UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

LÍVIA CÂMARA DE CARVALHO GALVÃO

CONSTRUÇÃO E CARACTERIZAÇÃO FENOTÍPICA DE CEPAS MUTANTES DE *Streptococcus mutans* DE GENES RELACIONADOS À SUA VIRULÊNCIA

CONSTRUCTION AND PHENOTYPIC CHARACTERIZATION OF *Streptococcus mutans* MUTANT STRAINS OF GENES RELATED TO VIRULENCE

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Odontologia na Área de Farmacologia, Anestesiologia e Terapêutica

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Orientador: Prof. Dr. Pedro Luiz Rosalen
Coorientador: Prof. Dr. Gilson César Nobre Franco

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RESUMO

O fato de *Streptococcus mutans* conseguir emergir como microrganismo dominante no biofilme dental mesmo sob condições de estresse faz com que ele seja alvo de múltiplos interesses não apenas para a saúde oral, mas também para a saúde sistêmica. O sistema proteolítico Clp (Protease caseinolítica dependente de ATP) detém o papel central na tolerância desse microrganismo frente ao estresse ambiental, por desempenhar um importante papel na homeostase celular e controlar a estabilidade de proteínas reguladoras. Além disso, observou-se que a proteína Spx serve de substrato para esse sistema Clp e por isso foi feita uma detalhada caracterização fenotípica de Spx, regulador global, capaz de regular a transcrição de muitos genes conhecidamente relacionados ao estresse oxidativo em *S. mutans* e outros genes ainda hipotéticos. Assim, o objetivo deste trabalho foi construir e caracterizar fenotípicamente, *in vitro* e *in vivo*, cepas mutantes de *S. mutans* UA159 com deleção de genes hipotéticos, que apresentaram expressão alterada nas mutantes dos genes *spxA1* e *spxA1spxA2*. Foram construídas as mutantes dos genes selecionados: *smu143c smu144c, smu247, smu248, smu540, smu569, smu570, smu929, smu1296, smu1497* e *smu1685*, hipotéticos e regulados por Spx. Foi feita a análise transcricional dos genes selecionados frente ao estresse oxidativo através de PCR quantitativo (RT-qPCR). As mutantes construídas foram caracterizadas fenotipicamente por meio da avaliação da sua susceptibilidade, sobrevivência e crescimento sob condições de estresse ácido, oxidativo, em presença de ferro e outros metais. Em acréscimo, foi feita uma avaliação quanto à capacidade de formação de biofilme em meio com sacarose e esses genes foram avaliados por meio de ensaio de transcrição *in vitro* de forma a confirmar a sua regulação direta ou indereta por Spx . As cepas selecionadas por apresentarem fenótipo que justificasse relação com estresse oxidativo foram também investigadas quanto a capacidade de colonização em modelo animal sujeito a alto desafio cariogênico. Além disso, foi pesquisada a capacidade de cepas mutantes de Spx em causar lesões cariosas em ratos e seu papel regulatório como ativador de competência, na capacidade de formar mutacinas, biofilmes e glucanos a partir de sacarose. Além disso, a cepa mutante *smu1784c,* também regulada por Spx, foi submetida a uma caracterização inicial em busca de fenótipos que justifiquem sua relação com o gene *eep* de *Enterococcus faecalis* ou com estresse oxidativo em *S. mutans*. Com este trabalho, por meio de uma caracterização fenotípica detalhadas de novos genes regulados por Spx pôde-se relacioná-los ao estresse oxidativo em *S. mutans*, além de que verificou-se

relação desses genes e de Spx com a homeostase de ferro. Pôde-se confirmar o papel de Spx na patogênese da doença cárie e virulência do *S. mutans* uma vez que as mutantes dos genes *spxA1* e *spxA2* reduziram a formação de cárie em modelo animal, mostraram-se menos competentes e *spxA1* mostrou-se incapaz de produzir mutacinas. E o gene *smu1784c*, também regulado por Spx, mostrou-se envolvido com respostas ao estresse ácido e oxidativo e na virulência de *S. mutans*, uma vez que a cepa mutante mostrou-se atenuada em modelo de *Galleria mellonella* e capaz de reduzir os parâmetros bioquímicos relacionados à formação de biofilme. Portanto, esses achados confirmam e elucidam o papel regulador de Spx, possibilitando a identificação de novos alvos terepêuticos para a prevenção/tratamento de doenças envolvendo este microrganismo.
palavras-chave: Cárie dental, Estresse Oxidativo, Biofilmes, Ativação transcricional.

ABSTRACT

Streptococcus mutans is abble to emerge as dominant flora in biofilms even during stress conditions and has been studied in dentistry and medicine. The *S. mutans* Clp proteolytic system (Caseinolytic protease ATP-dependent) holds a central role in stress tolerance by controlling cellular homeostasis and stability of regulatory proteins. Besides, it was shown that Spx is substrate of Clp what justified a detailed phenotypic characterization of Spx, a global regulator able to regulate transcription of well-known genes related to oxidative stress and others hypothetical genes. Thus, the goal of this study was to construct and phenotypically characterize, *in vitro* and *in vivo*, *S. mutans* knockout mutants of hypothetical genes that showed altered expression in the *ΔspxA1* and *ΔspxA1/sxpA2* strains. The mutants of the following genes, regulated by Spx and strong candidates to be related with survival of *S. mutans* in stress conditions were constructed: *smu143c smu144c, smu247, smu248, smu540, smu569, smu570, smu929, smu1296, smu1497* and *smu1685*. RT-qPCR was used to evaluate the responses of Spx-regulated genes during oxidative stress in the parental and *Δspx* strains. The mutant strains were characterized by evaluation of its susceptibility, survival and growth under oxidative and acid stresses conditions, as well as in presence of iron and other metals. In addition, their ability to form biofilm in presence of sucrose was evaluated. These genes were also assessed through *in vitro* transcription assay to confirm their direct regulation by Spx. Selected strains that showed phenotypes associated with oxidative stress management were also evaluated for their ability to colonize using an animal model subjected to high cariogenic challenges. Besides, Spx mutant strains were evaluated for their ability to cause dental caries using an animal model and their regulatory role as competency activator and mutacin, biofilm and glucan sinthesizer. Moreover, mutant for gene *smu1784c*, also regulated by Spx, was characterized to find phenotypes that justified its relation with *eep* in *Enterococcus faecalis* or with oxidative stress in *S. mutans*.

The detailed phenotipic characterization of novel Spx regulated genes lead to their involvment with oxidative stress in *S. mutans*. Furthermore, these genes and Spx were associated with iron homeostoasis. The role of Spx in caries pathogenesis was confirmed once *spxA1* and *spxA2* genes were able to reduce caries score in an animal model. Also, *smu1784c* gene, regulated by Spx, showed involvement with acid and oxidative stresses responses, and virulence of *S. mutans* once the mutant strain of this gene showed attenuated behavior in *Galleria mellonella* model. The mutant Δ*smu1784c* was also able to reduce biochemical parameters related with biofilm formation. Therefore, these findings support the regulatory role of Spx enabling the identification of new therapeutic targets usefull for prevention and treatment of *S. mutans* related diseases.

Key-words: Dental Caries, Oxidative Stress, Biofilms, Transcriptional Activation.

SUMÁRIO

1 INTRODUÇÃO

A cárie dental representa um grande problema de saúde coletiva bucal (Dinelli *et al*., 2000; Oliveira *et al*., 2006), tanto no Brasil (Brasil, 1988, 1996; Dantas *et al*., 2000) como na maior parte do mundo (Weyne, 1997; Loretto *et al*., 2000) e esses achados são confirmados pelos resultados do último Saúde Bucal (SB) Brasil (Brasil, 2010).

A cárie é uma doença infecciosa multifatorial biofilme dependente (Marsh, 2003), que se desenvolve quando ocorrem mudanças no ambiente da cavidade oral, que são muitas vezes substanciais podendo alterar rapidamente o pH, a disponibilidade de nutrientes, os tipos de carboidratos e a concentração de oxigênio (Lemos *et al*., 2005). Caracteriza-se por um aumento no crescimento de bactérias cariogênicas, que são altamente eficientes na conversão de carboidratos à ácidos orgânicos, que têm a capacidade de desmineralizar o esmalte dental (Lemos & Burne, 2008).

Normalmente centenas de espécies de bactérias coexistem na cavidade oral, formando um complexo biofilme em equilíbrio (Stoodley *et al*., 2002). Dentre essas bactérias, espécies do gênero *Streptococcus,* são largamente distribuídas na cavidade oral formando um biofilme dental multi-espécie que, juntamente com outras bactérias, protege o hospedeiro por evitar a colonização dos tecidos orais por espécies exógenas (Wade, 1999; Marsh, 2003; Lemos *et al*., 2005).

Em alguns casos, porém, a partir de perturbações provocadas nesse ambiente podem ocorrer alterações metabólicas e estruturais no biofilme o que leva a um maior crescimento de espécies odontopatogênicas, como *S. mutans*, principal agente etiológico da cárie (Loesche, 1986; Lemos *et al*., 2005;).

A virulência de *S. mutans* está diretamente relacionada com a sua habilidade de: 1) formar biofilme que adere na superfície do dente; 2) produzir grandes quantidades de ácidos orgânicos a partir de carboidratos ingeridos na dieta do hospedeiro (acidogenicidade), e 3) tolerar o estresse ambiental, principalmente pela sobrevivência em meio ácido (aciduricidade) (Quivey *et al*., 2000; Bowen, 2002; Lemos *et al*., 2005).

Somado a esses fatores de virulência já conhecidos, como capacidade de formação de biofilme, produção de ácidos e tolerância ao ácido, sabe-se que muitas cepas de *S. mutans* são capazes de produzir peptídeos antimicrobianos, bacteriocinas, que em *S. mutans* são chamados de mutacinas (Caufield *et al.,* 1985; Qi *et al.,* 2004). A atividade

antimicrobiana exibibida pelas mutacinas é contra Estreptococcus e outras bactérias gram-positivas que estão intimamente relacionadas com *S. mutans* (Gronroos *et al.,* 1998), o que facilita a sua adesão e permanência na superfície do dente assim como a formação de biofilme.

A atividade de mutacinas em *S. mutans* é coordenada por um sistema quorum-sensing e envolve um peptídeo estimulador de competência, dessa forma a produção de mutacinas está interligada com a ativação de competência nessa bactéria que além de facilitar a captação de DNA extracelular, tem influência sobre a formação de biofilme, tolerância ao estresse, persistência e virulência de *S. mutans* (van der Ploeg, 2005; Dufour *et al*., 2011 Leung *et al*., 2015)

Dentre os fatores de virulência de *S. mutans*, a sua capacidade de se aderir à superfície dental e formar biofilme é mediada por mecanismos sacarose-depenpende e independente, sendo que o primeiro é o mais importante. Quando a sacarose é consumida através da dieta do hospedeiro, *S. mutans* a usa como fonte de energia e também para formação de glucanos e frutanos, polissacarídeos extracelulares, que são de extrema importância para formação do biofilme dental e produzidos respectivamente por glucosiltransferases (GTFs) e frutosiltransferases (FTFs) (Hamada & Slade, 1980; Rivera-Ramos, 2015). Além das enzimas acima mencionadas, as Gbp (proteína de ligação ao glucano) produzidas por *S. mutans* também desempenham um papel crucial na formatação do biofilme oral. (Bowen & Koo, 2011; Rivera-Ramos, 2015).

Uma vez estabelecido na cavidade oral, *S. mutans* terá que lidar com diversos desafios para manter-se vivo já que este ambiente é uma rica fonte de estresse, principalmente oxidativo, e tanto o hospedeiro quanto as bactérias que coexistem na cavidade oral são capazes de gerar ERO (Espécies Reativas de Oxigênio), que causam danos às célula e as afetam mesmo quando estão organizadas em biofilmes (Rivera-Ramos, 2015).

O estresse oxidativo consegue afetar diversos fatores de virulência de *S. mutans*, como a capacidade de formação de biofilme, a atividade de GTFs, produção de bacteriocinas, competência, e o metabolismo de carboidratos (Ahn *et al.,* 2007). Para tentar minimizar tais danos, *S. mutan*s possui enzimas que são essenciais por conferirem proteção em situações de extresse oxidativo, dentre elas 1) a Dpr uma proteína que sequestra ferro do ambiente evitando a reação de Fenton, 2) Sod que previne o acúmulo

de O_2 no citoplasma, assim como, 3) Gor que ajuda a manter o equilíbrio pela redução da glutationa dissulfeto à glutationa (Yamamoto *et al.,* 1999) e a 4) Nox que converte H_2O_2 à H_2O (Higuchi *et al.,* 1999).

O fato de a virulência de *S. mutans* estar intimamente relacionada com a sua tolerância ao estresse ambiental (mudanças periódicas de pH, tensão de oxigênio e disponibilidade de nutrientes) faz com que se busque uma melhor compreensão dos mecanismos envolvidos nesta adaptação, a qual permitiria uma maior elucidação dos mecanismos de patogênese da doença cárie (Lemos & Burne, 2008). Além disso, o entendimento dos mecanismos envolvidos neste processo poderia facilitar o desenvolvimento de novos agentes terapêuticos úteis na prevenção de doenças associadas a *S. mutans*, como é o caso da cárie dental (Kajfasz *et al*., 2010).

Umas das principais consequências da exposição de *S. mutans* ao estresse ambiental é o acúmulo de proteínas anormais. Isso ocorre pois durante condições de estresse há um aumento de erros nos processos de transcrição e tradução protéica. Dessa forma, manter a homeostase protéica é de suma importância para viabilidade e crescimento de patógenos como *S. mutans*, por meio da estabilização das proteínas que executam funções essenciais e da degradação das proteínas defeituosas (Butler *et al*., 2006; Frees *et al.,* 2007). Nesse contexto, a Regulação de Proteínas via proteólise ganha destaque, seja para liberação de fatores responsáveis pelo início da transcrição gênica ou ativação de proteínas com função celular essencial (Hastie *et al*., 2014; Frank *et al.,* 2013)

Sabe-se que em bactérias gram-positivas, como *S. mutans*, o sistema Clp (Protease caseinolítica dependente de ATP) detém o papel central na tolerância ao estresse, uma vez que este complexo enzimático possui a função de degradação de cadeias polipeptídicas de proteínas mal-formadas. O sistema proteolítico Clp consiste de duas subunidades, um componente com atividade proteolítica ativa (ClpP) e um componente responsável pelo reconhecimento da proteína a ser degradada (ClpX, uma AAA^+ ATpase) (Butler *et al.,* 2006, Gribun *et al.,* 2005; Kajfasz *et al*., 2010; Lemos & Burne, 2002; Baker & Sauer, 2012).

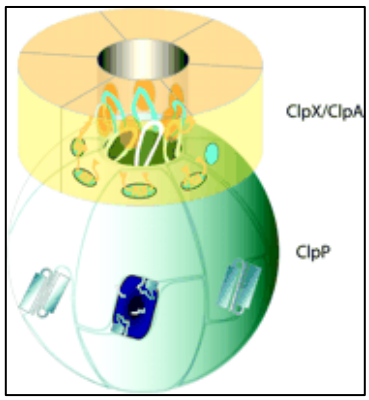

Figura 1: Representação esquemática da protease Clp com duas sub-unidades, um componente com atividade proteolítica ativa (ClpP) e um componente responsável pelo reconhecimento da proteína a ser degradada (ClpX/ClpA) (Gribun *et al.,* 2005).

Desta forma, Clp tem sido relacionada à patogênese bacteriana, uma vez que desempenha um importante papel na homeostase celular, e tem a capacidade de controlar a estabilidade de proteínas reguladoras (proteínas capazes de modular a expressão de múltiplos genes) (Frees *et al*., 2007; Kajfasz *et al*., 2010).

Mais especificamente, esse complexo proteítico tem como alvo proteínas defeituosas e reguladoras de forma a manter o controle da expressão gênica (Frees *et al*., 2004; Zuber, 2004). As proteases ClpP já foram identificadas em algumas bactérias gram-positivas, dentre elas: *Bacillus subtilis, Lactococcus lactis, Listeria monocytogenes, Staphylococcus aureus* and todas as espécies de Estreptpcoccus (Frees and Ingmer, 1999; Frees *et al.,* 2003; Frees *et al.,* 2007; Gaillot *et al.*, 2000; Lemos and Burne, 2002; Msadek *et al.,* 1998; Nair *et al.,* 2003; Robertson *et al.,* 2002).

Por meio de um mecanismo de degradação intrínseca, ClpXP mantém baixos os níveis de proteínas relacionadas ao estresse, mas uma vez que em situação de estresse, a transcrição de genes relacionados a esta situação é aumentada, permitindo acúmulo destas proteínas, até que a normalidade seja alcançada e ClpXP volte a degradar essas proteínas relacionadas ao estresse novamente (Gonzalez *et al.,* 2000; Neher *et al.,* 2003; Neher *et al.,* 2006).

Em decorrência da importância desta protease ClpP na manutenção da homeostase bacteriana frente ao estresse ambiental, diferentes autores vêm buscando um maior entendimento das funções desempenhadas por este sistema proteolítico.

Em experimento realizado por Nakano *et al* (2001), observaram um acúmulo da proteína Spx em cepas de *Bacillus subtilis* com deleções dos genes *clpP* ou *clpX*, concluindo, desta forma, que Spx possa representar um substrato para o sistema

proteolítico e o acúmulo desta proteína foi relacionado a fenótipos pleiotrópicos[1] de *ΔclpXP* (Nakano *et al.,* 2002). Assim, estudos em *Bacillus subtillis* revelaram que os fenótipos exibidos pelas cepas com mutações nos genes *clpP* ou *clpX* frente ao estresse foram aliviados quando da inativação de Spx (Nakano *et al*., 2001).

Spx é considerada um fator anti-α, pois em situações de estresse bloqueia o domínio α-C terminal da RNAP, proibindo sua interação com proteínas ativadoras responsáveis pela transcrição de genes relacionados ao crescimento e desenvolvimento, fazendo com que a bactéria fique estagnada nestas situações, e ao mesmo tempo Spx ativa a transcrição de genes em resposta ao estresse oxidativo (Kajfasz *et al.,* 2012; Nakano *et al.,* 2003).

Kajfasz *et al*., (2009) demonstraram que a inativação de um dos dois genes ortólogos (*spxA1 ou spxA2*) reguladores de transcrição da proteína Spx (Substrato de ClpP e ClpX) em *S. mutans*, promoveu um alívio nos fenotipos observados nas cepas que possuíam mutações nos genes *clpP* ou *clpX*. Além disso, em estudo conduzido por Kajfasz, *et al*. (2010) ficou claro que genes envolvidos em repostas ao estresse oxidativo têm sua expressão controlada por Spx em *S. mutans*, que é um regulador global de transcrição, fato comprovado também por testes de atividade enzimática que mostraram redução na expressão e atividade da NAD oxidase (Nox), glutationa oxidorredutase (Gor) e Superóxido dismutase (Sod), que são as principais enzimas protetoras contra estresses oxidativos em *S. mutans* (Marquis, 1995), em cepas mutantes com deleção do gene *SpxA1* e *SpxA1A2*.

Apesar da descoberta de que genes relacionados ao estresse oxidativo são regulados por Spx, estudos adicionais são necessários para avaliar o papel destes genes, para que se entenda de que forma são coordenados em direção à tolerância ao estresse oxidativo.

A partir dessas evidências, foi realizada uma caracterização detalhada da função das proteínas SpxA1 e SpxA2 em *S. mutans.* Interessantemente, análises moleculares através de microarray demonstraram que cepas com deleções destes genes (*ΔspxA1*, *ΔspxA2* e *ΔspxA1*/*ΔspxA2*) apresentaram alterações no padrão de expressão de genes relacionados com a tolerância ao estresse oxidativo, composição/estrutura de

[1] Pleiotropia é o nome dado aos multipos efeitos de um gene.

parede celular, virulência e hipotéticos (atividade ainda desconhecida) (Kajfasz, *et al*., 2010).

Além disso, as mutantes (*ΔspxA1* e *ΔspxA1/ΔspxA2*) mostraram-se com a virulência atenuada quanto à capacidade de infecção sistêmica em larvas de *Galleria mellonella* e quanto a capacidade de colonização em dentes de ratos. Desta forma, atribuiu-se um papel fundamental a Spx para a sobrevivência de *S. mutans* (Kajfasz *et al.,* 2010).

Assim, o objetivo geral deste estudo foi de dar continuidade a linha de pesquisa em andamento para confirmação do papel de Spx na patogenicidade da doença cárie, por meio de ensaio de competição *in vitro*, e ensaio de cárie *in vivo,* além da análise da competência de cepas mutantes de Spx e sua capacidade de formação de biofilme *in vitro*. Foram construídas cepas mutantes de *S. mutans* com deleção de genes hipotéticos, que apresentaram expressão alterada nas mutantes dos genes *spxA1* e *spxA1spxA2* e confirmou-se a importância de Spx como importante para conferir patogenicidade ao *S. mutans* e no controle de novos genes frente ao estresse oxidativo. Após construção e confirmação da obtenção das cepas mutantes de genes hipotéticos regulados por Spx, estas foram caracterizadas fenotipicamente em experimentações *in vitro* e *in vivo*. Este conhecimento é de fundamental importância para um melhor entendimento da função do regulador Spx em *S. mutans*, além de possibilitar a identificação de novos alvos terapêuticos para a prevenção/tratamento de doenças envolvendo este microrganismo.

2 ARTIGOS

2.1 ARTIGO: Inactivation of the *spxA1* or *spxA2* genes of *Streptococcus mutans* decreases virulence in the rat caries model

Artigo submetido ao periódico MOLECULAR ORAL MICROBIOLOGY **(Anexo 2).**

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Running title: Role of the *S. mutans* Spx in caries

Keywords: dental caries, *Streptococcus mutans*,Spx, oxidative stress

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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 (Δ*spxA1* and Δ*spxA1*Δ*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 D*spx*strains and the production of mutacin IV and V is virtually abolished in D*spxA1* strains. Unexpectedly, the Δ*spxA2* strain showed an enhanced ability to synthesize exopolysaccharides of glucan from sucrose, forming thicker biofilms than the parent strain. By using the rat caries model, we showed that the capacity of the D*spxA1* and D*spxA2* strains to cause caries on smooth tooth surfaces is significantly impaired. The D*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.

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* with the initiation and development of this disease (Lemos *et al*., 2005; Takahashi & 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 O_2 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 H_2O_2 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, H_2O_2 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*., 2010). 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*., 2010; Nakano *et al*., 2003a; Nakano *et al*., 2003b; Zuber, 2004). Physiologic characterizations of Δ*spxA1*, Δ*spxA2* and Δ*spxA1*/Δ*spxA2* strains revealed that SpxA1 plays a major role in survival under acid and oxidative stress conditions (Kajfasz *et al*., 2010). While stress tolerances were generally not impaired in the Δ*spxA2* strain, the stress sensitivities of the double Δ*spxA1*/Δ*spxA2*strain were more pronounced than in the single Δ*spxA1* strain (Kajfasz *et al*., 2010). In addition to activation of genes involved in thiol homeostasis and ROS scavenging (Kajfasz *et al*., 2010; 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 (Galvao *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*., 2010; 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 (Δ*spxA1* and Δ*spxA1*/Δ*spxA2*) showed a reduced ability to colonize the teeth of rats fed a highly cariogenic diet(Kajfasz *et al.*, 2010).

