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The ecology of *Fibrobacter* spp. in cellulose-
degrading microbial communities

Emma Ransom-Jones

September 2014



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A thesis submitted to Bangor University in candidature
for the degree
Philosophiae Doctor

School of Biological Sciences

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Abbreviations

| | |
|---------|--|
| % | Percent |
| µl | Microlitre |
| µm | Micrometer |
| °C | Degrees Celcius |
| A | Adenine |
| BLAST | Basic Local Alignment Search Tool |
| BLASTn | Nucleotide Basic Local Alignment Search Tool |
| bp | Base Pair |
| BR | Broad Range |
| BSA | Bovine Serum Albumin |
| C | Cytosine |
| CAZy | Carbohydrate Active Enzyme |
| CBM | Carbohydrate Binding Module |
| CD | Catalytic Domain |
| cDNA | Reverse-Transcribed Ribonucleic Acid |
| CMC | Carboxymethyl Cellulose |
| COG | Cluster of Orthologous Group |
| Ct | Threshold Cycle |
| dd | Double distilled |
| DGGE | Denaturing Gradient Gel Electrophoresis |
| DNA | Deoxyribonucleic Acid |
| dNTP | Dinucleotide Triphosphate |
| dsDNA | Double Stranded Deoxyribonucleic Acid |
| FISH | Fluorescent <i>in situ</i> Hybridisation |
| g | Gram |
| G | Guanine |
| GPP | Gross Primary Production |
| kb | Kilo Base Pair |
| l | Litre |
| MELiSSA | Micro-Ecological Life Support Alternative |
| ml | Millilitre |
| mM | Milimole |
| ND | Not Determined |

| | |
|---------|---|
| ng | Nanogram |
| OTU | Operational Taxonomic Unit |
| PCR | Polymerase Chain Reaction |
| qPCR | Quantitative Polymerase Chain Reaction |
| RFLP | Restriction Fragment Length Polymorphism |
| RISA | Ribosomal Intergenic Spacer Analysis |
| RNA | Ribonucleic Acid |
| rpm | Revolutions Per Minute |
| rRNA | Ribosomal Ribonucleic Acid |
| spp. | Species |
| subsp. | Sub-species |
| T | Thymine |
| TMTC | Too Many To Count |
| TRFLP | Terminal Restriction Fragment Length Polymorphism |
| TTGE | Temporal Thermal Gel Electrophoresis |
| V | Variable |
| vol/vol | Volume to Volume Ratio |
| wt/vol | Weight to Volume Ratio |

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Summary

The *Fibrobacteres* phylum contains only two characterised species, *Fibrobacter succinogenes* and *F. intestinalis*, both of which are important degraders of cellulose in the mammalian gut. Fibrobacters were thought to be restricted to the gut environment, but this was recently disproven via the detection of novel *Fibrobacter* spp. in landfill sites and freshwater lakes, with the implication that the distribution and diversity of members of the genus *Fibrobacter* is poorly understood. Here, the environmental distribution of members of the genus *Fibrobacter* was addressed; a total of 64 samples from contrasting environments were screened for the presence of *Fibrobacter* spp. via genus-specific 16S rRNA gene PCR primers. Fibrobacters were detected in 23 samples, with the first specific detection of fibrobacters in marine and estuarine sediments, and Arctic cryoconite. Phylogenetic analysis of 16S rRNA gene sequences revealed 63 *Fibrobacter* OTUs at 95% sequence similarity, representing a wealth of unclassified species contained within this genus. To address the lack of cultivated *Fibrobacter* isolates, their isolation from landfill leachate microcosms containing either Avicel or dewaxed cotton string as the sole carbon source for growth was attempted, resulting in the first isolation of *F. succinogenes* from a landfill site, and the first isolation of fibrobacters from outside the mammalian gut. This is the first phenotypic evidence that fibrobacters are active members of the landfill cellulolytic community. The importance of fibrobacters in landfill cellulose decomposition was further characterised via 454 pyrosequencing of 16S rRNA gene amplicons and shotgun metagenomic sequencing of heavily-degraded cotton samples from landfill leachate microcosm, where fibrobacters have previously been shown to predominate. *Fibrobacter* 16S rRNA gene amplicons were enriched on the heavily degraded cotton sample (14.2% of sequences, vs. 0.02% for a poorly degraded cotton sample), and metagenome sequencing of the heavily degraded cotton revealed that proteins most closely related to *F. succinogenes* strain S85 dominated the sequence output. Attempts to isolate *Fibrobacter* spp. from the same heavily degraded cotton sample revealed that *Fibrobacter* spp. were present in co-culture with *Clostridium sporogenes*, but attempts to purify these strains were unsuccessful. These data represent a significant contribution to our understanding of the ecology of members of the genus *Fibrobacter*, and their important role in the hydrolysis of cellulolytic biomass.

CHAPTER 1

Introduction

1.1 Cellulose

With the exception of cotton, which represents 100% crystalline cellulose, naturally occurring cellulose is typically associated with hemicellulose and lignin. The contribution of these compounds to the dry weight of lignocellulosic biomass varies between plant species, but typically lignin comprises 10 to 25%, hemicellulose 20 to 40% and cellulose 40 to 60% (Lin *et al.*, 2010). Cellulose is the major structural component of plants, although it is also present in bacteria (O'Sullivan, 1997, Watanabe and Tokuda, 2010), fungi (O'Sullivan, 1997; Watanabe and Tokuda, 2010), algae (O'Sullivan, 1997; Jarvis, 2003) and tunicates (O'Sullivan, 1997; Jarvis, 2003). Whilst there are six polymorphs of cellulose (I, II, III₁, III₂, IV₁ and IV₂) (O'Sullivan, 1997), only cellulose I and II, known collectively as native cellulose, are present in nature (Brown, 2004). Cellulose I is the most abundant of the two native polymorphs (Brown, 2004) and contains a mixture of two crystalline forms, I_α and I_β, with the proportions of each varying between organisms. Typically, I_α is more abundant in celluloses derived from bacteria and algae, whilst I_β is more prevalent in higher plant celluloses (Atalla and Vanderhart, 1984).

A single cellulose chain comprises of β-D-glucopyranose residues organised in alternating directions and joined together by β-1, 4 glycosidic bonds (Brown, 2004). These cellulose chains lie alongside each other to form sheets which are held together by O-HO hydrogen bonds (Jarvis, 2003). These sheets are then stacked on top of each other in a staggered pattern and fixed via C-OH hydrogen bonds (Fig. 1.1) (Jarvis, 2003). In cellulose I_α the glucose molecules alternate in conformation in each chain, with each of the chains and sheets identical in pattern. In contrast, cellulose I_β consists of chains and sheets consisting of a single conformation of cellulose, with sheets containing each conformation stacked alternately (Fig. 1.1) (Jarvis, 2003).

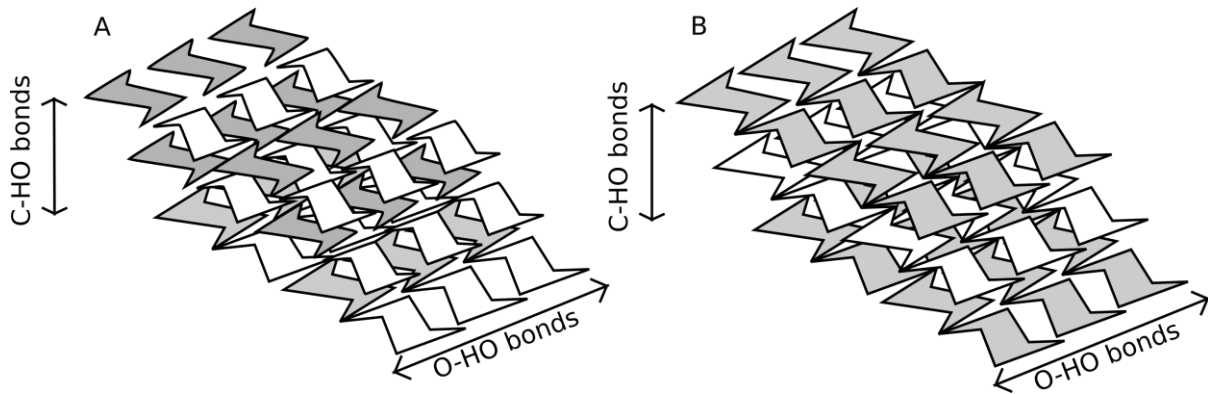


Figure 1.1 The structures of cellulose I.

(A) Cellulose I_{α} has identical chains and sheets, with the glucose units in the chains alternating between the two conformations (shaded grey and white). (B) Cellulose I_{β} , with each glucose molecule within the chain and sheet identical, and the sheets alternating in conformation. Modified from (Jarvis, 2003).

1.2 The global carbon cycle

Gross Primary Production (GPP) occurs when photosynthetic organisms fix CO_2 to form organic compounds, resulting in the largest global flow of carbon (Beer *et al.*, 2010) (Fig. 1.2). GPP also forms the basis for a number of other processes, and in conjunction with respiration, is critical in controlling the exchange of CO_2 between the atmosphere and terrestrial and aquatic environments (Reich, 2010). In order for the carbon cycle to be completed, cellulosic biomass must be degraded (Fig. 1.2). As it is estimated that the terrestrial GPP is between 115 and 131 petagrams of carbon per year, the amount of cellulosic biomass produced is substantial.

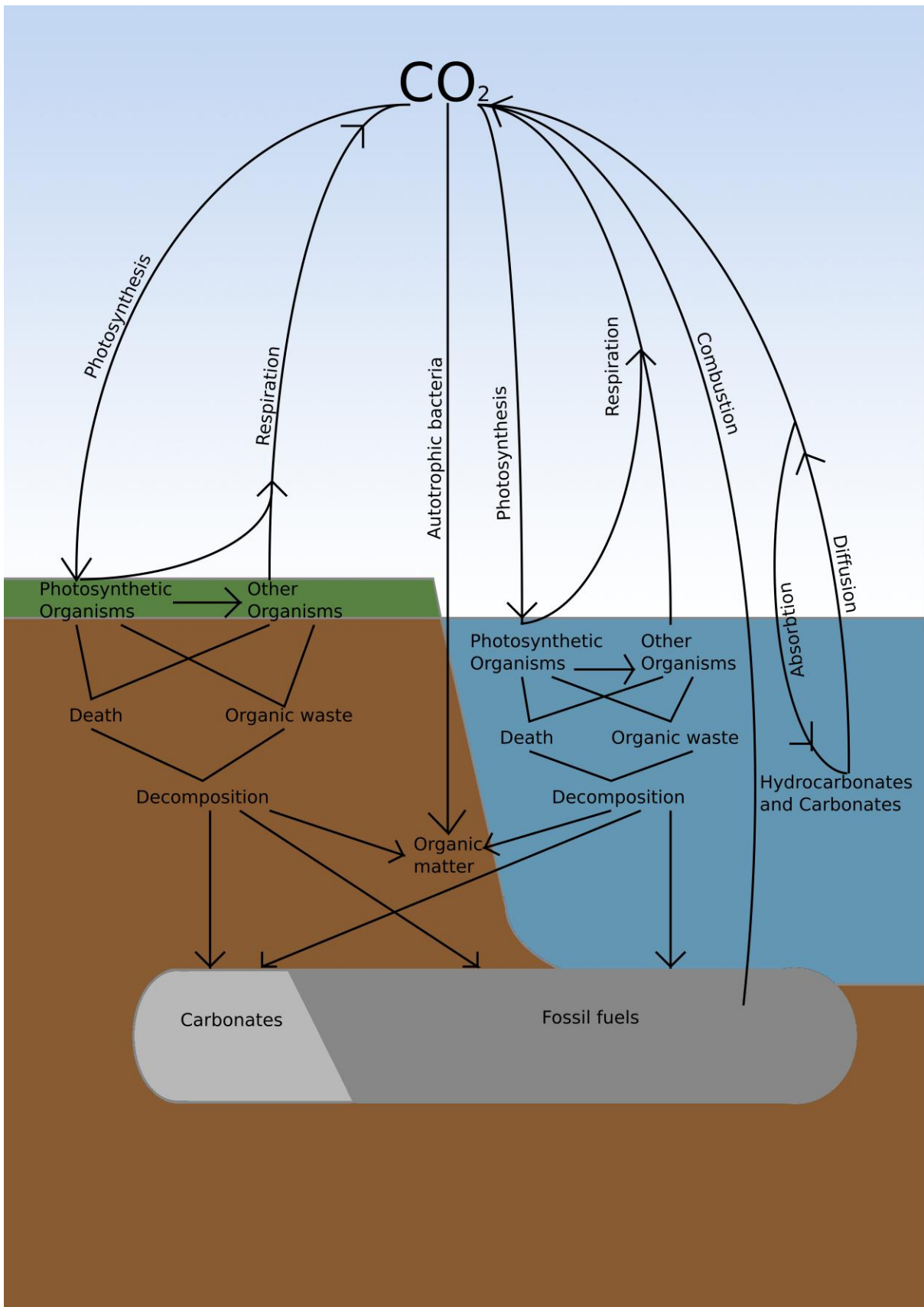


Figure 1.2. The global carbon cycle.

1.3 Degradation of cellulose

In the absence of enzymes, cellulose has a half-life of several million years (Wilson, 2011). Although some cellulose is oxidised via fire (Falkowski *et al.*, 2000), the majority is hydrolysed via enzymes. It was originally thought that animals were only capable of cellulose hydrolysis due to the presence of symbiotic microorganisms such as bacteria, fungi and protozoa (Watanabe and Tokuda, 2010; Tanimura *et al.*, 2013). Whilst the majority of higher animals, such as ruminants, do rely on this symbiotic relationship to degrade cellulose, certain members of Arthropoda (Tanimura *et al.*, 2013; Watanabe and Tokuda, 2001), Nematoda (Tanimura *et al.*, 2013; Watanabe and Tokuda, 2001), Mollusca (Tanimura *et al.*, 2013; Watanabe and Tokuda, 2001), Annelida (Tanimura *et al.*, 2013), Echinodermata (Tanimura *et al.*, 2013) and Chordata (Tanimura *et al.*, 2013) have endogenous cellulases. However, the majority of cellulose hydrolysis is mediated by cellulolytic microorganisms (Lynd *et al.*, 2002). Whilst approximately 90 to 95% of this hydrolysis occurs under aerobic conditions (Vogels, 1979; Jenkinson *et al.*, 1991; Perez *et al.*, 2002), due to the amount of cellulosic biomass produced each year the amount degraded under anaerobic conditions is nevertheless substantial.

1.3.1 Methods of microbial cellulose degradation

Cellulases are a diverse group of enzymes that hydrolyse the β -1, 4 glycosidic bond between two glucose molecules (Wilson, 2008; Wilson, 2011). There are three types of cellulases; endocellulases, also known as endoglucanases, exocellulases, also known as cellobiohydrolases, and processive endocellulases (Wilson, 2008; Wilson, 2011; Watanabe and Tokuda, 2010). The majority of cellulases are endocellulases (Wilson, 2011), which are capable of binding and cleaving molecules from any point of the cellulose chain (Spezio *et al.*, 1993). Exocellulases contain their active site within a 'tunnel-like structure' and bind to an end of the cellulose chain before cleaving molecules sequentially (Rouvinen *et al.*, 1990), with two different classes of exocellulases, those that work on the reducing end, and those that work on the non-reducing end (Barr *et al.*, 1996). To date, processive endocellulases appear to be unique to bacteria (Wilson, 2011). These act initially in an endocellulolytic manner in order to cleave the cellulose chain at a random point, before sequentially cleaving molecules from the non-reducing end (Wilson, 2011). Whilst the majority of microorganisms use either the free-cellulase or cellulosome based mechanisms of cellulose hydrolysis, there are at least five different methods by which microbially mediated cellulose degradation can occur (Wilson, 2011).

1.3.1.1 The free-cellulase mechanism

The majority of aerobic microorganisms utilise the free-cellulase mechanism to degrade cellulose (Wilson, 2008), the model for which is based on the cellulase system of the aerobic fungus *Trichoderma reesei* (Lynd *et al.*, 2005). This method of cellulose hydrolysis consists of the organism secreting several different cellulases (Fig. 1.3). The majority of these cellulases contain a carbohydrate binding module (CBM) attached to the catalytic domain (CD) via a linker peptide, in order to facilitate binding of the enzyme to the substrate (Wilson, 2008; Wilson, 2011). These cellulases often act synergistically, and this can increase the specific activity of these enzymes up to fifteen fold (Irwin *et al.*, 1993).

1.3.1.2 The cellulosomal mechanism

The 'cellulosome' mechanism of anaerobic bacteria and fungi (order *Neocallimastigales*) is based on the mechanisms identified within cellulolytic clostridia (Lynd *et al.*, 2005). A cellulosome is a complex of multiple cellulases bound to a scaffoldin unit (Bayer *et al.*, 2004) (Fig. 1.3). The scaffoldin unit contains the CBM in order to facilitate binding of the cellulosome to the substrate, as the majority of the cellulases involved in this mechanism do not contain individual CBMs (Bayer *et al.*, 2004; Wilson, 2008; Wilson, 2011). The scaffoldin also contains cohesion modules which bind to the dockerin domains of the enzymes to enable them to anchor to the scaffoldin (Bayer *et al.*, 2004).

1.3.1.3 Cellulose degradation by brown rot fungi

Brown rot fungi such as *Gloeophyllum trabeum* utilise free radicals and cellulases in order to hydrolyse cellulose. The free radicals cleave the cellulose chain, thus enabling degradation via the cellulases (Xu and Goodell, 2001) (Fig. 1.3). As the cellulases utilised by brown rot fungi lack CBMs and processive cellulases needed to hydrolyse untreated crystalline cellulose, the initial cleavage contained within this mechanism is critical (Wilson, 2011). This method consists of the hyphae of the fungus secreting oxalate and hydrogen peroxide into the plant cell lumen, where they can then diffuse into the cell wall. The oxalate lowers the pH generating a gradient that enables the fungus to transfer and sequester the insoluble, oxidised iron, and in turn to produce hydroxyl radicals within the plant cell wall. The oxalate forms a soluble complex with the Fe^{III}, which is then removed from this complex via the chelator and reduced to Fe^{II} via Fenton chemistry (Hammel *et al.*, 2002). The reduction of the Fe^{III} also results in the formation of the hydroxyl radical ($\bullet\text{OH}$), which can oxidise the cellulose chains and allow the cellulases to act (Xu and Goodell, 2001).

1.3.1.4 Cellulose degradation by *Saccharophagus degradans*

Saccharophagus degradans is an aerobic bacterium that appears to utilise an atypical mechanism of cellulose hydrolysis. The *S. degradans* genome contains none of the scaffoldin or dockerin homologues associated with the cellulosomal method (Weiner *et al.*, 2008). However, whilst *S. degradans* is capable of utilising cellulose as the sole carbon source (Taylor *et al.*, 2006), although there are a number of endoglucanases contained within the genome there is only one cellobiohydrolase (Weiner *et al.*, 2008), which is atypical of organisms that utilise the free cellulase mechanism. Furthermore, it has been proven that the sole cellobiohydrolase is actually an endoglucanase (Watson *et al.*, 2009), thus raising the question as to how *S. degradans* is capable of degrading cellulose. It has been suggested that this is due to the novel processive endoglucanases secreted by this organism, and that these are capable of acting both as endoglucanases and cellobiohydrolases (Watson *et al.*, 2009) (Fig. 1.3).

1.3.1.5 Cellulose degradation by *Fibrobacter* spp.

Members of the genus *Fibrobacter* are obligately anaerobic, cellulolytic bacteria that were first isolated from the bovine rumen (Hungate, 1947; Hungate, 1950). Electron microscopy was used to show that *F. succinogenes* adheres to plant cell walls, and on this material forms digestive pits (Cheng *et al.*, 1984). *Fibrobacter succinogenes* binds tightly to the surface of plant materials via adhesins, leading to extensive plant cell wall degradation (Miron *et al.*, 1989; Miron and Benghedalia, 1993c; Miron and Benghedalia, 1993b), and when adhesion cannot occur, either in non-adherent mutants (Gong and Forsberg, 1989) or due to the presence of the phenolic aldehyde vanillin (Varel and Jung, 1986), cellulose degradation does not occur. The outer membrane of *F. succinogenes* has been found to contain thirteen 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 (Jun *et al.*, 2007). 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 (Jun *et al.*, 2007). 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 (Suen *et al.*, 2011) (Fig. 1.3).

It has been suggested that *Fibrobacter* spp. utilise a novel mechanism of cellulose-degradation. This is based on the identification of genes encoding endocellulases, which randomly hydrolyse the cellulose chain and disrupt the crystalline structure, and the absence of 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 (Wilson, 2008). Furthermore, genome sequence data indicate that *Cytophaga hutchinsonii* may utilise a similar and novel mechanism (Wilson, 2009), and like *F. succinogenes*, also exhibits gliding motility on surfaces (Hungate, 1950). This is intriguing because *F. succinogenes* is an anaerobic rumen bacterium and *C. hutchinsonii* an aerobic soil bacterium, and they are phylogenetically distant from one another. 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 (Wilson, 2009) (Fig. 1.3). 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 (Suen *et al.*, 2011).

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 (COGs) analysis (Suen *et al.*, 2011). 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 (Suen *et al.*, 2011). A total of 134 genes encoded enzymes that were identified by carbohydrate-active enzyme (CAZy (Cantarel *et al.*, 2009)) 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 (Suen *et al.*, 2011). *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, therefore suggest that *F. succinogenes* S85 does not utilise the cellulosomal degradation mechanism (Suen *et al.*, 2011). Whilst *F. succinogenes* S85 possesses endo-hemicellulases capable of hydrolysing a variety of substrates, it apparently lacks the genes necessary to transport and metabolise any of these carbohydrates other than cellulose and its hydrolytic products (Suen *et al.*, 2011). *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 (Suen *et al.*, 2011), including cellulose II, which is highly stable (Weimer *et al.*, 1991). Forano and colleagues have studied the carbohydrate metabolism of *F. succinogenes* in detail, reviewed in Forano *et al.* (2008). NMR studies demonstrated the cycling of carbohydrates, notably glycogen, by *F. succinogenes*; these are 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 (Forano *et al.*, 2008).

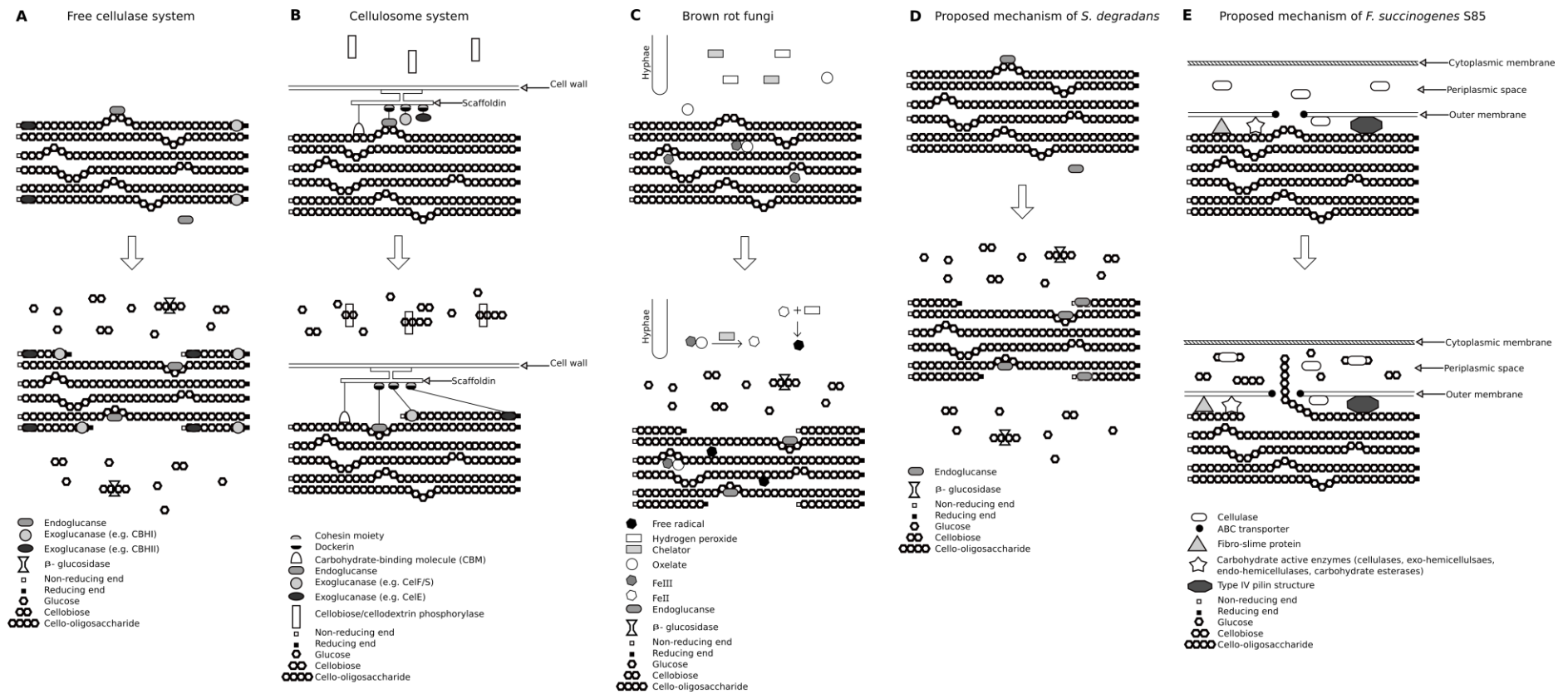


Figure 1.3. Microbial mechanisms of cellulose-degradation. Modified from Ransom-Jones *et al.* (2012).

(A) Aerobic cell-free cellulase system (based on Lynd *et al.* (2002)); typical of aerobic microorganisms including *Trichoderma reesei*. Cellulose is hydrolysed via the synergistic interaction of individual enzymes that are secreted from the cell.

(B) Anaerobic ‘cellulosome’ mechanism (based on Lynd *et al.* (2002)); 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 modules (CBMs) 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) Mechanism utilised by brown rot fungi (based on Xu and Goodell (2001)). Cellulose hydrolysis occurs via the interaction of free radicals and endoglucanases. (D) Proposed mechanism of *S. degradans* (based on Watson *et al.* (2009)) whereby cellulose hydrolysis is mediated solely via the activity of secreted endoglucanases. (E) Proposed cellulose degradation mechanism for *F. succinogenes* (based on Wilson (2009) and Suen *et al.* (2011)). 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.

1.4 Cellulases in biotechnology

Cellulases have a variety of industrial applications including those in food, animal feed, paper, textile, waste management, fuel and chemical industries (Mandels, 1985; Bhat, 2000). The first interest in microbial cellulases arose during World War II, when a fungus, *T. reesei*, attacked the tents and clothing of soldiers in Southeast Asia (Reese *et al.*, 1950). Whilst cellulases have a broad range of applications, growing concern over the global energy crisis has highlighted the potential for cellulases to be utilised for the production of second-generation biofuels. 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 (Lynd *et al.*, 2005), with the added benefit of providing an alternative waste disposal to landfill sites, and reducing greenhouse gas emissions (Bayer *et al.*, 2007). Cellulases are also 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 (Sun and Cheng, 2002).

1.4.1 *Fibrobacter* cellulases in biotechnology

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 (Chen and Wang, 2008). *F. succinogenes* has also been used to produce succinic acid (Li *et al.*, 2010), which is utilised in a variety of industries and chemical manufacturing processes (Isar *et al.*, 2006). 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 (MELiSSA) (Christophe *et al.*, 2009). 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 (Stewart and Flint, 1989). The display of *F. succinogenes* β -glucanase on the cell surface of *Lactobacillus reuteri* is the first example of the 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 (Huang *et al.*, 2011).

F. succinogenes cellulolytic enzymes also have the potential to be used in the production of biogas (Lissens *et al.*, 2004), and have significant potential for the refining of lignocellulosic biomass in the generation of bioethanol (Lynd *et al.*, 1991; Rubin, 2008). For these processes, cellulose from plant matter and municipal waste could be utilised, thus also providing an alternative waste disposal mechanism and so reducing the environmental impact of waste treatment sites (Bayer *et al.*, 2007). 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.

1.5 The genus *Fibrobacter*

Originally designated as *Bacteroides succinogenes*, *F. succinogenes* was first isolated from the bovine rumen in 1947 (Hungate, 1947; Hungate, 1950) and members of the genus are one of the predominant bacterial degraders of cellulosic material in the herbivore gut (Hungate, 1966; Stewart and Bryant, 1988; Kobayashi *et al.*, 2008). *Fibrobacter* is currently the sole formal genus of the bacterial phylum *Fibrobacteres*, which is phylogenetically related to the well-characterised *Bacteroidetes* and *Chlorobi* phyla (Cole *et al.*, 2003; Ludwig and Schleifer, 2001). *F. succinogenes* was initially classified as *B. succinogenes* due 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” (Cato and Salmon, 1976). 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 (Shah and Collins, 1983).

Subsequently, 16S rRNA oligonucleotide cataloguing methods were used to demonstrate that *B. succinogenes* and *B. anylophilus* were in fact not closely related to the other *Bacteroides* species (Paster *et al.*, 1985). In addition, the advent of 16S rRNA gene sequencing revealed that there were no organisms closely related to *B. succinogenes* and that its isolates formed a phylogenetically coherent group (Montgomery *et al.*, 1988). The genus *Fibrobacter* was circumscribed on this basis and contains only two recognised species, *F. succinogenes* and *F. intestinalis*, both Gram-negative, obligate anaerobes that are the predominant bacterial colonisers and degraders of lignocellulosic plant material in the herbivore gut (Montgomery *et al.*, 1988). *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 (Woese *et al.*, 1985). 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 (Suen *et al.*, 2011).

1.5.1 Phenotypic characteristics of *Fibrobacter* isolates

Members of the genus *Fibrobacter* are defined as obligately anaerobic, non-sporeforming, Gram-negative, rods or pleiomorphic ovoid cells (Montgomery *et al.*, 1988), 0.3 to 0.5 µm in diameter and 0.8 to 2.0 µm in length (Hungate, 1950; Stewart and Flint, 1989). The cells are able to migrate through agar medium by a mechanism comparable to that of *Cytophaga* spp. (Hungate, 1950). *Fibrobacter* spp. ferment xylan (Groleau and Forsberg, 1983; Sipat *et al.*, 1987; Miron and Benghedalia, 1993a), glucose, cellobiose and cellulose, producing succinic and acetic acids, and sometimes a small amount of formic acid (Montgomery *et al.*, 1988), although only cellulose and the hydrolytic products of cellulose can be metabolised (Suen *et al.*, 2011). Ammonium (Montgomery *et al.*, 1988), in addition to peptides and amino acids (Atasoglu *et al.*, 2001; Ling and Armstead, 1995), 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, B₁₂ (cyanocobalamine) or thiamine) are also required for growth (Montgomery *et al.*, 1988).

There are currently no definitive phenotypic characteristics that can be used to separate *F. succinogenes* and *F. intestinalis* taxonomically. Previously, it was considered that *F. succinogenes* is a rumen bacterium while *F. intestinalis* inhabits the caecum (Montgomery *et al.*, 1988). This was later discredited when the use of rRNA gene-targeted oligonucleotide probes demonstrated that *F. intestinalis* is present in the rumen (Stahl *et al.*, 1988), and *F. intestinalis* strains LH1 and JG1 were subsequently isolated from the ovine rumen (Table 1.1). Furthermore, *F. succinogenes* was thought likely to be present in the intestine due to the carriage from rumen digesta (Montgomery *et al.*, 1988), and this was confirmed by the isolation of strain GC5 from the bovine caecum (Table 1.1). 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 (Amann *et al.*, 1992). The absolute requirement for biotin exhibited by *F. succinogenes* strains was the only known distinguishing phenotypic characteristic between the two species (Montgomery *et al.*, 1988; Hungate, 1966). However, it was subsequently found that two strains of *F. intestinalis* (LH1 and JG1) also require biotin for growth (Table 1.1) (Amann *et al.*, 1992).

Table 1.1 Sources and growth characteristics of *Fibrobacter* isolates. From Ransom-Jones *et al.* (2012), modified from Amann *et al.* (1992). a. can also use maltose (Varel *et al.*, 1984). ND = Not Determined. V = Variable. PABA = Para-aminobenzoic acid.

| Strain | ATCC No. | Source | Morphology | Yellow Pigment | mol% G+C | Vitamin Requirements | | | | Energy Sources | | Reference(s) |
|--|--------------------|----------------|------------|----------------|----------|----------------------|------|-----|---------------|----------------|----------|----------------------------------|
| | | | | | | Biotin | PABA | B12 | Thia- mine | Glucose | Lactose | |
| <i>Fibrobacter succinogenes</i> strains: | | | | | | | | | | | | |
| Group 1. subsp. <i>succinogenes</i> strains | | | | | | | | | | | | |
| B1 | | Bovine Rumen | Cocoid | - | ND | + | ND | - | - | + | - | (Stewart <i>et al.</i> , 1981) |
| BL2 | | Bovine Rumen | Cocoid | - | ND | + | ND | - | - | + | - | (Stewart <i>et al.</i> , 1981) |
| A3c | | Bovine Rumen | Cocoid | - | 49 | + | - | - | - | + | - | (Dehority, 1963; Dehority, 1969) |
| S85 | 19169 ^T | Bovine Rumen | Cocoid | - | 48 | + | V | - | - | + | (slow) + | (Bryant <i>et al.</i> , 1959) |
| Group 2. | | | | | | | | | | | | |
| GC5 | | Bovine Caecum | Rod shaped | - | ND | + | ND | - | - | + | - | (Amann <i>et al.</i> , 1992) |
| REH9-1 | 53857 ^T | Bovine Rumen | Rod shaped | - | 51 | + | + | - | - | + | - | (Montgomery and Macy, 1982) |
| Group 3. | | | | | | | | | | | | |
| HM2 | 43856 ^T | Ovine Rumen | Rod shaped | + | ND | + | + | + | - | + | - | (Amann <i>et al.</i> , 1992) |
| MN4 | | Ovine Rumen | Rod shaped | + | ND | + | ND | + | - | + | - | (Amann <i>et al.</i> , 1992) |
| MB4 | | Ovine Rumen | Rod shaped | + | ND | + | ND | + | - | + | - | (Amann <i>et al.</i> , 1992) |
| Group 4. | | | | | | | | | | | | |
| MC1 | | Ovine Rumen | Rod shaped | - | ND | + | ND | - | - | + | - | (Amann <i>et al.</i> , 1992) |
| <i>Fibrobacter intestinalis</i> strains: | | | | | | | | | | | | |
| NR9 | 43854 ^T | Rat caecum | Rod shaped | - | 45 | - | + | + | + | + | - | (Montgomery and Macy, 1982) |
| C1a | | Porcine caecum | Rod shaped | - | ND | - | ND | + | + | + ^a | - | (Varel <i>et al.</i> , 1984) |
| DR7 | 43855 | Porcine caecum | Rod shaped | - | ND | - | + | + | - | + | - | (Amann <i>et al.</i> , 1992) |
| LH1 | | Ovine rumen | Rod shaped | + | ND | + | ND | - | - | + | - | (Amann <i>et al.</i> , 1992) |
| JG1 | | Ovine rumen | Rod shaped | + | ND | + | ND | - | - | + | - | (Amann <i>et al.</i> , 1992) |

1.5.2 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 (Amann *et al.*, 1992). 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 (Montgomery *et al.*, 1988) (Fig. 1.4). 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* (Montgomery *et al.*, 1988). The diversity of *Fibrobacter* isolates was further characterised by the use of DNA:DNA hybridisation and 16S rRNA gene sequencing of a greater number of isolates (Table 1.1) (Amann *et al.*, 1992). Comparisons of the 16S rRNA gene of *F. succinogenes* and *F. intestinalis* demonstrated approximately 91 to 93% similarity (Amann *et al.*, 1992; Jewell *et al.*, 2013), and genomic DNA similarity between the two species as determined by DNA:DNA hybridisation was less than 20% (Amann *et al.*, 1992). It is currently suggested that 20% DNA-DNA homology and approximately 95% 16S rRNA similarity (Ludwig *et al.*, 1998) 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 (Yilmaz *et al.*, 2010).

The study by Amann and colleagues (1992) demonstrated four distinct lines of descent within the *F. succinogenes* lineage, designated *F. succinogenes* subsp. *Succinogenes* (subgroup 1) (Montgomery *et al.*, 1988) and subgroups 2, 3 and 4 (Amann *et al.*, 1992). Of these, group 1 is considered to be the most important in cellulose degradation (Kobayashi *et al.*, 2008; Shinkai and Kobayashi, 2007; Shinkai *et al.*, 2009) due to its high metabolic activity and widespread presence on plant material. Koike *et al.* (2004) 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 (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 (Shinkai and Kobayashi, 2007). 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 (Qi *et al.*, 2005) and 41% of *F. succinogenes* S85 genes were either absent from, or exhibited low

similarity to, those of *F. intestinalis* DR7 (Qi *et al.*, 2008). However, as discussed above there is no known phenotypic difference between the two species and as such they remain within the same genus (Fig. 1.4). 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.

1.5.3 Fibrobacters are major degraders of plant biomass in the herbivore gut

Whilst cellulose degradation is fundamental to the global carbon cycle, only a small proportion of organisms are capable of complete cellulose hydrolysis. As a result many animals, including ruminants, rely on consortia of bacteria, protozoa and fungi in order to digest plant material (Moir, 1965). 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* (Halliwell and Bryant, 1963; Hungate, 1966), notwithstanding recent studies suggesting that other as yet uncultivated bacteria may also have a role in cellulose hydrolysis within the rumen (Koike and Kobayashi, 2009). 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 (Tajima *et al.*, 2001; Denman and McSweeney, 2006; Mosoni *et al.*, 2007; Shinkai and Kobayashi, 2007). 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 *F. succinogenes* 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* (Dehority, 1993). *F. succinogenes* is also capable of a growth rate on ball milled cellulose equivalent to that when cellobiose is used as substrate (Fields *et al.*, 2000).

F. succinogenes has been described as one of the major cellulolytic bacterial species present in the rumen (Forsberg *et al.*, 1997), and real-time PCR has been widely utilised to quantify *Fibrobacter* spp. in the rumen (Tajima *et al.*, 2001; Ozutsumi *et al.*, 2006; Koike *et al.*, 2007; Denman and McSweeney, 2006; McDonald *et al.*, 2008). *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 caecum (Hungate, 1947; Hungate, 1950; Bryant *et al.*, 1959; Dehority, 1963; Dehority, 1969; Stewart *et al.*, 1981), ovine rumen (Mosoni *et al.*, 2007; Stewart and Duncan, 1985), porcine caecum (Varel *et al.*, 1984), equine

caecum (Davies, 1964; Lin and Stahl, 1995; Julliard *et al.*, 1999; Daly and Shirazi-Beechey; 2003; Ley *et al.*, 2008), faeces of Grevy's zebra (Ley *et al.*, 2008), rat caecum (Macy *et al.*, 1982; Montgomery and Macy, 1982), black rhinoceros faeces (Ley *et al.*, 2008), ostrich caecum (Matsui *et al.*, 2010a; Matsui *et al.*, 2010b), faeces of snub-nosed monkeys (Wu *et al.*, 2010), yak rumen (An *et al.*, 2005), wild ass faeces (Ley *et al.*, 2008), goat rumen (Lin *et al.*, 1994), rock hyrax faeces (Ley *et al.*, 2008), capybara faeces (Ley *et al.*, 2008) and antelope rumen (Hungate *et al.*, 1959). The application of 16S rRNA gene-targeted oligonucleotide probes has provided an insight into *Fibrobacter* diversity and ecology in a number of gut ecosystems, with the use of these probes on RNA extracted from the intestinal contents of cattle and goats demonstrating a greater diversity of *Fibrobacter* spp. than previously thought (Lin *et al.*, 1994). Whilst the relative abundance of members of the genus *Fibrobacter* were found to be between 0.6 and 6% of the total 16S rRNA for cattle and 0.5 and 2% for goats, only half of this abundance was accounted for by the species-specific probes (Lin *et al.*, 1994). A similar study conducted on equine caecal samples also suggested the presence of novel *Fibrobacter* spp. similar to *F. succinogenes*, as whilst the genus specific probe demonstrated that fibrobacters comprised 12% of the 16S rRNA, neither the *F. intestinalis* probe, nor any of the *F. succinogenes* subspecies-specific probes hybridised with the RNA (Lin and Stahl, 1995). These novel *Fibrobacter* spp. affiliated with *F. succinogenes*, but representing novel lines of descent (Fig. 1.4 - lineage represented by sequence accession number L35547) were confirmed via PCR amplification of the 16S rRNA gene, cloning and sequencing (Lin and Stahl, 1995).

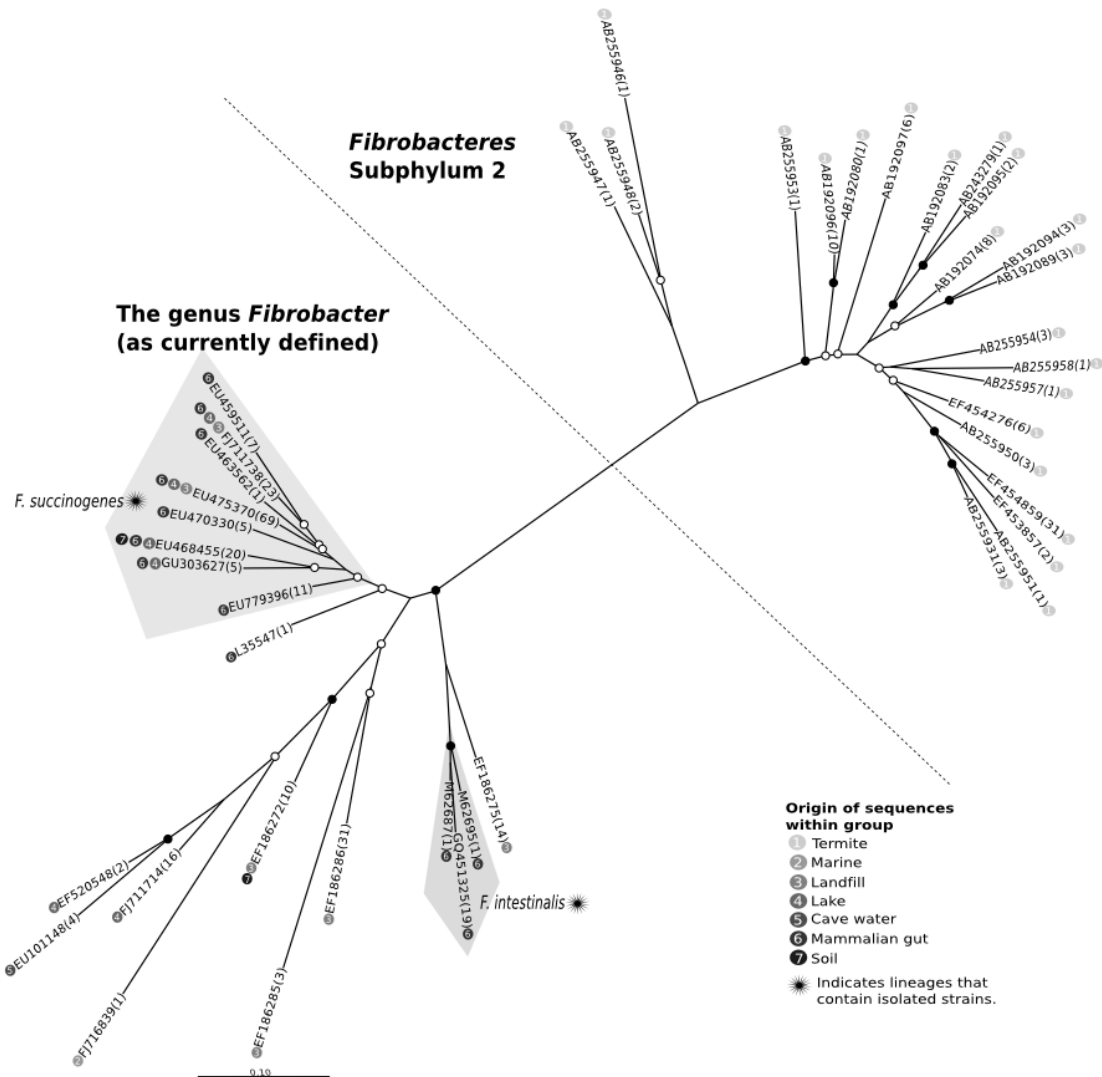


Figure 1.4. Phylogeny of the *Fibrobacteres* phylum. Maximum likelihood tree of 16S rRNA gene sequences belonging to the *Fibrobacteres* phylum. All sequences classified within the *Fibrobacteres* phylum and annotated as ‘good’ quality were downloaded from the Ribosomal Database Project (Cole *et al.*, 2007; Cole *et al.*, 2009) website in November 2010. An updated version of this tree is present in chapter 2 (Fig. 2.1). Sequences were aligned using the MUSCLE aligner (Edgar, 2004). 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 (OTU’s) at 95% similarity using CDHIT (Li and Godzik, 2006; Huang *et al.*, 2010). A number of putative chimeric sequences were removed from the dataset after analysis with the Pintail chimera check program (Ashelford *et al.*, 2005). The representative sequences of each OTU (n=42) were aligned using the Greengenes NAST aligner (DeSantis *et al.*, 2006)

and imported into Arb where the alignment was visually checked. A maximum likelihood tree was produced from the final alignment using PhyML online (Guindon *et al.*, 2010) with the HKY85 substitution model and the Shimodaira-Hasegawa (SH)-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 are 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. From Ransom-Jones *et al.* (2012).

1.5.4 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 (Hongoh *et al.*, 2005, 2006). 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 (Tokuda and Watanabe, 2007).

Hongoh and colleagues (2005), utilised terminal restriction fragment length polymorphism (T-RFLP) 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 10 termite colonies (six *Microcerotermes* colonies and four *Reticulitermes* colonies), 12 phlotypes 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 (Hongoh *et al.*, 2005) (Fig. 1.4). 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 (Hongoh *et al.*, 2006). 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 μm x 1.3-4.9 μm (Hongoh *et al.*, 2006).

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 were not detected in any of these environments, suggesting that this novel subphylum of the *Fibrobacteres* represents an autochthonous lineage of termite gut symbionts (Hongoh *et al.*, 2006). 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.* (2006)) 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 (Hongoh *et al.*, 2006), 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 (2006) 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* (Warnecke *et al.*, 2007). *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 (Warnecke *et al.*, 2007). 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.

1.5.5 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 (Montgomery *et al.*, 1988). 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 (Leschine, 1995). 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 (Skinner, 1960; Monserrate *et al.*, 2001), estuarine sediments (Madden *et al.*, 1982; Murray *et al.*, 1986) freshwater sediments (Leschine and Canaleparola, 1983), the bovine rumen (Hobson and Wallace, 1982), methanogenic bioreactors (Sleat *et al.*, 1984; Shiratori *et al.*, 2006), waste digesters (Benoit *et al.*, 1992), anoxic rice paddy field soils (Chin *et al.*, 1998; Weber *et al.*, 2001) and landfill sites (Westlake *et al.*, 1995). 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 (Nusslein and Tiedje; 1999, Saul *et al.*, 2005), peat bogs (Sizova *et al.*, 2003), mangrove sediments (Liang *et al.*, 2007) and the Atlantic and Pacific oceans (Gordon and Giovannoni, 1996). 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 (McDonald *et al.*, 2008) and freshwater lake sediments (McDonald *et al.*, 2009) 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.

1.5.5.1 Landfill sites

It has been suggested that anaerobic cellulose degradation in landfill sites is predominantly due to members of the genera *Clostridium* and *Eubacterium* (Van Dyke and McCarthy, 2002). This was first indicated by the work of Westlake *et al.* (1995), 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 (Burrell *et al.*, 2004). Studies utilising 454 pyrosequencing of PCR amplicons targeting 16S rRNA gene have also demonstrated the presence of *Firmicutes*, and more specifically *Clostridia* in landfill leachate (Bareither *et al.*, 2013; Xie *et al.*, 2014). Furthermore, both 16S rRNA gene clone libraries derived from the leachate of a closed municipal solid waste landfill (Huang *et al.*, 2005) and effluent leachate of a full-scale recirculating landfill (Huang *et al.*, 2004), as well as 454 pyrosequencing studies on an anaerobic bioreactor (Xie *et al.*, 2014) and a lab-scale bioreactor (Bareither *et al.*, 2013), both treating landfill leachate, did not identify any sequences belonging to the genus *Fibrobacter*. However, as stated above, even in the rumen where fibrobacters 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 (McDonald *et al.*, 2008). 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 (TTGE) 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. 1.4. Landfill *Fibrobacter* lineages 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. 1.4).

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 fibrobacters 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 (McDonald *et al.*, 2008). 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 (McDonald *et al.*, 2008); cellulose is the main biodegradable component of landfill, representing up to 63.4% of the total organic content (Bookter and Ham, 1982). Recently, the predominance of *Fibrobacter* spp. in a cellulolytic biofilm that colonised and degraded cotton in a landfill leachate microcosm has been demonstrated via qPCR, whereas *Fibrobacter* were not detected in the biofilm of an un-degraded cotton sample (McDonald *et al.*, 2012).

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 (McDonald *et al.*, 2008). Amann and colleagues (1992) suggested that *Fibrobacter* may in fact represent a supra-generic 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 in 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, as addressed in this thesis.

1.5.5.2 Freshwater lakes

Novel lineages of *Fibrobacter* have also been detected in freshwater lakes (Percent *et al.*, 2008; McDonald *et al.*, 2009). *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 U.K. freshwater lakes (McDonald *et al.*, 2009). This study identified two sets of sequences; those that were similar to *F. succinogenes*, (Fig. 1.4. 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. 1.4. 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* lineages, suggesting that there is no linkage between the *Fibrobacter* sequences in these environments (Fig. 1.4). 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 the equivalent depth, suggesting active colonisation of cellulosic substrates and metabolic activity (McDonald *et al.*, 2009). 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 (McDonald *et al.*, 2009).

1.5.6 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 (McDonald *et al.*, 2008). Undoubtedly, low cell numbers obtained by the anaerobic culture of *Fibrobacter* strains from the rumen has similarly resulted in the underestimation of their contribution to the degradation of cellulose (Hungate, 1966). Latham *et al.* (1971) isolated several hundred 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 (Jullian *et al.*, 1999), 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 (Whitford *et al.*, 1998; Daly *et al.*, 2001; Tajima *et al.*, 1999; Tajima *et al.*, 2000; Tajima *et al.*, 2001) and a ribosomal intergenic spacer clone library (Larue *et al.*, 2005). In a study by Larue and colleagues (2005), community DNA prepared from colonised plant biomass in the herbivore gastrointestinal tract was subjected to both ribosomal intergenic spacer analysis (RISA) 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 (Larue *et al.*, 2005). *Fibrobacter* spp. are often poorly represented in metagenomic studies, with some studies on the bovine rumen unable to detect any *Fibrobacteres* sequences at all (Hess *et al.*, 2011; Brulc *et al.*, 2009), although they have been detected in others (Nathani *et al.*, 2013) as well as other mammalian metagenomes (Ley *et al.*, 2008).

It is not known conclusively why *Fibrobacter* spp. are so poorly represented in general bacterial 16S rRNA gene libraries, but one hypothesis is that this is due to the presence of DNA associated molecules (Tajima *et al.*, 2001). When equal quantities (30 ng) of DNA extracted from pure cultures of 12 rumen bacteria, including *F. succinogenes*, were subjected to qPCR with a general bacterial primer set it was found that different bacterial species exceeded the threshold fluorescence at different cycle numbers. Whilst *Streptococcus bovis* exceeded the threshold at cycle 6.74, it was not until cycle 15.85 that the same occurred for the last species, *F. succinogenes* (Fig. 1.5). This delay in amplification was not due to fewer copies of the rRNA operon in *F. succinogenes* as it possesses three copies, whilst *S. bovis* has only one. In addition, once the threshold had been exceeded the *F. succinogenes* template behaved in the same manner (exponential amplification) as that of the other species. This led to the conclusion that the initial *Fibrobacter* genomic DNA template was the problem, rather than the PCR amplicons or primers themselves, and the possibility that molecules associated with the DNA imparted this inhibitory effect (Tajima *et al.*, 2001). It has since been suggested that where possible, reverse transcribed rRNA should be utilised in order to study the *Fibrobacteres* phylum (McDonald *et al.*, 2008). It is therefore possible that due to the under-representation of fibrobacters in rumen clone libraries and the difficulties in isolating these obligately anaerobic organisms, that their apparent absence from many terrestrial and aquatic anoxic environments

is erroneous, particularly in environments with a high cellulosic biomass content (addressed in chapter 2).

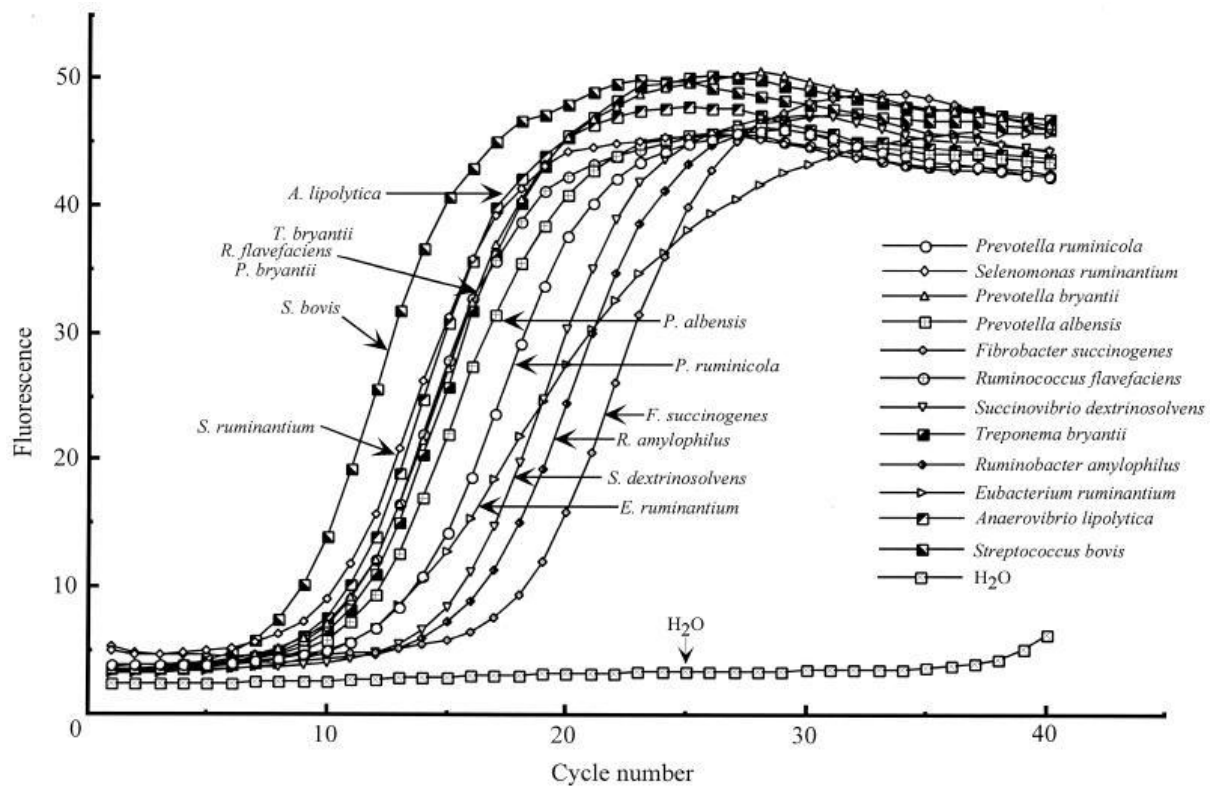


Figure 1.5. Differential amplification of rumen bacterial DNA templates with universal bacterial primers 27f and 1525r. Real-time PCR amplification was conducted essentially as described in Materials and Methods with 30 ng of each bacterial DNA template. PCR cycling was performed as follows: 95°C for 10 min of initial denaturation, then 40 cycles of 95°C for 15 s, 60°C for 5 s, and 72°C for 1 min. The fluorescence was captured at the end of the extension phase. The threshold fluorescence values were calculated with the LightCycler software and were as follows: *S. bovis*, 6.736 cycles; *S. ruminantium*, 8.375 cycles; *A. lipolytica*, 8.412 cycles; *P. bryantii*, 8.758 cycles; *R. flavefaciens*, 8.821 cycles; *T. bryantii*, 9.071 cycles; *P. albensis*, 9.592 cycles; *P. ruminicola*, 10.98 cycles; *E. ruminantium*, 10.28 cycles; *S. dextrinosolvens*, 12.59 cycles; *R. amylophilus*, 13.39 cycles; and *F. succinogenes*, 15.85 cycles. From Tajima *et al.* (2001).

1.6 Aims and objectives

Despite the diversity and potential functional importance of the members of the *Fibrobacteres* phylum, little is known concerning their ecology, phylogeny and physiology. The three key aims of this thesis are:

1. To determine the ecological range and taxonomic diversity of members of the *Fibrobacteres* phylum.
2. To attempt the isolation and cultivation of the *Fibrobacter* spp. present in landfill sites.
3. To determine the function of members of the *Fibrobacteres* phylum present in landfill sites.

