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Identification of *Enterococcus faecalis* on MSB Medium Selective for Mutans Streptococci

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Mitis-salivarius sucrose bacitracin (MSB) medium is widely used in the selective isolation of mutans streptococci (MS), a designation for a group of oral cariogenic species. Recently, we have isolated three bacterial strains grown on MSB agar from human dental plaques. The three strains exhibited biochemical characteristics similar to those of the biotype IV of MS, with the exception that they manifested a positive reaction for arginine deaminase. The objective of this study was to identify and characterize these three clinical isolates. The bacteria were identified with biochemical tests as well as by 16S rDNA cloning and sequencing. In order to compare the antibiotics susceptibility of the clinical isolates with that of type strain, the minimum inhibitory concentrations of 9 antibiotics were determined using broth dilution assays. The results identified all of our three clinical isolates as *Enterococcus faecalis*. All *E. faecalis* strains were found to be susceptible to penicillin G, amoxicillin, augmentin, and vancomycin, but were resistant to ciprofloxacin, cefuroxim axetil, and clindamycin. Our findings indicate that *E. faecalis* is capable of growing on MSB agar, and suggest that the MSB medium be improved so that only MS should be recoverable on the medium, as originally devised for their selection.

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Introduction

Several selective media have been developed for the isolation of mutans streptococci, a group of cariogenic species that include *Streptococcus mutans*, *S. sobrinus*, *S. downei*, *S. rattus*, and *S. cricetus* (Gold *et al.*, 1973; Tanzer *et al.*, 1984; Van Palenstein Helderman *et al.*, 1983; Whiley and Beighton, 1998). Of these media, mitis-salivarius sucrose bacitracin (MSB) agar is most frequently used for the selective isolation of mutans streptococci from humans. "Cariescreen SM (Butler Co., Chicago, IL, USA)" is a kit that is used to quantify the number of *S. mutans* present in a salivary sample. This kit is based on MSB agar and is used in the individual cariogenic activity test. Occasionally, non-mutans streptococci organisms (non-MSO) are isolated on MSB agar. In the previous study, we identified some non-MSO growing on MSB agar at the species level (Yoo *et al.*, 2005). The results indicated that we had succeeded in isolating such organisms as *S. anginosus*, *S. sanguinis*, and *Pantoea agglomerans*.

Recently, we isolated three strains of another non-MSO on MSB agar plates. The diameters of the colonies were approximately 0.8 to 1.2 mm after being cultured on MSB agar plates for 24 hours. All of the colonies were mucoid type. The morphology of the colonies was similar to that associated

with mucoid type of *S. sobrinus* or *P. agglomerans*. In addition, the biochemical characteristics of the three strains were similar to those exhibited by the biotype IV (*S. sobrinus*) of mutans streptococci, with the exception that our isolates manifested a positive reaction for arginine deaminase.

Therefore, in this study, we performed 16S rDNA sequencing and biochemical test using API 20 STREP (BioMerieux, Marcy l'Etoile, France) in order to identify the isolates at the species level. In addition, antibiotic susceptibility of the bacteria was evaluated in order to characterize the bacteria.

Materials and Methods

Bacteria strains and growth condition

E. faecalis KCTC 3206^T (ATCC 19433^T) and *S. sobrinus* ATCC 33478^T were obtained from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea) and American Type Culture Collection (ATCC, University Boulevand, Manassas, VA, USA), respectively. The clinically isolated strains, *E. faecalis* (ChDC YE1), (ChDC YE2), and (ChDC YE3), *P. agglomerans* (ChDC YP1), *S. sobrinus* (ChDC YS1), and *S. sobrinus* (ChDC YS9), were isolated from dental plaque samples obtained from Koreans. The bacterial strains were allowed to grow in a medium composed of mitis salivarius agar (Difco Laboratories, Detroit, MI, USA) supplemented with 0.0001% potassium tellurite, 0.2 units/ml of bacitracin (Sigma Chemical Co., St. Louis, MO, USA), and 20% (w/v) sucrose (CJ Co., Seoul, Korea) (MSB agar). Bacitracin was always prepared freshly prior to use. The MSB agar plates were stored at 4°C, and used within 7 days after preparation. The bacterial strains were allowed to grow in an incubator which contained air and 5% CO_2 at 37°C for 1-2 days.

