Description of Two Morphotypes of *Peptostreptococcus micros*

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Peptostreptococcus micros is often isolated from abscesses in several parts of the human body. The oral cavity is considered the natural habitat for the species, which has been implicated as a periodontal pathogen. In plaque samples from periodontitis patients we observed the presence of a rough morphotype of *P. micros* in addition to the previously recognized smooth morphotype. The rough morphotype has not been described previously. Both morphotypes are frequently isolated simultaneously from the same patient. In this paper strains of both morphotypes are described. The smooth morphotype, represented by the type strain, grew as small, dome-shaped, bright white, nonhemolytic colonies. The rough morphotype grew as equally white dry colonies which were hemolytic and had wrinkled edges. DNA-DNA reassociation studies revealed homology at the species level between the two morphotypes; in addition, no differences in physiological characteristics were observed when the organisms were tested with API-32A and API-ZYM kits. The rough cells had long, thin fibrillar structures outside the cell envelope when they were stained negatively for electron microscopy. In the smooth morphotype these structures were not present. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of whole-cell extracts were different for the two morphotypes. In xylene-water phase partition studies, the smooth morphotype was found to be hydrophobic, whereas the rough morphotype was found to be relatively hydrophilic. The distinct morphotypes were stable on blood agar; however, the rough morphotype changed to a nonfibrillar type with a smooth colony morphology after repeated subculturing in broth.

Gram-positive anaerobic cocci are commonly found on mucous membranes of the oral cavity, the intestines, and the vagina. These bacteria are isolated from a variety of human abscesses as well. Frequently, the strains isolated belong to the genus *Peptostreptococcus*. The presence of *Peptostreptococcus micros* is not restricted to a particular part of the human body. Polymicrobial pulmonary and cerebral abscesses (9, 12, 24), as well as female genital tract infections (7), can contain high percentages of *P. micros*. It is thought that the natural habitat of this species is the oral cavity (14). Plaque of healthy individuals contains low percentages of this bacterium. It is isolated more often and in increased percentages from patients with periodontitis (18). A correlation in patients has been reported between poor response to periodontal therapy and the percentage of *P. micros* present in the total cultivable anaerobic flora (17). Furthermore, the presence of the microorganism has been related to active sites of periodontitis (4, 30), to endodontic lesions, and to human immunodeficiency virus-related periodontitis (3, 25, 26, 36). For these reasons *P. micros* has been implicated as a periodontal pathogen (27).

P. micros usually forms smooth colonies. In samples obtained from periodontitis patients, we frequently isolated a rough morphotype of *P. micros* as well. In this paper we describe the results of a descriptive study of the two morphotypes. Strains representing both morphotypes are described on the basis of the results of electron microscopy studies and studies on DNA-DNA homology, hydrophobicity, biochemical reactions, interactions with erythrocytes, and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) profiles.

MATERIALS AND METHODS

Microorganisms. A total of 39 strains, including 15 isolates of the rough morphotype, 19 strains of the smooth morphotype, and 5 strains of other *Peptostreptococcus* species, were examined in this study. The *Peptostreptococcus magnus* strain, the *Peptostreptococcus anaerobius* strains, and five *P. micros* strains were kindly provided by T. Ezaki, Gifu, Japan, and S. Persson, Umeå, Sweden. Our own isolates were obtained from subgingival plaque samples from periodontitis patients. The bacterial strains were all cultivated on blood agar plates (Oxoid no. 2 agar supplemented with 5% defibrinated horse blood, 5 mg of hemin per liter, and 1 mg of menadione per liter) incubated in 80% N₂-10% CO₂-10% H₂ at 37°C.

Strains were identified on the basis of Gram staining, anaerobic growth, and the results of biochemical reactions, for which ATB-32A and API-ZYM kits (Analytab Products, Montalieu-Vercieu, France) were used. These kits were used according to the recommendations of the manufacturer. The stability of the morphotypes was tested by repeated subculturing, both on blood agar plates and in broth (Schaedler broth; BBL Microbiology Systems, Cockeysville, Md.) in an anaerobic glove box (Forma Scientific, Marietta, Ohio).

