Crystalline surface protein of *Peptostreptococcus anaerobius*

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The surface ultrastructure of three anaerobic Gram-positive cocci frequently encountered in oral infections, *Peptostreptococcus micros*, *P. magnus* and *P. anaerobius*, was studied. The type strains of *P. micros* (DSM 20468) and *P. anaerobius* (ATCC 27337), several clinical isolates of both species and the type strain of *P. magnus* (DSM 20470) were included. Thin-sectioned cells studied by electron microscopy revealed a homogeneous layer outside the peptidoglycan layer in *P. anaerobius*. In *P. micros* and *P. magnus* a more amorphous layer was present. No periodic structures were seen in negatively stained whole cells of these three species. However, in freeze-etched cells of *P. anaerobius* a crystalline surface protein layer (S-layer) was detected. No periodicity was seen in any of the *P. micros* strains or the *P. magnus* type strain by the methods used, but a periodic pattern was observed in negatively stained specimens of cell wall fragments of sonicated *P. anaerobius* cells. No capsular material was visible outside the S-layer in *P. anaerobius*. The cells of the *Peptostreptococcus* spp. were extracted for 30 min with detergents and urea. One per cent SDS and 6 M urea both extracted a major 78 kDa protein from all strains of *P. anaerobius*. Extraction of *P. micros* and *P. magnus* cells did not reveal any major protein bands comparable to that of *P. anaerobius*. Surface biotinylation of cells followed by Western blotting and detection by alkaline-phosphatase-conjugated extravidin showed strong staining of the 78 kDa band in *P. anaerobius*, further indicating that this molecule is located on the surface of the cell and is the S-protein. Another protein, of 127 kDa, was also detected by surface biotinylation in all *P. anaerobius* strains. No differences were detected in colony morphology or cell surface structures between the four strains. Isoelectric focusing of the proteins of *P. anaerobius* revealed that the S-proteins of individual strains have different pI values, ranging from 5.3 to 6.9.

Keywords: *Peptostreptococcus*, S-layer, surface protein, ultrastructure, oral bacteria

INTRODUCTION

Peptostreptococcus spp. are regularly isolated in infections of the oral region as well as in other parts of the human body (Sugita *et al.*, 1982; Sklavounos *et al.*, 1986; Topiel & Simon, 1986; Brook *et al.*, 1991; Sundqvist, 1992). Several studies have suggested that *Peptostreptococcus* spp., especially *P. magnus*, *P. micros* and *P. anaerobius*, may have an important role in the pathogenesis of various infections (Brook & Walker, 1984; Papasian *et al.*, 1986; Smith *et al.*, 1986a; Brook, 1988; Davies *et al.*, 1988; Hunter & Chow,

1988; Lewis *et al.*, 1989; Panagou *et al.*, 1991; Schenkein *et al.*, 1993). Some studies have also indicated that *P. micros* may be involved in cases of advancing adult periodontitis (Rams *et al.*, 1992; Tew *et al.*, 1985). Apart from their regular isolation as part of the infective flora, relatively little information is available about their potential virulence factors. Mucin degradation by *P. micros* strains isolated from rat intestine (Carlstedt-Duke *et al.*, 1986) and production of a variety of peptidases and hyaluronidases (Tam & Chan, 1983, 1985; Tam *et al.*, 1987) have been related to their virulence. Collagenase production (Krepel *et al.*, 1991) and expression of protein L, which has the capability to bind to the light chain of immunoglobulins (Kastern *et al.*, 1990), have been reported with some non-oral strains of *P. magnus*. In

Abbreviations: %HP, percentage hydrophobicity; NHS-biotin, biotin-amidocaproate *N*-hydroxysuccinimide ester.

addition to hydrolytic enzymes, bacterial surface properties are also regarded as important for the outcome of the interaction of the bacterial cell with the host's defences (van Steenberg & de Graaff, 1983; Absalom, 1988; Lämmler *et al.*, 1989). Messner & Buckel (1988) reported an S-layer in *P. asaccharolyticus*. Cowan *et al.* (1992) could not find any evidence of S-layer in *P. micros* in negatively stained samples. We have recently reported a crystalline surface layer in a clinical strain of *Peptostreptococcus* sp. isolated from purulent ear infection that resembled *P. magnus* (Lounatmaa *et al.*, 1988), but showed some differences from the type strain of *P. magnus*. The present study was undertaken to investigate the surface structure of the three *Peptostreptococcus* spp. frequently isolated in human infections: *P. micros*, *P. magnus* and *P. anaerobius*.

