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Differential oxidative stress tolerance of *Streptococcus mutans* isolates affects competition in an ecological mixed-species biofilm model

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Summary

Streptococcus mutans strongly influences the development of pathogenic biofilms associated with dental caries. Our understanding of *S. mutans* behavior in biofilms is based on a few well-characterized laboratory strains; however, individual isolates vary widely in genome content and virulence-associated phenotypes, such as biofilm formation and environmental stress sensitivity. Using an ecological biofilm model, we assessed the impact of co-cultivation of several *S. mutans* isolates with *Streptococcus oralis* and *Actinomyces naeslundii* on biofilm composition following exposure to sucrose. The laboratory reference strain *S. mutans* UA159 and clinical isolates Smu44 (most aciduric), Smu56 (altered biofilm formation) and Smu81 (more sensitive to oxidative stress) were used. Our data revealed *S. mutans* isolates varied in their ability to compete and become dominant in the biofilm after the addition of sucrose, and this difference correlated with sensitivity to H₂O₂ produced by *S. oralis*. Smu81 was particularly sensitive to H₂O₂ and couldn't compete with *S. oralis* in mixed-species biofilm, despite forming robust biofilms on its own. Thus, diminished oxidative stress tolerance in *S. mutans* isolates can impair their ability to compete in complex biofilms, even in the presence of sucrose, which could influence the progression of a healthy biofilm community to one capable of causing disease.

Keywords

Streptococcus mutans; *Streptococcus oralis*; ecological mixed-species biofilm; oxidative stress tolerance

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Conflict of interest

The authors declare no conflict of interest.

Introduction

Dental caries is the most prevalent and costly biofilm-associated oral infectious disease (Kassebaum *et al.*, 2015). The pathogenesis of dental caries is characterized by changes in the biofilm (or plaque) composition triggered by the host diet; transitioning from a microbiota dominated by health-associated commensals to one that contains greater proportions of highly acidogenic and acid tolerant organisms enmeshed in an exopolysaccharide (EPS) rich matrix (Paes Leme *et al.*, 2006; Takahashi and Nyvad, 2011). The human oral cavity is home to a diverse microbiome (Dewhirst, 2010) that constantly interacts with a salivary film (pellicle) formed on the tooth surface, to which a group of early colonizers (mostly streptococci and actinomyces species) adhere with high affinity. Many synergistic and antagonistic interactions between these bacterial species influence the composition of the colonizing community (Kuramitsu *et al.*, 2007; Kuboniwa, 2006). For example, health-associated commensal bacteria, mainly Mitis group strep, can antagonize the growth of potentially harmful species through the production of hydrogen peroxide (H₂O₂) and/or bacteriocins, thus providing protection to the host against disease (Kuramitsu *et al.*, 2007; Merritt and Qi, 2012; Qi and Kreth, 2017).

The transition from health to disease is driven in large part by the metabolism of dietary sugars (Paes Leme *et al.*, 2006). Frequent consumption of sucrose, in particular, dramatically influences cariogenic biofilm development by providing a substrate for both EPS and organic acid production. The EPS promote bacterial accumulation and cohesion on tooth surfaces, while creating a diffusion-limiting matrix (Bowen and Koo, 2011). The metabolic activity of the bacteria enmeshed within EPS-rich matrices can create localized acidic microenvironments, despite the nearly continuous supply of buffering saliva. The persistence of acidic microenvironments allows acidogenic and acid-tolerant species to out-compete the acid-sensitive commensal bacteria associated with health (Takahashi and Nyvad, 2011; Koo *et al.*, 2013). Furthermore, in transcriptomic studies that measured gene transcripts expressed in natural oral biofilm communities, about 2% of all transcripts identified were associated with oxidative stress tolerance genes, including those involved with detoxifying superoxides and peroxides (Peterson *et al.*, 2014), suggesting that the ability of bacteria to cope with oxidative stress is critical to their survival when residing in biofilms. Once established, cariogenic biofilms increases the potential for demineralization of tooth enamel, leading to the onset and progression of dental caries.

Streptococcus mutans is the bacterium that has been most closely and consistently associated (albeit not exclusively) with the initiation of dental caries (Bowen, 2016). *S. mutans* lives almost entirely in dental plaque on the tooth surface, where there are dramatic fluctuations in nutrient availability, pH and oxidative stress (Bitoun, *et al.*, 2011). The unique combined abilities of this pathogen to produce EPS and acids, to be acid-tolerant, and to rapidly adapt to environmental stresses are essential for establishment and persistence in biofilms and for competition with other oral bacteria when conditions are conducive to the development of dental caries (Lemos and Burne, 2008). However, diversity exists within the *S. mutans* species, which can be classified into at least four different serological groups (*c*, *e*, *f*, *k*) (Nakano and Ooshima, 2009); most strains isolated from the human oral cavity are serotype *c* (70-80%) (Palmer *et al.*, 2013). In addition, the carriage of particular genotypes of

S. mutans may correlate (or not) with the caries status of an individual (Napimoga *et al.*, 2004; Pieralisi *et al.*, 2010; Phattarataratip *et al.*, 2011).

Recently, whole genome sequencing was completed on a large collection of strains of *S. mutans* from across the globe, providing new insights into the core and accessory genomes of the organism (Cornejo *et al.*, 2013). In this study 73 unique-core genes (found in *S. mutans*, but absent in its closest relatives) were defined, of which 49% were hypothetical proteins, with the remaining involved in carbohydrate metabolism, acid resistance, adaptation to oxidative stress, metal and peptide translocation, and adhesion to host tissues (Cornejo *et al.*, 2013). In a subsequent study, phenotypic properties directly associated with the ability of *S. mutans* to establish, persist, and cause disease in humans were demonstrated to vary greatly among these genetically- and geographically-heterogeneous clinical isolates (Palmer *et al.*, 2013). These findings provide evidence that the genomic diversity of the species may translate to substantial variations in their ability to compete with physiologically similar organisms in human oral biofilms. Thus, a critical question to be addressed is whether genomic variability influences the way in which *S. mutans* interacts and competes with abundant commensal bacteria that dominate oral biofilms in a healthy state. Such information could provide powerful new tools to accurately assess caries risk and to develop new therapies that hinder the cariogenic potential of *S. mutans* and oral biofilms. In the present study, we compared the ability of four *S. mutans* isolates that vary considerably in genome composition and key phenotypic characteristics to compete with *Streptococcus oralis* and *Actinomyces naeslundii* under cariogenic conditions using an established mixed-species ecological biofilm model.

