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PHYSIOLOGY

Plasticity of the Pyruvate Node Modulates Hydrogen Peroxide Production and Acid Tolerance in Multiple Oral Streptococci

Xingqun Cheng,[a,b,c] Sylvio Redanz,[a] Nyssa Cullin,[a,d] Xuedong Zhou,[b,c] Xin Xu,[b,c] Vrushali Joshi,[e] Dipankar Koley,[e] Justin Merritt,[a] Jens Kreth[a]

[a]Department of Restorative Dentistry, Oregon Health and Science University, Portland, Oregon, USA

[b]The State Key Laboratory of Oral Diseases and the National Clinical Research Center for Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, China

[c]The Department of Cariology and Endodontics, West China Hospital of Stomatology, Sichuan University, Chengdu, China

[d]Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

[e]Department of Chemistry, Oregon State University, Corvallis, Oregon, USA

ABSTRACT Commensal *Streptococcus sanguinis* and *Streptococcus gordonii* are pioneer oral biofilm colonizers. Characteristic for both is the SpxB-dependent production of H_2O_2, which is crucial for inhibiting competing biofilm members, especially the cariogenic species *Streptococcus mutans*. H_2O_2 production is strongly affected by environmental conditions, but few mechanisms are known. Dental plaque pH is one of the key parameters dictating dental plaque ecology and ultimately oral health status. Therefore, the objective of the current study was to characterize the effects of environmental pH on H_2O_2 production by *S. sanguinis* and *S. gordonii*. *S. sanguinis* H_2O_2 production was not found to be affected by moderate changes in environmental pH, whereas *S. gordonii* H_2O_2 production declined markedly in response to lower pH. Further investigation into the pyruvate node, the central metabolic switch modulating H_2O_2 or lactic acid production, revealed increased lactic acid levels for *S. gordonii* at pH 6. The bias for lactic acid production at pH 6 resulted in concomitant improvement in the survival of *S. gordonii* at low pH and seems to constitute part of the acid tolerance response of *S. gordonii*. Differential responses to pH similarly affect other oral streptococcal species, suggesting that the observed results are part of a larger phenomenon linking environmental pH, central metabolism, and the capacity to produce antagonistic amounts of H_2O_2.

IMPORTANCE Oral biofilms are subject to frequent and dramatic changes in pH. *S. sanguinis* and *S. gordonii* can compete with caries- and periodontitis-associated pathogens by generating H_2O_2. Therefore, it is crucial to understand how *S. sanguinis* and *S. gordonii* adapt to low pH and maintain their competitiveness under acid stress. The present study provides evidence that certain oral bacteria respond to environmental pH changes by tuning their metabolic output in favor of lactic acid production, to increase their acid survival, while others maintain their H_2O_2 production at a constant level. The differential control of H_2O_2 production provides important insights into the role of environmental conditions for growth competition of the oral flora.

KEYWORDS *Streptococcus*, biofilms, microbial ecology

Oral biofilm bacteria are exposed to constantly changing environmental conditions. Some of these environmental changes are triggered directly by host behaviors and promote the development of oral diseases (1, 2). One striking example is the development of caries as the result of increased carbohydrate exposure due to nutritional

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Address correspondence to Jens Kreth, kreth@ohsu.edu.

choices of the host (3). Oral bacteria can respond to environmental changes by modifying their gene expression. For example, exposure of *Streptococcus mutans* to low pH leads to an acid tolerance response (4). In the oral biofilm, frequent exposure to low pH selects for aciduric species, resulting in a shift toward a dysbiotic, caries-promoting microbial community (5). The selection process that favors aciduric species leads to a decline in the abundance of several commensal species that can be found in healthy oral biofilms. For example, recent clinical studies observed that *Streptococcus sanguinis* is strongly associated with oral health, but its prevalence declines in individuals with caries and periodontal diseases (6, 7). Similarly, *Streptococcus gordonii* seems to be an abundant species in healthy dental plaque (3, 8, 9).

It is largely unknown how exposure to lower pH influences commensal bacterial species in the oral biofilm. *S. sanguinis* and *S. gordonii* are less aciduric than *S. mutans*, thus providing a possible explanation for their decreased abundance in acidic dental plaques (10–12). The competitive abilities of commensal streptococci likely also play an integral role in oral biofilm ecology. Both *S. sanguinis* and *S. gordonii* have been demonstrated to successfully antagonize competitors in dual-species biofilms (13, 14), but it is unclear to what degree, if any, pH influences these abilities.

Our group and others have demonstrated the importance of the pyruvate oxidase SpxB in the competitive abilities of commensal streptococci (14–16). Under aerobic conditions, as found during early stages of oral biofilm formation, the catalytic activity of SpxB generates H_2O_2, CO_2, and acetyl phosphate. Acetyl phosphate is further metabolized to acetate, generating ATP (14). Recent studies have demonstrated that the H_2O_2 produced by *S. sanguinis* and *S. gordonii* under aerobic conditions plays an important role in cell-cell aggregation and biofilm formation through the H_2O_2-mediated release of DNA (17, 18). Extracellular DNA (eDNA) not only has a structurally stabilizing role as part of the biofilm extracellular polymeric substances (EPS) but also serves as a source of DNA for horizontal gene transfer (17, 18). Moreover, H_2O_2 is the main inhibitory substance produced by *S. sanguinis* and *S. gordonii* to combat competitors such as *S. mutans*, one of the few oral streptococci that does not express SpxB (14). Thus, SpxB activity likely provides commensal colonizers with a competitive growth advantage during early oral biofilm formation.

Interestingly, *spxB* is encoded in the majority of oral streptococci with a high degree of sequence conservation (19) but little is known about its regulation. Initial investigations of *spxB* expression in *S. sanguinis* and *S. gordonii* revealed a striking difference between the species despite a largely conserved *spxB* promoter region. The presence of *cre* binding sites in the *spxB* promoter for the carbon catabolite regulator CcpA suggests carbohydrate-dependent regulation (20). As expected, deletion of CcpA in both species increased the expression of *spxB* (21, 22); however, carbohydrate-dependent repression of *spxB* was detected only in *S. gordonii*. Different carbohydrates had no influence on *spxB* expression in *S. sanguinis* (21, 22). The underlying mechanism for this differential expression regulation is currently unknown, which piqued our interest to further investigate how environmental factors influence *spxB* expression in the two commensal streptococci.

Here we demonstrate that *S. gordonii* and *S. sanguinis* exhibit vastly different H_2O_2 production phenotypes in response to pH. Although *S. sanguinis* produces less H_2O_2 overall, H_2O_2 production is similar at pH 6 and pH 7. In contrast, *S. gordonii* H_2O_2 production decreases significantly as the pH drops. Further investigations into the metabolic consequences of decreased SpxB activity in *S. gordonii* suggest a redirection of central metabolism toward lactic acid production at pH 6, contributing to the survival of *S. gordonii* at low pH. Additionally, we demonstrate that the differential responses to a change in pH are not confined to *S. sanguinis* and *S. gordonii* but also affect other oral streptococcal species.

RESULTS

Differential pH-dependent H_2O_2 production in *S. gordonii* and *S. sanguinis*. To determine whether H_2O_2 production in *S. sanguinis* and *S. gordonii* is influenced by

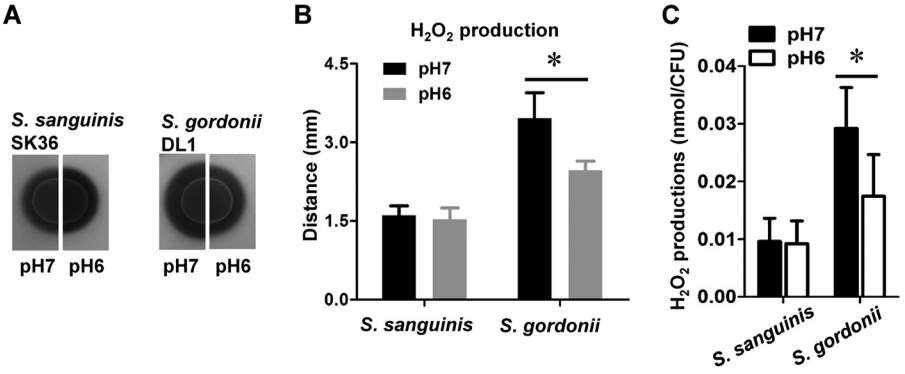

FIG 1 pH-dependent H_2O_2 production in *S. sanguinis* and *S. gordonii*. (A) Images of *S. sanguinis* and *S. gordonii* on H_2O_2 indicator plates adjusted to pH 7 and pH 6; representative images are shown. (B) Quantitative analysis of H_2O_2 production on indicator plates; production was determined by measuring the distance between the border of the colony and the border of the halo formed by the Prussian blue precipitate. (C) Quantitative analysis of H_2O_2 production in liquid cultures; production was determined enzymatically and normalized to CFU. All data were obtained in triplicate at separate times. *, significant difference ($P < 0.05$).

