Characterization of Smooth and Rough Morphotypes of Peptostreptococcus micros

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Isolation of the smooth (Sm) morphotype of Peptostreptococcus micros, a suspected oral pathogen, is sometimes accompanied by isolation of a rough (Rg) morphotype of P. micros. The Rg type readily changes to a Sm-like variant (Rg<sup>sm</sup>) in broth culture. Sm and Rg isolates and Rg<sup>sm</sup> variants were compared to determine whether these three types are the result of phase variation. The Rg<sup>sm</sup> variants resembled the Sm morphotype in colony morphology; furthermore, the Sm type and the Rg<sup>sm</sup> type did not have the fibrillar surface structures characteristic of the Rg type, and the Sm and Rg<sup>sm</sup> types were more hydrophobic than the Rg type. However, when we compared the sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of whole-cell proteins, serotyping data, pyrolysis mass spectrometry data, 16S ribosomal DNA sequences, and hemolytic activities, the Rg<sup>sm</sup> variants and the Rg isolates were very similar and were clearly distinct from the Sm isolates. These results suggest that the Rg and Rg<sup>sm</sup> types form a cluster distinct from the Sm type and thus provide evidence that P. micros can be differentiated into two groups, one consisting of the Sm type and the other consisting of the Rg and Rg<sup>sm</sup> types.

Peptostreptococcus micros is a natural commensal of the oral cavity (11, 27). However, it is also considered a suspected pathogen related to periodontal disease, as elevated levels of this gram-positive anaerobic coccus in subgingival plaque samples are associated with periodontal destruction, especially with active sites (4, 21, 22). P. micros is often isolated from other oral infections, such as endodontic lesions (3, 26, 38) and peritonsillar infections (20). This species is also commonly isolated from abscesses associated with mixed anaerobic infections throughout the human body (7, 24); cases of polymicrobial pulmonary and cerebral abscesses, female genital tract infections, and endocarditis infections containing high proportions of P. micros have been described (9, 14, 23).

Recent work in our laboratory revealed the existence of the following two morphotypes of P. micros (32): a smooth-colony (Sm) morphotype, which is recognizable by white, dome-shaped, mucous colonies; and a rough (Rg) morphotype, which produces dry white colonies with wrinkled edges. These two morphotypes are serologically distinguishable; the Sm morphotype represents serotype a, while the Rg morphotype represents serotype b (34). Both morphotypes can be isolated from subgingival plaque samples; the Rg type is always isolated in association with the Sm type, whereas the Sm type can also be isolated alone (33). This observation may indicate that phase variation occurs in P. micros.

van Dalen et al. (32) described the instability of the Rg morphotype in broth culture; the Rg morphotype readily changed to a Sm variant (Rg<sup>sm</sup>). Phase variation is uncommon in gram-positive bacteria and has been reported previously only for Streptococcus species (36) and Corynebacterium species (8, 13). This is in contrast to gram-negative organisms, where phase variation is common (1, 6, 30, 37). In the present study we characterized the Rg<sup>sm</sup> variant and compared its characteristics with those of Sm and Rg isolates in order to determine if the Sm, Rg, and Rg<sup>sm</sup> types are the result of phase variation or if they represent separate groups within P. micros.

MATERIALS AND METHODS

Microorganisms. A total of 11 pairs of Sm and Rg strains of P. micros were isolated from subgingival plaque samples from 11 periodontitis patients. All of the strains were identified by using anaerobic growth, Gram staining, and ATB-32A kits (Analytab Products, Montvale-Vereeck, France). Rg<sup>sm</sup> variants of all of the Rg strains were obtained after four passages in Schaedler broth (BBL Microbiology Systems, Cockeysville, Md.) and subsequent subculturing on blood agar plates. The reference strains included were P. micros type strain ATCC 33270, Peptostreptococcus magnus ATCC 14956 (kindly provided by S. Persson, Umeå, Sweden), and Peptostreptococcus anaerobius Gifu 7800 (kindly provided by T. Ezaki, Gifu, Japan). All bacterial strains were routinely cultured on blood agar plates (Oxoid no. 2 agar [1.5%] 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 for 4 days.

Hemolysis. All strains were grown anaerobically on agar plates supplemented with 5% heparinized blood from various sources (chickens, ducks, horses, humans [blood group O], bovines, rabbits, and sheep). The plates were examined for hemolysis after incubation for 7, 14, 21, and 28 days.

