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16S–23S rDNA internal transcribed spacer sequences for analysis of the phylogenetic relationships among species of the genus *Fusobacterium*

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The 16S–23S rDNA internal transcribed spacer (ITS) regions of all currently defined *Fusobacterium* species and related taxa such as *Leptotrichia buccalis*, *Sebaldella termitidis* and *Streptobacillus moniliformans*, were analysed to examine inter- and intraspecies as well as subspecies relationships. For the ITS-amplification, a new eubacterial universal primer pair was designed and used. The majority of the *Fusobacterium* strains, along with *L. buccalis* showed one major, and two to three weaker, distinct bands (short and long versions) with lengths of 800–830 bp and 1000–1100 bp. Nevertheless, six other patterns were also found within the genus *Fusobacterium*, demonstrating its heterogeneity. The ITS region was sequenced and found to consist both of conserved motifs, which functioned as a framework for alignment, and of variable sites, which provided high phylogenetic resolution. Analyses of the ITS-DNA sequences and ITS relative length (short version) allowed species and subspecies differentiation in most cases. The results confirmed the strikingly distant relationship between *Fusobacterium prausnitzii* and the genus *Fusobacterium*. *Fusobacterium nucleatum* subspecies, along with *Fusobacterium naviforme*, *Fusobacterium simiae* and *Fusobacterium periodonticum*, formed a cluster with an inherently high potential for diversification. Other clusters were formed by *Fusobacterium necrophorum* subspecies with *Fusobacterium gonidaformans* and by *Fusobacterium varium* with *Fusobacterium mortiferum* and *Fusobacterium ulcerans*. *Fusobacterium russii* as well as *Fusobacterium perfoetens* formed separate branches. *Fusobacterium necrophorum* subspp. *necrophorum* and *funduliforme* on the one hand, and *Fusobacterium varium* and *Fusobacterium mortiferum* on the other, were found to be very similar, even at the high-resolution ITS level.

Keywords: *Fusobacterium*, *Leptotrichia*, *Sebaldella*, *Streptobacillus*, 16S–23S rDNA internal transcribed spacer

INTRODUCTION

Fusobacteria are obligately anaerobic, non-spore-forming, Gram-negative, non-motile, pleomorphically rod-shaped bacilli. The DNA base composition within this genus is heterogeneous: whereas the majority of strains have a restricted range of 26–34 mol% G+C, strains of *Fusobacterium prausnitzii* (ATCC 27768^T and ATCC 27766; 49–57 mol%) and *Fusobacterium naviforme* (ATCC 25832; 49 mol%) are well outside this range. It was therefore suggested that they may belong to a different taxonomic group (Bennett & Eley, 1993; Gharbia & Shah, 1990; Wang *et al.*, 1996).

It is widely accepted that comparative analysis of small-subunit rRNA sequences is a powerful tool for

Abbreviation: ITS, internal transcribed spacer.

A similarity matrix table and an alignment file of DNA–DNA hybridization data for all 33 taxa sequenced are available in IJSEM Online (http://ijs.sgmjournals.org).

The GenBank/EMBL/DDBJ accession numbers for the sequences described in this work are AF342829–AF342861.

Table 1. Number and length of different amplificons as well as the 16S/spacer/23S composition of internal transcribed spacer amplificons in fusobacterial species and relatives

Data for *Actinobacillus actinomycetemcomitans* ATCC 33384^T, *Bacteroides forsythus* ATCC 43037^T, *Escherichia coli* ATCC 25922, *Eubacterium lentum* ATCC 43055, *Porphyromonas gingivalis* ATCC 33277^T and *Streptococcus mutans* ATCC 25175^T, used for contrast, are not shown.

