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dpr and *sod* in *Streptococcus mutans* Are Involved in Coexistence with *S. sanguinis*, and PerR Is Associated with Resistance to H_2O_2

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Large numbers of bacteria coexist in the oral cavity. *Streptococcus sanguinis*, one of the major bacteria in dental plaque, produces hydrogen peroxide (H_2O_2), which interferes with the growth of other bacteria. *Streptococcus mutans*, a cariogenic bacterium, can coexist with *S. sanguinis* in dental plaque, but to do so, it needs a means of detoxifying the H_2O_2 produced by *S. sanguinis*. In this study, we investigated the association of three oxidative stress factors, Dpr, superoxide dismutase (SOD), and AhpCF, with the resistance of *S. sanguinis* to H_2O_2. The knockout of *dpr* and *sod* significantly increased susceptibility to H_2O_2, while the knockout of *ahpCF* had no apparent effect on susceptibility. In particular, *dpr* inactivation resulted in hypersensitivity to H_2O_2. Next, we sought to identify the factor(s) involved in the regulation of these oxidative stress genes and found that PerR negatively regulated *dpr* expression. The knockout of *perR* caused increased *dpr* expression levels, resulting in low-level susceptibility to H_2O_2 compared with the wild type. Furthermore, we evaluated the roles of *perR*, *dpr*, and *sod* when *S. mutans* was cocultured with *S. sanguinis*. Culturing of the *dpr* or *sod* mutant with *S. sanguinis* showed a significant decrease in the *S. mutans* population ratio compared with the wild type, while the *perR* mutant increased the ratio. Our results suggest that *dpr* and *sod* in *S. mutans* are involved in coexistence with *S. sanguinis*, and PerR is associated with resistance to H_2O_2 in regulating the expression of Dpr.

The oral cavity is colonized by large numbers of bacteria, i.e., the indigenous microflorae (1, 2). The oral microflorae are considered to act as components of an innate immune system, which protects the host from infection by exogenous pathogenic bacteria. Oral bacteria, however, can sometimes cause infectious diseases, such as tooth decay, periodontitis, and aspiration pneumonitis. Among the oral bacteria, each bacterial species competes and/or coexists with other bacteria. To compete, bacteria produce antibacterial agents, such as bacteriolytic enzymes, bacteriocins, and hydrogen peroxide (3–5). In oral streptococci, especially *Streptococcus sanguinis* and *Streptococcus gordonii*, the production of hydrogen peroxide (H_2O_2) is well known as an antibacterial agent against other bacterial species (5–7).

H_2O_2 is one of the reactive oxygen species (ROS), which also include the superoxide anion (O_2^-) and hydroxyl radicals ($^{\bullet}OH$) (8, 9). These ROS cause serious damage to cellular macromolecules such as proteins and DNA. Although the toxicity of H_2O_2 is relatively weak compared with that of other ROS, H_2O_2 is converted nonenzymatically to the highly toxic hydroxyl radical via the Fenton pathway ($H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + -OH + Fe^{3+}$) in the presence of iron (Fe). Generally, aerobes and facultative anaerobes have efficient mechanisms for protection against ROS during aerobic respiration or encounters with oxidative stress due to neutrophils or bacteria. Several factors for protection against ROS, such as catalase, superoxide dismutase (SOD), Dps-like protein, alkylhydroperoxide reductase (AhpCF), glutathione reductase, and thiol reductase, have been identified in many bacterial species (9–14). Among them, catalase, AhpCF, and Dps-like protein are considered to be primarily responsible for resistance to H_2O_2 (Fig. 1) (15). Catalase and AhpCF directly decompose H_2O_2, and Dps-like protein inhibits the reaction of the Fenton pathway by capturing free Fe.

Streptococcus mutans, one of the commensal bacteria in the oral cavity, is known to be a cariogenic pathogen in humans (16, 17). *S. mutans* is able to attach to the smooth surfaces of teeth and to form biofilm known as dental plaque with other oral bacteria. Although *S. sanguinis* is a dominant bacterium in dental plaque, *S. mutans* coexists there with *S. sanguinis*. *S. sanguinis* is known to produce large amounts of H_2O_2, causing it to have antibacterial activity against several bacteria (5, 7, 18–20). Thus, to persist in dental plaque, *S. mutans* requires a resistance mechanism against H_2O_2 produced by *S. sanguinis*. To date, AhpCF and Dps-like protein (Dpr) have been identified and characterized in the resistance of *S. mutans* to ROS (11, 19, 21–23). In particular, Yamamoto et al. reported previously that Dpr was a major factor in resistance to oxidative stress during respiration (24). However, no comparative analysis of the contribution of the factors responsible for resistance to oxidative stress by *S. sanguinis* has been documented for *S. mutans*. Furthermore, the regulatory mechanism(s) for the expression of these factors has not been determined for *S. mutans*. To date, the peroxide regulator, PerR, has been demonstrated to be associated with resistance to oxidative stress in several bacteria, including other streptococci (25–28).

In this study, we investigated the contribution of three factors, Dpr, Sod, and AhpCF, to resistance against H_2O_2 produced by *S. sanguinis* and also identified a regulatory factor responsible for Dpr expression. Furthermore, we demonstrated that two factors

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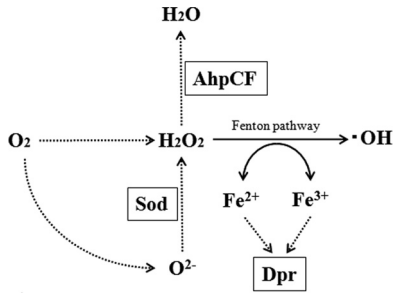

FIG 1 Function of Sod, AhpCF, and Dpr in *S. mutans*. Sod, superoxide dismutase; AhpCF, alkylhydroperoxide reductase; Dpr, Dps-like peroxidase resistance protein (15).

in *S. mutans* are critical for coexisting with *S. sanguinis in vivo* by using an established coculture method.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. mutans* strains used are listed in Table 1. *S. mutans* and *S. sanguinis* GTC217 were grown in Trypticase soy broth (TSB; Becton Dickinson Microbiology Systems, Cockeysville, MD) or Trypticase soy agar (TSA) at 37°C with 5% CO_2. Erythromycin (EM) (10 μg ml^{-1}) and spectinomycin (SPC) (600 μg ml^{-1}) were added when necessary for *S. mutans*.

