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Distribution and diversity of members of the bacterial phylum *Fibrobacteres* in environments where cellulose degradation occurs

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ABSTRACT

The *Fibrobacteres* phylum contains two described species, *Fibrobacter succinogenes* and *Fibrobacter intestinalis*, both of which are prolific degraders of cellulosic plant biomass in the herbivore gut. However, recent 16S rRNA gene sequencing studies have identified novel *Fibrobacteres* in landfill sites, freshwater lakes and the termite hindgut, suggesting that members of the *Fibrobacteres* occupy a broader ecological range than previously appreciated. In this study, the ecology and diversity of *Fibrobacteres* was evaluated in 64 samples from contrasting environments where cellulose degradation occurred. Fibrobacters were detected in 23 of the 64 samples using *Fibrobacter* genus-specific 16S rRNA gene PCR, which provided their first targeted detection in marine and estuarine sediments, cryoconite from Arctic glaciers, as well as a broader range of environmental samples. To determine the phylogenetic diversity of the *Fibrobacteres* phylum, Fibrobacter-specific 16S rRNA gene clone libraries derived from 17 samples were sequenced (384 clones) and compared with all available *Fibrobacteres* sequences in the Ribosomal Database Project repository. Phylogenetic analysis revealed 63 lineages of *Fibrobacteres* (95% OTUs), with many representing as yet unclassified species. Of these, 24 OTUs were exclusively comprised of fibrobacters derived from environmental (non-gut) samples, 17 were exclusive to the mammalian gut, 15 to the termite hindgut, and 7 comprised both environmental and mammalian strains, thus establishing *Fibrobacter* spp. as indigenous members of microbial communities beyond the gut ecosystem. The data highlighted significant taxonomic and ecological diversity within the *Fibrobacteres*, a phylum circumscribed by potent cellulolytic activity, suggesting considerable functional importance in the conversion of lignocellulosic biomass in the biosphere.

Introduction

Cellulose is Earth's most abundant organic polymer and, as such, the microbial-mediated degradation of cellulosic biomass is a fundamental mechanism in the global carbon cycle [29]. Cellulose hydrolysis occurs in both oxic and anoxic environments, where anaerobic decomposition usually occurs due to the synergistic interaction of a consortium of bacteria, rather than the activity of a single species [29]. This is best exemplified in the rumen, where the microbial decomposition of cellulosic plant biomass has been relatively well studied, with members of the genus *Fibrobacter* thought to be the main bacterial degraders of cellulose [8,26,27] in conjunction with *Ruminococcus albus* and *Ruminococcus flavefaciens* [8,39,43,48]. However, *Fibrobacter succinogenes* is considered to be the predominant bacterial degrader of cellulose in the rumen [26], since when *F. succinogenes* strains S85 and A3C were grown in pure culture alongside *R. albus* and *R. flavefaciens*, the *Fibrobacter* species degraded more of the cellulose from intact forage than the *Ruminococcus* species. [7]. This may be explained by the recent observation that *Fibrobacter* species do not appear to utilise either of the two well-established mechanisms of cellulose-decomposition; the aerobic cell-free cellulase mechanism [54], or the cellulosome system typified by anaerobic bacteria and fungi [9]. Instead, the superior efficiency of cellulolysis by *Fibrobacter* species [7] is thought to arise from a novel enzyme mechanism for cellulose decomposition that appears to be restricted to members of the *Fibrobacteres* phylum. The genome of the type strain, *F. succinogenes* S85 does not appear to contain exocellulases or processive endocellulases, and these enzymes are required for both the cellulosomal and free cellulase methods used for cellulose hydrolysis

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[52]. Furthermore, none of the predicted cellulase genes contain the carbohydrate binding molecules, dockerin domains or scaffoldin genes that are typically associated with cellulosomes [46]. Consequently, it has been suggested that the method by which *F. succinogenes* degrades cellulose involves adherence of a putative fibro-slime protein located on the outer membrane of the cell [46] before the severing of individual cellulose chains. These chains are thought to be subsequently transported into the periplasmic space where they are hydrolysed by endoglucanases [53].

F. succinogenes was first isolated from the rumen in 1947 and was originally designated as *Bacteroides succinogenes* [23,24]. However, the subsequent application of 16S rRNA gene-based phylogeny demonstrated that *B. succinogenes* belonged to a separate genus, *Fibrobacter*, that contained two species, the renamed *F. succinogenes* and the newly described *Fibrobacter intestinalis*, both of which were thought to be present only in the mammalian intestinal tract [38].