changes in environmental pH, we employed a colorimetric plate assay for the detection of H_2O_2. The plate assay ensures maximum H_2O_2 production, due to naturally efficient aeration, as reported previously (23). The amount of H_2O_2 produced by *S. gordonii* at pH 6 was noticeably decreased, compared to that produced at pH 7, as evident from the smaller halo surrounding the respective inoculum (Fig. 1A). No difference was observed for *S. sanguinis* (Fig. 1A). Quantification of the halo size confirmed a statistically significant difference in H_2O_2 production for *S. gordonii* (Fig. 1B). The observation that *S. sanguinis* is not influenced by the environmental pH while *S. gordonii* responds with decreased H_2O_2 production was also determined with different initial cell densities. A positive correlation between the increase in cell density and H_2O_2 production was observed for both species, but the overall observation that *S. sanguinis* produces less H_2O_2 remained consistent (see Fig. S1 in the supplemental material). The H_2O_2 concentration was also determined with planktonic cells in liquid cultures grown aerobically and was normalized to CFU counts. The *S. sanguinis* H_2O_2 production levels were similar at pH 6 and pH 7, whereas *S. gordonii* H_2O_2 production decreased significantly at pH 6, consistent with the results determined by the colorimetric indicator plate assays (Fig. 1C).

The growth of *S. sanguinis* and *S. gordonii* was slower at pH 6 than at pH 7, and both strains yielded fewer CFU than did cells grown at pH 7 after 24 h (Fig. 2A). Additionally, we found that the growth of both strains was largely inhibited at pH 5 (data not shown). To account for potential differences in cell densities at different pH values and to determine whether the reduced production of H_2O_2 in *S. gordonii* correlates with gene expression, the expression of *spxB* was determined with quantitative reverse transcription-PCR (qRT-PCR). *S. sanguinis spxB* gene expression was not affected by pH, in agreement with the phenotypic observations on H_2O_2 indicator plates. The expression of *spxB* in *S. gordonii* was decreased 3.5-fold at pH 6, compared to that at pH 7 (Fig. 2B). Furthermore, Western blot analysis was performed using isogenic strains of *S. sanguinis* and *S. gordonii* carrying engineered SpxB with FLAG tags. The FLAG epitope did not interfere with H_2O_2 production and was specific for SpxB (Fig. S2A). As predicted, SpxB in *S. sanguinis* exhibited the same abundance levels at pH 6 and pH 7. However, noticeably less SpxB was detected for *S. gordonii* at pH 6 versus pH 7 (Fig. 2C). The data indicate that *S. sanguinis* H_2O_2 production is largely insensitive to slightly acidic environmental pH, whereas the same pH reduces *S. gordonii spxB* gene expression, ultimately decreasing its H_2O_2 production.

Role of two-component signal transduction systems in the control of pH-dependent H_2O_2 adjustment. The two-component systems (TCSs) ComDE, VicKR, and

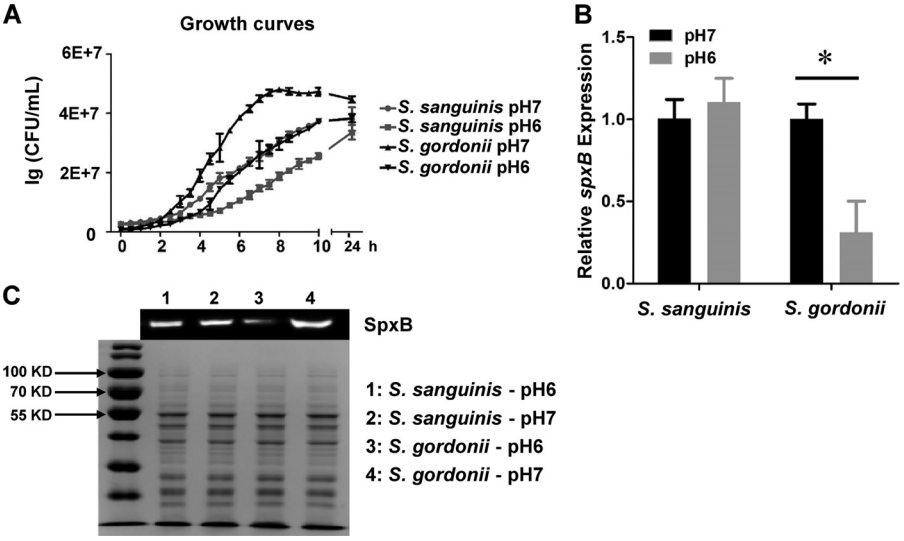

FIG 2 Growth, *spxB* expression, and SpxB abundance in *S. sanguinis* SK36 and *S. gordonii* DL1 at pH 7 and pH 6. (A) Growth curves for *S. sanguinis* SK36 and *S. gordonii* DL1 at pH 7 and pH 6. (B) Relative levels of expression of *spxB* genes in *S. sanguinis* SK36 and *S. gordonii* DL1 under different pH conditions; the relative cDNA abundance with SK36 and DL1 at pH 7 was set to 1. (C) Top, Western blot analysis of SpxB protein (72 kDa) abundance in *S. sanguinis* SK36 and *S. gordonii* DL1 at pH 7 and pH 6; bottom, SDS-PAGE gel with Coomassie blue staining, shown as a loading control. All data were obtained in triplicate at separate times. *, significant difference ($P < 0.05$).

CiaHR have been associated with pH-dependent stress response regulation (24–27). Therefore, we were curious regarding whether expression of *comC* (the first gene in the *comCDE* operon), *vicK*, and *ciaH* responded to pH changes in *S. sanguinis* and *S. gordonii*, as a potential mechanism to explain the observed differential regulation of *spxB* expression during acidification. Expression of all three TCSs was indeed influenced by changes in pH; however, the differences were moderate (Fig. S3A and B). Thus, we did not observe compelling evidence to implicate TCS regulation of *spxB* gene expression at pH 6.

pH-dependent redirection of central metabolism toward lactic acid production in *S. gordonii*. The pyruvate node of streptococcal central metabolism is responsible for the SpxB-dependent generation of acetyl-PO_4, which is further converted to acetate via acetate kinase (AckA) and to lactic acid via lactate dehydrogenase (Ldh), thereby generating ATP and NAD^+. Since these pathways are interconnected, we hypothesized that a decrease in SpxB activity, i.e., lower H_2O_2 production in *S. gordonii*, would potentially redirect pyruvate toward the Ldh pathway (see Fig. 7 for an overview of the pyruvate node). To test this, we compared the concentrations of lactic acid secreted from *S. sanguinis* and *S. gordonii*. As expected, a change from pH 7 to pH 6 or from pH 6 to pH 7 had no obvious impact on lactic acid production by *S. sanguinis*, whereas both conditions significantly altered lactic acid production by *S. gordonii* (Fig. 3A). Similarly, *ldh* gene expression in both strains correlated with their lactic acid production at pH 6 and pH 7 (Fig. 3B). Moreover, we measured lactic acid production and *ldh* gene expression when the pH was fixed to 7 or 6. The amount of lactic acid produced by *S. gordonii* at pH 6 was significantly increased, compared to that produced at pH 7, whereas no difference was observed for *S. sanguinis* 2 h or 4 h after the start of incubation (Fig. 3C). Consistently, *ldh* expression was noticeably upregulated in *S. gordonii* at pH 6, while no difference was observed for *S. sanguinis* (Fig. 3D). Interestingly, it was also possible to increase *S. gordonii* lactic acid production at pH 7 simply by deleting *spxB* (Fig. 3E). This is also reflected in increased *ldh* gene expression for the *S. gordonii* Δ*spxB* mutant at pH 6 (Fig. 3F). Interestingly, lactic acid production by the *S. sanguinis spxB* mutant was slightly decreased and *spxB* expression was unaffected during the pH shift (Fig. 3E and F). When the pH was fixed to 7 or 6, *ldh* gene expression

