DOCTOR OF PHILOSOPHY

Ecology of Fibrobacter spp. in cellulose-degrading microbial communities

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Award date:
2015

Awarding institution:
Bangor University

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

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September 2014

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

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Abbreviations

%	Percent
µl	Microlitre
µm	Micrometer
^{0}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 *in situ* 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

Acknowledgements

This thesis would not have been possible without the help and support of numerous people. My thanks to Bangor University for funding me with a 125th Anniversary Scholarship, and the Systematics Association's SynTax award scheme, supported by the Linnean Society of London, BBSRC and NERC, for the financial support. I would like to thank my supervisors, James and Davey, for your assistance, guidance and support. James in particular, I don't actually have the words to say thank you properly for everything that you have done, but please know how much I appreciate you as a mentor.

I would also like to thank my fellow colleagues, in no particular order: Carys for the tea and advice, Katie for the generous doses of common sense, James D for your help with the computer wizardry, and my fellow inmates Adam and Jess for keeping me (relatively) sane. Also Karen, Jasmine, Ben and Christoph for your support at various stages.

On a more personal note, my thanks to my Mum (who is way more awesome than me) and Dad, for your unconditional love and support, this would not have been possible without you both. Thanks to the people who have helped raise me, Alyse, Ian, Betty, Tony, Roy, Kathy, Jan and Brian. To Jo and my Liverpool girls, lab work is not the same without you! Allan and Xena, thank you so much for all you have done, I did warn you that if you fed me you kept me! Thanks to DeAnn and Rob for being my writing buddies and also not heeding the warning about feeding me (we did try to warn you!). My lovely WI ladies, thank you for the encouragement and cake. Also to Coco, normal minion service will resume shortly.

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, III_1, III_2, IV_1 and IV_2) (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).

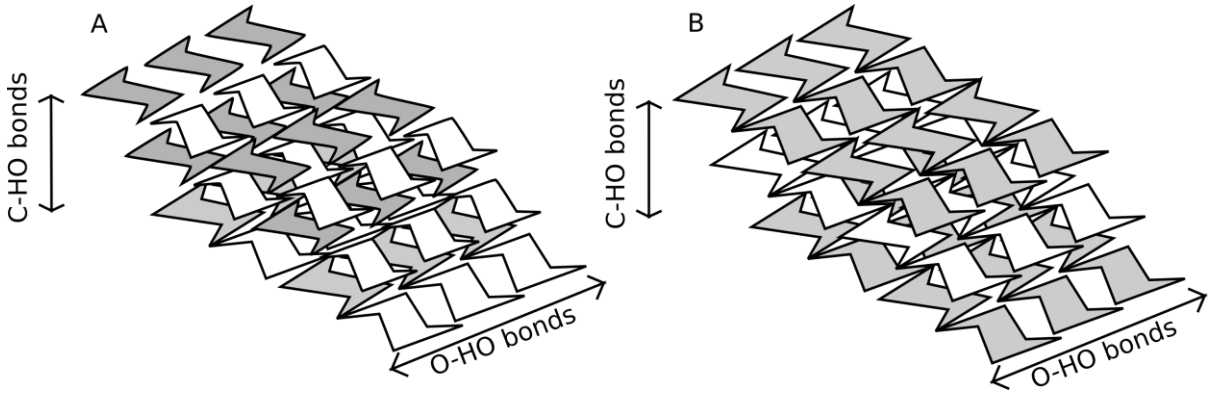

Figure 1.1 The structures of cellulose I.

(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 GGP is between 115 and 131 petagrams of carbon per year, the amount of cellulosic biomass produced is substantial.

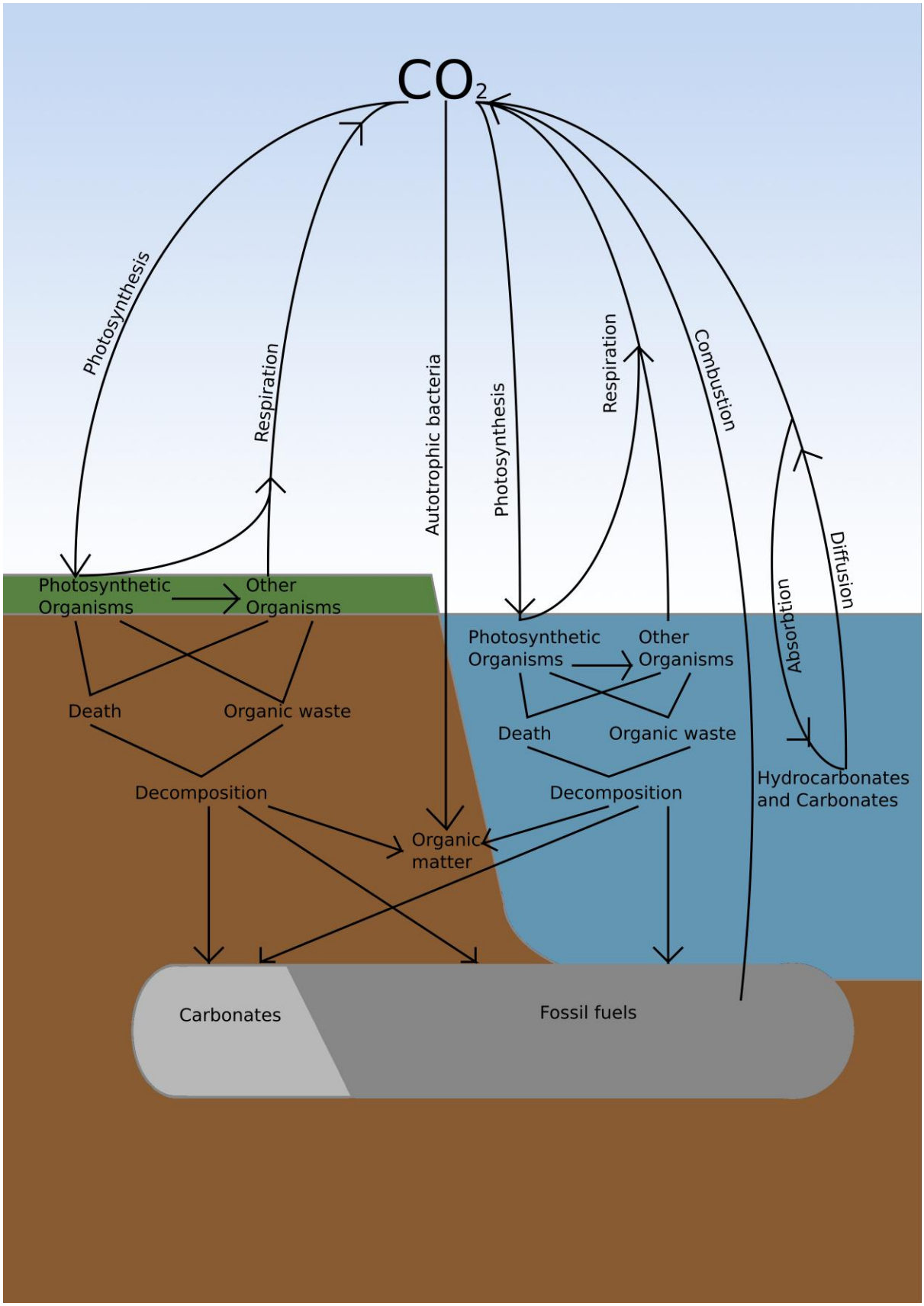

Figure 1.2. The global carbon cycle.

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 FeIII, which is then removed from this complex via the chelator and reduced to FeII via Fenton chemistry (Hammel *at al.*, 2002). The reduction of the FeIII also results in the formation of the hydroxyl radical (•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 cellulose 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).

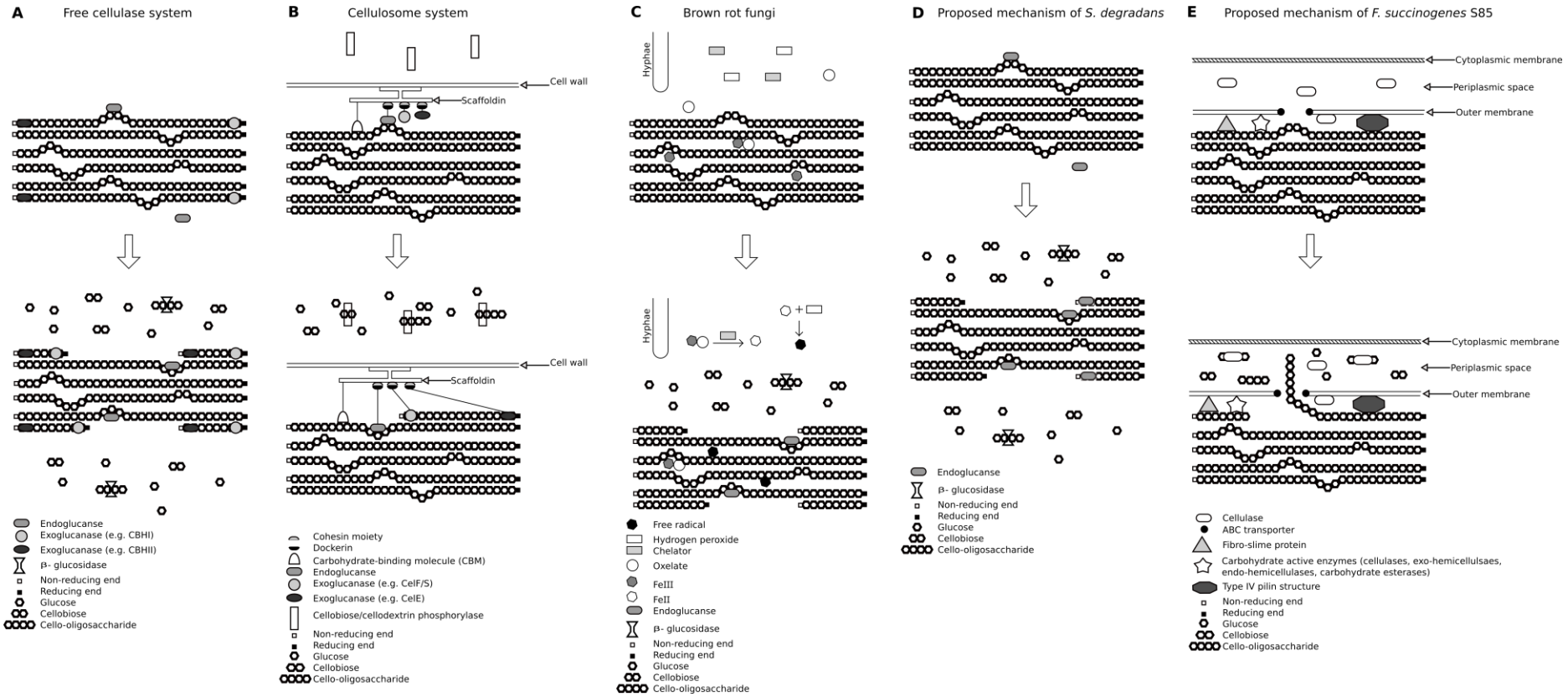

Figure 1.3. Microbial mechanisms of cellulose-degradation. Modified from Ransom-Jones *et al.* (2012).

(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 form 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. amylophilus* 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, B_{12} (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.

