Physico-chemical and structural properties of the surfaces of *Peptostreptococcus micros* and *Streptococcus mitis* as compared to those of mutans streptococci, *Streptococcus sanguis* and *Streptococcus salivarius*

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The surface properties of nine *Streptococcus mitis* and four *Peptostreptococcus micros* strains from the oral cavity were examined and compared with a large group of oral streptococci. Zeta potential and contact angle measurements were employed to determine physico-chemical cell surface properties. In addition, elemental surface concentration ratios were obtained via X-ray photoelectron spectroscopy, and surface structures were examined with transmission electron microscopy. The *S. mitis* and *P. micros* strains were found to have higher isoelectric points, higher hydrophobicities and higher N/C surface concentration ratios than some other oral streptococci. The combined data suggest that both species possess large amounts of surface protein. All the *S. mitis* strains displayed abundant surface fibrils in negative staining, but the *P. micros* strains were devoid of surface appendages indicating that surface protein is present in different forms in the two species. The surfaces of *S. mitis* and *P. micros* type strains differed significantly from the other strains examined.

Introduction

Streptococcus mitis and *Peptostreptococcus micros* are prevalent organisms in the oral cavity. *S. mitis* is one of the most frequently isolated bacteria in dental plaque, and also colonizes mucous membranes, most notably the cheek and tongue (Marsh & Martin, 1980). Although it may cause dental caries, in most cases it is considered to be an innocuous member of the oral microflora (Hardie & Marsh, 1978). Conversely, *Peptostreptococcus* is an anaerobic coccus found in substantial numbers in many forms of anaerobic mixed infections (Rogosa, 1971; Bourgault *et al.*, 1980) and though it is detectable in healthy mouths (Socransky, 1970; Slots, 1979) its numbers increase dramatically in the presence of periodontal disease (Moore *et al.*, 1982; Slots, 1979; Ter Steeg *et al.*, 1987). *Peptostreptococcus* strains resemble *Streptococcus* strains in that they are Gram-positive cocci with a tendency to grow in chains, yet are distinguished by their requirement for strictly anaerobic conditions (Hofstad, 1985). While *S. mitis* colonizes supragingival

regions of the tooth, *P. micros* is found primarily below the gingival margin where anaerobic conditions prevail and is characterized by the production of various tissue-damaging enzymes, such as hyaluronidase and peptidase (Tam *et al.*, 1987).

Successful colonization of most tissues requires adhesion of bacteria. The adhesion of a bacterium to a substratum is in turn influenced by the physico-chemical properties of its surface. In particular, the approach of a bacterium to a substratum surface is usually assumed to be mediated by electrostatic and Lifshitz–Van der Waals forces (Busscher & Weerkamp, 1987). Upon close approach, stereochemical groups on the interacting surfaces may be brought close together through the above mentioned forces yielding a so-called “specific bond”. Zeta potentials and hydrophobicity (assessed for instance by contact angle measurements) are the physico-chemical corollaries of the capacity of a surface to express these forces.

There are many examples of the use of zeta potential measurements to probe bacterial surfaces (Olsson *et al.*, 1976; Van Loosdrecht *et al.*, 1987; Mozes *et al.*, 1989; Van der Mei *et al.*, 1989a). In one study (Van der Mei *et*

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al., 1988a) the isoelectric point (IEP) of a *Streptococcus salivarius* fibrillated strain was much higher than the IEP found for a series of non-fibrillar mutants. Other work attests to the usefulness of contact angle studies (Van der Mei *et al.*, 1989a; Silva-Filho *et al.*, 1990) and X-ray photoelectron spectroscopy (XPS) (Millard *et al.*, 1976; Amory *et al.*, 1988; Van der Mei *et al.*, 1991; Busscher *et al.*, 1991) in determining microbial surface characteristics.

The aim of this study was to characterize the cell surface properties of *P. micros* and *S. mitis* by examining the hydrophobicity and surface charge characteristics of several strains of each species through the use of contact angle and zeta potential measurements, respectively. In addition, XPS has been performed to probe the elemental surface composition, and electron microscopy used to assess surface morphology of these bacteria. Results are compared with data obtained for a collection of oral streptococcal strains.

