molecular oral microbiology

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Salivary proteins promote proteolytic activity in *Streptococcus mitis* biovar 2 and *Streptococcus mutans*

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SUMMARY

A major function of the salivary pellicle on oral surfaces is to promote colonization of the commensal microbiota by providing binding sites for adherence. *Streptococcus mitis* is an early colonizer of the oral cavity whereas *Streptococcus mutans* represents a later colonizer. To survive and grow, oral bacteria produce enzymes, proteases and glycosidases, which allow them to exploit salivary proteins as a nutrient source. In this study, adherence and proteolytic activity of *S. mitis* biovar 2 and *S. mutans* were investigated in a flow-cell model in the presence of different populations of surface-associated salivary proteins. *Streptococcus mitis* biovar 2 adhered well to surfaces coated with both a MUC5B-enriched fraction and a pool of low-density proteins containing MUC7, amylase, cystatin, gp340, immunoglobulin A, lactoferrin, lysozyme and statherin, whereas adherence of *S. mutans* to these proteins was poor. In environments of MUC5B or the low-density proteins, both *S. mitis* biovar 2 and *S. mutans* showed high levels of proteolytic activity. For *S. mitis* in the MUC5B environment, most of this activity may be attributable to contact with the molecules in the fluid phase although activity was also enhanced by adherence to surface-associated MUC5B. These data suggest that although they differ in their capacity to adhere to surface-associated salivary proteins, in the natural environment exploitation of saliva as a nutrient source can contribute to survival and colonization of the oral cavity by both *S. mitis* biovar 2 and *S. mutans*.

INTRODUCTION

Saliva is a complex secretion containing a range of proteins and glycoproteins involved in lubrication and host defence in the oral cavity. The large salivary mucin, MUC5B, is known to form a gel-matrix, which binds innate defence proteins such as immunoglobulin A (IgA), lactoferrin and lysozyme (Iontcheva *et al.*, 1997; Wickström *et al.*, 2000) whereas the primary function of the smaller MUC7 mucin, is proposed to be the agglutination of microorganisms (Nieuw Amerongen & Veerman, 2002). Proteins originating from saliva form a pellicle covering the tooth and mucosal surfaces (for reviews, see Lendenmann *et al.*, 2000; Hannig & Joiner, 2006). One of the major functions of this pellicle is to promote colonization by the commensal microbiota and thereby limit access to pathogenic species. Surface-associated proteins provide binding sites for adherence of microorganisms and

the initial colonizers may differ between niches in the oral cavity because of differences in pellicle composition. Once the early colonizers are established, integration of other microorganisms leads to the formation of mixed-species microbial communities (Kolenbrander *et al.*, 2010). In plaque biofilms, bacteria produce proteases and glycosidases to exploit salivary proteins and glycoproteins as a nutrient source (Bradshaw *et al.*, 1994; Wickström *et al.*, 2009). Hence, interactions between microorganisms and between microorganisms and their environment become essential ecological determinants in driving the development of these complex communities (Marsh *et al.*, 2011).

Streptococcal species comprise an important part of the oral commensal flora (Aas *et al.*, 2005) and members of the mitis group such as *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus sanguinis* and *Streptococcus gordonii* are regarded as early colonizers in dental biofilms (Li *et al.*, 2004). Streptococci can adhere (Nobbs *et al.*, 2009) to a range of salivary proteins, including MUC5B (MG1), amylase, lysozyme and proline-rich proteins in the acquired enamel pellicle (Yao *et al.*, 2003; Siquiera *et al.*, 2007). For instance *S. gordonii*, the most studied member of this group, is known to interact with α-amylase and proline-rich proteins (Kilian & Nyvad, 1990; Murray *et al.*, 1992), as well as sialylated molecules such as gp340 (Jakubovics *et al.*, 2005) and MUC7 (Ruhl *et al.*, 2004). *Streptococcus oralis* shows a similar pattern of binding, suggesting that multiple salivary proteins probably contribute to the adherence of these species *in vivo* (Murray *et al.*, 1992). Although less well investigated, studies show that *S. mitis* can bind α-amylase (Kilian & Nyvad, 1990) and expresses an amylase-binding protein, AbpC (Vorassi *et al.*, 2010). Once the initial colonizers are established, they can provide co-aggregation sites for later colonizers such as *S. mutans*, which can represent a high proportion of the biofilm community under acidic conditions. Agglutination studies have shown that *S. mutans* can interact with several salivary proteins, including the large oligomeric mucin MUC5B (MG1), MUC7, IgA, salivary agglutinin (gp340) and lysozyme (for a review, see Scannapieco, 1994). However, *S. mutans* binds less well to saliva-coated surfaces than to uncoated ones (Pratt-Terpstra *et al.*, 1989; Lima *et al.*, 2008).

At the same time as providing sites for adhesion, complex salivary proteins have been proposed to represent the predominant source of nutrients for oral bacteria in developing biofilms (Bowden & Hamilton, 1998). MUC5B, MUC7 and gp340 are all large highly glycosylated proteins, the degradation of which requires the production of both proteolytic enzymes and glycosidases. As a group, oral streptococci can degrade both the peptide backbone and the glycan moieties of the human glycoprotein immunoglobulin A1 but few individual species are able to digest both (Cole *et al.*, 1999; Grønbaek Frandsen, 1999). For instance, *S. mitis* biovar 1 and *S. oralis* produce a human immunoglobulin A1 protease, while *S. mitis* biovar 2 is capable of removing sialic acid, galactose and *N*-acetylgalactosamine from the molecule (Reinholdt *et al.*, 1990). However, supragingival plaque from macaque monkeys, containing a mixed-species consortium of oral bacteria, has been reported to express both exoglycosidases as well as a wide range of proteolytic activities in the absence of dietary nutrients (Smith & Beighton, 1986, 1987). It therefore appears that cooperation between different species, which express complementary patterns of glycosidases and protease expression, is necessary to generate nutrients from complex salivary molecules (Bradshaw *et al.*, 1994; Wickström *et al.*, 2009).

In this study we compare the adherence of one initial colonizer, *S. mitis* biovar 2, and one late streptococcal colonizer, *S. mutans*, to selected surface-associated salivary proteins. We also investigate whether contact with salivary proteins can induce activity of proteases that could serve to degrade them. The MUC5B-enriched material used to coat the surfaces was prepared from whole human saliva using a density-gradient centrifugation technique in which the molecules were not exposed to chaotrophic agents (Raynal *et al.*, 2003). By preparing mucins in this way, we characterize streptococcal adhesion interactions with MUC5B maintained in a near-native conformation. These data are considered, therefore, to have high physiological relevance.