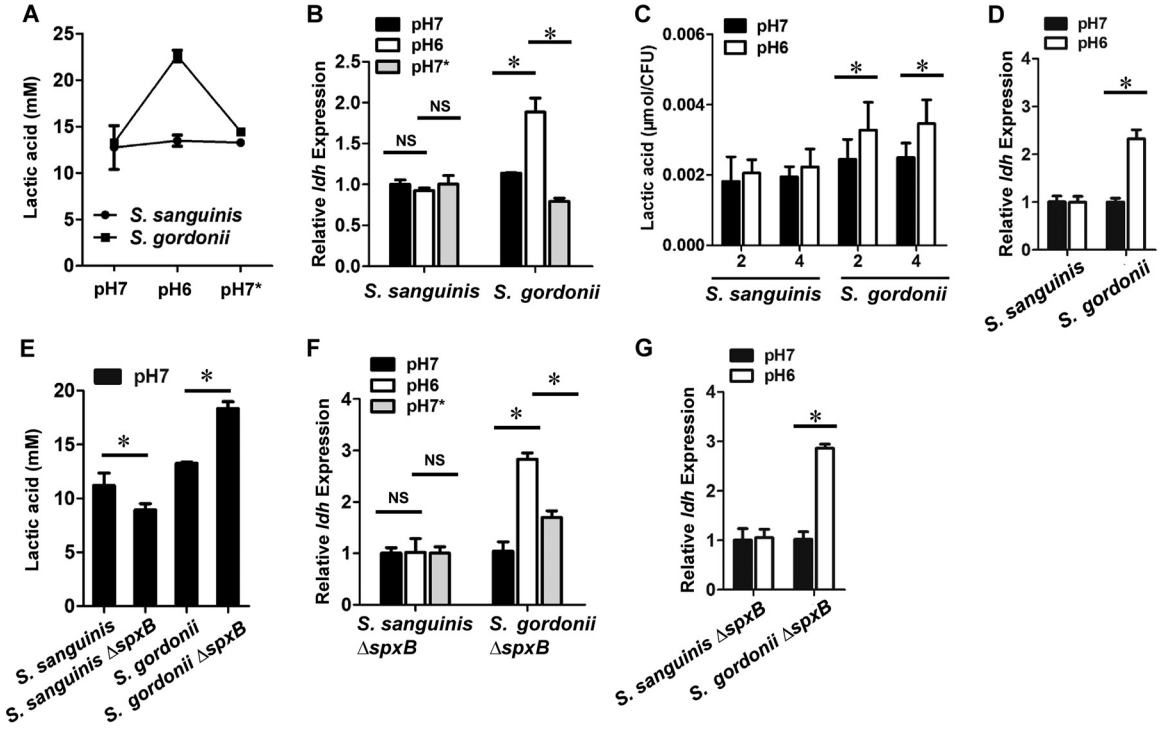

FIG 3 pH-dependent influence of lactic acid production in *S. sanguinis* and *S. gordonii*. (A) Lactic acid levels in *S. sanguinis* SK36 and *S. gordonii* DL1 under different pH conditions. pH 7, pH 6, and pH 7* represent different time points illustrated in Fig. S4 in the supplemental material. Bacteria were adjusted to the same cell density and volume before lactic acid production measurements. (B) Relative *ldh* gene expression in *S. sanguinis* SK36 and *S. gordonii* DL1 under different conditions. pH 7, pH 6, and pH 7* represent different time points illustrated in Fig. S4. (C) Lactic acid levels in *S. sanguinis* SK36 and *S. gordonii* DL1 at different pH conditions and different time points. The mid-log-phase bacteria were cultured at a fixed pH (pH 7 or pH 6) for 2 h or 4 h. Lactic acid concentrations were normalized to CFU. (D) Relative *ldh* gene expression in *S. sanguinis* SK36 and *S. gordonii* DL1 at pH 7 and pH 6. The pH was fixed to pH 7 or 6, and the relative cDNA abundance with SK36 and DL1 at pH 7 was set to 1. (E) Lactic acid levels in wild-type *S. sanguinis* SK36 and *S. gordonii* DL1 and their respective Δ*spxB* mutants. Bacteria were adjusted to the same cell density and volume before lactic acid production measurements. (F) Relative *ldh* gene expression in the *S. sanguinis* SK36 Δ*spxB* mutant and the *S. gordonii* DL1 Δ*spxB* mutant under different conditions. pH 7, pH 6, and pH 7* represent different time points illustrated in Fig. S4. (G) Relative *ldh* gene expression in the *S. sanguinis* SK36 Δ*spxB* mutant and the *S. gordonii* DL1 Δ*spxB* mutant at pH 7 and pH 6. The pH was fixed to pH 7 or 6, and the relative cDNA abundance with the SK36 Δ*spxB* mutant and the DL1 Δ*spxB* mutant at pH 7 was set to 1. All data were obtained in triplicate at separate times. *, significant difference ($P < 0.05$). NS, not significant.

in both strains was comparable to findings from the pH shift experiments, confirming the observation that *S. gordonii* adjusts its *ldh* expression to the lower pH condition (Fig. 3G).

The pyruvate node involves several other gene products, including pyruvate kinase (*pyk*), acetate kinase (*ackA*), pyruvate formate lyase (*pfl*), alcohol dehydrogenase (*adh*), and phosphate acetyltransferase (*pta*) (see Fig. 7 for an overview). To initially assess whether a change in pH leads to alteration in expression of those genes, expression in *S. sanguinis* and *S. gordonii* at pH 6 and pH 7 was determined. Interestingly, *pyk* was upregulated at pH 6 in *S. sanguinis*, suggesting increased availability of pyruvate. Since *S. sanguinis* does not produce more H_2O_2 or lactic acid at pH 6, the increased expression of *pfl* and *adh* suggests that the pyruvate might be metabolized to ethanol. No difference of >2-fold was observed for the other tested genes of *S. gordonii* and *S. sanguinis* (Fig. S5).

Effects of pH-dependent redirection of central metabolism toward lactic acid production on acid survival of *S. gordonii*. Acidogenic *S. mutans* can substantially lower the environmental pH via production of lactic acid, and it is able to survive this low pH for extended periods (4). Since *S. gordonii* seems to shift its metabolism toward lactic acid production at pH 6, we wondered whether this could be a strategy to activate its acid tolerance mechanisms. Cells of *S. gordonii* and *S. sanguinis* grown in brain heart infusion (BHI) broth at pH 7 were inoculated into BHI broth at pH 6 to trigger

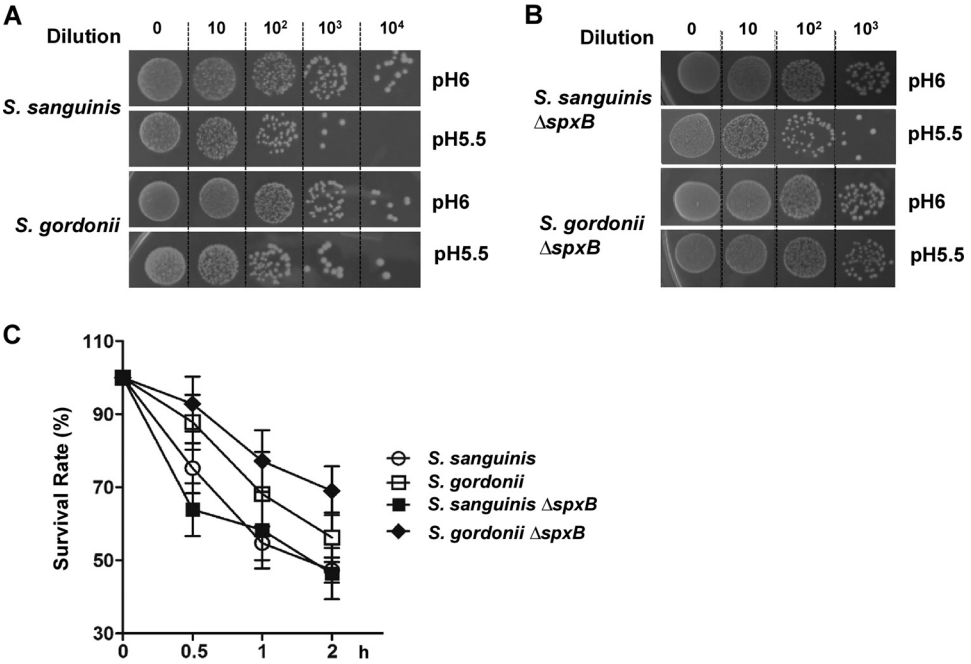

FIG 4 Acid survival of *S. sanguinis* SK36 and *S. gordonii* DL1 wild-type strains and Δ*spxB* mutants at pH 5.5. (A) Representative images of the wild-type SK36 and DL1 strains. Cells grown in BHI broth at pH 7 were inoculated into BHI broth at pH 6 to adjust their metabolic activity. Subsequently, serial dilutions were spotted on pH 5.5 plates. Photographs of the plates were taken after 24 h of incubation. (B) Representative images of the SK36 and DL1 Δ*spxB* mutants. (C) Acid survival rates of planktonic cells exposed to pH 5.5 over time (0, 0.5, 1, and 2 h). All data were obtained in triplicate at separate times.

acid adaptation and were subsequently spotted onto a pH 5.5 plate. *S. gordonii* acid tolerance seems to be more robust than that of *S. sanguinis*, as evident by the growth of colonies at dilutions of 10^3 and 10^4 (Fig. 4A). The acid survival rate assay confirmed a higher survival rate for *S. gordonii* versus *S. sanguinis* at low pH (Fig. 4C). Interestingly, the *S. gordonii* Δ*spxB* mutation further increased the acid survival rate, while the same mutation demonstrated no impact on *S. sanguinis* survival (Fig. 4B and C). This observation confirms that, as the pH drops, the modification of *S. gordonii* metabolism toward lactic acid production increases its survival at low pH.

