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NOTE

Bacterial Diversity in the Human Saliva from Different Ages

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To obtain primary idea on oral bacterium species that are generally present in periodotally healthy Koreans, the oral bacterial flora in the saliva of four periodontally healthy Koreans at different ages (5, 32, 35, 65) was investigated in this study. For this investigation, 16S rRNA gene clone libraries were generated from the saliva of the four healthy Koreans, and 50 clones were randomly selected from each saliva clone library and sequenced. Totally, 37 different kinds of bacterial 16S rRNA gene sequences were identified based on sequence homology search through GenBank database. The 37 kinds of saliva clone sequences were classified to 14 genera and 2 uncultured and 1 unidentified bacteria. Among the 14 identified genera, *Streptococcus*, *Prevotella*, and *Veillonella* were common genera, and *Streptococcus* was dominant genus that accounted for 7 different species. Among the seven *Streptococcus* species, *S. salivarius* appeared as the most common species. More numbers of species belonging to the genera *Streptococcus* and *Prevotella* was present in saliva from ages 32 and 35. While saliva from ages 5 and 65 showed more numbers of species belonging to the genera *Rothia*, including potential pathogenic species. Overall, saliva of a young child and a senior showed higher bacterial diversity than that of young adults.

Keywords: human saliva, oral bacterial diversity, 16S rRNA gene clone

It has been known that dental laboratories could be a source of bacterial infection for the people visiting to and/or working at dental laboratories. Except for infected patients or dental laboratory persons, gargle water and dental unit water lines have been concerned as major sources of bacterial contamination (Fitzgibbon *et al.*, 1984; Peters and McGaw, 1996). Thus the understating of biofilm of dental unit water systems is important to perform microbiological evaluation of a range of disinfectant products and to find proper ways of dental hygiene (Fayle and Pollard, 1996; Walker *et al.*, 2003). Investigation on dental unit water lines including dental hand-piece, air-water syringe, and ultrasonic scaler system has been performed by several researchers, and the presence of microorganisms including bacteria, fungi, and protozoa were reported (Challacombe and Fernandes, 1995; Pankhurst *et al.*, 1998; Singh *et al.*, 2003).

Currently, no information on the diversity of bacteria present in dental unit water systems is available in Korea. Therefore we have been investigating microbial diversity in dental unit water systems at several dental units in Korea. In order to properly analyze the results of the investigation, information on oral bacteria both in the patients and in non-patients who regularly visit dental units is required because the information can provide us with the clues to find exact origin of bacterial contamination in dental unit water systems. There have been several reports in Korea on the bacterial species from patients with oral diseases (Kim *et al.*, 2003; Lee *et al.*, 2005; Lim *et al.*, 2005), but there has been no comparative report from periodotally healthy visitors. To obtain primary idea on oral bacterium species that are generally present in periodotally healthy Koreans, in this paper we analyzed bacterial diversity in the saliva of four Koreans who visit a dental unit regularly to check the condition of their mouth health.

Saliva samples (5 ml) of four Koreans including a child (age 5), two young adults (age 31 and 35) and a senior (age 65) were collected using sterilized 10 ml plastic tubes at the Dental Unit in Dankook

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University where the Koreans have been regularly checking their oral health. Two hours before saliva sampling the subjects brushed and had no food and drinks. These subjects had no periodontal problems and no record of antibiotic use during the previous 3 months. Bacterial DNA was extracted basically according to the method described by Tsai and Olson (1991). The saliva sample (1 ml) was subject to lysis firstly with lysozyme solution (1 M NaCl, 1 M EDTA, [pH 8.0]) by incubating at 37°C for 1.5 h with periodical shaking and secondly with Protease K (200 μg/ml) by incubating at 37°C for 30 min. The lysed saliva sample was frozen at −70°C for 5 min and heated at 70°C for 5 min. After repeating this freezing and heating treatment five times, bacterial DNA was purified from the lysed saliva by standard phenol-chloroform extraction. The extracted DNA was quantified with a Gene Quant II spectrophotometer (Amersham Pharmacia) and its final concentration adjusted to 100 ng/μl. PCR amplification was carried out using two general 16S rRNA gene primers, 27F; 5'-AGAGTTTGATCMTGGCTCA G-3' and 1492R; 5'-GGYTACCTTGTTACGACTT-3', corresponding to nucleotide positions 8-27 and 1492-1510, respectively, in *Escherichia coli* 16S rRNA gene (Lane, 1991). PCR was performed using MJ Research PTC-100 thermal cycler according to the method of Lee *et al.* (2005).

