Roles and recruitment of *Streptomyces* species in the wheat root microbiome

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Abstract

The demand for staple food crops is rising alongside the world's population and matching supply to demand is one of the greatest challenges facing global food security. A key strategy is to better understand and exploit microorganisms that associate with plant roots, thus improving growth and reducing the incidence of disease. *Streptomyces* is a genus of bacteria that are consistently found in soil and are well known for their diverse and specialised metabolism. A growing body of literature documents their association with higher eukaryotes, including plants. Their ability to improve plant growth by alleviating stress and reducing disease has been documented, but factors determining their recruitment are enigmatic.

Many *Streptomyces* spp. produce specialised metabolites that kill the wheat take-all fungus, *Gaeumannomyces tritici*, but it is not known how widespread this trait is. This study shows that at least 17 out of 54 streptomycetes (31%) isolated from wheat roots can inhibit the growth of wheat take-all fungus. Isolates from the Paragon variety showed greater antifungal activity which may implicate host genotype with functional variation of the root microbiome. The genomes of two *Streptomyces* strains with exceptionally potent antifungal activity were sequenced and putative antifungal gene cluters were identified. Root exudates are hypothesised to play a key role in the recruitment of beneficial microbes and $^{13}CO_2$ DNA stable isotope probing (SIP) experiment revealed that many of the rhizosphere enriched bacterial taxa utilised wheat root exudates. Surprisingly, *Streptomycetaceae* were not metabolising root exudates in the rhizosphere but the data suggested that are feeding on plant metabolites in the endosphere, where they have less competition. Whole bacterial community analysis showed their abundance was significantly enriched in the endosphere compartment. Nitric oxide (NO) was implicated for the first time in the recruitment of *Steptomyces* bacteria to the plant rhizosphere using *S. coelicolor* as a model. Mutant strains with deletions in genes coding for NO detoxification colonised the rhizosphere better than the control, while bulk soil was survival was unaffected, suggesting NO enhances root colonisation by these bacteria.

Overall, this research gives new insight into the ecological roles of *Streptomyces* spp. and supports the hypothesis that they are useful plant growth promoting bacteria that could be exploited as plant probiotics.

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Prudence, Samuel M. M. †, **Newitt, Jake T.** †, Worsley, Sarah F., Murrell, J. Colin, Lehtovirta-Morley**,** Hutchings and Matthew I. (2020) 'Microbiome assembly in the Spring bread wheat *Triticum aestivum* variety Paragon.', **In prep**

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Worsley, Sarah F., **Newitt, Jake**, Rassbach, Johannes, Batey, Sibyl F. D. D., Holmes, Neil A., Murrell, J. Colin, Wilkinson, Barrie and Hutchings, Matthew I. (2020) '*Streptomyces* endophytes promote host health and enhance growth across plant species', Applied and environmental microbiology, (June).

† These authors contributed equally.

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µg	Microgram
µl	Microlitre
µM	Micromolar
%	Percent
°C	Degrees centigrade
ACC	1-aminocyclopropane-1-carboxylate
ANI	Average nucleotide identity
ANOVA	Analysis of variance
BX	Benzoxazinoid
BGC	Biosynthetic gene cluster
BLAST	Basic local alighment search tool
bp	Base pairs
cDNA	Complementary DNA
CDS	Coding sequence
CFU	Colony forming units
cm	Centimeter
CsCl	Cesium chloride
DGGE	Denaturing gradient gel electrophoresis
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
EtOH	Ethanol
g	Gram
GB	Gradient buffer
IAA	Indole-3-acetic acid
KAAS	KEGG Automatic Annotation Server
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	Kegg Orthology
L	Litre
LB	Lennox broth
m	Metre
M	Molar
MeOH	Methanol
mg	Milligrams
min(s)	Minutes(s)
ml	Milliliter
mM	Millimolar
mm	Millimeter
MRSA	Methicillin Resistant *Staphylococcus aureus*
MYM	Maltose, Yeast extract and Malt extract
nD-TC	Temperature adjusted average density

NCBI	National Centre for Biotechnology Information
ng	Nanograms
nm	Nanometre
ORF	Open Reading Frame
OTU	Operational Taxonomic Unit
NMDS	Non-metric multidimensional scaling
PBS-S	Phosphate Buffered Saline - Silwett L-77 amended
PCR	Polymerase chain reaction
PERMANOVA	Permutational analysis of variance
PGA	Potato glucose agar
PGP	Plant Growth Promoting
ppmv	Parts per million volume
qPCR	Quantitative polymerase chain reaction
RAST	Rapid annotation using subsystem technology
R.I	Refractive index
Rpm	Revolutions per minute
SE	Standard error of the mean
sec(s)	Second(s)
SFM	Soy flour mannitol
SIP	Stable isotope probing
SOM	Soil organic matter
TAE	Tris acetate EDTA
TSB	Tryptone soy broth
v/v	Volume to volume
w/v	Weight to volume

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Chapter 1 General Introduction

1.1 Sustainable agriculture is the foundation of our society

Before the development of society as we know it today, most people would spend the majority of their time hunting, gathering or growing food in order to survive. For the last 7,000 to 12,000 years, cereal crops have been a staple nutrient source for humanity, since groups of people began to settle (Paterson *et al.*, 1995; DeWet, 2009). Cereal varieties were domesticated all over the world: sorghum and millet in Africa, rice in Asia, maize in America and wheat, barley, oats and rye on the Near East Eurasian continent (Paterson *et al.*, 1995) Today, agricultural technologies have improved in ways that allow more people to be fed for the same amount of land and labour. Advances in farming machinery have maximised efficiencies of labour (Kerridge, 2013; Bochtis *et al.*, 2014), while improvements in crop breeding have increased effective land usage by increasing yields (Spears, 2019). Modern agricultural practices such as crop rotation (Bullock, 1992) as well as supplementation with fertilizer (Cardwell, 1982; Caliskan *et al.*, 2008) have also helped push this yield up to match the demand driven by increasing populations. Today, the limitations of these technologies to improve yields without limit is recognised. By 2050, the world's population is predicted to reach 9.8 billion people (United Nations, 2019)and so it is important that food production continues to grow, to meet this demand.

Researchers across broad-ranging disciplines are working to find a solution to this problem, by looking into novel ways of improving the effective yield of staple crops. Increased fertilizer usage may be a viable strategy, but often sees diminished returns and leads to environmental issues such as eutrophication (Good and Beatty, 2011). Today, yield improvement strategies are often crop specific, be it through selective breeding (Reynolds *et al.*, 2011), genetic manipulation (Beddington, 2010; Godfray *et al.*, 2010), or management of species specific pathogens (Cook, 2003; Hernandez-Restrepo *et al.*, 2016) that reduce yields. *Triticum aestivum* (common wheat) is one of the most grown crops world-wide, an estimated 2609 million tonnes were produced in 2018 alone (FAO, 2019b). It may come as no surprise then, that increasing the security of this staple food crop has become a focus of much research.

1.1.1 Wheat as a focal point of study for cereal crops

The genus *Triticum* encompasses a variety of grass species collectively known as 'wheat', the most common being *Triticum aestivum*. Wheat is the dominant crop in temperate climates used to feed humans and livestock (Shewry, 2009). The widespread adoption throughout these regions is

multifactorial. Nutrition is naturally, a strong predictor of the success of any food crop, although there are other factors which shall be explored. *Triticum aestivum* is often referred to as 'common wheat' or 'bread wheat', the latter of which hints at the properties which underpin its success as a food crop. Cereal crops in general, are cultivated for their grain; their seeds contain a starchy endosperm that is a vital calorific source (McKevith, 2004). Bread wheat differs from many other cereals, in the diversity of proteins present – most notably, glutens (Anjum *et al.*, 2007). These proteins form extensive networks that are responsible for much of the structural properties associated with food products made from wheat flour. During breadmaking, CO_2 is released by respiring yeast which gets trapped in the proteinaceous matrix, which leads to a starchy yet light food product.

Practicality is of additional note, wheat is predominantly grown in temperate regions but is cultivated in regions as far north as Russia and as far south as Argentina (Feldman *et al.*, 1995). This broad geographical scope of cultivation is partly due to its genetic diversity, there are 25,000 varieties of bread wheat alone (Feldman *et al.*, 1995). Yield density of wheat is also comparable to other cereals, in 2019 the UK average yield for wheat was 9 tonnes per hectare (Defra, 2019), although this can vary globally according to a range of factors. When it is considered that wheat can be readily harvested at scale with combine harvesters, it is clear to see why wheat dominated much of the arable landscape following the agricultural revolution.

Scientific research into the biology of wheat has been held back in many ways, due to the complexity of its genome. Common wheat is hexaploid, which means that it has 6 copies of each of its seven chromosomes and 42 in total. Aneuploidy like this occurs when parent strains hybridize, retaining multiple genomes in a single strain. It is easy to see how this may facilitate genetic diversity, but it poses challenges for understanding genomic interactions. Advances in sequencing technologies have revolutionised the way we look at biology, the human genome project sequenced the euchromatic regions of the human genome and was declared complete in 2003, a milestone in human history. Despite this, it was not until 2012 when the draft genome was published for bread wheat (Brenchley *et al.*, 2012) and a further 5 years before it was complete. This was, at least in part, due to the size and complexity of its genome. Now, the blueprints for the plant that accounts for 20% of the calories consumed by humans (Brenchley *et al.*, 2012), are complete and there has never been a better time to unlock the secrets of its biology.

For this thesis, the roles and recruitment of *Streptomyces* species in the wheat root microbiome will be interrogated for the elite spring bread wheat variety Paragon. This variety has emerged as a model organism and is commonly cited in the literature with extensive genomic references (Walkowiak *et al.*, 2020; Wilkinson *et al.*, 2020). The reasoning behind this is that Paragon

is frequently crossed with landrace varieties to explore the inherent diversity within the wheat pangenome (Moore, 2015). Intraspecific variation is known to affect root microbiome assembly (Mahoney *et al.*, 2017), this will be discussed in greater depth later. The study of the Paragon variety root microbiome is therefore crucial. A comprehensive library of gamma mutant lines with Paragon background is currently in development at the John Innes Centre, which will provide tools to further explore the root microbiome of this variety in future.

1.1.2 Emerging challenges for sustainable production of wheat

One of the biggest challenges faced in agriculture, is disease. Each year an estimated 20-40% of food crops are lost, as a result of pests and diseases caused by soil-borne phytopathogenic microorganisms (Oerke, 2006; Savary *et al.*, 2012). The most prevalent agents of disease in plants are viruses and fungi. While potential crop yields have been increased and optimised through selective breeding and crop management strategies, minimising yield losses from disease is potentially more challenging; the factors that determine the establishment of plant diseases are complex (Savary *et al.*, 2012) and in many cases are not fully understood. Plants, like all pathosystems, are in an evolutionary arms race with numerous disease-causing agents. While crop breeding can lead to varieties that are resistant to disease in some cases, it is not uncommon for pathogens to emerge just years later, that are able to bypass this resistance.

In some cases, there are no known varieties of a crop that are resistant to disease. This is the case for common wheat and 'wheat take-all' disease. *Gaumannomyces tritici* (formerly known as *Gaumannomyces graminis* var. *tritici*) is the fungal agent responsible for take-all of wheat, which is arguably the most important root disease of wheat worldwide (Cook, 2003; Freeman and Ward, 2004; Hernandez-Restrepo *et al.*, 2016). The disease attacks primarily through localised root rot, or 'lesions', which leads to reduced uptake of water and nutrients and can ultimately cause massive yield losses. Limited success of chemical fungicides coupled with a lack of resistant varieties have focussed efforts to control disease severity with agricultural practices and biological control. Wheat take-all can persist in soils, living saprophytically on straw leftover from harvest. As such, one of the key ways to mitigate this is to rotate land usage with non-cereal crops (Cook, 2003). The alternative solution, paradoxically, involves just the opposite. Continuous cultivation of wheat leads to a period of severe disease, followed by a spontaneous decline of wheat take-all. This is caused by the accumulation of specific microorganisms, that suppress pathogenesis by producing antifungal compounds (Raaijmakers and Weller, 1998; Weller, 2015). While this does not restore yield to the same degree as crop rotation, it is often more practical. For these reasons, spontaneous take-all decline (TAD) represents one of the

best model systems for the study of plant/microbe interactions and biological control. This thesis will explore the way wheat root associated microorganisms have the potential to combat this disease.

An additional threat to global food security is climate change. As extreme weather patterns become more frequent, arable farming may become subject to more challenging conditions, such as drought (Le Houérou, 1996). Yield losses under these circumstances are inevitable, as water is required for the generation of energy through photosynthesis (Blankenship, 2014). Plants additionally respond to drought and other abiotic stressors through signalling pathways regulated by the phytohormone ethylene (Abeles *et al.*, 1992; Glick *et al.*, 2007). Accumulation of this hormone inhibits root elongation, nodulation and auxin transport, additionally promoting senescence which compounds yield losses under these conditions (Glick *et al.*, 2007). Again, some plants are more resilient to such abiotic stressors as drought and hypersaline soils (Sorrells *et al.*, 1998; Van Oosten *et al.*, 2016), particularly those that have evolved in such conditions (Majidi *et al.*, 2011). Climate change may alter our world more rapidly than plants can adapt, however. Crop breeding can be used to select for plants that are more resilient (Sorrells *et al.*, 1998; Van Oosten *et al.*, 2016), but this becomes increasingly laborious as the number of desired traits in a plant increase. Irrigation may be used to mitigate acute drought conditions, but this is not a viable approach worldwide, particularly in areas that face chronic drought. This is just one of many reasons that there is such inequality in food security worldwide (FAO, 2018).

1.1.3 The plant root microbiome: ancient allies

The golden age of biology is upon us, and we have never been so aware of the influence of microorganisms on our world. DNA sequencing technologies allow us to probe deeper into this unseen world and it has become clear that almost every niche on this planet is awash with an incredible diversity of microorganisms (Portillo and Gonzalez, 2008; Varin *et al.*, 2010; Kimes *et al.*, 2013), that includes higher organisms such as animals (Yatsunenko *et al.*, 2012) and plants (Lundberg *et al.*, 2013). The microbial life that resides within a host associated niche is termed ‘the microbiome’ and the composition of this community is often subject to long standing coevolution (O’Brien *et al.*, 2019; Lewin-Epstein and Hadany, 2020). Host/microbe interactions can be pathogenic, but many are commensal, or even mutually beneficial. A host and its microbiota are sometimes collectively referred to as the ‘holobiont’, and the study of how these organisms interact is seeping its way into public consciousness. Microbes can have a profound effect on their host, take for example the ‘gut-brain axis’. The human gut is occupied by trillions of microorganisms and there is increasing evidence that they influence not only our gut physiology but our neurology as well (Mayer, 2011; Cryan *et al.*, 2019).

There are a variety of tools available for the study of microbiomes. Briefly, the most common approach is isolate genomic DNA from a community of microorganisms and amplify a barcode gene using PCR. Barcode genes are fairly conserved, but vary consistently between taxa. This approach is known as metabarcoding and the barcode sequences can be profiled in various ways to investigate how the microbial community changes. Next generation sequencing coupled with barcode gene databases provides a vital reference tool for characterisation of microbial communities. Metabarcoding can additionally be used in combination with more targeted methodologies such as nucleic acid stable isotop probing (SIP). In summary, plant fix CO_2 into sugars, some of which are exuded by plant roots and consumed by microorganisms that inhabit the root microbiome. By feeding the plant with labelled $^{13}CO_2$, microbes that feed on root exudates will incorporate that label into their nucleic acid. These labelled nucleic acids can be separated and profiled with metabarcode sequencing in order to determine which microbes are key in metabolising root metabolites. This approach underpins the work presented in Chapter 3, as it is a vital tool for understanding potential recruitment mechanisms of *Streptomyces* to the wheat root microbiome.

While plant microbiomes may not feature quite so prominently in the public interest, they are arguably no less important. Commensal microorganisms that occupy the root microbiome (Figure 1-1) play a key role in the suppression of pathogenic infection. Given that plant roots interface with such a rich and diverse environment as soil, commensal association is arguably crucial for the plant's survival; soil-borne pathogens are abundant (Reeleder, 2003; Pieterse *et al.*, 2016; Agtmaal *et al.*, 2017), and commensal organisms help exclude these pathogens from colonising root tissue. The plant root microbiome can ultimately be divided into two compartments. The endosphere is a term given to the microorganisms which colonise the inside of the roots, either inter or intracellularly. The rhizosphere is a somewhat more loosely defined term to describe the microorganisms that colonise the layer of soil closest to the root, a concept first described by (Hiltner, 1904). Although chemically and microbially distinct from the surrounding soil, there is a continuous gradient, which means that separation of these compartments is arbitrary. The rhizosphere is sometimes further subdivided, the rhizoplane can be used to describe the microbial community that adheres to the surface of the root, although from a practical standpoint this may also include rhizosphere members.

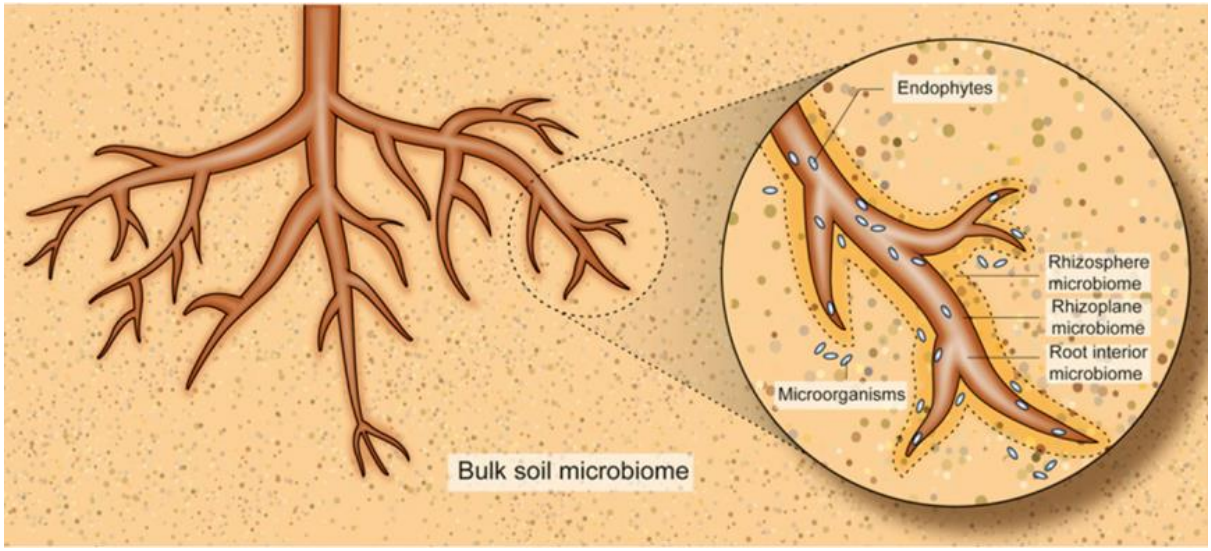

Figure 1-1 An overview of the root microbiome, image adapted from (Gaiero *et al.*, 2013). The image shows microorganisms colonising the insides of the roots (endosphere), the surface of the roots (rhizoplane) or the soil immediately surrounding the roots (rhizosphere). These microbes colonise from the surrounding bulk soil microbiome.

Recently, it has been shown that many of these microorganisms are able to confer wide-ranging benefits on their plant host (Haas and Défago, 2005; Tiwari *et al.*, 2018). The plant root microbiome is distinct from the surrounding soil, which suggests that microbes may be selected for by the unique environment that the plant host creates. The ramification of select occupation of this niche, is that it may be more challenging for root pathogens to infect their host, a concept known as general suppression. Select members of the root microbial community such as *Pseudomonas* spp., which are frequently enriched compared to bulk soil populations (Lundberg *et al.*, 2013; Bulgarelli *et al.*, 2015), are commonly cited as producers of antimicrobial compounds which are associated with reduced disease levels (Raaijmakers and Weller, 1998). This is known as specific suppression; both these concepts will be explored in greater detail in Chapter 1.

In addition to suppressing pathogens, many microbes that occupy the root niche can promote plant growth through various mechanisms, they are referred to as plant growth promoting rhizobacteria (PGPR). There are numerous ways this can be achieved; pathogen suppression can improve growth by mitigating the loss of resources caused by infection, but many PGPR have mechanisms for stimulating plant growth by hormone signalling. Physiologically, plant growth is regulated through dynamic temporal and spatial production of a group of phytohormones called auxins. One such example is Indole-3-acetic acid (IAA), many PGPR have been shown to produce this compound (Naveed *et al.*, 2015), PGP effects are partly attributed to this. Many have also been shown

to metabolise the precursor to phytohormone ethylene (Glick, 2004; Glick *et al.*, 2007), which has already been discussed as an inhibitor of growth under abiotic stress. It may come as no surprise that PGPR are a particular focus of research into plant/microbe interactions from a global food security standpoint. Studies that explore the link between plant/microbe signalling and plant health will be explored in greater depth in this thesis.

1.1.4 *Streptomyces*: champions of plant protection

As researchers peered into the microverse, observing ancient warfare that was for the most of human history unknown to us, the potential to exploit these interactions for agriculture became clear. It has already been discussed how wheat take-all disease can be managed passively, by facilitating the establishment of microbes antagonistic to plant pathogens, but there are plenty of examples of the active use of microbes in plant health. Finnish *Sphagnum* peat is known for its disease suppressive qualities and is often used as a potting mixture in glass houses (Tahvonen, 1982). Studies have identified *Streptomyces* species as key to this suppression and it has been shown that production of antimicrobial compounds leads to reduced disease incidence (Lahdenperä *et al.*, 1991). A *Streptomyces* strain isolated from this peat has been formulated and commodified to reduce disease incidence, this will be discussed later.

Soil represents great diversity of microbes, many have been used for decades for their medicinally and industrially relevant small molecules, arguable none more so than *Streptomyces* spp. Filamentous actinomycetes like *Streptomyces* are known for their production of an extraordinary diversity of antimicrobial natural products, producing 64% of known classes (Hutchings *et al.*, 2019). These saprophytic soil dwelling microorganisms can survive on a broad range of complex substrates, including chitin, which is one of the most abundant biopolymers on the planet (McCormick and Flärdh, 2012; Swiontek Brzezinska *et al.*, 2014).

While they have been recognised since their discovery as soil dwelling microorganisms, there is growing awareness in the literature today documenting their association with plant roots. Culture independent, whole community studies have been widely utilised to profile just about every place on earth including the root microbiome of many plant species (Bulgarelli *et al.*, 2012, 2015; Rascovan *et al.*, 2016; Gkarmiri *et al.*, 2017). What many of these studies show is that key bacteria are consistently enriched in the rhizosphere and endosphere microbiome compared to the surrounding soil, namely taxa belonging to the phyla Proteobacteria and Actinobacteria (Mendes *et al.*, 2013a; Schlaeppi and Bulgarelli, 2015; Rascovan *et al.*, 2016). *Pseudomonadaceae* and *Streptomycetaceae* (Fitzpatrick *et al.*, 2018a) are notable examples of lower level taxa that have been reported to be enriched in various

studies. Disease suppressive qualities of pseudomonads have been discussed, but there is also growing evidence that streptomycetes play a key role in maintaining plant health.

Strong pedigree in competitive secondary metabolism is attributed with much of *Streptomyces'* disease suppressive qualities, but there are other traits that promote plant health. The ecological relevance of chitin metabolism in *Streptomyces* spp. extends beyond efficient utilisation of resources, chitin is a key component of fungal cell walls, *Streptomyces* chitinases have been shown to exhibit mycocidal properties (Narayana and Vijayalakshmi, 2009). In addition to their role in the protective microbiome, streptomycetes are also PGPR. Like many microbes that improve plant growth through association, *Streptomyces* spp. are known to produce the plant growth promoting phytohormone IAA (Worsley *et al.*, 2020). There are currently no studies that document how widespread this metabolism is. Despite this, stimulation of plant growth by this mechanism remains poorly understood; auxin signalling is highly dynamic, and plant growth does not increase in a dose dependent manner as overproduction of IAA can lead to poor root development (Kochar *et al.*, 2011). *Streptomyces* spp. are also known to produce ACC deaminase (Jaemsaeng *et al.*, 2018b; Yoolong *et al.*, 2018), which may reduce yield loss through abiotic stressors, as discussed. Contrary to IAA, expression of the gene that encodes this protein may provide a dose dependent response with recovery from these abiotic stressors, as demonstrated by (Yoolong *et al.*, 2018). Ultimately, more studies are needed to fully understand these interactions.

1.1.5 Biocontrol: harnessing microbes for sustainable agriculture

The disease suppressive qualities of Finnish *Sphagnum* peat have been discussed briefly (Tahvonen, 1982), but it is even more relevant in the context of this thesis. This soil exhibits specific suppressive qualities, in other words, disease suppression is transferrable. Mycostop™ represents the first example of the commodification of *Streptomyces* bacteria, for promoting plant health (Lahdenperä *et al.*, 1991). Since it was first isolated from Finnish *Sphagnum* peat, its ability to mitigate disease in various plants hosts has been challenged by active root inoculation (Lahdenperä *et al.*, 1991; Suleman *et al.*, 2002; Hiltunen *et al.*, 2009; Khalil and Alsanius, 2010). To this date, just one other *Streptomyces* based biofungicide has been brought to market, Actinovate™ (Himmelstein *et al.*, 2014). These will be explored in greater detail later, but an important take home point for now, is that they are implemented with mixed success. The main reason for this is that their interactions with plants are not yet fully understood. Some disease phenotypes may be significantly reduced for some plants but not at all in others. What this demonstrates is the need for two things: a greater understanding of the chemical ecology of *Streptomyces* spp. and plants, as well as an increased diversity of commercially available *Streptomyces* based biocontrol products, this thesis aims to tackle both needs.

There are various reasons why *Streptomyces* spp. make good biocontrol agents. Diverse capacity for antimicrobial secondary metabolism clearly plays a big part in this, but there are more practical considerations for the deployment of biocontrol measures at scale (Fravel, 2005; Vincent *et al.*, 2007). One of the most considerable challenges of commercialising biocontrol agents is delivery. Crop-spraying represents one of the best ways to deliver microbial suspensions, especially for biocontrol of foliar pathogens (Jambhulkar *et al.*, 2016). This can be problematic, however, as mechanical shearing forces induced by this delivery method can reduce viability of the product. Microbial suspensions may also clog or damage spraying equipment. Delivery may also be less effective for the control of root pathogens. One of the key benefits of *Streptomyces* spp. is that they are spore-forming. In this state they are resistant to dessication (Martín *et al.*, 1986), the added bonus here is that seeds can be coated with a formulation of dried spores, minimising labour for the end user.

1.1.6 Unpicking root microbiome assembly cues

It has already been established that the root microbiome is chemically and microbially distinct, which suggests that plant hosts selectively recruit microorganisms from the soil environment. Additionally, there is evidence to suggest that the physiology of these organisms is different, when occupying this niche (Jousset *et al.*, 2011). The factors that drive this selection process are only partially understood, however. There are numerous cues that might drive the enrichment of specific microbial taxa, but it is thought that exudation of organic substrates largely facilitates this selective pressure (Sasse *et al.*, 2018), something which will be explored in Chapter 3.

With the revolution that next-generation sequencing brought, researchers have probed and interrogated the inter and intraspecific variation in the root microbial communities of plants (Fitzpatrick *et al.*, 2018a; Schlatter *et al.*, 2020). What became clear is that communities are often conserved at higher levels of taxonomy, particularly for different varieties of the same species (Bulgarelli *et al.*, 2015; Schlatter *et al.*, 2020). Variation is often apparent at lower levels of taxonomy, increasingly so with more distantly related plants (Bulgarelli *et al.*, 2015; Fitzpatrick *et al.*, 2018a). All of this suggests there is some genetic component to root microbial composition. Community structure for the root microbiome is also strongly driven by that of the surrounding soil (Edwards *et al.*, 2015; Dombrowski *et al.*, 2017), but this is especially true for the rhizosphere community (Singh *et al.*, 2007; Garbeva *et al.*, 2008), which can make unpicking these genetic factors challenging.

To break it down, it is necessary to understand how these genetic factors may influence communities. Root morphology is discussed (Sasse *et al.*, 2018), but it is consensus that community shaping is strongly influenced by exudation of organic compounds by the root (Haichar *et al.*, 2008;

Sasse *et al.*, 2018; Dastogeer *et al.*, 2020). It is estimated that between 20 and 40% of photosynthetically fixed carbon is exuded by the roots (Kuzyakov and Domanski, 2000; Derrien *et al.*, 2004; Haichar *et al.*, 2016), in the form of sugars, amino acids and other organic compounds. Exudates may act as signalling molecules between the plant host and soil microorganisms, but they also serve as substrates for the root associated microbial community. Root exudation is driven by many physiological factors and the interplay with microbial community formation is complex, something which will be discussed in greater detail.

A key question here is: if root exudates can be used as substrates and are driven by genetic components, which microorganisms are consuming these exudates in the root microbiome? Answering this question would help to unpick the influence of plant host on the root microbiome, from the surrounding soil. The take home point here is that if we hope to exploit the beneficial properties of root microorganisms in agriculture, it is crucial that we understand how plant hosts interact with them. Root exudation represents an intensive energy sink, something which can only be offset in evolutionary terms with an equal, if not greater benefit to the plant host. It may be then, that plants exude specific substrates to enrich select taxa. This is an attractive hypothesis when you consider that root exudate composition changes in response to phytopathogenesis (Rudrappa *et al.*, 2008), which is correlated with the transition to a more disease suppressive microbiome . When you consider that domestication of crop species has been linked with reduced root exudation (Brisson *et al.*, 2019; Preece and Peñuelas, 2020), it is clear to see how important it is to understand how crop breeding may interfere with these ancient allegiances.

This ecological link between root exudate chemistry and root microbial community can be probed with stable isotope labelling (Haichar *et al.*, 2016). As secreted root metabolites are ultimately derived from photosynthetically fixed carbon, plants fed with labelled $^{13}CO_2$, will incorporate that label into those compounds. Microbes that metabolise root exudates will then incorporate the label into their nucleic acids. Following the isolation of DNA from the root microbiome, labelled DNA can be separated from unlabelled DNA by mass. Profiling the communities in this fraction can identify key taxa involved in root exudate metabolism, yielding insights into the recruitment process of the root associated community.

Nucleic acid stable isotope probing (SIP) has already been used to probe communities associated with root exudate metabolism for various plant hosts (Lu and Conrad, 2005; Haichar *et al.*, 2008; Uksa *et al.*, 2017), this will be a focus for Chapter 3. It has proven to be a powerful technique which has shown how taxa implicated with disease suppression like *Pseudomonadaceae* and *Streptomycetaceae*, are fed by root exudates (Haichar *et al.*, 2008; Uksa *et al.*, 2017). SIP studies have

been conducted using various cereal crops, including wheat, these will be summarised. While studies may be limited in number, common trends have emerged. Key plant growth promoting and disease preventing taxa like pseudomonads and streptomycetes are often present in labelled communities, which suggest that they are consuming root exudates. While other taxa from the root associated community may be labelled, many are not – this suggests that plants exude substrates that are preferentially consumed by certain groups of organisms. Understanding which microbes are fed by root exudates is the first step to engineering defensive microbiomes on an agricultural scale. While there are DNA SIP studies in wheat, endosphere communities associated with the consumption of root metabolites have not yet been profiled with the resolution afforded by next-generation sequencing technologies. The question remains, are streptomycetes living in the endosphere fed by wheat plants? This thesis aims to tackle this question.

1.1.7 Microbial physiology in the rhizosphere: Nitric oxide as a universal signal in plant-microbe interactions

Microbes can provide benefits to their plant host; this has been discussed. Plants likely recruit these microorganisms through substrate secretion by the roots. This is only part of the story, however, there is clear evidence that rhizobacteria use specialised metabolic processes to occupy this niche which are only partially understood. Like most specialised metabolisms, regulation is key for optimal energy usage. This regulation is likely dependent on signals from the plant host, a universal language that has yet to be decoded. Microbes undergo physiological changes in the rhizosphere, as deduced by transcriptomic experiments, presumably in response to metabolomic stimuli (Jousset *et al.*, 2011). Various studies have shown how changes in plant physiology can lead to changes in microbial composition or physiology, but the holy grail is to reductively identify discreet signals that drive colonisation of select microorganisms. Numerous plant hormones have been implicated in the modulation of colonisation fitness, or physiology. IAA has been discussed already in the context of a plant growth promoting metabolite of rhizobacteria, but exogenous or plant derived IAA can also regulate the physiology of microorganisms. In some cases, IAA has been shown to stimulate production of antimicrobial secondary metabolites in *Streptomyces* species (van der Meij *et al.*, 2018; Worsley *et al.*, 2020).

A core theme of this thesis is the factors that drive defensive microbiome assembly, there are numerous plant metabolites that could be interrogated for their role in recruitment of beneficial streptomycetes but there are a few that stand out. Benzoxazinoids like 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) are defensive compounds produced by grasses and have been shown to increase colonisation of beneficial *Pseudomonas putida* in the rhizosphere of maize,

although it is likely that this is a selective pressure, as DIMBOA is antimicrobial. Chapter 4 will focus not on this class of compounds, but something far more fundamental to defensive metabolism in plants. Nitric oxide (NO) not only sits at the forefront of the plant's immune system (Bellin *et al.*, 2013) but is a widespread and universal effector across all domains of life, with diverse mechanisms of action (Thomas *et al.*, 2008). This short lived, highly reactive and diffusible molecule has been implicated in the formation of numerous symbioses in biology from *Vibrio fischeri* in the light producing organs of squid (Wang *et al.*, 2010b), as well as the egg sacs of beewolf wasps (Strohm *et al.*, 2019). *Streptomyces* are known to stimulate the plant immune system (Kurth *et al.*, 2014; Pieterse *et al.*, 2014), so resilience to NO may be crucial for their ability to colonise. On the other hand, NO is known to drive symbiotic associations with plant beneficial fungi (Espinosa *et al.*, 2014; Tian *et al.*, 2017), so it may be that it can behave as a recruitment signal for microbes. Chapter 4 will explore what is known about the role of NO in plant immunity, as well as symbiotic associations with fungi.

Studies suggest that endogenous NO in *Streptomyces* spp. may be involved in the regulation of diverse physiological processes, from development (Crack *et al.*, 2011; Fowler-Goldsworthy *et al.*, 2011) to the production of antimicrobial secondary metabolites (Sasaki *et al.*, 2016). Several genes have been implicated in the detection and metabolism of cytoplasmic NO. Despite this, NO metabolism in *Streptomyces* is only partly understood. Many studies focus on investigating the biology of *Streptomyces* in axenic systems (Sasaki *et al.*, 2016). While it is often crucial to precipitate at least a basic understanding of an organism in isolation, there is strong evidence to suggest it plays a role in communicating with other organisms and must be studied in this context in order to fully understand it. Chapter 4 will investigate the role of NO in wheat root colonisation by the model organism *Streptomyces coelicolor* M145. Ultimately, these kinds of mechanistic studies into the molecular interactions underpinning root colonisation by *Streptomyces* are incredibly scarce, this thesis will fill some of these gaps.

1.2 Outline of this thesis

The state of the world's food security depends on our intimate understanding of the numerous factors that drive crop yield. Perhaps the most significant innovation in the recent history of agriculture was the mass production of ammonia through the Haber-Bosch process, which led to a significant increase in the maximum yield attainable. Coupled with high-yield domesticated crop varieties, we are now able to feed more people than ever before. Despite this, it may still not be enough. As it happens, it would seem we may know more about what happens above the soil than below it. While crop varieties have been optimized and fine-tuned to produce higher yields with less waste, there is still much we do not understand about how soil microbes affect agricultural output. If

we wish to continue to improve crop yields to match demand, it is clear that we must thoroughly interrogate this complex system.

Specific groups of microbes seem to be adapted to provide benefits to plant hosts but there is still much that is not yet known about their interactions. There is clearly a need to scrutinise the biology of key organisms in the root microbiome, as well as broader host microbiome dynamics. *Streptomyces* spp. are promising beneficial root microbes and show great potential for commodification in the agricultural setting. Streptomycetes have been isolated from diverse environments, but it is crucial that we continue to isolate representatives from the root microbiome of staple crops like bread wheat, in order to unravel the secrets of their biology in this context. For example, *Streptomyces* spp. may be known for their secondary metabolic prowess, they may even be able to challenge economically relevant pathogens such as wheat take-all, but it is not clear how prevalent this trait is among root associated Streptomycetes. It is even less clear how host genotype may affect this. Much of what we know about the biology of these organisms is derived from studies that probe their metabolism in axenic culture. These studies are crucial to understanding the fundamentals of *Streptomyces* biology, yet they are the tip of the iceberg. The genomic era has made it clear just how many secrets are locked away beneath the surface; in a world where we are desperate to find new antimicrobials for clinical use, just a fraction of them are produced under laboratory conditions. Understanding the microbial ecology of these organisms may prove to be the key that unlocks these 'cryptic' gene clusters, the benefits of which can be exploited for both clinical and agricultural systems.

It is crucial that we understand how crops like bread wheat shape their microbiome through excretion of root exudates. It is not known whether endophytic Streptomycetes may utilise root exudates or how this may influence other metabolisms. While whole community structures are reasonably well defined for bread wheat, the influences of root exudates on microbial communities are less studied. The factors that govern successful colonisation of *Streptomyces* to the root microbiome have also yet to be elucidated.

Chapter 2 presents an initial exploratory study of the biology of root associated streptomycetes. Strains will be isolated from different compartments of the root microbiome of 3 different varieties of mature *Triticum aestivum*. Full length 16S rRNA gene sequences will be obtained to probe phylogenetic relatedness of these strains. The aim of this is to identify whether plant genotype has any influence on the selection of *Streptomyces* spp. at the 16S rRNA level. Antimicrobial capacity will also be investigated, testing each strain for bioactivity against a range of organisms, including the wheat take-all fungus. The ultimate goal is to narrow down the isolate library to those

with the highest potential for inhibiting wheat take-all and further probe them for plant beneficial traits. The capacity to degrade chitin will be tested, in addition to ethylene stress hormone precursor, ACC. Candidates will be selected for whole genome sequencing based on their performance in these assays, while maximising phylogenetic diversity between candidates. Genomic functional capacity will be analysed with both KEGG and AntiSMASH pipelines.

Chapter 3 will take a much broader look at the root microbiome of *Triticum aestivum* var. Paragon with a community survey of the 16S rRNA gene. This experiment will be conducted in soil sampled from the JIC field studies site at Church Farm, Bawburgh, to complement the culture dependent work carried out in Chapter 2. This community survey will also lay the foundation for exploring metabolically active microbes responsible for the consumption of root exudates with a $^{13}CO_2$ DNA stable isotope (SIP) probing experiment. This study is novel, the endosphere community responsible for the consumption of root metabolites has not been probed with 16S rRNA metabarcode sequencing. This will help unpick host genetics from other factors that may influence community assembly. Perhaps the most important question that is asked in this Chapter, is whether *Streptomyces* spp. are fed by root exudates, or whether they feed on alternative carbon sources – either saprophytically or on root activated carbon. Identifying the key taxa fed by wheat root exudates in this context will highlight potential interactions between *Streptomyces* and other microbes in this niche, something which is crucial to consider for the deployment of these organisms in a commercial context.

Finally, Chapter 4 will employ a combination of reverse genetics and a variety of ecological fitness studies, in order to probe NO metabolism in the soil and root microbiome. This Chapter ultimately steers away from the ecological studies of environmental microorganisms presented in this thesis, to investigate the physiology of model organism *S. coelicolor* M145 in the wheat root microbiome. Root and soil colonisation fitness will be assessed for mutant strains with increased sensitivity to NO. Soil type will be additionally considered. Some *Streptomyces* spp. generate NO with enzymes homologous to mammalian NO synthase. Enzymes from phytopathogenic and non-pathogenic representatives will be heterologously expressed in *S. coelicolor* to investigate ecological fitness effects. Exploring these fundamentals will provide a basis for further investigations in environmental streptomycete interactions with wheat.

Overall, this thesis aims to expand our understanding of the microbial ecology of wheat roots at several levels. By isolating a library of streptomycetes, this study will form the foundations of future investigations into their biology in the wheat root microbiome. Candidate strains with biocontrol potential will be identified. The metabarcoding and DNA-SIP Chapter will identify other key microbes

that associate with wheat plants, directing future studies into microbe-microbe interactions with *Streptomyces* and other root associated microbes. The DNA-SIP study will additionally yield insights into root carbon flux, which may influence microbiome engineering strategies. Mechanistic studies will investigate recruitment signals for *Streptomyces* species to the wheat root microbiome. All these things combined work towards a common goal, exploitation of beneficial microbe interactions with wheat for improved plant health and long-term food security.

Chapter 2 Isolation and characterisation of beneficial actinomycetes from the root microbiome of agricultural wheat

2.1 Introduction

2.1.1 The plant microbiome

The soil microbiome represents one of the most metabolically and ecologically diverse niches on the planet (Roesch *et al.*, 2007). Although there are often notable differences in microbial community composition between soil types, it is widely accepted that soil is generally a highly competitive, diverse, and dynamic environment. It is no coincidence then, that land plants associate with a variety of these microorganisms, particularly at the root/soil interface (Fitzpatrick *et al.*, 2018a). Plants, like many eukaryotes, have evolved to facilitate these associations for the benefit of improved physical fitness (Lambers *et al.*, 2009). Resistance to disease (Patel *et al.*, 2018; Oni *et al.*, 2019) and abiotic stress (Palaniyandi *et al.*, 2014), improved plant growth (Dinesh *et al.*, 2015) and access to nutrition (Jacoby *et al.*, 2017) are all benefits derived from specialised metabolisms which will be discussed in greater detail. Associating with soil microorganisms comes at a cost however, soil effectively acts as a reservoir for diverse phytopathogens (Reeleder, 2003). The plant immune system must therefore be highly tuned to maintain the balance of agonists and antagonistic microbes.

The plant microbiome is often described in one of two contexts: - the phyllosphere (aboveground/leaf microbiome), or the root microbiome. The root microbiome is essentially a subset of the microbial population found in the bulk soil, which can be further sub-divided into compartments. The rhizosphere is defined as a layer of soil that adheres to the plant root, which is usually distinct in both microbial and chemical composition from bulk soil (Lundberg *et al.*, 2013). Many microorganisms that occupy this niche are specifically adapted to do so. Some soil microbes are yet further adapted to adhere to the surface of the root known as the rhizoplane (Combes-Meynet *et al.*, 2011) and a smaller subset is adapted to enter the plant tissue and reside inter or intracellularly (Dai *et al.*, 2010), collectively known as the endosphere.

Over the last two decades, next generation sequencing of established microbial barcoding genes has enabled high resolution microbiome studies to define the microbial communities associated with various organisms (Lagier *et al.*, 2012; Lundberg *et al.*, 2013; Lanan *et al.*, 2016). In plant root microbiome studies, barcode amplicons are usually sequenced from DNA isolated from bulk soil, combined rhizosphere/rhizoplane, and endosphere compartments. These studies are important

because they show how different taxa can vary in abundance according to a wide range of environmental factors such as soil composition (Schreiter *et al.*, 2014) and host genotype (Bulgarelli *et al.*, 2012). Our understanding of the host genetic component in microbiome assembly is increasingly important for the breeding and development of robust and sustainable crops.

Metabarcoding studies of microbial populations can yield valuable insights into community structure, but it is important to recognise their limitations in terms of inferring metabolic functionality within a microbial community. In other words, we may correlate host genetic makeup with microbiome assembly, but it gives us limited information on the biology of these associations. These culture independent studies represent a valuable tool in probing the microbial ecology of a host-associated niche, but a more focussed approach is required to characterise the physiology and metabolism of microorganisms associated with a host.

2.1.2 *Streptomyces* ecology in the plant root microbiome

Many studies focus on isolating and cultivating key players in the plant root microbiome in order to better understand the mechanisms by which they improve plant fitness (Law *et al.*, 2017; Shahid *et al.*, 2017). From a functional perspective, there is a focus on characterising phyla that dominate the root microbiome. Although this naturally varies by plant and soil type, there are some trends. Proteobacteria and Actinobacteria often dominate root associated compartments, literature that demonstrates this was reviewed by (Mendes *et al.*, 2013a). Whilst the fast growing and diverse R-strategist genus *Pseudomonas* has long been a key focus of isolation studies involving Proteobacteria (de Weger *et al.*, 1987; Voisard *et al.*, 1989; Achouak *et al.*, 2000), *Streptomyces* is often cited as a dominant genus in the actinobacterial enriched community of the root microbiome (Lundberg *et al.*, 2013; Chaparro *et al.*, 2014) and is implicated in a similar role for plant health (Coombs *et al.*, 2004; Palaniyandi *et al.*, 2014; Chen *et al.*, 2016). Contrary to *Pseudomonas* species, streptomycetes are slow growing but exhibit greater diversity in primary and secondary metabolism.

A prime example of this is *Streptomyces* spp. ability to degrade chitin, utilising this complex polymer as both a source of carbon and nitrogen (Schrempf, 2001). This effectively serves multiple ecological functions. The first and most obvious is that it facilitates utilisation of an abundant and largely untapped resource. Insect detritus is a common component in many soils, and chitin is one of the most abundant polysaccharides in nature (Swiontek Brzezinska *et al.*, 2014). Despite this, chitin metabolism is rare in bacterial organisms as this complex polymer is challenging to break down. The second implication for the secretion of diverse chitinases is that it can potentiate antagonism of fungi (Narayana and Vijayalakshmi, 2009), chitin being a core component of the fungal cell wall. Ultimately

this combined metabolic and competitive strategy is a big part of the *Streptomyces* lifestyle, although its potential for microbial antagonism arguably underpins much of its ecological function in the plant root microbiome context and this will be explored in greater detail.

The evolution of *Streptomyces* species coincides with the emergence of land plants, and this has been discussed by (Chater and Chandra, 2006). We know that they associate with plant hosts today, and there is a reasonable possibility that *Streptomyces* and plants co-evolved, although such hypotheses present challenges for validation. Elucidating the molecular mechanisms of root colonisation remains one of the greatest challenges of the field, and Chapter 4 will take a closer look at studies that have advanced our understanding in this respect. What is clear, however, is that one of the key factors affecting successful colonisation of both rhizosphere and endosphere compartments is motility (Liu *et al.*, 2017a). Bacterial motility and chemotaxis genes are shown to be upregulated in root endophyte *Burkholderia kururiensis* M130 in the presence of rice extract (Coutinho *et al.*, 2015) and a transposon mutagenesis study by (Cole *et al.*, 2017) highlights a similar significance of motility genes in *Pseudomonas simians* for plant root colonisation.

Unfortunately, it is necessary to draw some conceptual parallels for *Streptomyces* spp., it must be acknowledged that there are large gaps in the literature alluding to their mechanisms of root colonisation. Due to the limited tractability of these organisms, forward genetic screens such as transposon mutagenesis are challenging and pose many limitations as discussed by (Baltz, 2016). Only two examples of transposon mutagenesis in the genus *Streptomyces* were found in the literature (Solenberg and Baltz, 1991; Ikeda *et al.*, 1993). Despite this, we can argue a strong case that motility is of similar importance for root colonisation by *Streptomyces* spp., and so it is important to establish some background in this respect.

Unlike many other prokaryotes, *Streptomyces* do not move with flagella, they are not even unicellular. Instead, they move by way of their somewhat unusual development and life cycle. Whilst many bacteria divide by non-polar growth, streptomycetes and other actinomycetes are filamentous organisms that grow by hyphal tip extension (Flärdh and Buttner, 2009). During vegetative growth *Streptomyces* species grow with branching hyphae, but under certain circumstances such as limited nutrients, the hyphae differentiate into the reproductive, aerial form. Vegetative hyphae give rise to aerial mycelium which undergoes rapid DNA replication and cell division to form long chains of spores which facilitate dispersal in the environment. In addition to the classical model of development, a distinct 'exploratory' phase of growth has been recently described for some organisms (Jones and Elliot, 2017), though it has not yet been shown that this behaviour is conserved among streptomycetes. Certain signals such as glucose depletion and increased pH are shown to stimulate

rapid extension of non-branching hyphae that appear to be specialised for covering distance as opposed to increasing in density (Jones *et al.*, 2017b). In the root microbiome context this could be relevant because glucose is a rare carbohydrate in the soil environment but is exuded by plant roots. Root exudate profile is linked to microbiome assembly as shown by several studies (Bais *et al.*, 2006; Berg and Smalla, 2009; Chaparro *et al.*, 2013), so it is not out of the realms of possibility that this primitive chemotaxis observed by (Jones *et al.*, 2017a) may be linked to rhizosphere recruitment and colonisation.

Given that studies have shown *Streptomyces* spp. to be enriched in not only the rhizosphere but also the endosphere (Fitzpatrick *et al.*, 2018a), it stands to reason that aspects of their lifestyle must be adapted to colonise these environments. Whilst it is not exactly clear how the root tissue is colonised by streptomycetes, various studies provide microscopical evidence to suggest they colonise intercellularly (Tokala *et al.*, 2002; Chen *et al.*, 2016). Some studies show that they can even colonise the intracellular space (van der Meij *et al.*, 2018), although this evidence is much more limited. These interactions are likely to vary between species of plant and *Streptomyces*, though we can infer from a combination of microscopical studies and bacterial development background, that polar growth likely facilitates endosphere colonisation by hyphal tip extension. Again, these studies offer us a mere glimpse into their lifestyle as a symbiont, but certainly hints at their coevolution with plants. There are various parts of a plant root that may provide an optimal entry point, streptomycetes are often seen clustered at root junctions and root hairs (Chen *et al.*, 2016), although a well-established root colonisation model is lacking. All things considered, there are still vast gaps in our understanding of the endophytic lifestyle of these organisms, and studies cited are still somewhat limited.

2.1.3 The role of *Streptomyces* species as beneficial symbionts

Given the body of evidence to support both the abundance and ecological relevance of *Streptomyces* spp. in the plant root microbiome, a key focus in this field of research is to understand not just how these organisms associate on a mechanistic level, but to understand and exploit the benefits that they confer. Natural selection underpins these beneficial interactions and so it is critical that we examine the way plants exploit these organisms before we can leverage this understanding with a more applied approach. Agricultural systems that don't see crop rotation often develop spontaneous disease resistance after continuous pathogen load (Weller *et al.*, 2002), a microbially driven phenomenon that *Streptomyces* spp. have been implicated in (Kinkel *et al.*, 2012). The exploitation of course goes both ways, organisms that live on the support of a host stand to benefit from host fitness. *Streptomyces* are among known root associated organisms that can stimulate plant growth, not just through antagonism of parasites but through production of phytohormones (Lin and

Xu, 2013), alleviation of salt stress (Yoolong *et al.*, 2018) and plant immune system priming (Suvala and Kokati, 2019). The culmination of this research sees the distillation of these benefits into formulations containing streptomycetes, for the translational improvement of plant health in agriculture. These benefits are discussed and the potential of *Streptomyces* species for biocontrol is explored.

2.1.3.1 Disease suppressive soils, the role of *Streptomyces* spp. in the protective microbiome

As previously mentioned, the soil microbiome is a diverse niche that is home to vast numbers of microorganisms that include plant pathogens. The rhizosphere community acts as a first line of defence, by competitive exclusion of these invading organisms. In addition, some members of the rhizosphere community are equipped not only to outcompete, but also to antagonise would be invaders of the plant host with antimicrobial secondary metabolites (Oni *et al.*, 2019). Whilst the plant host can influence assembly of the root associated community, it is still driven by bulk soil populations. When a pathogen is either unable to establish or thrive and cause a disease phenotype due to microbial community composition, this is termed disease suppressive soil (Schlatter *et al.*, 2017).

There are two different types of disease suppression which operate on a continuous scale. Antagonism by the collective soil community through competitive exclusion is known as general suppression, this was introduced in Chapter 1. Introducing this soil to disease conducive soil will not confer the same disease resistance, so it is non transferrable. By contrast, specific disease suppression by an individual organism is due to the accumulation of metabolites that antagonise the disease-causing organism. Specific suppression is transferrable and soils are used for just that quality (Tahvonen, 1982). *Streptomyces* spp. are often associated with specific suppression (Kinkel *et al.*, 2012; Cretoiu *et al.*, 2013), and one of the driving forces behind this research is to understand their importance in the plant's defence against phytopathogens, so that we can better exploit their potential for agriculture.

During the time period in which research was conducted for this thesis, the role of *Streptomyces* spp. in biocontrol of cereal crop diseases was reviewed (Newitt *et al.*, 2019). There is a strong focus in this review towards fungal pathogens of cereal crops, in part due to diverse host range and tissue pathologies that are documented (Dean *et al.*, 2012). Some of the most devastating crop diseases are caused by fungi and lead to massive yield losses. *Magnaporthe oryzae* for example which is responsible for 'rice blast', is estimated to cause 30% of rice yield loss worldwide (Nalley *et al.*, 2016). Similarly, *Gaumannomyces tritici* otherwise known as 'wheat take-all' is similarly notorious for high yield losses in wheat crops (Hernandez-Restrepo *et al.*, 2016) and is considered the most aggressive

pathotype of its species. Rice blast largely affects above ground sections of the plant, whereas wheat take-all causes necrotic lesions of the roots and feeds on dead tissue, eventually starving the plant of nutrients. The severity and impact of these diseases is a driving force for this research. We reviewed literature that implicates *Streptomyces* spp. in the protection of cereal crops from rice blast (Zarandi *et al.*, 2009; Li *et al.*, 2011; Law *et al.*, 2017), wheat take-all (Coombs *et al.*, 2004) and others.

Before we can discuss the effectiveness of streptomycetes as active biofungicidal agents, it is important to understand the context of its requirement. Conventional treatment for phytopathologies caused by fungi or similar organisms, involves somewhat crude and broadly biocidal agents. These commercially applied fungicides are usually copper based and quite toxic, which leads to off target effects. Copper fungicides have a non-specific mechanism of action, their toxicity is the result of copper ions disrupting metabolism by reacting with cellular components (Lyr, 2012). Studies have shown that soils contaminated with copper from fertiliser use (albeit within local regulation limits) can reduce both microbial biomass as well as earthworm biomass (Merrington *et al.*, 2002; Van Zwieten *et al.*, 2004). While It is not known whether earthworms avoid high copper soils due to reduced microbial biomass carbon (which constitutes a large portion of their diet), shifts in microbial community composition can clearly have vast and unseen ecological consequences. For this reason, there has been a peaking interest in the use of naturally disease suppressive and bioactive microorganisms in agricultural technology.

Streptomyces has been introduced already as a genus that has diverse secondary metabolism and produces a broad range of bioactive molecules. Many papers cite that *Streptomyces* spp. are responsible for the production of anywhere between 50 and 65% of antimicrobials used in the clinic to date (Chater, 2006; Hopwood, 2007; Hutchings *et al.*, 2019). This is certainly to undersell their industrial value because they additionally represent a considerable source of antitumour, antihelminthic, immunosuppressive and herbicidal compounds. A review by (Demain, 1998) explores in greater depth the diversity of high value natural products, far beyond that which is cited within the scope of this introduction, which will focus largely on the potential for antagonism of microorganisms.

Discussing the merits of *Streptomyces* spp. in a plant health context, is quite different from a human health context. Exploitation of their secondary metabolite natural products for health has involved a much more focussed strategy, beneficial compounds are isolated and administered. The consensus is, for the most part, that it is unreasonable from both an economic and regulatory standpoint to treat large portions of land with purified fungicidal compounds in this way. It is certainly plausible that one might expect similar off target effects to those reported by (Merrington *et al.*, 2002; Van Zwieten *et al.*, 2004). Instead, there is a focus on understanding the role of beneficial

microorganisms like *Streptomyces* spp. in disease suppressive soils. This strategy has multiple benefits, *Streptomyces* bacteria are generally quite abundant in the plant root microbiome, so finding way to better enhance the intrinsic protection they provide could be quite an efficient approach. Soil enhancement with streptomycetes also has the potential to mitigate off target effects from chemical applications, antimicrobial secondary metabolite production is highly controlled (Bertrand *et al.*, 2014; Tyc *et al.*, 2014). In an ecological setting, production of these compounds are likely 'switched on' by interaction with the target organism (van der Meij *et al.*, 2018; Worsley *et al.*, 2020).

From a perspective of mimicking this disease suppressive phenotype, there have been attempts to engineer the microbiome to enrich communities responsible for specific suppression. Some studies have achieved this with soil amendments (Inderbitzin *et al.*, 2018) such as broccoli and chitin. The rationale behind this is that chitin amendment can shift the microbiome towards substrate metabolisers, namely Actinobacteria (Cretoiu *et al.*, 2013), of which *Streptomyces* are key players. (Inderbitzin *et al.*, 2018) noted that chitin amendment increased relative abunances of *Pseudomonas* and *Streptomyces*, which correlated with reduced Verticillium wilt severity. The role of these taxa in disease suppression can be explored further by artificially increasing their abundance and will be discussed later. It has already been discussed that chitinolytic organisms can have antagonistic effects on fungal populations (Narayana and Vijayalakshmi, 2009), so it makes sense that selecting for these organisms can increase general suppression of fungal disease. Ultimately, understanding the driving forces in suppressive soil formation will help promote a healthier microbiome without resorting to aggressive treatments. Crucial to this is improved knowledge of the metabolism and ecological relevance of these organisms.

2.1.3.2 Additional fitness benefits through association with *Streptomyces*

It has been recognised for some time now that many plants rely on symbiotic microorganisms to provide access to nutrients (Ledgard and Steele, 1992) and maximise growth (Gosling *et al.*, 2006). While many of these associations are obligate in nature like the well-known but under-studied mycorrhizal fungi (Tisserant *et al.*, 2012), there are plenty which are able to improve resilience to abiotic stress factors (Ahemad and Kibret, 2014) and improve plant growth (Ingham *et al.*, 1985) facultatively. *Pseudomonas* and *Streptomyces* are genera which are both enriched in the root microbiome and implicated in disease suppression. It should perhaps come as no surprise then that both facilitate improved plant growth (Preston, 2004; Dias *et al.*, 2017).

The mechanisms by which they can do this vary but are largely common to both *Pseudomonas* and *Streptomyces* species. This work will focus on the latter. One way that growth can be indirectly

enhanced has already been discussed, disease suppression leads to improved utilisation of resources and lower yield losses (Palaniyandi *et al.*, 2013). *Streptomyces* spp. that associate with roots are able to mitigate not only biotic stressors such as pathogen load, but can also buffer abiotic stressors such as hypersaline soils (Palaniyandi *et al.*, 2014) and drought (Yandigeri *et al.*, 2012). Salt stress in plants triggers the production of phytohormone ethylene (Glick, 2004). This hormone is used for long distance multi-cellular signalling and is a normal part of plant physiology, but under stress conditions it accumulates to levels that can impede growth (Kawasaki *et al.*, 2001; Rocha *et al.*, 2007). The precursor for ethylene, 1-aminocyclopropane-1- carboxylate (ACC) can be metabolised by many plant growth-promoting rhizobacteria (PGPR) with the enzyme 1-aminocyclopropane-1- carboxylate deaminase (ACCD) (Jaemsaeng *et al.*, 2018b), which can restore ethylene to physiological levels and improving growth.

