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Streptococcal Antagonism in Oral Biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* Interference with *Streptococcus mutans*▽

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Biofilms are polymicrobial, with diverse bacterial species competing for limited space and nutrients. Under healthy conditions, the different species in biofilms maintain an ecological balance. This balance can be disturbed by environmental factors and interspecies interactions. These perturbations can enable dominant growth of certain species, leading to disease. To model clinically relevant interspecies antagonism, we studied three well-characterized and closely related oral species, *Streptococcus gordonii*, *Streptococcus sanguinis*, and cariogenic *Streptococcus mutans*. *S. sanguinis* and *S. gordonii* used oxygen availability and the differential production of hydrogen peroxide (H_2O_2) to compete effectively against *S. mutans*. Interspecies antagonism was influenced by glucose with reduced production of H_2O_2. Furthermore, aerobic conditions stimulated the competence system and the expression of the bacteriocin mutacin IV of *S. mutans*, as well as the H_2O_2-dependent release of heterologous DNA from mixed cultures of *S. sanguinis* and *S. gordonii*. These data provide new insights into ecological factors that determine the outcome of competition between pioneer colonizing oral streptococci and the survival mechanisms of *S. mutans* in the oral biofilm.

Bacterial biofilms are complex microbial communities found on virtually all mucosal surfaces in humans and animals (8). Bacterial communities indigenous to humans consist of dozens to hundreds of different species or phylotypes, as has been shown in gut, vaginal, and oral biofilms (11, 12, 46). Changes in biofilm ecology can lead to diseases such as inflammatory bowel disease (28), vaginitis (12), and caries (46). Interactions between the bacterial species that reside in the biofilm influence the composition of the communities (21, 45), a process termed bacterial interference (16). As a consequence, changes in the populating species influence whether the biofilm favors either a benign or otherwise beneficial homeostatic state or the development of disease. In the beneficial state, the bacterial community restricts potentially harmful bacteria from colonizing, protecting the host from disease. However, some disease-causing microorganisms are present under healthy conditions, and shifts in the population may affect the virulence of a species; e.g., virulence genes that require diffusible intraspecies signal molecules for expression might only be transcribed when a certain bacterial density is archived. For example, caries-causing *Streptococcus mutans* can become a predominant member of the community under appropriate conditions, leading to dental caries formation (24). How changes in the species composition of dental biofilms occur is not well understood.

The polymicrobial community dental plaque is one of the best-studied biofilms (for a recent review, see reference 22). In the oral biofilm, more than 500 species or phylotypes have been identified (20, 36). The cell density can reach 10^{11} CFU ml^{-1} (11). The biofilm is challenged by frequent changes in environmental conditions, e.g., food intake, temperature, pH change, and salivary flow. Perhaps as a response to environmental challenges, the oral biofilm community evolved with individual members assuming specialized functions, e.g., primary and secondary colonizers (reviewed in reference 15), including members that can metabolize excreted products (such as lactic acid) produced by other species (35).

Approximately 20% of the oral bacteria are streptococci (27, 47). The oral streptococci pioneer early dental plaque formation and have a specific temporal and spatial distribution that is crucial for the development of oral biofilms (44). Reflecting their genetic similarity, the oral streptococci have similar metabolic activities, which include the ability to metabolize a wide variety of carbohydrates (50). The streptococci compete for adhesion-binding sites on the saliva-coated tooth surface (31) and are able to produce antimicrobial compounds, such as bacteriocins and hydrogen peroxide (H_2O_2) (18). When present in the oral biofilm in high numbers, the pioneer colonizers *Streptococcus gordonii* and *Streptococcus sanguinis* can antagonize *S. mutans*, as suggested by clinical studies (5) and sequential inoculation experiments in germfree rats (5, 29).

S. mutans can, however, become dominant in oral biofilms, leading to dental caries development. *S. mutans* dominance depends on competition with *S. sanguinis* and is influenced by the production of antimicrobial compounds (18). For example, *S. mutans* shows cell density-dependent expression of bacteriocins mutacin I and mutacin IV when inoculated before *S. sanguinis* in an in vitro plate assay. Conversely, *S. sanguinis* produces H_2O_2 when inoculated before *S. mutans* (19). These compounds are able to inhibit the competing species to keep their numbers low.

In the present study, we tested the hypothesis that *S. sanguinis* and *S. gordonii* grown under aerobic conditions are better able to inhibit *S. mutans* than when grown under anaerobic conditions. Our results show that the interspecies interactions

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TABLE 1. Primers used in this study

Primer	Function	Nucleotide sequence (5′ to 3′)
poxCF	*pox* complementation	CGGGTACCTAGTTTCTTCGAAAGAACACAGC
poxCR	*pox* complementation	ACGCGTCGACTTATTTAATTGCGCGTGATTGC
poxKOF	*pox* inactivation	GGATCCCATGGGGCGTAGATACCATC
poxKOR	*pox* inactivation	AAGCTTACCACCAAATCCAGCGTAGA
nlmA RT F	Real-time PCR *nlmA*	TATCGGGTGGAGAAGCAGTC
nlmA RT R	Real-time PCR *nlmA*	TCCCCAAGTGCCTACACAAT
mutA RT F	Real-time PCR *mutA*	CTATTGTGGCAAGCAACGAC
mutA RT R	Real-time PCR *mutA*	AAACTAGGATTTTTCACCCCTGT
comE RT F	Real-time PCR *comE*	AGCCCATAAGCTCTGCCTTT
comE RT R	Real-time PCR *comE*	AGCGATGGCACTGAAAAAGT
comC RT F	Real-time PCR *comC*	GAGATTATATGGGCGGAAGC
comC RT R	Real-time PCR *comC*	TTTCCCAAAGCTTGTGTAAAA
16S RT F S.m	Real-time PCR 16S rRNA	GATAATTGATTGAAAGATGCAAGC
16S RT R S.m	Real-time PCR 16S rRNA	ATTCCCTACTGCTGCCTCCC
16S strep F	DNA release quantification	AAGCAACGCGAAGAACCTTA
16S strep R	DNA release quantification	GTCTCGCTAGAGTGCCCAAC

were influenced by the availability of oxygen and the differential production of H_2O_2 by *S. sanguinis* and *S. gordonii*. Furthermore, in the presence of oxygen, *S. sanguinis* and *S. gordonii* released more DNA into the growth medium. Stimulation of the competence system of *S. mutans* leads to the production of higher levels of antistreptococcal bacteriocins. Hence, oxygen availability appears to be a crucial factor in the interspecies competition between *S. mutans* and *S. sanguinis* and *S. gordonii* in the oral biofilm.

