

SO-LAAO, a Novel L-Amino Acid Oxidase That Enables *Streptococcus oligofermentans* To Outcompete *Streptococcus mutans* by Generating H₂O₂ from Peptone^{∇†}

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We previously demonstrated that *Streptococcus oligofermentans* suppressed the growth of *Streptococcus mutans*, the primary cariogenic pathogen, by producing hydrogen peroxide (H₂O₂) through lactate oxidase activity. In this study, we found that the *lox* mutant of *S. oligofermentans* regained the inhibition while growing on peptone-rich plates. Further studies demonstrated that the H₂O₂ produced on peptone by *S. oligofermentans* was mainly derived from seven L-amino acids, i.e., L-aspartic acid, L-tryptophan, L-lysine, L-isoleucine, L-arginine, L-asparagine, and L-glutamine, indicating the possible existence of L-amino acid oxidase (LAAO) that can produce H₂O₂ from L-amino acids. Through searching the *S. oligofermentans* genome for open reading frames with a conserved flavin adenine dinucleotide binding motif that exists in the known LAAOs, including those of snake venom, fungi, and bacteria, a putative LAAO gene, assigned as *ao_{so}*, was cloned and overexpressed in *Escherichia coli*. The purified protein, SO-LAAO, showed a molecular mass of 43 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and catalyzed H₂O₂ formation from the seven L-amino acids determined above, thus confirming its LAAO activity. The SO-LAAO identified in *S. oligofermentans* differed evidently from the known LAAOs in both substrate profile and sequence, suggesting that it could represent a novel LAAO. An *ao_{so}* mutant of *S. oligofermentans* did lose H₂O₂ formation from the seven L-amino acids, further verifying its function as an LAAO. Furthermore, the inhibition by *S. oligofermentans* of *S. mutans* in a peptone-rich mixed-species biofilm was greatly reduced for the *ao_{so}* mutant, indicating the gene's importance in interspecies competition.

The oral cavity harbors more than 500 microbial species (26), which maintain a dynamic balance through interacting with each other. This natural microflora contributes to the host defenses by excluding exogenous microorganisms. However, this stability can be perturbed by significant changes in the oral environment. Changes in diet, saliva flow, and general health, etc., can lead to overgrowth of some opportunistic pathogens, which cause such diseases as dental caries and bacterial endocarditis (23). Therefore, understanding the interactions among different oral microbial species is an important aspect of microbial ecology and essential for studies of oral microbial pathogenesis.

Streptococcus mutans is a primary pathogen causing dental caries (tooth decay) (18). The virulence attributes of *S. mutans* include biofilm formation (14, 24), lactic acid production and tolerance (18), and bacteriocin production (29, 40). It has also

been reported that some other oral streptococcal species, such as *Streptococcus sanguinis* and *Streptococcus oligofermentans*, a recently identified oral streptococcal species (34), can suppress the growth of *S. mutans* by producing hydrogen peroxide (H₂O₂) (16, 31, 36). These in vitro interactions were also substantiated by epidemiological studies, in which a reverse relationship between the level of *S. mutans* and that of *S. sanguinis* or *S. oligofermentans* has been demonstrated (3, 5; unpublished data for *S. oligofermentans*). This interspecies competition and inhibition probably help maintain the microbial homeostasis in the oral cavity.

S. oligofermentans has been isolated from dental plaques of caries-free human subjects in Beijing, China (34), and belongs to the “mitis” group of oral streptococci, which are believed to be the “pioneer” colonizers during the initial stages of dental biofilm formation. In a previous study, we demonstrated that *S. oligofermentans* inhibited the growth of *S. mutans* by producing abundant H₂O₂: more specifically, by converting the large amount of lactic acid produced by *S. mutans* through its enzymatic activity of lactate oxidase (Lox). The *lox* mutant of *S. oligofermentans* indeed lost its inhibitory activity toward *S. mutans* on peptone-deprived tryptone-yeast extract-glucose (TYG) plates (36); however, it was found that the *lox* mutant regained its inhibitory activity while growing on peptone-rich plates in this study. This suggests that *S. oligofermentans* may have other H₂O₂ production pathways independent of Lox. In

