Global Biogeography and Local Adaptation of *Streptomyces*

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Dedication

"It's a magical world, Hobbes 'ol buddy... Let's go exploring!"

-Calvin, from *Calvin and Hobbes*

I would like to dedicate this work to all those who have inspired in me a sense of wonder, curiosity, and adventure, and to those companions who join in the ride.

To my parents, Jan and Charlie, for making us play outside.

To my brothers, Lee and Max, for the many sheep roads to victory.

To Lacy, whose incredible patience and fortitude, as long as ample reading material is at hand, never cease to amaze.

To great friends, who continue to put up with me for some reason.

Table of Contents

List of Tables……………………………………………………………………..iv

List of Figures………………………………………………………………….....vi

Introduction………………………………………………………………………1

Chapter 1—Resource use of soilborne *Streptomyces* varies with location, phylogeny, and nitrogen amendment……………………………………………………………..6

Chapter 2— Tradeoffs structure antibiotic inhibition, resistance, and resource use among soil-borne *Streptomyces*………………………………………………………….......34

Chapter 3— *Streptomyces* inhibition and resource competition: Coevolutionary arms race and niche differentiation as alternative trajectories…………………………......54

Chapter 4— Evolutionary biogeography of *Streptomyces* antibiotic inhibition, resistance, and resource use…………………………………………………………....72

Chapter 5— Bacterial communities respond to plant species, plant community richness, soil characteristics, and microbial interactions in soil………………………………….100

Bibliography……………………………………………………………………..132

Appendix— Supplemental material………………………………………………...149

List of Tables

Chapter 1

Supplemental Table 1 – Plot and treatment of origin and accession numbers for isolates used in this study……………………………………………………………………149

Supplemental Table 2 – Resources most commonly used by *Streptomyces*………….156

Supplemental Table 3 – Resources used most efficiently by *Streptomyces*…………..157

Supplemental Table 4 – Soil characteristics and densities of plots…………………...157

Chapter 2

Table 1 – Correlations of *Streptomyces* inhibitory and resistance capacitites with soil properties……………………………………………………………………...……47

Chapter 3

Chapter 4

Table 1 – Description of sampling sites…………………………………………...……76

Table 2 – Antibiotic susceptibility of *Streptomyces* from different locatons…………...84

Supplemental Table 1 – Inhibition of standards by *Streptomyces* from different locatons…………………………………………………………………………………158

Supplemental Table 2 – Resource use of *Streptomyces* from different locatons……..159

Supplemental Table 3 – Origin and classification of isolates………………………...160

Chapter 5

Table 1 – Correlations of soil characteristics along non-metric multidimensional scaling axes…………………………………………………………………………………..120

Table 2 – Correlations of bacterial richness and diversity with soil characteristics…...121

Supplemental Table 1 – Abundances of major bacterial phyla……………………….166

Supplemental Table 2 – Abundances and classification of the largest OTUs……………………………………………………………………166(electronic)

Supplemental Table 3 – Abundances and classification of indicator OTUs for each plant species…………………………………………………………………166(electronic)

Supplemental Table 4 – Correlations among plant richness, biomass, and soil edaphic characteristics…………………………………………………………166(electronic)

Supplemental Table 5 – Correlation network module composition……...166(electronic)

Supplemental Table 6 – Correlations of major bacterial phyla and OTU abundances with soil characteristics…………………………………………………166(electronic)

List of Figures

Chapter 1

Figure 1 – Frequency distributions of Streptomyces niche widths……………………...15

Figure 2 – Resource use of Streptomyces from different locations and nitrogen treatments…………………………………………………………………………...18

Figure 3 – Non-metric multidimensional scaling of *Streptomyces* 16S rRNA gene sequences from non-amended and nitrogen amended communities……………………..20

Figure 4 – Resource use among *Streptomyces* OTUs…………………………….…. 22

Figure 5 – Resource use of *Streptomyces* OTUs among locations…………………......24

Figure 6 – Resource use of *Streptomyces* OTUs among nitrogen treatments……….…..26

Figure 7 – Relationship of *Streptomyces* resource use with soil carbon……………….…..27

Supplemental Figure 1 – *Streptomyces* phylogeny and heatmap of resource use……………………………………………………………………..157(electronic)

Chapter 2

Figure 1 – Frequency distribution of *Streptomyces* antibiotic inhibition and resistance capacities……………………………………………………………….…………...42

Figure 2 – Correlations between *Streptomyces* resource use and antibiotic inhibition and resistance capacities……………………………………………………………………...44

Figure 3 – Resource use among *Streptomyces* with distinct antibiotic inhibition and resistance strategies…………………………………………………………………45

Chapter 3

Figure 1 – Proportion of inhibitory interaction among sympatric *Streptomyces*………..61

Figure 2 – Intensity of inhibition among sympatric and allopatric *Streptomyces*………62

Figure 3 – Intensity of inhibition among sympatric and allopatric *Streptomyces* among locations in soil……………………………………………………………………..63

Figure 4 – Proportion of sympatric and allopatric resistant interactions………………..64

Figure 5 – Relationship between niche overlap and inhibition intensity among sympatric *Streptomyces*……………………………………………………………………………..65

Chapter 4

Figure 1 – Frequency distribution of *Streptomyces* inhibition capacities…………..…..80

Figure 2 – Proportion of *Streptomyces* resistant to clinical antibiotics………………….81

Figure 3 – Streptomyces antibiotic inhibition, resistance, and niche width among locations……………………………………………………………………………………83

Figure 4 – Niche overlap within *Streptomyces* communities from different locations…88

Figure 5 – Variation in inhibition, resistance, and resource use among Streptomyces belonging to the same OTU from different locations…………………………………...92

Supplemental Figure 1 – Phylogenetic tree of *Streptomyces* used in this chapter…..164

Chapter 5

Figure 1 – Similarity of bacterial communities from plant species and diversity treatments…………………………………………………………………………….111

Figure 2 – Bacterial diversity among plant richness treatments…………………….....115

Figure 3 – Dissimilarity in bacterial communities from the same and different plant host across plant richness treatments…………………………………………….....……117

Figure 4 – Non-metric multidimensional scaling of bacterial communities……..….…119

Figure 5 – Co-occurrence network and modulation of bacterial OTUs……………..…123

Figure 6 – Correlations of OTU abundances belonging to modules with soil and plant characteristics………………………………………………………………………..124

Figure 7 – Relationship between proportion of antagonists and bacterial diversity......126

Introduction

Soil microbial communities play crucial roles in key ecosystem processes such as nutrient cycling and availability, decomposition, and plant disease in natural and agricultural systems (Garbeva et al., 2004; Van Der Heijden et al., 2008). *Streptomyces* are believed to be important players in many of these processes, especially plant disease suppression (Menzies et al., 1959; Weller et al., 2002; Kinkel et al., 2012). The primary goals of my work are to expand our knowledge of *Streptomyces* diversity at both local (field) and global scales and shed light on the roles of local adaptation and coevolution in structuring *Streptomyces* phenotypes in soil communities. Specifically, this work focuses on three *Streptomyces* phenotypes believed to be critical to bacterial fitness and species interactions in soil: antibiotic inhibition, antibiotic resistance, and resource use.

Streptomyces are a large and diverse genus of filamentous, Gram (+) bacteria with complex lifestyles that are found ubiquitously in soils and sediments across the globe (Seipke et al., 2011; Kinkel et al., 2012). *Streptomyces* exist primarily as hardy, desiccation-resistant spores that can remain viable for many decades in soil (Morita, 1985; Keiser et al., 2000). However, when *Streptomyces* encounter favorable growth conditions and appropriate resources (Ensign, 1978), spores germinate and grow as filamentous vegetative hyphae to form a mycelium similar to fungi. When resources are exhausted, *Streptomyces* substrate mycelia undergo programmed cell death that provides nutrients for the formation of aerial hyphae (Chater, 2006). To complete the life cycle, aerial hyphae then differentiate into chains of spores. *Streptomyces* are non-motile and rely largely on wind, rain-splash, dust, and arthropods for long-distance dispersal (Lloyd,

1969; Seipke et al., 2011). Importantly, once established, *Streptomyces* are unable to avoid unfavorable conditions and must engage directly with the diverse biotic and abiotic environments that they encounter in soil.

In order to interact with coexisting microbes in soil communities *Streptomyces* produce a vast array of antibiotic compounds to inhibit competitors (Williams and Vickers, 1987; Hibbing et al., 2010; Kinkel et al., in press) or engage in chemical communication (Vaz Jauri et al., accepted; Goh et al., 2002; Yim et al., 2007; Romero et al., 2011). Notably, *Streptomyces* are the producers of the majority of naturally-occurring clinical antibiotics (Tanka and Omura, 1990). Additionally, the production of antibiotics is often credited to be the mechanism by which *Streptomyces* are able to suppress a variety of fungal (Weller et al., 2002; Hjort et al., 2010; Mendes et al., 2011), bacterial (Lorang et al., 1989; Meng et al., 2012), and nematode (Zuckerman et al., 1989) plant pathogens. In the best-studied cases of potato scab suppressive soils, suppressive soils are characterized by soil *Streptomyces* populations with high frequencies, intensities, and diversities of antibiotic phenotypes (Wiggins and Kinkel, 2005a, b; Kinkel et al., 2012). However, despite their importance in clinical and agricultural settings, there have been few systematic studies of the roles that antibiotics play in species interactions among *Streptomyces* or how antibiotic phenotypes vary across geographic locations.

In addition to inhibiting competitors, *Streptomyces* may cope with antibiotic-producing competitors by resisting inhibition (Hibbing et al., 2010). Indeed, *Streptomyces* are commonly resistant to a broad suite of natural and synthetic antibiotic compounds and are important contributors to the total antibiotic resistome of soils (D’Costa et al., 2006).

Moreover, the acquisition or evolution of antibiotic resistance genes within soil communities may drive a coevolutionary arms race between inhibition and resistance interactions of competing *Streptomyces*, which may play a role in generating the substantial diversity in antibiotic resistance and inhibition phenotypes observed among *Streptomyces* (Czárán et al., 2002; Davelos et al., 2004a; Kinkel et al., 2011; Kinkel et al., in press). Thus, understanding the forces that select for and generate diversity in antibiotic resistance phenotypes among *Streptomyces* will be especially important in the face of growing concern about environmentally-acquired antibiotic resistance in clinical pathogens and the potential presence of diverse resistance genes in agricultural systems.

