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Inactivation of the *spxA1* or *spxA2* gene of *Streptococcus mutans* decreases virulence in the rat caries model

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SUMMARY

In oral biofilms, the major environmental challenges encountered by *Streptococcus mutans* are acid and oxidative stresses. Previously, we showed that the transcriptional regulators SpxA1 and SpxA2 are involved in general stress survival of *S. mutans* with SpxA1 playing a primary role in activation of antioxidant and detoxification strategies whereas SpxA2 serves as a back up activator of oxidative stress genes. We have also found that *spxA1* mutant strains (*spxA1* and *spxA1 spxA2*) are outcompeted by peroxigenic oral streptococci *in vitro* and have impaired abilities to colonize the teeth of rats fed a highly cariogenic diet. Here, we show that the Spx proteins can also exert regulatory roles in the expression of additional virulence attributes of *S. mutans*. Competence activation is significantly impaired in *spx* strains and the production of mutacin IV and V is virtually abolished in *spxA1* strains. Unexpectedly, the *spxA2* strain showed increased production of glucans from sucrose, without affecting the total amount of bacteria within biofilms when compared to the parent strain. By using the rat caries model, we showed that the capacity of the *spxA1* and *spxA2* strains to cause caries on smooth tooth surfaces is significantly impaired. The *spxA2* strain also formed fewer lesions on sulcal surfaces. This report reveals that global regulation via Spx contributes to the cariogenic potential of *S. mutans* and highlights the essentiality of animal models in the characterization of bacterial traits implicated in virulence.

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Keywords

dental caries; *Streptococcus mutans*; Spx; oxidative stress

INTRODUCTION

Dental caries is one of the most prevalent infectious diseases worldwide (Marcenes *et al.*, 2013; Selwitz *et al.*, 2007). Even though the participation of other oral bacteria in the etiology of caries cannot be overlooked (Aas *et al.*, 2005; Jenkinson, 2011), clinical and laboratory studies clearly implicate the oral pathogen *Streptococcus mutans* in the initiation and development of this disease (Lemos *et al.*, 2005; Takahashi and Nyvad, 2011). The virulence of *S. mutans* is directly associated with its ability to form biofilms on tooth surfaces, to produce extracellular polysaccharides (EPS) and weak acid from sugars, and to efficiently adapt to large fluctuations in pH, oxygen tension and nutrient availability (Lemos *et al.*, 2005).

Work from a number of laboratories demonstrated that *S. mutans* is well equipped to adapt to low pH values by activation of a robust physiological response to acidification referred to as the acid tolerance response (ATR) (Lemos *et al.*, 2005). The ATR is accomplished by upregulation of the membrane-associated F-ATPase, induction of pathways that contribute to cytoplasm buffering and changes in membrane fatty acid composition, among other processes. While the *S. mutans* ATR has been studied in some detail (Lemos *et al.*, 2005), the importance of O₂ metabolism and the mechanisms to cope with reactive oxygen species (ROS) in *S. mutans* have received limited attention. The relevance of oxidative stress survival to the pathophysiology of *S. mutans* is supported by studies that show an inverse correlation between the total numbers of *S. mutans* and peroxigenic Streptococci (e.g. *S. sanguinis* and *S. gordonii*) in dental plaque (Becker *et al.*, 2002; Kreth *et al.*, 2008; Mikx *et al.*, 1972). Specifically, members of the mitis group are often associated with oral health; a series of elegant *in vitro* studies showed that H₂O₂ produced by *S. gordonii* or *S. sanguinis* via a pyruvate oxidase serves as a “chemical weapon” antagonizing the growth of *S. mutans* (Kreth *et al.*, 2008). In addition, H₂O₂ present in certain oral hygiene and tooth bleaching products may represent another source of peroxide stress for oral bacteria (Marquis, 1995).