To construct 16S rRNA gene clone libraries of oral bacteria from the four Korean saliva samples, PCR amplicons were separated on a 1% agarose gel, stained with ethidium bromide, and visualized under UV illumination. About 1.5 kb size of DNA band was observed from all the four PCR amplicons of saliva samples. The 1.5 kb DNA bands were purified with the Qiaquick Gel Extraction kit (Qiagen), cloned into the pCR®2.1-TOPO vector using the TOPO™ TA cloning kit (Invitrogen), and transformed into One-shot competent *E. coli* cells according to the manufacturer's protocols.

To identify the diversity of bacterial species present in the saliva of the four healthy Koreans, 50 clones were randomly selected from each saliva clone library and sequenced using M13F sequencing primer and the PRISM Ready Reaction DyeDeoxy termination cycle sequencing kit. DNA sequences were determined in an Applied Biosystems ABI 373 DNA sequencer. The determined sequences of the saliva clones were confirmed as bacterial 16S rRNA gene by homology search through GenBank nucleotide sequence database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Some of clones were recalcitrant to sequencing, thus they were omitted. The identity of the determined partial 16S rRNA gene sequences (about 500 bp) of the four saliva clones was accessed based sequence similarity (higher than 97%) with known 16S rRNA gene sequences of bacterial species and clones from different sources.

Totally, 37 different kinds of bacterial 16S rRNA gene sequences were identified based on sequence homology search through GenBank database and they are listed in Table 1. Sequence similarity between all the clones and closest species found in GenBank was higher than 97% except for 3 clones (C32, C36, and D39). When the clone showing lowest sequence similarity (C32) was compared through DNA databases such as GenBank, EMBL, and DDBJ, it shared sequence similarity only 88% with some known bacterial species such as *Selenomonas*. Thus, the clone C32 is considered as the sequence of a bacterium that has not been reported yet. The remained 36 kinds of saliva clone sequences (of having >97% sequence similarity) were classified to 14 genera and 2 uncultured bacteria (Table 1). Since there were redundant sequences among the identified species or clones from the four saliva clones, only a representative sequence of each species or clone was registered in the GenBank. The accession numbers (DQ677536-DQ677560) and clone code of the representative saliva clones are given at Table 1.

The major genera of the saliva clones were found to be *Streptococcus*, *Provetella*, and *Veillonella* that included more number of species than other genera (Table 1). *Streptococcus* was dominant genus that accounted for more than 6 different species. This result could be explained by the fact that *Streptococcus* is known to constitute about 20% of the normal human oral flora (Kolenbrander, 2000). Among the six *Streptococcus* species *S. salivarius* appeared as the most common species (Table 1). This shows *Streptococcus salivarius* is a predominant member of normal oral bacteria flora in the four healthy Koreans. Recently, Lim *et al.* (2005) surveyed the frequency of *Streptoccocus* species in the dental plaques of 47 Korean adolescent and adults. They found the prevalence of *S. mutans, S. sobrinus*, and *S. downei* were 93.6%, 12.8%, and 8.5%, respectively. *S. mutans* was found to be dominant species in dental plaques. These three *Streptoccocus* species found in the dental plaques are different from the six *Streptoccocus* species listed at Table1. Based on both the results of Lim *et al.* (2005) and Table 1, we may construe that the diversity of *Streptoccocus* species tends to vary depending on the status of oral health in Koreans.

In the detected *Streptoccocus* sp. oral clones at Table 1, three different sequences were identified. Thus, to better understand the diversity of *Streptoccocus* species found from the saliva of periodontally healthy Koreans, phylogenetic relationships of the tree different sequences of *Streptoccocus* sp. oral clones (clones A38, B21, and D17) to other known *Streptoccocus* species were analyzed. For the phylogenetic analysis,

Table 1. Bacterial species and clones identified in the saliva of four different ages of Koreans by the analysis of the 16S rRNA gene clone libraries

