Regulatory Involvement of the PerR and SloR Metalloregulators in the *Streptococcus mutans* Oxidative Stress Response

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ABSTRACT *Streptococcus mutans* is a commensal of the human oral microbiome that can promote dental caries under conditions of dysbiosis. This study investigates metalloregulators and their involvement in the *S. mutans* oxidative stress response. Oxidative stress in the human mouth can derive from temporal increases in reactive oxygen species (ROS) after meal consumption and from endogenous bacterial ROS producers that colonize the dentition. We hypothesize that the *S. mutans* PerR (SMU.593) and SloR (SMU.186) metalloregulatory proteins contribute to the regulation of oxidative stress genes and their products. Expression assays with *S. mutans* UA159 wild-type cultures exposed to H₂O₂ reveal that H₂O₂ upregulates *perR* and that PerR represses *sloR* transcription upon binding directly to Fur and PerR consensus sequences within the *sloR* operator. In addition, the results of Western blotting experiments implicate the Clp proteolytic system in SloR degradation under conditions of H₂O₂ stress. To reveal a potential role for SloR in the H₂O₂-resistant phenotype of *S. mutans* GMS802 (a *perR*-deficient strain), we generated a *sloR perR* double-knockout mutant, GMS1386, where we observed upregulation of the *tpx* and *dpr* antioxidant genes. These results are consistent with GMS802 H₂O₂ resistance and with a role for PerR as a transcriptional repressor. Cumulatively, these findings support a reciprocal relationship between PerR and SloR during the *S. mutans* oxidative stress response and begin to elucidate the fitness strategies that evolved to foster *S. mutans* persistence in the transient environments of the human oral cavity.

IMPORTANCE In 2020, untreated dental caries, especially in the permanent dentition, ranked among the most prevalent infectious diseases worldwide, disproportionately impacting individuals of low socioeconomic status. Untreated caries can lead to systemic health problems and have been associated with extended school and work absences, inappropriate use of emergency departments, and an inability for military forces to deploy. Together with public health policy, research aimed at alleviating *S. mutans*-induced tooth decay is important because it can improve oral health (and overall health), especially in underserved populations. This research, focused on *S. mutans* metalloregulatory proteins and their gene targets, is significant because it can promote virulence gene control in an important oral pathogen, and contributes to the development of an anticaries therapeutic that can reduce tooth decay.

KEYWORDS Fur, PerR, SloR, *Streptococcus*, hydrogen peroxide, metalloregulation, stress tolerance

Dental caries is the most common chronic infectious disease in the United States and around the world, affecting approximately 60% to 90% of school-age children and a vast majority of adults (1). As of 2020, untreated dental caries in the permanent dentition ranked as the most highly prevalent infectious disease worldwide (2). Maladies of the oral cavity, including dental caries, are also inextricably linked to systemic

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diseases, such as diabetes, cardiovascular disease, and low birth weight (3). Moreover, the social determinants of health widen the gap between those with access to quality oral health care and underresourced populations, namely, minority racial and ethnic groups and those of low socioeconomic status (4, 5).

Streptococcus mutans, one of the primary causative agents of dental caries in humans (6), uses dietary sucrose as a substrate for extracellular polysaccharide synthesis, facilitating formation of the plaque biofilm. Subsequent acid production demineralizes tooth enamel, which can lead to decay (7). In addition to its unique aciduric and acidogenic properties that allow *S. mutans* to withstand stressors, such as acid pH, are virulence factors that protect it against oxidative stress, both during and between mealtimes (8–10). Especially during mealtimes, reactive oxygen species (ROS) can reach high levels in the plaque environment that extraoral microbes are not equipped to detoxify (11). In contrast, *S. mutans* has evolved an oxidative stress response that can detoxify ROS that derive from exogenous sources as well as from within the dental plaque microbiome where the H₂O₂-producing *Streptococcus sanguinis* and *Streptococcus gordonii* reside (11, 12). H₂O₂ production may have evolved in these early colonizers of the plaque biofilm as a fitness strategy aimed at antagonizing neighboring microbes, such as the nonperoxigenic *S. mutans*, which are later colonizers of the dentition. As a constituent of the plaque microbiome, however, *S. mutans* can prompt the fine-tuning of its metal ion uptake machinery, thereby curtailing ROS production and toxicity (13–15). For instance, a Dps-like peroxide resistance protein (Dpr) protects *S. mutans* from oxidative stress by sequestering free Fe, thereby blocking the production of toxic hydroxyl radicals (HO·) via Fenton chemistry (16). In addition, the *S. mutans* *tpx* gene product, a thiol peroxidase, is an antioxidant enzyme that can convert peroxides into H₂O and so prevent the formation of toxic HO· (17). Thus, Dpr and Tpx are essential for maintaining an appropriate response to oxidative stress in *S. mutans*. Taken together, the overall fitness of *S. mutans* in the oral cavity is highly dependent on the expression of virulence factors, like Dpr and Tpx, that allow it to coordinate an appropriate response to ROS- (and more specifically, H₂O₂-) induced stress (18–20).

Bacterial metalloregulatory proteins are the “watchdogs” of intracellular metal ion homeostasis; they are essential for *S. mutans* survival and pathogenesis. Among the metalloregulatory proteins in *S. mutans* is PerR, an 18-kDa Fur family regulator that functions as a metal ion-dependent transcriptional repressor (21, 22). PerR is the sole Fur family regulator in *S. mutans*, consistent with *in silico* analysis of the *S. mutans* UA159 chromosome (www.homd.org), which reveals a single 477-bp *perR* gene (SMU.593). Multiple Fur homologs, including Fur and PerR, have been documented in other bacteria, such as *Bacillus subtilis* (21). While little is known about PerR in *S. mutans*, a recent report describes the transcriptomes of a *perR* insertion-deletion mutant and its wild-type progenitor in which a relatively small number of genes are differentially expressed (23, 24). In contrast, a 25-kDa SloR protein belonging to the DtxR family of metalloregulators modulates a plethora of *S. mutans* genes, including those that encode essential metal ion transport (25–27). Specifically, *S. mutans* SloR regulates an ABC-type metal ion transport operon comprised of the *sloABC* genes by mechanisms that have been previously described (9, 27–29). A more recent report describes an ancillary metal ion transporter, called MntH, that helps maintain intracellular manganese ion homeostasis in *S. mutans* and which bears similarity to the Nramp-type transporters of manganese, namely, MntH1 and MntH2 in *Enterococcus faecalis* and MntH in *Streptococcus agalactiae* (18, 30, 31). Taken together, the SloABC and MntH transporters, as well as their regulators, are essential for ensuring *S. mutans* fitness and survival in both healthy and dysbiotic plaque environments (9, 18, 32).