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CHAPTER 2

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

Abstract

The *Fibrobacteres* phylum contains two described species, *Fibrobacter succinogenes* and *F. 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 the *Fibrobacteres* occupy a broader ecological range than previously appreciated. Here, the ecology and diversity of *Fibrobacteres* was evaluated across 64 samples from contrasting environments where cellulose degradation occurs. Fibrobacters were detected in 23 of the 64 samples via *Fibrobacter* genus-specific 16S rRNA gene PCR, providing their first targeted detection in marine and estuarine sediments, and cryoconite from Arctic glaciers, in addition to 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), 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 fibrobacters, establishing *Fibrobacter* spp. as indigenous members of microbial communities beyond the gut ecosystem. These data highlight 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.

2.1 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 (Leschine, 1995). 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 (Leschine, 1995). 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 predominant bacterial degraders of cellulose (Denman and McSweeney, 2006; Koike and Kobayashi, 2001; Kobayashi *et al.*, 2008) in conjunction with *Ruminococcus albus* and *Ruminococcus flavefaciens* (Tajima *et al.*, 2001; Denman and McSweeney, 2006; Mosoni *et al.*, 2007; Shinkai and Kobayashi, 2007). However, *F. succinogenes* is considered as the predominant bacterial degrader of cellulose in the rumen (Kobayashi *et al.*, 2008), as when *F. succinogenes* strains S85 and A3C were grown in pure culture alongside *R. albus* and *R. flavefaciens*, *Fibrobacter* spp. degraded more of the cellulose from intact forage than *Ruminococcus* spp. (Dehority, 1993). This may be explained by the recent observation that *Fibrobacter* spp. do not appear to utilise either of the two well-established mechanisms of cellulose-decomposition; the aerobic cell-free cellulase mechanism (Wilson, 2011), or the cellulosome system typified by anaerobic bacteria and fungi (Ding *et al.*, 2008). Instead, the superior efficiency of cellulolysis by *Fibrobacter* spp. (Dehority, 1993) 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 for cellulose hydrolysis (Wilson, 2008). Furthermore, none of the predicted cellulase genes contain the carbohydrate binding molecules, dockerin domains or scaffoldin genes that are typically associated with cellulosomes (Suen *et al.*, 2011). Consequently, it has been suggested that the method by which *F. succinogenes* degrades cellulose involves adherence via a putative fibro-slime protein located on the outer membrane of the cell (Suen *et al.*, 2011) 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 (Wilson, 2009).

F. succinogenes was first isolated from the rumen in 1947 and was originally designated as *Bacteroides succinogenes* (Hungate, 1947, Hungate, 1950). 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 *F. intestinalis*, both of which were thought to be present only in the mammalian intestinal tract (Montgomery *et al.*, 1988).

The diversity of *Fibrobacter* spp. in the herbivore gut has been relatively well characterised, particularly via oligonucleotide probes and comparative sequencing of the 16S rRNA gene. Stahl *et al.* (1988) designed the first *Fibrobacter*-specific oligonucleotide probes. These three probes had varying levels of specificity, one designed to targeted all but one of the known *Fibrobacter* strains, one to target rumen isolates, and the other to target cecal strains, enabling the successful detection and quantification of fibrobacters where cultivation-based methods were unsuccessful (Stahl *et al.*, 1988). 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. (Amann *et al.*, 1990); five isolated from ovine rumen, two from bovine rumen and one from the bovine cecum (Amann *et al.*, 1990), with strain identification later confirmed by DNA:DNA hybridisation (Amann *et al.*, 1992). Consequently, the application of rRNA-targeted probes enabled the quantification of fibrobacters in the rumen (Stahl *et al.*, 1988; Lin *et al.*, 1994; Lin and Stahl, 1995), and the detection of novel *Fibrobacter* populations in the bovine (Stahl *et al.*, 1988; Lin *et al.*, 1994) caprine (Lin *et al.*, 1994) and equine (Lin and Stahl, 1995) intestinal tract. Significantly, Stahl *et al.* (1988) 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 cecal 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.* (1994), 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 cecum (Lin and Stahl, 1995), as the application of a *Fibrobacter* genus-specific probe suggested that fibrobacters comprised 12% of total 16S rRNA in the equine cecum, and 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, suggesting that presence of novel species or sub-species closely related to *F. succinogenes* (Lin and Stahl, 1995).

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 (Ransom-Jones *et al.*, 2012). However, members of a novel subphylum of the *Fibrobacteres*, designated subphylum 2, have since been detected in the gut of wood-feeding termites (Hongoh *et al.*, 2005; Hongoh *et al.*, 2006) and proteomic analyses confirmed that these novel *Fibrobacteres* are involved in cellulose hydrolysis in the termite hindgut (Warnecke *et al.*, 2007).

Using a genus-specific 16S rRNA gene primer set, members of the genus *Fibrobacter* were detected in landfill sites (McDonald *et al.*, 2008; McDonald *et al.*, 2012) and freshwater lakes (McDonald *et al.*, 2009), providing the first evidence of members of the genus *Fibrobacter* beyond the intestinal tract, and these environmental fibrobacters include novel phylogenetic lineages that represent as yet uncultivated species, in addition to *F. succinogenes*-like strains (Ransom-Jones *et al.*, 2012). It has been suggested that fibrobacters are active members of the cellulolytic microbial community in these environments, as it has been demonstrated via quantitative PCR that they become enriched on heavily degraded cotton string both in landfill sites (McDonald *et al.*, 2012) and freshwater lakes (McDonald *et al.*, 2009). 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 (McDonald *et al.*, 2008).

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, *Fibrobacter* genus-specific PCR primer sets were applied to DNA extracted from a range of natural and managed environments where cellulose decomposition occurs, 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 current *Fibrobacteres* diversity in the public databases, provides the most comprehensive analysis of the ecology and diversity of the *Fibrobacteres* to date.

2.2 Materials and methods

2.2.1 Sampling

Sixty-four samples were collected from a range of mammalian gut, terrestrial, aquatic and managed environments as listed in Table 2.1. Landfill leachate and water samples were processed by filtration through 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 to avoid the presence of air in the headspace, sealed and incubated in the laboratory at ambient temperature for three months. 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.* (2014). In brief, samples were collected aseptically in 15 ml tubes and stored at -20°C in field stations pending frozen transfer to the laboratory.

Table 2.1. PCR and qPCR analysis of environmental samples. ND = ‘Not Determined’. Insufficient nucleic acid was retrieved from the environmental sample to enable qPCR analysis with sufficient replication for the quantitative analysis of both general bacteria and *Fibrobacter* spp.

a. Percentage relative abundance of 16S rRNA genes of *Fibrobacter* spp. compared with total bacteria

b. An additional 24 soil samples from Conwy, North Wales were tested via nested PCR, but no *Fibrobacter* PCR amplicons were detectable.

| Sample | Sample Type | Location | Direct PCR product with <i>Fibrobacter</i> primers | Nested PCR product with <i>Fibrobacter</i> primers | Clone library sequencing of the <i>Fibrobacter</i> 16S rRNA gene PCR amplicons | qPCR (% of total bacterial rRNA gene copies)* |
|------------------------------|---------------|---|--|--|--|---|
| Equine faeces | Faecal matter | n/a | + | + | + | 1.31 |
| Ovine rumen fluid | Rumen fluid | n/a | + | + | + | 0.04 |
| Bovine rumen fluid | Rumen fluid | n/a | + | + | + | ND |
| Peat | Peat | Acid Erosion Complex, Migneint-Arenig-Dduallt, Conwy, Wales | - | - | - | ND |
| Soil from stable sand dune | Soil | Stable sand dune, Newborough, Anglesey | - | + | - | ND |
| Blanket bog soil | Soil | Migneint-Arenig-Dduallt, Conwy, Wales | - | + | + | ND |
| Buckley compost | Compost | Compost heap, Chester, England | - | - | - | ND |
| Cryoconite VB1 | Cryoconite | High Arctic (Svalbard) | - | + | + | ND |
| Cryoconite PF1 | Cryoconite | European Alps (Tyrol) | - | - | - | ND |
| Cryoconite RM1 | Cryoconite | European Alps (Tyrol) | - | - | - | ND |
| Cryoconite GB1 | Cryoconite | European Alps (Tyrol) | - | - | - | ND |
| Cryoconite ML6 | Cryoconite | High Arctic (Svalbard) | - | + | - | ND |
| Cryoconite AB6 | Cryoconite | High Arctic (Svalbard) | - | + | + | ND |
| Esthwaite (lake) Sediment | Sediment | Esthwaite Lake, Lake District, England | - | - | - | ND |
| Lake Ogwen sediment Sediment | Sediment | Lake Ogwen, Gwynedd, Wales | - | + | - | ND |
| Llyn Aled (lake) Sediment | Sediment | Llyn Aled, Conwy, Wales | - | + | + | ND |

| | | | | | | |
|--|----------------------|--|---|---|---|------|
| Aled Isaf (lake) Sediment | Sediment | Aled Isaf, Conwy, Wales | - | + | + | ND |
| Conwy Estuary microcosm 1 cotton | Cotton string | Mussel Bed, Conwy Estuary, Wales | - | - | - | ND |
| Conwy Estuary microcosm 2 cotton | Cotton string | Mussel Bed, Conwy Estuary, Wales | - | + | + | ND |
| Conwy Estuary microcosm 3 cotton | Cotton string | Mussel Bed, Conwy Estuary, Wales | - | + | + | ND |
| Conwy Estuary microcosm 4 cotton | Cotton string | Mud Flat, Conwy Estuary, Wales | - | + | - | ND |
| Conwy Estuary microcosm 5 cotton | Cotton string | Mud Flat, Conwy Estuary, Wales | - | - | - | ND |
| Marine off shore transect sediment 1km | Sediment | Conwy, Wales | - | - | - | ND |
| Marine off shore transect sediment 2km | Sediment | Conwy, Wales | - | - | - | ND |
| Marine off shore transect sediment 4km | Sediment | Conwy, Wales | - | + | + | ND |
| Marine off shore transect sediment 8km | Sediment | Conwy, Wales | - | - | - | ND |
| Marine off shore transect sediment 12km | Sediment | Conwy, Wales | - | - | - | ND |
| Brombrough Dock (landfill) microcosm | Filtered leachate | Brombrough Dock Landfill, Wirral, England | + | + | + | 3.90 |
| Buckley (landfill) leachate 1 (LC3) | Filtered leachate | Buckley Landfill, Flintshire, Wales | - | + | + | ND |
| Buckley (landfill) leachate 2 (LC1B) | Filtered leachate | Buckley Landfill, Flintshire, Wales | - | + | + | ND |
| Buckley (landfill) leachate 3 (LC2B) | Filtered leachate | Buckley Landfill, Flintshire, Wales | - | - | - | ND |
| Bidston Moss (landfill) microcosm cotton 1J | Cotton string | Bidston Moss Landfill, Wirral, England | - | + | + | 0.02 |
| Bidston Moss (landfill) microcosm cotton 3E | Cotton string | Bidston Moss Landfill, Wirral, England | - | - | - | ND |

| | | | | | | |
|---|--------------------|--|---|---|---|------|
| Bidston Moss (landfill) microcosm cotton 3F | Cotton string | Bidston Moss Landfill, Wirral, England | - | - | - | ND |
| Bidston Moss (landfill) microcosm 3F containing 0.1% (wt/vol) avicell | Filtered microcosm | Bidston Moss Landfill, Wirral, England | - | + | + | 1.43 |
| Bidston Moss (landfill) leachate 1J | Filtered leachate | Bidston Moss Landfill, Wirral, England | - | - | - | ND |
| Bidston Moss (landfill) leachate 3E | Filtered leachate | Bidston Moss Landfill, Wirral, England | - | - | - | ND |
| Bidston Moss (landfill) leachate 3F | Filtered leachate | Bidston Moss Landfill, Wirral, England | - | + | + | ND |
| Soil transect point 5 | Soil ^b | Conwy, Wales | - | + | - | ND |
| Soil transect point 8 | Soil ^b | Conwy, Wales | - | + | - | ND |

2.2.2 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 via the method of Griffiths *et al.* (2000) with the following modifications. Prior to precipitation with polyethelene 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 minutes before the addition of an equal volume of chloroform-isoamyl alcohol (24:1) (Sigma) and centrifugation and precipitation as described by Griffiths *et al.* (2000). The DNA was resuspended in 50 µl nuclease free water (Bioline) and visualised on a 1% agarose (Bioline) gel with HyperLadder 1kb (Bioline) before quantification with the Qubit Fluorometer (Life Technologies) and the Qubit dsDNA BR Assay Kit (Life Technologies). Cryoconite samples were subjected to PowerSoil (MoBio, Inc.) DNA extraction as specified by the manufacturer, with DNA extracted from 250 mg (fresh weight) of cryoconite and eluted in 100 µl Buffer C6. Purified DNA was stored at -80°C.

2.2.3 Amplification of the 16S rRNA gene via direct and nested PCR

PCR reactions contained 0.2 mM each primer (Table 2.2), 0.2 mM each dNTP, 1x SuperTaq Buffer (Cambio), 0.5 mM MgCl₂, 1x BSA, 1 unit SuperTaq (Cambio), 50 ng DNA and dd H₂O to a final volume of 50 µl. PCR reactions using the *Fibrobacter* primer set (Fib 1F and Fib 2AR, Table 2.2) contained an increased concentration of each primer (0.4 mM) and MgCl₂ (1.5 mM). PCR cycling conditions were as follows: initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 1 min at the specific annealing temperature for each primer set (Table 2.2) and 72°C for 1.5 min. The final extension was performed at 72°C for 10 min. For direct PCR, 50 ng extracted DNA was amplified with the *Fibrobacter* specific primers (Table 2.2). Nested PCR consisted of an initial round of PCR using the general bacterial primer set (pA and pH', Table 2.2), followed by a second round of PCR on the general bacterial amplification products (1 µl) with the *Fibrobacter* specific primers (Fib 1F and Fib 2AR, Table 2.2). PCR products were visualised on a 1% agarose (Bioline) gel with HyperLadder 1kb (Bioline) and stored at -20°C.

Table 2.2. 16S rRNA gene primers used for PCR and qPCR amplification and sequencing.

- a. Ambiguities: K=(GorT), S=(GorC), W=(AorT), Y=(CorT), H=(A,CorT), R=(AorG), D=(G,AorT), V=(A,CorG).
b. Primers used for qPCR analysis.
c. Primer used for sequencing.
d. QuantiFast™ SYBR® Green PCR assay (Qiagen) uses same annealing temperature (60°C) for all primer sets.
e. Primers based on those of Lin and Stahl (1995) and modified by McDonald *et al.* (2008).

| Primers | Sequence (5' - 3') ^a | Specificity | Annealing Temperature (°C) | Amplicon Size (bp) | Reference |
|---------------------------|---------------------------------|---------------------------------------|----------------------------|--------------------|---------------------------------|
| pA | AGAGTTTGATCCTGGCTCAG | General Bacteria | 55 | ~ 1534 | (Edwards <i>et al.</i> , 1989) |
| pH' | AAGGAGGTGATCCAGCCGCA | | | | |
| Fib 1F ^c | CCGKSCCAACGSSCGG | <i>Fibrobacter</i> genus | 60 | ~ 855 | (McDonald <i>et al.</i> , 2008) |
| Fib 2AR | ATCTCTCGCYGCGGCGWTYCC | | | | |
| 1369F ^b | CGGTGAATACGTTTCYCGG | General bacteria ^b | 60 ^d | ~ 151 | (Suzuki <i>et al.</i> , 2000) |
| Prok 1492R ^b | GGWTACCTTGTTACGACTT | | | | |
| FibroQ153F ^{b,c} | CCGKSCCAACGSSCGGHTAA | <i>Fibrobacter</i> ^b genus | 60 ^d | ~ 104 | (McDonald <i>et al.</i> , 2008) |
| FibroQ238R ^b | CSCCWACTRGTAAATCRGAC | | | | |
| M13 Forward ^c | GTTTTCCAGTCACGAC | M13 Vector | n/a | n/a | (Messing, 1983) |

2.2.4 Cloning and sequencing of *Fibrobacter* specific PCR amplification products

Seventeen of the *Fibrobacter* specific 16S rRNA-gene PCR amplification products from the nested PCR described above were extracted from a 1% agarose (Bioline) gel and purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The 16S rRNA gene PCR products were ligated and cloned into competent *E. coli* JM109 (Promega) using the pGEM-T Easy Vector System I (Promega) according to the manufacturer's protocol, and the plasmid DNA extracted and purified using the QIAEX II Gel Extraction Kit (Qiagen) prior to sequencing using the M13 forward primer (Table 2.2) by Source BioScience.

2.2.5 Quantification of *Fibrobacter* spp. via qPCR

For each of the five samples for which sufficient DNA template was achieved for qPCR analysis, triplicate qPCR assays were performed with both the general bacterial (1369F and Prok 1492Rb, Table 2.2) and *Fibrobacter* specific primer sets (FibroQ153F and FibroQ238R, Table 2.2) on the 7900HT Fast Real-Time PCR System (Applied Biosystems). Each reaction was performed in a 20 µl final volume, containing 10 ng DNA, 10 µl of 2x QuantiFast SYBR Green PCR Master Mix (Qiagen), 1 mM (final concentration) forward and reverse primer and dd H₂O. Cycling conditions were 95°C for 5 min, followed by 45 cycles of 95°C for 10 seconds, and 60°C for 30 seconds, with fluorescence detection in the combined annealing and extension step. A dissociation step was included at the end of every run to confirm the presence of single amplification products.

The amplified 16S rRNA gene of *F. succinogenes* S85 was used to generate standard dilution curves to determine the relative abundance of *Fibrobacter* spp. The almost full length 16S rRNA gene (~1534 bp) was amplified using the primers pA and pH' (Table 2.2) as described above and the amplification product excised from a 1% agarose (Bioline) gel and purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. The concentration of purified DNA was established with a Qubit Fluorometer (Life Technologies) using the Qubit dsDNA BR Assay Kit (Life Technologies) and the 16S rRNA gene copy number per microliter was calculated via the following equation: $(X \text{ g/}\mu\text{l DNA} / [\text{PCR product length in basepairs} \times 660]) \times 6.022 \times 10^{23} = Y \text{ molecules/}\mu\text{l}$.

Triplicate standard curves of the *F. succinogenes* S85 16S rRNA gene were generated using serial dilutions from 3×10^8 to 3×10^2 gene copies, with all three serial dilutions included on each plate with each primer set. Standard curves for each primer set were generated by plotting the Ct value against the log gene copy number, and a linear line of best fit used to

determine the r^2 value, amplification efficiency and y-intercept (Pfaffl, 2001). The relative abundance (%) of *Fibrobacter* spp. was determined by dividing the number of gene copies per sample from the *Fibrobacter* specific assay with the number of total bacterial gene copies per sample as determined by the standard curves for each primer set (Smits *et al.*, 2004).

2.2.6 Phylogenetic analysis of *Fibrobacteres* 16S rRNA gene sequences

All sequences classified within the *Fibrobacteres* phylum and annotated as ‘good’ quality were downloaded from the Ribosomal Database Project (Cole *et al.*, 2007; Cole *et al.*, 2009) website in July 2013, and combined with sequences derived from the clone libraries produced in this study. The resulting dataset was subsequently aligned using the MUSCLE aligner (Edgar, 2004). Sequences were trimmed to produce an alignment containing only complete sequences corresponding to the regions between positions 188 and 887 of the *E. coli* 16S rRNA gene. Sequences from the aligned dataset were subsequently clustered into Operational Taxonomic Units (OTUs) using a cutoff of 95% similarity using CDHIT (Li and Godzik, 2006; Huang *et al.*, 2010). Sequences were checked for chimeras using Bellerophon (Huber *et al.*, 2004) and putative chimeric sequences removed from the dataset. The representative sequences of each OTU (n=63) were aligned using the MUSCLE aligner (Edgar, 2004) and imported into ARB (Ludwig *et al.*, 2004) where the alignment was visually checked and manually optimised. A maximum likelihood tree was produced from the final alignment using ARB (Ludwig *et al.*, 2004). Nodes for which a bootstrap value of >95% was observed are marked with a filled circle, nodes for which the bootstrap value was between 75 and 95% are marked with an unfilled circle. Nucleotide sequence accession numbers for the representative sequence of each OTU are displayed on each node and the number of sequences clustering within each OTU are displayed in parentheses. 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.

2.3 Results and discussion

2.3.1 Genus-specific 16S rRNA gene PCR amplification of *Fibrobacter* spp. in environmental samples

Fibrobacter spp. were detected in 23 of the 64 samples studied via nested PCR, including equine faeces, ovine and bovine rumen fluids, soils, cryoconite, freshwater, estuarine and marine sediments and landfill sites (Table 2.1). This is the first targeted detection of *Fibrobacter* spp. in estuarine sediments, marine sediments and cryoconite. A direct PCR amplification product was detected in 4 of the 64 environmental samples screened (Table 2.1). A direct PCR result for the presence of *Fibrobacter* spp. usually only occurs in samples where there is a significant abundance of *Fibrobacter* spp. The four environments in which *Fibrobacter* spp. were detected via direct PCR (equine faeces, ovine rumen fluid, bovine rumen fluid and Bromborough Dock landfill) are known to have high numbers of fibrobacters, with qPCR demonstrating the relative abundance of *Fibrobacter* rRNA compared with total bacterial rRNA as 21-32% (Ovine gut) (McDonald *et al.*, 2008) and 28.9% (Brombrough Dock Riser 3) (McDonald *et al.*, 2012).

The relative rRNA gene abundance of *Fibrobacter* spp. in relation to total bacteria as determined by qPCR ranged from 0.02 to 3.9% in landfill sites (Table 2.1), which is comparable with previous studies that have shown that fibrobacters range from 0.2 to 40% of the total bacterial rRNA molecules in landfill sites (McDonald *et al.*, 2008) and 0.005 to 1% in lakes (McDonald *et al.*, 2009). These data suggest that fibrobacters can represent a significant and active proportion of the microbial population in these environments. There are however caveats when using DNA to detect fibrobacters via PCR, as it is thought that DNA associated molecules interfere with PCR amplification, thus resulting in the previous underestimation of their abundance within the rumen via general bacterial 16S rRNA gene libraries (Tajima *et al.*, 2001). Furthermore, the extraction method used can also introduce bias (Henderson *et al.*, 2013). Molecular analysis of reverse transcribed rRNA is thought to be a better approach to studying members of the *Fibrobacteres* (McDonald *et al.*, 2008), as the inhibitory molecules seem to only be associated with DNA. Tajima *et al.*, (2001) observed that *F. succinogenes* genomic DNA had a prolonged delay in amplification prior to the exponential amplification phase of the DNA template in qPCR assays; however, once PCR amplification surpassed the threshold of detection, the template amplified exponentially, suggesting that the initial genomic DNA was responsible for the poor amplification efficiency. Thus when cDNA is used for downstream applications this potential interference would be overcome. However, it was not possible to extract enough RNA from the samples for RT qPCR to be applied here.

2.3.2 Phylogenetic analysis

The *Fibrobacteres* phylum currently consists of two subphyla, subphylum 2, which contains only species detected in the termite gut, and subphylum 1 - the genus *Fibrobacter sensu stricto*, which contains the two characterised species, *F. succinogenes* and *F. intestinalis* (Fig. 2.1). The generation of new *Fibrobacter* 16S rRNA gene sequence data from several contrasting environments here expands our knowledge of the ecological range of this poorly studied phylum and comparative phylogenetic analyses of these data in addition to known *Fibrobacteres* diversity in the public databases makes this study of the *Fibrobacteres* phylum the most comprehensive to date. The Ribosomal Database Project repository (Cole *et al.*, 2007) previously contained only one *Fibrobacter* sequence that had been detected in marine and estuarine sediments, and as a result this study has added to the current understanding of the ecology of the phylum in these and other environments. This is also the first specific detection of *Fibrobacter* spp. in cryoconite, a microbe-mineral aggregate responsible for darkening glacial ice surfaces (Takeuchi *et al.*, 2001) and associated with high rates of microbial carbon production despite ambient temperatures between 0 and 1°C (Anesio *et al.*, 2009).

Previous phylogenetic studies have used 95% similarity to designate species-level diversity within the *Fibrobacteres* phylum due to the substantial 16S rRNA gene and genomic diversity between the two described species (Jewell *et al.*, 2013; Ransom-Jones *et al.*, 2012), with the sequences derived from *F. intestinalis* isolates forming a single OTU at 95%, below the commonly accepted 97% OTU cutoff used to cluster at species-level (Jewell *et al.*, 2013). When all sequences were clustered at 95% sequence similarity, 63 OTUs were generated, with *F. succinogenes* comprising 11 OTUs and *F. intestinalis* 3 OTUs clustering separately from *F. succinogenes*, with a bootstrap value of >95% (Fig. 2.1). This would suggest that the strains currently designated as *F. succinogenes* do not actually represent a single species. Previous studies have suggested that *F. succinogenes* and *F. intestinalis* may actually represent two distinct genera (Montgomery *et al.*, 1988; Ransom-Jones *et al.*, 2012), but in the absence of phenotypic data to distinguish between the two species (despite significant genomic diversity), elevating each taxon to genus status is premature. Consequently, *F. succinogenes* is currently separated into four sub-species.

In order to determine the extent of 16S rRNA gene diversity within the *F. succinogenes* lineage, all sequences designated as isolates of *F. succinogenes* were downloaded from the Ribosomal Database Project website and aligned as described previously. The alignment was then trimmed to create a near full length alignment of the 16SrRNA gene (1176 bp) (data not shown). When a similarity matrix was constructed for this alignment, it was found that a 91%

clustering value would be needed to group all members of *F. succinogenes* into the same node. Nevertheless, this value for interspecies variation is lower than the current 95% 16S rRNA similarity considered as the minimum allowable within a genus (Ludwig *et al.*, 1998). If clustered at 91% similarity, 29 OTUs are generated, suggesting that there are at least 27 potentially novel species contained within the phylum, demonstrating that there is greater diversity outside of the two recognised species than within.

The number of sequences contained within each of the 63 OTUs generated at 95% (Fig. 2.1) varies from 297 sequences to singleton sequences, with 18 OTUs containing only one sequence. As a result of this, it cannot be inferred if these lineages are exclusive to a particular niche, as further sequencing data may well reveal other as yet undetected *Fibrobacters* that would cluster within these OTUs, either from the same or different environments. Whilst there are lineages that contain species from a range of environments, others seem to be specific to one particular ecological niche. Seven of the 63 OTUs (95%) contained sequences derived from both mammalian gut and environmental samples. However, 24 of the 64 OTUs contained sequences detected exclusively in non-gut environmental samples. These data suggest that a significant proportion of the diversity detected within the *Fibrobacteres* phylum is derived from environmental (non-gut) fibrobacters (Fig. 2.1). Consequently, the isolation and cultivation of these potentially novel *Fibrobacter* spp. is an obvious priority, in order to further our understanding of their physiology and function in natural and managed environments. The OTU represented by FJ711711 contains species found solely in freshwater lakes, and lineages GQ139119, GQ132590, GQ133837, GQ135610, GQ135762, GQ135590, GQ135618, GQ134316, GQ138403, GQ135015 and GQ136636 all contain species from an Anaerobic Batch Sequencing Reactor (ABSR), utilised for treating swine waste (Fig. 2.1). In addition to this, EF186285, EF186275 and KJ364190 contain species found solely in landfill sites, with the separation of EF186275 supported by a bootstrap value of >95%, and KJ364274 containing only species present in cryoconite samples (Fig. 2.1). The OTU represented by KJ364274 is also distant to both its nearest neighbor, GQ134316, and any of the other lineages, suggesting that the sequences contained within this OTU may in fact be members of a novel subphylum (Fig. 2.1). Cryoconite fibrobacters are also present in lineage CP001702, which clusters within the *F. succinogenes* group, supported by a >95% bootstrap value, despite the geographic separation of this environment from grazing areas. In addition, cryoconite bacterial communities are distinct from adjacent habitats, and appear to assemble by deterministic processes (Edwards *et al.*, 2013), implying the selection of taxa involved in the functioning of cryoconite ecosystems. Both alpine and Arctic cryoconites receive allocthonous organic matter

from plant sources (Pautler *et al.*, 2013; Xu *et al.*, 2010). The detection of fibrobacters on all Svalbard glaciers sampled but none of the alpine glaciers examined in this study is interesting. Cryoconite aggregates on Arctic glaciers, including those sampled here, frequently mature to form granular structures (Langford *et al.*, 2010) while the cryoconite aggregates on the alpine glaciers sampled are poorly developed aggregates of cells, organic matter and mineral debris. The distribution of fibrobacters in Arctic cryoconite is therefore consistent with the evolution of anoxic microhabitats in the interiors of cryoconite granules (Hodson *et al.*, 2010; Telling *et al.*, 2012). The presence of both cosmopolitan and unique lineages illustrates the potential broad dispersal and diversity of fibrobacters in Arctic glacial environments. As such, the data presented suggests a role for fibrobacters in Arctic cryoconite carbon cycling, in particular as cryoconite community structure, respiration rates and organic matter profiles are closely related (Edwards *et al.*, 2011; Edwards *et al.*, 2014). The detection of fibrobacters within this environment is therefore suggestive of a greater role in the global carbon cycle than previously thought. This is supported by the fact that landfill site (McDonald *et al.*, 2008; McDonald *et al.*, 2012), freshwater lake (McDonald *et al.*, 2009) and estuarine fibrobacters were detected on colonised cotton in both this and previous studies.

However, the detection of novel *Fibrobacter* species is not limited to environmental samples, as 15 of the detected *Fibrobacter* lineages (95% OTUs) were exclusive to the termite gut (Fig. 2.1). In addition, the majority of previous mammalian intestinal tract studies have relied on *F. succinogenes* species-specific primers, thus potentially missing other novel members of the genus that may be present in these environments. Bovine, ovine and equine samples were therefore included in order to determine whether or not novel fibrobacters were also present in these environments. Seventeen of the 63 *Fibrobacter* OTUs (95%) observed were comprised exclusively of mammalian gut sequences (Fig. 2.1). Lineages M62682 and KJ364196 (Fig. 2.1) contain only bovine associated species, suggesting that in the bovine rumen there are also as yet unclassified novel species. Furthermore, a number of OTUs, EU470330, EU473529, EU473449, EU775761 and L35547 contain only equine associated species, with the separation of EU473449 and EU473529 supported by bootstrap values of >95%. Lin and Stahl (1995) used *Fibrobacter* genus and species specific probes in an rRNA hybridization study of equine cecal contents, suggesting that the *F. succinogenes* and *F. intestinalis* signal represented only a small proportion of the total *Fibrobacter* abundance generated with the genus specific probe. These data therefore suggested that novel *Fibrobacter* species were present in the equine cecum and our detection of five equine-specific *Fibrobacter* lineages here supports this assertion.

Further work should focus on the application of PCR-independent methods to investigate the abundance, ecology and physiology of fibrobacters in these environments. The sequence data and phylogenetic analysis presented here enable the design and application of lineage-specific *Fibrobacter* probes for both RNA and cellular quantification of fibrobacters. Previous studies have also utilised PCR-independent methods such as RNA hybridization and Fluorescence In Situ Hybridization (FISH) to provide important insights into the abundance, ecology and physiology of *Fibrobacter* lineages in the gut (Amann *et al.*, 1990; Amann *et al.*, 1992; Lin and Stahl, 1995; Lin *et al.*, 1994; Stahl *et al.*, 1988). For example, ecological and physiological differences between strains from phylogenetic subgroups 1-3 of *F. succinogenes* have been detected in the rumen using qPCR and FISH. *F. succinogenes* subgroup 1 were observed to predominate numerically and were highly active on plant material, particularly on the less degradable hay stems, whereas subgroups 2 and 3 were more often associated with other rumen bacteria on the more readily degradable leaf sheaths (Shinkai and Kobayashi, 2007; Kobayashi *et al.*, 2008). Consequently, such approaches may now be applied to determine the ecology and physiology of fibrobacters in their newly described ecological niches.

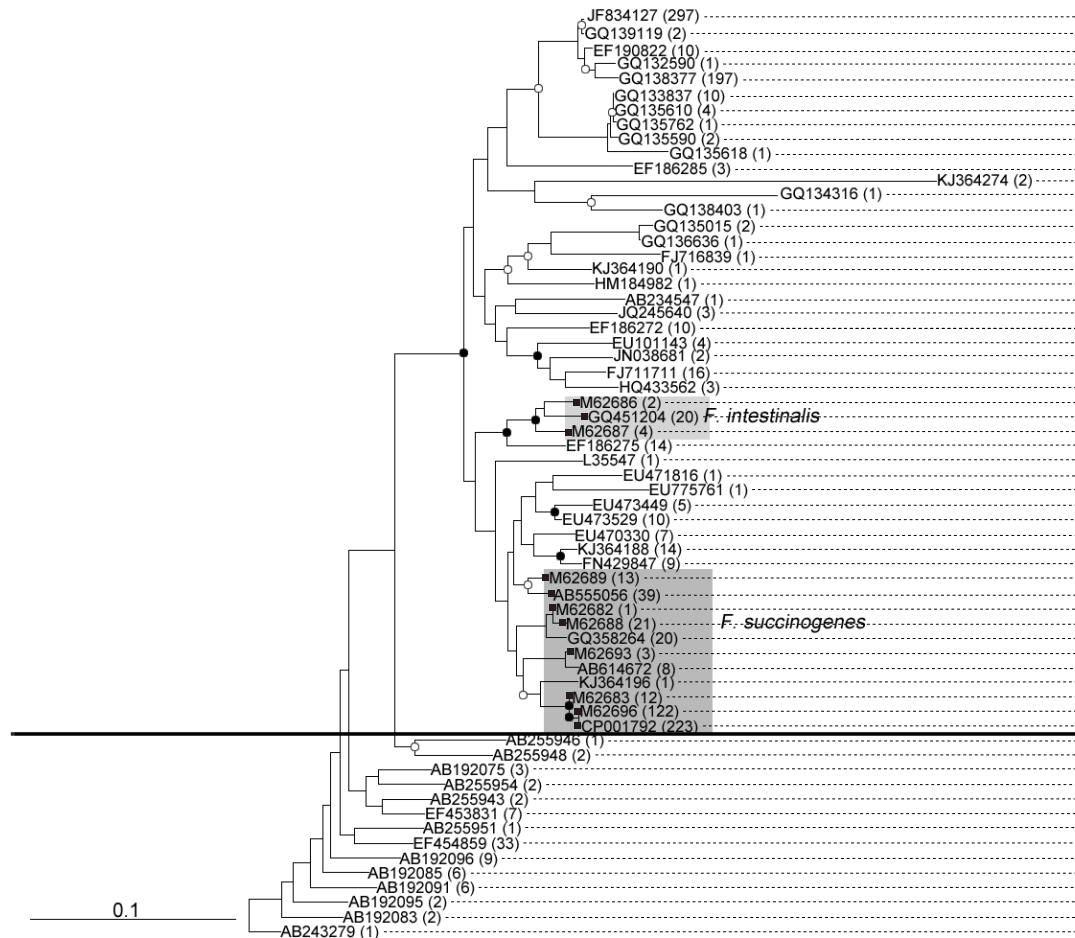
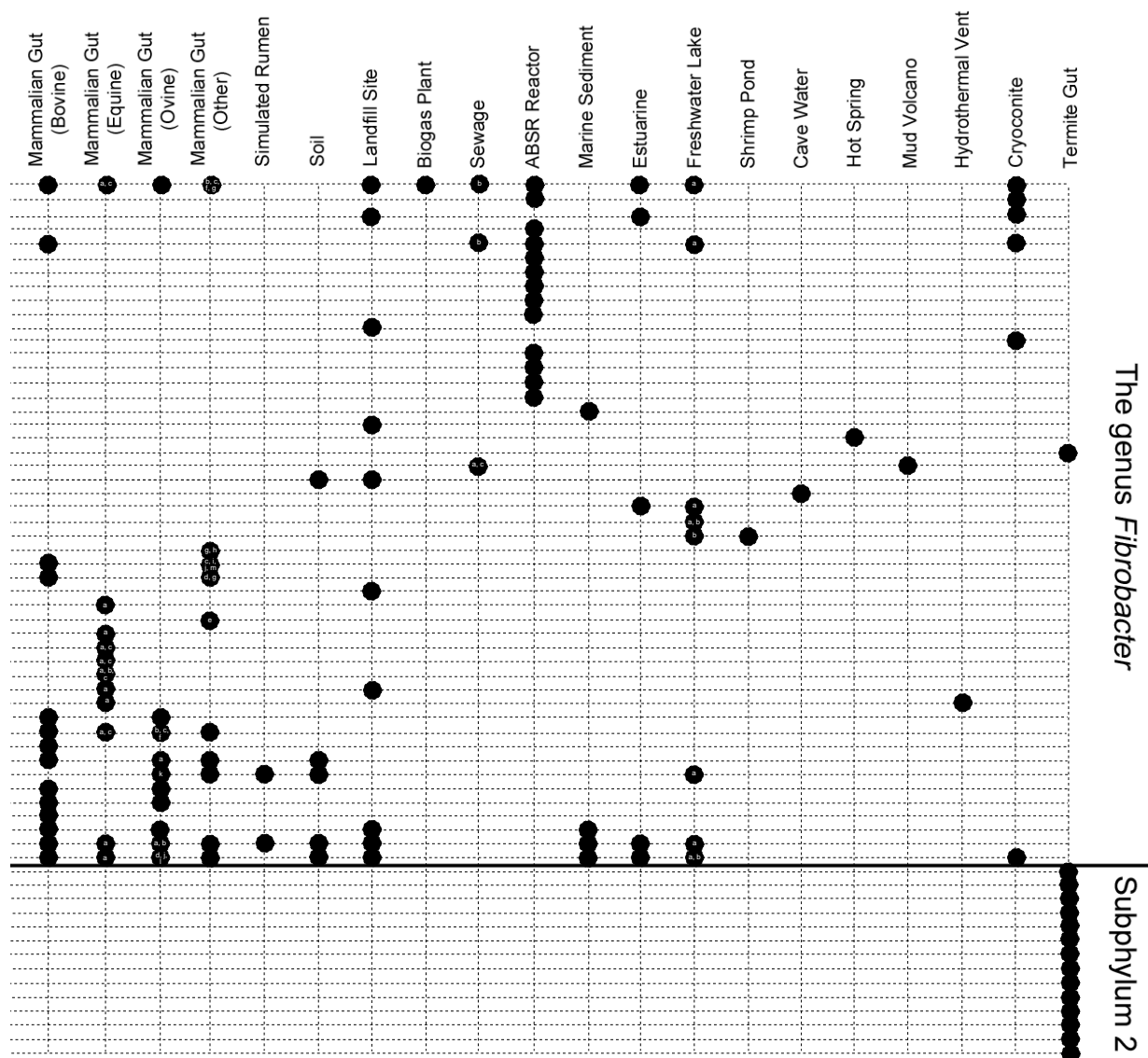


Figure 2.1. The ecology and taxonomy of the *Fibrobacteres* phylum (Ransom-Jones *et al.*, 2012). OTUs are labelled with the accession number of the representative sequence, and the number of sequences contained within each OTU is in brackets. OTUs containing cultivated species are designated by boxes on the end of the node. Please refer to Appendix 3 for a full data table describing the sequences contained in each OTU, their accession numbers and percentage environmental composition of each OTU.



Further information on the environmental distribution is as follows:

Mammalian Gut (Equine): a) Horse, b) Grevy’s Zebra, c) Wild Ass.

Mammalian Gut (Other): a) Buffalo, b) Capybara, c) Colobus, d) Dromedary Camel, e) Elephant, f) Goat, g) Pig, h) Rat, i) River Hog, j) Rock Hyrax, k) Tammar Wallaby, l) Yak, m) Yunnan Snub-nosed Monkey.

Sewage: a) Activated Sludge, b) Anaerobic Sludge Digester, c) Raw Sewage.

Freshwater lake: a) Lake Sediment, b) Lake Water.

2.4 Conclusions

There is a current impetus to better understand the diversity of cellulolytic microbes and their enzyme systems for biotechnological applications, particularly in the production of second-generation biofuels and in understanding biomass decomposition and nutrition in commercially important herbivores. Fibrobacters are prolific degraders of cellulose, however

most cultivation-based approaches for the isolation of cellulolytic microorganisms typically focus on aerobic or facultative anaerobic species that are easier to isolate and cultivate, thus disregarding obligate anaerobes such as fibrobacters. Furthermore, the problems associated with *Fibrobacter* DNA amplification have meant that until recently *Fibrobacter* spp. have remained undescribed in many environments due to the apparent biases against the detection of *Fibrobacter* DNA in microbial communities using general 16S rRNA gene and shotgun metagenomic approaches (Hess *et al.*, 2011; Brulc *et al.*, 2009). Consequently, the genomic diversity, physiology and metabolism of the *Fibrobacteres* is barely understood, despite the significant ecological, economical and biotechnological potential of this functionally diverse phylum.

Here, our understanding of the taxonomic diversity and ecological range of *Fibrobacter* spp. in natural and managed environments is extended to several newly described niches, all of which potentially promote adaptation and diversity, thus generating novel centres of variation within the *Fibrobacteres* that comprise enzymes and growth requirements that are favourable for biotechnological exploitation. Historically, the ecology of fibrobacters was thought to be restricted to the mammalian intestinal tract. However, the significant diversity of potentially novel *Fibrobacter* species described here, and in particular the large proportion of OTUs (n=24) derived exclusively from natural and managed environments, demonstrates their broad ecological range in the biosphere. Fibrobacters are therefore an important target for cultivation-based and omics approaches aiming to elucidate novel carbohydrate active enzymes and mechanisms. It has recently been suggested that *F. succinogenes* S85 utilises a novel mechanism for cellulose hydrolysis (Wilson, 2008), and with the observed taxonomic diversity within the *Fibrobacteres* it is likely that the phylum represents a significant source of unexplored diversity with respect to carbohydrate active enzymes.

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CHAPTER 3

Isolation and cultivation of *Fibrobacter spp.* from landfill leachate
cellulose enrichment cultures

Abstract

Whilst it was originally thought that fibrobacters were restricted to the mammalian intestinal tract, *F. succinogenes* and novel members of the genus *Fibrobacter* have recently been detected in natural and managed environments beyond the gut, confirming the broad ecological range of the *Fibrobacteres* phylum. Landfill sites are perhaps the best characterised of these environments, with the relative abundance of *Fibrobacter* rRNA representing as much as 40% of the total bacterial rRNA. However, despite the diversity and abundance of these *Fibrobacter* lineages, there remains only two characterised species within the genus, both isolated from the mammalian intestinal tract. Here, the isolation of *Fibrobacter* spp. from landfill sites was attempted by the use of microcosms containing landfill leachate supplemented with either glucose or Avicel, in order to enrich fibrobacters for isolation via the Hungate roll tube method. A total of 63 cellulolytic bacterial strains were isolated from landfill leachate/Avicel enrichment cultures; five of which contained *Fibrobacter* spp. as determined via genus specific 16S rRNA gene PCR. These *Fibrobacter*-containing cultures underwent seven further rounds of purification before purity was established. However, upon achieving purification of *Fibrobacter* strains, it was not possible to passage the isolates further. Sequencing and BLASTn analysis of *Fibrobacter* specific 16S rRNA gene PCR amplicons of each isolate demonstrated that these species were *F. succinogenes*. Phylogenetic analysis of each isolate demonstrated that landfill *F. succinogenes* isolates clustered within the *F. succinogenes* subsp. *succinogenes* lineage, suggesting that these are strains of *F. succinogenes* that are adapted to the landfill environment. This is the first isolation of *Fibrobacter* spp. from a non-gut environment (landfill sites) and their ability to propagate outside of the gut confirms their status as active members of cellulolytic microbial populations in landfill sites, and potentially, in other environments.

3.1 Introduction

Fibrobacter succinogenes (previously designated as *Bacteroides succinogenes*) was isolated from the bovine rumen in 1947 (Hungate, 1947) and represents one of only two formally described species of the genus *Fibrobacter*, the sole genus of the *Fibrobacteres* phylum. The genus *Fibrobacter* was established in 1988 to contain *F. succinogenes* (reclassified from *Bacteroides succinogenes*) and the newly described *F. intestinalis*, which to date remain the only characterised species within the *Fibrobacteres* phylum (Montgomery *et al.*, 1988). Members of the genus are described as Gram-negative, non-sporeforming, obligate anaerobes, that are either rod-shaped or pleiomorphic ovoid cells (Montgomery *et al.*, 1988), approximately 0.8-2.0 μm in length and 0.3-0.5 μm in diameter (Hungate, 1950; Stewart and Flint, 1989).

The Hungate method for isolating obligate anaerobes was first described in 1947 (Hungate, 1947) and is still the primary technique employed for the cultivation of *F. succinogenes* from the rumen (McDonald *et al.*, 2012b). The preferred medium, either liquid or solid, is prepared under anoxic conditions by boiling the medium for 10 minutes to remove excess oxygen, with CO_2 gas also flowing through the medium. A reducing agent such as cysteine hydrochloride is added to the medium and subsequently the redox indicator Resazurin, which possesses a dark blue colour under aerobic conditions, becomes colourless due to the reduction of the medium to an anoxic state (McDonald *et al.*, 2012b). The medium is then aliquoted into individual glass tubes under anaerobic conditions, which are maintained via the use of metal gas hooks connected to a CO_2 source, enabling the flow of CO_2 into the tubes, thus displacing the oxygen (Fig. 3.1). The tubes are subsequently sealed with butyl rubber stoppers and autoclaved to ensure sterility (Fig. 3.1). Further work with the cultures takes place aseptically under anaerobic conditions maintained by the flow of CO_2 via the gas hooks.

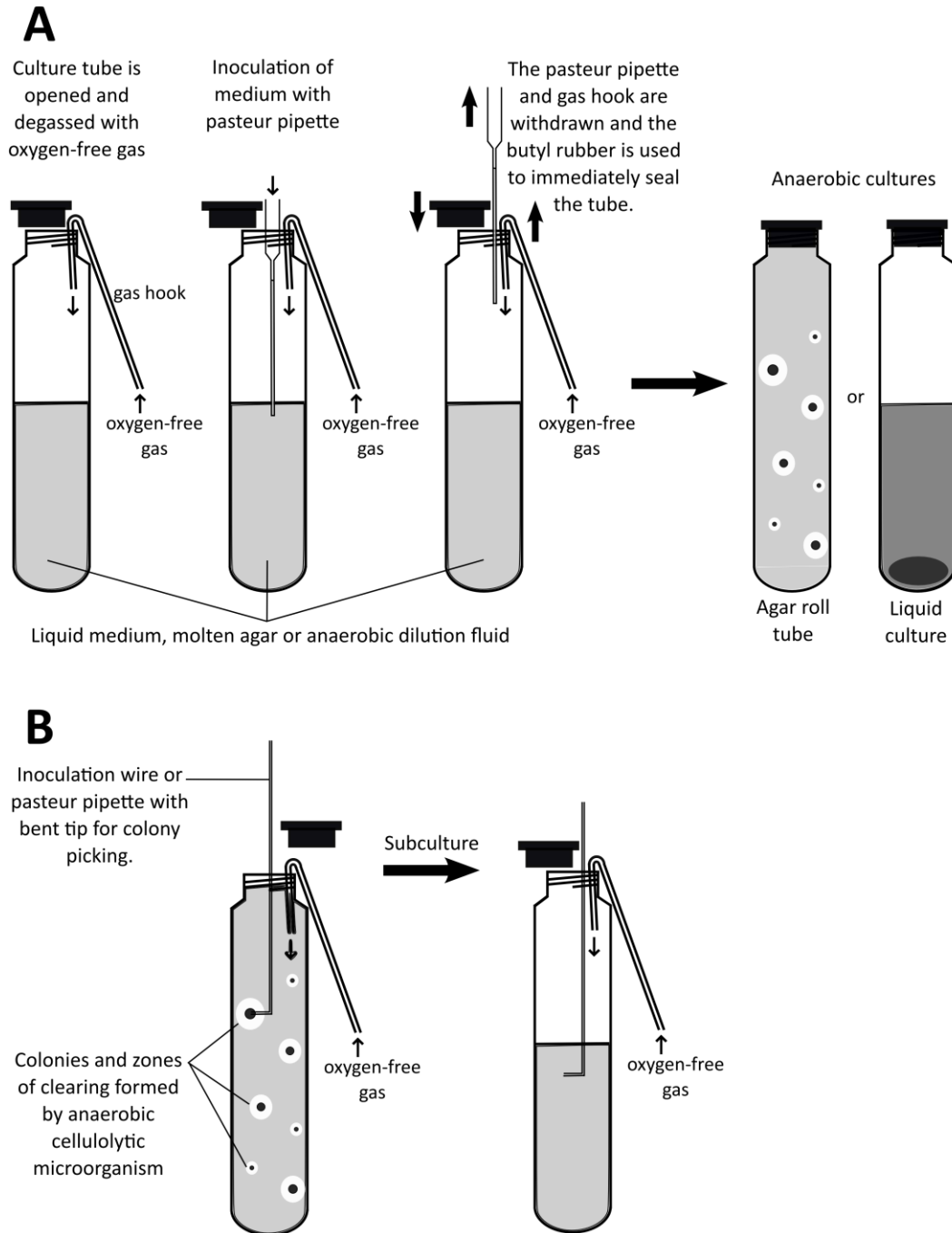


Figure 3.1. The Hungate anaerobic cultivation method demonstrating A) The inoculation of medium with microbial strains, and B) the isolation of individual colonies. From McDonald *et al.* (2012b).

In order to ensure that individual colonies can be picked from agar roll tubes, a dilution series may be required, which is subsequently used to inoculate molten agar roll tubes that have been held in a hot water bath after autoclaving. Any tubes that have a pink colour after autoclaving (due to the Resazurin redox indicator) are discarded, as they are not sufficiently

anaerobic (McDonald *et al.*, 2012b). Once inoculated, the tubes are spun in an ice bath in order to solidify the agar in a film around the outside of the tube. The roll tubes are then incubated and individual colonies can be picked using a sterile inoculating wire and sub-cultured into either sloppy agar or broth medium under a flow of CO₂ gas in order to maintain anaerobic conditions (Fig. 3.1).

Cellulose is an insoluble substrate, but soluble derivatives such as carboxymethyl cellulose (CMC) are also available (McDonald *et al.*, 2012b). The latter are often preferred for isolation and cultivation of cellulolytic microorganisms as growth is more readily visible. However, the degradation of these substrates may not always indicate true cellulolytic activity. CMC is a water soluble derivative of cellulose (Gelman, 1982), which although enables a more accurate visual estimation of growth in liquid and solid media, can also be utilised by organisms that do not possess a full cellulase system, including those that possess endocellulases, exocellulases and processive endocellulases but lack a carbohydrate binding module (CBM) (Wilson, 2011). Another soluble derivative of cellulose is cellobiose, which consists of two glucose molecules bound in alternating rotations. The hydrolysis of cellobiose is mediated by β -glucosidase (Wilson, 2008; Watanabe and Tokuda, 2010) and can also be used by microorganisms that are not capable of growth on crystalline cellulose. However, this does make cellobiose a useful substrate when determining the purity of cellulolytic isolates (McBee, 1950). Insoluble forms of cellulose commonly used as substrates for growth include Avicel, a microcrystalline form of acid-treated cellulose, bacterial cellulose, filter paper and dewaxed cotton string (McDonald *et al.*, 2012b).

Until recently, fibrobacters were thought to exclusively occupy the mammalian intestinal tract, where they represent one of the predominant bacterial degraders of cellulose (Hungate, 1966; Stewart and Bryant, 1988; Kobayashi *et al.*, 2008). Consequently, cultivation-based studies have focused entirely on this environment, and whilst there remains only two characterised species (*F. succinogenes* and *F. intestinalis*), *F. succinogenes* is currently separated into four subspecies (Amann *et al.*, 1992); *F. succinogenes* subsp. *succinogenes* (subgroup one) (Montgomery *et al.*, 1988) and subgroups two, three and four (Amann *et al.*, 1992). Of these four subgroups, *F. succinogenes* subsp. *succinogenes* (subgroup one) is considered to be the most important in the rumen. The use of competitive PCR and restriction fragment length polymorphism (RFLP) analysis on ruminally incubated hay stems demonstrated the presence of subgroups one and three of *F. succinogenes*, with the abundance of subgroup one greater than that of subgroup three (Koike *et al.*, 2004). Shinkai and Kobayashi (2007) utilised fluorescence *in situ* hybridization (FISH) to visualise *F. succinogenes*

subgroups one to three on ruminally incubated hay, with subgroup one and a few cells from subgroup two detected. The greater abundance of *F. succinogenes* subgroup one in comparison to other subgroups and the detection of this group on hay stems which are difficult to degrade, has led to the suggestion that subgroup one is the predominant subgroup of *F. succinogenes* involved in the cellulose hydrolysis that occurs within the rumen (Koiike *et al.*, 2004).

The first specific detection of *Fibrobacter* spp. outside of the gut environment was reported in 2008, when fibrobacters were detected via genus-specific 16S rRNA gene PCR in landfill leachate samples, including leachate from Bidston Moss landfill, the source of landfill leachate for this study (McDonald *et al.*, 2008). Whilst two of the 58 cloned PCR amplicons were identified as *F. succinogenes*, the remaining sequences represented novel species of *Fibrobacter*, with sequences from Bidston Moss residing within a general landfill cluster (containing fibrobacters derived from a variety of geographically distinct landfill sites) and a cluster comprising sequences entirely from this specific landfill site (McDonald *et al.*, 2008). Further phylogenetic analysis demonstrated that within the genus *Fibrobacter*, these landfill species occupied four distinct lineages that represented novel species within the genus *Fibrobacter* as currently defined (Ransom-Jones *et al.*, 2012; McDonald *et al.*, 2008). However, there is evidence to support the separation of *F. succinogenes* and *F. intestinalis* into two distinct genera (Montgomery *et al.*, 1988), suggesting that landfill *Fibrobacter* lineages may in fact represent novel genera of the phylum *Fibrobacteres*, rather than species.

In addition, qPCR targeting the 16S rRNA of cDNA generated from these landfill leachate samples demonstrated that the abundance of *Fibrobacter* 16S rRNA could represent up to 40% of the total bacterial rRNA, and this abundance was greater than the relative abundances of *Fibrobacter* spp. detected in ovine rumen fluid samples (21 to 32%) (McDonald *et al.*, 2008). Subsequently, evidence of a functional role for fibrobacters in cellulose hydrolysis in landfill sites was obtained by the comparison of cellulolytic biofilms from two separate landfill leachate microcosms, one of which contained heavily degraded cotton after 6 weeks incubation, with the second microcosm exhibiting poorly-degraded cotton. qPCR analysis of the cotton-associated biofilm demonstrated that fibrobacters represented 28.9% of the total bacterial 16S rRNA on the heavily degraded cotton, in comparison to the less-degraded cotton sample where *Fibrobacter* species were not detected (McDonald *et al.*, 2012a).

Given the level of diversity contained within the *Fibrobacteres* phylum (Ransom-Jones *et al.*, 2012) it is likely that several novel species/genera exist beyond the rumen, with phylogenetic analysis of the *Fibrobacteres* phylum demonstrating 24 OTUs (95%) that

contained sequences derived solely from natural and managed environments, as demonstrated in chapter 2 (Ransom-Jones *et al.*, 2014). As members of the genus are defined by their cellulolytic activity, and considering their broad ecological range in environments where cellulolysis occurs, the potential role of *Fibrobacter* spp. in cellulose decomposition within the carbon cycle cannot be underestimated. However, the lack of characterised species remains a critical gap in our understanding of the ecology of this phylum. The isolation of these novel environmental species is therefore a priority, with the high relative abundance and diversity of landfill derived fibrobacters implicating this environment as an obvious starting point. This study aims to isolate novel *Fibrobacter* spp. previously detected in Bidston Moss municipal waste landfill (McDonald *et al.*, 2008) via the anaerobic cultivation method of Hungate (Hungate, 1947).

3.2 Materials and methods

3.2.1 Collection of landfill leachate samples

Leachate from Bidston Moss municipal waste landfill site, Wirral, United Kingdom (riser 3F, leachate temperature on collection approximately 41°C) was collected in April 2011 and transported to the laboratory where microcosms were set up within 8 hours of sampling as described below. The remaining leachate was stored at 4°C for processing and use in M2GSC culture medium as described below.

3.2.2 Landfill leachate enrichment culture setup

Microcosms containing a total volume of 35 ml landfill leachate were set up in triplicate in sealed sterile glass bijou bottles. Each microcosm contained landfill leachate and either 0.1%, 1% or 10% (wt/vol) glucose (Sigma) or 0.1%, 1% or 10% (wt/vol) Avicel (Sigma), with a no substrate microcosm (leachate only) as a control. Cysteine hydrochloride at 0.01% (wt/vol) was added to each microcosm to act as a reducing agent. These microcosms were incubated (without shaking) at 41°C for 68 days, after which they were used as a source material for the isolation of *Fibrobacter* species via the Hungate anaerobic cultivation method described below.

3.2.3 Processing of landfill leachate for use in bacterial growth medium

Leachate for use as a growth supplement in M2GSC medium was prepared by straining the leachate through muslin cloth to remove large particles before centrifugation at 5000 rpm for 30 minutes. The supernatant was then removed and filtered through a membrane filter (0.2 µm pore diameter) (Whatman) before autoclaving. The processed fluid was stored at 4°C.

