



Streptococcus mutans SpxA2 relays the signal of cell envelope stress from LiaR to effectors that maintain cell wall and membrane homeostasis

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Abstract

Streptococcus mutans is a major etiologic agent of dental caries, which is the most common chronic infectious disease worldwide. *S. mutans* is particularly adept at causing caries due to its exceptional capacity to form biofilms and its ability to survive acidic conditions that arrest acid production and growth in many more benign members of the oral microbiota. Two mechanisms utilized by *S. mutans* to tolerate acid are: modulation of the membrane fatty acid content and utilization of the F₁F₀-ATPase to pump protons out of the cytosol. In this study, the role of the *spxA2* transcriptional regulator in these two pathways, and overall cell envelope homeostasis, was examined. Loss of *spxA2* resulted in an increase in the proportion of saturated fatty acids in the *S. mutans* membrane and altered transcription of several genes involved in the production of these membrane fatty acids, including *fabT* and *fabM*. Furthermore, activity of the F₁F₀-ATPase was increased in the Δ *spxA2* strain. Transcription of *spxA2* was elevated in the presence of a variety of membrane stressors, and highly dependent on the *liaR* component of the LiaFSR system, which is known to sense cell envelope stress in many Gram-positive bacteria. Finally, deletion of Δ *spxA2* led to altered susceptibility of *S. mutans* to membrane stressors. Overall, the results of this study indicate that *spxA2* serves a crucial role in transmitting the signal of cell wall/membrane damage from the LiaFSR sensor to downstream effectors in the SpxA2 regulon which restore and maintain membrane and cell wall homeostasis.

KEYWORDS

dental caries, environmental regulation, fatty acids, Spx, *Streptococcus mutans*

1 | INTRODUCTION

Dental caries is an oral disease affecting greater than 50% of the population worldwide, and is caused by biofilms of acid-producing

bacteria on the tooth surface which demineralize the enamel (Pitts et al., 2017). Dental caries is often associated with elevated numbers of *Streptococcus mutans* (Gross et al., 2012), which is considered a major etiologic agent of the disease. *S. mutans* contributes substantially to caries pathogenesis through its exceptional capacity to form biofilms and its ability to produce acid from a wide variety of carbohydrate sources (Bowen, Burne, Wu, & Koo, 2018; Lemos et al.,

Jonathon L. Baker and Sarah Saputo contributed equally to this work.

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2019). Furthermore, *S. mutans* employs an acid-tolerance response (ATR) that utilizes multiple mechanisms to survive better in acidic conditions than many of its neighbors in the oral microbiota (Baker, Faustoferri, & Quivey, 2017; Lemos & Burne, 2008). This allows *S. mutans* to contribute to the destruction of the underlying tooth even at low pH values that would arrest growth and glycolysis in many more benign species.

An important component of the *S. mutans* ATR is a shift from a cell membrane consisting of mainly saturated fatty acids (SFAs) to one comprised mainly of unsaturated fatty acids (UFAs) that is accompanied by an increase in fatty acid chain length by ~2 carbons (Fozo & Quivey, Jr., 2004b). These longer-chained UFAs are synthesized de novo by *S. mutans* using FabM, a trans-2/cis-3-decenoyl-ACP isomerase enzyme (Fozo & Quivey, Jr., 2004a). Blockage of this pathway through inhibition of the FabF enzyme (the next enzyme downstream of FabM in the UFA synthesis pathway) via the drug cerulenin impaired the ability of *S. mutans* to generate UFAs, which rendered it more sensitive to acid (Fozo & Quivey, Jr., 2004b). Alternatively, deletion of the *fabM* gene resulted in a strain unable to produce UFAs (0% UFA content), which was severely impaired in its ability to survive an acid challenge and, crucially, cause disease in a rodent model of dental caries (Fozo & Quivey, Jr., 2004a; Fozo, Scott-Anne, Koo, & Quivey, 2007). The acid-sensitive phenotype of the $\Delta fabM$ strain could be partially rescued by addition of exogenous fatty acids to the growth media, which were incorporated into the membrane and protective against acid stress (Fozo & Quivey, Jr., 2004a).

The transcriptional regulator denoted Spx, for suppressor of ClpX, has been described to play a major role in the general stress response in Gram-positive bacteria (Nakano, Hajarizadeh, Zhu, & Zuber, 2001). Canonical Spx regulators serve as sensors that monitor redox state and regulate transcription accordingly (Zuber, 2004). *S. mutans* UA159 encodes two Spx homologs, *spxA1* (SMU.1142) and *spxA2* (SMU.2084c) (Kajfasz et al., 2010). SpxA1 plays a major role in the oxidative stress response and survival in the presence of oxidative stressors, serving as a positive transcriptional regulator of a number of genes that ameliorate oxidative stress including superoxide dismutase, NADH oxidase, and glutathione oxidoreductase (Baker et al., 2014; Kajfasz et al., 2010, 2015). Interestingly, microarray analysis revealed that the regulons of SpxA1 and SpxA2 had little overlap, suggesting distinct regulatory duties for each Spx protein (Kajfasz et al., 2010). While the regulon of SpxA1 heavily featured genes involved in oxidative stress, the regulon of SpxA2 included many genes involved in cell division, the cell envelope, and fatty acid biosynthesis (FAB) (Kajfasz et al., 2010). Indeed, additional research illustrated that the SpxA2 deletion mutant ($\Delta spxA2$) could not form the chains of cells typical of Streptococci, suggesting a defect in the cell division process (Kajfasz et al., 2010, 2015). Furthermore, $\Delta spxA2$ exhibited enhanced biofilm formation due to increased expression of the glycosyltransferase enzymes that form the extracellular glucan matrix (Galvao et al., 2017). Finally, expression of $\Delta spxA2$ was also dependent on the LiaFSR three-component system (Shankar, Mohapatra, Biswas, & Biswas, 2015; Suntharalingam, Senadheera,

Mair, Levesque, & Cvitkovitch, 2009), which governs the response to cell envelope stress across a diverse number of Gram-positive bacteria (Mascher, Helmann, & Uden, 2006).

The hypothesis of this study was that *S. mutans* SpxA2 modulates membrane composition, which is likely to have significant downstream physiological effects on the ability of the organism to deal with cell envelope stress. This theory was well supported by the fact that loss of *spxA2* affects transcription of several genes in the fatty acid biosynthesis operon (*fab*) (Kajfasz et al., 2010), and that deletion of *spxA2* decreased colonization and virulence in a rodent model of dental caries (Galvao et al., 2017). The data presented below illustrates that SpxA2 is indeed required for *S. mutans* to have the full complement of UFAs in its membrane, and that SpxA2 influences UFA production at the transcriptional level. Furthermore, SpxA2 transcription is increased in the presence of membrane stressors, and the deletion of *spxA2* rendered *S. mutans* more sensitive to several peptidoglycan-targeting antibiotics, yet more resistant to osmotic stress and the fatty acid biosynthesis inhibitor, cerulenin.