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

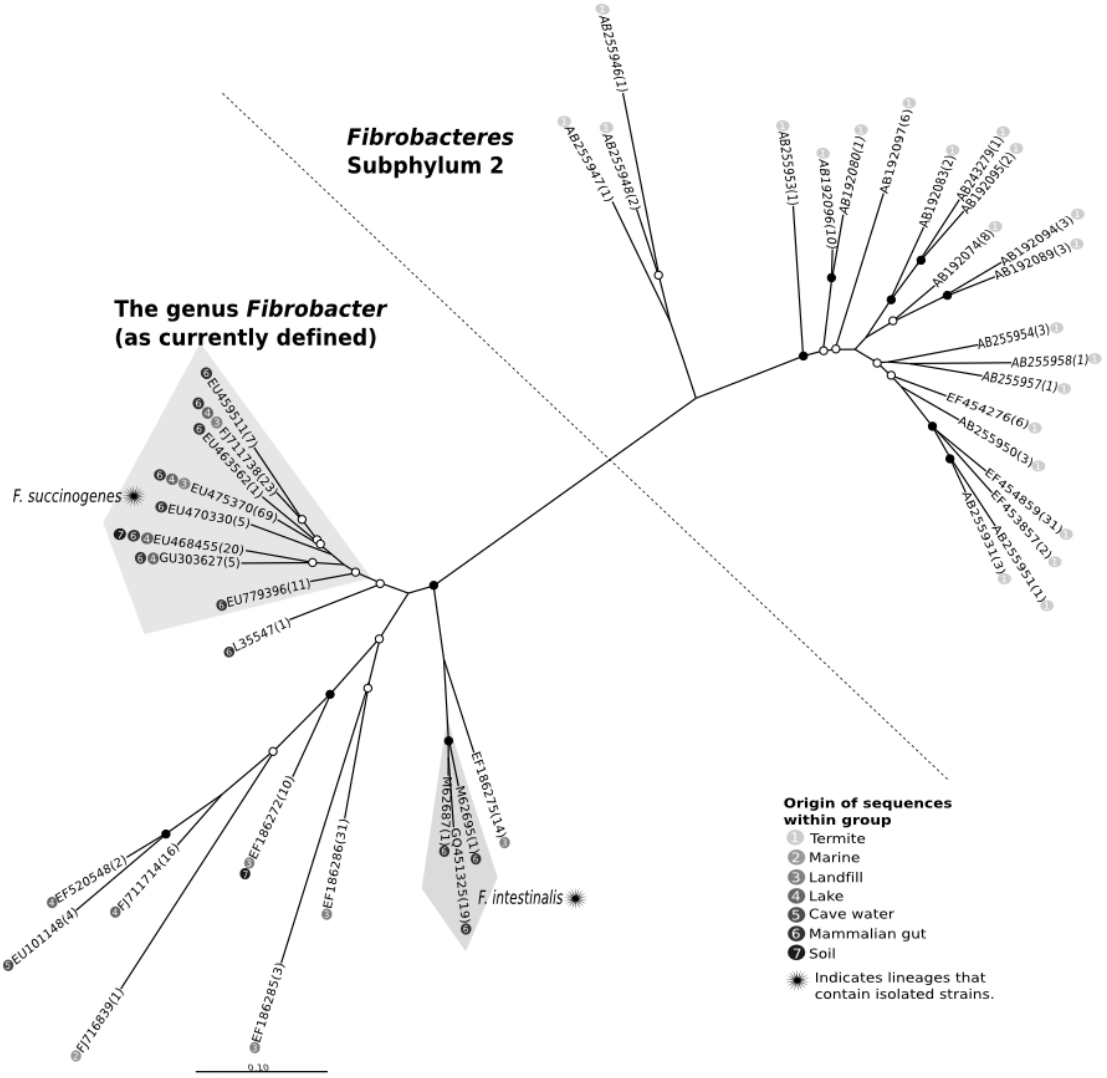

Figure 1.4. Phylogeny of the *Fibrobacteres* phylum. Maximum likelihood tree of 16S rRNA gene sequences belonging to the *Fibrobacteres* phylum. All sequences classified within the *Fibrobacteres* phylum and annotated as 'good' quality were downloaded from the Ribosomal Database Project (Cole *et al.*, 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 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 (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 anerobic 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 (Julliand *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).

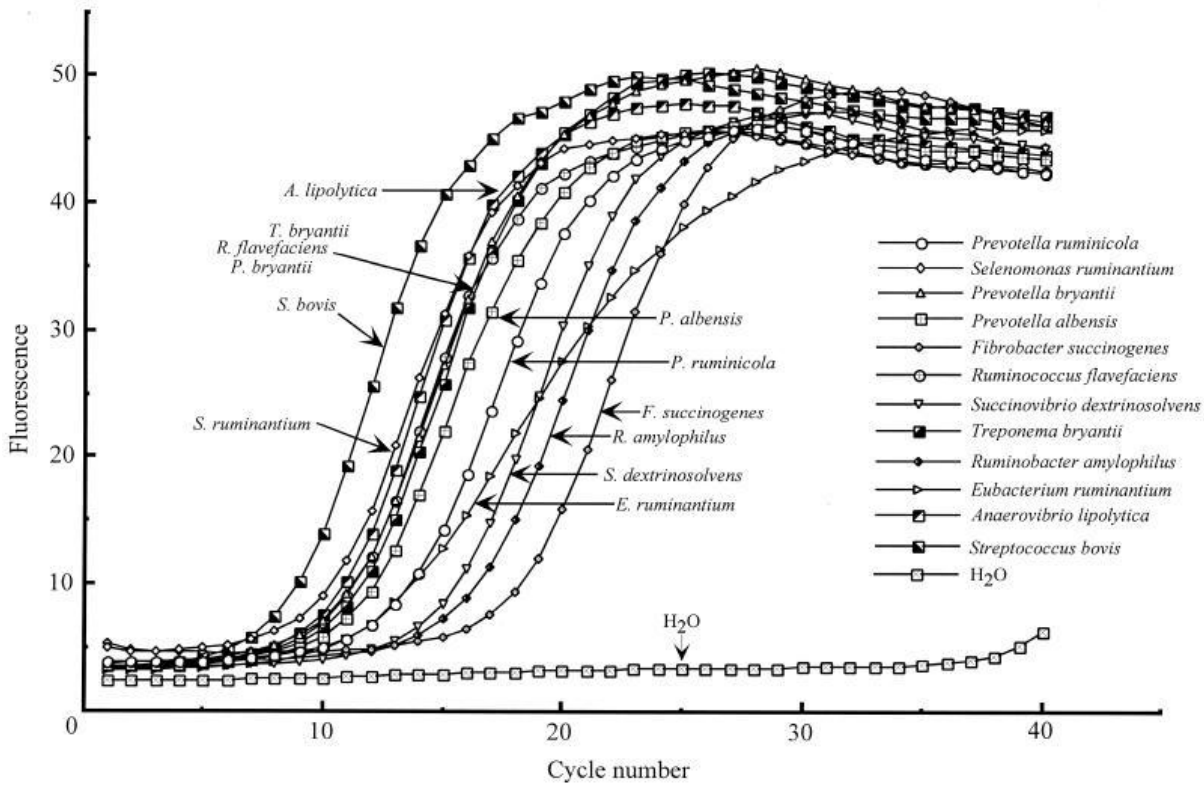

Figure 1.5. Differential amplification of rumen bacterial DNA templates with universal bacterial primers 27f and 1525r. Real-time PCR amplification was conducted essentially as described in Materials and Methods with 30 ng of each bacterial DNA template. PCR cycling was performed as follows: 95°C for 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 though a 0.2 μm pore diameter membrane. Landfill leachate microcosms were constructed by placing nylon mesh bags containing dewaxed cotton string in 1 l Duran bottles, sterilised by autoclaving and transported to the landfill site where they were filled to the top with leachate 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 *Fibrobacter* primers	Nested PCR product with *Fibrobacter* primers	Clone library sequencing of the *Fibrobacter* 16S rRNA gene PCR amplicons	qPCR (% of total bacterial rRNA gene copies)[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	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 $MgCl_2$, 1x BSA, 1 unit SuperTaq (Cambio), 50 ng DNA and dd H_2O 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 $MgCl_2$ (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. QuantiFastTM 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 *et al.*, 1989)
pH'	AAGGAGGTGATCCAGCCGCA				
Fib 1F[e]	CCGKSCCAACGSSCGG	*Fibrobacter* genus	60	~ 855	(McDonald *et al.*, 2008)
Fib 2AR	ATCTCTCGCYGCGGCGWTYCC				
1369F[b]	CGGTGAATACGTTCYCGG	General bacteria[b]	60[d]	~ 151	(Suzuki *et al.*, 2000)
Prok 1492R[b]	GGWTACCTTGTTACGACTT				
FibroQ153F[be]	CCGKSCCAACGSSCGGHTAA	*Fibrobacter*[b] genus	60[d]	~ 104	(McDonald *et al.*, 2008)
FibroQ238R[b]	CSCCWACTRGYTAATCRGAC				
M13 Forward[c]	GTTTTCCCAGTCACGAC	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 H_2O. 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 g/µl DNA / [PCR product length in basepairs x 660]) x 6.022 x 1023 = Y molecules/µl.

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 it's 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.

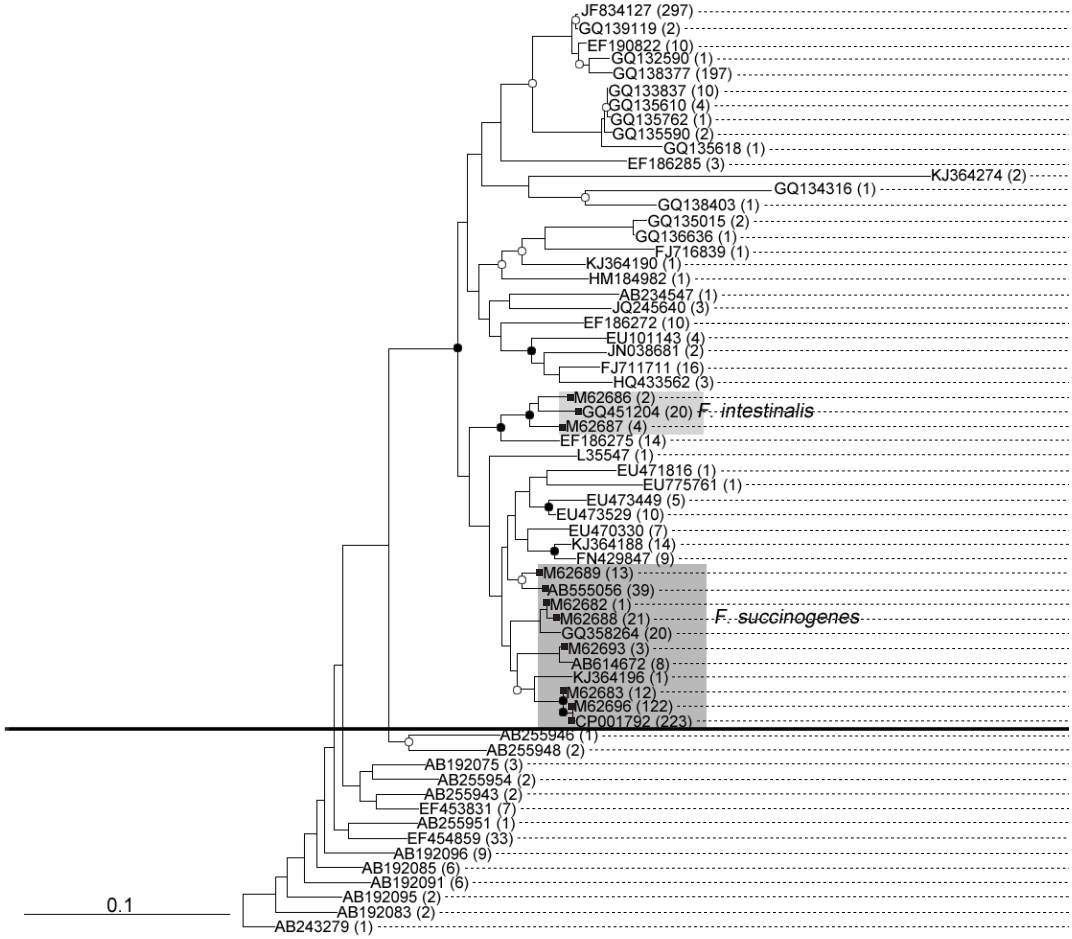
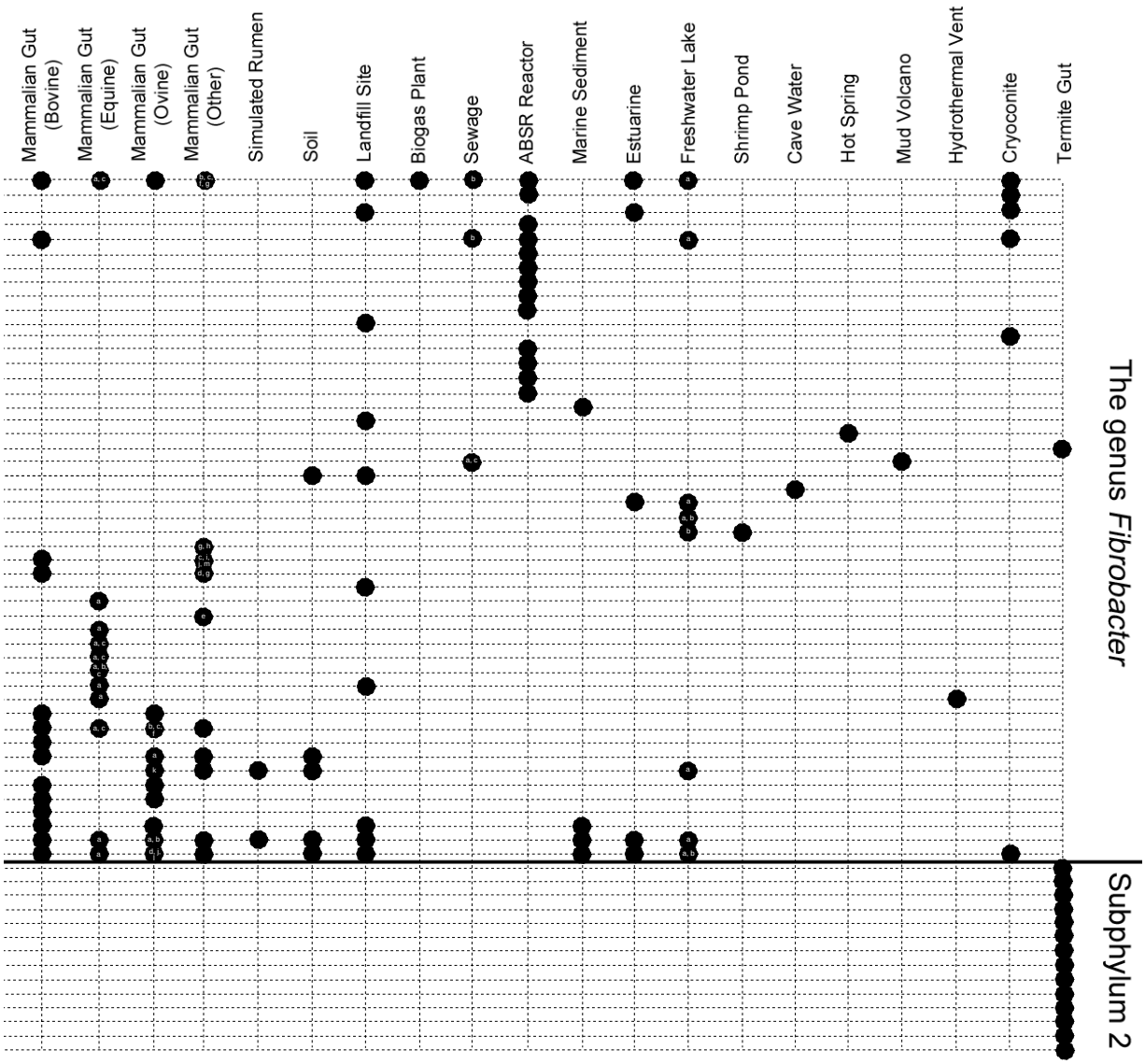