METHODS

Collection and preparation of saliva

Whole saliva was collected from six healthy volunteers over 30 min into containers placed on ice, in

compliance with the human subject review committee at The Faculty of Odontology, Malmö University. The saliva was pooled, mixed 1 : 1 with 0.2 M NaCl, and gently mixed overnight at 4°C. The sample was centrifuged in a Beckman Coulter Avanti J-E centrifuge (Beckman JA 20 rotor; Beckman Coulter, Fullerton, CA; 4°C, 20 min, 4400 × ***g***.) and the supernatant was subjected to isopycnic density-gradient centrifugation in CsCl/0.1 M NaCl in a Beckman Coulter Optima LE-80K Ultracentrifuge (Beckman 50.2 Ti rotor, starting density 1.45 g ml^{-1}, 15°C, 90 h, 36,000 r.p.m. as described previously (Wickström & Svensäter, 2008). Fractions were collected from the bottom of the tube and analysed for density by weighing, absorbance (A_{280}) and their content of MUC5B and MUC7 (see below).

Fractions 3–10 (rich in MUC5B) and 11–25 (rich in low-density proteins, e.g. MUC7, gp340 and non-mucin proteins) were pooled and dialysed against phosphate-buffered saline (0.15 M sodium chloride, 10 mM potassium phosphate, pH 7.2; PBS). The dry weights of solids were determined by extensive dialysis against water, freeze-drying and weighing. The pools were stored at −20°C until used in the adhesion and protease assays.

Enzyme-linked immunosorbent assay

After appropriate dilution in PBS, fractions were coated overnight onto microtitre plates (3912, Falcon, Franklin Lakes, NJ) maintained in a humid chamber at room temperature. Wells were blocked for 1 h with PBS containing 0.05% (volume/volume) Tween-20 and 0.5% (weight/volume) bovine serum albumin (blocking solution) and then incubated for 1 h with a polyclonal rabbit human α-amylase antiserum (Sigma-Aldrich, St Louis, MO) diluted 1 : 1000, a monoclonal mouse human cystatin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1 : 1000, a polyclonal rabbit human gp340 antiserum (a kind gift from Dr David Thornton, Manchester, UK) diluted 1 : 1000, a polyclonal rabbit human IgA antiserum (Dako, Glostrup, Denmark) diluted 1 : 600, a polyclonal rabbit human lactoferrin antiserum (Dako) diluted 1 : 400, a polyclonal rabbit human lysozyme antiserum (Dako A/S, Copenhagen, Denmark) diluted 1 : 400 or a polyclonal goat human statherin antiserum (Santa Cruz Biotechnology) diluted 1 : 1000. MUC5B and MUC7 were identified using polyclonal rabbit antisera recognizing the peptide backbone of the proteins [LUM5B-2 (Wickström *et al.*, 1998) diluted 1 : 1000 for MUC5B, or LUM7-1 (Wickström *et al.*, 2000) diluted 1 : 500 for MUC7]. All antibodies were diluted in blocking solution. Reactivity was detected with horseradish peroxidase (HRP) -conjugated polyclonal swine anti-rabbit, polyclonal rabbit anti-mouse or polyclonal rabbit anti-goat antisera (Dako) diluted 1 : 2000 in blocking solution or an alkaline phosphatase-conjugated swine anti-rabbit antiserum (Dako), diluted 1 : 2000 in blocking solution (LUM5B-2 and LUM7-1). Substrates used were α-phenylenediamine dihydrochloride for HRP-conjugated antibodies and nitrophenyl phosphate (Mallinckrodt Baker, Phillipsburg, NJ) (2 mg ml^{-1} in 1 M diethanolamine/HCl buffer, pH 9.8, containing 5 mM $MgCl_2$) for alkaline phosphatase-conjugated antibodies. Reactivity was expressed as absorbance at 450 or 405 nm after 1 h.

Characterization of conditioning films

Samples of MUC5B and low-density proteins were added to six-well Ibidi μ-Slide VI ibiTreat flow-cells (ibidi GmbH, Munich, Germany) and incubated at 37°C for 24 h. A non-conditioned flow-cell served as a control. The flow-cell channels were blocked for 1 h with ELISA blocking solution. Surface coverage of the conditioning films was investigated using a mixture of HRP-conjugated lectins – *Ulex europaeus-I* (recognizing fucose), *Erythrina cristagalli* (recognizing β-galactose), *Sambucus nigra* and *Maackia amurensis* (both recognizing *N*-acetyl neuraminic acid) (EY Laboratories Inc., San Mateo, CA) diluted 2 μg ml^{-1} in blocking solution containing 0.1 mM $CaCl_2$. Following addition of lectins, flow-cells were incubated for 1 h at 37°C and all channels were washed with PBS. The rabbit/mouse EnVision™ HRP system (Dako) was then added for 15 min at 37°C. The channels were washed with PBS and staining was visualized using transmitted light microscopy.

To identify specific proteins in the conditioning films, LUM5B-2 and LUM7-1 as well as antibodies against amylase, cystatin, gp340, IgA, lysozyme, lactoferrin and statherin (as above) were used. After incubation with primary antibodies, fluorescein isothiocyanate (FITC) -conjugated secondary antibodies were added for 1 h at 37°C. Unbound antibodies were removed by rinsing with PBS, after which the

samples were studied with confocal scanning laser microscopy (CSLM).

Bacterial strains and growth conditions

The *S. mitis* biovar 2 cells were a fresh isolate from dental plaque (Wickström *et al.*, 2009) and *S. mutans* UA159 was a kind gift from Dr Dennis Cvitkovitch (University of Toronto, Toronto, ON, Canada). Strains were stored at −70°C and recovered on blood agar in an atmosphere of 5% CO_2 in air at 37°C for 24 h. Colonies were suspended in PBS to an optical density of 0.25, corresponding to approximately 4×10^8 cells ml^{-1}.