Effects of pH on pyruvate node plasticity of clinical isolates. The *spxB* gene is highly conserved among H_2O_2-producing oral streptococci. Therefore, we were curious to compare pH-dependent H_2O_2 production using additional oral streptococci (19). Low-passage-number oral clinical isolates of *Streptococcus parasanguinis*, *Streptococcus dentisani*, and *S. gordonii* and an endocarditis *S. sanguinis* isolate were tested for H_2O_2 production using H_2O_2 indicator plates adjusted to pH 7 or pH 6. As shown in Fig. 5A, *S. sanguinis* 133-79 exhibited no significant differences in H_2O_2 production at pH 6 versus pH 7, consistent with our previous results with *S. sanguinis* SK36. The same observation was made with the *S. parasanguinis* isolate. In contrast, *S. gordonii* JM1 and *S. dentisani* both produced significantly less H_2O_2 at pH 6 than at pH 7 (Fig. 5B). The difference in H_2O_2 production also influenced the lactic acid production and the ability to survive an acid challenge. Compared to *S. sanguinis* 133-79 and *S. parasanguinis*, *S. gordonii* JM1 and *S. dentisani* increased lactic acid production at pH 6 (Fig. 5C) and survived the acid challenge from pH 6 to pH 5.5 to a greater extent (Fig. 5D).

Furthermore, testing of several other low-passage-number *S. parasanguinis* isolates with unchanged or slightly lower production of H_2O_2 at pH 6 demonstrated lower survival rates at pH 6, compared to low-passage-number isolates of *Streptococcus mitis*, *S. dentisani*, and *Streptococcus oralis* (Fig. 6A). Accordingly, *S. mitis*, *S. dentisani*, and *S. oralis* all showed reduced production of H_2O_2 with increased lactic acid concentration at pH 6 (Fig. 6B). This suggests that the ability to adjust H_2O_2 production with changing

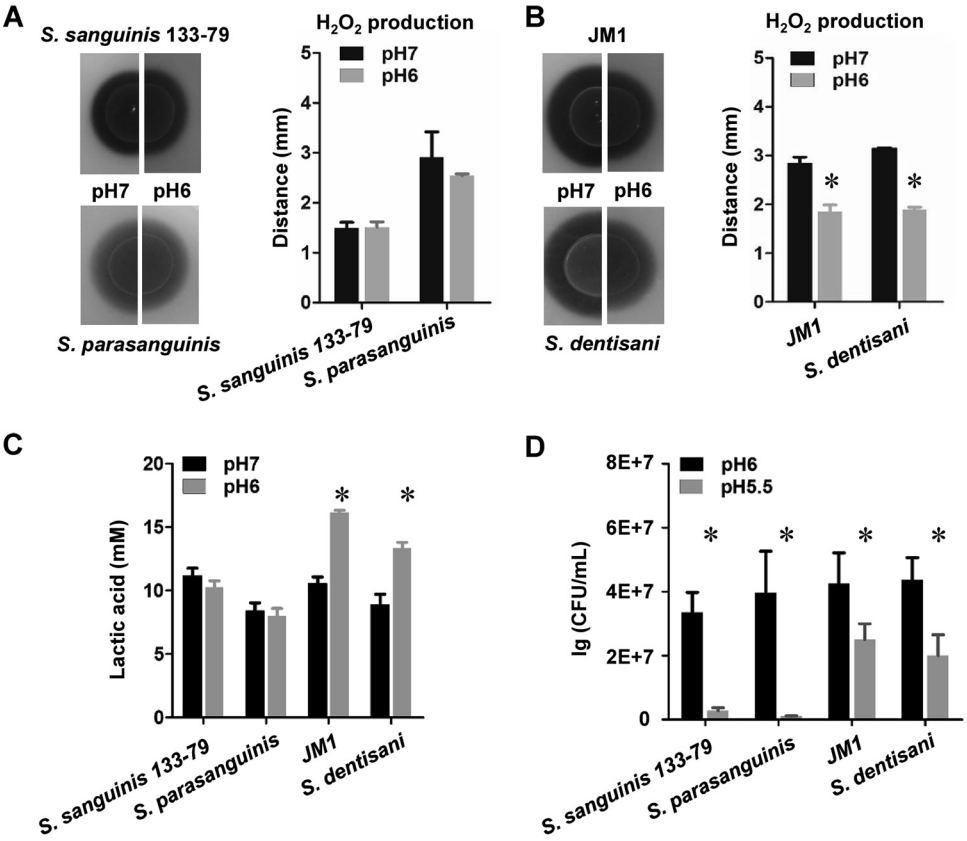

FIG 5 Effects of pH on H_2O_2 production in various oral streptococci. (A) H_2O_2 production of *S. sanguinis* 133-79 and *S. parasanguinis* at pH 7 and pH 6. (B) H_2O_2 production of *S. gordonii* JM1 and *S. dentisani* at pH 7 and pH 6. (C) Lactic acid levels in *S. sanguinis* 133-79, *S. parasanguinis*, *S. gordonii* JM1, and *S. dentisani* at pH 7 and pH 6. (D) CFU quantification of *S. sanguinis* 133-79, *S. parasanguinis*, *S. gordonii* JM1, and *S. dentisani* at pH 6 and pH 5.5 after overnight growth. All data were obtained at least three separate times. *, significant difference ($P < 0.05$).

environmental pH to increase survival at lower pH is likely characteristic of most or all *S. gordonii* strains and may be a feature shared by a variety of other oral streptococci as well.

DISCUSSION

Polymicrobial biofilms have been implicated in the development of several chronic infections. Such infections are a problem of bacterial ecology, and efficacious treatment approaches require a holistic view of the microbial community and its specific interactions. In the case of oral diseases such as periodontitis and caries, recent next-generation sequencing studies have shown that the disruption of oral biofilm homeostasis leads to dysbiosis through a decline in commensal species (28, 29). However, we lack a fundamental mechanistic understanding of the early events that ultimately favor a dysbiotic ecology in the flora. In this study, we were interested to determine how changes in environmental pH influence the competitive capabilities of the commensal streptococci *S. sanguinis* and *S. gordonii*. Their survival at pH 5.5 is particularly relevant to caries development, since exposure to a pH below this value favors tooth enamel demineralization and also is associated with a steep decline in the population of commensal streptococcal species, most of which encode SpxB (3, 6, 8).