Hydrophobicity. Cell surface hydrophobicity was assessed by performing the two-phase partition assay with hexadecane (26). All of the strains were suspended and washed twice in phosphate-buffered saline (PBS), and the optical density at 650 nm (OD<sub>650</sub>) was adjusted to 1.0 (~10<sup>8</sup> bacteria/ml). Triplicate samples were mixed with 7.5% (vol/vol) hexadecane and vortexed at the maximum setting for 45 s in 9-cm glass tubes. After separation of the phases for 30 min, the aqueous phase was transferred to a cuvette. The optical density was measured after the contaminating hexadecane was evaporated and the suspension was vortexed. The hydrophobicity was calculated as the percentage of decrease in optical density compared to the optical density of the initial suspension.

Electron microscopy. All P. micros strains were harvested from blood agar and washed once in water. After negative counterstaining with 2% methylamine tungstate, the bacteria were examined with a model EM301 electron microscope (Philips, Eindhoven, The Netherlands).

SDS-PAGE. Whole-cell protein patterns were determined by standard protein polyacrylamide gel electrophoresis (PAGE) (16). Cells harvested from blood agar were washed, resuspended in 0.5 M Tris-HCl (pH 6.8), and diluted 1:1 in sample buffer containing sodium dodecyl sulfate (SDS) (4% SDS, 2% mercaptoethanol, 20% glycerol, 125 mM Tris-HCl [pH 6.8], 0.1 mg of bromphenol blue per ml). The samples were heated for 10 min at 100°C, and the insoluble debris was removed by centrifugation at 14,000 × g for 10 min. Electrophoresis was performed on a 1.5-mm-thick, 10% homogeneous polyacrylamide gel at 100 V.

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for 2 h, and the proteins were stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, Calif.).

**Rabbit antiserum.** Male chinchilla rabbits (weight, 2 to 3 kg) were immunized with P. micros ATCC 33270* (T = type strain) or HG 1468 (Rg morphotype). A suspension of bacteria in PBS (approximately 10⁸ bacteria/ml) was administered intravenously every other day in increasing amounts (0.25× ml steps, eight times). One week after the last injection, a 2.0-ml booster was administered intravenously. Then sera, prepared from blood obtained by cardiac puncture, were inactivated by heating them for 30 min at 50°C and stored at −80°C until they were used.

For serotype analysis 1-ml serum aliquots were adsorbed with 400 mg (weight) of bacteria, the anti-Sm serum (o-Sm serum) was adsorbed either with Rg strain HG 1468 or with RgSm strain HG 1468Sm*, and the a-rabbit serum was adsorbed with Sm strain ATCC 33270* or with HG 1468Sm*. For these adsorptions the sera were subjected to four cycles of incubation, each consisting of 1 h at room temperature followed by 12 h at 4°C and centrifugation at 15,000 × g for 5 min. The specificities of the various sera were determined by an enzyme-linked immunosorbent assay (ELISA), as well as by immunoblotting.

ELISA. ELISAs were performed as described previously (33). After the bacteria were harvested and washed once in PBS, 100 μl of a bacterial suspension in PBS (OD₅₆₂ 0.1; =10⁸ bacteria) was added to each well of a 96-well flat-bottom plate (catalog no. 655003; Greiner BV, Alphen aan den Rijn, The Netherlands). The plates were incubated at 4°C for 1 h, washed with PBS supplemented with 0.05% Tween 80 (PBSST), and then incubated for 1 h at room temperature with PBSST supplemented with 0.5% bovine serum albumin. After washing with PBSST, the unadsorbed sera, adsorbed sera, and preimmune serum (used as a negative control) were added to the plates, and serial twofold dilutions in PBSST were performed. After incubation at 4°C for 1 h, the plates were washed three times with PBSST, and horseradish peroxidase-labelled goat anti-rabbit immunoglobulin G conjugate (1:1,000 in PBSST, Nordic, Tilburg, The Netherlands) was added. After 1 h of incubation with the conjugate and three washes with PBSST, antibody binding to the beads was detected by peroxidase substrate (AEC, Sigma BV, Poortrecht, The Netherlands) and the amount of binding was determined with a plate reader (Dynatech Laboratories, Bilingtshurst, United Kingdom) as the A₄₅₀.

