J Microbiol Immunol Infect.
2009;42:188-196

Original Article

Inhibiting effects of *Enterococcus faecium* non-biofilm strain on *Streptococcus mutans* biofilm formation

Masayuki Kumada[1], Mizuho Motegi[2], Ryoma Nakao[2], Hideo Yonezawa[2], Hideki Yamamura[3], Junji Tagami[1,4], Hidenobu Senpuku[2]

[1]Cariology and Operative Dentistry, Department of Restorative Sciences, Graduate School, Tokyo Medical and Dental University, Tokyo; [2]Department of Bacteriology, National Institute of Infectious Diseases, Tokyo; [3]Research & Development Department, Biofermin Pharmaceutical Co, Ltd, Kobe; and [4]Center of Excellence Program for Frontier Research on Molecular, Destruction and Reconstruction of Tooth and Bone, Tokyo Medical and Dental University, Tokyo, Japan

Received: October 22, 2007 Revised: January 18, 2008 Accepted: February 20, 2008

Background and purpose: *Enterococcus faecium* is a normal bowel commensal and lactic acid bacterium that is rarely found in the oral cavity. This study investigated whether a non-pathogenic and non-biofilm strain of *E. faecium* functioned as a probiotic strain toward biofilm formation by *Streptococcus mutans*, which is an etiological agent for dental caries.

Methods: The effects of *E. faecium* on streptococcal biofilm was evaluated by absorbance of safranin-stained biofilm at 492 nm in a 96-well microtiter plate.

Results: The *E. faecium* strain demonstrated cell-number–dependent inhibition of biofilm in dual cultures with 4 laboratory and 16 clinical *S. mutans* strains, as well as laboratory strains of *Streptococcus sobrinus* and *Streptococcus sanguinis*, in 96-well microtiter plates. The inhibiting effects of *E. faecium* were not dependent on the production of bacteriocin from streptococci and *E. faecium*, low pH after mix culture, or biofilm formation levels of *S. mutans*. A culture supernatant sample of more than 10 kDa from *E. faecium* showed direct inhibiting effects toward *S. mutans* biofilm formation. Treatment of heat, butanol, and phenol to a supernatant sample restored biofilm formation in culture of *S. mutans* with the sample. Moreover, the tendencies of inhibition levels by the supernatant sample were associated with those by bacterial cells of *E. faecium* to *S. mutans* strains.

Conclusion: The *E. faecium* non-biofilm strain produced an inhibiting protein to streptococci biofilm formation, showed various susceptibilities to inhibit streptococcal biofilm, and acted as a probiotic bacterial inhibitor of streptococcal biofilm formation.

Key words: Biofilms; Cell communication; Colony count, microbial; *Enterococcus faecium*; *Streptococcus mutans*

Introduction

Oral bacterial communities are known as biofilms, which are characterized by species composition, surface or substratum composition, and the conditioning films that coat the surfaces on which they form [1-3]. The interactions between oral streptococci and other bacteria are potentially beneficial for one or more species present in the biofilm through aggregation [4,5]. *Streptococcus mutans* is the predominant etiologic agent of human dental caries [1], and has been shown to be able to adhere to and form a biofilm on tooth surfaces, as well as to catabolize carbohydrates and generate acids, and survive in low pH and other environmental stress conditions, which are characteristics involved with its cariogenicity [6]. Further, extracellular polysaccharides synthesized by key bacteria such as *S. mutans* play an important role in biofilm formation by facilitating bacterial adherence and cell-cell communication on tooth surfaces [7,8].

Corresponding author: Dr. Hidenobu Senpuku, Department of Bacteriology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan.
E-mail: hsenpuku@nih.go.jp

Enterococci are Gram-positive cocci that form a part of the normal gastrointestinal tract flora in animals and humans [9], and are generally considered to be normal bowel commensals, although they are also recognized as opportunistic pathogens [10]. Enterococci have long been implicated in persistent root canal and dentin infections [11-13], as well as endocarditis and urinary tract infections [12,14]. In addition, enterococci occur in natural foods and are used as probiotics in dairy products [15]. The genus *Enterococcus* consists of at least 23 species, 2 of which, *Enterococcus faecalis* and *Enterococcus faecium*, account for more than 95% of the clinically important isolates. In clinical settings, the use of *E. faecium* and *E. faecalis* during food fermentation and as probiotics demands careful safety evaluation [11,15].

Lactic acid bacteria such as *Enterococcus* do not communicate usually with oral biofilm bacteria, because they are rarely found in the oral cavity. However, they have abilities to act or live in some conditions of root and dentin caries. Enterococci can survive in mono and mixed cultures in the root canal systems [16] and are hard to eliminate once present [17]. A recent report found that colonization of the healthy oral cavity by enterococci was caused by cheese contaminated with enterococci in the oral cavity [18]. Therefore, enterococci may develop an independent microbial community and have unique effects on oral biofilm formation. In this study, the biofilm inhibition of *S. mutans* in mixed cultures with a non-pathogenic and non-biofilm strain of *E. faecium* in vitro was assessed using a 96-well microtiter plate assay.

