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Involvement of antigen I/II surface proteins in *Streptococcus mutans* and *Streptococcus intermedius* biofilm formation

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Background/aim: Dental diseases are caused by microorganisms organized in biofilms. *Streptococcus mutans* and *Streptococcus intermedius* are commensals of the human oral cavity. *S. mutans* is associated with caries, whereas *S. intermedius* is associated with purulent infections. Oral streptococci including *S. mutants* and *S. intermedius* express a family of surface proteins termed antigen I/II (Ag I/II). Ag I/II is implicated in adhesion; however, its role in biofilm formation has not yet been investigated.
Methods: By using isogenic Ag I/II-deficient mutants of *S. mutans* and *S. intermedius* we studied the influence of Ag I/II on *in vitro* biofilm formation. Biofilm was quantified in polystyrene microtiter plates and visualized by scanning electron microscopy. Ag I/II expression in planktonic and biofilm cells, as well as in the presence or absence of saliva was investigated by immunoblotting.
Results: In the presence of saliva, the Ag I/II-deficient mutants formed 65% less biofilm than the wild-types. In the absence of saliva, no difference was observed in *S. mutans*, whereas the *S. intermedius* Ag I/II mutant formed 41% less biofilm. Ag I/II expression was reduced in the presence of saliva. No differences in expression were observed between biofilm and planktonic cells.
Conclusion: The results indicated that Ag I/II may be important during biofilm formation particularly in the presence of saliva. These findings may provide useful information regarding the importance of Ag I/II in biofilm formation and in the search of new strategies to control biofilm-mediated infections.

Key words: antigen I/II; biofilm; saliva; streptococcus

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Most infections are caused by microorganisms organized in biofilm communities (4, 35). Biofilm formation is a dynamic process that involves initial adhesion of free-floating cells, coadhesion, growth and maturation, and finally detachment of some microorganisms (3, 36). In a biofilm, microorganisms encounter environmental conditions that differ from the conditions of planktonic cultures. Consequently, microorganisms in biofilms are phenotypically different from their planktonic counterparts, and may demonstrate alterations in cellular components such as proteins, fatty acids, and phospholipids associated with the cell envelope, and in the production of extracellular enzymes and polysaccharides (2, 9).

Streptococcus intermedius and *Streptococcus mutans* form part of the normal flora found in oral biofilms. *S. intermedius* is an early colonizer (11), also associated with deep-seated purulent infections (39). *S. mutans* is a late colonizer of dental plaque (26) and is frequently associated with dental caries (23). Like other oral streptococci, *S. intermedius* and *S. mutans*

are implicated as causative agents of infective endocarditis (11, 12, 32, 39).

Bacterial interaction with the host depends on associations with surface proteins (16). The oral streptococci express a family of structurally and antigenically related surface proteins termed antigen I/II (Ag I/II) (15). These proteins have been given a variety of names according to the strains or species in which they were identified, such as antigen B (33), Sr (27), and PAc (28) from *S. mutans*; Spa A (20) from *Streptococcus sobrinus*; PAa from *Streptococcus cricetus* (38); Pas from *S. intermedius* (38); and SspA and SspB from two genes in *Streptococcus gordonii* arranged in tandem (6). The Ag I/II C-terminal includes a conserved LPXTG motif important for anchoring of the protein to the cell wall by the sortase enzyme (25). Ag I/II shows multifunctional activities, including binding to soluble extracellular matrix glycoproteins and host cell receptors, coaggregation with other bacteria, interactions with salivary glycoproteins, and activation of monocytic cells (15). Thus, the success of oral streptococci colonization and survival within the human host may be related to Ag I/II. Ag I/II has species-specific functions and Ag I/II gene expression is influenced by variable environmental conditions (8, 31). Isogenic mutants of *S. mutans* and *S. gordonii* that lack or express reduced amounts of Ag I/II exhibit reduced adhesion to saliva-coated surfaces as well as to salivary agglutinin glycoproteins (SAG) compared with the wild-type (5, 19, 22). Previous studies with *S. mutans* and *S. intermedius* show that the initial adhesion to salivary films under conditions of flow was reduced in *S. mutans* isogenic Ag I/II-deficient mutants, whereas no difference was observed between *S. intermedius* wild-type and its isogenic Ag I/II-deficient mutant (30).