MATERIALS AND METHODS

Bacterial strains and media. *S. mutans* UA140 (40), *S. sanguinis* SK36 (54), and *S. gordonii* DL1 (34) and their derivatives were routinely grown at 37°C anaerobically (90% N_2, 5% CO_2, 5% H_2) or aerobically (5% CO_2) in brain heart infusion broth (BHI; Difco, Sparks, MD) or on BHI agar plates. Filter-sterilized glucose was supplemented when indicated from a freshly prepared stock solution (20%). *Escherichia coli* DH5α cells were grown aerobically at 37°C in Luria-Bertani medium (Difco). When required, antibiotics were supplemented at the following concentrations: spectinomycin at 500 μg ml^{-1} for *S. sanguinis* and *S. gordonii* and at 100 μg ml^{-1} for *E. coli*, kanamycin at 300 μg ml^{-1} for *S. sanguinis* and *S. gordonii* and at 25 μg ml^{-1} for *E. coli*, and ampicillin 100 μg ml^{-1} for *E. coli*.

DNA manipulations. Standard recombinant DNA manipulations were used. Restriction enzymes and DNA ligase were obtained from New England Biolabs (Beverly, MA) and used as specified by the manufacturer. PCR products were cloned into the pGEM-T kit from Promega (Madison, WI). All plasmids were extracted and purified from *E. coli* with a Qiagen Miniprep kit (Valencia, CA). DNA extracted from agarose gels (1%) was purified with a Qiagen QIAquick gel extraction kit. PCR was performed with a Mastercycler thermocycler (Eppendorf, Westbury, NY) according to the manufacturer's protocol. GoTaq-DNA polymerase was obtained from Promega, and Phusion high-fidelity DNA polymerase was obtained from New England Biolabs. Primer sequences (Table 1) were designed using sequence data obtained from the Los Alamos National Laboratory Oral Pathogens Sequence Database (http://www.oralgen.lanl.gov) and synthesized by Integrated DNA Technologies (Coralville, IA).

Construction of Pox$^-$ mutants and complemented strains. For the insertional inactivation of the *spxB* gene, which encodes pyruvate oxidase (Pox), an internal DNA fragment of the *spxB* gene from *S. sanguinis* was amplified by PCR using the primers poxKOF and poxKOR (Table 1), which incorporated restriction sites for BamHI and HindIII. The PCR fragments were cloned into the vector pGEM-T. After digestion with BamHI/HindIII, the fragment was ligated into compatible sites on the suicide vector pFW5, which contains a spectinomycin resistance marker (gene *aad9*) (39), to generate pFW5-pox. The resulting constructs were confirmed by restriction analysis before integration into the chromosome of *S. sanguinis* and *S. gordonii* via single-crossover homologous recombination using a transformation protocol described previously (52). Antibiotic-resistant isolates were tested by PCR to confirm the genotypes. Representative clones were named JKH2 (SK36 Pox$^-$) and JKH1 (DL1 Pox$^-$) and were frozen for storage. To complement the Pox$^-$ mutants, the *spxB* gene, including the promoter, was PCR amplified from *S. sanguinis* chromosomal DNA by using Phusion high-fidelity DNA polymerase and the primers poxCF and poxCR. These primers incorporated restriction sites for KpnI and SalI. The PCR fragment was sequentially digested with KpnI and SalI and ligated into the streptococcal shuttle plasmid pDL276 (9). The resulting plasmid, pDL276-pox, was transformed into JKH2 and JKH1. Transformants were then tested for the ability to inhibit growth of *S. mutans*.

RNA isolation, cDNA synthesis, and real-time PCR. *S. mutans* was inoculated on BHI plates with a sterile cotton tip. The plates were incubated anaerobically or aerobically, and the cells were subsequently scraped from the plate after 7 h of growth. RNA was isolated as described previously (31), except that the DNase I (Promega) treatment was extended to 5 h. RNA concentration was determined spectrophotometrically. RNA (10 μg) was reverse transcribed into cDNA with random hexamer primers (Promega) as described previously (55) using M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. The expression of the mutacin I and IV genes, *mutA* and *nlmA*, and the *com* genes, *comC* and *comE*, was measured. Real-time PCR was performed using the FullVelocity Sybr Green QPCR master mix (Stratagene, Cedar Creek, TX) according to the manufacturer's instructions. Quantification was performed by using an Mx3000P real-time PCR system (Stratagene) for amplification and fluorescent detection. Total cDNA abundance between test samples was normalized by using the 16S rRNA gene as a housekeeping gene control.

H_2O_2 concentration. The concentration of H_2O_2 was determined according to a protocol described previously (33). Briefly, bacteria were grown in glucose-free tryptic soy broth (TSB; Difco) supplemented with 1% glucose when indicated. Samples (1 ml) were taken at the times indicated, centrifuged (16,000 × *g*) for 5 min, and transferred (0.2 ml) to a new incubation tube. A reaction solution (0.8 ml of 10 mM phosphate buffer [pH 7.4] with 0.16 mM *o*-dianisidine [ICN, Aurora, OH], 1.2 μg of horseradish peroxidase (Pierce, Rockford, IL) ml^{-1}, 0.02% Triton X-100) was added, and the reaction mixture was incubated at 37°C for 20 min. The absorbance at 570 nm was determined, and the concentration was calculated from a standard curve prepared for each experiment from a 30% H_2O_2 stock solution (Sigma, St. Louis, MO).

Preparation of *S. sanguinis* and *S. gordonii* conditioned media for *S. mutans* growth curves. *S. sanguinis* or *S. gordonii* were grown overnight in BHI media (containing 0.2% glucose in original formula) and inoculated into 40 ml of BHI in tubes to an A_{600} of 0.025. When indicated, 30 μg of catalase (Sigma) was added to the culture. The cells were then incubated aerobically on a rocking platform at 37°C until the A_{600} reached 0.6 (ca. 10^7 bacteria ml^{-1}). The cultures were harvested by centrifugation at 1,700 × *g*, and 20 ml of the supernatant (conditioned medium) was removed and filter sterilized. The sterile conditioned medium was supplemented with 0.25% glucose, *S. mutans* was inoculated to an A_{600} of 0.025, and the growth of static cultures was monitored by measuring the absorbance at the indicated time points.

