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Sequencing of the Ribosomal Intergenic Spacer Region for Strain Identification of *Porphyromonas gingivalis*

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The ribosomal intergenic spacer regions (ISRs) of 19 laboratory strains and 30 clinical samples of *Porphyromonas gingivalis* were amplified by PCR and sequenced to provide a strain identifier. The ISR is a variable region of DNA located between the conserved 16S and 23S rRNA genes. This makes it an ideal locus for differentiation of strains within a species: primers specific for the conserved flanking genes were used to amplify the ISR, which was then sequenced to identify the strain. We have constructed a *P. gingivalis* ISR sequence database to facilitate strain identification. ISR sequence analysis provides a strain identifier that can be easily reproduced among laboratories and catalogued for unambiguous comparison.

Porphyromonas gingivalis has been strongly implicated as a periodontal pathogen (13, 16, 34, 36, 42). Many studies have shown phenotypic differences, including differences in virulence, among strains of *P. gingivalis* (3, 4, 8, 9, 15, 21, 22, 31, 38). Accurate strain identification is a prerequisite for studies investigating the roles of specific strains of *P. gingivalis* in periodontitis and for studies tracking their transmission and distribution. Previous techniques for the identification of *P. gingivalis* strains include whole-genome restriction fragment length polymorphism analysis or DNA fingerprinting (10, 39), ribotyping (17, 39), arbitrarily primed (AP)-PCR (29, 39), serotyping (6, 18, 32), and multilocus enzyme electrophoresis (26). While these techniques have made it possible to track strains, none have provided a strain identifier that is easily reproduced among laboratories or that can be catalogued for unambiguous comparison. In addition, many of these techniques require culturing of the organisms prior to analysis. Not only is this time-consuming, but it also reduces sensitivity and may introduce bias.

The DNA sequence of the ribosomal small subunit (16S in bacteria and 18S in eukaryotes) has been employed extensively for both identification and phylogenetic resolution of bacteria at the species level (5, 7). This gene contains both conserved regions and areas of variability sufficient to resolve species. Within a species, however, this gene does not provide sufficient variability to resolve strains. In contrast, the ribosomal intergenic spacer region (ISR), a stretch of DNA that lies between the small and large (23S) ribosomal subunit genes (Fig. 1), is variable among strains. Analysis of the ISR has been employed for the resolution of strains within several species (14, 20, 33, 41). The location of the ISR makes it ideal for strain identification: the ribosomal operon can be amplified and sequenced with species-specific primers whose targets are located within the conserved 16S and 23S genes. The 16S gene can be sequenced to verify the species, and the sequence of the ISR can be used to distinguish among strains of a species. Here we demonstrate the utility of direct PCR amplification, without culturing, followed by sequencing of the ISR for strain identification of *P. gingivalis*. Using this technique, we have constructed a catalogue of ISR sequences for 19 known laboratory strains of *P. gingivalis* as well as 30 novel sequences obtained from clinical samples. Twenty-seven of these clinical samples were selected based on their failure to match any of the patterns obtained by heteroduplex analysis of the ISR for the 19 laboratory strains (24). The strains sequenced in this study are listed in Table 1.

The ribosomal DNA spacer regions from both cultured laboratory strains and clinical samples were amplified as described previously (23, 28). The sequences and locations of the primers are shown in Table 2 and Fig. 1. Genomic DNA isolated from plaque samples or laboratory strains was used as a template with universal prokaryotic primers 785 and 422. To generate species-specific DNA fragments from mixed clinical samples, a second amplification was performed. Aliquots consisting of 2% of the product from the first amplification served as templates for the second amplification with the *P. gingivalis* species-specific primer PG8R and the universal prokaryotic primer L189. This generated ISR DNA fragments specific to *P. gingivalis*.

PCR products were purified via the Geneclean protocol (Bio 101, Inc., La Jolla, Calif.) and sequenced with an ABI 310 automated DNA sequencer. Universal prokaryotic primers 317R and EricM were used for sequencing. Both strands were sequenced at least once to ensure accuracy. Direct sequencing of PCR products eliminated the problem of misincorporation that is associated with cloning PCR products. Because of the large number of templates available at the beginning of the amplification, a base change in any one molecule would have resulted in an insignificant fraction of the amplified products representing the misincorporation.

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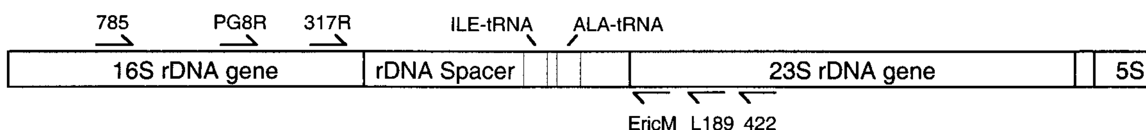

FIG. 1. Map of the ribosomal operon including the ISR and primer-binding locations. rDNA, ribosomal DNA.