In the present study, we set out to understand the mechanism(s) that allows *S. mutans* to tolerate oxidative stress in the plaque microbiome. Herein, we demonstrate that *perR*-deficient strains of *S. mutans* are more resistant to hydrogen peroxide stress than their wild-type progenitors, consistent with previous reports (24, 33), and that PerR directly represses *sloR* when challenged with H₂O₂-induced stress. In addition, we

FIG 1 Doubling times for *S. mutans* cultures grown in the presence of increasing concentrations of H₂O₂. The results of hydrogen peroxide titration experiments, expressed as doubling time for *S. mutans* cultures grown in the presence of up to 1 mM H₂O₂, revealed a linear relationship between growth rate and H₂O₂ concentration for the UA159 wild-type strain and for the GMS1386 *sloR perR* double-knockout mutant but not the *perR*-deficient mutant, GMS802.

describe a relationship between the PerR and SloR metalloregulatory proteins during the *S. mutans* response to H₂O₂ stress, in part by confirming their roles in controlling expression of the *S. mutans* *tpx* and *dpr* oxidative stress response genes.

RESULTS

***S. mutans* doubling time in the presence of H₂O₂.** The results of hydrogen peroxide titration experiments, expressed as doubling time for *S. mutans* cultures grown in the presence of up to 1 mM H₂O₂, revealed a linear relationship between growth rate and H₂O₂ concentration for the UA159 wild-type strain and the *perR sloR* double-knockout mutant, GMS1386. As expected, doubling times increased with increasing H₂O₂ concentrations (Fig. 1), reaching a maximum of 95 to 100 min. This linear increase was not observed for the GMS802 *perR*-deficient mutant, however, which maintained a significantly shorter doubling time (~85 min) regardless of H₂O₂ concentration. These findings corroborate the results of hydrogen peroxide antagonism assays that support heightened H₂O₂ tolerance in the GMS802 mutant compared to that in its UA159 wild-type progenitor and greatly diminish the likelihood that confounding factors in the bacteriological medium are responsible for the stress-resistant phenotype in *S. mutans* GMS802.

A *perR* insertion-deletion mutation renders *S. mutans* more resistant to H₂O₂ challenge than its PerR-proficient counterparts. To determine if disruption of the *perR* gene impacts the *S. mutans* response to H₂O₂ stress and to reveal potential involvement of the *S. mutans* *sloR* gene product in this protective response, we grew the *S. mutans* UA159 wild-type strain and its isogenic *perR* (GMS802), *sloR* (GMS584), and *perR sloR* (GMS1386) insertion-deletion mutants in Todd-Hewitt broth supplemented with 0.3% yeast extract (THYE) broth supplemented with a sublethal concentration of 0.5 mM H₂O₂. The results of these growth determination assays revealed doubling times for GMS802 and GMS1386 that were significantly shorter than that of the UA159 wild-type strain (analysis of variance [ANOVA], $P < 0.05$) but only in the presence of H₂O₂. Interestingly, the GMS584 doubling time was not significantly different from that of the wild type. To mimic conditions of hydrogen peroxide stress *in vivo*, we repeated these experiments in a conditioned medium containing *S. gordonii*-generated H₂O₂ where we likewise noted a significant decrease in GMS802 doubling time relative to that of UA159 (data not shown).

While GMS584, like *S. mutans* UA159, struggled to grow in the presence of 0.5 mM H₂O₂, this growth defect was rescued by introducing a *perR* mutation into the GMS584 background, generating the GMS1386 double mutant (Fig. 2). These data indicate that

FIG 2 *S. mutans* GMS802 and GMS1386 are more resistant to H₂O₂-induced stress than either the UA159 wild-type or GMS584 *sloR*-deficient strain. *S. mutans* strains were grown in THYE or in THYE supplemented with a sublethal concentration (0.5 mM) of hydrogen peroxide. GMS802 and GMS1386 reveal significantly shorter doubling times (~85 to 90 min) compared to the doubling times of the UA159 and GMS584 strains (~100 min). Univariate analysis of variance (ANOVA) and *post hoc* Bonferroni *t* tests were applied to these data using SPSS statistical software (SPSS for Windows, version 17.0).

disruption of the *S. mutans perR* gene confers a growth advantage on *S. mutans* GMS802 and GMS1386 when challenged with H₂O₂ stress and that disruption of *sloR* alone is not sufficient to do so.

To complement these *in vitro* growth assays and reveal the impact of *perR* disruption on *S. mutans* survival, we performed antagonism assays by growing *S. mutans* immediately adjacent to hydrogen peroxide-producing *S. gordonii* as previously described (34). The results of these assays reveal inhibition of the *S. mutans* UA159 wild-type strain when cultivated adjacent to *S. gordonii* cells that had been growing and producing H₂O₂ for 16 h (Fig. 3A). In contrast, the GMS802 *perR* mutant appeared to be unaffected by *S. gordonii* H₂O₂ challenge (Fig. 3B). In fact, the mutant appeared to infiltrate the growth zone of *S.*

FIG 3 The *S. mutans* GMS802 mutant is more resistant to hydrogen peroxide production by *S. gordonii* than its UA159 wild-type progenitor. Peroxigenic *S. gordonii* was spot inoculated onto the surface of a THYE agar medium and grown for 16 h before inoculating the *S. mutans* UA159 wild-type strain in close juxtaposition. (A) Following overnight incubation at 37°C and 5% CO₂, inhibition of *S. mutans* growth is evident. (B) Growth of the *perR*-deficient GMS802 mutant was not inhibited by *S. gordonii* hydrogen peroxide challenge under these same growth conditions. (C) Complementation of the *perR* mutation in *S. mutans* GMS803 restored its sensitivity to hydrogen peroxide challenge by *S. gordonii*. Each panel represents a single representative experiment among the eight total biological replicates performed.

FIG 4 Hydrogen peroxide challenge increases transcription of *perR* but not *sloR* in *S. mutans* UA159. Expression of *S. mutans perR* and *sloR* was determined in qRT-PCR experiments and normalized against an endogenous *hkl1* control gene. (A) Expression of *S. mutans perR* is increased 2.5-fold in wild-type UA159 cultures grown in the presence of 0.5 mM H₂O₂ ($n=4$; $P<0.05$). (B) Transcription of the *S. mutans* UA159 *sloR* gene is not significantly impacted under the same test conditions ($n=4$). Error bars depict standard error of the mean (SEM).

gordonii despite the elaboration of H₂O₂, consistent with a phenotype of H₂O₂ resistance. Complementation of the *perR* mutation in *cis* in the GMS803 strain restored H₂O₂ sensitivity to near wild-type levels (Fig. 3C).

