16S–23S rRNA gene internal transcribed spacer sequences for analysis of the phylogenetic relationships among species of the genus *Porphyromonas*

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The 16S–23S rRNA gene internal transcribed spacer (ITS) regions of 11 reference strains of *Porphyromonas* species, together with *Bacteroides distasonis* and *Tannerella forsythensis*, were analysed to examine interspecies relationships. Compared with the phylogenetic tree generated using 16S rRNA gene sequences, the resolution of the ITS sequence-based tree was higher, but species positioning and clustering were similar with both approaches. The recent separation of *Porphyromonas gulae* and *Porphyromonas gingivalis* into distinct species was confirmed by the ITS data. In addition, analysis of the ITS sequences of 24 clinical isolates of *Porphyromonas asaccharolytica* plus the type strain ATCC 25260^T divided the sequences into two clusters, of which one was α -fucosidase-positive (like the type strain) while the other was α -fucosidase-negative. The latter resembled the previously studied unusual extra-oral isolates of 'Porphyromonas endodontalis-like organisms' (PELOs) which could therefore be called 'Porphyromonas asaccharolytica-like organisms' (PALOs), based on the genetic identification. Moreover, the proposal of α -fucosidase-negative *P. asaccharolytica* strains as a new species should also be considered.

INTRODUCTION

The genus *Porphyromonas* currently includes 13 recognized species of asaccharolytic, obligately anaerobic, non-spore-forming, Gram-negative, non-motile, pleomorphic bacilli. Of human origin are three catalase-negative species: *Porphyromonas asaccharolytica*, *Porphyromonas endodontalis* and *Porphyromonas gingivalis*. Most of the known species are, however, of animal origin, including the catalase-positive *Porphyromonas canoris*, *Porphyromonas cangingivalis*,

Porphyromonas cansulci, *Porphyromonas circumdentaria*, *Porphyromonas gingivicanis* and *Porphyromonas macacae* (which includes *Porphyromonas salivosa* strains), and the catalase-negative *Porphyromonas levii* and *Porphyromonas crevioricanis* (Jousimies-Somer & Summanen, 2002). It has also been shown that *Oribaculum catoniae*, although saccharolytic, is phylogenetically a member of the genus *Porphyromonas*; thus, it has been reclassified as *Porphyromonas catoniae* (Willems & Collins, 1995). Finally, animal strains of *P. gingivalis* that were catalase-positive were reclassified as *Porphyromonas gulae*; however, the difference between *Porphyromonas gingivalis* and *Porphyromonas gulae* appeared questionable by analysis of 16S rRNA gene sequences (Fournier *et al.*, 2001).

There are two additional candidates for new species classification, both of which are from humans: PLLOs ('*Porphyromonas levii*-like organisms'; Jousimies-Somer, 1995, 1997; Jousimies-Somer *et al.*, 1995) and PELOs ('*Porphyromonas endodontalis*-like organisms', isolated from extra-oral sites, whereas *Porphyromonas endodontalis* is almost exclusively isolated from endodontic infections; Jousimies-Somer, 1997; Jousimies-Somer & Summanen, 2002; Vaisanen

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Abbreviations: ITS, internal transcribed spacer; PELO, '*Porphyromonas endodontalis*-like organism'.

The GenBank/EMBL/DDBJ accession numbers for the ITS and 16S rRNA gene sequences discussed in this study can be found in Figs 1 and 2.

A similarity matrix table (.xls), an alignment file (.aln) and a multi sequence file (.msf) of DNA–DNA hybridization data for all sequences discussed in this article are available as supplementary material in IJSEM Online. A figure showing representative gel-electrophoretic patterns of *Porphyromonas* species is also available.

et al., 1997). It should also be mentioned that there is a 16S rRNA gene sequence for '*Porphyromonas canis*' strain JCM 10100 in GenBank/EMBL/DBJ (accession no. AB034799), but this name has never been formally proposed or validated. Within the proposed order '*Bacteroidales*', *Tannerella forsythensis* (a species related to *Bacteroides distasonis* and *Bacteroides merdae*) is grouped within the proposed family '*Porphyromonadaceae*', so that all three might be close relatives of *Porphyromonas* species (Sakamoto *et al.*, 2002).