Figure 2.1. The ecology and taxonomy of the *Fibrobacteres* phylum (Ransom-Jones *et al.*, 2012). OTUs are labelled with the accession number of the representative sequence, and the number of sequences contained within each OTU is in brackets. OTUs containing cultivated species are designated by boxes 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* (re-classified 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.

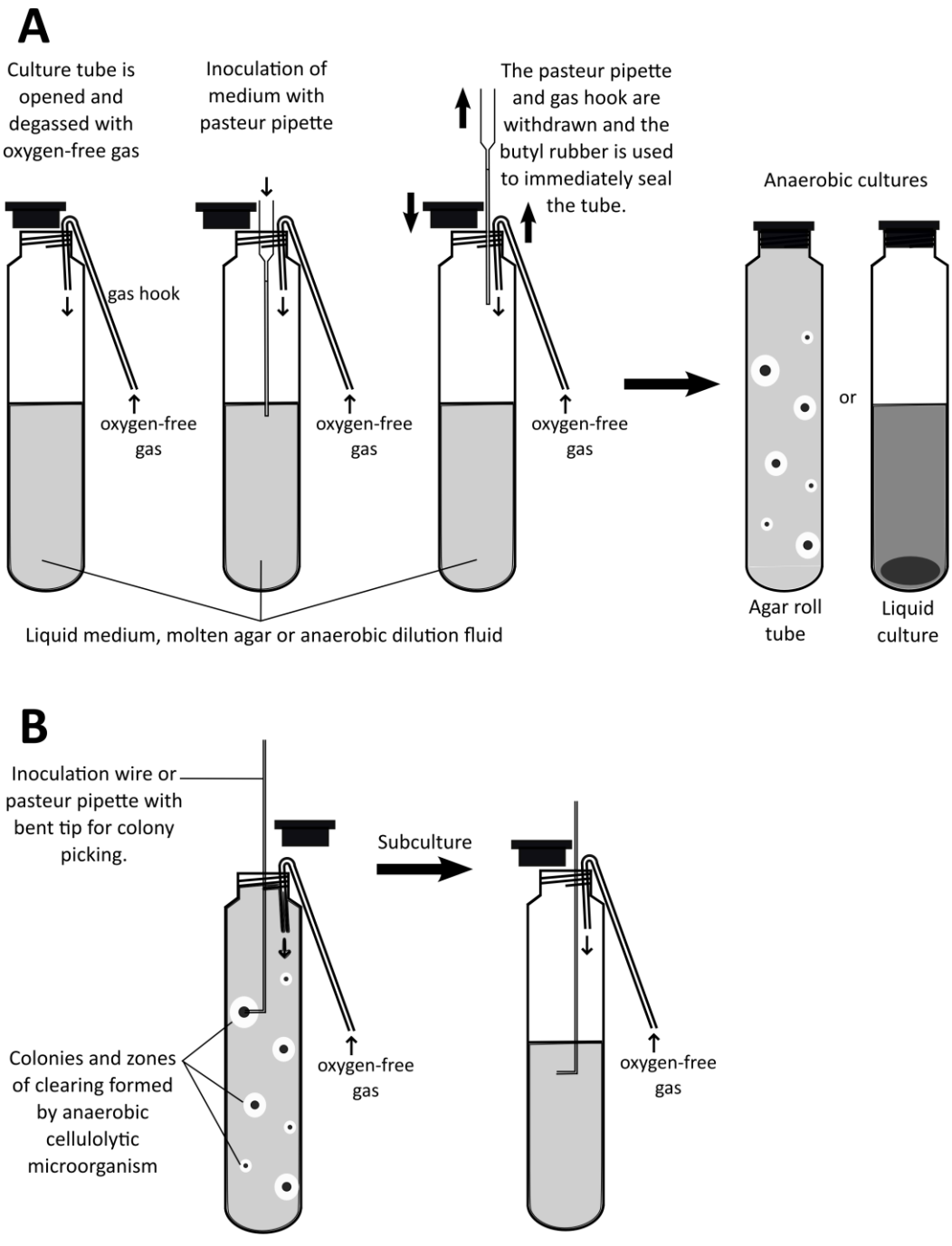


Figure 3.1. The Hungate anaerobic cultivation method demonstrating A) The inoculation of medium with microbial strains, and B) the isolation of individual colonies. From McDonald *et al.* (2012b).

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 Resasurin 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 CO_2 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 (Koike *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 microscosm (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% CO_2 (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.

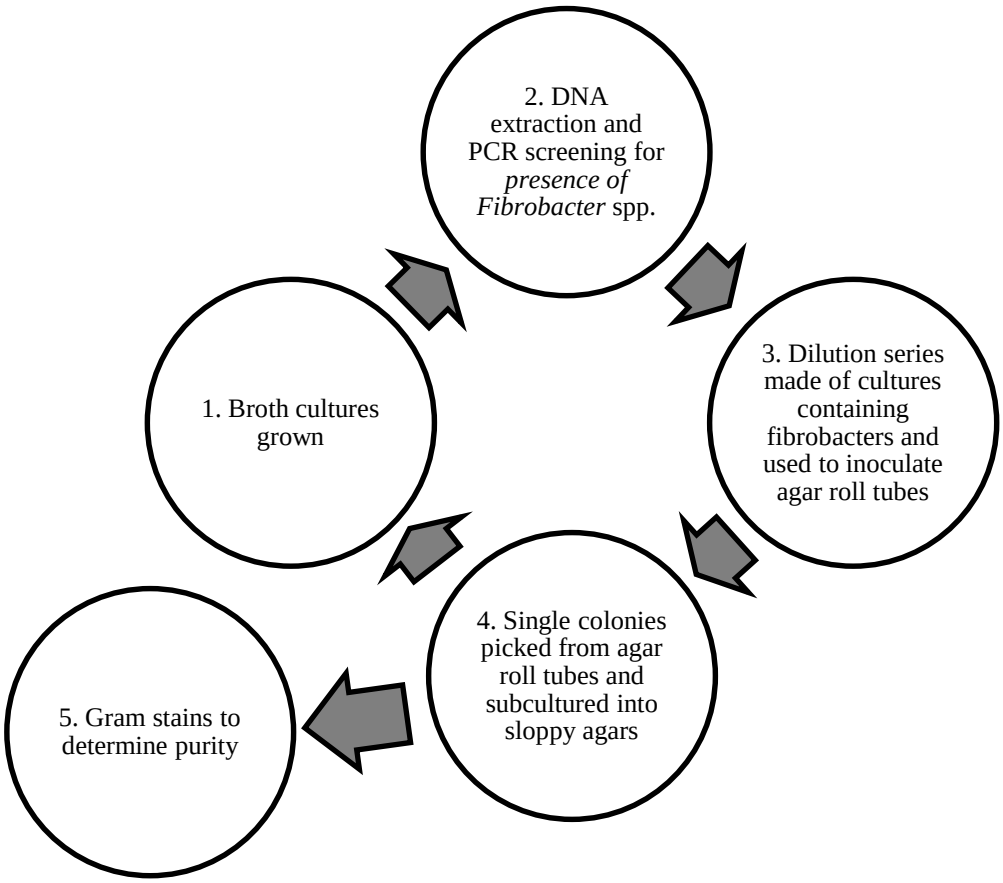


Figure 3.2. Processes contained within one round of strain purification.

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) $NaHCO_3$ (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) K_2HPO_4 (Sigma), 0.045% (wt/vol) KH_2PO_4 (Sigma), 0.09% (wt/vol) $(NH_4)_2SO_4$ (Sigma), 0.09% (wt/vol) NaCl (Sigma), 0.09% (wt/vol) $MgSO_4$ (Sigma), 0.09% (wt/vol) $CaCl_2$ (Sigma), 0.0001% (wt/vol) resazurin (Sigma) and H_2O 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, H_2O 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) $NaHCO_3$ (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) K_2HPO_4 (Sigma), 0.045% (wt/vol) KH_2PO_4 (Sigma), 0.09% (wt/vol) $(NH_4)_2SO_4$ (Sigma), 0.09% (wt/vol) NaCl (Sigma), 0.09% (wt/vol) $MgSO_4$ (Sigma), 0.09% (wt/vol) $CaCl_2$ (Sigma), 0.0001% (wt/vol) resazurin (Sigma) and H_2O 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 it's 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 $MgCl_2$, 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 $MgCl_2$ (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 *et al.*, 1989)
pH'	AAGGAGGTGATCCAGCCGCA		53[d]		
Fib 1F[b]	CCGKSCCAACGSSCGG	*Fibrobacter* genus	60[c]	~ 855	(McDonald *et al.*, 2008)
Fib 2AR	ATCTCTCGCYGCGGCGWTYCC		58[d]		
M13 Forward[c]	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 *Fibrobacter* PCR result	Nested *Fibrobacter* 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.

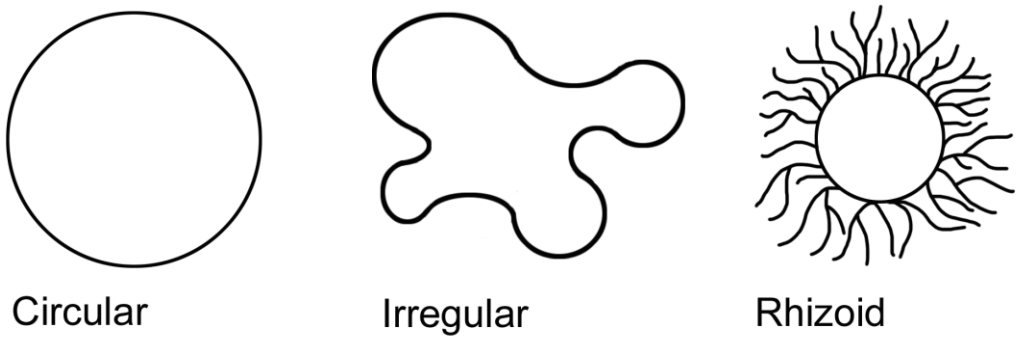


Figure 3.3. Colony morphologies as seen on agar roll tubes.

