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Isolation and Characterization of the Mutans Streptococci from the Dental Plaques in Koreans

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Mutans streptococci have been implicated as cariogenic bacteria in dental caries because they can produce high levels of dental caries-causing lactic acid and extracellular polysaccharide. The aim of this study was to isolate and characterize the mutans streptococci from the dental plaque obtained from Koreans. The dental plaque samples were collected from the anterior and molar teeth of both jaws in 155 subjects (aged 2 to 33.2 years, average age 13.7±4.7 years). The samples were diluted by 100-fold in 1x PBS and plated on mitis-salivarius bacitracin (MSB) agar plates. The mutans streptococci grown on MSB plates were screened by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) targeting dextranase gene (*dex*). The mutans streptococci were identified at the species level using a 16S rDNA sequencing comparison method. The biochemical tests were carried out to biotype the mutans streptococci. Ninety-five strains of the mutans streptococci out of 358 colonies, which were derived from 141 subjects, were isolated. Of them, 77 strains and 18 strains were *Streptococcus mutans* and *Streptococcus sobrinus*, respectively. The biotyping data showed that 62, 1, 20, 10, and 2 strains were biotypes I, II, IV, V and variant, respectively. Of the two strains of variant biotype, one strains was similar to biotype IV except that it was positive to the arginine hydrolysis test. We considered this one strain a new biotype, and classified it as biotype VII. In conclusion, *S. mutans* and its biotype I was most frequently isolated in Korean dental plaque. The mutans streptococci strains isolated in this study might be useful for the study of the pathogenesis and the prevention of dental caries.

Keywords: biotype, *dex* gene, mutans streptococci, 16S rDNA

Dental caries is one of the most common infectious diseases in the human oral cavity. The enamel and dentin are demineralized by acids, such as lactic acid, which are produced as a by-product of the carbohydrate metabolism by cariogenic bacteria in dental plaque (Gjermo *et al.*, 1973). Among the oral bacteria, mutans streptococci have been implicated as major cariogenic bacteria (Loesche, 1986). In a study of the genus *Streptococcus* based on the sequence comparisons of 16S ribosomal RNA gene (16S rDNA), a total of six species groups were demonstrated, the anginosus, mitis, mutans, salivalius, bovis, and pyogenic group (Kawamura *et al.*, 1995). Mutans streptococci are divided into seven species: *Streptococcus mutans*, *S. sobrinus*, *S. downei*, *S. rattus*, *S. cricetus*, *S. ferus*, and *S. macacae* (Kawamura *et al.*, 1995). Of these, *S. mutans* and *S. sobrinus* are strongly associated with human dental caries (Loesche *et al.*, 1986). It had been previously thought that *S. downei* was isolated only from monkeys. Interestingly, *S. downei* was isolated also from human dental plaque (Yoo *et al.*, 2005a). *S. downei* and *S. sobrinus* are closely related species with similar characteristics but they are distinct species phylogenetically (Whiley *et al.*, 1988). The mutans streptococci have been classified as 6 biotypes (I to VI) according to their ability to ferment 4 carbohydrates (mannitol, sorbitol, raffinose, and melibiose) and to deaminate arginine (Shklair *et al.*, 1974, 1976; Whiley *et al.*, 1988).

Recently, natural products have been investigated in an attempt to prevent dental caries (Ito *et al.*, 2003; Hwang *et al.*, 2004). However, most studies on dental caries had been performed using the strains of mutans streptococci derived from Westerns. It is unclear if the natural products used in previous studies would have a similar effect on the mutans streptococci of Korean population. Lim *et al.* (2003) reported that the leaf-extract from *Camellia sinensis* had an antimicrobial effect on mutans streptococci. There were differences in the susceptibility between the type strains and the clinical isolates of mutans streptococci. In addition, the effectiveness of the leaf-extract from *Camellia sinensis* differed among the clinical isolates. In order to test the anti-cariogenic effect of natural extracts, it would be necessary to evaluate the antibacterial activity against clinical strains of the mutans streptococci isolated from the dental plaque obtained from Korean population.

Various methods have been used to both detect and identify the oral streptococcal species including biochemical tests

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(Beighton *et al.*, 1991), immunological tests (de Soet *et al.*, 1990), DNA probes (Cangelosi *et al.*, 1994), Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Igarashi *et al.*, 2001), PCR (Igarashi *et al.*, 1996; Igarashi *et al.*, 2000) and 16S rDNA sequencing comparison methods (Kawamura *et al.*, 1995). Among them, the PCR method is currently being applied to the detection of putative pathogens and the identification of human cariogenic bacteria because it is rapid, sensitive, and simple. The target genes for the PCR are often related to virulence factors such as dextranase gene (*dex*) (Igarashi *et al.*, 1996; Igarashi *et al.*, 2000) or 16S ribosomal RNA gene (16S rDNA) (Rupf *et al.*, 2001; Choi *et al.*, 2006). In order to assess the epidemiological studies for dental caries in Korea, it is essential to confirm the specificity of the PCR primers against the clinical strains of mutans streptococci isolated from Koreans.

In this study, we isolated and identified the mutans streptococci from the dental plaques in Koreans using bacterial cultivation with selective medium and PCR-RFLP targeted *dex*. The mutans streptococci were identified using the 16S rDNA sequencing comparison method at the species level. The biotype of clinical isolates of mutans streptococci was determined by the biochemical test. In addition, we developed the *S. sobrinus*-specific PCR primers based on the 16S rDNA and validated the specificity of *S. mutans*- or *S. sobrinus*-specific PCR primers based on the *dex* (Igarashi *et al.*, 1996; Igarashi *et al.*, 2000) and 16S rDNA (Choi *et al.*, 2006) as well as the new PCR primers. The clinical isolates of mutans streptococci isolated from Koreans may be useful in the studies of the pathogenesis, epidemiology, and prevention of dental caries in Korea.

