ORAL MICROBIOLOGY AND IMMUNOLOGY

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# Analysis of *Streptococcus mutans* biofilm proteins recognized by salivary immunoglobulin A

**T. Sanui[1,2], R. L. Gregory[1,3]**

[1]Department of Oral Biology, School of Dentistry, Indiana University, Indianapolis, IN, USA, [2]Department of Periodontology, Faculty of Dental Science, Kyushu University, Fukuoka, Japan, [3]Department of Pathology and Laboratory Medicine, School of Medicine, Indiana University, Indianapolis, IN, USA

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**Introduction:** The purpose of this study was to examine the *Streptococcus mutans* biofilm cellular proteins recognized by immunoglobulin A (IgA) in saliva from various caries-defined populations.
**Methods:** Biofilm and planktonic *S. mutans* UA159 cells were prepared. The proteins were extracted, separated by two-dimensional gel electrophoresis, transferred to blotting membranes, and probed for IgA using individual saliva samples from three groups of subjects; those who developed 0 caries (no active caries), 5–9 caries (medium), or more than 10 caries (severe) over a 12-month interval.
**Results:** Several proteins were recognized by salivary IgA in all groups of saliva but spot distribution and intensity varied greatly between the groups, and some proteins were recognized more strongly in biofilm cells than in planktonic culture, and vice versa. Furthermore, 15 proteins were only recognized by saliva from the 'no active caries' group, and four proteins were recognized by saliva samples from subjects in all three groups. Specifically, antigen I/II was recognized less in biofilm cells by caries-free saliva compared with planktonic cells. However, salivary IgA antibody to antigen I/II was absent in blots using saliva from the 'medium caries' and 'severe caries' groups.
**Conclusion:** The bacterial molecules recognized by caries-free saliva are significant factors for *S. mutans* caries formation, and their inhibition could be a therapeutic target. In addition, saliva of caries-free subjects includes significant IgA antibody against antigen I/II of *S. mutans*, indicating a protective mechanism. However, microorganisms may protect themselves from host immune attack by forming biofilms and decreasing expression of antigen I/II.

Key words: antigen I/II; biofilm; salivary immunoglobulin A; *Streptococcus mutans*; two-dimensional gel electrophoresis

Richard L. Gregory, Department of Oral Biology, Indiana University School of Dentistry, 1121 West Michigan Street, Indianapolis, IN 46202, USA
Tel.: + 1 317 274 9949;
fax: + 1 317 278 1411;
e-mail: rgregory@iupui.edu

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Biofilms are significant in the human body because they account for the majority of microbial infections (9). Biofilms consist of microorganisms that adhere to each other or to a surface, and are buried in the extracellular substances they produce. In the biofilm mode of bacterial growth, microorganisms demonstrate increased resistance to environmental stresses, antimicrobial agents (29), and host immune defense mechanisms. Therefore, biofilm formation is often harmful to humans and the cells in the biofilm are more difficult to eradicate than free-living planktonic cells (8, 10). Oral microbial biofilms, known as dental plaque on tooth surfaces, are responsible for initiating dental caries. *Streptococcus mutans*, in particular, is considered the primary etiological agent of human dental caries (18). *S. mutans* is a gram-positive and facultative anaerobic bacterium and dental plaque that includes *S. mutans* grows as a biofilm on the tooth surface of humans (3). Several antigens of *S. mutans* are involved in the capacity to adhere and accumulate in biofilms. These antigens include antigen I/II, glucosyltransferases, and glucan-binding protein (21, 22, 31). Animal experiments have indicated that specific antibodies against

these antigens prevented dental caries development (17, 20). Salivary immunoglobulin A (IgA) reacts with *S. mutans*, and is considered an important factor for host defense against *S. mutans* for the prevention of dental caries through bacteriostasis (5, 7, 15). We have previously reported that the saliva of caries-resistant subjects contains higher levels of IgA antibody against many *S. mutans* epitopes than the saliva of caries-susceptible patients (14, 15, 25). In the present study, we attempted to identify the *S. mutans* cellular proteins recognized by IgA in human saliva from various caries-defined populations and to compare expression of biofilm proteins with planktonic proteins using two-dimensional gel electrophoresis.

## Materials and methods

### Bacterial strain and biofilm growth

*S. mutans* UA159 (serotype *c*), the strain used in this study, was isolated from dental plaque above a carious enamel surface. To obtain biofilm cells, *S. mutans* was cultured in 75-$cm^2$ polystyrene flasks (Corning Inc., Corning, NY) with Todd–Hewitt broth (Acumedia, Baltimore, MA) supplemented with 1% dextrose (Fisher Scientific, Pittsburg, PA) for 24 h in a 5% $CO_2$ air incubator at 37°C. Non-adherent cells were removed by decanting and then gently washing the flasks with phosphate-buffered saline (PBS); adherent cells were collected by scraping. Planktonic *S. mutans* was cultured in 15-ml polystyrene tubes (Corning Inc.) under the same conditions and non-adherent and loosely bound cells were harvested.

### Preparation of cellular proteins

After cells had been harvested by centrifuging and washed twice with PBS, they were resuspended in lysis buffer [(9 M urea, 4% nonidet-P40, 2% ampholytes pH 3–10, and 1% dithiothreitol (DTT)], and subjected to four cycles of ultrasonication at the highest setting in the presence of 0.2-mm microglass beads (B. Braun Melsungen AG., Melsungen, Germany) for 5 min each with cooling between cycles. Cellular proteins were harvested and protein concentration was determined by the Bradford Sigma protein assay (Sigma Chemical Co., St Louis, MO).

