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MINIREVIEW

The *Fibrobacteres*: an Important Phylum of Cellulose-Degrading Bacteria

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Abstract The phylum *Fibrobacteres* currently comprises one formal genus, *Fibrobacter*, and two cultured species, *Fibrobacter succinogenes* and *Fibrobacter intestinalis*, that are recognised as major bacterial degraders of lignocellulosic material in the herbivore gut. Historically, members of the genus *Fibrobacter* were thought to only occupy mammalian intestinal tracts. However, recent 16S rRNA gene-targeted molecular approaches have demonstrated that novel centres of variation within the genus *Fibrobacter* are present in landfill sites and freshwater lakes, and their relative abundance suggests a potential role for fibrobacters in cellulose degradation beyond the herbivore gut. Furthermore, a novel subphylum within the *Fibrobacteres* has been detected in the gut of wood-feeding termites, and proteomic analyses have confirmed their involvement in cellulose hydrolysis. The genome sequence of *F. succinogenes* rumen strain S85 has recently suggested that within this group of organisms a "third" way of attacking the most abundant form of organic carbon in the biosphere, cellulose, has evolved. This observation not only has evolutionary significance, but the superior efficiency of anaerobic cellulose hydrolysis by *Fibrobacter* spp., in comparison to other cellulolytic rumen bacteria that typically utilise membrane-bound enzyme complexes (cellulosomes), may be explained by this novel cellulase system. There are few bacterial phyla with potential functional importance for which there is such a paucity of phenotypic and functional data. In this review, we highlight current knowledge of the *Fibrobacteres* phylum, its taxonomy, phylogeny, ecology and potential as a source of novel glycosyl hydrolases of biotechnological importance.

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Introduction

The Genus *Fibrobacter*

Since Robert E. Hungate first isolated *Fibrobacter succinogenes* (formerly *Bacteroides succinogenes*) from the bovine rumen in 1947 [47, 48], members of the genus *Fibrobacter* have been considered to be major degraders of cellulosic plant biomass in the herbivore gut [49, 55, 114]. *Fibrobacter* is currently the sole formal genus of the bacterial phylum *Fibrobacteres*, which is phylogenetically related to the well-characterised *Bacteroidetes* and *Chlorobi* phyla [20, 71]. *F. succinogenes* was initially classified as *B. succinogenes*, and this was attributed to the historical broad genus definition for *Bacteroides*: "all anaerobic, Gram-negative, nonmotile or peritrichous, nonsporeforming rods that do not produce butyric acid from the fermentation of carbohydrates" [14]. However, this resulted in the accumulation of many unrelated species within the *Bacteroides* genus. It was suggested that, as *B. succinogenes* possessed

mainly straight-chain fatty acids and lacked the membrane sphingolipids observed in other *Bacteroides* spp., it should be excluded from the genus [105].

Subsequently, 16S rRNA oligonucleotide cataloguing methods were used to demonstrate that *B. succinogenes* and *Bacteroides amylophilus* were in fact not closely related to the other *Bacteroides* species [98]. Montgomery and colleagues [91] utilised 16S rRNA gene sequencing methods to assess the phylogenetic relationship of *B. succinogenes* and its closest relatives, demonstrating that *B. succinogenes* isolates formed a phylogenetically coherent group, having no closely related organisms for which 16S rRNA gene sequence data were available. The genus *Fibrobacter* was circumscribed on this basis and contains only two recognised species, *F. succinogenes* and *Fibrobacter intestinalis*, both Gram-negative, obligate anaerobes that are the predominant bacterial colonisers and degraders of lignocellulosic plant material in the herbivore gut [91]. *F. succinogenes* comprised rumen isolates and *F. intestinalis* was the name assigned to the caecal isolates of *B. succinogenes*. Moreover, a previous study suggested that *B. succinogenes* isolates were sufficiently distant from other species to represent a distinct phylum [135]. Most recently, taxonomic distribution analysis of the predicted proteins in the *F. succinogenes* S85 genome confirmed that this species is indeed correctly classified at the phylum level [118].

Phenotypic Characteristics of *Fibrobacter* Isolates

Members of the genus *Fibrobacter* are defined as obligately anaerobic, non-sporeforming, Gram-negative, rods or pleiomorphic ovoid cells [91], 0.3 to 0.5 μm in diameter and 0.8 to 2.0 μm in length [48, 116]. The cells are able to migrate through agar medium by a mechanism comparable to that of *Cytophaga* spp. [48]. *Fibrobacter* spp. ferment xylan [36, 85, 109], glucose, cellobiose and cellulose, producing succinic and acetic acids, and sometimes a small amount of formic acid [91]. Ammonia [91], in addition to peptides and amino acids [4, 69], can be utilised as a source of nitrogen, and carbon dioxide, straight-chain and branched-chain fatty acids and one or more vitamins (typically biotin, *p*-aminobenzoic acid, B_{12} (cyanocobalamine) or thiamine) are also required for growth [91].

There are currently no definitive phenotypic characteristics that can be used to separate *F. succinogenes* and *F. intestinalis*. Previously, it was considered that *F. succinogenes* is a rumen bacterium while *F. intestinalis* inhabits the caecum [91]. This was later discredited when the use of rRNA gene-targeted oligonucleotide probes demonstrated that *F. intestinalis* is present in the rumen [113], and *F. intestinalis* strains LH1 and JG1 were subsequently isolated from the ovine rumen (Table 1). Furthermore, *F. succinogenes* was thought likely to be present in the intestine due to the carriage from rumen digesta [91], and this was confirmed by the isolation of stain GC5 from the bovine cecum (Table 1). Although it is evident that a loose relationship exists between the isolation site and the species, this cannot be used to definitively identify a *Fibrobacter* species [1]. The absolute requirement for biotin exhibited by *F. succinogenes* strains was the only known distinguishing phenotypic characteristic between the two species [49, 91]. However, it was subsequently found that two strains of *F. intestinalis* (LH1 and JG1) also require biotin for growth (Table 1) [1].