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this study, a novel L-amino acid oxidase (LAAO) (SO-LAAO) was identified and was demonstrated to catalyze H₂O₂ production from peptone in *S. oligofermentans*. Thus, a novel H₂O₂ production pathway was determined for this oral streptococcus.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. All *Streptococcus* strains were routinely grown in brain heart infusion (BHI) medium (Difco, BD) or TYPG (0.5% tryptone–0.5% soy peptone–1% yeast extract–1% glucose) broth and incubated at 37°C as static culture unless indicated otherwise. For the biofilm assay, bacteria were grown in BHI or TYPG broth plus 0.5% sucrose and 1% glucose. For the antagonism assay on amino acids, bacteria were grown on an 0.8% modified chemically defined agar plate (33) containing the following components (per ml): 5 mg of glucose; 5 µg of L-glutamine; 100 µg of L-alanine, L-histidine, L-proline, L-cysteine, L-glycine and L-threonine; 200 µg of L-aspartic acid, L-isoleucine, L-phenylalanine, L-valine, L-methionine, L-serine, and L-leucine; 300 µg of L-glutamic acid; 400 µg of L-lysine; 100 µg of riboflavin, nicotinic acid, and pyridoxine; 50 µg of thiamine HCl and α-pantothenate; 10 µg of D-biotin, *p*-aminobenzoic acid, and folic acid; 208 mg of K₂HPO₄; 292 mg of KH₂PO₄; 120 mg of MgCl₂ [6H₂O]; 2 mg of MnCl₂ [4H₂O]; 11 mg of CaCl₂ [2H₂O]; 2 mg of FeSO₄ [7H₂O]; and 800 mg of agar.

H₂O₂ assay. Hydrogen peroxide in liquid culture was quantified using a modified method described previously (10, 32). Briefly, 1.3 ml of culture supernatant was added to 1.2 ml 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, and then horseradish peroxidase (Sigma) was added to a final concentration of 50 mU ml⁻¹ in 0.2 M potassium phosphate buffer (pH 7.2). After a 4-min incubation at room temperature, the A₅₁₀ was measured with a Beckman DU-800 spectrophotometer. A standard curve was generated with known concentrations of chemical H₂O₂.

In vitro interspecies interaction assays between oral streptococci. Overnight cultures of *Streptococcus* species were adjusted to the same optical density at 600 nm (OD₆₀₀) (~1.0). All 10-µl aliquots of *Streptococcus* species were inoculated adjacently on 0.8% agar plates. The plates were incubated in a candle jar at 37°C for 24 h.

Amplification and cloning of the entire L-amino acid oxidase gene of *S. oligofermentans* (*aoa*_{so}). The genomic DNA of *S. oligofermentans* was extracted and purified using the method of Marmur (22) with slight modification (8). Based on the known LAAOs, using flavin adenine dinucleotide (FAD) as a cofactor (37), the protein sequence of amino acid oxidase of *Rhodococcus opacus* was used as a probe to search the homologue against the complete genome of *S. sanguinis* SK36 because of its close relationship with *S. oligofermentans* and production of hydrogen peroxide with a similar amino acids profile (data not shown). The open reading frame (ORF) SSA-0323, annotated as “flavoprotein, putative” in the completed *S. sanguinis* SK36 genome, was selected as the candidate. To identify a conserved DNA sequence for primer design, SSA-0323 was used as a probe to search for homologous proteins in the *Streptococcus pyogenes* M1 GAS genome and an ORF, SPy_1866, was identified. By aligning the coding sequences of SPy_1866 (1,170 bp) and SSA-0323 (1,176 bp) using DNAMAN, a pair of degenerate primers, solaaof (5'-GCNGGNATCCNGGNAATGG-3') (nucleotide positions 186 to 205 of SPy_1866 and SSA-0323) and solaaor (5'-TCNANNCNCCNTTGGTCCAC-3') (nucleotide positions 999 to 1018 of SPy_1866 and SSA-0323) were designed and synthesized by Sangon Company (Shanghai, China). A partial *aoa*_{so} gene of *S. oligofermentans* was amplified by using the primer pair solaaof/solaaor and chromosomal DNA as a PCR template. The 25-µl PCR mixture contained 100 ng DNA, 0.4 µM (each) primer, 100 µM (each) deoxynucleoside triphosphate mix, 10× PCR buffer, and 0.1 U *Taq* DNA polymerase (Takara Company, Dalian, China). PCR was performed at 95°C for 5 min, followed by 30 cycles of 94°C for 30s, 55°C for 1 min, and 72°C for 1 min and then one cycle at 72°C for 10 min. The PCR product (832 bp) was cloned into pUCM-T (Shenergy Biocolor Company, Shanghai, China) and verified by DNA sequencing.

