Evolving Microbial Communities for Biofuel Production

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Abstract

Plant lignocellulose is the most abundant raw material on the planet and a promising substrate for biofuel production. While this complex polymer is efficiently degraded by a range of naturally occurring microbial communities, cost- and energy-efficient industrial use is hampered by its recalcitrance to degradation. By gaining a better understanding of how microbial lignocellulose degrading communities function we may be able to improve industrial processes. In this thesis, I used a combination of ecological and evolutionary approaches to uncover the species and functional traits that drive lignocellulolytic microbial community productivity. I found that the presence of key highly active cellulolytic bacteria increased the productivity of microbial consortia. Specifically, we identified two species, *Cellulomonas* sp. D13 and *Paenibacillus* sp. A8, with a range of cellulase and hemicellulase enzymes that have potential for application in industrial processes. Experimental evolution revealed that the rate of phenotypic adaptation of a focal bacterial species, *Stenotrophomonas* sp. D12, to growth on wheat straw was accelerated by the presence of other competing species. The trajectory of focal species evolution was determined by both the identity and the ecological and evolutionary responses of the competing species. Genome sequencing of evolved clones suggested that genetic adaptation by the focal species to degrade wheat straw involved mutations targeting regulatory genes involved in catabolite repression and carbon storage, two systems that may represent promising targets for the improvement of industrial strains. Overall these results suggest the ecological and evolutionary approaches can be used to design and improve microbial consortia for lignocellulose bioconversion.

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And finally to Rupert for never getting mad when I'm being infuriating but constantly making me laugh instead. Thank you for believing in me and giving me the confidence to pursue my goals (even if it is just so you can be a house husband one day).

Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

The following paper has been published and is included in this thesis:

Evans, R., Alessi, A.M., Bird, S., McQueen-Mason, S.J., Bruce, N.C., and Brockhurst, M.A. (2017). Defining the functional traits that drive bacterial decomposer community productivity. ISME J. 11, 1680–1687.

This manuscript was written by myself and Michael Brockhurst while the remaining authors provided guidance regarding experimental design.

1 Introduction

1.1 Lignocellulosic biofuel production

As global energy consumption continues to increase, the demand for sustainable and environmentally friendly fuel sources is growing (Demain, 2009; Van Dyk and Pletschke, 2012). The majority of liquid biofuels, known collectively as first-generation biofuels, are currently produced from sugar rich crops such as sugarcane and maize. These crops require dedicated land for growth and it is argued that this not only reduces food production but also increases prices (Tenenbaum, 2008). Plant lignocellulose, the most abundant organic material on Earth, offers an attractive alternative as a sustainable substrate for biofuel production due to its low cost and high sugar content (Bhatia et al., 2012; Shekhar, 2011). Composed primarily of cellulose, hemicellulose and lignin (Figure 1.1), lignocellulose evolved to provide plants with both their structural rigidity and resistance to microbial and enzymatic attack and as such, it is extremely recalcitrant to degradation (Cragg et al., 2015). Finding an energy-efficient and cost-effective method to depolymerise this complex substrate is a key challenge that must be overcome before lignocellulosic biofuel production can be achieved on a commercial scale (Naik et al., 2010).

Current industrial processing of lignocellulose relies on physiochemical pretreatments to disrupt the lignin-polysaccharide interactions thereby reducing the degree of recalcitrance (Van Dyk and Pletschke, 2012). These processes are expensive, require a large energy input and can produce compounds that are inhibitory to saccharification and fermentation (Agbor et al., 2011; Zheng et al., 2014). Following pretreatment, cellulose and hemicellulose polymers are exposed to hydrolysis enzymes to release monosaccharides which can then be converted into ethanol by fermentation. The saccharification enzymes currently used require high enzyme loading due to their low catalytic efficiencies contributing to the cost barrier associated with lignocellulosic biofuels (Lynd et al., 2008, 2017). For lignocellulose-derived biofuels to become economically competitive, more energy- and cost-effective processing methods need to be developed (Lynd et al., 2008; Naik et al., 2010).
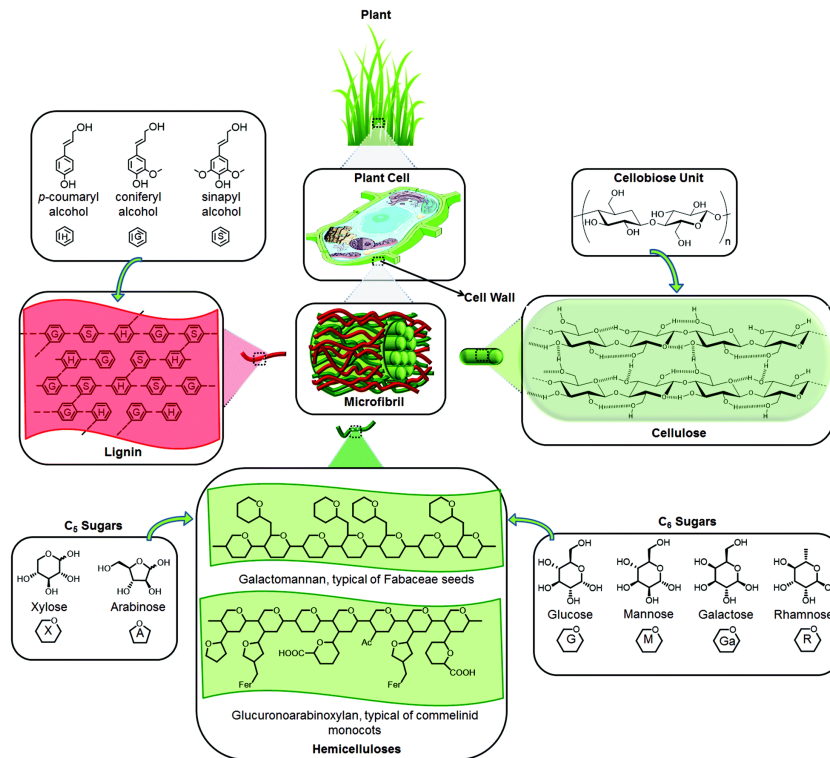
Figure 1.1 Structure of lignocellulose and its main components: lignin, hemicellulose and cellulose. "Fer" represents esterification with ferulic acid, which is characteristic of xylans in commelinid monocots such as wheat. Figure reproduced from (H. Isikgor and Remzi Becer, 2015).

Despite the recalcitrance of lignocellulose, its breakdown is achieved in a wide range of environments as part of the global carbon cycle. Microbial communities are responsible for a large portion of lignocellulose degradation in soil (Woo et al., 2014a), compost (Wei et al., 2012) and sediments (Cortes-Tolalpa et al., 2016). Most animals, including ruminants (Haitjema et al., 2014) and termites (Brune, 2014; Warnecke et al., 2007), also rely on specialised microbial communities in their guts to degrade plant materials and release sugars that can be absorbed by the host. These microbial communities are, therefore, a promising source of microorganisms and enzymes with high lignocellulolytic activity and potential industrial applications (Brune, 2014; Cragg et al., 2015).

1.2 Lignocellulose degradation by microbial communities

Microbial communities play an essential role in various natural and industrial processes. They are key to human health and digestion (Eloe-Fadrosh and Rasko, 2013), global nutrient cycling (Hättenschwiler et al., 2005) and industrial biotechnology (Kouzuma and Watanabe, 2014). In order to manage these communities effectively and predict how they will perform, we require a better

understanding of the features which drive microbial community function. Several studies have shown microbial communities to be more efficient than monocultures when degrading micropollutants (Johnson et al., 2015), resisting invasion (Elsas et al., 2012) and degrading lignocellulosic biomass (Halsall and Gibson, 1985; Ponce-Noyola and Torre, 1993; Szambelan et al., 2004). Studies examining the relationship between biodiversity and ecosystem functioning (BEF) often find a positive relationship which can be explained by two key mechanisms. First, the complementarity effect in which diverse communities possess a wider range of functional traits and are therefore able to occupy more of the available niche space (Salles et al., 2009; Singh et al., 2015). Second, the selection effect in which diverse communities are more likely to contain highly active species or species that are preadapted to environmental perturbations (Awasthi et al., 2014; Hooper et al., 2005). Bell et al. (2005) found a positive BEF relationship between bacterial strains isolated from beech treeholes and their productivity when grown on beech leaf extract (presumably containing lignocellulose). The selection effect played a minor role in driving this relationship with no single species dominating productivity. The results suggest that while it may be possible to maximise community functioning by selecting a particular consortium of species, an increase in function can also be achieved by increasing species richness (Bell et al., 2005). This may be particularly true for cellulose degrading communities which have been shown to achieve greater rates of degradation with increasing diversity, despite the constituent species having similar functional traits (Wohl et al., 2004).

Exploiting lignocellulolytic microbial communities could improve industrial lignocellulose degradation in two main ways. First, enzymes produced by these communities may be more efficient than those currently used by industry and numerous efforts are ongoing to identify and characterise the enzymes used by these communities (Mori et al., 2014). Microbial communities offer the added benefit of being able to adapt to specific substrates and experimental conditions. For example, Gladden et al. (2011) showed that compost-derived microbial communities that adapted to degrade switchgrass at high temperature (60°C) produced enzymes that were more thermotolerant and stable in the presence of by-products than

commercially available enzyme cocktails (Gladden et al., 2011). Second, microbial communities can be utilised through the consolidated bioprocessing (CBP) approach. In CBP, lignocellulose is converted into valuable products by a single engineered strain or a consortium of naturally occurring and/or engineered strains in a single step improving the efficiency and reducing the cost of bioethanol production from lignocellulose (Lynd et al., 2002; Zuroff and Curtis, 2012).

While current industrial bioprocessing is dominated by the single species approach, using a multispecies consortium for CBP has several benefits. Metabolic load means that single species are limited in the number of resources they can utilise and the number of products they can produce. In addition, it can be difficult to optimise individual processes, let alone multiple processes in a single species system [e.g. the expression of carbohydrate degradation enzymes and the production of ethanol (Brenner et al., 2008)]. By contrast, microbial consortia can contain species specialised at degrading different parts of a complex substrate and so the metabolic load is shared (Gerchman and Weiss, 2004; Shong et al., 2012). Cross-feeding, the process by which species utilise the by-products of the metabolism of others, can also slow the accumulation of inhibitory compounds in the media, increasing overall productivity (D'Souza et al., 2018; Estrela et al., 2012). One limiting factor to this approach is that it is often difficult to control and predict changes in community composition and functioning over time. Designing stable microbial communities capable of efficient, predictable CBP requires a better understanding of how microbial communities function and how they adapt over time.

1.3 Evolution of increased lignocellulose degradation

Experimental evolution allows various evolutionary questions to be rigorously studied in a controlled, laboratory environment. Microorganisms are particularly useful model organisms for these experiments as they typically have rapid generation times, they can be frozen to produce a complete living fossil record, and they readily adapt to various abiotic and biotic selection pressures (Elena and Lenski, 2003). Various experimental evolution studies have aimed to elucidate the mechanisms by which microbes adapt to growth in new environments, often in limiting resources or

novel substrates (Chubiz and Marx, 2017; Herring et al., 2006; Lee and Palsson, 2010). These studies are particularly powerful when coupled with omics approaches to uncover the genetic and transcriptional changes which underpin phenotypic adaptations (Barrick and Lenski, 2013; Brockhurst et al., 2011a). For example, genes involved in glycerol metabolism by *Saccharomyces cerevisiae* were discovered by a combination of experimental evolution and genome sequencing (Strucko et al., 2018). Introduction of mutations in these genes in industrial strains increased glycerol utilisation and have the potential to increase the efficiency of industrial processes.

Experimental evolution is being explored as a method to improve lignocellulose degradation by strains of both bacteria and fungi. Serial propagation of the fungi *Trichoderma citrinoviride* with filter paper (cellulose) as substrate led to a 2.5 increase in degradation of the substrate (Lin et al., 2016). Increased cellulase activity was attributed to changes in the regulatory strategies by the cells which increased the amount of total secreted cellulase with only small increases in metabolic load. Due to the complexity of regulatory networks that both bacteria and fungi possess, this phenotype would be difficult to design and engineer by synthetic biology approaches (Falke et al., 1997; Mira et al., 2012). Recently, the industrial fermentative bacteria *Corynebacterium glutamicum* was experimentally evolved to increase its tolerance of lignocellulose derived inhibitors (Wang et al., 2018). A 68.4% increase in glutamic acid production was achieved by the evolved populations and transcriptomic analysis revealed this was a result of upregulation of glucose transport and the pentose phosphate pathway.

One key benefit of experimental evolution is that the resulting phenotypes are the result of natural selection and therefore are likely to be more stable than synthetically engineered strains. For example, studies aiming to increase enzyme production often lead to impaired growth or eventual loss of viability (Dong et al., 1995; Eguchi et al., 2018). The negative effect that adding these engineered traits have on the fitness of the cell may eventually lead to the emergence of genotypes where the engineered trait has been lost (i.e. the emergence of non-producers). Non-

producers are likely to be fitter than the engineered strain and will therefore outcompete it over time (Rosano and Ceccarelli, 2014; Travisano and Velicer, 2004). By contrast, experimental evolution relies on natural selection and so for genotypes to reach fixation, they must be able to outcompete other genotypes and are likely to have undergone compensatory evolution to mitigate the costly pleiotropic effects of mutations (Park and Krug, 2007). For example, the tolerance of *C. glutamicum* to lignocellulose derived inhibitors increased until transfer 70 and thereafter its growth and glucose consumption remained constant until the end of the experiment (transfer 130) (Wang et al., 2018).

Industrially-focused applications of the experimental evolution approach have typically focused on single-species populations, however, as described above lignocellulose degradation is performed by communities in nature. Various studies have shown that the trajectory and rate of evolution can be shaped by the presence of competing strains (Fiegna et al., 2015a; Lawrence et al., 2012). These studies have revealed that when negatively interacting bacterial species are evolved in new environments, the overall community productivity increases even though the ability of species to grow alone in the selection environment decreases. This is as a result of the evolution of cross-feeding metabolic dependencies between the coexisting species, such that originally negative competitive interactions can become positive facilitatory interactions following community evolution (Barraclough, 2015; Fiegna et al., 2015a). It is reasonable therefore to assume that community experimental evolution may be a promising method to improve the stability and increase the efficiency of microbial communities with potential industrial applications (Brenner et al., 2008; Jimenez et al., 2014; Puentes-Téllez and Salles, 2018).

1.4 Thesis outline

In this thesis I investigate a range of these issues in detail using wheat straw degradation by bacterial compost communities as a model system. The various chapters of the thesis address the following research questions:

Chapter 2 – The isolation of lignocellulolytic bacteria from wheat straw compost

Various lignocellulosic substrates are degraded in compost by the action of a diverse microbial community. As our research focusses on wheat straw degradation, I used wheat straw adapted compost as a source for the bacterial strains used throughout this thesis. Compost enrichment cultures were grown for eight weeks with bacteria isolations each week. Isolates were assayed for their ability to degrade cellulose and hemicellulose and identified by 16S rRNA sequencing. We show that compost is a viable source of phylogenetically, functionally and phenotypically diverse culturable lignocellulolytic bacteria.

Chapter 3 - Defining the functional traits that drive lignocellulose degrading community productivity

We assembled 12 phylogenetically and functionally diverse species isolated from compost into communities of varying diversity and measured their ability to degrade wheat straw. Similar to several previous studies, we found a positive relationship between the diversity of the community and their ability to grow on wheat straw. We found that this positive relationship was driven by the increased likelihood of diverse communities containing two highly active species, *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13. Furthermore, we identified that the ability of these species to drive community productivity was due to their ability to degrade key components of cellulose.

Chapter 4 - Lignocellulolytic bacteria Paenibacillus sp. A8 and Cellulomonas sp. D13 possess a wide range of carbohydrate active enzymes

In this chapter, we investigate the lignocellulolytic potential of *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 further by measuring their ability to degrade wheat straw in monoculture and in coculture and by obtaining whole genome sequences for both species. We found that while both species were able to degrade wheat straw, *Cellulomonas* sp. D13 was more efficient, achieving the same degree of wheat straw degradation in monoculture as the coculture. Genome sequencing revealed that both these strains possess a diverse range of cellulase and hemicellulase enzymes.

Chapter 5 – The influence of competing species and their ecoevolutionary responses on the rate and trajectory of focal species evolution

We next experimentally evolved a compost isolate, *Stenotrophomonas* sp. D12, to increase its ability to utilise wheat straw as a substrate. This focal species was evolved in monoculture and in the presence of a community of competing species. The rate of phenotypic evolution increased in the presence of competing species however autonomous growth rates of the populations was reduced. Ecoevolutionary adaptations of the competing species altered the evolutionary trajectory of the focal species' phenotypic traits. Specifically, the focal species that had evolved against fixed competitors (i.e. in the absence of ecoevolutionary adaptations of the competing community) exhibited an increased ability to utilise the more readily digestible labile substrates of lignocellulose. However, in the presence of ecoevolutionary adaptations by the competing species, the focal species evolved to better utilise the more recalcitrant components of lignocellulose. In addition, the ecoevolutionary responses of the competing communities increased the phenotypic divergence between the replicate focal species populations. Our results suggest that both the ecological and evolutionary responses of competing species drives the rate and trajectory of evolution to new environments.

Chapter 6 – Genetic adaptation of Stenotrophomonas sp. D12 to growth on wheat straw involved multiple regulatory pathways

We obtained whole genome sequences for 84 experimentally evolved clones of *Stenotrophomonas* sp. D12 from chapter 5. We identify parallel mutations in ten genes, the majority of which occurred in regulatory genes, which are likely to play a role in adaption to growth on wheat straw. Specifically, we identified several loci targeted by natural selection which are likely to play key roles in catabolite repression and carbon storage. These mutations potentially allow the utilisation of a more diverse range of sugars and are promising candidates for improving the productivity of industrial strains.

2   The isolation of lignocellulolytic bacteria from wheat straw compost

2.1   Abstract

Industry struggles to degrade lignocellulose in a cost and energy effective manner, but this process is efficiently achieved by a wide range of natural microbial communities. Identifying the species and enzymes involved in natural lignocellulose degradation could help to improve industrial processes. Here, we isolated a range of bacteria from wheat straw compost and measured the ability of these isolates to utilise cellulose and hemicellulose as substrates. Our results indicate that compost is a viable source of culturable, phylogenetically diverse bacteria with lignocellulolytic potential.

2.2   Introduction

Lignocellulose is a complex, recalcitrant polymer that is a promising substrate for biofuel production due to its abundance and low cost (Naik et al., 2010). While industry struggles to efficiently degrade lignocellulose in a cost-effective manner, it is a process achieved in nature by various microbial communities (Cragg et al., 2015). Understanding both the enzymes and species involved in natural lignocellulose degradation could improve the efficiency and reduce the cost barriers associated with lignocellulose degradation (Lynd et al., 2002).

Lignocellulose is composed primarily of cellulose, hemicellulose and lignin and has evolved various chemical and structural characteristics that provide plants with structural rigidity and protection from microbial attack and grazing (Himmel et al., 2007). Cellulose, the major constituent of plant material, is a polysaccharide composed of chains of between 500 and 14,000 ᴅ-glucose monomers linked by β-1,4-glycosidic bonds (Somerville, 2006). These chains are held together by hydrogen bonds to form crystalline, cable-like structures known as microfibrils, typically composed of 36 parallel glucan chains (Somerville, 2006). Hemicelluloses are a diverse group of short-chain, branched heteropolysaccharides characterised by β-1,4-linked sugar backbones with various side chains (Zhao et al., 2012). The predominant hemicellulose in wheat straw is xylan, a polysaccharide composed of a

β-1,4-linked xylose backbone with various side chains, commonly arabinose to form arabinoxylan (Scheller and Ulvskov, 2010). Lignin is a large, complex three-dimensional polymer composed of three phenyl propane monomers: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Buranov and Mazza, 2008). This aromatic polymer is the major contributor to lignocellulose recalcitrance as it covalently binds to hemicellulose, and to a lesser extent cellulose, to form complexes which prevent saccharification enzymes accessing the polysaccharides (Buranov and Mazza, 2008; Draude et al., 2001; Ishizawa et al., 2009).

As lignocellulose is the most abundant organic substrate on Earth, its degradation is a vital part of the carbon and nitrogen cycles. Degradation of this recalcitrant material is primarily achieved by microbial communities in various terrestrial and aquatic ecosystems including soil (Woo et al., 2014a), compost (Mello et al., 2016), the rumen (Cai et al., 2010) and aquatic sediments (Cortes-Tolalpa et al., 2016). Due to its complexity, lignocellulose degradation requires the combined action of multiple enzymes produced by various species present in these communities (Cragg et al., 2015). Release of monosaccharides from cellulose requires the synergistic action of both endo- and exo-glucanases and β-glucosidase (Lynd et al., 2002). Due to the heterogeneous structure of hemicellulose it is less resistant to degradation than cellulose, but complete depolymerisation requires the synergistic action of multiple enzymes including endoxylanases, endomannanases, xylosidases, glucosidases, arabinosidases, galactosidases, mannosidases and glucuronidases (Cobucci-Ponzano et al., 2015). These enzymes are classified into one of the 152 glycoside hydrolase (GH) families which can be found in the CAZyme database (www.cazy.org).

Lignin degradation is less common among microbial species and is therefore less well understood (Brown and Chang, 2014). Basidiomycete white-rot fungi are the most active lignin degraders studied to date. They utilise various heme peroxidases (lignin peroxidase, manganese peroxidase and versatile peroxidase), laccases and small molecular mediators to depolymerise lignin (Dashtban et al., 2010). Several bacterial enzymes have also been identified which play a role in lignin degradation including

dye-decolourising peroxidases (DyPs), laccases, β-esterases, superoxide dismutases and catalase-peroxidases (Brown and Chang, 2014; de Gonzalo et al., 2016). These enzymes are produced by various bacterial strains including members of Streptomyces, Rhodococcus, Microbacterium, Pseudomonas and Sphingobacterium genera (Taylor et al., 2012) and efforts are ongoing to fully characterise bacterial lignin degradation (Brown and Chang, 2014; de Gonzalo et al., 2016).

Both culture-dependent and culture-independent techniques are being employed to enhance our understanding of the species and enzymes involved in natural lignocellulose degradation. In recent years, metagenomic, metaproteomic and metatranscriptomic approaches have allowed microbial communities to be mined for enzymes with potential industrial applications (Cragg et al., 2015). Comparative transcriptomics allows identification of enzymes that are expressed under specific growth conditions, such as at high temperatures, that have potential roles in industrial processes (Simmons et al., 2014). While these methods have the benefit of identifying species and enzymes out with the culturable fraction of microbial communities, the complexity of these communities makes it difficult to fully understand the role individual species and enzymes play in lignocellulose degradation (Widder et al., 2016). Culture based methods have identified several microbial species with high lignocellulolytic activity (Gupta et al., 2012; Haitjema et al., 2014; Taylor et al., 2012). The functional potential of isolated microbes can be fully interrogated using a combination of sequencing and activity assays to provide a better understanding of how these species function. These isolated species can also be studied in communities in an effort to understand how interactions between species impact their functioning (Widder et al., 2016; Wongwilaiwalin et al., 2010).

One source of highly active lignocellulolytic microbes is compost (Wei et al., 2012). Composting is the process by which organic materials (e.g. food waste, plant material, crop residues) are degraded by a diverse microbial community to produce a nutrient rich material that can be used to improve the fertility of soil (Ryckeboer JR, 2003; Wei et al., 2012). These natural communities provide a phylogenetically and functionally diverse inoculum source for enrichment cultures. Enrichment culture

methods exploit the ability of microbial communities to adapt to both abiotic conditions and the substrate they are degrading (Wei et al., 2009). The substrate a community is adapted to significantly affects the phylogenetic and functional composition of the resulting community (de Lima Brossi et al., 2016; Wong et al., 2016). For example, Allgaier *et al.* (2010) found significant differences in species abundance before and after adaptation to switchgrass. Adaptation resulted in species sorting with some species increasing in abundance >20 fold, though evolutionary adaptation may have also occurred. Adaptation to particular substrates is likely due to the structural and compositional differences between lignocellulosic feedstocks which result in slight differences between the niche spaces available to microbes. Another important factor defining enrichment culture composition is the source of the original inoculum. Cortes-Tolalpa *et al.* (2016) initiated enrichment cultures with wheat straw as the substrates but with inoculum from three distinct sources. While each community had similar functional traits following enrichment, the phylogenetic composition of the communities varied depending on the source of the inoculum (Cortes-Tolalpa et al., 2016).