The Phylogeny of the Genus *Fibrobacter*

Despite the fact that there are currently no distinct phenotypic traits to distinguish *F. succinogenes* and *F. intestinalis*, there is considerable genetic distance between the two formally recognised species [1]. Furthermore, it has been suggested that the phylogenetic difference between them based on 16S rRNA gene sequence comparison is sufficient to designate them as belonging to two distinct genera [91] (Fig. 1). This is compounded by the fact that the evolutionary distance between *F. succinogenes* and *F. intestinalis* (as determined by 16S rRNA gene analysis) is similar to that between the bacterial genera containing *Arthrobacter globiformis* and *Mycobacterium flavescens* and deeper than that between *Escherichia coli* and *Proteus vulgaris* [91]. The diversity of *Fibrobacter* isolates was further characterised using comparative 16S rRNA gene sequencing and DNA/DNA hybridisations of a larger number of isolates (Table 1) [1]. Comparisons of the 16S rRNA gene of *F. succinogenes* and *F. intestinalis* demonstrated approximately 91% to 93% similarity, and genomic DNA similarity between the two species as determined by DNA/DNA hybridisation was less than 20% [1]. It is currently suggested that 20% DNA/DNA homology and approximately 95% 16S rRNA similarity [72] are the minimum allowable with a genus. Advances in next-generation sequencing technologies now make the application of comparative genomics a tangible approach for the 'phylogenomic' analysis of the *Fibrobacteres* phylum [137].

The study by Amann and colleagues [1] demonstrated four distinct lines of descent within the *F. succinogenes* lineage, designated *F. succinogenes* subsp. *Succinogenes* (subgroup 1) [91] and subgroups 2, 3 and 4 [1]. Of these, group 1 is considered to be the most important in cellulose degradation [55, 106, 107] due to its high metabolic activity and widespread presence on plant material. Koike et al. [57] detected only subgroups 1 and 3 in rumen digesta and on hay stems incubated in the rumen, with subgroup 1 dominating the *Fibrobacter* population on the less degradable hay stems. A study using fluorescence in situ hybridization

Table 1 Sources and growth characteristics of *Fibrobacter* isolates (modified from Amann et al. [1])

Strain	ATCC number	Source	Morphology	Yellow pigment	Mol.% G+C	Vitamin requirements				Energy sources		Reference(s)
						Biotin	PABA	B12	Thiamine	Glucose	Lactose	
Fibrobacter succinogenes strains												
Group 1. subsp. *succinogenes* strains												
B1		Bovine rumen	Coccoid	−	ND	+	ND	−	−	+	−	[117]
BL2		Bovine rumen	Coccoid	−	ND	+	ND	−	−	+	−	[117]
A3c		Bovine rumen	Coccoid	−	49	+	−	−	−	+	−	[25, 26]
S85	19169^T	Bovine rumen	Coccoid	−	48	+	V	−	−	+	(slow) +	[11]
Group 2												
GC5		Bovine caecum	Rod shaped	−	ND	+	ND	−	−	+	−	[1]
REH9-1	53857^T	Bovine rumen	Rod shaped	−	51	+	+	−	−	+	−	[92]
Group 3												
HM2	43856^T	Ovine rumen	Rod shaped	+	ND	+	+	+	−	+	−	[1]
MN4		Ovine rumen	Rod shaped	+	ND	+	ND	+	−	+	−	[1]
MB4		Ovine rumen	Rod shaped	+	ND	+	ND	+	−	+	−	[1]
Group 4												
MC1		Ovine rumen	Rod shaped	−	ND	+	ND	−	−	+	−	[1]
Fibrobacter intestinalis strains												
NR9	43854^T	Rat caecum	Rod shaped	−	45	−	+	+	+	+	−	[92]
C1a		Porcine caecum	Rod shaped	−	ND	−	ND	+	+	+[a]	−	[125]
DR7	43855	Porcine caecum	Rod shaped	−	ND	−	+	+	−	+	−	[1]
LH1		Ovine rumen	Rod shaped	+	ND	+	ND	−	−	+	−	[1]
JG1		Ovine rumen	Rod shaped	+	ND	+	ND	−	−	+	−	[1]

ND not determined, *V* variable, *PABA* para-aminobenzoic acid

[a] Can also use maltose [125]

(FISH) to determine the attachment of bacteria to hay within the rumen detected only *F. succinogenes* subgroups 1 and 2, with subgroup 1 cells representing the largest proportion of the *Fibrobacter* population on the stems [106]. Suppressive subtractive hybridization has been used to compare the genes of *F. succinogenes* S85 and *F. intestinalis* DR7, suggesting that 33% of *F. intestinalis* DR7 genes were specific to this strain [100] and 41% of *F. succinogenes* S85 genes were either absent from, or exhibited low similarity to, those of *F. intestinalis* DR7 [101]. However, as discussed above, there is little phenotypic difference between the two species and as such they remain within the same genus (Fig. 1). It is envisaged that a phylogenetically coherent family will be established for what is currently the genus *Fibrobacter* and its close relatives when more taxa are detected and identified.

Cellulose Degradation

Cellulose is the main structural component of higher plant cell walls and represents approximately 35–50% of plant dry weight [76]. It is also present in bacteria, fungi and some animals such as marine tunicates [96]. The process of photosynthesis creates extensive amounts of plant biomass and therefore cellulose, which must be degraded by cellulolytic microorganisms that are present in the soil, marine and lake sediments, water and animal guts. As such, one of the largest material flows in the biosphere is controlled by cellulolytic microorganisms [75]. Cellulose hydrolysis can occur under both aerobic and anaerobic conditions, with anaerobic hydrolysis accounting for 5% to 10% of global cellulose degradation [52, 127], which is substantial in view of the absolute amount of cellulosic biomass present in the environment. The physiological capability to degrade cellulose is distributed widely across the universal phylogenetic tree of life [75]. Within the *Eubacteria*, cellulose-degrading bacteria are largely concentrated in the aerobic order *Actinomycetales* (phylum *Actinobacteria*) and the anaerobic order *Clostridiales* (phylum *Firmicutes*). There is significant diversity in the physiology of cellulolytic bacteria, and on this basis they can be placed into three diverse physiological groups: (1) fermentative anaerobes, typically Gram-positive, such as *Clostridium* and *Ruminococcus*, but with a few Gram-negative species (*Butyvibrio* and *Acetivibrio*) that are phylogenetically related