In order to acquire resources in oligotrophic soil environments, *Streptomyces* employ a diverse array of degraditive enzymes to break down complex and often recalcitrant organic substrates, including cellulose, lignin, and chitin (Chater et al., 2010; Schrempf et al., 2011). In this way, *Streptomyces* are able to occupy diverse niches in soils and contribute to decomposition and the global carbon cycle (McCarthy and Williamson, 1992). Competition among organisms with similar resource use niches can play a major role in community assembly, diversity, and function (Tilman, 1982). Indeed, managing resource inputs, which likely affects competitive interactions, has been an attractive approach for enhancing the activities of indigenous *Streptomyces* communities in laboratory and field settings (Wiggins and Kinkel, 2005a, b; Schlatter et al., 2009; Mazzola and Zhao, 2010). However, there are few data on the diversity of *Streptomyces* resource use niches within or among communities, or how resource phenotypes respond to amendments.

Chapter 1 of this thesis describes a detailed study of the resource use niche of *Streptomyces*. Specifically, we investigate the diversity of resource use phenotypes among *Streptomyces*, how they are distributed among communities within a single field site, the association between resource use and phylogeny, and the impacts of nitrogen amendment history on resource use.

Chapter 2 explores the role of tradeoffs between resource use and the accumulation of antibiotic inhibition and resistance capacities among *Streptomyces*. Specifically, we investigate the distribution of antibiotic inhibition and resistance capacities among *Streptomyces* and the relationships of resource use with antibiotic inhibition and resistance.

Chapter 3 investigates antibiotic inhibitory interactions among *Streptomyces* from the same and different locations in soil to assess coevolution as a driver of antibiotic inhibition and resistance among *Streptomyces*. This chapter explores resource competition (niche overlap) among coexisting *Streptomyces* from the same and different locations as a predictor of inhibition among interacting *Streptomyces*.

Chapter 4 expands on previous work by exploring the diversity of *Streptomyces* at a global scale and investigating biogeographic patterns in antibiotic inhibition, resistance, and resource use traits among *Streptomyces*. Moreover, this chapter addresses the role of adaptation and phylogeny in determining antibiotic inhibition, resistance, and resource use among a global collection of *Streptomyces*.

Chapter 5 evaluates the roles that plant species, plant community richness, soil

edaphic characteristics, spatial distance, and the antagonistic activity of soil *Streptomyces* play in structuring the composition and diversity of rhizosphere bacterial communities.

Chapter 1: Resource use of soilborne *Streptomyces* varies with location, phylogeny, and nitrogen amendment.

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Background

Recent advances in microbial ecology have greatly expanded our knowledge of microbial species distributions, community composition, and diversity. However, the ecological niches of microbial species within soil communities remain poorly characterized. Resource use is a critical aspect of an organism's ecological niche that can influence competitive interactions among species and the assembly, diversity, and functioning of communities (Tilman, 1982; Loreau and Hector, 2001; Tilman, 2004). Variation in resource use among soil bacteria is hypothesized to reflect adaptation to resource availability (Barret et al., 2005; Schlatter et al., 2009), microbial or plant-microbe interactions (Folman et al., 2001; Craig MaClean et al., 2005; Lawrence et al., 2012), or general life history strategies (eg. generalist vs specialist)(Garbeva et al., 2004;

Fierer et al., 2007). However, few studies have explored variation in resource use within natural bacterial populations in soil. Consequently, we have limited insight into the natural history of microbial resource use patterns and the environmental or management factors that influence variation in resource use among specific groups of soil microbes.

Streptomyces (phylum Actinobacteria) are filamentous, Gram-positive bacteria that are of great interest in agricultural systems for plant disease suppression (Wiggins and Kinkel, 2005a; Wiggins and Kinkel, 2005b) and in clinical settings as major producers of antibiotic compounds (Clardy et al., 2006). Additionally, *Streptomyces* employ an array of extracellular enzymes in order to break down complex resources, including cellulose, lignin, and chitin (Hodgson, 2000; Williamson et al, 2000; Chater et al., 2010; Schrempf et al., 2011). Because of their tremendous metabolic capacities, *Streptomyces* can occupy a wide variety of ecological niches in nature (Seipke et al., 2011; Kinkel et al., 2012) and are an ideal taxon for exploring variation and adaptation of resource use among soil bacteria. However, despite the long history of using resource use patterns for bacterial taxonomy, there are few systematic data on variation in resource use phenotypes among *Streptomyces* populations from an ecological perspective or correlates of resource use among natural *Streptomyces* communities.

Streptomyces are well known for their prolific production of antibiotics, which are thought to mediate species interactions (Williams and Vickers, 1986; Slattery et al., 2001; Davelos et al., 2004a). Antibiotic production by *Streptomyces* is often tied to the amount and identity of available resources (Rigali et al., 2008; Sánchez et al., 2010), consistent with the hypothesis that antagonistic interactions occur during resource competition.

Though spatial variation in antibiotic phenotypes among *Streptomyces* populations has been documented (Davelos et al., 2004a), little is known about how resource use varies among *Streptomyces* communities from different locations. Resource inputs and management practices have been implicated in selection for resource use and antibiotic inhibitory phenotypes among soil-borne *Streptomyces* (Schlatter et al., 2009; Wiggins and Kinkel, 2005a,b). Nitrogen (N) inputs from agricultural fertilizers or atmospheric deposition can significantly alter the composition of soil microbial communities and reduce soil enzyme activities, respiration rates, and decomposition (Frey et al., 2004; Eisenlord and Zak, 2010; Ramirez et al., 2010; Fierer et al., 2012; Ramirez et al., 2012). In this manner, N additions may alter the capacity of soils to serve as sinks for carbon storage (Frey et al., 2004; Carreiro et al., 2000). However, the impact of N-amendments on *Streptomyces* resource use has not been explored and it remains unknown if changes in soil bacterial community function associated with N-amendment result from shifts in community phylogenetic composition, adaptation of resource use preferences of resident communities to high N environments, or direct effects of N-amendment on bacterial activities in vivo (Fierer et al., 2012; Ramirez et al., 2012; Gallo et al., 2004; Otto-Hanson et al., 2013). More detailed information on *Streptomyces* resource use patterns and how they respond to long-term N-amendment will offer unique insight into the potential for resource competition within and among communities and enhance our ability to predict the effects of N-amendment on soil communities.

In this work we characterize *Streptomyces* collected from prairie soils to ask 1) how does resource use vary among *Streptomyces* isolates?; 2) is variation in resource use

among *Streptomyces* associated with space, phylogeny, or N-amendment history?; and 3) do soil edaphic characteristics or *Streptomyces* population densities correlate with resource use phenotypes among soil *Streptomyces* populations? These data provide important insight into variation in resource use niches among *Streptomyces* populations in soil and suggest that local adaptation, resource competition, and N-amendment impact *Streptomyces* resource use.

Materials and Methods

Soil Sampling and Processing

Soil samples were collected from long-term N-amended and non-amended plots (experiment E001) at the University of Minnesota Cedar Creek Ecosystem Science Reserve (www.cedarcreek.umn.edu), a NSF Long-Term Ecological Research site in East Bethel, MN. In this experiment N-fertilization (NH_4NO_3) treatments have been applied to field plots twice a year in early May and late June since 1982 (18 years prior to sampling). Six of these plots (4x4 m) were chosen for sampling so that three N-amended plots receiving the same NH_4NO_3 treatment were paired in space with three non-amended plots. Non-amended plots (E001 Field C plots 08-A, 26-A, and 47-A, referred to here as plots 1, 3, and 5, respectively) received a base nutrient treatment that lacks a source of N (10 g/m^2 P_2O_5, 10 g/m^2 K_2O, 20 g/m^2 $CaCO_3$, 15g/m^2 $MgSO_4$, and 0.0625 ml/m^2 trace mineral solution). N-amended plots (E001 Field C plots 10-D, 19-D, and 46-D, referred to here as 2N, 4N, and 6N, respectively) received the base nutrient treatment plus an

additional nitrogen treatment (NH_4NO_3 at 5 g/m^2) that has been found to impact plant diversity (Clark and Tilman, 2008).

Soil cores (3 tightly bundled 30 x 1 cm micro-cores) were taken from three randomly chosen locations within a 1 x 1 m section at the center of each plot. Soil samples from each micro-core were dried overnight under a double layer of sterile cheesecloth, dilution plated onto oatmeal agar amended with antibiotics (300 ug/L nystatin, 50 ug/L cycloheximide, 2.5 ug/L polymyxin B, 0.2 ug/L penicillin) , and incubated at 28 C for 7 days. Detailed description of micro-core sampling and culturing conditions can be found in (Davelos et al., 2004a). For each micro-core (n=54 cores), total culturable streptomycete densities were estimated (CFU/g soil) and twenty randomly selected *Streptomyces* colonies were isolated based on characteristic *Streptomyces* morphology for a total of ~1080 isolates. Streptomycete densities were averaged across micro-cores for each location and plot for subsequent analyses. From this collection, 459 *Streptomyces* isolates were characterized for resource utilization phenotypes. Most isolates characterized were from plots 1 and 2N (n=152 and 128, respectively); fewer isolates were characterized from plots 3, 4N, 5, and 6N (plot 3, n=47; plot 4N, n=47; plot 5, n=46; plot 6N, n=39). Isolates for each location within plots were chosen randomly. This approach allows for the characterization of resource use phenotypes among *Streptomyces* within and among plots. Sub-samples of micro-cores from each sampling location within each plot were bulked and submitted to the University of Minnesota Soil Testing Laboratory for determination of soil characteristics (pH, NO_3-N, Bray-P, K, and total C; http://ral.cfans.umn.edu).

