mechanical ventilation.[29] Considerable attention needs to be paid to oral biofilms and the oral hygiene of patients prior to surgery, even when the surgical site is some distance from the oral cavity. Our study suggests that good oral care of patients who are scheduled for surgery is important for satisfactory post-operative management.

Acknowledgement

This work was supported by a Grant-in Aid from the Ministry of Health, Labour and Welfare (H13-Iryo-001).

Conflicts of interest

No conflicts of interest were declared in relation to this article.

• Received for publication 1 September 2005
• Accepted subject to revision 26 September 2005 • Revised accepted 4 November 2005

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Relationships of anti-PAc (361–386) peptide salivary IgA antibody, eosinophils and basophils with periodontal status in the elderly

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Received 27 December 2005; revised 9 November 2006; accepted 10 November 2006
First published online January 2007.

DOI:10.1111/j.1574-695X.2006.00193.x

Editor: Jennelle Kyd

Keywords
eosinophil; PAc (361–386) peptide; salivary IgA; attachment loss; elderly.

Abstract

The amino acid residues 361–386 of *Streptococcus mutans* PAc includes an important region associated with the interaction between *S. mutans* and salivary components. We investigated the relationships between levels of the anti-PAc (361–386) peptide antibody (PPA) in saliva and periodontal status in 281 elderly subjects (mean age 77 years; 118 females, 163 males) by assessing dental calculus (CA), attachment loss (AL), pocket depth (PD), bleeding on probing (BOP) and various blood parameters. Enzyme-linked immunosorbent assay results revealed that subjects with a PPA level of greater than 0.1 (PPA detected group) showed a lower average value for number of sites with more than 6 mm of AL/6 points × 100/tooth (rAL6) than those with a PPA level of less than 0.1 (PPA not detected group). Furthermore, average values for rAL6 were significantly lower in the PPA detected group, and BOP, AL and rAL6 correlated positively and significantly with the percentage of eosinophils present in leukocytes in female subjects in both groups. PPA level had a negative correlation with percentages of basophils and eosinophils. The results indicate that systemic increases in numbers of eosinophils and basophils are associated with the development of periodontal diseases, while PPA level may be a useful indicator of periodontal status.

Introduction

In Japan, the majority of elderly people suffer from periodontitis (Dental Health Division of Health Policy Bureau Ministry of Health and Welfare Japan, 1999), which is a major factor in tooth loss (Ong, 1998; Olver & Brown, 2000). However, at present there is no known useful indicator or predictor of periodontal status. Periodontal diseases are chronic inflammatory conditions that affect the well-vascularized connective tissues of the periodontium (Egelberg, 1996). At the end of the 1990s, aggressive periodontitis was redefined as a complex disease exhibiting microbial alterations and cellular dysfunction that is differentiated from chronic periodontal disease by the underlying molecular mechanisms of its pathogenesis (Armitage, 1999). Periodontal inflammation may have an effect on and worsen systemic conditions associated with leukocyte migration from the bloodstream into tissues at the site of inflammation, and several prominent neutrophils have been implicated as amplifiers of that inflammatory response (Batino *et al.*, 1999; Yamalik *et al.*, 2000).

In the human oral cavity, gram-negative anaerobic organisms reside in a complex mixed-species biofilm that forms on tooth surfaces and in periodontal pockets, with *Porphyromonas gingivalis*, a gram-negative anaerobe, recognized as one of the primary pathogens in severe manifestations of adult periodontitis (Socransky & Haffajee, 1992; Kolenbrander & London, 1993). Recently, Lamont *et al.* reported that the surface protein antigen of *Streptococcus gordonii* (SspB), which is also a member of the highly conserved PAc (Okahashi *et al.*, 1989) in *Streptococcus mutans*, interacted with fimbriae from *P. gingivalis*, as shown by the results of *in vitro* assays (Lamont *et al.*, 1994; Park *et al.*, 2005). *Streptococcus gordonii* and *Streptococcus sanguinis* are early colonizers of the salivary pellicle, while *S. mutans* colonizes at later stages. However, the abilities of each to bind to salivary proteins and glycoproteins as well as the differences in affinity to the salivary pellicle between *P. gingivalis* and *S. mutans* may be related to the virulence of supra- or subgingival microbial communities in dental diseases.

Streptococcus mutans has been reported to have an association with the development of biofilm and dental

caries on tooth surfaces (Hamada & Slade, 1980; Loesche, 1986). The function of the cell-surface protein antigen of *S. mutans*, also known as PAc (Okahashi *et al.*, 1993), Ag I/II (Russell & Lehner, 1978), PI (Forester *et al.*, 1983) and B (Russell, 1979), is essential for colonization by the bacterium on tooth surfaces as well as its interaction with the salivary pellicle that coats dental enamel (Russell & Mansson-Rahemtulla, 1989; Demuth *et al.*, 1990; Senpuku *et al.*, 1996a). The alanine-rich repeating region (residue 219–464, A-region) of the PAc molecule, which is important for bacterial interaction with the salivary pellicle (Brady *et al.*, 1992; Nakai *et al.*, 1993; Timmerman *et al.*, 2000), has a strong immunogenicity in humans (Senpuku *et al.*, 1996b) and has been proposed as a candidate antigen for inducing the production of antibodies that inhibit the adherence of *S. mutans* to tooth surfaces (Senpuku *et al.*, 1995, 2001). The PAc (361–377) peptide in the A-region containing the epitope has also been shown to induce an antibody that inhibits the interactions of *S. mutans* with salivary components on tooth surfaces and is considered to be important for the adherence of *S. mutans* to tooth surfaces (Senpuku *et al.*, 2001; Takeuchi *et al.*, 2001). The overlapped area of the PAc (370–386) peptide to the PAc (361–377) peptide includes a multiple binding motif (L––V–K––A) that reacts with human leukocyte antigen (HLA)-*DRB1*0802*, **1101*, **1402* and **1405* genotypes, and is also found in the A-region (Senpuku *et al.*, 1998). The high production of salivary IgA antibody levels in reaction to the coupled PAc (361–386) peptide from residues 361–377 and 370–386 was reported to be a unique indicator of population and proportion of mutans streptococci (mS), such as *S. mutans* and *Streptococcus sobrinus*, because low and high concentrations of the salivary antibody were found to be correlated positively and negatively, respectively, with the concentrations of mS in saliva from human subjects (Tsuha *et al.*, 2004).

In our search for a new indicator or predictor of periodontal diseases, the present study analysed the relationships between the production of anti-PAc (361–386) peptide salivary IgA antibody (PPA), blood status and periodontal status. PPA level was found to be associated with attachment loss (AL) in both males and females, and bleeding on probing (BOP) and pocket depth (PD), as well as concentrations of total streptococci (tS), lactobacilli (LB) and basophils in females. The results may provide important information on PPA for the development of preventive medicines for periodontal diseases.

Materials and methods

Human subjects

In 1998, a longitudinal interdisciplinary study of ageing was initiated to evaluate the relationships between health status and dental diseases, such as root caries and periodontal disease, in Japan. Initially, questionnaires were sent to all 4542 residents aged 70 years (born in 1927) in Niigata City. After dividing by sex, 600 subjects were selected randomly, with approximately the same numbers of each sex chosen for the baseline survey (Yamaga *et al.*, 2002; Yoshihara *et al.*, 2003). The participants agreed to undergo medical and dental examinations, and signed informed consent forms regarding the protocol, which was approved by the Ethics Committee of Niigata University Graduate School of Medical Dental Science. The study was carried out in accordance with the Helsinki Declarations. Follow-up surveys have been carried out every year in June using the same methods as in the baseline survey. Among the participants ($n = 399$) in the follow-up survey conducted in June 2005, 281 subjects (average age 77 years old; 118 females, 163 males) participated in the present measurements of salivary antibodies and blood parameters.

Dental examinations were conducted under artificial white light by trained dentists. According to WHO criteria (WHO, 1986), decayed teeth (DT), missing teeth (MT) and filled teeth (FT) (DMFT) scores were recorded along with findings of dental caries. Four calibrated dentists assessed subject periodontal conditions based on the results from six measurements points (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, distolingual) around each tooth. Intra- and interexaminer reliability was confirmed using a kappa statistic ($k = 0.56$–0.92 for AL). Two hundred fifty-eight subjects (112 females and 146 males) participated in the present measurements of periodontal status. To estimate periodontal status, rCA (rate of occurrence of sites with dental calculus), rAL4 (rate of occurrence of sites with greater than 4 mm of AL), rAL6 (6 mm of AL), rPD4 (rate of occurrence of sites with greater than 4 mm of PD), rPD6 (6 mm of PD), AL [length (mm) of attachment loss] and BOP (rate of occurrence of sites with blooding on probing) were also measured at the same six points of each tooth (Ogawa *et al.*, 2002). Thereafter, the indicators were assessed and used to estimate the periodontal status of each subject, according to the methods explained below.

Synthetic peptide

The sequences of PAc (361–386) (NAKATYEAALKQYEADLAAVKKANAA) and PAc (346–364) (AALTAENTAIKQRNENAKA) were derived from the sequence of the PAc gene from *S. mutans* MT8148, as reported by (Okahashi *et al.*, 1989). The peptide was synthesized using a stepwise solid-phase procedure at Asahi Techno Glass Co. Inc. (Tokyo, Japan). Synthesized peptide samples were subsequently purified by reversed-phase HPLC on a TSK-GEL column (1 × 30 cm) (TOSO, Tokyo, Japan) with a 10–45% acetonitrile gradient in 0.1% trifluoroacetic acid (TFA) and developed

over 50 min at a flow rate of 5 mL min^{-1}. Purity was determined to be greater than 95% in each tube by HPLC analysis. To confirm the amino acid sequences of the synthetic peptides, several samples were randomly selected, and then analysed using a System 7300 Amino Acid Analyzer (Beckman, NJ) and a Model 477A Protein Sequencer (Applied Biosystems, Foster City, CA). The peptide was used as a coating antigen in enzyme-linked immunosorbent assay (ELISA) examinations to determine the antibody titre in the saliva samples.