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 biofilm formation in the presence of sucrose due to increased glucan production. We also assessed the virulence potential of the Δ*spxA1* and Δ*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 Δ*spx* derivatives (Δ*spxA*1, Δ*spxA*2 and Δ*spxA1*/Δ*spxA2*) were routinely grown in Brain Heart Infusion (BHI) at 37°C under anaerobic conditions (BBL Gaspack 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.

Table 1. Bacterial strains used in this study.

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

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 OD_{600} 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% CO_2 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 OD_{575} ofthe 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. PubMed 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). 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 Δ*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 OD_{600} 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% CO_2 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, including quantification of glucans and total biomass, as described elsewhere (Falsetta *et al*., 2012; Xiao & Koo, 2010).

Deferred antagonism assay

Cultures were grown in BHI to an OD_{600} of 0.3 and a 5 ml aliquot spotted onto BHI agar and incubated for 24 h. Following incubation, 500 ml of an overnight culture of *S. gordonii* DL-1 (mutacin IV sensitive) *or Lactococus 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 & Biswas, 2011).

Genetic competence assay

Overnight cultures were sub-cultured 1:20 into fresh BHI supplemented with 10% horse serum and grown to OD_{600} 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) followed by addition of 0.2 μg of the shuttle plasmid pMC340B. 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 % CO_2 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, *ΔspxA1* or *ΔspxA2*. 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 CO_2 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, *ΔspxA1* and *ΔspxA2* 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

Spx regulation has been previously shown to repress competence development in other bacterial species. In *B. subtilis*, Spx inhibits the activation of ComK which in turn inhibits the activation of late competence genes (Nakano *et al*., 2002). In *Streptococcus pneumoniae*, SpxA1 negatively regulates competence by repressing transcription of the early competence operon *comCDE* (Turlan *et al*., 2009). In *S. mutans* UA159, competence development is controlled by quorum-sensing mechanisms involving the s^X inducing peptide (XIP) and the competence stimulating peptide (CSP)(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; Reck *et al*., 2015; van der Ploeg, 2005). Previously, we showed that competence and production of at least two mutacins (mutacin IV and mutacin V) were significantly impaired in Δ*clpP* and Δ*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 Δ*clpP* and Δ*clpX* strains. Then, we tested the transformation efficiency of these strains after nisin induction (Spx overexpression). As observed in both Δ*clp* and Δ*spx* strains, overexpression of SpxA1 or SpxA2 also resulted in competence deficiency as compared to the parent strain (Fig. 1C-D).

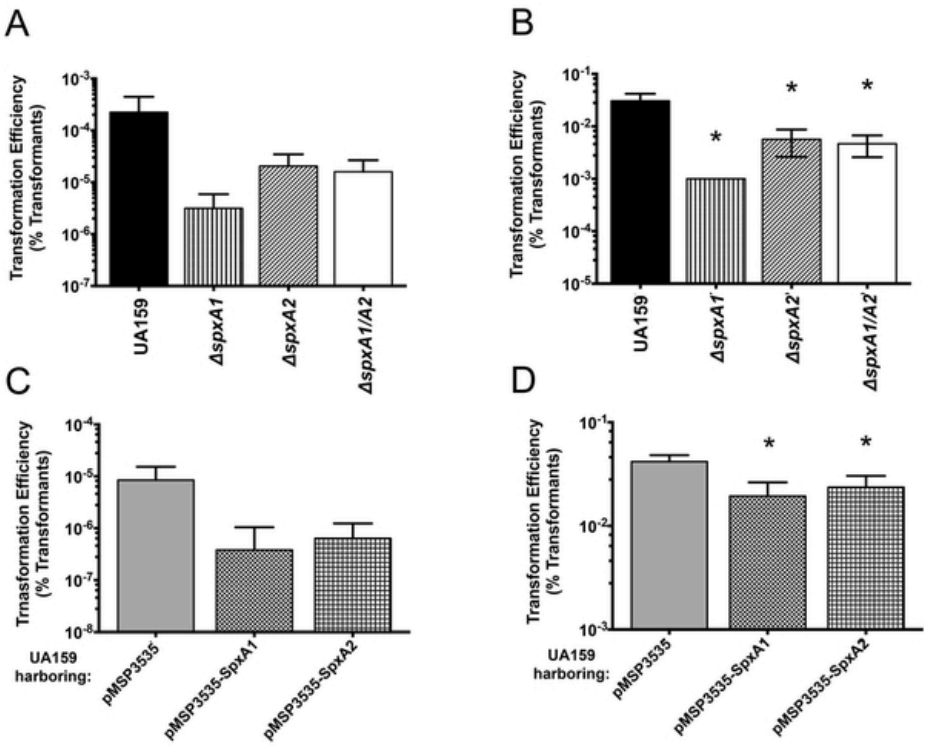


Fig. 1. Competence of *S. mutans* UA159 and its derivatives. Plasmid (pMC340A) was added to cells in early-logarithmic phase (OD_{600} of 0.15) with or without the addition of CSP or nisin. Cultures were incubated at 37°C in 5% CO_2 until entering stationary phase, serially diluted and plated in BHI for total CFU and in BHI containing spectinomycin for transformants. (A) pMC340A only (B) pMC340A + CSP, (C) pMC340A only, and (D) pMC340A + nisin.

Next, we measured bacteriocin production by the Δ*spx* strains using a deferred antagonism assay. Inhibition of the growth of *S. gordonii* or *L. lactis* was used to assess the ability of the wild-type and Δ*spxA* strains to produce, respectively, mutacin IV or mutacin V (Fig. 2). Based on our results, mutacin production in the Δ*spxA1* and Δ*spxA1*/Δ*spxA2* strains was diminished to the extent that both strains were completely unable to antagonize the growth of *S. gordonii* or *L. lactis*. Mutacin IV production was slightly increased in the Δ*spxA2* strain ($P \leq 0.05$) when compared to UA159, whereas no obvious differences in mutacin V production were observed between Δ*spxA2* and UA159.

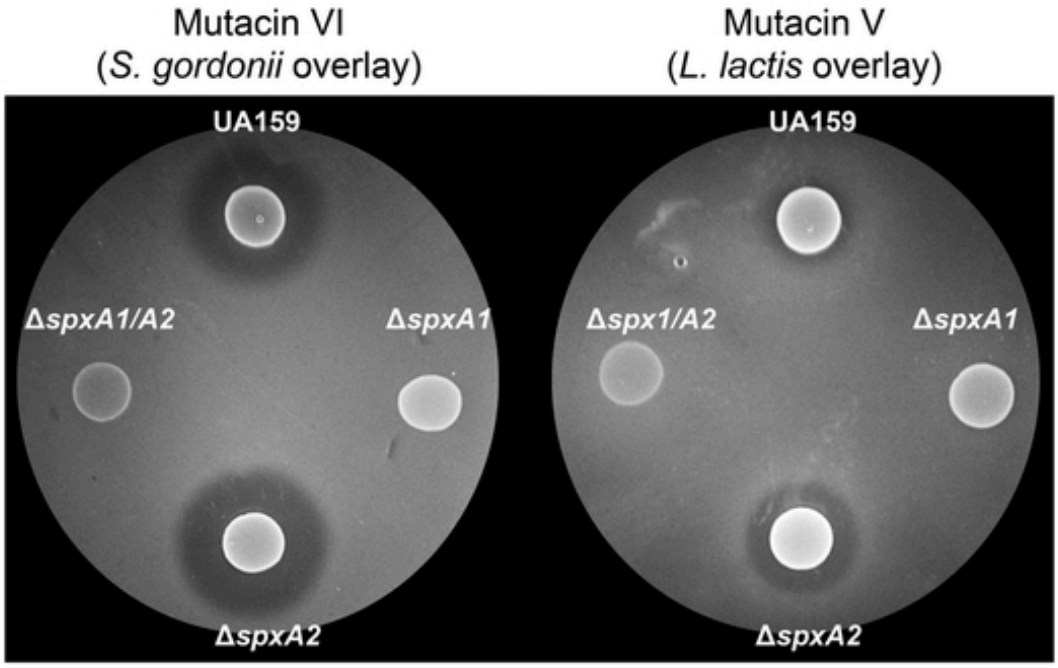
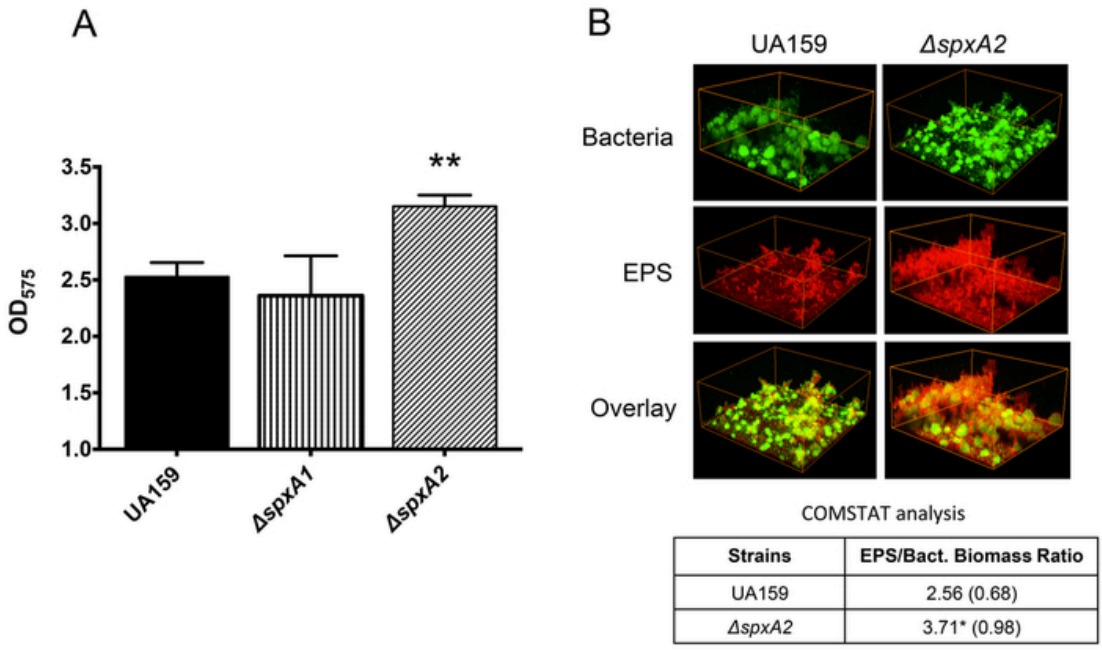


Fig. 2. Mutacin production by *S. mutans* UA159 and its derivatives. Cultures were grown in BHI to OD_{600} of 0.3 and 15 µl of each culture spotted on BHI plates followed by incubation at 37°C in 5% CO_2. After 24 h incubation, plates were exposed to UV light for 20 min and then overlayed with 5 ml soft BHI agar containing 500 µl of overnight cultures of *L. lactis* or *S. gordonii*. Plates were incubated for additional 48 h and the zone of inhibition recorded.

The Δ*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 Δ*spx* strains to form biofilms in media containing sucrose as the sole carbohydrate source. In saliva-coated microtiter plates, biofilm

formation by Δ*spxA2*, but not the Δ*spxA1* strain, was significantly enhanced (Fig. 3A). We also tried to measure the biofilm-forming capacity of the double Δ*spxA1*/Δ*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 Δ*spx* strains were due to small variations in the growth rates of the strains, the biofilm data were normalized by the total final OD_{600} comprising of both planktonic and biofilm cells. To confirm the enhanced sucrose-dependent biofilm phenotype of the Δ*spxA2* strain, a saliva-coated HA disc assay, a tooth enamel surrogate, was used to measure total biomass and to quantify glucan production in relation to total bacteria via confocal microscopy. In agreement with the results using microtiter plates, the Δ*spxA2* strain showed enhanced production of glucans (Fig. 3B). COMSTAT analysis revealed that the glucan/ biomass ratio of the Δ*spxA2* strain (3.71±0.98) was significantly higher ($P \leq 0.05$) than the ratios observed for the parent strain (2.56± 0.68).



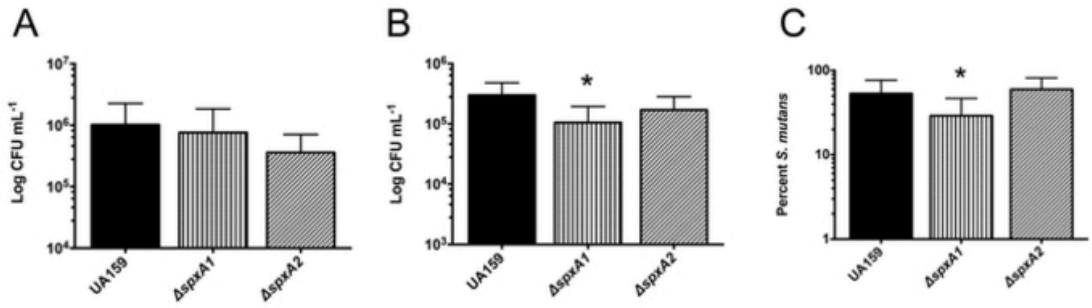
Strains	EPS/Bact. Biomass Ratio
UA159	2.56 (0.68)
ΔspxA2	3.71* (0.98)

Fig. 3. Biofilm fomation 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 (*, $P \leq 0.05$). (B) The imaging of matrix components in intact biofilms was conducted using a method based on incorporation of fluorescently labeled dextran (Alexa Fluor 647) by Gtf's during glucan synthesis. Bacterial cells were labeled with SYTO9. The images were acquired using an Olympus FV 1000 two-photon microscope (Olympus), and analyzed by AMIRA and COMSTAT (*, $P \leq 0.05$).

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

Previously, we showed that the ability of the Δ*spxA1* and Δ*spx1*/Δ*spxA2* strains to colonize the teeth of rats was significantly impaired (Kajfasz *et al*., 2010). As stated above, the Δ*spx1*/D*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-typeand Δ*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, Δ*spxA1* or Δ*spxA2* strains (Fig. 4A). However, when focusing on *S. mutans*, the numbers of Δ*spxA1* and Δ*spxA2*colonies recovered from the animals were lower when compared to the parent strain (Fig. 4B) but only the differences between parent and Δ*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 Δ*spxA1* strain (29.08 %± 17.61) but not for Δ*spxA2* (59.79 % ± 21.9) when compared to the proportions of the wild-type to total flora (53.35 % ± 23.42) (Fig. 4C).



The incidence and severity of smooth surface and sulcal surface caries in animals infected with the parent or Δ*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 Δ*spxA1* or Δ*spxA2* had reduced dental caries based on lower levels of average lesion extension [E] on smooth surfaces. The Δ*spxA2* strain also caused fewer sulcal carious lesions when compared to animals infected with the UA159 or Δ*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.

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

	Smooth surface				Sulcal surface			
Strains	**Total caries**	**Lesion severity**			**Total caries**	**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)
Δ*spxA1*	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)
Δ*spxA2*	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 ($P \leq 0.05$), ANOVA, using t-test without any transformation. Notes: Ds – slight dentinal caries; Dm – moderate dentinal caries; Dx – extensive dentinal caries.

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*., 2010; 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*., 2010; 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*., 2010) 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). Our previous observation that competence was impaired in *S. mutans* strains lacking *clpP* (Δ*clpP*) or *clpX* (Δ*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 Δ*clp* and Δ*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 microarray data (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 Δ*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 streptococci enabling its establishment and persistence in dental plaque (Merritt & Qi, 2012). Mutacins are divided in two types: lantibiotic and non-lantibiotic, with lantibiotics showing a higher spectrum of activity.than non-lantibiotics (Merritt & Qi, 2012). The genome of *S. mutans* UA159 encodes at least three non-lantibiotic mutacins; mutacin IV, V andVI. 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 Δ*spx* strains. Using *L. lactis*(sensitive to mutacin V) and *S. gordonii*(sensitive to mutacin IV) as indicators, we found that strains lacking *spxA1* could no longer inhibit the growth of both indicator strains. Different than competence activation that is influenced by both SpxA1 and SpxA2, mutacin production was not affected in the Δ*spxA2* single mutant. We also searched our previous transcriptome analysis (Kajfasz *et al*., 2010) for the differential expression of mutacin-coding genes in the Δ*spx* strains. Transcription of the genes responsible for mutacin V (*nlmC*) and mutacin VI (*nlmD*) was not significantly affected in theΔ*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 & Koo, 2011). The genome of *S. mutans* UA159 encodes for three Gtf genes; *gtfB*, *gtfC* and *gtfD*. The *gtfB* and *gtfC* genes are genetically linked and co-regulated by multiple transcriptional regulators (Bowen & Koo, 2011; Goodman & Gao, 2000; Yoshida & Kuramitsu, 2002). The *gtfD* gene is located elsewhere in the chromosome and also appears to be under complex transcriptional control (Bowen & Koo, 2011; Goodman & Gao, 2000). Our observations that glucan production and, as a result, biofilm formation, is enhanced in the Δ*spxA2* strain aligns well with previous microarray analysis showing that expression of *gtfB* and *gtfD* was enhanced in Δ*spxA2* (Kajfasz *et al*., 2010). Additionally, we observed the upregulation of the *lytTS* 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 Δ*spxA2* in our earlier microarray analysis, which may help to explain the enhanced biofilm formation seen for the Δ*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 Δ*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 Δ*spxA2* mutant, the CFU numbers recovered were similar to that 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 Δ*spxA1* or Δ*spxA2* strain had fewer carious lesions on smooth surfaces whereas the Δ*spxA2* strain alsocaused fewer sulcal caries. Interestingly, while the Δ*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 Δ*spxA2* strain was not significant. 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 Δ*spxA1* strain was a logical and expected extension of the oral colonization study and aligns well with the general stress sensitivity (Galvao *et al.,* 2015*;*Kajfasz *et al.,* 2010*;* Kajfasz *et al.,* 2015*)* and defects in competence and mutacin production of the Δ*spxA1* strain, the decreased cariogenicity of Δ*spxA2* was somewhat surprising. With the exception of competence and biofilm formation, the *in vitro* expression of all other virulence attributes investigated in the Δ*spxA2* strain were not affected. The decrease in formation of carious lesions by Δ*spxA2* is especially surprising considering the enhanced *in vitro* biofilm formation by this strain. Further, anatomy dictates that sulcal surfaces are more prone to caries formation than are smooth surfaces due to the increased likelihood for retention of acids and other contributors to caries formation. We previously reported diminished virulence of Δ*spxA2*, as well as the Δ*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 vivo* 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 Δ*spx*strains isolated directly from dental plaque of infected SPF rats.

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2.2 ARTIGO: **Transcriptional and phenotypic characterization of novel Spx-regulated genes in *Streptococcus mutans.***

Artigo aceito para publicação na PLoS ONE. PLoS ONE. 2015; DOI: 10.1371/journal.pone.0124969 **(Anexo 3)[2].**

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ABSTRACT

In oral biofilms, two of the major environmental challenges encountered by the dental pathogen *Streptococcus mutans* are acid and oxidative stresses. Previously, we showed that the *S. mutans* transcriptional regulators SpxA1 and SpxA2 (formerly SpxA and SpxB, respectively) are involved in stress survival by activating the expression of classic oxidative stress genes such as *dpr*, *nox, sodA* and *tpx*. We reasoned that some of the uncharacterized genes under SpxA1/A2 control are potentially involved in oxidative stress management. Therefore, the goal of this study was to use Spx-regulated genes as a tool to identify novel oxidative stress genes in *S. mutans*. Quantitative real-time PCR was used to evaluate the responses of ten Spx-regulated genes during H_2O_2 stress in the parent and Δ*spx* strains. Transcription activation of the H_2O_2-induced genes (8 out of 10) was strongly dependent on SpxA1 and, to a lesser extent, SpxA2. *In vitro* transcription assays revealed that one or both Spx proteins directly regulate three of these genes. The gene encoding the FeoB ferrous permease was slightly repressed by H_2O_2 but constitutively induced in strains lacking SpxA1. Nine genes were selected for downstream mutational analysis but inactivation of *smu127*, encoding a subunit of the acetoin dehydrogenase was apparently lethal. *In vitro* and *in vivo* characterization of the viable mutants indicated that, in addition to the transcriptional activation of reducing and antioxidant pathways, Spx performs an important role in iron homeostasis by regulating the intracellular availability of free iron. In particular, inactivation of the genes encoding the Fe-S biogenesis SUF system and the previously characterized iron-binding protein Dpr resulted in impaired growth under different oxidative stress conditions, increased sensitivity to iron and lower infectivity in rats. These results serve as an entryway into

the characterization of novel genes and pathways that allow *S. mutans* to cope with oxidative stress.

INTRODUCTION

Dental caries remains one of the most prevalent infectious diseases affecting billions of people worldwide [1]. Caries results from an ecological imbalance of the oral flora caused by interactions of specific bacteria (e.g. *Streptococcus mutans*) with salivary proteins and dietary carbohydrates (e.g. sucrose). The cariogenic potential of microorganisms is largely based on three factors: (i) the ability to form biofilms on the tooth surface, (ii) the ability to produce weak acids mainly lactic acid, and (iii) the ability to rapidly adapt to environmental stresses such as large and fluctuations in pH, oxygen tension and nutrient availability [2]. In addition to fulfilling these central requirements, *S. mutans* appears as one of the most dominant species during the early and intermediate stages of caries development, strengthening its association with dental caries initiation and progression [3,4].

Well-documented work from a number of laboratories has established that *S. mutans* is well-equipped to adapt to low pH values by activation of a robust physiological response to acidification that includes, among other responses, upregulation of the membrane-associated F-ATPase, induction of pathways that contribute to cytoplasm buffering and changes in membrane fatty acid composition [2]. In addition to acid tolerance, mounting evidence indicates that the ability of *S. mutans* to cope with the reactive oxygen species (ROS) generated by their own metabolism as well as by other oral species can also impact its pathogenic potential. In fact, it has been shown that, in dental plaque, there is an inverse correlation between the total numbers of *S. mutans* and of members of the mitis group (e.g. *S. sanguinis* and *S. gordonii*), known to produce large

quantities of H_2O_2 from the metabolic reduction of oxygen. Specifically, members of the mitis group are often associated with oral health; a series of elegant *in vitro* studies showed that H_2O_2 produced by *S. gordonii* or *S. sanguinis* serves as a "chemical weapon" antagonizing the growth of *S. mutans* [5]. Likewise, *S. oligofermentans*, a recently identified oral streptococci isolated from plaque of caries-free patients, was shown to antagonize the growth of *S. mutans* through a lactate oxidase that generates H_2O_2 from lactic acid [6]. Finally, H_2O_2 present in certain oral hygiene and tooth bleaching products may represent another source of peroxide stress for oral bacteria [7].