Hydrogen peroxide stress cues transcription of the *S. mutans perR* gene but not *sloR*. Reports in the literature describe PerR as a redox sensor in *Bacillus subtilis* and *Streptococcus pyogenes* (35, 36). Accordingly, we hypothesized that oxidative stress would induce *perR* transcription in *S. mutans*. To test this hypothesis, we challenged *S. mutans* wild-type UA159 cultures with 0.5 mM H₂O₂ and monitored *perR* expression in these and unchallenged controls in semiquantitative real-time PCR (qRT-PCR) experiments. The results indicate that *perR* transcription was upregulated 2.5-fold in *S. mutans* UA159 cultures challenged with H₂O₂ compared to that of unchallenged cultures ($n=4$; $P<0.05$) (Fig. 4A). In contrast, exposure of *S. mutans* UA159 to H₂O₂ did not have any impact on *sloR* transcription relative to controls ($n=5$) (Fig. 4B). Heightened *perR* transcription in response to H₂O₂ stress is further corroborated by the results of transcriptome sequencing (RNA-seq) experiments performed previously by Kajfasz et al. (24).

Intracellular SloR protein levels are sensitive to H₂O₂. To determine whether SloR is responsive to oxidative stress at the level of protein, we resolved whole-cell lysates (WCL) from *S. mutans* cultures that had been exposed to 0.5 mM H₂O₂ for 0, 15, and 30 min on SDS-PAGE gels and transferred them to a polyvinylidene difluoride (PVDF) membrane for immunoblotting with a polyclonal anti-SloR antibody. The resulting immunoblots reveal SloR concentrations that decrease as exposure time to H₂O₂ increases up to 30 min (see Fig. S1 in the supplemental material). We also performed Western blotting to test SloR stability in *S. mutans* cultures that were not H₂O₂ challenged but to which chloramphenicol had been added to halt protein synthesis. The results indicate that SloR levels are stable for at least 30 min after chloramphenicol addition. Together, these findings support an SloR protein that is labile within 15 min of H₂O₂ challenge but stable in the absence of the stressor for at least 30 min.

SloR accumulates in *clpE*-, *clpX*-, and *clpP*-deficient mutants of *S. mutans*. To determine whether the *S. mutans* Clp proteolytic system is involved in decreasing SloR concentrations as exposure time to H₂O₂ increases, we conducted Western blotting experiments with a polyclonal anti-SloR antibody and whole-cell lysates prepared from wild-type *S. mutans* and its isogenic *clpE*, *clpX*, and *clpP* mutant derivatives (37). The *clpP* gene encodes a cellular protease that, together with the ATPase activity encoded by the *clpE* and *clpX* genes, comprise the major stress-responsive proteolytic system in *S. mutans* (37, 38). The Western blot results reveal accumulation of SloR in all three *clp* mutants compared to wild-type SloR levels in the UA159 strain (see Fig. S2 in the supplemental material) and thus support posttranscriptional control of intracellular SloR concentration by the Clp proteolytic system in *S. mutans*.

FIG 5 The *sloR* promoter includes putative consensus binding sequences for an *S. mutans* PerR metalloregulator. Shown is the intergenic region between the *S. mutans sloC* and *sloR* genes on the *S. mutans* UA159 chromosome. The *S. mutans* UA159 genome (RefSeq accession number [NC_004350.2](https://www.ncbi.nlm.nih.gov/RefSeq/assembly/NC_004350.2)) from the NCBI GenBank database was used to generate the nucleotide sequence in this region. The sequence harbors the -35 and extended -10 promoter regions that drive *sloR* gene transcription from the $+1$ transcription start site (designated by the bent arrow above the sequence). Also shown is the predicted ribosome binding site (RBS) and the start codon (SC) for the *sloR* coding sequence. *In silico* analysis of the *sloR* promoter supports the presence of an Fur binding sequence in the -35 region, comprised of two forward hexamers separated by 1 bp from a third inverted hexamer (6-6-1-6) and a predicted 7-1-7 PerR binding sequence that shares overlap with the -10 promoter region (gray shading).

***In silico* prediction of PerR and Fur binding sites in *S. mutans*.** Since *S. mutans* contains only one Fur family metalloregulator (PerR) with an unknown binding motif, we used a combination of consensus binding weight matrices based on known PerR and Fur binding motifs in other bacteria to identify targets of *S. mutans* PerR. Specifically, we used four consensus binding weight matrices based on the binding sites of PerR homologs in *S. pyogenes* and *Streptococcus suis* (consensus, TTAGAATCATTWTAA), PerR (consensus, AWTTAKAATAATTATAATT) and Fur (consensus, NWAAATGATAATCATTATCAWT) homolog binding sites in *Bacillus subtilis*, *Staphylococcus aureus*, and streptococci species, and Zur, Fur, and PerR homolog binding sites (consensus, TNWWAWTKATAATMATTATMAWTTAN) in *B. subtilis*, *Listeria monocytogenes*, *Bacillus amyloliquefaciens*, *S. aureus*, *S. pyogenes*, and *S. suis*. All four matrices were used to screen 1,113 *S. mutans* intergenic regions with a length of at least 50 bp. We identified a total of 3,824 significant (P value < 0.001 ; false-discovery rate [q value] < 0.129) motif matches with a boundary localized to the putative promoter within 50 bp of the start codon. After purging redundancies by retaining only the match with the lowest P value for a given promoter region in a given matrix search, we identified a total of 779 genes with a match from one or more matrices in the adjacent promoter region (see Data Set S1 in the supplemental material). We further narrowed the number of putative PerR binding targets to a total of 191 gene promoters with matching binding motifs in all four matrices. The resulting output, representing an estimated 10% of annotated genes in the *S. mutans* genome, includes the *S. mutans sloR* gene promoter (SMU.186) (Fig. 5) as well as promoters that precede the *dpr* (SMU.540), *ahpC* (SMU.764) and *gor* (SMU.838) antioxidant genes.

FIG 6 Transcription of the *S. mutans sloR* and *perR* genes in the presence of 0.5 mM H₂O₂. (A) Expression of the *S. mutans sloR* gene is 2-fold greater in GMS802 compared to that in the wild-type UA159 strain ($n=4$; $P < 0.05$) when challenged with 0.5 mM H₂O₂ for 15 min. (B) Expression of the *S. mutans perR* gene is not significantly different in GMS584 compared to the wild type ($n=5$). Expression was normalized against that of an endogenous *hk11* control gene. Error bars depict SEM.

FIG 7 A PerR homolog from *B. subtilis* shifts an operator sequence within the *S. mutans sloR* promoter region in gel mobility shift experiments. A 17.4-kDa Fur protein from *Bacillus subtilis* (kind gift of John Helmann, Cornell University) was used in gel mobility shift experiments with a 53-bp DNA amplicon that harbors the wild-type *S. mutans sloR* promoter including the -35 and -10 regions (A) or a 54-bp oligonucleotide that harbors the *sloR* operator sequence with A-to-G transition mutations in the predicted Fur binding sequence (B). Reaction mixtures were resolved on a 12% nondenaturing polyacrylamide gel for 450 volt-hours. (A) As little as 60 nM Fur generated a robust shift of the *sloR* target sequence (closed arrow). (B) Shifting of the 54-bp mutated *sloR* target sequence was compromised even when as much as 100 nM Fur was added to the reaction mixture. Two band shifts are evident when 150 nM Fur was included in the reaction mixture, suggesting the presence of two protein binding sites on the *sloR* target probe (closed arrows). Addition of EDTA abrogated the band shifts, consistent with an Fur-DNA interaction that is metal ion-dependent. Open arrows, unbound target probe; closed arrow(s), protein-bound target probe.

