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NOTE

Identification of Non-mutans Streptococci Organisms in Dental Plaques Recovering on Mitis-Salivarius Bacitracin Agar Medium

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The objective of this study was to both isolate and identify non-mutans streptococci organisms (non-MSO) from dental plaques recovered on mitis-salivarius sucrose bacitracin agar (MSB) plates. The dental plaque samples, which had been collected from 63 human subjects, were diluted and plated on MSB. The bacteria growing on the MSB plates were then identified with biochemical tests, as well as with 16S rDNA cloning and sequencing techniques. Our data indicated that bacteria from 30 subjects had been recovered on the MSB plates. Among the 21 typical colonies selected from the 30 subjects, 12 colonies, derived from 10 subjects, were identified as non-MSO. These 12 colonies were determined to be *Streptococcus anginosus* (8 colonies), *S. sanguinis* (1 colony), and *Pantoea agglomerans* (3 colonies). These results strongly suggest that a new selective medium will be required for the reliable isolation of mutans streptococci.

Key words: dental plaque, mitis-salivarius bacitracin agar, non-mutans streptococci organism, 16S rDNA

The mutans streptococci (MS) constitute a group of oral cariogenic species, which includes the species, *Streptococcus mutans, S. sobrinus, S. cricetus, S. rattus, S. downei, S. macacae*, and possibly, *S. ferus* (Whiley and Beighton, 1998). Within this group of streptococci, *S. mutans* and *S. sobrinus* are most frequently found in humans, whereas *S. cricetus* and *S. rattus* are only rarely detected. Several media have been developed for the selective isolation of MS (Gold *et al.*, 1973; Van Palenstein Helderman *et al.*, 1983; Tanzer *et al.*, 1984). Among these media, mitis-salivarius sucrose bacitracin agar (MSB) is used most frequently. On MSB agar, MS can normally be recognized according to their colony morphology, with the aid of a dissecting microscope (Gold *et al.*, 1973). Occasionally, colonies other than MS are also recovered. Enterococci and yeasts have been encountered on MSB, when undiluted saliva or dental plaque samples were cultured (Gold *et al.*, 1973; Jensen and Bratthall, 1989). Although no studies have yet been published regarding the identification of non-mutans streptococcal organisms (non-MSO) at the species level, certain oral streptococci have been shown to grow on MSB. For instance, when *S. sanguinis* and *S. milleri* type 2 were inoculated into aged MSB, the bacteria were demonstrated to recover on the medium (Kimmel and Tinanoff, 1991). The paucity of species-level information regarding non-MSO that can grow on MSB led us to attempt to isolate and identify them from dental plaque samples, using biochemical tests as well as 16S rDNA cloning and sequencing techniques.

Ethical approval for this study was granted by the Institutional Research Board of Chosun University. 63 patients, aged 14 to 34 years (average age was 16 years) were enrolled in this study. None of the participants had received antibiotic therapy in the prior 3 months, and none had any history of systemic disease. Dental plaque samples were collected from at least 4 sites (buccal sites of deciduous molars or molars of upper jaws and palatal sites of deciduous molars or molars of lower jaw) in each subject, diluted 100-fold in 1X PBS, and plated with sterilized cotton balls, on a medium consisting of mitis salivarius agar (Difco, USA) supplemented with 0.0001%

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Table 1. Number of MS and non-MSO recovered on MSB from dental plaques of ten patients

Pt' No.	*S. mutans*	*S. sobrinus*	*S. anginosus*	*S. sanguinis*	*P. agglumerans*
1	1 (YM2)*	1 (YS1)	1 (YA1)	-	-
2	1 (YM3)	-	1 (YA3)	-	-
4	1 (YM9)	-	1 (YA4)	-	-
30	-	-	1 (YA11)	-	-
35	-	-	-	1 (YSA1)	-
43	1 (YM1)	-	2 (YA6, YA7)	-	-
52	1 (YM83)	-	-	-	1 (YP3)
53	1 (YM86)	-	1 (YM9)	-	1 (YP1)
55	1 (YM88)	-	-	-	1 (YP2)
62	1 (YM97)	-	1 (YA10)	-	-
Sum	8	1	8	1	3