Materials and Methods

Bacterial strains and growth conditions

The type strains of the bacteria used in this study are as follows: *Streptococcus mutans* ATCC 25175^T, *S. sobrinus* ATCC 33478^T, *S. downei* ATCC 33748^T, *S. rattus* KCTC 3655^T, *S. cricetus* KCTC 3640^T, *S. anginosus* ATCC 33397^T, *S. thermophilus* KCTC 3658^T, *S. mitis* KCTC 3556^T, *Staphylococcus aureus* KCTC 1621^T, *Actinobacillus actinomycetemcomitans* ATCC 33384^T, and *Fusobacterium nucleatum* ATCC 25586^T. All strains were obtained from the American Type Culture Collection (ATCC, USA) or the Korean Collection for Type Cultures (KCTC, Korea). The clinical strains of the mutans streptococci isolated from the dental plaque of Koreans were identified at the species level using a 16S rDNA cloning and sequencing method.

S. mutans, *S. sobrinus*, *S. downei*, *S. rattus*, and *S. cricetus* strains were cultured on a medium composed of mitis salivarius agar (Difco Lab., USA) supplemented with 0.0001% potassium tellurite, 0.2 units (2.8 μg/ml) of bacitracin (Sigma Chemical Co., USA), and 20% (w/v) sucrose (CJ Co., Korea) (MSB agar). The bacitracin was freshly prepared immediately before use. The MSB agar plates were stored at 4°C and were used within 7 days after preparation. *S. anginosus*, *S. thermophilus*, *S. mitis*, and *S. aureus* strains were cultured on Brain Heart Infusion agar (BHI) (Difco). The streptococci and staphylococci strains were also cultured on a BHI broth (Difco Laboratories). All of the above species were grown in a 37°C incubator in air containing 10% CO_2.

A. actinomycetemcomitans was grown in a medium containing Tryptic Soy Broth (TSB) (Difco) supplemented with 0.6% yeast extract, 5% horse serum, 75 g/ml of bacitracin, and 5 μg/ml of vancomycin (Sigma). *F. nucleatum* was grown in Schaedler broth (Difco). *A. actinomycetemcomitans* and *F. nucleatum* strains were grown at 37°C in an anaerobic chamber containing 10% H_2, 5% CO_2, and 80% N_2.

Dental plaque collection

The dental plaque samples were collected from the anterior and molar teeth of both jaws in 155 subjects (aged 2 to 33.2 years, average age 13.7±4.7 years). Ethical approval for this study was granted by the Institutional Research Board of to collect the dental plaque. The plaque samples were stored in a 1.5 ml Eppendorff tube containing 500 μl of 1X phosphate buffered saline (PBS) at -20°C before analysis. The samples were diluted 100-fold in 1 X PBS and plated onto MSB agar using a sterilized cotton ball. The plates were incubated for 2 days at 37°C in a CO_2 incubator.

Bacterial genomic DNA preparation

The bacterial genomic DNAs were prepared using a G-spin™ Genomic DNA Extraction kit (iNtRON Co., Korea) according to the manufacturer's instructions. The DNA concentrations were determined by measuring the OD at 260 and 280 nm using an UV spectrophotometer (Ultraspec 2000, Pharmacia Biotech., UK).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

PCR-RFLP based on *dex* was performed to screen the mutans streptococci growing on the MSB agar plate, as described elsewhere (Igarashi *et al.*, 2001). The PCR reaction was carried out using an *AccuPower*® PCR PreMix (Bioneer Corp., Korea) containing 5 nmole of each deoxynucleoside triphosphate, 0.8 μmole KCl, 0.2 μmole Tris-HCl (pH 9.0), 0.03 μmole $MgCl_2$, and 1 unit of *Taq* DNA polymerase. The bacterial genomic DNA and 20 pmoles of each primer were added to a PCR PreMix tube. The PCR was carried out in a final volume of 20 μl. The PCR reaction was run for 27 cycles on a Peltier thermal cycler (Model PTC-200 DNA engine™, MJ Research Inc., USA) under the following conditions: denaturation at 94°C for 2 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min. The final cycle included an additional extension time of 10 min at 72°C. Two μl of the reaction mixture was analyzed by 1.5% agarose gel electrophoresis in a Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA; pH 8.0) at 100 V for 30 min. The amplification products were stained with ethidium bromide and visualized using a UV transilluminator. The PCR products were digested with the restriction enzyme, *Hae*III (Bioneer Corp.).

16S rDNA cloning and sequencing

16S rDNA cloning and sequencing were also carried out to identify the mutans streptococci screened by the PCR-RFLP at the species level. In order to amplify the 16S rDNA from the bacteria, the PCR was performed with the 27F and

1492R primers (Lane *et al.*, 1985). The PCR conditions were the same as described elsewhere (Lane *et al.*, 1985). The PCR products were purified using an *Accu*Prep® PCR purification kit (Bioneer Corp., Korea) and were directly ligated using the pGEM-T easy vector (Promega, USA). The nucleotide sequencing of the 16S rDNA was determined using the dideoxy chain termination method with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). The sequencing primers used for the nucleotide sequencing of 16S rDNA were as follows: ChDC-GEM-F; 5'-TTC CCA GTC ACG ACG TTG TAA AA-3', ChDC-GEM-R; 5'-GTG TGG AAT TGT GAG CGG ATA AC-3', Seq-F1; 5'-CCT ACG GGA GGC AGC AG-3', Seq-R2; 5'-GAC TAC CAG GGT ATC TAA TCC-3', and F16; 5'-TAG ATA CCC YGG TAG TCC-3'. All of the sequences were compared with similar sequences from the reference organisms provided by BLAST (a genome database of the National Center for Biotechnology Information). The 16S rDNA nucleotide sequences of the mutans streptococci were registered on the GenBank database (National Center for Biotechnology Information).

