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Genotypic characterization of *Porphyromonas gingivalis* isolated from Swedish patients with periodontitis and from periodontal abscesses

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Yoshino T, Laine ML, van Winkelhoff AJ, Dahlén G. Genotypic characterization of Porphyromonas gingivalis *isolated from Swedish patients with periodontitis and from periodontal abscesses.*
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Introduction: A significant genetic polymorphism has been shown for *Porphyromonas gingivalis* isolates from different geographical areas. It is, however, possible that genetic similarities can be found among isolates obtained from a more specific population. The aim of the present study was to evaluate genetic heterogeneity among *P. gingivalis* isolates obtained from Swedish subjects with chronic periodontitis and from periodontal abscess lesions.
Methods: A total of 78 *P. gingivalis* strains, including 55 fresh clinical isolates obtained from 52 Swedish periodontitis subjects, eight isolates from eight Swedish periodontal abscess subjects and 15 reference strains, were subjected to amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) genotyping assays.
Results: A total of 62 AFLP genotypes and 70 RAPD genotypes were identified among the 78 *P. gingivalis* strains. Forty-six strains were clustered at 70% similarity level into 15 clusters. Six identical RAPD genotypes were identified among the strains. The AFLP/RAPD profiles were compared for identical genotypes. A total of 56 AFLP/RAPD genotypes were found. Four pairs of identical AFLP/RAPD genotypes were found for two strains obtained from two different periodontal pockets each of four subjects. Interestingly, two strains showed an RAPD/AFLP genotype, which was identical to the type strain W83.
Conclusion: The present study demonstrated that Swedish *P. gingivalis* isolates exhibit a wide variety of genotypes with only a weak clustering pattern. No predominant genotype at the whole chromosomal DNA level was present among Swedish *P. gingivalis* strains.

Key words: amplified fragment length polymorphism; genotype; periodontal disease; *Porphyromonas gingivalis*; random amplified polymorphic DNA

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Porphyromonas gingivalis is a gram-negative, black-pigmented anaerobe associated with destructive periodontal disease (12). It is also implicated in periodontal abscesses and endodontic infections (11, 31). *P. gingivalis* can be detected in healthy subjects (10, 33, 30), which could have a number of explanations, e.g. bacterial interactions, host defense mechanisms, genetic polymorphism and variable phenotypic expression of virulence factors. Different pathogenic properties within *P. gingivalis* are known; however, the molecular bases for this variation and the relation to the genetic diversity are not fully understood.

To examine the phenotypic heterogeneity of this organism, *P. gingivalis* isolates of various origins have been evaluated

(8, 18, 21, 25). Recently, we demonstrated that *P. gingivalis* isolates showed a consistent phenotypic homogeneity but differed in colony morphology and could be classified into serotypes A and B using monoclonal antibodies (4). However, this phenotypic approach may not distinguish genetically different isolates because of the limited phenotypic variation (19).

The heterogeneity of ribosomal or chromosomal DNA sequences within *P. gingivalis* species has been investigated using molecular typing methods such as restriction fragment length polymorphism (RFLP), ribotyping, multilocus enzyme electrophoresis (MEE), random amplified polymorphic DNA (RAPD) and multilocus sequence typing (MLST) (1, 7, 19, 20, 22). These studies have shown a considerable genotypic heterogeneity among *P. gingivalis* strains and no specific genotype could be related to pathogenicity. These studies investigated *P. gingivalis* isolates obtained from different geographical locations. Genetic polymorphism within *P. gingivalis* strains isolated from a more limited population has only been sparsely studied (15).

The purpose of this study was to evaluate genetic heterogeneity of *P. gingivalis* isolates from Swedish subjects with periodontal disease.

Material and methods

Bacterial strains

A total of 78 *P. gingivalis* strains were investigated. These included 55 fresh clinical isolates (labeled strain PgS 1–55) from 52 periodontitis Swedish subjects with deep periodontal pockets ($\geqslant$6 mm) and eight clinical isolates from subjects with periodontal abscesses (13). A further two reference strains (FDC381 or HG91 and W83 or HG66); five representative K-serotype strains [HG184(K2), HG1025(K3), HG1660(K4), HG1690(K5) and HG1661(K6)]; and eight other reference strains [OMGS 406 (from Kenyan periodontal pocket), OMGS 673 (from an infected necrotic root canal in Swedish subject), OMGS 769 (from Kenyan periodontal pocket), OMGS 788 (from Kenyan periodontal pocket), OMGS 984 (from dorsum of tongue in Swedish subject), OMGS 2104 (from Chinese periodontal pocket), OMGS 1577 (from Japanese periodontal pocket) and OMGS 1578 (from Japanese periodontal pocket)] were included and also one negative control strain (*Prevotella intermedia*) (Table 1) (4).

