Evidence that *Bacteroides nodosus* Belongs in Subgroup Gamma of the Class *Proteobacteria*, Not in the Genus *Bacteroides*: Partial Sequence Analysis of a *B. nodosus* 16S rRNA Gene

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The taxonomic status of the anaerobe *Bacteroides nodosus* has for some time been uncertain. To resolve this uncertainty, the distal portion of a 16S rRNA gene from this important ovine pathogen was cloned, mapped, and sequenced. A comparison of the sequence with the sequences of 16S rRNA molecules from other bacteria indicated that *B. nodosus* is more closely related to *Escherichia coli* and other members of the class *Proteobacteria* than to *Bacteroides fragilis* or the bacteroides-flavobacterium-cytophaga phylum. The evidence from the comparison of sequence signatures suggests that *B. nodosus* is not a member of the genus *Bacteroides* but that it belongs in subgroup γ of the class *Proteobacteria*.

Footrot in sheep is an economically important contagious disease, which is characterized by separation of a large portion of the hoof from the underlying soft tissues (3, 13) and leads to loss of body condition and reduced wool production (31). The essential causative agent of ovine footrot is *Bacteroides nodosus*, an obligately anaerobic, nonsporing, gram-negative, rod-shaped organism. This organism is nonsaccharolytic, has limited activity in standard biochemical tests, has a guanine-plus-cytosine content of 45 mol%, and is relatively slow growing. In electron micrographs large numbers of polar fimbriae (or pili) are observed. According to the amended genus description, *B. nodosus* belongs to a collection of anaerobic, gram-negative, nonsporing, rod-shaped bacteria, many of which were misclassified (22, 23, 26). It has recently been proposed that the genus be reclassified (26). This proposal has been supported by the results of 16S rRNA analysis (22, 23) and SS rRNA analysis (35). According to the amended genus description, *B. nodosus* does not belong in the genus and is therefore of uncertain taxonomic status.

We undertook the cloning and sequencing of a 16S rRNA gene from *B. nodosus* with the following two objectives: to obtain data to clarify the taxonomic status of *B. nodosus* and to determine species-specific rRNA sequences which can be used as probes for the diagnosis of ovine footrot. The results of this study support the conclusion that *B. nodosus* is not a member of the genus *Bacteroides*.

**MATERIALS AND METHODS**

Bacterial cultures. The *B. nodosus* strain which we used was strain A198, the most frequently studied virulent ovine isolate (8). This organism was cultured on hoof agar (33) and Trypticase-arginine-serine agar (28) and was incubated at 37°C for 5 to 7 days in an atmosphere containing 10% H₂ and 10% CO₂ in N₂. *B. fragilis* J102 was grown under anaerobic conditions on horse blood agar (9). All *Escherichia coli* strains were derivatives of strain DH5α (Bethesda Research Laboratories, Inc.) or strain MC1022 (7) and were cultured in 2× YT medium (36). Ampicillin (100 μg/ml) was added when appropriate.

RNA isolation and analysis. *E. coli* cultures were grown at 37°C with shaking to a turbidity of 600 nm of 0.4. To extract RNA from *B. nodosus* and *B. fragilis*, the cells were removed from Trypticase-arginine-serine agar and horse blood agar plates, respectively, with 1 to 2 ml of phosphate-buffered saline per plate. The cells were suspended to give a turbidity at 600 nm of approximately 0.4. Total RNA was extracted from all cultures as previously described (37). The RNA was suspended in Tris-EDTA buffer and stored at −70°C. Electrophoresis of RNA was carried out as described previously (15).

**DNA isolation and analysis.** Plasmid DNA from *E. coli* was prepared as previously described (4, 17). Total DNA was isolated from washed *B. nodosus* cells as described previously (1). DNA fragments to be radiolabeled were isolated from 0.8% (wt/vol) agarose gels by electrophoresis onto DEAE-cellulose paper (Whatman, Inc.), using the method described previously (18) for electrophoresis onto a dialysis membrane. All restriction endonuclease digestions were carried out under standard conditions (18).