DNA isolation from culture supernatants. To determine the amount of DNA released during growth in coculture, *S. sanguinis*, *S. gordonii*, and the isogenic Pox$^-$ mutants were separately grown overnight in BHI (with 500 μg of spectinomycin ml^{-1} for the mutants). Next, separate tubes for *S. sanguinis* wild-type plus *S. gordonii* wild-type and for JKH1 plus JKH2 were inoculated in 5 ml of

BHI medium (no antibiotic added) as a 1-to-1 mixed culture. The cells were incubated overnight anaerobically as static cultures or aerobically on a rocking platform. At harvest, the A_{600} and CFU were determined by serial dilution and plating. Cells were removed by centrifugation for 2 min at 16,000 × *g*, 1 ml of the supernatant was transferred to a new tube, and 0.5 ml of phenol was added and vortex mixed for 30 s. To precipitate DNA, the mixture was centrifuged for 5 min at 16,000 × *g*, and 0.8 ml of the aqueous solution was removed and mixed with 80 μl of sodium acetate (3 M, pH 5.2) and 500 μl of 100% 2-propanol. The solution was then centrifuged for 10 min at 16,000 × *g*, the liquid was decanted, and the precipitated sample was air dried and suspended in 25 μl of deionized H_2O. Using universal primers for 16S rRNA genes of *S. sanguinis* and *S. gordonii* (16S strep F and 16S strep R), the relative quantity of DNA was estimated to a standard curve of dilutions (1:1, 1:5, 1:25, 1:125, and 1:625) of chromosomal DNA of *S. sanguinis*. DNA from the standard dilutions and samples from *S. sanguinis* and *S. gordonii* were amplified by real-time PCR, and the relative quantity of DNA in the streptococcal samples was calculated in comparison to the cycle threshold (C_T) values of the DNA dilutions as described previously (17). Real-time PCR was performed by using the FullVelocity Sybr Green QPCR Master Mix (Stratagene) according to the manufacturer's instructions. The experiment was repeated three times on different days.

Competition assays on solid medium and in liquid medium. To assess competitive growth, a protocol described previously was used with modifications (18). Briefly, 8 μl of an overnight culture of each species in BHI medium was inoculated onto a BHI agar plate (supplemented with 1% glucose when indicated) as the pioneer colonizer. After incubation overnight (16 h) under the specified conditions, 8 μl of the competing species was inoculated next to the pioneer colonizer such that the colonies almost touched each other. The plate was incubated overnight. Growth inhibition was assessed by the presence of a proximal zone of inhibition at the intersection with the pioneer colony. For competition assays in liquid media, cells were grown in BHI medium overnight, and either *S. sanguinis* or *S. gordonii* (15 μl of each) and *S. mutans* (15 μl) was inoculated simultaneously into 1 ml of fresh BHI (supplemented with 1% glucose when indicated) in a Costar 24-well cell culture cluster (Corning, Inc., Corning, NY) for biofilm growth. The cells were incubated overnight in static culture in the presence of 5% CO_2. Cells were dispersed by vigorous pipetting, serially diluted (10^{-5} to 10^{-7}), and plated on BHI agar plates in duplicates, and the CFU counts were determined.

Statistical analysis. Statistical analysis of data was performed with the QuickCalcs online calculators (http://www.graphpad.com/quickcalcs/index.cfm) using the *t* test software to compare the means of two groups. The data were considered significantly different if the two-tailed *P* value was ≤0.05.

RESULTS

Influence of oxygen availability on interspecies competition. To test the hypothesis that competitiveness increases under aerobic conditions which favor H_2O_2 production, we analyzed the inhibitory potential of oral streptococci when grown with (aerobic: 5% CO_2) or without (anaerobic: 5% CO_2, 5% H_2, 90% N_2) oxygen. Under aerobic growth conditions, the first inoculated competing species inhibited the growth of the opposing strain (Fig. 1). During anaerobic growth, however, *S. gordonii* and *S. sanguinis* no longer inhibited *S. mutans* (Fig. 1A). *S. mutans* inhibited *S. gordonii* and *S. sanguinis* during anaerobic growth (Fig. 1B).

Pox-mediated inhibition of *S. mutans* under aerobic growth conditions. During aerobic growth, the Pox (pyruvate oxidase) converts pyruvate into acetyl phosphate, carbon dioxide, and H_2O_2. The Pox-expressing wild-type *S. sanguinis* (Fig. 2A;a) and *S. gordonii* (Fig. 2A;d) inhibited *S. mutans*. *S. mutans* was not inhibited by the tested Pox$^-$ mutants of *S. sanguinis* (Fig.2Ab) and *S. gordonii* (Fig.2Ae). Complementation of the *spxB* mutation restored the growth-inhibiting ability of both Pox$^-$ mutants (Fig. 2Ac and f), indicating that under aerobic conditions competitive growth inhibition caused by H_2O_2 production is Pox dependent.

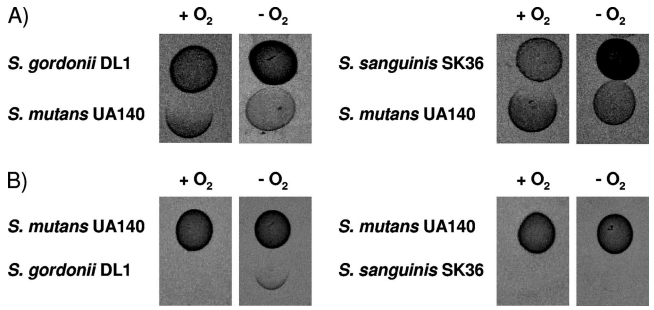

FIG. 1. Inhibition ability of *S. sanguinis*, *S. gordonii*, and *S. mutans* when grown with or without oxygen. (A) *S. gordonii* or *S. sanguinis* was inoculated first and grown for 16 h at 37°C with ($+O_2$) or without oxygen ($-O_2$), *S. mutans* was then inoculated next to the pioneer colonizer, and the plates incubated overnight. (B) *S. mutans* was inoculated first and grown for 16 h at 37°C with ($+O_2$) or without oxygen ($-O_2$), and then *S. gordonii* or *S. sanguinis* was inoculated next to the pioneer colonizer and the plates were incubated overnight.

Growth inhibition of *S. mutans* during planktonic growth in conditioned medium. To confirm that the inhibition of *S. mutans* was caused by H_2O_2, *S. mutans* was grown in filter-sterilized conditioned medium from aerobically grown wild-type cells in the presence or absence of catalase or from the Pox$^-$ mutants of *S. sanguinis* and *S. gordonii*. The conditioned medium from wild-type cells supported slow growth, with approximately two doublings of *S. mutans* in 5.5 h (Fig. 2B). In contrast, *S. mutans* grown in conditioned medium from the Pox$^-$ mutants or from wild-type cells grown in the presence of catalase showed growth after a lag phase, with approximately 4.5 doublings in 5.5 h.

