

Peptostreptococcus micros Smooth and Rough Genotypes in Periodontitis and Gingivitis

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Background: Two genotypes can be distinguished within the species *Peptostreptococcus micros*: a smooth (Sm) and a rough (Rg) type. To date no systematic study has been performed on the prevalence and proportion of both types in untreated periodontitis patients and subjects without destructive periodontal disease. Therefore, the present study was performed to investigate: 1) the relative importance of the Sm and the Rg genotype of *P. micros* in periodontitis and gingivitis; 2) the correlation between smoking and the 2 genotypes of *P. micros*; and 3) the systemic antibody response against the 2 genotypes in relation to the periodontal condition and smoking.

Methods: A total of 104 untreated periodontitis patients and 41 individuals with gingivitis underwent clinical examination and microbiological sampling. Pocket samples were cultured anaerobically on blood agar plates to determine the prevalence and proportion of the Sm and Rg types of *P. micros*. Serum antibody titers against both types of *P. micros* were determined in all subjects by enzyme-linked immunosorbent assay (ELISA) using whole bacterial cells as antigen. Additionally, in a representative group of subjects, the antigen specificity of the serum antibodies was assessed by immunoblotting experiments.

Results: The prevalence of the Sm genotype was higher in subjects with periodontitis (94%) compared to subjects with gingivitis (59%), whereas the prevalence of the Rg type was not significantly different (38% versus 29%). Similar analyses were performed for subgroups of smokers and non-smokers; within the periodontitis group, the prevalence of the Sm type was not different between smokers and non-smokers (96% and 92%, respectively), whereas the prevalence of the Rg type was higher in smokers (48%) compared to non-smokers (19%). No difference in prevalence of both types was observed between smokers and non-smokers within the gingivitis group. The titers and specificity of *P. micros*-specific immunoglobulins in periodontitis patients were not different from those in gingivitis subjects, nor were they related to smoking status or culture-positivity.

Conclusions: The results of this study suggest that both the Sm and the Rg genotypes of *P. micros* are part of the normal oral microbiota. However, the elevated prevalence of the Sm genotype in periodontitis and the elevated prevalence of the Rg type in periodontitis patients who smoke implies that both types can behave as opportunistic pathogens in destructive periodontal disease. *J Periodontol* 2000;71:209-218.

KEY WORDS

Peptostreptococcus micros/genetics; periodontitis/microbiology; gingivitis/microbiology; smoking/adverse effects.

The Gram-positive anaerobic coccus *Peptostreptococcus micros* is considered a periodontal pathogen by currently accepted criteria for implicating bacteria involved in the etiology of periodontitis.^{1,2} Several studies have associated this species with active progressive periodontal disease.³⁻⁷ For example, Rams et al.⁷ reported a higher prevalence of *P. micros* in disease-active than in disease-inactive periodontitis patients (47% versus 14%). Also, elimination of *P. micros* has been associated with clinical improvement.⁸ Furthermore, increased serum antibody levels against this species have been reported in young adult patients (<35 years) with generalized severe periodontitis as compared to subjects without destructive periodontal disease.^{9,10} However, in subjects with severe adult periodontitis older than 35 years, the serum immunoglobulin G (IgG) levels to *P. micros* appeared comparable to healthy subjects.^{9,10} Several potential virulence factors of *P. micros* have been reported, including adherence to gingival epithelial cells,^{11,12} expression of IgG Fc-binding

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proteins.¹³ production of hydrogen sulfide from glutathione,¹⁴ production of hyaluronidase,¹⁵ and the expression of fibril-like structures.¹⁶

P. micros can be differentiated into 2 types:¹⁶ a smooth (Sm) and a rough (Rg). These types are morphologically and serologically distinguishable¹⁷ and should be considered as distinct genotypes, as determined by 16S RNA analysis and pyrolysis mass spectrometry.¹⁸ Both the Sm type and the Rg type can be isolated from subgingival plaque samples from subjects with periodontitis.¹⁹ However, to date no systematic study has been performed on the prevalence and proportion of both types in untreated periodontitis patients and subjects without destructive periodontal disease.

Smoking is currently considered a risk factor for destructive periodontal disease.²⁰ From a growing number of studies, the correlation between smoking and periodontal breakdown is evident.²¹⁻²⁶ Preliminary investigations have shown that smoking may affect the composition of the subgingival microbiota.^{22,27,28} Interestingly, these studies showed higher proportions of *P. micros* in periodontitis subjects who smoke compared to non-smoking periodontitis subjects. Therefore, it is important to analyze the microbiological data regarding the 2 genotypes of *P. micros* in relation to smoking status. To better understand the role of the Sm and Rg types of *P. micros* in periodontitis, we also included analysis of the systemic antibody levels to both genotypes in the present study.

The purposes of the present study were to investigate: 1) the relative importance of the Sm genotype and the Rg genotype of *P. micros* in gingivitis and periodontitis; 2) the relationship between smoking and the 2 genotypes of *P. micros*; and 3) the systemic antibody response against the 2 genotypes in relation to the periodontal condition and smoking.