As a facultative anaerobe that lacks catalase and a full electron transport chain, oxygen metabolism in *S. mutans* is thought to occur through two flavin-based enzymes, Nox and AhpF [8]. However, biochemical and physiological characterization of AhpF (an H_2O_2 -forming NADH oxidase) and Nox (an H_2O-forming NADH oxidase) indicates that the latter is the stronger of the two implicated in oxygen metabolism [9,10]. Loss of Nox leads to a decreased ability to metabolize oxygen and elevated expression of genes involved in ROS detoxification [9]. In conjunction with the AhpC peroxidase, AhpF was shown to be part of an alkyl hydroperoxide reductase system working as a flavoprotein dehydrogenase, which supports peroxide reduction by AhpC [8]. Other ROS scavenging and protective systems are also present in *S. mutans* including superoxide dismutase (SodA), thiol peroxidase (Tpx), the thioredoxin reductase system (TrxA/TrxB), glutathione synthase (GshAB), glutathione oxidoreductase (Gor), and the iron-binding peroxide resistance protein Dpr [11-14].

Spx is a global regulator ubiquitously found in low GC Gram-positive bacteria that is involved in stress survival, principally by serving as a transcriptional activator of genes involved in thiol homeostasis and detoxification [15]. Spx lacks a DNA-binding domain

and its function depends on direct interactions with the RNA polymerase α-subunit, which can result in either positive or negative regulation [16,17]. There is now clear evidence that two or more Spx paralogs, with overlapping but also unique regulatory functions, contribute to adaptive stress responses of streptococcal species and, more recently, *Bacillus anthracis* [18-23].

Previously, we identified and characterized two Spx proteins (SpxA1 and SpxA2, previously named SpxA and SpxB, respectively) in *S. mutans* [18,19]. Inactivation of *spxA1* significantly impaired growth and survival under acid and oxidative stress conditions. Whereas stress tolerances were generally not impaired in the Δ*spxA2* strain, the stress sensitivities of the double Δ*spxA1*Δ*spxA2* strain was more pronounced than in the Δ*spxA1* strain [19]. Microarray profiling of the Δ*spxA1*, Δ*spxA2* and Δ*spxA1*Δ*spxA2* strains further indicated that SpxA1 plays a primary role in activating oxidative stress genes whereas SpxA2 appears to have a secondary role in the regulation of these same genes [19]. Notably, nearly every known gene of *S. mutans* with a proven role in oxygen metabolism or oxidative stress was found to be under Spx control. For example, transcription of *ahpC, ahpF*, *dpr*, *gor*, *nox*, *sodA*, *tpx* and *trxB* was downregulated in the Δ*spxA1* strain and, in most cases, further downregulated in the double mutant Δ*spxA1*Δ*spxA2* [19]. Despite the large number of genes identified in our microarray analysis, a relatively small number of genes followed this regulatory pattern, i.e. downregulated in both Δ*spxA1* and Δ*spxA1*Δ*spxA2* strains. We reasoned that some of the uncharacterized genes under SpxA1 control, and to a lesser extent SpxA2, might be involved in oxidative stress management. We performed transcriptional and phenotypic characterization of selected Spx-regulated genes previously identified in our microarray analysis to further investigate oxidative stress management under Spx regulation in *S.*

mutans. Our results strongly suggest that, in addition to reducing and antioxidant pathways, Spx also controls metal ion homeostasis, thus serving as a starting point for the characterization of novel genes and pathways that allows for *S. mutans* to cope with oxidative stresses.

Materials and Methods

Bacterial strains and stress growth conditions. The bacterial strains used in this study are listed in Table 1. *S. mutans* UA159 and its derivatives were routinely grown in brain heart infusion (BHI) or trypticase soy agar (TSA) at 37°C in a 5% CO_2 atmosphere or under anaerobic conditions (BBL Gaspack system, BD). Where appropriate, kanamycin (1 mg ml^{-1}), erythromycin (10 μg ml^{-1}) or spectinomycin (1 mg ml^{-1}) was added to the growth medium. For mRNA quantifications, cultures were grown to OD_{600} = 0.4, at which point control samples were harvested by centrifugation, while experimental samples were exposed to 0.5 mM H_2O_2 for 5 and 15 min before harvest.

Table 1. Bacterial strains used in this study.

Strains	Relevant genotype	Source or reference
UA159	Wild type	Laboratory stock
Δ*spxA1*	*spxA1*:: Sp^R	Kajfazs *et al*., 2010
Δ*smu143*	*smu143c*::Em^R	This study
Δ*smu144*	*smu144c*:: Km^R	This study
Δ*suf*	*smu248*::Km^R	This study
Δ*dpr*	*smu540*::Em^R	This study
Δ*feo*	*smu570*::Km^R	This study
Δ*smu929*	*smu929c*::Em^R	This study
Δ*smu1296*	*smu1296*::Km^R	This study
Δ*smu1645*	*smu1645*::Km^R	This study
Δ*spxA1*Δ*smu143*	*spxA1*:: Sp^R, *smu143c*::Em^R	This study
Δ*spxA1*Δ*smu144*	*spxA1*:: Sp^R, *smu144c*::Km^R	This study
Δ*spxA1*Δ*suf*	*spxA1*:: Sp^R, *smu248*::Km^R	This study
Δ*spxA1*Δ*smu929*	*spxA1*:: Sp^R, *smu929c*::Em^R	This study
Δ*spxA1*Δ*smu1296*	*spxA1*:: Sp^R, *smu1296*::Km^R	This study
Δ*spxA1*Δ*smu1645*	*spxA1*:: Sp^R, *smu1645*::Km^R	This study

Harvested pellets were stored at -80°C until use. To generate growth curves, strains were grown overnight under anaerobic conditions and diluted 20-fold in pH 7.0-buffered BHI containing H_2O_2 (0.2 mM), methyl viologen (MV, 5 mM), diamide (0.5 mM). Cultures were incubated at 37°C in a 5% CO_2 atmosphere and the OD_{600} recorded at selected intervals. To test the toxicity of iron to *S. mutans* strains, bacterial cultures grown to OD_{600} ~ 0.3 were diluted 1:100 in fresh BHI supplemented with 5 mM $FeSO_4$. After 18h of incubation at 37° in 5% CO_2, a 100 µl aliquot of each culture was serially diluted and plated onto BHI agar for CFU counting. To test the sensitivity of *S. mutans* UA159 and its derivatives to streptonigrin in disc diffusion assays, a uniform layer of exponentially-grown cells was spread onto BHI agar plates using a sterile swab, and filter papers (Whatman paper, 1 mm in diameter) saturated with 20 µl of a solution of 0.125 µg μl^{-1} of streptonigrin were placed onto the agar. All plates were incubated at 37°C in a 5% CO_2 for 48 h after which the diameter of the inhibition halo was measured.

Construction of mutant strains. Standard DNA manipulation techniques were used as previously described (Sambrook *et al*., 1989). The *S. mutans* strains lacking the various genes selected for this study (*smu143c smu144c, smu248, smu540, smu570, smu929, smu1296* and *smu1497*) were constructed using a PCR ligation mutagenesis approach (Lau et al., 2002). Briefly, PCR fragments flanking each target gene were obtained and ligated to non-polar kanamycin (NP-Km^R), polar kanamycin (Ω-Km^R), or polar erythromycin (Em^R) cassettes, and the ligation mix was used to transform *S. mutans* UA159. Double mutants were obtained by amplifying the mutated *spxA1* region in the previously constructed *ΔspxA1* strain (spectinomycin-resistant, Spc^R) and using this PCR product to transform the different single mutants. Mutant strains were isolated on BHI plates supplemented with the appropriate antibiotic. The deletions were confirmed as

correct by PCR sequencing of the insertion site and flanking region. Primers used to generate mutants are listed in S1 Table.

Real-time quantitative RT-PCR. Total mRNA was obtained using our standard protocols [24]. Briefly, RNA was isolated from homogenized *S. mutans* cells by repeated hot acid-phenol:chloroform extractions, and the nucleic acid precipitated with 1 volume of ice-cold isopropanol and one-tenth volume of 3 M sodium acetate (pH 5) at 4°C overnight. RNA pellets were resuspended in 80 μl nuclease-free H_2O, and treated with DNase I (Ambion) at 37°C for 30 minutes. The RNA was purified again using the RNeasy mini kit (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 qRT-PCR, cDNA templates were created from 0.4 μg of RNA using the SuperScript first-strand synthesis system (Invitrogen). Reactions were carried out on a StepOnePlus real-time PCR system (Life Technologies) according to protocols described elsewhere [24]. Gene-specific primers (S2 Table) were designed using Beacon Designer 2.0 (Premier Biosoft International) to amplify region of each gene 85 to 200 bp in length.

Protein Purification. The *S. mutans* SpxA1 and SpxA2 were expressed in *E. coli* as recombinant His-tagged fusion proteins using the pET-16B expression vector (EMD Millipore) as described elsewhere (Kajfasz *et al*, submitted). Purification of the recombinant proteins was performed by column chromatography using Ni-NTA resin (Qiagen) following the manufacturer's instructions. A crude extract of *S. mutans* RNAP was obtained based upon the methods described by Seepersaud *et al* for the extraction of RNAP from *Streptococcus agalactiae*, which utilizes the heparin binding properties to

enrich for RNAP [25]. Briefly, *S. mutans* cells were grown in BHI broth to OD_{600} ~ 0.5. Cells were harvested, resuspended in protoplast preparation buffer (0.3 M potassium phosphate buffer, pH 7.0; 40% sucrose; 0.5 U μl^{-1} mutanolysin) and incubated at 37°C for 90 min. Protoplasts were harvested, and the pellets resuspended in lysis buffer (50 mM Tris HCl, 10 mM $MgCl_2$, 0.1 M DTT, 0.1 mM EDTA, 10% glycerol, 1 mM PMSF, pH 8.0), followed by 3 rounds of 15 seconds of sonication, intersperced by 1 minute intervals on ice. Following centrifugation, supernatants were applied to Affi-Gel heparin resin (Bio-Rad) and eluted with a gradient of a NaCl gradient of 0.1 to 1 M. The elutions were run on 10% SDS-PAGE and the fractions containing a visible β subunit (134 kDa) were pooled, and the salt concentration 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 0.1-0.8 M NaCl. The desired fractions were again pooled and dialyzed (10 mM Tris HCl, 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mM EDTA, 50% glycerol, pH 8.0). Protein concentration was determined using the bicinchoninic acid assay (Pierce).

***In vitro* transcription assay.** A linear DNA template for each gene of interest was generated by PCR using primers designed to amplify from the promoter region to 70 to 200 base pairs of the coding region (S3 Table). Following amplification, the DNA fragments were purified with a QIAQuick PCR Purification Kit (Qiagen). The *in-vitro* transcription (IVT) reactions were performed as described elsewhere [26]. Briefly, 10 nM of each individual purified promoter template, 25 nM *S. mutans* RNAP, and 25 nM *B. subtilis* σ^A (a gift from Peter Zuber, Oregon Health Science Center) were incubated with or without purified 75 nM ofSpxA1 or SpxA2 in reaction buffer (10 mM Tris-HCl, 50 mM NaCl, 5 mM $MgCl_2$; pH 8.0) containing bovine serum albumin (BSA, 50 $\mu g\ ml^{-1}$) to a final volume of 20 μl for 10 min at 37°C. A nucleotide mixture (200 mM ATP, GTP,

and CTP, 10 mM UTP, and 5 μCi [α-^{32}P] UTP) was added and the incubation proceeded for an additional 3 to 5 minutes. Stop solution (1 M ammonium acetate, 0.1 mg ml^{-1} yeast RNA, 0.03 M EDTA) was added and the mixture was precipitated with ethanol at 4°C for 3 hours. The nucleotide pellet was resuspended in formamide dye (0.3% xylene cyanol, 0.3% bromophenol blue, 12 mM EDTA dissolved in formamide). The samples were heated at 90°C for 2 min, then placed on ice before applying to an 8% polyacrylamide urea gel. The transcripts were visualized with Bio-Rad Quantity One Fx software following overnight exposure to an Imaging Screen-K (Bio-Rad).

Competition assay on solid media. The ability of *Streptococcus gordonii* to inhibit growth of *S. mutans* via H_2O_2 production was assessed as described previously by Kreth *et. al*. [5]. Briefly, 8 μl of an overnight culture of *S. gordonii* DL-1 was spotted on the center of a BHI agar plate incubated at 37°C for 16 h. The following day, 8 μl 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 H_2O_2 by *S. gordonii*, a control condition was included in which 8 μl of catalase (0.75 μg μl^{-1}) was immediately spotted on top of the *S. gordonii* culture.

Rat colonization. The ability of the mutant strains to colonize the teeth of rats was evaluated using an established model of dental caries [27]. Pathogen-free Wistar rat pups 19 days of age were purchased from CEMIB/ University of Campinas (credited by ICLAS – International Council for Laboratory Animal Science). Rats were first screened to ensure a lack of indigenous mutans streptococci as previously described [27], and infected separately for three consecutive days by means of cotton swab with actively growing *S. mutans* strains. Animals were fed a cariogenic diet (Diet-2000 containing

56% sucrose) and 5% sucrose water (wt/vol) *ad libitum* to enhance the infection by *S. mutans*. The experiment proceeded for 15 days, at the end of which the animals were killed by CO_2 asphyxiation, and the lower jaws removed for microbiological assessment. The number of mutans streptococci recovered from the animals was expressed as CFU ml^{-1} of jaw sonicate. This study was reviewed and approved by the University of Campinas Committee on Animal Resources (CEUA Protocol #2639-1).

Biofilm assay. Biofilm formation of the constructed *S. mutans* mutants was assessed by growing cells in wells of polystyrene microtiter plates using a semi-defined biofilm medium (BM) [28]. Strains grown in BHI medium to OD_{600} ~ 0.5 were diluted 1:100 in BM containing 1% sucrose and added to the wells of the microtiter plate. Plates were incubated at 37°C in a 5% CO_2 atmosphere for 24 h. After incubation, plates were washed twice with water to remove planktonic and loosely bound bacteria, and adherent cells were stained with 0.1% crystal violet for 15 min. The bound dye was extracted with 33% acetic acid solution, and biofilm formation was then quantified by measuring the optical density at 575 nm. To exclude the possibility that differences in bound cells obesrved were due to variations in growth rates and growth yields, the biofilm data was normalized by the final OD_{600} growth.

Statistical analysis. Student's *t* tests were performed to assess the distributions of the real-time PCR quantifications. One-way analysis of variance (ANOVA) was performed to verify the significance of the growth curves, growth in $FeSO_4$, streptonigrin disc inhibition and biofilm quantifications. The animal study data were subjected to ANOVA in the Tukey-Kramer Honest Standard Deviation (HSD) test for all pairs. In all cases, *P*-values ≤ 0.05 were considered significant.

Results

Transcription of several Spx-regulated genes is induced by H_2O_2 stress. Nine genes selected for this study were previously identified in a microarray study as being positively regulated by the *S. mutans* SpxA1 protein [19]. In addition to the genes positively regulated by SpxA1, one gene under SpxA1 negative control, *smu570* predicted to encode the second gene (*feoB*) of the FeoABC iron transporter system [29], was also analyzed. The selected genes with their respective assigned functions and expression ratios from our previous microarray analysis are shown in Table 2. Recently, we showed that treatment with 5 mM H_2O_2 strongly induced the expression of known oxidative stress genes (e.g. *ahpC, gor, nox, sodA, tpx* and *trxB*), and that this regulation was strongly dependent on SpxA1 (Kajfasz et al., submitted). Here, we used quantitative real-time PCR (qPCR) to evaluate the transcriptional responses of the ten selected SpxA1-regulated genes (*smu127*, *smu143c, smu144c, smu248, smu540, smu570, mu929c, smu1296, smu1297* and *smu1645* to H_2O_2 stress (Fig. 1). A heat map depicting the transcript fold change in relation to the parent UA159 under control growth conditions (T0) is also shown (S1 Fig.). In the parent strain, transcription of *smu127*, *smu143c, smu144c, smu248* (*sufD*), *smu540* (*dpr*), *smu929, smu1296* and *smu1297* was significantly induced by H_2O_2 at both 5 and 15 min time points. Transcription of *smu1645* was also moderately induced by H_2O_2 but the differences were not statistically significant. Nevertheless, transcriptional levels of *smu1645* were significantly lower in all Δ*spx* stains confirming that this gene is also under Spx control. Among the H_2O_2-induced genes, *smu1296* and *smu1297* respectively, showed the most robust responses ranging from 10 to 100-fold induction after stress. Transcription of all H_2O_2-induced genes was strongly dependent on SpxA1 and, with the exception of *smu127*, SpxA2. In

agreement with our previous microarrays, expression of *smu570* (*feoB*) was induced in the absence of SpxA1 but slightly repressed in Δ*spxA2*. Also within the expected, *feoB* transcription was repressed by H_2O_2 in the two SpxA1-positive strains (UA159 and Δ*spxA2*). Altogether, these results reveal that transcription of the genes investigated is controlled by both SpxA1 and SpxA2, induced by H_2O_2 in an SpxA1-dependent manner and, therefore, likely to participate in oxidative stress responses.

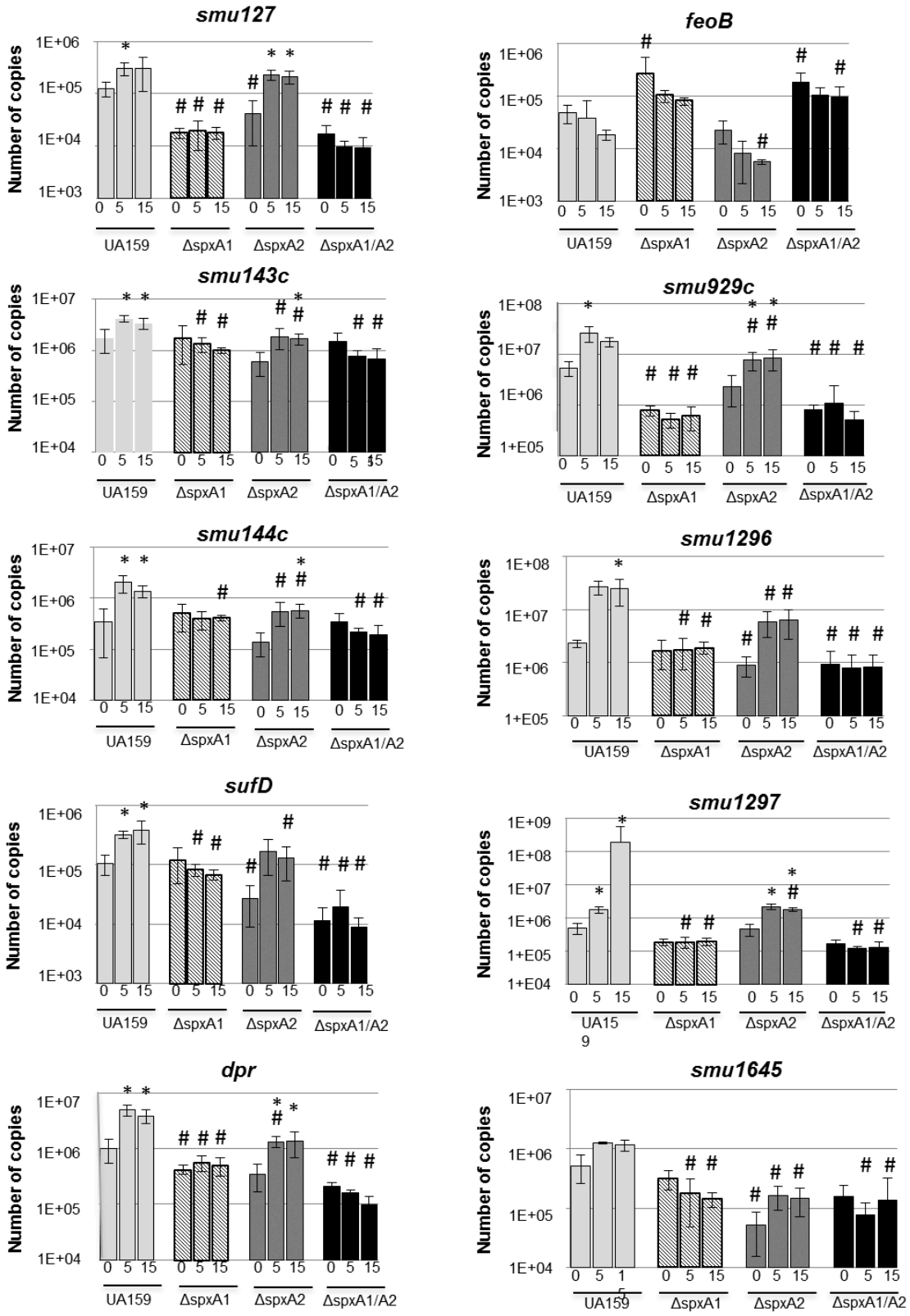

Figure 1. Transcriptional profile of Spx-regulated genes during H_2O_2 exposure. *S. mutans* UA159, Δ*spxA1*, Δ*spxA2*, or Δ*spxA1*Δ*spxA2* were grown in BHI to OD_{600} = 0.4.

At that point, control cultures (Time 0) were harvested, while the remaining samples were exposed to 0.5 mM H_2O_2 for 5 and 15 minutes before harvest. Bars represent the relative copy number detected for each gene. (*) indicates a significant difference ($p \leq 0.05$) from the same strain at Time 0; ($^{\#}$) indicates significant difference ($p \leq 0.05$) from UA159 at the corresponding condition.

IVT assays confirm direct regulation of *smu144c*, *dpr* and *smu929c* by Spx. To confirm whether the regulatory effects exerted by the two Spx proteins were direct or indirect, we performed IVT reactions in the presence or absence of purified SpxA1 or SpxA2 proteins. Our results demonstrate that the addition of purified SpxA1 to the reactions enhanced transcription of *smu144c/143c, dpr* and *smu929c* (Fig. 2). Addition of SpxA2 also enhanced *dpr* transcription. However, SpxA2, at equal or greater concentrations than SpxA1, had no apparent effects on *smu144c/143c* or *smu929c* transcript levels. In the case of the *sufA* operon, a transcript was seen in the absence of Spx protein, but the addition of SpxA1 or SpxA2 did not enhance transcription. Despite multiple attempts, we were unable to detect transcripts upstream *smu127*, *feoA*, *smu1296* and *smu1645* (data not shown). Given that transcripts for these genes have been detected by microarrays and qPCR, it is possible that these transcripts are not abundant and/or short-lived for detection via IVT assay. Another possible explanation is that co-factors or other regulatory proteins, not included in the *in vitro* system, may be required for efficient transcription of these genes.

