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 (RgSm) in broth culture. Sm and Rg isolates and RgSm variants were compared to determine whether these three types are the result of phase variation. The RgSm variants resembled the Sm morphotype in colony morphology; furthermore, the Sm type and the RgSm type did not have the fibrillar surface structures characteristic of the Rg type, and the Sm and RgSm 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 RgSm variants and the Rg isolates were very similar and were clearly distinct from the Sm isolates. These results suggest that the Rg and RgSm 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 RgSm 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 (RgSm). 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 RgSm variant and compared its

characteristics with those of Sm and Rg isolates in order to determine if the Sm, Rg, and RgSm 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). RgSm 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 (28). All of the strains were suspended and washed twice in phosphate-buffered saline (PBS), and the optical density at 650 nm (OD₆₅₀) was adjusted to 1.0 (≈10⁹ 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% 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

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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 33270^T (T = type strain) or HG 1468 (Rg morphotype). A suspension of bacteria in PBS (approximately 10¹⁰ 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 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 (α -Sm serum) was adsorbed either with Rg strain HG 1468 or with RgSm strain HG 1468Sm, and the α -Rg serum was adsorbed with Sm strain ATCC 33270^T 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 14,000 $\times$ 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 μ l of a bacterial suspension in PBS (OD₆₅₀, 0.1; $\approx$ 10⁷ bacteria) was added to each well of a 96-well flat-bottom plate (catalog no. 655001; Greiner BV, Alphen a/d 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 0.5% bovine 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 16 h, the plates were washed three times with PBST, and horseradish peroxidase-labelled goat anti-rabbit immunoglobulin G 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, Billingham, United Kingdom) as the A₄₉₂.

Immunoblotting. Whole-cell proteins were separated on a 10% polyacrylamide SDS-PAGE gel and transferred to a nitrocellulose membrane (pore size, 0.45 μ m; 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 GαR-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).

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 desiccator 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^T, HG 1256, and HG 1771), two Rg strains (HG 1467 and HG 1772), and two RgSm strains (HG 1467Sm 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 lysozyme (10 mg/ml) and proteinase K (1 mg/ml) and for 1 h at 56°C with SDS (4%). After two phenol-chloroform-isoamyl alcohol extractions and two chloroform-isoamyl alcohol extractions, sodium acetate (0.3 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 μ g/ml).

Cloning of 16S rDNA fragments. 16S rRNA gene (rDNA) 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 CC[A/G]-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 30 s, followed by a final extension step for 10 min at 55°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* DH5 α F' as recommended by the manufacturer. Plasmids were isolated from the white colonies by

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

<i>P. micros</i> type	No. of strains	Colony morphology	Fibrillar structures	Hydrophobicity (%) ^a	Hemolysis ^b	Protein pattern ^c
Sm	13	Smooth	—	68 $\pm$ 11	—	A
Rg	11	Rough	+	25 $\pm$ 7 ^d	+	B
Rg Sm	11	Smooth	—	58 $\pm$ 9	+	B

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

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

^c Protein patterns were determined by SDS-PAGE.

^d Significantly less hydrophobic than the Sm and RgSm types, as determined by the Student *t* test (*P* < 0.05).

the miniprep method (2) and were digested with *Nco*I and *Pst*I (New England Biolabs) to check the size of the insert.

Sequencing. The cloned 16S rDNA fragments were sequenced with forward primer M13. The sequence reactions were performed with a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.) as recommended by the manufacturer. After a sequence gel was examined with an Applied Biosystems model 373A DNA sequencing system (Applied Biosystems, Inc.), the sequence data were analyzed and compared with known sequences of *P. micros* GIFU 7701, *P. magnus* ATCC 15794^T, and *P. anaerobius* ATCC 27337^T from the National Center for Biotechnology Information GenBank. Phylogenetic analyses were performed with the PHYLIP package, version 3.57c (J. Felsenstein, University of Washington, Seattle).