3.2.4 Hungate method for isolating anaerobes

Bacterial strains were isolated using the anaerobic roll tube method of Hungate (Hungate, 1947, Bryant, 1972) and incubated at 41°C. The strains underwent seven rounds of purification with each round as described in (Fig. 3.2). The M2GSC medium described below was prepared anaerobically under an atmosphere of 99.999% CO₂ (BOC gases). Broths and sloppy agar cultures were made in 7.5 ml volumes, whilst roll tubes were made in 4.5 ml volumes and dilution fluid in 9 ml volumes. For subculture into either broth or roll tubes, 0.5 ml of broth/diluting fluid was used as the inoculum. For transfer of colonies from roll tubes and sloppy agars, a wire inoculating loop was used.

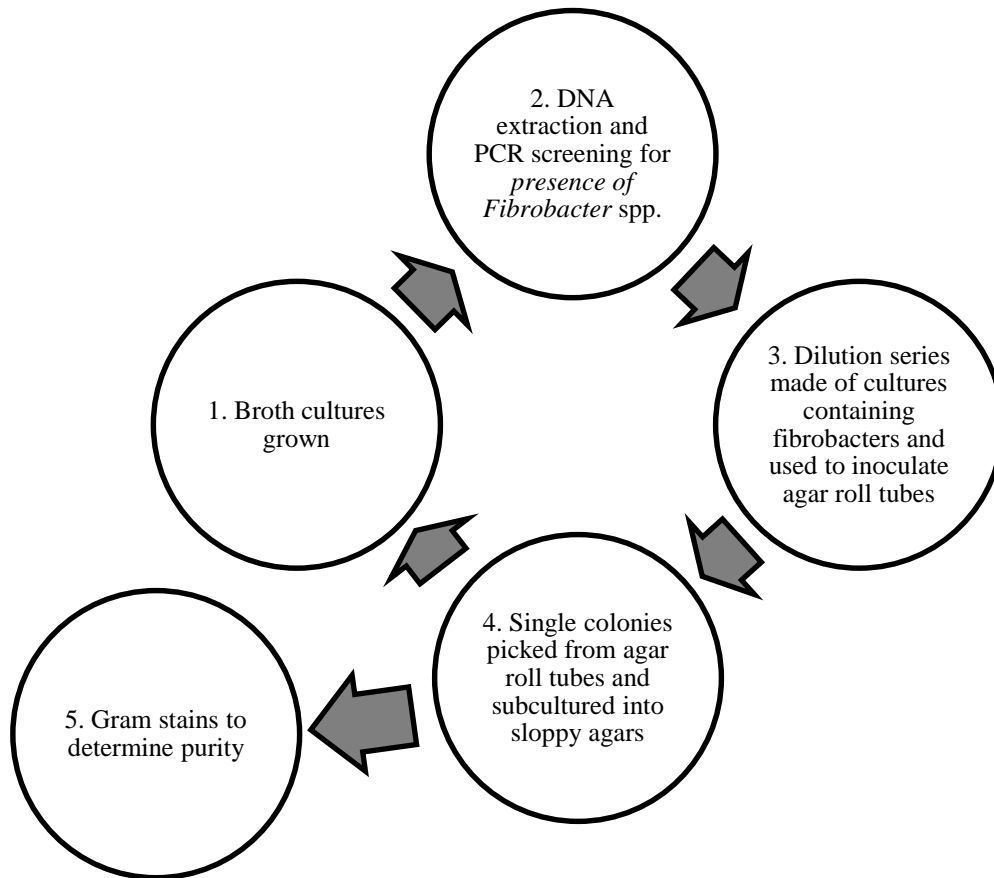


Figure 3.2. Processes contained within one round of strain purification.

3.2.5 Preparation of M2GSC medium

Liquid M2GSC medium was prepared as described by Miyazaki *et al.* (1997) with one modification; landfill leachate was used instead of rumen fluid. The medium contained; 1% (wt/vol) casitone (BD Biosciences), 0.25% (wt/vol) yeast extract (BD Biosciences), 0.4% (wt/vol) NaHCO₃ (Sigma), 0.2% (wt/vol) glucose (Sigma), 0.2% (wt/vol) cellobiose (Sigma), 0.2% (wt/vol) soluble starch (Sigma), 30% (vol/vol) filtered landfill leachate, 0.1% (wt/vol) cysteine hydrochloride (Sigma), 0.045% (wt/vol) K₂HPO₄ (Sigma), 0.045% (wt/vol) KH₂PO₄ (Sigma), 0.09% (wt/vol) (NH₄)₂SO₄ (Sigma), 0.09% (wt/vol) NaCl (Sigma), 0.09% (wt/vol) MgSO₄ (Sigma), 0.09% (wt/vol) CaCl₂ (Sigma), 0.0001% (wt/vol) resazurin (Sigma) and H₂O to the final volume. For sloppy agars, 0.75% (wt/vol) agar (Sigma) was added, whilst roll tubes contained 2% (wt/vol) agar (Sigma). Where carboxymethyl cellulose (CMC) (Sigma) was used as an alternative carbon source, it was added to the medium at a concentration of 0.2% (wt/vol), with glucose, cellobiose and soluble starch omitted. Where cellobiose was used as the sole carbon source, glucose and soluble starch were omitted. For medium that contained no leachate, H₂O was added as an alternative to leachate.

3.2.6 Preparation of dilution fluid for anaerobic cultures

Dilution fluid was used for the serial dilution of cultures (Miyazaki *et al.*, 1997) and contained 1% (wt/vol) casitone (BD Biosciences), 0.25% (wt/vol) yeast extract (BD Biosciences), 0.4% (wt/vol) NaHCO₃ (Sigma), 10% (vol/vol) filtered landfill leachate, 0.1% (vol/vol) Tween 80 (Sigma), 0.1% (wt/vol) cysteine hydrochloride (Sigma), 0.045% (wt/vol) K₂HPO₄ (Sigma), 0.045% (wt/vol) KH₂PO₄ (Sigma), 0.09% (wt/vol) (NH₄)₂SO₄ (Sigma), 0.09% (wt/vol) NaCl (Sigma), 0.09% (wt/vol) MgSO₄ (Sigma), 0.09% (wt/vol) CaCl₂ (Sigma), 0.0001% (wt/vol) resazurin (Sigma) and H₂O to a final volume.

3.2.7 Gram stain of bacterial isolates

Cell suspensions were obtained from culture tubes by inserting a sterile 10 µl inoculation loop into the sloppy agar culture and spreading onto a glass slide. The cells were heat fixed and Gram stained using the Gram Staining Kit (Sigma) according to the manufacturer's instructions before analysis under oil immersion microscopy (x1000 magnification).

3.2.8 Preparation of DNA for PCR via boiling of bacterial cultures

A 1.5 ml volume of broth culture was centrifuged for 5 minutes at 14,000 rpm, the supernatant removed and the cell pellet re-suspended with nuclease free water (Bioline) to a final volume of 100 µl. This suspension was boiled at 100°C in a dry heat block for 10 minutes before 1 µl was used as a template in each PCR reaction.

3.2.9 DNA extraction from bacterial cultures and leachate samples

Leachate samples (50 ml) were filtered through a 0.2 µm pore diameter membrane filter, which was then used for nucleic acid extraction. For extraction from broth cultures, 1.5 ml of the culture was centrifuged at 14,000 rpm for 5 minutes, the supernatant removed and the cell pellet re-suspended with nuclease free water (Bioline) to a final volume of 500 µl. Nucleic acid extraction occurred via the method of Griffiths *et al.* (2000) with the modifications as described in chapter 2. Extracted DNA was visualised via agarose gel electrophoresis on a 1% agarose (Bioline) gel with HyperLadder 1kb (Bioline), and quantified using the Qubit Fluorometer (Life Technologies) and the Qubit dsDNA BR Assay Kit (Life Technologies).

3.2.10 DNA and RNA extraction from bacterial cultures after round seven of purification

Total broth cultures (7.5 ml) were centrifuged at 5000 rpm for 30 minutes, the supernatant removed and the cell pellet re-suspended with nuclease free water (Bioline) to a final volume of 500 µl. DNA extractions were attempted via the methods of Griffiths *et al.* (2000) and the following DNA extraction kits: Isolate Genomic DNA Kit (Bioline), Wizard Genomic DNA Purification Kit (Promega), PowerSoil DNA Isolation Kit (MoBio) and the DNeasy Blood & Tissue Kit (Qiagen). RNA extractions were attempted via the method of Griffiths *et al.* (2000) and the RNeasy Midi Kit (Qiagen), however these were unsuccessful.

3.2.11 Direct and nested PCR targeting the 16S rRNA gene

MyTaq Red Mix (Bioline) was initially trialled due to its increased sensitivity for lower quantities of template DNA and reduced cycling time, however it was found that the SuperTaq enzyme used in previous studies (McDonald *et al.*, 2008; McDonald *et al.*, 2009; McDonald *et al.*, 2012a; Ransom-Jones *et al.*, 2012) and in chapter 2, was more appropriate for the detection of fibrobacters. MyTaq Red Mix was used for PCR up to and including round four of purification, after which SuperTaq was used. For the initial PCR, approximately 10 ng (MyTaq Red Mix) or 50 ng (SuperTaq) of the extracted DNA was used with the direct and nested PCR

protocols as described below. For both enzymes, the PCR products were visualised on a 1% agarose (Bioline) gel with HyperLadder 1kb (Bioline) and stored at -20°C.

3.2.12 PCR amplification using MyTaq Red Mix (Bioline)

Reactions were performed in 50 µl volumes containing 10 ng DNA, 0.2 mM of each primer (Table 3.1), 1 x MyTaq™ Red Mix (Bioline) and nuclease free water to 50 µl. PCR cycling conditions were as follows: initial denaturation at 95°C for 1 minute, 35 cycles of 95°C for 15 seconds, 15 seconds at the annealing temperature required by the primer set (Table 3.1) and 72°C for 10 seconds.

3.2.13 PCR using amplification SuperTaq (Cambio)

Each reaction (total volume 50 µl) contained 0.2 mM each primer (Table 3.1), 0.2 mM each dNTP, 1x SuperTaq Buffer (Cambio), 0.5 mM MgCl₂, 1x BSA, 1 unit SuperTaq (Cambio), 50 ng DNA and nuclease free water (Bioline). PCR reactions using the *Fibrobacter* primer set (Fib 1F and Fib 2AR, Table 3.1) contained an increased concentration of each primer (0.4 mM) and MgCl₂ (1.5 mM). The PCR cycling conditions were: initial denaturation at 94°C for 5 minutes, 35 cycles of 94°C for 1 minute, 1 minute at the specific annealing temperature for each primer set (Table 3.1) and an extension of 72°C for 1.5 minutes. The final extension was performed at 72°C for 10 minutes.

Table 3.1. 16S rRNA gene primers used for PCR and sequencing.

a. Ambiguities: K=(GorT), S=(GorC), W=(AorT), Y=(CorT), H=(A,CorT), R=(AorG), D=(G,AorT), V=(A,CorG).

b. Primer used for sequencing.

c. Annealing temperature used with SuperTaq (Cambio).

d. Annealing temperature used with MyTaq Red Mix (Bioline).

| Primers | Sequence (5' - 3') ^a | Specificity | Annealing Temperature (°C) | Amplicon Size (bp) | Reference |
|--------------|---------------------------------|--------------------------|----------------------------|--------------------|---------------------------------|
| pA | AGAGTTTGATCCTGGCTCAG | General Bacteria | 55 ^c | ~ 1534 | (Edwards <i>et al.</i> , 1989) |
| pH' | AAGGAGGTGATCCAGCCGCA | | 53 ^d | | |
| Fib 1F' | CCGKSCCAACGSSCGG | <i>Fibrobacter</i> genus | 60 ^c | ~ 855 | (McDonald <i>et al.</i> , 2008) |
| Fib 2AR | ATCTCTCGCYGCGGCGWTYCC | | 58 ^d | | |
| M13 Forward' | GTTTTCCCAGTCACGAC | M13 Vector | n/a | n/a | (Messing, 1983) |

3.2.14 Cloning and sequencing of *Fibrobacter*-specific PCR amplicons

PCR amplification products were excised from a 1% agarose (Bioline) gel and purified using the Bioline Isolate Gel Extraction Kit (Bioline) following the manufacturer's protocol. The purified PCR products were ligated and cloned into competent *E. coli* JM109 (Promega) using the pGEM-T Easy Vector System I (Promega) according to the manufacturer's instructions. The plasmid DNA was extracted and purified using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced using the M13 forward primer (Table 3.1) by GATC Biotech.

3.1.15 Phylogenetic analysis of *Fibrobacter* 16S rRNA gene sequences

Sequences from this study and cultivated *Fibrobacter* species from the Ribosomal Database Project website (Cole *et al.*, 2007; Cole *et al.*, 2009) were aligned using the MUSCLE aligner (Edgar, 2004), trimmed and used to generate a neighbour-joining tree with 1000 bootstrap samplings via PhyML (version 3.0) (Guindon *et al.*, 2010) which was viewed using Interactive Tree of Life (Letunic and Bork, 2007). Bootstrap values of >95% are marked by a filled circle on the node, bootstrap values between 75 and 95% are marked with an unfilled circle. The scale bar represents 0.1 base substitutions per nucleotide.

3.3 Results

3.3.1 Molecular detection of fibrobacters in landfill leachate microcosms

Prior to the construction of the landfill leachate microcosms, DNA extracted from 50 ml of the landfill leachate collected to set up the microcosms was filtered through a 0.2 µm pore diameter membrane filter and *Fibrobacter*-specific nested PCR confirmed the presence of fibrobacters in the leachate sample. After 68 days of incubation, all microcosms (excluding those containing 10% (wt/vol) Avicel) were filtered through 0.2 µm membrane filters, whilst the microcosms containing 10% (wt/vol) Avicel were vortexed, 1.5 ml of the liquid and Avicel suspension removed and prepared for extraction as described above for broth cultures (section 3.2.9). Both filters and cell pellets were subjected to DNA extraction using the method of Griffiths *et al.* (2000) and the extracts screened for the presence of fibrobacters via the use of *Fibrobacter* specific PCR primers (Table 3.1) and MyTaq Red Mix. No *Fibrobacter* amplification products were obtained via direct PCR, but all microcosms were positive for *Fibrobacter* spp. via nested PCR (Table 3.2).

Table 3.2. Detection of *Fibrobacter* spp. in landfill leachate samples and microcosms via PCR with genus specific 16S rRNA gene primers.

| Sample/microcosm | Direct <i>Fibrobacter</i> PCR result | Nested <i>Fibrobacter</i> PCR result |
|----------------------------|---|---|
| Landfill leachate inoculum | - | + |
| Microcosms: | | |
| No substrate control | - | + |
| 0.1% (wt/vol) glucose | - | + |
| 1% (wt/vol) glucose | - | + |
| 10% (wt/vol) glucose | - | + |
| 0.1% (wt/vol) Avicel | - | + |
| 1% (wt/vol) Avicel | - | + |
| 10% (wt/vol) Avicel | - | + |

3.3.2 First round of isolation using the Hungate anaerobic culture method

Landfill leachate microcosms containing Avicel were used as the source material for the inoculation of Hungate roll tubes containing M2GSC and leachate, as it was predicted that due to Avicel being the sole carbon source for microbial growth, there would be a greater chance of isolating true cellulolytic species such as *Fibrobacter*. A total of 63 individual colonies were picked from agar roll tubes; 18 from microcosms containing 0.1% (wt/vol) Avicel (strains A1-A9 and B1-B9), 14 from 1% (wt/vol) Avicel (strains C1-C9 and D1-D5) and 31 from 10% (wt/vol) Avicel (strains D6-D9, E1-E9, F1-F9 and G1-G9). *Fibrobacter*-specific PCR with MyTaq Red Mix (Bioline) on boil preps of these cultures detected five isolates/cultures that contained *Fibrobacter* species via direct PCR; cultures A7, B2, B4, D2 and E4.

3.3.3 Second round of isolation via the Hungate anaerobic culture method

Subsequently, the five strains testing positive for *Fibrobacter* after the initial round of isolation (A7, B2, B4, D2 and E4) were subjected to a second round of purification in Hungate agar roll tubes containing M2GSC medium and leachate. Each of the roll tubes contained only one colony morphology type for each culture (Table 3.3), with individual colonies selected due to sufficient separation to reduce the risk of contamination and picked from roll tubes containing approximately 30 to 300 colonies, before transfer to individual sloppy agar cultures.

Gram stains of the sloppy agar cultures from this round of purification showed that all cultures contained a mixture of Gram-negative and different sized Gram-positive rods, as the cultures were not pure.

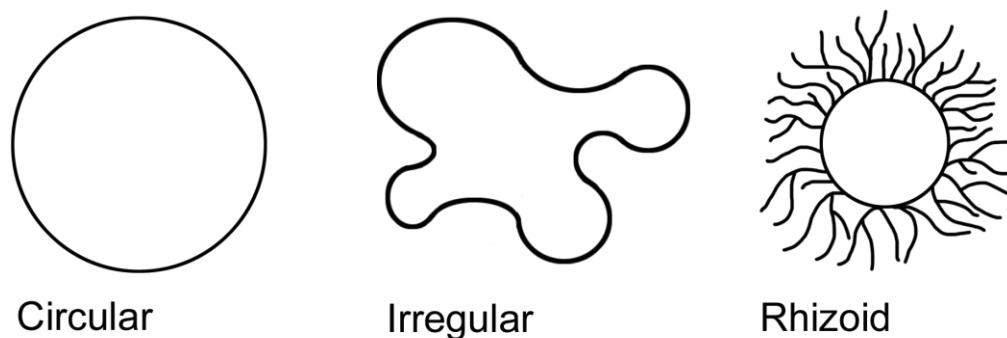


Figure 3.3. Colony morphologies as seen on agar roll tubes.

Table 3.3. Colony morphologies (as described in Fig. 3.3) present in cultures from round one of purification via the Hungate roll tube method.

| Culture | Size | Form | Colour |
|---------|--------|-----------|--------|
| A7 | Medium | Rhizoid | White |
| B2 | Medium | Irregular | White |
| B4 | Small | Irregular | White |
| D2 | Small | Circular | White |
| E4 | Small | Circular | White |

3.3.4 Testing media supplementation and substrate utilisation of isolates from the second round of purification

Although the leachate used to supplement the bacterial growth medium was filtered through a 0.2 µm pore membrane filter and autoclaved with the medium before inoculation with the isolated strains, it was conceivable that the endospores typically associated with Gram-positive bacteria were still present in the medium, thus resulting in contamination of the cultures. In order to determine whether or not these isolated strains required leachate for growth, and therefore potentially avoid this issue by removing leachate from the medium,

strains A7, B2, B4, D2 and E4 were grown in M2GSC both containing leachate, and with distilled water substituted for the leachate. Visible growth was observed in both media demonstrating that leachate was not required by these strains for growth.

Strains A7, B2, B4, D2 and E4 were also grown in M2GSC medium containing leachate, with the following substrates provided as the sole carbon sources for growth; cellobiose, Avicel, dewaxed cotton string and filter paper. Visible growth occurred on all substrates as determined by turbidity in the medium and visible degradation of the filter paper and dewaxed cotton string (with the exception of Avicel) (Table 3.4), proving that at least one of the organisms in these cultures is capable of cellulose hydrolysis. Whilst bacterial growth may certainly have occurred in the medium containing Avicel, this was difficult to determine due to the insoluble nature of the substrate.

Table 3.4. Growth of cultures in media containing different substrates as the sole carbon source as determined via visual examination. ND - not determined.

| Culture | Cellobiose | Avicel | Dewaxed cotton string | Filter paper |
|---------|------------|--------|--------------------------|--------------|
| A7 | + | ND | + | + |
| B2 | + | ND | + | + |
| B4 | + | ND | + | + |
| D2 | + | ND | + | + |
| E4 | + | ND | + | + |

3.3.5 16S rRNA gene sequencing of *Fibrobacter* specific PCR amplicons

PCR amplicons of strains A7, B4, and E4 generated with the *Fibrobacter* specific 16S rRNA gene primers (Table 3.1) and MyTaq Red Mix were cloned and sequenced with the M13 forward primer (Table 3.1). *Fibrobacter* specific 16S rRNA gene PCR amplicons generated from strains B2 and D2 were unable to be sequenced as the products were lost during the gel purification step. The sequences obtained showed that whilst strains A7 and B4 were 99% similar to *F. succinogenes*, strain E4 had a similarity of 97%, with strains A7 and E7 most closely related to fibrobacters previously detected on colonised cotton incubated in Esthwaite water lake (Table 3.5). This lake sequence was identified as a member of *F.*

succinogenes along with two previous landfill sequences, supporting the assertion that *F. succinogenes* is present outside of the mammalian intestinal tract (McDonald *et al.*, 2009).

Table 3.5. Blast matches of *Fibrobacter* 16S rRNA gene PCR amplicon sequences (~852bp)

| Strain | Closest match via NCBI Blast | Percentage similarity |
|--------|---|-----------------------|
| A7 | Uncultured <i>Fibrobacter</i> sp. clone EW-C-3.0-1 16S ribosomal RNA gene, partial sequence (lake) | 99 |
| B4 | <i>Fibrobacter succinogenes</i> subsp. <i>succinogenes</i> S85, complete genome (rumen) | 99 |
| E4 | Uncultured <i>Fibrobacter</i> sp. clone EW-C-3.0-1 16S ribosomal RNA gene, partial sequence (lake) | 97 |

3.3.6 Third round of isolation via the Hungate anaerobic culture method

The cultures of strains A7, B2, B4, D2 and E4 containing filter paper as the sole carbon source for growth were used as the inoculum for the third round of purification in M2GSC medium containing leachate and cellobiose as the sole carbon source. The filter paper cultures were utilised in order to select for cellulolytic species such as *Fibrobacter*, as although utilising inoculum from the Avicel cultures would also have enabled this, the amount of biomass present in the broth cultures was difficult to determine due to the insoluble nature of this substrate. This would also have made isolating single colonies in agar roll tubes difficult. From these five strain cultures, a total of seven colonies were isolated, A71, A72, B21, B22, B41, D21 and E41. Gram stains of these cultures suggested that none of the stains were pure, with all strains containing a mixture of different sized Gram-negative rods.

3.3.7 Fourth round of isolation via the Hungate anaerobic culture method

The seven cultures from the previous round of purification (strains A71, A72, B21, B22, B41, D21 and E41) were inoculated into roll tubes containing water instead of leachate and cellobiose as the sole carbon source, as the cultures were able to grow in the absence of leachate, as reported in 3.3.4 and the substitution with water removed any potential contamination of the cultures via spores present in the leachate. Of the 20 individual colonies selected for isolation due to varying morphology and sufficient separation (Table 3.6), three

were positive for *Fibrobacter* species via direct PCR with MyTaq Red Mix and the *Fibrobacter* specific 16S rRNA gene primer set (Table 3.1), cultures A711, A721 and B211. Gram stains of these cultures revealed that the cultures were not pure, with A711 containing a mixture of small Gram-negative ovoid cells and short, Gram-positive rods, and B211 containing a mixture of Gram-negative short rods and small ovoid cells (A721 did not stain). The presence of Gram-positive cells is interesting as the parent cultures were all Gram-negative, but this could be due to experimental stain variation or the presence of *C. botulinum* (Table 3.7) which can stain Gram variable (Brazier *et al.*, 2002).

Table 3.6. Morphologies (as described in Fig. 3.3) of cultures from round four of purification via the Hungate roll tube method.

| Parent culture name | Isolate | Size | Form | Colour |
|---------------------|---------|--------|----------|-------------|
| A71 | A711 | Small | Circular | White |
| | A712 | Medium | Rhizoid | White |
| | A713 | Medium | Rhizoid | Cream |
| | A714 | Medium | Rhizoid | Cream |
| A72 | A721 | Medium | Rhizoid | White |
| B21 | B211 | Medium | Rhizoid | White |
| | B212 | Medium | Rhizoid | White |
| | B213 | Medium | Rhizoid | White |
| | B214 | Small | Circular | White |
| B22 | B221 | Medium | Rhizoid | White |
| B41 | B411 | Small | Circular | White |
| D21 | D211 | Medium | Circular | White |
| | D212 | Medium | Rhizoid | Pale yellow |
| | D213 | Medium | Rhizoid | White |
| | D214 | Medium | Rhizoid | Pale yellow |
| E41 | E411 | Medium | Circular | Cream |
| | E412 | Medium | Rhizoid | White |
| | E413 | Medium | Rhizoid | White |
| | E414 | Medium | Rhizoid | White |
| | E415 | Small | Circular | White |

3.3.8 Sequencing of 16SrRNA gene PCR amplicons

General bacterial 16S rRNA gene PCR amplification products from strains A711, A721 and B211 were gel extracted, cloned and sequenced with the M13 forward primer (Table 3.1) generating sequences of approximately 852 bp. Despite a positive *Fibrobacter*-specific direct PCR result on DNA extracted from all of these cultures, of the twelve clones sequenced for each strain, none were related to *Fibrobacter* spp. when blasted against the NCBI nucleotide database (Table 3.7). Sequences for strain A711 were a mixture of *Bacillus thermoamylovorans* and *Clostridium botulinum*, whilst the sequences for B211 were all *B. thermoamylovorans* (Table 3.7). Further sequencing of amplicons from PCRs using the *Fibrobacter* specific primer

set (Table 3.1) confirmed the presence of fibrobacters in these cultures, and determined that these strains were 99% similar to *F. succinogenes* (Table 3.8), and specifically were most closely related to *F. succinogenes* sequences previously detected on colonised cotton baits in Esthwaite water, as observed in round 2 of purification (Table 3.5). Phylogenetic analysis of these sequences demonstrated that all of these isolates formed a cluster within *F. succinogenes* subgroup one, supported by a bootstrap value of 95% (Fig. 3.4).

Table 3.7. Blast matches of general bacterial clone library sequences.

| Strain | Clone number | Closest match via NCBI Blast | Percentage similarity |
|--------|--------------|--|-----------------------|
| A711 | 1 | <i>Bacillus thermoamylovorans</i> strain NBY26 16S ribosomal RNA gene, partial sequence | 98 |
| | 2 | <i>Bacillus thermoamylovorans</i> gene for 16S rRNA, partial sequence, strain: BHK180-4 | 99 |
| | 3 | <i>Bacillus thermoamylovorans</i> strain N12-2 16S ribosomal RNA gene, partial sequence | 99 |
| | 4 | <i>Bacillus thermoamylovorans</i> gene for 16S rRNA, partial sequence, strain: BHK180-3 | 99 |
| | 5 | <i>Bacillus thermoamylovorans</i> strain N12-2 16S ribosomal RNA gene, partial sequence | 97 |
| | 6 | <i>Bacillus thermoamylovorans</i> gene for 16S rRNA, partial sequence, strain: BHK180-4 | 99 |
| | 7 | <i>Bacillus</i> spp. R-31297 partial 16S rRNA gene, strain R-31297 | 99 |
| | 8 | <i>Bacillus thermoamylovorans</i> gene for 16S rRNA, partial sequence, strain: BHK180-4 | 99 |
| | 9 | <i>Bacillus thermoamylovorans</i> strain N12-2 16S ribosomal RNA gene, partial sequence | 98 |
| | 10 | <i>Bacillus thermoamylovorans</i> gene for 16S rRNA, partial sequence, strain: BHK180-4 | 99 |
| | 11 | <i>Clostridium botulinum</i> H04402 065, complete genome sequence | 99 |

| | | | |
|------|----|--|----|
| | 12 | <i>Clostridium botulinum</i> str. ATCC 3502 | 99 |
| | | complete genome | |
| A721 | 1 | <i>Clostridium botulinum</i> H04402 065, | 99 |
| | | complete genome sequence | |
| | 2 | <i>Clostridium botulinum</i> H04402 065, | 99 |
| | | complete genome sequence | |
| | 3 | <i>Clostridium botulinum</i> H04402 065, | 99 |
| | | complete genome sequence | |
| | 4 | <i>Clostridium botulinum</i> F str. 230613, | 99 |
| | | complete genome | |
| | 5 | <i>Clostridium botulinum</i> H04402 065, | 99 |
| | | complete genome sequence | |
| | 6 | <i>Clostridium botulinum</i> H04402 065, | 99 |
| | | complete genome sequence | |
| | 7 | <i>Clostridium botulinum</i> H04402 065, | 99 |
| | | complete genome sequence | |
| | 8 | <i>Clostridium botulinum</i> H04402 065, | 99 |
| | | complete genome sequence | |
| | 9 | <i>Clostridium botulinum</i> H04402 065, | 99 |
| | | complete genome sequence | |
| | 10 | <i>Clostridium botulinum</i> H04402 065, | 99 |
| | | complete genome sequence | |
| | 11 | <i>Clostridium botulinum</i> H04402 065, | 99 |
| | | complete genome sequence | |
| | 12 | <i>Clostridium botulinum</i> H04402 065, | 99 |
| | | complete genome sequence | |
| B211 | 1 | <i>Bacillus thermoamylovorans</i> strain N12-2 | 99 |
| | | 16S ribosomal RNA gene, partial sequence | |
| | 2 | <i>Bacillus thermoamylovorans</i> gene for 16S | 99 |
| | | rRNA, partial sequence, strain: BHK180-4 | |
| | 3 | <i>Bacillus thermoamylovorans</i> strain N12-2 | 99 |
| | | 16S ribosomal RNA gene, partial sequence | |
| | 4 | <i>Bacillus thermoamylovorans</i> strain N12-2 | 99 |
| | | 16S ribosomal RNA gene, partial sequence | |

| | | |
|----|--|----|
| 5 | <i>Bacillus thermoamylovorans</i> gene for 16S rRNA, partial sequence, strain: BHK180-4 | 99 |
| 6 | <i>Bacillus thermoamylovorans</i> strain N12-2 16S ribosomal RNA gene, partial sequence | 99 |
| 7 | <i>Bacillus thermoamylovorans</i> strain N12-2 16S ribosomal RNA gene, partial sequence | 99 |
| 8 | <i>Bacillus thermoamylovorans</i> strain N12-2 16S ribosomal RNA gene, partial sequence | 99 |
| 9 | <i>Bacillus thermoamylovorans</i> strain N12-2 16S ribosomal RNA gene, partial sequence | 99 |
| 10 | <i>Bacillus thermoamylovorans</i> gene for 16S rRNA, partial sequence, strain: BHK180-3 | 99 |
| 11 | <i>Bacillus thermoamylovorans</i> strain N12-2 16S ribosomal RNA gene, partial sequence | 99 |
| 12 | <i>Bacillus thermoamylovorans</i> strain N12-2 16S ribosomal RNA gene, partial sequence | 99 |

Table 3.8. Blast matches of 16S rRNA gene sequences generated using the *Fibrobacter* genus specific primer set.

| Strain | Clone number | Closest match via NCBI Blast | Percentage similarity |
|--------|--------------|---|-----------------------|
| A711 | 1 | Uncultured <i>Fibrobacter</i> spp. clone EW-C-3.0-1 16S ribosomal RNA gene | 99 |
| A721 | 1 | Uncultured <i>Fibrobacter</i> spp. clone EW-C-SED-4 16S ribosomal RNA gene | 99 |
| B211 | 1 | Uncultured <i>Fibrobacter</i> spp. clone EW-C-SED-4 16S ribosomal RNA gene | 99 |
| B211 | 2 | Uncultured <i>Fibrobacter</i> spp. clone EW-C-SED-4 16S ribosomal RNA gene | 99 |

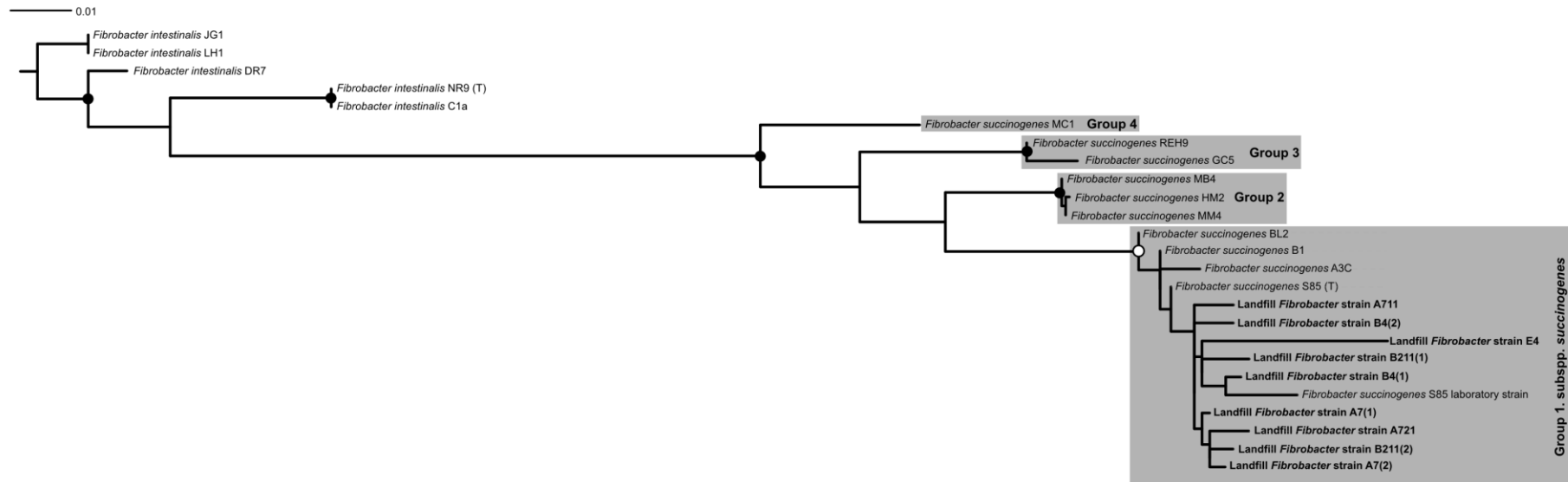


Figure 3.4. Phylogeny of *Fibrobacter* isolates from landfill sites.

Isolates from this study are in bold. Bootstrap values of >95% are marked by a filled circle on the node, bootstrap values between 75 and 95% are marked with an unfilled circle. The scale bar represents 0.1 base substitutions per nucleotide.

3.3.9 Fifth round of isolation via the Hungate anaerobic culture method

The three strains containing fibrobacters (A711, A721 and B211) were sub-cultured in M2GSC roll tubes containing water and cellobiose as the sole carbon source. A total of 8 colonies were picked, strains A711A, A711B, A711C, A721A, A721B, B211A, B211B and B211C. DNA extracted from these cultures was subjected to PCR with SuperTaq, as although the sequenced MyTaq products were fibrobacters, there had been some false positives given when this enzyme had been used in other experiments (data not shown). No direct positives were obtained with the *Fibrobacter* specific primer set (Table 3.1), however nested PCRs on strains A711C and B211C were positive for the presence *Fibrobacter* spp. (Table 3.9).

Table 3.9. Detection of *Fibrobacter* spp. in cultures via PCR with genus specific 16S rRNA gene primers.

| Sample | Direct <i>Fibrobacter</i> PCR result | Nested <i>Fibrobacter</i> PCR result |
|--------|---|---|
| A711A | - | - |
| A711B | - | - |
| A711C | - | + |
| A721A | - | - |
| A721B | - | - |
| B211A | - | - |
| B211B | - | - |
| B211C | - | + |

3.3.10 Sixth round of isolation via the Hungate anaerobic culture method

Strains A711C and B211C were sub-cultured in M2GSC roll tubes containing cellobiose as the sole carbon source and no leachate. A total of 8 colonies were picked, strains A711C1, A711C2, A711C3, A711C4, B211C1, B211C2, B211C3 and B211C4. Gram stains showed that these strains were not pure.

3.3.11 Testing the growth of isolated landfill fibrobacters in culture medium supplemented with landfill leachate or rumen fluid

Sequencing of the *Fibrobacter* specific 16S rRNA gene PCR amplicons demonstrated that these strains were 99% similar to *F. succinogenes*. In order to ascertain whether these

strains were landfill adapted *F. succinogenes*, or if they were simply rumen strains, A711C and B211C from the fifth round of purification were grown in M2GSC medium containing cellobiose as the sole carbon source, and supplemented with either rumen fluid, landfill leachate or distilled water. Roll tubes of the two stains were created using each of the three media and incubated for 48 hours at 41°C. For both strains, there was no discernable difference in the colony counts from any of the three media supplementations, although this was not conducted in triplicate (Table 3.10).

Table 3.10. Colony counts for strain dilution series in roll tubes supplemented with rumen fluid, landfill leachate or distilled water. TMTC- Too many to count.

| Strain | Dilution | Colony count (leachate) | Colony count (rumen fluid) | Colony count (water) |
|--------|------------------|----------------------------|-------------------------------|-------------------------|
| A711C | 10 ⁻¹ | TMTC | TMTC | TMTC |
| | 10 ⁻² | TMTC | TMTC | TMTC |
| | 10 ⁻³ | 14 | TMTC | 17 |
| | 10 ⁻⁴ | 5 | 2 | 2 |
| | 10 ⁻⁵ | 0 | 0 | 0 |
| | 10 ⁻⁶ | 0 | 0 | 0 |
| B211C | 10 ⁻¹ | TMTC | TMTC | TMTC |
| | 10 ⁻² | TMTC | TMTC | TMTC |
| | 10 ⁻³ | 46 | TMTC | 27 |
| | 10 ⁻⁴ | 3 | 3 | 4 |
| | 10 ⁻⁵ | 0 | 0 | 0 |
| | 10 ⁻⁶ | 0 | 0 | 0 |

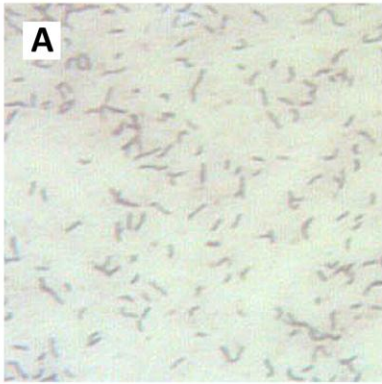
3.3.12 Seventh round of isolation via the Hungate anaerobic culture method

Strains A711C1, A711C2, A711C3, A711C4, B211C1, B211C2, B211C3 and B211C4 were purified in M2GSC medium with water substituted for leachate and cellobiose as the sole carbon source and selected due to variable morphology (Table 3.11), with Gram stains suggesting that some of these strains were pure (Fig. 3.5). All of the isolated strains from round seven of purification were Gram-negative rods and with the impure strains A711C2, A711C3,

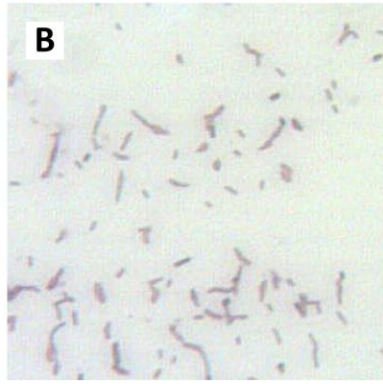
B211C1 and B211C3 containing a mixture of long and short rods (Fig. 3.5). All strains were able to grow on cellobiose as the sole carbon source and so strains A711C1, A711C4, B211C2 and B211C4 possess at least the enzymes required to hydrolyse β -1, 4 glycosidic bonds. Extraction of either DNA or RNA via any of the methods attempted was not possible from broth cultures and possibly reflects a lack of microbial biomass in the broth cultures, which had an incubation time of two months. In addition, attempts to subculture the strains from the sloppy agar cultures were unsuccessful suggesting that although the culture had grown sufficiently to enable Gram staining, it had subsequently died.

Table 3.11. Morphologies (as described in Fig. 3.3) of colonies from round seven of purification via the Hungate roll tube method.

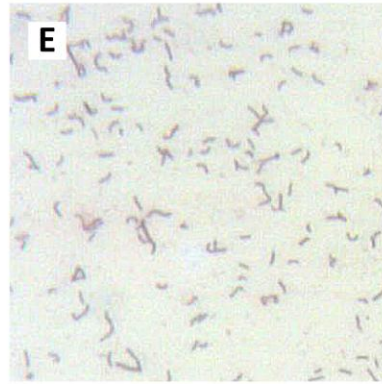
| Isolate name | Size | Form | Colour |
|--------------|-------|-----------|--------|
| A711C1 | Small | Circular | White |
| A711C2 | Small | Circular | Black |
| A711C3 | Large | Irregular | Cream |
| A711C4 | Large | Irregular | White |
| B211C1 | Small | Circular | White |
| B211C2 | Small | Circular | White |
| B211C3 | Small | Circular | White |
| B211C4 | Small | Circular | White |



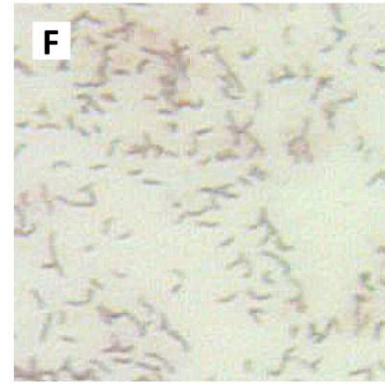
A. Strain A711C1. Gram negative rods, pure culture.



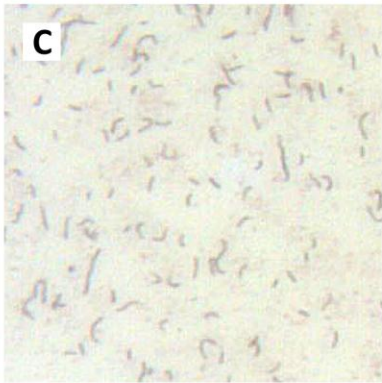
B. Strain A711C2. Gram negative rods, impure culture.



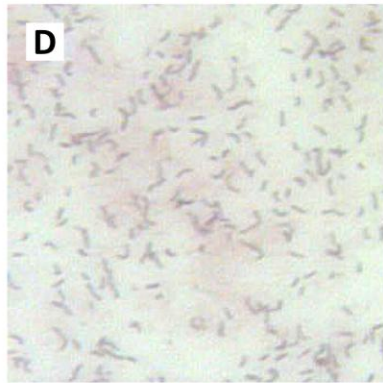
E. Strain B211C1. Gram negative rods, impure culture.



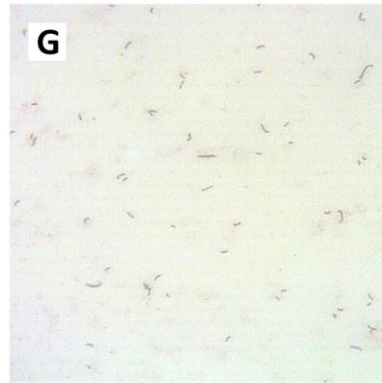
F. Strain B211C2. Gram negative rods, pure culture.



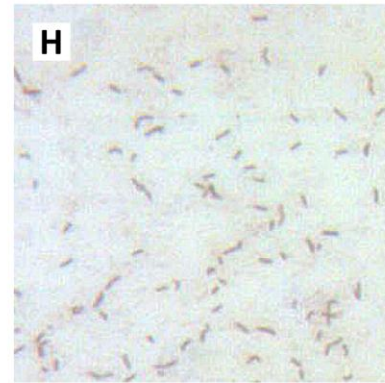
C. Strain A711C3. Gram negative rods, impure culture.



D. Strain A711C4. Gram negative rods, pure culture.



G. Strain B211C3. Gram negative rods, impure culture.



H. Strain B211C4. Gram negative rods, pure culture.

Figure 3.5. Gram stains of isolated strains from the seventh round of purification, viewed under oil immersion microscopy (x1000).

3.4 Discussion

Despite the fact that *F. succinogenes* (then *Bacteroides succinogenes*) was first isolated over 60 years ago from the bovine rumen (Hungate, 1947, Hungate, 1950), there are still only two characterised species contained within the phylum (Montgomery *et al.*, 1988). Due to the importance of *F. succinogenes* in the rumen, where it is considered to be one of the main cellulolytic bacterial species (Hungate, 1966; Stewart and Bryant, 1988; Kobayashi *et al.*, 2008), and the assumption that fibrobacters were restricted to the gut environment, attempts to cultivate members of this phylum have been limited to the mammalian intestinal tract. Of the 63 originally colonies isolated here, five were positive for the presence of *Fibrobacter* spp. via PCR using genus specific 16S rRNA gene primers, with the cultures containing these species comprising 7.9% of the first round of isolates. Previous cultivation based studies focusing on the rumen yielded few isolates of *F. succinogenes* despite the abundance of this species in the rumen, with only 2 of 214 (0.93%) (Nyonyo *et al.*, 2013) and 3 of 129 (2.3%) (Nyonyo *et al.*, 2014) bacterial isolates belonging to the *Fibrobacteres* phylum.

The inoculation source may determine the ease by which fibrobacters are isolated. In a study that isolated 339 bacterial strains from either rumen fluid, or hay that had been incubated in the rumen, 32 (9%) were *F. succinogenes*. Of these isolates, 30 were obtained from cultures that used the ruminally incubated hay as an inoculation source, compared to the 2 isolated from the rumen fluid (Shinkai *et al.*, 2009), suggesting that the fibrobacters were enriched on the solid substrate, thus making it a more efficient isolation source. Whilst this study utilised landfill leachate microcosms enriched with Avicel as an inoculation source, the use of filter paper or dewaxed cotton string may be a better alternative. The *Fibrobacter* spp., which bind rapidly to the substrate (Koike *et al.*, 2003), would thus be present in higher numbers on the cotton or filter paper in comparison with other organisms present., enabling easier transfer of fibrobacters from one culture to another as well as clearer visualisation of degradation

All of the *Fibrobacter* spp. isolated from landfill within this study are contained within *F. succinogenes* subsp. *succinogenes* (supgroup 1) (Fig. 3.4), the main subgroup involved in cellulolytic activity in the rumen (Kobayashi *et al.*, 2008; Shinkai and Kobayashi, 2007; Shinkai *et al.*, 2009; Koike *et al.*, 2004) and it is therefore likely that these landfill isolates are also active members of the cellulolytic community. This is further supported by previous work, which demonstrated via qPCR that fibrobacters comprised 28.9% of the total bacterial rRNA from the biofilm of a heavily degraded cotton sample, higher than that of *Clostridium* spp., compared to the poorly degraded cotton sample, where *Fibrobacters* were undetectable (McDonald *et al.*, 2012a). The degree of similarity of the isolated strains to *F. succinogenes* is

intriguing given that previous studies on landfill sites have demonstrated the presence of novel *Fibrobacter* populations (McDonald *et al.*, 2008). However, *F. succinogenes* has been detected in landfill sites (McDonald *et al.*, 2008) and on colonised cotton baits deployed in freshwater lakes (McDonald *et al.*, 2009).

Furthermore, the generation of PCR contamination from our *Fibrobacter* PCR control material is also unlikely given that negative PCR controls consistently demonstrated no contaminants in the PCR master mix, only a handful of cultures tested positive in some rounds of amplification, and sequence analysis demonstrated that there were 11 base pair mismatches between the *F. succinogenes* S85 DNA used as the control in PCR reactions and the most closely related isolate (B4(1)) in the 852 base pair alignment used to construct the phylogenetic tree. It therefore is unlikely that the detection of *F. succinogenes* in the cultures is due to PCR contamination. One explanation for the detection of *F. succinogenes* strains rather than the novel fibrobacters previously detected using molecular methods (McDonald *et al.*, 2008) could be that the medium used was designed for the isolation of anaerobes from the rumen, and so isolates related to *F. succinogenes* may have been inadvertently selected for. In later rounds of strain purification, qPCR quantification of *Fibrobacter* abundance in the cultures was attempted, but it was not possible to retrieve suitable quantities of DNA for qPCR analysis.

Modifications to the culture medium may enable the isolation of novel *Fibrobacter* spp. The use of a different gelling agent, such as phytigel or gellan gum instead of agar, has enabled the isolation of previously uncultivated rumen bacteria (Nyonyo *et al.*, 2013; Nyonyo *et al.*, 2014), along with the removal of potassium phosphate and the addition of magnesium chloride (Nyonyo *et al.*, 2013). Although *F. succinogenes* does not possess any motility genes it does exhibit a motility mechanism similar to that of *Cytophaga hutchinsonii* (Suen *et al.*, 2011). The modification of the media via either the reduction (Shinkai *et al.*, 2009) or change (Nyonyo *et al.*, 2013; Nyonyo *et al.*, 2014) of gelling agent may therefore be particularly helpful in enabling the bacteria to migrate through the agar and access the substrate, thus improving the isolation of *Fibrobacter* spp.

The lack of *Fibrobacter* sequences from the general bacterial 16S rRNA general clone library, despite a positive PCR result with the *Fibrobacter* specific primer set, is unsurprising given that a number of 16S rRNA gene clone library studies on ruminants have also not generated any *Fibrobacter* sequences (Whitford *et al.*, 1998; Daly *et al.*, 2001; Tajima *et al.*, 1999; Tajima *et al.*, 2000; Tajima *et al.*, 2001), despite the abundance of fibrobacters in this environment. The two species that were detected in co-culture with the *Fibrobacter* spp. were *B. thermoamylovorans*, a facultative anaerobe (Combetblanc *et al.*, 1995), and *C. botulinum*,

an anaerobe (Collins *et al.*, 1994) (Table 3.7). Clostridia have been previously isolated from landfill (Westlake *et al.*, 1995) and clusters III, IV and XIV of the clostridia detected in landfill sites (Burrell *et al.*, 2004; Van Dyke and McCarthy, 2002; McDonald *et al.*, 2012a). However *C. botulinum*, falls within cluster I of the clostridia (Collins *et al.*, 1994), and has previously been undetectable in landfill via PCR with subgroup specific primers (Van Dyke and McCarthy, 2002; McDonald *et al.*, 2012a), although it has since been isolated from a landfill site (Krishnamurthi and Chakrabarti, 2013). Unlike other members of the clostridia, *C. botulinum* is not capable of degrading cellulose (Sebaihia *et al.*, 2007), suggesting that for culture A721, *F. succinogenes* is responsible for the cellulose hydrolysis that occurred with potential cross-feeding with *C. botulinum*. This is less clear for cultures A711, which contained *C. botulinum*, *B. thermoamylovorans* and *F. succinogenes*, and B211, which contained *B. thermoamylovorans* and *F. succinogenes*. Whilst *F. succinogenes* is likely to have contributed to cellulose hydrolysis, *B. thermoamylovorans* is also cellulolytic (Chang *et al.*, 2008) thus making it difficult to determine the exact role of these organisms whilst they existed in co-culture.

Whilst after seven rounds of purification cultures A711C1, A711C4, B211C2 and B211C4 were pure, the broth cultures were slow growing with an incubation time of two months. Extraction of either DNA or RNA from these broth cultures via any of the attempted methods was unsuccessful. In addition, attempts to subculture the strains from the sloppy agar cultures into broth were unsuccessful, suggesting that although the culture had grown sufficiently to enable Gram staining it was no longer extant.

3.5 Conclusions

It has long been known that fibrobacters are the predominant bacterial degraders of cellulose within the rumen, and the genus *Fibrobacter* is circumscribed by cellulolytic activity. Of the 63 initial isolates from microcosms containing landfill leachate and Avicel, five were determined to contain *Fibrobacter* spp. via PCR with *Fibrobacter* specific primers. When sequenced it was demonstrated that these species were closely related to *F. succinogenes*, rather than novel landfill lineages previously described. Phylogenetic analysis revealed that these species formed a distinct group within *F. succinogenes* subsp. *succinogenes*, and their ability to grow in the absence of rumen fluid, usually a requirement, supports the assertion that these are novel landfill adapted isolates of *F. succinogenes*. This is the first isolation of *F. succinogenes* from outside of the mammalian intestinal tract, and the first isolation of fibrobacters from landfill sites. These data demonstrate that *Fibrobacter* spp. are metabolically

active within landfill sites and that they potentially play an important role in the cellulose hydrolysis that occurs within this environment.

3.6 References

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CHAPTER 4

Characterising cellulolytic microbial communities in landfill sites: a combined molecular and cultivation-based approach

Abstract

Whilst cellulolytic bacteria are critical to the global carbon cycle, their presence outside of the rumen has not been well studied, and this is particularly true for anaerobic environments where the isolation and cultivation of cellulolytic anaerobic bacteria and fungi has proven difficult. The material contained within landfill sites often has a high cellulosic content, and as such, understanding the diversity and function of members of the microbial community contained within this environment may improve both our approach to waste management, and elucidate novel species and enzymes for biotechnological applications. This study utilised a combination of molecular and cultivation-based methods to characterise the biofilm of a heavily degraded colonised cotton sample from a landfill leachate microcosm. A general bacterial 16S rRNA gene clone library detected members of 22 bacterial families, of which the predominant groups were members of the *Firmicutes* (54.5%), *Porphyromonadaceae* (16.5%), *Spirochaetaceae* (8.3%) and *Fibrobacteraceae* (5.8%), with sequence identities ranging from 76 to 98% similarity to their nearest neighbour, suggesting the presence of both novel cellulolytic species, genera and potentially phyla, in this environment. Novel members of the *Fibrobacteraceae* were detected despite known biases against the amplification of these species in general 16S rRNA gene clone libraries, supporting previous studies that identified fibrobacters as abundant members of the cellulolytic landfill bacterial community. Anaerobic isolation and cultivation yielded 55 bacterial isolates, with PCR and sequencing of the 16S rRNA gene demonstrating that all isolates were members of the *Firmicutes*. These isolates were related to one of three bacterial species, *C. leptum*, *C. sporogenes* or *S. acetigenes*, with the BLAST score of the isolates with *C. leptum* as their nearest neighbour demonstrating only 90 to 93% similarity, suggesting that these are potentially members of a novel genus. The isolation of *S. acetigenes*, a member of *Clostridium* cluster XII, represents the first isolation of this clostridia group from a landfill site. These data provide important insights into the composition of cellulolytic microbial communities in landfill sites.

4.1 Introduction

Members of the genus *Clostridium* are thought to be responsible for the majority of cellulose hydrolysis that occurs in landfill, due to their consistent isolation from this environment and their dominance amongst isolated strains (Westlake *et al.*, 1995; Benoit *et al.*, 1992; Krishnamurthi and Chakrabarti, 2013). However, the isolation and cultivation of anaerobic bacteria and fungi from landfill sites is a difficult and laborious process, and to date much of the work characterising the microbial populations of landfill sites has relied on the application of molecular biological techniques (Van Dyke and McCarthy, 2002; Li *et al.*, 2009; McDonald *et al.*, 2012; Burrell *et al.*, 2004). Of the 19 clusters of the clostridia (Collins *et al.*, 1994) clusters III and IV are the most commonly found in landfill sites (Van Dyke and McCarthy, 2002; Li *et al.*, 2009; McDonald *et al.*, 2012; Burrell *et al.*, 2004), although members of cluster XIV (McDonald *et al.*, 2012; Burrell *et al.*, 2004) and lineages associated with clusters VIII and XII (Burrell *et al.*, 2004) have also been detected. General bacterial 16S rRNA gene clone libraries have suggested the predominance of members of the genus *Clostridium* in landfill sites, and the absence of fibrobacters (Burrell *et al.*, 2004; Huang *et al.*, 2004; Huang *et al.*, 2005). Burrell *et al.* (2004) sequenced general bacterial 16S rRNA gene clone libraries derived from both biomass attached to cellulosic material (attached phase) and a mixture of solid and liquid material (mixed phase) contained within a landfill leachate bioreactor. The majority of these clones belonged to clusters III (65% attached phase, 48% mixed phase) and XIVa (16% attached phase, 10% mixed phase) of the clostridia, although members of cluster VI (11% attached phase, 10% mixed phase) were also present. However, as discussed previously in chapter 1, there is an apparent bias against fibrobacters and their detection in general 16S rRNA clone libraries.

Studies focussing on the *Fibrobacter* populations contained within landfill sites have determined that fibrobacters are ubiquitous in this environment (McDonald *et al.*, 2008; McDonald *et al.*, 2012), with many of these species representing novel lineages within the genus *Fibrobacter* (McDonald *et al.*, 2008). The relative abundance of these species also demonstrates the potential importance of fibrobacters in landfill, with qPCR illustrating that they may represent as much as 40% of the total bacterial population, greater than the 21 to 32% relative abundance of *Fibrobacter* spp. in ovine rumen samples, where they are known to predominate (McDonald *et al.*, 2008). The comparison of biofilms from heavily and poorly degraded cotton incubated in two microcosms containing landfill leachate also supports the potential importance of *Fibrobacter* spp. in this environment (McDonald *et al.*, 2012). The relative abundance of fibrobacters was determined via qPCR as 28.9% of the total bacterial

population on the heavily degraded cotton sample, and undetectable on the poorly degraded cotton, suggesting that they are active members of the cellulolytic community within this environment. Furthermore, the relative abundance of *Clostridium* cluster III, of which all known members are cellulolytic, was only 17.2% of the total bacterial population from the heavily degraded cotton (McDonald *et al.*, 2012).

Due to the potential issues both with isolating obligately anaerobic *Fibrobacter* spp. and relying solely on molecular approaches, it is important to utilise a combination of cultivation and molecular methods in order to study both this and members of the other phyla present in landfill sites. Also, whilst 16S rRNA gene clone library studies focussing on landfill sites have generated sequence data suggesting that novel cellulolytic species are present in this environment (Burrell *et al.*, 2004; Huang *et al.*, 2004; Huang *et al.*, 2005), the identity and function of these species can only be validated by cultivation. Here, heavily degraded cotton string retrieved from a landfill leachate microcosm (McDonald *et al.*, 2012) was used as source material for the isolation of cellulolytic anaerobic bacteria and the simultaneous analysis of taxonomic diversity via 16S rRNA gene clone library analysis. This study utilises colonised cotton from a landfill leachate microcosm, where fibrobacters were 28.9% of the total bacterial 16S rRNA (McDonald *et al.*, 2012), in order to characterise and isolate members of the cellulolytic microbial community, including *Fibrobacter* spp.