Fitness of wild-type streptococci and Pox$^-$ mutants in dual-species biofilms. Growth exclusion of the tested streptococci was most evident when the growth of a pioneer colonizer preceded a potentially competing species. To test whether H_2O_2 production gives *S. sanguinis* and *S. gordonii* a competitive advantage during growth, we determined the simultaneous growth of each species in dual-species biofilms with *S. mutans* (Fig. 2C). The wild-type species and Pox$^-$ mutants grew with similar generation times (data not shown), with the Pox$^-$ mutants yielding slightly higher final cell densities. When inoculated simultaneously at a 1:1 ratio with *S. mutans*, the Pox$^-$ mutants of *S. sanguinis* and *S. gordonii* showed less growth than their wild-type counterparts. For *S. sanguinis*, this difference was significant ($P \leq 0.05$).

Influence of glucose on the competitiveness of *S. sanguinis* and *S. gordonii*. To learn whether glucose might repress Pox as described for *Lactobacillus plantarum* (25) and influence the fitness of *S. gordonii* and *S. sanguinis* in competition with *S. mutans*, BHI was supplemented with 1% glucose. In the presence of 1% glucose, *S. gordonii* was unable to inhibit growth of *S. mutans*; *S. sanguinis* activity against *S. mutans* was slightly reduced (Fig. 3A). Conversely, the addition of glucose to *S. mutans* did not influence the ability to inhibit *S. sanguinis* and *S. gordonii* (data not shown). To confirm these results, conditioned media from *S. sanguinis* and *S. gordonii* cultures grown with or without 1% glucose were compared for inhibiting activity against *S. mutans*. In the presence of glucose, *S. gordonii* conditioned medium inhibited *S. mutans* growth less than in

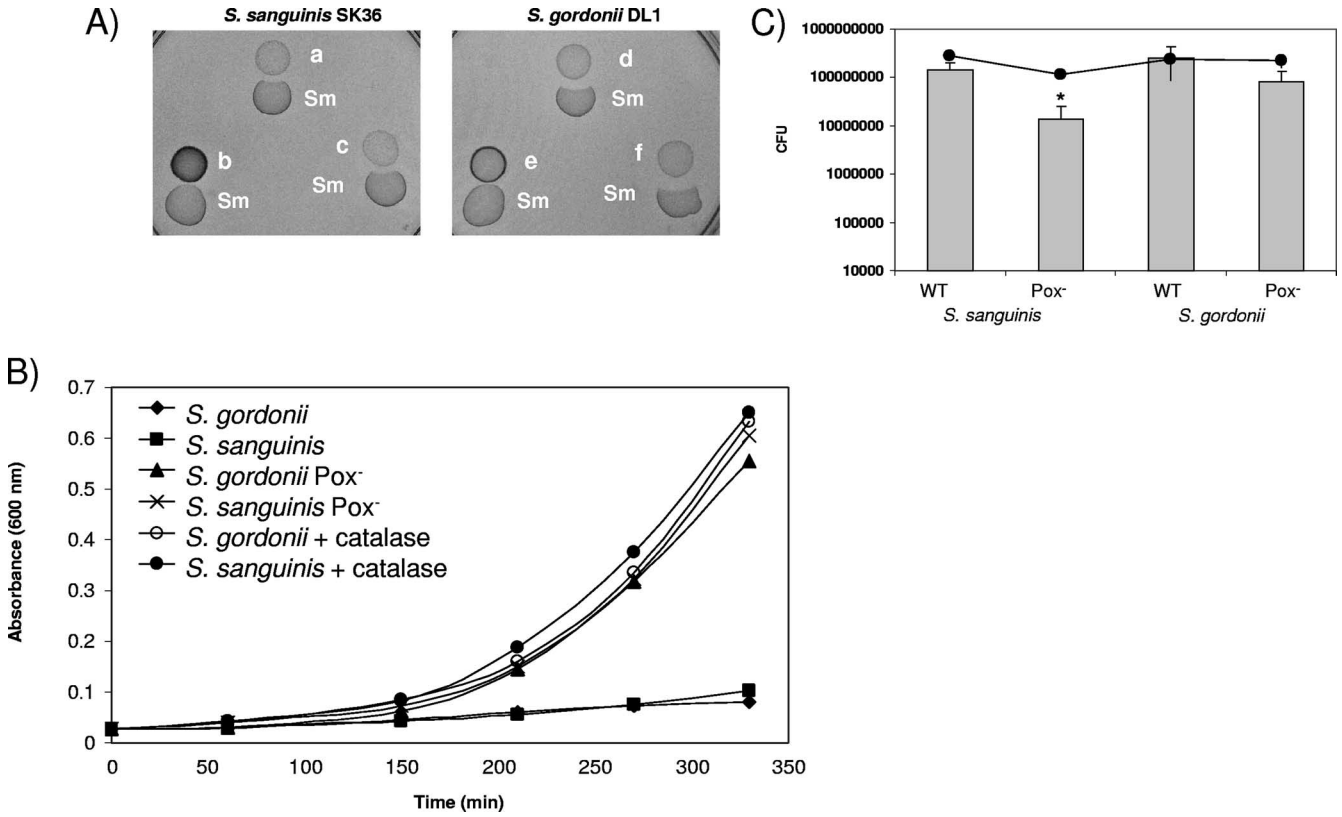

FIG. 2. Inhibition ability and competitiveness of wild-type and Pox$^-$ mutant of *S. sanguinis* and *S. gordonii*. (A) *S. sanguinis* or *S. gordonii* or their derivatives were inoculated first and incubated aerobically at 37°C. *S. mutans* was inoculated 16 h later, and the plates were incubated overnight. a, SK36; b, JKH2 Pox$^-$; c, JKH2/pDL276-pox; d, DL1; e, JKH1 Pox$^-$; f, JKH1/pDL276-pox. (B) Growth of *S. mutans* in sterile conditioned medium from *S. sanguinis* or *S. gordonii* cultures. The conditioned medium was prepared from exponentially growing *S. sanguinis* or *S. gordonii* cells or their derivatives, filter sterilized, supplemented with 0.25% glucose, and immediately inoculated with *S. mutans* at a titer of 10^7 bacteria ml^{-1}. Cells were incubated aerobically at 37°C, and 1 ml was removed to determine the A_{600} at the indicated time points. The data presented are representative of two independent experiments with similar results. (C) Competitiveness of *S. sanguinis* or *S. gordonii* wild-type and Pox$^-$ mutants in submerged dual-species biofilms with *S. mutans*. Dual-species biofilms were grown in BHI. After overnight growth, the cells were dispersed by vigorous pipetting, serially diluted, and plated. The CFU values for each strain and the standard deviations were calculated from three independent experiments performed on different days. *, $P \leq 0.05$. Gray bars, *S. sanguinis* or *S. gordonii*; black line, *S. mutans*.

the absence of glucose (Fig. 3B). Conditioned medium from *S. sanguinis* also inhibited growth of *S. mutans*, but glucose did not reverse the inhibition (Fig. 3B). Since changes in the generation time could explain the appearance of inhibition, growth was monitored for all strains with or without the addition of 1% glucose in liquid growth medium. *S. mutans*, *S. sanguinis*, and *S. gordonii* showed similar generation times with or without glucose, but the glucose-supplemented cultures yielded higher densities (data not shown). Interestingly, when tested as dual-species biofilms as described above, the addition of 1% glucose to the growth medium had an effect similar to that of the Pox$^-$ mutation on the outcome. *S. sanguinis* and *S. gordonii* grew significantly less in the presence of 1% glucose than in unsupplemented cultures, whereas *S. mutans* was unaffected (Fig. 3C).

