Catabolite Control Protein A Controls Hydrogen Peroxide Production and Cell Death in *Streptococcus sanguinis*[▽]

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Streptococcus sanguinis is a commensal oral bacterium producing hydrogen peroxide (H₂O₂) that is dependent on pyruvate oxidase (Spx) activity. In addition to its well-known role in bacterial antagonism during interspecies competition, H₂O₂ causes cell death in about 10% of the *S. sanguinis* population. As a consequence of H₂O₂-induced cell death, largely intact chromosomal DNA is released into the environment. This extracellular DNA (eDNA) contributes to the self-aggregation phenotype under aerobic conditions. To further investigate the regulation of *spx* gene expression, we assessed the role of catabolite control protein A (CcpA) in *spx* expression control. We report here that CcpA represses *spx* expression. An isogenic Δ ccpA mutant showed elevated *spx* expression, increased Spx abundance, and H₂O₂ production, whereas the wild type did not respond with altered *spx* expression in the presence of glucose and other carbohydrates. Since H₂O₂ is directly involved in the release of eDNA and bacterial cell death, the presented data suggest that CcpA is a central control element in this important developmental process in *S. sanguinis*.

Initial development of dental biofilms is dominated by oral streptococci, which produce specific adhesins that interact with salivary proteins bathing the teeth and oral mucosa surfaces (29). Biofilm development is a highly competitive process, and different mechanisms are used by individual bacteria to compete with other initial colonizers (17). For example, *Streptococcus gordonii* binding to salivary components via the surface protein Hsa has been shown to provide a competitive measure during niche competition with *Streptococcus sanguinis* (30). The excretion of antimicrobial components by oral streptococci as a more aggressive mode of competition has been known for several decades. Bacteriocins produced by cariogenic *Streptococcus mutans* are effective in inhibiting the growth of several other oral streptococci (10). Conversely, competitive hydrogen peroxide (H₂O₂) production by commensal *S. sanguinis* and *S. gordonii* during aerobic growth inhibits *S. mutans* (18, 20). The enzyme responsible for competitive H₂O₂ production has been identified as pyruvate oxidase (Spx, also referred to as Pox) (5, 20). Isogenic Spx[−] mutants of *S. sanguinis* and *S. gordonii* were unable to inhibit the growth of *S. mutans* in an *in vitro* competition assay (20). A similar effective role of pyruvate oxidase dependent H₂O₂ production has been shown in the *Streptococcus pneumoniae*-*Staphylococcus aureus* interference (38). Moreover, the inverse association between *S. sanguinis* and more cariogenic species has been shown in clinical studies, suggesting a protective effect of *S.*

sanguinis colonization resulting in lower caries incidence (1, 3, 6, 43). Although molecular mechanisms of this inverse relationship are not well defined, H₂O₂ production might play a role. The initial colonization process during early biofilm formation occurs when oxygen tension is high enough to allow for respiration and H₂O₂ production (25). With the consequence that H₂O₂ susceptible species might be outcompeted. This has a profound consequence on the overall composition of the biofilm because the initial colonization process influences the spatial and temporal development of the dental biofilm (15). Detailed knowledge of the regulation of pyruvate oxidase-mediated H₂O₂ production could therefore provide important insights into dental biofilm ecology and eventually lead to new ways to promote biofilm development toward a healthy composition. Initial results have shown that the pyruvate oxidases of *S. sanguinis* and *S. gordonii* are differentially regulated by glucose, despite a high homology of the promoter region. *S. gordonii* is not able to inhibit the growth of *S. mutans* in the presence of glucose, while *S. sanguinis* inhibiting ability is not affected (20). Furthermore, it was shown that the pyruvate oxidase dependent production of H₂O₂ is correlated with bacterial cell death and the release of extracellular DNA (eDNA). eDNA is an important component of the extracellular matrix in biofilms and in the case of *S. sanguinis* confers cell-cell adhesion to a certain extent, thus providing evidence that H₂O₂ production not only increases competitiveness but also promotes biofilm development (19).

In this report, the regulation of pyruvate oxidase gene expression was further investigated in *S. sanguinis*. Carbon catabolite control protein A (CcpA) plays a role in *spx* expression regulation, but the regulation is not influenced by glucose. Gene expression control was also verified on the protein level.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strain		
SK36	<i>S. sanguinis</i> wild type	47
JKH2	SK36; <i>spx::aad9</i> ; <i>Spc</i> ^r	20
SK36 CcpA ⁻	Δ <i>ccpA</i> ; <i>Erm</i> ^r	This study
SK36 CcpA ⁺	SK36 CcpA ⁻ ; <i>ccpA</i> restored; <i>Erm</i> ^s Kan ^r	This study
SK36 CcpA ⁻ <i>Spx</i> ⁻	SK36 CcpA ⁻ ; <i>spx::aad9</i> ; <i>Erm</i> ^r <i>Spc</i> ^r	This study
UA140	<i>S. mutans</i> wild type	35
DH5 α	<i>E. coli</i> ; cloning strain	11
BL21(DE3)(pLysS)	<i>E. coli</i> ; protein expression strains	27
Plasmid		
pFW5	Suicide vector; <i>Spc</i> ^r	34
pGEM-T Easy	Cloning vector; <i>Amp</i> ^r Kan ^r	Promega
pET-29b(+)	The pET-29b(+) vector, including C-terminal His tag; Kan ^r	Novagen
pGEM-T-spxB-flag	pGEM-T Easy, <i>spxB</i> -Flag tag; <i>Amp</i> ^r	This study
pFW5-spxB-flag	pFW5, <i>spxB</i> -Flag tag; <i>Spc</i> ^r	This study
pLZ1	pFW5 Φ (<i>spxB_p::luc</i>) <i>S. sanguinis</i> luciferase reporter; <i>Spc</i> ^r	This study
pLZ2	pFW5 Φ (<i>spxB_p::luc</i>) <i>S. gordonii</i> luciferase reporter; <i>Spc</i> ^r	This study

^a *Erm*^r, erythromycin resistance; *Erm*^s, erythromycin susceptibility; *Amp*^r, ampicillin resistance; *Spc*^r, spectinomycin resistance; Kan^r, kanamycin resistance.

Moreover, evidence of CcpA-dependent regulation of cell death is presented in the context of increased H₂O₂ production for a Δ *ccpA* mutant background.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains used in the present study are listed in Table 1. Strains were routinely grown aerobically as static cultures (5% CO₂) at 37°C in brain heart infusion (BHI; Difco, Sparks, MD) or on BHI agar plates unless otherwise stated. Cells were grown anaerobically in an anaerobic chamber (90% N₂, 5% CO₂, 5% H₂). Shaking cultures were grown in ambient air. Filter-sterilized glucose was added from a 20% stock solution when indicated. When required for selection, cultures were supplemented with the following antibiotics: spectinomycin at 500 μ g ml⁻¹, erythromycin at 2 μ g ml⁻¹, kanamycin at 300 μ g ml⁻¹ for *S. sanguinis*, and ampicillin at 100 μ g ml⁻¹ for *E. coli*.