Human saliva collection

Whole saliva samples were collected on swabs after stimulating by biting paraffin gum for 5 min and placed in transport fluid (0.4% agar, 0.15% thioglycolate/phosphate-buffered saline), to determine the numbers of mS and tS, which was performed by Bio Medical Laboratory (BML, Tokyo, Japan). Other saliva samples was also collected after stimulation by biting paraffin gum and placed into ice-chilled sterile bottles over a period of 5 min, which were then clarified by centrifugation at 10 000 *g* for 10 min, filter-sterilized and used immediately for measuring the antibody levels.

ELISA

For enumeration of the IgA specific to *S. mutans*, 96-well microtitre H-plates (Sumitomo Bakelite, Tokyo, Japan) were coated overnight at 4 °C with 100 μL of PAc (361–386) and PAc (346–364) peptides (concentration 20 μg mL^{-1}) or skimmed milk (as a control) in coating buffer at pH 9.6, and then subjected to ELISA (Senpuku *et al.*, 1996b). The plates were washed with phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20 (PBST) and blocked with 1% (w/v) skimmed milk in PBST for 1 h at 37 °C. Excess skimmed milk was removed by washing three times with PBST, and then a 100-μL aliquot of a 1 : 4 dilution of saliva was added to the wells and the mixtures were incubated for 1 h at 37 °C. The wells were then washed five times with PBST and further incubated for 1 h at 37 °C with 100 μL of alkaline phosphatase-conjugated goat antihuman immunoglobulin A (both heavy and light chains) antibodies (Zymed Laboratories, South San Francisco, CA). After five washes with PBST, bound antibodies were detected after the addition of 100 μL of para-nitrophenyl phosphate at 3 mg mL^{-1} as a substrate and incubation for 90 min at 37 °C. Absorbance at 405 nm was measured with a microplate reader (Multiskan Bichromatic Laboratory Japan, Tokyo, Japan). The experiments were performed independently three times, with similar results obtained in each.

Bacterial counts

Cotton swabs containing saliva samples from the elderly subjects were placed in transport fluid and taken to BML for analysis. Each sample was poured onto Mitis–Salivarius agar (Nippon Becton Dickinson Co. Ltd, Tokyo, Japan) or modified Mitis–Salivarius agar containing 0.2 U mL^{-1} of bacitracin (MMTSB) (Tsuha *et al.*, 2004) using an EDDY JET spiral plating system (IUL, S.A., Barcelona, Spain), and incubated at 37 °C under anaerobic conditions for 48 h, before counting tS and mS organisms. MMTSB is known to be extremely precise for the counting of mS colonies (Tsuha *et al.*, 2004). Following anaerobic inoculation for 48 h at 37 °C, numbers CFU were counted. Colonies of mS were identified by their characteristic appearance and the mS ratio was calculated as colony numbers of mS/colony numbers of tS × 100. All bacteria counting was performed by BML.

Blood parameters

Ten millilitres of blood was extracted from each of the elderly subjects, placed in a sterilized glass tube including heparin, and sent to Niigata Rinsyo Laboratory (Niigata, Japan) to determine the following blood parameters: leukocyte count, erythrocyte count, haemoglobin concentration, haematocrit count, platelet count, mean cellular volume (MCV), mean cellular haemoglobin (MCH), mean cellular haemoglobin concentration (MCHC) and total serum IgA. A percentage of the different types of leukocytes was generated based on the counts of eosinophils, basophils, lymphocytes, monocytes and neutrophils.

Group comparisons

The subjects were divided into two groups according to ELISA antibody titre, those with a PPA level $\leq$ 0.1 (PPA not detected group) and those with a PPA level >0.1 (PPA detected group). This grouping by antibody level has been used in a previous epidemiological study (Tsuha *et al.*, 2004). The Mann–Whitney *U*-test was used to compare mean periodontal status and blood parameters between the two groups. Fisher's correlation test was used for correlation among PPA level, oral bacterial status, basophils, eosinophils and periodontal status in females. Differences at $P = 0.05$ were considered to be significant. StatView for Macintosh (Version 10.0) was used for all statistical analyses.

Results

There were no significant differences for the various parameters (DMFT, tooth number, sound tooth number, saliva volume) between the two groups (data not shown). Table 1 shows the periodontal status of the two groups based on antibody level, as determined by ELISA. The average rAL6 result (8.7 ± 14.1) in the PPA detected group was significantly lower than that (14.7 ± 22.9) in the PPA not detected group. In addition, females in the PPA detected group had lower values for average BOP, AL, rAL6 and rPD (8.9 ± 11.1,

Table 1. Periodontal status of subjects in PPA detected and non-detected group

Periodontal status	PPA non-detected group ($n = 40$)	PPA detected group ($n = 218$)	P-value*
rCA	1.6 ± 4.6	1.1 ± 3.3	0.864
BOP	11.4 ± 13.1	8.9 ± 11.5	0.348
AL	3.7 ± 1.3	3.4 ± 1.0	0.220
rAL4	44.1 ± 32.6	37.1 ± 28.1	0.236
rAL6	14.7 ± 22.9	8.7 ± 14.1	0.035*
RPD	2.2 ± 0.1	2.2 ± 0.5	0.989
rPD4	11.6 ± 13.9	10.4 ± 11.9	0.882
rPD6	2.4 ± 4.4	2.0 ± 4.5	0.555

*Significant difference demonstrated by Mann–Whitney U test

3.4 ± 1.0, 9.0 ± 13.8 and 2.2 ± 0.5, respectively, $n = 99$) as compared with those in the nondetected group (16.8 ± 17.2, 4.1 ± 1.7, 22.1 ± 32.6 and 2.5 ± 0.7, respectively, $n = 13$) but these differences were not significant. Antibody specificity to the PAc (361–386) peptide was also confirmed by comparisons between PPA and the anti-PAc (346–364) peptide antibody (positive control) (Senpuku *et al.*, 1996b), and the antiskimmed milk antibody (negative control). The PAc (346–364) peptide has an antigenic epitope that recognizes human antibodies (Senpuku *et al.*, 1996b), but no correlation was found between this and numbers of mS, tS and LB organisms in saliva or the various periodontal status parameters (data not shown). There were no significant differences between males in the two groups for any of the periodontal parameters. For female subjects, to clarify the systemic association of PPA with periodontal status, various blood status parameters, such as leukocytes, erythrocytes, haemoglobin and haematocrit, were tested and compared between the two groups. The PPA detected group had significant lower values for eosinophils (2.5 ± 1.8%) and basophils (0.5 ± 1.3%) than the PPA not detected group (4.0 ± 2.0% and 0.8 ± 0.3%, respectively) (Table 2). However, there were no significant differences between the two groups when only males were analysed. For females, PPA showed a positive correlation with LB and tS counts and a negative correlation with proportion of basophils ($P = 0.0015$, 0.0433 and 0.0340, respectively) (Table 3). The number of tS organisms also showed a positive correlation with LB and mS numbers ($P = 0.0004$ and < 0.0001, respectively), while the number of LB organisms showed the largest correlation with number of mS in all comparisons ($P = 0.0170$). The percentage of eosinophils was positively correlated with that of basophils, as well as AL, BOP and rPL6 ($P = 0.029$, 0.0439, 0.0475 and 0.0168, respectively). However, there were no significant differences between PPA level and total serum IgA level (data not shown). Thus, it was clear that PPA level in saliva responded to the progression of periodontal disease, as well as to the proportions of eosinophils and basophils in female subjects.

Table 2. Blood status between PPA detected and non-detected female subjects

	PPA non detected group ($n = 13$)	PPA detected group (104)	P-value
Leukocyte ($\times 10^3\,\mu L^{-1}$)	5.9 ± 1.3	5.8 ± 1.4	0.118
Erythrocyte ($\times 10^6\,\mu L^{-1}$)	4.2 ± 0.3	4.0 ± 0.4	0.187
Hemoglobin ($g\,dL^{-1}$)	39.2 ± 3.2	12.3 ± 1.4	0.362
Hematocrit (%)	12.6 ± 1.1	38.0 ± 3.9	0.302
Platelet ($\times 10^4\,\mu L^{-1}$)	21.1 ± 5.7	21.1 ± 5.4	0.948
MCV (fl)	93.8 ± 3.7	94.4 ± 4.0	0.475
MCM (pg)	30.2 ± 1.4	30.4 ± 2.6	0.390
MCHC (%)	32.2 ± 0.7	32.4 ± 2.6	0.948
Eosinophil (%)	4.0 ± 2.0	2.5 ± 1.8	0.005*
Basophil (%)	0.8 ± 0.3	0.5 ± 0.3	0.003*
Lymphocyte (%)	33.7 ± 6.9	36.9 ± 6.9	0.192
Monocyte (%)	5.6 ± 1.0	5.6 ± 1.4	0.882
Neutrophil (%)	56.0 ± 8.2	54.5 ± 6.6	0.578

*Significant difference demonstrated by Mann–Whitney U test

Table 3. Correlation among PPA level, oral bacteria status, basophil, eosinophil and periodontal status in female

	Correlation	P-value
PPA vs. LB	0.297	0.0015*
PPA vs. tS	0.193	0.0433*
PPA vs. mS	0.044	0.6484
tS vs. LB	0.328	0.0004*
tS vs. mS	0.423	< 0.0001*
LB vs. mS	0.227	0.0170*
PPA vs. Eosinophil	−0.119	0.2160
PPA vs. Basophil	−0.195	0.0340*
Eosinophil vs. Basophil	0.272	0.0029*
AL vs. Eosinophil	0.192	0.0439*
BOP vs. Eosinophil	0.189	0.0475*
rAL6 vs. Eosinophil	0.227	0.0168*

*Significant differences demonstrated by Correlation Fisher' Test

Discussion

The present results suggest that the presence of PPA indicates immunological activities that induce the production of human IgA antibodies to tS, but not those to mS, in elderly subjects. A positive correlation between antibody levels in saliva and previous infection and colonization with tS containing *S. mutans* and *S. sobrinus* in the oral cavity is suggested. Furthermore, a high concentration of PPA has been speculated to play a role as a negative contributor in proportion to the numbers of mS organisms in the oral cavity (Tsuha *et al.*, 2004). However, in the present study, the mS ratio was higher in the PPA detected group than in the PPA not detected group for all subjects, as well as for female and male subjects separately (1.5 ± 3.7 vs. 1.0 ± 1.8, 1.7 ± 3.9 vs. 1.3 ± 1.9 and, 1.4 ± 3.4 vs. 0.8 ± 1.8, respectively),

although the differences between the two groups were not significant.

