

Transcription of Oxidative Stress Genes Is Directly Activated by SpxA1 and, to a Lesser Extent, by SpxA2 in *Streptococcus mutans*

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ABSTRACT

The SpxA1 and SpxA2 (formerly SpxA and SpxB) transcriptional regulators of *Streptococcus mutans* are members of a highly conserved family of proteins found in *Firmicutes*, and they were previously shown to activate oxidative stress responses. In this study, we showed that SpxA1 exerts substantial positive regulatory influence over oxidative stress genes following exposure to H₂O₂, while SpxA2 appears to have a secondary regulatory role. *In vitro* transcription (IVT) assays using purified SpxA1 and/or SpxA2 showed that SpxA1 and, less often, SpxA2 directly activate transcription of some of the major oxidative stress genes. Addition of equimolar concentrations of SpxA1 and SpxA2 to the IVT reactions neither enhanced transcription of the tested genes nor disrupted the dominant role of SpxA1. Substitution of a conserved glycine residue (G52) present in both Spx proteins by arginine (Spx_{G52R}) resulted in strains that phenocopied the Δ spx strains. Moreover, addition of purified SpxA1_{G52R} completely failed to activate transcription of *ahpC*, *sodA*, and *tpx*, further confirming that the G52 residue is critical for Spx functionality.

IMPORTANCE

Streptococcus mutans is a pathogen associated with the formation of dental caries in humans. Within the oral cavity, *S. mutans* routinely encounters oxidative stress. Our previous data revealed that two regulatory proteins, SpxA1 and SpxA2 (formerly SpxA and SpxB), bear high homology to the Spx regulator that has been characterized as a critical activator of oxidative stress genes in *Bacillus subtilis*. In this report, we prove that Spx proteins of *S. mutans* directly activate transcription of genes involved in the oxidative stress response, though SpxA1 appears to have a more dominant role than SpxA2. Therefore, the Spx regulators play a critical role in the ability of *S. mutans* to thrive within the oral cavity.

The oral cavity is colonized by hundreds of bacterial species, some of which contribute to overall oral health and others that are associated with disease, such as dental caries and periodontitis. Among the pathogenic oral bacteria, clinical evidence paired with *in vitro* and *in vivo* studies strongly associates *Streptococcus mutans* with dental caries onset and development (1, 2). For all organisms that inhabit the oral cavity, oxidative stresses are relevant threats for which defense mechanisms must be in place. Aside from the presence of hydrogen peroxide (H₂O₂) in oral care products, members of the mitis group of streptococci, which cohabit the dental plaque along with *S. mutans*, are net producers of H₂O₂ (3–5). Notably, there is an inverse correlation between the proportion of *S. mutans* and of members of the mitis group in health and disease, with high numbers of *S. mutans* organisms associated with disease and a high proportion of mitis streptococci associated with oral health (6, 7). Ultimately, the breakdown of H₂O₂ into other variants of reactive oxygen species (ROS) can disturb the integrity of DNA and proteins and thereby pose a significant threat to the viability of *S. mutans*.

Spx is a global regulator ubiquitously found in low-GC-content Gram-positive bacteria (*Firmicutes*), and it has been shown to function as a transcriptional activator of genes critical for oxidative stress survival (8). While the majority of work that has contributed to the understanding of Spx function has come from the model organism *Bacillus subtilis*, evidence is now accumulating that Spx proteins exert similar regulatory functions in bacterial pathogens, including *S. mutans* (9–15). Spx proteins do not have a DNA-binding domain but rather exert influence over transcription, positive or negative, by interacting with the C-terminal domain of the alpha subunit of the RNA polymerase (RNAP α -CTD)

(16, 17). The method of regulation of these so-called appropriator proteins is different from the more common mechanism of transcriptional regulators that physically interact with the regulatory region of a target gene via a DNA-binding domain (18–20).

In previous work, we identified and characterized two Spx proteins in *S. mutans*, earlier referred to as SpxA and SpxB (11, 21). We demonstrated that both proteins bear strong homology to the well-studied *B. subtilis* Spx (8, 17). To eliminate any confusion with gene nomenclature due to the existence of the SpxB pyruvate oxidase in certain streptococci, we now adopt the *Streptococcus pneumoniae* nomenclature (13) and refer to SpxA and SpxB as SpxA1 and SpxA2, respectively. Both SpxA1 and SpxA2 possess a CXXC redox disulfide motif typical of Spx family regulators described to date (8–10, 13, 16, 22). This motif is thought to facilitate disulfide bond formation when exposed to an oxidizing environment, triggering a conformational change that ultimately facilitates interaction of Spx with the RNAP. In addition, SpxA1 and

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SpxA2 possess a conserved glycine residue (G52), which may be one of several residues involved in Spx–RNAP α -CTD interaction.

We previously constructed mutant strains bearing deletions of the *spx* genes in *S. mutans*, each singly (Δ *spxA1* and Δ *spxA2*) or in combination (Δ *spxA1* Δ *spxA2*). We observed that the Δ *spxA1* strain exhibited strong stress-sensitive phenotypes, in particular toward oxidative stresses, which were often exacerbated in the double-mutant Δ *spxA1* Δ *spxA2* strain (11). Microarray analysis suggested that many genes involved in oxygen reduction and ROS detoxification were under positive regulation by SpxA1 and, to a minor extent, SpxA2 (11). Despite the association of Spx in oxidative stress responses in different bacteria, evidence that the regulatory effect exerted by Spx is direct has been limited to *B. subtilis* (23–25). Moreover, our previous studies suggest that the *S. mutans* SpxA1 is a more potent regulator than its SpxA2 paralogue (11, 26), although this observation had not been experimentally proven. In this study, we used quantitative reverse transcriptase real-time PCR (qRT-PCR) and *in vitro* transcription (IVT) assays to better understand the role of SpxA1 and SpxA2 in oxidative stress gene regulation. Our results provided unequivocal evidence that SpxA1 is required for optimal expression of some of the major oxidative stress genes through direct interaction with the RNAP. We also demonstrated that SpxA2 could contribute to transcription of oxidative stress genes, though its regulatory influence appears secondary to that of SpxA1. Finally, single amino acid substitutions confirmed the essentiality of the G52 residue for functionality of both SpxA1 and SpxA2 in *S. mutans*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *S. mutans* UA159 and its Δ *spx* derivatives were routinely grown in brain heart infusion (BHI) at 37°C in a 5% CO₂ atmosphere. When appropriate, erythromycin (Erm; 10 μ g ml⁻¹) or spectinomycin (1,000 μ g ml⁻¹) was added to the growth medium. For gene expression analysis, cultures were grown to an optical density at 600 nm (OD₆₀₀) of 0.4, at which point control samples were harvested by centrifugation, while experimental samples were exposed to 0.5 mM H₂O₂ for 5 min before harvest. Harvested pellets were stored at –80°C until use.

Creation of *spx*_{G52R} strains. A PCR-based site-directed mutagenesis strategy was used to create markerless point mutations in the *S. mutans* *spxA1* and *spxA2* genes. Specifically, primers (Table 2) were designed to introduce changes in the DNA sequence so that the GGG (*spxA1*) and GGA (*spxA2*) bases encoding glycine residues at position 52 of each protein were replaced by CGC and CGT, respectively, which both encode arginine. *S. mutans* UA159 was transformed with each PCR product in combination with the pSU20erm-lacG suicide plasmid encoding resistance to erythromycin (27). The plasmid was included for the purpose of identifying competent cells. Erm-resistant colonies were screened for a phenotype (changes in chain length) typical of Δ *spxA1* or Δ *spxA2* and then submitted for sequencing analysis of the appropriate gene to confirm the desired point mutation.

