減少しない．磨き残しがあると，たちまちのうちにミュータンスレンサ球菌量が元に戻ってしまう．通常の口腔ケアに加え歯科衛生士による専門的な口腔ケアを定期的に取り入れたり，クロルヘキシジンなどの薬剤を使用したりすると，ミュータンスレンサ球菌をよりよく減少させることができる[3, 4]．

3 歯周病発症に関わる微生物対する口腔ケアの効果

歯周病は歯周組織に起こる炎症が起点となり進行していく，と考えられている．その炎症を誘発しているのが口腔微生物といわれ，主にグラム陰性嫌気性桿菌（*Porphylomonas gingivalis, Aggregatibacter actinomycetemcomitans, Tannerella forsythesis* など）を含めた細菌群が関与していると考えられている．このような細菌群の増殖するきっかけとなるのが，歯肉縁下に形成される歯垢である．縁下歯垢は，好気性菌であるレンサ球菌に加え嫌気性桿菌など多くの種類の菌により構成されている（**表2**）．

磨き残しにより歯垢が歯表面に残り，時間の経過とともに歯垢内の微生物の一部は仮死化あるいは死化するようになる．唾液によりリン酸カルシウムの沈着後，石灰化が起こり歯石が形成される（その詳細なメカニズムは明らかになっていない）．その結果，歯肉溝内の嫌気度が高まり嫌気性菌の増殖しやすい環境になる．歯肉炎により，骨吸収，歯周組織の破壊が起こると歯周ポケットが形成され，さらに嫌気性菌の住みやすい環境となる．歯ブラシによる通常の口腔ケアのみでは，このような環境を改善していくのが難しくなってくる．よって歯科衛生士や歯科医師による歯石を除去するスケーリングを含めた口腔ケアが必要になってくる．これに加え，歯周ポケット内でバイオフィルムを形成し蓄積している細菌群を除去することも必要である．

歯周病を予防するためには，発症する前に歯肉縁下歯垢や歯石の除去を念頭において，定期的に口腔ケアを行うことが重要である．

表2　歯肉縁下歯垢から検出される細菌

病原性細菌
グラム陽性好気性球菌
Streptococcus oralis, Streptococcus sanguinis, Streptococcus mitis, Streptococcus gordonii, Streptococcus mutans, Streptococcus anginosus, Streptococcus intermedius, Rothia dentocariosa
グラム陽性嫌気性球菌
Peptostreptococcus micros
グラム陰性嫌気性球菌
Gemella morbillorum
グラム陽性嫌気性桿菌
Actinomyces naeslundii, Actinomyces odontplyticus, Eubacterium nodatum
グラム陰性嫌気性桿菌
Capnocytophaga ochracea, Capnocytophaga gingivalis, Capnocytophaga gracilis, Fusobacterium nucleatum

「口腔微生物学・免疫学　第2版」浜田茂幸著　医歯薬出版より改変

4 口腔日和見菌に対する口腔ケアの効果

口腔内の日和見菌は高齢者になると検出される割合が高まってくる．特に要介護高齢者になるとその検出率は上昇してくる（**図4**）[5]．誤嚥性肺炎に関わるような微生物はこれらの日和見菌にも含まれており，口腔ケアによる日和見菌の制御が誤嚥性肺炎の予防につながると考えられている．口腔ケアにより日和見菌を減少させることは，齲蝕，歯周病関連菌を減少させることよりも容易ではない．それは日和見菌が口腔内に検出される理由として，菌に対する抵抗力の低下が原因と考えられるからである．口腔常在菌は日和見菌が口腔内において増殖するのを抑えることに働くが，それに加え全身および局所の免疫応答も，日和見菌の口腔内感染および増殖抑制に関与してくる．しかし，その詳細なメカニズムは明らかになっていない．

米山らは，2年間の歯科衛生士および歯科医師による専門的な口腔ケアを行うことにより，発熱や死亡する割合が専門的な口腔ケアを行わない場合より

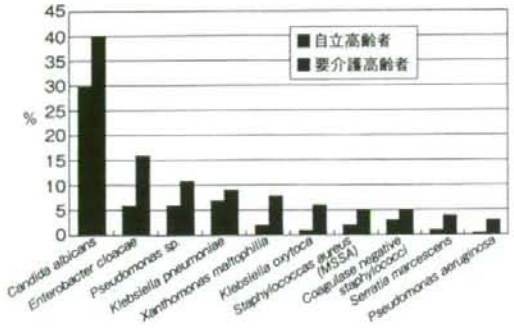

図4　歯垢における日和見菌検出率（自立高齢者と要介護高齢者の比較）.

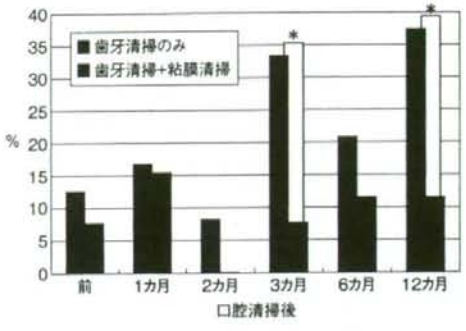

図5　Candida が検出された要介護高齢者の割合.

も減少することを報告した[6]．その報告では，開始してから数カ月では有意な効果が現れていないことがわかる．われわれの研究結果でも，口腔ケアの日和見菌に対する効果が現れてくるのは口腔ケアを始めて3カ月を過ぎてからであった（図5）．しかも，日和見菌は減少することがなかった．この研究で，歯牙表層の清掃に加えさらに粘膜の清掃を加えた場合に，日和見菌の増加を抑える効果が現れていた．もし主な口腔ケアの効果が物理的な微生物の除去であるならば，開始してから1カ月，2カ月でその減少効果が現れてもおかしくない．しかし，現実ではそうなっていない．

口腔ケアの日和見菌への効果は長期的に行うことによる日和見菌数の維持であり，減少効果ではない，と考えられる．微生物の物理的除去以外の効果が主に影響して，結果的に日和見菌の増加を抑制しているのではないかと推察する．

5 口腔ケアの自然免疫応答に対する効果

日和見菌が菌に対する抵抗力の減少により検出されることを考えれば，口腔ケアが免疫力を上昇させて，その結果として時間経過とともに間接的に日和見菌感染を制御したのではないか，という仮説が成り立つ．そこで，要介護高齢者に歯科衛生士による専門的な口腔ケアを行い，専門的な口腔ケアを行う前と後で血中の活性化ナチュラルキラー（NK）細胞の割合（自然免疫応答の指標）の変動を検討した．その結果，通常の口腔ケアでは活性化NK細胞は変動しないにもかかわらず，専門的な口腔ケアを行うと活性化NK細胞を有意に上昇させることが明らかとなった（図6）．専門的な口腔ケアを止めると3カ月で元の状態にもどることも明らかとなった．専門的な口腔ケアによる何らかの刺激により，特に自然免疫に関わる活性化NK細胞を増加させたのかもしれない．

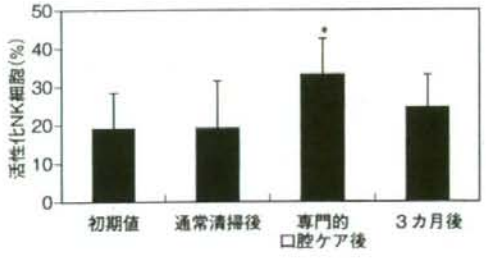

図6　要介護高齢者における口腔清掃後の血中活性化NK細胞の変化.
＊：初期値との比較，$p<0.05$で有意差があり.

東北大学の佐々木教授は，口腔ケアによる刺激が唾液中神経伝達物質であるサブスタンスPの産生を上昇させる効果があることを報告した[7]．口腔は脳に近く，三叉神経，迷走神経，顔面神経，舌咽神経など多くの運動および知覚神経による制御下にあ

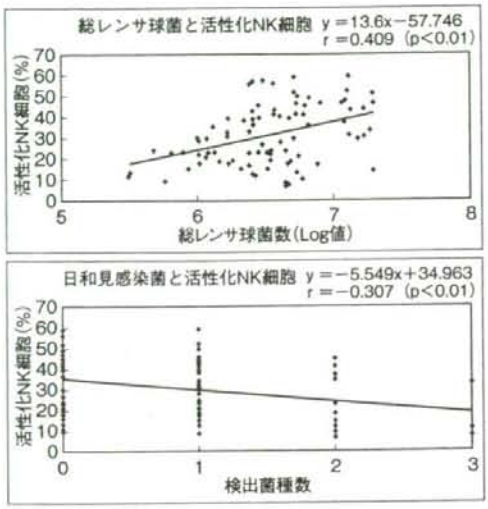

図7　口腔内微生物と活性化NK細胞との関係.