METHODS

Bacterial strains. *Peptostreptococcus* strains were obtained from reference culture collections (DSM, German Collection of Micro-organisms; ATCC, American Type Culture Collection) or isolated from infections of the oral cavity (Table 1). Identification of the clinical strains was based on Gram staining, oxygen tolerance, profiles of preformed enzymes (RapID ANA II system, Innovative Diagnostic Systems; and API AnIdent system, API Analytab Products) and comparison of the whole-cell protein profiles with the reference strains in SDS-PAGE (see below) (Smith *et al.*, 1986b). Pure cultures were preserved in skim milk (Difco) at -70°C . The bacterial strains were grown on Brucella horse blood agar plates supplemented with menadione (0.5 mg l^{-1}) and haemin (5 mg l^{-1}), in an anaerobic atmosphere ($85\% \text{ N}_2$, $10\% \text{ CO}_2$, $5\% \text{ H}_2$; mark 3 Anaerobic

Work Station, Don Whitley Ltd). Cultures 3–5 d old were used for the experiments.

Electron microscopy. For thin sections, the bacteria were collected from the plates, washed twice with phosphate buffer (pH 7.3) and fixed with 2.5% (v/v) buffered glutaraldehyde for 2 h at room temperature. The samples were then washed twice with the same buffer and prepared for electron microscopy as previously described (Lounatmaa, 1985). For negative staining, the cells were collected into phosphate-buffered saline (PBS, pH 7.2), washed once and stained with 2% (w/v) phosphotungstic acid. Cell wall fragments obtained by pulse sonication for 10 min in an ice bath (30% duty cycle, output 4; Branson Sonifier model 250) were also studied by negative staining. For freeze-etching, the cells were collected from the plates as described above, washed and centrifuged to form a thick suspension and frozen in liquid Freon 22 cooled by liquid nitrogen. The fracturing, in a Balzers freeze-etching unit BAF 400T, was performed at -100°C and the platinum shadowing after 1 min etching at an angle of 40° degrees (Lounatmaa, 1985). The electron micrographs were taken with JEM-1200EX and 100CX transmission electron microscopes at 60 kV.

Hydrophobicity assay. Cell surface hydrophobicities were measured as previously described (Rosenberg *et al.*, 1980; Haapasalo *et al.*, 1990). Cells were grown for 4 d on supplemented Brucella blood agar plates and collected in PBS. Cell suspensions were adjusted to $\text{OD}_{450} 0.5$ in PBS (Cary 210 spectrophotometer). n-Hexadecane ($200\text{ }\mu\text{l}$; Sigma) was thoroughly mixed with 3 ml of the cell suspension for 90 s with a Vortex mixer (Scientific Industries). Cells mixed in PBS without hexadecane were used as controls. The percentage hydrophobicity (%HP) was calculated as the change in the optical density of the PBS phase before and 15 min after mixing according to the formula

$$\% \text{HP} = \frac{(H_i - H_e) - (C_i - C_e)}{H_i} \times 100$$

where H_i is the initial optical density of the PBS phase before mixing (hexadecane tube), H_e the optical density after mixing, and C_i and C_e are the corresponding values in the control tubes. The higher the value, the more hydrophobic the cells. Two samples of the same bacterial cell suspensions were used for each measurement and all strains were tested at least twice, using new cultures each time.

Extraction of *Peptostreptococcus* surface proteins. Freshly cultured bacterial cells were washed twice in 20 mM Tris buffer (pH 8.0) and incubated in the same buffer in the presence of 6 M urea or one of the following detergents: 0.1% and 1.0% SDS (Sigma); 0.1% , 1.0% and 2.1% deoxycholate (Sigma). The cells were gently shaken at room temperature for 30 min with the detergents or urea and centrifuged ($16000g$, 10 min). The supernatant was analysed by protein electrophoresis as described below.