To obtain the entire *aoa*_{so} gene, inverse PCR was employed to amplify the flanked sequences of the 832-bp fragment. Genomic DNA from *S. oligofermentans* was digested with EcoRI, BamHI, NheI, StuI, and XbaI at 37°C for 2 h. The digestion mixture was extracted by chloroform, and DNA was precipitated by ethanol. The digested DNA was self-circularized using T₄ DNA ligase (Shenergy Biocolor Company, Shanghai, China) at 14°C for 24 h. The resultant ligation mixture was used as the template, and primers solaaoinverseF (5'-CGGAGAC

GATTTCAGTATTGGTG-3') and solaaoinverseR (5'-TTACCCAGCCCTTTCCCGAG-3') were applied for the inverse PCR. The PCR was performed as described above except that *LA Taq* DNA polymerase (Takara Company, Dalian, China) was used and the extension time was 5 min. The 5-kb PCR product obtained from XbaI-digested DNA mixtures was purified, ligated to pCR2.1-Topo (Invitrogen), and sequenced.

Construction of the expression vector of the *aoa*_{so} gene. The complete *aoa*_{so} gene was amplified using a pair of 5'-modified primers (restriction sites are underlined, and modified sequences are in italics): solaaoinverseF (with an NdeI restriction site) (5'-*ACATATGATGAACCATTTCGACAC*-3') and solaaoinverseR (with a BamHI restriction site) (5'-*AAGGATCCTTAATCATAATGCAA*ACTTC-3'). *Pfu* DNA polymerase (Promega) was used in a PCR to amplify the 1,176-bp *aoa*_{so} gene. This fragment was subsequently digested with NdeI-BamHI and then cloned into the NdeI-BamHI restriction sites of expression vector pET-15b (Novagen) to generate the pTH3 plasmid. The His₆-tag fusion sites and nucleotide sequences of double-stranded template DNA were confirmed by DNA sequencing using the ABI 3730xl sequencer.

Overexpression and purification of SO-LAAO protein. The pTH3 plasmid inserted with a PCR-amplified *aoa*_{so} gene fragment was transformed into *Escherichia coli* BL21(DE3)pLysS (Novagen) cells and cultured in LB medium supplemented with 100 µg ml⁻¹ of ampicillin and 34 µg ml⁻¹ of chloramphenicol. Cells were grown at 37°C to an OD₆₀₀ of 0.4 to 0.6. Overproduction of the SO-LAAO protein was induced by addition of 5 mM isopropyl-β-D-thiogalactopyranoside and 1.2 µM FAD. The culture was allowed to grow for an additional 3 to 4 h before being harvested. Cells were collected by centrifugation at 8,200 × g for 10 min, resuspended in a 1/10 volume of binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 30 mM imidazole, pH 7.4), and then broken by sonication for 10 min. After the cell lysate was spun down at 18,449 × g for 15 min, the supernatant was filtered through a 0.22-µm polyvinylidene difluoride membrane (Millipore) and then applied to a Ni²⁺-charged chelating column (GE Healthcare) previously equilibrated with binding buffer. Proteins were eluted by elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4), and the fractions of elution were run on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. The fractions with desired protein were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 8.0).

Assay of LAAO activity of SO-LAAO. LAAO activity of SO-LAAO was determined by measuring hydrogen peroxide production with the enzyme-coupled assay described above. The assay mixtures contained 1.2 ml of 2% L-amino acid solution, 12.5 U of the horseradish peroxidase (250 U mg⁻¹; Sigma), 1.2 ml of solution containing 2.5 mM 4-aminoantipyrine and 0.17 M phenol, and 200 nM SO-LAAO in Tris-HCl buffer, pH 8.0, and the incubation was performed at 25°C.

Construction of *aoa*_{so} insertional mutant of *S. oligofermentans*. A pair of primers, solaaoinverseF (5'-GAAAATTGCAGAGCTGGG-3') and solaaoinverseR (5'-AAAGTCTGCCAAATCCT-3'), were designed and synthesized by Sangon Company (Shanghai, China). The 447-bp internal fragment of *aoa*_{so} (located from 357 bp to 803 bp) was generated by PCR using the chromosomal DNA as a template and cloned into pCR2.1-Topo (Invitrogen). The fragment was subsequently excised using BamHI and XbaI (New England Biolabs), purified, and ligated to the compatible sites on the pFW5-luc (28) vector using T₄ DNA ligase (Shenergy Biocolor Company, Shanghai, China) to generate plasmid pTH4. The ligation mixture was then transformed into *E. coli*. Plasmid pTH4 was confirmed by restriction analysis and PCR and then transformed into *S. oligofermentans* using the method described previously (35). The transformants were then selected on BHI agar containing spectinomycin (800 µg ml⁻¹) and confirmed by PCR amplification of the spectinomycin-resistant gene *aad9* and by Southern blotting with probes for *aoa*_{so} and *aad9*.

