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ENVIRONMENTAL MICROBIOLOGY

Veillonella Catalase Protects the Growth of *Fusobacterium nucleatum* in Microaerophilic and *Streptococcus gordonii*-Resident Environments

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ABSTRACT The oral biofilm is a multispecies community in which antagonism and mutualism coexist among friends and foes to keep an ecological balance of community members. The pioneer colonizers, such as *Streptococcus gordonii*, produce H_2O_2 to inhibit the growth of competitors, like the mutans streptococci, as well as strict anaerobic middle and later colonizers of the dental biofilm. Interestingly, *Veillonella* species, as early colonizers, physically interact (coaggregate) with *S. gordonii*. A putative catalase gene (*catA*) is found in most sequenced *Veillonella* species; however, the function of this gene is unknown. In this study, we characterized the ecological function of *catA* from *Veillonella parvula* PK1910 by integrating it into the only transformable strain, *Veillonella atypica* OK5, which is *catA* negative. The strain (OK5-*catA*) became more resistant to H_2O_2. Further studies demonstrated that the *catA* gene expression is induced by the addition of H_2O_2 or coculture with *S. gordonii*. Mixed-culture experiments further revealed that the transgenic OK5-*catA* strain not only enhanced the growth of *Fusobacterium nucleatum*, a strict anaerobic periodontopathogen, under microaerophilic conditions, but it also rescued *F. nucleatum* from killing by *S. gordonii*. A potential role of catalase in veillonellae in biofilm ecology and pathogenesis is discussed here.

IMPORTANCE *Veillonella* species, as early colonizers, can coaggregate with many bacteria, including the initial colonizer *Streptococcus gordonii* and periodontal pathogen *Fusobacterium nucleatum*, during various stages of oral biofilm formation. In addition to providing binding sites for many microbes, our previous study also showed that *Veillonella* produces nutrients for the survival and growth of periodontal pathogens. These findings indicate that *Veillonella* plays an important "bridging" role in the development of oral biofilms and the ecology of the human oral cavity. In this study, we demonstrated that the reducing activity of *Veillonella* can rescue the growth of *Fusobacterium nucleatum* not only under microaerophilic conditions, but also in an environment in which *Streptococcus gordonii* is present. Thus, this study will provide a new insight for future studies on the mechanisms of human oral biofilm formation and the control of periodontal diseases.

KEYWORDS *Fusobacterium nucleatum*, oxidative stress, *Streptococcus gordonii*, veillonellae, anaerobes, transformable strain

The human oral biofilm is a multispecies community colonized by more than 700 bacterial species, and 200 species may present in each human mouth (1, 2). Formation of the dental biofilm involves a sequential process (3–5): on a newly emerged or professionally cleaned tooth surface, pioneer colonizers consisting mostly of the mitis streptococci (i.e., *Streptococcus gordonii* and *Streptococcus sanguinis*) attach

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to the tooth surface via specific interactions with the acquired pellicle on the surface. Growth and metabolic activity of the pioneer colonizers create an environment conducive to colonization by the bridging species, some of which are species of the *Veillonella* genus. Growth of the bridging species then modifies the local environment or generates nutrients, such as heme/hemin, facilitating the growth of later colonizers, many of which are periodontopathogens (6). Eventually, through cell growth and coadhesion, a mature biofilm is formed (3, 5). Under normal conditions, this biofilm community keeps an ecological homeostasis; however, environmental perturbation can disrupt this balance, leading to dysbiosis, and then oral diseases, such as dental caries and periodontitis, ensue (7–9). As bridging species play such an important role in oral biofilm development, understanding their interaction with both pioneer and later colonizers would generate a knowledge base leading to developments in disease prevention.

The mitis streptococci, such as *S. gordonii*, produce abundant hydrogen peroxide via multiple pathways (4, 10–12). Kreth et al. reported that the concentrations of H_2O_2 generated by *S. sanguinis* and *S. gordonii* are sufficient to inhibit the growth of many oral bacteria, such as the cariogenic *Streptococcus mutans* (13). Interestingly, our previous study demonstrated that adding *Veillonella parvula* strain PK1910 (formerly *Veillonella atypica* PK1910 [14]) to the *S. gordonii-S. mutans* mixed culture could rescue *S. mutans* from the inhibition of *S. gordonii* (15), implying that *V. parvula* PK1910 may employ some strategies to counteract the killing effect of H_2O_2.

Veillonellae, as some of the most predominant bacteria in oral microbiota (16–18), possess two characteristics that make them some of the most important bridging species in the oral biofilm community. One is the use of lactate, generated mainly by streptococci, as their primary carbon and energy source (19). Thus, it is not surprising that veillonellae might also encounter a high level of streptococcus-produced H_2O_2. Interestingly, our studies revealed that veillonellae, although anaerobic, have an extremely high capacity to tolerate oxygen stress (P. Zhou, unpublished data). The other characteristic is the ability to coaggregate with numerous initial, early, middle, and later colonizers (20–23). *In vitro*, *Veillonella* species have been shown to physically coaggregate with streptococci and other periodontal pathogens, such as *Fusobacterium nucleatum* (20, 23, 24).

F. nucleatum (25), a strict anaerobe and middle colonizer, is often present in the early biofilm community (17). How does this pathogen deal with the high concentration of H_2O_2 produced by mitis streptococci? It has been shown that fusobacteria play a role in protecting against atmospheric oxygen and hydrogen peroxide in the oral biofilm and even support the growth of *Porphyromonas gingivalis* under aerated conditions (26, 27). However, compared to fusobacteria, *Veillonella* species are more tolerant to oxygen stress (P. Zhou, unpublished data). Thus, we hypothesized that veillonellae might be able to protect downstream strict anaerobes, such as *F. nucleatum*, against oxygen stress. In this study, we identified a functional catalase in *V. parvula* PK1910 and hypothesized that the catalase activity of this strain may play a crucial role in protecting *F. nucleatum* from oxygen stress and in turn facilitate its persistence in early biofilm community. Recently, we have successfully developed a genetic transformation system in a clinical isolate of *Veillonella atypica* OK5 (28, 29), which made it possible to test the effect of *Veillonella* catalase in biofilm ecology. Due to the absence of catalase in the *V. atypica* OK5 strain, in this study, we transferred the catalase gene from *V. parvula* PK1910 to OK5 and tested its effect on *Veillonella* resistance to H_2O_2, as well as on the growth of *F. nucleatum* in the presence of *S. gordonii* under microaerophilic conditions.

