

Environmental Influences on Competitive Hydrogen Peroxide Production in *Streptococcus gordonii*[∇]

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Streptococcus gordonii is an important member of the oral biofilm. One of its phenotypic traits is the production of hydrogen peroxide (H₂O₂). H₂O₂ is an antimicrobial component produced by *S. gordonii* that is able to antagonize the growth of cariogenic *Streptococcus mutans*. Strategies that modulate H₂O₂ production in the oral cavity may be useful as a simple therapeutic mechanism to improve oral health, but little is known about the regulation of H₂O₂ production. The enzyme responsible for H₂O₂ production is pyruvate oxidase, encoded by *spxB*. The functional studies of *spxB* expression and SpxB abundance presented in this report demonstrate a strong dependence on environmental oxygen tension and carbohydrate availability. Carbon catabolite repression (CCR) modulates *spxB* expression carbohydrate dependently. Catabolite control protein A (CcpA) represses *spxB* expression by direct binding to the *spxB* promoter, as shown by electrophoretic mobility shift assays (EMSA). Promoter mutation studies revealed the requirement of two catabolite-responsive elements (CRE) for CcpA-dependent *spxB* regulation, as evaluated by *spxB* expression and phenotypic H₂O₂ production assays. Thus, molecular mechanisms for the control of *S. gordonii* *spxB* expression are presented for the first time, demonstrating the possibility of manipulating H₂O₂ production for increased competitive fitness.

Oral biofilm development follows distinct spatial-temporal patterns (28). The developmental process is influenced by environmental fluctuations in nutrient availability, oxygen tension, pH, saliva flow, and host innate immune components (29). Individual biofilm members need to adapt to these environmental fluctuations and compete with other species for the available space (20). The most abundant early oral biofilm formers belong to the genus *Streptococcus* (44). Oral streptococci can initiate biofilm development by expressing surface proteins that specifically interact with components of the salivary pellicle formed on the tooth surface (15, 36). Once the early biofilm has been established, other organisms can join via cell-cell interactions and/or binding to extracellular substances, such as glucans, proteins, and DNA produced or secreted by the early colonizers during biofilm formation (28). Two important pioneer colonizers are the oral commensals *Streptococcus sanguinis* and *Streptococcus gordonii*. In addition to their ability to bind to salivary components such as amylase, both species are known to produce bactericidal amounts of hydrogen peroxide (H₂O₂), possibly as a competitive measure during biofilm development (3, 32, 45). H₂O₂ originates mostly from the enzymatic activity of the pyruvate oxidase, SpxB (encoded by the *spxB* gene) (6, 7). SpxB catalyzes the conversion of pyruvate to acetyl phosphate, which is subsequently converted to acetate by acetate kinase (7). Acetyl phosphate is a central metabolite

also used in the transfer of phosphoryl groups to two-component response regulators and ADP in order to generate ATP for energy (52). The enzymatic reaction requires oxygen, and the end product H₂O₂ leaves the cell, probably through diffusion. H₂O₂ can accumulate to substantial amounts when *S. sanguinis* and *S. gordonii* are cultivated as aerobic batch cultures (32). Concentrations between 1 and 10 mM have been reported (45). The ability to produce H₂O₂ has also been reported for another related species, *Streptococcus pneumoniae*, and is dependent on SpxB activity (48). Interestingly, interspecies interference between *S. pneumoniae* and *Staphylococcus aureus* has been observed in epidemiologic studies (41) and was attributed to the bactericidal activity of H₂O₂ demonstrated in *in vitro* experiments (42, 47).

A similar antagonistic relationship between cariogenic species and oral commensal streptococci has been shown in several clinical studies. For example, subjects with a high abundance of *S. sanguinis* have a lower incidence of caries development, and vice versa (4). Furthermore, initial colonization with *S. sanguinis* delays colonization with cariogenic *Streptococcus mutans* (8). This suggests a protective role of oral commensal streptococci against oral disease development. The relevant *in vivo* mechanisms for this interspecies interference are not known, but several *in vitro* studies strongly suggest an important role for bacteriocins and H₂O₂ in this process (30). Interestingly, *S. mutans* can excrete bacteriocins to engage in interspecies competition with oral commensal streptococci under both aerobic and anaerobic conditions (30, 32). In contrast, *S. sanguinis* and *S. gordonii* produce growth-inhibiting amounts of H₂O₂ only during aerobic growth (32). Further studies with *S. gordonii* revealed that another environmental component is able to interfere with H₂O₂ production: when grown with high

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
DL1	Wild-type <i>S. gordonii</i>	39
DL1 <i>spxBp-luc</i>	<i>spxBp</i> luciferase reporter; Spc ^r	55
DL1 Δ <i>ccpA</i>	Δ <i>ccpA</i> ; Erm ^r	This study
DL1 compl	DL1 Δ <i>ccpA</i> ; <i>ccpA</i> complemented; Spc ^r	This study
DL1 Mut CRE 0	<i>S. gordonii</i> <i>spxB</i> CRE mutagenesis control; Kan ^r	This study
DL1 Mut CRE 1	<i>S. gordonii</i> <i>spxB</i> CRE 1 mutagenesis; Kan ^r	This study
DL1 Mut CRE 2	<i>S. gordonii</i> <i>spxB</i> CRE 2 mutagenesis; Kan ^r	This study
DL1 Mut CRE 1 + 2	<i>S. gordonii</i> <i>spxB</i> CRE 1 + CRE 2 mutagenesis; Kan ^r	This study
DL1 Mut CRE Δ	<i>S. gordonii</i> <i>spxB</i> CRE 1 + CRE 2 deletion; Kan ^r	This study
DH5 α	<i>E. coli</i> ; cloning strain	18
BL21(DE3)pLysS	<i>E. coli</i> ; protein expression strain	35
BL21(pET29b+ <i>ccpA</i>)	<i>E. coli</i> ; protein expression strain; pET29b+ <i>ccpA</i> ; Kan ^r	This study
Plasmids		
pFW5	Suicide vector; Spc ^r	40
pDL278	<i>E. coli-Streptococcus</i> shuttle vector; Spc ^r	13
pLZ2	pFW5 ϕ (<i>spxBp::luc</i>), luciferase reporter; Spc ^r	This study
pGEM-T Easy	Cloning vector; Amp ^r	Promega
pET-29b(+)	His ₆ fusion protein expression vector; Kan ^r	Novagen
pDL278+ <i>ccpA</i> _{compl}	<i>ccpA</i> cloned into pDL278; Spc ^r	This study
pET-29b+ <i>ccpA</i>	pET29b + <i>ccpA</i> ; <i>S. gordonii</i> <i>ccpA</i> overexpression; Kan ^r	This study
pGEM-T- <i>spxB</i> -FLAG	pGEM-T Easy, DL1 <i>spxB</i> -FLAG construction; Amp ^r	This study
pFW5- <i>spxB</i> -flag	pFW5, DL1 <i>spxB</i> -FLAG expression; Spc ^r	This study
pGEM-T-CRE 1	pGEM + <i>spxBp</i> ; CRE 1 mutagenesis; Amp ^r	This study
pGEM-T-CRE 2	pGEM + <i>spxBp</i> ; CRE 2 mutagenesis; Amp ^r	This study
pGEM-T-CRE 1 + 2	pGEM + <i>spxBp</i> ; CRE 1 + CRE 2 mutagenesis; Amp ^r	This study
pGEM-T- CRE Δ	pGEM + <i>spxBp</i> ; CRE 1 + CRE 2 deletion; Amp ^r	This study

concentrations of glucose, *S. gordonii* is no longer able to produce competitive amounts of H₂O₂ and can no longer inhibit H₂O₂-susceptible species, such as *S. mutans* (32). This suggests a carbon catabolite repression (CCR) of H₂O₂ production. CCR in the Gram-positive model species *Bacillus subtilis* is regulated mainly by catabolite control protein A (CcpA), belonging to the LacI/GalR transcriptional regulator family (16). The CcpA DNA binding activity is enhanced by the Hpr component of the phosphoenolpyruvate (PEP)-dependent carbohydrate phosphotransferase system (PTS), which is phosphorylated at Ser-46 (Hpr-Ser-P) during growth on glucose or other PTS carbohydrates. The interaction of CcpA and Hpr-Ser-P promotes binding to specific DNA sequences in the promoter region of CCR-controlled genes, designated catabolite-responsive elements (CRE) (10). CCR and CcpA are known to regulate genes involved in carbon and nitrogen utilization as well as virulence genes in several Gram-positive bacteria, including *S. gordonii* (5, 16, 19).

