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Taxon-Specific Probes for the Cellulolytic Genus *Fibrobacter* Reveal Abundant and Novel Equine-Associated Populations

CHUZHAO LIN AND DAVID A. STAHL*

Department of Veterinary Pathobiology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

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A total of six 16S rRNA targeted oligonucleotide probes were used to quantify *Fibrobacter* abundance and diversity in the gastrointestinal contents of a pony. Approximately 12% of the total 16S rRNA extracted from cecal contents hybridized with a *Fibrobacter* genus-specific probe and a *Fibrobacter succinogenes* species-specific probe. However, no significant hybridization was observed with a probe for the species *Fibrobacter intestinalis* or with three probes for *F. succinogenes* subspecies. This suggested the presence of a previously undescribed population of *F. succinogenes*-like organisms. Novel lineages of *F. succinogenes* were subsequently identified by using PCR primers specific for the genus to amplify sequences coding for 16S rRNA from DNA extracted from cecal contents. Sequences of the cloned amplification products were shown to be affiliated with *F. succinogenes* but represented two distinct, and novel, lines of descent within the species.

The current taxonomy of fiber-digesting bacteria of the genus *Fibrobacter* is based upon 16S rRNA sequence relationships (11). Comparative 16S rRNA sequencing initially served to reveal the tremendous genetic diversity among isolates previously classified as *Bacteroides succinogenes* and as a basis for the formation of a new genus (*Fibrobacter*) to accommodate them. The extent of this diversity was more fully evaluated by DNA homology (1). Although the members of this genus appeared to be closely related phenotypically, are all cellulolytic, and produce succinic acid as a major fermentation product, their genetic diversity implied far greater ecological and physiological range. In fact, the current classification is extremely conservative. Although DNA homology values justify taxonomic divisions at the genus rank (1), there is no phenotypic basis to do so at this time. Thus, we have formally described only a single genus and two species, *Fibrobacter succinogenes* and *Fibrobacter intestinalis.*

We have continued to examine the genetic diversity of *Fibrobacter* organisms with comparative sequence analysis and taxon-specific hybridization probes (10). The probes are DNA oligonucleotides complementary to regions within the 16S rRNA that correspond to the taxonomic ranks of genus, species, and subspecies. Since the taxonomy is based upon phylogenetic relationships, the probes encompass naturally defined assemblages of *Fibrobacter* spp. However, the probes were developed with sequence information obtained from *Fibrobacter* strains available in pure culture. Thus, the question of undescribed environmental diversity remained largely unanswered.

In this study, we evaluate the use of taxon-specific hybridization probes to explore the environmental diversity of *Fibrobacter* spp. Our observation of *F. succinogenes* species-specific hybridization that could not be accounted for by hybridization with any of three subspecies-specific probes suggested the presence of a novel *Fibrobacter* population. This was confirmed by using selective PCR amplification, cloning, and sequencing to identify two previously undescribed populations.

* Corresponding author. Present address: Department of Civil Engineering, Northwestern University, 2145 Sheridan Ave., Evanston, IL 60208-3109. Phone: (708) 491-4997. Fax: (708) 491-4011. Electronic mail address: d-stahl@nwu.edu.

MATERIALS AND METHODS

Sampling. Gut content samples, from ileum, cecum, and colon, were obtained from a female pony immediately following euthanasia by lethal injection. The animal had been maintained on an alfalfa hay diet following recovery from an experimental infection with a strain of *Ehrlichia*. Euthanasia was part of the original animal use protocol and unrelated to this study. *Ehrlichia* infection is restricted to the circulating leukocytes (12), and infection of this animal was naturally resolved. There are no records of antibiotic treatment during or following the experimental infection. Although prior infection is not anticipated to have influenced the gut microbiota, we note this for completeness of the animal description. Samples were immediately frozen in liquid nitrogen and held at −85°C until they were processed.