Amino acid residues 365–377 [PAc (365–377) peptide] in the A-region is an antigenic epitope for the induction of antibodies that inhibits the interactions of *S. mutans* PAc with human salivary components (Senpuku *et al.*, 1995, 2001). Furthermore, the common epitope (YEA-L-QY) between the surface protein antigen (PAg) of *S. sobrinus* (Okahashi *et al.*, 1993) and its PAc, as well as its core B-cell epitope (–Y–––L––Y–) are essential sequences in the antigenic epitopes of the surface proteins of oral streptococci that are specifically recognized by the antibody (Senpuku *et al.*, 1997). The antibody to the core epitope in PPA may serve as an indicator of infection by tS as well as mS colonization. Because PPA plays roles as a positive indicator and negative effector of mS infection, the noncorrelation between PPA level and mS count in saliva seen in our study might have been caused by the combination of positive and negative effects. In addition, the correlations between numbers of LB organisms and PPA, tS and mS indicate that LB may be incorporated with tS biofilm formation.

Periodontitis often induces bone absorption and gingival recession through gingival inflammation. In addition, gingival recession changes the oral condition, which may cause ecological changes, resulting in microbiological changes such as the development of supragingival plaque-containing streptococcal bacteria (Quirynen *et al.*, 1999; Reiker *et al.*, 1999). Marsh found that the optimal growth conditions for streptococci were different from those for other periodontal pathogens (Marsh, 2003), while other studies have shown that the growth of periodontal pathogens is not correlated with or inhibited by *S. mutans*, and also that the number of streptococci was important for growth conditions for periodontal pathogens (Drake *et al.*, 1993; Grenier, 1996). Therefore, an increase in tS number may produce PPA and inhibit the growth of microorganisms that are associated with the progression of periodontal diseases.

In the present study, the PPA detected group showed a significantly lower number of sites with rAL6 than the PPA not detected group, while the PPA detected group also showed a lower rate of AL in female subjects. Several studies have reported a relationship between CA and AL (Albandar *et al.*, 1996; Timmerman *et al.*, 2000; Neely *et al.*, 2001; Cobb, 2002); however, our PPA not detected group showed a significant progression of AL, but not of CA deposition. Therefore, there may be contrasting variables involved in the relationships between the antibody and AL increases and reduction with ageing. We performed a comparison between PPA and periodontal status in a small number of elderly subjects over a 4-year period (initial study in 2001; average age 73 years; 60 males, 27 females). Two years after obtaining baseline data, 62 of the subjects from the original cohort participated in follow-up examinations (2003 study; average age 75 years; 45 males, 17 females) and 1 year later 69 subjects from the original cohort (2004 study; average age 76 years; 46 males, 23 females) also participated, with periodontal status continuously observed in the PPA detected and PPA not detected groups. We found that AL was significantly lower in the PPA detected group in all subjects at every examination (data not shown), indicating a consistent association of AL with induction of PPA.

In the present study, eosinophils were correlated positively with periodontal status during the progression of periodontitis, while basophils were also correlated positively with eosinophil and negatively with PPA levels. Eosinophils and basophils are known to be associated with most inflammatory and infectious disorders associated with allergic manifestations. The eosinophil fraction in gingival crevicular fluid (GCF) from periodontitis patients has been reported to range from 6 to 10%, which is much higher than that in circulating blood (Sugita *et al.*, 1993), while GCF specifically yielded a higher rate of activated eosinophils in another study (Suzuki *et al.*, 1995). A striking common feature of many autoimmune and inflammatory diseases in humans is that females are more susceptible to specific immunological disorders than males (Ansan Ahmed *et al.*, 1985). Our results support those data, and also suggest that eosinophils and basophils play important roles in host immune and defensive systems in response to periodontitis in elderly females.

The present findings suggest that the anti-PAc (361–386) peptide antibody is responsible for physical status in periodontal tissues, as well as systemic condition with regard to eosinophil and basophil proportions among leukocytes during the development of periodontal disease. They also imply that the induction of PPA may be indirectly correlated with some of the factors that inhibit periodontal pathogens. We conclude that this antibody is useful as a predictor of periodontal diseases in elderly patients.

Acknowledgements

We thank Drs Yoshiaki Nomura, Yuzo Tsuha, Maki Kozu, and Nao Kusumoto for helpful discussions, advice and technical support. This work was supported in part by a Grant-in-Aid for Development of Scientific Research (15390571) from the Ministry of Education, Science, and Culture of Japan, and by a grant from the Japan Health Science Foundation to H.S.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2006, p. 6277–6287 Vol. 72, No. 9
0099-2240/06/$08.00+0 doi:10.1128/AEM.00614-06

Assessment of Genes Associated with *Streptococcus mutans* Biofilm Morphology

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Received 16 March 2006/Accepted 23 June 2006

***Streptococcus mutans*, the major pathogen responsible for dental caries in humans, is a biofilm-forming bacterium. In the present study, 17 different pulsed-field gel electrophoresis patterns of genomic DNA were identified in *S. mutans* organisms isolated clinically from whole saliva. The *S. mutans* isolates showed different abilities to form biofilms on polystyrene surfaces in semidefined minimal medium cultures. Following cultivation in a flow cell system in tryptic soy broth with 0.25% sucrose and staining using a BacLight LIVE/DEAD system, two strains, designated FSC-3 and FSC-4, showed the greatest and least, respectively, levels of biofilm formation when examined with confocal laser scanning microscopy. Further, image analyses of spatial distribution and architecture were performed to quantify the merged green (live cells) and red (dead cells) light. The light intensity of the FSC-3 biofilm was greater than that of the FSC-4 biofilm in the bottom area but not in the top area. *S. mutans* whole-genome array results showed that approximately 3.8% of the genes were differentially expressed in the two strains, of which approximately 2.2%, including bacitracin transport ATP-binding protein gene *glrA* and a BLpL-like putative immunity protein gene, were activated in FSC-3. In addition, about 1.6% of the genes, including those associated with phosphotransferase system genes, were repressed. Analyses of the *glrA*-deficient strains and reverse transcription-PCR confirmed the role of the gene in biofilm formation. Differential assessment of biofilm-associated genes in clinical strains may provide useful information for understanding the morphological development of streptococcal biofilm, as well as for colonization of *S. mutans*.**

Streptococcus mutans, a biofilm-forming bacterium considered to be the primary etiological agent of human dental caries (16), possesses a variety of abilities to colonize tooth surfaces. *S. mutans*, under certain conditions, is numerically significant in cariogenic biofilms and forms biofilms with other organisms in the oral cavity (6) following colonization and the eruption of primary teeth (3). Further, epidemiological surveys have confirmed that greater numbers of *S. mutans* organisms in children are associated with a higher incidence of decayed, missing, and filled (dmf) teeth (13, 25).

Recently, considerable research with a variety of microscopy techniques has focused on the structure of intact visible biofilm. In previous studies, the spatial arrangement of microorganisms in dental biofilm samples showed voids outlined by layers of vital bacteria, which themselves were packed in layers of dead materials (2, 18). Although the structure of biofilm has been reported, few studies have quantified the differences in structure, although studies of the cellular functions that are modified during the cellular transition from the planktonic to the biofilm state are in progress, with some findings recently reported (23).

Isolation and characterization of the genes involved with biofilm formation may contribute to elucidating how *S. mutans* responds to environmental signals in the oral cavity. Previous studies have indicated the roles of sucrose and glucosyltransferases in *S. mutans* biofilm formation (48). Others have implicated several genes that are associated with genetic competence (24), including *com* genes (25, 50), which play other regulatory roles, together with one or more other genes, including *ccpA* and *brpA* (*lytR*) (43) and *luxS* (29). Those genes have also been shown to function with putative two-component response regulators (4), which are involved in biofilm formation. DNA microarrays have been used to monitor global gene expression profiles in response to different stimuli (44), such as heat shock and other stresses (17, 45), quorum sensing (10, 35), anaerobic metabolism (49), sporulation (11), and biofilm formation (35, 44). For example, a recent study of *Pseudomonas aeruginosa* by using a DNA microarray technique showed that only 1% of the genes were differentially expressed in biofilms compared to those expressed in suspension cells (44). In contrast, a proteomics approach used to study *P. aeruginosa*-associated biofilms showed a greater number of changes than with transcription profiling (42). Although there have been studies on biofilm formation by a variety of bacteria by using DNA microarrays, little is known about that associated with *S. mutans*. In addition, many reports have compared planktonic cells with biofilm cells by using laboratory strains (36, 37, 44); however, there is scant information regarding differential gene expression in biofilm formation in clinical strains. Clinical strains can survive in severe conditions involving various antibacterial agents, such as seen in the oral cavity, which leads to the expression of several genes involved with biofilm formation. In contrast, laboratory strains may not require those genes while being cultured under mild and planktonic conditions and may lose their expression.

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TABLE 1. Oligonucleotide sequences of PCR primers

Primer	Direction[a]	Sequence[b]	Target gene product	Product size (bp)
PS0941-a	Fw (EcoRI)	5′-CCGAATTCACTTGTCCAAACGATATGGA-3′	Bacitracin transport ATP-binding protein GlrA	1,047
	Rev (PstI)	5′-CCCTGCAGTGTGGAAGCTGGATACCAA-3′		
PS0941-b	Fw	5′-GCGATCAAAGAATTTCGGC-3′		434
	Rev	5′-TTCTAAATCTTGGCGTTTG-3′		
PS1731	Fw	5′-AGACTTTTAACAGCGCAGC-3′	BLpL-like putative immunity protein	338
	Rev	5′-CCCCATAATAAAAGAAGAGAG-3′		
PS0092	Fw	5′-CTCATAATCATATGGCATCAG-3′	Putative PTS system IIA component	386
	Rev	5′-CTCATCCCCCTCATCATTT-3′		
PS1365	Fw	5′-GATGAAGAGGAAAGCGTATT-3′	Conserved hypothetical protein S1365	340
	Rev	5′-GCCTCCCGAAATGGTTTTA-3′		
LARNA5	Fw	5′-GTTGTCCGGATTTATTGGG-3′	Control primer	248
LARNA6	Rev	5′-GGGTATCTAATCCTGTTCGC-3′		
GTFB-F	Fw	5′-ACTACACTTTCGGGTGGCTTGG-3′	Glucosyltransferase GtfB	517
GTFB-R	Rev	5′-CAGTATAAGCGCCAGTTTCATC-3′		
GTFB-FIN	Fw	5′-AAAGCAGATTCTAATGAATCGA-3′	Glucosyltransferase GtfB (inner primer)	469
GTFB-RIN	Rev	5′-AATGTAAAATTTTGCCATCAGC-3′		
GTFI-F	Fw	5′-GATAACTACCTGACAGCTGACT-3′	Glucosyltransferase GtfI	712
GTFI-R	Rev	5′-AAGCTGCCTTAAGGTAATCACT-3′		
GTFI-FIN	Fw	5′-TGGTATCGTCCAAAATCAATCC-3′	Glucosyltransferase GtfI (inner primer)	664
GTFI-RIN	Rev	5′-AGATTTGCAGTTGGTCAGCATC-3		

[a] Fw, forward; Rev, reverse.
[b] Restriction site sequences are underlined.