Most adhesion studies are based on the interaction of cells grown in liquid cultures with various surfaces. Such conditions differ from those of biofilm formation, in which the cells interact with the surfaces during growth. The biofilm mode of growth involves communication between cells, expression of new phenotypic traits, and responses to the biofilm environment itself, as part of the dynamic nature of biofilm formation. The aim of this study was to investigate whether Ag I/II of *S. mutans* and *S. intermedius* influences the formation of biofilms in the presence and absence of saliva. We also evaluated whether expression of Ag I/II is affected during biofilm formation and by the presence of saliva.

Material and methods

Bacterial strains and culture media

The *S. intermedius* strains used in this study were type strain NCTC 11324 and its Ag I/II isogenic mutant IB08981 (31). The *S. mutans* strains were *S. mutans* LT11 and the Ag I/II isogenic mutant IB10991 (30). *S. gordonii* (Challis) was included as it showed no significant differences in levels of mRNA *spaA* expression in planktonic and biofilm mode of growth in previous studies (10). All strains were stored at −70°C in brain heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with 15% (v/v) glycerol. Streptococci were grown in 5% CO_2 at 37°C in Todd-Hewitt broth (Difco Laboratories) supplemented with 0.2% yeast extract (THY).

Saliva collection

Fresh stimulated whole saliva samples were collected and pooled from six non-medicated individuals who had refrained from eating and drinking at least 60 min prior to collection. Saliva secretion was stimulated by parafilm chewing, and the saliva was collected on ice. Dithiothreitol (2.5 mM final concentration) was added to the collected saliva, and the mixture was gently stirred for 10 min at 4°C. The saliva was centrifuged at 5000 × *g* for 20 min, and the clarified saliva supernatant was filtered through a 0.22-μm pore size polyethersulfone filter (Schleicher & Schuell, Dassel, Germany). The samples were kept frozen at −20°C until used.

Biofilm formation assay

The biofilm formation assay was adapted from the method of O'Toole & Kolter (29) and is based on the ability of bacteria to form biofilms on solid surfaces such as polystyrene. Biofilm formation by *S. mutans* and *S. intermedius* wild-types and their isogenic Ag I/II-deficient mutants was compared. Before inoculation the wells of flat-bottom, 24-well microtiter polystyrene plates (Nunc, Copenhagen, Denmark) were conditioned with 500 μl Tryptone Soya Broth (TSB) or TSB supplemented with 25% saliva (TSBS) overnight at 4°C. Cells grown overnight in THY were diluted 1 : 200 directly in the overnight plates of TSB and TSBS. The plates were incubated at 37°C for 24 h anaerobically (Anoxomat System, WS9000; Mart, Lichtenvoorde, the Netherlands). After 24 h, supernatants were replaced by the respective fresh media and the plates were then incubated for another 24 h. Spent medium, including the unattached bacteria, was then aspirated and the remaining planktonic cells were removed by rinsing with 1 ml double distilled water. After drying, the cells in the biofilm were stained for 10 min with a 0.1% solution of safranin and the wells were again rinsed with distilled water. Bound dye was released from stained cells using 30% glacial acetic acid. Biofilm formation was quantified by measuring the absorbance of the solution at 530 nm with Wallac VICTOR2 1420 Multilabel Counter (Perkin Elmer, Turku, Finland). Each assay was performed in triplicate in three independent experiments.

Scanning electron microscopy (SEM)

SEM was used to examine the architecture of *S. mutans* and *S. intermedius* biofilms formed in 3 h or 48 h. Polystyrene disks (Nunc) were immersed into each well of flat-bottom, 24-well microtiter polystyrene plates and inoculated with 500 μl TSBS. The plates were stored overnight at 4°C. Cells grown overnight in THY were diluted 1 : 200 directly in TSBS. Biofilms were allowed to form according to the biofilm assay described above. The polystyrene discs were removed after 3 h or 48 h, rinsed with distilled water, and fixed with 2.5% glutaraldehyde in 0.1 M Sørensen phosphate buffer. Samples were critical point dried with liquid CO_2 and palladium/gold sputtered. The samples were then examined by SEM (model XL 30 ESEM; Philips, Eindoven, Netherlands)