Four days before the experiment, the bacterial strains were plated on Brucella agar (BBL Microbiology Systems, Cockeysville, MD) enriched with 5% defibrinated horse blood, 0.5% hemolyzed blood and 5 μg/ml menadione and incubated at 37°C in jars with 95% H_2 and 5% CO_2. The cells were harvested and transferred to TE buffer (10 mM Tris–HCl, 1 mM EDTA; pH 8.0).

Table 1. Source of *P. gingivalis* strains used in this study

P. gingivalis strains	Source	Country
S 1-55	Periodontitis	Sweden
A 1-8	Periodontal abscess	Sweden
W83[1] (HG[2]66)	Unknown	Germany
FDC 381[3](HG91)	Periodontitis	USA
HG[3] 184	Periodontitis	the Netherlands
HG 1025	Periodontitis	USA
HG 1660	Periodontitis	the Netherlands
HG 1690	Periodontitis	the Netherlands
HG 1661	Periodontitis	the Netherlands
OMGS[4] 769	Periodontitis	Kenya
OMGS 788	Periodontitis	Kenya
OMGS 984	Dorsum of tongue	Sweden
OMGS 673	Infected necrotic root canal	Sweden
OMGS 1577	Periodontitis	Japan
OMGS 1578	Periodontitis	Japan
OMGS 406	Periodontitis	Kenya
OMGS 2104	Periodontitis	China

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[2]Obtained from Oral Microbiology, ACTA, Amsterdam, the Netherlands.
[3]Obtained from Forsyth Dental Center.
[4]Obtained from Oral Microbiology, Gothenburg, Sweden (OMGS).

Identification of *P. gingivalis* by checkerboard DNA–DNA hybridization

To confirm *P. gingivalis* species, all strains were subjected to checkerboard DNA–DNA hybridization with *P. gingivalis*-specific polymerase chain reaction (PCR) products of the central portion of the collagenase gene (*prt*C) after amplification using primers Coll-1 (5′-ACA ATC CAC GAG ACC ATC-3′) and Coll-2 (5′-TTC AGC CAC CGA GAC-3′) (2). Digoxigenin-labeled *prt*C gene amplified probes were prepared from genomic DNA of *P. gingivalis* FDC381 by using the DIG-High Prime labeling kit (Roche Diagnostics GmbH, Manheim, Germany).

In brief, samples were boiled for 5 min, neutralized, transferred to nylon membranes and immobilized by ultraviolet light and incubated at 120°C. After 2 h of prehybridization, the DNA probes were allowed to hybridize overnight with the sample DNA at 42°C. After stringency washes at 70°C hybrids were detected using an anti-digoxigenin antibody and enhanced chemiluminescence.

Amplified fragment length polymorphism (AFLP) genotyping

Chromosomal DNA from bacterial cells was isolated according to Boom et al. (3). Extracted DNA was resolved in 100 μl TE buffer supplemented with 10 μg RNase (Sigma, St Louis, MO). Purified DNA was aliquoted and stored at – 20°C. DNA concentrations were estimated by agarose gel electrophoresis against diluted samples of λ DNA (New England Biolabs Inc., Beverly, MA).

Twenty-five nanograms of DNA templates for AFLP were prepared as previously described by Janssen et al. (14) and Vos et al. (32) with the adaptation of Koeleman et al. (16). Briefly, purified DNA was digested and ligated simultaneously with *Pst*I (New England Biolabs Inc., Beverly, MA), *Mse*I (New England Biolabs Inc.), *Pst*I-O adapter, *Mse*-C adapter and T4 DNA ligase (Phamacia LKB Biotechnology, Uppsala, Sweden) for 4 h. A Texas Red fluorescent labeled *Pst*I-O primer (Isogen Bioscience, Bilthoven, the Netherlands) and unlabeled *Mse*-C primer were used for DNA amplification, which was perfomed in a GeneAmp PCR System 9700 thermal cycler (Perkin Elmer, Boston, MA) for 35 cycles of denaturation (30 s at 94°C), annealing (30 s at 65–56°C), and DNA molecule extension (60 s at 72°C). Fluorescent amplified fragments were separated on a denaturing polyacrylamide gel (RapidGel-XL-6%; Amersham Life Science, Cleveland, OH) according to the manufacturer's instructions in a Vista 725 automated DNA sequencer (Amersham Life Science, Cleveland, OH). Two microliters of each sample was loaded on the gel and the gels were run at 150 V for 6 h. Fluorescently labeled AFLP fingerprints were analyzed on the Vistra 725 DNA sequencer and stored as. tiff files with the VISTRA 2 TIFF

