Differential metabolic activity by dental plaque bacteria in association with two preparations of MUC5B mucins in solution and in biofilms

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Salivary mucin, MUC5B, is an oligomeric glycoprotein, heterogeneous in size and with a diverse repertoire of oligosaccharides, which differ in composition and charge. Since complex salivary glycoproteins are considered to be the major source of nutrients for the oral supragingival microbiota, the major aim of the current study was to determine whether different preparations of non-denatured MUC5B could be isolated exhibiting different biological properties in relation to the microflora associated with the surfaces of the oral cavity. Two preparations, solMUC5B and gelMUC5B, were isolated by density-gradient centrifugation and were shown to have different buoyant densities, carbohydrate content and surface-adsorbing characteristics. To ascertain differences in biological activity, the two mucin preparations, both in solution and adsorbed to a model surface, were incubated with freshly isolated dental plaque and assayed for metabolic (dehydrogenase) activity with the fluorescent substrate CTC (5-cyano-2,3-ditolyl tetrazolium chloride). The plaque bacteria exhibited higher metabolism with the solMUC5B preparation in solution, with 79.4 % active plaque cells compared to the controls without mucin (9.6 %), while gelMUC5B showed 48.2 % active cells with the same plaque population. In contrast, the same mucins adhered to a surface elicited a significantly lower metabolic response, with surface-associated plaque cells showing only 12.1 % active cells with solMUC5B and 29.2 % with gelMUC5B. These results suggested that the metabolism by the plaque cells adsorbed to surface-associated mucins was downregulated compared to the same cells suspended in mucin solution. This was confirmed in an experiment where active dispersed plaque/solMUC5B suspensions were shown to lose significant metabolic activity (e.g. 74.9 to 19.3 %) when allowed to interact with gelMUC5B adsorbed to a surface. Clearly, the solMUC5B and gelMUC5B preparations exhibited different biological activity when assayed with freshly plaque bacteria in suspension and in a biofilm.

INTRODUCTION

The human oral cavity supports growth of a complex microflora consisting of more than 500 species of microorganisms (Kroes et al., 1999). Dental plaque can be characterized both as a microbial community and as a biofilm (Marsh, 2005) and, in the latter, synergistic and competitive interactions amongst the bacteria lead to a biodiverse ecosystem in balance with its host. The oral commensal microbiota colonizing the epithelial and dental surfaces can be seen to have two opposing roles in the mouth. On the one hand, the microflora is a positive force acting with the host defences to prevent colonization by pathogenic microbes. On the other hand, this consortium comprises organisms associated with oral diseases, such as dental caries, periodontitis and root canal infections (Bowden, 1991; Socransky & Haffajee, 2002; Chavez de Paz, 2007).

Bacterial cells growing on surfaces in mature biofilms display a reduced susceptibility to antimicrobial agents and various host defence systems, and are known to be physiologically distinct from the same organism growing in a liquid or planktonic phase (Costerton et al., 1987; Fletcher, 1991; Goodman & Marshall, 1995; Welin et al., 2004). To the latter point, Lactobacillus salivarius and Streptococcus anginosus have recently been shown to differ in metabolic activity depending on whether the cells were in a biofilm or in a planktonic culture, with the planktonic cells being reactivated almost immediately but the biofilm cells requiring 72 h to show metabolic activity (Chávez de Paz et al., 2008). Similarly, starved dental plaque showed

Abbreviation: CTC, 5-cyano-2,3-ditolyl tetrazolium chloride.
very little metabolic activity when tested directly after removal from the tooth surface, but exhibited an immediate metabolic response after incubation with salivary glycoproteins in solution (Wickström & Svensäter, 2008).