TABLE 1 Strain and plasmids used in this study

	Description	Source or reference
Strains		
<i>Streptococcus mutans</i>		
UA159	genomic type strain	Murchison, Barrett, Cardineau, & Curtiss, (1986), Ajdic et al. (2002)
JL13	<i>spxA2</i> mutant strain	Kajfasz et al. (2009)
UR222	UA159 carrying <i>fabT-cat</i> construct	Faustoferri et al. (2015)
UR309	JL13 carrying <i>fabT-cat</i> construct	This study
UR211	UA159 carrying <i>fabM-cat</i> construct	Faustoferri et al. (2015)
UR312	JL13 carrying <i>fabM-cat</i> construct	This study
$\Delta liaR$	<i>liaR</i> deletion strain, Δ SMU.487	Quivey, Jr. et al. (2015)
UA159 (pJLspxA2)	UA159 carrying <i>spxA2-cat</i> construct	Rivera-Ramos (2015) and gift from Lemos Lab
UR378	<i>liaR</i> carrying <i>spxA2-cat</i> construct	This study
Plasmids		
pJLmarR	<i>fabT</i> (SMU.1745c) promoter- <i>cat</i> construct	Faustoferri et al. (2015)
pJLfabM	<i>fabM</i> (SMU.1746c) promoter- <i>cat</i> construct	Faustoferri et al. (2015)
pJLspxA2	<i>spxA2</i> (SMU.2084c) promoter- <i>cat</i> construct	Rivera-Ramos (2015) and gift from Lemos Lab

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and growth conditions

Strains in this study are listed in Table 1. Bacterial strains were grown in brain heart infusion (BHI) medium (BD/Difco) at 37°C in a 5% (vol/vol) CO₂/95% air atmosphere. Overnight cultures of *S. mutans* strains UA159 and Δ *spxA2* were grown in BHI medium, sub-cultured 1:20 into fresh BHI medium, and incubated at 37°C in a 5% (vol/vol) CO₂/95% air atmosphere until cultures reached an optical density at 600 nm (OD₆₀₀) ~0.3. Where indicated, cerulenin (10 µg/ml) was added to BHI medium and *S. mutans* strains were seeded to a final OD₆₀₀ of 0.05 in a 96 well plate (Corning). Growth was assessed through measurement of OD₆₀₀ in a Bioscreen C (Growth Curves USA).

2.2 | Chemostat culture conditions

S. mutans strains UA159 and Δ *spxA2* were grown under continuous culture conditions using a BioFlo 2000 fermenter (New Brunswick Scientific) in TY medium (3% tryptone, 0.1% yeast extract, 0.5% KOH, and 1 mM H₃PO₄) containing 1% glucose, as described previously (Faustoferri et al., 2015). Cultures were grown at a constant dilution rate of 0.24/hr under glucose-limiting (2.3 mM) conditions. Culture pH was continually maintained throughout the experiment via the addition of 2 N KOH and verified by a pH probe (Mettler Toledo). Cultures were harvested after 10 generations at steady-state pH 7, followed by the addition of excess glucose (20 mM), thereby lowering culture pH. Cultures were similarly harvested at pH 5 after 10 generations at steady-state and stored at -80°C.

2.3 | ATPase activity assay

ATPase activity from three independent cultures of *S. mutans* strains UA159 and Δ *spxA2* was measured as a function of release of inorganic phosphate from ATP, as previously described (Cross, Garcia, Faustoferri, & Quivey, 2016). Briefly, 25 ml cultures were grown in BHI medium overnight at 37°C in a 5% (vol/vol) CO₂/95% air atmosphere, harvested, and washed in membrane buffer (75 mM Tris pH 7, 10 mM MgSO₄). Cells were permeabilized by addition of toluene, followed by two freeze-thaw cycles in dry ice/ethanol, and pelleted in a microcentrifuge for 10 min at 16,100 g. The supernatant was decanted and cells were resuspended in 1 ml ATPase buffer (50 mM Tris maleate pH 6, 10 mM MgSO₄) and stored in 500 µl aliquots at -80°C.

An aliquot (300 µl) of the toluenized cell extract was added to 9 ml ATPase buffer. ATP (pH 6, 5 mM final concentration) was added and 500 µl aliquots were removed at time points 0, 5, 10, 20, 30, and 45 min, in triplicate. Reactions were stopped with 2 ml 20% trichloroacetic acid. Samples were centrifuged at 4°C for 15 min at 2,272 g. The supernatant (1 ml) was transferred to tubes containing 1.5 ml H₂O, followed by addition of 0.5 ml acid molybdate solution (1.25 g/dl ammonium molybdate tetrahydrate in

2.5 N sulfuric acid). Fiske and Subbarow Reducing solution (125 µl) (Fiske & Subbarow, 1925) was added to each tube, samples were incubated at room temperature for 10 min, and absorbance was measured at 660 nm. Protein concentration was estimated using the bicinchoninic acid (BCA) assay (Smith et al., 1985) (Sigma). ATPase activity was assessed relative to a phosphate standard curve, normalized to OD₆₀₀ at time zero, and expressed as mM PO₄ released per min per µg protein.

2.4 | Real-time quantitative RT-PCR

Real-time quantitative reverse transcription-PCR (qRT-PCR) was performed as described previously (Kovacs, Faustoferri, & Quivey, 2017) using a StepOnePlus real-time PCR system (Applied Biosystems) with gene-specific primers described previously (Faustoferri et al., 2015). RNA was isolated from three independent cultures of UA159 or Δ *spxA2* grown to steady-state in the chemostat to pH values of 7 and 5 (described above). The mRNA copy number was quantified based on a standard curve of PCR product, as previously described (Kovacs et al., 2017).

2.5 | Membrane fatty acid determination

Membrane fatty acid content was determined from aliquots of steady-state cultures of *S. mutans* UA159 and Δ *spxA2* grown to pH 7 and 5 (described above). Samples (20 ml) were stored at -80°C until sent to Microbial ID for analysis of membrane fatty acid composition by gas chromatography of fatty acid methyl esters (GC-FAME), as described elsewhere (Fozo & Quivey, 2004b).

