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Function of the Pyruvate Oxidase-Lactate Oxidase Cascade in Interspecies Competition between *Streptococcus oligofermentans* and *Streptococcus mutans*

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Complex interspecies interactions occur constantly between oral commensals and the opportunistic pathogen *Streptococcus mutans* in dental plaque. Previously, we showed that oral commensal *Streptococcus oligofermentans* possesses multiple enzymes for H_2O_2 production, especially lactate oxidase (Lox), allowing it to out-compete *S. mutans*. In this study, through extensive biochemical and genetic studies, we identified a pyruvate oxidase (*pox*) gene in *S. oligofermentans*. A *pox* deletion mutant completely lost Pox activity, while ectopically expressed *pox* restored activity. Pox was determined to produce most of the H_2O_2 in the earlier growth phase and log phase, while Lox mainly contributed to H_2O_2 production in stationary phase. Both *pox* and *lox* were expressed throughout the growth phase, while expression of the *lox* gene increased by about 2.5-fold when cells entered stationary phase. Since lactate accumulation occurred to a large degree in stationary phase, the differential Pox- and Lox-generated H_2O_2 can be attributed to differential gene expression and substrate availability. Interestingly, inactivation of *pox* causes a dramatic reduction in H_2O_2 production from lactate, suggesting a synergistic action of the two oxidases in converting lactate into H_2O_2. In an *in vitro* two-species biofilm experiment, the *pox* mutant of *S. oligofermentans* failed to inhibit *S. mutans* even though *lox* was active. In summary, *S. oligofermentans* develops a Pox-Lox synergy strategy to maximize its H_2O_2 formation so as to win the interspecies competition.

Humans harbor hundreds to thousands of different indigenous bacterial phylotypes which colonize various surfaces, such as the skin (11), gut (9), vagina (37), and oral cavity (1, 38). These microorganisms usually exist as complex biofilms (6, 12), and species composition in these communities varies in response to environmental changes, such as pH, temperature, nutrition supply (3, 5, 35), and interspecies interaction (15, 23, 46). Though the majority of the inhabitants are beneficial bacteria, some are opportunistic pathogens. When environmental changes favor the growth of opportunistic pathogens, infectious diseases, such as vaginitis (25) and dental caries (13, 31), could develop. Therefore, homeostasis among residential bacteria is critical for maintaining human health (24, 28).

So far, as many as 600 to 800 operational taxonomic units (OTUs) have been found in the human oral cavity (18, 38); some of these OTUs inhabit tooth surfaces and form the multispecies biofilm called dental plaque (20). Dental plaque is a site of dynamic interspecies interaction (34, 36). Oral streptococci are believed to be among the earliest colonizers on tooth surfaces and comprise about 80% of the earliest inhabitants (2, 43). It has been demonstrated that complex interspecies competitions also take place among oral streptococci to contend for the limited space and nutritional resources. Some oral commensal streptococci, such as *Streptococcus sanguinis*, *Streptococcus gordonii*, and *Streptococcus oligofermentans*, are capable of producing hydrogen peroxide (H_2O_2) and bacteriocins which inhibit the overgrowth of the cariogenic pathogen *Streptococcus mutans* (14, 21, 44, 50, 51); *S. mutans*, in turn, produces plentiful lactic acid and mutacin to suppress the growth of oral commensal streptococci (31, 40). Once the environmental parameters are favorable to *S. mutans* growth, e.g., frequent intake of large amounts of carbohydrates by the host, *S. mutans* could become prevalent in dental plaque, resulting in the eventual development of dental caries (31, 54). Conversely, if beneficial oral commensals, such as *S. sanguinis*, are predominant over *S. mutans*, good oral health might be achieved. This antagonistic relationship has been confirmed by epidemiological studies (4). Therefore, a prerequisite for building a healthy oral cavity is to better understand the molecular basis for such relationships.

Frequently isolated from healthy tooth surfaces of the human oral cavity (55), *S. oligofermentans* was first described in 2003 (52). Phylogenetically it belongs to the mitis group of genus *Streptococcus* (52). *S. oligofermentans* has been reported to not only inhibit the growth of *S. mutans* in a two-species biofilm model (50) but also diminish the capacity of *S. mutans* to demineralize hydroxyapatite (55). The inhibitory effect of *S. oligofermentans* on *S. mutans* is attributable to its abundant H_2O_2 production (50, 51). Previously, we reported that *S. oligofermentans* possesses two H_2O_2-generating enzymes, including a lactate oxidase (Lox) (50), by which the bacterium catalyzes the formation of pyruvate and H_2O_2 from L-lactate and oxygen and an L-amino acid oxidase that generates H_2O_2 from amino acids and peptone (51). Especially significant, the lactate oxidase enables *S. oligofermentans* to convert the abundant lactate produced by *S. mutans* into H_2O_2, which in turn inhibits the growth of *S. mutans* (50). Despite the fact that lactate tends to accumulate in the latter stages of bacterial growth, significant levels of H_2O_2 have been detected in an early-log-phase culture of *S. oligofermentans*. Furthermore, H_2O_2 can be detected