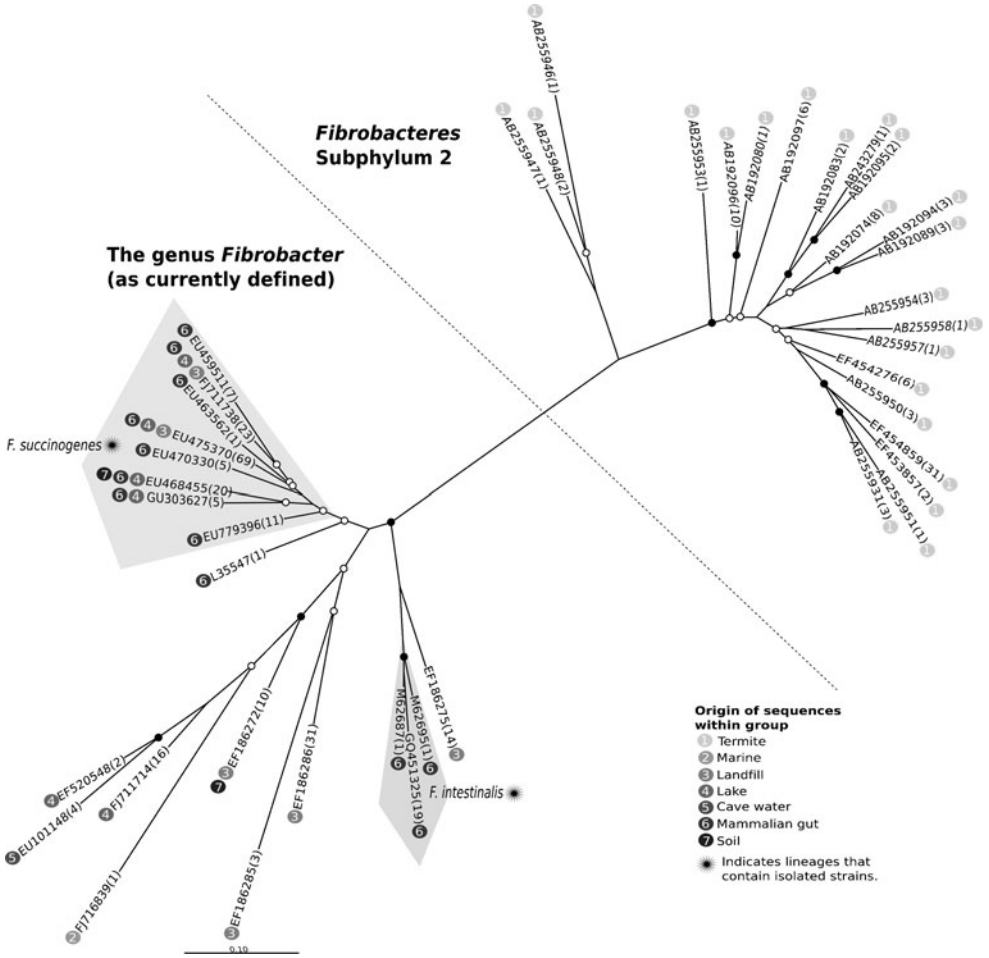

Figure 1 Phylogeny of the *Fibrobacteres* phylum. Maximum likelihood tree of 16S rRNA gene sequences belonging to the *Fibrobacteres* phylum. All sequences classified within the *Fibrobacteres* phylum and annotated as of 'good' quality were downloaded from the Ribosomal Database Project [19, 21] website in November 2010. Sequences were aligned using the MUSCLE aligner [30]. In order to compare the phylogeny of those sequences derived from environmental samples, termites and the herbivore gut, alignments were trimmed to include only sequences that contained positions corresponding to 153 to 1017 of the *E. coli* 16S rRNA gene. The remaining trimmed sequences were clustered into Operational Taxonomic Units (OTUs) at 95% similarity using CDHIT [46, 65]. A number of putative chimeric sequences were removed from the dataset after analysis with the Pintail chimera check program [3]. The representative sequences of each OTU (n=42) were aligned using the Greengenes NAST aligner [29] and imported into Arb where the alignment was visually checked. A maximum likelihood tree was produced from the final alignment using PhyML online [37] with the HKY85 substitution model and the Shimodaira–Hasegawa-like approximate likelihood ratio test (aLRT) branch support method. *Filled circles* indicate nodes at which an aLRT value of >95% was observed, and *unfilled circles* denote nodes with aLRT values between 75 and 95%. Nucleotide sequence accession numbers for the representative sequence of each OTU are displayed on each node. The number of sequences clustering within each OTU is displayed in *parentheses* and *numbered circles* indicate the environmental niches represented within each OTU. Clusters highlighted in *grey* represent sequences that are affiliated with the two known cultivated species within the genus, *F. succinogenes* and *F. intestinalis*. The *scale bar* indicates 0.1 base substitutions per nucleotide

to the *Clostridium* assemblage (fibrobacters are within this group despite being phylogenetically unrelated); (2) aerobic Gram-positive bacteria, e.g. *Cellulomonas* and *Thermobifida*; and (3) aerobic gliding bacteria, such as *Cytophaga* and *Sporocytophaga* [75].

The majority of characterised cellulolytic microorganisms use either the free cellulase mechanism [133] in which multiple secreted enzymes act synergistically or complexes of cellulolytic enzymes bound to the outer cell wall (cellulosomes) [5] to digest cellulose (Fig. 2). Brown rot fungi are exceptional in their ability to attack cellulose using coupled oxidative enzymes [80]. For both the free cellulase mechanism most commonly used by aerobic organisms and the cellulosomes associated with anaerobic organisms, the β-1,4 linkages within the cellulose are hydrolysed by cellulases. The model of aerobic cellulose hydrolysis via the cell-free enzyme mechanism is based on the cellulase system of the aerobic fungus *Trichoderma reesei* and the 'cellulosome' mechanism of anaerobic bacteria and fungi (order *Neocallimastigales*) is

based on the mechanisms of cellulolytic clostridia (reviewed by Lynd et al.) [74]. There are therefore substantial differences between the cellulose hydrolysis strategies employed by aerobic and anaerobic organisms [6]; the aerobic cell-free cellulase mechanism evolved in terrestrial microorganisms that colonise solid substrates and therefore secrete cellulases to enable penetration and utilisation of the substrate, whereas bacteria and fungi in aquatic environments would not benefit from a cell-free cellulase system and instead produce surface-bound cellulases to support their exclusive use of breakdown products as carbon and energy sources. However, evidence is emerging that in *F. succinogenes*, a separate and distinct mechanism is employed (Fig. 2) [118].