Confocal laser scanning microscopy of mixed-species biofilms. Overnight cultures of *S. oligofermentans*::Φ(*ldhp-gfp*) (35) and *S. mutans* UA140::Φ(*mutAp-mrfp*) (15) were diluted (1:10) into BHI-SG (BHI supplemented with 0.5% sucrose and 1% glucose) or TYPG-SG (TYPG supplemented with 0.5% sucrose and 1% glucose) broth and then inoculated into the Lab-Tek II chamber slide system (Nalge Nunc International, Naperville, IL). After a 16-h incubation at 37°C in a candle jar, the culture supernatant was removed from the chambers. Mature biofilms were exposed to air for 10 min in the dark at room temperature and then washed with phosphate-buffered saline buffer. The biofilms were observed using a confocal laser scanning microscope (LEICA TCS SP2).

Luciferase measurement. An overnight bacterial culture was diluted (1:30) into fresh culture medium. The culture was sampled every 60 min from early log phase to early stationary phase to measure luciferase activity and OD₆₀₀. 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, and luciferase assays were performed essentially as previously described (19) using a TD 20/20 luminometer (Turner system). The OD₆₀₀ was read with a 721 spectrophotometer (Shanghai

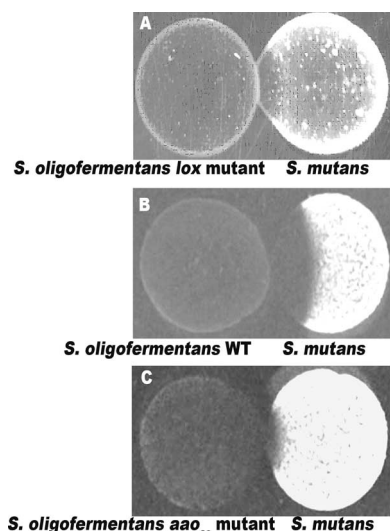


FIG. 1. Growth inhibition of *Streptococcus mutans* by *Streptococcus oligofermentans*. Each 10- μ l overnight culture of *S. oligofermentans* wild type or the *lox* or *aao_{So}* mutant of *S. oligofermentans* and *S. mutans* at the same OD₆₀₀ was spotted adjacently on plates and incubated in a candle jar at 37°C for 24 h. Inhibition by the *S. oligofermentans lox* mutant on peptone-present TPYG medium (A), by the *S. oligofermentans* wild type (WT) (B), or by the *S. oligofermentans aao_{So}* mutant on an amino acid-containing chemically defined agar plate (C) is shown.

Analytical Manufactory). All the measurements were done with duplicate samples, and all the readings were from three repeated experiments.

Nucleotide sequence accession number. The DNA sequence of the *aao_{So}* gene has been deposited in the GenBank database under accession number EU495328.

RESULTS

***S. oligofermentans* produces hydrogen peroxide from peptone and amino acids.** It had been demonstrated that *S. oligofermentans* inhibits *S. mutans* in carbohydrate-rich medium by generating pronounced H₂O₂ from lactate with Lox. While the *lox* mutant completely lost the inhibition on TYG (36), partial inhibition still was retained on 0.5% soy peptone (Difco, BD)-supplemented TYG (TPYG) (Fig. 1A). This suggested that *S. oligofermentans* might have other H₂O₂ production pathways in addition to Lox. Since peptone was the only different component between TYG and TPYG, it was speculated that peptone was involved in H₂O₂ generation by *S. oligofermentans* in addition to lactate. To verify this, H₂O₂ production from peptone by *S. oligofermentans* was measured. By suspending the BHI overnight culture of *S. oligofermentans* in 0.5% peptone and exposing it to air for 20 min at 37°C, the H₂O₂ yield was determined to be 33.11 \pm 0.22 μ mol mg⁻¹ cell mass.

It is well known that L-amino acids are the constitutive components of peptone; therefore, 20 L-amino acids were tested for hydrogen peroxide production by *S. oligofermentans*. It was determined that H₂O₂ was produced mainly from seven L-amino acids (final concentration, 2%) (Table 1), including L-aspartic acid, L-tryptophan, L-lysine, L-isoleucine, L-arginine, L-asparagine, and L-glutamine. Therefore, a conclusion could be drawn that the H₂O₂ produced from peptone was derived from the seven L-amino acids.