Differential H_2O_2 production by *S. sanguinis* and *S. gordonii*. *S. gordonii* grown in media with 1% glucose showed reduced ability to inhibit *S. mutans*. To determine whether the presence of 1% glucose reduces generation of H_2O_2, H_2O_2 production by *S. sanguinis* and *S. gordonii* was determined. In TSB growth medium, H_2O_2 production by *S. gordonii* was reduced more than *S. sanguinis* by the addition of 1% glucose (*S. gordonii*, $P \leq 0.05$; Fig. 4A). The Pox$^-$ mutants and cells grown with catalase did not produce detectable amounts of H_2O_2. H_2O_2 production was restored in *S. sanguinis* and *S. gordonii* by complementation of Pox (Fig. 4A). During growth in the presence of 1% glucose *S. gordonii* and *S. sanguinis* H_2O_2 production peaked during late log transition into stationary phase (after 300 min), followed by a 30% reduction in stationary phase for *S. gordonii* (Fig. 4B) and a slight reduction for *S. sanguinis* (Fig. 4C). In the presence of 1% glucose, *S. sanguinis* H_2O_2 production was maximal in stationary phase (Fig. 4C). H_2O_2 production by both species was generally higher in the absence of glucose (Fig. 4A to C).

Differential expression of *S. mutans* mutacin IV gene. The expression of the genes for mutacin I and IV, the two characterized mutacins of *S. mutans* UA140 (40), were compared when cells were grown aerobically or anaerobically. Expression of *mutA*, the structural gene for mutacin I, was similar under aerobic and anaerobic conditions (data not shown). In aerobic conditions, the expression of *nlmA*, the structural gene for mutacin IV, was at least fivefold higher (Fig. 5). The expression

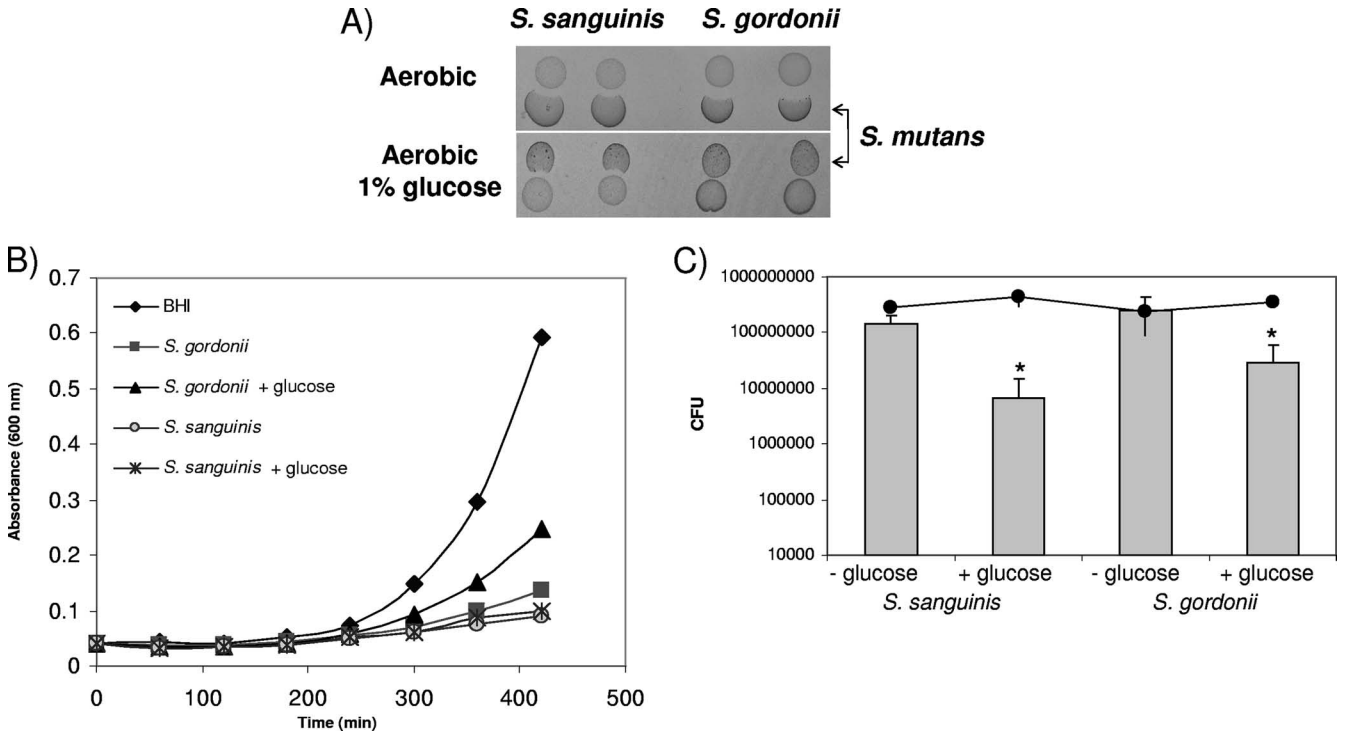

FIG. 3. Influence of glucose on the competitiveness of *S. sanguinis* or *S. gordonii*. (A) *S. mutans* growth inhibition assay in the presence or absence of 1% glucose. (B) Growth of *S. mutans* in sterile conditioned medium from *S. sanguinis* or *S. gordonii* cultures grown with or without 1% added glucose. The conditioned medium was prepared from cells growing to the mid log growth phase and immediately inoculated with *S. mutans*. Cells were incubated aerobically at 37°C, and 1 ml removed to determine the A_{600} at the indicated time points. The data presented are representative of two independent experiments with similar results. (C) Competitiveness of *S. sanguinis* or *S. gordonii* in submerged dual-species biofilms with *S. mutans*. Dual-species biofilms were grown in BHI plus 1% glucose when indicated. After overnight growth, the cells were dispersed by vigorous pipetting, serially diluted, and plated. CFU values and standard deviations were calculated from three independent experiments performed on different days. *, $P \leq 0.05$. Gray bars, *S. sanguinis* or *S. gordonii*; black line, *S. mutans*.

of *nlmA* is controlled by the ComDE two-component system in response to the competence-stimulating peptide CSP (17, 19). To learn whether oxygen-dependent regulation of *nlmA* is controlled by ComDE, the expression of *comC* and *comE* was measured in aerobic and anaerobic cultures. In aerobic conditions, *comC* and *comE* were also expressed at about fivefold higher levels than under anaerobic conditions (Fig. 5).