16S rDNA cloning and sequencing

16S rDNA cloning and sequencing were performed in an attempt to verify that the non-mutans streptococci were growing on the MSB agar plates. The bacterial genomic DNAs were prepared using a G-spin™ Genomic DNA Extraction kit (iNtRON Co., Seoul, Korea) according to the manufacturer's instructions. In order to amplify the 16S rDNA from the bacteria, we conducted polymerase chain reactions (PCR) with 27F and 1492R primers using an *AccuPower*® PCR PreMix (Bioneer Corp., Daejeon, Korea), which contained 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 PCR conditions were the same as described previously (Lane, 1991). The PCR products were purified with an *Accu*Prep™ PCR purification kit (Bioneer Corp.), and then directly ligated with pGEM-T easy vector (Promega Corp., Madison, WI, USA). The nucleotide sequencing of the 16S rDNA was determined by use of the dideoxy chain termination method, with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The primers used in nucleotide sequencing were as follows: ChDC-GEM-F (5'-TTC CCA GTC ACG ACG TTG TAA AA-3'), Seq-F1 (5'-CCT ACG GGA GGC AGC AG-3'), Seq-R2 (5'-GAC TAC CAG GGT ATC TAA TCC-3'), F16 (5'-TAG ATA CCC YGG TAG TCC-3'), and ChDC-GEM-R (5'-GTG TGG AAT TGT GAG CGG ATA AC-3'). All sequences were compared with similar sequences from the reference organisms, as provided by BLAST (a genome database of the National Center for Biotechnology Information).

Biochemical test

To confirm the result of identification of the three strains by 16S rDNA sequence comparison method, biochemical tests were performed using commercial identification kit, API 20 STREP (BioMerieux, Marcy l'Etoile, France) according to the manufacturer's instruction.

Antibiotic susceptibility test

In order to compare the antibiotics susceptibility of the clinical isolates with that of type strain, the minimum inhibitory concentrations of 9 antibiotics were determined using broth dilution assays (Murray and Jorgensen, 1981), using brain heart infusion broth that contained two-fold serial dilutions of each antibiotics. The following antibiotics were purchased from the Sigma Chemical Co.: penicillin G, amoxicillin, erythromycin, tetracycline, clindamycin, vancomycin, and bacitracin. Augmentin (amoxycillin+cavulanic acid, 5:1) was purchased from the SmithKline Beecham Company (Brentford, UK), ciprofloxacin from the Samchundang Pharmaceutical Co. (Seoul, Korea), and cefuroxim axetil from the Daewoong Pharmaceutical Co. (Seoul, Korea). The susceptibility breakpoint concentrations were determined to be in accordance with the interpretive standards provided by the National Committee for Clinical Laboratory standards (NCCLS) (1997; 1999).

Results and Discussion

Our data indicated that the three clinical isolates grown on the MSB agar were all variants of *E. faecalis*. The 16S rDNA sequences of the three clinical isolates were highly homologous (>98%) with that (GenBank accession number AB154827) of *E. faecalis* EC-12 (Fig. 1). The GenBank accession numbers of 16S rDNA sequences derived from *E. faecalis* ChDC YE1, ChDC YE2, and ChDC YE3 were AY942557, AY942558, and AY942559, respectively. The biological codes of API 20 STREP test for the three strains were 5173711. The three strains were identified as *E. faecalis* by 96.5%. It was reported that enterococci had been encountered on MSB when undiluted samples of saliva or dental plaque were cultured (Gold *et al.*, 1973). However,