### Saliva sampling

Volunteers were recruited from pediatric subjects in Puerto Rico. All of the saliva samples were collected from children ranging in age from 11 to 13 years. Equal numbers of boys and girls were recruited. These studies were carried out with informed consent and were approved by the Institutional Review Board of Indiana University-Purdue University at Indianapolis. Subjects were screened twice for the number of decayed, missing, and filled surfaces (DMFS) as described earlier (14) at baseline and 12 months later. Volunteers who remained free of carious lesions over the 12-month interval were designated 'no active caries' subjects. Patients who developed a DMFS score between 5 and 9 or ≥ 10 over the 12-month interval were designated 'medium' or 'severe' individuals, respectively. Individual saliva samples from five 'no active caries', four 'medium caries' and four 'severe caries' subjects were used for this study. Unstimulated whole saliva was collected by expectoration and was stored at −70°C until laboratory analysis.

### Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was used to separate the cellular proteins of *S. mutans*. Briefly, 500 μg protein from the two *S. mutans* UA159 cell extracts (biofilm and planktonic), diluted with rehydration buffer (8 M urea, 2% 3- ((3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid, 50 mM DTT, 0.2% biolyte 3–10 ampholytes, and a trace of bromophenol blue dye) and DNase-I (1 mg/ml), was loaded onto an isoelectric focusing (IEF) strip (7 cm, pH 3–10; BioRad Laboratories, Hercules, CA). The strip was covered with mineral oil, and rehydrated for 18 h at 50 V at 20°C. IEF was then carried out at 250 V for 20 min, 4000 V for 2.5 h, and then 4000 V for 10 KV-h. After focusing, the gel strip was incubated in fresh equilibration buffer 1 [6 M urea, 2% sodium dodecyl sulfate (SDS), 0.375 M Tris–HCl, 20% glycerol, and freshly made 2% DTT] and equilibration buffer 2 (6 M urea, 2% SDS, 0.375 M Tris–HCl, 20% glycerol, and freshly made 2.5% iodoacetamide) for 10 min each with shaking. The IEF strips were embedded on top of 20% polyacrylamide gradient gels (BioRad) using 0.5% (weight/volume) molten agarose. The SDS–polyacrylamide gel electrophoresis was performed at 120 V for 100 min at room temperature. The gels were stained with Coomassie Brilliant Blue G-250 or Silver Stain Plus (BioRad).

### Immunoblotting analysis

After the protein samples were separated by SDS–polyacrylamide gel electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA) at 90 mA for 16 h. Membranes were washed and blocked overnight at 4°C in PBS containing 5% non-fat milk. Saliva samples diluted 1 : 10 in PBS/milk were incubated at room temperature for 1 h. Goat IgG anti-human IgA conjugated with horseradish peroxidase (1 : 200 dilution in PBS/milk) was used as the secondary antibody. Antibody reactions were developed using the ECL Western Blotting Substrate (Pierce, Rockford, IL). For this purpose, immunoblots were incubated with enhanced chemiluminescence detection solution for 1 min, and then exposed to Fuji autoradiography film (Fisher Scientific) for an appropriate time.

### Identification of proteins by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy

Protein spots were selected for mass spectrometry analysis using the following criteria: (i) the proteins were present reproducibly in at least three of four Coomassie brilliant blue-stained gels of the biofilm cells compared with planktonic cells (Fig. 1C,D), and (ii) all protein spots that were found reproducibly in immunoblots except smudges were selected in silver-stained gels (Figs 2–4). The darkest spots were excised from the gel and individually digested with trypsin. Mass spectrometry (12) was provided by the Indiana University Protein Analysis and Research Center with support in part from the Indiana Genomics Initiative (INGEN) and the Indiana 21st Century Research and Technology Fund. Monoisotopic masses were sorted by density and the top 30 peptide monoisotopic masses were used for each protein with PROFOUND (http://prowl.rockefeller.edu/prowl-cgi/profound.exe). Proteins were identified as *S. mutans* proteins or homologues from other streptococcal or bacterial databases.

### Image analysis

Gels and the developed X-ray films were scanned with a scanner and the intensity of reactive spots were analysed using National Institutes of Health IMAGEJ software version 1.37. The number of spots and the intensity of each spot in gray values were recorded.

## Results

### Differentially-expressed proteins in biofilm and planktonic cells

Cellular proteins from biofilm or planktonic bacteria were separated by two-dimensional