4.2 Materials and methods

4.2.1 Sampling and set up of a microcosm containing landfill leachate and dewaxed cotton string

Dewaxed cotton string from a microcosm containing leachate from Brombrough Dock landfill site risers 3 and 4 was generated in a previous study (McDonald *et al.*, 2012) and the material had been stored at -80°C prior to use as the source material for DNA extraction and cultivation via the Hungate roll tube method, as described in chapter 2.

4.2.2 Hungate roll tube method for isolating obligate anaerobes

Bacterial strains were isolated from 0.5 g dewaxed cotton string used to inoculate M2GSC broths containing either glucose, soluble starch and cellobiose, or carboxymethyl cellulose (CMC) as the carbon sources, via the anaerobic roll tube method of Hungate (Hungate, 1947; Bryant, 1972) with static incubation at 41°C until visible growth occurred. The bacterial strains underwent two rounds of purification as described in chapter 3. The culture medium was prepared anaerobically under 99.999% CO₂ (BOC gases).

4.2.3 Preparation of M2GSC medium for the cultivation of anaerobes

Liquid M2GSC medium as described by Miyazaki *et al.*, (1997) was prepared with the removal of rumen fluid from the medium, which contained; 1% (wt/vol) casitone (BD Biosciences), 0.25% (wt/vol) yeast extract (BD Biosciences), 0.4% (wt/vol) NaHCO₃ (Sigma), 0.2% (wt/vol) glucose (Sigma), 0.2% (wt/vol) cellobiose (Sigma), 0.2% (wt/vol) soluble starch (Sigma), 30% (vol/vol) filtered landfill leachate, 0.1% (wt/vol) cysteine hydrochloride (Sigma), 0.045% (wt/vol) K₂HPO₄ (Sigma), 0.045% (wt/vol) KH₂PO₄ (Sigma), 0.09% (wt/vol) (NH₄)₂SO₄ (Sigma), 0.09% (wt/vol) NaCl (Sigma), 0.09% (wt/vol) MgSO₄ (Sigma), 0.09% (wt/vol) CaCl₂ (Sigma), 0.0001% (wt/vol) resazurin (Sigma) and H₂O to the final volume. Where CMC (Sigma) was used as an alternative carbon source this was added to the medium at a 0.2% (wt/vol) concentration, with glucose, cellobiose and soluble starch omitted from the medium. For sloppy agars, 0.75% (wt/vol) agar (Sigma) was added, whilst roll tubes contained 2% (wt/vol) agar (Sigma).

4.2.4 Gram stains of cultured isolates

Cells were collected using a sterile 10 µl inoculation loop inserted into the sloppy agar culture and streaked on a glass slide. The cells were heat fixed and Gram stained using the Gram Staining Kit (Sigma) according to the manufacturer's instructions before analysis under oil immersion (x1000 magnification).

4.2.5 DNA extraction of dewaxed cotton string and broth cultures

For DNA extraction, either 0.5 g colonised dewaxed cotton string (for general bacterial clone library analysis of the cotton sample) or the pellet of 1.5 ml broth culture centrifuged at 15000 rpm for 5 minutes and resuspended in 500 µl PCR water (Bioline) (for 16S rRNA gene sequencing of bacterial isolates) was subjected to the method of Griffiths *et al.*, (2000) with the modifications as described in chapter 2. The DNA was visualised on a 1% agarose (Bioline) gel with HyperLadder 1kb (Bioline) as a marker, and the DNA concentration quantified using the Qubit Fluorometer (Life Technologies) with the Qubit dsDNA BR Assay Kit (Life Technologies).

4.2.6 Direct and nested PCR targeting the 16S rRNA gene

Each reaction (total volume 50 µl) contained 0.2 mM each of general bacterial primers pA (5' - 3' sequence AGAGTTTGATCCTGGCTCAG) and pH' (5' - 3' sequence AAGGAGGTGATCCAGCCGCA) (Edwards *et al.*, 1989), 0.2 mM each dNTP, 1x SuperTaq

Buffer (Cambio), 0.5 mM MgCl₂, 1x BSA, 1 unit SuperTaq (Cambio), 50 ng DNA and nuclease free water (Bioline). Where nested PCR was utilised to determine the presence of *Fibrobacter* spp. the PCR reactions used the *Fibrobacter* primer set, Fib 1F (5' - 3' sequence CCGKSCCAACGSSCGG) and Fib 2AR (5' - 3' sequence ATCTCTCGCYGCGGCGWTY CC) (McDonald *et al.*, 2008) with each reaction containing 0.4 mM each primer, 1.5 mM MgCl₂ and 1 µl of the PCR product generated using the general bacterial primers pA/pH'. The PCR cycling conditions were: an initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 1 min at the specific annealing temperature for each primer set (55°C for pA/pH', 60°C for Fib 1F/FIB 2AR) and an extension of 72°C for 1.5 min. The final extension was performed at 72°C for 10 min. The PCR products were visualised on a 1% agarose (Bioline) gel with HyperLadder 1kb (Bioline) as a size marker, and stored at -20°C.

4.2.7 Cloning and sequencing of 16S rRNA gene PCR amplicons

PCR amplicons generated using the general bacterial primer set pA (5' - 3' sequence AGAGTTTGATCCTGGCTCAG) and pH' (5' - 3' sequence AAGGAGGTGATCCAGCCGCA) (Edwards *et al.*, 1989) were purified from a 1% agarose (Bioline) gel using the Bioline Isolate Gel Extraction Kit (Bioline) following the manufacturer's instructions. The PCR products were ligated and cloned into competent *E. coli* JM109 (Promega) using the pGEM-T Easy Vector System I (Promega) following the manufacturer's protocol. These clones were purified from the plasmid and sequenced using the M13 forward primer (5' - 3' sequence GTTTTCCCAGTCACGAC) (Messing, 1983) by Macrogen. General bacterial 16S rRNA gene PCR products from the isolated strains were purified and sequenced using the pA forward primer (5' - 3' sequence AGAGTTTGATCCTGGCTCAG) by Macrogen.

4.2.8 Phylogenetic analysis of 16S rRNA gene sequences from the general bacterial clone library

Sequences derived from the clone library were viewed using 4Peaks, quality clipped and subjected to a BLASTn search against the NCBI nucleotide database in order to determine their nearest neighbour. Sequences identified as members of the *Fibrobacteraceae* and cultivated *Fibrobacter* spp. downloaded from the Ribosomal Database Project website (Cole *et al.*, 2007; Cole *et al.*, 2009) were aligned using the MUSCLE aligner (Edgar, 2004), trimmed and used to generate a neighbour-joining tree with 1000 bootstrap samplings via

PhyML (version 3.0) (Guindon *et al.*, 2010) which was viewed using Interactive Tree of Life (Letunic and Bork, 2007).

4.2.9 Phylogenetic analysis of 16S rRNA gene sequences derived from isolates

The sequence trace files were viewed using 4Peaks, quality clipped and the nearest neighbour assigned by a BLASTn search against the NCBI 16S ribosomal RNA (Bacteria and Archaea) database. These sequences were aligned using the MUSCLE aligner (Edgar, 2004) before clustering at 97% similarity using CDHIT (Li and Godzik, 2006; Huang *et al.*, 2010). The nearest neighbours of the sequences as determined by BLAST were downloaded from the Ribosomal Database Project website (Cole *et al.*, 2007; Cole *et al.*, 2009) and these along with the representative sequences of each cluster were realigned using MUSCLE, trimmed and used to generate a neighbour-joining tree with 1000 bootstrap samplings with PhyML (version 3.0) (Guindon *et al.*, 2010), viewed using Interactive Tree of Life (Letunic and Bork, 2007).

4.3 Results

4.3.1 Diversity of 16S rRNA gene sequences derived from a landfill colonised cotton biofilm

The partial 16S rRNA gene sequence of all 121 clones was sequenced and the identity assigned via a BLASTn search against the NCBI 16S ribosomal RNA (Bacteria and Archaea) database (Altschul *et al.*, 1990), identifying members of 22 bacterial families (Table 4.1). Members of the *Ruminococcaceae* were most abundant (23.4%) followed by *Lachnospiraceae* (23.1%), *Porphyromonadaceae* (16.5%) and *Spirochaetaceae* (8.3%) (Table 4.1). These sequences varied in their similarity to their nearest neighbour, with the lowest sequence similarity to their nearest neighbour belonging to a sequence within the *Thermoanaerobacteraceae* (76%) and the highest within the *Spirochaetaceae* (98%) (Table 4.1). Members of *Clostridium* clusters XIVa (28 clones) III (15 clones) and IV (14 clones) were detected in the general bacterial 16S rRNA gene clone library, whilst members of cluster I were undetected (Table 4.1). The *Fibrobacteraceae* were the fifth most abundant family, comprising 5.8% of the total 16S rRNA gene sequences (n=7) (Table 4.1), with these sequences demonstrating 83 – 89% sequence similarity to their nearest cultivated neighbour (Fig. 4.1).

Table 4.1. Taxonomy of 16S rRNA gene clone library sequences derived from the biofilm of colonised cotton incubated in a landfill leachate microcosm.

a. Closest relative as determined by NCBI nucleotide BLAST (Altschul *et al.*, 1990)

| Putative assignment | Number of clones | Percentage of clone library sequences (%) | Similarity to closest relative (%) ^a | <i>Clostridium</i> cluster (number of sequences) |
|--|------------------|---|---|--|
| <i>Acholeplasmataceae</i> | 1 | 0.8 | 90 | |
| <i>Synergistaceae</i> | 1 | 0.8 | 90 | |
| <i>Anaerolineaceae</i> | 1 | 0.8 | 88 | |
| <i>Spirochaetaceae</i> | 10 | 8.3 | 86-98 | |
| <i>Fibrobacteraceae</i> | 7 | 5.8 | 83-89 | |
| <i>Peptococcaceae</i> | 2 | 1.7 | 88-89 | |
| <i>Clostridiales Incertae Sedis XI</i> | 5 | 4.1 | 89-95 | |
| <i>Ruminococcaceae</i> | 29 | 24.0 | 82-94 | III (15), IV (14) |
| <i>Lachnospiraceae</i> | 28 | 23.1 | 82-94 | XIVa (28) |
| <i>Porphyromonadaceae</i> | 20 | 16.5 | 82-96 | |
| <i>Flavobacteriaceae</i> | 1 | 0.8 | 85 | |
| <i>Marinilabiaceae</i> | 4 | 3.3 | 91-93 | |
| <i>Flammeovirgaceae</i> | 1 | 0.8 | 86 | |
| <i>Cytophagaceae</i> | 1 | 0.8 | 91 | |
| <i>Peptostreptococcaceae</i> | 2 | 1.7 | 84-87 | |
| <i>Lactobacillaceae</i> | 1 | 0.8 | 91 | |
| <i>Streptococcaceae</i> | 1 | 0.8 | 81 | |
| <i>Gracilibacteraceae</i> | 1 | 0.8 | 87 | |
| <i>Thermoanaerobacteraceae</i> | 2 | 1.7 | 76-84 | |
| <i>Cardiobacteriaceae</i> | 1 | 0.8 | 82 | |
| <i>Chromatiaceae</i> | 1 | 0.8 | 90 | |
| <i>Xanthobacteraceae</i> | 1 | 0.8 | 88 | |

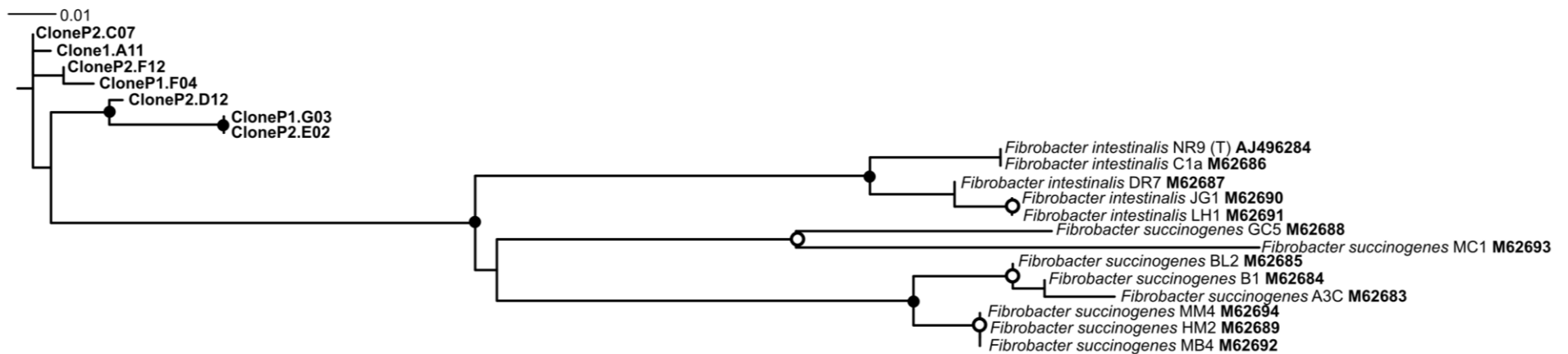


Figure 4.1. Phylogeny of 16S rRNA gene clone library sequences derived from the biofilm of heavily degraded colonised cotton and identified as fibrobacters via NCBI nucleotide blast. Accession numbers of cultivated isolates downloaded from the Ribosomal Database Project are in bold. Filled circles represent bootstrap values >95%, unfilled circles represent bootstrap values between 75 and 95%. The scale bar represents 0.1 base substitutions per nucleotide.

4.3.2 Isolation and 16S rRNA gene sequencing of isolates cultivated from the biofilm of cotton incubated in a landfill leachate microcosm

A total of 55 bacterial isolates were obtained from the cellulolytic biofilm and the 16S rRNA gene amplified via PCR and sequenced using a general bacterial primer set. Twenty-five (45%) of these isolates were between 90 and 93% similar to *Clostridium leptum*, whilst 17 isolates (31%) were 99 to 100% similar to *Clostridium sporogenes* and 13 isolates (24%) 99% identical to *Sporanaerobacter acetigenes* (Table 4.2) (Fig. 4.2). Of these strains, 29 (53%) were isolated using medium containing CMC as the sole carbon source for growth (suggesting a cellulolytic phenotype), whilst the remaining 26 (47%) strains were isolated using medium that contained glucose, cellobiose and soluble starch (GSC). All isolate cultures obtained from M2GSC medium, with the exception of one isolate, were pure (Table 4.2). All of the isolates obtained using M2MGSC medium were related to *C. leptum*, apart from the impure strain, which was identified as *S. acetigenes* (Table 4.2). The strains isolated from CMC containing medium were either *C. sporogenes* or *S. acetigenes*, with 13 of the strains remaining impure after two rounds of purification (Table 4.2).

Cultures of the 13 impure strains isolated from CMC medium and sequenced along with two strains for which no sequence data was available due to poor quality reads (S41A and U11A) were subjected to a third round of purification before DNA extraction and nested PCR using the *Fibrobacter* specific primer Fib 1F (5' - 3' sequence CCGKSCCAACGSSCGG) and Fib 2AR (5' - 3' sequence ATCTCTCGCYGCGGCGWTYCC) (McDonald *et al.*, 2008). Of these cultures, 6 were positive for the presence of *Fibrobacter* spp., with all of these cultures containing a mixture of Gram-negative rods and cocci (Table 4.3) (Fig. 4.3).

Table 4.2. Taxonomy of 16S rRNA gene sequences generated from isolates cultivated from a biofilm on colonised cotton from a landfill leachate microcosm.

a. Closest relative as determined by NCBI nucleotide BLAST (Altschul *et al.*, 1990).

b. GSC refers to media containing glucose, soluble starch and cellobiose, CMC refers to media containing carboxymethylcellulose.

| Putative assignment | Strain | Nearest cultivated neighbour ^a | Maximum Identity | Substrate used for isolation ^b | Gram stain | Pure |
|---|--------|---|------------------|---|--------------------|------|
| <i>Ruminococcaceae</i> (<i>Clostridium</i> cluster IV) | W41A | <i>Clostridium leptum</i> | 90% | GSC | Gram-negative rods | Y |
| | W41B | <i>Clostridium leptum</i> | 90% | GSC | Gram-negative rods | Y |
| | W42A | <i>Clostridium leptum</i> | 93% | GSC | Gram-negative rods | Y |
| | W51A | <i>Clostridium leptum</i> | 92% | GSC | Gram-negative rods | Y |
| | W62A | <i>Clostridium leptum</i> | 92% | GSC | Gram-negative rods | Y |
| | W62B | <i>Clostridium leptum</i> | 93% | GSC | Gram-negative rods | Y |
| | X41A | <i>Clostridium leptum</i> | 92% | GSC | Gram-negative rods | Y |
| | X42A | <i>Clostridium leptum</i> | 92% | GSC | Gram-negative rods | Y |
| | X42B | <i>Clostridium leptum</i> | 93% | GSC | Gram-negative rods | Y |
| | X71A | <i>Clostridium leptum</i> | 93% | GSC | Gram-positive rods | Y |
| | Y32A | <i>Clostridium leptum</i> | 92% | GSC | Gram-positive rods | Y |
| | Y52B | <i>Clostridium leptum</i> | 92% | GSC | Gram-negative rods | Y |
| | Y81B | <i>Clostridium leptum</i> | 93% | GSC | Gram-negative rods | Y |
| | Z11A | <i>Clostridium leptum</i> | 93% | GSC | Gram-negative rods | Y |
| | Z12A | <i>Clostridium leptum</i> | 93% | GSC | Gram-negative rods | Y |
| | Z21A | <i>Clostridium leptum</i> | 92% | GSC | Gram-negative rods | Y |
| | Z22B | <i>Clostridium leptum</i> | 93% | GSC | Gram-negative rods | Y |

| | | | | | | |
|-------------------------------|------|------------------------------------|------|-----|------------------------------|---|
| | Z31A | <i>Clostridium leptum</i> | 93% | GSC | Gram-negative rods | Y |
| | Z32A | <i>Clostridium leptum</i> | 93% | GSC | Gram-negative rods | Y |
| | Z41A | <i>Clostridium leptum</i> | 93% | GSC | Gram-negative rods | Y |
| | Z41B | <i>Clostridium leptum</i> | 93% | GSC | Gram-negative rods | Y |
| | Z81A | <i>Clostridium leptum</i> | 92% | GSC | Gram-negative rods | Y |
| | Z82A | <i>Clostridium leptum</i> | 93% | GSC | Gram-negative rods | Y |
| | Z82B | <i>Clostridium leptum</i> | 93% | GSC | Gram-negative rods | Y |
| | Z92A | <i>Clostridium leptum</i> | 92% | GSC | Gram-negative rods | Y |
| <i>Clostridiaceae</i> | R51A | <i>Clostridium sporogenes</i> | 99% | CMC | Gram-negative cocci and rods | N |
| (<i>Clostridium</i> | R61A | <i>Clostridium sporogenes</i> | 99% | CMC | Gram-negative cocci and rods | N |
| cluster I) | R62A | <i>Clostridium sporogenes</i> | 99% | CMC | Gram-negative cocci and rods | N |
| | R72A | <i>Clostridium sporogenes</i> | 100% | CMC | Gram-negative cocci and rods | N |
| | R92A | <i>Clostridium sporogenes</i> | 100% | CMC | Gram-negative rods | Y |
| | S11A | <i>Clostridium sporogenes</i> | 100% | CMC | Gram-negative cocci and rods | N |
| | S12A | <i>Clostridium sporogenes</i> | 100% | CMC | Gram-negative rods | Y |
| | S21A | <i>Clostridium sporogenes</i> | 99% | CMC | Gram-negative cocci and rods | N |
| | S22A | <i>Clostridium sporogenes</i> | 100% | CMC | Gram-negative cocci and rods | N |
| | S31A | <i>Clostridium sporogenes</i> | 99% | CMC | Gram-negative rods | Y |
| | S72A | <i>Clostridium sporogenes</i> | 100% | CMC | Gram-negative cocci and rods | N |
| | S81A | <i>Clostridium sporogenes</i> | 100% | CMC | Gram-negative cocci and rods | N |
| | T21A | <i>Clostridium sporogenes</i> | 100% | CMC | Gram-negative rods | Y |
| | T72A | <i>Clostridium sporogenes</i> | 99% | CMC | Gram-negative rods | Y |
| | T81A | <i>Clostridium sporogenes</i> | 99% | CMC | Gram-negative rods | Y |
| | T82A | <i>Clostridium sporogenes</i> | 99% | CMC | Gram-negative rods | Y |
| | T91A | <i>Clostridium sporogenes</i> | 100% | CMC | Gram-negative cocci | Y |
| <i>Clostridiales Incertae</i> | R12A | <i>Sporanaerobacter acetigenes</i> | 99% | CMC | Gram-negative rods | Y |

| | | | | | | |
|--|------|------------------------------------|-----|-----|---------------------------------|---|
| Sedis XI (Clostridium cluster XII) | R31A | <i>Sporanaerobacter acetigenes</i> | 99% | CMC | Gram-positive rods | Y |
| | R41A | <i>Sporanaerobacter acetigenes</i> | 99% | CMC | Gram-negative, mixed sized rods | N |
| | S52A | <i>Sporanaerobacter acetigenes</i> | 99% | CMC | Gram-negative rods | Y |
| | S82A | <i>Sporanaerobacter acetigenes</i> | 99% | CMC | Gram-positive rods | Y |
| | S91A | <i>Sporanaerobacter acetigenes</i> | 99% | CMC | Gram-positive rods | Y |
| | S92A | <i>Sporanaerobacter acetigenes</i> | 99% | CMC | Gram-negative cocci and rods | N |
| | T32A | <i>Sporanaerobacter acetigenes</i> | 99% | CMC | Gram-negative rods | Y |
| | T42A | <i>Sporanaerobacter acetigenes</i> | 99% | CMC | Gram-positive, mixed sized rods | N |
| | T52A | <i>Sporanaerobacter acetigenes</i> | 99% | CMC | Gram-negative rods | Y |
| | U12A | <i>Sporanaerobacter acetigenes</i> | 99% | CMC | Gram-positive, mixed sized rods | N |
| | U21A | <i>Sporanaerobacter acetigenes</i> | 99% | CMC | Gram-negative rods | Y |
| | W22A | <i>Sporanaerobacter acetigenes</i> | 99% | GSC | Gram-negative, mixed sized rods | N |

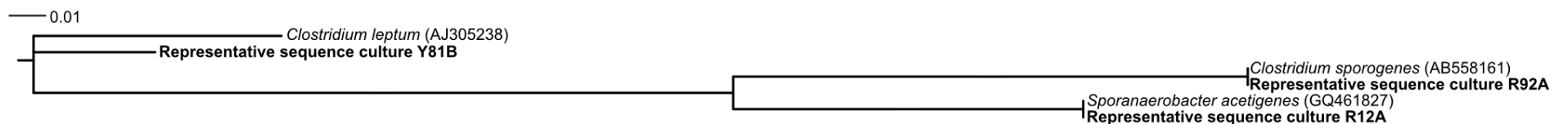
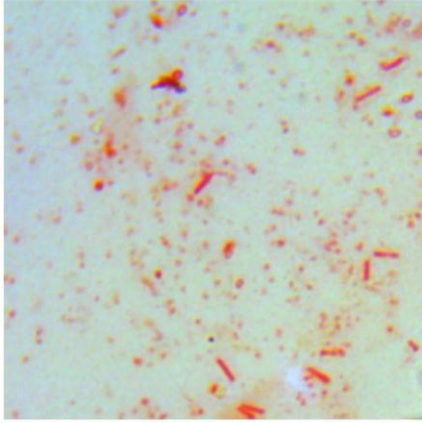


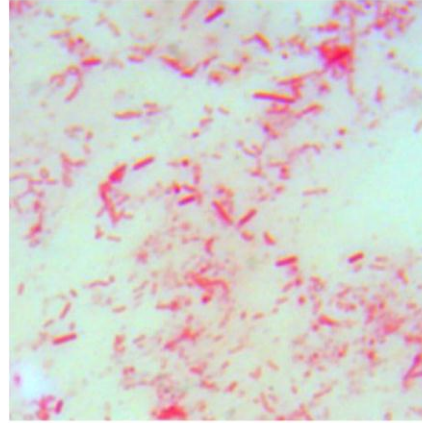
Figure 4.2. Phylogeny of 16S rRNA gene sequences derived from cultivated isolates. Filled circles represent bootstrap values >95%, unfilled circles represent bootstrap values between 75 and 95%. Accession numbers are in brackets. The scale bar represents 0.1 base substitutions per nucleotide.

Table 4.3. Detection of *Fibrobacter* spp. via nested PCR using genus specific 16S rRNA gene primers in impure cultures subjected to a third round of purification.

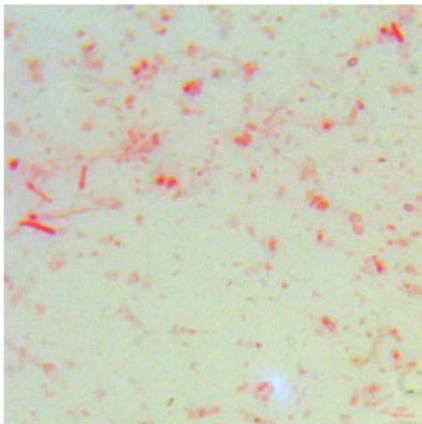
| Strain | Nested <i>Fibrobacter</i> PCR Result | Species in co-culture |
|--------|---|-----------------------|
| R51A | - | <i>C. sporogenes</i> |
| R61A | + | <i>C. sporogenes</i> |
| R62A | - | <i>C. sporogenes</i> |
| R72A | - | <i>C. sporogenes</i> |
| S11A | + | <i>C. sporogenes</i> |
| S21A | + | <i>C. sporogenes</i> |
| S22A | - | <i>C. sporogenes</i> |
| S41A | + | No sequence data |
| S72A | - | <i>C. sporogenes</i> |
| S81A | + | <i>C. sporogenes</i> |
| R41A | - | <i>S. acetigenes</i> |
| S92A | - | <i>S. acetigenes</i> |
| T42A | - | <i>S. acetigenes</i> |
| U11A | + | No sequence data |
| U12A | - | <i>S. acetigenes</i> |



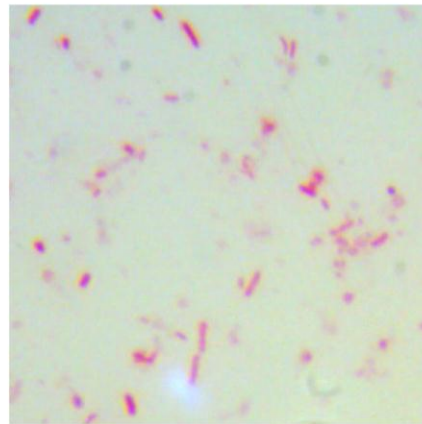
A) R61A Mixture of Gram negative small ovoid cells and larger Gram negative rods. Some background staining.



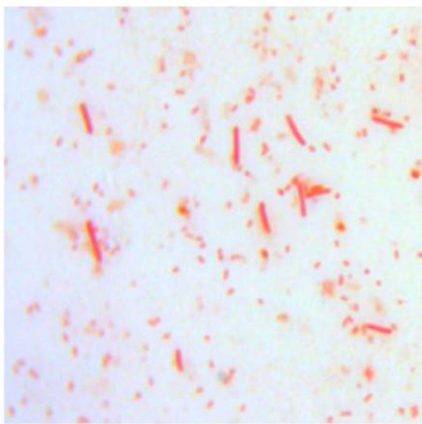
B) S11A Mixture of Gram negative small ovoid cells and larger Gram negative rods. Some background staining.



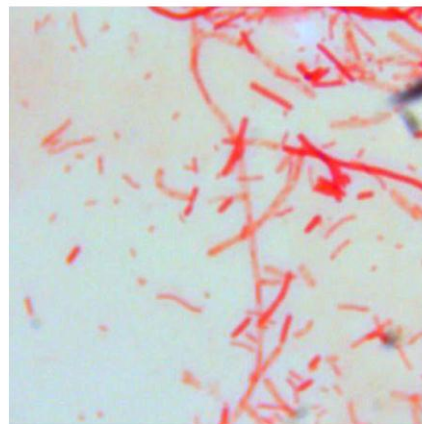
C) S21A Mixture of Gram negative small ovoid cells and larger Gram negative rods. Some background staining.



D) S41A Mixture of Gram negative small ovoid cells and larger Gram negative rods.



E) S81A Mixture of Gram negative small ovoid cells and larger Gram negative rods.



F) U11A Mixture of Gram negative small ovoid cells and both small and large Gram negative rods. Some background staining.

Figure 4.3. Gram stain images of the 6 isolates that were positive for the presence of *Fibrobacter* spp. as determined via 16S rRNA gene PCR.

4.4 Discussion

4.4.1 Comparing the composition of cellulolytic microbial communities in landfill as determined via molecular and cultivation-based methods

Molecular methods revealed the presence of 22 bacterial families present on the colonised cotton biofilm with members of the *Firmicutes* comprising the majority of sequences at 54.5% (Table 4.1). However, the cultivation-based approach yielded three species, *C. leptum*, *C. sporogenes* and *S. acetigenes*, all of which belong to the *Firmicutes* (Table 4.2), demonstrating that the application of molecular methods revealed a greater diversity than the cultivation based approach. This is unsurprising given that many organisms are intractable to study via conventional cultivation, and that the sample had been frozen. The diversity of species detected via molecular methods varied, with the highest sequence similarity belonging to a species related to a member of the *Spirochaetaceae* (98%) whilst the lowest sequence similarity belonging to a species loosely related to the *Thermoanaerobacteraceae* (76%) (Table 4.1). In contrast, the cultivation based approach yielded two species that were highly similar to their nearest neighbour, *Clostridium sporogenes* (99 - 100% sequence similarity) and *Sporanaerobacter acetigenes* (99% sequence similarity), whilst only the species related to *Clostridium leptum* was novel (90 - 93% sequence similarity) (Table 4.2). Although these results suggest that a solely molecular based approach would be best for studying an environment, the isolation of *C. sporogenes*, a member of cluster I of the clostridia both here and previously (Krishnamurthi and Chakrabarti, 2013) would suggest that this is not always the case. Members of *Clostridium* cluster I were not detected in the 16S rRNA gene clone library constructed in this or a previous study (Burrell *et al.*, 2004) and in addition to this, members of this cluster have not been detected in landfill sites via group-specific 16S rRNA gene PCR primers, (Van Dyke and McCarthy, 2002; McDonald *et al.*, 2012). This would suggest that for this group in particular, the use of molecular methods is biased against the detection of these organisms, and it would stand to reason that other species might also be either underrepresented or missed entirely by the reliance on only one method.

4.4.2 Diversity of 16S rRNA gene clone library sequences derived from a colonised cotton sample incubated in a landfill leachate microcosm

A previous study of cellulolytic bacterial populations in a landfill leachate microcosm, using the same colonised cotton material used as source material for the culture-based and general bacterial 16S rRNA analysis here, demonstrated via qPCR the presence of members of clostridia clusters III, IV, XIV, and the genus *Fibrobacter*, with the relative abundance of these species representing 17.2%, 3.2%, 0.9% and 28.9% of the total bacterial 16S rRNA gene respectively (McDonald *et al.*, 2012). This is consistent with other studies that also detected *Clostridium* clusters III, IV and XIV (Burrell *et al.*, 2004; Van Dyke and McCarthy, 2002; McDonald *et al.*, 2012) and fibrobacters (McDonald *et al.*, 2008) in landfill sites.

Interestingly, despite *Fibrobacter* spp. representing the greatest proportion of the total bacterial rRNA from this cotton as determined via qPCR (McDonald *et al.*, 2012), only 7 clones, 5.7% of the sequences from the general bacterial clone library, were determined to be members of the *Fibrobacteraceae* via NCBI nucleotide BLAST (Table 4.1). These species were between 83 and 89% similar to their nearest neighbour as determined via NCBI nucleotide BLAST (Table 4.1) (Fig. 4.1), which supports previous work that demonstrated the presence of novel *Fibrobacter* spp. in landfill sites (McDonald *et al.*, 2008; Ransom-Jones *et al.*, 2014). However, previous 16S rRNA gene clone libraries derived from a landfill leachate bioreactor (Burrell *et al.*, 2004), a closed municipal solid waste landfill (Huang *et al.*, 2005) and a recirculating landfill (Huang *et al.*, 2004), along with 454 pyrosequencing of simulated municipal solid waste bioreactors (Bareither *et al.*, 2013) failed to detect any *Fibrobacter* sequences. Therefore, the fact that fibrobacters were detected in this clone library, despite the bias against them, supports the qPCR data that *Fibrobacter* spp. are highly abundant in this environment (McDonald *et al.*, 2012), and demonstrates the use of dewaxed cotton string as a 'bait' for the enrichment of cellulolytic species.

Although fibrobacters were the predominant bacterial species as determined via qPCR, with their relative abundance of the total bacterial 16S rRNA exceeding the combined *Clostridium* cluster abundances (McDonald *et al.*, 2012), the *Firmicutes* comprise the majority of sequences (54.5%) from the general 16S rRNA gene clone library (Table 4.1), with the majority belonging to cluster XIVa of the clostridia (28 sequences), along with clusters III (15 sequences) and IV (14 sequences) (Table 4.1). The *Firmicutes*, also known as low G+C Gram-positive bacteria, are known to

dominate general 16S rRNA gene clone libraries derived from landfill leachate (Huang *et al.*, 2004; Burrell *et al.*, 2004; Huang *et al.*, 2005). Burrell *et al.*, (2004) constructed clone libraries from a mixture of leachate and solid substrate, and colonised cellulosic matter from a landfill leachate bioreactor. The *Firmicutes* comprised 100% of the sequences derived from the biofilm on the solid cellulose and 90% of the sequences from the leachate/solid substrate mix, with the majority of these sequences similar to *Clostridium* clusters III, IV and XIVa, although clusters VIII and XII were also detected (Burrell *et al.*, 2004). Studies utilising 16S rRNA gene targeted PCR primers specific to groups I, III, IV and XIV of the clostridia detected clusters III, IV and XIV in landfill sites, whilst cluster I was not detected in any of the samples (Van Dyke and McCarthy, 2002; McDonald *et al.*, 2012). Of the clusters that were detected, cluster III was found to be the most commonly detected in both studies (Van Dyke and McCarthy, 2002; McDonald *et al.*, 2012) along with either cluster IV (Van Dyke and McCarthy, 2002) or cluster XIV (McDonald *et al.*, 2012).

4.4.3 Taxonomy of isolates obtained from dewaxed cotton string incubated in a landfill leachate microcosm

Of the 55 isolates, all of the sequences were identified as having one of three species as their nearest neighbours via NCBI nucleotide BLAST, suggesting that these were multiple isolates of the same organisms (Table 4.2). Of the three strains identified, isolates related to *Clostridium sporogenes* (99 - 100% sequence similarity) and *Sporanaerobacter acetigenes* (99% sequence similarity) had a high similarity to their nearest neighbour, whilst isolates related to the other identified strain were determined as having 90 - 93% sequence similarity to their nearest neighbour, *Clostridium leptum* (Table 4.2), with the isolates related to *C. leptum* representing a novel species based on 16S rRNA gene sequence similarity.

In previous isolation studies from landfill sites, both a *Cellulomonas* species (Bagnara *et al.*, 1985) and members of the genus *Eubacterium* (Westlake *et al.*, 1995) have been isolated from landfill, as well as clostridia (Westlake *et al.*, 1995; Benoit *et al.*, 1992; Krishnamurthi and Chakrabarti, 2013). No eubacteria were isolated in this study, but that all of the isolates from this study belonged to the *Firmicutes* is unsurprising given their dominance both in clone libraries (Huang *et al.*, 2004; Burrell *et al.*, 2004; Huang *et al.*, 2005) and a cultivation based study on landfill, where 86.7% of species isolated belonged to this phylum (Krishnamurthi and Chakrabarti, 2013).

As discussed previously, members of *Clostridium* cluster IV are known to be present in landfill environments (Burrell *et al.*, 2004; McDonald *et al.*, 2012; Van Dyke and McCarthy, 2002), therefore the isolation of strains related to *Clostridium leptum* (cluster IV), and that these strains account for the 45.5% of the isolated species, is expected. These strains are between 90 and 93% similar to their nearest neighbour (Table 4.2) (Fig. 4.2), suggesting that these isolates are a novel species, and potentially belong to a novel genus within clostridium cluster IV, as were many of the other bacteria previously isolated from landfill sites (Krishnamurthi and Chakrabarti, 2013; Bagnara *et al.*, 1985; Westlake *et al.*, 1995).

The other isolates from this study belonged to clusters I and XII of the clostridia. Members of cluster XII are less readily detected in landfill than other groups of the clostridia, but they have been detected previously in a 16S rRNA gene clone library derived from a landfill leachate bioreactor (Burrell *et al.*, 2004), this is however the first isolation of members of cluster XII clostridia from a landfill site. Whilst members of cluster I have not been previously detected in landfill sites via group specific 16S rRNA gene PCR primers, (Van Dyke and McCarthy, 2002; McDonald *et al.*, 2012), or via a 16S rRNA gene clone library (Burrell *et al.*, 2004), *C. sporogenes*, belonging to cluster I of the clostridia (Collins *et al.*, 1994), has been isolated from landfill in both here (chapter 3), and previous studies (Krishnamurthi and Chakrabarti, 2013). The species isolated in this study were between 99 and 100% similar to their nearest neighbour, so it is unlikely that these species were undetected due to primer mismatches in the group specific PCR primers. It is possible that these species, whilst present in the landfill environment, are in much lower abundances than the members of other *Clostridium* clusters. As a result of this, it is only when they are enriched by cultivation, particularly using a substrate such as CMC, which requires the organism to be capable of producing at least some of the cellulolytic enzyme groups required for the degradation of crystalline cellulose, that they are detected.

The detection of *Fibrobacter* spp. in the impure cultures is unsurprising given their presence in both the general bacterial 16S rRNA gene clone library (Table 4.1), and their relative abundance of 28.9% of the total bacterial 16S rRNA on the colonised cotton as determined via qPCR (McDonald *et al.*, 2012). As demonstrated in chapter 3, fibrobacters are difficult to purify, thus the impurity of these cultures even after three rounds of purification (Table 4.3). The *Fibrobacter* spp. are likely to be the smaller, ovoid cells (Montgomery *et al.*, 1988) present in the Gram stain images (Fig. 4.3). The

fact that the fibrobacters were always associated with *C. sporogenes* is intriguing, especially due to the fact that in chapter 3, cultures containing *Fibrobacter* spp. also contained *C. botulinum*, which is closely related to *C. sporogenes* (Lee and Riemann, 1970). It is possible that this association is required by the *Fibrobacter* spp. in order for them to survive, thus potentially explaining the difficulties in obtaining pure isolates of these species.

4.5 Conclusions

The general bacterial 16S rRNA gene clone library generated in this study from the colonised cotton sample detected the presence of 22 bacterial families, with the species detected by the 16S rRNA gene clone library ranging in their similarity to their nearest neighbour, from 76% within the *Thermoanaerobacteraceae* to 98% within the *Spirochaetaceae*, demonstrating that there are potentially a number of novel organisms present in landfill sites. The detection of members of the *Fibrobacteraceae* in this general bacterial 16S rRNA gene clone library, despite the bias against these species, supports previous data to suggest that fibrobacters are abundant members of this community (McDonald *et al.*, 2012).

The cultivation-based approach detected only members of the *Firmicutes*, and isolated three strains related to *C. leptum* (90 - 93% sequence similarity), *C. sporogenes* (99 - 100% sequence similarity) and *S. acetigenes* (99 - 100% sequence similarity), with sequence similarity suggesting that only the *C. leptum* related isolates were novel. It has long been known that environmental characterisation studies focusing solely on cultivation fail to isolate all of the members of the community, however molecular methods also have their limitations and as such the cultivation of novel species remains important (Walker *et al.*, 2014). It is likely that a combination of molecular and cultivation based approaches will be needed in order to fully understand the taxonomy and function of members of any given community, with the isolation of novel species improved by the molecular data (Pope *et al.*, 2011; Renesto *et al.*, 2003). Given the diversity of bacteria detected in the biofilm on the colonised cotton, it is possible that the cellulolytic community contained within landfill sites is more diverse than previously thought, with the implication that this environment is a potential source of both novel species and enzymes for use in biotechnological applications.

4.6 References

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CHAPTER 5

Shotgun metagenomic analysis of a landfill cellulolytic microbial community

Abstract

Despite the fact that landfill waste contains a high cellulosic content, the taxonomic and functional composition of cellulose-degrading microbial communities in landfill is not well understood. In this study, we determined the composition of two bacterial communities associated with poorly or heavily degraded colonised cotton 'baits' incubated in landfill leachate via 454 pyrosequencing of 16S rRNA gene PCR amplicons. In addition, metagenomic sequencing of the heavily degraded colonised cotton sample was utilised in order to determine the taxonomic and functional diversity of the members of this community. Analysis of 16S rRNA gene PCR amplicons revealed a total of 22 and 24 phyla present on the poorly and heavily degraded colonised cotton, respectively. Members of the *Bacteroidetes* (72.7%), *Proteobacteria* (18.7%) and *Actinobacteria* (3.3%) dominated the poorly-degraded cotton biofilm, whereas *Firmicutes* (34.7%), *Bacteroidetes* (20.5%), *Spirochaetes* (14.8%) and *Fibrobacteres* (14.2%) dominated the heavily degraded cotton. These data imply that members of the *Firmicutes*, *Spirochaetes* and *Fibrobacteres* are key members of the cellulolytic community in landfill sites. Analysis of bacterial contigs from the metagenome dataset supported this observation, with the identification of 18 phyla including members of the *Firmicutes* (47.2%), *Bacteroidetes* (22.8%) and *Spirochaetes* (6.1%), whilst also potentially implicating *Proteobacteria* (6.4%) as having a role in cellulose degradation in landfill sites. Recruitment plot analysis of the heavily degraded colonised cotton metagenome also implicated members of the *Fibrobacteres* in cellulose hydrolysis, with the genome of *F. succinogenes* subsp. *succinogenes* S85 having the most protein matches in comparison to the metagenome. These data provide important insights into the cellulose degrading microbial community present in the landfill environment, with implications for our understanding of carbon cycling and waste management.

5.1 Introduction

Cellulose is the most abundant organic carbon polymer in landfill waste, and can comprise as much as 63.4% of the organic content present (Bookter and Ham, 1982). Despite this, little is known about the taxonomic and functional composition of microbial communities associated with the cellulose hydrolysis in landfill. Members of the *Firmicutes*, and more specifically the class *Clostridia*, are thought to be one of the predominant bacterial degraders of cellulose in landfills due to their isolation (Westlake *et al.*, 1995) and detection via molecular methods (Burrell *et al.*, 2004; Huang *et al.*, 2005; Huang *et al.*, 2004; Xie *et al.*, 2014; Bareither *et al.*, 2013); both a 16S rRNA gene clone library (Burrell *et al.*, 2004) and 454 pyrosequencing of 16S rRNA gene PCR amplicons (Bareither *et al.*, 2013; Xie *et al.*, 2014) have demonstrated the presence of members of the *Clostrida* in landfill leachate. A study of both leachate and solid material from a lab-scale landfill waste bioreactor demonstrated that 100% of *Firmicutes* sequences from the leachate and 85.8% of *Firmicutes* sequences from the solid material were assigned to the class *Clostridia* (Bareither *et al.*, 2013).

Bacterial 16S rRNA gene clone library studies of landfill leachate have also demonstrated the presence of members of the *Chlamydiae/Verrucomicrobia* group (Huang *et al.*, 2004; Huang *et al.*, 2005), the *Cytophaga-Flexibacter-Bacteroides* group (Huang *et al.*, 2004; Huang *et al.*, 2005), *Planctomycetes* (Huang *et al.*, 2004), *Spirochaetes* (Huang *et al.*, 2004; Huang *et al.*, 2005), *Proteobacteria* (Huang *et al.*, 2004; Huang *et al.*, 2005) and *Actinobacteria* (Huang *et al.*, 2004), with the members of these groups implicated in a variety of processes that occur within the landfill environment, including the degradation of cellulose. The use of next-generation sequencing has enabled further characterisation of the members of the microbial community present in landfill, with these data supporting the results of previous clone library studies. The use of 454 pyrosequencing targeting 16S rRNA gene PCR amplicons to determine the microbial community associated with anaerobic bioreactors that were treating landfill leachate demonstrated that *Firmicutes* (Xie *et al.*, 2014; Bareither *et al.*, 2013), *Bacteroidetes* (Bareither *et al.*, 2013; Xie *et al.*, 2014), *TM6* (Xie *et al.*, 2014), *Chloroflexi* (Bareither *et al.*, 2013; Xie *et al.*, 2014), *Actinobacteria* (Xie *et al.*, 2014), *Proteobacteria* (Bareither *et al.*, 2013; Xie *et al.*, 2014), *Lentisphaerae* (Bareither *et al.*, 2013), *Spirochetes* (Bareither *et al.*, 2013), *Synergistetes* (Bareither *et al.*, 2013) and *Thermotogae* (Bareither *et al.*, 2013) were the most abundant phyla detected in this environment.

However, the use of 16S rRNA gene-targeted sequencing approaches as the sole method for the characterisation of microbial communities is not without issue, including PCR bias, which can result in certain members of the community being undetected or under-represented, despite their presence in that environmental niche. One such example is the detection of novel *Fibrobacter* spp. in landfill sites via the use of genus specific 16S rRNA gene PCR primers (McDonald *et al.*, 2008; Ransom-Jones *et al.*, 2014), despite their absence from both 16S rRNA gene clone libraries (Burrell *et al.*, 2004; Huang *et al.*, 2004; Huang *et al.*, 2005) and studies utilising 454 pyrosequencing (Bareither *et al.*, 2013; Xie *et al.*, 2014). In addition, qPCR on cDNA utilising genus specific 16S rRNA gene PCR primers has demonstrated that these *Fibrobacter* spp. can comprise as much as 40% of the total bacterial community in landfill (McDonald *et al.*, 2008), and qPCR analysis of DNA extracted from the heavily degraded colonised cotton studied here demonstrated that fibrobacters represented 28.9% of the total bacterial 16S rRNA, in comparison to members of the clostridia for which the highest relative abundance was that of *Clostridium* cluster III at 17.2% (McDonald *et al.*, 2012).

Although PCR amplicon sequencing studies can generate a wealth of taxonomic information, inherent limitations do exist, including the reliance on PCR primer sets that are designed based on sequences already present in the public databases, and that the specificity of primer sets used is at best limited to one domain. Whole community metagenome sequencing studies can overcome some of these problems, as they do not rely on specific PCR primer sets and therefore can be utilised to simultaneously study bacterial, eukaryotic, archaeal and viral diversity present in the same sample, in addition to potentially novel members of the community that would be undetectable by PCR-based analysis. Metagenome sequencing can also be utilised in order to assign function to the members of the microbial community, and potentially inform further cultivation-based approaches (Pope *et al.*, 2011; Renesto *et al.*, 2003).

Here, 16S rRNA gene targeted 454 pyrosequencing was used to assess the taxonomic diversity of both heavily and poorly degraded colonised cotton incubated in two landfill leachate microcosms. The use of amplicon sequencing enabled a direct comparison of community composition between the two samples in order to determine the differences in the microbial communities, and gain a greater insight into which species are involved in the cellulose degradation that occurs in landfill sites. In addition, metagenome sequencing of the heavily degraded colonised cotton biofilm was

performed in order to determine both the taxonomy and function of landfill cellulolytic organisms. A further aim of this study was to investigate the role of *Fibrobacter* spp. as cellulolytic members of the landfill microbial community via the assembly of large metagenome contigs belonging to the novel *Fibrobacter* spp. present in this environment (McDonald *et al.*, 2008). The detection and annotation of *Fibrobacter* genome contigs in a highly cellulolytic biofilm could potentially indicate the genomic potential of fibrobacters for cellulose biodegradation in landfill sites, particularly as fibrobacters comprised 28.9% of the total bacterial 16S rRNA present on the heavily degraded colonised cotton biofilm sample studied here (as determined via qPCR) (McDonald *et al.*, 2012). These data would enable the determination of the function of the species, including fibrobacters, present in landfill, and potentially aid future cultivation approaches in order to characterise the members of this poorly studied genus (Pope *et al.*, 2011; Renesto *et al.*, 2003).

5.2 Materials and methods

5.2.1 Construction of landfill leachate microcosms containing dewaxed cotton dewaxed cotton string

Archive samples of dewaxed cotton string from microcosms 1, containing leachate from Brombrough Dock risers 3 and 4 (C1-R3) and 2, containing leachate from Brombrough Dock riser 5 (C2-R5) as described and previously studied by McDonald *et al.* (2012) was stored at -80°C prior to use as the source material for DNA extraction.

5.2.2 DNA extraction of colonised cotton from landfill leachate microcosms for 454 pyrosequencing and metagenome analysis

Both the poorly and heavily degraded cotton from the microcosms described in McDonald *et al.*, (McDonald *et al.*, 2012) were used as a source of material for DNA extraction via the method of Griffiths *et al.* (2000) as discussed in chapter 2.

5.2.3 Metagenome sequencing of heavily degraded colonised cotton (C1-R3)

Total DNA extracted from the heavily degraded colonised cotton was utilised to generate three sequencing libraries with insert sizes of 300, 400 and 600 bp, which were then sequenced on one lane of an Illumina HiSeq, generating paired-end libraries (2 x 100 bp), by the Centre for Genomic Research, Liverpool, UK.

5.2.4 Assembly of the heavily degraded colonised cotton (C1-R3) metagenome

Adapter sequences were removed using Cutadapt (version 1.2.1) (Martin, 2011) and trimmed via Sickle (version 1.2) (Joshi and Fass, 2011) with a minimum window quality score of 20 and reads shorter than 10 bp removed. The three sequence libraries were assembled via MetaVelvet (version 1.2.01, k-mer = 61) (Namiki *et al.*, 2012) using the HPC Wales computing network.

5.2.5 Metagenome analysis of the heavily degraded colonised cotton biofilm C1-R3 via MG-RAST

Contigs assembled from each library were uploaded to the MG-RAST server (version 3.3.6) (Meyer *et al.*, 2008) using the option for assembled sequences and the default quality control settings. Taxonomic profiling based on 16S/18S rRNA genes was performed against the M5RNA rRNA gene database via the best hit classification algorithm with an E-value cutoff of 1E-5, a minimum identity of 80% and a minimum alignment length of 50 bp. Functional annotation was determined via the hierarchical classification algorithm against the KEGG (KO) database, E-value cutoff of 1E-5, a minimum identity of 80% and a minimum alignment of 50 bp. Recruitment plot analysis identified genomes containing proteins similar to those contained in the metagenome libraries, and further analysis was conducted for each of the three libraries against the most similar genome, *Fibrobacter succinogenes* subsp. *succinogenes* S85, with an E-value cutoff of 1E-3.

5.2.6 454 pyrosequencing of general bacterial 16S rRNA gene PCR amplicons generated from poorly (C2-R5) and heavily (C1-R3) degraded colonised cotton samples

DNA extracted from both the heavily and poorly degraded colonised cotton resulted in extracts of comparable quantity and quality. These extracts were subjected to PCR with barcoded general bacterial primers that targeted the V1-V3 region of the 16S rRNA gene, forward primer B16S-F (5' - 3' sequence GAGTTTGATCMTGGCTCAG) and reverse primer B16 (5' - 3' sequence WTTACCGCGGCTGCTGG) by Chunlab Inc., Republic of Korea, resulting in sequences of approximately 500bp. These PCR amplicons were then purified via the QIAquick PCR Purification Kit (Qiagen) before sequencing with the 454 GS FLX Titanium Sequencing System by Chunlab Inc., Republic of Korea.

5.2.7 Analysis of the 454 pyrosequencing PCR amplicons generated from poorly (C2-R5) and heavily (C1-R3) degraded colonised cotton

The 16S rRNA gene sequences were processed to separate the samples via the barcodes before removal of the barcode, linker and PCR primer sequences via Chunlab Inc., Republic of Korea. Sequences were classified via CLcommunity against the ExTaxon database using the default parameters.

5.3 Results

5.3.1 Bacterial community composition of colonised cotton biofilms from landfill leachate microcosms as determined by 16S rRNA gene pyrosequencing

The colonised cotton from two landfill leachate microcosms, C1-R3 (microcosm one, heavily degraded cotton) and C2-R5 (microcosm two, poorly degraded cotton), analysed in a previous study (McDonald *et al.*, 2012) was subjected to DNA extraction and 454 pyrosequencing of 16S rRNA gene PCR amplicons (V1-V3 region). A total of 10,783 reads were generated for sample C2-R5 and 6690 reads for C1-R3 (Appendix 4), which were analysed against the EzTaxon database. A total of 22 and 24 phyla were identified for poorly (C2-R5) and heavily degraded (C1-R3) cotton respectively, with 0.01% of reads from the heavily degraded cotton unable to be classified at the phylum level, whilst all reads from the poorly degraded cotton were able to be assigned to a phylum (Fig. 5.1). The bacterial community composition varied between the two samples, with the dominant phyla within the poorly degraded cotton determined as *Bacteroidetes* (72.7%), *Proteobacteria* (18.7%) and *Actinobacteria* (3.3%), whilst for the heavily degraded cotton the dominant phyla were *Firmicutes* (34.7%), *Bacteroidetes* (20.5%), *Spirochaetes* (14.8%) and *Fibrobacteres* (14.2%) (Fig. 5.1).

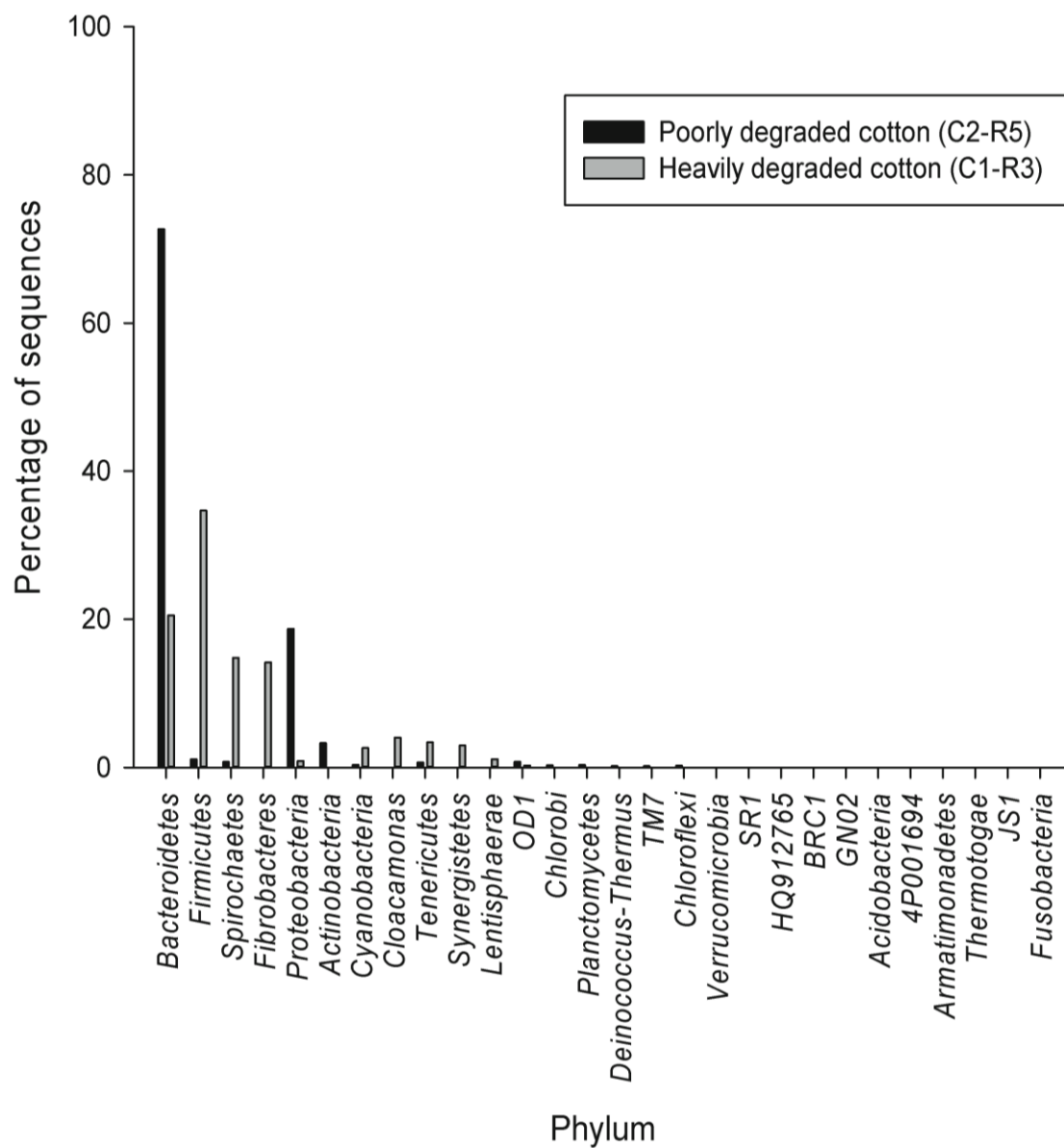


Figure 5.1. Comparison of bacterial phyla identified via 16S rRNA gene PCR amplicon sequences from poorly (black bars) and heavily (grey bars) degraded cotton from landfill leachate microcosms as determined via comparison of the sequences against the EzTaxon database.