MATERIALS AND METHODS

Study Population

A total of 145 adult subjects, who were systemically healthy and not pregnant, participated in the study. They were comprised of a consecutive series of 104 untreated periodontitis patients referred to the Department of Periodontology at the Academic Centre for Dentistry Amsterdam (ACTA) and had accepted a proposed treatment plan, and 41 subjects with gingivitis, who were registered at ACTA for restorative dentistry procedures or who visited ACTA for regular check-ups. For the gingivitis group, only subjects with a radiographic distance between the cemento-enamel junction and the alveolar bonecrest ≤ 2 mm on recent (<1 year old) bite-wing radiographs, and not missing more than 1 tooth per quadrant were recruited. All participants were informed verbally and in writing about the purposes of the study, and all signed an informed con-

sent form. An extensive medical history was taken from each subject by interview and written questionnaire. Additionally the history and amount of smoking was recorded and converted to cigarettes per day and pack-years (number of packs of cigarettes smoked per day \times years smoking). Smokers were defined as current smokers and subjects who stopped smoking less than 10 years ago (16 periodontitis patients and 4 subjects with gingivitis stopped smoking between 6 months and 10 years earlier). This approach was chosen since the effects of smoking on the periodontium can continue after cessation for at least 10 years.^{29,30} Subjects who stopped smoking more than 10 years before the study were included in the non-smokers group.

Clinical Procedures

From each subject, 10 ml blood was collected from the antecubital fossa into a vacuum tube containing K₂EDTA.[‡] The tube was immediately stored on ice and after 1 hour plasma was collected by centrifugation of the tube at 3,000 rpm for 10 minutes. The plasma was stored in 5 aliquots of 0.5 ml each at -80°C .

In periodontitis patients, the deepest site per quadrant was selected for microbiological sampling; the selection of sites was based on probing measurements at the first referral visit to the ACTA. In the subjects with gingivitis, the mesio- and distobuccal sites of all first molars were selected for microbiological sampling. The sample sites were isolated with cotton rolls and supragingival plaque was removed with curets and cotton pellets. Subsequently, 2 paper-points were inserted and left in place for 10 seconds. All paper-points were transferred to the same vial containing 2 ml of reduced transport medium.³¹ The pooled sample was transported to the laboratory and processed within 4 hours.

After microbiological sampling, the following clinical parameters were evaluated: 1) probing depth; 2) attachment loss (in patients only); and 3) bleeding on probing. Bleeding upon probing was recorded according to the sulcus bleeding index (scored as 0 = no bleeding; 1 = point bleeding within 30 seconds; 2 = immediate overt bleeding).³² For these data, a mean of the sampled sites was determined per subject.

Microbiological Analysis

Subgingival plaque samples were vortexed for 30 seconds and 10-fold serial dilutions were prepared in phosphate-buffered saline. One hundred μl of the 10^{-3} , 10^{-4} , and 10^{-5} dilutions were plated onto blood agar^{§§} plates supplemented with 5% (vol/vol) defibrinated horse blood, 5 mg/ml hemin, and 1 mg/ml menadione. The plates were incubated for 14 days in 80% N₂-10% CO₂-

‡ VenoJect II, Terumo Europe BV, Leuven, Belgium.

§§ Oxoid No. 2, Oxoid BV, Haarlem, The Netherlands.

10% H₂ at 37°C and examined on days 7 and 14. The prevalence of Rg and Sm morphotypes of *P. micros* was determined by stereomicroscopy as described previously⁷ (magnification 20 to 40×). Additionally, *P. micros* morphotypes were identified by Gram-staining and biochemical reactions using a commercially-available kit[§] for 2 isolates per genotype per subject. These tests results always confirmed the stereomicroscopic identifications.

Determination of Systemic Antibody Titers

To investigate the systemic antibody response to the Sm type and the Rg type of *P. micros*, patients in the periodontitis group and subjects in the gingivitis group were selected by an enzyme-linked immunosorbent assay (ELISA). The selection was based on the proportions of *P. micros*; representative numbers of subjects harboring total proportions of *P. micros* of 0% (n = 5 for periodontitis and n = 11 for gingivitis), 0 to 5% (n = 13 and n = 11, respectively), and >5% (n = 16 and n = 7, respectively) were selected. In the periodontitis subgroup (n = 32), 29 patients harbored the Sm type and 19 patients harbored the Rg type, whereas in the gingivitis subgroup (n = 29), 15 and 7 subjects were culture-positive for the Sm type and the Rg type, respectively. The selection resulted in test groups that were fully representative of the main population both in clinical and in microbiological parameters (data not shown). ELISAs were performed as described previously.³³ *P. micros* strains HG1253 (Sm type) and HG 1259 (Rg type)¹⁶ were cultured on blood agar plates for 4 days. After harvesting of the selected strains and washing once in PBS, 100 ml of bacterial suspension in PBS with an OD₆₅₀=0.1 (10⁷ bacteria) was added to each well of a 96-well flat-bottom plate^{||}. Plates were incubated at 4°C for 16 hours, washed with PBS supplemented with 0.05% Tween-20 (PBST), followed by a 1 hour incubation at room temperature in PBST with 0.5% bovine serum albumin (BSA). After 3 washes with PBST, the sera were added in starting dilutions of 1:100 for immunoglobulin G (IgG) measurements, and 1:20 for immunoglobulin A (IgA) and immunoglobulin M (IgM) measurements. Subsequently, 6 two-fold serial dilutions were made in PBST. After incubation at 4°C for 16 hours the plates were washed 3 times with PBST and horseradish peroxidase labeled goat-anti-human IgG, IgA, or IgM conjugates[¶] (1:1,000 in PBST) were added. After a 1 hour incubation with conjugate and 3 washes with PBST, antibody-binding was visualized with the use of o-phenylenediamine[#] as substrate and H₂O₂, and the absorbance at 492 nm was determined with a microtiter plate reader. Antibody titers were defined as the calculated reciprocal dilution of the plasma that resulted in an absorbance value that was twice the background A₄₉₂.