DNA manipulations. Standard recombinant DNA manipulations were used (42). Restriction enzymes and DNA ligase were obtained from New England Biolabs (Beverly, MA) or Promega (Madison, WI) and used as specified by the manufacturer. PCR products were cloned into the pGEM-T kit from Promega. All plasmids were extracted and purified from *E. coli* using 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 G-Storm GSI thermocycler (Gene Technologies, Essex, United Kingdom) 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. The primer sequences (Table 2) 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 a Δ *ccpA* deletion mutant. To study the role of CcpA in *S. sanguinis*, we constructed a Δ *ccpA* deletion mutant via double-crossover homologous recombination. To generate the construct, two fragments corresponding to ~800 bp of the upstream and downstream sequences of *ccpA* were generated by PCR, using Phusion Hot Start High-Fidelity DNA polymerase with the primer pair *ccpA* Up F/*ccpA* Up R and the primer pair *ccpA* Dn F/*ccpA* R Dn R (Table 2). Each of the primers listed as Up R and Dn F incorporated 25 bases complementary to the erythromycin resistance cassette, *ermAM*. The erythromycin resistance gene *ermAM* (26) was amplified by PCR using the primers *ermAM* F and *ermAM* R. All three PCR amplicons were purified with the Qiagen PCR purification kit and mixed in a 1:1:1 ratio. The mixture served as a template for a second round PCR with the appropriate Up F and Dn R primers. The resulting PCR amplicons were transformed into wild-type strain SK36 to generate the Δ *ccpA* deletion mutant (SK36 CcpA⁻). Deletion was confirmed by PCR.

Restoration of the Δ *ccpA* deletion mutant. To restore the Δ *ccpA* mutant with a functional *ccpA*, overlap extension PCR was performed. For the upstream fragment, ~1,700 bp of the entire *ccpA* was amplified from SK36 wild-type

chromosomal DNA using Phusion Hot Start High-Fidelity DNA polymerase with primer pairs *ccpA*-c Up F/*ccpA*-c Up R. About 800 bp downstream of the *ccpA* sequence were amplified by PCR with the primer pair *ccpA*-c Dn F/*ccpA*-c Dn R (Table 2). The downstream and upstream fragments were homologous to the surrounding sequence of the *ccpA* region still present in the Δ *ccpA* mutant. Each of the primers listed as Up R and Dn F incorporated 25 bases complementary to the kanamycin resistance gene *aphA*. The kanamycin resistance gene was amplified by PCR from plasmid pJH1 (46) using the primers Kan F and Kan R. All three PCR amplicons were purified with the Qiagen PCR purification kit and mixed in a 1:1:1 ratio. The mixture served as the template for a second round PCR with the appropriate Up F and Dn R primers. The resulting PCR amplicons were transformed into the SK36 CcpA⁻ strain to restore the Δ *ccpA* mutant strain (SK36 CcpA⁺). Restoration was verified by PCR. We also complemented the mutant with an *in trans* expressed *ccpA* copy from shuttle plasmid pDL278. The phenotypes of both, the restored and the complemented strains were similar when tested for the production of H₂O₂ and growth phenotypes as presented in Fig. 5D (data not shown).

Construction of *spx* luciferase reporter strains. A 0.7-kb fragment containing the *spxB* promoter region was amplified by PCR from chromosomal DNA of *S. sanguinis* using the primers Ss *spxB* F, including a SalI linker, and Ss *spxB* R, including a BamHI linker. The PCR product was cloned in front of the luciferase gene of plasmid pFW5-*luc* (16) to generate plasmid pLZ1. DNA sequencing of the respective promoter region confirmed the correct construct. The plasmid was transformed into wild-type SK36 and its isogenic Δ *ccpA* mutant. The same strategy was used for the construction of pLZ2 using the primers Sg *spxB* F and Sg *spxB* R and *S. gordonii* chromosomal DNA. The correct plasmid was also confirmed by DNA sequencing and subsequently transformed into *S. gordonii*.

Luciferase assay. Luciferase assays were performed using a method similar to one described previously (21). Briefly, 25 μ l of 1 mM D-luciferin (Sigma) suspended in 100 mM citrate buffer (pH 6) was added to 100 μ l of cell culture. To ensure sufficient levels of intracellular ATP, the cells were recharged with 1% glucose for 10 min prior to luciferin addition. The luciferase activity was measured with a Modulus luminometer (Turner Biosystems). The specific luciferase activity is measured as relative light units normalized to the absorption at 600 nm. Usually, three parallel cultures were measured at each time point, and the mean value was taken. Each experiment was repeated at least two times.

Construction of a pyruvate oxidase carrying a C-terminal FLAG epitope. About 700 bp from the 3' end of the pyruvate oxidase gene (*spxB*) was PCR amplified with specific primers incorporating a 6 FLAG epitope sequence (GA TTACAAAGACGATGATGACAAG) before the stop codon using the primer pair *spxB*-flag tag F/*spxB*-flag tag R. The PCR product was inserted into the pGEM-T Easy vector (Promega) to create pGEM-T-*spxB*-flag. The construct was sequenced to confirm the in-frame presence of the FLAG epitope sequence. The cloned fragment was released with XhoI/BglII and inserted into pFW5 digested with the same enzymes. pFW5 is not able to replicate in *S. sanguinis* and requires integration into the chromosome for propagation (34). The recombi-

TABLE 2. Primers used in this study

Primer	Sequence (5'-3')	Purpose
Erm F	GAAGGAGTGATTACATGAACAAAAA	<i>ccpA</i> knockout
Erm R	AAAGCGACTCATAGAATTATTTCTC	<i>ccpA</i> knockout
ccpA Up F	CATGCGGATTAGCTGCAITA	<i>ccpA</i> knockout
ccpA Up R	TTTTGTTTCATGTAATCACTCCTTC GGGCGACATCATAAATCGTT	<i>ccpA</i> knockout
ccpA Dn F	GAGGAAATAATTCTATGAGTCGCTTT TGC GCATGCTGACTAAGATT	<i>ccpA</i> knockout
ccpA Dn R	ACCTTCTGCCCCCTCTTCAAT	<i>ccpA</i> knockout
Kan F	AGG TGA TAG GTA AGA TTA TAC CG	<i>ccpA</i> knockin
Kan R	CCC TAT CTA GCG AAC TTT TAG A	<i>ccpA</i> knockin
ccpA-c Up F	GATGGCAAAGTCAATCTGAGC	<i>ccpA</i> knockin
ccpA-c Up R	CTCGGTA TAA TCT TAC CTA TCA CCT TCACCGCTTATTTCCGAGTT	<i>ccpA</i> knockin
ccpA-c Dn F	TTTTCTA AAA GTT CGC TAG ATA GGG CCGCAAGTCTGATTAGGAAT	<i>ccpA</i> knockin
ccpA-c Dn R	ACCTTCTGCCCCCTCTTCAAT	<i>ccpA</i> knockin
spxB-flag tag F	AGCGCTCGAGCGTGATTACATGAACAAACTCG	<i>spxB</i> -flag tag protein
spxB-flag tag R	CGCTAGATCTTTACTTGTCTATCATCGTCTTTGTAATCTTTAATTGCGCG TGATTGCA	<i>spxB</i> -flag tag protein
spxB RT F	AATTCGGCGGCTCAATCG	<i>spxB</i> RT-PCR
spxB RT R	AAGGATAGCAAGGAATGGAGTG	<i>spxB</i> RT-PCR
gyrA RT F	GCCGTGAGCGAATTGTCGTAAC	RT-PCR normalization
gyrA RT R	CGAACAGCAGTGATACCGTCAATG	RT-PCR normalization
Ss spxB F	AGCGGTGACCGCAGATCCAATTGC	eDNA detection
Ss spxB R	AGCGGGATCCTGCTGGAGATGCAGTA	eDNA detection
Ss spxB F	AGCGGTGACCGCAGATCCAATTGCTGT	Ss spxB reporter
Ss spxB R	AGCGGGATCCTGCTGCAGATGCAGTAAT	Ss spxB reporter
Sg spxB F	AGCGGTGACGGACTGGGATTGACCCTC	Sg spxB reporter
Sg spxB R	AGCGGGATCCTGCTGCAGATGCAGTAAT	Sg spxB reporter
5'RACE PCR adapter	Phos-TTT AGT GAG GGT TAA TAA GCG GCC GCG TCG TGA CTG GGA GCG C	5'RACE PCR
5'RACE PCR adapter primer	GCG GCC GCT TAT TAA CCC TCA CTA AA	5'RACE PCR
5'RACE PCR Ss spxB gene specific primer	AAGGATAGCAAGGAATGGAGTG	5'RACE PCR