Construction of *perR*, *dpr*, *sod*, and *ahpCF* single deletion mutants and *perR dpr*, *perR sod*, and *perR ahpCF* double deletion mutants. We constructed the *perR*, *dpr*, *sod*, and *ahpCF* single deletion mutants via an overlapping-extension PCR approach, as described previously (29). To generate the *dpr* deletion mutant, two fragments corresponding to approximately 500 bp of the upstream and downstream sequences of this gene were generated by PCR using KOD Plus (Toyobo, Tokyo, Japan) with primer pairs for the upstream fragment Δ*dpr-F1* and the downstream fragment Δ*dpr-F2* (Table 2). Each of the F1 reverse and F2 forward primers incorporated 11 bases that were complementary to the erythromycin resistance cassette Em^r, which was cloned by using pBlueScript-Em (Stratagene, La Jolla, CA) (30). The Em^r gene was amplified by PCR using primers for Em^r. All PCR amplicons were purified by using the Qiagen PCR purification kit (Qiagen KK, Tokyo, Japan), and the corresponding upstream and downstream amplicons were mixed in a 1:1:1 ratio with the Em^r PCR product. The amplicon mixture was then used as a template for a second PCR using appropriate forward Δ*dpr-F1* and reverse Δ*dpr-F2* primers. The resulting PCR products were then transformed into UA159, as described previously (30). Additionally, the fragments of ΔΔ*perR-F1* and ΔΔ*perR-F2* were mixed in a 1:1:1 ratio with the Spc^r PCR product cloned from pBSSK-Spc (29). This mixture was then used as a template for a second PCR using appropriate forward ΔΔ*perR-F1* and reverse ΔΔ*perR-F2* primers. The resulting PCR products were also transformed into the *dpr*, *sod*, and *ahpCF* single deletion mutants to construct double deletion mutants. The mutation was verified by PCR.

Construction of complement strains. For genetic complementation, we constructed a DNA fragment to insert the Spc^r and *perR* genes into the *ftf* gene, encoding fructosyltransferase. First, the plasmid and fused fragment were prepared. To prepare the plasmid, the *ftf* N-terminal fragment was transferred into the N terminus of the Spc^r gene in pBSSK-Spc using XhoI and SalI sites (pBBSK-Spc::*ftf*-N-terminal). To prepare the fused fragment, the *perR-compl.-F1* fragment and *ftf*-C-terminal fragment were generated by PCR using KOD Plus (Toyobo) with the primer pairs (Table 2). The *perR-compl.-F1* reverse primer and the *ftf*-C-terminal forward primer added an extra 12 nucleotides to anneal each PCR fragment, and the *perR-compl.-F1* forward primer and the *ftf*-C-terminal reverse primer contained BamHI and SacII restriction enzyme sites. The *perR-compl.-F1* and the *ftf*-C-terminal fragments were mixed at a 1:1 ratio, and the mixture was then used as a template for a second PCR using the appropriate *perR-compl.-F1* forward primer and *ftf*-C-terminal reverse primer. The BamHI- and SacII-digested amplified fragment was ligated with pBBSK-Spc::*ftf*-N-terminal digested with BamHI and SacII and then transformed into *Escherichia coli* XL-II (obtained plasmid, pBBSK-Spc::*ftf*-N-terminal+perR+*ftf*-C-terminal). The DNA fragment to insert the Spc^r and *perR* genes into the *ftf* gene was obtained from pBBSK-Spc::*ftf*-N-terminal+perR+*ftf*-C-terminal using the *ftf*-N-terminal forward primer and the *ftf*-C-terminal reverse primer. The resulting PCR product was then transformed into the *perR* deletion mutant to construct the *perR* complement strain. By selecting for erythromycin and spectinomycin resistance, complementation strains were isolated. Finally, in the strain obtained, Spc^r and *perR* gene insertion into the *ftf* gene was verified by PCR.

Quantitation of H_2O_2 concentration in culture medium. The concentration of H_2O_2 was measured by using a method described previously (31). A small portion (10^8 cells) of an *S. sanguinis* culture grown overnight was inoculated into 10 ml TSB and then incubated at 37°C with 5% CO_2. The culture supernatant at the appropriate phase was prepared by centrifugation (10,000 × *g* for 5 min) of the bacterial culture. Each culture supernatant (0.2 ml) was reacted with the reaction solution (0.8 ml) and incubated at 37°C for 20 min. The components of the reaction solution were 10 mM sodium phosphate buffer (pH 7.4), 0.16 mM *o*-dianisidine, 1.2 μg ml^{-1} horseradish peroxidase, and 0.02% Triton X-100. The absorbance at 570 nm was measured, and the concentration was calculated from a standard curve prepared from an experiment using various concentrations of H_2O_2.

Quantitative analysis of gene expression by quantitative PCR. A small portion of *S. mutans* cells cultured overnight was inoculated into fresh TSB. *S. mutans* cells were then grown at 37°C with 5% CO_2, and bacterial cells in the exponential phase were collected. Total RNA was extracted from bacterial cells with a FastRNA Pro Blue kit (MP Biomedicals, Cleveland, OH), according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed to cDNA by using a first-strand cDNA synthesis kit (Roche, Tokyo, Japan). Using the cDNA as a template, quantitative PCR was performed by using the MyiQ system (Bio-Rad, Tokyo, Japan). The primers used are shown in Table 2. Since the DNA gyrase A subunit (*gyrA*) was stably expressed and used as the internal control for quantitative PCR (32, 33), the amount of *gyrA* was used as an internal control in this study. Three independent experiments were performed, and the means ± standard deviations (SD) were calculated. Data were analyzed for statistically significant differences from the UA159 control by a two-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* tests.