RESULTS

***V. parvula* PK1910 produces active catalase.** In our previous study, we demonstrated that the presence of *V. parvula* PK1910 in a *S. mutans-S. gordonii* mixed culture could rescue *S. mutans* from inhibition by *S. gordonii* (15). To determine the cause of this effect, we first analyzed catalase activity of PK1910, because in nature, many bacteria have evolved to produce catalase as a protective strategy against H_2O_2

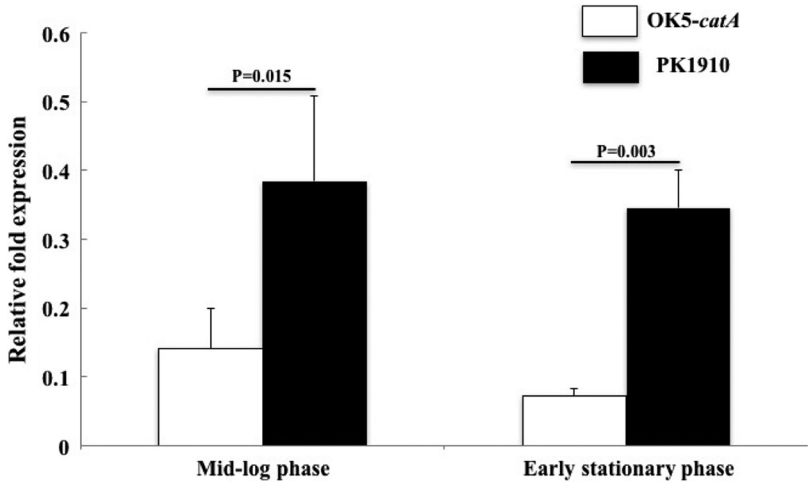

FIG 1 Detection of *catA* expression in OK5-*catA* and PK1910 using qPCR assay. Expression of the *catA* gene was normalized to the expression of the reference gene *gyrA*, and relative fold expression was calculated using the formula $2^{-\Delta CT\ (catA\text{-}gyrA)}$. All experiments were repeated at least 3 times, and the results are shown as mean ± standard deviation (SD).

produced by hosts or neighboring microbial species (30, 31). To detect catalase activity, *V. parvula* PK1910 cells in mid-log phase were pelleted by centrifugation, and H_2O_2 was dropped on top of the cell pellet. Bubbling was observed immediately after H_2O_2 addition, indicating possible catalase activity (data not shown).

Additional proof of the existence of catalase production came from analyzing the draft genome sequence of PK1910 (P. Zhou and F. Qi, unpublished data). The predicted functions of available genes in all contigs revealed a single copy of a putative catalase gene (*catA*). A quantitative PCR (qPCR) assay using PCR primers designed from the predicted sequence indicated that gene-specific mRNA was present in *V. parvula* PK1910 (Fig. 1). Having confirmed the presence and transcription of a *catA* gene, we measured enzyme activity produced by *V. parvula* PK1910 in liquid culture. Using mid-log cells grown in brain heart infusion supplemented with 0.6% sodium lactate (BHIL) broth, approximately 30 U/ml catalase enzyme activity was detected (data not shown).

***V. parvula catA* is functional in *V. atypica* OK5.** To study the function of *catA* in *Veillonella* species, we chose the only transformable strain, *V. atypica* OK5, as a model (28, 32), because a catalase gene does not exist in the *V. atypica* OK5 genome (33), and transformation is not feasible with *V. parvula* PK1910. Using the shuttle vector pBSJL2 for *V. atypica* (29), we constructed plasmid pBSJL2-Vp-*catA*, carrying the *catA* gene under the control of its native promoter, and transformed it into OK5. Transformants showed strong catalase activity when tested with externally added H_2O_2 (Fig. 2A), suggesting that the *V. parvula catA* is functional in *V. atypica*.

Having confirmed the function of *catA* in *V. atypica* by the shuttle plasmid pBSJL2-Vp-*catA*, we then wanted to integrate this gene into the chromosomal DNA of OK5, because putting *catA* into a multicopy shuttle vector is not suitable when studying gene expression and regulation. We chose an intergenic region to integrate the *catA* gene into the chromosome of *V. atypica* OK5 (see Materials and Methods and Fig. 2B). Using the previously mentioned H_2O_2 drop assay, obvious bubble formation was detected for this strain, but the effect was not as obvious as when the same assay was performed using the strain containing the shuttle plasmid pBSJL2-Vp-*catA* (data not shown). This is likely due to different copy numbers of *catA* between the two strains. To test if this strain, named OK5-*catA*, can be a surrogate for *V. parvula* PK1910 to study the function of catalase in the *Veillonella* genus, a qPCR assay was utilized to compare *catA* expression in OK5-*catA* and *V. parvula* PK1910. The data showed that the expressive pattern was similar in the two strains at mid-log- and early stationary phases, although

