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Fibrobacter communities in the gastrointestinal tracts of diverse hindgut-fermenting herbivores are distinct from those of the rumen

Anthony P. Neumann[1], **Caroline A. McCormick**[1], and **Garret Suen**[1,#]

[1]Department of Bacteriology, University of Wisconsin – Madison, Madison, Wisconsin, USA

Summary

The genus *Fibrobacter* contains cellulolytic bacteria originally isolated from the rumen. Culture-independent investigations have since identified *Fibrobacter* populations in gastrointestinal tracts of numerous hindgut-fermenting herbivores, but their physiology is poorly characterized due to few representative axenic cultures. To test the hypothesis that novel *Fibrobacter* diversity exists in hindgut fermenters, we performed culture and 16S rRNA gene amplicon sequencing on samples collected from phylogenetically diverse herbivorous hosts. Using a unique approach for recovering axenic *Fibrobacter* cultures, we isolated 45 novel strains from 11 different hosts. Full-length 16S rRNA gene sequencing of these isolates identified nine discrete phylotypes (cutoff = 0.03%) among them, including several that were only isolated from hindgut-fermenting hosts, and four previously unrepresented by axenic cultures. Our phylogenetic analysis indicated that six of the phylotypes are more closely related to previously described subspecies of *Fibrobacter succinogenes*, while the remaining three were more closely related to *F. intestinalis*. Culture-independent bacterial community profiling confirmed that most isolates were representative of numerically dominant phylotypes in their respective samples and strengthened the association of certain phylotypes with either ruminants or hindgut-fermenters. Despite considerable phylogenetic diversity observed among the *Fibrobacter* strains isolated here, phenotypic characterization suggests a conserved specialization for growth on cellulose.

Keywords

Fibrobacter; herbivore; hindgut-fermenter; cellulose; fiber digestion

Introduction

Herbivores depend on microorganisms living in their gastrointestinal tract for the efficient digestion of their fiber-rich diet (Mackie, 2002; Morrison *et al.*, 2009). The plant cell wall polysaccharides that constitute the bulk of this fiber represent a potentially plentiful source of carbon that is highly resistant to enzymatic decomposition (Flint *et al.*, 2008). Despite this recalcitrance, certain microbial taxa have evolved mechanisms to extract the sugars from these structural polysaccharides in order to ferment them for energy (Leschine, 1995; Lynd

[#]Address correspondence to Garret Suen, gsuen@wisc.edu, Department of Bacteriology, University of Wisconsin – Madison, 1550 Linden Drive, Room 5159, Madison, WI, 53706. Phone: (608) 890-3971, Fax: (608) 262-9865.

et al., 2002). By maintaining a community of these microbes in their gut, herbivores gain access to the chemical energy in their diet through absorption of the byproducts of this microbial fermentation. Animals engaged in this symbiosis over extended periods of evolutionary time have optimized their digestive physiology through enlargements in either the foregut or hindgut, which increase the capacity and retention time of feed through the gastrointestinal tract (Ley *et al.*, 2008). Although microbial consortia responsible for fiber digestion in foregut fermenters have been well characterized via studies of the rumen (Flint, 1997), less is known regarding the microbial taxa that perform this function in hindgut-fermenting herbivores.

Plant cell walls are primarily composed of cellulose, hemicellulose, pectin, and lignin (Cosgrove, 2005). Cellulose is the most abundant of these plant cell wall polysaccharides, but its crystalline structure makes it among the most difficult to hydrolyze (Beguin, 1990). Therefore, microorganisms that can efficiently deconstruct cellulose in the herbivore gut are critically important for optimal digestion. In the rumen, cellulose degradation is facilitated, in part, via the efforts of certain species of bacteria, including members of the genus *Fibrobacter* (Dehority and Scott, 1967; Hungate, 1975; Flint, 1997; Kobayashi *et al.*, 2008; Solomon *et al.*, 2016). Representatives were first isolated and described by Hungate during studies of cellulose digestion in the bovine rumen (Hungate, 1950). Although they were originally classified as *Bacteroides*, phylogenetic analyses have subsequently established their membership to a deeply rooted clade within the Bacteria designated as the phylum Fibrobacteres (Montgomery *et al.*, 1988; Ransom-Jones *et al.*, 2012). The type strain, *Fibrobacter succinogenes* S85, has been extensively investigated and is among the most actively cellulolytic of all strictly anaerobic, mesophilic bacteria known (Weimer, 1996; Qi *et al.*, 2007; Nouaille *et al.*, 2009; Suen *et al.*, 2011). *F. succinogenes* S85 ferments the breakdown products of cellulose to primarily succinic acid and lesser amounts of acetic acid. Production of these fermentation products, along with an apparent inability to ferment pentoses, appears to be a conserved feature among *Fibrobacter* spp. (Stewart and Flint, 1989; Amann *et al.*, 1992).

Since their original isolation from the rumen, culture-independent studies have suggested the presence of *Fibrobacter* populations in diverse herbivore gastrointestinal tracts (Lin and Stahl, 1995; Matsui *et al.*, 2010; Ilmberger *et al.*, 2014). However, cultured representatives from most of these hosts are rare, and as a result, our understanding of their physiology is limited. Currently, there are only two formally described species for the genus *Fibrobacter*: *F. succinogenes* and *F. intestinalis* (Amann *et al.*, 1992; Ransom-Jones *et al.*, 2012). Phylogenetic analysis based on full-length 16S rRNA gene sequences of all of the available isolates from these two species groups indicates that there are at least four distinct phylogenetic lineages of *F. succinogenes* (Amann *et al.*, 1992; Shinkai *et al.*, 2009), but culture-independent analyses suggest that this represents only a fraction of the true diversity in the *F. succinogenes* group (Jewell *et al.*, 2013; Ransom-Jones *et al.*, 2014). Similarly, poor cultural representation almost certainly exists for *F. intestinalis*, since fewer representative isolates have been described. Moreover, while the phylogenetic distance between *F. succinogenes* and *F. intestinalis* is satisfactory for segregation into separate genera, sufficient phenotypic differentiation has not yet been established to justify reclassification (Amann *et al.*, 1992).

The aim of this investigation was to gain insights into the ecology of *Fibrobacter* spp. in the gastrointestinal tracts of herbivores, particularly hindgut-fermenters. To achieve this goal, we complemented 16S rRNA gene amplicon sequencing with culturing and isolation, an approach that addresses some of the limitations associated with a solely culture-independent analysis (Walker *et al.*, 2017). We chose to focus on hindgut-fermenting herbivores because the bacterial communities involved in fiber-degradation in the gastrointestinal tracts of these hosts have generally been understudied, relative to analogous bacteria in ruminant livestock. We hypothesized that hindgut-fermenting herbivores would harbor phylogenetically distinct *Fibrobacter* populations in their gastrointestinal tracts, compared to those that have been observed in the rumen, but that their niche as cellulose-degrading specialists would be conserved. Here, we describe the isolation and ecological distribution of 45 novel *Fibrobacter* isolates, including four previously uncharacterized phylotypes, and further introduce a novel strategy for isolating these bacteria. Our results provide strong evidence that several relatively abundant *Fibrobacter* phylotypes found in hindgut fermenters are phylogenetically distinct from those typically observed in the rumen.

Results

A total of 95 samples, collected from animals housed at farms, university facilities, and two zoos in Wisconsin, USA, were examined for this study (Table S1). The samples included 78 fresh fecal samples, 14 samples of rumen contents, and three samples of cecal contents. Samples were collected from 23 different animal hosts representing seven orders of Mammals: Artiodactyla (even-toed ungulates), Perissodactyla (odd-toed ungulates), Rodentia, Primates, Carnivora, Proboscidea, and Pilosa. Samples from one bird (ostrich) and one reptile (Aldabra tortoise) were also examined. These hosts exhibited a primarily herbivorous dietary lifestyle (n = 84), although a small number of samples from omnivores (n = 8) and carnivores (n = 3) were also investigated for comparison. Most of the samples represented hosts with a gastrointestinal anatomy specialized for either hindgut-fermentation (n = 61) or foregut-fermentation (n = 29) of plant cell wall polysaccharides.

Isolation of novel *Fibrobacter* phylotypes

A total of 45 axenic cultures of *Fibrobacter* from 41 of the 95 (43.2%) samples were recovered after all isolation attempts (Table 1, Table S2). Cellulose-adherent cocci, commonly observed microscopically in primary enrichment cultures, were also occasionally isolated and identified through 16S rRNA gene sequencing as being closely related to either *Ruminococcus flavefaciens* or *Ruminococcus albus*, but were not pursued further as they were considered beyond the scope of the present investigation (data not shown). Isolates were recovered from 11 different host species including: Holstein cattle (n = 17), horse (n = 9), pig (n = 4), rhinoceros (n =4), tapir (n = 3), capybara (n = 2), sheep (n = 2), colobus monkey (n = 1), elephant (n = 1), ostrich (n = 1), and rhesus monkey (n = 1). Microscopic examination, as well as the recovery of single 16S rRNA gene sequences, using the universal bacterial primers 27F and 1492R (Weisburg *et al.*, 1991), from cultures grown in rich media supported the conclusion that these 45 cultures are axenic and are members of the phylum Fibrobacteres, with the closest cultured relatives in the genus *Fibrobacter*.