Nucleic acid extraction and hybridization. A slight modification of the extraction procedure of Stahl et al. (16) was used to extract nucleic acid from gut samples. Mechanical disruption on a reciprocating shaker (Mini-Beadbeater; Biospec Products, Bartlesville, Okla.) with zirconium beads (0.1 mm in diameter) was employed to extract total nucleic acid from about 1 g of each sample. Samples were processed in conical 2.2-ml screw-cap polypropylene vials (Sarstedt, Inc., Newton, N.C.). Approximately 0.3 g of beads was used for each sample, together with 50 μl of 20% (wt/vol) sodium dodecyl sulfate (SDS)–700 μl of phenol equilibrated with 50 mM sodium acetate–10 mM EDTA buffer (pH 5.1). The sample-containing tubes were "beadbeated" for 2 min at room temperature and transferred to a 60°C water bath for 10 min before an additional 2 min of beating. Samples were extracted again with buffer-equilibrated phenol and then extracted an additional five times with an equal volume of phenol-chloroform-isoamyl alcohol (100:24:1 [vol/vol/vol]) equilibrated with the same buffer. Total nucleic acid (primarily rRNA) was precipitated by the addition of ammonium acetate (2 M final concentration) and isopropanol (0.7 volume) and overnight incubation at −20°C. Following 10 min of centrifugation in a microcentrifuge (Eppendorf; Brinkman Instruments, Westbury, N.Y.) at 14,000 rpm, the supernatant was removed, and the RNA pellet was washed with 1 ml of 70% (vol/vol) ethanol and resuspended in double-distilled water.

Nucleic acid at 50 μg/ml was denatured by the addition of 3 volumes of 2% (vol/vol) glutaraldehyde. The samples were then diluted with dilution water [1 μg of poly(A) per ml (Sigma, St. Louis, Mo.), 0.004% bromophenol blue] to a final concentration of 4 μg/ml. A 100-μl volume sample (400 ng) was applied in duplicate to a nylon membrane (Micron Separations, Inc., Westborough, Mass.) with a slot blot device (Schleicher & Schuell Co., Keene, N.H.) under slight vacuum. A dilution series of rRNA isolated from an appropriate strain of *Fibrobacter* was included as a hybridization reference on each membrane. The membranes were air dried and baked at 80°C for 2 h before being used for hybridization.

DNA oligonucleotide probes were 5′ end labeled with [γ-^{32}P]ATP essentially as described by Stahl et al. (16). A 10-fold excess of the amount of probe necessary to bind to the total RNA applied to the nylon membrane was labeled and used in the hybridization reaction. The hybridization and washing conditions were as previously described (10). Baked membranes were prewetted in hybridization buffer [0.9 M NaCl, 50 mM sodium phosphate (pH 7.0), 5 mM EDTA, 10× Denhardt's solution (13), 0.5% SDS, 100 μg of poly(A) per ml] and placed in screw-cap hybridization tubes (Robbins Scientific, Sunnyvale, Calif.). Approximately 10 ml of hybridization buffer was added to each hybridization tube that contained a maximum of four membranes. Membranes were prehybridized for 2

TABLE 1. Primers used for PCR amplification

Primer	*E. coli* numbering	Sequence	Target group	Feature	Reference
Fibro	153–168	CCGTGCCAACGCGCGG	*Fibrobacter* sp.	5′ specific	This study
Eugen3R	1528–1542	AAAGGAGGTGGTCCA	Universal	3′ general	8 (modified)
Eugen3R-1	1528–1542	CCGCGGCCGCAAAGGAGGTGGTCCA	Universal	3′ general with restriction sites	8 (modified)
Bact1512bR	1492–1507	TACCTTGTTACGACTT	Bacterial domain	3′ general	This study
Univ	1392–1406	ACGGGCGGTGTGT(GA)C	Universal	3′ general	16
1510R	1510–1496	GGGTACCTTGTTACG	Universal	3′ general	This study

h at 40°C in a rotating incubator (Robbins Scientific) before the addition of labeled probe. Incubation was continued overnight (12 to 16 h) at 40°C, and the membranes were then washed once with about 100 ml of washing solution (1× SSC [0.15 M NaCl, 0.015 M sodium citrate, pH 7.0], 1% [wt/vol] SDS) for 30 min at 40°C. The membranes were removed from the hybridization tubes and washed twice with 500 ml of washing solution at a predetermined temperature for each probe (corresponding to the dissociation temperature) for 30 min each.