Results and Discussion

The clinical isolates utilized in this study are described in Table 1 and selection of these particular strains was based on specific phenotypic characteristics associated with the virulence potential of *S. mutans*, as previously described (Palmer *et al.*, 2013). Typically, *S. mutans* UA159 forms more biofilm in the presence of sucrose than glucose. However, Smu56 was able to form biofilms equally well in the presence of either carbohydrates. Smu44 stood out among *S. mutans* isolates in aciduricity (ability to grow and continue to produce acids at low pH), which is an important virulence attribute of *S. mutans*. Smu44 grew at a significantly faster rate compared to UA159 in media acidified to pH 5.5, whereas Smu56 and Smu81 grew more slowly than UA159 in acidic conditions (Palmer *et al.*, 2013). Smu81 was selected based on its enhanced sensitivity to oxidative stress and because it contains the *cnm* gene associated with the ability of *S. mutans* to invade epithelial and endothelial cells. This strain is unique among *S. mutans* isolates studied, as it is unable to initiate growth in the presence of 25 mM paraquat (Palmer *et al.*, 2013), which can generate superoxide anion. Importantly, the ability to tolerate oxidative stress is critical to the establishment, persistence and ecology of oral bacteria, and redox and oxygen levels affect the pathogenic potential of dental plaque in major ways (Bradshaw *et al.*, 1996; Marquis, 1995; Lemos and Burne, 2008). These strains also differ in the number of mutacin (bacteriocin) genes present. Mutacin genes encode peptide antibiotics that are important for the establishment of *S. mutans* in the complex oral microbiota (Merritt and Qi, 2012), and their expression has been linked to oxidative stress (Ahn *et al.*, 2007). Smu81 lacks mutacin

IV (*nImA/B*), while Smu56 and Smu44 contain only *nImA* and are missing *nImB*, as detailed in (Palmer *et al.*, 2013).

Differential response of clinical isolates towards other oral species in mixed-species system

S. mutans co-exists and associates with a number of other streptococci and bacterial species in the oral cavity (Jenkinson, 2011; Dewhirst *et al.*, 2010). Analysis of the microbial species composition in an initial colonizing community have shown that the majority (47 to 90%) of cultivable bacteria are non-mutans streptococci, such as *S. oralis*, and that one-third of the remaining bacteria are *Actinomyces* species, such as *A. naeslundii*. In the mixed-species ecological biofilm model, *S. mutans* is co-cultured with the early colonizers *S. oralis* and *A. naeslundii* to form an initial microbial consortium on pellicle-coated hydroxyapatite. Then, sucrose is introduced to induce the transition from a non-virulent microbial community (high levels of *S. oralis*, few *A. naeslundii* cells and *S. mutans* as the least abundant species) to a biofilm characterized by an EPS-rich matrix and acidic microenvironments that favor *S. mutans* survival (Koo *et al.*, 2010; Xiao *et al.*, 2012) (Fig. S1), mimicking the biochemical and microbiological changes associated with cariogenic biofilms. Using this model, we observed intriguing variation in the ability of the different *S. mutans* strains to compete with the other species during biofilm development.

Prior to introduction of 1% sucrose (Fig. 1) the bacterial populations on the sHA surface were comprised mainly of *S. oralis* and some *A. naeslundii* cells. However, after the introduction of 1% sucrose at 29 h, the proportions of *S. mutans* UA159 and Smu44 rapidly increased, and the *S. mutans* isolates became the dominant species in 91 h biofilms (Fig. 1A). While Smu56 displayed a much slower population shift, representing a noticeably lower proportion compared to UA159 at 67 h (Fig. 1B, $P<0.001$), it eventually became dominant at 91 h, suggesting that Smu56 is slow to adapt to environmental stress (i.e. low pH or increased sucrose). Remarkably, even in the presence of 1% sucrose, Smu81 was never able to overtake the other species in the biofilm, and made up a strikingly lower proportion of the bacteria present at 67 h and 91 h, compared to biofilms formed with the other *S. mutans* strains (Fig. 1B, $P<0.001$).

When individual isolates were compared in single-species biofilms, Smu44 produced significantly less biofilm, likely due to both reduced EPS and bacterial cells, compared to UA159 (Supplementary Fig. S2), but still became the dominant species within the mixed-species biofilm after 1% sucrose was added. While oxidative stress-sensitive strain Smu81 produced similar EPS to UA159 during single species biofilms (Supplementary Fig. S2), it could not out-compete *S. oralis* in the mixed-species biofilm. Collectively, these results suggest that the differences seen between the abilities of *S. mutans* strains to establish and survive in the mixed-species ecological biofilms are due to additional factors other than their capacity to form EPS. The glycosyltransferases (Gtfs) of *S. mutans* contribute to the accumulation of this pathogen in a mixed-species environment after introduction of sucrose by increasing the biomass and EPS content of the biofilms, triggering the formation of structured microcolonies (Koo *et al.*, 2010). However, variations in Gtf expression among *S. mutans* strains did not seem to contribute to their survival and dominance at the later stages

of the mixed-species biofilm development. Considering the inability of Smu81 to grow in the presence of paraquat (Palmer *et al.*, 2013), it is conceivable that the differences observed between the *S. mutans* isolates in the mixed-species biofilms may be determined by reduced resistance to oxidative stress, perhaps caused by the H₂O₂ produced by *S. oralis*, rather than differences in EPS production and biofilm formation.