Adhesion assay

MUC5B and the low-density proteins were added to the flow-cells and left overnight at room temperature to create conditioning films. Non-conditioned surfaces served as controls. Either *S. mitis* biovar 2 or *S. mutans* cells were flowed over the conditioned and non-conditioned surfaces for 2 h at a rate of 3.6 ml h^{-1}. The flow-cells were subsequently rinsed with sterile PBS (pH 7.5) for 1 h to remove non-adherent cells. A LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen, Carlsbad, CA) was added, and the cells were investigated using CSLM. These and all subsequent experiments were repeated three times with independent bacterial cultures. Image analysis was performed using the software package *BIO*IMAGE_L (Chávez de Paz, 2009).

Proteolytic activity

Adherent cells were prepared as described above and MUC5B-pool or the low-density protein (LDP) -pool added for 24 h at 37°C to induce proteolytic activity. After rinsing with sterile PBS for 1 h, the cells were incubated with an FITC-conjugated casein substrate (Thermo Fisher Scientific, Rockford, IL) for 1 h at 37°C (Wickström *et al.*, 2009). SYTO® 62 (Invitrogen) (DNA dye) was used as a counterstain and the flow-cells were viewed using CSLM. Because of the relatively poor adhesion of *S. mutans* to MUC5B compared with the *S. mitis* biovar 2, a greater number of fields was analysed for the former species to give at least 1000 cells for analysis.

To study protease activity over time, *S. mitis* biovar 2 and *S. mutans* cells were allowed to adhere to MUC5B-coated surfaces for 2 h. Fluid-phase MUC5B was then added and the flow-cells were incubated for 1, 2, 3, 4, 5 or 24 h before the addition of the FITC-conjugated casein substrate. After 1 h at 37°C, cells were viewed using CSLM and image analysis was carried out as described above.

To investigate the effect of fluid phase MUC5B on proteolytic activity in *S. mitis* biovar 2, cells were suspended in sterile PBS (control) or MUC5B-pool at an optical density of 0.25, placed in a flow-cell, incubated with FITC-casein for 1 h at 37°C. Cells were viewed using CSLM and image analysis was carried out as described above. To determine how interactions with surface-associated MUC5B affect proteolytic activity, *S. mitis* biovar 2 cells suspended in sterile PBS were allowed to adhere to unconditioned or MUC5B-coated surfaces under flow for 2 h at a rate of 3.6 ml h^{-1}. After rinsing with sterile PBS for 1 h, the cells were incubated with FITC-casein for 1 h at 37°C and the cells were viewed using CSLM. Image analysis was performed as above.

Confocal scanning laser microscopy

All microscopy was performed using an Eclipse TE2000 inverted confocal scanning laser microscope (Nikon Corp., Tokyo, Japan). Twenty randomly selected areas of each sample corresponding to 1 mm^2 were viewed using an objective with a numerical aperture of 1.4 and the confocal pinhole was set to a diameter of 30 mm. Illumination was provided by an argon laser (488 nm laser excitation) fitted with a long-pass 515/30 filter for the green fluorescence signal and a long-pass 605/75 filter for the red fluorescence signal.

Statistical analysis

Results were analysed using the Mann–Whitney *U*-test and a *P*-value < 5% was considered significant.

RESULTS

Separation of salivary proteins

To separate and provide different populations of salivary proteins, whole human saliva was fractionated using density-gradient centrifugation (Fig. 1). The two

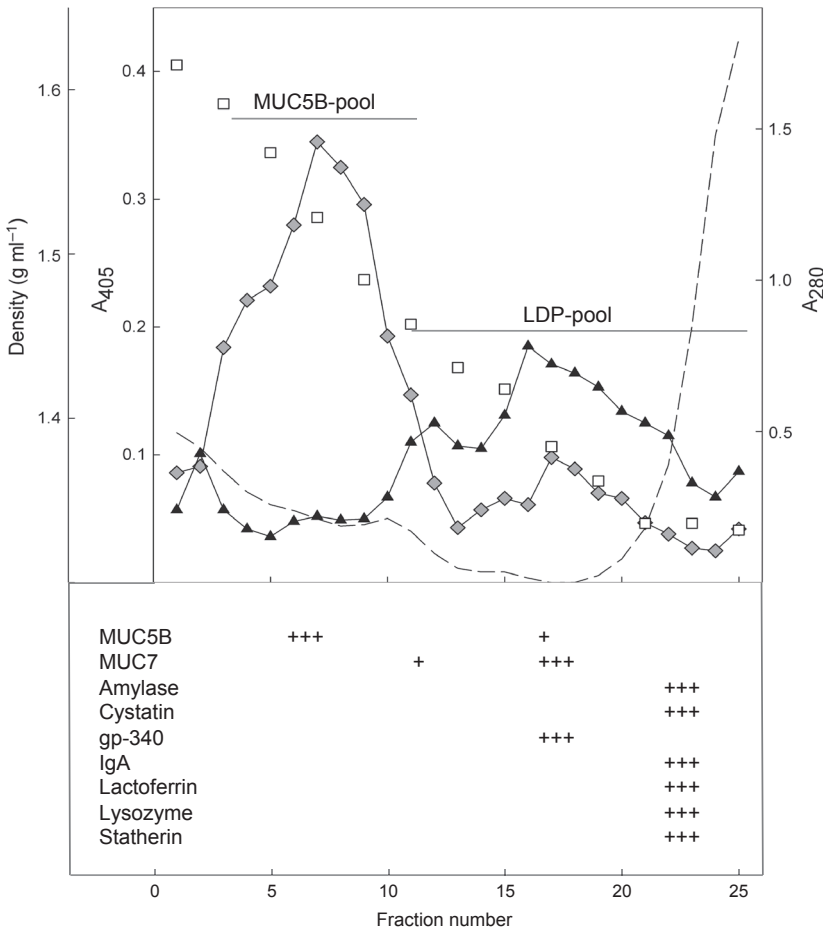


Figure 1 Fractionation of whole human saliva. Density-gradient centrifugation was performed in CsCl/0.1 M NaCl. The gradients were emptied from the bottom of the tubes and fractions were analysed for density (□), A_{280} (–) as well as reactivity with LUM5B-2 (◇) and LUM7-1 (▲) antisera using enzyme-linked immunosorbent assay (ELISA). Pools were made of fractions 3–10 (MUC5B-pool) and 11–25 [low-density proteins (LDP) –pool] and used as coatings in further experiments. The proteins identified in each pool using ELISA are shown beneath the graph.

major salivary mucins appeared to be well separated when analysed using the LUM5B-2 and LUM7-1 antisera. MUC5B was enriched between 1.48 and 1.60 g ml^{-1} (fractions 3–10) while MUC7 was found together with low-buoyant density proteins (showing absorbance at 280 nm) (fractions 11–25). Fractions 11–25 were also enriched in amylase, cystatin, gp340, IgA, lactoferrin, lysozyme and statherin as shown by additional analyses using specific antibodies. These proteins are less heavily glycosylated than MUC5B. Based on these analyses, two enriched pools were formed corresponding to high-density fractions 3–10 (MUC5B) and low-density protein fractions 11–25 (LDP). The MUC5B- and LDP-pools were dialysed against PBS for further use and because the dry weight of solids was essentially the same (0.4 and 0.6 mg ml^{-1}, respectively), equal volumes of each were used in subsequent experiments.

