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A Preliminary Study of Biological Characteristics of *Streptococcus oligofermentans* in Oral Microecology

J. Zhang[a] H.C. Tong[b] X.Z. Dong[b] L. Yue[a] X.J. Gao[a]

[a]Department of Cariology and Endodontology, Peking University School and Hospital of Stomatology, and [b]State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, PR China

Key Words

Acid production · Bacterial adherence · Demineralization · Streptococci · *Streptococcus oligofermentans*

Abstract

This study was designed to explore the biological characteristics of a new oral streptococcus species *Streptococcus oligofermentans*. Plaque samples were collected from caries-free and caries-active subjects. *S. oligofermentans* was selectively grown on Mitis salivarius agar plates and identified by using 2-step PCR and was isolated from 38% of 18 subjects. Isolates were found mostly on healthy tooth surfaces. *S. oligofermentans* had weaker abilities in acid production and demineralizing hydroxyapatite. When *Streptococcus mutans* was coincubated with *S. oligofermentans*, total number, acid production and calcium release were significantly inhibited. In conclusion, inhibition of *S. mutans* could be a beneficial biological characteristic of *S. oligofermentans* in oral microecology.

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Although mutans streptococci have been considered as the most important cariogenic bacteria [van Houte et al., 1990], non-mutans streptococci could be involved in the carious process in one way or another [Sansone et al., 1993]. For example, *Streptococcus sanguis*, one member of the non-mutans family, has been attributed to beneficial bacteria in the oral cavity. It could inhibit the growth of *Streptococcus mutans* by production of hydrogen peroxide [Kreth et al., 2005]. Recently, we reported a new species of non-mutans which had a similar function as *S. sanguis* regarding the ability to produce hydrogen peroxide and the ability to inhibit *S. mutans* [Tong et al., 2007]. The new species was first reported and named *Streptococcus oligofermentans* in 2003 [Tong et al., 2003]. In this paper, we further report the biological characteristics of *S. oligofermentans* and its possible effects on oral microecology.

Materials and Methods

Collection and Preparation of Plaque and Saliva Samples

The subjects (aged 22–32 years, 9 caries-free, 9 caries-active with DMFT 2–8) had not been treated with antibiotics and not received orthodontic treatment 3 months before the study. Informed consent was obtained and the study was approved by the ethics institution of Peking University.

J. Zhang and H.C. Tong contributed equally to this work.

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X.J. Gao
Department of Cariology and Endodontology
Peking University School and Hospital of Stomatology
No. 22 Zhongguancun Nan Dajie, Haidian District, Beijing, 100081 (PR China)
Tel. +86 10 6217 9977 ext. 2523, Fax +86 10 6217 3402, E-Mail kqxjgao@bjmu.edu.cn

Plaque samples were obtained from all tooth surfaces available in caries-free teeth and the dorsum of the tongue. Stimulated saliva by paraffin wax was collected at the same time. In caries-active subjects, more samples from carious lesions and healthy surfaces adjacent to caries were collected. Samples from the same tooth surfaces (approximal, occlusal or buccal-lingual) in each individual subject were separately placed in sterilized, capped microcentrifuge tubes, making 5 samples from caries-free individuals and 7 from caries-active individuals. Each tube contained 1,500 μl of PBSTC [Brailsford et al., 2001]. The samples were kept on ice and processed in the laboratory within 4 h.

Primary Bacteriological Analysis

Aliquots (0.1 ml) of appropriate dilutions of suspensions in PBSTC were spread on plates with 3 different agar media, i.e. MSAE (Mitis salivarius agar with 15 μg/ml erythromycin) for *S. oligofermentans,* TYCSB (tryptone-yeast-extract-cystine supplemented with 0.15 g/ml sucrose and 0.2 U/ml bacitracin) for *S. mutans* [Wan et al., 2002] and BHI (brain heart infusion agar supplemented with 5% defibrinated sheep blood) for the total cultivable sample flora. The plates were incubated in an anaerobic workstation with 95% N_2 and 5% CO_2 for 2–3 days at 37°C. The final quantity of *S. mutans* was expressed as percentage in the total cultivable sample flora.