Aerobic growth triggers increased DNA release from mixed *S. sanguinis* and *S. gordonii* cultures. Mutacin IV can lyse closely related streptococci, and the released DNA can be taken up by competent *S. mutans* cells (17). We next determined whether higher expression of the competence system under aerobic growth conditions correlates with DNA release from mixed cultures of *S. sanguinis* and *S. gordonii*. After overnight growth, the mixed anaerobic cultures of *S. sanguinis* and *S. gordonii* wild-type cells and the Pox$^-$ mutants released similar amounts of DNA (Fig. 6A) and reached comparable cell densities and CFU counts (Fig. 6B). Under aerobic conditions, however, the DNA released from the mixed wild-type cultures increased more than 10-fold (Fig. 6A), although the final cell density was less than under anaerobic conditions (Fig. 6B). Under aerobic conditions the release of DNA appeared to depend upon the expression of a functional Pox gene, since mixed cultures with the Pox$^-$ mutants released less DNA than the other mixed culture conditions tested (Fig. 6A). DNA release from aerobically grown wild-type cultures was reduced by the addition of catalase to the levels of the Pox$^-$ mutants (data not shown). Catalase addition also leads to higher wild-type cell densities, a finding consistent with a role for H_2O_2 in DNA release (data not shown).

DISCUSSION

The oral biofilm is a biological system of exceptionally high complexity. This complexity is largely reflected by the broad diversity of microbial species, the spatial and temporal arrangement of the individual members, and the high cell density. In addition, the oral environment is a habitat with constant changes in conditions, e.g., nutrient limitation, saliva flow, and oxygen availability. Members of the oral biofilm have evolved to cope with change and compete with other oral bacteria for this ecological niche. For example, adhesion through specific proteins enables *S. gordonii* to successfully compete with *S. sanguinis* during initial biofilm formation (31), and *Streptococcus oligofermentans* can use the lactic acid produced by *S. mutans* to increase its production of H_2O_2 to inhibit growth of the competitor (49). Hydrogen peroxide production by streptococci is well known to inhibit the growth of other bacterial species and contribute to the antagonism between *S. mutans* and *S. sanguinis* in oral biofilms (18, 43).

Oral biofilm formation and H_2O_2 production are influenced by oxygen (1, 2, 6). Oxygen in the oral biofilm allows for respiration of plaque bacteria and H_2O_2 production (26).

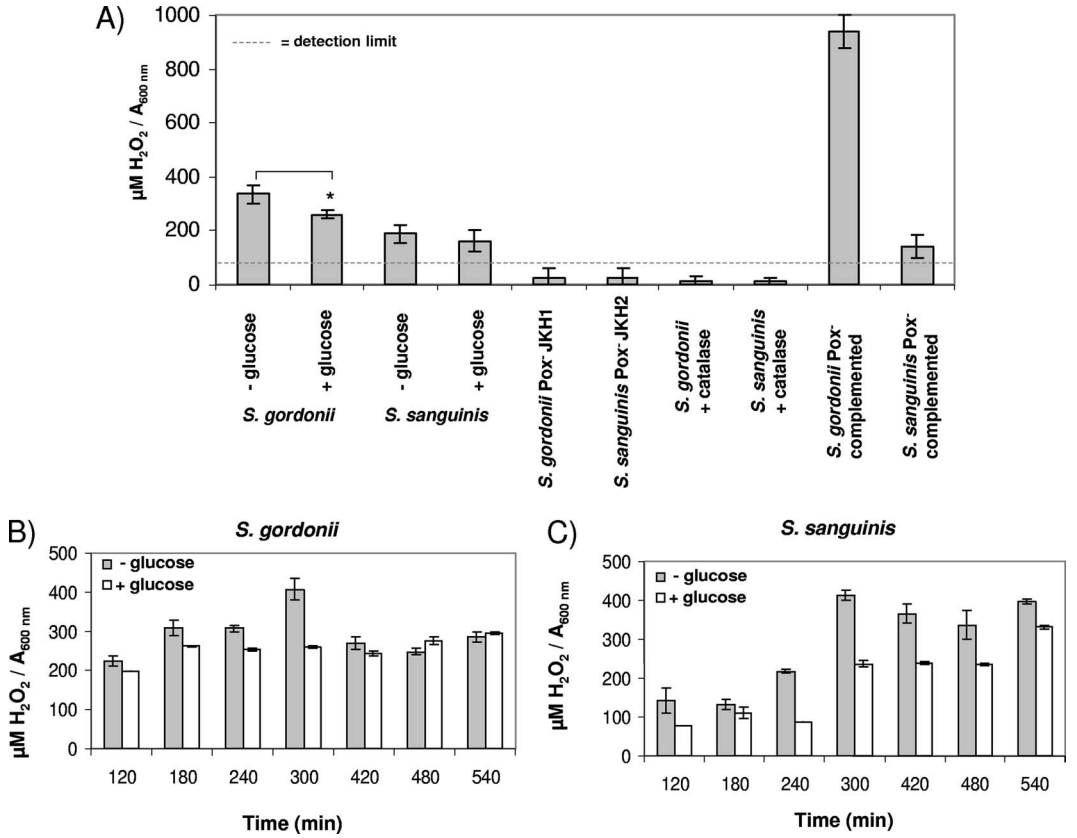

FIG. 4. Production of H_2O_2 during growth of *S. sanguinis* or *S. gordonii*. (A) SK36 or DL1 wild type with or without 1% glucose or catalase (30 μg), Pox$^-$ mutants JKH1 and JKH2, and complemented strains grown for 3 h until mid-exponential phase in TSB medium. (B) Time course of DL1 H_2O_2 production with or without glucose. (C) Time course of SK36 H_2O_2 production with or without glucose. Hydrogen peroxide concentration was adjusted to the absorbance at 600 nm. Gray bars, TSB; white bars, TSB plus 1% glucose. *, $P \leq 0.05$ ($n = 3$).