Immunoblotting

Ag I/II expression in planktonic and biofilm cells of *S. intermedius*, *S. mutans*, and *S. gordonii* were investigated by immunoblotting. Overnight, grown cells were diluted 1 : 200 in TSB and 1.5 ml were inoculated in either 24-well microtiter plates for biofilm formation or in glass tubes for planktonic growth. Plates and glass tubes were incubated at 37°C in an anaerobic atmosphere for 24 h. The biofilm was washed twice and suspended in 500 μl 0.9% saline by scraping the bottom and lateral walls of the wells and was then transferred to an Eppendorf tube. The planktonic cells were transferred directly to an Eppendorf tube. Biofilm cells and planktonic cells were harvested by centrifugation (5500 × *g*, 10 min, 4°C), washed twice in PBS (phosphate buffered saline), and pellets kept at −20°C until used. Biofilm cells and planktonic cells were

then adjusted with PBS to equal optical density (OD_{650}). Ag I/II expression was determined by immunoblot detection of Ag I/II in whole cells adsorbed to nitrocellulose membranes. The blots were incubated with rabbit anti-I/II immunoglobulin G (IgG) raised against purified Sr (anti-SR I/II) from *S. mutans* OMZ 175 (the kind gift of Prof. J. Ogier). Antibody binding was revealed with alkaline phosphatase-conjugated goat antirabbit IgG and enzyme substrate (5-bromo-4-chloro-3-indolylphosphate and Nitro Blue Tetrazolium; both from Sigma). The images of the membranes were captured by ProEXPRESS Proteomic Imaging System (Perkin Elmer) and analyzed by the TOTALLAB program (Nonlinear Dynamics, Newcastle, UK). For quantitative titration, the pellets were serially diluted in PBS and values above the saturation level were excluded.

To study the effect of saliva on Ag I/II expression, cells of *S. intermedius* (11324), *S. mutans* (LT11), and *S. gordonii* (Challis), grown in THY overnight, were diluted 1 : 10 in fresh TSB medium, TSBS or dilute saliva (1 : 4 saliva in sterile water) and incubated for 2 h anaerobically at 37°C. The microorganisms were then harvested by centrifugation (5500 × *g*, 10 min, 4°C) and washed twice in PBS. Pellets were kept at −20°C until further analysis. The cells in TSB, TSBS or saliva were then adjusted with PBS to equal optical density (OD_{650}). The Immunoblot method described above was used to compare the expression of Ag I/II under the various incubation conditions.

Statistical analysis

The Wilcoxon signed rank test was used for statistical analyses of data. The level of significance was set at $p \leq 0.05$.

Results

Effect of saliva on biofilm formation by *S. mutans* and *S. intermedius* and their respective Ag I/II isogenic mutants

Biofilm formation by *S. mutans* and *S. intermedius* wild-types or their Ag I/II-deficient mutants was approximately 85% lower in TSBS than in TSB. When inoculated with saliva (TSBS), the Ag I/II-deficient isogenic mutant *S. mutans* IB10991 produced 65% less biofilm than the wild-type *S. mutans* LT11. No significant difference was found in biofilm formation between the *S. mutans* LT11 and the Ag I/II-deficient isogenic mutant IB10991 when grown in plain TSB medium. Compared to *S. intermedius* NCTC 11324, the Ag I/II-deficient isogenic mutant IB08981 formed 67% less biofilm in the presence of saliva and 41% less biofilm in plain TSB medium (Table 1).

Table 1. S. mutans and S. intermedius biofilm formation in TSB or TSB with saliva (TSBS)

Strain	TSB	TSBS
S. mutans (LT11)	3.278 (1.989 – 3.545)	0.515 (0.381 – 0.699)
S. mutans (Ag I/II mutant)	2.878 (2.661 – 3.624)	0.159 (0.132 – 0.251)[a]
S. intermedius (11324)	5.234 (4.815 – 6.356)	1.397 (1.150 – 1.606)
S. intermedius (Ag I/II mutant)	3.770 (2.761 – 4.034)[a]	0.511 (0.457 – 0.611)[a]

Median values corresponding to optical density of the biofilm cells stained by safranin, with the 25th and 75th percentiles in parentheses. The data represent three samples from three independent experiments.
[a]Significantly different from the wild type ($P < 0.05$) as calculated by the Wilcoxon signed rank test.