PerR is a repressor of *sloR* transcription. To reveal whether *S. mutans* PerR modulates expression of *sloR* under conditions of H_2O_2 stress, qRT-PCR experiments were performed with GMS802 and UA159 cultures that had been exposed to H_2O_2 challenge. We observed a 2-fold increase in *sloR* transcription in GMS802 compared to that in UA159 when these *S. mutans* strains were challenged with 0.5 mM H_2O_2 , indicating that PerR functions as a repressor of *sloR* transcription in *S. mutans* wild-type cells grown under conditions of oxidative stress ($n=4$; $P<0.05$) (Fig. 6A). When the *S. mutans* GMS584 *sloR*-deficient strain was grown under the same test conditions, expression of *perR* was not significantly impacted, indicating that *perR* transcription is not subject to control by the SloR metalloregulator ($n=5$) (Fig. 6B).

A PerR homolog from *Bacillus subtilis* binds directly to the *S. mutans sloR* operator. To elucidate whether the impact of PerR on *sloR* transcription is direct, we performed gel mobility shift assays with a 17.4-kDa PerR homolog in *B. subtilis*, called Fur, and an *sloR* PCR product that harbors an operator sequence with predicted Fur- and PerR-like binding motifs (Fig. 5). The outcome of experiments performed with the PerR homolog supports direct Fur binding to the 53-bp *sloR* operator probe at protein concentrations as low as 60 nM (Fig. 7A). The addition of the EDTA metal ion-chelating reagent to a reaction mixture containing as much as 400 nM Fur protein abrogated the band shift, consistent with an Fur-DNA interaction that is metal ion dependent.

Binding of a PerR homolog from *Bacillus subtilis* to a mutated *sloR* operator in *S. mutans* is reduced. To validate sequence-specific binding of the heterologous Fur protein from *B. subtilis* (see Fig. S3 in the supplemental material) to the *S. mutans sloR* operator, we performed gel shift assays with a mutated 54-bp oligonucleotide containing two A to G transition mutations in the predicted Fur binding sequence that is proximal to the -35 region of the *sloR* promoter (AATTAAGGC [transitions indicated in boldface type]). Transition mutations were generated instead of transversions because we did not want to significantly alter the chemical composition of the DNA and confirm the specificity with which we believe the PerR homolog interacts with its target

FIG 8 *S. mutans dpr* and *tpx* gene transcription is upregulated in the *S. mutans* GMS1386 PerR/SloR double mutant compared to the UA159 wild-type strain when challenged with 0.5 mM H₂O₂. (A) *S. mutans dpr* expression is increased 1.6-fold in the *S. mutans* GMS1386 knockout mutant compared to its UA159 wild-type progenitor under conditions of hydrogen peroxide stress ($n=5$; $P<0.05$). (B) Transcription of the *S. mutans tpx* gene is heightened >3-fold in GMS1386 compared to that in UA159 in the presence of hydrogen peroxide stress ($n=3$; $P<0.05$). Expression of the *dpr* and *tpx* genes was normalized to that of an endogenous *hk11* control. Error bars depict SEM.

sequence. The results indicate that *in vitro* binding of 60 nM Fur to the mutated *sloR* target sequence was reduced when compared to the robust binding that we observed when 60 nM Fur was allowed to react with the wild-type target sequence (mean pixel area for the band shift with the mutated probe was 63% versus 83% with the wild-type probe) (Fig. 7B). Moreover, robust binding to the mutated AATTAAGGC sequence required at least 150 nM Fur protein, consistent with an attenuated Fur-DNA interaction, and an important role for adenine nucleotides in accommodating the binding of Fur to the wild-type consensus sequence in *S. mutans*.

***S. mutans* PerR and SloR repress *dpr* and *tpx* transcription.** We hypothesized that the heightened hydrogen peroxide resistance that we observed for the GMS802 *perR*-deficient strain might translate into repression of the antioxidant arsenal in the wild-type UA159 strain. To explore the putative impact of the PerR and SloR metalloregulators on two antioxidant genes, *dpr* and *tpx*, we performed qRT-PCR experiments with RNAs isolated from H₂O₂-challenged UA159 (wild type), GMS802 (*perR* deficient), GMS584 (*sloR* deficient), and GMS1386 (*perR* and *sloR* deficient) *S. mutans* strains. Most notably, expression of the *dpr* and *tpx* genes in *S. mutans* GMS1386 was significantly

FIG 9 Whole-cell lysates fail to shift the *S. mutans tpx* promoter in gel shift experiments. Whole-cell lysate prepared from *S. mutans* UA159 and GMS802 failed to shift a 242-bp amplicon that harbors the *S. mutans tpx* gene promoter. The 53-bp *sloR* operator was used as a positive control. [γ -³²P] end-labeled DNA amplicons were resolved on a 12% nondenaturing polyacrylamide gel for 225 volt-hours.

increased up to 3-fold when compared with that in the UA159 wild-type progenitor strain (Fig. 8A and B). Thus, under conditions of H₂O₂ stress, it seems that PerR and SloR work together to downregulate transcription of *S. mutans tpx*, *dpr*, and likely other antioxidant genes.

Regulation of *tpx* transcription by PerR is not direct. We performed gel mobility shift experiments with *S. mutans* UA159 and GMS802 whole-cell lysates (WCL) and a 242-bp target probe that harbors the *tpx* promoter to reveal whether the impact of PerR on *tpx* transcription is direct. The experimental results reveal no evidence of shifting the *tpx* promoter region even when as much as 20 μ g of protein lysate was included in the reaction mixture (Fig. 9). The *sloR* operator was used as a positive control in these experiments.

DISCUSSION

In the present study, we describe a GMS802 *perR*-deficient strain of *S. mutans* that is more resistant to hydrogen peroxide challenge than its wild-type UA159 progenitor, corroborating other published reports of H₂O₂ resistance in *perR*-deficient strains of *S. mutans* (24, 33). Yet, other reports in the literature (25) describe an *sloR*-deficient GMS584 strain of *S. mutans* that demonstrates compromised survivorship in the face of H₂O₂ challenge. Taken together, these findings support a role for both PerR and SloR in *S. mutans* aerotolerance. Herein, we extend these reported findings by characterizing the relationship between the PerR and SloR metalloregulators in the *S. mutans* response to H₂O₂ challenge and by elucidating binding of a PerR homolog to an *S. mutans sloR* operator region that harbors Fur- and PerR-like consensus binding sequences.