Species	Strain	No. of bands	Short version (bp)	Long version (bp)	Dimer (facultative) (bp)	16S part (bp)	Space (bp)	23S part (bp)
Fusobacterium mortiferum	ATCC 25557^T	3–4	830	1050	2100	112	168	501
Fusobacterium mortiferum	ATCC 9817	3–4	830	1050	2100	112	168	501
Fusobacterium ulcerans	NCTC 12111^T	3–4	830	1050	2100	112	171	496
Fusobacterium ulcerans	NCTC 12112	3–4	830	1050	2100	112	171	496
Fusobacterium varium	ATCC 8501^T	3–4	830	1050	2100	112	168	498
Fusobacterium varium	ATCC 27725	3–4	830	1050	2100	112	168	498
Fusobacterium necrogenes	ATCC 25556^T	3–4	880	1080	2200	112	208	490
Fusobacterium nucleatum subsp. *nucleatum*	ATCC 23726	3–4	800	1050	1900	112	124	496
Fusobacterium nucleatum subsp. *nucleatum*	ATCC 25586^T	3–4	810	1050	1950	112	135	498
Fusobacterium nucleatum subsp. *fusiforme*	ATCC 51190^T	3	800	1050	–	112	123	496
Fusobacterium nucleatum subsp. *polymorphum*	RMA 7159^T	1	850–920	–	–	112	151	479
Fusobacterium nucleatum subsp. *polymorphum*	ATCC 10953^T	1	850–920	–	–	112	151	479
Fusobacterium nucleatum subsp. *vincenti*	ATCC 49256^T	3	800	1050	–	112	121	496
Fusobacterium nucleatum subsp. *animalis*	RMA 6840	3	810	1050	–	112	132	497
Fusobacterium nucleatum subsp. *animalis*	RMA 6681	3	810	1050	–	112	132	498
Fusobacterium nucleatum subsp. *animalis*	ATCC 51191^T	3	810	1050	–	112	132	497
Fusobacterium simiae	ATCC 33568^T	3	810	1050	–	112	132	497
Fusobacterium periodonticum	ATCC 33693^T	1	900	–	–	112	186	498
Fusobacterium naviforme	ATCC 25832^T	1	800	–	1900	112	123	498
Fusobacterium russii	ATCC 25533^T	1–2	800	–	1900	112	115	497
Fusobacterium necrophorum subsp. *funduliforme*	ATCC 51357^T	1	830	–	–	112	139	495
Fusobacterium necrophorum subsp. *necrophorum*	ATCC 27852	1	830	–	–	112	139	495
Fusobacterium necrophorum subsp. *necrophorum*	NCTC 10575	1	830	–	–	112	139	495
Fusobacterium necrophorum subsp. *necrophorum*	ATCC 25286^T	1	830	–	–	112	139	495
Fusobacterium gonidiaformans	RMA 11660	3–4	800	1000	1900	112	168	462
Fusobacterium gonidiaformans	RMA 11653	3–4	800	1000	1900	112	168	462
Fusobacterium gonidiaformans	ATCC 25563^T	3–4	800	1000	1900	112	168	462
Fusobacterium perfoetens	ATCC 29250^T	1	830	–	–	112	115	518
Leptotrichia buccalis	RMA 2181	3	810	1100	–	112	108	496
Leptotrichia buccalis	ATCC 14201^T	3	810	1100	–	112	108	496
Fusobacterium prausnitzii	ATCC 27766	3	950	1150	–	112	262	480
Fusobacterium prausnitzii	ATCC 27768^T	3	950	1150	–	112	262	480
Streptobacillus moniliformans	ATCC 14647^T	3–4	900	1100	2300	112	207	497
Sebaldella termitidis	ATCC 33386^T	3–4	810	1000	2000	112	113	447

investigating the phylogenetic relationships of especially biochemically inert micro-organisms. Nevertheless, because of the limited resolution of 16S sequences, different data-mining approaches can lead to different results, as has been reported for *Fusobacterium alocis*. The 16S rRNA sequence-based distance matrix used by Lawson *et al*. (1991) confirmed the similarity between *Fusobacterium alocis* and the *Fusobacterium nucleatum* group, whereas the recent publication by Jalava & Eerola (1999) led to the reclassification of this species as the Gram-positive *Filifactor alocis*.

Sequence polymorphism and lengths found in the 16S–23S rDNA 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). The higher number of variable sites typical of the ITS sequence (Soller *et al*., 2000) seems to overcome some of the apparent limitations of the phylogenetic resolution of the 16S rDNA. Although variable, the spacer is sufficiently conserved to guarantee a stable classification (Anton *et al*., 1998; Gürtler, 1999; Iteman *et al*., 2000; Perez Luz *et al*., 1998).