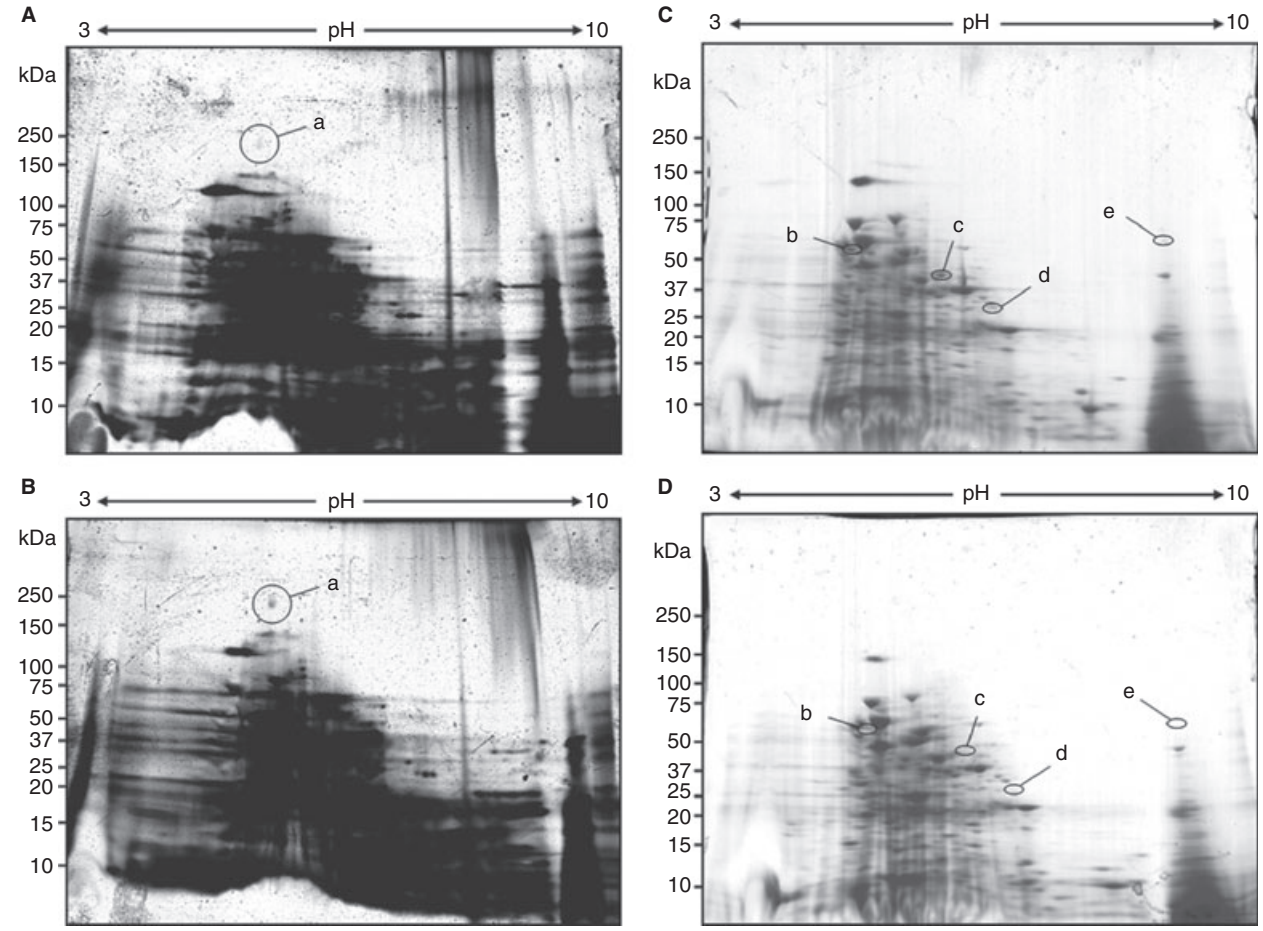


*Fig. 1.* Cellular proteins in representative silver-stained two-dimensional gel electrophoresis gels (approximately 185–200 kDa) from (A) biofilm (density: 33.7 gray values) and (B) planktonic (density: 71.7 gray values) *Streptococcus mutans* UA159 cells. Gels were overstained to allow antigen I/II spots marked with an (a) to be distinguished. Representative Coomassie brilliant blue-stained two-dimensional gel electrophoresis gels from biofilm (C) and planktonic (D) *S. mutans* UA159 cells; 500 $\mu$g protein was extracted, separated by two-dimensional gel electrophoresis and stained. The protein spot letter is the same ID as given in Table 1.

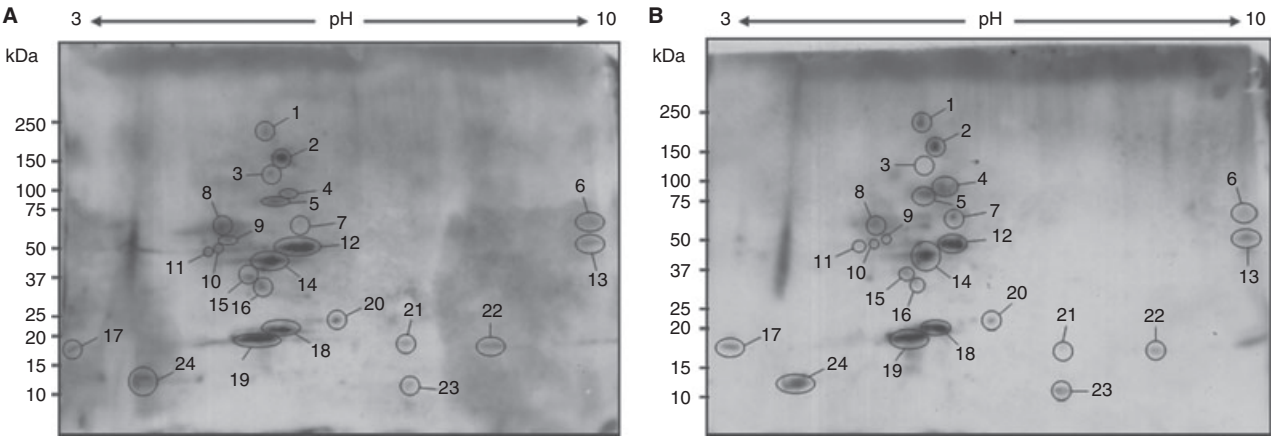


*Fig. 2.* Representative immunoglobulin A (IgA) immunoblots with a caries-free saliva prepared from biofilm (A) and planktonic (B) *Streptococcus mutans* UA159 cells: 500 $\mu$g protein was extracted, separated by two-dimensional gel electrophoresis, and transferred to a blotting membrane. Blots were probed with saliva from a subject who developed 0 caries (no active caries) followed by anti-human IgA secondary antibodies. The protein spot number is the same ID as given in Table 2.