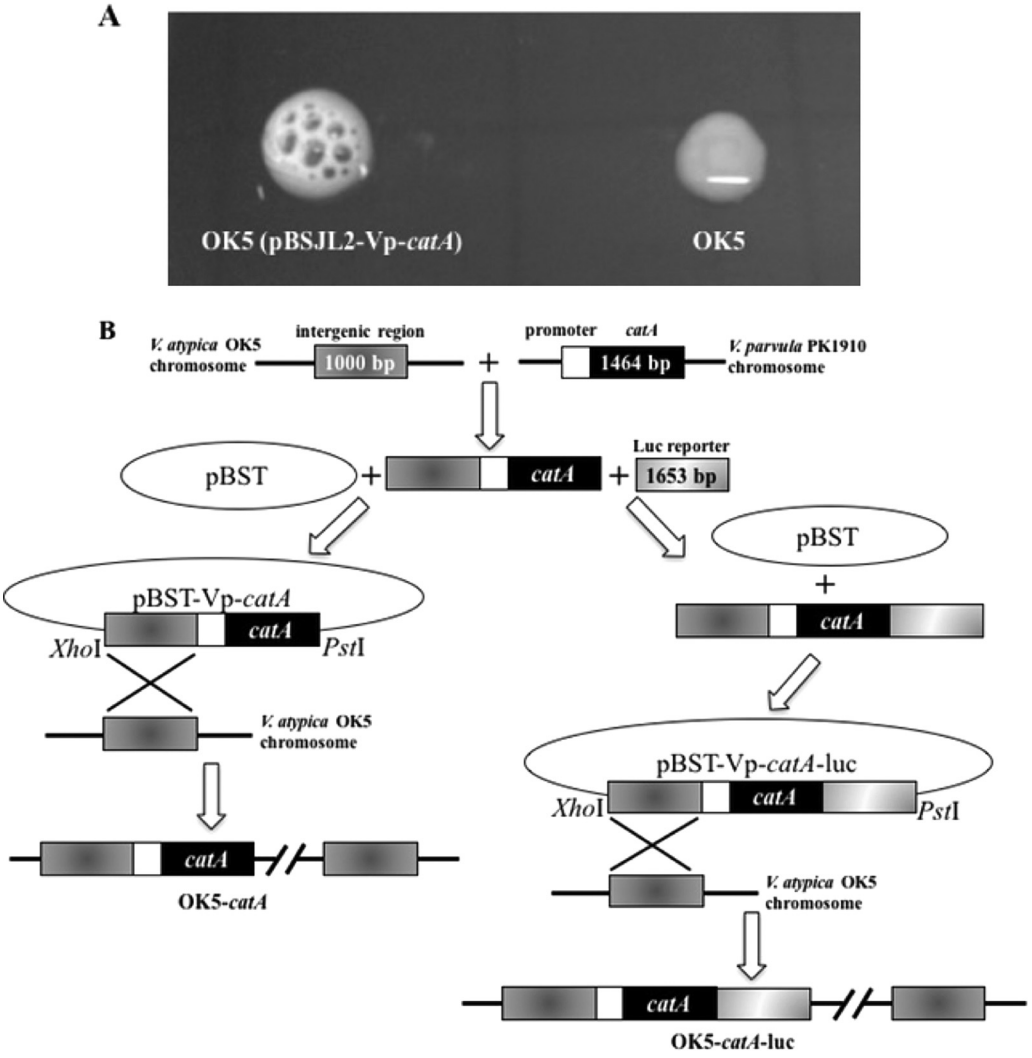

FIG 2 (A) Catalase test for OK5-pBSJL2-*catA* (left) and OK5 wild type. (B) Construction of OK5-pBSJL2-*catA*, *catA*-integrated, and *catA*-luc-integrated strains in *V. atypica*.

the expression of *catA* in OK5-*catA* was lower than that in *V. parvula* PK1910 (Fig. 1). Thus, OK5-*catA* was used in subsequent studies.

OK5-*catA* was more tolerant to H_2O_2 than its parental strain. To determine the sensitivities of OK5, OK5-*catA*, and PK1910 to H_2O_2, the MIC was measured using hydrogen peroxide challenge assays. All three *Veillonella* strains were treated as described in Materials and Methods. As expected, the strain most susceptible to H_2O_2 inhibition was the OK5 wild type (MIC, 15.63 μM). Similar to PK1910 (MIC, 31.25 μM), OK5-*catA* (MIC, 31.25 μM) showed more tolerance to hydrogen peroxide, thus suggesting that catalase might be an important factor contributing to the difference in H_2O_2 sensitivities between *V. atypica* and *V. parvula*.

Expression of *catA* was induced by chemical and *S. gordonii*-produced H_2O_2. As the function of catalase is to eliminate H_2O_2 in cells, we speculated that the expression of the *catA* gene in *Veillonella* species might be inducible by H_2O_2. To test this hypothesis, a luciferase reporter was transcriptionally fused with *catA* and then integrated into the same intergenic region as in OK5-*catA* (see Materials and Methods and Fig. 2B). The resulting strain, designated OK5-*catA*-luc, displayed the same level of catalase activity as in OK5-*catA* (data not shown), suggesting that luciferase gene fusion did not affect *catA* gene expression.

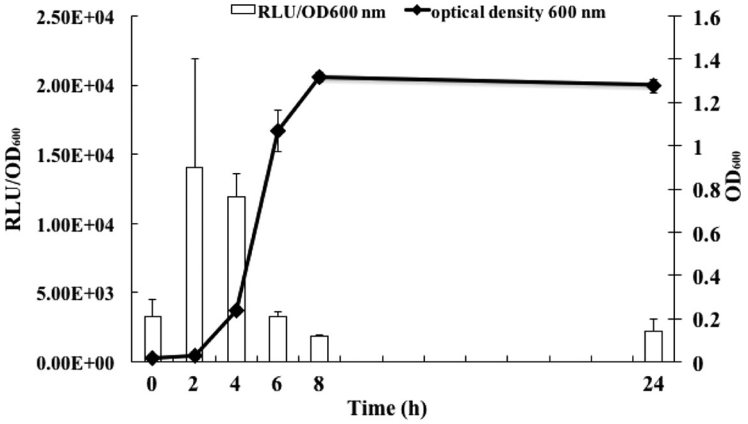

FIG 3 Expression of the *catA* gene in OK5-*catA*-luc. Cells were grown in BHIL anaerobically at 37°C, and samples were taken at 2-h intervals. Luciferase activity was measured and normalized to cell density (OD_{600}), shown as RLU/OD_{600}. The results are shown as the means ± SD of the results from three independent experiments.