A maximum likelihood phylogeny showing the relationships among these 45 *Fibrobacter* strains was inferred from the near-full length 16S rRNA gene sequences (Fig. 1). All but one (UW P2) of the 45 strains isolated in this study segregated into eight discrete and well resolved (≥ 98% bootstrap support) lineages. UW P2, isolated from capybara feces, did not cluster with any of the others, suggesting a ninth distinct lineage. Additionally, none of the 45 strains clustered closely with *Fibrobacter succinogenes* strain HM2, the type strain for *F. succinogenes* subsp. *elongatus* (Montgomery *et al.*, 1988), indicating at least ten discrete lineages represented by *Fibrobacter* isolates described to date. The phylogeny supported the placement of 39 of the 45 strains, representing seven lineages including strain HM2, in the *Fibrobacter succinogenes* species clade with high confidence (96% bootstrap support). The remaining six strains fell into three discrete lineages, two of which also contained previously described isolates of *F. intestinalis* (Amann *et al.*, 1992). Based on these results, and following the nomenclature previously reported (Amann *et al.*, 1992), these ten lineages are designated Fs I through Fs VII, for the seven lineages of the *F. succinogenes* clade, and Fi I through Fi III, for *F. intestinalis* and its closest relatives (Fig. 1, Table S2). Cultured representatives for three of the six *F. succinogenes* lineages represented by strains isolated in this study, Fs V, Fs VI, and Fs VII, have not been reported previously. Moreover, the strains UW RM and UW S4 are the first cultured representatives of Fi III, and although their closest previously cultured representative is *F. intestinalis* strain JG1, they share only 94% nucleotide identity with this strain across the corresponding 1,262 base pairs of 16S rRNA gene sequence examined (Table S2). Clustering using mothur (Schloss *et al.*, 2009) of the near-full length 16S rRNA gene sequences from the 45 *Fibrobacter* strains isolated in this study, along with the sequence from *F. succinogenes* strain HM2, identified ten distinct operational taxonomic units (OTUs) or phylotypes [average neighbor, cutoff = 0.03]. The grouping of the strains into OTUs was in absolute agreement with the grouping observed in the maximum likelihood phylogeny, providing further support for the ten distinct *Fibrobacter* phylotypes and their member strains.

Culture-independent analysis of bacterial communities

Total bacterial community profiling by 16S rRNA gene amplicon sequencing was performed in order to determine whether the *Fibrobacter* isolates we obtained were representative of the abundant *Fibrobacter* populations in their respective samples, as well as to provide additional ecological context for the *Fibrobacter* phylotypes. Data for 83 of the samples used for culturing was obtained by Illumina sequencing of the V4 region (Kozich *et al.*, 2013). A scatterplot of the two-dimensional NMDS analysis based on Bray-Curtis dissimilarities among the total bacterial communities from these samples showed general clustering by host and, more broadly, by host phylogeny, with hosts from the same taxonomic order typically placed within similar coordinates of the Cartesian plane (Fig. 2a). Most samples were dominated by the phyla Firmicutes and Bacteroidetes (Table S3). Other commonly observed phyla in gastrointestinal or fecal samples included: Spirochaetes, Verrucomicrobia, Proteobacteria, and Tenericutes. Classifiable genera that were commonly observed across samples include: *Prevotella*, *Oscillospira*, *Arcobacter*, *Ruminococcus*, and *Treponema* (Table S4).

Samples were considered positive for the presence of Fibrobacteres if the relative abundance of sequences classified to this phylum was greater than 0.01%. Overall, 67 of the 83 samples analyzed by 16S rRNA gene amplicon sequencing were positive for Fibrobacteres sequences, with a median percent relative abundance among these samples of 0.38% (Fig. 2b, Table S5). Most sequences (96.83%) that classified to the phylum Fibrobacteres were also classified to the genus *Fibrobacter*. All rumen samples (Holstein cows and sheep) and fecal samples from odd-toed ungulates (horses, tapirs, and rhinoceros) were positive for Fibrobacteres. Rumen samples had a median percent relative abundance of Fibrobacteres sequences of 1.03%, while fecal samples from odd-toed ungulates had an overall median of 0.41%. Less abundant Fibrobacteres populations were observed in fecal/cecal samples from even-toed ungulates (7 of 11 positive, median = 0.05%) and fecal samples from primates (11 of 15 positive, median = 0.05%). None of the fecal samples from carnivores (n = 5) met the criteria for positive detection of Fibrobacteres populations. The most extensively sampled hosts, rumen samples from Holstein cows (n = 7) and fecal samples from horses (n = 22), had similar median percent relative abundance of Fibrobacteres sequences of 0.65% and 0.54%, respectively (Fig. 2b). Relatively abundant Fibrobacteres populations were also observed for the limited number of fecal samples collected from the ostrich (2 of 2 positive, median = 2.00%), elephants (2 of 2 positive, median = 1.32%), and capybaras (2 of 2 positive, median = 0.57%).

Ecological differences among *Fibrobacter* phylotypes

We further analyzed those 16S rRNA amplicon sequences classified to the phylum Fibrobacteres by comparing them against the 16S rRNA gene sequences obtained from our 45 cultured strains and *F. succinogenes* subsp. *elongatus* strain HM2, to determine if they could be classified as belonging to any of the ten identified *Fibrobacter* phylotypes. The *Fibrobacter* phylotypes were then tested for associations with the dimensions of the NMDS ordination of Bray-Curtis dissimilarities among the total bacterial communities to identify possible relationships with total bacterial communities from certain hosts (Fig. 2a). Vectors representing sequence counts for *Fibrobacter* phylotypes showing a possible association (p-value < 0.2) with a certain area of the ordination are shown in Fig. 2a. Statistical support for the association of individual vectors and the ordination can be found in Table S6. Vectors for the phylotypes Fs I, Fs II, Fs III, Fs IV, and Fs VII were associated with an area of the plot that included total bacterial communities from rumen samples. In contrast, the vectors corresponding to sequence counts for *Fibrobacter* phylotypes Fs V and Fs VI, as well as a vector representing *Fibrobacter* sequences that did not classify to any of the ten phylotypes, were directed toward the region where total bacterial communities from the feces of odd-toed ungulates and elephants were plotted.

The fraction of Fibrobacteres 16S rRNA amplicon sequences, identified in the culture-independent analysis, assigned to the different *Fibrobacter* phylotypes, determined from the 45 isolates, in samples from the most extensively investigated hosts, Holstein cows (n = 7) and horses (n = 22), are shown in Figure 3a. Almost all (99.17%) of the Fibrobacteres sequences recovered from cow fecal samples could not be classified to any of the ten *Fibrobacter* phylotypes represented by the strains isolated in this study. In contrast, only 8.97% of the Fibrobacteres sequences recovered from cow rumen samples were not

represented by one of the ten phylotypes. Five *Fibrobacter* phylotypes were consistently observed in cow rumen samples: Fs I, Fs II, Fs III, Fs IV, and Fs VII (Table S5). None of the Fibrobacteres sequences recovered from rumen samples were classified as Fs V, Fs VI, or any of the three *F. intestinalis* phylotypes. The distribution of *Fibrobacter* phylotypes in horse fecal samples was distinct from what was observed for cow fecal samples. A total of 50.57% of all of the Fibrobacteres sequences from horse fecal samples could not be classified to any of the ten *Fibrobacter* phylotypes. However, sequences classified as either *Fibrobacter* phylotype Fs V or Fs VI were commonly observed in horse fecal samples, occurring in 72.73% and 81.82% of the samples examined, respectively (Table S5). Sequences classified as Fs II, Fs III, and Fs IV were rarely observed in horse fecal samples, whereas phylotypes Fs I, Fs VII, Fi I, Fi II, and Fi III were not observed at all. The fraction of Fibrobacteres sequences classified to the *Fibrobacter* phylotypes in other hosts for which Fibrobacteres sequences were detected is shown in Figure 3b. Phylotype Fs II exhibited the widest host range, with sequences detected in 11 different animal hosts. Sequences related to the *F. intestinalis* type strain, phylotype Fi I, dominated the Fibrobacteres community in pigs, gorillas, orangutans, and the ostrich. Sequences classified as Fi II or Fi III were rarely, if ever, observed.

