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Fine-tuned production of hydrogen peroxide promotes biofilm formation of *Streptococcus parasanguinis* by a pathogenic cohabitant *Aggregatibacter actinomycetemcomitans*

Dingyu Duan^{1,2}, Jessica A. Scofield¹, Xuedong Zhou², and Hui Wu^{1,*}

¹Departments of Pediatric Dentistry and Microbiology, Schools of Dentistry and Medicine, University of Alabama at Birmingham, Birmingham, AL 35294

²State Key Laboratory of Oral Diseases, Sichuan University, Chengdu 610041, China

Summary

Balanced bacterial biofilm communities help to maintain host health. Disturbance of such communities can lead to bacterial dysbiosis and pathogenesis. However, complex and dynamic bacterial interactions within the biofilm communities are poorly understood. In this study, we used a dual-species biofilm consisting of the periodontal pathogen *Aggregatibacter actinomycetemcomitans*, and a commensal *Streptococcus parasanguinis* to investigate bacterial interactions since the two organisms have been found to coexist during the development of localized aggressive periodontal disease. We report that *A. actinomycetemcomitans* promoted biofilm formation of *S. parasanguinis* *in vitro* and *in vivo*. Protein profiling of *S. parasanguinis* co-cultured with *A. actinomycetemcomitans* revealed a significant decrease in the protein level of pyruvate oxidase(PoxL), an enzyme required for the generation of hydrogen peroxide (H₂O₂). Consistently, the H₂O₂ concentration was concurrently decreased. However, the complete removal of H₂O₂ impaired the biofilm formation. H₂O₂ at a low concentration range regulated by *A. actinomycetemcomitans* enhanced the biofilm formation. These results demonstrate that *A. actinomycetemcomitans* promotes the *S. parasanguinis* biofilm formation through modulating the production of H₂O₂ by fine-tuning the expression of *poxL*, indicating that H₂O₂ functions as a signaling molecule. Taken together, this report revealed a previously unknown bacteria-bacteria interaction mechanism.

Keywords

Dual-species biofilm; hydrogen peroxide; pyruvate oxidase; *Streptococcus*; *Aggregatibacter actinomycetemcomitans*

Introduction

A large number of microbes live in the human oral cavity, and they often grow within well-organized multispecies microbial communities known as dental biofilms (Kolenbrander et

* Address for correspondence: Hui Wu, Department of Pediatric Dentistry, University of Alabama at Birmingham, School of Dentistry, 1919 7th Avenue South, Birmingham, AL 35294, hwu@uab.edu, Tel. (+1)205-966-2392, Fax (+1)205-975-4431.

al., 2002; Aas et al., 2005; Paster et al., 2006; Kolenbrander et al., 2010; Zijng et al., 2010). Dental caries and periodontal disease, the most prevalent oral diseases, are modulated by the biofilm formation. Biofilm-driven infectious diseases are more difficult to control and treat by routine oral hygiene procedures (Hall-Stoodley et al., 2004). The composition and interactions between diverse species are very important in shaping the microbial balance within the host (Kolenbrander et al., 2002; Jenkinson and Lamont, 2005; Kuramitsu et al., 2007; Kolenbrander et al., 2010). Often, the bacterial communities live in harmony with healthy hosts, however, pathogenic conditions disrupt the community balance, which promotes the development of oral infectious diseases. Many mono-species infection models have been established and investigated, which offer a wealth of basic insights into the mechanisms of bacterial pathogenesis, however, the complex and dynamic bacterial interactions within the multispecies community are poorly understood. Oral bacterial species exhibit metabolic cooperation as well as antagonistic competitions within the oral cavity (Hibbing et al., 2010; Ramsey et al., 2011). To investigate the specific mechanisms that mediate the interspecies interaction we established a two-species biofilm model composed of an oral commensal, *Streptococcus parasanguinis*, and the opportunistic pathogen, *Aggregatibacter actinomycetemcomitans*, to determine how the interspecies interactions impact their fitness and virulence.

The gram-negative, facultative anaerobic bacterium *A. actinomycetemcomitans* is a causative agent of localized aggressive periodontitis (LAP) (Ebersole et al., 1994; Meyer and Fives-Taylor, 1998). *A. actinomycetemcomitans* often resides in the subgingival crevice in the presence of numerous oral streptococci (Kolenbrander et al., 2002; Kreth et al., 2009a). Abundant oral streptococci are typically non-pathogenic, and are frequently associated with periodontal health (Paster et al., 2001). However, the presence of *Streptococcus gordonii* up-regulates the complement resistance protein ApiA of *A. actinomycetemcomitans* to enhancing *A. actinomycetemcomitans* virulence by promoting its resistance to host innate immunity (Ramsey and Whiteley, 2009). Moreover, *A. actinomycetemcomitans* prefers to utilize lactic acid produced by streptococci as an energy source (Brown and Whiteley, 2007). This metabolite cross-feeding is also critical for enhancing *A. actinomycetemcomitans* virulence (Ramsey et al., 2011). A clinical cohort study documented the coexistence of *A. actinomycetemcomitans* and *S. parasanguinis*, suggesting this bacterial consortium can be used as an indicator of future bone loss in LAP (Fine et al., 2013). Furthermore, recent studies suggest that *S. parasanguinis* might play a different role in the development of periodontal disease from other oral streptococci. Unlike other streptococci such as *S. gordonii*, *S. parasanguinis* was found more prevalent in refractory periodontitis patients than in both good responders and periodontal health patients (Colombo et al., 2009; Colombo et al., 2012). Therefore, the interactions between *A. actinomycetemcomitans* and *S. parasanguinis* are dynamic and may be different from the reported interaction between *A. actinomycetemcomitans* and *S. gordonii*.

In the present study, we report that *A. actinomycetemcomitans* promotes *S. parasanguinis* biofilm formation via a previously unknown mechanism. *A. actinomycetemcomitans* fine-tuned down-regulation of hydrogen peroxide (H₂O₂) production of *S. parasanguinis* to promote the biofilm formation, which is abolished by either deletion of the H₂O₂ producing pyruvate oxidase or removing H₂O₂ by catalase. These data suggest that H₂O₂ acts as a

signaling molecule. Our studies reveal a unique bacterial interaction between *S. parasanguinis* and *A. actinomycetemcomitans*.

Results

A. actinomycetemcomitans promotes biofilm formation of *S. parasanguinis*

To better understand how interspecies interactions between *S. parasanguinis* and *A. actinomycetemcomitans* impact their virulence properties, we established an *in vitro* two-species biofilm model and determined whether *A. actinomycetemcomitans* and *S. parasanguinis* have synergetic effects when co-cultured. Crystal violet staining of bacterial biofilms grown in 96-well microtiter plates was used to assess the biofilm formation of the dual-species model. Indeed, *A. actinomycetemcomitans* dose-dependently enhanced biofilm formation during the 16 h co-culture (Fig. 1A). On the other hand, adding higher numbers of *S. parasanguinis* to *A. actinomycetemcomitans* did not enhance the biofilm formation (Fig. S1). The effect of *A. actinomycetemcomitans* on *S. parasanguinis* appeared to be specific since *A. actinomycetemcomitans* did not promote biofilm formation of other streptococci such as *S. gordonii* and *S. sanguinis* (Fig. S2). To determine the ratio of each organism in the dual species biofilm, we examined the dual-species biofilms and enumerated *A. actinomycetemcomitans* and *S. parasanguinis*. Although 16 h mature biofilms were very stable and ideal for the biofilm biomass staining assay, fewer live cells were present and no live *A. actinomycetemcomitans* was recovered in the 16 h dual-species biofilm (Fig. S3). So we employed the 6 h biofilm that exhibited the same biofilm enhancement as the 16 h biofilm, and also reached to the exponential growth phase to assess bacterial viability. In 6 h biofilm, fewer *A. actinomycetemcomitans* were recovered (Fig. 1B), suggesting that the viability of *A. actinomycetemcomitans* was significantly reduced by *S. parasanguinis*. In contrast, the number of *S. parasanguinis* recovered was drastically increased by at least 40-fold in the dual-species biofilm (Fig. 1B). The growth of planktonic *S. parasanguinis* was also enhanced by *A. actinomycetemcomitans* (Fig. S4), but the increase is less dramatic in comparison with the biofilm (4-fold versus 40-fold).