Characterization of salivary conditioning films

Flow-cells were coated with the MUC5B- or LDP-pools and, as described in the Methods, a mixture of lectins was used to visualize glycosylated proteins in the conditioning film. In the absence of proteins, no staining was seen (Fig. 2A, insert). The MUC5B-enriched conditioning film covered evenly although occasional aggregates, probably corresponding to MUC5B, could be seen (Fig. 2A). On the surfaces covered with the LDP-pool, the aggregates were

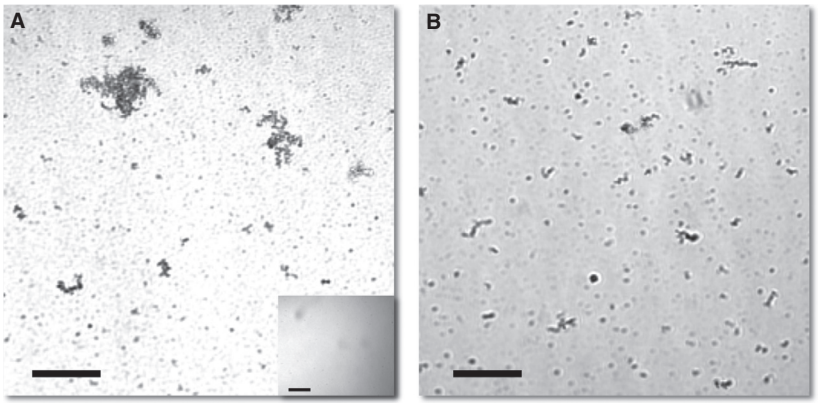


Figure 2 Characterization of salivary conditioning films. The distribution of (A) the MUC5B-pool and (B) the low-density protein (LDP) -pool on the flow-cell surfaces was studied *in situ* using a mixture of horseradish peroxidase (HRP) -conjugated lectins (*Ulex europaeus-I*, *Erythrina cristagalli*, *Sambucus nigra* and *Maackia amurensis*). Flow-cells were incubated with the lectins (2 μg ml^{-1} in blocking solution containing 0.1 mM $CaCl_2$) for 1 h at 37°C. Binding was visualized using the EnVision HRP™ system and staining was viewed using transmitted light microscopy. The insert shows an uncoated surface stained with the lectin mixture. The scale bar represents 10 μm.

absent (Fig. 2B). The presence of specific salivary proteins in the surface conditioning films was determined *in situ* using CSLM after staining with antibodies. After coating with the MUC5B-pool, MUC5B could be detected on the surface. Proteins previously detected in the LDP-pool (MUC7, amylase, cystatin, gp340, IgA, lactoferrin, lysozyme and statherin) were identified on the flow-cell surface after coating with this material (data not shown).

Adhesion of *Streptococcus mitis* biovar 2 and *Streptococcus mutans* to salivary conditioning films

Bacteria were passed over flow-cell surfaces conditioned with the MUC5B-pool or the LDP-pool for 2 h and the surface coverage was quantified after staining with the *Bac*Light™ viability stain. More than 90% of the attached *S. mitis* biovar 2 cells stained green, indicating a high level of viability (data not shown). Adhesion of *S. mitis* biovar 2 cells was more than three-fold higher to the MUC5B-coated surfaces (average surface coverage 7.3%) than to non-conditioned surfaces (average surface coverage, 2.0%) ($P < 0.0001$) or to those coated with the LDP-pool (average surface coverage, 1.6%) ($P < 0.005$) (Fig. 3 A). More than 90% of the attached *S. mutans* cells were viable based upon *Bac*Light™ viability staining (data not shown). *Streptococcus mutans* showed a slightly higher level of adherence to the non-conditioned surface (average surface coverage, 3.4%) than *S. mitis* biovar 2 but this difference was not significant (Fig. 3B). Unlike *S. mitis* biovar 2, *S. mutans* adherence was significantly reduced on surfaces coated with the MUC5B-pool (average surface coverage, 0.05%) as compared with the uncoated surface ($P < 0.05$) (Fig. 3B). When compared with the uncoated surface, *S. mutans* also adhered less to the

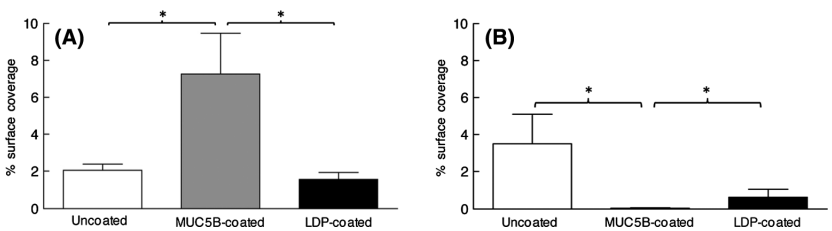


Figure 3 Adherence of (A) *S. mitis* biovar 2 and (B) *S. mutans* UA159 to surfaces coated with salivary proteins. Strains were grown on blood agar overnight and suspended in PBS at OD_{600} 0.25. The suspensions were flowed over the coated flow-cell surfaces at a rate of 3.6 ml h^{-1} for 2 h at 37°C. Adhered bacteria were stained using the LIVE/DEAD bacterial viability kit and visualized with CSLM. The graphs show mean ± standard error of the percentage surface coverage from three experiments using independent bacterial cultures (*$P < 0.05$).

LDP-pool (average surface coverage, 0.9%), although this difference was not significant at the 5% level.