Not surprisingly, we describe *perR* transcription that is upregulated in *S. mutans* cultures exposed to 0.5 mM H₂O₂. This result is consistent with previous reports (39) and RNA-seq data (24), and it suggests that PerR is a redox sensor in *S. mutans*. The PerR protein in *Bacillus subtilis* has also been implicated in redox sensing following protein activation (21, 40, 41). Specifically, Fuangthong et al. describe a role for H₂O₂ (or an H₂O₂ reaction product) and intracellular Mn²⁺:Fe²⁺ in PerR activation via oxidation of histidine residues at amino acid positions 37 and 91 in the PerR protein (40). While it is possible that a similar mechanism for PerR activation occurs in *S. mutans* given the presence of conserved histidine residues (H47 and H100) that localize to a predicted Fe²⁺ binding site where they can undergo metal-catalyzed oxidation (41), further investigations are warranted to elucidate the details of redox sensing that engage PerR-Fe²⁺ complexes with DNA binding and gene regulation in this oral pathogen.

Unlike *S. mutans* and *S. pyogenes* that harbor a single PerR protein, *B. subtilis* harbors two separate Fur family metalloregulators, PerR and Fur (16), the latter sharing 28.6% identity and 50.0% similarity with PerR from *S. mutans* (see Fig. S3 in the supplemental material). Our *in silico* analysis of the *S. mutans* UA159 genome revealed Fur- and PerR-like binding sequences at numerous predicted promoter regions across the UA159 chromosome (see Data Set S1 in the supplemental material), including gene promoters that mediate oxidative stress tolerance via ROS scavenging (*ahpC*, SMU.764) and thiol homeostasis (*gor*, SMU.838). Other binding targets of the PerR homolog were noted in regions upstream of metal ion transport genes (*zupT*, SMU.2069; *codY*, SMU.1824c), regulators of metal ion homeostasis (*sloR*; SMU.186), and yet other virulence genes that encode sucrose-dependent adherence (*gtfB*, SMU.1004; *ftf*, SMU.2028; *gbpA*, SMU.2112; *gbpC*, SMU.1396; and *gbpD*, SMU.772), acid tolerance (*fabM*; SMU.1746), and two-component signaling systems (*vicR*, SMU.1517) (see Fig. S4 in the supplemental material). A more comprehensive list of genes preceded by both binding motifs are listed in Data Set S1. Taken collectively, our bioinformatics approach reveals a subset of DNA loci that is potentially bound by PerR and identifies candidate nearby genes that are likely impacted by PerR mutation/deletion. We believe that these binding targets and their associated genes may be involved in producing the oxidative stress-resistant phenotype that we observe in the GMS802 strain and that these data can ultimately reveal the molecular basis for oxidative stress resistance in *S. mutans*.

Chromatin immunoprecipitation sequencing (ChIP-seq) experiments currently ongoing in our laboratory will facilitate investigations of these and other PerR targets throughout the *S. mutans* UA159 genome.

The Fur and PerR binding sequences at the *S. mutans sloR* locus share overlap with the -35 and -10 regions of the *sloR* promoter, respectively (Fig. 5), strongly implicating PerR in *S. mutans sloR* gene regulation. In fact, our follow-up investigations of *sloR* transcription in the presence versus absence of PerR support repression of *sloR* by PerR in *S. mutans* cultures challenged with 0.5 mM H_2O_2 but not in the absence of H_2O_2 . This finding is consistent with the results of immunoblotting experiments that reveal decreasing SloR protein concentrations with increasing exposure time to 0.5 mM H_2O_2 (up to 30 min) (see Fig. S1 in the supplemental material). Finally, while these results support an impact for PerR on *sloR* gene expression, computational analyses did not reveal an SloR recognition element (SRE) proximal to the *perR* promoter (27), consistent with the results of qRT-PCR experiments that indicate no significant impact of the SloR metalloregulator on *perR* gene transcription under conditions of hydrogen peroxide stress.

The *in silico* prediction of Fur- and PerR-like consensus sequences in the *sloR* promoter, combined with the results of expression profiling experiments that support PerR repression of *sloR*, led us to hypothesize direct binding of PerR to the *sloR* operator. To address this hypothesis, we used a heterologous *B. subtilis* Fur protein in gel mobility shift experiments with the *S. mutans sloR* operator as the target probe and noted direct Fur binding at protein concentrations as low as 60 nM (Fig. 7A). In addition, Fur binding to an *S. mutans* probe containing transition mutations in a predicted Fur binding sequence that is proximal to (and shares some overlap with) the -35 promoter was reduced by 20% when compared to wild type (Fig. 7B). Taken together, these results indicate that repression of *sloR* by the PerR homolog is both direct and sequence specific. In addition, the presence of two distinct band shifts, most notably at the 150 nM Fur concentration, indicates that the target probe can accommodate at least two distinct Fur binding species. Given that the Fur protein and its homologs in other bacteria are known to interact with DNA as homodimers (42, 43), we predict that the bottom band shift (Fig. 7B, designated by a solid arrow) represents a probe interaction with a single Fur dimer and that the upper band (Fig. 7B, designated by a solid arrow) represents a probe interaction with a double Fur dimer. This binding appears to be cooperative since only the lower band shift is abrogated upon addition of EDTA. Monette et al. (9) describe similar cooperative binding of the SloR metalloregulator to its cognate recognition element in *S. mutans*. Taken together, we propose that the PerR protein in *S. mutans* self-associates to form a dimer or higher order oligomer prior to DNA binding at a so-called "Fur box" in the -35 region of the *sloR* promoter and at a "PerR box" that is predicted to overlap the -10 promoter region. Such binding would be expected to displace the RNA polymerase holoenzyme, thereby repressing *sloR* gene transcription. An *S. mutans* PerR-DNA interaction may also help maintain low levels of *sloR* transcription in H_2O_2 challenged *S. mutans* cultures by obstructing positive *sloR* gene control from a promoter-distal SloR binding site described previously (9, 27).

Initial attempts to shift DNA probes with a recombinant PerR protein from *S. mutans* were unsuccessful, likely due to the absence of cofactors that were needed to facilitate PerR oligomerization and/or DNA binding *in vitro*. Reports in the literature describe ferrous iron and zinc as necessary cofactors for dimerization and DNA binding of the *B. subtilis* and *S. pyogenes* PerR orthologs (35, 36). The DNA binding activity of PerR is also sensitive to relevant hydrogen peroxide concentrations, which are needed to facilitate metal-dependent protein oxidation (41). We reasoned, however, that these and other putative cofactors would already be present and available to PerR in WCL preparations of *S. mutans* that we generated from cultures exposed to 0.5 mM H_2O_2 . Indeed, the outcome of gel mobility shift experiments performed with the *S. mutans* UA159 WCL revealed a band shift when allowed to react with the 53-bp *sloR* promoter target sequence (Fig. 7) (8, 26, 27).