Previously, we identified and characterized two genes, named *spxA1* (formerly *spxA*) and *spxA2* (formerly *spxB*) in *S. mutans*, which serve as global transcriptional regulators (Kajfasz *et al.*, 2009; Kajfasz *et al.*, 2010b). Spx regulators are highly conserved among Firmicutes and are directly involved in oxidative stress responses by positively affecting the transcription of genes involved in thiol homeostasis and detoxification (Kajfasz *et al.*, 2010b; Nakano *et al.*, 2003a; Nakano *et al.*, 2003b; Zuber, 2004). Physiologic characterizations of *spxA1*, *spxA2* and *spxA1/ spxA2* strains revealed that SpxA1 plays a major role in survival under acid and oxidative stress conditions (Kajfasz *et al.*, 2010b). While stress tolerances were generally not impaired in the *spxA2* strain, the stress sensitivities of the double *spxA1/ spxA2* strain were more pronounced than in the single *spxA1* strain (Kajfasz *et al.*, 2010b). In addition to activation of genes involved in thiol homeostasis and ROS scavenging (Kajfasz *et al.*, 2010b; Kajfasz *et al.*, 2015), we have recently shown that

Spx performs an important role in iron homeostasis by regulating the intracellular availability of free iron (Galvão *et al.*, 2015). Transcriptome and *in vitro* transcription analyses further supported that SpxA1 functions as the primary transcriptional activator of oxidative stress genes whereas SpxA2 appears to have a secondary but supportive role in the activation of oxidative stress responses (Kajfasz *et al.*, 2010b; Kajfasz *et al.*, 2015). Finally, inactivation of *spxA1*, *spxA2* or both attenuated the virulence of *S. mutans* in the *G. mellonella* invertebrate model but only strains lacking the *spxA1* gene (*spxA1* and *spxA1/ spxA2*) showed a reduced ability to colonize the teeth of rats fed a highly cariogenic diet (Kajfasz *et al.*, 2010b).

In this manuscript, we showed that loss of one or both *spx* genes affects the expression of important virulence attributes of *S. mutans*. Specifically, both Spx proteins were shown to mediate competence development and SpxA1 appears to be essential for mutacin production. While SpxA2 was not seemingly involved in mutacin production, inactivation of *spxA2* resulted in enhanced biomass without affecting bacterial amount. We also assessed the virulence potential of the *spxA1* and *spxA2* strains in the rat caries model to show that both mutants were less cariogenic than the parental strain.

METHODS

Bacterial strains and growth conditions

The strains used in this study are listed in Table 1. The *S. mutans* UA159 (wild-type) and its *spx* derivatives (*spxA1*, *spxA2* and *spxA1/ spxA2*) were routinely grown in Brain Heart Infusion (BHI) at 37°C under anaerobic conditions (BBL Gaspak system, BD, Franklin Lakes, NJ). For overexpression of SpxA1 or SpxA2, strains harboring the pMSP3535 empty plasmid (Bryan *et al.*, 2000) or pMSP3535 expressing *spxA1* or *spxA2* were grown in BHI broth containing 10 µg ml⁻¹ erythromycin. Overproduction of SpxA1 and SpxA2 was achieved by adding 16 ng ml⁻¹ nisin to the growth media.

Biofilm Assays

Biofilm development was measured in polystyrene 96-well (flat-bottom) microtiter plates (Costar .595; Corning Inc., Corning, NY). Briefly, cultures were grown in BHI to OD₆₀₀ of 0.5 and used to inoculate (1:100) the wells of a microtiter plate containing 200 µl of low molecular weight medium (LMW) (Koo *et al.*, 2005) containing 1% sucrose. Following incubation for 24 h at 37°C in a 5% CO₂ aerobic atmosphere, culture medium was removed by aspiration and wells were gently washed with 200 µl sterile deionized water. Subsequently, 50 µl of a 0.1% solution of crystal violet dissolved in 99% ethanol was applied to each well and incubated at room temperature for 15 min, followed by removal of the fluid by aspiration. Wells were washed twice with water as before and allowed to air dry. The plates were de-stained with 200 µl of an acetone:ethanol solution (2:8) for 30 min at room temperature. The de-staining procedure was repeated and the OD₅₇₅ of the pooled de-staining solution was measured. Background was determined from staining non-inoculated wells with crystal violet. Significance was established as a *P*value = 0.05. Experiments were conducted at least in triplicates.