The aim here was to isolate lignocellulolytic bacterial strains which could be used to study the ecology and evolution of synthetic microbial communities when grown on wheat straw. We used wheat straw adapted compost as an inoculum to improve the likelihood of isolating species able to degrade this substrate. Bacteria were isolated from batch enrichment cultures, assayed for activity against cellulose and hemicellulose and active strains were identified by 16S rRNA gene sequencing. Although these culturable isolates will not represent the full diversity of the compost community, they do represent a functionally and phylogenetically diverse range of co-occurring lignocellulolytic bacteria with industrial potential.

2.3   Methods

2.3.1   Compost enrichment cultures

Wheat straw compost was homogenised in a blender and 7 g was used to inoculate 700 ml M9 media (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 19 mM NH_4Cl, 1 mM $MgSO_4$,

0.09 mM CaCl$_2$, 9 mM NaCl) containing 5% (w/v) wheat straw. Cultures were grown on an orbital shaker (150 rpm) at 30°C for eight weeks. Six replicate cultures were initiated, three of which were treated with 25 μg/ml cycloheximide with the aim of inhibiting fungal growth. This enrichment culture method will favour species able to grow at 30°C in a well aerated environment which are required characteristics for further experiments. Each week serial dilutions were prepared and spread onto nutrient agar, potato dextrose agar and M9 minimal media containing 1.5% (w/v) agar and 1% (w/v) milled wheat straw. Single colonies that appeared morphologically distinct on agar plates were assayed for activity against carboxymethylcellulose (CMC) and xylan (both from Sigma-Aldrich, Dorset, UK) using Congo red staining assays (Teather and Wood, 1982). Briefly, plates containing 0.2% (w/v) CMC or xylan in M9 media and 1.5% (w/v) agar were prepared. Bacterial isolates were grown overnight in nutrient agar and 10 μl was spot plated onto the CMC and xylan plates. Plates were incubated at 30°C overnight then flooded with 1 mg/ml Congo red in 5 mM NaOH for 15 minutes. The Congo red solution was washed off and plates were destained with 1 M NaCl in 5 mM NaOH for 30 minutes. 5% acetic acid was added to plates for 2-3 seconds to darken stains and plates were photographed. Clear halos indicate CMC or xylan degradation (Teather and Wood, 1982).

2.3.2 Identification of active isolates

Isolates that appeared to have xylanase and/or CMCase activity were identified using 16S rRNA gene sequencing with primers UniF 7f 5'-AGAGTTTGATYMTGGCTCAG-3' and UniR 1510r 5'-ACGGYTACCTTGTTACGACTT-3' (Weisburg et al., 1991). A single colony was added to 50 μl ddH$_2$O and incubated at 95°C for 5 minutes to lyse cells. 1 μl of this mixture was used as the source for template DNA. Each 25 μl PCR reaction contained 5 μl 5X Phusion HF buffer, 0.5 μl 10 mM dNTPs, 1.25 μl each primer, 1 μl template DNA and 16 μl dH$_2$O. PCR conditions were as follows: 98°C for 30s; 30 cycles of 98°C for 10s, 56°C for 30s, 72°C for 90s; 72°C for 5 minutes. The resulting PCR fragments were purified and sent for Sanger sequencing to GATC Biotech (Cologne, Germany). Forward and reverse strand sequences were aligned and used in BLAST analysis with the National Centre for Biotechnology Information database to identify similar 16S rDNA sequences.

2.3.3 Quantitative functional trait assays

To quantify the xylanase and cellulase activity of isolates their productivity was measured when grown on arabinoxylan (the most abundant hemicellulose in wheat straw) and β-glucan. Isolates were also grown on nutrient broth as a positive control. Isolates were grown for 48 hours in nutrient broth then cells were harvested, washed and suspended in M9 media. Cultures were diluted to an OD_{600} of 0.5 and 5 μl was used to inoculate 495 μl M9 media with 0.2% (w/v) arabinoxylan, 0.2% (w/v) β-glucan or nutrient broth. Productivity of cultures was measured using the MicroResp system similar to the method used by Lawrence et al. (2012) (Campbell et al., 2003). Briefly, each well in the deepwell plate is sealed to a microplate well containing indicator dye which changes colour in response to CO_2 concentration. Microplates containing indicator gel were replaced every 24h to prevent cultures becoming anaerobic. Community productivity was quantified as cumulative respiration . Specifically, cultures were grown for 3 days at 30°C and productivity was measured as the cumulative change in absorbance (λ=570 nm) of the indicator gel immediately before and after being sealed to deep well cultures plates. The change in OD of the indicator gel from control wells containing no inoculum was used to account for atmospheric CO_2 concentration.
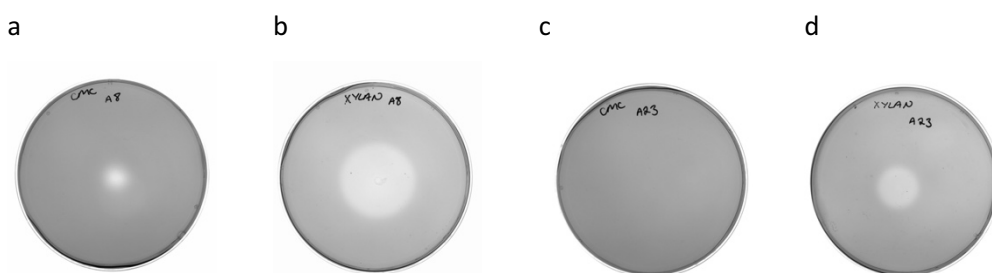
Figure 2.1 Representative qualitative cellulase and xylanase plate assays. 10 μl overnight culture spotted onto plates containing M9 minimal media, 1.5% agar and 0.2% CMC (a and c) or xylan (b and d). After 24h plates are stained with Congo red. Clear halos indicate CMC or xylan degradation. Shown are plates inoculated with Paenibacillus sp. A8 which can degrade both CMC (a) and xylan (b) and Luteimonas sp. A23 which can degrade xylan (d) but not CMC (c).

2.4 Results

2.4.1 Isolation and plate assays

One hundred and five colonies were isolated from the six enrichment cultures over eight weeks. Gram staining and microscopy revealed that 23 of these isolates were archaeal. The remaining isolates were assayed for cellulase and xylanase activity using plate assays which revealed that thirty-nine isolates appeared to be active on at least one of the CMC and xylan assay plates (Figure 2.1). Treating cultures with cycloheximide did not significantly increase the number of active bacterial strains isolated from cultures (21 from untreated, 18 from treated cultures). One active strain was isolated in week one, while eleven were isolated at week three suggesting cultures were efficiently enriched for bacterial strains able to degrade lignocellulose by this stage. The number of active strains isolated decreased after week three with only two active strains isolated in week eight. Colonies were isolated based on morphological differences and, as such, the reduction in the number of active strains isolated in the later stages of the experiment is likely due to colonies not being picked if they resembled already isolated strains rather than a reduction in the density of cellulolytic or xylanolytic strains.

2.4.2 Identification of active isolates

Active isolates were identified by 16S rRNA gene sequencing. Fifteen of the active isolates were from the Microbacterium genus and seven were Cellulomonas strains suggesting the enrichment and isolation process favoured these genera. Six isolates belonged to the Bacillus genus with an additional five belonging to the Paenibacillus genus. The additional species were identified as belonging to Cellulosimicrobium, Luteimonas, Paracoccus, Rheinheimera, Rhodococcus and Stenotrophomonas genera (Figure 2.3).

2.4.3 Quantitative functional traits

To gain a better understanding of isolates' metabolic functional traits and the diversity in metabolic functional traits between isolates of the same genera we used

quantitative growth assays with arabinoxylan or β-glucan as a sole carbon source. Linear models revealed significant variation between genera in terms of their growth on each substrate, although the relationship was stronger for β-glucan ($F_{9, 29}$ = 13.2 P < 0.0001, R^2 = 0.74) than arabinoxylan ($F_{2, 29}$ = 4.1, P < 0.01, R^2 = 0.43). Isolates belonging to the Paenibacillus, Cellulomonas and Bacillus genera (with the exception of two isolates) were able to utilise β-glucan efficiently while the remaining strains achieved only low productivity on this substrate (Figure 2.2). The ability to utilise arabinoxylan was more common than β-glucan utilisation which is unsurprising due to the labile nature of this polysaccharide. *Paenibacillus* strains A12 and E3 achieved the highest productivity while *Rheinheimera* sp. D14A was the least efficient on both substrates. These functional trait assays confirmed that a functionally diverse range of lignocellulolytic isolates was successfully isolated.
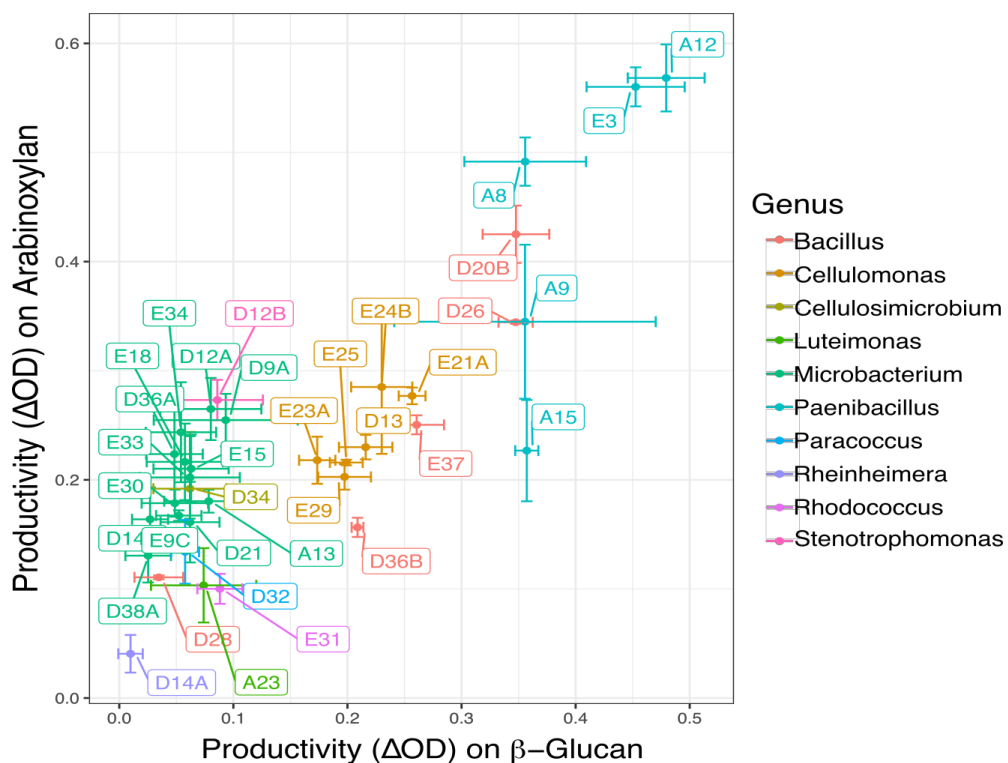
Figure 2.2 Productivity of isolates in M9 minimal media with 0.2% β-glucan (x-axis) or arabinoxylan (y-axis). Productivity is cumulative change in OD of MicroResp indicator plates three days as a measure of CO_2 production, points indicate mean of three reps with standard error represented by error bars.
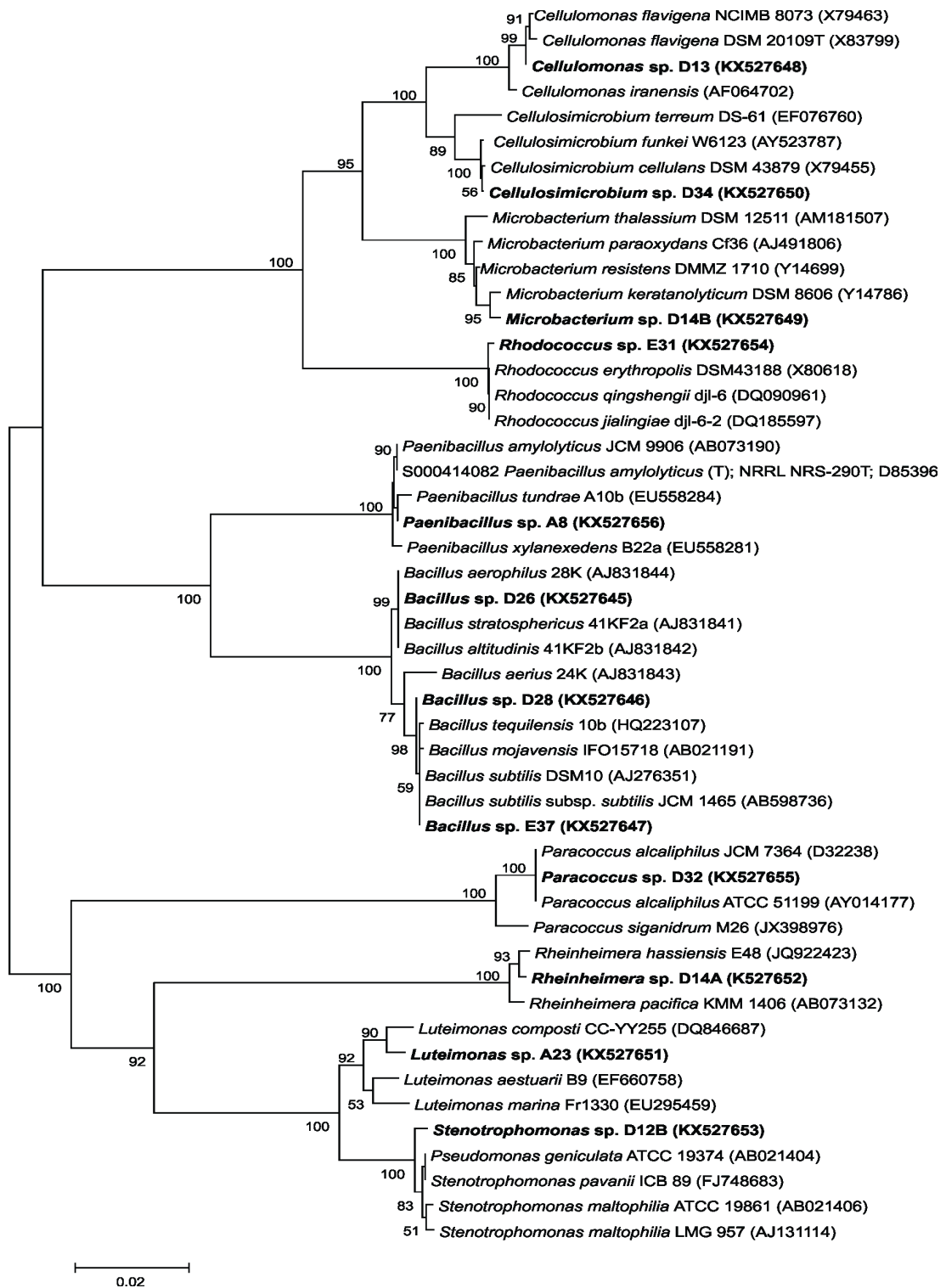
Figure 2.3 Neighbour-joining phylogenetic tree based on bacterial 16S rRNA gene partial sequences. Sequences were aligned using the SILVA Incremental Aligner (SINA) and analysed by MEGA6. Isolates from this study are highlighted in bold with accession numbers provided in brackets. Bootstrap values representing percentage of 1000 replicates are shown at nodes. If isolates had 100% 16S rRNA sequence identity, only a representative is shown (i.e. Microbacterium).

2.5 Discussion

This work aimed to isolate a selection of phylogenetically and functionally diverse bacterial strains from wheat straw compost. Enrichment cultures were initiated with wheat straw as a carbon source and wheat straw adapted compost as an inoculum. Thirty-nine bacterial isolates, belonging to ten genera, with the ability to degrade CMC and/or xylan were isolated.

The bacterial species isolated here do not represent the full diversity of the natural microbial community present in compost. Enrichment culture conditions selected for bacterial species able to grow at 30°C in well-aerated conditions which are required characteristics for future planned experiments. Although these conditions would have selected against anaerobic species, it has been reported that 90-95% of cellulose degradation in nature is achieved aerobically (Carere et al., 2008). In addition, metagenomics has revealed Cellulomonas, Cellulosimicrobium and Stenotrophomonas among the dominant genera in lignocellulose degrading communities growing on poplar wood or xylan (Carlos et al., 2018) and 16S amplicon sequencing found Paenibacillus and Cellulomonas to be among the most dominant genera in sugarcane bagasse compost (Mello et al., 2016). This suggests that although the full diversity of the compost community has not been captured, we have successfully isolated a phylogenetically diverse range of species that are abundant in these natural communities and representative of the ecologically important taxa for lignocellulose degradation in natural communities.

Quantitative growth assays on β-glucan and arabinoxylan revealed that the most productive strains on these substrates belonged to the Paenibacillus genus. Strains of this genus have been isolated from pulp mill waste (Mathews et al., 2016) and soil (Puentes-Téllez and Salles, 2018; Woo et al., 2017) and studied for various functions including nitrogen fixation, phosphate solubilisation (Weselowski et al., 2016) and lignocellulose degradation (Mathews et al., 2016). Interestingly, strains of this genera have been shown to be capable of degrading lignin in addition to cellulose and hemicellulose however ligninolytic potential was not measured here (Mathews et al., 2016).

Four of the six Bacillus strains grew well on both β-glucan and arabinoxylan. *Bacillus subtilis* is a promising candidate for CBP as there are several very well characterised strains, it can utilise both pentose and hexose sugars and it is already established as an industrial strain (Zhang and Zhang, 2010). While most common laboratory strains of Bacillus are not cellulolytic, various environmental strains are and these may provide a source of enzymes to enhance the lignocellulolytic ability of industrial strains (Amore et al., 2013a). The Bacillus strains isolated here are culturable and grew well on both cellulose (β-glucan) and hemicellulose (xylan) making them promising targets for the discovery of cellulases and hemicellulases.

Cellulomonas is a well-known genus of cellulose degrading bacteria that has been studied for more than 40 years (Han and Srinivasan, 1968). The genome sequences of *Cellulomonas fimi* ATCC 484 and *Cellulomonas flavigena* ATCC 482 revealed 89 and 76 GHs respectively (Christopherson et al., 2013) and efforts are ongoing to understand the functions of these enzymes and the conditions under which they are expressed (Wakarchuk et al., 2016). Consistent with previous work investigating the functional traits of members of the Cellulomonas genus, the strains isolated here appeared to be highly active on cellulose and were also able to utilise xylan as a sole substrate.

Fifteen (39%) of the active isolates were identified as members of the Microbacterium genus suggesting the isolation protocol favoured these strains. Microbacterium strains have previously been shown to have high cellulolytic and xylanolytic activities (Okeke and Lu, 2011) and some strains are able to depolymerise Kraft lignin (Taylor et al., 2012) though this was not measured here.

Stenotrophomonas has been identified in several lignocellulose degrading communities (Montella et al., 2017; Puentes-Téllez and Salles, 2018; Qi et al., 2011). Using metatranscriptomic analysis to study a highly active five species consortium, Jiménez et al. (2018) found that three species, including one Paenibacillus strain, were active during the initial stages of the culture while Stenotrophomonas was

more active (higher expression of RNA polymerase *rpoA*) towards the end of the culture without significantly contributing to lignocellulolytic enzyme production. The authors of that study suggested that Stenotrophomonas may be acting as a 'sugar-cheat', i.e. it benefits from the release of sugars by other taxa without contributing to lignocellulose degradation itself (Jiménez et al., 2018b). Alternatively, Montella et al. (2017) found that Stenotrophomonas was a dominant strain in natural energy crop degradation and also contributed a significant proportion of the GHs identified by metagenomics. Identifying the conditions under which Stenotrophomonas strains express GH enzymes may help to understand the role this species plays in lignocellulose degradation. Stenotrophomonas strains are metabolically diverse and can grow well under a wide variety of environments conditions and so they are also under investigation for their potential industrial applications (Mukherjee and Roy, 2016).

Rhodococcus strains have been well researched for their role in bioremediation as they are able to metabolise a wide range of hydrocarbons (Alvarez et al., 2017) including lignin (Mahan et al., 2017). *Cellulosimicrobium cellulans* is a recognised lignocellulose degrading bacterium and has been studied for its ability to produce bioflocculants directly from lignocellulose (Liu et al., 2015). The lignocellulolytic capability of *Paracoccus*, *Rheinheimera* and *Luteimonas* species has not been well studied. Cellulolytic *Paracoccus* strains have been isolated from the termite gut (Ferbiyanto et al., 2015) and salt marshes (Deng and Wang, 2016) while *Luteimonas* strains have been shown to degrade cellulose and have the enzymes necessary for xylan degradation (Zhang et al., 2015). To the best of my knowledge, *Rheinheimera* has not previously been isolated from lignocellulolytic communities. This is perhaps unsurprising as it achieved the lowest productivity on both xylan and β-glucan growth assays and produced only a faint halo on xylan assay plates. However, *Rheinheimera* strains have been studied in recent years as potential industrial laccase producers (Sharma et al., 2017). Laccase is involved in bacterial lignin degradation and may improve the overall efficiency of converting lignocellulose to ethanol (Moreno et al., 2016).

In conclusion, a functionally and phylogenetically diverse range of bacterial strains have been isolated from wheat straw compost enrichment cultures. While the enrichment culture conditions appear to have favoured strains well adapted to the isolation conditions, several of the strains isolated have previously been identified as playing a significant and ecologically important role in lignocellulose degrading communities.

3 Defining the functional traits that drive lignocellulose degrading community productivity

This chapter is adapted from the published article: Evans, R., Alessi, A.M., Bird, S., McQueen-Mason, S.J., Bruce, N.C., and Brockhurst, M.A. (2017). Defining the functional traits that drive bacterial decomposer community productivity. ISME J. 11, 1680–1687.

3.1 Abstract

Microbial communities are essential to a wide range of ecologically and industrially important processes. To control or predict how these communities function, we require a better understanding of the factors which influence microbial community productivity. Here, we combine functional resource use assays with a biodiversity-ecosystem functioning (BEF) experiment to determine whether the functional traits of constituent species can be used to predict community productivity. We quantified the abilities of 12 bacterial species to metabolise components of lignocellulose and then assembled these species into communities of varying diversity and composition to measure their productivity growing on lignocellulose, a complex natural substrate. A positive relationship between diversity and community productivity was caused by a selection effect whereby more diverse communities were more likely to contain two species that significantly improved community productivity. Analysis of functional traits revealed that the observed selection effect was primarily driven by the abilities of these species to degrade β-glucan. Our results indicate that by identifying the key functional traits underlying microbial community productivity we could improve industrial bioprocessing of complex natural substrates.