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.

CultureSize	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 *Fibrobacter* sp. clone EW-C-3.0-1 16S ribosomal RNA gene, partial sequence (lake)	99
B4	*Fibrobacter succinogenes* subsp. *succinogenes* S85, complete genome (rumen)	99
E4	Uncultured *Fibrobacter* 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	*Bacillus thermoamylovorans* strain NBY26 16S ribosomal RNA gene, partial sequence	98
	2	*Bacillus thermoamylovorans* gene for 16S rRNA, partial sequence, strain: BHK180-4	99
	3	*Bacillus thermoamylovorans* strain N12-2 16S ribosomal RNA gene, partial sequence	99
	4	*Bacillus thermoamylovorans* gene for 16S rRNA, partial sequence, strain: BHK180-3	99
	5	*Bacillus thermoamylovorans* strain N12-2 16S ribosomal RNA gene, partial sequence	97
	6	*Bacillus thermoamylovorans* gene for 16S rRNA, partial sequence, strain: BHK180-4	99
	7	*Bacillus* spp. R-31297 partial 16S rRNA gene, strain R-31297	99
	8	*Bacillus thermoamylovorans* gene for 16S rRNA, partial sequence, strain: BHK180-4	99
	9	*Bacillus thermoamylovorans* strain N12-2 16S ribosomal RNA gene, partial sequence	98
	10	*Bacillus thermoamylovorans* gene for 16S rRNA, partial sequence, strain: BHK180-4	99
	11	*Clostridium botulinum* H04402 065, complete genome sequence	99

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

5	*Bacillus thermoamylovorans* gene for 16S rRNA, partial sequence, strain: BHK180-4	99
6	*Bacillus thermoamylovorans* strain N12-2 16S ribosomal RNA gene, partial sequence	99
7	*Bacillus thermoamylovorans* strain N12-2 16S ribosomal RNA gene, partial sequence	99
8	*Bacillus thermoamylovorans* strain N12-2 16S ribosomal RNA gene, partial sequence	99
9	*Bacillus thermoamylovorans* strain N12-2 16S ribosomal RNA gene, partial sequence	99
10	*Bacillus thermoamylovorans* gene for 16S rRNA, partial sequence, strain: BHK180-3	99
11	*Bacillus thermoamylovorans* strain N12-2 16S ribosomal RNA gene, partial sequence	99
12	*Bacillus thermoamylovorans* 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 *Fibrobacter* spp. clone EW-C-3.0-1 16S ribosomal RNA gene	99
A721	1	Uncultured *Fibrobacter* spp. clone EW-C-SED-4 16S ribosomal RNA gene 99	99
B211	1	Uncultured *Fibrobacter* spp. clone EW-C-SED-4 16S ribosomal RNA gene 99	99
B211	2	Uncultured *Fibrobacter* spp. clone EW-C-SED-4 16S ribosomal RNA gene 99	99

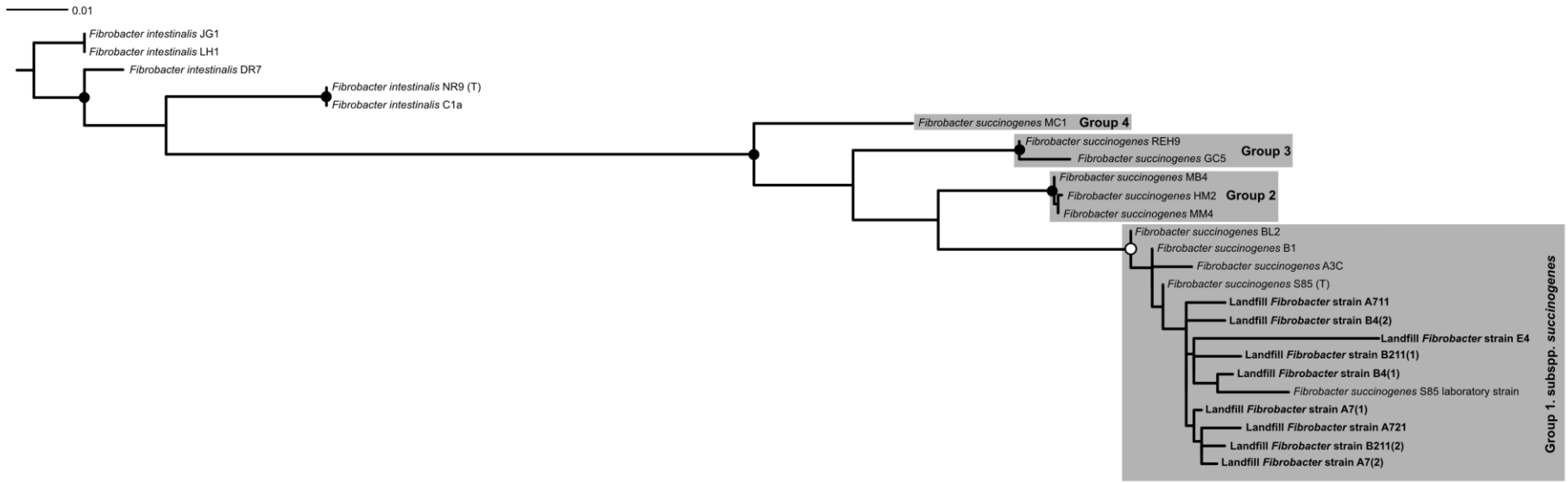


Figure 3.4. Phylogeny of *Fibrobacter* isolates from landfill sites.

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 *Fibrobacter* PCR result	Nested *Fibrobacter* 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

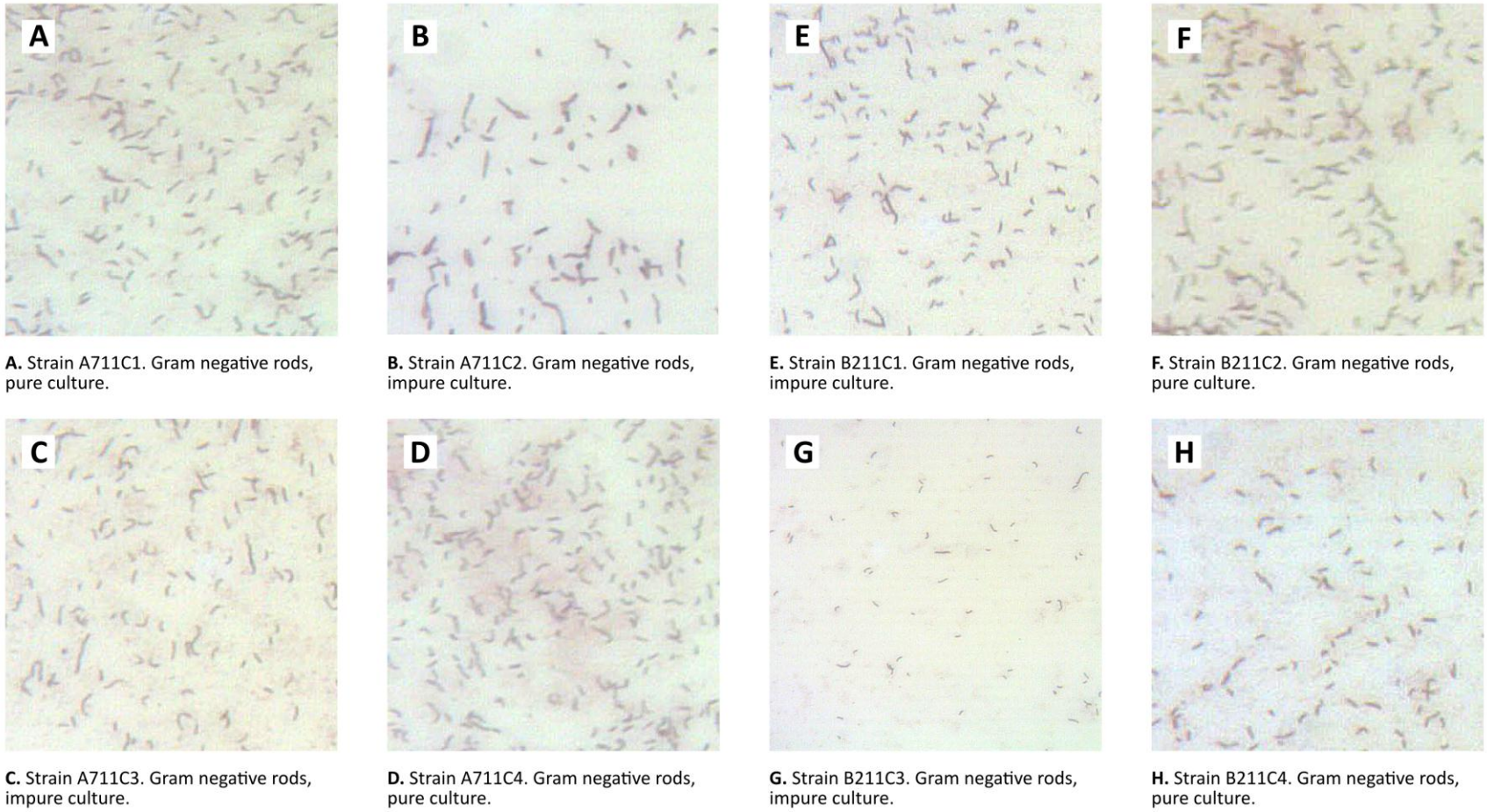
3.3.12 Seventh round of isolation via the Hungate anaerobic culture method

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 phytagel 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.

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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 cellulolyltic 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% CO_2 (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) $NaHCO_3$ (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) K_2HPO_4 (Sigma), 0.045% (wt/vol) KH2PO4 (Sigma), 0.09% (wt/vol) $(NH_4)_2SO_4$ (Sigma), 0.09% (wt/vol) NaCl (Sigma), 0.09% (wt/vol) $MgSO_4$ (Sigma), 0.09% (wt/vol) $CaCl_2$ (Sigma), 0.0001% (wt/vol) resazurin (Sigma) and H_2O 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 $MgCl_2$, 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 $MgCl_2$ 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 determine 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 *Clostridrum* 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]	*Clostridium* cluster (number of sequences)
Acholeplasmataceae	1	0.8	90	
Synergistaceae	1	0.8	90	
Anaerolineaceae	1	0.8	88	
Spirochaetaceae	10	8.3	86-98	
Fibrobacteraceae	7	5.8	83-89	
Peptococcaceae	2	1.7	88-89	
Clostridiales Incertae Sedis XI	5	4.1	89-95	
Ruminococcaceae	29	24.0	82-94	III (15), IV (14)
Lachnospiraceae	28	23.1	82-94	XIVa (28)
Porphyromonadaceae	20	16.5	82-96	
Flavobacteriaceae	1	0.8	85	
Marinilabiaceae	4	3.3	91-93	
Flammeovirgaceae	1	0.8	86	
Cytophagaceae	1	0.8	91	
Peptostreptococcaceae	2	1.7	84-87	
Lactobacillaceae	1	0.8	91	
Streptococcaceae	1	0.8	81	
Gracilibacteraceae	1	0.8	87	
Thermoanaerobacteraceae	2	1.7	76-84	
Cardiobacteriaceae	1	0.8	82	
Chromatiaceae	1	0.8	90	
Xanthobacteraceae	1	0.8	88	

