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Short communication

Isolation and characterization of *Lactobacillus* species inhibiting the formation of *Streptococcus mutans* biofilm

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Oral bacterium S11 was isolated from the saliva of young children without dental caries and with little or no visible supragingival plaque. The S11 strain showed 99.5% similarity with *Lactobacillus fermentum*, and was identified on the basis of biochemical characteristics and a 16S rDNA sequence. S11 strain and its culture supernatant significantly inhibited the formation of the insoluble glucan produced by *Streptococcus mutans* Ingbritt. S11 did not affect the multiplication of *S. mutans* Ingbritt, but the adherence of *S. mutans* Ingbritt onto cuvette walls was inhibited completely.

Key words: insoluble glucan; *Lactobacillus fermentum*; saliva; *Streptococcus mutans*

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It is well established that dental caries and periodontal diseases are infectious diseases associated with microorganisms residing in dental plaque biofilm (11). Insoluble glucan synthesized by *Streptococcus mutans* plays an important role in the development of dental plaque biofilm by facilitating bacterial adherence and accumulation on tooth surfaces (14).

Lactobacilli are considered to play a secondary or opportunistic role in the development of dental caries by producing lactic acid and extracellular polysaccharides (10). However, lactobacilli also exert beneficial aspects in relation to health. Lactic acid bacteria seem to protect the gastrointestinal system of humans and animals from various pathogenic infections (4, 5) and the organisms have been vigorously studied for use in probiotics. However, few data exist on the ability of lactobacilli to inhibit the formation of oral biofilms. The purpose of this study was to isolate and characterize lactobacilli that inhibit the formation of glucan by *S. mutans* Ingbritt.

An oral examination was carried out in 160 kindergarten children aged 4–7 years living in Pusan, South Korea. A 0.5-ml sample of saliva was taken from children with little supragingival plaque and without oral diseases, including dental caries. Lactobacilli were isolated using MRS (deMan, Rogosa and Sharpe, Difco Laboratories, Detroit, MI) or Rogosa (Difco) agar plates. *S. mutans* Ingbritt and lactobacilli were grown in BHI (Brain heart infusion, Difco) broth and MRS broth, respectively.

The test system for the isolation of lactobacilli with the ability to inhibit the formation of *S. mutans* biofilm was a modification of the method of McCabe et al. (12). In brief, equal amounts of *S. mutans* Ingbritt and lactobacilli were placed in each beaker containing 40 ml of medium A (a mixture of equal volume of BHI and MRS with 5% sucrose and 0.1 M of MES (2-(N-Morpholino) ethanesulfonic acid monohydrate, pH 6.5). Each of three 0.016-inch stainless steel wires (Ormco, Glendora, CA) prepared in 4-cm lengths were hung on three metal frameworks inserted in cork stoppers and were immersed into the medium. As the control, *S. mutans* Ingbritt without lactobacilli was inoculated in another beaker containing medium A. After incubation at 37°C for 24 h, each wire was weighed. To confirm the inhibitory effects of the selected lactobacilli strain, each experiment was repeated three times.

Isolated strains were identified based on a biochemical test and 16S rDNA sequence. In addition, a carbohydrate fermentation assay (API-50 CHL system, BioMérieux, Marcy l′Etoile, France) was carried out according to the manufacturer's instructions and analyzed by APILAB PLUS software version 3.2.2.

Chromosomal DNA was extracted from the isolated strain as described previously (1) and the 16S rDNAs were amplified using polymerase chain reaction. Polymerase chain reaction primers were designed from highly conserved regions between species (8). The sequences were 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-AAGGAGGTGWTCCARCC-3'. The polymerase chain reaction was performed using a thermal cycler (PE Applied Biosystems, Foster City, CA) for 30 cycles. The amplification program was as follows: denaturation at 94°C for 30 s, primer annealing at 50°C

for 30 s, and extension at 72°C for 5 min. The amplified polymerase chain reaction products were recovered and purified using a Wizard polymerase chain reaction Preps DNA Purification System (Promega, Madison, WI). The purified polymerase chain reaction products were sequenced with an automatic DNA sequencer (ABI 310, Perkin-Elmer) using the primers 27F (5'-AGAGTTTG-ATCMTGGCTCAG-3'), 1522R (5'-AAG-GAGGTGWTCCARCC-3'), and 1088R (5'-GCTCGTTGCGGGACTTAACC-3'). The 16S rDNA sequences were aligned with selected sequences obtained from the Ribosomal Database Project release 4 (9). A phylogenetic tree was constructed using the neighbor-joining method (18).

To examine the effect of lactobacilli on the formation of insoluble glucan and on the replication and adherence of *S. mutans* Ingbritt, *S. mutans* Ingbritt was cultured with and without the isolated lactobacilli strains in 3 ml of medium A in disposable cuvettes. After 24 h, the amount of insoluble glucan produced was measured at 550 nm by spectrophotometer (Pharmacia, Cambridge, UK) as described previously (7, 15). Viable cells and detached cells of *S. mutans* Ingbritt were counted using the method described previously (7) on Mitis-Salivarius agar (Difco) containing 0.2 U/ml of bacitracin.