Fibrobacter phylotypes in the Global Rumen Census

16S rRNA gene amplicon sequence data from the Global Rumen Census (GRC) was analyzed (Henderson *et al.*, 2015) in order to determine whether the absence of *Fibrobacter* phylotypes Fs V and Fs VI in rumen samples extended beyond the nine individual rumen samples investigated in our study. We classified 96,331 sequences out of 3,601,905 total bacterial sequences in the GRC to the phylum Fibrobacteres (2.67%). These Fibrobacteres sequences were then classified to our defined *Fibrobacter* phylotypes. Figure 3c shows the fraction of GRC Fibrobacteres sequences classified to our *Fibrobacter* phylotypes for each of eight different ruminant hosts. Overall, 89.02% of the GRC Fibrobacteres sequences could be classified to one of the ten *Fibrobacter* phylotypes. As was observed for rumen samples collected and analyzed for this study, sequences classified to phylotypes Fs I, Fs II, Fs III, and Fs IV were common in rumen samples analyzed for the GRC. Of these, Fs I and Fs II typically dominated the Fibrobacteres community, accounting for more than 60% of the Fibrobacteres sequences, on average, regardless of host (except in giraffes), as well as 72.79% of all Fibrobacteres sequences from the GRC. Lesser amounts of *Fibrobacter* phylotypes Fs VII, Fi II, and Fi III were also observed, including a moderate percentage of the Fibrobacteres sequences from giraffe rumen samples being classified as Fi III. Despite the substantial number of Fibrobacteres sequences recovered and analyzed from the GRC dataset, none of the sequences were classified to the *Fibrobacter* phylotypes Fs V, Fs VI, or Fi I.

Ecology of unclassified Fibrobacteres phylotypes

An OTU-based analysis of the Fibrobacteres sequences identified among the 16S rRNA gene amplicon data generated in this study was performed in order to gain additional insights into the large proportions of unclassified Fibrobacteres sequences observed in fecal samples from Holstein cows and horses. After clustering Fibrobacteres sequences into OTUs

(average neighbor, cutoff = 0.03), representative sequences were used to construct a maximum likelihood neighbor-joining tree of those OTUs observed in more than one sample as well as at a percent relative abundance of at least 0.1% in at least one sample (Figure 4). Of these 13 commonly observed Fibrobacteres OTUs, six represent phylogenetic lineages not represented by the ten phylotypes described in this study (Table 2). Two of the unrepresented Fibrobacteres OTUs, Otu011 and Otu012, are close relatives of the cultured phylotypes Fs V and Fs VI and exhibit a similar pattern of ecological distribution being solely observed in horse fecal samples. Our OTU-based analysis also identified three distinct phylogenetic lineages located outside of the *F. succinogenes* group. Otu010 is likely representative of a separate phylotype within the *F. intestinalis* group, as it exhibited close sequence similarity to *F. intestinalis* strain JG1 (Table 2). *F. intestinalis* strain JG1 was not included in our reference data set for classification to *Fibrobacter* phylotypes because of several ambiguous base calls in the full-length 16S rRNA gene sequences, however it was included in the maximum likelihood phylogeny inferred from near-full length 16S rRNA gene sequences (Figure 1). Otu003 evaded isolation, despite representing one of the most commonly observed Fibrobacteres OTUs in horse fecal samples. Otu003 was observed in 14 of 22 (63.64%) horse fecal samples, but also at a relatively low abundance in four of the seven (57.14%) Holstein cow rumen samples. Lastly, Fibrobacteres Otu008, likely representing the most phylogenetically divergent uncultured Fibrobacteres population observed in this study, was observed in four fecal samples from Holstein cows and in one fecal sample from a horse.

Phenotypic characteristics of *Fibrobacter* isolates

All of the 45 *Fibrobacter* strains exhibited growth on crystalline cellulose as the sole carbon source in the media formulations used (Table S2, Table S7). However, strains UW P2 and UW R4 consistently produced lower concentrations of fermentation products and quantities of genomic DNA after extraction. In fact, UW R4 grew so poorly after isolation that fermentation products could not be reliably quantified for this strain. Attachment of at least a fraction of the population to cellulose during growth was observed via light microscopy for all strains. Generally, cultures reached cell densities between $1 \times 10^8 – 1 \times 10^{10}$ viable cells per ml when grown on cellulose based on the results of dilution to extinction in fresh media. No growth of any of the 45 strains was observed when xylan (from beechwood) was used as the sole carbon source in the growth medium. All strains produced succinate as the major fermentation product (5.78 ± 1.20 mM), with lesser amounts of acetate (3.42 ± 0.80 mM), and in some cases small amounts of formate (0.70 ± 0.42 mM). No lactate, propionate or butyrate production was detected (< 0.05 mM) for any of the 45 strains.

Discussion

Although the first isolates of *Fibrobacter* were reported over 50 years ago (Hungate, 1950), cultured representatives for much of the apparent phylogenetic diversity of this group are lacking (Amann *et al.*, 1992; Jewell *et al.*, 2013; Ransom-Jones *et al.*, 2014). As a result, knowledge regarding their ecology and physiology is limited. One barrier to achieving a more representative *Fibrobacter* culture collection is the technical challenge of the traditional anaerobic culture techniques used in the past to successfully recover isolates

(Stewart *et al.*, 1981; Macy *et al.*, 1982; Varel *et al.*, 1984). Another is their preference for growth on an insoluble substrate, and requirement for attachment (Suen *et al.*, 2011), which makes using agar-based media problematic. For this study, we developed a novel methodology for isolating these bacteria in order to alleviate some of these difficulties. Our approach leverages the tendency of *Fibrobacter* to adhere to cellulose (Gong and Forsberg, 1989), which can be used for enrichment, followed by dilution to extinction, which has demonstrated utility for the isolation of bacteria that do not efficiently form colonies on agar plates (Kenters *et al.*, 2011). Using this method, we successfully purified 45 axenic cultures of *Fibrobacter*, including the first isolated strains conclusively representative of four distinct phylotypes (Fs V, Fs VI, Fs VII, and Fi III). Although they were not the focus of the present investigation, and were therefore not pursued further, cellulolytic *Ruminococcus* spp. were also isolated using our approach supporting this method's broader utility for isolating diverse cellulose-degrading anaerobic bacteria.

Our method proved very reliable for isolating relatively abundant *F. succinogenes* phylotypes from Holstein cow rumen samples, as isolates of either phylotype Fs I or Fs II were recovered from all 14 cow rumen samples subjected to isolation. Isolates of phylotype Fs III and Fs IV, typically present but at lower abundance in the rumen (Amann *et al.*, 1992; Shinkai *et al.*, 2009), were not recovered from cow rumen samples, suggesting a potential bias towards the most numerically abundant strains in a given sample. Although representatives of Fs IV were not recovered from rumen samples, they were isolated from rhinoceros, tapir, and colobus monkey fecal samples, suggesting a broad host range for this phylotype. Isolates representing two phylotypes commonly observed in fecal samples from large hindgut-fermenting mammals, Fs V and Fs VI, were recovered from horse, rhinoceros, and elephant fecal samples. These isolates represent the first reported cultures of *Fibrobacter* from any rhinoceros and elephant hosts. Isolation of bacteria that fit the description of *Fibrobacter* have been previously reported for horses (Davies, 1964), and molecular evidence has suggested phylogenetically distinct populations in the hindgut of these animals (Lin and Stahl, 1995). However, the horse isolates reported here provide the first conclusive synthesis of these previous observations. Representatives of *F. intestinalis* were also successfully isolated using our approach, with three phylotypes of *F. intestinalis* recovered from pigs, rhesus monkeys, and an ostrich. Strains of *F. intestinalis* have been previously isolated from pigs (Varel *et al.*, 1984), but UW OS from the ostrich is the first reported isolate of *Fibrobacter* from a non-mammal.

Culture-independent analysis of the total bacterial community was also performed in order to investigate whether the *Fibrobacter* isolates we recovered accurately represent the dominant populations in their respective samples. In most instances, this was the case, particularly for the most extensively sampled hosts: horses and Holstein cows. Our culture-independent analysis did, however, provide strong evidence for *Fibrobacter* phylotypes in horse fecal and Holstein cow fecal samples that were not represented by any of our 45 isolates. A possible explanation for our inability to isolate these phylotypes is that the media formulation, which is based on a composition originally optimized for culturing rumen *Fibrobacter* strains (Scott and Dehority, 1965), lacked specific growth factors. In particular, sterilized rumen fluid, or cecal extract, which have previously been used to aid in *Fibrobacter* isolations (Davies, 1964; Stewart *et al.*, 1981; Macy *et al.*, 1982), were not

included in our media in order to facilitate reproducibility by researchers who may lack access to these additives. As a result, we cannot exclude the possibility of missing or suboptimal concentrations of growth factors essential for certain strains or phylotypes. Most samples for which we were unable to obtain isolates did not display any signs of cells resembling *Fibrobacter* colonizing cellulose in primary enrichment cultures. Insufficient nutrition to stimulate and/or sustain growth would explain these observations, and might also explain why strains UW R4 and UW P2 consistently exhibited poor growth under the conditions used in this study despite their axenic status. Lastly, consideration must also be given to the possibility that at least some of these uncultured phylotypes are unable to degrade crystalline cellulose, which could also explain why axenic cultures were not recovered.