Methods

Bacterial strains and growth conditions. Bacteria used in this study are listed in Table 1 along with their sources. *P. micros* strains were grown in 0.06% peptone, 0.2% yeast extract, 2.5% (v/v) horse serum. *S. mitis* strains were grown in Todd Hewitt Broth. *P. micros* strains were grown in an anaerobic cabinet under 10% H₂, 85% N₂, 5% CO₂ (by vol.). *S. mitis* strains were cultured aerobically. Growth temperature was 37 °C for all strains. Cultures were grown to stationary phase and harvested by centrifugation at 5000 g. Cultures for use in zeta potential measurements were then washed twice and resuspended in potassium phosphate buffer (10 mM, pH 7.0). For XPS, contact angle measurements and electron microscopy, cultures were washed twice in demineralized water.

Zeta potential measurements. Bacterial suspensions (1 × 10⁶ cells ml⁻¹) were prepared in potassium phosphate buffer (10 mM), the pH of which had been adjusted to 2, 3, 4, 5, 7 or 9 by the addition of HCl or KOH. Addition of cells did not affect pH. A Lazer Zee Meter 501 (PenKem, Bedford Hills, NY) set at 150 V was used to determine the electrophoretic mobility of the cells which was then converted to apparent zeta potentials via the Smoluchowski equation (Abramson *et al.*, 1942).

Contact angle measurements. The method for measuring contact angles on bacterial layers has been described (Busscher *et al.*, 1984). Briefly, bacteria suspended in water were deposited on membrane filters to a thickness of 50–100 cells. After a standard drying time of 30 min advancing-type contact angles were measured using the sessile drop technique with the following liquids: water, α -bromonaphthalene, formamide and diiodomethane. Water and formamide are used to probe polar hydrogen-donating and -accepting groups while α -bromonaphthalene and diiodomethane probe apolar groups on the cell surface.

X-ray photoelectron spectroscopy (XPS). Bacterial pellets were quickly frozen in stainless steel cups by immersion in liquid nitrogen and lyophilized at -10 °C in a Lyovac GT1 lyophilizer (Leybold Heraeus, Cologne). The freeze dryer contained a liquid nitrogen cooled plate positioned next to the bacterial sample which prevented carbonaceous contamination during freeze drying. A magnesium anode was used for

Table 1. Bacterial strains used in this study

Species	Collection no.	Source
<i>P. micros</i>	HG 1108	A. J. van Winkelhoff, ACTA Amsterdam
	HG 1109	
	HG 1111 ATCC 33270	ATCC
<i>S. mitis</i>	244 biovar 2*	P. S. Handley Univ. of Manchester
	272 biovar 1	
	357 biovar 1	
	398 biovar 1	
	BA biovar 1	own isolate
	BMS biovar 1	
	T9 biovar 1	ATCC
	ATCC 9811 biovar 1	
	ATCC 33399 biovar 2	ATCC

* Biovar assessed by checking for arginine hydrolysis (Kilian *et al.*, 1989) as part of the API-20 STREP system.

Fig. 1. Zeta potentials of (a) *S. mitis* and (b) *P. micros* as a function of pH. Each curve represents the mean of two experiments with separate bacterial cultures, results of which coincided within 4 mV. *S. mitis*: ▼, ATCC 9811; ★, ATCC 33399; ●, BMS; ☆, 272; ▲, 244; □, 398; ■, 357; ●, BA; ○, T9. *P. micros*: ■, HG 1111; ▲, HG 1109; ●, HG 1108; ★, ATCC 33270.

X-ray production (14 kV, 20 mA). After a scan of the overall spectrum, peaks were recorded in the following order: C_{1s} , O_{1s} , N_{1s} , P_{2p} , and C_{1s} again. The area under each peak after linear background subtraction was used for calculation of the peak intensities, yielding the elemental surface concentration ratios O/C, N/C, and P/C. The C_{1s} peak was decomposed into three components, assuming a pure Gaussian shape and using a best-fit program. The components were set at 285.0 eV (C–C and C–H bonds), 286.6 eV (C–O and C–N groups) and 288.4 eV (HNC=O groups) (Ratner & McElroy, 1985).