The regulation of H₂O₂ production in *S. gordonii* is of considerable interest because of the potential protective nature of oral commensals against cariogenic species. Increasing the fitness of oral commensal bacteria could be a valuable alternative approach to caries prevention. In this report, the consequences of oxygen and glucose availability for *spxB* expression and SpxB abundance are further investigated. In addition, evidence is provided that CCR is mediated by several carbohydrate sources and relies on the binding of CcpA to the *spxB* promoter. This is in contrast to the recently reported observation for *S. sanguinis* that CcpA-mediated *spxB* repression is carbohydrate independent (55).

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are listed in Table 1. Strains were routinely grown aerobically (5% CO₂) or in an anaerobic chamber (90% N₂, 5% CO₂, 5% H₂) at 37°C on brain heart infusion (BHI) agar plates but in some cases (as stated) were grown in TYE medium (1% tryptone, 0.5% yeast extract, 0.3% K₂HPO₄). The medium was supplemented with the carbohydrate glucose, galactose, sucrose, maltose, fructose, or lactose from filter-sterilized 20% stock solutions where indicated. For antibiotic selection, cultures were supplemented with the following antibiotics: spectinomycin at 500 µg ml⁻¹, erythromycin at 2 µg ml⁻¹, kanamycin at 300 µg ml⁻¹ for *S. gordonii*, and ampicillin at 100 µg ml⁻¹ for *Escherichia coli*.

DNA manipulations. Standard recombinant DNA techniques were used as described previously (55). Restriction enzymes and DNA ligase were obtained from New England Biolabs (Beverly, MA) or Promega (Madison, WI) and were used as specified by the manufacturer. PCR products were cloned into the pGEM-T Easy kit from Promega. All plasmids were extracted and purified from *E. coli* using a Qiagen (Valencia, CA) miniprep kit. DNA extracted from agarose gels (1%) was purified with a Qiagen QIAquick gel extraction kit. PCR was performed with a G-Storm GS1 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 were synthesized by Integrated DNA Technologies (IDT; Coralville, IA).

Construction of a Δ *ccpA* mutant. A Δ *ccpA* deletion mutant was constructed via double-crossover homologous recombination. To generate the construct, two fragments corresponding to ~800 bp of the upstream and downstream sequences of *ccpA* were amplified 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* Dn R. Each of the primers listed as Up R or Dn F incorporated 25 bases complementary to the erythromycin resistance cassette *ermAM* (34). The erythromycin resistance cassette *ermAM* was amplified by PCR using primers *ermF* and *ermR* as described previously (55). All three PCR amplicons were purified with the Qiagen PCR purification kit and were mixed in a 1:1:1 ratio. The mixture served as a template for a second round of PCR with the appro-

TABLE 2. Primers used in this study

Primer	Sequence (5'–3')	Purpose
16S RNA RT F	AAGCAACGCGAAGAACCTTA	RT-PCR
16S RNA RT R	GTCTCGCTAGAGTGCCCAAC	RT-PCR
Sg. <i>spxB</i> RT F	GGATGCTTTGGCTGAAGAC	RT-PCR
Sg. <i>spxB</i> RT R	GGACCACCTGAACCTACTG	RT-PCR
Sg. <i>spxB</i> F	AGCGGTGACGACTGGGATTGACCCTC	<i>spxB</i> reporter
Sg. <i>spxB</i> R	AGCGGGATCCTGCTGCAGATGCAGTAAT	<i>spxB</i> reporter
Sg. <i>spxB</i> -flag F	AGCGCTCGAGCGTGATTACATGAACAACTCG	<i>spxB</i> -FLAG tag
Sg. <i>spxB</i> -flag R	CGCTAGATCTTTACTTGTGCATCATCGTCTTTGTAATCTTTAATTGC GCGTGATTGCA	<i>spxB</i> -FLAG tag
Sg CcpA-his tag F	AGCGCATATGAACACAGACGATACAGTAAC	CcpA-His tag
Sg CcpA-his tag R	CGCTCTCGAGTTTCTAGTTGAGTTCCGTTT	CcpA-His tag
Sg <i>ccpA</i> Up F	CTTGCTTGATGGCAAAGTCA	<i>ccpA</i> knockout
Sg <i>ccpA</i> Up R	GACAACCCCGACTTTGAA CCATAGAAACCCCTGCTTCA	<i>ccpA</i> knockout
<i>ermF</i>	TTCAAAGTCGGGTGGTTGTC	<i>ccpA</i> knockout
<i>ermR</i>	GAAGCTGTCAGTAGTATACC	<i>ccpA</i> knockout
Sg <i>ccpA</i> Dn F	GGTATACTACTGACAGCTTC GGAAGAGTTGGAAGAACGTGA	<i>ccpA</i> knockout
Sg <i>ccpA</i> Dn R	TGGCATCCTTCTAGCCACTT	<i>ccpA</i> knockout
Sg <i>ccpA</i> compl F	GATGGATCCTGAAACCTTCTTTCTATGCCTCT	<i>ccpA</i> complementation
Sg <i>ccpA</i> compl R	GATGAATTCTGGCAAAGCAAGTCTGTCT	<i>ccpA</i> complementation
Kan F	AGG TGA TAG GTA AGA TTA TAC CG	CRE mutagenesis
Kan R	CCC TAT CTA GCG AAC TTT TAG A	CRE mutagenesis
Sg <i>spxB</i> cre mut up F	CAGCTCAAAAGAAGCGATCA	CRE mutagenesis
Sg <i>spxB</i> cre mut up R	GGTATAATCTTACCTATCACCTGAGGCTGGGACAAAAGTCCT	CRE mutagenesis
Sg <i>spxB</i> cre mut Dn F	TAAAAGTTCGCTAGATAGGG CAGCTACAAGTCTTAGAGGTGCAT	CRE mutagenesis
Sg <i>spxB</i> cre mut Dn R	TTTCAACAACAGCTGGACCTT	CRE mutagenesis
mut Cre1 R	GAACGGAGATACCTCTGGAACTTTACTATATCAATTTACCA	CRE 1 mutagenesis
mut Cre1 F	CCAGAGGTATCTCCGTTTCAAA ATGGAAATGTTTTCAA	CRE 1 mutagenesis
mut Cre2 R	TAGCAGATATGTCCGTTTGTGAATGAAAACGCTTCAAG	CRE 2 mutagenesis
mut Cre2 F	AAACGGACATATCTGCTATAACTGAAAATTTATTTTATTGAAG	CRE 2 mutagenesis
mut Cre3 (both) F	CCAGAGGTATCTCCGTTTCAAA ACGGACATATCTGCTA	CRE 1 + CRE 2 mutagenesis
delete both Up R	CAATAAAATAAAATTTTCAGTAACTTTACTATATCAATTTACCA	CRE 1 + CRE 2 deletion
delete both Dn F	ACTGAAAATTTATTTTATTGAAGGAGA	CRE 1 + CRE 2 deletion
5' RACE PCR adapter	PHOS-TTT AGT GAG GGT TAA TAA GCG GCC GCG TCG TGA CTG GGAGCG C	5' RACE PCR
5' RACE PCR adapter primer	GCG GCC GCT TAT TAA CCC TCA CTA AA	5' RACE PCR
5' RACE PCR <i>spxB</i> gene-specific primer	GTATGAACCTGAACCGTAGTATGAGTTT	5' RACE PCR
EMSA F primer	CAGCTACAAGTCTTAGAGGTGCAT	EMSA
EMSA R primer	Biotin-TGC AGA TGC AGT AAT TTT TCC TTG AG	EMSA