As expected, the expression of the *catA*-luc gene was low when grown anaerobically (Fig. 3) and obviously less than that with exposure to oxygen. To test if H_2O_2 could induce the *catA* gene, strain OK5-*catA*-luc was allowed to preincubate with various concentrations of H_2O_2 in the range of 0 to 2,400 μM for 30 min at room temperature. The highest increase in *catA*-luc expression was observed at 300 μM H_2O_2, indicating that the expression of the *catA* gene in OK5 could be induced by a low concentration of H_2O_2. In contrast, *catA*-luc expression was unable to be detected at a high concentration of H_2O_2 (more than 1,200 μM), perhaps due to *Veillonella* dying under this detrimental condition (Fig. 4).

Having demonstrated that *catA*-luc gene expression was induced by chemical H_2O_2, we reasoned that coculture with *S. gordonii*, which generates plentiful H_2O_2 via different pathways, should also upregulate the expression of *catA*-luc gene. To test this, OK5-*catA*-luc was cocultured with *S. gordonii* under microaerophilic conditions, samples were taken at 0 h and 6 h after coincubation, and luciferase activity was measured. A mixed culture with *S. mutans* was used as a control because *S. mutans* was known not to produce H_2O_2. At 6 h postincubation, luciferase activity in the mixed culture with *S.*

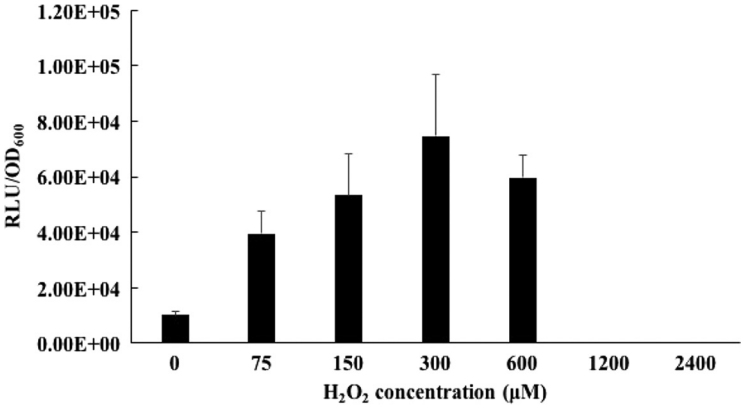

FIG 4 Expression of the *catA* gene by H_2O_2 treatment. OK5-*catA*-luc was precultured overnight in BHIL medium, centrifuged to remove supernatant, and resuspended in fresh BHIL broth to an OD_{600} of ~1.0. The suspension was diluted 1:20 into fresh BHIL and the culture grown to mid-log phase (OD_{600}, 0.7 to ~0.8). The cells were treated with different concentrations of H_2O_2 for 30 min at room temperature, the cells were centrifuged to remove the supernatants, and the cell pellets were resuspended into fresh BHIL broth. Luciferase activity was measured and normalized to the OD_{600}, expressed as RLU/OD_{600}. The culture without H_2O_2 treatment was used as a negative control. The results are shown as the means ± SD of the results from three independent experiments.

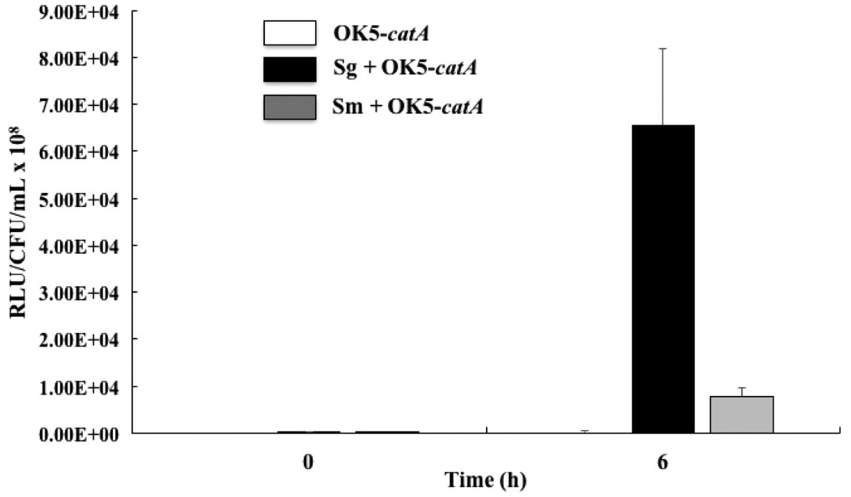

FIG 5 Expression of the *catA* gene in the presence of different streptococcal species. Luciferase activity was measured at 0 h and 6 h. The CFU per milliliter of OK5-*catA* was obtained by plate counting on the BHIL plate supplied with tetracycline (2.5 μg/ml). Luciferase activity was expressed as RLU/CFU/ml $\times$ 10^8. The results are shown as the means $\pm$ SD of the results from three independent experiments. Sg, *S. gordonii*; Sm, *S. mutans*.

gordonii increased 180-fold compared with OK5-*catA*-luc monoculture and 8.5-fold compared with mixed culture with *S. mutans* (Fig. 5), suggesting that the expression of *catA* was induced by coculturing with *S. gordonii*.

OK5-*catA* rescued the growth of *F. nucleatum* under microaerophilic conditions. *F. nucleatum*, known as a strict anaerobe, can also be found in early biofilm plaque, which is primarily an aerobic or microaerophilic niche (17). How does this strict anaerobe deal with oxygen stress? Based on our previous observation that *Veillonella* could rescue *S. mutans* from the inhibitory effect of *S. gordonii* (15), we speculate that *Veillonella* might exert a crucial impact that facilitates the growth of *F. nucleatum* under hyperoxic conditions. To test this assumption, we first measured *catA* expression in OK5-*catA*-luc under different conditions, and a noticeable increased expression was observed when the strain was grown in a candle jar compared to growth in the anaerobic chamber (data not shown). Next, we carried out *V. atypica*-*F. nucleatum* coculture assays, as described in Materials and Methods. As shown in Fig. 6, monoculture of *F. nucleatum* grew to 5.30 $\times$ 10^7 CFU/ml, and a 3-fold increase in *F. nucleatum*