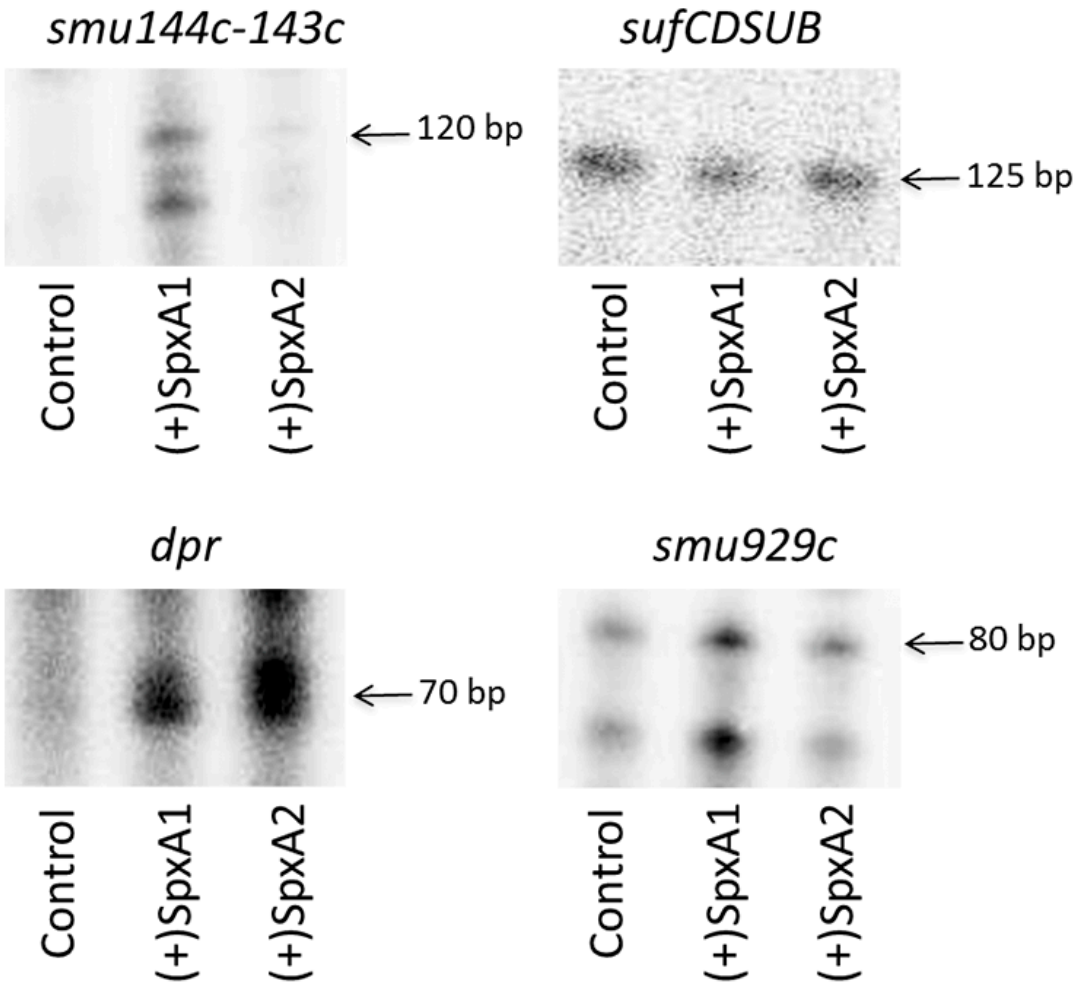

Figure 2. One or both Spx proteins specifically enhance transcription of *smu144c*, *dpr* and *smu929c* but not of the *suf* operon. *In-vitro* transcription reactions were performed by incubating the regulatory regions of stress genes with RNAP and nucleotides including [α-^{32}P] UTP in the absence (control lane) or presence of 75 nM of purified SpxA1 or SpxA2 proteins at 37°C. Reactions were performed at 37°C. Radiolabeled RNA transcripts were precipitated, applied to 8% urea PAGE, and visualized by exposure to phosphorimager screen.

Table 2. List of selected genes under SpxA1/A2 control identified in microarray analysis.

Gene ID	Assigned function	Fold change in expression in Δ*spx* strains relative to UA159[a]		
		Δ*spxA1*	Δ*spxA2*	Δ*spxA1/A2*
smu127	acetoin dehydrogenase, *adhA*	0.206	ND[b]	0.131
smu143c	polypeptide deformylase	0.430	ND	0.425
smu144c	transcriptional regulator	0.255	ND	0.201
smu247	Fe-S assembly ATPase, *sufC*	0.578	ND	0.397
smu540	peroxide resistance protein, *dpr*	0.133	ND	0.198
smu569	ferrous ion transport, *feoA*	3.135	ND	2.253
smu929c	hypothetical protein	0.135	ND	0.108
smu1296	glutathione S-transferase	0.301	ND	ND
smu1297	pAp phosphatase	0.232	ND	0.325
smu1645	tellurite resistance protein, *tehB*	0.502	ND	0.245

[a] [19]

[b] ND, no difference

Growth characteristics of mutant strains. Upon confirmation that the selected genes were transcriptionally regulated by Spx and, in most cases, induced by H_2O_2 stress, we sought to determine the effect that deletion of each individual gene would have upon *S. mutans* ability growth under oxidative stress. The *smu1297* gene, encoding an enzyme with 3'-phosphoadenosine-5'-phosphate (pAp) activity, was previously identified in a transposon mutagenesis library to be involved in the superoxide stress response [30]. Because Smu1297 already has an assigned role in oxidative stress, the *smu1297* gene was

not further investigated. Deletion mutations of eight of the genes described above were readily obtained but a viable *smu127* mutant strain was not. The *smu127* gene is predicted to encode the E1 component of the subunit α of the acetoin dehydrogenase (Adh) complex. The genetic organization of the eight mutated genes is depicted in (S2 Fig.). The *smu143c* and *smu144c* genes, encoding, respectively, polypeptide deformylase and a putative transcriptional regulator, are separated by only 25 bp and co-transcribed (S3 Fig.). Likewise, *smu1296* encoding a glutathione S-transferase (GST) homologue was shown to be co-transcribed with *smu1297* [30]. To avoid downstream polar effects, *smu144c* and *smu1296* were inactivated with a non-polar cassette that contains a promoterless *aphA3* Km^R gene without transcription termination sequences to allow transcription readthrough into downstream sequences [31]. Real-time PCR quantifications confirmed expression of the downstream *smu143c* and *smu1297* genes in the Δ*smu144* and Δ*smu1296* strains, respectively (S4 Fig.). The Em^R marker was used to replace the *smu143c* and *smu929c* genes; *smu929c* is the last gene of a bicistronic operon (*smu930c-smu929c*). The *smu569-571* and *smu247-251* genes are arranged in operons with other genes involved in iron transport (FeoABC) and iron-sulfur (Fe-S) cluster assembly (SUF), respectively. To disrupt the FeoABC and Suf systems, the *feoA*/*feoB* genes (*smu569-570*) was replaced by the nonpolar Km^R cassette (Δ*feo*) whereas the first two genes in the *suf* operon (*sufC*/*sufD*) were individually inactivated using a polar Km^R cassette such that transcription of the downstream genes was interrupted (Δ*suf*). The *smu1645* gene, encoding a putative tellurite resistance protein, is transcribed in the opposite orientation of its surrounding genes and was also inactivated using the polar Km^R marker. Finally, we used the Em^R marker to inactivate the *smu540* gene, encoding the iron-binding peroxidase resistance Dpr protein. The importance of Dpr to *S. mutans*

oxidative stress tolerance has been relatively well documented [11,32,33], and the Δ*dpr* strain was used as a benchmark in downstream studies.

The growth characteristics of the mutant strains under different conditions are shown in Fig. 3**.** Under standard non-stressful conditions, e.g. brain heart infusion (BHI) broth at 37°C in a 5% CO_2 atmosphere, all strains, with the exception of the Δ*suf* strain with a doubling time of 2.56 ± 0.23 h, grew as well as the parent UA159 strain (doubling time 1.32 ± 0.07 h). Next, we evaluated the ability of the mutant strains to grow in a 5% CO_2 atmosphere in the presence of H_2O_2, methyl viologen (MV, a quaternary ammonium compound that generates superoxide radicals) and diamide (a specific oxidant of thiols). In the presence of 0.2 mM H_2O_2, the Δ*suf* strain showed an extended lag phase and slow growth rates whereas the Δ*dpr* strain was completely unable to grow under this condition. In the presence of diamide, growth rates and growth yields of the Δ*suf* were severely affected (doubling time 3.54 ± 0.41 h compared to 1.48 ± 0.08 for the parent strain). The Δ*suf* strain also displayed long lag and slow growth rates in MV. Surprisingly, the Δ*dpr* strain that failed to grow in H_2O_2 was able to grow in the presence of diamide or MV, albeit with extended lag phases. Growth kinetics of the remaining strains, Δ*smu143c,* Δ*smu144c,* Δ*feo*, Δ*smu929,* Δ*smu1296* and Δ*smu1645*, in the presence of the different oxidative stress agents was nearly identical to that of the parent strain.

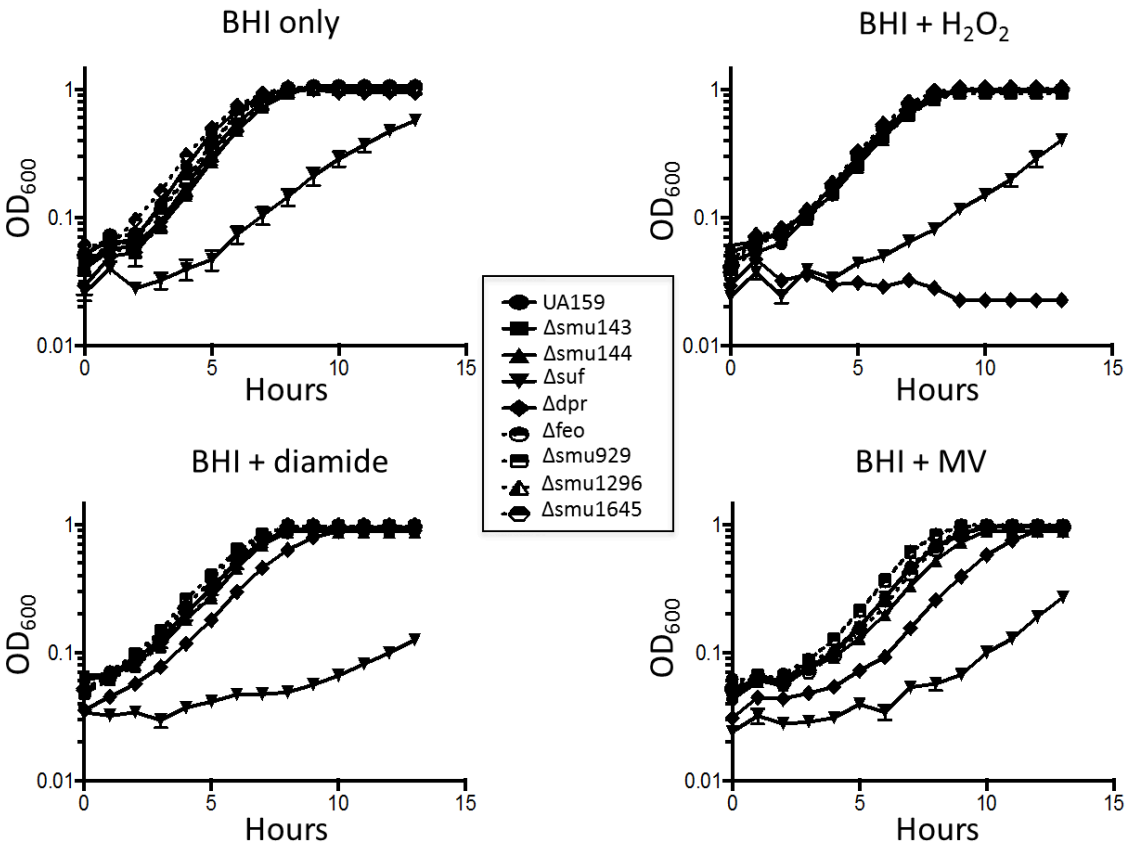

Figure 3. Growth curves of *S. mutans* UA159 and its derivatives under different environmental conditions. The curves shown are the means with standard deviations of the results from five independent cultures.

We also tested the ability of our panel of mutants to grow in the presence of the peroxigenic commensal *Streptococcus gordonii*. In agreement with its inability to grow in the presence of H_2O_2 (Fig. 3), growth the Δ*dpr* strain was inhibited by *S. gordonii* (Fig. 4). Despite an extended lag phase in media containing H_2O_2, growth of the Δ*suf* strain was not inhibited by *S. gordonii*. Also in agreement with growth curve results, the remaining mutant strains were not inhibited by *S. gordonii*, at least under the conditions tested (Fig. 4 or data not shown).

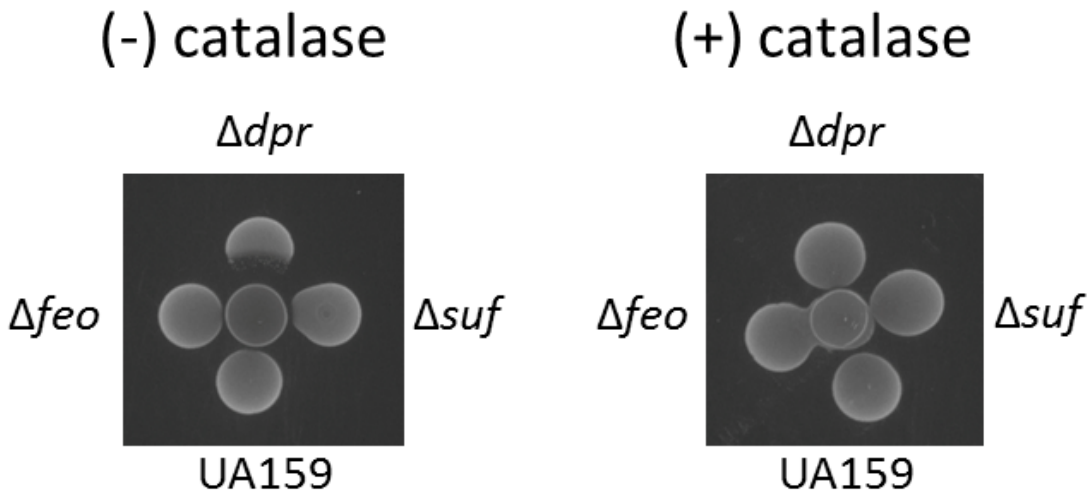

Figure 4. Growth competition on solid media between the peroxigenic *S. gordonii* and *S. mutans* UA159 and its derivatives. Competition assay reveals that *S. mutans* Δ*dpr* is sensitive to the H_2O_2 produced by *S. gordonii* (center spot). Under the conditions tested, growth of UA159, Δ*feo* and Δ*suf* strains (shown) as well as Δ*smu143*, Δ*smu144*, Δ*smu929*, Δ*smu1296* and Δ*smu1645* (not shown) was not inhibited by *S. gordonii.* The assay was repeated with catalase overlaid onto the *S. gordonii* spot to inactivate the H_2O_2, resulting in complete loss of sensitivity for the Δ*dpr* strain.

Iron sensitivity of the mutant strains. Functional annotation of genes selected for this study suggest that some of the genes may be involved in metal ion homeostasis, which includes genes encoding a polypeptide deformylase metalloprotein (*smu143c*), an Fe-S cluster assembly system (*smu247-smu251*), iron-binding protein (*dpr*), iron transport system (*feoABC*) and tellurite resistance protein (*smu1645*). Metal ions, in particular iron, are essential for life functions, but cytoplasmic levels must be tightly controlled as iron can serve as a catalyst for the production of damaging hydroxyl radicals in the presence of H_2O_2 via Fenton reaction. Next, we tested the ability of our panel of mutant strains to cope with iron by assessing their ability to grow in the presence of 5 mM $FeSO_4$ or streptonigrin, an iron-activated antibiotic [34] (Fig. 5). Strains

Δsmu143, Δsuf and Δ*dpr* displayed a growth impairment in the presence of $FeSO_4$, whereas *Δsmu144, Δsuf,* Δ*dpr, Δsmu1296* and *Δsmu1645* showed increased sensitivity to streptonigrin based on disc diffusion assays. In agreement with its assigned role in iron transport, the *Δfeo* strain showed increased tolerance to streptonigrin but not to $FeSO_4$. Because *smu1645* encodes a putative tellurite resistance protein, we also compared the minimum inhibitory concentration (MIC) of tellurite between the parent UA159 and *Δsmu1645* strains. In accordance with its predicted function, the Δ*smu1645* strain had a lower MIC for tellurite than UA159 (3.9 μM versus 15.6 μM for UA159, data not shown).

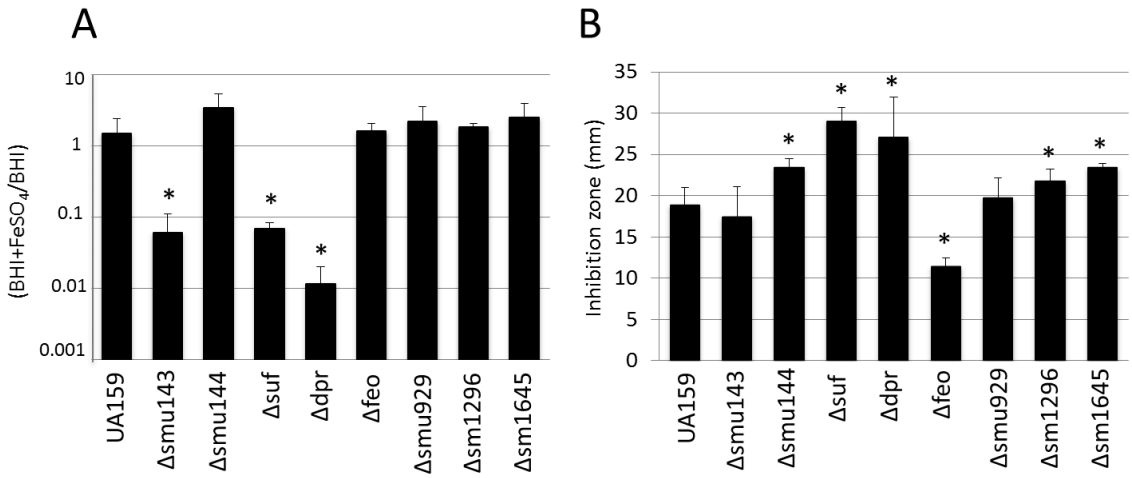

Figure 5. Iron homeostasis in *S. mutans* UA159 and its derivatives. (A) Growth of *S. mutans* UA159 and single mutant strains in the presence of 5 mM $FeSO_4$. (B) Inhibition zones (in mm) of cultures in the presence of filter disks soaked with streptonigrin. (*) $p \leq 0.05$.

The Δ*suf* and Δ*dpr* strains showed an impaired ability to colonize the teeth of rats. Next, we tested the ability of the Δ*smu143,* Δ*suf,* Δ*dpr*, Δfeo*,* Δ*smu1296* and Δ*smu1645* strains to colonize the teeth of Wistar rats (Fig. 6). Following a 15-day infection period, colonies of *S. mutans* were recovered from the jaws of infected animals

by plating the jaw sonicates on MS agar. In agreement with the growth defect and general stress sensitivity, the infectivity of the Δ*dpr* and Δ*suf* strains was significantly reduced ($P < 0.05$). The differences observed between the remaining mutant strains were not statistically significant. Because the infectivity of *S. mutans* in the oral cavity has been shown to be directly tied to its ability to form biofilms on tooth surfaces through a sucrose-dependent mechanism [35], we also evaluated the capacity of each strain to form biofilms on microtiter plates in the presence of sucrose. The Δ*smu143*, Δ*dpr* and Δ*suf* and Δ*feoB* strains accumulated more biofilm ($P < 0.05$) when grown in sucrose when compared to UA159 (Fig. 7). Therefore, the lower infectivity of the Δ*dpr* and Δ*suf* strains in the teeth of rats does not appear to correlate with a deficiency in biofilm formation.

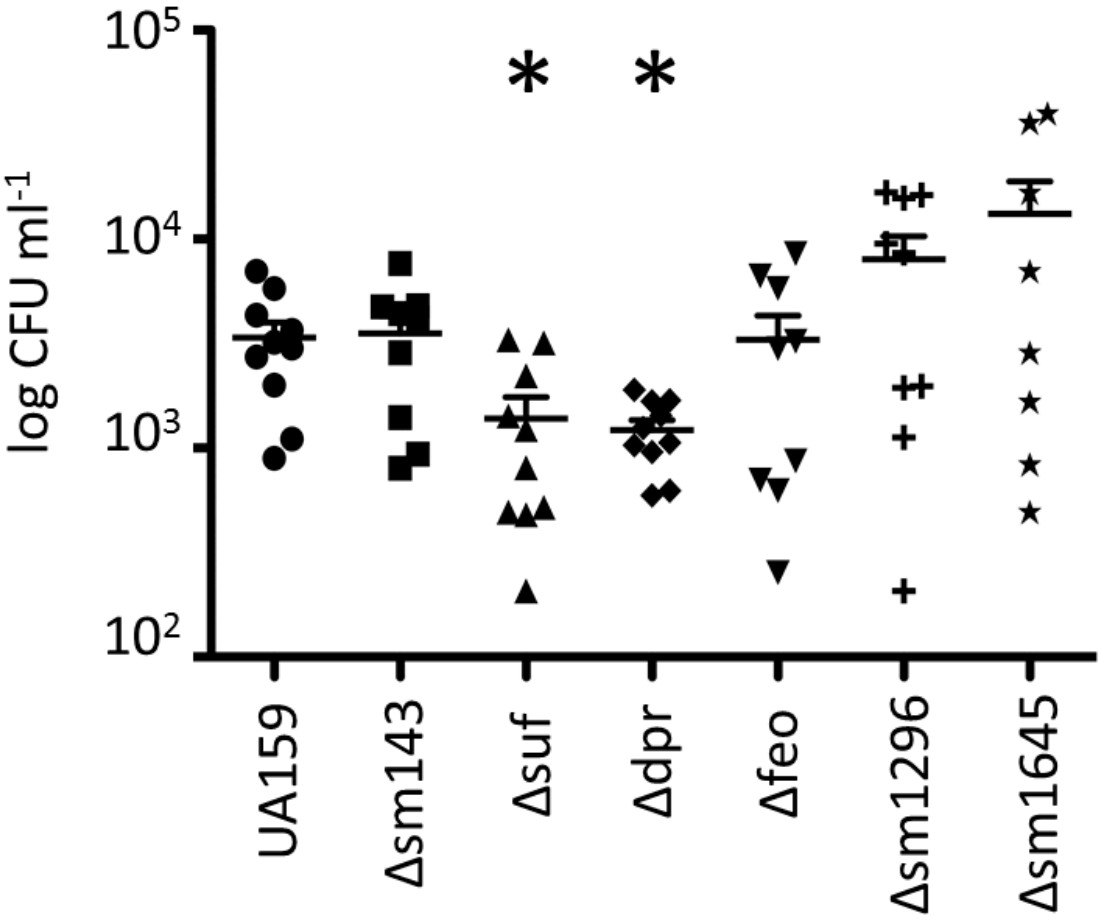

Figure 6. Colonization of *S. mutans* UA159 and derivative strains on the teeth of rats 15 days post-infection. The symbols represent the recovered bacterial colonies from each individual rat while the horizontal line represents the mean recovery per bacterial strain. (*) $p \leq 0.05$.

