Comparison of 16S rRNA Sequences of Segmented Filamentous Bacteria Isolated from Mice, Rats, and Chickens and Proposal of “*Candidatus Arthromitus*”

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Segmented filamentous bacteria (SFB) are nonpathogenic bacteria that are commonly found attached to the intestinal walls of many animals. Until now, these bacteria have not been cultured *in vitro*. Recently, a 16S rRNA sequence analysis revealed that SFB isolated from mice represent a distinct subline within the *Clostridium* subphylum of the gram-positive bacteria. Since SFB isolated from mice, rats, and chickens are known to be host specific, we investigated the phylogenetic relationships among SFB obtained from these three hosts. Total DNAs from the intestinal floras of chickens and rats were used as templates for PCR amplification of 16S rRNA genes. PCR products were cloned and screened by a dot blot hybridization procedure to identify homologous sequences that cross-reacted with mouse SFB-specific oligonucleotide probes. A phylogenetic analysis of these 16S ribosomal DNA sequences revealed that SFB isolated from these three hosts form a natural group, which is peripherally related to the genus *Clostridium sensu stricto* (group I *Clostridium*). The SFB obtained from chickens, rats, and mice had closely related, albeit different, 16S rRNA gene sequences. The observed levels of 16S rRNA sequence divergence, ca. 1.5 to 3%, together with host specificity, suggest that SFB isolated from mice, rats, and chickens represent different species and that coevolution of the SFB and their hosts occurred. “*Candidatus Arthromitus*” is proposed as the provisional generic name for this group of organisms.

The small intestines of many animals (e.g., mice, rats, pigs, and other mammals, as well as chickens, amphibians, and insects) are inhabited by segmented filamentous bacteria (SFB), which are also referred to as long segmented filamentous organisms (reviewed in reference 10). These bacteria cannot be cultured *in vitro* and do not have official taxonomic names. Until recently, SFB could be recognized only on the basis of their morphology and their ecological niches. In intestines, these gram-positive, endospore-forming organisms are attached via holdfasts to the epithelial walls. In rodents, colonization by SFB is restricted to the ileum (4). In birds, SFB are also found in the ceca (9). No pathologic effects of SFB have been found, even in immunodeficient animals. SFB found in different animal species are morphologically very similar. However, attempts to transfer bacteria from one host species to another have revealed that SFB found in mice, rats, and chickens exhibit host specificity (1, 13, 21). It is not known if SFB that inhabit different hosts are taxonomically closely related or whether they belong to different species.

In a recent study, the 16S rRNA-encoding gene sequence of SFB obtained from mice was determined (19). This study was greatly facilitated by the use of a monoculture of these organisms in formerly germfree mice (12). A comparative 16S rRNA

analysis revealed that there is a phylogenetic relationship between the SFB found in mice and the genus *Clostridium*. Furthermore, mouse SFB-specific oligonucleotide probes were developed on the basis of 16S rRNA sequence data to facilitate the detection of SFB in mixed populations. The purpose of this study was to use these specific probes to select for 16S rRNA gene sequences of SFB found in other animal species. Analysis of such sequences should reveal information about the relationships among the SFB found in different hosts. Since SFB cannot be cultured *in vitro* and no pure cultures in germ-free animals other than mice exist, sequences were obtained from mixed bacterial populations. To do this, DNA from intestinal bacteria was isolated and used as a template for PCR amplification with universal 16S rRNA primers. Then cloned 16S ribosomal DNA (rDNA) was dot blotted and probed with the SFB-specific oligonucleotides developed previously. The sequences of positive clones were determined and used in a phylogenetic analysis.

MATERIALS AND METHODS

Animals. Cpb:WU rats were derived from a home-bred colony maintained under specified-pathogen-free conditions until the rats were used. These rats received RMH-TM pellets (Hope Farms) and tapwater *ad libitum*. The chickens which we used were 10-day-old Sex Sals (White Rock × Rhode Island Red) chickens; they were given unsterilized Broiler Starter Feed (Southern States, Inc.) and tap water *ad libitum*.

Extraction of nucleic acids. The end of the small intestine of each chicken and rat was removed. In addition, the ceca of chickens were removed. The intestinal

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walls were flushed with a saline solution to remove most of the unattached bacteria. A smear was Gram stained and microscopically examined for the presence of SFB. Approximately 150 μ l of intestinal contents was diluted with a saline solution so that the final volume was 500 μ l. Then 5 μ l of a proteinase K solution (20 mg/ml) and 25 μ l of a 10% sodium dodecyl sulfate (SDS) solution were added. The resulting mixture was incubated at 55°C for 1 h. The solutions prepared in this way were extracted with phenol-chloroform (1:1) several times. Nucleic acids were precipitated by adding 0.1 volume of 3 M sodium acetate and 1 volume of isopropanol. After incubation for 1 h at -20°C and centrifugation at 5,000 $\times$ g for 10 min, the resulting pellet was washed with 70% ethanol and dissolved in 100 μ l of TE buffer.

