Phylogenetic Relationship of *Fusobacterium necrophorum* A, AB, and B Biotypes Based upon 16S rRNA Gene Sequence Analysis

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The 16S rRNA genes from virulent (AB) and benign (B) biotype strains of *Fusobacterium necrophorum*, the causative agent of ovine foot abscess, were cloned and sequenced. Notwithstanding the distinct phenotypic differences between the AB and B biotypes, a phylogenetic analysis in which the 16S rRNA gene sequences were used revealed the close relationship of these taxa. Comparison of the virulent and benign biotypes of *F. necrophorum* may therefore be a legitimate way to identify key virulence factors useful in vaccine development.

Fusobacteria are obligately anaerobic, nonsporing, gram-negative, spindle-shaped rods that are either nonmotile or motile by means of peritrichous flagella (8, 17). Acetic and butyric acids are the characteristic major end products of carbohydrate metabolism (17). The DNA base compositions of *fusobacteria* range from 26 to 52 mol% G+C (average, 30 mol%) (10).

The ranges of hosts and tissues infected by *fusobacteria*, either alone or in synergy with other bacteria, vary widely, but not all species are pathogenic. Pathogenic species of *fusobacteria* have been recovered from humans with oral and dental infections, brain, hepatic, or intraabdominal abscesses, empyema, and endocarditis (9). *Fusobacterium* spp., specifically *Fusobacterium necrophorum*, infect domestic animals, causing footrot in cattle, foot abscesses in sheep, and hepatic abscesses in both species (15).

Historically, *F. necrophorum* has been divided into the following three biotypes: the virulent A biotype, the benign B biotype, and the nonpathogenic C biotype. There is also a fourth intermediate biotype designated AB (8). The biotypes differ in cellular and colonial morphology and in hemolysin and hemagglutinin production (Table 1). The A biotype is predominantly isolated from bovine hepatic abscesses, the AB biotype is most common in ovine and bovine foot lesions (6), and the B biotype is recovered consistently from ruminal contents but can also be isolated from lesions primarily caused by A and AB biotype strains (3, 13). The C biotype, the nonpathogenic biovar of *F. necrophorum*, can also be isolated from abscesses or fecal material (22).

The current taxonomic positions of the *F. necrophorum* biotypes, based on DNA-DNA homology data and biochemical and morphological differences (21, 22), are as follows: biotype A strains are placed in *Fusobacterium necrophorum* subsp. *necrophorum*, biotype B strains are placed in *Fusobacterium necrophorum* subsp. *funduliforme*, and biotype C strains have been reclassified in a new species, *Fusobacterium pseudonecrophorum*. Although cellular morphology and total DNA homology data can provide compelling evidence for establishing bacterial relatedness, it is recognized that comparisons of 16S rRNA sequences provide a highly sensitive method for substantiating taxonomic identity (23). Therefore, to clarify the

intraspecies relationships of the *F. necrophorum* biotypes and the relative positions of these biotypes on the *Fusobacterium* phylogenetic tree, we compared the 16S rRNA sequences of members of the AB and B biotypes with the 16S rRNA sequences of other *fusobacteria*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Representative *F. necrophorum* biotype AB and B strains were isolated from ovine foot abscess lesions and were designated FnS1 (5) and FnS40, respectively (22a). These isolates were initially identified by characteristics described by Fievez (8) and by standard biochemical tests (17). Cultures were grown anaerobically for 18 h at 37°C on brain heart infusion blood (bovine) agar plates (2) and then subcultured in modified Eugon broth and grown anaerobically for 18 h at 37°C (6). *Escherichia coli* DH5 α was the host strain for plasmid pTZ19U (Bio-Rad). *E. coli* transformants were grown in Luria broth (19) supplemented with 50 μ g of ampicillin (Sigma) per ml.

DNA techniques. Unless indicated otherwise, DNA manipulations were performed by the methods described by Sambrook et al. (19). *F. necrophorum* genomic DNA was extracted as previously described (11).

Cloning of the 16S rRNA genes. Portions (20 ng) of chromosomal DNAs from the *F. necrophorum* biotype AB and B strains were used as templates to amplify the 16S rRNA genes by using 200 ng of each of the following conserved eubacterial 16S rRNA primers: primer 27F (5' AGAGTTTGATCMTG GCTCAG 3') and primer 1525R (5' AAGGAGGTGWTG CARCC 3') (M = A or C, W = A or T, and R = A or G [15]). One unit of Vent DNA polymerase (New England BioLabs) was used per 50 μ l of reaction mixture. PCRs were carried out with a Perkin-Elmer thermocycler as previously described (11). The PCR product (5 μ l) was electrophoresed on a 1% agarose gel, and a 1,500-bp fragment was excised, purified by using GeneClean (Bresatec, Adelaide, Australia), resuspended in 8 μ l of distilled water, and blunt end ligated into the *Sma*I site of pTZ19U before being transformed into *E. coli* DH5 α .