Immunoblotting. Whole-cell proteins were separated on a 10% polyacrylamide SDS-PAGE gel and transferred to a nitrocellulose membrane (pore size 0.45 μm, Schleicher & Schuell, Dassel, Germany) by Western blotting (31). After blotting at 100 V for 1 h, the nitrocellulose sheets were incubated for 1 h at room temperature with blocking buffer TTBS (100 mM Tris-HCl [pH 7.5], 0.9% NaCl supplemented with 0.1% [vol/vol] Tween 20), and this was followed by overnight incubation with sera diluted 1:1,000 in TTBS. The blots were washed four times with TTBS, incubated for 1 h with the conjugate, and then washed four times in TTBS. Afterward, antibody binding antigens were visualized by using H₂O₂ and 4-chloro-1-naphthol (Bioread).

PMS. Principles and methods of bacterial pyrolysis mass spectrometry (PMS) have been described previously (18, 19). Strains were grown on a single batch of blood agar plates with an additional 0.2% agar added to increase gel strength. Colony material was smeared onto pyrolysis foils; four replicate foils per plate and two plates per strain were used. Blind and known culture replicates were included to assess reproducibility. Within 10 min after sampling, the foils were heated at 80°C for 10 min, and then they were stored in a vacuum desicator for 16 h before processing. Curie point pyrolysis was performed for 4 s at 530°C with a model PYMS 200X automated pyrolysis mass spectrometer (Horizon Instruments, Heathfield, Sussex, United Kingdom). Data for pyrolysis products with mass/charge (m/z) ratios of 50 to 150 were collected. Mathematical analysis and classification of the spectra obtained were performed as described previously (12) by using the SPSS-PC and Clustan-PC version 2 programs with the unweighted pair group with mathematical average strategy.

**DNA Isolation.** Three Sm strains (ATCC 33270*, HG 1252*, and HG 1771*), two Rg strains (HG 1468* and HG 1772*), and two RgSm strains (HG 1468Sm* and HG 1772Sm*) were harvested from blood agar plates, washed twice in PBS, and resuspended in 25 mM Tris-HCl-10 mM EDTA-50 mM glucose (pH 8.0). The bacteria were disrupted by four cycles of freeze-thawing, and this was followed by incubation for 1 h at 37°C with lysosome (10 mg/ml) and proteasine K (1 mg/ml) and for 1 h at 55°C with SDS (4%). After two phenol-chloroform-isooamyl alcohol extractions and two chloroform-isooamyl alcohol extractions, sodium acetate (100 M, pH 7.6) was added before ethanol precipitation. The DNA was air dried and dissolved in 10 mM Tris-1 mM EDTA (pH 8.0) containing RNase (20 μg/ml).

**Cloning of 16S rDNA fragments.** 16S rRNA gene (DNA) fragments were amplified by a PCR with a PrimeZyme kit (Biometra, Inc., Tampa, Fla.) by using 25 ng of purified chromosomal DNA as the template. The reactions were performed according to the recommendations of the manufacturer with primers F1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and R7 (5′-AAG GAG GTG ATC CAG GG(GA)-3′) designed for the conserved regions of the 16S rDNA gene (35). Each PCR consisted of 30 cycles of denaturation for 30 s, annealing at 55°C for 30 s, and extension for 2 min at 75°C. The PCR products were checked for size and purity on a 1.2% agarose minigel. After concentration of the fragments by ethanol precipitation, the fragments were ligated in the pGEM-T vector (pGEM-T Vector System I; Promega Corp., Madison, Wis.) and transformed into Escherichia coli DHS08* as recommended by the manufacturer. Plasmids were isolated from the white colonies by

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**TABLE 1. Characteristics of the Sm type, the Rg type, and the RgSm variant of P. micros**

<table>
<thead>
<tr>
<th>P. micros type</th>
<th>No. of strains</th>
<th>Colony morphology</th>
<th>Fibre structures</th>
<th>Hydrophobicity (%)*</th>
<th>Hemolysis</th>
<th>Protein pattern</th>
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<tbody>
<tr>
<td>Sm</td>
<td>13 Smooth</td>
<td></td>
<td></td>
<td>68 ± 11</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Rg</td>
<td>11 Rough</td>
<td></td>
<td></td>
<td>25 ± 7</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>RgSm</td>
<td>11 Smooth</td>
<td></td>
<td></td>
<td>58 ± 9</td>
<td>B</td>
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* Hydrophobicity is expressed as the percent decrease in optical density compared with the optical density of the initial suspension.