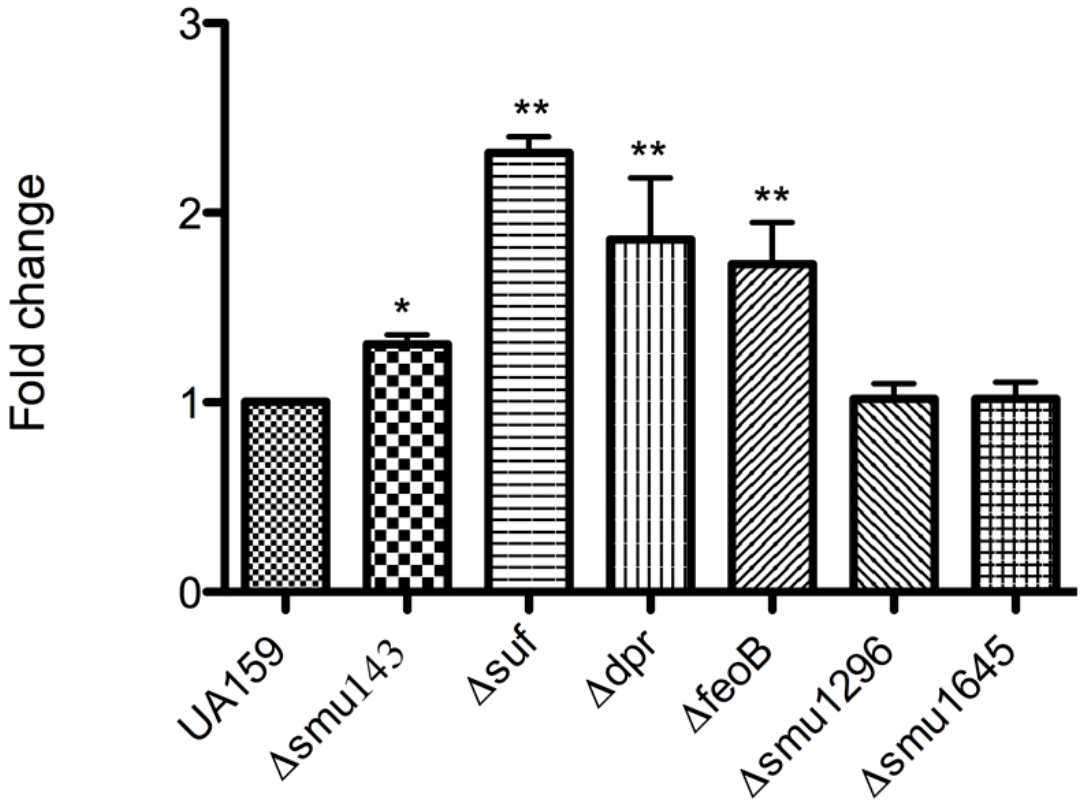

Figure 7. Biofilm formation by *S. mutans* UA159 and its derivatives. Cultures were gown for 24-h in BM supplemented with sucrose on the surface of 96-well microtiter plates. The graph shows the average and standard deviation for at least three independent experiments. (*) $p \leq 0.05$.

A phenotype enhancement screen confirmed the physiologic relevance of the Fe-S cluster assembly system but failed to assign new roles to the other Spx-regulated genes. Due to functional redundancy and genetic buffering, it is not unusual

that single gene deletions do not have any detectable phenotype. This is readily observed in systematic gene deletion libraries [36,37] whereby gene functions are rarely assigned based on single deletions. A useful way to overcome this limitation is the construction of paired mutations that may eliminate or reduce genomic buffering effects [38,39]. Recently, the Zuber lab utilized such an approach to uncover the role of Spx-regulated genes of unknown function in *B. subtilis* [40]. Specifically, double mutant strains were created by introducing mutations on selected Spx-regulated genes into the *spx* mutant background followed by a screen for enhanced oxidative stress sensitivity when compared to the *spx* single mutant. Here, we utilized a similar approach by inactivating our genes of interest, one at a time, in the Δ*spxA1* strain. We have previously shown that the Δ*spxA1* strain grew poorly under oxidative stress conditions [19], thus we evaluated the ability of Δ*spxA1* single and double mutants to grow under these conditions. While we were able to isolate a Δ*spxA1*Δ*suf* double deletion strain, this strain grew very poorly under standard laboratory conditions and was not amenable to further phenotypic characterization. In most cases, the double mutants did not display slower growth rates or hypersensitivity against the oxidative stress agents tested (H_2O_2, MV or diamide) when compared to the Δ*spxA1* single mutant (Fig. 8). The only exceptions were the Δ*spxA1*Δ*smu143c* and Δ*spxA1*Δ*smu929c* strains that, unexpectedly, grew better than Δ*spxA1* in the presence of diamide and the Δ*spxA1*Δ*smu144c* strain that showed slower growth than Δ*spxA1* in the presence of MV. We tested whether the increased diamide resistance of the Δ*spxA1*Δ*smu143c* and Δ*spxA1*Δ*smu929c* strains was a result of elevated *spxA2* expression but in, both case, the transcriptional levels of *spxA2* were identical to the parent UA159 strain (S5 Fig.). At this time, the reasons for mutations on either the *smu143* or the *smu929* genes serving to alleviate the diamide sensitivity of the

Δ*spxA1*strain are unknown. One possible explanation is that loss of Smu143 or Smu929 triggered some yet to be determined oxidative stress mechanism that compensates for the loss of SpxA1.

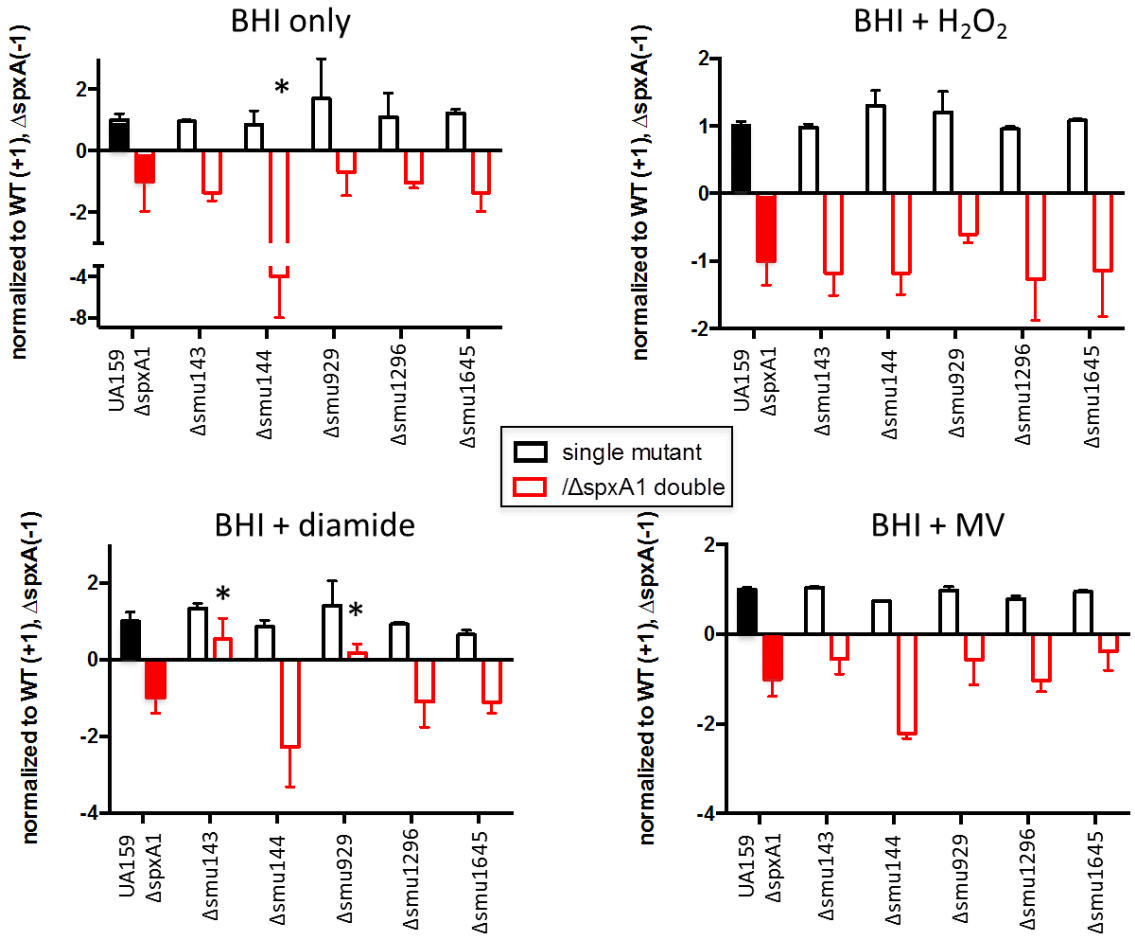

Figure 8. Phenotype enhancement screen of Spx-regulated genes. Growth of single mutants (Δ*smu143,* Δ*smu144,* Δ*smu929,* Δ*smu1296* and Δ*smu1645*, open black bars) and double (Δ*smu/*Δ*spxA1,* open red bar) in relation to wild-type UA159 and Δ*spxA1* single mutant. Solid bars represent normalized growth of Δ*spxA1* strains (grey bar, set to -1) in relation to UA159 (black bar, set to 1). (*) $p \leq 0.05$.

Discussion

While the significance of acid production, acid tolerance and biofilm formation to the cariogenic potential of *S. mutans* has been well established and have been studied in

great detail [2], the role of bacterial oxygen metabolism and oxidative stress survival in caries have thus far received limited attention. Over the years, the primary notion that the dental plaque environment was virtually anaerobic has been replaced by evidence that, as a whole, the microbial plaque community has a high capacity to reduce oxygen thereby generating a variety of toxic byproducts. The relevance of oxidative stress survival to the pathophysiology of *S. mutans* has been further supported by *in vitro*, *in vivo* and clinical studies, showing an inverse relationship between the presence of H_2O_2-generating streptococci from the mitis group and *S. mutans* [5,41-43].

Spx proteins are conserved global transcriptional regulators of Gram-positive bacteria that function as positive regulators of oxidative stress genes [15] that, more recently, have been linked to the virulence potential of *E. faecalis, S. mutans* and other streptococcal species [19,22,23,44]. Previously, using microarrays, we showed that most genes with a proven role in the oxidative stress response of *S. mutans* were under SpxA1 positive regulation, and, to a lesser extent, SpxA2 [19]. Specifically, expression of genes involved in oxygen metabolism (*nox*), thiol stress (*trxA* and *trxB*) and ROS detoxification (*ahpC*, *ahpF*, *sodA* and *tpx*) among others was significantly lower in strains lacking *spxA1*, *spxA2* or both. Here, we sought to uncover the function of previously uncharacterized genes that, in the microarrays, displayed the same regulatory profile of the known oxidative stress genes, namely *smu127*, *smu143*, *smu144, smu247*, *smu929c*, *smu1296*, *smu1297*, and *smu1645*. In addition, we included one previously characterized oxidative stress gene, *smu540* (*dpr*), to serve as a phenotypic benchmark as well as to gain additional knowledge about the function of Dpr in *S. mutans*. An exception to the trend of positive regulation by Spx was the inclusion of the *smu569/570/571* transcriptional unit encoding the FeoABC iron transport system, which was by our

microarrays to be negatively regulated by SpxA1. Given the well-defined role of iron in hydroxyl radical formation as a catalyst of the Fenton reaction, it is therefore expected that negative regulation of iron uptake by Spx is an integral part of the oxidative stress response.

The transcriptional data obtained from our microarrays provided insight into the role of Spx proteins as regulators under basal conditions, when the cells were not subjected to oxidative stress. Here, quantitative real time PCR analysis of cells exposed to H_2O_2 further supported that the genes studied herein are regulated by Spx and encode bona fide oxidative stress proteins. Exposure to H_2O_2 resulted in increased expression of each of these genes (with the exception of, as expected, *feoB*), demonstrating a role for each of them in defending the cell against oxidative stresses. However, in the absence of SpxA1, exposure to oxidative stress did not trigger increased expression of these genes, demonstrating their regulatory dependence upon SpxA1. With the exception of *smu143c* (polypeptide deformylase), *smu144c*, (transcriptional regulator) and *smu929c*, which encodes a hypothetical membrane protein conserved among Gram-positive cocci, all other genes are predicted to encode proteins involved in oxidative stress responses in other bacteria. Among the latter group of genes, the aforementioned *dpr* and *feoB* are predicted to participate in oxidative stress defenses by binding to iron (Dpr) or by limiting iron uptake (FeoB). The FeoABC system can be found in both Gram-positive and Gram-negative bacteria and has been implicated in virulence of several Gram-negative species as well as *Streptococcus suis* [45,46]. With the exception of the increased resistance to streptonigrin, an antibiotic whose toxicity depends on the availability of intracellular free iron, the Δ*feo* strain phenocopied the parent strain including a nearly identical ability to colonize the teeth of rats fed a cariogenic diet. This

may be explained, at least in part, by iron being non-specifically transported by other metal-binding ABC transporters. The iron-binding Dpr has been extensively studied in bacteria and shown to mitigate H_2O_2 lethality by preventing the Fenton reaction [11,33]. In agreement with previous reports [11,14,47], the Δ*dpr* strain was hypersensitive to H_2O_2 and, based on indirect evidence, including growth in the presence of iron and tolerance to streptonigrin, cannot maintain iron homeostasis. Interestingly, growth of the Δ*dpr* strain was not affected by diamide, and only modestly inhibited by MV. However, the different behavior of the Δ*dpr* mutant in the presence of H_2O_2, diamide or MV is on par with a proteomic study in *S. aureus* that revealed a small overlap in the kinds of proteins produced when cells are exposed to these oxidants [48]. Diamide specifically oxidizes thiol groups, causing an increase in formation of intra- or extramolecular disulfide bonds leading to the accumulation of misfolded and aggregated proteins as a result of thiol oxidation. In this case, hydroxyl radical formation due to Fenton reaction should play an indirect role in cell viability. MV, also known as paraquat, generates O_2^- radicals in the cell that can directly mobilize Fe-S clusters or can be converted to H_2O_2. It is conceivable that the Δ*dpr* strain can partially overcome the detrimental effects of MV by protecting Fe-S cluster enzymes, or by activating multidrug efflux systems. In fact, an ABC transporter, dubbed VltA/VltB for viologen transporter, was shown to mediate resistance to MV as well as other quaternary ammonium compounds in *S. mutans* [49]. Most importantly, the Δ*dpr* mutant showed reduced ability to colonize the teeth of rats thereby providing, for the first time, direct evidence that Dpr is involved in the virulence of *S. mutans*.

The most dramatic phenotypes were observed in the Δ*suf* strain, in which the entire *suf* operon was inactivated with a polar kanamycin cassette. The Δ*suf* strain

displayed impaired growth under all conditions tested, increased sensitivity to iron and lower infectivity in rats. The SUF machinery is one of three Fe-S cluster assembly systems identified in bacteria but can also be found in plant chloroplasts [50]. Proteins containing Fe-S clusters as a prosthetic group are widely distributed in nature and perform essential biological processes including electron transfer, substrate binding/activation, transcriptional and translational regulation and iron storage [51]. ROS cause destabilization of Fe-S clusters affecting important cellular processes and stimulating the Fenton reaction due to an increase in intracellular free iron. Two Fe-S assembly systems are present in *E. coli*: the housekeeping ISC and the stress-inducible SUF, whereas only the SUF machinery is present in *S. mutans* and other members of the Firmicutes phylum [52]. Considering that the SUF system is the only pathway for assembly and repair of Fe-S clusters in *S. mutans*, it was not entirely unexpected that fitness and viability of the Δ*suf* strain was significantly impaired. Interestingly, while transcriptionally induced by H_2O_2 stress and dependent on the SpxA1 and SpxA2 regulators for optimal expression (see Fig. 1), our IVT assays indicate that neither SpxA1 nor SpxA2 directly induce transcription of the *suf* operon. The most logical interpretation of these results is that Spx exerts indirect control over *suf* gene expression perhaps by controlling the transcription of another regulator. Of note, the *suf* operon of *E. coli* is induced by oxidative stress and is under the control of three transcriptional regulators, IscR, Fur and OxyR [53,54].

Although strains lacking either *smu144c* or *smu143c* were able to grow as well as the parental strain under oxidative stress conditions, it is important to note that the *smu144c*/*143c* genes, which are likely co-transcribed, were: (i) induced by H_2O_2 treatment, (ii) directly regulated by SpxA1, and apparently (iii) unable to maintain iron

homeostasis. The *smu144c* genes encode a putative transcriptional regulator of the Crp/Fnr family conserved in many streptococcal species. Members of the Crp/Fnr family utilize Fe-S clusters to sense and respond to oxygen. The *smu43c* codes for a polypeptide deformylase (PDF) metalloenzyme, which removes the formyl group from the N-terminal methionine of newly synthesized proteins. The crystal structure of Smu143c has been solved (pdb3L87) and shown to coordinate binding to ferrous ion. Given that the PDF process is essential in bacteria but not in eukaryotes, PDF inhibitors are considered promising targets for the development of new antimicrobials [55].

In conclusion, we have shown that the Spx proteins of *S. mutans* control the expression of several additional genes involved in oxidative stress management. It appears that in addition to the transcriptional activation of oxygen metabolism, ROS scavengers and thiol repair enzymes, Spx performs an important role in iron homeostasis by regulating the intracellular availability of free iron. As part of a continued effort to assign a functional role for the genes characterized in this study, an expanded phenotype enhancement screen based on the double *spx* strain (Δ*spxA1*Δ*spxA2*) is currently under way.

Acknowledgments

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analysis, decision to publish, or preparation of the manuscript.

Supplemental Material
S1 Table. Real-time PCR primers.

Primer	Sequence	Application
5'143Arm1	5'-GCAGTAAGACCGCTAATATAGC-3'	*smu143c deletion*
3-143Arm1	5'-AATAGCAGGTACCTTTTTCTCC-3'	
5'143Arm2	5'-TTGATTGAAGCTTGGTACTTAGC-3'	
3'143Arm2	5'-CGTCAGTGACACGGC-3'	
5'144Arm1	5'-CCATAATGATTAGCCTGC-3'	*smu144c* deletion
3'144Arm1KpnI	5'-CCATTCTTTGGTACCAGCCACAAG-3'	
5'144Arm2KpnI	5'-CGCTGGCAAGGTACCGAATAAGGC-3'	
3'144Arm2	5'-CCCTAGTTCTGTCCTTTGT-3'	
5'248Arm1	5'-GTTTAGAGATTTGGGGAC-3'	*smu247/248 deletion*
3'248Arm1BamHI	5'-GCTCTGCCTGGATCCGTGAAAATG-3'	
5'248Arm2BamHI	5'-GTGACGAAAGGATCCCTGTTTTGG-3'	
3'248Arm2	5'-GGTCCATAGACTGTCAAC-3'	
5’540Arm1	5'-GCCAGCTAATGTCAGAAACAC-3'	*smu540 deletion*
3’540Arm1HindIII	5'-GCAGAATCAAGCTTTTTGACC-3'	
5’540Arm2HindIII	5'-CGTGGTTAAAGCTTGACCTTG-3'	
3’540Arm2	5'-CGCGATTAGTCACAATTTTATC-3'	
5'570Arm1	5'-CGTACTTCTTTTTGATGAG-3'	*smu569/570* deletion
3'570Arm1BamHI	5'-CGCTATTGGGGATCCCAATCAAAG-3'	
5'570Arm2BamHI	5'-CCACGTCAGGGATCCGAAAAAGTG-3'	
3'570Arm2	5'-CACCTTCTTTGACAAATTC-3'	
5'929Arm1	5'-CAGGTTCTTTTAGTGAAGCTGC-3'	*smu929c deletion*
3'929Arm1KpnI	5'-GCATTTTAATAATTGGTACCATTGCC-3'	
5'929Arm2HindIII	5'-CGCTGCAAAGCTTTATCTCTT-3'	
3'929Arm2	5'-ACAGAAGAACAGGCAGAGTC-3'	
5'1296Arm1	5'-GGTTCCGAAATAATCCACA-3'	*smu1296* deletion
3'1296Arm1KpnI	5'-GACCAAACTTTGGTACCAATAAGTAAG-3'	
5'1296Arm2KpnI	5'-GGCACAAAGGGTACCAGTTCTTAAC-3'	

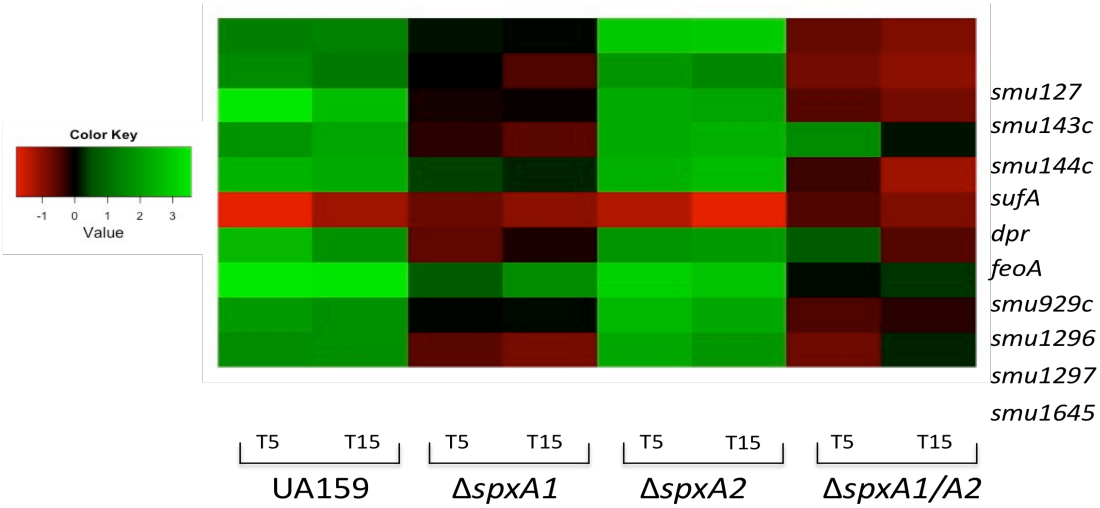
3'1296Arm2	5'-GGGAAAGAATCCATTTGACGT-3'	
5'1645Arm1	5'-GACCAGGACATTTGTATAG-3'	*smu1645* deletion
3'1645Arm1KpnI	5'-GCCAGCAGCATAGGTACCTTTCTGTTAG-3'	
5'1645Arm2KpnI	5'-CCTTGGATCGGTACCAGGACGTAA-3'	
3'1645Arm2	5'-GCTCTAAAATGCATCGTC-3'	

S2 Table. Primers used for gene inactivation.