Immunoblotting

To investigate the serological reactivity to specific antigens of the Sm type and the Rg type of *P. micros* in periodontitis and gingivitis, whole-cell preparations of *P. micros* strain HG1253 (Sm type) or HG1259 (Rg type) were incubated with plasma from the subset subjects of both study groups in immunoblotting experiments. After 4 days of growth, bacterial cells were harvested from blood agar, washed in PBS, resuspended in 0.5 M Tris-HCl (pH 6.8), and diluted 1:1 (vol:vol) in sample buffer containing sodium dodecyl sulphate (SDS) (4% SDS, 2% 2-mercaptoethanol, 20% glycerol, 125 mM Tris-HCl [pH 6.8], 0.1 mg/ml bromophenol blue). The samples were heated for 10 minutes at 100°C and insoluble debris was removed by centrifugation at 14,000 × g for 10 minutes. Electrophoresis was performed on a 1.5 mm thick, 10% homogeneous polyacrylamide gel³⁴ at 100 V for 2 hours, and part of the gel was stained with Coomassie brilliant blue^{**} to confirm the separation of the proteins. The separated proteins from the other part of the gel were transferred onto a nitrocellulose membrane^{††} (pore size 0.45 mm) by Western blotting³⁵ at 100 V for 1 hour. The nitrocellulose sheets were subsequently incubated with washing buffer (TTBS: 100 mM Tris-HCl [pH 7.5], 0.9% NaCl, and 0.1% [vol/vol] Tween-20) containing 0.5% BSA as blocking reagent, at room temperature for 1 hour. After 2 washes in TTBS, the blots were cut into small strips that were incubated overnight with plasma from selected subjects, diluted in TTBS. The blots were washed 4 times with TTBS, incubated with goat-anti-human IgG horseradish peroxidase-labeled conjugates[¶] (1:1,000 in TTBS) for 1 hour, and washed another 4 times in TTBS. Afterwards, antibody-binding antigens were visualized using H₂O₂ and 4-chloro-1-naphthol^{**} as substrate.

Statistical Analysis

Statistical analyses were performed with the SPSS 7.5 package for Windows. Chi-square (χ²) tests were used to assess differences in prevalence of *P. micros* types between the subject groups. The Mann-Whitney U test was used for comparing clinical data, proportions of the two genotypes, and antibody titers, in various subject groups. Correlations were analyzed with the non-parametric Spearman correlation test.

RESULTS

The periodontal patients and gingivitis subjects did not differ in age (42.5 years and 39.9 years, respectively; Table 1). The percentage of subjects smoking in the periodontitis group was significantly higher than in the

§ Rapid ID 32A, Analytab Products, Montalieu-Vercieu, France.

|| Greiner BV, Alphen a/d Rijn, The Netherlands.

¶ American Qualex, San Clemente, CA.

Sigma BV, Zwijndrecht, The Netherlands.

** R-250, BioRad Laboratories, Hercules, CA.

†† Schleicher & Schuell, Dassel, Germany.

Table 1.
Characteristics (mean ± SD) of the Study Population and Clinical Description of Sampled Sites

	Periodontitis (n = 104)		Gingivitis (n = 41)
Mean age (years)	42.5 ± 9.9		39.9 ± 12.3
N smokers	67 (64%)	*	17 (41%)
N teeth	26.4 ± 3.3		27.7 ± 1.8
Probing depth (mm)	6.3 ± 1.3	†	3.0 ± 0.4
Attachment loss (mm)	6.9 ± 1.5		—
Bleeding-on-probing score	1.1 ± 0.4	†	0.6 ± 0.3

* $P < 0.05$.
 † $P < 0.001$.

gingivitis group ($P < 0.05$; Table 1). Both groups had equal numbers of teeth, but the mean value of the probing depth of 6.3 mm and the mean bleeding on probing score of 1.1 in the periodontitis group were significantly higher than the mean value of the probing depth of 3.0 mm and the mean bleeding on probing score of 0.6 in the gingivitis group ($P < 0.001$; Table 1).