Identification of S. oligofermentans

After 2 days of growth on the MSAE plates, viscid milk white colonies 0.5–1.0 mm in diameter, similar to *S. oligofermentans* type strain, were picked out and checked under microscope. After 18 h of growth at 37°C in BHI, strains with characteristics of Gram-positive nonmotile and in short chains alignment under microscope were selected for further identification by 2-round PCR reactions. A pair of 16S rDNA-specific primer of *S. oligofermentans* 88F (5′-CATTTTACTGCATGGTAAGATG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′) were designed and synthesized by Sangon company (Shanghai, China). First-round PCR reaction was performed at 95°C/6 min, 30 cycles at 94°C/30 s, 55°C/1 min, 72°C/1.5 min, then 1 cycle at 72°C/10 min. The positive colonies with amplified fragment of 1,450 bp were identified by second-round PCR using lactate oxidase gene fragment, LoxF/LoxR as primers. LoxF (5′-CACCNCATTGGTTTCAATT-3′) and LoxR (5′-CCAGNNANTTGCATAAC-3′) were synthesized by Sangon company. The second-round PCR was performed at 95°C/6 min, 30 cycles at 94°C/30 s, 40°C/1 min, 72°C/1.5 min, then one cycle at 72°C/10 min (626 bp). The details of the technique were reported elsewhere [Zhang et al., 2007].

Biochemical Characteristics of S. oligofermentans *Isolated Strains*

Seven representative isolated strains of *S. oligofermentans* were progressed into fermentation experiments. Biochemical traits were determined by using the back part of the API 20 Strep system (bioMérieux). API 20 Strep was used by following the manufacturer's instructions. Fermentations were determined at 4 and 24 h, respectively.

Adhesion to Smooth Hard Glass

Strains of streptococcus species: *S. oligofermentans* LMG 21535^T was freeze-dry preserved in the laboratory. *S. sanguinis* ATCC 10556^T and *S. salivarius* ATCC 7073^T in freeze-dried cultures were provided by the China Microbiological Culture Collection Center. *S. gordonii* ATCC 10558^T was from the China General Microbiological Culture Collection Center. *S. mutans* NCTC 10449^T was kindly given by Professor Samaranayake of the University of Hong Kong.

The experiments were carried out according to Mukasa and Slade [1973]. Briefly, bacteria were inoculated in 6 ml of BHI. After 18 h of incubation at 37°C, the cultures were collected by centrifugation at 4,000 rpm for 5 min, then washed 3 times with PBS buffer. The bacterial deposit was suspended in PBS buffer to make the optical density at 550 nm to be 0.75. Two milliliters of cell suspension and 0.4 ml 5% sucrose in PBS were added and mixed in glass tubes. The final volume was 2.4 ml. The tubes were incubated at 37°C at a 30° angle for 18 h. After swirling 5 times by hand, the contents were poured out, and 3 ml of PBS was added. After vortex for 10 min, optical density values at 550 nm were obtained. The relative adhering ability of experimental strains was expressed as a mean percentage of adhering bacteria in the total bacterial amount of 3 independent, repeated experiments.

Demineralization of Hydroxyapatite

The same strains as above were used. The strains were first grown in BHI agar for 2 days. Single colonies were inoculated in 4 ml of BHI and incubated anaerobically at 37°C for 18 h. The cultures were collected by centrifugation at 8,000 rpm for 5 min and washed 3 times in 2 ml of PBS buffer. Each pellet was resuspended in BHI to yield an optical density value of 0.65 at a wavelength of 630 nm. Then, 150 μl of culture of every strain was mixed with 9 ml of BHI, 1% sucrose and 0.09 g of powdered hydroxyapatite (BDH Chemicals, England), and 150 μl culture of *S. oligofermentans* and 150 μl of *S. mutans* were mixed with 18 ml BHI, 1% sucrose and 0.09 g powdered hydroxyapatite. Negative controls were without adding bacteria. The mixtures were added into plates and incubated anaerobically with 95% N_2 and 5% CO_2 at 37°C for 24 h. Supernatant was aspirated and measured for calcium release and acid production. The calcium content was determined with atomic absorption spectrophotometer (PE-Zeemam-5100). Acid production was evaluated by measured pH values (Ks701, Shindengen, Japan). The precipitated biofilm was suspended in 2 ml of PBS and dispersed by vortex-mixing for 30 s and sonication for 10 s. After serial dilution with PBS, aliquots were plated out on the BHI agar plates. The plates were incubated anaerobically with 95% N_2 and 5% CO_2 at 37°C for 2 days. The amounts of bacteria in biofilm were counted.