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TABLE 1 Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Relevant characteristics and description	Reference or source
Strains		
E. coli DH5α	*supE44* Δ(*lacZYA-argF*)*U169* ϕ80d*lacZ*Δ M15 *hsdR17 recA1 endA1 gyrA96 thi-1 relA1 luxS*	48
S. oligofermentans wild type	AS 1.3089, wild type, Kan^s Sp^s	52
S. oligofermentans pox mutant	AS 1.3089 *pox*::*kan*, AS 1.3089 with *pox* deletion	This study
S. oligofermentans pox-com	AS 1.3089 *pox*::*kan* pDL278-*pox*, AS 1.3089 *pox* mutant with *pox* complement, Kan^r Sp^r	This study
S. oligofermentans lox mutant	AS 1.3089::pFW5-*lox*-luc, AS 1.3089 with *lox* insertion inactivation, Sp^r	50
S. oligofermentans pox lox double mutant	AS 1.3089 *pox*::*kan* pFW5-*lox*-luc, AS 1.3089 with *pox* deletion and *lox* insertion inactivation, Sp^r Kan^r	This study
S. oligofermentans P*pox-luc*	AS 1.3089::pFW5-P*pox*-luc, *luc* under AS 1.3089 *pox* promoter, Sp^r	This study
S. oligofermentans P*lox-luc*	AS 1.3089::pFW5-P*lox*-luc, *luc* under AS 1.3089 *lox* promoter, Sp^r	This study
S. mutans UA140	Wild type	40
Plasmids		
pALH124	Kan^r	30
pDL278	Sp^r	27
pDL278-*pox*	Sp^r, pDL278 with AS 1.3089 *pox* gene under its inherent promoter	This study
pFW5-luc	Sp^r *luc*	39
pFW5-P*pox*-luc	Sp^r *luc*, *luc* under AS 1.3089 *pox* promoter	This study
pFW5-P*lox*-luc	Sp^r *luc*, *luc* under AS 1.3089 *lox* promoter	This study

[a] Kan, kanamycin; Sp, spectinomycin.

even in cultures of *lox* mutants (data not shown). Therefore, other H_2O_2 production pathways may be present. In this study, by using biochemical and genetic approaches, we determined that *S. oligofermentans* possesses another H_2O_2-forming enzyme, pyruvate oxidase (Pox), and demonstrated that Pox contributed to a large portion of H_2O_2 production during the log phase, while Lox generation of H_2O_2 was mainly in the stationary phase. More importantly, inactivation of Pox also largely abolished lactate-dependent H_2O_2 production, suggesting that a Pox-Lox synergy is required for *S. oligofermentans* to best engage in interspecies competition. According to the prevalence of *pox* and *lox* homologues in the oral streptococci, the ecological impact of this synergy can be predicted.

MATERIALS AND METHODS

Experimental strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All *Streptococcus* strains were routinely grown in brain heart infusion (BHI) broth (Difco, Detroit, MI) at 37°C as static cultures, whereas plate cultures were incubated in a candle jar. To grow mutant strains, kanamycin (1 mg ml^{-1}) and spectinomycin (800 μg ml^{-1}) were added to BHI medium when necessary. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium, and when needed, spectinomycin (250 μg ml^{-1}) was supplemented for recombinant selection.

Genetic manipulations. The genomic DNA of *S. oligofermentans* was extracted and purified using the method of Marmur with slight modifications (33). Due to the cell wall of *S. oligofermentans* being resistant to lysozyme breakage and sodium dodecyl sulfate (SDS) treatment, penicillin G (1.25 μg ml^{-1}) was added to the culture at the late exponential phase to make the cell wall development imperfect. The cultures were incubated for another 3 h before being subjected to the DNA extraction procedure. The PCR primers were designed according to the unreleased draft genome of *S. oligofermentans* and synthesized by Sangon Company (Shanghai, China). PCR amplifications were performed with KOD-Plus-Neo (Toyobo, Japan), and purification of PCR products was carried out by using a Qiagen QIAquick PCR purification kit (Valencia, CA). DNA extracted from agarose gels was purified with a Tiangen TIANgel purification midikit (Beijing, China), and plasmids were extracted and purified with a Tiangen TIANprep plasmid minikit. Restriction enzymes and DNA ligase were purchased from New England BioLabs (Beverly, MA). All procedures were carried out as recommended by the suppliers.

A pyruvate oxidase gene (*pox*) deletion mutant was constructed by using the PCR ligation method (26). Briefly, two fragments corresponding to about 600 bp of the upstream and downstream sequence, respectively, of the *pox* gene were amplified by PCR with the following two pairs of primers (restriction sites are underlined, and modified sequences are in italics): *pox*upF (5′-GGCATTCACTAGCATAACCC-3′)/*pox*upR (with a BamHI restriction site) (5′-*AA*GGATCCTTGAGTCATAATGATAAC-3′) and *pox*dnF (with a BamHI restriction site) (5′-*AA*GGATCCGGAAGAAGAAGGTTTGC-3′)/*pox*dnR (5′-CAAGCAATGGACAGACGATAG-3′) using *S. oligofermentans* genomic DNA as the template. The purified PCR products were digested with BamHI. The nonpolar kanamycin resistance gene cassette was released from plasmid pALH124 (30) by digestion with BamHI. All three fragments were purified and mixed at a 1:1:1 molar ratio. A fused fragment was formed by T4 DNA ligase treatment and transformed into the *S. oligofermentans* wild-type strain using the method described previously (53). Transformants were selected on BHI agar plates containing 1 mg ml^{-1} kanamycin; *pox* deletion was confirmed by PCR and sequencing.