The probes used and their approximate dissociation temperatures were described by Lin et al. (10). The abundance of *Fibrobacter* cells was expressed as the fraction of the total rRNA in the sample. Total rRNA abundance was inferred by the use of a universal hybridization probe (Univ) complementary to all characterized 16S rRNAs (16). Following exposure, the film (Kodak XRP; Eastman Kodak Co., Rochester, N.Y.) was quantified with an LKB laser scanning densitometer and Gel-Scan XL software (Pharmacia LKB Biotechnology, Piscataway, N.J.).

DNA extraction, amplification, and cloning. In order to isolate DNA suitable for PCR, the RNA extraction procedure was modified as follows. TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)-buffered phenol and phenol-chloroform replaced the low-pH sodium acetate-EDTA-buffered phenol. The time of bead beating was reduced to 1 min. RNase A (Sigma) was added to a final concentration of 50 μg/ml, and the sample was incubated at 37°C for 30 min to digest RNA. The DNA was recovered by isopropanol precipitation as described above for the recovery of total nucleic acid.

A *Fibrobacter* sp.-specific 5′ primer and a bacterial domain 3′ primer for 16S rRNA genes were used for the PCR amplification (Table 1). The general primer is complementary to a region of sequence common to the 3′ end of most bacterial 16S rRNAs. The reaction volume was 50 μl and contained 15 ng of DNA, 10 pmol each of both primers, 2.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 100 μg of nuclease-free bovine serum albumin per ml, 0.2 mM (each) deoxynucleoside triphosphates, and 2.5 U of *Taq* polymerase. Amplification was accomplished with a thermal cycler (Perkin-Elmer, Norwalk, Conn.). The first cycle was 3 min at 94°C, 2 min at 40°C, and 2.5 min at 72°C. The remaining 29 cycles were run for 1 min, 2 min, and 2.5 min at the respective temperatures. The PCR product was filled in by using the Klenow fragment of DNA polymerase (GIBCO BRL, Gaithersburg, Md.) to improve cloning efficiency (15). The phage cloning vectors M13mp18 and -mp19 (GIBCO BRL) were cut with the restriction enzyme *Sma*I (GIBCO BRL) according to manufacturer's specifications. Five microliters of PCR product was ligated with 200 ng of cut vector in a final volume of 20 μl at 16°C overnight. Half of the ligated product (10 μl) was used to transform *Escherichia coli* JM109 in the presence of X-Gal and (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) for blue and white recombinant selection (13). White plaques were grown in LB broth (10 g of Bacto Tryptone, 5 g of Bacto Yeast Extract, 10 g of NaCl per liter) for 5 h at 37°C. Single-stranded phage DNA was isolated by polyethylene glycol-NaCl precipitation, followed by phenol-CH_3Cl extraction, CH_3Cl extraction, and ethanol precipitation (13).

Sequence analysis. Nucleotide sequences were determined by the dideoxynucleotide method with a Sequenase kit from U.S. Biochemical (Cleveland, Ohio). In addition to the M13 universal primer, 16S rRNA-specific reverse and forward primers were used as previously described (8). Sequence similarities and evolutionary distances were determined as described previously (11), considering only homologous positions. A phylogenetic tree was constructed with a distance algorithm (2).

Nucleotide sequence accession number. Sequences have been deposited in GenBank under accession numbers L35547 and L35548.