Oligonucleotides and PCR amplification. Conserved 16S rRNA-targeted oligonucleotides were used as primers for PCR. The sense primer 27f (5'-CACG GATCCAGAGTTTGAT[C/T][A/C]TGGCTCAG-3') is homologous to *Escherichia coli* 16S rRNA positions 8 to 27 (3) and contains an internal *Bam*HI site. The antisense primer 1492r (5'-GTGAAGCTTACGG[C/T]TACCTTGTAC GACTT-3') is complementary to *E. coli* 16S rRNA positions 1492 to 1513 and contains an internal *Hind*III site. A standard PCR was performed in a 100- μ l (total volume) reaction mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 5 mM MgCl₂, each deoxynucleoside triphosphate at a concentration of 200 μ M, 0.2 μ l of *Taq* polymerase (5 U/ μ l; Gibco-BRL), 10 pmol of each primer, and 1 μ l of diluted nucleic acid preparation. A total of 35 rounds of temperature cycling (93°C for 1 min, 54°C for 2 min, and 72°C for 3 min) in a DNA thermal cycler (Perkin-Elmer, Norwalk, Conn.) were followed by 7 min of incubation at 72°C. An aliquot of each PCR product was dot blotted with a mouse SFB-specific oligonucleotide probe, SFB1008 (5'-GCGAGCTTCCTCATTACAAGG-3') (19), and a universal bacterial probe, EUB338 (2), by using standard methods (18).

Blotting. DNA samples were spotted on a nylon membrane (Hybond N⁺; Amersham International) by using a standard procedure and were covalently bound to the membrane by UV irradiation. The membrane was placed in prehybridization buffer (0.5 M Na₂HPO₄ [pH 7.2], 1% bovine serum albumin, 1 mM EDTA, 7% SDS). The probes were 5'-labelled with [γ -³²P]ATP by a standard procedure (18). Hybridization was performed in prehybridization buffer to which labelled probe was added, and then the mixture was incubated at 37°C for 16 h. The blots were washed twice (30 min each) at a fixed stringency in 2 $\times$ SSC-0.1% SDS (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The wash temperatures used and the stringency of the wash solution were determined empirically. Blots were analyzed with a phosphor imager (B&L Systems, Zoetermeer, The Netherlands).

Cloning and sequencing. The amplification products obtained from two PCR per host species were digested with restriction enzymes *Bam*HI and *Hind*III and cloned in M13mp18 by using standard methods (18). Vector DNA was isolated from recombinant clones and screened by a dot blot procedure by using probes SFB1008 and EUB338. Cloned amplification products that reacted positively with probe SFB1008 were selected for sequencing. When M13mp18 was used, the sequencing reactions were performed with a single-stranded DNA sequencing kit (PRISM Sequenase Terminator; Perkin-Elmer) as recommended by the manufacturer. Sequences were determined with a model 373A automatic sequencer (Applied Biosystems).

Analysis of sequence data. The sequences which we determined and the sequences of clostridial species obtained from the EMBL Data Library were aligned by using the program PILE UP (6), and the alignment was corrected manually. A distance matrix was produced by using the program DNADIST of the PHYLIP package (7), and a tree was constructed by the neighbor-joining method with the program NEIGHBOR of the same package. The stability of the groups was assessed by performing a bootstrap analysis with the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE (7).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences which we determined have been deposited in the EMBL Data Library under accession numbers X80834 (SFB from chickens) and X87244 (SFB from rats). The 16S rRNA nucleotide sequence accession number for the SFB obtained from mice is X77814.

RESULTS

Isolation of bacteria, cloning of PCR products, and selection of clones. The intestinal samples were flushed with a saline solution. Since SFB were attached to the epithelial cells, most of the nonadhering bacteria were removed, thereby making the SFB the most dominant organisms in each sample. Cloning of the PCR products revealed that there were several clones, and high percentages of these clones reacted with the SFB-specific probe, SFB1008; 6 of 12 and 8 of 19 the clones selected from chickens and rats, respectively, hybridized with the SFB-specific probe.