2.6 | Chloramphenicol acetyltransferase (CAT) activity assay

S. mutans strains carrying a promoter-*cat* fusion were grown overnight and sub-cultured in BHI medium, BHI medium buffered to pH 7 (+50 mM KPO₄ buffer), or BHI titrated to pH 5 (with HCl), where indicated, in conditions described above. Cultures were harvested at OD₆₀₀ ~0.5 and pellets were stored at -80°C. For experiments measuring *spxA2* transcription in cultures exposed to cell wall stress agents, overnight cultures were sub-cultured in BHI medium and grown to an OD₆₀₀ ~0.4. Test agents were added to a final concentration of 0.5× MIC [i.e., 4 µg/ml tunicamycin; 1 µg/ml vancomycin; 2.5% NaCl; or 10 µg/ml cerulenin] and cultures were incubated at 37°C for 30 min. Cells were harvested and pellets stored at -80°C.

CAT assays for the measurement of transcriptional activity were performed according to the method of Shaw et al., as modified by Kuhnert et al. (Kuhnert, Zheng, Faustoferri, & Quivey, 2004; Shaw, 1975). In brief, pellets from 3 independent 50 ml cultures per test strain were resuspended in 1 ml 10 mM Tris-Cl, pH 7.8 and lysed for two 1-min intervals using 0.1-mm glass beads in a Mini-Bead Beater

(BioSpec Products). Cell debris was removed by centrifugation at 4°C, 16,100 g for 10 min. The lysates were removed and used for CAT assays and total protein quantification using the Bradford method and assayed in triplicate. Each CAT assay reaction mixture consisted of 50 µl whole-cell lysate, 100 mM Tris-Cl, pH 7.8, 0.1 mM acetyl-coenzyme A (acetyl-CoA), and 0.4 mg/ml 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) in a total volume of 1 ml. Reactions were initiated by addition of 0.1 mM chloramphenicol. The reaction rate was determined at an optical density of 412 nm over a period of 3 min and is presented as µmol of chloramphenicol acetylated per min per mg total protein.

2.7 | MIC determination

Compounds used in MIC testing were as follows: bacitracin, chloramphenicol, daptomycin, penicillin, polymixin B, tunicamycin, and vancomycin (Sigma-Aldrich). To determine the MICs of test

compounds against *S. mutans* UA159 and Δ *spxA2*, overnight cultures were grown in BHI medium, diluted 1:50 in fresh TYG medium, and grown to exponential phase ($OD_{600} \sim 0.3$). A 96-well plate (Corning, Inc.) containing fresh TYG medium was inoculated with 10^5 CFU. A dilution series of the test compound (concentrations ranged from 0 to 64 µg/ml) was added to the plate. The plates were incubated at 37°C in a 5% (vol/vol) $CO_2/95\%$ air atmosphere for 24 hr. The MIC was considered the lowest compound concentration that inhibited bacterial growth, as measured by the OD_{600} . MICs are presented as a concentration in µg/ml from 3 independent cultures assayed in triplicate.

2.8 | Statistical analysis

Statistical significance ($p < .05$) was determined by pairwise comparison using either a paired *t*-test, or a two-way ANOVA with a Tukey's Multiple Comparisons Post Test, where indicated.