Prevalence and Proportions of *P. micros* Types in Relation to Periodontal Disease

The Sm type of *P. micros* was isolated from a significantly larger percentage of subjects in the periodontitis group; i.e. 94% compared to the 59% of subjects in the gingivitis group ($P < 0.05$; Table 2A). No statistically significant difference in prevalence of the Rg type was found between the periodontitis group and the gingivitis group (38% versus 29%; Table 2A). The prevalence of *P. micros* Sm and Rg types combined was 95% in the periodontitis group and 66% in the gingivitis group (Table 2A).

The proportions of the Sm type and the Rg type

Table 2A.
Prevalence and Total (number and % of culture-positive subjects) of *P. micros* Sm and Rg Genotypes in Study Population

<i>P. micros</i>	Periodontitis (n = 104)		Gingivitis (n = 41)
Sm	98 (94%)	*	24 (59%)
Rg	39 (38%)		12 (29%)
Total	99 (95%)	*	27 (66%)

* $P < 0.05$.

Table 2B.
Proportions and Total of Cultivable Microbiota (mean % ± SD) of *P. micros* Sm and Rg Genotypes in Culture-Positive Study Population

<i>P. micros</i>	Periodontitis	Gingivitis
Sm type	3.7 ± 3.8	5.4 ± 4.4
Rg type	2.7 ± 5.6	6.6 ± 11.8
Total	4.8 ± 5.5	7.8 ± 9.3

of *P. micros* in culture-positive samples were not significantly different in periodontitis and gingivitis; the Sm type accounted for 3.7 ± 3.8% and 5.4 ± 4.4% of the total cultivable microbiota in periodontitis and gingivitis respectively, whereas the proportions of the Rg type were 2.7 ± 5.6% in periodontitis and 6.6 ± 11.8% in gingivitis (Table 2B). The proportion that total *P. micros* isolates accounted for was 4.8 ± 5.5% in periodontitis and 7.8 ± 9.3% in gingivitis (Table 2B).

In the present study, none of the 98 smooth colony isolates from periodontitis patients (one isolate per culture-positive patient) was identified as the RgSm variant, as determined by ELISA using type-specific rabbit antisera as described by van Dalen et al;¹⁷ serologically, all of them were Sm type *P. micros*.

***P. micros* Genotypes in Relation of Smoking**

To investigate the prevalence of the *P. micros* genotypes in relation to smoking status, the 2 subject groups were separated into smokers and non-smokers. Within both the periodontitis group and the gingivitis group, no significant differences were observed between the prevalence of the Sm type of *P. micros* in smokers and non-smokers (Table 3A). In contrast, the

Table 3A.
Prevalence and Total (number and % of culture-positive subjects) of *P. micros* Sm and Rg Genotypes in Smokers and Non-Smokers

<i>P. micros</i>	Periodontitis		Gingivitis	
	Smoker (n = 67)	Non-Smoker (n = 37)	Smoker (n = 17)	Non-Smoker (n = 24)
Sm	64 (96%)	34 (92%)	10 (59%)	14 (58%)
Rg	32 (48%)*	7 (19%)	4 (24%)	8 (33%)
Total	65 (97%)	34 (92%)	11 (65%)	16 (67%)

* Significantly different between smoker and non-smoker periodontitis patients, $P < 0.01$.

Table 3B.
Proportions of Cultivable Microbiota (mean % ± SD) of Sm and Rg and Total *P. micros* in Culture-Positive Smokers and Non-Smokers

<i>P. micros</i>	Periodontitis		Gingivitis	
	Smoker	Non-Smoker	Smoker	Non-Smoker
Sm	4.2 ± 4.2	3.0 ± 3.0	5.6 ± 4.4	5.3 ± 4.6
Rg	2.9 ± 6.2	1.7 ± 2.1	6.9 ± 8.8	6.5 ± 13.6
Total <i>P.</i>	5.5 ± 6.3*	3.3 ± 3.2	7.6 ± 9.2	7.9 ± 9.6

* Significantly different between smoker and non-smoker periodontitis patients, $P < 0.05$.

Rg type was cultured from a significantly larger percentage of smokers (48%) than non-smokers (19%) within the periodontitis group ($P < 0.01$; Table 3A). In the gingivitis group the prevalence of the Rg type was similar in smokers and non-smokers. Both the Sm type and the Rg type accounted for similar proportions of the cultivable microbiota in smokers and non-smokers within both subject groups (Table 3B). The total proportion of *P. micros* in the smokers with periodontitis was significantly higher than in non-smokers with periodontitis ($P = 0.026$).

The proportions of the *P. micros* types were examined for correlation with parameters representing the amount of smoking. Within both subject groups the proportions of *P. micros* Sm type and Rg type were not correlated to the number of cigarettes per day (r values ranging from 0.12 to 0.29) nor to the pack-years (r values ranging from 0.00 to 0.29), although trends

were observed in these correlations within the periodontitis group (P values ranging from 0.07 to 0.12). The proportion of total *P. micros* within the periodontitis group was significantly correlated to both the number of cigarettes per day ($r = 0.22$, $P = 0.03$) and pack-years ($r = 0.22$, $P = 0.03$).