It is also noted that many PGPR are equipped to synthesise plant growth hormones such as Indole-3-acetic acid (IAA) (Persello-Cartieaux *et al.*, 2003). Genomic analysis of plant derived *Streptomyces* strains isolated by previous lab members indicate the presence of several genes involved IAA synthesis (Worsley, 2019), colorimetric assays confirmed production *in vitro* also. Several studies have in fact shown that streptomycetes are able to produce IAA (Khamna *et al.*, 2010; Lin and Xu, 2013; Jaemsaeng *et al.*, 2018b) and enhance plant growth. IAA production does not increase plant growth in a dose dependent manner, however (Xie *et al.*, 1996). *Pseudomonas putida* strains which produced 4 times more IAA than the wild-type strain lost their plant growth promoting potential. It is not entirely clear why this is the case, but it is likely that IAA signalling in the regulation of plant growth is a complex and finely tuned process. Understanding the ecological relevance of PGPR derived IAA could thus prove challenging. This trait is so common amongst PGPR however, and apparently so finely tuned as to exert improvements in plant growth, that it seems unlikely to be ecologically irrelevant.

Streptomyces spp. can enhance plant growth by improving access to nutrition. The diverse metabolic potential of *Streptomyces* bacteria has been discussed briefly; their lifestyle allows them to grow on a variety of substrates. In microbial ecosystems and host symbioses, the benefits of advanced metabolisms can be shared. It is well known that while many bacteria utilise siderophore production to scavenge trace metals, there are cheaters that are able to utilise heterologous siderophores (Raaijmakers *et al.*, 1995). It would seem that this benefit can influence the plant host as well as other microbes, (Rungin *et al.*, 2012) showed that plant growth promoting effects of root associated *Streptomyces* were lost after knocking out siderophore production. It is not clear whether this is a direct effect of plant growth improvement or whether increased streptomycete fitness may potentiate other mechanisms of plant growth promotion. Nevertheless, mechanisms of enhanced

nutrient acquisition are numerous, even if their symbiotic relevance is less extensively characterised. Some literature suggests *Streptomyces* spp. may be able to fix nitrogen (Ribbe *et al.*, 1997; Dahal *et al.*, 2017), although literature documenting this interaction is scarce. Other, niche plant beneficial traits include phosphate solubilisation (Gupta *et al.*, 2010; Jog *et al.*, 2014) and oxidation of elemental sulphur to thiosulphate (Yagi *et al.*, 1971)

Finally, *Streptomyces* are somewhat unique in that they are exceptional at 'priming' the plant immune system in ways that other PGPR cannot (Tarkka *et al.*, 2008). Many beneficial microorganisms that associate with plants can trigger Induced Systemic Resistance (ISR) (Pieterse *et al.*, 2014). Essentially, this means that a plant responds to Microbe Associated Molecular Patterns (MAMPs), which primes the plant immune system via the jasmonate and ethylene pathways to reduce disease severity upon infection (Choudhary *et al.*, 2007). Accumulation of defence-related compounds and reinforced cell wall components is characteristic of systemic resistance induced by rhizobacteria (Kurth *et al.*, 2014) but most PGPR microbes cannot trigger Systemic Acquired Resistance (SAR). *Streptomyces* spp. on the other hand have been shown to trigger SAR in oak trees through the salicylic acid pathway in combination with ISR (Kurth *et al.*, 2014), priming resistance against powdery mildew. This combination of ISR and SAR, along with the associated upregulation of defence genes, is also reported in a study involving *Arabidopsis thaliana* and *Streptomyces* spp. (Conn *et al.*, 2008). The fact that SAR is usually triggered by pathogenic microorganisms (Van Wees *et al.*, 2008) suggests plants may initially recognise streptomycetes as pathogens during the early phases of colonisation, thereby priming the immune system further. The implication of this is that *Streptomyces* spp. may exert biocontrol even when they do not produce antimicrobial compounds.

2.1.3.3 *Streptomyces* as a biocontrol agent in agriculture

Whilst next generation of sequencing has facilitated extensive probing into agricultural microbiomes, we may never fully grasp the dizzying complexities of plant/microbial dynamics. This is because there are so many factors that govern a population, from soil composition (Schreiter *et al.*, 2014), plant genetics (Bulgarelli *et al.*, 2015) and environmental conditions. For this reason, many researchers and agricultural technology companies have shifted focus toward isolating beneficial microorganisms, which are often enriched for in disease suppressive conditions, for the purpose of reintroduction to different agricultural systems. Strains are isolated and screened for beneficial properties, then formulated for long term storage and application. Soils can then be amended with these strains to improve their resilience to disease and convey other benefits. Validating pathogenic suppression in field conditions is the gold standard, although this can prove challenging, and the majority of biocontrol studies interrogate glasshouse systems like (Nourozian *et al.*, 2006).

Streptomyces make good biocontrol agents for several reasons. Their metabolic prowess has already been discussed at length and their association with plants has been shown to confer many different benefits. Using beneficial microorganisms as agricultural technology however is not quite so straightforward as 'copy and paste', there are lots of factors that need to be considered. Application of the biocontrol agent needs to be straightforward and cost effective. Foliar spray is often used for biocontrol of phytopathogens such as *Fusarium* spp. (Jambhulkar *et al.*, 2016) but soil inoculation is the most used strategy for combatting root pathogens. Delivery through irrigation is the primary introduction method for *Pseudomonas* spp., as described by (Steddom and Menge, 2001). This poses challenges however, microbial inoculum can block irrigation leading to equipment downtime and lost productivity, and these are limitations of working with desiccation sensitive agents. *Streptomyces* spp. on the other hand, are more resilient to desiccation as spore forming bacteria (Martín et al., 1986). This means spores can be dried and formulated for long term storage (Lahdenperä *et al.*, 1991), which makes it more cost effective. Studies have shown this can be achieved with starch granules or talcum powder (Fravel, 2005; Palaniyandi *et al.*, 2013; Palazzini *et al.*, 2017). These formulations can be mixed with the soil when planting or applied as a seed coating. The latter is often preferable as less inoculum is required; efficiency issues related to soil competition are circumvented, though this issue is widely debated and largely pathosystem dependent (Fravel, 2005).

Another issue to consider is pathogenicity. There is often a fine line between mutualists/commensals and pathogens. The genus *Pseudomonas* for example contains many species that are PGPR, but there are 21 recognised species of plant pathogens (Höfte and De Vos, 2006) and likely many more. The most important of these species is *P. syringae* with over 50 different pathovars, causing a range of pathophysiologies on a range of host plants. There are a handful of *Streptomyces* spp. that are also pathogenic (Bignell *et al.*, 2010, 2014; Fyans *et al.*, 2016), though it is generally thought that the acquisition of their pathogenicity island only happened once (Kers *et al.*, 2004a), as only 10 species have been identified as phytopathogenic out of more than 600 species (Seipke *et al.*, 2012; Viaene *et al.*, 2016). This means that the diversity of select beneficial strains from this genus is high, with a relatively low risk of selecting strains detrimental for plant health.

It has already been discussed that disease resistance conferred by specific suppression is transferrable. Disease suppressive soils are excellent ways to enrich for the isolation of beneficial organisms. Finnish *Sphagnum* peat is a well-known source of these organisms (Tahvonen, 1982; Weller *et al.*, 2002) and is often adopted for glasshouse cultivation of plants. A broad-spectrum bioactive strain belonging to the species *Streptomyces griseoviridis* was isolated from this soil. The strain was formulated for application and is sold under the brand name Mycostop™ (Lahdenperä *et al.*, 1991).

As noted above, *Streptomyces* spores are resilient to desiccation (Martín et al., 1986) so the inoculum is relatively stable as a dry powder (Lallemand Plant Care, 2014). The product can be homogenised with soil, or used as a seed coating (Fravel, 2005; O'Callaghan, 2016). The primary bioactive agent in Actinovate™ is *S. lydicus* strain WYEC 108 (Himmelstein *et al.*, 2014). This strain has also been shown to antagonise root pathogens and was originally isolated from the Linseed rhizosphere by (Crawford *et al.*, 1993), demonstrating the power of harnessing a plant host's natural selective ability, to enrich for beneficial microorganisms.

These biofungicidal agents have been shown to inhibit a range of phytopathogens *in vivo* (Lahdenperä *et al.*, 1991; Tokala *et al.*, 2002; Chen *et al.*, 2016). This is particularly important to note because secondary metabolism is often tightly controlled, and in many cases secondary metabolites remain cryptic, or not produced, *in vitro*. Whilst various strategies have been devised to maximise industrial scale antimicrobial production in axenic cultures (Hu *et al.*, 2002; Liu *et al.*, 2013), robust antimicrobial production in environmental systems are somewhat less validated. Actinovate™ and Mycostop™ are the only *Streptomyces* based biofungicides on the market but are both listed with the organic materials review institute (OMRI), for use in organic farming. The biofungicidal mechanisms for these products are likely derived from antifungal metabolites produced by the active organism, although there is no direct evidence to support this. There is likely plenty of market potential for further biofungicidal formulations of *Streptomyces* spp., particularly because neither product offers complete protection against fungal pathogens. Both work in laboratory and glasshouse settings but are less reliable in agricultural systems (Zhang *et al.*, 2011; Himmelstein *et al.*, 2014). Other studies have highlighted the potential of novel isolates for biocontrol, and selected strains are presented in Table 2-1.

Table 2-1 Select biocontrol studies involving *Streptomyces*, adapted from (Newitt et al., 2019)

Strain	Host protection	Pathogen protection	Condition of study
Streptomyces griseoviridis (Mycostop) Commercial strain	Date palm, carnation, cauliflower, sweet pepper, wheat	*Ceratocystis radicicola*, *Fusarium* spp., *Pythium* spp., *Rhizoctonia* spp.,	Sterile soil (Suleman *et al.*, 2002) Greenhouse and Field (Lahdenperä *et al.*, 1991)
Streptomyces lydicus WYEC108 (Actinovate) Commercial strain	Pea, lettuce, soybean	*Rhizoctonia solani*, *Pythium ultimum*, *Sclerotinia sclerotiorum*,	Glasshouse (Yuan and Crawford, 1995; Chen *et al.*, 2016) Field (Zeng *et al.*, 2012; Chen *et al.*, 2016)
Streptomyces S4-7	strawberry	*Fusarium oxysporum*	Chamber and field (Cha *et al.*, 2016)
Streptomyces caviscabies	wheat	*Gaumannoymyces graminis* (Take-all)	Sterile soil and field (Coombs *et al.*, 2004)
Streptomyces spp. strain BN1	wheat	*Fusarium graminearum*	Greenhouse (Jung *et al.*, 2013)
Streptomyces spp. DAUFPE 11470 and 14632	maize	*Stenocarpella maydis*	Greenhouse (Bressan and Figueiredo, 2005)

The current state of the biocontrol field highlights a need for three things: a greater number of biocontrol products, a greater understanding of the factors that control recruitment of *Streptomyces* spp. to the root microbiome and a greater understanding of the role/regulation of antimicrobial natural products in the root microbiome. The first two points are addressed in the research conducted for this thesis.

2.1.4 Wheat take-all as a model for disease suppression

This study focuses primarily on the interactions between *Triticum aestivum* (common wheat) and *Streptomyces* species, the logic behind this focus is two-fold. Firstly, it is hard to overstate the economic importance of this staple food crop. In 2019, the Food and Agriculture Organization (FAO) predicted 2719 million metric tonnes of cereal grain was produced worldwide (FAO, 2019a), wheat

accounts for 763 million tonnes of this production – that's 28%. As we face challenges like climate change and increasing susceptibility to plant diseases (FAO, 2018), applied knowledge of microbial interaction dynamics in the wheat root microbiome will play a crucial part in stabilising food security for the future. Secondly, spontaneous decline of wheat take-all disease is a model system for understanding microbial suppression of plant diseases (Cook, 2003), *Streptomyces* spp. have been identified as key players in this process (Andrade *et al.*, 1994). Studying the interactions of *Streptomyces* spp. associated with wheat and wheat take-all disease, will help guide the development of new strains for biocontrol in agriculture.

G. tritici is one of the best studied fungal plant pathogens. (Kwak and Weller, 2013) discuss its history, citing its description as early as 1858. Today It is seen largely as a model for disease suppressive soils and contributed to the development of an entire field of study into the beneficial associations between plants and microorganisms (Cook, 2003). The wheat root microbiome is dominated by pseudomonads and streptomycetes (Rascovan *et al.*, 2016; Liu *et al.*, 2017b; Araujo *et al.*, 2019) which contribute significantly to the natural suppression of root rot diseases like wheat take-all (Thomashow and Weller, 1988; Harrison *et al.*, 1993; Tahvonen *et al.*, 1995; Raaijmakers and Weller, 1998; Loper and Gross, 2007)

To understand why these organisms have been so well studied in this context, it is important to consider that there are no varieties of wheat that are resistant to take-all (Kwak and Weller, 2013), nor are there economically viable chemical control options (Tahvonen *et al.*, 1995). In lieu of these strategies, growers worldwide take advantage of a phenomenon known as Take-all decline (TAD). This is a term used to describe a spontaneous decline in disease severity, following a period of high disease load of monoculture crops, this is the most widely known example of induced disease suppression (Weller *et al.*, 2007). Whilst the crop yield is ultimately still lower than disease free crops, alternative strategies for disease prevention such as crop rotation are not always viable. Whilst TAD is ultimately associated with the accumulation of antagonistic microorganisms, we are only just beginning to understand the process.

It has been shown that *Streptomyces* spp. play a crucial role in plant health through antagonism of disease-causing agents, and additionally convey numerous benefits. While promising plant beneficial traits can be identified in these organisms, there are still few *Streptomyces* based biofungicides on the market today. In part, this is due to a strong reliance on chemical fungicides, although increasing regulations will likely see a shift to biofungicidal use in the future. Perhaps the key reason, however, is that the root microbiome is quite genotype specific; plants selectively filter and recruit organisms to the root microbiome. *Streptomyces* spp. isolated from Finnish *Sphagnum* peat is

likely to share only distant co-evolutionary history with modern wheat varieties grown in Norfolk. This study aims to isolate novel biocontrol strains from paragon wheat roots grown in local agricultural soil and to assess their potential for application. This study will provide an initial screen, and platform for further development of strains for commercial use.

2.2 Aims

One of the key aims of this Chapter was to explore the biology of *Streptomyces* spp. isolated from the root microbiome of three different wheat varieties. Strains were isolated from the rhizosphere, rhizoplane and endosphere compartments. Bioactivity was assayed against a range of microbial indicator strains, including plant pathogen *G. tritici* (wheat take-all). The 16S rRNA gene was amplified and sequenced to compare phylogenetic relatedness of strains. The data were analysed to determine whether properties such as bioactivity grouped by relatedness, host variety, or root compartment origin. Plant beneficial metabolisms such as chitinolysis and ACC metabolism, were screened on a smaller scale with strains that showed bioactivity against wheat take-all. Two strains that were bioactive against wheat take-all were selected for whole genome sequencing. The genomes underwent further *in silico* analysis to determine their functional capacity. AntiSMASH analysis was used to predict BGCs that might encode for antifungal compounds responsible for antifungal activity. KEGG pathway analysis predicted metabolic pathways involved in promoting plant growth.

2.3 Materials and methods

2.3.1 Growth and isolation of wheat root associated *Streptomyces*

Wheat plants were grown in field conditions at The John Innes Centre field site from the John Innes Centre (JIC) Church Farm cereal crop research station in Bawburgh (Norfolk, United Kingdom) (52°37'39.4"N 1°10'42.2"E). Plants were grown until plants had reached maturity (growth stage 11). In July 2018, wheat plants were removed from the ground in clusters of 4 or 5, around 10cm of soil was removed with the plants to preserve as much of the root system as possible. Clusters of plants were sampled from 3 different varieties: - Paragon, Soissons and a commercial variety called Kerin (the surrounding farmer's crop). The plants were sampled immediately for microbial isolation.

2.3.2 Sampling of wheat root compartments

Microorganisms were isolated from rhizosphere, rhizoplane and endosphere compartments. Rhizosphere soil (R) was sampled by removing loosely attached soil from the roots of the plant using sterile cotton buds. The rhizoplane compartment was isolated by separating the stem of the plant aseptically and vortexing the root in PBS-S (See Table 2-3) for 30 seconds, following bulk soil removal. The root was removed, and the rhizoplane soil was pelleted by centrifugation at 3500RCF for 10 minutes. Following the removal of tightly adhering soil particles with tweezers, the root was washed and vortexed a further 2 times in PBS-S. Roots were washed with 70% (v/v) ethanol (EtOH) for 1 minute then 3% (v/v) bleach (6% sodium hypochlorite) for 10 minutes. Sterile dH_2O was used to wash the roots 10 times before transferring to a sterile mortar and pestle. Roots were then homogenised with 4ml of 10% glycerol (v/v) to generate the endosphere (E) sample. Serial dilutions were generated for rhizosphere, rhizoplane and endosphere compartments, which were then plated on SFM or ISP-2 (see Table 2-3) amended with nystatin (5µg/ml) and cycloheximide (100µg/ml), to reduce fungal growth. Some plates were also amended with phosphomycin (50µg/ml) in order to enrich for *Streptomyces* spp. Plates were incubated at 30°C for 2-4 days. Colonies were subsequently isolated from these plates and purified by restreaking. SFM and ISP-2 media both with and without phosphomycin (50µg/µl) were used in the isolation of microorganisms, plates were grown at 30°C.

2.3.3 Cultivation and maintenance of *Streptomyces* species

Regardless of isolation media, environmental strains suspected to be *Streptomyces* spp. were cultivated on SFM. All isolates were purified by restreaking at least twice and grown at 30°C for 3-7 days. In order to stock *Streptomyces* species, a single colony was picked from a plate grown for 7 days and homogenised in 150µl sterile dH_2O. The colony was then plated using sterile cotton buds soaked

in dH_2O. Strains were grown for anywhere between 5 and 14 days. Onset of sporulation was assessed visually. Typically, sporulation was identified with a colour change from white (aerial hyphae), to a darker greyish colour associated with spore pigment. Plates were then agitated with 10ml of sterile dH_2O using sterile cotton buds to remove the spores. The spore suspension was then pelleted in a centrifuge at 3500RCF and resuspended in sterile 20% Glycerol (v/v).

2.3.4 Cultivation and maintenance of *G. tritici*

A small plug of agar was removed from a plate of PGA (Table 2-3) using the end of a sterile glass pasteur pipette. A similar sized plug of agar was removed from an existing culture of *G. tritici* using the same technique and transferred to fill the plug of agar that was removed from the fresh plate. The plate was left in the dark at room temperature until the fungus had expanded to cover the entire plate, at which point it was replated.

2.3.5 Colony morphology microscopy

Streptomyces strains were spot plated three times (4µl) in a triangle around the centre of a 90mm petri dish containing approximately 33ml SFM. Plates were incubated at 30°C for 7 days. Micrographs were obtained using a Leica MZ16 stereo microscope equipped with a fibre coupled Leica CLS100x white light source and Leica SFC420 colour camera. Camera mount magnification was equal to 0.63x while the objective magnification was variable.

2.3.6 Soft nutrient agar overlay bioactivity assay

Streptomyces spores (4µl) were pipetted onto 50mm petri dishes containing 10ml of agar media. Strains were left to grow for 7 days. On the fifth day, indicator strains (see Table 2-4) were streaked out to single colonies on LB agar and incubated at 30°C for 24h. On the sixth day, single colonies were picked with a toothpick and used to inoculate 10ml of LB broth (Table 2-3). Cultures were grown for 24h at 30°C to reach stationary phase. These cultures were mixed with SNA (Table 2-3) at a ratio of 1 to 20. Of this mixture, 1ml was added to each plate in such a way that it surrounds the colony of *Streptomyces*. The assay was left to grow at 30°C overnight, before recording the results and imaging the plates.

2.3.7 *Streptomyces* bioassays against *G. tritici*

A petridish containing approximately 33ml of PGA (Table 2-3) was innoculated with 4µl of *Streptomyces* spores at roughly 1/3 of the way across the plate and incubated for 7 days at 30°C. A small plug of agar was removed from the opposite third of the plate with a flame sterilised glass

pasteur pipette and wooden toothpick. The section was replaced with similar plug removed from the edge of a freshly grown plate of take-all. The assays were left at room temperature in the dark and imaged at 14 days.

2.3.8 DNA isolation for 16S gene amplification

Strains were innoculated into 10ml TSB in a 50ml Falcon tube with 10µl of. Cultures were grown for 3 days at 30°C. Liquid cultures were pelleted in a centrifuge at 3500RCF for 10 mins. Mycelia were suspended in 500µl SET buffer (See Table 2-3). Samples were incubated at 37°C for 2-3h following the addition of 10µl of lyzozyme solution (60mg/ml), inverting occasionally. After the solutions had cleared, 25µl of 20% (w/v) SDS was added with 500µl phenol/chloroform. Samples were vortexed for 10 seconds and centrifuged at 20000RCF for 5 minutes. The aqueous phase was transferred to a fresh tube and the phenol/chloroform step was repeated a further 2 times. The final aqueous phase was transferred and precipated in 3 volumes of 100% EtOH at -20°C. Samples were left at -20°C for at least 30 minutes before pelleting at 20000RCF for 20 minutes at 4°C. Pellets were washed with ice cold 80% EtOH and left to air dry at 20°C.

2.3.9 16S rRNA gene PCR and sequencing

DNA isolated from strains was used as a template for 16S rRNA gene PCR and sequencing. Table 2-2 describes the components and thermocycler conditions required for amplication.

Table 2-2 Components and thermocycler conditions for amplification of the bacterial 16S rRNA gene using colony PCR.

Component	**Volume (ul)**
BioMix™ red (Bioline)	12.5
dH_2O	8.75
Forward primer, 27F – 5'-AGAGTTTGATCMTGGCTCAG-3	1.25
Reverse primer, 1492R - 5'-TACGGYTACCTTGTTAGGACTT-3'	1.25
DNA template	1.25
PCR thermocycler reaction steps	
95° for 2 minutes	
30 cycles of 95° for 15s, 55° for 15s, 72° for 15s	
72° for 2 minutes	

2.3.10 16S rRNA gene sequence analysis

16S rRNA gene PCR products were sequenced by Eurofins Genomics, German and the resulting sequences were submitted to the NCBI BLAST web server (Agarwala *et al.*, 2018), searching both the nucleotide collection database as well as the RefSeq genome database. A phylogenetic tree was then generated using the SILVA alignment, classification and tree service web server. Bases remaining unaligned at the end of sequences were removed and the phylogenetic tree was calculated using FastTree. The resulting tree was further processed and exported using the interactive Tree of Life (iTOL) web app.

2.3.11 *In vitro* test for ACC deaminase production

To test for the use of 1-aminocyclopropane-1-carboxylic acid (ACC) as a sole nitrogen source, 4µl of spores were spot plated onto minimal media (MM) in which 0.2% (w/v) $(NH_4)_2SO_4$ or 0.051% (w/v) ACC was added as a sole nitrogen source. Plates contraining no nitrogen source were also innoculated as controls. Plates were left for 14 days at 30°C before imaging. Different strains preferred different carbon sources; this was determined in a prior trial with MM containing $(NH_4)_2SO_4$.

2.3.12 *In vitro* test for chitinase production

Selected strains were spot plated using 4µl on minimal media (MM) with chitin as a carbon source. Strains were plated on media with either L-asparagine or $(NH_4)_2SO_4$. Plates were grown for 14 days at 30°C and then flooded with 0.1% (w/v) congo red. After incubating for 10 minutes, the congo red was discarded and the plates flooded with Lugol's iodine solution. After another 10 minutes the reagent was discarded, and the plates were imaged on a light box.

2.3.13 High molecular weight DNA isolation for PacBIO whole genome sequencing

For whole genome sequencing, DNA was extracted using an established “salting out” protocol (Kieser et al 2000). Briefly, spores of *Streptomyces* isolates were inoculated into 250ml Erlenmeyer flasks containing 50 ml of TSB (Table 2-3) and sterile metal springs. Cultures were incubated at 30°C, shaking at 220 rpm, for 3 days. Cell material was then pelleted by centrifuging at 3500RCF for 10 minutes. Pellets were then resuspended in 10 ml of SET buffer (75 mM NaCl, 25 mM EDTA pH 8, 20 mM Tris-HClpH 7.5) containing 1 mg ml^{-1} of lysozyme, 40 µg ml^{-1} achromopeptidase and 30 µg ml^{-1} RNaseA, before incubating for 3h at 37°C. Proteinase K (0.5 mg ml^{-1}) and 600 µl of 1% (w/v) sodium dodecyl sulfate (SDS) were then added to the solution, before incubating at 55°C for 2 hours. After incubation, 2 ml of 5 M NaCl was added, along with 5 ml of chloroform. The solution was then mixed

continuously for 30 minutes, before centrifuging for 15 minutes at 3500RCF. The top layer of supernatant was placed into a fresh tube on ice before extracting again with chloroform. Mixing and centrifugation steps were repeated, and the top layer was transferred to a fresh tube. 0.6 volume of isopropanol was added, and the DNA was spooled onto a glass rod. After several minutes of spooling, the glass pipette was dipped into 6 ml of ice cold 70% (v/v) EtOH before being air-dried. Once dry, 500 µl of sterile dH_2O was used to gently wash the DNA into a 1.5 ml microcentrifuge tube. DNA was left at 4°C overnight to resuspend, before being transferred to -20°C for storage. The quality of DNA was assessed using a nanodrop spectrophotometer and the DNA concentration was established using a Qubit™ fluorometer with the DNA broad sensitivity assay kit (Invitrogen™).

2.3.14 Whole genome sequence analysis

The automated Multi-Locus Species Tree (autoMLST) server (Alanjary et al 2019) was used to phylogenetically classify the newly isolated streptomycete strains CRS3 and CRS4. Genomes were uploaded to the server, which performs a multi-locus sequence analysis against a curated genome database from NCBI, to produce high resolution species trees, as well as average nucleotide identity scores for isolates compared to sequenced strains (Alanjary *et al.*, 2019). AutoMLST automates the process of gene selection for multi-locus comparison by screening for gene homologues using the tool HMMER and essential gene models (Finn *et al.*, 2011; Alanjary *et al.*, 2019). It also automates the process of finding reference genomes for tree assembly and the de novo construction of maximum-likelihood trees (Alanjary *et al.*, 2019). The antiSMASH 5.0 server (Blin *et al.*, 2019) was used to predict the presence of Biosynthetic Gene Clusters (BGCs) in genome sequences, and genomes were additionally annotated using RAST (Aziz *et al.*, 2008). Genome sequences were uploaded to the KEGG Automatic Annotation Server (KAAS) for the functional annotation of genes and metabolic pathway mapping using the bi-directional best hit (BBH) method to assign orthologues. KAAS assigns K numbers to genes via BLAST comparisons against a manually curated KEGG GENES database. (Moriya *et al.*, 2007) K numbers then act as unique identifiers in the KEGG Orthology (KO) database which classifies genes by molecular function into pathway categories (Kanehisa *et al.*, 2016).

2.3.15 Media and buffers

Table 2-3 Media and buffer used in this study.

Medium	Ingredient	$g.L^{-1}$ dH2O
SFM (Soy flour medium) agar	Soy flour	20.00
	Mannitol	20.00
	Agar	20.00

Medium	Ingredient	g.L^{-1} dH2O
MYM (Maltose, Yeast extract, Malt extract) agar	Maltose	4.00
	Yeast extract	4.00
	Malt extract	10.00
	Agar	20.00
ISP-2 (International *Streptomyces* Project - 2) agar	Yeast extract	4.00
	Maltose	20.00
	D-glucose	4.00
	Agar	20.00
MM (Minimal medium)	L-asparagine	0.50
	K_2HPO_4	0.50
	$MgSO_4*7H_2O$	0.20
	0.01g $FeSO_4*7H_2O$	0.01
	Carbon source	10.00
	agar	10.00
PGA (Potato Glucose Agar)	Potato extract	4.00
	D-glucose	20.00
	Agar	20.00
SNA (Soft Nutrient Agar)	Difco Nutrient Broth Powder	4.00
	Agar	5.00
PBS-S (Silwett L-77 amended Phosphate Buffered Saline)	NaH2PO4.H2O	6.33
	Na2HPO4.H2O	16.50
	200 µl Silwet L-77 added after autoclaving	
LB (Lysogeny broth)	Tryptone	10.00
	NaCl	10.00
	Yeast extract	5.00
	agar (if required)	15.00

Table 2-4 Strains used in this study.

Species/strain name	Description	Origin
Methicillin resistant *Staphylococcus aureus* (MRSA)	Clinical isolate	Norfolk and Norwich University Hospital (UK)
***Klebsiella aerogenes* (KE)**	ESKAPE strain	Lab Stock
Candida albicans (CA)	Clinical isolate	Gift from Neil Gow, University of Exeter
***Gaumannomyces tritici* (wheat take-all)**	Environmental isolate	John Innes Centre, Norwich, UK
Triticum aestivum	Wild-type, variety Paragon	John Innes Centre, Norwich, UK
Triticum aestivum	Wild-type, variety Soisson	John Innes Centre, Norwich, UK
Triticum aestivum	Wild-type, commercial variety	John Innes Centre, Norwich, UK

2.4 Results

2.4.1 Isolation of wheat root associated microorganisms

Microorganisms were isolated from different compartments associated with the roots of mature wheat plants grown at the JIC field site at Church Farm, Bawburgh, Norfolk. Throughout this Chapter, strains are identified by a three-part code to determine plant accession, compartment origin and the culture media used for isolation, followed by a uniquely identifying number (Table 2-5). For example, the strain PES5 is the fifth strain to be isolated from the endosphere of the paragon variety of wheat on SFM agar.

Table 2-5 Codes used to identify strain origin from discreet nomenclature.

1st code – plant accession isolated from	2nd code – compartment isolated from	3rd Code – media isolated on
Paragon – (P)	Endosphere – (E)	SFM – (S)
Soissons – (S)	Rhizoplane – (Rw)	ISP-2 – (I)
Commercial (Kerin) – (C)	Rhizosphere – (R)	SFM/ISP-2 + phosphomycin – (Sp/Ip)

A total of 75 strains were isolated, 54 of which showed morphology consistent with *Streptomyces*. Of the 54 suspected streptomycetes, 15 were from the rhizosphere (R), 27 from the rhizoplane (Rw) and 18 were isolated from surface sterilised roots otherwise known as endosphere (E). The roots of three different plant varieties were sampled: 22 suspected streptomycetes were isolated from Paragon, 19 from Soisson, and 19 from a proprietary commercial variety.

2.4.2 Morphological diversity of wheat root associated *Streptomyces* spp.

Stereo microscopy was initially used to examine the colony morphology of wheat root isolates on SFM agar and identify streptomycetes from root-wash (Rw) and endosphere (E) compartments (Figure 2-1). Isolates were morphologically distinct, and no two strains appeared identical. Strains SES2 and SES4 were initially thought to be *Streptomyces*, although their morphology was distinct from the others and likely belonged to a different genus.

Figure 2-1 Colony morphology of the 54 putative streptomycetes isolated from rhizosphere, rhizoplane and endosphere compartments. Images obtained after 7 days growth. Scale bar = 3mm

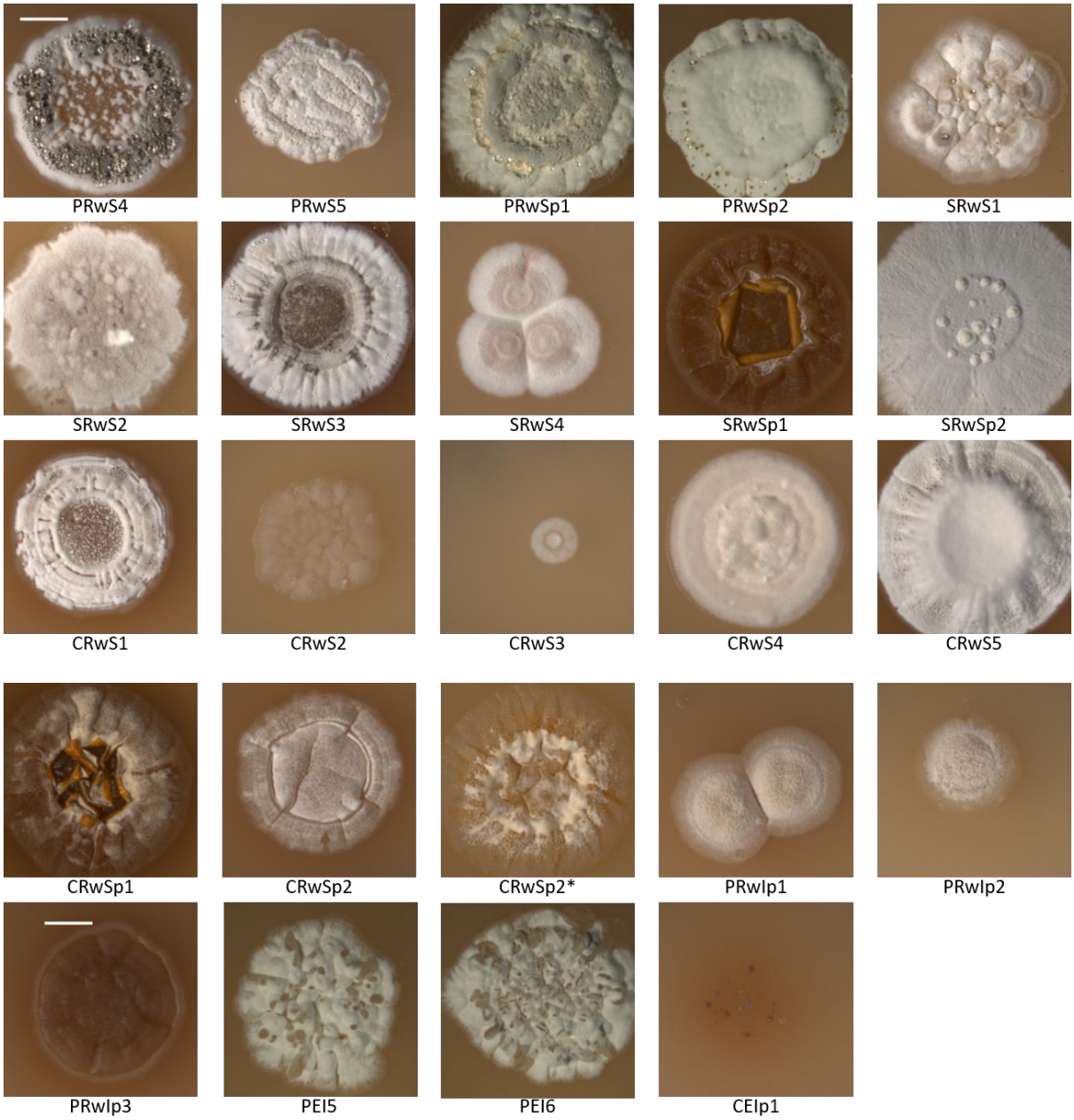

Figure 2.1 (cont.) Colony morphology of the 54 putative streptomycetes isolated from rhizosphere, rhizoplane and endosphere compartments. Images obtained after 7 days growth. Scale bar = 3mm

2.4.3 Phylogenetic and bioactive diversity of wheat root associated *Streptomyces* spp. and related actinomycetes

The full length 16S rRNA gene was amplified from genomic DNA isolated from wheat root isolates and sequenced using Sanger sequencing. BLAST analysis identified 52 of the isolates as *Streptomyces* spp. while SES2 and SES4 belonged to the genus *Promicromonospora*, which shares the order Actinomycetales with the genus *Streptomyces*. A complete table of BLAST results for the 16S

rRNA gene sequence for each isolate can be found in the supplementary data (Supplementary data S1)

A 16S rRNA gene tree was plotted to investigate patterns of relatedness within the metadata (Figure 2-2). The tree was rooted using the 16S rRNA gene sequence for *Arthrobacter crystallopoieties*. Taxa labels were colour coded to denote the wheat variety from which they originated. Symbols were used to denote the root microbiome compartment that each strain was isolated from. Based on these factors, no clustering could be observed, which suggested that 16S rRNA gene relatedness had no impact on recruitment to specific varieties. It might also suggest that it had no impact on which compartment was colonised, although this is challenging to confirm as strains may be distributed throughout the root microbiome.

Streptomyces are well studied for their extensive production of bioactive secondary metabolites, which likely contributes to their fitness in natural ecosystems. The bioactivity of wheat root associated streptomycetes was determined with a basic screen against bacterial indicator strains, with Gram positive (methicillin resistant *Staphylococcus aureus*, MRSA*)* and Gram negative (*Klebsiella aerogenes*, EA) bacteria, as well as the fungi *Candida albicans* (CA). Bioactivity against *G. tritici* (wheat take-all, TA) is also presented here and was carried out by Sarah Worsley. Bioactivity profiles are presented for each strain on soya flour + mannitol (SFM) and maltose, yeast extract + malt extract medium (MYM) agar (Figure 2-2). Take-all bioactivity was determined on SFM and potato glucose agar (PGA) instead. PGA was used in this instance, as it is the standard cultivation medium for wheat take-all. Bioactivities were plotted alongside the 16S rRNA gene tree, no clustering could be observed. This suggested that the relatedness of 16S rRNA gene sequences had no influence on bioactivity for the conditions tested.

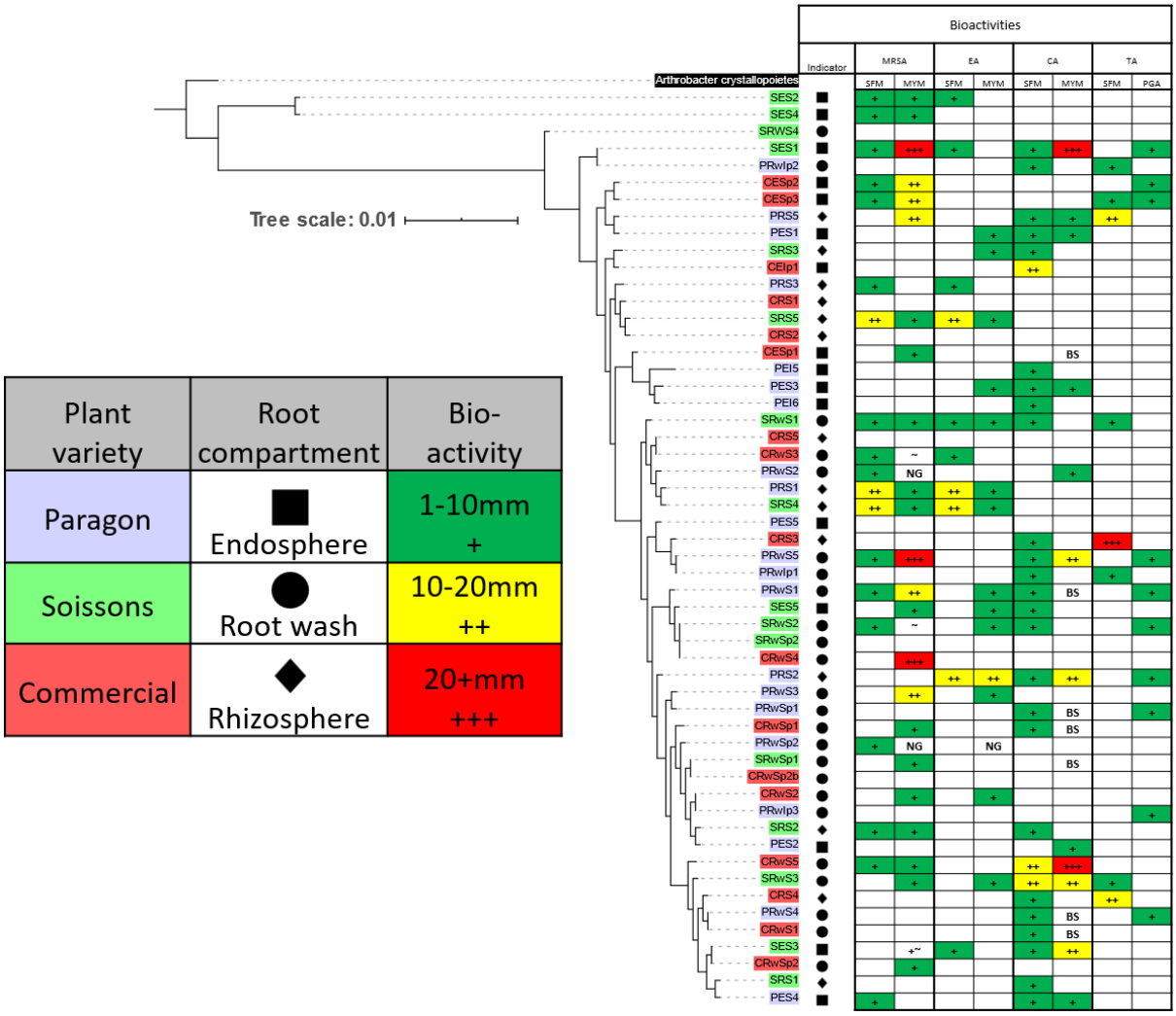

Figure 2-2 Full length 16S rRNA gene tree for 54 actinomycetes isolated from the wheat root microbiome. The rRNA gene sequence for *Arthrobacter crystallopoieties*, was used to root the tree. Strain origin is shown for both plant variety and root compartment. Bioactivities are shown on SFM and MYM agar against methicillin resistant *Staphylococcus aureus* (MRSA, Gram +ve), *Klebsiella aerogenes* (EA, Gram -ve), *Candida albicans* (CA, fungus) and Wheat take-all (TA, fungus).

Bioactivity was explored by root compartment and variety of origin (Table 2-6). Of the 54 strains, 29 killed MRSA on SFM or MYM agar, while 19 of them inhibited *K. aerogenes*. Out of the 54 isolates, 30 were bioactive against *C. albicans*. Bioactivity against wheat root pathogen take-all was less common, only 17 out of 54 strains could inhibit. Overall, compartment origin had little effect on whether a strain showed bioactivity or not. Fungal bioactivities were highest for strains isolated from paragon, 71% were bioactive against *C. albicans*, compared to the average of 56% for all strains. 43% inhibited take-all, compared to the average of 31%. Soissons isolates showed the greatest antibacterial activity, 69% and 63% inhibited MRSA and *K. aerogenes* respectively, compared to the average of 54% and 35%.

Table 2-6 Bioactivities shown as a proportion of strains that either share origin root compartment or host accession. Indicator strains: - methicillin resistant *S. aureus* (MRSA), *K. aerogenes* (EA), *C. albicans* (CA), *G. tritici* (wheat take-all, TA)

Number of bioactive strains		Compartment	MRSA	EA	CA	TA	% of bioactive strains		Compartment	MRSA	EA	CA	TA
Variety	Commercial (Kerin)	Rhizosphere	0 / 5	0 / 5	2 / 5	2 / 5	Variety	Commercial (Kerin)	Rhizosphere	0.00	0.00	40.00	40.00
		Rhizoplane	6 / 8	2 / 8	3 / 8	0 / 8			Rhizoplane	75.00	25.00	37.50	0.00
		Endosphere	3 / 4	0 / 4	1 / 4	2 / 4			Endosphere	75.00	0.00	25.00	50.00
		Commercial total	**9 / 17**	**2 / 17**	**6 / 17**	**4 / 17**			**Commercial total**	**52.94**	**11.76**	**35.29**	**23.53**
	Soissons	Rhizosphere	3 / 5	3 / 5	3 / 5	0 / 5		Soissons	Rhizosphere	60.00	60.00	60.00	0.00
		Rhizoplane	4 / 6	3 / 6	3 / 6	3 / 6			Rhizoplane	66.67	50.00	50.00	50.00
		Endosphere	4 / 5	4 / 5	3 / 5	1 / 5			Endosphere	80.00	80.00	60.00	20.00
		Soissons total	**11 / 16**	**10 / 16**	**9 / 16**	**4 / 16**			**Soissons total**	**68.75**	**62.50**	**56.25**	**25.00**
	Paragon	Rhizosphere	3 / 4	3 / 4	2 / 4	2 / 4		Paragon	Rhizosphere	75.00	75.00	50.00	50.00
		Rhizoplane	5 / 10	2 / 10	7 / 10	7 / 10			Rhizoplane	50.00	20.00	70.00	70.00
		Endosphere	1 / 7	2 / 7	6 / 7	0 / 7			Endosphere	14.29	28.57	85.71	0.00
		Paragon total	**9 / 21**	**7 / 21**	**15 / 21**	**9 / 21**			**Paragon total**	**42.86**	**33.33**	**71.43**	**42.86**
All varieties by compartment		Rhizosphere	6 / 14	6 / 14	7 / 14	4 / 14	All varieties by compartment		Rhizosphere	42.86	42.86	50.00	28.57
		Rhizoplane	15 / 24	7 / 24	13 / 24	10 / 24			Rhizoplane	62.50	29.17	54.17	41.67
		Endosphere	8 / 16	6 / 18	10 / 18	3 / 18			Endosphere	50.00	33.33	55.56	16.67
Total		**Total**	**29 / 54**	**19 / 54**	**30 / 54**	**17 / 54**	**Total**		**Total**	**53.70**	**35.19**	**55.56**	**31.48**

Of the 17 strains that showed bioactivity against wheat take-all, zones of inhibition (ZOI) varied greatly. While many showed ZOI of 1-2mm, others were far more potent. CRS3 demonstrated the highest potency against wheat take-all, mycelial plugs consistently failed to establish in PGA or SFM plates inoculated with this strain (Figure 2-3). It was identified as a prime candidate for long read PacBIO genome sequencing and *de novo* assembly.

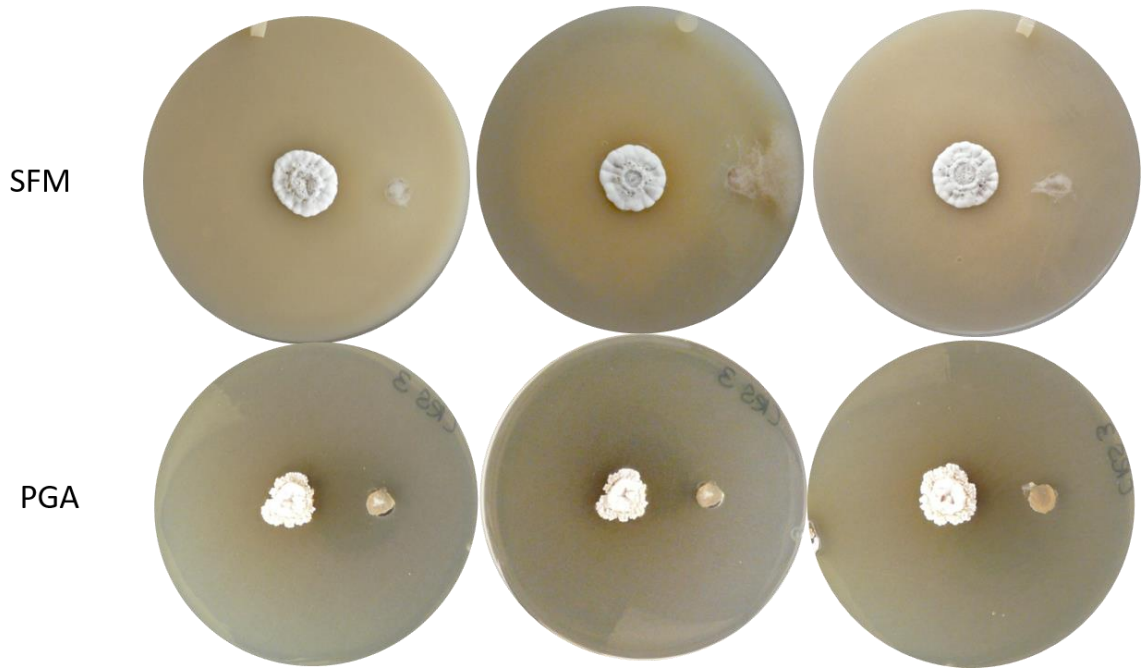

Figure 2-3 CRS3 (left of plate) challenged against wheat take-all fungus (right of plate) in 3 separate experiments. Wheat take-all failed to establish in all instances. This image was obtained by Sarah Worsley, who screened the isolates presented in this study for bioactivity against wheat take-all.

2.4.4 Chitin degradation by Take-all killing *Streptomyces* species

Representative wheat take-all killing strains of *Streptomyces* from either rhizoplane (Rw) or endosphere (E) compartments were chosen further characterisation. Chitinase activity has been shown to serve ecological functions in *Streptomyces*, from diversifying nutrient sources to conferring competitive advantage from antifungal activity. To test chitinase activity of isolates, they were grown on a minimal medium with chitin as the primary carbon source. Ammonium sulphate $(NH_4)_2SO_4$ or L-asparagine was provided as the nitrogen source. The model organism *Streptomyces coelicolor* was challenged for chitinolytic activity, in addition to three isolates from rhizoplane (Rw) and two isolates from endosphere (E) compartments. Strains that best utilised chitin could be identified, as plates would appear brighter when imaged on a lightbox after staining, due to increased translucency from

chitin breakdown. All strains were able to utilise chitin to some degree. Strains grown with L-asparagine as a nitrogen source utilised chitin better than they did on $(NH_4)_2SO_4$.

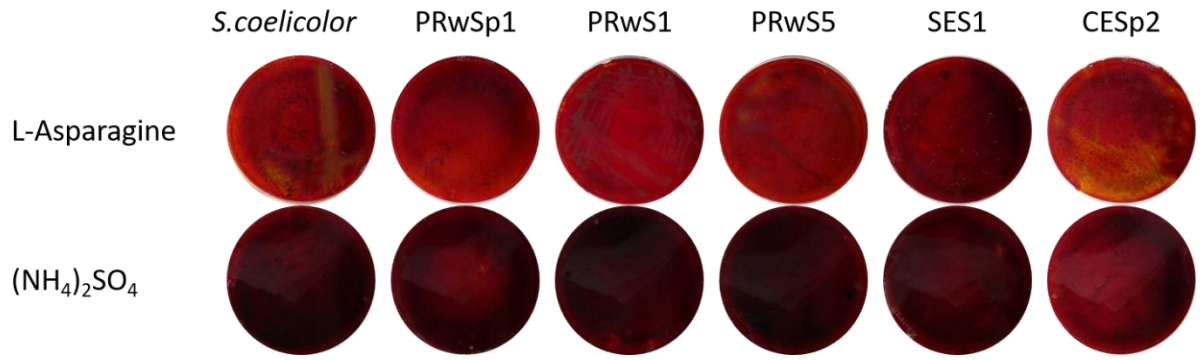

Figure 2-4 Chitinase activity determined qualitatively by Congo red and Lugol's iodine assay. Plates were backlit for imaging, increased translucency indicated higher levels of chitin degradation.

2.4.5 ACC deaminase activity by root associated *Streptomyces* species

Streptomyces can mitigate abiotic stresses in plant hosts through association, which is most often attributed to the degradation of 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor for plant stress hormone ethylene. Production of the ACC deaminase (ACCd) enzyme responsible for catalysing this reaction can be demonstrated by culturing isolates on minimal medium with ACC as the sole nitrogen source. Optimum carbon source was determined for each strain assayed in a pilot study; these preferences are presented in supplementary data S2. ACC metabolism assays were conducted for a selection of *Streptomyces* strains isolated from different compartments, that demonstrated wheat take-all bioactivity. Spores were spot plated onto minimal medium containing either an absence of nitrogen (negative control), $(NH_4)_2SO_4$ (positive control) or ACC. Strains showing improved growth on ACC compared to the negative control were confirmed to metabolise ACC. Most strains showed minimal growth on the negative control plates, but SRwS3 and CRS4 were able to grow almost as well as the $(NH_4)_2SO_4$ positive control. This is surprising, as this media used agarose in place of agar as the gelling agent to reduce contaminating nitrogen, yet these strains were clearly able to grow. The other strains assayed demonstrated improved growth on ACC plates compared to the negative control, which suggested they had the potential to utilise it as a nitrogen source. All strains demonstrated at least slight growth on the negative control, which suggests that these strains are either highly efficient at metabolising trace nitrogen, or that some component of the medium was contaminated.

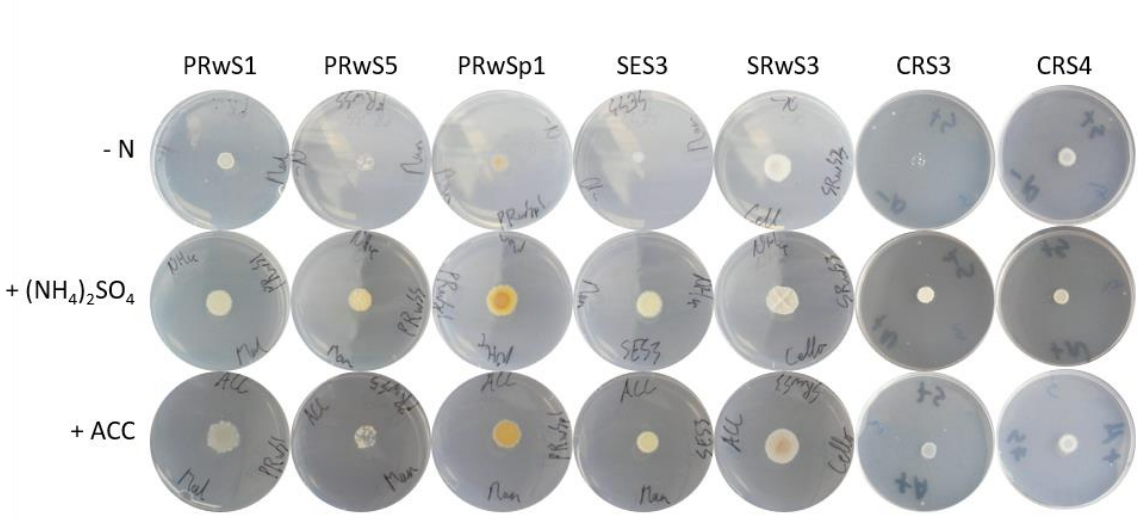

Figure 2-5 Spot plate images of wheat root isolates growing on minimal medium with ACC as the sole nitrogen source. All seven streptomycetes were able to metabolise ACC, apart from SRwS3 and CRS4, due to the anomalous growth on neative control plates.

2.4.6 Genome sequencing and *de novo* assembly of wheat take-all killing *Streptomyces* strains CRS3 and CRS4

Strains were selected for genome sequencing using the following criteria: Strains must consistently demonstrate bioactivity against wheat take-all, sporulate well under laboratory conditions and show distant relatedness from one another. Candidates must also demonstrate genetic tractability; this was determined by successful conjugation with the hygromycin resistant integrative vector pSS170, a derivative of pMS82 (Gregory *et al.*, 2003). CRS3 and CRS4 were chosen as they fit all these criteria. The genomes were sequenced by Novogene using the PacBio Sequel platform and assembled to a high level of completion, details are presented in (Table 2-7) and (Table 2-8) The N_{50} for CRS3 was 8,938,267 with a total genome size of 9,342,798. There was a total of 10 linear contigs after the final assembly of CRS3. The main chromosome (8,938,283bp) and a 237,316bp contig comprised most of the genome. The remaining 8 contigs were less than 10kbp each. These contigs could either represent linear plasmids or inadequate assembly. The genome for CRS4 was larger than CRS3 at 9,985,339bp and was assembled more completely with an N_{50} of the same value and 4 contigs. Final assembly reduced this to a total of 3 contigs, the chromosome was 9,985,339bp while the remaining linear contigs were 1323bp and 845bp. Again, these may represent either plasmids or regions of DNA that could not be assembled to the genome.

Table 2-7 Initial assembly statistics for PacBIO whole genome sequencing of wheat root isolates CRS3 and CRS4.

Sample ID	Polished Contigs	Max Contig Length (bp)	N50 Contig Length (bp)	Sum of Contig Lengths (bp)
CRS3	25	8,938,267	8938267	9342798
CRS4	4	9,985,305	9985305	10004409

Table 2-8 Assembled contig information for PacBIO whole genome sequencing of wheat root isolated CRS3 and CRS4.

Sample ID	Predicted contig type	Contig ID	Size(bp)	GC%	Circular?
CRS3	Chromosome	Chr1	8938283	71.08	Linear
CRS3	Plasmid	Plas1	1139	53.12	Linear
CRS3	Plasmid	Plas2	1318	63.28	Linear
CRS3	Plasmid	Plas3	237316	67.73	Linear
CRS3	Plasmid	Plas4	2870	49.58	Linear
CRS3	Plasmid	Plas5	6520	52.85	Linear
CRS3	Plasmid	Plas6	6682	45.57	Linear
CRS3	Plasmid	Plas7	7142	51.37	Linear
CRS3	Plasmid	Plas8	8181	49.11	Linear
CRS3	Plasmid	Plas9	8811	55.81	Linear
Sample ID	**Predicted contig type**	**Contig ID**	**Size(bp)**	**GC%**	**Circular?**
CRS4	Chromosome	Chr1	9985339	71.27	Linear
CRS4	Plasmid	Plas1	1323	63.04	Linear
CRS4	Plasmid	Plas2	845	63.08	Linear

Phylogenetic analysis determined species identity for CRS3 and CRS4, using the automated multi-locus species tree (AutoMLST) web server tool (Alanjary *et al.*, 2019). The estimated average nucleotide identities (ANI) for the ten most similar organisms are presented in (Table 2-9). The ANI of the closest type strain was below 95% for both strains sequenced. CRS3 was most similar to *Streptomyces platensis* with an estimated average nucleotide identity (ANI) of 93.4. The second closest type strain was *Streptomyces lydicus* (Figure 1.6) with an estimated ANI of 89.4%. This is interesting because the active microorganism in commercial biofungicide is also a strain of *S. lydicus* (Himmelstein *et al.*, 2014). CRS4 was less closely related to its nearest type strain, which shared an estimated ANI of

88.5% with *Streptomyces griseoruber*. A concatenated multi-gene phylogenetic tree was used to visualise the relatedness of CRS3 and CRS4 with similar organisms (Figure 2-6).

Table 2-9 Most similar organisms to wheat root associated *Streptomyces* species CRS3 and CRS4, as determined by AutoMLST analysis (Alanjary *et al.*, 2019).