software (Amersham Life Science). Images were processed using GelCompar 3.1 software (Applied Maths, Kortrijk, Belgium). Levels of similarity between fingerprints were calculated with the Pearson product moment correlation coefficient (r). Cluster analysis was performed with the non-weighted pair group method using average linkages (UPGMA).

To make the cut-off point for identical AFLP genotypes, the two reference strains (FDC 381 and W83) were analysed in duplicate cultures.

RAPD genotyping

Chromosomal DNA from bacterial cells was extracted according to Smith et al. (27) and resolved in 50 μl TE buffer overnight at 4°C. The resuspended DNA was treated with 5 μg RNase for 45 min at 37°C and stored at −20°C until further use. The DNA concentrations were quantified by measurement of the absorbance at 260 nm (GeneQuant, Pharmacia Biotech Ltd, Cambridge, UK).

Prior to the RAPD analysis, twenty 10-base oligonucleotide primers (RAPD primer KIT 01-01 to KIT 01-20, USBiological, Swampscott, MA) were tested for their capacity to discriminate between four different *P. gingivalis* strains (W83, FDC 381, OMGS 1579, OMGS 438). The base sequences (5′–3′) of the 20 primers (from KIT 01-01 to KIT 01-20) were 01, CAG GCC CTT C; 02, TGC CGA GCT G; 03, AGT CAG CCA C; 04, AAT CGG GCT G; 05, AGG GCT CTT G; 06, GTT CCC TGA C; 07, GAA ACG GGT G; 08, GTG ACG TAG G; 09, GGG TAA CGC C; 10, GTG ATC GCA G; 11, CAA TCG CCG T; 12, TCG GCG ATA G; 13, CAG CAC CCA C; 14, TCT GTG CTG G; 15, TTC CGA ACC C; 16, AGC CAG CGA A; 17, GAC CGC TTG T; 18, AGG TGA CCG T; 19, CCA ACG TCG G; 20, GTT GCG ATC C.

Primer KIT 01-05 generated the most discriminatory RAPD profile for these bacterial strains (data not shown). Thus, KIT 01-05 primer was chosen as an optimal primer for the analysis of genetic heterogeneity of *P. gingivalis* strains in the present study.

The RAPD amplification reaction was performed in a total volume of 25 μl, consisting of 2.5 μl 10× Stoffel Buffer, 0.4 mM dNTPs, 3 U Ampli*Taq* DNA polymerase, Stoffel Fragment (Applied Biosystems, Foster City, CA), 2 μM primer (10 μM) (USbiological), 4 mM $MgCl_2$ and 100 ng DNA template.

DNA was amplified using a PTC-100 thermal controller (MJ Research, Watertown, MA). The thermal controller temperature profile comprised an initial denaturation at 95°C for 2 min followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 2 min and primer extension at 72°C for 2 min. The final extension step was carried out at 72°C for 7 min. The PCR products were separated by polyacrylamide gel electrophoresis (10% polyacrylamide gel, Criterion TBE-gel 345-0053, Bio-Rad, Hercules, CA) for 10 min at 20 V, followed by 90 V for 95 min by applying a mixture of 12 μl PCR products and 2 μl loading buffer (Gel loading solution, Sigma-Aldrich, Stockholm, Sweden) and run in TBE (Tris-Borale-EDTA) buffer. DNA was visualized by silver-staining (plusOne DNA silver staining kit, Pharmacia Biotech, Uppsala, Sweden). Then, the polyacrylamide gel was dried (Dry Ease Gel Drying System, Invitrogen Co., Carlsbad, CA).