A. actinomycetemcomitans alters three-dimensional structure of *S. parasanguinis* biofilms

To further characterize the interaction, we examined the effect of *A. actinomycetemcomitans* on the 16 h biofilm structure of *S. parasanguinis* using confocal laser scanning microscopy. The biofilm of *S. parasanguinis* or *A. actinomycetemcomitans* alone was very thin and did not cover the entire biofilm surface area. The dual-species biofilm was much thicker and covered the majority of the surface area (Fig. 2A). The number of *S. parasanguinis* was significantly increased in the dual-species biofilm, while only small numbers of *A. actinomycetemcomitans* clusters were observed in the dual-species biofilm. Quantitative analysis of these biofilm images further confirmed increased biomass and biovolume of the dual species biofilm (Fig. 2B).

Physical interaction contributes to but is not essential for enhanced biofilm formation of *S. parasanguinis* by *A. actinomycetemcomitans*

To determine if the direct physical contact between *S. parasanguinis* and *A. actinomycetemcomitans* is required for the enhanced biofilm formation, we separated *A.*

actinomycetemcomitans from *S. parasanguinis* in a transwell insert system by a permeable membrane, which would only allow the growth media and small molecules to permeate. Crystal violet staining was used to measure the 16 h biofilm formation in transwells. Without the direct contact, *A. actinomycetemcomitans* was still able to promote the biofilm formation of *S. parasanguinis*, however the degree of the increase was significantly reduced (Fig. 3A).

Co-culture with *A. actinomycetemcomitans* down-regulates *S. parasanguinis* pyruvate oxidase

To identify factors mediating the interaction, we examined the protein profile of each organism in the presence of its partner using the transwell system. The presence of *S. parasanguinis* did not significantly alter the protein profile of *A. actinomycetemcomitans* cell lysates (Fig. S5). The protein profile of *S. parasanguinis* was also examined and compared. The presence of *A. actinomycetemcomitans* did not alter overall protein profile, but the intensity of a ~65 kDa protein band was decreased (Fig. 3B). LC-MS/MS analysis revealed it was pyruvate oxidase (PoxL). PoxL is responsible for production of H₂O₂. We also performed the protein profile analysis of the 6 h biofilm on the SDS-PAGE gel, but didn't observe any changes (data not shown). This might be due to only low concentration of PoxL accumulated at this immature biofilm stage. To further evaluate the effect of *A. actinomycetemcomitans*, we determined the expression of *poxL* with the 6 h biofilm using real-time RT-PCR. We determined that the expression of *poxL* was downregulated (Fig. 3C). These data indicate that the production of H₂O₂ by the *poxL* regulation mediates the interaction between *A. actinomycetemcomitans* and *S. parasanguinis*. Such a regulation is specific for *S. parasanguinis* as PoxL of other oral streptococci was not downregulated by *A. actinomycetemcomitans* in 16 h transwell co-culture (Fig. S6).

H₂O₂ is crucial for the dual-species biofilm formation

To test whether H₂O₂ is involved in this two-species interaction, we first measured the concentration of H₂O₂ produced by single- and dual-species biofilms. Consistent with the down-regulation of *poxL*, the concentration of H₂O₂ in the co-culture was significantly reduced (Fig. 4A), suggesting that down-regulation of the H₂O₂ production is crucial. To our surprise, when we evaluated the biofilm formation of the *S. parasanguinis* using a PoxL deficient mutant (*poxL*), we found the mutant lost its ability to form the biofilm with *A. actinomycetemcomitans*, while the complemented strain partially restored the phenotype (Fig. 4B). These data suggest the requirement of H₂O₂ for the biofilm formation. Since PoxL plays a role in metabolic activities in addition to producing H₂O₂, catalase was used to directly deplete H₂O₂ to further confirm the role of H₂O₂ in the biofilm formation (Fig. 4C, 4D). The addition of catalase at a low concentration did not have any impact on the dual-species biofilm formation. As the concentration of catalase increased, which decreased H₂O₂ levels gradually, a dose-dependent reduction in the dual-species biofilm formation was evident. Eventually, the biofilm was abolished when H₂O₂ was completely depleted by the addition of a higher concentration (500 U/mL) of catalase. Catalase did not inhibit bacterial growth at tested concentrations (Fig. S7). To determine if the modulation of H₂O₂ production is the major factor that drives the biofilm formation of *S. parasanguinis*, we added catalase to *S. parasanguinis* mono-species culture and measured the biofilm

formation. As the catalase concentrations increased, the mono-species biofilm formation was first increased and then decreased (Fig. S8) although the degree of the biofilm enhancement is different from the dual-species biofilms. Taken together, these results suggest that H₂O₂ plays an important role in modulating the dual-species biofilm formation, and appropriate concentrations of H₂O₂ are important for the process.

Fine-tuned production of H₂O₂ promotes biofilm formation by *S. parasanguinis*

To further validate the hypothesis that fine-tuned down-regulation of H₂O₂ is crucial for biofilm formation, a regulatable gene induction system (Xie et al., 2013) was used to fine-tune the expression of *poxL* by *S. parasanguinis*. The *poxL* gene was introduced into the xylose-inducible plasmid pZX9 to generate plasmid pZX9-*ipoxL* and transformed into the *poxL* deficient mutant. Various concentrations of xylose were used to induce the expression of *poxL*. Consistent with our previous data, low concentrations (between ~10 to 40 μM/A₄₇₀) of H₂O₂ promoted biofilm formation of the *poxL* mutant (Fig. 5). Since higher concentration of xylose started to inhibit the growth of *S. parasanguinis*, it cannot be used to generate higher concentration of H₂O₂. We added exogenous H₂O₂ to the *poxL* mutant to assess the effect of higher concentrations of H₂O₂ on the *poxL* biofilm (Fig. S9). The biofilm formation of *poxL* was increased when 0.2mM of H₂O₂ was used, but decreased at 0.5mM of H₂O₂, again suggesting appropriate concentrations of H₂O₂ are needed. As the external H₂O₂ cannot be added in the same manner that mimics H₂O₂ produced by *S. parasanguinis*, and H₂O₂ is not a stable molecule, it undergoes degradation all the time, a higher concentration of H₂O₂ (0.2 mM) was required to be added to promote the biofilm formation. Together these data demonstrate that fine-tuned production of appropriate concentrations of H₂O₂ mediate *S. parasanguinis* biofilm formation.