appropriate Up F and Dn R primers. The resulting PCR amplicons were transformed into wild-type (WT) strain DL1 to generate the *ΔccpA* deletion mutant (DL1 *ΔccpA*). Deletion was confirmed by PCR. The mutant was complemented with a copy of *ccpA* expressed in *trans* from shuttle plasmid pDL278. The complete *ccpA* open reading frame and 250 bp of the upstream intergenic region encoding the promoter were amplified by PCR with primer pair Sg *ccpA* compl F/Sg *ccpA* compl R; the amplicon was digested with BamHI/EcoRI and was ligated into pDL278 digested with the same enzymes to generate the complementation plasmid pDL278+*ccpA*_{compl}.

Construction of a pyruvate oxidase carrying a C-terminal FLAG epitope. For the construction of an SpxB 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 (GATTACAAAGACGATGATGA CAAG) upstream of the stop codon using primer pair *spxB*-flag F/*spxB*-flag R. The PCR product was inserted into the pGEM-T Easy vector 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 was inserted into pFW5 digested with the same enzymes. Plasmid pFW5 is not able to replicate in *S. gordonii* and requires integration into the chromosome to confer antibiotic resistance (40). The recombinant plasmid was transformed into wild-type DL1; transformants were selected on BHI plates containing spectinomycin antibiotics and were further analyzed by PCR for integration of the plasmid at the correct chromosomal position.

Western immunoblotting. Western immunoblotting was performed essentially as described previously (55). Briefly, after cell disruption, cytoplasmic extracts were obtained by centrifugation, and the proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was

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 a primary antibody solution (anti-FLAG antibody M2; Stratagene) overnight at 4°C, followed by three wash steps with TBST, and was then incubated with horseradish peroxidase-conjugated secondary antibodies (Thermo Scientific) for 1 h at room temperature. After washing with TBST, the blots were developed using the ECL chemiluminescent detection system (Thermo Scientific). Images of Western immunoblots were obtained by scanning of the respective films. Images were adjusted with GIMP photo processing software, version 2.6.10 (<http://www.gimp.org/>), applied to all parts of the image.

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 previously (38). RACE-specific primers are listed in Table 2.

RNA isolation, cDNA synthesis, and real-time PCR. RNA was isolated using the Qiagen RNeasy kit. cDNA was synthesized using StrataScript reverse transcriptase (Stratagene) according to the manufacturer's protocol. Real-time reverse transcriptase PCR (RT-PCR) was performed to detect specific transcripts by the comparative threshold cycle (C_T) method using the Bio-Rad (Hercules, CA) MyiQ cycler. Relative changes in gene expression were calculated using the ΔC_T method described previously (55). The 16S rRNA gene was used as the housekeeping reference gene. All primers used for real-time RT-PCR are listed in Table 2.

Luciferase assay. The protocol for the construction of an *spxB* luciferase reporter strain was described previously (55). Luciferase assays were performed using a method described previously (33). Three parallel cultures were measured

at each time point, and the mean value was calculated. Each experiment was repeated at least twice.

Overexpression and purification of CcpA. PCR amplification of *ccpA* with the primer pair Sg CcpA-his tag F/Sg CcpA-his tag R was performed using DL1 chromosomal DNA as the template. The *ccpA* PCR fragment was inserted into the pGEM-T Easy vector, creating plasmid pGEM-T-*ccpA*. The construct was confirmed by sequencing and was subsequently digested with NdeI/XhoI to release *ccpA*. The *ccpA* fragment was ligated into appropriate restriction enzyme sites in pET-29b(+) to generate a CcpA C-terminal His tag. For overexpression, the confirmed plasmid was transformed into *E. coli* BL21(DE3)pLysS. The overexpressed CcpA His tag protein was purified under non-denaturing conditions using the Ni-nitrilotriacetic acid (NTA) purification system according to the manufacturer's protocol (Invitrogen). The protein concentration was determined using the Bradford protein assay (Bio-Rad protein assay) with bovine serum albumin (BSA) as a standard, and the samples were stored at -80°C for future use.

CRE site mutagenesis. To study the roles of the two conserved catabolite-responsive elements (CRE) in the *S. gordonii* *spxB* promoter, overlap extension PCR was used to introduce mutations into the respective CRE. For the mutation of CRE 1, we used primer pairs cre mut up F/mut Cre1 R and mut Cre1 F/cre mut Dn R to generate two DNA fragments with overlapping ends containing the desired CRE 1 mutation. The two PCR fragments were purified with the Qiagen PCR purification kit, mixed in a 1:1 ratio, and used as a template for a second-round PCR with primers cre mut Dn F/cre mut Dn R. The mutated PCR fragment was purified and ligated into pGEM-T, resulting in plasmid pGEM-T-CRE 1. The same strategy was used to mutate CRE 2 (with primer pairs cre mut up F/mut Cre2 R and mut Cre2 F/cre mut Dn R), to mutate both CRE 1 and CRE 2 [with primer pairs cre mut up F/mut Cre2 R and mut Cre3 (both) F/cre mut Dn R], and to completely delete both CRE (with primer pairs cre mut up F/delete both Up R and delete both Dn F/cre mut Dn R). The plasmids constructed, pGEM-T-CRE 1, pGEM-T-CRE 2, pGEM-T-CRE 1 + 2, and pGEM-T-CRE Δ, were all sequenced to confirm the mutations introduced. The respective CRE mutations were transferred to DL1 using an overlap extension PCR strategy, with subsequent transformation of the PCR fragment by chromosomal integration via homologous recombination. Briefly, ~700 bp of the upstream sequences of the CRE using wild-type chromosomal DNA as a template was PCR amplified with the primer pair cre mut up F/cre mut up R. The downstream fragments containing the mutated CRE sites were PCR amplified using the respective plasmids with the primer pair cre mut Dn F/cre mut Dn R. As a control, the same region was amplified from DL1 chromosomal DNA. Each of the primers listed as Up R or Dn F incorporated 25 bases complementary to the kanamycin resistance gene *aphAIII*. The kanamycin resistance gene was amplified by PCR from plasmid pJH1 (50) using primers Kan F and Kan R. All three PCR amplicons (the upstream fragment, kanamycin cassette, and downstream fragment) were purified with the Qiagen PCR purification kit and were 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 the wild-type strain DL1 to generate mutagenesis strains with various CRE sites (DL1 Mut CRE 1, DL1 Mut CRE 2, DL1 Mut CRE 1 + 2, DL1 Mut CRE Δ; strain DL1 Mut CRE 0 works as a control to exclude a polar effect from the introduced kanamycin cassette). All strains were confirmed by PCR.