S. sanguinis exhibited no change in the production of H_2O_2 at pH 6; in sharp contrast, the amount of H_2O_2 produced by *S. gordonii* was significantly reduced at pH 6. These phenotypes were strongly correlated with both reduced *spxB* gene expression and lower SpxB protein abundance. The different responses to decreased environmental pH suggest that, compared with *S. gordonii*, *S. sanguinis* attempts to maintain its competitiveness via production of H_2O_2 as the pH becomes acidic. However, species

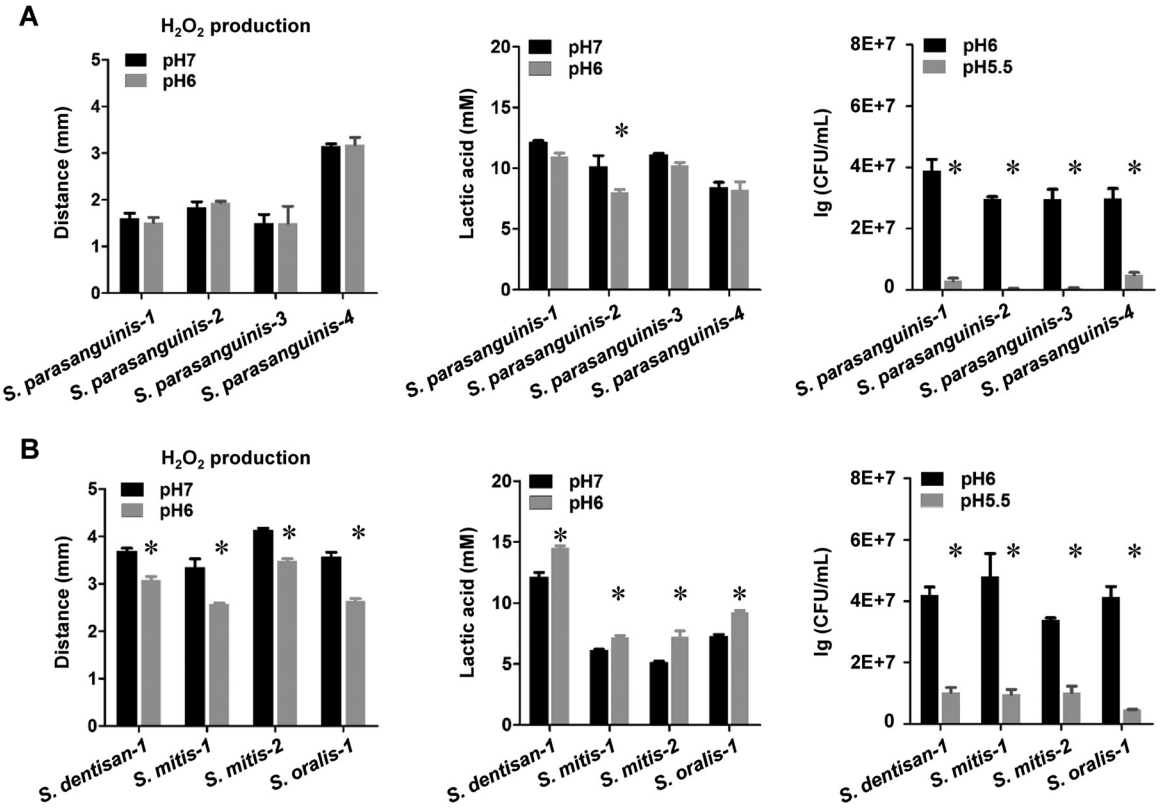

FIG 6 Effects of pH on H_2O_2 production in low-passage-number clinical isolates. (A) H_2O_2 production, lactic acid production, and acid stress survival of 4 *S. parasanguinis* isolates at pH 7 and pH 6. (B) H_2O_2 production, lactic acid production, and acid stress survival of additional *S. dentisani*, *S. mitis*, and *S. oralis* low-passage-number isolates at pH 7 and pH 6. *, significant difference ($P < 0.05$).

such as *S. gordonii* adjust their metabolic output toward lactic acid production as the environment becomes more acidic. Part of this adjustment requires reduced activity from SpxB to favor pyruvate fermentation via lactate dehydrogenase (Fig. 7).

Since the response to low pH was a transcriptional adjustment in *S. gordonii*, our initial focus centered on gene expression control by TCSs. TCSs are known to integrate environmental information to modulate cellular behavior (30). TCSs ComDE, VicKR, and CiaHR of *S. mutans* (24, 26, 27) and *Streptococcus pneumoniae* (25) have been associated with stress response regulation as a result of pH changes. Therefore, we investigated whether *comC*, *vicK*, and *ciaH* expression responded to pH changes in *S. sanguinis* and *S. gordonii* (see Fig. S3 in the supplemental material). Overall, the expression responses to pH changes were moderate but more prominent in *S. gordonii*, suggesting that the involvement of the TCSs tested here is limited.

Based on our previous findings (14, 20), H_2O_2 production in *S. gordonii* seems to be strongly influenced by environmental cues. For example, the presence of carbohydrates can elicit a carbon catabolite repression of *spxB* expression (22). Considering that, in general, *S. gordonii* produces more H_2O_2 than does *S. sanguinis*, adaptation to diverse environmental conditions could be a strategy to ensure overall fitness. Interestingly, *S. gordonii* is also lower in abundance than *S. sanguinis*, which is one of the most abundant streptococci in the oral biofilm (31). We speculate that high H_2O_2 production is important for initial competitiveness but adaptation is more important than antagonism for *S. gordonii* to counter the environmental stress. Hence, *S. gordonii* is willing to sacrifice H_2O_2 production for acid protection at low pH. Alternatively, it could be that the acidification of the environment is a result of increased population growth and therefore is indicative of an established biofilm population, where H_2O_2 is unlikely to be produced due to an ever-increasing anoxic environment. Thus, *S. gordonii* may respond accordingly by decreasing *spxB* gene expression to further bias its metabolism toward

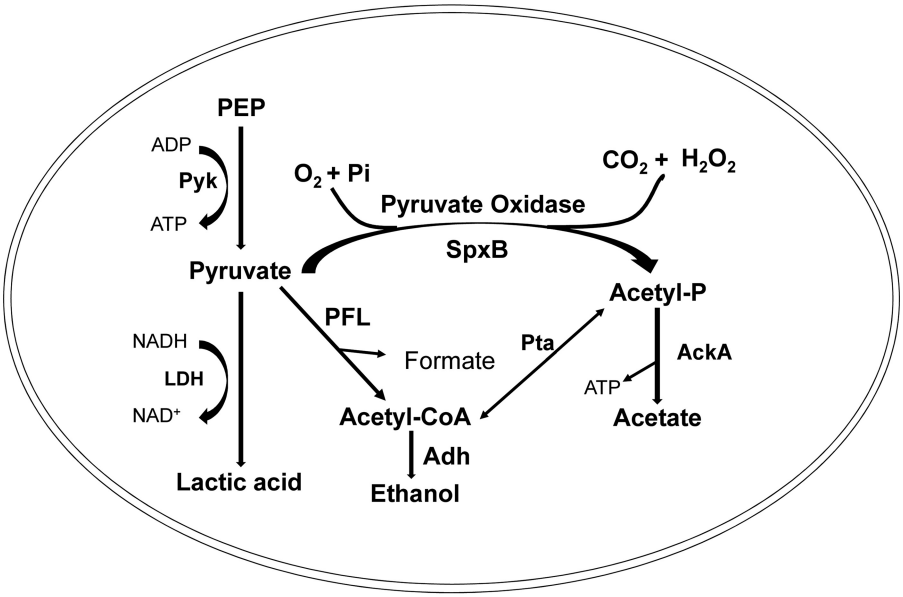

FIG 7 Schematic overview of the pyruvate node. Pyruvate is generated through the glycolytic pathway from phosphoenolpyruvate (PEP) via pyruvate kinase (Pyk). As relevant here, pyruvate can be channeled through pyruvate oxidase (SpxB), generating acetyl-phosphate (Acetyl-P), CO_2, and H_2O_2, with subsequent generation of acetate by acetate kinase (AckA), or alternatively through lactate dehydrogenase (LDH), generating lactic acid and NAD^+ from NADH, as well as through pyruvate-formate lyase (PFL), generating acetyl-CoA and formate, with subsequent generation of ethanol by alcohol dehydrogenase (Adh). Acetyl-CoA and acetyl-P can be converted into each other by phosphate acetyltransferase (Pta).

pyruvate fermentation. In either scenario, the net result is increased acid production and acid tolerance for *S. gordonii* and various other oral streptococci. For *S. sanguinis*, no change was observed at pH 6, compared with pH 7, regarding *spxB* expression, H_2O_2 production, and *vicK* and *ciaH* expression. We did observe increased expression of *pyk*, *pfl*, and *adh*, suggesting that the change in pH has an effect on the pyruvate node but potentially directs the metabolic flow toward ethanol production. This is somewhat surprising, since in general the pyruvate formate lyase is considered an oxygen-sensitive enzyme (32) and we performed our experiments aerobically. However, gene expression does not necessarily reflect protein production. In addition, the pyruvate-formate lyase of *S. sanguinis* was shown to be less oxygen sensitive than other streptococci (33). Overall, the observation that *S. sanguinis* does not change *spxB* expression in response to changes in pH is analogous to our previous finding that, unlike *S. gordonii*, *S. sanguinis* does not adjust *spxB* expression in response to changes in carbohydrate concentrations (20–22). The finding that the major carbon catabolite regulatory protein CcpA represses *spxB* expression in a carbohydrate-independent manner in *S. sanguinis* suggests that H_2O_2 production has a slightly different ecological purpose in this species. *S. sanguinis* is an abundant early colonizer and this potentially affects how *S. sanguinis* responds to environmental changes and stresses. If its overall abundance is high and the cells reside in a biofilm, then perhaps biofilm-mediated protection ensures *S. sanguinis* survival when it is temporarily exposed to lower pH. Alternatively, it is possible that *S. sanguinis* never developed a significant acid tolerance response, since high sugar consumption is, from an evolutionary perspective, a recent development (34).