Methods

Bacterial strains and culture conditions

S. mutans MT8148, MT6229, GS5, and clinical strains, as well as *Streptococcus sobrinus* American Type Culture Collection (ATCC) 33478, AHT, and 6715, *E. faecium* 129 BIO 3B (classified previously as *Streptococcus faecalis*), which was provided by Biofermin Pharmaceutical Co (Kobe, Japan), and JCM8727, and *E. faecalis* JCM8726 were used in this study. The *S. mutans* clinical strains are listed in Table 1, and each

Table 1. Inhibitory effects of *Enterococcus faecium* to streptococcal biofilm formation.

Bacterial strain	Minimal dilution to inhibition effects	Biofilm formation	Bacteriocin production level	Clinical or laboratory strain
Streptococcus mutans				
MT8148	0.2[a]	1.23 ± 0.08[b]	-	L
MT6229	3.1	1.16 ± 0.08	-	L
GS5	0.8	1.72 ± 0.02	-	L
FSC-1	25.0	1.27 ± 0.21	-	C
FSC-2	1.6	1.84 ± 0.08	-	C
FSC-3	12.5	1.99 ± 0.21	-	C
FSC-5	0.1	2.20 ± 0.09	-	C
FSC-6	6.3	1.98 ± 0.06	-	C
FSC-8	6.3	1.82 ± 0.26	-	C
FSC-9	0.4	1.70 ± 0.20	-	C
FSC-10	<0.1	2.48 ± 0.02	-	C
FSC-11	6.3	1.85 ± 0.07	-	C
FSM-2	1.6	2.23 ± 0.09	-	C
FSM-3	25.0	1.46 ± 0.19	+	C
FSM-6	0.1	1.95 ± 0.05	-	C
FSM-7	6.3	2.71 ± 0.12	++	C
FSM-11	<0.1	2.55 ± 0.05	-	C
Streptococcus sobrinus				
ATCC 33478	0.1	2.96 ± 0.23	-	L
AHT	0.1	0.90 ± 0.02	-	L
6715	<0.1	2.78 ± 0.07	-	L
Streptococcus sanguinis				
ATCC 10556	0.4	0.87 ± 0.05	-	L
ST205	0.4	0.78 ± 0.05	-	L

[a]Minimum ratio of cell number of *E. faecium* to streptococci (x1.0) in the inhibition effects on streptococcal biofilm formation.

[b]Biofilm formation level of each *Streptococcus* sp. in a single culture.

Abbreviations: ATCC = American Type Culture Collection; C = clinical; L = laboratory.

revealed different types of genomic DNA and biofilm formation [19]. The streptococci, as well as *E. faecium* and *E. faecalis*, were grown in brain heart infusion BHI medium (Difco Laboratory, Detroit, MI, USA).

Human saliva collection

Whole saliva samples were collected from 5 healthy human participants (age, 28-30 years) after stimulation by chewing paraffin gum, and pooled into ice-chilled sterile bottles for 5 min. The samples were clarified by centrifugation at 10,000 × g for 20 min at 4°C, filter sterilized, and used immediately for biofilm assays, using 96-well microtiter plates and a flow chamber system.

Biofilm formation assay in 96-well microtiter plates

Biofilm formation by each strain was assayed using a method described previously [19]. To evaluate biofilm formation by single cultures of an oral *Streptococcus* sp. and lactobacilli or in a coculture of *S. mutans*, *S. sobrinus*, or *Streptococcus sanguinis* with *E. faecium*, 20 μL (4.0×10^4 colony forming units [CFUs]) of a bacterial cell suspension along with 20 μL (4.0×10^4 CFUs) of the other bacterial cell suspension were mixed in the wells of a 96-well (flat bottom) microtiter plate (Sumitomo Bakelite, Tokyo, Japan), along with 160 μL of tryptic soy broth without dextrose supplemented with 0.25% sucrose (TSBS), after coating with whole saliva for 30 min. *E. faecium* treated with 10% formalin (Wako Pure Chemical Industries, Ltd, Osaka, Japan) was used for comparison between live and non-live cells. The plates were incubated at 37°C for 16 h under anaerobic conditions, then the liquid medium was removed and the wells were rinsed a second time with sterile distilled water (dH_2O). The plates were then air dried and stained with 0.25% safranin for 15 min. After being stained, the plates were rinsed with dH_2O to remove excess dye and then air dried. The biofilm mass was measured without desolvation by a solvent using an enzyme-linked immunosorbent assay microplate reader (Multiskan Bichromatic Laboratory Japan, Tokyo, Japan), as the biofilms were uniformly formed on the bottoms of the wells in the 96-well plates [19]. Quantification of stained biofilm on the bottom was performed by measuring the absorbance at 492 nm. After culturing for 16 h, pH of the supernatant was determined. Photographs of biofilm formation were taken using a digital camera connected to a microscope (Olympus, Tokyo, Japan) with low (× 40) and high (× 400) magnification.

Bacteriocin assay

Loopfuls of stationary-phase cultures of streptococci or *E. faecium* strains were stabbed into BHI agar on a plate, and then incubated at 37°C for 24 h. An indicator strain of *E. faecium* or streptococci was grown to an optical density of 0.3 at 550 nm. Each culture was then diluted to 1:100 and 0.2 mL of the dilution was transferred by pipette into a tube containing 4 mL of molten BHI with 1% agar (Wako). This solution was mixed and poured evenly onto the surfaces of the plates and incubated at 37°C for an additional 24 to 48 h, after which the diameters of the zones of inhibition were measured.