Competition assay on agar plates. To investigate the growth inhibition ability of *S. sanguinis* GTC217 against *S. mutans*, a competition assay was performed, as described previously (19). Briefly, 10 μl of an *S. sanguinis* culture grown overnight was spotted onto TSA as the pioneer colonizer. After incubation for 16 h at 37°C with 5% CO_2, 10 μl of an *S. mutans* culture grown overnight, as the competitor, was spotted at a distance where the bacterial cells almost touched each other. After incuba-

TABLE 1 *S. mutans* strains used in this study

Strain	Description[b]	Reference
UA159	Laboratory strain	41
Δ*dpr*	SMU.540[a] deletion mutant; Em^r	This study
Δ*sod*	SMU.629 deletion mutant; Em^r	This study
Δ*ahpCF*	SMU.764–765 deletion mutant; Em^r	This study
Δ*perR*	SMU.593 deletion mutant; Em^r	This study
Δ*perR*::*perR* complement	*perR* complemented in Δ*perR*; Em^r Spc^r	This study
ΔΔ*perR dpr*	*perR dpr* double deletion mutant; Em^r Spc^r	This study
ΔΔ*perR sod*	*perR sod* double deletion mutant; Em^r Spc^r	This study
ΔΔ*perR ahpCF*	*perR ahpCF* double deletion mutant; Em^r Spc^r	This study

[a] The GenBank locus tag was from the *S. mutans* UA159 genome at the NCBI database.
[b] Em^r, erythromycin resistance; Spc^r, spectinomycin resistance.

TABLE 2 primers used in this study

Primer	Description	Sequence[a]	
		Forward	Reverse
Δ*dpr-F1*	Upstream of *dpr* for Δ*dpr*	AAGCCTATCCACAGTGT	CAGTCGAGGATCCTCCTTAAAAAATATTCTT
Δ*dpr-F2*	Downstream of *dpr* for Δ*dpr*	GCTGACCTAGTAAAAGACAATCTGGATGTT	CATCTTCCAAAATATTGGT
Δ*sod-F1*	Upstream of *sod* for Δ*sod*	GTCTTCAGGGAGAAGATG	CAGTCGAGGATTCATTTCCTCTTTTCTTTT
Δ*sod-F2*	Downstream of *sod* for Δ*sod*	GCTGACCTAGTCTGTTGCTCGTCTTTATG	AACGAGCTATTCCATTCT
Δ*ahpCF-F1*	Upstream of *ahpC* for Δ*ahpCF*	GAATTGTGGAAAGTCGC	CAGTCGAGGATACACTTGTCCTCCTTCT
Δ*ahpCF-F2*	Downstream of *ahpF* for Δ*ahpCF*	GCTGACCTAGTGCTTTAGGTGCCTTTGA	TCGGAGTTCTCTCAACT
Δ*perR-F1*	Upstream of *perR* for Δ*perR*	TTGAGTGCTTGGTTGAAT	CAGTCGAGGATTTGACTCCTTCGTTATTC
Δ*perR-F2*	Downstream of *perR* for Δ*perR*	GCTGACCTAGTATTTGTCCAGACTGTCAA	GCCAATGTTCATGCTTTT
perR-compl.-F1	*perR* complementation	TGCGGATCCTTGATGATGCTAGGCAC	TCTTTGTTTCTTACCTCCTATTTCCCATAT
Em[r]	Erythromycin resistance gene for deletion	ATCCTCGACTGGAAGCAAACTTAAGAGTG	ACTAGGTCAGCTTATTTCCTCCCGTTAAA
Spc[r]	Spectinomycin resistance gene for complementation, double deletion	ATCCTCGACTGATCGATTTTCGTTCGTGA	ACTAGGTCAGCTTCCACCATTTTTTCAATTT
ftf-N-terminal	Complementation	ATCTCGAGTTTACTAAGTTCAACAATGG	AAGTCGACCCACCAATAACATTCCAAT
ftf-C-terminal	Complementation	AAGAAACAAAGAAAGCTCATCATGTTTCAAC	CGGCCGCGGTTCGTCTTGTTTCTCTCA
ΔΔ*perR-F1*	Upstream of *perR* for double deletion	TTGAGTGCTTGGTTGAAT[b]	CAGTCGAGGATTTGACTCCTTCGTTATTC[b]
ΔΔ*perR-F2*	Downstream of *perR* for double deletion	GCTGACCTAGTATTTGTCCAGACTGTCAA[b]	GCCAATGTTCATGCTTTT[b]
dpr	Primers for quantitative PCR for *dpr*	GTGGTTCAGGCTTCCTTTAT	ACTGTTTCTTCAAGTCTGGA
sod	Primers for quantitative PCR for *sod*	GTTTTGGCTCAGGTTGGGCT	ATAGTTTGGACGAACATTAC
ahpC	Primers for quantitative PCR for *ahpC*	TGGTTTAGCACAACGTGGAA	AGGGCAAACTTCTCCTGGAT
sloR	Primers for quantitative PCR for *sloR*	ACTGTCTCTGATGTGTTTGT	TCAACAAATATACTCCCATC
gyrA	Primers for quantitative PCR for *gyrA*	TCTCGCTGGACTTGTCACTG	CATCTAGGCGCATCACTTTG

[a] Restriction enzyme sequences are underlined.
[b] Same primer as Δ*perR-F1* or Δ*perR-F2*.

tion overnight, the growth inhibition of *S. mutans* was evaluated. For the control, 10 μl of each *S. mutans* strain was spotted onto TSA without *S. sanguinis* to confirm the growth of each strain. This assay was performed under anaerobic conditions by using the GasPack system (Mitsubishi Gas Chemical Company Inc., Tokyo, Japan). When necessary, before spotting, the competitor strain with catalase solution (100 μg ml^{-1}) was spotted at the same site where the competitor strain was spotted.

H_2O_2 was also used for evaluating susceptibility. An H_2O_2 solution (0.35 or 0.5%) was spotted onto TSA, and a bacterial culture of *S. mutans* was then spotted near the H_2O_2 solution. After incubation overnight, the growth inhibition of *S. mutans* was evaluated. We preliminarily used various concentrations of H_2O_2 and found that 0.35% H_2O_2 was the minimal concentration which did not inhibit the growth of the wild type and that 0.5% H_2O_2 was the concentration which showed 50% growth inhibition of the wild type.