In microbial biofilms, available nutrients are a major ecological determinant for the microbial composition of surface ecosystems. In the human mouth, salivary proteins and glycoproteins are continuously available to support growth and adhesion. In the latter process, salivary mucins contribute substantially to the formation of the acquired pellicle, or conditioning film, in the oral cavity (Nieuw Amerongen et al., 1989; Tabak, 1995) and can influence the establishment and selection of the oral microflora by promoting the adhesion of certain bacteria and facilitating the clearance of other species (e.g. Marsh, 2003; Scannapieco, 1994). The attached bacteria then grow and modify the local environment, making the site suitable for the colonization of more fastidious species. The complexity of the substrate has also been shown to influence the diversity of oral streptococcal species in the mouth (van der Hoeven & Camp, 1991). Early research has demonstrated that while the concentration of free sugar in saliva is too low to support the growth of many individual oral strains, selected groups of organisms possessing the requisite cell-bound hydrolytic enzymes can degrade salivary glycoproteins for energy and growth (De Jong et al., 1984; De Jong & van der Hoeven, 1987). Oral bacteria have been shown to express a wide variety of glycosidases that permits the degradation of complex substrates, such as salivary glycoproteins (e.g. Beighton & Whiteley, 1990; Byers et al., 2000). This metabolic activity was clearly shown by Bradshaw et al. (1994) in a study employing hog gastric mucin as the major carbon and energy source in a chemostat inoculated with a mixture of 10 organisms selected for their hydrolytic enzyme activity. A basic group possessing glycosidase and protease activity was supplemented with organisms possessing additional enzymes, such as sialidase, α-fucosidase and endopeptidase, to form a stable diverse community with an overlapping pattern of enzymic activity capable of the complete degradation of the mucin.

The major gel-forming mucin in saliva is MUC5B (MG1) and thus constitutes the major framework for the mucus film in the mouth. The MUC5B mucin is a complex molecule due to oligomerization and glycosylation, as well as putative cleavage sites at both the C- and N-termini (Thornton et al., 1997; Veerman et al., 1992; Wickström et al., 1998; Wickström & Carlstedt, 2001). The ultracentrifugation of saliva results in a sol and a gel phase; hence MUC5B can be isolated from the two phases using density-gradient centrifugation, with the distribution of MUC5B between the phases revealing subpopulations of the mucin (Wickström et al., 2000). The biological significance of this discovery is not yet clear, but it could suggest that the various subpopulations have different biological functions in the oral cavity.

Mucus on other epithelial surfaces in the body (e.g. the stomach and the respiratory tract) has been shown to be organized in layers, with one layer being more surface-associated and more ‘insoluble’, while a second layer is more porous (Taylor et al., 2004; Thornton & Sheehan, 2004; Sheehan et al., 2006; Hidaka et al., 2001). In the case of the stomach and the respiratory tract, different mucin gene products, MUC5AC and MUC6, MUC5B and MUC5AC, respectively, may fulfill these two roles. Currently, salivary MUC5B is the only known gel-forming, oligomeric mucin, which might suggest that differently ‘processed’ MUC5B molecules can display similar functions in the oral cavity. Apart from its proposed function of providing lubrication to the oral epithelia, little is known about the molecule’s specific function, such as the role it might play in the modulation of the oral bacterial community.

In this study, we undertook to examine whether different species of non-denatured salivary MUC5B might exist possessing different biological functions in the oral cavity, particularly in relation to the metabolic activity of the resident oral microflora in human dental plaque. Two preparations of salivary MUC5B were derived by density-gradient centrifugation and shown to differ in buoyant density, carbohydrate content and surface-adherent characteristics. These two preparations were further shown to support differing levels of metabolic activity by freshly harvested dental plaque cells using a fluorescent assay differentiating between metabolically active and inactive cells. These differences were further accentuated when comparing the metabolic activity of plaque cells in suspension with those adsorbed to a surface in a biofilm.