Preparation of culture supernatant

Culture supernatant samples from *E. faecium* 129 BIO 3B incubated in extra BHI medium after dialysis by cellulose membrane (passage molecular weight, 14,000; Viskase Companies, Inc., Darien, IL, USA) overnight were precipitated by salting out using ammonium sulfate. The precipitates were suspended and dialyzed in phosphate-buffered saline (PBS), then further centrifuged at 6000 × g for 20 min at 4°C. The sample of more than 10 kDa was condensed by ultrafiltration using a centrifugal filter device (Amicon Ultra; Millipore, Billerica, MA, USA). Protein concentrations in the sample solution were determined using a protein assay kit (BioRad Laboratories, Hercules, CA, USA) and the samples were also used as a supernatant sample in the biofilm experiment. To confirm whether the inhibiting substance was a protein, the culture supernatant sample was treated with heat (boiled for 5 min), with an equal volume of l-butanol (Sigma Aldrich, St Louis, MO, USA) for 20 min or hot phenol (Wako) for 20 min. The lower aqueous phases were dialyzed using hydrate membrane (Slide-A-Lyzer® Dialysis Cassettes, 10 kDa cutoff; Pierce, Rockford, IL, USA) in PBS and used for the biofilm inhibition assay.

Native polyacrylamide gel electrophoresis

Prior to electrophoretic analysis, the culture supernatant samples were diluted with an equal volume of native polyacrylamide gel electrophoresis (PAGE) sample buffer (0.06 M trisaminomethane hydrochloride [Tris-HCl], pH 6.8; Amersham Pharmacia Biotech, Uppsala, Sweden) with 20% glycerol (Wako) and 0.0012% bromophenol blue (Wako). The samples were subjected to 10% polyacrylamide gel (PAGEL-mini, ATTO Corp, Tokyo, Japan) in the presence of

25 mM of Tris-HCl and 192 mM of glycine (Wako). Electrophoretic separation of the proteins was carried out in the native polyacrylamide gels for 80 min at 40 mA. The Precision Plus Protein Standard molecular weight marker (BioRad) was used as a protein size marker for native PAGE. When electrophoresis was completed, proteins in the gels were stained with 0.1% (w/v) Coomassie Brilliant Blue.

Application of inhibiting substance for biofilm formation assay

A sonic extract, culture supernatant sample has been identified as able to inhibit *S. mutans* biofilm formation. To evaluate the effects of samples from *E. faecium*, 40 μL (8.0×10^4 CFUs) of cell suspension of *S. mutans* was mixed and simultaneously cultivated in a 160-μL mixture containing various concentrations of samples in the wells of a 96-well (flat bottom) microtiter plate (Sumitomo Bakelite), after coating with saliva for 30 min. To evaluate the direct effects of the culture supernatant sample, an *S. mutans* cell suspension

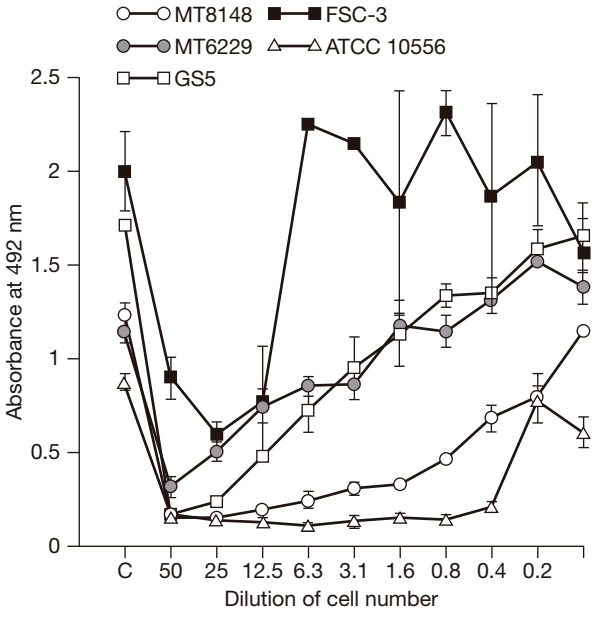

Fig. 1. Inhibition of streptococcal biofilm by *Enterococcus faecium*. Cocultures were performed with various cell number ratios of *E. faecium* to various streptococci strains (range, 0.1:1 to 50:1). Formed biofilms were stained with safranin and absorbance measured at 492 nm. Streptococci strains included *Streptococcus mutans* MT8148, MT6229, GS5, and FSC-3, and *Streptococcus sanguinis* American Type Culture Collection 10556. Data are representative of 3 independent experiments with similar results. Results are expressed as mean ± standard deviation of triplicate assays. Abbreviation: C = control.

(20 μL, 8×10^4 CFUs) was preincubated with 3 mg/mL (20 μL, 6 mg/mL in PBS) of culture super-natant sample for 1 h at 37°C and cultivated in 160 μL TSBS (final concentration, 600 μg/mL; culture supernatant sample). To clarify the dominance effects of the culture supernatant sample toward biofilm inhibition, 20 μL of culture supernatant sample 6 mg/mL was initially incubated for 1 h at 37°C in the wells of a 96-well microtiter plate prior to the cell culture, after which cell suspensions of *S. mutans* were added and cultivated in 160 μL TSBS (final concentration, 600 μg/mL; culture supernatant sample). In another experiment, before adding the culture supernatant sample, *S. mutans* was initially incubated for 1 h at 37°C and, after adding the sample, the mixture was cultivated for 16 h at 37°C. Thereafter, the biofilm was evaluated by the methods previously described [19].