( )*: Name of the strain: for example, (YM2) means *S. mutans* ChDC YM2.

potassium tellurite, 0.2 units (2.8 μg)/ml of bacitracin (Sigma, USA), and 20% (w/v) sucrose (CJ Co., Korea) (MSB agar). The bacitracin was freshly prepared immediately before use. The MSB agar was stored at 4°C, and was used within 7 days of preparation. The plates were incubated at 37°C for 1-2 days in a CO_2 incubator. In order to collect all of the organisms that were recoverable on MSB, whether MS or non-MSO, we randomly selected typical colonies from the 30 plates exhibiting bacterial growth (a total of 21 strains), based on their morphology, size, and color.

In order to identify MS, we conducted biochemical tests according to the methods described by Shklair and Keen (1974, 1976). A phenol red broth base was used as the basal medium for the fermentation of mannitol, sorbitol, raffinose, and melibiose (BBL, USA). The carbohydrates were sterilized via Millipore filtration (0.22 μm pore size) and were added aseptically to warm basal media. The final concentration of the carbohydrates was 1.0%. The media were then dispensed into sterile screw cap tubes inoculated with the organisms to be tested. After 48 h of either aerobic (for all tubes of carbohydrates) or anaerobic (for the mannitol tube) incubation, we read the tubes. In order to determine the level of ammonia generated from L-arginine, we used the appropriate medium, which was previously described (Niven *et al.*, 1942). After 48 h incubation, 0.1 ml of Nessler's reagent was directly added to the medium. The development of an orange-yellow color indicated the generation of ammonia. These biochemical tests were repeated with cultures of the reference strains, in order to determine reproducibility and reliability.

The bacteria, which were not included in the biochemical scheme developed by Shklair and Keen (1974, 1976), were designated non-MSO. We performed cloning and sequencing of the 16S rDNA in order to confirm our identification of MS as well as non-MSO. We prepared the bacterial genomic DNA with a G-spin™ Genomic DNA Extraction kit (iNtRON, Korea), in accordance with the manufacturer's instructions. In order to amplify the 16S rDNA of the bacteria, we conducted polymerase chain reactions (PCR) with the 27F and 1492R primers. The PCR conditions used in this phase of the study were the same as those described previously (Lane, 1991). The

Table 2. Results from biochemical tests of bacteria recovered on MSB

Strain	Man[1]	Sor[2]	Raf[3]	Mel[4]	Arg[5]
ChDC* YM2	+	+	+	+	-
ChDC YM3	+	+	+	+	-
ChDC YM9	+	+	+	+	-
ChDC YM71	+	+	+	+	-
ChDC YM83	+	-	+	+	-
ChDC YM86	+	+	+	+	-
ChDC YM88	+	+	+	+	-
ChDC YM97	+	+	+	+	-
ChDC YS1	+	-	-	-	-
ChDC YA1	+	-	-	-	+
ChDC YA3	-	-	-	-	+
ChDC YA4	-	-	-	-	+
ChDC YA6	-	-	-	-	-
ChDC YA7	-	-	-	-	+
ChDC YA9	-	-	-	-	+
ChDC YA10	-	-	-	-	-
ChDC YA11	-	-	-	-	-
ChDC YSA1	+	+	+	+	+
ChDC YP1	-	-	+	-	+
ChDC YP2	-	-	-	-	-
ChDC YP3	+	-	-	-	+

ChDC*: Department of Oral Biochemistry, College of Dentistry, Chosun University
1, Man: fermentation of mannitol
2, Sor: fermentation of sorbitol
3, Raf: fermentation of raffinose
4, Mel: fermentation of melibiose
5, Arg: hydrolysis of arginine