RESULTS

Nucleic acid hybridization. *Fibrobacter* rRNA represented about 12% of total rRNA extracted from the cecal sample and about 4% of total rRNA from the colon sample but was undetectable in the ileum (Fig. 1). Of the 12% present in the cecum, virtually all was accounted for by quantification with the *F. succinogenes* species-specific probe (Fig. 1). Similarly, most of the *Fibrobacter* cells in the colon were *F. succinogenes* (ca. 4 to 5%), with some detectable *F. intestinalis*. However, no hybridization was observed with any of the three subspecies-specific probes for *F. succinogenes* in either the cecum or the colon. This observation suggested the presence of either an undescribed *F. succinogenes* strain or one related to strain MC1, for which a probe is not yet available.

Identification of novel *Fibrobacter* organisms in the equine cecum. PCR amplification product from primers Fibro (5′) and 1510R (3′) was cloned into M18mp18 and -mp19. The sequences of five cloned PCR amplification products were determined (Fig. 2). Sequence comparisons revealed that four of the five clones were affiliated with *Fibrobacter* organisms. Three of these clones were identical in sequence (Fibro-A). The fourth was designated Fibro-B. The fifth clone contained a smaller amplification product, and its sequence was affiliated with the *Bacteroides* line of descent (data not shown). Phylogenetic relationships inferred from the sequence similarity values of these and previously characterized *Fibrobacter* 16S rRNA sequences are presented as a phylogenetic tree (Fig. 3). The new sequences fall within the radiation of *Fibrobacter* species. The target sequence for the genus probe (Fig. 1) is present in both. Their specific relationship with *F. succinogenes* is supported by two criteria. Both are affiliated with the *F. succinogenes* side of the inferred root of the tree (Fig. 3). Both have a signature sequence characteristic of the species (10). The species-specific sequence for *F. succinogenes* is present in Fibro-A (complementary to the succ probe [Fig. 1]). Although Fibro-B contains a single nucleotide change in this region, members of *F. intestinalis* demonstrate three to five nucleotide changes. Neither is closely related to any of the described subspecies. Fibro-A is peripherally related to group 3 of *F. succinogenes*. The Fibro-B sequence now defines the deepest branch within *F. succinogenes*. Even so, total *Fibrobacter* diversity remains encompassed by two primary lines of evolutionary

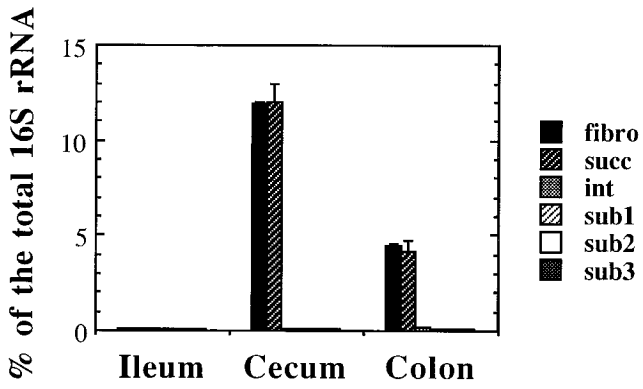

FIG. 1. Relative abundance of *Fibrobacter* spp. in equine inferred by oligonucleotide probe hybridization to total RNA extracted from gut contents. The bar graph represents the average of duplicate hybridization experiments. The error bar defines the range. The legend included in the figure corresponds to the phylogenetically defined target groups for each probe as defined in Fig. 3: fibro, *Fibrobacter* genus; succ, *F. succinogenes*; int, *F. intestinalis*; and sub1 to sub3, *F. succinogenes* subspecies.