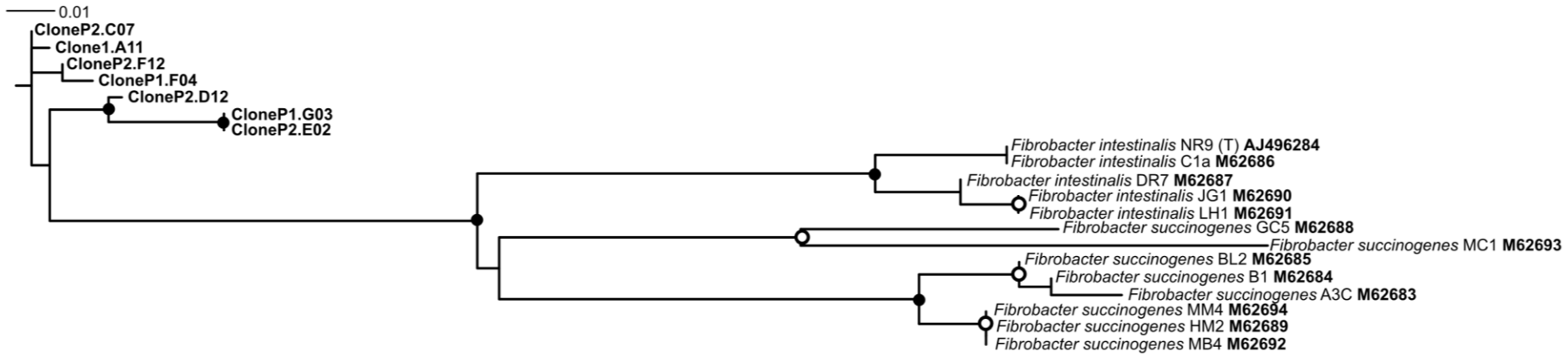


Figure 4.1. Phylogeny of 16S rRNA gene clone library sequences derived from the biofilm of heavily degraded colonised cotton and identified as fibrobacters via NCBI nucleotide blast. Accession numbers of cultivated isolates downloaded from the Ribosomal Database Project are in bold. Filled circles represent 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
Ruminococcaceae (*Clostridium* cluster IV)	W41A	*Clostridium leptum*	90%	GSC	Gram-negative rods	Y
	W41B	*Clostridium leptum*	90%	GSC	Gram-negative rods	Y
	W42A	*Clostridium leptum*	93%	GSC	Gram-negative rods	Y
	W51A	*Clostridium leptum*	92%	GSC	Gram-negative rods	Y
	W62A	*Clostridium leptum*	92%	GSC	Gram-negative rods	Y
	W62B	*Clostridium leptum*	93%	GSC	Gram-negative rods	Y
	X41A	*Clostridium leptum*	92%	GSC	Gram-negative rods	Y
	X42A	*Clostridium leptum*	92%	GSC	Gram-negative rods	Y
	X42B	*Clostridium leptum*	93%	GSC	Gram-negative rods	Y
	X71A	*Clostridium leptum*	93%	GSC	Gram-positive rods	Y
	Y32A	*Clostridium leptum*	92%	GSC	Gram-positive rods	Y
	Y52B	*Clostridium leptum*	92%	GSC	Gram-negative rods	Y
	Y81B	*Clostridium leptum*	93%	GSC	Gram-negative rods	Y
	Z11A	*Clostridium leptum*	93%	GSC	Gram-negative rods	Y
	Z12A	*Clostridium leptum*	93%	GSC	Gram-negative rods	Y
	Z21A	*Clostridium leptum*	92%	GSC	Gram-negative rods	Y
	Z22B	*Clostridium leptum*	93%	GSC	Gram-negative rods	Y

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

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

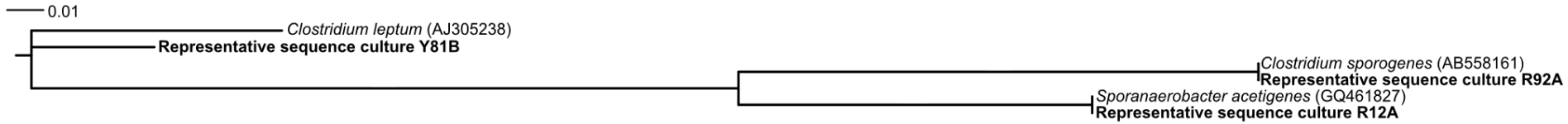
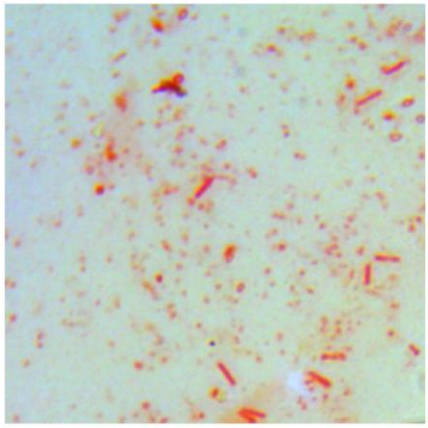


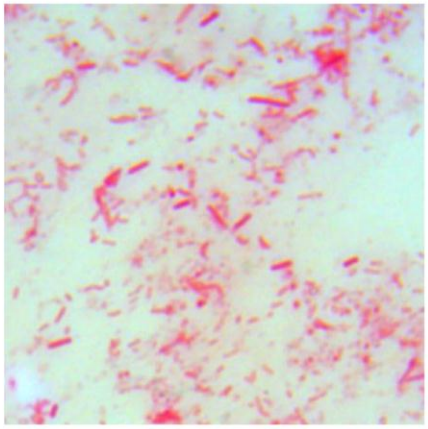
Figure 4.2. Phylogeny of 16S rRNA gene sequences derived from cultivated isolates. Filled circles represent bootstrap values >95%, unfilled circles represent bootstrap values between 75 and 95%. Accession numbers are in brackets. The scale bar represents 0.1 base substitutions per nucleotide.

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.

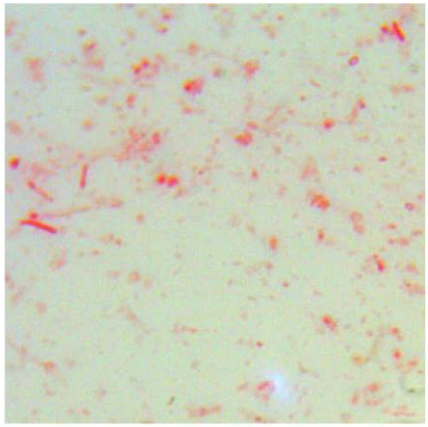
Strain	Nested *Fibrobacter* PCR Result	Species in co-culture
R51A	-	*C. sporogenes*
R61A	+	*C. sporogenes*
R62A	-	*C. sporogenes*
R72A	-	*C. sporogenes*
S11A	+	*C. sporogenes*
S21A	+	*C. sporogenes*
S22A	-	*C. sporogenes*
S41A	+	No sequence data
S72A	-	*C. sporogenes*
S81A	+	*C. sporogenes*
R41A	-	*S.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



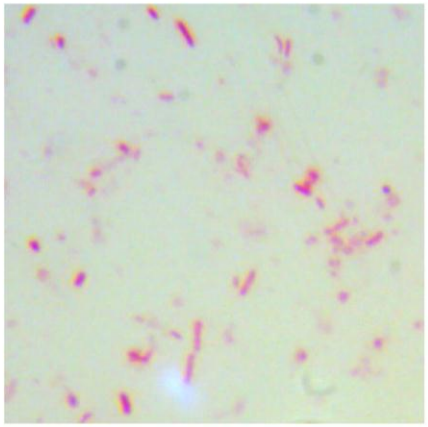
A) R61A Mixture of Gram negative small ovoid cells and larger Gram negative rods. Some background staining.



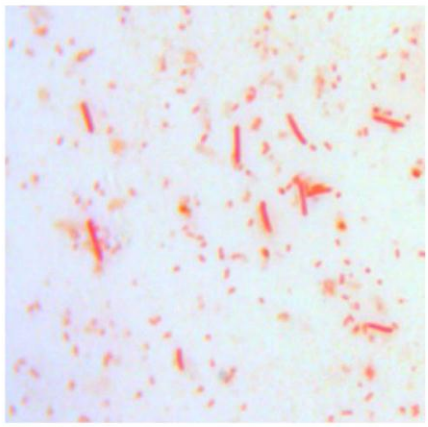
B) S11A Mixture of Gram negative small ovoid cells and larger Gram negative rods. Some background staining.



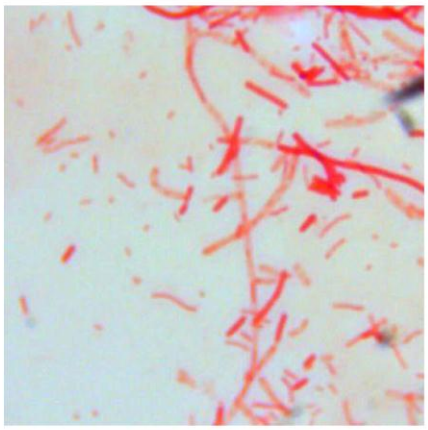
C) S21A Mixture of Gram negative small ovoid cells and larger Gram negative rods. Some background staining.



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 loosley 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 in 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.

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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 quntitly 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 datatbase 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).

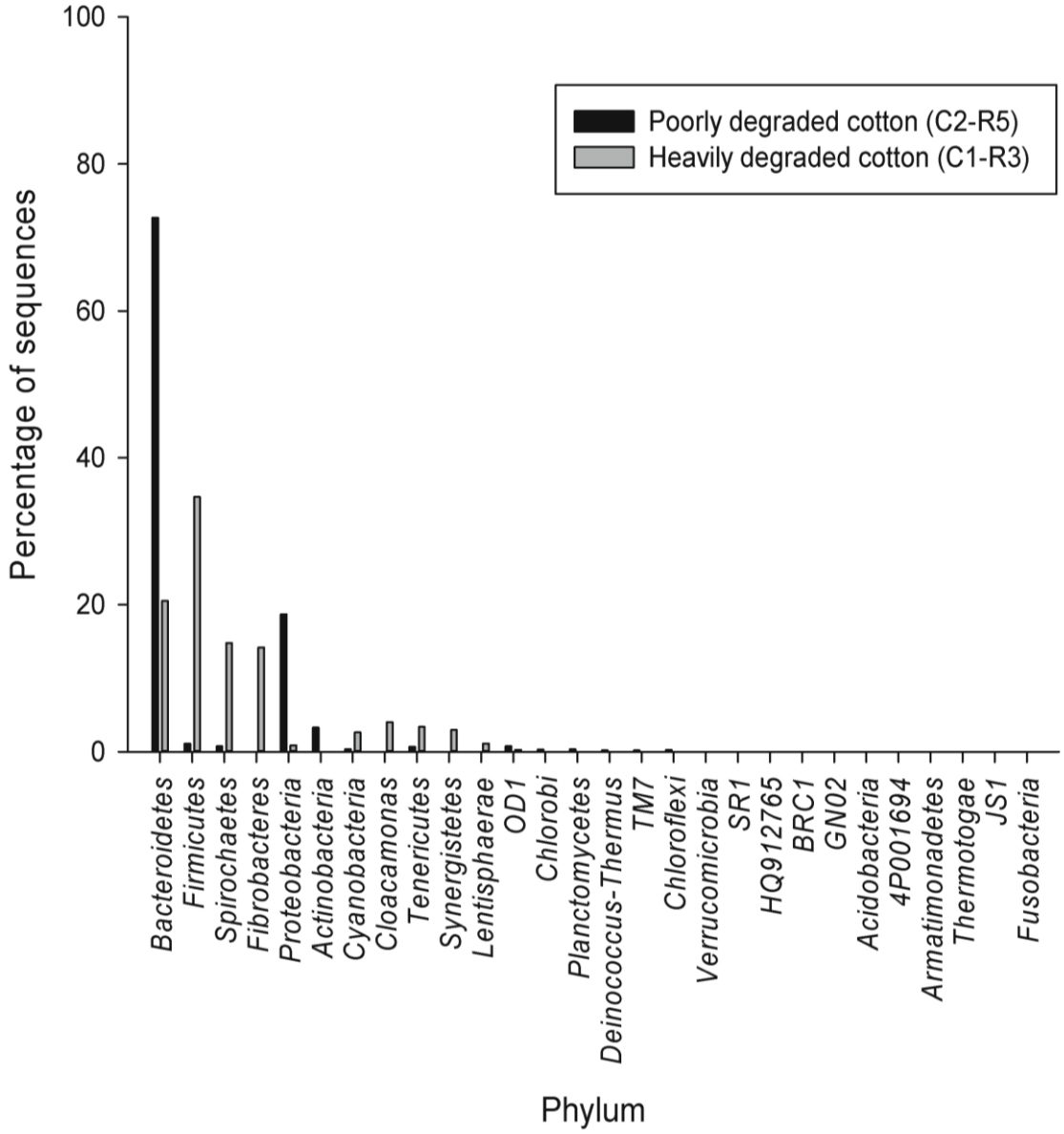

Figure 5.1. Comparison of bacterial phyla identified via 16S rRNA gene PCR amplicon sequences from poorly (black bars) and heavily (grey bars) degraded cotton from landfill leachate microcosms as determined via comparison of the sequences against the EzTaxon database.