Among the antioxidant genes that belong to the *S. mutans* PerR regulon are *tpx* and *dpr*, which encode a thiol peroxidase and a ferric iron binding protein, respectively,

FIG 10 Model of PerR and SloR involvement in the *S. mutans* oxidative stress response. H₂O₂ stress and redox sensing by baseline levels of cytosolic PerR upregulate *S. mutans perR* gene transcription. As intracellular concentrations of the *perR* gene product accumulate, PerR binds directly to the *sloR* operator at conserved Fur- and/or PerR-like consensus sequences (GATAATAAACTTTATC and ATATAATGATTTGA, respectively) to repress *sloR* gene transcription. SloR is also subject to posttranslational degradation by the *S. mutans* Clp proteolytic system under conditions of H₂O₂ stress. The accumulation of PerR in the cell combined with decreasing intracellular SloR concentrations repress transcription of the *S. mutans* antioxidant genes, *dpr* and *tpx*, consistent with the proposed involvement of both SloR and PerR in *S. mutans* stress adaptation.

both essential contributors to oxidative stress tolerance. However, the results of gel mobility shift studies support indirect control of *tpx* and *dpr* transcription by PerR and so implicate additional regulators in their control (Fig. 7). Not surprisingly, the regulation of these and other oxidative stress genes in *S. mutans* is likely subject to tight control since stress gene transcription in the absence of stress can compromise fitness, and failure to express antioxidant genes in the face of oxidative stress can prove lethal. Given such fine-tuning of the *S. mutans* oxidative stress response, profound redundancies in the PerR- and SloR-mediated pathways that converge on *tpx* and *dpr* gene expression can be expected. In a recent report, Kajfasz et al. (24) propose corepression of the *S. mutans* antioxidant arsenal by both PerR and SloR. Since redundancies in gene regulation can mask a phenotype in a single mutant, we constructed the *S. mutans* GMS1386 *perR sloR* double mutant for inclusion in expression profiling experiments. The double mutation in this strain revealed an increase in *dpr* transcription that was not observable in the GMS584 single-knockout mutant and supports interplay between the PerR and SloR metalloregulatory proteins and the mechanisms that regulate *S. mutans* oxidative stress tolerance.

Consistent with resistance of the *perR*-deficient GMS802 strain of *S. mutans* to hydrogen peroxide stress and sensitivity of the *sloR*-deficient GMS584 strain to the same stressor (25), we hypothesize a relationship of reciprocity between the PerR and SloR metalloregulators in *S. mutans* cultures challenged with H₂O₂ (Fig. 10). Specifically, our experimental findings support a regulatory model that begins with heightened *perR* expression above baseline levels in response to H₂O₂ stress that we believe promotes the metal ion-catalyzed activation of the PerR protein via histidine oxidation (39–41). Subsequent PerR binding to Fur- and/or PerR-like consensus sequences is then enabled within the –35 and –10 regions of the *sloR* promoter, respectively, which represses *sloR* transcription, causing cytosolic SloR concentrations to fall. These findings are consistent with the results of immunoblotting experiments presented herein that support intracellular SloR concentrations that decrease as exposure time to H₂O₂ increases (see Fig. S1). Moreover, we observed accumulation of the 25-kDa SloR protein in *S. mutans* ClpX, ClpP, and ClpE deletion mutants on Western blots (see Fig. S2 in the supplemental material), which implicates the Clp proteolytic machinery in post-translational SloR degradation. In sum, we believe a decline in cytosolic SloR levels in the face of hydrogen peroxide challenge compromises the positive influence that SloR would otherwise have on the *S. mutans* oxidative stress response (25). Low levels of SloR and high levels of PerR exacerbate downregulation of the oxidative stress response, in part by repressing the antioxidant genes *dpr* and *tpx*. Consistent with these findings are reports implicating the *S. mutans* PerR regulator as a repressor of *dpr* and *sod* transcription (33). In addition,

a recent report describes corepression of the *S. mutans* *spxA1* gene by the PerR and SloR metalloregulators (44). The *spxA1* gene product has been previously described as a positive regulator of oxidative stress tolerance in *S. mutans* (44–46). Clearly, further investigation is warranted to decipher the complexities of antioxidant gene regulation involving SpxA1.

In summary, we propose that PerR is a prevalent redox-sensing metalloregulator in *S. mutans* and that H₂O₂ at noncytotoxic concentrations (20 to 50 μ M) (47) is a possible signal that activates PerR and promotes *S. mutans* stress adaptation. In addition, we describe stress adaptation that involves PerR-mediated repression of the *S. mutans* *dpr* and *tpx* antioxidant genes under conditions of oxidative stress, as well as SloR repression at the transcriptional and posttranscriptional levels. Why the *S. mutans* PerR metalloregulator evolved to repress expression of its antioxidant arsenal in the face of hydrogen peroxide stress is an interesting question. We propose that the answer resides in the two faces of reactive oxygen species (ROS)—redox biology, which activates signaling pathways to initiate normal biological processes (48, 49), and oxidative stress when elevated levels of ROS can incur damage to cellular DNA, protein, and/or lipids (13, 15). Taken together with our experimental findings, we hypothesize that PerR repression and the regulatory mechanisms that maintain low levels of SloR in the cell cytosol likely evolved to maintain beneficial redox biology in the face of H₂O₂ challenge. In contrast, mobilizers of *S. mutans* *dpr* and *tpx* transcription, which may involve SpxA1 as a positive regulator, likely evolved as part of an oxidative stress response to prevent ROS-induced cell death. Taken collectively, continuing investigations of the mechanisms that repress the oxidative stress response in *S. mutans* combined with the regulatory events that facilitate oxidative stress tolerance can elucidate *S. mutans* fitness and survival strategies in healthy versus dysbiotic plaque and so inform an anticaries therapeutic.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. The bacterial strains and plasmids used in this study are presented in Table S1 in the supplemental material. Primers and oligonucleotides are presented in Table S2 in the supplemental material. Primers were designed using MacVector 7.0 software and purchased from Eurofins Genomics (Louisville, KY).

Bacteriological media and reagents. All *S. mutans* strains were grown as standing cultures at 37°C and 5% CO₂ in 15-ml Falcon tubes (Fisher Scientific, Pittsburgh, PA) in Todd-Hewitt broth supplemented with 0.3% yeast extract (THYE) or in brain heart infusion (BHI) medium. Erythromycin (10 μ g/ml) was added when growing *S. mutans* GMS584 and GMS802 cultures to maintain selective pressure. Erythromycin (10 μ g/ml) and kanamycin (250 μ g/ml) were added when growing *S. mutans* GMS1386.