Differences in oxidative stress tolerance between *S. mutans* strains

To determine whether the growth of *S. mutans* strains is inhibited by *S. oralis*, or vice versa, a plate-based growth inhibition test was performed. Actively growing *S. mutans* were spotted next to *S. oralis* on agar plates and allowed to grow for 48 h. While UA159 was unaffected by *S. oralis*, the growth of Smu81 was strongly inhibited by the commensal (Fig. 2A). Smu44 and Smu56 were slightly sensitive to growth inhibition by *S. oralis*, but more resistant than Smu81. Importantly, when the plates were pretreated with catalase, which neutralizes H₂O₂, Smu81, Smu44 and Smu56 were no longer inhibited by *S. oralis* (Fig. 2A). It's also important to note that growth of *S. oralis* was not inhibited by the *S. mutans* strains, as would be expected if *S. oralis* were sensitive to mutacins produced by *S. mutans*. Thus, Smu81 may aberrantly express, be lacking in, or contain key mutations in, gene products required for H₂O₂ tolerance. To explore in more detail the differences between the *S. mutans* isolates to cope with H₂O₂, growth in the presence of H₂O₂ was monitored using an automated growth monitoring machine (Bioscreen C, LabSystem). The growth rate, lag time and maximum yield were calculated based on the growth curves. Table 2 shows the results for each *S. mutans* strain grown under non-stress conditions compared to growth in the presence of H₂O₂. In the presence of H₂O₂, UA159, Smu44, and Smu81 all had significantly slower growth rates compared to non-stress conditions, while Smu56's growth rate was not significantly different ($P>0.05$). In addition, UA159, Smu44, and Smu56 had similar yields when grown with H₂O₂ compared to non-stress conditions. Consistent with the sensitivity of Smu81 to growth inhibition by *S. oralis*, this strain showed the most pronounced decrease in growth yield, and greatest increase in lag time (320 min for Smu81), compared to the other *S. mutans* strains tested (UA159- 50 min, Smu44- 80 min and Smu56- 310 min). To further define the level of oxidative stress sensitivity among the *S. mutans* strains, a H₂O₂ killing assay was completed, where the percent viable cells was determined after exposure to H₂O₂ for 15, 30, and 45 min. Consistent with data from the growth curve assays and the agar spot test, Smu81 was the most sensitive to H₂O₂ of the four strains tested. It took ~25 min of exposure for Smu56, and 45 min for UA159 or Smu44, to see the same level of killing as Smu81 experienced after only 15 min exposure to assay conditions (Fig. 2B).

While *S. mutans* does not produce catalase, it contains several pathways that neutralize reactive oxygen species (ROS), including an alkylhydroperoxide reductase AhpCF (Smu.764 and Smu.765), a Dps-like iron binding protein Dpr (Smu.540), a Flavin-based NAD (NADH) oxidase (*nox*, Smu.1117), and a superoxide dismutase (Sod, Smu.629) (Higuchi *et al.*, 2000; Yamamoto *et al.*, 2000). To further explore the differences in ROS resistance between *S. mutans* strains, we compared the expression of *ahpC* (Smu.764), *ahpF* (Smu.765), *dpr* (Smu.540), and *nox* (Smu.1117) by qRT-PCR in cells grown with or without a 15 min exposure to H₂O₂. While there were differences in expression of oxidative stress genes

between the clinical isolates and *S. mutans* UA159 (Fig. 3A), results were contrary to what we expected: the strains that were more sensitive to H₂O₂ (Smu56 and Smu81) actually expressed significantly higher levels of *aphF* and/or *aphC* compared to the more resistant strains, UA159 and Smu44 (Fig. 3B). The reason for this disparity could be due to differences in expression of other genes not evaluated, or from differences in enzymatic activity due to polymorphisms in the oxidative stress genes themselves. Therefore, the NADH oxidase enzyme activity of the *S. mutans* strains was determined after a 30 or 45 min exposure to H₂O₂ and compared to untreated samples (no H₂O₂) (Fig. 3C). While Smu44 was the only strain that did not see a significant increase in NADH oxidase activity after 30 min, all strains saw a significant increase after the 45 min exposure to H₂O₂. In addition, despite Smu81 being the most sensitive to oxidative stress, we were surprised to find this strain produced more NADH oxidase activity upon H₂O₂ treatment than any other *S. mutans* strains tested, including UA159 (Fig. 3D). Furthermore, there was no correlation between the NADH oxidase activity observed and expression of genes responsible for NADH oxidase activity (*nox* and *aphFC*).

Next, we compared the protein sequences of oxidative stress genes to see if there was any incongruence between strains that could explain the differences in H₂O₂ sensitivity. Smu.629 (*sod*) and Smu.764 (*ahpC*) were 100% conserved among all *S. mutans* strains; however, Smu81 had two polymorphisms in Smu.765 (*ahpF*), and one in the iron binding protein *dpr* (Table 3), both of which contribute to *S. mutans* resistance to H₂O₂ (Fujishima, *et al.*, 2013). Further, when the sequences for *spxA* (Smu.1142c), a global regulator of oxidative stress genes in *S. mutans* (Kajfasz *et al.*, 2015), was compared, Smu81 was the only strain out of the 57 included in the analysis that harbored mutations in this gene (Table 3). Given the high level of conservation of this gene product among sequenced strains of *S. mutans*, it is possible that these polymorphisms significantly affect the ability of SpxA to sense oxidative stress and appropriately regulate oxidative stress genes and, therefore, could partially explain why Smu81 is so sensitive to H₂O₂. SpxA functions as a transcriptional regulator by binding to RNA polymerase, either inhibiting or activating transcription of genes required for adaptation to oxidative stress. Previous work found that Smu.764 (*ahpC*) and Smu.765 (*ahpF*) are regulated by SpxA in *S. mutans* UA159, and that *spxA* deletion mutants display significantly less NADH oxidase activity than the parental strain (Kajfasz *et al.*, 2015). However, Smu81 had 4-fold greater expression of the Smu.764 gene upon treatment with H₂O₂, compared to UA159, suggesting that decreased expression of Smu.764 is not the reason for the oxidative stress sensitivity of this strain. Compared to UA159, Smu81 also produced more than twice as much NADH oxidase activity. Therefore, aberrant expression of other oxidative stress genes regulated by SpxA, not evaluated here, may explain our results. Further, the polymorphism in the iron binding protein Dpr, which prevents Fenton reactions, may also be responsible for the sensitivity to H₂O₂ observed in Smu81. Accordingly, future studies will investigate the complete transcriptomes of the *S. mutans* strains under oxidative stress to gain additional insights into how genetic differences between strains affect global gene regulation in *S. mutans*. We will also examine the enzymatic activities of oxidative stress response pathways and activity of the transcriptional regulator SpxA to further dissect the exact mechanism behind the differences in oxidative stress tolerance among *S. mutans* strains.