3.2 Introduction

Microbial communities underpin the functioning of natural ecosystems (Soliveres et al., 2016) and the efficiency of a wide range of industrial bioprocesses (e.g. waste bioreactors) (Cydzik-Kwiatkowska and Zielińska, 2016; Widder et al., 2016). The form of the biodiversity-ecosystem functioning (BEF) relationship is therefore an important property of microbial communities both in nature and the simpler

communities used in a range of industrial bioprocesses. Several studies have identified positive BEF relationships for microbial community productivity (Bell et al., 2005; Gravel et al., 2011), stability (Awasthi et al., 2014), micropollutant degradation (Johnson et al., 2015) and resistance to invasion (Elsas et al., 2012), suggesting that for a range of functions microbial community performance improves with increasing species richness. Positive BEF relationships can arise via the complementarity effect, whereby diverse communities use more of the available resource space through niche differentiation or facilitation (Salles et al., 2009; Singh et al., 2015), or the selection effect (also termed the sampling effect), whereby diverse communities are more likely to contain species which have a large impact on community functioning (Awasthi et al., 2014; Hooper et al., 2005; Langenheder et al., 2010, 2012). Both complementarity and selection effects depend on the functional traits of constituent species and several studies have now shown functional diversity to be a better predictor of community function than phylogenetic diversity (Krause et al., 2014; Mokany et al., 2008; Salles et al., 2009). However, for many ecologically and biotechnologically important microbial communities it is still unclear how the functional traits of individual species scale-up to determine the performance of a diverse community.

One of the most important ecosystem functions microbial communities perform is the decomposition of plant material and subsequent nutrient cycling (McGuire and Treseder, 2010; Van Der Heijden et al., 2008). Understanding how natural microbial communities achieve efficient lignocellulose degradation could inform both the prediction of nutrient cycling in natural systems and the design of efficient microbial communities for industrial processes (Wei et al., 2012). Both biodiversity and the presence of certain species have been shown to influence the rate of decomposition by bacterial communities (Bell et al., 2005; Bonkowski and Roy, 2005; Langenheder et al., 2012) but the mechanisms which determine community decomposition performance remain poorly understood (McGuire and Treseder, 2010). A key question therefore is to what extent community functioning is predictable from the combined functional traits of constituent species?

Using culturable bacterial strains isolated from compost we performed a random partition design BEF experiment (Bell et al. 2009) to test the contributions of species richness and composition to productivity of communities when grown on wheat straw. Although using only the culturable fraction of the community is likely to overlook some functionally important species in the natural community, culturability is a key feature of microbes that could feasibly be used in industrial bioprocessing. Next, we tested how the functional traits of individual species shaped the productivity of these communities to determine the extent to which community productivity was predictable from the functional traits of the constituent species and to determine the contribution of each functional trait to overall productivity. We quantified the functional resource use traits of each species by their ability to utilise a range of known components of lignocellulose (i.e. cellulose, hemicellulose, pectin and lignin).

3.3   Methods

3.3.1   Bacterial isolates

Bacterial strains used in this study were isolated as described in Chapter 2.2.1. The twelve species included in this study were chosen as they represent phylogenetic or functional diversity based on 16S rRNA sequences (Figure 2.3) and growth assays (Figure 2.2).

3.3.2   Biodiversity ecosystem functioning experiment

Communities for the BEF experiment were designed using the random partition design described by Bell et al. (2009). Species were randomly divided into communities with species richness levels of 1, 2, 3, 4, 6 and 12 species with each isolate represented an equal number of times at each richness level. This process was repeated to produce 12 monocultures, 66 two-isolate communities, 58 three-isolate communities, 63 four-isolate communities, 68 six-isolate communities and one twelve isolate community. Each community was replicated five times to give a total of 1340 communities. The twelve species were grown for two days in 5 ml nutrient broth on an orbital shaker (150rpm) at 30°C. Cultures were harvested by

centrifugation, washed and suspended in M9 minimal media and left at room temperature for 2h to metabolise remaining nutrients before OD600 was standardised to 0.1 to ensure similar starting densities. Deep well plates containing 380 μl M9 minimal media with 1% (w/v) milled wheat straw per well were inoculated with a total of 120 μl cultures, e.g. monocultures were inoculated with 120 μl single species culture whereas the 12-species community was inoculated with 10 μl of each culture. The MicroResp system was used to measure respiration of cultures (Campbell et al., 2003). Briefly, each well in the deepwell plate is sealed to a microplate well containing indicator dye which changes colour in response to CO_2 concentration. Microplates containing indicator gel were replaced every 24h to prevent cultures becoming anaerobic. Community productivity was estimated as cumulative respiration (Armitage, 2016; Tiunov and Scheu, 2005). Specifically, cultures were grown for 7 days at 30°C and productivity was measured as the cumulative change in absorbance (λ=595nm) of the indicator gel immediately before and after being sealed to deep well cultures plates. The change in OD of the indicator gel from control wells containing no inoculum was used to account for atmospheric CO^2 concentration. Note that due to the presence of particles of wheat straw in the growth medium it was not possible to measure change in microbial biomass by absorbance.

3.3.3 Functional trait assays

To quantify the fundamental niche of each species, growth assays were performed on several polysaccharides present in lignocellulose. Hemicellulose substrates included xylan (Sigma-Aldrich), arabinoxylan (P-WAXYL, Megazyme, Bray, Ireland) and galactomannan (P-GALML, Megazyme); cellulose substrates included β-glucan (P-BGBL, Megazyme) and Whatman filter paper; additional substrates included pectin (Sigma-Aldrich) and Kraft lignin (Sigma-Aldrich). Cultures were prepared as described for the BEF experiment. These cultures (5 μl) were used to inoculate 495 μl of M9 minimal media with 0.2% (w/v) of each carbon source or one 6mm sterile filter paper disc in 96-well deepwell plates. Cultures were replicated six times and several blank wells containing no inoculum were included as negative controls.

Cultures were grown for 7 days at 30°C and the MicroResp system was used to measure culture respiration as described above.

3.3.4 Statistical analysis

The biodiversity and ecosystem functioning relationship was analysed using the linear model method described by Bell et al. (2009). The species coefficients provided by this method give a measure of the effect of each species on community productivity relative to an average species: values of >1 indicate an above average contribution while values of <1 indicate a below average contribution to community productivity. To assess the effect of *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 on community productivity, communities containing both species, *Paenibacillus* sp. A8 only, *Cellulomonas* sp. D13 only or neither of these species were compared using analysis of variance (ANOVA) followed by post hoc Tukey tests. Linear models were used to compare the ability of species richness and the presence or absence of *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 to predict community productivity.

To standardise measures of functional traits across diverse substrates, performance on each substrate was normalised by dividing by the maximum observed respiration on that substrate. For each bacterial isolate we can then calculate its fundamental niche (along the carbon degradation axis) by summing performance on all substrates. To estimate the niche space of each community we used the community niche (CN) metric described by Salles et al. (2009), which sums the maximal performance on each substrate: $CN = \sum_{i=1}^{7} \max_{j=1}^{n}(P_{ij})$, where Pij is the performance of species j on carbon source i and n is the number of species in each community.

The ability of each functional trait to predict community productivity was analysed by summing performance of all species in a community on each carbon source to give a measure of the total fundamental niche space of that community. To approximate the realised niche space of communities we also assessed the ability of the maximum performance on each carbon source in a community to predict community productivity; this metric assumes that the species best able to grow on a given carbon source in a community dominates consumption of that carbon source providing a

conservative estimate of realised niche. Linear regressions were used to analyse how well CN and functional trait performance predicted community productivity. It is important to note that because all species can grow on several carbon sources, summing functional trait use may act as a proxy of species richness. To control for this effect, we analysed whether summed community functional traits remained significant when fitted to the residuals of the species richness model (i.e. community productivity predicted by species richness). Competing models were compared using the Akaike information criterion (AIC).

3.4   Results

3.4.1   Biodiversity-ecosystem function relationship

We observed a positive relationship between species richness and community productivity ($F_{1, 264}$ = 60.1, P < 0.001, Figure 3.1A) with species richness explaining 19% of variation in productivity. As highlighted by the variance in productivity within species richness levels, species identity also had a significant effect on community productivity ($F_{12, 254}$ = 45.3, P < 0.001). The linear model coefficient for each species provides the estimated contribution of that species to community productivity relative to an average species (Bell et al., 2009). Two species, *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13, made significantly greater contributions to community function relative to an average species ($F_{1, 254}$ = 73.1, P < 0.001 and $F_{1, 254}$ = 256.3, P < 0.001 respectively, Figure 3.1B). Of the remaining species, the contribution of *Rheinheimera* sp. D14A and *Stenotrophomonas* sp. D12, did not significantly differ from the average species while the remaining eight species made significantly below average contributions to community functioning (Figure 3.1B).

To further investigate the effects of *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13, the productivity of communities containing either one, both or neither of these species was compared. Communities that contained both *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 were significantly more productive than communities containing either one or neither of these species (post-hoc Tukey tests, P < 0.001, Figure 3.1A). The productivity of communities containing both *Paenibacillus* sp. A8

and *Cellulomonas* sp. D13 did not significantly differ across species richness levels suggesting additional species within these communities are not contributing to community productivity ($F_{1,\,28}$ = 0.42, *P* > 0.05, green line Figure 3.1b). Communities containing only *Cellulomonas* sp. D13 were more productive than those containing only *Paenibacillus* sp. A8 (post-hoc Tukey test, *P* < 0.001), while communities which did not contain these species were significantly less productive than communities containing either one of these species (post-hoc Tukey test, *P* < 0.001). These results indicate that the positive BEF relationship is predominantly driven by the selection effect, i.e. more diverse communities are more likely to contain the highly performing species *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 and are therefore more productive.
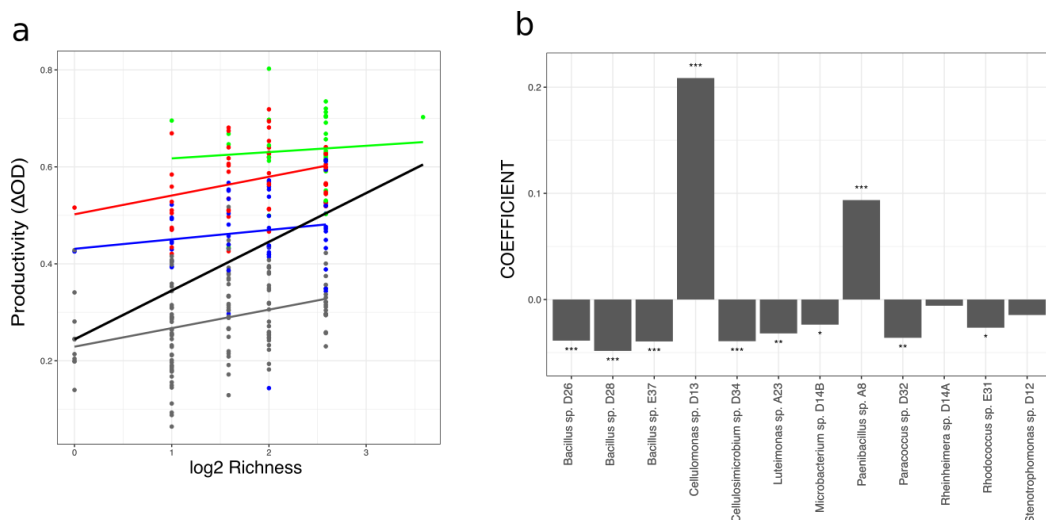
Figure 3.1 a) Relationship between community productivity and species richness. Black line shows linear regression for all data points ($F_{1,\,264}$ = 60.1, R^2 = 0.19, P < 0.001). Each point is the mean productivity of five replicate communities. Green points represent communities containing both *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 ($F_{1,\,28}$ = 0.42, P > 0.05); red points represent communities containing *Cellulomonas* sp. D13 ($F_{1,\,50}$ = 4.43, P < 0.05); blue points represent communities containing *Paenibacillus* sp. A8 ($F_{1,\,50}$ = 1.01, P > 0.05); grey points represent communities containing neither of these species ($F_{1,\,129}$ = 60.1, P < 0.001). b) Linear model coefficients for each species in the BEF experiment. Positive or negative coefficients indicate species contribute more or less to community productivity than an average species (Bell et al., 2009).

3.4.2 Quantification of functional traits

To determine if differences in productivity could be explained by the functional traits of species we assayed the ability of species to utilise various components of lignocellulose. All species were able to grow to varying degrees on the labile

substrates, hemicellulose (xylan, arabinoxylan and galactomannan) and pectin, whereas growth on recalcitrant substrates (β-glucan, filter paper and lignin) was less universal (Figure 3.2). This pattern is consistent with the hypothesis that functional groups that degrade recalcitrant substrates are not as common as those that degrade labile substrates (Schimel and Gulledge, 1998; Waldrop and Firestone, 2004). A linear model revealed significant main effects of both species ($F_{11, 336}$ = 30.3, P < 0.001) and carbon source ($F_{6, 336}$ = 105.8, P < 0.001) on productivity and a significant interaction between these factors ($F_{66, 336}$ = 6.8, P < 0.001), suggesting niche differentiation in resource use among the species. It is notable that some species, in particular *Rhodococcus* sp. E31, displayed generalist resource use, being able to grow on recalcitrant substrates like lignin as well as on the more labile substrates.
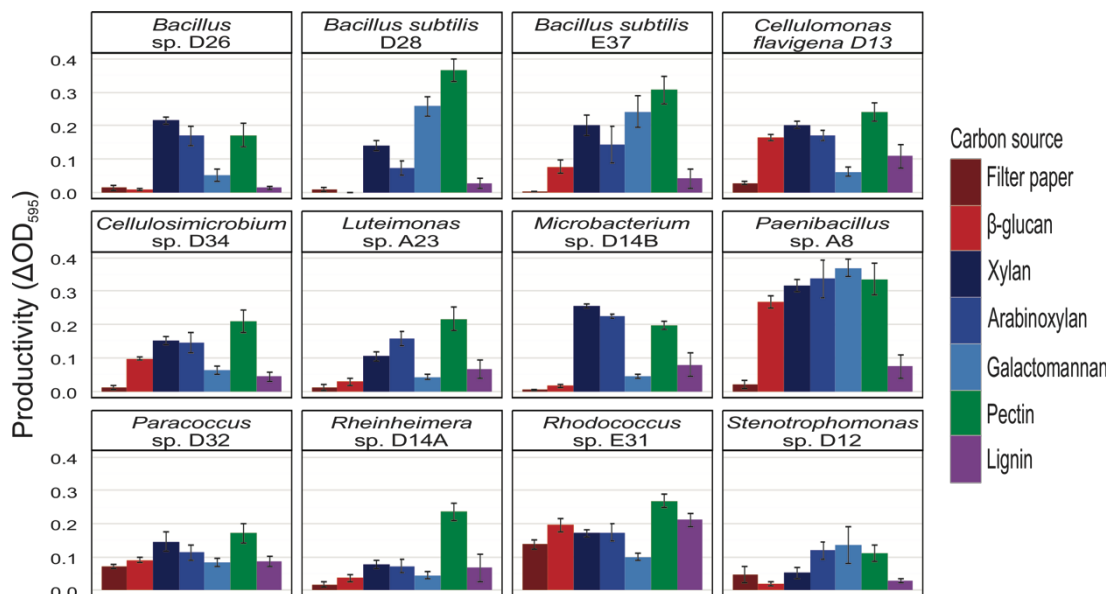
Figure 3.2 Productivity of species grown on each carbon source. Filter paper and β-glucan represent cellulose like substrates (red); xylan, arabinoxylan and galactomannan represent hemicelluloses (blue). Productivity is measured as the cumulative change in OD of MicroResp indicator plates over 7 days.

3.4.3 Community productivity and functional traits

To determine if the functional niche of communities could be used to predict productivity we calculated community niche as described by Salles et al. (2009). This index sums the maximum growth achieved by a constituent species on each substrate. We found a significant positive relationship between community niche and community productivity ($F_{1, 264}$ = 73.31, P < 0.001, Figure 3.3A). Similar to the

results of Salles et al. (2009), community niche explained more variation in community productivity than species richness (22% and 19% respectively).
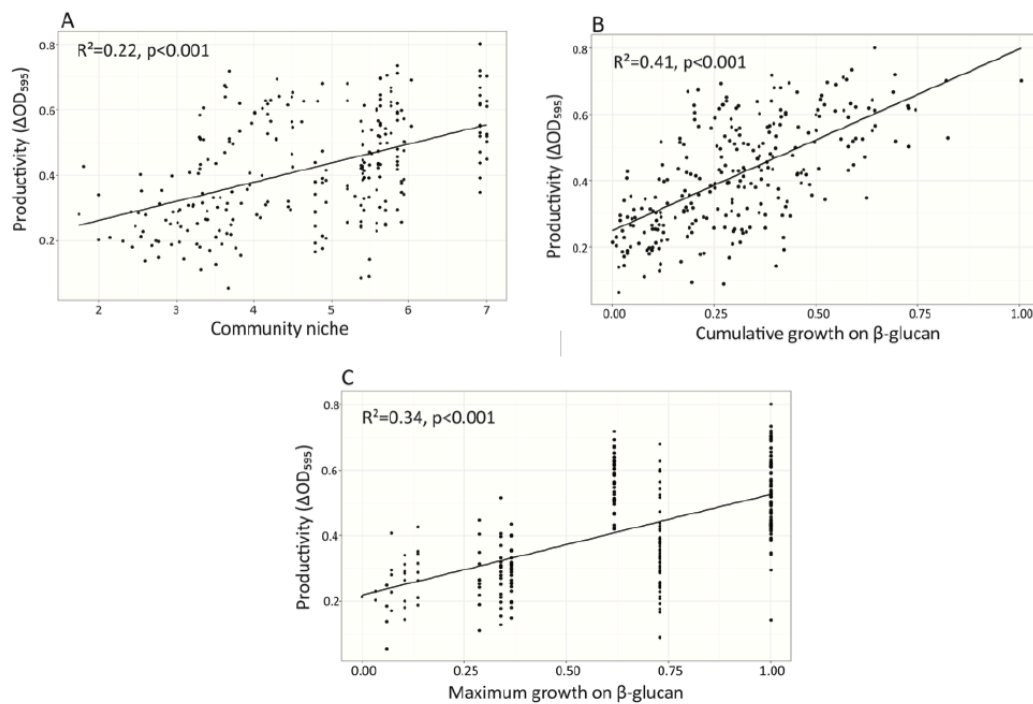
Figure 3.3 Relationship between community productivity and (A) community niche, (B) cumulative ability of constituent species to utilise β-glucan and (C) maximum ability of constituent species to utilise β-glucan. Higher community niche indicates communities can utilise more resources more efficiently. The ability of constituent species to utilise β-glucan was calculated from their ability to grow on this substrate in functional trait assays (Figure 3.2). Each point represents the mean productivity of five replicate communities.

When calculating community niche, each functional trait is weighted equally despite differences in the abundances of substrates in wheat straw lignocellulose, e.g. cellulose constitutes 40-50% whereas pectin only constitutes 1-2%. To determine which functional traits were important for predicting community productivity we summed the growth of constituent species on each carbon source used in functional trait assays to calculate the total fundamental niche of that community. The summed activity on β-glucan had a significant positive relationship with productivity ($F_{1, 264} = 182.7$, $P < 0.001$) and was the best predictor of community productivity, explaining 41% of variation (Figure 3.3B). The ability to utilise arabinoxylan and xylan also had significant positive relationships with productivity ($F_{1, 264} = 105.8$, $P < 0.001$ and $F_{1, 264}$

= 98.6, $P < 0.001$ respectively), explaining 29% and 27% of variation respectively. There were significant positive relationships between the remaining carbon sources and community productivity though these explained less variation than community richness and were not significant when species richness was included in models. The fundamental niche space of community is unlikely to be achieved due to interactions between species such as competition for resources. Therefore, to approximate the realised niche space of each community we also analysed the maximum performance per carbon source in a community. Consistent with the analysis of summed performance, maximum performance on β-glucan, arabinoxylan and xylan had significant positive relationships with productivity ($F_{1, 264} = 134.8$, $P < 0.001$, $F_{1, 264} = 76.2$, $P < 0.001$ and $F_{1, 264} = 74.5$, $P < 0.001$ respectively, Supplementary Figure 3.1) explaining 34%, 23% and 22% of variation respectively. There were significant positive relationships between the maximum performance on lignin ($F_{1, 264} = 7.4$, p<0.01), pectin ($F_{1, 264} = 20.8$, p<0.001) and galactomannan ($F_{1, 264} = 47.1$, p<0.001) and community productivity though these explained less variation than community richness. There was no significant relationship between the maximum ability to degrade filter paper and community productivity. This suggests that identifying and measuring key functional traits could be a better predictor of community productivity than either species richness or community niche.

3.5 Discussion

Understanding the factors that influence microbial community productivity has potentially important ecological and industrial applications (Widder et al., 2016). The ability of community niche to predict functioning in well-defined media has been demonstrated previously (Salles et al., 2009). Here, we define for communities growing in complex undefined media, the key functional resource use traits that predict decomposer community productivity. Crucially, functional resource use traits explained more variation in productivity than either species richness or measures of community niche. Indeed, a single function, the ability to degrade β-glucan, explained a larger proportion of variation than community niche. This key functional

trait was shared by two dominant strains which were shown to significantly increase the productivity of communities.

As with several previous BEF studies (Awasthi et al., 2014; Bell et al., 2009; Gravel et al., 2011), we identified a positive relationship between species richness and community productivity. By analysing the effect of community composition, we found that the presence of two highly functioning species, *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13, significantly increased community productivity suggesting this positive BEF relationship is driven by the selection effect. To determine if the dominance of these two species could be explained by their functional traits, we compared the ability of these species to utilise the various carbon sources used in functional trait assays to the other species. With the exception of *Rhodococcus* sp. E31, *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 were the highest performing species on β-glucan (Figure 3.2). The ability to utilise β-glucan may suggest these species are able to metabolise the cellulose portion of wheat straw in addition to the more labile hemicellulose and pectin fractions. Interestingly, when the productivity of communities containing either one, both or neither of these species is compared across each day of the experiment (Figure 3.4), it is noticeable that communities containing neither of these species have very low productivity during the later days of the experiment. A possible explanation is that easily-accessible labile substrates are being used within the first two days of growth after which only recalcitrant and inaccessible substrates remain. The ability to degrade cellulose would allow *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 to maintain higher levels of growth when labile substrates become depleted.

Interestingly, *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 have similar functional traits which would indicate they occupy overlapping niche space and may be in direct competition with each other. However, communities containing both these species were significantly more productive than communities containing only one or neither suggesting complementarity or facilitation effect between these species, i.e. they are able to exploit a wider niche space when grown together potentially because they each produce enzymes or by-products that improve the overall community

productivity. Wohl et al. (2004) found a similar result whereby functionally redundant cellulose degrading bacteria were more productive in communities than in monoculture.
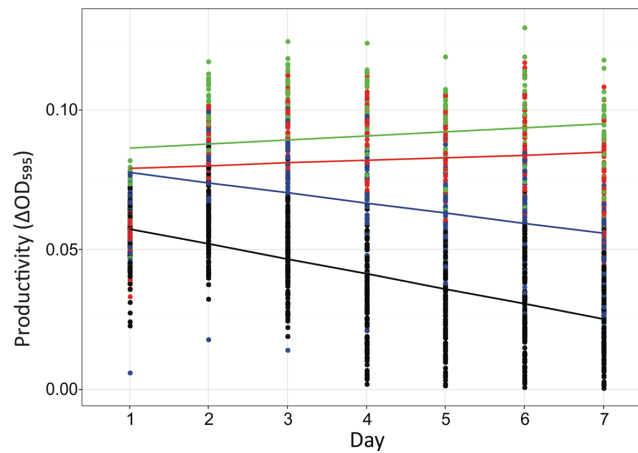
Figure 3.4 Productivity of communities on each day of the BEF experiment. Points represent mean of five replicate communities and are coloured by the presence of *Paenibacillus* sp. A8 (blue), *Cellulomonas* sp. D13 (red), both these species (green) or neither of these species (black). Productivity is the change in OD595 of MicroResp indicator plates after 24h.