Statistical analysis

Comparisons of biofilm formation levels among the various cultures of single and dual species in 96-well microtiter plates were performed using Mann-Whitney *U* test. Differences at the 0.05 level or less were considered to be statistically significant.

Results

Effects of *Enterococcus faecium* on *Streptococcus mutans* biofilm in 96-well microtiter plate

Biofilm formations were not observed in monocultures of all tested *Enterococcus* strains and they inhibited *S. mutans* biofilm formation. *E. faecium* 129 BIO 3B did not produce a variety of toxins, such as bacteriocin and hemolysin, and functioned as a lactic acid and non-pathogenic bacteria. *E. faecium* 129 BIO 3B did not demonstrate antibiotic resistance and was proposed as a beneficial probiotic strain for intestinal flora conditions [20]. *E. faecium* 129 BIO 3B was used as a typical non-pathogenic and non-biofilm *Enterococcus* strain for further experiments.

Biofilms produced by *S. mutans*, *S. sobrinus*, and *S. sanguinis* were inhibited by *E. faecium* in cocultures in a cell-number–dependent manner, with various inhibition activities (Fig. 1, Table 1). A 0.1:1 cell ratio of *E. faecium* to *S. mutans* FSC-5, FSM-6, *S. sobrinus* ATCC 33478, or AHT caused significant inhibition of absorbance at 492 nm in comparison with a single culture of the streptococcal strain, although that relative ratio was the minimum to inhibit absorbance. A 0.4 or 0.8 cell ratio of *E. faecium* to *S. mutans*

GS-5, FSC-9, *S. sanguinis* ATCC 10556, or ST205 also caused significant inhibition. In contrast, a 25-fold greater number of *E. faecium* cells to *S. mutans* were necessary to cause significant inhibition of absorbance at 492 nm in cocultures with *S. mutans* FSC-1 and FSM-3. Bacteriocin activities by streptococci to *E. faecium* were only observed in *S. mutans* FSM-3 and FSM-7, while none were seen in the other streptococci (Table 1). Bacteriocin activities by *E. faecium* were not observed with all tested streptococci (data not shown). Taken together, the biofilm inhibition activities were not dependent on the bacteriocin production from