A. actinomycetemcomitans catalase is not involved in H₂O₂-mediated biofilm formation

Since H₂O₂-mediated biofilm formation by *S. parasanguinis* in the dual-species biofilm with *A. actinomycetemcomitans* is altered by exogenous catalase, an enzyme that detoxifies H₂O₂, we questioned whether the catalase enzyme that *A. actinomycetemcomitans* harbors (Ramsey and Whiteley, 2009) would impact the biofilm formation in the dual species. Therefore, we constructed an *A. actinomycetemcomitans* *katA* mutant (Fig. S10) and examined the effect of the *A. actinomycetemcomitans* catalase deficient mutant (*katA*) on the dual-species biofilm formation. Interestingly, the catalase deficient *A. actinomycetemcomitans* still down-regulated H₂O₂ level and promoted the biofilm formation of *S. parasanguinis* (Fig. 6AB). In addition, the PoxL level of *S. parasanguinis* was also decreased by the *katA* mutant (Fig. 6C), indicating that degradation of H₂O₂ by *A. actinomycetemcomitans* catalase did not contribute to the modulation of the PoxL level, and an alternative mechanism exists. Notably, the *katA* mutant is more sensitive to killing by *S. parasanguinis* (Fig. S11A), despite the fact it does not have any impact on the dual species biofilm formation.

A. actinomycetemcomitans enhanced the ability of *S. parasanguinis* to colonize *Drosophila melanogaster*

A *Drosophila melanogaster* model was used to investigate colonization of *A. actinomycetemcomitans* and *S. parasanguinis* *in vivo*. *Drosophila* flies were fed with single

species cultures of *A. actinomycetemcomitans* and *S. parasanguinis*, and a mixture of both organisms respectively. Single species or dual species colonization did not affect the survival rate of *Drosophila* (data not shown). However, 24 h and 48 h post-infection, *A. actinomycetemcomitans* significantly promoted the ability of *S. parasanguinis* to colonize. In addition, the deletion of *poxL* from *S. parasanguinis* reduced its ability to colonize with *A. actinomycetemcomitans*. At 24 h post-infection, the *poxL* mutant exhibited reduced colonization. At the 48 h post-infection, the mutant no longer colonized (Fig. 7A). Moreover, the number of *A. actinomycetemcomitans* was significantly reduced in flies when co-colonized with *S. parasanguinis* (Fig. 7B). These *in vivo* data are consistent with the *in vitro* findings, demonstrating the importance of H₂O₂ in modulating the enhanced biofilm formation of *S. parasanguinis* by *A. actinomycetemcomitans* *in vivo*.

Discussion

Oral streptococci are abundant bacterial residents, and often associated with periodontal health in the oral cavity (Paster et al., 2001). *S. parasanguinis* is one of the primary colonizers (Carlsson et al., 1970) and is also an opportunistic pathogen that can cause infective endocarditis (Burnette-Curley et al., 1995; Kitten et al., 2002). A growing body of evidence suggests that *S. parasanguinis* plays more complicated roles than we previously understood in a periodontal biofilm community (Colombo et al., 2009; Colombo et al., 2012; Fine et al., 2013), it may contribute positively to the pathogenesis of the disease. *A. actinomycetemcomitans*, a late colonizer and periodontal pathogen, coexists with *S. parasanguinis*, which has been associated with the onset of LAP (Fine et al., 2013). Little is known about the detailed interaction between the two bacteria when the late colonizer fights its way to be incorporated into the earlier community.

In this study, we reported the involvement of *A. actinomycetemcomitans* in promoting biofilm formation of *S. parasanguinis* by modulating the production of H₂O₂. In the *S. parasanguinis* mono-species biofilm, growth and biofilm formation was inhibited and limited by the overproduction of H₂O₂. However, the presence of *A. actinomycetemcomitans* significantly altered the microenvironment, and down-regulated the H₂O₂ production of *S. parasanguinis* by mediating the PoxL level. Interestingly, completely elimination of the H₂O₂ by either deleting the *poxL* gene in *S. parasanguinis* or using excessive amounts of exogenous catalase impaired the dual-species biofilm formation, suggesting that H₂O₂ is crucial for *S. parasanguinis* to interact with *A. actinomycetemcomitans*. Low concentrations of H₂O₂, which was either fine-tuned by *A. actinomycetemcomitans*, or modulated by the use of an inducible promoter, dose-dependently promoted the *S. parasanguinis* biofilm formation, suggesting a signaling nature of H₂O₂ in the co-culture.

H₂O₂ has long been regarded as a bactericide. H₂O₂-based disinfectants are widely used to control bacterial colonization and contamination in a number of medical applications (Linley et al., 2012). Previous studies have documented the role of H₂O₂ produced by commensal streptococci in the control of dental pathogens (Kreth et al., 2008; Qi and Kreth, 2010; Giacaman et al., 2015; Herrero et al., 2016). H₂O₂ was also considered as a potentially harmful byproduct of aerobic metabolism since it generates reactive oxygen species. However, mounting evidence has now shown that at low concentrations, H₂O₂ can function

as a signaling molecule that is distinct from its traditional role in the oxidative stress in eukaryotic cells (Lara-Ortíz et al., 2003; Stone and Yang, 2006; Veal et al., 2007; Nasution et al., 2008; Semighini and Harris, 2008; Veal and Day, 2011). Consistent with this notion, we reported that production of H₂O₂ by *S. parasanguinis* was tightly regulated by *A. actinomycetemcomitans*, and the appropriate concentrations of H₂O₂ promote biofilm formation dose-dependently.

In a mono-species biofilm model, H₂O₂ produced by another metabolic enzyme, lysine oxidase, mediates bacterial cell death during the biofilm development (Mai-Prochnow et al., 2008). The cell death modulated by the production of H₂O₂ through *S. parasanguinis* PoxL is unlikely to be involved in such biofilm formation, as the *poxL* mutant is still capable of killing *A. actinomycetemcomitans* in the co-culture (Fig. S11B), however, it failed to form a robust dual-species biofilm with *A. actinomycetemcomitans*. In addition, increased killing of catalase deficient *A. actinomycetemcomitans* in the co-culture did not lead to any change in the formation of the dual species biofilm. H₂O₂ can also induce extracellular DNA (eDNA) release from oral streptococci without causing autolysis (Kreth et al., 2009b). Thus, it is possible that increased eDNA release of the dual-species biofilm modulated by H₂O₂ may promote bacterial biofilm formation. However, the increased biofilm formation of *S. parasanguinis* and *A. actinomycetemcomitans* was not accompanied by an increase in eDNA release. In fact, the released eDNA was reduced in the dual-species community (Fig. S12). Our studies indicate a novel eDNA-release-independent function of H₂O₂ in promoting biofilm formation.

A. actinomycetemcomitans dispersin (DspB), an enzyme that degrades biofilm matrix and disperses biofilms, was up-regulated by H₂O₂ when co-cultured with *S. gordonii* (Stacy et al., 2014). However, expression of *dspB* was not significantly changed by *S. parasanguinis* (data not shown). *A. actinomycetemcomitans* increased activity of catalase that degrades H₂O₂ during the co-culture with *S. gordonii* (Ramsey and Whiteley, 2009). Intriguingly, we found that the catalase deficient mutant didn't exhibit any defect in lowering H₂O₂ production by *S. parasanguinis* in the co-culture (Fig. 6B). Paradoxically, exogenous addition of catalase reduced H₂O₂ levels and concurrently altered the biofilm formation. It is possible that the amount of catalase produced by *A. actinomycetemcomitans* in the presence of *S. parasanguinis* is not sufficient to degrade H₂O₂, and *A. actinomycetemcomitans* developed an alternative mechanism to efficiently down regulate the expression of *S. parasanguinis* *poxL*. Furthermore, this regulation is specific for *S. parasanguinis* as the amount of PoxL produced by other oral streptococci in the presence of *A. actinomycetemcomitans* was not altered (Fig. S6). The mechanism by which *A. actinomycetemcomitans* down-regulates the expression of *poxL* is under active investigation.