In the present study, we attempted to quantify the intensity of biofilm formation by *S. mutans* clinical strains by measuring biofilm volume with confocal laser scanning microscopy (CLSM). Further, we analyzed how gene expression differs between various isolated *S. mutans* strains that showed either high or low levels of ability to form biofilm, and we compared the results with those regarding biofilm formation ability by using DNA microarray and reverse transcription-PCR (RT-PCR) methods.

MATERIALS AND METHODS

Subjects. One hundred seventy-four pairs of mothers and children (3 years old) from the city of Yokohama in Japan participated in this study. The aim and details of the experiments were explained, and consent was obtained from all subjects prior to obtaining the samples. The study was conducted according to the ethical guidelines of Asahikawa Medical College and the Helsinki Declaration. Dental examinations were conducted under artificial white light by trained dentists. According to WHO criteria (47), scores for dmf teeth were recorded, along with findings of dental caries.

Human saliva collection. Whole saliva samples were collected on cotton swabs from the subjects, after stimulation by biting paraffin gum for 5 min, and then placed in transport fluid (0.4% agar, 0.15% thioglycolate/phosphate-buffered saline [PBS]) and sent to Bio Medical Laboratory (BML) (Tokyo, Japan) for measurement of mutans streptococci (mS) and total streptococci (tS). Other saliva samples from three healthy human subjects (28 to 42 years old) were also collected after stimulation by biting paraffin gum and placed into ice-chilled sterile bottles over a period of 5 min. The samples were then clarified by centrifugation at 10,000 × *g* for 10 min, filter sterilized, and used immediately for biofilm assays, utilizing a flow cell system and a 96-well plate.

Sampling and bacteriological methods. At BML, each saliva sample were poured onto mitis salivarius agar (MTS) (Nippon Becton Dickinson Co. Ltd., Tokyo, Japan) or modified MTS containing 0.2 U/ml of bacitracin (MMSB) (38), using an Eddy Jet spiral plating system (IUL, S.A., Barcelona, Spain), and incubated at 37°C under anaerobic conditions for 48 h. MTS and MMSB were used to count tS and mS, respectively. tS and mS colonies were identified by their characteristic appearance, and the mS ratio was calculated as the number of mS colonies/number of tS colonies × 100. Further, colonies were collected at random and tested using a PCR method with the GTF primers listed in Table 1, as previously reported (32). DNA from the bacteria was extracted using a DNeasy tissue kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The PCR assays were designed to discriminate between *S. mutans* and *Streptococcus sobrinus* by targeting the genes encoding the glucan-synthesizing enzyme (*gtfB* and *gtfI*). *S. mutans* isolates were propagated overnight in brain heart infusion (BHI) (Difco Laboratories, Detroit, MI) broth and frozen at −80°C, after the addition of glycerol to 25%, for subsequent use in genomic DNA preparations that were analyzed by pulsed-field gel electrophoresis (PFGE).

PFGE. PFGE analysis of mutans streptococcal DNA was performed as described elsewhere (19, 20), with minor modifications. Frozen stock cultures were poured onto BHI agar plates and incubated at 37°C under anaerobic conditions (BBL GasPak Systems; Becton Dickinson and Company, Franklin Lakes, NJ). Colonies on the BHI agar plates were scraped off using a sterilized spoon and resuspended in 200 μl of distilled water (DW) (optical density of 0.2). An equal amount of 1% low-melting-point agarose (Bio-Rad Laboratories, Richmond,

CA) was then prepared in DW, mixed gently, melted in a microwave oven until liquid, and added to each cell suspension, after which the mixture was transferred to Bio-Rad plug molds (two wells per sample) with a pipette. Two plugs were prepared for each isolate and refrigerated on ice for 20 min.

For restriction endonuclease digestion, one half of a plug slice from each isolate was suspended overnight in a mixed solution (3 μl NotI or SmaI, 10 μl H or T buffer, 10 μl 0.1% bovine serum albumin, and up to 100 μl DW) at 37°C. The plug slices were then loaded and run on a 1% pulsed field certified agarose (Bio-Rad) gel in 45 mM Tris-borate–1 mM EDTA buffer. PFGE was performed using a CHEF-DRII apparatus (Bio-Rad Laboratories) in 0.5× 45 mM Tris-borate–1 mM EDTA buffer at 10 to 12°C. The switch times used for directional change of the electrical fields for NotI and SmaI were 40 seconds for 20 h at 180 V and 1 to 30 seconds for 20 h at 200 V, respectively. A lambda liner DNA ladder (Bio-Rad) ranging from 48.5 to 970 kbp was used as the size standard. Following PFGE, the gels were stained with ethidium bromide (0.2 μg/ml) and photographed under UV transillumination.

Biofilm formation assay. Biofilm formation by all strains was assayed using a method described previously (24, 26), with some modifications. To evaluate the biofilms formed by the isolated *S. mutans* strains, 40 μl of cell suspension (8×10^6 CFU) and 160 μl of semidefined minimal medium (SDM) (chosen a minimal medium) (24) or tryptic soy broth without dextrose (Difco) supplemented with 0.25% sucrose (TSB) (chosen a rich medium) were mixed in the wells of 96-well (flat-bottom) microtiter plates (Sumitomo Bakelite, Tokyo, Japan). Before addition of the cell suspension, the wells were coated with whole saliva in TSB culture but were not coated in SDM, as biofilms are unable to form in saliva-coated wells in the presence of SDM. After the plates were incubated at 37°C for 16 h under anaerobic conditions, the liquid medium was removed and the wells were rinsed a second time with sterilized DW. The plates were then air dried and stained with 0.25% safranin for 15 min. After being stained, the plates were rinsed with DW to remove excess dye and then air dried. Each biofilm was examined without dissolving with solvent, using an enzyme-linked immunosorbent assay microplate reader (Multiskan Bichromatic; Laboratory Japan, Tokyo, Japan), as the biofilm was applied uniformly onto the bottoms of the wells in the 96-well plates. Quantification of the stained biofilm was performed by measuring A_{492} absorbance.

Flow chamber experiments. Biofilm samples were cultivated at 37°C in three-channel flow cells (46) with individual channel dimensions of 1 by 4 by 40 mm and supplied with a flow of TSB, as biofilms under flow show better formation when cultured in TSB than in SDM. The flow system (Stovall Flowcell; Stovall Life Science Inc., Greensboro, NC) was assembled and prepared as described by Christensen et al. (8). The substratum consisted of a microscope glass coverslip, and the flow cells were covered with filter-sterilized human saliva samples and left for 30 min. After the saliva was carried away, the cells were inoculated with an overnight culture of each strain diluted to 1×10^6 to 10×10^6 CFU/ml. Following inoculation, the 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/hour for 20 h using a peristaltic pump (Ismatec; IDEX Corp., Glattbrugg-Zürich, Switzerland).

CLSM. Noninvasive confocal imaging of fully hydrated biofilms was performed using a Fluoview CLSM (Olympus, Tokyo, Japan) fitted with a water immersion dipping objective lens (100×) and a Kr-Ar laser. The specimens were stained for 30 min with BacLight LIVE/DEAD bacterial viability kit solution (4 ml of DW 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*) optodigital sections, each 5.0 μm thick, with the intervening gaps between the horizontal sections ranging from 0 to 50 mm over the entire height (*z* axis) of the biofilm. In addition, we analyzed the vertical (*xz*) sections, which were recorded from the center of each biofilm, to determine the architecture. Each biofilm was scanned at five randomly selected positions away from the disk edge. The digital images were processed using Fluoview software version 2.0 (Olympus).

Image analysis. The intensity profiles of both viable (488-nm) and nonviable (568-nm) channels for the image stacks were calculated using Olympus Fluoview software. This application is able to save images in several formats and also allows numerical analysis of images, such as average pixel counts, and then saves the data as an Excel file for later analysis (28). The data were normalized against the maximum image intensity for the total intensities of both channels. Briefly, the software divided the optional area (141.145 μm by 141.145 μm) into a grid of 64 by 64 masses in *xy* planes and presented light intensity as a value of 0 to 4,095 for each mass (see Fig. 3C). The rate of light intensity per highest intensity value (4,095) was calculated in each mass. If the average of intensity rate or area (μm^2) was more than 25%, the intensity rate was recalculated as the volume of biofilm formation of green light (live cells) and red light (dead cells).

Cell harvest and total RNA isolation. To analyze the expression of mRNA in the *S. mutans* biofilms, biofilm cells were harvested from two sources: the microscope glass coverslips after removal from the flow cell chambers and the culture plates. As in the production of biofilm on six-well culture plates (Corning Incorporated, Corning, NY), 400 μl of cell suspension (8×10^6 CFU) and 1,600 μl of SDM were mixed and cultivated in the wells at 37°C for 16 h under anaerobic conditions. The biofilm cells were then scraped with a sterilized scraper after being washed three times with sterilized PBS. The planktonic cells were also cultivated in BHI. All cells were also harvested at 500 × *g* and 4°C and washed with sterilized PBS three times. Total RNA was isolated from the biofilm and planktonic cells by using a modified protocol with an RNeasy minikit (QIAGEN), which included an on-column DNase digestion with RNase-free DNase I (QIAGEN). Briefly, the harvested cells were suspended in PBS and sonicated, and then the samples were centrifuged at 5,000 × *g* at 4°C for 5 min. Next, the precipitate was resuspended in 100 μl of Tris-EDTA buffer containing 3 mg/ml lysozyme and 40 U of mutanolysin, followed by incubation at 37°C for 1 h, according to a modification of the manufacturer's instructions reported previously (51). Total RNA from extractions of flow cell biofilm samples performed four times was pooled and mixed to provide a sufficient quantity of RNA for analysis in the microarray. For the DNA microarray and RT-PCR analyses, 50 μg of total RNA from the flow cell biofilms and 1 mg of total RNA from biofilms or planktonic cells cultured in six-well plates were used.