る．定期的な口腔ケアが免疫力の低下した高齢者において刺激となり，免疫力を上昇させるのかもしれない．

血中の活性化NK細胞が上昇すると口腔内微生物に対してどのような影響を及ぼすのか，高齢者を対象に検討を行った．その結果，活性化NK細胞の割合と唾液中レンサ球菌の量が正の相関を示し，日和見菌の検出菌数と負の相関を示すことが明らかとなった(図7)．これらのことは，自然免疫応答とレンサ球菌のような口腔常在菌との間に何らかの関係があり，それが日和見菌感染を制御する効果として働いていることが考えられた．免疫力が低下した患者に対して，口腔のみならず全身の健康を維持するために口腔ケアは有用な手段になりうるかもしれない．

6 口腔ケアと獲得免疫応答との関係

ヒトは微生物が感染すると，獲得免疫応答が起こり微生物に作用する抗体が分泌され，その抗体が微生物を駆逐するために働く．口腔に感染した常在菌に対しても粘膜免疫応答が起こり，唾液中に常在菌

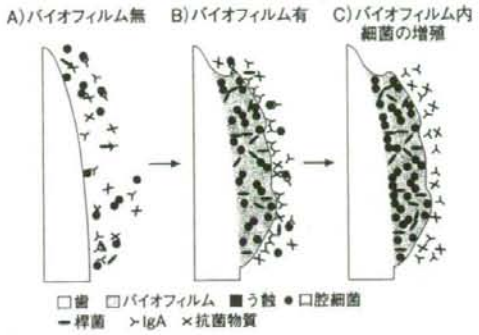

図8　細菌は唾液中の抗体および抗菌物質からバイオフィルムにより守られる.
(泉福英信，花田信弘：う蝕の原因と対策．感染・炎症・免疫．30：2-10，1999．より改変)

に対するIgA抗体が分泌され，常在菌に反応し口腔内微生物量の調節に関与している，と考えられる．しかし，バイオフィルムが形成されるとその抗体がバイオフィルム内部に行きわたらなくなり，細菌はそのバイオフィルムにより抗体の攻撃から守られることになる（図8）．そこで，バイオフィルムを剝がしてしまえば，抗体が口腔細菌によく反応できる環境になり，抗体の効果がよく現れてくるのではないか，という仮説をたて検討を行った．

S. mutans の表層蛋白質の中で歯表面の唾液ペリクル成分に結合する領域を明らかにし，その領域が含まれるペプチドを合成した．唾液中にこの合成ペプチドに反応するsIgA抗体を有する要介護高齢者と有しない要介護高齢者のグループに分け，それぞれ専門的口腔ケアによるミュータンスレンサ球菌への効果について検討を行った．その結果，抗体を有するグループは，専門的口腔ケアを始めて1カ月目から唾液におけるミュータンスレンサ球菌量が有意に減少したが，抗体をもっていないグループは減少する時期が遅くなり，6カ月目にミュータンスレンサ球菌が減少する結果となった．すなわち唾液中にその抗体を有するヒトは，専門的口腔ケアにより抗体のないグループよりも早くミュータンスレンサ球菌を減少させることが明らかとなった（図9）．こ

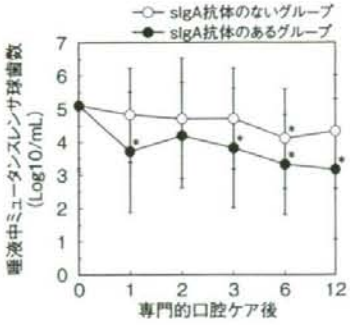

図9　唾液 IgA 抗体と口腔ケアの効果との関係.
＊：vs 0 month, $p < 0.05$

図10　口腔ケアの効果のまとめ.

れはバイオフィルムのないほうが，口腔内に誘導された抗体が細菌に対して反応しやすくなり，その結果，口腔ケアに加えその抗体の効果が強く現れてくることを示している.

7 口腔ケアの効果のまとめ

口腔ケアの効果は，微生物の物理的除去に加え，全身の自然免疫への刺激効果，唾液中に分泌された特異抗体の効果促進により，口腔常在菌および日和見菌に影響を与え，健康的な細菌叢へと導いていると考えられる（**図10**）．これは，口腔ケアによりバイオフィルムや菌が除去されると，口腔各所に残って浮遊している常在菌が口腔表面に再付着し，元の菌量に戻る際に自然免疫および獲得免疫にも何らかの作用を及ぼしている可能性が考えられる．この結果，病原性をもたない常在菌が増えることによって，日和見菌のような病原性をもつ菌のいる場所がなくなり，健康的な細菌叢が形成されていくことになる．よって，口腔ケアの重要な効果は，病原性をもたない常在菌を増やすこと，およびその維持にあると考えられる.

口腔ケアは，先に挙げた効果以外にも，口臭予防，コミュニケーションの一環，爽快感など，口腔疾患の直接的な予防以外にも多様に働く．口腔ケアは比較的容易に行える手段であり，多様な効果の側面をもつことからも，今後の歯科医療の重要な役割を演じる手段の一つになると考えられる.

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ORIGINAL

Effects of *Enterococcus faecium* on *Streptococcus mutans* Biofilm Formation Using Flow Cell System

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

[1] *Cariology and Operative Dentistry, Department of Restorative Sciences, Graduate School, Tokyo Medical and Dental University*

[2] *Department of Bacteriology, National Institute of Infectious Diseases*

[3] *Research & Development Department, Biofermin Pharmaceutical Co., Ltd.*

[4] *Center of Excellence Program for Frontier Research on Molecular, Destruction and Reconstruction of Tooth and Bone, Tokyo Medical and Dental University*

[1] *1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8549, Japan*

[2] *1-23-1, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan*

[3] *7-3-4, Ibukidai-Higashimachi, Nishi-ku, Kobe 651-2242, Japan*

[4] *1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8549, Japan*

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Key words : *Streptococcus mutans* / *Enterococcus faecium* / *Lactobacillus casei* / biofilm / flow cell system

Abstract : *Enterococcus faecium*, a lactic acid bacterium, is a normal bowel commensal inhabitant that is rarely found in the oral cavity. We investigated whether *E. faecium* functions as a probiotic strain inhibiting biofilm formation by *Streptococcus mutans*, which is an etiological agent for dental caries, using a flow cell system. Cell suspensions were cultured in flow cell systems coated with salivary components in tryptic soy broth including 0.25% sucrose without dextrose. The resultant biofilm formation was stained using a LIVE/DEAD® *Bac*Light™ Viability Kit, and examined using confocal laser scanning microscopy. *E. faecium* showed cell density-dependent inhibition of biofilm formation in dual species culture with *S. mutans* in flow cell cultures at ratios of 100 : 1 and 10 : 1. Biofilm formation with increased numbers of voids and hollows was observed at the base of the culture using a confocal microscope. In contrast, increasing the ratio of *L. casei* or *L. salivarius*, other lactic acid bacteria, to *S. mutans* did not affect biofilm formation. In addition, a sonic extract sample of *E. faecium* was sub-purified by salting out and gel filtration, and its inhibitory effects on *S. mutans* biofilm were similarly observed in the same assays. Together, our results suggest that *E. faecium* possesses an inhibitory substance and functions as a probiotic bacterial inhibitor of streptococcal biofilm formation. Further, more they provide important information regarding bacterial communication and diversity, as well as for potential therapies and materials for the prevention of biofilm development in the oral cavity.

§ Corresponding author
E-mail : hsenpuku@nih.go.jp

Introduction

Oral lactic acid bacteria have been reported to occur at high numbers in both superficial and deep caries[1], and are known to play secondary or opportunistic roles in caries development by producing lactic acid and extracellular polysaccharides[2]. Recently, lactic acid bacteria have been utilized as probiotics in dietary supplementation and medicine, as they are known to be useful for the enhancement of immunological activities[3] and adjustment of serum cholesterol[4], as well as for their anti-allergy effects[5]. Their potential mechanisms include the formation of an enhanced barrier to the translocation of bacteria and bacterial products across the mucosa[6,7], and the competitive exclusion of potential pathogens[8], along with modification of host responses to microbial products[9,10] that inhibit the growth of pathogens[11,12], such as *Klebsiella pneumoniae*[13], *Escherichia coli*[14], and *Candida albicans*[12]. In the field of dentistry, it was reported that a water-soluble extract of *Lactobacillus fermentum* completely inhibited the growth of *S. mutans*[15], while another report showed that the clinical strain S11 of *L. fermentum* and its culture supernatant significantly inhibited insoluble glucan formation by *S. mutans*[16]. However, it is not clear whether lactic acid bacteria possess activities to regulate oral biofilm formation.

Streptococcus mutans, the predominant etiologic agent of human dental caries[17] has been shown to be able to adhere to and form a biofilm on tooth surfaces, catabolize carbohydrates and generate acids, and survive at a low pH and under other environmental stress conditions, which are characteristics involved in its cariogenicity[2]. *S. mutans* interacts with other organisms including gram-positive streptococci and bacteria such as *Actinomyces, Neisseria*, and *Vellonela* in biofilm development[2,18,19]. Therefore, it is likely that cooperative and competitive interactions between *S. mutans* and other organisms play important roles in the development of dental biofilm and caries in the oral cavity[20].

Enterococci are gram-positive cocci that form a part of the normal gastrointestinal tract flora in animals and humans[21], function as lactic acid bacteria, and are generally considered to be normal bowel commensals, though they are also recognized as opportunistic pathogens[22]. Enterococci have long been implicated in persistent root canal and dentin infections[23–25], as well as endocarditis and urinary tract infections[24,26]. In addition, enterococci occur in natural foods and are used as probiotics in dairy products[27]. The genus *Enterococcus* consists of at least 23 species, 2 of which, *Enterococcus faecalis* and *E. faecium*, account for greater than 95% of the clinically important isolates.

Lactic acid bacteria such as *E. faecalis* and *E. faecium* are rarely found on oral surfaces. However, they are able to survive under conditions of root and dentin caries. Therefore, they may be associated with the development of microbiological communities in difficult-to-access areas of the oral cavity, and have unique effects on oral biofilm formation with streptococci. In the present study, we observed biofilm formation of *S. mutans* in mixed cultures with lactic acid bacteria and non-biofilm bacteria such as *E. faecium*, *Lactobacillus casei*, and *L. salivarius in vitro* using a flow cell system[28]. Our results further clarify the role of lactic acid bacteria in oral biofilm formation and provide useful information for the development of preventive medicines for oral diseases.