Resource use characterization

Resource use phenotypes were determined for *Streptomyces* isolates on 95 sole carbon sources using Biolog SF-P2 plates (Biolog, Inc. Hayward, CA) as described previously (Schlatter et al., 2009). Briefly, fresh spore suspensions of each *Streptomyces* isolate were adjusted to an optical density of 0.22 at 590 nm, diluted according to the manufacturer's instructions (1.5mL spore suspension in 13.5 mL 0.2% carrageenan), and inoculated into 96-well Biolog plates. The absorbance (au) of each well was determined after 3 days of incubation at 28 C using a Multiskan EX microplate reader (Labsystems, Helsinki, Finland) at 590 nm. For each plate, the absorbance of the water control well was subtracted from the absorbance of all other wells before analysis.

16S rRNA Gene Sequencing

The 16S rRNA gene was sequenced for a random subset of 323 of the 459 isolates as described previously (Davelos et al., 2004c) (plot 1, n=146; plot 2N, n=51, plot 3, n=39; plot 4N, n= 27; plot 5, n= 42; plot 6N, n=18). Briefly, genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega. Madison, WI) according to the manufacturer's instructions with minor modification and 16S rRNA genes were amplified using the universal bacterial primers 27F (5'- AGAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCCGCA-3') in a 50ul reaction volume using PCR Supermix High Fidelity master mix (Invitrogen, Carlsbad, CA) with 10pM each primer and 100 ng template DNA following the thermocycling protocol of Takeuchi et al.,

(1996). Amplicons were sequenced using the forward primer (27F) at the University of Minnesota Biomedical Genomics Center (Saint Paul, MN). Sequences were edited manually, aligned, and trimmed to 703 bp of good quality alignment using BioEdit (Hall, 1999) for further analyses. Sequence accessions are available in GenBank (Supplemental Table 1).

Analyses

Resource use: We considered used resources to be those on which a *Streptomyces* isolate grew to an absorbance greater than 0.005 above the water control well. Using this definition, niche width, resource use efficiency, and efficiency on preferred resources were determined for each isolate. We defined the niche width of an isolate as the number of used resources, the resource use efficiency of each isolate was defined as the mean absorbance value for used resources, and the efficiency on preferred resources was defined by calculating the mean absorbance on the 5 resources on which each isolate grew best (largest absorbance values). Similarity in resource use profiles among *Streptomyces* isolates was calculated using the Bray-Curtis index in the vegan package for R (Oksanen et al., 2011). All subsequent statistical analyses were conducted in R version 2.14 (R Development Core Team, 2011).

Phylogenetic analyses: 16S rRNA gene sequences were used to compute pairwise distances between isolates and construct a neighbor-joining (NJ) tree using 1000 bootstraps. The NJ tree was analyzed using the unweighted unifrac metric and P-test using 1000 bootstraps to test for significant differences in *Streptomyces* community structure among different plots and locations and among nitrogen treatments. Non-metric

multidimensional scaling (NMDS) was conducted on the distance matrix to visualize similarity among isolates. Further, isolates were binned into operational taxonomic units (OTUs) at 99% sequence similarity using mothur version 1.22 (Schloss et al., 2009).

Comparing resource use and phylogeny: A Mantel test was performed to test for a relationship between genetic distance and resource use phenotypes. The distance matrix of 16S sequences and the Bray-Curtis dissimilarity of resource use profiles of *Streptomyces* isolates were correlated using 999 permutations in the vegan software package of R. Also, niche width, resource use efficiency, and efficiency on preferred resources were compared among large OTUs ($n \geq 16$) and among isolates from the same OTU but from different plots and locations.

Results

Resource use among soil-borne Streptomyces

Streptomyces grew on a wide variety of carbohydrates, carboxylic acids, polymers, amines/amides, and amino acids as sole carbon sources. Glycerol was most frequently used as a sole carbon source overall (used by 98% of isolates), followed by alpha-D-glucose and adenosine (each used by 96% of isolates; Supplemental table 2). However, the best growth on average occurred on Tween 40 and L-Malic acid, followed more distantly by glycerol (Supplemental table 3). On average, *Streptomyces* isolates used 68.7 of 95 possible resources, though niche widths (the number of resources used for growth) among individual *Streptomyces* demonstrated substantial variability and ranged from 11 to 95 resources. Frequency distributions of niche widths for each plot

(Figure 1) tend to be skewed left: *Streptomyces* communities were generally composed of many generalists with relatively large niche widths and fewer specialists with narrow niche widths. Across all isolates, resource use efficiency (the mean growth achieved across all used resources) ranged from 0.06 to 0.11 au with an average growth efficiency of 0.08 ±0.02 au. Growth efficiency on preferred resources (the mean growth achieved on the top 5 resources for each isolate) ranged from 0.93 to 1.91 au with an average of 1.34 ±0.33 au. Thus, the catabolic potential of *Streptomyces* varies substantially among individual isolates. Moreover, resource use patterns for individual *Streptomyces* were very diverse. Considering used resources using a discrete (+/-) approach, there were 453 unique patterns of resource utilization among 459 isolates. These data highlight the wide diversity of *Streptomyces* and their capacity to metabolize a range of resource sources and suggest that *Streptomyces* have substantial potential to adapt to different resource conditions in soil.

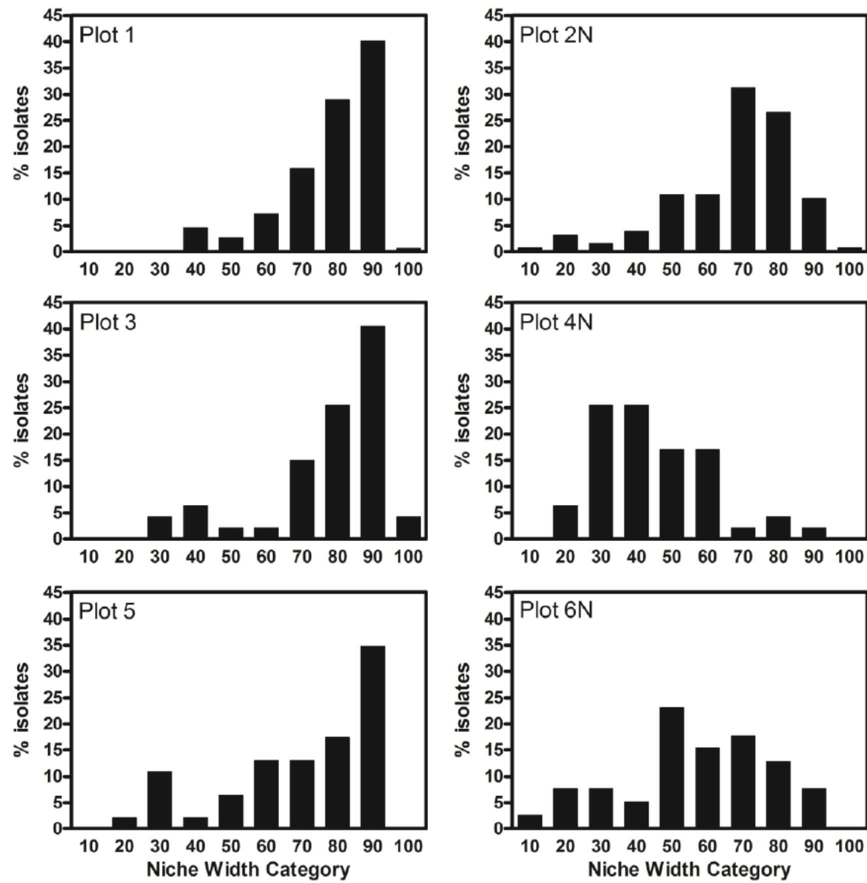

Fig. 1: Frequency distributions of *Streptomyces* niche widths among plots. Isolates from non-amended plots (plots 1, 3, and 5) tended to have larger niche widths than isolates from N-amended plots (plots 2N, 4N, and 6N).

Spatial variation in Streptomyces *resource use*

Among individual *Streptomyces*, isolates from the same plots had more similar resource use phenotypes than *Streptomyces* from different plots (mean Bray-Curtis dissimilarity= 0.43 and 0.52 for same vs. different plots, respectively; Welch's t-test $p<0.0001$, $t=81.22$). Within plots (sampling locations <1 m^2 apart) *Streptomyces* from the same location consistently had more similar resource use phenotypes than those from

different locations in that plot (Welch's t-test of mean Bray-Curtis dissimilarity among locations vs. within location: Plot 1, $p<0.0001$, $t=5.20$; Plot 2N, $p<0.0001$, $t=4.53$; Plot 3, $p<0.0001$, $t=8.76$; Plot 4N, $p=0.058$, $t=1.90$; Plot 5, $p<0.0001$, $t=4.50$), though differences were not statistically significant for *Streptomyces* from plot 6N ($p=0.61$, $t=0.51$). Thus, isolates from the same locations in soil were more likely to be able to metabolize the same resources and use them at similar growth efficiencies when compared to isolates from different locations.

Niche width, resource use efficiency, and growth efficiency on preferred resources varied among spatially distinct *Streptomyces* communities. Niche width differed significantly among *Streptomyces* from different plots (Figure 2a; ANOVA: $F=36.8$, $p<0.0001$). *Streptomyces* from non-amended plots 1 and 3 had the largest niche widths on average, while those from N-amended plots 4N and 6N used the fewest resources. There were also significant differences in mean niche widths among *Streptomyces* communities from different locations within the same plot for 4 of the 6 plots (plots 1, 2N, 3, and 4N, but not 5 or 6N; ANOVA, $p\leq0.03$, $F\geq3.57$; $p\geq0.09$, $F\leq2.57$, respectively). Similarly, mean resource use efficiency varied among *Streptomyces* from different plots (Figure 2b; ANOVA: $F=35.0$, $p<0.0001$). *Streptomyces* from plots 3, 5, and 6N grew more efficiently than those from plots 1, 2N, and 4N (TukeyHSD, $p\leq0.05$). However, there were no significant differences in mean growth efficiency of *Streptomyces* among locations within plots (data not shown). When considering only the 5 most-preferred resources for each isolate, *Streptomyces* from plots 1 and 2N were less efficient than *Streptomyces* from all other plots (Figure 2c: ANOVA, $F=45.85$, $p<0.0001$;

TukeyHSD, $p<0.0001$). Further, among locations within plots there were significant differences in growth efficiency on preferred resources for plots 3 (ANOVA, $F=10.27$, $p=0.0002$) and 5 (ANOVA, $F=11.77$, $p<0.0001$), but not plots 1, 2N, 4N, or 6N (ANOVA, $p\geq0.14$, $F\leq2.12$ for each plot). Together these data demonstrate variation in *Streptomyces* resource use phenotypes at spatial scales ranging from <1 m^2 to 50 m^2.