Electron microscopy. Bacteria suspended in demineralized water were negatively-stained with 2% (w/v) methylamine tungstate according to Hancock & Poxton (1988). Briefly, a suspension of bacteria was applied to a carbon film-coated copper grid and the excess liquid removed. Stain was then applied for 30 s, after which the excess was removed. Preparations were viewed with a Philips 201 transmission electron microscope operating at 80 kV.

Results

Zeta potentials of *S. mitis* and *P. micros*

Fig. 1 depicts the pH-dependence of the zeta potentials of the nine *S. mitis* and four *P. micros* strains. All strains displayed a pH-dependence which is characteristic of mixed amino/carboxyl surfaces (James, 1982) with positive zeta potentials at low pH values and negative zeta potentials at pH values higher than 4. Both genera showed a tendency to be highly positively charged at low pH values.

The isoelectric points (IEP; the point at which the zeta potential is zero) of three of the four *P. micros* and eight of the nine *S. mitis* strains were greater than 3.5. At higher pH values, *S. mitis* strains displayed more rapidly increasing negative zeta potentials than did *P. micros* strains.

Contact angles on *S. mitis* and *P. micros*

Data obtained from contact angle measurements on lawns of the 13 strains are shown in Table 2. Wide variation in the liquid contact angles can be seen within as well as between the two genera. On average, however, *S. mitis* strains were more hydrophobic than *P. micros* strains.

Fig. 2 depicts the relationships between contact angles obtained with the two polar liquids (Fig. 2a) and with the

Table 2. Contact angles of different pure liquids on lawns of *P. micros* and *S. mitis* strains

Contact angles are in degrees. Data are mean values of four experiments performed on two separate bacterial cultures. Standard deviation $\leq 9^\circ$ unless otherwise indicated.

Bacterium	θ_{H_2O}	$\theta_{formamide}$	$\theta_{diiodomethane}$	$\theta_{\alpha-br}^*$
<i>P. micros</i>				
HG 1108	66 ³	69	51	34
HG 1109	33	49	58	43
HG 1111	58	70	55	34†
ATCC 33270	39	49	72	51
<i>S. mitis</i>				
244	60	38	47	30
272	54	39	51	30
357	53	31	42	33
398	59	33	48	26
BA	103	55	49	35
BMS	100	43	50	34
T9	91	47	55	36
ATCC 9811	68	53	48	31
ATCC 33399	56	40	45	31

* α -br, alpha-bromonaphthalene.

† SD = 17° .

Table 3. X-ray photoelectron spectroscopy of *S. mitis* and *P. micros*

Data are means of 2 determinations on separate bacterial cultures and were reproducible within $\pm 10\%$.

Bacterium	Surface concentration ratios			Peak components of C_{1s} at		
	N/C	O/C	P/C	285.0 eV	286.6 eV	288.4 eV
<i>P. micros</i>						
HG 1108	0.139	0.306	0.003	0.49	0.36	0.17
HG 1109	0.125	0.277	0.003	0.58	0.32	0.10
HG 1111	0.137	0.295	0.004	0.61	0.24	0.15
ATCC 33270	0.074	0.215	0.014	0.74	0.19	0.08
<i>S. mitis</i>						
244	0.097	0.314	0.008	0.54	0.32	0.14
272	0.131	0.348	0.008	0.51	0.35	0.15
357	0.119	0.339	0.011	0.50	0.36	0.14
398	0.116	0.261	0.008	0.57	0.29	0.14
BA	0.129	0.326	0.007	0.60	0.28	0.13
BMS	0.124	0.312	0.009	0.57	0.29	0.14
T9	0.125	0.259	0.006	0.63	0.25	0.13
ATCC 9811	0.110	0.303	0.008	0.60	0.28	0.12
ATCC 33399	0.106	0.343	0.008	0.58	0.31	0.12

Fig. 2. Relationship between the contact angles of water and formamide (a) and α -bromonaphthalene and diiodomethane (b) on lawns of *P. micros* (■) and *S. mitis* (●) strains.

two apolar liquids (Fig. 2b). All *S. mitis* and *P. micros* strains displayed the same relationship between α -bromonaphthalene and diiodomethane contact angles. This was not the case with the two polar liquids. The four *P. micros* strains displayed a relationship between contact angles found with water and formamide which clearly differed from that of the *S. mitis* strains.