Biochemical tests

The biochemical tests were carried out to determine the biotypes of the mutans streptococci, as described previously (Shklair *et al.*, 1974, 1976). A phenol red broth base (BBL, Becton Dickinson Microbiology System, USA) was used as the basal medium used for fermenting mannitol, sorbitol, raffinose, and melibiose. The carbohydrates were sterilized by Millipore filtration (0.22 μm pore size) and added aseptically to the warm basal media. The final concentration of the carbohydrates was 1.0%. The media were dispensed into sterile screw cap tubes that had been inoculated with the organisms to be tested, and read after 48 h of either aerobic (for all tubes of carbohydrates) or anaerobic (for the mannitol tube) incubation. The level of ammonia production from L-arginine was determined using the medium described by Facklam (1977). After 48 h incubation, 0.1 ml of the Nessler's reagent was added directly to the medium, and the production of ammonia was indicated by the development of an orange-yellow color. The biochemical tests were repeated with the cultures of type strains to determine the reproducibility and reliability.

Validation of the specificity of the species-specific PCR primers

In this study, a new *S. sobrinus*-specific PCR primer was developed. The oligonucleotide forward ChDC-SsF2; 5'-CAT TGG TAA CAC CGG ACT TGC-3' and reverse primers ChDC-SsR2; 5'-CGC CTG CGC TCC CTT TAC-3' were designed based on the 16S rDNA genes of *S. sobrinus* using the PrimerSelect program (DNASTAR, USA). The 16S rDNA sequences of *S. sobrinus*, which were stored in the GenBank database, were used as DNA templates. The predicted size of amplicon is 500 bp. The specificity of the PCR primers was evaluated using the type strains of 11 oral bacteria species, *S. sobrinus* ATCC 33478^T, *S. mutans* ATCC 25175^T, *S. downei* KCTC 3634^T, *S. rattus* KCTC 3655^T, *S. cricetus* KCTC 3640^T, *S. anginosus* ATCC 33397^T, *S. thermophilus* KCTC 3658^T, *S. aureus* KCTC 1621^T, *S. mitis* KCTC 3556^T, *F. nucleatum* ATCC 25586^T, and *A. actinomycetemcomitans* ATCC 33384^T. The sensitivity test was determined by serial dilution of a genomic DNA mixture from *S. sobrinus* ATCC 33478^T. The sensitivities ranged from 4 fg to 4 ng using 10-fold dilutions. The PCR was performed using an *AccuPower*® PCR PreMix (Bioneer, Korea). The PCR was carried out in a final volume of 20 μl. The PCR reaction was run for 30 cycles on a Peltier thermal cycler (Model PTC-200 DNA engine™, MJ Research Inc.) using the following conditions: denaturation at 95°C for 1 min, primer annealing at 66°C for 30 sec, and extension at 72°C for 1 min. The final cycle included an additional extension time of 10 min at 72°C. A 2 μl aliquot of the reaction mixture was then analyzed by 1.5% agarose gel electrophoresis in a Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA; pH 8.0) at 100 V for 30 min. The amplification products were stained with ethidium bromide and visualized using a UV transilluminator. PCR was carried out to compare the species-specificity of the PCR primers based on the *dex* reported previously with those based on the 16S rDNA gene developed in this study using the genomic DNAs of the mutans streptococci strains isolated from Koreans. The pairs of PCR primers based on the dextranase gene, SD1 and SD2 (Igarashi *et al.*, 1996) and SOF14 and SOR1623 (Igarashi *et al.*, 2000), were used to detect the clinical isolates of *S. mutans* and *S. sobrinus*, respectively. The PCR primer pairs targeting the 16S rDNA gene, ChDC-SmF2 and ChDC-SmR2 (Choi *et al.*, 2006) and ChDC-SsF2 and ChDC-SsR2 (in this study) were used to detect *S. mutans* and *S. sobrinus*, respectively. The PCR conditions were the same as those reported elsewhere (Igarashi *et al.*, 1996; Igarashi *et al.*, 2000; Choi *et al.*, 2006).

Results

Isolation of the mutans streptococci

Three hundred and fifty-eight bacteria were recovered on MSB from 141 out of 155 persons (aged 2 to 33.2 years old). In order to isolate the mutans streptococci, 358 colonies were selected based on the colony morphology (data not

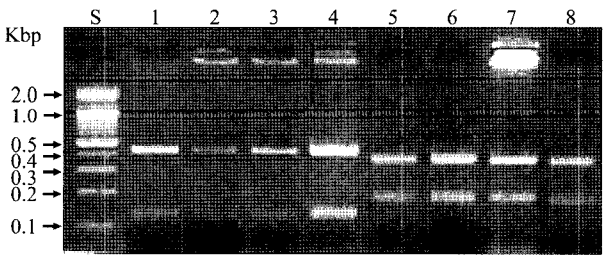

Fig. 1. Dextranase gene PCR-RFLP profiles of mutans streptococcal species from the dental plaque samples. The PCR products amplified with primers MSSD1467F and MSSD2000R were digested with *Hae*III, and then electrophoresed in 3% agarose gel. Lanes; S, size maker (100 bp DNA ladder); 1, *S. mutans* ATCC 25175^T; 2, *S. mutans* YM210; 3, *S. mutans* YM211; 4, *S. mutans* YM215; 5, *S. sobrinus* ATCC 33478^T; 6, *S. sobrinus* YS1; 7, *S. sobrinus* YS206; 8, *S. sobrinus* YS210. The Sizes (bp) of the PCR products digested with *Hae*III were 412 and 122 (for *S. mutans*) or 351 and 174 (for *S. sobrinus*).