gel electrophoresis and stained with silver-staining (Fig. 1A,B). Coomassie brilliant blue G-250 staining (Fig. 1C,D) was also performed. The same concentrations of protein (500 $\mu$g) from the *S. mutans* biofilm and planktonic cells were applied in the original separation but the silver-stained gels were overdeveloped to visualize antigen I/II. More than 200 protein spots

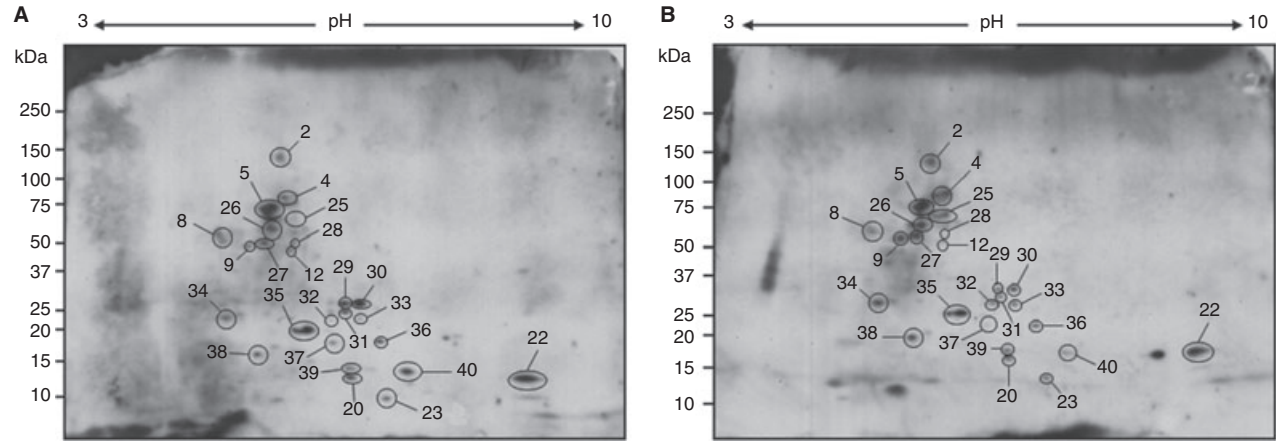


*Fig. 3.* Representative immunoglobulin A (IgA) immunoblots with a 'medium caries' saliva prepared from biofilm (A) and planktonic (B) *Streptococcus mutans* UA159 cells: 500 $\mu$g protein was extracted, separated by two-dimensional gel electrophoresis, and transferred to a blotting membrane. Blots were probed with saliva from a subject who developed 5–9 grade caries (medium caries) followed by anti-human IgA secondary antibodies. The protein spot number is the same ID as given in Table 3.

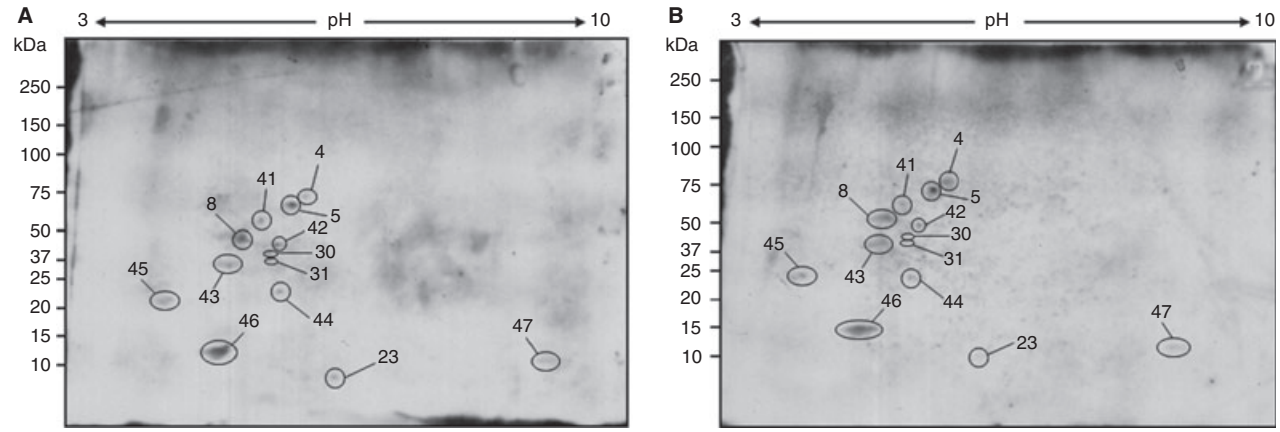


*Fig. 4.* Representative immunoglobulin A (IgA) immunoblots with 'severe caries' saliva prepared from biofilm (A) and planktonic (B) *Streptococcus mutans* UA159 cells: 500 $\mu$g protein was extracted, separated by two-dimensional gel electrophoresis, and transferred to a blotting membrane, and blots were probed with saliva from a subject who developed > 10 grade caries (severe caries) followed by anti-human IgA secondary antibodies. The protein spot number is the same ID as given in Table 4.