This study was performed to differentiate, and construct a tree for, strains of the genus *Fusobacterium* and for other related members of the 'fusobacterial group'. To amplify the 16S–23S ribosomal ITS, a new eubacterial PCR primer set was designed. The length and pattern of the amplificons, together with patterns of DNA sequence variability, were used to clarify the phylogenetic relationship of *Fusobacterium* subspp. and related genera.

METHODS

Bacterial strains, culture conditions and DNA extraction. The bacterial strains used in the present study are listed in Table 1 and were either directly received from a type culture collection or phenotypically characterized at the R.M. Alden Research Laboratory according to the original description in *Bergey's Manual* (Murray *et al*., 1984). Fusobacterial strains and *Leptotrichia buccalis* were cultivated at 37 °C on Brucella agar (Anaerobe Systems) under anaerobic conditions, using an anaerobic chamber; aerobes (used for contrast) were cultivated on blood-agar plates (Hardy Diagnostics). DNA was extracted using the DNeasy Tissue Kit (Qiagen).

PCR amplification and DNA sequence analysis. The 16S primer SPF (5′-GGT GTG ACG GGC GGT GTG TAC-3′, *Escherichia coli* position 1391–1411) was designed on the basis of universal 16S sequence information (Ribosomal Database Project II). The target sequence of the degenerated 23S primer SPR [5′-GGT (TG)CT TTT C(GA)C CTT TCC-3′, *Escherichia coli* position 468–485] was highly conserved among eubacterial large-subunit sequences (Ribosomal Database Project). A PCR was carried out using a Biometra Uno I (Biometra) thermocycler in a volume of 100 µl containing 1 × PCR buffer, 1·5 mM $MgCl_2$, 2 U *Taq*-polymerase (Boehringer Mannheim), 0·2 mM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim), 10 pmol SPF forward primer, 100 pmol reversed primer SPR, and 100 ng template nucleic acids. Primer oligonucleotides were synthesized using a DNA synthesizer (OLIGO 1000; Beckman). The amplification was performed using the following temperature profile and 30 cycles: denaturation for 1 min at 94 °C; annealing for 1 min at 50 °C; elongation for 2·5 min at 72 °C.

Amplification products (aliquots of 10 µl) were separated electrophoretically on a 2 % (w/v) macro agarose gel in 1 × 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 *Taq* Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic capillary DNA sequencer (API PRISM 310; Applied Biosystems). Sequences were assembled using the program VECTOR NTI Suite (InforMax) and aligned using the program GENEDOC (Nicholas & Nicholas, 1997). A phylogenetic tree was constructed by using the neighbour-joining method and the program CLUSTAL W (Jeanmougin *et al*., 1998) using *Fusobacterium prausnitzii* as an outgroup.

RESULTS

Approximations of ITS lengths were obtained from agarose gels, as demonstrated in Fig. 1. Table 1 gives the number and lengths of different amplificons found in each species and strain tested. In some species (e.g. *Fusobacterium mortiferum*, *Fusobacterium ulcerans* or *Fusobacterium gonidioformans*), larger amplificons, probably ITS dimers 1900–2200 bp long, occurred facultatively in addition to the monomeric ITS (800–1080 bp).

According to this, only a few species, such as *Fusobacterium nucleatum* subsp. *polymorphum*, *Fusobacterium periodonticum* and *Fusobacterium prausnitzii*, showed a unique pattern of PCR bands in gel electrophoresis. In addition, *Fusobacterium nucleatum* subsp. *animalis* and *Fusobacterium necrogenes* could be identified either by small differences in the lengths of amplificons or by the comparative intensities of their bands. In general, however, it was not possible to differentiate fusobacterial species by comparing ITS gel-electrophoretic profiles alone. Further discrimination without the need for sequencing might be possible by ITS restriction, since we found variations in the following restriction sites: *Eco*RI (bp 676–731), *Hin*dIII (bp 303–454) and *Ava*I (bp 358–473). *Fusobacterium prausnitzii* was the only *Fusobacterium* species without an *Eco*RI restriction site. Reference strains of the chosen relatives *Sebaldella termitidis* or *Streptobacillus moniliformans* and the more distantly related species, such as *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, *Escherichia coli*, *Eubacterium lentum*, *Porphyromonas gingivalis* and *Streptococcus mutans*, demonstrated gel-electrophoretic patterns that were completely different from those for fusobacteria when the ITS-directed PCR method described was used (data are shown only for *Sebaldella termitidis* and *Streptobacillus moniliformans*; Table 1).