Confocal microscopy analysis of biofilms was performed on saliva-coated hydroxyapatite discs (sHA) (diameter, 1.25 cm; Clarkson Chromatography Products, Inc., South Williamsport, PA). Stimulated human saliva obtained from healthy subjects (RSRB 00030432, University of Rochester) was pooled, clarified by centrifugation and filter-sterilized prior to use. Cells of *S. mutans* UA159 or *spxA2* were grown in ultrafiltered (10-kDa-cutoff membrane; Prep/Scale; Millipore, MA) buffered tryptone-yeast extract broth (UFTYE) containing 2.5% tryptone and 1.5% yeast extract (pH 7.0) with 1% glucose to OD₆₀₀ of 0.5. Biofilms were formed on sHA discs placed vertically using a disc holder in a 24-well plate containing batch (static) cultures (Falsetta *et al.*, 2012; Koo *et al.*, 2005). The bacterial cells were grown in 2.8 ml (per well) of UFTYE containing 1% sucrose and an sHA disc at 37°C in 5% CO₂ for 24 h. At the end of the experimental period, the biofilms were dip-washed three times and then gently swirled in physiological saline to remove loosely adherent material. The biofilms were subjected to confocal imaging analysis as described elsewhere (Falsetta *et al.*, 2012; Xiao and Koo, 2010). Briefly, Alexa Fluor 647-dextran conjugate (10 kDa; absorbance/fluorescence emission maxima, 647/668 nm; Molecular Probes, Invitrogen Corp., Carlsbad, CA) was used to label glucans in the EPS matrix, while total microbial biomass was stained with Syto 9 (485/498 nm; Molecular Probes). Imaging was performed using an Olympus FV 1000 two-photon laser scanning microscope (Olympus, Tokyo, Japan). Each biofilm was scanned at five positions randomly selected at the microscope stage for a total of 15 stacks per sample from 3 independent experiments. The confocal images were analyzed using COMSTAT software for the quantitation of EPS and microbial cells biomass within intact biofilms (Falsetta *et al.*, 2012; Xiao and Koo, 2010).

Deferred antagonism assay

Cultures were grown in BHI to an OD₆₀₀ of 0.3 when a 15 µl aliquot was spotted onto BHI agar and incubated for 24 h. Following incubation, plates were exposed to UV light for 20 min to ensure that subsequent antagonism was not due to actively growing *S. mutans* cells. Then, 500 µl of an overnight culture of *S. gordonii* DL-1 (mutacin IV sensitive) or *Lactococcus lactis* ATCC 11454 (mutacin V sensitive) was added to 5 ml soft (0.75 %) BHI agar, spread as an overlay and incubated for another 24 h before zones of growth inhibition around the *S. mutans* spots were measured (Hossain and Biswas, 2011).

Genetic competence assay

Overnight cultures were sub-cultured 1:20 into fresh BHI supplemented with 10% horse serum and grown to OD₆₀₀ of 0.125. At this point, cultures were split in two and incubated for an additional 15 min with or without 5 µM of the synthetic competence stimulating peptide (CSP) (Li *et al.*, 2002) followed by addition of 0.2 µg of the shuttle plasmid pMC340B that confers resistance to kanamycin. Cells were then incubated until stationary phase was reached and plated in duplicate on BHI agar plates with or without 1 mg ml⁻¹ kanamycin. Transformation efficiency was determined after 48 h incubation at 37°C in a 5 % CO₂ atmosphere and was expressed as the percentage of transformants (kanamycin plates) among the total viable recipient cells (antibiotic-free plates).

Rat caries model

The animal experiment protocol was reviewed and approved by the Ethical Committee on Animal Research at the University of Campinas, SP, Brazil (Protocol # 2637-1) and was performed according to methods previously described (Falsetta *et al.*, 2012). Female pups free of *S. mutans* and SDA virus from 10 litters of SPF Wistar rats were provided by CEMIB (UNICAMP). At the age of 19 days, pups were weaned and randomly divided into three groups of 10 animals. Animals were orally infected for three successive days by means of a cotton swab containing mid-exponential cultures of *S. mutans* strains UA159, *spxA1* or *spxA2*. The oral infection of the pups was confirmed 1 week later by plating on Mitis-Salivarius (MS) agar plus bacitracin (MSB; Sigma-Aldrich, St Louis, MO). Each group received highly cariogenic diet 2000 and 5% sucrose water *ad libitum* (Bowen *et al.* 1988). The animals were weighed weekly and their behavior and physical appearance noted on a daily basis. After five weeks, animals were killed by CO₂ asphyxiation. The lower left jaw was aseptically dissected, suspended into 5.0 ml of sterile saline solution (0.9%, w/v), and sonicated (three 10 s pulses at 5 s intervals, at 30 W; Vibracell, Sonics and Material Inc). The suspension was plated on MSB to estimate the populations of *S. mutans* UA159, *spxA1* and *spxA2* and on blood agar to determine the total cultivable microorganisms. Smooth-surface and sulcal caries and their severities were evaluated according to Larson's modification of Keyes' system (Keyes, 1958; Larson, 1981) by a single calibrated examiner blinded to the study. Statistical significance was determined by ANOVA in the Tukey-Kramer HSD test for all pairs. The statistical software GraphPad PRISM version 5.0 was used to perform the analyses. The level of significance was set at 5%.