No. of clones obtained from				Clone no.	Closest species (similarity)	Accession no.
age 5	age 31	age 35	age 65			
0	1	0	0	B53	*Atopobium* sp. oral clone (97%)	DQ677526
0	0	0	1	D40	*Campylobacter* sp. oral clone (99%)	DQ677527
1	0	0	1	D27	*Granulicatella* sp. oral clones (98%)	DQ677528
0	0	0	2	D32	*Haemophilus influenzae* (99%)	DQ677529
1	0	0	0	A32	*Haemophilus parainfluenzae* (97%)	DQ677530
1	1	0	4	D49	*Lautropia* sp. oral clones (98%)	DQ677531
0	3	0	0	B25	*Megasphaera* sp. clone (98%)	DQ677532
1	1	0	0	A04	*Neisseria weaveri* (99%)	DQ677533
2	0	0	3	A18	*Neisseria* sp. oral clones (99%)	DQ677534
0	0	1	0	C06	*Peptostreptococcus* sp. oral clone (97%)	DQ677535
0	0	1	1	D39	*Prevotella intermedia* (92%)	DQ677536
4	2	5	0	B08	*Prevotella melaninogenica* (98%)	DQ677537
0	0	1	0	C21	*Prevotella pollens* (99%)	DQ677538
0	0	0	1	D44	*Prevotella salivae* (99%)	DQ677540
1	0	2	0	A02	*Prevotella* sp. (98%)	DQ677541
3	9	2	0	B09	*Prevotella* sp. oral clones (99%)	DQ677539
0	0	0	2	D24	*Pseudomonas* sp. (100%)	DQ677542
1	0	0	4	D30	*Rothia dentocariosa* (100%)	DQ677544
2	0	0	0	A25	*Rothia mucilaginosa* (97%)	DQ677545
2	0	2	5	A13	*Rothia* sp. oral clones (99%)	DQ677546
3	4	5	4	D01	*Streptococcus mitis* (99%)	DQ677551
1	2	1	0	C25	*Streptococcus parasanguinis* (99%)	DQ677552
0	0	0	1	B40	*Streptococcus pneumoniae* (99%)	DQ677547
2	11	9	1	B24	*Streptococcus salivarius* (100%)	DQ677553
2	0	0	3	D45	*Streptococcus sanguinis* (99%)	DQ677554
0	1	0	1	D46	*Streptococcus bovis* (99%)	DQ677555
1	0	0	0	A38	*Streptococcus* sp. oral clones (98%)	DQ677548
0	5	0	0	B21	*Streptococcus* sp. oral clones (99%)	DQ677549
0	0	0	2	D17	*Streptococcus* sp. oral clones (98%)	DQ677550
4	3	3	1	C10	*Veillonella atypical* (98%)	DQ677556
0	1	0	0	B01	*Veillonella dispar* (99%)	DQ677557
2	1	0	1	A42	*Veillonella* sp. oral clones (99%)	DQ677558
0	0	1	3	C26	Uncultured bacteria clone (95%)	DQ677559
1	0	0	1	A03	Uncultured eubacteria clone (95%)	DQ677560
0	0	1	0	C32	Unidentified bacteria (88%)	DQ677542

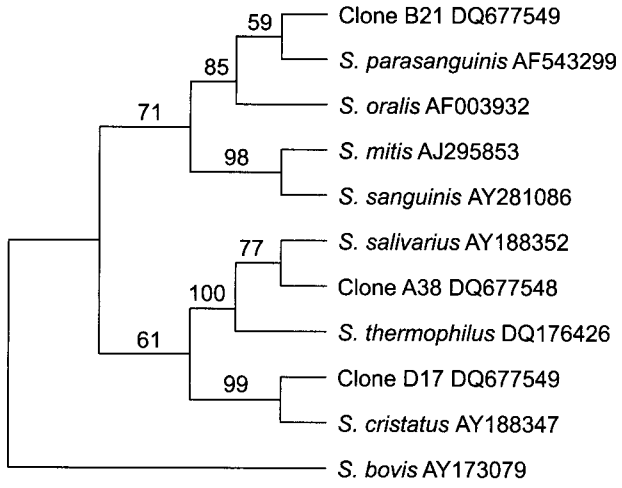

Fig. 1. Phylogenetic relationships of *Streptococcus* sp. saliva clones (A38, B21, D17) to other *Streptococcus* spp. Cladogram was generated based on 16S rRNA gene analysis using PAUP 4.0 program. Bootstrap values >50% are on the node.