METHODS

Purification and carbohydrate analysis of human salivary MUC5B. Two preparations of human salivary MUC5B were generated from whole saliva collected on ice from eight individuals and purified in such a way as to preserve as much as possible the native, non-denatured state of the molecules. The saliva was pooled and then subjected to ultracentrifugation at 36 000 r.p.m. for 30 min at 4 °C in a Beckman Optima LE-80K centrifuge fitted with a 70.1Ti rotor. The supernatant was recovered and subjected to isopycnic density-gradient centrifugation at 15 °C in CsCl/0.1M NaCl (start density 1.45 g ml⁻¹) for 96 h at 36 000 r.p.m. in the above centrifuge fitted with a 50.2Ti rotor. Fractions were collected from the bottom of the tube and analysed for density (weighing) and A₂₈₀. MUC5B-containing fractions were identified using an antiserum against the peptide backbone of the protein (LUM5B-2) described by Wickström et al. (1998), and MUC5B-containing fractions were pooled and dialysed against 10 mM PBS (7 mM K₂HPO₄, 2.5 mM KH₂PO₄, 70 mM NaCl, pH 7.2) and stored in −20 °C until used. This pooled MUC5B fraction was designated solMUC5B (Fig. 1). The pellet from the original 30 min centrifugation was homogenized in 0.1M NaCl using a Dounce homogenizer and the solubilized pellet gently centrifuged (4400 g, 30 min, 4 °C). The supernatant from this latter centrifugation was subjected to isopycnic density-gradient centrifugation and the fractions containing MUC5B were pooled and dialysed as described above to constitute the second preparation, designated gelMUC5B (Fig. 1). The pools of MUC5B were subjected to SDS-
PAGE in order to examine whether low-molecular-mass proteins were associated with the gel network to contaminate the preparation. The concentrations of solMUC5B and gelMUC5B were determined following lyophilization and weighing, and each was adjusted to a final concentration of 0.4 mg ml⁻¹. Carbohydrate analyses were performed investigating both the neutral monosaccharide composition and the sialic acid content of the two mucin preparations. The analyses were carried out by M-Scan (Wokingham, UK).

ELISA and SDS-PAGE. ELISA was performed as previously described (Wickström et al., 1998). In short, samples were coated onto multiwell assay plates (3912, Falcon) overnight at room temperature. Plates were blocked for 1 h with 0.15 M NaCl, 0.1 M Tris/HCl buffer, pH 7.4 containing 0.05% (v/v) Tween 20 and 1% (w/v) blocking solution) and incubated (1 h) with the LUM5B-2 antiserum diluted 1 : 1000. Detection was carried out using an alkaline phosphatase-conjugated swine anti-rabbit antiserum (Dako), diluted 1 : 5000. 2-Antiserum was then incubated (1 h) with alkaline phosphatase-conjugated swine anti-rabbit antiserum (Dako), diluted 1 : 5000. Detection was carried out using an alkaline phosphatase-conjugated swine anti-rabbit antiserum (Dako), diluted 1 : 5000. The gel was stained with silver nitrate as described by the manufacturer (Pharmacia Biotech).

Detection of MUC5B on mucin-coated flow-cells. The two mucin preparations were coated onto the flow-chamber system μ-Slide VI for Live Cell Analysis (IBIDI, Integrated BioDiagnostics) with and without 10 mM CaCl₂ overnight at room temperature. Flow-cells were incubated with blocking solution for 1 h, followed by incubation (1 h) with the monoclonal antibody EU-MUC5Bα (Rousseau et al., 2003) diluted 1 : 100 in blocking solution. Flow-cells were then incubated (1 h) with a FITC-conjugated sheep anti-mouse IgG antibody (Dako) and analysed by epifluorescence microscopy.

Experimental protocol. Freshly harvested, 24-h-old supragingival dental plaque was pooled from buccal and lingual surfaces of the premolar and molar regions from one 38-year-old healthy male before breakfast. The individual had refrained from normal dental hygiene procedures the night before and on the morning of the collection, and the plaque sample was used for the experiments on the same day. The plaque was mixed by vortexing for 10 s and approximately 10 μg plaque, as measured using a sterile spoon modified from Jordan et al. (1968), was added to 1 ml PBS/MUC5B solution.

To test the metabolic activity of plaque in suspension (planktonic culture), an aliquot of the plaque suspension was added to 1 ml solMUC5B or gelMUC5B in 1.5 ml tubes and incubated in air for 1 h in 37 °C. As a control, a similar aliquot was incubated in PBS under the same conditions. Following incubation, separate cell samples were subjected to staining using the RedoxSensor CTC Vitality kits (Molecular Probes). CTC staining involved the redox fluorescence dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), measuring dehydrogenase activity (red), together with a DNA-staining dye (SYTO24) (green). For each assay, a 30 μl aliquot of the plaque suspension was incubated with 20 μl dye (20 mM CTC and 1 μl SYTO24 ml⁻¹) for 30 min in the dark at room temperature before microscopic analysis.