Growth inhibition assay. The ability of *Streptococcus gordonii* to inhibit growth of *S. mutans* via H₂O₂ production was assessed as described previously by Kreth et al. (28). Briefly, 8 μ l of an overnight culture of *S. gordonii* DL-1 was spotted on the center of a BHI agar plate and incubated at 37°C for 16 h. The following day, 8- μ l quantities of *S. mutans* cultures were spotted near the *S. gordonii* spot and incubated for an additional 16 h before visualizing the ability of the different *S. mutans* strains to grow in proximity of *S. gordonii*. To ascertain that any growth inhibition was due to the production of H₂O₂ by *S. gordonii*, a control condition was included in which 8 μ l of catalase (0.75 μ g μ l⁻¹) was immediately spotted on top of the *S. gordonii* culture.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype ^a or function	Source or reference
<i>Streptococcus mutans</i> strains		
UA159	Wild type	Laboratory stock
JL12 Δ <i>spxA1</i>	<i>spxA1</i> ::Sp ^r	21
JL13 Δ <i>spxA2</i>	<i>spxA2</i> ::Erm ^r	11
JL21 Δ <i>spxA1</i> Δ <i>spxA2</i>	<i>spxA1</i> ::Sp ^r <i>spxA2</i> ::Erm ^r	11
JL32 <i>spxA1</i> _{G52R}	SpxA1 _{G52R}	This study
JL33 <i>spxA2</i> _{G52R}	SpxA2 _{G52R}	This study
JL34 Δ <i>spxA1</i> <i>spxA2</i> _{G52R}	<i>spxA</i> ::Sp ^r SpxA2 _{G52R}	This study
Other bacterial strains		
<i>B. subtilis</i> MH5636	His-tagged RNAP	42
<i>E. coli</i> BL21(DE3)	Expression host strain	New England BioLabs
<i>E. coli</i> DH5 α	Expression host strain	Invitrogen
<i>S. gordonii</i> DL-1	Wild type	Laboratory stock
Plasmids		
pET16B-SpxA1	His-tagged SpxA1	This study
pET16B-SpxA2	His-tagged SpxA2	This study
pSU20erm-lacG	Competency control	27
pMSP3535	Expression in <i>S. mutans</i>	31
pMSP3535-SpxA1-His	Expression of SpxA1-His	This study
pMSP3535-SpxA2-His	Expression in SpxA2-His	This study
pMALc2x-SpxA1	MBP-tagged SpxA1	This study
pMALc2x-SpxA1 _{G52R}	MBP-tagged SpxA1 _{G52R}	This study

^a Sp^r, spectinomycin resistance; Erm^r, erythromycin resistance.

Disc sensitivity assays. Bacterial cultures were grown in BHI to an OD₆₀₀ of 0.2, and aliquots (25 μ l) of each culture were spread evenly over a quadrant of a 30-ml BHI plate with a sterile swab. Next, 6-mm filter paper discs (Whatman) were soaked with 20 μ l of a 0.25% H₂O₂ or 0.2 M diamide solution and aseptically placed on top of the swabbed plate. Diameters of zones of growth inhibition were measured after 24 h of incubation at 37°C in a 5% CO₂ atmosphere. Student's *t* test was performed to verify significant differences in sensitivity.

RNA analysis. Total RNA was isolated as described previously (1). Briefly, RNA was isolated from homogenized *S. mutans* cells by repeated hot acid-phenol-chloroform extractions, and the nucleic acid was precipitated with 1 volume of ice-cold isopropanol and 1/10 volume of 3 M sodium acetate (pH 5) at 4°C overnight. RNA pellets were resuspended in nuclease-free H₂O and treated with DNase I (Ambion) at 37°C for 30 min. The RNA was purified again using the RNeasy minikit (Qiagen), including a second on-column DNase treatment that was performed as recommended by the supplier. RNA concentrations were determined in triplicate using a Nanovue spectrophotometer (GE Healthcare) and run on an agarose gel to verify RNA integrity. For mRNA analysis, gene specific primers (Table 2) were designed using Beacon Designer 2.0 software (Premier Biosoft International). Reverse transcription and qRT-PCR were carried out according to protocols described elsewhere (1, 29). Student's *t* test was performed to verify significance of the qRT-PCR results.

Spx purification. SpxA1 and SpxA2 were produced as recombinant His-tagged fusion proteins using the pET-16B expression vector (EMD Millipore). Both *spxA1* and *spxA2* were cloned using NdeI and XhoI restriction sites that had been engineered into primers flanking the coding regions of each gene (Table 2). Protein expression was induced by growing *Escherichia coli* DH5 α in Luria-Bertani (LB) broth supplemented with 100 μ g ml⁻¹ of ampicillin at 37°C with agitation (200 rpm). After reaching OD₆₀₀ of 0.5, cultures were transferred to 15°C and 0.4 mM isopropyl

TABLE 2 Primers used in this study

Name	Sequence (5'–3')	Application
5'SpxA1pET-16B	AGGGGTAGTCATATGATGGTTACC	His-SpxA1 express
3'SpxA1pET16B	GGGTAAGCGCTCGAGTTAGTCATCTTC	His-SpxA1 express
5'SpxA2pET16B	GAAAGAGAGATCATATGATGATTAATAATTTA	His-SpxA2 express
3'SpxA2pET16B	TCATTACTTCGACTCGAGTTATAAAGCTGC	His-SpxA2 express
5'pET16B BamHI	TTGTTTAACTGGATCCGGAGATATAACCATG	Release His-tag Spx
3'pET16B XhoI	CGGGCTTTGTCTGCAAC	Release His-tag Spx
5'spxA1pMAL	AAGGGGTAGTGAATTCATGGTTACC	MBP-SpxA1 express
3'spxA1pMAL	GGGTAAGCGGGATCCTTAGTCATCTTC	MBP-SpxA1 express
5'A1G52R-PCR1	CTTCCAGAGTCTCCTCAATTAGC	<i>spxA1</i> G52R mutation
3'A1G52R-PCR1	GTCGAAATAATATCTTCTGTGCGGTTTTCTGTATAGG	<i>spxA1</i> G52R mutation
5'A1G52R-PCR2	CCTATACAGAAACCGCACAGAAATATTATTTCGAC	<i>spxA1</i> G52R mutation
3'A1G52R-PCR2	GGTTTGACGACCGAAATCGGC	<i>spxA1</i> G52R mutation
5'A2G52R-PCR1	GCAGGGAAATTGATTGATTCTGGCGC	<i>spxA2</i> G52R mutation
5'A2G52R-PCR1	GCAGGGAAATTGATTGATTCTGGCGC	<i>spxA2</i> G52R mutation
3'A2G52R-PCR1	GGATACAATGCTTTCAATACGATTTTCTGTTTTTTG	<i>spxA2</i> G52R mutation
5'A2G52R-PCR2	CAAAAACAGAAAATCGTATTGAAAGCATTGTATCC	<i>spxA2</i> G52R mutation
3'A2G52R-PCR2	CAACATCATACTATAGTTATAGTACAAGC	<i>spxA2</i> G52R mutation
sodART Fwd	GATGCTGAAACGATGACCCCTTC	qRT-PCR, <i>sodA</i>
sodART Rev	CACATCAGCCAAAAGCACTTCC	qRT-PCR, <i>sodA</i>
tpxRT Fwd	CTCCATCTGCTTGGACGTGCTG	qRT-PCR, <i>tpx</i>
tpxRT Rev	GCAAGGGCAGCGTCATAGTTG	qRT-PCR, <i>tpx</i>
ahpCRT Fwd	ATGGTTTAGCACAAACGTCGAAC	qRT-PCR, <i>ahpC</i>
ahpCRT Rev	TTGGCAGGGCAAACCTTCTCC	qRT-PCR, <i>ahpC</i>
gorRT Fwd	ACCTGTAATGTTGGCTGTG	qRT-PCR, <i>gor</i>
gorRT Rev	CCTGACGATTTTGTCTCAAGAC	qRT-PCR, <i>gor</i>
noxRT Fwd	GGGTGTGGAATGGCACTTTGG	qRT-PCR, <i>nox</i>
noxRT Rev	CAATGGCTGTCACTGGCGATTC	qRT-PCR, <i>nox</i>
dprRT Fwd	GAAGAAAACAGTTGGCACATGGG	qRT-PCR, <i>dpr</i>
dprRT Rev	TTCCGTTTGAGCTGCTGTAAAG	qRT-PCR, <i>dpr</i>
trxART Fwd	TTGAAGCTGAAACGGCTAAGGG	qRT-PCR, <i>trxA</i>
trxART Rev	GCCTGCATAAGACATGGACCAC	qRT-PCR, <i>trxA</i>
trxBRT Fwd	AGTTGTTGGTGGTGGCGATTC	qRT-PCR, <i>trxB1</i>
trxBRT Rev	CATTGGCAAAGGCACGTTCTTG	qRT-PCR, <i>trxB1</i>
trxB2RT Fwd	GGAGAGGCACCAACAGCAG	qRT-PCR, <i>trxB2</i>
trxB2RT Rev	ATGATGTGGAATGAACGACACG	qRT-PCR, <i>trxB2</i>
5'IVTsodA	GCTATTAAGAGCGGGACTTAC	IVT, <i>sodA</i>
3'sodA	GCATGATGCTTATCATGATG	IVT, <i>sodA</i>
5'tpx	GAGTGGTCAAAAATAAACATATTTTTATTG	IVT, <i>tpx</i>
3'tpx	CTGTCAGAGAAAATCAGGTGCTGTATC	IVT, <i>tpx</i>
5'ahpC	CTCTTTTGTTTTATGCTTCAAATTTTATTTTTAG	IVT, <i>ahpC</i>
3'ahpC	GTTAACCGTAACAAATCTCCTTGATG	IVT, <i>ahpC</i>
5'mleS	GAATACAAGTTTAAAAGCAAATAGTTAAC	IVT, <i>mleS</i>
3'mleS	GGCAAAAGACCAATAAGTCC	IVT, <i>mleS</i>