Sequences were assembled in SeqPup (11) and aligned via Clustal X (19, 37) for automated alignment and via SeqApp for final manual alignment. A total of 830 bases were sequenced and aligned for each strain examined. The ISR sequences for strains W50, ATCC 49417, and ATCC 33277 are available from GenBank (see below); complete ISR alignments for the 19 laboratory strains and 30 clinical samples are available in National Biomedical Research Foundation format (15a).

The 19 laboratory strains were resolved into 17 unique groups based on their ISR sequences. Strains W50 and W83 were indistinguishable from one another, as were strains ATCC 49417 and HG445. Also, strains W50 and W83 were unresolved by techniques such as AP-PCR (2, 30), fimbrial restriction fragment length polymorphism analysis (25), genomic DNA fingerprinting (27), and serotyping (6, 40). It is possible that they are either the same strain or two very closely related strains. Strains ATCC 49417 and HG445 were not compared in any of the previous strain-typing studies; therefore, the difficulty of distinguishing between these two isolates by using other methods is unknown. Strains 381 and ATCC 33277, which have been previously unresolvable by techniques such as Southern blotting (1), serotyping (6), genomic DNA fingerprinting (27), and AP-PCR (2) but were separable based on infectivity and metabolic requirements (12), were distinguishable by ISR sequencing. A previous study has also been able to distinguish between these two strains via AP-PCR (35).

Twenty-seven clinical samples were selected for sequencing because they showed ISR heteroduplex patterns distinct from that of any of the 19 laboratory strains (24). As expected, their sequences did not match that of any of the laboratory strains, although of the 830 bases compared, some of the sequences differed from those of the laboratory strains by as little as a single indel (insertion or deletion event). Three samples that matched either strain W50 or 381 by ISR heteroduplex type were sequenced and found to be between 99.28 and 99.76% identical to their heteroduplex type strain. The existence of laboratory strains with perfect ISR sequence homology (e.g., W50 and W83) suggests that although the ISR is variable, it is sufficiently stable within an existing strain to make it a useful marker for strain identification.

Sequence analysis of the *P. gingivalis* ISR provides a strain identifier that can be easily reproduced among laboratories and catalogued for unambiguous comparison. The ISR sequence alignment is available for downloading and comparison (15a). We will continue to add additional ISR sequences to the catalogue as they become available.

Nucleotide sequence accession numbers. The ISR sequences for strains W50, ATCC 49417, and ATCC 33277 are avail-

TABLE 1. Strains of *P. gingivalis* included in the ISR sequence database

Strain	Source[a]
Cultured lab strains	
381	J. Zambon
3492	D. Mayrand
17-5	C. Cutler
22KN612	D. Mayrand
23A4	D. Mayrand
817H	C. Cutler
A7A1 (28)	J. Zambon
ATCC 33277	ATCC
ATCC 49417	ATCC
B57	C. Cutler
DCR2011	C. Cutler
ESO/27	C. Cutler
HG1691	R. Schifferle
HG445	C. Cutler
HG564	T. J. M. van Steenbergen
JKG7	C. Cutler
MSM3	C. Cutler
W50	J. Zambon
W83	M. Duncan
Clinical amplification products	
7.4	Periodontally healthy subject
35	Unidentified subject
36	Unidentified subject
37	Unidentified subject
61.2	Periodontally healthy subject
62	Unidentified subject
A102	Periodontitis patient
A111	Periodontitis patient
A116	Periodontitis patient
A117	Periodontitis patient
A119	Periodontitis patient
A120	Periodontitis patient
A134	Periodontitis patient
A140	Periodontitis patient
A151	Periodontitis patient
A17	Periodontitis patient
A198	Periodontitis patient
A211	Periodontitis patient
A27	Periodontitis patient
A39	Periodontitis patient
A50	Periodontitis patient
A52	Periodontitis patient
A62	Periodontitis patient
A64	Periodontitis patient
A8	Periodontitis patient
FS8	Student from the People's Republic of China
FS106	Student from France
FS155	Student from the People's Republic of China
FS159	Student from Taiwan
FS170	Student from the People's Republic of China

[a] The sources for all clinical amplification products were sampled in Columbus, Ohio. ATCC, American Type Culture Collection.

TABLE 2. Primers used for ISR amplification and sequencing

Primer	Specificity	Sequence	Target gene
785	Universal	GGATTAGATACCCTGGTAGTC	16S
PG8R	*P. gingivalis*	TGTAGATGACTGATGGTGAAAACC	16S
317R	Universal	GGCTGGATCACCTCCTT	16S
EricM	Universal	GCCAAGGCATCCACCG	23S
L189	Universal	GGTACTTAGATGTTTCAGTTC	23S
422	Universal	GGAGTATTTAGCCTT	23S

able from GenBank (accession no. AF118633, AF118634, and AF118635, respectively).

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