Systemic Antibodies Against *P. micros* Genotypes

To investigate the systemic immune response evoked against the Sm and Rg type of *P. micros*, IgG, IgA, and IgM titers were determined in 34 periodontitis patients and 29 gingivitis subjects. No significant differences were observed in the titers of IgG, IgA, and IgM antibodies binding to the Sm type or the Rg type between the periodontitis group and the gingivitis group for all subjects. When culture-positivity was regarded as a grouping characteristic, the following observations were made. Within both subgroups, the antibody titers against the Sm type were comparable (Fig. 1A). The values of the antibody titers against the Rg type were also comparable, except for the IgA titers within the gingivitis subgroup (Fig. 1B); the IgA titers in the gingivitis group were significantly higher in the Rg type culture-positive subjects as compared to the culture-negative subjects ($P < 0.05$). To further investigate the systemic antibody titers in relation to colonization with the Sm and Rg types of *P. micros*, correlations of the individual titers with corresponding proportions of the cultivable microbiota were performed. In general, the antibody titers were not significantly correlated to the proportions of the Rg and Sm types (r values for the Sm type ranging from -0.07 to 0.24 ; r values for the Rg type ranging from -0.03 to 0.13). However, the IgA titers against the Rg type in gingivitis were significantly correlated to the proportions of the Rg type ($r = 0.41$; $P < 0.05$).

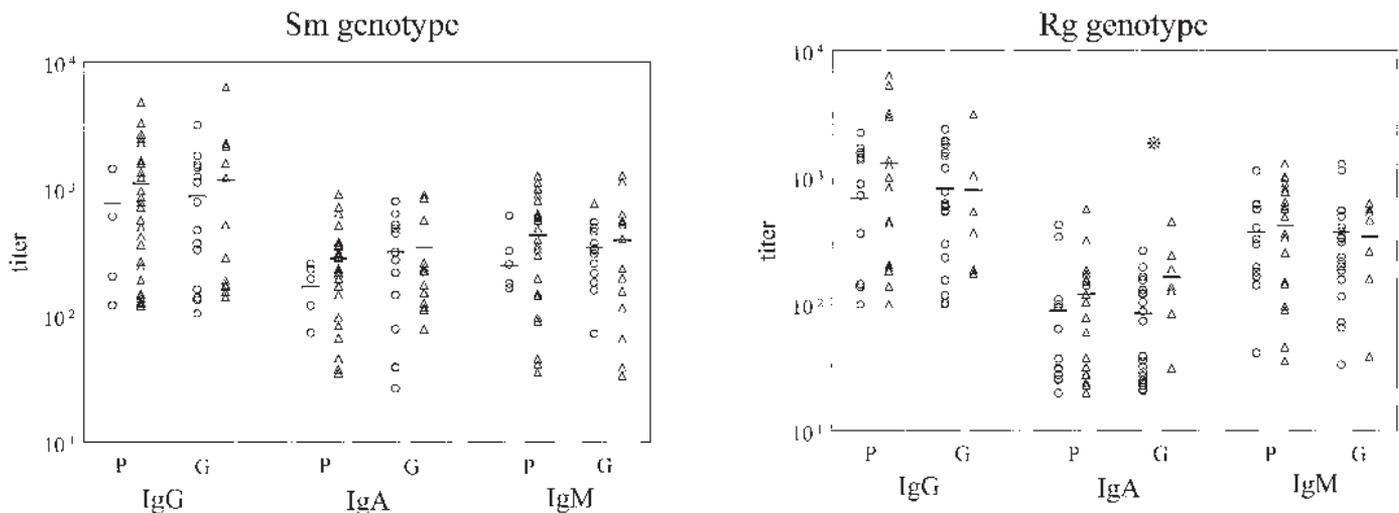


Figure 1. Systemic antibody titers against the Sm genotype and the Rg genotype of *P. micros* in culture-positive (Δ) and culture-negative (O) subjects with periodontitis (P) and gingivitis (G) as determined by ELISA. Mean titers of the subjects per study group are presented as horizontal bars. * $P < 0.05$.

Since smoking has been shown to influence the host immune system unfavorably,^{36,37} we also analyzed the systemic antibody response to both genotypes of *P. micros* in relation to smoking status. However, within the subsets of both study groups, the antibody titers against the Sm type and the Rg type were comparable in smoking and non-smoking subjects (Fig. 2).

Antigen Recognition by Systemic Antibodies

The antigen specificity of the systemic immunoglobulins of the subsets of the study groups, 34 periodontitis patients and 29 subjects with gingivitis, was investigated by immunoblotting. Representative Sm type antigen patterns obtained with plasma of periodontitis (plasma 1-12) and gingivitis subjects (plasma 13-24) are shown in Fig. 3A. All tested plasmas contained IgG antibodies binding to 4 major antigens with a molecular weight of 20 to 30 kD. Additionally, a ladder pattern of 40 to 70 kD was recognized by IgG antibodies of all subjects. About half of the plasmas contained IgG antibodies that strongly bind to several high molecular weight antigens of the Sm type. In the Rg type immunoblots (Fig. 3B), a 20 to 30 kD antigen pattern, identical to that in Sm type whole-cell lysate, was observed in 53 out of 63 plasmas tested. High molecular weight proteins of approximately 110 and 120 kD were recognized by 50 and 42 out of 63 plasmas, respectively. Finally, another major antigen of the Rg type of approximately 42 kD was recognized by systemic antibodies of 58 out of 63 plasmas.