DNA microarrays. DNA microarray analyses were performed using a NimbleGen system (NimbleGen Systems, Inc., Madison, WI). These arrays can detect 1,960 of the 1,963 *S. mutans* UA159 open reading frames (ORFs) (1). An array with three sets of *S. mutans* ORFs was used to precisely detect the relative gene expressions of the FSC-3 and -4 strains. Data analysis and determination of absent and present cells were performed using raw fluorescence intensity values with Microarray Suite version 5.0 (Affymetrix, Santa Clara, CA). Gene expressions of mRNA were normalized to frequency values by using the robust multichip average (5). The gene functions were obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/).

RT-PCR. For RT-PCR, cDNA templates were created from 1 μg of RNA by using the SuperScript first-strand synthesis system (Invitrogen Corp., Carlsbad, CA) according to the recommended procedure, with the primers shown in Table 1. To check for DNA contamination, purified total RNA without reverse transcriptase served as a negative control. The resulting cDNA and negative control were amplified using a TaKaRa PCR amplification kit (Takara Bio Inc). We assayed the FSC-3 and -4 strains by using cDNA synthesized from RNA extracted from two independent biofilms grown in SDM and TSB cultures, as well as from planktonic cells grown in BHI in triplicate for each gene. The results were normalized using the conserved primers LARNA5 and LARNA5, which were selected on the basis of a comparison between the available 16S rRNA sequences of lactobacilli and gram-positive bacteria, including oral streptococci (27), as internal controls. To analyze the gene expression quantitatively in RT-PCR, PCR was performed at various cycles (24 to 50). The gene expression between FSC-3 and FSC-4 was assessed at the acceptable PCR cycle to each primer for PCR production before stationary phase.

Construction of *glrA* mutant. An ORF for the *glrA* gene region was identified in the *S. mutans* UA159 database at the University of Oklahoma Advanced Center for Genomic Technology (http://www.genome.ou.edu/smutans.html). A mutant of the *glrA* gene was created using a double-crossover homologous recombination by insertion of an erythromycin resistance determinant into the gene. The plasmid used for inactivation of the *glrA* gene was prepared as follows. The PCR fragment of the *glrA* region containing the BamHI site within the gene was amplified with the forward primer for *glrA* (EcoRI) and the reverse primer for *glrA* (PstI) (Table 1, PS0941-a), using chromosomal DNA from *S. mutans* FSC-3 as the template. The amplified fragment was then inserted into multiple cloning sites of pUC19 (Invitrogen) following EcoRI-PstI digestion. The resultant plasmid was digested with BamHI, after which the erythromycin cassette isolated from pResEmMCS10 was inserted (34). The plasmid was linearized with SphI, and *S. mutans* FSC-3 was transformed with the resultant linear plasmid. Confirmation that plasmid insertion caused gene disruption was by either Southern blotting or PCR (data not shown).

Statistical analysis. Comparisons of biofilm formation levels between FSC-3 and -4 and between FSC-3 and FSC-3D *glrA* were performed using a Mann-Whitney U test. Differences of 0.05 were considered statistically significant.

RESULTS

Isolation of *S. mutans* from human subjects. *S. mutans* was isolated from both mother and child in 19 (10.9%) of 174

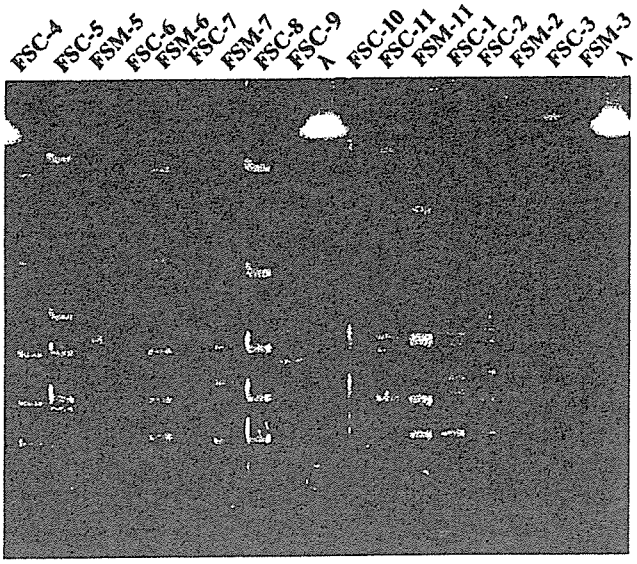

FIG. 1. PFGE patterns of genomic DNA from *S. mutans* isolates. At least 17 different PFGE patterns were found in the paired samples from mothers and children. NotI was used for digestion. λ, lambda DNA ladder molecular size standards; FSC, strain from child; FSM, strain from mother; 1 to 11, subject numbers. FSC and FSM strains with the same number are child-mother pairs. The data shown are representative of three independent experiments.

mother-child pairs, of which 11 (57.9%) paired samples were identified by PCR and cultivated for use in the following experiments. Some of the *S. mutans* organisms from the other eight pairs died during transit to our laboratory from BML. A single colony was randomly taken from one MMSB plate per subject in our laboratory. The PFGE pattern of DNA digested with NotI or SmaI from the *S. mutans* sample from a mother was identical to that for the *S. mutans* sample from her child for 5 (45.5%) of the 11 mother-child pairs. Following digestion with NotI from *S. mutans*, 17 different patterns of genomic DNA were found, including the 5 fragment patterns of genomic DNA from children that were identical to those from their mothers (FSC-1, FSC-4, FSC-8, FSC-9, and FSC-10) (Fig. 1). Thus, various *S. mutans* genetic types were isolated from our human subjects.

Evaluation of biofilm formation by isolated *S. mutans*. Seventeen different types of *S. mutans* on polystyrene surfaces in SDM were characterized in an in vitro biofilm formation assay (Fig. 2A). In SDM, strains FSC-3, FSM-5, FSC-6, and FSC-7 showed greater levels of biofilm formation, while FSC-1, FSM-2, FSM-3, FSC-4, FSC-5, FSC-8, and FSC-10 showed lower levels of biofilm formation, than a laboratory strain (*S. mutans* MT8148) (Fig. 2A). In the TSB cultures, all strains except FSC-1 and FSM-3 showed greater levels of biofilm formation than *S. mutans* MT8148 (Fig. 2B), while *S. mutans* MT8148 showed a level of biofilm formation similar to that of the other laboratory strains, *S. mutans* MT6229 and OMZ175, in both SDM and TSB cultures (data not shown). The growth rates in planktonic cell culture were comparable for all clinical strains and laboratory strains (data not shown). These results indicated multiple and medium nutrition-dependent variations in the abilities of *S. mutans* with different PFGE patterns to form biofilms on polystyrene surfaces. The 11 strains from the FSC child group in SDM cultures could be equivalently divided into two groups: those with high biofilm formation (greater than that of MT8148) (FSC-2, -3, -6, -7, and -11) and those with low biofilm formation (lower than that of MT8148) (FSC-1, -4, -5, -8, -9, and -10). Comparisons between the high and low biofilm formation groups were performed for incidence of dmf teeth, mS number, and mS ratio in saliva. The incidence of dmf teeth, mS number, and mS ratio were each greater in the high biofilm formation group (3.0 ± 4.7, $17.2 \times 10^3 \pm 27.8 \times 10^3$ CFU/ml, and $3.1\% \pm 5.5\%$, respectively) than in the low biofilm formation group (1.2 ± 2.2, $9.6 \times 10^3 \pm 18.1 \times 10^3$, and $0.8\% \pm 0.9\%$, respectively), whereas there were no significant differences in clinical status. Thereafter, the FSC-3 and FSC-4 strains were selected and used as typical strains showing greater and lower, respectively, biofilm formation in the following experiments using the flow cell system, while *S. mutans* MT8148 served as a control.

Image analysis of FSC-3 and FSC-4 biofilms. The biofilm formations of FSC-3 and FSC-4 in the flow cell system were observable in the condition using TSB but not in the condition using SDM. Therefore, TSB was chosen as a better experimental medium to compare biofilm formation between FSC-3 and FSC-4 in the flow cell system. Optical sections 5 μm apart on two axes were analyzed in CLSM images of the FSC-3 and FSC-4 biofilms. Eleven sections (0 to 50.0 μm) and image analysis of the bottom area (0 μm) are shown in Fig. 3. FSC-4 and *S. mutans* MT8148 biofilms had voids similar to those described by Auschill et al. (2). In contrast, FSC-3 showed a greater level of biofilm formation than the others and did not show a void (Fig. 3A and B). In the *xy* planes, we analyzed the average light area and light intensity to determine the quality of biofilm formation and found that the light area and intensity decreased from the bottom to the top in all of the samples. Although there was a clear difference between the volumes of biofilm formation by FSC-3 and FSC-4 in the bottom portions of the biofilms (approximately 1.8-fold), there was no difference in the top areas (Fig. 4). Further, the relative tendency of light intensity was similar to that of light area for all three strains (data not shown).