Caries development is clearly associated with the overgrowth of a few bacterial species, including *S. mutans*, certain lactobacilli and bifidobacteriae, and correlated with acidification of the biofilm microenvironment and a reduction in overall species diversity (Gross *et al.*, 2012; Caufield *et al.*, 2007; Takahashi and Nyvad, 2011). Interspecies interactions play an important role in this shift. Many beneficial early-colonizers are sensitive to low pH conditions, while the disease-associated mutans streptococci are more acid tolerant, but sensitive to H₂O₂. Therefore, the ability of commensal streptococci (i.e. *S. oralis*, *S. gordonii*, and *S. sanguinis*) to produce H₂O₂, which can antagonize cariogenic *S. mutans* (Kreth *et al.*, 2005), is regarded as a key protective mechanism against competing species in early healthy biofilm communities (Bitoun, *et al.*, 2011; Zhu *et al.*, 2012). However, perturbations in the environment triggered by dietary sugars promote the growth of opportunistic pathogenic species, which can be present in health at low numbers, resulting in changes in cariogenic potential of the biofilm community overall (Nyvad and Kilian, 1990; Takahashi and Nyvad, 2011). As a consequence, the populating species can influence whether the biofilm behaves benignly by restricting potential pathogens from either colonizing or accumulating in sufficient proportions to elicit disease. Although, most *S. mutans* strains have some capacity to deal with oxidative stress caused by damaging reactive oxygen species (e.g. O₂^{•-}) and unfavorable cellular redox potential (Fujishima *et al.*, 2013; Marquis, 1995), Smu81 was distinct from the other strains and displayed an unusually high degree of sensitivity to H₂O₂, and therefore was unable to dominate the biofilm even in the presence of 1% sucrose. Thus, in order to compete within biofilms, *S. mutans* must be able to cope with various oxidative stresses (Marquis, 1995), either through enzymatic deactivation, or by associating with other oxidative stress tolerant bacteria, before it can become numerically significant and modify the biofilm milieu to become more pathogenic.

The results presented here demonstrate that the considerable genomic heterogeneity and disparate phenotypic behaviors in virulence-related properties translate to clear differences in the way in which various *S. mutans* isolates interact and compete with common oral commensal bacteria. While much attention has been paid to determining the ecology of dental biofilms in health and disease, simply knowing which species are there tells only part of the story. Understanding intra-species variability, such as virulence characteristics or variations in stress tolerance within a species and its effect on biofilm ecology, are also vital to understanding disease etiology. Approximately 28% of *S. mutans* strains isolated from dental biofilm are naturally competent and able to take up DNA from their environment (Westergren and Emilson, 1983), which contributes to the genomic and phenotypic plasticity of this species. Based on gene content clustering of the *S. mutans* strains used in this study, Smu44 and UA159 differed by ~23%, Smu56 and UA159 by ~22%, and Smu81 and UA159 by ~19% (Cornejo *et al.*, 2013), thus small differences in gene content can result in great differences in virulence properties. A previous study, based on the genome sequence of 57 *S. mutans* isolates, revealed a core genome of 1,490 genes (present in all *S. mutans* strains), and a pan-genome of 3,296 genes (total number of genes found in all strains) (Cornejo *et al.*, 2013). The *S. mutans* genome contains ~ 2.0 Mb of DNA and encodes roughly 2,000 genes (Ajdic *et al.*, 2002). Thus, in any single isolate of *S. mutans*, about 500 genes could be transient or dispensable, and fluctuate based on the particular environment or niche of that isolate; so not all *S. mutans* isolates are likely to be equally virulent in any particular host.

While, it is understood that inter-species interactions are crucial to community dynamics and the development of polymicrobial diseases, such as dental caries (Mira et al., 2017; Marsh and Zaura, 2017), a better understanding of bacteria-bacteria interactions, particularly phenotypically diverse strains of *S. mutans* and commensal bacteria, and how these interactions influence the establishment of pathogenic microenvironments, could contribute to the design of novel preventive and therapeutic strategies against human dental caries. Furthermore, understanding how *S. mutans* promotes genomic heterogeneity and disparate phenotypic behaviors could be exploited to modulate its pathogenicity and perhaps turn-off *S. mutans* virulence.

Experimental procedures

Mixed-species biofilm model

The mixed-species biofilm model was based on a batch culture approach using saliva-coated hydroxyapatite (sHA) disks designed to mimic the formation of biofilms according to the “ecological plaque-biofilm” concept (Marsh, 2003), as recently described by (Koo *et al.*, 2010; Xiao *et al.*, 2012). Briefly, *S. mutans* UA159, *S. oralis* ATCC 35037 and *A. naeslundii* ATCC 12104 were grown in ultrafiltered (10-kDa-cutoff membrane; Prep/Scale; Millipore, MA) buffered (pH 7.0) tryptone-yeast extract broth (UFTYE) containing 2.5% tryptone and 1.5% yeast extract with 1% (wt/vol) glucose to mid-exponential phase (optical density at 600 nm [OD₆₀₀] = 0.5). All isolates of *S. mutans* except Smu56 had similar growth rates, as determined by monitoring OD₆₀₀ (Spectronic 20D+, Thermo Fisher Scientific Inc., Madison, WI). The bacterial suspensions were mixed to obtain an inoculum containing a defined microbial population consisting of *S. mutans* (10² CFU/ml), *S. oralis* (10⁷ CFU/ml), and *A. naeslundii* (10⁶ CFU/ml). Biofilms were formed on hydroxyapatite disks (diameter, 1.25 cm; Clarkson Chromatography Products, Inc., South Williamsport, PA) coated with filter-sterilized, clarified, human whole saliva, with the discs held in a vertical position using a custom-made holder (Xiao *et al.*, 2012). The mixed population of *S. mutans* isolates, *S. oralis*, and *A. naeslundii* was inoculated in 2.8 ml of UFTYE with 0.1% (wt/vol) sucrose and incubated at 37°C in a 5% CO₂ aerobic atmosphere. During the first 19 h, the cells were grown undisturbed to allow initial biofilm formation. At the 19 h time point, spent media was replaced with fresh culture medium containing 0.1% (wt/vol) sucrose and the biofilms were grown until the 29 h time point to allow establishment of the initial biofilm community (Xiao *et al.*, 2012). The biofilms were then transferred to UFTYE containing 1% (wt/vol) sucrose to induce environmental changes that simulate a cariogenic challenge. The culture medium was then replaced twice daily at 8 am and 6 pm until the end of the experimental period (91 h) (Supplementary Fig. S1). *S. mutans* single-species biofilm was formed under the same condition as described above. The biofilms were analyzed at specific time points (29, 67 and 91 h) using biochemical and microbiological assays. Results are presented as mean ± standard deviation (SD). The experimental results presented are representative of three separate experiments. One-way ANOVA was performed, followed by post-hoc Tukey HSD test and statistical analysis was performed using the Prism-Graphpad software.