Sequencing and phylogenetic analysis. Four chicken and two rat SFB rDNA insertions were characterized by perform-

FIG. 1. Unrooted tree showing the phylogenetic position of the SFB within the *Clostridium* subphylum of the gram-positive bacteria. Abbreviation: C., *Clostridium*.

ing a sequence analysis. A single SFB rDNA from chickens was completely sequenced, and the sequence consisted of approximately 1,440 nucleotides (representing approximately 94% of the complete 16S rRNA primary sequence). The identities of the other host-specific SFB rDNA insertions were confirmed by partial sequencing (approximately positions 50 to 500; 16S rRNA *E. coli* numbering) in which three diagnostic variable regions (regions V1 to V3) were included. Not a single base difference was observed in the chicken rDNA insertions. The two rat SFB sequences were both completely sequenced and were found to be identical. Our SFB 16S rRNA sequences were compared with previously published sequences of mouse SFB and clostridial species. Figure 1 is a phylogenetic tree that was constructed from a matrix of derived evolutionary distances and shows the relationships of the three SFB which we studied and related clostridia.

DISCUSSION

The 16S rRNA gene sequences of chicken and rat SFB were found to be very similar to each other and to the sequence of a previously described mouse SFB (18). A comparative sequence analysis revealed that the SFB obtained from the three different hosts form a natural group (level of intragroup 16S rRNA sequence similarity, >97%; bootstrap value, 100) within the *Clostridium* subphylum. Phylogenetically, the SFB which we studied form a distinct line that branches proximal to the periphery of a large cluster designated *Clostridium sensu stricto* (formerly group I *Clostridium* of Johnson and Francis

[5]). The bootstrap data indicated that the SFB exhibit no significant affinity with any other species or group and belong to a long-isolated lineage which exhibits a level of intragroup divergence of <3% and is worthy of separate generic status.

It is evident from the comparative sequence analysis results that SFB obtained from chickens, rats, and mice represent a group containing highly related, albeit genetically different, organisms. Previous reports from independent research groups have shown that the SFB obtained from these three host species exhibit host specificity (1, 13, 21), which indicates that these organisms belong to different species. In this context it is pertinent to note that mouse and rat SFB are more closely related to each other (level of relatedness, 98.7%) than to chicken SFB (97.2 to 97.7%). Although a precise correlation between level of 16S rRNA sequence relatedness and species differentiation is not possible (8, 20), the level of sequence divergence exhibited by the SFB obtained from these different hosts is greater than the level of sequence divergence generally exhibited by strains of a species and in our opinion indicates that these organisms belong to different, albeit closely related, species. SFB have been found in about 20 vertebrate animals, including humans, and several invertebrate species (10, 11). The presence of SFB with characteristic rRNA sequences in chickens, mice, and rats indicates that there has been coevolution of the SFB and their hosts, but comparisons of sequences of SFB from a greater number of animals will be required to confirm this. In addition, more sequences need to be obtained from SFB found in other hosts, including invertebrates, to confirm this hypothesis.

The phylogenetic analysis described above supplemented the previous analysis of Snel et al. (19), and our results clearly demonstrated that SFB obtained from chickens, mice, and rats belong to a distinct lineage within the *Clostridium* subphylum at the rank of genus. Since these bacteria cannot be cultured in vitro, this genus can now be described mainly on the basis of its phylogeny and morphology. Therefore, we propose that this group of organisms should be given the provisional status *Candidatus* (17). We propose the genus name *Arthromitus* (Ar. thro'mi.tus. Gr. n. *arthron*, a joint; Gr. masc. n. *mitos*, a thread; N. L. masc. n. *Arthromitus*, a jointed thread) for this group of organisms; this name was originally coined by Leidy (14, 15) for spore-forming, filamentous organisms found in the intestines of myriapods and termites. Later, SFB obtained from rodents and fowl were thought to be related to these bacteria (4, 16). "*Candidatus* Arthromitus" contains gram-positive, endospore-forming bacteria which grow as septate filaments. One end of each filament is attached to the intestinal wall. "*Candidatus* Arthromitus" represents a new subline within the *Clostridium* subphylum. The members of this group of organisms can be divided into different species on the basis of host specificity and 16S rRNA sequences. Therefore, in addition to the *Candidatus* genus name, we propose that the host-specific SFB obtained from chickens, mice, and rats should be designated "*Candidatus* Arthromitus galli" (gal'li), "*Candidatus* Arthromitus muris" (mu'ris), and "*Candidatus* Arthromitus ratti" (rat'ti), respectively. These *Candidatus* species can be recognized on the basis of their 16S rRNA sequences and the host species in which they are found.