*Table 1.* Proteins strongly expressed in biofilm grown cells compared with their planktonic counterparts in Coomassie brilliant blue-stained gels

| Spot[1] | Species with homologous protein[2] | Putative description[3] | MW (kDa) | pI | %[4] |
|---|---|---|---|---|---|
| b | *S. mutans* | Peptidyl-prolyl isomerase RopA (trigger factor) | 47.47 | 4.5 | 30 |
| c | *S. mutans* | Putative NADP-specific glutamate dehydrogenase | 48.30 | 5.4 | 38 |
| d | *S. mutans* | Putative cystein synthetase A; *O*-acetylserine lyase | 32.40 | 5.8 | 21 |
| e | *B. subtilis* | Modifier protein of major autolysin | 76.26 | 9.5 | 13 |

[1]Spot is the protein spot letter mark on Fig. 1(C,D).
[2]The bacterial species with highest protein sequence similarity to the stated *Streptococcus mutans* protein. *B. subtilis*, *Bacillus subtilis*.
[3]Putative functions were assigned from the NCBInr database.
[4]% Minimal sequence coverage (the percentage of matched peptides covering the whole protein sequence of streptococcal species in the Profound database).

idnetified by Coomassie staining were observed in both biofilm and planktonic cells (Fig. 1C,D; respectively). However, some proteins were reproducibly upregulated in the biofilm-grown cells (Table 1) compared with planktonic culture, and vice versa. For example, antigen I/II was located at approximately 200 kDa in silver-stained gels and was expressed less in biofilms than in planktonic cultures (more than two-fold difference; Fig. 1A,B: marked with an 'a'). These 200-kDa spots were identified as antigen I/II by using anti-antigen I/II (data not shown) and by performing MALDI-TOF analysis.

#### Reactivity of IgA in caries-free saliva

A number of proteins recognized by salivary IgA were detected in all saliva samples but spot distribution varied greatly between samples. However, there was consistency between saliva samples within the same group. The number of immunoblot spots was almost equal between biofilm and planktonic cells, but some proteins were recognized more strongly in biofilm cells compared with planktonic

*Table 2.* Comparison of reactive proteins enhanced or diminished in biofilm cells with their planktonic counterparts in representative immunoglobulin A (IgA) immunoblots with caries-free saliva

| Spot no.[1] | Species with homologous protein[2] | Putative description[3] | MW (kDa) | pI | %[4] | Fold change[5] |
|---|---|---|---|---|---|---|
| *Enhanced* | | | | | | |
| 3 | *S. mutans* | Fructan hydrolase; exo-beta-D-fructosidase; fructanase, FraA | 158.80 | 5.0 | 17 | 2.51 ± 2.77 |
| 5 | *S. mutans* | Putative Clp-like ATP dependent protease, ATP-binding subunit | 77.17 | 5.0 | 44 | 1.25 ± 0.31 |
| 6 | *B. subtilis* | Modifier protein of major autolysin | 76.26 | 9.5 | 13 | 1.74 ± 0.17 |
| 8 | *S. mutans* | Peptidyl-prolyl isomerase RopA (trigger factor) | 47.47 | 4.5 | 30 | 1.82 ± 0.32 |
| 10 | *S. mutans* | putative enolase | 46.90 | 4.7 | 68 | 1.53 ± 0.11 |
| 11 | *S. mutans* | Cell division protein FtsG | 45.73 | 4.2 | 21 | 2.93 ± 1.47 |
| 12 | *S. mutans* | Translation elongation factor EF-Tu | 43.90 | 4.8 | 32 | 1.11 ± 0.27 |
| 15 | *C. tetani* | Hypothetical protein CTC00757 | 35.89 | 5.8 | 24 | 1.47 |
| 16 | *S. mutans* | Ketol-acid reductoisomerase | 37.32 | 5.0 | 34 | 1.87 ± 0.69 |
| 20 | *C. tetani* | hypothetical protein CTC01032 | 27.71 | 5.1 | 25 | 1.86 |
| 21 | *S. mutans* | Hypothetical protein Chte02003382 | 29.47 | 5.9 | 25 | 1.69 |
| *Diminished* | | | | | | |
| 1 | *S. mutans* | Antigen I/II | 171.33 | 6.0 | 13 | 1.86 ± 0.23 |
| 2 | *G. kaustophilus* | DNA-directed DNA polymerase III alpha chain | 163.95 | 5.5 | 10 | 1.13 |
| 4 | *S. epidermidis* | Putative ATPase TraE | 75.00 | 5.7 | 17 | 1.13 |
| 7 | *S. mutans* | Glucosamine-fructose-6-phosphate aminotransferase 1 | 65.50 | 5.2 | 13 | 3.15 ± 2.10 |
| 9 | *S. mutans* | Chaperonin GroEL | 52.02 | 4.7 | 26 | 2.13 ± 1.33 |
| 13 | *S. mutans* | Putative ABC transporter, branched chain amino acid-binding protein | 41.26 | 9.0 | 24 | 1.13 ± 0.69 |
| 14 | *S. mutans* | Putative translation elongation factor TS | 37.60 | 4.9 | 15 | 1.01 ± 0.12 |
| 17 | *S. aureus* | Hypothetical protein SAS0038 | 32.79 | 9.2 | 25 | 1.41 |
| 18 | *M. thermoacetica* | Phosphate transport regulator (distant homolog of phoU) | 25.12 | 9.1 | 15 | 1.06 |
| 19 | *S. mutans* | Putative co-chaperonin GroES | 10.08 | 4.9 | 38 | 1.23 ± 0.33 |
| 22 | *S. mutans* | Putative mannose-specific IIB component | 18.25 | 7.7 | 66 | 1.08 ± 0.19 |
| 23 | *O. theyensis* | Hypothetical protein OB1961 | 16.93 | 5.0 | 26 | 1.11 ± 0.24 |
| 24 | *S. pyogenes* | Plasmid stabilization system antitoxin protein | 13.38 | 8.0 | 40 | 1.69 |