The combination of culture-dependent and culture-independent analyses used in this study provided new insights into the ecology of *Fibrobacter* spp. in the gastrointestinal tracts of herbivores. As expected, the highest relative abundances of Fibrobacteres sequences were observed in samples from strictly herbivorous hosts with large body weights. Importantly, the *Fibrobacter* populations in these animals were typically dominated by phylotypes from the *F. succinogenes* species group, and not *F. intestinalis*, despite most of these hosts being hindgut fermenters. In fact, no sequences classified to any of the *F. intestinalis* phylotypes were observed in our 22 horse fecal samples. Although the highest abundance Fibrobacteres populations were typically composed of *F. succinogenes*-related phylotypes, an exception was the ostrich, which had a population almost entirely made up of phylotype Fi I. Lower relative abundances of *Fibrobacter* were observed in samples from pigs and several primates, with phylotype Fi I being the most commonly observed phylotype in these phylogenetically diverse hosts. It is not known why phylotype Fi I, which is represented by the *F. intestinalis* type strain NR9, is particularly suited for the hindguts of these hosts. No sequences classified as phylotype Fi I were observed in rumen samples examined here, or in those from the GRC. It has been suggested that, despite its name, *F. intestinalis* is also present in the rumen (Amann *et al.*, 1992). Sequences likely representative of *F. intestinalis* strain JG1, originally isolated from the rumen of sheep (Amann *et al.*, 1992), were observed in two sheep rumen contents examined here, as were sequences classified to phylotype Fi II in the GRC dataset, particularly in the rumen samples from giraffes. These observations suggest that some strains of *F. intestinalis* live in the rumen, but that they are likely distinct from those closely related to the *F. intestinalis* type strain and that their presence may depend on an association with certain host species. Two phylotypes of *F. intestinalis*, Fi II and Fi III, isolated here from pigs and a rhesus monkey were rarely observed in the culture-independent analysis, including the samples from which they were isolated. This indicates that the rare biosphere harbors more *Fibrobacter* diversity than is currently appreciated, and that culture-based methods represent an effective means of accessing this diversity for further characterization.

A clear difference in the *Fibrobacter* phylotypes commonly observed in the bovine rumen and horse hindgut was observed in our 16S rRNA gene amplicon data. *Fibrobacter* phylotypes typically observed in the rumen, Fs I-IV, were either not detected, or rarely observed in horse fecal samples. Recovery and phylotyping of *Fibrobacter* isolates from horse fecal samples confirmed previously reported culture-independent evidence of

phylogenetically distinct *Fibrobacter* populations in the hindgut of horses (Lin and Stahl, 1995). We observed no evidence for populations of *Fibrobacter* phylotypes Fs V or Fs VI in rumen samples, although our culture-independent data indicated that these phylotypes make up a substantial fraction of the Fibrobacteres population in the horse hindgut. Additionally, the total absence of sequences corresponding to these phylotypes in the GRC (Henderson *et al.*, 2015), a global dataset that includes hundreds of samples from diverse ruminant host species consuming different diets, across a broad geographic range, strongly reinforces that this is a general phenomenon. Based on these observations, we hypothesize that *Fibrobacter* phylotypes Fs V and Fs VI are specifically adapted to the hindgut, and that they do not compete effectively enough with the typical rumen phylotypes (Fs I-IV) to be readily observed in that environment. The phylogenetic and ecological attributes, separating Fs V and Fs VI from previously characterized strains of *F. succinogenes*, suggest that these phylotypes may warrant classification as a distinct species in the genus *Fibrobacter*.

An underappreciated aspect of *Fibrobacter* ecology is the co-occurrence of multiple *Fibrobacter* phylotypes within a given host, as was demonstrated here for rumen samples as well as most fecal samples from horses. In Holstein cow rumen samples, sequences classified as *Fibrobacter* phylotypes Fs I, Fs II, Fs III, and Fs IV were consistently observed together within a given sample, although Fs I and Fs II were typically dominant. This pattern of *Fibrobacter* phylotype co-occurrence was found across multiple ruminant host species in the GRC dataset, and is in agreement with previous observations (Amann *et al.*, 1992; Shinkai *et al.*, 2009; Shinkai *et al.*, 2010). We hypothesize that these *Fibrobacter* phylotypes occupy distinct ecological niches *in vivo*, but data supporting this supposition is sparse. Despite considerable phylogenetic diversity within the genus *Fibrobacter*, a general lack of phenotypic variation has been reported (Amann *et al.*, 1992). All of the isolates examined in our study grew on crystalline cellulose, produced succinate and acetate as major fermentation products in similar ratios, and failed to exhibit any growth on beechwood xylan. Although it has been reported that some *Fibrobacter* strains are capable of growth on xylan (Miron and Ben-Ghedalia, 1993), recent genomic evidence suggests that the inability of grow on pentoses is a unifying feature for the entire phylum (Suen *et al.*, 2011; Abdul Rahman *et al.*, 2016). The expansion of cultured *Fibrobacter* representatives resulting from this work will aid future efforts to identify the specific niches of distinct and co-occurring *Fibrobacter* phylotypes in the herbivore gastrointestinal tract.

In conclusion, this study not only expands our phylogenetic and physiological understanding of bacteria in the genus *Fibrobacter*, but also presents a novel and generalizable isolation method for recovering axenic cultures of these bacteria from herbivore hosts. Our study focused on hindgut-fermenting herbivores, as these hosts represent a rich source of uncultured *Fibrobacter* diversity and harbor microbial communities that are distinct and less well characterized than those of ruminants. Importantly, we present the first cultured representatives for four distinct *Fibrobacter* phylotypes, and our culture-independent analyses revealed insights into their ecology. Future work, including whole-genome sequencing and further phenotypic characterizations, promises to shed additional light on the ecology and evolution of these relatively abundant, but enigmatic, fiber-degrading bacterial symbionts of herbivores. Given the presence of Fibrobacteres within the gastrointestinal tracts of a broad diversity of herbivores, our work not only expands our understanding of this

phylum, but also provides access to cultured isolates from which hypotheses generated from culture-independent, sequence-based studies can be effectively tested.

Experimental Procedures

Sample collection and processing

Animal hosts sampled for this study were housed at the Henry Vilas Zoo (Madison, WI), the Milwaukee County Zoo (Milwaukee, WI), the US Dairy Forage Research Center farm (Prairie du Sac, WI), and several other research facilities managed by the University of Wisconsin – Madison (Madison, WI) (Table S1). Institutional Animal Care and Use Committee (IACUC) approval was obtained from the proper authority where applicable. Fresh feces, or gastrointestinal contents where available, were collected aseptically in sterile 50 ml conical tubes, immediately placed on wet ice, and transported back to the lab for same day processing, typically less than four hours post collection. Upon arrival at the lab, samples were transferred to an anaerobic glovebag (atmosphere: 5% H_2, 20% CO_2, balance N_2) and preserved for future isolation attempts and culture-independent bacterial community analysis. Preservation of samples for culturing consisted of preparing a suspension by mixing one part sample with four parts sterile anaerobic buffer (155 mM NaCl, 3 mM Na_2HPO_4-$7H_2O$, 1.5 mM KH_2PO_4, 25 mM $NaHCO_3$, 6 mM L-cysteine HCl, pH=6.8) followed by 2× dilution in an equal volume of sterile glycerol, distribution to sterile serum vials with butyl rubber stoppers and aluminum crimp seals via needle and syringe, and storage at −80°C. Preservation of samples for culture-independent analysis involved 10× dilution in $T_{50}E_{50}$ sucrose buffer (50 mM Tris-HCl, 50 mM EDTA, 15% sucrose, pH = 8.0) and storage at −20°C.