Both the Sm and the Rg antigen patterns as recognized by serum antibodies from patients were comparable to those recognized by antibodies from subjects with gingivitis (Fig. 3). Furthermore, no differences

were observed between antigen patterns recognized by sera of culture-positive subjects as compared to culture-negative subjects. The antigen patterns were also not related to smoking, since antigen patterns obtained with sera of smokers and non-smokers were similar.

DISCUSSION

Several reports have indicated that *P. micros* is a normal resident of the oral cavity,^{1,7} and it should thus be defined as a commensal. However, evidence has also been provided for considering this species as a periodontal pathogen.³⁻¹⁰ In the present study we determined the presence of both the Sm and the Rg genotype of *P. micros*¹⁸ in periodontitis and gingivitis. The prevalence in periodontitis patients was 94% for the Sm type and 38% for the Rg type. The prevalence in the present study is higher than reported in other studies.⁷ This might be explained by a difference in detection level, possibly due to careful microscopic examination of the primary isolation plates. Our data are comparable to those of van Dalen et al.,¹⁹ who reported prevalences of 91% for the Sm type and 45% for the Rg type in a series of microbiological samples from periodontitis patients. Interestingly, in the gingivitis group, the prevalence of the Sm type was 59%, which was significantly lower than in periodontitis patients. The prevalence of the Rg type in the gingivitis group was not different from that in periodontitis patients (about 30% in both groups). These observations indicate that both the Sm and the Rg types of *P. micros* are microorganisms associated with periodontitis. The proportions of both *P. micros* genotypes in culture-positive subjects were not significantly dif-