Fibrobacters are Major Degraders of Plant Biomass in the Herbivore Gut

Cellulose is the most abundant energy source on the planet, yet vertebrate herbivores do not possess the enzymes capable of degrading cellulose and other complex plant polysaccharides [89]. Consequently, herbivorous animals have evolved symbiotic relationships with bacteria, protozoa and fungi that possess the enzymes necessary for plant polymer degradation. Previous studies have indicated that the predominant species of cellulose-degrading bacteria detected via cultivation-based approaches in the herbivore gut are *F. succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* [38, 49], notwithstanding recent studies suggesting that

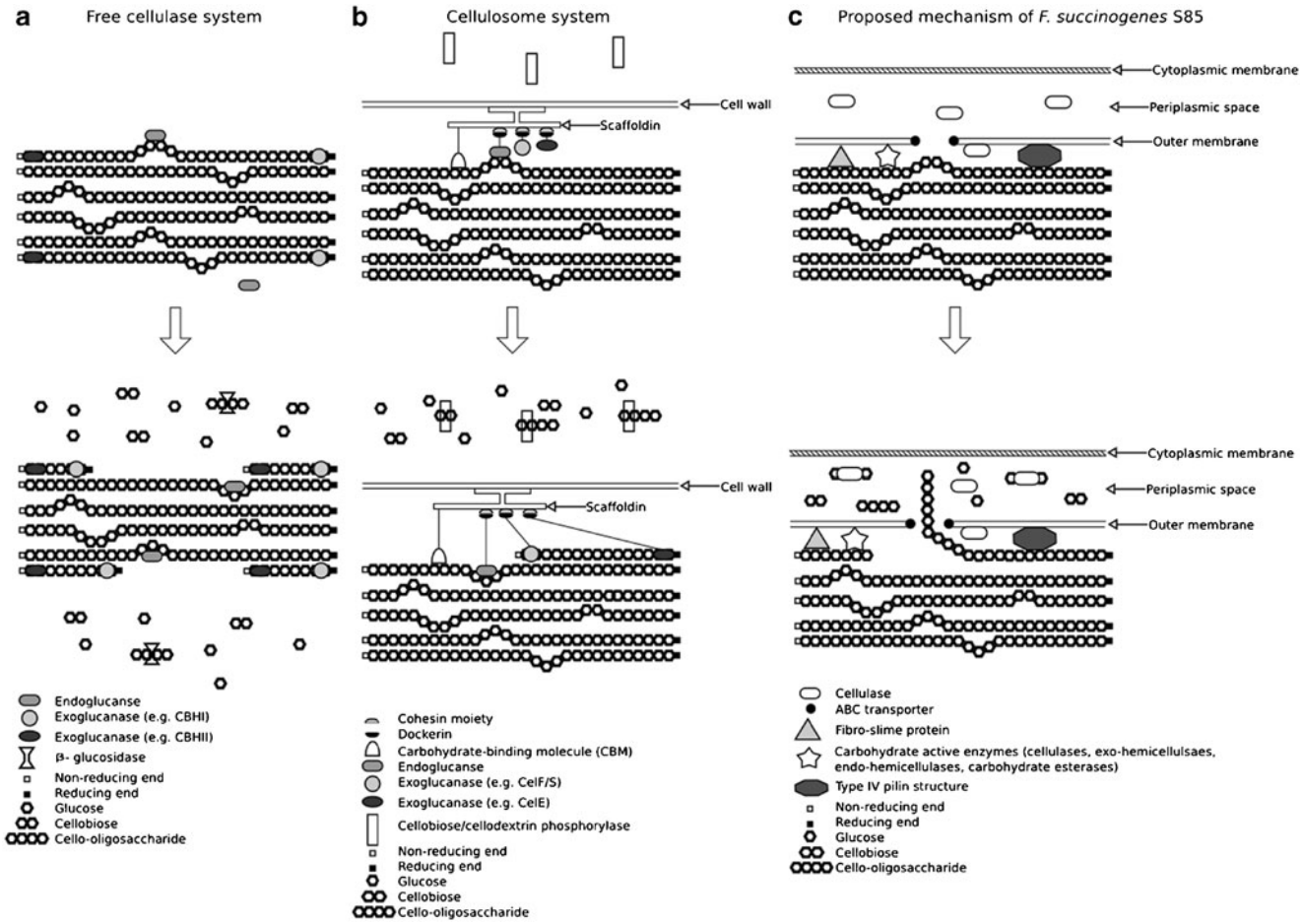

Figure 2 Microbial mechanisms of cellulose degradation. **a** Aerobic cell-free cellulase system (based on [75]); typical of aerobic microorganisms including *T. reesei*. Cellulose is hydrolysed via the synergistic interaction of individual enzymes that are secreted from the cell. **b** Anaerobic 'cellulosome' mechanism (based on [75]); typical of anaerobic bacteria (e.g. *Clostridium thermocellum*) and fungi. Cellulosomes consist of the catalytic enzymes capable of cellulose hydrolysis in addition to scaffoldin molecules, which anchor the enzymes to the cellulosome, and carbohydrate binding molecules (CBM) to maintain close contact with the substrate. The close proximity between the bacterial cell wall and cellulose substrate is a major benefit, resulting in concerted enzymatic activity arising from optimal synergy between cellulases. **c** Proposed cellulose degradation mechanism for *F. succinogenes* (based on [118, 134]). Attachment to the substrate is mediated by fibro-slime proteins and type IV pilin structures attached to the outer membrane. Cellulose fibres are disrupted by carbohydrate-active enzymes and individual cellulose chains are transported through the outer membrane via an ABC transporter. Current data suggests that the degradation of cellulose chains occurs in the periplasmic space

other as yet uncultivated bacteria may also have a role in cellulose hydrolysis within the rumen [56]. More recently, molecular biological techniques targeting the 16S rRNA gene of cellulolytic rumen bacteria have further supported the importance of *F. succinogenes*, *R. albus* and *R. flavefaciens* in cellulose hydrolysis [28, 93, 106, 120]. It is possible that the enzymatic system of *F. succinogenes* is more effective at degrading cellulose than the mechanisms used by the other cellulolytic organisms that occupy the same environment. For example, it was found that when strains S85 and A3C were grown in pure cultures, they were able to degrade a greater amount of cellulose from intact forage than the two other predominant rumen cellulolytic bacteria, *R. albus* and *R. flavefaciens* [27]. *F. succinogenes* is also capable of growth rate on ball-milled cellulose equivalent to that when cellobiose is used as substrate [31].