**RESULTS**

**Characteristics of the P. micros RgSm type.** All 11 P. micros Rg morphotype strains changed easily to RgSm variants. In blood agar subcultures of broth cultures after the fourth passage, the RgSm type accounted for more than 90% of the population. The conversion frequency when the organisms were cultured continuously on blood agar plates was about 10⁻¹. The RgSm variants obtained by subculturing broth cultures were compared to the Rg and Sm periodontal isolates (Table 1). The RgSm variants were morphologically indistinguishable from the Sm isolates; both types formed white, dome-shaped, relatively mucous colonies. Electron microscopy revealed that the RgSm variants, like the Sm type, did not express the large fibrillar surface structures that are characteristic of the Rg type. The RgSm variants were significantly more hydrophobic than the Rg parental strains (58% ± 6%, versus 25% ± 7%, P < 0.01). The hydrophobicity of the Sm isolates (68% ± 11%) was not significantly different from that of the RgSm variants. The RgSm variants and their Rg parent strains both were clearly beta-hemolytic, in contrast to the Sm type; after 7 to 14 days the hemolytic zones around the colonies became visible. Hemolysis by both Rg and RgSm strains was most pronounced with human erythrocytes (data not shown). As shown in Fig. 1, the SDS-PAGE whole-cell lysate patterns of the RgSm variants were more similar to the patterns of the Rg type than to the patterns of the Sm type, although there were slight differences in the levels of expression of some proteins between the Rg isolates and the RgSm variants. The patterns of independent Rg (and RgSm) strains were relatively homogeneous and clearly distinguishable from the homogenously patterns of independent Sm strains. The SDS-PAGE patterns of P. micros strains were easily distinguishable from the patterns of other Peptostreptococcus species (Fig. 1, lanes 8 and 9).
The 330 bases determined (level of homology, 99.1%). When compared, they differed at 2 to 6 of the 330 bases (levels of homology, 98.2 to 98.5%). The sequences of three independent Sm strains differed at 9 to 16 nucleotides at the 330 base positions were observed (levels of homology, 95.2 to 97.2%). Comparable differences were observed with a known sequence of P. micros GIFU 7701 (5). Large differences were found with known 16S rDNA sequences of P. magnus and P. anaerobius (56 to 61 of 330 bases and 81 to 85 of 330 bases, respectively).

**Discussion**

In gram-positive bacteria, phase variation based on the presence of pili or fibrillar surface structures has been described previously only for Corynebacterium species (8, 13, 15). Previously, van Dalen et al. (32) have described two phenotypically different types of P. micros, the Sm type and the Rg type, which can be isolated from patients with periodontal disease. In a prevalence study performed in our laboratory (33), the Rg morphotype could be isolated only from patients that were also infected with Sm type P. micros. As the Rg type was found to be unstable in broth culture, changing to Rgsm-type colonies, we determined whether the existence of Rg and Sm morpho-

**Serotype analysis.** The results of the ELISA serotype analysis are shown in Fig. 2. Unadsorbed a-Rg and a-Sm sera exhibited strong cross-reactions with both the Sm type and the Rgsm type; the titers were all ca. 2^2 (Fig. 2A). Adsorption of this serum with Rg variants reduced all reactions to preimmune serum levels (titers, ca. 2^2). Adsorption with Sm variants removed only the cross-reactive antibodies against the Sm type. When the a-Sm serum was used (Fig. 2B), only Sm-reactive antibodies could be detected after adsorption with either Rgsm or Rg bacteria. Immunoblotting of SDS-soluble proteins with unadsorbed and adsorbed a-Rg and a-Sm sera produced results that agreed with the ELISA results. Identical antigen patterns for the Rg and Rgsm types were obtained with a-Rg sera adsorbed with Sm type bacteria; a-Sm sera adsorbed with either Sm or Rgsm bacteria recognized the identical Sm-specific antigens. Adsorption with Rgsm bacteria removed all antibodies that recognized Rg antigens from both a-Rg and a-Sm sera.