The ability of species within communities to utilise β-glucan was a better predictor of community productivity than measures of community niche or species richness. The significance of this activity is consistent with the composition of wheat straw lignocellulose, which is made up of 40-50% cellulose. Interestingly, functional trait assays revealed that *Rhodococcus* sp. E31 achieved the second highest growth on β-glucan but this species did not significantly increase community productivity compared to an average species. In addition, *Rhodococcus* sp. E31 was able to utilise lignin as well as the more labile hemicellulose substrates (Figure 3.2). It might have reasonably been expected that as lignin is the major contributing factor to recalcitrance, species able to degrade it would increase community productivity by increasing accessibility of saccharification enzymes to cellulose. The limited contribution of *Rhodococcus* sp. E31 to community productivity may be explained in part by structural differences between Kraft lignin used in functional trait assays and native lignin present in lignocellulose (Vishtal and Kraslawski, 2011). Alternatively, although able to achieve efficient degradation of all substrates in monoculture growth assays, *Rhodococcus* sp. E31 may be outcompeted in communities and

unable to achieve the functional potentials revealed by trait assays. Recalcitrant substrates may require more energy expensive breakdown pathways than labile substrates (Lynd et al., 2002) which may put species that are specialised to degrade such substrates, e.g. *Rhodococcus* sp. E31, at a competitive disadvantage in communities. Measuring the abundance of species in each community would allow us to better determine the functional traits present in communities assuming that enzyme expression does not differ between monoculture and communities. Alternatively, it may be possible to match functional traits to community productivity by comparing the transcriptome and proteome of focal communities, although any such approach is necessarily limited by the correct annotation of functional genes and/or proteins.

Rivett et al. (2016) found that the ability of species to degrade labile resources could be explained by metabolic plasticity whereas the ability to degrade more recalcitrant substrates required evolutionary adaptation. Species best adapted to utilise the accessible labile substrates may be able to dominate communities during initial growth stages, but as labile substrates become depleted, species able to adapt to utilise the remaining recalcitrant substrates will become more dominant in communities. When comparing the contribution of species across each day of the BEF experiment, we found that the contribution of species did not noticeably differ throughout the seven days of growth. *Paenibacillus* sp. A8 significantly improved community productivity relative to the average species on each day while *Cellulomonas* sp. D13 made a significantly higher contribution than the average species from day two onwards. The presence of *Rheinheimera* sp. D14A made a significantly above average contribution to community productivity on day one of the experiment, though for the remaining six days the contribution of this species did not significantly differ from that of an average species. Of the remaining 9 species, contributions remained lower than or did not significantly differ from the average species throughout the 7 days. The ability of *Cellulomonas* sp. D13 and *Paenibacillus* sp. A8 to efficiently degrade both recalcitrant and labile substrates may allow them to outcompete other species before they are able to adapt to utilise recalcitrant substrates. Allowing the species used here a period of evolutionary adaptation to the

wheat straw substrate may increase their ability to degrade recalcitrant substrates and alter the dominance hierarchy within these communities and is an interesting topic for future study.

In conclusion, we have identified key functional traits that define the productivity of communities degrading lignocellulose. We found that the degradative abilities of communities against β-glucan, arabinoxylan and xylan were able to predict community productivity more effectively than either measures of community niche or species richness. Furthermore, we found that two species, *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13, made greater than average contributions to community productivity suggesting a key role for the selection effect in driving the observed positive BEF relationship. Our results suggest that, using simple experiments, it is possible to identify the important functional traits and species that drive microbial community productivity on complex natural substrates like wheat straw, potentially simplifying efforts to predict the functioning of natural communities and the assembly of highly performing communities for biotechnological industrial applications.

3.6   Supplementary figures
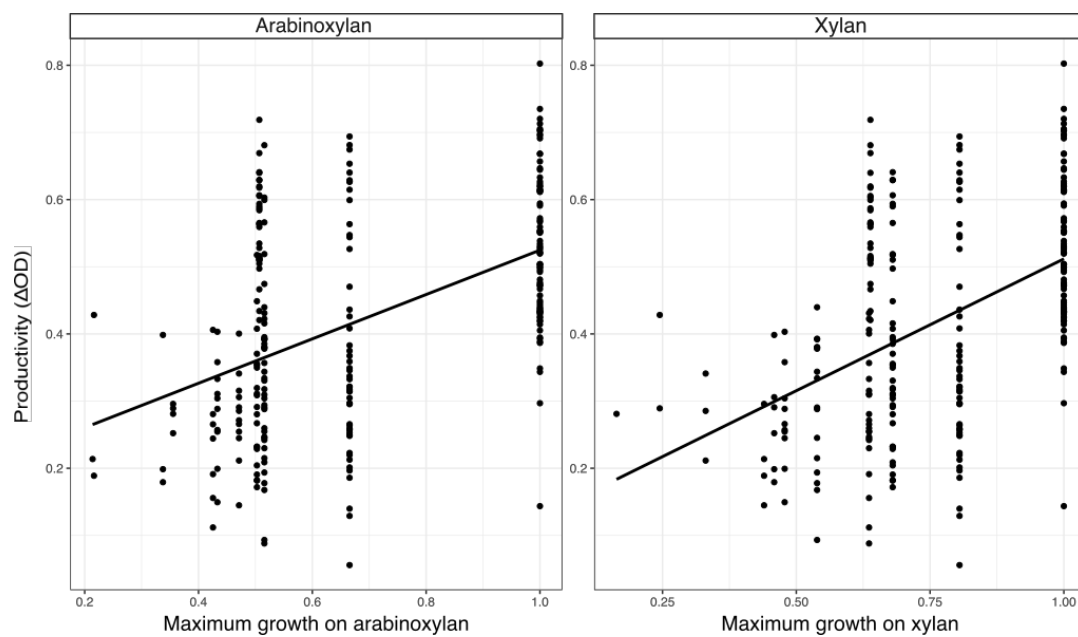
Supplementary Figure 3.1 Relationship between community productivity and maximum ability of constituent species to utilise arabinoxylan (left) and xylan (right). The ability of constituent species to utilise arabinoxylan and xylan was calculated from their ability to grow on these substrates in functional trait assays (Figure 3.2). Each point represents the mean productivity of five replicate communities.

4 Lignocellulolytic bacteria *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 possess a wide range of carbohydrate active enzymes

4.1 Abstract

A promising solution to tackle the problem of lignocellulose recalcitrance is to identify the microorganisms and enzymes which achieve efficient lignocellulosic degradation in nature. In the previous chapter we identified two species, *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 which significantly improved the productivity of lignocellulose degrading microbial communities. Here, we measure the ability of these species to degrade wheat straw lignocellulose in monoculture and in coculture. In addition, we use genome sequencing to identify the carbohydrate active enzymes encoded by these species. We found that while both species were able to degrade wheat straw, *Cellulomonas* sp. D13 was more efficient and achieved an equivalent extent of mass loss in monoculture as was achieved by the coculture. We also discovered a diverse range of cellulase and hemicellulase enzymes in the genomes of both species indicating that they have the potential to degrade a range of lignocellulosic substrates.

4.2 Introduction

The long-term viability of second-generation biofuels is reliant on improving the cost-effectiveness and efficiency of lignocellulose degradation (Lynd et al., 2008; Naik et al., 2010). The major limiting factors currently affecting biofuel production are the requirement of energy-intensive physiochemical pretreatment to remove lignin, and the high cost of saccharification enzyme production coupled with the requirement of high enzyme loadings due to low catalytic efficiencies (Johnson, 2016; Lynd et al., 2017; Van Dyk and Pletschke, 2012).

One of the approaches being investigated to tackle these issues is the identification of both the microorganisms and enzymes involved in efficient lignocellulose degradation in natural systems (Brune, 2014; Cragg et al., 2015). Highly active individual or communities of microbes could play a role in consolidated bioprocessing, the direct conversion of lignocellulose to valuable products

eliminating the requirement for pretreatment (Lynd et al., 2002; Zuroff and Curtis, 2012). Alternatively, the identification of more efficient enzymes could reduce the cost of current biofuel production processes through reduced enzyme-loading requirements (Lynd et al., 2017). Specifically, the discovery of efficient bacterial cellulases and hemicellulases could dramatically reduce the cost of saccharification as these are cheaper and more straight forward to express than the fungal enzymes currently used (Himmel et al., 2010; Mori et al., 2014).

One promising source of lignocellulolytic microbes and enzymes under investigation is compost (Wei et al., 2012). Composting is the process by which various forms of lignocellulose are degraded by a diverse microbial community to produce a nutrient rich material that can be used to improve the fertility of soil (Ryckeboer JR, 2003; Wei et al., 2012). In chapters 2 and 3, we found that wheat straw compost is a promising source of microorganisms capable of degrading the components of lignocellulose. We found a positive relationship between the diversity of these communities and their ability to utilise wheat straw as a carbon source. Interestingly, the productivity of communities was significantly improved by the presence of two dominant species, *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13, with the most productive communities containing both these species (Evans et al., 2017). Species from the Paenibacillus and Cellulomonas genera are frequently identified in studies of lignocellulose degradation and efforts are ongoing to uncover the enzymatic machinery that the genomes of these species encode (Ahmed et al., 2018; de Lima Brossi et al., 2016).

Paenibacillus is an industrially relevant genus of bacteria owing to the ability of some strains to produce antimicrobials, promote plant growth and protect against insect pests and phytopathogens (Grady et al., 2016). In addition, Paenibacillus species produce a range of enzymes involved in bioremediation and lignocellulose degradation (Bohra et al., 2018). The production of various oxygenases, dehydrogenases, and ligninolytic enzymes by Paenibacillus strains suggests a promising role in bioremediation and these enzymes may also be able to depolymerise lignin (Abbasian et al., 2015; Chandra et al., 2008; Haritash and

Kaushik, 2009). As a result of the various enzymatic activities identified in Paenibacillus strains, they are being studied for roles in biofuel production (Weselowski et al., 2016). For example, pretreatment of water hyacinth with a Paenibacillus strain isolated from a millipede gut significantly improved biogas production (Barua et al., 2018). Due to their potential agricultural and industrial applications, several Paenibacillus genomes have been sequenced revealing the presence of multiple carbohydrate active enzymes (CAZymes) including various cellulases and hemicellulases (Bohra et al., 2018; Eastman et al., 2014).

The ability of the Cellulomonas genus to produce extracellular lignocellulolytic enzymes was first studied 50 years ago (Han and Srinivasan, 1968) and the cellulases produced by this genus are among the best studied (Brumm, 2013). Both genomic and proteomic approaches have identified a diverse range of hemicellulase and cellulases encoded by the type strains *Cellulomonas fimi* ATCC 484 and *Cellulomonas flavigena* ATCC482, and shown substrate-specific expression of these enzymes (Abt et al., 2010; Christopherson et al., 2013; Sánchez-Herrera et al., 2007; Wakarchuk et al., 2016). Efforts to characterise the full range of lignocellulolytic enzymes produced by Cellulomonas species and assess their industrial potential are ongoing.

Here, we further characterise *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 by measuring their ability to degrade wheat straw lignocellulose in monoculture and in coculture. We found that while cocultures achieved higher wheat straw degradation than the monocultures over short timescales, the *Cellulomonas* sp. D13 monoculture is able to degrade wheat straw as well as the coculture over longer timescales. We sequenced the genomes of these stains and describe here the range of lignocellulolytic enzymes they possess. Similar to previous genomic analysis of these genera, we found that both these species contain a wide range of carbohydrate enzymes (CAZymes). Identifying which of these enzymes are required for wheat straw degradation could provide industrially relevant insights.

4.3 Methods

4.3.1 Growth on wheat straw

To measure the ability of *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 to degrade wheat straw lignocellulose we grew these strains in monoculture and coculture in 10 ml M9 minimal media with 1 g wheat straw lignocellulose. *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 were grown overnight in nutrient broth at 30°C then diluted to an abundance of approximately 10^6 cells/ml. 10ml of M9 minimal media containing 1 g wheat straw were inoculated with 50 µl each strain for cocultures or 100 µl each strain in monocultures. A negative control was included which contained no inoculum. There were three independent replicates destructively sampled at each treatment for each transfer to give a total of 48 microcosms. Microcosms were incubated at 30°C, 150 rpm. At days 4, 7, 14 and 21 three replicates from each treatment were removed. Serial dilutions were prepared and spread onto nutrient agar plates to allow the density of *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 to be counted. Biomass was harvested by centrifugation and media was removed. Samples were freeze-dried and weighed to determine the amount of mass loss. Microbial biomass was not removed and so mass loss values are likely to be conservative.

4.3.2 Genome Sequencing

To identify the CAZymes possessed by these species we sequenced the genomes of *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13. A single colony of each species was resuspended and grown overnight in nutrient broth at 30°C, 150 rpm. Cells were harvested, and genomic DNA was extracted using Qiagen Genomic Tips 20G following the manufacturer's instructions. The genome was sequenced on the Pacific Biosciences Sequel System by NERC Biomolecular Analysis Facility at the University of Sheffield. PacBio sequencing of the *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 genomes produced 217,443 reads at an average length of 5,740 bp and 237,833 reads at an average length of 5,627 bp, respectively. Trimming and correction by Canu (Koren et al., 2017) produced 185,632 reads at an average length of 5,174 bp and 199,449 reads at an average length of 4,858 bp for the *Paenibacillus* sp. A8 and

Cellulomonas sp. D13 genomes which represents 135x and 233x coverage respectively. Reads were assembled into a single contig by Canu which were circularised by Circlator (Hunt et al., 2015) and polished with Pilon (Walker et al., 2014) using 2x250 paired-end Illumina reads generated by MicrobesNG. Genome annotation was performed using Prokka (Seemann, 2014) and CAZymes were annotated by HMMER, Diamond and Hotpop using the dbCAN meta server (Yin et al., 2012). CAZymes annotated by at least two methods are included in results. Visualisation of the genome was performed in DNA Plotter which is part of the Artemis platform (Carver et al., 2012).

4.3.3   Statistical analysis

All statistical analysis was performed in R version 3.5.1 (R Core Team, 2018). Linear mixed effects models using the nlme package (Pinheiro et al., 2018) were used to compare the density of *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13. The quantity of mass loss through time was also analysed using a linear mixed effects model. To identify differences in the extent of mass loss in the presence or absence of each species we used the binary presence and absence of each species and time as the independent variables.

4.4   Results

4.4.1   Degradation of wheat straw by *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13

We first assessed the ability of *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 to degrade wheat straw lignocellulose. Comparison of growth in monoculture and coculture indicates that competition with *Paenibacillus* sp. A8 inhibited the growth of *Cellulomonas* sp. D13 (linear mixed effects model, main effect of diversity, $F_{1,4}$ = 102.6, $P < 0.001$, Figure 4.1a) while *Paenibacillus* sp. A8 benefited from the presence of *Cellulomonas* sp. D13 (linear mixed effects model, main effect of diversity, $F_{1,4}$ = 12.8, $P < 0.05$, Figure 4.1a).

To determine the extent of lignocellulose degradation we measured the mass loss of wheat straw following degradation by *Paenibacillus* sp. A8 and/or *Cellulomonas* sp. D13 in monoculture and in coculture. The presence of *Cellulomonas* sp. D13

significantly increased the rate of lignocellulose mass loss (linear mixed effects model, *Cellulomonas* sp. D13 presence by time interaction, $F_{1,25} = 5.0$, $P < 0.05$, Figure 4.1b).
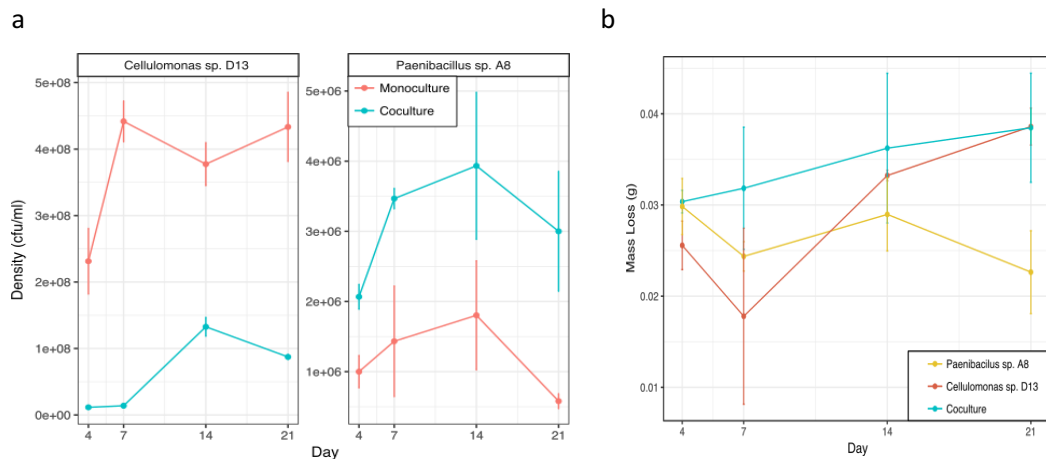
Figure 4.1 (a) The density of *Cellulomonas* sp. D13 (left panel) and *Paenibacillus* sp. A8 (right panel) when grown in monoculture (red) and coculture (cyan) in 10 ml M9 minimal media with 1 g wheat straw as the sole substrate. (b) The mass loss of wheat straw following degradation by *Paenibacillus* sp. A8 in monoculture (yellow), *Cellulomonas* sp. D13 (red) and both these species in coculture (cyan).

4.4.2 Genome sequences for *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13

De novo assembly of *Paenibacillus* sp. A8 produced a single circular contig 7,136,835 bp in size with an average GC content of 46.1%. The genome contained 6,487 coding sequences (CDS) with 99 tRNAs and 35 rRNAs. Prokka annotated 62.9% of the genes with the remaining genes annotated as hypothetical proteins. Comparison of the *Paenibacillus* sp. A8 genome to previously sequenced genomes using average nucleotide identity (ANI) revealed 82% ANI to the type strain *Paenibacillus polymyxa*, 94% ANI to *Paenibacillus amylolyticus* and 95% ANI to *Paenibacillus antarcticus*.

The genome assembly of *Cellulomonas* sp. D13 also produced a single circular contig 4,166,311 bp in size with an average GC content of 74.5%. Prokka annotation predicted the presence of 3,777 CDS, 59% of which were annotated by Prokka with 56 tRNAs and 6 rRNAs. ANI suggested that *Cellulomonas* sp. D13 is most closely related to *Cellulomonas composti* (96% ANI) and is relatively diverged from the type strains *C. fimi* (ANI 81%) and *C. flavigena* (ANI 89%).
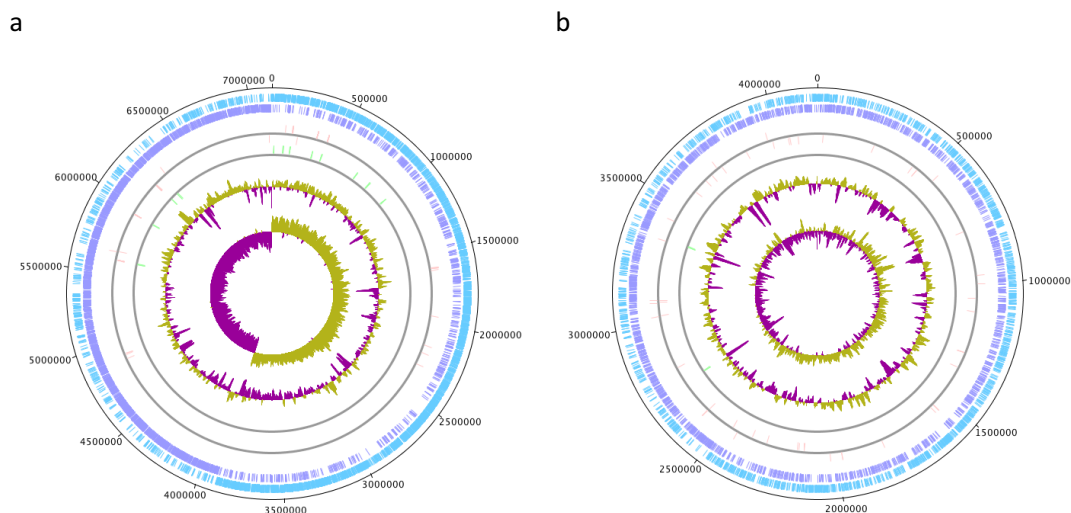
Figure 4.2 Graphical representation of the chromosome of (a) *Paenibacillus* sp. A8 and (b) *Cellulomonas* sp. D13. From outer to inner circles: genes on forward strand (blue); genes on reverse strand (purple); tRNA genes on forward and reverse strands respectively (pink); rRNA genes on forward and reverse strands respectively (green); GC content; GC skew. Figure produced using DNA Plotter from Artemis.

4.4.3 Carbohydrate utilisation enzymes

To assess the carbohydrate degradation capacity of *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 we annotated the genome for CAZymes using dbCAN2. As with previous analysis of Paenibacillus and Cellulomonas genomes, we found multiple CAZYme domains including glycoside hydrolases (GHs), carbohydrate-bindings modules (CBMs), glycosyl transferases (GTs), carbohydrate esterases (CEs), auxiliary activities (AAs) and polysaccharide lyases (PL) with a total of 333 and 217 CAZyme hits in *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 respectively (Figure 4.3). The genomes of both species contained a high number of GH enzymes which is a common trait amongst lignocellulolytic bacteria (Woo et al., 2014b). Cellulolytic enzymes are predominantly classed into families GH1, GH3, GH5, GH6, GH7, GH8 GH9, GH12, GH45 and GH48 while hemicellulolytic enzymes are predominantly found in families GH2, GH10, GH11, GH16, GH26, GH30, GH31, GH36, GH43, GH51, GH74 and GH95 (López-Mondéjar et al., 2016a).

The genome of *Paenibacillus* sp. A8 contains 333 CAZyme domains in 281 genes, approximately 4.3% of CDS, 63 of which have signal peptides. Similar to other sequenced Paenibacillus genomes, the majority of CAZyme hits (56.2%) belonged to

76 GH families. Ninety-six genes were assigned to GH families predicted to be involved in cellulose and hemicellulose degradation (Table 4.1). As is typical of multiple bacterial species there was an abundance of GH1 and GH3 genes, 12 and 14 respectively, which encode β-glucosidases (Berlemont and Martiny, 2013). The presence of these genes is not considered to be sufficient for cellulose degradation with members of the other cellulolytic GH families also required for growth on cellulose (Koeck et al., 2014). The presence of 14 genes belonging to the remaining cellulolytic GH families suggests *Paenibacillus* sp. A8 possesses the enzymes required for cellulose degradation. Similar to other Paenibacillus genomes (Yadav and Dubey, 2018), we found the highest number of CAZymes (23) belonged to GH43, a family of arabino/xylosidases. In addition to multiple members of the other GH families related to hemicellulose degradation, *Paenibacillus* sp. A8 had 19 carbohydrate esterases (CE1, CE4, CE10, DE14) that are predicted to act on xylan.
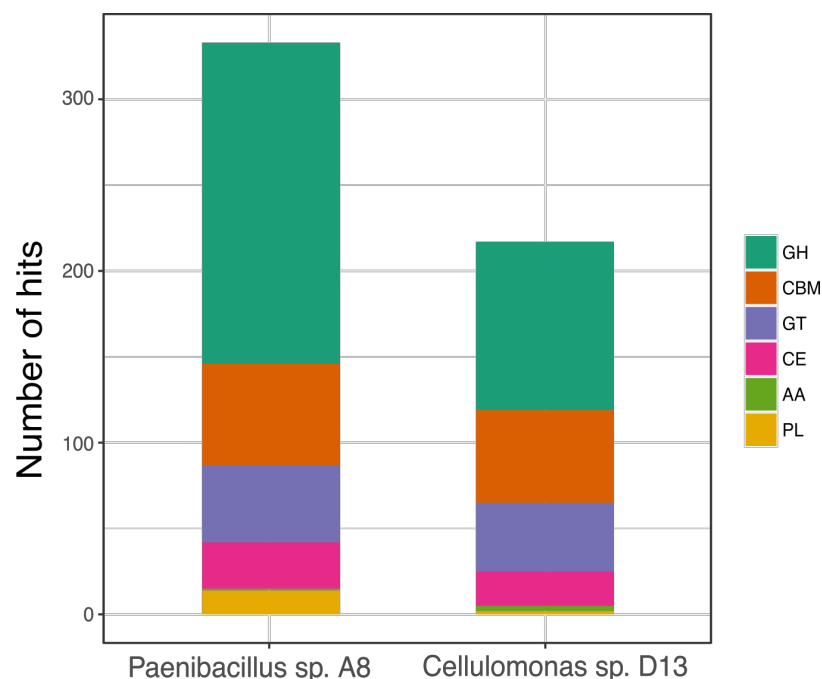
Figure 4.3 Number of hits identified by at least two programs/databases by dbCAN2. Hits are annotated as glycoside hydrolases (GHs), carbohydrate-bindings modules (CBMs), glycosyl transferases (GTs), carbohydrate esterases (CEs), auxiliary activities (AAs) and polysaccharide lyases (PL).