Table 1. The determination of the species, biotype and PCR of mutans streptococci isolated from the dental plaques in Koreans

Species and strains (Accession no.)[a]	KCTC[b] no.	KCOM[c] no.	Biotype	PCR-RFLP	PCR		Sample no.	Gender (year, month)
				dex	*dex*	16S rDNA		
S. mutans ChDC YM1 [DQ677758]	5218	1074	I	M	M	M	1	Male (19, 10)
S. mutans ChDC YM3 [DQ677758]	5124	1054	I	M	M	M	2	Male (26, 2)
S. mutans ChDC YM6 [DQ677777]	5220	1076	I	M	M	M	3	Male (28, 0)
S. mutans ChDC YM9 [AY691527]	5128	1055	I	M	M	M	4	Female (29, 4)
S. mutans ChDC YM12 [DQ677756]	5224	1080	I	M	M	M	5	Female (14, 4)
S. mutans ChDC YM14 [DQ677755]	5225	1081	I	M	M	M	9	Female (15, 0)
S. mutans ChDC YM16 [DQ677787]	5227	1083	VII	M	M	M	9	Female (15, 0)
S. mutans ChDC YM20 [DQ677786]	5229	1085	I	M	M	M	10	Female (21, 3)
S. mutans ChDC YM22 [DQ677785]	5230	1086	I	M	M	M	13	Male (20, 9)
S. mutans ChDC YM25 [DQ677784]	5408	1087	I	M	M	M	14	Male (27, 11)
S. mutans ChDC YM26 [DQ677782]	5232	1088	I	M	M	M	16	Male (18, 11)
S. mutans ChDC YM29 [DQ677783]	NS[d]	2760	I	M	M	M	17	Male (27, 2)
S. mutans ChDC YM30 [DQ677780]	5235	1091	I	M	M	M	18	Female (18, 1)
S. mutans ChDC YM31 [DQ677781]	5236	1092	I	M	M	M	20	Male (15, 3)
S. mutans ChDC YM34 [DQ677779]	5410	2761	V	M	M	M	20	Male (15, 3)
S. mutans ChDC YM37 [DQ677778]	5239	1095	IV	M	M	M	21	Female (15, 3)
S. mutans ChDC YM40 [DQ677774]	5241	1097	I	M	M	M	21	Female (15, 3)
S. mutans ChDC YM41 [DQ677773]	5242	1098	V	M	M	M	22	Male (17, 1)
S. mutans ChDC YM44 [DQ677775]	5244	1100	V	M	M	M	25	Male (19, 4)
S. mutans ChDC YM45 [DQ677772]	5245	1111	I	M	M	M	27	Male (16, 0)
S. mutans ChDC YM47 [DQ677771]	5246	1112	I	M	M	M	28	Female (31, 5)
S. mutans ChDC YM49 [DQ677770]	5247	1113	I	M	M	M	30	Female (22, 9)
S. mutans ChDC YM51 [DQ677769]	NS[b]	1114	I	M	M	M	31	Female (21, 7)
S. mutans ChDC YM53 [DQ677768]	5252	1116	II	M	M	M	32	Female (22, 7)
S. mutans ChDC YM54 [DQ677767]	5257	1117	I	M	M	M	32	Female (22, 7)
S. mutans ChDC YM55 [DQ677766]	5249	1118	V	M	M	M	32	Female (22, 7)
S. mutans ChDC YM57 [DQ677765]	5250	1120	I	M	M	M	33	Female (24, 3)
S. mutans ChDC YM60 [DQ677764]	5251	1121	V	M	M	M	33	Female (24, 3)
S. mutans ChDC YM61 [DQ677763]	5253	1123	I	M	M	M	36	Female (21, 3)
S. mutans ChDC YM62 [DQ677762]	5254	1124	V	M	M	M	36	Female (21, 3)
S. mutans ChDC YM63 [DQ677761]	5255	1125	I	M	M	M	37	Female (15, 9)
S. mutans ChDC YM64 [DQ677760]	5256	1126	IV	M	M	M	38	Male (14, 4)
S. mutans ChDC YM67 [DQ677759]	5395	1127	I	M	M	M	40	Female (20, 6)
S. mutans ChDC YM69 [DQ677757]	5258	1128	I	M	M	M	41	Female (22, 7)
S. mutans ChDC YM70 [DQ677754]	5259	1129	I	M	M	M	42	Female (26, 10)
S. mutans ChDC YM71 [AY691528]	5411	2762	V	M	M	M	43	Male (24, 1)
S. mutans ChDC YM72 [DQ677753]	5286	1131	I	M	M	M	44	Female (14, 3)
S. mutans ChDC YM75 [DQ677752]	NS[d]	1132	I	M	M	M	45	Female (20, 11)
S. mutans ChDC YM77 [DQ677751]	5261	1133	I	M	M	M	48	Male (14, 1)
S. mutans ChDC YM79 [DQ677750]	5262	1134	I	M	M	M	49	Female (33, 2)
S. mutans ChDC YM81 [DQ677749]	5260	1136	I	M	M	M	50	Female (22, 7)
S. mutans ChDC YM82 [DQ677748]	5398	1137	I	M	M	M	52	Female (23, 2)
S. mutans ChDC YM85 [DQ677747]	5400	1139	I	M	M	M	53	Female (16, 4)