H_2O_2 is produced by Pox as a by-product of aerobic metabolism (7). Using genomic analysis, the genes encoding the Pox of *S. sanguinis* (54) and *S. gordonii* (51) showed 95% homology when compared at the DNA level (data not shown). In agreement with its inability to produce H_2O_2 (1), the *S. mutans* genome contains no ortholog of a Pox gene (4). The production of H_2O_2 during aerobic respiration is considered an aggressive by-product that may eliminate competitors. H_2O_2 is strongly suggested to be a competitive factor in oral biofilms based upon in vitro studies (18) and our results demonstrating the Pox-dependent inhibition of *S. mutans* growth under aerobic conditions. Interspecies inhibition mediated by H_2O_2 has also been reported for *Streptococcus pneumoniae* (43) and *Lactobacillus paracasei* (32) toward *Staphylococcus aureus*.

The production of H_2O_2 by *S. sanguinis* and *S. gordonii* is likely to have other important consequences to the biofilm community. Under aerobic conditions, H_2O_2 production by mixed wild-type *S. sanguinis*/*S. gordonii* cultures caused release of 10-fold more DNA than cells grown under anaerobic conditions. Pox$^-$ mutant mixed cultures grown under aerobic conditions also released DNA, but less DNA than when H_2O_2 was produced. In aerobic conditions, wild-type cocultures yielded fewer viable cells correlating with the increased DNA release. Consistent with our data, spontaneous cell death and lysis of the bacterial pathogen *S. pneumoniae* has recently been shown to be H_2O_2 dependent (42). Indeed, H_2O_2 induced an apoptosis-like response, resulting in the loss of DNA content from H_2O_2-damaged cells (42). Besides triggering the lysis of competitors during initial colonization, H_2O_2-dependent cell lysis might confer an alternative competitive advantage. The biofilm matrix consists of proteins, carbohydrates, water, lipids, and DNA. The addition of DNase to *S. mutans* or *Staphylococcus epidermidis* cultures reduces the ability to form a biofilm (38, 41), suggesting that DNA stabilizes the biofilm. Moreover, the availability of DNA in the biofilm increases the chance for competence-dependent DNA uptake and genetic exchange, since competence increases when *S. mutans* grows in a biofilm (23). Hence, H_2O_2 is likely to regulate interspecies interactions of oral streptococci in the oral biofilm in several ways that were previously unknown.

In *S. sanguinis* and *S. gordonii* the promoter and gene sequences of the *spxB* genes share high homology. In the presence of glucose, which is a major nutritional carbon source whose concentration increases up to 1,000-fold during food intake (1), only *S. sanguinis* was able to inhibit *S. mutans*. During planktonic growth, both species produced less H_2O_2 in the presence of 1% glucose. To explain regulation in response to glucose, a putative *cre* box was identified in the promoter regions of the *spxB* genes of *S. gordonii* and *S. sanguinis* (data not shown). The *cre* box is the binding site for the major regulator of carbon catabolite repression in gram-positive bacteria, CcpA (13). In *L. plantarum*, CcpA

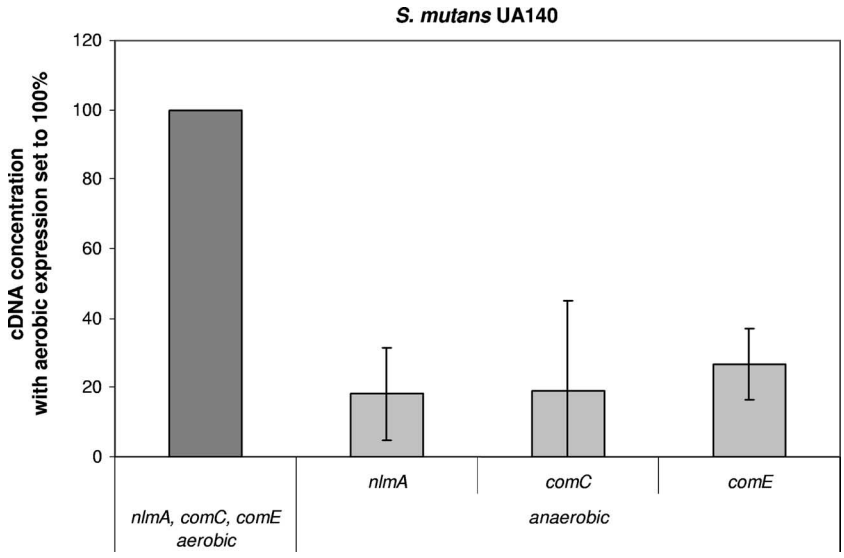

FIG. 5. Differential expression of *nlmA*, *comC*, and *comE* from *S. mutans* during aerobic and anaerobic growth. Gene expression was compared from cells growing on BHI agar plates for 7 h with or without oxygen. The values are given as relative cDNA abundance, with the expression under aerobic conditions set to 100%. Transcript levels were measured by real-time PCR using the 16S RNA as a housekeeping control. cDNA abundance was normalized against the 16S cDNA. The data presented represent the means and standard deviations of two (*comC* and *comE*) or three (*nlmA*) independent experiments performed on different days.

represses the Pox gene *poxB* by binding to the experimentally confirmed *cre* box in the *poxB* promoter (25). However, a transcript for *poxB* could be measured in cells grown in the presence of 2% glucose. Transcription increased markedly when glucose was exhausted (25). In the results presented here, *S. sanguinis* was able to inhibit *S. mutans* in the presence of 1% glucose. CcpA may regulate differently in *S. gordonii*, allowing for more complete repression of the *spxB* gene. Differences in Pox half-life and the H_2O_2-degrading abilities of *S. sanguinis* and *S. gordonii* might also contribute to this process. Interestingly, the Pox-complemented strain of *S. gordonii* produced more H_2O_2 and slightly greater inhibition ability than the wild type (Fig. 2Ad and f and Fig. 4A), suggesting the presence of a potential regulator, which is titrated in the complemented strain by the presence of several *spxB* promoter sequences on the low-copy shuttle plasmid. Further work is needed to verify that the *spxB* genes in *S. gordonii* and *S. sanguinis* are in fact repressed by the binding of CcpA to the putative *cre* boxes and that the transcription is indeed controlled differently.

The glucose-mediated repression of H_2O_2 production shown in vitro might have a larger impact on the in vivo oral biofilm community. *S. mutans*, *S. sanguinis*, and *S. gordonii* all utilize glucose and possess multiple transport systems for this substrate (50). When BHI medium was supplemented with glucose, the growth rates of the three species were similar (data not shown). The reduced production of H_2O_2, however, could impair *S. gordonii* and *S. sanguinis* inhibition of *S. mutans* in vivo. *S. mutans* would acquire a competitive advantage, because the production of mutacins is not influenced by glucose. In fact, our biofilm experiments with mixed *S. mutans* and *S. gordonii*/*S. sanguinis* wild types grown simultaneously with or without glucose showed that *S. gordonii* and *S. sanguinis* CFU counts were significantly reduced in the presence of glucose in vitro.

