* mutants demonstrated drastic reduction in the ability to take up exogenous DNA (2, 54). In addition, CiaH also represses expression of *htrA* in *S. mutans* up to 100-fold (2), in contrast to what was observed in *S. pneumoniae*.

Inactivation of *ciaH* resulted in multiple growth defects under a variety of stress conditions. Most importantly, we found that CiaH was required for growth under aerobic conditions, as well as for superoxide radical stress tolerance. Therefore, it is plausible that CiaH activates the expression of genes encoding SOD and NOXase. Interestingly, we did not find any reduction of *sod* expression using reverse transcription-PCR analysis in this mutant strain compared to the wild type (Biswas, unpublished). While the result is surprising, a recent study indicates that at least 10 gene products are necessary for defense against oxidative stress in *S. thermophilus* (62). Thus, CiaH could be involved in the regulation of any of those 10 genes.

Δ*ciaH* mutants displayed slower growth rates when grown in CDM but not during growth on THY agar medium. Moreover, mutacin production was reduced in the Δ*ciaH* mutant. This strain also showed sensitivity to thermal stress, acid stress, and mitomycin C. Interestingly, the Cia system was previously shown to be associated with O_2-mediated regulation of competence in *S. pneumoniae* (19). The exact mechanism by which the Cia system is involved in this process is not yet known. At present, it is quite premature to contemplate how CiaH might be involved in recognizing such diverse stresses, but one possibility may be that CiaH regulates an important stress-dependent protease, such as HtrA. However, in contrast to other organisms, CiaH negatively regulates *htrA* expression in *S. mutans* (2), indicating that HtrA may not be directly involved in CiaH-mediated stress tolerance. We speculate that CiaH in *S. mutans* may serve to regulate other intracellular proteases, such as ClpP; experiments are under way to evaluate this possibility.

In addition to LiaS and CiaH, VicK also has a significant role in the stress tolerance response of *S. mutans* UA159. The Vic system, which is encoded by the three-gene operon *vicRKX*, is essential for cell viability in many low-G+C, gram-positive bacteria. In *S. pneumoniae*, this system regulates cell wall and fatty acid biosynthesis, as well as a putative mureinhydrolase encoded by *pcsB* (50, 51). PcsB is essential for cell viability, and the Vic system is needed for *pcsB* expression. Downregulation of *pcsB* expression results in defects in cell separation, synthesis, and morphology (50). Interestingly, GbpB, the PcsB homolog in *S. mutans*, has recently been shown to be positively regulated by expression of the *vic* genes, including *vicK* (57). Although the physiological role of GbpB in *S. mutans* is currently unknown, it may function as a cell wall hydrolase (46). Since the expression of *gbpB* is induced under a variety of cellular stresses (16), GbpB may participate in a number of bacterial stress responses.

We observed that inactivation of *vicK* resulted in an increased sensitivity to various antibiotics that interfere with cell wall synthesis. VicK mutants (IBS349) also grew consistently faster compared to the wild type, producing yellow colonies with increased biomass on THY agar medium, and formed clumps when grown in THY broth. Although further studies are necessary to gain a better understanding of these phenotypes, we believe that these phenotypes are associated with the altered cell wall structure due to reduced GbpB production.

Among all of the sensor kinases present in *S. mutans*, only VicK contains a characteristic domain at the N-terminal region, known as the PAS domain. The PAS domain-containing sensor kinases typically recognize O_2 tension and intracellular redox potential (61, 67). However, our results indicated that the *vicK* mutant (IBS349) is not sensitive to oxidative or superoxide stress, suggesting that the mechanisms for O_2 sensing vary in different bacteria. In *S. mutans*, the CiaH system appears to play a major role in sensing O_2 tension.

Many other novel findings have also emerged from our systematic study. The newly discovered putative sensor kinase Smu45 was associated with the recognition of DNA-damaging agents. This is surprising, since Smu45/46 is not universally present in all *S. mutans* isolates. Another finding is that while both LaiS and CiaH are involved in oxidative stress response, only CiaH appears to be involved in superoxide radical stress response (Fig. 4). Surprisingly, we found that the PAS domain-containing VicK is not involved in the oxidative stress response of *S. mutans* UA159. We also found that none of the sensor kinases participate in osmotic stress response, which is contrary to what has been observed in other bacteria. Most importantly, we observed strain-dependent phenotypic differences among various strains of *S. mutans*. For example, while LiaS is not involved in the acid tolerance response in strain UA159, LiaS is required for the acid tolerance response in the NG-8 strain (38). Additional studies are warranted not only to understand the reason for strain-dependent phenotypic differences but also to reveal the network of genes regulated by various TCS in this pathogen.

In conclusion, the present study clearly demonstrates that the number of TCS varies among various *S. mutans* isolates and that a number of the putative TCS encoded by this organ-

ism are essential for the recognition and response to various environmental stresses, antibiotic resistance, and bacteriocin production. Since TCS are involved in the regulation of virulence genes and biofilm formation, a complete deciphering of the networks of TCS-regulated virulence genes may reveal novel signaling pathways in this pathogen. The ability of *S. mutans* to colonize teeth is paramount to the initiation and progression of dental caries. Therefore, interrupting the pathogen's ability to sense and respond to external stimuli, using various sensor kinases, should prove extremely useful in preventing the formation of dental caries by *S. mutans*.

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