Sequence polymorphisms and length variations found in the 16S–23S rRNA gene internal transcribed spacer (ITS) are increasingly being used as tools for the differentiation of bacterial species and subspecies (Guasp *et al.*, 2000; Motoyama & Ogata, 2000; Conrads *et al.*, 2002). This is because the higher number of variable sites typical for the ITS sequence (Soller *et al.*, 2000) can overcome the apparent limitations of the resolution of 16S rRNA-based phylogenies in some genera where amplification and sequencing of this region is easy, almost free of ambiguities and reproducible, as has been described for *Fusobacterium* (Conrads *et al.*, 2002).

The current study was performed to generate ITS data for most of the type strains of the genus *Porphyromonas* and for *T. forsythensis*, *B. distasonis* and *Prevotella melaninogenica* (outgroup), and to compare a phylogenetic tree deduced from these data with one deduced from corresponding 16S rRNA gene data. The ITS sequences were further used to clarify the phylogenetic relationship between *Porphyromonas gingivalis* and *Porphyromonas gulae*, as well as between atypical α -fucosidase-negative isolates of *Porphyromonas asaccharolytica* (which are phenotypically indistinguishable from the PELO group; Vaisanen *et al.*, 1997) and typical α -fucosidase-positive *Porphyromonas asaccharolytica*.

METHODS

Bacterial strains, culture conditions and DNA extraction. The following bacterial strains were used: *Porphyromonas asaccharolytica* ATCC 25260^T; RMA (R. M. Alden Research Laboratory) 7115 (sacral wound); RMA 7120 (toe); RMA 7178 (endocervix); RMA 8631 (rectal abscess); RMA 9240 (peritoneal); RMA 9603 (abdominal); RMA 9674 (appendiceal fluid); RMA 10263 (peritoneal); RMA 10884, RMA 10898, RMA 10955, RMA 10966, RMA 10997, RMA 11049, RMA 11138 and RMA 11258 (all from pelvic fluid); RMA 11290 (vaginal cupule); RMA 11582 (endometrial pus); RMA 11690 (endometrium); RMA 11666 (endometrial pus); RMA 11805 (pelvic fluid); RMA 12959, RMA 12984 and RMA 13273 (all from diabetic foot infections); *Porphyromonas cangingivalis* NCTC 12856^T; *Porphyromonas cansulci* NCTC 12858^T; *Porphyromonas circumdentaria* NCTC 12469^T; *Porphyromonas endodontalis* ATCC 35406^T; *Porphyromonas gingivalis* ATCC 33277^T; RMA 3725 (oral, mandible); RMA 4165 (oral, maxilla); RMA 10371 (peritoneal/abdominal fluid); *Porphyromonas gulae* ATCC 51700^T; *Porphyromonas gingivicanis* ATCC 55562^T; *Porphyromonas levii* ATCC 29147^T; *Porphyromonas macacae* ATCC 33141; *Porphyromonas salivosa* ATCC 49407^T; *B. distasonis* ATCC 8503^T; *T. forsythensis* ATCC 43037^T; and *Prevotella melaninogenica* ATCC 25845^T. The last three strains were used for contrast. All strains were cultivated

at 37 °C on Brucella agar (Anaerobe Systems, Morgan Hill, CA, USA) under anaerobic conditions using an anaerobic chamber. Genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen).

PCR amplification and DNA sequence analysis. The 16S primer SPFPorph (5'-GTA CAC ACC GCC CGT CAA GCC-3', corresponding to *Escherichia coli* positions 1390–1411) and the 23S primer SPRPorph (5'-TCG CAG CTT ATC ACG TCC TTC-3', corresponding to *E. coli* positions 62–42) were designed based on the complete genome of *P. gingivalis* W83 (GenBank/EMBL/DBJ accession no. NC_002950); however, the respective regions among bacterial small-and large-subunit sequences (RDP) are relatively conserved. PCR was carried out using an Uno I (Biometra) thermocycler in a volume of 100 μ l containing 1 $\times$ PCR buffer, 1.5 mM MgCl₂, 2 units *Taq* polymerase, 0.2 mM each of dATP, dCTP, dGTP and dTTP (Roche Biochemicals), 10 pmol SPFPorph forward primer, 10 pmol SPRPorph reverse primer and 100 ng template DNA. Primer oligonucleotides were synthesized using a DNA synthesizer (OLIGO 1000; Beckman). The amplification was performed using the following temperature profile and 30 cycles: denaturation, 1 min at 94 °C; annealing, 1 min at 52 °C; elongation, 2.5 min at 72 °C. Amplification products (aliquots of 10 μ l) were separated electrophoretically on a 2% macro agarose gel in 1 $\times$ TPE (80 mM Tris/phosphate, 2 mM EDTA, pH 7.5) for a minimum of 18 h at 30 V.