β -D-1-thiogalactopyranoside (IPTG) was added to induce production of the tagged protein overnight. SpxA1 protein was purified from the soluble fraction of cell lysate. Cell pellets were collected by centrifugation, resuspended in lysis buffer S (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole [pH 8.0]), and subjected to three 30-s cycles of homogenization using a bead beater (BioSpec Products), with chilling on ice between cycles. After centrifugation, purification of the recombinant proteins in the soluble supernatant was performed by column chromatography using nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen). Recombinant SpxA1 was released from the resin with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole [pH 8.0]) and dialyzed in phosphate-buffered saline (PBS) containing 10% glycerol. For SpxA2, reasonable yields were obtained only when the protein was purified from the insoluble fraction and refolded. Cell pellets were resuspended in lysis buffer I (50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM dithiothreitol [DTT], 100 mM NaCl, 5% glycerol [pH 8.0]) and subjected to homogenization as described above. After centrifugation, the pellet was retained and resus-

pended in lysis buffer B containing 1% Triton X-100. The resuspended protein was denatured by the addition of 8 M urea and subjected to column chromatography using Ni-NTA resin, with 8 M urea included in the wash buffers. The purified SpxA2-His protein was refolded by stepwise dialysis with decreasing concentrations of urea, with a final dialysis step in the absence of urea. Protein concentrations were determined using the bicinchoninic acid assay (Sigma) (30).

To facilitate *in vivo* complementation and verify that the His-tagged Spx proteins retained activity, the coding region for the protein and N-terminal His tag were amplified from their respective constructs (pET16B-SpxA1 or pET16B-SpxA2). The amplicons were cloned into pMSP3535 (31) using BamHI and XhoI restriction sites that were engineered into the amplifying primers. The resulting plasmids, pMSP3535-SpxA1-His and pMSP3535-SpxA2-His, were confirmed by sequencing and then transformed into Δ *spxA1* and Δ *spxA2* *S. mutans*, respectively. Empty pMSP3535 was transformed into *S. mutans* UA159 as a control. Protein expression in pMSP3535 is driven by the *nisA* promoter upon

addition of nisin to the culture. However, it has been shown that low-level protein expression may occur in the absence of nisin (31).

SpxA1 and SpxA1_{G52R} were also produced as recombinant proteins fused to a maltose-binding protein (MBP) tag using the pMALc2X expression vector (New England BioLabs). The *spxA1* and mutated *spxA1*_{G52R} genes were cloned into pMALc2X using BamHI and EcoRI restriction sites that had been engineered into primers flanking the coding regions of each gene (Table 2). Protein expression was induced by growing *E. coli* strain DH5 α in LB broth supplemented with 1% (wt/vol) glucose and 100 μ g ml⁻¹ of ampicillin at 37°C with agitation (200 rpm). The resulting cultures were grown until an OD₆₀₀ of 0.5 was reached, at which point the cultures were transferred to 15°C, and 0.3 mM IPTG was added to induce production of the tagged protein overnight. Cell pellets were collected by centrifugation, resuspended in column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA), and homogenized in a bead beater as described above. Purification of the recombinant proteins was performed by column chromatography using amylose resin (New England BioLabs). Recombinant SpxA1-MBP and SpxA1_{G52R}-MBP were eluted in column buffer containing 10 mM maltose and dialyzed in PBS containing 10% glycerol.

RNAP purification. A crude extract of *S. mutans* RNAP was obtained based upon the methods described by Seepersaud et al. for isolation of *Streptococcus agalactiae* RNAP, which utilizes the binding properties of heparin to enrich for RNAP (32). Briefly, *S. mutans* cells were grown in BHI to an OD₆₀₀ of 0.5, harvested by centrifugation, resuspended in protoplast preparation buffer (0.3 M potassium phosphate buffer, pH 7.0; 40% sucrose; 0.5 U μ l⁻¹ of mutanolysin), and incubated at 37°C for 90 min. Protoplasts were harvested, and the pellets were resuspended in lysis buffer (50 mM Tris HCl, 10 mM MgCl₂, 0.1 mM DTT, 0.1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride [PMSF] [pH 8.0]) and then subjected to three 15-s rounds of sonication, with chilling on ice between cycles. Following centrifugation, supernatants were applied to an Affi-Gel heparin resin (Bio-Rad) and eluted with a gradient of 0.1 to 1 M NaCl. The elutions were then run on 10% SDS-PAGE, the fractions containing a visible β subunit (~134 kDa) were pooled, and the salt concentration was adjusted to 0.1 M NaCl by buffer exchange. The protein was then applied to Macro-Prep High-Q ion-exchange resin (Bio-Rad) and eluted with a gradient of 0.1 to 0.8 M NaCl. The desired fractions were again pooled, dialyzed in 10 mM Tris HCl, 10 mM MgCl₂, 100 mM NaCl, 0.1 mM EDTA, and 50% glycerol (pH 8.0), and stored at -20°C.

His-tagged, σ^A -depleted RNAP was purified from *B. subtilis* strain MH5636 as described by Lin and Zuber (23). Briefly, cultures were grown in 2 \times yeast extract-tryptone (2 \times YT) medium supplemented with 5 μ g ml⁻¹ of chloramphenicol at 37°C under agitation to an OD₆₀₀ of 0.9, harvested, and kept at -80°C until use. Protein purification was performed in multiple steps using Ni-NTA resin, an Affi-Gel heparin column, and a High-Q ion-exchange column (23). The purified RNAP was stored at -20°C in buffer containing 10 mM Tris-HCl (pH 7.8), 100 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, and 50% glycerol.