Data Analysis

Statistical analyses were performed with SPSS (version 10.0). After a primary analysis, the plaque samples were regrouped as with or without *S. oligofermentans* group. Differences in percentage of *S. mutans* between the 2 groups were analyzed with nonparametric tests. To compare the amount and ability of adhering to smooth glass and the ability of producing acid and demineralizing hydroxyapatite, 1-way ANOVA was used.

Results

General Findings

Twelve isolates were finally identified as *S. oligofermentans* out of the colonies obtained from the cultivation on

MSAE plates. The isolates were from 38% of the subjects, from 3 caries-active and 4 caries-free individuals. The isolates were more often isolated from dental plaques at healthy noncarious tooth surfaces from both caries-free and caries-active subjects and from only 1 carious lesion.

The detection ratio of *S. mutans* was 9/9 in the caries-active group and 8/9 in the caries-free group. Taking the tooth surface sites with *S. oligofermentans* as one group and the tooth surface sites without *S. oligofermentans* as another group, the percentage of *S. mutans* in total bacteria was 0.14 ± 0.27% in the tooth surfaces with *S. oligofermentans*, while it was 1.22 ± 0.43% when *S. oligofermentans* was absent ($p = 0.780$).

Fermentation Characteristics of S. oligofermentans *Isolated Strains*

Fermentation with the API 20 Strep system was carried out with 7 isolates identified as *S. oligofermentans*. Table 1 shows these biochemical characteristics.

Ability of Adhering to Smooth Glass

The adherence of *S. oligofermentans* type strain LMG 21535^T to smooth glass was stronger than other experimental non-mutans strains (table 2).

Ability of Producing Acid and Demineralizing Hydroxyapatite

The experimental strains were coincubated with hydroxyapatite and BHI in the presence of sucrose in biofilm forms to compare their acidogenic and demineralizing ability. Biofilms formed with *S. mutans*, *S. sanguinis* and mixture of *S. mutans*/*S. oligofermentans* were firmly adherent and compact. Biofilms formed with *S. oligofermentans, S. gordonii* and *S. salivarius* were easily dislodged by washing with water. Acid production and calcium release were determined at the end of incubation. The results are also presented in table 2. With regard to acid production, *S. mutans* was the strongest, while *S. sanguinis* and *S. oligofermentans* were the weakest. A similar trend could be found with regard to calcium release. When *S. mutans* was co-incubated with *S. oligofermentans,* both acid production and calcium release were reduced in comparison with *S. mutans* alone.

Amount of Experimental Strains in Biofilm

The numbers of each experimental strain recovered after 48 h of biofilm formation are shown in table 2. *S. mutans* was the best biofilm former of the species tested

Table 1. Biochemical characteristics of the 7 strains identified as *S. oligofermentans*

Acid produced from sugar of	Strains from						
	Ca21b	Ca24p	Cf28t	Cf2l	Ca28o	Cf31p	Ca28a
Ribose	–	–	–	–	–	–	–
Arabinose	–	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–	–
Sorbitol	–	–	–	–	–	–	–
Lactose	–	–	+	+	–	–	–
Trehalose	–	–	–	–	–	+	–
Inulin	–	–	–	–	–	–	–
Raffinose	+	–	–	–	–	–	–
Starch	–	–	+	–	–	–	–
Glycogen	–	–	–	–	–	–	–

Table 2. Adhering ability, acid and calcium release and the amounts of experimental strains in biofilm