Electrophoretic mobility shift assays (EMSA). Biotin-labeled DNA probes were generated by PCR using Phusion Hot Start high-fidelity DNA polymerase with primer pair EMSA F/EMSA R; primer EMSA R was biotin labeled at the 5' end by IDT. Chromosomal DNA from the wild type or the respective cre site-mutated strains (DL1 Mut CRE 1, DL1 Mut CRE 2, DL1 Mut CRE 1 + 2, and DL1 Mut CRE Δ) served as a template. All five PCR amplicons were purified with the Qiagen PCR purification kit, and biotin-labeled DNA probes (1 ng) were incubated with varying concentrations of purified CcpA in binding buffer [10 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 1 mM EDTA, 50 mM KCl, 0.05 μg/μl poly(dI-dC), 1 mM MgCl] for 30 min at room temperature. After incubation, samples were mixed with bromophenol blue loading buffer and were separated by electrophoresis using 6% acrylamide gels in 0.5× Tris-borate-EDTA buffer (pH 8.0) that had been prerun for 30 min prior to sample loading. The samples were electroblotted from the gels onto positively charged Zeta-Probe nylon membranes (Bio-Rad). The membranes were UV cross-linked (Stratagene), and the biotinylated probes were detected according to the manufacturer's protocol for the LightShift chemiluminescent EMSA kit (Thermo Scientific).

Measurement of H₂O₂ production. The production of H₂O₂ during planktonic cultivation was performed essentially as described previously (55). H₂O₂ indicator plates were prepared as described elsewhere (46).

RESULTS

Growth and expression of *spxB* in *S. gordonii* strain DL1.

The growth of *S. gordonii* was tested in BHI and TYE media under conditions of low (0.2%) or high (2%) glucose concentrations. No significant difference in growth was observed between 0.2% and 2% glucose (data not shown). The expression of *spxB* over time was determined by quantitative real-time PCR. Planktonic cells were grown in TYE medium plus 0.2% glucose, and cDNA abundance was determined at the time points indicated in Fig. 1. The initial cDNA abundance was low but increased steadily during exponential growth. The transition from late-logarithmic phase to early-stationary phase resulted in a decrease of cDNA abundance (Fig. 1). Further overnight incubation gave the lowest cDNA abundance, about 20-fold lower than that in logarithmic phase. We also used an *spxB* luciferase reporter and confirmed the results from real-time PCR (data not shown). The expression profile of *spxB* suggests that the *spxB* promoter is most actively transcribed during the logarithmic growth phase of *S. gordonii*. Subsequent experiments were therefore performed with cells grown to mid-logarithmic phase. Cells were grown in TYE medium to test for carbohydrate effects; for all other experiments, cells were grown in BHI.

Oxygen influences *spxB* transcription and SpxB abundance.

SpxB-dependent H₂O₂ production requires aerobic conditions (32). Oxygen is one of the SpxB substrates for the conversion of pyruvate into acetyl phosphate (7). The oxygen dependence of *spxB* expression has a profound effect on the competitiveness of *S. gordonii*. During anaerobic growth, *S. gordonii* is not able to produce growth-inhibiting amounts of H₂O₂ to antagonize H₂O₂-susceptible species such as *S. mutans* (32). To test if *S. gordonii* adjusts *spxB* gene expression and SpxB abundance according to oxygen availability, expression and protein production were determined under aerobic and anaerobic growth conditions. Cells were grown in BHI with 0.2% glucose. Real-time PCR was used to compare the *spxB* expression of cells grown with and without oxygen. As shown in Fig. 2A, *spxB* expression increased about 5-fold in the presence of oxygen. To correlate gene expression with protein production, a FLAG epitope was engineered onto the C-terminal end of SpxB. This allows for chromosomal *spxB*-FLAG expression from its own promoter. The FLAG epitope did not influence the production of H₂O₂ under aerobic conditions, which confirmed the functionality of the protein (data not shown). The band intensity in Western immunoblot analysis appeared to be increased with aerobically grown cells (Fig. 2B). Interestingly, a band was detected for anaerobically grown cells, although no H₂O₂ is produced under these conditions (32). This suggests that *S. gordonii* couples *spxB* expression and protein production but ensures the presence of SpxB even under anaerobic conditions, possibly so as to produce competitive H₂O₂ instantaneously when oxygen becomes available.

Glucose represses *spxB* expression and SpxB abundance.

Nutrient availability routinely fluctuates tremendously within the oral cavity. For example, the concentration of glucose can increase as much as 1,000 fold during food intake (1). For *S. mutans*, it has been shown that growth under conditions of nutrient excess represses the production of bacteriocins. It was suggested that the hostile bacteriocin-producing behavior is

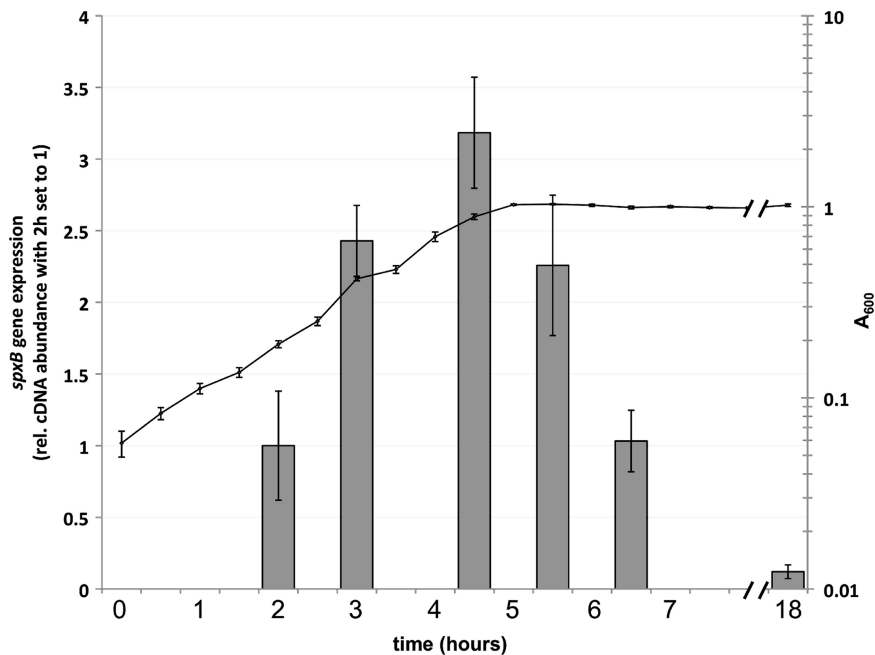


FIG. 1. Growth phase-dependent regulation of *spxB* expression. DL1 was grown in TYE medium plus 0.2% glucose. cDNA abundance and optical density were measured at the indicated time points. The 16S rRNA gene was used as the housekeeping reference gene. The means and standard deviations from two independent experiments are presented. rel., relative.

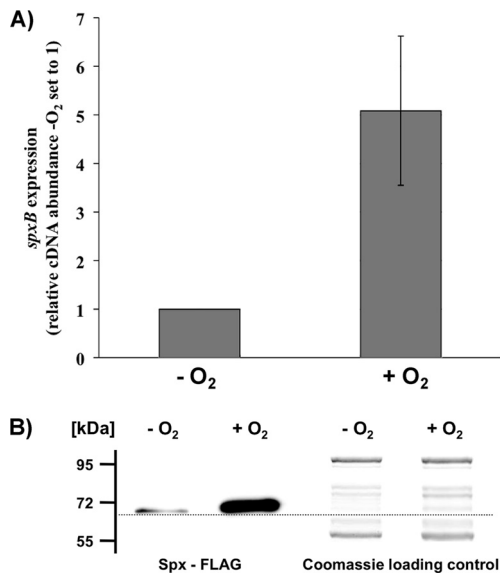


FIG. 2. Effects of oxygen on *spxB* transcription and SpxB abundance. (A) *spxB* transcription was measured by real-time RT-PCR analysis in wild-type DL1. Cells were grown anaerobically or aerobically on BHI agar plates for 7 h at 37°C before RNA isolation. The expression level for *spxB* from cells grown aerobically is presented relative to that from cells grown anaerobically, which was arbitrarily assigned a value of 1. The 16S rRNA gene was used as the housekeeping reference gene. Averages and standard deviations from three independent experiments are presented. (B) Western blot analysis of SpxB protein abundance. Cells were grown anaerobically or aerobically on BHI agar plates for 7 h at 37°C. Pyruvate oxidase production was detected using Western immunoblot analysis with an antibody against the FLAG epitope. A replicate sodium dodecyl sulfate gel was prepared and stained with Coomassie blue as a loading control. The PageRuler prestained protein ladder (Fermentas) was used as a size reference. Relative protein sizes are given on the left.