nant plasmid was transformed into SK36 wild type, Δ *ccpA* mutant, and the restored strain, respectively. Transformants were selected on BHI plates containing appropriate antibiotics and were further analyzed by PCR for integration of the plasmid at the correct locus. Overnight cultures of strains carrying the Spx-FLAG construct were grown in the presence of spectinomycin.

Western immunoblots. The protein abundance of Spx-FLAG in the SK36 wild type, Δ *ccpA* mutant, and restored strain was investigated with Western blot analysis. Strains were grown in BHI medium (20 ml) as planktonic cultures until mid-logarithmic phase ($A_{600} = 0.7$), collected by centrifugation at $11,000 \times g$ for 15 min at 4°C, and resuspended in 1 ml of phosphate-buffered saline (PBS) buffer. Cytoplasmic extracts were generated by using a FastPrep FP210 Homogenizer with Lysing Matrix A (Thermo Scientific) by mechanical cell disruption. Cell debris was separated by centrifugation. Aliquots of the supernatants were adjusted to approximately the same protein concentration spectrophotometrically using the Bradford protein assay (Bio-Rad protein assay) and an appropriate volume separated by SDS-10% PAGE. For Western immunoblotting, the proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were blocked with a solution of 5% skim milk dissolved in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h. Subsequently the membrane was incubated in primary antibody solution (anti-FLAG M2 antibody; Stratagene) overnight at 4°C, followed by three washes with TBST, and then incubated with horseradish peroxidase-conjugated secondary antibodies (Thermo Scientific) for 1 h at room temperature. After being washed with TBST, the blots were developed using the ECL chemiluminescent detection system (Thermo Scientific).

Determination of transcription start sites. The FirstChoice RLM random amplification of cDNA ends (RACE) kit (Ambion) was used to determine transcription start sites essentially as described earlier (32). RACE-specific primers are listed in Table 2.

RNA isolation, cDNA synthesis, and real-time PCR. For sample collection, overnight cultures (1 ml) grown in BHI medium were inoculated into 40 ml of fresh BHI medium in a 50-ml conical tube. Growth was monitored spectrophotometrically at A_{600} . Cells were collected at mid-logarithmic phase ($A_{600} = 0.7$) by centrifugation at $11,000 \times g$ for 15 min at 4°C, and the cell pellet stored at -80°C until further use. For cells grown on BHI plates, overnight cultures were

inoculated on BHI plates or BHI plates supplied with 1% glucose using a sterile cotton swab (6 plates for each sample). After 6 h of incubation at 37°C, 5 ml of BHI medium was poured on each plate, and the cells were scraped from the plates with a sterile cotton swab. The cell suspensions were collected in 50-ml conical tubes, followed by centrifugation at $11,000 \times g$ for 15 min at 4°C, and the cell pellet was stored at -80°C until further use. To isolate RNA, the cell pellet was resuspended in 778 μl of RLT buffer (Qiagen RNeasy kit; Qiagen) and 222 μl of nuclease-free water. The cells were immediately disrupted twice for 1 min each using Lysing Matrix B (MP Biomedicals, Solon, OH) and a FastPrep FP210 Homogenizer (Thermo Scientific). RNA was purified by using a Qiagen RNeasy kit according to the manufacturer's protocol. Isolated RNA was treated with DNase I (Invitrogen, Carlsbad, CA) to remove traces of chromosomal DNA. After the treatment, RNA samples were cleaned with the Qiagen RNeasy kit. The concentration and quality of RNA samples was confirmed using NanoDrop spectrophotometer measurements and gel electrophoresis. cDNA was synthesized from 2 μg of RNA using the SuperScript II reverse transcriptase (Invitrogen) according to manufacturer's instructions.

Specific transcripts were quantified with the comparative threshold cycle (C_T) method using the Bio-Rad (Hercules, CA) MyiQ real-time reverse transcription-PCR (RT-PCR) detection system. The RT-PCR protocol included one cycle of 95°C for 90 s, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primer sequences are listed in Table 2. Real-time PCR was performed using 1 $\times$ SYBR green master mix (Bio-Rad) according to the manufacturer's instructions. Changes of gene expression were calculated using the C_T method as follows: $\Delta C_T = C_T(\text{target}) - C_T(\text{housekeeping gene})$. The fold changes are calculated as $2^{-\Delta C_T}$. The *gyrA* gene was used as the housekeeping reference gene.

Measurements of H_2O_2 production. The concentration of H_2O_2 in BHI liquid culture was determined by using a modification of the protocol described by Gilliland (8). A portion (40 μl) of cell-free culture supernatant from cells grown to mid-logarithmic phase ($A_{600} = 0.7$) was mixed in a 96-well microtiter plate (Falcon) with 160 μl of freshly prepared 0.1 M sodium acetate (pH 4.5) containing 0.1 μg of horseradish peroxidase (Thermo Scientific) and 10 μl of 1 mg of *o*-dianisidine (Alfa Aesar)/ml in methanol. The reaction mixture was incubated at room temperature for 10 min protected from light, and the absorbance at 570 nm was determined by using a microplate reader (model 680; Bio-Rad).

FIG. 1. *S. sanguinis* *spx* promoter region and *spx* expression in the presence of glucose. (A) Sequence of the *spx* promoter region of *S. sanguinis*. The putative *cre* is underlined, the start ATG is in boldface, and the transcription-start is in boldface and underlined. (B) For comparative real-time RT-PCR analysis, *S. sanguinis* cells were grown on BHI agar plates with or without 1% glucose for 6 h and subsequently scraped from the plate for RNA isolation. The expression level for *spx* grown with 1% glucose is presented relative to that of non-glucose-grown cells, which was arbitrarily assigned a value of 1. The *gyrA* gene was used as the housekeeping reference gene. Presented are averages and standard deviations of three independent experiments. (C) Luciferase activity of *S. sanguinis* and *S. gordonii* cells grown as planktonic culture to mid-logarithmic phase in TYE plus 0.2 or 2% glucose.