ACKNOWLEDGMENTS

This project was supported in part by the European Community (BRIDGE-HRAMI project BIOT-CT91-0294) and by the Ministry of

Agriculture, Fisheries and Food (United Kingdom). The assistance of S. Stubbs is gratefully acknowledged. A. D. L. Akkermans (Wageningen Agricultural University) is acknowledged for providing advice during the experiments and for critically reading the manuscript.

REFERENCES

- Allen, P. C. 1992. Comparative study of long segmented filamentous organisms in chickens and mice. *Lab. Anim. Sci.* **42**:542-547.
- Amann, R. I., B. J. Binder, R. J. Olsen, S. W. Chisholm, R. Devereux, and D. A. Stahl. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* **56**:1919-1925.
- Brosius, J., J. L. Palmer, H. P. Kennedy, and H. F. Noller. 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **75**:4801-4805.
- Chase, D. G., and S. L. Erlandsen. 1976. Evidence for a complex life cycle and endospore formation in the attached, filamentous, segmented bacterium from murine ileum. *J. Bacteriol.* **127**:572-583.
- Collins, M. D., P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. E. Farrow. 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int. J. Syst. Bacteriol.* **44**:812-826.
- Devereux, J., P. Haeberli, and D. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387-395.
- Felsenstein, J. 1989. PHYLIP—phylogeny inference package (version 3.2). *Cladistics* **5**:164-166.
- Fox, G. E., J. D. Wisotzkey, and P. Jurtshuk. 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int. J. Syst. Bacteriol.* **42**:166-170.
- Glick, B., K. A. Holbrook, I. Olah, W. D. Perkins, and R. Stinson. 1978. A scanning electron microscope study of the caecal tonsil: the identification of a bacterial attachment to the villi of the caecal tonsil and the possible presence of lymphatics in the caecal tonsil. *Poult. Sci.* **57**:1408-1416.
- Klaasen, H. L. B. M., J. P. Koopman, F. G. J. Poelma, and A. C. Beynen. 1992. Intestinal, segmented, filamentous bacteria. *FEMS Microbiol. Rev.* **88**:165-180.
- Klaasen, H. L. B. M., J. P. Koopman, M. E. van den Brink, M. H. Bakker, F. G. J. Poelma, and A. C. Beynen. 1993. Intestinal, segmented, filamentous bacteria in a wide range of vertebrate species. *Lab. Anim.* **27**:141-150.
- Klaasen, H. L. B. M., J. P. Koopman, M. E. van den Brink, H. P. N. van Wezel, and A. C. Beynen. 1991. Mono-association of mice with non-cultivable, intestinal, segmented, filamentous bacteria. *Arch. Microbiol.* **156**:148-151.
- Koopman, J. P., H. M. Kennis, M. P. C. Hectors, A. Lankhorst, A. M. Stadhouders, and H. de Boer. 1984. Reciprocal 'normalization' of intestinal parameters by indigenous intestinal microflora of the rat and mouse. *Z. Versuchstierkd.* **26**:289-295.
- Leidy, J. 1849. On the existence of entophyta in healthy animals, as a natural condition. *Proc. Natl. Acad. Sci. Phila.* **4**:225-233.
- Leidy, J. 1881. The parasites of termites. *J. Acad. Nat. Sci. Phila.* **8**:425-447.
- Margulis, L., L. Olendzenski, and B. A. Afzelius. 1990. Endospore-forming filamentous bacteria symbiotic in termites: ultrastructure and growth in culture of *Arthromitus*. *Symbiosis* **8**:95-116.
- Murray, R. G. E., and E. Stackebrandt. 1995. Taxonomic note: implementation of the provisional status *Candidatus* for incompletely described prokaryotes. *Int. J. Syst. Bacteriol.* **45**:186-187.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1990. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Snel, J., H. J. Blok, H. M. P. Kengen, W. Ludwig, F. G. J. Poelma, J. P. Koopman, and A. D. L. Akkermans. 1994. Phylogenetic characterization of *Clostridium* related segmented filamentous bacteria in mice based on 16S ribosomal RNA analysis. *Syst. Appl. Microbiol.* **17**:172-179.
- Stackebrandt, E., and B. M. Goebel. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44**:846-849.
- Tannock, G. W., J. R. Miller, and D. C. Savage. 1984. Host specificity of filamentous, segmented microorganisms adherent to the small bowel epithelium in mice and rats. *Appl. Environ. Microbiol.* **47**:441-442.