not required when carbohydrate abundance allows for optimal cell proliferation (30). To test if *S. gordonii spxB* expression is influenced by glucose, cells were grown in TYE medium containing 0.2% or 2% glucose under aerobic conditions until the mid-logarithmic growth phase. To exclude any pH-dependent effect on *spxB* expression, the pHs were measured and found to be similar (pH 6.8 with 0.2% glucose and pH 6.7 with 2% glucose). As shown in Fig. 3A, *spxB* expression decreased about 5-fold in the presence of 2% glucose as measured by RT-PCR (RT-PCR). SpxB protein abundance appeared to decrease as well under the same growth conditions (Fig. 3B). This is in agreement with the inability of *S. gordonii* to inhibit *S. mutans* in an antagonism assay performed in the presence of 1% glucose (32). As with anaerobic conditions, the immunoblot band intensity appeared reduced for 2% glucose, but the enzyme was still present. The amount of H₂O₂ produced under these conditions is not high enough to inhibit *S. mutans* (32). These results strongly suggest that *spxB* expression is regulated by CCR.

CCR of *spxB* expression is not restricted to glucose. To further characterize the extent of CCR, other carbohydrates were tested for their abilities to repress *spxB* expression. Cells were grown under aerobic conditions in TYE medium supplemented with either 0.2% or 2% glucose, galactose, sucrose, maltose, fructose, or lactose. No significant difference in growth was observed between low and high carbohydrate concentrations (data not presented). Growth in the presence of 2% glucose confirmed the RT-PCR results, showing 4-fold reduction in luciferase activity (Fig. 4). Growth in the presence of 0.2% galactose resulted in the highest luciferase activity measured. However, at 2% galactose, there was a 3-fold decrease in luciferase activity. This result is consistent with earlier reports of galactose exerting CCR in *S. gordonii* (49), whereas

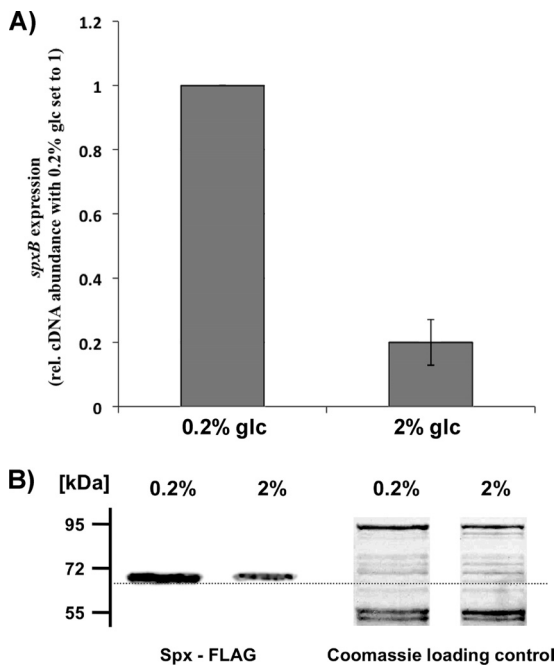


FIG. 3. Glucose-dependent repression of *spxB* expression and SpxB abundance. (A) Comparative real-time RT-PCR analysis was used to determine the effect of glucose on *spxB* transcription. Wild-type DL1 was grown in TYE medium supplied with 0.2% glucose or 2% glucose. The expression level for *spxB* from cells grown with 2% glucose is presented relative to that from cells grown with 0.2% glucose, which was arbitrarily assigned a value of 1. The 16S rRNA gene was used as the housekeeping reference gene. Averages and standard deviations from three independent experiments are presented. (B) Western blot analysis of SpxB protein abundance in cells grown with 0.2% or 2% glucose. Pyruvate oxidase production was detected using Western immunoblot analysis with an antibody against the FLAG epitope. A replicate sodium dodecyl sulfate gel was prepared and stained with Coomassie blue as a loading control. The PageRuler prestained protein ladder (Fermentas) was used as a size reference. Relative protein sizes are given on the left.

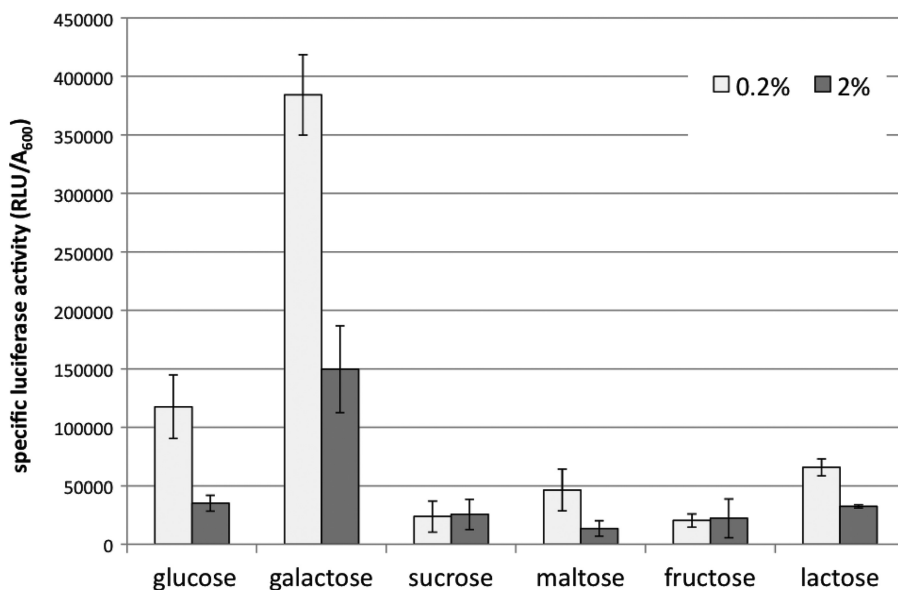


FIG. 4. Carbohydrate-dependent repression of *spxB* expression. The DL1 *spxBp-luc* reporter strain was grown in TYE medium supplemented with the indicated carbohydrate at 0.2% or 2%. Luciferase activity and optical density were measured for cells grown to mid-logarithmic phase (A_{600} , ≈ 0.6). Means and standard deviations from three independent experiments, normalized to optical density, are presented.

in *S. mutans*, galactose is nonrepressing (49). Reductions in luciferase activity were also observed with 2% maltose and 2% lactose, while luciferase activity was not changed with increasing concentrations of sucrose and fructose (Fig. 4). The decrease in luciferase activity suggests that *spxB* expression is subject to CCR by a broad range of carbohydrate sources. This is in contrast to a recent study with *S. sanguinis* demonstrating that *spxB* expression was not significantly repressed by any of the carbohydrates tested, including glucose, maltose, and lactose (55).

CCR of *spxB* expression is mediated through CcpA. CCR is usually mediated through CcpA (51). CcpA-dependent CCR in *S. gordonii* has been demonstrated for the gene encoding amylase-binding protein A and the arginine deiminase operon (12, 43). To test if CCR is mediated through CcpA, the promoter sequence was analyzed for the presence of a catabolite-responsive element (CRE), the binding site for CcpA. As shown in Fig. 5A, two potential CRE, CRE 1 and CRE 2, could be identified upstream of the ribosome binding site. The transcriptional start site for *spxB* was therefore determined using RACE PCR to confirm a potential overlap with CRE 1 and CRE 2. Transcription starts right after a potential extended -10 region, but before CRE 1 (Fig. 5A).