CRS3				
Reference assembly ID	**Reference name**	**Mash distance**	**estimated ANI**	**Type strain**
GCF_000375625	*Streptomyces* sp. FxanaC1	0.0153	98.50%	FALSE
GCF_900090095	*Streptomyces* sp. SceaMP-e96	0.0179	98.20%	FALSE
GCF_000829715	*Streptomyces* sp. NBRC 110027	0.0612	93.90%	FALSE
GCF_002119195	*Streptomyces platensis*	0.066	93.40%	TRUE
GCF_001270575	*Streptomyces decoyicus*	0.0833	91.70%	TRUE
GCF_000719605	*Streptomyces* sp. NRRL S-1813	0.0848	91.50%	FALSE
GCF_900105775	*Streptomyces* sp. 2112.3	0.0909	90.90%	FALSE
GCF_900104865	*Streptomyces* sp. 2112.2	0.0914	90.90%	FALSE
GCF_900105525	*Streptomyces* sp. 2314.4	0.0914	90.90%	FALSE
GCF_000280865	*Streptomyces auratus* AGR0001	0.0914	90.90%	FALSE
GCF_000383635	*Streptomyces* sp. 303MFCol5.2	0.0259	97.40%	FALSE
GCF_001426425	*Streptomyces* sp. Root1310	0.0404	96.00%	FALSE
GCF_002081495	*Streptomyces* sp. B9173	0.061	93.90%	FALSE
GCF_001184025	*Streptomyces* sp. NBRC 110468	0.0611	93.90%	FALSE
GCF_900129855	*Streptomyces* sp. 3124.6	0.0937	90.60%	FALSE
GCF_900110755	*Streptomyces* sp. yr375	0.0968	90.30%	FALSE

CRS3				
Reference assembly ID	**Reference name**	**Mash distance**	**estimated ANI**	**Type strain**
GCF_001746365	*Streptomyces* sp. LUP30	0.0991	90.10%	FALSE
GCF_001428885	*Streptomyces* sp. Root264	0.1017	89.80%	FALSE
GCF_001514205	*Streptomyces griseoruber*	0.1151	88.50%	TRUE
GCF_002019855	*Streptomyces antibioticus*	0.1206	87.90%	FALSE

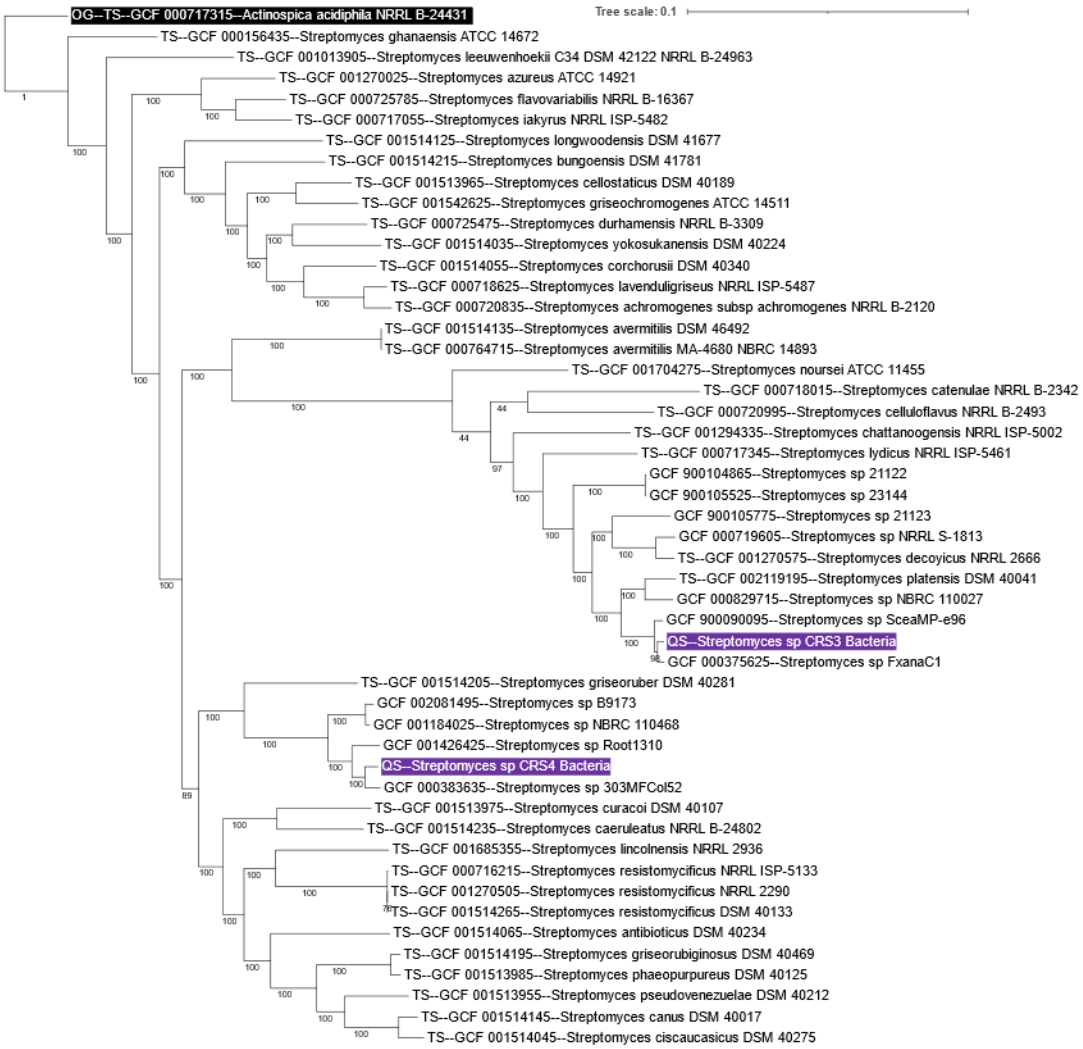

Figure 2-6 Concatenated multi-gene tree showing phylogenetic relatedness of wheat root isolates CRS3 and CRS4 (highlighted in yellow) with similar organisms. The tree was rooted using the outgroup actinomycete, *Actinospica acidiphila* (highlighted in grey). Mash distances were calculated by AutoMLST (Alanjary *et al.*, 2019).

2.4.7 KEGG pathway analysis of CRS3 and CRS4 genomes for plant growth promoting metabolism

It has been discussed that *Streptomyces* spp. may improve plant growth through production of plant growth hormone IAA, and by producing ACCd which can additionally mitigate plant stress by reducing ethylene levels. While there are a few known pathways and enzymes involved in the production of IAA, only a single enzyme so far is known to degrade ACC. In order to determine whether CRS3 or CRS4 encode enzymes implicated in any of these pathways, genomes were annotated using the KEGG Automatic Annotation Server (KAAS) (Moriya *et al.*, 2007). KEGG pathway analysis showed that both strains had the genomic capacity to produce ACC deaminase (Table 2-10), which supported *in vitro* ACC metabolism data presented earlier in this Chapter. While CRS3 had all three genes required to produce IAA through the TPM pathway, CRS4 had only the genes required for the final two steps of the pathway.

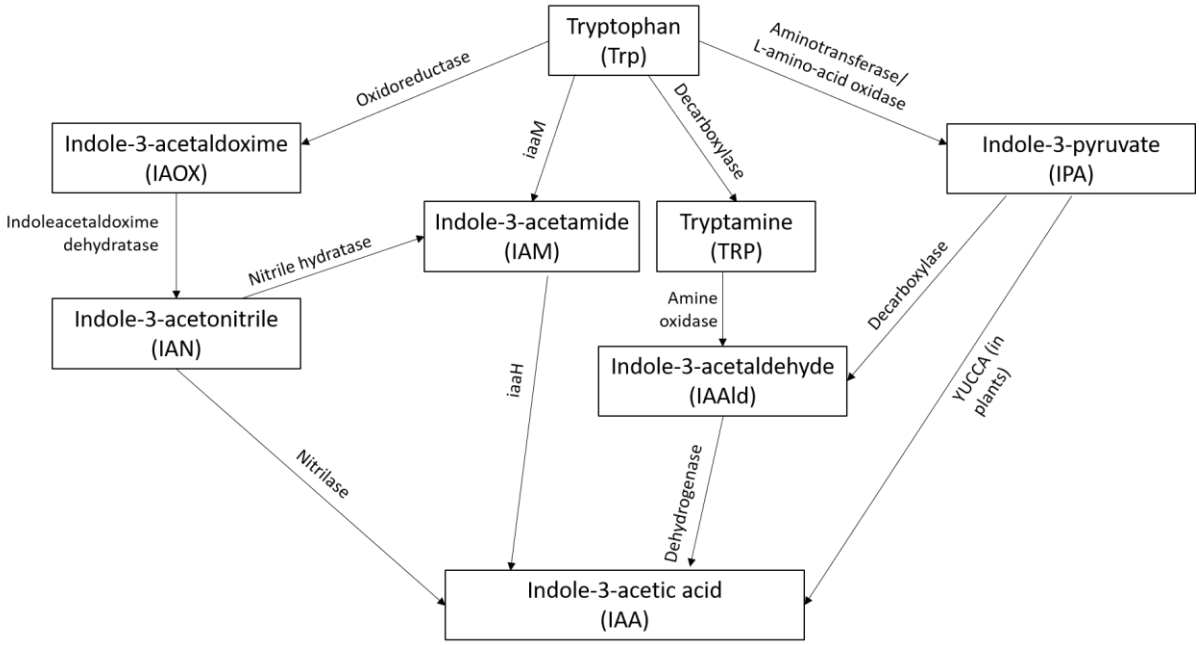

Figure 2-7 The main microbial tryptophan dependent IAA synthesis pathways. Image adapted from (Zhang *et al.*, 2019)

Table 2-10 A visualisation of KEGG pathway analysis for wheat root isolates CRS3 and CRS4. Genes present that encode for enzymes linked to either IAA production or ACC degradation are highlighted in green if present and red if absent.

Pathway	Enzyme	Reaction step	Enzyme K number	CRS3	CRS4
IPA-ipdC	Aminotransferase/L-amino-acid oxidase	1	K03334,K14265,K00838,K16903,K00832,K00838,K05821		
	Indole-3-pyruvate decarboxylase	2	K04103		
	Acetaldehyde oxidase/dehydrogenase	3	K00128,K14085,K00149,K11817,K22417		
IPA-YUCCA	Aminotransferase/L-amino-acid oxidase	1	K03334,K14265,K00838,K16903,K00832,K00838,K05821		
	Indole-3-pyruvate decarboxylase	2	K04103		
	YUCCA	3	K11816		
TPM	Tryptophan decarboxylase	1	K01593,K22433		
	Amine oxidase	2	K11182,K00274		
	Indole-3-acetaldehyde dehydrogenase	3	K00128,K14085,K00149,K11817,K22417		
IAM	iaaM	1	K00466		
	iaaH	2	K01426,K18541		
IAN-nitrilase-IAOX	indoleacetaldoxime dehydratase	1	K11868		
	nitrilase	2	K01501		
IAN-nitrilase-Gluco	myrosinase	1	K01237		
	nitrilase	2	K01501		

Pathway	Enzyme	Reaction step	Enzyme K number	CRS3	CRS4
IAN-nitrile-IAOX	indoleacetaldoxime dehydratase	1	K11868		
	nitrile hydratase	2	K01721, K20807		
	amidase	3	K01426, K18541		
IAN-nitrile-Gluco	myrosinase	1	K01237		
	nitrile hydratase	2	K01721, K20807		
	amidase	3	K01426, K18541		
ACC degradation	1-aminocyclopropane-1-carboxylic acid (ACC) deaminase	1	K01505		

2.4.8 Secondary metabolite BGC analysis of CRS3 and CRS4 using AntiSMASH

As these strains were selected for whole genome sequencing for their bioactivity against wheat take-all fungus, AntiSMASH 5.0 analysis (Blin *et al.*, 2019) was used to predict the presence of BGCs. For CRS3, 30 of its 32 BGCs were located on the main chromosome and the remaining two were on the largest of the remaining contigs, which was designated "plasmid". All 32 BGCs were located on the main chromosome for CRS4. Figure 2-8 summarises the type of BGCs predicted by AntiSMASH for CRS3 and CRS4

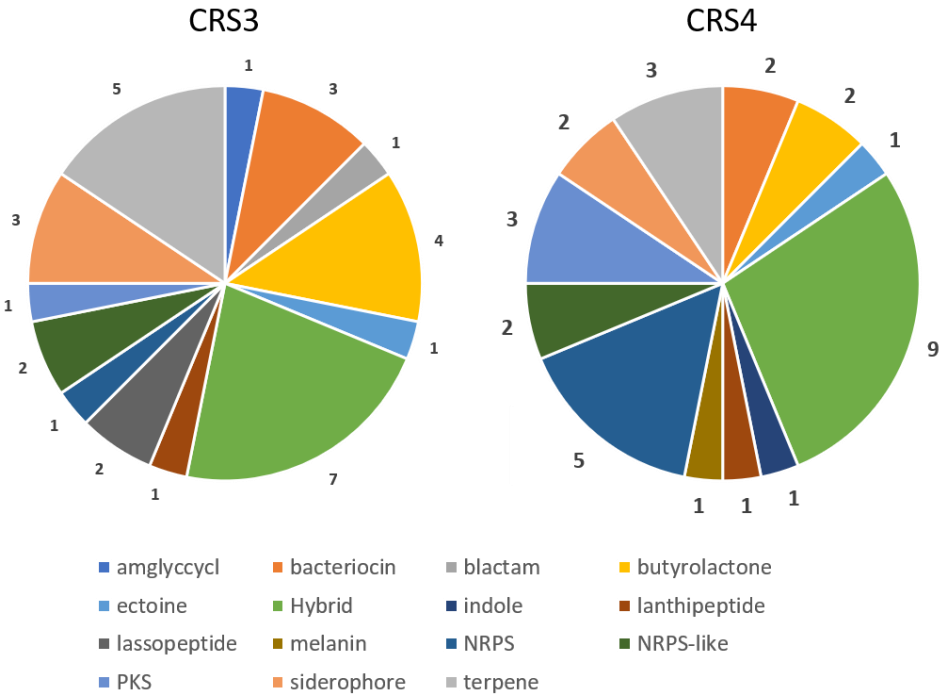

Figure 2-8 A summary of the type of BGCs predicted for *Streptomyces* strains CRS3 and CRS4. BGCs were predicted using by *in silico* analysis using the AntiSMASH web server. Numbers represent the abundance of each cluster in respective genomes.

Secondary metabolic capacity was diverse, predicted BGCs for various antimicrobial compounds were present for both strains. Full lists of predicted BGCs are presented in Table 2-11 and Table 2-12. The genome of CRS3 encodes a cluster which is predicted to produce a compound similar to lydicamycin. This further supports its close phylogenetic relationship with *S. lydicus*, after which the compound is named. Lydicamycin is antagonistic to Gram positive bacteria as well as certain yeasts. As such, it is not likely to be responsible for the antagonism of take-all fungus demonstrated in this study. Cluster 27 was identified as a candidate BGC responsible for the potent antifungal activity of CRS3. This hybrid cluster, predicted by AntiSMASH, contains a type-1 polyketide synthase (T1-PKS)

cluster. 60% of genes in the hybrid cluster showed similarities with the caniferolide gene cluster found in *Streptomyces caniferus* (Pérez-Victoria *et al.*, 2019). Caniferolide, like many other macrolides (Alvarino *et al.*, 2019), has been shown to have antifungal properties (Pérez-Victoria *et al.*, 2019).

Cluster 31 was highlighted as a candidate BGC responsible for antifungal activity (Table 2-12), as it shares 100% of the genes encoded by the BGC for antimycin production in *Streptomyces argillaceus* (Becerril *et al.*, 2018). This compound is widely used for its piscicidal properties in aquaculture but is additionally known for its ability to inhibit fungi and other organisms by blocking the respiratory electron transport chain. The identity of compounds responsible for the antifungal properties of CRS3 and CRS4 could not be determined during the timeframe of this study.

Table 2-11 BGCs in the genome of *Streptomyces* strain CRS3 as predicted by AntiSMASH analysis. Clusters were predicted using 'relaxed' strictness setting. Cluster 27, which was most similar to the BGC for caniferolide, is predicted to be the BGC responsible for antifungal bioactivity and is highlighted.

Cluster	Type	From	To	Most similar known cluster	Cluster type	Similarity
1	butyrolactone	46430	56445	BE-14106	Polyketide:Modular type I	7
2	amglyccycl	73560	97505	streptomycin	Saccharide	55
3	terpene	311522	336460	isorenieratene	Terpene	100
4	other,NRPS	392139	453118	deimino-antipain	NRP	66
5	butyrolactone	540897	551666	coelimycin P1	Polyketide:Modular type I	12
6	bacteriocin	866005	877073			
7	lanthipeptide	950946	974910	sceliphrolactam	Polyketide	8
8	T1PKS,hglE-KS	1113077	1164484	geosmin	Terpene	66
9	terpene	1308663	1334151	hopene	Terpene	69
10	bacteriocin	1929029	1938652			
11	butyrolactone	2006005	2016101			
12	siderophore	2176293	2189451	ficellomycin	NRP	3
13	NRPS,lassopeptide,LAP, thiopeptide,TfuA-related	3722607	3819106	A54145	NRP	13

Cluster	Type	From	To	Most similar known cluster	Cluster type	Similarity
14	terpene	3965518	3987515	salinomycin	Polyketide:Modular type I	6
15	terpene	4425935	4442184	pentamycin	Polyketide	20
16	terpene	5464372	5485433	ebelactone	Polyketide	5
17	T1PKS	5554954	5601019	melanin	Other	40
18	ectoine	6547301	6557717	ectoine	Other	100
19	siderophore	6641864	6650615	desferrioxamine E	Other	100
20	lassopeptide	6736653	6757909	citrulassin D	RiPP	40
21	blactam	7217962	7241463	valclavam	Other:Non-NRPS beta-lactam	71
22	siderophore	7324076	7338948	vibrioferrin	Other	18
23	T1PKS,NRPS,melanin,T2PKS	7484274	7717774	lydicamycin	NRPS + Polyketide:Modular type I	100
24	bacteriocin	7751036	7761263			
25	NRPS-like	7789584	7832406	stenothricin	NRPS:Cyclic depsipeptide	13
26	lanthipeptide,T3PKS	7935258	7985168	SapB	RiPP:Lanthipeptide	100

Cluster	Type	From	To	Most similar known cluster	Cluster type	Similarity
27	transAT-PKS,T1PKS, NRPS-like, oligosaccharide, PKS-like	8027687	8271333	caniferolide family	Polyketide:Modular type I	59
28	betalactone,NRPS, thiopeptide,LAP	8389732	8464198	muraymycin C1	NRP + Polyketide	23
29	butyrolactone	8759476	8770396			
30	lassopeptide	8886322	8908951	citrulassin D	RiPP	80
31	NRPS-like	Plasmid - 4163	Plasmid - 47705	cyclothiazomycin	RiPP:Thiopeptide	9
32	NRPS	Plasmid - 164382	Plasmid - 218877	glycinocin A	NRPS	9

Table 2-12 BGCs in the genome of *Streptomyces* strain CRS4 as predicted by AntiSMASH analysis. Clusters were predicted using 'relaxed' strictness setting. Cluster 31, which was most similar to the BGC for antimycin, is predicted to be BGC responsible for antifungal bioactivity and is highlighted.

Cluster	Type	From	To	Most similar known cluster	Cluster type	Similarity
1	NRPS	108835	193119	glycinocin A	NRP	11
2	lanthipeptide	221248	243201			
3	other,T3PKS,phenazine	299911	396257	merochlorin family	Terpene + Polyketide:Type III	56
4	butyrolactone	401004	408494	griseoviridin / fijimycin A	NRP:Cyclic depsipeptide + Polyketide:Trans-AT type I	8
5	T3PKS	642683	682231	germicidin	Other	100
6	NRPS	955788	1017674	diisonitrile antibiotic SF2768	NRP	61
7	bacteriocin	1161734	1172412			
8	lanthipeptide,bacteriocin	1383719	1409489	informatipeptin	RiPP:Lanthipeptide	85
9	hglE-KS,T1PKS	1734258	1785761	glycinocin A	NRP	13
10	terpene	1855037	1879495	hopene	Terpene	92
11	indole	2249258	2270385	kanamycin	Saccharide	61

Cluster	Type	From	To	Most similar known cluster	Cluster type	Similarity
12	NRPS,siderophore	2359319	2421234	scabichelin	NRP	100
13	terpene	2605484	2627313	geosmin	Terpene	100
14	bacteriocin	2667836	2679305			
15	siderophore	2875156	2886954			
16	terpene	3595348	3612747	albaflavenone	Terpene	100
17	siderophore	6214603	6226372	desferrioxamin B / desferrioxamine E	Other	83
18	melanin	6330780	6340160	lactonamycin	Polyketide	8
19	NRPS	6682033	6739872	SCO-2138	RiPP	64
20	NRPS	7399586	7452257	tetronasin	Polyketide	11
21	ectoine	7482883	7493287	ectoine	Other	100
22	NRPS-like	7707242	7749792			
23	T2PKS	8264728	8337243	spore pigment	Polyketide	83
24	T1PKS	8452012	8494992	chlorothricin / deschlorothricin	Polyketide:Modular type I + Polyketide:Iterative type I + Saccharide:Oligosaccharide	4
25	T3PKS,terpene	8622235	8663293	naringenin	Terpene	100

Cluster	Type	From	To	Most similar known cluster	Cluster type	Similarity
26	terpene,melanin	8932990	8954160	melanin	Other	71
27	NRPS	9033249	9078358	paenibactin	NRP	83
28	T1PKS,NRPS-like	9118008	9213139	streptovaricin	Polyketide	73
29	NRPS-like,T1PKS	9510610	9599055	mycotrienin I	NRP + Polyketide	57
30	NRPS-like	9736312	9778734	abyssomicin family	Polyketide	6
31	NRPS,T1PKS	9836999	9929428	antimycin	NRP + Polyketide	100
32	butyrolactone	9969272	9980264	griseoviridin / fijimycin A	NRP:Cyclic depsipeptide + Polyketide:Trans-AT type I	8

AutoMLST analysis classified the *Streptomyces* strain CRS3 in the same phylogenetic clade as *S. lydicus*, which is well studied for its plant beneficial traits and biocontrol properties. AntiSMASH analysis was used to predict BGCs encoded for by *S. lydicus* WYEC 108 (Actinovate™) in order to compare secondary metabolic capacities with CRS3. Table 2-13 shows BGCs that are shared between the strains, full antiSMASH output for *S. lydicus* WYEC 108 is presented in supplementary S3.

Table 2-13 A comparison of BGCs predicted by AntiSMASH analysis for *Streptomyces* strain CRS3 and *S. lydicus* WYEC 108. A full list of predicted BGCs for *S. lydicus* WYEC 108 can be found in supplementary data S3.

		CRS3			*S. lydicus* WYEC 108		
Most similar known cluster	**Cluster type**	**Cluster**	**Type**	**Similarity**	**Cluster**	**Type**	**Similarity**
A54145	NRP	13	NRPS,lassopeptide ,LAP, thiopeptide,TfuA-related	13%	19	TfuA-related,LAP, thiopeptide, NRPS,lassopeptide	13%
caniferolide A / caniferolide B / caniferolide C / caniferolide D	Polyketide: Modular type I	27	transAT-PKS, T1PKS, NRPS-like, oligosaccharide, PKS-like	59%	4	T1PKS,NRPS-like, PKS-like,oligosaccharide, transAT-PKS	61%
deimino-antipain	NRP	4	other,NRPS	66%	32	NRPS,other	66%
desferrioxamine E	Other	19	siderophore	100%	12	siderophore	100%
ectoine	Other	18	ectoine	100%	13	ectoine	100%
ficellomycin	NRP	12	siderophore	3%	21	siderophore	3%
hopene	Terpene	9	terpene	69%	27	terpene	69%
isorenieratene	Terpene	3	terpene	100%	33	terpene	100%
lydicamycin	NRP + Polyketide: Modular type I	23	T1PKS,NRPS, melanin, T2PKS	100%	9	NRPS,melanin,T1PKS	100%
salinomycin	Polyketide:Modular type I	14	terpene	6%	18	terpene	6%
SapB	RiPP:Lanthipeptide	26	lanthipeptide, T3PKS	100%	5	T3PKS,lanthipeptide	100%
stenothricin	NRP:Cyclic depsipeptide	25	NRPS-like	13%	6	NRPS-like	13%

		CRS3			*S. lydicus* WYEC 108		
Most similar known cluster	**Cluster type**	**Cluster**	**Type**	**Similarity**	**Cluster**	**Type**	**Similarity**
streptomycin	Saccharide	2	amglyccycl	55%	34	amglyccycl	55%
valclavam / (-)-2-(2-hydroxyethyl)clavam	Other:Non-NRP beta-lactam	21	blactam	71%	11	blactam	71%
vibrioferrin	Other	22	siderophore	18%	10	siderophore	18%

2.4.9 Candidate antifungal cluster analysis

In order to determine how similar cluster 27 from CRS3 was to the BGC for caniferolide, a schematic representation of each cluster was exported from antiSMASH and adapted (Figure 2-9). Analysis showed that there were strong similarities between clusters; type I PKS genes were alike in size and organisation. Additional genes were present in cluster 27. These genes may encode an additional secondary metabolite or metabolites, as this region was classified as a hybrid cluster by antiSMASH.

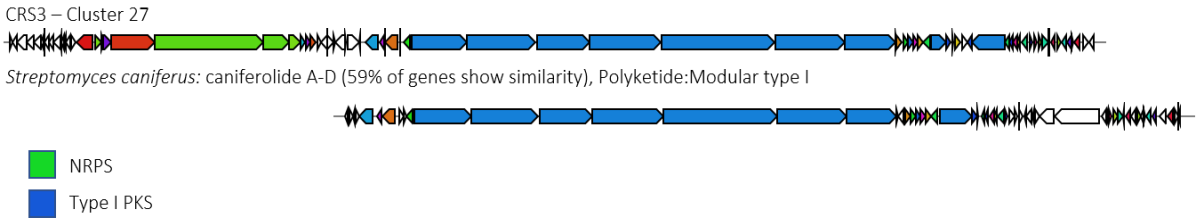

Figure 2-9 A schematic representation of the caniferolide BGC from *S. caniferus* and cluster 27 from *Streptomyces* CRS3.

AntiSMASH analysis showed that 100% of genes in cluster 31 of CRS4 shared similarity with antimycin clusters found in both *S. agrillaceus* and *S. sp. S4* (Figure 2-10). The size and organisation of NRPS and type I PKS genes were similar for all strains compared. Much like cluster 27 from CRS3, cluster 31 from CRS4 is a predicted hybrid cluster. As such, additional genes were present which may encode an additional secondary metabolite or metabolites.

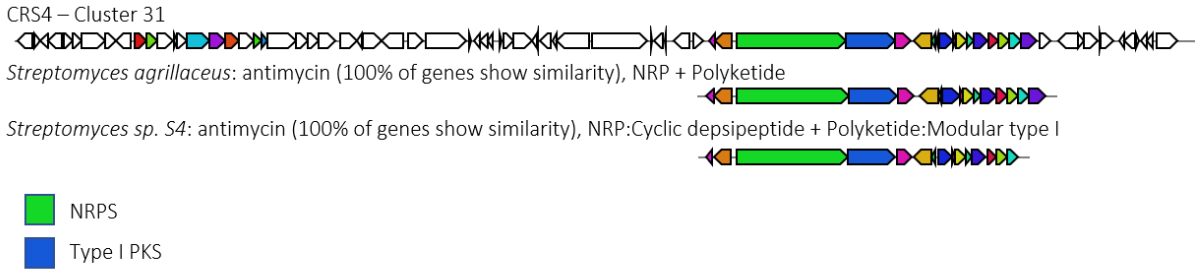

Figure 2-10 A schematic representation of the antimycin BGCs from *S. agrillaceus* and *S. sp.* S4 as well as cluster 31 from *Streptomyces* CRS4.

2.5 Discussion

This study set out to explore the biology of *Streptomyces* spp. associated with the roots of mature wheat plants. Studies have shown that these bacteria can provide benefits to their plant hosts, ranging from disease resistance to stimulation of plant growth. While commercially available products seek to exploit this association with the commodification of high-performance strains, they are limited. This study showed that more than a quarter of streptomycetes isolated from a range of host plants and compartments demonstrated potential to inhibit the wheat take-all fungus. No phylogenetic clustering could be observed between strains of similar origin, or bioactivity.

Additionally, a key aim was to screen and identify suitable strains for further study as model root associated streptomycetes. This study identified several strains with the potential to mitigate abiotic stressors through metabolism of ethylene precursor ACC. Chitinolysis, which is linked to the antagonism of pathogenic fungi, was also a common trait. Studies have shown that bacterial chitinolytic enzymes can attack fungal cell walls (Nagpure *et al.*, 2014; Swiontek Brzezinska *et al.*, 2014; Schönbichler *et al.*, 2020). Genome sequences were assembled *de novo* following PacBIO sequencing for 2 strains, one of which showed exceptional bioactivity against wheat take-all. KEGG pathway analysis confirmed that both strains have the functional capacity to metabolise ACC. Complete and partially complete metabolic pathways were identified for IAA production, which suggests that these strains have the potential to enhance plant growth. Both strains could be transformed with the integrative plasmid pSS170, this tractability highlighted their potential as model root associated streptomycetes as genetic manipulation facilitates deeper interrogation of their relationship with their plant host.

Initial screening demonstrated high morphological diversity of *Streptomyces* spp. isolated from wheat roots. This diversity was partly due to the initial selection process, colonies were picked that seemed to be the most unique. Similar isolation studies have also shown a range of visual phenotypes for *Streptomyces* spp. isolated from *Arabidopsis thaliana* (van der Meij *et al.*, 2018). Differing patterns of hyphal growth and differentiation may go some way to explain the super-structural differences between strains, while secondary metabolomic disparity could explain colour variations. Many isolates seem to be sporulating when the image was taken, the production of a grey pigment is characteristic of spore production in *S. coelicolor* (Kelemen *et al.*, 1998). BGCs that encode this pigment are related to those responsible for the production of antimicrobial compounds, it is suggested that these clusters diverged early in the evolutionary history of *Streptomyces* spp. (Blanco *et al.*, 1993). Robust sporulation is an important trait for streptomycetes used in agriculture, as seeds

are often coated with formulations in which the active ingredient is desiccated spores (Singh and Mehrotra, 1980; El-Abyad *et al.*, 1993)

Streptomyces isolates seemed to be unique from one another, not just at the morphological level, but the metabolomic level; all strains demonstrated a variety of bioactivity profiles for the growth conditions tested. Despite this, actinobacterial rRNA gene sequence similarity was high, many strains differing by only a few nucleotides and 9 duplicate sequences were observed. While the 16S rRNA gene is a widely used marker for barcoding and identification of bacteria at the genus level, the resolution for species level classification is low. The 16S rRNA gene may not be the best taxonomic marker for the *Streptomyces* genus; one study showed that 10 streptomycetes with identical actinobacterial rRNA gene sequences actually belonged to five different species, with diverse metabolic phenotypes (Antony-Babu *et al.*, 2017). Metabarcoding with this marker is useful for measuring the diversity of a sample, but it more limited for identification purposes. Despite this, phylogenetic analysis with 16S rRNA gene sequences suggested that there was no evolutionary divergence between strains isolated from different compartments or wheat accessions. Biologically, this might suggest that there is no fundamental difference between endosphere colonising streptomycetes and those found in the rhizosphere and rhizoplane. Additionally, strains do not seem to have coevolved with specific varieties of wheat. Due to metabarcode limitations, however, this theory would need to be validated further. Multi-locus sequence alignment with additional gene sequences such as *gyrB* or *trpB* (Law *et al.*, 2018) would help further resolve these phylogenetic relationships.

While no phylogenetic patterns could be observed between streptomycetes from different wheat accessions, a greater proportion of Paragon isolates demonstrated antifungal potential than strains from other varieties. Intraspecies variation in host genotype is known to alter microbial communities; studies have shown how richness and diversity, inferred from phospholipid fatty acid (PFLA) profiles, can vary between spring wheat types (Nelson *et al.*, 2011). PFLAs have been used as a biomarker for microbial diversity for many years now (White *et al.*, 1979). Briefly, fatty acids are extracted from environmental samples using organic solvents and fractionated into neutral, glycol and phospholipids (Willers *et al.*, 2015). These phospholipids are subjected to mild alkaline methanolysis to produce fatty acid methyl esters (FAMEs) which are profiled and quantified using gas chromatography mass spectrometry (GCMS). Chemical characteristics such as fatty acid chain length, saturation and branching can be used as a fingerprint for the microbial community (Steer and Harris, 2000). PFLA is a particularly useful biomarker in stable isotope probing (SIP) experiments. An environmental sample is fed with an isotopically labelled substrate, this label is incorporated into

microbial PFLAs by taxa responsible for the utilisation of this substrate. PFLA-SIP is more sensitive than other profiling methods such as DNA-SIP, this will be discussed in greater detail later. Taxonomic resolution of PFLA profiling is poor, however, species level identification of microbial communities is not possible. This is partly due to the fact that fatty acid biomarkers are not known for all microbial species, and are additionally sensitive to environmental changes (Watzinger, 2015). As such, PFLA is best used in conjunction with other profiling techniques.

Richer communities might suggest a weaker 'rhizosphere effect', in other words the plant host may exert a weaker selective pressure on its root microbiome. This could lead to increased competition, which in turn could select for streptomycetes with a greater capacity to produce antimicrobials. One issue with this hypothesis is that Paragon isolates did not show the same kind of dominance for antibacterial capacity. Fungal taxa may be higher abundance relative to bacteria in the Paragon root microbiome, this hypothesis could be validated with single copy gene quantification by qPCR. In this way, fungal taxa could be quantified relative to bacterial or archaeal taxa. Differences in functional capacity of the root microbiome between wheat accessions could be resolved with metagenomic analyses (Sessitsch *et al.*, 2012; Mendes *et al.*, 2014; Ofek-Lalzar *et al.*, 2014).

Indicator strains were chosen carefully for the bioactivity screening experiment. MRSA was chosen as a robust indicator for bioactivity against Gram positive bacteria. This strain has been used previously in this lab to reduce the rediscovery of known natural products. Gram negative bioactivity is less common, so *K. aerogenes* was used as an indicator to try and capture a wider range of bioactive strains. *Candida* species have been used to determine the antifungal activity of Actinobacteria associated with a range of environments, from marine sponges to fungus-farming ants (Dharmaraj and Sumantha, 2009; Mendes *et al.*, 2013b). *C. albicans* was used in this study and is a popular indicator of fungal bioactivity for a few different reasons. It is not only easy to culture in yeast form, which is optimal for overlay technique bioassays, it is also an opportunistic pathogen of humans. Actinomycetes such as *Streptomyces* spp. have been a focus in the field of drug discovery for some time, owing to the diversity of natural products they create. It makes sense that this strain is well reported in the literature as a bioindicator, given the relative shortage of clinical antifungals, compared to antibacterials (Carrillo-Muñoz *et al.*, 2005; Hof, 2006).

Mixed or dual-culture of an organism with a bioindicator is often cited as a viable approach for the induction of secondary metabolism (Rateb *et al.*, 2013; Okada and Seyedsayamdost, 2017), due to the inherent nature of competitive signals to drive defensive metabolisms. In this study, isolates are grown for 7 days before overlaying with the indicator culture, variations of this method have been employed in other studies that investigate the bioactivity of streptomycetes (Aparicio *et*

al., 1999; Barke *et al.*, 2010). Co-culture is important as it replicates some factor of environmental conditions in a very simple way. While simple indicator strains may be practical from a throughput level for screening, it is important to consider bioactivity against environmentally relevant organisms. *G. tritici* (wheat take-all) was chosen for this reason. The overlay method could not be employed to challenge strains against wheat take-all, instead a mycelial plug was transferred from an agar plate culture of Wheat take-all fungus in active growth. This is a common cultivation method (Asher, 1980) for filamentous fungi like *Fusarium* (Cha *et al.*, 2016). This technique has been established for working with wheat take-all (Worsley *et al.*, 2020) but there are some methodological considerations when using this strain as a bioindicator. While *Streptomyces* plates may be inoculated at the same time, mycelial plugs may differ in the time taken to establish in the new media. This leads to variation in the timing of interaction with streptomycetes, leading to variation in bioactivity. Triplicate bioassays helped to mitigate the issue within this study.

Once strains were identified with bioactivity against wheat take-all, chitin degrading activity was determined. All strains that were assayed showed chitinolytic activity to varying degrees. Chitinolysis is a well described property among streptomycetes (Quecine *et al.*, 2008; Narayana and Vijayalakshmi, 2009; Nagpure *et al.*, 2014; Yandigeri *et al.*, 2015), although it is not clear whether it is a universal trait. As saprophytes, it is the nature of *Streptomyces* spp. to live on complex substrates in the soil environment (Saito *et al.*, 1999; Peacock *et al.*, 2003; Borodina *et al.*, 2005; D'Huys *et al.*, 2012), so it is likely that metabolism of chitin derived from fungal and insect detritus is widespread. Studies have suggested that the presence of chitin switches on production of enzymes that target its degradation (Schrempf, 2001). Since then, additional studies have shown that chitin supplementation can enrich for streptomycetes, which suggests that they are responsive to this substrate. Supplementation with chitin can also increase the disease suppressiveness of soil (Cretoiu *et al.*, 2013). The authors additionally observed enriched actinobacterial and oxalobacterial communities, this might implicate a plethora of biological processes with the suppressiveness they observed. Despite this, chitinases have been shown to elicit antifungal effects directly (Wang *et al.*, 2002). As such, it is likely that these enzymes at least contribute to fungal bioactivities, especially in combination with chitin synthase inhibitors such as nikkomycin (Liao *et al.*, 2009). Given that this study has shown that many *Streptomyces* species demonstrate capacity for chitin metabolism, future studies should investigate how this may alter fungal bioactivity profiles, given that it represents a strategy for the enhancement of biocontrol properties in an agricultural setting.

The ACC utilisation experiment determined that ACC metabolism was common among take-all killing *Streptomyces* species. One of the earliest papers describing an assay for determination of

ACC metabolism used a similar method to that employed by this study (Dworkin and Foster, 1958), in which growth was determined qualitatively on minimal media agar with ACC as the sole nitrogen source. While the recipe described in this study has been used in previous experiments by our lab group (Worsley, 2019), the work presented in this Chapter adopted a medium more frequently used in *Streptomyces* experimental biology (Kieser *et al.*, 2000), in order to maintain continuity with the chitin metabolism experiment. Variations on this recipe have been used in other studies that have interrogated metabolism of ACC in streptomycetes, while the concentration of ACC remained the same (Jaemsaeng *et al.*, 2018b, 2018a). The results from this study showed that many of the streptomycetes tested were able to grow at least partially, in the absence of supplied nitrogen. Ideally, a complete absence of growth should be observed for these negative control plates, it is possible that isolates were able to grow on trace nitrogen which had contaminated media components. Despite this, growth was visibly improved for strains grown on media containing ACC relative to the negative control, which suggested they had the capacity to metabolise it. The implication of this finding is that these strains may be able to mitigate abiotic stress in association with plants, by preventing pathologic accumulation of ethylene (Jaemsaeng *et al.*, 2018b).

CRS3 was able to metabolise ACC, KEGG pathway analysis for the assembled genome additionally confirmed that ACCd was encoded by the genomes of both CRS3 and CRS4. While metabolism was confirmed for CRS3 *in vitro* and *in silico* for both CRS3 and CRS4, the question remains, what effect would this have in the wheat root microbiome? While studies have linked ACC metabolism in *Streptomyces* spp. with salt stress protection in various plant such as tomato (Palaniyandi *et al.*, 2014) and rice (Yoolong *et al.*, 2018), the parameters required for inducing salt stress and determining recovery may differ. As such, this would require optimisation for future studies.

Genome assembly for CRS3 and CRS4 facilitated further insights into the biology and genomic capacity of wheat root associated strains. Both genomes were shown to encode the enzyme for metabolising ACC, by KEGG pathway analysis. The same analysis showed that model streptomycete *S. coelicolor* also encodes this gene (SCO5247), reciprocal BLASTP analysis of an actinobacterial genomes database suggests that this gene may be relatively conserved in streptomcyetes (Chandra and Chater, 2014). All bacterial genomes of the order Streptomycineae that were analysed were predicted to encode homologues of this gene (Table 2-14). This may hint at a specialised metabolism resulting from long-standing coevolution between plants and streptomycetes. This theory is supported by (Chater and Chandra, 2006), who state that the emergence of land plants coincides with the emergence of *Streptomyces*. As such it is likely that plants have exerted a strong selective pressure on streptomycetes along with other root associated microbes, to utilise plant metabolites such as ACC,

especially when it may confer a benefit to the plant to do so. Nevertheless, genomic capacity is not always equal to functionality. ACC deaminase may not always be sufficiently expressed or translated to yield the stress recovery phenotype. Other studies have shown that salt stress recovery is linked to expression of ACC; constitutive expression in *Streptomyces venezuelae* led to increased rice growth under saline stress, relative to the control group which was inoculated with wild-type *S. venezuelae*. Future studies should seek to investigate the expression of ACC deaminase in the root microbiome, not just for plant derived strains, but for other streptomycetes as well.

Table 2-14 Conservation of the ACC deaminase gene SCO5247 (K01505) among bacterial genomes of the order Streptomycineae. Gene conservation was determine by reciprocal blastP analysis of 100 bacterial genomes (Chandra and Chater, 2014)

Streptomycineae	
Species	**Present**
S. lividans TK24,	Y
S. viridochromogenes DSM 40736,	Y
S. scabiei 87.22,	Y
S. sviceus ATCC 29083,	Y
S. avermitilis MA-4680,	Y
S. griseoflavus Tu4000,	Y
S. venezuelae ATCC 10712,	Y
S. griseus subsp. *griseus* NBRC 13350,	Y
S. hygroscopicus ATCC 53653,	Y
S. pristinaespiralis ATCC 25486,	Y
S. roseosporus NRRL 15998,	Y
S. albus G J1074,	Y
S. clavuligerus ATCC 27064,	Y
Kitasatospora setae KM-6054.	Y

Full genome sequences for root associated strains are useful for generating hypotheses about BGCs that may be responsible for bioactivity observed for CRS3 and CRS4. One candidate for antifungal bioactivity in CRS3 that was identified with antiSMASH analysis is cluster 27, which showed 59% of genes were similar to the caniferolide gene cluster found in *Streptomyces caniferus* (Pérez-Victoria *et al.*, 2019). This compound belongs to a group called macrolides, which are known for their antifungal activities, particularly in the clinical setting (Zotchev, 2012). Cluster 31 of CRS4 shared 100% of genes with the BGC for antimycin (Seipke *et al.*, 2014), a compound which has been described in the literature as early as 1949 (Dunshee *et al.*, 1949).

While these may not be novel compounds, there are clear benefits to the implication of a biocontrol product that produces well characterised bioactive metabolites, especially if the plant is

for human consumption. For example, if CRS3 produces caniferolide in the wheat microbiome, trace levels may be present in the grain. The effects of consumption on human biology are likely to be more predictable, as it has already been studied in a clinical context (Alvarino *et al.*, 2019). Antimycin on the other hand is not currently used in the clinical setting, but it is known for its broad piscicidal, pesticidal and fungicidal properties (Seipke and Hutchings, 2013). If CRS4 produces this compound, food products may need to be monitored, should the strain be used for biocontrol. This may not be a problem, one of the key benefits of biocontrol is the targeted delivery of bioactive compounds through enrichment of producing microbes in the root microbiome, which means that levels should be far lower than for direct chemical application by crop spraying. Should CRS4 produce antimycin in the wheat root microbiome, toxicity studies can help shape regulations on safe working limits (Kotiaho *et al.*, 2008), due to its use in aquaculture.

Genome mining of streptomycetes, often from unusual places, is also a popular approach for the discovery of novel antimicrobials and other natural products (Aigle *et al.*, 2014; Iftime *et al.*, 2016). Both strains sequenced in this shared less than 95% estimated ANI with type strain organisms, which may suggest that they are new species. Several novel BGCs were additionally identified. While it is important to recognise that these clusters may not be responsible for the production of antimicrobial compounds, many of them have low similarity to other known BGCs, which suggests that they may produce molecules with novel structures or ecological functions. For example, while cluster 25 from CRS3 is most similar to the non-ribosomal peptide synthase (NRPS) stenothricin, only 13% of genes are shared with the cluster found in *Streptomyces filamentosus*. This might suggest that the compound produced by cluster 25 is completely different from anything observed in the literature. By exploring the chemical diversity found within competitive niches, like the root microbiome, it may be possible to discover new and beneficial compounds.

Various studies have laid the foundation for using *Streptomyces* species as biocontrol agents in an agricultural setting. In particular, the plant beneficial properties of *S. lydicus* WYEC 108 are well described (Zeng *et al.*, 2012; Himmelstein *et al.*, 2014; Chen *et al.*, 2016). The work presented in this Chapter cannot be compared directly with these studies, rather this work intends to the lay the foundations for discovery of additional biocontrol products such as WYEC 108. Additionally, this study sought to investigate whether all streptomycetes can confer these benefits to plants and what links those that confer a greater benefit than others. In conclusion, some isolates seemed better equipped to suppress wheat take-all disease than others, but there did not seem to be an evolutionary link between them. Streptomycetes isolated from Paragon showed greater bioactivity against fungi than

other varieties, but the sample size was too low to determine the statistical significance of this observation. A more extensive media screen may even out these disparities.

A more restricted screen showed similar potential between isolates, for plant beneficial traits such as ACC metabolism. Further studies would seek to interrogate plant beneficial properties in a more relevant context, plant growth promotion and disease prevention would be challenged *in vivo* with greenhouse trials and field studies. These studies would ideally scale to screen all streptomycetes isolated in this study, to further elucidate what make specific strains more suited to biocontrol.

S. lydicus WYEC 108 is the primary ingredient in the commercially available biocontrol product Actinovate™. Interestingly, CRS3 shares 89.4% ANI with *S. lydicus* and was identified as the second most closely related type strain from the AutoMLST analysis. AntiSMASH analysis of WYEC 108 showed that both strains had similar profiles of BGCs. Most notably, both strains encode clusters with high similarity to lydicamycin and caniferolide gene clusters. A comparison of these gene clusters suggests that cluster 27 from CRS3 may have all the genes required to produce caniferolide. While purified caniferolide was not available for general purchase at the time of publishing, future studies may seek to disrupt these putative antifungal BGCs in both *S. lydicus* WYEC 108 and CRS3. Continued exploration of the biology of CRS3 will benefit from literature documenting such closely related organisms from the *S. lydicus* group (Anderson and Wellington, 2001; Komaki *et al.*, 2020).

There are broader considerations for the development of a biocontrol strain. While many of the isolates in this study showed promising qualities for improvement of plant health, there are concerns that large-scale inoculation of these strains in the environment may have unintended consequences, this has been discussed in the literature (Winding *et al.*, 2004). The abundance of mycorrhizal fungi is often cited as an indicator of plant health (Jeffries *et al.*, 2003; Abbott and Lumley, 2014), so there are often concerns that antifungal compounds in the root microbiome may target these organisms. DAPG, an antifungal produced by *Pseudomonas fluorescens* has been shown to affect the mycelial development of *Glomus mosseae* (Barea *et al.*, 1998), which is an AMF known for its positive role in plant health. What is clear from the review is that there is a distinct lack of studies describing the abundance of antifungal compounds in the root microbiome, where biocontrol measures are implicated. Inoculation controls for these studies are similarly sparse (Winding *et al.*, 2004).

The off-target effects may be even greater, for candidate biocontrol strains with the capacity to produce much broader biocidal compounds, such as CRS4. Antimycins inhibit respiration by binding to the mitochondrial bc_1 complex (Li-shar *et al.*, 2005), which means they are toxic to all organisms,

including higher eukaryotes. As such, the presence of these compounds in the root microbiome may antagonise nematode populations, many of which are pathogenic to plants (Butterworth *et al.*, 1989). Further studies would challenge CRS4 with a model bioindicator such as *Caenorhabditis elegans* (Burns *et al.*, 2015), in order to probe for nematocidal activity.

In conclusion, this study set out with two key goals: to explore the biology of root associated streptomycetes and to identify candidate strains for the promotion of plant health in agriculture. No evolutionary divergence was found between strains isolated from different compartments of the wheat root microbiome. Additionally, there was no clear distinction between strains isolated from different wheat genotypes. Many of these strains were able to kill wheat take-all fungus, several of these exhibited other plant beneficial metabolisms such as chitinolysis and ACC degradation. This work lays the foundation for further study into the biology of root associated *Streptomyces*. Deeper phylogenetic analyses using a multi-locus sequence tree would further resolve the relationships between streptomycetes that associate more intimately with plant roots. This study provides a platform to test additional hypotheses; do any of these streptomycetes improve plant growth? If so, what links those that do. Further screening will help identify strains that can provide multiple benefits for plants. In addition, strains that demonstrate tractability may serve as models for investigating the mechanisms of root colonisation by *Streptomyces* species, such as those which shall be explored in Chapter 4.

This study also set out to identify potential strains for use in agriculture, for the promotion of plant health. Strong candidates with dissimilar 16S rRNA gene sequences and strong bioactivity against wheat take-all were identified, whole genome sequences were obtained to explore their functional capacity further. Candidate BGCs were proposed to account for antifungal activity, gene deletion studies would confirm this. These are promising strains that require more characterization *in vivo*, in an agricultural setting.

Chapter 3 Identification of wheat root associated microbial communities active in consumption of exuded carbon

3.1 Introduction

While the plant microbiome was introduced in Chapter 1, this Chapter will take a closer look at the roots specifically, diving deeper into culture independent approaches to look at community dynamics. There is an extraordinary amount of diversity present in the root microbiome; complex and dynamic forces shape microbial populations. Soil conditions have a significant impact on microbiome assembly; microbial composition is strongly influenced by a range of variables in soil properties (Schreiter *et al.*, 2014). Plants are also shown to a varying extent, to selectively filter and recruit microorganisms from the bulk soil community to the root microbiome. It is widely accepted that this happens through exudation of organic compounds, which can change according to development (Houlden *et al.*, 2008) and plant health.

Despite the considerable number of variables that impact microbial composition in the root microbiome, many studies show that select genera such as *Pseudomonas* and *Streptomyces* are commonly found to be enriched in the wheat root microbiome, compared to the surrounding bulk soil (Germida and Siciliano, 2001; Hayden *et al.*, 2018; Chen *et al.*, 2019). These core communities are better defined for the model plant *Arabidopsis thaliana* (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2013) but are also described for various plant species (Costa *et al.*, 2006; Garbeva *et al.*, 2008; Philippot *et al.*, 2013). Understanding the factors that drive microbiome assembly to the benefit or detriment of plant health is the first step towards exploiting microbial communities in agriculture.

3.1.1 Factors driving root microbiome assembly

Soil microbial composition is well known to vary according to the physical and chemical properties of the soil substrate itself. Plant root associated communities, as subsets of bulk soil communities, are similarly influenced. (Schreiter *et al.*, 2014) observed differing lettuce rhizosphere communities between soil types in the same location, thereby controlling for a range of confounders such as climate and agricultural practices. Bulk soil and plant root microbe composition are both sensitive to agricultural practices such as fertilisation (Wang *et al.*, 2019b) and tilling regimes, though unpicking these effects are challenging when there is a distinct lack of studies controlling for these confounding variables. Long term nitrogen fertilisation is known to increase the exudation of photosynthetically fixed organic compounds by the roots, which itself is known to influence the

community composition (Zhu *et al.*, 2016; Chen *et al.*, 2019). Nitrogen supplementation may also enrich denitrifying species of microorganisms as well.

The plant root associated microbial community may be under external pressure from the soil, but it is also driven by the biological chemistry of organic compounds exuded by the plant's roots. There are a range of factors that determine the types and quantities of plant root exudates, this will be discussed in detail, but a key determinant is plant genotype. Different species of plants usually host communities from common phyla (Bulgarelli *et al.*, 2013) but differ at lower level taxonomies (Lundberg *et al.*, 2013; Sousa *et al.*, 2020; Zhang *et al.*, 2020). Even at an intraspecies level, microbiome assembly is shown to differ between varieties (Nelson *et al.*, 2011; Mahoney *et al.*, 2017; Sousa *et al.*, 2020). These differences are most evident when comparing domesticated and wild-type variant crops (Bulgarelli *et al.*, 2015), wild crop varieties tend to associate more strongly with a more varied microbial community, demonstrating the dynamic and evolving process of microbiome assembly. Microbial community analysis combined with genome wide association studies can even yield insight into genomic regions associated with microbiome assembly (Bulgarelli *et al.*, 2015).

Many wheat varieties have been generated by crossing the elite spring bread wheat variety Paragon with various land races, it has already been established that this is the rationale behind its usage as a model in this thesis. The choice of wheat variety is important to consider, as various studies demonstrate wheat variety dependent root microbiome variation (Germida and Siciliano, 2001; Mahoney *et al.*, 2017). For example, greater complexity is observed for the endosphere community of modern wheat varieties than for ancient land races (Germida and Siciliano, 2001), *Aureobacter* species also differed significantly between the rhizosphere communities, of the three different cultivars that they explored. (Mahoney *et al.*, 2017) sampled rhizosphere communities from 54 different samples across 9 different wheat cultivars. 26 phyla were identified, 95% of which were part of the core community, they were present in all 9 cultivars. Differential relative abundances were observed for certain phyla, however. Actinobacteria were more abundant in Finch, Madsen and PI561722 cultivars. Eltan, PI561722, PI561725 cultivars had higher abundances of Planctomycetes. While these studies present no functional analyses of the microbial community, they do serve to demonstrate how plant genotype can lead to divergence of root microbial communities from the core microbiome, however subtle they may be.

(Pieterse *et al.* 2016) have discussed that although soil type can often exert a greater pressure on a host microbiome than plant genotype for artificial ecosystems such as commercial farming (Philippot *et al.*, 2013), the reverse is predicted to be true for naturally occurring plant/microbe communities as a result of a longer standing co-evolution. To understand why this is the case, it is

important to recognise that plants dedicate between up to 40% of the carbon fixed by photosynthesis to secretion from the root, in the form of organic compounds called root exudates (Badri and Vivanco, 2009; Vives-Peris *et al.*, 2020). The cost of this is outweighed by several benefits; root exudates can impact the soil environment in favour of the plant in various ways. Compounds that modulate pH can solubilise nutrients for plant uptake, chelators can mitigate the impact of toxic compounds and antimicrobials can combat plant pathogens (Vives-Peris *et al.*, 2020). All of these things can affect the root associated community, but plants arguably exert the greatest influence on the root microbiome by providing substrate, to supplement or maintain select co-evolved microbial communities (Badri and Vivanco, 2009), this will be discussed in greater detail. As all these benefits come at a cost of fixed carbon, domesticated plant varieties that are better nourished are generally less reliant on the benefits associated with high levels of root exudation and often exhibit less diverse microbial communities.

3.1.2 Plant root exudates

The way that plant roots alter their environment was first termed the 'rhizosphere effect' by (Hiltner, 1904). Since then, it has been established that a major component driving this sphere of influence is a diverse and complex array of secreted compounds. Fatty, organic and amino acids are secreted along with sugars, phenolics and alcohols (Badri and Vivanco, 2009; Preece and Peñuelas, 2020; Vives-Peris *et al.*, 2020). The exact makeup of these compounds has been shown to shift according to plant development (Chaparro *et al.*, 2014) and health (Jousset *et al.*, 2011). The root associated community is also known to shift in both composition and function, in response to these changes.

There are various ways to investigate the influence of root exudates in plant/microbe systems. Compounds can be isolated and purified, often from sterile or gnotobiotic systems. Specific compounds can then be correlated with microbial community structure and discrete developmental stages (Chaparro *et al.*, 2013). The caveat is that root exudate profiles will vary depending on the method of isolation. Harvesting root exudates from sterile hydroponic systems is more reproducible (and easier) than isolation from field systems (Vives-Peris *et al.*, 2020). Plants respond to microbial communities in the soil, and this would impact the resulting exudate profile.

Other studies use genetically modified plant lines that shift root exudate profiles (Bressan *et al.*, 2013; Carvalhais *et al.*, 2015), these studies often focus on tractable model organisms such as *Arabidopsis thaliana*. Pleiotropic effects are a crucial factor to consider when drawing conclusions from studies involving mutant plant lines, however. A study by (Lebeis *et al.*, 2015) used *cpr5* null

mutants to correlate salicylic acid production with colonisation of *Streptomyces* spp. Work published by (Worsley, 2019) demonstrated flaws in this approach, *cpr5* null mutants are associated with early onset senescence and cell death (Kirik *et al.*, 2001; Jing *et al.*, 2008). (Worsley, 2019) showed that these lines suffer fitness defects, the implication here is that increased colonisation by *Streptomyces* species could well be due to confounding effects caused by these mutations.

3.1.2.1 The microbial ecology of plant root exudates

While there are various studies demonstrating the correlation between root exudate profile and microbial community, the isolation, purification and subsequent application of individual compounds to plant microbe systems can offer more robust insights. (Badri *et al.*, 2013) showed that repeated application of purified exudates to *A. thaliana* grown in soil, led to enrichment or depletion of specific OTUs. Gamma-aminobutyric acid (GABA) reduced Streptomycineae members but increased various proteobacterial genera. Salicylic acid treatment of soil showed an increase in actinobacterial species from Streptomycineae, Corynebacterineae and Pseudonocardineae. Secondary metabolites produced by wheat plants and other cereal crop have also been implicated as chemotactic nutrient sources for rhizobacteria. (Neal *et al.*, 2012) showed that exogenous application of benzoxazinoids (BXs) like 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) altered gene regulation in rhizobacteria *Pseudomonas putida*; transcriptomic data showed upregulation of catabolism and chemotaxis genes. (Jacqueline M Chaparro et al., 2013) took a systems level approach to infer the metabolic capacity of the rhizosphere community of *A. thaliana*, by sequencing the mRNA transcript of the microbial population. Genes associated with catabolism of specific compounds correlated with the abundance of those compounds in collected root exudates, suggesting the microbial community can respond to changes in root exudate production. All the evidence combined strongly suggests that individual root exudates can be utilised as a nutrient source or act as a signal to modulate colonisation (Chaparro *et al.*, 2013).

The idea that plants feed root associated microbial communities is additionally supported by various studies that interrogate mutant plant lines. More robust studies aim to mitigate pleiotropic confounding effects, with a combined experimental approach. (Neal *et al.*, 2012) showed that DIMBOA deficient *bx1* null mutants of wheat were less colonised by *P. putida* than the wild type accession, validating the transcriptomic study from the same publication. Transgenic studies using cereal crops are scarce, but those conducted with model organisms such as *A. thaliana* support this link between root exudate production and microbial community assembly. Jasmonate pathway loss-of-function mutations in *A. thaliana* led to a shift in root exudate profile, namely a reduction in the amino acids asparagine, ornithine and tryptophan but an increase in various sugars, including fructose

(Carvalhais *et al.*, 2015). This led to increased colonisation of select genera of Actinobacteria and Firmicutes, this is interesting because fructose is a known chemotactic stimulant for *Bacillus* spp. (Ordal *et al.*, 1979). Again, this study followed on from prior work that showed enrichments of similar taxa, with exogenous activation of the jasmonate pathway (Carvalhais *et al.*, 2013).