Results

Identification of *P. gingivalis* by *prt*C DNA probe

All 78 *P. gingivalis* strains were found to harbor the *prt*C gene specific for *P. gingivalis*.

AFLP genotypes

A dendrogram of AFLP genotyping of 78 *P. gingivalis* strains, including reference *P. gingivalis* strains and one *P. intermedia* strain, is shown in Fig. 1. The majority of the *P. gingivalis* strains, with the exception of strain S28, fell into a single group at a similarity level of more than 45%. Strain S28 was clearly different from the other *P. gingivalis* strains. *P. intermedia* was also distinguished from *P. gingivalis* strains at this level. When all the *P. gingivalis* strains were clustered at a similarity level of 70%, *P. gingivalis* strains revealed 15 (I–XV) clusters. Most of the clusters were provided by small groups consisting of only two or three clones. The non-Swedish isolates did not cluster separately from the Swedish isolates. A majority of the strains were assigned into 11 groups (A–K) at a 55% similarity. Forty-nine *P. gingivalis* isolates from Swedish subjects fell into five major groups; group A (six isolates), group B (11 isolates), group C (nine isolates); group D (17 isolates), and E (six isolates) respectively.

Duplicate AFLP genotype patterns of strain FDC 381 and W83 were used to evaluate the similarity level within the same strain (Fig. 1). The FDC 381 and W83 had similarities of 89% and 81%, respectively. A similarity of 81% as the cut-off point was therefore used to detect same or similar AFLP genotype profiles. Consequently, 62 AFLP genotypes were detected among the 78 *P. gingivalis* strains.

RAPD genotypes

A total of 78 *P. gingivalis* strains and a *P. intermedia* strain were subjected to RAPD profile analysis to evaluate identity based on RAPD profile similarities. The RAPD profiles of each strain were compared with the banding patterns of their counterparts and strains with the same or similar banding patterns were regarded as identical genotypes. The 78 *P. gingivalis* strains were classified into 70 RAPD genotypes. The majority of *P. gingivalis* strains showed distinct single RAPD banding patterns and provided an extensive genetic heterogeneity (data not shown). Thirteen strains were grouped into six identical RAPD genotypes. Of these, two strains S3 and A7 revealed genotypes identical to that of the type strain W83 (Fig. 2). Moreover, four pairs of identical genotypes (e.g. S7 and S8, S22 and S23, S36 and S37, S52 and S53) were obtained each from two deep periodontal pockets in four subjects. Another three pairs of identical genotypes were isolated from unrelated subjects.

AFLP/RAPD-matched genotypes

A total of 70 AFLP/RAPD-matched genotypes were found among the 78 *P. gingivalis* strains. In addition, 56 AFLP/RAPD genotypes were found among 63 clinical isolates from Swedish subjects. The majority of Swedish isolates showed rather different individual genotypes and diversity of *P. gingivalis*. Six identical genotypes were also detected in AFLP/RAPD genotype analysis, in accordance with the observation from RAPD profiling. Interestingly, strains S3 and A7 showed genotype similarity with the type strain W83 in the combined AFLP/RAPD profiling. No identical genotype corresponding to type strain 381 was found in this population.

Discussion

The present study demonstrates a considerable genetic diversity of *P. gingivalis* in Swedish periodontitis and periodontal abscess subjects. However, two isolates exhibited a genotypic similarity with the virulent strain W83.