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)	Largestcontig 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).

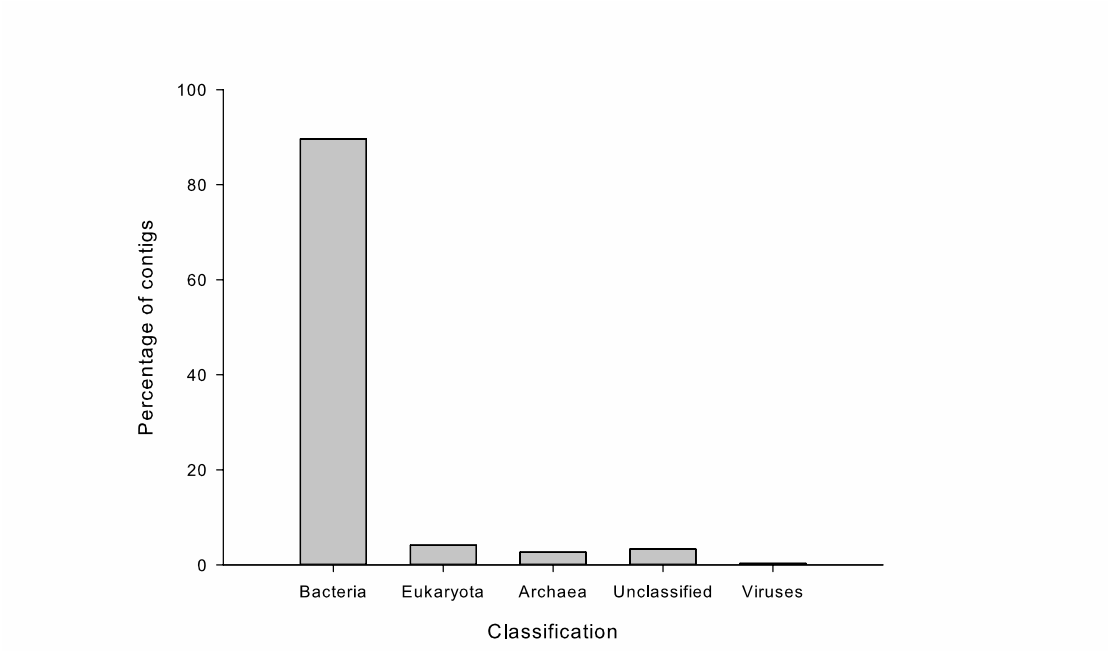

Figure 5.2. Classification of contigs derived from the metagenome of heavily degraded colonised cotton incubated in a landfill leachate microcosm. Domains were assigned via analysis of 16/18S rRNA gene sequences by MG-RAST against the M5RNA database, utilising the best hit classification algorithm with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

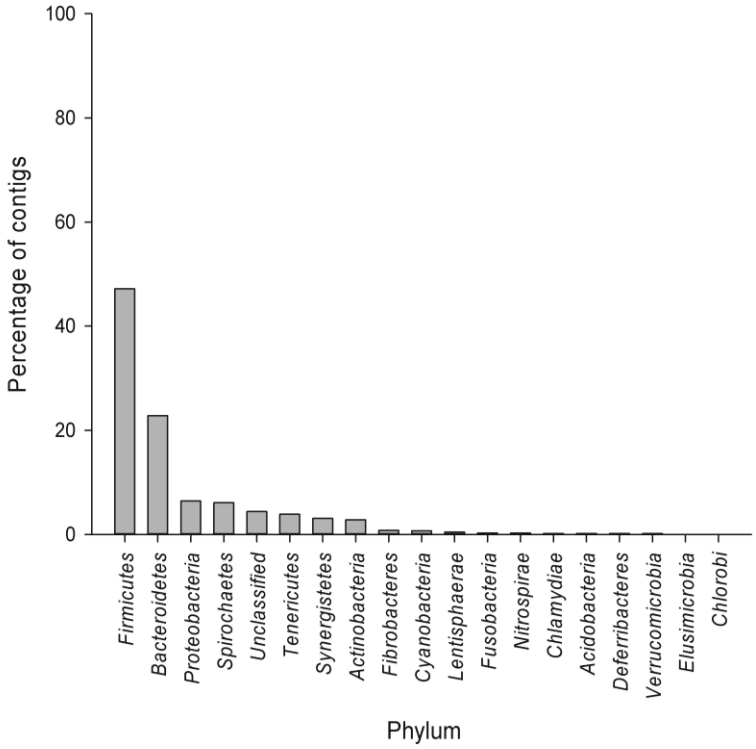

Figure 5.3. Classification of bacterial phyla via analysis of contigs derived from the metagenome of heavily degraded colonised cotton incubated in a landfill leachate microcosm. Assignments were determined via analysis of 16S rRNA gene sequences by MG-RAST against the M5RNA database, utilising the best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

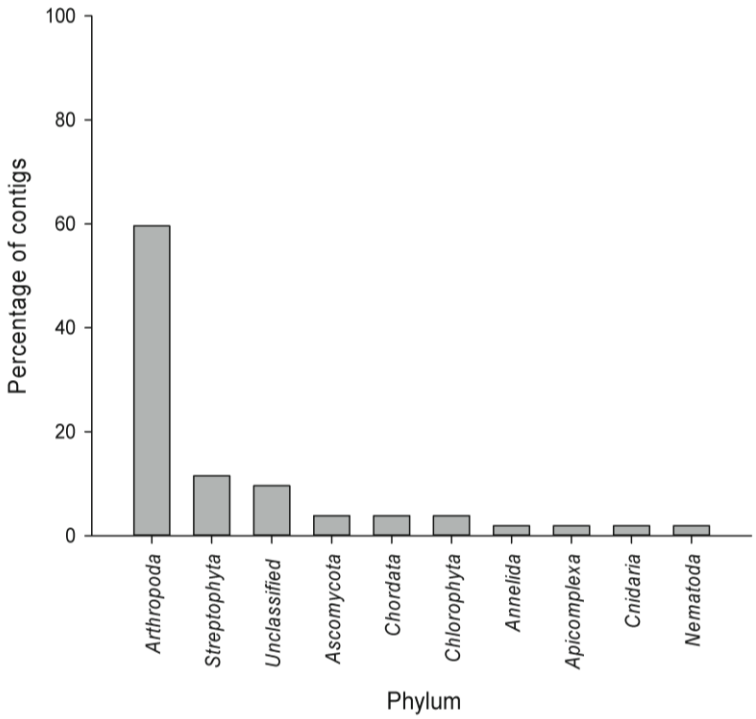

Figure 5.4. Classification of phyla assigned as eukaryota via analysis of contigs derived from the metagenome of heavily degraded colonised cotton incubated in a landfill leachate microcosm. Assignment determined via analysis of 18S rRNA gene sequences by MG-RAST against the M5RNA database, utilising 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.

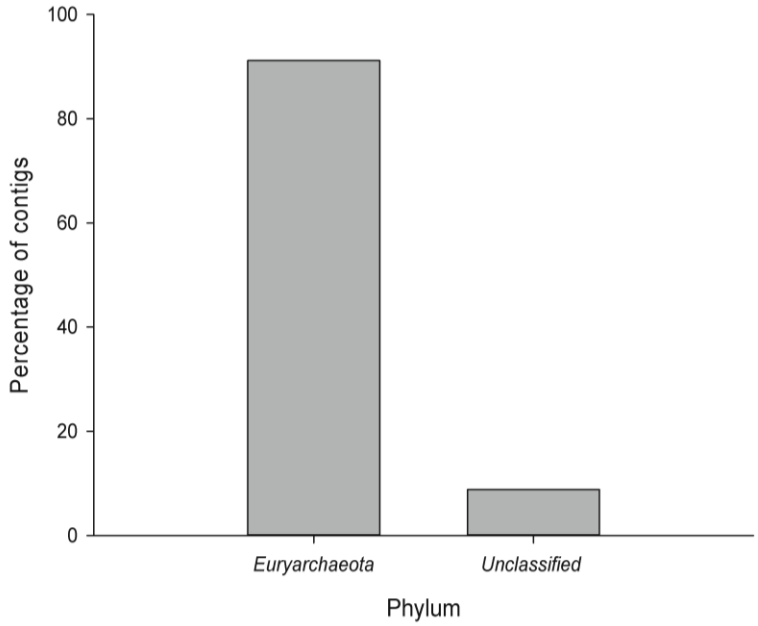

Figure 5.5. Classification of phyla assigned as archaea via analysis of contigs derived from the metagenome of the heavily degraded colonised cotton incubated in a landfill leachate microcosm. Assignment determined via analysis of 16S rRNA gene sequences by MG-RAST against the M5RNA database, utilising 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 againt 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 *Streptohyta* (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 *Streptohyta* 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).

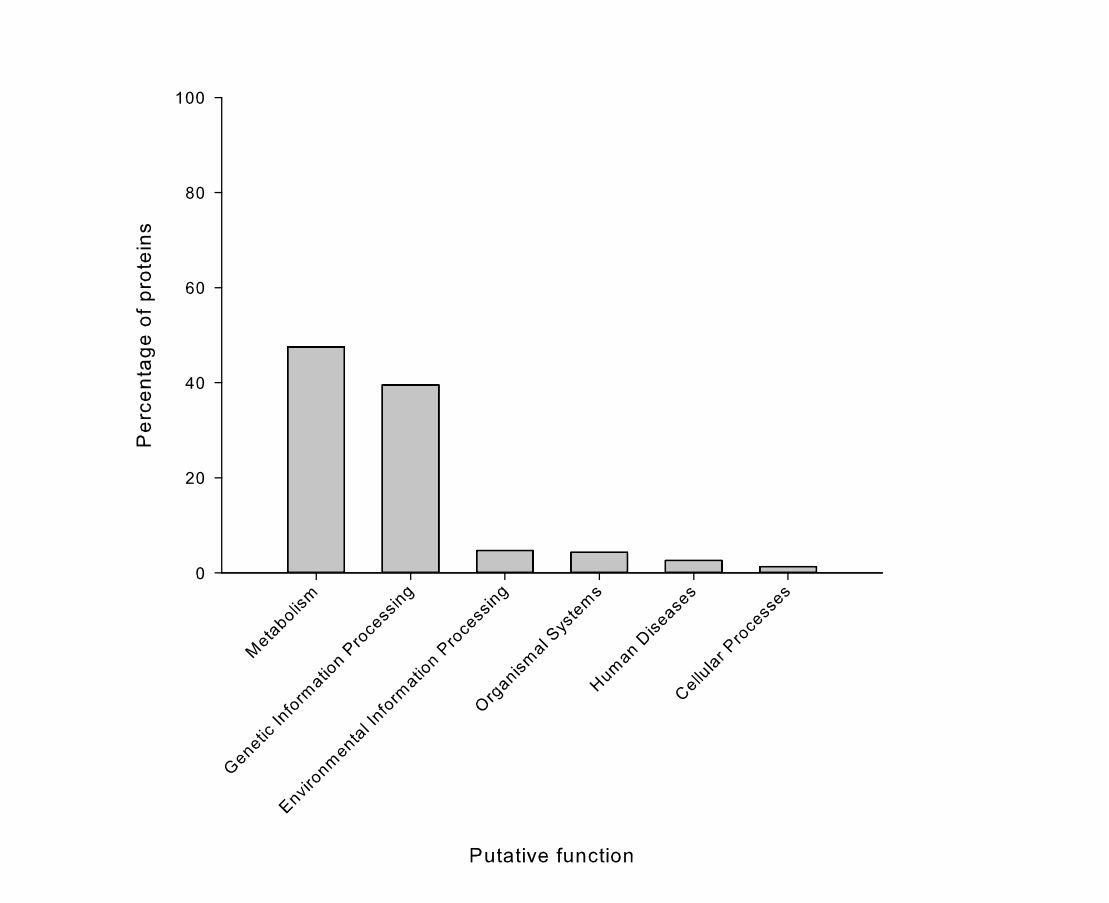

Figure 5.6. Predicted function of proteins as identified via MG-RAST in metagenome contigs derived from the heavily degraded colonised cotton sample.