While many of these studies have looked at microbial community structure, it is important to ask questions about the function of microbial communities in response to root exudates. (Jousset *et al.*, 2011) linked not only primary metabolic function of the root associated microbiome but secondary metabolism. Barley plants switched on antifungal metabolite gene expression in root associated *Pseudomonas fluorescens* when challenged with phytopathogen *Pythium ultimum*, further reinforcing the link between microbiome function and plant health. Although there are few *in situ* studies investigating the regulation of microbial secondary metabolism in response to plant root exudates, they have been explored as elicitors of secondary metabolism in *Streptomyces* spp. (van der Meij *et al.*, 2018; Worsley *et al.*, 2020). A deeper look at secondary metabolite regulation in the root microbiome is necessary.

3.1.2.2 Plant root exudates are linked to development

Genotype is one of the primary drivers of root exudate profile, but it is known to vary throughout a plant's life according to two key factors. The most noticeable shift occurs as a plant advances through development stages (Houlden *et al.*, 2008; Chaparro *et al.*, 2013, 2014; Chen *et al.*, 2019). In *A. thaliana*, these changes can be summarised; exudation of sugars are high at early stages of development and decline over time, amino acids and phenolics are low early on but increase over time (Chaparro *et al.*, 2013). Root exudate profiles were shown to correlate with the expression of genes encoding the relevant exudate transporters, in root tissue. Another study from the same group demonstrated distinct microbial communities at the 4 different stages of plant development (seedling, vegetative, bolting, flowering) (Chaparro *et al.*, 2014). The authors showed that although there was a shift in community over different development stages, there was an overlap between adjacent stages, which implied a gradual and continuous shift. Species richness was greatest at the seedling stage, which suggests that the plant is refining the root microbiome over time. The authors also used metatranscriptomic profiling to investigate the function of the microbial community, the transcripts encoding biosynthesis of antimicrobial streptomycin were shown to be upregulated during bolting and flowering, presumably to support disease resistance (Chaparro *et al.*, 2013). What is important to note here is that actinobacterial abundance was lowest at these stages, offering a cautionary tale of inferring function from 16S rRNA gene community profiles.

There is a distinct lack of literature exploring the relationship between wheat development and root exudate profile at this level, or even related plant species. The one study that has been published is quite comprehensive however, investigating the influence of not just development, but nitrogen fertilisation on both root exudate profile and root microbial community (Chen *et al.*, 2019). Development stages are characterised slightly differently than (Chaparro *et al.*, 2013), with sampling at tillering (vegetative), jointing (stem formation) and ripening (pre-harvest) stages. An important thing to take away from this study is that released organic carbon (ROC) is low at the tillering stage but spikes at the jointing stage, succinic acid dominates exuded organic acids. The ROC drops at the ripening stage and undergoes a shift toward citric acid exudation. Whilst the authors present no richness or diversity analysis, there is shown to be a significant shift in the microbial community through different development stages. Actinobacterial abundance increases at tillering as Bacteroidetes drops. The abundance of actinobacterial order Micrococcales was shown to be strongly correlated with succinic acid, which was highly produced at jointing. *Streptomyces* and *Promicromonospora* were among the actinobacterial genera that were more abundant at jointing and ripening stages. The study conducted by (Chen *et al.*, 2019) offers a useful insight into how factors such as development and agronomic practices correlate with microbial community composition, but it is important to recognise that it is just that. Correlative studies explore the relationship between factors and observations but do not implicate microbial taxa with a function, such as metabolism of rhizodepositions.

3.1.2.3 Plant root exudates are linked to plant health

Studies have shown that antimicrobial production can be switched on at specific development stages, which has been correlated to the exudation of specific metabolites (Chaparro *et al.*, 2013), so it seems fitting that plants would benefit from exploiting this mechanism. Root exudation profile does in fact shift according to biotic stressors, which has been shown to alter the microbial community. This sits at the core of the 'cry for help' hypothesis, which is reviewed by (Rolfe et al., 2019). *A. thaliana* has been shown to respond to foliar pathogenesis with production of L-malic acid (MA) in the roots (Rudrappa *et al.*, 2008), which was independently shown to increase colonisation and biofilm formation of the beneficial *Bacillus subtilis* FB17. (Liu *et al.*, 2017c) performed a similar screen to identify cucumber root metabolites increased in response to colonisation by either pathogen *Fusarium oxysporum* or beneficial *Bacillus amyloliquefasciens* SQR9. It was found that tryptophan production strongly correlated with SQR9 colonisation. The same group also demonstrated a 27-fold increase in plant hormone IAA gene expression by SQR9 (Liu *et al.*, 2016), when treated with tryptophan. This shows that root exudates are not only involved in recruiting microorganisms in response to biotic stresses, but also stimulating beneficial traits in them. Other groups have similarly published root

metabolomic shifts associated with pathogenesis. (Yuan *et al.*, 2018) showed that infection of *A. thaliana* with foliar pathogen *Pseudomonas syringae* caused a shift in the root exudate profile from sugars, alcohols and short-chain organics acids to amino acids, nucleotides and long chain amino acids. Remarkably, community profiling showed that this caused a shift not only in the rhizosphere community, but the bulk soil as well. This suggests that plants can respond to pathogenesis to protect not only themselves, but future generations.

BXs have already been discussed as root metabolites responsible for recruiting beneficial microorganisms to the root microbiome (Neal *et al.*, 2012), but they have long been recognised in the broader context as key defence metabolites in cereal crops (Niemeyer, 1988, 2009). (Cotton *et al.*, 2019) took a multi-omic approach using genetically modified maize lines with disruption to genes involved in BX biosynthesis. Using metabolomics and a metabarcoding approach, the authors showed that BXs are global regulators of the root metabolome and subsequent microbial communities. This suggests that BX compounds may play a key role in grass species rhizobiome assembly. Interestingly, these compounds are tryptophan derived. Given that other studies have shown increased production of tryptophan under biotic stressors, in addition to stimulating colonisation of beneficial microorganisms and promote symbiotic metabolisms, it is quite possible that it has a central role in defence signalling and root microbiome assembly.

3.1.3 Identifying microbial consumers of root exudates with Stable Isotope Probing

The key technique underpinning most of these studies is metabarcoding. When asking questions related to the microbial ecology, it is important to understand the function of microorganisms. It is also important to understand the limitations of metabarcoding for this purpose, It has been shown with meta-transcriptomic studies, that reduced abundance does not necessarily mean reduced function of a particular taxa (Chaparro *et al.*, 2013). Function cannot accurately be inferred from community surveys in this way. The studies reviewed thus far have correlated root metabolomic profiles or host development checkpoints with microbial community profiles but lack *in vivo* evidence to directly support the feeding of microorganisms with root exudates.

The studies that have probed deeper into the function and chemical ecology of specific organisms have demonstrated high complexity in host-microbiome dynamics. To answer with greater confidence whether root microbe associated communities are fed by root metabolites, metabarcoding can be combined with nucleic acid stable isotope probing (SIP), in order to track the flow of metabolites from the host to the microbiome. This technique utilises an isotopically labelled substrate to feed a population of microorganisms. Metabolism of labelled substrate leads to the

incorporation of this label into nucleic acids and other biomolecules. These biomolecules are then isolated from a microbial community and separated by mass; isotopically labelled molecules are heavier. Profiling this heavy fraction yields insights into which community members are active in metabolism the substrate of interest. While this technique was first used to probe communities with simple substrates using enrichment cultures in microcosms (Radajewski *et al.*, 2000), SIP can also be used to probe the microbial ecology of host associated microorganisms such as plants (Haichar *et al.*, 2008; Uksa *et al.*, 2017). When interrogating microbial communities responsible for consuming root exudates, $^{13}CO_2$ is injected into sealed plant growth chambers during the photosynthesis period. Labelled carbon is fixed by photosynthesis into sugars and other organic compounds, a portion of which is exuded by plant roots. As microorganisms consume these labelled root exudates, ^{13}C is incorporated into their DNA as they grow and divide. Following isolation of total DNA from rhizosphere soil and surface sterilised roots (endosphere), labelled (^{13}C) heavy DNA is separated from unlabelled (^{12}C) light DNA using density gradient ultracentrifugation. The identity of root exudate metabolisers can be determined by amplifying and profiling metabarcode genes, such as the 16S rRNA gene, from this labelled fraction.

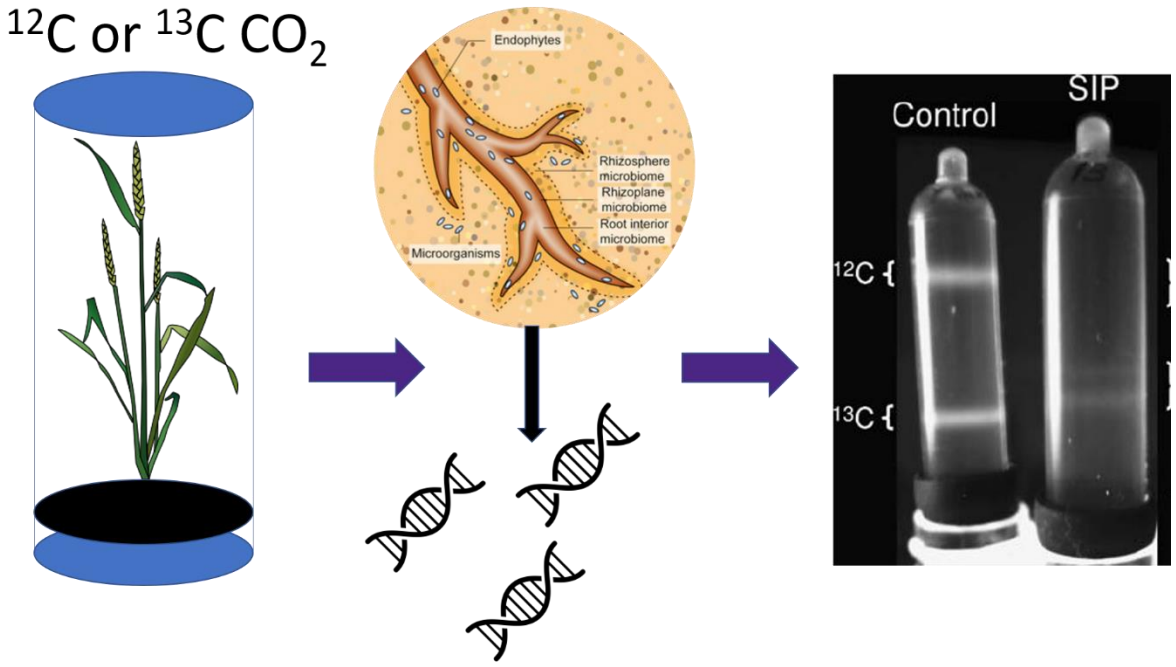

Figure 3-1 An overview of Stable Isotope Probing (SIP) in plants. Plants are grown inside sealed chambers that are injected with either $^{13}CO_2$, or $^{12}CO_2$ for control group plants. The ^{13}C label is fixed into organic metabolites that are exuded by the plant's roots and consumed by microorganisms that inhabit the root microbiome. DNA is then extracted from these microbes and separated by CsCl DNA gradient ultracentrifugation. The "heavy" ^{13}C labelled DNA and "light" unlabelled DNA are sequenced

to determine which taxa are metabolising root exudates. Root microbiome image (centre) is adapted from (Gaiero *et al.*, 2013), image visualising DNA separated by ultracentrifugation is adapted from (Neufeld *et al.*, 2007a).

Early studies using this technique utilised qualitative approaches to analyse community shifts in labelled DNA, such as denaturing gradient gel electrophoresis (DGGE) (Ikenaga *et al.*, 2003; Costa *et al.*, 2006). This technique offers a crude visualisation of the richness and diversity of a community but cannot provide quantitative insights. Other qualitative techniques used restriction fragment length polymorphism (RFLP) (Lu *et al.*, 2006) in order to assess community shifts. More recent approaches often still utilise metabarcode amplification with PCR, but instead use next generation sequencing to provide a higher resolution and more robust, quantitative analysis (Uksa *et al.*, 2017). Metagenomics has been coupled with SIP to probe the genomic capacity of communities metabolising specific substrates (Eyice *et al.*, 2015). In some cases this has led to the discovery of entirely new bacterial families (Jones *et al.*, 2019). SIP has also been used in this way to probe microbial consumers of root derived compounds (Verastegui *et al.*, 2014), although this has not been done *in vivo*. SIP performed with ribosomal RNA can be used to resolve more active consumers of root exudates (Rangel-Castro *et al.*, 2005; Lu *et al.*, 2006), and meta-transcriptomics can even be performed on active communities using mRNA SIP (Haichar *et al.*, 2012) .

Researchers have probed the active communities associated with the roots of numerous plant species from model organisms like *Arabidopsis thaliana* (Haichar *et al.*, 2012) to cereal crops like wheat (Uksa *et al.*, 2017) and rice (Lu *et al.*, 2006), using various permutations of nucleic acid SIP. A summary of SIP studies conducted in cereal crops is presented in Table 3-1. These studies are useful as they offer insights into how specific microbial taxa may become enriched in the root microbiome, by identifying key organisms involved in the metabolism of root exudates.

3.1.4 Stable isotope probing of wheat plants

Nucleic acid SIP is a tool that has been used to investigate microbial community dynamics for some 20 years now (Radajewski *et al.*, 2000), but studies that interrogate root associated communities with this technique are less common than microcosm enrichments. This is in part due to the additional complexities associated with probing the metabolism of multi-trophic, host associated communities. (Worsley, 2019) summarised the 14 published nucleic acid SIP studies that investigated microbial metabolisers of plant root exudates. Only five of these publications profiled actively metabolising communities with next generation metabarcoding techniques, with the remainder using more qualitative analysis such as DGGE and RFLP. Since these publications were summarised, several new

SIP studies have been published. Table 3-1 is adapted from (Worsley, 2019), with a focus on the investigations of cereal crops, publications are brought up to date.

Table 3-1 A summary of SIP studies conducted in cereal crops that investigate microbial metabolism of root exudates. Table adapted from (Worsley, 2019).

Plant species	Compartment	Nucleic acid	Profiling technique	Key findings	reference
Rice	Rhizosphere and bulk soil	RNA	T-RFLP from RNA and sequencing of 16S rRNA clone libraries	^{13}C was incorporated into archaeal RNA in soil surrounding rice roots and plant derived carbon was suggested to play a key role in archaeal CH_4 production in anoxic rice soils	(Lu and Conrad, 2005)
Rice	Endosphere and rhizosphere	RNA	Bacterial T-RFLP from RNA and sequencing of 16S rRNA clone libraries	Alpha and Beta Proteobacteria dominated rice roots as well as Firmicutes. Proteobacteria assimilated root exudates most actively	(Lu *et al.*, 2006)
Wheat Maize	Rhizosphere	DNA	DGGE	Different bacterial phyla metabolised root exudates in the rhizosphere of different plant species. Root exudates activated communities of SOM degraders	(Haichar *et al.*, 2008)
Rice	Roots and rhizosphere	RNA	16 rRNA gene amplicon sequencing with Illumina 454	Proteobacteria and Verrucomicrobia were most highly labelled. Different bacterial groups were labelled in the root and rhizosphere. A greater proportion of OTUs were labelled in the roots versus the rhizosphere	(Hernández *et al.*, 2015)

Wheat	Rhizosphere	DNA	16S rRNA gene amplicon sequencing with Illumina 454	Actinobacteria and Proteobacteria dominate metabolism of root exudates. Actinobacterial root exudate usage reduces with soil fertilisation.	(Ai *et al.*, 2015)
Wheat	Rhizosphere	DNA	16S rRNA gene amplicon sequencing with Illumina 454	Different bacterial communities use plant-derived resources in the rhizosphere of wheat at different soil depths. Proteobacteria were important in topsoil, whereas Firmicutes and Bacteriodetes were active in the subsoil. *Streptomyces* were ^{13}C enriched at all depths	(Uksa *et al.*, 2017)
Maize	Hyphosphere	DNA	16S rRNA gene amplification and T-RFLP	Phosphate solubilising bacteria associated with arbuscular mycorrhizal fungi (but not the rhizosphere) of maize plants, assimilated labelled carbon	(Wang *et al.*, 2016)
Wheat	Rhizosphere	DNA	ITS amplicon sequencing with Illumina HiSeq	There was a lower diversity of ^{13}C labelled fungi than ^{12}C. Ascomycota and Basidiomycota dominated the fungal community and were higher in abundance in ^{13}C labelled fractions. ^{13}C labelled communities were less driven by soil chemical properties	(Wang *et al.*, 2019b)

Rice	Bulk	DNA	16S rRNA and *pmoA* gene sequencing with NGS	A diverse community of methane oxidisers were found to be labelled by ^{13}C-methanol	(Sultana *et al.*, 2019)
Wheat	Root (decomposition)	DNA	16S rRNA gene sequencing with ION torrent	The most dominant phyla or root degraders were Proteobacteria and Actinobacteria. Xanthomonadaceae was most enriched in ^{13}C labelled samples	(Kaplan *et al.*, 2019)
Rice	Rhizosphere	DNA	16S rRNA gene sequencing with NGS	Transgenic rice did not significantly change the bacterial community structure from the parental type. Bacteria from the order Opitutales was most enriched in ^{13}C samples	(Wang *et al.*, 2019a)
Rice	Crop residue	DNA	16S rRNA gene and fungal ITS1 sequencing with Illumina MiSeq	*Lysobacter* and *Streptomyces* are key bacteria involved in the utilisation of labelled rice residues	(Kong *et al.*, 2020)
Wheat	Rhizosphere (soil isolated for microcosm study)	DNA	16S rRNA gene sequencing and metagenome assembly	^{13}C-Methanol degraders enriched from wheat and pea rhizospheres were more diverse than those isolated from unplanted soil	(Macey *et al.*, 2020)

In addition to the six cereal crop studies initially summarised by (Worsley, 2019), seven more recent publications are presented Table 3-1. This list is comprehensive at the time of publishing and demonstrates the different ways in which SIP can be utilised to probe and characterise microbial interactions with plant roots. Studies have interrogated not just rhizosphere (Haichar *et al.*, 2008) and endosphere communities (Lu *et al.*, 2006) but also bulk soil (Lu *et al.*, 2006; Sultana *et al.*, 2019). SIP has additionally been used to investigate which microbial communities are involved with the degradation of plant residues (Kong *et al.*, 2020). *Streptomyces*, well-known saprophytes, were shown to dominate utilisation of labelled rice residues. One of the big questions that underpin this Chapter is, do *Streptomyces* spp. use wheat root exudates as a carbon source?

There are some conflicting reports in the literature; while some studies suggest *Streptomyces* spp. do not feed on root exudates (Ai *et al.*, 2015), others suggest they do (Uksa *et al.*, 2017). Differences between taxa labelling could be due to various factors. For example, labelling occurred at different developmental stages between these studies; while (Ai *et al.*, 2015) labelled during active vegetative growth at 40 days post-sowing, (Uksa *et al.*, 2017) labelled after 75 days. It has already been discussed plant development can influence both root exudation profiles and microbial community (Chaparro *et al.*, 2013, 2014), so it is plausible that it may also affect the labelling of taxa in these experiments. Soil type may additionally determine which taxa are labelled, as the studies were conducted using soil sampled from different parts of the world. While root metabolite consumers have been probed in the endosphere community of various crops (Haichar *et al.*, 2008), there have been no studies published to date that investigate these microbes with high throughput amplicon sequencing. Thus, the power to quantify their enrichment is limited. Consensus is lacking for which microbial taxa are central to wheat root exudate metabolism, data for the endosphere is additionally insufficient. This highlights a gap in our understanding of wheat root microbiome assembly, something which is crucial to the future of food security.

The literature has shown extensively that SIP is a powerful technique for examining the complexities of the plant root microbiome, scrutinising the way carbon flux in the holobiont can drive microbial community assembly. Understanding this critical component of root microbiome assembly is key to the breeding and development of plants with stronger associations to microbes that promote plant health. Chapter 2 demonstrated that that the actinobacterial genus *Streptomyces*, which are enriched in the wheat root microbiome, have the potential to convey a range of benefits to their plant hosts. The challenges associated with the exogenous application of biological agents for disease control in agricultural systems were discussed. There are notable discrepancies in the literature, however. *Streptomyces* spp. are not always enriched in plant roots, understanding which taxa

dominate the wheat root microbiome and how root exudate metabolism influences community structure, is likely to be key for the development of inoculation strategies for *Streptomyces* biocontrol agents. This Chapter aims to address that.

3.2 Aims

This study has explored the bacterial community structure associated with bulk soil, rhizosphere and endosphere compartments of the wheat *var.* Paragon root microbiome. 16S rRNA gene sequencing and analysis have identified key taxa that are enriched in the rhizosphere and endosphere compartments.

A $^{13}CO_2$ DNA SIP study was conducted to determine which taxa were metabolising root exudates in the rhizosphere and endosphere. This has built on previous SIP studies in wheat and other cereal crops, with a focus specifically on understanding the role of exuded carbon, as opposed to the broader influence that a plant can have, on associated communities.

Maximising both agricultural and contextual relevance with Chapter 1 was key, so wheat *var.* Paragon was grown in agricultural soil for this experiment. Plants were labelled by growing under $^{13}CO_2$, while a control group was grown with $^{12}CO_2$. Plants were destructively sampled to isolate DNA. Heavy (labelled) and light (unlabelled) DNA was separated by caesium chloride density gradient ultracentrifugation. The 16S rRNA gene was amplified from these fractions, denaturing gradient gel electrophoresis (DGGE) and quantitative PCR was be used to forecast shifts in community composition before amplicon sequencing and analysis.

3.3 Materials and Methods

3.3.1 Buffers used in this study

Table 3-2 Buffers used in this study.

Buffer	Ingredient	Concentration
GB (Gradient buffer)	Tris	0.1M
	KCl	0.1M
	EDTA	1mM
PEG-NaCL	PEG 6000 (Polyethylene glycol 6000)	30% w/v
	NaCl	1.6M
TAE (Tris, acetic acid, EDTA)	Tris	40mM
	Acetic acid	20mM
	EDTA	1mM
TBE (Tris, boric acid, EDTA)	Tris HCl	90mM
	Boric Acid	90mM
	EDTA	2mM
PBS-S (Silwett L-77 amended Phosphate Buffered Saline)	NaH2PO4.H2O	6.33g.L^{-1}
	Na2HPO4.H2O	16.50g.L^{-1}
	200 µl Silwet L-77 added after autoclaving	

3.3.2 Wheat plant growth and $^{13}CO_2$ labelling

Soil was sampled from JIC field site. The top 5cm of soil was removed and set aside, the subsequent 10-20cm was sampled for the experiment. The soil was homogenized and any stone larger than around 3cm were removed. Wheat seeds were sterilized with 70% (v/v) EtOH for 30 seconds followed by 3% (v/v) bleach for 10 minutes. Seeds were washed in sterile distilled H_2O 10 times and left to soak for 90 minutes. Seeds were sown at soil surface level. Plants were grown in pots placed in 4.25l PVC chambers. Plants were grown for a total of 5 weeks for 12h light conditions and 12h dark conditions at 21°C. Plants were labelled for weeks 4 and 5 by sealing chambers with vacuum grease and maintaining CO_2 headspace conditions at 800ppmv (twice atmospheric CO_2) by pulse injection every hour. During photoperiod, 3 plants were injected with $^{13}CO_2$ (99% Cambridge isotopes, Massachusetts, USA), 3 plants were injected with $^{12}CO_2$ and 3 pots of unplanted soil were injected with $^{13}CO_2$ to control for autotrophic fixation of CO_2 by soil microorganisms.

The rate of plant CO_2 uptake was determined every 4 days by monitoring headspace concentrations using gas chromatography (Figure 3-2). This enabled the volume of CO_2 to be added at each 1h interval to be calculated, in order to maintain the concentration at 800 ppmv within tubes. CO_2 concentrations were measured using an Agilent 7890A gas chromatography instrument with a flame ionization detector and a Poropak Q (6ft x 1/8") HP plotQ column (30 m x 0.530 mm, 40 µm film) with a nickel catalyst and a helium carrier gas. The instrument was run with the following settings: injector temperature 250°C, detector temperature 300°C, column temperature 115°C and oven temperature 50°C. The injection volume was 100 µl and the run time was 5 mins, with CO_2 having a retention time of 3.4 mins. Peak areas were compared to a standard curve to convert them to CO_2 concentrations in ppmv. Standards of known CO_2 concentration were prepared in 120 ml serum vials that had been flushed with nitrogen prior to the introduction of CO_2. The volume of CO_2 to be added at each 20-minute interval could then be calculated using the formula: -

$$Volume\ of\ CO_2\ to\ inject = \left(\frac{800\ ppmv - measured\ ppmv}{1000}\right) * 4.25L$$

At the end of the light period each day, tube lids were removed to prevent the build-up of respiratory CO_2 during the dark period. Just before the next light period, tubes were flushed with the 80%/20% nitrogen/oxygen mix to remove any residual CO_2 before replacing the lids and beginning the first injection of 4.25 ml of CO_2.

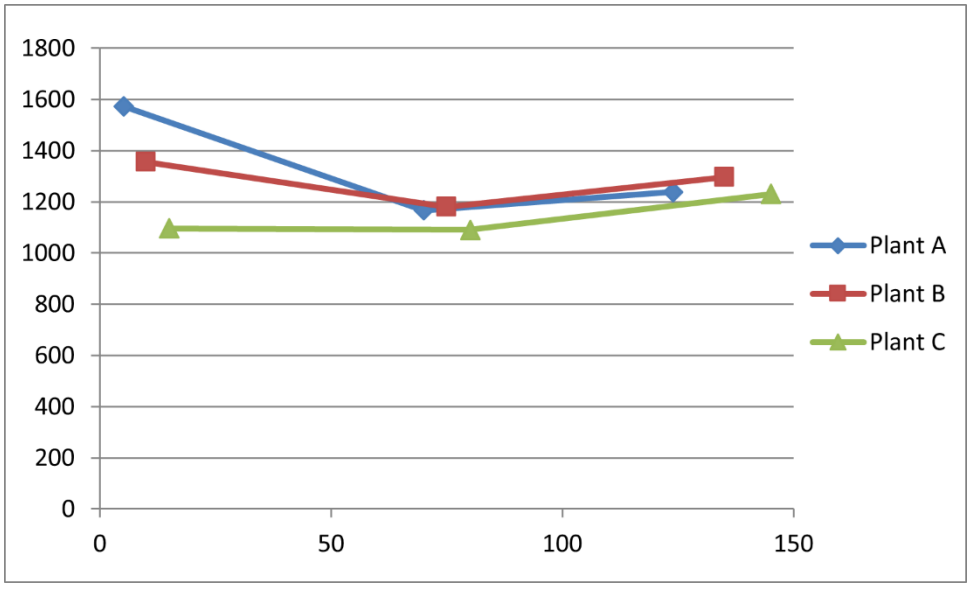

Figure 3-2 Plant growth champer headspace CO_2 measurements as determined by GC. Chambers were injected with 200PPMV every hour and measured shortly after.

3.3.3 Sampling bulk soil, endosphere and rhizosphere compartments

At the end of the CO_2 labelling experiment samples of the bulk soil, rhizosphere and endosphere compartments were taken from each pot for total DNA extraction and further downstream analysis. Approximately 300mg of root-free homogenized "bulk soil" was collected from each of the planted and unplanted pots; samples were snap-frozen in liquid nitrogen and stored at -80°C. For the planted pots, roots were shaken firmly to remove the bulk soil, apart from the particles adhering firmly to the root surface. This soil was defined as the rhizosphere fraction. To collect this, roots were vortexed for 30s in 25 ml of PBS-S (See Table 3-2). After the roots were transferred to a separate tube, the remaining sample was treated as rhizosphere. Following centrifugation at 3500RCF for 15 minutes, the pellet was snap-frozen and stored at -80°C. The rhizosphere-free roots were then washed again in fresh PBS and any remaining soil removed with tweezers. Roots were soaked for 30 seconds in 70% EtOH (v/v), 5 minutes in 3% bleach (v/v) and rinsed 10 times with sterile water. Roots were subsequently sonicated for 20 minutes in a sonicating water bath. Roots were then snap frozen in liquid nitrogen and stored at -80°C. A modified version of the manufacturer's protocol for the FastDNA™ SPIN Kit for Soil (MP Biomedicals) was used to extract DNA from soil, rhizosphere, and root samples. Modifications included pre-homogenization of the root material by grinding in a mortar and pestle with liquid nitrogen before addition of lysis buffer, an extended incubation time for samples (10 minutes) in the DNA matrix buffer, and elution in 150 µl of sterile dH_2O. DNA yields were quantified using a Qubit fluorimeter combined with a Qubit™ dsDNA High Sensitivity (HS) or Broad Range (BR) Assay kit (Invitrogen™) depending on the expected concentration.

3.3.4 Density gradient ultracentrifugation and fractionation of DNA

In order to separate ^{13}C labelled DNA (belonging to metabolisers of root exudates), from ^{12}C DNA, DNA samples from the rhizosphere and roots of planted pots as well as from the soil of unplanted pots, underwent density gradient separation according to an established protocol (Neufeld *et al.*, 2007b). A 7.163 M caesium-1 chloride (CsCl) solution was made, which had a measured density of 1.858g.ml^{-1}. GB (Table 3-2) and DNA were then mixed and added to 4.8 ml of the CsCl solution. The total volume of the GB/DNA mixture to add to the CsCl solution depends upon the initial CsCl stock solution density and should result in a solution with an approximate density of 1.725 g ml^{-1} to give optimal separation of DNA. The volume of GB/DNA to add was quantified using the following calculation from (Neufeld *et al.*, 2007b):

$$Combine\frac{GB}{DNA}volume = (CsCl\ stock\ density - 1.725\ g.ml^{-1}) * 4.8ml\ CsCl * 1.52$$

For each of the replicate samples from both the $^{12}CO_2$ and $^{13}CO_2$ incubated plants, or the $^{13}CO_2$ unplanted control pots, 700ng of DNA was loaded into the GB and added to the CsCl solution. The refractive index (R.I, measured as the temperature adjusted RI in nD-TC) of each complete gradient solution was checked using a refractometer (Reichert Analytical Instruments, NY, USA) which had been calibrated with nuclease free water. Gradient buffer (if the nD-TC was too high) or CsCl solution (if the nD-TC was too low) was added in order to achieve an nD-TC value of 1.4038 (this approximates a density of 1.725 g ml^{-1}); this was done to normalise the density of solutions between samples. Once solutions were complete, they were loaded into polyallomer quick seal centrifuge tubes (Beckman Coulter) and heat-sealed. Tubes were then placed into a vti 65.2 rotor (Beckman Coulter), balancing them to within 10 mg. The rotor was then loaded into a 180 Beckman Optima XL-100K ultracentrifuge and set to run at 20°C and 44,100 rpm for a minimum of 62h. A vacuum was applied during the run and breaks were not used for deceleration to ensure that the gradient remained intact at the end of the run. After ultracentrifugation, each sample underwent gradient fractionation. For this, a 0.6 mm sterile needle was attached to the tubing of a peristaltic pump (cleaned with sterile dH_2O before use) and pierced into the top of the ultracentrifuge tube, which was held in place by a clamp stand. The bottom of the centrifuge tube was then pierced swiftly with a sterile needle and dH_2O was pumped into the centrifuge tube at a rate of 450 µg per minute, displacing the gradient into 1.5 ml microcentrifuge tubes that were placed beneath the clamp stand. Tubes were exchanged every minute until the water had fully displaced the gradient solution, resulting in 12 x 450 µl fractions being collected in total. The RI of 60 µl of each fraction was measured to confirm successful gradient formation. DNA was then precipitated by adding 4 µl of Co-precipitant Pink Linear Polyacrylamide (Bioline) and 2 volumes of PEG-NaCl solution (see Table 3-2) to each fraction, before leaving them overnight at 4°C. DNA was then pelleted by centrifuging at 13,000 RCF for 30 minutes and the supernatant was removed. The pellet was then washed in 500 µl 70% (v/v) EtOH, before centrifuging again at 21130 RCF for 10 minutes. The resulting pellet was then air-dried and resuspended in 30 µl sterile dH_2O. Fractions were then stored at - 20°C before use as a template in qPCR and PCR reactions.

3.3.5 Ethidium bromide gel electrophoresis

Prior to DGGE analysis of 16S rRNA gene products amplified from fractions, concentration was equalised by ethidium bromide gel electrophoresis. Gels for PCR products were made with 1% (w/v) agarose in TBE buffer (Table 3-2) with 2 µg/ml ethidium bromide, gels for running plasmids were made with 0.5% agarose instead. DNA samples and loading buffer (5x) (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene-cyanol blue, 40% (w/v) sucrose in water) were run alongside a 1 kb plus DNA ladder (Invitrogen) plus loading buffer for size determination. Electrophoresis occurred at 120 V (Sub-Cell GT

electrophoresis system, BIOLINE) for 40 minutes for PCR products and 60 minutes for plasmids. DNA was visualised by UV-light using a Molecular Imager Gel Doc System (BIO-RAD).

3.3.6 16S rRNA gene amplification and DGGE analysis of fractions

In order to qualitatively analyse the distribution of the 16S rRNA gene across fractions, DGGE was carried out for samples under the different labelling treatments. For DGGE, the 16S rRNA gene was amplified from each fraction using PCR (Table 3-3) with the DGGE primers 341F-GC and 518R (

Table 3-4). The forward primer introduces a GC clamp to the 5' end of products which further modifies the melting properties of fragments and allows for greater resolution (Muyzer et al 1993). PCR products were visualised using ethidium bromide gel electrophoresis (3.3.5), in order to equalise the amount of product loaded on the DGGE gel.

Table 3-3 Components and thermocycler conditions for the amplification of the 16S rRNA gene using PCR.

Component	Volume (µl)
BioMix™ red (containing BioTaq™ DNA polymerase, Bioline) for DGGE or 2 x Ultra mix (containing Ultra DNA polymerase, PCRBIO)	10
dH_2O	12
Forward Primer	0.5
Reverse Primer	0.5
Sample DNA	2
PCR thermocycler reaction steps	
1/ 95°C for 1 min 2/ 30 x cycles of 95°C for 15 secs; 55°C for 15 secs, 72°C for 15 secs 3/ 72°C for 2 mins	

Table 3-4 Primers used in stable isotope probing experiments.

Primer (10mM)	Sequence	Reference
341F-GC	5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3'	(Muyzer et al 1993)

Primer (10mM)	Sequence	Reference
518R	5'- ATTACCGCGGCTGCTGG -3'	
PRK341F	5'-CCTACGGGAGGCAGCAG-3'	(Yu et al 2005)
MPRK806R	5'-GGACTACHVGGGTWTCTAAT-3'	

An 8% (v/v) polyacrylamide denaturing gel with a linear denaturant gradient of 40-80% (2.8 M urea/16% (v/v) formamide, to 5.6M urea/32% (v/v) formamide) was made along with a top loading gel of 0% denaturant (Table 3-5). For each sample, 1-10 µl of PCR product from each fraction was loaded consecutively into each well of the gel. The gel was then loaded into an electrophoresis tank filled with TAE buffer (Table 3-2). Electrophoresis was run at 0.2 Amps, 75 Volts and 60°C for 16h. Gels were then stained for one hour in the dark, using a solution of 400 ml 1 x TAE buffer with 4 µl of SYBR Gold Nucleic Acid Gel stain (Invitrogen™). Gels were then washed twice with dH_2O and imaged using a Bio- Rad Gel Doc XR imager. Bands of interest that were enriched in heavy fractions were sliced from the gel using a sterile scalpel blade and placed into a microcentrifuge tube containing 50 µl of sterile dH_2O. Tubes were then vortexed vigorously, before spinning briefly in a centrifuge. The supernatant was then used as a template in a PCR reaction (Table 3-3, using BioMix™) to amplify the 16S rRNA gene, using the primers PRK341F, 518R (

Table 3-4). PCR products were then purified from a 1% agarose gel using the Qiagen™ QIAquick gel extraction kit. Products were sent for sequencing at Eurofins Genomics (Germany) and the taxonomic identity of bands was confirmed using BLASTn.

Table 3-5 Components of an 8% polyacrylamide denaturing gel, with a linear denaturant gradient of 40-80% (2.8 M urea/16% (v/v) formamide, to 5.6M urea/32% formamide). APS and TEMED are added to the solution just before the gel is cast.

Gel component	40% denaturing solution (100ml)	80% denaturing solution (100ml)	Top loading gel (5ml)
40% (w/v) Bis- Acrylamide (37:5:1) (ml)	20	20	0.75
50 x TAE buffer (ml)	2	2	0.1
Formamide (ml)	16	32	0
Urea (g)	16.8	33.6	0
dH_2O (ml)	Up to 100ml	up to 100ml	4.1
10% (w/v) Ammonium Persulfate (APS) (µl)	675	675	50
Tetramethylethylenedi- amine (TEMED) (µl)	67.5	67.5	5

Analysis of the 16S rRNA gene copy number across fractions using qPCR to further identify fractions containing heavy (^{13}C) and light (^{12}C) DNA for each sample, 16S rRNA gene copy number was quantified across fractions using qPCR. qPCR assays were carried out using Applied Biosystems Step One Plus Real Time PCR System. Reactions were carried out in 15 µl volumes. 2µl of template DNA (either sample DNA or standard DNA), or dH_2O as a control, was added to 13 µl of reaction mix containing 7.5 µl of 2x Precision Plus qPCR Master Mix (Primer Design), 0.375 µl of each of the primers PRK341F and MPRK806R (

Table 3-4), 4.75µl dH_2O. Sample DNA, standards (a dilution series of the target 16S rRNA gene at known molecular quantities), and negative controls were quantified in duplicate. Reactions were run under the following conditions: 95°C for 2 mins; 40 cycles of 95°C for 10 sec, 60°C for 70 sec; 95°C for 15 sec; 100 cycles at 75°C-95°C for 10 secs, ramping 0.2°C per cycle. Reactions were performed in 96-well plates (Applied Biosystems). The threshold cycle (C_T) for each sample was then converted to target molecule number by comparing to C_T values of a dilution series of target DNA standards.

3.3.7 16S rRNA gene amplicon sequencing

Following quantification of 16S rRNA gene copy number in each fraction using qPCR and DGGE, fractions spanning the peaks in copy number were identified. Equal quantities of each of these fractions were combined to create a labelled “heavy” (H) and unlabelled “light” (L) fraction for each sample, respectively. H and L fractions were combined for each of the rhizosphere and endosphere samples, from ^{12}C and ^{13}C treatments (deriving from the three replicate pots under each labelling treatment). For the unplanted control, three replicate H and L fractions were combined from each of the ^{13}C unplanted control pots.

For each H and L fraction, the 16S rRNA gene was amplified by PCR using the universal primers PRK341F and MPRK806R; these amplify the V3 and V4 variable region of the 16S rRNA gene (

Table 3-4). These primers were chosen to amplify an extended region of the gene, compared to qPCR and DGGE experiments. Primer pairs are optimised for each assay, sequencing the V3-V4 region yields improved resolution of microbial taxa, whereas this would not be appropriate for qPCR which works best using short sequences of DNA. DGGE is similarly optimised for short sequences. PCR was performed using an Ultra DNA polymerase according to the protocol defined in Table 3-3. The 16S rRNA gene was also amplified from three replicate unfractionated DNA samples of the unplanted control, rhizosphere and endosphere of wheat plants grown under the $^{13}CO_2$ treatment in order to profile complete bacterial communities in each of these compartments. The resulting PCR products

were purified using the Qiagen™ QIAquick gel extraction kit and submitted for 16S rRNA gene amplicon sequencing using Illumina MiSeq technology at Mr DNA (Molecular Research LP), Shallowater, Texas, USA.

The initial SIP sequencing reads were analyzed using the software package quantitative insights into microbial ecology 2 (Qiime2) version 2019.7. Subsequent SIP resequencing was analyzed using Qiime2 version 2020.2. In both cases, paired-end sequencing reads were demultiplexed and then quality filtered and denoised using the DADA2 plugin. Reads were trimmed to remove the first 17-20 base pairs, dependent on the length of the primer. Reads were also truncated to 150-230 base pairs to remove low quality base calls. Chimeras were removed using the consensus method and default settings were used for all other analyses. For taxonomic assignments 16S sequence classifiers were trained against the SILVA database version 128 using a 97% similarity cut off.

Sequence data was further processed and statistical analyses were carried out using *R* 3.2.3 (R Core Team, 2019), using the packages phyloseq, ggplot2 (for plotting graphs and heatmaps, respectively) and vegan (for calculating Bray-Curtis dissimilarities and PERMANOVA analyses).

3.4 Results

3.4.1 Investigating the total bacterial community structure of wheat *var.* Paragon root microbiome

To determine the total bacterial community associated with rhizosphere and endosphere compartments, DNA samples were collected from each of the $^{13}CO_2$ treated plants. DNA was collected from each of the $^{13}CO_2$ unplanted control pots to interrogate the bulk soil community. These samples underwent 16S rRNA gene sequencing and analysis, prior to fractionation. In order to generate samples for amplicon sequencing, primers were used to amplify 341-806 region of the 16S rRNA gene. This meant that paired-end sequencing data could probe the V3-V4 region. By looking at this region of the 16S rRNA gene, family level taxonomic identification can be assigned with greater confidence than each variable region alone.

16S rRNA gene products were amplified with peptide nucleic acid (PNA) blockers (Fitzpatrick *et al.*, 2018b) prior to sequencing. These were used to limit polymerase binding and amplification of host DNA sequences. This approach is used to enrich for microbial sequences, host reads from mitochondria and chloroplast can otherwise dominate sequencing data; as much as 99.9% of reads obtained from endosphere DNA samples can originate from the plant host (Sam Prudence, unpublished). As this was the first time these blockers had been used experimentally in the lab, their efficacy was determined. Figure 3-3 displays the percentage of reads assigned as archaeal, bacterial and host after quality filtering and paired end joining. Bulk soil and rhizosphere compartment showed very low numbers of host reads at 1.1%-1.4%, this was to be expected. The endosphere compartment showed a much greater number of host reads, between 34.5% and 57.6%. This still represents a considerable reduction of host reads from the endosphere compartment, compared to previous experiments in our lab.

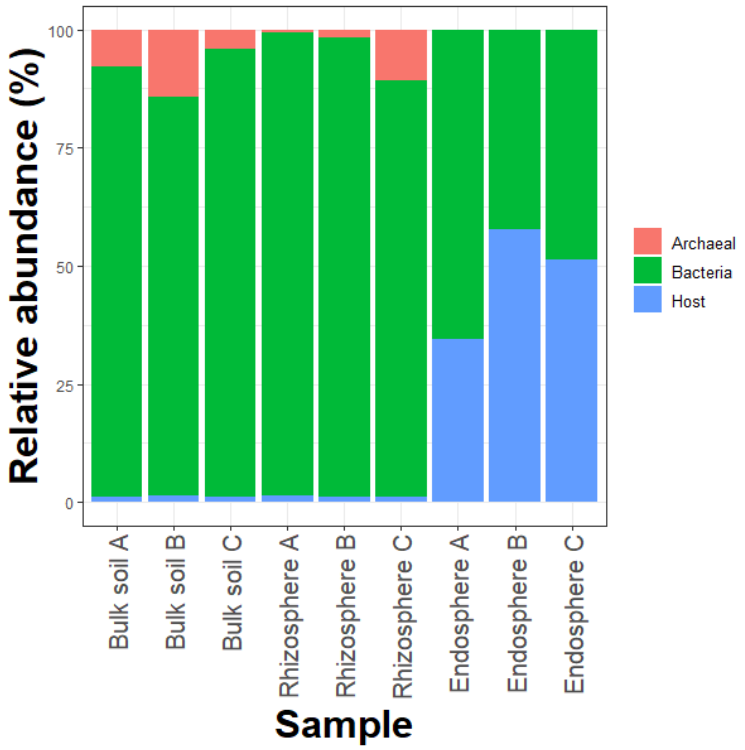

Figure 3-3 Relative abundance of archaeal, plant host and bacterial reads from endosphere sequence data. Host reads were calculated based on taxonomic assignment "mitochondria" and "chloroplast" following quality filtering and paired end joining for endosphere samples.

Following removal of host and Archaea derived sequenced, Bray-Curtis dissimilarity matrices were calculated using Vegan package in R and plotted using Non-Metric Multidimensional Scaling (NMDS) (Figure 3-4). The function of this was two-fold; the analysis served both to visualise the consistency of replicates, as well as qualitatively assess the influence of root compartment, on bacterial community structure. The analysis showed that Bulk soil samples were very similar to one another, as coordinates were clustered closely together. Rhizosphere samples showed greater variation in community structure as the coordinates were more loosely clustered. Endosphere samples showed the greatest variation, points were highly dispersed. Compartment significantly influenced the separation of Bray-Curtis distances, this was determined with Permutational multivariate analysis of variance (PERMANOVA, Permutations=999, R^2=0.5531, P=0.003).

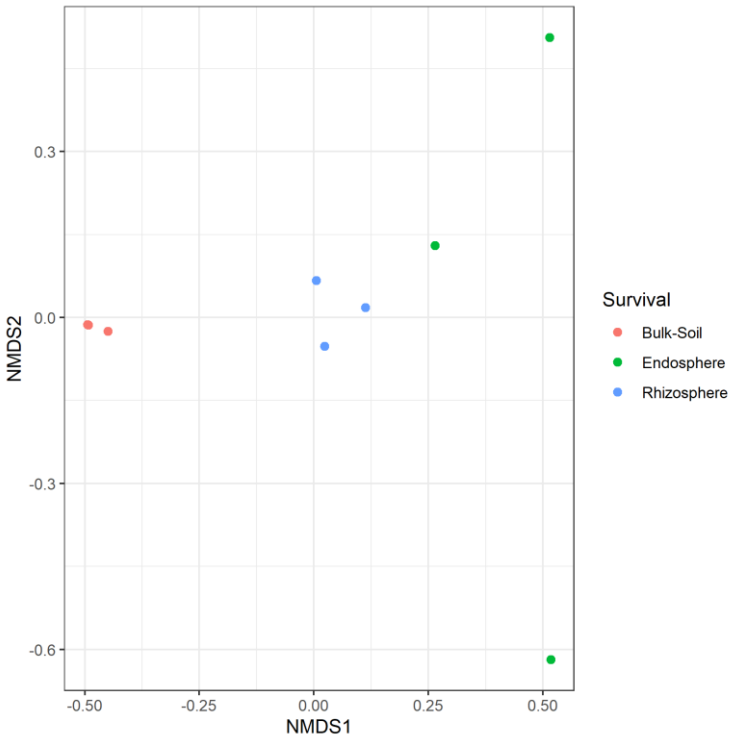

Figure 3-4 NMDS plot showing Bray-Curtis distances between bacterial families present in bulk soil, rhizosphere and endosphere compartments. Each compartment was sampled from N=3 plants. Root compartment significantly influenced the separation of Bray-Curtis distances (PERMANOVA, Permutations=999, R^2=0.5531, P=0.003).

A bar plot showing the relative abundance of bacterial families present in each sample was used to explore differences in bacterial community structure further (Figure 3-5). Taxa with relative abundance not higher than 1% in any sample was grouped as "other". The data supports the variability presented in the NMDS analysis Figure 3-4. Bulk soil samples show consistent community composition between replicate samples. Rhizosphere samples are distinct from bulk soil samples but show greater variation between replicates. Endosphere community structure showed very little similarity between replicates, with each sample being dominated by a small number of taxa. The bacterial community of Endosphere A was almost entirely composed of *Enterobacteriaceae*, while *Streptomycetaceae*, *Paenibacillaceae* and *Comamonadaceae* co-dominated Endosphere B. Endosphere C was dominated by *Bacillaceae*, but *Streptomycetaceae* were still present in high abundance. It is not known what the cause of this variability is, but it is possibly due to a founder's effect, whereby certain taxa establish early in the life cycle of an individual plant and are able to dominate the microbial community.

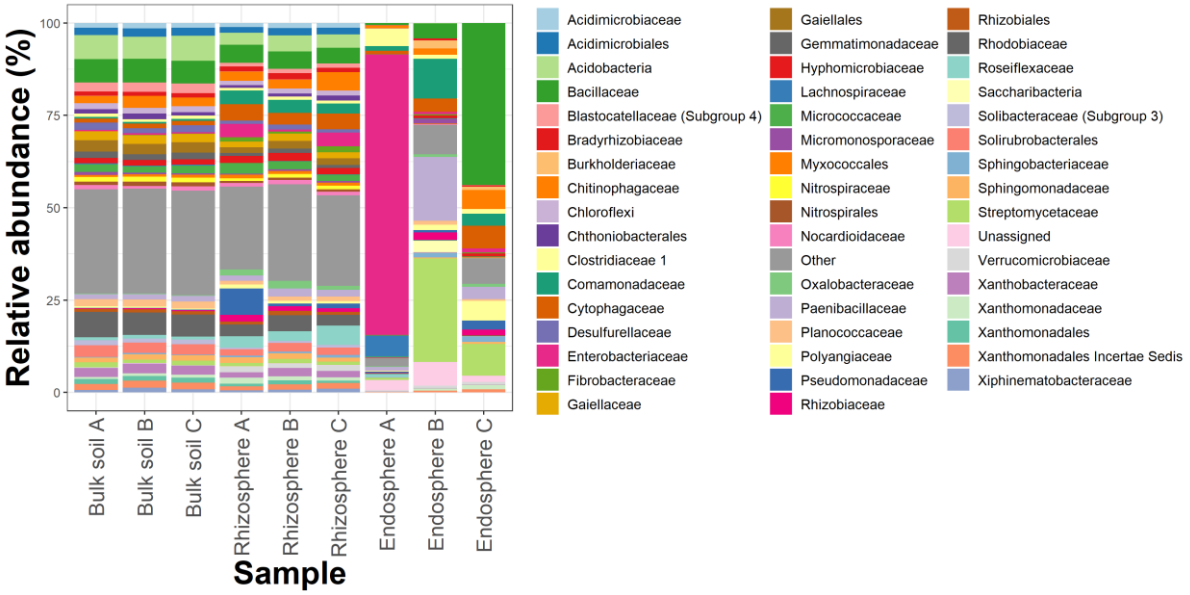

Figure 3-5 A bar plot showing relative abundance of bacterial families for each sample. Families not present at more than 1% abundance in any of the samples were grouped categorically as 'Other'. Bulk soil, rhizosphere and endosphere compartments were sampled from N=3 plants.

Different root microbiome compartments showed different community structure and while the barplot suggested certain taxa may be differentially abundant between these compartments, further analysis was required. Quantitative analysis of differential abundance was determined with the Deseq2 package in R. Taxa with a base mean lower than 300 was omitted from the analysis, to mitigate spurious correlations. 18 taxa were enriched from bulk soil to the rhizosphere, while 21 taxa were enriched from the rhizosphere to endosphere. 10 taxa showed enrichment in both cases. An important question that underpinned the focus of this research was, are *Streptomyces* spp. enriched in the wheat root microbiome? The analysis shows that they are not enriched from bulk soil to rhizosphere, there was an overall log2-fold reduction of *Streptomycetaceae* in the rhizosphere compared to bulk soil, although it was not significant (-0.04458, padj = 0.889024). There was, however, a significant log2-fold increase in abundance of this taxon from the rhizosphere to endosphere compartment (5.9655, padj = 2.29E-09). Taxa that were significantly upregulated from bulk soil to rhizosphere, or rhizosphere to endosphere compartments, were plotted to show log2-fold differential abundance as calculated by Deseq Figure 3-6a. Mean relative abundance for each compartment was plotted in parallel Figure 3-6b. Taxa with base mean lower than 400 were removed from the visualisation. A summary of enriched taxa for each compartment is presented in Table 3-6, while full statistical output can be found in supplementary table S4.

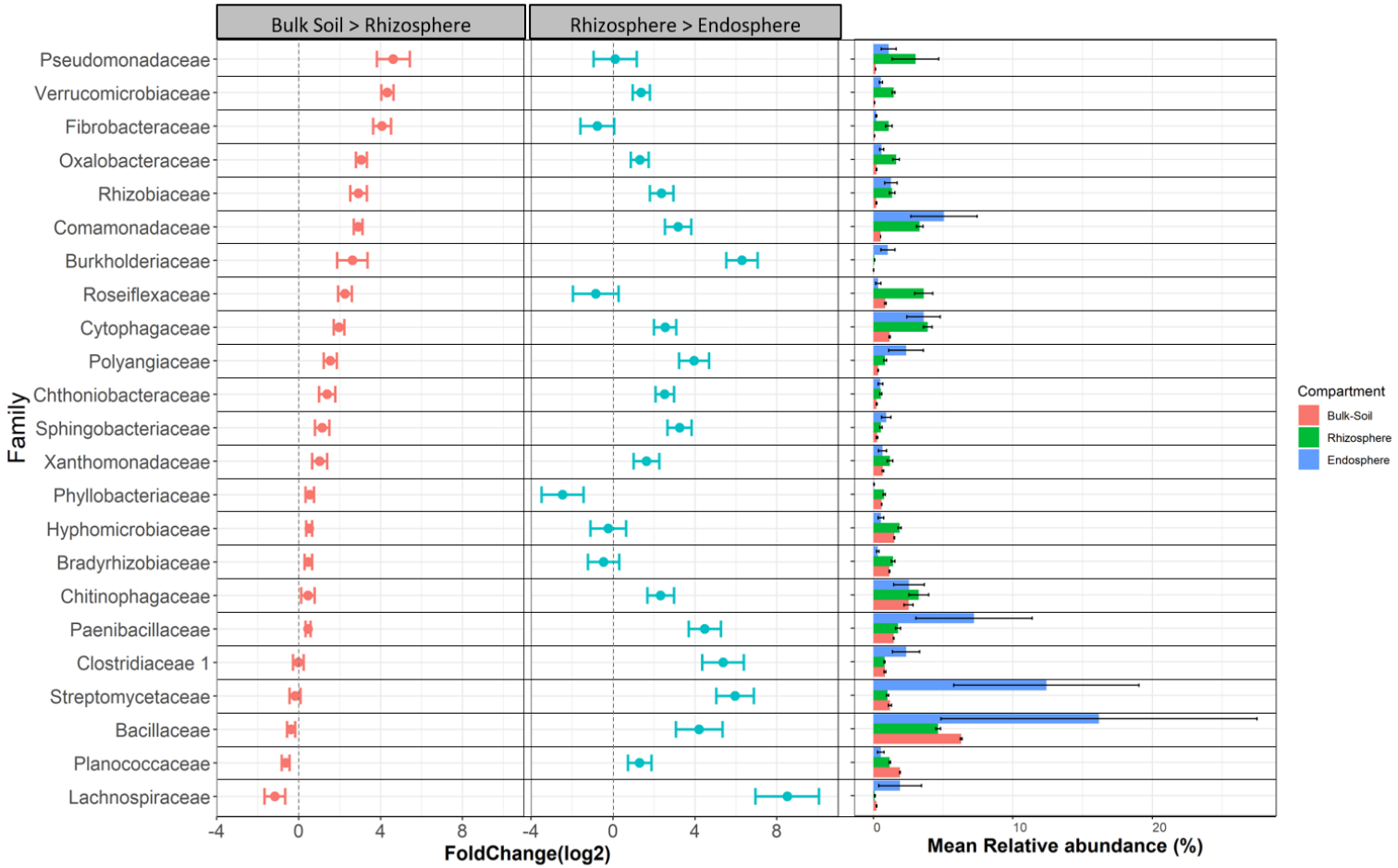

Figure 3-6 (A) A dotplot showing log2 fold-change for taxa significantly enriched from bulk soil to rhizosphere, or rhizosphere to endosphere, determined by Deseq analysis. Taxa with base mean lower than 400 were omitted. Error bars show log-fold change standard error. **(B)** Mean relative abundance in each compartment, for significantly enriched taxa. Bars show standard error of the mean.

Table 3-6 A list of taxa showing differential abundance of taxa significantly enriched from bulk soil to rhizosphere, or rhizosphere to endosphere compartments. Taxa with base mean lower than 300 were removed to mitigate spurious correlations.

Bulk soil > Rhizosphere				Rhizosphere > Endosphere			
Phylum	**Family**	**baseMean**	**log2FoldChange**	**Phylum**	**Family**	**baseMean**	**log2FoldChange**
Proteobacteria	Pseudomonadaceae	1415.2576	4.6226	Proteobacteria	Enterobacteriaceae	55820.8681	7.4522
Verrucomicrobia	Verrucomicrobiaceae	633.3492	4.3269	Firmicutes	Bacillaceae	10635.0945	4.2097
Fibrobacteres	Fibrobacteraceae	492.4774	4.0739	Actinobacteria	Streptomycetaceae	7548.6264	5.9655
Proteobacteria	Oxalobacteraceae	743.6033	3.0635	Firmicutes	Paenibacillaceae	4814.7072	4.4808
Proteobacteria	Enterobacteriaceae	1284.5518	3.0354	Firmicutes	Lachnospiraceae	4141.8211	8.5068
Proteobacteria	Rhizobiaceae	638.5884	2.9253	Firmicutes	Clostridiaceae 1	3974.4288	5.3772
Proteobacteria	Comamonadaceae	1558.4328	2.9023	Proteobacteria	Comamonadaceae	3913.3003	3.1752
Chloroflexi	Roseiflexaceae	1799.7646	2.2633	Bacteroidetes	Cytophagaceae	3151.4550	2.5473
Bacteroidetes	Cytophagaceae	2048.8044	1.9693	Bacteroidetes	Chitinophagaceae	2299.2182	2.3287
Proteobacteria	Polyangiaceae	473.0929	1.5386	Proteobacteria	Polyangiaceae	1634.9730	3.9583
Verrucomicrobia	Chthoniobacteraceae	304.1029	1.3959	Proteobacteria	Rhizobiaceae	979.0443	2.3750
Bacteroidetes	Sphingobacteriaceae	312.4487	1.2305	Proteobacteria	Oxalobacteraceae	660.7432	1.3084
Proteobacteria	BIrii41	333.6190	1.2152	Bacteroidetes	Sphingobacteriaceae	660.3309	3.2544
Proteobacteria	Xanthomonadaceae	746.2113	1.0294	Proteobacteria	Burkholderiaceae	628.1072	6.2962
Proteobacteria	Phyllobacteriaceae	526.5742	0.5473	Verrucomicrobia	Verrucomicrobiaceae	614.9336	1.3812
Proteobacteria	Hyphomicrobiaceae	1309.8988	0.5153	Proteobacteria	Xanthomonadaceae	572.9690	1.6296
Proteobacteria	Bradyrhizobiaceae	994.7585	0.4744	Firmicutes	Planococcaceae	475.3605	1.3035
Firmicutes	Paenibacillaceae	1241.6357	0.4539	Verrucomicrobia	Chthoniobacteraceae	418.4190	2.5231
				Actinobacteria	Micromonosporaceae	384.4599	3.0267
				Bacteroidetes	Flavobacteriaceae	372.1323	9.1781
				Proteobacteria	Cellvibrionaceae	324.8956	2.5979

3.4.2 An investigation of wheat exudate root exudate metabolisers using Stable Isotope Probing (SIP)

A key aim of this experiment was to elucidate microorganisms responsible for the consumption of root exudates. ^{13}C DNA SIP was the chosen method for this investigation. The experiment had to be carefully designed to ensure that only utilisers of roots exudates were labelled, label for too long and microbial cross feeding may skew our understanding of the microbial ecology of this carbon source. Wheat plants were grown for 3 weeks in agricultural soil under atmospheric condition. This time point was chosen as it represents a crucial development point for the plant. While root exudation may be higher when the plant is more mature, doing the experiment later in the plant's life offers a more limited insight into how root exudates influence early root microbiome assembly. As the plants were only pulse-labelled with either $^{13}CO_2$ or $^{12}CO_2$ for a short period of time following this initial growth phase, it was expected that labelled carbon would dominate root exudates but be kept to a minimum in plant cell components. This was crucial for discriminating exudate metabolisers from saprophytes, we are asking questions specifically pertaining to the microbial community that is actively fed by the plant. CO_2 uptake was measured intermittently between 3- and 6-weeks growth during a pilot experiment. After 3 weeks, wheat plants were shown to use 200ppmv CO_2 per hour, when maintained at 800-1000 ppmv (roughly twice atmospheric CO_2). A 12h photoperiod with 14d labelling was chosen as sufficient labelling was observed using similar conditions by (Macey *et al.*, 2014).