Materials and Methods

1. Bacterial strains and culture conditions

S. mutans MT8148, *E. faecium* 129 BIO 3B (provided by Biofermin Phamaceutical Co., Kobe, Japan), *L. casei* ATCC 393, and *L. salivarius* JCM1231 were used in this study. A strain of *E. faecium*, 129 BIO 3B (classified previously as *Streptococcus faecalis* 129 BIO 3B), reportedly does not produce various toxins, such as bacteriocin and hemolysin, and was proposed as a beneficial probiotic strain for intestinal flora conditions[29]. *S. mutans* and *E. faecium* were grown in Brain Heart Infusion medium (BHI ; Difco Laboratory, Detroit, MI, USA), while *L. casei* and *L. salivarius* were grown in Lactobacilli MRS medium (Difco).

2. Human saliva collection

Whole saliva samples were collected from 5 healthy human subjects (28—30 years old) after stimulation by chewing paraffin gum, and pooled in ice-chilled sterile bottles for 5 min. The samples were clarified by centrifugation at 10,000×*g* for 20 min at 4℃, filter-sterilized, and used immediately for biofilm assays, employing a flow cell system.

3. Biofilm formation assay using flow chamber system

Biofilm formation by all strains was assayed using a method described previously, with some modifications[28,30]. Biofilm samples were cultivated at 37℃ in 3-channel flow cells[31] with individual channel dimensions of 1×4×40 mm and supplied with a flow of tryptic soy broth without dextrose including 0.25% sucrose (TSBS). The flow cell system (The Stovall Flowcell ; Stovall Life Science Inc,. Greensboro, NC, USA) was assembled and prepared as described by Christensen *et al.*[32]. The substratum consisted of a microscope glass cover slip. The flow cells were covered with filter-sterilized human saliva samples and left for 30 min. After the saliva was removed, 0 or 1×10^7 of *S. mutans* and 0, 1, 10, or 100×10^7/mL of *E. faecium*, *L. casei*, or *L. salivarius* were inoculated together in the flow cell. Following that inoculation period, medium flow was stopped for 1 h. Next, the flow was started again and the medium was pumped through the flow cells at a constant rate of 3 mL/h for 16 h using a peristaltic pump (Ismatec ; IDEX Corp., Glattbrugg-Zürich, Switzerland).

4. Confocal laser scanning microscopy

Non-invasive confocal imaging of fully hydrated biofilm samples was performed using a Fluoview CLSM (Olympus, Tokyo, Japan), fitted with a water immersion dipping objective lens (×100) and a Kr-Ar laser[30]. The specimens were stained for 30 min with LIVE DEAD® *Bac*Light™ Bacterial Viability Kit solution (4 mL of distilled water containing 6 μL each of components A and B, Molecular Probes, Leiden, The Netherlands). The biofilm structure was analyzed using a series of horizontal (*xy*) opt digital sections, each being 5.0 μm thick, with the intervening gaps between the horizontal sections ranging from 0 to 20 μm over the entire height (*z*-axis) of the biofilm. In addition, we analyzed vertical (*xz*) sections, which were recorded from the center of each biofilm, to determine the architecture. Each biofilm was scanned at 5 randomly selected positions away from the disk edge. The digital images were processed using Fluoview software version 2.0 (Olympus, Tokyo, Japan).

5. SE preparation

E. faecium was obtained by centrifugation (10,000×*g*), and then washed 3 times with phosphate-buffered saline (PBS, pH 7.2). Sonic extracts (SEs) from the harvested bacterial cells were prepared by a method reported previously[33]. Briefly, the bacterial cells were re-suspended in PBS and sonicated 40 times for 30 s on ice with a sonicator (Sonifier 250D, Branson Ultrasonics. Co., Danbury, CT, USA). Disruption of the bacteria was confirmed by microscopy. The samples were centrifuged at 8,000×*g* for 30 min and cell-free supernatants were stored at −20℃ until use in the experiments. Protein concentrations in the SEs were estimated using a BioRad protein assay kit (BioRad, Richmond, CA, USA).

6. Gel filtration

Proteins in the SE samples were precipitated by salting out, utilizing 60% ammonium sulfate. The precipitates were suspended and dialyzed in 20-mM Tris buffer (pH 7.4). The prepared SE sample was then applied to a Sepharose 4B column of 26×100 cm (Amersham Pharmacia Biotech, Buckinghamshire, UK) pre-equilibrated with the same buffer. Nine-mL fractions were collected, then monitored for UV absorbance and inhibitory effects on biofilm formation by *S. mutans* MT8148. Active fractions were pooled and the proteins were again precipitated by salting out with ammonium sulfate. The precipitates were collected by centrifugation at 10,000×*g*, then suspended and dialyzed in PBS (pH 7.4). Protein concentrations in the sample solution were determined using a protein assay kit (BioRad). The samples were also used in other experiments.

Results

1. Effects of *E. faecium* on *S. mutans* biofilm formation in flow cell system

Significant *S. mutans* biofilm formation was shown in both the flow cell system and CLSM images (Fig. 1A and B), whereas none was observed with *E. faecium* (Fig. 1A), *L. casei* (Fig. 2B), and *L. salivarius* (data not shown) mono-cultures. Optical sections 5 μm apart and showing *xy* planes were analyzed in CLSM images of mixed biofilms formed by *S. mutans* and *E. faecium*. In 5 sections (0 to 20 μm along the *z* axis), the biofilm volumes in the CLSM images were assessed, as shown in Fig. 1. *E. faecium* showed cell number-dependent inhibition of biofilm formation on co-culture with *S. mutans* (Fig. 1A). Irregular biofilms with increased numbers of voids and hollows were observed in cultures of *E. faecium* and *S. mutans* at ratios of 100 : 1 and 10 : 1, as compared to the biofilm of mono-cultured *S. mutans* (Fig. 1B). The diameters of circular voids in each section were measured at the base (0 μm) of 6 sites selected randomly in the mixed and single biofilm samples, and found to be significantly larger in the mixed culture biofilms of *E. faecium* and *S. mutans* at ratios of 100 : 1 and 10 : 1 (28.9 ± 4.0 and 20.3 ± 1.9 μm, respectively), as compared to those of the *S. mutans* mono-cultured biofilm (14.4 ± 2.4 μm). However, increasing the ratio of *L. casei* or *L. salivarius* with *S. mutans* did not significantly affect biofilm formation (Fig. 2A, B).

2. Inhibitory effects of SE sample

To confirm the inhibitory activities of *E. faecium*, an SE sample was extracted. The SE sample showed a significant inhibitory activity against biofilm formation of *S. mutans* MT8148 in the 96-well microtiter plate assay (unpublished data). Moreover, to clearly observe the inhibitory effects, the sample was sub-purified from its SE by salting out and gel filtration. A 4-fold purification of the inhibitory substance was achieved, and a high molecular weight complex >600 kDa was observed by SDS and native-PAGE in the sub-purified SE sample (unpublished data). The sub-purified sample was further applied to the biofilm formation assay using the flow cell. Applications of 25 and 50 μg/mL of sub-purified SE samples inhibited biofilm formation by *S. mutans* MT8148 in comparison with 0 μg/mL, and the level of inhibition was greater with the higher concentration in the flow cell system (Fig. 3). Morphological inhibition by each sub-purified SE sample shown was similar to that in biofilm with a larger void induced by the *E. faecium* cells in CLSM images.

Discussion

Oral streptococci have a strong tendency to colonize tooth surfaces, and the subsequent bacterial multiplication, adherence of additional bacterial species, and production of extracellular polymers on colonized tooth surfaces is referred to as oral biofilm[2,5,11]. Mutans streptococci in particular are able to induce a low-pH environment and so associate with other species such as lactic acid bacteria during biofilm formation. In contrast, lactic acid bacteria may control the environment during the development of dental caries[1,2,6,7].

Under the present test conditions, mono-cultures of *E. faecium*, *L. casei*, and *L. salivarius* failed to form substantial biofilms. Further, more mixed cultures of *S. mutans* and *E. faecium* formed irregular biofilms with an increased number of voids and hollows, observed in ratios of *E. faecium* to *S. mutans* of 10 : 1 and 100 : 1. *E. faecium* did not co-aggregate with *S. mutans* or form biofilm in the present flow cell system; thus, other diverse mechanisms are likely to be involved. It is possible that *E. faecium* produces competitive factors for binding to a saliva-coated glass surface or anti-bacterial agents against *S. mutans*. In fact, our results showed that *E. faecium* produced a substance which inhibited biofilm formation by *S. mutans*. Previous studies have reported that some strains of *Enterococcus* produce cytolysin, which has hemolytic (lysing a broad spectrum of cells, including human, horse, and rabbit erythrocytes) and bactericidal activities against gram-positive bacteria[34,35]. However, the strain used in the present experiments does not produce cytolysin. Thus, it is considered that the SE sample may have included other inhibi-