In this study, we also observed that *S. parasanguinis* antagonized *A. actinomycetemcomitans*, however, H₂O₂ did not contribute significantly to this phenotype (Fig. S11), indicating there is an alternative mechanism by which *S. parasanguinis* competes with *A. actinomycetemcomitans*. The ability of *S. parasanguinis* to become more competitive in the dual-species setting could have significant clinical implications. In a healthy periodontal condition, such mechanism would allow *S. parasanguinis* to attenuate *A. actinomycetemcomitans* virulence. However, under dysbiotic circumstances, the

interaction is altered drastically by the influence of other bacteria or the host environment. Overgrowth of *A. actinomycetemcomitans* could explain why *S. parasanguinis* is more prevalent in LAP or refractory periodontitis (Colombo et al., 2009; Colombo et al., 2012; Fine et al., 2013) since *A. actinomycetemcomitans* can promote the biofilm formation of *S. parasanguinis*. Studies are ongoing to investigate the molecular details of the interaction. For instance, what components from *A. actinomycetemcomitans* mediate the H₂O₂ production and biofilm formation of *S. parasanguinis*, how production of H₂O₂ promotes biofilm formation, and what factors from *S. parasanguinis* are modulated by fine tuned H₂O₂ to promote the biofilm formation. The transwell biofilm assay revealed that the direct contact between *S. parasanguinis* and *A. actinomycetemcomitans* also contributes to the dual-species biofilm formation (Fig. 3A). Further study is required to explore the underlying contact-dependent mechanism. In conclusion, this study revealed a previously unknown mechanism by which an oral commensal responds to and hijacks an opportunistic pathogen to increase its own biofilm formation and fitness.

Experimental Procedures

Bacterial strains and growth conditions

A. actinomycetemcomitans VT 1169 strain used in this study is derived from the clinical strain SUNY465. VT 1169 is a spontaneous rifampicin and nalidixic acid resistant strain, which was utilized as the recipient strain in conjugation experiments. All *A. actinomycetemcomitans* strains were grown aerobically (5% CO₂) at 37°C in Tryptic soy broth (TSB, MP Biomedicals) supplemented with 0.5% yeast extract, termed TSBYE. *S. parasanguinis* FW213 was routinely grown aerobically (5% CO₂) at 37°C in Todd-Hewitt broth (THB, Difco). All *E. coli* strains were grown in Luria-Bertani (LB) broth at 37°C with aeration or on LB agar plates. Antibiotics were used at following concentrations: 10 µg/ml erythromycin, 125 µg/ml kanamycin, 100 µg/ml spectinomycin in THB liquid or agar plates for *S. parasanguinis*; 50 µg/ml spectinomycin, 100 µg/ml kanamycin in LB broth or agar plates for *E. coli*; 50 µg/ml spectinomycin, 5 µg/ml vancomycin, 100 µg/ml rifampicin, 50 µg/ml nalidixic acid in TSB liquid or agar plates for *A. actinomycetemcomitans*. The bacterial strains and plasmids used in this study are listed in Table 1.

Biofilm formation assay

Biofilm formation of *S. parasanguinis* and *A. actinomycetemcomitans* was assessed by crystal violet staining method. The optical density of cells at 470 nm (A₄₇₀) was used to monitor bacterial growth. Briefly, overnight cultures of *S. parasanguinis* and *A. actinomycetemcomitans* were subcultured in THB and TSB respectively and grown to exponential phases (A₄₇₀ 0.6~0.8). Since *A. actinomycetemcomitans* has a much slower growth rate than *S. parasanguinis*, we added more *A. actinomycetemcomitans* to the co-culture to make sure there was a considerable number of *A. actinomycetemcomitans* in the dual-species biofilm. *S. parasanguinis* cells were inoculated at 1:1000 dilution (1×10⁵ CFU/ml) while *A. actinomycetemcomitans* cells were inoculated at 1:100 dilution (1×10⁶ CFU/ml) in TSBYE containing 1% sucrose separately or mixed together for mono-species or dual-species biofilm assays. 200 µl of each culture were inoculated into wells of sterile 96-well plates (Nunc) and incubated at 37 °C in 5% CO₂ for 16 h. As designed, *A.*

actinomycetemcomitans was serially diluted into different concentrations and inoculated (10^4 to 10^6 CFU/ml). As indicated, bovine liver catalase (Sigma-Aldrich) was suspended in 50 mM potassium phosphate buffer (pH 7.0) and added to the medium to designated concentrations. For the 6 h biofilm, *S. parasanguinis* and *A. actinomycetemcomitans* were inoculated 1:100 and 1:10 respectively and incubated for 6 h. The biofilms were stained with 0.1% crystal violet and measured at 562 nm as previously described (Zhou et al., 2010). Each assay was carried out with duplicate wells and was repeated three times.

Viability of *S. parasanguinis* and *A. actinomycetemcomitans* in co-cultures

Planktonic *S. parasanguinis* and *A. actinomycetemcomitans* cells from mono-species or dual-species cultures were serially diluted and spread on TSBYE agar plates. *S. parasanguinis* colonies appeared after 1-day incubation whereas *A. actinomycetemcomitans* colonies were visible after 2-day incubation. To enumerate *S. parasanguinis*, colony forming units (CFU) were counted at 24 h. To enumerate *A. actinomycetemcomitans*, properly diluted cultures were plated on selective TSBYE agar containing 5 µg/ml vancomycin and CFUs were counted at 48 h. Biofilms were washed twice using PBS before being re-suspended and serially diluted and spread on agar plates to count CFUs.

Confocal laser scanning microscopy (CLSM) analysis

Bacterial strains were grown in TSBYE in wells of a sterile µ-Slide 8 Well plate (Ibidi) under 5% CO₂ at 37°C for 16 h. The biofilm samples were gently washed with PBS three times to remove unattached cells, and dried for 5 min. *S. parasanguinis* can be detected by F51, a monoclonal antibody that recognizes the surface protein Fap1 (Wu et al., 1998), *A. actinomycetemcomitans* was detected with a polyclonal antibody that binds to a surface protein Aae (Rose et al., 2003). Both antibodies were introduced into mono-species biofilms of *S. parasanguinis* or *A. actinomycetemcomitans* in 5% BSA PBS and incubated for 1 h, so the mono-species biofilms can serve as the controls for the specificity of the antibodies. Subsequently, the biofilms were washed 3 times with PBS to remove the unattached primary antibodies and then incubated with fluorescent-conjugated secondary antibodies (Molecular Probes) for 30 min. Alexa Fluor 594 (red)-conjugated goat anti-mouse IgG was used to stain *S. parasanguinis*. Alexa Fluor 488 (green)-conjugated goat anti-rabbit IgG was used to stain *A. actinomycetemcomitans*. The stained samples were then washed 3 times prior to detection by CLSM. Biofilms were examined by CLSM (LSM 710; Zeiss) with a 63x oil immersion objective as previously described (Peng et al., 2015). At least three independent experiments were performed by the same person on different days, and image stacks were acquired randomly. Thickness and bio-volume of biofilm were quantified by the program COMSTAT (Heydorn et al., 2000; Kreth et al., 2004).

Quantification of hydrogen peroxide

To detect the production of H₂O₂ by *S. parasanguinis*, bacterial cultures were centrifuged and harvested culture supernatants were filter-sterilized before quantification. H₂O₂ was measured immediately by using the Amplex red hydrogen peroxide/peroxidase assay kit (Molecular Probes). Standard curve established from given concentrations of H₂O₂ was used to determine the absolute concentration of H₂O₂.