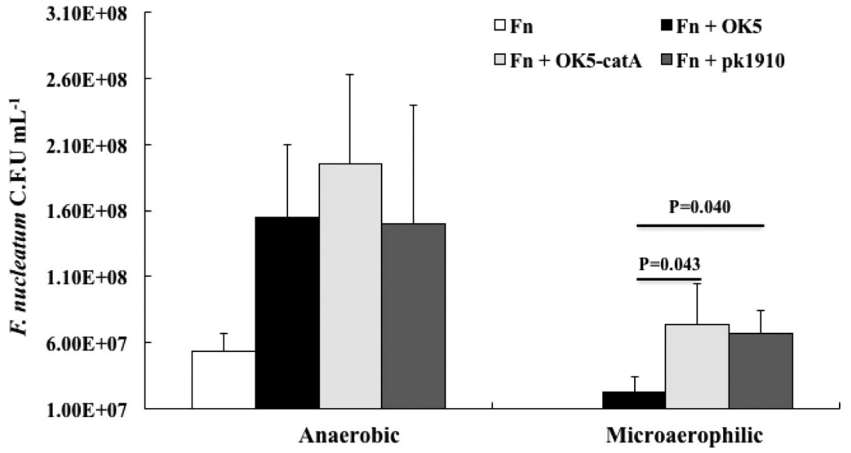

FIG 6 Growth of *F. nucleatum* in monoculture and cocultures with OK5 wild type, OK5-*catA*, and PK1910 under anaerobic (left) and microaerophilic (right) conditions. The CFU per milliliter of *F. nucleatum* were obtained by plate counting on blood agar plates. The results are shown as the means $\pm$ SD of the results from at least three independent experiments. Fn, *F. nucleatum*.

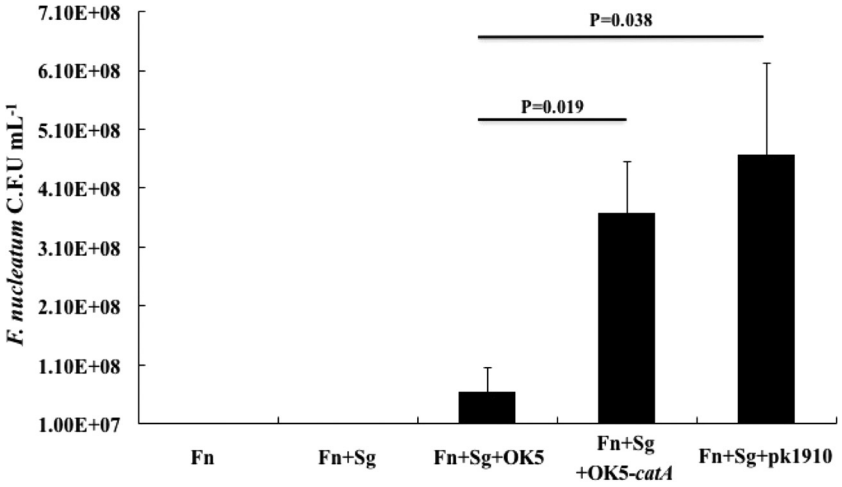

FIG 7 Growth of *F. nucleatum* in monoculture, coculture with *S. gordonii*, and 3-species culture with different *Veillonella* strains under microaerophilic conditions. The CFU per milliliter of *F. nucleatum* were obtained by plate counting on blood agar plates. The results are shown as the means ± SD of the results from at least three independent experiments. Fn, *F. nucleatum*; Sg, *S. gordonii*.

CFU/ml was observed when grown in the presence of each of the three tested *Veillonella* strains/species under anaerobic conditions. In contrast, *F. nucleatum* single culture was unable to grow under microaerophilic conditions, but as expected, the presence of OK5-*catA* or PK1910 rescued *F. nucleatum* growth. Interestingly, the OK5 wild-type strain also enhanced the survival rate of *F. nucleatum*, although not as well as the catalase-positive strains (Fig. 6), suggesting the catalase in *Veillonella* plays a protective role for *F. nucleatum* against oxidative stress.

OK5-*catA* protected *F. nucleatum* from inhibition by *S. gordonii*. It has been well documented that *Veillonella*, as an early colonizer, coaggregates with initial, early/middle, and later colonizers, such as *S. gordonii* and *F. nucleatum* (14, 23, 24), so this microorganism was believed not only to play a "bridging" role in the formation of oral biofilm community, but it can also optimize the local microniche, for instance, by reducing an oxidative environment, to facilitate the growth of later colonizers. To further confirm that catalase in *Veillonella* species can protect *F. nucleatum* from the inhibitory effect of H_2O_2 generated by initial colonizers, a 3-species mixed culture system, including *S. gordonii-V. atypica-F. nucleatum*, was used in this study. Under microaerophilic conditions, *F. nucleatum* failed to grow in either monoculture or as a *S. gordonii-F. nucleatum* coculture. As expected, the addition of OK5-*catA* or PK1910 in 3-species cultures supported the growth of *F. nucleatum* (Fig. 7). This demonstrated that veillonellae utilize catalase to eliminate *S. gordonii*-produced H_2O_2 and generate a reducing environment required for the growth of *F. nucleatum*. Interestingly, the presence of OK5 protected *F. nucleatum* from killing by *S. gordonii* (Fig. 7), implying that another strategy might be employed by *V. atypica* to antagonize oxidative stress.

DISCUSSION

All organisms living in an aerobic environment have to confront oxidative stress. Oxygen molecules, which are relatively inert, can be easily converted into reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot -}$), and hydroxyl radicals ($OH^{\cdot -}$). The damaging effects of ROS on various biological macromolecules are known to be associated with aging, mutagenesis, and tumorigenesis (34, 35). Aerobes generally use two enzymes, superoxide dismutase (SOD) and catalase, to deal with oxidative stress. Superoxide anions are dismuted by SOD to H_2O_2, which is then decomposed to water and oxygen by catalase (30, 36). The antioxidative mechanisms employed by anaerobes involve the reduction of superoxide or H_2O_2 using reduced rubredoxin as an electron donor. Superoxide reductase (SOR) reduces super-

oxide to H_2O_2, and afterward, rubrerythrin can catalyze the reduction of hydrogen peroxide to water (37–40). In fact, intact H_2O_2 is relatively harmless to cells, but Fenton reactions, which can be triggered by ferrous or copper, can result in the conversion of H_2O_2 into hydroxyl radicals, which are extremely deleterious due to its ability to indiscriminately destroy all biological macromolecules (34, 41, 42). Therefore, catalase and rubrerythrin play vital roles in defense against ROS through the elimination of H_2O_2.