Gel electrophoresis and surface biotinylation. SDS-PAGE was performed in 10% (w/v) gels at 200 V as described by Laemmli (1970) (Mini Protean II, Bio-Rad). Molecular masses of the polypeptides were determined by comparison with standards (Bio-Rad). In some samples whole cells were boiled in sample buffer for 5 or 15 min. The supernatant obtained after centrifugation was used for the electrophoresis. Surface biotinylation was performed as described by Dooley & Trust (1988). The cells ($50\text{ }\mu\text{l}$ cells in 1 ml PBS) were incubated for 2 min at room temperature with $2\text{ }\mu\text{l}$ NHS-biotin (biotin-amidocaproate *N*-hydroxysuccinimide ester, 50 mg in 1 ml dry DMSO; Sigma) that was diluted 1:20 in PBS. The reaction was

Table 1. *Peptostreptococcus* strains and their hydrophobicities

Strain	Source	%HP (±SD)
<i>P. anaerobius</i>		
ATCC 27377 ^T	ATCC	35.5 ± 15.7
AHC5715	NPHI	NT
AHC5756	NPHI	37.0 ± 8.9
AHC5765	NPHI	NT
AHC5742	NPHI	58.2 ± 8.9
AHC5734	NPHI	5.3 ± 1.7
<i>P. micros</i>		
DSM 20468 ^T	DSM (root canal isolate)	44.3 ± 6.9
MPP-36	Authors' isolates; from root canal	47.3 ± 4.9
MPP-9		62.5 ± 3.1
T324	Authors' isolates; from periodontal pocket	50.5 ± 4.2
T326 ^R		9.7 ± 4.2
T330		58.7 ± 7.4
T523 ^R		13.8 ± 1.4
<i>P. magnus</i>		
DSM 20470 ^T	DSM	NT

Abbreviations: ATCC, American Type Culture Collection; DSM, German Collection of Micro-organisms, NPHI, National Public Health Institute of Finland; T, type strain; R, rough colony variant; NT, not tested.

stopped with Tris buffer (pH 7.5, final concentration 100 mM), and the cells were washed twice with 50 mM Tris buffer (pH 7.5). Sample preparation and electrophoresis were performed as above. Polypeptides were transferred to nitrocellulose membrane (Bio-Rad) in Tris/glycine ($5.8 \text{ g l}^{-1}/2.9 \text{ g l}^{-1}$) blotting buffer with 20% (v/v) methanol for 1 h at 0.8 mA cm^{-2} (Nova-Blot, Pharmacia LKB Biotechnology). The membranes were blocked with 3% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (TBS; 20 mM Tris, 0.5 M NaCl, pH 7.5) for 1 h. The membranes were incubated with alkaline-phosphatase-labelled extravidin (1:20000 in 1% BSA in TBS; Sigma) for 1 h, washed and stained. The colour-developing substrates were 44 μl nitroblue tetrazolium (Sigma) (75 mg ml^{-1} in 75%, v/v, dimethylformamide) and 33 μl 5-bromo-4-chloro-3-indolyl phosphate (Sigma) (50 mg ml^{-1} in dimethylformamide) in 10 ml substrate buffer (0.1 M Tris, 0.1 M NaCl, 50 mM MgCl_2 , pH 9.5).

Two-dimensional PAGE. Isoelectric focusing (IEF) of *P. anaerobius* proteins was performed by using a Multiphor II electrophoretic unit (Pharmacia) using precast Immobiline Drystrips (Pharmacia) according to the manufacturer's directions. Briefly, *P. anaerobius* cells were disrupted in lysis buffer (27 g urea, 1 ml Triton X-100, 1 ml 2-mercaptoethanol, 1 ml Pharmalyte 3–10 and 70 mg phenylmethylsulfonyl fluoride in 50 ml distilled water) and dissolved in sample buffer (24 g urea, 1 ml 2-mercaptoethanol, 1 ml Pharmalyte 3–10, 0.25 ml Triton X-100 and a few grains of bromophenol blue in 50 ml distilled water). A 50 μl portion of each sample was applied at the anode end of the rehydrated Immobiline Drystrips (pH 4.0–7.0) using sample cups. Sample cups and strips were covered with silicone oil and IEF was carried out on a cooling plate as a stepwise run giving 35 kV h. After running, the strips were incubated for $2 \times 10 \text{ min}$ in equilibrating solution (50 mM Tris buffer, pH 6.8, urea 360 g l^{-1} , glycerol 300 ml l^{-1} and SDS 10 g l^{-1}). For the second dimension the strips were embedded into the stacking gel and SDS-PAGE was run in a 8–18% (w/v) gradient gel (ExcelGel SDS; Pharmacia) according to manufacturer's directions. The proteins were stained with Coomassie blue and the pI was determined by comparison with standards (high pI kit; Pharmacia).