A *pox* gene complementary strain was constructed as described below. The *pox* coding region with its inherent promoter was amplified using a pair of 5′-end-modified primers (restriction sites are underlined, and modified sequences are in italics): Pox-com-up (with an EcoRI restriction site) (5′-*AG*GAATTCCTTTTCACCTACTATTTTG-3′) and Pox-com-dn (with a SalI restriction site) (5′-*ATAT*GTCGACTTATTTAATTGCGCGTGATTG-3′). The 2,112-bp PCR product was purified and double digested with EcoRI and SalI. After digestion, the purified fragment was inserted into compatible sites on an *E. coli-Streptococcus* shuttle vector pDL278 (27), which was treated with the same enzymes. Recombinant plasmid pDL278-*pox* was transformed into the *pox* mutant; transformants were selected on BHI agar containing kanamycin (1 mg ml^{-1}) and spectinomycin (800 μg ml^{-1}). The positive transformant was further confirmed by PCR and sequencing.

P*pox-luc* and P*lox-luc* reporter strains were constructed by the following procedures. Two pairs of 5′-modified primers were designed and synthesized, PpoxF (with a BamHI restriction site) (5′-*AA*GGATCCTGTTTTCAATACGTTGAGC-3′)/PpoxR (with an NheI restriction

site) (5′-*AA*GCTAGCGCTGCCAGACCTTTTGTAAG-3′) and PloxF (with a BamHI restriction site) (5′-*AA*GGATCCCATTGCTTGTTTTGTATG-3′)/PloxR (with an NheI restriction site) (5′-*AA*GCTAGCAAGACCCACCTAGGAAAATG-3′). The *pox* and *lox* promoter regions, each including approximately 600 bp of sequence upstream of the gene start codon, were amplified from chromosome DNA of *S. oligofermentans* by PCR with the primer pairs PpoxF/PpoxR and PloxF/PloxR, respectively. Purified *pox* and *lox* promoter fragments were subsequently digested with NheI and BamHI, gel purified, and ligated to compatible sites on the pFW5-luc (39) vector using DNA ligase. Correct recombinants (pFW5-P*pox*-luc and pFW5-P*lox*-luc) were confirmed by restriction analysis, PCR, and sequencing. Plasmids containing P*pox-luc* and P*lox-luc* fusions were then transformed into the *S. oligofermentans* wild-type strain. Transformants were selected on BHI agar containing 800 μg ml^{-1} spectinomycin and confirmed by PCR and luciferase activity.

Hydrogen peroxide determination. Hydrogen peroxide (H_2O_2) in liquid culture was quantified using a modified method described previously (10, 45). Briefly, 650 μl of culture supernatant was added to 600 μl of solution containing 2.5 mM 4-amino-antipyrine (4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one; Sigma) and 0.17 M phenol. The reaction proceeded for 4 min at room temperature; horseradish peroxidase (Sigma) was then added to a final concentration of 50 mU/ml in 0.2 M potassium phosphate buffer (pH 7.2). After 4 min of incubation at room temperature, the optical density at 510 nm (OD_{510}) was measured with a Unico 2100 visible-light spectrophotometer (Shanghai, China). A standard curve was generated with known concentrations of chemical H_2O_2.

Luciferase activity assay. Twenty-five microliters of 1 mM D-luciferin (Sigma) solution (suspended in 1 mM citrate buffer, pH 6.0) was added to 100-μl samples. Luciferase assays were performed essentially as previously described (53) using a TD 20/20 luminometer (Turner system). The OD_{600} of the sample was read with a 2100 visible light spectrophotometer (Unico). All the measurements were done with duplicate samples, and all experiments were repeated at least three times.

Lactic acid determination. The lactic acid assay was performed using an EnzyChrome lactate assay kit (ECLC-100; BioAssay Systems) according to the manufacturer's instructions. Culture samples or reaction mixtures were centrifuged at 12,000 × *g* for 2 min, and the supernatant was diluted to a suitable concentration to perform the assay.

Hydrogen peroxide-generating oxidase activity assay. Cells were collected and washed twice with 1 ml of phosphate-buffered saline (PBS) by centrifugation at 10,000 × *g* for 10 min. The pelleted cells were resuspended in 1 ml of the same buffer and then permeabilized by mixing the suspensions with 0.02 volume of toluene-acetone (1:9, vol/vol) with a vortex mixer for 2 min. Pyruvate oxidase activity was determined by assaying the acetyl phosphate production as described previously with a slight modification (29, 49). The reaction mixture consisted of 0.5 ml of the permeabilized cell suspension and 0.5 ml of a solution containing 50 mM potassium phosphate buffer (pH 6.0), 10 μM $MgCl_2$, 0.2 μM thiamine pyrophosphate (Sigma), 50 mM potassium pyruvate, and 12 μM flavin adenine dinucleotide (FAD; Sigma). The mixtures without the permeabilized cells or potassium pyruvate were used as negative controls. After incubation at 37°C for 20 min with shaking, 1 ml each of 4 M hydroxylamine (pH 6.4) and 0.1 M acetate buffer (pH 5.4) was added, and the mixtures were kept standing for 10 min at room temperature. Then, 1 ml each of 36% HCl, 12% (wt/vol) trichloroacetic acid, and 5% (wt/vol) ferric chloride in 0.1 N HCl was added to the mixtures. After being kept at room temperature for 20 min, the mixtures were centrifuged at 12,000 × *g* for 5 min. The absorbance of the supernatant was measured at 540 nm. A standard curve was generated with known concentrations of acetyl phosphate.