As well as probing both the endosphere and rhizosphere communities of plants grown with labelled or unlabelled CO_2, autotrophic microorganisms were controlled for by treating unplanted soil with the same levels of labelled $^{13}CO_2$. This meant that were a total of 30 samples to analyse, with heavy and light fractions pooled for each combination of compartment and treatment. These fractions were generated by separating DNA by caesium chloride density gradient ultracentrifugation. Each of the 15 samples were separated into 12 distinct fractions and initially analysed qualitatively with DGGE, or quantitatively with qPCR. Heavy and light fractions were pooled for amplicon sequencing following this analysis.

3.4.3 Qualitative assessment of microbial community labelling with Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was used to separate unique 16S rRNA gene amplicons from individual samples, offering early insights into the community composition of DNA samples over different fractions. The buoyant densities of acquired fractions ranged from 1.3956 to 1.7545. A rapid decline in buoyant density was

observed for the 12th fraction, which suggested no further fractions needed to be collected. Following the amplification of 341-508 region of the 16S rRNA gene with a 5' GC-clamp on the forward primer, PCR products were qualitatively equalised, with ethidium bromide gel electrophoresis images. Equalising was essential for optimising contrast across the fractions in subsequent DGGE gel images.

Key to the interpretation of these results is the understanding that banding patterns observed for a sample correlate with microbial community composition. As the gel denatures the double stranded DNA of amplified gene products, the migration slows. As this denaturant is at a gradient across the gel, PCR products more resilient to denaturation (often containing higher GC content) can migrate further before this happens. The GC-clamp mediates the rate at which products denature, which contributes to efficient separation and resolution of bands. As a result, PCR products from a specific taxon or groups of taxa will migrate to the same height across a single gel. When the bands that represent these products appear to 'shift' from lighter fractions in the $^{12}CO_2$ treatment to heavy fractions in the $^{13}CO_2$ treatment, it implies the incorporation of labelled carbon into DNA. Biologically, this suggests that these taxa are consuming root exudates.

DGGE gel images of PCR products amplified from endosphere fractions (Figure 3-7) gave the first indication that labelling had been successful. Several of the bands which appeared in the higher fractions (lighter/lower buoyant density) were shown to appear in lower fractions (heavier/higher buoyant density). These bands represent PCR products from taxa that are potentially labelled with ^{13}C. It is important to recognise that mitochondria and chloroplast sequences will be amplified from host DNA. As such, some of these bands which appear to represent labelled taxa in heavier fractions, will instead represent PCR products amplified from labelled host DNA. The presence of a "high mobility" band was observed in the heavier fractions of ^{13}C samples than ^{12}C samples. This was interesting as they had the potential to represent PCR products from high GC organisms such as *Streptomyces* spp., as high mobility suggests resilience to denaturing associated with G-C bonds. Banding patterns did vary between replicates of the same group, however. This is likely due to the aforementioned variability in community structure observed in Figure 3-4 and Figure 3-5, which may also be exacerbated through fractionation of the DNA.

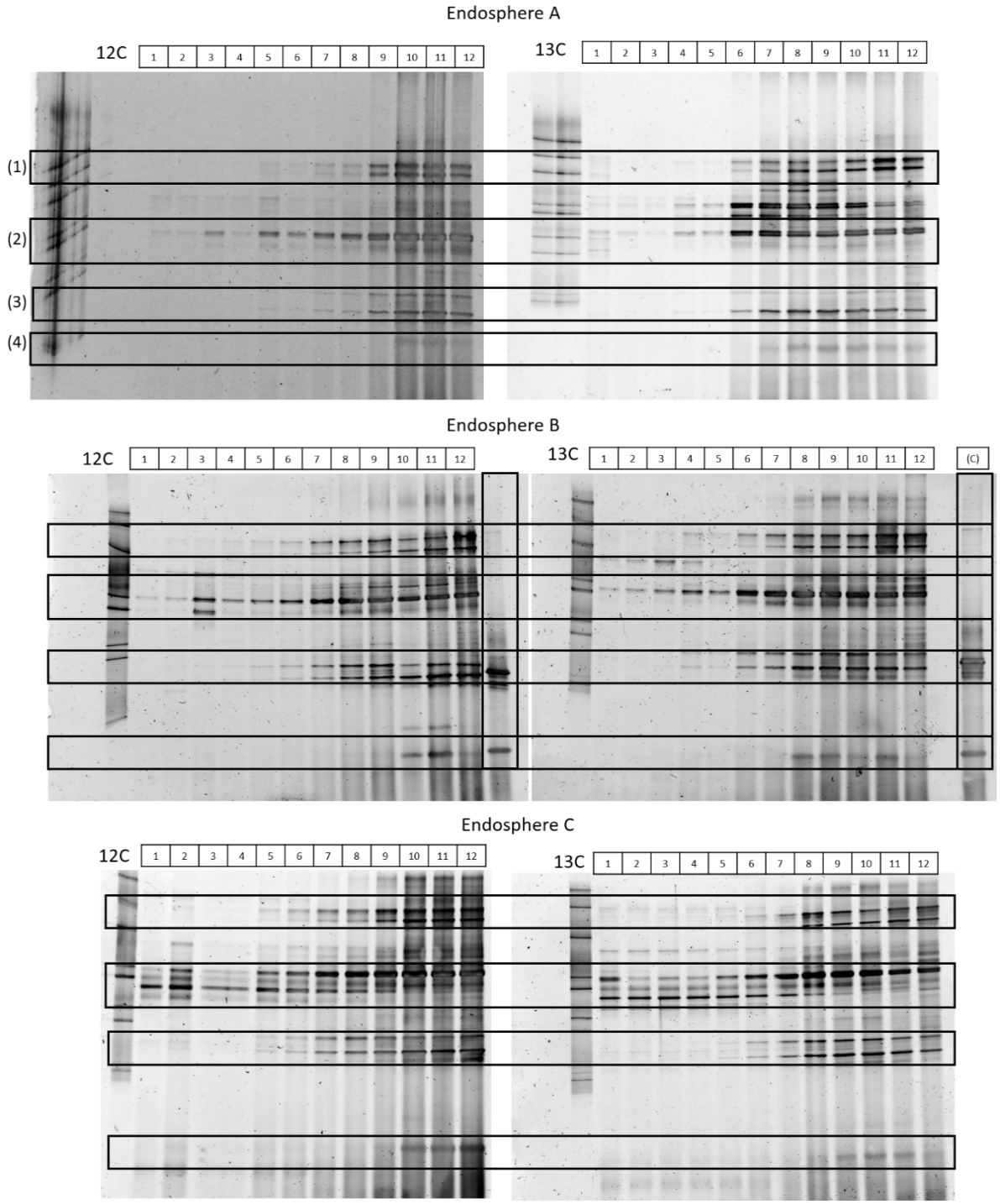

Figure 3-7 DGGE gel images of 16S rRNA gene PCRs amplified from density gradient fractions of DNA isolated from the Wheat endosphere. Each band represents a PCR product from a single taxon or group of taxa. PCR products (1)-(4) were isolated for further analysis. Genomic DNA from *S. coelicolor* was used to generate a reference product and was run in lane (C) for Endosphere B.

These bands of interest along with others that potentially represented labelled taxa (shown in Figure 3-8) were excised. The extracted DNA was used as a template for further amplification, this

time without using a GC clamp. These PCR products were then sent for Sanger sequencing to identify them. Blast analysis classified the sequences of PCR products (1) and (2) as "*Triticum*" and "plastid" (chloroplast), which indicated that they were amplified from host DNA. The sequence for product (3) was classified as to Proteobacteria while the sequence for product (4), which was the "high mobility" band of interest, was unfortunately not identifiable.

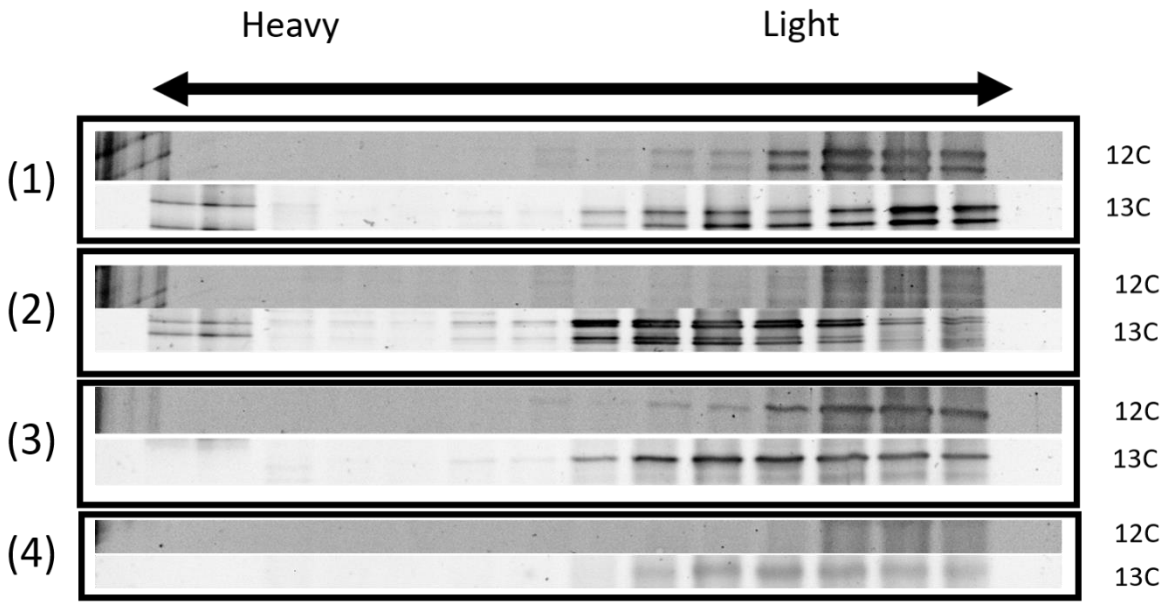

Figure 3-8 DGGE gel image showing bands which represent 16S rRNA gene PCR products, amplified from $^{12}C/^{13}C$ endosphere A sample. Bands appear in heavier fractions for the ^{13}C treated sample which suggests they were amplified from labelled DNA.

In order to further investigate whether this high mobility product may be amplified from *Streptomyces* spp., a small amount of GC clamped 16S rRNA gene PCR product was generated using *S. coelicolor* genomic DNA. This was loaded as a control, pictured in Figure 3-7 for replicate B. Curiously, despite amplifying from purified DNA of a single clone, multiple bands were present. In order to understand whether this phenomenon was specific to *S. coelicolor*, 16S rRNA gene PCR products for eight different environmental streptomycetes isolated in Chapter 1, were run on a DGGE gel. Figure 3-9 demonstrated that the same banding pattern was observed for these environmental isolates as for *S. coelicolor*. Multiple bands were again observed despite being a pure and homogenous template. High-mobility (H) and low-mobility (L) bands were excised, and the extracted DNA was used as PCR template. These products were then run on an agarose gel to confirm that were all a single product of the same size (Figure 3-9). Following purification, Sanger sequencing confirmed that high and low running bands were identical sequences. This clearly demonstrated the importance of exercising caution, when inferring diversity from DGGE gels, they offer limited insights into the identification of

specific taxa. This study shows that that multiple bands on a gel can represent PCR products from a single taxon. Sanger sequencing also suggested that a single band can represent multiple taxa, as the sequence for band 4 was unidentifiable. Nevertheless, these results imply that ^{13}C heavy enriched PCR products may be amplified from streptomycetes that are utilising root exudates.

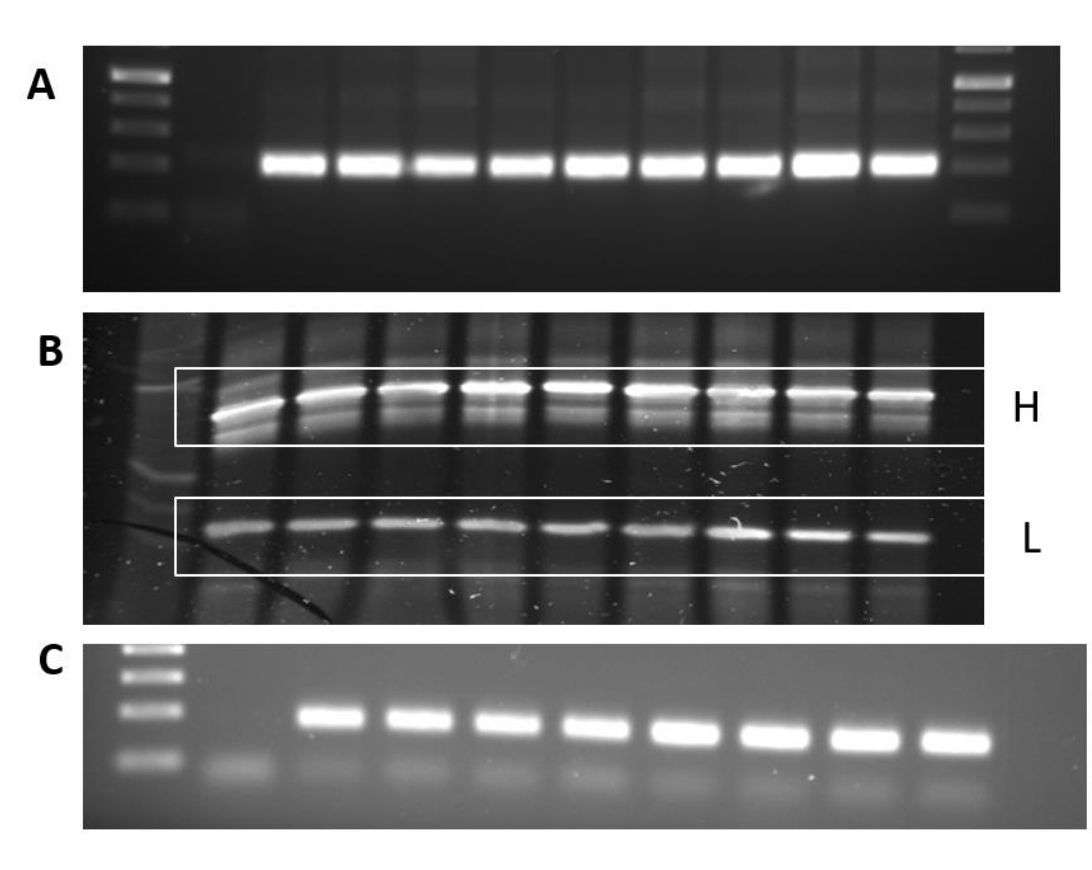

Figure 3-9 (A) Ethidium bromide-stained gel used to separate PCR products of the 16S rRNA gene of eight different environmental streptomycetes in addition to *S. coelicolor*. **(B)** DGGE gel image of the same PCR products. **(C)** Ethidium bromide-stained gel used to separate PCR products amplified from 4 different high and low running bands extracted from the DGGE gel.

3.4.4 Quantification of 16S rRNA gene copy with qPCR

Following qualitative assessment of microbial community shifts, it was necessary to quantify the gene copy number in each fraction, to identify more accurately which were the 'heavy' and 'light' fractions. The refractive index (RI) and buoyant density (BD) were measured for solutions of varying CsCl concentration. A standard curve was plotted using these values prior to fractionation, so that BD could be calculated from the RI of fractions. 16S rRNA gene copy number within a fraction was determined using qPCR and used to calculate gene copy number as a percentage of the total unfractionated samples. These values are plotted against fraction number, to compare across

different samples (Figure 3-10). A peak at a lower fraction numbers (higher buoyant density) in ^{13}C treatments, indicates labelling of 16S rRNA gene sequences.

Practically, this abundance peak shift was most pronounced for endosphere fractions. A peak shift was observed from fractions 9 and 10 for ^{12}C to 7 through 9 for ^{13}C samples, this is what was expected. The heavy fractions contain a greater relative quantity of DNA for labelled samples than for unlabelled samples. This labelled peak likely included significant quantities of host DNA amplicons, which were indiscernible from Prokaryote amplicons at this stage. Nevertheless, this experiment clearly defined which fractions should be pooled for sequencing.

While endosphere samples showed distinct peaks at different fraction numbers for different treatments, $^{13}C/^{12}C$ treatment appeared to have a much lower influence over which fractions the peak appeared for rhizosphere samples. Instead of separate peaks with minimal overlap, both treatments exhibit a single, much broader peak. This was not expected, it is not clear why this pattern is observed but may be due to partial or intermediate labelling of taxa. Nevertheless, there is still a shift observed in the peak between treatments, the proportion of sample DNA found in heavy fractions is higher for labelled samples than for unlabelled samples Table 3-7.

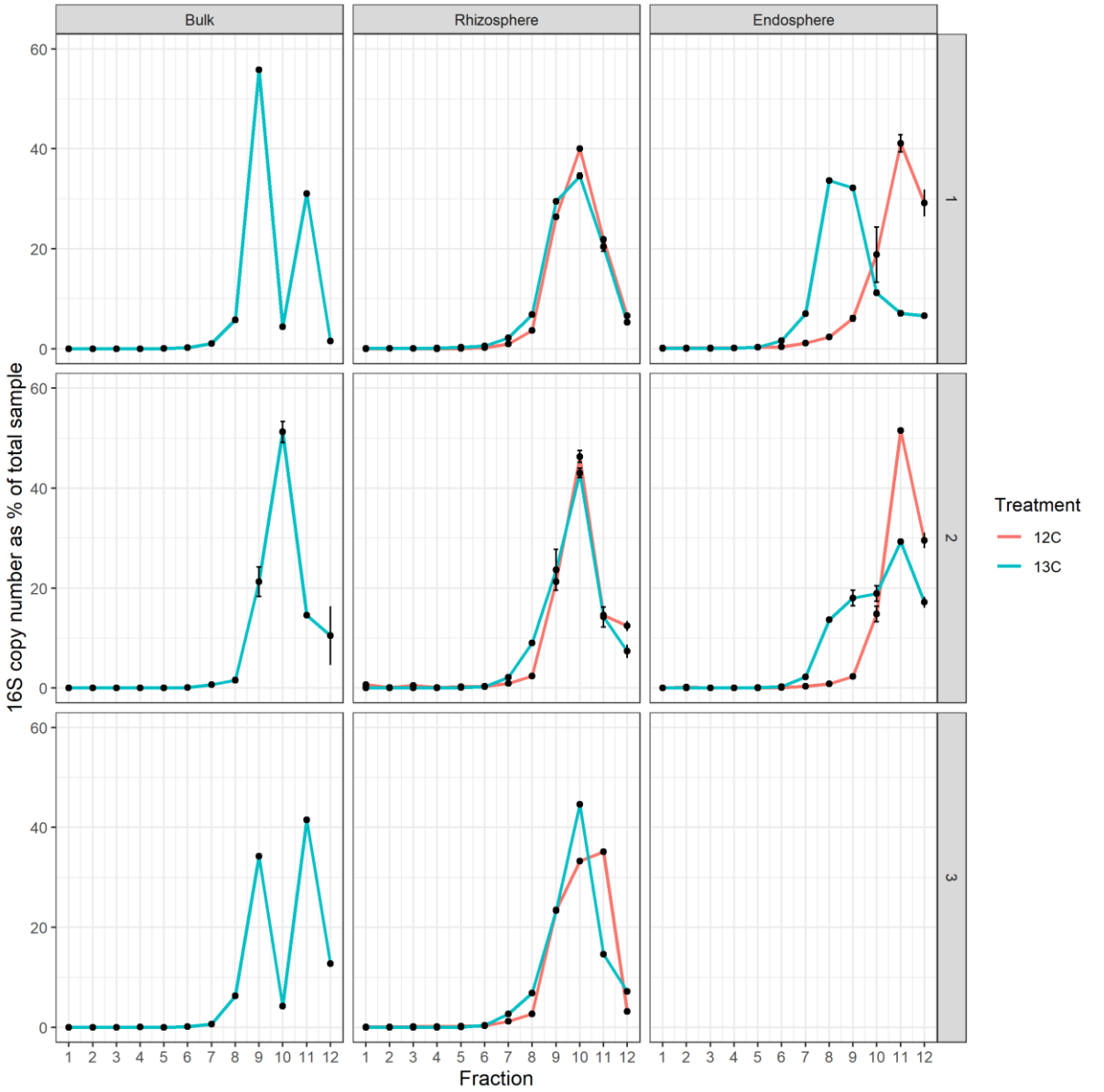

Figure 3-10 16S rRNA gene copy number in each fraction as percentage of total copy number per sample, plotted against fraction number. Each plot shows ^{12}C and ^{13}C treated samples for rhizosphere and endosphere, while plots showing bulk data show a single ^{13}C treated sample. Buoyant densities of each fraction are presented in supplementary information. 16S rRNA Gene copy numbers were quantified by using each fraction as a template for qPCR, N=2 replicate measurements on the same fraction.

These data were then used to define fractions to be pooled as either heavy or light. As mentioned, in some cases, selection was straightforward due to clear separation of peaks between treatments. Where peak separation was less clear, such as for rhizosphere samples, fraction choices were validated by confirming differential abundance between treatments. Table 3-7 shows how the relative copy number for ^{13}C treatment is higher for heavy fractions and lower for light fractions, than

^{12}C treatment. Equal quantities of selected fractions were used as a template for 16S rRNA gene PCR amplification. These products were sent for Illumina paired-end sequencing. It was noted that the densities for the 12th and final fraction of each sample dropped sharply. This was due to the fact that the water which was used for fractionation carried over into the sample. Sterile water was used, and the peristaltic pump was sterilised thoroughly beforehand, so DNA contamination was not a major concern.

Table 3-7 A list defining heavy (H) and light (L) pooled fractions for 16S rRNA gene sequencing. Fractions were generated by CsCl density gradient ultracentrifugation. Following DGGE and qPCR analysis, fractions were selected for pooling. Heavy/Light fractions demonstrated differential abundances of 16S gene copy number relative to the sum of fractions, between $^{13}C/^{12}C$ treated samples.

Origin of fractions	Fraction classification	Pooled fractions	Relative proportion of gene copies in pooled samples (%)	fraction density range (g.ml^{-1})
E1	12H	7,8,9	3.23	1.7086-1.7204
	12L	11,12	35.30	1.3956-1.6910
	13H	7,8,9	24.26	1.7098-1.7227
	13L	11,12	6.82	1.6004-1.6992
E2	12H	7,8,9	1.19	1.7098-1.7204
	12L	11,12	40.54	1.5097-1.6957
	13H	7,8,9	11.75	1.7098-1.7216
	13L	11,12	25.06	1.5745-1.6992
E3	12H	7,8,9		1.7086-1.7204
	12L	11,12		1.5427-1.6957
	13H	7,8,9		1.7110-1.7216
	13L	11,12		1.6310-1.7004
Rz1	12H	7,8	2.32	1.7169-1.7227
	12L	10,11	14.26	1.6992-1.7051
	13H	7,8	4.53	1.7169-1.7216
	13L	10,11	12.85	1.6992-1.7039
Rz2	12H	7,8	1.67	1.7157-1.7204
	12L	10,11	13.50	1.7004-1.7051
	13H	7,8	5.60	1.7157-1.7216
	13L	10,11	10.75	1.7004-1.7051
Rz3	12H	7,8	1.96	1.7169-1.7227
	12L	10,11	19.17	1.7016-1.7051

Origin of fractions	Fraction classification	Pooled fractions	Relative proportion of gene copies in pooled samples (%)	fraction density range ($g.ml^{-1}$)
	13H	7,8	4.79	1.7157-1.7216
	13L	10,11	10.92	1.7004-1.7051
Soil1	13H	8,9		1.7086-1.7145
	13L	11		1.6992
Soil2	13H	8		1.7145
	13L	10,11		1.7004-1.7039
Soil3	13H	8,9		1.7098-1.7157
	13L	11,12		1.6745-1.7004

3.4.5 Determining bacterial community members involved with root exudate metabolism with bacterial 16S rRNA gene amplicon sequencing

16S rRNA gene products amplified from heavy and light fractions (as detailed in Table 3-7) were sent for amplicon sequencing. The V3-V4 regions were probed with the 341-806 primer set, in concordance with the analysis of unfractionated DNA samples in section 3.4.1. This contrasted with the 341-506 primer pair that was more suitable for DGGE and qPCR analysis. As with the unfractionated samples, heavy and light fractions of DNA isolated from endosphere samples were sequenced from gene products amplified with PNA blockers (Fitzpatrick *et al.*, 2018b). Bulk soil and rhizosphere samples were not amplified with PNA blockers as host DNA contamination was expected to be minimal. For the DNA-SIP sequencing, the number of reads assigned to mitochondria or chloroplast was more variable than for unfractionated samples, ranging from 27.7% to 91.6% (Figure 3-11). The sample with fewest reads following host sequence removal had 7723 representative sequences. Alpha rarefaction curves showed that sampling at this depth did not lead to loss of diversity, according to Shannon's index (Supplementary S5).

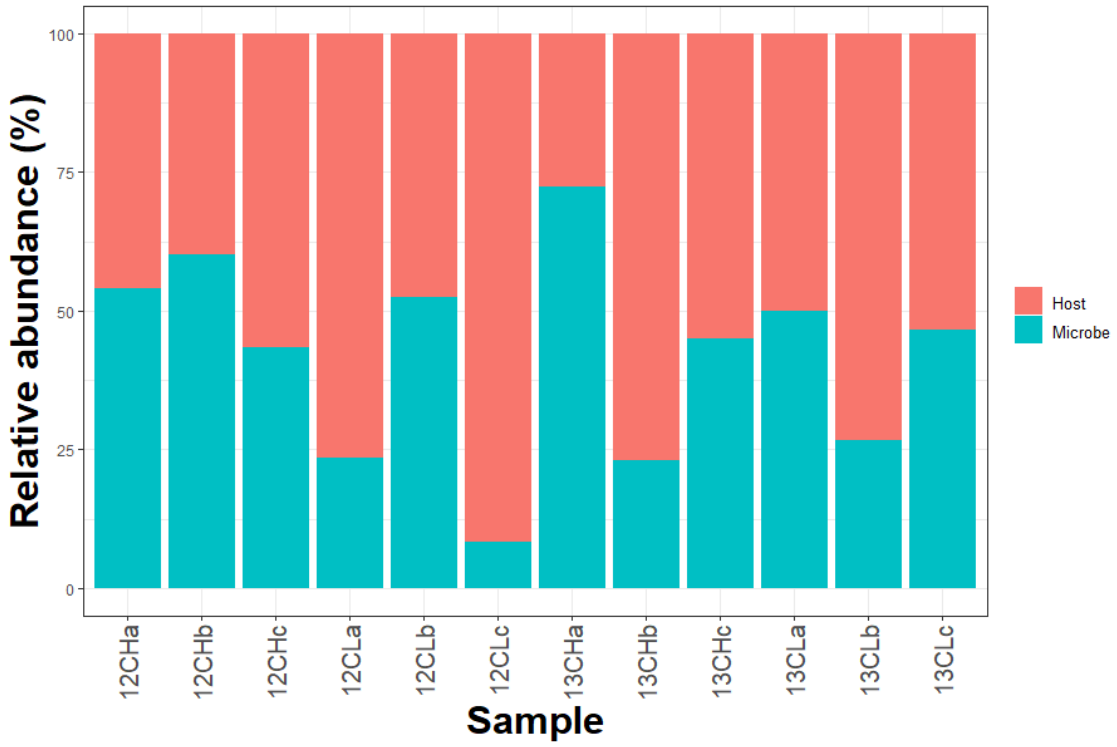

Figure 3-11 Relative abundance of host and microbial reads from endosphere sequence data. Host reads were calculated based on taxonomic assignment "mitochondria" and "chloroplast" following quality filtering and paired end joining for endosphere samples. Host reads were discarded along with those assigned as "Archaea" prior to downstream analysis.

It was expected that the final read count would be higher for rhizosphere and bulk soil samples, as less host derived sequences would have to be discarded. Despite this, the smallest sample read count following taxonomic filtering was 8452, as many reads were removed due to poor sequence quality. Although higher diversity was expected for these samples, alpha rarefaction analysis showed that suitable diversity was captured at this sampling depth (supplementary S5). As such, paired-end sequence data were carried forward for further analyses.

3.4.6 Determining bacterial community members involved with root exudate metabolism in the endosphere compartment

Bray-Curtis dissimilarity matrices were calculated using Vegan package in R and plotted using Non-Metric Multidimensional Scaling (NMDS). The function of this was two-fold; the analysis served both to visualise the consistency of replicates, as well as qualitatively assess the impact of labelling treatment, on community structure. Figure 3-12 showed that high variability was observed for endosphere samples, with poor clustering between replicates. Separation was additionally quite poor between sample treatments. Permutational multivariate analysis of variance (PERMANOVA) confirmed that treatment did not have a significant impact on the separation of Bray-Curtis distances (N=999, R^2=0.29443, P=0.326)

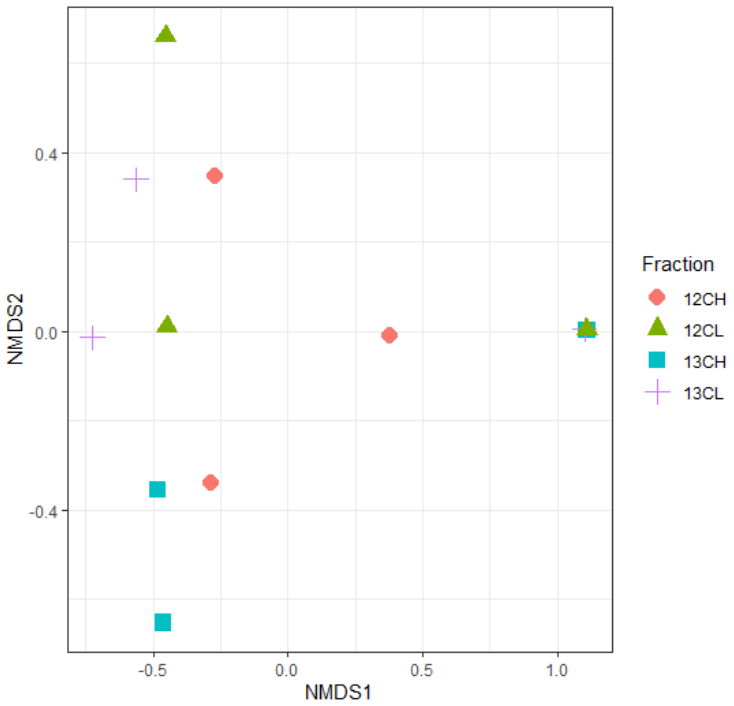

Figure 3-12 NMDS plot showing Bray-Curtis distances between bacterial families present in the heavy and light fractions of endosphere samples. N=3 plants were sampled for each fraction of ^{13}C

and ^{12}C treatments. PERMANOVA test showed that treatment/fraction did not significantly affect Bray-Curtis distances between groups (N=999, R^2=0.29443, P=0.326)

Coordinates were poorly separated, there was also overlap from different groups, which suggested communities were extremely similar. This was quite unexpected as these samples are not only from different treatments, but different fractions. A bar plot showing the mean relative abundance of bacterial families present in each sample was used to explore this further (Figure 3-13). Individual samples from all treatments were shown to be dominated by *Enterobacteriaceae*. The data supports the variability presented in the NMDS analysis (Figure 3-12). The dominance of this taxa across different fractions and treatments also explains the overlapping distances.

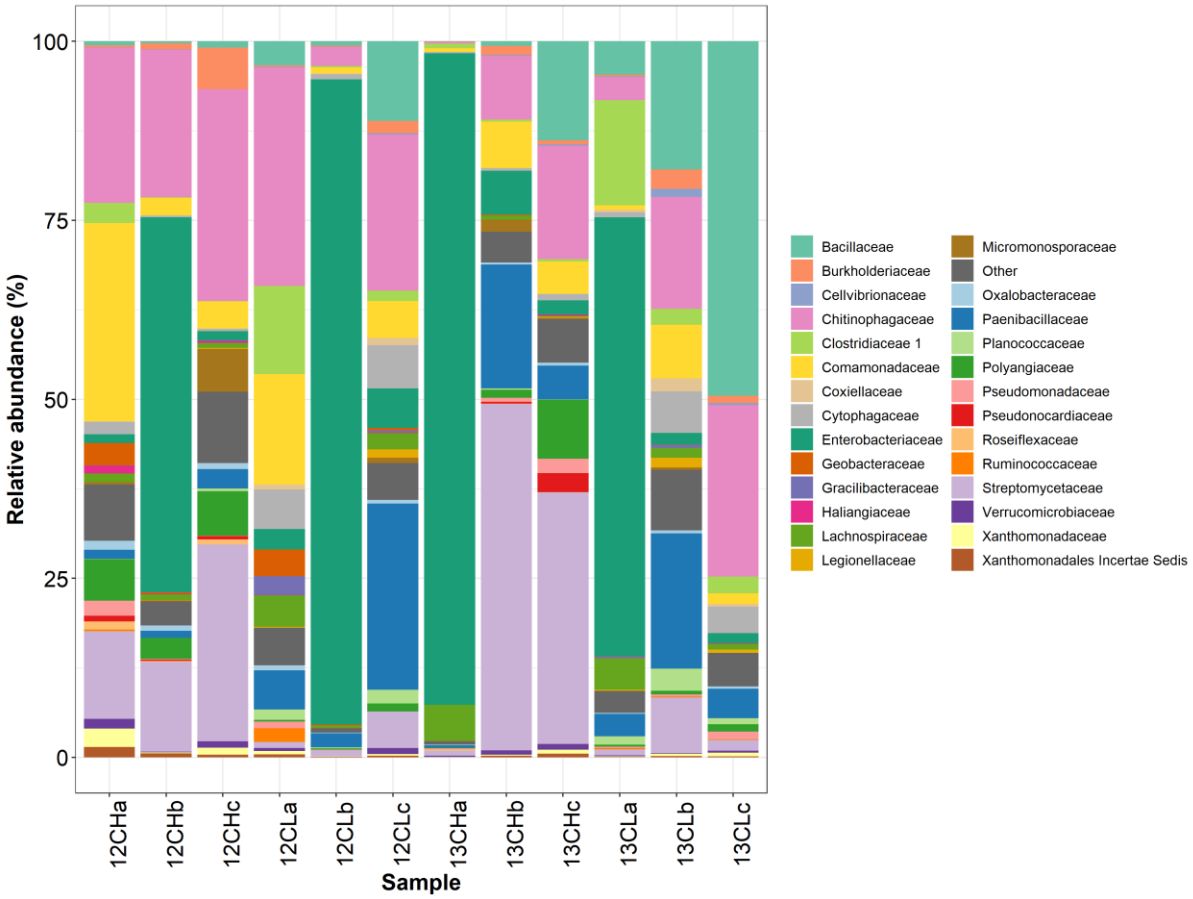

Figure 3-13 A bar plot showing relative abundance of bacterial families for each sample. Families not present at more than 1% abundance in any of the samples were grouped categorically as 'Other'. N=3 replicates are shown for Heavy (H) and light (L) fractions of samples treated with $^{13}CO_2$ or $^{12}CO_2$. Samples are grouped by treatment then by fraction.

Relative abundance analysis (Figure 3-6) showed that *Streptomycetaceae* were enriched in the endosphere relative to the rhizosphere of unfractionated samples. A key aim of this Chapter was to determine whether *Streptomyces* bacteria may utilise root exudates in the wheat root microbiome.

The barplot (Figure 3-13) suggested that *Streptomycetaceae* may be present in higher abundance for ^{13}C heavy fractions than ^{12}C heavy fractions, as well as ^{13}CL fractions, which would indicate consumption of root exudates. To demonstrate this more clearly, a barplot was used to visualise the mean relative abundance of *Streptomycetaceae* for each endosphere fraction (Figure 3-14). This confirmed that the mean relative abundance was higher for labelled fractions (^{13}CH) than control samples (^{13}CL/^{12}CH).

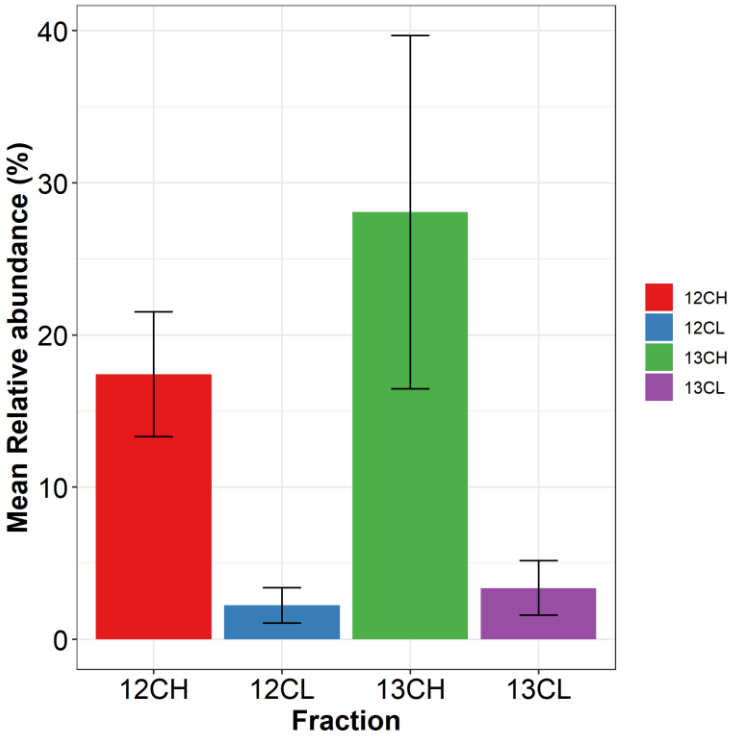

Figure 3-14 A bar plot showing mean relative abundance of *Streptomycetaceae* for endosphere samples. N=3 replicates are shown for Heavy (H) and light (L) fractions of samples treated with $^{13}CO_2$ or $^{12}CO_2$.

Differential abundance analysis was used to determine taxa that were statistically enriched in ^{13}CH fractions, relative to ^{13}CL and ^{12}CH fractions. The analysis did not return any significant taxa, which suggested that the variability was too high between samples. To determine whether the observed variation in bacterial community structure was due to some unforeseen artefact in the process of sequencing and analysis, 16S rRNA gene products were again generated using fractions selected in Table 3-7 and sent for amplicon sequencing. Barplots were used to visualise mean relative abundance of bacterial families across samples, for both sequencing runs (Figure 3-15). The datasets were comparable, with only slight variation between them. A greater number of replicates may be required to determine statistical enrichments of taxa in labelled fractions, for future experiments.

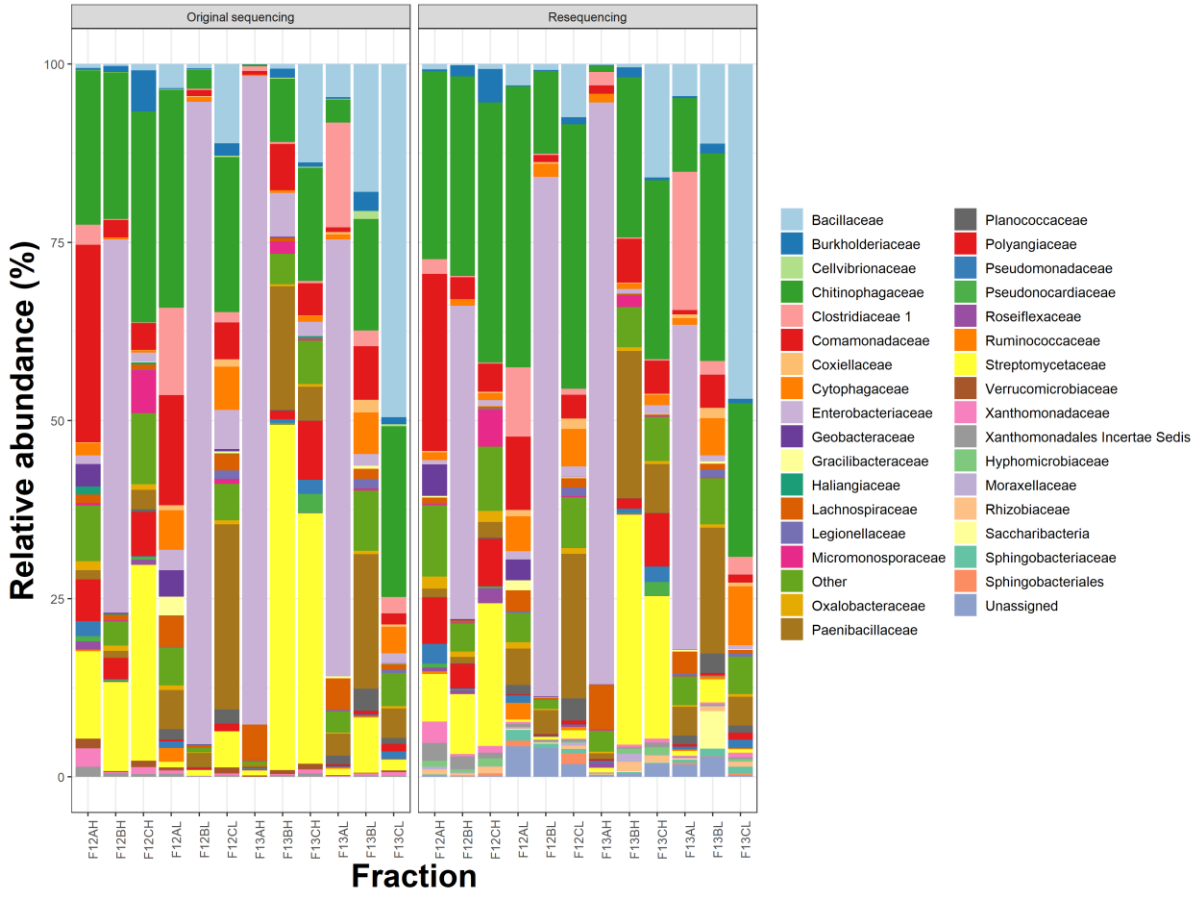

Figure 3-15 Bar plots showing mean relative abundance of taxa across samples for separate sequencing runs. For each dataset, heavy (H) and light (L) fractions were sequenced for N=3 plants treated with $^{13}CO_2$ or $^{12}CO_2$.

3.4.7 Determining bacterial community members involved with root exudate metabolism in the rhizosphere compartment

NMDS analysis suggested rhizosphere and bulk soil communities were more consistent than endosphere communities (Figure 3-16), Bray-Curtis ordinations showed clear separation of samples from different fractions and compartments. PERMANOVA analysis showed that fraction significantly predicted the separation of Bray-Curtis distances for rhizosphere samples (permutations=999, $P<0.001$, $R^2=0.59509$). While NMDS analysis showed separation of Bray-Curtis distances for heavy and light fractions of bulk soil, this was not significant (PERMANOVA, permutations=719, $R^2=0.56333$, $P=0.1$). This may be due to poor clustering of ^{13}C light fraction samples, in addition to low sample size.

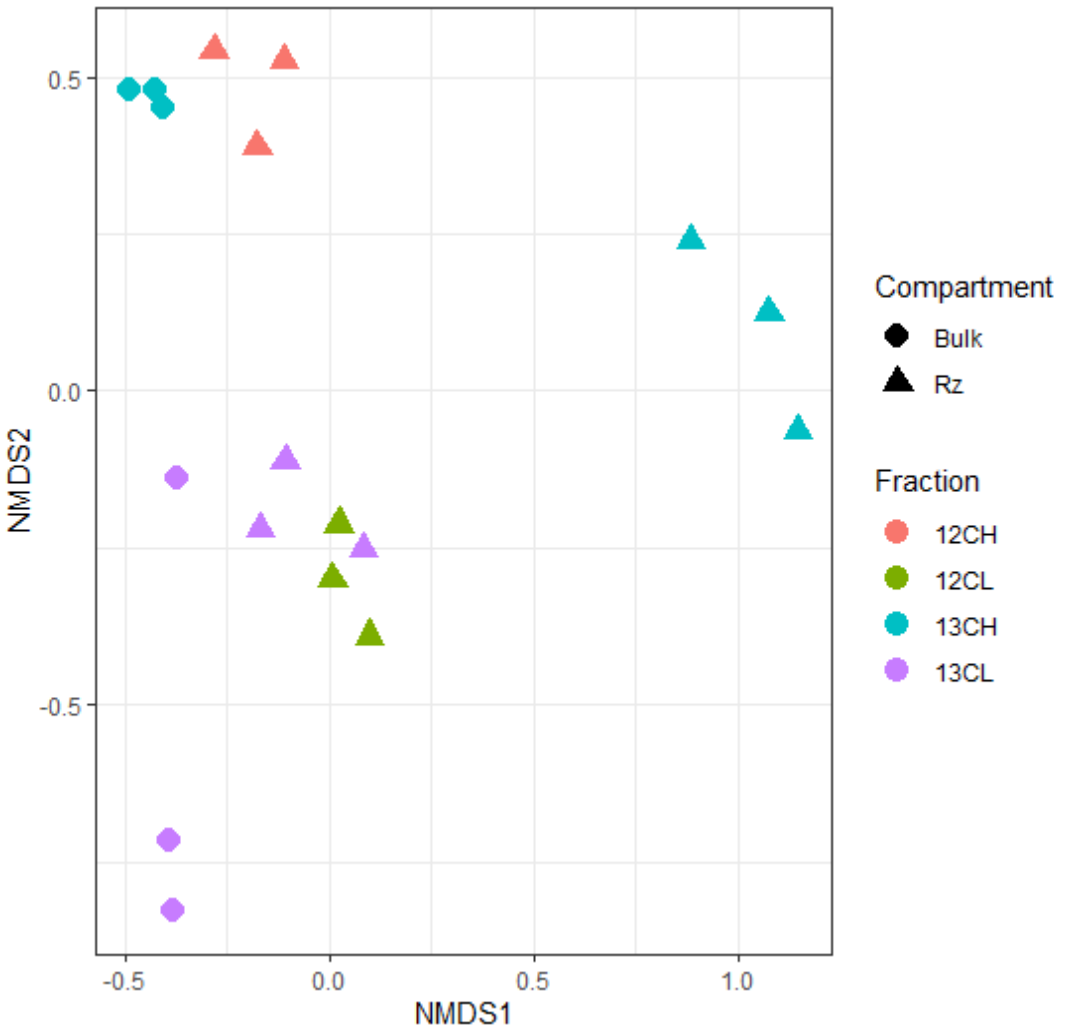

Figure 3-16 NMDS plot showing Bray-Curtis distances between bacterial families present in the heavy and light fractions of rhizosphere and bulk soil samples. N=3 replicates were sampled for each fraction of ^{13}C and ^{12}C treatments.

Mean relative abundance of taxa was compared between samples (Figure 3-17). The barplot suggested that some taxa were likely enriched in ^{13}CH fractions relative to both ^{12}CH and ^{13}CL, suggesting the incorporation of labelled metabolites for these taxa.

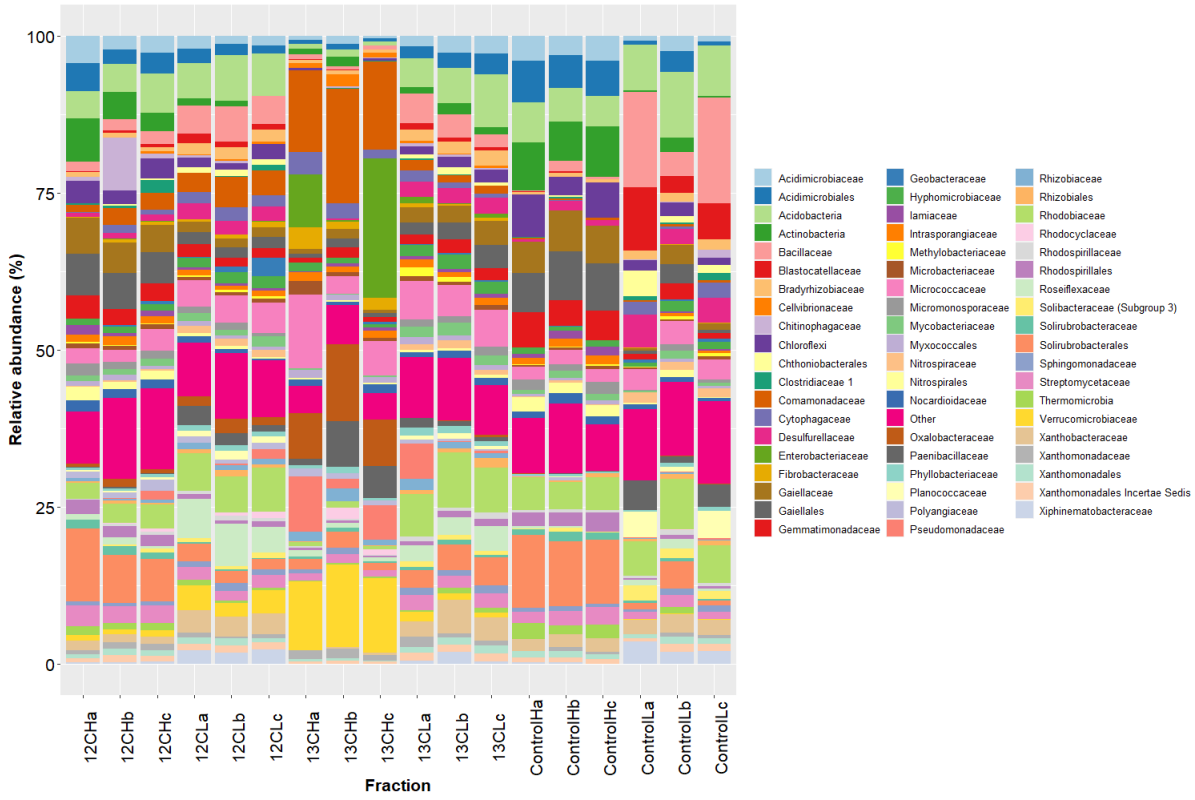

Figure 3-17 A bar plot showing relative abundance of bacterial families for each sample from the rhizosphere and unplanted control dataset. Families present at less than 1% abundance in any of the samples were grouped into 'Other'. N=3 replicates are shown for Heavy (H) and light (L) fractions of samples treated with $^{13}CO_2$ or $^{12}CO_2$. Samples are grouped by treatment then by fraction.

Observations of potential enrichment preceded quantitative analysis of differential abundance using the Deseq2 package in R. The enrichment of taxa in ^{13}CH fractions, compared to ^{12}CH and ^{13}CL fractions, was determined. These families are presented in Table 3-8. Any taxa that showed enrichment with a lower base mean than 100 was removed from the analysis, to eliminate spurious correlations due to poor sample size. After data pruning, the Deseq output showed that 16 bacterial families fulfilled the criteria for root exudate metabolism.

Table 3-8 A curated list of families enriched in ^{13}C heavy fractions compared to ^{12}C heavy and ^{13}C light fractions. Significant enrichment was determined with Deseq2 package in R. Taxa with base mean of less than 100 were removed from the Table to mitigate spurious correlations. The table is arranged in descending order of log2 fold change of ^{13}CH compared to ^{12}C.

		^{13}CH vs ^{12}CH		^{13}CH vs ^{13}CL	
Phylum	**Family**	**baseMean**	**log2FoldChange**	**baseMean**	**log2FoldChange**
Proteobacteria	Enterobacteriaceae	1525.5852	9.2255	1193.5578	5.6631
Firmicutes	Paenibacillaceae	542.7148	6.1312	474.7391	3.4503
Verrucomicrobia	Verrucomicrobiaceae	1540.4763	5.5257	1240.2326	4.8175
Proteobacteria	Pseudomonadaceae	747.4943	5.2964	640.9324	2.8280
Proteobacteria	Oxalobacteraceae	1121.7981	5.0936	882.3909	6.8662
Proteobacteria	Cellvibrionaceae	143.5597	4.5639	122.6921	3.3599
Proteobacteria	Comamonadaceae	1965.0959	4.5540	1557.0644	4.8109
Fibrobacteres	Fibrobacteraceae	308.4267	4.2510	247.2869	3.5586
Proteobacteria	Rhizobiaceae	200.6142	3.5866	194.8885	1.6767
Bacteroidetes	Cytophagaceae	333.3919	3.4852	283.9659	2.5201
Actinobacteria	Micrococcaceae	1002.9197	3.1889	918.5754	1.6739
Actinobacteria	Microbacteriaceae	182.5424	2.3165	144.6834	2.1570
Proteobacteria	Xanthomonadaceae	191.2474	2.2102	167.3075	1.5652
Proteobacteria	BIrii41	130.5447	2.2020	111.3784	1.6377
Actinobacteria	Intrasporangiaceae	196.5958	1.6795	158.6062	1.5069
Proteobacteria	Polyangiaceae	174.8114	1.4411	119.7725	2.4243

Mean relative abundances for labelled rhizosphere families in each fraction and treatment were plotted (Figure 3-18a). Taxa with a base mean lower than 250, as defined by differential abundance analysis, were omitted from the plot. Log-fold changes from ^{12}CH to ^{13}CH as well as ^{13}CL to ^{13}CH were also presented (Figure 3-18b), as determined by differential abundance analysis. Figure 10b was generated by co-author of Prudence *et al.* (in prep), Sam Prudence. Enterobacteriaceae showed the greatest enrichment from ^{12}CH to ^{13}CH, with a 9.2255 log-fold change, while *Oxalobacteriaceae* showed greatest enrichment from ^{13}CL to ^{13}CH with a 6.8662 log-fold change.

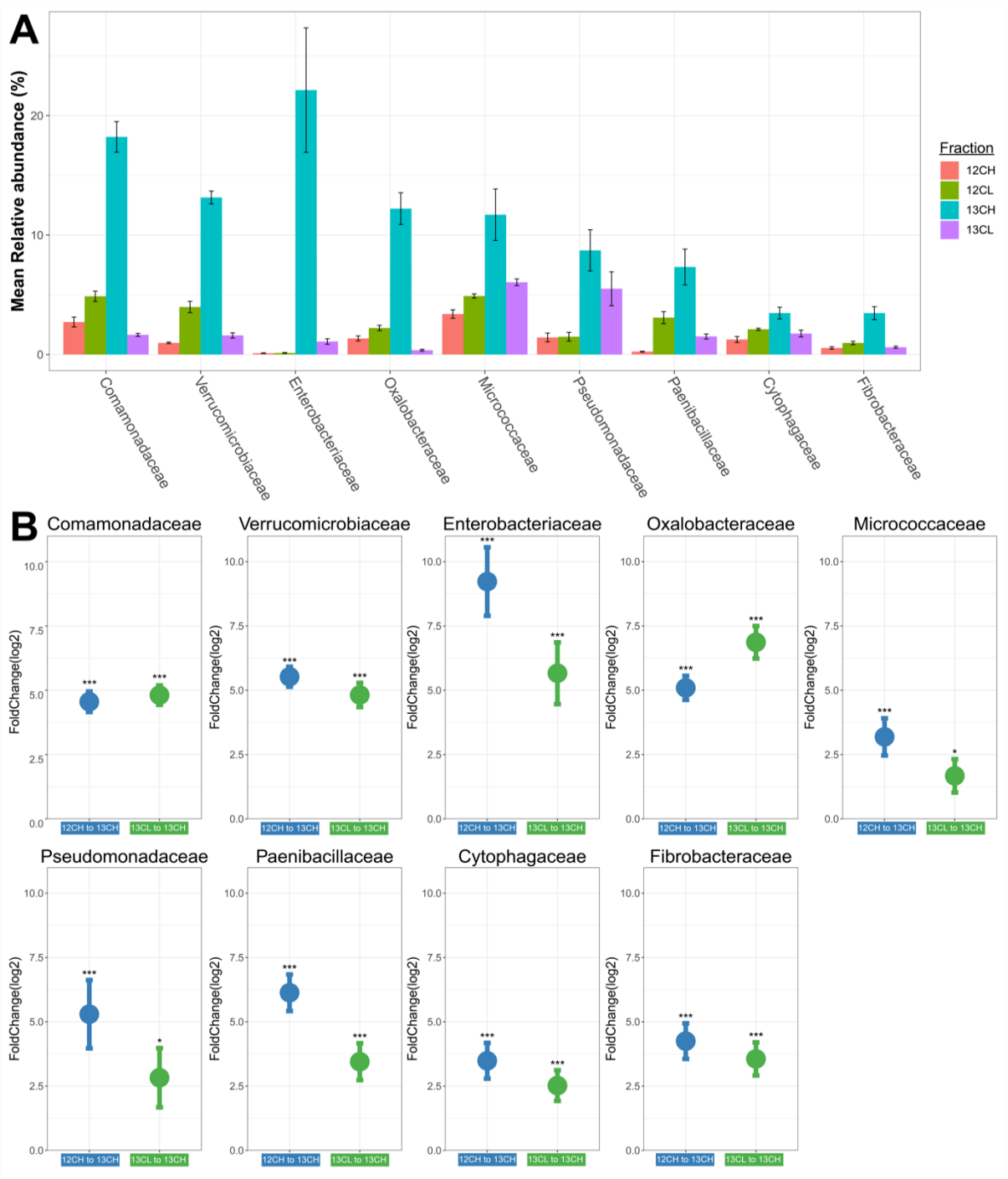

Figure 3-18 Data presented for ^{13}C labelled rhizosphere families with a base higher than 250, according to the differential abundance analysis. (A) Mean relative abundance for each bacterial family in the rhizosphere compartment of N=3 plants incubated with $^{12}CO_2$ or $^{13}CO_2$, within the heavy or the light fractions. Error bars represent standard error of the mean. (B) Differential abundance analysis. Dots show the fold change of different bacterial families from the $^{12}CO_2$ heavy to the $^{13}CO_2$ heavy fraction (blue) or from the $^{13}CO_2$ light to the $^{13}CO_2$ heavy fraction (green). N=3 plants. Error

bars represent log-fold change standard error. Panel B was generated by Sam Prudence, co-author of Prudence *et al.* (in prep).