PCR product was then purified with an *Accu*Prep® PCR purification kit (Bioneer, Korea), and was directly ligated with pEZ-T Easy vector (RNA, Korea). 16S rDNA nucleotide sequences were 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 primers used for nucleotide sequencing were as follows: ChDC-F (5'-AAT ACG ACT CAC TAT AGG GCG AA-3'), ChDC-R (5'-CCT CAC TAA AGG GAA CAA AAG C-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 obtained sequences were then compared with similar sequences from the reference organisms, with the BLAST database (a genome database of the National Center for Biotechnology Information).

Bacteria from 30 out of 63 dental plaque samples were recovered on MSB. Non-MSO were isolated from 10 of these 30 samples. The number of total bacterial strains isolated from the colonies recovered on the MSB plates from the dental plaque samples of 10 patients, are shown in Table 1. Identification of these strains, with regard to whether they were MS or non-MSO, was predicated on the results of the biochemical tests described in the Materials and Methods section (Table 2). Subsequently, all of the organisms were identified at the species level by 16S rDNA cloning and sequencing (Table 3). It remains unclear as to why we witnessed such a low bacterial isolation rate from the dental plaque samples , but it may be attributable either to the relatively high dilution of plaque samples, or to the fact that the patients had brushed their teeth before visiting the dental clinic.

Among the 21 strains collected from the 30 subjects, 12 of the strains were identified as non-MSO. The majority of these non-MSO were ultimately identified as *S. anginosus.* After 36 h of culturing on MSB plates, these *S. anginosus* colonies all formed colonies of approximately 0.1 mm in diameter. All of the *S. anginosus* colonies were mucoid-type. The results of biochemical tests on these *S. anginosus* isolates indicated that most of the strains exhibited similar characteristics, but were not necessarily identical. For instance, the *S. anginosus* strains ChDC YA6 and YA7 had been isolated from the same patient, but their ability to hydrolyze arginine was not identical. This clearly demonstrates that multiple strains of *S. anginosus,* with different phenotypes, can be isolated from a single patient. *S. anginosus* was also reported to be relatively resistant to bacitracin, as the organism was observed to grow on MS-SOB medium containing 0.02 units/ml of bacitracin, together with other antimicrobial agents (Hirasawa and Takada, 2002). It may not, therefore, be incredibly surprising that all of the *S. anginosus* strains isolated in this study exhibited bacitracin resistance. Their

Table 3. Homology analysis of 16S rDNA sequences of bacteria recovered on MSB

Strains	GenBank accession number	Species match [GenBank accession number]	Homology (%)
ChDC* YM2	AY691525	*S. mutans* ATCC 25175 [AY188348]	98
ChDC YM3	AY691526	*S. mutans* ATCC 25175 [AY188348]	99
ChDC YM9	AY691527	*S. mutans* ATCC 25175 [AY188348]	99
ChDC YM71	AY691528	*S. mutans* ATCC 25175 [AY188348]	99
ChDC YM83	AY691529	*S. mutans* ATCC 25175 [AY188348]	99
ChDC YM86	AY691530	*S. mutans* ATCC 25175 [AY188348]	99
ChDC YM88	AY691531	*S. mutans* ATCC 25175 [AY188348]	99
ChDC YM97	AY691532	*S. mutans* ATCC 25175 [AY188348]	99
ChDC YS1	AY691533	*S. sobrinus* ATCC 33478 [AY188349]	98
ChDC YA1	AY691534	*S. anginosus* ATCC 33397 [AF104678]	98
ChDC YA3	AY691535	*S. anginosus* ATCC 33397 [AF104678]	99
ChDC YA4	AY691536	*S. anginosus* ATCC 33397 [AF104678]	99
ChDC YA6	AY691537	*S. anginosus* ATCC 33397 [AF104678]	99
ChDC YA7	AY691538	*S. anginosus* ATCC 33397 [AF104678]	99
ChDC YA9	AY691539	*S. a nginosus* ATCC 33397 [AF104678]	99
ChDC YA10	AY691540	*S. anginosus* strain 367 [AF145239]	99
ChDC YA11	AY691541	*S. anginosus* strain 367 [AF145239]	99
ChDC YSA1	AY691542	*S. sanguinis* ATCC 10556 [AF003928]	99
ChDC YP1	AY691543	*P. agglomerans* SP1 [AF199029]	99
ChDC YP2	AY691544	*P. agglomerans* SP1 [AF199029]	99
ChDC YP3	AY691545	*P. agglomerans* SP1 [AF199029]	99