DNA isolation. Bacterial strains (four strains of the smooth morphotype and five strains of the rough morphotype) were inoculated into 100-ml portions of Schaedler broth and incubated in an anaerobic glove box for 48 h without shaking. The cells were harvested by centrifugation at 2,000 × g for 15 min at 4°C. Then the cells were washed twice in TES (10 mM Tris, 5 mM EDTA, 50 mM NaCl; pH 8.0) and finally resuspended in 1 ml of the same buffer. Each suspension was ultrasonically treated for 5 s at the maximum power setting with an MSE Soniprep 150 apparatus. The cells were then treated with lysozyme (1 mg/ml, 1 h, 37°C), proteinase K (250 µg/ml, 1 h, 37°C), and SDS (1%, 16 h, 60°C) in the

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presence of RNase (0.5 µg/ml). The DNA was isolated by two phenol-chloroform extractions, two chloroform-isoamyl alcohol extractions, an ether extraction, and RNase treatment. After this, the DNA was purified by two phenol-chloroform extractions and two chloroform-isoamyl alcohol extractions and ethanol precipitation. The DNA was washed in ethanol, vacuum dried, and dissolved in 100 µl of H₂O. The DNA of *Bacteroides fragilis* was kindly provided by B. Otto, Amsterdam, The Netherlands.

A mini agarose gel (0.8% agarose in 0.04 M Tris-acetate–0.001 M EDTA [pH 8.0]) was used to check the integrity of the DNA. The DNA concentration was calculated from the optical density at 260 nm.

Preparation of labelled DNA. DNAs from the type strain of *P. micros* (ATCC 33270) and a representative rough strain (HG 1439) were labelled by using [³H]dTTP (NEN Research Products, Du Pont, Dreiech, Germany) and a random-primed DNA labelling kit (Boehringer, Mannheim, Germany). A total of 30 ng of DNA was labelled according to the recommendations of the manufacturer. The remaining single nucleotides were removed from the labelled DNA by using Quick Spin columns containing Sephadex G-50 (Boehringer). Samples containing 1 µl of labelled DNA were suspended in 2 ml of Ultima Gold scintillation fluid (Packard) and counted with a Packard model 1900TR liquid scintillation analyzer to determine the specific activity of the probe.

DNA-DNA reassociation. Cross-reassociation studies were performed by using randomly selected smooth and rough strains. The unlabelled DNA solutions were brought to an NaCl concentration of 0.42 M and sheared by sonication to give fragment sizes of approximately 500 bp. Each reaction mixture contained 0.5 ng of labelled DNA and 5.8 µg of unlabelled DNA. The total volume was adjusted to 50 µl with 0.42 M NaCl, and the preparation was layered with 100 µl of mineral oil (catalog no. M-5904; Sigma). As negative controls, we used calf thymus DNA (catalog no. D-8661; Sigma) and *B. fragilis* DNA at the same concentration as the unlabelled DNAs from the *P. micros* strains. The DNA was denatured by boiling it for 10 min. After this the vials were cooled to 0°C. The reassociation reactions were carried out at 61.4°C for 72 h. These optimum renaturation conditions were calculated by using the average G+C content of *P. micros* given by Ezaki et al. (6) in the formulas published by Brenner and Falkow (2) and Gillis et al. (8).

To stop renaturation, the vials were cooled to 0°C again. S1 nuclease (100 U/ml) in a buffer containing 0.1 mM ZnSO₄, 0.15 M NaCl, 30 mM sodium acetate (pH 4.5), and 20 mg of sheared and denatured calf thymus DNA per ml was added to all vials. The resulting mixture was incubated for 20 min at 50°C, after which single nucleotides were removed by using Quick Spin columns containing Sephadex G-50. Samples of the reaction mixtures and of the void volumes of the columns were analyzed by using Ultima Gold scintillation fluid and a Packard liquid scintillation analyzer. The level of reassociation in each reaction mixture was determined as a percentage of the value for the homologous reaction corrected for the calf thymus DNA control.