RESULTS

Cell surface ultrastructure of *P. micros*, *P. magnus* and *P. anaerobius* was studied by electron microscopy. A homogeneous outer layer about 10 nm thick outside the peptidoglycan layer was present in *P. anaerobius*. In *P. micros* and *P. magnus* (not shown) a more amorphous layer was seen (Fig. 1). Negative staining of whole cells revealed no periodic structures in *P. anaerobius*. However, when cells of *P. anaerobius* were disrupted by sonication, cell wall fragments with periodic structures were detectable in negatively stained samples (Fig. 2). Freeze-etching of whole cells followed by platinum and carbon shadowing clearly showed a periodic crystalline surface layer in *P. anaerobius* (Fig. 3a, b). The width of the repeating unit was 12 nm. None of the electron microscopic methods used in this study revealed any periodic structures in *P. micros* strains or in the type strain of *P. magnus*.

Whole cells of *P. anaerobius* ATCC 27337 were extracted with 0.1 and 1% SDS, 0.1–2.1% deoxycholate and 6 M urea to identify the probable S-layer protein. A major 78 kDa band was obtained with 6 M urea and 1% SDS

Fig. 1. Thin-sectioned cells of *P. micros* DSM 20468 (a) and *P. anaerobius* ATCC 27337 (b). A thin layer of polymeric substance outside the peptidoglycan covers the cells of *P. anaerobius* (arrows). The bars in these and subsequent micrographs represent 0.1 μm .

(Fig. 4, lanes 1 and 2), but not with 0.1% SDS or deoxycholate. The 78 kDa band also appeared in SDS-PAGE when whole cells were boiled in sample buffer containing 0.7% SDS and the supernatants were examined. There were differences in the protein profiles of different *P. anaerobius* strains, but the 78 kDa protein was present in every strain as the major protein (Fig. 5). Urea and detergent extraction of *P. micros* (Fig. 6) and *P. magnus* (not shown) followed by SDS-PAGE revealed no corresponding dominant protein band.

Whole cells of the three *Peptostreptococcus* species were biotinylated for a short period with NHS-biotin. Immediately after biotinylation, Tris buffer was added to

Fig. 2. Negative staining of a cell wall fragment obtained by sonication of *P. anaerobius* ATCC 27337. Cell-surface periodicity can be seen (arrows).

bind all unbound biotin. SDS-PAGE of the biotinylated cells followed by Western blotting and detection by alkaline-phosphatase-conjugated extravidin revealed strong staining of the 78 kDa band in *P. anaerobius*. In addition, a sharp 127 kDa band appeared in all *P. anaerobius* strains (Fig. 7). Western blotting of biotinylated *P. micros* (Fig. 7) and *P. magnus* (not shown) revealed no corresponding major protein band.

Two-dimensional PAGE was performed with *P. anaerobius* strains to obtain further information on the characteristics of the crystalline surface protein. In all four strains studied, the S-protein appeared as a row of dots, probably because of deamination, typically seen in IEF of bacterial proteins (Fig. 8a, b). The pI ranges of the S-proteins differed between strains: the pI of the S-protein of *P. anaerobius* AHC5734 and AHC5742 was between 5.3 and 5.7 (5.4–5.7 and 5.3–5.6 respectively), while the corresponding values for strains ATCC 27377 and AHC5756 were 6.1–6.6 and 6.7–6.9. The pI range for each strain was stable between repeated runs.