Species and strains (Accession no.)[a]	KCTC[b] no.	KCOM[c] no.	Biotype	PCR-RFLP	PCR		Sample no.	Gender (year, month)
				dex	*dex*	16S rDNA		
S. mutans ChDC YM87 [DQ677746]	5263	1140	I	M	M	M	55	Female (14, 6)
S. mutans ChDC YM89 [DQ677744]	5287	1142	I	M	M	M	56	Female (22, 5)
S. mutans ChDC YM90 [DQ677745]	5266	1143	I	M	M	M	57	Female (16, 0)
S. mutans ChDC YM95 [DQ677742]	5268	1145	I	M	M	M	61	Female (18, 11)
S. mutans ChDC YM96[DQ677741]	5269	1146	I	M	M	M	62	Male (18, 5)
S. mutans ChDC YM97 [AY691532]	5129	1060	V	M	M	M	62	Male (18, 5)
S. mutans ChDC YM99 [DQ677740]	5270	1147	I	M	M	M	63	Male (16, 10)
S. mutans ChDC YM101 [DQ677739]	5394	1122	I	M	M	M	35	Female (21, 8)
S. mutans ChDC YM201 [DQ677737]	5299	1178	I	M	M	M	PD4	Male (8, 10)
S. mutans ChDC YM202 [DQ677736]	5300	1179	I	M	M	M	PD9	Male (11, 3)
S. mutans ChDC YM203 [DQ677735]	5302	1181	I	M	M	M	PD24	Male (8, 4)
S. mutans ChDC YM204 [DQ677734]	5303	1183	I	M	M	M	PD32	Male (8, 4)
S. mutans ChDC YM205 [DQ677733]	5306	1186	I	M	M	M	PD59	Female (11, 3)
S. mutans ChDC YM206 [DQ677732]	5307	1187	I	M	M	M	PD65	Male (3, 8)
S. mutans ChDC YM207 [DQ677731]	5308	1188	V	M	M	M	PD69	Female (15, 1)
S. mutans ChDC YM209 [DQ677730]	5313	1194	I	M	M	M	PD1018	Female (2, 3)
S. mutans ChDC YM210 [DQ677729]	5314	1195	I	M	M	M	PD1019	Male (3, 2)
S. mutans ChDC YM211 [DQ677728]	5316	1197	I	M	M	M	PD1022	Female (5, 9)
S. mutans ChDC YM212 [DQ677727]	5365	1200	I	M	M	M	PD1026	Male (7, 4)
S. mutans ChDC YM213 [DQ677726]	5353	1201	I	M	M	M	PD1027	Male (5, 0)
S. mutans ChDC YM214 [DQ677725]	5366	1202	I	M	M	M	PD1035	Male (4, 6)
S. mutans ChDC YM215 [DQ677724]	5354	1203	I	M	M	M	PD1036	Male (2, 4)
S. mutans ChDC YM216 [DQ677723]	5402	1207	V	M	M	M	PD1045	Male (3, 8)
S. mutans ChDC YM217 [DQ677722]	5409	1208	VT[e]	M	M	M	PD1047	Female (3, 9)
S. mutans ChDC YM218 [DQ677721]	5356	1209	I	M	M	M	PD1049	Male (2, 4)
S. mutans ChDC YM219 [DQ677720]	5358	1212	I	M	M	M	PD1053	Female (3, 1)
S. mutans ChDC YM220 [DQ677719]	5367	1214	I	M	M	M	PD1056	Male (3, 2)
S. mutans ChDC YM222 [DQ677718]	5368	1216	I	M	M	M	PD1062	Male (3, 8)
S. mutans ChDC YM223 [DQ677717]	5369	1217	I	M	M	M	PD1063	Female (3, 5)
S. mutans ChDC YM225 [DQ677716]	5361	1219	I	M	M	M	PD1111	Female (3, 6)
S. mutans ChDC YM226 [DQ677715]	5370	1220	I	M	M	M	PD1115	Male (3, 5)
S. mutans ChDC YM228 [DQ677714]	5372	1223	I	M	M	M	PD1140	Female (4, 0)
S. mutans ChDC YM229 [DQ677713]	5364	1225	I	M	M	M	PD1145	Male (5, 5)
S. mutans ChDC YM230 [DQ677712]	5403	1226	I	M	M	M	PD1163	Male (3, 7)
S. sobrinus ChDC YS1 [AY691533]	5134	1061	IV	S	S	S	1	Male (19, 10)
S. sobrinus ChDC YS4 [DQ677801]	5273	1150	IV	S	S	S	57	Female (16, 0)
S. sobrinus ChDC YS5 [DQ677805]	5274	1151	IV	S	S	S	58	Female (14, 8)
S. sobrinus ChDC YS6 [DQ677804]	5275	1152	IV	S	S	S	59	Female (14, 0)
S. sobrinus ChDC YS7 [DQ677803]	5276	1153	IV	S	S	S	59	Female (14, 0)
S. sobrinus ChDC YS11 [DQ677802]	5279	1157	IV	S	S	S	45	Female (20, 11)
S. sobrinus ChDC YS12 [DQ677800]	5280	1158	IV	S	S	S	40	Female (20, 6)
S. sobrinus ChDC YS13 [DQ677799]	5281	1159	IV	S	S	S	40	Female (20, 6)
S. sobrinus ChDC YS201 [DQ677798]	5301	1180	IV	S	S	S	PD14	Female (6, 1)
S. sobrinus ChDC YS202 [DQ677797]	5304	1184	IV	S	S	S	PD54	Male (14, 0)
S. sobrinus ChDC YS203 [DQ677796]	5305	1185	IV	S	S	S	PD57	Male (9, 0)

Species and strains (Accession no.)[a]	KCTC[b] no.	KCOM[c] no.	Biotype	PCR-RFLP	PCR		Sample no.	Gender (year, month)
				dex	*dex*	16S rDNA		
S. sobrinus ChDC YS205 [DQ677795]	5311	1191	IV	S	S	S	PD1010	Male (5, 6)
S. sobrinus ChDC YS206 [DQ677794]	5312	1193	IV	S	S	S	PD1017	Female (5, 2)
S. sobrinus ChDC YS207 [DQ677793]	5315	1196	IV	S	S	S	PD1021	Female (4, 1)
S. sobrinus ChDC YS209 [DQ677792]	5357	1210	IV	S	S	S	PD1051	Female (3, 7)
S. sobrinus ChDC YS210 [DQ677791]	5362	1221	IV	S	S	S	PD1135	Female (4, 6)
S. sobrinus ChDC YS211 [DQ677790]	5364	1228	IV	S	S	S	PD1164	Male (2, 6)
S. sobrinus ChDC YS212 [DQ677789]	5360	1218	IV	S	S	S	PD1109	Male (2, 1)

M, *S. mutans*; S, *S. sobrinus*
[a] GenBank accession number for the nucleotide sequencing of 16S rDNA gene
[b] Korean Collection for Type Culture
[c] Korean Collection for Oral Microbiology
[d] not submitted
[e] Variant type

Table 2. The frequency of the mutans streptococci species isolated from the dental plaques in Koreans

	Mutans streptococci	
	S. mutans	*S. sobrinus*
Percent frequency (Number of individuals)	86.3% (69)	20.0% (16)