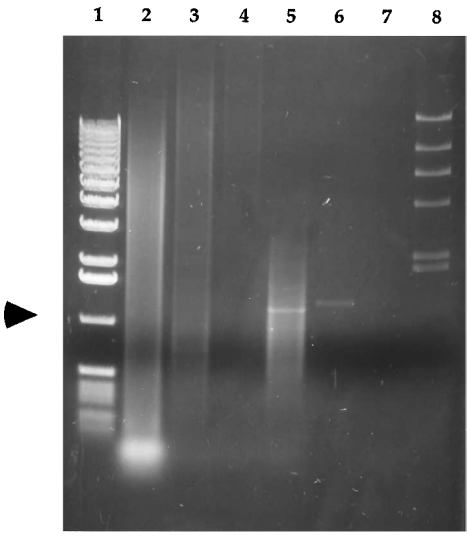

FIG. 2. PCR amplication of *Fibrobacter* sp.-specific genes encoding 16S rRNA. Primers used are listed in Table 1. Lanes 1 and 8 are a 1-kb ladder and *Hin*dIII-digested lambda DNA, respectively. The 3′ general primers used in lanes 2 to 6 were Eugen3R-1, Eugen3R, 1510R, Univ, and Bact1512bR, respectively. Lane 7 is a negative control containing no 3′ primer. The arrowhead indicates the 1-kb band (lane 1).

descent, supporting the original division of the genus *Fibrobacter* into two species. However, we again call attention to the very conservative character of the present classification. Although DNA-DNA homology values are sufficient to elevate subspecies within *F. succinogenes* to genus rank (1), there is no phenotypic basis to do so at this time.

DISCUSSION

Past cultural enumerations of *Fibrobacter* spp. lumped members of this genetically diverse group together without recognizing functional or genetic differences (17). This was a consequence of the constraints imposed by cultural enumeration and the relatively few differentiating characteristics for this assemblage of microorganisms. However, the increasing use of nucleic acid hybridization techniques in determinative and environmental microbiology is now alleviating complete reliance on cultural enumeration and phenotypic identification. The use of the rRNAs as targets for determinative hybridization probes has added a phylogenetic dimension. It was this dimension that initially served to highlight the diversity within the group and now serves to identify novel populations of *Fibrobacter* spp. in equine gut compartments.

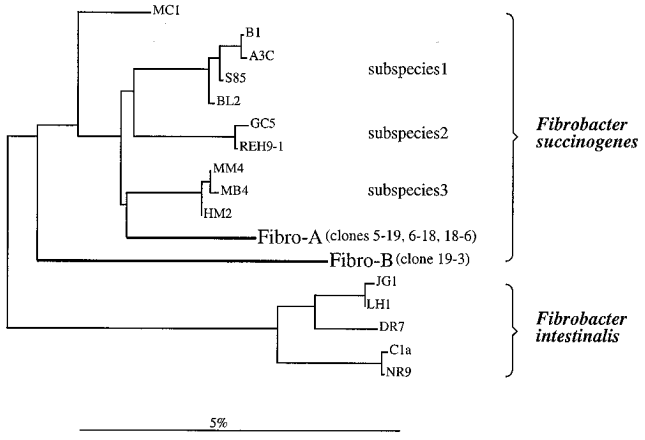

FIG. 3. Phylogenetic tree based on 16S rRNA sequence comparison for *Fibrobacter* spp. The 5% bar represents 0.05 estimated nucleotide changes per position with reference to the horizontal distances within the tree. The root was inferred by using the 16S rRNA sequences of *Bacteroides fragilis* and *Flavobacterium heparinum* as outgroup organisms (9).

The question of undescribed environmental diversity of *Fibrobacter* spp. was addressed by the combined use of probes having different specificities. As we have previously discussed (16), consistency between quantification with probes for different target groups provides an internal check on the analysis. For example, quantification with a set of species-specific probes should give the same value as that obtained with a single genus-specific probe. An inconsistency could imply unrecognized diversity within the larger phylogenetic assemblage. The present study demonstrated the general utility of this approach. Total amount of *Fibrobacter* organisms approximately equaled the sum of *F. intestinalis* and *F. succinogenes* organisms in both the cecum and colon. However, inconsistency was also apparent. Although the abundant *Fibrobacter* organisms identified in the horse cecum could be identified as *F. succinogenes*, they could not be identified with the subspecies probes.