Proteolytic activity of *Streptococcus mitis* biovar 2 and *Streptococcus mutans* in different salivary protein environments

To determine the effects of salivary proteins on proteolytic activity, cells were incubated in a MUC5B environment (a surface coating of MUC5B-pool + MUC5B-pool in the fluid phase) or an LDP environment (a surface coating of LDP-pool + LDP-pool in the fluid phase). *Streptococcus mitis* showed proteolytically active cells in both the MUC5B (Fig. 4A) and LDP (Fig. 4B) environments and in all cases, the activity appeared to be associated with the bacterial cell surface (see insert in Fig. 4A). Quantification revealed that in the absence of salivary proteins (no surface coating + PBS), approximately 10% of the *S. mitis* biovar 2 cells were proteolytically active whereas in the MUC5B or LDP environments, this proportion increased significantly to 80% ($P < 0.05$). and 50% ($P < 0.05$), respectively (Fig. 5). For *S. mutans* UA159, approximately 50% of the cells were proteolytically active in the absence of salivary proteins. In either the MUC5B or the LDP environments, the activity in the *S. mutans* population increased to over 80% ($P < 0.05$). The presence of salivary proteins therefore appears to enhance proteolytic activity in both *S. mitis* biovar 2 and *S. mutans* cells.

To study the induction of proteolytic activity over time, *S. mitis* biovar 2 and *S. mutans* cells were incubated in a MUC5B-environment over 24 h and the number of active cells was investigated at intervals (Fig. 6). For both species, the percentage of proteolytically active cells reached a maximum after 2 h, which was maintained over the following 24 h.

Effect of soluble and surface-associated MUC5B

The proteolytic activity seen in the MUC5B environment could be the result of both fluid phase and surface-associated MUC5B, so the effect of fluid phase MUC5B alone was investigated by comparing the activity of *S. mitis* cells suspended in MUC5B with that of cells suspended in buffer (Table 1). In PBS, 13 ± 1% of the cells were active whereas in the presence of MUC5B, the level was 70 ± 6% indicating that the presence of MUC5B in the fluid phase enhanced proteolytic activity in the population. To investigate the contribution of surface-associated MUC5B to the effect seen in the MUC5B environment, the activity of *S. mitis* biovar 2 cells on a MUC5B-coated surface was compared with that on an unconditioned one. On the MUC5B coating, the proportion of proteolytically active *S. mitis* biovar 2 cells was 25 ± 2%, compared with 6 ± 1% on an unconditioned surface suggesting that surface-associated MUC5B enhances proteolytic activity. For *S. mutans* the pattern was the same as that seen for

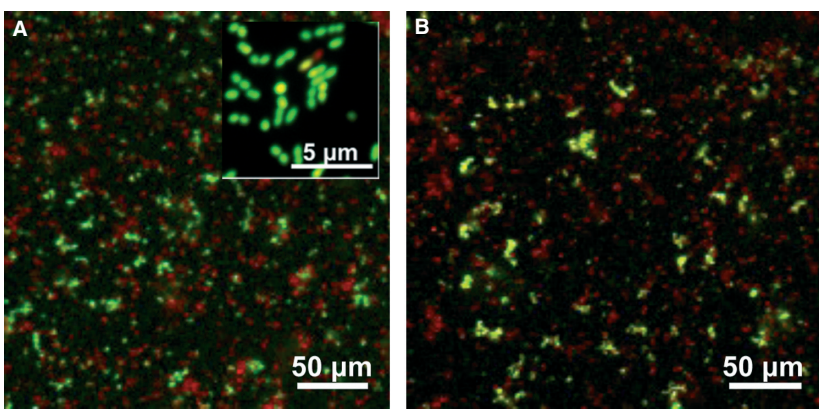


Figure 4 Protease activity of adherent *Streptococcus mitis* biovar 2 cells. Bacteria were exposed to (A) a MUC5B environment (a surface coating of MUC5B-pool + MUC5B-pool in the fluid phase) or (B) a low-density protein (LDP) environment (a surface coating of LDP-pool + LDP-pool in the fluid phase). Protease activity was assessed *in situ* after 24 h by incubation with a fluorescein isothiocyanate-casein substrate for 1 h at 37°C and representative confocal laser scanning microscopy images are shown. Green staining shows protease-active cells while staining with the red counterstain (SYTO 62) indicates an absence of protease activity. The insert shows activity associated with the bacterial cell surface.

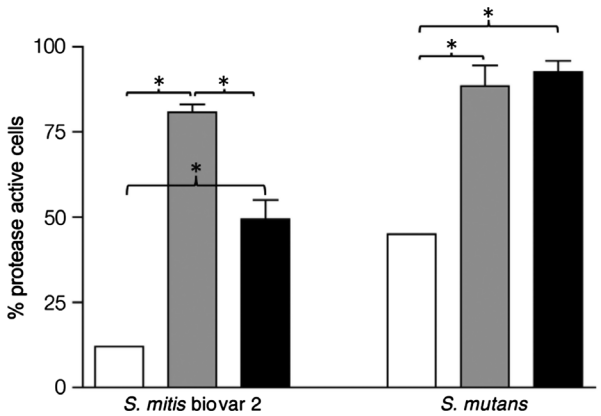


Figure 5 Protease activity of *Streptococcus mitis* biovar 2 or *Streptococcus mutans* cells exposed to different salivary protein environments. The proportion of protease-active *S. mitis* biovar 2 or *S. mutans* cells exposed to a phosphate-buffered saline environment (no surface coating + PBS, white bars), a MUC5B environment (a surface coating of MUC5B-pool + MUC5B-pool in the fluid phase, grey bars) or a low-density protein (LDP) environment (a surface coating of LDP-pool + LDP-pool in the fluid phase, black bars) was assessed *in situ* after 24 h by incubation with a fluorescein isothiocyanate-casein substrate for 1 h at 37°C. The results are presented as mean ± standard error of three independent experiments (*$P < 0.05$).