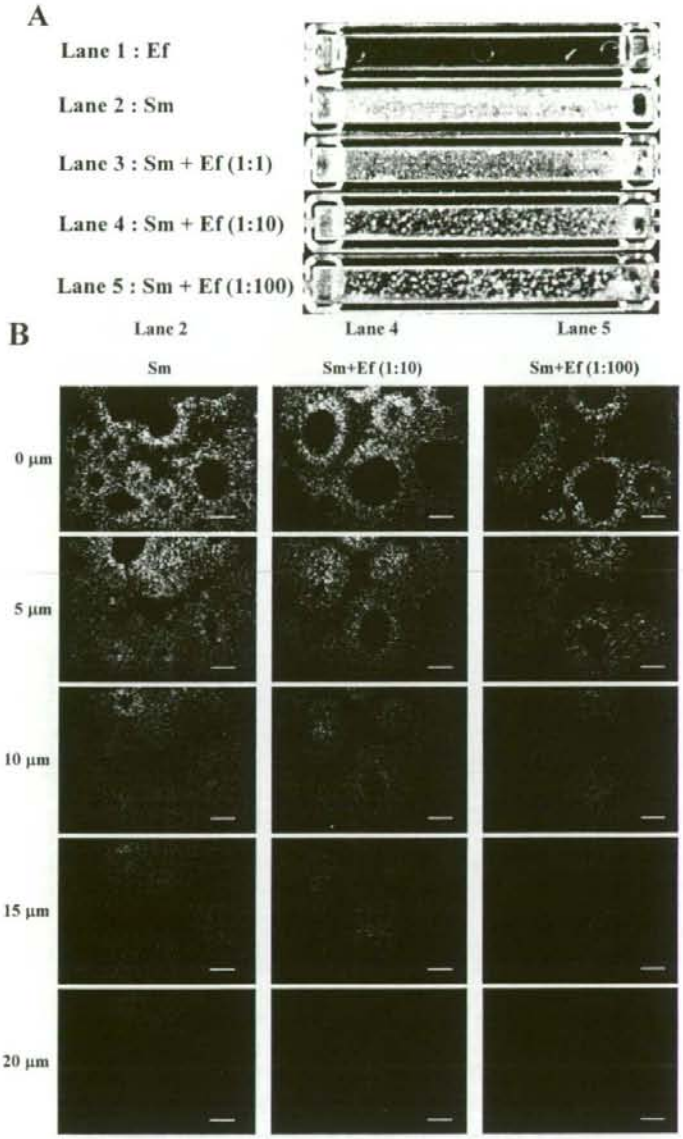

Fig. 1 Biofilm formation by *E. faecium* and *S. mutans* in a flow cell system

(A) : Biofilm formation following mono- and co-cultures of *S. mutans* (Sm) and *E. faecium* (Ef) in a flow cell culture system. Lane 1 : Ef (5×10^6 CFU/lane), Lane 2 : Sm (5×10^6 CFU/lane), Lane 3 : Sm (5×10^6 CFU/lane) + Ef (5×10^6 CFU/lane), Lane 4 : Sm (5×10^6 CFU/lane) + Ef (5×10^7 CFU/lane), Lane 5 : Sm (5×10^6 CFU/lane) + Ef (5×10^8 CFU/lane). The images shown are representative of 5 independent experiments, with similar results obtained in each. (B) : CLSM *xy* images of biofilms formed by mono- and co-cultures of *S. mutans* (Sm) and *E. faecium* (Ef). Live (green) and dead (red) cells in the biofilm were stained with SYTO9 and propidium iodide, respectively. Lanes 2, 4, and 5 in Fig. 1A are presented. Marks from 0—20 μm show the distance from the bottom of the biofilm. Bars = 20 μm. Images shown are representative of 5 independent experiments, with similar results obtained in each.

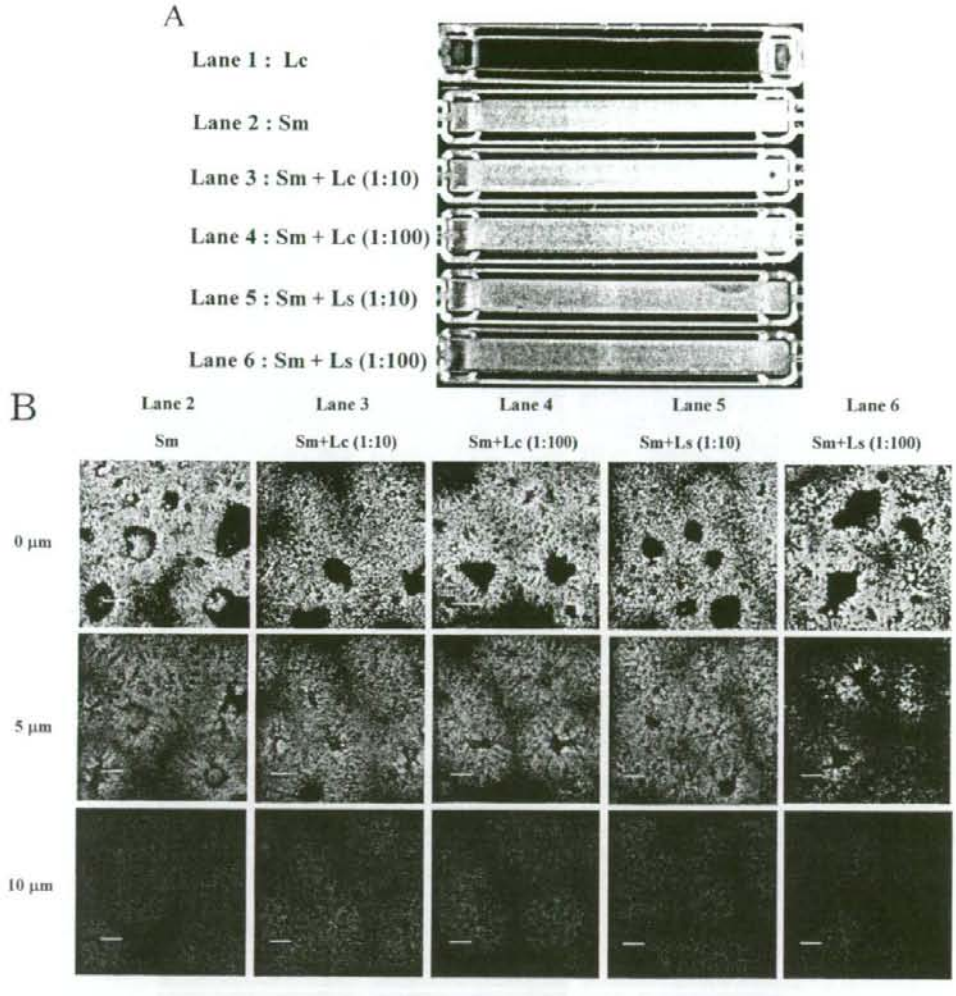

Fig. 2 Biofilm formation by *Lactobacillus* and *S. mutans* in a flow cell system

(A) : Biofilm formation with mono- and co-cultures of *S. mutans* (Sm) and *L. casei* (Lc) or *L. salivarius* (Ls) in a flow cell culture system. Lane 1 : Lc (5×10^6 CFU/lane), Lane 2 : Sm (5×10^6 CFU/lane), Lane 3 : Sm (5×10^6 CFU/lane) + (5×10^7 CFU/lane), Lane 4 : Sm (5×10^6 CFU/lane) + Lc (5×10^8 CFU/lane), Lane 5 : Sm (5×10^6 CFU/lane) + Ls (5×10^7 CFU/lane), Lane 6 : Sm (5×10^6 CFU/lane) + Ls (5×10^8 CFU/lane). Images shown are representative of 3 independent experiments, with similar results obtained in each. (B) : CLSM *xy* images of biofilms formed by mono- and co-cultures of *S. mutans* and Lactobacilli. Dead (red) and live (green) cells in the biofilm were stained with propidium iodide and SYTO9, respectively. Lanes 2, 3, 4, 5, and 6 in Fig. 2A are presented. Marks from 0—10 μm show the distance from the bottom of the biofilm. Bars = 20 μm. Images shown are representative of 3 independent experiments, with similar results obtained in each.

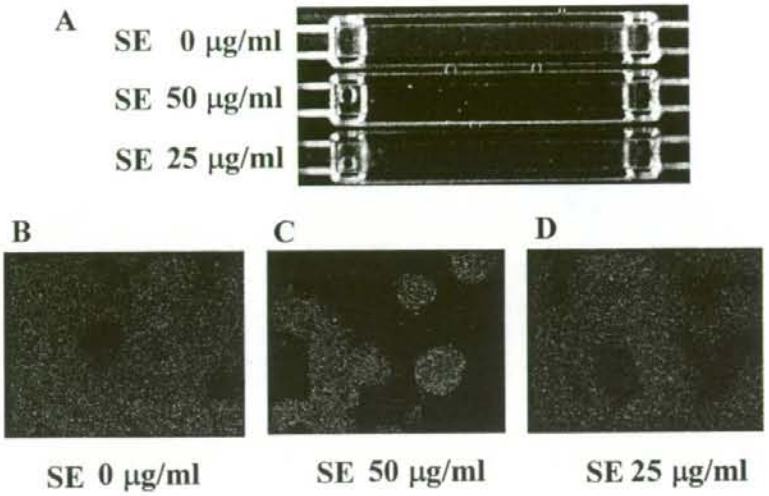

Fig. 3 Inhibitory effects of HMWC on *S. mutans* biofilm formation in a flow cell system

(A) : Photographs of biofilm formation by *S. mutans* MT8148 with 0, 25, and 50 μg/mL of HMWC in a flow cell culture system (actual size). (B), (C), (D) : CLSM *xy* images of biofilms formed by *S. mutans* cultured with 0, 50 and 25 μg/mL, respectively, of sub-purified SE sample. The images shown are representative of 3 independent experiments, with similar results obtained in each.

tors and reacted with biofilm-producing bacteria to reduce attachment of the organisms to the glass surface as well as colonization, leading to a poor quality biofilm with an increased number of voids and hollows (Figs. 1, 2). Culture supernatant from *E. faecium* did not show a significant inhibition of *S. mutans* biofilm formation. Therefore, these inhibitory effects of *E. faecium* may require cell density-dependent bacterial contact and cell lysis, and the binding of products to a salivary receptor of biofilm bacteria on a hard surface during biofilm development.

In the present study, the lactic acid bacteria *L. casei* and *L. salivarius* did not show abilities to produce biofilm in mono-cultures, nor did they inhibit biofilm formation or the attachment of *S. mutans* in co-cultures with increasing ratios. In general, lactobacilli are major odonto-pathogens[36]. Filoche *et al*[37]. reported that *S. mutans* markedly promoted the growth of lactobacilli-based biofilm, and suggested that the ability of individual bacteria to form a mono-culture biofilm was not necessarily an indicator of their survival or pathogenic potential in a complex multi-species biofilm community. However, soluble extracts of *L. fermentum* were previously shown to be able to inhibit the growth of *S. mutans* and formation of insoluble glucan produced by *S. mutans*[15,16]. *Lactobacillus rhamnosus* GG, another well-known probiotic strain, was also recently reported to be able to promote oral health in children and adults[38]. Further, more enhanced biofilm growth was observed in experiments with *Lactobacillus plantarum*, which has been implicated in the development of dental caries[36,39].