Table 3. The frequency of the mutans streptococci biotypes isolated from the dental plaques in Koreans

Species	Percent frequency of biotypes (n[a])					
	I	II	IV	V	VII	VT[b]
S. mutans	65.26 (62)	1.05 (1)	2.11 (2)	10.53 (10)	1.05 (1)	1.05 (1)
S. sobrinus	-	-	18.95 (18)	-	-	-
Total	65.26 (62)	1.05 (1)	21.06 (20)	10.53 (10)	1.05 (1)	1.05 (1)

[a] Number of strains
[b] Variant type

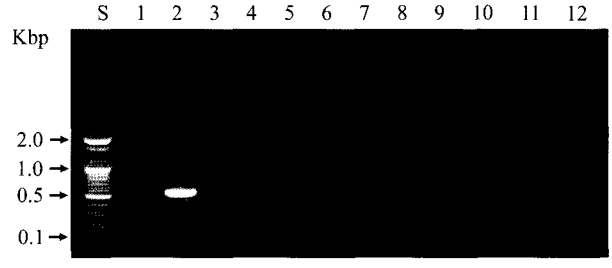

Fig. 2. Specificity test of PCR with ChDC-SsF2 and ChDC-SsR2 primers and purified genomic DNA of type strains. 4 ng of each bacterial genomic DNA was used as PCR templates. The PCR reactions were electrophoresed in 1.5% agarose gel. Lanes: S, size maker (100 bp DNA ladder); 1, negative control (distilled water); 2, *Streptococcus sobrinus* ATCC 33478[T]; 3, *S. mutans* ATCC 25175[T]; 4, *S. downei* KCTC 3634[T]; 5, *S. rattus* KCTC 3655[T]; 6, *S. cricetus* KCTC 3640[T]; 7, *S. anginosus* ATCC 33397[T]; 8, *S. thermophilus* KCTC 3658[T]; 9, *S. aureus* KCTC 1621[T]; 10, *S. mitis* KCTC 3556[T]; 11, *Fusobacterium nucleatum* ATCC 25586[T]; 12, *Actinobacillus actinomycetemcomitans* ATCC 33384[T].

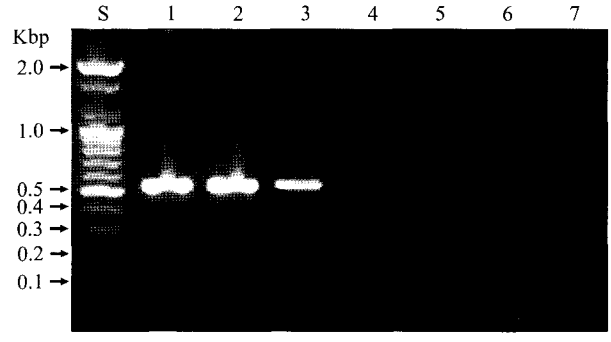

Fig. 3. The detection limits of PCR amplification with ChDC-SsF2, ChDC-SsR2 primers and purified genomic DNA of *S. sobrinus* ATCC 33478[T]. The PCR reactions were electrophoresed in 1.5% agarose gel. Lanes: S, size maker (100 bp DNA ladder); 1 through 7, purified genomic DNA serially diluted 10-fold from 4 ng to 4 fg. 1, 4 ng; 2, 400 pg; 3, 40 pg; 4, 4 pg; 5, 400 fg; 6, 40 fg; 7, 4 fg.

shown).

The mutans streptococci were screened using PCR-RFLP targeting dextranase gene (*dex*). Of the 358 clinical isolates, 95 strains (from 80 persons) were mutans streptococci (Table 1). The PCR-RFLP profile resulting from the *Hae*III-digested 530 bp DNA fragments clearly differentiated each clinical

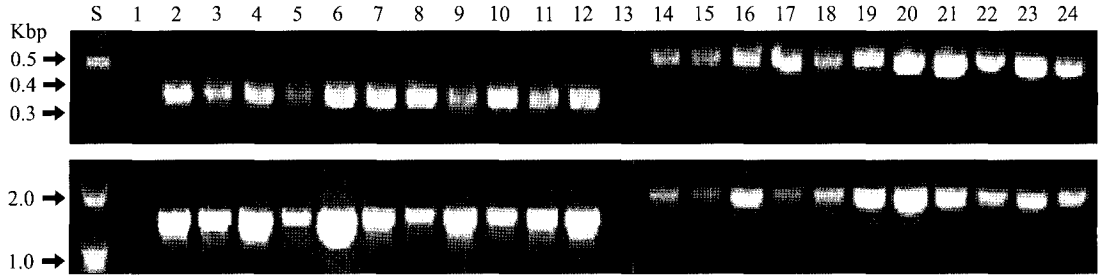

Fig. 4. Detection of *S. mutans* and *S. sobrinus* with PCR primers based on the 16S rDNA (A) and dextranase gene (B). The PCR products amplified with ChDC-SmF2, ChDC-SmR2 primer (for *S. mutans*) and ChDC-SsF2, ChDC-SsR2 primer (for *S. sobrinus*) (A). The PCR products amplified with SD1-SD2 (for *S. mutans*) and SOF14-SOR1623 primers (for *S. sobrinus*) (B). The PCR reactions were electrophoresed in 1.5% agarose gel. Lanes: S, size maker (100 bp DNA ladder); 1, negative control (distilled water); 2, *S. mutans* ATCC 25175^T; 3, *S. mutans* YM201; 4, *S. mutans* YM204; 5, *S. mutans* YM213; 6, *S. mutans* YM225; 7, *S. mutans* YM230; 8, *S. mutans* YM1; 9, *S. mutans* YM20; 10, *S. mutans* YM49; 11, *S. mutans* YM89; 12, *S. mutans* YM96; 13, distilled water; 14, *S. sobrinus* ATCC 33478^T; 15, *S. sobrinus* YS201; 16, *S. sobrinus* YS203; 17, *S. sobrinus* YS205; 18, *S. sobrinus* YS206; 19, *S. sobrinus* YS210; 20, *S. sobrinus* YS1; 21, *S. sobrinus* YS7; 22, *S. sobrinus* YS9; 23, *S. sobrinus* YS11; 24, *S. sobrinus* YS14.

isolate of mutans streptococci at the species level (data not shown). According to the PCR-RFLP data, there were 77 and 18 strains of *S. mutans* and *S. sobrinus*, respectively (Table 1). The size of the PCR products and their *Hae*III-fragments were all matched to those of the type strains (Fig. 1 and Table 1).