Lactate oxidase and NADH oxidase activities were assessed by assaying the H_2O_2 production as described previously with a slight modification (45). Two hundred microliters of the permeabilized cells was added to 2 ml of the 0.1 M sodium phosphate buffer (pH 7.0) containing 20 mM sodium L-lactate (Shang Hai SSS Reagent Co., Ltd., China) and 13 mM NADH (Sigma), respectively, and the reaction mixtures without sodium L-lactate or NADH were used as negative controls. The mixtures were incubated at 37°C with shaking for 20 min and then centrifuged at 12,000 × *g* for 5 min. The supernatant was used for H_2O_2 determination using the method mentioned above.

Interspecies antagonism assay between *S. oligofermentans* and *S. mutans*. The interspecies antagonism experiment was performed as described previously with a slight modification (50). Briefly, overnight BHI cultures of *S. mutans* and various *S. oligofermentans* strains were adjusted to the same optical density at 600 nm (~1.0). The cultures were diluted at a 1:10 ratio into fresh BHI broth plus 0.5% sucrose in a 12-well cell culture plate (Corning) as a mixed-species or single-species culture. The plate was incubated at 37°C as a standing culture for 8 h. After incubation, cells were dispersed by vigorous pipetting. Serial dilutions of cells were then plated on BHI agar, and CFU of *S. oligofermentans* and *S. mutans* were counted based on their different colony morphologies; the former is white and slimy and the latter is yellow and dry.

Nucleotide sequence accession number. The DNA sequence of the *pox* gene has been deposited in the GenBank database under accession number JQ004920.

RESULTS

Determination of pyruvate oxidase activity and identification of a *pox* gene in *S. oligofermentans*. To find extra H_2O_2 production pathways in *S. oligofermentans*, the well-known bacterial enzymatic activities for H_2O_2 production were tested; those included pyruvate oxidase and NADH oxidase activities. Using the permeabilized cells as the crude enzyme and pyruvate as substrate, the Pox activity of 1.84 ± 0.13 (mean ± standard deviation) μmol/min/mg protein was measured. NADH oxidase activity was barely detected (data not shown); this is probably because the detection limit (1.90 μM) of the H_2O_2 assay makes the assay incapable of detecting the lower H_2O_2 level generated by NADH oxidase.

Next, by using the pyruvate oxidase gene (SGO_0292) from *S. gordonii* as a probe, a putative pyruvate oxidase gene (*pox*; open reading frame [orf] 2213) was hit in the draft genome of *S. oligofermentans*. orf 2213 showed high degrees of identity (96, 96, and 95%, respectively) with pyruvate oxidase genes from *Streptococcus oralis*, *S. gordonii*, and *Streptococcus mitis*.

To further verify the function of the putative *pox* gene, a *pox* deletion mutant was constructed by allelic exchange with a nonpolar kanamycin resistance cassette (*kan*). Meanwhile, a strain ectopically expressing *pox*, designated *pox*-com, was constructed by transforming the shuttle vector pDL278 carrying the *pox* gene with its inherent promoter into the *S. oligofermentans pox* mutant. Both *pox* mutant and *pox*-com strains were confirmed by PCR and sequencing; they were then subjected to pyruvate oxidase (Pox) assay by measuring acetyl phosphate production as described in Materials and Methods. The results showed that the *pox* mutant lost almost all the acetyl phosphate production (0.09 ± 0.02 μmol/min/mg protein), while an activity of 2.41 ± 0.03 μmol/min/mg protein was determined for the complementary strain *pox*-com.

Pox and Lox contribute the majority of hydrogen peroxide production in *S. oligofermentans* but act in different growth phases. To determine the relative contributions of Pox and Lox activities to H_2O_2 production during different growth phases, *S. oligofermentans* wild type and *pox* and *lox* mutants were grown in BHI broth aerobically, with their optical density and H_2O_2 production monitored every hour. As shown in Fig. 1A, the growth results showed that the *lox* mutant displayed a growth profile similar to that of the wild type, while the *pox* mutant grew significantly