Taken together, these reports and results show that lactic acid bacteria including lactobacilli and *E. faecium* play multiple roles as unique modulators of both pathogenic and normal microflora on tooth surfaces. Additional investigations are required to clarify whether the products inhibit colonization or attachment in a physical or chemical manner during *S. mutans* biofilm formation on a hard surface, as well as to purify the tested products, before a definitive conclusion regarding the mechanism of early-onset inhi-

bition can be presented. Nevertheless, the present results should provide useful information to help understand oral biofilm formation, as well as for the development of therapies and materials for the prevention of biofilm development in the oral cavity.

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Differential Expression of the Smb Bacteriocin in *Streptococcus mutans* Isolates[▽]

Hideo Yonezawa,[1] Howard K. Kuramitsu,[2] Shu-ichi Nakayama,[1] Jiro Mitobe,[1] Mizuho Motegi,[1] Ryoma Nakao,[1] Haruo Watanabe,[1] and Hidenobu Senpuku[1]*

Department of Bacteriology, National Institute of Infectious Diseases, Tokyo, Japan,[1] and Department of Oral Biology, State University of New York at Buffalo, Buffalo, New York[2]

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The two-component lantibiotic Smb is produced by *Streptococcus mutans* GS5. In the present study, we identified seven strains of *S. mutans* containing the *smb* gene cluster. These strains could be classified into high- and low-level Smb producers relative to the levels of Smb production by indicator strains in vitro. This classification was dependent upon the transcription levels of the structural *smbA* and *smbB* genes. Sequence analysis upstream of *smbA* in the high- and low-level Smb-producing strains revealed differences at nucleotide position −46 relative to the *smbA* start codon. Interestingly, the transcription start site was present upstream of the point mutation, indicating that both groups of strains have the same promoter constructs and that the differential expression of *smbA* and *smbB* mRNA occurred subsequent to transcription initiation. In addition, *smbA::lacZ* fusion expression was higher when it was regulated by the sequences of strains with high-level Smb activity than when it was regulated by the comparable region from strains with low-level Smb activity. Taken together, we conclude that high- or low-level Smb expression is dependent on the presence of a G or a T nucleotide at position −46 relative to the *smbA* translational start site in *S. mutans* Smb producers.

Streptococcus mutans, the principal etiological agent of human dental caries, is present along with other oral bacteria in heterogeneous biofilms termed dental plaque (7). Since different strains of *S. mutans* have been shown to produce bacteriocins, also termed mutacins, some of which are active against other oral bacteria (16). One of them, Smb, which is regulated by a competence-stimulating peptide (CSP)-dependent quorum-sensing system, is a two-component lantibiotic produced by *S. mutans* strain GS5 (16, 24). The biosynthetic apparatus of lantibiotics is generally organized in gene clusters (20); and the *smb* operon consists of seven open reading frames (ORFs) in the order *smbM1*, *-F*, *-T*, *-M2*, *-G*, *-A*, and *-B*, flanked by putative transposase genes (24). The *smbF* and *smbG* genes are thought to be involved in immunity to Smb. A recent report also indicated that the *smbG* gene appears to play a role in the sensitivity of strain GS5 to a variety of antimicrobial agents, such as tetracycline and triclosan (10). The *smbT* gene is presumably the ATP binding cassette transporter for pre-Smb processing and secretion. The structural genes for the precursor preproSmb consist of *smbA* and *smbB*. These genes encode the two peptides SmbA and SmbB, respectively, within a single operon (16, 24). The two-component lantibiotic systems utilize two peptides that are each posttranslationally modified to an active form and that act in synergy to produce antibacterial activity (3). The gene cluster encoding Smb expression also encodes two putative modification enzymes, which have been designated *smbM1* and *smbM2* (24). Recently, predicted lanthionine and methyllanthionine ring forms were proposed for other known two-component lantibiotics similar to Smb (11).

S. mutans is thought to use Smb production as a means to compete with other oral bacteria present in dental plaque (22). Some reports indicated that the bacteriocins produced by this organism play an important role in the regulation and composition of dental biofilms (7, 9, 14). On the other hand, some reports failed to show a relationship between the presence of bacteriocin genes in different strains of this organism and caries status (4, 6). In the present study, we demonstrate that a range of Smb activities are present in *S. mutans* clinical isolates harboring the *smb* operon. In addition, we describe evidence that a single base mutation in the upstream region of *smbA* can account for these differences.

MATERIALS AND METHODS

Bacterial strains. The *S. mutans* strains used in this study are listed in Table 1. *S. mutans* wild-type strains were assessed for the presence of the *smb* genes as well as the expression of Smb activity. RP66 (group C streptococcus, which is sensitive to Smb) and oral streptococci (*Streptococcus sanguinis* ATCC 10556, ST205, and ST134; *Streptococcus mitis* ATCC 903 and ATCC 6249; *Streptococcus gordonii* ATCC 10558 and Challis; and *Streptococcus salivarius* HT9R, JSM5707, and ATCC 9759) were used as indicator strains for Smb activity. These strains were grown in brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI) in an anaerobic atmosphere of 85% N_2, 10% CO_2, and 5% H_2 at 37°C. Transformants of *S. mutans* were selected following their growth on BHI agar plates supplemented with 10 μg of erythromycin per ml or 500 μg of kanamycin per ml.

Agar plate bacteriocin assays. Loopfuls of stationary-phase cultures of *S. mutans* strains were stabbed into BHI agar plates. The plates were incubated at 37°C for 16 h. Indicator strains were grown to an optical density of 0.2 at 550 nm. Each culture was then diluted 1:100, and 0.2 ml of this solution was pipetted into a tube containing 4 ml of molten BHI broth containing 1% agar. This solution was mixed and poured evenly onto the surfaces of the plates, the plates were incubated at 37°C for an additional 24 h, and the diameters of the zones of inhibition were measured.

Construction of *smbAB and nlmAB* mutants. The mutants with defective *smbAB* genes were constructed by double-crossover homologous recombination

* Corresponding author. Mailing address: Department of Bacteriology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81-3-5285-1111. Fax: 81-3-5285-1163. E-mail: hsenpuku@nih.go.jp.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
S. mutans strains		
GS5	Wild type Km^s	SUNY at Buffalo
BM71	Wild type Km^s	G. H. Bowden, University of Manitoba
FSC-4	Wild type Km^s	23
FSM-6	Wild type Km^s	23
FSC-8	Wild type Km^s	23
FSC-1	Wild type Km^s	23
FSM-3	Wild type Km^s	23
FSM-6-lacL	FSM-6 carrying pSmblacL Km^r	This study
FSM-6-lacH	FSM-6 carrying pSmblacH Km^r	This study
BM71-lacL	BM71 carrying pSmblacL Km^r	This study
BM71-lacH	BM71 carrying pSmblacH Km^r	This study
BM71-lacLAGC	BM71 carrying pSmblacLAGC Km^r	This study
BM71-lacHATC	BM71 carrying pSmblacHATC Km^r	This study
Plasmids		
pDL276	*E. coli-Streptococcus* shuttle vector	4
pSmblacL	pDL276 harboring *upsmbA* (derived from FSM-6)-*lacZ* fragment	This study
pSmblacH	pDL276 harboring *upsmbA* (derived from BM71)-*lacZ* fragment	This study
pSmblacLAGC	Site-directed change plasmid from pUpsmblacLATC to AGC	This study
pSmblacHATC	Site directed change plasmid from pUpsmblacHAGC to ATC	This study
pSmbABL	pDL276 harboring *upsmbA-smbB* (derived from FSM-6)	This study
pSmbABH	pDL276 harboring *upsmbA-smbB* (derived from BM71)	This study

by insertion of an erythromycin resistance determinant into the genes, as described previously (24). In addition, the nonlantibiotic mutacin (19) *nlmAB* mutant was also constructed by the same method. Confirmation that plasmid insertion caused gene disruption was determined either by Southern blotting or by PCR.

Extraction of RNA, real-time quantitative RT-PCR, and primer extension analysis. The *S. mutans* strains were grown on BHI agar plates. After incubation at 37°C 10 h, the cells were scraped from the plates and resuspended in phosphate-buffered saline (PBS). After centrifugation and washing of the cells with PBS three times, the cells were suspended in 0.3 ml of diethylpyrocarbonate-treated water. RNA extraction was carried out as described previously (24). The RNA samples were then treated for 15 min at 37°C with 1.0 U of RNase-free DNase (Amersham Biosciences Corp., Piscataway, NJ) per ml to remove contaminating DNA. Reverse transcription (RT) was carried out with a SuperScript III kit (Invitrogen, Corp., Carlsbad, CA), according to the directions of the supplier. The real-time RT-PCR was performed with cDNA samples with either the 16S rRNA-specific primers (primers LARNA5 and LARNA6 as internal controls) or *smb*-specific primers (SmbABRTFw and SmbABRTRev) with Power SYBR green PCR master mixture (Applied Biosystems, Foster City, CA) in an ABI Prism 7700 sequence detection system (Applied Biosystems). The final results were expressed as the level of *smb* gene expression relative to the level of 16S rRNA gene expression, i.e., relative expression = level of *smbAB* gene expression/level of 16S rRNA gene expression. A similar approach was used for the *smbG* to *smbA* transcript (with primer pair SmbGARTFw and SmbGARTRev).