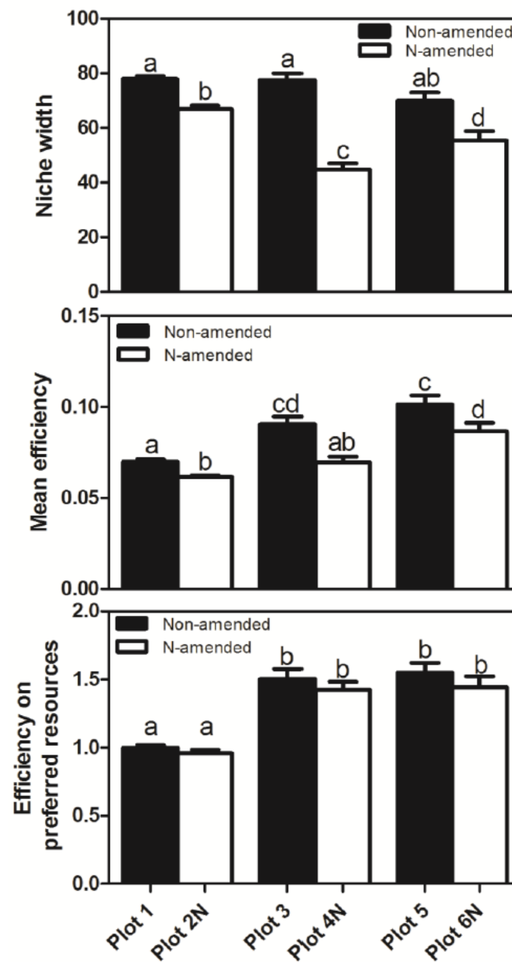

Fig. 2: Mean niche width (top row), mean growth efficiency (middle row), and efficiency on preferred resources (bottom row) of *Streptomyces* from different plots in soil. Non-amended plots (black bars) are grouped with their paired N-amended plot (white bars). Significant differences ($p<0.05$) among plots are indicated by different letters above bars and letters found in common above bars indicate no significant difference ($p>0.05$).

Long-term nitrogen amendment and Streptomyces *resource use*

Long-term N-amended plots had *Streptomyces* communities that differed significantly in resource use from communities from non-amended plots. Among paired

plots (1 and 2N; 3 and 4N; 5 and 6N), *Streptomyces* from N-amended plots had consistently smaller average niche widths than those from non-amended plots (Figure 2a; Welch's t-test, $p \leq 0.002$ and $t \geq 3.16$ for each plot pair). *Streptomyces* from N-amended plots used from 14-42% fewer resources than *Streptomyces* from their paired non-amended plot. Moreover, *Streptomyces* communities from N-amended plots also had significantly reduced resource use efficiency compared to communities from non-amended plots (Figure 2b; Welch's t-test, $p \leq 0.03$ and $t \geq 2.12$ for each pair), with mean growth efficiency over all used resources reduced 12-21% in N-amended plots. *Streptomyces* from N-amended plots also grew consistently less efficiently on preferred resources than those from paired non-amended plots, though differences were not statistically significant (Figure 2c; Welch's t-test, $p \geq 0.17$, $t \geq 1.39$). Additionally, *Streptomyces* from the same nitrogen treatment (N-amended and non-amended) but different plots had significantly more similar resource use phenotypes than those from different treatments (Bray-Curtis dissimilarity =0.49 and 0.52, respectively, $p<0.0001$, $t=32.26$). These results indicate that N-amended soils support *Streptomyces* communities that use fewer resources, have distinct resource use profiles, and grow less efficiently than communities in non-amended soils.

Streptomyces phylogeny

Streptomyces community composition varied among plots and among locations within plots (unweighted unifrac and P-test, $p<0.001$). Moreover, N-amended and non-amended communities differed significantly in their phylogenetic composition

(unweighted unifrac and P-test, p<0.001; Figure 3). Thus, *Streptomyces* community composition in soils varied across small spatial scales and soil communities under long-term N amendment supported *Streptomyces* that were phylogenetically distinct from those found among non-amended soils.

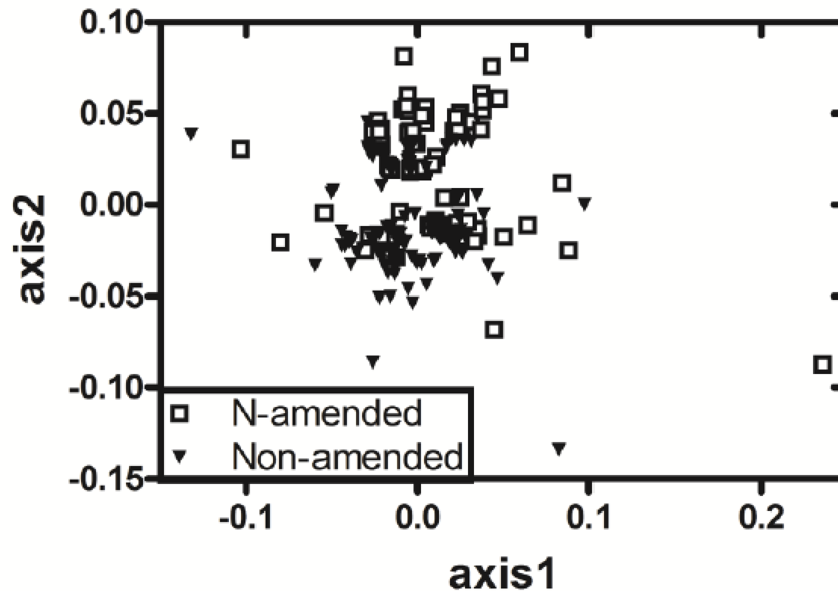

Fig. 3: Non-metric multidimensional scaling (NMDS) plot of 16S rRNA gene sequences (Bray-Curtis dissimilarity) among *Streptomyces* from N-amended (open squares, n=96) and non-amended (closed triangles, n=227) plots.

Relationships between resource use and phylogeny

Isolates that were more closely related had more similar resource use phenotypes (Supplemental figure 1). Among all isolate pairs (n= 52,003 pairwise combinations) there was a significant positive correlation between 16S sequence distance and dissimilarity in resource use patterns (Mantel r=0.24, p=0.001). The 323 *Streptomyces* isolates for which the 16S rRNA gene was sequenced formed 66 OTUs at a 99% similarity cutoff.

Streptomyces OTUs differed in their capacity to utilize resources. Among the 5 largest OTUs (Figure 4, n≥16 isolates per OTU) there were significant differences in mean niche width (ANOVA, p=0.002, F=4.48), mean resource use efficiency (ANOVA, p<0.001, F=24.3), and mean efficiency on preferred resources (ANOVA, p<0.001, F=21.6). Moreover, among all non-unique OTUs (n>1 isolates per OTU), *Streptomyces* from the same OTU had significantly more similar resource use phenotypes to each other than to isolates from different OTUs (Welch's t-test, p<0.0001, t=47.53; Bray-Curtis index=0.37 vs 0.48, respectively). Thus, some *Streptomyces* OTUs could use a broader array of resources (eg. OTUs 3 and 4) or grow more efficiently (eg. OTUs 3 and 16) than others, suggesting that OTUs may have different life history strategies and be adapted to distinct ecological niches in the soil environment.

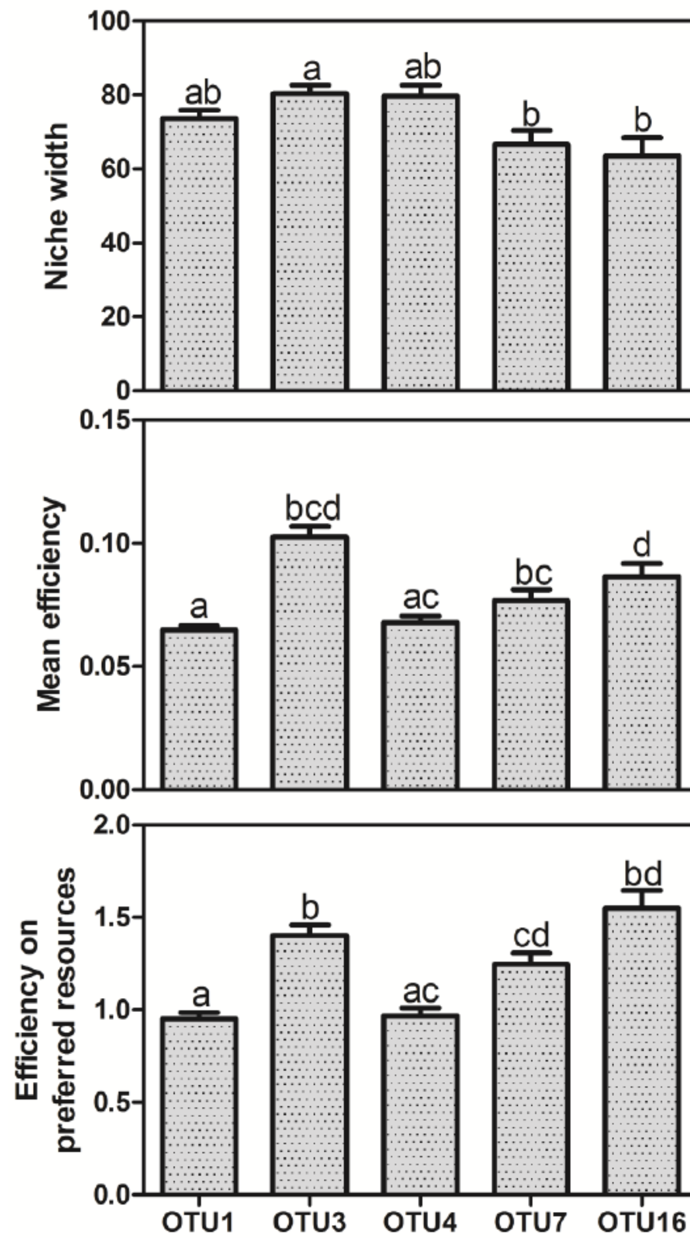

Fig. 4: Niche width (top row), mean growth efficiency (middle row), and growth efficiency on preferred resources (bottom row) among the 5 largest *Streptomyces* OTUs. In each category, significant differences among OTUs are indicated by different letters above bars ($p<0.05$) and letters found in common above bars indicate no significant difference ($p>0.05$).