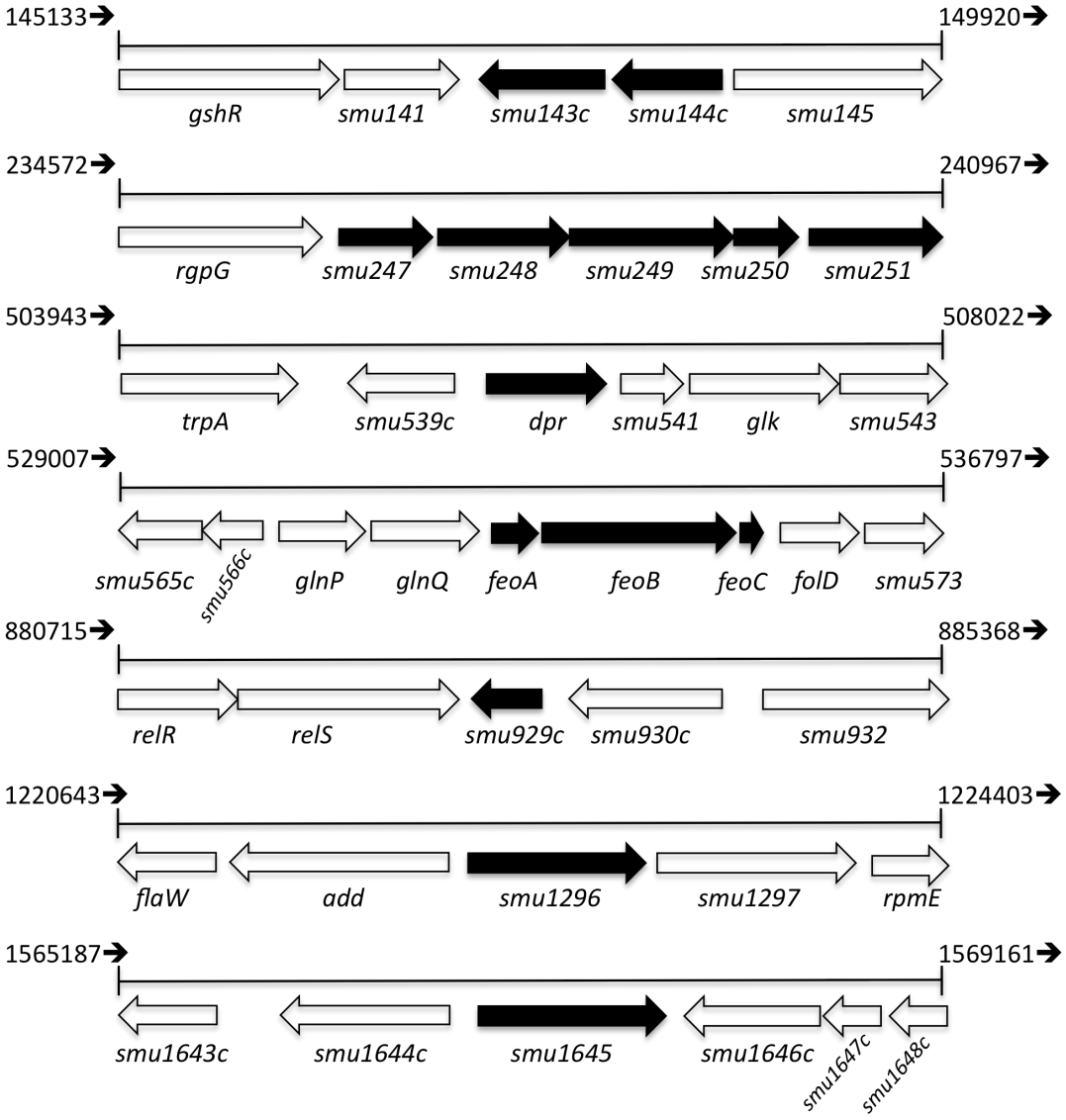
Gene ID	Primer	Product size (bp)	Sequence
smu127	5'127Arm1	150	5'-CGTGCTGATGCTTATGGTATTC-3'
	3'127Amr2		5'-TCCGAACCAACGATAAGATTCC-3'
smu143c	5'143Arm1	178	5'-TTGCTGTTCTCATTCCTAAT-3'
	3'143Arm2		5'-CGGATAACATAGCCTTCAA-3'
smu144c	5'144Arm1	168	5'-TTATTGGAGATATTGAACTGCTAA-3'
	3'144Arm2		5'-TGGTAGAGTGAATACAA-3'
smu248	5'248Arm1	193	5'-ACCAACTACGGCAATAAC-3'
	3'248Arm2		5'-CATCAATAAGAAGAATAGGATTAGC-3'
smu540	5'540Arm1	162	5'-GAAGAAACAGTTGGCACATGGG-3'
	3'540Arm2		5'-TTCCGTTTGAGCTGCTGTAAAG-3'
smu570	5'feoBArm1	131	5'-TTTAACAGGGACAAGTCAG-3'
	3'feoBArm2		5'-ATTATACCATTGCCGATA-3'
smu929c	5'929Arm1	123	5'-TCTACAAAGACATCTCAAGTT-3'
	3'929Arm2		5'-GCCATAAAGCAGACCTAA-3'
smu1296	5'1296Arm1	116	5'-ATTATACCATTGCCGATA-3'
	3'1296Arm2		5'-GTCTAATAAGTGTTGATAGG-3'
smu1297	5'1297Arm1	119	5'-GGGATTCTTGGTGATACAG-3'
	3'1297Arm2		5'-TCCATTTGACGTGCTAAG-3'
smu1645	5'1645Arm1	107	5'-TAGACCAGTTGTACCAGATA-3'
	3'1645Arm2		5'-TGGGACAAGGATAGGATT-3'

S3 Table. *In vitro* transcription primers.

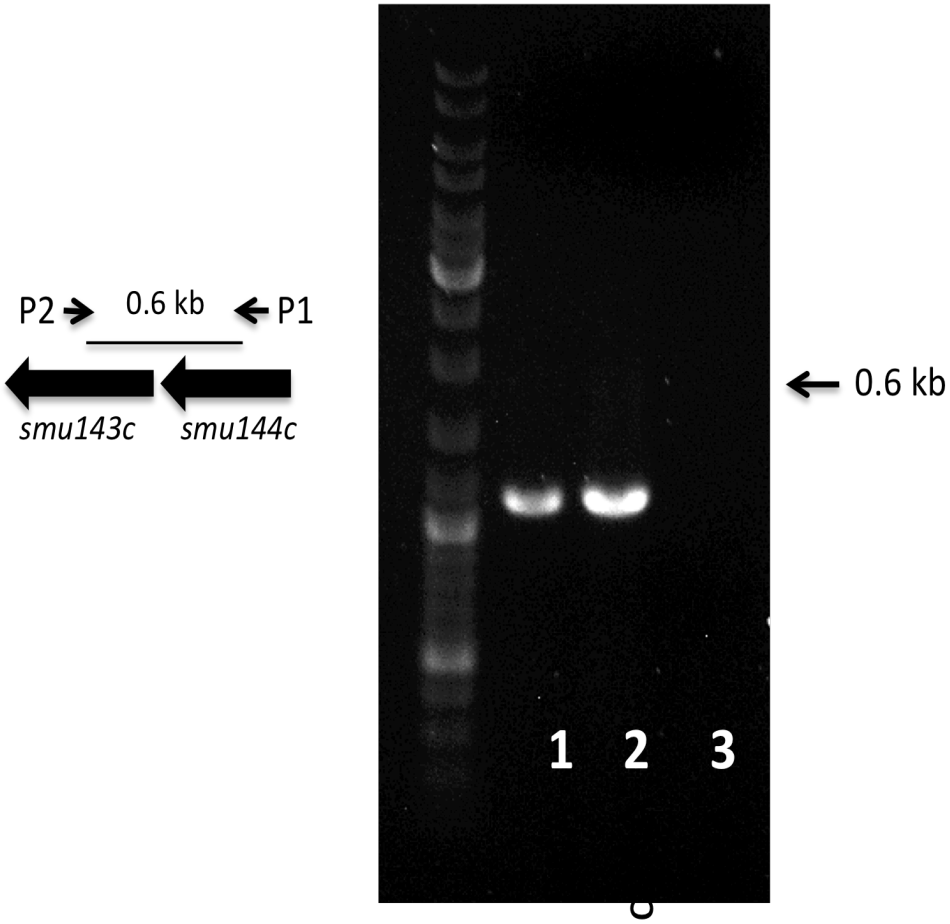
Gene ID	Transcript size (bp)	Primer name	Sequence
smu127	80	5'127Arm1IVT	GACTGTTATAGTGGGATTCTTATCTG
		3'127Arm2IVT	CGCTGCATTTTTAAAAACATATCC
smu144c	322	5'144Arm1IVT	CTCCTTAAACACAAAACTTG
		3'144Arm2IVT	CGGCAATTGGACAACCC
smu247	118	5'247Arm1IVT	CAAGAAGAAGTCCAATTG
		3'247Arm2IVT	GCATGGCAAGAAAAAGCC
smu540	70	5'540Arm1IVT	GCCAAGAGAAGCTCCCAAC
		3'540Arm2IVT	CTGAATTTTCTTTTTCTCCACTTGATG
smu569	184	5'569Arm1IVT	CGGTGAGTACACTCAGC
		3'569Arm2IVT	GAATCATCAAAAGCTAAACG
smu929c	144	5'929Arm1IVT	GGCAGAAAAAGTACAAAAATGTTCAC
		3'929Arm2IVT	GTGGCTAACGTTTCCAAATAC
smu1296	96	5'1296Arm1IVT	CACATTTCAGTTGATTTTCTC
		3'1296Arm2IVT	GTCAAAACGACTGCCAG
smu1645	72	5'1645Arm1IVT	CCACTCGTTTTGATTCATG
		3'1645Arm2IVT	GATGCTATTGGCAGTCC



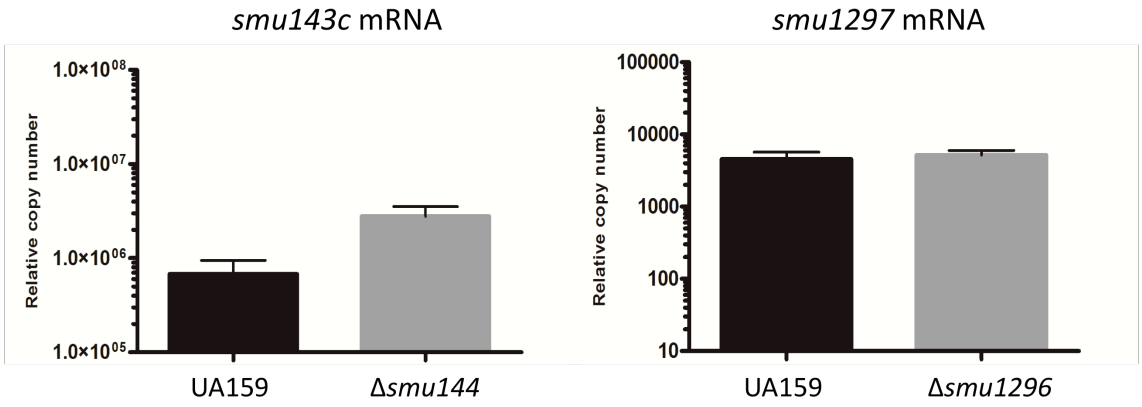
S1 Fig. Transcriptional shift profiles derived from RT-qPCR after 5 and 15 minute exposures to % H_2O_2, presented as the log base 2 average fold change from pre-treatment transcript copy numbers of at least three replicates. Heat map construction was performed in R (http://www.R-project.org/) version 2.1.0, using packages 'gplots' (2.14.1) and 'RColorBrewer' (1.0-5).



S2 Fig. Schematic representation of the *smu143c/144c, sufCDSUB, dpr, feoABC, smu929c, smu1296* and *tehB* loci in *S. mutans* UA159.

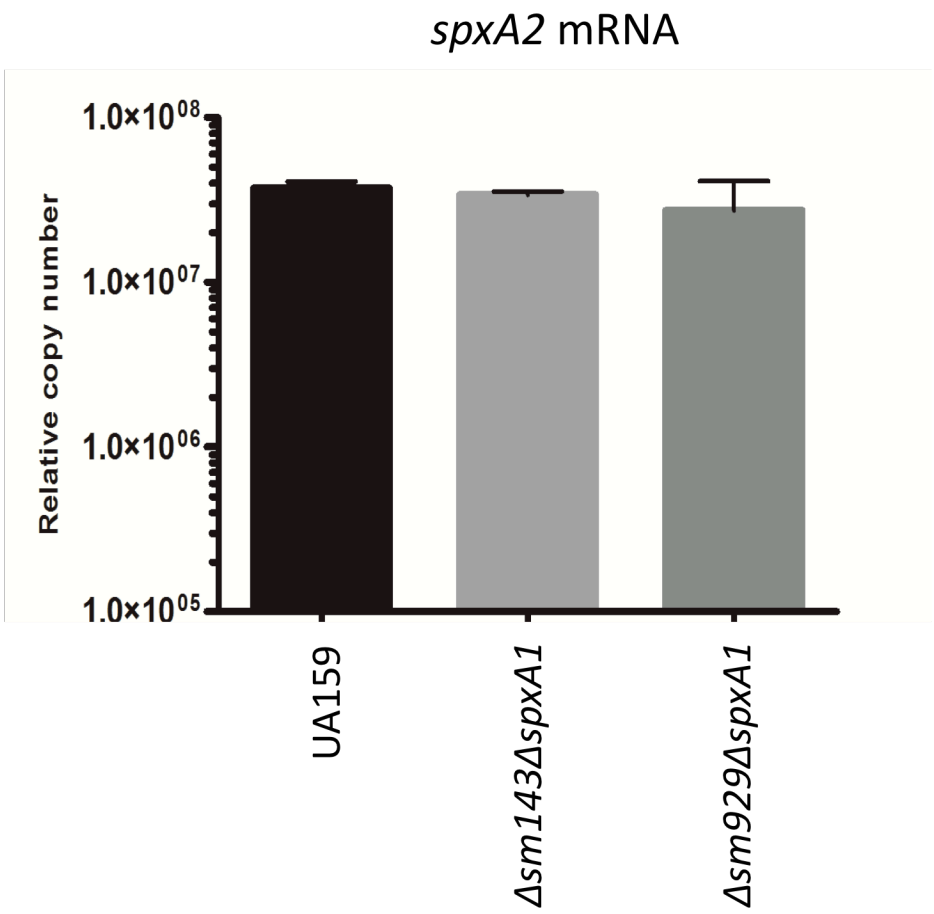


S3 Fig. RT-PCR analysis of the *smu144c-smu143c* transcriptional unit. Products from the PCR were derived from *S. mutans* UA159 chromosomal DNA (positive control, lane 1), cDNA obtained from total mRNA (lane 2) and a negative control using total mRNA but omitting RT (negative control, lane 3).



S4 Fig. RT-qPCR analysis of (A) *smu143c* gene expression in the Δ*smu144* strain and

(B) *smu1297* expression in the Δ*smu1296* strain. Total RNA was isolated from mid-exponential phase cultures grown in BHI at 37°C. Bars represent the relative copy number detected for each gene.



S5 Fig. RT-qPCR analysis of *spxA2* gene expression in the Δ*smu143*/Δ*spxA1* and Δ*smu929*/Δ*spxA1* strains. Total RNA was isolated from mid-exponential phase cultures grown in BHI at 37°C. Bars represent the relative copy number detected for each gene.

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2.3 ARTIGO 3: Characterization of a putative eep in *Steptococcus mutans*

Artigo em fase de submissão ao periódico MOLECULAR ORAL MICROBIOLOGY (MOM).

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SUMMARY

One consequence of bacterial exposure to environmental stresses is the accumulation of abnormal proteins due to increased errors in transcription and translation processes. Thereby the maintenance of proteic homeostasis is very important to keep viability of pathogens as *Streptococcus mutans* through proteins that perform a quality check in the cell and target abnormal proteins for degradation via proteolysis. The Regulated Intramembrane Proteolysis (RIP) is known to be widespread in organisms and happens when transmembrane proteins are cleaved within the plane of the membrane to release cytosolic fragments to start gene transcription. Two different proteins are involved in this process and site-2 proteases homologous are well conserved and found in mammals as well as in many bacterial species. In *Bacillus subtilis* and *Streptococcus uberis* it is called Eep (Enhanced expression of pheromone) and it also has been implicated in the processing of signal peptides of some lipoproteins. After a *S. mutans* BLAST search analysis it was indicated that the putative hypothetical protein encoded by the *smu1784c* gene, regulated by the global regulator Spx, shares high levels of similarity to *E. faecalis* Eep. Therefore, we performed an initial phenotypic characterization of *smu1784c* to further investigate it response to cell, membrane envelope, oxidants agents, its toxicity and ability to form biofilm, *in vitro* and to cause toxicicty *in vivo*. We showed that *smu1784c* is involved in oxidative and acid stress management and has an important role in the virulence of *S. mutants* once it was attenuated in the *Galleria mellonella* model and showed biofilm defects.

INTRODUCTION

Despite the implementation of measures to control and treat dental caries with fluoride, it remains the most prevalent dental disease in many countries (Kassebaun *et al*., 2015). Caries is a multifactorial infectious disease caused by accumulation of biofilm on tooth surface characterized by increased growth of cariogenic bacteria, such as *Streptocuccus mutans*, which is highly efficient to convert carbohydrates into organic acids able to demineralize the dental enamel (Lemos & Burne 2008).

Another key *S. mutans'* virulence factor is its ability to tolerate external stresses such as acid and oxidative environments (Quivey *et al*., 2000; Bowen *et al*., 2002; Lemos *et al*., 2005). One consequence of exposure to environmental stresses is the accumulation of abnormal proteins due to increased errors in transcription and translation processes (Lemos & Burne 2008). Thus, the maintenance of proteic homeostasis is very important to keep viability and growth of pathogens as *S. mutans*. This is possible through proteins that perform a quality check in the cell and target abnormal proteins for degradation via proteolysis (Butler *et al.,* 2006; Frees *et al.,* 2007).

Among proteolytic reactions that occur in order to control the levels of regulators or functional proteins, the Regulated Intramembrane Proteolysis (RIP) is known to be widespread in organisms. It happens when transmembrane proteins are cleaved within the plane of the membrane to release cytosolic fragments to start gene transcription (Browm *et al*., 2000). Usually RIP is involved in degradation of transmembrane proteins with the purpose of proteolytic activation, involving among other proteins, a Zn-metalloprotease which is very conserved in mammals and in a variety of organism including bacteria.

RIP mechanism involves an intramembrane protein called anti-σ factor whose function is to bind to the extracytoplasmic σ factor keeping it inactive in non-stressful

situations (Brown *et al*., 2000). The activation of transcription factor is governed by two, successive proteolytic events, known as site-1 and site-2 cleavage. The site-2 cleavage is the step involving Intramembrane proteolysis, generally occurring as a passive consequence of the first cleavage (Hastie *et al,* 2014). The proteins involved in site-1 proteolysis are not very conserved in different species, although site-2 proteases homologous are well conserved, belonging to the same family of membrane-embedded, Zn-metalloproteases (Schobel *et al.,* 2004; Kanehara *et al.,* 2003; Chen *et al.,* 2005). The site-2 homologous of S2P found in mammals are: YaeL (ResP) in *E. coli*; RasP in *B. subtilis;* Eep (Enhanced expression of pheromone) in *Enterococcus feacalis*; and they were also found in insects and invertebrates (Rawson, 2013).

Dispite Eep-like protein have been enrolled in many bacterial species with a known function to release ECF σ factor, the *S. mutans* genome contains only two sigma factors, σ^{70} and σ^{X}, to coordinate their gene expression in response to environmental stresses (Gong *et al.,* 2009), indicating that the presence of eep-like proteins in *S. mutans* could be related with another biological function.

Eep in *E. faecalis* and *S. uberis* has been implicated also in the processing of signal peptides of some lipoproteins (Deham *et al.,* 2008; Juncker *et al.*, 2003) which consist of a large group of proteins with many different functions (Hayashi & Wu, 1990; Juncker *et al.,* 2003). In *E. faecalis* case it has been related with cleavage of signal peptides of lipoproteins to yield octapeptides that act as bacterial pheromones, inducing conjugation between different strains (Dehan *et al.,* 2008)

In *S. mutans*, BLAST search analysis indicated that the putative hypothetical protein encoded by *smu1784c* gene is well conserved among Gram-positive bacteria and it also shares high levels of similarity with *E. faecalis* Eep. Also, *smu1784c* is regulated

by Spx (Kajfasz *et al.,* 2010), a transcriptional regulator which controls expression of known genes against oxidative stress, and several additional genes involved in oxidative stress management. In addition to the transcriptional activation of oxygen metabolism, ROS scavengers and thiol repair enzymes, Spx performs an important role in iron homeostasis by regulating the intracellular availability of free iron (Galvão *et al.,* 2015).

Here, we reseoned to search evidences that justify control of *smu1784c* by Spx and characterize this putative Eep-like protein in *S. mutans*, performing an initial phenotypic characterization of this gene to further investigate response to cell, membrane envelope and oxidants agents once Eep in *E. feacalis* is responsible for lysozyme resistance (Varahan *et al.,* 2013).

METHODS

Bacterial strains and stress growth conditions

The bacterial strains used in this study are listed in Table 1. *S. mutans* UA159 and its derivatives were routinely grown in brain heart infusion (BHI) or trypticase soy agar (TSA) at 37°C in a 5% CO_2 atmosphere or under anaerobic conditions (BBL Gaspack system, BD). Where appropriate, kanamycin (1 mg ml^{-1}) or erythromycin (10 μg ml^{-1}) were added to the growth medium. To generate growth curves, strains were grown overnight under anaerobic conditions and diluted 20-fold in pH 7.0-buffered BHI containing H_2O_2 (0.2 mM), methyl viologen (MV, 5 mM), diamide (0.5 mM). Cultures were incubated at 37 °C in a 5 % CO_2 atmosphere and the OD_{600} recorded at selected intervals.