Total RNA was prepared as described above, and primer extension was carried out essentially as described previously (13). RNA (20 μg) was annealed to 2.0 pmol of [γ-^{32}P]ATP-labeled primer SmbAPE (Table 2; see also Fig. 3A) at 80°C for 2 min and then at 37°C for 45 min in RT buffer (Omniscript RT kit; Qiagen, Valencia, CA). Then, 0.5 mM of the four deoxynucleoside triphosphates and reverse transcriptase from an Omniscript RT kit were added to 60 μl of the solution. The primer extension reaction was done at 37°C for 1 h. The synthe-

TABLE 2. Oligonucleotide sequences of PCR primers

Primer	Sequence (5′–3′)[a]	Use or target gene product	Amplicon size (bp)
LARNA5	GTTGTCCGGATTTATTGGG	Internal control for RT-PCR	248
LARNA6	GGGTATCTAATCCTGTTCGC		
SmbABRTFw	AAGATATGTTAGCTGGGGG	*smbA* and *smbB*	199
SmbABRTRev	GCATCATCCATGAGAATTG		
SmbGARTFw	GCTTGATGGCTCAAAAGG	*smbG* and *smbA*	239
SmbGARTRev	CCCCCAGCTAACATATCTT		
UpsmbAFwBam	GC**GGATCC**TATATTGAGCAATAACTTTTGGG	Upstream of *smbA* (promoter region)	192
UpsmbARevTag	**TTCGCTCATTTTATATTCCTTCC**TATTCTTTATAAAC TACC		
LacFwTag	**GGAAGGAATATAAAATGAGCGAA**AAATACATCGTCA	*lacZ*	3,285
LacRevXba	GG**TCTAGA**TTATTTTTGACACCAGACCAACT		
SiteGtoT	CAAGGAGAAGATAGGATCATTATGATGATAGG	pUpsmbAlacH for site-directed mutagenesis	10,435
SiteGtoTcomp	CCTATCATCATAATGATCCTATCTTCTCCTTG		
SiteTtoG	CAAGGAGAAGATAGGAGCATTATGATGATAGG	pUpsmbAlacL for site-directed mutagenesis	10,435
SiteTtoGcomp	CCTATCATCATAATGCTCCTATCTTCTCCTTG		
RevSmbABKpn	GC**GGTAC**CGGAATATTTATCATCATGTAAGT AGTC	Downstream of *smbB* (with primer UpsmbAFwBam)	650
SmbAPE	GTTACATTATTTATTTTTAAT	For primer extension analysis	

[a] Restriction site sequences are indicated in boldface. The tag sequences for secondary PCR are indicated in boldface and underlined.

sized DNA was extracted with phenol-chloroform, precipitated with ethanol, dissolved in DNA-sequencing load buffer (SequiTherm Excel II DNA sequencing kit; Epicentre Biotechnologies, Madison, WI), and analyzed by electrophoresis on an 8% polyacrylamide gel containing 50% urea, followed by autoradiography. As a standard, the same ^{32}P-labeled primer was annealed to alkaline-denatured DNA from pSmbABH, and the dideoxy chain termination sequencing reaction was carried out with the SequiTherm Excel II DNA sequencing kit. The sample was loaded on the gel along with the synthesized DNA described above.

Construction of *upsmbA::lacZ* fusion plasmids. To construct a fusion fragment of the *upsmbA* region and *lacZ*, the upstream regions of *smbA* containing the promoter regions from strain FSM-6 or BM71 and the *lacZ* gene from the pSV-beta-galactosidase control vector (Promega, Madison, WI) were used as templates with each pair of specific primers, each of which contained a 9- or 14-bp tag fragment (Table 2). The first step of PCR amplification was performed with each template and each primer pair (primers UpsmbAFwBam and UpsmbARevTag or primers LacFwTag and LacRevXba). After amplification, the 183-bp *upsmbA* region and the 3,285-bp *lacZ* amplification fragments were treated with a PCR cleanup kit (Qiagen GmbH, Hilden, Germany) and were used as templates. In addition, the amplified fragments served as primers for the amplification of each other. The secondary PCR mixture contained 1 μl of each PCR fragment, 5 μl of 10× PCR buffer (Takara Shuzo, Tokyo, Japan), 200 mM $MgCl_2$, and 1.25 U of high-fidelity DNA polymerase Pyrobest (Takara Shuzo). The temperature profile included, first, denaturation at 94°C for 5 min, followed by 15 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 3 min and then heating at 72°C for 5 min. The 3,454-bp amplified fragments were purified and used as templates for the third PCR with primers UpsmbAFwBam and LacZRevXba. The amplified 3,454-bp PCR fragments were digested with BamHI and XbaI and ligated into the BamHI-XbaI sites of *Escherichia coli-Streptococcus* shuttle vector pDL276 (2). The plasmids containing the *upsmbA-lacZ* fusions were then transformed into *E. coli* DH5α and the transformants were selected on LB agar plates containing 50 μg of kanamycin per ml. The relevant structures of the resultant plasmids, named pSmblacL and pSmblacH, were confirmed by DNA sequencing.

Determination of β-galactosidase activity. Plasmids pSmblacL and pSmblacH and control plasmid pDL276 were transformed into *S. mutans* strain FSM-6 or BM71. Transformants of the *S. mutans* strains were selected on mitis salivarius agar plates containing 500 μg of kanamycin per ml. For measurement of β-galactosidase activity, each transformant was incubated on a BHI agar plate containing kanamycin. After incubation at 37°C for 10 h, the cells were scraped from the agar plate and resuspended in PBS. The cells were centrifuged and assayed for β-galactosidase activity, as described previously (5).

In vitro mutagenesis. A PCR-based oligonucleotide-directed mutagenesis strategy was carried out for site-directed mutagenesis of the *smbA* promoter region. The synthetic oligonucleotides used as mutagenic primers are listed in Table 2. Briefly, plasmid pSmblacL was used as a template for mutagenesis with primer pair SiteTtoG and SiteTtoGcomp. Plasmid pSmblacH was also used as a template in mutagenesis with primer pair SiteGtoT and SiteGtoTcomp. The PCR products were treated with the restriction enzyme DpnI (New England Biolabs, Beverly, MA), and the treated products were transformed into *E. coli* DH5α. The mutagenic plasmids were then subjected to DNA sequencing to confirm that the desired mutation but no additional mutation was present (data not shown). These mutagenized plasmids, named pSmblacLAGC and pSmblacHATC, respectively, were transformed into *S. mutans* strain FSM-6 or BM71, and the transformants were used for β-galactosidase activity assays.

Construction of *smbA* and *smbB* expression vectors. To construct the vectors expressing the *smbA* and *smbB* genes, the *smbA* and *smbB* regions downstream from the *upsmbA* region were amplified with primer pair UpSmbAFwBam and RevSmbABKpn (Table 2). The chromosomal DNA from strain FSM-6 or BM71 was used as the template. The amplified 573-bp fragments containing the BamHI and KpnI sites were then digested with BamHI and KpnI and ligated into the BamHI and KpnI sites of pDL276 (2). The plasmids containing the *upsmbA-smbB* region were then transformed into *E. coli* DH5α and the transformants were selected on LB agar plates containing 50 μg of kanamycin per ml. The relevant structures of the resultant plasmids, named pSmbABL (with fragments amplified from strain FSM-6) and pSmbABH (with fragments amplified from BM71), were confirmed by DNA sequencing. These plasmids were transformed into the *S. mutans* BM71 *smbAB* mutant, and the transformants were used for bacteriocin agar plate assays with the *S. salivarius* strain.

RESULTS AND DISCUSSION

Identification of *smb* genes in *S. mutans* clinical isolates. By screening 17 clinical isolates of *S. mutans* (12) by PCR and Southern blotting for the presence of the *smb* genes, we found five positive strains (strains FSC-4, FSM-6, FSC-8, FSC-1, and FSM-3). In addition, laboratory strains GS5 and BM71, which are known to be producers of Smb, were confirmed to harbor these genes (22, 24). We previously determined that the *smb* operon consists of seven ORFs (24). We observed that all of these positive strains possess all of the ORFs by PCR or Southern blotting analysis (data not shown). In order to assess the antimicrobial activities of these Smb-producing strains, bacteriocin agar plate assays were performed with some potential indicators strains (the strains are noted in the Materials and Methods section). *S. mutans* strain BM71 exhibited the maximum inhibitory zones against most of the indicator strains (data not shown). Variations in the antimicrobial activities of the *S. mutans* strains against these indicator strains were also observed. The most uniform results, separation into high- and low-level Smb-producing groups, was accomplished with *S. salivarius* JCM5907 as an indicator strain in the bacteriocin agar plate assays (Fig. 1A). Strains GS5, BM71, FSC-1, FSM-3, and FSM-6 exhibited bacteriocin activity; on the other hand, strains FSC-4 and FSC-8 did not produce inhibitory zones.

Previous reports indicated that some of the *S. mutans* strains possess two or more bacteriocins (7, 19, 21). To determine whether these Smb producers express other bacteriocins (mutacins I to IV) (1, 17, 18, 19), PCR analysis, Southern blot analysis, and sequence examination were carried out. We determined that the mutacin I genes (18) were present in the genome of strain FSM-6 (data not shown). Mutacin I belongs to the lantibiotic family and has a high level of activity against oral streptococci (7, 18, 19). We disrupted the *smbAB* genes in FSM-6 by inserting an erythromycin cassette within the genes and examined its antimicrobial activity with *S. salivarius* strain JCM5907 (Fig. 1B). The *smbAB* mutant of strain FSM-6 was as inhibitory as the parental strain against the indicator strains. We determined the transcription levels of the *smbAB* genes and found that the level of expression was low (Fig. 2), as described in more detail below. These results suggest that the bacteriocin activity of FSM-6 against the *S. salivarius* strain might be due to the expression of mutacin I or another antimicrobial agent.