		Paenibacillus sp. A8	Penibacillus polymyxa ND24	Paenibacillus sp. O199	Cellulomonas sp. D13	Cellulomonas flavigena ATCC 482	Cellulomonas fimi ATCC 484
GH Total		**187**	116	231	**98**	76	98
Cellulases	GH1	**12**	7	11	**1**	1	1
	GH3	**14**	2	11	**3**	8	7
	GH5	**5**	5	7	**1**	2	4
	GH6	**1**	1	1	**3**	4	4
	GH8	**1**	0	0	**0**	0	0
	GH9	**1**	0	1	**4**	5	4
	GH48	**1**	1	1	**1**	1	1
	GH51	**4**	3	4	**2**	4	4
	GH74	**1**	7	4	**3**	0	1
Hemicellulases	GH2	**8**	2	10	**1**	0	0
	GH10	**4**	2	3	**18**	9	15
	GH11	**1**	1	1	**5**	2	3
	GH16	**3**	2	5	**0**	0	1
	GH26	**4**	5	2	**3**	1	3
	GH30	**2**	1	6	**0**	0	0
	GH31	**1**	1	2	**1**	0	0
	GH39	**2**	0	0	**2**	2	2
	GH42	**5**	3	4	**2**	2	2
	GH43	**23**	8	17	**5**	3	4
	GH53	**3**	1	3	**1**	0	1
CBM		**59**	55	84	**54**	59	72
GT		**45**	65	61	**40**	19	28
CE		**27**	43	79	**20**	10	11
AA		**1**	5	10	**3**	2	4
PL		**14**	9	15	**2**	3	4

Table 4.1 Summary of carbohydrate active enzymes (CAZymes) in Paenibacillus sp. A8 and Cellulomonas sp. D13 (in bold). The total number of CAZyme hits in each group are given as well as the number of hits in each family for cellulase and hemicellulase containing families. The number of CAZyme hits for two Paenibacillus and two Cellulomonas strains are given for comparison.

The *Cellulomonas* sp. D13 genome contained 217 CAZyme domains in 152 genes (4% CDS), 60 of which contained signal peptides. The majority of these genes (45%) were GHs which were classified into 55 families. There are three predicted GH3 genes and one GH1 along with 14 other genes assigned to cellulase families suggesting the *Cellulomonas* sp. D13 contains the necessary enzymes for autonomous cellulose

degradation. Similar to *C. flavigena*, the genome contained a high number (16) of GH10 genes which encode endo-1,4-β-xylanase (Wakarchuk et al., 2016). There were 16 genes assigned to xylan acting CEs (CE1, CE3, CE4, CE10, CE14) and three AA10s which encode lytic polysaccharide monooxygenases (AA10) which hydrolyse lignocellulose by direct oxidative attack (Hemsworth et al., 2014).

Our results suggest that both *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 possess the genes required for degradation of both cellulose and hemicellulose. However, transcriptomic and/or proteomic work would be required to identify which of these genes are expressed and under what conditions.

4.5   Discussion

The identification of microorganisms and enzymes capable of efficient lignocellulose degradation is one of the most promising solutions to optimise second generation biofuel production (Harris et al., 2014). Using a biodiversity ecosystem functioning experiment we previously identified two species which significantly improve community productivity during growth on wheat straw. These species belonged to the Paenibacillus and Cellulomonas genera which have previously been identified as promising candidates for industrial applications (Christopherson et al., 2013; López-Mondéjar et al., 2016a).

Here we confirmed our previous finding that both of these species were able to grow in minimal media with wheat straw lignocellulose as the sole carbon source. While *Paenibacillus* sp. A8 was able to grow on wheat straw in monoculture, mass loss did not significantly increase throughout time. The density of *Paenibacillus* sp. A8 and the degradation of wheat straw was increased by the presence of *Cellulomonas* sp. D13 with cocultures and *Cellulomonas* sp. D13 monocultures resulting in the same amount of mass loss after 21 days. Interestingly, despite exhibiting the same degree of mass loss as monocultures, cocultures contained significantly lower densities of *Cellulomonas* sp. D12 suggesting that *Paenibacillus* sp. A8 is also contributing to mass loss in these cultures. One consideration that should be taken into account is that

this experiment measured the total biomass following several days of bacterial growth and so the bacterial biomass as well as the remaining lignocellulose biomass is included leading to conservative estimates of mass loss.

Despite the apparent ability of *Cellulomonas* sp. D13 to more efficiently degrade wheat straw than *Paenibacillus* sp. A8, analysis of the genomes indicated they contain a similar number of CAZymes relative to their genome size. Paenibacillus species are widespread in nature and have been studied for various industrial applications including their ability to produce lignocellulolytic enzymes (López-Mondéjar et al., 2016a; Song et al., 2014; Yadav and Dubey, 2018). Here we show that *Paenibacillus* sp. A8 possesses 40 genes assigned to GH families related to cellulose degradation and 56 assigned to GH families related to hemicellulose degradation. Similar to the genomes of various Cellulomonas species (Christopherson et al., 2013; Lisov et al., 2017; Sánchez-Herrera et al., 2007), *Cellulomonas* sp. D13 contains 18 genes assigned to GH families with cellulase activity and 36 assigned to GH families with hemicellulase activity. As with previous studies, analysis of the *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 genomes predicts the presence of multiple genes in the same family, however these enzymes may catalyse different reactions and further characterisation would be required to determine specific functions. However, it has been shown that enzymes involved in both cellulose and hemicellulose degradation often exhibit redundancy which may explain the presence of multiple enzymes in the same family (Amore et al., 2013b; Stricker et al., 2006). While analysis of the genomes suggests *Paenibacillus* sp. A8 and *Cellulomonas* sp. D13 possess the genes required for cellulase and hemicellulose degradation, the presence of these genes does not necessarily mean they are expressed. López-Mondéjar et al. (2016) found that while *Paenibacillus* sp. O199 possesses a wide arsenal of CAZymes, only about 30% of these are expressed during growth on cellulose or lignocellulose. However, the proteins that were expressed represented the full set required for cellulose degradation, i.e. endoglucanases, exoglucanases, β-glucosidases and cellobiohydrolases (Brumm, 2013). Therefore, in order to identify enzymes with potential industrial applications, annotations of CAZymes in a genome is often coupled with proteomic analysis to determine the

expression patterns of enzymes under different growth conditions (López-Mondéjar et al., 2016b; Takasuka et al., 2013; Wilson, 2012).

In summary, we have identified two lignocellulolytic bacteria able to degrade wheat straw. *Cellulomonas* sp. D13 grew better in monoculture and achieved the same amount of wheat straw degradation (mass loss) as the coculture containing both *Cellulomonas* sp. D13 and *Paenibacillus* sp. A8. Analysis of the genomes of these bacteria indicate that they both possess multiple enzymes required for degradation of cellulose and hemicellulose. Proteomic analysis to determine which of these enzymes are expressed would help to uncover how these species achieve lignocellulose degradation.

5 The influence of competing species and their ecoevolutionary responses on the rate and trajectory of focal species evolution

5.1 Abstract

The evolutionary adaptation of species to new environments are typically studied in single-species populations but in nature these processes occur in complex communities. Here I tested the effect of competing species on the phenotypic evolution of a focal species using experimental evolution of simple lignocellulose degrading communities grown on wheat straw. To determine the effect of ecological and evolutionary responses of the other species upon focal species evolution these species were either held fixed or allowed to dynamically respond to changes in the focal species population. Evolution in the presence of a competing community accelerated metabolic phenotype evolution in the focal species relative to monoculture controls, but also led to reduced autonomous growth performance on wheat straw. Species sorting and evolutionary responses of the competing communities led to greater between population divergence of the metabolic phenotype of the focal species in the dynamic polyculture treatment compared to fixed polyculture treatment, but also limited the fitness gains of the focal species against the ancestral community. Taken together these data suggest that the combined ecological and evolutionary responses of competing species shape both the rate and trajectory of evolution of a focal species adapting to a new environment.

5.2 Introduction

Understanding how species adapt to new environments has applications in managing human health (Eloe-Fadrosh and Rasko, 2013), predicting responses to climate change (Berg et al., 2010) and industrial biotechnology (Kouzuma and Watanabe, 2014). Most theoretical and experimental studies on evolutionary dynamics consider single species responding to abiotic selection pressures in isolation. However, in nature almost all species exist as part of complex and dynamic communities and so to understand evolution in natural systems the influence of community context on the adaptation of constituent species must be taken into account (Barraclough, 2015).

When communities encounter new or changed abiotic environments, both evolutionary and ecological responses may occur. Evolutionary responses of species can be either promoted or hindered by species interactions depending on the nature of the ecological interaction (Barraclough, 2015). Competition can reduce a species' abundance thereby limiting genetic variation and evolutionary potential, which may in turn reduce the rate of evolution (Johansson, 2008; Lanfear et al., 2014; Rich et al., 1979). If trait variation is greater between species than within species, abiotic selection pressures can lead to changes in the relative abundance of competing species (i.e. species sorting) before selection can act on the genetic variation within species, hindering the evolutionary response (Barraclough, 2015; De Mazancourt et al., 2008). Theory predicts that as a result of species sorting, species that are preadapted to the prevailing environmental conditions will more readily occupy the available niche space and increase in relative abundance at the expense of less well adapted species, which consequently evolve at a slower rate than they would in monoculture (De Mazancourt et al., 2008). However, the occupation of a niche by a preadapted species may force competing species to evolve the ability to occupy different niche space, referred to as character displacement (Grant and Grant, 2006), or to occupy new niche space created by the activity of additional species in the community, referred to as facilitation or cross-feeding (Jousset et al., 2016; Osmond and de Mazancourt, 2013; Zhang et al., 2012). An increased rate of evolution due to the presence of biotic interactions is supported by Lawrence et al. (2012) who found that four species adapted by a combination of niche partitioning and cross-feeding when evolving in polyculture. Populations that had evolved in polyculture evolved more but achieved lower growth when in monoculture than those that had evolved in monoculture suggesting a trade-off between adaptation to biotic and abiotic selection pressures (Lawrence et al., 2012).

In addition to their ecological effects on the focal species, interacting species may themselves evolve which in turn could shape the evolutionary response of the focal species. Such reciprocal evolutionary change is termed coevolution (Janzen, 1980; Thompson, 2016). The Red Queen hypothesis proposes that for antagonistic species

interactions, each adaptation made by a species will be matched by counter-adaptations of interacting species driving continual coevolution without change in the relative fitnesses of species over time (Brockhurst et al., 2014; Stenseth and Smith, 1984; Van Valen, 1973). Experimental evolution using microorganisms allows the effects of coevolution to be studied in a controlled laboratory setting (Brockhurst and Koskella, 2013). Such studies generally provide support for the central tenet of the Red Queen hypothesis that reciprocal evolution accelerates evolutionary rates compared to controls where only one of the species is allowed to evolve (Paterson et al., 2010; Schulte et al., 2010). However, studies of coevolution and the Red Queen hypothesis have predominantly focussed on pairs of interacting species and consequently little is known about how these pairwise coevolutionary processes scale-up in more complex communities (Brockhurst et al., 2014). Recently, Betts et al. (2018) found that when a bacterial host was coevolved with a community of phage parasites, the rate of evolution and divergence among replicate populations was greater than when coevolved with a single phage parasite. Similarly, Fiegna et al. (2015) found that species interactions evolved more, becoming less negative, in more diverse communities. Further work is required to understand how community context shapes the adaptation of a species to new environments and whether this process is affected by coevolution with the community.

Here, we investigate how adaptation of a focal species (*Stenotrophomonas* sp. D12) to a new resource environment is affected by the presence and the evolution of a community of competing species. Specifically, we tracked the evolutionary response of a focal species to a novel wheat straw environment both in monoculture and in polyculture where the competing species were either held constant (fixed polyculture treatment) or allowed to themselves make ecological and evolutionary responses (dynamic polyculture treatment). At the end of the experiment we measured the autonomous growth performance of the focal species on wheat straw to test for abiotic adaptation and estimated relative fitness against the ancestral community to test for biotic adaptation. We also quantified change in the metabolic phenotype of the focal species over time to estimate differences in the rate and trajectory of phenotypic evolution within and between treatments. Finally, we

evolved the focal species in pairwise coculture with each of the constituent species to distinguish the evolutionary trajectories driven by each competitor species alone. We report that evolution in the presence of a competing community accelerated metabolic phenotype evolution relative monoculture controls, but also led to reduced autonomous growth performance on wheat straw. Species sorting and evolutionary responses of the competing communities led to greater between population divergence of the metabolic phenotype of the focal species in the dynamic polyculture treatment compared to fixed polyculture treatment, but also limited the fitness gains of the focal species against the ancestral community. Taken together these data suggest that the combined ecological and evolutionary responses of competing species shape both the rate and trajectory of evolution of a focal species adapting to a new environment.

5.3   Methods

5.3.1   Bacterial isolates and experimental design

All isolates used in this experiment were isolated from wheat straw compost enrichment cultures as described in Chapter 2. *Stenotrophomonas* sp. D12 was used as the focal species as it was found to be resistant to high concentrations of kanamycin in minimum inhibitory concentration assays. Five additional strains from the Bacillus, Paenibacillus, Microbacterium, Cellulomonas and Rhodococcus genera were chosen as they were susceptible to kanamycin and exhibited varying functional metabolic traits (Figure 3.2).

The focal species was evolved in monoculture and in polyculture with all additional strains with wheat straw lignocellulose as the sole carbon source. In half of the polyculture replicates both the focal species and additional species were allowed to adapt to the environment (referred to hereafter as dynamic) and in the other half only the focal species was allowed to adapt while the additional species were held in evolutionary stasis (referred to hereafter as fixed). Cultures were serially transferred to new media each week for 16 weeks. To identify the evolutionary trajectories

driven by each of the five competing species we also evolved the focal species in the presence of dynamic and fixed cocultures with each of the additional species.

5.3.2   Experimental set up

All isolates were grown overnight in nutrient broth at 30°C then diluted to an abundance of approximately 10^6 cells/ml. These cultures were used to inoculate 6 ml M9 media with 60 mg wheat straw. Monocultures were inoculated with 60 µl of the focal species, polycultures were inoculated with 10 µl of each species and cocultures with 30 µl of each species. Each community was replicated six times to yield 18 microcosms which were incubated at 30°C, 150 rpm for 6 days. Cultures were then diluted to 10^{-5} in M9 media and 100 µl was spread onto nutrient agar plates (dynamic polyculture and monocultures) or nutrient agar plates containing 50 µg/ml kanamycin (fixed polyculture). Plates were incubated overnight at 30°C then 1 ml M9 media was added to plates and colonies were disrupted using a spreader. 100 µl was transferred to a microplate and cells were pelleted by centrifugation, then washed and suspended in M9 media. Dynamic communities and monocultures were inoculated with 60 µl of these cultures. Fixed communities were inoculated with 10 µl of the evolved focal species population and 10 µl of each additional species grown overnight in nutrient broth from ancestral glycerol stocks and diluted to 10^6 cells/ml.

5.3.3   Relative fitness assays

To measure the fitness of the focal species relative to the ancestral community we inoculated microcosms with 10 µl of the community of additional species (10^6 cfu/ml) grown from ancestral glycerol stocks and 10 µl of the ancestral, monoculture evolved, coevolved or evolved focal species population. Cultures were grown for 6 days at 30°C, 150rpm. Cultures were serially diluted and plated onto nutrient agar to count the total community density and nutrient agar containing 50 µg/ml kanamycin to count the focal species density at the beginning of the experiment and after 6 days of incubation. Relative fitness (w) was calculated as $w = x_2(1-x_1)/ x_1(1-x_2)$ where x_1 is the proportion of focal species in the community at the start of the experiment and x_2 is the final proportion of focal species (Ross-Gillespie et al., 2007). Relative fitness was standardised to the relative fitness of the ancestor to control for variation

between starting replicates and one-tailed t-tests were used to test for significant differences between the relative fitness of the evolved and the ancestral populations.

5.3.4   Activity assays

To quantify the functional traits of the ancestral and evolved *Stenotrophomonas* sp. D12 populations, growth assays were performed on several polysaccharides present in lignocellulose as described in Chapter 3. Hemicellulose substrates included xylan (Sigma-Aldrich), arabinoxylan (P-WAXYL, Megazyme) and galactomannan (P-GALML, Megazyme); cellulose substrates included β-glucan (P-BGBL, Megazyme) and Whatman filter paper; additional substrates included pectin (Sigma-Aldrich) and Kraft lignin (Sigma-Aldrich). Ancestral *Stenotrophomonas* sp. D12 strains were grown from glycerol stocks overnight in nutrient broth at 30°C, 150 rpm. Cultures were harvested by centrifugation, washed and suspended in M9 minimal media and left at room temperature for 2 hours to metabolise remaining nutrients. Communities from transfers 4, 8, 12 and 16 were diluted to 10^{-5} and 100 μl was spread onto nutrient agar plates with 50 μg/ml kanamycin. Plates were incubated at 30°C for 24 hours then 1 ml M9 media was added to plates and colonies were disrupted with a spreader. 100 μl of culture was added to 900 μl M9 minimal media and cells were harvested by centrifugation, washed and suspended in 1ml M9 minimal media then left for 2 hours at room temperature to metabolise remaining nutrients. All cultures were standardised to an OD_{600} of 0.1 and 5 μl of culture was used to inoculate 495 μl of M9 minimal media with 0.2% (w/v) of each carbon source or one 6mm sterile filter paper disc in 96-well deepwell plates. Cultures were grown for 5 days at 30°C and the MicroResp system was used to measure culture respiration as described in Chapter 3.

5.3.5   Amplicon sequencing

To confirm the presence of each species in the coevolved polycultures and quantify their relative abundances over time we isolated total genomic DNA from dynamic polycultures from every fourth transfer using Qiagen DNeasy Blood and Tissue kits following the protocol for Gram-positive bacteria. 16S-EZ amplicon sequencing of the

V3 and V4 hypervariable regions of the 16S rRNA gene and all data analysis was performed by Genewiz (New Jersey, USA). Briefly, the V3/V4 regions were amplified by PCR and sequenced using the Illumina platform with 2x250 bp paired end reads. Raw reads were optimised by assembling read pairs and removing undetermined bases and primer and adapter sequences. Chimera sequences were also removed. Qiime was used to assemble reads into OTU clusters (similarity = 97%) and these were identified and relative abundance was calculated.

5.3.6 Statistical analysis

Phenotypic diversification from the ancestor was quantified using the Bray-Curtis dissimilarity (BCD). BCD between the evolved populations and the centroid of each treatment was used to quantify within treatment divergence. Linear mixed effects models were used to test for differences in phenotypic divergence from the ancestor and within treatments. Analysis of variance (ANOVA) followed by post hoc Tukey tests were used to test for differences between the BCD of the evolved populations at each transfer. To assess whether phenotypic traits or community composition varied between treatments or polycultures we conducted permutational multivariate analysis of variance (MANOVA) with 999 permutations using the 'adonis' function in the vegan package in r (Oksanen et al., 2018). *Post hoc* pairwise comparisons of the results were conducted using the pairwise.perm.manova function from the RVAideMemoire package (Hervé, 2018). Linear models were used to compare phenotypic divergence between the focal species populations and community composition for the dynamic polycultures.

5.4 Results

5.4.1 Abiotic and biotic adaptation of the evolved focal species populations

We first compared the growth performance of the evolved focal species populations when grown autonomously on wheat straw. Focal species populations evolved in fixed or dynamic polycultures had lower autonomous growth on wheat straw than focal species populations that had evolved in monoculture (t(5) = -2.6, *P* = 0.05 and t(5) = -3.7, *P* < 0.05, respectively), and moreover had declined in autonomous growth

compared to the ancestor (t(5) = -4.0, *P* < 0.05 and t(5) = -4.9, *P* < 0.01, respectively, Figure 5.1a). This suggests that polyculture-adapted populations did not adapt to the abiotic environment. This lack of abiotic adaptation could have been caused by a trade-off with biotic adaptation, potentially due to the evolution of metabolic dependencies upon other species in the polyculture. Such metabolic dependencies could allow the focal species to exploit the products of the metabolism of competing species, increasing the fitness of the focal species in competition with the polyculture. To test this, we next directly competed the evolved focal species populations from the end of the experiment against the ancestral polyculture community on wheat straw. The focal species populations evolved in fixed polyculture had evolved higher fitness against the ancestral polyculture than those evolved in dynamic polyculture (t(5) = 4.0, *P* < 0.05) or those evolved in monoculture (t(5) = 6.4, *P* < 0.01), neither of which showed fitness significantly different to the ancestor (t(5) = 0.36, *P* > 0.05 and t(5) = -2.6, *P* > 0.05, respectively, Figure 5.1b). This suggests that biotic adaptation of the focal species populations evolved in fixed polyculture had evolved to exploit metabolites produced by the other species in the ancestral polyculture. In contrast, this outcome was prevented where the polyculture community was absent or where it was able to make ecological or evolutionary responses. Taken together these data are consistent with the existence of a trade-off between biotic and abiotic adaptation but suggest that neither biotic nor abiotic adaptation was achieved by the focal species when evolving in dynamic polyculture.
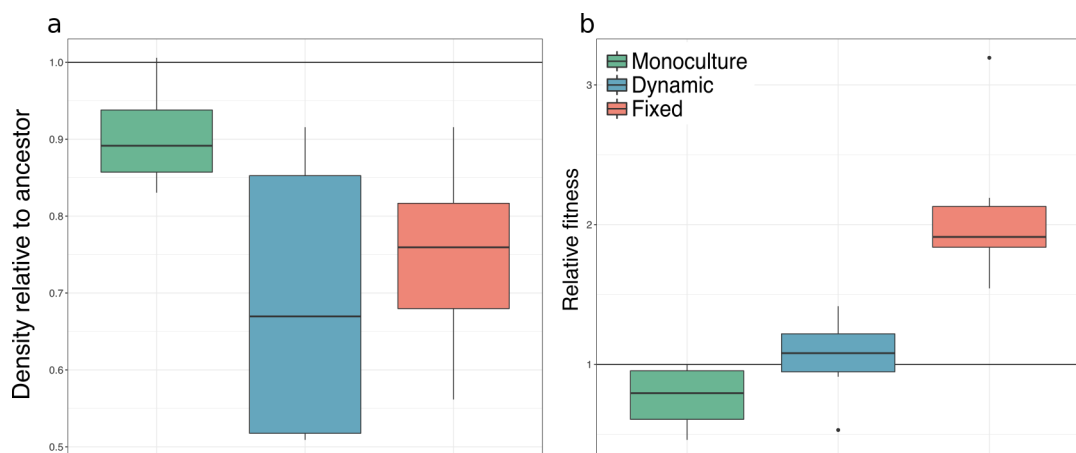

Figure 5.1 (a) Density of the evolved focal species populations when grown in monoculture relative to ancestral density (y = 1). (b) Relative fitness of evolved focal species populations when grown in polyculture with the ancestral community presented relative to the fitness of the ancestor (y = 1).