After purification using the Wizard DNA Clean-up system (Promega), the spacer DNA was directly sequenced in duplicate using a Big Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems) and an automated capillary DNA sequencer (API Prism 310; Applied Biosystems). Sequences were assembled using the program Vector NTI Suite 9.0 (InforMax) and aligned using the program GeneDoc (Nicholas & Nicholas, 1997). A phylogenetic tree was constructed by using the neighbour-joining method and the programs CLUSTAL W (Chenna *et al.*, 2003) and TreeView (Page, 1996), using *Prevotella melaninogenica* as an outgroup. The robustness of tree topologies and the statistical significance levels of interior nodes were determined by performing bootstrap analyses (1000 iterations) using the ARB software package (Ludwig *et al.*, 2004).

RESULTS AND DISCUSSION

Approximations of ITS lengths were obtained from agarose gels, as demonstrated in Fig. Aa (available as supplementary material in IJSEM Online). All *Porphyromonas* reference strains showed a single band between 970 bp (*Porphyromonas gingivalis* ATCC 33277^T) and 710 bp (*Porphyromonas circumdentaria* NCTC 12469^T). The four strains of *Porphyromonas gingivalis* analysed were almost identical by ITS amplicon length (970–960 bp) and sequence (97–99% similarity). In contrast, among 24 clinical isolates of *Porphyromonas asaccharolytica* and the type strain ATCC 25260^T, the length of the ITS amplicons was more variable and ranged from 1044 bp (*Porphyromonas asaccharolytica* RMA 10263, α -fucosidase-negative strain) to 960 bp (*Porphyromonas asaccharolytica* ATCC 25260^T, α -fucosidase-positive strain) (Fig. Ab, available as supplementary material in IJSEM Online).

In general, it was not possible to differentiate *Porphyromonas* species by comparing ITS gel-electrophoretic profiles alone (for length of amplicons see supplementary material). Further discrimination without need of sequencing might be possible by ITS restriction digest with

endonucleases, since we found considerable variation in restriction sites (e.g. *Ava*I, *Apa*LI, *Cla*I, *Eco*RI, *Hind*III and *Sma*I).

Sequencing the purified ITS amplicons of the *Porphyromonas* strains using SPFPorph and SPRPorph as primers led to nearly ambiguity-free sequence determination by comparing both runs and directions. Transfer RNA genes (type Ile and Ala) were found in all '*Porphyromonadaceae*' ITS sequences but not in *Prevotella melaninogenica*, using the program tRNAscan-SE, version 1.21 (Lowe & Eddy, 1997).

Phylogenetic tree reconstruction based on the ITS sequences (short version only in the case of *Prevotella melaninogenica*) is demonstrated in Fig. 1. As mentioned above, the different strains of *Porphyromonas gingivalis* matched on a 97–99 % level and the two *Porphyromonas macacae* ATCC strains (ATCC 49407^T is the type strain of *Porphyromonas salivosa*, which has been reclassified) matched on a 94 % level; however, *Porphyromonas asaccharolytica* was more heterogeneous (80–99 % range in similarity level). Even more interesting, this latter species, which phenotypically differed in α -fucosidase activity, showed two main clusters. Intercluster similarity was only

Fig. 1. Phylogram (neighbour-joining method) showing the genetic relationships among *Porphyromonas* species based on the DNA sequences of their 16S–23S rRNA gene spacer regions. *B. distasonis* ATCC 8503^T, *T. forsythensis* ATCC 43037^T and *Prevotella melaninogenica* ATCC 25845^T (outgroup, based on the short version of spacer) were included for contrast. The numbers at the nodes indicate the percentage recovery in 1000 bootstrap resamplings. Only bootstrap values above 50 % are shown. Bar, 0.1 substitution per nucleotide.

80–87 %, whereas intracluster similarity was 92–99 % and thus more comparable with the phylogenetic relatedness of *Porphyromonas gingivalis* strains.

Based on the spacer sequences, four groups were distinguished. *Porphyromonas gingivalis* and *Porphyromonas gulae* represented an individual branch with 84–85 % similarity to each other, justifying their separation into two different species. A second group was formed by *Porphyromonas endodontalis*/*Porphyromonas gingivicanis* and *Porphyromonas circumdentaria* (75–81 %) and a third group by *Porphyromonas levii* and *Porphyromonas cangingivalis* (69 %). The latter two groups were again related by ITS but not by 16S information (Fig. 2). The fourth group was formed by *Porphyromonas macacae*, *Porphyromonas*

asaccharolytica and *Porphyromonas cansulci*, which showed a match between 66 and 68 %.