Assays for metabolic activity of the collected plaque in biofilms on mucin-coated surfaces were carried out with the flow-chamber system μ-Slide VI for Live Cell Analysis coated with solMUC5B or gelMUC5B in the presence of 10 mM CaCl₂ overnight at room temperature. Control flow-cells were coated with PBS containing 10 mM CaCl₂. After rinsing with PBS, plaque was added to all surfaces and the bacteria were allowed to adhere to the surfaces for 2 h. After an additional rinsing with PBS, the adhered bacteria were stained with CTC/SYTO 24 for 30 min in the dark at room temperature before microscopic analysis. Fluorescent microscopic images were captured using the model DS-2Mv Nikon Digital Sight system with a digital camera connected to a PC with the software NIS-Elements BR 2.30 (Nikon).

Each experiment consisted of analysing 15 different microscopic fields displaying clusters of metabolically active and inactive cells of plaque bacteria. The calculation of the proportion of metabolically active and active cells involved determining the percentage surface coverage of fluorescent green and red cells using the software MATLAB, v.7.0 for Windows. Each experiment was repeated three times and one representative image of each triplicate experiment is shown in the results.

Since there is evidence for the metabolic downregulation of cells once they adhere to a surface in a biofilm (e.g. Chávez de Paz et al., 2008), we undertook to test this by interacting metabolically active planktonic solMUC5B/plaque complexes with a gelMUC5B-coated surface. For this, freshly harvested dental plaque was added to 1 ml solMUC5B in 1.5 ml tubes and incubated in air for 1 h in 37 °C. As a control, a 30 μl aliquot of the plaque suspension was incubated in PBS with 20 μl CTC/SYTO24 dye as described above. A second 30 μl aliquot of the solMUC5B/plaque mixture was added to a gelMUC5B-coated flow-cell and bacteria were allowed to adhere to the surface for 2 h. After rinsing with PBS, adhered bacteria were stained with CTC/SYTO 24 for 30 min in the dark at room temperature before microscopic analysis. Each experiment analysed 10 different microscopic fields for active and inactive cells in clusters of plaque bacteria and the proportion of metabolically inactive and active cells was calculated as described above. The experiment was repeated twice and a representative image of the 20 total images in the planktonic and biofilm samples is shown in the results.

Statistical analysis. To detect any significant difference in proportion of metabolically active cells of dental plaque an unpaired non-parametric test (two-tailed P value), Mann–Whitney U test, was
used. Standard errors were also calculated. InStat 3.0 for Macintosh was used.

RESULTS

Characterization of the two MUC5B preparations

Two different preparations of salivary MUC5B, solMUC5B and gelMUC5B, were isolated from human whole saliva by isopycnic density-gradient centrifugation as outlined in Fig. 1. Using the peptide-specific antiserum LUM5B-2, the MUC5B containing fractions were identified in the two density-gradient preparations with the UV-absorbing, low-molecular-mass material enriched at the top of the gradients (Fig. 2a, b). The two preparations showed slightly different buoyant densities, with solMUC5B banding between 1.54 and 1.46 g ml\(^{-1}\), and gelMUC5B between 1.53 and 1.42 g ml\(^{-1}\). MUC5B-containing fractions were pooled and subjected to SDS-PAGE in order to establish purity (insets, Fig. 2a, b). Staining was evident only in the well, the expected position of MUC5B. Carbohydrate analyses showed higher levels of fucose, galactose, N-acetylgalactosamine and N-acetylgalactosamine in the gelMUC5B preparation, whereas the levels of mannose and specifically N-acetylenuraminic acid were higher in the solMUC5B preparation (Table 1). In addition to differences in buoyant density and carbohydrate composition, the two MUC5B preparations also differed in surface adherence characteristics. Since one of the aims of the present study was to examine whether mucin could act as a food source for supragingival plaque on mucin-coated surfaces in comparison with the mucin in solution, each mucin preparation was coated onto flow-cells to assess adhesion patterns by epifluorescence microscopy. As shown in Fig. 3(a), solMUC5B showed an uneven distribution, exhibiting streaks of large aggregates, whereas gelMUC5B coated the surface in a more homogeneous pattern (Fig. 3b). Calcium was essential for the adherence of both mucin preparations, as seen by the absence of mucin on the surfaces (Fig. 3, insets).