5.4.2 Evolution of the focal species' multivariate metabolic phenotype

To estimate how the rate and trajectory of evolution varied between treatments and the extent of phenotypic diversification between evolved replicate populations, we quantified multivariate metabolic phenotypes for each focal species population over time. Specifically, the performance of each focal species population was quantified on seven components of lignocellulose at every fourth transfer. Phenotypic divergence from the ancestor was quantified using Bray-Curtis dissimilarity (BCD) which gives an estimate of the rate of evolution in each population. Divergence from the ancestor increased over time and occurred at different rates between treatments (linear mixed effects model, treatment by time interaction, $F_{1,51} = 6.6$, $P < 0.01$). The focal species populations that had evolved in the polyculture treatments displayed higher rates of phenotypic evolution than the populations evolved in monoculture suggesting that biotic interactions increased the rate of evolutionary divergence from the ancestral metabolic phenotype in the focal species (Figure 5.2, BCD from ancestor represents distance between evolved population points and ancestral point in grey).
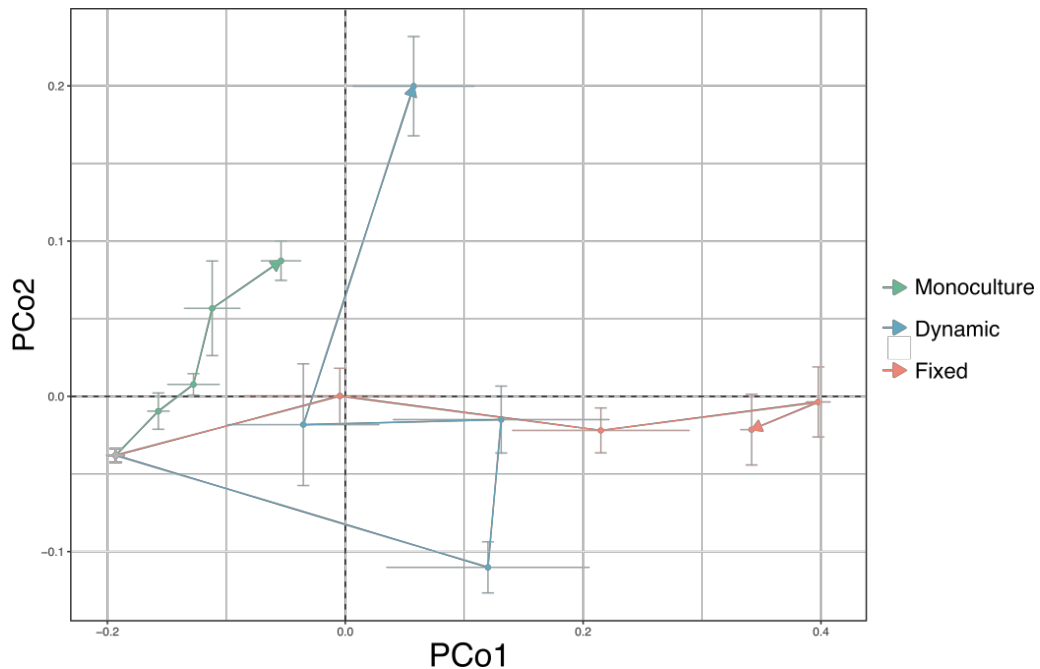
Figure 5.2 Trajectory of evolution of multivariate phenotypic traits. Points indicate mean PCoA coordinates of six replicate populations with error bars indicating standard error at each time point. Larger error bars indicate greater phenotypic divergence within treatments. Arrows join points in sequential order, i.e. transfers 4, 8, 12 and 16. Starting point (grey) indicates ancestral phenotypic traits. Increase in principal coordinate 1 indicates increased ability to utilise arabinoxylan, xylan and pectin while increase in principal coordinate 2 indicates an increased ability to utilise filter paper, β-glucan and lignin.

The trajectory of evolution of the focal species' metabolic phenotype varied between treatments and diverged over time (permutational MANOVA, treatment by time interaction $F_{6,55}$ = 5.0, P < 0.001, Figure 5.2). The focal species populations that evolved in the polyculture treatments followed similar evolutionary trajectories up to transfer 8, increasing their productivity on xylan, arabinoxylan and pectin. These substrates are protected from saccharification by lignin but once liberated are the more readily digestible, or labile, components of lignocellulose. By contrast, thereafter, whereas the focal species populations that had evolved in fixed polyculture continued to adapt to better exploit these labile substrates, the focal species populations that had evolved in dynamic polyculture instead switched from labile substrates to increase their productivity on the more recalcitrant substrates (β-glucan, filter paper and lignin) by transfer 16. Notably, the extent of this change in evolutionary trajectory varied among replicate populations and we therefore observed greater diversification between replicate focal species populations evolved in dynamic polyculture than those evolved in fixed polyculture or in monoculture (linear mixed effects model, treatment by time interaction $F_{3,68}$ = 6.5, P < 0.001, Figure 5.2, within treatment diversification represented by error bars). This suggests that biotic interactions *per se* potentiated the specialisation of the focal species on labile substrates, presumably because these were liberated from wheat straw by the action of one or more of the additional polyculture species. However, in the dynamic polyculture, ecological or evolutionary responses by the polyculture community appear to have favoured alternate evolutionary trajectories between replicate populations, leading to greater diversification and enhanced utilisation of more recalcitrant substrates by the focal species.

5.4.3 Species sorting in dynamic polycultures

Diversification between replicate populations in the dynamic polyculture treatment could have been driven by changes in community composition (species sorting) or evolution of the non-focal competing species. To estimate the contribution of species sorting we quantified the relative abundance of the species every fourth transfer in each community from the dynamic polyculture treatment by amplicon sequencing of

the 16S rRNA gene. Community composition varied between replicates: all six species coexisted throughout the experiment in four of the six replicates, whereas at transfer 16 replicate 38A had lost *Paenibacillus* sp. A8, *Microbacterium* sp. D14B and *Rhodococcus* sp. E31 and replicate 39A had lost *Cellulomonas* sp. D13 (Figure 5.3a). Using the relative abundance data, we next calculated the BCD for community composition and tested whether this explained variation in metabolic phenotype between the replicate focal species populations that had evolved embedded in these communities. The effect of community composition on the metabolic phenotype of the focal species strengthened over time (community composition x transfer interaction; $F_{1,87} = 6.4$, $P < 0.05$, Figure 5.3b), such that there was a significant positive relationship between functional metabolic divergence and community compositional divergence only at transfer 16 ($F_{1, 28} = 8.2$, $P < 0.01$, $R^2 = 0.20$, purple line Figure 5.3b). At transfer 16, the replicates formed 2 clusters with distinct community compositions. The first cluster was formed of the dynamic polycultures 37A and 38A which were dominated by the focal species, *Stenotrophomonas* sp. D12 (relative abundance of 94% and 97% respectively), but also contained *Cellulomonas* sp. D13 at relative abundances of 2-3%. These focal species populations followed a similar evolutionary trajectory with increased utilisation of the labile substrates at transfer 4 followed by a decrease in productivity on these substrates. However, unlike the other four populations evolved in dynamic polyculture which form the second cluster, the focal species in polycultures 37A and 38A showed only a small increase in productivity on lignin and filter paper relative to the ancestor at transfers 12 and 16. The key difference in community composition between the two clusters appears to have been that whereas 37A and 38A were dominated by the focal species *Stenotrophomonas* sp. D12 after transfer 8, the other coevolved polycultures retained relatively higher abundances of other species. In particular, all 4 replicates in cluster 2 had high relative abundance of *Bacillus* sp. D26, whilst replicates 40A, 41A and 42A also contained appreciable relative abundances of *Microbacterium* sp. D14B and *Paenibacillus* sp. A8. Furthermore, replicates 37A and 38A maintained higher relative abundances of *Cellulomonas* sp. D13 (2-3%) compared to the other 4 replicates in the second cluster where this species declined to very low relative abundance (<0.05%). Our results indicate that species sorting significantly influenced

the evolutionary trajectory of the focal species populations in the dynamic polyculture treatment, and that this effect strengthened over time as community composition diverged between replicates.
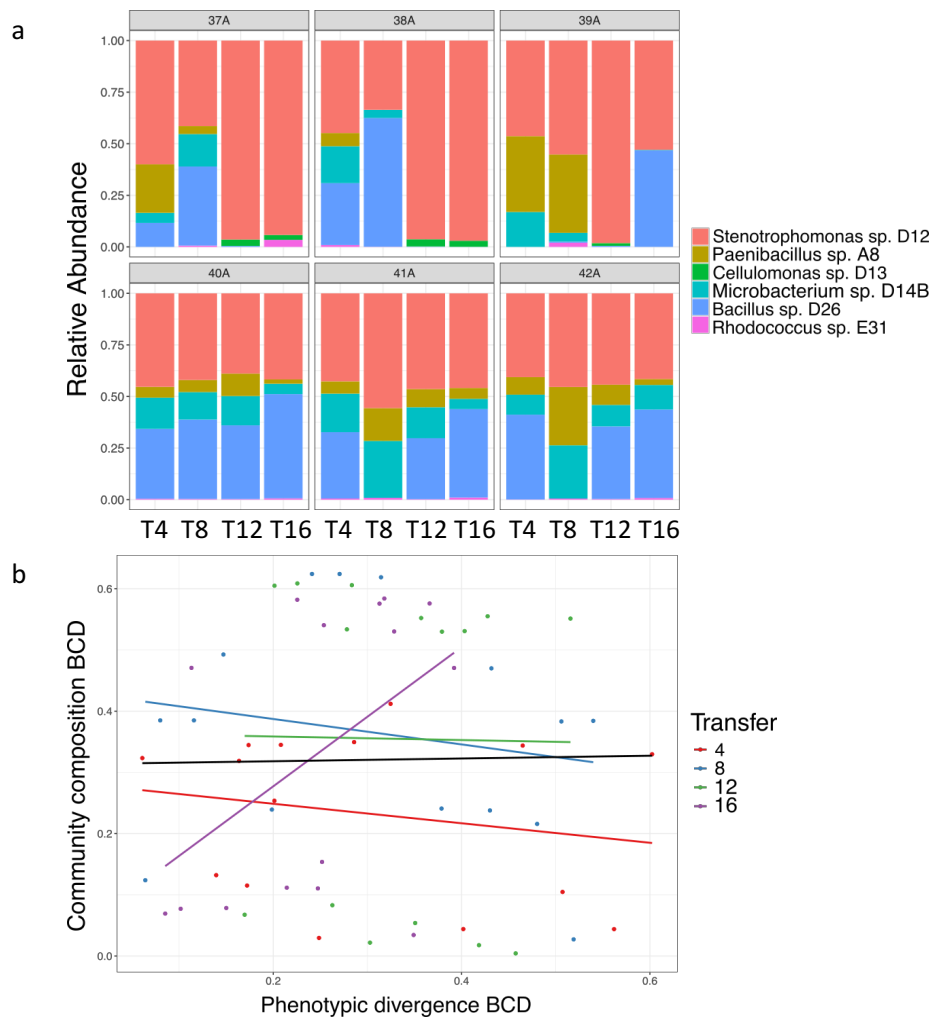
Figure 5.3 (a) Relative abundance of the six species in dynamic polycultures at transfer 16. (b) Relationship between phenotypic BCD and community composition BCD between replicate populations at transfers 4, 8, 12 and 16. Black line shows linear regression for full data set while coloured lines show linear regression of data points from each transfer.

5.4.4 Evolution of functional traits in coculture

To determine whether the different competitor species drove distinct evolutionary trajectories of the focal species' metabolic phenotype, and the contribution of competitor species evolution to this, we also evolved the focal species with each of the constituent species in fixed or dynamic coculture. The metabolic phenotype of the focal species evolved over time with the trajectory of evolution depending on the

identity of the competitor species and whether the competitor was itself evolving (permutational MANOVA, coculture by fixed/dynamic by time interaction, $F_{30,200} = 6.5$, $P < 0.001$, Figure 5.4). Four out of five competitor species drove increased utilisation of recalcitrant substrates by the focal species, whereas one competitor species drove increased utilisation of labile substrates by the focal species. Specifically, focal species populations evolved with *Bacillus* sp. D26 or *Cellulomonas* sp. D13 increased their utilisation of lignin while focal species populations evolved with *Rhodococcus* sp. E31 or *Paenibacillus* sp. A8 increased their utilisation of β-glucan and filter paper, respectively. In contrast, *Microbacterium* sp. D14B drove large increases in utilisation of xylan (8 to 9-fold increase relative to ancestor) and arabinoxylan (5 to 6-fold increase relative to ancestor) by the focal species while the other competitor species drove less than a 4-fold or 2-fold increase in utilisation by the focal species of these substrates, respectively.
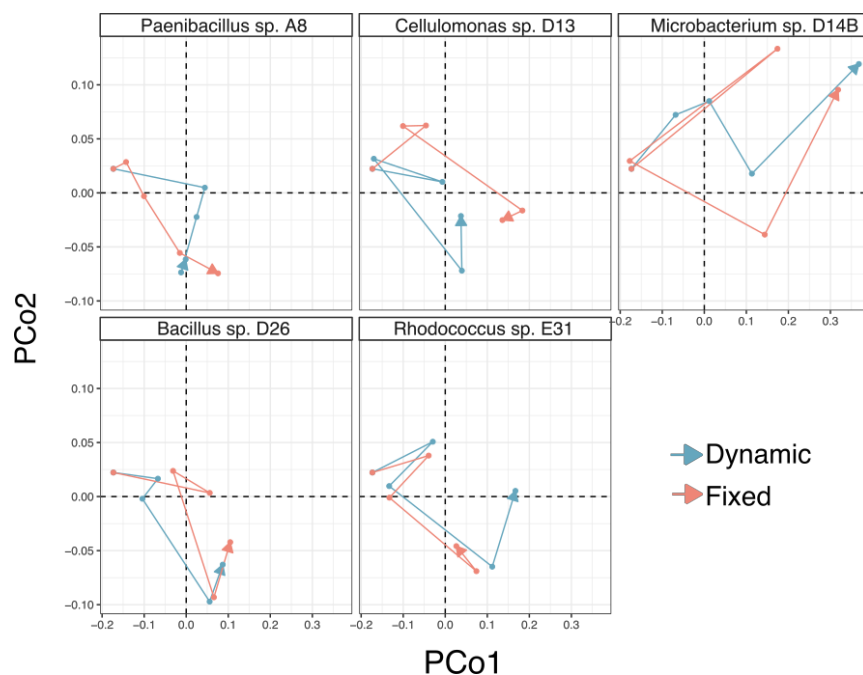
Figure 5.4 Evolutionary trajectory of focal species phenotypic traits when evolved in dynamic and fixed cocultures. An Increase in PCo1 indicates an increased ability to utilise arabinoxylan, xylan and pectin (labile substrates) while a decrease in PCo2 indicates increased ability to utilise recalcitrant substrates lignin, filter paper and β-glucan.

Significant effects of competitor evolution on the evolutionary trajectory of metabolic phenotype of the focal species were observed when the focal species was cocultured with *Bacillus* sp. D26 (main effect of fixed/dynamic; permutational

MANOVA, $F_{1,40}$ = 4.1, $P < 0.05$), *Rhodococcus* sp. E31 (main effect of fixed/dynamic; permutational MANOVA, $F_{1,40}$ = 3.1, $P < 0.05$), *Paenibacillus* sp. A8 (fixed/dynamic by time interaction; permutational MANOVA, $F_{3,40}$ = 4.0, $P < 0.01$) or *Microbacterium* sp. D14B (fixed/dynamic by time interaction; permutational MANOVA $F_{3,40}$ = 3.6, $P < 0.01$ respectively). Relative to the evolutionary trajectories driven by non-evolving competitors: the evolution of *Bacillus* sp. D26 inhibited focal species adaptation to utilise labile substrates; the evolution of *Rhodococcus* sp. E31 drove greater adaptation of the focal species to utilise both labile and recalcitrant substrates; the evolution of *Paenibacillus* sp. A8 drove greater focal species adaptation to utilise both labile and recalcitrant substrates at transfers four and eight; while the evolution of *Microbacterium* sp. D14B delayed adaptation of the focal species to utilise the labile substrates.

These data suggest that the observed differences in the relative abundance of competitor species in the dynamic polycultures is likely to have driven divergent evolutionary trajectories of the focal species populations. Specifically, higher relative abundance of *Bacillus* sp. D26 and *Paenibacillus* sp. A8 in the dynamic polycultures 40A, 41A and 42A at transfers 12 and 16 is likely to have driven the focal species adaptation to utilise recalcitrant substrates. Similarly, while the dynamic polyculture 39A had a low abundance of these species at transfer 12 (relative abundance of all three <0.5%), reinvasion of the community by *Bacillus* sp. D26 by transfer 16 may have driven the observed focal species adaptation to better utilise recalcitrant substrates. In contrast, the dynamic polycultures where these species were at low abundance after transfer eight, 37A and 38A, followed an alternate evolutionary trajectory specialising on labile substrates. Competitor evolution, especially in the case of *Bacillus* sp. D26, appears to reinforce the adaptive trajectory towards improved utilisation by the focal species of recalcitrant substrates. It is notable that in three of the four coevolved polycultures where the focal species adapted to recalcitrant substrates, *Bacillus* sp. D26 reinvades the community after falling to low relative abundance, suggesting that adaptive evolution of *Bacillus* sp. D26 could be playing an important role in determining the evolutionary trajectory of the focal species in these dynamic polycultures.

5.5 Discussion

Microbial communities are diverse and dynamic, with each constituent species engaged in a complex network of ecological interactions. By tracking the evolution of a focal bacterium embedded within a multispecies bacterial community we show that both the rate and trajectory of focal species evolution is shaped by the ecological and evolutionary responses of the competing community. Evolving in a competitive community increased the rate of phenotypic evolution of the focal species but prevented its adaptation to the abiotic environment relative to monoculture control populations, suggesting a trade-off with abiotic adaptation. Eco-evolutionary responses by the competing community changed the evolutionary trajectory of the focal species, leading to increased utilisation of recalcitrant substrates when evolved in dynamic polycultures in contrast to improved utilisation of labile substrates when evolved in fixed polycultures. Consequently, only those focal species populations evolved in fixed polyculture adapted to the ancestral biotic environment, evolving to outcompete the ancestral polyculture, presumably by exploiting the metabolic activities of competitors that would have been required to liberate these labile substrates from lignocellulose. The combined effects of species sorting and competitor evolutionary responses increased evolutionary divergence of focal species metabolic phenotype between replicate populations evolved in dynamic polyculture, suggesting that eco-evolutionary dynamics in competitive communities make the outcome of evolution less predictable.

Previous studies suggest that species interactions can either promote or limit evolution depending on the nature of the ecological interaction. Competition can reduce the rate of evolution of a species by reducing its abundance and therefore the limiting supply of genetic variation for natural selection to act upon (Barraclough, 2015; Johansson, 2008). Alternatively, the presence of biotic selection pressures may enhance the rate of evolution and alter the evolutionary trajectory of species by altering the ecological opportunities available to the focal species or by strengthening selection (Barraclough, 2015; Osmond and de Mazancourt, 2013). In

common with several other studies (Fiegna et al., 2015a, 2015b; Lawrence et al., 2012) we report that biotic interactions accelerated phenotypic evolution relative to monoculture controls. This suggests that competition with a multispecies community strengthened selection upon the metabolic phenotype of the focal species relative to abiotic selection alone. In contrast to other studies (Lawrence et al., 2012), monocultures here showed no improvement relative to the ancestor in their autonomous growth performance on wheat straw, perhaps due to this solid substrate being inherently more challenging for single species to metabolise compared to a liquid medium. However, while the monoculture evolved populations showed no improvement in their autonomous growth on wheat straw they did not lose adaptation to this abiotic environment unlike the polyculture evolved populations. This loss of abiotic adaptation when evolved in polyculture suggests a trade-off between biotic and abiotic adaptation.

When evolved in a fixed polyculture, the focal species evolved increased fitness against the ancestral polyculture community. Functional trait assays revealed that these populations evolved to better utilise xylan and arabinoxylan, the hemicellulosic components of lignocellulose. In the native lignocellulose structure these components are protected from enzymatic hydrolysis by lignin and depolymerisation of lignin is required to allow efficient degradation (Chen and Dixon, 2007; Ding et al., 2012; Zhao et al., 2012). Taken together these findings suggest that the metabolic activities of other species in the fixed polyculture liberated these labile substrates allowing the focal species to exploit them and adapt to improve its utilisation of them. Our co-culture experiments suggest that *Microbacterium* sp. D14B may have played a particularly important role in driving this evolutionary trajectory, since only those focal species populations evolved in co-culture with *Microbacterium* sp. D14B increased their utilisation of these hemicellulosic components of lignocellulose. To liberate these requires breakdown of lignin, and in common with other *Microbacterium* species (Li et al., 2005; Taylor et al., 2012; Wang et al., 2013) we have previously shown that this isolate has the ability to depolymerise lignin (Evans et al., 2017).

In contrast to the focal species populations evolved in fixed polyculture, those evolved in dynamic polyculture showed no fitness gains in competition against the ancestral polyculture community despite extensive evolutionary divergence of their metabolic phenotype. This is consistent with the Red Queen hypothesis which predicts continual evolution without concomitant increases in competitive fitness (Brockhurst et al., 2014). Increased fitness in competition with the ancestral community was associated with improved exploitation of labile substrates in focal species populations evolved in fixed polycultures. In contrast, while focal species populations evolved in dynamic polyculture improved in their utilisation of labile substrates to begin with, these populations later switched evolutionary trajectory towards improved utilisation the recalcitrant substrates (i.e. cellulose and lignin). This suggests that eco-evolutionary responses by the other species in the dynamic polyculture prevented focal species adaptation to exploit labile substrates. The labile substrates are likely to represent the most favoured and productive ecological niche in a lignocellulose degrading community because these substrates require less energy to degrade than the more recalcitrant cellulose (Gupta et al., 2012). In fixed polycultures the lack of eco-evolutionary response by the community allowed the focal species to adapt to the labile substrate niche and outcompete the other species. However, in the dynamic polycultures, it is likely that adaptation of one or more of the other species to occupy this niche prevented its exploitation by the focal species driving niche differentiation towards improved utilisation of the recalcitrant substrates. Interestingly, Rivett et al. (2016) similarly found that during bacterial succession, resource use switched from labile substrates during early succession to recalcitrant substrates later in succession. This shift in resource use was coupled with a reduction in the strength of negative interactions, potentially as a result of niche partitioning.

We observed greater diversification in metabolic phenotype among focal species populations evolved in dynamic polyculture as a result of eco-evolutionary changes by the other species in the competitive community. Specifically, dynamic polycultures formed two clusters that varied according to their community composition at the end of the experiment. Where these were dominated by the focal

species we observed less evolution towards recalcitrant substrate use. Notably, these communities contained higher relative abundances of *Cellulomonas* sp. D13 compared to other replicates, which is a species that we have previously shown to be highly effective at degrading lignocellulose (Evans et al., 2017). *S. maltophilia* has previously been shown to dominate a five species lignocellulose degrading community after 96 hours growth without significantly contributing to enzyme production (Jiménez et al., 2018a) by exploiting monosaccharides released by the enzymatic activity of other species. It may be the case that in these two replicate communities, selection on the focal species to adapt to utilise recalcitrant substrates was weaker due to the high effectiveness of *Cellulomonas* sp. D13 in lignocellulose metabolism. The four other dynamic polyculture communities were dominated by both the focal species and *Bacillus* sp. D26 at the end of the experiment and had higher relative abundances of *Paenibacillus* sp. A8 and *Microbacterium* sp. D14B. The focal species populations from these replicates evolved to better utilise recalcitrant substrates (cellulose and lignin). Our co-culture experiments revealed that evolution in coculture with *Bacillus* sp. D26 and *Paenibacillus* sp. A8 led to improved utilisation of the recalcitrant substrates by the focal species, suggesting that these competitors played an important role in driving this evolutionary trajectory in more complex communities. Moreover, the reinvasion by *Bacillus* sp. D26 observed in 3 dynamic polycultures coincided with the shift of the focal species evolutionary trajectory towards the recalcitrant substrates, suggesting that counter-adaptations of *Bacillus* sp. D26 in particular favoured improved use of recalcitrant substrates by the focal species.