Adhesion and biofilm formation on saliva-coated surfaces

Scanning electron microscope images of adherent cells are shown in Fig. 1. In *S. mutans*, after 3 h of inoculation, the wild-type formed larger cell aggregates than the Ag I/II mutant. After 48 h incubation, more cells and more cell aggregates were still present in the wild-type compared to the mutant. *S. intermedius* showed no clear difference between the wild-type and Ag I/II mutant after 3 h of inoculation. After 48 h, however, the biofilm formed by the wild-type appeared to form more aggregates than the Ag I/II mutant.

Expression of Ag I/II by biofilm and planktonic cells

No statistically significant differences were found in Ag I/II expression on the cell surface of *S. intermedius*, *S. mutans* and *S. gordonii* when we compared biofilm-grown cells with their respective planktonic counterparts (data not shown).

Effect of saliva on Ag I/II expression

Ag I/II expression on the cell surface of *S. mutans* was slightly higher when cells were incubated in TSB medium rather than diluted saliva or TSB supplemented with saliva. *S. intermedius* showed no difference in Ag I/II expression during incubation in TSB or TSB supplemented with saliva. These values were, however, higher than in diluted saliva. No differences were observed in *S. gordonii* Ag I/II expression exposed to any of the three conditions (Table 2). During the 2 h incubation period, bacterial growth was similar in TSB and TSBS, whereas in saliva alone, growth was reduced by 50% and 16% in *S. mutants* and *S. intermedius,* respectively. It is possible therefore that the effects observed with saliva alone may have been influenced by the inhibitory effect on growth.

Discussion

Despite the high degree of sequence and structural conservation that exists among the Ag I/II family, the individual members of this family of polypeptides are functionally distinct. Previous studies of *S. mutans* and *S. intermedius* have shown that initial adhesion to salivary film under conditions of flow was reduced in the *S. mutans* isogenic Ag I/II-deficient mutant, whereas no differences were observed between *S. intermedius* wild-type and its Ag I/II isogenic mutant (30). These results were confirmed in our static model by scanning electron microscopy of *S. mutans* and *S. intermedius* after 3 h of growth in TSB supplemented with saliva. However, after 48 h biofilm formation in the presence of saliva, both *S. mutans* and *S. intermedius* wild-types showed increased levels of biomass compared with their Ag I/II-deficient mutant. While Ag I/II may be unimportant for adhesion of *S. intermedius* to saliva-coated surfaces, the present results indicated that Ag I/II may be relevant in later stages of biofilm formation. In contrast, *S. mutans* Ag I/II seems to be important in both adhesion and biofilm formation. Other surface proteins have been implicated in streptococcal biofilm formation, including the *S. mutans* glucan binding protein C (GbpC) (13). This is in agreement with findings in most other bacteria where multiple strategies may be involved in biofilm formation. Interestingly, when saliva is absent in the medium no differences were observed between the wild-type and the Ag I/II-deficient mutant in *S. mutans*. However, the differences were significant in *S. intermedius*. Ag I/II segments C terminal of the proline-rich region and N-terminal sequences, including the alanine-rich region, have been identified as being