Experimental strains	Adherent percentage (mean ± SD)	pH value (mean ± SD)	Calcium release, μg/ml (mean ± SD)	Total count, CFU/ml (mean ± SD)
S. mutans	$34.22 \pm 2.04^{b–e}$	$4.5 \pm 0.1^{b, c, e}$	$367.92 \pm 31.70^{b–f}$	$(140 \pm 39) \times 10^{7\ b–f}$
S. salivarius	$5.07 \pm 1.75^{a–c, e}$	$4.6 \pm 0.2^{b, c, e}$	$234.74 \pm 22.98^{a–c, e, f}$	$(58 \pm 24) \times 10^{6\ a}$
S. sanguinis	$12.40 \pm 0.13^{a, b, d}$	$6.3 \pm 0.3^{a, b, d–f}$	$8.8 \pm 0.52^{a, d–f}$	$(39 \pm 11.14) \times 10^{6\ a}$
S. gordonii	$14.71 \pm 0.78^{a, d}$	$5.7 \pm 0.3^{a, c, d, f}$	$39.31 \pm 4.78^{a, c, d, f}$	$(76.33 \pm 21.56) \times 10^{5\ a}$
S. oligofermentans	$18.18 \pm 1.69^{a, c, d}$	$5.6 \pm 0.3^{a, c, d, f}$	$20.17 \pm 1.29^{a, d, f}$	$(49.67 \pm 13.01) \times 10^{5\ a}$
S. mutans + *S. oligofermentans*	–	$4.9 \pm 0.1^{b, c, e}$	$100.26 \pm 10.36^{a–e}$	only *S. oligofermentans* $(67 \pm 16) \times 10^{6\ a}$

[a] Significant compared with that of *S. mutans* ($p < 0.05$). [b] Significant compared with that of *S. oligofermentans* ($p < 0.05$). [c] Significant compared with that of *S. sanguinis* ($p < 0.05$). [d] Significant compared with that of *S. salivarius* ($p < 0.05$). [e] Significant compared with that of *S. gordonii* ($p < 0.05$). [f] Significant compared with that of *S. mutans* + *S. oligofermentans* ($p < 0.05$).

when incubated separately. However, when biofilms were formed with both *S. mutans* and *S. oligofermentans*, the total numbers of bacteria in the biofilms was significantly reduced with no detectable *S. mutans* recovered.

Discussion

Isolated strains of *S. oligofermentans* could be identified by 2-step PCR. With the first step, *S. oligofermentans* was differentiated from *S. gordonii* and *S. mutans,* by amplified 16S rDNA fragments. The amplified fragment for *S. oligofermentans* was 1,450 bp, compared to 1,500 and 750 bp respectively for *S. gordonii* and *S. mutans*. However, *S. oligofermentans* may not be identified by the first-step PCR alone, because a similar length fragment could also be derived from *S. intermedius*. To differentiate *S. oligofermentans* from *S. intermedius*, a pair of primers complemented with lactate oxidase gene *(lox)* was designed [Tong et al., 2007]. The PCR could produce a 626-bp fragment from *S. oligofermentans* but not from *S. intermedius*. By this second step, *S. oligofermentans* could eventually be differentiated from all other oral streptococci [Zhang et al., 2007].

It has been established that *S. mutans* may be related to early onset of dental caries, as it was found abundant in carious lesion, but is less frequently isolated from healthy tooth surfaces of caries-active or caries-free individuals [van Houte et al., 1990]. In contrast to *S. mutans*, *S. oligofermentans* was mostly frequently isolated in dental plaque from healthy tooth surfaces, which suggested that *S. oligofermentans* may not be an important cariogenic species. We found that the adherence of *S. oligofermentans* to smooth glass surface was weaker than that of *S. mutans* and also produced less acid than all other oral streptococci examined but apparently inhibited the growth of *S. mutans* in the biofilm model used here.

Among the 5 tested strains of oral streptococci, *S. oligofermentans* showed weaker acid production ability and consequently weaker ability to demineralize hydroxyapatite. Furthermore, when *S. mutans* was incubated with *S. oligofermentans*, the acid production and calcium release of *S. mutans* were reduced. Inhibition of *S. mutans* may be a beneficial biological characteristic of *S. oligofermentans* in oral microecology and in this model system this inhibitory activity may be due to the production of hydrogen peroxide by *S. oligofermentans* [Tong et al., 2007].

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