Transwell assay

A 6-well transwell insert system was used. *A. actinomycetemcomitans* was separated from *S. parasanguinis* using a 0.4 µm pore polycarbonate membrane (Corning). *S. parasanguinis* was added to the bottom of the well, and *A. actinomycetemcomitans* was introduced to the top of the membrane. For 16 h biofilm assay in the transwell system, *S. parasanguinis* of the exponential phase culture was added at 1:1000 dilution (1×10^5 CFU/ml) and *A. actinomycetemcomitans* was added at 1:100 dilution (1×10^6 CFU/ml). When preparing bacterial cells for RNA purification and RT-PCR, *S. parasanguinis* was added at 1:100 dilution (1×10^6 CFU/ml), and *A. actinomycetemcomitans* was added at 1:10 dilution (1×10^7 CFU/ml) and incubated at 37 °C in 5% CO₂ for 6 h. As the 6 h dual species culture exhibited a similar biofilm enhancement phenotype to the 16 h co-culture, we used it to isolate total RNA and evaluated gene expression profile.

SDS-PAGE and mass spectrometry analysis

S. parasanguinis cells from the transwell system were collected and treated with LambdaSa2 lysin (Zhou et al., 2012) in NETN buffer to lyse the cells. Cell lysates were boiled in sample buffer (0.0625M Tris [pH6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.01% bromophenol blue) for 10min before being loaded onto 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). *A. actinomycetemcomitans* cells were harvested and directly boiled in sample buffer before loading onto 10% SDS-PAGE. Protein bands were visualized using Coomassie brilliant blue staining. The reduced abundance of a protein band in the co-culture, which migrated at ~65 kDa, was evident from SDS-PAGE analysis. This band was excised from the gels and digested with trypsin. The digested peptide fragments were analyzed for protein identification by liquid chromatography-tandem mass spectrometry (LC/MS/MS) as described previously (Bu et al., 2008). We repeated the SDS-PAGE experiment three times, the only reproducible change was this 65 kDa band. We also excised the bands from three biologic replicates and analyzed by mass spectrometry. Data from three different replicates all identified this band as the PoxL protein.

Transcriptional analysis of *poxL* by qRT-PCR

S. parasanguinis in transwells incubated with or without *A. actinomycetemcomitans* for 6 h were collected and total RNA was purified using RNeasy Mini Kit (Qiagen) and further treated with RNase-free DNase (Promega) to remove trace amounts of contaminated DNA. The isolated RNA was then reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-rad). The cDNA samples were then used to quantify the relative gene expression of *poxL* using a primer pair of QpoxLF and QpoxLR (Table 2) by real-time PCR using the iQ SYBR green supermix kit (Bio-Rad). 16S rRNA gene, a housekeeping gene, was used as a control to normalize the *poxL* expression. Relative quantitation of gene expression was calculated using comparative C_T method (C_T). We first, calculated the C_T value, $C_T = C_{T \text{ } poxL} - C_{T \text{ housekeeping}}$, and then C_T, using the equation of $C_T = C_{T \text{ sample}} - C_{T \text{ control sample}}$. Changes in the gene expression level (fold) was determined by 2^{-C_T} .

Construction of the xylose-inducible *poxL* expression plasmid

A regulatable gene induction system was employed to construct inducible *poxL* expression plasmid, as described (Xie et al., 2013). A xylose-inducible cassette Xyl-S1 with a streptococcal replicon was amplified from pZX9 with a primer pair of ISF-*poxL* and ISR-*poxL* (Table 2). Using the genomic DNA of *S. parasanguinis* FW213 as a template, the *poxL* open reading frame (ORF) was amplified with a primer pair of *poxLF*-IS and *poxLR*-IS. Concatemers were generated by prolonged overlap extension PCR (POE-PCR) using the two amplicons as described (Xie et al., 2013). The PCR product (plasmid) was purified with QIAGEN QIAprep Spin Miniprep Kit, and then transformed into the *poxL* strain. Transformants were selected on THB agar plates containing 100 µg/ml spectinomycin and confirmed by PCR. Inducible production of H₂O₂ by the transformant was validated by the hydrogen peroxide assay.

Construction of the *A. actinomycetemcomitans* catalase deficient mutant (*katA*)

To generate *katA* mutant of *A. actinomycetemcomitans*, a spectinomycin resistance gene (*aad9*) was inserted to replace the *katA* gene via an allelic replacement approach as previously described (Mintz et al., 2002; Mintz, 2004). The fragments of 1000 bp upstream and 1000 bp downstream of the *katA* coding sequence was PCR amplified from *A. actinomycetemcomitans* genomic DNA. Fusion PCR was performed to ligate the upstream, *aad9* and the downstream fragment together. The ~3k bp fusion PCR product was restriction digested and ligated to pVT1460 and transformed. The resulting plasmid was introduced into *A. actinomycetemcomitans* via conjugation. Nalidixic acid, rifampicin and spectinomycin resistant colonies were replica-plated on TSBYE plates containing 100 µg/ml kanamycin. Colonies that were spectinomycin resistant and kanamycin sensitive were analyzed by colony PCR to confirm the allelic replacement of *katA* in the chromosome (Fig. S10). In brief, *katA* cannot be amplified from the *katA* mutant we constructed, while the spectinomycin resistant cassette *aad9* was amplified from the mutant. And the primer pair flanking *katA* amplified different sizes of products from wild type and the *katA* mutant, respectively. We sequenced the mutant allele from the *katA* mutant, and confirmed that the *katA* gene was deleted. We also carried out the catalase test using 30% H₂O₂. 30% H₂O₂ was dropped onto bacterial colonies of wild type *A. actinomycetemcomitans* and *katA* mutant, bubbles were evident from the wild type bacteria while the *katA* mutant did not produce any bubbles, suggesting that the mutant had a negative catalase activity (data not shown).

Colonization of *Drosophila melanogaster* by *S. parasanguinis* and *A. actinomycetemcomitans*

S. parasanguinis and *A. actinomycetemcomitans* infection of *Drosophila melanogaster* flies was performed as previously described (Schofield and Wu, 2015). Briefly, *Drosophila* flies were maintained on Jazz-Mix *Drosophila* food (Fisher). To infect flies, *S. parasanguinis* was grown to an A₄₇₀ of 2.0 and 1.5 ml of the culture was harvested. *A. actinomycetemcomitans* was grown to an A₄₇₀ of 0.8 and 4 ml of the culture was harvested. The harvested cell pellets of the two species were re-suspended respectively or mixed together in 100 µl sterile 5% sucrose to desirable cell density (10⁹~10¹⁰ CFU/mL). The re-suspended cells were spotted

onto a sterile 21-mm filter paper disc (Whatman) that was placed on the surface of 5 ml of solidified 5% sucrose agar in a plastic vial (FlyBase). Male *Drosophila* flies (1 to 3 days old) were treated with antibiotics (erythromycin, vancomycin, and ampicillin at 50 µg/ml) for 3 days and transferred to fresh medium for 3 days to remove residual antibiotics. All the flies were starved for 4 h before being added to vials (10 flies per vial). To determine the number of viable bacterial cells inside the flies, the surface of the flies were sterilized by treating with 70% ethanol for 30s and washed 3 times with sterile PBS. Flies were crushed with tips in an Eppendorf tube with 100 µl of saline. Serial dilutions of the homogenate were spread on TSBYE agar plates. Colonies of *S. parasanguinis* appeared after overnight incubation at 37°C. Colonies of *A. actinomycetemcomitans* were visible after 2 days incubation at 37°C. Vancomycin (5µg/ml) was added to TSBYE agar plate to selectively grow *A. actinomycetemcomitans* colonies.