5.3.1.1 Taxonomic classification at the class level of 454 pyrosequenced 16S rRNA gene PCR amplicons derived from poorly degraded colonised cotton from landfill leachate microcosm C2-R5

Of the 22 phyla detected from the poorly degraded colonised cotton sample via analysis of the 16S rRNA gene sequences against the EzTaxon database, the three most abundant phyla were *Bacteroidetes* (72.7%), *Proteobacteria* (18.7%) and *Actinobacteria* (3.3%) (Fig. 5.1). Within the *Bacteroidetes* phylum, five classes were detected of which the predominant was *Bacteroidia*, comprising 92.5% of sequences assigned to *Bacteroidetes*, as well as *Flavobacteria* (4.7%), *Sphingobacteria* (1.0%), *Cytophagia* (0.8%), *Balneola* (0.9%) and 0.04% of sequences belonging to the *Bacteroidetes* phylum that were unable to be classified at the class level (Appendix 4). Within the *Proteobacteria*, four classes, *Gammaproteobacteria* (67.7%), *Betaproteobacteria* (26.9%), *Alphaproteobacteria* (4.0%) and *Epsilonproteobacteria* (1.5%) were detected whilst *Micrococcales* (96.1%), *Bifidobacteriales* (1.7%), *Frankiales* (1.1%) and *Propionibacteriales* (0.8%) were the classes detected within the *Actinobacteria* with 0.3% of sequences assigned to this phylum unable to be assigned to a particular class (Appendix 4).

5.3.1.2 Taxonomic classification at the class level of 454 pyrosequenced 16S rRNA gene PCR amplicons derived from heavily degraded colonised cotton from landfill leachate microcosm C1-R3

A total of 24 phyla were detected on the heavily degraded colonised cotton sample, with the most abundant phyla comprising *Firmicutes* (34.7%), *Bacteroidetes* (20.5%), *Spirochaetes* (14.8%) and *Fibrobacteres* (14.2%) (Fig. 5.1). Members of the *Clostridia* (96.6%) were the most abundant class within the *Firmicutes* phylum, with *Erysipelotrichi* (2.0%), *Bacilli* (1.2%) and an uncharacterised class for which the representative sequence accession number was AB476673 (0.04%) also detected (Appendix 4). Within the *Bacteroidetes*, *Bacteroidia* were the predominant class detected comprising 99.3% of the sequences assigned to this phylum. Members of the *Flavobacteria* (0.4%), *Sphingobacteria* (0.2%) and *Cytophagia* (0.1%) were also present, with the remaining 0.1% of sequences unable to be assigned at the class level (Appendix 4). Of the sequences assigned to the *Spirochaetes*, 99.9% were members of the class *Spirochaetes* and the remaining 0.1% unclassified whilst those assigned as members of

the *Fibrobacteres* phylum comprised entirely of those designated as belonging to the class *Fibrobacteria* (Appendix 4).

5.3.2 Metagenome assembly of heavily degraded colonised cotton sample

The three trimmed sequence libraries generated via Illumina HiSeq technology were assembled via MetaVelvet, generating a total of 546,248 contiguous sequences (contigs) (Table 5.1).

Table 5.1. Assembly of heavily degraded colonised cotton metagenome sequences.

a. Paired end reads consist of both a forward and reverse sequence, therefore each paired end read represents two of the sequences after processing.

| Library | Initial number of reads | Number of reads after processing | Number of paired-end reads ^a | K-mer length used | Number of contigs | Average contig length (bp) | Largest contig length (bp) |
|---------|-------------------------|----------------------------------|---|-------------------|-------------------|----------------------------|----------------------------|
| 300 bp | 135,007,994 | 134,585,268 | 67,087,235 | 61 | 115,162 | 780.4 | 134,352 |
| 400 bp | 103,519,620 | 102,970,873 | 51,216,304 | 61 | 328,447 | 497.7 | 103,882 |
| 600 bp | 93,776,958 | 92,853,901 | 45,970,929 | 61 | 102,639 | 826.5 | 91,326 |

5.3.3 Analysis of heavily degraded colonised cotton metagenome contigs against 16S/18S

rRNA gene data

The taxonomy of contigs assembled via MetaVelvet from the heavily degraded cotton sample was determined via comparison against the M5RNA rRNA gene database via MG-RAST. Bacteria were the dominant domain comprising 85.6% of the community, with 4.1% assigned to eukaryota, 2.7% to archaea and 0.3% to viruses, with 3.3% of contigs unable to be classified at the domain level (Fig. 5.2). A total of 18 bacterial phyla were identified with the predominant phyla determined as *Firmicutes* (47.2%), *Bacteroidetes* (22.8%), *Proteobacteria* (6.4%) and *Spirochaetes* (6.1%), with 4.4% of these contigs unable to be assigned to a specific phylum (Fig. 5.3) (Appendix 5). The eukaryota and archaea consisted of 9 and 1 phyla respectively, with the dominant eukaryota determined as *Arthropoda* (59.6%) and *Streptophyta* (11.5%) (Fig. 5.4), whilst *Euryarchaeota* (91.18%) dominated the reads assigned to the archaea (Fig. 5.5).

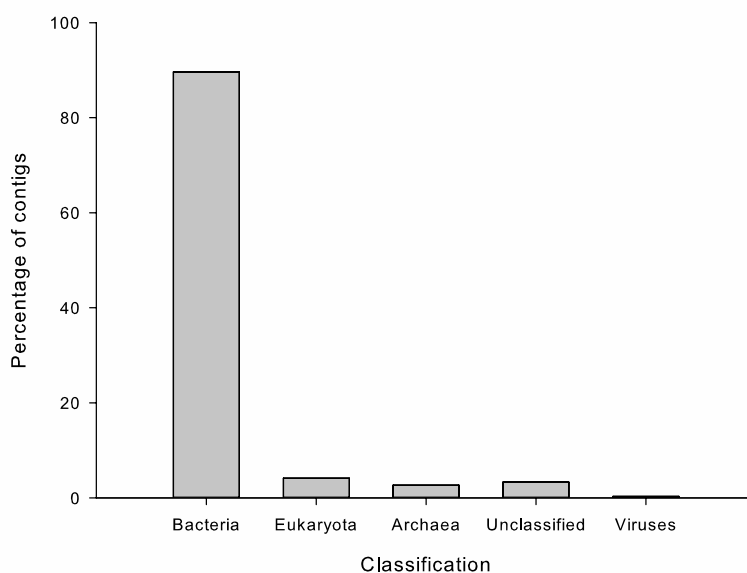


Figure 5.2. Classification of contigs derived from the metagenome of heavily degraded colonised cotton incubated in a landfill leachate microcosm. Domains were assigned via analysis of 16/18S rRNA gene sequences by MG-RAST against the M5RNA database, utilising the best hit classification algorithm with an E-value cutoff of $1E-5$, a minimum identity of 80% and a minimum alignment length of 50 bp.

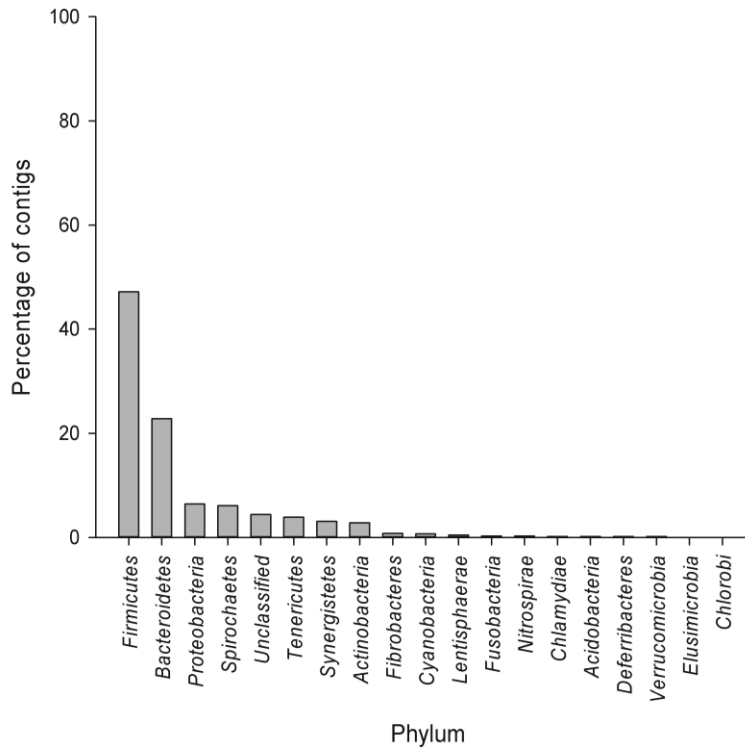


Figure 5.3. Classification of bacterial phyla via analysis of contigs derived from the metagenome of heavily degraded colonised cotton incubated in a landfill leachate microcosm. Assignments were determined via analysis of 16S rRNA gene sequences by MG-RAST against the M5RNA database, utilising the best hit classification algorithm with an E-value cutoff of 1E-5, a minimum identity of 80% and a minimum alignment length of 50 bp.

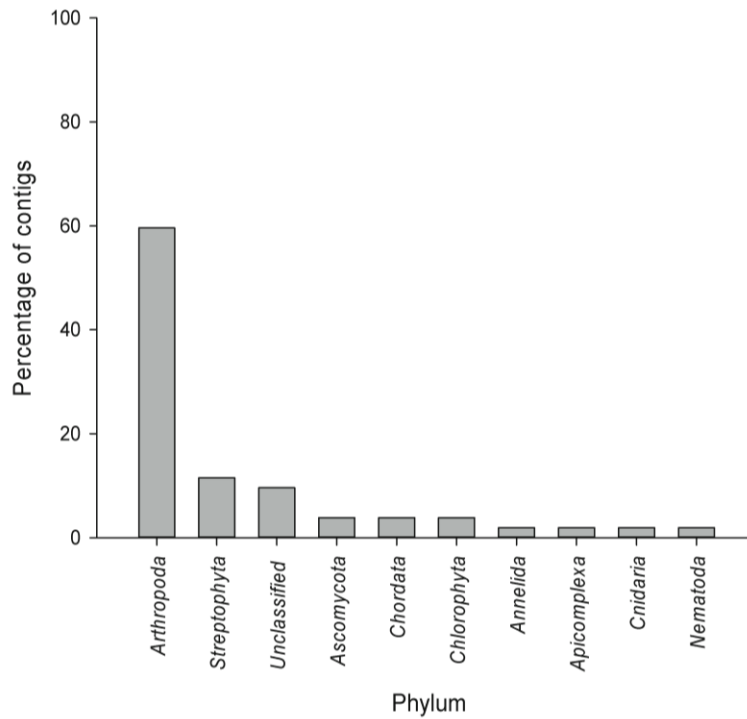


Figure 5.4. Classification of phyla assigned as eukaryota via analysis of contigs derived from the metagenome of heavily degraded colonised cotton incubated in a landfill leachate microcosm. Assignment determined via analysis of 18S rRNA gene sequences by MG-RAST against the M5RNA database, utilising the best hit classification algorithm with an E-value cutoff of $1E-5$, a minimum identity of 80% and a minimum alignment length of 50 bp.

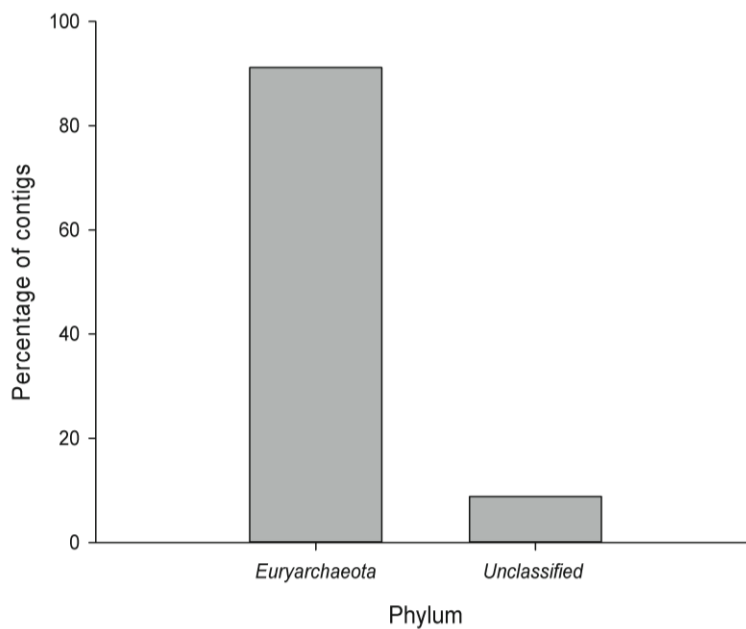


Figure 5.5. Classification of phyla assigned as archaea via analysis of contigs derived from the metagenome of the heavily degraded colonised cotton incubated in a landfill leachate microcosm. Assignment determined via analysis of 16S rRNA gene sequences by MG-RAST against the M5RNA database, utilising the best hit classification algorithm with an E-value cutoff of $1E-5$, a minimum identity of 80% and a minimum alignment length of 50 bp.

5.3.3.1 Classification of metagenome contigs derived from heavily degraded colonised cotton from landfill leachate microcosm C1-R3 at the class level via comparison against 16S/18S rRNA gene data

Of the 18 bacterial phyla identified via analysis of contigs against the M5RNA database with MG-RAST, the *Firmicutes* (47.2%), *Bacteroidetes* (22.8%), *Proteobacteria* (6.4%) and *Spirochaetes* (6.1%) were the most abundant phyla (Fig. 5.3). Within the *Firmicutes* phylum four classes were identified, *Clostridia* (79.7%), *Bacilli* (15.7%), *Erysipelotrichi* (2.6%) and *Negativicutes* (2.1%) (Appendix 5). Contigs identified as *Bacteroidetes* were also assigned to one of four classes, *Bacteroidia* (57.5%), *Flavobacteriia* (23.9%), *Sphingobacteriia* (9.3%) or *Cytophagia* (3.9%), with 5.4% of these contigs unable to be assigned to a class (Appendix 5). Members of the *Proteobacteria* belonged to one of six classes, *Gammaproteobacteria* (30.1%), *Alphaproteobacteria* (23.3%), *Deltaproteobacteria* (23.3%), *Betaproteobacteria* (16.4%), *Epsilonproteobacteria* (4.1%) or *Zetaproteobacteria* (2.7%), whilst the members of the *Spirochaetes* phylum were all assigned to the class *Spirochaetia* (Appendix 5).

Contigs designated as members of the eukaryota via MG-RAST were assigned to one of nine phyla, of which *Arthropoda* (59.6%) and *Streptophyta* (11.5%) were the most abundant (Fig. 5.4). Within the *Arthropoda* two classes were present, *Insecta* (90.3%) and *Maxillopoda* (9.7%), whilst *Liliopsida* (66.7%) was the only class detected within the *Streptophyta* phylum with the remaining 33.3% of contigs unable to be classified at the class level (Appendix 5). Only one phylum (*Euryarchaeota*) was identified within the archaea (Fig. 5.5) for which *Methanomicrobia* (93.6%) was the sole class identified with the remainder of the contigs (6.5%) unassigned to a class (Appendix 5).

5.3.4 Functional analysis of heavily degraded colonised cotton metagenome contigs

Functional affiliation of predicted proteins from the heavily degraded colonised cotton metagenome was determined via comparison against the KEGG (KO) database using MG-RAST. These proteins were assigned to one of six processes, of which those assigned to functions relation to metabolism had the greatest abundance (47.5%) (Fig. 5.6). Within this category a further ten putative functions could be determined with amino acid metabolism comprising 35.2% of proteins, energy metabolism 31.3% and carbohydrate metabolism 16.8% (Fig. 5.7).

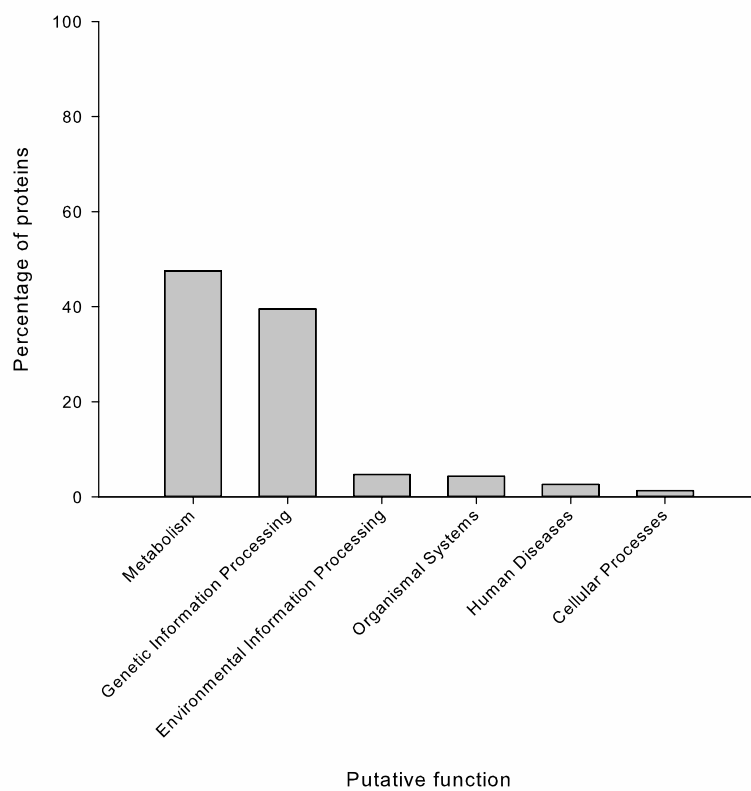


Figure 5.6. Predicted function of proteins as identified via MG-RAST in metagenome contigs derived from the heavily degraded colonised cotton sample.

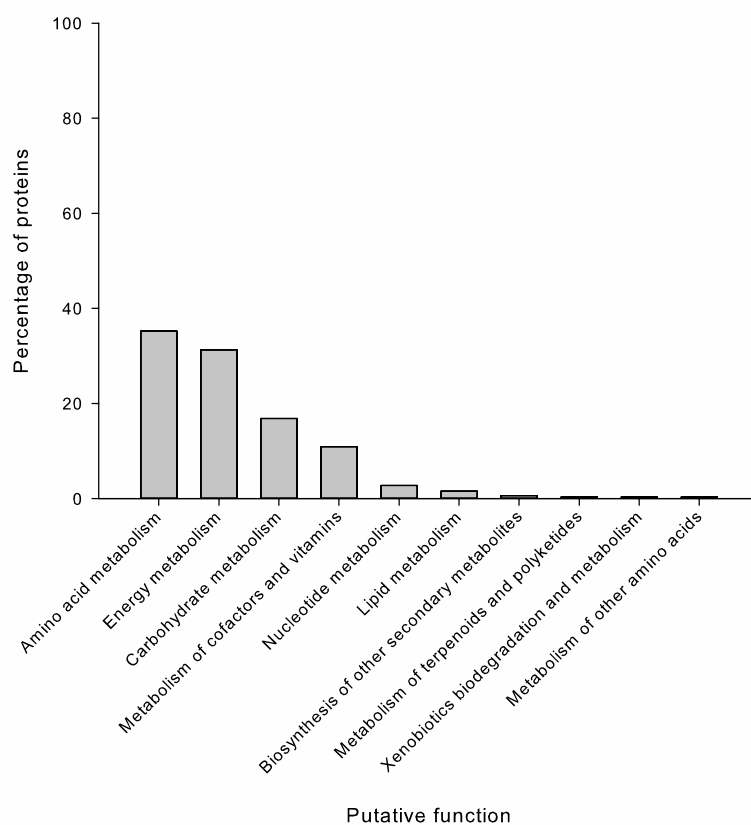


Figure 5.7. Classification of proteins from the heavily degraded colonised cotton metagenome assigned to the metabolism category via MG-RAST

5.3.5 Comparison of the heavily degraded colonised cotton metagenome contigs against the genome sequence of *Fibrobacter succinogenes* subsp. *succinogenes* S85

The three libraries were compared against related reference genomes via MG-RAST via recruitment plot analysis. For each of the libraries, *F. succinogenes* S85 was the genome with the most similarities to the metagenome contigs (Table 5.2). For each of the libraries, glycoside hydrolase 9 was within the 10 most prevalent matches when compared with *F. succinogenes* S85 (Table 5.3), and both carbohydrate binding family 11 and glycosyl hydrolase family 98 putative carbohydrate were within the top 20 protein matches.

Table 5.2. Genomes most closely related to the heavily degraded colonised cotton metagenome as determined via analysis with MG-RAST.

| Library | Genome | Number of protein matches |
|---------|--|---------------------------|
| 300 bp | <i>Fibrobacter succinogenes</i> subsp. <i>succinogenes</i> S85 | 5931 |
| | <i>Treponema vincentii</i> ATCC 35580 | 2153 |
| | <i>Treponema denticola</i> ATCC 35405 | 1934 |
| | <i>Paludibacter propionicigenes</i> WB4 | 1554 |
| | <i>Clostridium thermocellum</i> ATCC 27405 | 1131 |
| 400 bp | <i>Fibrobacter succinogenes</i> subsp. <i>succinogenes</i> S85 | 7460 |
| | <i>Methanoculleus marisnigri</i> JR1 | 4239 |
| | <i>Paludibacter propionicigenes</i> WB4 | 4017 |
| | <i>Treponema vincentii</i> ATCC 35580 | 2681 |
| | <i>Clostridium thermocellum</i> ATCC 27405 | 2551 |
| 600bp | <i>Fibrobacter succinogenes</i> subsp. <i>succinogenes</i> S85 | 5783 |
| | <i>Treponema vincentii</i> ATCC 35580 | 1900 |
| | <i>Treponema denticola</i> ATCC 35405 | 1708 |
| | <i>Paludibacter propionicigenes</i> WB4 | 1544 |
| | <i>Clostridium thermocellum</i> ATCC 27405 | 1045 |

5.3.6 Identification of carbohydrate-active enzymes (CAZymes) present within the heavily degraded colonised cotton metagenome

Each of the three libraries were searched for the presence of proteins identified as glycoside hydrolases via MG-RAST. For all three libraries the most abundant classification was for proteins that were unable to be assigned to a specific family, followed by the presence of family 9 glycoside hydrolases in the 300 and 400 bp libraries and family 43 glycoside hydrolases in the 600 bp library (Table 5.3).

Table 5.3. Glycoside hydrolase families identified in the metagenome libraries generated from the heavily degraded colonised cotton.a. Function as identified via the Carbohydrate Active Enzymes database (Lombard *et al.*, 2014)

| Glycoside hydrolase | Function of enzymes in this category ^a | Number of protein matches (300 bp) | Number of protein matches (400 bp) | Number of protein matches (600 bp) |
|-----------------------------|---|------------------------------------|------------------------------------|------------------------------------|
| Family protein | Unclassified | 151 | 382 | 128 |
| Family 9 | Mainly cellulose degradation, second largest cellulase family | 85 | 110 | 84 |
| Family 3 domain protein | Cellulose degradation, cell wall modification, pathogen defence | 83 | 177 | 51 |
| Family 43 | Plant cell wall degradation, includes xylanases | 69 | 170 | 73 |
| Family 8 | Enzymes that degrade cellulose, chitin, lichen and xylan | 27 | 42 | 21 |
| Family 31 | Enzymes that degrade numerous substrates including starch | 24 | 47 | 17 |
| Family 16 | Enzymes that target numerous substrates | 20 | 30 | 25 |
| Family 5 | Largest family, includes cellulases and xylanases | 17 | 41 | 12 |
| Family 65 central catalytic | Phosphorylases targeting various substrates | 16 | 18 | 16 |
| Family 3 protein | Cellulose degradation, cell wall modification, pathogen defence | 16 | 86 | 21 |

5.4 Discussion

5.4.1 Bacterial community composition as determined via 16S rRNA gene amplicon sequencing of the poorly degraded colonised cotton

A total of 22 phyla were detected on the poorly degraded colonised cotton sample, of which the dominant phyla were *Bacteroidetes* (72.7%), *Proteobacteria* (18.7%) and *Actinobacteria* (3.3%) (Fig. 5.1). Members of the *Bacteroidetes* have been previously identified in landfill sites both via general bacterial 16S rRNA gene clone libraries (Huang *et al.*, 2004; Huang *et al.*, 2005) and 454 pyrosequencing of 16S rRNA gene PCR amplicons (Bareither *et al.*, 2013; Xie *et al.*, 2014). At the class level, *Bacteroidia* comprised 92.5% of the sequences assigned to the *Bacteroidetes* phylum, supporting a previous 454 pyrosequencing study on 16S rRNA gene PCR amplicons derived from an anaerobic bioreactor that was treating landfill leachate, which demonstrated that *Bacteroidia* were the dominant class of *Bacteroidetes* identified (Xie *et al.*, 2014). *Bacteroidetes* are known to occupy a variety of ecological niches including activated sludge, decaying plant material and compost, and are capable of degrading both polysaccharides and proteins (Thomas *et al.*, 2011), though given that this cotton was poorly degraded it is more likely that the species identified here were involved in protein degradation and the hydrolysis of simple polysaccharides that were present in the landfill leachate.

Members of the *Proteobacteria* were the second most abundant phylum comprising 18.7% of the sequences (Fig. 5.1), of which the dominant classes were *Gammaproteobacteria* (67.7%) and *Betaproteobacteria* (26.9%) (Appendix 4). *Proteobacteria* have been detected in 16S rRNA gene clone libraries from landfill leachate (Huang *et al.*, 2004; Huang *et al.*, 2005), with *Gammaproteobacteria* (22 clones), and *Betaproteobacteria* (14 clones) identified as the most abundant classes of *Proteobacteria* (Huang *et al.*, 2005). Members of the *Proteobacteria* have also been detected in landfill leachate via 454 pyrosequencing of 16S rRNA gene PCR amplicons (Bareither *et al.*, 2013; Xie *et al.*, 2014) in which *Gammaproteobacteria* (Bareither *et al.*, 2013) or *Betaproteobacteria* (Xie *et al.*, 2014) were the predominant class of *Proteobacteria*. The detection of *Proteobacteria* as a key member of the landfill biofilm is therefore unsurprising, and it is possible that the poorly degraded colonised cotton sample simply provided an inert surface for which members of this and other phyla present could colonise whilst metabolising the nutrients already present in the landfill leachate.

Within the poorly degraded colonised cotton 16S rRNA gene PCR amplicon library, members of the phylum *Actinobacteria* comprised 3.3% of sequences, with 96.1% of these assigned to the class *Micrococcales* (Appendix 4). *Actinobacteria* have previously been detected

in landfill leachate via a clone library (Huang *et al.*, 2004) and 454 pyrosequencing (Xie *et al.*, 2014), both targeting the 16S rRNA gene. Given the broad ecological range of members of the *Actinobacteria*, including both natural and managed environments, with the majority of species having a saprophytic role and contributing to nutrient cycling in this manner (Goodfellow and Williams, 1983), their detection in landfill leachate is unsurprising.

5.4.2 Bacterial community composition as determined via 454 pyrosequencing of the heavily degraded colonised cotton

Of the 24 phyla detected via 454 pyrosequencing of the 16S rRNA gene, the four most abundant phyla were *Firmicutes* (34.7%), *Bacteroidetes* (20.5%), *Spirochaetes* (14.8%) and *Fibrobacteres* (14.2%) (Fig. 5.1). Members of the *Firmicutes* have previously been identified as 100% and 90% of the 16S rRNA gene clones in libraries derived from solid cellulosic material and mixed cellulosic/leachate material respectively from a bioreactor treating landfill leachate (Burrell *et al.*, 2004), and have also been detected in other 16S rRNA gene clone library studies (Huang *et al.*, 2004; Huang *et al.*, 2005). 454 pyrosequencing studies targeting the 16S rRNA gene have also detected both *Firmicutes*, and more specifically *Clostridia*, within both an anaerobic bioreactor (Xie *et al.*, 2014) and a lab-scale reactor (Bareither *et al.*, 2013) treating landfill leachate, with *Clostridia* identified as the most abundant class within the *Firmicutes* (Bareither *et al.*, 2013; Xie *et al.*, 2014). The identification of members of the *Clostridia* supports previous qPCR analysis of this heavily degraded colonised cotton sample, which determined that *Clostridium* clusters III, IV and XIV totalled 21.3% of the bacterial community. Of the 19 *Clostridium* clusters, four (I, III, IV and XIVab) contain cellulolytic species (Collins *et al.*, 1994), and it is likely that the members of these clusters have played a role in the degradation of the cotton sampled here, with members of clusters III and IV most commonly identified in landfill (Van Dyke and McCarthy, 2002; Li *et al.*, 2009; McDonald *et al.*, 2012; Burrell *et al.*, 2004), along with cluster XIV (McDonald *et al.*, 2012; Burrell *et al.*, 2004). Members of cluster I have also been isolated from the landfill environment both in chapters 3 and 4, and in a previous study (Krishnamurthi and Chakrabarti, 2013), despite their absence in a previous landfill 16S rRNA gene clone library (Burrell *et al.*, 2004) and the lack of detection of this cluster using cluster-specific 16S rRNA gene PCR primers (Van Dyke and McCarthy, 2002; McDonald *et al.*, 2012), therefore suggesting a potential role for cluster I clostridia in landfill cellulose decomposition.

Members of the *Bacteroidetes* were also detected on the heavily degraded cotton, as well as on the poorly degraded sample (Fig. 5.1), although they comprised 20.5% of sequences

of the heavily degraded cotton in comparison to 72.7% on the poorly degraded sample. For both samples, *Bacteroidia* was the most prevalent class and as discussed previously this is unsurprising given the previous dominance of this class within a 16S rRNA gene 454 pyrosequencing dataset derived from landfill leachate (Xie *et al.*, 2014). Both proteins and polysaccharides can be utilised by *Bacteroidetes* (Thomas *et al.*, 2011), thus accounting for these presence of this phylum in both samples despite the differences in the degradation of the cotton, and it is likely that more cellulolytic members of this genus are present on the heavily degraded cotton sample than on the poorly degraded sample.

Sequences assigned to the *Spirochaetes* were the third most dominant phylum (14.8%) and have been identified in landfill both by 16S rRNA gene clone libraries (Huang *et al.*, 2004; Huang *et al.*, 2005) and 454 pyrosequencing targeting the 16S rRNA gene (Bareither *et al.*, 2013; Xie *et al.*, 2014). Spirochetes have also been isolated from the bovine rumen, (Stanton and Canaleparola, 1979) and although are not cellulolytic, they are capable of utilising polymers such as xylan, pectin, starch and cellobiose and may act in a symbiotic manner with cellulolytic organisms in order to improve the hydrolysis of cellulose (Leschine, 1995). When *Spirochaeta caldaria* was grown in co-culture with *Clostridium thermocellum*, a cellulolytic bacteria, the rate of cellulose degradation in the co-culture was higher than that observed in the pure culture of *C. thermocellum* (Pohlschröder *et al.*, 1994). This is possibly due to the removal of cellobiose by *S caldaria*, as high concentrations of cellobiose can have an inhibitory effect on cellulose hydrolysis (Ljungdahl and Eriksson, 1985). The importance of spirochetes in the rumen environment and their detection both here and in previous studies may suggest that they have an important role in the cellulose degradation that occurs in landfill sites, especially in light of the fact that two species of *Treponema*, *T. vincentii* and *T. denticola*, were the second and third genomes with the most protein matches against the 300 and 600 bp metagenome libraries via recruitment plot analysis (Table 5.2), and *T. vincentii* was also the fourth genome with the most protein matches against the 400 bp library (Table 5.2).

The detection of members of the *Fibrobacteres* as 14.2% of the sequences at the phylum level is perhaps more surprising. Despite the detection of fibrobacters in landfill sites via genus specific 16S rRNA gene PCR primers (McDonald *et al.*, 2008, McDonald *et al.*, 2012), they remained undetected in this environment via either 16S rRNA gene clone libraries (Huang *et al.*, 2004; Huang *et al.*, 2005; Burrell *et al.*, 2004) or 454 pyrosequencing approaches (Bareither *et al.*, 2013; Xie *et al.*, 2014). Given the absence of *Fibrobacter* spp. in other landfill leachate studies (Burrell *et al.*, 2004; Huang *et al.*, 2004; Huang *et al.*, 2005; Bareither *et al.*, 2013; Xie *et al.*, 2014), the fact that they were both identified and comprised the fourth most

abundant phylum within this 16S rRNA gene amplicon library (Fig. 5.1) supports the assertion that fibrobacters are one of the prevalent members of the bacterial community within this environment (McDonald *et al.*, 2012). Given their low abundance on the poorly degraded cotton as determined in the 16S rRNA gene PCR amplicon library generated in this study (Fig. 5.1), in comparison to the heavily degraded colonised cotton, it is evident that members of the genus *Fibrobacter* are an important member of the landfill cellulolytic microbial community.

5.4.3 Taxonomic profile of metagenome contigs derived from heavily degraded colonised cotton

Of the 85.6% of contigs that were designated as bacteria at the domain level, 18 phyla were identified, with members of the *Firmicutes* (47.2%), *Bacteroidetes* (22.8%), *Proteobacteria* (6.4%) and *Spirochaetes* (6.1%) the most abundant (Fig. 5.3). The presence of the *Firmicutes*, *Bacteroidetes* and *Spirochaetes* is similar to that observed in the heavily degraded colonised cotton 16S rRNA gene PCR amplicon library, however *Proteobacteria* were more readily detected on the poorly degraded colonised cotton (18.7%) than the heavily degraded cotton (0.9%) (Fig. 5.1). In addition, *Fibrobacteres* comprised 0.8% of the metagenome contigs (Fig. 5.3) in comparison to 14.2% of the sequences from the heavily degraded cotton 454 pyrosequencing dataset (Fig. 5.1). The disparity between these results may be explained by the nature of the different sequencing approaches used. The amplicon approach sequenced only the 16S rRNA gene, thus ensuring that all sequences could be compared against each other and differences in the members of the bacterial community between the poorly and heavily degraded colonised cotton inferred with more accuracy, although as discussed previously there are issues with PCR bias, particularly in relation to fibrobacters (Tajima *et al.*, 2001). In contrast, the metagenome sequencing approach resulted in the generation of both 16/18S rRNA gene and other gene sequences, many of which were protein sequences that could therefore not be assigned to a particular taxonomic rank via analysis against a 16/18S gene database, thus potentially resulting in fewer sequences being assigned as belonging to the *Fibrobacteres*.

5.4.4 Functional analysis of the heavily degraded colonised cotton metagenome

Of the predicted proteins that were assigned putative functions via MG-RAST, the largest number (47.5%) were involved in metabolism (Fig. 5.6), with further analysis of this group revealing proteins involved in amino acid metabolism (35.2%), energy metabolism (31.3%) and carbohydrate metabolism (16.8%) (Fig. 5.7). The proteins assigned to energy and

carbohydrate metabolism are unsurprising given the degraded nature of this cotton sample, and the detection of high numbers of members of the *Clostridia*, of which many are known cellulose degraders (Collins *et al.*, 1994) and *Bacteroidetes*, which can also utilise complex polysaccharides as well as proteins and amino acids (Thomas *et al.*, 2011).

Recruitment plot analysis demonstrated that the genome of *F. succinogenes* S85 had the most proteins that matched proteins encoded within the metagenome (Table 5.2). This is intriguing given the low abundance of members of the *Fibrobacteres* as determined via comparison of 16S rRNA gene sequences, however, as discussed previously this could be due to the nature of metagenome sequencing, which sequences random fragments of the entire genome rather than one specific region, and it is therefore more likely that more protein coding regions will have been sequenced than 16S rRNA gene regions. The abundance of proteins similar to that of *F. succinogenes* subsp. *succinogenes* S85 present in the heavily degraded colonised cotton metagenome as determined via recruitment plot analysis (Table 5.2) also demonstrates that fibrobacters are an important member of the cellulolytic microbial community, especially due to the fact that for each of the libraries compared against *F. succinogenes* S85, enzymes involved in cellulose hydrolysis, including members of the glycoside hydrolase family 9, carbohydrate binding family 11 and glycosyl hydrolase family 98 putative carbohydrate, were within the top 20 hits.

Further analysis of the glycoside hydrolases present within the heavily degraded colonised cotton metagenome revealed that for each of the three libraries, many of the glycoside hydrolases could not be assigned to a family, with glycoside hydrolases belonging to family 9 the second most prevalent in the 300 and 600 bp libraries, and with the glycoside hydrolases assigned as family 3 domain proteins the second most prevalent in the 400 bp library (Table 5.3). The presence of these glycoside hydrolase, especially family 9 which is the largest cellulose family, supports the assertion that the dominant members of this microbial community are responsible for the cellulolytic activity that occurs in landfill sites.

5.5 Conclusions

Although landfill sites are a repository of cellulosic matter, little is understood about the taxonomic and functional diversity of the microbial community. The use of high throughput 454 pyrosequencing of 16S rRNA gene PCR amplicons from two colonised cotton samples at different stages of decomposition from landfill leachate microcosms implicates the involvement of members of the *Firmicutes*, *Bacteroidetes*, *Spirochaetes* and *Fibrobacteres* as having a role in the cellulose hydrolysis that occurs within landfill sites. In addition, the use of metagenome

sequencing on the heavily degraded colonised cotton sample demonstrated the abundance of members of the *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Spirochaetes*, with analysis of the glycoside hydrolases present demonstrating the cellulolytic capabilities of the members of this microbial community, and recruitment plot analysis on the proteins encoded in the heavily degraded colonised cotton metagenome contigs further supported the cellulolytic role of members of the *Fibrobacteres*. These data provide an important insight into the role of members of the microbial community within the landfill environment, with these organisms playing an important role in both carbon cycling and waste management.

5.6 Acknowledgements

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CHAPTER 6

Synthesis

6.1 Synthesis

The most abundant organic polymer on Earth is cellulose, and the hydrolysis of this compound represents a critical step in the global carbon cycle (Leschine, 1995). The degradation of cellulosic biomass is best characterised in the rumen, where fibrobacters are one of the predominant bacterial species that mediate the degradation of cellulose (Denman and McSweeney 2006; Koike and Kobayashi 2001; Kobayashi *et al.*, 2008). One possible explanation for the superior cellulolytic activity of *Fibrobacter* spp. is the apparent evolution of a novel mechanism by which *Fibrobacter* spp. degrade cellulose. The genome sequence of *F. succinogenes* S85 demonstrated that this organism did not utilise either the free cellulase or cellulosomal mechanisms typically associated with cellulose hydrolysis (Suen *et al.*, 2011). It is therefore suggested that *F. succinogenes* attaches to the cellulose via fibro-slime proteins and type IV pilin structures present on the outer membrane of the cell, before using carbohydrate-active enzymes to cleave the cellulose into individual chains that can be transported through the outer membrane into the periplasmic space, where they are further degraded (Wilson, 2009; Suen *et al.*, 2011).

F. succinogenes (then *Bacteroides succinogenes*) was first isolated from the bovine rumen in 1947 (Hungate, 1947; Hungate, 1950), and since then only two species (*F. succinogenes* and *F. intestinalis*) have been formally described, with fibrobacters thought to be present solely in the mammalian intestinal tract (Montgomery *et al.*, 1988). This is due to the obligately anaerobic phenotype of known *Fibrobacter* strains, which makes progress towards the isolation and cultivation of new *Fibrobacter* isolates a challenging process. Consequently, only a handful of additional *Fibrobacter* strains have been isolated in the subsequent 60 years, and all have been obtained from mammalian intestinal tracts (Amann *et al.*, 1992).

However, the paradigm that fibrobacters are restricted to the gut environment has since been disproven via the detection of novel members of the genus *Fibrobacter* in landfill sites (McDonald *et al.*, 2008; McDonald *et al.*, 2012) and freshwater lakes (McDonald *et al.*, 2009) via genus specific 16S rRNA gene PCR primers, which provided the first evidence for fibrobacters existing outside of the gut environment. In addition, these environments contained both species similar to *F. succinogenes*, and novel lineages that may represent as yet uncultivated species, suggesting that the true diversity of this phylum was not yet characterised (Ransom-Jones *et al.*, 2012). The paucity of characterised species also hinders our understanding of both the ecology of the members of this phylum, and the mechanism by which they hydrolyse cellulose. Therefore, the aims of this thesis as stated in chapter 1 were:

1. To determine the ecological range and taxonomic diversity of members of the *Fibrobacteres* phylum.
2. To attempt the isolation and cultivation of the *Fibrobacter* spp. present in landfill sites.
3. To determine the function of members of the *Fibrobacteres* phylum present in landfill sites.

6.2 Results of objective 1; to determine the ecological range and taxonomic diversity of members of the *Fibrobacteres* phylum

As discussed throughout this thesis, much of the previous work on members of the genus *Fibrobacter* has been conducted on the rumen, resulting in a lack of information on both the environmental distribution and diversity contained within this genus. This is compounded by that fact that fibrobacters are obligate anaerobes, thus making them difficult to isolate and cultivate (Hungate, 1966) and that they are poorly represented via molecular methods using general bacterial primers targeting the 16S rRNA gene, even in the rumen where they are known to predominate (Whitford *et al.*; 1998; Daly *et al.*, 2001; Tajima *et al.*, 1999; Tajima *et al.*, 2000; Tajima *et al.*, 2001). However, novel *Fibrobacter* spp. have been detected in landfill sites (McDonald *et al.*, 2008) and freshwater lakes (McDonald *et al.*, 2009) via *Fibrobacter*-specific 16S rRNA gene PCR primers, suggesting that the use of specific primers may be a better method for studying the members of this genus. Therefore, in order to identify the ecological range of fibrobacters, these genus specific 16S rRNA gene PCR primers were used to determine the presence of fibrobacters in 64 environmental samples, as outlined in chapter 2.

Fibrobacters were detected in 23 samples, including equine faecal, ovine and bovine rumen, soil, cryoconite, freshwater, estuarine and marine sediment and landfill leachate samples, demonstrating the broad ecological range of the members of this genus. In addition, the detection of *Fibrobacter* spp. in estuarine and marine sediments and cryoconite represents the first specific detection of members of this genus in these environments, and adds to the current body of knowledge concerning the ecological range of this phylum. These data demonstrate the broad ecological diversity of members of the genus *Fibrobacter*, with a significant proportion of this diversity present outside of the mammalian gut, thus demonstrating that *Fibrobacter* spp. have a wider ecological distribution and greater role in carbon cycling in the biosphere than previously thought. It is therefore likely that fibrobacters are present in a wide variety of anoxic environments in which there is cellulosic biomass.

These data yielded important insights into the ecological distribution of members of the genus *Fibrobacter*, and provided the basis for taxonomic analysis. Clone library sequences of the *Fibrobacter*-specific 16S rRNA gene PCR amplicons were constructed for 17 environmental samples, and these sequences, in addition to all *Fibrobacter* sequences in the Ribosomal Database Project database, were used to determine the phylogeny of the members of this phylum, resulting in the most comprehensive study the *Fibrobacteres* to date. A total of 63 OTUs were generated at 95% sequence similarity, suggesting that there are a number of novel species contained within the *Fibrobacteres* phylum. Of these OTUs, 18 OTUs contained only one sequence, and therefore it cannot be inferred as to whether or not these lineages are exclusive to a particular environment or if they would in actual fact contain sequences derived from several environments if the sequencing coverage was increased. The use of next generation sequencing, such as 454 pyrosequencing or Illumina amplicon sequencing, would enable a better study of the taxonomic diversity present in these environmental samples, however although 454 pyrosequencing was attempted as part of the work contained within chapter 2, it was unsuccessful and so clone library sequencing was utilised instead. At the time, 454 pyrosequencing was selected due to the greater read length than sequencing on the Illumina platform, in order to enable more accurate taxonomic analysis, but this would still have resulted in shorter reads than the clone library sequencing, which generated almost full length *Fibrobacter*-specific 16S rRNA gene PCR amplicon sequences. Since then advances have been made in the Illumina sequencing capability, with the V3 Miseq kit enabling the generation of 2x300 bp paired-end reads (Tang *et al.*, 2014), which would enable both the generation of sufficient length sequences to perform accurate taxonomic analysis and greater coverage to enable further identification of the *Fibrobacter* spp. present in different environments.

The taxonomic analysis of the *Fibrobacteres* phylum also revealed the unexplored diversity contained within the two characterised species, with *F. succinogenes* comprising 11 OTUs (95%) and *F. intestinalis* 3 OTUs (95%), suggesting that the current species designations actually represent distinct genera that comprise a collection of several different species. The cultivation of these novel species is therefore critical to our understanding of the taxonomy and function of the members of this phylum, with the adaptation and diversity of fibrobacters in these environments likely to have also resulted in the evolution of novel enzymes and growth conditions, that may be favourable for biotechnological applications.

6.3 Results of objective 2; to attempt the isolation and cultivation of the *Fibrobacter* spp. present in landfill sites

Whilst the presence and function of fibrobacters is relatively well characterised in the rumen, there are no isolates of *Fibrobacter* spp. from other environments despite the diversity contained within this phylum, described in chapter 2. Because of this lack of isolates it difficult to determine precisely the role of these species in these environments, and it is therefore important to attempt to isolate *Fibrobacter* spp. in order to be able to ascertain their function. Additionally, given the previous characterisation work on landfill sites, including the detection of novel lineages in chapter 2, and the potential abundance of fibrobacters within this environment (McDonald *et al.*, 2008; McDonald *et al.*, 2012), landfill sites were selected as the environment for attempts to isolate and cultivate fibrobacters as outlined in chapters 3 and 4.

In chapter 3, microcosms containing landfill leachate and Avicel were used as a source for the isolation and cultivation of *Fibrobacter* spp. via the method of Hungate (Hungate, 1947). Initially 63 cultures were isolated, of which five were positive for the presence of fibrobacters via PCR using genus specific 16S rRNA gene primers, and these five strains underwent seven rounds of purification resulting in four pure cultures, although these were slow growing and attempts at both nucleic acid extraction and further sub-culturing were unsuccessful. The use of *Fibrobacter* specific 16S rRNA gene PCR primers to identify cultures for further purification is unusual, as normally cultivation studies focus on the purification of strains and then classify the isolates once they are pure. However, given the laborious nature of anaerobic cultivation, and the difficulties in isolating fibrobacters, this screening was required in order to maximise the likelihood of isolating and purifying *Fibrobacter* spp. The difficult nature of these species was demonstrated when it was impossible to further passage or extract DNA from the pure cultures, and without the PCR derived data from previous rounds, it would have been impossible to determine the presence of fibrobacters in the cultures, and utilise 16S rRNA gene sequencing in order to determine the taxonomy of these isolates.

Sequencing of genus specific 16S rRNA gene PCR amplicons from previous rounds of purification determined that these fibrobacters were closely related to *F. succinogenes*, and formed a distinct group within *F. succinogenes* subsp. *succinogenes*. This represents the first isolation of *F. succinogenes* from outside of the mammalian gut, and the first isolation of fibrobacters from a landfill site. The isolation of *F. succinogenes*, rather than the novel fibrobacters detected in landfill sites is intriguing in light of the fact that a previous clone library of genus-specific 16S rRNA gene PCR amplicons, derived from landfill leachate, detected only two *F. succinogenes* clones out of a total of 58 (McDonald *et al.*, 2008). The isolation of *F.*

succinogenes may therefore be due to the use of culture medium designed for the isolation of anaerobic rumen bacteria, thus inadvertently selecting for species related to those present in the rumen. However, the fact that *F. succinogenes* could be isolated and propagated from landfill leachate, combined with its molecular detection in landfill, suggest that it is metabolically active in this environment, rather than being present solely due to faecal pollution.

In order to attempt the isolation of novel *Fibrobacter* spp. from landfill, a combined molecular and cultivation-based study was conducted on dewaxed cotton string from a landfill leachate microcosm. Previous analysis of this cotton had determined that fibrobacters comprised 28.9% of the total bacterial 16S rRNA gene copies, as determined via qPCR (McDonald *et al.*, 2012), with this enrichment potentially resulting the isolation of novel *Fibrobacter* spp. In addition, the total community of the colonised cotton string was characterised in order to determine the potential role of fibrobacters in this environment. In chapter 4, 55 bacterial strains were isolated from dewaxed cotton string that had been incubated in a landfill leachate microcosm, with cultures most closely related to either *C. leptum*, *C. sporogenes* or *S. acetigenes* purified, and the strains related to *C. leptum* representing a novel species, and possibly genus, based on 16S rRNA gene sequence similarity (90 - 93%). A total of 13 of the 55 strains remained impure after three rounds of purification via the Hungate roll tube method (Hungate, 1947), with six of these strains containing fibrobacters as determined via PCR using genus specific 16S rRNA gene primers. Given that to date only two species of the genus, *F. succinogenes* and *F. intestinalis*, have been isolated and characterised, it is understandable that further attempts at cultivation would be difficult. The modification of the inoculum source in order to utilise solid cellulosic matter may be a more efficient method for isolating *Fibrobacter* spp. (Shinkai *et al.*, 2009). Other modifications, such as the use of a gelling agent that enables the migration of the less motile *Fibrobacter* spp. through the agar, may also prove useful in cultivating these species (Nyonyo *et al.*, 2013; Nyonyo *et al.*, 2014; Shinkai *et al.*, 2009). It may also be possible to utilise a different purification method, such as dilution to extinction, in order to purify *Fibrobacter* spp. (Kenters *et al.*, 2011), with qPCR using *Fibrobacter* specific and general bacterial 16S rRNA gene primers utilised in order to track the purity of the cultures.

The fact that the fibrobacters were associated with either *C. sporogenes* (chapter 3) or *C. botulinum* (chapter 4) is intriguing, especially due to the fact that these two species are both members of cluster I of the clostridia and are closely related (Lee and Riemann, 1970). It is possible that this association is required by the *Fibrobacter* spp. in order for them to survive, thus potentially explaining the difficulties in obtaining pure isolates of these species.

It is likely that further isolation attempts will be enhanced by molecular data, such as genome or metagenome sequencing (Pope *et al.*, 2011; Renesto *et al.*, 2003). The use of a combined molecular and cultivation approach would also be useful both for confirming the cellulolytic phenotype of fibrobacters outside of the gut environment, and for elucidating the mechanism by which members of the genus *Fibrobacter* are able to degrade cellulose.

6.4 Results of objective 3; to determine the function of members of the *Fibrobacteres* phylum present in landfill sites

Due to the fact that the *Fibrobacter* spp. isolated in chapter 3 were unable to be sub-cultured and that those isolated in chapter 4 were impure, it was not possible to confirm the cellulolytic phenotype of the landfill fibrobacters based solely on this work. However, the fact that these strains were able to be isolated from microcosms containing either Avicel or dewaxed cotton string as the sole carbon source does suggest fibrobacters are capable of degrading cellulose, especially in light of the fact that although these *Fibrobacter* spp. were in co-culture with *C. sporogenes* (chapter 3) or *C. botulinum* (chapter 4), neither *C. sporogenes* or *C. botulinum* are capable of degrading cellulose (Sebahia *et al.*, 2007). In order to confirm the role of fibrobacters in landfill, two next generation sequencing approaches were used. The first was 454 pyrosequencing of 16S rRNA gene PCR amplicons from poorly and heavily degraded colonised cotton samples incubated in microcosms containing landfill leachate, and the second was shotgun metagenome sequencing of the heavily degraded colonised cotton, both outlined in chapter 5.

The comparison of the 16S rRNA gene amplicon libraries from the heavily and poorly degraded colonised cotton implicated members of the bacterial phyla *Firmicutes*, *Bacteroidetes*, *Spirochaetes* and *Fibrobacteres* as the major bacterial cellulose degraders in landfill sites. The metagenome sequences derived from the heavily degraded colonised cotton supported the assertion that members of the *Firmicutes*, *Bacteroidetes* and *Spirochaetes* are involved in cellulose hydrolysis, with the additional involvement of members of the *Proteobacteria*.

The *Firmicutes* has previously been identified as the dominant phylum in a bioreactor treating landfill leachate via a 16S rRNA gene clone library (Burrell *et al.*, 2004), and has also been detected in other landfill 16S rRNA gene clone library studies (Huang *et al.*, 2004; Huang *et al.*, 2005) and 16S rRNA gene 454 pyrosequencing studies on an anaerobic bioreactor (Xie *et al.*, 2014) and a lab-scale reactor (Bareither *et al.*, 2013) treating landfill leachate. The involvement of members of the *Bacteroidetes*, which are able to degrade polysaccharides

(Thomas *et al.*, 2011) is also expected due to their previous detection in landfill leachate via general bacterial 16S rRNA gene clone libraries (Huang *et al.*, 2004; Huang *et al.*, 2005) and 16S rRNA gene PCR amplicon 454 pyrosequencing studies (Bareither *et al.*, 2013; Xie *et al.*, 2014). Both *Proteobacteria* and *Spirochaetes* have previously been detected in landfill leachate 16S rRNA gene clone libraries (Huang *et al.*, 2004; Huang *et al.*, 2005), and *Spirochaetes* have also been identified in landfill leachate via 454 pyrosequencing targeting the 16S rRNA gene (Bareither *et al.*, 2013; Xie *et al.*, 2014). The detection of members of the *Fibrobacteres* via 454 pyrosequencing of 16S rRNA gene PCR amplicons is more surprising, as although they have been previously detected in landfill sites via genus-specific 16S rRNA gene PCR primers (McDonald *et al.*, 2008, McDonald *et al.*, 2012), previous landfill leachate studies utilising either clone libraries (Huang *et al.*, 2004; Huang *et al.*, 2005; Burrell *et al.*, 2004) or previous 454 pyrosequencing approaches (Bareither *et al.*, 2013; Xie *et al.*, 2014) targeting the 16S rRNA gene have failed to detect members of this phylum. Therefore these data not only implicate fibrobacters in the cellulose degradation that occurs in landfill sites, but also demonstrates that they are more readily detected on the colonised cotton string that had been used as a 'bait' for cellulolytic species than in the landfill leachate, suggesting that this enrichment may be a better method for studying the fibrobacters present in environmental samples.

Further functional analysis of the heavily degraded colonised cotton metagenome in chapter 5 determined that the genome of *F. succinogenes* subsp. *succinogenes* S85 had the most protein matches against each of the three metagenome libraries, with *T. vincentii* and *T. denticola* (phylum *Spirochaetes*) the second and third genomes with the most protein matches against the 300 and 600 bp metagenome libraries. These data implicate both fibrobacters and spirochetes as being important members of the landfill cellulolytic microbial community. Recruitment plot analysis of the genome of *F. succinogenes* subsp. *succinogenes* S85 against the metagenome contigs confirmed this via the detection of proteins involved in cellulose hydrolysis by *F. succinogenes* in the metagenome. These included members of glycoside hydrolase family 9, the largest cellulase family, but a large number of glycoside hydrolases were unable to be assigned to a specific family, further supporting the novelty of the mechanism by which *Fibrobacter* spp. degrade cellulose. These data, combined with the isolation of fibrobacters in chapter 4 from the dewaxed cotton string, demonstrates that the fibrobacters present in landfill sites are active members of the cellulolytic community.

6.5 Key findings of this study

The key findings from this study are:

- Fibrobacters have a broad ecological range that includes mammalian and termite guts, terrestrial, aquatic and managed environments (landfill) where cellulose decomposition occurs; here, the first specific detection of fibrobacters in marine and estuarine sediments, and Arctic cryoconite samples is also reported.
- The *Fibrobacter* genus contains 63 OTUs at 95% clustering of the 16S rRNA gene sequences, demonstrating the molecular detection of several new species of fibrobacters.
- Members of *F. succinogenes* comprised 11 OTUs and *F. intestinalis* 3 OTUs at 95% clustering of the 16S rRNA gene sequences, suggesting that these two formally described species may in fact be separate genera.
- *F. succinogenes* has been isolated from a landfill site, providing the first evidence that *F. succinogenes* is metabolically active outside of the mammalian intestinal tract.
- *Fibrobacter* spp. have been isolated from dewaxed cotton string incubated in a landfill leachate microcosm, demonstrating the cellulolytic nature of fibrobacters in landfill.
- A novel species of bacteria, related to *C. leptum*, was isolated from a landfill site and its 16S rRNA gene similarity (90-93%) suggests that this is potentially a member of a new genus within the *Firmicutes* phylum.
- *S. acetigenes* was isolated from a landfill site, representing the first isolation of a member of *Clostridium* cluster XII from landfill.
- Fibrobacters were determined to be important members of the landfill cellulolytic community via comparative 454 pyrosequencing targeting 16S rRNA gene PCR amplicons, which determined that members of the *Fibrobacteres* comprised 14.2% of sequences from a heavily degraded colonised cotton sample in comparison to 0.02% of sequences from the poorly degraded sample.
- The cellulolytic phenotype of landfill fibrobacters was confirmed via functional analysis of the metagenome from a heavily degraded colonised cotton sample, which demonstrated that *F. succinogenes* S85 had the most protein matches to those encoded within the metagenome, including members of glycoside hydrolase family 9.

6.6 Future work

The detection of novel *Fibrobacter* spp. in a range of environmental niches demonstrates that fibrobacters have a broader ecological range and are more diverse than previously thought. Given that this diversity has likely also resulted in the evolution of novel enzyme systems, it is inconceivable that so little is known with regards to the taxonomy and function of the members of the genus *Fibrobacter*.

Further attempts to isolate and cultivate the novel *Fibrobacter* spp. present in a variety of environments would enable both the characterisation of their taxonomy, and function within their environmental niche. Given the level of diversity contained within the phylum based solely on 16S rRNA gene sequence data, it is also likely that different *Fibrobacter* isolates would have different growth conditions and different enzymes of potential interest. Comparative genomic analysis of new *Fibrobacter* isolates derived from contrasting environments, and *F. intestinalis* and *F. succinogenes* subspecies against the genome of *F. succinogenes* S85, would also further improve our understanding of the taxonomy, ecology and mechanism of cellulose hydrolysis exhibited by the members of this phylum.