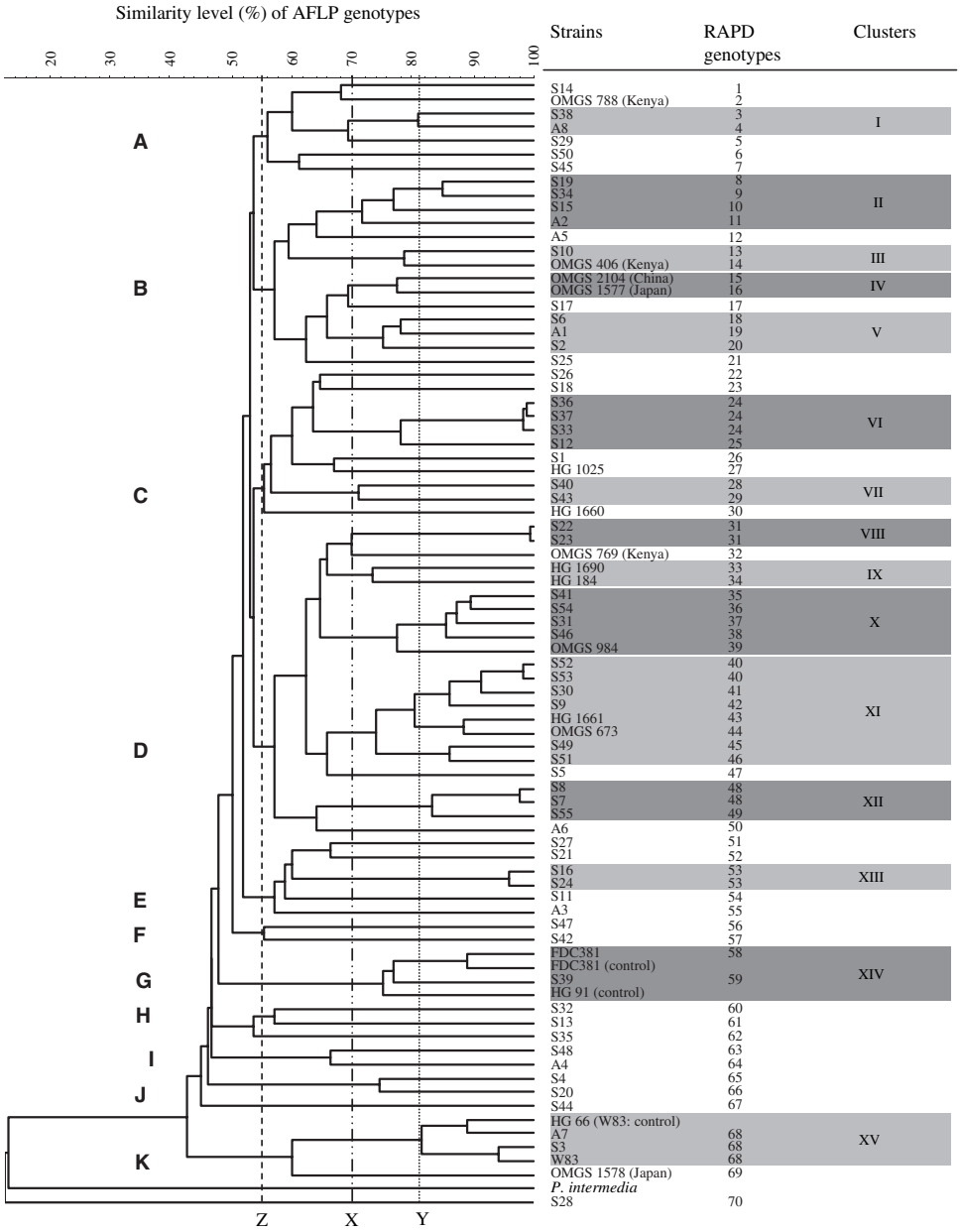

Fig. 1. Similarity level (%) of AFLP genotypes. $X = 70\%$ similarity, $Y = 81\%$ and $Z = 55\%$ (see text for full explanation).

Recently, the AFLP genotyping method has been introduced as a novel DNA fingerprinting method based on PCR technique with restriction fragments of digested genomic DNA (32, 34). The advantages of this method are the great discriminatory power and the reproducibility compared with other genetic typing methods (23). However, poor reproducibility of AFLP genotype profiling may occur as a result of incomplete digestion of the chromosomal DNA (17). Furthermore, the RAPD method based on the PCR technique has been extensively used as a genetic typing method to evaluate bacterial genomic profiles, being a distinguishable, reproducible, rapid and simple technique (5). The reproducibility of genotyping analysis based on PCR technology is extremely dependent on the condition of the PCR; moreover, the discrimination power is also, in general, dependent on the genetic typing method and the PCR primers (26). Therefore, we applied, in the present study, both AFLP and RAPD profiling assays to the evaluation of a more precise genetic polymorphism of *P. gingivalis* strains.

