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\textsuperscript{sm}) in broth culture. Sm and Rg isolates and Rg\textsuperscript{sm} variants were compared to determine whether these three types are the result of phase variation. The Rg\textsuperscript{sm} variants resembled the Sm morphotype in colony morphology; furthermore, the Sm type and the Rg\textsuperscript{sm} type did not have the fibrillar surface structures characteristic of the Rg type, and the Sm and Rg\textsuperscript{sm} 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\textsuperscript{sm} variants and the Rg isolates were very similar and were clearly distinct from the Sm isolates. These results suggest that the Rg and Rg\textsuperscript{sm} 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\textsuperscript{sm} 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\textsuperscript{sm}). 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 bacteria, where phase variation is common (1, 6, 30, 37). In the present study we characterized the Rg\textsuperscript{sm} variant and compared its characteristics with those of Sm and Rg isolates in order to determine if the Sm, Rg, and Rg\textsuperscript{sm} 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, Montalieu-Vercieu, France). Rg\textsuperscript{sm} 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 strain ATCC 33270, Peptostreptococcus magnus ATCC 14956 (kindly provided by S. Persson, Umeå, Sweden), and Peptostreptococcus anaerobius CIFU 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% N\textsubscript{2}-10% CO\textsubscript{2}-10% H\textsubscript{2} 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 (28). All of the strains were suspended and washed twice in phosphate-buffered saline (PBS), and the optical density at 650 nm (OD\textsubscript{650}) was adjusted to 1.0 (~10\textsuperscript{8} bacteria/ml). Triplicate samples were mixed with 7.5% (vol/vol) hexadecane and vortexed at the maximum setting for 4.5 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 contrast staining 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% 2-mercaptoethanol, 20% glycerol, 125 mM Tris-HCl [pH 6.8], 0.1 mg of bromophenol 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
for 2 h, and the proteins were stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, Calif.).

**Rabbit antisera.** Male chinchilla rabbits (weight, 2 to 3 kg) were immunized with P. micros ATCC 33270T (T = type strain) or HG 1468 (Rg morphotype). A suspension of bacteria in PBS (approximately 10^8 bacterial/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 56°C and stored at −80°C until they were used.

For serotype analysis 1-ml serum aliquots were adsorbed with 400 mg (wet weight) of bacteria; the anti-Sm serum (0.5-ml volume) was adsorbed either with Rg strain HG 1468 or with Rg^sm strain HG 1468^sm, and the a-Rg serum was adsorbed with Sm strain ATCC 33270T or with HG 1468^Sm. 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 10,000 × 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 (OD600 nm, 0.1; = 10^9 bacteria) was added to each well of a 96-well flat-bottom plate (catalog no. 65500; Greiner BV, Alphen aan den Rijn, the Netherlands). The plates were incubated at 4°C for 16 h, washed with PBS supplemented with 0.05% Tween 80 (PBST), and then incubated for 1 h at room temperature with PBST supplemented with 0.05% Tween 80 bacterial serum albumin. After washing with PBST, the unadsorbed sera, adsorbed sera, and preimmune serum (used as a negative control) were added to the plates, and serial twofold dilutions in PBST were prepared. After incubation at 4°C for 1 h, the plates were washed three times with PBST, and horseradish peroxidase-conjugated goat anti-rabbit monoclonal antibody (anti-HyG conjugate (1:1,000 in PBST; Nordic, Tilburg, The Netherlands) was added. After 1 h of incubation with the conjugate and three washes with PBST, antibody binding was detected with H2O2 and o-phenylenediamine (Sigma BV, Zwijndrecht, The Netherlands) and the amount of binding was determined with a plate reader (Dynatech Laboratories, Béblinghoven, United Kingdom) as the A405 nm.

**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 GaR-horseradish peroxidase (1:1,500 in TTBS; Nordic), and then washed four times in TTBS. Afterward, antibody binding antigens were visualized by using H2O2 and 4-chloro-1-naphthol (Bio-Rad).