Elemental surface composition

Table 3 contains data representing elemental surface concentration ratios for the surfaces of *S. mitis* and

Fig. 3. Transmission electron microscopy of negatively-stained *S. mitis* (a) and *P. micros* (b) cells. Stain is 2.0% (w/v) methylamine tungstate. Bar, 100 nm.

Fig. 4. N/C vs. water contact angle and isoelectric point for *S. mitis* (●), *P. micros* (■), and other oral streptococci (▲).

P. micros strains. Also listed are the fractions of components of which the C_{1s} peak was thought to be composed. Noteworthy are the relatively high values for N/C for the three clinical isolates of *P. micros* and for the *S. mitis* strains. Also, in all 13 strains the component of the C_{1s} peak set at 285.0 eV (representing C–C and C–H bonds) was the most abundant of the three carbon-containing components.

Electron microscopy

Negative staining revealed long abundant fibrillar extensions on all *S. mitis* strains (Fig. 3a). No fibrils of any sort could be detected on any of the *P. micros* strains (Fig. 3b).

Relationships among data obtained with different techniques

Fig. 4 depicts the relationships between N/C and hydrophobicity (stated here as the water contact angle, Fig. 4a) as well as between N/C and IEP (Fig. 4b) for the four *P. micros* strains, the nine *S. mitis* strains, and a range of oral streptococci. The ratio N/C is seen to

correlate positively with IEP and with water contact angle. In addition, these graphs clearly illustrate the relatively high N/C surface concentration ratio found for the *P. micros* and *S. mitis* strains.

Discussion

The two most notable features of the *S. mitis* and *P. micros* strains examined here are their exceptionally high N/C surface concentration ratios and relatively high IEPs as compared to other oral streptococci which stimulated us to make a more detailed comparison of the surface properties of these two species. Table 4 summarizes these and other physico-chemical and compositional properties for the *S. mitis* and *P. micros* strains as well as for a group of 27 other oral streptococci (see Table footnote for list of strains). Values for the strains *P. micros* ATCC 33270 and *S. mitis* ATCC 33399 are excluded from the analysis since they were found to be atypical of their species (see below). In addition, previously unpublished elemental surface concentration ratios found by XPS are listed for freeze-dried preparations of α -amylase and a glycoprotein isolated from human whole saliva and commercially obtained dextran and albumin. Comparison of the N/C and O/C values found for these macromolecules with those of the bacterial cell surfaces provide a clear indication that *P. micros* and *S. mitis* possess surfaces which are very rich in nitrogen and thus probably predominantly proteinaceous. XPS values for *P. micros*, and to a somewhat lesser extent for *S. mitis*, most closely approximate those of the salivary glycoprotein preparation. The surfaces of the group of other oral streptococci appeared to contain much less nitrogen and more oxygen than *P. micros* and *S. mitis*. Thus their surfaces are probably made out of oxygen rich polysaccharides (see dextran data) to a greater extent than those of *P. micros* and *S. mitis*. This conclusion is also supported by the percentage of carbon found at 286.6 eV (Table 4). The value of 0.30 found for *P. micros* and *S. mitis* strains is comparable to the values found for all three of the macromolecules which consist either totally or in large part of protein (α -amylase, albumin and the salivary glycoprotein). In the case of both dextran and the group of oral streptococci, the 286.6 eV fraction is much larger. The high isoelectric points found for the *S. mitis* and *P. micros* strains provide further evidence that high levels of surface protein are responsible for the high N/C surface concentration ratios, since previous investigations (Amory *et al.*, 1988; Van der Mei *et al.*, 1988b) have found IEP to be closely correlated with amount of exposed protein.

Low O/C surface concentration ratios were also found for both genera studied as compared to other oral

Table 4. Physico-chemical and compositional data for oral bacteria, albumin, dextran and isolated freeze-dried salivary components

Isolation methods as in Pratt-Terpstra *et al.* (1991). *P. micros* differs significantly from *S. mitis* only in P/C, but 'other oral streptococci' differ significantly from *P. micros* and *S. mitis* in IEP, θ_{H_2O} , N/C, O/C and C_{1s} at 285.0 and 286.6 eV (Student's *t*, $P < 0.01$).