Additionally, the use of mRNA transcriptome sequencing on these *Fibrobacter* strains whilst they are grown on different substrates would also improve our understanding of the novel mechanism by which fibrobacters are thought to degrade cellulose, by elucidating the genes, and therefore enzymes, required for various cellulosic substrates with differing levels of complexity. These data would provide an important insight into the cellulolytic mechanism of *Fibrobacter* spp., with the potential for the enzymes to be utilised in biotechnological applications such as the improvement of ruminant nutrition, waste management and the production of second-generation biofuels.

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APPENDIX I

Ransom-Jones E, Jones DL, McCarthy AJ, McDonald JE (2012) The
Fibrobacteres: an important phylum of cellulose-degrading bacteria.
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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.

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

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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 pleomorphic 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, B₁₂ (cyanocobalamin) 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 strain GCS from the bovine caecum (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 | |
| <i>Fibrobacter succinogenes</i> strains | | | | | | | | | | | | |
| Group 1. subsp. <i>succinogenes</i> strains | | | | | | | | | | | | |
| B1 | | Bovine rumen | Cocoid | - | ND | + | ND | - | - | + | - | [117] |
| BL2 | | Bovine rumen | Cocoid | - | ND | + | ND | - | - | + | - | [117] |
| A3c | | Bovine rumen | Cocoid | - | 49 | + | - | - | - | + | - | [25, 26] |
| S85 | 19169 ^T | Bovine rumen | Cocoid | - | 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] |
| <i>Fibrobacter intestinalis</i> strains | | | | | | | | | | | | |
| NR9 | 43854 ^T | Rat caecum | Rod shaped | - | 45 | - | + | + | + | + | - | [92] |
| C1a | | Porcine caecum | Rod shaped | - | ND | - | ND | + | + | + | - | [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 (*Butyivibrio* and *Acetivibrio*) that are phylogenetically related

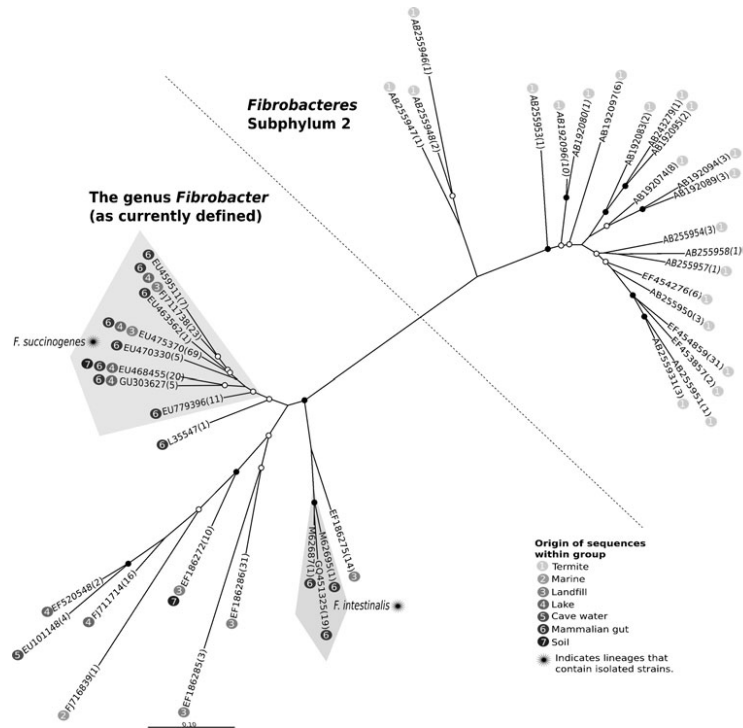


Figure 1 Phylogeny of the *Fibrobacteres* phylum. Maximum likelihood tree of 16S rRNA gene sequences belonging to the *Fibrobacteres* phylum. All sequences classified within the *Fibrobacteres* phylum and annotated as of ‘good’ quality were downloaded from the Ribosomal Database Project [19, 21] website 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

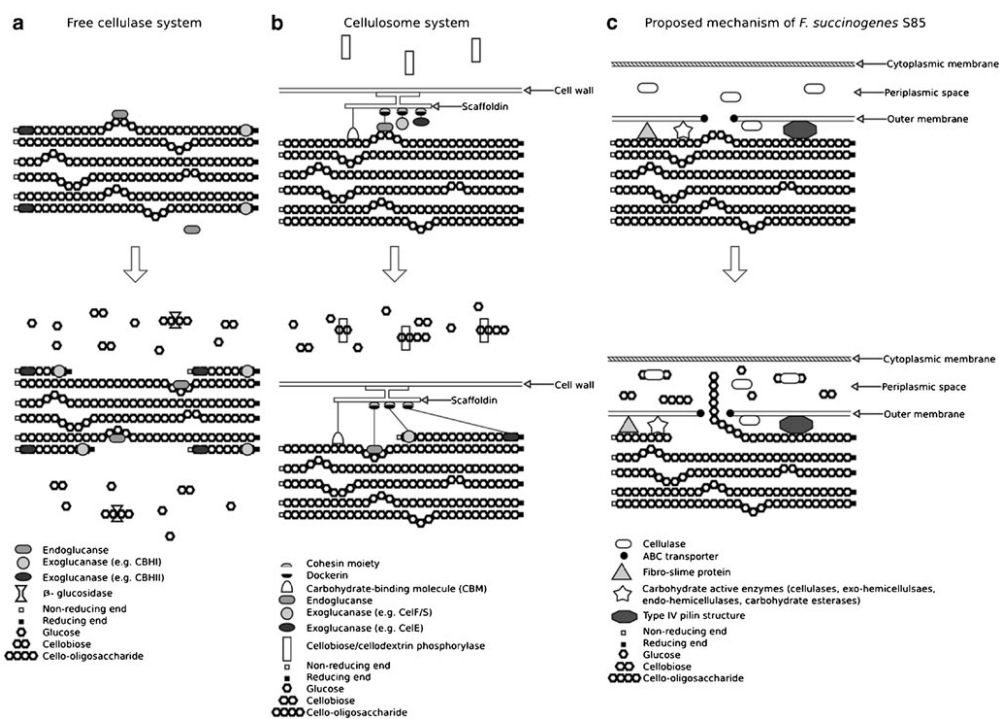


Figure 2 Microbial mechanisms of cellulose degradation. **a** Aerobic cell-free cellulase system (based on [75]); typical of aerobic microorganisms including *T. reesei*. Cellulose is hydrolysed via the synergistic interaction of individual enzymes that are secreted from the cell. **b** Anaerobic ‘cellulosome’ 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 $0.2\text{--}0.3 \times 1.3\text{--}4.9 \mu\text{m}$ [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 supra-generic 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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APPENDIX II

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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. *Fibrobacteres* 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.

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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 celulosomal 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 through 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

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Table 1
PCR and qPCR analysis of environmental samples.

| Sample | Sample type | Location | Direct PCR <i>Fibrobacter</i> product | Nested PCR <i>Fibrobacter</i> product | Clone library sequenced | qPCR (% relative rRNA abundance) ^a |
|---|--------------------|---|---|---|-------------------------------|---|
| Equine faeces | Faecal matter | n/a | + | + | + | 1.31 |
| Ovine rumen fluid | Rumen fluid | n/a | + | + | + | 0.04 |
| Bovine rumen fluid | Rumen fluid | n/a | + | + | + | ND |
| Peat | Peat | Acid erosion complex, Migneint-Arenig-Dduallt, Conwy, Wales | – | – | – | ND |
| Soil from stable sand dune | Soil | Stable sand dune, Newborough, Anglesey | – | + | – | ND |
| Blanket bog soil | Soil | Migneint-Arenig-Dduallt, Conwy, Wales | – | + | + | ND |
| Buckley compost | Compost | Compost heap, Chester, England | – | – | – | ND |
| Cryoconite VB1 | Cryoconite | High Arctic (Svalbard) | – | + | + | ND |
| Cryoconite PF1 | Cryoconite | European Alps (Tyrol) | – | – | – | ND |
| Cryoconite RM1 | Cryoconite | European Alps (Tyrol) | – | – | – | ND |
| Cryoconite GB1 | Cryoconite | European Alps (Tyrol) | – | – | – | ND |
| Cryoconite ML6 | Cryoconite | High Arctic (Svalbard) | – | + | – | ND |
| Cryoconite AB6 | Cryoconite | High Arctic (Svalbard) | – | + | + | ND |
| Esthwaite (lake) sediment | Sediment | Esthwaite Lake, Lake District, England | – | – | – | ND |
| Lake Ogwen sediment | Sediment | Lake Ogwen, Gwynedd, Wales | – | + | – | ND |
| Llyn Aled (lake) sediment | Sediment | Llyn Aled, Conwy, Wales | – | + | + | ND |
| Aled Isaf (lake) sediment | Sediment | Aled Isaf, Conwy, Wales | – | + | + | ND |
| Conwy Estuary microcosm 1 cotton | Cotton string | Mussel Bed, Conwy Estuary, Wales | – | – | – | ND |
| Conwy Estuary microcosm 2 cotton | Cotton string | Mussel Bed, Conwy Estuary, Wales | – | + | + | ND |
| Conwy Estuary microcosm 3 cotton | Cotton string | Mussel Bed, Conwy Estuary, Wales | – | + | + | ND |
| Conwy Estuary microcosm 4 cotton | Cotton string | Mud Flat, Conwy Estuary, Wales | – | + | – | ND |
| Conwy Estuary microcosm 5 cotton | Cotton string | Mud Flat, Conwy Estuary, Wales | – | – | – | ND |
| Marine off shore transect sediment 1 km | Sediment | Conwy, Wales | – | – | – | ND |
| Marine off shore transect sediment 2 km | Sediment | Conwy, Wales | – | – | – | ND |
| Marine off shore transect sediment 4 km | Sediment | Conwy, Wales | – | + | + | ND |
| Marine off shore transect sediment 8 km | Sediment | Conwy, Wales | – | – | – | ND |
| Marine off shore transect sediment 12 km | Sediment | Conwy, Wales | – | – | – | ND |
| Brombrough Dock (landfill) microcosm | Filtered leachate | Brombrough Dock Landfill, Wirral, England | + | + | + | 3.90 |
| Buckley (landfill) leachate 1 (LC3) | Filtered leachate | Buckley Landfill, Flintshire, Wales | – | + | + | ND |
| Buckley (landfill) leachate 2 (LC1B) | Filtered leachate | Buckley Landfill, Flintshire, Wales | – | + | + | ND |
| Buckley (landfill) leachate 3 (LC2B) | Filtered leachate | Buckley Landfill, Flintshire, Wales | – | – | – | ND |
| Bidston Moss (landfill) microcosm cotton 1J | Cotton string | Bidston Moss Landfill, Wirral, England | – | + | + | 0.02 |
| Bidston Moss (landfill) microcosm cotton 3E | Cotton string | Bidston Moss Landfill, Wirral, England | – | – | – | ND |
| Bidston Moss (landfill) microcosm cotton 3F | Cotton string | Bidston Moss Landfill, Wirral, England | – | – | – | ND |
| Bidston Moss (landfill) microcosm 3F containing 0.1% (w/v) avicell | Filtered microcosm | Bidston Moss Landfill, Wirral, England | – | + | + | 1.43 |
| Bidston Moss (landfill) leachate 1J | Filtered leachate | Bidston Moss Landfill, Wirral, England | – | – | – | ND |
| Bidston Moss (landfill) leachate 3E | Filtered leachate | Bidston Moss Landfill, Wirral, England | – | – | – | ND |
| Bidston Moss (landfill) leachate 3F | Filtered leachate | Bidston Moss Landfill, Wirral, England | – | + | + | ND |
| Soil transect point 5 | Soil ^b | Conwy, Wales | – | + | – | ND |
| Soil transect point 8 | Soil ^b | Conwy, Wales | – | + | – | ND |

ND, 'not determined'. Insufficient nucleic acid was retrieved from the environmental sample to enable qPCR with sufficient replication for the quantitative analysis of both general bacteria and *Fibrobacter* spp.
n/a, not applicable.

^a Percentage relative abundance of 16S rRNA genes of *Fibrobacter* spp. compared with total bacteria.

^b An additional 24 soil samples from Conwy, North Wales were tested with nested PCR, but no *Fibrobacter* PCR amplicons were detectable.

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Table 2
16S rRNA gene primers used for PCR and qPCR amplification and sequencing.

| Primers | Sequence (5'–3') | Specificity | Annealing temperature (°C) | Amplicon size (bp) | Reference |
|---------------------------|-----------------------|---------------------------------------|----------------------------|--------------------|-----------|
| pA | AGAGTTTGATCCTGGCTCAG | General bacteria | 55 | ~ 1534 | [14] |
| pH' | AAGGAGTGTATCCAGCCGCA | | | | |
| Fib 1F ^a | CCGKSCCAACGSSCGG | <i>Fibrobacter</i> genus | 60 | ~855 | [36] |
| Fib 2AR | ATCTCTCGCYCGGGCGWTYCC | | | | |
| 1369F ^b | CGGTGAATACGTTCCYCGG | General bacteria ^b | 60 ^d | ~ 151 | [47] |
| Prok 1492R ^b | GGWTACCTTGTTACGACTT | | | | |
| FibroQ153F ^{b,c} | CCGKSCCAACGSSCGGHATA | <i>Fibrobacter</i> ^b genus | 60 ^d | ~104 | [36] |
| FibroQ238R ^b | CSCCWACTRGYTAATCRGAC | | | | |
| M13 Forward ^c | GTTTCCCAGTACGAC | M13 Vector | n/a | n/a | [37] |

n/a, not applicable.

^a Ambiguities: K = (GorT), S = (GorC), W = (AorT), Y = (CorT), H = (A,CorT), R = (AorG), D = (G,AorT), V = (A,CorG).^b Primers used for qPCR analysis.^c Primer used for sequencing.^d QuantiFast™ SYBR® Green PCR assay (Qiagen) uses the same annealing temperature (60 °C) for all primer sets.^e Primers based on those of Lin and Stahl [32] as modified by McDonald et al. [36].

with HyperLadder™ 1 kb (Bioline) before quantification with the Qubit® Fluorometer (Life Technologies) and the Qubit® dsDNA BR Assay Kit (Life Technologies). Cryoconite samples were subjected to PowerSoil® (MoBio Inc.) DNA extraction, as specified by the manufacturer, with DNA being extracted from 250 mg (fresh weight) of cryoconite and eluted in 100 µL Buffer C6. Purified DNA was stored at –80 °C.

Amplification of the 16S rRNA gene with direct and nested PCR

PCR reactions contained 0.2 mM of each primer (Table 2), 0.2 mM of each dNTP, 1 × SuperTaq Buffer (Cambio), 0.5 mM MgCl₂, 1 × BSA, 1 U SuperTaq (Cambio), 50 ng DNA and ddH₂O to a final volume of 50 µL. PCR reactions using the *Fibrobacter* primer set (Fib 1F and Fib 2AR, Table 2) contained an increased concentration of each primer (0.4 mM) and MgCl₂ (1.5 mM). PCR cycling conditions were as follows: initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 1 min at the specific annealing temperature for each primer set (Table 2) and 72 °C for 1.5 min. The final extension was performed at 72 °C for 10 min. For direct PCR, 50 ng of extracted DNA were amplified with the *Fibrobacter*-specific primers (Table 2). Nested PCR consisted of an initial round of PCR using the general bacterial primer set (pA and pH, Table 2), followed by a second round of PCR on the general bacterial amplification products (1 µL) using the *Fibrobacter*-specific primers (Fib 1F and Fib 2AR, Table 2). PCR products were visualised on a 1% agarose (Bioline) gel with HyperLadder™ 1 kb (Bioline) and stored at –20 °C.

Cloning and sequencing of *Fibrobacter*-specific PCR amplification products

Seventeen of the *Fibrobacter*-specific 16S rRNA gene PCR amplification products from the nested PCR described above were extracted from a 1% agarose (Bioline) gel and purified using the QIAquick® Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. The 16S rRNA gene PCR products were ligated and cloned into competent *Escherichia coli* JM109 (Promega) using the pGEM®-T Easy Vector System 1 (Promega), according to the manufacturer's protocol. Plasmid DNA was then extracted and purified using the QIAEX® II Gel Extraction Kit (Qiagen) prior to sequencing using the M13 forward primer (Table 2) by Source Bio-Science.

Quantification of *Fibrobacter* spp. using qPCR

For each of the five samples for which sufficient DNA template was achieved for qPCR analysis, triplicate qPCR assays were performed with both the general bacterial (1369F and Prok 1492Rb,

Table 2) and *Fibrobacter*-specific primer sets (FibroQ153F and FibroQ238R, Table 2) using the 7900HT Fast Real-Time PCR System (Applied Biosystems). Each reaction was performed in a 20 µL final volume, containing 10 ng DNA, 10 µL of 2 × QuantiFast SYBR® Green PCR Master Mix (Qiagen), 1 mM (final concentration) forward and reverse primer and ddH₂O. Cycling conditions were 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, and 60 °C for 30 s, with fluorescence detection in the combined annealing and extension step. A dissociation step was included at the end of every run in order to confirm the presence of single amplification products.

The amplified 16S rRNA gene of *F. succinogenes* S85 was used to generate standard dilution curves in order to determine the relative abundance of *Fibrobacter* spp. The almost full-length 16S rRNA gene (~1534 bp) was amplified using the primers pA and pH' (Table 2), as described above, and the amplification product was excised from a 1% agarose (Bioline) gel and purified using the QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. The concentration of purified DNA was established with a Qubit® Fluorometer (Life Technologies) using the Qubit® dsDNA BR Assay Kit (Life Technologies), and the 16S rRNA gene copy number per microlitre was calculated with the following equation: $(X \text{ g } \mu\text{L}^{-1} \text{ DNA}) / [\text{PCR product length in base pairs} \times 660] \times 6.022 \times 10^{23} = Y \text{ molecules } \mu\text{L}^{-1}$.

Triplicate standard curves of the *F. succinogenes* S85 16S rRNA gene were generated using serial dilutions from 3×10^8 to 3×10^2 gene copies, with all three serial dilutions included on each plate with each primer set. Standard curves for each primer set were generated by plotting the Ct value against the log gene copy number, and a linear line of best fit was used to determine the r² value, amplification efficiency and y-intercept [41]. The relative abundance (%) of *Fibrobacter* spp. was determined by dividing the number of gene copies per sample from the *Fibrobacter*-specific assay with the number of total bacterial gene copies per sample, as determined by the standard curves for each primer set [44].

Phylogenetic analysis of *Fibrobacteres* 16S rRNA gene sequences

All sequences classified within the *Fibrobacteres* phylum and annotated as 'good' quality were downloaded from the Ribosomal Database Project [5,6] website in July 2013, and they were combined with sequences derived from the clone libraries produced in this study. The resulting dataset was subsequently aligned using the MUSCLE aligner [10]. Sequences were trimmed to produce an alignment containing only complete sequences corresponding to the regions between positions 188 and 887 of the *E. coli* 16S rRNA gene. Sequences from the aligned dataset were subsequently clustered into operational taxonomic units (OTUs) with a 95% similarity cut-off using CDHIT [21,30]. Sequences were checked for

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chimaeras using Bellerophon [22] and putative chimeric sequences were removed from the dataset. The representative sequences of each OTU ($n=63$) were aligned using the MUSCLE aligner [10] and imported into ARB [33] where the alignment was visually checked and manually optimised. A maximum likelihood tree was produced from the final alignment using ARB [33]. Nodes for which a bootstrap value of >95% was observed are marked with a filled circle, nodes for which the bootstrap value was between 75% and 95% are marked with an unfilled circle. Nucleotide sequence accession numbers for the representative sequence of each OTU are displayed on each node and the number of sequences clustering within each OTU is displayed in parentheses. 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.

Nucleotide sequence accession numbers

The sequence data have been submitted to the GenBank database under accession numbers KJ364183–KJ364484.

Results and discussion

Genus-specific 16S rRNA gene PCR amplification of *Fibrobacter* spp. in environmental samples

Fibrobacter spp. were detected using nested PCR in 23 of the 64 samples studied, including equine faeces, ovine and bovine rumen fluids, soils, cryoconite, freshwater, estuarine and marine sediments, and landfill sites (Table 1). This is the first targeted detection of *Fibrobacter* spp. in estuarine sediments, marine sediments and cryoconite. A direct PCR amplification product was detected in 4 of the 64 environmental samples screened (Table 1). This was due to the fact that *Fibrobacter* genomic DNA expresses poor PCR amplification efficiencies [36] and nested PCR greatly improves the sensitivity of detection. Consequently, a direct PCR result for the presence of *Fibrobacter* spp. usually only occurs in samples where there is a significant abundance of *Fibrobacter* spp. The four environments in which *Fibrobacter* spp. were detected with direct PCR (equine faeces, ovine rumen fluid, bovine rumen fluid and Bromborough Dock landfill) are known to have high numbers of fibrobacters, since qPCR has demonstrated that the relative abundance of *Fibrobacter* rRNA compared with total bacterial rRNA was 21–32% (ovine gut) [36] and 28.9% (Bromborough Dock Riser 3) [35].

The relative rRNA abundance of *Fibrobacter* spp. in relation to total bacteria, as determined by qPCR, ranged from 0.02% to 3.9% in landfill sites, which was comparable with previous studies that have shown fibrobacters range from 0.2% to 40% of the total bacterial rRNA molecules in landfill sites [36] and 0.005–1% in lakes [34]. These data suggested that fibrobacters can represent a significant and active proportion of the microbial population in these environments. There are however caveats when using DNA to detect fibrobacters with PCR, as it is thought that DNA-associated molecules interfere with PCR amplification, thus resulting in the previous underestimation of their abundance within the rumen using general bacterial 16S rRNA gene libraries [48]. Furthermore, the extraction method used can also introduce bias [16]. Molecular analysis of reverse transcribed rRNA is thought to be a better approach for studying members of the *Fibrobacteres* [36], as the inhibitory molecules only seem to be associated with DNA. Tajima et al. [48] observed that *F. succinogenes* genomic DNA had a prolonged delay in amplification prior to the exponential amplification phase of the DNA template in qPCR assays; however, once PCR amplification surpassed the threshold of detection, the template

amplified exponentially, suggesting that the initial genomic DNA was responsible for the poor amplification efficiency. Thus, when cDNA is used for downstream applications, this potential interference would be overcome. However, it was not possible to extract enough RNA from the samples for RT qPCR to be applied here.

Phylogenetic analysis

The *Fibrobacteres* phylum currently consists of two subphyla, subphylum 2, which only contains species detected in the termite gut, and subphylum 1 (the genus *Fibrobacter sensu stricto*), which contains the two characterised species *F. succinogenes* and *F. intestinalis* (Fig. 1). The generation of new *Fibrobacter* 16S rRNA gene sequence data from several contrasting environments in this study expands our knowledge of the ecological range of this poorly studied phylum, and comparative phylogenetic analyses of these data in addition to the known *Fibrobacteres* diversity in the public databases makes this study of the *Fibrobacteres* phylum the most comprehensive to date. The Ribosomal Database Project repository [5] previously contained only one *Fibrobacter* sequence that had been detected in marine and estuarine sediments, and as a result this study has added to the current understanding of the ecology of the phylum in these and other environments. This is also the first specific detection of *Fibrobacter* spp. in cryoconite, a microbe-mineral aggregate responsible for darkening glacial ice surfaces [49] that is associated with high rates of microbial carbon production despite ambient temperatures between 0 and 1 °C [3].

Previous phylogenetic studies have used 95% similarity to designate species-level diversity within the *Fibrobacteres* phylum due to the substantial 16S rRNA gene and genomic diversity between the two described species [25,42], with the sequences derived from *F. intestinalis* isolates forming a single OTU at 95%, which is below the commonly accepted 97% OTU cut-off used to cluster at the species level [25]. When all sequences were clustered at 95% sequence similarity, 63 OTUs were generated, with *F. succinogenes* comprising 11 OTUs and *F. intestinalis* 3 OTUs that clustered separately from *F. succinogenes* with a bootstrap value of >95% (Fig. 1). This would suggest that the strains currently designated as *F. succinogenes* do not actually represent a single species. Previous studies have suggested that *F. succinogenes* and *F. intestinalis* may actually represent two distinct genera [38,42], but in the absence of phenotypic data to distinguish between the two species (despite significant genomic diversity), elevating each taxon to genus status is premature. Consequently, *F. succinogenes* is currently separated into four sub-species.

In order to determine the extent of 16S rRNA gene diversity within the *F. succinogenes* lineage, all sequences designated as isolates of *F. succinogenes* were downloaded from the Ribosomal Database Project website and aligned as described previously. The alignment was then trimmed to create a near full-length alignment of the 16SrRNA gene (1176 bp) (data not shown). When a similarity matrix was constructed for this alignment, it was found that a 91% clustering value would be needed to group all members of *F. succinogenes* into the same node. Nevertheless, this value for inter-species variation is lower than the current 95% 16S rRNA similarity considered as the minimum allowable within a genus [33]. Clustering at 91% similarity generated 29 OTUs, suggesting that there are at least 27 potentially novel species contained within the phylum, and demonstrating that there is greater diversity outside the two recognised species than within.

The number of sequences contained within each of the 63 OTUs generated at 95% similarity (Fig. 1) varied from 297 sequences to singleton sequences, with 18 OTUs containing only one sequence. As a result, it could not be inferred if these lineages were exclusive to a particular niche, since further sequencing data may well have revealed other as yet undetected fibrobacters that would cluster within these OTUs, either from the same or different environments.

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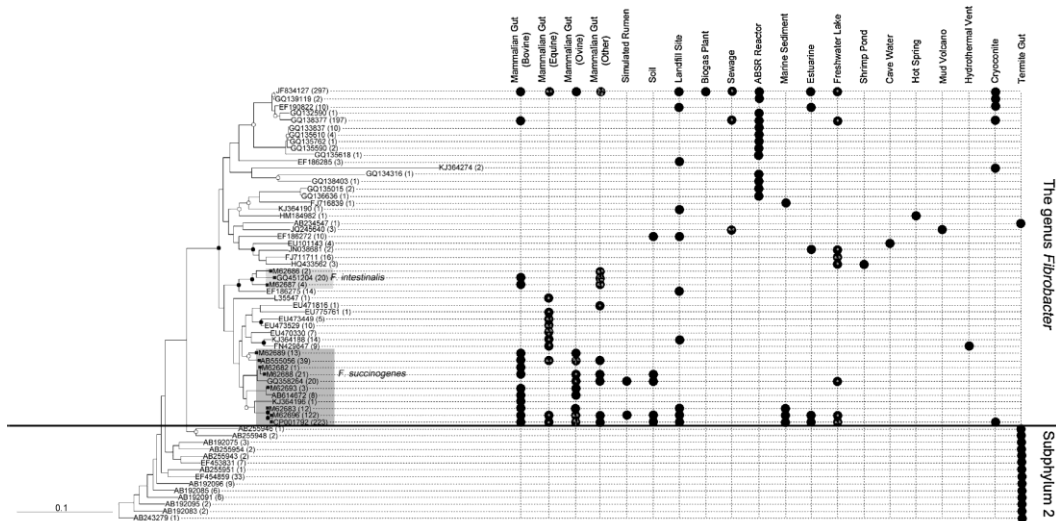


Fig. 1. The ecology and taxonomy of the *Fibrobacteres* phylum. OTUs containing cultivated species are designated by boxes on the end of the node. Refer to Supplementary Table S1 for a full data table describing the sequences contained in each OTU, their accession numbers and percentage environmental composition of each one. Further information on the environmental distribution is as follows: *Mammalian Gut (Equine)*: (a) Horse, (b) Grevy's zebra, and (c) Wild ass. *Mammalian Gut (Other)*: (a) Buffalo, (b) Capybara, (c) Colobus, (d) Dromedary camel, (e) Elephant, (f) Goat, (g) Pig, (h) Rat, (i) River hog, (j) Rock hyrax, (k) Tammar wallaby, (l) Yak, and (m) Yunnan snub-nosed monkey. *Sewage*: (a) activated sludge, (b) anaerobic sludge digester, and (c) raw sewage. *Freshwater lake*: (a) lake sediment and (b) lake water.

Whilst there were lineages that contained species from a range of environments, others seemed to be specific to one particular ecological niche. Seven of the 63 OTUs (at 95%) contained sequences derived from both mammalian gut and environmental samples. However, 24 of the 64 OTUs contained sequences detected exclusively in non-gut environmental samples. These data suggested that a significant proportion of the diversity detected within the *Fibrobacteres* phylum was derived from environmental (non-gut) fibrobacters (Fig. 1). Consequently, the isolation and cultivation of these potentially novel *Fibrobacter* spp. is an obvious priority, in order to further our understanding of their physiology and function in natural and managed environments. The OTU represented by FJ711711 contained species found solely in freshwater lakes, and lineages GQ139119, GQ132590, GQ133837, GQ135610, GQ135762, GQ135590, GQ135618, GQ134316, GQ138403, GQ135015 and GQ136636 all contained species from an anaerobic batch sequencing reactor (ABSR) used for treating swine waste (Fig. 1). In addition, EF186285, EF186275 and KJ364190 contained species found solely in landfill sites, with the separation of EF186275 supported by a bootstrap value of >95%, and KJ364274 contained only species present in cryoconite samples (Fig. 1). Cryoconite fibrobacters were also present in lineage CP001702, which clustered within the *F. succinogenes* group and was supported by a >95% bootstrap value, despite the geographic separation of this environment from grazing areas. In addition, cryoconite bacterial communities have been shown to be distinct from adjacent habitats, and appear to assemble by deterministic processes [13], implying the selection of taxa involved in the functioning of cryoconite ecosystems. Both alpine and Arctic cryoconites receive allochthonous organic matter from plant sources [40,55]. The detection of fibrobacters on all Svalbard glaciers sampled but none of the alpine glaciers examined in this study is interesting. Cryoconite aggregates on Arctic glaciers, including those sampled here, frequently mature to form granular structures [28], while the cryoconite aggregates on the alpine

glaciers sampled were poorly developed aggregates of cells, organic matter and mineral debris. The distribution of fibrobacters in Arctic cryoconite is therefore consistent with the evolution of anoxic microhabitats in the interiors of cryoconite granules [18,50]. The presence of both cosmopolitan and unique lineages illustrates the potentially broad dispersal and diversity of fibrobacters in Arctic glacial environments. As such, the data presented suggests a role for fibrobacters in Arctic cryoconite carbon cycling, especially since cryoconite community structure, respiration rates and organic matter profiles are closely related [11,12]. The detection of fibrobacters within this environment is therefore suggestive of a greater role in the global carbon cycle than previously thought. This is supported by the fact that landfill site [35,36], freshwater lake [34] and estuarine fibrobacters were detected on colonised cotton in both this and previous studies.

Nevertheless, the detection of novel *Fibrobacter* species was not limited to environmental samples, as 15 of the detected *Fibrobacter* lineages (95% OTUs) were exclusive to the termite gut (Fig. 1). In addition, the majority of previous mammalian intestinal tract studies have relied on *F. succinogenes* species-specific primers, thus potentially missing other novel members of the genus that may be present in these environments. Bovine, ovine and equine samples were therefore included in order to determine whether or not novel fibrobacters were also present in these environments. Seventeen of the 63 *Fibrobacter* OTUs (at 95%) observed were comprised exclusively of mammalian gut sequences (Fig. 1). Lineages M62682 and KJ364196 (Fig. 1) contained only bovine-associated species, suggesting that there are also as yet unclassified novel species in the bovine rumen. Furthermore, a number of OTUs, EU470330, EU473529, EU473449, EU775761 and L35547, contained only equine-associated species, with the separation of EU473449 and EU473529 supported by bootstrap values of >95%. Lin and Stahl [32] used *Fibrobacter* genus- and species-specific probes in an rRNA hybridisation study of equine caecal contents,

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and the results suggested that the *F. succinogenes* and *F. intestinalis* signal represented only a small proportion of the total *Fibrobacter* abundance generated with the genus-specific probe. These data therefore indicated that novel *Fibrobacter* species were present in the equine caecum, and our detection of five equine-specific *Fibrobacter* lineages supports this assertion.

Further work should focus on the application of PCR-independent methods to investigate the abundance, ecology and physiology of fibrobacters in these environments. The sequence data and phylogenetic analysis presented here now enable the design and application of lineage-specific *Fibrobacter* probes for both RNA and cellular quantification of fibrobacters. Previous studies have also utilised PCR-independent methods, such as RNA hybridisation and fluorescence *in situ* hybridisation (FISH), in order to provide important insights into the abundance, ecology and physiology of *Fibrobacter* lineages in the gut [1,2,31,32,45]. For example, ecological and physiological differences between strains from *F. succinogenes* phylogenetic subgroups 1–3 have been detected in the rumen using qPCR and FISH. Members of *F. succinogenes* subgroup 1 were observed to predominate numerically and were highly active on plant material, particularly on less degradable hay stems, whereas subgroups 2 and 3 were more often associated with other rumen bacteria associated with the more readily degradable leaf sheaths [26,43]. Consequently, such approaches may now be applied to determine the ecology and physiology of fibrobacters in their newly described ecological niches.

Conclusions

There is a current impetus towards better understanding the diversity of cellulolytic microbes and their enzyme systems for biotechnological applications, particularly in the production of second-generation biofuels, and in understanding biomass decomposition and nutrition in commercially important herbivores. Fibrobacters are prolific degraders of cellulose, however, most cultivation-based approaches for the isolation of cellulolytic microorganisms typically focus on aerobic or facultative anaerobic species that are easier to isolate and cultivate, thus disregarding obligate anaerobes such as fibrobacters. Furthermore, the problems associated with *Fibrobacter* DNA amplification have meant that until recently *Fibrobacter* spp. have remained undescribed in many environments due to the apparent biases against the detection of *Fibrobacter* DNA in microbial communities using general 16S rRNA gene and shotgun metagenomic approaches [4,17]. Consequently, the genomic diversity, physiology and metabolism of *Fibrobacteres* members are barely understood, despite the significant ecological, economical and biotechnological potential of this functionally diverse phylum.

In this current study, the understanding of the taxonomic diversity and ecological range of *Fibrobacter* spp. in natural and managed environments has been extended to several newly described niches, all of which potentially promote adaptation and diversity. This has generated novel centres of variation within the *Fibrobacteres* phylum that contain enzymes and growth requirements favourable for biotechnological exploitation. Historically, the ecology of fibrobacters was thought to be restricted to the mammalian intestinal tract. However, the significant diversity of potentially novel *Fibrobacter* species described here and, in particular, the large proportion of OTUs ($n=24$) derived exclusively from natural and managed environments, demonstrates their broad ecological range in the biosphere. Fibrobacters are therefore an important target for cultivation-based and omics approaches aiming to elucidate novel carbohydrate-active enzymes and mechanisms. It has recently been suggested that *F. succinogenes* S85 utilises a novel mechanism for cellulose hydrolysis [52], and with the observed

taxonomic diversity within the *Fibrobacteres* it is likely that the phylum represents a significant source of unexplored diversity with respect to carbohydrate-active enzymes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.syapm.2014.06.001>.

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APPENDIX III

Supplementary Table for Chapter 2

Supplementary Table 1: Sequences contained within each OTU, the environment from which they were derived and the percentage of sequences for each OTU that are associated with each environment (*RS = representative sequence for that OTU).

| Number of sequences in OTU (95% sequence identity) | Sequence number | NCBI sequence accession number | Similarity to representative sequence for OTU (%) | Environmental source | Sequence represents a cultivated species | Environmental composition of OTU: % of sequences from each environment. |
|--|-----------------|--------------------------------|---|----------------------------|--|---|
| 1 | 0 | FJ716839 | RS* | Marine Sediment | | Marine Sediment 100 |
| 2 | 0 | M62686 | RS* | Porcine Cecum | <i>Fibrobacter intestinalis</i> ; C1a | Other Mammal 100 |
| | 1 | M62695 | 97 | Rat Cecum | <i>Fibrobacter intestinalis</i> ; NR9 | |
| 4 | 0 | M62687 | RS* | Porcine Cecum | <i>Fibrobacter intestinalis</i> ; DR7 | Ovine 50 |
| | 1 | M62690 | 95 | Ovine Rumen | <i>Fibrobacter intestinalis</i> ; JG1 | Other Mammal 50 |
| | 2 | M62691 | 95 | Ovine Rumen | <i>Fibrobacter intestinalis</i> ; LH1 | |
| | 3 | HQ008626 | 96 | Dromedary Camel | | |
| 12 | 0 | M62688 | RS* | Bovine Rumen | <i>Fibrobacter succinogenes</i> ; A3C | Bovine 75 |
| | 1 | M62684 | 96 | Bovine Rumen | <i>Fibrobacter succinogenes</i> ; B1 | Bovine/Lake Sediment 8.3 |
| | 2 | M62685 | 96 | Bovine Rumen | <i>Fibrobacter succinogenes</i> ; BL2 | |
| | 3 | EF186237 | 98 | Bovine Rumen | | Marine Sediment 8.3 |
| | 4 | JF629951 | 95 | Bovine Rumen/Lake Sediment | | |
| | 5 | JF657541 | 97 | Bovine Rumen | | |
| | 6 | KJ364216 | 100 | Landfill | | |
| | 7 | KJ364259 | 97 | Marine Sediment | | |
| | 8 | KJ364262 | 98 | Bovine Rumen | | |
| | 9 | KJ364263 | 95 | Bovine Rumen | | |
| | 10 | KJ364269 | 98 | Bovine Rumen | | |
| 11 | KJ364270 | 97 | Bovine Rumen | | | |
| 122 | 0 | M62696 | RS* | Bovine Rumen | <i>Fibrobacter succinogenes</i> ; S85 | Landfill 24 |

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|----|----------|----|-----------------|--|------------------------|
| 1 | DQ054633 | 97 | Bovine Rumen | | Bovine 18 |
| 2 | DQ054634 | 98 | Bovine Rumen | | Lake 14 |
| 3 | AB275487 | 98 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; OS117 | Soil 11 |
| 4 | AB275488 | 98 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; AS206 | Estuarine 9 |
| 5 | AB275489 | 98 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; AS213 | Bovine/Lake Sediment 8 |
| 6 | AB275509 | 98 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; RS223 | Ovine 7 |
| 7 | AB275513 | 98 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; RS233 | Simulated Rumen 3 |
| 8 | AM493696 | 99 | Simulated Rumen | | Marine Sediment 3 |
| 9 | AM493698 | 97 | Simulated Rumen | | Other Mammal 2 |
| 10 | AM493701 | 99 | Simulated Rumen | | Equine <1 |
| 11 | AM493708 | 99 | Simulated Rumen | | |
| 12 | EF186240 | 98 | Bovine Rumen | | |
| 13 | EF186242 | 98 | Bovine Rumen | | |
| 14 | EF186234 | 99 | Bovine Rumen | <i>Fibrobacter succinogenes</i> subsp. <i>succinogenes</i> S85 | |
| 15 | EF186235 | 98 | Bovine Rumen | <i>Fibrobacter succinogenes</i> subsp. <i>succinogenes</i> S85; 2 | |
| 16 | EU719256 | 98 | Bovine Rumen | | |
| 17 | EU459511 | 95 | Capibara Faeces | | |
| 18 | EU981979 | 98 | Buffalo Rumen | | |
| 19 | FJ711720 | 98 | Lake Sediment | | |
| 20 | FJ711721 | 98 | Lake Sediment | | |
| 21 | FJ711722 | 98 | Lake Sediment | | |
| 22 | FJ711724 | 98 | Lake Sediment | | |
| 23 | FJ711725 | 98 | Lake Sediment | | |
| 24 | FJ711726 | 98 | Lake Sediment | | |
| 25 | AB549935 | 96 | Equine Faeces | | |
| 26 | GQ327172 | 98 | Bovine Rumen | | |
| 27 | HM104722 | 96 | Bovine Rumen | | |
| 28 | HM104756 | 95 | Bovine Rumen | | |

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|----|----------|----|-------------------------------|
| 29 | JF619947 | 96 | Bovine Rumen |
| 30 | JF628603 | 97 | Bovine Rumen |
| 31 | JF631124 | 97 | Bovine Rumen |
| 32 | JF631487 | 96 | Bovine Rumen |
| 33 | JF643198 | 97 | Bovine Rumen/Lake Sediment |
| 34 | JF654521 | 96 | Bovine Rumen/Lake Sediment |
| 35 | JF655714 | 97 | Bovine Rumen/Lake Sediment |
| 36 | JF655875 | 97 | Bovine Rumen/Lake Sediment |
| 37 | JF659371 | 97 | Bovine Rumen/Lake Sediment |
| 38 | JF659783 | 98 | Bovine Rumen/Lake Sediment |
| 39 | JF662483 | 97 | Bovine Rumen/Lake Sediment |
| 40 | JF663034 | 97 | Bovine Rumen/Lake Sediment |
| 41 | JF665372 | 98 | Bovine Rumen/Lake Sediment |
| 42 | JF667541 | 97 | Bovine Rumen/Lake Sediment |
| 43 | JF667934 | 97 | Bovine Rumen/Lake Sediment |
| 44 | KJ364185 | 97 | Lake Sediment |
| 45 | KJ364187 | 95 | Landfill |
| 46 | KJ364191 | 98 | Landfill |
| 47 | KJ364193 | 96 | Landfill |

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|----|----------|----|-----------------|
| 48 | KJ364195 | 97 | Bovine Rumen |
| 49 | KJ364197 | 97 | Bovine Rumen |
| 50 | KJ364213 | 99 | Landfill |
| 51 | KJ364214 | 98 | Landfill |
| 52 | KJ364217 | 98 | Estuarine |
| 53 | KJ364219 | 98 | Estuarine |
| 54 | KJ364242 | 98 | Lake Sediment |
| 55 | KJ364253 | 98 | Soil |
| 56 | KJ364254 | 98 | Soil |
| 57 | KJ364258 | 98 | Marine Sediment |
| 58 | KJ364261 | 98 | Bovine Rumen |
| 59 | KJ364264 | 98 | Bovine Rumen |
| 60 | KJ364265 | 99 | Bovine Rumen |
| 61 | KJ364271 | 96 | Bovine Rumen |
| 62 | KJ364310 | 97 | Ovine Rumen |
| 63 | KJ364312 | 98 | Ovine Rumen |
| 64 | KJ364315 | 98 | Ovine Rumen |
| 65 | KJ364325 | 99 | Soil |
| 66 | KJ364326 | 99 | Soil |
| 67 | KJ364327 | 97 | Soil |
| 68 | KJ364328 | 99 | Soil |
| 69 | KJ364329 | 97 | Soil |
| 70 | KJ364330 | 98 | Soil |
| 71 | KJ364331 | 98 | Soil |
| 72 | KJ364333 | 98 | Soil |
| 73 | KJ364335 | 99 | Soil |
| 74 | KJ364336 | 99 | Soil |
| 75 | KJ364338 | 98 | Soil |
| 76 | KJ364339 | 98 | Soil |
| 77 | KJ364342 | 98 | Lake Sediment |

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|-----|----------|----|-----------------|
| 78 | KJ364344 | 99 | Lake Sediment |
| 79 | KJ364345 | 98 | Lake Sediment |
| 80 | KJ364346 | 99 | Lake Sediment |
| 81 | KJ364354 | 97 | Lake Sediment |
| 82 | KJ364358 | 98 | Landfill |
| 83 | KJ364359 | 98 | Landfill |
| 84 | KJ364361 | 99 | Estuarine |
| 85 | KJ364363 | 99 | Estuarine |
| 86 | KJ364365 | 98 | Estuarine |
| 87 | KJ364367 | 98 | Estuarine |
| 88 | KJ364369 | 99 | Estuarine |
| 89 | KJ364370 | 97 | Estuarine |
| 90 | KJ364371 | 98 | Estuarine |
| 91 | KJ364376 | 96 | Estuarine |
| 92 | KJ364380 | 97 | Estuarine |
| 93 | KJ364390 | 98 | Lake Sediment |
| 94 | KJ364394 | 98 | Lake Sediment |
| 95 | KJ364397 | 99 | Lake Sediment |
| 96 | KJ364402 | 98 | Lake Sediment |
| 97 | KJ364404 | 99 | Marine Sediment |
| 98 | KJ364409 | 97 | Marine Sediment |
| 99 | KJ364412 | 98 | Marine Sediment |
| 100 | KJ364418 | 98 | Landfill |
| 101 | KJ364419 | 97 | Landfill |
| 102 | KJ364420 | 99 | Landfill |
| 103 | KJ364421 | 98 | Landfill |
| 104 | KJ364422 | 98 | Landfill |
| 105 | KJ364423 | 98 | Landfill |
| 106 | KJ364424 | 98 | Landfill |
| 107 | KJ364426 | 99 | Landfill |

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|-------|----------|----------|----------|---------------------|--|
| 108 | KJ364432 | 99 | Landfill | | |
| 109 | KJ364447 | 97 | Landfill | | |
| 110 | KJ364451 | 98 | Landfill | | |
| 111 | KJ364452 | 97 | Landfill | | |
| 112 | KJ364453 | 98 | Landfill | | |
| 113 | KJ364454 | 98 | Landfill | | |
| 114 | KJ364456 | 98 | Landfill | | |
| 115 | KJ364461 | 99 | Landfill | | |
| 116 | KJ364476 | 98 | Landfill | | |
| 117 | KJ364480 | 98 | Landfill | | |
| 118 | KJ364481 | 99 | Landfill | | |
| 119 | KJ364482 | 99 | Landfill | | |
| 120 | KJ364483 | 99 | Landfill | | |
| 121 | KJ364484 | 99 | Landfill | | |
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| 223 | 0 | AF018454 | 98 | Bovine Rumen | Bovine 33 |
| | 1 | AJ496032 | 99 | Bovine Rumen | <i>Fibrobacter succinogenes</i> subsp. <i>Succinogenes</i> ; S85 Ovine 14 Lake 13 |
| | 2 | AJ496186 | 96 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; HM2 Landfill 13 |
| | 3 | AJ496447 | 99 | Bovine Rumen | <i>Fibrobacter succinogenes</i> ; H Estuarine 9 |
| | 4 | AJ496448 | 99 | Bovine Rumen | <i>Fibrobacter succinogenes</i> strain; U Marine Sediment 5 |
| | 5 | AJ496566 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; FE Bovine/Lake Sediment 5 |
| | 6 | AJ505937 | 99 | Bovine Rumen | <i>Fibrobacter succinogenes</i> ; BL2 Soil 4 |
| | 7 | AJ505938 | 99 | Bovine Rumen | <i>Fibrobacter succinogenes</i> ; R Other Mammal 3 |
| | 8 | AY311623 | 99 | Yak Rumen | Cryoconite <1 |
| | 9 | AY311716 | 99 | Yak Rumen | Equine <1 |
| | 10 | AY315348 | 96 | Jinman Cattle Rumen | |
| | 11 | AB113694 | 99 | Ovine Rumen | |
| | 12 | AB275486 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; OS109 |
| | 13 | AB275490 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; AS216 |
| | 14 | AB275491 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; AS220 |

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|----|----------|----|--------------|--|
| 15 | AB275492 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; AS221 |
| 16 | AB275493 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; AS225 |
| 17 | AB275494 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; AS226 |
| 18 | AB275495 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; AS228 |
| 19 | AB275496 | 98 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; OS102 |
| 20 | AB275497 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; OS103 |
| 21 | AB275498 | 98 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; OS112 |
| 22 | AB275500 | 98 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; OS118 |
| 23 | AB275501 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; OS119 |
| 24 | AB275502 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; OS120 |
| 25 | AB275503 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; OS128 |
| 26 | AB275504 | 98 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; RS*209 |
| 27 | AB275505 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; RS*214 |
| 28 | AB275506 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; RS*215 |
| 29 | AB275507 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; RS*216 |
| 30 | AB275508 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; RS*220 |
| 31 | AB275510 | 98 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; RS*224 |
| 32 | AB275511 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; RS*225 |
| 33 | AB275512 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; RS*230 |
| 34 | AB275514 | 99 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; RS*235 |
| 35 | EF445213 | 99 | Bovine Rumen | |
| 36 | EF186236 | 98 | Bovine Rumen | |
| 37 | EF186238 | 98 | Bovine Rumen | |
| 38 | EF186239 | 99 | Bovine Rumen | |
| 39 | EF186243 | 99 | Bovine Rumen | |
| 40 | EF190826 | 99 | Landfill | |
| 41 | EF190828 | 99 | Landfill | |
| 42 | EU381787 | 98 | Bovine Rumen | |
| 43 | EU381803 | 97 | Bovine Rumen | |
| 44 | EU381811 | 97 | Bovine Rumen | |

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|----|----------|-----|-------------------|---|
| 45 | EU381836 | 98 | Bovine Rumen | |
| 46 | EU381840 | 98 | Bovine Rumen | |
| 47 | EU381857 | 97 | Bovine Rumen | |
| 48 | EU381861 | 97 | Bovine Rumen | |
| 49 | EU381922 | 98 | Bovine Rumen | |
| 50 | EU381936 | 96 | Bovine Rumen | |
| 51 | EU381958 | 98 | Bovine Rumen | |
| 52 | EU381968 | 98 | Bovine Rumen | |
| 53 | EU381993 | 98 | Bovine Rumen | |
| 54 | EU382022 | 98 | Bovine Rumen | |
| 55 | EU382049 | 97 | Bovine Rumen | |
| 56 | EU463562 | 95 | Equine Faeces | |
| 57 | EU475370 | 96 | Rock Hyrax Faeces | |
| 58 | EU475376 | 97 | Rock Hyrax Faeces | |
| 59 | EU606019 | 98 | Bovine Rumen | <i>Fibrobacter succinogenes</i> |
| 60 | FJ711723 | 98 | Lake Sediment | |
| 61 | FJ711733 | 99 | Lake Water | |
| 62 | FJ711734 | 99 | Lake Water | |
| 63 | FJ711735 | 99 | Lake Water | |
| 64 | FJ711736 | 99 | Lake Water | |
| 65 | FJ711738 | 99 | Lake Sediment | |
| 66 | FJ711739 | 98 | Soil | |
| 67 | FJ711740 | 99 | Soil | |
| 68 | CP001792 | 99 | Bovine Rumen | <i>Fibrobacter succinogenes</i> subsp. <i>Succinogenes</i> ; S85 |
| 69 | CP001792 | 100 | Bovine Rumen | <i>Fibrobacter succinogenes</i> subsp. <i>Succinogenes</i> ; S85 |
| 70 | CP001792 | 99 | Bovine Rumen | <i>Fibrobacter succinogenes</i> subsp. <i>Succinogenes</i> ; S85 |
| 71 | GU269553 | 96 | Bovine Rumen | <i>Fibrobacter succinogenes</i> (T); HM2 |

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|----|----------|-----|--------------------------|--|
| 72 | GU303546 | 96 | Bovine Rumen | |
| 73 | CP002158 | 99 | Bovine Rumen | <i>Fibrobacter succinogenes</i> subsp. <i>Succinogenes</i> ; S85. |
| 74 | CP002158 | 99 | Bovine Rumen | <i>Fibrobacter succinogenes</i> subsp. <i>Succinogenes</i> ; S85 |
| 75 | CP002158 | 99 | Bovine Rumen | <i>Fibrobacter succinogenes</i> subsp. <i>Succinogenes</i> ; S85 |
| 76 | HM104735 | 97 | Bovine Rumen | |
| 77 | HM104984 | 96 | Bovine Rumen | |
| 78 | CP001792 | RS* | Bovine Rumen | <i>Fibrobacter succinogenes</i> subsp. <i>Succinogenes</i> ; S85 |
| 79 | CP001792 | 99 | Bovine Rumen | <i>Fibrobacter succinogenes</i> subsp. <i>Succinogenes</i> ; S85 |
| 80 | CP001792 | 99 | Bovine Rumen | <i>Fibrobacter succinogenes</i> subsp. <i>Succinogenes</i> ; S85 |
| 81 | HQ616118 | 99 | Bovine Rumen | |
| 82 | HQ008623 | 99 | Dromedary Camel Rumen | |
| 83 | JF619353 | 97 | Bovine Rumen | |
| 84 | JF624919 | 97 | Bovine Rumen | |
| 85 | JF626877 | 97 | Bovine Rumen | |
| 86 | JF628349 | 98 | Bovine Rumen | |
| 87 | JF628876 | 98 | Bovine Rumen | |
| 88 | JF631630 | 97 | Bovine Rumen | |
| 89 | JF632152 | 97 | Bovine Rumen | |
| 90 | JF632279 | 98 | Bovine Rumen | |
| 91 | JF633413 | 96 | Bovine Rumen | |
| 92 | JF634082 | 97 | Bovine Rumen | |
| 93 | JF636497 | 98 | Bovine Rumen | |
| 94 | JF636594 | 98 | Bovine Rumen | |

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|-----|----------|----|-------------------------------|---------------------------------------|
| 95 | JF636625 | 98 | Bovine Rumen | |
| 96 | JF638129 | 97 | Bovine Rumen | |
| 97 | JF639431 | 97 | Bovine Rumen | |
| 98 | JF640254 | 97 | Bovine Rumen | |
| 99 | JF646765 | 97 | Bovine Rumen/Lake Sediment | |
| 100 | JF653661 | 98 | Bovine Rumen/Lake Sediment | |
| 101 | JF655355 | 97 | Bovine Rumen/Lake Sediment | |
| 102 | JF656038 | 97 | Bovine Rumen/Lake Sediment | |
| 103 | JF656888 | 98 | Bovine Rumen/Lake Sediment | |
| 104 | JF657159 | 98 | Bovine Rumen/Lake Sediment | |
| 105 | JF657955 | 98 | Bovine Rumen/Lake Sediment | |
| 106 | JF657961 | 97 | Bovine Rumen/Lake Sediment | |
| 107 | JF658129 | 95 | Bovine Rumen/Lake Sediment | |
| 108 | JF659551 | 97 | Bovine Rumen/Lake Sediment | |
| 109 | JF665653 | 97 | Bovine Rumen/Lake Sediment | |
| 110 | JF666539 | 98 | Bovine Rumen/Lake Sediment | |
| 111 | JF970205 | 99 | Bovine Rumen | <i>Fibrobacter succinogenes</i> ; H23 |
| 112 | AB665863 | 98 | Ovine Rumen | |

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|-----|----------|----|---------------|---|
| 113 | AB665895 | 99 | Ovine Rumen | |
| 114 | AB665948 | 98 | Ovine Rumen | |
| 115 | HQ634725 | 98 | Yak Rumen | |
| 116 | JQ346742 | 99 | Bovine Rumen | <i>Fibrobacter succinogenes</i> subsp. <i>Succinogenes</i> ; S85 <i>Fibrobacter</i> ; RM1 |
| 117 | AB730669 | 98 | Bovine Rumen | |
| 118 | AB555230 | 98 | Bovine Rumen | |
| 119 | AB555376 | 97 | Bovine Rumen | |
| 120 | AB612345 | 95 | Bovine Rumen | |
| 121 | AB612778 | 99 | Bovine Rumen | |
| 122 | AB614696 | 99 | Bovine Rumen | |
| 123 | AB614894 | 97 | Bovine Rumen | |
| 124 | AB614945 | 97 | Bovine Rumen | |
| 125 | AB615044 | 97 | Bovine Rumen | |
| 126 | AB615174 | 98 | Bovine Rumen | |
| 127 | AB615195 | 98 | Bovine Rumen | |
| 128 | AB616290 | 99 | Bovine Rumen | |
| 129 | AB616322 | 99 | Bovine Rumen | |
| 130 | AB616408 | 98 | Bovine Rumen | |
| 131 | KJ364183 | 98 | Lake Sediment | |
| 132 | KJ364184 | 98 | Lake Sediment | |
| 133 | KJ364186 | 98 | Landfill | |
| 134 | KJ364192 | 98 | Landfill | |
| 135 | KJ364204 | 98 | Landfill | |
| 136 | KJ364205 | 98 | Landfill | |
| 137 | KJ364206 | 98 | Landfill | |
| 138 | KJ364212 | 98 | Landfill | |
| 139 | KJ364215 | 98 | Landfill | |
| 140 | KJ364218 | 98 | Estuarine | |
| 141 | KJ364220 | 99 | Estuarine | |

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|-----|----------|----|-----------------|
| 142 | KJ364221 | 97 | Estuarine |
| 143 | KJ364222 | 98 | Estuarine |
| 144 | KJ364223 | 98 | Estuarine |
| 145 | KJ364224 | 97 | Estuarine |
| 146 | KJ364240 | 98 | Lake Sediment |
| 147 | KJ364241 | 98 | Lake Sediment |
| 148 | KJ364243 | 98 | Lake Sediment |
| 149 | KJ364247 | 97 | Ovine Rumen |
| 150 | KJ364250 | 98 | Soil |
| 151 | KJ364251 | 98 | Soil |
| 152 | KJ364252 | 98 | Soil |
| 153 | KJ364255 | 98 | Marine Sediment |
| 154 | KJ364256 | 98 | Marine Sediment |
| 155 | KJ364257 | 98 | Marine Sediment |
| 156 | KJ364267 | 99 | Bovine Rumen |
| 157 | KJ364288 | 96 | Cryoconite |
| 158 | KJ364320 | 99 | Ovine Rumen |
| 159 | KJ364322 | 98 | Ovine Rumen |
| 160 | KJ364332 | 99 | Soil |
| 161 | KJ364334 | 99 | Soil |
| 162 | KJ364337 | 99 | Soil |
| 163 | KJ364340 | 99 | Lake Sediment |
| 164 | KJ364341 | 98 | Lake Sediment |
| 165 | KJ364343 | 98 | Lake Sediment |
| 166 | KJ364347 | 98 | Lake Sediment |
| 167 | KJ364348 | 98 | Lake Sediment |
| 168 | KJ364349 | 98 | Lake Sediment |
| 169 | KJ364350 | 99 | Lake Sediment |
| 170 | KJ364351 | 98 | Lake Sediment |
| 171 | KJ364352 | 98 | Lake Sediment |