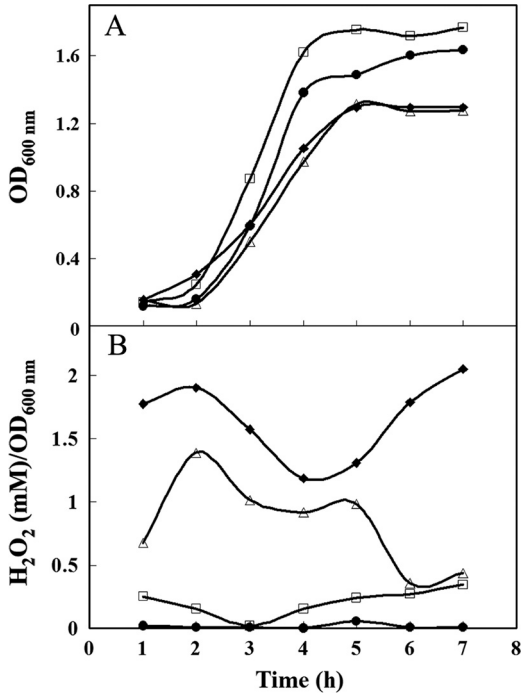

FIG 1 Hydrogen peroxide production by various *S. oligofermentans* strains growing in BHI cultures. Overnight cultures of *S. oligofermentans* wild type and various mutant strains were diluted at a 1:40 ratio into fresh BHI medium. Subsequently, cultures were sampled every hour and then measured for optical density at 600 nm and H_2O_2 production upon shaking at 200 rpm/min for 20 min. (A) Growth curve of various *S. oligofermentans* strains expressed as OD_{600}. (B) Hydrogen peroxide production throughout the growth phase. Data are expressed as H_2O_2 concentration (mM) per optical density at 600 nm. ♦, wild type; △, *lox* mutant; □, *pox* mutant; ●, *pox lox* double mutant. Data are representative of three independent experiments.

faster than the wild type and accumulated more cell mass in the end. Also, as shown in Fig. 1B, the wild-type strain produced H_2O_2 throughout the growth phase, with the highest concentrations of H_2O_2 detected at early log phase and stationary phase. Compared to the H_2O_2 production of the wild type, the *pox* mutant produced markedly smaller amounts of H_2O_2 overall, which increased slightly after cells entered stationary phase. On the other hand, H_2O_2 production by the *lox* mutant was only slightly less than that of the wild type during the exponential phase, and it then quickly diminished to the level produced by the *pox* mutant at the start of the stationary phase. These results indicated that Pox contributes the majority of H_2O_2 production by *S. oligofermentans* when growing exponentially in BHI, while Lox seems to function primarily in the stationary phase.

To determine whether other glucose-originated H_2O_2 production pathways besides Pox and Lox were present in *S. oligofermentans*, a *pox lox* double mutant was constructed by transformation of the *lox* mutant (50) with a PCR fragment containing a *pox* deletion. When tested in the same growth/H_2O_2 production assays as described above, the *pox lox* double mutant also grew faster than the wild type and reached a higher final cell mass (Fig. 1A), while its H_2O_2 level was extremely low (average, 3 μM) throughout the growth phase (Fig. 1B). Together with the phenotype of the *pox* and *lox* mutants, these results indicate that the gene products of *pox* and *lox* are the primary producers of glucose-derived H_2O_2 in *S. oligofermentans*. Therefore, the H_2O_2 from the *pox* mutant can be taken as the product of Lox, while the *lox* mutant can be taken as a Pox active strain.

Gene expression level and substrate availability determine Pox and Lox activities for hydrogen peroxide production. Based on the differential H_2O_2 production mediated by Pox and Lox in different growth periods, the levels of growth phase-related expression were assayed for the two genes. Each of the P*pox-luc* and P*lox-luc* fusions was constructed by the fusion of a DNA fragment containing the *pox* or *lox* promoter to a promoterless luciferase gene (*luc*) on the pFW5-luc plasmid (39); the plasmids pFW5-P*pox*-luc and pFW5-P*lox*-luc were then transformed into *S. oligofermentans* wild type. Overnight BHI cultures of the reporter strains, *S. oligofermentans* P*pox-luc* and *S. oligofermentans* P*lox-luc*, were diluted 1:40 into fresh BHI broth, and the cultures were sampled at 1-h intervals to measure the optical density at 600 nm and luciferase activity as described in Materials and Methods. As shown in Fig. 2A, luciferase activity of the P*pox-luc* fusion could be detected from the early growth phase and was maintained at a somewhat-constant level throughout the growth phase, indicating constitutive expression of the *pox* gene. While the expression of the *lox* gene was minimal at early log phase, it increased by about 2.5-fold when cells entered stationary phase. To further examine the expression of these two genes at the protein level, the enzyme activities of Pox and Lox were measured throughout the growth