Assay to determine the antibacterial activity of H_2O_2. *S. mutans* strains at the appropriate phase were harvested, washed with phosphate-buffered saline (PBS), and suspended in 10 mM sodium phosphate buffer (PB). The bacterial suspension was diluted to 10^7 cells ml^{-1} with PB, and 10 μl of the bacterial suspension (10^5 cells) was inoculated into 500 μl of TSB with or without 0.04% H_2O_2 and incubated for 30 min at 37°C with 5% CO_2. We preliminarily investigated the effect of various concentrations of H_2O_2 on susceptibility to H_2O_2 and found that the wild type showed 50% survival when exposed to 0.04% H_2O_2. Dilutions of the reaction mixture (100 μl) were plated onto agar medium and incubated at 37°C overnight. The CFU were determined as the total number of colonies identified on each plate. The antibacterial effect was calculated as the ratio of the number of surviving cells (percent survival rate) to the total number of bacteria incubated in TSB without 0.04% H_2O_2. Also, the dilutions of the mixture without 0.04% H_2O_2 prior to incubation were plated onto TSA and incubated at 37°C overnight. The CFU of each strain were determined as the total cell numbers prior to the reaction. Three independent experiments were performed, and the mean ± SD was calculated. Data were analyzed for statistically significant differences from the UA159 control by a two-way ANOVA followed by Dunnett's *post hoc* tests.

Coculture of *S. mutans* with *S. sanguinis*. Cultures of *S. mutans* (wild type and *perR* mutant) and *S. sanguinis* grown overnight were adjusted to an optical density at 660 nm (OD_{660}) of 1.0 and diluted 10-fold. One hundred microliters of each bacterial culture (10- and 100-fold-diluted cultures for *S. sanguinis* and 10-fold-diluted culture for *S. mutans*) was then mixed well. For the control without coculture, 100 μl of *S. mutans* culture was mixed with 100 μl of TSB. A 20-μl aliquot of the nonmixed culture (*S. mutans* only) or the mixed culture was spotted onto 50% TSA plates. When necessary, 10 μl of catalase solution (100 μg ml^{-1}) was also spotted after the bacterial solution was spotted. After 8 h of incubation at 37°C with 5% CO_2, the bacterial colonies growing on the agar plate were scraped and suspended in 500 μl of PBS. Appropriate dilutions were plated onto TSA and TSA containing 32 μg ml^{-1} of bacitracin. After incubation at 37°C with 5% CO_2 for 24 h, the CFU were counted, and the ratio of *S. mutans* with CFU from TSA containing bacitracin in the mixed culture/CFU from TSA in the nonmixed culture was then calculated. We also extracted total RNA from scraped cells and performed cDNA synthe-

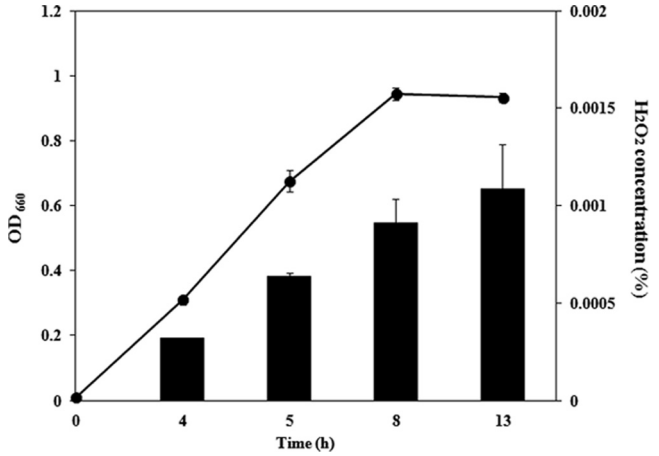

FIG 2 H_2O_2 production in *S. sanguinis* during growth. A small portion (10^8 cells) of an *S. sanguinis* culture grown overnight was inoculated into 10 ml TSB and then incubated at 37°C with 5% CO_2. Culture supernatant at the appropriate phase was prepared, and the H_2O_2 concentration was measured as described in Materials and Methods (black bar). The line represents the growth curve of *S. sanguinis*.

sis using the method described above, and gene expression analysis was conducted by quantitative PCR. Three independent experiments were performed, and the mean ± SD was calculated. The statistical difference between UA159 and the *perR* mutant was determined by two-sided Student's *t* test. A difference with a *P* value of <0.05 was considered significant.

Next, the coculture of *S. mutans perR*, *dpr*, *sod*, and *ahpC* mutants with *S. sanguinis* was investigated by using the method described above. The concentration of bacterial cells used in this assay was 10^8 cells ml^{-1} *S. mutans* mutant and 10^7 cells ml^{-1} *S. sanguinis*. For the coculture of *S. mutans* with *S. sanguinis*, TSA containing 32 μg ml^{-1} of bacitracin was used for *S. mutans* selection. Data were analyzed for statistically significant differences from the UA159 control by a two-way ANOVA followed by Dunnett's *post hoc* tests.

RESULTS

Susceptibility of *sod*, *ahpCF*, and *dpr* mutants to *S. sanguinis* and H_2O_2. We first investigated whether the *S. sanguinis* strain used had the capability to produce H_2O_2. We found that *S. sanguinis* could produce H_2O_2 during growth (Fig. 2). Next, we investigated the association of three factors, *dpr*, *sod*, and *ahpCF*, which are known to be involved in resistance to oxidative stress. We constructed *dpr*, *sod*, and *ahpCF* mutants for further experiments. First, we checked the growth of the mutants and found that the *dpr*, *sod*, and *ahpCF* mutants grew well in TSB or on 50% TSA under anaerobic and aerobic conditions, although the *dpr* and *sod* mutants grew slowly compared to the wild type under aerobic conditions (data not shown). In a competition assay, the growth of *S. mutans* UA159 was partially inhibited by *S. sanguinis* (Fig. 3A), and this inhibition was abolished when the bacterial suspension was mixed with catalase (100 μg ml^{-1}) before spotting onto the TSA plate (data not shown).

We then performed the competition assay using the mutants (Fig. 3A). When the *dpr*, *sod*, and *ahpCF* mutants without *S. sanguinis* were spotted onto TSA, we confirmed that the growth of these mutants was similar to that of the wild type. The *dpr* and *sod* mutants increased the growth-inhibitory area compared with the wild-type strain. In fact, the *dpr* and *sod* mutants showed almost no growth against *S. sanguinis*. As a result of the competition assay using 0.35% H_2O_2, the *dpr* mutant showed almost no growth, while the *sod* mutant displayed slight growth compared with that of the *dpr* mutant (Fig. 3A). In a quantitative assay using TSB containing 0.04% H_2O_2, we investigated the susceptibility of the mutants to H_2O_2 (Fig. 3B). The results were almost the same as those of the competition assay. In the absence of H_2O_2, the cell numbers of the wild type and the *dpr*, *sod*, and *ahpCF* mutants were not changed during 30 min of incubation. In the presence of 0.04% H_2O_2, the wild-type strain demonstrated almost 50% survival, while the *dpr* and *sod* mutants showed 0% and 1.0% survival, respectively. The *ahpCF* mutant displayed susceptibility (55% survival) similar to that of the wild type.