**Blotting and hybridization methods.** For Northern hybridizations monodirectional transfer of RNA onto nitrocellulose was performed as previously described (18). Monodirectional transfer of DNA from agarose gels to nitrocellulose filters was performed by the method of Southern (29). For DNA dot blots, 0.5-μg DNA samples were applied to nitrocellulose filters (approximately 1 to 2 μl).

The hybridization conditions used for Northern and Southern blots were the conditions described previously (20) for the hybridization of nick-translated probes, with the modifications described below. Prehybridization and hybridization of Northern and Southern blots were carried out overnight at 42 and 37°C, respectively. For Northern blots the stringency washes consisted of two 15-min washes at 45°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.5% sodium dodecyl sulfate. Stringency washes for Southern blots consisted of two 30-min washes in 0.16× SSC containing 0.1% sodium dodecyl sulfate at 65°C. The conditions used for hybridization of RNA probes to Southern and DNA dot blot filters were identical except that prehybridization and hybridization were carried out at 55°C. The filters were then washed twice at room temperature in 2× SSC containing 0.5% sodium dodecyl sulfate for 5 min.

DNA probes (0.5 to 1.0 μg of DNA) were radiolabeled

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with 50 μCi of [α-32P]dATP by nick translation (Amersham Corp.). The reaction was terminated by adding 0.25 M EDTA, and removal of proteins was achieved by ammonium acetate-ethanol precipitation (10). RNA probes were prepared from total B. nodosus RNA (10 μg) by hydrolysis (2) and then end-labeling with 20 μCi of [γ-32P]ATP and T4 polynucleotide kinase (New England Biolabs, Inc.).

**DNA cloning.** A B. nodosus gene library, in the form of a ligation mixture, was obtained from E. K. Moses and was constructed essentially as previously described (21), except that the genome was digested with PstI and then ligated with PstI-digested, dephosphorylated plasmid vector pUC18 (41). The ligation mixture was used to transform E. coli DH5α cells to ampicillin resistance. Recombinants were selected as white colonies on medium containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and were screened by a modified colony hybridization method (16), using total RNA from B. nodosus as a probe.

A 0.4-kilobase (kb) BamHI-EcoRI fragment and a 0.7-kb EcoRI-PvuII fragment from pJIR284 were separately subcloned into vector pUC18 to form pJIR288 and pJIR290, respectively. The latter fragment was ligated into SmaI-digested pUC18. Ligation was carried out in a low-melting-temperature gel matrix (SeaPlaque; FMC Corp., Marine Colloids Div.) (32). The ligation mixtures were used to transform E. coli MC1022 cells, and recombinant clones were screened by restriction analysis for the presence of the required fragment.

**DNA sequence analysis.** A sequence analysis of supercoiled plasmids was carried out by using a type T7 sequencing kit (Pharmacia) and M13 universal and reverse primers. Sequence data were analyzed by using the MELDBDBSYS system.

**RESULTS AND DISCUSSION**

**Cloning of a B. nodosus 16S rRNA gene.** Screening of the B. nodosus gene library revealed two clones which hybridized with total RNA from B. nodosus. Dot blots carried out on purified plasmid DNAs from these clones confirmed that they hybridized with the RNA probe, and restriction endonuclease digestions showed that the two plasmids were indistinguishable.

Although the 8.6-kb plasmid, designated pJIR284, was from a PstI-derived gene bank, it had only one PstI site. Southern blots of chromosomal digests of strain A198 DNA, in which pJIR284 DNA was used as the probe, indicated that the cloned region was located on a large (approximately 20-kb) PstI fragment in B. nodosus (data not shown). Recombination between multiple rRNA genes on the original fragment may account for the reduced size of the recombinant clones and the alteration of one PstI site. Further hybridization analysis showed that, in DNA from strain A198, there were several fragments which hybridized with the probe and were the same size as the equivalent fragments of pJIR284. These results confirmed that pJIR284 was derived from B. nodosus A198.