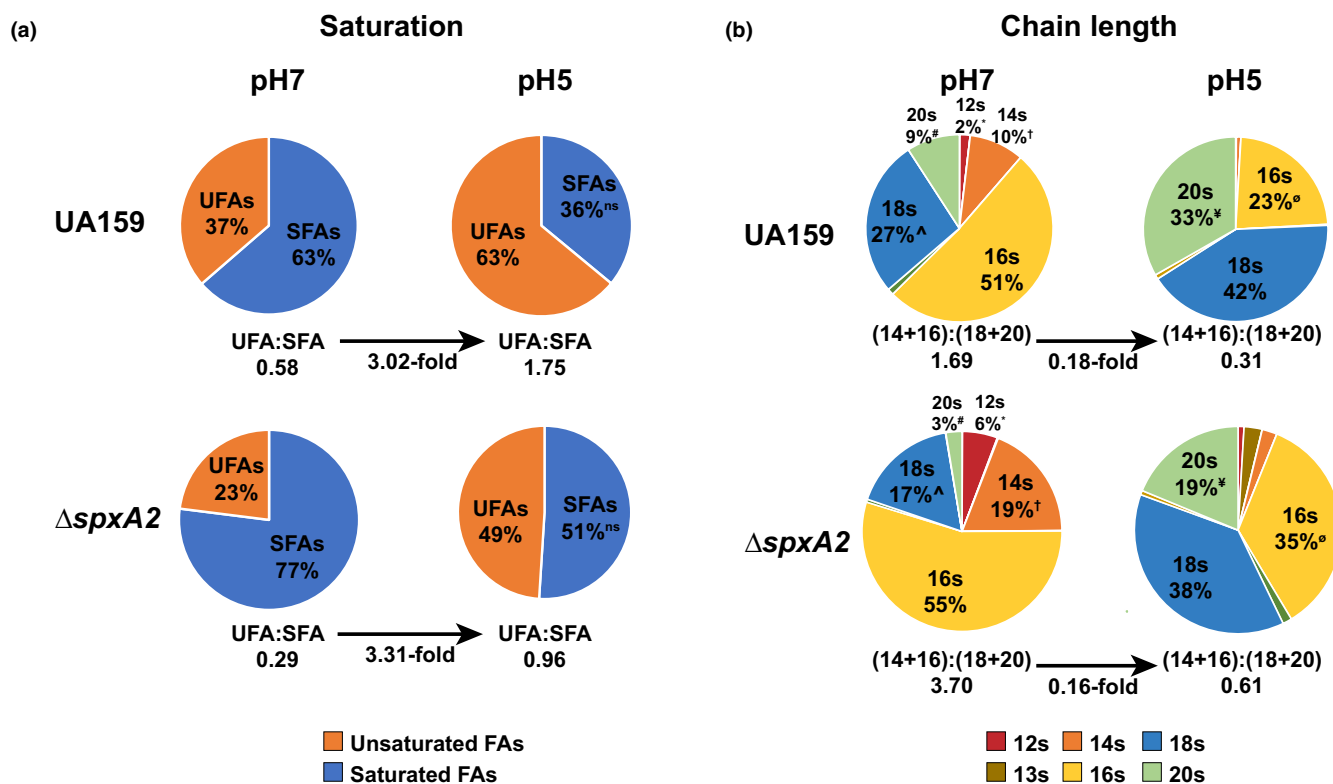


FIGURE 1 Membrane fatty acid composition is altered in Δ *spxA2*. (a) GC-FAME analysis was used to determine membrane fatty acid composition from cultures of *S. mutans* UA159 and Δ *spxA2* grown to steady-state pH values of 7 and 5 as described in Materials and Methods. Percentage of membrane composition is denoted in each sector where saturated fatty acids are shown in blue and unsaturated fatty acids in orange. All values are statistically significant in pairwise comparisons, except for ns, as determined by Student's *t*-test, $p < .05$. Below each pie graph is the UFA:SFA ratio, and values are statistically significant in pairwise comparisons, the fold-change between pH 7 and pH 5 is also indicated. (b) Fatty acid chain lengths are displayed for cultures of *S. mutans* UA159 and Δ *spxA2* grown to steady-state pH values of 7 and 5. Percentage of membrane composition representing fatty acids composed of indicated carbon chain-lengths is denoted in each sector. Only chain lengths representing at least 1% of the total are shown. Statistical significance is designated for major chain length groups, between pairs of like symbols, determined by Student's *t*-test, $p < .05$. Below each pie graph is the ratio of C14 + C16 carbon chains to C18 + C20 carbon chains. The fold-change in this ratio between pH 7 and pH 5 is also indicated. Ratios were statistically significant in pairwise comparisons

3 | RESULTS

3.1 | Deletion of *spxA2* results in aberrant membrane composition

Previous transcriptional analysis indicated that the *fabF* and *fabG* genes within the *fab* operon were down-regulated in the Δ *spxA2* strain, compared to the UA159 parent strain, suggesting that SpxA2 may be an enhancer of their transcription (Kajfasz et al., 2010). To examine whether this dysregulation of FAB in the Δ *spxA2* strain affected the membrane composition of *S. mutans*, GC-FAME was performed on cultures of UA159 or Δ *spxA2* in steady-state growth at pH 7 and pH 5, as described in the Materials and Methods. Δ *spxA2* exhibited a significant reduction in the UFA:SFA ratio, compared to UA159, at both pH 7 and 5 (Figure 1a). Similarly, the length of the fatty acid chains was reduced in the Δ *spxA2* strain compared to UA159 at both pH 7 and pH 5. Specifically, increased proportions of 12-, 14-, and 16-carbon chains and decreased proportions of 18- and 20-carbon chains were observed in Δ *spxA2* compared to UA159 (Figure 1b). This increased chain length may be reflective of the increase in transcription of *fabF* and *fabG* observed in Δ *spxA2* (Kajfasz et al., 2010). The full list of fatty acid types detected by the GC-FAME analysis is available in Table S1. Although both fatty acid saturation and chain length were affected by the loss of *spxA2*, the Δ *spxA2* strain was still able to significantly increase the percentage of UFAs, and 18 and 20 carbon chains, in its membrane through the transition from pH 7 to pH 5. The fold-change in UFA:SFA ratio at pH 5, compared to pH 7, in Δ *spxA2* (3.31-fold) was comparable to that observed in UA159 (3.02-fold), as was the fold-change in the (C14 + C16)/(C18 + C20) ratio from pH 5 to pH 7 (0.18-fold in UA159 and 0.16-fold in Δ *spxA2*). Overall, these data indicate that in Δ *spxA2*, the ATR is still able to increase the proportion of UFAs upon a reduction in environmental pH; however, the proportion of UFAs at both neutral and acidic pH is significantly lower. The

reduction of the UFA:SFA ratio in the Δ *spxA2* strain, compared to UA159, concurs with a previous study that examined membrane composition in batch culture growth conditions (Rivera-Ramos, 2015).

3.2 | SpxA2 influences transcription of *fabT* and *fabM*

Since previous microarray transcriptomics indicated that SpxA2 may be a positive regulator of transcription of the *fabF* and *fabG* components of the fatty acid biosynthesis elongation module (Kajfasz et al., 2010), and loss of *spxA2* led to an increase in SFAs, we examined the role of SpxA2 on transcription of *fabT* and *fabM*, two genes that are involved specifically with UFA biosynthesis. qRT-PCR and a chloramphenicol acetyltransferase (CAT) assay were utilized, which are more sensitive than microarray analysis. qRT-PCR of steady-state cultures of UA159 and Δ *spxA2* at pH 7 and pH 5 indicated a modest reduction in transcription of *fabT* in the Δ *spxA2* strain (Figure 2a). Transcription of *fabM* was also slightly reduced in the Δ *spxA2* background, however, the decrease was not statistically significant (data not shown). *fabT* encodes a transcriptional regulator of the *fab* operon (Faustoferri et al., 2015), while *fabM* encodes the isomerase that allows for UFA production (Fozo & Quivey, Jr., 2004a). To provide further evidence for this regulation by SpxA2, and in a batch culture setting, transcriptional activity from the *fabT* and *fabM* promoters was measured using a chloramphenicol acetyltransferase (CAT) reporter system. In this assay, transcription of *fabT* was decreased in Δ *spxA2* compared to UA159 in media buffered either to pH 7 or pH 5 (Figure 2b). *fabT* transcription at pH 5 was elevated compared to pH 7 in both genetic backgrounds (Figure 2b), in agreement with the qRT-PCR data (Figure 2a). Transcription of *fabM* was increased in Δ *spxA2*, compared to UA159, at pH 7, but was similar to UA159 at pH 5. The discrepancy in results between the CAT