The concentration was calculated from a standard curve prepared in BHI using a 1:1,000 dilution of a commercial 30% H₂O₂ solution (BDH) in MilliQ water. The concentration of the dilution was determined spectrophotometrically ($\epsilon_{240} = 43.6/\text{M} \cdot \text{cm}$) using a SmartSpec Plus UV-visible spectrophotometer (Bio-Rad). Indicator plates for H₂O₂ production were prepared and used as described previously (41).

Antagonism assay. To assay the antagonistic H₂O₂ effect of *S. sanguinis* against *S. mutans*, overnight cultures of the *S. sanguinis* wild type, ΔccpA mutant, and the restored strain in liquid cultures grown under standard conditions as static cultures (cell densities of between A_{600} 1.2 and 1.3) were adjusted to the same cell density ($A_{600} = 0.5$). Portions (12 μl) were spotted onto a BHI plate and, after the liquid evaporated, the plate was incubated for 6 h at 37°C aerobically. Subsequently, 3 ml of an *S. mutans* overnight culture was mixed with 3 ml of hand warm BHI agar and immediately poured onto the BHI plate. After overnight incubation, the plate was photographed for documentation.

Confocal laser scanning microscopy of biofilms. To visualize static biofilms, the wild type, ΔccpA mutant, and restored strain were inoculated from an overnight culture in a 1:50 dilution in six-well plates (Becton Dickinson, Franklin Lakes, NJ) containing 5 ml of BHI plus 1% sucrose. The sucrose was added to increase the stability of the biofilms, since biofilms grown without sucrose showed disruption during preparation for imaging. After overnight incubation at 37°C in ambient air, the medium was replaced with PBS buffer containing Live/Dead BacLight bacterial viability fluorescent stain (Invitrogen) according to the manufacturer's instructions. Biofilm images were captured with a Leica SP2 MP confocal microscope equipped with fluorescent filters for red and green fluorescence detection ($\times 40$ HCX APO 0.8 NA dipping lens). Image projection was performed with Velocity software (version 5.2.1; Cellular Imaging).

edNA quantification and semiquantitative PCR. Both procedures were performed essentially as described before (19).

RESULTS

Increased glucose concentration does not influence *spx* expression. In a previous study the influence of glucose addition on the competitiveness of the two oral commensals, *S. sanguinis* and *S. gordonii*, was compared. Competitive inhibition is caused by H₂O₂ production when grown under aerobic conditions. While the addition of 1% glucose diminished the ability of *S. gordonii* to inhibit *S. mutans* in a dual-species competition assay, *S. sanguinis* was not affected (18). A similar result was obtained when H₂O₂ production was measured (20). Interestingly, the *spx* promoter regions of *S. sanguinis* and *S. gordonii* show high homology. A putative *cre* site (catabolite responsive element) for CcpA binding was identified in both species. The

position in *S. sanguinis* (Fig. 1A) is similar to the *cre* site found in the promoter of *Lactobacillus plantarum*, known to be repressed by glucose in a CcpA-dependent process (22). The *cre* site matched the consensus sequence determined for *Streptococcus pyogenes* (14), indicating that CcpA might directly repress *spx* expression. A second potential binding site was identified overlapping the -10 region, but with less identity to the consensus sequence (data not shown). A similar prediction for CcpA binding sites in *S. sanguinis* and *S. gordonii* has been made by the RegPrecise database for capturing, visualization, and analysis of transcription factor regulons (31).

Therefore, the influence of glucose on the expression of the *spx* gene in *S. sanguinis* was determined with real-time RT-PCR. Cells were grown on BHI plates containing 0.2 or 1% glucose and collected after 6 h for RNA isolation. No significant difference was observed (Fig. 1B). Furthermore, the influence of glucose on *spx* expression was compared between *S. sanguinis* and *S. gordonii* grown as planktonic cultures. Luciferase reporter strains were constructed for both species, and the luciferase activity measured dependent on the glucose concentration. To control the amount of glucose, tryptone-yeast extract (TYE) medium was used. In contrast to *S. sanguinis*, *S. gordonii* showed a clear repression of *spx* expression when grown in the presence of 2% glucose (Fig. 1C). Taken together, the results suggest a constant repression of *spx* in *S. sanguinis*, whereas *S. gordonii* shows carbon catabolite repression of *spx* expression. The results are consistent with the observed influence of glucose addition on interspecies competitiveness (20).

A ΔccpA mutant shows increased expression of *spx* in *S. sanguinis*. To further assess the potential role of CcpA in the expression control of *spx*, a ΔccpA mutant was constructed. The genomic organization of *ccpA* in *S. sanguinis* is similar to other streptococcal species, e.g., *Streptococcus suis*, *Streptococcus galloyticus*, and *Streptococcus equi* although not conserved in all streptococci (data not shown). A divergent transcribed *pepQ* gene is sharing the same intergenic region with *ccpA*. The gene following *ccpA* is separated by an intergenic space, and it

FIG. 2. Comparative *spx* gene expression analysis of the wild type, the $\Delta ccpA$ mutant, and the restored strain using real-time RT PCR and luciferase reporter gene fusion. (A) Construction of the $\Delta ccpA$ and restored strain. (B) For comparative real-time RT-PCR analysis, cells were grown in BHI until mid-logarithmic phase. The expression level for wild-type *spx* was arbitrarily assigned a value of 1. The *gyrA* gene was used as the housekeeping reference gene. Presented are averages and standard deviations of three independent experiments. (C) Luciferase activity of *S. sanguinis* grown as planktonic culture to mid-logarithmic phase in TYE plus 0.2 or 2% glucose, maltose, sucrose, or lactose. Grey bars, wild type; black bars, $\Delta ccpA$ mutant.

was shown for a *S. pyogenes* serotype M1 strain that both genes are transcriptionally linked (14). A promoterless, nonpolar *ermAM* gene cassette was therefore used to delete *ccpA* by allelic replacement to avoid any polar effects on the downstream gene. The $\Delta ccpA$ mutant was restored by a knockin strategy reintroducing the *ccpA* gene transcribed from its own promoter (Fig. 2A).

Expression analysis using real-time RT-PCR was performed with planktonic cultures grown to mid-logarithmic phase ($A_{600} \sim 0.7$). The expression of *spx* in the $\Delta ccpA$ mutant was

increased >6-fold, while expression by the restored mutant was comparable to the wild-type expression (Fig. 2B).

Furthermore, we addressed the question whether other carbohydrate sources influence expression of *spx* in *S. sanguinis*. The luciferase reporter gene fusion to the *spx* promoter was introduced into the wild type and the $\Delta ccpA$ mutant. Cells were grown in the presence of 0.2 and 2% glucose, sucrose, maltose, and lactose in TYE medium until mid-logarithmic phase, and the luciferase activity was measured. As shown in Fig. 2C, there was no difference of *spx* expression depending on

the carbohydrate concentration. When compared to the other carbohydrates, lactose showed a 2-fold increase in luciferase activity (Fig. 2C). The luciferase activity in the $\Delta ccpA$ mutant was increased with all carbohydrates tested compared to the wild type, a finding consistent with the real-time PCR results. Collectively, the results suggest that CcpA controls the expression of *spx* independent from the carbohydrate source. Under normal growth conditions, *spx* seems to be repressed by CcpA.