Table 1. Bacterial strains used in this study.

Strains	Relevant genotype	Source of reference
UA159	Wild type	Laboratory stock
Δsmu1784c	*Smu1784c*::Km^R	This study
Δsmu1784c complement strain (*cΔsmu1784c*)	*Δsmu1784c* with pMSP3535 harboring *smu1784c*	This study
UA159/pMSP3535	UA159 with pMSP3535 without insert	This study
Δsmu1784c/pMSP3535	*Δsmu1784c with pMSP3535 without insert*	This study

Construction of knockout strains and complementation strain.

S. mutans UA159 strains had the gene *smu1784c* disrupted by insertion of a nonpolar kanamycin marker (Kramer *et al.,* 2001) 1000 bp downstream of the ATG start codon, using a PCR-ligation mutagenesis strategy (Lau *et al.,* 2002). Briefly, the 1000-bp 5' of *smu1784c* was amplified from strain UA159 by use of primers smu1784c-F (5'-CTTACCTTTACCGTGATC-3') and smu1784cKpnI-R (5'-CTATTAGTCGGTACCTTCTTTCCTC-3'), and the 1000-bp 3' of the gene was amplified using primers smu1784cKpnI-F (5'-CGCTGGTGTGGTACCTATGGTTGC-3') and smu1784c-R (5'-CTATTTCTAAAAAGGCAGCA-3'). After amplification, the two PCR fragments were digested with KpnI and ligated to a nonpolar kanamycin resistance gene cassette that was obtained as a KpnI fragment. The ligation mixture was used to transform *S. mutans* UA159, followed by plating onto BHI containing kanamycin (1 mg ml^{-1}). The insertional inactivation of *smu1784c* was confirmed by PCR sequencing.

To express the *smu1784c* gene *in trans*, the full-length *smu1784c* gene, including the ribosomal binding site, was amplified by PCR with primers containing BamHI (5'-GGAGGAGGGATCCATGTCAGGAC-3') and XbaI (5'-

CTCCGTATCTCTAGATTAGAAAAATG-3') restriction sites and ligated into pMSP3535 which had been digested with BamHI and XbaI. A ligation mixture containing pMSP3535 expressing *smu1784c* (p1784) was used to directly transform the *S. mutans smu1784c* knockout strain UA159 to generate a complementation strain carrying the gene. Expression of *smu1784c* from p1784 was induced with 10 μg ml^{-1} of erythromycin as described elsewhere (Abranches *et al.,* 2011) Also, *S. mutans smu1784c* knockout strain and UA159 strains were transformed with pMSP3535 without insert just to acquire the ERM resistance cassette and serve as negative controls in the experiments described below.

Acid and H_2O_2 killings

For killing experiments, exponentially grown cultures (OD_{600} = 0.5) were washed once with one culture volume of 0.1 M glycine buffer (pH 7) and resuspended in one-fifth of the original volume in 0.1 M glycine buffer (pH 2.85) or for the hydrogen peroxide killing assays, washed cells were resuspended in 0.1 M glycine buffer, pH 7.0, and hydrogen peroxide was added at a final concentration of 0.5% for up to 60 min. Every 30 min, aliquots were serially diluted, plated on BHI agar, and incubated for 48 h before colonies were counted (Wen & Burne, 2004).

***Galleria mellonella* infection**

For the *G. mellonella* killing assays, insects in the final instar larval stage were purchased from Vanderhorst, Inc. (St. Marys, OH), stored at 4 °C in the dark, and used within 7 days of shipment. Groups of 20 larvae, ranging from 200 to 300 mg in weight and with no signs of melanization, were randomly chosen and used for subsequent infection. A 10 μl Hamilton® syringe was used to inject 5-μl aliquots of bacterial inoculum into the hemocoel of each larva via the last left proleg. Bacterial colony counts

on BHI plates were used to confirm initial inoculum. Groups injected with saline solution or with heat-inactivated (10 min at 75 °C) *S. mutans* UA159 were used as controls in each experiment. After injection, larvae were incubated at 37 °C, and appearance (signs of melanization) and survival were recorded at selected intervals. Larvae were scored as dead when they displayed no movement in response to touch. Kaplan-Meier killing curves were plotted, and estimations of differences in survival were compared using a Log-rank test. A p value of < 0.05 was considered significant. All data were analyzed with GraphPad Prism, version 4.0, software. Experiments were repeated three independent times with similar results (Kajfasz *et al.,* 2010).

Minimum Inhibitory Concentrations (MIC)

To determine the MIC for drugs that target the cell envelope (bacitracin, lysozyme, chlorhexidine, triclosan, ampicilin, vancomycin, daptomycin and cefalexyn), cultures of *S. mutans* UA159 and its derivatives were grown in BHI broth to an OD_{600} = 0.3 and a two-fold serial dilution was used to inoculate antibiotic containing media. Plates were incubated overnight and an automated reader (BioRad, Benchmark Plus Microplate Spectrophotometer or Asys, UVM340 biochrom Microplate Spectrophotometer) was used to measure culture turbidity at OD_{600} nm. The MIC was determined as lowest antibiotic concentration that inhibited growth for each strain. Significance was established as a P value ≤ 0.05. GraphPad Prism, version 4.0 software was used for all data analysis. Experiments were conducted at least in triplicates (Ramos, 2015).

Biofilm formation assay

To assess the effect of *S. mutans* UA159 and its derivates on biofilm formation, 115 hours-old biofilms were formed. During the 5 days of biofilm formation, the culture medium was changed every day (Cunha *et al.,* 2013). At the end of the experimental

period (115 h) for biochemical collection data, the biofilms were removed and were subjected to ultrasound bath, sonication (30 s pulse; output 7 W), to provide the maximum recoverable viable counts. The homogenized suspension was analyzed for biomass (dry weight), bacterial viability (colony forming units CFU/mL), polysaccharide, and protein content. The extracellular water soluble polysaccharides (WSP), alkali soluble polysaccharides (IP), and intracellular iodophilic polysaccharides (IPS) were extracted and quantified by colorimetric assays as detailed by Koo *et al.* (2003) and Duarte *et al.* (2008); the exopolysaccharides were quantified by the phenol sulfuric method using glucose as standard (Dubois *et al.,* 1956), whereas IPS was quantified using 0.2% I2/2%KI solution and glycogen as standard, as described by DiPersio *et al.* (1974). The total protein was determined by colorimetric assays as detailed by Smith *et al.* (1985).

Statistical analysis

One-way analysis of variance (ANOVA) in the Tukey-Kramer Honest Standard Deviation (HSD) was used to verify the significance of biofilm analysis and growth curves. Student' s t tests were performed to assess the distributions of Killings. In all cases, P-values ≤ 0.05 were considered significant.

RESULTS

Growth characteristics of strains

Since it has been established that Spx has a role in transcriptional control of genes involved in oxidative stress management (Nakano *et al.,* 2003), and Δ*smu1784c* is under its control (Kajfasz et al., 2010), we examined whether the absence of Δ*smu1784c* gene had an effect in the growth of mutant or wild type strains in presence of oxidative stress agents.

Cultures of UA159 and Δ*smu1784c* were grown in BHI medium containing 0.2 mM H_2O_2 or 0.25 mM diamide, a thiol specific oxidative stress agent that oxidizes cysteines, resulting in the erroneous formation of disulfide bonds. In presence of 2.5 mM metyl viologen, a quaternary ammonium compound that generates superoxide radicals, and in presence of BHI with pH adjusted to 7.0 and 6.0. Δ*smu1784c* strain showed a slow-growth pattern as compared to the parental strain in all conditions tested (Table 2). However, the complemented strain, cΔ*smu1784c,* behaved similarly to the parental strain harboring the empty *pMSP3535* UA159/pMSP3535 when grown in BHI broth (Table 3).

Table 2. Doubling time of parental strain and Δ*smu1784c* under different conditions.

	STRAINS	
CONDITIONS	UA159	Δ*smu1784c*
BHI	58.71 ± 3	82.17 ± 0.2***
BHI pH 6	100.7 ± 10.5	157.9 ± 6.7*
H2O2 0.2 mM	66.57 ± 2.3	73.33 ± 1.1*
Diamide 0.25 mM	67.47 ± 5.5	79.67 ± 5*
Metyl viologen 2.5 mM	62.10 ±12.1	79.2 ± 7*

* indicate statistically significant differences. ANOVA, using t-test without any transformation. * $p \leq 0.05$, *** $p \leq 0.0005$.

Table 3. Doubling time of *S. mutans* UA159/pMSP3535, Δ*smu1784c/*pMSP3535 and cΔ*smu1784c.*

	STRAINS		
CONDITIONS	UA159/pMSP3535	Δ*smu1784c/*pMSP3535	cΔ*smu1784c*
BHI	50.33 (±3.5) a	64.67 (±1.15) b	56.67 (±1.52) c

Different letters indicate statistically significant differences. $p \leq 0.05$.

Also, when observed under the microscope during all stages of growth (early-log, mid-log, late-log and stationary) Δ*smu1784c* formed longer chains than the parent UA159 strain upon entry into exponential and stationary growth phases (Figure 1). Interestingly, the altered chain length was also reverted in the complemented strain as observed in figure 2.

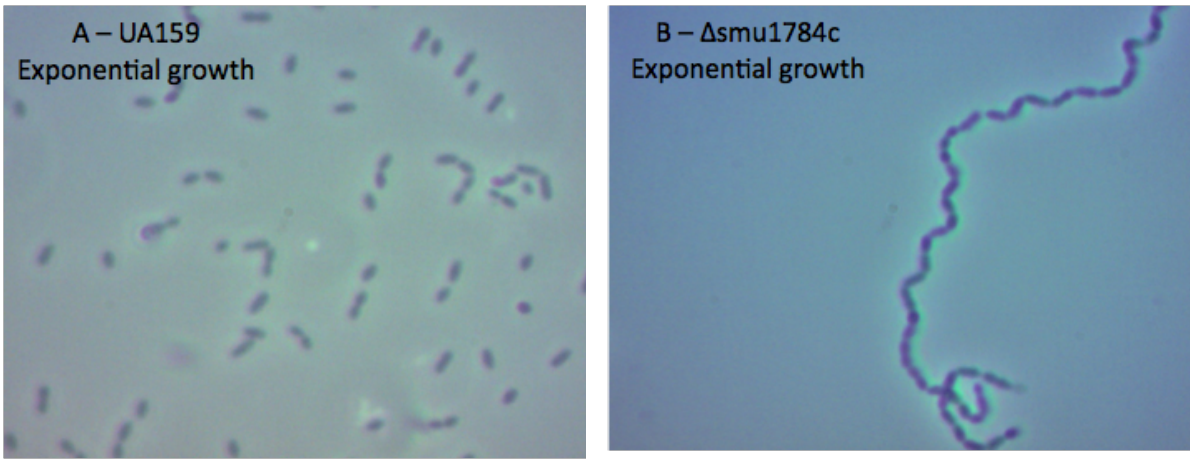

Figure 1. Phase-contrast microphotographs of *S. mutans* UA159 and Δ*smu1784c*.

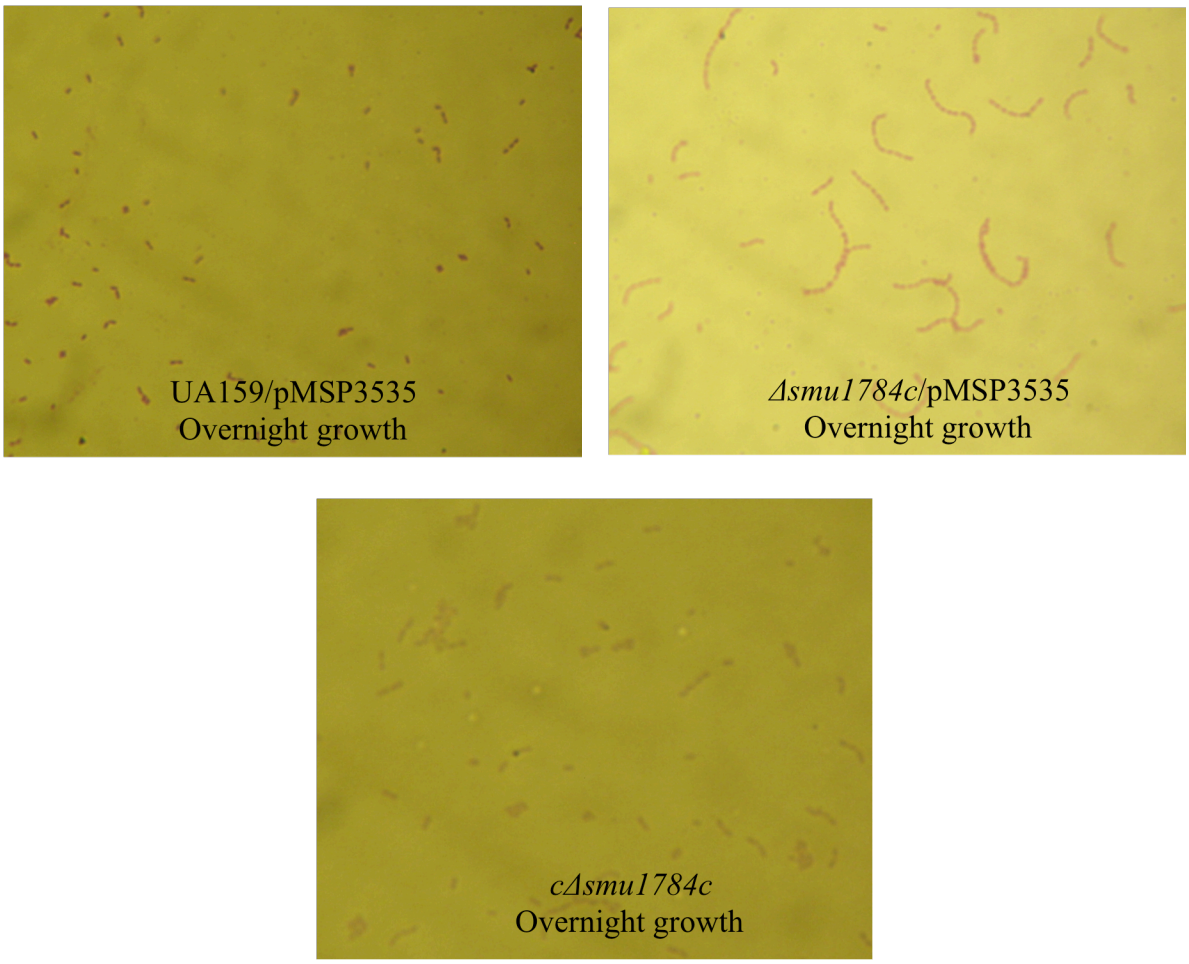

Figure 2. Microphotographs of Gram stained *S. mutans* UA159/pMSP3535, Δ*smu1784c*/pMSP3535 and cΔ*smu1784c*.

Eep-like protein is involved in sensitivity to acid and H_2O_2

The impact of *Δsmu1784c* deficiency on the ability of *S. mutans* to withstand acid and hydrogen peroxide stress was determined by using acid killing and hydrogen peroxide challenge assays as described elsewhere (Wen & Burne, 2004). The percent survival after 30 minutes of acid exposure was lower for *Δsmu1784c* when compared with percent survival of wild type and after exposure to H_2O_2 it was higher, but these differences were not statistically significants at 30 min (Figure 3). Although after 60 min

of acid and H_2O_2 exposure, the percent survival was approximately 2 and 1.5 logs lower for *Δsmu1784c* respectively, when compared to the percent survival of parental strain.

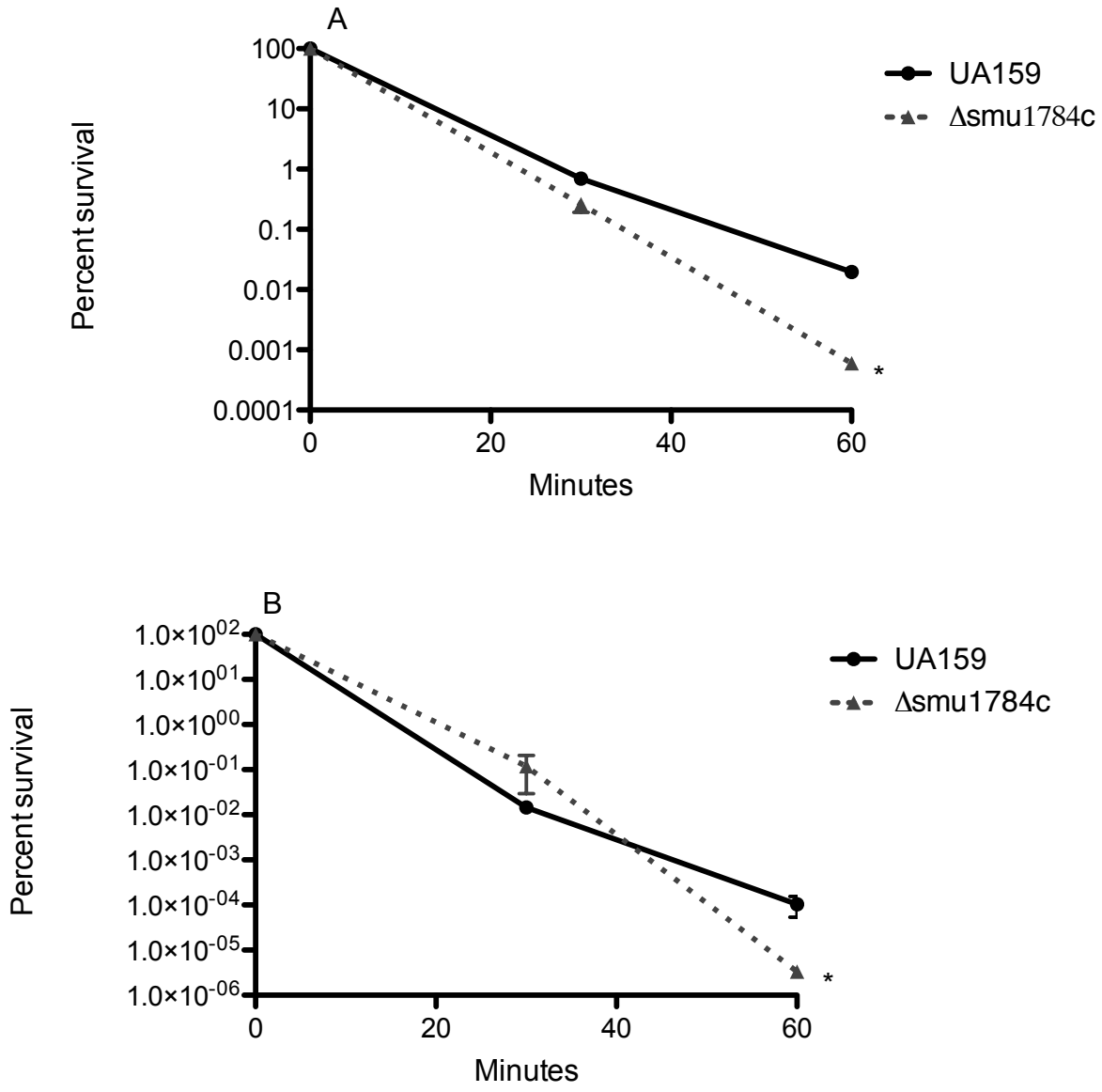

Figure 3. Survival percent to (A) Acid and (B) H_2O_2 killing of *S. mutans* strains bearing mutation in the *smu1784c* gene. The curves shown are averages with standard deviations of the results from three independent experiments. $p \leq 0.05$ (t-test).

Δ*smu1784c* strain is less virulent than the parental strains.

We tested the ability of *smu1784c* mutant strain to kill larvae of *G. mellonella*. The virulence of the *smu1784c* knockout strain in the UA159 background was attenuated and this difference was statistically significant (Figure 4).

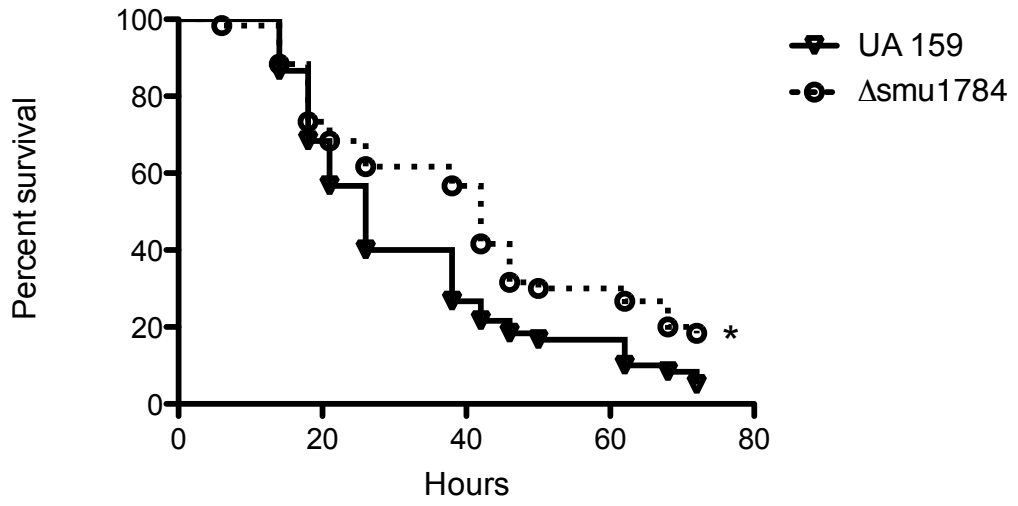

Figure 4. Survival of *G. mellonella* larvae infected with UA159 and Δ*smu1784c*. The experiments were repeated three times, and the results are representative of a typical experiment. $P \leq 0.05$, Log-rank test.

Δ*smu1784c* does not show sensitivity to cell envelope agents

In *E. feacalis,* Eep is associated with cell envelope homeostasis as it confers resistance to lysozyme, a cell envelope agent. Thus, here we tested the ability of *Δsmu1784c* strain to withstand with cell envelope stressors. We determined MIC for bacitracin, lysozyme, chlorhexidine, triclosan, daptomycin, ampicylin and vancomycin. Despite the indication that *Δsmu1784c* is involved in cell division, cell envelope, or fatty acid metabolism, the mutant strain did not show significant alterations in relation to the different cell envelope-targeting agents tested in this study (Table 4).

Table 4. Cultures of *S. mutants* UA159/pMSP3535, *Δsmu1784c*/pMSM3535 and c*Δsmu1784c* were tested against cell wall agents in order to find the lowest concentration able to inhibit bacterial growth.