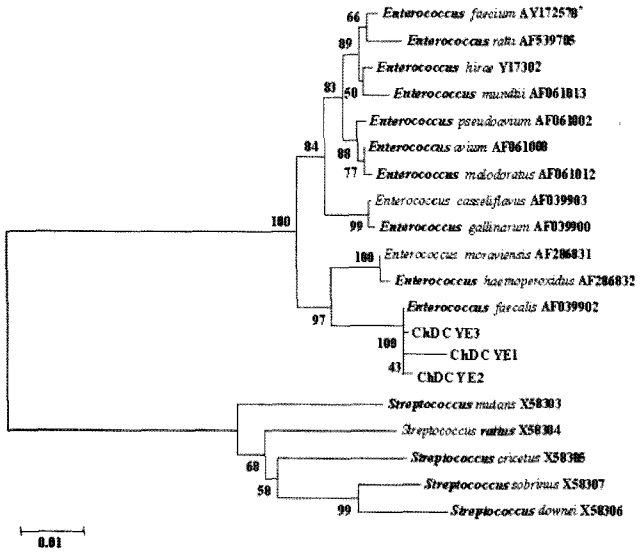

Fig. 1. Dendrogram based on the alignment of the nucleotides of the 16S rDNA of three clinical isolates of *E. faecalis*, 17 representative species of mutans streptococci and enterococci. The tree was inferred from the similarity values obtained using the Clustal X method and Mega2 software (http://www.megasoftware.net.). *The strains studied were designated by their GenBank accession number.

the three *E. faecalis* strains were isolated from plaque samples diluted 100-fold in 1X PBS. To our knowledge, this is the first report on the identification at the species level of enterococci from dental plaques grown on MSB medium.

The colony morphology of *E. faecalis* is similar to that of *S. sobrinus* or *P. agglomerans* on MSB agar, but is not strictly identical (Fig. 2). It was reported that *S. sobrinus* colonies exhibit a creamy, marzipan consistency with a dull granular surface, gray to brown in color, and often with some liquid around the top of the colony (Emilson, 1983). However, in our experience, the colony morphology of the clinical isolates derived from Koreans was variable among the strains (data not shown). Our data also showed that *E. faecalis* colonies exhibit a creamy consistency, but have a smooth surface and are dark blue in color (Fig. 2A & 2B). The colony morphology and color of *E. faecalis* are similar to those of *P. agglomerans*. However, the size of *P. agglomerans* colonies ranges from 1.2 to 1.5 mm after 24 hours of culturing on MSB agar. The growth rate of *P. agglomerans* is faster than that of *E. faecalis*. However, *E. faecalis* can be easily distinguished from *P. agglomerans* by Gram staining. These data indicate that *E. faecalis* can be differentiated from *S. sobrinus* or *P. agglomerans* by biochemical tests and Gram staining.

The MIC of the type strain and clinically isolated strains of *E. faecalis* for the 9 antibiotics are listed in Table 1. All *E.*

Table 1. Minimal inhibitory concentration of several antibiotics for *E. faecalis* growing on an MSB agar plate

Antibiotics	Minimal inhibitory concentration (μg/ml)			
	KCTC 3206	ChDC YE1	ChDC YE2	ChDC YE3
Penicillin G	2	8	4	8
Amoxicillin	1	2	4	2
Augmentin	2	4	2	2
Tetracycline	2	>32	>32	>32
Ciprofloxacin	4	16	16	16
Cefuroxim axetil	32	>64	>64	>64
Erythromycin	0.125	0.5	0.5	1
Clindamycin	4	32	32	32
Vancomycin	4	8	8	8