Increased pyruvate oxidase abundance in the $\Delta ccpA$ mutant.

To learn whether the increase in *spx* expression in the $\Delta ccpA$ mutant is reflected in higher production of the pyruvate oxidase, a C-terminal fusion to the FLAG epitope with the pyruvate oxidase was constructed. The construct was introduced into the wild type, the $\Delta ccpA$ mutant, and the restored strain. The epitope tag did not influence the production of H_2O_2 in the wild-type background (Fig. 3A), confirming a functional fusion protein. The cells were grown as planktonic cultures until mid-logarithmic phase and subsequently mechanically disrupted, and the cell debris was separated by centrifugation. An appropriate volume was separated by SDS-PAGE, followed by Western blotting. Production of the pyruvate oxidase was detected using an anti-FLAG M2 antibody. After detection and development of the immunoblot, a band corresponding to a molecular mass of about 65 kDa was visible for all three strains analyzed. The band intensity for the $\Delta ccpA$ mutant was appreciably increased over the wild type and the restored strain (Fig. 3B), confirming the elevated expression of the *spx* gene in the $\Delta ccpA$ mutant and indicating coupling between gene transcription and protein production for the pyruvate oxidase. Close investigation of the corresponding Coomassie blue stain loading control gel revealed that some bands appeared to be changed in intensity. This is to be expected since CcpA is one of the major global regulators of gene expression affecting more than 100 genes in other firmicutes examined (2).

FIG. 4. Increased H_2O_2 production of surface biofilms and planktonic grown cells in the $\Delta ccpA$ mutant. (A) H_2O_2 production of surface-grown biofilms was evaluated on H_2O_2 indicator plates. Reaction with H_2O_2 leads to the formation of a blue precipitate (Prussian blue). A Spx^- and a $Spx^-/\Delta ccpA$ mutant were as controls for diminished H_2O_2 production. (B) The specific production of H_2O_2 was determined with planktonic cells grown under conditions of maximal H_2O_2 production. Detection limit for the assay was 0.125 mM H_2O_2 . Presented are averages and standard deviations of two independent experiments.

Increased H_2O_2 production in the $\Delta ccpA$ mutant. Expression of *spx* and pyruvate oxidase abundance was increased in the $\Delta ccpA$ mutant. To learn if the production of H_2O_2 is affected under aerobic growth conditions, surface biofilms were grown on H_2O_2 indicator plates. Plates were inoculated with overnight grown static cultures adjusted to the same cell numbers and immediately incubated. The plates were removed at 2, 5, and 7 h postinoculation and photographed to document color development over time. After 2 h of incubation, an obvious difference between the wild type and the $\Delta ccpA$ mutant was visible, with dark blue color development for the mutant and only slight development for the wild type. This was also observed after longer incubation, but the wild type started to produce H_2O_2 , and blue color development was visible. The $\Delta ccpA$ mutant, however, always showed an increased diameter of the blue area. The restored mutant behaved like the wild type, while the Spx^- and the $Spx^-/\Delta ccpA$ mutant showed a slight blue color development only after 7 h, indicating other sources of intracellular H_2O_2 (Fig. 4A). The specific production of H_2O_2 was determined with planktonic cells grown under conditions of maximal H_2O_2 production. Under these conditions the H_2O_2 production was ~3.5-fold increased in the $\Delta ccpA$ mutant (Fig. 4B). The production of H_2O_2 in the Spx^- mutant and the $Spx^-/\Delta ccpA$ mutant was below detection level. The data suggest that CcpA directly controls the production of H_2O_2 under planktonic and biofilm growth conditions.

The $\Delta ccpA$ mutant showed altered cell aggregation, altered colony morphology, and increased interspecies inhibition. To further characterize the consequences of a $\Delta ccpA$ mutation, cell aggregation was measured with planktonic cells grown as static cultures and under H_2O_2 -producing conditions. Although the static grown cultures did not show any sign of aggregation, the shaking cultures of the $\Delta ccpA$ mutant formed visible aggregates and no detectable cell turbidity (Fig. 5A). The cells, however, could be brought into suspension by vortexing. We reported a similar phenotype when increasing amounts of H_2O_2 were added to an Spx^- mutant that normally

FIG. 5. Increased H_2O_2 production leads to cell aggregation, altered colony morphology and increased interspecies inhibition. (A) Cells were grown as static culture and under shaking conditions to maximize aeration and H_2O_2 production. (B) Terminal absorption of cultures grown under shaking conditions. (C) Corresponding CFU counts. Presented are averages and standard deviations of two independent experiments. (D) Colony phenotype of cells grown under aerobic and anaerobic conditions. (E) Antagonism assay of cells overlaid with the H_2O_2 -sensitive *S. mutans* strain UA140. The contrast and brightness was adjusted to the entire field of all pictures. Presented are representative images of three independent experiments.

would not form aggregates (19). The wild type showed precipitation of cells, indicating aggregation, but a visible amount of cells remained in suspension, consistent with our earlier observations. Aggregation of the restored strain was visible but less obvious compared to the wild type (Fig. 5A).

When the cell densities of resuspended cells were compared, the $\Delta ccpA$ mutant showed a 5-fold reduction compared to the wild type and restored mutant. The Spx^- and the $Spx^-/\Delta ccpA$ mutant increased in cell density, probably due to diminished H_2O_2 production (Fig. 5B). Interestingly, when cell viability

was determined, CFU counts were consistent with the optical densities, except for the $\text{Spx}^-/\Delta\text{ccpA}$ mutant (Fig. 5C). The double mutant had CFU counts comparable to those of the ΔccpA mutant, although no detectable H_2O_2 was produced. Why the double mutant can exceed the optical density of the non- H_2O_2 -producing Spx^- mutant but shows an increase in cell death is currently under investigation. These results suggest that CcpA is involved in the control of H_2O_2 induced aggregation and that increased H_2O_2 production leads to a reduced cell biomass under aerobic conditions.

The colony morphology changed obviously when cells of the ΔccpA mutant were incubated in the presence of oxygen. The colonies of the ΔccpA mutant appeared smaller in diameter and more translucent compared to those of the wild type (Fig. 5D). The restored mutant produced colonies like the wild type. The smaller colony size of the ΔccpA mutant is most likely a result of the growth inhibition by the increased production of H_2O_2 . No obvious difference was observed with cells incubated under anaerobic conditions (Fig. 5D).

Although conditions where the CcpA mediated repression is lifted are yet to be identified, a possible advantage of derepressed *spx* expression was tested in an interspecies antagonism assay. A clear advantage is reflected in the size of the inhibition zone of the ΔccpA mutant. Despite reduced cell density after 6 h of growth prior to the overlay with *S. mutans*, the ΔccpA mutant showed an increased inhibition zone compared to the wild type and the restored mutant (Fig. 5E).

In summary, increased production of H_2O_2 has a profound impact on the phenotypic appearance of the ΔccpA mutant, causing growth inhibition and cell aggregation. Increased H_2O_2 production seems to be counterproductive regarding cell growth but might actually confer some ecological advantage under competitive growth conditions.