[1]Spot no. is the protein spot number in Fig. 2.
[2]The bacterial species with highest protein sequence similarity to the stated *Streptococcus mutans* protein. *B. subtilis*, *Bacillus subtilis*; *C. tetani*, *Clostridium tetani*; *G. kaustophilus*, *Geobacillus kaustophilus*; *M. thermoacetica*, *Moorella thermoacetica*; *O. itheyensis*, ; *S. aureus*, *Staphylococcus aureus*; *S. epidermidis*, *Staphylococcus epidermidis*; *S. pyogenes*, *Streptococcus pyogenes*;
[3]Putative functions were assigned from the NCBInr database.
[4]% Minimal sequence coverage (the percentage of matched peptides covering the whole protein sequence of streptococcal species in the Profound database).
[5]Fold change calculated as the gray value of protein recognized by caries-free salivary IgA in planktonic vs. biofilm cells in saliva from five subjects. Proteins enhanced or diminished in biofilm cells compared with their planktonic counterparts were analysed. Fold change which lacks ± SD indicates that the proteins were detected in only one representative immunoblot.

cultures, and vice versa. In the experiments using the 'no active caries' saliva (Fig. 2-A,B), nine proteins from biofilm cells were recognized by IgA antibodies significantly more strongly than for the planktonic cells (more than 1.4-fold). In contrast, reactivity to five biofilm proteins was diminished compared with planktonic cells (Table 2). In particular, the approximately 200-kDa proteins (antigen I/II) were recognized less (> 1.4-fold decrease) in biofilm cells compared with planktonic cells. In addition, antigen I/II proteins were detected by all five caries-free saliva samples.

#### Reactivity of salivary IgA in 'medium caries' saliva

Of 25 proteins recognized by 'medium caries' saliva, reactivity to seven proteins was enhanced (> 1.4-fold) in biofilm cells compared with planktonic cells in the experiments using the 'medium caries' saliva (Fig. 3A,B). On the other hand, reactivity to six biofilm proteins was diminished compared with planktonic cells (Table 3). Furthermore, antigen I/II was detected on these salivary IgA immunoblots from only one of the four 'medium caries' saliva samples.

#### Reactivity of salivary IgA in 'severe caries' saliva

The overall number and the gray-scale of spots were significantly decreased in immunoblots with 'severe caries' saliva compared with caries-free saliva (Fig. 4A,B). Reactivity to three proteins was enhanced (> 1.4-fold) in biofilm cells compared with planktonic cells out of a total of 13 proteins recognized by 'severe caries' saliva. In contrast, reactivity to one biofilm protein was diminished compared with planktonic cells (Table 4). Salivary IgA antibody to antigen I/II was totally absent in blots using the four 'severe caries' salivas.

## Discussion

In this study we examined the *S. mutans* biofilm and planktonic cellular proteins recognized by IgA in human saliva from various caries-defined populations. Numerous studies have established salivary IgA antibody specificity for several *S. mutans* antigens prepared from planktonically grown cells but no studies have identified immune responses to the different biofilm-associated *S. mutans* antigens. The response to *S. mutans* antigen I/II was of significant interest because of its role in sucrose-independent colonization. Our results indicated that antigen I/II may be not as necessary after the formation of biofilms as in the free-living planktonic state because antigen I/II was down-regulated in biofilms compared with planktonic proteins. Recent results from our laboratory indicated that the *spaP* gene is down-regulated in biofilm cells compared with planktonically grown cells as well as in statically grown cels compared with biofilm cells grown in a flow cell (Z. Chen, D. Galli and R.L. Gregory, manuscript submitted). This suggests that antigen I/II is differentially expressed under various conditions including after the bacterium

*Table 3.* Comparison of reactive proteins enhanced or diminished in biofilm cells with their planktonic counterparts in representative immunoglobulin A (IgA) immunoblots with 'medum caries' saliva