The diversity of *Fibrobacter* spp. in the herbivore gut has been relatively well characterised, particularly using oligonucleotide probes and comparative sequencing of the 16S rRNA gene. Stahl et al. [45] designed the first *Fibrobacter*-specific oligonucleotide probes. Their three probes had varying levels of specificity, one designed to target all but one of the known *Fibrobacter* strains, one to target rumen isolates, and the other to target caecal strains, enabling the successful detection and quantification of fibrobacters where cultivation-based methods were unsuccessful [45]. Fluorescently labelled oligonucleotide probes were subsequently designed for *F. succinogenes*, *F. intestinalis* and *F. succinogenes* subsp. *succinogenes*, which when used alongside comparative sequencing enabled the characterisation of eight previously uncharacterised *Fibrobacter* strains [1]; five isolated from ovine rumen, two from bovine rumen and one from the bovine caecum [1], with strain identification later confirmed by DNA:DNA hybridisation [2]. Consequently, the application of rRNA-targeted probes enabled the quantification of fibrobacters in the rumen [31,32,45], and the detection of novel *Fibrobacter* populations in the bovine [31,45] caprine [31] and equine [32] intestinal tract. Significantly, Stahl et al. [45] determined that the probe designed to target all but one of the currently isolated, putative *Fibrobacter* strains (then members of *Bacteroides*) detected a greater number of fibrobacters than the combination of rumen- and caecal-specific probes, leading to the suggestion that the bovine rumen contained previously uncharacterised species similar to *F. succinogenes*. This was later supported by the work of Lin et al. [31] which demonstrated that only half of the species detected by general *Fibrobacter* probes in cattle and goats could be detected by probes targeting the two specific species. A further study suggested the presence of novel *Fibrobacter* populations in the equine caecum [32], since application of a *Fibrobacter* genus-specific probe indicated that fibrobacters comprised 12% of the total 16S rRNA in the equine caecum. In addition, while the species-specific probe designed to target *F. succinogenes* suggested that the majority of these sequences belonged to *F. succinogenes*, there was no hybridisation with any of the three *F. succinogenes* subspecies-specific probes, indicating the presence of novel species or subspecies closely related to *F. succinogenes* [32].

F. succinogenes and *F. intestinalis* remain the only two formally described *Fibrobacter* species to date, possibly because fibrobacters are difficult to isolate and cultivate, and their ecology was previously thought to be restricted to the mammalian gut [42]. However, members of a novel subphylum of the *Fibrobacteres*, designated subphylum 2, have since been detected in the gut of wood-feeding termites [19,20] and proteomic analyses has confirmed that these novel *Fibrobacteres* were involved in cellulose hydrolysis in the termite hindgut [51].

Using a genus-specific 16S rRNA gene primer set, members of the genus *Fibrobacter* were detected in landfill sites [35,36] and freshwater lakes [34], providing the first evidence of members of the genus *Fibrobacter* beyond the intestinal tract. These environmental fibrobacters included novel phylogenetic lineages that represented as yet uncultivated species, in addition to *F. succinogenes*-like strains [42]. It has been suggested that fibrobacters are active members of the cellulolytic microbial community in these environments, since it has been demonstrated using quantitative PCR that they become enriched on heavily degraded cotton string both in landfill sites [35] and freshwater lakes [34]. In landfill sites, fibrobacters can comprise up to 40% of the total bacterial rRNA and reach relative rRNA abundances that exceed those detected in the ovine rumen [36].

The molecular detection of novel lineages of the *Fibrobacteres* phylum in landfill sites and freshwater lakes suggests that the true ecology and diversity of this poorly studied, but functionally important phylum, is not fully understood. To address the ecological range and diversity of fibrobacters, we applied *Fibrobacter* genus-specific PCR primer sets to DNA extracted from a range of natural and managed environments where cellulose decomposition occurred, expanding the range of ecological niches for which the presence of fibrobacters has previously been described. Cloning, sequencing and phylogenetic analysis of fibrobacters from seventeen of these environments, in addition to the current diversity of *Fibrobacteres* in the public databases, provides the most comprehensive analysis of the ecology and diversity of the phylum to date.

Materials and methods

Sampling

Sixty-four samples were collected from a range of mammalian gut, terrestrial, aquatic and managed environments, as listed in Table 1. Landfill leachate and water samples were processed by filtration though a 0.2 μm pore diameter membrane. Landfill leachate microcosms were constructed by placing nylon mesh bags containing dewaxed cotton string in 1 L Duran bottles, sterilised by autoclaving and transported to the landfill site where they were filled to the top with leachate in order to avoid the presence of air in the headspace, sealed and incubated in the laboratory at ambient temperature. For solid sample matrices, such as equine faeces, soils and sediments, samples were collected in sterile containers and transported to the laboratory where they were frozen at −80 °C. Samples of cryoconite were collected from three High-Arctic valley glaciers on Svalbard (Austre Brøggerbreen [AB], Midtre Lovénbreen [ML], and Vestre Brøggerbreen [VB]) and three alpine valley glaciers in Austria (Gaisbergferner [GB], Pfaffenferner [PF], and Rotmoosferner [RM]), as detailed by Edwards et al. [12]. In brief, samples were collected aseptically in 15 mL tubes and stored at −20 °C in field stations pending frozen transfer to the Aberystwyth laboratory.

DNA extraction

Either a complete membrane filter (0.2 μm pore diameter) or 0.5 g of sample material was subjected to nucleic acid extraction with phenol-chloroform-isoamyl alcohol and mechanical bead beating using the method of Griffiths et al. [15] with the following modifications. Prior to precipitation with polyethylene glycol, RNase A (Sigma) was added to the aqueous layer at a final concentration of 100 mg mL^{-1} and incubated at 37 °C for 30 min before the addition of an equal volume of chloroform-isoamyl alcohol (24:1) (Sigma), with centrifugation and precipitation as previously described [15]. The DNA was resuspended in 50 μL nuclease-free water (Bioline) and visualised on a 1% agarose (Bioline) gel