**Nucleotide sequence accession numbers.** The 16S DNA sequences of P. micros ATCC 33270 (Sm type) and P. micros HG 1467 (Rg type) have been deposited in the GenBank database under accession numbers U60326 and U60327, respectively.

### RESULTS

**Characteristics of the P. micros Rg^sm type.** All 11 P. micros Rg morphotype strains changed easily to Rg^sm variants. In blood agar subcultures of broth cultures after the fourth passage, the Rg^sm 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^-4. The Rg^sm variants obtained by subculturing broth cultures were compared to the Rg and Sm periodontal isolates (Table 1). The Rg^sm variants were morphologically indistinguishable from the Sm isolates; both types formed white, dome-shaped, relatively mucous colonies. Electron microscopy revealed that the Rg^sm variants, like the Sm type, did not express the large fibrillar surface structures that are characteristic of the Rg type. The Rg^sm strains were significantly more hydrophobic than the Rg parental strains (58% ± 2%; P < 0.01). The hydrophobicity of the Sm isolates (68% ± 1%) was not significantly different from that of the Rg^sm variants. The Rg^sm 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 Rg^sm strains was most pronounced with human erythrocytes (data not shown). As shown in Fig. 1, the SDS-PAGE whole-cell lysate patterns of the Rg^sm 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 Rg^sm variants. The patterns of independent Rg (and Rg^sm) strains were relatively homogeneous and clearly distinguishable from the homogenous 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).

### TABLE 1. Characteristics of the Sm type, the Rg type, and the Rg^sm variant of P. micros

<table>
<thead>
<tr>
<th>P. micros</th>
<th>No. of strains</th>
<th>Colony morphology</th>
<th>Fibrillar structures</th>
<th>Hydrophobicity (% of optimal)</th>
<th>Hemolysis</th>
<th>Protein pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sm</td>
<td>13</td>
<td>Smooth</td>
<td>–</td>
<td>68 ± 11</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Rg</td>
<td>11</td>
<td>Rough</td>
<td>+</td>
<td>25 ± 7</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Rg^sm</td>
<td>11</td>
<td>Smooth</td>
<td>+</td>
<td>58 ± 9</td>
<td>B</td>
<td></td>
</tr>
</tbody>
</table>

* Hydrophobicity is expressed as the percent decrease in optical density compared with the optical density of the initial suspension.

* Hemolysis was determined on blood agar plates after 7 days of anaerobic growth.

**Protein patterns were determined by SDS-PAGE.**

* Significantly less hydrophobic than the Sm and Rg^sm types, as determined by the Student t test (P < 0.05).
Serotype analysis. The results of the ELISA serotype analysis are shown in Fig. 2. Unadsorbed α-Rg serum exhibited strong cross-reactions with both the Sm type and the Rg\textsuperscript{sm} type; the titers were all ca. 2\textsuperscript{2} (Fig. 2A). Adsorption of this serum with Rg\textsuperscript{sm} variants reduced all reactions to preimmune serum levels (titers, ca. 2\textsuperscript{2}). Adsorption with Sm type bacteria removed only the cross-reactive antibodies against the Sm type. When the α-Sm serum was used (Fig. 2B), only Sm-reactive antibodies could be detected after adsorption with either Rg\textsuperscript{sm} or Rg bacteria. Immunoblotting of SDS-soluble proteins with unadsorbed and adsorbed α-Rg serum and α-Sm serum produced results that agreed with the ELISA results. Identical antigen patterns for the Rg and Rg\textsuperscript{sm} types were obtained with α-Rg serum adsorbed with Sm type bacteria; α-Sm serum adsorbed with either Rg or Rg\textsuperscript{sm} bacteria recognized the identical Sm-specific antigens. Adsorption with Rg\textsuperscript{sm} bacteria removed all antibodies that recognized Rg antigens from both α-Rg serum and α-Sm serum.