Loos et al. (20) investigated the genetic diversity of *P. gingivalis* by MEE profiling assay in 88 human and 12 animal strains obtained from geographically different locations. A total of seventy-eight genotypes were identified, and it was concluded that the population structure of this organism was basically clonal, and that no specific predominant genotype was found. This heterogeneity of whole chromosomal genotypes was confirmed by RAPD profiling of 97 human strains and 32 animal strains by Ménard and Mouton (22). In the present study, the results in AFLP/RAPD genotype profiles confirmed that this heterogeneity also occurred among *P. gingivalis* strains obtained from a smaller geographical area. Furthermore, our findings showed that although 15 clusters were detected at a similarity level of 70%, the

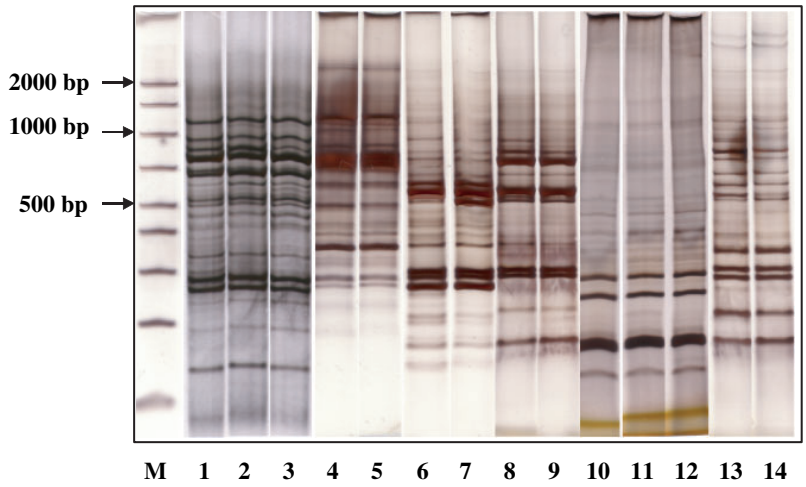

Fig. 2. Identical RAPD genotype profiling patterns of *P. gingivalis*. M, molecular size marker; lane 1, type strains W83; lane 2, strain S3; lane 3, A7; lane 4, S7; lane 5, S8; lane 6, S16; lane 7, S24; lane 8, S22; lane 9, S23; lane 10, S33; lane 11, S36; lane 12, S37; lane 13, S52; lane 14, S53.

majority was provided by small clusters consisting of two or three clones. However, we demonstrated that a majority of Swedish clinical isolates (43 isolates) fell into five major groups (Group A, B, C, D and E) at 55% similarity. This may indicate that the majority of Swedish *P. gingivalis* isolates have a weak genetic relationship and are generally non-clonal. Our data thus support previous multilocus sequence studies on several DNA gene fragments that suggest a low clonality for *P. gingivalis* and are characterized by recombination including horizontal gene transfer of this organism (6, 7, 15). However, *P. gingivalis* isolates from the same individual seem to be of the same genotype.

Recently, consistent phenotypic properties among the same *P. gingivalis* isolates were reported (4). In the present study, however, strain S28 exhibited a <20% similarity against other *P. gingivalis* strains. Although this strain was phenotypically indistinguishable from other *P. gingivalis* strains, it does not genotypically fit into the *P. gingivalis* taxon. Fournier and Mouton (8) reported on the phenotypic diversity between human and animal *P. gingivalis* strains and described that human strains can be distinguished from animal strains by their negative enzymatic activities such as catalase, β-galactosidase (β-GAL) and glutamyl-glutamic acid arylamidase (GGA) in human strains. Strain S28 proved to be β-GAL-negative but catalase-positive (data not shown). However, it is unclear whether this strain is transmitted from animal to human or not.

Previous experimental abscess studies in animals have demonstrated that the type strains W83 and W50 belong to a more virulent phenotype than most other strains and induce severe generalized abscess formation with a risk of animal death (9, 24, 28, 29). In the present study, the strains S3 and A7 were found to be of the same clonal type as strain W83. Previously, we have shown that these two strains and W83 also belong to the same serotype (serotype B) using monoclonal antibodies (4). This may indicate that a virulent clone of *P. gingivalis* can be detected from periodontitis subjects. However, the virulence is probably characterized by a limited number of specific virulence and other genes and cooperation between them. Therefore, further studies should be directed to more specific genes related to the pathogenicity of this organism.

In conclusion, the present study demonstrated that Swedish *P. gingivalis* isolates exhibited a wide genetic heterogeneity. Although the study also confirmed that there was no predominant *P. gingivalis* genotype among these isolates, it is likely that a limited number of periodontally diseased subjects harbor a more virulent genotype of *P. gingivalis*. Further research is needed to evaluate the diversity of more specific virulence genes.

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