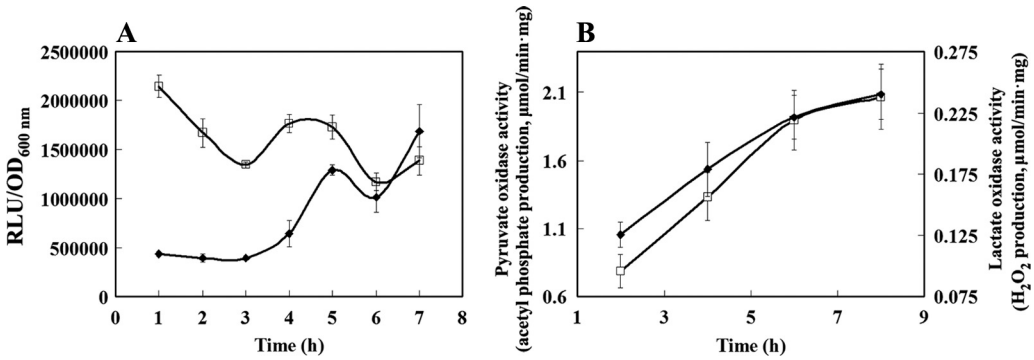

FIG 2 Expression profiles and enzymatic activities of pyruvate oxidase and lactate oxidase in *S. oligofermentans*. Cells were collected at different growth phases and then assayed for luciferase, pyruvate oxidase, and lactate oxidase activities as described in Materials and Methods. (A) Luciferase activities of P*pox-luc* and P*lox-luc* fusions throughout the growth phase. Data are expressed as the relative light units (RLU) per optical density at 600 nm. □, P*pox-luc*; ♦, P*lox-luc*. (B) Pyruvate oxidase (□) and lactate oxidase (♦) activities. Results are expressed as the means ± standard deviations of three independent experiments.

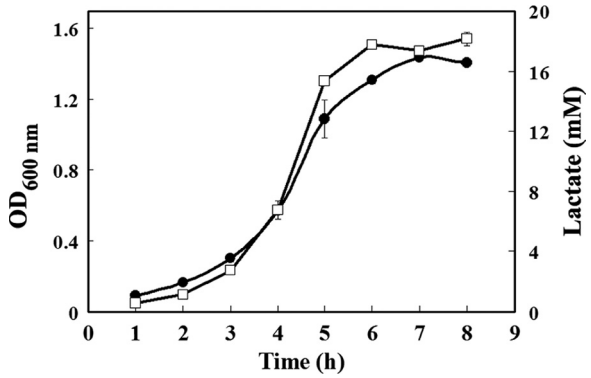

FIG 3 Time course of lactate production by *S. oligofermentans*. Overnight cultures of *S. oligofermentans* were diluted at a 1:40 ratio into fresh BHI medium and incubated as a static culture; samples were taken every hour to measure optical density at 600 nm and lactate concentration. ●, growth expressed as OD_{600}; □, lactate concentration (mM). Results are expressed as means ± standard deviations of three independent experiments.

period in *S. oligofermentans* wild type. The results showed that their enzymatic activities increased steadily during the entire growth period (Fig. 2B).

Since Pox- and Lox-mediated H_2O_2 production occurred differentially during these growth phases, substrate availability for each enzyme at different growth periods could be predicted. Pyruvate, the substrate for Pox, is the intermediate of glucose fermentation via the Embden-Meyerhof-Parnas (EMP) pathway, while lactate, the substrate of Lox, is the end product of the EMP pathway and accumulates in late log phase. As shown in Fig. 3, lactate was only detected at about 2 to 3 mM in early log phase, while the yield increased to about 6 mM in the mid-log-phase culture and increased quickly to about 18 mM in the stationary-phase culture. Therefore, both gene expression level and substrate availability coordinately influence H_2O_2 generation by Pox or Lox in *S. oligofermentans*.

Inactivation of Pox dramatically reduces lactate oxidase activity. Although low H_2O_2 production by Lox in the earlier growth phase might be due to the low lactate level, reduced H_2O_2 production (about 0.50 mM) in the stationary culture of the *pox* mutant suggests a possible effect of Pox on the regulation or function of Lox. To investigate this possibility, lactate levels were measured for the stationary cultures of the wild-type strain and the *pox* and *lox* mutants. As shown in Fig. 4A, lactate yields in the static cultures were similar in all three strains, with 18.3, 18.1, and 18.4 mM in the wild type and the *pox* and *lox* mutants, respectively. Surprisingly, upon shaking the cultures for 20 min to maximize lactate oxidase activity, the lactate levels decreased 14% in the wild type ($P < 0.05$), whereas little change was seen in either the *pox* or *lox* mutant. In shaken cultures of the wild-type strain and the *pox* and *lox* mutants, the increased H_2O_2 levels were measured at 1.73 mM, 0.31 mM, and 0.33 mM (Fig. 4B), respectively. This indicates that Pox activity could exert an effect on Lox activity.

To clarify the effect of Pox on Lox, Lox activity in the wild type was compared with that in the *pox* mutant as described in Materials and Methods. Lactate oxidase activity, i.e., H_2O_2 formation from lactate, in the *pox* mutant was found to be only 8% of that in the wild-type strain. According to Taniai et al. (49), lactate is oxidized to acetyl phosphate and H_2O_2 by a coupled reaction of Pox and Lox; i.e., lactate is first oxidized by Lox into H_2O_2 and pyruvate, which is then converted into acetyl phosphate and H_2O_2 by Pox. Therefore, inactivation of *pox* would in theory disrupt the complete conversion of lactate into H_2O_2. Since the Pox activity in the *lox* mutant (1.95 ± 0.43 μmol/min/mg) was about the same as that in the wild-type strain (1.84 ± 0.14 μmol/min/mg), Lox activity does not seem to be required for the function of Pox.