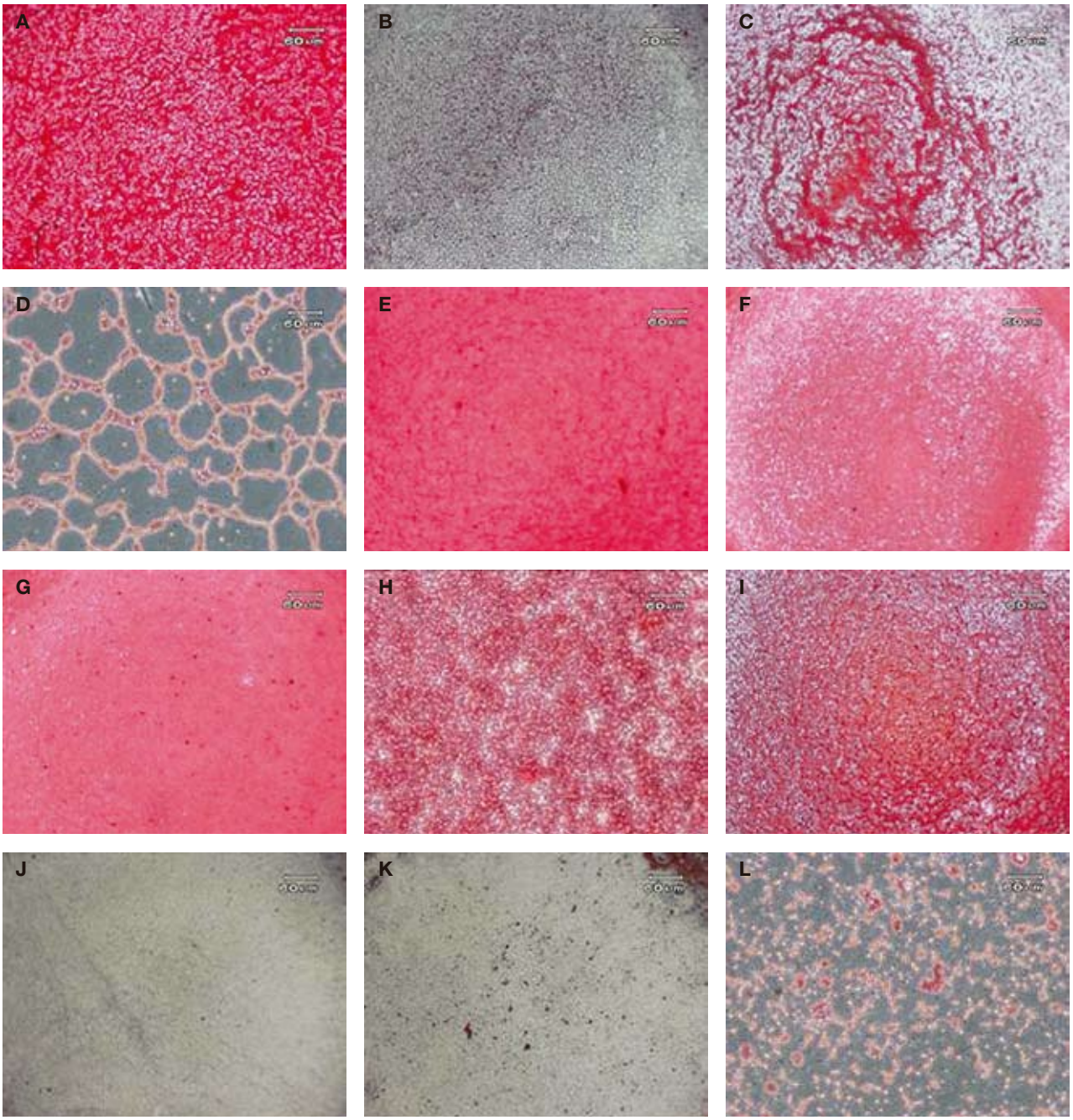

Fig. 2. Biofilm formation by (A-D) *Streptococcus mutans* MT8148, (E-H) *Streptococcus mutans* GS5, and (I-L) *Streptococcus sanguinis*. Photographs were taken at low (× 40: A-C, E-G, I-K) or high (× 400; D, H, L) magnification. Biofilm formation in a single culture is shown (A, E, I). The cell number ratio of *Enterococcus faecium* to streptococci was 3.2:1 (B, F, J) or 0.8:1 (C, D, G, H, K, L). Biofilm formations were taken from the results shown in Fig. 1. Photographs are representative of independent experiments with similar results.

E. faecium or streptococci or with the level of biofilm formation by the streptococci. Biofilm inhibition was confirmed in photographs (Fig. 2), and irregular biofilms with increased numbers of voids and hollows were observed in cocultures of *E. faecium* and *S. mutans* MT8148 or *S. sanguinis* ATCC 10556 at ratios of 0.8:1, as compared with the biofilms of monocultured *S. mutans* and cocultured *S. mutans* GS5 and *E. faecium* in high magnification photographs (Fig. 2D and Fig. 2L). The inhibiting effects of *E. faecium* were lost by treatment of 10% formalin (data not shown). The final pH levels after incubation in all experiments ranged from 4.8 to 5.2.

Inhibiting effects of culture supernatant

The culture supernatant sample of more than 10 kDa also suppressed biofilm formation by *S. mutans* GS5 in the simultaneous culture (Fig. 3), and the suppression was observed in a dose-dependent manner (data not shown). Addition of 600 μg/mL of the sample caused the inhibition of *S. mutans* by 52%. Treatment by heat, butanol, and phenol to the supernatant samples restored biofilm formation in the culture of *S. mutans* with the sample (Fig. 3). Therefore, it is considered that the more than 10-kDa protein is an inhibiting substance in the culture supernatant sample. The inhibitions were also observed in pretreatment culture of *S. mutans* GS5, MT8148, and FSC-2 but not in FSM-7, FSC-11, and FSM-3 with the supernatant sample (Fig. 4). The inhibition cell ratios of *E. faecium* to *S. mutans* GS5, MT8148, and FSC-2 were 0.8, 0.2, and 1.6, respectively, but those to FSM-7, FSC-11, and FSM-3 were 6.3, 6.3, and 25.0, respectively (Table 1). The tendencies of inhibition levels by the supernatant sample were associated with those by bacterial cells of *E. faecium*. Initial culture of the culture supernatant sample or *S. mutans* did not show significant inhibition (data not shown).

Discussion

Antimicrobial and bacteriocin-like substances have roles in the interactions of *S. mutans* with other bacteria during biofilm formation, and may provide a selective advantage for initial or sustained colonization in an environment of diversely packed and competing organisms [21,22]. In addition, lactic acid bacteria may control the microbial environment during the development of dental caries [6,23-25]. In this study, the inhibiting effects of the *E. faecium* strain toward biofilm development