Genes differentially expressed in two clinical strains. Microarray analysis was performed to compare gene expression in the FSC-3 and FSC-4 biofilms. The array contained 1,960 of the 1,963 *S. mutans* UA159 ORFs. Of the total number of predicted ORFs, 63% are considered to have a putative function, 21% have homologs with different species (though their function is unknown), and 16% are unique to *S. mutans* (1). A few of these genes showed differential expression in FSC-3 biofilms compared with FSC-4 biofilms (Fig. 5). In total, 74 genes (3.8%) showed differential expression, 43 (2.2%) were activated (genes for bacteriocin secretion protein, bacitracin transport ATP-binding protein GlrA, and BLpL-like putative immunity protein, as well as others), and 31 (1.6%) were repressed (genes for phosphoenolpyruvate:sugar phosphotransferase system [PTS] IIA, B, C, and D and the Na^+-driven multidrug efflux pump, as well as others) (genes exhibiting a four- to eightfold difference are shown in Table 2, and those exhibiting a greater-than-eightfold difference are shown in Table 3). Further, 30 genes (12 of those showing a four- to eightfold difference and 18 of those showing a greater-than-eightfold difference) selected randomly were examined by

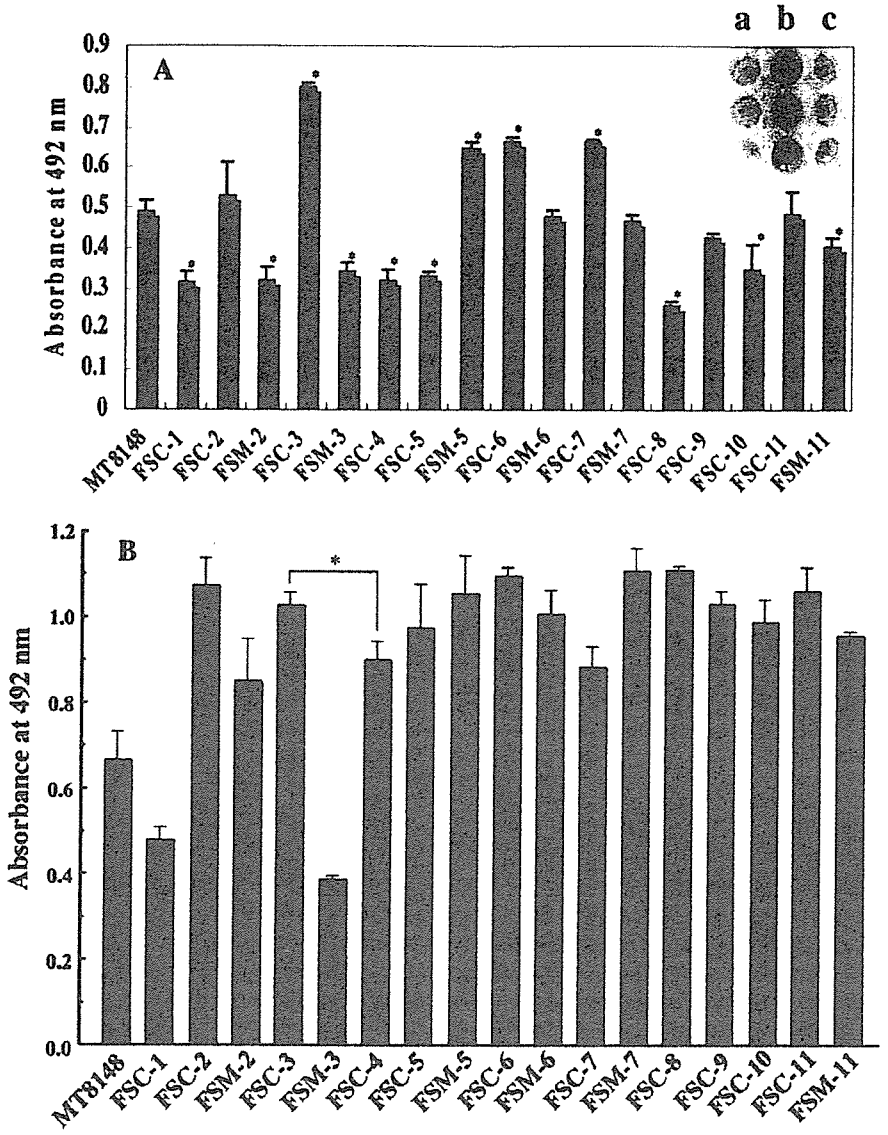

FIG. 2. Biofilm formation by 17 *S. mutans* genotypes. Graphs show quantification of biofilms formed after 16 h of culture in SDM (A) and TSB (B). The upper right photograph shows typical biofilms grown on polystyrene microtiter plates (a, FSM-2; b, FSC-3; c, FSM-3). The results are expressed as the means ± standard deviations from three independent assays. MT8148, *S. mutans* MT8148. Asterisks denote significantly different ($P < 0.05$) relative levels of biofilm formation (A, other clinical strains versus MT8148; B, FSC-3 versus FSC-4).

RT-PCR to confirm the gene expression shown by the microarray results. The gene expressions in the RT-PCR results showed tendencies of difference between FSC-3 and FSC-4 that were similar to those in the microarray results (data not shown).

RT-PCR analysis of biofilm and planktonic cells. The genes for GlrA, the BLpL-like protein, PTS IIA, and hypothetical protein S1365 were randomly selected from the protein groups with positive and negative fold changes and the conserved hypothetical protein group (Table 3), respectively, as target genes to confirm their contribution to biofilm formation in the experimental stage, with two paired primers (PS0941-b, PS1731, PS1365, and PS0092) designated for these genes (Table 1). Amplifications with PS1731 and with PS1365 and PS0092 resulted in both higher and lower expressions of FSC-3 than of FSC-4 in planktonic cell (Fig. 6). Further, amplifications with PS1731, PS1365, and PS0092 showed a significant and similar expression of mRNA in the biofilm cells from both the FSC-3 and FSC-4 strains (Fig. 6). Amplification with PS0941-b showed a higher expression of FSC-3 compared to FSC-4 in biofilm cells and a similar expression of FSC-3 and FSC-4 in planktonic cells (Fig. 6). No PCR products were obtained from total RNA preparations that had not been initially reverse transcribed (data not shown).

Biofilm formation by *glrA* deficient mutant. The *glrA* was enhanced ninefold or greater in FSC-3 biofilms compared to the FSC-4 biofilms in the DNA microarray results (Table 3). Further, a higher expression of *glrA* was also shown in FSC-3 biofilms compared to FSC-4 biofilms in our RT-PCR analysis (Fig. 6). These results suggest that *glrA* interacts with this stage

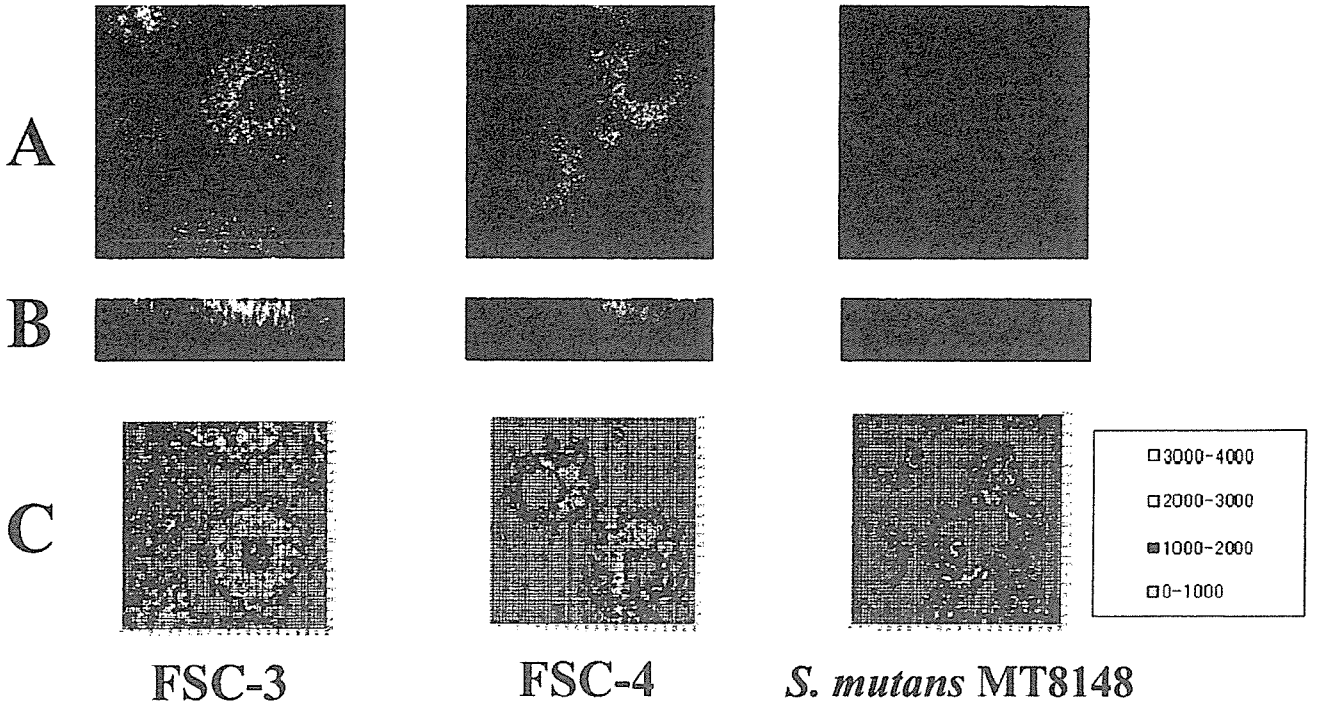

FIG. 3. (A) CLSM images showing *xy* planes (141.145 μm by 141.145 μm) of the biofilm bottom area. Viable cells are colored green, and nonviable cells are colored red. (B) CLSM images showing *xz* planes (141.145 μm by 50.0 μm) recorded from the center of a single biofilm shown in panel A. (C) Two-dimensional contour map generated from the CLSM image shown in panel A. As a control, *S. mutans* MT8148 organisms were analyzed. Light intensities were divided into four color groups as shown in the box. The data are representative of three independent experiments.

of biofilm bottom formation. To confirm whether *glrA* contributes to the increased biofilm volume of FSC-3 in comparison with FSC-4, a *glrA*-deficient mutant of FSC-3 was constructed and compared with FSC-3 (wild type), using a 96-well microplate and flow cell biofilm formation (Fig. 7). FSC-3 Δ*glrA* showed significant differences in biofilm formation in comparison with FSC-3 in both TSB and SDM in 96-well microplate cultures (Fig. 7A). The growth rates were comparable in the deficient and wild-type strains (data not shown). In the flow cell system, horizontal biofilm formation volumes at 5, 10, and 15 μm from the bottom of the biofilm were significantly lower, while those at 25, 30, 35, 40, 45, and 50 μm from the bottom were significantly higher, in FSC-3 Δ*glrA* compared to FSC-3 (Fig. 7B).