Strains	**Bacitracin** (μg/mL)	**Triclosan** (μg/mL)	**Ampicylin** (ng/mL)	**Vancomycin** (μg/mL)	**Lysozyme** (mg/mL)	**Cefalexyn** (μg/mL)
UA159/pMSP3535	62,5	15.62	37.5	0.48	12.5	0.31
*Δ*smu1784c/ pMSM3535	62,5	15.62	37.5	0.48	12.5	0.31
c*Δsmu1784c*	62,5	15.62	37.5	0.48	12.5	0.31

The *Δsmu1784c* biofilm biomass was reduced

Here it was investigated the ability of these mutants to form biofilm, using hydroxyapatite discs. Table 5. shows the influence of *Δsmu1784c* in biofilm formation on saliva-coated hydroxyapatite surface. The number of viable cells recovered from biofilm formed by *Δsmu1784c* was similar to that formed by UA159 ($p > 0.05$). However, the formation and accumulation of biomass by *Δsmu1784c* was significantly reduced ($p \leq 0.05$) when compared to that of UA159. The biofilms formed by *Δsmu1784c* exhibited approximately 30% less alkali soluble polysaccharide than those formed by UA159 ($p \leq 0.05$). Even showing a great reduction for the water soluble polysaccharide (WSP) and intracellular polysaccharide (IPS), only the other glucan and protein analyzed in this study did differ statistically from biofilms formed by *Δsmu1784c* compared to those formed by UA159 ($p \leq 0.05$).

Table 5. Effect of UA159 and its derivate on the biochemical composition and viability of biofilm of *S. mutans*.

GROUPS	Mean (SD)					
	DW (mg)	**ASP (ug)**	**IPS (ug)**	**WSP (ug)**	**BV (logCFU/mL)**	**Total Protein (ug)**
UA159	6.029 a (±0.86)	1269 a (±346.3)	2078 a (±786.2)	112.9 a (±29.14)	8.018 a (±0.1)	73.34 a (±12.29)
Δsmu1784c	3.766 b (±0.63)	905.9 b (±393.6)	1243 a (±457.5)	94.1 a (±41.69)	7.942 a (±0.17)	53.69 b (±5.84)

Different letters in the same columm indicate statistically significant differences. ($p \leq 0.05$). DW: dry weight; ASP: alkali-soluble polysaccharide; IPS: intracellular iodophilic polysaccharide;WSP: water soluble polysaccharide; BV: bacterial viability. ANOVA One-Way, $p \leq 0.05$.

DISCUSSION

As dental caries is the most prevalent chronic disease worldwide (Kassebaun *et al.,* 2015), it is interesting to investigate how this microorganism is able to cope with many kind of stress sources present in the oral cavity (Koo *et al.,* 2013; Galvão *et al.,* 2015).

Ageing cells present in mature biofilms and exposure to environmental stresses result in accumulation of abnormal proteins. In this context, molecular chaperones, proteases and RIP, mechanism of signal trasduction, are central to physiological homeostasis, by modulating the stability of proteins, by controlling gene transcription and by preventing accumulation of misfolded proteins (Lemos and Burne, 2008; Brown *et al.,* 2000).

The Site-2 proteases (S2Ps) form a large family of membrane-embedded metalloproteases that participate in cellular signaling pathways through sequential

cleavage of membrane-adhered substrates. These proteases have also been described and are well characterize in *E. feacalis*, *E. coli* and *B. subtilis* (Kinch *et al.,* 2005). In order to identify homologs proteins, Kinch *et al*., (2005) used conserved S2P sequence elements to do an exhaustive PSI-BLAST search, and as a result they found it present in all major phylogenetic lineages.

In *S. mutans, smu1784c* gene was found as having a conserved domain DPZ, also present in all site-2 proteases, related with organization of signaling complexes at the level of cellular membranes. Proteins containing PDZ domains play a key role in anchoring receptor proteins in the membrane (Ponting, 1997; Kennedy, 1995).

Also using a BLAST tool it was possible to verify that *S. mutans smu1784c* gene shares similarity with *E. feacalis* gene responsible for encoding Eep protein, a site-2 protease related with activation of extracytoplasmic sigma-factor (Varahan *et al.,* 2013; Frank *et al.,* 2013). Besides, in *E. faecalis* and *S. uberis* Eep it has been implicated in the processing of signal peptides of some lipoproteins to yield active proteins with different functions (Deham *et al*., 2008; Juncker *et al.,* 2003).

Another relevant information is that *smu1784c* gene is regulated by Spx, a global regulator ubiquitously found in low GC Gram-positive bacteria that is involved in stress survival, principally by serving as a transcriptional activator of genes involved in thiol homeostasis and detoxification (Kajfasz *et al.,* 2010). In addition, Spx performs an important role in iron homeostasis by regulating the intracellular availability of free iron (Galvão *et al.,* 2015).

Therefore, is important to seek for indications that *smu1784c* is indeed involved in regulated intramembrane proteolysis, cleavage of signal peptides of lipoproteins and/or

management of oxidative stress by *S. mutants* UA159. Consequently, we performed an initial phenotypical characterization *Δsmu1784c* mutant strain.

As the oral cavity is colonized by hundreds of different bacterial species, there is intense competition among the bacteria for colonization and survival (Aas *et al.,* 2005). Other species of oral streptococci such as the primary colonizers *Streptococcus gordonii* and *Streptococcus sanguinis* produce, as part of their metabolism, large quantities of H_2O_2 (Kreth *et al.,* 2008), for this reason and because Spx regulates *Δsmu1784c*, here we investigated the growth characteristics of the mutant strains under different conditions.

Δsmu1784c mutant strain was more sensitive to acid and oxidative stressors as demonstrated by growth curves and by killings at low pH or using H_2O_2 at higher concentrations. Also, the *Δsmu1784c* showed the tendency to form long chains. Similar phenotypes were observed by *S. mutans* mutant strains of BrpA, biofilm regulatory protein A, once its deletion resulted in strains with decreased acid and oxidative stress tolerance, presenting defects in biofilm formation and alterations in lenght of chains and in morphology of its colonies. These similarities suggest that *Δsmu1784c* gene is related with regulation of cell division, stress tolerance response and cell envelope biogenesis/homeostasis as well *brpA* is (Bitoun *et al.,* 2014).

However, unexpectedly, this strain was not sensitive to any of the cell envelope agents tested in this study suggesting that *Δsmu1784c* is a paralog of *E. faecalis* Eep (Varahan *et al.,* 2013). On the other hand, *E. faecalis eep* mutant makes biofilms with an aberrant distribution of cells and matrix, resulting in attenuated phenotypes in the rabbit endocarditis model and impaired ability to colonize kidney using a mouse model of catheter-associated urinary tract infection (Frank *et al., 2012; Frank et al.,* 2013).

Since *Δsmu1784c* mutant strain was attenuated in the *G. mellonella* waxworm model it appears that this gene has an important role in the virulence of *S. mutans*. This is supported by Kajfasz *et al*., (2010) whom demonstrated the usefulness of systemic infection of *G. mellonella* as an adjunct model to study the virulence of *S. mutans*. *G. mellonella* is used as a model of systemic bacterial infection as insects posses a complex, multicomponent innate immune system able to kills pathogens by production of lysozymes, reactive oxygen species and antimicrobial peptides (Kavanagh & Reeves, 2004).

Another important *S. mutans*' virulence factor is its ability to form biofilm. The role of biofilms in the pathogenesis of some chronic human infections, such as tooth decay, is now very accepted worldwide (Parsek & Signhn, 2003), and because caries disease is a chronic disease biofilm-associated, here we sought to deepen our understanding on the contribution of *smu1784* to the ability to form biofilms using saliva-coated HA discs and compare the data obtained with those from biofilms formed by UA159.

We did not observed a decrease in the number of viable cells in the biofilms formed by the mutant strain, comparing with biofilm formed by UA159 but important differences were noticeable, as reduction of biomass and reduction of the alkali soluble polysaccharides and proteins amounts. Reduction in the amounts of polysaccharides and protein leads to a structural shift in the matrix, as these biochemical compounds are responsible for a three dimensional conformation of the biofilm (Koo *et al*., 2010; da Cunha *et al*., 2013), which makes it a more vulnerable environment to environmental stressors.

The reduction in biomass verified in biofilms formed by *Δsmu1784c* can be justified once there was a reduction in the amount of polysaccharides, which are produced by exoenzymes from *S. mutans* and are the main constituents in the matrix of cariogenic plaque-biofilms recognized as essential virulence factors associated with dental caries (Bowen & Koo, 2011).

In conclusion we showed that *smu1784c,* regulated by Spx, is involved in oxidative and acid stress management. In addition, we showed that *smu1784c* may play an important role in the virulence of *S. mutants* once it was attenuated in the *G. mellonella* model and presented biofilm defects, which could make the bacterium more susceptible to the host's immune defense.

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3 DISCUSSÃO

A cárie é uma doença infecciosa, multifatorial, biofilme dependente, causada pelo acúmulo de biofilme bacteriano na superfície dental capaz de metabolizar carboidratos ingeridos na dieta, levando a formação de ácidos que culminam com mudanças no ambiente da cavidade oral e com o aparecimento da doença cárie (Marsh, 2003; Lemos e Burne, 2008; Galvão *et al.,* 2012).

Essas alterações que ocorrem na cavidade oral, que é um ambiente dinâmico, são rápidas, mas muitas vezes substanciais e podem alterar rapidamente o pH; a disponibilidade de nutrientes, carboidratos e oxigênio (Lemos *et al*., 2005).

Por isso o *S. mutans*, principal agente etiológico da cárie dental, é desafiado a sobreviver e emergir como flora dominate em um meio onde o estresse ambiental é presente e predominante, fazendo com que se busque uma melhor compreensão dos mecanismos envolvidos nesta adaptação, a qual permitiria uma maior elucidação da patogênese da doença cárie (Lemos & Burne, 2008). Além disso, o entendimento dos mecanismos envolvidos neste processo poderia facilitar o desenvolvimento de novos agentes terapêuticos úteis na prevenção de doenças *S. mutans* dependente, como é o caso da cárie dental (Kajfasz *et al*., 2010).

A virulência de *S. mutans* se dá pelo fato desse microrganismo ser capaz de 1) se aderir à superfície do dente formando o biofilme, 2) de produzir ácidos capazes de desmineralizar o esmalte dental e 3) de sobreviver em meio ácido (Lemos *et al*., 2005). Além disso, *S. mutans* é capaz de produzir peptídeos bacterianos, chamados de mutacinas, que o tornam capazes de competir com bactérias que estão em íntimo contato no biofilme dental (Gronroos *et al*., 1998).

Em *S. mutans*, a produção de mutacinas e o processo de competência são interligados, ambos são coordenados por um mecanismo quorum-sensing de comunicação intercelular e envolvem um peptídio de estimulação de competência (CSP, em inglês, Competence Stimulating Peptide) (van der Ploeg, 2005; Dufuir *et al*., 2011; Reck *et al*., 2015).

No processo de competência, os genes que constituem este sistema de comunicação intercelular envolvem a produção e translocação de pequenos peptídeos, que ao atingir concentrações ótimas no meio extracelular, o que é obtida com o aumento

da densidade celular, ativam histidina-quinases da superfície bacteriana, transmitindo um sinal intracelular que ativa a expressão de diversos genes, não somente os envolvidos na captação de DNA extracelular mas também aqueles envolvidos na capacidade das espécies de responderem aos estímulos como pressão osmótica, diminuição de pH e outras condições de estresse (Shapiro, 1998; de Kievit & lglewski, 2000).

Diante da importância do controle de microrganismos como *S. mutans*, principal causador da doença cárie, e do fato de que este microrganismo consegue emergir como dominante em situações de estresse ácido e oxidativo, deve-se entender o papel do metabolismo bacteriano de oxigênio e como o *S. mutans* lida com situações de estresse oxidativo, já que este assunto ainda não é tao explorado quanto o estresse ácido o é (Galvão *et al.,* 2015).

Sabe-se que em *S. mutans*, as proteínas Spx, reguladores globais de transcrição em bactérias Gram-positivas, funcionam como reguladores positivos de genes relacionados ao estresse oxidativo. Dessa forma, analisou-se fenotipicamente alguns genes hipotéticos sob controle de Spx que mostraram-se relacionados com o estresse oxidativo em *S. mutans* uma vez que suas mutantes mostraram-se atenuadas em situações de estresse ácido e oxidativo em comparação com a cepa UA159. Além disso, as cepas mutantes dos genes estudados nesse trabalho e regulados por Spx também mostraram-se incapazes de manter a homeostase de ferro intracelular, tornando-se mais sensíveis (Galvão *et al*., 2015) (Capítulo 2).

Para confirmar o papel de Spx na patogenicidade da doença cárie e virulência do *S. mutans*, foi testada a competência (eficiência de transformação) de cepas mutantes de Spx, a capacidade de produzir mutacinas tipo IV e V e foi conduzido um experimento de cárie em ratas, onde cepas mutantes de *S. mutans* com deleções dos genes *spxA1* e *spxA2* foram inoculadas na cavidade oral de ratas por três dias e ao final de 5 semanas a quantidade de bactérias mutantes, selvagem e totais foram recuperadas e o score de cárie foi registrado (Capítulo 1).

Assim, percebeu-se que mutações nos genes de Spx resultaram em deficiência de competência quando comparadas capacidade de tranformação da cepa selvagem (capítulo 1) e as mutantes de SpxA1 e SpxA1/A2 mostraram-se incapazes de produzir mutacinas dos tipos IV e V uma vez que essas cepas foram incapazes de antagonizar o crescimento de *Streptococcus gordonii* or *Lactococcus lactis* (Capítulo 1).

Além disso, houve uma redução do score de cárie em cepas mutantes de Spx em comparação com a cepa UA159, o que confirma o papel de Spx na patogênese da doença cárie. Da mesma forma, outros genes/proteínas também foram implicados após utilização de modelo similar ao utilizado neste estudo. Dentre os genes também implicados na patogênese da cárie destaca-se *gtf* (Yamashita *et al.,* 1993), *gbp* (Lynch *et al.,* 2013; Matsumura *et al.,* 2003; Banas *et al.,* 2004 and Hazlett *et al.,* 1998), *fur* and *ftf* (Burne *et al.,* 1996), fabM (Fozo *et al.,* 2007), spaP (Crowley *et al.,* 1999) e ldh (Fitzgerald *et al.,* 1989) (Capítulo 1).

Buscando elucidar o papel de outro gene também regulado por Spx, foi conduzido um estudo de caracterização fenotípica em *smu1784c* (Capítulo 3), que também apresentou alto grau de homologia com gene de *E. faecalis* responsável por codificar a proteína Eep que confere resistência à lisozima nesta bacteria (Varahan *et al*., 2013).

Eep em *E. faecalis*, assim como em *B. subtilis* e *E. coli*, é uma protease relacionada liberação de fator sigma extracitoplasmático, que uma vez livre no citoplasma se liga à RNA polymerase e ativa a transcrição de genes em resposta ao estresse ambiental e a agentes que causam danos à perede celular e envelope (Schobel *et al.*, 2004; Ellermeier e Losick, 2006).

Foi mostrado no Capítulo 3 que a proteína homóloga à Eep em *S. mutans* não parece compartihar a característica de resistência à lisozima conferida a *E. faecalis*. Mas ao mesmo tempo, ela mostrou ter um papel importante na virulência de *S. mutans*, uma vez que em modelo animal de toxicidade em larvas de *G. mellonella* a cepa com mutação no gene *smu1784c* mostrou-se atenuada em relação à cepa selvagem e em modelo de formação de biofilme maduro de 115 horas a mesma cepa conseguir reduzir a formação de biomassa, de proteínas e polissacarídeos álcali-solúveis.

Esses resultados corroboram com os encontrados por Frank *et al*., (2012 e 2013) que mostrarem que a Eep em *E. faecalis* é responsável pela alterações em modelo de biofilme resultando em fenótipos atenuados em modelo de endocardite em coelhos e dificuldade de colonizar rins utilizando um modelo de infecção utilizando cateter associado ao trato urinário de ratos.

4 CONCLUSÃO

Com base nos resultados pode-se concluir que:

1) Inativação dos genes *spxA1* e *spxA2* interferem com um importante fator de virulência do *S. mutans* que é sua capacidade de formar bacteriocinas e na sua efetividade de transformação, o que reflete na competência desse microrganismo. Além disso, a cepa Δ*spxA2* formou mais biofilme e glucanos na presença de sacarose *in vitro* e inativação de Spx faz que as cepas mutantes resultantes tenham menor capacidade de formar cárie em ratas, o que confirma o papel significativo de Spx na cariogênese induzida por *S. mutans*.

2) Spx também é responsável pelo controle da expressão de muitos genes nunca antes caracterizados envolvidos em resposta contra o estresse oxidativo em *S. mutans*. Além disso, em acréscimo à ativação transcricional de genes relacionados ao metabolismo de oxigênio e à depleção de espécies reativas de oxigênio, Spx desempenha também um importante papel da homeostase de ferro, por regular a disponibilidade intracelular deste íon.

3) O gene *smu1784c* também regulado por Spx, mostra-se envolvido em respostas ao estresse ácido e oxidativo. Além disso, embora esse gene não tenha mostrado conferir resistência à lisozima ou a outros agentes que interferem na membrana/parece celular de *S. mutans*, como o faz em *E. faecalis*, ele mostrou ter um papel importante an virulência de *S. mutans* uma vez que a cepa mutante mostrou-se atenuada em modelo de *G. mellonella* e capaz de reduzir os parâmetros bioquímicos relacionados à formação de biofilme.

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ANEXOS

Anexo 1. Certificado de aprovação do Comitê de Ética no Uso de Animais FOP/UNICAMP.

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O,

Certificamos que o projeto **"Construção e caracterização fenotípica, *in vivo* e *in vitro*, de cepas mutantes de *Streptococcus mutans* de genes relacionados à sua virulência em ratos"** (protocolo nº **2637-1**), sob a responsabilidade de **Prof. Dr. Pedro Luiz Rosalen / Lívia Câmara de Carvalho Galvão**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **26 de março de 2012**.

Campinas, 26 de março de 2012.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

CEUA/UNICAMP
Caixa Postal 6109
13083-970 Campinas, SP – Brasil

Telefone: (19) 3521-6359
E-mail: comisib@unicamp.br
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Anexo 2. Comprovação de submissão do artigo "Inactivation of the spxA1 or spxA2 genes of Streptococcus mutans decreases virulence in the rat caries model" ao periódico Molecular Oral Microbiology (MOM).

Assunto:	Molecular Oral Microbiology - Account Created in ScholarOne Manuscripts [email ref: SE-4-a]
De:	MOM_journal@wiley.com (MOM_journal@wiley.com)
Para:	liviagalvao@ymail.com;
Data:	Sexta-feira, 22 de Janeiro de 2016 18:43

22-Jan-2016

Dear Dr Galvao:

A manuscript titled Inactivation of the spxA1 or spxA2 genes of Streptococcus mutans decreases virulence in the rat caries model (MOM-01-16-0747) has been submitted by Dr Livia Galvao to Molecular Oral Microbiology.

You are listed as an author for this manuscript. The online peer-review system, ScholarOne Manuscripts, automatically creates a user account for you. Your Molecular Oral Microbiology - ScholarOne Manuscripts account information is as follows:

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Thank you for your participation.

Sincerely,
Molecular Oral Microbiology Editorial Office

Anexo 3. Comprovação de aceite do artigo "Transcriptional and phenotipic characterization of novel Spx-regulated genes in *Streptococcus mutans*" ao periódico PLoS ONE.

Assunto: FW: PONE-D-15-03267R1: Final Decision Being Processed - [EMID:9125ae1071974e8f]

De: Lemos, Jose A (Jose_Lemos@URMC.Rochester.edu)

Para: liviagalvao@ymail.com; Jacqueline_Abranches@URMC.Rochester.edu; James_Miller@URMC.Rochester.edu; Jessica_Kajfasz@URMC.Rochester.edu; Irlan_Freires@URMC.Rochester.edu; rosalen@fop.unicamp.br; Kathy_ScottAnne@URMC.Rochester.edu;

Data: Terça-feira, 24 de Março de 2015 13:09

Good news!

On 3/24/15, 10:15 AM, "PLOS ONE" <em@editorialmanager.com> wrote:

>Transcriptional and phenotypic characterization of novel Spx-regulated
>genes in Streptococcus mutans
>
>PONE-D-15-03267R1
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>Dear Dr. Lemos,
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>
>We are pleased to inform you that your manuscript has been judged
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>lqspfb4&s=IhbpIot8Um_d5vyS042BBt1NvJNdrW7t7ZM46lqAMUw&e= , click the
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Anexo 4: Autorização da editora para inclusão do artigo "Transcriptional and phenotipic characterization of novel Spx-regulated genes in *Streptococcus mutans*" na tese.

Assunto: Editorial permission PLOS ONE [ref:_00DU0lfis._500U0Qppoj:ref]

De: plosone (plosone@plos.org)

Para: liviagalvao@ymail.com;

Data: Domingo, 17 de Janeiro de 2016 4:47

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Kind regards,

Amy

Amy Sutherland
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From: Livia Galvao [liviagalvao@ymail.com]
Sent: 15/01/2016
To: plosone@plos.org
Subject: Editorial permission

Editorial Office,
My research group published a Plos One paper and I would like to use this as a chapter in my P.h.D thesis. I was the first author:Article Source:"Transcriptional and PhenotypicCharacterization of Novel Spx-Regulated Genes in Streptococcus mutans". Lívia C. C. Galvão, James H. Miller, Jessica K. Kajfasz, Kathy Scott-Anne, Irlan A. Freires, Gilson C. N. Franco, Jacqueline Abranches, Pedro L. Rosalen (2015). PLoS ONE 10(4): e0124969. doi:10.1371/ journal.pone.0124969.

I would be very grateful for your permission. If you require any additional information, do not hesitate to contact me at this address.

Sincerely,

Lívia Câmara de Carvalho GalvãoDoutoranda do Programa de Pós-Graduação em Odontologia/ PhD student of Dentistry graduate program.Universidade Estadual de Campinas / State University of Campinas - UNICAMPFaculdade de Odontologia de Piracicaba / Piracicaba School of DentistryÁrea Farmacologia, Anestesiologia e Terapêutica/ Pharmacology, Anesthesiology and Therapeutic area.
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Anexo 5: Declaração de direitos autorais.

Declaração

As copias dos documentos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congresso sujeitos a arbitragem, qu constam da minha Tese de Doutorado, intitulada "**CONSTRUÇÃO E CARACTERIZAÇÃO FENOTÍPICA DE CEPAS MUTANTES DE *Streptococcus mutans* DE GENES RELACIONADOS À SUA VIRULÊNCIA**" não infringem os dispositivos da Lei no. 9.610/98, nem o direito autoral de qualquer editora.

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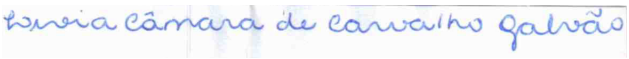

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Pedro Luiz Rosalen

RG 11185975-SSP/SP