Metabolic activity of dental plaque in suspension

To provide a comparison with the mucin-surface experiments, the metabolic activity of the dental plaque in liquid phase or planktonic suspension was tested by fluorescent assay. For this, freshly collected plaque was dispersed and incubated with solMUC5B or gelMUC5B for 1 h after which CTC/SYTO24 (metabolic activity) dye was added. Cells incubated in PBS acted as the control. The metabolic activity in the control, as measured by the CTC/SYTO24 dye, was low or absent in all fields examined (Fig. 4a). The analysis indicated that only 9.6 % (± 4.0) of the cells in the 45 fields examined were coloured red, indicating low dehydrogenase activity. In contrast, plaque bacteria incubated with solMUC5B showed that an average of 79.4 % (± 4.7) of the cells were metabolically active (red) in all fields examined (Fig. 4b). Plaque bacteria were, however, much less active with gelMUC5B than with solMUC5B: only an average of 48.2 % (± 6.4) of the cells stained red. The differences seen were statistically significant both in comparison to the control (P<0.0001) and between the preparations (P<0.0001).

Metabolic activity of dental plaque on mucin-coated surfaces

Metabolic activity of supragingival plaque cells adhered to mucin-coated surfaces was tested by using flow-cells coated with solMUC5B or gelMUC5B, using an uncoated surface as a control. When dental plaque adhered to the uncoated surface a low metabolic activity was seen, with an average of only 9.4 % (± 3.9) of the cells displaying dehydrogenase activity (Fig. 4d). This is in line with the result from the control sample for the plaque cells incubated in suspension (Fig. 4a). Surprisingly, an equally low metabolic activity was seen when the plaque cells were adhering to a solMUC5B-coated surface (Fig. 4e), as indicated by the fact that an average of only 12.1 % (± 3.9) of the cells in

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**Fig. 2.** Fractional analysis of isopycnic density-gradient centrifugation in CsCl/0.1M NaCl of (a) the supernatant (solMUC5B) and (b) the homogenized pellet (gelMUC5B) derived from whole human saliva following of ultracentrifugation. The gradients were emptied from the bottom of the tubes and fractions analysed for density (■), \(A_{280} (\longrightarrow)\) and antibody reactivity with LUM5B-2 (◇). The MUC5B-containing fractions were pooled (two samples), dialysed against PBS and subjected to SDS-PAGE followed by staining with silver nitrate (insets).
this experiment were shown to be active. Statistical analysis indicated no difference between the control and the solMUC5B preparation \( (P < 0.0991) \). This is in stark contrast to the result obtained with the same plaque samples in suspension with solMUC5B \( (P < 0.0001) \) (Fig. 4b). When supragingival plaque was introduced to a gelMUC5B surface, 29.2 % \( \pm 5.6 \) of the cells showed metabolic activity (Fig. 4f), a statistically significant increase in active cells in comparison to the control \( (P < 0.0003) \) and the solMUC5B preparation \( (P < 0.00212) \), while at the same time displaying a significant decrease in activity \( (P < 0.0134) \) compared to the results for gelMUC5B/plaque in suspension (Fig. 4c).