Test subject	XPS							
	IEP	θ_{H_2O}	Surface concn. ratios			Peak components of C_{1s} at		
			N/C	O/C	P/C	285.0 eV	286.6 eV	288.4 eV
<i>P. micros</i> *	3.2 $\pm$ 0.5	52 $\pm$ 14	0.134 $\pm$ 0.006	0.293 $\pm$ 0.012	0.003 $\pm$ 0.000	0.57 $\pm$ 0.05	0.30 $\pm$ 0.04	0.14 $\pm$ 0.03
<i>S. mitis</i> †	3.6 $\pm$ 0.4	74 $\pm$ 20	0.119 $\pm$ 0.010	0.308 $\pm$ 0.030	0.008 $\pm$ 0.001	0.56 $\pm$ 0.04	0.30 $\pm$ 0.04	0.14 $\pm$ 0.01
Other oral streptococci‡	2.1 $\pm$ 0.7	34 $\pm$ 16	0.074 $\pm$ 0.013	0.427 $\pm$ 0.042	0.004 $\pm$ 0.003	0.45 $\pm$ 0.07	0.41 $\pm$ 0.05	0.14 $\pm$ 0.03
Salivary α -amylase	—	—	0.221	0.382	0.008	0.47	0.32	0.21
Albumin§	—	—	0.240	0.292	0.001	0.50	0.29	0.21
Salivary glycoprotein	—	—	0.182	0.432	0.003	0.52	0.32	0.16
Dextran	—	—	ND	0.712	ND	0.20	0.69	0.12

ND, not detected.

* Mean values for *P. micros* strains HG 1108, HG 1109, HG 1111.

† Mean values for *S. mitis* strains 244, 272, 357, 398, BA, BMS, T9 and ATCC 9811.

‡ Mean values for 27 oral streptococcal strains: *S. sobrinus* strains HG 970, HG 1025 and HG 977; *S. cricetus* AHT, E49 and H56; *S. rattus* BHT, BHT_{sm}, ATCC 19645 and FA1; *S. mutans* NS, HG 982, HG 983, HG 1003, HG 979 and HG 985; *S. salivarius* HB, HBC12, HBV 5, HB 7 and HBV 51; *S. crista* (previously *S. sanguis*) CR 311, CR 311V₁, CR 311V₂ and CR 311V₃; *S. sanguis* PSH and CH3 (Van der Mei *et al.*, 1988a; Van der Mei *et al.*, 1991; Busscher *et al.*, 1991).

§ Albumin Fraction V, Sigma.

|| Dextran 500; molecular mass 500–700 kDa (serva).

streptococci. This observation corresponds with their elevated IEP and suggests that the two bacteria possess little or no extracellular capsular-type material, which is often rich in negative charges (especially phosphate and carboxyl groups) and can mask underlying wall or fibril proteins.

Protein may not be the only abundant macromolecule on the surface of *P. micros* and *S. mitis* strains. Decomposition of the carbon peak obtained from XPS revealed that the first carbon component situated at 285.0 eV, and attributed to the position of carbon involved in hydrocarbon bonds, is much more dominant in *S. mitis* and *P. micros* strains than in the other streptococci studied (Tables 3 and 4). This suggests that lipid, which is readily found in Gram-positive organisms in the form of lipoteichoic acids and other amphiphilic wall polymers (Wicken & Knox, 1983), may be present either in a bound form or as a secreted polymer at the cell surface. It could then be expected to have a variable influence on physico-chemical surface properties depending on its orientation.