CcpA controls cell death in biofilms. To test whether the increase in H_2O_2 production influences cell survival of *S. sanguinis* ΔccpA mutant biofilms, the bacterial viability was assessed with the Live/Dead BacLight bacterial viability fluorescent stains. Cells were grown as static biofilms in BHI with 1% sucrose. The wild type, the ΔccpA mutant, and the restored strain all formed readily biofilms under these conditions. The increased H_2O_2 production seemed to affect the ΔccpA mutant showing an increase in membrane compromised cells (increase in red fluorescence) compared to the wild type (Fig. 6A), whereas the cell density was only marginally affected, as determined spectrophotometrically with resuspended cells (data not shown). The restored strain behaved like the wild type. The Spx^- mutant formed a dense biofilm with a few red cells visible in agreement with no H_2O_2 production as shown previously (19). Interestingly, the $\text{Spx}/\Delta\text{ccpA}$ strain formed a dense biofilm but showed an increase red fluorescent cells similar to what was observed with the planktonic cultures, indicating that the double mutant might be impaired in other cellular processes. This is currently under investigation. In summary, this suggests that CcpA controls cell viability in monospecies biofilms, as demonstrated with an increase in membrane-compromised cells in the ΔccpA mutant.

H_2O_2 induces eDNA release, potentially leading to membrane compromised cells as demonstrated earlier (19). To test whether the ΔccpA mutant releases more eDNA, as predicted by an increase in membrane compromised cells,

eDNA was isolated from biofilms and analyzed with agarose gel electrophoresis and semiquantitative PCR, as described previously (19). As shown in Fig. 6B, the relative amount of eDNA was highest in the ΔccpA mutant compared to the wild type and the restored strain. This was confirmed with semiquantitative PCR using the eDNA and specific primers for the *spx* promoter. The results corroborate the correlation of CcpA-controlled H_2O_2 production, membrane-compromised cells, and eDNA release.

DISCUSSION

The pyruvate oxidase metabolic function catalyzes the formation of acetyl-phosphate from pyruvate. This process produces CO_2 and H_2O_2 (4, 5). While the role of acetyl-phosphate is clear, being converted to acetate by the acetate kinase generating ATP, the function of H_2O_2 might be more diverse and not yet entirely understood. We previously showed that the oral streptococci *S. sanguinis* and *S. gordonii* generate growth-inhibiting amounts of H_2O_2 when tested in an *in vitro* interspecies competition assay with *S. mutans* (18, 20). These results suggest an important function of H_2O_2 in oral biofilm ecology since initial colonizers, such as *S. sanguinis* and *S. gordonii*, influence spatial and temporal biofilm development (15). Their successful initial colonization might result in the production of H_2O_2 thus preventing H_2O_2 susceptible conditional pathogens from reaching critical, disease-inducing cell densities. Moreover, H_2O_2 seems to be involved in an important developmental process in *S. sanguinis* and *S. gordonii*. When grown under aerobic conditions, about 10% of cells enter a membrane-compromised state, as shown with the BacLight Bacterial viability stain, indicating cell death. This state is dependent on the functional expression of the pyruvate oxidase and H_2O_2 generation (19). Correspondingly, there is strong evidence that these cells release DNA into the environment (eDNA). A pyruvate oxidase mutant unable to produce H_2O_2 only shows about 1% of membrane-compromised cells and ~10-fold less eDNA when grown under aerobic conditions as a shaken culture. Under the conditions tested here, the Spx^- mutant did show red cells in amounts comparable to those of the wild type. This is most likely due to the fact that the biofilms were grown as static cultures, which limits oxygen availability over time due to oxygen consumption. Therefore, the biofilms are more comparable to an anaerobic culture. Since the eDNA has been shown for *S. sanguinis*, as well as other bacteria, to be important in cell-cell adherence and biofilm formation (36, 40, 45), the pyruvate oxidase-dependent H_2O_2 -mediated release of eDNA might be regarded as a developmental process to ensure survival of the population (19). Oral streptococci require attachment to the tooth surface or face extinction by swallowing.

Our initial finding that the *spx* promoter of *S. sanguinis* contains a *cre* sequence for CcpA binding was unexpected, since the expression is only marginally influenced by glucose. We therefore determined the transcription start site in the wild type and the ΔccpA mutant. No difference was detected. Transcription starts 9 bp upstream of the potential CcpA binding site (Fig. 1A). The location of the *cre* is in agreement with a repressive function of CcpA and might block binding of the DNA polymerase. During the preparation of the present study,

FIG. 6. Confocal laser scanning microscopic analysis to determine cell viability in biofilms and the release of eDNA. (A) Cells were grown overnight in BHI plus sucrose to promote adhesion. The bacterial viability was assessed with the Live/Dead BacLight bacterial viability fluorescent stains. Images were captured with a Leica SP2 MP confocal microscope equipped with fluorescent filters for red and green fluorescence detection ($\times 40$ HCX APO 0.8 NA dipping lens). White bar, 50 μ m. (B) Agarose gel electrophoresis (1%) stained with ethidium bromide (1 μ g/ml) of high-molecular-weight DNA (lanes 1 to 3) and semiquantitative PCR products using the precipitated eDNA as a template for PCR amplification with the primers Ss spxB F and Ss spxB R. PCR cycles were set to 25 repeats. The DNA size marker used was 1 Kb Plus DNA ladder (Invitrogen). A black arrow depicts the relevant DNA band size. The photograph is representative of three independent experiments with similar results.

a screen for hydrogen peroxide production-related genes in *S. sanguinis* did identify three genes with decreased H_2O_2 production. These colonies appeared more opaque compared to the wild type. This is similar to the observation we made with cells grown under anaerobic conditions that appeared more white and opaque compared to aerobically grown cells. CcpA was not identified as regulator, primarily due to the screening for better-growing cells (7).

The observed constant repression of *spx* is in contrast to the well-studied catabolite repression-like regulation for the pyruvate oxidase gene *poxB* in *Lactobacillus plantarum*. Growth in the presence of glucose resulted in nondetectable transcripts, as determined by Northern blot analysis. Once glucose was exhausted, stable transcripts were detected for several hours. Expression of *poxB* in the *L. plantarum* CcpA⁻ mutant was not subject to glucose repression and showed comparable levels to the wild type grown without glucose (22).

H_2O_2 -producing enzymes such as the pyruvate oxidases of *S.*

sanguinis, *L. plantarum* and *S. gordonii* (unpublished data) all seem to be under the control of CcpA or have a potential *cre* site such as *S. pneumoniae* (37, 44). Interestingly, *Streptococcus pyogenes*, although not encoding a pyruvate oxidase, produces significant amounts of H_2O_2 by the lactate oxidase (gene *lox*). Kietzman and Caperon recently demonstrated that *lox* gene expression is under the control of carbon catabolite repression. These authors identified a *cre* in the *lox* promoter and showed decreased expression of *lox* when grown in the presence of various amounts of glucose (12).