There was also extensive variation in resource use within individual OTUs. For example, niche widths ranged from 11 to 93 of 95 possible resources within OTU 3. *Streptomyces* belonging to the same OTU but originating from different plots differed significantly in resource use phenotypes. When resource use phenotypes among four OTUs with $n \geq 4$ *Streptomyces* each from at least two plots were compared (OTUs 1, 3, 7, and 16), there were significant differences in resource use phenotypes among *Streptomyces* from different plots for 3 of the 4 OTUs (Figure 5). Growth efficiency on preferred resources varied among plots for 3 *Streptomyces* OTUs (OTUs 1, 3, and 7), whereas mean growth efficiency and niche width differed among plots for a single OTU each (OTUs 3 and 7, respectively). Additionally, within each of the four OTUs, *Streptomyces* from the same plot had significantly more similar resource use patterns to each other than *Streptomyces* from different plots (Welch's t-test of mean Bray-Curtis index; $p < 0.003$, $t \geq 3.04$ for each OTU). This suggests that variation in resource use phenotypes within *Streptomyces* genetic groups is associated with space and that closely related *Streptomyces* are differentially adapted to local environments across the landscape.

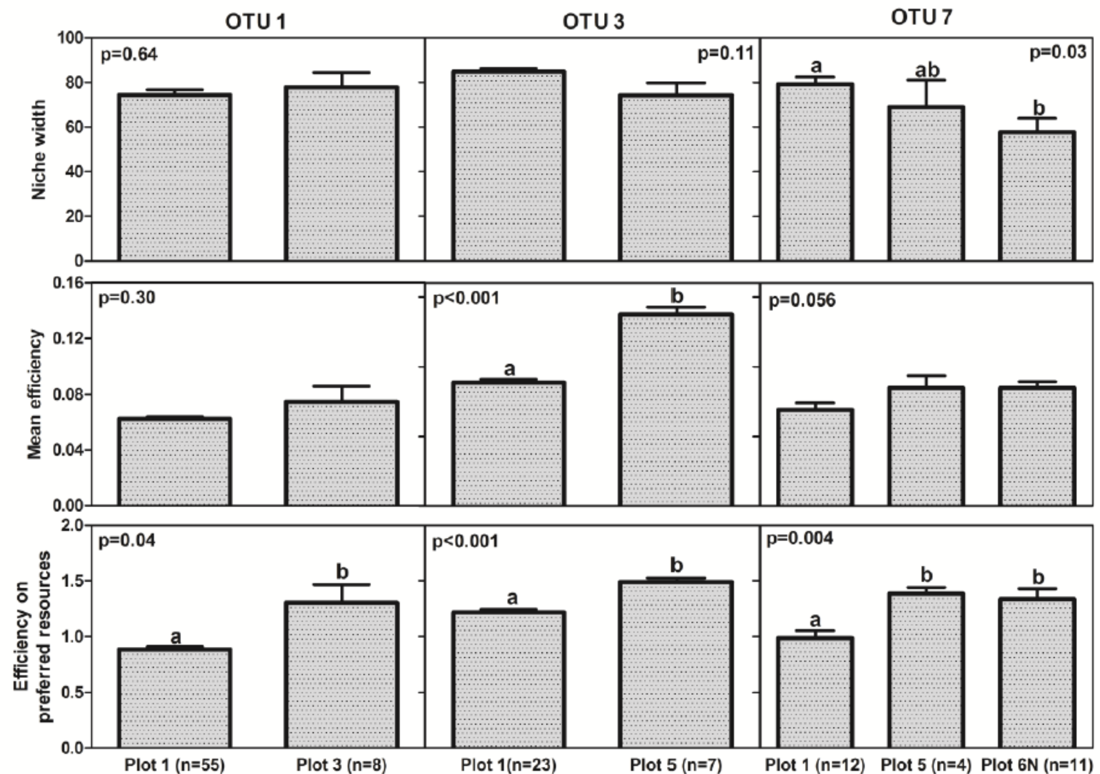

Fig. 5: Niche width (top row), mean growth efficiency (middle row), and growth efficiency on preferred resources (bottom row) among *Streptomyces* from the same OTU but isolated from different plots. P-values for OTUs 1 and 3 are from Welch's t-test between *Streptomyces* from different plots. P-values for OTU 7 represent an ANOVA comparing *Streptomyces* among plots 1, 5, and 6N. Within each category, different letters above bars represent significant differences ($p<0.05$) among plots for each OTU and letters found in common above bars indicate no significant difference ($p>0.05$).

Because N-amended plots differed in composition from non-amended plots, there were only two OTUs (OTU 7 and OTU 16) with sufficient numbers of *Streptomyces* isolates in both treatments to test for differences in resource use across nitrogen treatments among isolates from the same OTU (Figure 6). Among *Streptomyces* belonging to OTU 7 (N-amended, n=14; non-amended, n=17), isolates from N-amended

plots were significantly more efficient over all resources (Welch's t-test $p=0.03$, $t=2.30$) and on preferred resources (Welch's t-test, $p=0.03$, $t=2.36$), but had smaller niche widths than those from non-amended plots (Welch's t-test, $p=0.002$, $t=3.47$). Similarly, niche widths among *Streptomyces* from OTU 16 (N-amended, $n=10$; non-amended, $n=14$) were significantly smaller among isolates from N-amended plots than those from non-amended plots (Welch's t-test, $p=0.03$, $t=2.41$). However, mean efficiency and efficiency on preferred resources did not differ significantly between treatments for isolates in OTU 16 (Welch's t-test, $p \geq 0.12$, $t \leq 1.69$ in each case). Thus, N-amendment consistently selected for more narrow niche widths among *Streptomyces* from the same 16S group but had variable effects on resource use efficiency. Further, when resource use patterns among isolates from the same OTU were compared between N-treatments, resource use patterns were significantly more similar among isolates from the same vs different treatments (Welch's t-test, $p<0.0001$, $t=6.20$ and $p=0.0015$, $t=3.21$ for OTU7 and OTU16, respectively). These data suggest that even when the same OTU is considered, resource use patterns among *Streptomyces* shift significantly in soils under long-term N-amendment.

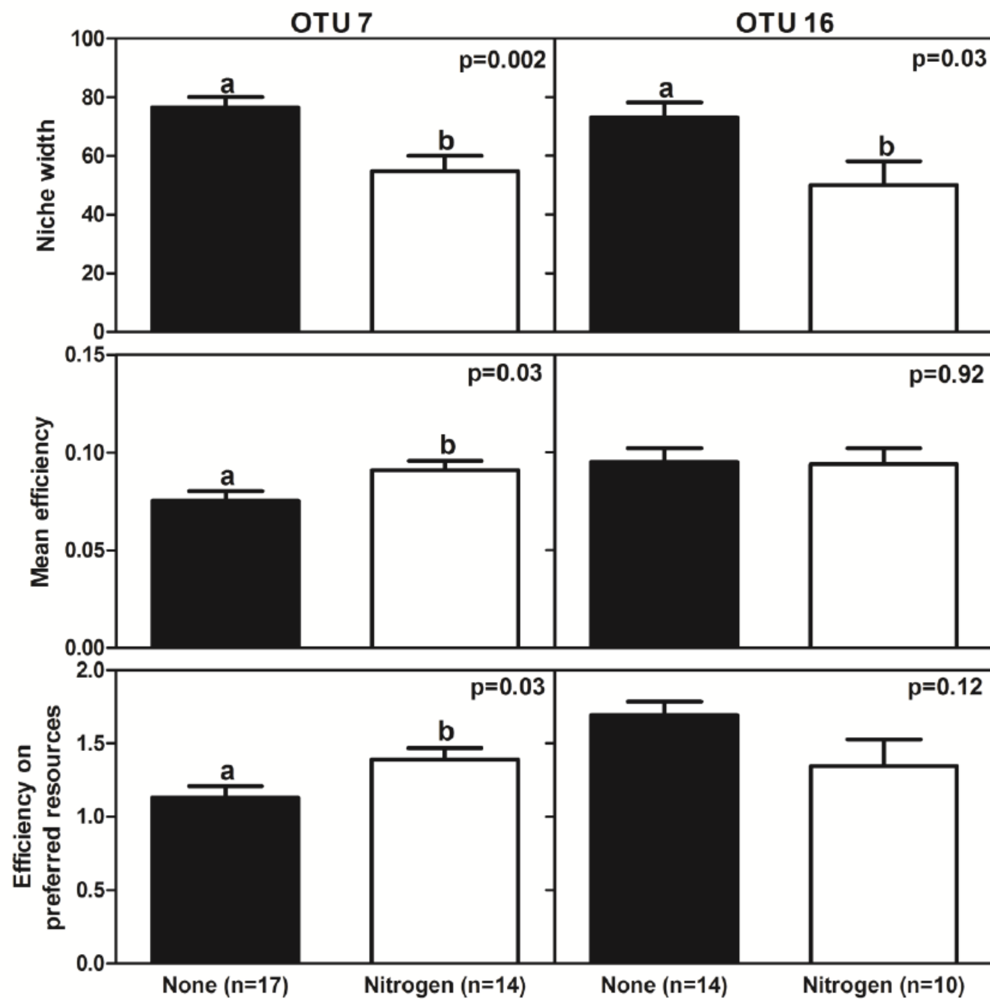

Fig. 6: Niche width (top row), mean growth efficiency (middle row), and efficiency on preferred resources (bottom row) among *Streptomyces* from the same OTU but different nitrogen treatments. P-values from Welch's t-tests comparing *Streptomyces* from non-amended plots (black bars) and N-amended plots (white bars) are presented. In each category, significant differences among OTUs are indicated by different letters above bars ($p<0.05$).