The development of human oral biofilm is a sequential process (3–5). As initial colonizers, streptococci play a vital role in the colonization of early/middle and later colonizers (3, 43); however, the mitis streptococci, such as *S. gordonii*, produce plentiful H_2O_2 to inhibit the growth of downstream bacteria, most of which are extremely sensitive to hyperoxic stress. Therefore, how do these anaerobes deal with the high concentration of H_2O_2 produced by streptococci? By searching the genome database of veillonellae on the Human Oral Microbiome Database (HOMD) (44), we found most *Veillonella* species, except for all *V. atypica* strains, to possess a putative catalase. Our previous study showed that *V. parvula* PK1910 could reduce the growth-inhibitory effect of *S. gordonii* on *S. mutans* (15), implying that catalase, encoded by the *catA* gene in *V. parvula* PK1910, might play a crucial role not only in the competition between the two streptococcal species but also in supporting the growth of downstream obligate anaerobes. To prove our hypothesis, we used the only transformable strain of *Veillonella*, *V. atypica* OK5 (29), to study the function of catalase in the *Veillonella* genus.

In the present study, we integrated the *V. parvula* PK1910 *catA* gene into *V. atypica* OK5 chromosomal DNA and confirmed that the *catA* gene expresses functional catalase in *V. atypica*. Although *catA* expression in *V. atypica* is lower than that in original species, expressive patterns in both species are similar, indicating OK5-*catA* can be a surrogate for studying the function of *Veillonella* catalase in human oral biofilm.

The oral biofilm plays a crucial role in human health and disease. Numerous epidemiological studies have suggested that homeostasis of the biofilm community contributes to oral health, while dysbiosis of the community gives rise to the development of diseases. Thus, studying the biological and environmental elements that affect the ecological balance of the biofilm has significant implications in disease prevention. As some of the bridging species, veillonellae are biologically significant for the development of the oral biofilm community, because they not only provide "site and food" to oral bacteria, such as streptococci, *F. nucleatum*, and *P. gingivalis* via cell-cell coaggregation (6, 22–24), but they can also optimize the microenvironment to ensure the survival of downstream pathogens. For example, we have shown in this study that OK5-*catA* and *V. parvula* PK1910 can improve the growth of *F. nucleatum* under low-oxygen conditions; however, *F. nucleatum* alone failed to survive under the same condition. Interestingly and unexpectedly, the OK5 wild type supported *F. nucleatum* growth as well. This is likely due to the antioxidant role of rubrerythrin in *V. atypica* OK5. Rubrerythrins, encoded by the *rbr* gene, are nonheme iron proteins that play vital roles in antioxidant defense in anaerobic bacteria and archaea and catalyze the reduction of H_2O_2 to H_2O (45). The genome database shows that the *rbr* gene exists in all *Veillonella* species, implying it has an important role for antioxidant defense in these microorganisms. The expression of *rbr* in OK5 and OK5-*catA* was measured by qPCR and showed at least 1,000-fold higher in transcript abundance than in PK1910, indicating that rubrerythrin rather than catalase plays a vital role in defense against oxygen stress, especially hydrogen peroxide, in *V. atypica* species (P. Zhou, unpublished data). Furthermore, the extremely high expression of *rbr* (P. Zhou, unpublished data) in OK5 can be an explanation for its support with the growth of *F. nucleatum* in a low-oxygen environment. The synergy of catalase and rubrerythrin should render OK5-*catA* a higher capability of eliminating and then tolerating to H_2O_2; thus, this could explain why OK5-*catA* was better than PK1910 in supporting *F. nucleatum* growth under microaerophilic conditions (Fig. 6).

When grown anaerobically, *F. nucleatum* alone can grow and reach to 5.30×10^7 CFU/ml, while both *V. atypica* and *V. parvula* can boost the growth of *F. nucleatum*

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
Strains		
E. coli DH5α	Cloning strain	
V. atypica OK5	Wild type	29
OK5-pBSJL2-*catA*	OK5 transformed by shuttle plasmid pBSJL2-*catA*; Tc^r	This work
OK5-*catA*	OK5 *catA*-integrated strain; Tc^r	This work
OK5-*catA*-luc	OK5 *catA*-integrated strain with luc reporter; Tc^r	This work
V. parvula PK1910	Wild type	43
S. gordonii DL1	Wild type	48
S. mutans UA140	Wild type	49
F. nucleatum ATCC 23726	Wild type	This work
Plasmids		
pBST	Suicide vector of *V. atypica*, the beta-lactamase gene in pBluescript II KS(+) was replaced by *tetM* cassette; Tc^r	23
pBSJL2	Shuttle vector of *V. atypica*, for complementation; Tc^r	29
pBSJL2-Vp-*catA*	pBSJL2+ *catA*; Tc^r	This work
pBST-Vp-*catA*	See Materials and Methods for *catA* integration	This work
pBST-Vp-*catA*-luc	See Materials and Methods for *catA* reporter; Tc^r	This work

[a]Tc^r, tetracycline resistance.

growth by 3-fold (Fig. 6). This increase in growth output is likely ascribed to the nutrients produced by *Veillonella* supporting the growth of *F. nucleatum*. A similar phenomenon was also reported by Periasamy and Kolenbrander (46): *Veillonella* sp. strain PK1910 increased *F. nucleatum* biomass in a two-species biofilm using a flow cell system.