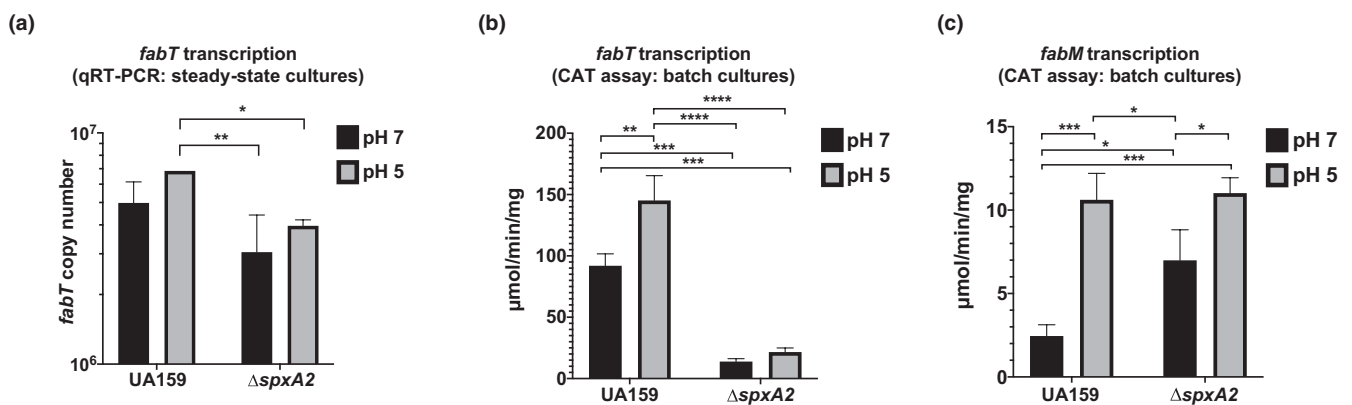


FIGURE 2 *spxA2* affects transcription of *fabT* and *fabM*. (a) qRT-PCR enumerating *fabT* transcripts in RNA extracted from the indicated strains during steady-state growth at pH 7 or pH 5. (b) CAT assay quantifying transcription of *fabT* in the indicated strain from batch cultures buffered to either pH 7 or pH 5. (c) CAT assay quantifying transcription of *fabM* in the indicated strain from batch cultures buffered to either pH 7 or pH 5. All data are derived from 3 independent cultures assayed in triplicate. Asterisks indicate expression levels which are statistically significant between indicated conditions based on a Tukey's Multiple Comparisons Test following a two-way ANOVA (* $p < .05$; ** $p < .01$; *** $p < .001$; **** $p < .0001$; $n = 3$)

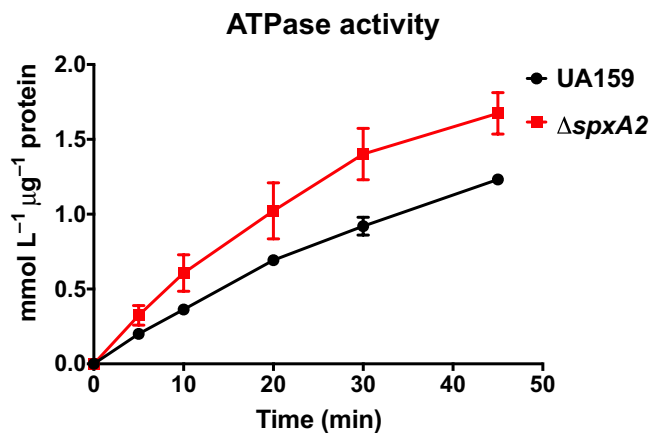


FIGURE 3 ATPase activity is increased in the $\Delta spxA2$ strain. Graph illustrating ATPase activity, measured as described in Materials and Methods, in the indicated strains over time. Data are derived from 3 independent cultures assayed in triplicate. The ATPase activities of UA159 and $\Delta spxA2$ were significantly different based on a paired Student's *t*-test ($p = .0162$; $n = 3$)

assay and qRT-PCR in *fabM* transcription may either result from the physiological differences between steady-state and batch growth or subtle differences between qRT-PCR and the CAT assay.

3.3 | Loss of *spxA2* results in elevated ATPase activity

In addition to an increase in the UFA:SFA ratio, another major component of the *S. mutans* ATR is increased expression and activity of the F_1F_0 -ATPase (Baker et al., 2017; Kuhnert et al., 2004). This complex hydrolyzes ATP to extrude protons from the cell interior in order to maintain a relatively alkaline cytoplasm during growth

of the organism in acidic conditions (Kuhnert et al., 2004). The transcriptional profile of $\Delta spxA2$ revealed that expression of the genes encoding 3 ATPase subunits (*atpC*, *atpD*, and *atpG*) was elevated, relative to UA159, during growth in standard, non-stressed conditions (Kajfasz et al., 2010). To examine whether the increased gene expression of ATPase subunits in $\Delta spxA2$ was correlated with changes in enzyme activity, cultures of UA159 and $\Delta spxA2$ were grown and ATPase activity was measured from permeabilized cells by assaying for release of inorganic phosphate relative to a standard curve. The ATPase activity measured in the *spxA2* mutant strain ($1.67 \pm 0.14 \text{ mmol L}^{-1} \mu\text{g protein}^{-1}$) was elevated, compared to UA159 ($1.23 \pm 0.02 \text{ mmol L}^{-1} \mu\text{g protein}^{-1}$) (Figure 3). These results suggest that the higher transcript levels of ATPase subunits observed in $\Delta spxA2$ are indeed directly correlated with an overall increase in ATPase activity.

3.4 | Expression of *spxA2* is dependent on LiaR, and is elevated in the presence of membrane and cell wall stressors

Previous work illustrated that transcription of *spxA2* is highly dependent on the LiaFSR three-component system (Suntharalingam et al., 2009). This phenomenon was confirmed here using the CAT reporter system. In batch cultures buffered to either to pH 7 or pH 5, expression of *spxA2* was reduced to background levels in a $\Delta liaR$ strain (Figure 4a). Since the LiaFSR system plays a major role in the cell envelope stress response, and induces transcription in the presence of membrane stressors (Suntharalingam et al., 2009), it was likely that *spxA2* responded to membrane stressors as well. To examine this hypothesis, a CAT assay was used to examine the expression of *spxA2* in the presence of membrane-targeting antibiotics tunicamycin and vancomycin, the osmotic stressor NaCl, the osmotic stressor NaCl, Cerulenin