3.4.7.1 Determining candidate autotrophic bacterial taxa in the rhizosphere and unplanted controls

For endosphere and rhizosphere compartments, samples were sequenced from plants labelled with $^{13}CO_2$, as well as a control group which was grown under $^{12}CO_2$. An additional, unplanted control pot containing bulk soil was maintained under the same conditions as $^{13}CO_2$ labelled plants. The purpose of this control was highlight candidate photoautotrophic taxa, to distinguish from those that may be utilising root exudates in the rhizosphere. An important caveat to note here is that chemoautotrophic taxa, which can assimilate CO_2 from carbon oxidation, may also be labelled. Candidate photoautotrophs were determined by significant enrichment from ^{13}CL to ^{13}CH samples. Taxa with base mean lower than 100 was omitted from the analysis. Following the removal of these taxa, 11 bacterial families were identified, which are presented in Table 3-9

Table 3-9 Candidate photoautotrophic taxa as determined by enrichment in rhizosphere heavy fractions, relative to the light fractions of $^{13}CO_2$ unplanted control samples. Highlighted in yellow is the only taxon to be labelled in the rhizosphere analysis.

Phylum	Order	Class	Family	baseMean	log2FoldChange
Unknown	Unknown	Unknown	uncultured	1201.013	-2.18107
Actinobacteria	Thermoleophilia	Solirubrobacterales	Elev-16S-1332	533.7676	-2.62678
Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	523.2174	-2.42321
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	463.0262	-2.07108
Actinobacteria	Acidimicrobiia	Acidimicrobiales	Acidimicrobiaceae	366.6392	-1.76032
Nitrospirae	Nitrospira	Nitrospirales	0319-6A21	177.7571	-2.27997
Actinobacteria	Thermoleophilia	Solirubrobacterales	288-2	175.2058	-2.42222
Proteobacteria	Alphaproteobacteria	Rhodospirillales	MSB-1E8	161.641	-3.19409
Actinobacteria	Actinobacteria	Micromonosporales	Micromonosporaceae	152.0272	-1.92326
Actinobacteria	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	114.7633	-1.91417
Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	111.1246	-2.43516

3.4.7.2 The influence of root exudates on root microbiome formation

A key aim was to determine the influence of root exudate feeding on wheat root microbiome assembly. Taxa associated with root exudate metabolism, as determined by the SIP experiment, were compared with taxa that were differentially abundant in the root compartments of unfractionated samples (Table 3-10). Out of 15 families that were shown to utilise root exudates in the rhizosphere, 12 were enriched in the rhizosphere compartment of unfractionated samples, compared to bulk soil. In addition, 10 of the 15 families were also enriched from rhizosphere to endosphere compartments.

Table 3-10 Differential abundance of root exudate utilising taxa, between different compartments of the wheat root microbiome. Significant enrichments from bulk soil to rhizosphere or rhizosphere to endosphere are highlighted.

		^{12}CH > ^{13}CH		^{13}CL > ^{13}CH		Bulk > Rhizosphere		Rhizosphere > Endosphere	
Phylum	**Family**	**Base Mean**	**log2Fold Change**	**Base Mean**	**log2Fold Change**	**Base Mean**	**log2Fold Change**	**Base Mean**	**log2Fold Change**
Proteobacteria	Enterobacteriaceae	1525.5852	9.2255	1193.5578	5.6631	1284.5518	3.0354	55820.8681	7.4522
Firmicutes	Paenibacillaceae	542.7148	6.1312	474.7391	3.4503	1241.6357	0.4539	4814.7072	4.4808
Verrucomicrobia	Verrucomicrobiaceae	1540.4763	5.5257	1240.2326	4.8175	633.3492	4.3269	614.9336	1.3812
Proteobacteria	Pseudomonadaceae	747.4943	5.2964	640.9324	2.8280	1415.2576	4.6226	1178.3398	-1.1575
Proteobacteria	Oxalobacteraceae	1121.7981	5.0936	882.3909	6.8662	743.6033	3.0635	660.7432	1.3084
Proteobacteria	Cellvibrionaceae	143.5597	4.5639	122.6921	3.3599	492.4774	4.0739	324.8956	2.5979
Proteobacteria	Comamonadaceae	1965.0959	4.5540	1557.0644	4.8109	1558.4328	2.9023	3913.3003	3.1752
Fibrobacteres	Fibrobacteraceae	308.4267	4.2510	247.2869	3.5586	492.4774	4.0739	295.4643	-0.3308
Proteobacteria	Rhizobiaceae	200.6142	3.5866	194.8885	1.6767	638.5884	2.9253	979.0443	2.3750
Bacteroidetes	Cytophagaceae	333.3919	3.4852	283.9659	2.5201	2048.8044	1.9693	3151.4550	2.5473
Actinobacteria	Micrococcaceae	1002.9197	3.1889	918.5754	1.6739	1651.2314	0.4297	469.3043	0.4077
Actinobacteria	Microbacteriaceae	182.5424	2.3165	144.6834	2.1570	358.5089	0.6992	150.6791	-0.5229
Proteobacteria	Xanthomonadaceae	191.2474	2.2102	167.3075	1.5652	746.2113	1.0294	572.9690	1.6296
Proteobacteria	BIrii41	130.5447	2.2020	111.3784	1.6377	333.6190	1.2152	166.8192	-0.5887
Actinobacteria	Intrasporangiaceae	196.5958	1.6795	158.6062	1.5069	317.1009	0.2048	50.2107	5.0514
Proteobacteria	Polyangiaceae	174.8114	1.4411	119.7725	2.4243	473.0929	1.5386	1634.9730	3.9583

3.5 Discussion

This study aimed to identify bacterial wheat root metabolite consumers in the rhizosphere and endosphere microbiomes and to determine whether root exudates may contribute to the assembly of a beneficial microbiome. Most taxa implicated in root exudate metabolism were shown to be enriched in the rhizosphere compartment, relative to the bulk soil. Similarly, many of these taxa were enriched in the endosphere, relative to the rhizosphere. This data supports the consensus that bacterial community assembly is mediated by exudation of organic substrates (Huang *et al.*, 2014; Sasse *et al.*, 2018). While taxa were implicated in root exudate metabolism in the rhizosphere, labelled taxa could not be determined in the endosphere by differential abundance analysis. A key aim of this Chapter was to determine whether *Streptomyces* were using wheat root metabolites in the rhizosphere or endosphere microbiome. This study showed that *Streptomycetaceae* were enriched in the endosphere, preliminary data also showed that they may utilise root exudates, as they were present at a higher mean relative abundance in labelled fractions than control samples. This could be validated further by performing qPCR using primers that amplify a gene which is specific to *Streptomyces*. This would then confirm that *Streptomyces* were enriched in labelled samples, relative to control samples. It is important to recognise the limitations of this data however, this study has shown that bacterial communities in the endosphere can be highly variable. The extent of this variability was unexpected. Although the reasons behind this are unknown, it is possible that the variability observed is due to a founder's effect, whereby early colonisers of the plant tissue are able to quickly replicate to fill the niche before other taxa are able to do so. This variability might be verified using other profiling techniques, such as PFLA. Future experiments should sequence more replicates to to leverage improved statistical power in order to test meaningful hypotheses.

The community composition of the wheat root microbiome was consistent with the literature, the list of enriched taxa was comprised mostly of Proteobacteria (Schlatter *et al.*, 2020). Actinobacteria were less enriched than some studies might suggest (Rascovan *et al.*, 2016), although the abundance of this phylum is shown to be more soil type dependent. Most families identified as wheat root exudate consumers in this study were representatives of these 2 phyla, which supported the findings of other nucleic acid SIP studies of the wheat rhizosphere microbiome (Uksa *et al.*, 2017). Proteobacteria seem consistently labelled in the rhizosphere of wheat plants (Haichar *et al.*, 2008; Ai *et al.*, 2015; Uksa *et al.*, 2017), while Actinobacteria is more variably labelled. This may be due to either substrate preference or microbial competition.

Numerous taxa were shown to be mutually labelled in this study and others (Haichar *et al.*, 2008); *Enterobacteriaceae* and several members of the order Burkholderiales, such as

Comamonadaceae and *Oxalobacteriaceae* utilised root exudates. Key players in the metabolism of root-derived Carbon varied between studies however, these families were not shown to be labelled in a later study by (Uksa *et al.*, 2017). While congruence with the findings of (Haichar *et al.*, 2008) are promising, comparing NGS-derived metagenetic data with these earlier SIP studies such should be done with caution, due to limitations of the profiling technique. The authors used Sanger sequencing of DGGE bands, this offers a qualitative insight into microbial communities that precludes the kind of statistical enrichment analysis used in this study and others (Uksa *et al.*, 2017). Data presented in this Chapter has also explored how DGGE can overestimate diversity in environmental samples, it has been stated that such artefacts limit the power of such methods for quantitative analysis (Neilson *et al.*, 2013).

As members of the *Comamonadaceae* family were shown to be labelled, they may serve an important function in the wheat root microbiome. While they are a diverse taxon (Willems, 2014) some members have been implicated in methanol metabolism (Macey *et al.*, 2020). The authors used DNA-SIP to probe methanol enrichments of wheat rhizosphere communities. Methanol degraders such as those from *Comamonadaceae* were shown to be more diverse in the wheat rhizosphere, and it is suggested that this taxon may utilise plant derived methanol, although no SIP experiments were conducted *in planta* to confirm this. The hypotheses and methodology are notably different from this study, yet the results are discussed here as it is the only published study to investigate the bacterial rhizosphere community of wheat *var.* Paragon, in soil sampled from JIC church farm field studies site. The enrichment of *Comamonadaceae* in the labelled fractions of wheat rhizosphere presented in this study supports the authors' hypothesis that they metabolise methanol exuded by wheat roots.

Plant derived atmospheric methanol is known to play important role in biogeochemical processes, acting as a net source or sink for free radicals (Sargeant, 2013) which are known to contribute to acid rain (Galbally and Kirstine, 2002). A large proportion of plant derived methanol never reaches the atmosphere, as it is degraded by soil-borne methanol utilising (methanotrophs) prokaryotes. Greater understanding of methanotrophs like *Comamonadaceae* in the root microbiome of this widespread, staple crop is vital.

The results of this Chapter also shares common labelled taxa with the DNA-SIP study published by (Uksa *et al.*, 2017), which used next-generation amplicon sequencing to profile the wheat rhizosphere microbiome. The authors showed that *Pseudomonadaceae* were active in metabolising root exudates, additionally reporting a previously undescribed enrichment of the Firmicute family *Paenibacillaceae* in the labelled rhizosphere community. ^{13}C labelling of *Paenibacillaceae* suggests that it metabolises root exudates and by extension may serve an important ecological function in the

wheat root microbiome. This is especially true, as differential abundance analysis showed sequential enrichment in both rhizosphere and endosphere compartments. Representatives from this family are known to have PGP properties (Grady *et al.*, 2016), although they remains less characterised than many plant-beneficial rhizobacteria like *Streptomyces* or *Pseudomonas*. This family represents a good candidate for further study, with cultured representatives.

One of the key challenges with nucleic acid SIP/amplicon sequencing is the effective resolution for identification. Key taxa have been identified at the family level, as the amplicon sequenced covers V3/V4 regions of the 16S rRNA gene. As long read amplicon sequencing technology improves, it will be possible to probe these taxa at genus, or even species level. It is also important to recognise however, that the limitations of 16S rRNA gene amplicon community surveys still apply; root exudate metabolism may be inferred but metabarcoding can offer little in the way of functional characterisation. Nevertheless, this study offers key insight into microbial community members involved with plant-derived carbon dynamics and helps to focus future lines of enquiry to investigate wheat root interactions, with specific groups of bacteria. Understanding how plant beneficial taxa like *Streptomycetaceae* feed in the root microbiome may drive strategies for their enrichment in agriculture. Targeted metagenomics may also prove to be a valuable tool in further probing the functional capacity of root exudate metabolising organisms, by sequencing whole genomes from labelled fractions. Bacterial identities may additionally be resolved to a lower taxonomic level with this method.

Although several labelled taxa were mutually identified with other studies, some were uniquely implicated in root carbon metabolism by this study, such as *Verrucomicrobiaceae*. Something to be noted as the results of this study are presented in the context of others, while there are often similarities in the findings between studies, labelled taxa vary. This shows that although certain microbes may be more likely to metabolise root exudates, the metabolically active community may vary. Just as soil type and genotype both affect total microbiome composition (Fitzpatrick *et al.*, 2018a), they are likely to influence the exudate metabolising community, either directly or indirectly. This was core to the rationale of using agricultural soil for this SIP experiment in this Chapter. Although plants likely shape their root microbiome to varying degrees through rhizodeposition, the rhizosphere community and root microbiome as a whole, is shaped by soil type and microbial composition (Kuźniar *et al.*, 2020; Schlatter *et al.*, 2020). Bacteria can only be labelled if they are present in the bulk soil, increased abundance of certain taxa may also lead to labelling through a founder's effect, which may not be observed if they are outcompeted. This may go some way to explain the variation of labelled taxa present in endosphere samples. Difference in host genotype will also affect root exudate profile

(Fan *et al.*, 2001; Iannucci *et al.*, 2017), this is likely an important driver of the variation in exudate metabolisers reported, as different taxa will have preferential substrates.

Given that beneficial *Streptomyces* in wheat root microbiomes is the focus for this thesis, a key question was whether Streptomycetes use root exudates. This study showed that *Streptomycetaceae* were not labelled in rhizosphere samples, which suggested that they were not using root exudates. Differential abundance analysis also showed no significant enrichment in the rhizosphere, compared to bulk soil. This was surprising as they were shown to be significantly enriched in rhizosphere of the same variety of wheat grown in JIC field centre soil in previous studies (Prudence, unpublished). The same study did show however, that *Streptomycetaceae* were enriched in the endosphere compared to rhizosphere, which is something that this study validates. Some studies have shown that *Streptomyces* species can grow on root exudates as the sole nutrient source (Worsley, 2019), while others have demonstrated wheat root exudates metabolism *in planta* using DNA-SIP (Uksa *et al.*, 2017). *Streptomycetaceae* were more abundant in labelled endosphere fractions, which might suggest they are feeding on root exudates. While it is crucial to recognise the limitations of such preliminary data, contemporary studies lend credence to the hypothesis.

There are various reasons that *Streptomycetaceae* may not be labelled in the rhizosphere compartment. If they are dormant in the rhizosphere, cells will not actively divide and would not assimilate the ^{13}C label. This may be the case if they are not enriched in the rhizosphere relative to bulk soil. Taxa such as *Streptomyces* are spore forming organisms, if conditions are not favourable for growth and reproduction, spores will not germinate (Flärdh and Buttner, 2009). The alternative hypothesis is that they are utilising other nutrient sources, this DNA-SIP study was designed to investigate which communities metabolise exudates during the 2-week labelling period. *Streptomyces* may be metabolising root detritus, (Kong *et al.*, 2020) showed that they dominated utilisation of labelled rice residues (Kong *et al.*, 2020). Taxa metabolising root detritus would not be labelled in the study presented for this Chapter. If further experimental evidence were to support the hypothesis that *Streptomycetaceae* subsist predominantly on dead root material, then it could still help drive microbiome engineering strategies. Studies have already shown that chitin supplementation shifts root microbiome composition towards actinobacterial dominance (Cretoiu *et al.*, 2013). Continued research into factors reinforcing beneficial taxa will likely prove key to the success of microbial bio enhancement of crop systems.

Another possibility is that they are metabolising exudates in the rhizosphere, but they are too slow growing to be labelled under the experimental conditions tested. This could be addressed by profiling the microbial community of labelled sample using a more sensitive biomarker. As an example,

RNA-SIP would enable the identification of metabolically active organisms, even if they were not actively dividing. For example, if a community of Streptomycetes were to divide an average of twice over the course of labelling, then a significant proportion of microbes would not be labelled even if root metabolites were the sole substrate. Streptomycetes are well known examples of K-strategists (Karpovich-Tate and Rebrikova, 1991) in the microbial world. While some organisms' life strategy is to grow and divide exponentially, others like *Streptomyces* invest more heavily in secondary metabolism, in order to establish a stronger position in their niche. The trade-off is that fast growing *r*-strategists in the same niche such as *Pseudomonas*, may dominate resources, which in this case would be root exudates. Labelling is likely be more complete for such bacteria, which often have a shorter lifecycle. This study also showed that wheat demonstrates a weak rhizosphere effect. Certain taxa are enriched in the rhizosphere, but the community is still fairly complex, which shows the plant has limited scope for shaping microbial community. *Streptomycetaceae* were present at low abundance which means their opportunity to utilise root exudates in this niche would also be limited.

This chapter has established that *Streptomycetaceae* are significantly enriched in the endosphere of wheat, relative to the rhizosphere and surrounding bulk soil and while no statistical conclusions could be made, the DNA-SIP experiment suggested that they were able to metabolise root exudates. Combined, this data supports the hypothesis that *Streptomyces* have a beneficial role to play in the wheat root microbiome. These roles were explored briefly in chapter 2. Many of the strains isolated were capable of inhibiting wheat take-all fungus *in vitro* in dual culture assays and whilst *in planta* bioactivity could not be established, it seems likely that these strains could confer the benefit of disease resistance to their host. Select *Streptomyces* strains were all able to metabolise chitin, which may contribute to this bioactivity against pathogenic fungi. Under stressful conditions, ACC could be readily metabolised by endophytic streptomycetes, which could prevent the formation of excess ethylene and reduce the chances of pathologic accumulation.

The early stages of a plant's development see the greatest richness of the root microbiome community (Chaparro *et al.* 2014). Over time, there is a richness decline, and it is suggested that the plant host selectively recruits beneficial microorganisms. Numerous studies have shown that root exudation plays a strong role in this process (Houlden *et al.*, 2008; Badri *et al.*, 2013; Chaparro *et al.*, 2013). The data presented in this study supports the hypothesis that root exudates recruit bacteria in order to exploit their beneficial properties. *Pseudomonadaceae* is perhaps one of the best characterised plant-beneficial rhizobacterial families, so it is interesting to see that this taxon was both the most enriched in the rhizosphere microbiome and shown to utilise root exudates *in situ.*

Unlike *Streptomyces*, *Pseudomonas* are *r*-strategists. High growth rate might mean they are able to assimilate root exudates rapidly, outcompeting other slow-growing bacteria. The alternative hypothesis, which is attractive given their beneficial properties, is that *Pseudomonadaceae* are selectively recruited by root exudates. Plants were grown for this study in agricultural soil. As such, plant pathogens may be present. Organisms such as *Pseudomonas* spp. are known to contribute to spontaneous decline of wheat take-all through production of secondary metabolites (Raaijmakers and Weller, 1998; Weller *et al.*, 2007). In addition, they are known plant growth promoters (Patten and Glick, 2002; Dinesh *et al.*, 2015) and can prime the plant immune system through ISR (Pieterse *et al.*, 2001; Choudhary *et al.*, 2007). It may be that *Pseudomonadaceae* are recruited for their benefits by root exudates early in plant development while other slower-growing plant beneficial taxa establish over time to provide protection through maturity.

Greater understanding of this process is required. It is important to recognise, however, that this study offers a snapshot of the exudate metabolising community at this life stage. If wheat plants are recruiting *Pseudomonadaceae* between 4 and 6 weeks, as suggested by the incorporation of the ^{13}C label, it shows that they may be a key group of microorganisms for the plant's health at that developmental timepoint. The implication is that if *Pseudomonadaceae* are fed by specific root exudates that leads to their enrichment, these compounds may represent a viable pharmacological strategy for bioaugmentation of this plant beneficial taxa. Microbial communities are known to shift throughout developmental stages (Chaparro *et al.*, 2014), a study into microbiome of wheat roots over its life cycle showed that *Streptomycetaceae* increased in abundance during the later jointing and ripening stages (Chen *et al.*, 2019). Pseudomonadales on the other hand, were early colonisers. The data presented in this thesis showed *Pseudomonadaceae* were not the most abundant taxon present in rhizosphere samples, but they were enriched in the rhizosphere and they were utilising root exudates. It could be that *Pseudomondaceae* bacteria are recruited early as rapid colonisers, in order to protect the plant from pathogens by competitive exclusion, while K-strategists such as *Streptomycetaceae* establish with maturity. This theory does not fit entirely, however, as *Streptomycetaceae* were enriched in the endosphere compartment, even though they were not established in the rhizosphere. It may be that slow growing bacteria like *Streptomycetaceae* are just more likely to be present later in a plant's development. This may go some way to explain why the enrichment and labelling of such key plant beneficial Actinobacteria lacks consistency between studies. While it is not possible to identify consumers of mature wheat root exudates within the scope of this study, it would be useful to conduct a longitudinal nucleic acid SIP study in future, in order to probe carbon dynamics further in wheat and other economically relevant cereal crops.

Of the 16 enriched taxa from the labelled rhizosphere samples and the 11 from labelled bulk soil samples, *Intrasporangiaceae* was the only one that was mutually enriched. The purpose of the labelled soil control was to identify candidate autotrophic taxa which were labelled in bulk soil, to distinguish them from root exudate metabolisers in the rhizosphere. The fact that this was the only candidate autotroph to be labelled in the rhizosphere, suggests that this niche was well occupied, as most autotrophs were excluded. An additional consideration is that the soil may have been too dense to allow light to penetrate to the rhizosphere compartment, so less photoautotrophs may occupy the niche. There are difficulties distinguishing photoautotrophs and chemoautotrophs from the root exudate metabolisers in this way, however. $^{12}CO_2$ controls were not sequenced for unplanted soil samples. The caveat to this approach is that some taxa may be naturally more abundant in heavier fractions of bulk soil if their genome contains higher G/C%. This is the case for *Intrasporangium*, the type genus of *Intrasporangiaceae* (del Rio *et al.*, 2010). As such, it is possible that candidate photoautotrophs may be naturally more abundant in heavier fractions of bulk soil without fixing labelled carbon. As previously stated, there is additionally no conclusive way of distinguishing candidate photoautotrophs from chemoautotrophic taxa.

Although a conclusive table of root exudate utilisers was calculated by differential abundance from both ^{12}CH and ^{13}CL to ^{13}CH fractions, it is also important to recognise that numerous taxa may be partially labelled, due to a mixed utilisation of root exudates and other substrates such as soil organic carbon (SOC). Increased abundance of taxa in ^{13}CH fractions compared to the ^{12}CH fraction alone would suggest partial usage of labelled substrate in this way, as a lack of shift from ^{13}CL would suggest ^{12}C uptake as well.

The metabolism of root exudates and the microbial ecology of root microbiomes are complicated. It is important to recognise that there are caveats to this study, as there is with any other. Although it is not possible to robustly resolve nuances such as partial labelling, the insight offered by this study is robust for the conditions tested. Statistical analysis with the Deseq2 package offers key insights about root Carbon dynamics of prokaryote taxa. Similarly, the timeframe of this study was carefully chosen. While it may not offer insights into root exudate metabolism in mature plant microbiomes, key taxa are implicated for plants at the vegetative stage. Not only is this development stage most comparable to other wheat root microbiome studies, it is perhaps the most important time for microbiome formation, rhizosphere species richness was shown to be lowest in *Arabidopsis thaliana* at the vegetative growth stage (Chaparro *et al.*, 2014). If this correlation holds true for wheat plants, disease suppressive taxa may be far less established. It has also been reported that 3 week old wheat plants are more susceptible to disease than at 6 weeks old (Farber and Mundt, 2017), which

also shows how important early microbiome formation may be. Overall, while community surveys are important tools for understanding how the microbial community composition shifts between environments, SIP studies such as this one and others like it help narrow down the list of taxa to those that are more functionally relevant from an agricultural perspective.

Careful consideration was given to the choice of labelled biomarker. Nucleic acid SIP can be performed with DNA or ribosomal RNA, both have their benefits and their drawbacks (Neufeld *et al.*, 2007b, 2007a). DNA-SIP is the least sensitive technique, but it is currently the most versatile. Labelling of DNA requires division, it has been discussed how this may lead to a bias against slow growing organisms, but isolation of labelled DNA can be used to probe the function of microbial communities in numerous ways. Metagenome sequencing of labelled DNA is becoming increasingly popular as a functional metagenomic technique (Ziels *et al.*, 2018). rRNA-SIP is a more sensitive technique in that it can detect metabolically active microorganisms that are not actively dividing. One of the key drawbacks of this technique is that with increased sensitivity, the chance of cross-feeding may also increase, although this can be mitigated to a degree with shorter labelling times. Cross-feeding is a significant issue where labelled metabolites are released from organisms and utilised by other microbes (Manefield *et al.*, 2007). The result is that some taxa may be labelled without directly metabolising the resource of interest. Both options were considered carefully, DNA-SIP was chosen due to additional technical challenges imposed by rRNA-SIP, as a far less stable biomarker with reduced flexibility for further analyses.

It was noted that the density of light fractions dropped off sharply compared to denser fractions, this was due to the fact that the water used for fractionation of the CsCl gradient was collected in these lighter fractions. DNA contamination from the water was not a major concern, as sterile dH_2O was used and the peristaltic pump was sterilised prior to fractionation by flushing with 70% (v/v) EtOH followed by sterile dH_2O. In order to eliminate this concern entirely, it may be useful to shift the gradient such that the DNA is collected from heavier fractions. This could be done by separating DNA with a lower concentration of $CsCl_2$.

This study has contributed to our understanding of root exudate metabolism in the wheat rhizosphere, once published it will be one of only three studies that profile these communities with next-generation amplicon sequencing, along with (Ai *et al.*, 2015; Uksa *et al.*, 2017). In addition to probing root exudate metabolism of wheat rhizosphere communities, endosphere communities were also investigated, this has not been done before using next generation metabarcoding. Sample variability between different plants was unfortunately too high to infer any statistical enrichment of taxa in ^{13}C labelled fractions. Despite this, there was reasonable evidence to suggest that

Streptomycetaceae were using root exudates in the endosphere, which was a key question of this Chapter. Variability was high enough that the possibility of contamination during processing was considered. This theory was refuted following regeneration and sequencing of 16S rRNA gene products from endosphere SIP fractions. This study determined that future investigations into root wheat root exudate metabolism in the endosphere should sample a greater number of plants to account for this variability.

In conclusion, this study shows that plant derived carbon is metabolised by a range of families, mostly from the phylum Proteobacteria. It both supports and expands the observations of other studies into root exudate metabolism in the wheat rhizosphere. Numerous prokaryote families were implicated with functions ranging from the plant-beneficial, to involvement with biogeochemical cycles like methane cycling. Families like *Paenibacillaceae* have been identified as key metabolisers of root exudates and while their beneficial properties are known (Grady *et al.*, 2016), they remain understudied compared to taxa such as *Streptomycetaceae* and *Pseudomondacaeae*. This study has broad reaching implications for understanding plant-derived Carbon dynamics and plant microbiome engineering. Moving forward, this study could be used to direct *in vitro* metabolic studies, to determine substrate specificity for key taxa, using fractionated root exudates. SIP studies for other cereal crops could prove valuable, to determine core taxa in cereal root exudate metabolism generally. While the research questions asked by this Chapter have been answered, several new ones have emerged. Firstly, why is the wheat endosphere community so variable and what factors determine the microbial community? *Streptomycetaceae* were enriched in the endosphere and preliminary data suggested they might use root exudates. Despite this, their abundance in the rhizosphere was not significantly different from bulk soil and they were not shown to be labelled. Our understanding of the molecular mechanisms driving the recruitment of *Streptomyces* to the wheat root microbiome are poor. Research presented in Chapter 4 will explore this.

Chapter 4 Elucidating the role of nitric oxide (NO) in wheat root colonisation by *Streptomyces coelicolor*

4.1 Introduction

Within the plant root microbiome, studies have shown that individual community members can function to benefit the plant host, and this has been explored for the case of *Streptomyces* species in Chapter 2. The genomic era has additionally yielded key insights into the microbial dynamics of the root microbiome, it has been shown that 16S rRNA gene amplicon surveys can be powerful tools to understand how communities are assembled and in response to what stimuli (Bulgarelli *et al.*, 2012; Lareen *et al.*, 2016). What remains elusive in many cases, however, are the molecular mechanisms that operate in microorganisms to facilitate colonisation of the root soil or tissue.

We are beginning to understand plant derived factors that drive root microbiome assembly, this was explored in Chapter 3 (Houlden *et al.*, 2008; Rolfe *et al.*, 2019), yet the lower level molecular mechanisms remain less clear. Community level surveys are not suited to this kind of investigation, as the resolution of data that implicates development cues, biotic stressors, or even specific metabolites in the recruitment of specific microbial taxa is poor.

Ultimately, the limitations are partly due to current community profiling techniques, short read metabarcode amplicon sequencing can only reliably resolve family level taxonomy. In addition, a single OTU can represent a wide range of organisms, and bacteria are also known to have multiple 16S rRNA gene copies that vary in sequence (Coenye and Vandamme, 2003). This means that there is potential to miscalculate diversity. Other 16S rRNA gene profiling techniques such as DGGE were shown to be similarly error prone in Chapter 3, as species richness can be overestimated. Community surveys are additionally limited by the insight they provide into the function and lifestyle of microorganisms.

This Chapter will address the question of how *Streptomyces* species interact with wheat roots at the molecular level and try to identify the mechanisms that influence successful colonisation of the rhizosphere and endosphere microbiome. The methods and paradigms utilised for investigation of microbial gene function in the root microbiome context will be explored.

4.1.1 Established root colonisation mechanisms of bacteria

When considering the holobiont functional capacity, studies utilising integrative approaches to understanding the chemical ecology of plant microbe systems often yield valuable insights into microbial colonisation mechanisms. Numerous studies reviewed thus far have discussed the way in which plants likely use signals and nutrients to communicate with multiple taxa within microbial communities to coordinate mutualism, but it is also important to consider the way microbial physiology influences these interactions. For instance, inferences about recruitment can be made with the correlation of root exudates known to drive chemotactic motility of individual taxa (Carvalhais *et al.*, 2015), but it is important to recognise that the authors provide no direct evidence that fructose chemotaxis in *Bacillus* spp. is vital for colonisation. Such mechanistic studies are significantly lacking in the literature but have the potential to provide fundamental insights.

There are a few different approaches for interrogation of genetic factors affecting the microbial physiology of root colonisation. Forward genetic screens can be used to identify genes responsible for host-microbe interactions (Cole *et al.*, 2017), as discussed in Chapter 2. Randomly barcoded transposon mutagenesis sequencing (RB-TnSeq) generates a library of uniquely identified mutants with gene disruptions across the genome, so that relative fitness can be determined. It was established that there are a range of factors that are important to root colonisation by bacteria, but motility was highlighted as particularly important, concurrent with other studies (Coutinho *et al.*, 2015; Liu *et al.*, 2017a). Carbohydrate metabolism and cell wall biosynthesis gene were also important, supporting the theory explored in Chapter 3, that the plant host recruits this organism with root secreted sugars. Increased defence may also be important when transitioning to a more competitive niche. Forward genetic screens like RB-TnSeq used by (Cole *et al.*, 2017) are not without their issues however. Numerous important genes were implicated in colonisation, such as motility, carbohydrate, and amino acid metabolism. Many were also hypothetical, with unknown function, however. This of course represents a great starting point for the investigation of novel gene targets but determining physiological function without prior characterisation poses challenges. Nevertheless, for root associated organisms with suitable genetic toolkits, these studies are crucial for expanding the frontiers of our understanding.

Reverse genetic studies target candidate gene(s) of interest, based on prior characterisation. For example, motility has been discussed as a factor in root colonisation and *cheA* is a gene implicated in motility through chemotaxis in *Pseudomonas fluorescens* (De Weert *et al.*, 2002). Root colonisation assays showed that disruption of the *cheA* gene led to reduced colonisation fitness for tomato roots, both in soil and sterile sand systems. This suggested motility and chemotaxis are important for

colonisation, in both the presence and absence of microbial competition. This Chapter will take a similar approach, using prior knowledge of plant-microbe interactions to identify a potential mechanistic target, which will be interrogated using gene deletion mutagenesis and root colonisation assays.

4.1.2 The plant immune system mediates microbial colonisation

While the root metabolome and microbial physiology have been considered as factors governing root/microbe associations, the role of the plant immune system as a whole cannot be understated, in selectively filtering beneficial microorganisms from the bulk soil community (Zipfel and Oldroyd, 2017). The molecular target that will be explored in this Chapter is nitric oxide (NO) and it is linked to both microbial physiology and the plant immune system, so it is necessary to give a brief overview of plant immunity. The plant immune system is well studied and has been reviewed by (Jones and Dangl, 2006; De Wit, 2007; David *et al.*, 2019). Plants sense general microbe associated molecular patterns (MAMPs) or the more specific pathogen associated molecular patterns (PAMPs) using transmembrane pattern recognition receptors (PRRs). This pattern triggered immunity (PTI) is akin to the innate immune system in humans and occurs at the interface of plant cells and the environment. Pathogens often secrete compounds known as effectors which act as virulence factors, inducing susceptibility to disease. Effectors can be detected by cytoplasmic receptors called NB-LRRs, which are usually encoded by disease resistance genes (R genes), this is known as effector triggered immunity (ETI). These two branches of the immune system feed into a highly complex and finely tuned molecular network of defense responses. Pathogenesis activates a range of defense related genes through reactive oxygen and nitrogen species, as well as phytohormone production, e.g. salicylates and jasmonates.

The important thing to take away is that the plant host initially detects microbes through MAMP sensing PRRs. It is hypothesised that beneficial microbes with a long-standing evolutionary history with the host can pass through this filter. This clearly isn't the whole story though because there is plenty of evidence to show even beneficial microbes are flagged by the plant immune system. Many rhizobacteria have been shown to induce systemic resistance (ISR) (Pieterse *et al.*, 2001, 2014), this acts in a way to prime the immune response to respond quicker to PTI or ETI. *Streptomyces* have even been shown to trigger a more advanced systemic acquired response (SAR), this has been discussed briefly in prior Chapters. This effect was demonstrated with foliar treatment of cucumber plants with *Streptomyces* culture filtrates, an 85% reduction of cucumber mosaic virus symptoms (CMV) was observed (Galal, 2006). While antimicrobial activity is characteristic of *Streptomyces* spp., they are not known to have antiviral activity and so the authors suggest that disease resistance is due

to SAR. This is no doubt beneficial, but the fact that *Streptomyces* species can elicit this response, does suggest that plants may initially view *Streptomyces* spp. as pathogenic.

It seems rational then, that resilience to plant defences may be key for root colonisation, given that *Streptomyces* spp. have been shown to elicit an advanced immune response. Following the activation of plant host defenses by PTI or ETI, a cascade of transcriptional and translational changes occurs at the primary infection site. These responses are initially localised (David *et al.*, 2019). Alkalinisation, ion flux changes and increased reactive oxygen species (ROS) and nitrogen species (RNS) are all characteristic of PTI. NO is known to play a pivotal role, altering plant physiology, as well as regulation in numerous ways. It can additionally behave as an antimicrobial at higher concentrations. Over the last two decades or so, it has become a point of focus for research into plant immunity (Hausladen and Stamler, 1998; Bellin *et al.*, 2013; Domingos *et al.*, 2015; Thalineau *et al.*, 2016) and holobiont interactions. NO will be the focus of this Chapter.

4.1.3 Nitric Oxide (NO)

Nitric oxide (NO) is a highly reactive and readily diffusible gaseous free radical that is ubiquitous in nature. It has been implicated in a broad range of physiological functions in organisms as diverse as bacteria (Crane *et al.*, 2010), plants (Stöhr and Stremlau, 2006) and humans (Hibbs *et al.*, 1988; Maiorana *et al.*, 2003). NO has long been recognised as an antimicrobial in natural systems, human macrophage cells are known to use it to kill invading pathogens (Epstein *et al.*, 1993). High levels of NO are also produced by Beewolf digger wasps to protect themselves from pathogenic fungi (Strohm *et al.*, 2019). Mutualist *Streptomyces* species are known to colonise the antennal glands of beewolves and produce antimicrobials when sprayed onto the larval cocoons (Kroiss *et al.*, 2010), contributing to disease resistance. In order to do this, they must be exceptionally resilient to nitrosative stress. It is suggested that such tolerance is the result of long-standing coevolution. Model streptomycetes with less specialised lifestyles are also known to be equipped with mechanisms for detoxification of NO, which will be discussed below.

The antimicrobial function of NO is hypothesised to mediate symbiosis in certain organisms, excluding undesired microorganisms. Selective recruitment of *Vibrio fischeri* to light-producing organs of squid is driven by NO (Wang *et al.*, 2010b; Thompson *et al.*, 2019). It is thought that NO excludes undesired microorganisms, while *Vibrio fischeri* is itself protected by a flavohaemoglobin (HmpA), a NO dioxygenase protein under the control of an NO sensor (NsrR) (Wang *et al.*, 2010b). Interestingly, *Streptomyces* use a similar system to resist nitrosative stress, *nsrR*/*hmpA* are fairly well conserved in this genus (Table 4-2).

NO seems to mediate symbiosis in ways that extend beyond its antimicrobial nature, studies show that genes implicated in biofilm formation and subsequent colonisation, are also regulated by NO (Thompson *et al.*, 2019). NO seems to serve several roles in this system, it is likely that the potential for such diverse biological functions underpins its ubiquity in the natural world. For example, it has been implicated in the regulation of blood flow, through relaxation of endothelial smooth muscle tissues in humans (Maiorana *et al.*, 2003). A broader role for NO as a signaling molecule in the nervous system is also discussed (Esplugues, 2002).

The fact is, NO can act as such a versatile biological effector due to various properties. Unlike many other signaling mediators, this simple molecule exerts biological effects through directly modifying the chemicals that drive them (Thomas *et al.*, 2008). Intracellular NO at concentrations lower than 200mM can lead to the formation of lipid and DNA radicals, as well as alter protein function through R-group nitrosylation. Reported nitrosylation of methylation enzymes introduces further potential for regulatory inception (Frungillo and Spoel, 2017). The cytotoxic effects of NO are generally associated with concentrations higher than 400nM (Thomas *et al.*, 2008). Cytotoxic products form when NO reacts with ROS, this underpins the hypersensitive response (HR), a vital process in plant immunity which shall be discussed.

4.1.3.1 The role of NO in plant immunity

It is clear from the literature that NO has a big part to play in plant immunity. This will be discussed, but it is important to recognise that this molecule is involved with plant physiology on a grand scale. It is implicated in a range of plant tissues at various development checkpoints (Corpas *et al.*, 2006) and is reported to underpin lateral root development (Correa-Aragunde *et al.*, 2004) as well seed germination (Beligni and Lamattina, 2000). NO is produced either by L-arginine dependent NO synthase (NOS) enzymes (Del Río *et al.*, 2004) or by molybdenum dependent nitrate reductase (NR) (Chamizo-Ampudia *et al.*, 2017). A growing body of evidence supports the hypothesis that NR is a vital source of NO, particularly in root tissue (Lu *et al.*, 2014; Chamizo-Ampudia *et al.*, 2017). Despite this, *in vitro* efficiencies of NO production by this complex multi-domain enzyme are poor, especially in biologically relevant conditions. This suggests that there is much we don't yet know. Tungstate has been used to inhibit NR *in vivo*, through competitive exclusion of the Molybdenum cofactor with conflicting results between studies. Many show that NO production is knocked down with the inhibition of NR (Zhao *et al.*, 2007; Bouchard and Yamasaki, 2008), while others demonstrate the opposite (Xiong *et al.*, 2009; Wang *et al.*, 2010a). Molybdenum is a vital co-factor for numerous plant enzymes, which likely confounds the outcome of these studies.

In relation to the plant immune system, NO is best understood for its interaction with reactive oxygen species such as H_2O_2, to trigger the hypersensitive response (HR) through reactive intermediates (Foissner *et al.*, 2000; Prats *et al.*, 2005; Mur *et al.*, 2017). This has been known for some 20 years, as the inhibition of plant NOS was shown to compromise HR (Delledonne *et al.*, 1998). This process by which individual plant cells undergo programmed cell death, is similar in function to the apoptotic pathway in animals. HR is vital for protection from biotrophic pathogens which require living tissue to colonise, but it must be very finely tuned, as it leaves plants open to infection from necrotrophic pathogens (Govrin and Levine, 2000). HR is often seen then as the "breaking point" in the plant's defence, at which a rapid burst of ROS and RNS triggers an irreversible cascade. Because the effects of this process are so permanent, there are many layers of regulation (Balint-Kurti, 2019; Salguero-Linares and Coll, 2019), discrimination of friend from foe is key.

The role of NO in plant immunity is by no means limited to this "kamikaze" defence however, as it has been mentioned before, NO can act as a dynamic signal with varying responses at different levels. Transcriptome analysis of *Arabidopsis* roots showed a multitude of changes in gene expression in response to the addition of various elicitors, which included NO (Badri *et al.*, 2008). Transcriptional shifts were characterised by altered carbohydrate metabolism and defense related genes. Upregulation of transporters was additionally demonstrated, in addition to increased root exudation. This suggests that root exudates may be used to recruit beneficial microorganisms to defend from pathogens, the 'cry for help' hypothesis is a view that is shared by many in the field (Pieterse *et al.*, 2016; Rolfe *et al.*, 2019).

4.1.3.2 The interplay between NO and microbiome assembly

This idea that NO may be implicated in the recruitment of beneficial organisms is gaining ground (Calcagno *et al.*, 2012; Tian *et al.*, 2017). Although literature relating to NO and plant/microbe interactions is dominated by examples of microbial suppression through HR or other defence related pathways, there is a growing body of evidence to support NO as a mediator of root colonisation by beneficial microorganisms, particularly mycorrhizal fungi (Calcagno *et al.*, 2012; Tian *et al.*, 2017). It has already been discussed that necrotrophic pathogens may seek to exploit HR for their own benefit (Govrin and Levine, 2000) and the mechanisms seem fairly apparent, but a smaller subsection of studies document a range of beneficial interactions that seem to be linked to NO, the exact mechanisms remain somewhat elusive however. (Martínez-Medina *et al.*, 2019) recently reviewed the literature. The authors cited 33 studies implicating NO in pathogenic interactions and 5 in beneficial interactions. A modified version of this is presented in Table 4-1 with an additional study that was found.

Table 4-1 A list of studies documenting beneficial plant fungal interactions that are driven by or drive NO signaling in plant roots. Adapted from (Martínez-Medina *et al.*, 2019)

Fungus	Plant	NO level (technique)	Time Scale	NO source	Gene expression	Pharmacological approrach	Genetic approach	Suggested function	Reference
F. mosseae (AMF)	*Trifolium repens*	DAF-FM DA	5-9 weeks	-	*PAL*/*CHS*	-	-	AMF increases NO levels in roots	(Zhang *et al.*, 2013)
F. mosseae (AMF)	*Trifolium repens*	DAF-FM DA	5-9 weeks	-	*PAL*/*CHS*	-	-	AMF increases NO levels in roots	(Zhu *et al.*, 2015)
Trichoderma asperelloides	*Arabidopsis thaliana*	DAF-2DA	10-120 minutes	-	78 NO-modulated genes	cPTIO (0.1mM), L-NAME (2.5mM)	*nia1nia2*	*T. asperelloides* suppresses NO generation elecited by *F. oxysporum*	(Gupta *et al.*, 2014)
Gigaspora margarita (exudates)	*Medicago trunculata*	DAF-2DA	0-15 minutes	NR	*NR*/*NiR*	cPTIO 1mM	Transgenic roots, (*DMI1-1*, *DMI-2*, *DMI3-1*)	NO specific signature related to AM interactions, distinct from NO signature in response to general elicitation	(Calcagno *et al.*, 2012)
Rhizophagus irregularis	*Olea europea*	DAF-2DA	1-24 hours	-	-	PTIO (400mM)	-	NO may be key in symbiosis establishment	(Espinosa *et al.*, 2014)

Fungus	Plant	NO level (technique)	Time Scale	NO source	Gene expression	Pharmacological approrach	Genetic approach	Suggested function	Reference
Diversispora versiformis	Poncirus trifoliata	-	16 weeks	-	-	SNP (0-0.2mM)	-	Exogenous NO increases AMF colonisation and plant growth	(Tian *et al.*, 2017)

The studies summarised in Table 4-1 demonstrate the numerous methods used to implicate NO in root colonisation. Studies have shown that *NR*/*NiR* gene expression is increased in response to colonisation of mycorrhizal fungi. With the numerous challenges associated with quantifying levels of such a reactive and unstable molecule, capturing the transcriptional state of genes responsible for its biological production is a common approach (Calcagno *et al.*, 2012). Similarly, transcriptomic studies can probe the downstream response of NO regulated genes, in response to association with microbes (Gupta *et al.*, 2014). These provide critical resolution into the biological responses, as it becomes increasingly apparent that the outcome of plant host NO production can vary considerably, due its transient and dynamic nature (Calcagno *et al.*, 2012).

Of the 6 studies presented, four of them adopted a pharmacological approach. Exogenous application of NO releasing compounds (SNP) or NO scavengers (cPTIO, PTIO) shows great potential for interrogating the role of NO in such interactions, although inferences should be made with caution. Many studies choose to focus on scavengers rather than donors, this is likely because donors are a seen as a 'blunt' tool for investigating such a complex and dynamic signal transduction systems. L-NAME is used similarly to scavengers in order to knock down NO signaling, the difference being that this compound competitively inhibits plant NOS enzymes, which is useful for identifying the source of NO.

4.1.3.3 NO and *Streptomyces* physiology

So far, presented plant/microbe interaction studies have been limited to fungi, but it should be clear that NO is an important signaling molecule that underpins important biological processes in many organisms – this is no exception for *Streptomyces*. Nitrosative stress is commonplace for microorganisms living in a variety of environments, and as such they are adapted to respond to that stress (Hausladen *et al.*, 1998; Plate and Marletta, 2013; Crack *et al.*, 2015). Plants and other eukaryotes may contribute to this stress in the soil environment, but numerous microorganisms can generate NO by similar means, either through nitrite reduction (Loick *et al.*, 2016) or bacterial NO synthases (bNOS) (Sudhamsu and Crane, 2009). Prokaryotes therefore deploy a wide range of detection mechanisms (Isabella *et al.*, 2009; Plate and Marletta, 2013), which often directly regulate mechanisms that mitigate cytotoxic effects.

Streptomyces species are highly resilient to nitrosative stress, many are known to utilise regulators from the Rrf2 family for detection of NO. The best characterised system in *Streptomyces* spp. is encoded by *nsrR* and *hmpA* (Crack *et al.*, 2015; Volbeda *et al.*, 2017). Gene homologues are found in numerous other bacterial species, including *Vibrio fischeri*, which has been previously

discussed. NsrR is an iron sulphur cluster (4Fe-4S) containing transcriptional repressor protein which binds to DNA in its holo form (Crack *et al.*, 2015; Volbeda *et al.*, 2017), preventing transcription of the *nsrR* and *hmpA*. When NO is present, it nitrosylates the 4Fe-4S clusters at the active site of the protein causing a conformational shift, releasing transcription of downstream genes. *S. coelicolor* NsrR has a small regulon consisting of *hmpA1*, *hmpA2* and *nsrR* itself. *hmpA1* and *hmpA2* encode flavohaemoglobin dioxygenases which convert NO into NO_3 (Crack *et al.*, 2015), and maintain NO homeostasis. Whilst this is the best-known system of NO detection and oxidation in *Streptomyces*, there are likely others, given how abundant this molecule is in its environment.

Fe-S clusters are a common feature among NO sensing proteins (Mettert and Kiley, 2015). They are a core feature of WhiB Like (Wbl) proteins, which are highly conserved in *Streptomyces* spp. as well as numerous other Actinobacteria, such as the clinically relevant *Mycobacterium tuberculosis* (Kudhair *et al.*, 2017; Bush, 2018). Unlike NsrR in *Streptomyces coelicolor*, Wbl proteins are global regulators. Some Wbl proteins appear to be essential and are known to regulate development and secondary metabolite production (Fowler-Goldsworthy *et al.*, 2011). In addition to bNOS, bacteria can also reduce nitrates in a similar manner to plants, using enzyme complexes with molybdenum cofactors (Philippot and Højberg, 1999). Bacterial nitrate reductses (bNARs) are thought to be the source of NO that drives these signaling events (Sasaki *et al.*, 2016), while NsrR and HmpA sense NO and maintain homeostasis, respectively. It is clear to see that NO underpins the biology of these organisms, yet there is much we still do not know about these vast regulatory networks.

To further reiterate the level to which this fundamental effector is understudied, some streptomycetes encode bNOS enzymes and their function is only partially understood. Such enzymes are best characterised in the plant pathogenic strain *Streptomyces scabei* (Johnson *et al.*, 2008). The consensus is that enzyme derived NO activates phytotoxin thaxtomin through S-nitrosylation (Kers *et al.*, 2004a; Wach *et al.*, 2005; Johnson *et al.*, 2008). There may be alternative functions for bNOS in *Streptomyces* species, however, since homologues can be found in non-pathogenic streptomycetes. Proteins involved with NO metabolism in numerous streptomycetes are presented in Table 4-2

Table 4-2 Gene identities and homologies for proteins involved with NO metabolism across 10 established *Streptomyces* species found on StrepDB. Green cells indicate a presence of protein with positive identity of greater than 50%. N/A = no hits found.

	***Streptomyces scabei* bacterial Nitric Oxide Synthase (bNOS)**			***Streptomyces coelicolor* NsrR (NO sensor)**			***Streptomyces coelicolor* HmpA (NO dioxygenase)**		
***Streptomyces* species**	**bNOS gene ID**	**identities**	**positives**	***nsrR* gene ID**	**NsrR ident**	**NsrR +ve**	***hmpA* gene ID**	**HmpA ident**	**HmpA +ve**
S. venezuelae	vnz_32420	60	70	32375	26	46	vnz_00825	28	42
S. coelicolor	N/A	0	0	7427	100	100	SCO7425	100	100
S. albus S4	N/A	0	0	STRS4_04406	71	79	STRS4_04493	52	70
S. avermitilis	SAV_1531	61	73	SAV_5952	66	76	SAV_4194	27	42
S. formicae	N/A	0	0	KY5_2236c	66	77	KY5_5266c	53	71
S. scabei	SCAB31841	100	100	SCAB86361	70	80	SCAB83441	32	52
S. griseus	N/A	0	0	SGR_2265	72	82	SGR_2366	52	69
S. leeuwenhoekii	N/A	0	0	sle_06320	26	45	sle_25830	52	71
S. clavuligerus ATCC 27064	N/A	0	0	SCLAV_0207	71	75	SCLAV_2608	27	44
S. lividans	N/A	0	0	SLI_7647	99	99	SLI_7645	99	99
CRS3	N/A	0	0	296	70	77	297	57	67
CRS4				6304	66	78			

So far, it has been demonstrated that *Streptomyces* spp. can provide extensive benefits to wheat plants through association with their roots. While the stable isotope labelling study in Chapter 3 showed that *Streptomyces* species were not metabolising root exudates in the rhizosphere, they were enriched in the endosphere. Their relative abundance in labelled fractions additionally suggested they were metabolising root metabolites in the endosphere compartment. While consistency in the literature is poor, their enrichment in the root microbiome of wheat and others have been well established (Edwards *et al.*, 2015; Liu *et al.*, 2017b). While the metabolic lifestyle of *Streptomyces* spp. in the root microbiome remains enigmatic, plant roots appear to provide an important ecological niche and streptomycetes represent an untapped tool for improving yield and resilience to disease as well as abiotic stressors like drought. Widespread adoption of these organisms for commercial biocontrol and bioaugmentation is held back by our limited understanding of the molecular mechanisms that drive colonisation of plant roots. NO is a widespread and universal biological effector, a likely candidate to facilitate signaling between a plant host and bacterial symbiont. There is an overwhelming body of evidence to support diverse roles for NO in plant immunity, but its role (if any) in mediating beneficial plant microbe interactions is less well studied. This study sets out to explore the role of NO in recruiting *S. coelicolor* to the wheat root microbiome

4.2 Aims

The aim of this study was to investigate the role of NO in wheat root colonisation in both compost and agricultural soil, by the model organism *S. coelicolor*. Initially, the NO donor compound DETA-NONOate ((Z)-1-[N-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate) was used to explore the effect of NO on development in axenic cultures. A reverse genetics approach was utilised to assess the rhizosphere and endosphere colonisation fitness of mutants lacking the NO detoxification genes *hmpA1/2*, as well as the Rrf2 family regulator NsrR. Colony morphology and bulk soil survival was used to determine whether these mutations had any deleterious effects.

This study additionally investigated heterologous expression of bNOS genes from two model streptomycetes, *S. scabei* (phytopathogenic) and *S. venezuelae* (non-pathogenic), under native and constitutive transcription levels in *S. coelicolor*. The function of the putative *S. venezuelae* bNOS, which shares 79% amino acid identity to *S. scabies* NOS, was investigated for the first time. Soil survival of the mutants was interrogated in addition to colony morphology and bioactivity.

4.3 Materials and Methods

4.3.1 Materials used in this study

Table 4-3 Strains used in this study.

Strain	Description	Plasmid	Resistance	Source/reference
E. coli				
Top10	F– *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G			Invitrogen™
ET12567	*dam*$^-$ *dcm*$^-$ *hsdS*$^-$	pUZ8002	Cml^R/Tet^R	(MacNeil et al., 1992)
***S. coelicolor* M145 derivative strains**				
M145	Wild-type			
M145 + pSS170	Wild-type	pSS170	Hyg^R	This work
ΔNHH	Δ*nsrR* Δ*hmpA1* Δ*hmpA2*		Apr^R Hyg^R	(Munnoch, 2016)
ΔH2	Δ*hmpA2*		Hyg^R	(Munnoch, 2016)
ΔNH	Δ*nsrR* Δ*hmpA1*			This work
ΔNH + pSS170	Δ*nsrR* Δ*hmpA2* + pSS170	pSS170	Hyg^R	This work
ΔNH partial complement	Δ*nsrR* Δ*hmpA1* + *ermEp*-hmpA1*	pIJ10257	Hygromycin	This work
Sc NOS1	ϕBT1 *ermEp*-txtD*	pSS170	Hygromycin	This work
Sc NOS2	ϕBT1 *txtDp-txtD*	pIJ10257	Hygromycin	This work
Sc NOS3	ϕBT1 *vnz_32420p-vnz_32420*	pSS170	Hygromycin	This work
Sc NOS4	ϕBT1 *ermEp*-vnz_32420*	pIJ10257	Hygromycin	This work

Table 4-4 Plasmids used in this study.

Plasmid	Description	Resistance	Reference
pSS170	*ori*, pUC18, *hyg*, *oriT*, RK2, int ΦBT1	HygR	
pIJ10257	*oriT*, ΦBT1 *attB-int*, Hygr, *ermEp**, pMS81 backbone	HygR	(Hong et al., 2005)
pCRISPomyces-2	AprR, oriT, reppSG5(ts), oriColE1, sSpcas9, synthetic guide RNA cassette	AprR	(Cobb et al., 2015)
pJN001	pCRISPomyces-2 *nsrR-hmpA* flanking DNA and gRNA	AprR	This work
pJN002	pSS170 *txtD* ptxtD	HygR	This work
pJN003	pIJ10257 *txtD*	HygR	This work
pJN004	pSS170 *vnz_32420 vnz_32420p*	HygR	This work
pJN005	pIJ10257 *vnz_32420*	HygR	This work
pJN006	pIJ10257 *hmpA*	HygR	This work

Table 4-5 Antibiotics and reagents used in this study.

Antibiotic/reagent	Selection concentration (μg/ml)
Apramycin	50
Chloramphenicol	30
Kanamycin	50
Hygromycin	50
Naladixic acid	25
Cyclohexamide	100
Nystatin	10
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGal)	80
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	119

Table 4-6 Media and buffers used in this study.

Medium	Ingredient	$g.L^{-1}$ dH_2O
SFM (Soy flour medium) agar	Soy flour	20.00
	Mannitol	20.00
	Agar	20.00
MYM (Maltose, Yeast extract, Malt extract) agar	Maltose	4.00
	Yeast extract	4.00
	Malt extract	10.00
	Agar	20.00
2xYT (2 x Yeast, Tryptone)	Bacto-tryptone	16.00
	Yeast extract	10.00
	NaCl	5.00
TSB (Tryptone soy broth)	Tryptone Soy Broth (Oxoid)	30.00
LB (Lysogeny Broth) + LB-NaCL (No salt)	Yeast extract	5.00
	NaCl (do not add for LB-NaCl)	10.00
	Tryptone	10.00
	Agar (for LB agar only)	15.00
PBS-S (Phosphate buffered saline + Silwet L-77)	$NaH_2PO_4.H_2O$	6.33
	$Na_2HPO_4.H_2O$	16.50
	200 µl Silwet L-77 added after autoclaving	

4.3.2 Cultivation of *Streptomyces coelicolor*

SFM was used as the standard cultivation media for all *Streptomyces coelicolor* strains. In order to stock strains, a single colony was picked from a plate grown for 7 days and homogenised in 150µl sterile dH_2O. The colony was then plated using sterile cotton buds soaked in dH_2O. Strains were grown for 7 days. Plates were then agitated with 10ml of sterile dH_2O using sterile cotton buds to remove the spores. The spore suspension was then pelleted in a centrifuge at 3500RCF and resuspended in 20% Glycerol (v/v).

4.3.3 Generating mutant strains

4.3.3.1 Genomic DNA preparation with phenol/chloroform extraction

10ml of *Streptomyces* spores were inoculated into 10ml TSB media and grown for 48h at 30°C, shaking at 220 rpm. Cells were isolated by centrifugation at 3,345 x g for 5 min. The supernatant was discarded, and the cells resuspended in 500µl Solution I (50 mM Tris/HCl, pH8; 10 mM EDTA) and transferred to a micro-centrifuge tube. Aliquots of 10µl of filter-sterilised lysosyme solution (30 mg/ml) and 5 µl DNase-free RNase (10 mg/ml) were added to the samples and incubated for 1h at 30°C followed by addition of 5µl of 20% (w/v) SDS, with samples mixed in by inversion. Approximately 500µl (one volume) of 1:1 phenol-chloroform was added, and samples were mixed thoroughly by vortexing for 1 min and then centrifuged at 21,130 RCF for 5 min. The upper aqueous phase (containing the DNA) was removed and transferred to a fresh microfuge tube. The phenol chloroform step was repeated until the upper phase was clear (i.e., protein free). The clear aqueous layer was transferred to a fresh microfuge tube. Then 1 ml of EtOH was added to the tube and it was mixed by inversion followed by centrifugation as above. EtOH was removed and the DNA pellet was washed in 70% (v/v) EtOH and centrifuged for a further 2 min at 21,130 RCF. The DNA pellet was dried at RT ensuring all EtOH was removed and the pellet was finally resuspended in 50µl sterile dH_2O and stored at 4°C.

4.3.3.2 Primers used in this study

Table 4-7 Primers used in this study.