To confirm the CcpA-dependent repression of *spxB* expression, a CcpA mutant was constructed. Initially, the effect of the CcpA mutation on the ability to produce H₂O₂ was tested phenotypically using a colorimetric plate assay (Fig. 5B). As expected, under low-glucose conditions (0.2% glucose), no obvious difference in H₂O₂ production was detected between the wild type, the CcpA mutant, and a complemented strain expressing *ccpA* in *trans* from a shuttle plasmid. When grown in the presence of 2% glucose, the wild type produced less H₂O₂ than the CcpA mutant, confirming that CcpA represses *spxB* expression. The complemented mutant showed very low H₂O₂ production under the conditions tested (Fig. 5B), possibly due

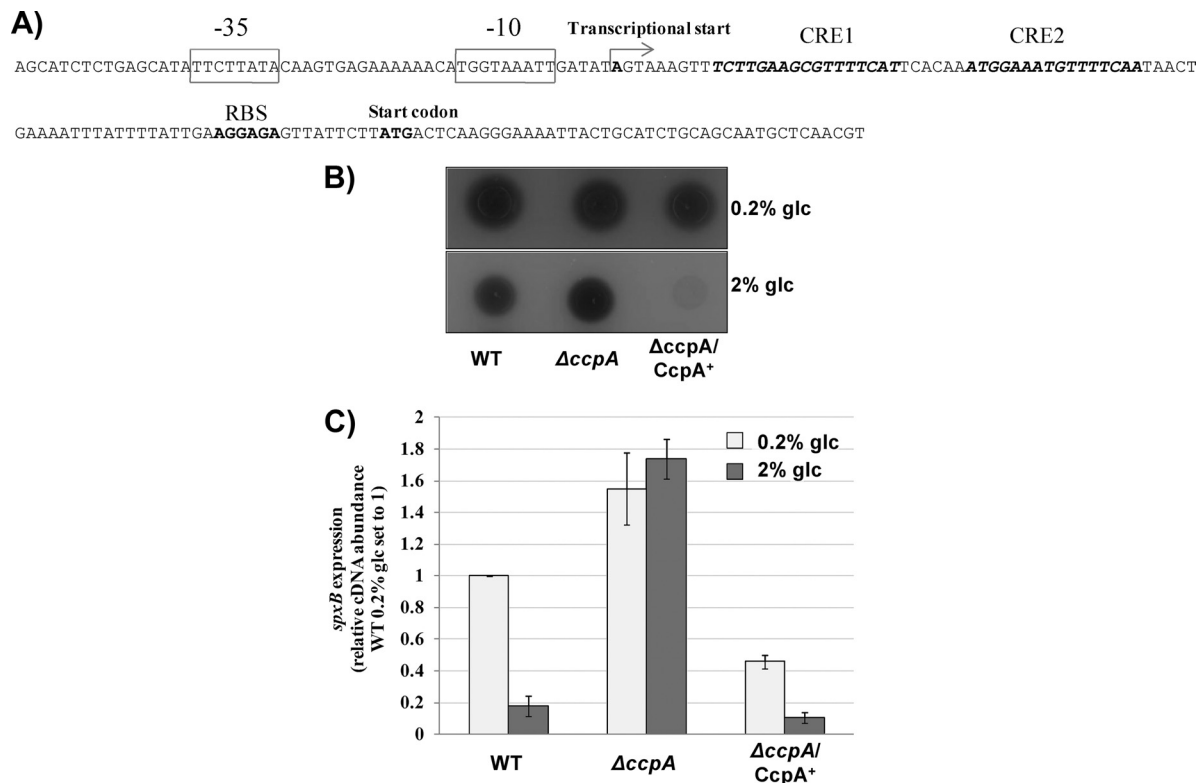


FIG. 5. CCR of *spxB* expression is mediated through CcpA. (A) Organization of the *spxB* promoter region. Presented is the sequence of the promoter region showing the transcriptional start, the predicted -35 and -10 regions, the ribosome binding site (RBS), and the translation start site of *spxB*. The putative CRE are shown in italics. The transcriptional start site was mapped by RACE PCR and is indicated by an arrow. (B) The influence of CcpA on H_2O_2 production was determined with indicator plates. A reaction with H_2O_2 leads to the formation of a blue precipitate (Prussian blue). H_2O_2 production was determined for cells grown with 0.2% glucose or 2% glucose. (C) Real-time RT-PCR analysis of the wild type, the Δ ccpA mutant, and the complemented strain. Cells were grown on BHI agar plates supplemented with 0.2% glucose or 2% glucose for 7 h aerobically at 37°C, and RNA was subsequently isolated. The data are presented as *spxB* expression levels relative to that of the wild type grown on BHI agar plates supplemented with 0.2% glucose, which was arbitrarily assigned a value of 1. The 16S rRNA gene was used as the housekeeping reference gene. Averages and standard deviations from three independent experiments are presented.

to the increased abundance of CcpA as a result of multicopy plasmid-encoded *ccpA*.

The expression of *spxB* was measured to confirm the phenotype. Cells were grown aerobically in TYE medium with low or high glucose concentrations. In agreement with H_2O_2 production, the most obvious difference was observed in the presence of 2% glucose. *spxB* expression in the CcpA mutant increased about 10-fold over that in the wild type, but that in the complemented strain was reduced about 2-fold (Fig. 5C). The results suggest a CcpA-dependent repression of *spxB* expression when the preferred carbohydrate sources are available. To exclude any CcpA-mediated regulation during the switch from aerobic to anaerobic growth, *spxB* abundance was determined in the CcpA mutant under conditions of anaerobic growth. *spxB* expression was similarly reduced in anaerobically grown CcpA mutant and wild-type cells. CcpA therefore seems not to be the transcriptional regulator mediating *spxB* repression under anaerobic conditions (data not shown).

Functional characterization of CcpA regulation. The presence of two or more CRE in CcpA-controlled promoter regions is not unusual (17). It has been demonstrated previously that CcpA binding covers more than the chromosomal region harboring the respective CRE, ranging from 20

to 40 bp (the predicted sizes of CRE 1 and CRE 2 are 16 bp) (17, 25, 26). The predicted CRE 1 and CRE 2 are located in close proximity, separated by 6 bp, suggesting that binding of CcpA to either one could sterically block the binding of an additional CcpA to the free CRE. To determine whether CcpA directly repressed *spxB* expression, EMSA analysis was performed. CcpA was overexpressed with an engineered C-terminal His tag and was purified using nickel affinity chromatography. To assess the importance of the individual CRE in the binding of CcpA, nucleotide exchanges were introduced into CRE 1 (Mut CRE 1), CRE 2 (Mut CRE 2), or CRE 1 and CRE 2 (Mut CRE 1 + 2). In addition, both CRE 1 and CRE 2 were deleted (Mut CRE Δ) (Fig. 6A). As shown in Fig. 6B, Mut CRE 1 + 2 prevented the binding of CcpA to the *spxB* promoter sequence used for EMSA; no shift in the DNA band was observed. The wild-type DNA sequence enabled CcpA binding, and a shift was observed, with increasing band intensity concomitant with larger amounts of CcpA. Mut CRE Δ prevented CcpA binding as well. The individual Mut CRE 1 or Mut CRE 2 mutant still exhibited a mobility shift (Fig. 6B). In conclusion, CcpA likely regulates *spxB* expression in *S. gordonii* by directly binding to the CRE sites in the *spxB* promoter, with both