| Spot no.[1] | Species with homologous protein[2] | Putative description[3] | MW (kDa) | pI | % [4] | Fold change[5] |
|---|---|---|---|---|---|---|
| *Enhanced* | | | | | | |
| 5 | *S. mutans* | Putative Clp-like ATP dependent protease, ATP-binding subunit | 77.17 | 5.0 | 44 | 1.22 ± 1.54 |
| 28 | *S. mutans* | NADP-dependent glyceraldehyde-3-phosphate dehydrogenase 1 | 51.20 | 5.1 | 61 | 4.57 ± 3.69 |
| 8 | *S. mutans* | Peptidyl-prolyl isomerase RopA (trigger factor) | 47.47 | 4.5 | 30 | 1.35 ± 1.12 |
| 29 | *T. tengcongensis* | Predicted transcriptional regulator | 49.59 | 5.6 | 22 | 1.90 |
| 12 | *S. mutans* | Translation elongation factor EF-Tu | 43.90 | 4.8 | 32 | 3.48 ± 2.93 |
| 30 | *S. mutans* | Extracellular glyceraldehyde-3-phosphate dehydrogenase | 36.16 | 5.7 | 45 | 1.24 ± 0.14 |
| 31 | *S. mutans* | 6-phosphofructokinase | 35.81 | 5.4 | 41 | 1.55 ± 0.27 |
| 35 | *S. mutans* | Fructose-1,6-bisphosphate aldolase | 31.58 | 5.0 | 40 | 1.01 |
| 36 | *M. genitalium* | Cell division protein (ftsY) | 39.01 | 9.1 | 23 | 1.09 ± 0.16 |
| 37 | *S. mutans* | Glycerophosphoryl diester phosphodiesterase | 35.24 | 8.7 | 16 | 1.99 ± 4.87 |
| 38 | *S. mutans* | Alkyl hydroperoxide reductase | 28.98 | 5.5 | 27 | 2.39 ± 2.74 |
| 20 | *C. tetani* | Hypothetical protein CTC01032 | 27.71 | 5.1 | 25 | 2.05 ± 4.46 |
| 22 | *S. mutans* | Putative PTS system, mannose-specific IIB component | 18.25 | 7.7 | 66 | 1.09 ± 0.11 |
| 23 | *O. theyensis* | Hypothetical protein OB1961 | 16.93 | 5.0 | 26 | 1.05 ± 0.39 |
| *Diminished* | | | | | | |
| 2 | *G. kaustophilus* | DNA-directed DNA polymerase III alpha chain | 163.95 | 5.5 | 10 | 1.12 |
| 4 | *S. epidermidis* | Putative ATPase TraE | 75.00 | 5.7 | 17 | 1.86 ± 0.56 |
| 25 | *S. mutans* | Transketolase | 71.00 | 5.7 | 17 | 3.05 ± 4.25 |
| 26 | *S. epidermidis* | Putative phosphoglucomutase | 63.10 | 4.9 | 14 | 1.13 |
| 27 | *S. mutans* | Pyruvate kinase | 54.41 | 4.9 | 18 | 1.11 ± 0.75 |
| 9 | *S. mutans* | Chaperonin GroEL | 52.02 | 4.7 | 26 | 1.92 ± 0.51 |
| 32 | *S. mutans* | DNA-directed RNA polymerase alpha chain | 35.02 | 4.8 | 47 | 3.51 |
| 33 | *S. mutans* | Putative trans-2-enoyl-ACP reductase | 33.57 | 4.7 | 22 | 1.88 ± 0.47 |
| 34 | *S. mutans* | Putative manganese-dependent inorganic pyrophosphatase | 33.38 | 4.7 | 26 | 1.54 ± 0.43 |
| 39 | *L. lactis* | Ribonuclease HII | 25.22 | 4.6 | 43 | 1.09 |
| 40 | *S. mutans* | Phosphoribosylaminoimidazolesuccinocarboxamide synthase | 20.63 | 5.7 | 40 | 1.10 |

[1]Spot no. is the protein spot number in Fig. 3.
[2]The bacterial species with highest protein sequence similarity to the stated *Streptococcus mutans* protein. *C. tetani*, *Clostridium tetani*; *G. kaustophilus*, *Geobacillus kaustophilus*; *L. lactis*, *Lactococcus lactis*; *M. genitalium*, *Mycoplasma genitalium*; *O. itheyensis*, *S. epidermidis*, *Staphylococcus epidermidis*; *T. tengcongensis*, *Thermoanaerobacter tengcongensis*.
[3]Putative functions were assigned from the NCBInr database.
[4]% Minimal sequence coverage (the percentage of matched peptides covering the whole protein sequence of streptococcal species in the Profound database).
[5]Fold change calculated as the gray value of protein recognized by medium caries salivary IgA in planktonic vs. biofilm cells in saliva from four subjects. Proteins enhanced or diminished in biofilm cells compared with their planktonic counterparts were analysed. Fold change which lacks ± SD indicates that the proteins were detected only in one representative immunoblot.

has become established in a biofilm. Antigen I/II (16, 25, 26, 28) polypeptides [also known as P1 (2, 4, 6, 13), protein B (4, 6), PAc (23), MSL-S (11) and SR(1)] are a major cell surface protein antigen in oral streptococci; they function primarily for colonization of bacteria on tooth surfaces and are strongly immunogenic in humans (27). *S. mutans* antigen I/II in planktonic cells seems to be important in adhesion and biofilm formation; however, expression of antigen I/II after biofilm formation may be less necessary.

Our results also suggested that the saliva of caries-free subjects includes significant IgA antibody against *S. mutans* antigen I/II because antigen I/II was detected in all immunoblots with caries-free saliva. Our laboratory has previously reported that caries-free saliva contains higher levels of IgA antibody against many *S. mutans* epitopes, including antigen I/II, than caries-active saliva (14, 15, 25). Conversely, microorganisms may protect themselves from host immune attack by forming biofilms and decreasing their expression of antigen I/II, which has strong immunogenic properties in humans.

In our MALDI-TOF mass spectroscopy analysis, proteins highly expressed in biofilm-grown cells were relevant to glycolytic enzymes (enolase: Table 2, and glyceraldehyde-3-phosphate dehydrogenase: Tables 3 and 4), cell division (cell division protein: Tables 2 and 3), translation (elongation factor: Tables 2 and 3) and protein folding (trigger factor: Tables 2–4), whereas other antigens (antigen I/II: Table 2 and GroEL: Tables 2 and 3) were repressed. These findings are similar to results of Svensäter et al. (30) in which biosynthetic processes were enhanced in biofilm cells. However, Rathsam et al. (24) indicated that nucleotide biosynthesis and cell division were downregulated in biofilm-grown cells.