The repression of Pox by glucose could have additional consequences for the metabolism of *S. sanguinis* and *S. gordonii* beyond those identified in the present study. The product of Pox enzymatic activity is acetyl-phosphate. Acetyl-phosphate is a potential phosphoryl donor for intracellular processes (14) and has been shown to be a global signal during biofilm development of *E. coli*. Mutants in the generation of acetyl-phosphate were able to form biofilms, but the architecture and physiological properties of the biofilm were changed (53). Similarly, *S. pneumoniae* $SpxB^-$ yields significantly less acetyl-phosphate (37) and is impaired in its ability to adhere to human endothelial cells (48). Adhesion ability is restored, however, when acetate is supplied in the growth medium and acetyl-phosphate can be generated by acetate kinase (48). Since inactivation of the Pox influences production of H_2O_2 and acetyl-phosphate, the role of acetyl-phosphate in biofilm development and interspecies competition requires further investigation.

Surprisingly, the competence system of *S. mutans* appears to be optimized for aerobic conditions, given that heterologous DNA is present in aerobic cultures of *S. gordonii* and *S. sanguinis*. Consistent with our data, *S. mutans* transformability is higher under aerobic conditions (38). Furthermore, *S. pneumoniae* controls the expression of the competence operon *comCDE* in an oxygen-dependent process via the two-component signal transduction system CiaRH (10). The expression of the competence genes in *S. mutans* is coordinated with the expression of a group of bacteriocins and bacteriocin-like genes (19); accordingly, the expression of the bacteriocin mutacin IV was elevated under aerobic conditions in our studies. During the preparation of the present study, Ahn et al. (3) reported the oxygen-dependent upregulation of the mutacin IV gene and the competence system for *S. mutans* using DNA microarrays, further supporting our results. Mutacin IV was shown to be involved in the lysis and release of plasmid DNA from *S. gordonii* (17). Interspecies interference by *S. mutans* appears to be equally effective in aerobic and anaerobic growth

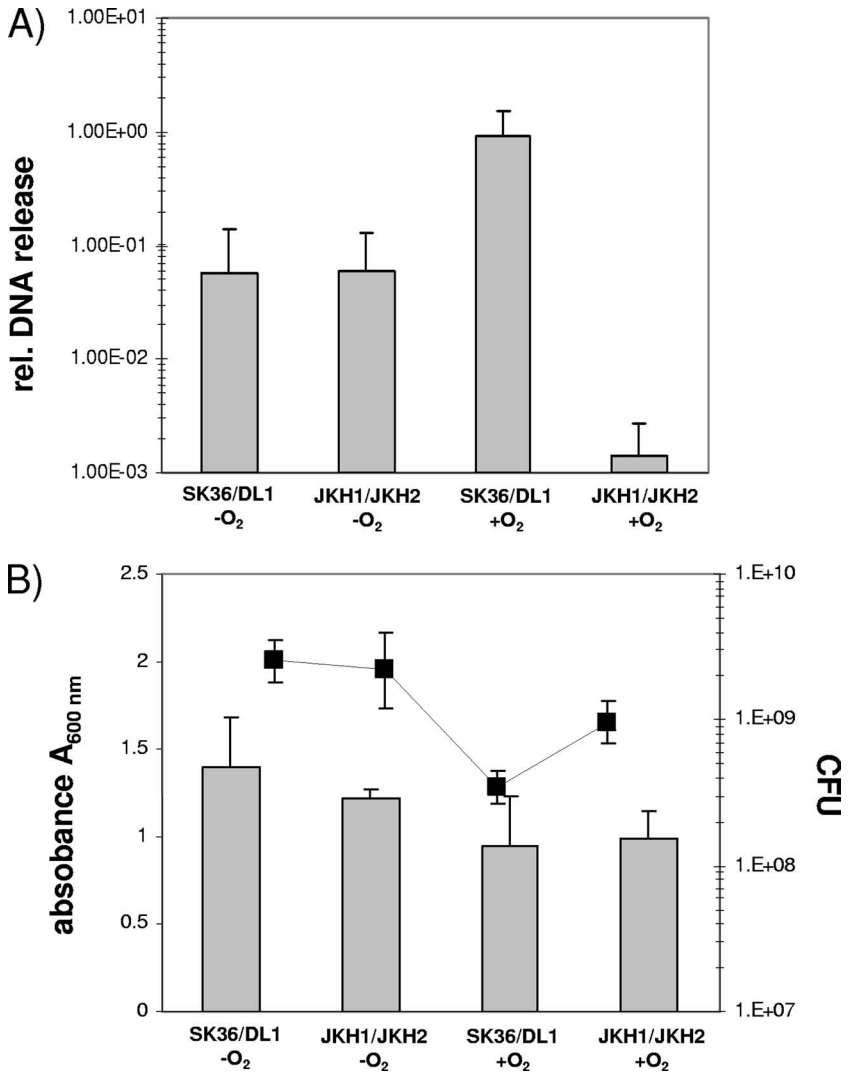

FIG. 6. Quantification of DNA release and cell densities from mixed cultures of *S. sanguinis* and *S. gordonii* during growth under aerobic and anaerobic conditions. (A) Real-time PCR quantification of DNA release from mixed cultures of wild-type SK36/DL1 and Pox$^-$ mutants JKH1/JKH2 after overnight incubation with or without oxygen using universal primers for 16S RNA genes of *S. gordonii* and *S. sanguinis* (16S strep F and 16S strep R). The DNA release in each individual sample is reported relative to a serial dilution of chromosomal DNA from *S. sanguinis* (see Materials and Methods for details) by comparison of the C_T values for the standard curve and the samples. (B) CFU counts and A_{600} values of the overnight cultures prior to DNA quantification. The data presented represent the means and standard deviations of three independent experiments performed on different days. Gray bars, CFU; black line, absorbance (A_{600}).

conditions and in the presence of glucose, based upon inhibition of the competitors *S. gordonii* and *S. sanguinis*. This preliminary result might partially explain the ability of *S. mutans* to increase in numbers and promote caries development when the diet is rich in glucose, a cariogenic carbohydrate (30), and the environment is anaerobic, even when no sucrose is available.

In conclusion, we have demonstrated that the environmental factor oxygen and the nutritional compound glucose substantially influence the interspecies competitiveness of the tested streptococci in our in vitro assays. The presented experiments were performed in liquid cultures and as plate assays, and the main outcomes were comparable between both experimental conditions. It is noteworthy that the oxygen availability is reduced in static liquid cultures due to rapid consumption. The experimental outcome could therefore as well be influenced by a specific biofilm phenotype. In future experiments, other ecological and environmental factors need to be incorporated into our model to understand the interplay between the members of the oral biofilm. The greatest challenge will be to confirm the basis of interspecies competition in vivo.

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