The data indicate that the metabolism of the plaque bacteria adhering to a surface in a biofilm is lower, or downregulated, compared to that seen with cell suspensions of the same plaque sample incubated with solMUC5B and gelMUC5B. To further confirm this observation, freshly harvested dental plaque was incubated with solMUC5B in solution and the sample divided into two. Aliquots of the first subsample acted as the control and were analysed for metabolic activity as described in Methods. The solMUC5B/plaque cells in the second subsample were introduced to a gelMUC5B-coated surface and subsequently analysed for metabolic activity. As seen in Fig. 4(g), the cells in the first sample tested directly for metabolic activity exhibited the same high ratio of dehydrogenase activity \( [74.9 \% \pm 4.7] \) as that seen in Fig. 4(b). However, as seen in Fig. 4(h), when the solMUC5B/plaque cells in suspension were allowed to adhere to the gelMUC5B-covered surface prior to the fluorescent assay, the majority of the biofilm cells on analysis were found to be dehydrogenase inactive, with only 19.3 % \( \pm 6.7 \) cells active \( (P < 0.0017) \), indicating that surface adherence had downregulated plaque metabolism.

### DISCUSSION

The oligomeric mucins, including MUC5B, are polydisperse in size, have molecular masses ranging between \( 10 \times 10^6 \) and \( 44 \times 10^6 \) Da (Carlstedt et al., 1983) with a subunit mass of approximately 2000–2500 kDa, containing mucin domains heavily substituted with oligosaccharides. Subpopulations of salivary MUC5B have been identified

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>solMUC5B*</th>
<th>gelMUC5B*</th>
<th>Ratio solMUC5B/gelMUC5B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucose</td>
<td>400</td>
<td>530</td>
<td>0.76</td>
</tr>
<tr>
<td>Mannose</td>
<td>36.7</td>
<td>26.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Galactose</td>
<td>580</td>
<td>713</td>
<td>0.81</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>200</td>
<td>233</td>
<td>0.86</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>387</td>
<td>507</td>
<td>0.76</td>
</tr>
<tr>
<td>N-Acetyleneuraminic acid</td>
<td>144</td>
<td>54.3</td>
<td>2.65</td>
</tr>
</tbody>
</table>

*nmol detected per mg sample.

**Fig. 3.** Adhesion patterns of the two isolated preparations of salivary MUC5B to a model surface: (a) solMUC5B + 10 mM CaCl₂ (b) gelMUC5B + 10 mM CaCl₂. Insets: solMUC5B and gelMUC5B without CaCl₂, respectively. MUC5B was identified using the monoclonal antibody EU-MUC5Ba followed by a FITC-conjugated secondary antibody.
based on different charged oligosaccharides (e.g. Thornton et al., 1997; Wickström et al., 1998) and the distribution of the mucins following specific separation techniques. When separated into a sol and a gel phase using ultracentrifugation, different MUC5B subpopulations were enriched in three ‘phases’: ‘soluble’, ‘soluble’ using high concentrations of guanidinium hydrochloride, with the remaining ‘insoluble’ fraction solubilized by the reduction of the disulphide bonds (Wickström et al., 2000). Although MUC5B is the major gel-forming component in saliva, guanidinium hydrochloride-purified MUC5B solutions do not seem to ‘reconstitute’ the properties of the native secretion (Raynal et al., 2002). In addition, Raynal and co-workers revealed that MUC5B in saliva is assembled into large structures through calcium-mediated protein cross-links, leading to an increase in the intrinsic viscosity of saliva (Raynal et al., 2003). This might explain the observation in this study, that mucins needed calcium to avidly bind to the model surface used. Hence, the gel-matrix in which MUC5B constitutes the backbone displays a multivalent complexity.

**Fig. 4.** Metabolic activity of freshly collected plaque incubated for 1 h in suspension without mucins (a), with solMUC5B (b) and with gelMUC5B (c) as well as in a biofilm without mucins (d), with solMUC5B (e) and with gelMUC5B (f). The images are representative of 45 different microscopic fields from three separate experiments, each with 15 fields. In addition, freshly harvested dental plaque cells incubated with solMUC5B in suspension (g) and the same cells adhered to a gelMUC5B-coated surface (h). The experiment was repeated twice and in each experiment 10 different microscopic fields were analysed for active and inactive cells in clusters of plaque bacteria. Representative images of the 20 total images in the planktonic and biofilm samples are shown. All samples were stained with CTC (dehydrogenase activity)/SYTO24, red cells being active and green cells being inactive.
which should be taken into account when studying the interactions between this molecule and surfaces or bacteria.