In vitro transcription assays. A linear DNA template for each gene of interest was generated by PCR using primers designed to amplify the promoter region as well as about 70 to 100 bp of the coding region (Table 2). Following amplification, the DNA fragments were purified with a QIAquick PCR purification kit (Qiagen). The *In vitro* transcription (IVT) reactions were performed as described by Lin and Zuber (23). Briefly, a 10 nM concentration of each individual purified promoter template, 95 nM *S. mutans* crude RNAP or 25 nM *B. subtilis* RNAP, and 25 nM *B. subtilis* σ^A (a gift from P. Zuber, Oregon Health Science Center) were incubated for 10 min at 37°C with or without purified His- or MBP-tagged SpxA1, His-tagged SpxA2, His-tagged SpxA1_{G52R}, or His-tagged *S. mutans* CodY (a gift from R. Quivey, University of Rochester) in 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM MgCl₂, and bovine serum albumin (BSA; 50 μ g ml⁻¹) supplemented with 2 U of RNase inhibitor (Applied Biosystems) to a final volume of 20 μ l. The Spx or CodY proteins were provided at 75 nM unless otherwise specified. A nucleotide mixture (200 mM ATP, GTP, and

CTP, 10 mM UTP, and 5 μ Ci of [α -³²P]UTP) was added, and the incubation proceeded for an additional 3 to 12 min. Stop solution (1 M ammonium acetate, 0.1 mg ml⁻¹ of yeast RNA, 0.03 M EDTA) was added and the mixture was precipitated with ethanol at 4°C overnight. The nucleotide pellet was resuspended in formamide dye (0.3% xylene cyanol, 0.3% bromophenol blue, and 12 mM EDTA dissolved in formamide). The samples were heated at 90°C for 2 min and then placed on ice before application to an 8% polyacrylamide-8 M urea gel. The transcripts were visualized with Bio-Rad Quantity One Fx software following overnight exposure to an Imaging Screen-K (Bio-Rad). Densitometry was performed using ImageJ software (<http://imagej.nih.gov/ij/>).

RESULTS

Deletion of SpxA1 and, to a lesser extent, SpxA2 impairs the ability of *S. mutans* to respond to oxidative stress. Previously, we demonstrated that several genes critical for responding to oxidative stresses were expressed at significantly reduced levels in the Δ *spxA1* and Δ *spxA1* Δ *spxA2* strains under nonstressful conditions compared to the parent UA159 strain (11). While these genes were mostly absent in the microarray analysis, qRT-PCR validation revealed that many of these genes are also repressed in the Δ *spxA2* mutant, though typically not to the same extent as in the Δ *spxA1* mutants. These initial data suggested that SpxA1 is the major regulator of oxidative stress genes in *S. mutans*, while SpxA2 appears to perform a backup regulatory role. Alternatively, SpxA2 may exert a more prominent control over oxidative stress gene expression under conditions that are yet to be determined. In this study, we asked if these differences in transcription were restricted to basal expression levels or whether the differences would become more noticeable following exposure to an oxidative stressor. To assess this possibility, we exposed mid-logarithmic cultures of the UA159, Δ *spxA1*, Δ *spxA2*, and Δ *spxA1* Δ *spxA2* strains to 0.5 mM H₂O₂ for 5 min and compared the transcription patterns of key oxidative stress genes with untreated (control) cultures (Fig. 1). Transcription expression levels of nine well-established oxidative stress genes were studied for all four strains (*sodA*, superoxide dismutase [SMU.629]; *tpx*, thiol peroxidase [SMU.924]; *ahpC*, alkyl hydroperoxide reductase [SMU.764]; *gor*, glutathione reductase [SMU.838]; *nox*, NADH oxidase [SMU.1117]; *dpr*, peroxide resistance protein [SMU.540]; *trxA*, thioredoxin [SMU.1869]; *trxB1*, thioredoxin reductase [SMU.463]; and *trxB2*, thioredoxin reductase [SMU.869]). As expected, the parent strain showed increased transcription of almost all of the genes following exposure to H₂O₂; the *trxA* and *trxB2* genes were the only exceptions to this trend. Additionally, among the untreated samples, UA159 showed the greatest expression level for all of these genes, with the exception of *trxB2*, which was not regulated by Spx. This trend among the untreated samples reveals that SpxA1 and SpxA2 are important for maintaining basal expression levels of the major oxidative stress genes even in the absence of stress. For simplicity, genes that showed similar patterns of expression have been grouped together and are described below.

Group I (*sodA*, *tpx*, *ahpC*, and *gor*). Among the group I genes, basal levels of expression in both the Δ *spxA1* and Δ *spxA1* Δ *spxA2* strains were noticeably reduced compared to those in the parent and, for *sodA* and *tpx*, the Δ *spxA2* strain. The most extreme example of this trend was seen for *ahpC*, in which basal level expression was decreased 40-fold (Δ *spxA1*) or 65-fold (Δ *spxA1* Δ *spxA2*) compared to that in the parent strain. For all genes in this grouping, exposure to H₂O₂ always resulted in increased expression in both UA159 and Δ *spxA2*. The preeminent role of SpxA1 in tran-