Isolation of Fibrobacter spp

Enrichment for anaerobic bacteria capable of growth on crystalline cellulose was achieved by serial dilution of the glycerol stocks prepared from feces, or gastrointestinal contents, in a slightly modified version of a medium originally described by Scott and Dehority (Scott and Dehority, 1965) with 0.5% (wt/vol) Sigmacell 50 (Sigma-Aldrich, St. Louis, MO) as the primary carbon source. The exact formula, minus the carbon source, of the modified Dehority medium (MDM) used for enrichment and isolation can be found in Table S7. The final volume of media used was 10 ml in 18 × 150 mm anaerobic tubes, with a gas phase of 100% CO_2, sealed with gas impermeable butyl rubber stoppers and 20 mm aluminum crimp seals (Bellco Glass, Vineland, NJ). The enrichments were incubated at 39°C and monitored via light microscopy daily for bacterial colonization of the cellulose crystals. Simple staining of the cells using crystal violet was employed to aid in microscopic examinations. Upon observing dense colonization, 1 ml of the enrichment was used to further enrich for cellulose-adherent bacteria using a modified version of the assay of adhesion described by Gong and Forsberg (Gong and Forsberg, 1989) performed in an anaerobic glovebag with a gas phase of 5% H_2, 20% CO_2, balance N_2. Briefly, 1 ml of sterile anaerobic buffer containing 2% (wt/vol) Sigmacell 50 (Sigma-Aldrich) was added to a 1.5 ml microcentrifuge tube and the cellulose was allowed to settle on the bottom of the tube. The liquid phase was discarded and 1 ml of enrichment culture was added to the cellulose pellet. The sample was mixed and bacterial attachment was promoted by incubating the tube at

39°C for 30 min. After the initial incubation period, the liquid phase was discarded and the cellulose pellet was washed with 1 ml of sterile anaerobic buffer (prewarmed to 39°C). The cellulose was allowed to settle, three min, while maintaining 39°C. Once again, the liquid phase was discarded and the process was repeated for a total of ten washes to remove non-adherent bacteria. Upon completion of the washes, the sample was serially diluted in MDM + cellulose and incubated at 39°C. The enrichments were monitored daily for signs of growth characteristic of *Fibrobacter* cultures, including visual observation of a reduction in the size of the cellulose sediment, often accompanied by a surface layer of slime, and microscopic observation of dense colonization of the cellulose crystals by rod-shaped cells. The most dilute culture exhibiting the desired characteristics was selected and the process was repeated until a culture suspected to be axenic was obtained, typically 3–5 rounds of washes followed by dilution to extinction. Isolation was confirmed through the recovery of a single 16S rRNA gene sequence using the universal bacterial primers 27F and 1492R (Weisburg *et al.*, 1991) (see below).

Phylogenetic analysis of Fibrobacter isolates

Genomic DNA (gDNA) extraction from suspected axenic *Fibrobacter* cultures was performed using a combination of bead-beating and SDS for cell lysis followed by phenol:chloroform extraction and alcohol precipitation (Stevenson and Weimer, 2007). Cell pellets from late log phase cultures, 24 – 48 hours post inoculation, were resuspended in 1 ml DNA extraction buffer (100 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, pH = 8.0) and combined with 0.5 g 0.1 mm zirconium beads, 700 µl equilibrated phenol (pH = 8.0) and 50 µl 20% SDS. The samples were subjected to bead-beating for 2 min followed by incubation at 60°C for 10 min followed by additional bead-beating for 2 min. The organic and aqueous phases were separated via centrifugation and the aqueous phase extracted with 500 µl equilibrated phenol:chloroform:isoamyl alcohol (25:24:1). A third, and final, extraction of the aqueous phase with 500 µl phenol:chloroform:isoamyl alcohol was performed followed by precipitation of the gDNA with 3 M Na acetate and isopropanol, then dried and subsequently resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 8.0). gDNA was quantified using the BR dsDNA assay kit and Qubit® Fluorometer (Invitrogen, Carlsbad, CA).

Near full-length 16S rRNA gene sequences were identified for each isolate by PCR and Sanger sequencing using the universal bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Weisburg *et al.*, 1991). PCR was performed using the Herculase II Fusion DNA Polymerase with dNTPs Combo kit (Agilent Technologies, Santa Clara, CA). Each 20 µl reaction contained: 4 µl 5× reaction buffer, 1 µl each of forward and reverse primers at a stock concentration of 10 µM, 0.2 µl 100 mM dNTPs, 0.4 µl Herculase II Fusion DNA Polymerase, 12.4 µl sterile H_2O, and 1 ng DNA template. The cycling conditions were as follows: 1 min initial denaturation at 95°C followed by 30 cycles consisting of 30 s at 95°C, 45 s at 50°C, and 1 min at 72°C after which a final extension step for 10 min at 72°C was performed. The PCR products, size ~ 1465 bp, were separated via electrophoresis through a 1% low-melt agarose (National Diagnostics, Atlanta, GA) gel and visualized using SYBR® Safe DNA Gel Stain (Invitrogen). PCR products of appropriate size were excised from the

gel and purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corp., Irvine, CA). Purified PCR products were submitted for Sanger sequencing (Functional Biosciences, Inc., Madison, WI).

A maximum likelihood phylogenetic tree was inferred from near full-length 16S rRNA gene sequences in MEGA 7.0 (Kumar *et al.*, 2016) using the Tamura-Nei substitution model (Tamura and Nei, 1993) and 1,000 bootstrap replicates. The initial tree for the heuristic search was generated automatically by applying Neighbor-Join and BioNJ algorithms to the matrix of pairwise distances estimated using maximum composite likelihood followed by selection of the phylogeny with the highest log likelihood. Prior to constructing the tree, the sequences were aligned in MEGA using ClustalW, with default parameters, and trimmed to an equal number of nucleotide positions (1268 sites). A 16S rRNA gene sequence from the *Bacteroides fragilis* type strain NCTC 9343 (NCBI Ref. CR626927.1) was included as an outgroup. The operational taxonomic units (OTUs) represented by the *Fibrobacter* isolates, henceforth referred to as *Fibrobacter* phylotypes, were determined using mothur v.1.38 (Schloss *et al.*, 2009). A distance matrix was created from the aligned and trimmed 16S rRNA gene sequences with the *dist.seqs* command and used as the input for the *cluster* command, which was run using the average neighbor algorithm with a distance cutoff of 0.03.

Culture-independent bacterial community profiling

Total DNA was extracted from gastrointestinal contents or feces that had been diluted and frozen in $T_{50}E_{50}$ sucrose buffer. The samples were thawed and the cells resuspended in 1 ml DNA extraction buffer. DNA was recovered using bead-beating and phenol:chloroform as described above for suspected isolates. PCR amplification of the bacterial 16S rRNA gene was performed using universal primers flanking the variable 4 (V4) region (Kozich *et al.*, 2013). Reactions contained 2 ng DNA template, 0.4 μM each primer, 12.5 μl 2× HotStart ReadyMix (KAPA Biosystems, Wilmington, MA), and H_2O to 25 μl. Cycling conditions were as follows: initial denaturation of 95°C for 3 min followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The PCR products were separated via electrophoresis through a 1% low-melt agarose (National Diagnostics) gel and visualized using SYBR® Safe DNA Gel Stain (Invitrogen). PCR products of appropriate size were excised from the gel and purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corp.). Purified PCR products were quantified using the HS dsDNA assay kit and Qubit® Fluorometer (Invitrogen) and equimolar pooled. The pool plus 5% PhiX control DNA was sequenced with the MiSeq 2×250 v2 kit (Illumina, San Diego, CA) using custom sequencing primers (Kozich *et al.*, 2013).

Sequences were demultiplexed on the Illumina MiSeq and processed using mothur v.1.38 (Schloss *et al.*, 2009) in general accordance with the MiSeq S.O.P. (https://www.mothur.org/wiki/MiSeq_SOP). Individual contigs were assembled from the corresponding paired-end sequences and poor quality sequences were removed. Sequences were aligned to the SILVA 16S rRNA gene reference database (Pruesse *et al.*, 2007) to check for alignment to the V4 target region. Chimeras were detected, and subsequently removed, using the *chimera.uchime* command (http://drive5.com/usearch/manual/uchime_algo.html) on a trimmed version of the

alignment that had been reduced using *unique.seqs* and *pre.cluster* (diffs = 2). Sequences that could not be classified to domain Bacteria, such as those classified to domain Eukaryota, domain Archaea, or domain "unknown" were removed from the dataset. Sequences suspected to be either host-cell or diet derived due to their classification as mitochondria and chloroplast, respectively, were also removed.