We further determined that strains FSC-1, GS5, and BM71 possessed the nonlantibiotic mutacin IV (data not shown), which has been classified as a nonlantibiotic bacteriocin encoded by the *nlmA* and the *nlmB* genes (24). To determine which bacteriocin is required for inhibition of the growth of the *S. salivarius* indicator strain, the levels of bacteriocin production by *smbAB* and *nlmAB* mutants of these strains were compared. We attempted several times to construct an *smbAB* mutant of FSC-1. However, we were not successful, since this strain apparently is not genetically competent. Therefore, the zones of inhibition of strains BM71 and GS5 and their mutants in plate assays with the indicator strain were compared. Both *smbAB* mutant strains were completely devoid of bacteriocin activity against the indicator strain, while the mutation in the *nlmAB* genes had no influence on inhibition of the growth of the *S. salivarius* strain compared to that of their parental

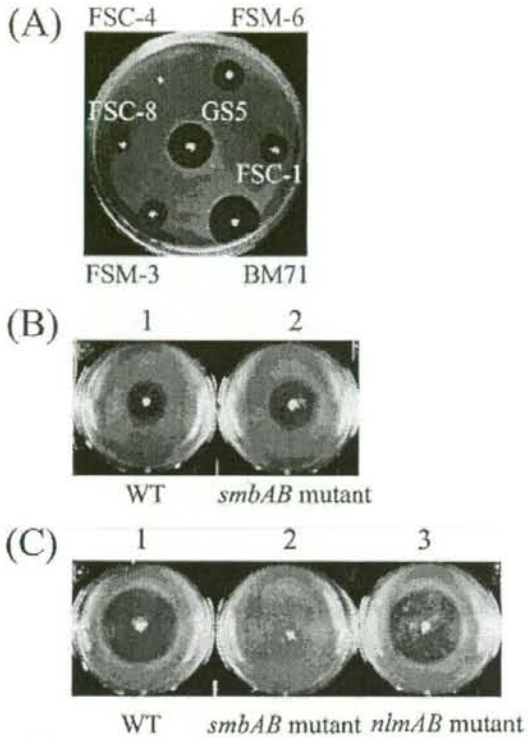

FIG. 1. (A) *S. mutans* production of agents with activity against *S. salivarius* strain JCM5907 as an indicator strain determined by bacteriocin agar plate assays; (B) activities of *S. mutans* wild-type (WT) strain FSM-6 (lane 1) and its *smbAB* mutant (lane 2) against *S. salivarius* determined by bacteriocin agar plate assays; (C) activities of wild-type strain BM71 (lane 1), its *nlmAB* mutant (lane 2), as well as its *smbAB* mutant (lane 3) against *S. salivarius* strain JCM5907.

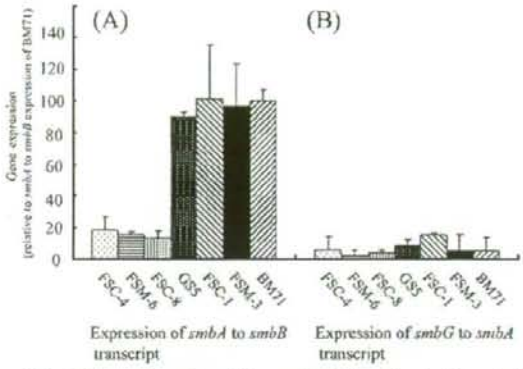

FIG. 2. Expression of *smbA* to *smbB* (A) and *smbG* to *smbA* (B) transcription by *S. mutans* strains. The quantity of cDNA corresponding to these genes was determined by real-time RT-PCR and was normalized to that of the 16S rRNA gene in each unique reaction. Each experiment was repeated three times with duplicate samples from each independently isolated RNA preparation. Data are expressed as the means of all of experiments ± standard deviations.

strains (Fig. 1C, strain BM71). These results indicated that Smb, but not mutacin IV, is the major bacteriocin in these strains that is active against the *S. salivarius* strain. A recent study has also indicated that Smb, but not mutacin IV, was isolated from the GS5 culture medium (16). This suggests that strain GS5 might not express mutacin IV under normal laboratory conditions.

Other mutacin genes are not present in the genomes of several Smb producers (strains FSM-3, FSC-4, and FSC-8). Strains FSC-1 and FSM-3 exhibited moderate inhibitory zones relative to those for strains GS-5 and BM71 (Fig. 1A). The growth of strains FSC-1 and FSM-3 was relatively slow, and their final titers in liquid culture, as determined from the optical density at 600 nm, were approximately 55% of the titers of strains GS5 and BM71. These results suggest that the relatively slower growth and final cell numbers of strains FSC-1 and FSM-3 may be responsible for the apparent relatively modest Smb activity of these strains. Although we did not obtain direct evidence for the role of Smb in inhibiting the growth of the *S. salivarius* indicator strain by strain FSC-1, we concluded that strain FSC-1 expresses Smb activity at levels similar to those for strains GS5 and BM71 under these conditions. Therefore, these results suggested that the Smb producer strains could be divided into high-level producers (strains GS5, BM71, FSC-1, and FSM-3) and low-level producers (strains FSC-4, FSM-6, and FSC-8) of Smb activity.

RT-PCR analysis of *smbAB* transcription. We hypothesized that there were sequence differences in the *smb* structural genes between the groups with high- and low-level Smb activity. Therefore, we analyzed the sequences of the *smbA* and *smbB* genes from the two groups. However, there were no differences in the *smbA* and *smbB* gene sequences of strains FSC-4, FSM-6, and FSC-8 compared with the comparable sequences in high-level Smb-producing strain GS5. We then determined if there were differences in the levels of transcription of the *smbA* and *smbB* genes between the two groups using RT-PCR. A comparison of *smbA* and *smbB* gene expression in these strains (Fig. 2A) revealed that the expression of both genes was significantly elevated in the strains with higher levels of activity than in the strains with lower levels of activity.

We previously reported that there is a single *smb* operon with two promoters in strain GS5. One is upstream of *smbM1*, which is the first gene of the *smb* gene cluster, and the other is immediately upstream of *smbA* and has a terminator sequence downstream of *smbB* (24). This suggests that *smbA* and *smbB* transcription might be regulated by one or two promoters in the *smb* operon. In order to analyze the effects of the promoter upstream of the *smbM1* gene on the expression of *smbA* and *smbB*, real-time RT-PCR was carried out with primer pair SmbGARTFw and SmbGARTRev (Table 2 and Fig. 3A), which detects the transcript from *smbG* to *smbA* (Fig. 2B). No difference in the level of expression of this transcript was detected between strains in the two Smb expression groups. Since this transcript is regulated by the promoter immediately upstream of the *smbM1* gene (24), this promoter does not appear to be responsible for the differential expression of Smb in the two groups. However, the levels of transcription of the *smbA*

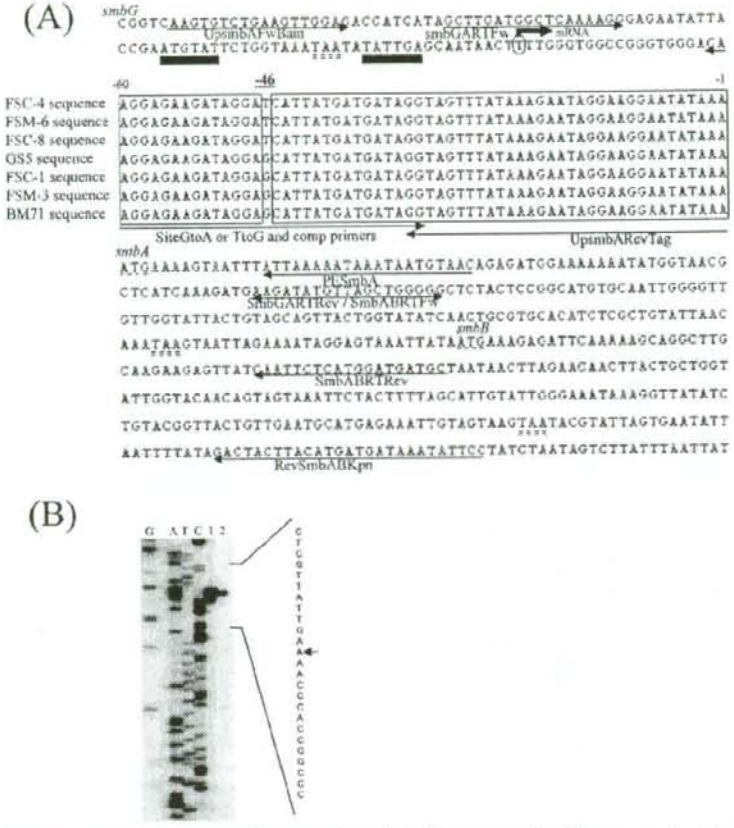

FIG. 3. (A) Sequence alignment of the upstream regions of *smb*-containing *S. mutans* strains. The uppermost sequence is that corresponding to the *smbG* C-terminal sequence, and the bottom sequence is that of the regions flanking the *smbB* gene derived from strain GS5 Smb (GenBank accession no. AB179778). The boxes indicate identical sequences in all strains. The primers for the PCR amplification of this region are marked with arrows under their nucleotide sequences. Dashed lines under these sequences denote the *smbA* or *smbB* ATG start codon. Double dashed lines denote the *smbG*, *smbA*, or *smbB* stop codon. The numbers −1, −46, and −60 indicate the position relative to the start codon of *smbA*. At the top, the circle with the arrow indicates the mapped transcription initiation site of *smbA*. The heavy bars represent the putative −10 and −35 regions of a candidate promoter region. (B) Primer extension analyses confirmed the 5′ end of the *smbA* transcript.

and *smbB* genes in all strains were higher than those of *smbG* and *smbA* (Fig. 2). Furthermore, there was a clear difference in the levels of transcription of the *smbAB* genes between the groups with high and low levels of Smb activity. These results suggest that the promoter region immediately upstream from the *smbA* gene is primarily responsible for the differential expression of these two genes in the two groups.