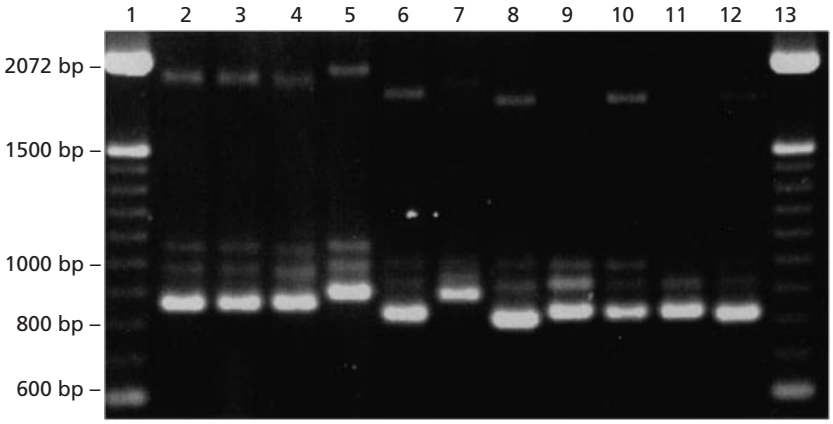

Fig. 1. Representative gel-electrophoretic ITS amplification patterns of fusobacterial species. Lanes: 1, marker; 2, *F. mortiferum* ATCC 25557^T; 3, *F. ulcerans* NCTC 12111^T; 4, *F. varium* ATCC 8501^T; 5, *F. necrogenes* ATCC 25556^T; 6, *F. nucleatum* subsp. *nucleatum* ATCC 25586^T; 7, *F. periodonticum* ATCC 33693^T; 8, *F. russii* ATCC 25533^T; 9, *F. necrophorum* subsp. *necrophorum* ATCC 25286^T; 10, *F. gonidiaformans* ATCC 25563^T; 11, *F. perfoetens* ATCC 29250^T; 12, *F. naviforme* ATCC 25832^T; 13, marker.

16S rRNA gene

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001GAAGTAGCAGGCCTAACCGTAAGGAGGGATGTTCCGAGGGTGTGATTAGC
051GATTGGGGTGAAGTCGTAACAAGGTATCCGTACGGGAACGTGCGGATGGA
101TCACCTCCTTTCTAAGGAGAATGTGTCTTTCTCTATTCTATTGGTAATGT
151TCTTACATTACTTCTGAACATTGGAAACTATATAGTAGAACAAACAAGAA
201AAAAATTAACTCTAAACAATTTCTTTAGAGTTAGCTTGNCAAAAAATAGG
251TTAAAATAATTAAGGGCACACAAAGGATGCCTAGGTAGTAAGAGCCGATG
301AAGGACGTGGTAAGCCTGCGATAAGCCTAGATAAGTTGCAATCGAACGTA
351AGAGTCTAGGATTTCCGAATGGAGCAATCTATTAAGATGGAGTCTTAATA
401CGAAAGAGGGAACCGCGTGAACTGAAACATCTAAGTACCGCGAGGAAAAG
451AAAGTAAAAACGATACCCAAAGTAGCGGCGAGCGAACTGGGTCAAGCCTA
501AACCTTAAATATGTCAAGGATACAGCCGTTGTATTTAAGGGGTAGAGGGA
551CAAAGTAGTGAAGAACTGTAAGATATTCAATATAGTGTATTGATGAATTA
601GAATTGTCTGGAAAGATGAACCGCAGAAGGTGAAAGTCCTGTATAAGTAA
651ATCCTTACACATATAACTTTGCTCCCAAGTAACATGGAACACGAGGAATT
701CTGTGTGAATCAGTGAGGACCAAATCTCATAAGGCTAAATACTCT
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23S rRNA gene

Fig. 2. Representative ITS region deduced from the type species *Fusobacterium nucleatum* subsp. *nucleatum* (ATCC 25586^T). Highlighted: conserved regions (the *Eco*RI restriction site is underlined). Bold: 16S rRNA–23S rRNA spacer.