Biochemical and microbiological analyses

The single- and mixed-species biofilms were removed at specific time points and dispersed by sonication in a sterile 0.89% (wt/vol) NaCl solution (30 sec pulse at an output of 7 W; Branson Sonifier 150; Branson Ultrasonics, Danbury, CT); the sonication procedure does not kill any of the bacterial species used in this study (Xiao *et al.*, 2012). The cell suspensions were used to determine the dry weight and for plating on blood agar using an automated EddyJet Spiral Plater (IUL, SA, Barcelona, Spain) to determine the total CFU per biofilm. The three species were differentiated by colony morphology in conjunction with microscopic examination of cells from selected colonies (Guggenheim *et al.*, 2001)

Growth inhibition test

The ability of *S. oralis* to inhibit growth of *S. mutans* strains was determined by growth on agar plates, and performed similarly to experiments described by (Huang *et al.*, 2016). For assay, 6 µl of overnight cultures of *S. mutans* strains and *S. oralis* were spotted next to one another on BHI agar plates and allowed to grow for 48 h in a 5% CO₂ aerobic incubator at 37°C. Experiments were also performed in which 300 units of catalase (3000 U/ml in 50 mM potassium phosphate buffer, pH 7.0) was applied to agar surface and allowed to air dry before strains were spotted on the plates. The experimental results presented are representative of four separate experiments.

Growth experiments

Mid-exponential phase cultures of each *S. mutans* strain grown in brain heart infusion (BHI) broth (BBL, BD Difco) were sub-cultured 1:100 into BHI broth or BHI broth supplemented with 250 µM H₂O₂. A 300 µl aliquot of each culture was dispensed into triplicate wells in a Bioscreen C Honeycomb plate, and overlaid with 50 µl sterile mineral oil to limit oxygen exposure. The OD₆₀₀ was measured every 30 min for 48 h in a Bioscreen C Automated Microbiology Growth Curve Analysis System (Growth Curves USA, Piscataway, NJ) set to 37°C, with shaking for 10 sec and a 5 sec pause before each reading. Doubling time (T_d) was calculated based on the slope of the line of the logarithmic growth phase using the formula $T_d = (t_2 - t_1) \ln(2) / (\ln(OD_2) - \ln(OD_1))$, as described in (Khalichi *et al.*, 2004), where OD₆₀₀ was graphed on a semi-log plot against time and MATLAB was used to fit to the logarithmic growth phase. Data are based on triplicate biological samples. Statistically significant differences were determined based on Student's *t*-test. Lag (the time from inoculation until exponential growth began) and yield (maximum OD reached) are based on growth curve results.

Hydrogen peroxide killing assay

Mid-exponential cells grown in BHI were resuspended in 0.1 M glycine (pH 7) containing 58.8 mM (0.2%) hydrogen peroxide. Aliquots of cell suspension were taken at 0, 15, 30, and 45 min and serially diluted in tryptone phosphate buffer (pH 7.0) containing 1 mg/ml catalase. Percent survival was determined by dividing the CFUs of treated samples by CFU of untreated controls × 100. Assays were performed on triplicate biological samples and repeated twice. Statistical analysis was performed in the GraphPad Prism program using a

Two-way ANOVA with post-hoc Tukey HSD test, and compared each clinical isolate at each time point to *S. mutans* reference strain UA159.

qRT-PCR

Briefly, total RNA was isolated using the RNeasy® Mini Kit (Qiagen) as described previously (Palmer and Burne, 2015), from cultures grown in BHI broth and treated with 200 μ M H₂O₂ and incubated at 37°C in a 5% CO₂ environment. cDNA was synthesized using the Maxima™ H Minus cDNA Synthesis Master Mix and dsDNase (Thermo Scientific™) according to manufacturer's protocol. qRT-PCR was performed using the QuantStudio 3 Real-Time PCR system (Applied Biosystems™) with the Power-Up™ SYBR™ Green Master Mix (Applied Biosystems™) according to supplier's directions. Absolute quantification of each gene was determined based on standard curves. Results are presented as mean $\pm$ standard error (SE). Experiment was performed with triplicate biological samples. Student's *t*-test was used to determine significant differences in expression of oxidative stress sensitive genes between H₂O₂ treated and untreated samples for each clinical isolate (Figure 3A). One-way ANOVA was also performed, followed by post-hoc Tukey HSD test, to compare the level of expression of the individual genes in the *S. mutans* clinical isolates to that of reference strain UA159 (Figure 3B). Statistical analysis was performed using the Prism-Graphpad software. Primers used can be found in supplemental information (Supplementary Table S1).