Cloning of putative LAAO gene (*aao_{So}*) from *S. oligofermentans*. According to the previous biochemical studies (9), LAAO was the enzyme most likely to implement the reaction of oxidizing amino acids to form ketoacids, ammonia, and H₂O₂. To further verify the capability of *S. oligofermentans* to produce H₂O₂ from amino acids, a possible amino acid oxidase gene(s) in this oral streptococcus was searched for as described in Materials and Methods, and an open reading frame, named the *aao_{So}* gene, was identified. A BLASTP search at the NCBI website revealed the presence of the deduced amino acid sequence (SO-LAAO) of *aao_{So}* homologues in the genomes of five other streptococci (Fig. 2), at the identity levels of 96% with “hypothetical protein TIGR00275” of *Streptococcus gordonii*, 95% with “putative flavoprotein SSA-0323” of *S. sanguinis*, 85% with “hypothetical protein SP_0741” of *Streptococcus pneumoniae*, 79% with “hypothetical protein SPY_1866” of *S. pyogenes*, and 75% with “hypothetical protein SMU.392c” of *S. mutans*. This indicated that SO-LAAO homologous proteins might be widely present in streptococci. Partial sequence alignment (Fig. 2) of six streptococcal ORFs and four known LAAOs from bacteria, snakes, and fungi revealed that all the sequences contained a typical FAD binding site for the flavoprotein GxGxxG (37), whereas SO-LAAO had two amino acid difference (RxGKK) from the LAAOs’ characteristic sequence motif (RxGGR) (37), so that SO-LAAO could represent a novel LAAO.

Amino acid oxidase activity of *aao_{So}* gene product. To test the amino acid oxidase activity of the *aao_{So}* gene product, it was overexpressed in the *E. coli* BL21(DE3)pLysS strain. A His₆-tag sequence was fused to the N terminus of SO-LAAO to facilitate subsequent purification by immobilized metal ion affinity chromatography. Purified recombinant protein was shown as a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with a molecular mass of about 43 kDa (data not shown), slightly lower than the predicated molecular mass (44,973.42 Da) of the recombinant SO-LAAO protein. The purified protein was analyzed for amino acid oxidase activity using 20 L-amino acids as substrates as described in Materials and Methods. The reaction mixture without either purified enzyme, L-amino acid substrate, or horseradish peroxidase was included as a blank control. Enzyme assays showed that SO-LAAO catalyzed H₂O₂ production from L-aspartic acid, L-tryptophan, L-lysine, L-isoleucine, L-arginine, L-asparagine, and L-glutamine (each at a 2% concentration) (Table 1), while no measurable H₂O₂ was detected

TABLE 1. Hydrogen peroxide production by *Streptococcus oligofermentans* and heterogeneous expressed SO-LAAO^a

L-Amino acid (2%)	H ₂ O ₂ production (nmol mg ⁻¹ cell mass)	H ₂ O ₂ production (nmol mg ⁻¹ SO-LAAO protein)
L-Aspartic acid	40 \pm 1.4	469.67 \pm 79.36
L-Tryptophan	414.0 \pm 4.9	434.83 \pm 19.86
L-Lysine	50.0 \pm 0.78	382.5 \pm 45.92
L-Isoleucine	381.4 \pm 0.7	351.8 \pm 69.36
L-Arginine	92.6 \pm 1.36	295 \pm 46.15
L-Asparagine	365.6 \pm 1.4	260.17 \pm 34.68
L-Glutamine	25.2 \pm 5.6	238.5 \pm 20.13

^a Values are means \pm standard deviations.

	GxGxxG	RxGGR
AY053450	55 ..GSHSVVVLGGGPAGLXSAFELQKAGYK----VTVLEAR TRPGGRVWT--	
AAY89681	50.NPKHVVVVVGAGIAGLVAGDLLTRAGHE--- NVTLEASER VGGRVNT--	
YP_171306	39....KRSVLVLGAGMAGLTAALSLLRRGHQ-----VTVIEYQ NRIGGRLLS--	
BAC55901	47 . KNTTIAIGAGMSGLMPTYLCLTQAGMTN----ILEASNNR LGGGRFRT--	
SO-LAAO	2.....NHFDTIII GGGPAGMMAA ISSSFY-----TLLLEKNR LGK KL LAG	
TIGR00275	2.... NHFDTIII GGGPAGMMAA ISSSFY----- TLLLEKNR LGK KL LAGTG	
SSA-0323	2.... KHFDTIII GGGPAGMMAA ISSSFY-----TLLLEKNR LGK KL LAGT	
SP_0741	2.... KHFDTIV GGGPAGMMA ISSSFY----- TLLIEKNR LGK KL LAGTG	
SPy_1866	2.....TQYDTIII GGGPAGMMAA ISSSY-----TLLIEKNR LGK KL LAGT	
SMU.392c	2.....HFDTIV GGGPAGMMA CISAGF-----ALLLEKNR LGK KL LAGTG	