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| 172 | KJ364353 | 98 | Lake Sediment |
| 173 | KJ364355 | 98 | Landfill |
| 174 | KJ364356 | 98 | Landfill |
| 175 | KJ364357 | 98 | Landfill |
| 176 | KJ364360 | 99 | Estuarine |
| 177 | KJ364362 | 99 | Estuarine |
| 178 | KJ364364 | 99 | Estuarine |
| 179 | KJ364366 | 99 | Estuarine |
| 180 | KJ364368 | 99 | Estuarine |
| 181 | KJ364372 | 97 | Estuarine |
| 182 | KJ364374 | 99 | Estuarine |
| 183 | KJ364375 | 97 | Estuarine |
| 184 | KJ364378 | 98 | Estuarine |
| 185 | KJ364379 | 99 | Estuarine |
| 186 | KJ364381 | 98 | Estuarine |
| 187 | KJ364382 | 97 | Estuarine |
| 188 | KJ364383 | 98 | Estuarine |
| 189 | KJ364391 | 99 | Lake Sediment |
| 190 | KJ364392 | 98 | Lake Sediment |
| 191 | KJ364395 | 98 | Lake Sediment |
| 192 | KJ364396 | 99 | Lake Sediment |
| 193 | KJ364398 | 98 | Lake Sediment |
| 194 | KJ364399 | 98 | Lake Sediment |
| 195 | KJ364400 | 99 | Lake Sediment |
| 196 | KJ364401 | 98 | Lake Sediment |
| 197 | KJ364403 | 98 | Lake Sediment |
| 198 | KJ364405 | 98 | Marine Sediment |
| 199 | KJ364406 | 98 | Marine Sediment |
| 200 | KJ364407 | 98 | Marine Sediment |
| 201 | KJ364408 | 99 | Marine Sediment |

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| 202 | KJ364410 | 98 | Marine Sediment |
| 203 | KJ364411 | 98 | Marine Sediment |
| 204 | KJ364413 | 99 | Marine Sediment |
| 205 | KJ364414 | 98 | Marine Sediment |
| 206 | KJ364425 | 99 | Landfill |
| 207 | KJ364427 | 99 | Landfill |
| 208 | KJ364428 | 99 | Landfill |
| 209 | KJ364429 | 98 | Landfill |
| 210 | KJ364430 | 99 | Landfill |
| 211 | KJ364431 | 98 | Landfill |
| 212 | KJ364448 | 99 | Landfill |
| 213 | KJ364449 | 99 | Landfill |
| 214 | KJ364450 | 99 | Landfill |
| 215 | KJ364455 | 98 | Landfill |
| 216 | KJ364457 | 99 | Landfill |
| 217 | KJ364458 | 99 | Landfill |
| 218 | KJ364459 | 99 | Landfill |
| 219 | KJ364460 | 99 | Landfill |
| 220 | KJ364477 | 99 | Landfill |
| 221 | KJ364478 | 99 | Landfill |
| 222 | KJ364479 | 99 | Landfill |

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| 1 | 0 | M62682 | RS* | Bovine Rumen | <i>Fibrobacter succinogenes</i> ; REH9-1 | Bovine 100 |
| 21 | 0 | M62688 | RS* | Bovine Rumen | <i>Fibrobacter succinogenes</i> ; GC5 | Bovine 71 |
| | 1 | AB275483 | 96 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; AL227 | Ovine 19 |
| | 2 | AB275484 | 97 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; AL225 | Other Mammal 5 |
| | 3 | AB275485 | 98 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; AS211 | Soil 5 |
| | 4 | AB275499 | 98 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; OS114 | |
| | 5 | EF186241 | 97 | Bovine Rumen | | |
| | 6 | EU981941 | 97 | Buffalo Rumen | | |
| | 7 | FJ711742 | 96 | Soil | | |

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| | 8 | HM104731 | 95 | Bovine Rumen | | |
| | 9 | HM104754 | 96 | Bovine Rumen | | |
| | 10 | HM104767 | 95 | Bovine Rumen | | |
| | 11 | HM104806 | 96 | Bovine Rumen | | |
| | 12 | HM104828 | 96 | Bovine Rumen | | |
| | 13 | HM104911 | 96 | Bovine Rumen | | |
| | 14 | HM104958 | 95 | Bovine Rumen | | |
| | 15 | HM105476 | 95 | Bovine Rumen | | |
| | 16 | KJ364194 | 97 | Bovine Rumen | | |
| | 17 | KJ364260 | 97 | Bovine Rumen | | |
| | 18 | KJ364266 | 97 | Bovine Rumen | | |
| | 19 | KJ364268 | 97 | Bovine Rumen | | |
| | 20 | KJ364272 | 96 | Bovine Rumen | | |
| 13 | 0 | AY578474 | 97 | Bovine Rumen | | Bovine 15 |
| | 1 | AY578638 | 97 | Bovine Rumen | | Ovine 85 |
| | 2 | M62689 | RS* | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; HM2 | |
| | 3 | M62692 | 96 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; MB4 | |
| | 4 | M62694 | 97 | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; MM4 | |
| | 5 | KJ364246 | 98 | Ovine Rumen | | |
| | 6 | KJ364248 | 97 | Ovine Rumen | | |
| | 7 | KJ364316 | 98 | Ovine Rumen | | |
| | 8 | KJ364317 | 98 | Ovine Rumen | | |
| | 9 | KJ364318 | 98 | Ovine Rumen | | |
| | 10 | KJ364319 | 99 | Ovine Rumen | | |
| | 11 | KJ364323 | 98 | Ovine Rumen | | |
| | 12 | KJ364324 | 96 | Ovine Rumen | | |
| 3 | 0 | M62693 | RS* | Ovine Rumen | <i>Fibrobacter succinogenes</i> ; MC1 | Ovine 100 |
| | 1 | FJ711751 | 98 | Ovine Faeces | | |
| | 2 | FJ711752 | 98 | Ovine Faeces | | |

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|----|----|----------|-----|---|--|---------------------------|
| 3 | 0 | EF520548 | 99 | Acid-impacted Lake Water | | Lake 67 Shrimp Pond 33 |
| | 1 | EF520549 | 99 | Acid-impacted Lake Water | | |
| | 2 | HQ433562 | RS* | Shrimp Pond | | |
| 1 | 0 | HM184982 | RS* | Hot Spring | | Hot Spring 100 |
| 20 | 0 | AJ496284 | 96 | Rat Cecum | <i>Fibrobacter intestinalis</i> (T); NR9 | Other Mammal 80 |
| | 1 | EU474873 | 97 | Red River Hog Faeces | | Bovine 20 |
| | 2 | EU475285 | 97 | Rock Hyrax Faeces | | |
| | 3 | EU774496 | 97 | Eastern Black and White Colobus Faeces | | |
| | 4 | GQ451204 | RS* | Yunnan Snub-nosed Monkey Faeces | | |
| | 5 | GQ451231 | 99 | Yunnan Snub-nosed Monkey Faeces | | |
| | 6 | GQ451246 | 99 | Yunnan Snub-nosed Monkey Faeces | | |
| | 7 | GQ451248 | 99 | Yunnan Snub-nosed Monkey Faeces | | |
| | 8 | GQ451260 | 99 | Yunnan Snub-nosed Monkey Faeces | | |
| | 9 | GQ451284 | 99 | Yunnan Snub-nosed Monkey Faeces | | |
| | 10 | GQ451292 | 99 | Yunnan Snub-nosed Monkey Faeces | | |
| | 11 | GQ451306 | 100 | Yunnan Snub-nosed Monkey Faeces | | |
| | 12 | GQ451307 | 99 | Yunnan Snub-nosed Monkey Faeces | | |
| | 13 | GQ451318 | 96 | Yunnan Snub-nosed | | |

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| | | | | Monkey Faeces | |
| | 14 | GQ451324 | 99 | Yunnan Snub-nosed Monkey Faeces | |
| | 15 | GQ451325 | 99 | Yunnan Snub-nosed Monkey Faeces | |
| | 16 | AB555427 | 97 | Bovine Rumen | |
| | 17 | AB555445 | 97 | Bovine Rumen | |
| | 18 | AB555456 | 96 | Bovine Rumen | |
| | 19 | AB555470 | 96 | Bovine Rumen | |
| 4 | 0 | EU101143 | RS* | Frasassi Sulfidic Cave Stream Biofilm | Cave Water 100 |
| | 1 | EU101148 | 99 | Frasassi Sulfidic Cave Stream Biofilm | |
| | 2 | EU101166 | 99 | Frasassi Sulfidic Cave Stream Biofilm | |
| | 3 | EU101192 | 99 | Frasassi Sulfidic Cave Stream Biofilm | |
| 297 | 0 | EF686988 | 99 | Bovine Faeces | Bovine/Sediment 54 |
| | 1 | EF186244 | 98 | Landfill | Landfill 22 |
| | 2 | EF186247 | 98 | Landfill | Cryoconite 10 |
| | 3 | EF186249 | 98 | Landfill | ASBR Reactor 8 |
| | 4 | EF186250 | 98 | Landfill | Sewage 4 |
| | 5 | EF186251 | 98 | Landfill | Biogas Plant 1 |
| | 6 | EF186253 | 98 | Landfill | Bovine <1 |
| | 7 | EF186259 | 98 | Landfill | Other Mammal <1 |
| | 8 | EF186260 | 99 | Landfill | Lake Sediment <1 |
| | 9 | EF186261 | 98 | Landfill | Estuarine <1 |
| | 10 | EF186262 | 99 | Landfill | |
| | 11 | EF186263 | 99 | Landfill | |
| | 12 | EF186264 | 97 | Landfill | |

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| 13 | EF186265 | 98 | Landfill |
| 14 | EF186266 | 97 | Landfill |
| 15 | EF186267 | 98 | Landfill |
| 16 | EF186270 | 98 | Landfill |
| 17 | EF186278 | 98 | Landfill |
| 18 | EF186282 | 98 | Landfill |
| 19 | EF186283 | 99 | Landfill |
| 20 | EF186284 | 98 | Landfill |
| 21 | EF186286 | 98 | Landfill |
| 22 | EF186291 | 99 | Landfill |
| 23 | EF186292 | 98 | Landfill |
| 24 | EF186293 | 98 | Landfill |
| 25 | EF190823 | 99 | Landfill |
| 26 | EF190824 | 98 | Landfill |
| 27 | EF190825 | 99 | Landfill |
| 28 | EF190827 | 99 | Landfill |
| 29 | EF190829 | 96 | Landfill |
| 30 | AM982635 | 98 | Porcine Faeces |
| 31 | DQ261258 | 99 | Biogas Plant |
| 32 | AB494338 | 99 | Anaerobic Digester Sludge |
| 33 | FN985259 | 99 | Biogas Reactor |
| 34 | JF541098 | 97 | Bovine Rumen/Sediment |
| 35 | JF541203 | 97 | Bovine Rumen/Sediment |
| 36 | JF541333 | 98 | Bovine Rumen/Sediment |
| 37 | JF541377 | 99 | Bovine Rumen/Sediment |

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| 38 | JF541389 | 99 | Bovine Rumen/Sediment |
| 39 | JF541495 | 97 | Bovine Rumen/Sediment |
| 40 | JF541543 | 97 | Bovine Rumen/Sediment |
| 41 | JF541618 | 99 | Bovine Rumen/Sediment |
| 42 | JF541636 | 98 | Bovine Rumen/Sediment |
| 43 | JF541697 | 98 | Bovine Rumen/Sediment |
| 44 | JF541813 | 98 | Bovine Rumen/Sediment |
| 45 | JF541863 | 98 | Bovine Rumen/Sediment |
| 46 | JF541917 | 98 | Bovine Rumen/Sediment |
| 47 | JF541954 | 98 | Bovine Rumen/Sediment |
| 48 | JF541985 | 99 | Bovine Rumen/Sediment |
| 49 | JF542048 | 98 | Bovine Rumen/Sediment |
| 50 | JF542170 | 98 | Bovine Rumen/Sediment |
| 51 | JF542223 | 98 | Bovine Rumen/Sediment |
| 52 | JF542229 | 98 | Bovine Rumen/Sediment |
| 53 | JF542428 | 98 | Bovine |

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| | | | Rumen/Sediment |
| | | | Bovine |
| 54 | JF542431 | 99 | Rumen/Sediment |
| | | | Bovine |
| 55 | JF542458 | 99 | Rumen/Sediment |
| | | | Bovine |
| 56 | JF542468 | 99 | Rumen/Sediment |
| | | | Bovine |
| 57 | JF542530 | 98 | Rumen/Sediment |
| | | | Bovine |
| 58 | JF542567 | 98 | Rumen/Sediment |
| | | | Bovine |
| 59 | JF542601 | 99 | Rumen/Sediment |
| | | | Bovine |
| 60 | JF542691 | 98 | Rumen/Sediment |
| | | | Bovine |
| 61 | JF542774 | 99 | Rumen/Sediment |
| | | | Bovine |
| 62 | JF542814 | 98 | Rumen/Sediment |
| | | | Bovine |
| 63 | JF542824 | 99 | Rumen/Sediment |
| | | | Bovine |
| 64 | JF542890 | 99 | Rumen/Sediment |
| | | | Bovine |
| 65 | JF542899 | 98 | Rumen/Sediment |
| | | | Bovine |
| 66 | JF543024 | 98 | Rumen/Sediment |
| | | | Bovine |
| 67 | JF543044 | 98 | Rumen/Sediment |
| | | | Bovine |
| 68 | JF543133 | 98 | Rumen/Sediment |

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| 69 | JF543204 | 98 | Bovine Rumen/Sediment |
| 70 | JF543225 | 98 | Bovine Rumen/Sediment |
| 71 | JF543302 | 98 | Bovine Rumen/Sediment |
| 72 | JF543359 | 99 | Bovine Rumen/Sediment |
| 73 | JF543393 | 98 | Bovine Rumen/Sediment |
| 74 | JF543564 | 99 | Bovine Rumen/Sediment |
| 75 | JF543634 | 98 | Bovine Rumen/Sediment |
| 76 | JF543662 | 98 | Bovine Rumen/Sediment |
| 77 | JF543786 | 98 | Bovine Rumen/Sediment |
| 78 | JF543841 | 98 | Bovine Rumen/Sediment |
| 79 | JF543842 | 99 | Bovine Rumen/Sediment |
| 80 | JF544020 | 98 | Bovine Rumen/Sediment |
| 81 | JF544038 | 99 | Bovine Rumen/Sediment |
| 82 | JF544044 | 98 | Bovine Rumen/Sediment |
| 83 | JF544047 | 98 | Bovine Rumen/Sediment |
| 84 | JF544065 | 98 | Bovine |

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| | | | Rumen/Sediment |
| | | | Bovine |
| 85 | JF544072 | 98 | Rumen/Sediment |
| | | | Bovine |
| 86 | JF544093 | 98 | Rumen/Sediment |
| | | | Bovine |
| 87 | JF544129 | 98 | Rumen/Sediment |
| | | | Bovine |
| 88 | JF544160 | 99 | Rumen/Sediment |
| | | | Bovine |
| 89 | JF544197 | 99 | Rumen/Sediment |
| | | | Bovine |
| 90 | JF544218 | 98 | Rumen/Sediment |
| | | | Bovine |
| 91 | JF544255 | 99 | Rumen/Sediment |
| | | | Bovine |
| 92 | JF544302 | 98 | Rumen/Sediment |
| | | | Bovine |
| 93 | JF544415 | 98 | Rumen/Sediment |
| | | | Bovine |
| 94 | JF544430 | 99 | Rumen/Sediment |
| | | | Bovine |
| 95 | JF544496 | 97 | Rumen/Sediment |
| | | | Bovine |
| 96 | JF544507 | 98 | Rumen/Sediment |
| | | | Bovine |
| 97 | JF544527 | 99 | Rumen/Sediment |
| | | | Bovine |
| 98 | JF544669 | 98 | Rumen/Sediment |
| | | | Bovine |
| 99 | JF544690 | 98 | Rumen/Sediment |

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|-----|----------|----|--------------------------|
| 100 | JF544694 | 99 | Bovine Rumen/Sediment |
| 101 | JF544734 | 99 | Bovine Rumen/Sediment |
| 102 | JF544820 | 98 | Bovine Rumen/Sediment |
| 103 | JF544927 | 98 | Bovine Rumen/Sediment |
| 104 | JF545000 | 98 | Bovine Rumen/Sediment |
| 105 | JF545077 | 98 | Bovine Rumen/Sediment |
| 106 | JF545138 | 98 | Bovine Rumen/Sediment |
| 107 | JF545139 | 99 | Bovine Rumen/Sediment |
| 108 | JF545196 | 98 | Bovine Rumen/Sediment |
| 109 | JF545245 | 99 | Bovine Rumen/Sediment |
| 110 | JF545297 | 99 | Bovine Rumen/Sediment |
| 111 | JF545342 | 99 | Bovine Rumen/Sediment |
| 112 | JF545419 | 99 | Bovine Rumen/Sediment |
| 113 | JF545440 | 99 | Bovine Rumen/Sediment |
| 114 | JF545457 | 99 | Bovine Rumen/Sediment |
| 115 | JF545530 | 99 | Bovine |

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| | | | Rumen/Sediment |
| | | | Bovine |
| 116 | JF545640 | 98 | Rumen/Sediment |
| | | | Bovine |
| 117 | JF545648 | 99 | Rumen/Sediment |
| | | | Bovine |
| 118 | JF545668 | 98 | Rumen/Sediment |
| | | | Bovine |
| 119 | JF545679 | 99 | Rumen/Sediment |
| | | | Bovine |
| 120 | JF545807 | 99 | Rumen/Sediment |
| | | | Bovine |
| 121 | JF545879 | 99 | Rumen/Sediment |
| | | | Bovine |
| 122 | JF545999 | 98 | Rumen/Sediment |
| | | | Bovine |
| 123 | JF546121 | 98 | Rumen/Sediment |
| | | | Bovine |
| 124 | JF546148 | 99 | Rumen/Sediment |
| | | | Bovine |
| 125 | JF546192 | 98 | Rumen/Sediment |
| | | | Bovine |
| 126 | JF546284 | 98 | Rumen/Sediment |
| | | | Bovine |
| 127 | JF546403 | 98 | Rumen/Sediment |
| | | | Bovine |
| 128 | JF546408 | 98 | Rumen/Sediment |
| | | | Bovine |
| 129 | JF546428 | 99 | Rumen/Sediment |
| | | | Bovine |
| 130 | JF546442 | 99 | Rumen/Sediment |

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| 131 | JF546445 | 98 | Bovine Rumen/Sediment |
| 132 | JF546491 | 99 | Bovine Rumen/Sediment |
| 133 | JF546526 | 98 | Bovine Rumen/Sediment |
| 134 | JF546550 | 98 | Bovine Rumen/Sediment |
| 135 | JF546640 | 99 | Bovine Rumen/Sediment |
| 136 | JF546690 | 98 | Bovine Rumen/Sediment |
| 137 | JF546700 | 99 | Bovine Rumen/Sediment |
| 138 | JF546748 | 98 | Bovine Rumen/Sediment |
| 139 | JF546770 | 99 | Bovine Rumen/Sediment |
| 140 | JF546809 | 98 | Bovine Rumen/Sediment |
| 141 | JF546971 | 99 | Bovine Rumen/Sediment |
| 142 | JF547113 | 98 | Bovine Rumen/Sediment |
| 143 | JF547263 | 98 | Bovine Rumen/Sediment |
| 144 | JF547289 | 99 | Bovine Rumen/Sediment |
| 145 | JF547344 | 98 | Bovine Rumen/Sediment |
| 146 | JF547382 | 98 | Bovine |

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| | | | Rumen/Sediment |
| | | | Bovine |
| 147 | JF547390 | 99 | Rumen/Sediment |
| | | | Bovine |
| 148 | JF547486 | 99 | Rumen/Sediment |
| | | | Bovine |
| 149 | JF547511 | 98 | Rumen/Sediment |
| | | | Bovine |
| 150 | JF547521 | 99 | Rumen/Sediment |
| | | | Bovine |
| 151 | JF547567 | 98 | Rumen/Sediment |
| | | | Bovine |
| 152 | JF547579 | 98 | Rumen/Sediment |
| | | | Bovine |
| 153 | JF547615 | 98 | Rumen/Sediment |
| | | | Bovine |
| 154 | JF547649 | 98 | Rumen/Sediment |
| | | | Bovine |
| 155 | JF547668 | 98 | Rumen/Sediment |
| | | | Bovine |
| 156 | JF547681 | 98 | Rumen/Sediment |
| | | | Bovine |
| 157 | JF547732 | 99 | Rumen/Sediment |
| | | | Bovine |
| 158 | JF547754 | 98 | Rumen/Sediment |
| | | | Bovine |
| 159 | JF547757 | 99 | Rumen/Sediment |
| | | | Bovine |
| 160 | JF547759 | 98 | Rumen/Sediment |
| | | | Bovine |
| 161 | JF547875 | 98 | Rumen/Sediment |

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| 162 | JF547905 | 98 | Bovine Rumen/Sediment |
| 163 | JF547973 | 99 | Bovine Rumen/Sediment |
| 164 | JF548050 | 98 | Bovine Rumen/Sediment |
| 165 | JF548055 | 98 | Bovine Rumen/Sediment |
| 166 | JF548135 | 99 | Bovine Rumen/Sediment |
| 167 | JF548216 | 98 | Bovine Rumen/Sediment |
| 168 | JF548298 | 98 | Bovine Rumen/Sediment |
| 169 | JF548329 | 98 | Bovine Rumen/Sediment |
| 170 | JF548397 | 99 | Bovine Rumen/Sediment |
| 171 | JF548553 | 98 | Bovine Rumen/Sediment |
| 172 | JF548560 | 98 | Bovine Rumen/Sediment |
| 173 | JF548562 | 98 | Bovine Rumen/Sediment |
| 174 | JF548626 | 98 | Bovine Rumen/Sediment |
| 175 | JF548710 | 98 | Bovine Rumen/Sediment |
| 176 | JF548771 | 99 | Bovine Rumen/Sediment |
| 177 | JF548794 | 98 | Bovine |

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| | | | Rumen/Sediment |
| | | | Bovine |
| 178 | JF548886 | 98 | Rumen/Sediment |
| | | | Bovine |
| 179 | JF549027 | 98 | Rumen/Sediment |
| | | | Bovine |
| 180 | JF549111 | 98 | Rumen/Sediment |
| | | | Bovine |
| 181 | JF549211 | 98 | Rumen/Sediment |
| | | | Bovine |
| 182 | JF549241 | 99 | Rumen/Sediment |
| | | | Bovine |
| 183 | JF549278 | 99 | Rumen/Sediment |
| | | | Bovine |
| 184 | JF549283 | 98 | Rumen/Sediment |
| | | | Bovine |
| 185 | JF549316 | 99 | Rumen/Sediment |
| | | | Bovine |
| 186 | JF549417 | 98 | Rumen/Sediment |
| | | | Bovine |
| 187 | JF549437 | 98 | Rumen/Sediment |
| | | | Bovine |
| 188 | JF549524 | 98 | Rumen/Sediment |
| | | | Bovine |
| 189 | JF549792 | 98 | Rumen/Sediment |
| | | | Bovine |
| 190 | JF550122 | 98 | Rumen/Sediment |
| | | | Bovine |
| 191 | JF550176 | 99 | Rumen/Sediment |
| | | | Bovine |
| 192 | JF550179 | 99 | Rumen/Sediment |

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| 193 | JF550297 | 98 | Bovine Rumen/Sediment |
| 194 | JF834127 | RS* | Biogas Slurry |
| 195 | GQ132252 | 99 | ASBR Reactor |
| 196 | GQ132261 | 99 | ASBR Reactor |
| 197 | GQ132442 | 98 | ASBR Reactor |
| 198 | GQ132478 | 98 | ASBR Reactor |
| 199 | GQ132499 | 99 | ASBR Reactor |
| 200 | GQ132501 | 98 | ASBR Reactor |
| 201 | GQ132609 | 98 | ASBR Reactor |
| 202 | GQ133123 | 97 | ASBR Reactor |
| 203 | GQ133225 | 97 | ASBR Reactor |
| 204 | GQ133237 | 97 | ASBR Reactor |
| 205 | GQ133240 | 98 | ASBR Reactor |
| 206 | GQ133287 | 99 | ASBR Reactor |
| 207 | GQ133293 | 98 | ASBR Reactor |
| 208 | GQ133295 | 95 | ASBR Reactor |
| 209 | GQ133301 | 99 | ASBR Reactor |
| 210 | GQ133369 | 98 | ASBR Reactor |
| 211 | GQ134182 | 99 | ASBR Reactor |
| 212 | GQ134365 | 98 | ASBR Reactor |
| 213 | GQ134392 | 99 | ASBR Reactor |
| 214 | GQ134395 | 98 | ASBR Reactor |
| 215 | GQ134401 | 98 | ASBR Reactor |
| 216 | GQ138292 | 99 | ASBR Reactor |
| 217 | GQ138400 | 99 | ASBR Reactor |
| 218 | JQ089558 | 98 | Anaerobic Sludge Digester |
| 219 | JQ108760 | 98 | Anaerobic Sludge Digester |

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| 220 | JQ110680 | 99 | Anaerobic Sludge Digester |
| 221 | JQ126817 | 99 | Anaerobic Sludge Digester |
| 222 | JQ129974 | 99 | Anaerobic Sludge Digester |
| 223 | JQ139566 | 98 | Anaerobic Sludge Digester |
| 224 | JQ149873 | 97 | Anaerobic Sludge Digester |
| 225 | JQ151287 | 98 | Anaerobic Sludge Digester |
| 226 | JQ151288 | 99 | Anaerobic Sludge Digester |
| 227 | JQ151319 | 99 | Anaerobic Sludge Digester |
| 228 | JQ151385 | 99 | Anaerobic Sludge Digester |
| 229 | KJ364199 | 99 | Landfill |
| 230 | KJ364200 | 99 | Landfill |
| 231 | KJ364201 | 98 | Landfill |
| 232 | KJ364202 | 98 | Landfill |
| 233 | KJ364203 | 98 | Landfill |
| 234 | KJ364207 | 98 | Landfill |
| 235 | KJ364208 | 98 | Landfill |
| 236 | KJ364209 | 98 | Landfill |
| 237 | KJ364210 | 98 | Landfill |
| 238 | KJ364211 | 97 | Landfill |
| 239 | KJ364225 | 98 | Cryoconite |
| 240 | KJ364226 | 98 | Cryoconite |

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| 241 | KJ364227 | 99 | Cryoconite |
| 242 | KJ364228 | 99 | Cryoconite |
| 243 | KJ364229 | 98 | Cryoconite |
| 244 | KJ364230 | 97 | Cryoconite |
| 245 | KJ364231 | 97 | Cryoconite |
| 246 | KJ364232 | 99 | Cryoconite |
| 247 | KJ364233 | 98 | Cryoconite |
| 248 | KJ364234 | 98 | Cryoconite |
| 249 | KJ364244 | 98 | Lake Sediment |
| 250 | KJ364276 | 99 | Cryoconite |
| 251 | KJ364277 | 98 | Cryoconite |
| 252 | KJ364278 | 96 | Cryoconite |
| 253 | KJ364280 | 98 | Cryoconite |
| 254 | KJ364281 | 99 | Cryoconite |
| 255 | KJ364283 | 98 | Cryoconite |
| 256 | KJ364285 | 99 | Cryoconite |
| 257 | KJ364286 | 99 | Cryoconite |
| 258 | KJ364287 | 99 | Cryoconite |
| 259 | KJ364289 | 99 | Cryoconite |
| 260 | KJ364290 | 99 | Cryoconite |
| 261 | KJ364291 | 98 | Cryoconite |
| 262 | KJ364292 | 95 | Cryoconite |
| 263 | KJ364293 | 98 | Cryoconite |
| 264 | KJ364294 | 98 | Cryoconite |
| 265 | KJ364295 | 96 | Cryoconite |
| 266 | KJ364373 | 98 | Estuarine |
| 267 | KJ364377 | 98 | Estuarine |
| 268 | KJ364385 | 99 | Cryoconite |
| 269 | KJ364388 | 98 | Cryoconite |
| 270 | KJ364389 | 99 | Cryoconite |

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|----|-----|----------|-----|-----------------------------|--------------|
| | 271 | KJ364393 | 99 | Lake Sediment | |
| | 272 | KJ364433 | 98 | Landfill | |
| | 273 | KJ364434 | 99 | Landfill | |
| | 274 | KJ364435 | 99 | Landfill | |
| | 275 | KJ364437 | 99 | Landfill | |
| | 276 | KJ364438 | 98 | Landfill | |
| | 277 | KJ364439 | 99 | Landfill | |
| | 278 | KJ364440 | 99 | Landfill | |
| | 279 | KJ364441 | 99 | Landfill | |
| | 280 | KJ364442 | 99 | Landfill | |
| | 281 | KJ364443 | 97 | Landfill | |
| | 282 | KJ364444 | 99 | Landfill | |
| | 283 | KJ364445 | 99 | Landfill | |
| | 284 | KJ364446 | 99 | Landfill | |
| | 285 | KJ364462 | 99 | Landfill | |
| | 286 | KJ364463 | 98 | Landfill | |
| | 287 | KJ364464 | 98 | Landfill | |
| | 288 | KJ364465 | 99 | Landfill | |
| | 289 | KJ364466 | 99 | Landfill | |
| | 290 | KJ364467 | 96 | Landfill | |
| | 291 | KJ364468 | 99 | Landfill | |
| | 292 | KJ364469 | 99 | Landfill | |
| | 293 | KJ364471 | 99 | Landfill | |
| | 294 | KJ364473 | 99 | Landfill | |
| | 295 | KJ364474 | 98 | Landfill | |
| | 296 | KJ364475 | 99 | Landfill | |
| 2 | 0 | AM409807 | 98 | Lake Sediment | Lake 50 |
| | 1 | JN038681 | RS* | Wetland (Estuarine) Soil | Estuarine 50 |
| 39 | 0 | AY315287 | 97 | Bovine Rumens | Equine 46 |

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|----|----------|----|---|-----------------|
| 1 | L35548 | 95 | Equine Cecum | Bovine 38 |
| 2 | EU381839 | 95 | Bovine Rumen | Other Mammal 13 |
| 3 | EU463463 | 96 | Equine Faeces | Ovine 3 |
| 4 | EU473476 | 97 | Somali Wild Ass Faeces | |
| 5 | EU473520 | 97 | Somali Wild Ass Faeces | |
| 6 | EU473538 | 97 | Somali Wild Ass Faeces | |
| 7 | EU473539 | 97 | Somali Wild Ass Faeces | |
| 8 | EU473542 | 96 | Somali Wild Ass Faeces | |
| 9 | EU473558 | 96 | Somali Wild Ass Faeces | |
| 10 | EU473600 | 97 | Somali Wild Ass Faeces | |
| 11 | EU473604 | 97 | Somali Wild Ass Faeces | |
| 12 | EU473606 | 97 | Somali Wild Ass Faeces | |
| 13 | EU773721 | 98 | Capybara Faeces | |
| 14 | EU774414 | 96 | Eastern Black and White Colobus Faeces | |
| 15 | EU774452 | 96 | Eastern Black and White Colobus Faeces | |
| 16 | EU779347 | 95 | Somali Wild Ass Faeces | |
| 17 | EU779383 | 97 | Somali Wild Ass Faeces | |
| 18 | EU779394 | 97 | Somali Wild Ass | |

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| | | | | Faeces | |
| | 19 | EU779396 | 97 | Somali Wild Ass | |
| | | | | Faeces | |
| | 20 | EU779399 | 97 | Somali Wild Ass | |
| | | | | Faeces | |
| | 21 | AB549942 | 97 | Equine Faeces | |
| | 22 | GU303627 | 95 | Bovine Rumen | |
| | 23 | GU999988 | 96 | Goat Rumen | <i>Fibrobacter succinogenes</i> ; FGL 01 |
| | 24 | GU999989 | 96 | Goat Rumen | <i>Fibrobacter succinogenes</i> ; FGL 25 |
| | 25 | HM104821 | 97 | Bovine Rumen | |
| | 26 | HM105466 | 97 | Bovine Rumen | |
| | 27 | AB665809 | 98 | Ovine Rumen | |
| | 28 | AB555039 | 96 | Bovine Rumen | |
| | 29 | AB555056 | RS* | Bovine Rumen | |
| | 30 | AB555099 | 96 | Bovine Rumen | |
| | 31 | AB612349 | 96 | Bovine Rumen | |
| | 32 | AB612387 | 96 | Bovine Rumen | |
| | 33 | AB612730 | 95 | Bovine Rumen | |
| | 34 | AB614748 | 95 | Bovine Rumen | |
| | 35 | AB615064 | 96 | Bovine Rumen | |
| | 36 | AB615083 | 96 | Bovine Rumen | |
| | 37 | AB616232 | 96 | Bovine Rumen | |
| | 38 | KJ364303 | 95 | Equine Faeces | |
| 8 | 0 | HM104816 | 97 | Bovine Rumen | Bovine 75 |
| | 1 | HM104820 | 97 | Bovine Rumen | Ovine 25 |
| | 2 | HM104868 | 96 | Bovine Rumen | |
| | 3 | JF628563 | 96 | Bovine Rumen | |
| | 4 | AB665797 | 100 | Ovine Rumen | |
| | 5 | AB614672 | RS* | Bovine Rumen | |
| | 6 | AB614851 | 98 | Bovine Rumen | |

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| | 7 | KJ364314 | 96 | Ovine Rumen | |
| 33 | 0 | AB255931 | 95 | Termite Gut | Termite 100 |
| | 1 | AB255933 | 96 | Termite Gut | |
| | 2 | AB255934 | 96 | Termite Gut | |
| | 3 | AB255935 | 95 | Termite Gut | |
| | 4 | AB255937 | 96 | Termite Gut | |
| | 5 | AB255945 | 96 | Termite Gut | |
| | 6 | EF453857 | 95 | Termite Gut | |
| | 7 | EF454783 | 98 | Termite Gut | |
| | 8 | EF453821 | 97 | Termite Gut | |
| | 9 | EF453822 | 96 | Termite Gut | |
| | 10 | EF453826 | 97 | Termite Gut | |
| | 11 | EF454021 | 97 | Termite Gut | |
| | 12 | EF454057 | 97 | Termite Gut | |
| | 13 | EF454275 | 98 | Termite Gut | |
| | 14 | EF454284 | 98 | Termite Gut | |
| | 15 | EF454314 | 96 | Termite Gut | |
| | 16 | EF454318 | 98 | Termite Gut | |
| | 17 | EF454325 | 98 | Termite Gut | |
| | 18 | EF454418 | 97 | Termite Gut | |
| | 19 | EF454434 | 96 | Termite Gut | |
| | 20 | EF454459 | 98 | Termite Gut | |
| | 21 | EF454461 | 98 | Termite Gut | |
| | 22 | EF454475 | 98 | Termite Gut | |
| | 23 | EF454506 | 97 | Termite Gut | |
| | 24 | EF454585 | 98 | Termite Gut | |
| | 25 | EF454604 | 96 | Termite Gut | |
| | 26 | EF454628 | 98 | Termite Gut | |
| | 27 | EF454859 | RS* | Termite Gut | |
| | 28 | EF454888 | 98 | Termite Gut | |

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| | 29 | EF454924 | 97 | Termite Gut | |
| | 30 | EF454949 | 97 | Termite Gut | |
| | 31 | EF454981 | 96 | Termite Gut | |
| | 32 | EF455006 | 96 | Termite Gut | |
| 7 | 0 | AB255932 | 95 | Termite Gut | Termite 100 |
| | 1 | EF453831 | RS* | Termite Gut | |
| | 2 | EF454276 | 98 | Termite Gut | |
| | 3 | EF454303 | 99 | Termite Gut | |
| | 4 | EF454610 | 99 | Termite Gut | |
| | 5 | EF454823 | 99 | Termite Gut | |
| | 6 | EF454908 | 99 | Termite Gut | |
| 1 | 0 | EU471816 | RS* | Asiatic Elephant Faeces | Other Mammal 100 |
| 5 | 0 | EU473449 | RS* | Somali Wild Ass Faeces | Equine 100 |
| | 1 | AB549937 | 98 | Equine Faeces | |
| | 2 | AB549941 | 98 | Equine Faeces | |
| | 3 | AB549945 | 95 | Equine Faeces | |
| | 4 | AB549950 | 96 | Equine Faeces | |
| 1 | 0 | GQ134316 | RS* | ABSR Reactor | ABSR Reactor 100 |
| 2 | 0 | GQ135590 | RS* | ABSR Reactor | ABSR Reactor 100 |
| | 1 | GQ135614 | 99 | ABSR Reactor | |
| 4 | 0 | GQ135610 | RS* | ABSR Reactor | ABSR Reactor 100 |
| | 1 | GQ135709 | 99 | ABSR Reactor | |
| | 2 | GQ135746 | 99 | ABSR Reactor | |
| | 3 | GQ136142 | 100 | ABSR Reactor | |
| 10 | 0 | GQ133837 | RS* | ABSR Reactor | ABSR Reactor 100 |
| | 1 | GQ135134 | 99 | ABSR Reactor | |
| | 2 | GQ135584 | 99 | ABSR Reactor | |
| | 3 | GQ135690 | 99 | ABSR Reactor | |

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| | 4 | GQ135695 | 99 | ABSR Reactor | |
| | 5 | GQ135710 | 99 | ABSR Reactor | |
| | 6 | GQ135744 | 99 | ABSR Reactor | |
| | 7 | GQ135753 | 100 | ABSR Reactor | |
| | 8 | GQ135795 | 99 | ABSR Reactor | |
| | 9 | GQ135895 | 99 | ABSR Reactor | |
| 2 | 0 | GQ139119 | RS* | ABSR Reactor | ABSR Reactor 50 |
| | 1 | KJ364273 | 99 | Cryoconite | Cryoconite 50 |
| 3 | 0 | FJ353483 | 97 | Raw Sewage | Sewage 67 |
| | 1 | GU915580 | 96 | Activated Sludge | Mud Volcano 33 |
| | 2 | JQ245640 | RS* | Mud Volcano | |
| 1 | 0 | GQ135762 | RS* | ABSR Reactor | ABSR Reactor 100 |
| 7 | 0 | EU470330 | RS* | Grevy's Zebra Faeces | Equine 100 |
| | 1 | EU470375 | 98 | Grevy's Zebra Faeces | |
| | 2 | EU470410 | 98 | Grevy's Zebra Faeces | |
| | 3 | EU473545 | 96 | Somali Wild Ass Faeces | |
| | 4 | AB549946 | 97 | Equine Faeces | |
| | 5 | AB549947 | 96 | Equine Faeces | |
| | 6 | AB549949 | 97 | Equine Faeces | |
| 10 | 0 | EU463400 | 96 | Equine Faeces | Equine 100 |
| | 1 | EU473518 | 96 | Somali Wild Ass Faeces | |
| | 2 | EU473529 | RS* | Somali Wild Ass Faeces | |
| | 3 | EU473605 | 98 | Somali Wild Ass Faeces | |
| | 4 | AB549932 | 99 | Equine Faeces | |
| | 5 | AB549934 | 99 | Equine Faeces | |
| | 6 | AB549938 | 98 | Equine Faeces | |

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| | 7 | AB549948 | 97 | Equine Faeces | |
| | 8 | KJ364298 | 95 | Equine Faeces | |
| | 9 | KJ364301 | 95 | Equine Faeces | |
| 1 | 0 | GQ132590 | RS* | ABSR Reactor | ABSR Reactor 100 |
| 1 | 0 | GQ138403 | RS* | ABSR Reactor | ABSR Reactor 100 |
| 20 | 0 | DQ054636 | 97 | Bovine Rumen | Ovine 30 |
| | 1 | AB113670 | 98 | Ovine Rumen | Soil 30 |
| | 2 | AM493699 | 97 | Simulated Rumen | Other Mammal 15 |
| | 3 | EU461440 | 98 | Black Rhinoceros Faeces | Lake 15 Bovine 5 |
| | 4 | EU468455 | 97 | Black Rhinoceros Faeces | Simulated Rumen 5 |
| | 5 | FJ711741 | 97 | Soil | |
| | 6 | FJ711743 | 97 | Soil | |
| | 7 | FJ711744 | 97 | Lake Sediment | |
| | 8 | FJ711745 | 97 | Lake Sediment | |
| | 9 | FJ711746 | 97 | Lake Sediment | |
| | 10 | FJ711747 | 97 | Soil | |
| | 11 | FJ711748 | 97 | Soil | |
| | 12 | FJ711749 | 98 | Soil | |
| | 13 | FJ711750 | 98 | Soil | |
| | 14 | GQ358264 | RS* | Tammar Wallaby Gut | |
| | 15 | KJ364245 | 96 | Ovine Rumen | |
| | 16 | KJ364249 | 97 | Ovine Rumen | |
| | 17 | KJ364311 | 97 | Ovine Rumen | |
| | 18 | KJ364313 | 97 | Ovine Rumen | |
| | 19 | KJ364321 | 97 | Ovine Rumen | |
| 2 | 0 | GQ135015 | RS* | ABSR Reactor | ABSR Reactor 100 |
| | 1 | GQ136564 | 96 | ABSR Reactor | |
| 1 | 0 | AB234547 | RS* | Termite Gut | Termite Gut 100 |

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| 10 | 0 | EF190822 | RS* | Landfill | Landfill 50 |
| | 1 | KJ364198 | 97 | Landfill | Cryoconite 40 |
| | 2 | KJ364275 | 99 | Cryoconite | Estuarine 10 |
| | 3 | KJ364284 | 99 | Cryoconite | |
| | 4 | KJ364384 | 99 | Estuarine | |
| | 5 | KJ364386 | 99 | Cryoconite | |
| | 6 | KJ364387 | 99 | Cryoconite | |
| | 7 | KJ364436 | 99 | Landfill | |
| | 8 | KJ364470 | 99 | Landfill | |
| | 9 | KJ364472 | 99 | Landfill | |
| 2 | 0 | AB192095 | RS* | Termite Gut | Termite Gut 100 |
| | 1 | AB192093 | 96 | Termite Gut | |
| 6 | 0 | AB192085 | RS* | Termite Gut | Termite Gut 100 |
| | 1 | AB192086 | 99 | Termite Gut | |
| | 2 | AB192087 | 98 | Termite Gut | |
| | 3 | AB192088 | 100 | Termite Gut | |
| | 4 | AB192097 | 96 | Termite Gut | |
| | 5 | AB243275 | 100 | Termite Gut | |
| 9 | 0 | AB192079 | 95 | Termite Gut | Termite Gut 100 |
| | 1 | AB192081 | 96 | Termite Gut | |
| | 2 | AB192082 | 96 | Termite Gut | |
| | 3 | AB192096 | RS* | Termite Gut | |
| | 4 | AB243276 | 96 | Termite Gut | |
| | 5 | AB248829 | 99 | Termite Gut | |
| | 6 | AB248830 | 96 | Termite Gut | |
| | 7 | AB255941 | 95 | Termite Gut | |
| | 8 | AB255942 | 96 | Termite Gut | |
| 3 | 0 | AB192075 | RS* | Termite Gut | Termite Gut 100 |
| | 1 | AB192077 | 98 | Termite Gut | |

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| | 2 | AB255938 | 98 | Termite Gut | |
| 2 | 0 | AB192083 | RS* | Termite Gut | Termite Gut 100 |
| | 1 | AB192084 | 98 | Termite Gut | |
| 6 | 0 | AB192089 | 96 | Termite Gut | Termite Gut 100 |
| | 1 | AB192090 | 97 | Termite Gut | |
| | 2 | AB192091 | RS* | Termite Gut | |
| | 3 | AB192092 | 99 | Termite Gut | |
| | 4 | AB192094 | 96 | Termite Gut | |
| | 5 | AB243277 | 100 | Termite Gut | |
| 1 | 0 | AB243279 | RS* | Termite Gut | Termite Gut 100 |
| 2 | 0 | AB255943 | RS* | Termite Gut | Termite Gut 100 |
| | 1 | AB255952 | 96 | Termite Gut | |
| 1 | 0 | EU775761 | RS* | Equine Faeces | Equine 100 |
| 1 | 0 | GQ135618 | RS* | ABSR Reactor | ABSR Reactor 100 |
| 1 | 0 | L35547 | RS* | Equine Faeces | Equine 100 |
| 197 | 0 | JF531980 | 98 | Bovine Rumen/Sediment | Bovine/Sediment 97 Sewage 2 |
| | 1 | JF532546 | 98 | Bovine Rumen/Sediment | ABSR Reactor <1 Cryoconite <1 |
| | 2 | JF533869 | 99 | Bovine Rumen/Sediment | |
| | 3 | JF534568 | 98 | Bovine Rumen/Sediment | |
| | 4 | JF534724 | 98 | Bovine Rumen/Sediment | |
| | 5 | JF534917 | 99 | Bovine Rumen/Sediment | |
| | 6 | JF535075 | 99 | Bovine Rumen/Sediment | |
| | 7 | JF535488 | 99 | Bovine | |

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| | | | Rumen/Sediment |
| 8 | JF535659 | 98 | Bovine |
| | | | Rumen/Sediment |
| 9 | JF535681 | 99 | Bovine |
| | | | Rumen/Sediment |
| 10 | JF535696 | 99 | Bovine |
| | | | Rumen/Sediment |
| 11 | JF535727 | 98 | Bovine |
| | | | Rumen/Sediment |
| 12 | JF535730 | 98 | Bovine |
| | | | Rumen/Sediment |
| 13 | JF535789 | 99 | Bovine |
| | | | Rumen/Sediment |
| 14 | JF535830 | 99 | Bovine |
| | | | Rumen/Sediment |
| 15 | JF535997 | 98 | Bovine |
| | | | Rumen/Sediment |
| 16 | JF536057 | 99 | Bovine |
| | | | Rumen/Sediment |
| 17 | JF536331 | 99 | Bovine |
| | | | Rumen/Sediment |
| 18 | JF536779 | 98 | Bovine |
| | | | Rumen/Sediment |
| 19 | JF537054 | 99 | Bovine |
| | | | Rumen/Sediment |
| 20 | JF537774 | 99 | Bovine |
| | | | Rumen/Sediment |
| 21 | JF538050 | 98 | Bovine |
| | | | Rumen/Sediment |
| 22 | JF538234 | 99 | Bovine |
| | | | Rumen/Sediment |

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| 23 | JF538450 | 99 | Bovine Rumen/Sediment |
| 24 | JF538631 | 99 | Bovine Rumen/Sediment |
| 25 | JF538841 | 99 | Bovine Rumen/Sediment |
| 26 | JF539023 | 99 | Bovine Rumen/Sediment |
| 27 | JF539141 | 99 | Bovine Rumen/Sediment |
| 28 | JF539648 | 99 | Bovine Rumen/Sediment |
| 29 | JF539900 | 99 | Bovine Rumen/Sediment |
| 30 | JF541373 | 98 | Bovine Rumen/Sediment |
| 31 | JF542950 | 98 | Bovine Rumen/Sediment |
| 32 | JF543045 | 98 | Bovine Rumen/Sediment |
| 33 | JF543570 | 98 | Bovine Rumen/Sediment |
| 34 | JF543936 | 99 | Bovine Rumen/Sediment |
| 35 | JF544473 | 99 | Bovine Rumen/Sediment |
| 36 | JF544665 | 99 | Bovine Rumen/Sediment |
| 37 | JF545458 | 98 | Bovine Rumen/Sediment |
| 38 | JF545788 | 99 | Bovine |

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| | | | Rumen/Sediment |
| | | | Bovine |
| 39 | JF545833 | 99 | Rumen/Sediment |
| | | | Bovine |
| 40 | JF546354 | 99 | Rumen/Sediment |
| | | | Bovine |
| 41 | JF546563 | 99 | Rumen/Sediment |
| | | | Bovine |
| 42 | JF546814 | 98 | Rumen/Sediment |
| | | | Bovine |
| 43 | JF547023 | 98 | Rumen/Sediment |
| | | | Bovine |
| 44 | JF547792 | 98 | Rumen/Sediment |
| | | | Bovine |
| 45 | JF548146 | 99 | Rumen/Sediment |
| | | | Bovine |
| 46 | JF548554 | 98 | Rumen/Sediment |
| | | | Bovine |
| 47 | JF550142 | 98 | Rumen/Sediment |
| | | | Bovine |
| 48 | JF550832 | 97 | Rumen/Sediment |
| | | | Bovine |
| 49 | JF551115 | 99 | Rumen/Sediment |
| | | | Bovine |
| 50 | JF551780 | 99 | Rumen/Sediment |
| | | | Bovine |
| 51 | JF552279 | 98 | Rumen/Sediment |
| | | | Bovine |
| 52 | JF552475 | 98 | Rumen/Sediment |
| | | | Bovine |
| 53 | JF552527 | 98 | Rumen/Sediment |

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| 54 | JF552711 | 98 | Bovine Rumen/Sediment |
| 55 | JF552965 | 98 | Bovine Rumen/Sediment |
| 56 | JF553866 | 99 | Bovine Rumen/Sediment |
| 57 | JF554623 | 98 | Bovine Rumen/Sediment |
| 58 | JF554673 | 98 | Bovine Rumen/Sediment |
| 59 | JF555194 | 99 | Bovine Rumen/Sediment |
| 60 | JF555517 | 99 | Bovine Rumen/Sediment |
| 61 | JF556595 | 99 | Bovine Rumen/Sediment |
| 62 | JF557536 | 98 | Bovine Rumen/Sediment |
| 63 | JF557839 | 98 | Bovine Rumen/Sediment |
| 64 | JF557884 | 98 | Bovine Rumen/Sediment |
| 65 | JF558895 | 99 | Bovine Rumen/Sediment |
| 66 | JF559653 | 98 | Bovine Rumen/Sediment |
| 67 | JF561764 | 98 | Bovine Rumen/Sediment |
| 68 | JF561953 | 99 | Bovine Rumen/Sediment |
| 69 | JF562405 | 97 | Bovine |

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| | | | Rumen/Sediment |
| | | | Bovine |
| 70 | JF563069 | 99 | Rumen/Sediment |
| | | | Bovine |
| 71 | JF563769 | 99 | Rumen/Sediment |
| | | | Bovine |
| 72 | JF563801 | 98 | Rumen/Sediment |
| | | | Bovine |
| 73 | JF564019 | 98 | Rumen/Sediment |
| | | | Bovine |
| 74 | JF564430 | 99 | Rumen/Sediment |
| | | | Bovine |
| 75 | JF566211 | 98 | Rumen/Sediment |
| | | | Bovine |
| 76 | JF566576 | 98 | Rumen/Sediment |
| | | | Bovine |
| 77 | JF566592 | 99 | Rumen/Sediment |
| | | | Bovine |
| 78 | JF569925 | 98 | Rumen/Sediment |
| | | | Bovine |
| 79 | JF570487 | 98 | Rumen/Sediment |
| | | | Bovine |
| 80 | JF570876 | 98 | Rumen/Sediment |
| | | | Bovine |
| 81 | JF571055 | 98 | Rumen/Sediment |
| | | | Bovine |
| 82 | JF571741 | 98 | Rumen/Sediment |
| | | | Bovine |
| 83 | JF572088 | 98 | Rumen/Sediment |
| | | | Bovine |
| 84 | JF572705 | 98 | Rumen/Sediment |

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|-----|----------|----|--------------------------|
| 85 | JF574227 | 99 | Bovine Rumen/Sediment |
| 86 | JF574870 | 99 | Bovine Rumen/Sediment |
| 87 | JF575965 | 99 | Bovine Rumen/Sediment |
| 88 | JF576526 | 98 | Bovine Rumen/Sediment |
| 89 | JF577354 | 99 | Bovine Rumen/Sediment |
| 90 | JF577907 | 99 | Bovine Rumen/Sediment |
| 91 | JF578900 | 99 | Bovine Rumen/Sediment |
| 92 | JF578965 | 99 | Bovine Rumen/Sediment |
| 93 | JF579008 | 99 | Bovine Rumen/Sediment |
| 94 | JF579080 | 98 | Bovine Rumen/Sediment |
| 95 | JF579834 | 98 | Bovine Rumen/Sediment |
| 96 | JF579897 | 99 | Bovine Rumen/Sediment |
| 97 | JF580142 | 99 | Bovine Rumen/Sediment |
| 98 | JF580205 | 98 | Bovine Rumen/Sediment |
| 99 | JF580500 | 98 | Bovine Rumen/Sediment |
| 100 | JF580903 | 98 | Bovine |

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|-----|----------|----|----------------|
| | | | Rumen/Sediment |
| 101 | JF583391 | 98 | Bovine |
| | | | Rumen/Sediment |
| 102 | JF583770 | 96 | Bovine |
| | | | Rumen/Sediment |
| 103 | JF584324 | 98 | Bovine |
| | | | Rumen/Sediment |
| 104 | JF585065 | 98 | Bovine |
| | | | Rumen/Sediment |
| 105 | JF585898 | 98 | Bovine |
| | | | Rumen/Sediment |
| 106 | JF586657 | 99 | Bovine |
| | | | Rumen/Sediment |
| 107 | JF586945 | 98 | Bovine |
| | | | Rumen/Sediment |
| 108 | JF587075 | 98 | Bovine |
| | | | Rumen/Sediment |
| 109 | JF587208 | 98 | Bovine |
| | | | Rumen/Sediment |
| 110 | JF589024 | 98 | Bovine |
| | | | Rumen/Sediment |
| 111 | JF589244 | 98 | Bovine |
| | | | Rumen/Sediment |
| 112 | JF589553 | 98 | Bovine |
| | | | Rumen/Sediment |
| 113 | JF597551 | 97 | Bovine |
| | | | Rumen/Sediment |
| 114 | JF597589 | 99 | Bovine |
| | | | Rumen/Sediment |
| 115 | JF597612 | 98 | Bovine |
| | | | Rumen/Sediment |

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|-----|----------|----|--------------------------|
| 116 | JF597664 | 99 | Bovine Rumen/Sediment |
| 117 | JF597669 | 98 | Bovine Rumen/Sediment |
| 118 | JF597763 | 99 | Bovine Rumen/Sediment |
| 119 | JF597809 | 98 | Bovine Rumen/Sediment |
| 120 | JF597906 | 98 | Bovine Rumen/Sediment |
| 121 | JF597933 | 99 | Bovine Rumen/Sediment |
| 122 | JF598021 | 99 | Bovine Rumen/Sediment |
| 123 | JF598072 | 99 | Bovine Rumen/Sediment |
| 124 | JF598101 | 98 | Bovine Rumen/Sediment |
| 125 | JF598137 | 98 | Bovine Rumen/Sediment |
| 126 | JF598191 | 98 | Bovine Rumen/Sediment |
| 127 | JF598289 | 99 | Bovine Rumen/Sediment |
| 128 | JF598487 | 99 | Bovine Rumen/Sediment |
| 129 | JF598527 | 98 | Bovine Rumen/Sediment |
| 130 | JF598570 | 98 | Bovine Rumen/Sediment |
| 131 | JF598589 | 99 | Bovine |

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|-----|----------|----|----------------|
| | | | Rumen/Sediment |
| | | | Bovine |
| 132 | JF598650 | 99 | Rumen/Sediment |
| | | | Bovine |
| 133 | JF598737 | 98 | Rumen/Sediment |
| | | | Bovine |
| 134 | JF598783 | 99 | Rumen/Sediment |
| | | | Bovine |
| 135 | JF598885 | 98 | Rumen/Sediment |
| | | | Bovine |
| 136 | JF598997 | 99 | Rumen/Sediment |
| | | | Bovine |
| 137 | JF599024 | 99 | Rumen/Sediment |
| | | | Bovine |
| 138 | JF599211 | 99 | Rumen/Sediment |
| | | | Bovine |
| 139 | JF599303 | 98 | Rumen/Sediment |
| | | | Bovine |
| 140 | JF599344 | 99 | Rumen/Sediment |
| | | | Bovine |
| 141 | JF599598 | 98 | Rumen/Sediment |
| | | | Bovine |
| 142 | JF599749 | 99 | Rumen/Sediment |
| | | | Bovine |
| 143 | JF599775 | 99 | Rumen/Sediment |
| | | | Bovine |
| 144 | JF599792 | 98 | Rumen/Sediment |
| | | | Bovine |
| 145 | JF599909 | 98 | Rumen/Sediment |
| | | | Bovine |
| 146 | JF599926 | 98 | Rumen/Sediment |

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|-----|----------|----|--------------------------|
| 147 | JF599955 | 98 | Bovine Rumen/Sediment |
| 148 | JF600126 | 99 | Bovine Rumen/Sediment |
| 149 | JF600196 | 98 | Bovine Rumen/Sediment |
| 150 | JF600383 | 96 | Bovine Rumen/Sediment |
| 151 | JF600387 | 98 | Bovine Rumen/Sediment |
| 152 | JF600579 | 99 | Bovine Rumen/Sediment |
| 153 | JF600866 | 99 | Bovine Rumen/Sediment |
| 154 | JF600902 | 99 | Bovine Rumen/Sediment |
| 155 | JF601277 | 99 | Bovine Rumen/Sediment |
| 156 | JF601280 | 99 | Bovine Rumen/Sediment |
| 157 | JF670601 | 97 | Bovine Rumen/Sediment |
| 158 | JF670822 | 98 | Bovine Rumen/Sediment |
| 159 | JF671013 | 98 | Bovine Rumen/Sediment |
| 160 | JF671023 | 98 | Bovine Rumen/Sediment |
| 161 | JF671126 | 98 | Bovine Rumen/Sediment |
| 162 | JF671330 | 99 | Bovine |