Another notable finding in the current study is that the lactic acid levels in *S. gordonii* increased with reduced pH, whereas the levels in *S. sanguinis* showed no difference. Lactate dehydrogenase converts pyruvate to lactic acid with concomitant oxidation of NADH to NAD^+, which is critical for metabolic fitness (35). The amount of lactic acid production by the *S. gordonii spxB* mutant was significantly increased, compared to the wild type. A similar result was demonstrated with an *spxB* mutant of

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference
SK36	Wild-type *S. sanguinis*	45
DL1	Wild-type *S. gordonii*	46
SK36 Δ*spxB*	SK36, Δ*spxB*; Erm^r	This study
DL1 Δ*spxB*	DL1, Δ*spxB*; Erm^r	This study
SK36 *spxB*-FLAG	SK36 with *spxB*-FLAG tag (markerless); Erm^r	This study
DL1 *spxB*-FLAG	DL1 with *spxB*-FLAG tag (markerless); Erm^r	This study
133-79	Wild-type *S. sanguinis*, endocarditis isolate	47
JM1	*S. gordonii*, low-passage-number oral isolate	Unpublished data
S. parasanguinis	Five low-passage-number oral isolates	19
S. dentisani	Two low-passage-number oral isolates	19
S. mitis	Two low-passage-number oral isolates	19
S. oralis	Low-passage-number oral isolate	19
pDL278	Shuttle vector; Spc^r	48

[a]Erm^r, erythromycin resistant; Spc^r, spectinomycin resistant.

S. pneumoniae, compared to the wild type (36). At pH 6, the increase in lactic acid production from *S. gordonii* and its isogenic *spxB* mutant improves acid stress survival. This may partially explain why, in subjects with caries, *S. gordonii* levels diminish much less dramatically than those of *S. sanguinis* (6). Interestingly, we demonstrated that other SpxB-encoding oral streptococci behave like *S. sanguinis* and *S. gordonii*. In particular, our low-passage-number clinical isolates of *S. mitis*, *S. oralis*, and *S. dentisani*, like *S. gordonii*, reduce the production of H_2O_2 when exposed to pH 6 and exhibit enhanced survival at pH 5.5. Consistent with our results, *S. dentisani* was recently reported to grow better at pH 6 than at pH 6.5. The authors speculated that *S. dentisani* is able to activate the arginolytic pathway at pH 6, which aides in its acid survival (37). Overall, our results suggest that plasticity in the pyruvate node might give certain streptococci an advantage at lower pH. The mechanisms governing the physiological changes that occur due to changes in the pyruvate node await further investigation.

Finally, it should be noted that, although H_2O_2 production in *S. gordonii* was compromised at pH 6, the amount produced was still comparable to that of *S. sanguinis*. It is known that the abundance of *S. gordonii* in the oral cavity is typically much lower than that of *S. sanguinis* (38), but the two species are commonly isolated from the same intraoral sites. From an ecological perspective, it is conceivable that *S. sanguinis* and *S. gordonii* might coordinate H_2O_2 production against their competitors in the biofilm. Therefore, when the biofilm community encounters a temporal change in pH, both species might still produce constant levels of H_2O_2 that are capable of inhibiting their main competitors. This ability, however, is severely affected when the acid challenge changes from temporary fluctuations to more permanent changes in pH (for example, through frequent carbohydrate consumption), leading to a more consistently acidic environment favoring dysbiosis and cariogenesis.

MATERIALS AND METHODS

Bacterial species and culture conditions. Bacterial strains used in this study are listed in Table 1. *S. sanguinis* SK36, *S. gordonii* DL1, and their derivatives were routinely grown aerobically as static cultures (in 5% CO_2 at 37°C) in BHI broth (Difco, Sparks, MD) or on BHI agar plates. The pH of the plates was adjusted to pH 7 or pH 6 with HCl prior to autoclaving; the pH of the liquid medium was adjusted to pH 7 or pH 6 after autoclaving, and the medium was filtered with 0.45-μm filters (VWR). *Escherichia coli* cells were grown aerobically at 37°C in LB medium (Difco). For antibiotic selection, 500 μg ml^{-1} spectinomycin (Sigma-Aldrich, St. Louis, MO) or 10 μg ml^{-1} erythromycin (Fisher Scientific, Pittsburgh, PA) was used for *S. sanguinis* and *S. gordonii*, and 100 μg ml^{-1} spectinomycin was used for *E. coli*. To measure cell growth at pH 7 and pH 6, overnight cultures were diluted 1:30 into fresh medium, and then CFU were determined every 30 min for 10 h and again after 24 h of growth.

DNA manipulations. PCR was performed using a Bio-Rad thermocycler (Bio-Rad, Hercules, CA). DNA polymerases were purchased from New England BioLabs (Ipswich, MA) and used according to the manufacturer's protocol. Oligonucleotides (Table 2) were designed using *S. sanguinis* SK36 and *S. gordonii* DL1 sequence data obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/genome) and were synthesized by Integrated DNA Technologies (Coralville, IA).

Design and synthesis of in-frame deletion cassette. To create in-frame markerless deletions and insertions, an in-frame deletion cassette (IFDC) for use in *S. sanguinis* (NCBI reference sequence

TABLE 2 Primers used in this study

Primer name	Sequence (5′ to 3′)
IFDC_1_F	GCCGGATCCCGAGCCACGAT
IFDC_1_R	GATCCTTAGGCAGATTCATC
IFDC_2_F	ACTTTGAGCGGATGAATCTG
IFDC_2_R	ACCTGTTCCAATTTCGTAAAC
IFDC_3_F	ACCGATACCGTTTACGAAATTGG
IFDC_3_R	GCCAAGCTTTTATTTCCTC
spxB_up_del_S_F	TCTGAACGAAGAAGGTCAGC
spxB_up_seq_GPa_F	CTAGCAGGTCTTGCTATGG
spxB_up_del_GS_R	CCACCCGACTTTGATGAGTGAGCTGAGTGTTCC
spxB_dn_del_S_F	CTGACAGCTTCGACATCGATGCAGTTGTTGC
spxB_dn_del_G_F	CTGACAGCTTCGAACTGTTGTTATCGATGCTCG
spxB_dn_seq_R	AGGCAATGACTGCCAATCG
ermR_spxB_SG_del_F	CAGCTCACTCATCAAAGTCGGGTGGTTGTC
ermR_spxB_S_del_R	CTGCATCGATGTCGAAGCTGTCAGTAGTATACC
ermR_spxB_G_del_R	CGATAACAACAGTTCGAAGCTGTCAGTAGTATACC
Ss_spxB_Pex_U_F	CCTGATAGGTACCAATGAC
spxB_ml_del_SG_U_R	CGTTATCGTGGCTCGTTGAGCATTGCTGCAGATGC
spxB_ml_del_SG_D_F	GGAGGAAATAAGATGCAGTTGTTGCAGAAGC
spxB_IFDC_SG_F	GCAGCAATGCTCAACGAGCCACGATAACGCTCATCG
spxB_IFDC_SG_R	CTGCAACAACTGCATCTTATTTCCTCCCGTTAAATAATAGATAAC
SG_flag_spxB_R	CTTGTCATCATCGTCTTTGTAATCTTTAATTGCGCGTGATTGCAATCC
S_flag_spxB_dn_F	GATTACAAAGACGATGATGACAAGTAATTCCTCTCGCCGAAAATC
G_flag_spxB_dn_F	GATTACAAAGACGATGATGACAAGTAATTCTTCTCGTCGAAAATC
spxB_checker_R	AGCACCAAGCTCTTTCAACC
16S rRNA RT_F	AAGCAACGCGAAGAACCTTA
16S rRNA RT_R	GTCTCGCTAGAGTGCCCAAC
SK36/DL1 spxB RT_F	GGATGCTTTGGCTGAAGAC
SK36/DL1 spxB RT_R	GGACCACCTGAACCTACTG
SK36 comC RT_F	TGAAAATCTATTCTTTTCAAATTGC
SK36 comC RT_R	CCATGGATTTGGAACACCTC
DL1 comC RT_F	TCTATTGCCAAAAGAGTTACAACA
DL1 comC RT_R	GCGAACATCTCCACCAGTA
SK36 vicK RT_F	ATTATCAAGCAGCATCAG
SK36 vicK RT_R	ACACCATCATTCTCATAAG
DL1 vicK RT_F	ATCATCAAGCAGCACCAT
DL1 vicK RT_R	TTATCATACGGCAGGACAAT
SK36 ciaH RT_F	GACAATGCTATCAAGTAT
SK36 ciaH RT_R	AATCTTCTTCTTATCTTCAT
DL1 ciaH RT_F	CCACAATATCAAGACAGACA
DL1 ciaH RT_R	GTTATCGGACACTCGTAAG
SK36 ldh RT_F	GCATACTCAATCATCAACAA
SK36 ldh RT_R	ACTGACAATGGCAATACT
DL1 ldh RT_F	TGATTATGTGTTGATTGATACTA
DL1 ldh RT_R	ATTCTAATGCTGACCGAT
SK36 pyk RT_F	CTCGTTCAGAAGTATCAGA[a]
SK36 pyk RT_R	TAGTCGCCATTGTTGTTA[a]
DL1 pyk RT_F	CTCGTTCAGAAGTATCAG[a]
DL1 pyk RT_R	TTGTAGCCATTGTTGTTA[a]
SK36 ackA RT_F	AGCCATCATTCCTTACCT[a]
SK36 ackA RT_R	GCCAGATTCACGATTCAA[a]
DL1 ackA RT_F	CCTGATATTACAAGTGTGGTAGTG[a]
DL1 ackA RT_R	GGCAATGGATAGCGATAAGC[a]
SK36 pta RT_F	ACCGATATTACACCAGTTC[a]
SK36 pta RT_R	TCAACGAATGCTTCTACC[a]
DL1 pta RT_F	ATTGCTGAAGGAAGATGTC[a]
DL1 pta RT_R	GCTGCTGTTGAGTGAATA[a]
SK36 pfl RT_F	CGTTCTCTGGACTGATAA[a]
SK36 pfl RT_R	GTTACACCTTCGTATTGG[a]
DL1 pfl RT_F	GGATACATCTACGATTACGA[a]
DL1 pfl RT_R	CTTCAACCAACCATTCAG[a]
SK36 adh RT_F	CCAGAAGGAACCAATGTC[a]
SK36 adh RT_R	ACCGCAGTATTCAATGTC[a]
DL1 adh RT_F	GCTGAACAATGTATCGTAA[a]
DL1 adh RT_R	GCACAAGTGATAGAAGAAG[a]