The hydrophobicity of *P. micros* (a total of seven strains including two rough colonial variants) and *P. anaerobius* (four strains) was measured by the hexadecane method. The %HP of all *P. micros* strains with a smooth colony type ranged from 44.3 to 62.5, whereas the two rough strains were hydrophilic (%HP 9.7 and 13.8) (Table 1). The %HP of three of the *P. anaerobius* strains was 35.5–58.2; however, the fourth strain tested, AHC 5734, was very hydrophilic (%HP 5.3) (Table 1). No differences could be detected in colony morphology or in electron microscopic appearance between the hydrophilic and hydrophobic strains of *P. anaerobius*.

DISCUSSION

The present study is the first description of a crystalline surface protein (S-layer) on *Peptostreptococcus anaerobius*. We were unable to see cells with a continuous S-layer in the freeze-etched specimens. The periodicity of the S-layer was not very clear at all sites even in freeze-etched specimens. It is therefore possible that a thin layer of some other polymeric material covers the S-protein. This could partly explain the difficulty in observing the S-layer in negatively stained specimens, when material occupying/covering the possible pits in the S-protein sheet may leave no physical room for the stain. It is, however, also possible that the three-dimensional structure of the outer surface of the S-layer is unfavourable for negative staining. No periodic structures were detected on *P. micros* and *P. magnus*. Lounatmaa *et al.* (1988) reported an S-layer on a *Peptostreptococcus* sp. strain AHC5155 that resembled *P. magnus*. However, close comparison with the type strain of *P. magnus* in this study showed that strain AHC5155 cannot be identified as *P. magnus*. Classification of species in the genus *Peptostreptococcus* has recently undergone several changes and many new species have been established (Ezaki *et al.*, 1983; Li *et al.*, 1992). So far we have not been able to identify strain AHC5155 as any of the currently recognized species.

A 78 kDa major protein was detected in all *P. anaerobius* strains. This finding is in accordance with the results of Smith *et al.* (1986b). Biotinylation of bacteria in suspension for a short period with NHS-biotin followed by the addition of Tris buffer to bind all unbound biotin to the amine groups of the buffer labels proteins exposed to the surface of the bacterial cells (Dooley & Trust, 1988).

Fig. 3. (a, b). Freeze-etched and metal-shadowed cells of *P. anaerobius* ATCC 27337. The periodicity of the crystalline surface layer on the cells can be seen (thick arrows). Direction of shadowing is indicated by thin arrows.

Two proteins stained strongly after blotting in all *P. anaerobius* strains, a major 78 kDa and a 127 kDa band. A 95 kDa band and several lower molecular mass proteins that stained in SDS-PAGE did not stain at all in Western blotting after surface biotinylation (Figs 4, 5 and 7). This further indicates that the 78 kDa and the 127 kDa molecules are located on the surface of the cell. It is obvious that, as the major protein, the 78 kDa protein

rather than the 127 kDa protein is the S-protein. The molecular mass difference of the two proteins suggests that the 127 kDa protein is not a dimer of the 78 kDa protein. However, the relationship of these two proteins on the surface of *P. anaerobius* remains unclear. Biotinylation of *P. micros* revealed no major protein band in Western blots. This is in line with the electron microscopic examination, which gave no indication of a

Fig. 4. SDS-PAGE analysis of cell-surface proteins of *P. anaerobius* ATCC 27337. Whole cells were extracted with urea and different detergents for 30 min. Lanes: 1, 6 M urea extract; 2, 1% SDS extract; 3, 0.1% SDS extract; 4, 2.1% deoxycholate extract; 5, 1% deoxycholate extract; 6, 0.1% deoxycholate extract; 7, standard proteins (ovalbumin, 42.7 kDa; glutamate dehydrogenase, 55.5 kDa; ovotransferrin, 76–78 kDa; phosphorylase *b*, 97.4 kDa; β -galactosidase, 116.3 kDa; myosin, 200 kDa).

Fig. 5. Whole-cell protein profiles of *P. anaerobius* strains in SDS-PAGE. Lanes: 1, AHC5734; 2, AHC5742; 3, AHC5765; 4, AHC5756; 5, AHC5715; 6, ATCC 27337; 7, standard proteins (same as Fig. 4). The 78 kDa protein is a major protein in all strains.

presence of an S-protein in *P. micros* but suggested the presence of a capsule.