F. succinogenes has been described as one of the major cellulolytic bacterial species present in the rumen [33], and real-time polymerase chain reaction (PCR) has been widely utilised to quantify *Fibrobacter* spp. in the rumen [28, 58, 84, 97, 120]. *Fibrobacter* spp. have been detected in the intestinal tracts of a number of herbivorous species using both molecular and culture-based approaches including the bovine rumen and cecum [11, 25, 26, 47, 48, 117], ovine rumen [93, 115], porcine cecum [125], equine cecum [22, 24, 53, 63, 68], faeces of Grevy's zebra [63], rat cecum [77, 92], black rhinoceros faeces [63], ostrich cecum [81, 82], faeces of snub-nosed monkeys [136], yak rumen [2], wild ass faeces [63], goat rumen [67], rock hyrax faeces [63], capybara faeces [63] and antelope rumen [50]. The application of 16S rRNA gene-targeted oligonucleotide probes has provided an insight into *Fibrobacter* diversity and ecology in a number of gut ecosystems. Lin et al. [67] applied a suite of oligonucleotide probes for quantification of *Fibrobacter* spp. at genus, species and subspecies level. The application of these probes to RNA extracted from cattle and goat intestinal contents indicated a greater diversity of *Fibrobacter* as only ca. 50% of the total *Fibrobacter* genus abundance could be accounted for by the species-specific probes [67]. The relative abundances of the *Fibrobacter* genus in this study were 0.6–6% and 0.5–2% of the total 16S rRNA for cattle and goats, respectively. A similar study of equine-associated *Fibrobacter* populations also demonstrated the presence of a previously undescribed population of *F. succinogenes*-like species in caecal contents as the genus *Fibrobacter* represented 12% of the total 16S rRNA, yet none of the *F. succinogenes* subspecies-specific probes, or the *F. intestinalis* probe, hybridised with RNA derived from caecal contents [68]. Bacterial 16S rRNA gene PCR amplification, cloning and sequencing of DNA extracted from the caecal contents demonstrated the presence of novel *Fibrobacter* spp. affiliated with *F. succinogenes*, but representing novel lines of descent (Fig. 1—lineage represented by sequence accession number L35547) [68].

Cellulose Degradation by *Fibrobacter* spp.

Electron microscopy was used to show that *F. succinogenes* adheres to plant cell walls and on this material forms digestive pits [16]. *F. succinogenes* binds tightly to the surface of plant materials via adhesins, leading to extensive plant cell wall degradation [86–88], and when adhesion cannot occur, either in non-adherent mutants [34] or due to the presence of the phenolic aldehyde vanillin, [126], cellulose degradation does not occur. The outer membrane of *F. succinogenes* has been found to contain 13 cellulose binding proteins, and in a mutant strain where two of these were absent the strain was able to bind to amorphous cellulose, but not crystalline cellulose [54]. When seven of these cellulose-binding proteins were absent in another mutant strain, the strain was unable to bind to either of the two forms of cellulose and no growth was detected [54]. Proteins designated as fibro-slime domain-containing proteins present on the outer membrane of *F. succinogenes* S85 and type IV pili may also be involved in the adherence of *F. succinogenes* to crystalline cellulose [118] (Fig. 2).

It is suggested that *Fibrobacter* spp. utilise a novel mechanism of cellulose degradation because there are genes for endocellulases, which randomly hydrolyse the cellulose chain and disrupt the crystalline structure, but not for exocellulases or processive endocellulases, both of which release cellobiose from the ends of the cellulose chains and are crucial to the established free cellulase and cellulosomal mechanisms [133]. Furthermore, genome sequence data indicate that *Cytophaga hutchinsonii* may utilise a similar and novel mechanism [134] and, like *F. succinogenes*, also exhibits gliding motility on surfaces [48]. This is intriguing because *F. succinogenes* is an anaerobic rumen bacterium and *C. hutchinsonii* an aerobic soil bacterium, and both are phylogenetically distant. This 'third' mechanism of cellulose depolymerisation may involve a protein complex that is present in the outer membrane of the cell, cleaving individual cellulose chains from the bound cellulose fibres and transporting them into the periplasmic space through the outer membrane. Once in the periplasmic space, the cellulose chains would then be cleaved by endoglucanases, thus eradicating the need for exocellulases or processive endocellulases [134] (Fig. 2). This would explain the requirement for the *Fibrobacter* cells to be bound to the cellulose as the removal and binding of the individual cellulose chains would be a key step in the mechanism. This novel mechanism has both evolutionary and biotechnological significance and may be the explanation for the superior cellulolytic ability of *Fibrobacter* spp. compared to that of other rumen bacteria.

The recently sequenced genome of *F. succinogenes* strain S85 revealed that there are numerous proteins unique to *F. succinogenes*; 37% of proteins could not be attributed to a

known metabolic or physiological function using clusters of orthologous groups analysis [118]. Furthermore, up to 26% of the predicted proteins in the proteome of *F. succinogenes* did not have a known ortholog, suggesting a high content of genus- or species-specific proteins [118]. A total of 134 genes encoded enzymes that were identified by carbohydrate-active enzyme [13] analysis, representing carbohydrate esterases, carbohydrate binding modules (CBMs), polysaccharide lyases and glycosyl hydrolases derived from 49 different families. Of these, the majority were predicted to contain signal peptides, indicating that these enzymes are not targeted within the cytoplasm [118]. *F. succinogenes* strain S85 is predicted to have 31 cellulase genes, of which none contain the CBMs that are typically found in cellulosomes associated with adherence to crystalline cellulose. The absence of known dockerin domains in the cellulase genes and the absence of known scaffoldin genes within the genome suggest that *F. succinogenes* S85 does not utilise the cellulosomal degradation mechanism [118]. Whilst *F. succinogenes* S85 possesses endohemicellulases capable of hydrolysing a variety of substrates, it lacks the genes necessary to transport and metabolise any of these carbohydrates other than cellulose and its hydrolytic products [118]. *F. succinogenes* S85 is specialised for utilising only cellulose as growth assays utilising cellulose, pectin, starch, glucomannan, arabinogalactan and various forms of xylan found that, although all of the polysaccharides were hydrolysed, only cellulose was metabolised [118], including cellulose II, which is highly stable [130]. Forano and colleagues have studied the carbohydrate metabolism of *F. succinogenes* in detail (reviewed in [32]). NMR studies demonstrated the cycling of carbohydrates, notably glycogen, by *F. succinogenes*, in addition to several reversible metabolic pathways that enabled both the degradation and synthesis of carbohydrates. This ability to accumulate and rapidly degrade storage compounds such as glycogen may represent a strategy for rapid adaptation of *F. succinogenes* to changing environmental conditions. Surprisingly, *F. succinogenes* was found to synthesise maltodextrins and maltodextrin-1-phosphate, possibly in association with glycogen metabolism, and it is likely that the excretion of maltodextrins may support the cross-feeding of non-cellulolytic bacteria in co-culture in addition to other planktonic *F. succinogenes* cells [32].