NADH oxidase assay

Whole cell lysates were prepared from mid-exponential cultures treated with 250 μ M H₂O₂, or untreated controls, and incubated for 30 or 45 min. Assay reactions contained 0.1 M K₃PO₄ buffer (pH 7), 0.3 mM EDTA, 0.16 mM NADH, and cell lysates, and the decrease in absorbance at 340 nm was monitored over 4 min. NADH oxidase activity was calculated by multiplying the change in OD₃₄₀ over time by the extinction coefficient for NADH at 340 nm ($6.22 \times 10^3 \text{M}^{-1}, \text{cm}^{-1}$). NADH activity was standardized by protein concentration determined using the Pierce BCA Protein Assay Kit (Thermo Scientific™), with BSA as a standard. Assay was performed with triplicate biological samples. Results represent the mean $\pm$ standard error (SE). Student's *t*-test was used to determine significant differences in NADH oxidase activity between H₂O₂ treated and untreated samples for each strain (Figure 3C), and One-way ANOVA with post-hoc Tukey HSD test, was used to test differences between clinical isolates and *S. mutans* reference strain UA159 (Figure 3D). Statistical analysis was performed using Prism-Graphpad software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol.* 2005; 43:5721–5732. [PubMed: 16272510]
- Abdallah AM, Gey van Pittius NC, Champion PA, Cox J, Luirink J, Vandenbroucke-Grauls CM, et al. Type VII secretion—mycobacteria show the way. *Nat Rev Microbiol.* 2007; 5:883–891. [PubMed: 17922044]
- Ajdic D, McShan WM, McLaughlin RE, Savic G, Chang J, Carson MB, et al. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *Proc Natl Acad Sci U S A.* 2002; 99:14434–14439. [PubMed: 12397186]
- Ahn SJ, Wen ZT, Burne RA. Effects of oxygen on virulence traits of *Streptococcus mutans*. *J Bacteriol.* 2007; 189:8519–8527. [PubMed: 17921307]
- Bitoun JP, Nguyen AH, Fan Y, Burne RA, Wen ZT. Transcriptional repressor Rex is involved in regulation of oxidative stress response and biofilm formation by *Streptococcus mutans*. *FEMS Microbiol Lett.* 2011; 320:110–117. [PubMed: 21521360]
- Bowen WH. Do we need to be concerned about dental caries in the coming millennium? *Crit Rev Oral Biol Med.* 2002; 13:126–131. [PubMed: 12097355]
- Bowen WH. Dental caries-not just holes in teeth! A perspective. *Mol Oral Microbiol.* 2016; 31:228–233. [PubMed: 26343264]
- Bowen WH, Koo H. Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. *Caries Res.* 2011; 45:69–86.
- Bradshaw DJ, Marsh PD, Allison C, Schilling KM. Effect of oxygen, inoculum composition and flow rate on development of mixed-culture oral biofilms. *Microbiology.* 1996; 142:623–629. [PubMed: 8868437]
- Burts ML, Williams WA, DeBord K, Missiakas DM. EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of *Staphylococcus aureus* infections. *Proc Natl Acad Sci U S A.* 2005; 102:1169–1174. [PubMed: 15657139]
- Caufield PW, Li Y, Dasanayake A, Saxena D. Diversity of lactobacilli in the oral cavities of young women with dental caries. *Caries Res.* 2007; 41:2–8. [PubMed: 17167253]
- Clermont O, Couffignal C, Blanco J, Mentré F, Picard B, Denamur E, et al. Two levels of specialization in bacteraemic *Escherichia coli* strains revealed by their comparison with commensal strains. *Epidemiol Infect.* 2016; 28:1–11.
- Cornejo OE, Lefébure T, Bitar PD, Lang P, Richards VP, Eilertson K, et al. Evolutionary and population genomics of the cavity causing bacteria *Streptococcus mutans*. *Mol Biol Evol.* 2013; 20:881–893.
- Derr AM, Faustoferri RC, Betzenhauser MJ, Gonzalez K, Marquis RE, Quivey RG Jr. Mutation of the NADH oxidase gene (*nox*) reveals an overlap of the oxygen- and acid-mediated stress responses in *Streptococcus mutans*. *Appl Environ Microbiol.* 2012; 78:1215–1227. [PubMed: 22179247]
- Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, et al. The human oral microbiome. *J Bacteriol.* 2010; 192:5002–5017. [PubMed: 20656903]
- Fujishima K, Kawada-Matsuo M, Oogai Y, Tokuda M, Torii M, Komatsuzawa H. *dpr* and *sod* in *Streptococcus mutans* are involved in coexistence with *S. sanguinis*, and PerR is associated with resistance to H₂O₂. *Appl Environ Microbiol.* 2013; 79:1436–1443. [PubMed: 23263955]
- Gross EL, Beall CJ, Kutsch SR, Firestone ND, Leys EJ, Griffen AL. Beyond *Streptococcus mutans*: dental caries onset linked to multiple species by 16S rRNA community analysis. *PLoS One.* 2012; 7:e47722. [PubMed: 23091642]
- Guggenheim B, Giersten E, Schupbach P, Shapiro S. Validation of an *in vitro* biofilm model of supragingival plaque. *J Dent Res.* 2001; 80:363–370. [PubMed: 11269730]
- Han YW. *Fusobacterium nucleatum*: a commensal-turned pathogen. *Curr Opin Microbiol.* 2015; 23:141–147. [PubMed: 25576662]
- Higuchi M, Yamamoto Y, Kamio Y. Molecular biology of oxygen tolerance in lactic acid bacteria: functions of NADH oxidases and Dpr in oxidative stress. *J Biosci Bioeng.* 2000; 90:484–493. [PubMed: 16232897]