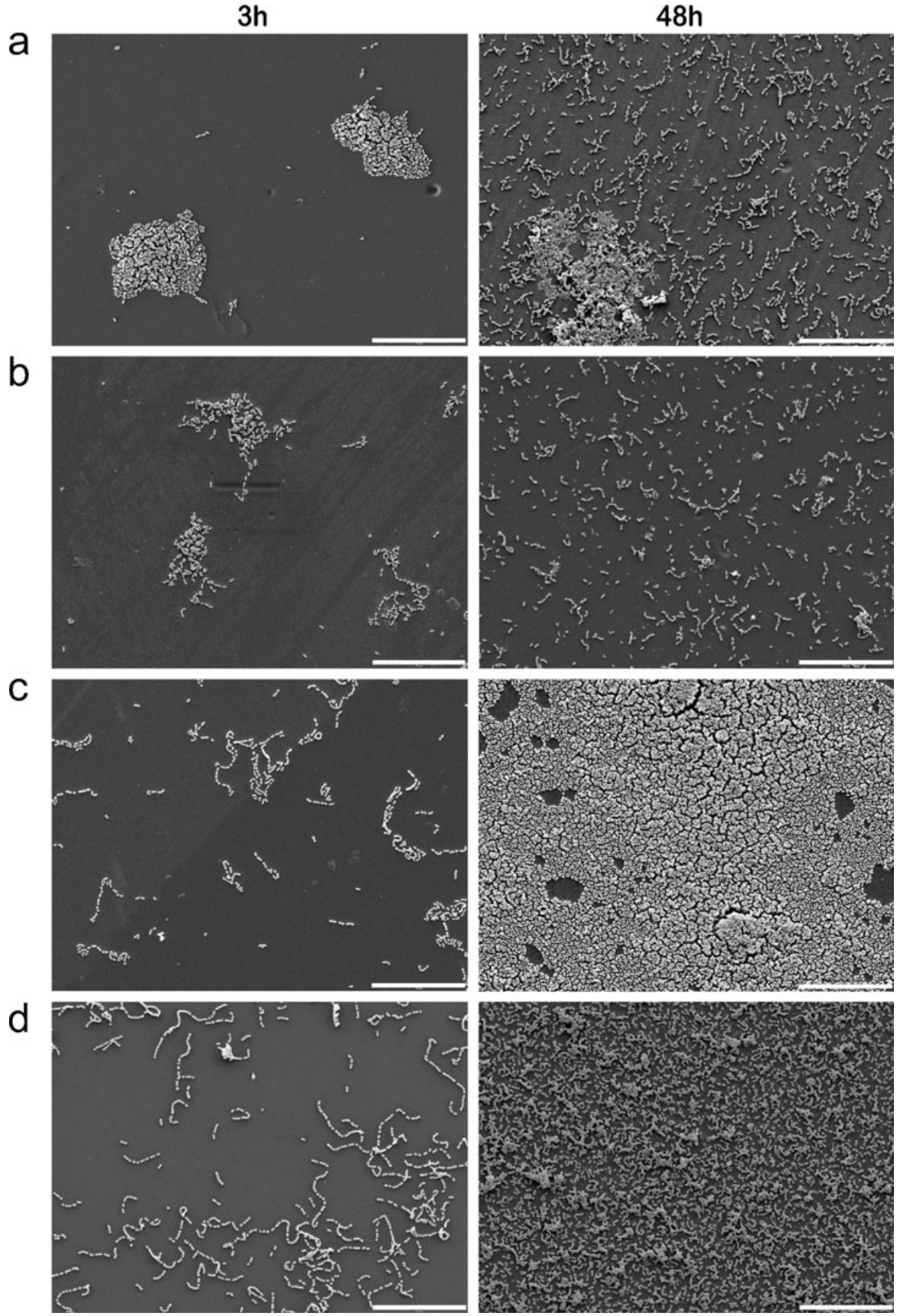

Fig. 1. Scanning electron micrographs of biofilm grown in TSBS formed on polystyrene discs. a) *S. mutans* LT11. b) *S. mutans* Ag /I/II defective mutant. c) *S. intermedius* 11324. d) *S. intermedius* Ag /I/II defective mutant. The discs were collected after 3 h or 48 h. Scale bar, 20 μm.

Table 2. Antigen I/II expression in TSB, TSBS or saliva[a]

Strain	TSB	TSBS	Saliva
S. mutans (LT11)	42 (20–92)	40 (19–69)[b]	35 (17–75)[b]
S. intermedius (11324)	11 (7–12)	10 (6–11)	5 (6–7)[b]
S. gordonii (Challis)	7 (2–8)	4 (0–8)	3 (1–8)

[a]Median values ($\times 10^{-4}$) corresponding to pixel intensities of the blot images analyzed by the TOTALLAB program, with the 25th and 75th percentiles in parentheses. The data presented correspond to five samples from three independent experiments.
[b]Significantly different from TSB ($P < 0.05$) as calculated by the Wilcoxon signed rank test.

involved in *S. mutans* binding to salivary components (17, 18). Protein sequence homology search with BLAST (1) reveals that the *S. mutans* segment C terminal of the proline-rich region (amino acid residues 1025–1044) shows homology to one of the sequenced segments in *S. intermedius* (38). However, 4–5-amino-acid substitutions are observed. One of the substitutions is in the E1037 residue, being K in *S. intermedius*. Site-directed mutagenesis studies have identified E1037 as one of the two residues important for *S. mutans* binding to salivary components. It is also possible that the difference in size of the alanine-rich sequence in *S. intermedius*, compared to *S. mutans*, may have an effect on the capacity of the *S. intermedius* Ag I/II to recognize salivary receptors. To verify these possibilities, further studies including genetic complementation are warranted. In recent work, Jakubovics et al. (14) showed different levels of binding of *S. gordonii*, *S. intermedius*, and *S. mutans* Ag I/II to immobilized SAG by expressing the Ag I/II on the surface of the surrogate host *Lactococcus lactis*.