Hydrophobicity. Cell surface hydrophobicity was assessed as described by Rosenberg et al. (28). *Peptostreptococcus* strains were grown on blood agar plates for 96 h. Cultures were checked for purity by Gram staining and cultivation on blood agar plates. Cells were harvested by centrifugation at 4°C and 2,000 × g and washed twice in phosphate-buffered saline (PBS). Bacterial suspensions were prepared in PBS to an optical density of 1.0 (λ, 650 nm). Duplicate (3-ml) samples of each suspension were placed in test tubes.

Xylene was added to each suspension at concentrations of 0, 2, 4, 8, and 16%. The tube contents were mixed for 45 s with a vortex mixer at the maximum setting. The aqueous and organic phases were allowed to separate for 30 min, after which the aqueous phase was carefully removed with a Pasteur pipette and transferred to cuvettes. Contaminating xylene was allowed to evaporate for 60 min. After this, each cuvette was vortexed for 1 s to resuspend the remaining bacteria. The level of absorption of the cells to the xylene droplets was calculated as the loss in optical density at 650 nm of the aqueous phase compared with the initial cell suspension, expressed as a percentage.

It is known that xylene can be cytotoxic. This might have influenced the results of the spectrophotometric assay described above. Therefore, we checked 12 strains (6 smooth morphotype strains and 6 rough morphotype strains) with the same method by using hexadecane (7.5 and 20%) instead of xylene (28).

SDS-PAGE. SDS-PAGE profiles were obtained by using the PhastSystem (Pharmacia LKB Biotechnology, Uppsala, Sweden). Samples of soluble proteins were prepared as described by Slayne et al. (29). Cells from five blood agar plates which had been incubated for 96 h were harvested in 0.5 M Tris-HCl (pH 6.8). The cells were washed twice in the same buffer. After the final wash the cells were suspended in 200 µl of a denaturation buffer containing 13.2 ml of water, 1.2 ml of glycerol, 4.8 ml of 10% SDS, 1.2 ml of 2-β-mercaptoethanol, 3.0 ml of 0.5 M Tris-HCl (pH 6.8), and 1 mg of bromphenol blue per ml. After the samples were heated for 10 min at 100°C, the cell debris was removed by centrifugation at 16,000 × g for 10 min. Before SDS-PAGE the proteins in the supernatant were alkylated as described by Heukeshoven and Dernick (10). The running conditions used for the 12.5% homogeneous acrylamide gel were the conditions described by Slayne et al. (29). Low-molecular-weight standard proteins were obtained from Pharmacia. The gels were silver stained manually as described by Morrissey (21).

Hemolysis. Cells were plated on agar plates containing heparinized blood from chickens, sheep, horses, rabbits, and humans (blood group O). The plates were incubated in an anaerobic glove box, as described above. The plates were examined for hemolysis on days 7, 17, and 21 after inoculation.

Electron microscopy. For electron microscopic investigations, cells of strains HG 1179 and HG 1439 were incubated on an agar plate for 96 h or in a broth culture for 48 h and were harvested in PBS and washed in water once. The cells were then resuspended in water, negatively stained with 1% methylamine tungstate, and examined with a Philips model EM 301 electron microscope. All experiments were performed in duplicate with the same batch of cells and at least three times with independent batches of cells.

RESULTS

Two different *P. micros* morphotypes are described below; one produced smooth colonies on blood agar, and the other produced rough colonies. The cells of both types were gram-positive anaerobic cocci arranged in short chains and clumps. All strains were asaccharolytic and strongly peptidase positive on API preformed substrates.

The smooth morphotype was represented by the type strain and was characterized by small, dome-shaped, bright white, nonhemolytic colonies on horse blood agar plates (Fig. 1A). The rough morphotype was represented by clini-

FIG. 1. (a) Characteristic smooth colonies of *P. micros* on a horse blood agar plate. (b) Characteristic rough colonies of *P. micros* on a horse blood agar plate. Magnification, $\times 80$.

cal isolates obtained from our laboratory and was characterized by equally white, dry, hemolytic colonies with wrinkled edges (Fig. 1B).