The majority of experimental evolution to date has focussed on the evolution of species in isolation. Here we add to the growing body of evidence showing that the rate and trajectory of evolution is influenced by interactions with other species (Barraclough, 2015). We show that in communities, adaptation to the abiotic environment is likely to be constrained due to trade-offs with adaptation to biotic selection. Moreover, adaptation to the biotic environment is likely to be counter-acted by eco-evolutionary responses of the other species in the community. These Red Queen evolutionary dynamics in communities lead to greater divergence among

populations and make their evolutionary trajectories less predictable. Natural microbial communities are much more complex than those studied here, and it remains to be tested whether these patterns translate to even more diverse, natural communities. However the communities studied here are of a similar complexity to those being used in industrial applications (Cortes-Tolalpa et al., 2016; Jimenez et al., 2014) suggesting that it will be important to account for biotic interactions and their eco-evolutionary dynamics when selecting microbial communities to perform bio-industrial functions.

6 Genetic adaptation of *Stenotrophomonas* sp. D12 to growth on wheat straw involves multiple regulatory pathways

6.1 Abstract

Experimental evolution combined with genome sequencing allows the genetic basis of evolved phenotypes associated with adaptation to be identified. We obtained whole genome sequences for 84 evolved clones of *Stenotrophomonas* sp. D12 that had been experimentally evolved in Chapter 5 on wheat straw in monoculture or in the presence of other competing species. Despite displaying higher rates and different trajectories of phenotypic evolution, focal species clones evolved in the presence of competing species did not differ in the number or targets of mutations compared to clones evolved in monoculture. Instead, we identified several genetic loci targeted by parallel mutations that were associated with adaptation to growth on wheat straw *per se*. The majority of parallel mutations were in regulatory genes, including genes predicted to regulate catabolite repression suggesting the focal species may have evolved to better exploit a wider range of the carbon sources liberated from lignocellulose.

6.2 Introduction

The experimental evolution of microorganisms allows the identification of the strategies that species employ to adapt to their environment. While traditionally these studies focussed on the phenotypic adaptations of species, the development of high-throughput and low-cost sequencing technologies now allow the genetic adaptations underlying these phenotypic changes to be uncovered (Barrick and Lenski, 2013; Brockhurst et al., 2011b; Bruger and Marx, 2018; Dettman et al., 2012). The ability to include multiple replicate populations allows the identification of genes mutated in several replicate populations, i.e. parallel mutations. The probability of these parallel mutations occurring by chance is extremely low and so the presence of parallel mutations is usually attributed to natural selection acting on a specific gene (Bailey et al., 2017).

One area where experimental evolution is being used to uncover genetic responses is the adaptation of species to new or different resource environments. A common adaptive response to selection in a constant resource environment is the loss of metabolic functions. When essential metabolites are available in the growth media some bacterial species quickly lose the ability to synthesise these metabolites (Nilsson et al., 2005). Experimental evolution followed by genome sequencing revealed parallel mutations in both regulatory and structural genes lead to these auxotrophic phenotypes in *E. coli* mutants (D'Souza and Kost, 2016). Lee and Palsson (2010) identified four nonsynonymous mutations which allowed *E. coli* K-12 MG1655 to utilise the novel substrate L-1,2-propanediol. Two of the four mutations occurred in genes directly involved in L-1,2-propanediol catabolism while the remaining two occurred in a hypothetical protein and the 23S rRNA subunit. The ability of *Shewanella oneidensis* to catabolise the non-native substrate glucose has been shown to be achieved by deletions in the proximity of the transcriptional repressor *nagR* (Chubiz and Marx, 2017).

Adaptive mutations have also been identified which allow species to improve existing metabolic traits. Herring et al. (2006) identified parallel mutations in three genes which played a role in enhancing *E. coli*'s ability to utilise glycerol as a substrate. One of these genes was directly involved in glycerol catabolism while the other two affected global transcriptional patterns. A similar study identified the genes involved in efficient glycerol utilisation by *Saccharomyces cerevisiae* which were then introduced into an industrial strain to improve efficiency (Strucko et al., 2018). Two of the affected genes were metabolic proteins while two were global signalling or regulatory proteins. Perhaps the most striking example of the gain of a metabolic function during experimental evolution is from long-term evolution experiment (LTEE) where *Escherichia coli* has been evolved in a glucose limited medium for over 69,000 generations (Barrick et al., 2009; Good et al., 2017). Here, 1 of the 12 replicate populations evolved the ability to utilise citrate after 30,000 generations even-though the inability to metabolise citrate is a diagnostic characteristic of the species *E. coli* (Blount et al., 2008). Genomic analysis revealed that the emergence of this new phenotypic trait relied on multiple sequential mutations and the duplication of

a promoter to induce expression of a citrate transporter (Blount et al., 2012). While mutations in genes associated with metabolic functions are often clearly linked to the evolved phenotype (Dettman et al., 2012), in some cases the way in which genetic adaptations cause observed changes in phenotypic changes is not initially clear. For example, a mutation in the large subunit of carbamoyl-phosphate synthetase (carB) in *Pseudomonas fluorescens* caused colony-morphology switching through disruption of pyrimidine biosynthesis (Beaumont et al., 2009; Gallie et al., 2015) . Thus, evolutionary adaptation to simple environments can give rise to surprisingly complex innovations.

Genomic analyses of experimentally evolved species have identified mutations in both structural and regulatory genes (as per examples above) and several studies have addressed which type of gene is most commonly targeted by natural selection. The evolution of auxotrophy in *E. coli* was found to be more commonly caused by mutations in regulatory genes than structural genes, which was hypothesised to be due to more pervasive negative epistatic among structural gene mutations (D'Souza and Kost, 2016; D'Souza et al., 2015). It has been suggested that the type of gene targeted by adaptive evolution may depend on the environment in which the species has evolved (Dettman et al., 2012). In experiments conducted in nutrient limiting environments, such as where *E. coli* and *S. cerevisiae* were adapted to better utilise glycerol (Herring et al., 2006; Strucko et al., 2018), the ancestral genomes already possessed the necessary pathways for substrate catabolism and so mutations in the regulatory genes controlling expression of these pathways alone can be sufficient to produce a fitness advantage. However, adaptation to a novel substrate, such as the emergence of citrate utilisation in the LTEE (Blount et al., 2008), may require adaptive neo-functionalisation of structural genes.

Most experimental evolution studies focused on abiotic adaptation have been carried out under conditions where a single species was propagated alone in a simple defined environment. The effect on genome evolution of more complex ecological settings that better represent the conditions experienced by microbes in nature are rarely studied. While several studies have observed the phenotypic evolutionary

responses of bacteria evolving in communities adapting to a complex natural resource (Fiegna et al., 2015a; Lawrence et al., 2012), the genetic bases of the observed adaptation have not been characterised. Nevertheless, selection arising from biotic interactions is expected to alter evolutionary rates and trajectories, giving rise to different patterns of molecular evolution. For example, studies of bacteria-phage interactions have found that phage genes involved in host infection evolve at a higher rate in the presence of a coevolving bacterial host compared to a fixed host genotype (Paterson et al., 2010). Similarly, bacterial hosts that coevolved with more diverse phage communities showed higher rates of molecular evolution than those coevolving with a single phage (Betts et al., 2018). Understanding the genomic response to selection during adaptation to utilise complex natural substrates and how community context affects this response is a key next step.

We previously evolved *Stenotrophomonas* sp. D12 in environments where wheat straw was the sole carbon source, either with or without competing species, that were either fixed or dynamic (i.e. allowed to change ecologically or evolutionarily). At the phenotypic level, interspecific competition increased the rate of evolution of the *Stenotrophomonas* metabolic phenotype, while ecoevolutionary responses of the competing community drove greater diversification between populations and the different competing species drove different metabolic adaptations in *Stenotrophomonas* sp. D12. To understand the underlying genetic response to selection and how this varied among treatments we obtained the whole genome sequence for an evolved clone from each replicate population. Specifically, we sequenced the genomes of 84 evolved clones, of which: 12 had evolved in monoculture, 60 had evolved in coculture together with one of *Paenibacillus* sp. A8, *Cellulomonas* sp. D13, *Microbacterium* sp. D14B, *Bacillus* sp. D26, or *Rhodococcus* sp. E31, and 12 had evolved in polycultures containing all species. In half of the coculture and polyculture replicates the competing species had been fixed (i.e. replaced at each transfer with the ancestral genotype) whereas in the other half of replicates the competing species were ecoevolutionarily dynamic. Surprisingly, despite the higher rates of phenotypic evolution that were driven by interspecific competition, we observed no significant differences in either the number or targets of mutations

between monoculture, coculture or polyculture treatments. This suggests that the main genetic response to selection was associated with adaptation to wheat straw. Consistent with this, we observed parallel mutations in a number of genes involved in metabolism, with the highest number of parallel mutations occurring in genes controlling catabolite repression (i.e. the preferential use of glucose as a substrate).

6.3   Methods

6.3.1   Genome sequencing ancestral *Stenotrophomonas* sp. D12

To identify mutations in the evolved *Stenotrophomonas* sp. D12 clones, we first obtained the complete closed genome sequence of the ancestral strain. A single colony was resuspended and grown overnight in nutrient broth at 30°C shaken at 150 rpm. Cells were harvested, and genomic DNA was extracted using Qiagen Genomic Tips 20G following the manufacturer's instructions (Qiagen Genomic DNA handbook, 2015). The genomic DNA was sequenced on the PacBio Sequel System (Pacific Biosciences) by NERC Biomolecular Analysis Facility at the University of Sheffield, and on Illumina MiSeq by MicrobesNG at the University of Birmingham (www.microbesng.com). PacBio sequencing produced 377,905 reads with an average length of 5,597 bp. Illumina MiSeq sequencing produced 337,653 2x250 paired-end reads with a median insert size of 529 bp representing 59x genome coverage. Canu (Koren et al., 2017) was used for de novo assembly of the PacBio reads using the default settings. Following correction and trimming, 334,634 reads with an average length of 5,148 bp representing 370x genome coverage were assembled into two contigs with a total length of 4,682,283 bp. Circlator (Hunt et al., 2015) using the default settings combined these into a single contig which was polished with Pilon (Walker et al., 2014) using Illumina Miseq reads. The assembled genome was annotated using Prokka (Seemann, 2014). The KEGG automated annotation server (KAAS) (Moriya et al., 2007) and InterPro (Apweiler et al., 2001) were used for functional annotation.

6.3.2 Genome sequencing evolved clones

A single clone from each evolved population was grown overnight in nutrient broth at 30°C then spread onto nutrient agar and incubated at 30°C overnight. Cells were scraped from half the agar plate and transferred to bead tubes provided by MicrobesNG. Tubes were then sent to MicrobesNG for genomic DNA extraction and genome sequencing on Illumina Miseq using 2x250 paired end reads. Forward and reverse reads were aligned to the Prokka annotated reference genome and variant were called using breseq with default settings (Deatherage and Barrick, 2014).

6.3.3 Statistical analysis

Analysis of variance (ANOVA) was used to compare the number of mutations between different evolutionary treatments. To account for the unbalanced design, we used weighted means and Type I Sums of Squares. Diversity (monoculture, coculture, polyculture), community (each coculture and polyculture) and type of biotic interaction (dynamic, fixed) were added to the model sequentially. Altering the order of factors did not alter the result of the ANOVA. Multivariate analysis of variance (MANOVA) was used to compare the type of mutations between diversity, community and biotic interaction treatments. Permutational MANOVA was used to test whether different community and biotic interaction treatments resulted in different sets of mutated genes. Statistical analyses and figures were generated in R version 3.5.1 (R Core Team, 2018) using the vegan (Oksanen et al., 2018) and ggplot2 (Wickham, 2016) packages.

6.4   Results

6.4.1   Genome sequence of *Stenotrophomonas* sp. D12

The genome sequence of *Stenotrophomonas* sp. D12 consisted of a single circular chromosome 4,659,921 bp in size and with a GC content of 66.2%. Annotation identified 4180 coding sequences (CDS) with 13 rRNAs and 78 tRNAs. Whole genome based average nucleotide identity (ANI) revealed 87.2% similarity to *Stenotrophomonas maltophilia* K279a and 98.4% similarity to *Stenotrophomonas sp.*

MYb57, a strain isolated from the microbiome of *Caenorhabditis elegans* (Zhang et al., 2017).

6.4.2 Genetic basis of *Stenotrophomonas* sp. D12 adaptation to wheat straw

Eleven of the 84 clones sequenced contained no mutations. In the remaining 73 clones, we found 138 mutations of which 89.2% were in coding regions. There were 82 non-synonymous single-nucleotide polymorphisms (SNP), 24 synonymous SNPs, and 17 small insertions and deletions. There was no difference in the number of mutations (ANOVA with total number of mutations as the dependent variable: main effects of diversity $F_{2,81} = 0.6$, $P > 0.05$, community $F_{6,77} = 1.3$, $P > 0.05$ and treatment $F_{2,81} = 0.6$, $P > 0.05$) or type of mutation (MANOVA with the number of each type of mutation [intergenic, indel, nonsynonymous, synonymous] as the dependent variables, main effects of diversity $F_{2,81} = 0.7$, $P > 0.05$, community $F_{6,77} = 1.0$, $P > 0.05$ and treatment $F_{2,81} = 1.0$, $P > 0.05$, Figure 6.1) between evolutionary treatments.
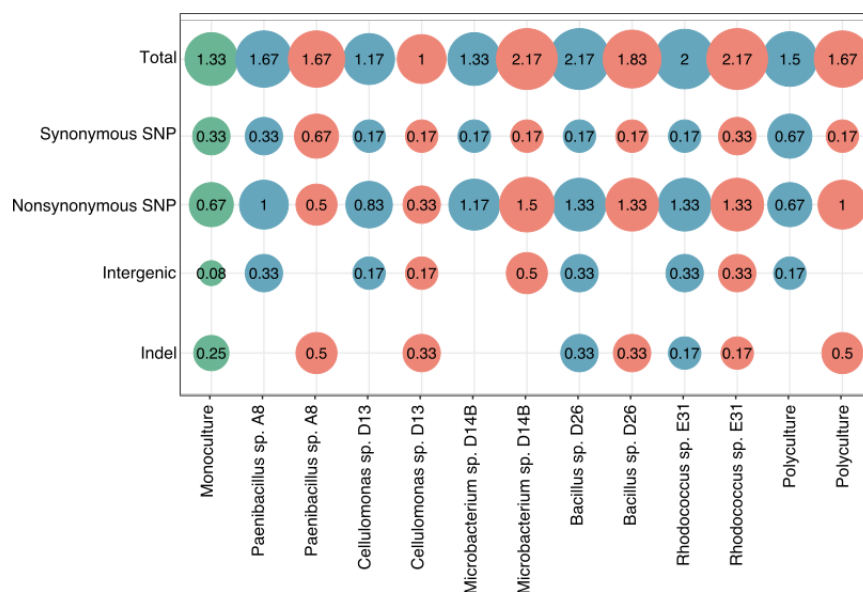
Figure 6.1 Average number of each type of mutation across the replicate populations (N = 12 for monocultures, N = 6 for all other treatments).

Mutations occurred in 84 genes of which 23 contained only synonymous mutations and were not analysed further. Among the 61 genes targeted by nonsynonymous mutations, we observed multiple cases of gene level parallelism with 48 of these nonsynonymous mutations occurring in just ten genes (Figure 6.2, Table 6.1). Of

these, 31 displayed nucleotide level parallelism (NLP) with mutations occurring at the exact same nucleotide location in multiple clones. Loci targeted by parallel mutations in multiple independent evolved clones suggests that these genes were the targets of natural selection. The occurrence of parallel mutations did not significantly differ between evolutionary treatments (permutational MANOVA, main effects of diversity, treatment and community: $F_{2, 71} = 1.1$, $P > 0.05$, $F_{1, 71} = 1.1$, $P > 0.05$ and $F_{4, 71} = 1.2$, $P > 0.05$) suggesting these genes may be involved in general adaptations to growth on wheat straw rather than specific responses to the presence of competing species.
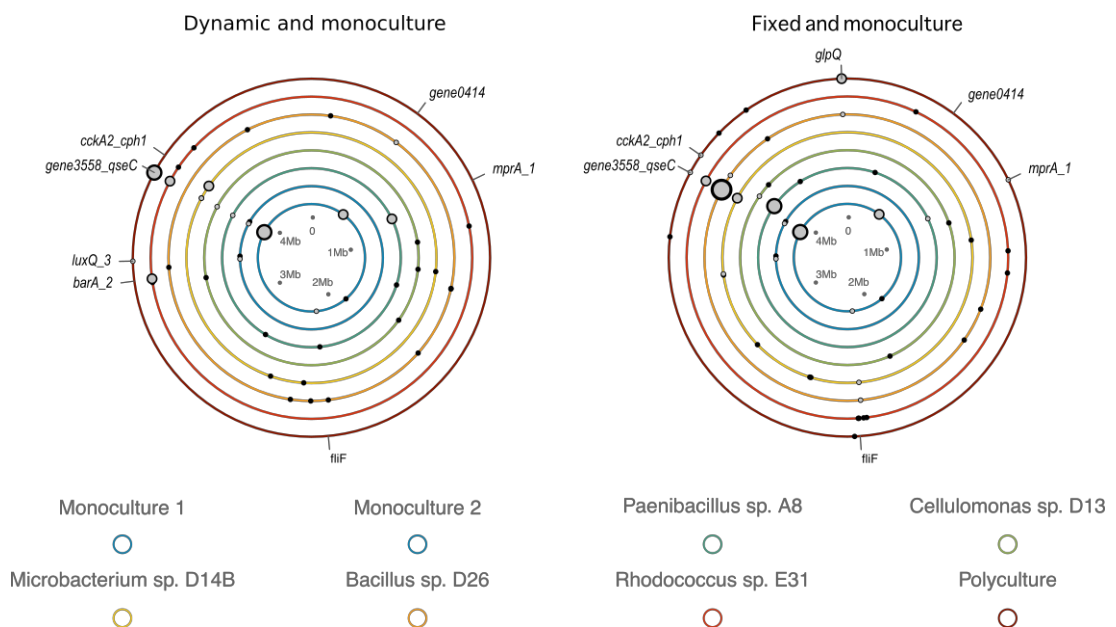
Figure 6.2 Summary of genes containing mutations in each evolutionary treatment. Rings represent the Stenotrophomonas sp. D12 genome with each ring representing the six clones sequenced from each evolutionary treatment. Nonsynonymous SNPs and indels are represented by dots with the size of the dots scaled by the number of mutations in that gene within each treatment. Genes with parallel mutations in more than one clone are labelled (using annotation generated by Prokka) and coloured grey. Rings are coloured by the community clones were evolved in. Circle plots showing individual replicates are provided in Supplementary Figure 6.1

Hereafter, the analysis is focused on the ten loci most affected by parallel mutations across all treatments. Six of the ten genes, containing 31 (64.6%) of the parallel mutations, were predicted to be involved in phosphorelay signal transduction systems (histidine kinases and response regulators, Table 6.1). This suggests an important role for changes in gene regulation in adaptation to the wheat straw environment.

Fourteen parallel mutations occurred in gene *3559* (originally annotated as *qseC* encoding a sensory histidine kinase, Figure 6.2) while eight mutations occurred in the neighbouring gene *3558* (Figure 6.2) which has sequence similarity to an ABC transporter substrate-binding protein. Analysis of the *Stenotrophomonas* sp. D12 genome suggests that these genes form a three-gene operon with *tctD* (mutated in one clone) similar to that found in *Xanthomonas campestris* pv. *vesicatoria* (Tamir-Ariel et al., 2011). Gene *3559* shares 100% sequence identity with *Stenotrophomonas* sp. 92mfcol6.1 gene *tctE* and this, along with its proximity to *tctD*, led us to reannotate this gene as a *tctE*. There were eight mutations in gene *3558*, half of which were in clones evolved in fixed coculture with *Bacillus* sp. D26 (Figure **6.2**, Supplementary Figure 6.1). Fourteen clones contained mutations in *tctE* including four clones evolved in monoculture, four clones evolved in coculture with *Rhodococcus* sp. E31 and four clones evolved in polyculture. There was at least one clone containing a mutation in either gene *3558* or *tctE* in all treatments except clones evolved in fixed coculture with *Paenibacillus* sp. A8 or *Cellulomonas* sp. D13, or in dynamic coculture with *Bacillus* sp. D26.

We observed four parallel mutations in each of the genes *3633* (annotated *cckA_2*) and *3635* (cph1) which are members of the same operon and encode a sensor histidine kinase and phytochrome-like protein, respectively. All eight of these mutations occurred in clones that had evolved in the presence of competing species. Three of the four clones with mutations in *cph1* evolved in fixed coculture with *Paenibacillus* sp. A8 while no clones evolved in dynamic coculture with *Paenibacillus* sp. A8 contained mutations in this gene. In contrast, two clones evolved in dynamic coculture with *Microbacterium* sp. D14B contained mutations in *cckA_2* while no mutations were found in clones evolved in fixed coculture with *Microbacterium* sp. D14B.

gene#	#mutations	Prokka annotation	BLASTP top hit		Functional annotations: GO terms
			Non-redundant protein sequence	UniProtKB SwissProt (identity, E-value)	
3558	8	hypothetical	ABC transporter substrate-binding protein	pgtC (33%, 0.002)	No GO terms
3559	14	qseC	tctE	qseC (29%, 9e-35)	F:phosphorelay sensor kinase activity P:phosphorelay signal transduction system F:ATP binding C:intracellular C:integral component of membrane P:signal transduction by protein phosphorylation
3633	4	cckA_2	Hybrid histidine kinase/response regulator	cckA (29%, 2e-34)	F:phosphorelay sensor kinase activity P:phosphorelay signal transduction system P:signal transduction P:phosphorylation F:transferase activity, transferring phosphorus-containing groups
3635	4	cph1	PAS domain S-box protein	cph1 (34%, 3e-36)	F:phosphorelay sensor kinase activity P:phosphorelay signal transduction system F:ATP binding C:intracellular C:integral component of membrane P:signal transduction by protein phosphorylation
0731	4	mprA_1	DNA-binding response regulator	mprA (43%, 5e-50)	P:phosphorelay signal transduction system F:DNA binding C:intracellular P:regulation of transcription, DNA-templated
3164	3	barA_2	Hybrid histidine kinase/response regulator	luxO (30%, 2e-8)	P:phosphorelay signal transduction system F:protein histidine kinase activity C:intracellular F:protein-glutamate methylesterase activity P:peptidyl-histidine phosphorylation
0414	3	hypothetical	hypothetical	No hits	C:integral component of membrane
4266	3	glpQ	glpQ	glpQ (36%, 8e-54)	P:lipid metabolic process F:phosphoric diester hydrolase activity
2090	3	fliF	fliF	fliF	F:motor activity C:bacterial-type flagellum basal body, MS ring C:integral component of membrane P:bacterial-type flagellum-dependent cell motility
3236	2	luxQ_3	Hybrid histidine kinase/response regulator	luxQ (35%, 3e-60)	F:phosphorelay sensor kinase activity P:phosphorelay signal transduction system C:intracellular C:integral component of membrane P:peptidyl-histidine phosphorylation P:signal transduction by protein phosphorylation

Table 6.1 Summary of genes which contained parallel mutations. Functional annotations were conducted using InterPro and predicted gene ontology (GO) terms are provided.

Four clones contained mutations in gene *0731* (annotated as *mprA_1*, Figure 6.2). Sequence identity and functional annotation suggests this gene is a DNA-binding response regulator with 43% sequence identity to Mycobacterial persistence regulator A (mprA, Table 6.1). Clones with mutations in this gene evolved in the presence of competing species, two NLP mutations were observed in clones evolved in dynamic coculture with *Paenibacillus* sp. A8.