A Cellulolytic Subphylum of the *Fibrobacteres* in the Termite Gut

It was originally thought that members of the genus *Fibrobacter* were restricted to the mammalian intestinal tract, but the occurrence and distribution of members of the *Fibrobacteres* phylum has recently been extended to include termite intestinal contents where cellulose is again the primary carbon source for the host organisms [41, 42]. However, data to support the role of symbiotic gut bacteria in the direct hydrolysis of cellulose and xylan in the termite gut were only recently reported [123].

Hongoh and colleagues [42] utilised terminal restriction fragment length polymorphism analysis in addition to general bacterial 16S rRNA gene clone libraries derived from colonies of the wood-feeding higher termite genus *Microcerotermes* and the lower termite genus *Reticulitermes* to create molecular community profiles of the bacterial gut microflora. Of 960 sequenced 16S rRNA gene clones derived from ten termite colonies (six *Microcerotermes* colonies and four *Reticulitermes* colonies), 12 phylotypes of clone sequences affiliated with the phylum *Fibrobacteres* were identified, and all of these sequences were from members of the higher termite genus *Microcerotermes*, representing approximately 10% of the total 16S rRNA clones from this group. These cloned *Fibrobacteres* sequences represented a novel sub-phylum cluster within the phylum, designated as *Fibrobacteres* subphylum 2 [42] (Fig. 1). Further work using a *Fibrobacteres* subphylum 2-specific probe in FISH experiments on samples of luminal fluid from the higher termite hindgut demonstrated that *Fibrobacteres* were the second most dominant group of the gut microflora, representing between 10.8% and 16.0% of the total bacterial cells and around 1.3×10^7 cells per gut [41]. Interestingly, FISH analysis demonstrated that the morphology of bacteria belonging to *Fibrobacteres* subphylum 2 differed from that of the known rumen strains of the genus *Fibrobacter* in that they represented undulate forms with a tapered end and a typical cell size of 0.2–0.3 × 1.3–4.9 μm [41].

Fibrobacteres subphylum 2-specific PCR primers were used to survey for these novel termite sequences in a variety of environments beyond the termite gut, including the gut of cockroaches, lake and deep sea sediments and rice paddy soil. However, *Fibrobacteres* subphylum 2 was not detected in any of these environments, suggesting that this novel subphylum of the *Fibrobacteres* represents an autochthonous lineage of termite gut symbionts [41]. Phylogenetic analysis of 16S rRNA gene sequences derived from *Fibrobacteres* subphylum 2 and members of the genus *Fibrobacter* sensu stricto (described as *Fibrobacteres* subphylum 1 by Hongoh et al. [41]) demonstrated 16S rRNA gene sequence similarities of 81.3% to 84.3% between subphyla 1 and 2 against 85.3% 16S rRNA gene similarity within subphylum 2 [41], again highlighting the profound genetic diversity that circumscribes this phylum. As the two currently described species of the *Fibrobacteres*, *F. succinogenes* and *F. intestinalis*, are known anaerobic degraders of lignocellulosic biomass in the herbivore gut, Hongoh and colleagues [41] suggested that the detection of novel lineages of *Fibrobacteres* in anoxic termite guts where cellulose again represents the

primary carbon source for growth implies a role for these organisms in cellulolysis.

This was later confirmed when a metagenomic and functional analysis of the microbiota of a wood-feeding higher termite demonstrated the presence of a broad diversity of bacterial genes responsible for cellulose degradation, and these were identified as belonging to the phyla *Spirochaetes* and *Fibrobacteres* [128]. *Fibrobacteres* were detected in 16S rRNA gene inventories from the higher termite hindgut and also represented 13% of the identifiable DNA fragments from a shotgun metagenome derived from the same sample. Many of these metagenomic sequences identified as belonging to *Fibrobacteres* encoded glycosyl hydrolases or carbohydrate-binding modules, and proteomic analysis confirmed that some of these genes were expressed in vivo or their cloned gene modules possessed cellulase activity in vitro, implicating them in lignocellulose degradation in this environment [128]. As molecular biological and 'omics' techniques continue to improve our ability to characterise such communities, it is likely that the role of fibrobacters in cellulose degradation in other anoxic environments will be definitively established.

Difficulties in the Isolation and Molecular Detection of *Fibrobacter* spp.

Although *F. succinogenes* was first characterised in 1947, fibrobacters are notoriously difficult to isolate and cultivate in the laboratory, and consequently their presence in other environments has probably been greatly underestimated [84]. Undoubtedly, low cell numbers obtained by the anaerobic culture of *Fibrobacter* strains from the rumen have similarly resulted in the underestimation of their contribution to the degradation of cellulose [49]. Latham et al. [60] isolated several hundreds of rumen bacteria strains, but only one of these was *F. succinogenes*, leading them to conclude that only a small amount of the cellulolytic activity that occurred in the rumen could be ascribed to this species. Furthermore, despite ecological and physiological evidence of the importance of fibrobacters as a major degrader of plant biomass in the herbivore gut [53], it has become apparent that the nucleic acid sequences of *Fibrobacter* spp. are poorly represented both in 16S rRNA gene clone libraries in a number of studies on ruminant microflora [23, 120–122, 132] and a ribosomal intergenic spacer clone library [59]. In a study by Larue and colleagues [59], community DNA prepared from colonised plant biomass in the herbivore gastrointestinal tract was subjected to both ribosomal intergenic spacer analysis and denaturing gradient gel electrophoresis (DGGE). Although *Fibrobacter* spp. were not detected in any of the clone libraries, genus-specific PCR-DGGE for *Fibrobacter* spp. confirmed their presence in all community DNA samples used to generate the libraries, with the cloned sequences showing between 91% and 98% identity to previously identified *F. succinogenes* sequences. Furthermore, the *F. succinogenes* sequences were found to have no mis-matches with the oligonucleotide primers used to produce the library, indicating an inherent bias against the PCR amplification of *Fibrobacter* 16S rRNA gene sequences [59]. *Fibrobacter* spp. are often poorly represented in metagenomic studies, with some studies on the bovine rumen unable to detect any *Fibrobacteres* sequences at all [10, 39], although they have been detected in a number of other mammalian metagenomes [63].