As shown in Table 1, the smooth and rough strains of *P. micros* exhibited high levels of DNA-DNA homology (64 to 132%) when DNA from the type strain was used as the probe. When DNA from a rough strain was used as the

TABLE 1. Levels of reassociation between genomic DNAs and DNA from the *P. micros* type strain or a representative rough strain^a

Strain	Morpho- type	% Reassociation with [³ H]DNA from ATCC 33270 ^{Tb}	% Reassociation with [³ H]DNA from HG 1439
ATCC 33270 ^T	Smooth	100	49
HG 1451	Smooth	115	52
HG 1454	Smooth	132	58
HG 1459	Smooth	67	65
HG 1468	Rough	79	99
HG 1467	Rough	82	89
HG 1439	Rough	64	100
HG 1442	Rough	87	96
HG 1257	Rough	77	103
<i>B. fragilis</i> BE1		0	7

^a Percentages were calculated by defining the value for the homologous reaction as 100% and the value for the reaction with calf thymus DNA as 0%.

^b T = type strain.

TABLE 2. Biochemical reactions of *Peptostreptococcus* species determined by using ATB-32A and API-ZYM kits^a

Reaction	<i>P. micros</i>		<i>P. magnus</i>	<i>P. anaerobius</i>
	Smooth morpho- type	Rough morpho- type		
β -Glucosidase	— ^b	—	—	+
Alkaline phosphatase	+	+	—	—
Arginine arylamidase	+	+	+	—
Proline arylamidase	V	V	—	+
Leucyl-glycine arylamidase	V	V	+	—
Phenylalanine arylamidase	+	+	—	—
Pyroglutamic acid arylamidase	+	+	+	—
Tyrosine arylamidase	+	+	±	—
Alanine arylamidase	+	+	+	—
Glycine arylamidase	+	+	+	—
Glutamic acid decarboxylase	V	V	—	—
α -Fucosidase	V	V	—	—
Histidine arylamidase	+	+	±	—
Glutamyl-glutamic acid arylamidase	+	+	—	—
Serine arylamidase	+	+	±	—

^a Results are given only for reactions which discriminated between species.

^b +, positive reaction; —, negative reaction; V, variable reaction; ±, weak reaction.

probe, the five rough strains gave reassociation values of 89 to 103%, whereas the levels of reassociation with DNAs from the smooth strains were lower (49 to 65%). *B. fragilis* did not exhibit homology to both labelled strains of *P. micros*. In addition, the *P. micros* strains produced almost identical preformed enzyme profiles when the ATB-32A kit was used (Table 2). All smooth and rough strains were confirmed to be *P. micros* strains. All sugar reactions were negative, and the strains were strongly alkaline phosphatase positive. Most of the proteolytic reactions were positive; the exceptions were the proline arylamidase and leucyl-glycine arylamidase reactions, which were negative or weakly positive for some strains. This variability did not correspond with morphotype. Other *Peptostreptococcus* species produced profiles completely distinct from the profile of the *P. micros* strains (Table 2). When the API-ZYM kit was used, the two morphotypes produced identical enzyme profiles.

We checked the stability of the morphotypes of the strains. On blood agar plates, both morphotypes proved to be stable after more than 25 subcultures. All smooth morphotype strains remained smooth after four passages in Schaedler broth. All rough morphotype strains changed to a nonfibrillar type with a smooth colony morphology after one to four subcultures in Schaedler broth.

The cell surface hydrophobicities of three smooth and three rough strains were tested with four different concentrations of xylene (Fig. 2). At all concentrations, the smooth strains were more hydrophobic than the rough strains. Because the greatest differences between the smooth and rough morphotypes were found at a xylene concentration of 2%, additional strains were tested at this concentration.

A significant difference between smooth and rough strains was found. The percentages of cells in the aqueous phase were $22\% \pm 9\%$ for the smooth strains and $70\% \pm 7\%$ for the rough strains (Table 3).