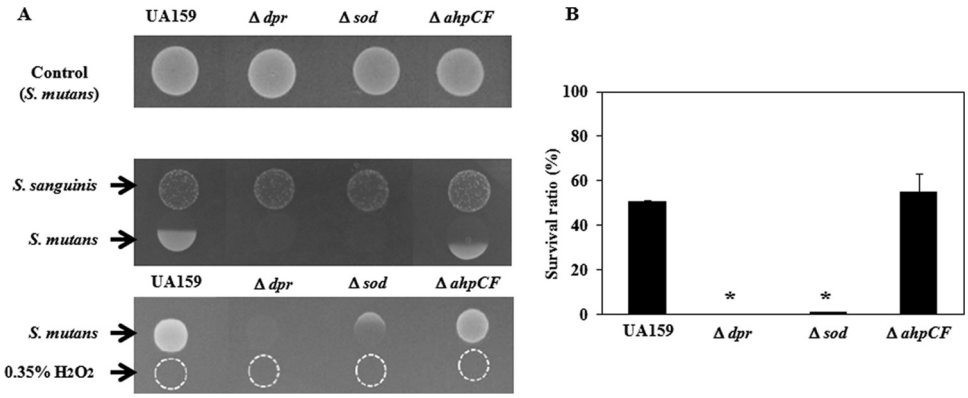

FIG 3 Susceptibility of *S. mutans dpr*, *sod*, and *ahpCF* mutants to *S. sanguinis* and H_2O_2. (A) Competition assay. A culture of *S. sanguinis* grown overnight (10 μl) was spotted onto TSA as the pioneer colonizer. After incubation for 16 h at 37°C with 5% CO_2, 10 μl of a culture of *S. mutans* grown overnight as the competitor was spotted at a distance where both bacterial cells almost attached to each other. After incubation overnight, the growth inhibition of *S. mutans* was evaluated. Also, a 0.35% H_2O_2 solution was spotted onto TSA, and the *S. sanguinis* culture was then spotted using the same method as that described above. In the top panel, only *S. mutans* strains were spotted onto TSA and incubated for 16 h at 37°C with 5% CO_2. (B) Quantitative assay. The bacterial suspension (10^5 cells) was inoculated into 500 μl of TSB with or without 0.04% H_2O_2 and incubated at 37°C with 5% CO_2 for 30 min. Dilutions of the reaction mixture (100 μl) were plated onto agar medium and incubated at 37°C with 5% CO_2 overnight. The CFU were determined as the total number of colonies identified on each plate. The antibacterial effect was calculated as the ratio of the number of surviving cells (percent survival rate) to the total number of bacteria incubated in control PB solution after exposure to H_2O_2. *, $P < 0.005$ compared to wild-type strain UA159, as determined by Dunnett's tests.

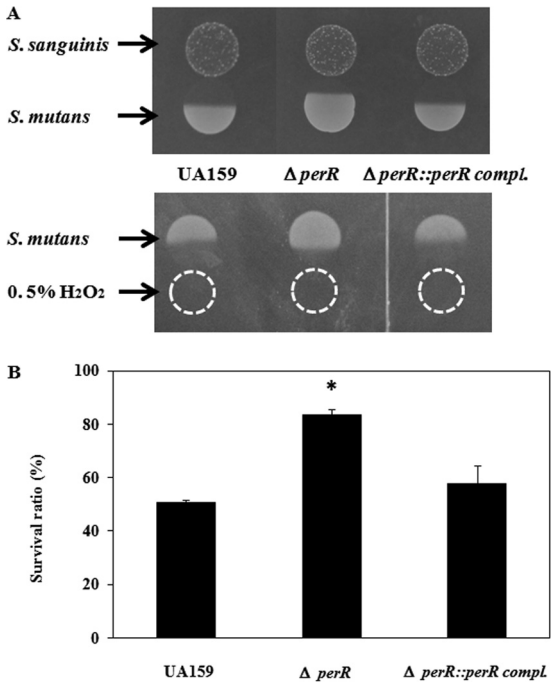

FIG 4 Susceptibility of the *S. mutans perR* mutant and its complemented strain to *S. sanguinis* and H_2O_2. Using wild-type *S. mutans* strain UA159 and the *perR* mutant and complemented strain, a competition assay (A) and a quantitative assay (B) were performed as described in Materials and Methods. *, $P < 0.05$ compared to wild-type strain UA159, as determined by Dunnett's tests.

Susceptibility of the *perR* mutant to H_2O_2. The *perR* mutant showed a decrease in the growth-inhibitory zone compared with that of the wild type, while the growth of the *perR* complementation strain showed an inhibition zone similar to that of the wild type (Fig. 4A). Instead of *S. sanguinis*, an H_2O_2 solution (0.5%) was used for the competition assay, and a similar tendency was found. The *perR* mutant demonstrated decreased susceptibility compared with that of the wild type, while the *perR* complementation strain showed susceptibility similar to that of the wild type. In a quantitative assay for susceptibility to H_2O_2, the wild type and the complementation strain showed 50.8% and 57.5% survival against 0.04% H_2O_2, respectively, while the *perR* mutant displayed 83.3% survival (Fig. 4B).

Expression of factors responsible for resistance to oxidative stress in the *perR* mutant. We investigated the expression of *sod*, *ahpC*, and *dpr* in the *perR* mutant (Fig. 5). The expression level of *dpr* in the *perR* mutant increased 2.5-fold compared with that of the wild type. The expression levels of *sod* and *ahpC* were increased 1.5- and 1.6-fold, respectively, compared with that of the wild type. In the *perR*-complemented strain, the expression level was similar to that of the wild type.

We also investigated the *perR* box upstream of the *dpr* open reading frame (ORF), which has been reported to have a binding region for PerR. Based on the consensus sequence (NTANAANN ATTNTAN) in *Streptococcus pyogenes* and *Bacillus subtilis* (25, 27), we found that a PerR box was found at 57 to 43 bases upstream of the *dpr* start codon, with the consensus sequence TTAGAATCGT TCTAA (data not shown). We also found a *perR* box at 121 to 107 bases upstream of the *sod* ORF, with the sequence TTAGAATTA TTTTAC (data not shown). However, we found no consensus sequence upstream of the *ahpC* ORF (data not shown).