RESULTS

Inactivation of either SpxA1 or SpxA2 impairs competence development and loss of SpxA1 abolishes mutacin production

Previously, we showed that competence and production of at least two mutacins (mutacin IV and mutacin V) were significantly impaired in *clpP* and *clpX* strains (Kajfasz *et al.*, 2011). As Spx proteins are targeted for degradation by the ClpXP proteolytic system (Kajfasz *et al.*, 2009), this result indirectly suggests that the Spx proteins negatively regulate competence and mutacin production in *S. mutans*. Unexpectedly, transformation efficiency was also lower in strains lacking one or both *spxA* genes, regardless of the presence of exogenously added CSP, which boosted the number of transformants in all strains (Fig. 1A-B). In an attempt to understand the conflicting results involving Spx levels and competence activation, we used a nisin-inducible plasmid to overexpress SpxA1 or SpxA2 in the parental strain thereby mimicking the high Spx levels observed in *clpP* and *clpX* strains. Then, we tested the transformation efficiency of these strains after induction with a sub-inhibitory concentration of nisin that does not affect growth rates of any of the strains. As observed in both *clp* and *spx* strains, overexpression of SpxA1 or SpxA2 also resulted in competence deficiency as compared to the parent strain (Fig. 1C-D).

Next, we measured production of two competence-coordinated bacteriocins by the *spx* strains using a deferred antagonism assay (Fig. 2). Inhibition of the growth of *S. gordonii* was used to assess the ability of the wild-type and *spxA* strains to produce mutacin IV,

which is active against the mitis group of streptococci (Qi et al., 2001). Inhibition of *L. lactis* was used to assess the ability of the strains to produce mutacin V, which has broader antimicrobial activity, ranging from mitis streptococci to lactococci and micrococci (Hale et al., 2005). Based on our results, mutacin production by the *spxA1* and *spxA1/ spxA2* strains was diminished to the extent that both strains were completely unable to antagonize the growth of *S. gordonii* or *L. lactis*. The link to mutacin production appears to be limited to *spxA1*, as the *spxA2* strain showed antagonism similar to that of UA159, generating average zone diameters of 12.3mm (UA159) to 14.3mm (*spxA2*) against *S. gordonii* and 19.7mm (UA159) to 20.7mm (*spxA2*) against *L. lactis*.

The *spxA2* strain shows enhanced glucan production

The ability to form biofilms via production of EPS such as glucans in the presence of sucrose is a major virulence factor of *S. mutans* (Koo et al., 2005; Xiao et al., 2012; Yamashita et al., 1992). Because *spx* genes have been linked to biofilm formation in Staphylococci (Pamp et al., 2006; Wang et al., 2010), we used *in vitro* assays to determine the ability of the single *spx* strains to form biofilms in media containing sucrose as the sole carbohydrate source. In saliva-coated microtiter plates, biofilm formation by *spxA2*, but not the *spxA1* strain, was significantly enhanced (Fig. 3A). We also tried to measure the biofilm-forming capacity of the double *spxA1/ spxA2* strain. However, growth of this strain in biofilms was dramatically impaired and the biofilm quantifications were not reproducible. To exclude any possibility that the differences observed between the parent and single *spx* strains were due to small variations in the growth rates of the strains, the biofilm data were normalized by the total final OD₆₀₀ comprised of both planktonic and biofilm cells. To confirm the enhanced sucrose-dependent biofilm phenotype of the *spxA2* strain, a saliva-coated HA disc assay (a tooth enamel surrogate) was used to measure total biomass of bacteria and EPS glucans as well as EPS/bacteria ratio via confocal microscopy. In agreement with the results using microtiter plates, the *spxA2* strain showed total biomass by increasing production of EPS glucans (Fig. 3B) without, however, affecting the amount of bacterial biomass. COMSTAT analysis revealed that the glucan/ bacteria ratio of the *spxA2* strain (3.71 ± 0.98) was significantly higher ($P < 0.05$) than the ratios observed for the parent strain (2.56 ± 0.68).