Pox contributes more in the interspecies competition between *S. oligofermentans* and *S. mutans* in biofilm. As Pox was determined to contribute the most to H_2O_2 production in *S. oligofermentans* and to be required for Lox activity, the relative roles of Pox and Lox in the interspecies competition were determined in the two-species biofilm model. To do this experiment, overnight BHI cultures of *S. oligofermentans* wild type, *pox* mutant, *lox* mutant, or *pox lox* double mutant were inoculated with *S. mutans* pairwise as a mixed-species biofilm, with monocultures of each strain included as controls. After 8 h, the biofilm cells were scraped and spun down. The cell numbers of each strain in mixed or monocultures were counted on BHI agar plates. *S. oligofermentans* and *S. mutans* cells were enumerated based on their different colony morphologies; however, when necessary, *S. oligofermentans* mutants were counted on BHI agar plates supplemented with antibiotics. As shown in Fig. 5A, compared to the number of *S. mutans* cells in monoculture, *S. mutans* cells decreased the most when cocultured with *S. oligofermentans* wild type, followed by the *lox* mutant, the *pox* mutant, and lastly, the *pox lox* double mutant. Meanwhile, the abundance of *S. oligofermentans* wild-type cells

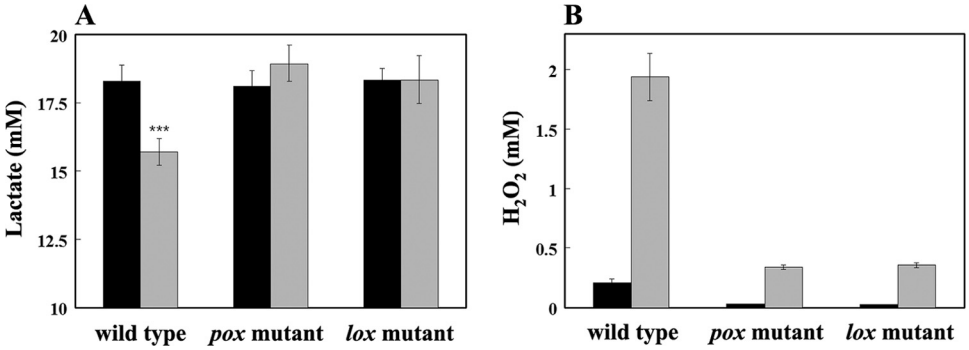

FIG 4 Lactate oxidase activities of *S. oligofermentans* wild type and *pox* and *lox* mutants. Lactate concentration (A) and H_2O_2 production (B) were measured in the stationary-phase cultures before (black bar) and after (gray bar) shaking at 200 rpm/min for 20 min. Data are expressed as the means ± standard deviations of three independent experiments. ***, data are statistically significant in comparison to values before shaking, as verified by Student's *t* test ($P < 0.05$).

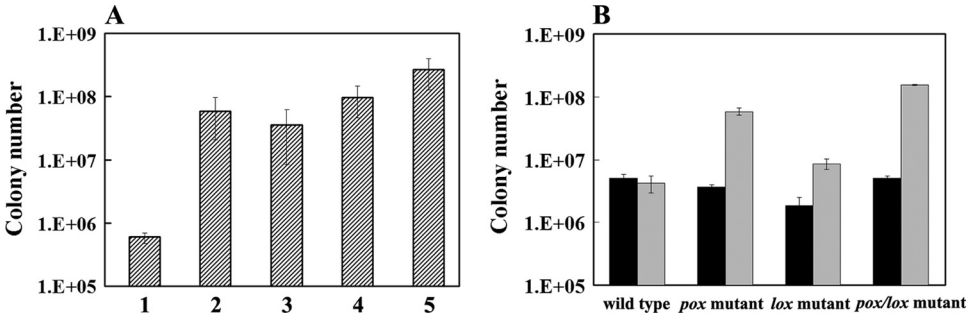

FIG 5 *pox* and *lox* mutant strains of *S. oligofermentans* lost antagonism against *S. mutans* in the two-species biofilms formed in BHI-sucrose. Overnight cultures of various *S. oligofermentans* strains and *S. mutans* UA140 were adjusted to the same optical density at 600 nm (~1.0) and then were inoculated (1:10) into a 12-well plate containing fresh BHI broth supplemented with 0.5% sucrose to form mono- or mixed-species biofilms. After 8 h of incubation, the biofilms were harvested and cell numbers enumerated by plating. (A) CFU of *S. mutans* in mixed cultures with various *S. oligofermentans* strains (bars 1 to 4) and in a monoculture (bar 5). Bars: 1, with wild type; 2, with *pox* mutant; 3, with *lox* mutant; 4, with *pox lox* double mutant. (B) CFU of various *S. oligofermentans* strains in mixed cultures with *S. mutans* (black bar) and in monocultures (gray bar). Data are expressed as the means ± standard deviations of three independent experiments.

was comparable in both monoculture and coculture with *S. mutans*, while the numbers of the *pox* mutant, *lox* mutant, and *pox lox* double mutant in the coculture with *S. mutans* were reduced to about 3, 21, and 6%, respectively, of their levels in monoculture (Fig. 5B). These results suggest that inactivation of Lox and, especially, loss of Pox resulted in diminished inhibition of *S. mutans* or, conversely, reduced resistance against *S. mutans*.