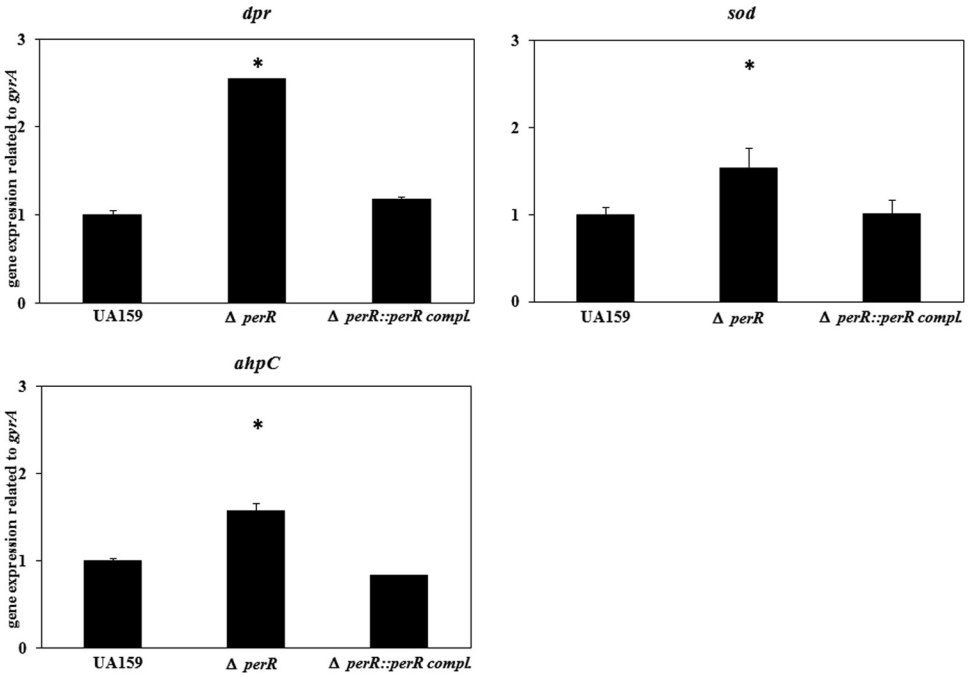

FIG 5 Expression of *dpr*, *sod*, and *ahpC* in the *perR* mutant. A small portion of *S. mutans* cells cultured overnight was inoculated into fresh TSB. *S. mutans* cells were then grown at 37°C with 5% CO_2, and bacterial cells at an OD_{660} of 0.3 were collected. After extraction of total RNA and cDNA synthesis, quantitative PCR was performed as described in Materials and Methods. The amount of *gyrA* was used as an internal control. *, $P < 0.05$ compared to wild-type strain UA159, as determined by Dunnett's tests.

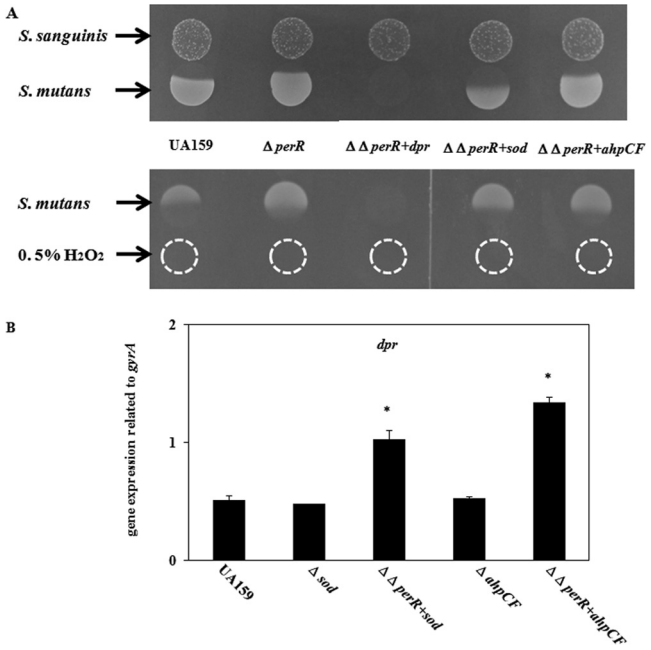

FIG 6 Susceptibility of *S. mutans* double mutants to *S. sanguinis* and H_2O_2. (A) Using wild-type *S. mutans* strain UA159, the *perR* mutant, and double mutants (*perR* with *dpr*, *sod*, or *ahpCF*), a competition assay was performed as described in Materials and Methods. (B) Expression of *dpr* in the double mutants was investigated as described in Materials and Methods. *, $P < 0.005$ compared to wild-type strain UA159, as determined by Dunnett's tests.

H_2O_2 susceptibility of the *perR dpr*, *perR sod*, and *perR ahpCF* double-knockout mutants. We investigated the susceptibility of double mutants (*perR dpr*, *perR sod*, and *perR ahpCF*) to clarify the intrinsic factor for decreased resistance in the *perR* mutant. Before the competition assay, we confirmed that wild-type *S. mutans* strain UA159 and the double deletion mutants on TSA without *S. sanguinis* grew well. The *perR dpr* double-knockout mutant showed a phenotype very similar to that of the *dpr* mutant, with almost no growth in the competition assay (Fig. 6A). In the *perR sod* double mutant, the susceptibility to H_2O_2 decreased compared with that of the *sod* mutant, while the susceptibility of this double mutant to H_2O_2 increased compared with that of the *perR* single mutant. The *perR ahpCF* double mutant showed decreased susceptibility to H_2O_2 compared with that of the wild type. We also investigated the expression of *dpr* in the *sod* single mutant, *ahpCF* single mutant, *perR sod* double mutant, and *perR ahpCF* double mutant (Fig. 6B). For both of the double mutant strains, the *dpr* expression level increased compared with that of the single mutant.