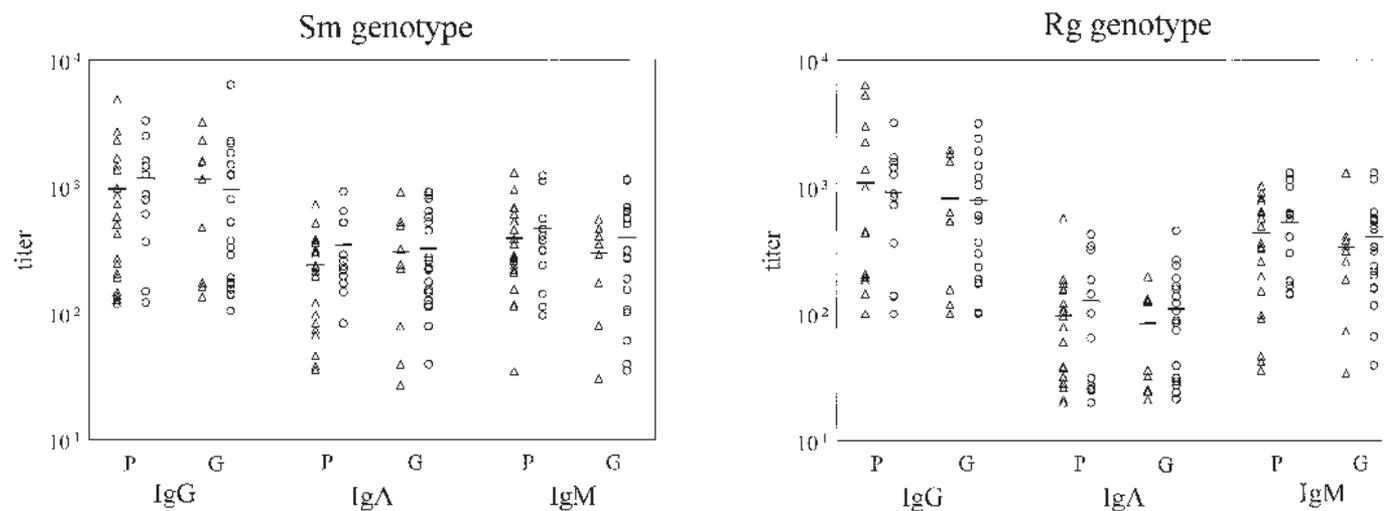
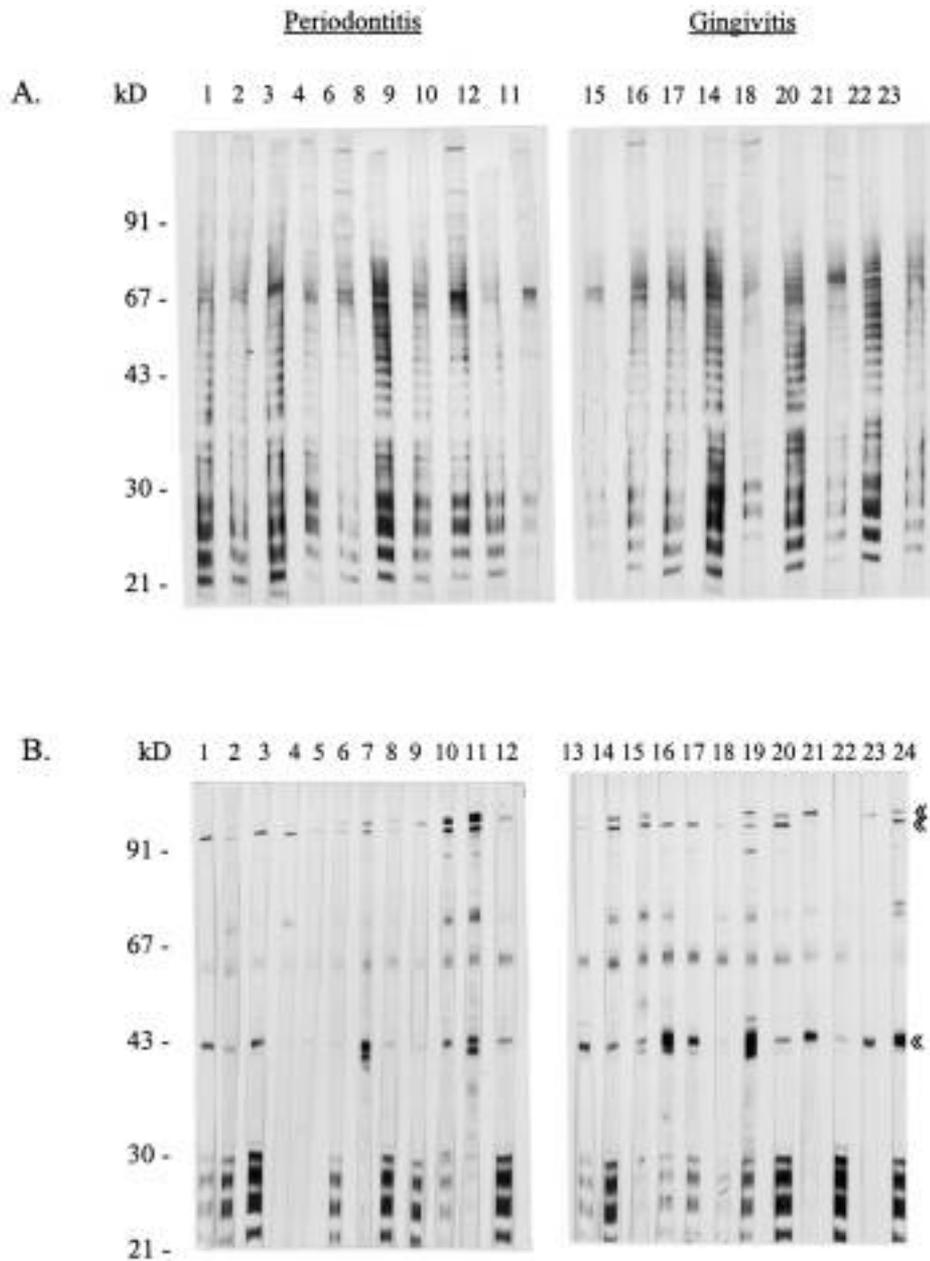


Figure 2. Systemic antibody titers against the Sm genotype and the Rg genotype of *P. micros* in smokers (Δ) and non-smokers (O) with periodontitis (P) and gingivitis (G) irrespective of the presence of *P. micros*, as determined by ELISA. Mean titers of the subjects per study group are presented as horizontal bars.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
<i>P. micros</i> Sm:	-	+	+	+	+	-	+	+	+	+	+	+	-	+	+	-	+	-	-	-	+	+	+	+
<i>P. micros</i> Rg:	-	+	+	-	-	-	+	-	+	-	+	-	-	-	-	-	-	+	+	-	+	-	-	+
Smoking:	-	+	+	-	-	+	+	+	+	-	+	-	-	+	-	-	+	-	+	+	-	-	-	+

Figure 3.

Representative Sm type and Rg type *P. micros* antigens recognized on a Western blot by plasma IgG of periodontitis patients and subjects with gingivitis. SDS-soluble antigens of HG1253 (A, Sm) and HG1259 (B, Rg) were transferred to nitrocellulose and incubated with plasmas of subsets of the periodontitis group (plasma 1-12) and the gingivitis group (plasma 13-24). The prominent antigens as discussed in the results are indicated with arrows. The panel summarizes the presence of *P. micros* genotypes and the smoking status per subject of which the plasma has been used: Sm, the presence of the Sm type in subgingival plaque; Rg, the presence of the Rg type in the subgingival plaque.

ferent between the periodontitis and gingivitis groups. They always ranged between 1 to 7% of the cultivable gingival microbiota, indicating that once these bacteria colonize the subgingival microbiota, they rarely dominate this niche.