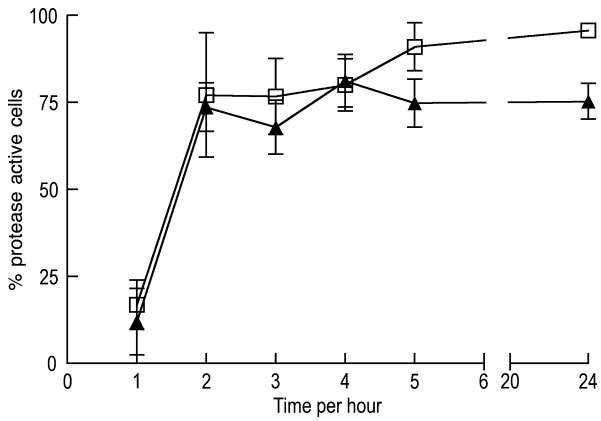


Figure 6 Protease activity of *Streptococcus mitis* biovar 2 and *Streptococcus mutans* cells in a MUC5B environment over time. *Streptococcus mitis* biovar 2 (□) or *S. mutans* (▲) cells were allowed to adhere to MUC5B-coated surfaces under flow conditions (3.6 ml h^{-1} at 37°C) for 2 h. Fluid-phase MUC5B was then added and the flow-cells were incubated for 1, 2, 3, 4, 5 or 24 h before the addition of the fluorescein isothiocyanate-conjugated casein substrate. After a further 1 h at 37°C, cells were viewed using confocal laser scanning microscopy and image analysis was carried out as described. Each time-point represents the mean ± standard error of at least three independent experiments.

S. mitis biovar 2. In the presence of fluid phase MUC5B the proteolytic activity in the population was 85 ± 2% compared with the level in PBS (52 ± 2%). On the MUC5B coating, the proportion of proteolytically active *S. mutans* cells was 50 ± 3%, compared with 4 ± 1% on the unconditioned surface.

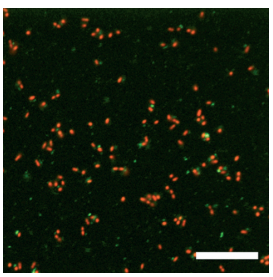
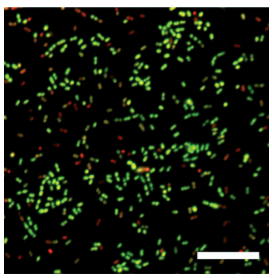
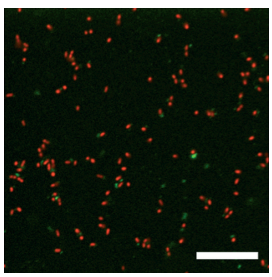
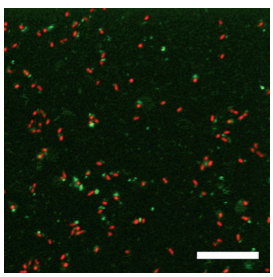
Overall these data suggest that although most of the proteolytic activity seen in the MUC5B environment may be attributed to interaction with MUC5B molecules in the fluid phase, surface-associated MUC5B may further contribute to the activity of the adherent cells.

DISCUSSION

Bacteria colonizing the oral cavity encounter surfaces covered with a salivary pellicle. Here, we show that *S. mitis* biovar 2 binds better to *ex vivo* pellicles of salivary proteins than *S. mutans* thereby providing a possible explanation for why *S. mitis* biovar 2 is an early colonizer of oral surfaces *in vivo* whereas *S. mutans* is not. Salivary proteins were separated into two pools, one was enriched in large MUC5B mucins and the other contained low-density proteins including MUC7, amylase, cystatin, gp340, IgA, lactoferrin, lysozyme and statherin. When compared with *S. mutans*, the *S. mitis* biovar 2 showed a significantly higher level of binding to surfaces coated with either protein pool. Indeed, the MUC5B-coated surface supported *S. mitis* biovar 2 binding at seven-fold greater levels than *S. mutans* binding The ability of *S. mitis* to bind well to surface-associated salivary proteins is consistent with *in vivo* data characterizing the early stages of microbial colonization on tooth surfaces. After 4 h, *S. mitis* (arginine-negative strains) comprised up to 35% of the cultivable microbiota whereas *S. mutans* was not detected (Nyvad & Kilian, 1987).

Salivary MUC5B mucins as well as MUC7 and gp340 carry a wide array of *O*-linked glycans, which contain α-2,3-linked or α-2,6-linked sialic acid residues (Thomsson *et al.*, 2002; Hartshorn *et al.*, 2006; Karlsson & Thomsson, 2009) and serve as binding sites for bacterial adhesion. *Streptococcus mitis* expresses at least one surface protein (PblA/PblB) that can mediate attachment to the sialic acid residues found on platelet membrane gangliosides (Bensing *et al.*, 2001; Mitchell & Sullam, 2009). PblA/PblB is therefore a candidate adhesin for binding of *S.*

Table 1 Effect of soluble and adsorbed MUC5B on proteolytic activity in *Streptococcus mitis* biovar 2 cells

Planktonic cells		Adherent cells	
In buffer	In MUC5B	No coat	MUC5B coat
13 ± 1%	70 ± 6%	6 ± 1%	25 ± 2%
			

Representative CSLM images are shown for each condition. The scale bars represent 50 μm.

mitis to sialylated salivary molecules. In addition, *S. mitis* express an α-amylase-binding protein C (AbpC) (Vorassi *et al.*, 2010), which interacts with fluid phase or surface-associated α-amylase (Kilian & Nyvad, 1990; Murray *et al.*, 1992; Brown *et al.*, 1999). In this study, the level of binding of *S. mitis* biovar 2 to surface-associated MUC5B was at least four-fold greater than to the pool containing MUC7, gp340 and amylase, suggesting that interaction with MUC5B via the PblA/PblB adhesin would be the predominant interaction allowing *S. mitis* to colonize saliva-coated oral surfaces *in vivo.*

It is well known that high-molecular-weight salivary mucins can interact with, and aggregate, *S. mutans* in the fluid phase (Levine *et al.*, 1978). In this study, however, *S. mutans* UA159 showed poor adherence to adsorbed MUC5B. *Streptococcus mutans* appears therefore to bind to sites on MUC5B that are not exposed when the molecules are presented on a surface. When compared with MUC5B, the level of *S. mutans* binding to the surface-associated low-density proteins was greater, most probably because of interactions with gp340. This salivary glycoprotein promotes the aggregation of *S. mutans* when present in the fluid phase but can also act as a binding site when adsorbed to a surface (Brady *et al.*, 1992; Danielsson Niemi *et al.*, 2009). Binding to gp340 can be mediated by *S. mutans* AgI/II (Crowley *et al.*, 1993; Jakubovics *et al.*, 2005). *Streptococcus mutans* could be divided into strains that were both aggregated by and adherent to gp340 and those that were preferentially aggregated and not adherent (Loimaranta *et al.*, 2005). *Streptococcus mutans* UA159 appeared to belong to the latter group.