DISCUSSION

The production of H_2O_2 is thought to be the principal strategy used by oral commensals to win the interspecies competition over *S. mutans* (21, 44, 50, 51). Enzymes that oral bacteria employ to produce H_2O_2 include NADH oxidase (17, 32), pyruvate oxidase (Pox) (22), lactate oxidase (Lox) (41, 50), and L-amino acid oxidase (LAAO) (51). Oral commensal *S. oligofermentans* can produce copious amounts of H_2O_2 from lactate through Lox (50) or from L-amino acids via LAAO (51). As demonstrated by its potent ability to inhibit *S. mutans* growth via release of H_2O_2, *S. oligofermentans* has great potential to be developed into a probiotic for prevention of dental caries. In this study, we showed that *S. oligofermentans* develops a smart strategy to maximize its advantage in interspecies competition over *S. mutans*, using a synergy of two H_2O_2-producing enzymes, pyruvate oxidase and lactate oxidase.

Pyruvate oxidase (Pox) catalyzes the oxidation of pyruvate to produce acetyl phosphate and H_2O_2. Pox has been identified in several lactic acid bacteria (16, 42, 47), including *S. sanguinis* and *S. gordonii* (22). Both *S. sanguinis* and *S. gordonii* are believed to be among the first colonizers in dental biofilm. They influence biofilm development through coaggregation and competition with other bacterial species (7), with Pox playing a critical role in their interaction with *S. mutans* (22). Hence, Pox can be predicted to be part of the primary defense mechanism against dental pathogen *S. mutans* in the initial stage of biofilm formation. In this study, a *pox* homologue was identified in *S. oligofermentans* and verified to encode a functional pyruvate oxidase. The relatively constitutive expression of *pox* throughout the growth (Fig. 2) and the sufficient substrate (pyruvate produced through glycolysis) enable the Pox-generated H_2O_2 to account for the major portion of the H_2O_2 pool (Fig. 1), especially in the earlier growth phase when little lactate is present. Therefore, Pox could be especially important in helping *S. oligofermentans* to outcompete H_2O_2-susceptible dental pathogens and inhabit the multispecies community as an earlier colonizer.

More importantly, inactivation of Pox causes a significant reduction in the amount of H_2O_2 produced by Lox. Lactate, the bacterial metabolite present in dental plaque, is the main cause of tooth demineralization and caries (31) but can be converted by the Lox-possessing *S. oligofermentans* into H_2O_2, which inversely inhibits *S. mutans* growth (50). Therefore, the activity of Lox can exert a special ecological impact in the oral environment. In this study, we found that through the biochemical cascade with Pox, Lox can achieve its maximal potential in converting the cariogenic lactic acid into H_2O_2 (Fig. 4). Affected by *pox* inactivation on Lox activity, the quantity of H_2O_2 produced by the *pox* mutant is very low in the stationary phase (Fig. 1B), even if a large amount of lactate accumulates (Fig. 3) and *lox* is active (Fig. 2); hence, the sum of H_2O_2 produced in *pox* and *lox* mutants is much lower than the wild-type level (Fig. 1B). Therefore, active Pox is critical for *S. oligofermentans* to win the interspecies competition against *S. mutans* (Fig. 5). Moreover, H_2O_2 production via Pox is coupled to the generation of acetyl phosphate, and the conversion of acetyl phosphate to acetate generates ATP. The Pox-Lox cascade has been shown to play a role in energy recruitment from lactate oxidation in *Streptococcus pneumoniae* (49). Hence, in addition to maximal H_2O_2 production from lactate, the Pox-Lox interplay might supply extra energy for oral commensals in dental biofilm.

Bioinformatics analysis showed that *pox* and *lox* homologues are also present in the genomes of four other streptococci—*S. mitis*, *Streptococcus cristatus*, *S. oralis*, and *Streptococcus infantis*. They all belong to the mitis group and are prominent members of the oral commensal streptococci (8). However, no *pox* and *lox* homologues can be found in the genome of *S. mutans*. Therefore, the synergistic action between the two oxidases can be crucial for oral streptococci in interspecies competition in plaque biofilm, thus affecting dental health.

Reportedly, the pyruvate oxidase gene is subjected to repression by catabolite control protein A (CcpA) in *S. gordonii* (57) and *S. sanguinis* (56). The isogenic *ccpA* mutant in the two species shows elevated pyruvate oxidase gene expression and H_2O_2 production. Similarly, *lox* gene expression is under the control of CcpA in *Streptococcus pyogenes* (19), and no Lox activity is detected in the glucose-containing culture of *S. pyogenes* (45),

whereas in *S. oligofermentans*, *lox* expression and, more importantly, Lox activity and H_2O_2 production are readily detected in the glucose-containing BHI culture (Fig. 2). This may help *S. oligofermentans* win the competition over *S. mutans* by using Pox and Lox as soon as it colonizes in the dental biofilm. Therefore, further studies are needed to explore the detailed characterization of these two genes, including their interaction and regulation in the context of different carbon sources, as well as oxygen tension.

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