**Localization and sequence analysis of the 16S rRNA gene.** Southern hybridization analysis of pJIR284 was carried out to determine the extent of homology between the cloned insert and the RNA probe. The plasmid was digested with a variety of restriction enzymes and probed with labeled, total RNA from B. nodosus. Hybridization was essentially restricted to a 1.3-kb PvuII fragment, which contained 1.1 kb of B. nodosus DNA.

Northern blotting of rRNAs purified from E. coli, B. fragilis, and B. nodosus was done to determine which rRNA species was homologous to the hybridizing fragment. The nitrocellulose filter was hybridized with the labeled 1.3-kb PvuII fragment from pJIR284. Intense hybridization signals were observed with the 16S rRNAs from all three species, with a particularly strong signal produced with B. nodosus 16S rRNA. No hybridization was evident with 5S rRNA, and, compared with the hybridization observed with 16S rRNA, significantly less hybridization was observed with the 23S rRNA band from B. nodosus (data not shown).

The hybridizing region of pJIR284, which clearly contained at least part of a 16S rRNA gene, was subcloned to form pJIR288 and pJIR290. The complete nucleotide sequence of the inserted DNA from both plasmids was then determined on both strands, with the exception of a small region downstream of the EcoRI site in pJIR290. The results showed that the PstI site from pUC18 had been subjected to a single guanine-to-adenine base change at the end of the recognition sequence, which explained why pJIR284 had only one PstI site.

**Comparison of 16S rRNA sequences.** Comparative sequence analysis of 16S rRNA molecules is one of the most useful tools with which to determine phylogenetic relationships between bacterial species (23, 39). Therefore, we decided to compare the B. nodosus sequence with equivalent sequences from other bacteria.

The B. nodosus sequence was aligned (Fig. 1) with the sequences of 16S rRNA genes from B. fragilis (38), Flavobacterium heparinum (38), E. coli (5), Proteus vulgaris (6), Pseudomonas aeruginosa (34), Rubinbacterium amylophilus (19) (formerly Bacteroides amylophilus), and Neisseria gonorhoeae (24). Alignment of the B. nodosus sequence commenced from the modified PstI site, which corresponded to approximately position 600 in the other sequences. Termination of alignment occurred at position 1,542 (E. coli numbering). This corresponded to the 3' end of the 16S rRNA genes of the species being compared. A sequence corresponding to the consensus sequence of the 3' terminus of 16S rRNA (5'-ACCUCUUUA-3') (27) was identified in the B. nodosus sequence and was found to differ by one base pair from the predicted E. coli sequence (an adenine-to-thymine substitution at the 3' end). There was approximately 130 base pairs of sequence that was distal to the putative 3' end of the gene and did not show any homology with the other 16S rRNA genes.

Similarity values for each pair of sequences were also calculated (Table 1). A similarity value of 86.4% was obtained by comparing the B. nodosus and Pseudomonas putida (S. J. Giovannoni, D. J. Lane, and N. R. Pace, personal communication) sequences. The values obtained for comparisons involving the E. coli, B. fragilis, and F. heparinum sequences, the E. coli and P. vulgaris sequences, and the E. coli and R. amylophilus sequences were consistent with those reported previously (6, 19, 38).

However, the results indicated that on the basis of similarity between 16S rRNA genes, B. nodosus was more closely related to E. coli and other members of the class Proteobacteria, which were formerly referred to as the "purple bacteria and their relatives" (30), than to B. fragilis; and that B. fragilis was more closely related to F. heparinum than to B. nodosus. The sequence similarity value between any member of the class Proteobacteria considered in this study and either B. fragilis or F. heparinum was of approximately the same magnitude as the sequence similarity value between B. nodosus and the latter species.

Additional comparisons were made between the B. no-
FIG. 1. Alignment of 16S rRNA gene sequences. The available sequence of the 16S rRNA gene from B. nodosus (BN) is aligned with the corresponding sequences from *B. fragilis* (BF) (38), *F. hepaticum* (FH) (38), *E. coli* (EC) (5), *P. vulgaris* (PV) (6), *Pseudomonas aerugiosa* (PA) (34), *R. amylophilus* (RA) (19), and *N. gonorrhoeae* (NG) (24). Coordinates are shown on the right. Dots represent nucleotides identical to those of *B. nodosus*. Dashes indicate gaps that were inserted to align the sequences. Nucleotides of unknown or uncertain identity are indicated by an X.