Sequencing of the purified amplificons using SPF and SPR as primers was performed in duplicate and led to nearly ambiguity-free sequence determination by comparing both runs and directions. Fig. 2 shows the sequence deduced from *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586^T with conserved regions highlighted for orientation. A similarity matrix table and an alignment file of DNA–DNA hybidization data for all 33 taxa sequenced are available in IJSEM Online (http://ijs.sgmjournals.org).

In the spacer region, approximately 3·6 ambiguities appeared. Species demonstrating ITS patterns either with confluent bands (such as *Fusobacterium nucleatum* subsp. *polymorphum*) or with four bands (such as *Fusobacterium gonidiaformans*) had more unresolvable ambiguities (13 maximum) in the deduced sequence. Since the short ITS version was obviously favoured in the PCR and amplified in much higher numbers relative to the long fragments (which probably contain tRNA gene insertions), the resulting sequence clearly represents the tRNA-free version only. A database search of tRNA consensus sequences and comparison with our fusobacterial spacer DNA revealed no matches.

The phylogram deduced from the ITS sequences is demonstrated in Fig. 3. From one type strain (*Fusobacterium varium* ATCC 8501^T), two cultures from different sources (the strain collection of the University of Leipzig, Germany and the R.M. Alden Research Laboratory, Santa Monica, CA) were sequenced as an internal control and (Seq. 1 and Seq. 2 in Fig. 3) found to be $>$ 99 % identical. The different strains of most *Fusobacterium* species matched at a 96–100 % level. Excluding Ns, relevant differences were found only among strains within the *Fusobacterium nucleatum* subspecies *nucleatum* and *animalis*, respectively. According to the spacer sequences, three major groups could be discriminated.

The species of the *Fusobacterium mortiferum/varium/ulcerans* group were found to be very closely related (93–97 % similarity), and related to *Fusobacterium necrogenes* (79–81 %). A second group was formed by all *Fusobacterium nucleatum* subspecies along with *Fusobacterium simiae*, *Fusobacterium periodonticum* and *Fusobacterium naviforme*, which showed a match between 81 and 95 %. A third group was formed by both of the *Fusobacterium necrophorum* subspecies and *Fusobacterium gonidiaformans* (79–98 %).

Fusobacterium russii represents an individual evolutionary branch within the main fusobacterial cluster, and *Fusobacterium perfoetens* is distantly related to all other core species with a similarity of only 71–78 %.

Fusobacterium prausnitzii, represented in this study by two reference strains, appeared to be unrelated to fusobacteria (showing only 48 % similarity to the type species *Fusobacterium nucleatum* subsp. *nucleatum*). Representatives of the most closely related genera (16S rRNA data), *Leptotrichia* (*L. buccalis* ATCC 14201^T), *Sebaldella* (*Sebaldella termitidis* ATCC 33386^T) and *Streptobacillus* (*Streptobacillus moniliformans* ATCC 14647^T) showed a closer relationship (66, 66 and 63 %, respectively) to the fusobacterial type species than did *Fusobacterium prausnitzii*. Even *Escherichia coli* (se-