Tajima et al., [120] have offered the only hypothesis thus far to explain the poor representation of *Fibrobacter* sequences in general bacterial 16S rRNA gene libraries. They grew pure cultures of 12 common rumen bacteria (including *F. succinogenes*) and added equal quantities (30 ng) of pure culture DNA to separate quantitative PCR assays with general bacterial primers. They observed that *F. succinogenes* was the last organism to exceed the threshold fluorescence at cycle 15.85 compared to *Streptococcus bovis* DNA, which surpassed the threshold fluorescence at cycle 6.74, demonstrating a prolonged amplification lag phase when compared with the other organisms. This observation was not a consequence of rRNA operon copy number as *F. succinogenes* possesses at least three rRNA operons compared to one copy in *S. bovis*. As annealing and extension of the *F. succinogenes* template was not affected once the threshold cycle was surpassed, the problem appears to be with the initial DNA template and it was concluded that this is possibly an effect due to DNA-associated molecules [120]. Therefore, in view of the under-representation of fibrobacters in rumen clone libraries and the difficulties in isolating these obligately anaerobic organisms, it is possible that their apparent absence from many terrestrial and aquatic anoxic environments is erroneous, particularly in environments with high cellulosic biomass content.

Molecular Detection of *Fibrobacter* spp. in Non-gut Environments

Members of the genus *Fibrobacter* are established as major degraders of lignocellulosic biomass in the herbivore gut, and the failure to detect fibrobacters in terrestrial and aquatic environments beyond this highly specialised and restricted environment supported the notion that they were in fact obligate 'gut' anaerobes [91]. However, the microbial-mediated depolymerisation of lignocellulose is also a feature of many other anoxic habitats in the biosphere, such as waterlogged soils, wetlands, landfill sites and the anoxic water column and sediments of freshwater, estuarine and marine systems [61]. Cellulolytic clostridia are ubiquitous

within the biosphere and have been isolated from numerous environments in which cellulose is hydrolysed under anaerobic conditions, such as soils [90, 111], estuarine sediments [78, 94] freshwater sediments [62], the bovine rumen [40], methanogenic bioreactors [108, 112], waste digesters [8], anoxic rice paddy field soils [17, 129] and landfill sites [131]. This leads to the suggestion that clostridia are the predominant degraders of cellulose in the open environment. However, a number of sequences related to the *Fibrobacteres* phylum have been detected in general bacterial 16S rRNA gene clone libraries derived from potentially anoxic cellulose-rich environments including soils [95, 104], peat bogs [110], mangrove sediments [66] and the Atlantic and Pacific oceans [35]. Despite this, 16S rRNA gene sequences affiliated with the genus *Fibrobacter* (as currently defined) have until recently evaded detection, possibly due to the associated difficulties in both the isolation and molecular detection of fibrobacters. The recent detection of novel centres of variation belonging to the genus *Fibrobacter* in landfill sites [84] and freshwater lake sediments [83] using a genus-specific 16S rRNA gene primer set represented the first detection of fibrobacters beyond the gut. These data indicate that fibrobacters occupy a much wider ecological range than previously acknowledged and suggest a role in cellulose hydrolysis in anaerobic environments in general.

Landfill Sites

It has been suggested that anaerobic cellulose degradation in landfill sites is predominantly due to members of the genera *Clostridium* and *Eubacterium* [124]. This was first indicated by the work of Westlake et al. [131], who isolated a number of cellulolytic bacteria from landfill sites and identified them as members of these genera. Furthermore, the advent of molecular biological techniques, and specifically the use of 16S rRNA gene PCR primers, enabled further characterisation of the landfill microbiota. General bacterial 16S rRNA gene clone libraries from anaerobic landfill leachate bioreactor samples demonstrated that of those microorganisms attached to cellulosic material and in the mixed fraction, 100% and 90%, respectively, belonged to the *Firmicutes* and the majority of these clones fell into clusters III and XIVa of the clostridia [12]. Furthermore, 16S rRNA gene clone libraries derived from the leachate of a closed municipal solid waste landfill [44] and from the effluent leachate of a full-scale recirculating landfill [43] also did not identify any sequences belonging to the genus *Fibrobacter*. However, as stated above, even in the rumen where *Fibrobacter* are known to predominate, 16S rRNA gene clone library analysis using general bacterial primers appears to bias against the detection of fibrobacters.

Recently, novel lineages belonging to the genus *Fibrobacter* (as currently defined) were detected in landfill leachate samples, providing the first evidence that *Fibrobacter* spp. existed outside of the gut ecosystem [84]. This study utilised genus-specific 16S rRNA gene PCR primer sets targeting all known *Fibrobacter* spp. to detect novel sequences from the community DNA of leachate drawn from five landfill sites. Cloned PCR products were further analysed using temporal thermal gel electrophoresis and phylogenetic analysis of 58 clone sequences revealed that only two sequences could be identified as a named *Fibrobacter* species, and both were *F. succinogenes*. The remaining sequences represented novel centres of variation within the genus *Fibrobacter* as currently defined, occupying four distinct clusters within the genus, all of which exclusively comprised novel landfill *Fibrobacter* sequences (Fig. 1). Landfill *Fibrobacter* lineages were represented by sequence accession numbers EF186272, EF186275, EF186285 and EF186286. Of these four clusters, one contained sequences that were identified across all of the sampled sites, two contained site-specific sequences from one of two landfill sites and the fourth predominantly consisted of sequences identified from a low-level radioactive waste site in which cellulosic material was the only source of organic carbon (Fig. 1).

In this study, reverse-transcribed community RNA from landfill leachate samples was subjected to 16S rRNA gene-targeted quantitative PCR (qPCR) assays, demonstrating that the abundance of reverse-transcribed *Fibrobacter* 16S rRNA in landfill samples relative to total bacterial 16S rRNA could be as much as 40%. Significantly, the abundance of *Fibrobacter* in one landfill sample (40%) was higher than that of ovine rumen fluid samples analysed in the same way (21% to 32%). Data from this study suggested that fibrobacters are more readily detected when environmental RNA samples were used as they were detected in a greater proportion of samples when reverse-transcribed RNA was utilised in PCR reactions compared to extracted DNA [84]. As *Fibrobacter* spp. are considered to be predominant bacterial degraders of cellulose in the herbivore gut, it is likely that these novel lineages play a role in the degradation of cellulose that occurs in landfill environments [84]; cellulose is the main biodegradable component of landfill, representing up to 63.4% of the total organic content [9]. Recently, we have demonstrated the predominance of *Fibrobacter* in a cellulolytic biofilm that colonised and degraded cotton in a landfill leachate microcosm using qPCR, whereas *Fibrobacter* were not detected in the biofilm of an un-degraded cotton sample (unpublished data).

Although only partial *Fibrobacter* 16S rRNA gene sequences were obtained from landfill samples (ca. 855 bp), phylogenetic analyses suggested that these four landfill lineages represent novel centres of variation within the genus

Fibrobacter as currently defined [84]. Amann and colleagues [1] suggested that *Fibrobacter* may in fact represent a suprageneric taxon, and the subsequent detection of novel lineages of *Fibrobacteres* in the termite gut and in landfill sites certainly supports this assertion. It remains necessary however, and a significant gap to our knowledge, to determine the physiology and true phylogeny of this group of organisms via the application of 'omic' techniques in addition to the targeted isolation and cultivation of representatives of these new taxa.