Colony screening of PCR clones. A total of 200 *E. coli* 16S rRNA recombinants derived from the biotype AB and B strains were patched onto Hybond N nylon filters (Amersham) and hybridized with randomly labelled PCR products (7). The filters were hybridized for 16 h at 37°C with DNA probes in buffer containing 50% formamide, 1.0% sodium dodecyl sulfate (SDS), 0.5% skim milk powder, and 0.5% SSPE (SSPE is

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TABLE 1. Characteristics of *F. necrophorum* biotypes

Characteristic	Biotype A	Biotype B	Biotype AB	Biotype C	Reference(s)
Colony morphology ^a					
Color	Metallic grey	Yellow	Grey or yellow	Grey or transparent	6, 8, 21, 22
Surface	Flat	Convex	Convex/Flat	Flat	6, 8, 21, 22
Diam (mm)	2–4	1–2	2–4	2.5	8
Cell morphology					
Cell type ^b	Long GNR and F	Short GNR and F	Long GNR and F	Thick GNR and F	6, 8, 21, 22
Cell length (µm)	30–70	0.5–20	NT ^c	NT	8, 20, 22
Growth in broth	Turbid	Sedimentary	Turbid	Turbid	6, 8, 21, 22
Biochemistry					
Diam of hemolysis zone (mm) ^d	8–12	2–4	8–12	—	6, 8, 21, 22
Hemagglutinin ^e	+	—	+	—	6, 8, 21, 22
Lipase	+	—	+	—	6, 8, 21, 22
Pathogenicity ^f	+	+	NT	—	6, 21, 22
Lethality for mice	+	—	NT	—	6, 21, 22
Molecular biology					
G + C content (mol %)	28–31	27–31	NT	28–30	10, 21
No. of 16S rRNA genes on genome	4	4	5	NT	11
Repeat DNA sequence	+	—	+	NT	11
Taxonomy					
Species	<i>F. necrophorum</i>	<i>F. necrophorum</i>	<i>F. necrophorum</i>	<i>F. pseudonecrophorum</i>	21, 22
Subspecies	<i>F. necrophorum</i> subsp. <i>necrophorum</i>	<i>F. necrophorum</i> subsp. <i>funduliforme</i>	NA ^h	NA	21, 22

^a Colony morphology characteristics were determined by observing growth on brain heart infusion media supplemented with 5% bovine blood.

^b GNR, gram-negative rods; F, filaments.

^c NT, not tested.

^d Diameter of hemolytic zone on brain heart infusion plates supplemented with 5% bovine blood.

^e Hemagglutination of chicken erythrocytes.

^f +, present; —, absent.

^g Development of liver abscesses when organisms are injected into mice.

^h NA, not applicable.

0.18 M NaCl, 10 mM NaPO₄, and 1 mM EDTA [pH 7.7]) (18) and then washed at 65°C in 0.1 × SSC–0.1% SDS (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and exposed to Fuji type RX X-ray film.

DNA sequencing and analysis. The DNA sequences of the cloned 16S rRNA gene PCR products were determined on both strands by using double-stranded templates, the dideoxy-nucleotide chain termination method (20), modified T7 DNA polymerase (AMRAD Pharmacia Biotech), and a set of conserved 16S rRNA sequencing primers, including primers 27F, 357F, 342R, 530F, 519R, 926F, 907R, 1114F, 1406F, and 1525R (14). Two PCR clones from each biotype were sequenced. The sequence data were stored, assembled, and analyzed by using DNASIS software (AMRAD Pharmacia Biotech).

Phylogenetic analysis. 16S rRNA sequences from 24 *Fusobacterium* species were obtained from the GenBank and EMBL data bases. These sequences were aligned with the sequences determined in this study for *F. necrophorum* biotypes AB and B; the *E. coli* sequence was included as an outgroup. *Propionigenium modestum* and *Bacteroides termitidis* 16S rRNA sequences were included in the analysis on the basis of similarities identified during BLAST (1) data base searches. Gaps and ambiguous nucleotides were excluded from the analysis. Pairwise evolutionary distances were computed from the fractional sequence similarity values by the method of Jukes and Cantor (12) as implemented in the GJO Sequence Editor and Analysis program, version 3.0.4 Gary J. Olsen.