Although there is wide variation in the wettability of the surfaces of all the strains studied here, the overall high water contact angles as compared to those for the other streptococcal strains is consistent with a comparatively large amount of surface protein. Since no fibrillar appendages were found on *P. micros* strains, it appears

that the protein is integral to the cell envelope itself, perhaps in the form of cell wall protein or protein adsorbed from the growth medium. No evidence could be found for the presence of a surface array or S-layer structure which could account for high protein levels. The lower degree of hydrophobicity compared to *S. mitis* strains, in combination with approximately equivalent IEPs and a N/C surface concentration ratio that is even higher than that for *S. mitis*, suggests that protein may have been masked in the semi-hydrated preparations used in contact angle measurements but not under the fully hydrated zeta potential conditions. Such a possible masking effect would be least likely to influence the XPS measurements because XPS probes more deeply (approximately 5 nm) than do the other techniques (zeta potentials probe at the plane of shear between the bacterial cell and the fluid (James, 1982); contact angles measure the outermost 0.4–0.5 nm of the semi-hydrated bacterial pellet (Bain & Whitesides, 1988)).

The relationships between the contact angles of two polar liquids for *P. micros* is not the same as that for the *S. mitis* strains (Fig. 2) and may be further evidence for fundamental differences in the type and/or structural arrangement of exposed surface protein present in the two genera.

It should be noted that two strains considered to be type strains of their species differ in significant ways

from the majority of clinical isolates of these strains. *S. mitis* ATCC 33399 had a lower IEP than the other eight *S. mitis* bacteria (Fig. 1, Table 2). Also, the three clinical isolates of *P. micros* displayed N/C surface concentration ratios almost twice as high as ATCC strain 33270 and P/C surface concentration ratios that were a fraction of that found for the type strain (Table 3). This is an important indication that while type strains may be biochemically representative of their species, cell surface properties, which are characteristically labile, are often grossly altered.

It is also worth mentioning that the three *S. mitis* strains with extremely high water contact angles (BA, BMS, and T9, see Table 2) were all isolated from buccal mucosa. *S. mitis* ATCC 33399, on the other hand, was originally isolated from the dentition and displays a lower water contact angle (the origin of the other *S. mitis* strains is not known). It is interesting to speculate that even though all *S. mitis* strains possess abundant surface fibrils, the composition of these fibrils and other surface components may differ in such a way as to correlate with their original intra oral isolation site. Other authors have also observed that strains isolated from oral soft tissue may be more hydrophobic than strains isolated from dental hard tissues (Beighton, 1984). Alternatively, it might be anticipated that the various hydrophobicities observed for the *S. mitis* strains correspond with their biovar, as recently described for *Fusobacterium necrophorum* (Shinjo *et al.*, 1987). Comparison of Table 1 with the data on water contact angles of the *S. mitis* strains presented in Table 2 shows that this observation does not hold for the present collection of *S. mitis* strains and hydrophobicity as assessed by water contact angles.

Originally, horse serum was added to peptone yeast extract medium for the growth of *P. micros* strains to better mimic the situation in the periodontal pocket, the predominant niche of this organism. Later on, addition of serum to peptone yeast extract medium turned out to be essential for growth of *P. micros* cells in this medium. Some *P. micros* strains (HG 1109 and ATCC 33270), however, have also been grown in Schaedler's broth with and without serum added. The zeta potentials of these two *P. micros* strains grown in Schaedler's broth with serum added were not greatly different than those of cells grown in peptone yeast extract medium with serum added. However, deletion of serum from Schaedler's broth resulted in slightly more hydrophobic cells with a lower N/C surface concentration ratio. This suggests that *P. micros* cells adventitiously adsorb serum proteins, consistent with the lack of microscopically visible surface structures on these strains. For supragingival oral streptococci, such an adventitious adsorption of proteins upon bathing cells in saliva has not been observed, as their N/C surface concentration ratios by XPS increased

only slightly upon adsorption of salivary components (Van der Mei *et al.*, 1989b).

In summary, the surfaces of *P. micros* and *S. mitis* strains possessed much higher IEPs and contained higher N/C and lower O/C surface concentration ratios than other oral streptococci studied. The data obtained with various techniques correlated with each other and suggest that the surfaces of both species are dominated by protein. *S. mitis* strains possess surface fibrils which are the probable location of the abundant surface protein. *P. micros* strains were found to be devoid of surface appendages and are therefore most likely to be rich in cell wall protein, either intrinsic to the cells or adsorbed from their growth environment. The surfaces of type strains of both species were found not to be representative of the majority of strains.

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