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. *Journal of clinical microbiology*. 2005; 43:5721–5732. [PubMed: 16272510]
- Brown SA, Whiteley M. A novel exclusion mechanism for carbon resource partitioning in *Aggregatibacter actinomycetemcomitans*. *Journal of bacteriology*. 2007; 189:6407–6414. [PubMed: 17586632]
- Bu S, Li Y, Zhou M, Azadin P, Zeng M, Fives-Taylor P, Wu H. Interaction between two putative glycosyltransferases is required for glycosylation of a serine-rich streptococcal adhesin. *J Bacteriol*. 2008; 190:1256–1266. [PubMed: 18083807]
- Burnette-Curley D, Wells V, Viscount H, Munro CL, Fenno JC, Fives-Taylor P, Macrina FL. FimA, a major virulence factor associated with *Streptococcus parasanguis* endocarditis. *Infection and immunity*. 1995; 63:4669–4674. [PubMed: 7591121]
- Carlsson J, Grahén H, Jonsson G, Wikner S. Establishment of *Streptococcus sanguis* in the mouths of infants. *Arch Oral Biol*. 1970; 15:1143–1148. [PubMed: 5280120]
- Colombo APV, Bennet S, Cotton SL, Goodson JM, Kent R, Haffajee AD, et al. Impact of periodontal therapy on the subgingival microbiota of severe periodontitis: comparison between good responders and individuals with refractory periodontitis using the human oral microbe identification microarray. *Journal of periodontology*. 2012; 83:1279–1287. [PubMed: 22324467]
- Colombo APV, Boches SK, Cotton SL, Goodson JM, Kent R, Haffajee AD, et al. Comparisons of subgingival microbial profiles of refractory periodontitis, severe periodontitis, and periodontal health using the human oral microbe identification microarray. *J Periodontol*. 2009; 80:1421–1432. [PubMed: 19722792]
- Ebersole J, Cappelli D, Sandoval MN. Subgingival distribution of *A. actinomycetemcomitans* in periodontitis. *Journal of clinical periodontology*. 1994; 21:65–75. [PubMed: 8144736]
- Fine DH, Markowitz K, Fairlie K, Tischio-Bereski D, Ferrendiz J, Furgang D, et al. A consortium of *Aggregatibacter actinomycetemcomitans*, *Streptococcus parasanguinis*, and *Filifactor alocis* is present in sites prior to bone loss in a longitudinal study of localized aggressive periodontitis. *Journal of clinical microbiology*. 2013; 51:2850–2861. [PubMed: 23784124]

- Fives-Taylor PM, Thompson DW. Surface properties of *Streptococcus sanguis* FW213 mutants nonadherent to saliva-coated hydroxyapatite. *Infection and immunity*. 1985; 47:752–759. [PubMed: 2857684]
- Giacaman RA, Torres S, Gómez Y, Muñoz-Sandoval C, Kreth J. Correlation of *Streptococcus mutans* and *Streptococcus sanguinis* colonization and ex vivo hydrogen peroxide production in carious lesion-free and high caries adults. *Arch Oral Biol*. 2015; 60:154–159. [PubMed: 25455129]
- Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology*. 2004; 2:95–108. [PubMed: 15040259]
- Herrero ER, Slomka V, Bernaerts K, Boon N, Hernandez-Sanabria E, Passoni BB, et al. Antimicrobial effects of commensal oral species are regulated by environmental factors. *Journal of dentistry*. 2016; 47:23–33. [PubMed: 26875613]
- Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersbøll BK, Molin S. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology*. 2000; 146:2395–2407. [PubMed: 11021916]
- Hibbing ME, Fuqua C, Parsek MR, Peterson SB. Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology*. 2010; 8:15–25. [PubMed: 19946288]
- Jenkinson HF, Lamont RJ. Oral microbial communities in sickness and in health. *Trends in microbiology*. 2005; 13:589–595. [PubMed: 16214341]
- Kitten T, Munro CL, Wang A, Macrina FL. Vaccination with FimA from *Streptococcus parasanguis* protects rats from endocarditis caused by other viridans streptococci. *Infection and immunity*. 2002; 70:422–425. [PubMed: 11748213]
- Kolenbrander PE, Palmer RJ, Periasamy S, Jakubovics NS. Oral multispecies biofilm development and the key role of cell–cell distance. *Nature Reviews Microbiology*. 2010; 8:471–480. [PubMed: 20514044]
- Kolenbrander PE, Andersen RN, Blehert DS, Eglund PG, Foster JS, Palmer RJ. Communication among oral bacteria. *Microbiology and molecular biology reviews*. 2002; 66:486–505. [PubMed: 12209001]
- Kreth J, Zhang Y, Herzberg MC. Streptococcal antagonism in oral biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* interference with *Streptococcus mutans*. *Journal of bacteriology*. 2008; 190:4632–4640. [PubMed: 18441055]
- Kreth J, Merritt J, Qi F. Bacterial and host interactions of oral streptococci. *DNA and cell biology*. 2009a; 28:397–403. [PubMed: 19435424]
- Kreth J, Vu H, Zhang Y, Herzberg MC. Characterization of hydrogen peroxide-induced DNA release by *Streptococcus sanguinis* and *Streptococcus gordonii*. *Journal of bacteriology*. 2009b; 191:6281–6291. [PubMed: 19684131]
- Kreth J, Hagerman E, Tam K, Merritt J, Wong D, Wu B, et al. Quantitative analyses of *Streptococcus mutans* biofilms with quartz crystal microbalance, microjet impingement and confocal microscopy. *Biofilms*. 2004; 1:277–284. [PubMed: 16429589]
- Kuramitsu HK, He X, Lux R, Anderson MH, Shi W. Interspecies interactions within oral microbial communities. *Microbiology and molecular biology reviews*. 2007; 71:653–670. [PubMed: 18063722]
- Lara-Ortiz T, Riveros-Rosas H, Aguirre J. Reactive oxygen species generated by microbial NADPH oxidase NoxA regulate sexual development in *Aspergillus nidulans*. *Molecular microbiology*. 2003; 50:1241–1255. [PubMed: 14622412]
- Linley E, Denyer SP, McDonnell G, Simons C, Maillard JY. Use of hydrogen peroxide as a biocide: new consideration of its mechanisms of biocidal action. *Journal of antimicrobial Chemotherapy*. 2012; 67:1589–1596. [PubMed: 22532463]
- Mai-Prochnow A, Lucas-Elio P, Egan S, Thomas T, Webb JS, Sanchez-Amat A, Kjelleberg S. Hydrogen peroxide linked to lysine oxidase activity facilitates biofilm differentiation and dispersal in several gram-negative bacteria. *Journal of bacteriology*. 2008; 190:5493–5501. [PubMed: 18502869]
- Meyer DH, Fives-Taylor PM. Oral pathogens: from dental plaque to cardiac disease. *Curr Opin Microbiol*. 1998; 1:88–95. [PubMed: 10066462]