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TABLE 1. Similarity of 16S rRNA sequences

<table>
<thead>
<tr>
<th>Organism</th>
<th>% Similarity witha:</th>
</tr>
</thead>
<tbody>
<tr>
<td>BN</td>
<td>100</td>
</tr>
<tr>
<td>BF</td>
<td>77.1 100</td>
</tr>
<tr>
<td>FH</td>
<td>77.5 83.6 100</td>
</tr>
<tr>
<td>EC</td>
<td>84.6 74.3 74.5 100</td>
</tr>
<tr>
<td>PV</td>
<td>84.2 72.8 74.2 94.1 100</td>
</tr>
<tr>
<td>PA</td>
<td>85.9 74.8 74.9 86.3 85.0 100</td>
</tr>
<tr>
<td>RA</td>
<td>84.8 74.0 75.6 87.8 87.4 86.1 100</td>
</tr>
<tr>
<td>NG</td>
<td>85.3 72.0 73.4 83.0 82.8 85.0 82.6 100</td>
</tr>
</tbody>
</table>

a Percent similarity values were calculated for each of the sequences analyzed. These values represent the percentage of bases in the alignment from position 604 to 1,542, with respect to the E. coli sequence, in which each pair of sequences had the same nucleotide. Positions where deletions or ambiguities were present in any of the sequences are not included. Abbreviations: BN, Bacteroides nodosus; BF, Bacteroides fragilis; FH, Flavobacterium hepaticum; EC, Escherichia coli; PV, Proteus vulgaris; PA, Pseudomonas aeruginosa; RA, Ruminobacter amylophilus; NG, Neisseria gonorrhoeae.

dosus 16S rRNA sequence and the Cardiobacterium hominis 16S rRNA sequence (F. E. Dewhirst and B. J. Paster, personal communication). The results showed that these species had 92.8% similarity sequence similarity. Therefore, it appears that B. nodosus and C. hominis may have a close phylogenetic relationship. Recent studies have shown that C. hominis belongs in the γ subdivision of the class Proteobacteria (12).

The findings presented above are in good agreement with recent biochemical and chemical evidence which led to the proposal that B. nodosus should be excluded from the genus Bacteroides (25, 26). If B. nodosus were a true member of this genus, then a similarity value that is at least equal to or greater than the similarity value between 16S rRNA genes of B. fragilis and F. heparinum would have been expected between the B. nodosus and B. fragilis sequences.

Comparative sequence analysis of 16S rRNAs from different microorganisms has provided the basis for dividing the eubacteria into 10 major phyla (38, 39). One of these phyla is the bacteroides-flavobacterium-cytophaga cluster, with bacteroides forming one subdivision and flavobacteria and cytophagae forming another (23, 38). Oligonucleotide cataloging of 16S rRNA sequences from this phylum has shown that these organisms are distinct from any other eubacteria previously characterized by this method (23, 38). 16S rRNA sequence signatures have been defined for the bacteroides-flavobacterium-cytophaga group and for the remaining eubacterial sequences (38). These signatures are unique and common to a given group of sequences and should be indicative of true phylogenetic relationships among organisms of a given group.

We scored the B. nodosus sequence for the positions which defined the sequence signature of the bacteroides-flavobacterium-cytophaga phylum. Within the region sequenced, the composition was found to be identical to the compositions in the remaining eubacterial sequences and different from the compositions in the B. fragilis and F. heparinum sequences (Table 2). In addition, some of the bacteroides-flavobacterium sequence signatures can be traced by unique oligonucleotides (38). Several of these sequences were identified in the B. nodosus 16S rRNA sequence and were found to differ from the corresponding sequences in B. fragilis and F. heparinum. These observations all support the conclusion that B. nodosus is not a member of the genus Bacteroides but belongs in the class Proteobacteria.