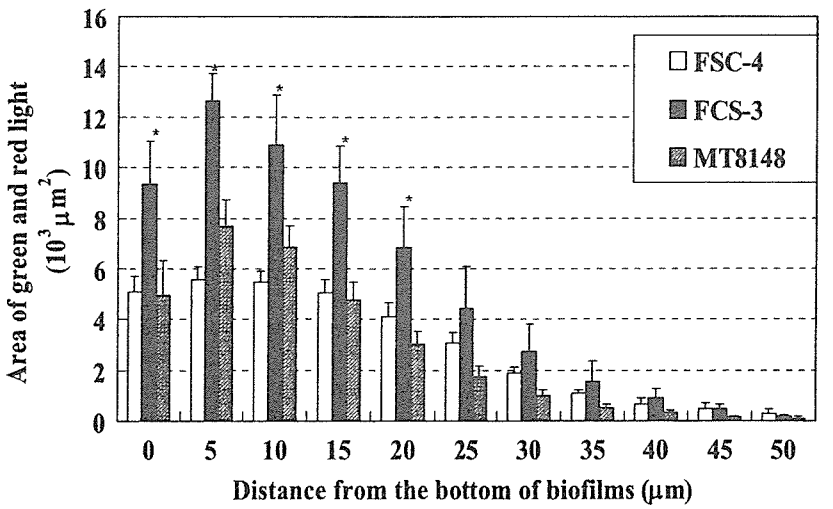

FIG. 4. Quantification of merged light areas of green and red in *xy* planes of CLSM images. Each biofilm was scanned at five randomly selected positions. Data are representative of three independent experiments. The results are expressed as the means ± standard deviations of triplicate assays. Asterisks denote significantly different relative levels of biofilm formation ($P < 0.05$; versus MT8148).

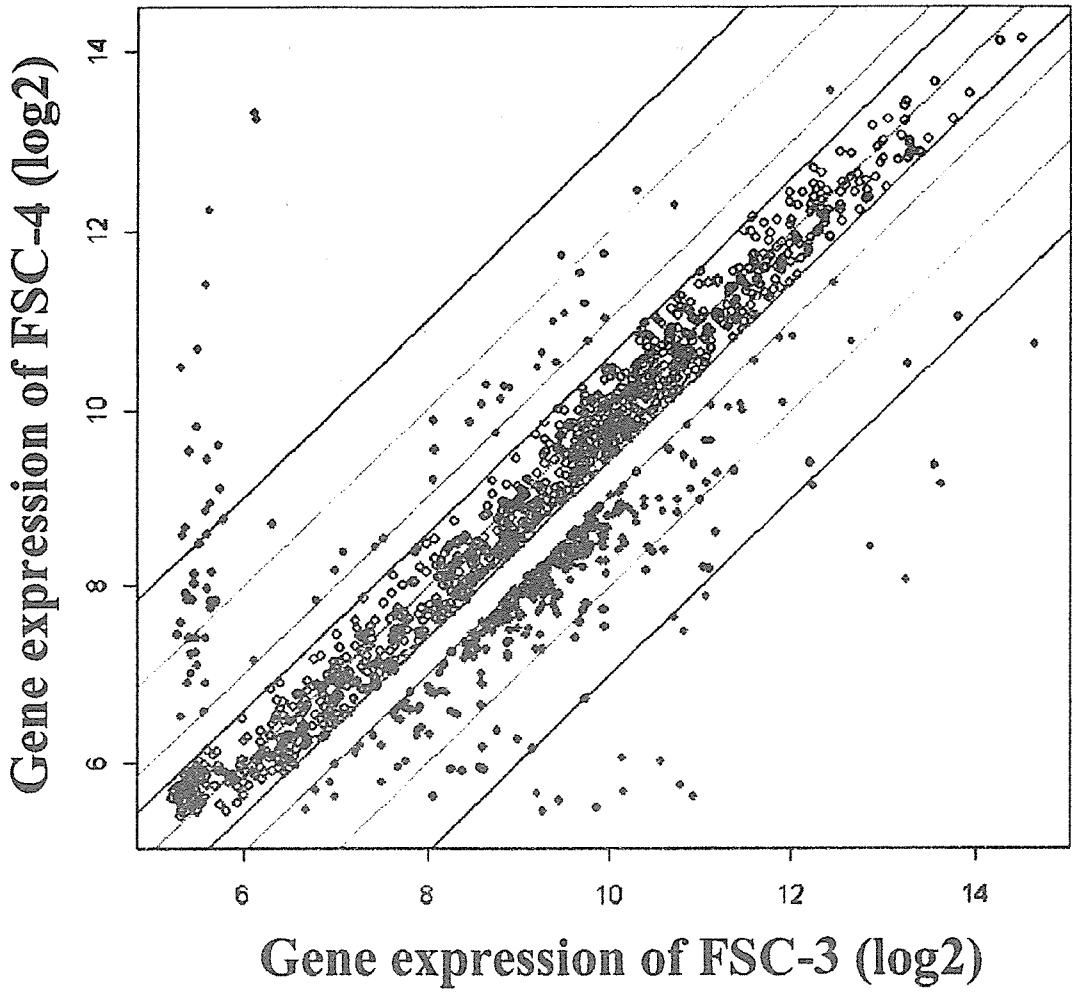

FIG. 5. Scatter plot showing intensities of the spots on an *S. mutans* microarray. Each gene expression was normalized using the robust multichip average, estimates of which were based on a robust average of $\log_2$ [B(PM)], where B(PM) represented background corrected PM (perfect match) intensities. Black dots, genes that showed less than 1.5-fold regulation; yellow dots, 1.5- to 2.0-fold; red dots, 2.0- to 4.0-fold; violet dots, 4.0- to 8.0-fold; green dots, more than 8.0-fold.

DISCUSSION

In spite of recent increased interest in biofilm research, little information is available on the physiology of the organisms that comprise surface-associated communities. Biofilm cells growing on surfaces exhibit properties distinct from those of planktonic cells, such as increased resistance to biocides and antimicrobial agents. In the present study, we investigated these mechanisms by using morphological and genetic analyses of clinical isolates of *S. mutans* as a means to analyze their biofilms. Because the clinical isolated strains might have been transmitted from mother to child and had survived in the severe conditions of the oral cavity, they were considered superior for testing compared to laboratory strains and were thought to express important genes for biofilm formation. DNA microarray and RT-PCR analyses were used to obtain gene expression profiles for the biofilms. We attempted to observe the biofilm samples in a state where the medium was flowing, with fresh nutrition continuously supplied. We utilized a flow cell system (8, 46) that provided a suitable in vitro method of growing reproducible biofilms for modeling dental plaque.

Regardless of the state of the medium, i.e., with (TSB) or without (SDM) added sucrose, FSC-3 showed a greater ability to form a biofilm than FSC-4 in both the 96-well microplates cultivated with SDM and flow cell systems cultivated with TSB (Fig. 2 to 4). However, the amount of FSC-4 biofilm increased and approached that of FSC-3 cultivated with TSB in the 96-well microtiter plate (Fig. 2). It was suspected that glucan-dependent biofilm formations were essential in FSC-3 and FSC-4 in the plate culture with rich medium supplemented with sucrose. The relative difference in biofilm formation between FSC-3 and FSC-4 in the 96-well microtiter plates was not similar to that in the flow cell system with TSB. Therefore, it was considered that biofilm formation and development in the nonflow 96-well microplate system were different from those in the flow cell system, even when the medium nutrition components were identical and included sucrose. Biofilm formation by FSC-3 was higher than that by FSC-4 in SDM, which

TABLE 2. Genes differentially expressed (four- to eightfold difference) in two clinical strains

S. mutans ORF		Fold change[a]
Product	No.	
Transmembrane protein Tmp5	S1870	4.1
Dipeptidyl aminopeptidase/ acylaminoacyl-peptidase-related protein	S0669	4.2
Hemolysin III	S0852	4.2
Murein hydrolase export regulator	S0522	4.3
SUN protein	S0435	4.6
UDP-glucose 4-epimerase	S1869	4.7
DNA-damage-inducible protein J, putative	S1611	5.0
Citrate transporter	S0921	5.3
PTS system, cellobiose-specific IIC component	S1445	5.3
Sterol biosynthesis methyltransferase related (*Pyrococcus abyssi*)	S0338	5.5
PTS system, lactose-specific IIA component	S1447	6.1
Putative ABC transporter, ATP-binding protain	S1704	6.5
Acetylornithine aminotransferase	S0341	6.5
BlpU (*S. pneumoniae*)	S1725	6.7
Bactoprenol glucosyl transferase	S1871	7.1
Transport protein ComB	S1720	−4.0
Putative transposase, ISSmu1	S1713	−4.5
Transcriptional regulator, MutR family	S1367	−4.6
Dihydrolipamide acetyltransferase component of pyruvate dehydrogenase complex	S0115	−4.7
Putative site-specific DNA-methyltransferase restriction-modification protein	S0039	−4.8
Phage-related protein	S1273	−5.3
Putative transposase, ISSmu1	S1279	−5.5
PTS system, IIC component	S0090	−5.6
Putative transposase, ISSmu1	S0395	−5.9
Sucrose operon repressor	S0094	−7.9
Hypothetical proteins	S0043	−7.8
	S0149	6.7
	S0150	7.0
	S0229	5.9
	S0670	4.6
	S1362	4.6
	S1712	−7.8
Conserved hypothetical proteins	S040	−4.5
	S0174	5.4
	S0354	7.6
	S0657	5.3
	S0743	−4.4
	S1446	7.9

[a] Fold change calculated as the $\log_2$ of gene expression in FSC-3 versus FSC-4. Positive values represent activation; negative values represent repression.