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|-----|----------|----|----------------|
| | | | Rumen/Sediment |
| | | | Bovine |
| 163 | JF671433 | 98 | Rumen/Sediment |
| | | | Bovine |
| 164 | JF671438 | 98 | Rumen/Sediment |
| | | | Bovine |
| 165 | JF671439 | 98 | Rumen/Sediment |
| | | | Bovine |
| 166 | JF671605 | 99 | Rumen/Sediment |
| | | | Bovine |
| 167 | JF671619 | 98 | Rumen/Sediment |
| | | | Bovine |
| 168 | JF671640 | 99 | Rumen/Sediment |
| | | | Bovine |
| 169 | JF671689 | 98 | Rumen/Sediment |
| | | | Bovine |
| 170 | JF671773 | 99 | Rumen/Sediment |
| | | | Bovine |
| 171 | JF671808 | 98 | Rumen/Sediment |
| | | | Bovine |
| 172 | JF671924 | 99 | Rumen/Sediment |
| | | | Bovine |
| 173 | JF671951 | 99 | Rumen/Sediment |
| | | | Bovine |
| 174 | JF671992 | 98 | Rumen/Sediment |
| | | | Bovine |
| 175 | JF672007 | 98 | Rumen/Sediment |
| | | | Bovine |
| 176 | JF672386 | 98 | Rumen/Sediment |
| | | | Bovine |
| 177 | JF672462 | 99 | Rumen/Sediment |

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|-----|----------|-----|------------------------------|
| 178 | JF672588 | 98 | Bovine Rumen/Sediment |
| 179 | JF672613 | 99 | Bovine Rumen/Sediment |
| 180 | JF672659 | 99 | Bovine Rumen/Sediment |
| 181 | JF672756 | 97 | Bovine Rumen/Sediment |
| 182 | JF672880 | 98 | Bovine Rumen/Sediment |
| 183 | JF672882 | 98 | Bovine Rumen/Sediment |
| 184 | JF672887 | 98 | Bovine Rumen/Sediment |
| 185 | JF672957 | 99 | Bovine Rumen/Sediment |
| 186 | JF673007 | 99 | Bovine Rumen/Sediment |
| 187 | JF673116 | 98 | Bovine Rumen/Sediment |
| 188 | JF673163 | 99 | Bovine Rumen/Sediment |
| 189 | JF673312 | 98 | Bovine Rumen/Sediment |
| 190 | JF673667 | 98 | Bovine Rumen/Sediment |
| 191 | JF673991 | 98 | Bovine Rumen/Sediment |
| 192 | GQ138377 | RS* | ABSR Reactor |
| 193 | JQ133101 | 98 | Anaerobic Sludge Digester |

| | | | | | |
|----|-----|----------|-----|------------------------------|----------------------|
| | 194 | JQ137036 | 99 | Anaerobic Sludge Digester | |
| | 195 | JQ148874 | 98 | Anaerobic Sludge Digester | |
| | 196 | KJ364282 | 99 | Cryoconite | |
| 1 | 0 | AB255951 | RS* | Termite Gut | Termite Gut 100 |
| 1 | 0 | AB255946 | RS* | Termite Gut | Termite Gut 100 |
| 2 | 0 | AB255948 | RS* | Termite Gut | Termite Gut 100 |
| | 1 | AB255949 | 98 | Termite Gut | |
| 2 | 0 | AB255954 | RS* | Termite Gut | Termite Gut 100 |
| | 1 | AB255956 | 96 | Termite Gut | |
| 1 | 0 | GQ136636 | RS* | ABSR Reactor | ABSR Reactor 100 |
| 9 | 0 | FN429847 | RS* | Hydrothermal Vent | Equine 89 |
| | 1 | KJ364235 | 95 | Equine Faeces | Hydrothermal Vent 11 |
| | 2 | KJ364296 | 97 | Equine Faeces | |
| | 3 | KJ364297 | 98 | Equine Faeces | |
| | 4 | KJ364299 | 96 | Equine Faeces | |
| | 5 | KJ364304 | 98 | Equine Faeces | |
| | 6 | KJ364306 | 98 | Equine Faeces | |
| | 7 | KJ364308 | 97 | Equine Faeces | |
| | 8 | KJ364309 | 98 | Equine Faeces | |
| 10 | 0 | EF186254 | 98 | Landfill | Landfill 90 |
| | 1 | EF186255 | 98 | Landfill | Soil 10 |
| | 2 | EF186256 | 98 | Landfill | |
| | 3 | EF186257 | 98 | Landfill | |
| | 4 | EF186258 | 98 | Landfill | |
| | 5 | EF186272 | RS* | Landfill | |
| | 6 | EF186288 | 98 | Landfill | |
| | 7 | EF186289 | 98 | Landfill | |

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|----|----|----------|-----|---------------|--------------|
| | 8 | EF186290 | 98 | Landfill | |
| | 9 | FJ711753 | 98 | Soil | |
| 14 | 0 | EF186245 | 99 | Landfill | Landfill 100 |
| | 1 | EF186246 | 99 | Landfill | |
| | 2 | EF186248 | 99 | Landfill | |
| | 3 | EF186268 | 99 | Landfill | |
| | 4 | EF186269 | 99 | Landfill | |
| | 5 | EF186271 | 99 | Landfill | |
| | 6 | EF186273 | 99 | Landfill | |
| | 7 | EF186274 | 99 | Landfill | |
| | 8 | EF186275 | RS* | Landfill | |
| | 9 | EF186276 | 99 | Landfill | |
| | 10 | EF186277 | 99 | Landfill | |
| | 11 | EF186279 | 99 | Landfill | |
| | 12 | EF186280 | 99 | Landfill | |
| | 13 | EF186281 | 99 | Landfill | |
| 3 | 0 | EF186252 | 99 | Landfill | Landfill 100 |
| | 1 | EF186285 | RS* | Landfill | |
| | 2 | EF186287 | 98 | Landfill | |
| 16 | 0 | FJ711708 | 99 | Lake Water | Lake 100 |
| | 1 | FJ711709 | 99 | Lake Water | |
| | 2 | FJ711710 | 99 | Lake Water | |
| | 3 | FJ711711 | RS* | Lake Water | |
| | 4 | FJ711712 | 99 | Lake Water | |
| | 5 | FJ711713 | 99 | Lake Water | |
| | 6 | FJ711714 | 99 | Lake Water | |
| | 7 | FJ711715 | 99 | Lake Water | |
| | 8 | FJ711716 | 99 | Lake Sediment | |
| | 9 | FJ711727 | 99 | Lake Water | |

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|----|----|----------|-----|---------------|----------------|
| | 10 | FJ711728 | 99 | Lake Water | |
| | 11 | FJ711729 | 98 | Lake Water | |
| | 12 | FJ711730 | 99 | Lake Water | |
| | 13 | FJ711731 | 99 | Lake Water | |
| | 14 | FJ711732 | 99 | Lake Water | |
| | 15 | FJ711737 | 99 | Lake Sediment | |
| 14 | 0 | AB549939 | 97 | Equine Faeces | Equine 64 |
| | 1 | KJ364188 | RS* | Landfill | Landfill 36 |
| | 2 | KJ364189 | 96 | Landfill | |
| | 3 | KJ364236 | 97 | Equine Faeces | |
| | 4 | KJ364237 | 97 | Equine Faeces | |
| | 5 | KJ364238 | 99 | Equine Faeces | |
| | 6 | KJ364239 | 97 | Equine Faeces | |
| | 7 | KJ364300 | 96 | Equine Faeces | |
| | 8 | KJ364302 | 99 | Equine Faeces | |
| | 9 | KJ364305 | 98 | Equine Faeces | |
| | 10 | KJ364307 | 99 | Equine Faeces | |
| | 11 | KJ364415 | 96 | Landfill | |
| | 12 | KJ364416 | 97 | Landfill | |
| | 13 | KJ364417 | 99 | Landfill | |
| 1 | 0 | KJ364190 | RS* | Landfill | Landfill 100 |
| 1 | 0 | KJ364196 | RS* | Bovine Rumen | Bovine 100 |
| 2 | 0 | KJ364274 | RS* | Cryoconite | Cryoconite 100 |
| | 1 | KJ364279 | 95 | Cryoconite | |

APPENDIX IV

454 Pyrosequencing Supplementary Table for Chapter 5

Supplementary Table 2. Classification of 454 pyrosequencing 16S rRNA gene PCR amplicons derived from heavily and poorly degraded colonised cotton via analysis against the EzTaxon database.

| Domain | Phylum | Class | Order | Family | Poorly Degraded Cotton (% of Sequences) | Heavily Degraded Cotton (% of Sequences) |
|--------------|---------------------|-------------|-------|--------|---|--|
| Bacteria | | | | | 100.00 | 99.99 |
| Unclassified | | | | | 0.00 | 0.01 |
| | Bacteroidetes | | | | 72.69 | 20.54 |
| | Firmicutes | | | | 1.08 | 34.66 |
| | Spirochaetes | | | | 0.75 | 14.80 |
| | Fibrobacteres | | | | 0.02 | 14.18 |
| | Proteobacteria | | | | 18.70 | 0.85 |
| | Actinobacteria | | | | 3.30 | 0.06 |
| | Cyanobacteria | | | | 0.36 | 2.63 |
| | Cloacamonas | | | | 0.00 | 4.01 |
| | Tenericutes | | | | 0.65 | 3.39 |
| | Synergistetes | | | | 0.00 | 2.99 |
| | Lentisphaerae | | | | 0.06 | 1.11 |
| | OD1 | | | | 0.74 | 0.28 |
| | Chlorobi | | | | 0.29 | 0.03 |
| | Planctomycetes | | | | 0.32 | 0.10 |
| | Deinococcus-Thermus | | | | 0.19 | 0.00 |
| | TM7 | | | | 0.18 | 0.01 |
| | Chloroflexi | | | | 0.24 | 0.07 |
| | Verrucomicrobia | | | | 0.13 | 0.07 |
| | SR1 | | | | 0.11 | 0.00 |
| | HQ912765 | | | | 0.05 | 0.06 |
| | BRC1 | | | | 0.06 | 0.01 |
| | GN02 | | | | 0.06 | 0.01 |
| | Acidobacteria | | | | 0.00 | 0.03 |
| | 4P001694 | | | | 0.03 | 0.00 |
| | Armatimonadetes | | | | 0.00 | 0.04 |
| | Thermotogae | | | | 0.00 | 0.03 |
| | JS1 | | | | 0.00 | 0.01 |
| | Fusobacteria | | | | 0.01 | 0.00 |
| | Bacteroidetes | Bacteroidia | | | 92.54 | 99.27 |

| | | | | | |
|---------------|-----------------|--------------------|--------------------|--------|--------|
| | Flavobacteria | | | 4.66 | 0.44 |
| | Cytophagia | | | 0.80 | 0.07 |
| | Balneola | | | 0.93 | 0.00 |
| | Sphingobacteria | | | 1.03 | 0.15 |
| | Unclassified | | | 0.04 | 0.07 |
| Bacteroidetes | Bacteroidia | Bacteroidales | | 99.92 | 99.71 |
| | | Unclassified | | 0.08 | 0.29 |
| | Flavobacteria | Flavobacteriales | | 99.73 | 100.00 |
| | | Unclassified | | 0.27 | 0.00 |
| | Cytophagia | Cytophagales | | 100.00 | 100.00 |
| | Balneola | Balneola | | 100.00 | 100.00 |
| | Sphingobacteria | Sphingobacteriales | | 100.00 | 100.00 |
| Bacteroidetes | Bacteroidia | Bacteroidales | Marinilabiliaceae | 87.88 | 6.62 |
| | | | Porphyromonadaceae | 4.97 | 54.34 |
| | | | Paludibacter | 4.35 | 8.24 |
| | | | FJ437992 | 1.34 | 0.74 |
| | | | EU845084 | 0.06 | 6.54 |
| | | | HQ183936 | 0.00 | 5.59 |
| | | | Prolixibacter | 0.07 | 4.19 |
| | | | Bacteroidaceae | 0.26 | 2.87 |
| | | | EF148839 | 0.00 | 3.90 |
| | | | AM982614 | 0.03 | 3.53 |
| | | | Prevotellaceae | 0.06 | 0.66 |
| | | | GQ480115 | 0.12 | 0.15 |
| | | | Anaerocella | 0.00 | 0.66 |
| | | | FJ437753 | 0.00 | 0.44 |
| | | | DQ206420 | 0.00 | 0.44 |
| | | | GQ357022 | 0.04 | 0.07 |
| | | | EF602759 | 0.00 | 0.15 |
| | | | Rikenellaceae | 0.00 | 0.07 |
| | | | FN658701 | 0.00 | 0.07 |
| | | | EU864494 | 0.00 | 0.07 |
| | | | Unclassified | 0.83 | 0.66 |
| Bacteroidetes | Flavobacteria | Flavobacteriales | Flavobacteriaceae | 59.63 | 3.33 |
| | | | EF572459 | 24.87 | 0.00 |
| | | | Brumimicrobiaceae | 4.01 | 0.00 |
| | | | GQ349278 | 2.94 | 0.00 |

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|---------------|-----------------|------------------------|---------------------|--------|--------|
| | | | Cryomorphaceae | 1.34 | 0.00 |
| | | | GU454927 | 1.07 | 0.00 |
| | | | FJ628329 | 0.27 | 0.00 |
| | | | Unclassified | 3.21 | 66.67 |
| Bacteroidetes | Cytophagia | Cytophagales | Cytophagaceae | 1.59 | 100.00 |
| | | | Unclassified | 98.41 | 0.00 |
| Bacteroidetes | Balneola | Balneola | HM129785 | 53.42 | 0.00 |
| | | | Balneola | 36.99 | 0.00 |
| | | | Unclassified | 9.59 | 0.00 |
| Bacteroidetes | Sphingobacteria | Sphingobacteriales | Chitinophagaceae | 44.44 | 100.00 |
| | | | Saprosiraceae | 28.40 | 0.00 |
| | | | EU234264 | 4.94 | 0.00 |
| | | | Unclassified | 22.22 | 0.00 |
| Firmicutes | Clostridia | | | 59.48 | 96.64 |
| | Bacilli | | | 35.34 | 1.21 |
| | Erysipelotrichi | | | 4.31 | 2.03 |
| | AB476673 | | | 0.00 | 0.04 |
| | Unclassified | | | 0.86 | 0.00 |
| Firmicutes | Clostridia | Clostridiales | | 76.81 | 99.11 |
| | | Thermoanaerobacterales | | 20.29 | 0.09 |
| | | DQ887962 | | 0.00 | 0.45 |
| | | Anaerobranca | | 0.00 | 0.04 |
| | | Unclassified | | 2.90 | 0.31 |
| Firmicutes | Bacilli | Bacillales | | 92.68 | 7.14 |
| | | Lactobacillales | | 7.32 | 92.86 |
| Firmicutes | Erysipelotrichi | Erysipelotrichales | | 100.00 | 100.00 |
| Firmicutes | AB476673 | AB476673 | | 0.00 | 100.00 |
| Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | 7.55 | 69.47 |
| | | | Lachnospiraceae | 11.32 | 11.26 |
| | | | Desulfonisporea | 0.00 | 4.77 |
| | | | Sedimentibacter | 0.00 | 3.65 |
| | | | Natranaerovirga | 0.00 | 2.21 |
| | | | Christensenellaceae | 0.00 | 1.62 |
| | | | HM124151 | 0.00 | 1.13 |
| | | | Mogibacterium | 0.00 | 2.48 |
| | | | Tissierella | 18.87 | 0.36 |
| | | | Anaerovirgula | 20.75 | 0.14 |

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|----------------|---------------------|------------------------|-----------------------|--------|--------|
| | | | Clostridiaceae | 13.21 | 0.14 |
| | | | Clostridium g7 | 16.98 | 0.00 |
| | | | Syntrophomonadaceae | 0.00 | 0.27 |
| | | | Gracilibacteraceae | 0.00 | 0.36 |
| | | | Peptostreptococcaceae | 0.00 | 0.18 |
| | | | Eubacteriaceae | 0.00 | 0.18 |
| | | | Veillonellaceae | 3.77 | 0.05 |
| | | | Thermincola | 0.00 | 0.05 |
| | | | AB118592 | 0.00 | 0.14 |
| | | | Unclassified | 7.55 | 1.58 |
| Firmicutes | Clostridia | Thermoanaerobacterales | DQ346486 | 100.00 | 0.00 |
| | | | HM041937 | 0.00 | 100.00 |
| Firmicutes | Clostridia | DQ887962 | DQ887962 | 0.00 | 90.00 |
| | | | FN436103 | 0.00 | 10.00 |
| Firmicutes | Clostridia | Anaerobranca | Anaerobranca | 0.00 | 100.00 |
| Firmicutes | Bacilli | Bacillales | Paenibacillaceae | 92.11 | 50.00 |
| | | | Planococcaceae | 0.00 | 50.00 |
| | | | Unclassified | 7.89 | 0.00 |
| Firmicutes | Bacilli | Lactobacillales | Lactobacillaceae | 66.67 | 100.00 |
| | | | Streptococcaceae | 33.33 | 0.00 |
| Firmicutes | Erysipelotrichi | Erysipelotrichales | EU009800 | 80.00 | 91.84 |
| | | | Allobaculum | 0.00 | 4.08 |
| | | | Erysipelotrichaceae | 20.00 | 2.04 |
| | | | Unclassified | 0.00 | 2.04 |
| Firmicutes | AB476673 | AB476673 | AY221604 | 0.00 | 100.00 |
| Spirochaetes | Spirochaetes | | | 100.00 | 99.90 |
| | | | Unclassified | 0.00 | 0.10 |
| Spirochaetes | Spirochaetes | Spirochaetales | | 100.00 | 98.79 |
| | | | Unclassified | 0.00 | 1.21 |
| Spirochaetes | Spirochaetes | Spirochaetales | Spirochaetaceae | 2.47 | 98.46 |
| | | | Leptospiraceae | 93.83 | 0.00 |
| | | | Unclassified | 3.7 | 1.54 |
| Fibrobacteres | Fibrobacteria | | | 100.00 | 100.00 |
| Fibrobacteres | Fibrobacteria | Fibrobacterales | | 100.00 | 100.00 |
| Fibrobacteres | Fibrobacteria | Fibrobacterales | Fibrobacteraceae | 100.00 | 99.68 |
| | | | Unclassified | 0.00 | 0.32 |
| Proteobacteria | Gammaproteobacteria | | | 67.67 | 22.81 |

| | | | | |
|----------------|-----------------------|-------------------|--------|--------|
| | Betaproteobacteria | | 26.87 | 22.81 |
| | Alphaproteobacteria | | 3.97 | 36.80 |
| | Epsilonproteobacteria | | 1.49 | 1.75 |
| | Deltaproteobacteria | | 0.00 | 15.79 |
| Proteobacteria | Gammaproteobacteria | Oceanospirillales | 60.51 | 23.08 |
| | | Chromatiales | 29.81 | 7.69 |
| | | Alteromonadales | 6.81 | 7.69 |
| | | Pseudomonadales | 1.61 | 15.38 |
| | | Xanthomonadales | 0.36 | 15.38 |
| | | OM182 | 0.29 | 0.00 |
| | | Methylococcales | 0.00 | 15.38 |
| | | Thiotrichales | 0.14 | 7.69 |
| | | Vibrionales | 0.07 | 0.00 |
| | | Enterobacteriales | 0.00 | 7.69 |
| | | HQ191045 | 0.07 | 0.00 |
| | | Steroidobacter | 0.15 | 0.00 |
| | | Marinicella | 0.00 | 0.00 |
| Proteobacteria | Betaproteobacteria | Rhodocyclales | 59.78 | 0.00 |
| | | Burkholderiales | 39.85 | 76.92 |
| | | Methylophilales | 0.37 | 0.00 |
| | | DQ009366 | 0.00 | 7.69 |
| | | Zoogloea | 0.00 | 7.69 |
| | | EU786132 | 0.00 | 7.69 |
| Proteobacteria | Alphaproteobacteria | Rhodobacterales | 67.50 | 4.76 |
| | | Rhizobiales | 17.50 | 42.86 |
| | | Micavibrio | 13.75 | 0.00 |
| | | SAR11 | 0.00 | 28.57 |
| | | Sphingomonadales | 0.00 | 9.52 |
| | | Rickettsiales | 0.00 | 4.76 |
| | | Rhodospirillales | 1.25 | 0.00 |
| | | EU939387 | 0.00 | 4.67 |
| | | Unclassified | 0.00 | 4.67 |
| Proteobacteria | Epsilonproteobacteria | Campylobacterales | 100.00 | 100.00 |
| Proteobacteria | Deltaproteobacteria | FM253572 | 0.00 | 55.56 |
| | | EU861868 | 0.00 | 22.22 |
| | | Desulfobacterales | 0.00 | 11.11 |
| | | GU112205 | 0.00 | 11.11 |

| | | | | | |
|----------------|---------------------|-------------------|--------------------|--------|--------|
| Proteobacteria | Gammaproteobacteria | Oceanospirillales | Halomonadaceae | 99.64 | 0.00 |
| | | | Oceanospirillaceae | 0.00 | 100.00 |
| | | | Unclassified | 0.36 | 0.00 |
| Proteobacteria | Gammaproteobacteria | Chromatiales | Sedimenticola | 99.51 | 0.00 |
| | | | Thiobios | 0.00 | 100.00 |
| | | | Nitrosococcus | 0.25 | 0.00 |
| | | | Unclassified | 0.25 | 0.00 |
| Proteobacteria | Gammaproteobacteria | Alteromonadales | Cellvibrio | 54.26 | 0.00 |
| | | | Idiomarinaceae | 42.55 | 0.00 |
| | | | Marinobacter | 1.06 | 0.00 |
| | | | Alishewanella | 1.06 | 0.00 |
| | | | Teredinibacter | 1.06 | 0.00 |
| | | | Porticoccus | 0.00 | 100.00 |
| Proteobacteria | Gammaproteobacteria | Pseudomonadales | Pseudomonadaceae | 100.00 | 100.00 |
| Proteobacteria | Gammaproteobacteria | Xanthomonadales | Xanthomonadaceae | 100.00 | 100.00 |
| Proteobacteria | Gammaproteobacteria | OM182 | GU474872 | 100.00 | 0.00 |
| Proteobacteria | Gammaproteobacteria | Methylococcales | Methylomonas | 100.00 | 100.00 |
| Proteobacteria | Gammaproteobacteria | Thiotrichales | Methylophaga | 100.00 | 0.00 |
| | | | Leucothrix | 0.00 | 100.00 |
| Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | 100.00 | 0.00 |
| Proteobacteria | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | 0.00 | 100.00 |
| Proteobacteria | Gammaproteobacteria | HQ191045 | HQ191045 | 100.00 | 0.00 |
| Proteobacteria | Gammaproteobacteria | Steroidobacter | AB013829 | 50.00 | 0.00 |
| | | | Unclassified | 50.00 | 0.00 |
| Proteobacteria | Gammaproteobacteria | Marinicella | Marinicella | 100.00 | 0.00 |
| Proteobacteria | Betaproteobacteria | Rhodocyclales | Rhodocyclaceae | 99.07 | 0.00 |
| | | | Unclassified | 0.93 | 0.00 |
| Proteobacteria | Betaproteobacteria | Burkholderiales | Alcaligenaceae | 71.30 | 0.00 |
| | | | Comamonadaceae | 25.46 | 30.00 |
| | | | Lautropia | 2.31 | 0.00 |
| | | | Sphaerotilus | 0.00 | 50.00 |
| | | | AY234747 | 0.93 | 0.00 |
| | | | FJ755754 | 0.00 | 20.00 |
| Proteobacteria | Betaproteobacteria | Methylophilales | Methylophilaceae | 100.00 | 0.00 |
| Proteobacteria | Betaproteobacteria | DQ009366 | AM990454 | 0.00 | 100.00 |
| Proteobacteria | Betaproteobacteria | Zoogloea | Zoogloea | 0.00 | 100.00 |
| Proteobacteria | Betaproteobacteria | EU786132 | EU786132 | 0.00 | 100.00 |

| | | | | | |
|----------------|-----------------------|---------------------|---------------------|--------|--------|
| Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | 85.19 | 100.00 |
| | | | Hyphomonadaceae | 14.81 | 0.00 |
| Proteobacteria | Alphaproteobacteria | Rhizobiales | Devosia | 28.57 | 0.00 |
| | | | Phyllobacteriaceae | 28.57 | 0.00 |
| | | | Xanthobacteraceae | 14.29 | 0.00 |
| | | | Rhodoligotrophos | 0.00 | 22.22 |
| | | | Rhizobiaceae | 7.14 | 1.11 |
| | | | Methylobacteriaceae | 7.14 | 0.00 |
| | | | Bradyrhizobiaceae | 0.00 | 1.11 |
| | | | Hyphomicrobiaceae | 0.00 | 1.11 |
| | | | Unclassified | 14.29 | 4.44 |
| Proteobacteria | Alphaproteobacteria | Micavibrio | AY945895 | 54.55 | 0.00 |
| | | | Micavibrio | 45.45 | 0.00 |
| Proteobacteria | Alphaproteobacteria | SAR11 | SAR11-1 | 0.00 | 83.33 |
| | | | SAR11-2 | 0.00 | 16.67 |
| Proteobacteria | Alphaproteobacteria | Sphingomonadales | Sphingomonadaceae | 0.00 | 100.00 |
| Proteobacteria | Alphaproteobacteria | Rickettsiales | EU800706 | 0.00 | 100.00 |
| Proteobacteria | Alphaproteobacteria | Rhodospirillales | Thalassobaculum | 100.00 | 0.00 |
| Proteobacteria | Alphaproteobacteria | EU939387 | AB270041 | 0.00 | 100.00 |
| Proteobacteria | Epsilonproteobacteria | Campylobacterales | Sulfurimonas | 90.00 | 100.00 |
| | | | Campylobacteraceae | 6.67 | 0.00 |
| | | | Sulfurovum | 3.33 | 0.00 |
| Proteobacteria | Deltaproteobacteria | FM253572 | FM253572 | 0.00 | 100.00 |
| Proteobacteria | Deltaproteobacteria | EU861868 | EU861868 | 0.00 | 100.00 |
| Proteobacteria | Deltaproteobacteria | Desulfobacterales | Desulfobacteraceae | 0.00 | 100.00 |
| Proteobacteria | Deltaproteobacteria | GU112205 | GU112205 | 0.00 | 100.00 |
| Actinobacteria | Actinobacteria | | | 100.00 | 100.00 |
| Actinobacteria | Actinobacteria | Micrococcales | | 96.07 | 75.00 |
| | | Bifidobacteriales | | 1.69 | 0.00 |
| | | Frankiales | | 1.12 | 0.00 |
| | | Propionibacteriales | | 0.84 | 25.00 |
| | | Unclassified | | 0.28 | 0.00 |
| Actinobacteria | Actinobacteria | Micrococcales | Demequinaceae | 64.33 | 0.00 |
| | | | Intrasporangiaceae | 33.04 | 33.33 |
| | | | Cellulomonadaceae | 1.75 | 0.00 |
| | | | Microbacteriaceae | 0.29 | 66.67 |
| | | | Unclassified | 0.58 | 0.00 |

| | | | | | |
|----------------|----------------|---------------------|----------------------|--------|--------|
| Actinobacteria | Actinobacteria | Bifidobacteriales | Bifidobacteriaceae | 100.00 | 0.00 |
| Actinobacteria | Actinobacteria | Frankiales | Nakamurellaceae | 100.00 | 0.00 |
| Actinobacteria | Actinobacteria | Propionibacteriales | Propionibacteriaceae | 100.00 | 0.00 |
| | | | Nocardioideaceae | 0.00 | 100.00 |
| Cyanobacteria | Vampirovibrio | | | 0.00 | 100.00 |
| Cyanobacteria | JF737898 | | | 100.00 | 0.00 |
| Cyanobacteria | Vampirovibrio | Vampirovibrio | | 0.00 | 99.43 |
| | | AF544207 | | 0.00 | 0.57 |
| Cyanobacteria | JF737898 | JF417809 | | 76.92 | 0.00 |
| | | GU174155 | | 15.38 | 0.00 |
| | | JF417809 | | 7.69 | 0.00 |
| Cyanobacteria | Vampirovibrio | AF544207 | AF544207 | 0.00 | 100.00 |
| | | Vampirovibrio | Unclassified | 0.00 | 100.00 |
| Cyanobacteria | JF737898 | JF417809 | AY212703 | 9.09 | 0.00 |
| | | JF417809 | Unclassified | 90.91 | 0.00 |
| | | GU174155 | GU444060 | 100.00 | 0.00 |
| Cloacamonas | Cloacamonas | | | 0.00 | 100.00 |
| Cloacamonas | Cloacamonas | EF031090 | | 0.00 | 60.07 |
| | | Cloacamonas | | 0.00 | 39.55 |
| | | Unclassified | | 0.00 | 0.37 |
| Cloacamonas | Cloacamonas | EF031090 | AJ853569 | 0.00 | 96.89 |
| | | | Unclassified | 0.00 | 3.11 |
| Cloacamonas | Cloacamonas | Cloacamonas | Cloacamonas | 0.00 | 100.00 |
| Tenericutes | Mollicutes | | | 100.00 | 100.00 |
| Tenericutes | Mollicutes | AM275436 | | 0.00 | 65.20 |
| | | Acholeplasmatales | | 100.00 | 7.93 |
| | | GU196243 | | 0.00 | 25.11 |
| | | Unclassified | | 0.00 | 1.76 |
| Tenericutes | Mollicutes | AM275436 | AM275436 | 0.00 | 83.78 |
| | | | EF445272 | 0.00 | 3.38 |
| | | | Unclassified | 0.00 | 12.84 |
| Tenericutes | Mollicutes | Acholeplasmatales | Acholeplasmataceae | 98.57 | 100.00 |
| | | | Unclassified | 1.43 | 0.00 |
| Tenericutes | Mollicutes | GU196243 | FJ367735 | 0.00 | 56.14 |
| | | | GU196243 | 0.00 | 3.51 |
| | | | Unclassified | 0.00 | 40.35 |
| Synergistetes | Synergistia | | | 0.00 | 100.00 |

| | | | | | |
|----------------|------------------|-------------------|-------------------|--------|--------|
| Synergistetes | Synergistia | Synergistales | | 0.00 | 40.50 |
| | | Dethiosulfovibrio | | 0.00 | 59.50 |
| Synergistetes | Synergistia | Synergistales | Synergistaceae | 0.00 | 77.78 |
| | | | Thermovirga | 0.00 | 22.22 |
| Synergistetes | Synergistia | Dethiosulfovibrio | Aminobacterium | 0.00 | 52.94 |
| | | | Dethiosulfovibrio | 0.00 | 47.06 |
| Lentisphaerae | Lentisphaeria | | | 0.00 | 77.02 |
| | GU196224 | | | 100.00 | 21.62 |
| | Unclassified | | | 0.00 | 1.35 |
| Lentisphaerae | Lentisphaeria | Oligosphaerales | | 0.00 | 94.74 |
| | | EU885056 | | 0.00 | 3.51 |
| | | Victivallales | | 0.00 | 1.75 |
| Lentisphaerae | GU196224 | GU196224 | | 100.00 | 100.00 |
| Lentisphaerae | Lentisphaeria | Oligosphaerales | Oligosphaeraceae | 0.00 | 100.00 |
| Lentisphaerae | Lentisphaeria | EU885056 | EU885056 | 0.00 | 100.00 |
| Lentisphaerae | Lentisphaeria | Victivallales | Unclassified | 0.00 | 100.00 |
| Lentisphaerae | GU196224 | GU196224 | EF574345 | 66.67 | 0.00 |
| | | | GU196224 | 0.00 | 93.75 |
| | | | JF747850 | 22.33 | 0.00 |
| | | | Unclassified | 0.00 | 6.25 |
| OD1 | FJ547054 | | | 87.50 | 0.00 |
| | OD1 | | | 12.50 | 100.00 |
| OD1 | FJ547054 | AB504963 | | 98.57 | 0.00 |
| | | Unclassified | | 1.43 | 0.00 |
| OD1 | OD1 | OD1 | | 100.00 | 100.00 |
| OD1 | FJ547054 | AB504963 | EU050865 | 59.42 | 0.00 |
| | | | AY168743 | 28.99 | 0.00 |
| | | | EU735622 | 8.70 | 0.00 |
| | | | FJ710698 | 1.45 | 0.00 |
| | | | AJ853574 | 1.45 | 0.00 |
| OD1 | OD1 | OD1 | AM982633 | 100.00 | 100.00 |
| Chlorobi | OPB56 | | | 100.00 | 100.00 |
| Chlorobi | OPB56 | OPB56 | | 100.00 | 100.00 |
| Chlorobi | OPB56 | OPB56 | EF648021 | 100.00 | 0.00 |
| | | | EU245114 | 0.00 | 100.00 |
| Planctomycetes | Phycisphaerae | | | 85.29 | 57.14 |
| | Planctomycetacia | | | 2.94 | 42.86 |

| | | | | | |
|-----------------|-------------------|-------------------|--------------------|--------|--------|
| | Unclassified | | | 11.76 | 0.00 |
| Planctomycetes | Phycisphaerae | Phycisphaerales | | 100.00 | |
| | | HQ697838 | | 0.00 | |
| | | Phycisphaerales | | 0.00 | |
| Planctomycetes | Planctomycetacia | Planctomycetales | | 100.00 | |
| Planctomycetes | Phycisphaerae | Phycisphaerales | FJ936783 | 100.00 | 0.00 |
| | | HQ697838 | HQ697838 | 0.00 | 100.00 |
| | | Phycisphaerales | Phycisphaeraeaceae | 0.00 | 100.00 |
| Planctomycetes | Planctomycetacia | Planctomycetales | Planctomycetaceae | 100.00 | 100.00 |
| TM7 | TM7 | | | 100.00 | 100.00 |
| TM7 | TM7 | JF421159 | | 68.42 | 100.00 |
| | | TM7 | | 31.58 | 0.00 |
| TM7 | TM7 | JF421159 | JF421159 | 100.00 | 0.00 |
| | | | AF269001 | 0.00 | 100.00 |
| | | TM7 | TM7 | 83.33 | 0.00 |
| | | | Unclassified | 16.67 | 0.00 |
| Chloroflexi | Anaerolineae | | | 92.31 | 40.00 |
| | Dehalococcoidetes | | | 0.00 | 20.00 |
| | Ktedonobacteria | | | 0.00 | 20.00 |
| | GQ396871 | | | 0.00 | 20.00 |
| | Thermomicrobia | | | 7.69 | 0.00 |
| Chloroflexi | Anaerolineae | AF234733 | | 54.17 | 0.00 |
| | | Anaerolinaeles | | 45.85 | 100.00 |
| | Dehalococcoidetes | Dehalococcoidales | | 0.00 | 100.00 |
| | Ktedonobacteria | AY673403 | | 100.00 | 100.00 |
| | GQ396871 | GQ396871 | | 0.00 | 100.00 |
| | Thermomicrobia | DQ129389 | | 100.00 | 0.00 |
| Chloroflexi | Anaerolineae | AF234733 | AB478660 | 92.31 | 0.00 |
| | | | AF234733 | 7.69 | 0.00 |
| | | Anaerolinaeles | GU455152 | 100.00 | 0.00 |
| | | | Anaerolinaceae | 0.00 | 100.00 |
| | Dehalococcoidetes | Dehalococcoidales | GU553783 | 0.00 | 100.00 |
| | Ktedonobacteria | AY673403 | AY673403 | 0.00 | 100.00 |
| | GQ396871 | GQ396871 | GQ396871 | 0.00 | 100.00 |
| | Thermomicrobia | DQ129389 | DQ129389 | 100.00 | 0.00 |
| Verrucomicrobia | Opitutae | | | 100.00 | 60.00 |
| | Verrucomicrobiae | | | 0.00 | 40.00 |

| | | | | | |
|-----------------|------------------|--------------------|-------------------|--------|--------|
| Verrucomicrobia | Opitutae | Puniceococcales | | 85.71 | 100.00 |
| | | Opitutales | | 14.29 | 0.00 |
| | Verrucomicrobiae | Verrucomicrobiales | | 0.00 | 100.00 |
| Verrucomicrobia | Opitutae | Puniceococcales | FJ825556 | 91.67 | 0.00 |
| | | | AY695840 | 8.33 | 100.00 |
| | | Opitutales | Opitutaceae | 100.00 | 0.00 |
| | Verrucomicrobiae | Verrucomicrobiales | Akkermansia | 0.00 | 100.00 |
| SR1 | AB015542 | | | 100.00 | 0.00 |
| SR1 | AB015542 | AB015542 | | 25.00 | 0.00 |
| | | Unclassified | | 75.00 | 0.00 |
| SR1 | AB015542 | AB015542 | GU410548 | 100.00 | 0.00 |
| HQ912765 | HQ912765 | | | 100.00 | 100.00 |
| HQ912765 | HQ912765 | HQ912765 | | 100.00 | 100.00 |
| HQ912765 | HQ912765 | HQ912765 | HQ912765 | 100.00 | 100.00 |
| BRC1 | FP245541 | | | 100.00 | 0.00 |
| BRC1 | FP245541 | FP245541 | | 100.00 | 0.00 |
| BRC1 | FP245541 | FP245541 | EF683079 | 83.33 | 0.00 |
| | | | AM490696 | 16.67 | 0.00 |
| GN02 | GN02 | | | 100.00 | 0.00 |
| GN02 | GN02 | AM086106 | | 83.33 | 0.00 |
| | | Unclassified | | 16.67 | 0.00 |
| GN02 | GN02 | AM086106 | AM086106 | 100.00 | 0.00 |
| Acidobacteria | Acidobacteria | | | 0.00 | 50.00 |
| | HM243779 | | | 0.00 | 50.00 |
| Acidobacteria | Acidobacteria | Acidobacteriales | | 0.00 | 100.00 |
| | HM243779 | JF718667 | | 0.00 | 100.00 |
| Acidobacteria | Acidobacteria | Acidobacteriales | Acidobacteriaceae | 0.00 | 100.00 |
| | HM243779 | JF718667 | JF718667 | 0.00 | 100.00 |
| 4P001694 | 4P001694 | | | 100.00 | 0.00 |
| 4P001694 | 4P001694 | Unclassified | | 100.00 | 0.00 |
| Armatimonadetes | Fimbriimonadia | | | 0.00 | 100.00 |
| Armatimonadetes | Fimbriimonadia | Fimbriimonadales | | 0.00 | 100.00 |
| Armatimonadetes | Fimbriimonadia | Fimbriimonadales | Unclassified | 0.00 | 100.00 |
| Thermotogae | Thermotogae | | | 0.00 | 100.00 |
| Thermotogae | Thermotogae | Thermotogales | | 0.00 | 100.00 |
| Thermotogae | Thermotogae | Thermotogales | Kosmotoga | 0.00 | 100.00 |
| JS1 | JS1 | | | 0.00 | 100.00 |

| | | | | | |
|--------------|--------------|-----------------|------------------|--------|--------|
| JS1 | JS1 | | | 0.00 | 100.00 |
| JS1 | JS1 | JS1 | JS1 | 0.00 | 100.00 |
| Fusobacteria | Fusobacteria | | | 100.00 | 0.00 |
| Fusobacteria | Fusobacteria | Fusobacteriales | | 100.00 | 0.00 |
| Fusobacteria | Fusobacteria | Fusobacteriales | Leptotrichiaceae | 100.00 | 0.00 |

APPENDIX V

Metagenome Supplementary Table for Chapter 5

Supplementary Table 3. Classification of metagenome contigs derived from heavily degraded colonised cotton via analysis using MG-RAST against the M5RNA database.

| Domain | Phylum | Class | Order | Family | Percentage of Contigs (%) |
|--------------|-----------------|------------------|-------|--------|---------------------------|
| Bacteria | | | | | 89.59 |
| Eukaryota | | | | | 4.10 |
| Archaea | | | | | 2.68 |
| Unclassified | | | | | 3.31 |
| Viruses | | | | | 0.32 |
| Bacteria | Firmicutes | | | | 47.18 |
| | Bacteroidetes | | | | 22.80 |
| | Proteobacteria | | | | 6.43 |
| | Spirochaetes | | | | 6.07 |
| | Unclassified | | | | 4.40 |
| | Tenericutes | | | | 3.87 |
| | Synergistetes | | | | 3.08 |
| | Actinobacteria | | | | 2.82 |
| | Fibrobacteres | | | | 0.79 |
| | Cyanobacteria | | | | 0.70 |
| | Lentisphaerae | | | | 0.44 |
| | Fusobacteria | | | | 0.26 |
| | Nitrospirae | | | | 0.26 |
| | Chlamydiae | | | | 0.18 |
| | Acidobacteria | | | | 0.18 |
| | Deferribacteres | | | | 0.18 |
| | Verrucomicrobia | | | | 0.18 |
| | Elusimicrobia | | | | 0.09 |
| | Chlorobi | | | | 0.09 |
| Bacteria | Firmicutes | Clostridia | | | 79.67 |
| | | Bacilli | | | 15.67 |
| | | Erysipelotrichi | | | 2.61 |
| | | Negativicutes | | | 2.05 |
| | Bacteroidetes | Bacteroidia | | | 57.52 |
| | | Flavobacteriia | | | 23.94 |
| | | Sphingobacteriia | | | 9.27 |
| | | Unclassified | | | 5.41 |
| | | Cytophagia | | | 3.86 |

| | | | | |
|----------|-----------------|------------------|-------------------------|--------|
| | Proteobacteria | Gamma | Gamma | 30.13 |
| | | Alpha | Alpha | 23.29 |
| | | Delta | Delta | 23.29 |
| | | Beta | Beta | 16.44 |
| | | Epsilon | Epsilon | 4.11 |
| | | Zeta | Zeta | 2.74 |
| | Spirochaetes | Spirochaetia | | 100.00 |
| | Tenericutes | Mollicutes | | 100.00 |
| | Synergistetes | Synergistia | | 91.43 |
| | | Unclassified | | 8.57 |
| | Actinobacteria | Actinobacteria | | 100.00 |
| | Fibrobacteres | Fibrobacteria | | 100.00 |
| | Cyanobacteria | Unclassified | | 100.00 |
| | Lentisphaerae | Unclassified | | 100.00 |
| | Fusobacteria | Fusobacteriia | | 100.00 |
| | Nitrospirae | Nitrospira | | 100.00 |
| | Chlamydiae | Chlamydiia | | 100.00 |
| | Acidobacteria | Acidobacteriia | | 100.00 |
| | Deferribacteres | Deferribacteres | | 100.00 |
| | Verrucomicrobia | Verrucomicrobiae | | 100.00 |
| | Elusimicrobia | Elusimicrobia | | 100.00 |
| | Chlorobi | Chlorobia | | 100.00 |
| Bacteria | Firmicutes | Clostridia | Clostridiales | 99.06 |
| | | | Thermoanaerobacteriales | 0.94 |
| | | Bacilli | Bacillales | 59.52 |
| | | | Lactobacillales | 40.48 |
| | | Erysipelotrichi | Erysipelotrichales | 100.00 |
| | | Negativicutes | Selenomonadales | 100.00 |
| | Bacteroidetes | Bacteroidia | Bacteroidales | 100.00 |
| | | Flavobacteriia | Flavobacteriales | 96.77 |
| | | | Unclassified | 3.23 |
| | | Sphingobacteriia | Sphingobacteriales | 100.00 |
| | | Cytophagia | Cytophagales | 100.00 |
| | Proteobacteria | Gamma | Unclassified | 50.00 |
| | | | Chromatiales | 13.64 |
| | | | Enterobacteriales | 13.64 |
| | | | Pseudomonadales | 13.64 |

| | | | | | |
|----------|-----------------|-----------------------|--------------------|----------------|--------|
| | | | Xanthomonadales | | 4.54 |
| | | | Thiotrichales | | 4.54 |
| | | Alphaproteobacteria | Rhizobiales | | 29.42 |
| | | | Unclassified | | 17.65 |
| | | | Rhodobacterales | | 17.65 |
| | | | Rickettsiales | | 11.76 |
| | | | Rhodospirillales | | 11.76 |
| | | | Sphingomonadales | | 11.76 |
| | | Deltaproteobacteria | Desulfovibrionales | | 35.30 |
| | | | Unclassified | | 29.41 |
| | | | Myxococcales | | 23.53 |
| | | | Bdellovibrionales | | 11.76 |
| | | Betaproteobacteria | Burkholderiales | | 66.67 |
| | | | Unclassified | | 25.00 |
| | | | Neisseriales | | 8.33 |
| | | Epsilonproteobacteria | Unclassified | | 66.67 |
| | | | Nautiliales | | 33.33 |
| | | Zetaproteobacteria | Mariprofundales | | 100.00 |
| | Spirochaetes | Spirochaetia | Spirochaetales | | 100.00 |
| | Tenericutes | Mollicutes | Acholeplasmatales | | 68.18 |
| | | | Mycoplasmatales | | 18.18 |
| | | | Entomoplasmatales | | 13.64 |
| | Synergistetes | Synergistia | Synergistales | | 100.00 |
| | Actinobacteria | Actinobacteria | Actinomycetales | | 71.88 |
| | | | Coriobacteriales | | 15.62 |
| | | | Acidimicrobiales | | 9.38 |
| | | | Rubrobacterales | | 3.12 |
| | Fibrobacteres | Fibrobacteria | Fibrobacterales | | 100.00 |
| | Fusobacteria | Fusobacteriia | Fusobacteriales | | 100.00 |
| | Nitrospirae | Nitrospira | Nitrospirales | | 100.00 |
| | Chlamydiae | Chlamydiia | Chlamydiales | | 100.00 |
| | Acidobacteria | Acidobacteriia | Acidobacteriales | | 100.00 |
| | Deferribacteres | Deferribacteres | Deferribacterales | | 100.00 |
| | Verrucomicrobia | Verrucomicrobiae | Verrucomicrobiales | | 100.00 |
| | Elusimicrobia | Elusimicrobia | Elusimicrobiales | | 100.00 |
| | Chlorobi | Chlorobia | Chlorobiales | | 100.00 |
| Bacteria | Firmicutes | Clostridia | Clostridiales | Clostridiaceae | 36.17 |

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|---------------|-----------------|------------------------|---|--------|
| | | | Ruminococcaceae | 13.48 |
| | | | Lachnospiraceae | 9.93 |
| | | | Clostridiales Family XI. Incertae Sedis | 9.69 |
| | | | Eubacteriaceae | 9.22 |
| | | | Peptostreptococcaceae | 7.57 |
| | | | Peptococcaceae | 4.96 |
| | | | Unclassified | 4.49 |
| | | | Heliobacteriaceae | 2.13 |
| | | | Clostridiales Family XII. Incertae Sedis | 1.18 |
| | | | Syntrophomonadaceae | 1.18 |
| | | Thermoanaerobacterales | Thermoanaerobacterales Family III. Incertae Sedis | 50.00 |
| | | | Thermodesulfobiaceae | 25.00 |
| | | | Thermoanaerobacteraceae | 25.00 |
| | Bacilli | Bacillales | Bacillaceae | 40.00 |
| | | | Paenibacillaceae | 20.00 |
| | | | Alicyclobacillaceae | 12.00 |
| | | | Listeriaceae | 12.00 |
| | | | Staphylococcaceae | 8.00 |
| | | | Unclassified | 4.00 |
| | | | Thermoactinomycetaceae | 4.00 |
| | | Lactobacillales | Lactobacillaceae | 41.19 |
| | | | Streptococcaceae | 29.41 |
| | | | Carnobacteriaceae | 8.82 |
| | | | Enterococcaceae | 8.82 |
| | | | Leuconostocaceae | 5.88 |
| | | | Aerococcaceae | 5.88 |
| | Erysipelotrichi | Erysipelotrichales | Erysipelotrichaceae | 100.00 |
| | Negativicutes | Selenomonadales | Veillonellaceae | 100.00 |
| Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | 43.62 |
| | | | Bacteroidaceae | 31.55 |
| | | | Rikenellaceae | 12.75 |
| | | | Prevotellaceae | 5.37 |
| | | | Marinilabiaceae | 4.03 |
| | | | Unclassified | 2.68 |
| | Flavobacteriia | Flavobacteriales | Flavobacteriaceae | 91.67 |
| | | | Unclassified | 6.67 |
| | | | Blattabacteriaceae | 1.66 |

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|----------------|-----------------------|--------------------|------------------------|--------|
| | Sphingobacteria | Sphingobacteriales | Sphingobacteriaceae | 87.50 |
| | | | Unclassified | 12.50 |
| | Cytophagia | Cytophagales | Cytophagaceae | 100.00 |
| Proteobacteria | Gamma proteobacteria | Chromatiales | Chromatiaceae | 66.67 |
| | | | Ectothiorhodospiraceae | 33.33 |
| | | Enterobacteriales | Enterobacteriaceae | 100.00 |
| | | Pseudomonadales | Pseudomonadaceae | 66.67 |
| | | | Moraxellaceae | 33.33 |
| | | Xanthomonadales | Xanthomonadaceae | 100.00 |
| | | Thiotrichales | Francisellaceae | 100.00 |
| | Alphaproteobacteria | Rhizobiales | Rhodobiaceae | 20.00 |
| | | | Brucellaceae | 20.00 |
| | | | Rhizobiaceae | 20.00 |
| | | | Methylobacteriaceae | 20.00 |
| | | | Bradyrhizobiaceae | 20.00 |
| | | Rhodobacterales | Rhodobacteraceae | 66.67 |
| | | | Unclassified | 33.33 |
| | | Rickettsiales | Anaplasmataceae | 100.00 |
| | | Rhodospirillales | Acetobacteraceae | 50.00 |
| | | | Rhodospirillaceae | 50.00 |
| | | Sphingomonadales | Sphingomonadaceae | 100.00 |
| | Deltaproteobacteria | Desulfovibrionales | Desulfohalobiaceae | 66.67 |
| | | | Desulfovibrionaceae | 33.33 |
| | | Myxococcales | Nannocystaceae | 75.00 |
| | | | Kofleriaceae | 25.00 |
| | | Bdellovibrionales | Bacteriovoracaceae | 50.00 |
| | | | Bdellovibrionaceae | 50.00 |
| | Betaproteobacteria | Burkholderiales | Burkholderiaceae | 50.00 |
| | | | Unclassified | 25.00 |
| | | | Alcaligenaceae | 12.50 |
| | | | Comamonadaceae | 12.50 |
| | | Neisseriales | Neisseriaceae | 100.00 |
| | Epsilonproteobacteria | Nautiliales | Nautiliaceae | 100.00 |
| | Zetaproteobacteria | Mariprofundales | Mariprofundaceae | 100.00 |
| Spirochaetes | Spirochaetia | Spirochaetales | Spirochaetaceae | 95.65 |
| | | | Leptospiraceae | 4.35 |
| Tenericutes | Mollicutes | Acholeplasmatales | Acholeplasmataceae | 100.00 |

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|-----------|-----------------|------------------|--------------------|-----------------------|--------|
| | | | Mycoplasmatales | Mycoplasmataceae | 100.00 |
| | | | Entomoplasmatales | Entomoplasmataceae | 50.00 |
| | | | | Spiroplasmataceae | 50.00 |
| | Synergistetes | Synergistia | Synergistales | Synergistaceae | 100.00 |
| | Actinobacteria | Actinobacteria | Actinomycetales | Streptomycetaceae | 17.39 |
| | | | | Nocardiaceae | 13.03 |
| | | | | Propionibacteriaceae | 13.03 |
| | | | | Micrococcaceae | 8.70 |
| | | | | Corynebacteriaceae | 8.70 |
| | | | | Mycobacteriaceae | 8.70 |
| | | | | Unclassified | 8.70 |
| | | | | Microbacteriaceae | 4.35 |
| | | | | Frankiaceae | 4.35 |
| | | | | Promicromonosporaceae | 4.35 |
| | | | | Beutenbergiaceae | 4.35 |
| | | | | Actinomycetaceae | 4.35 |
| | | | Coriobacteriales | Coriobacteriaceae | 100.00 |
| | | | Acidimicrobiales | Acidimicrobiaceae | 100.00 |
| | | | Rubrobacterales | Rubrobacteraceae | 100.00 |
| | Fibrobacteres | Fibrobacteria | Fibrobacterales | Fibrobacteraceae | 100.00 |
| | Fusobacteria | Fusobacteriia | Fusobacteriales | Fusobacteriaceae | 100.00 |
| | Nitrospirae | Nitrospira | Nitrospirales | Nitrospiraceae | 100.00 |
| | Chlamydiae | Chlamydiia | Chlamydiales | Parachlamydiaceae | 50.00 |
| | | | | Simkaniaceae | 50.00 |
| | Acidobacteria | Acidobacteriia | Acidobacteriales | Acidobacteriaceae | 100.00 |
| | Deferribacteres | Deferribacteres | Deferribacterales | Deferribacteraceae | 100.00 |
| | Verrucomicrobia | Verrucomicrobiae | Verrucomicrobiales | Verrucomicrobiaceae | 100.00 |
| | Elusimicrobia | Elusimicrobia | Elusimicrobiales | Elusimicrobiaceae | 100.00 |
| | Chlorobi | Chlorobia | Chlorobiales | Chlorobiaceae | 100.00 |
| Eukaryota | Arthropoda | | | | 59.62 |
| | Streptophyta | | | | 11.54 |
| | Unclassified | | | | 9.61 |
| | Ascomycota | | | | 3.85 |
| | Chordata | | | | 3.85 |
| | Chlorophyta | | | | 3.85 |
| | Annelida | | | | 1.92 |
| | Apicomplexa | | | | 1.92 |

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|-----------|---------------|------------------|-------------------|----------------------|--------|
| | Cnidaria | | | | 1.92 |
| | Nematoda | | | | 1.92 |
| Eukaryota | Arthropoda | Insecta | | | 90.32 |
| | | Maxillopoda | | | 9.68 |
| | Streptophyta | Liliopsida | | | 66.67 |
| | | Unclassified | | | 33.33 |
| | Ascomycota | Sordariomycetes | | | 100.00 |
| | Chordata | Actinopterygii | | | 100.00 |
| | Chlorophyta | Trebouxiophyceae | | | 50.00 |
| | | Prasinophyceae | | | 50.00 |
| | Annelida | Polychaeta | | | 100.00 |
| | Apicomplexa | Aconoidasida | | | 100.00 |
| | Cnidaria | Hydrozoa | | | 100.00 |
| | Nematoda | Chromadorea | | | 100.00 |
| Eukaryota | Arthropoda | Insecta | Isoptera | | 100.00 |
| | | Maxillopoda | Siphonostomatoida | | 100.00 |
| | Streptophyta | Liliopsida | Poales | | 100.00 |
| | Ascomycota | Sordariomycetes | Glomerellales | | 100.00 |
| | Chordata | Actinopterygii | Cypriniformes | | 100.00 |
| | Chlorophyta | Trebouxiophyceae | Chlorellales | | 100.00 |
| | | Prasinophyceae | Prasinococcales | | 100.00 |
| | Annelida | Polychaeta | Flabelligerida | | 100.00 |
| | Apicomplexa | Aconoidasida | Unclassified | | 100.00 |
| | Cnidaria | Hydrozoa | Hydroida | | 100.00 |
| | Nematoda | Chromadorea | Rhabditida | | 100.00 |
| Eukaryota | Arthropoda | Insecta | Isoptera | Rhinotermitidae | 100.00 |
| | | Maxillopoda | Siphonostomatoida | Caligidae | 100.00 |
| | Streptophyta | Liliopsida | Poales | Poaceae | 100.00 |
| | Ascomycota | Sordariomycetes | Glomerellales | Plectosphaerellaceae | 100.00 |
| | Chordata | Actinopterygii | Cypriniformes | Cyprinidae | 100.00 |
| | Chlorophyta | Trebouxiophyceae | Chlorellales | Unclassified | 100.00 |
| | | Prasinophyceae | Prasinococcales | Unclassified | 100.00 |
| | Annelida | Polychaeta | Flabelligerida | Flabelligeridae | 100.00 |
| | Cnidaria | Hydrozoa | Hydroida | Hydridae | 100.00 |
| | Nematoda | Chromadorea | Rhabditida | Rhabditidae | 100.00 |
| Archaea | Euryarchaeota | | | | 91.18 |
| | Unclassified | | | | 8.82 |

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|---------|---------------|-----------------|--------------------|-----------------------|-------|
| Archaea | Euryarchaeota | Methanomicrobia | | | 93.55 |
| | | Unclassified | | | 6.45 |
| Archaea | Euryarchaeota | Methanomicrobia | Methanomicrobiales | | 82.76 |
| | | | Methanosarcinales | | 17.24 |
| Archaea | Euryarchaeota | Methanomicrobia | Methanomicrobiales | Methanomicrobiaceae | 50.00 |
| | | | | Methanocorpusculaceae | 16.67 |
| | | | | Methanospirillaceae | 16.67 |
| | | | | Unclassified | 16.67 |
| | | | Methanosarcinales | Methanosarcinaceae | 80.00 |
| | | | | Methanosaetaceae | 20.00 |