Soil characteristics and Streptomyces *resource utilization*

Plots differed significantly in soil pH, N, and C but not P or K (Supplemental table 4). Among measured soil resources only C and K were correlated significantly with resource use phenotypes among *Streptomyces* communities. Specifically, total C was negatively correlated with mean niche width (Figure 7; r=-0.51, p=0.03) and mean resource use efficiency (Figure 7; r=-0.52, p=0.03). Thus, *Streptomyces* from soils with more C used fewer resources and grew less efficiently than soils with less C, suggesting that C limitation may select for *Streptomyces* with more efficient growth and the capacity to use a broader range of resources. In contrast, soil K was positively correlated with efficiency on preferred resources (r=0.47, p=0.05). *Streptomyces* from high-potassium soils grew more efficiently on preferred resources than *Streptomyces* from low-potassium soils.

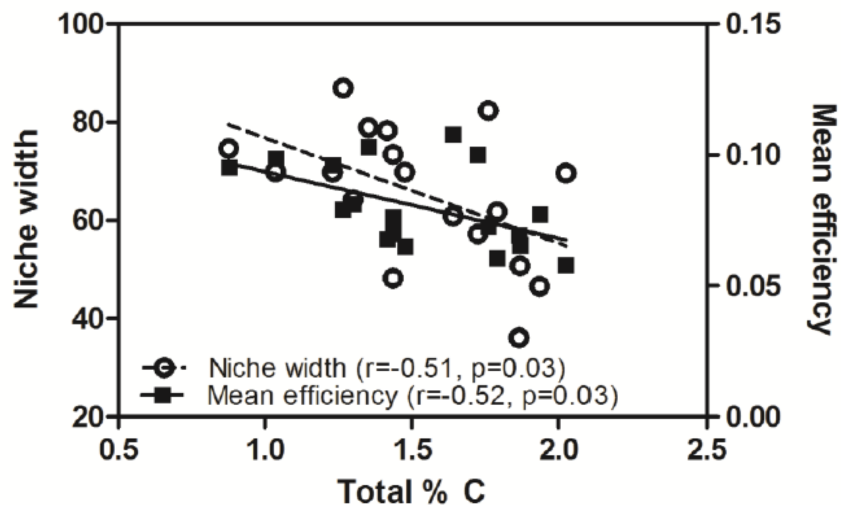

Fig. 7: Relationship between average niche widths (open circles, dashed line) and mean growth efficiency (closed squares, solid line) of *Streptomyces* from different locations with soil total carbon content (Total % C).

Relationships between Streptomyces *densities and resource use.*

Relationships between *Streptomyces* densities and resource use phenotypes differed among treatments. Among N-amended plots there were no significant correlations between *Streptomyces* densities and niche width (r=0.22, p=0.57), mean efficiency (r=-0.02, p=0.96), or efficiency on preferred resources (r=-0.38, p=0.32). In contrast, among non-amended plots *Streptomyces* densities were negatively correlated with mean efficiency (r=-0.68, p=0.05) and efficiency on preferred resources (r=-0.66, p=0.05), but not niche width (r=0.21, p=0.58). Thus, higher density communities supported *Streptomyces* with less efficient growth than lower density communities in non-amended soils but not in N-amended soils. Among all locations, there were no significant correlations between *Streptomyces* densities and niche width (r=0.16, p=0.53) or mean growth efficiency (r=-0.24, p=0.24), though there was a marginally significant correlation between *Streptomyces* densities and mean growth efficiency on preferred resources (r=-0.46, p=0.057). *Streptomyces* from high-density communities tended to be less efficient on their preferred resources than those from low-density communities.

Discussion

Resource use patterns among soilborne *Streptomyces* are highly diverse. Almost every isolate (453 of 459) grew on a unique suite of resources. The ability of *Streptomyces* isolates to use a wide range of resources and their extensive variation in

growth efficiencies suggests great potential for *Streptomyces* to adapt to local environments in soil. Indeed, the greater similarity in resource use phenotypes among *Streptomyces* from the same versus different soil locations less than 1m apart suggests that adaptation of resource use phenotypes among *Streptomyces* populations is highly localized across the landscape. Localized patterns in resource use phenotypes may result from ecological species sorting, where *Streptomyces* best adapted to exploit local resource pools are able to colonize and survive (Langenheder and Székely, 2011), or ongoing selection for indigenous *Streptomyces* to use available resources (Kassen, 2002). Significant variation in community phylogenetic composition among locations in soil suggests that species sorting may contribute to local patterns in resource use. However, *Streptomyces* belonging to the same OTU but isolated from different locations in soil also differed in resource use phenotypes, providing evidence that *Streptomyces* within an OTU are locally-adapted. This study provides empirical data to support the hypothesis that adaptation of resource use phenotypes contributes to the ability of *Streptomyces* to occupy a wide variety of soil niches (Antony-Babu et al., 2008) and that resource use patterns among *Streptomyces* are shaped in part by local selection pressures among locations in soil. Local adaptation of *Streptomyces* OTUs is likely to contribute to the large diversity of resource use phenotypes found within and among phylogenetic groups.

Adaptation of resource use patterns in natural communities is hypothesized to reflect the quantity and types of available resources (Schlatter et al., 2009; Lawrence et al., 2012). The negative relationship of soil C and *Streptomyces* niche width and mean growth efficiency suggests that the availability of C has a significant selective effect on

resource use phenotypes among *Streptomyces* in soil. Specifically, *Streptomyces* in high-C environments tended to be niche specialists and on average grew less efficiently than *Streptomyces* from low-C environments. In contrast, since C availability in soil is often considered to limit microbial growth (Aldén et al., 2001), low-C environments may select for generalist *Streptomyces* that are better able to exploit a broad range of carbon sources for nutrition (large niche widths) and use them more efficiently for growth. These data seem contrary to the predictions of r- and K-selection theory that niche specialists should be able to grow more efficiently than generalists (Kassen and Rainey, 2004). However, tradeoffs between niche width and resource use efficiency potentially ignore fitness costs incurred by microbial investment in species interaction phenotypes, such as antibiotic production or resistance (Czárán et al., 2002; Hibbing et al., 2010; Garbeva et al., 2011). For example, high-C environments, which are likely to support higher microbial population densities and more intense competition, may favor *Streptomyces* that grow less efficiently but invest more energy in antagonistic antibiotic production or resistance phenotypes (Lopez-Pascua and Buckling, 2008; Wloch-Salomon et al., 2008; Lopez-Pascua et al., 2010). In contrast, low-C environments may favor *Streptomyces* that are able to use a wide variety of resources and grow efficiently but invest little in species interaction phenotypes. However, while total carbon may be one predictor of resource use or inhibitory phenotypes among *Streptomyces* (Wiggins and Kinkel, 2005a,b; Cohen and Mazzola, 2006; Mazzola and Zhao, 2010; Lenc et al., 2011), recent work suggests that resource diversity may also play a significant role in local selection (Kinkel et al., 2011; Kinkel et al., 2012).

Plants are a major source of carbon for heterotrophic soil microbes and changes in plant community diversity and productivity can impact soil microbial communities (De Deyn et al., 2011; Bakker et al., 2013a,b; Bulgarelli et al., 2013). At Cedar Creek, long-term N-amendments have resulted in decreased plant diversity and increased productivity (Tilman, 1987; Clark and Tilman, 2008). Low-diversity plant communities in N-amended plots may supply a less diverse array of plant-derived resources to microbes in soil than high-diversity plant communities. As a result, *Streptomyces* in N-amended soils may have adapted to the more limited suite of available resources, as reflected in narrower niche widths among *Streptomyces* from N-amended versus non-amended plots. Greater primary productivity among plant communities in N-amended versus non-amended soils and the subsequent increases in total soil C may also have contributed to the negative relationships between niche width and growth efficiency with soil C described above.

Selection for *Streptomyces* with different resource use strategies in soil under N-amendment may reflect a reduced need for *Streptomyces* to decompose compounds to access N in soil organic matter (Craine et al., 2007). Alternatively, N-amendment may alter the recalcitrance or quality of organic compounds in soil via changes in plant-derived carbon inputs (Meier and Bowman, 2008). It has been hypothesized that N-amendments shift the composition of soil microbial communities to favor more specialized and less efficient (copiotrophic) microbes (Fontaine et al., 2003; Fierer et al., 2007; Ramirez et al., 2012). Different community composition in combination with greater similarity in resource use patterns among *Streptomyces* in N-amended versus non-amended soils is consistent with the hypotheses that reductions in microbial activity

frequently observed under N-amendment are due in part to shifts in communities towards copiotrophic microbes. However, among *Streptomyces* from the same OTU, those from N-amended soils had smaller niches and less efficient growth than *Streptomyces* from non-amended soils. This suggests that, in addition to shifts in community composition towards OTUs with smaller niche widths and less efficient growth, *Streptomyces* in long-term N-amended soils have been selected to use fewer resources in comparison to closely-related isolates from non-amended soils. In total, these data suggest that reductions in microbial activities under long-term N-amendment result from both shifts in phylogenetic composition and selection for more specialized resource use phenotypes both within and among phylogenetic groups (OTUs).

Nitrogen fertilizers are routine in most agricultural systems, yet little is known about how N-amendments influence interactions among soil bacteria (Otto-Hanson et al., 2013). Smaller *Streptomyces* niche widths and altered resource use preferences under long-term N-amendment are likely to significantly impact species interactions among *Streptomyces*, especially competition for resources. In particular, if smaller niche widths lead to more intense resource competition then N-amendment may indirectly select for *Streptomyces* that produce antibiotics to defend resources. Alternatively, if smaller resource use niches increase the likelihood of *Streptomyces* strains having distinct resource use patterns, *Streptomyces* may be less likely to engage in resource competition (ie. niche differentiation). However, N-amended plots did not always harbor *Streptomyces* with smaller niche widths and less efficient growth patterns than every non-amended plot. Rather, reductions in niche widths and growth efficiency were observed

between plots paired in space. This highlights the importance of understanding drivers of microbial phenotypes at small spatial scales and suggests that variation in bacterial phenotypes prior to N-amendment may be important for predicting the outcomes of N-amendment on soil communities.