Thus far, all Gram-positive species investigated encoding a pyruvate oxidase homolog show decreased survival rates when the enzymatic activity leads to the production and accumulation of H_2O_2 *in vitro*. A similar observation was made with the H_2O_2 -producing lactate oxidase of *S. pyogenes*. Interestingly, *Staphylococcus aureus* encodes a pyruvate oxidase, CidC, involved in cell survival but with a different mechanism. Patton et al. showed that CidC does perform the expected enzymatic

conversion of pyruvate to acetyl-phosphate but did not detect any H_2O_2 production. CidC is therefore considered as functionally analog to the *E. coli* pyruvate oxidase, yielding acetate and CO_2 as end products. Nonetheless, when *cidC* was inactivated, cells survived significantly longer compared to the wild type under growth conditions with a high glucose concentration. The increased cell death in the wild type was caused by the accumulation of acetate in the growth medium with subsequent drop in pH diminishing viability (33). Gram-negative bacteria such as *Pseudoalteromonas tunicata* control cell death during biofilm formation dependent on the production of H_2O_2 . The H_2O_2 production is dependent on the lysine-oxidase AlpP (24). Several other Gram-negative species encode for homologs of AlpP, suggesting a similar function of H_2O_2 (23).

The accumulated evidence suggests that the pyruvate oxidase and H_2O_2 production has an important function in the control of bacterial cell death in several species. Furthermore, it was suggested that bacterial cell death is related to the metabolic status of the cell (39). CcpA would be a good candidate to control this process since CcpA is one of the major regulators able to monitor the cellular metabolic status (9). However, why is the CcpA-controlled *spx* expression not influenced by glucose? One possible explanation could be that *S. sanguinis* shifts the control of CcpA-dependent repression toward other key metabolites. For example, a glucose-independent carbon catabolite regulation has been described for *Bacillus subtilis* before (28), but at this point we can only speculate that CcpA might bind to other metabolites such as NADP, which has been identified as corepressor for CcpA in *B. subtilis* (13). In this way *S. sanguinis* is still able to monitor the level of key metabolite via CcpA as a measure of its metabolic status and regulate H_2O_2 production, cell death, and eDNA release. Studies are under way to test whether purified CcpA is able to bind to key metabolites and the potential *cre* in the *spx* promoter.

In summary, we have shown here the important regulatory role of CcpA in the control of H_2O_2 production, H_2O_2 -dependent cell death, and eDNA release. CcpA represses *spx* expression and is not influenced by glucose. Derepression in a CcpA mutant leads to increased expression of *spx*, a greater abundance of Spx, and elevated H_2O_2 production. The ecological advantage of this unusual CcpA-dependent regulation might have led to increased numbers of *S. sanguinis* compared to *S. gordonii* in the dental biofilm and could be one of the reasons why *S. sanguinis* is able to successfully compete with conditional pathogens such as *S. mutans* in the oral biofilm.

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REFERENCES

1. Aas, J. A., B. J. Paster, L. N. Stokes, I. Olsen, and F. E. Dewhirst. 2005. Defining the normal bacterial flora of the oral cavity. *J. Clin. Microbiol.* **43**:5721–5732.
2. Abranches, J., M. M. Nascimento, L. Zeng, C. M. Browngardt, Z. T. Wen, M. F. Rivera, and R. A. Burne. 2008. CcpA regulates central metabolism and virulence gene expression in *Streptococcus mutans*. *J. Bacteriol.* **190**:2340–2349.
3. Becker, M. R., B. J. Paster, E. J. Leys, M. L. Moeschberger, S. G. Kenyon, J. L. Galvin, S. K. Boches, F. E. Dewhirst, and A. L. Griffen. 2002. Molecular analysis of bacterial species associated with childhood caries. *J. Clin. Microbiol.* **40**:1001–1009.
4. Carlsson, J., and M. B. Edlund. 1987. Pyruvate oxidase in *Streptococcus sanguis* under various growth conditions. *Oral Microbiol. Immunol.* **2**:10–14.
5. Carlsson, J., M. B. Edlund, and S. K. Lundmark. 1987. Characteristics of a hydrogen peroxide-forming pyruvate oxidase from *Streptococcus sanguis*. *Oral Microbiol. Immunol.* **2**:15–20.
6. Caufield, P. W., A. P. Dasanayake, Y. Li, Y. Pan, J. Hsu, and J. M. Hardin. 2000. Natural history of *Streptococcus sanguinis* in the oral cavity of infants: evidence for a discrete window of infectivity. *Infect. Immun.* **68**:4018–4023.
7. Chen, L., X. Ge, Y. Dou, X. Wang, J. R. Patel, and P. Xu. 2010. Identification of hydrogen peroxide production related genes in *Streptococcus sanguinis* and their functional relationship with pyruvate oxidase. *Microbiology* doi: 10.1099/mic.0.039669-0.
8. Gilliland, S. E. 1969. Enzymatic determination of residual hydrogen peroxide in milk. *J. Dairy Sci.* **52**:321–324.
9. Gorke, B., and J. Stulke. 2008. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat. Rev. Microbiol.* **6**:613–624.
10. Hamada, S., and T. Ooshima. 1975. Inhibitory spectrum of a bacteriocinlike substance (mutacin) produced by some strains of *Streptococcus mutans*. *J. Dent. Res.* **54**:140–145.
11. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**:557–580.
12. Kietzman, C. C., and M. G. Caparon. 2010. CcpA and LacD.1 affect temporal regulation of *Streptococcus pyogenes* virulence genes. *Infect. Immun.* **78**:241–252.
13. Kim, J. H., M. I. Voskuil, and G. H. Chambliss. 1998. NADP, corepressor for the *Bacillus* catabolite control protein CcpA. *Proc. Natl. Acad. Sci. U. S. A.* **95**:9590–9595.
14. Kinkel, T. L., and K. S. McIver. 2008. CcpA-mediated repression of streptolysin S expression and virulence in the group A streptococcus. *Infect. Immun.* **76**:3451–3463.
15. Kolenbrander, P. E., R. J. Palmer, Jr., S. Periasamy, and N. S. Jakubovics. 2010. Oral multispecies biofilm development and the key role of cell-cell distance. *Nat. Rev. Microbiol.* **8**:471–480.
16. Kreth, J., D. C. Hung, J. Merritt, J. Perry, L. Zhu, S. D. Goodman, D. G. Cvitkovitch, W. Shi, and F. Qi. 2007. The response regulator ComE in *Streptococcus mutans* functions both as a transcription activator of mutacin production and repressor of CSP biosynthesis. *Microbiology* **153**:1799–1807.
17. Kreth, J., J. Merritt, and F. Qi. 2009. Bacterial and host interactions of oral streptococci. *DNA Cell Biol.* **28**:397–403.
18. Kreth, J., J. Merritt, W. Shi, and F. Qi. 2005. Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. *J. Bacteriol.* **187**:7193–7203.
19. Kreth, J., H. Vu, Y. Zhang, and M. C. Herzberg. 2009. Characterization of hydrogen peroxide-induced DNA release by *Streptococcus sanguinis* and *Streptococcus gordonii*. *J. Bacteriol.* **191**:6281–6291.
20. Kreth, J., Y. Zhang, and M. C. Herzberg. 2008. Streptococcal antagonism in oral biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* interference with *Streptococcus mutans*. *J. Bacteriol.* **190**:4632–4640.
21. Loimaranta, V., J. Tenovou, L. Koivisto, and M. Karp. 1998. Generation of bioluminescent *Streptococcus mutans* and its usage in rapid analysis of the efficacy of antimicrobial compounds. *Antimicrob. Agents Chemother.* **42**:1906–1910.
22. Lorquet, F., P. Goffin, L. Muscariello, J. B. Baudry, V. Ladero, M. Sacco, M. Kleerebezem, and P. Hols. 2004. Characterization and functional analysis of the *poxB* gene, which encodes pyruvate oxidase in *Lactobacillus plantarum*. *J. Bacteriol.* **186**:3749–3759.
23. Mai-Prochnow, A., P. Lucas-Elio, S. Egan, T. Thomas, J. S. Webb, A. Sanchez-Amat, and S. Kjelleberg. 2008. Hydrogen peroxide linked to lysine oxidase activity facilitates biofilm differentiation and dispersal in several Gram-negative bacteria. *J. Bacteriol.* **190**:5493–5501.
24. Mai-Prochnow, A., J. S. Webb, B. C. Ferrari, and S. Kjelleberg. 2006. Ecological advantages of autolysis during the development and dispersal of *Pseudoalteromonas tunicata* biofilms. *Appl. Environ. Microbiol.* **72**:5414–5420.
25. Marquis, R. E. 1995. Oxygen metabolism, oxidative stress and acid-base physiology of dental plaque biofilms. *J. Ind. Microbiol.* **15**:198–207.
26. Martin, B., G. Alloing, V. Mejean, and J. P. Claverys. 1987. Constitutive expression of erythromycin resistance mediated by the *ermAM* determinant of plasmid pAM beta 1 results from deletion of 5' leader peptide sequences. *Plasmid* **18**:250–253.
27. Moffatt, B. A., and F. W. Studier. 1987. T7 lysozyme inhibits transcription by T7 RNA polymerase. *Cell* **49**:221–227.
28. Moreno, M. S., B. L. Schneider, R. R. Maile, W. Weyler, and M. H. Saier, Jr. 2001. Catabolite repression mediated by the CcpA protein in *Bacillus subtilis*: novel modes of regulation revealed by whole-genome analyses. *Mol. Microbiol.* **39**:1366–1381.
29. Nobbs, A. H., R. J. Lamont, and H. F. Jenkinson. 2009. *Streptococcus* adherence and colonization. *Microbiol. Mol. Biol. Rev.* **73**:407–450.
30. Nobbs, A. H., Y. Zhang, A. Khammanivong, and M. C. Herzberg. 2007.