Our earlier studies have indicated that S-layers have a major effect on the hydrophobicity of the cells and that the variation between different strains of the same species with an S-layer is considerably smaller than in species with no S-layer (Haapasalo *et al.*, 1990). This is obviously not the case with *P. anaerobius*, as one of four *P. anaerobius* strains studied was very hydrophilic while the others were relatively hydrophobic. Recently van Dalen *et al.* (1993) reported that *P. micros* strains with a rough colony type

Fig. 6. SDS-PAGE analysis of whole-cell proteins and urea or detergent extracts of *P. micros* DSM 20468 (lanes 1–4: lane 1, whole cells; lane 2, 6 M urea extract; lane 3, 1% SDS extract; lane 4, 0.1% SDS extract) and *P. micros* MPP-36 (lane 5: whole cells).

are hydrophilic as compared to strains with a smooth colony type. This was also confirmed in the present study. However, in the case of *P. anaerobius* we were unable to find any differences in colony morphology or ultra-structure between the hydrophilic and hydrophobic strains. An S-protein of the same molecular size was found in all strains; however, the pI values of the S-proteins of *P. anaerobius* strains were different. Strains AHC5734 and AHC5742, the most hydrophilic and the most hydrophobic strain, had clearly lower pI values (5.3–5.7) than strains AHC5756 and ATCC 27377 (6.1–6.9) (Fig. 8a, b). The biological relevance of the differences in pI of the S-proteins remains unclear.

Crystalline surface protein layers are present in several oral bacteria, in both Gram-negative and Gram-positive species (Messner & Sleytr, 1992; Lounatmaa *et al.*, 1993), and bacteria with S-layers are regularly present in oral infections. The role of the S-layer in virulence and microbial ecology for the majority of species possessing an S-layer is not known. In the case of the fish pathogen *Aeromonas salmonicida* it has been shown that only strains with an S-layer are virulent (Trust & Kay, 1992). Blaser *et al.* (1988) reported that the S-layer of *Campylobacter fetus* may render the cell resistant to phagocytosis. We have shown that the S-layers of bacteria from the human oral cavity seem to be of major importance in determining the cell surface hydrophobicity (Haapasalo *et al.*, 1990), which in turn plays a role in bacteria-leucocyte interaction (Absalom, 1988). We have also studied the non-opsonophagocytosis of a new oral anaerobe, *Eubacterium yurii*, of which all three subspecies possess an S-layer. Only one subspecies, subsp. *margaretiae*, was resistant to phagocytosis, while the two others were readily ingested by the leucocytes (Kerosuo *et al.*, 1993). The importance and function of the S-layers of the *Peptostreptococcus* species

Fig. 7. Western blotting of biotinylated cell surface proteins of *P. anaerobius* and *P. micros* strains. Lanes: 1 and 10, prestained standard proteins (ovalbumin, 49.5 kDa; bovine serum albumin, 80 kDa; β -galactosidase, 116.5 kDa; myosin, 20.5 kDa); 2, *P. anaerobius* ATCC 27337; 3, *P. anaerobius* AHC5734; 4, *P. anaerobius* AHC5742; 5, *P. anaerobius* AHC5756; 6, *P. micros* DSM 20468; 7, *P. micros* I-60; 8, *P. micros* MPP-9; 9, *P. micros* MPP-36. Strong staining of a major 78 kDa band and a minor 127 kDa band is clearly seen in all *P. anaerobius* strains. Corresponding staining cannot be detected in *P. micros* strains.

Fig. 8. Two-dimensional gel electrophoresis of *P. anaerobius* strains: (a) *P. anaerobius* ATCC 27337; (b) AHC5734.

is not known. It is worth noting that of the four species studied so far, *P. asaccharolyticus*, *P. magnus*, *P. micros* and *P. anaerobius*, two species, *P. asaccharolyticus* and *P. anaerobius*, possess an S-layer (Messner & Buckel, 1988; this study). It is obvious that the S-layer is a more common structure in oral anaerobic bacteria than was previously realized. The results of the present study also demonstrate that the S-layer may remain undetected by the conventional electron microscopic methods usually employed in studies of the ultrastructure of bacteria. Work is now in progress in our laboratory to investigate the role of the S-layers from different oral bacteria, as the outermost structures of the cell, in attachment to host proteins and to other bacteria.

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