FIG. 2. Sequence alignment of SO-LAAO and homologous proteins of five streptococci and four known LAAOs. The highly conserved dinucleotide-binding central sequence GxGxxG and LAAO's characteristic sequence motif RxGGR are shown in bold characters. The amino acid differences in LAAO's characteristic sequence motif from those of the other four LAAOs in six streptococcal sequences are underlined. AY053450, LAAO of *Rhodococcus opacus*; AAY89681, LAAO of *Notechis scutatus*, YP_171306, LAAO of *Synechococcus elongates*; BAC55901, LAAO of *Aspergillus oryzae*; SO-LAAO, LAAO of *S. oligofermentans*; TIGR00275, conserved hypothetical protein of *S. gordonii*; SSA-0323, putative flavoprotein of *S. sanguinis*; SP_0741, hypothetical protein of *S. pneumoniae*; SPy_1866, hypothetical protein of *S. pyogenes*; SMU.392c, hypothetical protein of *S. mutans*.

from the other tested L-amino acids and the derivatives, such as N-acetyl-L-cysteine and *cis*-4-hydroxyl-L-proline.

***ao_{so}* mutant of *S. oligofermentans* abolishes H₂O₂ production from amino acids.** Since the SO-LAAO protein exhibited LAAO activity, to test the *in vivo* role of SO-LAAO, an *ao_{so}* gene knockout mutant of *S. oligofermentans* was constructed by single-crossover homologous recombination with the suicide vector pFW5, fused with a *luc* reporter gene (pFW5-*luc* [28]). Hydrogen peroxide production from amino acids was then measured for the *ao_{so}* mutant. The result demonstrated that the *ao_{so}* mutant no longer produced H₂O₂ from the seven L-amino acids shown in Table 1, indicating that SO-LAAO did function in H₂O₂ production from amino acids in *S. oligofermentans*. Furthermore, the contribution of SO-LAAO in *S. oligofermentans*' competition with *S. mutans* was tested by using the *ao_{so}* mutant of *S. oligofermentans* in an interspecies antagonism experiment. To do this, the TPYG overnight cultures of *S. oligofermentans* or *S. oligofermentans* Δ *ao_{so}* and *S. mutans* UA140 (29) with the same OD₆₀₀ were spotted side by side on an 0.8% chemically defined agar plate containing amino acids. After growth for 16 h in a candle jar, growth of *S. mutans* UA140 beside the *S. oligofermentans* wild-type strain was obviously inhibited (Fig. 1B); however, the inhibition zone beside *S. oligofermentans* Δ *ao_{so}* was greatly reduced (Fig. 1C). This demonstrated that SO-LAAO contributed to the competitive potential of *S. oligofermentans* against *S. mutans* in growth on amino acid-rich plates.

Peptone up-regulates expression of *ao_{so}*. To get insight into the newly identified enzyme in *in vivo* H₂O₂ production, the expression profile of the *ao_{so}* gene was determined. Since the insertional mutation of the *ao_{so}* gene also resulted in an *ao_{so}*-*luc* fusion (see Materials and Methods), we used this strain to determine *ao_{so}* gene expression in cells grown in BHI (peptone absent) and TPYG (peptone-rich) broth by recording luciferase activity, expressed as relative light units per OD₆₀₀. The results indicated that the *ao_{so}* gene was constitutively expressed throughout growth in both media; however, the expression level in TPYG broth was nearly triple that in BHI broth.

SO-LAAO contributes to competitive edge of *S. oligofermentans* over *S. mutans* in mixed-species biofilm with peptone. It has been known that saliva is a great reservoir of proteinaceous

materials, while the oral streptococci are dominant species in the dental biofilm. Therefore, it would be interesting to test the role of SO-LAAO in the competition of *S. oligofermentans* against *S. mutans* in the artificial two-species biofilm formed in a medium supplemented with peptone. A *gfp* reporter strain of *S. oligofermentans* (35) was mixed with an *mrfp* reporter strain of *S. mutans*, UA140 (15), in either BHI (peptone absent) or TPYG (peptone-rich) broth to form mixed-species biofilms. After 16 h of incubation, growth inhibition in both culture media was visualized under a confocal laser scanning microscope (Fig. 3A-1 and A-2). The images showed that *S. mutans* (red cells) was more suppressed in peptone-rich TPYG broth than in BHI. Similar results were obtained from quantitative determination of colonies. Though cell numbers of *S. mutans* in mixed-species biofilms with *S. oligofermentans* decreased dramatically in both media by comparison with its single-species one, the number was almost 10 times lower in TPYG than in BHI broth (Fig. 3B). In comparison with its single-species biofilm, the cell numbers of *S. oligofermentans* in the mixed-species biofilm did not decrease significantly under both culture conditions (data not shown). These results indicated that the amino acid-derived H₂O₂ could also contribute to the inhibition of *S. mutans* by *S. oligofermentans*.