the nucleotide sequences were aligned with the multiple sequence alignment program CLUSTAL W (Thompson *et al.*, 1994) and rearranged manually. Regions of the sequences that could not be aligned with certainty were excluded from further analysis. Sequence identity was calculated by pair-wise comparison. Phylogenetic analysis was performed using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford, 2002). Alignment gaps were treated as missing data. Distance analysis employed the F84 neighbor-joining method. Trees were rooted using *S. bovis* as the outgroup for the 16S rRNA gene analyses. Bootstrap analyses were performed through heuristic searches with 1,000 replicates to evaluate confidence intervals at the branch points in phylogenetic tree. Cladogram displaying the relationship of *Streptococcus* sp. oral clones to other known *Streptococcus* species is given in Fig. 1. The three saliva clones clearly separated each other and grouped with different *Streptococcus* species. Saliva clone B21 closely grouped with *S. parasanguinis*, clone A38 with *S. salivarius*, and clone D17 with *S. cristatus*, respectively. These separated grouping demonstrates that at least 7 species of *Streptococcus* are present in the saliva of the four healthy Koreans. Doel *et al.* (2005) reported that *Veillonella* spp. were found to be the most prevalent taxa isolated from the human tongue. It seems the prevalence of bacterial taxa in the human mouth is variable depending on sampling resources in the human mouth.

It has been hypothesized that the enterosalivary nitrate circulation might lead nitrate-reducing bacteria to reside within the oral cavity. Consequently, nitrate reductase active bacteria have been searched. *Veillonella atypica, V. dispar, Actinomyces odontolyticus, A. naeslundii, Rothia mucilaginosa, R. dentocariosa*, and *Staphylococcus epidermidis* from the human tongue were identified as nitrate reductase positive (Doel *et al.*, 2005). These identified bacteria are assumed to make a major contribution to nitrate reduction in the oral cavity. In this study, we also identified the presence of *Veillonella atypica, V. dispar, Rothia dentocariosa*, and *R. mucilaginosa* in the human saliva. Especially, *Veillonella atypica* was commonly detected from the saliva of all the four different Koreans. The exact role of nitrogen reductive bacteria in the human saliva is not known yet. It is assumed that nitrite production may limit the growth of acidogenic bacteria as a result of the production of antimicrobial oxides of nitrogen, including nitric oxide (Doel *et al.*, 2005). Further work would be needed to confirm the ability of nitrogen reductive bacteria detected from the human saliva and to explore the importance of nitrogen reduction in oral microbial environment.

Sakamoto *et al.* (2000) did not isolate pathogenic bacteria in the salivary sample from the periodontally healthy human subjects. Whereas Paster *et al.* (2001) extensively analyzed bacterial diversity human subgingival plaque and found that many bacterial species are commonly present both in the healthy and diseased subjects. This agreed with our work because the four healthy Korean saliva clones contained sequences of several potential oral pathogens such as *Rothia dentocariosa* (causing ostiomyelitis of jaw), *Provetella intermedia* (causing periodontal disease), *Granulicatella* sp. (causing symptomatic endodontic infections). Thus it should be emphasized that although the four Koreans were periodontally healthy, their saliva contain possible source of oral pathogens.

For the comparison of bacterial diversity among saliva of the four different ages, the numbers of different clones found was compared. Saliva from age 65 showed the highest diversity followed by saliva from age 5, age 35 and age 32, respectively. Saliva from age 32 and 35 showed more number of common species belonging to the genera *Streptococcus* and *Prevotella*. While saliva from ages 5 and 65 showed more number of species belonging to the genera *Rothia* and less common species including potential pathogenic species. Overall, in our investigation, saliva of ages 5 and 65 contained more diverse bacteria than that of young adults.

In conclusion, by using a culture-independent molecular method this study has produced some basic information on the diversity of bacteria that are present in the saliva of four periodontally healthy Koreans. Saliva is thought to have a significant influence on the colonization of bacteria in the oral cavity. During conversation, oral health observation

and treatment at the dental units, saliva can also be a resource of bacterial contamination in the air, water systems and apparatus present in the dental units. Since we have been investigating the microflora in the dental unit water systems in Korea, this study will allow a better understating of the microflora in the saliva-associated environments. We expect the exploration of microbial diversity both in the saliva and saliva-associated environments will direct the development of efficient and noble prevention methods of oral bacteria contamination in dental water units and/or in dental units in Korea

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