T. forsythensis and *B. distasonis* showed 57 % similarity to each other and were related by 43–70 % to the *Porphyromonas* species and by 43–57 % to *Prevotella melaninogenica* (43–57 %). With the exception of *Porphyromonas cansulci*, cluster analysis by ITS and 16S information led to the same qualitative result. However, whereas the similarities calculated by ITS information were between 44 % (*Porphyromonas levii* versus *Porphyromonas gulae*) and 85 % (*Porphyromonas gingivalis* versus *Porphyromonas gulae*), similarities calculated by 16S information were found to be much higher and between 83 % (*Porphyromonas asaccharolytica* versus *Porphyromonas catoniae*) and 97 %

Fig. 2. Phylogram (neighbour-joining method) showing the genetic relationships among *Porphyromonas* species based on information from their 16S rRNA gene sequences. *B. distasonis* ATCC 8503^T, *T. forsythensis* ATCC 43037^T and *Prevotella melaninogenica* ATCC 25845^T (outgroup) were used for contrast. The numbers at the nodes indicate the percentage recovery in 1000 bootstrap resamplings. Only bootstrap values above 50 % are shown. Bar, 0.1 substitution per nucleotide.

(*Porphyromonas gingivalis* versus *Porphyromonas gulae*), underlining the higher resolution potential by ITS sequence analyses. The higher resolution was further used to analyse the relationship between nine α -fucosidase-positive and 16 α -fucosidase-negative strains of *Porphyromonas asaccharolytica* and clearly showed that both groups diverged into individual phylogenetic branches (Fig. 3a). While the α -fucosidase-negative strains phenotypically resemble the previously studied extra-oral isolates of PELOs, it is obvious that they are not genetically related to *Porphyromonas endodontalis* (Fig. 3b).

As we have previously reported for fusobacterial species, deducing bacterial phylogenetic relationships from 16S–23S rRNA gene ITS sequences seems to have several advantages over using 16S rRNA/DNA alone (Conrads *et al.*, 2002), especially when comparing species that are closely related. The phylogenetic differences are not only expressed in the sequence information itself but also by the different lengths of amplicons and, in some cases, the formation of distinct band patterns by gel electrophoresis resulting from variations among the *rrn* operons in the same strain (Christensen *et al.*, 2000; Iteman *et al.*, 2000). However, in some members of the *Proteobacteria* and in low-G+C Gram-positive genera such as *Bacillus* and *Clostridium*, six to ten rRNA operons exist, interfering with the reproducibility of ITS sequencing (Boyer *et al.*, 2001). Furthermore, it was demonstrated by the same group that one strain might possess several ITS regions which are identical in length but differ by sequence. As our ITS sequences – deduced from 38 *Porphyromonas* strains – were almost ambiguity-free, we conclude that the existence of multiple rRNA operons with significant difference in sequence in this genus is not very likely, which is in accordance with the findings of Nadkarni *et al.* (2002), who studied *Porphyromonas endodontalis* and other species using rRNA-operon-directed real-time quantitative PCR.

PCR amplification of the ITS region using newly designed primers, and subsequent gel electrophoresis of 11 different *Porphyromonas* reference strains plus three clinical isolates of *Porphyromonas gingivalis* and 24 of *Porphyromonas asaccharolytica*, showed larger heterogeneity in length of amplicons in comparison to *Fusobacterium* species (Conrads *et al.*, 2002). Furthermore, only one distinct amplification band was produced with *Porphyromonas* species as well as with the relatives *T. forsythensis* and *B. distasonis*, unlike *Fusobacterium* species (one to four bands; Conrads *et al.*, 2002), *Prevotella melaninogenica*, *Prevotella intermedia* (two bands, data not shown) or many other genera analysed so far (Graham *et al.*, 1997; Gurtler *et al.*, 1999; Motoyama & Ogata, 2000). However, judging by the electrophoretic pattern of *Porphyromonas asaccharolytica* strains, where a 50 bp shorter, ‘shadow-like’ second band can be seen (Fig. Ab, available as supplementary material in IJSEM Online), a second 16S–23S rRNA gene ITS sequence might exist. Within a species, the length of amplicons and the deduced sequence is relatively constant as we have shown