Inactivation of *spxA1* or *spxA2* decreases the colonization and cariogenic potentials of UA159

Previously, we showed that the ability of the *spxA1* and *spxA1/ spxA2* strains to colonize the teeth of rats was significantly impaired (Kajfasz et al., 2010b). As stated above, the *spxA1/ spxA2* strain shows poor growth in biofilms, making it virtually impossible to separate a low cariogenic potential from an overall loss of bacterial fitness. As in the case of the biofilm analysis, an animal caries study was conducted only with the wild-type and *spx* single mutant strains. During the course of the study, no differences were observed in the pattern of meals consumed by the animals and consistent with this was the finding that there were no significant differences observed in the weight gains of animals. Counts of total cultivable microflora revealed no significant differences among the groups infected with the wild-type, *spxA1* or *spxA2* strains (Fig. 4A). However, when focusing on *S. mutans*, the numbers of *spxA1* and *spxA2* colonies recovered from the animals were lower when

compared to the parent strain (Fig. 4B) but only the differences between parent and *spxA1* strains were statistically significant ($P = 0.05$). Likewise, the proportions of *S. mutans* to total flora were significantly lower ($P = 0.05$) for the *spxA1* strain ($29.08 \% \pm 17.61$) but not for *spxA2* ($59.79 \% \pm 21.9$) when compared to the proportions of the wild-type to total flora ($53.35 \% \pm 23.42$) (Fig. 4C).

The incidence and severity of smooth surface and sulcal surface caries in animals infected with the parent or *spx* strains are shown in Table 2. Scoring of carious lesions on smooth and sulcal surfaces was divided into parameters evaluating either lesion extension (enamel [E]), or lesion severity (slight [Ds], moderate [Dm], or severe [Dx]) as defined by Larson (Larson, 1981). Rats infected with *spxA1* or *spxA2* had reduced dental caries based on lower levels of average lesion extension [E] on smooth surfaces. The *spxA2* strain also caused fewer sulcal carious lesions when compared to animals infected with the UA159 or *spxA1* strains ($P = 0.05$). Despite the differences in lesion extension, the differences in lesion severity among strains were not significant at any level of dentin damage.

DISCUSSION

While the majority of work that has contributed to the understanding of Spx function has come from the model Gram-positive organism *Bacillus subtilis*, evidence is now accumulating that Spx proteins have similar regulatory functions in other bacteria (Chen et al., 2012; Kajfasz et al., 2010a; Kajfasz et al., 2012; Pamp et al., 2006b; Turlan et al., 2009; Turner et al., 2007; Zheng et al., 2014). After our initial discovery of the existence of two Spx proteins in *S. mutans*, evidence that two Spx paralogs, with overlapping but also unique regulatory functions, contribute to adaptive stress responses of other Streptococci and, more recently, *Bacillus anthracis* has emerged (Barendt et al., 2013; Chen et al., 2012; Kajfasz et al., 2009; Kajfasz et al., 2010b; Turlan et al., 2009; Zheng et al., 2014). From our previous studies, it becomes clear that SpxA1 and SpxA2 are global regulators and, while SpxA1 has a primary role in the activation of oxidative stress genes, both Spx proteins appear to modulate the expression of a variety of other cellular traits, including cell envelope homeostasis by SpxA2 (Kajfasz et al., 2010b) and, as shown here, competence development (SpxA1 and SpxA2), mutacin production (SpxA1) and biofilm formation (SpxA2).

The association of Spx regulation with competence development was first demonstrated in *B. subtilis*. The *B. subtilis* Spx was shown to negatively affect competence by assisting the formation of a complex with the ClpC ATPase and the MecA adaptor protein that sequesters ComK, a transcriptional regulator required for late competence gene activation (Nakano et al., 2002). More recently, the *S. pneumoniae* SpxA1 was shown to repress transcription of the early competence operon *comCDE* thereby also acting as a negative regulator of competence (Turlan et al., 2009). In *S. mutans* UA159, competence development is controlled by quorum-sensing mechanisms involving the σ^X inducing peptide (XIP) and the competence stimulating peptide (CSP) (Mashburn-Warren et al., 2010; van der Ploeg, 2005). Competence activation has been shown to influence biofilm formation, stress tolerance, persistence and virulence (Kaspar et al., 2015; Leung et al., 2015; Li et al., 2008; Perry et al., 2009a; Perry et al., 2009b; Wenderska et al., 2012), and is intertwined with antagonism of other species, as bacteriocin (mutacin) production is controlled by the CSP quorum-