Sequence analysis of *smbA* promoter region. In order to determine whether sequence differences in the *smbA* promoter region are responsible for the differential expression of the Smb structural genes in the two groups, sequence analysis of this region was carried out (Fig. 3A). Sequencing of this region of the strains with high and low levels of Smb activity revealed the presence of a transversion mutation (G to T) at nucleotide position −46 relative to the *smbA* ATG start codon in the strains with low levels of Smb activity (strains FSC-4, FSM-6, and FSC-8) compared with the sequences of the strains with high levels of Smb activity, including laboratory strain BM71, which is a reference strain for Smb in the GenBank nucleotide sequence database (GenBank accession no. AB179778), as well as strain GS5. No other sequence differences were detected in this region, which is partially shown in Fig. 3A, in any of the strains. These results indicate that a G nucleotide at position −46 results in a relatively higher level of expression of the *smbAB* genes compared with the level of expression when a T nucleotide is present at this position. We next analyzed the *smbA* and *smbB* transcripts by primer extension mapping in order to determine the transcription start site. The template RNAs were extracted from the groups with high (strain BM71) and low (strain FSM-6) levels of Smb antimicrobial activity and were used for the experiment with primer PEUpsmbA (Table 2 and Fig. 3A). The results of the mapping are shown in Fig. 3B. We observed a single major extended band at the same position in both strains. Comparison of the band with a DNA

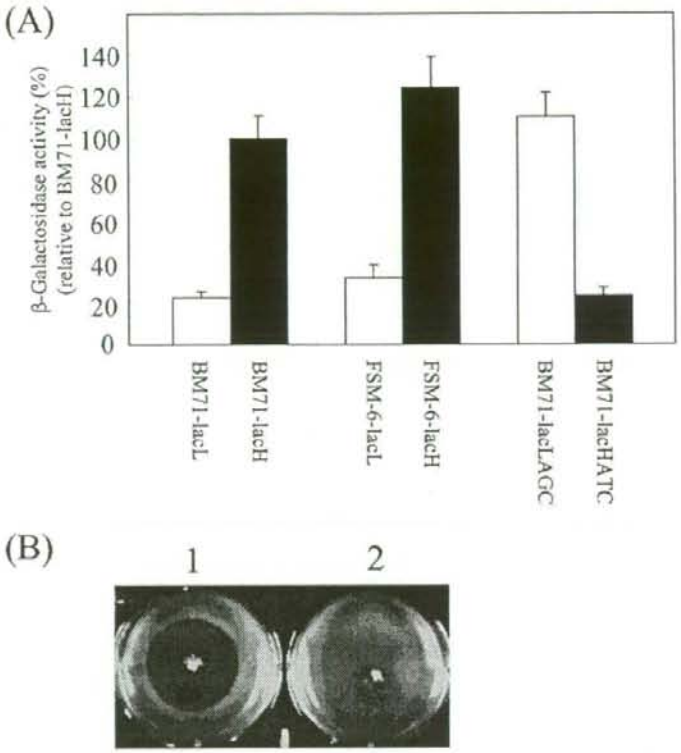

FIG. 4. (A) β-Galactosidase activities of strains BM71-lacL and BM71-lacH, strains FSM-6-lacL and FSM-6-lacH, and strains BM71-lacHATC and BM71-lacLAGC. The activity (percent) of each strain was calculated by use of the activity of strain BM71-lacH, which was set equal to 100%. Strains BM71-lacHATC and BM71-lacLAGC were generated from the BM71 *smbAB* mutant with plasmids pSmblacH and pSmblacL, respectively, following site-directed mutagenesis, as described in the text. The experiments were repeated three times with duplicate samples. Data are expressed as the means of all of experiments ± standard deviations. (B) Bacteriocin activity of the BM71 *smbAB* mutant complemented with plasmids carrying the *smbAB* region from strains with low and high levels of Smb activity. The bacteriocin activity was measured by the agar plate assay with *S. salivarius* strain JCM5907. *smbA*- and *smbB*-containing *upsmbA* was amplified from BM71 (lane 1) or FSM-6 (lane 2), and the constructs were transformed into the BM71 *smbAB* mutant.

sequencing ladder as a standard showed that the 5′ end of the mRNA corresponded to the T nucleotide at position −81 bp relative to the start codon of *smbA*, which is within the *smbG* ORF (Fig. 3A). The putative candidate of the promoter structure is shown in Fig. 3A (−35 sequences are ATGTAT, and −10 sequences are TATTGA, as indicated by the heavy bars). The intensities of the bands from the primer extension reaction with RNA from strain BM71 were much higher than those with RNA from FSM-6, although the same amount of RNA from each strain was used for this reaction (compare lanes 1 and 2 in Fig. 3B). This is consistent with the observation of the RT-PCR results (Fig. 2A). In this promoter region, all strains contained the same sequences, indicating that all of the strains have the same promoter constructs and that the events which discriminate between the strains with high and low levels of Smb activity likely occurred after the transcription starts. To explain these differences, we first considered that the regulation of mRNA transcription might be dependent upon a decrease in the stability of the mRNA of the *smbA* and *smbB* genes in the group with a low level of Smb activity as a result of the nucleotide transversion. However, there were no detectable differences in mRNA stability between the high- and low-level Smb producers following analysis of the cDNA synthesized from rifampin-treated cells (data not shown). Next, we hypothesized that the transversion may involve a palindrome construct. Indeed, the prediction obtained by sequence analysis with Genetyx (version 7.0.3) software suggested that a G-to-T nucleotide change might result in a higher probability of formation of a palindromic sequence in this region, suggesting that this construct might decrease the possibility that the gene will proceed with subsequent transcription. There is also a possibility that the transversion region may be an operator region which serves as a binding site for a regulatory protein(s) that has yet to be identified. Further research is now in progress to examine the mechanism of this regulation.

Characterization of the *smbA* flanking region and its influence on *smbA* and *smbB* translation. In order to confirm the effects of nucleotide changes at position −46 on the translation

of the *smbA* and *smbB* genes, the primer structure of low-level Smb-producing strain FSM-6 and high-level Smb-producing strain BM71 were fused to the promoterless *lacZ* gene of pDL276 to produce chimeric plasmids pSmblacL and pSmblacH, respectively. These plasmids were transformed into strains FSM-6 and BM71, and the strains were assayed for their β-galactosidase activities (Fig. 4A). For strains harboring pSmblacL (strains FSM-6-lacL and BM71-lacL) or pSmblacH (strains FSM-6-lacH and BM71-lacH), the results clearly indicated that the β-galactosidase activities of strains FSM-6-lacH and BM71-lacH were significantly higher than those of strains FSM-6-lacL and BM71-lacL, respectively (β-galactosidase activity of strain BM71-lacH, 850 ± 72 U).

To confirm the effect of the nucleotide transversion on the level of Smb expression, site-directed mutagenesis of these two plasmids was carried out. In plasmid pSmblacL, the T nucleotide at position −46 was changed to G. Likewise, the G nucleotide in pSmblacH was changed to a T in the comparable flanking region (named pSmblacLAGC and pSmblacHATC, respectively), and strain BM71 with these plasmids (BM71-lacLAGC and BM71-lacHATC, respectively) was tested for β-galactosidase activity. The β-galactosidase activities of BM71-lacL and BM71-lacHATC were similar. Furthermore, the activities of BM71-lacH and BM71-lacLAGC also covered a similar range (Fig. 4A), indicating that the difference in the levels of gene expression at both the transcriptional and the translational levels is dependent upon the single base mutation at nucleotide position −46. In addition, these results indicate that nonspecific factors (i.e., unexpected mutations) did not occur in the plasmids.

Expression of *smbA* and *smbB* genes in the BM71 *smbAB* mutant. In order to directly verify that the Smb-positive phenotype is influenced by the G nucleotide at position −46, the *smbA* and *smbB* gene expression plasmid, named pSmbABH, was constructed by using the fragment amplified from strain BM71. In addition, Smb-negative phenotype expression vector pSmbABL was also constructed with FSM-6. These plasmids were introduced into the BM71 *smbAB* mutant, and the Smb activities of these strains were analyzed by the bacteriocin agar plate assays with the *S. salivarius* indicator strain (Fig. 4B). The BM71 *smbAB* mutant was completely devoid of bacteriocin activity (Fig. 1C). In the equivalent strain containing plasmid pSmbABH, the Smb activity was restored to the wild-type level. In contrast, the complemented strain harboring pSmbABL did not have enhanced bacteriocin activity. These results indicate that the nucleotide at position −46 upstream of the *smbA* gene directly influences Smb activity via the transcription of the *smbA* and *smbB* genes.

In conclusion, we characterized the differential antimicrobial activities of Smb in *S. mutans* strains. The Smb-positive strains were shown to possess all of the *smb* ORFs. The differential expression of the *smbA* and *smbB* genes resulted from a single nucleotide transversion. Although a recent report suggested a relationship between mutacin production and caries status (4, 6), statistical analysis failed to show a positive correlation. However, this analysis was based upon the genotypes of the bacteriocins. Our present results suggest that it is important to assess not only the genotypes of the bacteriocins but also the actual antimicrobial activities of the bacteriocins. The production of high levels of bacteriocin, including Smb, may kill some neighboring streptococcal species, and subsequently, *S. mutans* may predominate within dental plaque, leading to dental caries. Moreover, Smb production may induce the release of DNA from killed competing streptococci. The liberated DNA could then function as a component of a biofilm structure (8, 15). Subsequently, increased biofilm formation could also induce the expression of additional Smb via *S. mutans* quorum-sensing regulation (16, 23, 24, 25). However, the relationship between Smb production by *S. mutans* and caries status remains to be determined.

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