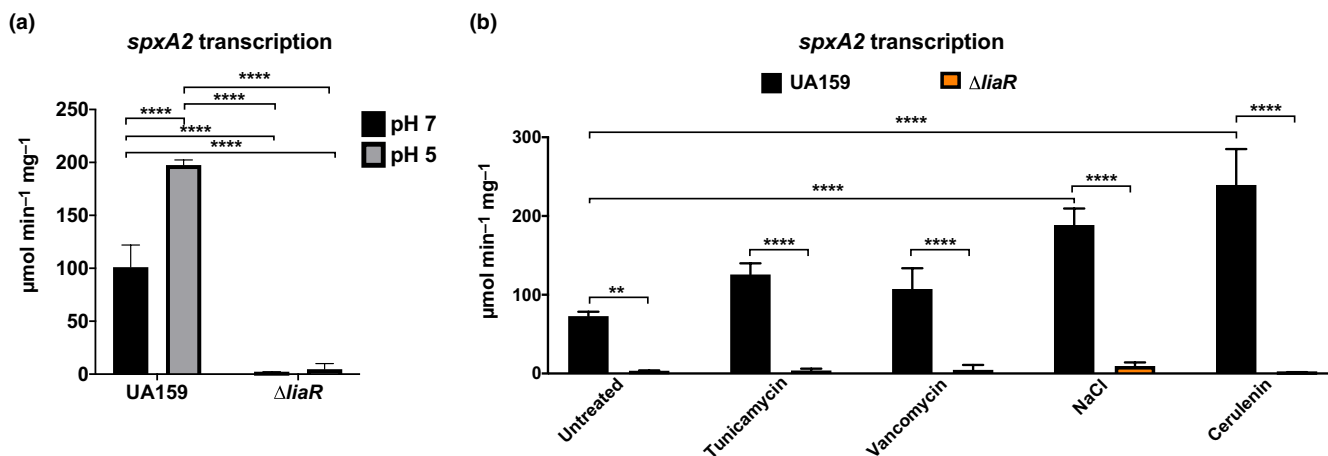


FIGURE 4 *spxA2* transcription is dependent on LiaR, and responds to membrane stressors. (a) CAT assay quantifying transcription of *spxA2* in the indicated strain from batch cultures buffered to either pH 7 or pH 5. (b) CAT assay quantifying transcription of *spxA2* in the indicated strain from batch cultures containing the indicated membrane stressor, as described in Materials and Methods. Data are derived from 3 independent cultures assayed in triplicate. Asterisks indicate expression levels which are statistically significant between indicated conditions based on a Tukey's Multiple Comparisons Test following a two-way ANOVA (** $p < .01$; **** $p < .0001$; $n = 3$)

TABLE 2 Minimum inhibitory concentrations (MIC) of *S. mutans* strains, presented in µg/ml ($n = 3$)

	UA159	$\Delta spxA2$
Bacitracin	256	256
Chloramphenicol	8	8
Daptomycin	64	64
Penicillin	0.25	0.25
Polymixin B	64	32
Tunicamycin	8	0.125
Vancomycin	2	1

and the fatty acid synthesis inhibitor, cerulenin. Transcription of *spxA2* was significantly increased in the presence of all four of these membrane perturbing agents (Figure 4b). When this experiment was repeated in a $\Delta liaR$ background, transcription of *spxA2* was reduced to background levels in the presence of all stressors (Figure 4b), further indicating that the expression of *spxA2* is dependent on *liaR*.

3.5 | Loss of *spxA2* results in increased sensitivity to certain peptidoglycan-targeting antibiotics, but increased resistance to osmotic stress and the FAB inhibitor, cerulenin

Since *spxA2* expression was increased in the presence of the membrane stressors tested above, the susceptibility of the $\Delta spxA2$ strain to these agents, and several others, was examined. The MIC of UA159 and $\Delta spxA2$ was determined for a number of antibiotics that target the cell membrane or peptidoglycan through various modes of action, and chloramphenicol, a protein synthesis inhibitor (Table 2). $\Delta spxA2$ had the same MIC as UA159 for

chloramphenicol, bacitracin, daptomycin, and penicillin. $\Delta spxA2$ did have an increased susceptibility to polymyxin B, tunicamycin, and vancomycin, which may indicate a defect in peptidoglycan homeostasis. The fact that bacitracin and penicillin, which also target the peptidoglycan layer, and daptomycin, which targets the membrane, did not have lowered MIC values in the $\Delta spxA2$ strain may seem contradictory; however, likely explanations are described below in the Discussion section.

The susceptibility of $\Delta spxA2$ to osmotic stress in the form of 2.5% NaCl was examined. The hyperosmotic stress induced by the addition of NaCl (2.5%) to log-phase cultures resulted in severe growth inhibition of UA159, but only moderate inhibition of $\Delta spxA2$, suggesting that $\Delta spxA2$ is resistant to osmotic stress (Figure 5a). Finally, the susceptibility of $\Delta spxA2$ was investigated in the presence of the fatty acid biosynthesis inhibitor, cerulenin. While cultures of both UA159 and $\Delta spxA2$ were inhibited by cerulenin, the $\Delta spxA2$ strain exhibited a faster growth rate and achieved a higher cell density than UA159 (Figure 5b).

4 | DISCUSSION

Spx proteins are transcriptional regulators that are widespread across Gram-positive bacteria (Zuber, 2004). Spx is most well-understood in *B. subtilis*, where the protein utilizes an N-terminal CXXC motif, a conserved glycine residue at position 52, and a C-terminal RPI motif to sense redox state and subsequently interact with a specific DNA sequence and the RNA polymerase to influence transcription of its regulon (Nakano, Erwin, Ralle, & Zuber, 2005). *S. mutans* encodes two Spx homologs, encoded by *spxA1* and *spxA2* (Kajfasz et al., 2009). SpxA1 plays a major role in sensing oxidative stress and positively regulating the transcription of genes that alleviate oxidative stress, such as superoxide dismutase, NADH oxidase, and glutathione oxidoreductase (Kajfasz et al., 2010, 2015). Deletion of *spxA1* results in increased

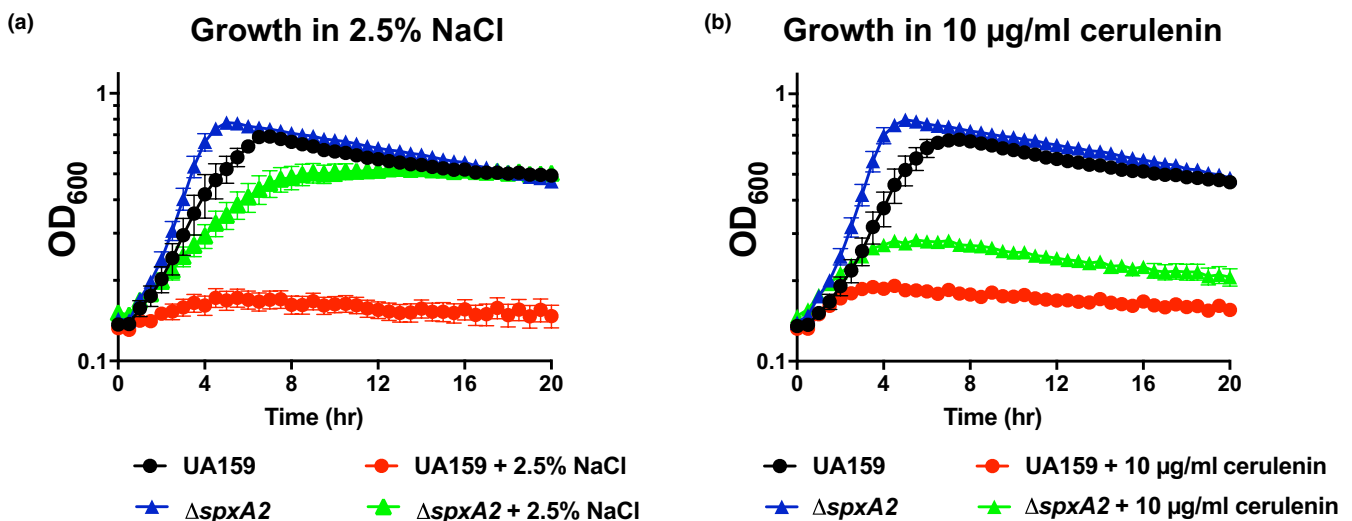


FIGURE 5 The $\Delta spxA2$ strain is resistant to osmotic stress (NaCl) and the fatty acid biosynthesis inhibitor, cerulenin. Growth of the indicated strain with or without 2.5% NaCl (a) or 10 µg/ml cerulenin (b). $n = 10$ for each condition