sensing factor (Dufour et al., 2011; Kreth et al., 2005; Reck et al., 2015; van der Ploeg, 2005). Our previous observation that competence was impaired in *S. mutans* strains lacking *clpP* (*clpP*) or *clpX* (*clpX*) (Kajfasz et al., 2011), known to accumulate the Spx proteins (Kajfasz et al., 2010), was in line with the literature. Unexpectedly, deletion of either *spxA1* or *spxA2* negatively affected competence suggesting a much more complex relationship between Spx and competence development in *S. mutans*. Given these conflicting results, we took a further step and assessed competence efficiency in wild-type strains (ClpXP⁺) overproducing *spxA1* or *spxA2* through an inducible plasmid. Surprisingly, competence efficiency was impaired to similar levels as seen in the *clp* and *spx* strains. Thus, our results indicate that any fluctuations in Spx pools can have a detrimental effect in *S. mutans* competence. By perusing our previous microarrays comparing the transcriptome of the wild-type UA159 and *spxA* strains grown in BHI medium to an OD₆₀₀ of 0.5 (Kajfasz et al., 2010), we found that expression of *comR* (*smu61*) and *comE* (*smu1917*), the transcriptional regulators involved in activation of the XIP competence and CSP competence/bacteriocin pathways, respectively, was downregulated (~ 2-fold each) in the *spxA1* strain. However, the transformation efficiency of all strains in this study was improved upon addition of CSP to the cultures, suggesting that cells lacking the SpxA proteins are able to respond to the presence of CSP. Additional studies to obtain mechanistic insight into the significance of Spx in competence development are underway.

The production of bacteriocins by *S. mutans* strains, the so-called mutacins, is thought to play an important role in oral colonization. Mutacin production helps *S. mutans* to compete with other oral bacteria enabling its establishment and persistence in dental plaque (Merritt and Qi, 2012). Mutacins are divided in two types: lantibiotic and non-lantibiotic, with lantibiotics showing a higher spectrum of activity than non-lantibiotics (Merritt and Qi, 2012). The genome of *S. mutans* UA159 encodes at least three non-lantibiotic mutacins; mutacin IV, V and VI. These mutacins are regulated by the ComCDE quorum-sensing system, initially identified as a competence activator (Li et al., 2002). The observed defects in competence of strains with abnormal Spx levels and the intimate relationship between the regulatory networks controlling competence and mutacin production led us to assess mutacin production in the *spx* strains. Using *S. gordonii* (sensitive to mutacin IV) and *L. lactis* (sensitive to mutacin V) as indicators, we found that strains lacking *spxA1* could no longer inhibit the growth of both indicator strains. Different from competence activation that is influenced by both SpxA1 and SpxA2, mutacin production was not affected in the *spxA2* single mutant. We also searched our previous transcriptome analysis (Kajfasz et al., 2010) for the differential expression of mutacin-coding genes in the *spx* strains. Transcription of the genes responsible for mutacin IV (*nImAB*) or mutacin V (*nImC*) was not significantly affected in the *spx* strains albeit this result remains to be validated using targeted approaches (e.g., qRT RT-PCR). While the specific association of Spx regulation with mutacin production is still unclear, it seems unlikely that it occurs through direct regulation of the mutacin-encoding genes by Spx.

It is well recognized that glucosyltransferases (Gtf) from *S. mutans* play critical roles in the development of highly cariogenic biofilms through formation of an insoluble polysaccharide matrix comprised primarily of glucans (Bowen and Koo, 2011). The genome of *S. mutans* UA159 encodes three Gtf genes; *gtfB*, *gtfC* and *gtfD*. The *gtfB* and *gtfC* genes are