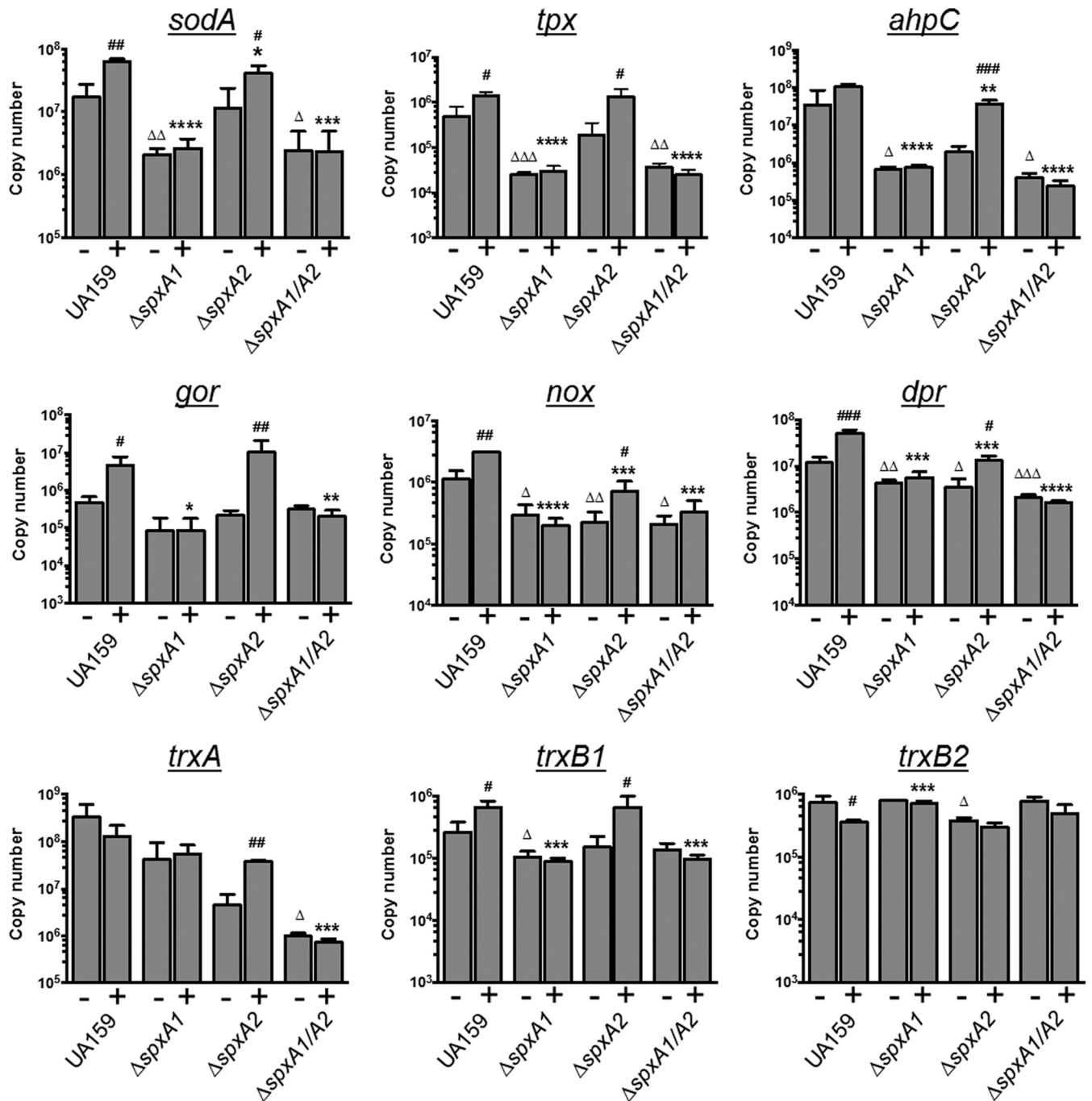


FIG 1 Mutation of *spxA1* abolishes the ability of *S. mutans* to transcriptionally respond to the presence of H_2O_2 . *S. mutans* UA159, $\Delta spxA1$, $\Delta spxA2$, and $\Delta spxA1 \Delta spxA2$ strains were grown in BHI to an OD_{600} of 0.4. At that point, control cultures (–) were harvested, while the remaining samples (+) were exposed to 0.5 mM H_2O_2 for 5 min before harvest. RNA was extracted from the cell pellets and used in qRT-PCR analysis to detect transcription of selected oxidative stress genes. Bars represent the relative copy number detected for each gene. Student's *t* test was performed to verify significance of the data. #, significant difference from the same strain when untreated; Δ , significant difference from untreated UA159; *, significant difference from UA159 after H_2O_2 treatment. Data shown represent averages and SDs from three replicate samples (#, Δ , and *, $P \leq 0.05$; ##, $\Delta\Delta$, and ***, $P \leq 0.005$; ###, $\Delta\Delta\Delta$, and ****, $P \leq 0.0005$).

scriptional activation of these genes becomes even more striking due to the complete lack of transcriptional activation in the $\Delta spxA1$ and $\Delta spxA1 \Delta spxA2$ strains after H_2O_2 exposure.

Group II (*nox* and *dpr*). One trend that separates *nox* and *dpr* from the previous grouping of genes is that the levels of expression among the untreated Δspx strains, including the $\Delta spxA2$ strain,

were not dramatically different. These mutants showed lower basal levels of expression than did UA159, a minimum of a 2.8-fold difference. Similar to the trends seen in the previous grouping of genes, exposure to H_2O_2 increased expression of both *nox* and *dpr* in the UA159 and $\Delta spxA2$ strains. Although expression of *nox* and *dpr* was induced in the $\Delta spxA2$ strain following exposure to

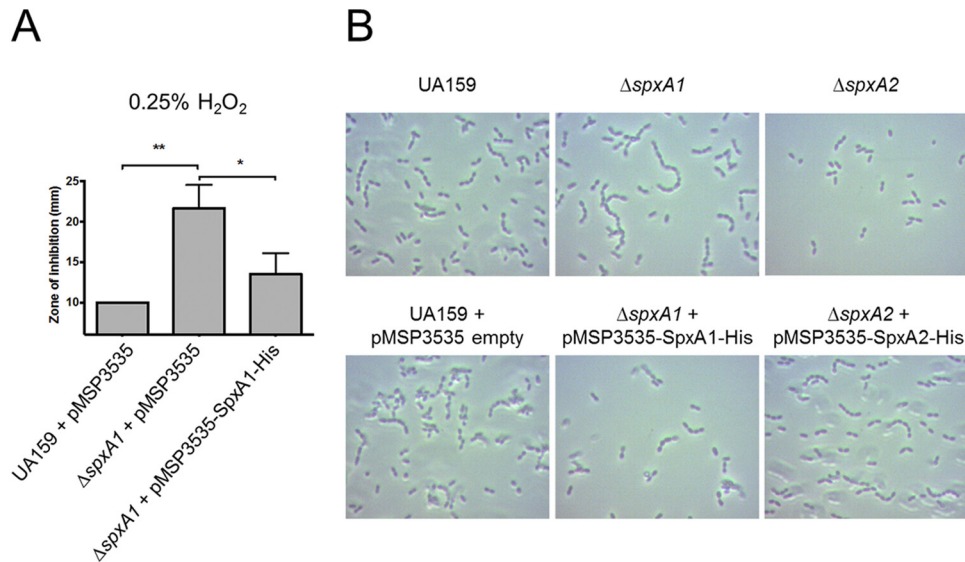


FIG 2 His-tagged SpxA proteins are active *in vivo*. His-tagged SpxA1 or SpxA2 was cloned into pMSP3535, allowing complementation of Δ spxA1 or Δ spxA2 in *S. mutans*. (A) Early logarithmic-phase cultures of *S. mutans* UA159 plus pMSP3535, the Δ spxA1 mutant plus pMSP3535, or the Δ spxA1 mutant plus pMSP3535-SpxA1-His were swabbed onto a BHI agar plate, upon which filter paper discs impregnated with 0.25% H₂O₂ were placed. Diameters of zones of growth inhibition were measured after 24 h of incubation at 37°C and 5% CO₂. Data shown represent averages and SDs from three replicate samples (*, $P \leq 0.05$; **, $P \leq 0.005$). (B) Chain lengths of mid-logarithmic phase cultures of *S. mutans* UA159, UA159 + pMSP3535, the Δ spxA1 mutant, the Δ spxA1 mutant plus pMSP3535-SpxA1-His, the Δ spxA2 mutant, and the Δ spxA2 mutant plus pMSP3535-SpxA2-His were observed by phase-contrast microscopy.

the stress, the mRNA copy numbers were significantly reduced compared to those with the parent strain. These differences may be attributed to the low basal expression for *nox* and *dpr* in the Δ spxA2 strain. As in the previous grouping of genes, the Δ spxA1 and Δ spxA1 Δ spxA2 strains were essentially unresponsive to H₂O₂.

Group III (*trxA*). Expression levels of *trxA* were unique in that basal levels of expression were quite different among all four strains: expression was greatest in UA159, followed by the Δ spxA1 strain (8-fold decrease), the Δ spxA2 strain (74-fold decrease), and, finally, the Δ spxA1 Δ spxA2 strain (337-fold decrease). The *trxA* gene is the only gene included in this study for which the basal level of expression was notably greater in the Δ spxA1 strain than in the Δ spxA2 strain. Unexpectedly, transcription of *trxA* did not increase in the parent UA159 strain upon exposure to H₂O₂, but rather it showed a slight decrease (2.6-fold). While the Δ spxA1 and Δ spxA1 Δ spxA2 strains were essentially unresponsive to H₂O₂, expression of *trxA* increased 8.4-fold in the Δ spxA2 strain.

Group IV (*trxB1* and *trxB2*). The group IV genes from the thioredoxin system each showed unique patterns in expression and were not easily grouped with the other genes included in this study. Although basal levels of *trxB1* were not statistically different between strains, H₂O₂ induction was, as in the other cases, SpxA1 dependent. Finally, the second putative thioredoxin reductase *trxB2* does not seem to be under the regulation of any of the two Spx proteins, and its expression was not altered during exposure to H₂O₂.