- Huang X, Palmer SR, Ahn SJ, Richards VP, Williams ML, Nascimento MM, et al. A highly arginolytic *Streptococcus* species that potently antagonizes *Streptococcus mutans*. *Appl Environ Microbiol*. 2016; 82:2187–2201. [PubMed: 26826230]
- Jenkinson HF. Beyond the oral microbiome. *Environ Microbiol*. 2011; 13:3077–3087. [PubMed: 21906224]
- Kajfasz JK, Rivera-Ramos I, Scott-Anne K, Gregoire S, Abranches J, Lemos JA. Transcription of Oxidative Stress Genes Is Directly Activated by SpxA1 and, to a Lesser Extent, by SpxA2 in *Streptococcus mutans*. *J Bacteriol*. 2015; 197:2160–2170. [PubMed: 25897032]
- Kassebaum NJ, Barnabé E, Dahiya M, Bhandari B, Murray CJ, Marcenes W. Global burden of untreated caries: a systematic review and meta-regression. *J Dent Res*. 2015; 94:650–658. [PubMed: 25740856]
- Khalichi J, Cvitkovitch DG, Santerre JP. Effect of composite resin biodegradation products on oral streptococcal growth. *Biomaterials*. 2004; 25:5467–5472. [PubMed: 15142727]
- Koo H, Falsetta ML, Klein MI. The exopolysaccharide matrix: a virulence determinant of cariogenic biofilm. *J Dent Res*. 2013; 92:1065–1073. [PubMed: 24045647]
- Koo H, Xiao J, Klein MI, Jeon JG. Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. *J Bacteriol*. 2010; 192:3024–3032. [PubMed: 20233920]
- Kreth J, Merritt J, Shi W, Qi F. Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. *J Bacteriol*. 2005; 187:193–203. [PubMed: 15601703]
- Kuboniwa M, Tribble GD, James CE, Kilic AO, Tao L, Herzberg MC, et al. *Streptococcus gordonii* utilizes several distinct gene functions to recruit *Porphyromonas gingivalis* into a mixed community. *Mol Microbiol*. 2006; 60:121–139. [PubMed: 16556225]
- Kuramitsu HK, He X, Lux R, Anderson MH, Shi W. Interspecies interactions within oral microbial communities. *Microbiol Mol Biol Rev*. 2007; 71:653–670. [PubMed: 18063722]
- Lemos JA, Burne RA. A model of efficiency: stress tolerance by *Streptococcus mutans*. *Microbiology*. 2008; 154:3247–3255. [PubMed: 18957579]
- Marquis RE. Oxygen metabolism, oxidative stress and acid-base physiology of dental plaque biofilms. *J Ind Microbiol*. 1995; 15:198–207. [PubMed: 8519478]
- Marsh PD. Are dental diseases examples of ecological catastrophes? *Microbiology*. 2003; 149:279–294. [PubMed: 12624191]
- Marsh PD, Zaura E. Dental biofilm: ecological interactions in health and disease. *J Clin Periodontol*. 2017; 44:S12–S22. [PubMed: 28266111]
- Merritt J, Qi F. The mutacins of *Streptococcus mutans*: regulation and ecology. *Mol Oral Microbiol*. 2012; 27:57–69. [PubMed: 22394465]
- Mira A, Simon-Soro A, Curtis MA. Role of microbial communities in the pathogenesis of periodontal diseases and caries. *J Clin Periodontol*. 2017; 44:S23–S38. [PubMed: 28266108]
- Nakano K, Ooshima T. Serotype classification of *Streptococcus mutans* and its detection outside the oral cavity. *Future Microbiol*. 2009; 4:891–902. [PubMed: 19722842]
- Napimoga MH, Kamiya RU, Rosa RT, Rosa EA, Höfling JF, de Oliveira Mattos-Graner R, et al. Genotypic diversity and virulence traits of *Streptococcus mutans* in caries-free and caries-active individuals. *J Med Microbiol*. 2004; 53:697–703. [PubMed: 15184543]
- Nyvad B, Kilian M. Comparison of the initial streptococcal microflora on dental enamel in caries-active and in caries-inactive individuals. *Caries Res*. 1990; 24:267–272. [PubMed: 2276164]
- Paes Leme AF, Koo H, Bellato CM, Bedi G, Cury JA. The role of sucrose in cariogenic dental biofilm formation—new insight. *J Dent Res*. 2006; 85:878–887. [PubMed: 16998125]
- Palmer SR, Miller JH, Abranches J, Zeng L, Lefebure T, Richards VP, et al. Phenotypic heterogeneity of genomically-diverse isolates of *Streptococcus mutans*. *PLoS one*. 2013; 8:e61358. [PubMed: 23613838]
- Palmer SR, Burne RA. Post-transcriptional regulation by distal Shine-Dalgarno sequences in the grpE-dnaK intergenic region of *Streptococcus mutans*. *Mol Microbiol*. 2015; 98:302–317. [PubMed: 26172310]

- Peterson SN, Meissner T, Su AI, Snesrud E, Ong AC, Schork NJ, et al. Functional expression of dental plaque microbiota. *Front Cell Infect Microbiol*. 2014; 4:108. [PubMed: 25177549]
- Pieralisi FJ, Rodrigues MR, Segura VG, Maciel SM, Ferreira FB, Garcia JE, et al. Genotypic Diversity of *Streptococcus mutans* in Caries-Free and Caries-Active Preschool Children. *Int J Dent*. 2010; 2010:824976. [PubMed: 20351760]
- Phattarataratip E, Olson B, Broffitt B, Qian F, Brogden KA, Drake DR, et al. *Streptococcus mutans* strains recovered from caries-active or caries-free individuals differ in sensitivity to host antimicrobial peptides. *Mol Oral Microbiol*. 2011; 26:187–199. [PubMed: 21545696]
- Qi F, Kreth J. Methods to study antagonistic activities among oral bacteria. *Methods Mol Biol*. 2017; 1537:203–218. [PubMed: 27924596]
- Takahashi N, Nyvad B. The role of bacteria in the caries process: ecological perspectives. *J Dent Res*. 2011; 90:294–303. [PubMed: 20924061]
- Westergren G, Emilson CG. Prevalence of transformable *Streptococcus mutans* in human dental plaque. *Infect Immun*. 1983; 41:1386–1388. [PubMed: 6885168]
- Xiao J, Klein MI, Falsetta ML, Lu B, Delahunty CM, Yates JR, et al. The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. *PLoS Pathog*. 2012; 8:e1002623. [PubMed: 22496649]
- Yamamoto Y, Higuchi M, Poole LB, Kamio Y. Identification of a new gene responsible for the oxygen tolerance in aerobic life of *Streptococcus mutans*. *Biosci Biotechnol Biochem*. 2000; 64:1106–1109. [PubMed: 10879495]
- Zhu L, Kreth J. The role of hydrogen peroxide in environmental adaptation of oral microbial communities. *Oxid Med Cell Longev*. 2012; 2012:717843. [PubMed: 22848782]

Fig. 1. Mixed-species biofilm formation by clinical isolates in UFTYE

A) Bacterial species were inoculated in the culture medium supplemented with 0.1% (w/v) sucrose until 29 h for establishment of initial biofilm community. The biofilms were then challenged with an environmental change by introducing 1% (w/v) sucrose. Viable population of *S. mutans*, *S. oralis* and *A. naeslundii* recovered from the biofilms were counted (number of CFU recovered per biofilm) over time and the proportion of each strain at each time point was calculated based on CFU data. B) The percentage of *S. mutans* after exposure to 1% sucrose at 67 h and 91 h. After the introduction of 1% sucrose at 29 h, the proportions of *S. mutans* UA159 and Smu44 rapidly increased, and became the dominant species in 91 h biofilms. Smu56 displayed a much slower population shift, representing a noticeably lower proportion compared to UA159 at 67 h. While, Smu81 was never able to overtake the other species, with a strikingly lower proportion of the bacteria present at 67 h and 91 h, compared to biofilms formed with the other *S. mutans* strains. Results represent the mean $\pm$ standard deviation. Statistical analysis was performed using One-way ANOVA with post-hoc Tukey HSD test. Each strain was compared to UA159 at each time point. ** P 0.001.