S. gordonii Challis CH1 cells were propagated in THYE at 37°C with gentle aeration to achieve “aerobic” growth. The “conditioned medium” used in growth rate determination assays was formulated as previously described (34) with the following modifications. First, *S. gordonii* grown overnight in THYE broth was used to inoculate 40 ml of prewarmed THYE to an optical density at 600 nm (OD₆₀₀) of 0.025. The subculture was then grown with aeration on a shaking platform (150 rpm) to mid-logarithmic phase (OD₆₀₀ ~0.6), after which the cells were harvested by centrifugation at 1,700 $\times$ g for 10 min in a Sorvall SS-34 rotor. Twenty milliliters of the resulting culture supernatant was supplemented with 0.25% glucose and filter sterilized. The culture supernatant is referred to as “conditioned medium” in subsequent growth determination assays.

Construction of *S. mutans* *perR* insertion-deletion mutants. A 980-bp amplicon harboring the 477-bp *perR* coding sequence (SMU.593), along with its upstream promoter region, was amplified with primers 593.smal.F and 593.smal.R (see Table S2), digested with SmaI (Promega), and ligated into a unique SmaI restriction site on the pBGK2 integration vector (50). The resulting ligation mixture was introduced into chemically competent *Escherichia coli* GC5 by natural transformation in accordance with the instructions of the manufacturer (Gene Choice), and transformants were selected on L-agar supplemented with kanamycin. Plasmid DNA was isolated from select transformants using a miniprep kit according to the instructions of the manufacturer (Qiagen), and the recombinant construct, called pJS2, was confirmed via restriction mapping.

PCR ligation mutagenesis (51) was then performed to disrupt the *perR* gene on the wild-type *S. mutans* UA159 chromosome. Specifically, primers 593.P1/593.P2, and 593.P3/593.P4 (see Table S2) were used to amplify the 5′ and 3′ flanking regions of the SMU.593 gene from the *S. mutans* genome, thereby generating a 103-bp deletion in the *perR* coding sequence. The resulting P1/P2 and P3/P4 amplicons were digested with AclI and FseI (New England BioLabs) and ligated in separate bimolecular reaction mixtures to an *ermAM* erythromycin resistance cassette with compatible 5′ AclI and 3′ FseI overhangs. The ligation mixtures were used as a template for a second round of PCR (as described above) with primers 593.P1 and 593.P4 (Table S2) to generate a P1/P2-*ermAM*-P3/P4 tripartite construct. The final amplification mixture was used to transform *S. mutans* UA159 in the presence of competence-

stimulating peptide (CSP) as described previously (52). Candidate transformants were screened for erythromycin resistance on THYE-erm agar, and disruption of the SMU.593 coding sequence was confirmed by PCR amplification and sequencing of the *perR* locus using primers 593.P1 and 593.P4. The resulting insertion-deletion mutant was named GMS802.

The pJ52 integration construct was used to complement the SMU.593 mutation in *S. mutans* GMS802 in *cis*. The recombinant plasmid was introduced into the *S. mutans* UA159 genome in single copy by CSP-induced transformation as previously described (52). The pBGK2 parent vector was used to transform GMS802 in parallel as a negative control. Successful complementation of the SMU.593-specific mutation in GMS802 was confirmed phenotypically in *S. gordonii* antagonism assays, and the complemented *S. mutans* strain was named GMS803.

To generate a double *perR* and *sloR* insertion-deletion mutant, an allelic exchange strategy similar to that described for GMS802 was employed to disrupt the *perR* gene in the *S. mutans* SloR-deficient GMS584 strain generated previously in our laboratory (26). A 1,500-bp *aphA3* cassette was inserted into the *perR* coding sequence, and the resulting insertion-deletion mutant, called GMS1386, was confirmed by PCR and nucleotide sequencing across the *perR-aphA3* and *sloR-ermAM* junctions.

Growth determination assays. *S. mutans* UA159, GMS802, GMS584, and GMS1386 were monitored for growth in a Bioscreen C microbiology plate reader (Growth Curves, USA) as previously described (26). To assess H₂O₂ tolerance, mid-exponential phase cells (OD₆₀₀ 0.4 to 0.6) were diluted 1:50 in either THYE; THYE supplemented with 0.15 mM, 0.3 mM, 0.5 mM, 0.75 mM, or 1 mM H₂O₂; or in a “conditioned medium” prepared as described above (34). Wells containing uninoculated THYE were included as negative controls. The Bioscreen reader was programmed with BioLink software (LabSystems) to monitor growth at 37°C for 24 h with low-amplitude shaking every 15 min. To assess the growth of UA159 and GMS802 under aerobic conditions, the Bioscreen plate reader was programmed for continuous low-amplitude shaking and monitored over a 24-h period. Doubling times were calculated using the equation $dt = \ln(2)/[15/(OD_{600}B - OD_{600}A)]$. The resulting data were interpreted after applying Univariate ANOVA and *post hoc* Bonferroni *t*-tests with SPSS statistical analysis software (SPSS for Windows).

Hydrogen peroxide challenge in broth culture. *S. mutans* cultures were grown as described above in THYE or BHI broth to an OD₆₀₀ of 0.5 (± 0.05) as determined by spectrophotometry (GENESYS 20; Thermo Scientific). A total of 0.5 mM H₂O₂ was added to select broth cultures for a cell exposure time of 15 or 30 min, after which the cells were immediately harvested by centrifugation and lysed by mechanical disruption in a BIO 101 FastPrep machine.

Antagonism assays. The sensitivity of *S. mutans* UA159 and GMS802 to H₂O₂ antagonism by *S. gordonii* was assessed in plate assays as described previously (34) with the following modifications. A total of 8 μ l of an overnight culture of *S. gordonii* was spot inoculated onto the surface of a THYE agar plate and incubated at 37°C and 5% CO₂ for 16 h to allow for growth and hydrogen peroxide production, after which 8 μ l of an *S. mutans* UA159, GMS802, or GMS803 culture was spot inoculated proximal to *S. gordonii*. Plates were incubated as described above for an additional 14 h, and the resulting zones of growth and growth inhibition were qualitatively assessed.

In silico prediction of *S. mutans* PerR and Fur binding sites. The CollecTF database of bacterial transcription factor binding sites (53) was used to identify consensus sequences for Fur homologs (including PerR) in *Bacillus* sp. TRANSFAC position weight matrices for each consensus sequence were then converted to MEME format and used to search extracted intergenic regions with a minimum length of 50 bp from the *S. mutans* UA159 genome. The search for intergenic regions was performed using FIMO version 5.3.0 (54) at the MEME suite webserver (55), a *P* value threshold of 10^{−3}, and a bootstrap implementation (56) to estimate false discovery rates as *q* values (57). Genes bordering intergenic regions were identified, and distances of motif matches from start codons were calculated. Matches with the closest boundary within 50 bp of the 5′ end of a neighboring gene were retained and then filtered with the lowest *P* value motif match reserved for each matrix search.