sensitivity to acid and oxidative stress, as well as attenuated virulence, and a reduced ability to produce mutacins and compete with neighbouring health-associated bacteria (Galvao et al., 2017). Deletion of *spxA2* resulted in a more subtle phenotype, and its role remains poorly understood. Microarray analysis indicated that the regulons of *spxA1* and *spxA2* have little overlap, suggesting a distinct role for each regulator (Kajfasz et al., 2010). The regulon of *SpxA2* primarily consisted of genes involved in cell division, the cell membrane, and cell wall homeostasis (Kajfasz et al., 2010). Further evidence of a role of *spxA2* in these pathways has accumulated. The *spxA2* deletion mutant was unable to form cell chains and also displayed increased biofilm formation due to elevated expression of glucosyltransferase enzymes (Galvao et al., 2017; Kajfasz et al., 2010, 2015). Lastly, transcription of *spxA2* was significantly decreased upon disruption of the LiaFSR three-component system, which is known to sense, and respond to, cell envelope stress (Shankar et al., 2015; Suntharalingam et al., 2009).

To cause disease, *S. mutans* must be able to survive and continue to produce acid below the critical pH of 5.5, the point where the adjacent enamel of the tooth begins to demineralize (Bowen et al., 2018; Pitts et al., 2017). To this end, *S. mutans* employs a complex ATR that allows it to survive in acidic conditions that many of the more health-associated dental plaque residents cannot tolerate (Lemos & Burne, 2008; Lemos et al., 2019). A crucial component of the ATR is a coordinated increase in the proportion of UFAs in the cell membrane, concurrent with an increase in the length of these fatty acid chains (Fozo & Quivey, Jr., 2004b). Blockage of this response via deletion of *FabM*, the enzyme responsible for unsaturated fatty acid production, resulted in greatly increased acid sensitivity and significantly reduced virulence in a rodent model of dental caries (Fozo & Quivey, Jr., 2004a; Fozo et al., 2007). Although the transcription of *fabM* is regulated in part by the *FabT* regulator (Faustoferri et al., 2015), the precise induction mechanism of this shift has not been elucidated. Another well-established component of the *S. mutans* ATR is increased expression of the F_1F_0 ATPase, which is used to actively pump protons out of the cytosol to maintain a more neutral pH (Kuhnert et al., 2004; Sheng & Marquis, 2006). Previous examination of the *SpxA2* regulon using microarrays suggested that *SpxA2* may serve as a positive regulator of genes responsible for both of these components of the ATR (Kajfasz et al., 2010). The goal of this study was to further explore the role of *SpxA2* in these ATR pathways and overall membrane homeostasis.

Deletion of *spxA2* resulted in a membrane fatty acid composition that was distinct from the parent strain, UA159 (Figure 1). Although the Δ *spxA2* strain was able to increase the proportion of UFAs in its membrane upon environmental acidification, the level of UFAs was significantly reduced compared to the parent strain in both neutral and acidic conditions. The reduced level of UFAs in Δ *spxA2* agreed with a preliminary experiment examining the UFA:SFA ratio of Δ *spxA2* in batch cultures (Rivera-Ramos, 2015). This result suggests that although the ATR is largely intact in the absence of *SpxA2*, normal FAB is disrupted. Typically, the UFAs present in the *S. mutans* membrane are mainly 18 and 20 carbon chains, while the SFAs are 14 and 16 carbon chains. This was observed here, indicating that loss of *spxA2* did not decouple unsaturation from the increase in chain

length. Although it is not known why the increase in UFAs in *S. mutans* is protective against acid, a hypothesis is that the fluidity of the membrane is altered by this shift. A change in fluidity could alter the activity of transmembrane proteins, such as ATPases, which are known to be required for maintenance of a neutral cytosol in an acidic extracellular environment (Sheng & Marquis, 2006). Indeed, the Δ *fabM* strain displayed aberrant ATPase activity in the complete absence of UFAs (Fozo & Quivey, Jr., 2004a). As opposed to the Δ *fabM* strain, which was extremely sensitive to acid, the Δ *spxA2* strain was only slightly more acid-sensitive than the parent strain UA159 (Kajfasz et al., 2010). This makes sense, as the Δ *fabM* strain has no UFAs in its membrane, while the Δ *spxA2* strain does have UFAs, but fewer than UA159. It is also worth noting that an Δ *spxA1/A2* double mutant was significantly more acid-sensitive than either the Δ *spxA1* or the Δ *spxA2* single mutant strains (Kajfasz et al., 2010).

Because the fatty acid composition was disrupted in the Δ *spxA2* strain, and microarray analysis had suggested that *SpxA2* may regulate genes in the *fab* operon (Kajfasz et al., 2010), transcription of *fabT* and *fabM*, specific genes involved in UFA production, was examined in the genetic background of the Δ *spxA2* strain. Similar to the microarray data that showed a decrease in *fabF* and *fabG* expression in the Δ *spxA2* strain (Kajfasz et al., 2010), qRT-PCR of the Δ *spxA2* strain grown in steady-state cultures showed a decrease in *fabT* transcription (Figure 2a). Data from a CAT assay performed using batch cultures confirmed the decrease in *fabT* expression in the absence of *spxA2* (Figure 2b). It appears that *SpxA2* positively regulates *fabT*, therefore the elevated *fabM* expression in the Δ *spxA2* background observed in the CAT assay could be explained by the fact that *FabT* represses *fabM* transcription (Faustoferri et al., 2015). Although it is clear that *SpxA2* regulates FAB, the collective transcriptional data examined here do not yet provide a coherent picture of why UFA production is reduced in the Δ *spxA2* strain. This is not terribly surprising given that the transcriptome of UA159 has been examined through the acid-induced shift of membrane saturation, and an overt mechanistic induction of UFA biosynthesis was not seen at the transcriptional level (Baker et al., 2015). Expression of *fab* genes appears to be dependent on a complex network of regulators including *FabT*, *CcpA*, and *SpxA2* (Faustoferri et al., 2015). Furthermore, it is likely that production of UFAs is also governed at least in part at the proteomic and metabolomic levels; therefore, more in-depth transcriptional, as well as biochemical, studies are needed to completely elucidate how the shift to a UFA-dominant membrane occurs, how UFA production is disrupted in Δ *spxA2*, and whether *spxA2* regulates the FAB machinery expression in a direct or indirect manner.