genetically linked and co-regulated by multiple transcriptional regulators (Bowen and Koo, 2011; Goodman and Gao, 2000; Yoshida and Kuramitsu, 2002). The *gtfD* gene is located elsewhere in the chromosome and also appears to be under complex transcriptional control (Bowen and Koo, 2011; Goodman and Gao, 2000). Our observations that glucan production and, as a result, biofilm biomass, is enhanced in the *spxA2* strain aligns well with previous microarray analysis showing that expression of *gtfB* and *gtfD* was enhanced in *spxA2* (Kajfasz et al., 2010). Additionally, we observed the upregulation of the *lytS* genes that are involved in autolysis and biofilm formation through the release of eDNA which contributes to the structure of the extracellular matrix (Klein et al., 2010). When compared to the parent strain, *lytS* was found to be upregulated more than 2-fold in *spxA2* in our earlier microarray analysis, which may help to explain the enhanced biofilm formation seen for the *spxA2* strain. Aside from serving as a structural component, the presence of eDNA may further contribute to matrix formation by stimulating glucan synthesis by GtfB on the *S. mutans* cell surface (Klein et al., 2015).

In a previous oral colonization study, we showed that 15 days post-infection, the *spxA1* strain was recovered in significantly fewer numbers from the teeth of specific pathogen-free (SPF) rats (Kajfasz et al., 2010). However, when SPF rats were infected with the *spxA2* mutant, the CFU numbers recovered were similar to those obtained from the parental strain. Here, in a 5-week caries study, when compared to animals infected with the parent strain, we showed that SPF rats infected with either the *spxA1* or *spxA2* strain had fewer carious lesions on smooth surfaces whereas the *spxA2* strain also caused fewer sulcal caries. Interestingly, while the *spxA1* strain was recovered at fewer numbers upon termination of the study, the difference between the number of *S. mutans* recovered from animals infected with the parent or *spxA2* strain was not significant, consistent with *in vitro* data showing unchanged bacterial amounts. Thus, it appears that loss of *spxA2* does not affect colonization of the tooth surface, as observed in our colonization study (Kajfasz et al., 2010), but rather affects the cariogenic potential of *S. mutans in vivo*. While the result of the caries study with the *spxA1* strain was a logical and expected extension of the oral colonization study and aligns well with the general stress sensitivity (Galvão et al., 2015; Kajfasz et al., 2010; Kajfasz et al., 2015) and defects in competence and mutacin production of the *spxA1* strain, the decreased cariogenicity of *spxA2* was somewhat surprising. With the exception of competence and biofilm formation, the *in vitro* expression of all other virulence attributes investigated in the *spxA2* strain were not affected. At first glance, the decrease in formation of carious lesions by *spxA2* may appear surprising considering the enhanced *in vitro* biofilm biomass due to increased EPS production by this strain. However, it is noteworthy that the bacterial accumulation within the biofilm was not significantly enhanced. Thus, it appears that the ability of *S. mutans* themselves, within the EPS-rich matrix milieu, to cause the disease was attenuated in *spxA2*. Whether this aberrant glucan accumulation affects the mechanical stability of the biofilm, proton diffusion, or proton retention of *S. mutans* as suggested by others (Guo et al., 2015; Hata and Mayanagi, 2003), it still needs further evaluation.

We previously reported diminished virulence of *spxA2*, as well as the *spxA1* strain, in the *G. mellonella* systemic infection model (Kajfasz et al., 2010). In addition, the fitness of the strain lacking both *spx* genes has consistently proven to be severely compromised in

physiologic assays and animal models (Kajfasz et al., 2010; Kajfasz et al., 2015), which highlights the cooperative nature of these two Spx paralogs. Thus, it appears that SpxA2 performs important functions in *S. mutans* and that further characterization of the regulatory activity of SpxA2 during infection and the nature of its cooperative relationship with SpxA1 is warranted. For example, it will be interesting to obtain the global transcriptional profile of cells from *spx* strains isolated directly from dental plaque of infected SPF rats.

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Fig. 1.

Competence of *S. mutans* UA159 and its derivatives. Transformation efficiency of (A, B) parent UA159 and $\Delta spxA$ strains and (C, D) UA159 harboring the nisin-inducible pMSP3535 (control) or pMSP3535-Spx plasmids (Spx-overexpressing strains). The integration plasmid pMC340A (donor DNA) was added to cells in early-logarithmic phase (OD_{600} of 0.15) with or without the addition of CSP or nisin. Cultures were incubated at 37°C in 5% CO_2 until entering stationary phase, serially diluted and plated in BHI for total CFU and in BHI containing kanamycin for transformants. (A) pMC340A only (B) pMC340A + CSP, (C) pMC340A only, and (D) pMC340A + nisin. The results represent the mean and standard deviations of at least three independent experiments. Asterisks indicate differences were statistically significant ($P \leq 0.05$) when compared to UA159 (A, B), or UA159 harboring the empty pMSP3535 vector (C, D).