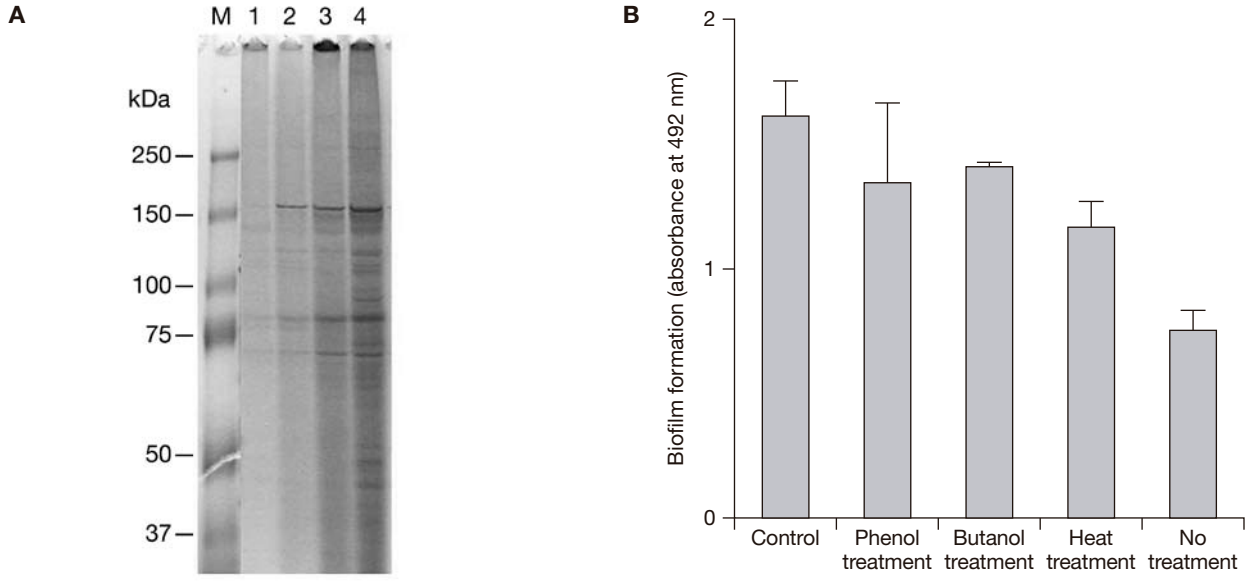

Fig. 3. (A) Native polyacrylamide gel electrophoresis of the culture supernatant from *Enterococcus faecium* 129 BIO 3B stained with Coomassie Brilliant Blue and (B) inhibiting effects of culture supernatant samples treated with phenol, butanol, and heat on biofilm formation by *Streptococcus mutans*. Each sample was simultaneously added to a cell suspension of *S. mutans* GS5 and cultured in TSBS for 16 h. Data are representative of 3 independent assays with similar results. Results are expressed as mean ± standard deviation of the triplicate assays.

Lane 1, phenol-treated sample; lane 2, butanol-treated sample; lane 3, heat-treated sample; lane 4, non-treated culture supernatant sample prepared by ammonium sulfate and super filtration.

Abbreviations: M = molecular weight marker; TSBS = tryptic soy broth without dextrose supplemented with 0.25% sucrose.

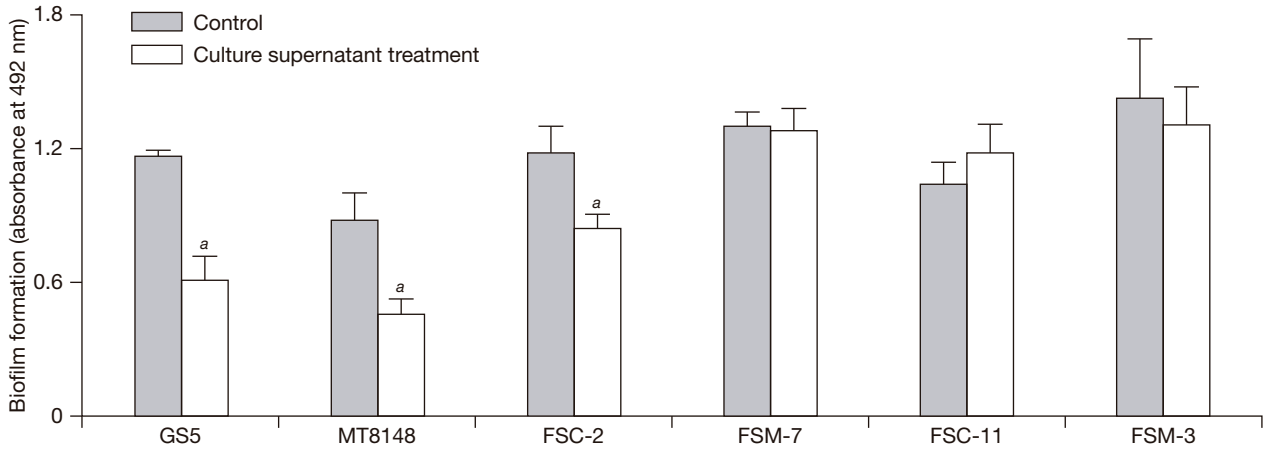

Fig. 4. Inhibiting effects of culture supernatant samples on biofilm formation by various *Streptococcus mutans* strains. Supernatant samples were simultaneously added to cell suspensions of *S. mutans* strains GS5, MT8148, FSC-2, FSM-7, FSC-11, or FSM-3 and precultured for 1 h. TSBS was added and cells cultured for 16 h. Data are representative of 3 independent assays with similar results. Results are expressed as mean ± standard deviation of the triplicate assays.
[a]$p < 0.01$ versus non-treated control.
Abbreviation: TSBS = tryptic soy broth without dextrose supplemented with 0.25% sucrose.

by *S. mutans* strains were observed at various ratios in 96-well microtiter plates and were not dependent on the production of bacteriocin, low pH, or biofilm formation levels. The effects were also observed in dual-species culture with *S. sobrinus* and *S. sanguinis.* Therefore, biofilm development is regulated by multiple cell-to-cell interactions between biofilm bacteria and others, as there are various susceptibilities of streptococci to inhibition by non-biofilm bacteria such as the tested strain of *E. faecium*.