Fig. 3. (a) Cladogram showing the genetic relationships among 25 strains of *Porphyromonas asaccharolytica* including 16 α -fucosidase-negative strains, eight α -fucosidase-positive strains and the type strain ATCC 25260^T. *Porphyromonas macacae* ATCC 33141^T was used as outgroup. (b) The phylogram (neighbour-joining method) gives an overview about the genetic (ITS-based) distance of the α -fucosidase-negative strains also known as PELOs and *Porphyromonas endodontalis* (ATCC 35406^T). They build a separate cluster in the *Porphyromonas asaccharolytica* branch of the genus *Porphyromonas*.

for *Porphyromonas gingivalis* (four strains), *Porphyromonas macacae* (two strains) and for fusobacterial species and subspecies. Thus, the heterogeneity found between the 25 *Porphyromonas asaccharolytica* strains could harbour an unrecognized species.

The high resolution of ITS sequences led to a separation of *Porphyromonas asaccharolytica* strains into two clusters: one cluster was α -fucosidase-positive, as is typical of the type strain, and the other was α -fucosidase-negative and biochemically resembled the previously studied unusual extra-oral isolates of PELOs. PELOs have been isolated as part of the mixed flora from extra-oral infections in adults (for example, from appendicitis with or without peritonitis, infected sacral decubitus ulcer, infected mastoid bone and pilonidal abscess) and have also been found in faecal specimens from children (Finegold & Jousimies-Somer, 1997). However, the isolation of PELOs from extra-oral sites is in contrast to the oral origin of *Porphyromonas endodontalis* but correlates with the *Porphyromonas asaccharolytica*-related cluster. By current biochemical methods, α -fucosidase-negative *Porphyromonas asaccharolytica* strains cannot be differentiated from *Porphyromonas endodontalis* (Jousimies-Somer *et al.*, 2002). Both are indole-positive, lack the typical glycosidase and peptidase enzymes, demonstrate fluorescence at 365 nm and do not ferment carbohydrates. In peptone/yeast/glucose (PYG) medium, acetic, propionic, butyric and isovaleric acids are produced as major metabolic products as well as isobutyric and succinic acids as minor products. The main cellular fatty acid is iso-C_{15:0}, followed by 3-OH iso-C_{17:0}, C_{16:0}, iso-C_{13:0}, 3-OH iso-C_{15:0}, C_{14:0} and minor amounts of anteiso-C_{15:0}. This profile is not distinctively different from that of *Porphyromonas asaccharolytica* (Vaisanen *et al.*, 1997) but it is different from that of *Porphyromonas endodontalis*, which produces neither C_{14:0} nor 3-OH iso-C_{15:0}, and less iso-C_{13:0}. Again, this supports our theory that PELOs are not related to *P. endodontalis* but are identical to α -fucosidase-negative *P. asaccharolytica* strains.

The separation between *Porphyromonas gingivalis* and *Porphyromonas gulae* as distinct species was supported by our ITS data; thus, *Porphyromonas gulae* should not be referred to as the 'animal strain of *Porphyromonas gingivalis*' as it is genetically related but not identical to *Porphyromonas gingivalis*. Fournier *et al.* (2001), describing *Porphyromonas gulae*, pointed out the paradox that although this species could be distinguished from *Porphyromonas gingivalis* phenotypically and by DNA–DNA similarity, the differences between genes encoding 16S rRNA were small. They also concluded that the recent divergences of ancestral phyla (for example, after colonizing different mammalian hosts) could not be sufficiently discerned by 16S information. Again, at least in some genera, ITS data give additional information and enhance phylogenetic resolution if discrepancies between DNA–DNA hybridization and 16S sequencing results are observed.

In conclusion, the ITS region is being used increasingly as an important tool for classification and differentiation of bacterial species. Our study is the first to provide this sequence information for most of the *Porphyromonas* species and their relatives. The higher resolution of ITS sequences helped clarify some of the current problems in

molecular taxonomy. In addition, we found that the name PELO should no longer be used for α -fucosidase-negative strains of *Porphyromonas asaccharolytica* that resemble *Porphyromonas endodontalis*. Instead, the name PALO ('*Porphyromonas asaccharolytica*-like organism') would be more suitable. Moreover, because of the reduced ITS similarity (80–85%) between the α -fucosidase-positive *Porphyromonas asaccharolytica* ATCC 25260^T and the α -fucosidase-negative cluster, the proposal of a new species seems to be warranted (Finegold *et al.*, 2004).

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