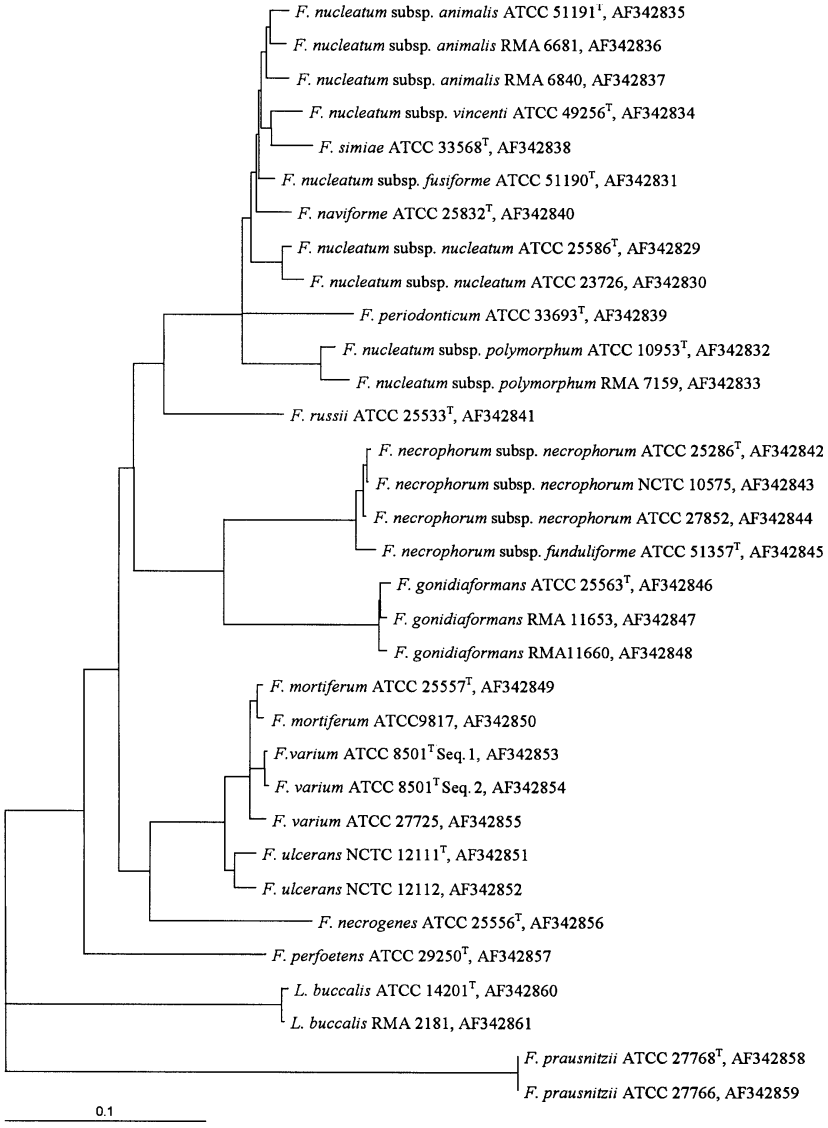

Fig. 3. Phylogram (constructed using the neighbour-joining method) showing the genetic relationships among fusobacterial species, based on the DNA sequences of their short 16S–23S rDNA spacer regions (GenBank accession numbers are included). *Fusobacterium prausnitzii* strains ATCC 27766 and ATCC 27768[T] were used as an outgroup.

quence imported from strain RIMD 0509952; GenBank accession no. AB035920) demonstrated a higher similarity (50%) to the fusobacterial type species after deletion of the alanine tRNA from the spacer region.

DISCUSSION

Deducing bacterial phylogenetic relationships from 16S–23S rDNA ITS sequences seems to have several advantages over using 16S rRNA–DNA alone. The phylogenetic differences are not only expressed in the sequence information itself but also by the different lengths of amplificons and, in some cases, in the formation of distinct band patterns (in gel electrophoresis) resulting from variations among the *rrn* operons in the same strain (Christensen *et al.*, 2000; Iteman *et al.*, 2000).

The taxonomy of fusobacterial species and some related genera and species is still a scientific riddle, especially with respect to the five controversial subspecies in *Fusobacterium nucleatum* (Gharbia & Shah, 1992) and the two in *Fusobacterium necrophorum* (Okwumabua *et al.*, 1996; Shinjo *et al.*, 1991). *Fusobacterium alocis* and *Fusobacterium sulci* have already been reclassified as *Filifactor alocis* and *Eubacterium sulci*, on the basis of 16S rDNA sequences (Jalava & Eerola, 1999).

PCR amplification of the ITS region by using newly designed primers and 33 fusobacterial strains showed striking differences after gel electrophoresis in only a limited number of species. Nevertheless, patterns consisting of one band or up to a maximum of four bands were produced with each strain tested. Thus, most *Fusobacterium* species exhibit different variants of the 16S–23S rDNA spacer as described for other taxa (Graham *et al.*, 1997; Gürtler *et al.*, 1999; Motoyama & Ogata, 2000). Within a species or subspecies, as we have seen in our testing, the pattern and the deduced sequence is relatively constant and

matches at the 97–100 % level, *Fusobacterium nucleatum* subsp. *nucleatum* being the only exception (< 97 %).