The SO-LAAO contribution in the interspecies competition was also verified by comparing the survival rate of *S. mutans* in mixed-species biofilm with that of the wild-type strain and *ao_{so}* mutant strain of *S. oligofermentans* after 16 h of growth in TPYG broth. The single-species biofilms of *S. oligofermentans* Δ *ao_{so}* and *S. mutans* were included as controls. As shown in Fig. 3B, about 10 times more cells of *S. mutans* survived in a biofilm mixed with the *ao_{so}* mutant than in that with the *S. oligofermentans* wild type. These results indicated that SO-LAAO conferred on *S. oligofermentans* an extra ability to compete with *S. mutans*, especially in peptone-abundant environments.

DISCUSSION

Hydrogen peroxide is widely used by lactic acid bacteria as a biological weapon to compete with other bacteria inhabiting

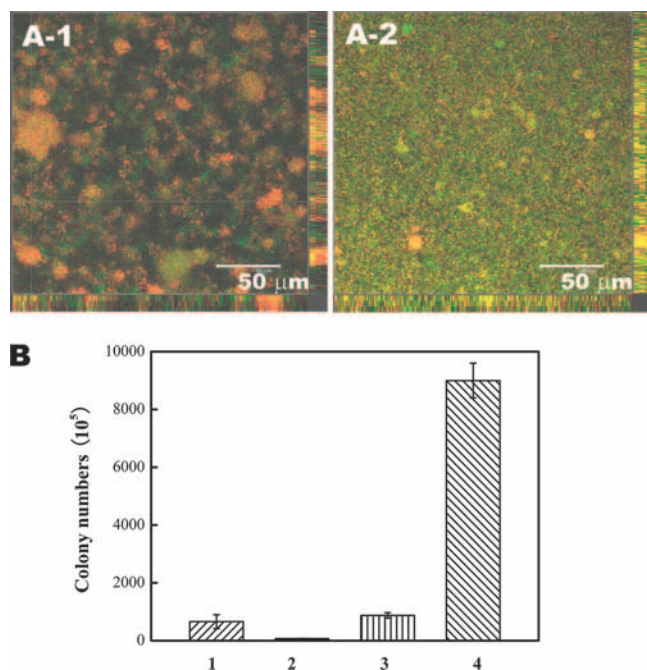


FIG. 3. Interspecies competition between *S. oligofermentans* and *S. mutans* in the biofilm formed in BHI-SG and TPYG-SG. (A) Interspecies competition assay using confocal laser scanning microscopy. Overnight cultures of *S. oligofermentans* *ldhp-gfp* and *S. mutans* UA140 *mutAp-mrfp* were adjusted to the same OD₆₀₀ and then were inoculated (1:10) into chambers to form mixed-species biofilms. After 12 h of incubation, the biofilms were visualized under a confocal laser scanning microscope. The pictures were 1,000-fold magnified. A-1, mixed-species biofilm in BHI-SG broth; A-2, mixed-species biofilm in TPYG-SG broth. Green cells, *S. oligofermentans* *ldhp-gfp*; red cells, *S. mutans* UA140 *mutAp-mrfp*. (B) Interspecies competition assay by colony counting of *S. mutans*. Mixed-species biofilms of the *S. oligofermentans* wild type or *aao_{so}* mutant and *S. mutans* UA140 were formed by the same procedure described for panel A, and then colony numbers of *S. mutans* were counted after 16 h of incubation. 1, colony numbers in *S. oligofermentans* wild type-*S. mutans* mixed-species biofilm in BHI-SG broth; 2, colony numbers in *S. oligofermentans* wild type-*S. mutans* mixed-species biofilm in TPYG-SG broth; 3, colony numbers in *aao_{so}* mutant-*S. mutans* mixed-species biofilm in TPYG-SG broth; 4, colony numbers in *S. mutans* single-species biofilm. The results were expressed as means \pm standard errors from three independent experiments.