There were three parallel mutations in gene *3164* and two parallel mutations in *3236* (annotated *barA_2* and *luxQ_3* respectively, Figure 6.2). Gene *3164* shares 92% sequence similarity to the signal transduction histidine kinase protein barA (BLASTP non-redundant sequence search) which forms a two-component regulatory system with uvrY (where we also observed a singleton mutation in a monoculture evolved clone, Supplementary Figure 6.1) while gene *3236* shares 80% sequences identity to luxQ (BLASTP non-redundant sequence search) an autoinducer 2 (AI-2) response regulator involved in quorum sensing.

Three clones carried parallel mutations in gene *2090* encoding the flagellar M-ring protein fliF. In addition to parallel mutations in this gene, we also observed singleton mutations in *fliG* (flagellar motor switch protein), *fliM* (flagellar motor switch protein), *flgI* (flagellar P-ring protein) and *flgK* (flagellar hook-associated protein 1). In total, we found seven mutations in flagellum-associated genes. The impact of these mutations, specifically whether they impede cell motility, remains to be determined. As clones were evolved in liquid, well-mixed cultures, flagellum mediated motility may not have been necessary, and loss of function may have provided a competitive advantage.

The remaining two out of the ten genes containing parallel mutations were in gene *0414*, a membrane bound hypothetical protein, and gene *4266*, a periplasmic glycerophosphodiesterase phosphodiesterase (GP-PDE). Functional annotation of gene *0414* indicates that this is a membrane bound protein though it has no significant similarity to proteins in the SwissProt database. The presence of three parallel mutations in this gene, two in clones evolved in monoculture, suggests a role

in adaptation to growth on wheat straw. Gene *4266*, annotated as *glpQ* which encodes a GP-PDE, contained mutations in three clones, two of which evolved in fixed polycultures. GP-PDEs catalyse the hydrolysis of glycerophosphodiesters to produce an alcohol and glycerol-3-phosphate which plays a major role in glycolysis and phospholipid biosynthesis. We also observed a singleton mutation in *glpD* (Supplementary Figure 6.1) which encodes an aerobic glycerol-3-phosphate dehydrogenase. Both these enzymes play central roles in various biosynthetic pathways suggesting that these mutations would have substantively altered the metabolism of the cell.

6.5 Discussion

To identify the genetic basis of adaptation to wheat straw we sequenced a randomly chosen evolved clone of *Stenotrophomonas* sp. D12 from each of 84 independently evolved populations that had evolved with wheat straw as the sole carbon source either with or without competing species. Among the 84 sequenced evolved clones, we found that 11 clones contained no mutations while the remaining 73 contained an average of 1.89 mutations each. Despite significant differences in the rate and trajectory of the evolution of the metabolic phenotype of the *Stenotrophomonas* sp. D12 populations, we did not find differences in the number or genes targeted by mutations among evolutionary treatments. This suggests that the majority of the observed genomic evolution was in response to the wheat straw environment *per se*. Ten genes were repeatedly targeted by mutation across multiple independently evolving populations, and such parallel evolution suggests that these mutated loci were targets of natural selection and associated with adaptation to the wheat straw environment.

The operon containing the two-component signal transduction genes *tctD/tctE* and gene *3558* was mutated in 23 of the 84 sequenced evolved clones. The remaining parallel evolving genes were mutated in a maximum of four clones suggesting either that *tctE/3558* genes were under stronger selection or possess an intrinsically higher mutation rate (Moxon et al., 1994). The *tctD/tctE* operon has previously been shown

to positively regulate citrate uptake in *Xanthomonas campestris* pv. *vesicatoria* with the presence of only *tctD/tctE* sufficient to allow citrate uptake while the presence of gene *3558* was not essential for function (Tamir-Ariel et al., 2011). It has also been reported that marker exchange mutagenesis of *tctD/tctE* leads to reduced virulence of *Xanthomonas oryzae* pv. *oryzae* which was associated with a reduction in xylanase and cellulase production by the mutants (Cho et al., 2010). The function of gene *3558* is yet to be determined and it would be interesting to compare the phenotypic effects of *tctE* mutants to *3558* mutants. The *tctD/tctE* two-component system is predicted to be involved in catabolite repression, i.e. preferential utilisation of glucose (or the most energy efficient carbon source available) and repression of metabolism of other sugars, in *Salmonella typhimurium* (Widenhorn et al., 1989). It is likely therefore that mutations in *tctD/tctE* may reduce catabolite repression, allowing utilisation of the various five and six carbon sugars present in lignocellulose in addition to glucose. Interestingly, negation of catabolite repression is one of the routes being explored to increase the efficiency of lignocellulose bioconversion of engineered strains (Flores et al., 2017; Vinuselvi et al., 2012), so understanding the effects of mutations in these genes may provide insights relevant to industrial processes.

Four mutations were observed in genes annotated as *barA* and *uvrY*, which likely form a two-component signal transduction system. Orthologs of this system regulate carbon metabolism through the carbon storage regulation (csr) system (Pernestig et al., 2003), as well as being involved in regulating virulence, biofilm formation, motility, stress resistance and quorum sensing (Mondragón et al., 2006; Zere et al., 2015). The csr system controls the expression of more than 700 genes and has been identified as a promising target to improve biofuel production (Edwards et al., 2011; McKee et al., 2012). Mutations in *barA* and *uvrY* have previously been shown to reduce the fitness of *E. coli* in glucose environments by inhibiting the switch between glycolytic and gluconeogenic pathways. However, in more complex environments containing alternative carbon sources, these mutants were able to outcompete the wild type strain (Pernestig et al., 2003). It seems likely therefore that mutations in *uvrY/barA* may increase *S. maltophilia* D12 utilisation of sugars other than glucose by inhibiting glycolysis. Consistent with this, in addition to *barA* and *uvrY* mutations, we

also identified parallel and single mutations in two genes involved in glycerol metabolism, *glpQ* and *glpD*. These mutations are predicted to cause loss of function and may therefore also reduce the efficiency of glycolysis leading to enhanced metabolism of other sugars.

Aside from carbon metabolism, another function targeted by parallel mutation was flagellar motility, including three mutations in the M-ring protein fliF. Such mutations were likely to lead to loss of flagellar motility, and similar loss of flagellar function has been previously observed in multiple experimental evolution studies (Ensminger et al., 2012; Maughan and Nicholson, 2011; Sandberg et al., 2014). Biosynthesis and use of the flagellar machinery incurs high energy costs e(Martínez-García et al., 2014)s and loss of function mutations allow this energy to be reallocated to other functions if motility is not required. Here, populations were grown in well mixed liquid media where the lack of chemical gradients may have selected against maintenance of a functioning flagellum. Alternatively, a functioning flagellum may have been selected against if the focal species formed a biofilm on the surface of wheat straw. Experimental evolution of *Cellulomonas fimi* under similar growth conditions, i.e. aerobic growth on wheat straw, appeared to lead to an increased ability of *C. fimi* to bind to wheat straw particles (Ihsan, 2017). This phenotype is difficult to measure quantitatively but if a similar adaptation has occurred by *Stenotrophomonas* sp. D12, this may explain the presence of multiple mutations in flagellum genes.

In common with previous experimental evolution studies performed in a range of environments (Barbosa et al., 2017; O'Rourke et al., 2015; Zhou et al., 2015), we observed a predominance of adaptive mutations in genes encoding two-component or phosphorelay signal transduction pathways, which typically regulate the expression of many other genes (Hoch, 2000). Evolution of regulatory genes is consistent with the idea that the early stages of adaptation to a new environment is driven by a small number of mutations which have large phenotypic effects (Dettman et al., 2012). Previous studies of single species adapting to single carbon source environments often identify mutations in a single regulatory system (Herring et al., 2006; Strucko et al., 2018). By contrast, we identified parallel mutations in five

distinct signal transduction pathways suggesting that more complex environments conditions impose selection upon a wider range of functions. While two of these signalling pathways are predicted to be involved in carbon metabolism, further characterisation is required to determine the functions of the other three signal transduction systems in the cell. Some clones contained mutations in more than one signalling pathway and it may be an interesting topic for future study to identify the effects of multiple regulatory mutations on fitness by reconstruction of single and double mutants in the ancestral genotype, in particular whether there are epistatic effects between mutations in these multiple signalling pathways.

Surprisingly, we did not find significant differences in the genetic response of *Stenotrophomonas* sp. D12 to the evolutionary treatments. This stands in contrast to the differences in metabolic phenotype previously observed at the population level, which showed greater evolutionary divergence from the ancestor due to interspecific competition compared to monocultures. There are two possible explanations for this discrepancy: first the differences in metabolic phenotype could be due to phenotypic responses and thus did not require mutational changes, or second, they could be an emergent property of a diverse population that was not captured through sequencing of single clones. Sequencing of additional clones or the populations will be required to distinguish between these possibilities.

Taken together these findings suggest that adaptation to better utilise wheat straw involves regulatory mutations whose effects are likely to impair catabolite repression and carbon storage processes, allowing evolved *Stenotrophomonas* sp. D12 to utilise a wider range of the sugars liberated from lignocellulose. It is interesting that both of these metabolic functions have been identified as potential targets for engineering improved strains for biofuel applications, suggesting that strain improvement by natural selection could readily achieve the same outcome.

6.6   Supplementary Figures

Paenbacillus A8

Cellulomonas sp. D13

Microbacterium sp. D14B

Bacillus sp. D26

Rhodococcus sp. E31

Polyculture

Monoculture

Dynamic   Fixed

Monoculture
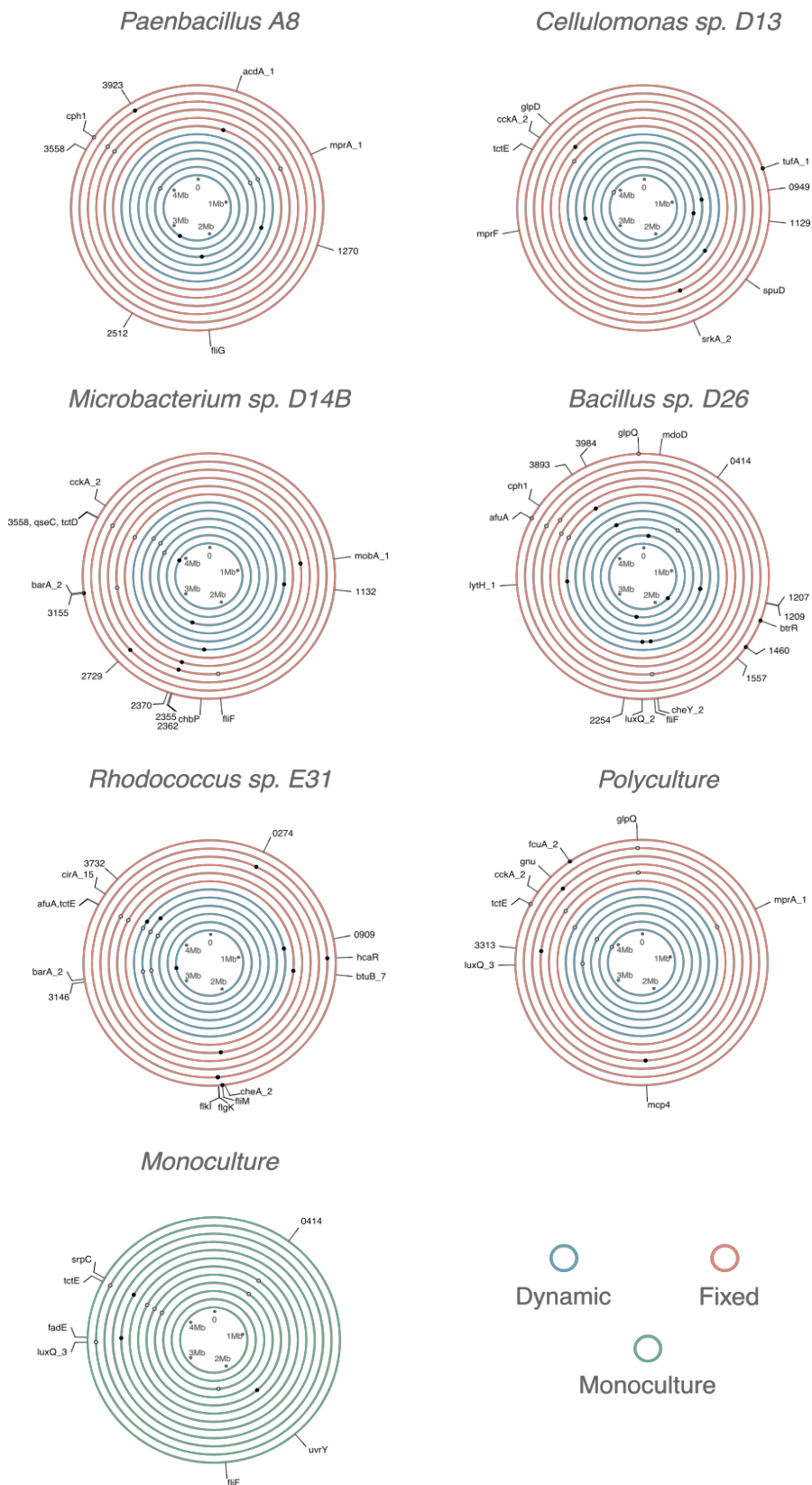
Supplementary Figure 6.1 Plot of *Stenotrophomonas sp.* D12 genomes of each sequenced clone. Genes with parallel mutations in more than one clone are labelled (using annotation generated by Prokka) and coloured grey. Rings are coloured by treatment

7   General Discussion

Lignocellulose represents the most abundant source of fixed carbon on the planet and is a promising substrate to produce biofuels. Uncovering methods to efficiently degrade this complex polymer remains a key challenge that must be overcome for the long-term viability of these promising fuel sources. A promising approach is to study naturally occurring microbial communities to uncover their lignocellulolytic potential (Brune, 2014; Wei et al., 2012; Woo et al., 2014b). In this thesis, various lignocellulolytic bacteria were isolated from wheat straw compost enrichment cultures and assayed for their ability to degrade the components of lignocellulose (chapter 2). I found that while communities of these isolated bacteria were more efficient lignocellulose degraders than any of the species in monoculture, two species in particular drove community productivity (chapter 3). My results indicated that the ability of these species to drive community productivity was due to their ability to degrade cellulose, this was supported by genome sequencing which revealed both these species possess a range of enzymes required for degradation of both cellulose and hemicellulose (chapter 4). I have also presented data that supports the utility of experimental evolution as a tool to improve lignocellulose degradation (chapter 5). The presence of competing species accelerated the phenotypic evolution of *Stenotrophomonas* relative to monocultures. Importantly the trajectory of focal species metabolic evolution was determined by the composition of the community and by the eco-evolutionary responses of the competing species. This led, in dynamic polycultures, to improved growth performance on recalcitrant components of lignocellulose by *Stenotrophomonas*, suggesting that community experimental evolution could be a powerful tool for directing the metabolic evolution of industrial strains. Genome sequencing of evolved genotypes revealed the genetic bases of improved lignocellulose degradation in *Stenotrophomonas* (chapter 6). Several genetic loci were repeatedly targeted by mutations, including genes involved in the regulation of carbon storage and carbon metabolism. This suggests that these genes may be useful targets for engineering more efficient industrial strains. Overall, these results indicate that compost is a promising source of culturable, lignocellulolytic bacteria and further suggest that community experimental evolution is a powerful tool for strain improvement. I have discovered key regulatory genes involved in

wheat straw adaptation that should be investigated further as potential targets for engineering industrial strains.

7.1   Design of communities for consolidated bioprocessing

Consolidated bioprocessing (CBP) seeks to replace physiochemical pretreatments and enzymatic hydrolysis of lignocellulose with the direct bioconversion to valuable products by a single engineered strain or a community of microorganisms. CBP has the potential to reduce costs and improve the efficiency of industrial processes (Xu et al., 2009). While traditionally industry utilises the single strain approach, the use of microbial communities in CBP is gaining more attention (Brenner et al., 2008; Shong et al., 2012). As with previous studies (Bell et al., 2005; Wohl et al., 2004) we found that lignocellulose degradation increased with community diversity. As an insoluble substrate, the degradation of lignocellulose requires the action of multiple enzymes with distinct activities that are believed to operate synergistically (Cobucci-Ponzano et al., 2015; Lynd et al., 2002). The design of efficient communities for CBP requires a better understanding of how species function and adapt when interacting with other species (Widder et al., 2016).

It has been suggested that identifying species which possess complementary enzyme activity are likely to produce more productive communities compared to species which compete for the same resources (Shong et al., 2012). However, similar to previous studies (Fetzer et al., 2015; Wohl et al., 2004), we found that the productivity of lignocellulose degrading communities increased with species diversity despite the presence of functional redundancy between constituent species. Particularly, we found that productivity was driven by the ability of constituent species to utilise cellulose as a substrate. Thus maximising species complementarity may not necessarily yield more highly performing communities.

Using a biodiversity and ecosystem functioning (BEF) experiment we identified two species, *Cellulomonas* sp. D13 and *Paenibacillus* sp. A8, which significantly improved community productivity. Genome sequencing revealed that these species both possess a diverse range of cellulase and xylanase enzymes with similar numbers of

carbohydrate active enzymes as previously sequenced members of these genera (Christopherson et al., 2013; López-Mondéjar et al., 2016a). While these results indicate that BEF experiments are useful for identifying highly performing, it may be the case that many of the culturable lignocellulolytic bacteria have already been identified by other methods, and that the discovery of novel lignocellulolytic bacteria and enzymes requires culture independent methods (Mori et al., 2014).

Genome sequencing and BEF experiments could be combined to identify highly functioning communities/individuals and define the functional traits required for efficient lignocellulose degradation. One issue associated with predicting species functions from the genome sequence is that while enzymes may be present in a species' genome, they may not necessarily be expressed (López-Mondéjar et al., 2016a) . A promising approach may be to combine genomics with proteomics and transcriptomics to identify the genes/enzymes that are expressed under different growth conditions and that result in increased community productivity (López-Mondéjar et al., 2016a; Salvachua et al., 2013; Wakarchuk et al., 2016). This may help to ensure growth conditions are used which maximise the expression of the broadest range of enzymes. In addition, identifying the regulatory pathways that control the expression of enzymes which significantly improve productivity could help to increase enzyme yields and improve the efficiency of industrial strains.

7.2   Adaptive evolution as tool to improve lignocellulose degradation

Microbes adapt rapidly in response to a range of abiotic and biotic selection pressures. Researchers can use this to their advantage in directed evolution experiments in which specific selection pressures are applied that result in the improvement of a desirable trait (Lin et al., 2016; Wang et al., 2018). I found that a focal species, *Stenotrophomonas* sp. D12, evolved more when grown on lignocellulose in the presence of competing species than when in isolation. Interestingly, the trajectory of evolution was altered by the identity and the ecoevolutionary responses of the competing species. These results have two main implications. First, evolving species in the presence of competitors increases the rate of adaptation and may enhance the extent of functional trait improvement by

directed evolution experiments. Second, by carefully designing the structure of the competing community, the direction of evolution can be altered to produce favourable outcomes that may not occur in monoculture evolution. For example, here we have shown that in the absence of ecoevolutionary adaptations, the focal species evolved enhanced utilisation of the more readily degradable labile substrates. Alternatively, in the presence of ecoevolutionary adaptations by the competing species, the focal species exhibited increased utilisation of the recalcitrant substrates. By including the appropriate competitors, my results suggest that it is possible to drive species to evolve enhanced utilisation of substrates that are resistant to degradation.

While total community productivity was not measured here, it has previously been shown that as communities evolve, they tend to become more productive as a result of niche partitioning and cross-feeding (Fiegna et al., 2015a; Lawrence et al., 2012). It is reasonable to assume therefore that experimental evolution could help to improve the efficiency and the stability of microbial communities for CBP. However, here we found that ecoevolutionary adaptations of competing species can cause replicate populations to diverge in terms of their structure and phenotypic traits. Community composition changed drastically throughout the experiment likely due to both species sorting and the evolution of constituent species. These changes in community composition were coupled with alterations in the evolutionary trajectory of the focal species. Microbial communities are complex and several ecoevolutionary outcomes may be possible. However, we found that dynamic communities followed two main trajectories suggesting these outcomes may be predictable.

Experimental evolution has already been applied to improve the native lignocellulolytic activities of various microorganisms (Lin et al., 2016; Wang et al., 2018). However, these experiments traditionally evolve the species of interest in monoculture. Our results suggest that the rate of evolution may be increased by evolving these species in the presence of competitors. As well as increasing the rate of evolution, the identity of the competitors led to distinct evolutionary trajectories. Specifically, the focal species evolved increased utilisation of labile substrates when

evolved against *Microbacterium* sp. D14B and increased utilisation of recalcitrant substrates when evolved against *Bacillus* sp. D26. Determining the functional characteristics of competitors which cause specific evolutionary trajectories may allow directed evolution of desirable traits.

Using genome sequencing of single clones we were not able to clearly link the observed phenotypic evolution to genomic adaptations. There are two main explanantions for this. First, we only sequenced a single clone from each replicate population and will have missed mutations present in other clones in the population. This is particuarly likely in populations that had evolved with dynamic communities as these popualtions are likely to be more geentically diverse. Alternatively, the phenotypic adaptations we observed may be a result of phenotypic plasticity. Rivett et al. (2016) identified a shift from use of labile to recalcitrant substrates during bacterial succession and suggested that adaptation to labile substrates was a result of phenotypic plasticity while adaptation to recalcitrant substrates required evolutionary (i.e. genetic) adaptation. Although we did not identify any genes that were targeted by specific evolutionary treatments, we did identify genes involved in adaptation of *Stenotrophomonas* sp. D12 to growth on wheat straw. The majority of parallel mutations we identified occurred in genes predicted to be involved in catabolite repression, i.e. the preferential use of glucose as a substrate. Mutations in these genes potentially allowed *Stenotrophomonas* sp. D12 to utilise more of the sugars liberated during lignocellulose degradation. It has been suggested that catabolite repression regulatory systems are one of the main contributors to the low efficiency of lignocellulose bioconversion as multiple sugars present in ligncoellulose remain unutilised (Flores et al., 2017; Vinuselvi et al., 2012). It is important to note however that catabolite repression involves a range of transcriptional, post-transcriptional, translational and biochemical regulations and so the effect these mutations have on catabolite repression must be further investigated. In addition, previous studies have found that modifications to catabolite repression are often deleterious to growth (Beg et al., 2007; Brückner and Titgemeyer, 2002), potentially explaining why evolved clones grew less well in monoculture than the ancestor. The

potential of these genes as targets to improve the efficiency of lignocellulose degradation requires further study.

There were also several mutations in genes predicted to be involved in carbon storage regulation (csr) which has also been identified as a target to improve biofuel production (McKee et al., 2012). Similar to catabolite repression, disruption of genes involved in csr have been shown to decrease growth rate but also reduce the yield of acetone, butanol and ethanol produced by fermentation (Tan et al., 2015). In contrast, the presence of parallel mutations in these genes suggests they confer a fitness advantage to *Stenotrophomonas* sp. D12 and detemining the nature of these fitness benefits may indicate ways to disrupt these systems and maximise lignocellulose degradation.

7.3   Concluding remarks

The search for novel lignocellulosic microorganisms by culturable methods often identify the same species. It is likely therefore that to find new enzymes, the application of culture independent 'omics techniques are required. However, here we suggest that more efficient lignocellulose degradation can be achieved by maximising and improving the functional traits of those culturable species already isolated. Using ecological experiments we can identify productive species combinations for use in CBP and furthermore, experimental evolution can then be used both to improve the productivity of these communities further and to identify gene targets for engineering industrial strains. In conclusion, in order to achieve efficient CBP, a detailed understanding of both the ecology and evolution of lignocellulolytic microorganisms is required in addition to a mechanistic understanding of lignocellulose degradation.

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