[a]Chromosomal locus tags can be found in Table S1 in the supplemental material.

NC_009009.1) and *S. gordonii* (NCBI reference sequence NC_009785.1) was created on the basis of an IFDC created previously for *S. mutans* (39). The IFDC was designed with the 278-bp *S. sanguinis ldh* promoter region. Downstream of this region, the *pheS* gene of *S. sanguinis* was engineered with an A316G point mutation (*pheS**); *pheS** was controlled by its native ribosome binding site. Silent mutations were created immediately following the point mutation within *pheS**, to prevent homologous recombination with the chromosomal *pheS*. An erythromycin resistance cassette (*ermAM*) was placed directly following *pheS**. The IFDC was flanked by BamHI and HindIII restriction sites at the 5′ and 3′ ends, respectively, to enable ligation and cloning for storage in *E. coli* DH10b cells (Lucigen). The IFDC was synthesized by Integrated DNA Technologies in three gBLocks and pieced together via PCR with the IFDC primers listed in Table 2.

Deletion of *spxB* in *S. gordonii* DL1 and *S. sanguinis* SK36. The deletion of *spxB* in *S. gordonii* DL1 and *S. sanguinis* SK36 was performed via allelic replacement. An upstream fragment and a downstream fragment of *spxB* were amplified by PCR using Accuprime Pfx DNA polymerase (Invitrogen). Subsequently, the upstream and downstream fragments were combined in an overlapping PCR with an *erm* resistance cassette, and the resulting amplicons were transformed into DL1 and SK36, generating DL1 Δ*spxB* and SK36 Δ*spxB*, respectively. In detail, the primer pairs spxB_up_del_S_F and spxB_up_del_GS_R (SK36) and spxB_up_seq_GPa_F and spxB_up_del_GS_R (DL1) were used for the upstream fragments. The downstream fragments were amplified using the primer pairs spxB_dn_del_S_F and spxB_dn_seq_R (SK36) and spxB_dn_del_G_F and spxB_dn_seq_R (DL1). The *erm* resistance cassette was amplified by using the primer pair ermR_spxB_SG_del_F and ermR_spxB_S_del_R (deletion of *spxB* in SK36) or the primer pair ermR_spxB_SG_del_F and ermR_spxB_G_del_R (deletion of *spxB* in DL1). For overlap PCR, 0.5 μl of the unpurified PCR products was added directly to 22.5 μl of Accuprime Pfx DNA polymerase reaction mixture. After 10 initial cycles with 80 s of elongation, the primer pairs spxB_up_del_S_F and spxB_dn_seq_R (SK36) and spxB_up_del_GPa_F and spxB_dn_seq_R (DL1) were added, followed by 30 additional amplification cycles (150 s of elongation). The resulting amplicon was transformed into wild-type SK36 and DL1 strains induced for competence with their respective competence-stimulating peptides (CSPs). The correct deletion of *spxB* was verified by PCR and sequencing. Prussian blue agar plates (23) were used to detect H_2O_2 production in in *S. gordonii* Δ*spxB* mutant and the *S. sanguinis* Δ*spxB* mutant. The representative images demonstrated that the *spxB* mutants of both strains did not produce H_2O_2 (see Fig. S2A in the supplemental material).

Construction of *S. gordonii* DL1 and *S. sanguinis* SK36 with *spxB*-FLAG tags. To measure the abundance of SpxB protein, a FLAG tag epitope (DYKDDDDK) was attached to the SpxB C terminus. In the first step, *spxB* was replaced with an IFDC cassette using allelic replacement. This cassette contained *ermAM* for positive selection and a site-directed mutant of *pheS* (SK36) for negative selection (39). In the second step, the IFDC cassette was replaced with *spxB*-FLAG. To introduce the IFDC cassette into SK36 and DL1, an upstream fragment was amplified using Accuprime Pfx DNA polymerase (Invitrogen) and the following primer pairs: Ss_spxB_Pex_U_F and spxB_ml_del_SG_U_R (SK36) and spxB_up_seq_GPa_F and spxB_ml_del_SG_U_R (DL1). The respective downstream fragment was amplified with spxB_ml_del_SG_D_F and spxB_dn_seq_R. The IFDC cassette was amplified with spxB_IFDC_SG_F and spxB_IFDC_SG_R. Overlap PCR was performed as described previously, utilizing the primer pairs Ss_spxB_Pex_U_F and spxB_dn_seq_R (SK36) and spxB_up_seq_GPa_F and spxB_dn_seq_R (DL1). After transformation, IFDC-positive clones (SK36 Δ*spxB*-IFDC and DL1 Δ*spxB*-IFDC) were selected with erythromycin. The correct integration of IFDC was verified by PCR and sequencing. For IFDC replacement, *spxB* was amplified with the primer pairs spxB_up_del_S_F and SG_flag_spxB_R (SK36) and spxB_up_seq_GPa_F and SG_flag_spxB_R (DL1). The downstream fragment was amplified with the primer pairs S_flag_spxB_dn_F and spxB_checker_R (SK36) and G_flag_spxB_dn_F and spxB_checker_R (DL1). Overlap PCR was performed using the primer pairs spxB_up_del_S_F and spxB_checker_R (SK36) and spxB_up_seq_GPa_F and spxB_checker_R (DL1). The resulting amplicons were transformed into SK36 Δ*spxB*-IFDC and DL1 Δ*spxB*-IFDC generating SK36 *spxB*-FLAG and DL1 *spxB*-FLAG, respectively. Transformants were selected with DL-4-chlorophenylalanine (39) and verified by PCR and sequencing.