Coculture of *S. mutans* mutants with *S. sanguinis*. Cocultures of the *S. mutans* wild type and mutants with *S. sanguinis* were analyzed. Figure 7A shows the percent ratios of the *S. mutans* population (wild type and the *perR* mutant) when various cell numbers (10^7, 10^6, and 10^5 cells) of *S. sanguinis* were mixed with 10^6 cells of *S. mutans* and spotted onto TSA plates, followed by incubation for 8 h at 37°C. For the mixed ratios of *S. mutans* with *S. sanguinis* at 1:10, 1:1, and 10:1, the population of the wild type accounted for approximately 0.3%, 3.4%, and 46.0%, respectively, while that of *perR* mutant accounted for approximately 0.8%, 15.3%, and 100%, respectively. When *S. mutans* was cocultured with *S. sanguinis* in the presence of catalase (100 μg ml^{-1}), the population ratios of the *S. mutans* wild type and *perR* mutant were 100% and 99.7%, respectively.

We analyzed the expression of *dpr* under coculture conditions (Fig. 7B). The expression level of *dpr* in the *perR* mutant significantly increased ($P < 0.05$) compared with that in the wild type when *S. mutans* was cocultured with *S. sanguinis* at various ratios.

Next, we investigated the population ratio of *S. mutans* mutants cocultured with *S. sanguinis* (Fig. 7C). In the mixed *S. mutans*-*S. sanguinis* population at a ratio of 10:1, the populations of the wild type and the *dpr*, *sod*, and *ahpCF* mutants were 46.0%, 0.2%, 1.8%, and 40.2%, respectively. When *S. mutans* mutants were cocultured with *S. sanguinis* in the presence of catalase (100 μg ml^{-1}), the population ratios of the *S. mutans* wild type and *dpr*, *sod*, and *ahp* mutants were 71.3%, 75.3%, 81.2%, and 81.4%, respectively, showing the increased population ratio of the mutants compared with that without catalase.

DISCUSSION

In this study, we investigated the roles of the oxidative stress-related factors Dpr, SOD, and AhpCF in resistance to H_2O_2. Although Dpr and AhpC have been reported to be involved in resistance to oxidative stress in *S. mutans* (22, 24), this is the first comprehensive analysis of these three factors against H_2O_2. Previous reports demonstrated that Dpr played a central role in resistance to ROS. Dpr binds to free Fe and suppresses the Fenton reaction pathway, which mediates the generation of $^{\bullet}OH$ (24, 34). Notably, Sod was also significantly associated with H_2O_2 resistance, although the *dpr* mutant showed more susceptibility to H_2O_2 than did the *sod* mutant. As shown in Fig. 1, Sod is involved in the reaction that mediates the conversion of O_2^- to H_2O_2 (12). When *sod* is deleted, O_2^- accumulates in the cytoplasm. By the addition of H_2O_2 to the medium, the accumulated O_2^- in the *sod* mutant reacts with H_2O_2, causing the formation of $^{\bullet}OH$, which is considered to be highly toxic to bacteria (35). Thus, the *sod* mutation caused an increase in H_2O_2 susceptibility. In contrast, inactivation of *ahpC*, which decomposes H_2O_2 to H_2O, had no effect on H_2O_2 susceptibility. Higuchi et al. also demonstrated previously that the *ahpC* deletion had no effect on the sensitivity of *S. mutans* to H_2O_2 (11). These results suggest that AhpC cannot decompose excess H_2O_2 completely. As a result, the presence of H_2O_2 in the cytoplasm has a cytotoxic effect on bacteria. In conclusion, Dpr and Sod are involved in H_2O_2 resistance in *S. mutans*.

PerR is known as an oxidative stress-responsive repressor and has been demonstrated to be associated with resistance to oxidative stress in Gram-positive and -negative bacteria (25–28, 36, 37). In *Streptococcus pyogenes*, PerR regulates *perR* itself, *ahpC*, and *mrgA* (Dps-like peroxide resistance protein) and contributes to resistance to ROS (25). Also, Ricci et al. reported previously that the *perR* mutant in *S. pyogenes* caused reduced transcription of *sodA*, encoding superoxide dismutase, which is involved in resistance to superoxide anions (38). For *Streptococcus suis*, *perR* was reported to regulate two oxidative stress response factors, *dpr* and *metQIN* (27). From our results, in *S. mutans*, the *perR* deletion caused significantly increased expression levels of *dpr* and slightly increased *sod* and *ahpC* expression levels. We also investigated the *perR* box, based on the consensus sequence in *S. pyogenes* and *Bacillus subtilis*, upstream of the *dpr* ORF (25, 27); we found that a PerR box was found upstream of the *dpr* and *sod* start codons. However, no consensus sequence was found upstream of the *ahpC*