Collectively, these results demonstrate that both SpxA1 and SpxA2 participate in the regulation of oxidative stress genes. Largely, the data confirm our previous microarray analysis (11) and point to SpxA1 as the major transcriptional regulator of oxidative stress genes, while SpxA2 may play a more relevant role in maintaining basal levels of expression.

SpxA1 directly regulates transcription of oxidative stress genes. The qRT-PCR analysis supported a role for SpxA1 as a major regulator of oxidative stress genes. However, it did not rule out a role for SpxA2, which appears to be important for maintaining basal levels of transcription. With the exception of the *nox* gene, which we have recently showed is directly activated by SpxA1 (26), it is not known whether the regulatory effects exerted by the *S. mutans* Spx proteins over other genes are direct or indirect. To answer this question, we performed IVT reactions using either a crude extract of the *S. mutans* RNAP or purified *B. subtilis* RNAP (results were not influenced by RNAP source), and the promoter regions of three major oxidative stress genes (*ahpC*, *sodA*, and *tpx*), in the presence or absence of purified His-tagged SpxA1 or SpxA2.

To confirm that the addition of the His tag did not have a negative impact upon function of Spx, we expressed the His-tagged SpxA1 and SpxA2 proteins in *S. mutans* using the pMSP3535 plasmid. Expression of His-tagged SpxA1 in the Δ spxA1 strain resulted in alleviation of the oxidative stress phenotype, as measured by sensitivity to filter paper discs impregnated with H₂O₂ (Fig. 2A). We previously reported distinctive chain length phenotypes in the Δ spx deletion strains when observed under the microscope whereby the Δ spxA1 strain had a tendency to form longer chains than UA159, while the Δ spxA2 strain was impaired in chain formation (11). Expression of SpxA1-His or SpxA2-His in the respective deletion mutant strain reverted the chain length phenotype, confirming that both His-tagged Spx proteins retain their normal activities *in vivo* (Fig. 2B).

Results of the IVT reactions undoubtedly demonstrate that addition of SpxA1 dramatically enhanced transcription of all three genes tested (Fig. 3A). In fact, in the cases of *sodA* and *tpx*, the transcript was barely detectable unless SpxA1 was added to the reaction mixture. IVT reactions for the selected genes were also

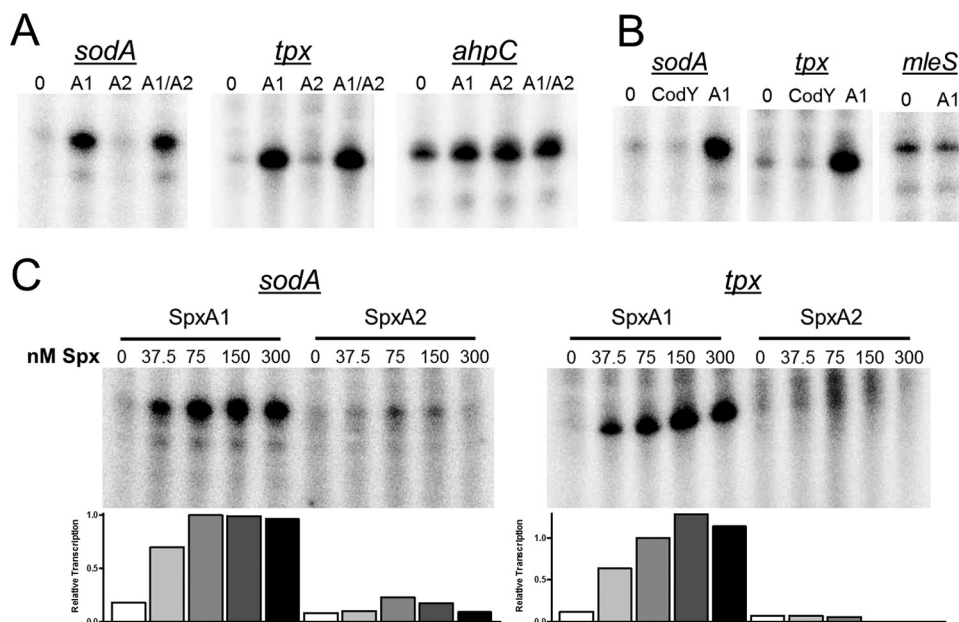


FIG 3 SpxA1 and SpxA2 specifically enhance transcription of oxidative stress genes. *In vitro* transcription (IVT) reactions were performed by incubating selected DNA templates with *S. mutans* RNAP and nucleotides including [α - 32 P]UTP in the absence (0) or presence of purified His-tagged protein: SpxA1 (A1), SpxA2 (A2), and SpxA1 and SpxA2 combined (A1/A2). Reactions were performed at 37°C. Radiolabeled RNA transcripts were precipitated, applied to 8% urea PAGE, and visualized by exposure to a phosphorimager screen. (A) IVT reactions performed with template DNA for *ahpC*, *sodA*, or *tpx*. (B) His-tagged CodY failed to enhance transcription of *sodA* or *tpx*, and SpxA1 failed to enhance transcription of *mleS*. (C) IVT reactions were performed with increasing amounts of SpxA1 or SpxA2 using the *sodA* or *tpx* DNA templates. Representatives of three or more independent reactions are shown. Densitometry was performed with ImageJ software.

performed with SpxA2 supplied instead of SpxA1. Under the initial conditions tested, SpxA2 increased transcript abundance for *ahpC* but not *sodA* or *tpx* (Fig. 3A). To consider the possibility that SpxA1 and SpxA2 either compete or cooperate with each other, the proteins were also provided together in equimolar concentrations, each at half the concentration as when provided singly. Our results indicate that the two Spx proteins do not cooperate or compete with each other, at least under the conditions tested. To be certain that enhanced transcription by Spx was specific, control reactions using recombinant His-tagged *S. mutans* CodY were performed. CodY is a regulator involved in branched-chain amino acid biosynthesis and is not known to have a role in oxidative stress gene regulation. As expected, CodY did not enhance transcription of *sodA* or *tpx* (Fig. 3B). Finally, IVT reactions were also performed with the promoter region of the *mleS* gene, involved in malolactic fermentation and not expected to be under Spx regulation. Transcription of *mleS* was not enhanced by SpxA1 addition (Fig. 3B).

To rule out the possibility that simply a greater amount of SpxA2 is required to induce transcription from the tested genes, additional IVT reactions with *sodA* and *tpx* were performed using increasing concentrations of Spx. By adding increasing amounts of SpxA2, a very small increase in *sodA* transcription but not *tpx* could be observed in these reactions (Fig. 3C). Despite the high level of homology between SpxA1 and SpxA2, it is possible that SpxA2 requires a yet-to-be-determined environmental cofactor or partner protein to exert strong regulatory effects on gene transcription. Alternatively, the control and scope of SpxA2 regulation of oxidative stress genes may be much more limited than those with SpxA1. Our previous phenotypic observations using the Δ *spxA1* and Δ *spxA2* strains (11) support the latter.

Substitution of a single residue of the Spx proteins results in strains that behave similarly to the Δ *spx* strains. The Spx proteins in Firmicutes are highly similar, and a conserved glycine residue at position 52 (G52) appears to be critical for interaction with the RNAP (16, 17). To investigate the importance of this residue in *S. mutans*, we created point mutations in SpxA1 and SpxA2, such that the G52 residue of each protein was replaced with an arginine (respectively, *spxA1*_{G52R} and *spxA2*_{G52R} strains). If the G52 residue is in fact essential, the *spxA1*_{G52R} and *spxA2*_{G52R} strains should phenocopy the Δ *spxA1* and Δ *spxA2* strains. As a means of verifying disrupted Spx function in these new strains, we again took advantage of the chain length phenotypes that we have described for the Δ *spx* strains (11). Microscopic observations indicated that the *spxA1*_{G52R} and *spxA2*_{G52R} strains displayed chain length phenotypes similar to those described for their respective deletion mutant strains (Fig. 4), suggesting that substitution of the G52 residue by an arginine renders the Spx proteins inactive. In fact, the *spxA1*_{G52R} strain was as sensitive to diamide or to the peroxigenic oral commensal *S. gordonii* as the Δ *spx* strain (Fig. 5).