Freshwater Lakes

Novel lineages of *Fibrobacter* have also been detected in freshwater lakes [83, 99]. *Fibrobacter* genus-specific PCR and qPCR primers targeting the 16S rRNA gene demonstrated the detection of novel members of the genus *Fibrobacter* in lake water, sediment and colonised cotton (cellulose) samples taken from different depths of two UK freshwater lakes [83]. This study identified two sets of sequences: those that were similar to *F. succinogenes* (Fig. 1; lake *Fibrobacter* clusters similar to *F. succinogenes* represented by accession numbers EU468455, GU303627, EU475370 and FJ711738) and a separate and novel cluster of *Fibrobacter* sequences that were similar to other sequences previously observed in clone libraries from freshwater environments (Fig. 1; novel lake *Fibrobacter* clusters represented by accession numbers EF520548 and FJ711714).

To determine if the detection of fibrobacters in freshwater lake sediments originated from the percolation of faecal contaminants from grazing ruminants, soil and ovine faecal samples from the adjacent fields were analysed in the same way and these did not contain any sequences related to the novel 'aquatic' *Fibrobacter* lineage, suggesting that there is no linkage between the *Fibrobacter* sequences in these environments (Fig. 1). Furthermore, all *Fibrobacter* sequences clustering within the aquatic group were detected on colonised cotton samples, many of which were obtained using reverse-transcribed RNA, and both qPCR and PCR demonstrated that fibrobacters were more readily detected in colonised cotton baits than in the surrounding water or sediment sample at equivalent depth, suggesting active colonisation of cellulosic substrates and metabolic activity [83]. In addition, *Fibrobacter* sequences were more readily detected in the anoxic regions of the water column and sediment, consistent with the obligate anaerobic physiology of all cultivated fibrobacters. Quantitative PCR analysis of reverse-transcribed bacterial community RNA suggested low metabolic activity of *Fibrobacter* spp. on the colonised cotton baits (0.005% to 0.02%) and on the sediment surface (ca. 1%), although the *Fibrobacter* sequences were enriched on the colonised cotton baits in comparison to the surrounding water column. The preference of these aquatic *Fibrobacter* spp. for colonised cotton baits and lake sediment provides further support for the suggestion that these organisms contribute to the degradation of plant and algal biomass in aquatic environments [83].

Fibrobacter Cellulases in Biotechnology

Microbial cellulases have been of industrial interest for over 60 years. Initially, a fungal attack on the clothing and tents of soldiers in Southeast Asia during World War II provided the impetus to understand the mechanism of cellulase action [102]. However, the industrial focus of cellulase enzymology has recently shifted to biofuel production in the light of the current energy crisis. Cellulose is the most abundant organic polymer both in the biosphere, as a major component of plant cell walls, and in human-generated wastes and therefore represents a valuable resource. The microbial conversion of cellulose (and similar polymers) from plant matter and municipal wastes to hydrolysis products such as ethanol and glucose is an attractive vision for nations seeking alternative fuel options [74]. In addition, cellulose conversion technologies offer disposal alternatives for municipal wastes otherwise deposited in landfill sites whilst reducing the environmental impact of greenhouse gases generated from municipal waste treatment and gasoline-fuelled transport [7]. Cellulases are increasingly being utilised in second-generation biofuel pilot plants for the optimal hydrolysis of lignocellulosic materials, maximising the yield of sugars that are available for fermentation to ethanol [119].

Cellulases have a variety of industrial applications including those in food, animal feed, paper, textile, waste management, fuel and chemical industries [79]. To date, there has been research into the application of *F. succinogenes* cellulolytic enzymes for use in detergent additives where cellulases are utilised to brighten and soften garments [15]. *F. succinogenes* has also been used to produce succinic acid [64], which is utilised in a variety of industries and chemical manufacturing processes [51]. The degradative capabilities of *Fibrobacter* spp. are also being utilised for waste decomposition in life support systems for long-term space missions such as the Micro-Ecological Life Support Alternative [18]. Cellulolytic enzymes of *Fibrobacter* spp. may also be cloned into non-cellulolytic bacteria in order to improve silage production and the pretreatment of animal feeds [116]. The display of *F. succinogenes* β-glucanase on the cell surface of *Lactobacillus reuteri* is the first example of successful cloning of *Fibrobacter* cellulolytic enzymes into a non-cellulolytic bacterium, which was shown to improve the capability of *L. reuteri* to adhere to and degrade β-glucan in barley [45].

F. succinogenes cellulolytic enzymes also have the potential to be used in the production of biogas [70] and have

significant potential for the refining of lignocellulosic biomass in the generation of bioethanol [73, 103]. For these processes, cellulose from plant matter and municipal waste would be utilised, thus also providing an alternative waste disposal mechanism and so reducing the environmental impact of waste treatment sites [7]. As the current work on the cellulolytic enzymes of *Fibrobacter* spp. is restricted to *F. succinogenes*, it is possible that the novel centres of variation detected in terrestrial and aquatic environments may contain cellulolytic enzymes with extended potential for applications in a variety of industrial processes, particularly in the area of second-generation biofuel production.

Final Comments

The *Fibrobacteres* is a diverse and functionally important phylum of bacteria, and yet there is a paucity of information on their ecology, phylogeny and physiology. This can be ascribed to the difficulties associated with the cultivation and molecular detection of members of this phylum. However, the recent application of more targeted molecular-based techniques and 'omics' approaches, including the use of environmental RNA rather than DNA as the starting material, has provided some important and novel observations on the *Fibrobacteres* phylum. *Fibrobacteres* are not restricted to the herbivore gut, with novel lineages detected in other anoxic environments where cellulose degradation occurs (termite gut, landfill sites and freshwater lakes). At least one species has evolved an atypical cellulose degradation mechanism, which may explain the superior hydrolytic capabilities of fibrobacters compared to other anaerobic bacterial groups. The detection of novel lineages of *Fibrobacteres* in termite guts, landfill sites and freshwater lakes has significant implications for their role in carbon flow in the biosphere, and their hydrolytic enzyme systems represent potential targets for novel catalysts with industrial application, such as the refining of lignocellulosic biomass for biofuel production. Isolation and cultivation of the *Fibrobacteres* we now know to be present and active in a number of different environments is an obvious priority.

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