In vitro the Rg type easily changes into a smooth variant, designated as RgSm type.¹⁸ The colony morphology of this variant resembles that of the Sm type. However, it is serologically and phylogenetically identical to the Rg type.¹⁸ In a previous study, none of the clinical isolates with a smooth colony morphology were identified as RgSm type.¹⁸ Again, in the present study, the RgSm variants were not isolated. Although we cannot exclude that this variant of the Rg type exists in very small proportions in the subgingival plaque in vivo, we consider the RgSm type as an in vitro variant.

Preliminary reports have indicated that the subgingival microbiota may be different in smokers and non-smokers. It has been suggested that the proportions of *Bacteroides forsythus* and *P. micros* in periodontitis patients are elevated in smokers as compared to non-smokers, whereas *Porphyromonas gingivalis* accounts for lower proportions of the cultivable microbiota in smokers.^{27,28} Other studies reported no differences in the recovery of periodontal bacteria such as *Actinobacillus actinomycetemcomitans*, *P. gingivalis*, and *Prevotella intermedia* from smokers as compared to non-smokers.^{38,39} In the present study, no relation between smoking and *P. micros* genotypes was observed in the subjects with gingivitis. In contrast, in periodontitis patients, we observed a higher prevalence of the Rg type in smokers as compared to non-smokers, whereas this difference was not found for the Sm type. Although no significant differences in the proportions of both genotypes were observed between smokers and non-smokers, the total proportion of *P. micros* (Sm plus Rg) in smokers with periodontitis was significantly higher than in non-smokers. Moreover, the proportion of total *P. micros* in culture-positive periodontitis patients was positively correlated to the pack-years and to the number of cigarettes per day, whereas trends were observed for the proportions of the Sm type and the Rg type separately. These results imply a relationship between smoking and *P. micros* in destructive periodontal disease. This relation may be the result of indirect effects of smoking such as the alteration of the local host response or changes in composition of the local environment,³⁷ perhaps affecting the growth and establishment of *P. micros* in this ecosystem.

As an increased antibody level against a microorganism in destructive periodontal disease is one of the criteria for implicating the microorganism as being important in the etiology of periodontitis,² the systemic immunoglobulin titers against the 2 genotypes of *P. micros* were investigated. In our study the systemic

IgG, IgA, and IgM titers against the Sm and Rg types of *P. micros* in subjects with periodontitis were not different from the titers in subjects with gingivitis. The antibody titers in culture-positive and culture-negative subjects were not statistically significantly different, except for the IgA titer in culture-positive gingivitis subjects. These results are in line with the study of Gunsolley et al.,⁹ which shows comparable levels of *P. micros*-specific serum antibodies in chronic adult periodontitis patients and subjects without destructive periodontal disease. Many reports reveal that antibody levels to periodontal pathogens such as *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, and *B. forsythus* are elevated in periodontitis patients as compared to control subjects, as reviewed by Wilton et al.⁴⁰ and Zambon.⁴¹ Our results imply that both the Sm and the Rg types of *P. micros* are part of the normal microbiota of the oral cavity, since the systemic antibody levels are comparable in both subject groups and in culture-positive and culture-negative subjects. Both types may be present in a large part of the human population, although often present in very small proportions (<0.1%) in the subgingival plaque. Alternatively, the systemic antibody response may be evoked by *P. micros* genotypes located at other ecological niches throughout the human body.⁴² Thirdly, the antibodies binding to the *P. micros* types may be in part cross-reactive antibodies evoked by other bacterial species.

The antigen specificity of the systemic antibody response to the *P. micros* genotypes was not different between periodontitis patients and gingivitis subjects, as the antigen patterns were comparable. The antigen specificity was not dependent on the presence of the Sm or Rg genotypes in the subgingival plaque. Finally, the antigen specificity was not different in smokers and non-smokers. These results are consistent with the hypothesis that both *P. micros* types are commensal bacteria. The antigen patterns of the Rg type recognized by systemic IgG in the present study were comparable to the antigen pattern obtained with a rabbit anti-Rg serum.¹⁷ This indicates that the immunogenic components of this genotype are not host-specific. The majority of the selected plasmas contained antibodies recognizing a major Rg type antigen of approximately 42 kD (arrow); most likely, this is the antigen that has recently been identified as FibA, which is an antigenic constituent of the fibril-like surface structure of the Rg type.⁴³ Interestingly, the serum antibodies of the selected periodontitis patients and gingivitis subjects recognized a ladder antigen pattern in Sm type preparations, which was not observed with a rabbit anti-Sm serum.¹⁷ This may be due to a host-specific antibody response to these immunogenic components; alternatively, it may be the result of host-specific expression of these antigens. The identity of these antigens remains to be elucidated.

In conclusion, the finding that the proportions of both genotypes of *P. micros* and the titers and specificity of the systemic antibodies against these genotypes did not differ between periodontitis patients and gingivitis subjects suggests that the Sm and Rg types of *P. micros* are commensal bacteria of the subgingival ecosystem. The elevated prevalence of the Sm genotype in periodontitis and the elevated prevalence of the Rg type in periodontitis patients who smoke implies that both types can behave as opportunistic pathogens in destructive periodontal disease.

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