After the processing steps outlined above, the "cleaned" sequences were clustered into OTUs using the average neighbor algorithm with a distance cutoff of 0.03 with the *dist.seqs* and *cluster.split* commands in mothur. All OTUs represented by only a single sequence were subsequently removed using the *remove.rare* command. OTUs were classified using the Greengenes database (DeSantis *et al.*, 2006) with the *classify.seqs* command (consensus confidence level ≥ 80%) followed by *classify.otu*. A matrix of OTU counts by sample, normalized to 10,000 sequences per sample, was generated using the *make.shared* and *normalize.shared* commands. A representative sequence for each OTU was extracted using the *get.oturep* command. The count data and relevant metadata were then imported into R (version 3.3.1) for statistical analyses and plotting (https://www.r-project.org/). A non-metric multidimensional scaling (nMDS) ordination analysis of Bray-Curtis dissimilarities among the total bacterial communities was performed using the *metaMDS* function from the VEGAN package (Dixon, 2003). Standard error ellipses were generated for select groups of samples from the ordination results using the VEGAN function *ordiellipse*.

In silico analysis of Fibrobacter spp. ecology

The V4 16S rRNA gene sequences classified to the phylum Fibrobacteres using Greengenes (DeSantis *et al.*, 2006) were extracted from the "cleaned" fasta file using the *get.lineage* command in mothur. These Fibrobacteres sequences were then clustered into OTUs using the same methods described for the total bacterial data. The Fibrobacteres OTUs were then classified in mothur, as described above, to *Fibrobacter* phylotypes using the 16S rRNA gene sequences for the isolates described in this study, as well as *F. succinogenes* subsp. *elongatus* strain HM2 (NCBI Ref. NR_104844.1), and a taxonomy file which included their designated phylotypes. No other *Fibrobacter* sequences from previously reported isolates were included in the reference taxonomy due to either a lack of sequences of sufficient quality or because they failed to add phylotype diversity to the dataset. As a result, only sequences containing no ambiguous base calls from cultured isolates were included. A matrix of the Fibrobacteres OTU counts by sample was generated using the *make.shared* command. The Fibrobacteres OTU counts were then summed according to phylotype and imported into R for statistical analysis and plotting. Normalized sequence counts for the *Fibrobacter* phylotypes were fit onto the total bacteria NMDS plot, described above, using the *envfit* function from VEGAN. Vectors corresponding to phylotypes exhibiting a fit with p-value < 0.20 were plotted onto the ordination. However, only vectors exhibiting a fit with p-value < 0.05 after adjustment using the Bonferroni correction were deemed statistically significant. Representative sequences for Fibrobacteres OTUs were extracted in mothur with the *get.oturep* command, followed by the creation of a distance matrix from the representative sequences using the *dist.seqs* command. The representative sequences and distance matrix were then imported into R using the *read.phyDat* function from the PHANGORN (Schliep, 2010) package and the *import_mothur_dist* function from the PHYLOSEQ (McMurdie and Holmes, 2013)

package. A maximum likelihood neighbor-joining tree was inferred from the representative sequences and distance matrix in R using the *bionj* function from the APE (Paradis *et al.*, 2004) package followed by the *pml*, *optim.pml*, and *bootstrap.pml* functions from the PHANGORN package. The phylogenetic tree and Fibrobacteres OTU count table were then used to create a "phyloseq" object using *phyloseq* from the PHYLOSEQ package, and the phylogenetic tree plotted with the function *plot_tree*. Closest BLAST hits for representative sequences for Fibrobacteres OTUs were determined by running *blastn* from the command line (Camacho *et al.*, 2009) using the near-full length 16S rRNA gene sequences of *Fibrobacter* isolates from this study and previously described reference strains (Amann *et al.*, 1992) as the reference database.

16S rRNA gene amplicon sequence data from the Global Rumen Census (GRC) (Henderson *et al.*, 2015) was downloaded from the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA), https://www.ncbi.nlm.nih.gov/sra (PRJNA272135, PRJNA272136, PRJNA273417), using *fastq-dump* from the SRA toolkit. Raw sequences from the GRC were processed in mothur using a pipeline similar to the one described for the Illumina data generated in this study. Sequences classified to the phylum Fibrobacteres using Greengenes were extracted from the "cleaned" GRC fasta file using the *get.lineage* command. The Fibrobacteres sequences were then clustered into OTUs and classified in mothur to *Fibrobacter* phylotypes using the *Fibrobacter* custom taxonomy file, described above. The matrix of GRC Fibrobacteres OTU counts by sample was generated using the *make.shared* command. The GRC Fibrobacteres OTU counts were then summed according to phylotype and imported into R for statistical analysis and plotting.

Phenotypic characterization

Growth on crystalline cellulose and xylan was investigated using MDM with either 0.3% (wt/vol) Sigmacell 20 (Sigma-Aldrich, St. Louis, MO) or 0.3% (wt/vol) xylan from beechwood (Megazyme, Bray, Ireland) as the primary carbon source. All growth tests were performed in triplicate, with 1 ml of medium in 1.5 ml microcentrifuge tubes in an anaerobic glovebag with a gas phase of 5% H_2, 20% CO_2, balance N_2. The cultures were incubated at 39°C for 48 hours. Growth was assessed by visually monitoring the cultures for increased turbidity. Fermentation products present in the supernatants of each triplicate 48 hour culture for strains exhibiting growth on a particular carbon source were quantified using high-performance liquid chromatography as described previously (Weimer *et al.*, 1991). Concentrations of fermentation products for positive cultures are summarized as the average ± one standard deviation.

Accession numbers

Near full-length 16S rRNA gene sequences for the 45 *Fibrobacter* strains isolated in this study have been deposited in the NCBI Nucleotide database under accessions numbers KY463324 – KY463368 (Table S2). Raw Illumina sequencing reads corresponding to the V4 16S rRNA gene amplicons for the 83 animal host gut and feces microbiotas have been deposited in the National Center for Biotechnology Information's Short Read Archive database under Bioproject accession PRJNA362214.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Originality-Significance Statement

Bacteria in the genus *Fibrobacter* are important for cellulose digestion in the rumen, but their relationship with hindgut-fermenting herbivores remains poorly characterized. Here we report an ecological analysis of *Fibrobacter* populations associated with diverse hindgut-fermenting herbivores. We describe a novel approach for recovering axenic *Fibrobacter* cultures from gastrointestinal samples and demonstrate its utility by isolating 45 novel *Fibrobacter* strains, several of which represent previously uncultured phylotypes (cutoff = 0.03%). This work provides the strongest evidence to date that the dominant *Fibrobacter* populations in ruminants and hindgut-fermenting herbivores are phylogenetically distinct, but that their proficient growth on crystalline cellulose is a conserved feature among these bacteria.