Compared to a *Veillonella-F. nucleatum* coculture assay, the growth of *F. nucleatum* was better in a 3-species (*S. gordonii-Veillonella-F. nucleatum*) mixed culture in the presence of *Veillonella* species/strains (Fig. 6 and 7). There could be two explanations for this phenomenon: (i) *S. gordonii* can produce lactate, a major nutrient for *Veillonella* growth, and this increase in growth of *Veillonella* species might in turn contribute to *F. nucleatum* blooming; (ii) *Veillonella catA* and *rbr* genes can be induced by *S. gordonii* under low-oxygen conditions (Fig. 5, and P. Zhou, unpublished data), thus establishing a better anaerobic niche for luxuriant growth of *F. nucleatum*.

In sum, as crucial bridging species, *Veillonella* spp. not only coaggregate with various bacteria in the oral cavity but can also optimize the microniche to support the growth of late periodontopathogens, such as *Fusobacterium* species. Thus, this study provided new insights for future studies on the mechanisms of human oral biofilm formation and the control of periodontal diseases.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used or constructed in this study are listed in Table 1. *V. atypica* OK5 and *V. parvula* PK1910 were grown in brain heart infusion broth (BHI; autoclave 20 min at 121°C; Difco) supplemented with 0.6% sodium lactate (BHIL), a semichemically defined medium (47) without glucose but supplemented with 0.6% sodium lactate and 0.1% peptone (SCDM; sterilized through a 0.22-μm filter), or on BHIL agar plates. *F. nucleatum* ATCC 23726 was grown in Columbia broth (CB; autoclave 20 min at 121°C; Difco) supplemented with vitamin K (1.2 μM) and hemin (7.7 μM). *S. gordonii* DL1 and *S. mutans* UA140 were grown in BHI broth. All bacterial strains were grown anaerobically (85% N_2, 10% CO_2, 5% H_2) at 37°C. The plasmids were transformed into *V. atypica* OK5 by using electroporation, as described previously (32). For transformant selection, cells were grown in Todd-Hewitt (TH) broth (autoclave 20 min at 121°C; Difco) with 0.6% sodium lactate (THL) plus tetracycline (2.5 $\mu g \cdot ml^{-1}$; Sigma-Aldrich). *Escherichia coli* DH5α cells were grown in Luria-Bertani (LB; autoclave 20 min at 121°C; Difco) medium with aeration at 37°C. *E. coli* strains carrying plasmids were grown in LB medium containing tetracycline (10 $\mu g \cdot ml^{-1}$).

General DNA manipulation. Phusion DNA polymerase, *Taq* DNA polymerase, restriction enzymes, and T4 DNA ligase were all purchased from New England BioLabs. Phusion DNA polymerase was used for overlapping PCR. *Taq* DNA polymerase was used for screening clones.

RNA isolation and real-time RT-PCR analysis. Overnight cultures of *Veillonella* spp. were centrifuged, adjusted to an optical density at 600 nm (OD_{600}) of ~1.0, and then diluted 1:100 into fresh BHIL

TABLE 2 Primers used in this study

Primer	Sequence (5′ to 3′)	Purpose
gyrA-q-F	CAGAAGCAGGTTCCCGTAACTC	Reference gene for qPCR
gyrA-q-R	GCCTACCGCAAGTGGCAATA	Reference gene for qPCR
catA-q-F	AAACTAACAACAGCCTTTGGTG	qPCR for *catA*
catA-q-R	AGAATTTTACGGCAAAGCCA	qPCR for *catA*
pBSJL2-*catA*-F	CCGCTCGAGATTGAATGAAAACACCTTG	*V. parvula catA* amplification
pBSJL2-*catA*-R	AAACTGCAGTTATTTTACATCGCTAAAATC	*V. parvula catA* amplification
catA-in-up-F	CCGCTCGAGCTGTACATTGTACATCGTCA	*catA* integration
catA-in-up-R	CTTGTAGATCACAAGGTGTTTTCATTCAATATTAGGTAGTGTTAATAATATTGTG	*catA* integration
catA-in-dn-R	AAACTGCAGTTATTTTACATCGCTAAAATC	*catA* integration
catA-luc-in-up-R	TCTTCCATATTTACCTCCTCGATTATTTTACATCGCTAAAATCGA	*catA*-luc integration
catA-luc-in-dn-F	TCGATTTTAGCGATGTAAAATAATCGAGGAGGTAAATATGGAAGA	*catA*-luc integration
catA-luc-in-dn-R	TTACTGCAGTTACAATTTGGACTTTCCGCCCTTCTTGGCCTTTA	*catA*-luc integration

broth. Cultures were incubated anaerobically at 37°C, and 10-ml samples were taken at an OD_{600} of ~0.5 and 1.0. Cells were harvested by centrifugation. Total RNA isolation and real-time RT-PCR were performed as previously described (47). The primers used are shown in Table 2.

Construction of the shuttle plasmid with *catA* gene. The PCR primers used in this study are listed in Table 2. To construct the shuttle plasmid with the *catA* gene, the *catA* coding region with its native promoter was amplified using the chromosome DNA of *V. parvula* PK1910 as the template and the primer pair pBSJL2-*catA*-F/pBSJL2-*catA*-R. The PCR product was double digested with XhoI and PstI and then ligated with plasmid pBSJL2 (29), which was digested with the same enzymes. The recombinant plasmid pBSJL2-Vp-*catA* was confirmed by sequencing. The confirmed plasmid was then transformed into *V. atypica* OK5 using the established protocol (29). Positive transformants were confirmed by extracting the plasmids and restriction enzyme digestions.

Construction of integrated suicide plasmid for *catA* gene. To construct the *catA* integrated plasmid, a 1,000-bp intergenic region of *V. atypica* OK5 chromosome DNA was amplified by PCR using the primer pair *catA*-in-up-F/*catA*-in-up-R. This PCR amplicon and plasmid pBSJL2-Vp-*catA* were used as the templates, and overlapping PCR was implemented to create a *catA* integrated cassette using the primer pair *catA*-in-up-F/*catA*-in-dn-R. The PCR product was double digested with XhoI and PstI and then ligated with plasmid pBST (23), which was digested with the same enzymes. The recombinant plasmid pBST-Vp-*catA* was confirmed by sequencing.