In addition to adherence, the complex process of biofilm formation by streptococci on oral surfaces involves environmental sensing, nutritional adaptation, growth and survival (Nobbs *et al.*, 2009). When compared with cells in the fluid phase in this study, contact with salivary proteins induced an additional protease activity in both *S. mitis* biovar 2 and *S. mutans*. Both species are therefore able to sense surface-associated salivary proteins and respond by increasing activity. Extracellular protease activity has been demonstrated previously for *S. mutans* (Cowman *et al.*, 1976) and has been proposed to be a mechanism for the generation of nutrients through the degradation of salivary proteins (Bowden & Hamilton, 1998). Analysis of the genome, suggests that *S. mutans* U159 possesses several proteases, including the serine protease HtrA, which can act as chaperones and degrade misfolded proteins (Diaz-Torres & Russell, 2001). The best characterized is the *S. mitis* cell wall-anchored zinc metalloprotease, IgA1 protease (Senior & Woof, 2006). However several other, as yet uncharacterized, proteases are predicted from genome sequences (Denapaite *et al.*, 2010).

In conclusion, although this may vary between strains, the early colonizer *S. mitis* biovar 2 and the later colonizer *S. mutans* appear to differ in their binding to surface-associated salivary proteins. *Streptococcus mitis* most probably binds to MUC5B and to a lesser extent low-density proteins whereas *S. mutans* adheres poorly. These differences may, in

part, explain why *S. mitis* is an early colonizer of oral surfaces while *S. mutans* colonizes later. We have demonstrated a proteolytic activity in both *S. mitis* and *S. mutans* in contact with salivary proteins but further studies are required to understand how this contributes to survival of streptococci in the oral environment.

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REFERENCES

Aas, J.A., Paster, B.J., Stokes, L.N., Olsen, I. and Dewhirst, F.E. (2005) Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol* **43**: 5721–5732.

Bensing, B.A., Siboo, I.R. and Sullam, P.M. (2001) Proteins pblA and pblB of *Streptococcus mitis*, which promote binding to human platelets, are encoded within a lysogenic bacteriophage. *Infect Immun* **69**: 6186–6192.

Bowden, G.H. and Hamilton, I.R. (1998) Survival of oral bacteria. *Crit Rev Oral Biol Med* **9**: 54–85.

Bradshaw, D.J., Homer, K.A., Marsh, P.D. and Beighton, D. (1994) Metabolic cooperation in oral microbial communities during growth on mucin. *Microbiology* **140**: 3407–3412.

Brady, L.J., Piacentini, D.A., Crowley, P.J., Oyston, P.C. and Bleiweis, A.S. (1992) Differentiation of salivary agglutinin-mediated adherence and aggregation of mutans streptococci by use of monoclonal antibodies against the major surface adhesin P1. *Infect Immun* **60**: 1008–1017.

Brown, A.E., Rogers, J.D., Haase, E.M., Zelasko, P.M. and Scannapieco, F.A. (1999) Prevalence of the amylase-binding protein A gene (abpA) in oral streptococci. *J Clin Microbiol* **37**: 4081–4085.

Chávez de Paz, L.E. (2009) Image analysis software based on color segmentation for characterization of viability and physiological activity of biofilms. *Appl Environ Microbiol* **75**: 1734–1739.

Cole, M.F., Evans, M., Fitzsimmons, S. *et al.* (1999) Pioneer oral streptococci produce immunoglobulin A1 protease. *Infect Immun* **62**: 2165–2168.

Cowman, R.A., Perrella, M.M. and Fitzgerald, R.J. (1976) Caseinolytic and glycoprotein hydrolase activity of *Streptococcus mutans*. *J Dent Res* **55**: 391–399.

Crowley, P.J., Brady, L.J., Piacentini, D.A. and Bleiweis, A.S. (1993) Identification of a salivary agglutinin-binding domain within cell surface adhesin P1 of *Streptococcus mutans*. *Infect Immun* **61**: 1547–1552.

Danielsson Niemi, L., Hernell, O. and Johansson, I. (2009) Human milk compounds inhibiting adhesion of mutans streptococci to host ligand-coated hydroxyapaptite *in vitro*. *Caries Res* **43**: 171–178.

Denapaite, D., Bruckner, R. and Nuhn, M. *et al.* (2010) The genome of *Streptococcus mitis* B6 – what is a commensal? *PLoS ONE* **5**: e9426.

Diaz-Torres, M.L. and Russell, R.R.B. (2001) HtrA protease and processing of extracellular proteins of *Streptococcus mutans*. *FEMS Microbiol Lett* **204**: 23–28.

Grønbaek Frandsen, E.V. (1999) Bacterial degradation of immunoglobulin A1 in relation to periodontal diseases. *APMIS Suppl* **87**: 1–54.

Hannig, M. and Joiner, A. (2006) The structure, function and properties of the acquired pellicle. *Monogr Oral Sci* **19**: 29–64.

Hartshorn, K.L., Ligtenberg, A., White, M.R. *et al.* (2006) Salivary agglutinin and lung scavenger receptor cysteine-rich glycoprotein 340 have broad anti-influenza activities and interactions with surfactant protein D that vary according to donor source and sialylation. *Biochem J* **393**: 545–553.

Iontcheva, I., Oppenheim, F.G. and Troxler, R.F. (1997) Human salivary mucin MG1 selectively forms heterotypic complexes with amylase, proline-rich proteins, statherin, and histatins. *J Dent Res* **76**: 734–743.

Jakubovics, N.S., Strömberg, N., van Dolleweerd, C.J., Kelly, C.G. and Jenkinson, H.F. (2005) Differential binding specificities of oral streptococci antigen I/II family adhesins for human or bacterial ligands. *Mol Microbiol* **55**: 1591–1605.

Karlsson, N.G. and Thomsson, K.A. (2009) Salivary MUC7 is a major carrier of blood group I type O-linked oligosaccharides serving as the scaffold for sialyl Lewis x. *Glycobiology* **19**: 288–300.