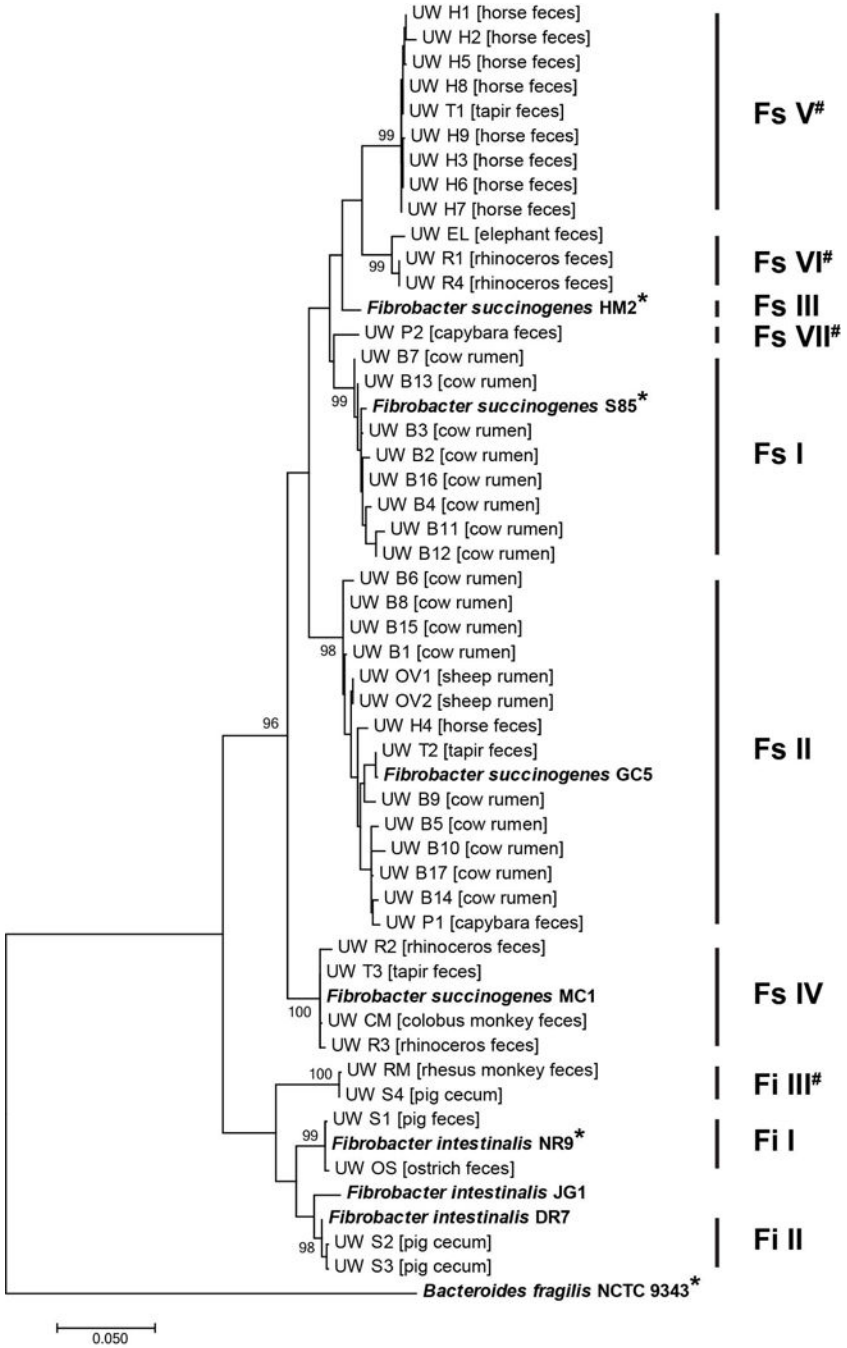

Figure 1. Maximum likelihood inferred phylogeny of *Fibrobacter* isolates

The phylogeny was constructed using near-full length 16S rRNA gene sequences (1268 sites). Reference sequences for previously described *Fibrobacter* strains, including the type strains for *F. succinogenes*, *F. succinogenes* subsp. *elongatus*, and *F. intestinalis*, are shown in bold with the type strains marked with an asterisk (*). Strain designations can be found at the branch tips, followed by the isolation source in brackets. *Fibrobacter* phylotypes are displayed on the far right along with a vertical line indicating their coverage of the phylogeny. Phylotypes with no previous cultural representation are denoted with a hash (#).

Bootstrap values, over 90% (1,000 replicates), for clades representing *Fibrobacter* phylotypes are displayed at their respective branch points. The *Bacteroides fragilis* type strain NCTC 9343 was included as an outgroup.

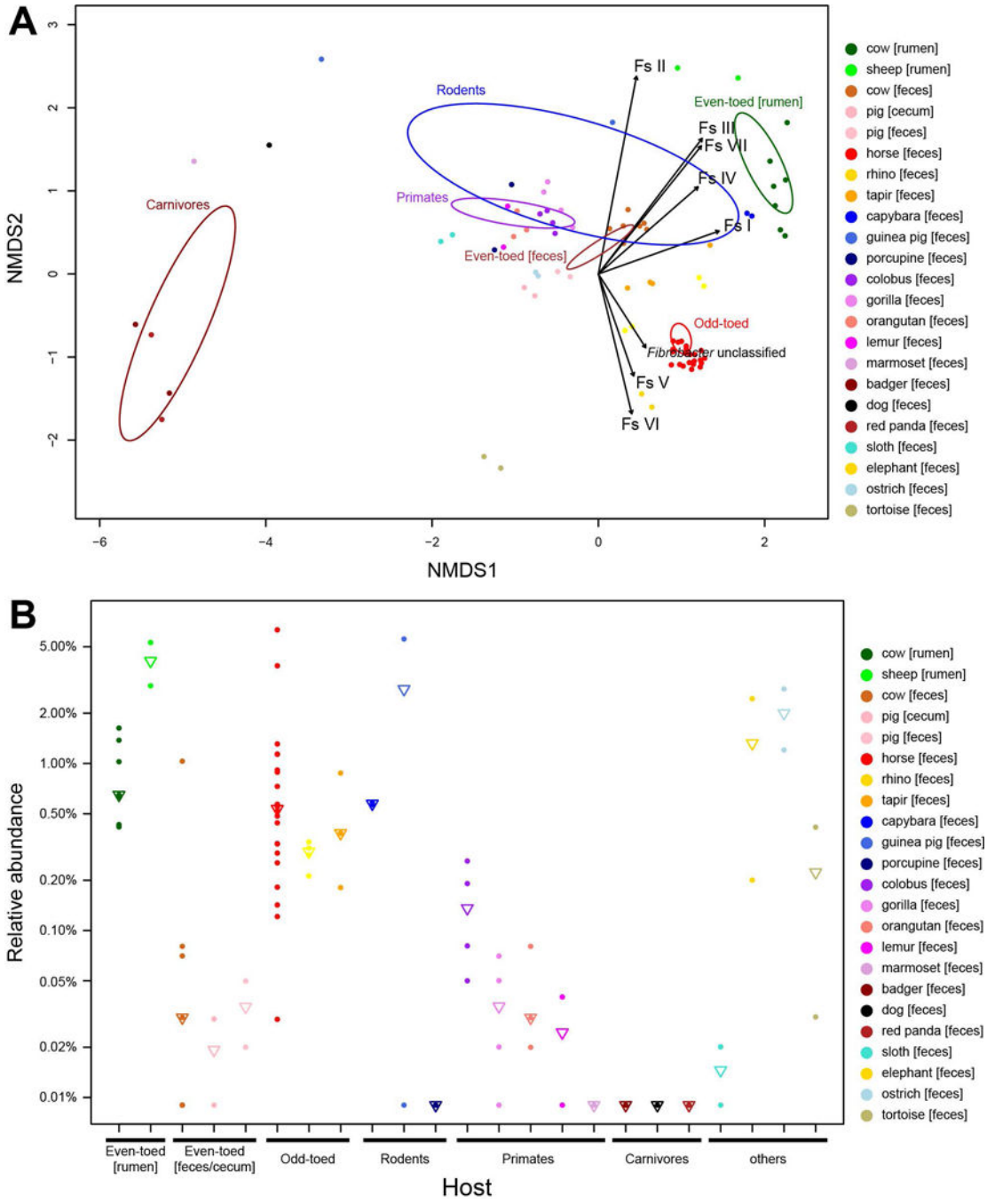

Figure 2. Culture-independent analysis of gastrointestinal tract samples

(a) NMDS ordination plot of Bray-Curtis dissimilarity among the total bacterial communities. Total bacterial communities are represented by dots colored according to host of origin. Sets of samples corresponding to specific host taxonomic orders are plotted as open standard error ellipses (95% confidence interval), and colored/labeled accordingly. Vectors representing sequence counts for *Fibrobacter* phylotypes showing a possible association (p-value < 0.2) with a certain area of the ordination are shown and labeled accordingly. See Table S6 for more details regarding the statistical support for the individual

vectors. (b) Percent relative abundance of Fibrobacteres sequences in samples by host. Each dot represents an individual sample, colored according to host of origin, and plotted on a logarithmic scale (y-axis). Median percent relative abundances for each host are plotted with open triangles. Samples are arranged by host taxonomic order, which are labeled along the x-axis.