The class Proteobacteria can be divided into four subdivisions, each of which is distinguished by a 16S rRNA sequence signature (39). When the B. nodosus sequence was scored for the positions that define the sequence signature of the γ subdivision, it was found to be identical in 40 of 46 positions. This level of similarity was not obtained with the other subdivisions of the Proteobacteria. Of the six positions which did not match, one was unknown in the B. nodosus sequence and the remaining five were identical to the β-sequence signatures. At locations where the β and γ sequences differ, B. nodosus had the same sequence as the γ subdivision at 11 sites. There were five locations where there was identity with the β subdivision.

The γ subdivision is divided into the following three subdivisions: photosynthetic bacteria; species associated with Legionnaires disease; and a mixture of enteric bacteria (including E. coli), pseudomonads and relatives, and others (40). Similarity values between B. nodosus and the members of the γ-3 subgroup considered in this study ranged between 84 and 86%. These values were comparable to the values obtained between any pair of bacteria in the γ-3 subgroup except E. coli and Proteus vulgaris, for which the values were significantly higher. In addition, the B. nodosus sequence contained several oligonucleotides which are highly conserved in this subgroup (40). Therefore, B. nodosus appears to belong in the γ-3 subdivision of the class Proteobacteria.

Although the phylogenetic position of B. nodosus was uncertain, some clues were provided previously by studies on the organization and evolution of the type 4 fimbriae which are produced by B. nodosus (11). Type 4 fimbriae are also produced by organisms such as N. gonorrhoeae and Pseudomonas aeruginosa, which belong to the β and γ subdivisions of the class Proteobacteria, respectively. Some regions of the fimbrial sequence appear to be conserved between B. nodosus and Neisseria species, which led to the suggestion that either these sequences are closest to the ancestral sequence or B. nodosus may be a misclassified member of the family Neisseriaceae, possibly related to Neisseria species (11). Recent evidence also suggests that B. nodosus is the only "Bacteroides" species which has the enterobacterial type of lipopolysaccharides (14). These data

TABLE 2. Comparison of the B. nodosus sequence with sequence signatures for bacteroides and flavobacteria

<table>
<thead>
<tr>
<th>Position(s)</th>
<th>B. fragilis and F. heparinum sequences</th>
<th>Other sequences</th>
<th>B. nodosus sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>680, 710</td>
<td>G.C</td>
<td>C.G</td>
<td>C.G</td>
</tr>
<tr>
<td>724</td>
<td>U</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>866</td>
<td>A</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>943, 1,340</td>
<td>A.U</td>
<td>U.A</td>
<td>U.A</td>
</tr>
<tr>
<td>975</td>
<td>G</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>995</td>
<td>A</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>1,475</td>
<td>A</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>1,532</td>
<td>A</td>
<td>U</td>
<td>U</td>
</tr>
</tbody>
</table>

a The B. nodosus sequence was scored for the positions defining the sequence signature of the bacteroides-flavobacterium group. The latter were taken from reference 38. Listed are the bases found at positions in the B. fragilis and F. heparinum sequences, in the remaining eubacterial sequences (other sequences), and in the available B. nodosus sequence. Only signature sequences from positions 604 to 1,542, with respect to the E. coli sequence, are included.
support our conclusion that *B. nodosus* is more closely related to the enteric bacteria and pseudomonads than to the genus *Bacteroides*. Further taxonomic studies will be required to determine whether *B. nodosus* belongs in an existing genus within the class *Proteobacteria* or in a new genus.

After comparison of the *B. nodosus* sequence with the sequences of the other bacteria, it was possible to identify many regions which were highly conserved. However, regions where the *B. nodosus* sequence was found to differ considerably from the other sequences could also be identified. These regions and others like them have considerable potential for use as *B. nodosus*-specific gene probes. Current studies in our laboratory are aimed at testing these potential probes and assessing their suitability for use in the diagnosis of ovine footrot.

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LITERATURE CITED