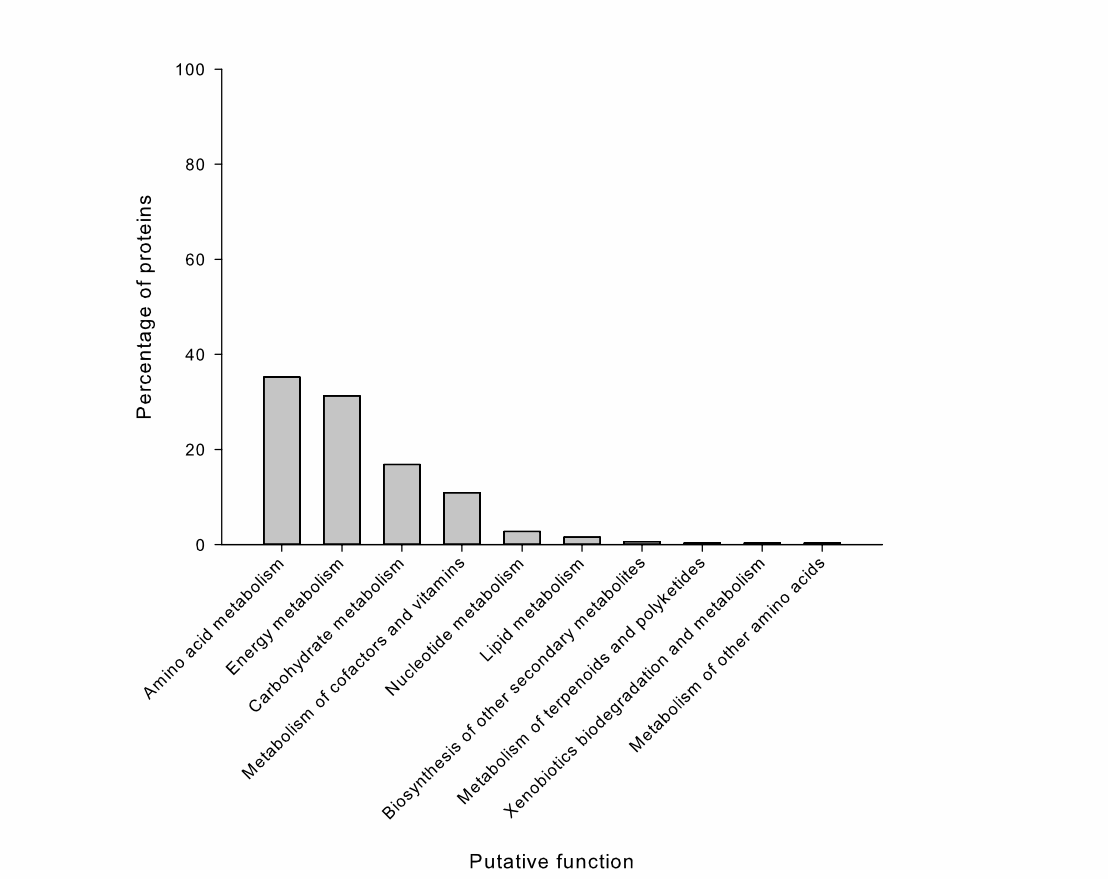

Figure 5.7. Classification of proteins from the heavily degraded colonised cotton metagenome assigned to the metabolism category via MG-RAST

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	*Fibrobacter succinogenes* subsp. *succinogenes* S85	5931
	Treponema vincentii ATCC 35580	2153
	Treponema denticola ATCC 35405	1934
	Paludibacter propionicigenes WB4	1554
	Clostridium thermocellum ATCC 27405	1131
400 bp	*Fibrobacter succinogenes* subsp. *succinogenes* S85	7460
	Methanoculleus marisnigri JR1	4239
	Paludibacter propionicigenes WB4	4017
	Treponema vincentii ATCC 35580	2681
	Clostridium thermocellum ATCC 27405	2551
600bp	*Fibrobacter succinogenes* subsp. *succinogenes* S85	5783
	Treponema vincentii ATCC 35580	1900
	Treponema denticola ATCC 35405	1708
	Paludibacter propionicigenes WB4	1544
	Clostridium thermocellum 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 anerobic 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 anerobic 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 degragded 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 metageome

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

Microcosm set up, sampling and DNA extractions were all performed by Dr. James McDonald. All other work was performed by Emma Ransom-Jones unless otherwise stated.

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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 fibrobaters 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 at 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 *Fibrobacters* 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. though 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 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 (Sebaihia *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 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 anerobic 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 sting 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 *Spriochaetes*) the second and third genomes with the most protein matches against the 300 and 600 bp metagenome libraries. These data implicate both fibrobacters and spriochetes as being important members of the landfill cellulolytic microbial community. Recruitment plot analysis of the genome of *F. succinogenes* subsp. *succinogenes* S85 against the metagemome 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. Microb Ecol 63:267-281

Microb Ecol (2012) 63:267–281
DOI 10.1007/s00248-011-9998-1

MINIREVIEW

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

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Received: 20 May 2011 / Accepted: 12 December 2011 / Published online: 3 January 2012

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

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Introduction

The Genus *Fibrobacter*

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

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

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

Phenotypic Characteristics of *Fibrobacter* Isolates

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

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

The Phylogeny of the Genus *Fibrobacter*

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

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

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

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

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

[a] Can also use maltose [125]

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

Cellulose Degradation

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

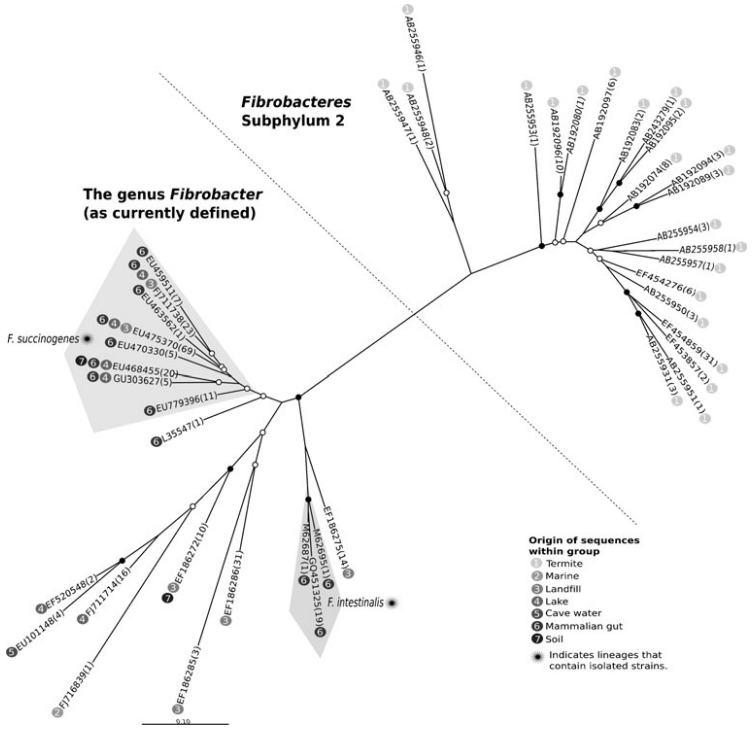

Figure 1 Phylogeny of the *Fibrobacteres* phylum. Maximum likelihood tree of 16S rRNA gene sequences belonging to the *Fibrobacteres* phylum. All sequences classified within the *Fibrobacteres* phylum and annotated as of 'good' quality were downloaded from the Ribosomal Database Project [19, 21] website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

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

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

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

Fibrobacters are Major Degraders of Plant Biomass in the Herbivore Gut

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

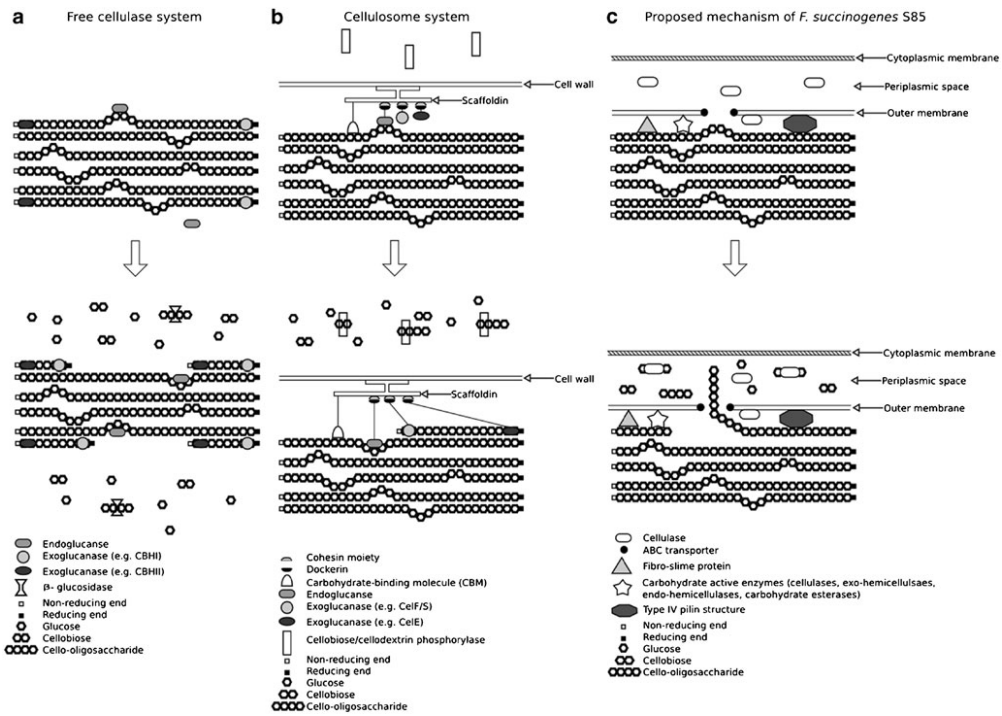

Figure 2 Microbial mechanisms of cellulose degradation. **a** Aerobic cell-free cellulase system (based on [75]); typical of aerobic microorganisms including *T. reesei*. Cellulose is hydrolysed via the synergistic interaction of individual enzymes that are secreted from the cell. **b** Anaerobic 'cellulosome'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

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

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

Cellulose Degradation by *Fibrobacter* spp.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Landfill Sites

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

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

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

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

Fibrobacter as currently defined [84]. Amann and colleagues [1] suggested that *Fibrobacter* may in fact represent a 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.

Acknowledgements Research on fibrobacters by the authors has been funded by the Natural Environment Research Council (AJM and JEM) and the Systematics Association's SynTax award scheme, supported by the Linnean Society of London, BBSRC and NERC (JEM). ERJ is supported by a 125th Anniversary Scholarship at Bangor University.

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APPENDIX II

Ransom-Jones E, Jones DL, Edwards A, McDonald JE (2014) Distribution and diversity of members of the bacterial phylum *Fibrobacteres* in environments where cellulose degradation occurs. Syst Appl Microbiol DOI: 10.1016/j.syapm.2014.06.001

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SYAPM-25635; No. of Pages 8
ARTICLE IN PRESS

Systematic and Applied Microbiology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

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

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ARTICLE INFO

Article history:
Received 6 April 2014
Received in revised form 10 June 2014
Accepted 10 June 2014

Keywords:
Fibrobacter
Fibrobacteres
Cellulose degradation
Diversity
Phylogeny
Environment

ABSTRACT

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

Introduction

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

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http://dx.doi.org/10.1016/j.syapm.2014.06.001
0723-2020/

Please cite this article in press as: E. Ransom-Jones, et al., Distribution and diversity of members of the bacterial phylum *Fibrobacteres* in environments where cellulose degradation occurs, Syst. Appl. Microbiol. (2014), http://dx.doi.org/10.1016/j.syapm.2014.06.001

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

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

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

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

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

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

Materials and methods

Sampling

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

DNA extraction

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

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Table 1
PCR and qPCR analysis of environmental samples.

Sample	Sample type	Location	Direct PCR *Fibrobacter* product	Nested PCR *Fibrobacter* 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′)[a]	Specificity	Annealing temperature (°C)	Amplicon size (bp)	Reference
pA	AGAGTTTGATCCTGGCTCAG	General bacteria	55	~ 1534	[14]
pH′	AAGGAGGTGATCCAGCCGCA				
Fib 1F[e]	CCGKSCCAACGSSCGG	*Fibrobacter* genus	60	~855	[36]
Fib 2AR	ATCTCTCGCYGCGGCGWTYCC				
1369F[b]	CGGTGAATACGTTCYCGG	General bacteria[b]	60[d]	~ 151	[47]
Prok 1492R[b]	GGWTACCTTGTTACGACTT				
FibroQ153F[be]	CCGKSCCAACGSSCGGHTAA	*Fibrobacter*[b] genus	60[d]	~104	[36]
FibroQ238R[b]	CSCCWACTRGYTAATCRGAC				
M13 Forward[c]	GTTTTCCCAGTCACGAC	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] QuantiFastTM 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 $MgCl_2$, 1× BSA, 1 U SuperTaq (Cambio), 50 ng DNA and ddH_2O 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 $MgCl_2$ (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 I (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 BioScience.