Kilian, M. and Nyvad, B. (1990) Ability to bind salivary α-amylase discriminates certain viridans group streptococcal species. *J Clin Microbiol* **28**: 2576–2577.

Kolenbrander, P.E., Palmer, R.J. Jr, Periasamy, S. and Jakubovics, N.S. (2010) Oral multispecies biofilm development and the key role of cell–cell distance. *Nat Rev Microbiol* **8**: 471–480.

Lendenmann, U., Grogan, J. and Oppenheim, F.G. (2000) Saliva and the dental pellicle – a review. *Adv Dent Res* **14**: 22–28.

Levine, M.J., Herzberg, M.C., Levine, M.S. *et al.* (1978) Specificity of salivary–bacterial interactions: role of terminal sialic acid residues in the interaction of salivary glycoproteins with *Streptococcus sanguis* and *Streptococcus mutans*. *Infect Immun* **19**: 107–115.

Li, J., Helmerhorst, E.J., Leone, C.W. *et al.* (2004) Identification of early microbial colonizers in human dental biofilm. *J Appl Microbiol* **97**: 1311–1318.

Lima, E.M., Koo, H., Vacca Smith, A.M., Rosalen, P.M. and Del Bel Cury, A.A. (2008) Adsorption of salivary and serum proteins, and bacterial adherence on titanium and zirconia ceramic surfaces. *Clin Oral Implants Res* **19**: 780–785.

Loimaranta, V., Jakubovics, N.S., Hytönen, J., Finne, J., Jenkinson, H.F. and Strömberg, N. (2005) Fluid- or surface-phase human salivary scavenger protein gp340 exposes different bacterial recognition properties. *Infect Immun* **73**: 2245–2252.

Marsh, P.D., Moter, A. and Devine, D.A. (2011) Dental plaque biofilms: communities, conflict and control. *Periodontol 2000* **55**: 16–35.

Mitchell, J. and Sullam, P.M. (2009) *Streptococcus mitis* phage-encoded adhesins mediate attachment to 2-8-linked sialic acid residues on platelet membrane gangliosides. *Infect Immun* **77**: 3485–3490.

Murray, P.A., Prakobphol, A., Lee, T., Hoover, C.I. and Fisher, S.J. (1992) Adherence of oral streptococci to salivary glycoproteins. *Infect Immun* **60**: 31–38.

Nieuw Amerongen, A.V. and Veerman, E.C.I. (2002) Saliva – the defender of the oral cavity. *Oral Dis* **8**: 12–20.

Nobbs, A.H., Lamont, R.J. and Jenkinson, H.F. (2009) Streptococcus adherence and colonization. *Microbiol Mol Biol Rev* **73**: 407–450.

Nyvad, B. and Kilian, M. (1987) Microbiology of the early colonization of human enamel and root surfaces *in vivo*. *Scand J Dent Res* **95**: 369–380.

Pratt-Terpstra, I.H., Weerkamp, A.H. and Busscher, H.J. (1989) The effects of pellicle formation on streptococcal adhesion to human enamel and artificial substrata with various surface free-energies. *J Dent Res* **68**: 463–467.

Raynal, B.D.E., Hardingham, T.E., Sheehan, J.K. and Thornton, D.J. (2003) Calcium-dependent protein interactions in MUC5B provide reversible cross-links in salivary mucus. *J Biol Chem* **278**: 28703–28710.

Reinholdt, J., Tomana, M., Mortensen, A.B. and Kilian, M. (1990) Molecular aspects of immunoglobulin A1 degradation by oral streptococci. *Infect Immun* **58**: 1186–1194.

Ruhl, S., Sandberg, A.L. and Cisar, J.O. (2004) Salivary receptors for the proline-rich protein-binding and lectin-like adhesins of oral Actinomyces and Streptococci. *J Dent Res* **83**: 505–510.

Scannapieco, F.A. (1994) Saliva–bacterium interactions in oral microbial ecology. *Crit Rev Oral Biol Med* **5**: 203–248.

Senior, B.W. and Woof, J.M. (2006) Sites in the CH3 domain of human IgA1 that influence sensitivity to bacterial IgA1 proteases. *J Immunol* **177**: 3913–3919.

Siquiera, W.L., Zhang, W., Helmerhorst, E.J., Gygi, S.P. and Oppenheim, F.G. (2007) Identification of protein components in *in vivo* human acquired enamel pellicle using LC-ESI-MS/MS. *J Proteome Res* **6**: 2152–2160.

Smith, K. and Beighton, D. (1986) The effects of the availability of diet on the levels of exoglycosidases in the supragingival plaque of macaque monkeys. *J Dent Res* **65**: 1349–1352.

Smith, K. and Beighton, D. (1987) Proteolytic activities in the supragingival plaque of monkeys (*Macaca fascicularis*). *Arch Oral Biol* **32**: 437–476.

Thomsson, K.A., Prakobphol, A., Leffler, H. *et al.* (2002) The salivary mucin MG1 (MUC5B) carries a repertoire of unique oligosaccharides that is large and diverse. *Glycobiology* **12**: 1–14.

Vorassi, J., Chauduri, B., Haase, E.M. and Scannapiecom, F.A. (2010) Identification and characterization of amylase binding protein C (AbpC) from *Streptococcus mitis* NS51. *Mol Oral Microbiol* **25**: 150–155.

Wickström, C. and Svensäter, G. (2008) Salivary gel-forming mucin MUC5B – a nutrient for dental plaque bacteria. *Oral Microbiol Immunol* **23**: 177–182.

Wickström, C., Davies, J.R., Eriksen, G.V., Veerman, E.C. and Carlstedt, I. (1998) MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage. *Biochem J* **334**: 685–693.

Wickström, C., Christersson, C., Davies, J.R. and Carlstedt, I. (2000) Macromolecular organization of saliva: identification of 'insoluble' MUC5B assemblies and non-mucin proteins in the gel phase. *Biochem J* **351**: 421–428.

Wickström, C., Herzberg, M.C., Beighton, D. and Svensäter, G. (2009) Proteolytic degradation of human salivary MUC5B by dental biofilms. *Microbiology* **155**: 2866–2872.

Yao, Y., Berg, E.A., Costello, C.E., Troxler, R.F. and Oppenheim, F.G. (2003) Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches. *J Biol Chem* **278**: 5300–5308.