- Streptococcus gordonii* Hsa environmentally constrains competitive binding by *Streptococcus sanguinis* to saliva-coated hydroxyapatite. *J. Bacteriol.* **189**: 3106–3114.
31. Novichkov, P. S., O. N. Laikova, E. S. Novichkova, M. S. Gelfand, A. P. Arkin, I. Dubchak, and D. A. Rodionov. 2010. RegPrecise: a database of curated genomic inferences of transcriptional regulatory interactions in prokaryotes. *Nucleic Acids Res.* **38**:D111–D118.
 32. Okinaga, T., Z. Xie, G. Niu, F. Qi, and J. Merritt. 2010. Examination of the *hdrRM* regulon yields insight into the competence system of *Streptococcus mutans*. *Mol. Oral Microbiol.* **25**:165–177.
 33. Patton, T. G., K. C. Rice, M. K. Foster, and K. W. Bayles. 2005. The *Staphylococcus aureus* *cidC* gene encodes a pyruvate oxidase that affects acetate metabolism and cell death in stationary phase. *Mol. Microbiol.* **56**: 1664–1674.
 34. Podbielski, A., B. Spellerberg, M. Woischnik, B. Pohl, and R. Luttmann. 1996. Novel series of plasmid vectors for gene inactivation and expression analysis in group A streptococci (GAS). *Gene* **177**:137–147.
 35. Qi, F., P. Chen, and P. W. Caufield. 2001. The group I strain of *Streptococcus mutans*, UA140, produces both the lantibiotic mutacin I and a nonlantibiotic bacteriocin, mutacin IV. *Appl. Environ. Microbiol.* **67**:15–21.
 36. Qin, Z., Y. Ou, L. Yang, Y. Zhu, T. Tolker-Nielsen, S. Molin, and D. Qu. 2007. Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. *Microbiology* **153**:2083–2092.
 37. Ramos-Montanez, S., H. C. Tsui, K. J. Wayne, J. L. Morris, L. E. Peters, F. Zhang, K. M. Kazmierczak, L. T. Sham, and M. E. Winkler. 2008. Polymorphism and regulation of the *spxB* (pyruvate oxidase) virulence factor gene by a CBS-HotDog domain protein (SpxR) in serotype 2 *Streptococcus pneumoniae*. *Mol. Microbiol.* **67**:729–746.
 38. Regev-Yochay, G., K. Trzcinski, C. M. Thompson, R. Malley, and M. Lipsitch. 2006. Interference between *Streptococcus pneumoniae* and *Staphylococcus aureus*: in vitro hydrogen peroxide-mediated killing by *Streptococcus pneumoniae*. *J. Bacteriol.* **188**:4996–5001.
 39. Rice, K. C., and K. W. Bayles. 2008. Molecular control of bacterial death and lysis. *Microbiol. Mol. Biol. Rev.* **72**:85–109.
 40. Rice, K. C., E. E. Mann, J. L. Endres, E. C. Weiss, J. E. Cassat, M. S. Smeltzer, and K. W. Bayles. 2007. The *cidA* murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. U. S. A.* **104**:8113–8118.
 41. Saito, M., M. Seki, K. Iida, H. Nakayama, and S. Yoshida. 2007. A novel agar medium to detect hydrogen peroxide-producing bacteria based on the Prussian blue-forming reaction. *Microbiol. Immunol.* **51**:889–892.
 42. Sambrook, J., E. F. Fritsch, and T. Maniatis (ed.). 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
 43. Stingl, C. S., K. Eschrich, A. C. Rodloff, R. Schaumann, and H. Jentsch. 2008. Periodontitis is associated with a loss of colonization by *Streptococcus sanguinis*. *J. Med. Microbiol.* **57**:495–499.
 44. Taniai, H., K. Iida, M. Seki, M. Saito, S. Shiota, H. Nakayama, and S. Yoshida. 2008. Concerted action of lactate oxidase and pyruvate oxidase in aerobic growth of *Streptococcus pneumoniae*: role of lactate as an energy source. *J. Bacteriol.* **190**:3572–3579.
 45. Thomas, V. C., L. R. Thurlow, D. Boyle, and L. E. Hancock. 2008. Regulation of autolysis-dependent extracellular DNA release by *Enterococcus faecalis* extracellular proteases influences biofilm development. *J. Bacteriol.* **190**: 5690–5698.
 46. Trieu-Cuot, P., and P. Courvalin. 1983. Nucleotide sequence of the *Streptococcus faecalis* plasmid gene encoding the 3′5′-aminoglycoside phosphotransferase type III. *Gene* **23**:331–341.
 47. Xu, P., J. M. Alves, T. Kitten, A. Brown, Z. Chen, L. S. Ozaki, P. Manque, X. Ge, M. G. Serrano, D. Puiu, S. Hendricks, Y. Wang, M. D. Chaplin, D. Akan, S. Paik, D. L. Peterson, F. L. Macrina, and G. A. Buck. 2007. Genome of the opportunistic pathogen *Streptococcus sanguinis*. *J. Bacteriol.* **189**:3166–3175.