The high resolution of ITS sequences led to a striking departure of *Fusobacterium prausnitzii* from the core fusobacterial group in our analysis. This confirms the finding of a previous study that used 16S rDNA sequences to demonstrate that *Fusobacterium prausnitzii* clusters with *Eubacterium* and *Clostridium* groups III and IV (Wang *et al*., 1996). Clearly, *Fusobacterium prausnitzii* must be reclassified.

Because of its 49 mol % G+C content, it has been suggested that *Fusobacterium naviforme* may also be outside the fusobacterial group (Gharbia & Shah, 1990). However, the original *Fusobacterium naviforme* strain (ATCC 25832) tested in our study was related (93 % sequence similarity) to *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586^T; it showed a typical morphology (boat-shaped), a typical biochemical profile (weak glucose fermentation and a positive indole reaction) and was resistant to the 5 μg vancomycin disk. Interestingly enough, '*Fusobacterium naviforme* strain ATCC 25588' obtained from other laboratories showed *Eubacterium*-like spacer sequences, a different biochemical profile from our strain, and was susceptible to vancomycin, suggesting a Gram-positive organism (sequence data are not included in this study). Therefore, we recommend that the identity of *Fusobacterium naviforme* strains other than those received directly from the American Type Culture Collection (ATCC) or the R.M. Alden Research Laboratory (RMA) collection be confirmed according to the original description in *Bergey's Manual* (Murray *et al*., 1984).

Basically, our ITS data support the validity of subspecies within *Fusobacterium nucleatum* and its separation from *Fusobacterium periodonticum*. However, the ITS sequences in this cluster, which includes *Fusobacterium simiae* and *Fusobacterium naviforme*, showed a 'fan-like' branching. Together with the intra-subspecies diversity at a 96 % level in *Fusobacterium nucleatum* subsp. *nucleatum*, this fusobacterial branch seems to have an increased potential for genetic diversity in general. Further subtyping will undoubtedly occur after additional strains are sequenced, but a reintegration of all or some subspecies should also be considered to limit the number of fusobacterial taxa.

In contrast, ITS-sequencing could barely differentiate between *Fusobacterium necrophorum* subspecies *necrophorum* and *funduliforme*, or between *Fusobacterium varium* and *Fusobacterium mortiferum*, since the spacer sequences of these are up to 98 % identical to each other; yet *Fusobacterium varium* and *Fusobacterium mortiferum* can be differentiated by cell morphology and a few biochemical tests, such as the aesculin reaction and lactose fermentation (Claros *et al*., 1999).

The species *Fusobacterium russii* and *Fusobacterium perfoetens* formed individual branches quite separate from the clusters formed by *Fusobacterium nucleatum* subspecies/*Fusobacterium naviforme*/*Fusobacterium simiae*/*Fusobacterium periodonticum*, by *Fusobacterium necrophorum* subspecies/*Fusobacterium gonidaformans*, and by *Fusobacterium varium*/*Fusobacterium mortiferum*/*Fusobacterium ulcerans*. This might explain some very unique features found in these species, such as the unusual antimicrobial susceptibility we have found in *Fusobacterium russii* (Goldstein *et al*., 1999), or the coccoid morphology in *Fusobacterium perfoetens*, from which it got its original name, *Coccobacillus perfoetens*.

Whereas the ITS segments we have sequenced were found to consist of a 16S rRNA gene segment of constant length 112 bp, the lengths of the spacer itself (115–262 bp) and of the 23S rRNA gene part (462–518 bp) have variations specific to species or subspecies (Table 1, columns 8 and 9). As a possible new tool for bacterial detection, primers could be designed from corresponding flanking and conserved regions and used to identify fusobacteria without further restriction or sequencing.

In conclusion, the ITS spacer region is being increasingly used as an important tool for classification and differentiation of bacterial species. Our study is the first to provide this sequence information for all species of an obligately anaerobic genus. Its higher resolution resolves some of the current problems in molecular taxonomy. Some discrepancies exist, however, and a synergism between phenotypic and genotypic approaches is still needed.

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