Our earlier characterizations revealed that the Δ *spxA2* strain does not display the hypersensitivity to oxidative stresses that was observed for the Δ *spxA1* strain. However, we had also demonstrated that the Δ *spxA1* Δ *spxA2* double mutant was even more sensitive to oxidative stress than the Δ *spxA1* single mutant (11). To further test if replacement of the G52 residue was just as disruptive to the normal functioning of SpxA2, we deleted the *spxA1* gene in the *spxA2*_{G52R} strain, thereby creating a double Δ *spxA1* *spxA2*_{G52R} strain. We then used disc diffusion assays to test the sensitivity of the strains to H₂O₂ or diamide. In both cases, as previously observed, the Δ *spxA2* strain showed a similar level of resistance as UA159. The Δ *spxA1* and Δ *spxA1* Δ *spxA2* strains,

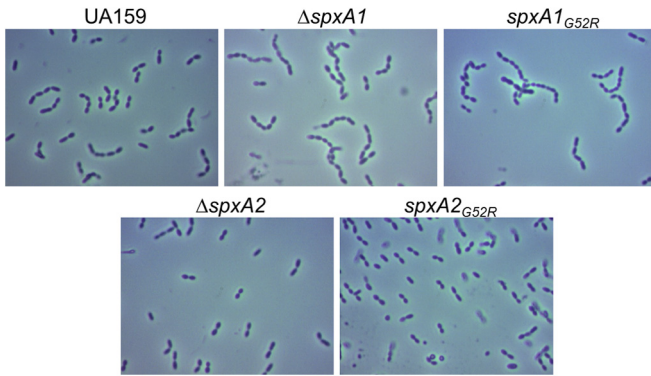


FIG 4 Chain length phenotype of $spxA_{G52R}$ strains is identical to that of $\Delta spxA$ strains. Overnight cultures of *S. mutans* UA159, $\Delta spxA1$, and $\Delta spxA2$ strains and variants with substitutions of the G52 residue ($spxA1_{G52R}$ and $spxA2_{G52R}$) were visualized by light microscopy.

however, demonstrated statistically significant heightened sensitivity to both compounds. The $spxA1_{G52R}$ and $\Delta spxA1$ $spxA2_{G52R}$ strains showed sensitivities nearly identical to those of the corresponding single and double deletion strains. In the $\Delta spxA1$ background, replacement of the G52 residue in SpxA2 resulted in a strain that was indistinguishable from the double Δspx strains (Fig. 5). Therefore, our results convey the importance of a single residue by demonstrating that replacing the glycine residue at po-

sition 52 with arginine disrupts the functionality of both SpxA1 and SpxA2.

The G52 residue is essential for SpxA1-dependent regulation. The physiological characterization of the strains bearing SpxA_{G52R} point mutations (Fig. 5) demonstrated the importance of the G52 residue for Spx functionality. Next, we tested whether the G52 residue was indeed critical for SpxA1 to exert its effect upon gene transcription. To this end, we performed the IVT reactions with MBP-tagged versions of SpxA1 or the SpxA1_{G52R} variant. Despite the presence of the large MBP tag, addition of SpxA1 but not of SpxA1_{G52R} enhanced transcription of *ahpC*, *sodA*, and *tpx* (Fig. 6). In fact, cleavage of the MBP tag did not augment transcription (data not shown). These results confirmed that, as observed in *B. subtilis*, an intact G52 residue is critical for SpxA1 to exert its transcriptional influence over oxidative stress genes.

DISCUSSION

Mounting evidence indicates that transcriptional regulation by Spx is critical for the ability of low-GC Gram-positive bacteria to cope with oxidative stress (9, 11, 12, 15, 16, 22, 26, 33–36). However, proof of direct regulation by Spx in bacteria other than *B. subtilis* has been limited to the *nox* gene of *S. mutans* (26). Moreover, while some species, such as *Enterococcus faecalis* and *Streptococcus aureus*, appear to encode only one copy of a bona fide Spx regulator, streptococcal species and, more recently, *Bacillus anthracis* were shown to encode two Spx proteins, dubbed SpxA1 and SpxA2 (11, 15, 33). From these studies, transcriptomic and

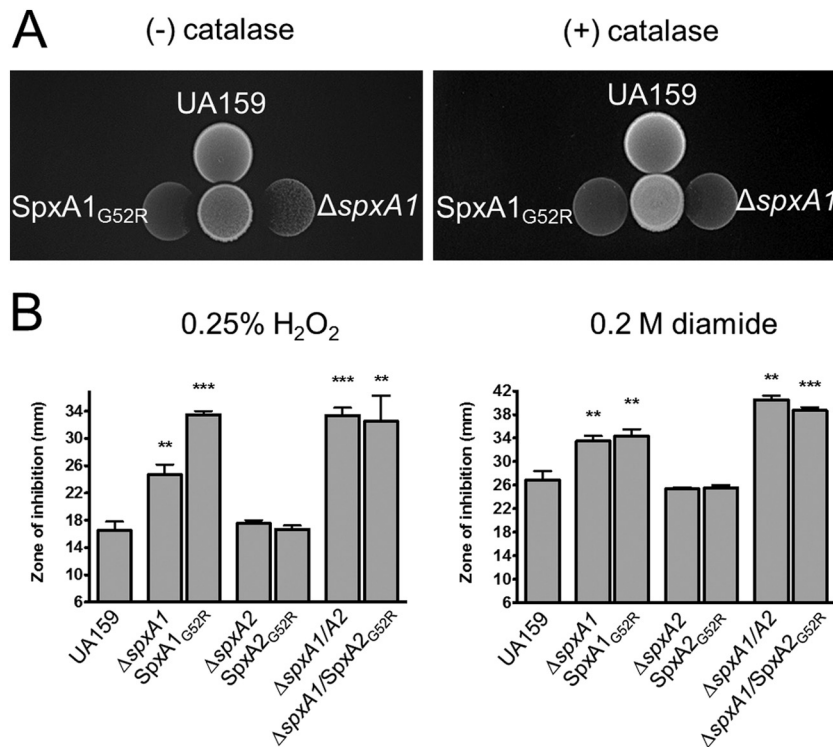


FIG 5 The $spxA_{G52R}$ strains phenocopied the $\Delta spxA$ strain in oxidative stress assays. (A) Inhibition assay reveals that $\Delta spxA1$ *S. mutans* is sensitive to the H₂O₂ produced by *S. gordonii* (center spot). A strain in which the G52 of SpxA1 has been replaced with arginine ($spxA1_{G52R}$) is equally sensitive. The assay was repeated with catalase overlaid onto the *S. gordonii* spot to inactivate the H₂O₂, resulting in loss of sensitivity. (B) Early logarithmic-phase cultures of *S. mutans* UA159, $\Delta spxA1$, $spxA1_{G52R}$, $\Delta spxA2$, $spxA2_{G52R}$, $\Delta spxA1$ $\Delta spxA2$, and $\Delta spxA1$ $spxA2_{G52R}$ strains were swabbed onto a BHI agar plate, upon which filter paper discs impregnated with 0.25% H₂O₂ or 0.2 M diamide were placed. Diameters of zones of growth inhibition were measured after 24 h of incubation at 37°C and 5% CO₂. Data shown represent averages and SDs from three replicate samples. **, $P \leq 0.005$; ***, $P \leq 0.0005$.