Resource use is a critical aspect of the ecological niche of microbes in soil and plays a key role in determining species interactions, adaptation, and community assembly. Here we document that *Streptomyces* have diverse resource use patterns that vary with space, phylogeny, and N-amendment. Moreover, soil C and *Streptomyces* population densities were correlated with niche width and growth efficiency, suggesting that resource use among *Streptomyces* is linked resource competition. Further study of resource use, adaptation, and competition among *Streptomyces* and other microbial groups will provide critical insight into microbial population dynamics and species interactions in natural systems.

Chapter 2: Tradeoffs structure antibiotic inhibition, resistance, and resource use among soil-borne *Streptomyces*.

Background

Soil bacteria produce an astounding array of antimicrobial compounds (Watve et al., 2001). Antibiotic production is perceived to provide a fitness benefit to the producer by inhibiting the growth of competing microbes (Williams and Vickers, 1986; Firn and Jones, 2000; Davelos et al., 2004a). In response, competitors may overcome inhibition by becoming resistant to antibiotics through mutation or horizontal gene transfer (Andersson and Hughes, 2011). Antibiotic inhibition and resistance phenotypes are extremely diverse and highly variable among soil bacteria (Davelos et al., 2004a; Vetsigian et al., 2011). However, it is not well understood how this diversity is maintained in soil microbial communities. For example, what limits the accumulation of antibiotic production and resistance genes among bacterial populations in soil?

It is commonly assumed that, because antibiotic production and resistance likely require energy expenditure, they are accompanied by tradeoffs with growth or other fitness components. Tradeoffs occur when the maximal fitness of an organism cannot be realized due to competing demands of distinct traits. Thus, tradeoffs preclude an organism's ability to optimize multiple competing traits (Kneitel and Chase, 2004; Tilman, 2011). As a result of tradeoffs, antibiotic-producing microbes are expected to be out-competed by non-producing counterparts in the absence of susceptible competitors (Czárán et al., 2002; Garbeva et al., 2011; Kinkel et al., 2011). Similarly, microbes that carry resistance to antibiotics are expected to be out-competed by susceptible

counterparts in the absence of antibiotic producing competitors (Czárán et al., 2002; Andersson and Hughes, 2011; Kinkel et al., 2011). Although tradeoffs between growth and antibiotic resistance have been studied in clinical settings (Andersson and Hughes, 2011), the lack of analogous data for antibiotic production or resistance among naturally-occurring microbial populations limits our understanding of the dynamics of antibiotic inhibition and resistance within soil communities.

Tradeoffs associated with antibiotic production and resistance may be reflected in growth reductions for antibiotic-producing vs. non-producing bacteria in the absence of competition (Garbeva et al., 2011) or in negative relationships among distinct phenotypic traits (Kneitel and Chase, 2004). Since bacterial strains often produce and resist many antibiotics in tandem (Challis and Hopwood, 2003; D'Costa et al., 2006; D'Costa et al., 2007a), tradeoffs that are important for the fitness of individuals will encompass the total or cumulative profile of antibiotic production and resistance. For example, production of or resistance to multiple antibiotic compounds is likely to require a greater total allocation of resources compared to production of or resistance to a single antibiotic (growth-inhibition and growth-resistance tradeoffs). It has been hypothesized that the accumulation of antibiotic production and resistance among bacteria is driven by a coevolutionary arms race (Challis and Hopwood, 2003; Kinkel et al., 2011). Arms race dynamics are hypothesized to result in extreme traits in populations (Thompson, 2005). For example, among microbes, arms race coevolution is considered to be responsible for the accumulation of virulence or resistance within individuals (Bohannon and Lenski, 2002; Thrall and Burdon, 2003; Hall et al., 2011), and may also generate a preponderance

of bacteria with highly antagonistic or resistant phenotypes (Kinkel et al., 2011). However, there are limited data on the distribution of cumulative inhibition and resistance phenotypes among soil bacteria.

Streptomyces are filamentous, Gram-positive bacteria that are prolific producers of antibiotics and are also a significant reservoir of antibiotic resistance in soils (D'Costa et al., 2006, D'Costa et al., 2007a). It is common for individual *Streptomyces* strains to have the capacity to both produce and resist inhibition by many antibiotic compounds (Challis and Hopwood, 2003; Davelos et al., 2004a; D'Costa et al., 2006). Additionally, antibiotic production by *Streptomyces* is often dependent on resource availability (Rigali et al., 2008, Sánchez et al., 2010). *Streptomyces* vary extensively in the resources on which they can grow and in their relative growth efficiencies on different resources (Schlatter et al., 2013). When resource limitation begins to restrict growth, *Streptomyces* are hypothesized to produce antibiotics as a means to protect their resource supply from competitors (Challis and Hopwood, 2003). There may be significant fitness tradeoffs associated with antibiotic inhibition or resistance that constrain the evolution of *Streptomyces* that are both highly antagonistic and capable of efficient growth on a wide array of resources (niche-inhibition or niche-resistance tradeoffs). Specific data on the relationships among *Streptomyces* resource use, antibiotic inhibition, and antibiotic resistance are crucial to understanding the potential for fitness tradeoffs to constrain the accumulation of antibiotic inhibition or resistance capacities in soil microbes.

Selection for antibiotic inhibition, resistance, and resource use within soil communities will further depend on biotic and abiotic characteristics of the soil

environment. In particular, since antibiotics will only confer an advantage in the presence of susceptible competitors, communities with high *Streptomyces* densities may have greater frequencies of species interactions and thus confer consistently greater fitness benefits on antibiotic inhibitory phenotypes than communities with low *Streptomyces* densities (Kinkel et al., 2012). Further, the benefit of producing antibiotic compounds may also depend on resource availability (Wloch-Salamon et al., 2008; Kinkel et al., 2011). Specifically, resource availability is likely to influence the cost:benefit ratios of antibiotic production and resistance against resource competitors. However, few studies have examined the soil edaphic conditions associated with *Streptomyces* communities having high or low capacities to produce or resist antibiotics in soil (Bakker et al., in press).

In this work we examine patterns of antibiotic inhibition and resistance phenotypes in relation to resource use among soil *Streptomyces*. Specifically, we 1) characterize antibiotic inhibition and resistance among *Streptomyces* and quantify cumulative inhibition and resistance phenotypes within communities; 2) determine relationships between antibiotic inhibition, resistance, and resource use among *Streptomyces* isolates and explore tradeoffs among *Streptomyces* exhibiting distinct inhibition and resistance strategies; and 3) evaluate soil characteristics and *Streptomyces* densities as correlates of cumulative antibiotic inhibition and resistance among *Streptomyces* communities. These data suggest that tradeoffs are critical in structuring antibiotic production and resistance strategies among *Streptomyces* and that soil

characteristics influence the accumulation of *Streptomyces* antibiotic inhibitory and resistance phenotypes in soil.

Materials and Methods

Soil sampling, processing, and isolation

Soil samples were collected at the University of Minnesota Cedar Creek Ecosystem Science Reserve (www.cedarcreek.umn.edu), a NSF Long-Term Ecological Research site. Detailed information on soil collection, processing, and isolation is published elsewhere (Davelos et al., 2004a). Briefly, three soil cores were taken from random locations within a $1m^2$ sections of two plots in experiment E001 (plots 08-A and 10-D) for a total of six soil cores. Soil cores were transported to the lab on ice and processed immediately. Soils were dried overnight under a double-layer of sterile cheesecloth, serially diluted in phosphate buffer (0.5M K_2HPO_4, 0.4M KH_2PO_4, pH=7.0), and plated on oatmeal agar amended with antibiotics. Plates were incubated at 28C for 7 days and *Streptomyces* densities were estimated based on characteristic colony morphology. *Streptomyces* colonies were randomly picked with a sterile toothpick, purified, and stored in 20% glycerol at -80C for further study. Subsamples of each soil were submitted to the University of Minnesota Soil Testing Laboratory for determination of soil characteristics (pH, NO_3-N, Bray-P, K, and C).

Characterization of *Streptomyces* antibiotic inhibition and resistance

Streptomyces antibiotic inhibition and resistance profiles were determined using 10 standard reference isolates described in Davelos et al. (2004b). These standard isolates vary in their antibiotic inhibition and resistance phenotypes and can differentiate up to 1024 different inhibition and resistance phenotypes (Davelos et al., 2004b). *Streptomyces* isolates (n=269) were characterized for antibiotic inhibition profiles by evaluating their ability to inhibit each standard isolate using an agar-overlay method (Davelos et al., 2004a). Briefly, spore suspensions (~10^8 spores/ml) were dotted (10ul) onto 15ml starch-casein agar (SCA). After incubation for 3 days at 28C, isolates were killed by inverting each plate over a watch glass containing 4ml chloroform. Plates were then left open in a laminar flow hood for 30 min to allow residual chloroform to evaporate, then overlaid with 15ml of fresh 1% water agar. After the agar solidified 100ul of each test standard spore stock (~10^8 spores/ml) was spread on the plate. Plates were incubated for 3 days at 28C and the size of inhibition zones around dotted isolates were measured. Each *Streptomyces*-standard interaction was replicated three times and only inhibition zones greater than 1 mm were considered to be inhibitory. The same approach was used to characterize *Streptomyces* isolates (n=273) for resistance to antibiotic inhibition by each standard isolate.

Resource use

Resource use was evaluated on Biolog SF-P2 microplates (Biolog, Inc. Hayward, CA) as described in Schlatter et al. (2009). Fresh spore suspensions of each *Streptomyces* isolate were quantified to an absorbance of 0.22 at 590nm, diluted according to the

manufacturer's instructions, and inoculated into Biolog SF-P2 plates. Plates were incubated at 28 C for 3 days and the absorbance of each well was measured (590nm). The absorbance (au) from the water control well was subtracted from all 95 substrate-containing wells. After correction, all wells with absorbances <0.005 were adjusted to 0 prior to analyses. We defined niche width as the number of substrates on which an isolate could grow (positive absorbance after adjustments). Growth efficiency was defined as the mean growth on used substrates (mean of absorbance values >0).