Description	Primer	Sequence	Notes
Δ*nsrR/hmpA1* in *S. coelicolor* template generation	176 JN	gctcggttgccgccgggcgtttttaTCTAGACTCGACC GGCTCGGAGGCATG	F1
	186 JN	gcgagctcgcctggtcgcagcagcAGGCCATCCACTA CGAGGTCTTCGGC	F2
	188 JN	gctgctgcgaccaggcgagctcgcTCAACCGCACACC AGGACGCTATC	R1
	187 JN	gcaacgcggccttttttacggttcctggccTCTAGATCGTT CCGGGCGGCCCGG	R2
Δ*nsrR/hmpA1* in *S. coelicolor* guide RNA	204 JN	acgcAGGCGTGCTTGTGCGAGATA	Spacer 4
	205 JN	aaacTATCTCGCACAAGCACGCCT	
Δ*nsrR/hmpA1* in *S. coelicolor*	196 JN	GTCAAAACGCCCGCGCGCAG	IntSeq1
	197 JN	GTCCGTCGCCGGGCGCATTG	IntSeq2

Description	Primer	Sequence	Notes
sequencing and confirmation primers	198 JN	GATCTGAGCGTCCCCTTCTA	long forward test
	199 JN	CACCTGCACCGTCGTACTC	short forward test
	200 JN	GGCACCTTCACTGAACACAA	short reverse test
	201 JN	CGGCTACATCTTCCTCGACT	long reverse test
	207 JN	GAGCACAGCGACTCCTTAAT	nsrR internal reverse
hmpaA1 overexpression	219 JN	GATTACAcatatgCTCTCCGAACAGTCCGTTC	Overexpression F1
	220 JN	AGATGCCaagcttGTGTCCTGATGTCCGTCGT	Overexpression R1
*S. scabie*s NOS in PSS170	189 JN	GATTACAggtaccCTACAACGCCTCGAACCAG	Forward
	190 JN	AGATGCCaagcttGGGGTTGTAGACGCCTCAT	Reverse
S. scabies NOS in PIJ10257	191 JN	GATTACAcatatgCCCGCCCCGTCCCCGAC	Forward
	192 JN	AGATGCCaagcttGGGGTTGTAGACGCCTCATC ACTTCACT	Reverse
S. venezuelae NOS in PSS170	193 JN	GATTACAggtaccGCTGGCCTGGAACGACTA	Forward
	194 JN	AGATGCCaagcttTGGAGATCTTCGACACCTGG	Reverse
S. venezuelae NOS in PIJ10257	195 JN	GATTACAcatatgATCTTCAGCCTGAGCCGTCG	Forward
		Use 194 JN as reverse primer	Reverse

4.3.3.3 PCR components and conditions

PCR components and conditions were dependent on the application. BioMix™ red (Table 4-8) was used routinely for diagnostic purposes like confirming the size of a PCR product with DNA gel electrophoresis. Q5 high fidelity polymerase was used for generating a product that would be cloned. PCRs were conducted using either a Biorad® DNA engine PTC 300 or Techne Prime machine.

Table 4-8 Components and conditions used for BioMix™ red PCRs.

Component	Volume (ul) – 25µl reaction volume total
BioMix™ red (Bioline)	12.5
dH_2O	8.75
Forward primer, 27F – 5'-AGAGTTTGATCMTGGCTCAG-3	1.25
Reverse primer, 1492R - 5'-TACGGYTACCTTGTTAGGACTT-3'	1.25
DNA template	1.25
PCR thermocycler reaction steps	
95° for 2 minutes	
30 cycles of: - • 95° for 15s • 55° for 15s • 72° for 15s	
72° for 2 minutes	

Table 4-9 Components and conditions used for Q5 high fidelity PCRs.

Component	Volume (ul) – 50µl reaction volume total
5x Q5 reaction buffer	10
5x Q5 high GC enhancer	10
10mM dNTPs	1
Forward primer (10µM)	2.5
Reverse primer (10µM)	2.5
DMSO	2.5
Template DNA	2.5
Q5 DNA polymerase	0.5
dH_2O	18.5
PCR thermocycler reaction steps	
98° for 2 minutes	
30 cycles of: - • 98° for 10s	

Component	Volume (ul) – 50µl reaction volume total
• 55-70° for 1 minute • 72° for 2 minutes	
72° for 2 minutes	

4.3.3.4 Agarose gel electrophoresis

Gels for PCR products were made with 1% (w/v) agarose in TBE buffer (90 mM Tris HCl, 90 mM Boric Acid, 2 mM EDTA) with 2 µg/ml ethidium bromide, gels for running plasmids were made with 0.5% agarose instead. DNA samples and loading buffer (5x) (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene-cyanol blue, 40% (w/v) sucrose in water) were run alongside a 1 kb plus DNA ladder (Invitrogen) plus loading buffer for size determination. Electrophoresis occurred at 120V (Sub-Cell GT electrophoresis system, BIOLINE) for 40 minutes for PCR products and 60 minutes for plasmids. DNA was visualised by UV-light using a Molecular Imager Gel Doc System (BIO-RAD).

4.3.3.5 Plasmid DNA preparation

Plasmid DNA was prepared using Wizard® Plus SV Minipreps DNA Purification System from 5-10ml overnight cultures as per manufacturer's instructions. Plasmids were eluted from the column routinely using 50µl of autoclaved distilled water (dH_2O) unless otherwise stated.

4.3.3.6 Gel extraction

Gel fragments containing DNA bands of interest were excised using a scalpel and extracted using a QiaQuick Gel Extraction Kit (QIAGEN), according to the manufacturer's instructions. DNA was eluted in 30 µl autoclaved dH_2O.

4.3.3.7 Restriction digestion

Either Roche or NEB restriction enzymes were used to digest plasmid DNA and PCR fragments in 100 µl total volumes in accordance with manufacturer's guidelines. Digests were carried out with optimal buffer, which was outlined by Roche or NEB. Digestion of 4 µg of DNA was typically performed at 37°C for 1h by adding 4 units of the appropriate restriction enzyme. Restriction enzymes were then heat inactivated at 65°C for 10 minutes. For plasmid restriction digestions, 2 µl shrimp alkaline phosphatase was added and the reaction was incubated for 1h at 37°C, in order to dephosphorylate the digested plasmid DNA and prevent re-ligation. Digests were then analysed by gel electrophoresis; the desired bands were excised, and gel extracted according to the protocol described in 4.3.3.6.

4.3.3.8 Ligations

Ligation reactions were carried out using T4 DNA Ligase according to the manufacturers' instructions. Typically, plasmid and insert DNA were added to the reaction at a ratio of 1:3. Volumes were calculated using the University of Dusseldorf ligation calculator (http://www.insilico.uni-duesseldorf.de/Lig_Input.html)

4.3.3.9 Golden gate assembly

Golden gate reaction was used for cloning guide RNA spacers into the P-CRISPomyces vector (Table 4-4) as described in (Cobb *et al.*, 2014) Constructs were assembled in 20 µl reactions with 100 ng purified backbone and 0.3 µl insert in the presence of 2 µl T4 ligase buffer (NEB) and 1 µl T4 ligase (NEB) with 1 µl BbsI (NEB) and dH_2O. This was run in a thermocycler under the following conditions: -

10 cycles of:- 10 minutes at 37°C, 10 minutes at 16°C
5 minutes at 50°C
20 minutes at 65°C
4°C hold

Assembly was confirmed by blue/white screening using X-Gal. The spacer region was confirmed by Sanger sequencing (Eurofins).

4.3.3.10 Gibson Assembly

Multiple DNA fragments were assembled into digested vector backbones using designed overlaps of between 18 and 24 nucleotides. Gel extracted DNA fragments were incubated in a ratio of 1:3 of plasmid to insert in the presence of Gibson Assembly master mix (NEB) at 50°C for 1h.

4.3.3.11 Constructing mutants with site directed mutagenesis using PCR targetting: -

Mutants generated through site directed mutagenesis using PCR targetting (*S. coelicolor* Δ*nsrR-hmpA1-hmpA2* and Δ*hmpA2*) were generated by John Munnoch (Munnoch, 2016) according to the the protocol outlined by (Gust *et al.*, 2002).

4.3.3.12 Preparation and transformation of electrocompetent *E. coli*

Single colonies of *E. coli* ET12567/pUZ8002 or Top10 were grown as 10 ml overnight cultures in LB broth containing appropriate antibiotics (Table 4-5) and sub-cultured to an OD_{600} of

approximately 0.4. Cultures were pelleted by centrifugation at 3500 RCF for 5 minutes at 4°C and resuspended in 10 ml ice-cold 10% (v/v) glycerol. Pellets were washed in ice-cold 10% (v/v) glycerol twice before being either flash frozen in liquid nitrogen and stored at -80°C or being used for transformation immediately. For transformation, 20-100µg of DNA was added to 50 µl of cells and the mixture transferred to an ice-cold electroporation cuvette. Electroporation was carried out using the BioRad Electroporator set to 200Ω, 25µF and 2.5kV. The electroporated cells were diluted in LB and transferred to a micro-centrifuge tube for recovery at 37°C for 1h with 220 rpm shaking before plating on selective media for overnight incubation and colony selection.

4.3.3.13 Colony PCR in *E. coli*

After overnight incubation, single colonies were picked with a sterile pipette tip and transferred to the base of a 0.2ml PCR Tube containing 25µl BioMix™ red reaction (Table 4-8). The tip was then discarded into an Eppendorf containing 500µl sterile LB broth, which was used as an inoculum following confirmation. BioMix™ red PCR reactions were analysed by gel electrophoresis. Positive colonies were grown in 10ml LB supplemented with required antibiotics overnight.

4.3.3.14 Conjugation

Single colonies of non-methylating *E. coli* ET12567/pUZ8002 containing the required plasmid were selected from plates and grown in 10ml LB broth plus antibiotics at 37°C overnight at 220 rpm. Subcultures of OD_{600} between 0.4 and 0.6 were washed twice in ice cold LB to remove antibiotics. Between 10 and 200 µl of *Streptomyces* spores were heat shocked at 50°C for 10 minutes in 500µl 2YT to encourage germination and added to the washed *E. coli* cells. Conjugation with integrative vectors typically required less spores, while conjugation with p-CRISPomyces for gene deletion required more spores. The cell mixture was pelleted by centrifugation at 20 for 1 minute, the supernatant was removed, and cells were resuspended in the residual liquid. This was plated on SFM + 10 mM $MgCl_2$ at various dilutions and incubated at 30°C for 16-20h. For selection of desired ex-conjugants, 0.5 mg Nalidixic acid and an appropriate volume of selection antibiotic (to equal final concentrations described in Table 4-5) was added in 1 ml dH_2O to each plate and cultures returned to the 30°C incubator until colonies appeared.

4.3.3.15 Generation of CRISPR/Cas9 mutants: -

Clean deletions were made using the pCRISPomyces-2 system as described previously (Cobb et al., 2015). Approximately 20 base pair protospacers for use in the synthetic guide RNA (sgRNA) were designed so that the last 15 nucleotides, including the adjacent NGG sequence, were unique in the genome to minimise off target effects. The forward and reverse sequences were ordered as oligos

from IDT and annealed by heating to 95°C for 5 minutes followed by ramping to 4°C at 0.1°C/second. Annealed protospacers were then assembled into the BbsI site of the pCRISPomyces-2 vector using golden gate assembly (4.3.3.9). This vector was then digested with XbaI (4.3.3.7) and a 2kb PCR-amplified homology repair template (1 kb from either side of the target region) was assembled into the vector using Gibson assembly (4.3.3.10). The final vector was transformed and cloned in *E. coli* (4.3.3.12), isolated, confirmed by PCR (4.3.3.3) and sequencing, and transformed into the desired *Streptomyces* strain by conjugation (4.3.3.14) via the non-methylating *E. coli* strain ET12567/pUZ8002 (Table 4-3). Once the required deletion event had taken place, loss of the temperature-sensitive pCRISPomyces-2 plasmid was encouraged by plating mutants on media lacking antibiotic selection at 37°C for multiple generations.

To confirm the desired deletion event had occurred within the genomic DNA, PCR products were generated that span across both arms of the homology repair template and genomic DNA. Primers for this confirmation PCR were designed so that mutants could be confirmed with the plasmid still present in the strain.

4.3.4 Experimental methods

4.3.4.1 Colony microscopy

Between five and ten CFUs were plated on a petri dish containing approximately 33ml SFM. Plates were incubated at 30°C for 3 days. Brightfield images were acquired using a Zeiss M2 Bio Quad SV11 stereomicroscope equipped with an AxioCam HRc CCD camera. Objective lens magnification was equal to 0.63, lens magnification at 0.63 and mount magnification 5.0.

4.3.4.2 Wheat seed inoculation with *S. coelicolor* strains

Wheat seeds were sterilized with 70% (v/v) EtOH for 30 seconds followed by 3% (v/v) bleach for 10 minutes. Seeds were washed in sterile distilled H_2O 10 times. 10^7 $CFU.ml^{-1}$ of *Streptomyces* spores in 2xYT broth (Table 4-6) was incubated for 10 minutes at 50°C. This heat shock phase was to encourage germination. Seeds were added to the *Streptomyces* spore suspension and tubes were inverted approximately 30 times per second for 90 minutes. Seeds were removed from the spore suspension and left to dry at room temperature under sterile conditions. Seeds were sown in 90mm pots containing either Levington's F2 compost or agricultural soil which had been watered with 50-100ml sterile dH_2O. Seeds were placed sideways so that they were flush with the soil and an additional 2ml of heat shocked 2xYT spore suspension ($1*10^7 cfu.ml^{-1}$) was added directly to the seed and surrounding soil.

4.3.4.3 Reisolation of *Streptomyces* strains from wheat roots

Wheat plants were removed from their pots and the bulk soil was removed by shaking gently. The roots were tapped gently with sterile tweezers to remove loosely attached soil from the roots. The rhizosphere was isolated by separating the stem of the plant aseptically and vortexing the root in PBS-S (See Table 4-6) for 30 seconds. The root was transferred to a separate tube and the rhizosphere/rhizoplane soil was pelleted by centrifugation at 3500RCF for 10 minutes. Following the removal of tightly adhering soil particles with tweezers, the root was washed and vortexed a further 2 times in PBS-S. Roots were washed with 70% (v/v) EtOH for 1 minute then 3% (v/v) bleach for 10 minutes. Sterile dH_2O was used to wash the roots 10 times before transferring to a sterile mortar and pestle. Roots were then homogenised with 4ml of 10% (v/v) glycerol to generate the endosphere (E) sample. Serial dilutions were generated for rhizosphere and endosphere samples, which were then plated on SFM amended with 50µg/ml hygromycin (to select for modified *S. coelicolor*) in addition to nystatin (5µg/ml) and cycloheximide (100µg/ml) to reduce fungal growth. Reisolation from plants grown in agricultural soil required additional amendment with nalidixic acid (25µg/ml). Samples were dried at 75°C overnight in an oven, so that colony counts could be standardized by the dry weight of the sample. Plates were incubated at 30°C for 3 days. Colonies were subsequently counted and recorded.

4.3.4.4 Soil survival assay

1g of Levington's F2 compost or 2g agricultural soil was added to each well of a 6-well plate. $5*10^5$ cfu of *Streptomyces* spores were added in sterile dH_2O to each well. Plates were left to incubate at room temperature on the bench for 7d. At the end of this period, the soil from each well was homogenised and the wet mass was measured. Soil was diluted and plated according to 4.3.4.3. Plates were incubated at 30°C for 3 days and colonies were counted.

4.3.4.5 Statistical analysis

For ecological fitness assays (4.3.4.3, 4.3.4.4) an analysis of variance (ANOVA) model was generated, using the package MASS in R 3.2.3 (R Core Team, 2019), to model the effect of strain treatment, experimental repeat and their interaction term (strain*experiment) on log-transformed $cfu.gram^{-1}$ recovered from samples.

4.4 Results

4.4.1 The effects of exogenous NO on colony morphology and development

Previous work has shown that exogenous NO can alter the development of *S. coelicolor* (Munnoch, 2016). Introduction of a NO donor (DETA-NONOate) to growth media led to a delay in sporulation. This experiment was repeated here using DETA-NONOate concentrations of 0µM and 400µM at 7 days. The experiment corroborated the results presented by (Munnoch, 2016); sporulation was inhibited on media containing deta-NONOates. This was apparent due to the white coloration of colonies, characteristic of aerial hyphae. Colonies grown on control plates contrasted this, grey pigmentation was observed which was characteristic of sporulation. This demonstrates that NO can alter the physiology of *S. coelicolor*. Due to the technical challenges of quantifying NO in biological systems, it is not entirely clear what the physiologically relevant concentrations of NO might be, but it is generally though that nitrosative stress occurs in the µM range (Thomas *et al.*, 2008).

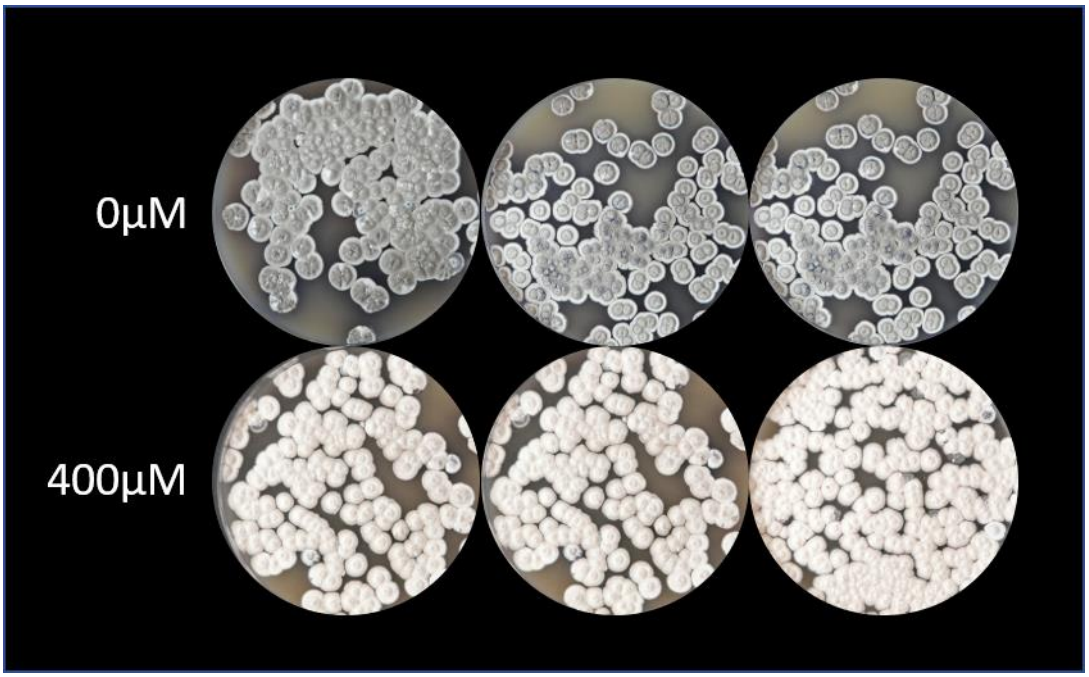

Figure 4-1 Colony morphology microscopy of *S. coelicolor* M145 growing on SFM supplemented with 0µM or 400µM deta-NONOate. Images were obtained after seven days growth. Spores were diluted to plate approximately 100 colonies per plate on N=3 plates. Plates with 0µM deta-NONOate show grey colonies which indicate sporulation. Plates with 400µM show white colonies which indicates development was halted at the aerial sporulation stage.

4.4.2 Investigating *S. coelicolor* Δ*nsrR* Δ*hmpA1/2* phenotypes

The hypothesis to be tested is that NO is involved in wheat root colonisation by *S. coelicolor*, although no predictions could be made as to whether it may improve or impair it. Root colonisation assays were used to test this hypothesis. In order to minimise confounded observations from root colonisation assays, strains were first morphologically characterised to screen for fitness defects.

The first mutant analysed was *S. coelicolor* Δ*nsrR-hmpA1* Δ*hmpA2*. This mutant is missing the genes that encode NsrR, as well as HmpA1 and HmpA2. NsrR detects NO, while HmpA1 oxidises NO to NO_3. HmpA2 is a homologue of HmpA1. The mutant is unable to detect and detoxify NO using these proteins and its increased sensitivity could be used to interrogate the role of NO in root colonisation. There were some limitations to working with this strain imposed by the gene deletion method, however. Site directed mutagenesis using PCR targeting had been performed at multiple sites, since *nsrR-hmpA1* and *hmpA2* sit at different regions of the genome. This meant that both available antibiotic resistance markers (apramycin and hygromycin) were used in the generation of the mutant and further genetic modification was prevented. Using an isogenic control for plant root colonisation assays is important, to ensure that any observed effects were not due to variation in the genetic tools that were used, so the *S. coelicolor* Δ*hmpA2* single mutant was chosen for this purpose. While it would be interesting to look at individual and double deletion mutant combinations of *nsrR*, *hmpA1*, *hmpA2*, throughput limitations of root colonisation assays meant that only the triple mutant and Δ*hmpA2* control mutants were tested for this group of mutants.

Initial characterisation of the mutant corroborated the findings of (Munnoch, 2016). No morphological differences were observed between the wild-type strain or the isogenic Δ*nsrR-hmpA1* Δ*hmpA2* and Δ*hmpA2* mutants, when grown for seven days on SFM agar. No data is presented here, although (Munnoch, 2016) also demonstrated that no morphological difference could be observed between wild-type *S. coelicolor* and these mutants when grown on SFM agar in the presence of NO donor deta-NONOate, at either 100µM or 400µM.

4.4.3 Investigating the fitness of *S. coelicolor* Δ*nsrR-hmpA1 ΔhmpA2* mutants to colonise the wheat root microbiome in Levington F2 compost

Rhizosphere and endosphere colonisation fitness were assessed for *S. coelicolor* M145 Δ*nsrR-hmpA1 ΔhmpA2* and the Δ*hmpA2* strains which are both resistant to hygromycin. Seeds were inoculated with the same quantity of spores, so that treatments could be compared. Fitness was

determined by normalising the number of colonies recovered on antibiotic selective media against dry sample weight. No colonies were recovered from the uninoculated control group.

The results showed that there was no significant difference in the endosphere colonisation fitness (measured in cfu/gram of root) between the strains across three experiments (Figure 4-2); strain treatment was not a significant predictor of log-transformed CFU g^{-1} (F=0.158, p=0.695) in an ANOVA test.

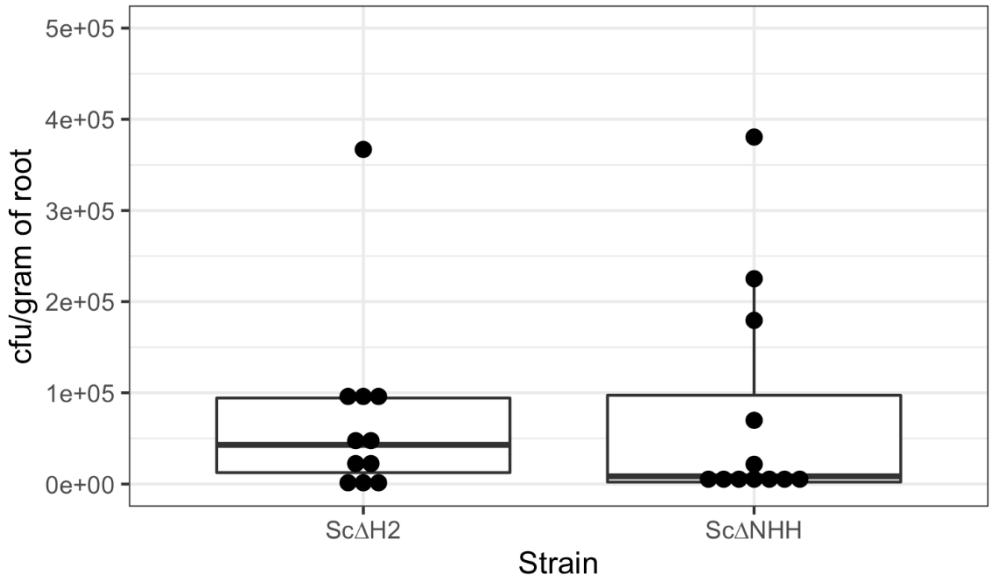

Figure 4-2 Wheat endosphere colonisation fitness of control strain *S. coelicolor* Δ*hmpA2* (ScΔH2) and mutant strain *S. coelicolor* Δ*nsrR-hmpA1* Δ*hmpA2* (ScΔNHH). There was no significant difference in the log-transformed cfu.g^{-1} between the 2 strains (F=0.158, p=0.695) in an ANOVA test. Results are presented for N=3 experiments.

The mutant strain (*S. coelicolor* Δ*nsrR-hmpA1 ΔhmpA2*) was significantly better at colonising the rhizosphere (measured in cfu/gram of rhizosphere dry mass) than the isogenic control (*S. coelicolor* Δ*hmpA2*), across three experiments; strain genotype was shown to be a significant predictor of log-transformed CFU.g^{-1} (ANOVA F=9.444, P=0.00626) which was neither affected by experiment (F=0.180 P=0.67639) nor the strain/experiment interaction term (F=0.910, P=0.35213).

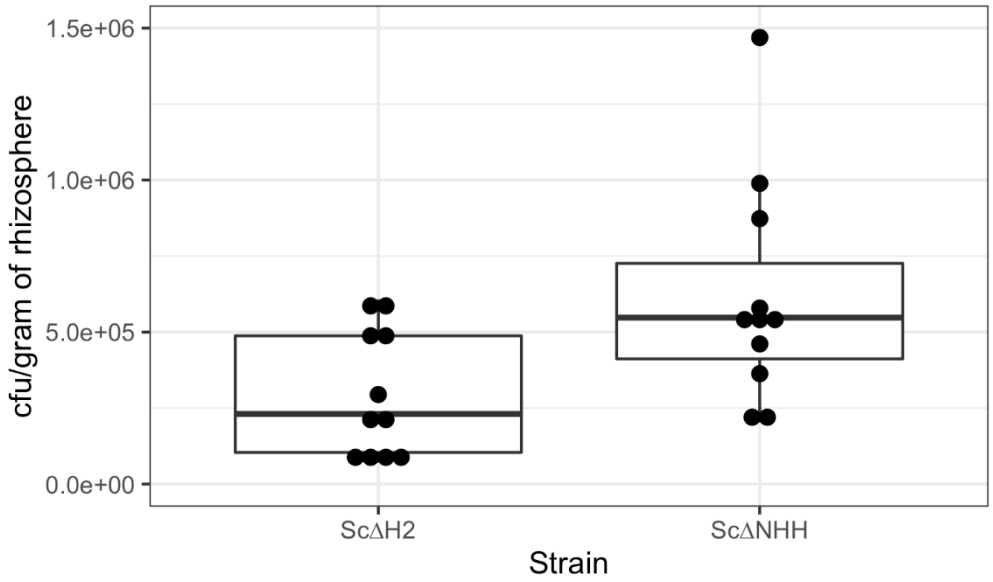

Figure 4-3 Wheat rhizosphere colonisation fitness of isogenic control strain *S. coelicolor* Δ*hmpA2* (ScΔH2) and *S. coelicolor* Δ*nsrR-hmpA1* Δ*hmpA2* (ScΔNHH). Log-transformed cfu.g^{-1} was significantly higher for the mutant strain that the isogenic control (ANOVA F=9.444, P=0.00626). Results are presented for N=3 experiments.

4.4.4 Investigating the survival of *S. coelicolor* mutants in Levington's F2 compost

In order to determine whether increased rhizosphere colonisation fitness was a plant dependent effect, microcosms were used to assess strain fitness in compost in the absence of a plant host. No significant difference was observed; strain genotype was not found to be a predictor of log-transformed cfu.g^{-1} recovered from compost (F=1.302 P=0.2643) in an ANOVA test. Log-transformed cfu.g^{-1} did however vary significantly between experiments (F=4.255, P=0.0492), which suggested that survival may be sensitive to uncontrolled environmental variables in these assays. Despite this, the strain/experiment interaction term was not significant (F=1.268, P=0.2704), which suggested the findings were robust.

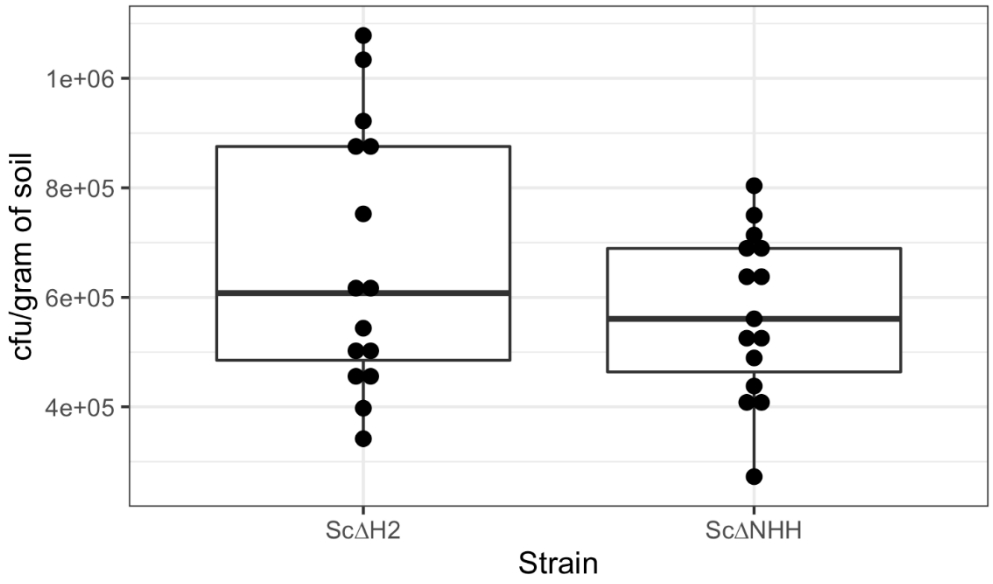

Figure 4-4 Fitness of isogenic control strain *S. coelicolor* Δ*hmpA2* (ScΔH2) and mutant strain Δ*nsrR-hmpA1-2* (ScΔNHH) in Levington F2 compost microcosms. Log-transformed cfu.g-1 was not significantly different between strains in an ANOVA test (F=1.302 P=0.2643). Results are presented for N=3 experiments.

4.4.5 Investigating the fitness of a CRISPR generated *S. coelicolor* Δ*nsrR-hmpA1* mutant to colonise the wheat rhizosphere in compost

The fact that the *S. coelicolor* Δ*nsrR-hmpA1 ΔhmpA2* mutant showed greater rhizosphere colonisation fitness supports the hypothesis that NO plays a role in rhizosphere colonisation by *Streptomyces* species. These data further suggest that NO promotes colonisation of the wheat rhizosphere by *S. coelicolor* but not the endosphere. The isogenic control strain was limited in this experiment as it was also missing *hmpA2*. In order to investigate the role of *nsrR-hmpA1*, without this as a confounding factor, an unmarked, in-frame deletion mutant was generated using CRISPR/Cas9. This way, both the mutant and the wild-type control could be transformed with a hygromycin resistance marker in the same way, through the introduction of an integrative vector. An additional benefit is that the gene deletions can be complemented. A partial complement strain was generated through introduction of *hmpA* under the constitutive promoter *ermEp**. Mutants were generated in biological triplicate and colony morphology was analyzed (Figure 4-5) to see if there were any obvious fitness defects, alongside the wild-type and complemented strains.

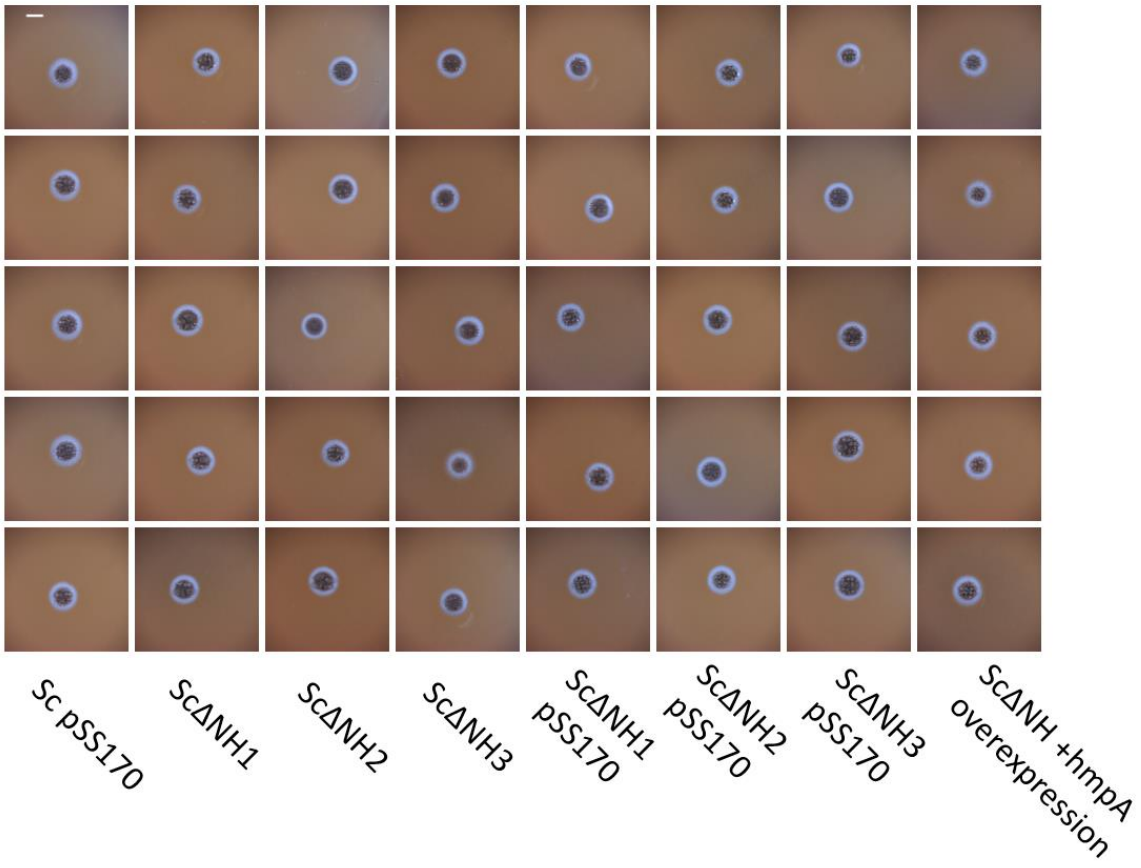

Figure 4-5 Colony morphology (N=5) for *S. coelicolor* M145 wild-type + pSS170 (Sc pSS170) and the Δ*nsrR-hmpA1* (mutant) strain generated in biological triplicate (ScΔNH1-3). Deletion mutants were transformed by conjugation with hygromycin resistant vector pSS170 (ScΔNH1-3 pSS170). ScΔNH1 was additionally transformed with pSS170 containing *hmpA1* under the constitutive promoter *ermEp** (ScΔNH +hmpA overexpression), to partially complement the deletion mutation. Scale bar = 2mm (top left).

Having confirmed that there were no obvious fitness defects which might suggest off-target mutations, wheat rhizosphere colonisation assays were performed using *S. coelicolor* Δ*nsrR-hmpA* + pSS170, to see if the same phenotype could be observed as for *S. coelicolor* Δ*nsrR-hmpA1 ΔhmpA2*. In addition to the wild-type control (*S. coelicolor* + pSS170), the partially complemented mutant (*S. coelicolor* Δ*nsrR-hmpA* + *hmpA* overexpression) was also challenged. Endosphere compartment fitness was not tested.

The results showed that *S. coelicolor* Δ*nsrR-hmpA1* + pSS170 colonised the wheat rhizosphere better than the wild-type control (Figure 4-6), while the partially complemented strain recovered the wild-type phenotype. An ANOVA test showed that strain was a significant predictor of log-transformed cfu.g^{-1} ($F=17.712$, $P= 6.95*10^{-7}$). Tukey's honestly significant difference (HSD) test was performed post-

hoc and confirmed that while colonisation by the wild type and the partially complemented strain was significantly lower than the mutant (adjusted P=1.27*10^{-5} and 3.14*10^{-4} respectively), they were not different from each other (adjusted P=0.6518).

With this we can conclude two things: (1) the *ΔnsrR-hmpA1* mutant colonises the wheat rhizosphere better than the wild-type control despite the presence of *hmpA2* and (2) reintroduction of *hmpA1* under the control of the high level, constitutive promoter *ermEp** reduces root colonisation back to wild-type levels. Thus, deleting *hmpA1* increases rhizosphere colonisation by *S. coelicolor* M145. The ANOVA test showed that experiment was a predictor of log-transformed cfu.g^{-1} (F=15.746, P=1.81*10^{-4}), but the strain/experiment interaction term was not significant (F=2.152, P=0.1244). This reaffirms the sensitivity of these assays to uncontrolled environmental conditions but supports a consistent predictor effect for strain type.

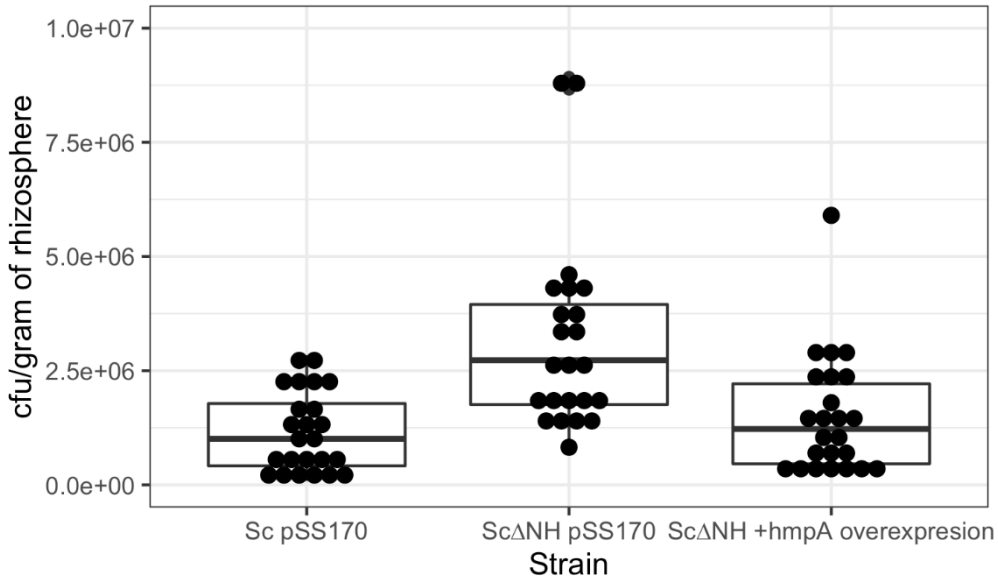

Figure 4-6 wheat rhizosphere colonisation fitness in compost for *S. coelicolor* pSS170, ΔnsrR-hmpA1 pSS170 mutant and partially complemented mutant with constitutive expression of hmpA1. An ANOVA test showed that experiment was a significant predictor of log-transformed cfu.g^{-1} (F=15.746, P=1.81*10^{-4}). Results are presented for N=3 experiments.

The mutant strain additionally showed no difference in its ability to survive in compost microcosms (Figure 4-7); strain was not a predictor of log-transformed cfu.g^{-1} (ANOVA, F=0.488, P=0.495). This demonstrated that deleting *nsrR-hmpA1* does not affect *S. coelicolor* survival in compost.

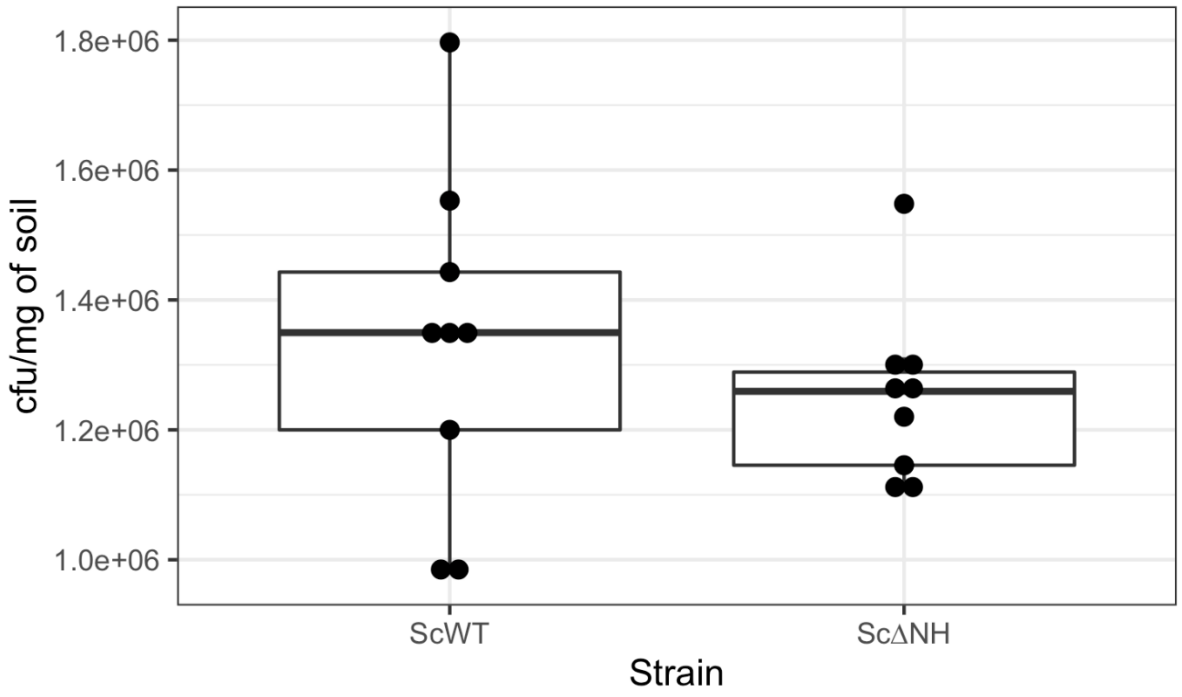

Figure 4-7 Compost fitness assay in compost for *S. coelicolor* pSS170 (ScWT) and *S. coelicolor* Δ*nsrR-hmpA1* + pSS170 mutant (ScΔNH). strain was not a predictor of log-transformed cfu.g^{-1} (ANOVA, F=0.488, P=0.495). Results are presented for N=1 experiment.

4.4.6 Investigating the ability of the *S. coelicolor* Δ*nsrR-hmpA1* mutant to colonise the wheat rhizosphere in agricultural soil

To see whether the mutant phenotype is soil type dependent, the experiment was repeated using agricultural soil collect from the John Innes Centre (JIC) field site at Church Farm in Bawburgh, Norfolk. Interestingly, there was no significant difference in rhizosphere colonisation between the wild-type and *ΔnsrR-hmpA1* strains under these conditions; strain was not shown to be a predictor of log-transformed cfu.g^{-1} (F=0.328, P=0.7233) in an ANOVA test (Figure 4-8). Experiment was a significant factor (F=10.371, P=3.66*10^{-3}) while the strain/experiment interaction term was not (F=2.678, P=0.1148). For any given experiment, germination rate was found to be lower (data not presented) in agricultural soil than for compost. This limited recoverable sample size for some treatments, and larger scale experiments may be required.

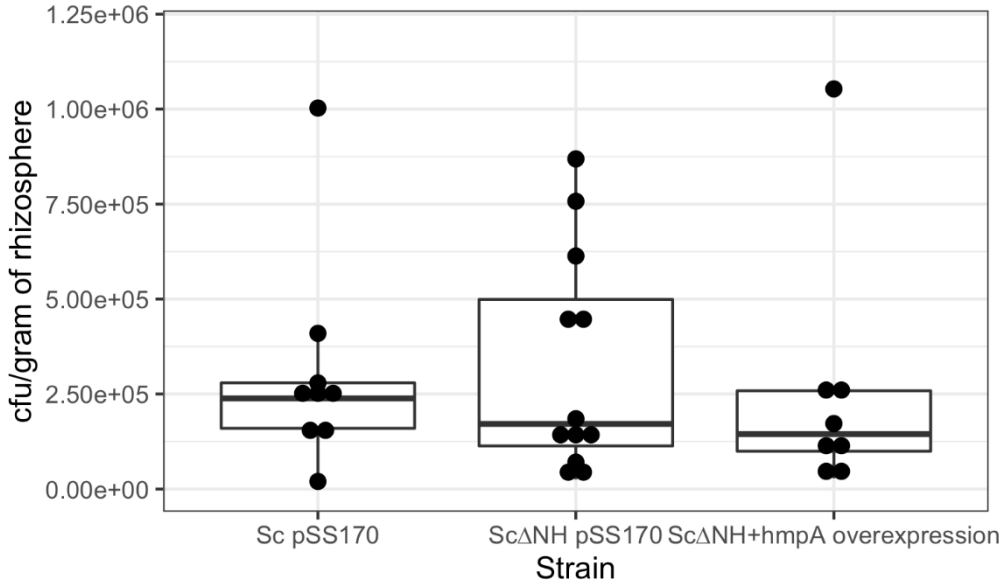

Figure 4-8 Wheat rhizosphere colonisation in agricultural soil for *S. coelicolor* M145 + pSS170 (Sc pSS170), Δ*nsrR-hmpA1* + pSS170 (ScΔNH pSS170) and Δ*nsrR-hmpA1* + pSS170-*ermEp*hmpA1* (ScΔNH+hmpA overexpression). strain was not shown to be a predictor of log-transformed cfu.g^{-1} (F=0.328, P=0.7233) in an ANOVA test.

4.4.7 Heterologous expression of *Streptomyces* NO synthases in *S. coelicolor*

Deletion of *nsrR-hmpA1* was shown to promote colonisation in Levington's F2 compost, suggesting a positive role for NO in wheat rhizosphere association, since a mutant lacking HmpA1 is predicted to be less able to detoxify NO. To probe this connection further, NO synthases from *Streptomyces scabei* (bNOS$_{ssc}$) and *Streptomyces venezuelae* (bNOS$_{sven}$) were cloned into an integrative vector (pSS170) downstream of the constitutive promoter *ermEp**. These vectors were introduced by conjugation into *S. coelicolor* M145. No observable difference between the colony morphologies of the wild-type strain and bNOS-containing strains after seven day's growth on SFM, which suggests that there is no growth defect under lab conditions.

4.4.8 Soil survival assays with *S. coelicolor* NOS mutants

Although NO levels could not be determined for bNOS producing strains, microcosm experiments were performed to investigate their soil survival (Figure 4-9). Both mutant strains demonstrated poor survival in agricultural soil, compared to the wild-type control; strain was a significant predictor of log-transformed cfu.g^{-1} in an ANOVA test (F= 15.43, P=2.29*10^{-4}). Tukey's HSD test found that both *S. coelicolor* with bNOS$_{ssc}$ and *S. coelicolor* with bNOS$_{sve}$ showed significantly lower log-transformed cfu.g^{-1} than the control strain (Padj.=1.61*10^{-4}, Padj.=1.48*10^{-2} respectively).

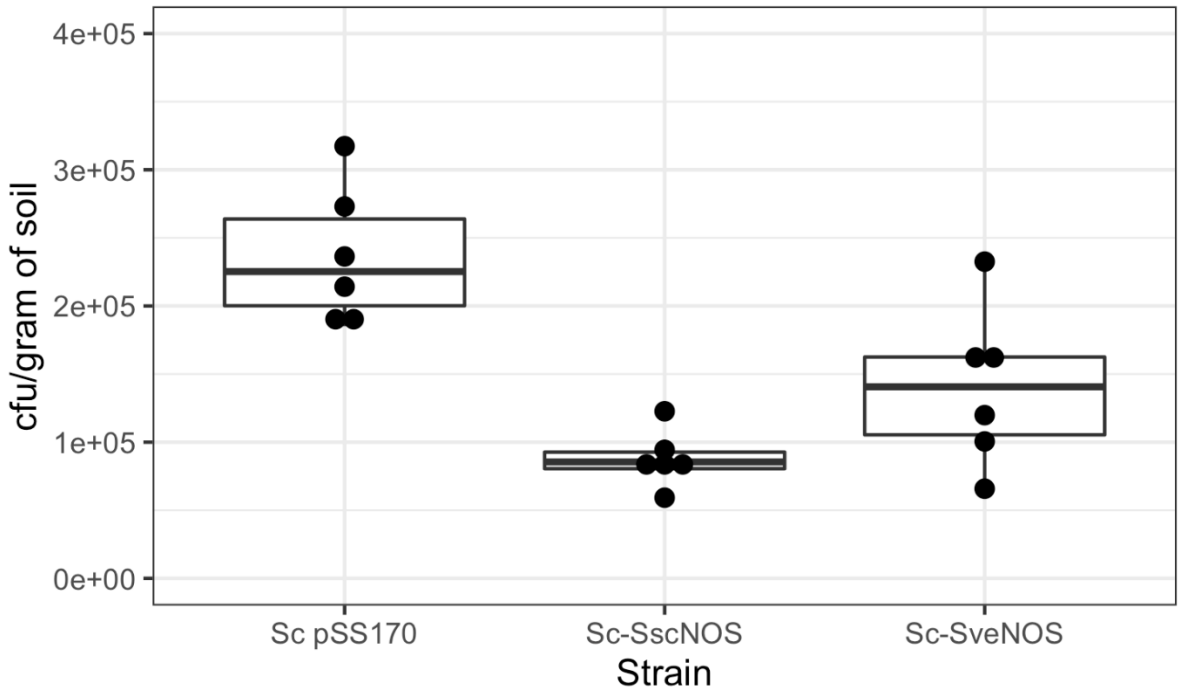

Figure 4-9. Wheat rhizosphere colonisation in agricultural soil for *S. coelicolor* M145 + pSS170 (Sc pSS170), *S. coelicolor* + *ermEp**-bNOS$_{Ssc}$ (Sc-SscNOS) and *S. coelicolor* + *ermEp**-bNOS$_{Sve}$ (Sc-SveNOS). Strain was found to be a significant predictor of log-transformed cfu.g^{-1} (F= 15.43, P=2.29*10^{-4}) in an ANOVA test. Results are shown for N=1 experiment.

The experiment was repeated with compost mesocosms, although results varied significantly over two different experiments (Figure 4-10). While strain was a significant predictor of log-transformed cfu.g^{-1} (F=5.612, P=7.56*10^{-3}), experiment was also a factor (F=137.006, P=7.89*10^{-14}), as was the strain/experiment interaction term (F=8.34, P=1.06*10^{-3}).

In the first experiment, *S. coelicolor* with bNOS$_{Sve}$ colonised worse than the control (Tukey's HSD, Padj.=1.63*10^{-2}), while *S. coelicolor* with bNOS$_{Ssc}$ did not demonstrate a significant fitness defect compared to the control strain (Padj.=0.825). In the second experiment, *S. coelicolor* with bNOS$_{Ssc}$ colonised better than the control (Tukey's HSD, Padj.=3.10*10^{-2}) while *S. coelicolor* with bNOS$_{Sve}$ showed no difference (Padj.=0.9997).

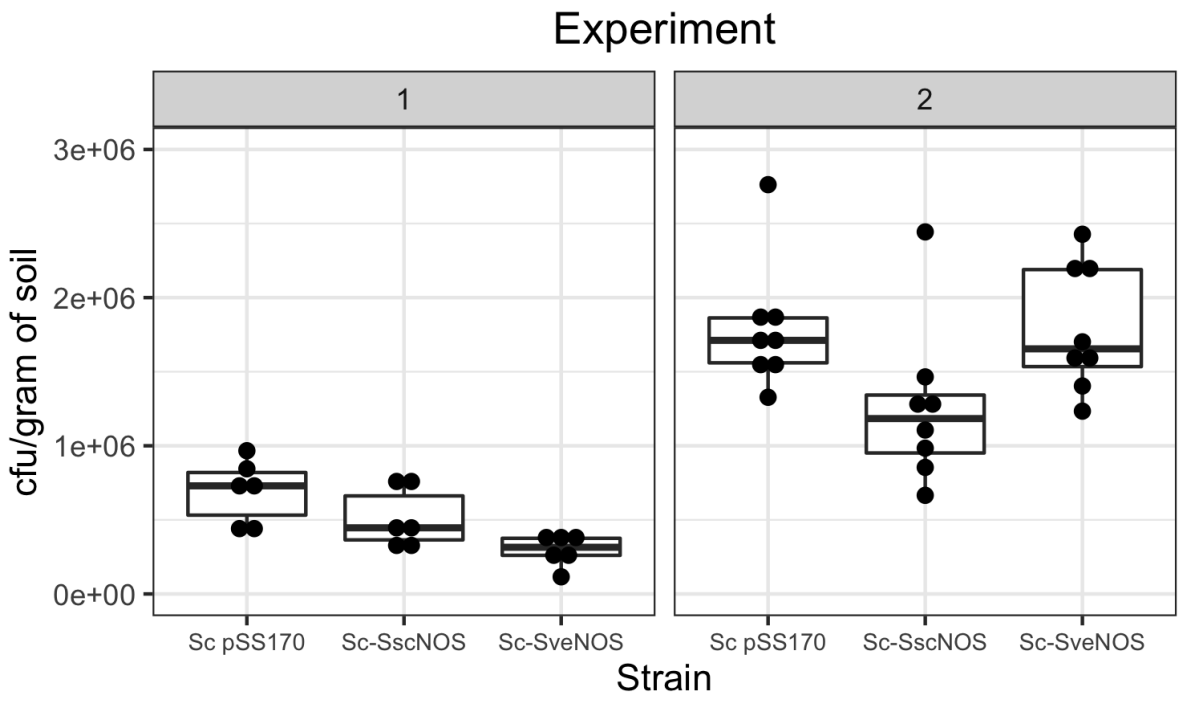

Figure 4-10 Survival in compost represented by cfu.g^{-1} recovered for *S. coelicolor* M145 + pSS170 (Sc pSS170), *S. coelicolor* + *ermEp**-bNOS$_{Ssc}$ (Sc-SscNOS) and *S. coelicolor* + *ermEp**-bNOS$_{Sven}$ (Sc-SveNOS). Strain was found to be a significant predictor of log-transformed cfu.g^{-1} ($F=5.612$, $P=7.56*10^{-3}$) in an ANOVA test. Results are shown for N=2 experiments.

4.4.9 Antibiotic activity of *S. coelicolor* bNOS strains

Bioactivity was tested for the *S. coelicolor* Δ*nsrR-hmpA1* Δ*hmpA2*, Δ*hmpA2* and Δ*nsrR-hmpA1* mutants carrying pSS170 and their isogenic parent strain. *S. coelicolor* bNOS mutants were also tested. Each strain was challenged with methicillin-resistant *Staphylococcus aureus* (MRSA), *Klebsiella aerogenes* (KA) and *Candida albicans* (CA) on separate plates. Bioactivities were recorded for all strains on both SFM and MYM with N=2 replicate plates per condition. *Streptomyces* strains demonstrated bioactivity against MRSA when grown on MYM. Zones of clearing were similar for each *Streptomyces* strain, with zones of clearing nearing the lip of the plate. No other bioactivities were observed.

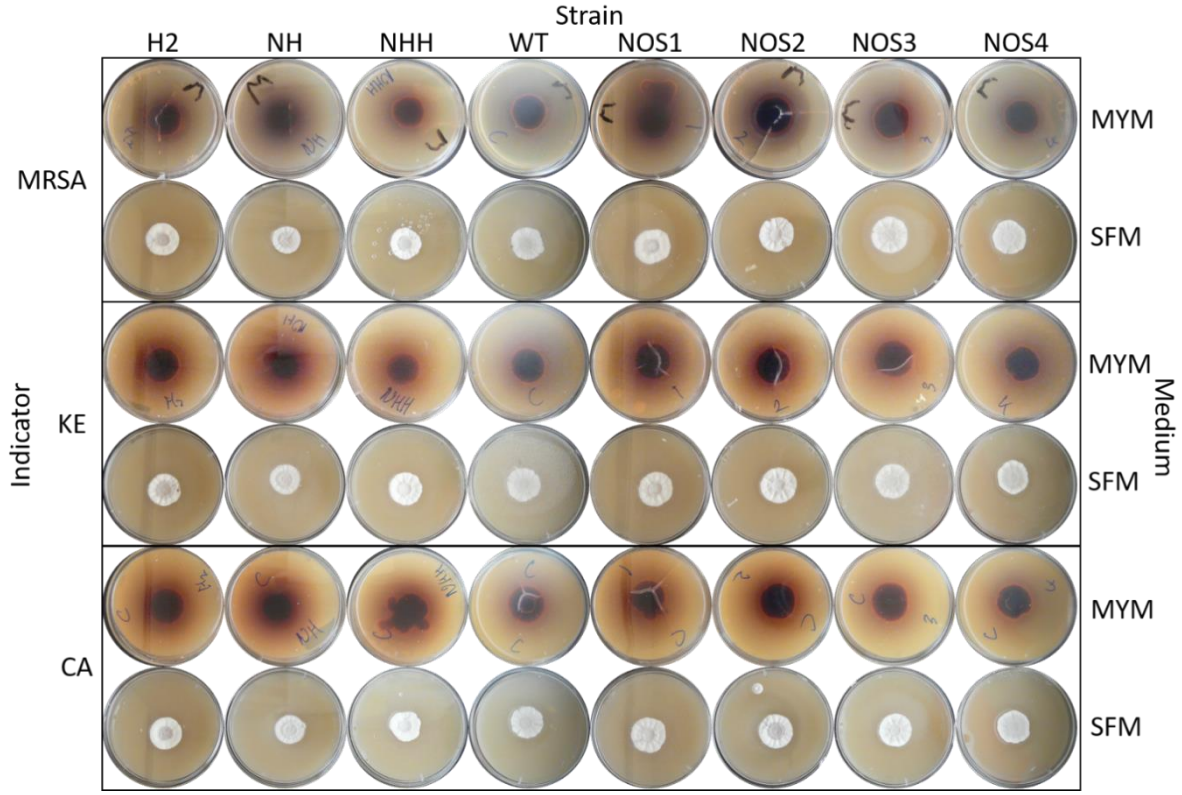

Figure 4-11 Bioactivity of *S. coelicolor* strains Δ*hmpA2* + pSS170 (H2), Δ*nsrR/hmpA* + pSS170 (NH), Δ*nsrR-hmpA1* Δ*hmpA2* (NHH), pSS170 (WT), $bNOS_{Ssc}$ under native expression (NOS1) and *ermEp** (NOS2), $bNOS_{Sve}$ under native expression (NOS3) and *ermEp** (NOS4). Bioactivity was tested against methicillin resistant *S. aureus* (MRSA), *K. aerogenes* (KE) and *C. albicans* (CA) on either MYM or SFM agar.

4.5 Discussion

While the benefits of *Streptomyces* spp. association with plant roots are well established, the molecular mechanisms of colonisation are poorly understood. This study set out to investigate the role of NO, in wheat root colonisation by model organism *S. coelicolor* M145. The aims of the study were met, increased rhizosphere fitness was observed with the deletion of known NO sensing and detoxification genes *nsrR-hmpA*. Soil survival was unchanged, which suggested a role for plant derived NO in the recruitment of beneficial streptomycetes. Increased rhizosphere fitness was dependent on soil type, however; the phenotype was only observed in Levington F2 compost which is both richer in nitrogen and organic material as well as lower in pH (Table 4-10). Nevertheless, this is the first time that NO has been implicated in the association of *Streptomyces* with wheat roots.