To eliminate the possible interference of the cytotoxic side effects of xylene, we measured hydrophobicities with hexa-

FIG. 2. Percentages of cells (i.e., turbidity at 650 nm compared with the control) in the aqueous phase after a two-phase partition test was performed.

decane as the organic phase at two different concentrations. As in the experiment performed with xylene, the smooth strains were hydrophobic, whereas the rough strains were relatively hydrophilic (Table 3).

Figure 3 shows SDS-PAGE profiles of representative strains of *P. magnus*, *P. anaerobius*, and the smooth and rough morphotypes of *P. micros*. The *P. micros* strains produced profiles that were clearly different from the profiles of the other *Peptostreptococcus* species tested. There were similarities between the profiles of the smooth and rough morphotypes of *P. micros*. However, there were bands that were unique for each morphotype. Minor differences among the strains of one group, especially among the smooth strains, were also observed.

The results of the hemolysis experiments are shown in Table 4. The smooth strains were not hemolytic except for limited activity on rabbit blood agar plates after 17 days. In contrast, the rough strains were hemolytic with a variety of erythrocytes. There were no noticeable differences in hemolysis between the different strains tested. Cells on chicken, horse, and rabbit blood agar plates were moderately positive after 7 days of incubation; after 21 days, the cells lysed human erythrocytes as well. Sheep erythrocytes were not attacked to any visible extent. Colony morphology and size were not influenced by the type of erythrocytes.

In electron micrographs all negatively stained strains

TABLE 3. Hydrophobicities of *Peptostreptococcus* species as determined by a two-phase partition test

Test conditions	Hydrophobicity (%) ^a			
	<i>P. micros</i>		<i>P. magnus</i> (n = 1)	<i>P. anaerobius</i> (n = 4)
	Smooth morpho- type (n = 19) ^b	Rough morpho- type (n = 15)		
2% Xylene	22 ± 9	70 ± 7	11	60 ± 11
7.5% Hexadecane	4 ± 1	40 ± 6	ND ^c	ND
20% Hexadecane	5 ± 1	17 ± 4	ND	ND

^a Percentage of cells in the aqueous phase compared with the control. Mean ± standard deviation.

^b n is the number of strains studied.

^c ND, not determined.

FIG. 3. SDS-PAGE patterns of different *Peptostreptococcus* species. (a) *P. micros* smooth morphotype. (b) *P. micros* rough morphotype. (c) Lanes 1 and 2, *P. micros* smooth morphotype; lanes 3 through 5, *P. micros* rough morphotype; lane 6, *P. anaerobius*; lane 7, *P. magnus*; lane 8, marker (molecular weights [$\times 10^3$] are indicated on the right).

exhibited the typical characteristics of gram-positive cells (Fig. 4). The cells of both morphotypes had thick cell walls. However, the cells of the rough morphotype had fibrillar structures, whereas the cells of the smooth morphotype did not. The fibrillar structures were long and appeared to aggregate with each other.

DISCUSSION

In this paper the results of a descriptive study of two morphotypes of *P. micros*, isolated from subgingival plaque of periodontitis patients, are presented. Both types exhibited significant DNA reassociation with the DNA of the type

TABLE 4. Hemolysis of erythrocytes by *P. micros* morphotypes on agar plates

Morphotype	No. of strains tested	Origin of erythrocytes	Hemolysis after:		
			7 days	17 days	22 days
Smooth	4	Chickens	— ^a	—	—
		Sheep	—	—	—
		Horses	—	—	—
		Rabbits	—	— ^b	— ^b
		Humans	—	—	—
Rough	3	Chickens	+	+	+
		Sheep	—	—	—
		Horses	—/+	+	+
		Rabbits	—/+	+	+
		Humans	—	—	+

^a —, no hemolysis; —/+, light zone around each colony; +, transparent zone around each colony.