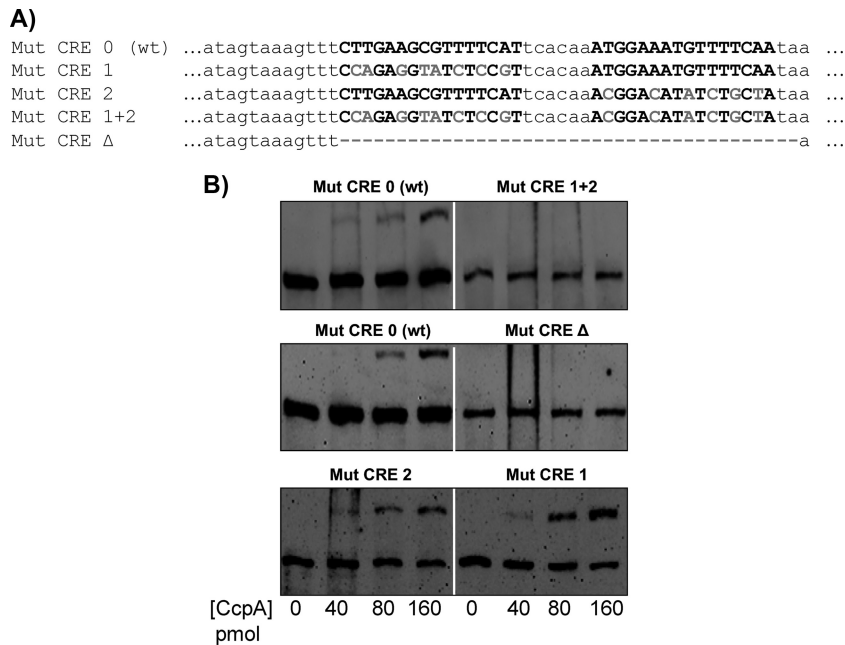


FIG. 6. EMSA for CcpA DNA binding studies. (A) Sequence of the *spxB* promoter used for site-directed mutagenesis. The putative CRE are in boldface (wild-type CRE or mutated CRE). Gray letters represent exchanged nucleotides, and dashes indicate deletions. All other bases in the DNA fragments are identical. (B) EMSA analysis was performed using purified recombinant CcpA and a biotin-labeled DNA probe containing wild-type CRE or various mutated CRE. Increasing concentrations of CcpA (40, 80, and 160 pmol) were incubated with 1 ng of the indicated DNA probe before the reactions were run on a nondenaturing polyacrylamide gel.

CRE 1 and CRE 2 promoting CcpA protein-DNA interactions.

Consequences of CRE site mutations for H₂O₂ production.

To further characterize the roles of the individual CRE sites in the regulation of *spxB* expression, the Mut CRE 1 and Mut CRE 2 mutations, as well as the Mut CRE 1 + 2 and Mut CRE Δ mutations, were transferred to the chromosome of *S. gordonii* using double-crossover homologous recombination. This required the introduction of a kanamycin resistance cassette upstream of the *spxB* promoter for selection. To prove that the kanamycin cassette did not cause any adverse effects on *spxB* expression, the same strategy for homologous recombination with the mutated promoter sequences was used with the wild-type *spxB* promoter (Mut CRE 0). Compared to the wild-type CRE, the wild-type CRE carrying the kanamycin cassette (Mut CRE 0) produced equal amounts of H₂O₂ in the colorimetric plate assay when grown with 0.2% and 2% glucose, respectively. An increase in H₂O₂ production was observed for the Mut CRE 1 + 2 mutant grown with 2% glucose, confirming the observation from the EMSA showing no binding of CcpA if both sites are mutated. Therefore, no repression occurred. Surprisingly, the individual Mut CRE 1 or Mut CRE 2 mutant seemed to promote CcpA repression. Both mutants showed decreased H₂O₂ production, even when grown in the presence of 0.2% glucose (Fig. 7A and B).

When grown with 2% glucose, the Mut CRE Δ mutant did not show increased H₂O₂ production over that for the Mut CRE 1 and Mut CRE 2 strains (Fig. 7B). This was unexpected, since no binding of CcpA was observed during EMSA. Therefore, it was expected that *spxB* was not repressed under any growth condition. The deletion of a substantial *spxB* promoter

part did not abolish the potential of *spxB* expression, as shown for H₂O₂ production during growth in the presence of 0.2% glucose (Fig. 7B). One possible explanation for this observation is that the promoter truncation leads to mRNA instability and therefore faster degradation, although *spxB* expression might be derepressed in a manner similar to that for the Mut CRE 1 + 2 mutant.

To further quantify the differences in H₂O₂ production caused by the CRE mutations, the strains were grown under planktonic conditions on a horizontal shaker. H₂O₂ production was shown to be maximal in the wild type under these conditions (31). Confirming the results from the plate assay, H₂O₂ production was under the control of CCR; production was reduced in the wild type and the Mut CRE 0 strain during growth with 2% glucose. The Mut CRE 1 and Mut CRE 2 mutants showed a 2-fold reduction in H₂O₂ production, confirming the unexpected repression when only one binding site is present. The Mut CRE 1 + 2 mutant was not responsive to the increased glucose concentration, and therefore CcpA did not repress *spxB* expression. The Mut CRE Δ mutant did produce less H₂O₂ than the wild type but was not derepressed like the Mut CRE 1 + 2 mutant (Fig. 7C). We also measured the expression of *spxB* in the respective mutants. Similar results were obtained, showing that the individual mutants Mut CRE 1 and Mut CRE 2 were lower in *spxB* expression, while the inability of CcpA to bind showed the expected derepression of *spxB* expression (Fig. 7D).

DISCUSSION

In the present study, the regulation of *spxB* expression and subsequent SpxB abundance in *S. gordonii* were further ex-