The failure to account for the *F. succinogenes* population with the available subspecies probes served as the impetus to further define the contributing population(s). To this end, PCR provided a convenient bridge between the hybridization results and a more complete characterization by sequence analysis. A genus-specific 5′ PCR primer together with 3′ general primers was used to selectively amplify, and clonally retrieve, the corresponding 16S rRNA genes. The combination of probe hybridization and PCR provided a systematic method to define *Fibrobacter* population diversity within this gut community. We anticipate that this approach should be of general utility in the characterization of other natural communities.

Equine digestive physiology: relative abundance of equine *Fibrobacter* organisms. A notable result of this study was the remarkably high abundance of cecal *Fibrobacter* organisms, composing approximately 12% of the total microbial content, as inferred by rRNA abundance. Rumina content values for forage-fed ruminants average around 3% (10). Although we recognize that these results are restricted to a single animal, we consider this observation in the context of equine digestive physiology. In nonruminant herbivores, such as equines, fermentation takes place in the cecum or colon; they are hindgut (colon) fermenters (5, 6). In contrast to ruminants, a sizable proportion of the available protein and carbohydrate is digested and absorbed prior to reaching the cecum, and volatile fatty acid production within the cecum accounts for only about 30% of the digestible energy intake (3). Total plant cell wall digestion in the cecum is about 30% less than in the rumen, although the digestion of soluble components is about the same (4, 7).

The observation of elevated abundance of *Fibrobacter* organisms in the cecum, as inferred from fractional representation of rRNA, is consistent with the removal of easily digested or soluble substrates prior to the entry into the cecum. Plant fiber represents a greater fraction of incoming substrate. Thus, a greater contribution by fiber-digesting bacteria is anticipated, as was observed in this study. However, the significance of this single observation must be established by studies of additional hindgut fermenters. Furthermore, we have yet to evaluate the abundance of other important cellulolytic organisms, such as species of *Ruminococcus.*

It is also of considerable interest that the cecal populations

are genetically distinct from those generally encountered in the rumina. Past studies have shown subspecies 1 and 2 to be most abundant in rumina (10). If the observed population differences between rumina and this equine cecum are representative, it could reflect habitat differences. Factors contributing to habitat distinction may include the character of the fiber and fiber retention time within these distinct gut compartments. The presence of bile acids also makes the large intestine and cecum very different from the rumen (18). Bile acids are known to inhibit the growth of different bacteria in vitro and may influence the composition of the biota in the large intestine and cecum (14).

The apparent habitat distinction may also be reflected in the phylogeny of the genus. Assuming unbiased PCR amplification and cloning, Fibro-A (represented by three identical clones) is the major *Fibrobacter* population within the equine cecum. Fibro-A is most closely related to subspecies 3 of *F. succinogenes*. This subspecies was shown to be generally absent in rumina of cattle and goat (10). Preliminary studies have also shown a similar lack of representation in ovine rumina (data not shown), although this is the site of original isolation (1). Thus, it is tempting to suggest that the phylogenetic group defined by subspecies 1 and 2 corresponds to *Fibrobacter* populations adapted to rumina, whereas the group composed of subspecies 3 and Fibro-A corresponds to cecal or colonic populations. This must be evaluated by more extensive surveys of different animals and different gut compartments.

The results of these genetic and phylogenetic studies now serve as a foundation for more defined studies of the ecology and physiology of *Fibrobacter* spp. For example, probes designed for the novel sequences could be used to screen enrichment cultures for their isolation. More generally, this study has highlighted the extensive environmental diversity of this phylogenetic group of fiber-digesting bacteria. However, our understanding of phylogenetic and genetic diversity now greatly exceeds appreciation of the underlying phenotypic diversity. The genus exhibits few phenotypic traits useful for traditional classification, most notably an extremely limited number of soluble substrates (glucose and cellobiose). Although we anticipate that the genetic diversity must be reflected in greater physiological and ecological diversity than now appreciated, these differentiating characteristics have yet to be defined.

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