Dendrograms were constructed by using the Phylogenetic Tree Inference program (18).

Nucleotide sequence accession numbers. The sequences of the strain FnS1 (biotype AB) and FnS40 (biotype B) 16S rRNA genes have been deposited in the EMBL Data Library under accession numbers X74407 and X74408, respectively. The GenBank accession number for the *Propionigenium modestum* 16S rRNA sequence is X54275, the EMBL accession number for the *B. termitidis* 16S rRNA sequence is M58678, and the EMBL accession number for the *Fusobacterium prausnitzii* 16S rRNA sequence is M58682. The GenBank and EMBL accession numbers for the *Propionibacterium acnes* 16S rRNA sequence are X53218 and M61903, respectively, and the GenBank accession number for the *Actinomyces israelii* serotype 1 16S rRNA sequence is X53228.

RESULTS AND DISCUSSION

Cloning of the 16S rRNA genes from the AB and B biotypes of *F. necrophorum*. Conserved 16S rRNA gene primers 27F and 1525R (14) were used to generate 1,500-bp PCR products from genomic DNAs isolated from *F. necrophorum* biotype AB and B strains. These products were cloned, and *E. coli* transformants containing inserts were selected by colony hybridization, using labelled PCR products as probes. Two PCR clones of each biotype were sequenced by using conserved 16S rRNA primers (14), and the identities of approximately 1,400 bases of DNA sequence were determined on both strands for each

TABLE 2. Similarity matrix for members of the genus *Fusobacterium*

*sp	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1																										
2	0.993																									
3	0.993	0.993																								
4	0.995	0.995	0.994																							
5	0.995	0.995	0.991	0.987																						
6	0.995	0.993	0.989	0.998																						
7	0.958	0.959	0.963	0.962	0.963																					
8	0.959	0.961	0.965	0.964	0.965	0.998																				
9	0.950	0.950	0.954	0.953	0.954	0.961	0.963	1.000																		
10	0.947	0.948	0.950	0.948	0.949	0.962	0.962	0.990	0.990																	
11	0.945	0.947	0.948	0.947	0.948	0.963	0.965	0.989	0.989	0.993																
12	0.944	0.943	0.946	0.945	0.946	0.967	0.968	0.985	0.985	0.989	0.991															
13	0.943	0.945	0.947	0.946	0.946	0.961	0.963	0.989	0.989	0.989	0.993	0.991														
14	0.943	0.944	0.947	0.947	0.947	0.962	0.964	0.989	0.989	0.990	0.991	0.988	0.989													
15	0.947	0.948	0.950	0.950	0.950	0.962	0.964	0.985	0.985	0.982	0.981	0.989	0.991	0.984												
16	0.961	0.960	0.965	0.964	0.966	0.963	0.965	0.958	0.958	0.950	0.952	0.949	0.951	0.954	0.955											
17	0.955	0.956	0.958	0.959	0.961	0.964	0.968	0.968	0.958	0.951	0.955	0.950	0.954	0.954	0.955	0.973										
18	0.962	0.961	0.968	0.965	0.966	0.964	0.966	0.958	0.958	0.950	0.953	0.950	0.952	0.955	0.955	0.959	0.974									
19	0.956	0.957	0.959	0.960	0.962	0.965	0.966	0.957	0.957	0.950	0.953	0.950	0.953	0.953	0.954	0.954	0.976	0.976								
20	0.956	0.957	0.959	0.960	0.962	0.965	0.966	0.958	0.958	0.952	0.956	0.951	0.955	0.955	0.956	0.974	0.974	0.999	0.976	0.998						
21	0.955	0.958	0.960	0.961	0.963	0.965	0.966	0.958	0.958	0.951	0.955	0.950	0.953	0.954	0.955	0.973	0.973	0.998	0.973	0.986	0.987					
22	0.935	0.934	0.937	0.937	0.937	0.937	0.937	0.944	0.944	0.937	0.935	0.934	0.935	0.935	0.935	0.945	0.945	0.939	0.948	0.940	0.939					
23	0.783	0.780	0.784	0.787	0.789	0.786	0.784	0.780	0.780	0.781	0.778	0.775	0.775	0.779	0.779	0.780	0.783	0.790	0.784	0.784	0.783	0.780				
24	0.820	0.822	0.824	0.824	0.824	0.820	0.820	0.818	0.818	0.812	0.813	0.811	0.812	0.814	0.817	0.832	0.827	0.833	0.827	0.828	0.822	0.784				
25	0.950	0.951	0.951	0.951	0.951	0.958	0.958	0.961	0.961	0.963	0.963	0.960	0.963	0.960	0.963	0.955	0.953	0.956	0.952	0.954	0.953	0.957	0.783	0.807		
26	0.766	0.766	0.765	0.766	0.767	0.762	0.761	0.760	0.760	0.767	0.768	0.765	0.766	0.766	0.766	0.770	0.769	0.769	0.771	0.770	0.767	0.771	0.775	0.777	0.780	