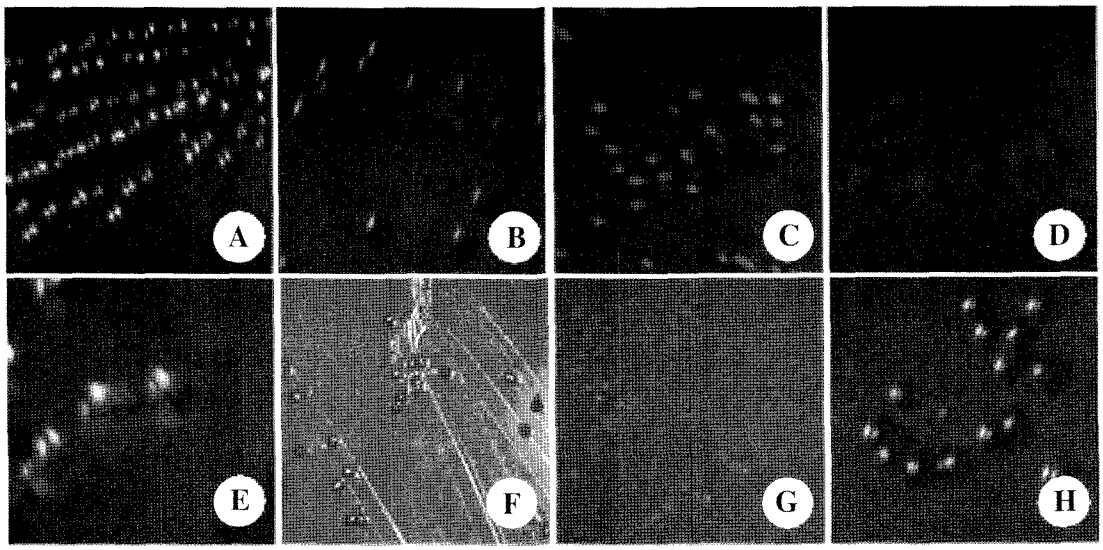

Fig. 2. The colony morphology of (A) *Entercoccus faecalis* KCTC 3206[T], (B) *E. faecalis* ChDC YE 1, (C) *E. faecalis* ChDC YE 2, (D) *E. faecalis* ChDC YE 3, (E) *Pantoea agglomerans* ChDC YP1, (F) *Streptococcus sobrinus* ATCC 33478, (G) *Streptococcus sobrinus* ChDC YS1, and (H) *Streptococcus sobrinus* ChDC YS9 grown on MSB agar plates in an incubator which contained air and 5% CO_2 at 37°C for 1 day (2 days for *S. sobrinus*).

faecalis strains were shown to be susceptible to penicillin G, amoxicillin, augmentin, and vancomycin. The susceptitility for vancomycine was confirmed by PCRs using PCR primers for *vanA* (Dutka-Malen *et al.*, 1995), *vanB* (Dutka-Malen *et al.*, 1995), *vanD* (Perichon *et al.*, 1997), and *vanG* (McKessar *et al.*, 2000) genes. None of the vancomycine-resistance genes was detected by the PCR from all of the three strains of *E. faecalis* (data not shown). Conversely, all strains were also demonstrated to be resistant to ciprofloxacin, cefuroxim axetil, and clindamycin. The type strain, KCTC 3206^T, proved susceptible to tetracycline, but the three clinical isolates were resistant. *E. faecalis* KCTC 3206^T, ChDC YE1, and ChDC YE2 were susceptible to erythromycin, but *E. faecalis* ChDC YE3 exhibited an intermediate level of susceptibility.

E. faecalis, a facultatively anaerobic Gram-positive coccus, is a normal bacterium, and is commensally adapted to the ecologically complex environments associated with the oral cavity and the gastrointestinal and vaginal tracts (Siqueira *et al.*, 2002). The prevalence of *E. faecalis* in 100 dental students has been reported as 1%, and the rate of detection of *E. faecalis* in oral rinse samples of 100 patients receiving endodontic treatment was 11% (Sedgley *et al.*, 2004). It has also been reported that the prevalence of *E. faecalis* in primary root canal infections was 11.5% (4/26) (Siqueira *et al.*, 2002). These reports indicate that *E. faecalis* may be isolated at a higher frequency in patients with persistent infections, including cases in which prior treatment has failed.

In summary, our results demonstrate that *E. faecalis* was able to grow on MSB agar. In view of the fact that *E. faecalis* was recoverable from routine dilutions of dental plaques along with another non-MSO, MSB medium needs to be improved so that only MS should be selected on it.

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