The present strains of *E. faecium* did not coaggregate with *S. mutans* and form a biofilm in 96-well microtiter plates, thus other diverse mechanisms are likely to be involved in the mechanism of inhibition. It is possible that *E. faecium* produces unknown or known inhibiting factors related to the colonization and biofilm formation of streptococci, as well as binding to saliva-coated surfaces. In this study, a heat-, butanol-, and phenol-unstable substance able to inhibit biofilm formation by *S. mutans* was found, which was considered to be a protein and not a polysaccharide or lipoteichoic acid. The *E. faecium* strain produced a small amount of the inhibiting protein outside of culturing with *S. mutans*, because non-concentrated culture supernatant sample from *E. faecium* could not inhibit biofilm formation of *S. mutans*. However, formalin-treated *E. faecium* did not show such inhibiting activities. The protein acted as an inhibiting factor to precolonized *S. mutans* in the early stage of *E. faecium* growth and did not have an effect on attached or colonized *S. mutans*. The inhibition effects of *E. faecium* may require streptococcal strain-dependent bacterial density, but not binding of the products to a salivary receptor on the hard surface during the development of biofilm. Previous studies have reported that some strains of *E. faecium* produce cytolysin, which has hemolytic (lysing a broad spectrum of cells, including human, horse, and rabbit erythrocytes) and bactericidal activities against Gram-positive bacteria [26,27]. However, the present strain did not produce cytolysin and showed inhibiting effects to streptococcal biofilm formation. Thus, it is considered that the inhibiting protein reacts directly with biofilm bacteria in the early growth phase of *E. faecium* to cause various susceptibilities of cell-to-cell communication leading to a poor-quality biofilm.

The primary habitat of *Enterococcus* organisms is the large intestine [24,28], although low numbers have been recovered from the oral cavity, mainly from the dental plaque of individuals with underlying diseases in whom opportunistic transmission may have occurred [29-31]. When *E. faecium* is increased in the oral cavity in certain environmental conditions, as well as because of an underlying disease and intake of diet contaminated with *E. faecium*, it might be more infective in a competitive state with streptococci. Then, the inhibiting protein may play an important role in the interaction of *E. faecalis* with streptococcal biofilm formation. In general, the biofilm matrix is composed of diverse extracellular polymeric substances, including exopolysaccharide,

proteins, and DNA, while it may also contain other non-cellular materials, depending on the environment in which the biofilm developed [5]. In particular, DNA has been identified as a key structural component of the biofilm extracellular matrix [32] and, more recently, biofilm formation was shown to involve a functional DNA-binding uptake system [33]. DNA-binding protein is also involved in biofilm formation by *Pneumococcus* app. [34]. These findings were confirmed by another study that found a significant inhibitory effect on biofilm formation when DNase I or protease was added, either before or after beginning the culture [35]. In this study, the culture supernatant sample did not imitate these result for the activity for inhibition, because the addition of *S. mutans* after a 1-h culture did not inhibit biofilm formation. Therefore, it is considered that the inhibitory phenotype of the culture supernatant sample is different from that of DNase and protease.

In additional experiments, a sonic extract sample was prepared from the whole cell of this strain of *E. faecium* and the sonic extract sample significantly suppressed biofilm formation by *S. mutans* MT8148 [36]. Purification of the inhibiting protein in the sonic extract sample and culture supernatant samples was performed by gel filtration and ion exchange chromatography. The inhibiting protein indicates a high molecular weight complex, and was not purified completely by various purification procedures in both samples. It is possible that the protein may function as a high molecular weight complex and play an important role in the unique inhibition of *S. mutans* biofilm formation.

Additional investigations are required to clarify which molecules in the inhibiting proteins participate in the inhibition of colonization in a physical or chemical manner during streptococcal biofilm formation, and whether the inhibiting proteins from other non-biofilm bacteria can also inhibit biofilm formation. Nevertheless, it seems feasible to employ such agents as an *E. faecium* strain as local therapy for oral infection in the future. The present results may also provide new information with regard to bacterial communication and diversity, as well as useful data for prevention of biofilm development in the oral environment.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (15390571 and 19659559) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a grant from the Ministry of Health, Labor and Welfare (H16-Medical Services-014 and H19-Medical Services-007).

References

1. Hamada S, Slade HD. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol Rev. 1980; 44:331-84.
2. McEldowney S, Fletcher M. Adhesion of bacteria from mixed cell suspension to solid surfaces. Arch Microbiol. 1987;148:57-62.
3. Paster B, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, et al. Bacterial diversity in human subgingival plaque. J Bacteriol. 2001;183:3770-83.
4. Kolenbrander PE, Andersen RN, Blehert DS, Egland PG, Foster JS, Palmer RJ Jr. Communication among oral bacteria. Microbiol Mol Biol Rev. 2002;66:486-505.
5. Palmer RJ Jr, Gordon SM, Cisar JO, Kolenbrander PE. Coaggregation-mediated interactions of streptococci and *Actinomyces* detected in initial human dental plaque development. J Bacteriol. 2003;185:3400-9.
6. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev. 1986;50:353-80.
7. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999;248: 1318-22.
8. Mukasa H, Slade HD. Mechanism of adherence of *Streptococcus mutans* to smooth surfaces. I. Roles of insoluble dextran-levan synthetase enzymes and cell wall polysaccharide antigen in plaque formation. Infect Immunol. 1973; 8:555-62.
9. Baker CN, Stocker SA, Culver DH, Thursberry C. Comparison of the E test to agar dilution, broth dilution, and agar diffusion susceptibility testing techniques by using a special challenge set of bacteria. J Clin Microbiol. 1991;29:533-8.
10. Murray BE, Weinstock GM. Enterococci: new aspects of an old organism. Proc Assoc Am Physicians. 1999;111:328-34.
11. Franz CM, Muscholl-Silberhorn AB, Yousif NM, Vancanneyt M, Swings J, Holzaphel WH. Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. Appl Environ Microbiol. 2001;67:4385-9.
12. Sundqvist G. Associations between microbial species in dental root canal infections. Oral Microbiol Immunol. 1992;7:257-62.
13. Nakajo K, Nakayama F, Iwaku M, Hoshino E. Alkali-resistant bacteria in root canal system. Oral Microbiol Immunol. 2004;19:390-4.
14. Megran DW. Enterococcal endocarditis. Clin Infect Dis. 1992;15:63-71.
15. Eaton TJ, Gasson MJ. Molecular screening of *Enterococcus*