For the preparation of the culture supernatant of the isolated strain, the bacterium was grown in MRS broth at 37°C for 24 h. The culture supernatant was centrifuged at $1000 \times g$ for 10 min, adjusted to pH 6.5 by adding 5N of NaOH, and sterilized by filtration with a 0.45 μm pore-sized filter. *S. mutans* Ingbritt was inoculated in 3 ml of M17 broth (Difco) both with and without the prepared supernatant. The amount of insoluble glucan and viable cells and detached cells of *S. mutans* Ingbritt were measured as described previously (7). Experimental values were analyzed statistically using a Mann–Whitney test.

Two strains of 150 lactobacilli isolates showed excellent and similar inhibitory effects on the formation of insoluble glucan by *S. mutans* Ingbritt. Thus, only the characteristics of S11 strain will be described. S11 strain was a non-spore forming, nonmotile, Gram-positive, and catalase-negative bacillus. It fermented D-glucose, D-fructose, lactose, sucrose, galactose, D-mannose and maltose. The 16S rDNA sequence of S11 strain, containing 1473 nucleotides, exhibited a high level of similarity (99.52%) to *Lactobacillus fermentum* ATCC 14931 sequence (accession no. M58819). Based on these results, S11 strain was identified as *L. fermentum*.

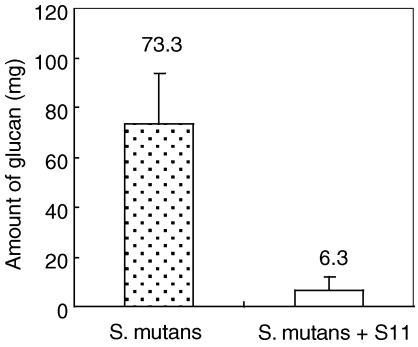

Fig. 1. Inhibitory effect of S11 strain on glucan formation by *S. mutans* Ingbritt on orthodontic wires.

As shown in Fig. 1, the mean weights of glucan produced on the orthodontic wires were 73.3 ± 20.6 mg in the control group and 6.3 ± 5.7 mg in the group with S11 strain. S11 strain inhibited the formation of glucan by 91% ($P < 0.01$). The group with S11 strain or its supernatant showed significantly lower mean optical densities ($P < 0.01$) for insoluble glucan than those of the control group (Table 1). The numbers of detached *S. mutans* Ingbritt were greatly decreased (31.3 ± 10 colony-forming units/ml, $P < 0.01$) in the group with S11 strain compared to the control group ($6.5 \times 10^6 \pm 3.5 \times 10^6$ colony-forming units/ml), while S11 strain or its supernatant had no apparent inhibitory effect on the replication of *S. mutans* Ingbritt. These data indicate that the inhibitory effects of S11 strain on the formation of insoluble glucan result from the inhibition of the adherence of *S. mutans* Ingbritt to the wires or cuvette walls.

There have been many efforts to inhibit the formation of plaque biofilm, including chemical methods using chlorhexidine, triclosan, sanguinarine, fluoride and antibiotics. However, some of these agents have undesirable side effects when used in the oral cavity for an extended period of time, and some require professional application. The use of probiotics, defined as ''microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well being of the host'', can offer an attractive alternative approach to inhibit the formation of oral biofilm and to protect against oral diseases.

L. fermentum is a well known probiotic species in urogenital infections because of its ability to adhere to urogenital cells and inhibit the growth and adhesion of uropathogenic bacteria (16, 17). Ishihara et al. (6) reported that a water-soluble extract of *L. fermentum* completely inhibited the growth of *S. mutans*. Recently, it was reported that *L. fermentum* inhibited *Staphylococcus aureus* infections of surgical implants (3) and reduced the amount of yeast in oropharyngeal biofilms (13). In our study, *L. fermentum* S11 was isolated from the saliva of young children without dental caries and with little or no visible supragingival plaque. Similarly, Colloca et al. (2) suggested *L. fermentum* to be a predominant species in healthy mouths. The present results are encouraging for the probiotic use of *L. fermentum* in the oral cavity. However, it should be borne in mind that this study was an *in vitro* investigation, and inhibiting the adherence of one species may have little effect on oral biofilm development. In addition, the effects of S11 strain may be abrogated in the presence of other dental plaque species, so its effects on *S. mutans* may be minimal. Future studies are needed to delineate the usefulness of employing *L. fermentum* as a replacement therapy to prevent oral diseases.

Table 1. Optical densities (OD) of the insoluble glucan produced on disposable cuvette walls when S11 strain or its culture supernatant was added

Group	Optical densities (OD) at 550 nm					Average OD
SM	0.995	0.82	0.94	0.929	1.037	0.9442 ± 0.08
SM + S11 strain	0.236	0.423	0.183	0.299	0.315	0.2912 ± 0.09
SM (without SUP)	0.937	0.864	0.971	1.051	1.043	0.9732 ± 0.07
SM (with SUP)	0.29	0.284	0.149	0.233	0.154	0.222 ± 0.07

SM, *S. mutans* Ingbritt. SUP, culture supernatant of S11 strain grown in MRS broth.

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