Fig. 2.

Mutacin production by *S. mutans* UA159 and its derivatives. Cultures of *S. mutans* were grown in BHI to OD₆₀₀ of 0.3 and 15 µl of each culture spotted on BHI plates followed by incubation at 37°C in 5% CO₂. After 24 h incubation, plates were exposed to UV light for 20 min and then overlayed with 5 ml soft BHI agar containing 500 µl of overnight cultures of *S. gordonii* or *L. lactis*. Plates were incubated for additional 48 h and the zone of inhibition recorded.

Fig. 3. Biofilm formation and biomass by *S. mutans* UA159 and its derivatives. (A) Biofilm formation on microtiter plates. Cultures were grown in LMW containing 1% sucrose for 24 h. Biofilm formation was normalized by total growth to exclude apparent differences due to the growth abilities of each strain (*, $P = 0.05$). (B) The imaging of EPS and bacteria within intact 3D biofilms was conducted using a method based on incorporation of fluorescently labeled dextran (Alexa Fluor 647, red) by Gtfs during glucan synthesis. Bacterial cells were labeled with SYTO9 (green). The images shown are representative of $n = 15$. The confocal images were analyzed by AMIRA and COMSTAT (*, $P = 0.05$).

Fig. 4.

Total cultivable microflora (A) and *S. mutans* (B) recovered from left jaws of animals five weeks after infection with UA159, *spxA1* or *spxA2* strains. (C) Proportion (%) of *S. mutans* per total flora. The results represent the mean and standard deviations of one experiment ($n=10$). Asterisks indicate differences were statistically significant ($P < 0.05$) when compared to UA159 * $P < 0.05$ (ANOVA).

Table 1

Bacterial strains used in this study.

Strains	Relevant genotype	Source or reference
<i>S. mutans</i>		
UA159	Wild-type	Laboratory stock
JL12 (<i>spxA1</i>)	<i>spxA1</i> :: Sp ^R	Kajfasz <i>et al.</i> , 2009
JL13 (<i>spxA2</i>)	<i>spxA2</i> :: Erm ^R	Kajfasz <i>et al.</i> , 2009
JL21 (<i>spxA1/A2</i>)	<i>spxA1</i> :: Sp ^R , <i>spxA2</i> :: Erm ^R	Kajfasz <i>et al.</i> , 2009
UA159+pMSP3535	Erm ^R	Kajfasz <i>et al.</i> , 2015
UA159+ <i>spxA1</i>	Wild-type harboring pMSP3535- <i>spxA1</i> , Erm ^R	This study
UA159+ <i>spxA2</i>	Wild-type harboring pMSP3535- <i>spxA2</i> , Erm ^R	This study
Other species		
<i>L. lactis</i> ATCC 11454	Wild-type	Laboratory stock
<i>S. gordonii</i> DL-1	Wild-type	Laboratory stock

Table 2
Influence of different strains on development of dental caries in Wistar rats (Keyes' score).

Strains	Smooth surface			Sulcal surface				
	Lesion extension (E)	Lesion severity		Lesion extension (E)	Lesion severity			
		Ds	Dm		Dx	Ds	Dm	Dx
UA159	40.8 (±9.05)	16.5 (±6.58)	4.8 (±4.75)	0.7 (±1.49)	47.2 (±3.04)	41 (±2.82)	11.6 (±6.73)	1.2 (±1.47)
<i>spxA1</i>	30.3* (±12.01)	16.8 (±9.18)	6.9 (±5.98)	0.1 (±0.31)	45.8 (±4.8)	40.1 (±3.34)	11.3 (±6.58)	4 (±5.07)
<i>spxA2</i>	25.2* (±8.16)	14.3 (±6.75)	4.7 (±3.88)	0	40.4* (±9.41)	35 (±8.65)	6.6 (±7.9)	1 (±1.15)

Asterisks denote statistically significant differences when compared to the wild-type (UA159) values in the same column, *p*≤0.05. ANOVA, using Tukey-Kramer HSD test for all pairs, was used to determine statistical significance. Notes: E – enamel; Ds – slight dental caries; Dm – moderate dental caries; Dx – extensive dental caries.