Table 4-10 Chemical composition of agricultural soil from JIC field site and Levington's F2 compost. NO_3 and NH_4 was calculated by Sam Prudence (Prudence *et al.* in progress). All other data was generated by the James Hutton Ltd. soil analysis service.

	Agricultural soil	**Compost**	**Fold change**
NO_3 (g/kg)	0.05	4.3	86
NH_4 (mg/kg)	3.47	96.09	28
P (mg/kg)	81.63	880.50	11
K (mg/kg)	103.80	2508.00	24
Mg (Mg/kg)	34.80	6021.00	173
Organic matter (%)	2.26	91.08	40
pH	7.97	4.98	

A review of the literature has made it clear that the roles for NO in plant microbe interactions generally, are very complex and not well understood. NO is produced by the plant host in response to pathogenic microbes, but this response can be exploited by necrotrophic pathogens, in order to trigger cell death (Govrin and Levine, 2000). While most literature implicates NO as a mediator of negative plant/microbe interactions, there is some evidence to suggest that it promotes the establishment of beneficial root associated fungi, as detailed in Table 4-1. None of these studies have investigated the role of NO in beneficial root/microbe interactions in wheat however, nor do they implicate NO as an interaction mediator with prokaryotes like *Streptomyces* spp. Nevertheless, there are some concepts that are shared with this study, which will be discussed.

While two studies took a reverse genetics approach by using modified plant lines in their experiments (Calcagno *et al.*, 2012; Gupta *et al.*, 2014), none of the studies probed NO metabolism in microbial partners. This study utilised tractable model organism *S. coelicolor* M145. This approach may be less viable for studies cited inTable 4-1, due to the increased complexity of working with eukaryotic microorganisms.

Many of the studies cited show how the plant host responds to colonisation by the microbial partner, using NO sensitive fluorescent probes such as DAF-2DA. This approach is useful, studies have

shown how NO mediates the defense response to invasion by pathogenic microorganisms, but they also show that beneficial microbes can trigger NO production. While the data in this Chapter does not explore NO level using fluorescent probes in this way, the involvement of NO is inferred from prior characterisation of the genes investigated (Crack *et al.*, 2015; Sasaki *et al.*, 2016). Consequently, extra validation is important to implicate the plant host in NO production, as numerous soil dwelling microorganisms may act as a source of nitrosative stress through NOS enzymes (Crane *et al.*, 2010) or denitrification (Loick *et al.*, 2016). Data presented in this Chapter showed that Increased fitness of the mutant occurs in the rhizosphere but not bulk soil, which suggests NO signaling is at least plant dependent. It is important to recognise that while increased rhizosphere fitness is likely due to plant derived NO, microbial production of NO in the rhizosphere cannot be ruled out.

The transient nature of reactive signaling molecules such as NO has been discussed (Thomas *et al.*, 2008), the outcome of signalling events can vary due to ranging concentrations. AMF interactions can trigger NO signalling with a unique contact related timing that differs from that of pathogenic interactions (Calcagno *et al.*, 2012), adding an extra layer of complexity. The data from this Chapter showed that while rhizosphere colonisation can increase with a deletion of *nsrR-hmpA*, constitutive expression of *Streptomyces* NO synthases led to an overall reduction in soil fitness, although this effect was extremely variable. This suggests that NO plays a more nuanced role in the ecological function of *Streptomyces* spp. in the soil and root microbiome. Accumulation of NO in the absence of HmpA may trigger downstream signaling changes, while constitutive expression of NO synthases may lead to the accumulation of NO at cytotoxic levels, despite the presence of HmpA in the wild-type background of these mutants. In addition, if Δ*nsrR-hmpA* leads to increased colonisation due to the amplification of plant-derived NO signaling, then the timing and intensity of the signal may be different than one would observe with constitutive expression of NO synthase.

While data presented in this Chapter fits with a small number of studies that investigate colonisation cues for AMF, NO is more often described in the context of host defence gene regulation, or as an antimicrobial. In the broader context, NO mediated symbioses are similarly described in the context of repressing colonisation. While NO is reported to act as a specificity determinant in squid-*Vibrio* symbiosis (Wang *et al.*, 2010b), Δ*nsrR-hmpA* mutants which are more sensitive to NO, exhibit poor biofilm formation and colonisation fitness. In other words, colonisation occurs despite the presence of NO, due to the detoxifying activity of NsrR and HmpA. Further studies showed that biofilm formation and subsequent colonisation was negatively regulated by the NO sensor protein HnoX (Thompson *et al.*, 2019). While this may have a broader ecological function, NO seems to negatively

mediate colonisation in this instance, even for symbiotic partners. This is contrary to the data presented in this Chapter, which suggests NO promotes colonisation.

The results presented in this Chapter were unexpected. Most studies investigating the role of NO in host-microbe interactions show that it acts as a specificity determinant, or a deterrent from colonisation. This work presents novel data that challenges this paradigm, it suggests that NO may stimulate colonisation, by regulation of an unknown physiological function in *S. coelicolor*. WhiB-like proteins (Wbl) are global regulators that are widely conserved across actinobacterial species and are prime candidates for signal transduction. It has been shown *in vitro* that they have a high binding affinity for NO (Crack *et al.*, 2011, 2014; Kudhair *et al.*, 2017) and have been implicated in the regulation of development in *Streptomyces* (Fowler-Goldsworthy *et al.*, 2011; Chater, 2016). While the model for NO signaling and homeostasis in *S. coelicolor* is far from complete, nitrate and nitrite reductase enzymes are likely utilised to generate endogenous NO (Sasaki *et al.*, 2016), which then interacts with NO sensing proteins like WblE (Fowler-Goldsworthy *et al.*, 2011). The results of this Chapter showed that exogenous application of an NO donor abrogated sporulation, in support of the model that NO regulates development through Wbl proteins like WblA. If plant derived NO inhibits sporulation in this way, recruitment of *S. coelicolor* to the wheat rhizosphere may be driven by developmental control. This theory makes biological sense, the consensus is that sporulation aids dispersal of *Streptomyces* spp. in the environment until they find a suitable niche (de Lima Procópio *et al.*, 2012), like the root microbiome of a plant.

Increased environmental fitness might also imply a competitive advantage, this may be achieved through production of antimicrobial secondary metabolites. It has been discussed in Chapter 3, that secondary metabolism increases in the root microbiome of *Arabidopsis thaliana* throughout plant development (Chaparro *et al.*, 2013), so it is likely that plants can influence the production of antimicrobial compounds. Secondary metabolism is tightly regulated and can be influenced by a range of factors (Bibb, 2005), in *Streptomyces coelicolor* it is linked to the development of aerial hyphae, however. If the development cycle is arrested at this stage due to plant derived NO, it might increase antimicrobial production. In theory, this effect may be exacerbated for the Δ*nsrR/hmpA* mutant. Unfortunately, the data does not support this *in vitro*, no difference in bioactivity could be observed between any of the mutants tested and the wild-type control. These results contrast with the findings of (Sasaki *et al.*, 2016), who show that *hmpA* deletion increases production of the antibiotic undecylprodigiosin , although it should be noted that they use a different media which was spiked with a nitrite source. It is also important to recognise the limitations of *in vitro* bioassays like these for inferring the ecological relevance of such metabolic disruptions; the transcriptional landscape for any

of the strains used in this study are likely quite different in the wheat rhizosphere or bulk soil. In order to conclude that plant derived NO activates secondary metabolic gene clusters, transcriptomic studies like mRNA sequencing should be performed, in order to look for differences between the wild-type strain and the Δ*nsrR/hmpA* mutant.

Perhaps one of the most interesting findings of this study was that increased rhizosphere colonisation by *S. coelicolor* Δ*nsrR-hmpA* was soil type dependent; increased rhizosphere fitness for the mutant was observed in Levington F2 compost but not Church Farm soil. An important thing to note here is that the chemical properties of these soils are quite different. Soil analysis conducted at the James Hutton institute (Table 4-10) indicated that phosphorous was slightly high, while potassium and magnesium were moderate and low respectively. Levington F2 compost on the other hand was excessively high in all nutrients tested. Nitrogen content, which was determined in house (Table 4-10), further reinforced the differences in chemical composition between soil types; Levington F2 compost had 27 times more ammonium and 78 times more nitrate than the agricultural soil. Soil properties have been discussed as a driving factor in root microbiome assembly (Chen *et al.*, 2019). Inorganic nitrogen level variation, particularly nitrate, may be an important factor in the phenotype difference between soils; nitrate reduction is a key source of NO in *Streptomyces* spp. (Sasaki *et al.*, 2016) and plants (Chamizo-Ampudia *et al.*, 2017). Denitrification, the process by which nitrogen is converted from oxidised forms like NO_3 to gaseous N_2 via NO_2, NO and N_2O, can be influenced by pH (Pan *et al.*, 2012). While it is not entirely clear how pH may affect NO levels may vary due to the influence of denitrification in agricultural soil and Levington's compost, it is a factor that could easily be controlled for in future experiments. If low pH is responsible for the soil dependent colonisation phenotype with the Δ*nsrR*/*hmpA1*/*2* and Δ*nsrR*/*hmpA1* mutants, then it can potentially be mimiced in agricultural soil through acidification. The influence of nitrate levels on the increased colonisation of Δ*nsrR*/*hmpA1*/*2* and Δ*nsrR*/*hmpA1* mutants in Levington's compost could additionally be investigated by controlling nitrate levels in agricultural soil.

The root microbiome can vary between soils due to starting composition but soil nutritional amendments such as inorganic nitrogen (which is a common agricultural practice) can alter root exudation and root microbial composition. Increased nitrogen fertilization in maize crops altered root exudation profiles and led to significant shifts in community composition (Zhu *et al.*, 2016). The number of N-cycle genes present was also shown to increase. A similar study in wheat showed that long-term N-fertilization regime influenced microbial community (Chen *et al.*, 2019). If nitrogen levels can increase denitrifying species in the soil, it is possible that increased rhizosphere colonisation by *S. coelicolor* Δ*nsrR-hmpA* is dependent on prokaryote derived NO. To reiterate, no difference was

observed between the mutant and control for bulk soil fitness assays, so the effect is still plant dependent. Supplementation of agricultural soil with nitrates and ammonium may resolve this issue further, increased mutant rhizosphere fitness rhizosphere fitness under these conditions would suggest nitrate reduction by the plant host, assuming the active microbial community remains unchanged.

The exact mechanisms driving microbial community composition changes are complicated but one theory is that nitrogen can affect plant physiology and root microbial community through cross-regulation with NO signalling (Thalineau *et al.*, 2016). It is noted that NO is generated in part through nitrate reductase enzymes. Using the model system *Medicago trunculata* and oomycete *Alphanomyces euteiches*, the authors showed that NO homeostasis was important for pathogen resistance. They also showed that NO homeostasis was influenced by nitrogen availability. This suggests that *S. coelicolor* Δ*nsrR-hmpA* may colonise the wheat rhizosphere better than the control in compost, as a result of increased plant NO levels from elevated nitrogen. It is also possible that *S. coelicolor* is subject to further elevated NO levels, due to the endogenous production by its own nitrate and nitrite reductase enzymes (Sasaki *et al.*, 2016). The theory that rhizosphere colonisation by *S. coelicolor* is mediated by plant nitrate reductase derived NO is unfortunately challenging to test. Tungstate salts can knock down nitrate reductase activity through competitive inhibition of its molybdenum cofactor (Deng *et al.*, 1989; Calcagno *et al.*, 2012; Ding *et al.*, 2020), but results may be confounded, as Tungstate can interfere with various plant enzymes in this way.

The potential for *Streptomyces* spp. to generate NO through denitrification has been discussed, studies have even demonstrated complete and co-denitrification in *Streptomyces antibioticus* (Kumon *et al.*, 2002). While the concept of nitrate reductase derived NO signaling in *Streptomyces* spp. may be gaining ground, characterisation of *Streptomyces* bNOS is limited to plant pathogenic strains from the *S. scabies* group. Species from this group possess a large pathogenicity island which encodes thaxtomin, a nitrated dipeptide phytotoxin (Kers *et al.*, 2004a). Production of thaxtomin was shown to be NO dependent; production could be knocked down with NO synthase inhibitors and recovered with NO donors (Wach *et al.*, 2005). The enzyme TxtD is homologous to mammalian NO synthases and catalyses NO formation from L-arginine (Kers *et al.*, 2004b). Homologous proteins are present for both *S. venezuelae* and *S. avermitilis* as detailed in Table 4-2, but the biological function has yet to be investigated for either. While this study does not confirm their functionality, constitutive heterologous expression of NOS_{Sven} and NOS_{Ssc} in *S. coelicolor* demonstrate similar soil growth fitness phenotypes, they are inconsistently poor. This suggests that *S. venezuelae* also encodes a functional NOS enzyme. Further biochemical characterisation of this protein is needed,

in order to confirm that NO is being produced. The genomic context of NOS_{Sven} is different from that of NOS_{Ssc} (*txtD*), in that it is not part of a thaxtomin biosynthesis cluster. As such, it is uncertain what biological function NO derived from this enzyme may serve.

No morphological defects were observed in lab conditions, but these observations do not necessarily preclude growth fitness defects. Unfortunately, while time course OD_{600} measurements is a suitable method for the establishment of a growth curve for many unicellular microorgansms, this is not possible for filamentous organisms such as *Streptomyces*. As such, poor soil and rhizosphere colonisation could be due to a growth defect caused by the constitutive expression of bNOS genes. Even if there is no fitness penalty for constitutive expression under laboratory conditions, it is likely that such a penalty may be incurred in more complex and stressful environments. Any fitness defects may be mitigated by expressing bNOS encoding genes at lower levels, this could be achieved using promoters of different strengths, or even a promoter that can be induced with the addition of a specific reagent.

While it is not yet clear how NO is involved in wheat rhizosphere colonisation by *S. coelicolor*, this study has shown that it may play a role in recruitment to the root microbiome. The observation was dependent on the use of compost as a growth medium, which suggests that NO signalling at the host-microbe interface was nitrogen dependent. The implications of this are far reaching. The consequences of widespread agricultural use of nitrogen fertilisation has been discussed (Good and Beatty, 2011; Loick *et al.*, 2016). The interplay between nitrogen levels, plant immunity (Thalineau *et al.*, 2016) and microbiome assembly (Chen *et al.*, 2019) have been explored to some degree but the effect on plant-*Streptomyces* spp. interactions has not yet been discussed in the literature. Throughout this thesis, the disease suppressive and plant-beneficial traits of *Streptomyces* spp. have been well established. This study has now yielded insights into the mechanisms underpinning their association, as well as some agricultural context. Further exploration could lead to improved strategies for stimulating beneficial microbiome formation.

High reactivity and short half-life make NO a challenging molecule to work with, especially in the biological context. As a free radical, NO can form intermediates with other reactive species and interact with numerous biological molecules. This means that it can be difficult to determine physiological functions underpinning NO-dependent phenotypes. Nevertheless, selective recovery of *Streptomyces* spp. from the root microbiome offers a robust and economical method for assessing colonisation fitness, for the conditions tested. Determination of root colonisation fitness by *Streptomyces* species using cfu counts is an established method in the literature (Kortemaa *et al.*, 1994; Zeng *et al.*, 2012; Clark *et al.*, 2013; Chen *et al.*, 2016), which is why it was adopted in this study

for investigating the role of NO in wheat root colonisation by *Streptomyces coelicolor*. While cfu counts are a relatively cost-efficient and straightforward way of determining colonisation fitness there are some caveats. It assumes that the number of CFUs recovered correlates linearly with microbial biomass. For *Streptomyces*, this may prove challenging in a couple of ways. *Streptomyces* are multinuclear filamentous organisms, so CFUs recovered may vary depending on the way hyphae fragment when they are reisolated from the root microbiome. Additionally, *Streptomyces* may be present as mononuclear spores, equal biomass of spores may therefore be overrepresented by cfu counts when compared to hyphae. Alternative methods for quantifying root colonisation could utilise qPCR to determine actual abundance, by targeting a single copy region of DNA that is specific to the organism in question in an environmental DNA sample. Targetting a region of DNA present on a single copy integrative vector such as pSS170 may be the most suitable way of quantifying a specific organism with environmental DNA samples, such as those isolated from plant roots.

Model organisms like *S. coelicolor* offer valuable insights into root colonisation dynamics. High quality genomic resources and well optimised genetic toolkits facilitate interrogation of the environmental function of well characterised genes. The caveat is that the biology of lab domesticated strains like these may not accurately reflect that of environmental strains. It would be valuable to investigate the role of *nsrR* and *hmpA* in host/microbe NO signalling with wheat root associated strains CRS3 and CRS4, now that full genome sequences are available. Moving forward, there are several studies that would be useful to further validate this hypothesis and explore in greater depth the relationship between NO signalling and *Streptomyces* physiology in the root microbiome. This study is a preliminary look at an interaction that has not been observed before. The challenges of working with NO in biological systems are clear, but this study provides the foundation for moving forward to address them. Further validation is required to confirm that NO signalling is plant derived. A review of the literature suggests that NO-fluorescent probes combined with fluorescence Confocal Laser Scanning Microscopy (fCLSM) is the most suitable approach for resolving this hypothesis (Calcagno *et al.*, 2012; Gupta *et al.*, 2014). The limitation of this approach is that it requires a well-optimised and robust method for imaging wheat/microbe interactions. While preliminary studies showed that *S. coelicolor* could be grown axenically with sterilised wheat roots, reliable imaging could not be optimised in the time frame of this study.

With more time, further genetic variations of the *nsrR*/*hmpA1*/*hmpA2* should be interrogated. *S. coelicolor* Δ*nsrR*/*hmpA1*/*hmpA2* showed improved colonisation compared to *S. coelicolor* Δ*hmpA2*, which suggested that improved colonisation was a result of the absence of *nsrR*/*hmpA1*. The CRISPR/Cas9 generated mutant confirmed this but it would be useful to explore the effects of Δ*nsrR*

without deletion of either *hmpA1* or *hmpA2*. It could be hypothesised that without the repression of *nsrR*, *hmpA1/2* expression would be higher, leading to less NO in the system and perhaps reduced colonisation. This does not seem likely however, given that overexpression of *hmpA1* in the Δ*nsrR*/*hmpA1* mutant simply led to the recovery of wild type levels of root colonisation, as opposed to a reduction. It would also be interesting to see if constitutive expression of *hmpA* could recover the phenotype caused by Δ*nsrR*/*hmpA1*.

Other methods could be used for the determination of intracellular NO. The expression of *hmpA* is normally repressed by NsrR, increased levels of endogenous NO release this transcription to restore homeostasis (Crack *et al.*, 2015; Sasaki *et al.*, 2016). Transcriptomic studies using environmental cDNA could determine *S. coelicolor hmpA* expression in the rhizosphere with qPCR, as a measure of endogenous NO levels. This approach could be validated *in vitro* using a NO donor. Preliminary data showed that *S. coelicolor hmpA1* could be amplified from DNA isolated from agricultural soil inoculated 2 weeks prior, whereas the product could not be obtained for control samples. This suggested RT-qPCR is a valid approach.

In conclusion, *S. coelicolor* Δ*nsrR-hmpA* demonstrated increased rhizosphere fitness in Levington F2 compost, while bulk soil growth fitness remained the same as the wild-type control. Increased rhizosphere fitness was not observed for the mutant strain in Church Farm soil. Our hypothesis is that the mutant phenotype is dependent on plant-derived NO from the reduction of excess nitrates in the compost, which are not present in such quantities for agricultural soil. This has profound impacts for the management of arable land on which cereal crops like wheat are grown. As the world's population continues to grow and the demand for food increases, yields must also increase to match this. Understanding how beneficial microorganisms like *Streptomyces* spp. associate with their plant hosts in order to improve growth and resistance to disease is vital, in order to maximise the potential of such environmental resources.

Chapter 5 Final conclusions

This thesis set out to explore the biology of streptomycetes in the wheat root microbiome, in the context of plant beneficial metabolisms. A key aim was to understand if *Streptomyces* species are enriched in the rhizosphere or endosphere of wheat variety Paragon grown in soil from the JIC field studies site at Church Farm in Bawburgh and to ascertain the influence of host root exudates on root microbiome assembly. Nitric oxide (NO) metabolism was also investigated as a factor in wheat root colonisation by *Streptomyces coelicolor* since it is implicated in root colonisation by fungi (Calcagno *et al.*, 2012; Zhang *et al.*, 2013; Tian *et al.*, 2017), which have a similar life cycle and filamentous mode of growth. The purpose behind this work was to explore the way in which wheat plants assemble microbial communities and determine if *Streptomyces* spp. are defensive mutualists in wheat. The breadth of approaches employed in this thesis reflect the complexity of the system they were intended to explore. Successful exploitation of beneficial plant/microbe interactions will likely be dependent on an intimate understanding of not only discreet groups of microbes like *Streptomyces* species, but the microbial community as a whole.

Streptomyces spp. have been documented to provide benefits to the plant host, including disease suppression through antimicrobial production and plant growth promoting metabolisms. While other studies have identified streptomycetes with beneficial properties towards plants, the work in this thesis is unique in that a large number of isolates were collected from different wheat varieties, in order to determine how widespread is the capacity for wheat take-all antagonism among these microorganisms.

Another driving force behind this work was to understand how specialised metabolism is controlled in a natural ecosystem. The molecular machinery employed by streptomycetes to create these natural products is complex and the majority of secondary metabolites encoded by *Streptomyces* spp. are not made under standard laboratory growth conditions. This is a problem, because there is an urgent need to discover new antimicrobial compounds, now more than ever. The discovery of novel secondary metabolites for use in medicine is often hampered by stringent regulation of the clusters that encode their biosynthesis. The key to unlocking the secrets of these organisms and their biosynthetic gene clusters is likely hidden in the environment in which they have been evolving for millions of years. The data presented in Chapter 2 reported the isolation and 16S rRNA gene sequencing of *Streptomyces* spp. from the rhizosphere and endosphere of three different wheat varieties and hinted that streptomycetes isolated from wheat variety Paragon were more bioactive against not just wheat take-all fungus but also the human pathogenic fungus *Candida albicans*. Similarly, a high proportion of *Streptomyces* spp. were isolated from wheat variety Soisson

had bioactivity against MRSA. Numerous studies have found that looking for streptomycetes in new environments, particularly those associated with higher organisms such as *Tetraponera penzigi* ants (Qin *et al.*, 2017) and coral reef (Hodges *et al.*, 2012), has proven to be a promising approach. This study has highlighted the potential of plant roots to enrich for these organisms.

Ultimately, however, the aim of this research was to understand roles and recruitment of *Streptomyces* to the wheat root microbiome for the improvement of agriculture. The aim of the isolation study in Chapter 2 was to explore the interplay between host genotype, root compartment and microbial bioactivity. The observation that Paragon streptomycete isolates were more bioactive against fungi should precede further studies into the influence of host genotype, on the metabolism of key groups of associated microbes like *Streptomyces* spp. This study also showed that promising new bioactive strains could be derived from local agricultural environments. CRS3 and CRS4 provide a promising platform for further interrogating the interactions of wheat plants and their associated streptomycetes, in addition to their potential commercial value.

Chapter 3 demonstrated that taxa belonging to the family *Streptomycetaceae* were abundant and enriched in the endosphere compartment, preliminary data also suggested that they may utilise root exudates in this niche. While the SIP experiment showed that *Streptomycetaceae* were not using root exudates in the rhizosphere, this is most likely because they were outcompeted for this resource by fast growing proteobacterial taxa like *Pseudomonadaceae* or *Enterobacteriaceae*. The unfractionated metabarcoding study showed that while *Streptomycetaceae* were enriched from rhizosphere to endosphere compartments, they were not enriched in the rhizosphere relative to the surrounding bulk soil.

Work presented in Chapter 3 also showed a high correlation between taxa metabolising root exudates and taxa enriched in the root microbiome. While it may be hard to infer cause and effect from this observation, the chemical ecology of plant host/microbe interactions clearly has an important role to play in microbiome formation. Studies have shown that plant hormones can improve antimicrobial production *in vitro* for *Streptomyces* spp. (van der Meij *et al.*, 2018; Worsley *et al.*, 2020). This may not be surprising when these metabolites may signal the occupation of a resource-rich host niche, which can support the energy requirements for expensive secondary metabolism while still attracting competition. This study has shown that *Streptomycetaceae* are highly abundant in the endosphere compartment relative to the surrounding soil and that they are likely consuming root metabolites. Members of Streptomycetaceae are known to be relatively slow growing microorganisms. To dominate so much of the endosphere community as this study shows, suggests they have a strong advantage, which is less apparent in the surrounding soil/rhizosphere. As such, it

is not out of the realms of possibility to suggest that *Streptomyces* produce more antibiotics in the root microbiome, some of which may be novel to medicine. This study has identified the need to explore this further. Bioactivity against root exudate metabolising taxa may provide a useful measure of predicting the success of a *Streptomyces* strain in colonising the wheat root microbiome. Preliminary data from Chapter 3 suggests that *Streptomyces* spp. utilise plant metabolites in the endosphere. Collection of these metabolites *in vitro* from hydroponic systems would enable further investigation into which streptomycetes shows the greatest metabolic responsiveness.

Ultimately, however, the successful deployment of *Streptomyces* spp. for the improvement of crop yield in agriculture will be dependent on fully understanding the factors that underpin all levels of wheat root colonisation by streptomycetes. To address this, Chapter 4 demonstrated for the first time that wheat rhizosphere colonisation by *S. coelicolor* could be increased by deleting genes involved with NO detoxification, while there were no observable differences in bulk soil survival. This study also showed that the effect was soil type dependent, differences which were observed in plants grown in Levington F2 compost were absent when agricultural soil was used to grow the plants. One theory is that this is caused by the abundance of nitrogen in the form of NH_4 and NO_3, which is known to affect NO metabolism in plants, and which are 86- and 29-fold more abundant, respectively in compost compared to soil. Understanding how soil type and nutrition may affect the root microbiome in wheat will likely prove key to the successful improvement of crop yields in the future. While *S. coelicolor* colonised the endosphere poorly, Chapter 3 showed that *Streptomycetaceae* were shown to be highly abundant in the endosphere compartment, yet rhizosphere enrichment was poor. The observation that NO seems to control the gateway to the root microbiome shows promise for improving both the efficiency of *Streptomyces* spp. that are naturally present in soil, as well as specially selected and optimised streptomycetes for commercial bioaugmentation. While there is still much to learn about the role of NO in recruitment of *Streptomyces* spp. to the wheat root microbiome, this study has offered key insights into the process, something which has been largely alluded to up until now.

To summarise, the roles of *Streptomyces* spp. in the wheat root microbiome are diverse. Many streptomycetes isolated from a common bread wheat variety in agricultural soil demonstrated antagonistic properties against wheat take-all and select strains were able to demonstrate other plant beneficial metabolisms. The recruitment of *Streptomyces* spp. has always been more of an enigma, however. The $^{13}CO_2$ DNA-SIP study shows that wheat plants are likely recruiting specific organisms to the root microbiome by exuding organic substrates. Carbon dynamics in this niche are clearly complicated, however, and there are other factors at work. Complex signalling pathways occur

between the roots and microorganisms from the surrounding soil. NO and the plant immune has a strong part to play in that.

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Supplementary data

S1 Closest hit identities for *Streptomyces* 16S rRNA gene sequences

Strain	Best match (RefSeq genome database blast)	% identity
PRS1	*Streptomyces* anulatus strain ATCC 11523 chromosome, whole genome shotgun sequence	100
PRS2	*Streptomyces* sp. CG 926 Ga0105840_133, whole genome shotgun sequence	99.78
PRS3	*Streptomyces* anulatus strain ATCC 11523 chromosome, whole genome shotgun sequence	99.93
PRS5	*Streptomyces* sp. 1121.2 Ga0151191_12, whole genome shotgun sequence	99.86
SRS1	*Streptomyces* sp. M41(2017) strain M41 M41_contig2, whole genome shotgun sequence	99.85
SRS2	*Streptomyces* cyaneochromogenes strain MK-45 chromosome, complete genome	99.35
SRS3	*Streptomyces* sp. Tu 2975 chromosome, complete genome	99.43
SRS4	*Streptomyces* anulatus strain ATCC 11523 chromosome, whole genome shotgun sequence	100
SRS5	*Streptomyces* anulatus strain ATCC 11523 chromosome, whole genome shotgun sequence	99.93
CRS1	*Streptomyces* anulatus strain ATCC 11523 chromosome, whole genome shotgun sequence	99.93
CRS2	*Streptomyces* anulatus strain ATCC 11523 chromosome, whole genome shotgun sequence	99.93
CRS3	*Streptomyces* libani subsp. libani strain NBRC 13452 sequence1, whole genome shotgun sequence	100
CRS4	*Streptomyces* sp. SLBN-115 Ga0314646_12, whole genome shotgun sequence	100
CRS5	*Streptomyces* anulatus strain ATCC 11523 chromosome, whole genome shotgun sequence	100
PES1	*Streptomyces* sp. 1121.2 Ga0151191_12, whole genome shotgun sequence	99.86
PES2	*Streptomyces* canus strain DSM 40275 PRJNA299222_s036, whole genome shotgun sequence	100
PES3	*Streptomyces* mutomycini strain NRRL B-65393 NRRL_B-65393_contig_102, whole genome shotgun sequence	99.93
PES4	*Streptomyces* sp. M41(2017) strain M41 M41_contig2, whole genome shotgun sequence	99.86
PES5	*Streptomyces* sp. Tu 2975 chromosome, complete genome	98.99
SES1	*Streptomyces* acidiscabies strain a2, whole genome shotgun sequence	99.64
SES2	Promicromonospora sukumoe strain SAI-064 Ga0222405_11, whole genome shotgun sequence	99.64

Strain	Best match (RefSeq genome database blast)	% identity
SES3	*Streptomyces* sp. M41(2017) strain M41 M41_contig2, whole genome shotgun sequence	99.93
SES4	Promicromonospora sukumoe strain SAI-064 Ga0222405_11, whole genome shotgun sequence	99.64
SES5	*Streptomyces* venezuelae ATCC 10712 chromosome, complete genome	100
CESp1	*Streptomyces* candidus strain DSM 40141 Ga0415311_46, whole genome shotgun sequence	99.42
CESp2	*Streptomyces* sp. NRRL WC-3618 P435contig282.2, whole genome shotgun sequence	100
CESp3	*Streptomyces* sp. NRRL WC-3618 P435contig282.2, whole genome shotgun sequence	100
PRwS1	*Streptomyces* sp. adm13(2018) Scaffold_129, whole genome shotgun sequence	100
PRwS2	*Streptomyces* anulatus strain ATCC 11523 chromosome, whole genome shotgun sequence	100
PRwS3	*Streptomyces* canus strain DSM 40275 PRJNA299222_s036, whole genome shotgun sequence	99.93
PRwS4	*Streptomyces* rishiriensis strain NBRC 13407, whole genome shotgun sequence	99.78
PRwS5	*Streptomyces* caniferus strain NBRC 15389 sequence01, whole genome shotgun sequence	99.93
PRwSp1	*Streptomyces* canus strain DSM 40275 PRJNA299222_s036, whole genome shotgun sequence	99.93
PRwSp2	*Streptomyces* canus strain DSM 40275 PRJNA299222_s036, whole genome shotgun sequence	100
SRwS1	*Streptomyces* anulatus strain ATCC 11523 chromosome, whole genome shotgun sequence	100
SRwS2	*Streptomyces* sp. adm13(2018) Scaffold_129, whole genome shotgun sequence	100
SRwS3	*Streptomyces* sp. SLBN-115 Ga0314646_12, whole genome shotgun sequence	100
SRwS4	*Streptomyces* coelicolor strain O-10.1 Ga0206415_163, whole genome shotgun sequence	100
SRwSp1	*Streptomyces* canus strain DSM 40275 PRJNA299222_s036, whole genome shotgun sequence	100
SRwSp2	*Streptomyces* venezuelae ATCC 10712 chromosome, complete genome	100
CRwS1	*Streptomyces* rishiriensis strain NBRC 13407, whole genome shotgun sequence	99.78
CRwS2	*Streptomyces* cyaneochromogenes strain MK-45 chromosome, complete genome	99.28
CRwS3	*Streptomyces* anulatus strain ATCC 11523 chromosome, whole genome shotgun sequence	100
CRwS4	*Streptomyces* sp. Root431 contig_1, whole genome shotgun sequence	100
CRwS5	*Streptomyces* sp. SLBN-115 Ga0314646_12, whole genome shotgun sequence	100
CRwSp1	*Streptomyces* canus strain DSM 40275 PRJNA299222_s036, whole genome shotgun sequence	99.93
CRwSp2	*Streptomyces* sp. SID14446 contig-3000002, whole genome shotgun sequence	100

Strain	Best match (RefSeq genome database blast)	% identity
CRwSp2b	*Streptomyces* canus strain DSM 40275 PRJNA299222_s036, whole genome shotgun sequence	100
PRwIp1	*Streptomyces* libani subsp. libani strain NBRC 13452 sequence1, whole genome shotgun sequence	100
PRwIp2	*Streptomyces* acidiscabies strain a2, whole genome shotgun sequence	99.64
PRwIp3	*Streptomyces* canus strain DSM 40275 PRJNA299222_s036, whole genome shotgun sequence	99.93
PEI5	*Streptomyces* mutomycini strain NRRL B-65393 NRRL_B-65393_contig_102, whole genome shotgun sequence	100
PEI6	*Streptomyces* mutomycini strain NRRL B-65393 NRRL_B-65393_contig_102, whole genome shotgun sequence	99.63
CEIp1	*Streptomyces* sp. WM6378 P402contig199.1, whole genome shotgun sequence	99.42

Strain	Closest match (nucleotide collection database)	% identity
PRS1	*Streptomyces* griseus strain S10-TSA-15 16S ribosomal RNA gene, partial sequence	100
PRS2	*Streptomyces* avidinii strain IHBA 9994 16S ribosomal RNA gene, partial sequence	99.93
PRS3	*Streptomyces* griseus strain S10-TSA-15 16S ribosomal RNA gene, partial sequence	99.93
PRS5	*Streptomyces* sp. ME02-6987.2a 16S ribosomal RNA gene, complete sequence	100
SRS1	*Streptomyces* sp. strain BV9 16S ribosomal RNA gene, partial sequence	99.78
SRS2	*Streptomyces* sp. strain W53 16S ribosomal RNA gene, partial sequence	99.85
SRS3	Bacterium strain YD201804IPB3 16S ribosomal RNA gene, partial sequence	99.86
SRS4	*Streptomyces* griseus strain S10-TSA-15 16S ribosomal RNA gene, partial sequence	100
SRS5	*Streptomyces* griseus strain S10-TSA-15 16S ribosomal RNA gene, partial sequence	99.93
CRS1	*Streptomyces* griseus strain S10-TSA-15 16S ribosomal RNA gene, partial sequence	99.93
CRS2	*Streptomyces* griseus strain S10-TSA-15 16S ribosomal RNA gene, partial sequence	99.93
CRS3	*Streptomyces* sp. NEAU-S7GS2 chromosome, complete genome	100
CRS4	*Streptomyces* sp. 18-7 16S ribosomal RNA gene, partial sequence	100
CRS5	*Streptomyces* griseus strain S10-TSA-15 16S ribosomal RNA gene, partial sequence	100
PES1	*Streptomyces* sp. ME02-6987.2a 16S ribosomal RNA gene, complete sequence	100
PES2	*Streptomyces* sp. strain ME10 16S ribosomal RNA gene, partial sequence	100
PES3	*Streptomyces* sp. strain MMun154 16S ribosomal RNA gene, partial sequence	100

Strain	Closest match (nucleotide collection database)	% identity
PES4	*Streptomyces* aureus strain Fist3 16S ribosomal RNA gene, partial sequence	99.86
PES5	*Streptomyces* sp. HBUM 78524 16S ribosomal RNA gene, partial sequence	99.5
SES1	*Streptomyces* acidiscabies strain Strep101 16S ribosomal RNA gene, partial sequence	99.64
SES2	Promicromonospora umidemergens strain 14614 16S ribosomal RNA gene, partial sequence	100
SES3	*Streptomyces* sp. strain BV9 16S ribosomal RNA gene, partial sequence	99.86
SES4	Promicromonospora umidemergens strain 14614 16S ribosomal RNA gene, partial sequence	100
SES5	*Streptomyces* venezuelae strain ATCC 10595 chromosome, complete genome	100
CESp1	*Streptomyces* spiroverticillatus 16S ribosomal RNA gene, partial sequence	99.93
CESp2	*Streptomyces* turgidiscabies strain 08-04-02-1a (#7) 16S ribosomal RNA gene, partial sequence	99.57
CESp3	*Streptomyces* turgidiscabies strain 08-04-02-1a (#7) 16S ribosomal RNA gene, partial sequence	99.57
PRwS1	*Streptomyces* sp. adm13(2018) strain adm13 16S ribosomal RNA gene, partial sequence	100
PRwS2	*Streptomyces* griseus strain S10-TSA-15 16S ribosomal RNA gene, partial sequence	100
PRwS3	*Streptomyces* sp. strain ME10 16S ribosomal RNA gene, partial sequence	99.93
PRwS4	*Streptomyces* sp. 08-06-03-1b(#K1) 16S ribosomal RNA gene, partial sequence	100
PRwS5	*Streptomyces* sp. D-15 gene for 16S ribosomal RNA, partial sequence	100
PRwSp1	*Streptomyces* sp. strain ME10 16S ribosomal RNA gene, partial sequence	99.93
PRwSp2	*Streptomyces* sp. strain ME10 16S ribosomal RNA gene, partial sequence	100
SRwS1	*Streptomyces* griseus strain S10-TSA-15 16S ribosomal RNA gene, partial sequence	100
SRwS2	*Streptomyces* zaomyceticus strain QMA47 16S ribosomal RNA gene, partial sequence	100
SRwS3	*Streptomyces* sp. 18-7 16S ribosomal RNA gene, partial sequence	100
SRwS4	*Streptomyces* coelicolor strain M1154/pAMX4/pGP1416 chromosome, complete genome	100
SRwSp1	*Streptomyces* sp. strain ME10 16S ribosomal RNA gene, partial sequence	100
SRwSp2	*Streptomyces* venezuelae strain ATCC 10595 chromosome, complete genome	100
CRwS1	*Streptomyces* sp. 08-06-03-1b(#K1) 16S ribosomal RNA gene, partial sequence	100
CRwS2	*Streptomyces* sp. strain W53 16S ribosomal RNA gene, partial sequence	99.93
CRwS3	*Streptomyces* griseus strain S10-TSA-15 16S ribosomal RNA gene, partial sequence	100

Strain	Closest match (nucleotide collection database)	% identity
CRwS4	*Streptomyces* zaomyceticus strain 174477 16S ribosomal RNA gene, partial sequence	100
CRwS5	*Streptomyces* sp. RJA2910 16S ribosomal RNA gene, partial sequence	100
CRwSp1	*Streptomyces* sp. strain ME10 16S ribosomal RNA gene, partial sequence	99.93
CRwSp2	*Streptomyces* sp. strain BV9 16S ribosomal RNA gene, partial sequence	100
CRwSp2b	*Streptomyces* sp. strain ME10 16S ribosomal RNA gene, partial sequence	100
PRwIp1	*Streptomyces* sp. NEAU-S7GS2 chromosome, complete genome	100
PRwIp2	*Streptomyces* acidiscabies strain Strep101 16S ribosomal RNA gene, partial sequence	99.64
PRwIp3	*Streptomyces* sp. strain ME10 16S ribosomal RNA gene, partial sequence	99.93
PEI5	*Streptomyces* sp. strain MMun154 16S ribosomal RNA gene, partial sequence	100
PEI6	*Streptomyces* sp. strain MMun154 16S ribosomal RNA gene, partial sequence	99.93
CEIp1	*Streptomyces* wistariopsis gene for 16S ribosomal RNA, partial sequence, strain: JCM 4688	99.42

S2 Optimum carbon source determination for ACC deaminase experiment. Strains were challenged on different carbon sources with and without $(NH_4)_2SO_4$. Strains which showed growth on a particular carbon source are marked with '+' for good growth and '++' for good growth. Carbon source was chosen that showed best growth on plates with nitrogen and minimal growth on plates without.

	Carbon Source				
Strain	**Glucose**	**Maltose**	**Mannitol**	**Sucrose**	**Cellobiose**
PRwS1	+	++			+
PRwS5	+	+	++	+	+
PRwSp1	+	+	++	+	+
SES3	++	++	+		++
SRwS3	+	+	+	+	++
CRS3		+		++	
CRS4	+			++	

S3 AntiSMASH output for *S. lydicus* WYEC 108

Overview	Region	Type	From	To	Most similar known cluster	Type	Similarity
1.1	Region 1	nucleoside,NRPS-like,butyrolactone	17,169	72,901	herboxidiene	Polyketide	7%
1.2	Region 2	lassopeptide	384,099	405,946			
1.3	Region 3	NRPS,LAP,thiopeptide,CDPS	430,915	499,770	muraymycin C1	NRP + Polyketide	23%
1.4	Region 4	T1PKS,NRPS-like,PKS-like,oligosaccharide,transAT-PKS	594,119	828,818	caniferolide A / caniferolide B / caniferolide C / caniferolide D	Polyketide: Modular type I	61%
1.5	Region 5	T3PKS,lanthipeptide	863,875	906,994	SapB	RiPP:Lanthipeptide	100%
1.6	Region 6	NRPS-like	1,004,695	1,046,642	stenothricin	NRP:Cyclic depsipeptide	13%
1.7	Region 7	bacteriocin	1,078,962	1,086,367			
1.8	Region 8	T2PKS	1,118,464	1,190,979	spore pigment	Polyketide	83%
1.9	Region 9	NRPS,melanin, T1PKS	1,211,344	1,388,016	lydicamycin	NRP + Polyketide: Modular type I	100%

Overview	Region	Type	From	To	Most similar known cluster	Type	Similarity
1.1	Region 10	siderophore	1,527,062	1,539,513	vibrioferrin	Other	18%
1.11	Region 11	blactam	1,615,118	1,638,619	valclavam / (-)-2-(2-hydroxyethyl)clavam	Other:Non-NRP beta-lactam	71%
1.12	Region 12	siderophore	2,221,748	2,230,573	desferrioxamine E	Other	100%
1.13	Region 13	ectoine	2,319,228	2,329,644	ectoine	Other	100%
1.14	Region 14	T1PKS	3,279,881	3,324,607	tetronasin	Polyketide	5%
1.15	Region 15	lanthipeptide	3,938,672	3,959,954	pentalenolactone	Terpene	15%
1.16	Region 16	lanthipeptide	4,039,967	4,063,199			
1.17	Region 17	terpene	4,520,042	4,537,633			
1.18	Region 18	terpene	4,956,617	4,978,225	salinomycin	Polyketide: Modular type I	6%
1.19	Region 19	TfuA-related,LAP,thiopeptide,NRPS, lassopeptide	5,134,934	5,219,055	A54145	NRP	13%
1.2	Region 20	terpene	5,848,082	5,867,980			
1.21	Region 21	siderophore	6,747,644	6,762,338	ficellomycin	NRP	3%
1.22	Region 22	butyrolactone	6,948,186	6,958,456			

Overview	Region	Type	From	To	Most similar known cluster	Type	Similarity
1.23	Region 23	bacteriocin	7,029,325	7,038,692			
1.24	Region 24	butyrolactone	7,432,679	7,439,760			
1.25	Region 25	indole	7,469,843	7,491,138	CDA1b / CDA2a / CDA2b / CDA3a / CDA3b / CDA4a / CDA4b	NRP:Ca+-dependent lipopeptide	12%
1.26	Region 26	NRPS-like	7,516,652	7,558,548	mycotrienin I	NRP + Polyketide	7%
1.27	Region 27	terpene	7,808,551	7,834,001	hopene	Terpene	69%
1.28	Region 28	hglE-KS,T1PKS	7,953,819	8,005,747	herboxidiene	Polyketide	4%
1.29	Region 29	lanthipeptide	8,163,025	8,187,573			
1.3	Region 30	bacteriocin	8,254,651	8,266,585			
1.31	Region 31	butyrolactone	8,556,760	8,567,731	merochlorin A / merochlorin B / deschloro-merochlorin A / deschloro-merochlorin B / isochloro-merochlorin B / dichloro-merochlorin B / merochlorin D / merochlorin C	Terpene + Polyketide: Type III	4%
1.32	Region 32	NRPS,other	8,626,208	8,687,263	deimino-antipain	NRP	66%
1.33	Region 33	terpene	8,720,529	8,746,092	isorenieratene	Terpene	100%
1.34	Region 34	amglyccycl	8,945,434	8,969,380	streptomycin	Saccharide	55%

S4 Statistical output for enriched taxa

Bulk soil to Rhizosphere enrichment												
Family	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	UF13BSA	UF13BSB	UF13BSC	UF13RZA	UF13RZB	UF13RZC
Pseudomonadaceae	1415.258	4.622582	0.808028	5.720819	1.06E-08	9.64E-08	113	97	138	5706	607	899
Verrucomicrobiaceae	633.3492	4.326882	0.297943	14.52254	8.72E-48	8.72E-46	77	52	59	1302	1055	1072
Fibrobacteraceae	492.4774	4.073909	0.434876	9.36797	7.39E-21	1.48E-19	55	54	63	941	513	1108
Oxalobacteraceae	743.6033	3.063469	0.271127	11.299	1.33E-29	3.32E-28	151	136	217	1319	1834	806
Enterobacteriaceae	1284.552	3.035377	0.649675	4.67215	2.98E-06	2.13E-05	283	260	331	2905	499	2691
Rhizobiaceae	638.5884	2.925346	0.400634	7.301783	2.84E-13	3.16E-12	104	149	214	1460	1112	666
Comamonadaceae	1558.433	2.902269	0.212758	13.64115	2.28E-42	1.14E-40	401	320	436	2948	3100	1954
Roseiflexaceae	1799.765	2.263302	0.331078	6.836165	8.13E-12	8.13E-11	626	612	689	2445	2248	3688
Cytophagaceae	2048.804	1.969272	0.256732	7.67053	1.71E-14	2.45E-13	834	747	1042	3533	2736	2976
Polyangiaceae	473.0929	1.538576	0.320658	4.798189	1.60E-06	1.23E-05	306	181	282	870	636	499
Chthoniobacteraceae	304.1029	1.395871	0.400125	3.488587	0.000486	0.001798	148	202	157	446	313	474
Sphingobacteriaceae	312.4487	1.230512	0.384536	3.199992	0.001374	0.004739	140	232	194	312	420	525
BIrii41	333.619	1.215248	0.366503	3.31579	0.000914	0.003264	166	120	377	467	452	420
Xanthomonadaceae	746.2113	1.029429	0.369583	2.785382	0.005346	0.016201	421	467	654	1333	911	609
Phyllobacteriaceae	526.5742	0.547293	0.204033	2.68238	0.00731	0.020306	469	359	524	614	810	425
Hyphomicrobiaceae	1309.899	0.515275	0.142788	3.608671	0.000308	0.001231	1164	949	1277	1473	1850	1196
Bradyrhizobiaceae	994.7585	0.47436	0.180199	2.63243	0.008478	0.022616	974	649	1021	1078	1509	838
Paenibacillaceae	1241.636	0.453862	0.124319	3.650792	0.000261	0.001137	1142	866	1310	1126	1846	1273

Rhizosphere to endosphere enrichment												
Family	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	UF13EA	UF13EB	UF13EC	UF13RZA	UF13RZB	UF13RZC
Flavobacteriaceae	372.1323	9.178098	2.143583	-4.28166	1.86E-05	8.07E-05	402	25	1	1	1	10
Lachnospiraceae	4141.821	8.506831	1.55481	-5.4713	4.47E-08	4.47E-07	4578	18	24	27	112	94
Enterobacteriaceae	55820.87	7.452203	2.045686	-3.64289	0.00027	0.000817	61376	326	568	2905	499	2691
Burkholderiaceae	628.1072	6.296202	0.757503	-8.31178	9.43E-17	9.43E-15	41	1035	459	68	64	31
Streptomycetaceae	7548.626	5.965518	0.91449	-6.52333	6.88E-11	2.29E-09	512	13288	4539	774	1039	637
Clostridiaceae 1	3974.429	5.377185	1.015438	-5.29543	1.19E-07	9.89E-07	3814	491	678	596	759	555
Paenibacillaceae	4814.707	4.480803	0.787231	-5.69185	1.26E-08	2.09E-07	771	8207	1790	1126	1846	1273

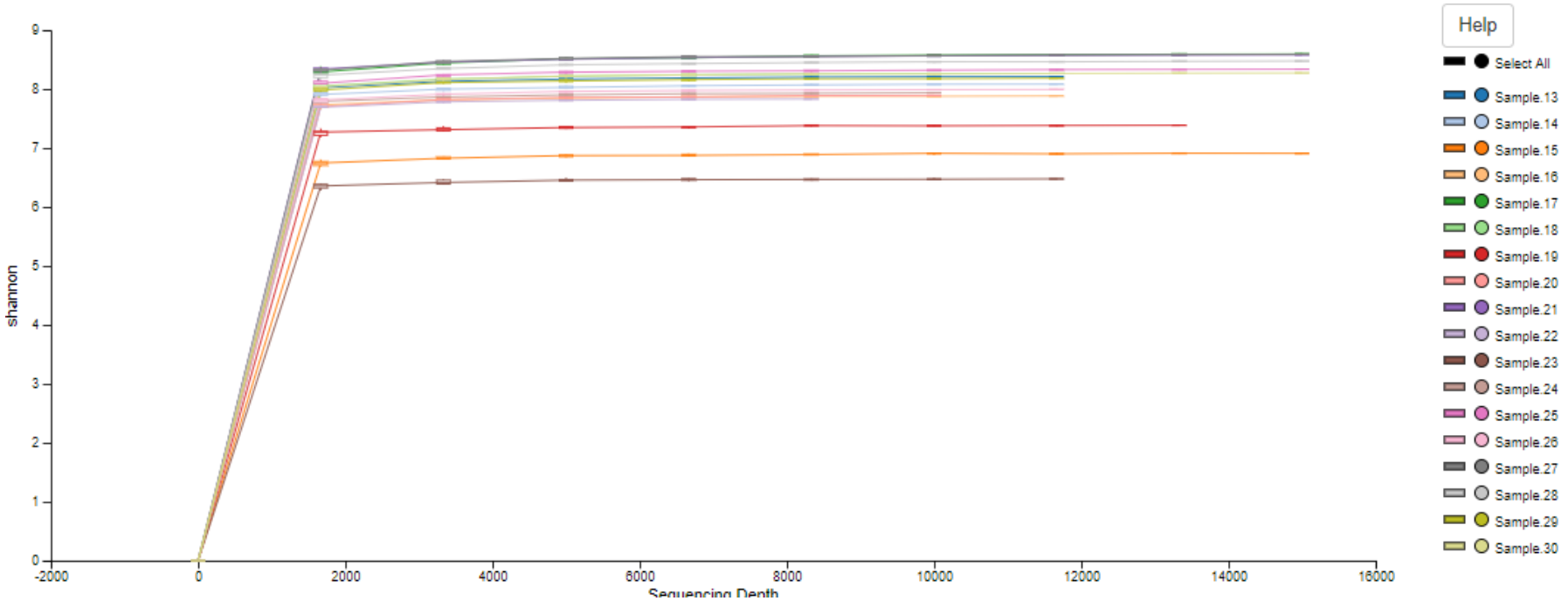
Rhizosphere to endosphere enrichment												
Family	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	UF13EA	UF13EB	UF13EC	UF13RZA	UF13RZB	UF13RZC
Bacillaceae	10635.09	4.209686	1.145645	-3.67451	0.000238	0.000745	427	1967	23259	3965	4126	3037
Polyangiaceae	1634.973	3.958297	0.735236	-5.38371	7.30E-08	6.63E-07	200	696	2819	870	636	499
Sphingobacteriaceae	660.3309	3.254433	0.588419	-5.53081	3.19E-08	3.54E-07	86	611	729	312	420	525
Comamonadaceae	3913.3	3.17519	0.645754	-4.91702	8.79E-07	5.86E-06	958	5102	1707	2948	3100	1954
Micromonosporaceae	384.4599	3.026698	0.793492	-3.81441	0.000137	0.000471	96	619	33	220	349	289
Cellvibrionaceae	324.8956	2.597896	0.614106	-4.23037	2.33E-05	9.72E-05	33	430	208	346	367	235
Cytophagaceae	3151.455	2.547296	0.546623	-4.66006	3.16E-06	1.86E-05	855	1712	3253	3533	2736	2976
Chthoniobacteraceae	418.419	2.523105	0.452025	-5.58178	2.38E-08	3.40E-07	99	373	314	446	313	474
Rhizobiaceae	979.0443	2.375026	0.578131	-4.10811	3.99E-05	0.00016	144	947	830	1460	1112	666
Chitinophagaceae	2299.218	2.328715	0.651818	-3.57264	0.000353	0.000982	600	820	2749	1963	2025	3546
Xanthomonadaceae	572.969	1.629636	0.624932	-2.6077	0.009115	0.017873	65	280	688	1333	911	609
Verrucomicrobiaceae	614.9336	1.381218	0.42636	-3.23956	0.001197	0.00292	207	303	363	1302	1055	1072
Oxalobacteraceae	660.7432	1.308362	0.438186	-2.98586	0.002828	0.005771	190	343	430	1319	1834	806
Planococcaceae	475.3605	1.303471	0.573219	-2.27395	0.022969	0.042535	49	508	245	847	1066	885

Rhizosphere ^{13}CL to ^{13}CH enrichment												
Family	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	13CHA	13CLA	13CHB	13CLB	13CHC	13CLC
Oxalobacteraceae	882.3909	6.866217	0.630972	-10.882	1.41E-27	6.88E-26	1133	11	1682	12	909	39
Enterobacteriaceae	1193.558	5.663078	1.200853	-4.71588	2.41E-06	1.47E-05	1305	132	42	13	2704	58
Verrucomicrobiaceae	1240.233	4.817514	0.470757	-10.2336	1.40E-24	4.58E-23	1705	193	1810	101	1437	74
Comamonadaceae	1557.064	4.810896	0.375168	-12.8233	1.21E-37	1.19E-35	2043	199	2508	118	1728	141
Fibrobacteraceae	247.2869	3.558584	0.645096	-5.51636	3.46E-08	3.77E-07	542	71	167	29	230	65
Paenibacillaceae	474.7391	3.450277	0.717541	-4.80847	1.52E-06	1.06E-05	165	183	1009	85	624	77
Cellvibrionaceae	122.6921	3.359934	0.60249	-5.57675	2.45E-08	3.00E-07	131	49	264	13	96	32
Pseudomonadaceae	640.9324	2.82797	1.152315	-2.45416	0.014121	0.034597	1363	663	196	23	679	38
Cytophagaceae	283.9659	2.520107	0.595382	-4.23276	2.31E-05	0.000113	543	212	334	88	171	68
Polyangiaceae	119.7725	2.42427	0.530117	-4.57309	4.81E-06	2.62E-05	201	87	140	38	89	38
Microbacteriaceae	144.6834	2.157009	0.693944	-3.10833	0.001881	0.006585	341	95	94	45	73	86
Rhizobiaceae	194.8885	1.676668	0.519658	-3.22648	0.001253	0.004549	245	222	288	99	116	83
Micrococcaceae	918.5754	1.673919	0.649494	-2.57727	0.009959	0.025268	1829	728	386	518	667	618

Rhizosphere ^{13}CL to ^{13}CH enrichment												
Family	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	13CHA	13CLA	13CHB	13CLB	13CHC	13CLC
BIrii41	111.3784	1.637668	0.487202	-3.36137	0.000776	0.00304	180	87	100	64	76	79
Xanthomonadaceae	167.3075	1.565205	0.537111	-2.91412	0.003567	0.011041	224	209	199	70	112	89
Intrasporangiaceae	158.6062	1.506893	0.517692	-2.91079	0.003605	0.011041	225	149	114	81	139	122

Rhizosphere ^{12}CH to ^{13}CH enrichment												
Family	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	12CHA	13CHA	12CHB	13CHB	12CHC	13CHC
Enterobacteriaceae	1525.585	9.225479	1.330868	6.931926	4.15E-12	8.30E-11	1	1305	22	42	14	2704
Paenibacillaceae	542.7148	6.131222	0.710965	8.623808	6.48E-18	1.62E-16	18	165	48	1009	33	624
Verrucomicrobiaceae	1540.476	5.525653	0.389862	14.17336	1.34E-45	1.34E-43	107	1705	137	1810	152	1437
Pseudomonadaceae	747.4943	5.296358	1.327441	3.989902	6.61E-05	0.000348	1	1363	36	196	222	679
Oxalobacteraceae	1121.798	5.093564	0.466396	10.9211	9.14E-28	3.05E-26	69	1133	259	1682	99	909
Cellvibrionaceae	143.5597	4.563866	0.692666	6.588845	4.43E-11	6.33E-10	7	131	18	264	52	96
Comamonadaceae	1965.096	4.553968	0.399632	11.39541	4.41E-30	2.20E-28	146	2043	504	2508	422	1728
Fibrobacteraceae	308.4267	4.250982	0.691292	6.149331	7.78E-10	9.73E-09	22	542	102	167	86	230
Rhodocyclaceae	104.054	3.649859	1.497193	2.437802	0.014777	0.037889	1	1	24	239	83	102
Rhizobiaceae	200.6142	3.586599	0.543772	6.595782	4.23E-11	6.33E-10	54	245	40	288	87	116
Cytophagaceae	333.3919	3.485163	0.695143	5.013594	5.34E-07	4.45E-06	27	543	242	334	122	171
Micrococcaceae	1002.92	3.188858	0.722796	4.411838	1.02E-05	6.41E-05	307	1829	368	386	525	667
Microbacteriaceae	182.5424	2.316547	0.708211	3.270982	0.001072	0.004466	101	341	138	94	130	73
Xanthomonadaceae	191.2474	2.210212	0.381146	5.798852	6.68E-09	6.68E-08	80	224	204	199	156	112
BIrii41	130.5447	2.202001	0.563662	3.906599	9.36E-05	0.000468	92	180	98	100	81	76
Roseiflexaceae	138.9378	1.739553	0.63377	2.744769	0.006055	0.01682	117	178	204	94	67	80
Intrasporangiaceae	196.5958	1.679542	0.52217	3.216465	0.001298	0.005191	147	225	253	114	176	139
Hyphomicrobiaceae	158.0022	1.556421	0.510171	3.050784	0.002282	0.00762	133	206	195	134	159	65
Polyangiaceae	174.8114	1.441128	0.521693	2.762408	0.005738	0.016393	141	201	152	140	277	89

S5a Shannon's diversity alpha rarefaction for paired end rhizosphere SIP data



S5b Shannon's diversity alpha rarefaction for paired end endosphere SIP data