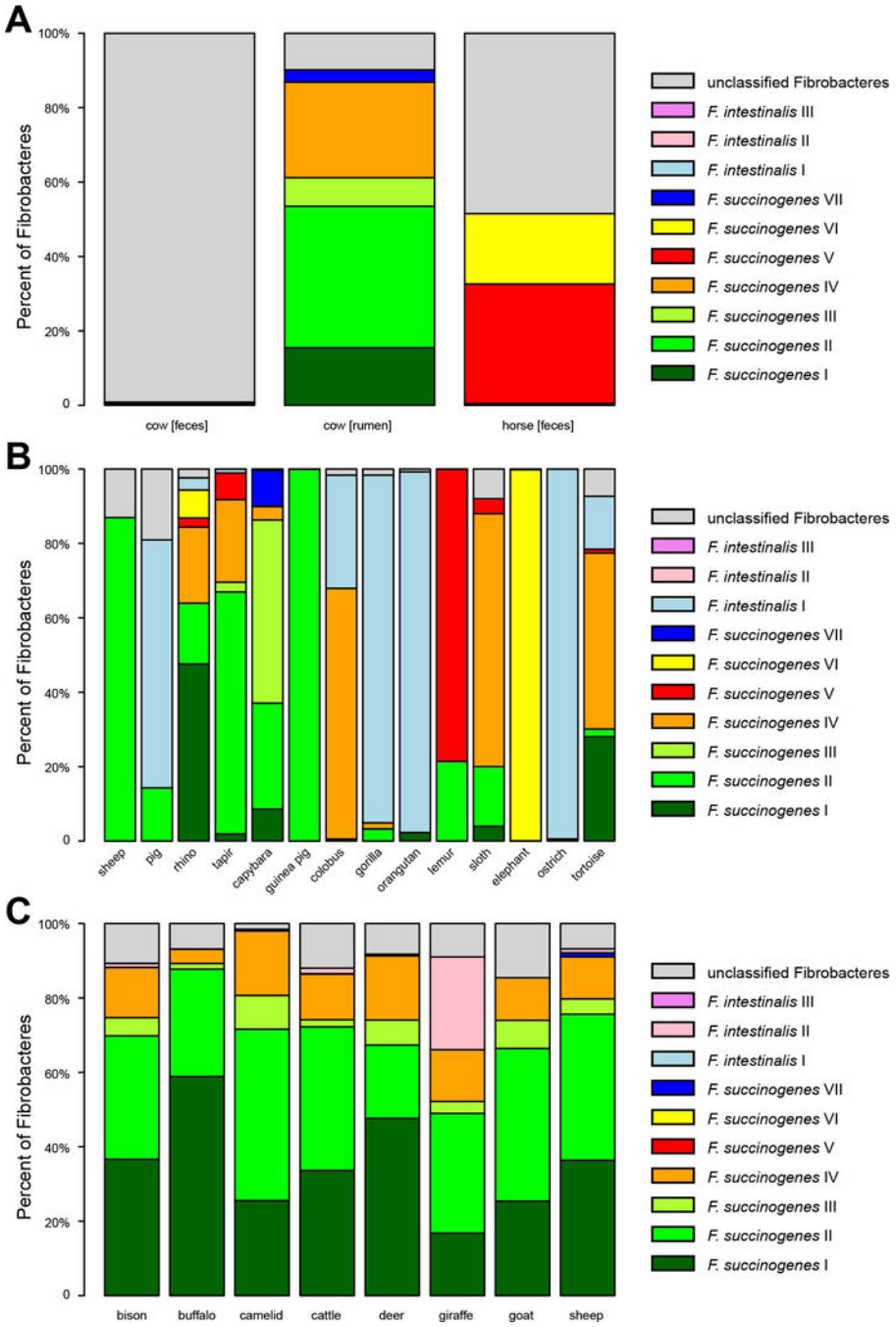

Figure 3. Fraction of Fibrobacteres sequences assigned to a specific *Fibrobacter* phylotype by host

(a) Distribution of *Fibrobacter* phylotypes in samples from the most extensively sampled hosts Holstein cows and horses. (b) Distribution of *Fibrobacter* phylotypes in other host samples for which Fibrobacteres sequences were observed. (c) Distribution of *Fibrobacter* phylotypes in rumen samples from various hosts, as determined by analysis of the 16S rRNA gene amplicon total bacterial dataset from the Global Rumen Census (GRC) (Henderson *et al.*, 2015).

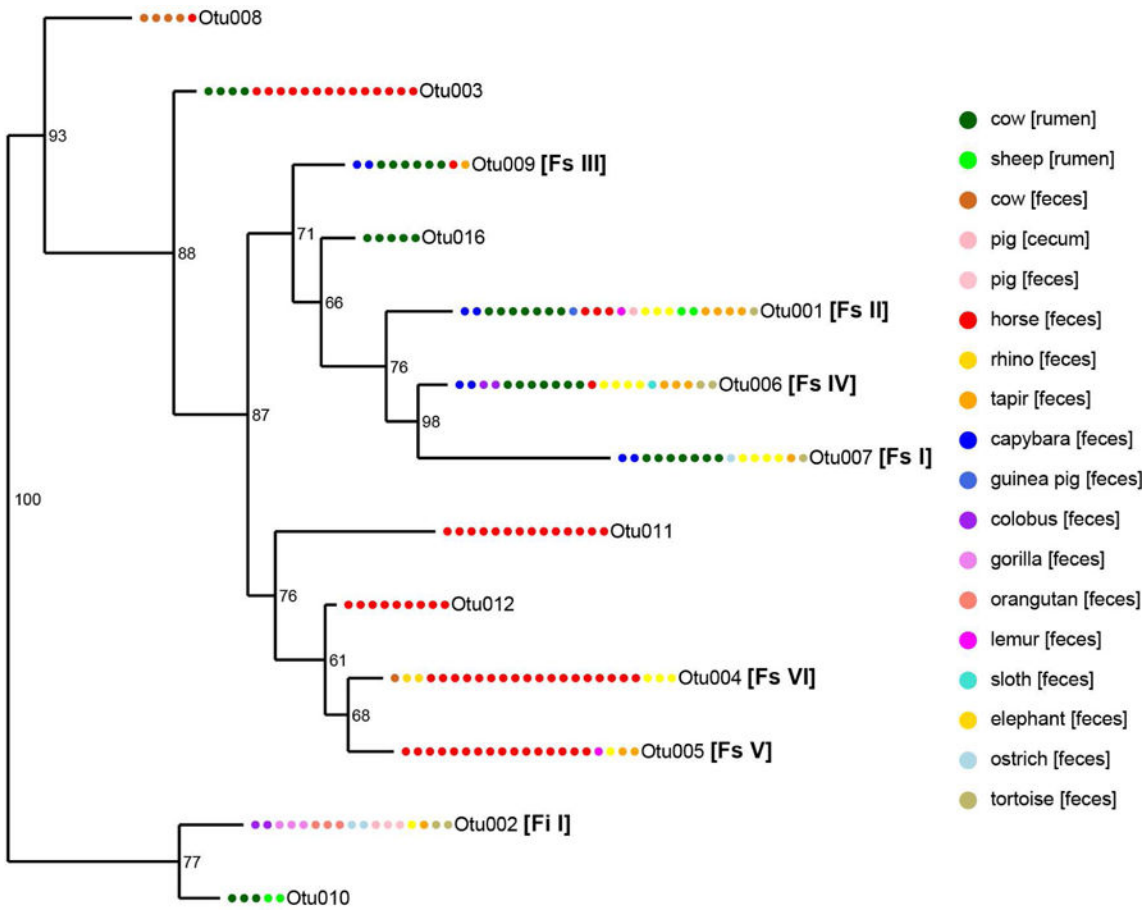

Figure 4. Phylogenetic relationships among common Fibrobacteres OTUs

A maximum likelihood neighbor-joining tree was inferred from the aligned nucleotide sequences representing commonly observed Fibrobacteres OTUs in the culture-independent analysis of total bacterial communities. Common OTUs were defined as those that were observed in more than one sample along with having a percent relative abundance of at least 0.1% in at least one sample. Nodes are labeled with the level of bootstrap support for their respective clades. Branch tips are labeled with dots, colored according to host of origin, indicating the occurrence of that particular OTU in a given microbiota sample, the OTU ID, and the corresponding *Fibrobacter* phylotype designation when appropriate (see Table 2).

Table 1

Sources of *Fibrobacter* isolates

Host Species	Sample Type	# of Isolates	# of Animals	# of sample sites	# of phylotypes
Cow (cattle)	R	17	14	3	2
Horse	F	9	8	4	2
Pig	F, C	4	4	2	3
Rhinoceros	F	4	2	2	2
Tapir	F	3	2	2	3
Capybara	F	2	unknown*	1	2
Sheep	R	2	2	1	1
Colobus monkey	F	1	unknown*	1	1
Elephant	F	1	1	1	1
Ostrich	F	1	1	1	1
Rhesus monkey	F	1	1	1	1

R = rumen contents, F = feces, C = cecal contents

* group sample

Table 2

16S rRNA sequence similarity values for common Fibrobacteres OTUs

OTU	Closest reference	Identity	Length	Mismatch	Gap	e-value
Otu001	*F. succinogenes* GC5 [Fs II]	100%	253	0	0	1.64E-134
Otu002	*F. intestinalis* NR9* [Fi I]	100%	253	0	0	1.64E-134
Otu003	UW H9	96.84%	253	8	0	3.60E-121
Otu004	UW R1 [Fs VI]	99.61%	253	1	0	7.62E-133
Otu005	UW H9 [Fs V]	100%	253	0	0	1.64E-134
Otu006	UW T3 [Fs IV]	100%	253	0	0	1.64E-134
Otu007	*F. succinogenes* S85* [Fs I]	100%	253	0	0	1.64E-134
Otu008	UW P2	93.33%	255	14	3	3.67E-106
Otu009	*F. succinogenes* HM2* [Fs III]	99.61%	253	1	0	7.62E-133
Otu010	*F. intestinalis* JG1	99.61%	253	1	0	7.62E-133
Otu011	F. succinogenes HM2	92.49%	253	19	0	7.95E-103
Otu012	UW H9	97.23%	253	7	0	7.73E-123
Otu016	UW P2	96.46%	254	9	0	4.67E-120

Common OTUs were defined as those that were observed in more than one sample along with having a percent relative abundance of at least 0.1% in at least one sample.

Fibrobacter phylotype designations, if known, as well as type strain status (*) are indicated for references corresponding to OTUs containing less than, or equal to, one mismatch over the length of the sequence examined.