**PMs.** A classification analysis of the P. micros spectra revealed that the 11 Rgsm variants clustered in one group with their Rg parental strains (Fig. 3). There was no evidence of subclusters of the two types within this cluster. All Sm strains grouped in one cluster that was clearly distinct from the Rgsm cluster. The reference strains of two other Peptostreptococcus species, P. magnus ATCC 14956 and P. anaerobius GIFU 7800, both produced markedly different spectra at great distances from the spectra of the two P. micros clusters.

**16S rDNA sequence analysis.** The PCR performed with P. micros chromosomal DNA and primers F1 and R7 resulted in a fragment that was ca. 1,500 bases long, which is the approximate known length of the 16S rDNA. A phylogenetic tree based on the first 330 nucleotides of the 16S rDNA fragments is shown in Fig. 4. A sequence analysis of these 330 nucleic acids at the 5' end of the 16S rDNA PCR fragments for three Sm strains, two Rg strains, and two Rgsm strains revealed that these strains were highly homologous. The Rgsm variants exhibited nearly 100% homology with their Rg parental strains; one pair (HG 1467 and HG 1467sm) was 100% homologous, and the other pair (HG 1772 and HG 1772sm) differed at 3 of the 330 bases determined (level of homology, 99.1%). When two independent Rg strains or their Rgsm variants were compared, they differed at 5 of the 330 bases (level of homology, 98.5%). The sequences of three independent Sm strains differed at 2 to 6 of the 330 bases (levels of homology, 98.2 to 99.4%). When the Rg or Rgsm strains were compared with the Sm strains, differences of 9 to 16 nucleotides at the 330 base positions were observed (levels of homology, 95.2 to 97.2%). Comparable differences were observed with a known sequence of P. micros GIFU 7701 (5). Large differences were found with known 16S rDNA sequences of P. magnus and P. anaerobius (56 to 61 of 330 bases and 81 to 85 of 330 bases, respectively).

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encapsulation. The Sm and Rg isolates of *P. micros* are serologically distinguishable (34); the Sm type has been recognized as serotype a, and the Rg type has been recognized as serotype b. The antigenic composition of the Rg<sup>Sm</sup> variant of *P. micros*, as determined by ELISA and immunoblotting, was identical to that of the Rg type, as the Rg<sup>Sm</sup> variants adsorbed all of the antibodies induced in rabbits by the Rg type. As the variants did not adsorb all Sm-specific antibodies from α-Sm serum, we concluded that the Rg<sup>Sm</sup> variants, like the Rg type, are serologically different from the Sm type. PMS results in mass spectra that are representative of the whole-cell composition. The cell composition of Rg<sup>Sm</sup> variants was very similar to that of the parental Rg strains. The Sm type strains clustered as a clearly distinct and separate group from the Rg and Rg<sup>Sm</sup> strains. However, the Euclidian distance (a measure of dissimilarity in PMS determined by the Normix strategy [12]) between these two clusters was relatively small compared to the distance from *P. micros* to *P. magnus* and *P. anaerobius*.

On the basis of both serotyping results and PMS data the Rg<sup>Sm</sup> variants were indistinguishable from the Rg isolates. This is remarkable as the fibrillar surface structures which are characteristic of the Rg type seem to be absent on the surfaces of
were small when the organisms were compared to reference strains. The levels of reassociation between Rg strain DNA and Sm strain DNA, either from the same pocket or from unrelated pockets, were 50 to 80%; these levels are subspecies homology levels. In conclusion, our results show that P. micros can be subdivided into two groups; one group consists of the Sm strains, and the other group consists of the Rg strains and their RgSm variants. These groups are distinguishable by phylogenetic analysis, PMS, SDS-PAGE, serology, and hemolytic activity data. This implies that the presence of both Sm type and Rg type P. micros in pocket samples from periodontal patients is not due to phase variation. Therefore, it may be necessary to reassess the role of both types of P. micros in periodontal disease, as well as in nonoral anaerobic mixed infections. Although Sm type P. micros and Rg type P. micros can be quite easily differentiated by morphological examination, SDS-PAGE seems to be a good method to verify the differentiation, as the RgSm variant cannot be differentiated from the Sm type by colony morphology alone. Our study revealed other tools that may be useful in future prevalence studies of Sm and Rg type P. micros; adsorbed antisera can be used to divide Sm and Rg or RgSm isolates by serotyping, and type-specific primers for use in PCR typing may be derived from the known 16S rDNA sequences.

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