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MICs were above 32 μg/ml of bacitracin (data not shown). In the future, we plan to isolate more *S. anginosus* from patients, and to determine their susceptibility to bacitracin. This will enable us to clarify the degree to which this organism is susceptible to antibiotics.

Two *S. sanguinis* strains from one patient were isolated on MSB, and these two strains exhibited identical biochemical characteristics. Therefore, we considered them to represent the same strain, and so only one of the two strains was actually cited in this study. In our laboratory, 4 strains of *S. sanguinis*, ChDC OS38, ChDC B304, ChDC B259, and ChDC B294, were isolated from sites of mandibular osteitis or maxillary sinusitis lesions (unpublished data). All of these strains exhibited resistance to 20% sucrose. Two strains (ChDC OS38, ChDC B304) grew on MSB and exhibited bacitracin resistance. The other strains were determined to be sensitive to bacitracin, and failed to grow on MSB. These data indicate that *S. sanguinis*, which acquired resistance to both 20% sucrose and to bacitracin, was capable of growth on MSB.

Interestingly, three *P. agglomerans* strains were also isolated from the dental plaques of three patients. To our knowledge, this is the first report of the isolation of this species from the human oral cavity. *P. agglomerans*, an environmental bacterium, is a Gram (-) bacillus, and is frequently isolated from the leaves of plants. Currently, remains unclear as to whether *P. agglomerans* is a normal constitutent of oral flora, or simply a transient bacterium. However, it is clearly possible that these patients had ingested vegetables, such as celery, cabbage, or lettuce, prior to the dental plaque sampling. The isolated *P. agglomerans* colonies appeared shiny and mucoid, and the sizes of the colonies ranged from 1.2 mm to 1.5 mm after 24 h of culturing on MSB. The biochemical characteristics of the three *P. agglomerans* isolates also differed (Table 2). In addition, this result was clearly inconsistent with the previous report regarding the biochemical characteristics of *P. agglomerans*, which held that it was positive for mannitol fermentation and negative for arginine dihydrolase (Farmer III, 1996). The reason for this discrepancy remains, at present, unclear, but it may be attributable to variances in the habitat of the organism.

MSB has been used extensively in the isolation of MS. This is primarily due to its ability to distinguish and select for this group of microorganisms, when used in conjunction with conventional dilution and plating techniques. However, in caries activity tests, in which undiluted dental plaque directly inoculated into the medium, such as with the dip-slide method used in the Cariescreen protocol (APO Diognostics, Canada), MSB does not suppress sufficient non-MSO to allow for the ready identification of MS. On this medium, some non-MSO colonies can be visually confused with MS, especially when counted by a non-microbiologist. High numbers of non-MSO, which could easily be visually confused with MS on MSB medium, tend to increase the incidence of false positive results. Due to this, subjects can often be inappropriately categorized as having higher levels of MS.

To our knowledge, this is the first report regarding the species-level identification of non-MSO growing on MSB. Recently, investigators have developed new selective media for *S. sobrinus* and *S. mutans,* by modifying the MSB medium (Hirasawa and Takada, 2002, 2003). The identification of the non-MSO species that can be recovered on MSB will contribute greatly to future improvements to this medium.

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