* The values on the upper right are the proportions of nucleotide differences for members of the *F. necrophorum* group when the sequence of the type subspecies, *F. necrophorum* subsp. *necrophorum*, was used as the outgroup; 1,287 bases were used in the analysis. The values on the lower left are the proportions of nucleotide differences for members of the genus *Fusobacterium* when *E. coli* was used as the outgroup; 1,131 bases were used in the analysis. 1, *F. necrophorum* biotype AB; 2, *F. necrophorum* biotype B; 3, *F. necrophorum* biotype A strain ATCC 25286^T (GenBank accession number X55411) (T = type strain); 4, *Fusobacterium gonidiaformans* ATCC 25563^T (EMBL accession number M58679); 5, *F. gonidiaformans* ATCC 25563^T (GenBank accession number X55410); 6, *Fusobacterium russii* ATCC 25533^T (EMBL accession number M58681); 7, *F. russii* ATCC 25533^T (GenBank accession number X55409); 8, *Fusobacterium periodonticum* ATCC 33693^T (GenBank accession number X55405); 9, *F. necrophorum* subsp. *necrophorum* ATCC 25586^T (GenBank accession number X55403); 10, *F. necrophorum* ATCC 25586^T (EMBL accession number M58683); 11, *Fusobacterium siniae* ATCC 33568 (EMBL accession number M58685); 12, *F. necrophorum* subsp. *animalis* NCTC 12276^T (GenBank accession number X55404); 13, *Fusobacterium allosis* ATCC 35896^T (GenBank accession number X55406); 14, *F. necrophorum* subsp. *fusiforme* NCTC 11326^T (GenBank accession number X55403); 15, *F. necrophorum* subsp. *polymorphum* ATCC 10953^T (GenBank accession number X55402); 16, *F. moritiferum* ATCC 25557^T (EMBL accession number M58680); 17, *F. moritiferum* ATCC 25557^T (GenBank accession number X55414); 18, *Fusobacterium necrogenes* ATCC 25556^T (GenBank accession number X55401); 19, *Fusobacterium varium* ATCC 8501 (EMBL accession number M58686); 20, *F. varium* NCTC 10560^T (GenBank accession number X55413); 21, *Fusobacterium ulcerans* NCTC 12111^T (GenBank accession number X55408); 22, *Fusobacterium perfoetans* ATCC 29250 (EMBL accession number M58684); 23, *F. prausnitzii* ATCC 27166 (EMBL accession number M58682); 24, *Propionigenium modestum* (GenBank accession number X54275); 25, *B. termittidis* ATCC 33386 (EMBL accession number M58678); 26, *E. coli* ATCC, American Type Culture Collection; NCTC, National Collection Type Cultures.

FIG. 1. Phylogenetic tree of *Fusobacterium* 16S rRNA gene sequences. The tree is based on a comparison of 1,131 bases, and *E. coli* was used as the outgroup. Each scale bar division represents a 2% difference in sequences, as determined by measuring the lengths of the horizontal lines connecting two species. The numbers in parentheses are the bacterial strain numbers in Table 2.

clone. Sequence comparisons of the two PCR clones of each biotype revealed that variation was present only in the AB biotype; the variable bases represented 1.3% of the 1,400-bp sequence. It is unclear whether the differences were the result of mutations introduced during the PCR (although Vent polymerase has a 3'-to-5' proofreading exonuclease activity) or reflected bona fide nucleotide differences between copies of the 16S rRNA genes on the *F. necrophorum* chromosome. Comparisons of both strands of a DNA sequence should minimize sequencing discrepancies, in contrast to RNA sequence comparisons, in which the sequence of only one strand can be determined and thus there is no way to confirm data. Despite the observed differences between individual PCR clones, both sequences were included in the initial phylogenetic analysis.