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 ddH_2O. 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: $(X\ g\ \mu L^{-1}\ DNA/[PCR\ product\ length\ in\ base\ pairs \times 660]) \times 6.022 \times 1023 = Y\ molecules\ \mu L^{-1}$.

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 r^2 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% (Brombrough 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 interspecies 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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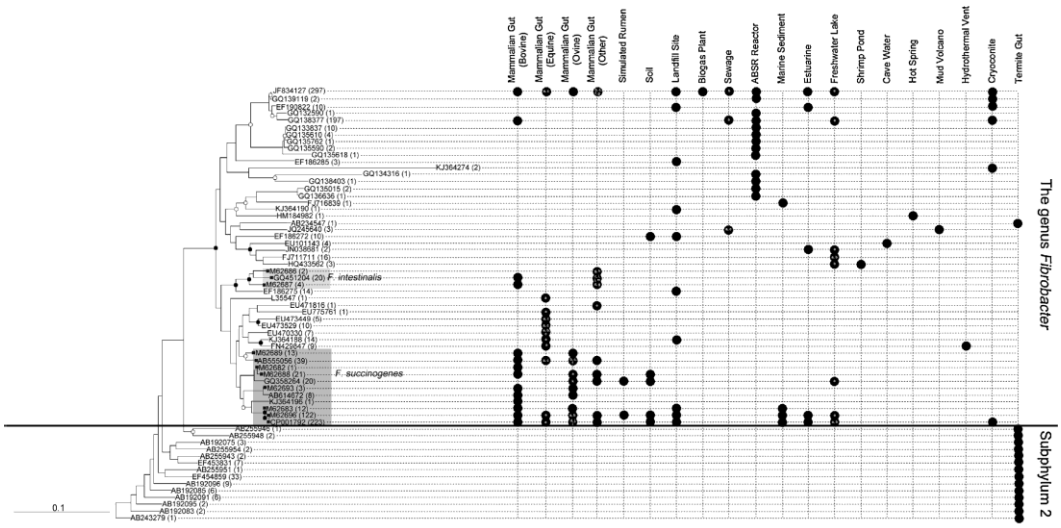

Fig. 1. The ecology and taxonomy of the *Fibrobacteres* phylum. OTUs containing cultivated species are designated by boxes on the end of the node. Refer to Supplementary Table S1 for a full data table describing the sequences contained in each OTU, their accession numbers and percentage environmental 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.

Acknowledgements

This research was funded by the Systematics Association's SynTax award scheme, supported by the Linnean Society of London, BBSRC and NERC. ERJ was supported by a 125th Anniversary Scholarship at Bangor University. AE acknowledges NERC (NE/K000942/1) and the Society for General Microbiology (PFRV 10/4).

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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Please cite this article in press as: E. Ransom-Jones, et al., Distribution and diversity of members of the bacterial phylum *Fibrobacteres* in environments where cellulose degradation occurs, Syst. Appl. Microbiol. (2014), http://dx.doi.org/10.1016/j.syapm.2014.06.001

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	*Fibrobacter intestinalis;* C1a	Other Mammal 100
	1	M62695	97	Rat Cecum	*Fibrobacter intestinalis;* NR9	
4	0	M62687	RS*	Porcine Cecum	*Fibrobacter intestinalis;* DR7	Ovine 50
	1	M62690	95	Ovine Rumen	*Fibrobacter intestinalis;* JG1	Other Mammal 50
	2	M62691	95	Ovine Rumen	*Fibrobacter intestinalis;* LH1	
	3	HQ008626	96	Dromedary Camel		
12	0	M62683	RS*	Bovine Rumen	*Fibrobacter succinogenes;* A3C	Bovine 75
	1	M62684	96	Bovine Rumen	*Fibrobacter succinogenes;* B1	Bovine/Lake Sediment 8.3
	2	M62685	96	Bovine Rumen	*Fibrobacter succinogenes;* BL2	Landfill 8.3
	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	*Fibrobacter succinogenes;* S85	Landfill 24

1	DQ054633	97	Bovine Rumen		Bovine 18
2	DQ054634	98	Bovine Rumen		Lake 14
3	AB275487	98	Ovine Rumen	*Fibrobacter succinogenes;* OS117	Soil 11
4	AB275488	98	Ovine Rumen	*Fibrobacter succinogenes;* AS206	Estuarine 9
5	AB275489	98	Ovine Rumen	*Fibrobacter succinogenes;* AS213	Bovine/Lake Sediment 8
6	AB275509	98	Ovine Rumen	*Fibrobacter succinogenes;* RS223	Ovine 7
7	AB275513	98	Ovine Rumen	*Fibrobacter succinogenes;* 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	*Fibrobacter succinogenes* subsp. *succinogenes* S85	
15	EF186235	98	Bovine Rumen	*Fibrobacter succinogenes* subsp. *succinogenes* S85; 2	
16	EU719256	98	Bovine Rumen		
17	EU459511	95	Capybara 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		

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

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

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

	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		
223	0	AF018454	98	Bovine Rumen		Bovine 33
	1	AJ496032	99	Bovine Rumen	*Fibrobacter succinogenes* subsp. *Succinogenes;* S85	Ovine 14 Lake 13
	2	AJ496186	96	Ovine Rumen	*Fibrobacter succinogenes;* HM2	Landfill 13
	3	AJ496447	99	Bovine Rumen	*Fibrobacter succinogenes;* H	Estuarine 9
	4	AJ496448	99	Bovine Rumen	*Fibrobacter succinogenes strain;* U	Marine Sediment 5
	5	AJ496566	99	Ovine Rumen	*Fibrobacter succinogenes;* FE	Bovine/Lake Sediment 5
	6	AJ505937	99	Bovine Rumen	*Fibrobacter succinogenes;* BL2	Soil 4
	7	AJ505938	99	Bovine Rumen	*Fibrobacter succinogenes;* R	Other Mammal 3
	8	AY311623	99	Yak Rumen		Cryoconite <1
	9	AY311716	99	Yak Rumen		Equine <1
	10	AY315348	96	Jinnan Cattle Rumen		
	11	AB113694	99	Ovine Rumen		
	12	AB275486	99	Ovine Rumen	*Fibrobacter succinogenes;* OS109	
	13	AB275490	99	Ovine Rumen	*Fibrobacter succinogenes;* AS216	
	14	AB275491	99	Ovine Rumen	*Fibrobacter succinogenes;* AS220	

15	AB275492	99	Ovine Rumen	*Fibrobacter succinogenes;* AS221
16	AB275493	99	Ovine Rumen	*Fibrobacter succinogenes;* AS225
17	AB275494	99	Ovine Rumen	*Fibrobacter succinogenes;* AS226
18	AB275495	99	Ovine Rumen	*Fibrobacter succinogenes;* AS228
19	AB275496	98	Ovine Rumen	*Fibrobacter succinogenes;* OS102
20	AB275497	99	Ovine Rumen	*Fibrobacter succinogenes;* OS103
21	AB275498	98	Ovine Rumen	*Fibrobacter succinogenes;* OS112
22	AB275500	98	Ovine Rumen	*Fibrobacter succinogenes;* OS118
23	AB275501	99	Ovine Rumen	*Fibrobacter succinogenes;* OS119
24	AB275502	99	Ovine Rumen	*Fibrobacter succinogenes;* OS120
25	AB275503	99	Ovine Rumen	*Fibrobacter succinogenes;* OS128
26	AB275504	98	Ovine Rumen	*Fibrobacter succinogenes;* RS*209
27	AB275505	99	Ovine Rumen	*Fibrobacter succinogenes;* RS*214
28	AB275506	99	Ovine Rumen	*Fibrobacter succinogenes;* RS*215
29	AB275507	99	Ovine Rumen	*Fibrobacter succinogenes;* RS*216
30	AB275508	99	Ovine Rumen	*Fibrobacter succinogenes;* RS*220
31	AB275510	98	Ovine Rumen	*Fibrobacter succinogenes;* RS*224
32	AB275511	99	Ovine Rumen	*Fibrobacter succinogenes;* RS*225
33	AB275512	99	Ovine Rumen	*Fibrobacter succinogenes;* RS*230
34	AB275514	99	Ovine Rumen	*Fibrobacter succinogenes;* 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	

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	*Fibrobacter succinogenes*
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	*Fibrobacter succinogenes* subsp. *Succinogenes;* S85
69	CP001792	100	Bovine Rumen	*Fibrobacter succinogenes* subsp. *Succinogenes;* S85
70	CP001792	99	Bovine Rumen	*Fibrobacter succinogenes* subsp. *Succinogenes;* S85
71	GU269553	96	Bovine Rumen	*Fibrobacter succinogenes (T);* HM2

72	GU303546	96	Bovine Rumen	
73	CP002158	99	Bovine Rumen	*Fibrobacter succinogenes* subsp. *Succinogenes;* S85.
74	CP002158	99	Bovine Rumen	*Fibrobacter succinogenes* subsp. *Succinogenes;* S85
75	CP002158	99	Bovine Rumen	*Fibrobacter succinogenes* subsp. *Succinogenes;* S85
76	HM104735	97	Bovine Rumen	
77	HM104984	96	Bovine Rumen	
78	CP001792	RS*	Bovine Rumen	*Fibrobacter succinogenes* subsp. *Succinogenes;* S85
79	CP001792	99	Bovine Rumen	*Fibrobacter succinogenes* subsp. *Succinogenes;* S85
80	CP001792	99	Bovine Rumen	*Fibrobacter succinogenes* subsp. *Succinogenes;* 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	

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	*Fibrobacter succinogenes*; H23
112	AB665863	98	Ovine Rumen	

113	AB665895	99	Ovine Rumen	
114	AB665948	98	Ovine Rumen	
115	HQ634725	98	Yak Rumen	
116	JQ346742	99	Bovine Rumen	*Fibrobacter succinogenes* subsp. *Succinogenes*; S85 *Fibrobacter;* 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	

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

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

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

	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	*Fibrobacter succinogenes;* HM2	
	3	M62692	96	Ovine Rumen	*Fibrobacter succinogenes;* MB4	
	4	M62694	97	Ovine Rumen	*Fibrobacter succinogenes;* 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	*Fibrobacter succinogenes;* MC1	Ovine 100
	1	FJ711751	98	Ovine Faeces		
	2	FJ711752	98	Ovine Faeces		

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	*Fibrobacter intestinalis* (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		

	14	GQ451324	99	Monkey Faeces 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	

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

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

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

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

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

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

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

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

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

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

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

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

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

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

	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 Rumen	Equine 46

1	L35548	95	Equine Cecum
2	EU381839	95	Bovine Rumen
3	EU463463	96	Equine Faeces
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

Bovine 38
Other Mammal 13
Ovine 3

				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	*Fibrobacter succinogenes;* FGL 01
	24	GU999989	96	Goat Rumen	*Fibrobacter succinogenes*; 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	

	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	

	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	

	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	

	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

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	

	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	

			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

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

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

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

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

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

			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

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

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

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

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

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	

	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	

	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

			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
			Saprospiraceae	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
			Desulfonispora	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

			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
			Nocardioidaceae	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	Phycisphaeraceae	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	Puniceicoccales		85.71	100.00
		Opitutales		14.29	0.00
	Verrucomicrobiae	Verrucomicrobiales		0.00	100.00
Verrucomicrobia	Opitutae	Puniceicoccales	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	Gammaproteobacteria		30.13
		Alphaproteobacteria		23.29
		Deltaproteobacteria		23.29
		Betaproteobacteria		16.44
		Epsilonproteobacteria		4.11
		Zetaproteobacteria		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
			Thermoanaerobacterales	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	Gammaproteobacteria	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

			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

	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	87.50
			Unclassified	12.50
	Cytophagia	Cytophagales	Cytophagaceae	100.00
Proteobacteria	Gammaproteobacteria	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

			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

	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

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