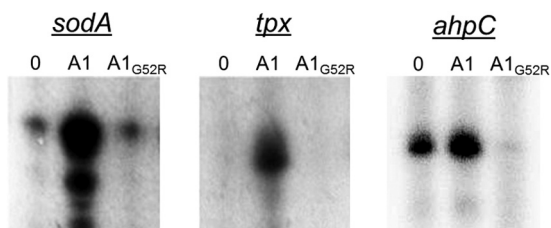


FIG 6 Enhanced gene transcription requires intact SpxA1 protein. *In vitro* transcription reactions were performed with the regulatory regions of *ahpC*, *sodA*, or *tpx* incubated with *B. subtilis* RNAP without SpxA1 (0), with MBP-tagged SpxA1 (A1), or with MBP-tagged SpxA1_{G52R} (A1_{G52R}). Radiolabeled RNA transcripts were precipitated, applied to 8% urea PAGE, and visualized by exposure to a phosphorimager screen. Representatives of three or more independent reactions are shown.

phenotypic characterization suggests that the two Spx paralogues have overlapping regulatory functions, although SpxA1 appears to exert a dominant role in oxidative stress gene regulation. Despite this progress, an understanding of how the two Spx proteins interact with the RNAP to activate transcription and even definitive proof that SpxA2 can bind to the RNAP are missing.

In this study, we exposed cells of the parent and Δ *spx* strains to H₂O₂ stress, which allowed for a better appreciation of the role of each Spx protein in oxidative stress gene regulation. The results confirmed not only that the basal expression levels of the bulk of these genes (*ahpC*, *dpr*, *gor*, *nox*, *sodA*, *tpx*, *trxA*, and *trxB1*) are reduced when SpxA1 is absent but also that the strains lacking SpxA1 are completely unable to mount a transcriptional response toward oxidative stress. The main function of SpxA2 appears to be in the maintenance of basal levels of expression, rather than during acute oxidative stress conditions. These results fit well with our own previous data and recent studies with the bacterial pathogens *Streptococcus suis* and *Bacillus anthracis*, both possessing two Spx proteins (11, 15, 33). In the case of *S. suis*, an Δ *spxA1* strain was considerably more sensitive to growth under oxidative conditions, while the Δ *spxA2* strain was more sensitive to detergents or salt (15). Similarly, SpxA1 of *B. anthracis* was most critical for tolerance of H₂O₂, though, as in *S. mutans*, simultaneous disruption of both *spxA1* and *spxA2* resulted in extreme sensitivity to diamide (33).

By using IVT assays, we obtained direct evidence that both Spx proteins can serve as positive transcriptional regulators. In agreement with our previous microarray study (11) and transcriptional profiling of H₂O₂-treated cells, SpxA1 is the major regulator governing transcription of oxidative stress genes. Among the three genes included in the IVT analysis, *ahpC*, *sodA*, and *tpx*, the contribution of SpxA2 to transcription was evident only for *ahpC*, although increasing amounts of SpxA2 resulted in modest transcription of *sodA*. It is possible that SpxA2 requires a cofactor(s), an adaptor protein(s), or specific environmental conditions that were lacking in our *in vitro* assays. Another plausible scenario is that the affinity of the SpxA2-RNAP complex to the regulatory region of oxidative stress genes is weak compared to that of the SpxA1-RNAP complex. Along these lines, the primary targets of *S. mutans* SpxA2 transcriptional control may lie beyond the boundaries of the oxidative stress response. For example, our microarray data suggested a role for SpxA2 in cell wall homeostasis (11), and Spx regulation has been implicated in antibiotic resistance, biofilm formation, and salt and SDS stress (10, 12, 15, 35, 37, 38). It is

conceivable that SpxA2 evolved to control genetic traits that are distinct from SpxA1, and yet it retained low affinity for the regulatory region of oxidative stress genes, thereby serving as a backup regulator when SpxA1 is not available. In the future, efforts to identify gene targets strongly dependent on SpxA2 for expression may shed new light on the cellular significance of SpxA2.

Another interesting possibility is that the two Spx proteins could work in concert in the activation of oxidative stress genes. However, recent work with *B. subtilis* revealed that Spx binds to the RNAP α -CTD as monomer (23), different than the initial belief that it bound as a dimer (24). If this is also true in *S. mutans*, then another possibility is that the two Spx proteins compete for binding to the RNAP. IVT assays in which SpxA1 and SpxA2 were provided in equal amounts indicated that neither cooperation nor competition between the two Spx proteins for RNAP binding seems to occur, although these observations are based on *in vitro* evidence and limited to only three genes.

In *B. subtilis*, replacement of the G52 residue by arginine (G52R) abolished the ability of Spx to exert transcriptional control, due to an inability to interact with the RNAP (24, 39). This interaction also requires conservation on the part of RNAP, and work in the Zuber lab demonstrated that certain amino acid substitutions in the alpha subunit of the RNAP also abolished this interaction (23, 40). The present study extends the importance of the G52 residue for Spx-stimulated transcription in *S. mutans*, as the *spxA1*_{G52R} and *spxA2*_{G52R} strains were phenotypically identical to their respective *spxA1* and *spxA2* deletion strains. As expected, no increase in transcription was observed in IVT reactions performed with the SpxA1_{G52R} variant. In this case, it is possible that steric disruption of the G52R variant alters the conformation of the protein so that upon interaction with the RNAP, the SpxA1_{G52R} variant causes a repositioning of the RNAP that does not favor transcription. Given that arginine is a considerably larger amino acid than glycine, it is possible that replacement of the G52 residue with a smaller amino acid such as alanine or valine would not result in the same outcome. Nevertheless, the substitution described in this study clearly demonstrates that interfering with the architecture of the *S. mutans* Spx proteins disrupts their normal function, likely due to inability to properly interact with the RNAP.

While the CXXC motif is central for redox control of Spx, recent work uncovered additional critical residues in the *B. subtilis* Spx. For example, mutational analysis suggests that the R92 residue is also important for redox control (24, 41). While the *S. mutans* SpxA1 possesses the R92 residue, the arginine is replaced by a serine (S92) in SpxA2. This is also the case in other streptococcal species such as *S. gordonii*, *S. pneumoniae*, and *S. pyogenes*, with one Spx protein (SpxA1) containing an R92 residue and the second (SpxA2) containing S92. We have hypothesized that Spx proteins harboring the R92 residue respond more strongly to environmental redox conditions and could be the basis for the differing roles that we have observed for the two Spx proteins of *S. mutans*. Studies to address the importance of the CXXC motif as well as the R92 residue to Spx functionality are under way.

A major challenge in the identification of Spx-regulated gene targets is the lack of a consensus DNA binding motif. In *B. subtilis*, an AGCA motif, located at the -44 region, was shown to be required for Spx-dependent transcription of *trxA* and *trxB*, with the less stringent a/tGCa/t motif (letters in lowercase denote less conserved residues) associated with a greater number of genes (20, 24,

41). In a separate study, standard computational searches for consensus Spx-RNAP binding motifs in *B. subtilis* were unsuccessful (36). The second study concluded that in lieu of a traditional consensus motif, promoters of genes regulated by Spx displayed extended -35 and -10 elements. Especially noticeable was an extension of the -35 element in genes that were positively, as opposed to negatively, regulated by Spx. That study also noted the AGCA element upstream of the -35 element, but only for about 10% of the Spx-regulated genes (36). We searched for the AGCA motif in *S. mutans* and found it in only three of the genes involved in the present study (*nox*, *sodA*, and *trxA*), suggesting that the *cis*-acting site directing SpxA gene regulation may vary from species to species, or even from one gene to another. Mutational analysis of the promoter region of targeted genes will likely prove useful in identifying sequences that are critical for Spx transcriptional activation of *S. mutans*.

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