Phylogenetic analysis of 16S rRNA sequences. To determine the intragenetic relationships among various fusobacteria and the AB and B biotypes of *F. necrophorum*, 16S rRNA sequences were manually aligned with the *E. coli* 16S rRNA sequence and the related *Propionigenium modestum* and *B. termitidis* sequences (data not shown). Compared with the *E. coli* 16S rRNA sequence, the *F. necrophorum* AB and B biotype sequences lacked 22 bases from position 458 to position 479 (*E. coli* numbering). In a previous study, this deletion was regarded as a *Fusobacterium* synapomorphism (shared derived characteristic) (16). However, the facts that the 22-bp segment was also absent from the 16S rRNA genes of *Propionigenium modestum* and *B. termitidis* (4) and was modified in *F. prausnitzii* to a 20-bp deletion suggest that this is probably not the case.

A total of 1,131 positions were included in the analysis; these positions were between coordinates 101 and 1450 (*E. coli* numbering). The phylogenetic relationships of the *F. necrophorum* biotypes did not alter using the two different 16S rRNA gene sequences obtained for each biotype; therefore, only one sequence of each biotype was used for the similarity matrix analysis (Table 2). This analysis revealed that *F.*

prausnitzii is more distantly related to other *Fusobacterium* spp. (less than 80% similar to all other members of the genus) than to either *Propionigenium modestum* (about 92% similar) or *B. termitidis* (86% similar) (Table 2 and Fig. 1). Thus, *F. prausnitzii* may not be a *Fusobacterium* species since all other members of the genus examined were more than 93% similar (Table 2). The results of a BLAST search of the GenBank and EMBL data bases suggested that *F. prausnitzii* was similar to *Propionibacterium acnes* and *A. israelii* serotype 1 (1).

Our analysis of the biotype AB and B sequences and the previously published *Fusobacterium* 16S rRNA sequences, in which we used the phylogenetic tree inference program (Fig. 1), revealed groupings similar to those described previously (16). Variation did occur in the clustering results for the two independently derived sequences of *Fusobacterium mortiferum* analyzed; this variation probably reflected differences between DNA and RNA reverse transcriptase sequencing results.

To further investigate relationships within the *F. necrophorum* group, the data were reanalyzed (Table 2 and Fig. 2). The type subspecies of the genus, *Fusobacterium nucleatum* subsp. *nucleatum*, was used as the outgroup. Two previously published *F. nucleatum* 16S rRNA sequences were available, and a total of 1,287 positions between positions 100 and 1450 (*E. coli* numbering) were included in the analysis. The clustering of the *F. necrophorum* biotypes in Fig. 2 is a reflection of homogeneity at the 16S rRNA level, a finding not mirrored by the bacterial phenotypes (Table 1). As our analysis revealed homogeneity of the *F. necrophorum* biotypes at the 16S rRNA level, we do not propose to alter the current taxonomic positions of the *F. necrophorum* biotypes despite the fact that these taxa are phenotypically distinct. Instead, it is important to note that studying specific phenotypic differences between virulent biotype AB strains and benign biotype B strains may well provide a legitimate way to identify key virulence factors useful in vaccine development.

FIG. 2. Intragenetic relationships of *F. necrophorum* biotypes. *F. nucleatum* was used as the outgroup. Each scale bar division represents a 1% difference in the 1,287 nucleotides analyzed. The numbers in parentheses are the bacterial strain numbers in Table 2.

ACKNOWLEDGMENTS

This work was supported in part by Wool Research and Development Corporation grant CHP25.

We thank Nanette Bold and Bob Campbell of the CSIRO Division of Animal Health for their assistance in the preparation of the tables and figures.

ADDENDUM IN PROOF

The results of our analysis of 16S rRNA sequences indicated that *F. prausnitzii* is more closely related to the gram-positive organisms *Propionibacterium acnes* and *Actinomyces israelii* serotype 1 than to the fusobacteria, thus aligning a gram-negative bacterium with the gram positives. Since submitting our manuscript, J.-M. Neefs, Y. Van de Peer, P. De Rijk, S. Chapelle, and R. De Wachter (Nucleic Acids Res. **21**:3025–3049, 1993) reported their analysis of all recorded SSU rRNA species and grouped *F. prausnitzii* among the "Gram positives and their relatives," which includes *Propionibacterium acnes* and *Actinomyces israelii* serotype 1. This independent datum is therefore consistent with our findings.

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