^b Erythrocytes were affected to a small extent.

strain. Therefore, we conclude that the smooth and rough strains belong to the same species (33). The reassociation data seemed to be more complex when DNA from a rough strain was used as the probe. Although the levels of reassociation with the other rough strains were approximately 100%, the levels of reassociation with the DNAs of the smooth strains ranged from 49 to 65%. The Gram stain reaction, anaerobic growth, and biochemical characteristics were identical for all strains.

Recently, the ATB-32A kit has been evaluated; the level of agreement between results obtained with this method and gas-liquid chromatography results was more than 99% (23). Therefore, we used this kit for identification of our strains. The profiles of preformed enzymes which we found with both ATB-32A and API-ZYM kits were identical to the profiles published previously (6, 15, 23).

Adhesion (32) and phagocytosis (1) of bacteria are influenced by the hydrophobicity of the bacterial surface. Therefore, we measured the hydrophobicities of cells of the two morphotypes.

The smooth morphotype was hydrophobic, whereas the rough morphotype was relatively hydrophilic in tests performed with both xylene and hexadecane. Other methods used to determine bacterial cell surface hydrophobicity include measurement of the contact angle of water on a film of bacteria (22). In a pilot experiment, the rough strains had a smaller contact angle than the smooth strains, which confirmed the lower hydrophobicity of the rough strains (data not shown).

A number of studies in which bacteria were identified by SDS-PAGE have been performed. Slayne et al. (29) demonstrated the possibilities of the PhastSystem for this technique. We characterized the two morphotypes of *P. micros* and showed that with this method, it was possible to differentiate between the two morphotypes despite minor differences between strains of one group.

Colonies of *P. micros* have been reported to be nonhemolytic (11). We showed that this is true only for the smooth morphotype. The smooth morphotype strains lysed rabbit erythrocytes only after prolonged incubation. In contrast, the rough morphotype strains hemolyzed a variety of erythrocytes in a relatively short period of time. In particular, the nucleus-containing chicken erythrocytes were very sensitive to the rough strains. The sheep erythrocytes were not lysed to a visible extent.

In contrast to gram-negative bacteria, only a few gram-

FIG. 4. Electron micrographs of negatively stained (1% methylamine tungstate) cells of *P. micros*. (A) Smooth morphotype. (B) Rough morphotype. Magnification, $\times 21,750$. Bar = 0.4 μm .

positive bacteria are known to express fibrillar structures. *Streptococcus sanguis*, for example, has adhesins (5, 13), fibrillar glycoproteins (20), and cell surface components related to aggregation, adherence, and hydrophobicity (19). *Streptococcus salivarius* expresses three different classes of fibrils, which have distinct adhesive functions (34, 35). It has been suggested that subunits of the fibrils of these oral streptococci range in size from 70 to 90 kDa (19) to more than 300 kDa (20). These different fibrils have distinct hydrophobic characteristics. Their subunits are considerably larger than the subunits of fimbriae described for gram-negative bacteria (16). Fibrillar structures with a possible function in adherence have been described for *Staphylococcus epidermidis* as well (31).

We found fibrillar structures in *P. micros* rough morphotype strains when cells were negatively stained with methylamine tungstate. These structures were not seen on smooth morphotype cells. As far as we know, this is the first description of these structures in gram-positive anaerobic cocci.

Expression of these structures was influenced by environmental conditions. The rough strains lost their rough appearance after they were subcultured in broth, which paralleled loss of their fibrillar structures, as determined with negatively stained cells by electron microscopy.

In summary, we found two different *P. micros* morphotypes. The presence of fibrillar structures is the major difference between the two morphotypes. The function and composition of the fibrillar structures are not clear yet, although a function in adhesion is likely. It is possible that these structures are responsible for the differences in the SDS-PAGE patterns and the hydrophilic character of the rough strains (19). Hemolysis is the only physiological difference which we found between the morphotypes. How this relates to the morphology of the cells is not yet known. Current investigations are concentrated on these phenomena. It is possible that the difference in appearance of these morphotypes is correlated with virulence. Therefore, we are currently investigating the virulence factors of both morphotypes as well.

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