the same ecological niche (16, 27), as with lactobacilli, which can prevent pathogens causing bacterial vaginosis from colonizing the vagina by forming H₂O₂ (11). H₂O₂ could be produced via NADH oxidase, pyruvate oxidase, or Lox (4, 20, 32). However, it was found in our study that *S. oligofermentans*, an oral streptococcus, produces large amounts of H₂O₂ not only from lactate but also from peptone (Fig. 1A) and from seven L-amino acids (Table 1). By an integrated approach using physiology, biochemistry, and genetics, an ORF protein assigned as SO-LAAO, which is the homologue of flavoprotein SSA-0323 in *S. sanguinis*, was verified to possess LAAO activity and to be responsible for H₂O₂ production from L-amino acids in *S. oligofermentans*.

LAAOs, which catalyze the oxidative deamination of amino acids to yield ammonia, hydrogen peroxide, and ketoacids with oxygen consumed (9), have been widely detected in snake and

insect venoms (1, 30) and in some fungi, algae, and bacteria (17, 25, 38). According to the substrate spectra, LAAOs can be divided into two categories, one with a broad spectrum of substrates, like the LAAO of *Rhodococcus opacus* DSM 43250, which catalyzes not only almost all the 20 L-amino acids but also some derivatives (9), and another with a restricted substrate spectrum, like lysine oxidase of *Marinomonas mediterranea*, which uses lysine exclusively (21). However, SO-LAAO of *S. oligofermentans* in this study can represent a novel amino acid oxidase by showing detectable activity against only seven L-amino acids (Table 1).

So far, all the described LAAOs, except the lysine oxidase of *Marinomonas mediterranea* (21), are flavoproteins possessing a FAD binding domain (37). It is reported that LAAOs possess another characteristic sequence motif, RxGGR, and this motif is also present in several families of flavoproteins, including achacin and aplysianin A, monoamine oxidase, corticosteroid-binding proteins, and tryptophan 2-monooxygenases (37). Great differences have been shown between the deduced SO-LAAO sequence and those of the known LAAOs, at identities from 12% to 16% (data not shown), and the characteristic amino acid differences in the RxGGR motif of SO-LAAO, as well as its highly homologous ORFs (75 to 96% identities), present in five other streptococci (Fig. 2), have been shown, so that SO-LAAO can represent a novel type of LAAO commonly existing in oral streptococci like *S. sanguinis* and *S. gordonii*. Although a SO-LAAO homologue (SMU.392c) also exists in the genome of *S. mutans*, no detectable hydrogen peroxide production from peptone has been measured for this streptococcus (data not shown). This implies that either SMU.392c does not function as an amino acid oxidase, possibly due to the relatively lower sequence identities (75%) to SO-LAAO, or a NADH-dependent peroxidase (AhpC) present in *S. mutans* (12) can scavenge the endogenous H₂O₂.

Although the intact cells of *S. oligofermentans* and SO-LAAO showed the same substrate profile, a large difference (3- to 10-fold) in hydrogen peroxide yield was detected for four L-amino acids, namely L-aspartic acid, L-lysine, L-arginine, and L-glutamine (Table 1). This can possibly be attributed to the fact that charged L-amino acids need a transporting apparatus, such as amino acid permease (13), to get into the cell, while neutral L-amino acids (L-tryptophan, L-isoleucine, and L-asparagine) might permeate cells freely.

In comparison with that mediated by Lox, SO-LAAO catalyzes a relative lower level of H₂O₂ production, and Lox-mediated inhibition on *S. mutans* (difference between the colony numbers of lanes 1 and 4 in Fig. 3B) is about 10 times higher than that mediated by SO-LAAO (difference between the colony numbers of lanes 1 and 2 of Fig. 3B) in *S. oligofermentans*. Therefore, *lox* can play the predominant role in H₂O₂ formation by *S. oligofermentans* when lactate is abundant. However, oral saliva is also rich in polypeptides (39), and oral streptococci take up salivary oligopeptides and then degrade them into free amino acids by intracellular aminopeptidase (2, 6, 7). Aminopeptidase activity has been detected in *S. oligofermentans* (data not shown), implying that it might follow a path similar to that of other oral streptococci to obtain amino acids from the environment. Thus, SO-LAAO can give oral streptococci a competitive advantage in dental plaque biofilm. In addition, we also found that the *aao_{so}* mutant of *S. oligofer-*

mentans not only lost peptone derivate H₂O₂ production but also reduced the growth to some extent (data not shown), implying that this protein can be involved in other biological process, probably amino acid metabolism.

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