Saliva as a growth source has been shown to strongly inhibit binding of *S. intermedius* to saliva-coated hydroxyapatite (40). We observed in this study that when saliva is present in the growth medium, less biofilm was formed. However, when plates were coated with saliva and inoculated with bacteria diluted in plain TSB the quantity of biofilm formed was similar to uncoated plates (data not shown). These results suggest that differences in biofilm formation are due to the presence of saliva in the fluid phase and not to the coating itself. This situation is closer to that found in the oral cavity, in which surfaces for attachment and bacterial cells are conditioned by salivary films. Aggregation of streptococci by saliva may facilitate clearance of the cells from the oral cavity. In our model it is possible that aggregation by saliva may have facilitated removal of the cell aggregates when the surfaces were washed, leading to less biofilm formation.

Bacteria growing in biofilms are phenotypically quite distinct from their planktonic counterparts. To adapt to a community lifestyle such as biofilms, bacteria undergo extensive changes and a number of genes are differentially expressed compared to the respective planktonic cultures (34). A wide range of genes was identified whose products generally fell into one of the following categories: peptidoglycan synthesis, cell–cell communication, environmental sensing and signaling, or adhesion (2). Compared with its planktonic counterpart, approximately 20% of the proteins that are expressed in *S. mutans* biofilms were either up- or down-regulated (37). In recent work, Black et al. (2) reported that in *Streptococcus sanguis*, surface expression of putative fibronectin and collagen adhesins were up-regulated in biofilms. However, although collagen binding by oral streptococci has, in part, been attributed to the Ag I/II family (24), no difference in Ag I/II expression was observed. In our study, similar levels of expression of Ag I/II on the cell surface were observed in *S. mutans*, *S. intermedius*, and *S. gordonii* whether grown planktonically or in biofilms. This is in line with previous findings showing no difference in gene expression of the *S. gordonii sspA* Ag I/II in planktonic and biofilm cultures (10). These results indicate that the oral streptococci studied do not regulate Ag I/II expression differently in biofilms and planktonic cells.

Protein expression may be regulated by the environmental conditions the cells are exposed to (8). Up-regulation of the *sspA* and *sspB* genes of *S. gordonii* in the presence of saliva has been shown (7). In the present study, no differences were observed in Ag I/II expression in the presence of saliva in *S. gordonii*. This may be explained by the fact that in this study we measured the differences in protein level and not differences in mRNA levels. Furthermore, in this study the proteins anchored to the cells and not the ones that are released to the medium were analyzed. Release of 40% of the *S. mutans* Ag I/II was observed by Lee (21) when the cells were washed and incubated in pH 6 buffer at 37°C. We can therefore not exclude the possibility that the levels of cell-free Ag I/II may have been altered by exposure to saliva.

Ag I/II expression in *S. mutans* was higher in TSB than in TSB supplemented with saliva or in saliva alone. Interestingly, the Ag I/II isogenic-deficient mutant in *S. mutans* formed less biofilm in TSB supplemented with saliva than the wild-type, whereas no differences were observed in TSB alone. In *S. intermedius*, no differences in Ag I/II expression were observed between TSB and TSB supplemented with saliva. Less biofilm was formed by the Ag I/II-deficient mutant in TSB and TSB supplemented with saliva than by the wild-type. This may indicate that the presence of saliva does not regulate protein expression in *S. intermedius*. This illustrates that both the genetic requirements and the environmental factors for biofilm formation are distinct.

Many studies were done to show the role of Ag I/II in the initial adhesion to different surfaces; however, this is the first study to demonstrate the involvement of Ag I/II in a dynamic and complex process as biofilms. Studies combining immunological and molecular approaches are important to better understand the biofilm phenotype and may facilitate the development of new strategies to control biofilm-mediated infections.

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