virulence determinants and potential for genetic exchange between food and medical isolates. Appl Environ Microbiol. 2001;67:1628-35.

16. Fabricius L, Dahlén G, Holm SE, Möller AJ. Influence of combinations of oral bacteria on periapical tissues of monkeys. Scand J Dental Res. 1982;90:200-6.
17. Engström B. The significance of enterococci on root canal treatment. Odontol Revy. 1964;15:87-106.
18. Razav A, Gmur R, Imfeld T, Zehnder M. Recovery of *Enterococcus faecalis* from cheese in the oral cavity of healthy subjects. Oral Microbiol Immunol. 2007;22:248-51.
19. Motegi M, Takagi Y, Yonezawa H, Hanada N, Terajima J, Watanabe H, et al. Assessment of genes associated with *Streptococcus mutans* biofilm morphology. Appl Environ Microbiol. 2006;72:6277-87.
20. Ishara M, Homma M, Kuno E, Watanabe M, Konda Y. Combination use of kampo-medicine and drug affecting intestinal bacterial flora. Yakugaku Zasshi. 2002;122:695-701. [Article in Japanese.]
21. Rogers AH, van der Hoeven JS, Mikx FH. Effect of bacteriocin production by *Streptococcus mutans* on the plaque of gnotobiotic rats. Infect Immunol. 1979;23:571-6.
22. van der Hoeven JS, Rogers AH. Stability of the resident microflora and the bacteriocinogeny of *Streptococcus mutans* as factors affecting its establishment in specific pathogen-free rats. Infect Immunol. 1979;23:206-13.
23. Hahn CL, Falker WA Jr, Minah GE. Microbiological studies of carious dentine from human teeth with irreversible pulpitis. Arch Oral Biol. 1991;36:147-53.
24. Mattar AF, Drongowski RA, Coran AG, Harmon CM. Effect of probiotics on enterocyte bacterial translocation in vitro. Pediatr Surg Int. 2001;17:265-8.
25. Quivey RG Jr, Kuhnert WL, Hahn K. Adaptation of oral streptococci to low pH. Adv Microb Physiol. 2000;42:239-74.
26. Ike Y, Hashimoto H, Clewell DB. Hemolysin of *Streptococcus faecalis* subspecies Zymogenes contributes to virulence in mice. Infect Immunol. 1984;45:528-30.
27. Jett BD, Jensen HG, Nordguist RE, Gilmore MS. Contribution of the pAD1-encoded cytolysin to the severity of experimental *Enterococcus faecalis* endophthalmitis. Infect Immunol. 1992;60:2445-52.
28. Chenoweth C, Schaberg D. The epidemiology of enterococci. Eur J Clin Microbiol Infect Dis. 1990;8:80-9.
29. Campbell J, McGowan DA, Macfarlane TW. The prevalence of enterococci in the dental plaque of chronic hospital patients. Br J Oral Surg. 1983;21:171-4.
30. Kurrle E, Bhaduri S, Krieger D, Gaus W, Heimpel H, Pflieger H, et al. Risk factors for infections of the oropharynx and the respiratory tract in patients with acute leukemia. J Infect Dis. 1981;144:128-36.
31. Smyth CJ, Halpenny MK, Ballagh SJ. Carriage rates of enterococci in the dental plaque of haemodialysis patients in Dublin. Br J Oral Maxillofac Surg. 1987;25:21-33.
32. Moscoso M, Garcia E, Lopes R. Biofilm formation by *Streptococcus pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. J Bacteriol. 2006;188:7785-95.
33. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. Extracellular DNA required for bacterial biofilm formation. Science. 2002;295:1487.
34. Petersen FC, Tao L, Scheie AA. DNA binding-uptake system: a link between cell-to-cell communication and biofilm formation. J Bacteriol. 2005;187:4394-400.
35. Peterson FC, Pecharki D, Scheie AA. Biofilm mode of growth of *Streptococcus intermedius* favored by a competence-stimulating signaling peptide. J Bacteriol. 2004;186:6327-31.
36. Kumada M, Senpuku H, Motegi M, Nakao R, Yonezawa H, Yamamura H, et al. Effects of *Enterococcus faecium* on *Streptococcus mutans* biofilm formation using flow cell system. J Oral Biosci. 2008;50:68-76.