Nucleotide sequence accession numbers. The 16S rDNA 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* 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 10⁻³. 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 strains were significantly more hydrophobic than the Rg parental strains (58% $\pm$ 9% versus 25% $\pm$ 7%; *P* < 0.01). The hydrophobicity of the Sm isolates (68% $\pm$ 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 homogeneous 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).

FIG. 1. Whole-cell protein patterns of *P. micros* strains determined by SDS-PAGE. Lane 1, HG 1256 (Sm type); lane 2, HG 1467Sm (RgSm variant); lane 3, HG 1467 (Rg type); lane 4, HG 1771 (Sm type); lane 5, HG 1772Sm (RgSm variant); lane 6, HG 1772 (Rg type); lane 7, *P. magnus* ATCC 14956; lane 8, *P. anaerobius* GIFU 7800. kD, kilodalton.

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 RgSm type; the titers were all ca. 2^{21} (Fig. 2A). Adsorption of this serum with RgSm variants reduced all reactions to preimmune serum levels (titers, ca. 2^9). 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 RgSm 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 RgSm types were obtained with α -Rg serum adsorbed with Sm type bacteria; α -Sm serum adsorbed with either Rg or RgSm bacteria recognized the identical Sm-specific antigens. Adsorption with RgSm 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 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 Rg-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).

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-

FIG. 2. Serotype analysis of *P. micros* by ELISA. The bars represent the antibody titers of α -Rg serum (A) and α -Sm serum (B) against Sm, Rg, and RgSm *P. micros* strains. Titers are expressed as the log₂ serum dilution giving values that are two times the background A_{450} . (A) PI, preimmune serum; α -Rg unads, unadsorbed α -Rg (HG 1468) serum; α -Rg Sm-ads, α -Rg serum adsorbed with Sm type bacteria; α -Rg RgSm-ads, α -Rg serum adsorbed with RgSm type bacteria. (B) PI, preimmune serum; α -Sm unads, unadsorbed α -Sm (ATCC 33270^T) serum; α -Sm Rg-ads, α -Sm serum adsorbed with Rg type bacteria; α -Sm RgSm-ads, α -Sm serum adsorbed with RgSm type bacteria.

FIG. 3. Dendrogram representing *P. micros* classification based on PMS data. *P. magnus* ATCC 14956 and *P. anaerobius* GIFU 7800 were included for comparison. The dissimilarity measure used is the squared Euclidian distance in the interstrain discriminant space. The clustering method used was the unweighted pair group with mathematical average method. dof, degrees of freedom.

types is due to phase variation by comparing in vitro-derived Rg^{Sm} variants with periodontal Sm and Rg type isolates.

The Rg^{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^{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^{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 *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^{Sm} variant of *P. micros*, as determined by ELISA and immunoblotting, was identical to that of

the Rg type, as the Rg^{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 α -Sm serum, we concluded that the Rg^{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^{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^{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 *P. micros* to *P. magnus* and *P. anaerobius*.

On the basis of both serotyping results and PMS data the Rg^{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

FIG. 4. Phylogenetic tree for *P. micros* based on 16S rDNA sequences. The sequence data for three Sm strains (ATCC 33270^T, HG 1256, and HG 1771), two Rg strains (HG 1467 and HG 1772), and two RgSm variants (HG 1467Sm and HG 1772Sm) were analyzed and compared with known sequences of *P. micros* GIFU 7701, *P. magnus* ATCC 15794^T, and *P. anaerobius* ATCC 27337^T. The phylogenetic tree was inferred from sequence alignments determined with the Clustal version V program.

the RgSm 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 RgSm variants, although these structures have not been observed by electron microscopy.

The phylogenetic relationship among the three types (Sm, Rg, and RgSm) 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 RgSm 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 RgSm 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-RgSm 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-RgSm 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 unre-

lated Sm strains were ca. 100%. 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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