PMS. A classification analysis of the P. micros spectra revealed that the 11 Rg\textsuperscript{sm} 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 Rg-Rg\textsuperscript{sm} 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 Rg\textsuperscript{sm} strains revealed that these strains were highly homologous. The Rg\textsuperscript{sm} variants exhibited nearly 100% homology with their Rg parental strains; one pair (HG 1467 and HG 1467\textsuperscript{sm}) was 100% homologous, and the other pair (HG 1772 and HG 1772\textsuperscript{sm}) differed at 3 of the 330 bases (level of homology, 99.4%). When two independent Rg strains or their Rg\textsuperscript{sm} 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 Rg\textsuperscript{sm} 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).

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 Rg\textsuperscript{sm}-type colonies, we determined whether the existence of Rg and Sm morpho-
The Rg\textsuperscript{Sm} type was morphologically indistinguishable from the Sm morphotype; both types lacked the fibrillar structures which are characteristic of the Rg type, and both types were relatively hydrophobic compared to the Rg type. However, the Rg\textsuperscript{Sm} variants had hemolytic activity and SDS-PAGE whole-cell lysate patterns similar to those of the Rg type strains. The SDS-PAGE protein patterns of the Rg and Rg\textsuperscript{Sm} type strains differed only in the levels of expression of some proteins. There was no indication that there are specific Rg proteins that could be part of the fibrillar structure.

The Sm and Rg isolates of \textit{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\textsuperscript{Sm} variant of \textit{P. micros}, as determined by ELISA and immunoblotting, was identical to that of the Rg type, as the Rg\textsuperscript{Sm} variants adsorbed all of the antibodies induced in rabbits by the Rg type. As the variants did not adsorb all Sm-specific antibodies from \(\alpha\)-Sm serum, we concluded that the Rg\textsuperscript{Sm} 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\textsuperscript{Sm} 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\textsuperscript{Sm} 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 \textit{P. micros} to \textit{P. magnus} and \textit{P. anaerobius}.

On the basis of both serotyping results and PMS data the Rg\textsuperscript{Sm} 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...
the Rg<sup>Sm</sup> variants, as determined by electron microscopy. Previously, both unadsorbed α-Rg serum and α-Rg serum adsorbed with Sm type bacteria recognized epitopes on the fibrillar surface structures of Rg bacteria, as determined by immunogold labelling (33). This indicates that the fibrillar structures have antigenic potential. Our serotyping and PMS data imply that the (antigenic) constituents of the fibrillar surface structures are also present on the Rg<sup>Sm</sup> variants, although these structures have not been observed by electron microscopy.

The phylogenetic relationship among the three types (Sm, Rg, and Rg<sup>Sm</sup>) was determined by analyzing 16S rDNA sequences. 16S rDNA is commonly used for phylogenetic analysis and for judging interspecies similarity, because it is present in all bacteria, it is highly conserved, and it is large enough for making inferences (10, 29, 39, 40). A homology analysis of known 16S rDNA sequences (5, 17) revealed that the first 330 nucleotides at the 5′ end of our PCR 16S rDNA fragments was highly discriminative for various Peptostreptococcus species, since variable regions were included. Our sequence data indicate that the Rg, Sm, and Rg<sup>Sm</sup> strains are closely related, although the possibility that more significant differences are present in the other variable parts of the 16S rDNA cannot be eliminated. Phylogenetic analysis with the Phylip program revealed clustering of the Rg and Rg<sup>Sm</sup> strains in one group and the Sm strains in another group; both of these groups were different from other Peptostreptococcus species (Fig. 4).

A classification analysis based on both 16S rDNA sequence data and PMS data showed that the Sm isolate cluster and the Rg-Rg<sup>Sm</sup> cluster are clearly distinct and separate groups. However, in both experiments the differences between the groups were small when the organisms were compared to reference strains of P. magnus and P. anaerobius, which are a relatively closely related species and a more distinct species within the genus Peptostreptococcus, respectively (17, 25). The differences between the Sm and Rg-Rg<sup>Sm</sup> groups are just within the accepted taxonomic limits for a species. The PMS and 16S rDNA sequence results are consistent with DNA reassociation results obtained previously in our laboratory (32). The levels of DNA reassociation between unrelated Rg strains or between unrel-