- Mintz KP. Identification of an extracellular matrix protein adhesin, EmaA, which mediates the adhesion of *Actinobacillus actinomycetemcomitans* to collagen. *Microbiology*. 2004; 150:2677–2688. [PubMed: 15289564]
- Mintz KP, Brissette C, Fives-Taylor PM. A recombinase A-deficient strain of *Actinobacillus actinomycetemcomitans* constructed by insertional mutagenesis using a mobilizable plasmid. *FEMS microbiology letters*. 2002; 206:87–92. [PubMed: 11786262]
- Nasution O, Srinivasa K, Kim M, Kim Y-J, Kim W, Jeong W, Choi W. Hydrogen peroxide induces hyphal differentiation in *Candida albicans*. *Eukaryotic cell*. 2008;7.
- Paster BJ, Olsen I, Aas JA, Dewhirst FE. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. *Periodontology* 2000. 2006; 42:80–87. [PubMed: 16930307]
- Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, et al. Bacterial diversity in human subgingival plaque. *Journal of bacteriology*. 2001; 183:3770–3783. [PubMed: 11371542]
- Peng X, Zhang Y, Bai G, Zhou X, Wu H. Cyclic di-AMP mediates biofilm formation. *Molecular Microbiology*. 2015 n/a–n/a.
- Qi, F.; Kreth, J. *Oral Biology*. Springer; 2010. Characterization of anti-competitor activities produced by oral bacteria; p. 151-166.
- Ramsey MM, Whiteley M. Polymicrobial interactions stimulate resistance to host innate immunity through metabolite perception. *Proceedings of the National Academy of Sciences*. 2009; 106:1578–1583.
- Ramsey MM, Rumbaugh KP, Whiteley M. Metabolite cross-feeding enhances virulence in a model polymicrobial infection. *PLoS pathogens*. 2011; 7:e1002012. [PubMed: 21483753]
- Rose JE, Meyer DH, Fives-Taylor PM. Aae, an autotransporter involved in adhesion of *Actinobacillus actinomycetemcomitans* to epithelial cells. *Infection and immunity*. 2003; 71:2384–2393. [PubMed: 12704108]
- Scofield JA, Wu H. Oral Streptococci and Nitrite-Mediated Interference of *Pseudomonas aeruginosa*. *Infection and immunity*. 2015; 83:101–107. [PubMed: 25312949]
- Semighini CP, Harris SD. Regulation of apical dominance in *Aspergillus nidulans* hyphae by reactive oxygen species. *Genetics*. 2008; 179:1919–1932. [PubMed: 18689883]
- Stacy A, Everett J, Jorth P, Trivedi U, Rumbaugh KP, Whiteley M. Bacterial fight-and-flight responses enhance virulence in a polymicrobial infection. *Proceedings of the National Academy of Sciences*. 2014; 111:7819–7824.
- Stone JR, Yang S. Hydrogen peroxide: a signaling messenger. *Antioxidants & redox signaling*. 2006; 8:243–270. [PubMed: 16677071]
- Veal E, Day A. Hydrogen peroxide as a signaling molecule. *Antioxidants & redox signaling*. 2011; 15:147–151. [PubMed: 21375475]
- Veal EA, Day AM, Morgan BA. Hydrogen peroxide sensing and signaling. *Molecular cell*. 2007; 26:1–14. [PubMed: 17434122]
- Wu H, Mintz KP, Ladha M, Fives-Taylor PM. Isolation and characterization of Fap1, a fimbriae-associated adhesin of *Streptococcus parasanguis* FW213. *Molecular microbiology*. 1998; 28:487–500. [PubMed: 9632253]
- Xie Z, Qi F, Merritt J. Development of a tunable wide-range gene induction system useful for the study of streptococcal toxin-antitoxin systems. *Applied and environmental microbiology*. 2013; 79:6375–6384. [PubMed: 23934493]
- Xing Y. The Role of a Surface Glycoprotein in the Interaction of *Aggregatibacter actinomycetemcomitans* with Dendritic Cells. 2015
- Zhou M, Zhu F, Dong S, Pritchard DG, Wu H. A novel glucosyltransferase is required for glycosylation of a serine-rich adhesin and biofilm formation by *Streptococcus parasanguinis*. *Journal of Biological Chemistry*. 2010; 285:12140–12148. [PubMed: 20164186]
- Zhou M, Zhu F, Li Y, Zhang H, Wu H. Gap1 functions as a molecular chaperone to stabilize its interactive partner Gap3 during biogenesis of serine-rich repeat bacterial adhesin. *Molecular microbiology*. 2012; 83:866–878. [PubMed: 22251284]
- Zijne V, van Leeuwen MBM, Degener JE, Abbas F, Thurnheer T, Gmür R, Harmsen HJ. Oral biofilm architecture on natural teeth. *PloS one*. 2010; 5:e9321. [PubMed: 20195365]

Fig. 1. *A. actinomycetemcomitans* (*Aa*) promotes biofilm formation of *S. parasanguinis* (*Sp*)
 (A) Dual-species biofilm formation was promoted by *Aa* in a dose-dependent manner. 16 h biofilm formation was determined by using crystal violet staining assay. (B) Quantification of bacteria in 6 h biofilms. Colony formation units (CFUs) of *Sp* and *Aa* in mono- and dual-species biofilms were enumerated respectively. Means of three independent experiments are shown. Error bars denote the standard deviations (SD). **, $P < 0.01$ (Student's t test)

B

Fig. 2. *S. parasanguinis* and *A. actinomycetemcomitans* mono- and dual-species biofilm formation analyzed by confocal laser scanning microscopy (CLSM)

(A) *Sp* forms more biofilms in the presence of *Aa*. Representative CLSM images of 16 h mono and dual-species biofilms of *Sp* (red) and *Aa* (green). Biofilms were stained with specific antibody to a surface protein of *Sp* and *Aa* respectively. A monoclonal antibody F51 was used to recognize the surface protein Fap1 of *Sp*. A polyclonal antibody against a surface protein Aae was used to stain *Aa*. Corresponding fluorescent conjugated secondary antibodies were used to label bacteria in different colors. Alexa Fluor 594-conjugated goat anti-mouse IgG was used to stain *Sp*, Alexa Fluor 488-conjugated goat anti-rabbit IgG was used to stain *Aa*. (B) COMSTAT analysis of bio-volume and depths of biofilm depicted in (A). Total biomass and thickness were analyzed. Data shown are the means with SD of results from 6 representative images of each group taken from two different days by the same person **, $P < 0.01$ (Student's t test)

Fig. 3. *A. actinomycetemcomitans* affects *S. parasanguinis* biofilm formation and PoxL expression without the physical interaction in a 6-well transwell insert system

(A) Influence of *Aa* on *Sp* biofilm formation in 6-well transwell plates. Biofilm formation of *Sp* at 16 h co-incubated with *Aa* directly or separated by a permeable membrane were determined by using crystal violet staining assay. (B) Protein profile of cell lysates of *Sp* co-cultured with or without *Aa* by SDS-PAGE analysis. A ~65 kDa band (arrow) was decreased in the presence of *Aa* and identified as PoxL by LC/MS/MS. (C) Expression of *poxL* determined by real time PCR. Gene expression of *poxL* in *Sp* was down-regulated in the presence of *Aa*. *Sp* cells in tranwell coincubated for 6 h with or without *Aa* were harvested for RNA preparation and real-time PCR analysis. Means of three independent experiments are shown. Error bars denote the standard deviation. (A) **, $P < 0.01$ (ANOVA, Tukey's multiple comparisons) (C) **, $P < 0.01$ (Student's t test)

Fig. 4. H₂O₂ is crucial for the biofilm formation of *S. parasanguinis*.