Fig. 2. Susceptibility of *S. mutans* clinical isolates to growth inhibition by *S. oralis* and to killing by hydrogen peroxide

A) Growth inhibition of *S. mutans* clinical isolates (Smu44, Smu56, Smu81) in response to *S. oralis*. *S. mutans* UA159 is resistant to growth inhibition by *S. oralis* and is included for comparison (left). Growth inhibition of *S. mutans* clinical isolates by *S. oralis* is not observed in the presence of 300 Units of Catalase, which degrades H₂O₂ (right). Images are representative results of quadruplicate experiments. B) Results of hydrogen peroxide killing assay. Aliquots of cells suspended in glycine buffer (pH 7) containing H₂O₂ were removed at indicated times (y-axis), and percent survival (x-axis) was determined based on CFU. Assay was performed with triplicate biological samples and repeated one time with similar results. Statistical analysis was performed using Two-way ANOVA with post-hoc Tukey test. Each strain was compared to UA159 at each time point. * $P < 0.01$, ** $P < 0.001$, and *** $P < 0.0001$.

Fig. 3. Expression of oxidative stress genes and NADH oxidase activity in *S. mutans* clinical isolates

(A) Expression of *dpr*, *ahpF*, *ahpC*, and *nox* genes, quantitated by real-time PCR (qRT-PCR), were compared between *S. mutans* strains treated with or without H_2O_2 . Results represent triplicate biological samples with three technical replicates. Student's *t*-test was used to determine significance. * = $P < 0.01$. (B) Results of statistical analysis comparing expression of oxidative stress genes in each clinical isolate to reference strain UA159. * = $P < 0.05$, determined by One-way ANOVA and post-hoc Tukey HSD test. * denotes clinical isolates that had significantly different level of expression of indicated genes compared to UA159. (C) NADH oxidase activity produced by each strain after a 30 or 45 min exposure to H_2O_2 compared to untreated controls (no H_2O_2). Results represent the mean $\pm$ standard error of nmol NADH oxidized $\text{min}^{-1} \text{mg}^{-1}$ protein. * = $P < 0.05$, determined by Student's *t*-test comparing H_2O_2 treated to untreated samples. Assay results represent triplicate biological samples. (D) Statistical analysis comparing *S. mutans* isolates to reference strain UA159 grown under the same conditions. Statistical differences indicated by * ($P < 0.05$) determined by One-way ANOVA and post-hoc Tukey HSD test. * denotes clinical isolates that expressed significantly different level of NADH oxidase activity compared to UA159.

Table 1

S. mutans clinical isolates used in this study.

Accession number	Lab ID	Strain name	Notable characteristics compared to reference strains <i>S. mutans</i> UA159*	Host/Sample	Origin/MLST	Serotype
ASM33925	Smu44	11VS1	More aciduric, forms poor biofilms in sucrose, forms more biofilms in glucose, contains type VII secretion system	30 months, initial stages of oral colonization, caries free	Brazil/ST28	C
ASM33933	Smu56	N29	Slow growth, forms equal biofilms in sucrose and glucose, sensitive to oxidative stress	Dental plaque	UK/SK11	C
ASM33943	Smu81	SF14	Oxidative stress sensitive, <i>cmm+</i>	Supragingival plaque	US/ST58	C

* Information adapted from Palmer *et al.*, 2013 *PLoS ONE*

Table 2

Growth characteristics of different *S. mutans* strains, grown with or without H₂O₂.

Growth	Condition	UA159	Smu44	Smu56	Smu81
Non-stress	T _d	49±2	52±6	119±4	60±3
	Lag	210	210	300	210
	Yield	0.94	0.95	0.82	1.00
250 µM H ₂ O ₂	T _d	55±1 [‡]	79±2 [‡]	135±11	71±3 [‡]
	Lag	260 [‡]	290 [*]	610 [*]	530 [*]
	Yield	0.96	0.89	0.86	0.80 [*]

Mean growth rate (T_d) and growth lag with standard deviation are expressed as min, and were calculated based on growth curves. Yield is the mean maximum OD₆₀₀ reached during growth. Significance is indicated by

^{*} = *P* 0.001,

[‡] = *P* 0.01, and

[‡] = *P* 0.05, and compares non-stress growth to growth with H₂O₂.

Table 3

S. mutans clinical isolates contain polymorphisms within oxidative stress genes when compared to UA159

Gene	Enzyme	Function	Polymorphisms*
<i>ahpF</i>	Nox-1 NADH oxidase	Reduces 2NADH + O ₂ -> H ₂ O ₂	Smu81 A195 to T; P197 to S
<i>ahpC</i>	Alkyl hydroperoxide reductase	Deactivates H ₂ O ₂	100 % identical
<i>dpr</i>	Dps-like peroxide resistance gene	Fe+ binding protein - prevents Fenton reactions	Smu81 T43 to A
<i>nox-2</i>	NADH oxidase	H ₂ O forming NADH oxidase	Smu81 R130 to C; H324 to Y; T459 to I Smu56 D233 to N; R430 to H Smu44 T430 to I
<i>spxAI</i>	Transcriptional regulator	Major regulator of oxidative stress genes	Smu81 A18 to T; S35 to N; K99 to R

Polymorphisms are based on sequence alignments performed using shotgun genome sequence for clinical isolates compared to *S. mutans* reference strain UA159. NCBI accession numbers for each strain are listed in Table 1. Note: The gene for superoxide dismutase (Smu.620) was 100% identical in all strains.