The 95 clinical isolates of the mutans streptococci were identified at the species level by 16S rDNA sequencing comparison method. Of the 95 isolates, 77 and 18 isolates were *S. mutans* and *S. sobrinus* (Table 1 and 2), respectively. 16S rDNA nucleotide sequences of the mutans streptococci were registered on the GenBank database (National Center for Biotechnology Information). Table 1 shows the GenBank accession numbers. The strains were submitted in KCTC and Korean Collection for Oral Microbiology (KCOM) (Table 1).

Biochemical characteristics of mutans streptococci

The biotyping data of the 95 strains showed that there were 62, 1, 20, 10, and 2 strains of biotypes I, II, IV, V and variant, respectively. Of two strains of variant biotype, one strain (ChDC YM16) was similar to biotype IV except that it was positive to the arginine hydrolysis test (Table 1 and 3). For this one strain, we created a new biotype "biotype VII". In addition, *S. mutans* ChDC YM217 could not ferment mannitol, sorbitol, raffinose, and melibiose and could not hydrolyze arginine (Table 1 and 3).

New PCR primers based on 16S rDNA sequence for the detection of S. sobrinus

The specificity and sensitivity of the new PCR primers for the detection of *S. sobrinus* were tested by performing PCR with the genomic DNAs of the type strains of 5 mutans streptococci species and *S. anginosus* ATCC 33397^T, *S. thermophilus* KCTC 3658^T, *S. aureus* KCTC 1621^T, *S. mitis* KCTC 3556^T, *F. nucleatum* ATCC 25586^T, and *A. actinomycetemcomitans* ATCC 33384^T. The specificity data of the *S. sobrinus*-specific primers (ChDC-SsF2 and ChDC-SsR2 primers) showed that the PCR product amplified only the *S. sobrinus* type strain (ATCC 33478^T) (Fig. 2). The detection limit with ChDC-SsF2 and the ChDC-SsR2 primers was 40 pg of the purified genomic DNA of *S. sobrinus* ATCC 33478^T (Fig. 3).

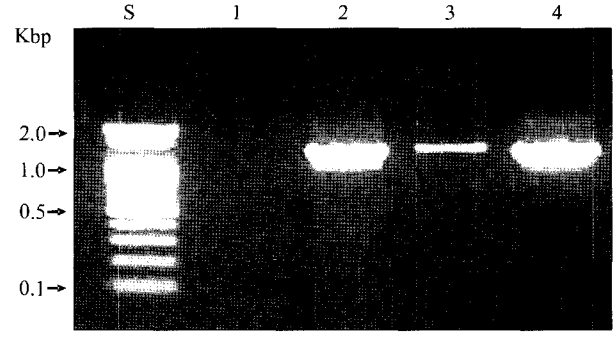

Fig. 5. Two clinical isolates of *S. sobrinus* were detected with the SD1 and SD2 primers. The PCR reactions were electrophoresed in 1.5% agarose gel. Lanes: S, size maker (100 bp DNA ladder); 1, negative control (distilled water); 2, *S. mutans* ATCC 25175^T; 3, *S. sobrinus* YS11; 4, *S. sobrinus* YS201.

Validation of specificity of the species-specific PCR primers for the detection of S. mutans or S. sobrinus using clinical isolates from Koreans

The specificity of the *S. mutans*- or *S. sobrinus*-specific PCR primers reported previously and the new PCR primers prepared in this study for the *S. sobrinus* in this study was confirmed by PCR with the genomic DNA of the clinical isolates from Koreans.

The *S. mutans*-specific PCR primers (ChDC-SmF2 and ChDC-SmR2) and *S. sobrinus*-specific PCR primers (ChDC-SsF2 and ChDC-SsR2) detected *S. mutans* and *S. sobrinus* isolated from Koreans, respectively (Fig. 4A and Table 1). The PCR primers (SOF14 and SOF1623), which were based on *dex* for the detection of *S. sobrinus*, also detected the type strain and clinical isolates of *S. sobrinus* (Fig. 4B and Table 1). The PCR primers (SD1 and SD2), which were based on *dex* for the detection of *S. mutans*, detected the two *S. sobrinus* strains (YS11 and YS201) as well as the *S. mutans* strains used in this study (Fig. 5).

Discussion

The results showed that the most frequent mutans streptococci detected in Korean dental plaque (aged 2-33.2 years) was *S. mutans* (86.3%), followed by *S. sobrinus* (20.0%) (Table 1 and 2). According to the other epidemiological data in Korea, *S. mutans* and *S. sobrinus* were detected in 93.1 and 33.1% of the dental plaque samples, respectively, from 137 Korean school children (10-11 years old) by biochemical tests (biotyping) (Kim *et al.*, 1983). The detection frequency of the two organisms was thus higher than in our data. This might be due to the different ages of the subjects employed. In a recent study, the prevalence of *S. mutans* and *S. sobrinus* was reported to be 72.8% and 61.1% in 77 Japanese aged 3-5 years old, respectively, using a PCR method (Okada *et al.*, 2002). Wu *et al.* (2003) also reported the prevalence of *S. mutans* and *S. sobrinus* in 126 Chinese dental plaque samples (aged 25-55 years) to be 75.4% and 57.1%, respectively, using biochemical tests and PCR. In addition, *S. mutans* and *S. sobrinus* were detected in 65-95% and 0.4-36% of pre-school children aged 5-13 years old of the United States and Canada, respectively (Qureshi *et al.*, 1977). Although the ages of the subjects and detection methods used were different, the distribution and prevalence of *S. mutans* and *S. sobrinus* reported in the studies cited above, including ours show a similar tendency to those of the present study. However, 90% of the mutans streptococci isolated from 5- to 8-year-old schoolchildren in the UK was *S. mutans* with rare cases of *S. sobrinus* being detected (Beighton *et al.*, 1987). In Australians aged 10-25 years, *S. mutans* and *S. sobrinus* were detected in 30% and 35%, respectively (Rogers, 1973). Therefore, the distribution of *S. mutans* and *S. sobrinus* appears to be associated with differences in the geographical human populations.