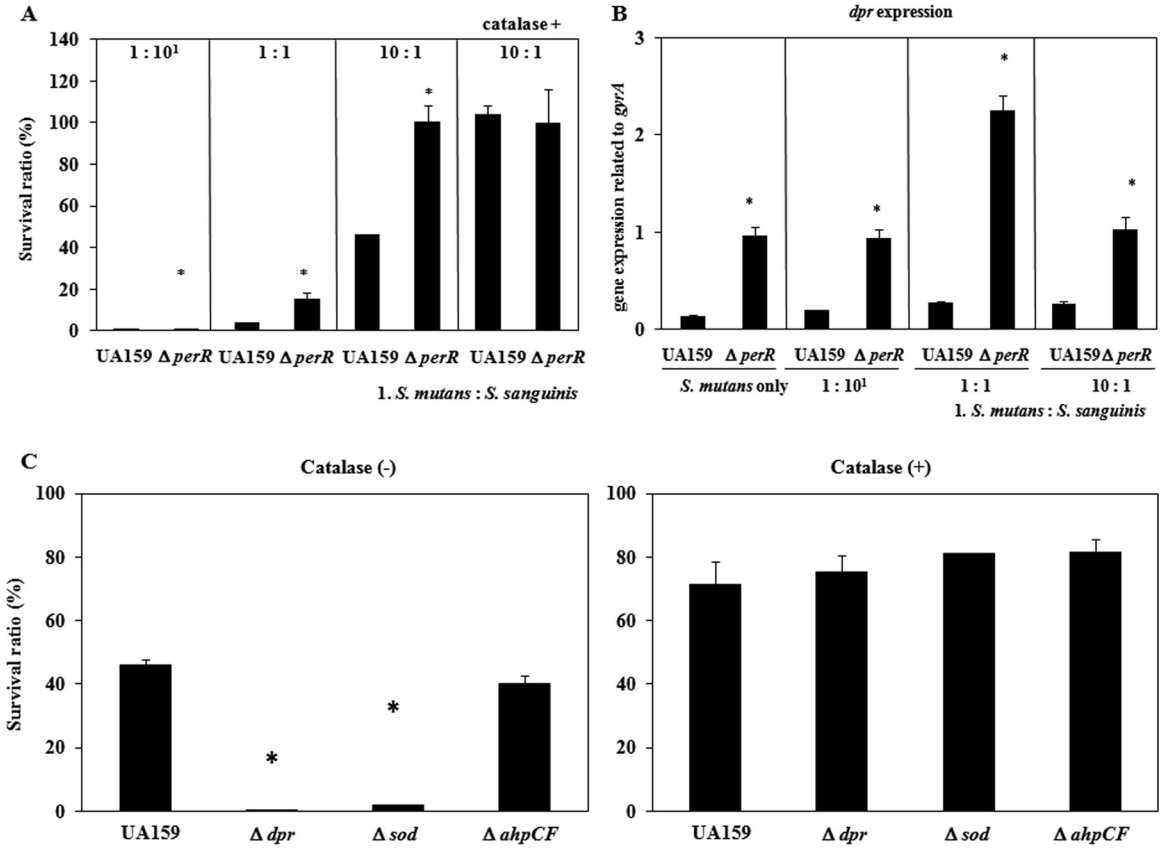

FIG 7 Coculture of *S. mutans* with *S. sanguinis*. Cultures of *S. mutans* and *S. sanguinis* grown overnight were adjusted to an OD_{660} of 1.0 and diluted 10- to 100-fold. One hundred microliters of *S. mutans* was then mixed with 100 μl *S. sanguinis* or 100 μl TSB. A 20-μl aliquot of the nonmixed culture and mixed culture was spotted onto 50% TSA. When necessary, 10 μl of catalase solution (100 μg/ml) was also spotted after the bacterial solution was spotted. After 8 h of incubation at 37°C with 5% CO_2, the bacterial colonies growing on the agar plate were scraped and suspended in 1 ml TSB. Appropriate dilutions were plated onto TSA and TSA containing antibacterial agents. After 2 days of incubation, the CFU on the plates containing TSA and TSA containing antibiotics were counted, and the ratio of *S. mutans* with CFU from TSA containing bacitracin in mixed culture/CFU from TSA in nonmixed culture was determined. In addition, total RNA was extracted from the scraped cells, and cDNA synthesis was performed by using the method described above. Finally, a gene expression analysis was conducted by using quantitative PCR. (A) Percent ratio of the *S. mutans* population when mixed with various concentrations of *S. sanguinis*. *, $P < 0.05$ compared to wild-type strain UA159, as determined by *t* test for the percent ratio of the *S. mutans* population. (B) *dpr* expression of *S. mutans* UA1159 and the *perR* mutant. *, $P < 0.05$ compared to wild-type strain UA159, as determined by *t* test. (C) Percent ratio of the *S. mutans* population with or without the addition of catalase solution when mixed with *S. sanguinis* (1:0.1). *, $P < 0.05$ compared to wild-type strain UA159, as determined by Dunnett's tests.

ORF. Thus, we suggest that PerR regulates *dpr* and *sod* directly. As described above, PerR is considered to regulate many genes, mainly factors responsible for oxidative stress resistance and metal transporters (10, 39, 40). ABC-type metal ion transporters are responsible for efflux or incorporation of metal ions, Fe and Mn, which are associated with oxidative stress responses because PerR and other Fur-like proteins bind metal ions for activation (10, 26). Thus, the increased resistance of the *perR* mutant to H_2O_2 may be due to factors other than *dpr*. However, the analysis of double-knockout mutants revealed that the *dpr* deletion in the *perR* mutant showed almost the same (increased) susceptibility to H_2O_2 as the *dpr* single mutant, suggesting that Dpr is significantly associated with H_2O_2 resistance in the *perR* mutant. Also, the *perR sod* double mutant showed increased susceptibility to H_2O_2 compared with that of the *perR* single mutant, indicating that *sod* is also partially associated with H_2O_2 resistance. In this study, we established a method for the coculture of two bacterial species. When a 1/10 dilution of *S. sanguinis* was cocultured with the *S. mutans* wild-type strain, the ratio of *S. mutans* (81.6%) was reduced significantly. In contrast, when catalase was added to the mixed culture, the ratio of *S. mutans* was slightly higher (91.2%). This result indicates that H_2O_2 produced by *S. sanguinis* affects competition with *S. mutans*. Next, we investigated the population ratio of *S. mutans* mutants cocultured with *S. sanguinis*. The results showed the same tendency as that seen in the competition and quantitative experiments. The *sod* and *dpr* genes mainly functioned in resistance to H_2O_2 when *S. mutans* was cocultured with *S. sanguinis*. Also, because the *perR* deletion, which caused significantly increased *dpr* expression levels, showed lower sensitivity to H_2O_2, the *perR* mutant showed a larger population than the wild type. Kreth et al. previously investigated coexistence between *S. mutans* and *S. sanguinis* in biofilms and also highlighted the importance of mutacin and H_2O_2 in the coexistence of the strains (19). Together with this result, we suggest that H_2O_2 produced by *S. sanguinis* is one of the factors affecting the growth of *S. mutans* within multispecies bacterial communities. As for *S. mutans* cocultured with *S. sanguinis*, we found that the *dpr* or *sod* mutants drastically reduced the population compared with that of the wild

type. To evaluate the coculture assay, we confirmed that the expression of *nlmA*, encoding mutacin IV, showed no change among UA159 and the *dpr*, *sod*, and *ahpCF* mutants, suggesting that mutacin IV from *S. mutans* had no effect on the result of survival ratios of individual strains. Thus, we conclude that *dpr* and *sod* are responsible for resistance to H_2O_2 in *S. mutans* cocultured with *S. sanguinis*.

In conclusion, we analyzed three factors in the oxidative stress response, *dpr*, *sod*, and *ahpCF*, and found that Dpr and Sod are involved in H_2O_2 resistance. We also demonstrated that these two factors had key roles in coexistence with *S. sanguinis*. Furthermore, we identified the factor, PerR, which negatively regulated the expression of oxidative stress factors and demonstrated that PerR was associated with resistance to H_2O_2 in *S. mutans*. Our findings may indicate the mechanism of the coexistence of *S. mutans* and *S. sanguinis* in dental biofilms.

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