Measurement of H_2O_2 production. For the quantification of H_2O_2 production at different pH values, overnight cultures of *S. sanguinis* and *S. gordonii* were diluted 1:30 in fresh BHI medium and grown to an A_{600} of ~0.5. Cells were collected by centrifugation and resuspended in an equal volume of fresh BHI broth to remove residual H_2O_2 that might have accumulated in the supernatant. Fifteen microliters of cells was then spotted onto Prussian blue agar plates (adjusted to pH 7 or pH 6 as described above) essentially as described previously (23). After 16 h of aerobic growth at 37°C, H_2O_2 production was quantified (in millimeters) by measuring the blue halos on the indicator plates with a digital caliper (Digimatic Caliper, Mitutoyo, Japan). The means ± standard deviations (SDs) of three replicates are presented. The same method was used to determine H_2O_2 production of low-passage-number oral clinical isolates collected in a previous study (19). The concentrations of H_2O_2 in liquid cultures were determined using a modification of the protocol described by Gilliland (40). Bacteria grown to mid-logarithmic phase were centrifuged, and 40 μl of cell-free supernatant was mixed with 160 μl of freshly prepared 0.1 M sodium acetate (pH 4.5) containing 0.1 μg horseradish peroxidase (Thermo Scientific) and 10 μl of 1 mg/ml *o*-dianisidine (Alfa Aesar) in methanol. The reaction mixture was protected from light and incubated at room temperature for 10 min. The absorbance at 570 nm was determined using a microplate reader (model 680; Bio-Rad). A standard curve was determined using serial dilutions of a commercial 30% H_2O_2 solution in Milli-Q water; the detectable range was 0.1 to 4.0 mM H_2O_2. To determine cell numbers for normalization, cells were serially diluted and 100-μl aliquots were spotted onto BHI agar plates. CFU were counted after 24 h of incubation at 37°C. The H_2O_2 concentrations were

calculated according to the standard curves and were normalized to the CFU counts. All experiments were performed in triplicate.

Western blotting. Western blotting was performed to investigate the protein abundance of SpxB-FLAG in *S. sanguinis* and *S. gordonii* (21). SK36 SpxB-FLAG and DL1 SpxB-FLAG strains were grown in BHI medium as planktonic cultures until mid-logarithmic phase (A_{600} values of ~0.5), collected by centrifugation at 11,000 × *g* for 15 min at 4°C, and resuspended in 1 ml of phosphate-buffered saline (PBS). Cytoplasmic extracts were generated by mechanical disruption using a Precellys homogenizer (Bertin Corp.), with 0.1-mm silica beads (Thermo Fisher Scientific). Cell debris was separated by centrifugation at 11,000 × *g* for 15 min at 4°C. Aliquots of the supernatants were adjusted to approximately the same protein concentration using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific), and appropriate volumes were separated by 10% SDS-PAGE. For Western immunoblotting, the proteins were transferred to nitrocellulose membranes (Thermo Fisher Scientific), and the membranes were blocked for 1 h with a solution of 5% skim milk dissolved in Tris-buffered saline containing 0.1% Tween 20 (TBST). The membranes were incubated overnight at 4°C with primary antibody solution (anti-FLAG M2 antibody; Stratagene), followed by three washes with TBST, and then were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (Thermo Fisher Scientific). After being washed with TBST, the blots were developed using the ECL chemiluminescence detection system (Thermo Fisher Scientific).

Lactic acid measurement after pH challenge. Lactic acid levels were measured essentially as described previously (41). Lactic acid assays were performed following a specific incubation protocol, as outlined in Fig. S4. Briefly, 10 ml of mid-log-phase cells of *S. sanguinis* SK36 and *S. gordonii* DL1 were harvested by centrifugation at 4,000 × *g* for 15 min at 4°C, suspended in fresh BHI broth (pH 7), and incubated at 37°C for 2 h (Fig. S4, 1, pH 7). The bacteria were adjusted to the same cell density (A_{600} values of ~0.5), and 10 ml of each culture was used for centrifugation. Aliquots were collected for lactic acid measurements, and the cells were resuspended in fresh BHI broth (pH 6) to a final A_{600} value of ~0.5. After 2 h of cultivation at 37°C (Fig. S4, 2, pH 6), the cultures were adjusted to A_{600} values of ~0.5, and 10 ml of each culture was centrifuged. Aliquots were collected, the cells were transferred to fresh BHI broth (pH 7) (A_{600} values of ~0.5) and incubated at 37°C for 2 h, the cultures were adjusted again to A_{600} values of ~0.5, and 10 ml was centrifuged. The supernatants were collected for lactic acid measurements after centrifugation (Fig. S4, 3, pH 7*). The cells from each time point were collected and stored at −80°C for RNA extraction, to determine *ldh* gene expression. All supernatants were filter-sterilized through a 0.22-μm-pore-size filter (EMD Millipore, Billerica, MA). Lactic acid concentrations were determined using an enzymatic (lactic dehydrogenase) method (41). Standard curves were prepared fresh each time, using a standard lactic acid solution (Supelco Analytical, Bellefonte, PA). All samples and standards were prepared in triplicate.

Additionally, overnight cultures of *S. sanguinis* SK36 and *S. gordonii* DL1 were inoculated in fresh BHI broth. Mid-log-phase cells were harvested by centrifugation at 4,000 × *g* for 15 min at 4°C, suspended in fresh BHI broth (pH 7 or pH 6), and incubated at 37°C for 2 h or 4 h. The CFU of each culture were determined by serial dilution; the cultures were subsequently centrifuged, and aliquots were collected for lactic acid measurements. CFU were counted after 24 h of incubation at 37°C. All supernatants were filter-sterilized and used for determination of lactic acid concentrations. The lactic acid concentrations were calculated according to the standard curves and normalized by CFU. All experiments were performed in triplicate.

RNA isolation, cDNA synthesis, and quantitative PCR. Overnight cultures of *S. sanguinis* SK36 and *S. gordonii* DL1 were inoculated into fresh BHI medium. Cells at mid-log phase were divided into two groups. For condition 1, cells were collected by centrifugation and resuspended in 20 ml fresh BHI broth adjusted to pH 7 or pH 6. After an additional 2 h of aerobic incubation, cells were collected by centrifugation (Fig. S4, a). For condition 2, 100 μl of log-phase cells was inoculated onto BHI agar plates (6 plates/group, pH 7 or pH 6). All plates were further incubated for 2 h at 37°C, and cells were collected by addition of 5 ml BHI broth to the plates and scraping with sterile cotton swabs, followed by collection of the liquid into 50-ml conical tubes and centrifugation at 11,000 × *g* for 15 min at 4°C (Fig. S4, b). Cell pellets were stored at −80°C until further use. Total RNA was extracted by following the TRIzol method, according to the manufacturer's protocol. Isolated RNA was treated with DNase I (Invitrogen) to remove traces of chromosomal DNA. RNA samples were cleaned with the Qiagen RNeasy kit. The concentrations and integrity of RNA samples were confirmed using NanoDrop spectrophotometer measurements and gel electrophoresis. cDNA was synthesized from 2 μg of RNA using SuperScript II reverse transcriptase (Invitrogen). Real-time PCR was performed using SYBR green master mix (Bio-Rad), according to the manufacturer's instructions. Threshold cycle (C_T) values were determined, and data were analyzed with Bio-Rad CFX MANAGER software (version 2.0), using the $2^{-\Delta\Delta CT}$ method (42). The 16S rRNA gene was used as the housekeeping reference control.

Acid survival assay. Acid survival of *S. sanguinis* SK36 and *S. gordonii* DL1 and their Δ*spxB* mutants at pH 6 or pH 5.5 was determined by modification of a previously published method (43, 44). Briefly, mid-log-phase cells were inoculated into fresh BHI broth (pH 7) for 24 h. Subsequently, cells were collected by centrifugation and resuspended in fresh BHI broth (pH 6). After 24 h of incubation at 37°C, cells were washed twice with PBS (pH 6), adjusted to the same A_{600} values, and divided into two groups. For condition 1, cells were serially diluted and 15 μl was spotted onto BHI agar plates adjusted to pH 6 or pH 5.5. After 24 h of incubation at 37°C, plates were photographed for documentation. For condition 2, cells were resuspended in 0.1 M glycine buffer (pH 6 or pH 5.5). CFU of cells incubated for 0, 0.5, 1, or 2 h were determined. Survival rates were defined as the ratio of CFU per milliliter at pH 5.5 to CFU per milliliter at pH 6.

Statistical analysis. All data were obtained in triplicate at separate times and statistically analyzed with SPSS (version 18.0 for Windows; SPSS Inc., Chicago, IL). Student's *t* test was used to compare the data for two groups, followed by the Student-Newman-Keuls test. Data were considered significantly different if the two-tailed *P* value was <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01697-17.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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We declare no conflicts of interest.

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