Construction of integrated suicide plasmid for luciferase reporter of *catA* gene. To construct the integrated plasmid of *catA*-luc, the *catA* integrated cassette and luciferase region were amplified by PCR using plasmids pBST-Vp-*catA* and pFW5-luc as the templates and primer pairs *catA*-in-up-F/*catA*-luc-in-up-R and *catA*-luc-in-dn-F/*catA*-luc-in-dn-R, respectively. The two amplicons were then ligated by overlapping PCR to create a *catA*-luc integrated cassette using the primer pair *catA*-in-up-F/*catA*-luc-in-dn-R. The PCR product was double digested with XhoI and PstI and then ligated with plasmid pBST (23), which was digested with the same enzymes. The recombinant plasmid pBST-Vp-*catA*-luc was confirmed by sequencing.

Generation of the *catA* and *catA*-luc integrated strains. The confirmed plasmids pBST-Vp-*catA* and pBST-Vp-*catA*-luc were transformed into *V. atypica* OK5 using the established protocol (29). The resulting transformants were selected on tetracycline plates and confirmed by PCR and sequencing.

H_2O_2 tolerance assays of OK5-*catA*. A 4,000 μM H_2O_2 solution in BHIL was prepared, and 100 μl of BHIL medium was added into each well of a 96-well plate (Falcon). Next, 100 μl of H_2O_2 solution (4,000 μM) was added into the first well, and then different concentrations of H_2O_2 were prepared by 2-fold serial dilution. As a control, the last well had no H_2O_2. The overnight monocultures of OK5, OK5-*catA*, and PK1910 were centrifuged and resuspended with fresh BHIL medium to an OD_{600} of ~1.0. The optical density at 600 nm was measured with a spectrophotometer (SmartSpec 3000; Bio-Rad). Resuspended bacterial cultures were diluted 1:500 into fresh BHIL medium, and then 100-μl cultures were added into the H_2O_2 solution in 96-well plate (final dilution ratio is 1:1,000). The plates were incubated in the anaerobic chamber at 37°C for 24 h. The MIC to inhibit the growth of *Veillonella* strains was determined. The experiments were performed three times.

Luciferase assays in OK5-*catA*-luc and streptococcus mixed cultures. Overnight cultures of the OK5-*catA*-luc reporter strain, *S. gordonii*, and *S. mutans* were centrifuged and resuspended with fresh BHI broth to an OD_{600} of ~1.0. As a control, resuspended OK5-*catA*-luc culture was diluted 1:100 into fresh BHIL. For coculture tests, resuspended OK5-*catA*-luc culture was diluted 1:100 into fresh BHI medium, and then *S. gordonii* or *S. mutans* was diluted into the same culture with streptococci–OK5-*catA*-luc at a ratio of 5:1. All cultures were grown in a candle jar, and the luciferase activity of OK5-*catA*-luc was measured at 6 h. Luciferase assays were performed by adding 25 μl of 1 mM D-luciferin (Sigma-Aldrich) solution (suspended in 0.1 M citrate buffer [pH 6.0]) into 100-μl samples, and luciferase activities were measured using a TD 20/20 luminometer (Turner Biosystems, Sunnyvale, CA). For mixed-species cultures, the CFU per milliliter of OK5-*catA*-luc were obtained by plate counting on the BHIL plate supplied with tetracycline (2.5 μg/ml). Luciferase activity was expressed in relative light units (RLU)/CFU/ml $\times$ 10^8.

Assay of OK5-*catA* supporting the growth of *F. nucleatum*. Overnight, the cultures of the OK5 wild-type strain, OK5-*catA*, *V. parvula* PK1910, and *F. nucleatum* ATCC 23726 were centrifuged to remove the supernatants, and the cell pellets were washed by SCDM twice and then resuspended in fresh SCDM

supplemented with vitamin K (1.2 μM) and hemin (7.7 μM). The cell suspensions were adjusted to an OD_{600} of ~1.0, *F. nucleatum* was 1:50 diluted into fresh SCDM supplemented with vitamin K (1.2 μM), hemin (7.7 μM) and peptone (0.1%), and then *Veillonella* species were mixed with *F. nucleatum* at a ratio of 2:1. The monocultures of species were included as a control, and 0.6% lactate was added to the cocultures to support *Veillonella* growth. All cultures were incubated in an anaerobic chamber or candle jar at 37°C for 24 h. Ten-microliter aliquots of all cultures were spotted on blood agar plates (Hardy Diagnostics). The plates were incubated in the anaerobic chamber at 37°C for 72 h. *F. nucleatum* colonies were obviously distinguished from *Veillonella* spp. by colony morphology and color. The number of CFU was determined from at least three independent experiments.

Assay of OK5-*catA* supporting *F. nucleatum* growth in three-species culture. The overnight cultures of the OK5 wild-type strain, OK5-*catA*, *V. parvula* PK1910, *F. nucleatum* ATCC 23726, and *S. gordonii* were centrifuged to remove the supernatants, and the cell pellets were washed by SCDM twice and then resuspended in fresh SCDM supplemented with vitamin K (1.2 μM) and hemin (7.7 μM). The cell suspensions were adjusted to an OD_{600} of ~1.0, *F. nucleatum* was diluted 1:50 into fresh SCDM supplemented with vitamin K (1.2 μM), hemin (7.7 μM), and peptone (0.1%), and then a monoculture of *F. nucleatum* was included as a control. *S. gordonii* was mixed with *F. nucleatum* at a ratio of 5:1, and 0.5% glucose was added to the coculture medium to support the growth of *S. gordonii*. Next, *Veillonella* species were mixed with *F. nucleatum* at a ratio of 2:1, resulting in a final ratio of a 3-species culture (*S. gordonii*/*Veillonella* spp./*F. nucleatum*) of 5:2:1. After 24 h of incubation in a candle jar at 37°C, each 10-μl aliquot of each cultures was spotted on blood agar plates. The plates were incubated in the anaerobic chamber at 37°C for 72 h. *F. nucleatum* colonies were distinguished from *Veillonella* and *S. gordonii* by colony morphology and color. The number of CFU was determined from at least three independent experiments.

Statistical analysis. The Student's *t* test was used for statistical analyses, and significance was determined at a *P* value of $\leq$0.05.

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