In this study, the mutans streptococci were screened using the *dex*-targeted PCR-RFLP and biochemical tests, and the screened strains were identified at the species level using the 16S rDNA sequencing comparison method. Interestingly, the result of 16S rDNA sequencing comparison analysis was clearly consistent with that of PCR-RFLP analysis. Therefore, *dex*-targeted PCR-RFLP could be used to identify the mutans streptococci without the need for 16S rDNA sequencing comparison analysis. The clinical isolates that were not identified as mutans streptococci by PCR-RFLP were classified into non-mutans streptococcal organisms (non-MSO). According to previous studies, the majority of the non-MSO was *S. anginosus, S. sanguinis, Enterococcus faecalis,* and *Pantoea agglomerans* (Yoo *et al.*, 2005b; Lee *et al.*, 2006).

The biotyping data showed biotype I (65.26%) to be the most common one in 95 strains of the mutans streptococci from Koreans (aged 2-33.2 years old), followed by IV (21.06%), V (10.53%), and II (1.05%) (Table 3), which is similar to the results of a previous study in which biotype I (78.8%), IV (33.1%), V (19.5%), and II (1.7%) were isolated from 137 Korean school children (10-11 years old) (Kim *et al.*, 1983). On the other hand, the detection frequency of biotypes isolated from the saliva of 114 preschool children in Taiwan were biotype I (72.9%), III (7.3%), IV (7.3%), V (7.3%), and II (5.2%) (Chen *et al.*, 1990). Several studies have reported biotype I to be the one detected most commonly detected in dental plaques of 49 San Diegan (17-22 years old), 25 Californian (17-22 years old), 55 Hawaiian school children (3-13 years old), and 217 Saudi Arabian Naval personnel (16-26 years old) (Bratthall, 1972; Keene *et al.*, 1977).

Generally, the biotype II is *S. rattus*, and the biotype IV is *S. sobrinus* (Coykendall and Alan, 1989). Interestingly, in this study, 2 strains of *S. mutans* were biotype IV and 1 strain of *S. mutans* was biotype II (Table 1 and 3). Another 2 strains of *S. mutans* were variable types. Of them, 1 strain was named as biotype VII. The reason for these observations is unclear but the oral environment of the hosts may cause changes in the bacterial metabolic characteristics.

Of the mutans streptococci, *S. rattus* (biotype II) has the ability to hydrolyze arginine (Coykendall, 1974). In a recent study, the operon of the arginine deiminase system (ADS) was cloned from *S. rattus* FA-1 (Griswold *et al.*, 2004). The catabolism of arginine using the ADS produced ammonia (NH_3) and ATP (Curran *et al.*, 1995; Griswold *et al.*, 2004). The generation of ATP allows the use of arginine as the sole energy source. NH_3 produced within the cell combines with a proton to yield NH_4^+, and this reaction increases the pH value in plaque. PCR with the degenerative PCR primers based on the ADS operon of *S. rattus* FA-1 was performed to determine if the biotype II and VII strains isolated in this study have the ADS operon (data not shown). The PCR data did not detect any PCR amplicon (data not shown). In order to confirm the ability of the arginine hydrolysis in the biotype II and VII strains, the cloning and characterization of the ADS from the strains may be needed.

In this study, 10 of the *S. mutans* strains were biotype V (10.53%) (Table 1 and 3). It was reported that approximately 12% of *S. mutans* clinical isolates were melibiose negative (Beighton *et al.*, 1991). The PCR primers based on the GTF A gene (*gtf*A) were used to detect the melibiose-negative isolates (biotype V) (Colby *et al.*, 1995). The *gtf*A gene, which lies within the multiple sugar metabolism (msm) operon, disappeared in the melibiose-negative isolates. The results suggest that PCR with the primers based on the genes of the enzymes corresponding to the biochemical tests may be useful for determining the biotype of mutans streptococci without resorting to those tests.

The results of the specificity tests of the PCR showed that the PCR primers for the *S. sobrinus* based on the 16S rDNA gene (ChDC-SsF2 and ChDC-SsR2) and *dex* (SOF14 and SOF1623) have the same specificity. For the clinical isolates of *S. mutans* in Koreans, the PCR primers based on the 16S rDNA (ChDC-SmF2 and ChDC-SmR2) were more specific than those based on the dextranase gene (SD1 and SD2). The PCR primers (ChDC-SsF2 and ChDC-SsR2) reported in this study will constitute a valuable addendum to the list of primers which are already available for the identification of *S. sobrinus*. In our laboratory, two or three PCR primer sets are routinely used to identify bacteria grown on agar plates at the species level, and the resulting PCR data generally tend to be consistent with the 16S rDNA cloning and sequencing data (Kim *et al.*, 2005). These results suggest that the species-specific PCR primers based on the 16S rDNA gene can be used to both detect and

identify mutans streptococci at the species level.

In this study, we isolated, identified, and characterized the 96 strains of mutans streptococci from the dental plaques in Koreans using bacterial cultivation with selective media, molecular biological methods, and biochemical tests. Among the strains, *S. mutans* and its biotype I was most frequently detected in Korean dental plaques. The mutans streptococci strains isolated in this study may be useful for the studies of the pathogenesis, epidemiology, and prevention of dental caries in Korean population.

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