TABLE 3. Genes differentially expressed (more-than-eightfold difference) in two clinical strains

S. mutans ORF		Fold change[a]
Product	No.	
Putative bacteriocin secretion protein	S1724	8.6
Bacitracin transport ATP-binding protein GlrA	S0941	9.0
Contains similarity to (p)ppGpp synthase (GTP pyrophosphokinase)	S1099	13.9
Phosphoglycerol transferase	S0754	14.5
Glucosyltransferase (side chain biosynthesis)	S0755	16.9
Putative immunity protein, BLpL-like	S1731	21.2
Glycosyltransferase involved in cell wall biogenesis	S0757	22.4
L-type calcium channel, alpha 1 subunit	S1729	22.4
Transcriptional regulator	S0344	23.4
Large-conductance mechanosensitive protein	S0742	32.5
Bactoprenol glucosyltransferase	S0756	39.2
Na^+-driven multidrug efflux pump	S1364	−9.4
Putative PTS system, IID component	S0091	−9.5
PTS system, IIB component	S0089	−9.8
Alpha-glucosidase	S0093	−9.9
Heme biosynthsis protein	S1366	−17.3
Putative PTS system, IIA component	S0092	−20.2
BlpJ (*S. pneumoniae*)	S1715	−97.0
BlpU (*S. pneumoniae*)	S1710	−137.5
BlpU (*S. pneumoniae*)	S1716	−147.8
Hypothetical proteins	S0029	−14.3
	S1098	14.5
	S1437	20.7
	S1610	11.6
	S1705	10.0
	S1711	−14.5
	S1721	−56.3
	S1722	−10.2
	S1727	18.2
	S1728	14.9
	S1730	35.8
Conserved hypothetical proteins	S0042	−10.2
	S0048	−35.6
	S0049	−36.2
	S0261	8.4
	S1365	−19.8

[a] Fold change calculated as the $\log_2$ of gene expression in FSC-3 versus FSC-4. Positive values represent activation; negative values represent repression.

did not contain sucrose, in a 96-well microtiter plate. Therefore, it was considered that genes not related to sucrose contribute to *S. mutans* biofilm formation, as well as genes, such as *gtf*, related to sucrose.

In regard to clinical status, the high biofilm formation group showed a possible relationship, with increases in incidence of dmf teeth, mS number, and mS ratio in saliva in comparison with the low biofilm formation group. Therefore, the differences in volume between the FSC-3 and FSC-4 biofilms indicated that distinctive strains of *S. mutans* might have been transmitted to and involved in pathogenic activity during the development of oral cavities in the 3-year-old children. A number of studies that used a serotyping method have shown that the initial acquisition of mutans streptococci by a child comes from the mother during infancy (14, 15), as have studies that used bacteriocin typing (14, 41) and genotyping (14, 22).

The PTS is the primary means of sugar transport in oral streptococci, especially under carbohydrate-limited conditions, and it plays important roles in global control of gene expression (33, 39). A previous study of *S. mutans* strains demonstrated that glucose PTS activity was markedly decreased in cells grown at pH 5.5 compared to those grown at neutral pH and suggested that repression occurs at the level of EII synthesis (40). On the other hand, in a study of *Streptococcus parasanguinis*, the addition of glucose to different types of media enhanced biofilm formation (12), whereas enriched media inhibited biofilm formation by *Streptococcus gordonii* (26) and *S. mutans* (50) in other studies.

Our results showed that further induction of the PTS system gene components (IIA, B, C, and D) was not correlated with

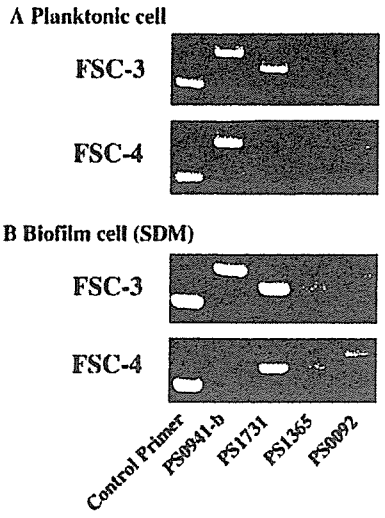

FIG. 6. RT-PCR analyses of biofilm and planktonic cells from *S. mutans* clinical strains. For FSC-3 and -4, we used the primers PS0941-b, PS1731, PS1365, and PS0092 to amplify the target genes described in Table 1. A control primer was used to normalize the expression of the test genes. Total bacterial RNA was isolated from biofilm and planktonic samples, and RT-PCR was performed as described in Materials and Methods. The data shown are representative of two independent assays.

increased biofilm formation in the bottom area in a comparison between the FSC-3 and -4 strains in culture medium that included sucrose (Fig. 4; Tables 2 and 3). However, RT-PCR analysis demonstrated that the PTS IIA gene was expressed in biofilm cells from both FSC-3 and -4 strains cultured in SDM, which was similar to that for FSC-3 and FSC-4 biofilm cells cultured in the nonflow system (Fig. 6). In contrast, the gene was expressed in planktonic cells of FSC-4 but not those of FSC-3 in BHI culture. Thus, PTS activity may depend on the various states of biofilm formation in in vitro nonflow and flow systems.

S. mutans is known to be cariogenic and resistant to bacitracin, and a number of mechanisms of bacitracin resistance have been reported for various bacteria (7, 31, 37). In the bacitracin-producing organism *Bacillus licheniformis*, resistance is encoded by the *glrABC* genes, which encode a putative heterodimeric ATP-binding cassette (ABC) transporter that has been proposed to mediate the active efflux of bacitracin (30). Homologs of this transporter have been identified in *Bacillus subtilis* (31) and *S. mutans* (9, 37). In the present study, *glrA* was enhanced ninefold or greater in FSC-3 biofilms compared to the FSC-4 biofilms in the DNA microarray analysis (Table 3). Further, a higher expression of *glrA* was also shown in FSC-3 biofilms compared to FSC-4 biofilms in our RT-PCR analysis (Fig. 6). To confirm the contribution of *glrA* to biofilm formation, FSC-3 Δ*glrA* was constructed and its biofilm formation was compared with that of FSC-3. FSC-3 Δ*glrA* demonstrated a lower level of biofilm formation at the bottom area than FSC-3, whereas the mutant, surprisingly, showed a higher level of biofilm formation at the top (Fig. 7). These findings indicate that *glrA* has a relationship to the morphology of *S. mutans* biofilm.

In the present study, we found that several regulatory genes of the Blp protein and the transport protein ComB were particularly repressed in FSC-3, which showed a high ability to form biofilm (Tables 2 and 3). Those results demonstrate that these genes do not have an association with the stage of greater biofilm formation by *S. mutans* that occurs at the bottom. In addition, a recent study investigated the role of the *S. mutans relA* gene, which codes for guanosine tetraphosphate and guanosine pentaphosphate [(p)ppGpp] synthetase/hydrolase, in both biofilm formation and acid tolerance. It was also found that the expression of the *luxS* gene was increased by as much as fivefold in the *relA* mutant, suggesting a link between AI-2

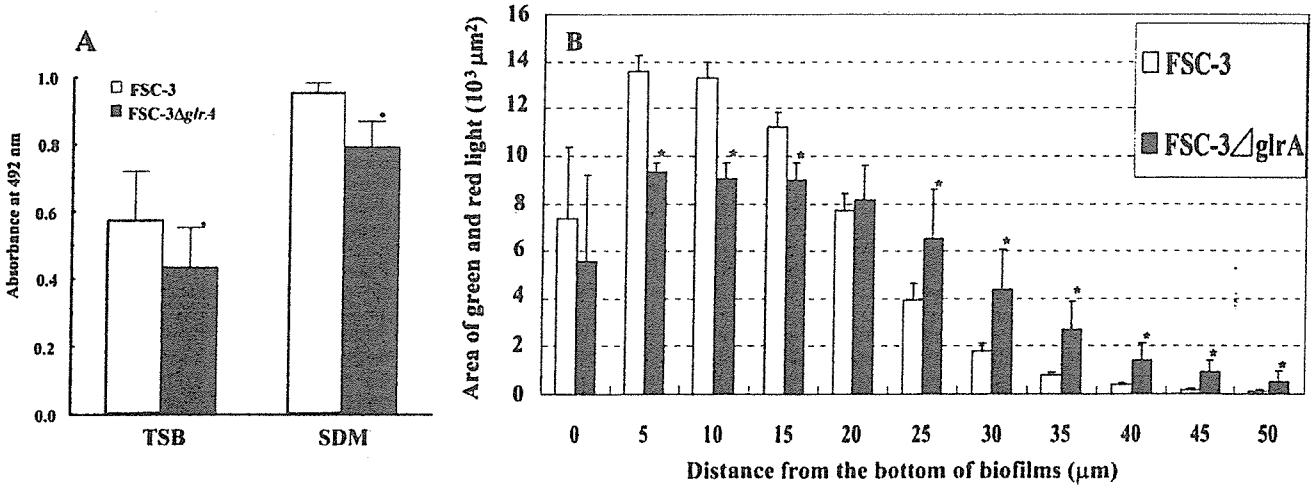

FIG. 7. Biofilm formation by FSC-3 Δ*glrA* and FSC-3. (A) Quantification of biofilm after 16 h of culture in TSB and SDM in 96-well microtiter plates. The results are expressed as the means ± standard deviations from three independent assays. (B) Quantification of biofilm after 20 h of culture in TSB using the flow cell system and fluorescence area in *xy* planes of CLSM images. Each biofilm was scanned at five randomly selected positions. The results are expressed as the means ± standard deviations from three independent assays. Asterisks denote significantly different relative levels of biofilm formation ($P < 0.05$; versus FSC-3).

quorum sensing and the stringent response (21). Our findings revealed that genes similar to the (p)ppGpp synthase gene were up-regulated in FSC-3.

Several genes involved in biofilm formation have been identified in a variety of organisms (24, 50). In the present study, we also found several genes that were associated with the ability of biofilm morphological formation in experiments using different clinical strains. However, little is known regarding the molecular interactions that are involved with transducing signals between the genes that trigger biofilm development. Therefore, definitive conclusions regarding how these signals are sensed, interacted with, and transduced in biofilm-forming bacteria, as well as definitions of the molecular mechanisms utilized to initiate the development of biofilm, require further investigation using genes from knockout and complement strains of *S. mutans*. Nevertheless, the present results provide useful information for understanding and assessing streptococcal biofilm, as well as determination of the potential risk for mother-child transmission and dental caries.

ACKNOWLEDGMENTS

We thank Tsutomu Sato and Fusao Nishikawara for their technical support.

This work was supported in part by grants-in-aid for development of scientific research from the Ministry of Education, Science, and Culture of Japan (15390571) and from the Ministry of Health, Labor and Welfare (H16-Medical Services-014).

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