In the current study, we employed a novel non-dissociative separation technique (Fig. 1) to isolate two different preparations of salivary MUC5B, thus providing further evidence for the existence of different subpopulations of this mucin. The procedures involved ultracentrifugation in the absence of denaturing agents followed by density-gradient centrifugation, generating two preparations with slightly different buoyant densities, and differences in carbohydrate composition and adhesion patterns when applied to a surface. The differences in carbohydrate content, particularly with respect to sialic acid, could explain the differences in buoyant density. Although this requires further research, we suggest that the two preparations presented in this paper represent two distinct subpopulations of the salivary MUC5B mucin.

To elucidate the potential biological significance of these two mucin preparations, we were interested in assessing the extent to which these preparations could act as an energy source for dental plaque bacteria. The results (Fig. 4) indicate significant metabolic differences between the two preparations, and also differences depending on whether the mucin–plaque reactions were carried out in suspension or in a biofilm. Clearly, plaque cells in suspension were more active than the same plaque cells adsorbed in a biofilm, with cells suspended with solMUC5B being the most active (79.4 % active), while gelMUC5B supported lower activity (48.2 % active cells). Of interest was the fact that while the cells associated with solMUC5B and gelMUC5B biofilms were less active than the planktonic cells, in this case, the cells incubated with gelMUC5B were more active than those incubated with solMUC5B. An immediate rise in metabolic activity by plaque biofilm cells when interacting with solMUC5B and gelMUC5B biofilms demonstrated two distinct mucin gel secretions in the oral cavity, with different biological activity.

Perhaps the key finding in this paper was the observation that both mucin preparations elicited a significantly lower metabolic response by the plaque bacteria in biofilms than the same plaque cells in planktonic suspension. That dehydrogenase-associated metabolism was downregulated when the cells became associated in a biofilm was clearly demonstrated when active solMUC5B/plaque suspensions were allowed to adhere to gelMUC5B-coated biofilm surfaces (Fig. 4g, h). The planktonic suspension exhibited 74.9 % active cells, but only 19.3 % were active in the biofilm. Convincing evidence exists that the physiological properties of bacteria in biofilms are different from those of the same cells in liquid culture, particularly with respect to resistance to adverse environmental conditions and to antimicrobial agents (Costerton et al., 1987; Fletcher, 1991; Goodman & Marshall, 1995; Welin et al., 2004). In a recent study, Lactobacillus salivarius and Streptococcus anginosus displayed striking differences in metabolic activity when investigated in biofilm and planktonic cultures, respectively. When provided with fresh nutrients after starvation, the planktonic bacteria metabolically reactivated within 2 h, whereas the biofilm cells required 72 h to show metabolic activity (Chávez de Paz et al., 2008). Adhesion of micro-organisms to a surface in itself triggers a number of changes, including quantitative alteration of protein synthesis and expression of adhesion molecules (O’Toole & Kolter, 1998; Burne, 1998). It should be pointed out that the downregulating effects seen here could, in part, be due to the fact that attachment of mucins to a surface results in steric effects that reduce the number of carbohydrate moieties available to the adherent bacteria.

We propose that the two preparations of salivary MUC5B described here have distinct biological functions in the oral cavity in how they interact with the oral microbiota, with one preferentially associated with the oral surfaces and the other enriched in the fluid phase. Taylor et al. (2004) have demonstrated two distinct mucus gel secretions in the stomach, an adherent and shear-resistant gel and a superficial shear-compliant gel; they speculate that the first one constitutes the mucus barrier and the other provides lubrication. The fact that the solMUC5B had a slightly higher density compared to the gelMUC5B fits nicely with the findings that the surface layer of stomach mucus displayed a lower density than the superficial layer. In the airways, it has been suggested that the two mucins MUC5B and MUC5AC form spatially distinct mucus gels, one tuned to flow and the other to coughing (Sheehan et al., 2006). In the oral situation, one could postulate that gelMUC5B would act primarily on a surface constituting the mucus barrier, while solMUC5B would constitute a more solubilized lubricating mucus interacting with oral bacteria not yet attached to a surface.

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