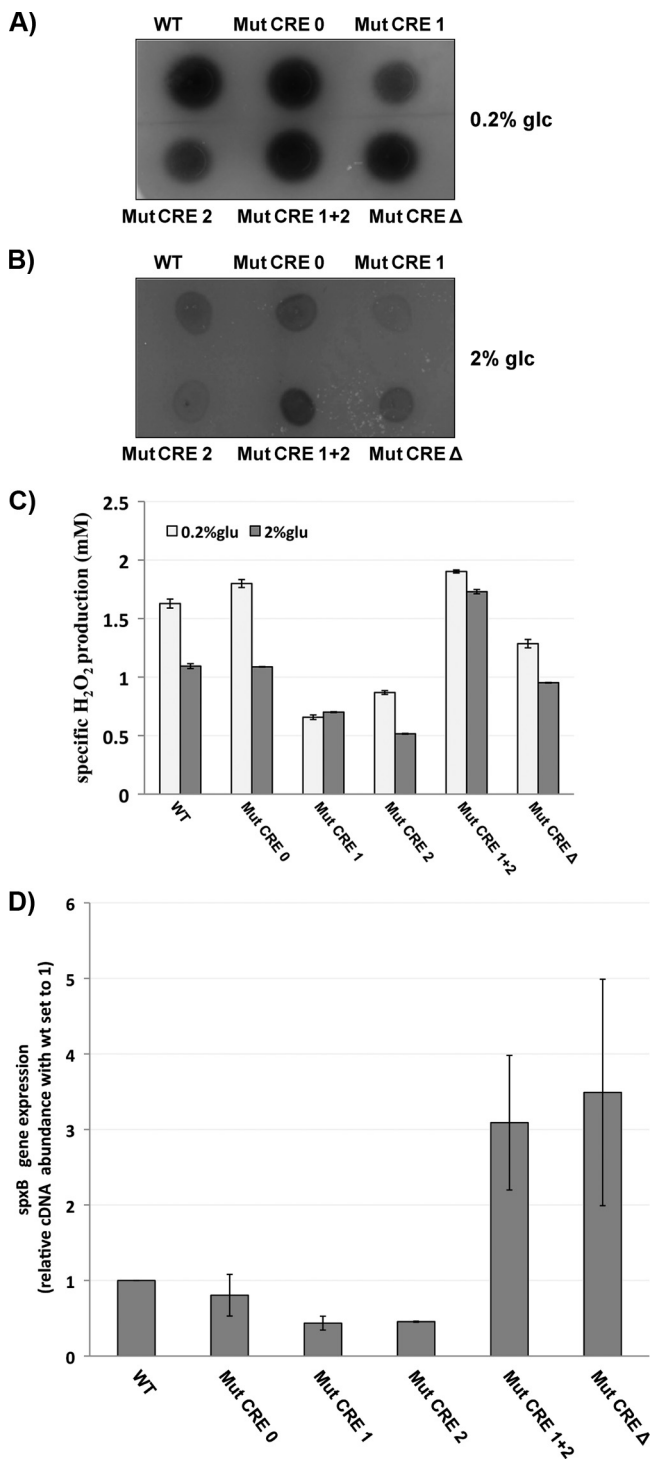


FIG. 7. Phenotypic consequences of CRE site mutations. The effects of CRE site mutations on H₂O₂ production were detected using H₂O₂ indicator plates. (A) Growth in the presence of 0.2% glucose. (B) Growth in the presence of 2% glucose. (C) The specific production of H₂O₂ was determined with planktonic cells grown under conditions of maximal H₂O₂ production. The detection limit for the assay was 0.125 mM H₂O₂. Averages and standard deviations from two independent experiments are presented. (D) The expression of *spxB* in the wild type and mutants grown in TYE medium with 0.2% Glc was determined by real-time PCR. The 16S rRNA gene was used as the house-keeping reference gene. Averages and standard deviations from two independent experiments are presented.

plored. SpxB-dependent H₂O₂ production is an important modulator of interspecies competition and oral biofilm ecology (21, 22, 30, 32). *S. gordonii* is able to inhibit the growth of oral biofilm bacteria, including cariogenic *S. mutans* and *Actinomyces naeslundii*, as demonstrated in deferred dual-species antagonism assays (22, 30). Furthermore, *S. sanguinis* and *S. gordonii* SpxB mutants are outcompeted by *S. mutans* in a dual-species biofilm model, whereas an H₂O₂ overproduction mutant of *S. sanguinis* increased its inhibiting activity toward *S. mutans* (32, 55). The production of H₂O₂ is coupled to the generation of acetyl phosphate. Acetyl phosphate by itself is an important component of central metabolism. It can serve as a phosphoryl donor for two-component response regulators involved in the regulation of diverse cellular processes (52). Furthermore, the conversion of acetyl phosphate to acetate generates ATP. Therefore, acetyl phosphate has been discussed as an important intracellular signal to couple energy metabolism with cellular signaling (52). A legitimate question, therefore, is whether H₂O₂ is merely a side product of energy metabolism under aerobic conditions. Compared to the ability of *S. mutans* to produce bacteriocins under aerobic and anaerobic conditions, the limited H₂O₂ production during anaerobic growth seems to argue against H₂O₂ production as a competitive antimicrobial component. However, an alternative explanation may be simply that H₂O₂ production under aerobic conditions is sufficient to function in interspecies competition. For example, an environment of high oxygen availability is most likely to occur under conditions of low biofilm abundance during the earliest stages of biofilm development. As the cells start to produce H₂O₂, they would naturally favor the growth of the H₂O₂-tolerant species at the expense of sensitive species, such as *S. mutans*. Another consequence of H₂O₂ production is the release of extracellular DNA (eDNA) (31). eDNA has been shown to be an important structural component of biofilms (14), and H₂O₂ is the sole agent required to induce eDNA release in *S. gordonii* (A. Itzek et al., unpublished data). Likewise, it has been shown for several species that eDNA is required for biofilm formation (9, 11, 27), and it can promote cell-cell aggregation in *S. sanguinis* (31). The production of eDNA might therefore promote the initial adhesion to the tooth surface and subsequent biofilm formation.

The initial observation of a glucose-effect on H₂O₂ production suggested CCR of *spxB* expression in *S. gordonii*. CCR in oral streptococci seems to be mediated by two different mechanisms, one that is CcpA dependent and one that is CcpA independent. In a recent report, Tong et al. demonstrated that the EIIAB^{Man} domain (ManL) of the PTS controls CCR in *S. gordonii* (49). Furthermore, CCR in *S. mutans* is mediated by the general PTS component Hpr-Ser-P, and the carbohydrate-specific EIIAB^{Man}, FruI, and EII^{Lev} and CcpA seem to play only a minor role (53, 54). Earlier reports for *S. gordonii* showed CcpA-dependent control of several other genes, including the arginine deiminase operon and the *abpA* gene, encoding amylase binding protein A, as well as the α -amylase gene *amyB* (12, 23, 43). The RegPrecise database, a collection of manually curated inferences of regulons in prokaryotic genomes (37), identified 151 genes in 72 operons regulated by CcpA in *S. gordonii*, while in *S. mutans* only 92 genes in 41 operons seemed to be regulated by CcpA. Further experiments are needed to identify the extent of CcpA- and EIIAB^{Man}-

mediated CCR in *S. gordonii* in order to identify the dominant mechanism of CCR. This will also help to precisely define the differences in the CCR network between *S. gordonii* and *S. mutans*. This is important considering that *S. gordonii* as a commensal is able to compete with *S. mutans* and is therefore a promising candidate for enhancement of competitiveness toward cariogenic species in general.

The difference in the glucose response between *S. sanguinis* and *S. gordonii* initiated the current investigation. CcpA permanently represses *spxB* expression in *S. sanguinis* independently of carbohydrate content (55). Interestingly, the Reg-Precise database (37) predicted only one CcpA binding site in the promoter region of *spxB* in *S. sanguinis*, while two were predicted for *S. gordonii*. CcpA *in vitro* bound both predicted CRE sites in *S. gordonii*. However, it was surprising that the deletion of either CRE 1 or CRE 2 decreased H₂O₂ production, suggesting that CcpA mediates *spxB* repression functions only when one CRE is present. This was true even under nonrepressive conditions, such as low glucose. This resembles the observation reported for *S. sanguinis*, that the presence of only one binding site confers permanent repression (55). One possible explanation for the regulatory role of two CRE sites in *spxB* expression in *S. gordonii* could be steric hindrance. Under low-glucose conditions, other coregulatory elements, such as phosphorylated Hpr-Ser-46 (24), would not be available to modulate the binding activity of CcpA. It is possible that these cofactors are required to alleviate the steric hindrance created when both CRE sites are bound. A survey of promoter sequences encoding two CRE sites in *S. gordonii* showed that the close association in the *spxB* promoter is unique. Usually, an extended spacer separates the CRE sites in other promoters under CCR. This was also found for *S. sanguinis* and *S. mutans*. Further experimental validation is required to confirm this unusual regulation.

In summary, the present study has provided evidence for the environmental control of *spxB* expression in *S. gordonii*. Given the potential role of *S. gordonii* *spxB* expression in oral biofilm homeostasis, it is possible that oral commensals such as *S. gordonii* and *S. sanguinis* could be exploited for their probiotic potential. For example, salivary lactoperoxidase is an important innate immune component that uses inorganic ions and H₂O₂ as oxidants to generate antimicrobials generally believed to be more effective than H₂O₂ itself (2). Thus, strategies that modulate SpxB activity in the oral cavity may be useful as a simple therapeutic mechanism to improve oral health.

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