Microarray analysis also indicated that *SpxA2* may be a repressor of the operon encoding the F_1F_0 ATPase (Kajfasz et al., 2010), which is another crucial component of the *S. mutans* ATR (Baker et al., 2017). We confirmed that ATPase activity was indeed significantly increased in the Δ *spxA2* strain concurrent with the increased transcriptional expression (Figure 3). This may suggest a number of different hypotheses. It is possible that the increased expression of the ATPase may mitigate the acid-sensitive phenotype that might be expected due to the decreased UFAs in

the Δ spxA2 strain, leading to the mild acid-sensitivity observed. Alternatively, increased ATPase activity was also seen in the Δ fabM strain, despite that strain being severely acid-sensitive (Fozo & Quivey, Jr., 2004a). As the ATPase assay utilized in both studies is not performed on intact cells, it is possible that in vivo, the altered membrane composition observed in both Δ fabM and Δ spxA2 renders the ATPase less effective at providing protection from acid, despite its increased expression. Despite these uncertainties, the research presented here does provide concrete evidence that SpxA2 regulates multiple arms of the ATR and overall membrane homeostasis.

This study confirmed that transcription of *spxA2* is dependent on LiaR. Like Spx, the Lia system is widespread among Gram-positive bacteria, and has been shown to sense cell envelope stress in the form of disruption in Lipid II cycling and subsequently regulate transcription of genes that alleviate membrane and cell wall stress (Mascher et al., 2006). In *S. mutans*, *liaR* transcription is elevated in the presence of several antibiotics, therefore the expression of *spxA2* in the presence of membrane stressors was examined in both UA159 and a *liaR* deletion mutant (Suntharalingam et al., 2009). Indeed, expression of *spxA2* was significantly increased in the presence of all of the membrane stressors tested (Figure 4b). In the absence of *liaR*, this response was not observed and expression of *spxA2* was nearly ablated, indicating that *spxA2* requires LiaR to sense membrane stress.

Because deletion of *spxA2* would be likely to disrupt signalling from LiaFSR to the cell envelope repair machinery in the SpxA2 regulon, the sensitivity of the Δ spxA2 strain to a number of envelope stressors was examined. The Δ spxA2 strain had increased sensitivity to polymyxin B, tunicamycin, and vancomycin, and no change in sensitivity to bacitracin, daptomycin, penicillin, and chloramphenicol (Table 2). Chloramphenicol was included as essentially a negative control since it targets protein synthesis, and not the cell envelope or peptidoglycan synthesis. Therefore, it was not surprising that Δ spxA2 did not have an altered susceptibility against this antibiotic. The increased susceptibility of Δ spxA2 to polymyxin B, tunicamycin, and vancomycin was expected, given that these antibiotics target cell wall synthesis and that increased susceptibility to vancomycin was also observed in a Δ liaR strain (Suntharalingam et al., 2009). Since bacitracin, daptomycin, and penicillin also target the cell wall and/or membrane, it was initially surprising that the Δ spxA2 strain was not more sensitive to these drugs. Although bacitracin disrupts the Lipid II step, which is sensed by LiaFSR, *S. mutans* is generally recalcitrant to bacitracin, which is included in a media formulation commonly utilized to isolate *S. mutans* (Gold, Jordan, & Van Houte, 1973). Although the Δ liaR strain was more susceptible to bacitracin than UA159 (Suntharalingam et al., 2009), it is possible that there is a compensatory mechanism at play in Δ spxA2, such as increased expression of the bacitracin resistance genes. Penicillin targets the transpeptidation step of peptidoglycan synthesis, which is downstream of the Lipid II step, the step sensed by LiaFSR. Furthermore, the Δ liaR strain showed a similar lack of increased susceptibility to penicillin (Suntharalingam et al., 2009).

Thus, the lack of increased penicillin susceptibility in Δ spxA2 is perhaps not unanticipated. Daptomycin creates pores in the cell envelope, thus it was unexpected that Δ spxA2 was not more sensitive to daptomycin than UA159. It is unclear why this is the case, however, it is worth noting that the Δ liaR strain was not more susceptible to nisin (Suntharalingam et al., 2009), which also creates pores in the membrane. Furthermore, in *Enterococcus faecalis*, exogenous fatty acids protect against daptomycin-induced membrane stress independent of LiaR (Harp et al., 2016). The Δ spxA2 strain was more resistant to osmotic stress in the form of 2.5% NaCl (Figure 5a). This was surprising given that the Δ liaR strain was more sensitive to NaCl than UA159 (Suntharalingam et al., 2009). This result illustrates that although the Δ spxA2 and Δ liaR strains have many overlapping phenotypes, there are subtle differences, highlighting the need for further work exploring the effect of the Lia system on membrane composition and other traits. The Δ spxA2 strain was also more resistant to the FabF inhibitor, cerulenin. It is presently unclear why this would be the case, however, the reduced abundance of longer chained UFAs in Δ spxA2 could indicate reduced flux through the FabF elongation enzyme, which is the target of cerulenin, causing a reduction in sensitivity.

Overall, the results of this study illustrate that SpxA2 responds to a variety of membrane stresses in a LiaR-dependent manner. Furthermore, SpxA2 plays a crucial role in two elements of the *S. mutans* ATR that are intertwined with cell membrane/wall homeostasis: modulation of the fatty acid composition of the cell membrane, and ATPase expression and activity. The growth phenotypes observed in the Δ spxA2 strain in the presence of membrane stressors indicate that SpxA2 is an integral part of the *S. mutans* cell envelope stress response. The conserved Spx RPI motif is encoded as SPI in *S. mutans* SpxA2, thus it has been postulated that SpxA2 may not be able to sense redox state in the same manner as SpxA1 and other canonical Spx proteins (Kajfasz et al., 2010). Perhaps this is why SpxA2 has evolved a distinct regulatory role (Kajfasz et al., 2010). The number of genes predicted to be directly regulated by SpxA2 is much larger than the predicted direct regulon of LiaR, further supporting the idea that SpxA2 transmits the signal of membrane stress detected by the Lia system to downstream effectors. Additional investigations to further examine and refine this regulatory network and its relationship to virulence in this important oral pathogen are currently in progress.

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CONFLICT OF INTEREST

The authors do not declare any conflicts of interest.

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SUPPORTING INFORMATION

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