In addition, four proteins were recognized by saliva samples from all three groups. Two of the *S. mutans* cellular proteins were adenosine triphosphate (ATP) -dependent protease and trigger factor. In most microbes, molecular chaperone and trigger factor protects proteins against the incorrect folding, while ATP-dependent protease functions in the degradation of useless translated proteins using ATPs. These proteins regulate protein biosynthesis and degradation. This finding indicated that all human saliva includes the antibody to bind to metabolic system proteins and the salivary IgA which recognizes these proteins plays the most fundamental role in the bioregulation of oral microbes. On the other hand, we also found that 15 proteins, including antigen I/II, were only recognized by saliva from the 'no-active caries' group (Table 2). Ten of these proteins were *S. mutans* fructan hydrolase, enolase, cell division protein, reductoisomerase, hypothetical protein, antigen I/II, aminotransferase, ATP-binding cassette transporter, elongation factor, and GroES. Enolase is a glycolytic enzyme and GroEL/GroES functions as not only a molecular chaperone but also has strong immunogenicity similar to antigen I/II.

*Table 4.* Comparison of reactive proteins enhanced or diminished in biofilm cells with their planktonic counterparts in representative immunoglobulin A (IgA) immunoblots with 'severe caries' saliva

| Spots no.[1] | Species with homologous protein[2] | Putative description[3] | MW (kDa) | pI | %[4] | Fold change[5] |
|---|---|---|---|---|---|---|
| *Enhanced* | | | | | | |
| 41 | *S. mutans* | Heat shock protein, DnaK (HSP-70) | 65.27 | 4.6 | 39 | 1.26 ± 0.1 |
| 42 | *S. mutans* | Putative NADP-specific glutamate dehydrogenase | 48.33 | 5.4 | 38 | 1.19 ± 1.86 |
| 8 | *S. mutans* | Peptidyl-prolyl isomerase RopA (trigger factor) | 47.47 | 4.5 | 30 | 1.20 ± 0.47 |
| 30 | *S. mutans* | Extracellular glyceraldehyde-3-phosphate dehydrogenase | 36.16 | 5.7 | 43 | 1.58 ± 0.36 |
| 31 | *S. mutans* | 6-phosphofructokinase | 35.81 | 5.4 | 41 | 2.00 |
| 44 | *D. hafniense* | Malate/L-lactate dehydrogenases | 38.48 | 5.5 | 16 | 1.23 |
| 46 | *M. hyopneumoniae* | 50s ribosomal protein L23 | 19.02 | 9.3 | 26 | 1.09 ± 1.65 |
| 47 | *L. delbrueckii* | Prolyl-tRNA synthetase | 10.51 | 5.3 | 50 | 2.06 |
| *Diminished* | | | | | | |
| 4 | *S. epidermidis* | Putative ATPase TraE | 75.00 | 5.7 | 17 | 2.55 ± 2.71 |
| 5 | *S. mutans* | Putative Clp-like ATP dependent protease, ATP-binding subunit | 77.17 | 5.0 | 44 | 1.02 ± 1.33 |
| 43 | *C. thernocellum* | COG1484: DNA replication protein | 33.30 | 8.9 | 15 | 1.02 |
| 45 | *S. mutans* | Putative ribonucleotide reductase, small subunit | 36.70 | 4.4 | 15 | 1.13 ± 3.44 |
| 23 | *O. theyensis* | Hypothetical protein OB1961 | 16.93 | 5.0 | 26 | 1.10 ± 0.98 |

[1]Spot no. is the protein spot number in Fig. 4.
[2]The bacterial species with highest protein sequence similarity to the stated *Streptococcus mutans* protein. *C. thermocellum*, *Clostridium thermocellum*; *D. hafniense*, *Desufitobacterium hafniense*; *L. delbrueckii*, *Lactobacillus delbrueckii*; *M. hyopneumoniae*, *Mycoplasma hyopneumoniae*; *O. itheyensis*, *S. epidermidis*, *Staphylococcus epidermidis*.
[3]Putative functions were assigned from the NCBInr database.
[4]% minimal sequence coverage (the percentage of matched peptides covering the whole protein sequence of streptococcal species in the Profound database).
[5]Fold change calculated as the gray value of protein recognized by 'severe caries' salivary IgA in planktonic vs. biofilm cells in saliva from four subjects. Proteins enhanced or diminished in biofilm cells compared with their planktonic counterparts were analysed. Fold change which lacks ± SD indicates that the proteins were detected only in one representative immunoblot.

(19) These results suggested that the proteins detected by the 'no-active caries' saliva samples included proteins associated with amino acid biosynthesis, glycolysis, cell division, translation, transport and antigen I/II, while those recognized by the 'medium caries' and 'severe caries' saliva were often identified as dehydrogenase and reductase proteins. In other words, these bacterial molecules are significant factors in the role of *S. mutans* in caries formation, and they could become a therapeutic target.

These studies indicate the complexity of oral biofilms and the resultant immune responses directed towards *S. mutans* epitopes. The presence of higher salivary IgA antibody responses from the caries-free subjects to several biofilm proteins provides an initial analysis of critical immune responses to these proteins and may direct future work towards the development of immune modifications against these proteins. The fact that caries-free subjects have been successful in not developing caries argues that the strong response to at least 10 proteins including antigen I/II seen in these individuals may be a protective mechanism. In particular, the downregulation of antigen I/II in biofilm cells supports previous work from this laboratory and others that antigen I/II appears to be critical in the initial formation of the biofilm but once the biofilm is established the antigen I/II is not as important and is down-regulated.

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