(A) *Aa* down-regulates H₂O₂ production in the dual-species culture. The Amplex Red reagent was used to measure H₂O₂ concentrations in 16 h biofilm cultures. The concentrations of H₂O₂ were normalized by the growth of the bacteria (A₄₇₀). The *poxL* deficient mutant *poxL* was used as a negative control. (B) *Sp poxL* mutant lost the ability to form the enhanced biofilm with *Aa*. Wild type *Sp*, *poxLSp* and complemented *Sp* were co-cultured with *Aa* to form biofilms respectively. 16 h biofilm formation was determined by crystal violet staining assay. (C) Catalase depletes H₂O₂ in the co-culture. Concentrations of H₂O₂ in the *Sp* and *Aa* 16 h co-cultures were decreased by addition of catalase in a dose-dependent manner. (D) Catalase reduced biofilm formation of *Sp* and *Aa* co-cultures dose-dependently. 16 h biofilm formation of *Sp* and *Aa* dual-species cultures were monitored under different concentrations of catalase. Means of three independent experiments are shown. Error bars denote the SD. (A), (B) **, $P < 0.01$ (Student's *t* test). (C)(D) *, $P < 0.05$ (One way ANOVA and Dunnett's multiple comparisons)

Fig. 5. Fine-tuned production of H_2O_2 affects the biofilm formation of *S. parasanguinis*
 (A) Biofilm formation of *poxL* transformed with pZX9 or pZX9-*ipoxL* were measured by crystal violet staining at 16 h. Different concentrations of xylose were used to induce expression of *poxL* and production of H_2O_2 . The biofilm formation of *poxL* transformed with pZX9-*ipoxL* under a given inducing condition was normalized by subtracting the background biofilm formation of *poxL*::pZX9. (B) Xylose dose-dependent induces production of H_2O_2 . Concentrations of H_2O_2 in the culture of *poxL*::pZX9-*ipoxL* induced by xylose were measured and normalized using bacterial growth ($\mu M/A_{470}$). All data represent the means of three independent experiments with SD *, $P < 0.05$ (One way ANOVA and Tukey's multiple comparisons)

Fig. 6. Catalase of *A. actinomycetemcomitans* is not involved in H₂O₂-mediated dual-species biofilm formation

(A) Catalase deficient *Aa* still promotes biofilm formation of *Sp* as wild type *Aa*. *Sp* was cultured with either wild type *Aa* or catalase deficient *Aa* (*katA*), and 16 h biofilm formation was assessed using crystal violet staining. (B) Catalase deficiency in *Aa* does not affect the down-regulation of H₂O₂ concentration. H₂O₂ concentration of *Sp* and dual-species biofilms were measured as described in the experimental procedures. (C) Catalase deficiency in *Aa* still down-regulates PoxL of *Sp*. Protein profile of cell lysates prepared from *Sp*, *Sp* cultured with wild type *Aa* or catalase deficient *Aa* (*katA*) was determined by SDS-PAGE analysis and Comassie blue staining. Means of three independent experiments are shown. Error bars denote the SD. ns, $P > 0.05$ (Student's *t* test)

Fig. 7. *A. actinomycetemcomitans* enhances the ability of *S. parasanguinis* to colonize flies
 Flies were divided into five experimental groups and fed with WT *Sp*, *poxL Sp*, *Aa*, WT *Sp* plus *Aa*, and *poxL Sp* plus *Aa*. After 24h and 48h, flies were crushed and suspended in sterile saline, and enumerated to determine colony formation units (CFUs) of *Sp* or *Aa*. (A) Effects of *Aa* on colonization of *Sp* in flies. *Aa* promotes colonization of WT *Sp*. PoxL deficiency inhibited colonization of the *poxL* mutant *Sp* promoted by *Aa*. (B) The number of *Aa* is reduced when co-colonized with *Sp* in flies. Means of three independent experiments are shown. Error bars denote the SD. **, $P < 0.01$ (One way ANOVA, Tukey's multiple comparisons).

Table 1**Bacterial strains and plasmids**

Strain or plasmid	Relevant characteristics	Reference or Source
Strains		
<i>E. coli</i>		
Top10	Host strain for cloning	Invitrogen
DH5 α λ pir	Host strain for cloning	(Mintz, 2004)
KM482	Host strain for conjugation	(Xing, 2015)
<i>A. actinomycetemcomitans</i>		
VT1169	Wild type strain, Nal ^r , Rif ^r	(Mintz et al., 2002)
VT1169 <i>katA</i>	<i>katA</i> mutant, Spc ^r	This study
<i>S. parasanguinis</i>		
FW213	Wild type strain	(Fives-Taylor and Thompson, 1985)
FW213 <i>poxL</i>	<i>poxL</i> mutant, Kan ^r	(Schofield and Wu, 2015)
FW213 <i>poxL comp.</i>	<i>poxL</i> containing plasmid pVPT- <i>poxL</i> , Kan ^r , Erm ^r	(Schofield and Wu, 2015)
FW213 <i>poxL::pZX9</i>	<i>poxL</i> containing plasmid pZX9, Kan ^r , Spc ^r	This study
FW213 <i>poxL::pZX9/ipoxL</i>	<i>poxL</i> containing plasmid pZX9/ <i>ipoxL</i> , Kan ^r , Spc ^r	This study
Plasmids		
pVT1460	<i>E. coli</i> - <i>A. actinomycetemcomitans</i> shuttle vector Kan ^r	(Mintz et al., 2002)
pVT1460- <i>katA-aad9</i>	pVT1460 vector containing <i>katA</i> up and down with <i>aad9</i> insertion Kan ^r , Spc ^r	This study
pZX9	Streptococcal replicon with luc ORF controlled by Xyl-S1	(Xie et al., 2013)
pZX9- <i>ipoxL</i>	Streptococcal replicon with <i>poxL</i> ORF controlled by Xyl-S1	This study

Table 2

Primers used in this study

Primers	Sequence (5'-3')	Description
katAupSphIF	GCCAGCATGCTAGCCGCACTGATGGAAATC	<i>KatA</i> deletion
katAupR	ATTCACGAACGAAAATCGATAATCGTTTCCTTAATTTTGAG	<i>KatA</i> deletion
katAdnF	ACAATAAACCCCTTGCATATGTTCAAATTTAACTCAAGAACCG	<i>KatA</i> deletion
katAdnSalIR	GCAGTCGACAAGCTAGTCACCGAGCAGGA	<i>KatA</i> deletion
katA F	ATGTCAGCAAAATGCCCTTTCG	<i>KatA</i> deletion
katA R	TCAAGCAAGTTTTGTGCGAA	<i>KatA</i> deletion
katA flanking F	GATTGAGAACTTTTGTCTGGTA	<i>KatA</i> deletion
katA flanking R	CAACATTTGCACACTGGGCATT	<i>KatA</i> deletion
aad9F	ATCGATTTTCGTTTCGTGAAT	Amplify aad9
aad9R	CATATGCAAGGTTTATTGT	Amplify aad9
Q16SrRNAF	GAGAGATGGACCTGCGTTGT	qRT-PCR
Q16SrRNAR	GCCGAAGATTCCCTACTGCT	qRT-PCR
QpoxLF	ACTGGAAGTGGACGGTGTG	qRT-PCR
QpoxLR	AAGCACAAGGAGCTGTTGGA	qRT-PCR
poxLF-IS	ACTTGTTCACTTAAATCAAAGGAGGAGTTAAAATGACTCAAGGAAAAATT	Inducible poxL
poxLR-IS	GATGCCCTGGCAGTTTATGGCGG TTATTTGATTGCGCGTGATTGCAAC	Inducible poxL
ISF-poxL	AATTTTTCCTTGAGTCATTTTAACT CCTCCTTTGATTTAAGTGAACAAGT	Inducible poxL
ISR-poxL	GTTGCAATCACGCGCAATCAAATAA CCGCCATAAACTGCCAGGCATC	Inducible poxL