RNA isolation and cDNA synthesis. Total RNA was isolated from mid-logarithmic *S. mutans* cultures grown in THYE as described previously (58). RNA was assessed for integrity on a 0.8% agarose gel, adjusted to a concentration of 100 ng/ μ l using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and immediately reverse transcribed into cDNA using a RevertAid first strand cDNA synthesis kit (Thermo Scientific) according to the manufacturer's instructions. Reaction mixtures with nuclease-free water instead of RNA served as no-template controls, and reaction mixtures without reverse transcriptase were prepared as RT[−] controls. cDNA integrity was examined on a 0.8% agarose gel and compared to the RT[−] controls.

Semiquantitative real-time PCR. qRT-PCR was performed with cDNA generated as described above. Amplification was performed in a CFX96 real-time system (Bio-Rad) with SYBR green as the intercalating dye (Bio-Rad) and 500 nM *perR*-, *sloR*-, *dpr*-, *tpx*-, and *hk11*-specific primers (Table S2). RT[−] controls were run in parallel with the same primers for each cDNA template to confirm the absence of contaminating genomic DNA. The thermal cycling conditions were 95°C for 3 min followed by 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 15 s. Primer efficiency was calculated using a standard curve that derived from dilutions of UA159 cDNA. Gene expression was normalized against the expression of the endogenous control gene *hk11*, the expression of which did not change under the experimental test conditions.

***S. mutans* whole-cell lysate preparation.** *S. mutans* UA159 was grown to mid-exponential phase (OD₆₀₀ 0.5) in 30 ml of prewarmed THYE or BHI broth and with selective pressure when appropriate. To test the stability of the SloR protein, we added 150 μ g/ml of chloramphenicol to mid-logarithmic phase cultures to halt *de novo* protein synthesis. The cells were harvested by centrifugation at 8,000 $\times g$ for 10 min in a Fiberlite F13S-14 $\times$ 50cy rotor and then washed with 30 ml of 10 mM Tris-Cl, pH 7.8. Cells were resuspended in 750 μ l of 10 mM Tris-Cl, pH 7.8, transferred to an ice-cold 2-ml screw-cap tube containing 300 μ l of 0.1-mm zirconium beads, and mechanically disrupted at 4°C for 30 s at a speed of 5.0 in

a BIO 101 FastPrep machine. The samples were then centrifuged at $18,000 \times g$ for 10 min at 4°C, and whole-cell lysates were collected as supernatants, quantified for total protein in a bicinchoninic acid assay (Pierce), and stored at −20°C.

SDS-PAGE and Western blotting. Total protein (25 to 30 µg) in 2× Laemmli sample buffer (Bio-Rad) was denatured at 95°C for 5 min and resolved on 12% Tris-glycine SDS-PAGE gels at 150 V before transfer to a Protran 0.2-µm nitrocellulose membrane (Schleicher & Schuell). Protein transfer proceeded overnight at 90 mA in semidry transfer buffer, after which the immobilized proteins were washed in a Ponceau S staining solution (0.1% wt/vol) for 1 h with gentle shaking and examined for equal loading across samples. The membrane was then blocked in 5% skim milk for 1 h and washed three times in Tris-buffered saline with Tween 20 (TBST) for up to 15 min per wash. The membrane was incubated with a polyclonal rabbit anti-SloR antiserum diluted 1:3,000 in Tris-buffered saline (TBS) for 1 h at room temperature with gentle shaking, followed by three washes in TBST as described above. A horseradish peroxidase-labeled donkey anti-rabbit conjugate antiserum diluted 1:8,400 was then added for an additional hour of incubation with gentle shaking. The blot was washed again in TBST as described and processed with an Amersham ECL Prime Western blotting detection reagent (GE Healthcare Life Sciences) according to the manufacturer's instructions. The blot was imaged in a ChemoDoc XRS+ system (Bio-Rad) or exposed to Kodak XAR film for up to 60 s and developed by autoradiography.

Preparation of DNA target probes for gel mobility shift experiments. Q5 high-fidelity DNA polymerase (New England Biolabs) was used to amplify target probes from the *S. mutans* UA159 chromosome with *sloR*- and *tpx*-specific primers (Table S2) according to the recommendations of the supplier. The NEBtools T_m calculator was used to facilitate primer design and define appropriate annealing temperatures. PCR products were confirmed on a 0.8% agarose gel and visualized after staining with SYBR safe DNA gel stain (Invitrogen, Carlsbad, CA). The amplicons were purified with an Invitrogen PCR purification kit (Thermo Fisher, Waltham, MA) according to the manufacturer's instructions prior to end-labeling.

Wild-type and mutated versions of the predicted PerR binding region in the *S. mutans sloR* promoter region were prepared as oligonucleotides (Table S2) and purchased from Eurofins Genomics (Louisville, KY). The forward (5' to 3') oligonucleotide was annealed to its reverse complement in 10× annealing buffer containing 100 mM potassium acetate and 30 µM HEPES at pH 7.5. The annealing conditions were set in a thermal cycler at 95°C for 2 min, followed by −0.1°C every cycle for 450 cycles, and then held at 4°C.

Gel mobility shift assay. Gel mobility shift assays were performed as previously described (25, 28). Purified PCR products or annealed oligonucleotides were prepared as described above and end labeled with 10 µCi [γ -³²P]ATP (Perkin-Elmer) and 10 U T4 polynucleotide kinase according to established protocols (New England Biolabs). Binding reaction mixtures included 1 µl (~30 ng) end-labeled DNA and up to 400 nM *B. subtilis* Fur or 6.5 µg of UA159 or GMS802 whole-cell lysate in a total reaction volume of 16 µl. Reaction mixtures without a source of protein were included as negative controls. All samples were resolved on 12% nondenaturing polyacrylamide gels for up to 70 min at 300 V. Gels were exposed to Carestream BioMax XAR film and stored at −80°C for up to 48 h in the presence of an intensifying screen. Autoradiography followed in the dark room according to established protocols. ImageJ software, version 2.0.0 (National Institutes of Health) was used to quantify the band shift intensities on autoradiograms. The band shift, expressed as a percentage, was calculated by comparing the mean pixel area of the 60 nM Fur band shift with that of the unshifted target sequence in the same lane and then expressed as a percentage of the 100% band shift that we observed when 400 nM Fur was used to shift the target probe.

Pairwise and multiple-sequence alignments. Nucleotide and amino acid sequence data were analyzed using Clustal Omega EMBOS Match and MView, respectively, services of EMBL-EMBL. For the multiple-sequence alignment, the gap opening penalty of 6 bits and gap extension of 1 bit were used. For the pairwise protein sequence alignment, the default parameters were used.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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We declare that we have no conflicts of interest to report.

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