Statistical analyses

All statistical analyses were performed using in R statistical package (Version 2.15; R Core Team, 2012). Pearson correlations were used to test for relationships between aggregate *Streptomyces* inhibitory, resistance, and resource use phenotypes. Slopes of regressions between antibiotic inhibitory and resistance capacities with growth efficiency were compared using Fisher's z-transformations of correlation coefficients. Because antibiotic inhibitory phenotypes among *Streptomyces* fell into two distinct categories, those that did not inhibit any standards (non-inhibitors) and those that inhibited all 10 standards (super-killers), Student's t-tests were used to compare differences in resource use of *Streptomyces* among these categories. Similarly, although antibiotic resistance was normally distributed, resource use among *Streptomyces* with low (resisting 3-5 standards) and high (resisting 8-10 standards) resistance capacities were compared using Student's t-tests. Variation in inhibitory and resistance capacities (number of standards inhibited or resisted) among *Streptomyces* communities from different soil samples was evaluated using analysis of variance. Finally, Pearson

correlations were used to evaluate relationships among mean inhibitory and resistance capacities of *Streptomyces* communities with soil characteristics and culturable *Streptomyces* densities.

Results

Streptomyces varied in their abilities to inhibit and resist standard isolates. Patterns of inhibition were diverse: considering discrete inhibitory phenotypes (+/-) there were 76 unique inhibition profiles among 269 isolates. Individual *Streptomyces* inhibited an average of 3.1 standards, or an average of 5.3 standards when considering only *Streptomyces* that could inhibit at least one standard. However, most isolates had either very little or very high inhibitory capacities, as reflected in the distinct bimodal distribution of isolate frequencies (Figure 1a). The majority of *Streptomyces* either lacked any inhibitory capacity (41% of isolates did not inhibit any standard; Figure 1a) or had extremely broad inhibitory phenotypes (15% of isolates inhibited all 10 standards; Figure 1a).

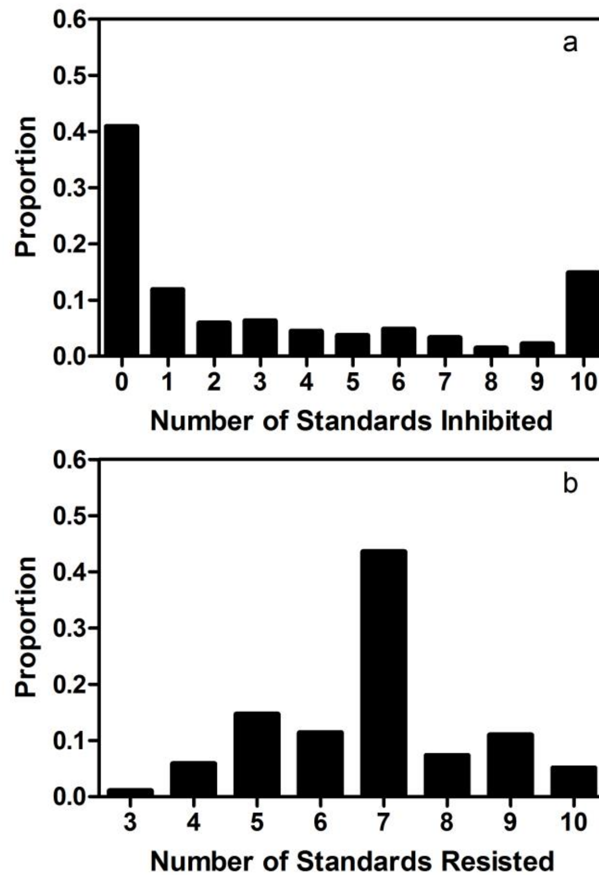

Figure 1. Frequency distribution of cumulative antibiotic inhibition (a) and resistance (b) phenotypes of *Streptomyces* against standard test isolates.

Resistance phenotypes among *Streptomyces* were less diverse than inhibition phenotypes; among 273 isolates there were 34 unique resistance profiles. *Streptomyces* isolates resisted 6.8 standards on average (Figure 1b). Resistance to inhibition by multiple standards was common among soil-borne *Streptomyces*. Every standard inhibited at least two *Streptomyces* isolates, indicating that each standard had some inhibitory capacity. Although each *Streptomyces* isolate resisted inhibition by at least 3 standards, only a

small proportion (5%) of isolates was resistant to all 10 standards. In contrast to the bimodal distribution of inhibitory phenotypes, frequencies of resistance were approximately normally distributed among isolates and fell around a single optimum.

Streptomyces capacities to inhibit and resist standards were significantly related, though correlations were small. There was a weak but marginally significant positive correlation between the number of standards inhibited and the number of standards resisted among individual *Streptomyces* (Pearson R=0.12, p=0.052), suggesting that *Streptomyces* with broad antibiotic inhibition capacities are very slightly more likely to have broad resistance capacities.

The number of standards that isolates inhibited was weakly but significantly negatively correlated with niche width (Figure 2; Pearson R=-0.15, p=0.015), but not growth efficiency (Figure 2; Pearson R=-0.03, p=0.62). Thus, although better inhibitors had smaller niche widths than poor inhibitors, correlations between antibiotic inhibition phenotype and resource use were weak. However, non-inhibitory isolates (inhibited none of the standards) and highly inhibitory 'super-killers' (inhibited all 10 standards) differed significantly in resource use. Super-killer *Streptomyces* had significantly smaller niche widths and grew less efficiently than non-inhibitors (Figure 3). Non-inhibitory *Streptomyces* had on average 12.5% larger niche widths (t-test, t=2.84, p=0.006) and 7.5% more efficient growth (t-test, t=2.23, p=0.03) than super-killers. Thus, highly inhibitory super-killers had more restricted niches and grew less efficiently than non-inhibitory *Streptomyces*. In total, *Streptomyces* with broad-spectrum inhibitory capacities are more likely to have smaller niches than *Streptomyces* with little inhibitory capacity.

More importantly, these data suggest that there are significant growth efficiency-inhibition and niche width-inhibition tradeoffs among *Streptomyces*.

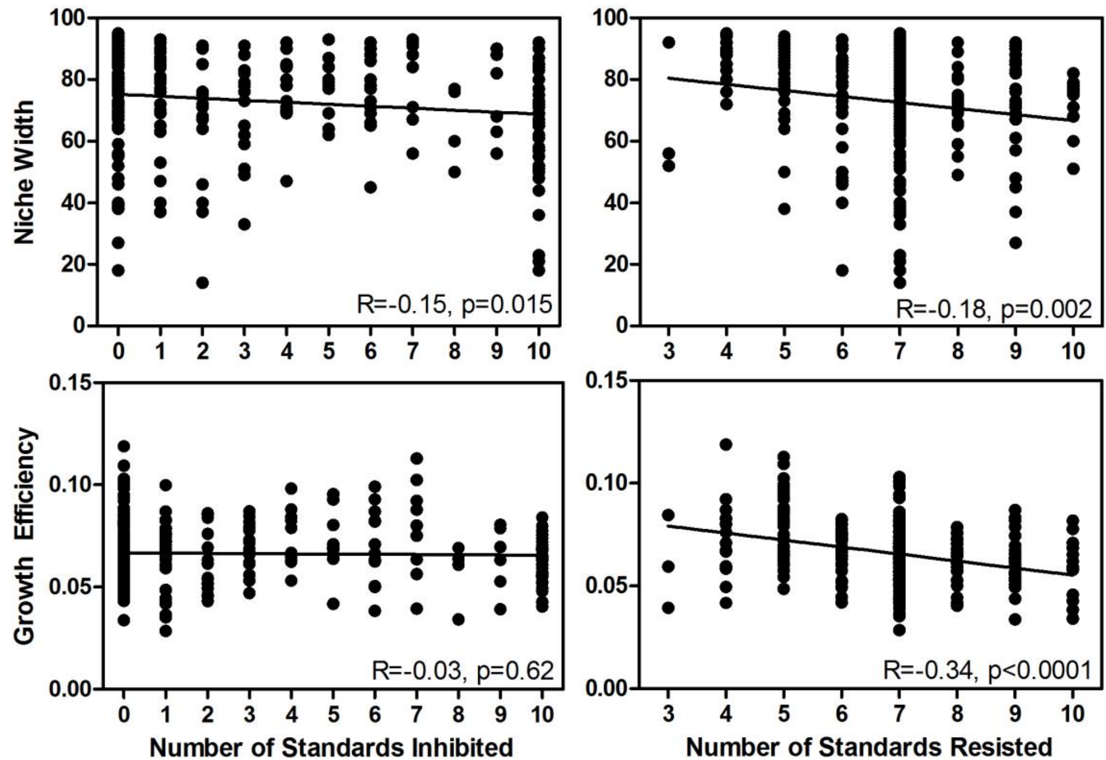

Figure 2. Relationships between cumulative inhibition (left panels) and resistance (right panels) phenotypes with niche width (top panels) and growth efficiency (bottom panels) among *Streptomyces* isolates. Pearson correlation coefficients and p-values are presented for relationships in each panel.

Similarly, the number of standards that *Streptomyces* isolates could resist was negatively correlated with niche width (Figure 2; Pearson R=-0.18, p=0.002) and growth efficiency (Figure 2; Pearson R=-0.34, p<0.0001). *Streptomyces* with higher resistance had on average smaller niche widths and grew less efficiently than more susceptible isolates. *Streptomyces* with very high versus low resistance also differed significantly in

resource use. Isolates with little resistance to inhibition by standards (those that could resist inhibition by ≤5 standards) had significantly larger niche widths and higher growth efficiency than highly resistant *Streptomyces* (those that resisted inhibition by ≥8 standards; Figure 3). On average, *Streptomyces* with little resistance had 11.6% larger niche widths (t-test, $t=4.28$, $p<0.0001$), and 30.9% greater growth efficiency (t-test, $t=6.36$, $p<0.0001$) than *Streptomyces* with a high number of resistances. Thus, there are substantial growth efficiency-resistance and niche width-resistance tradeoffs among *Streptomyces*.

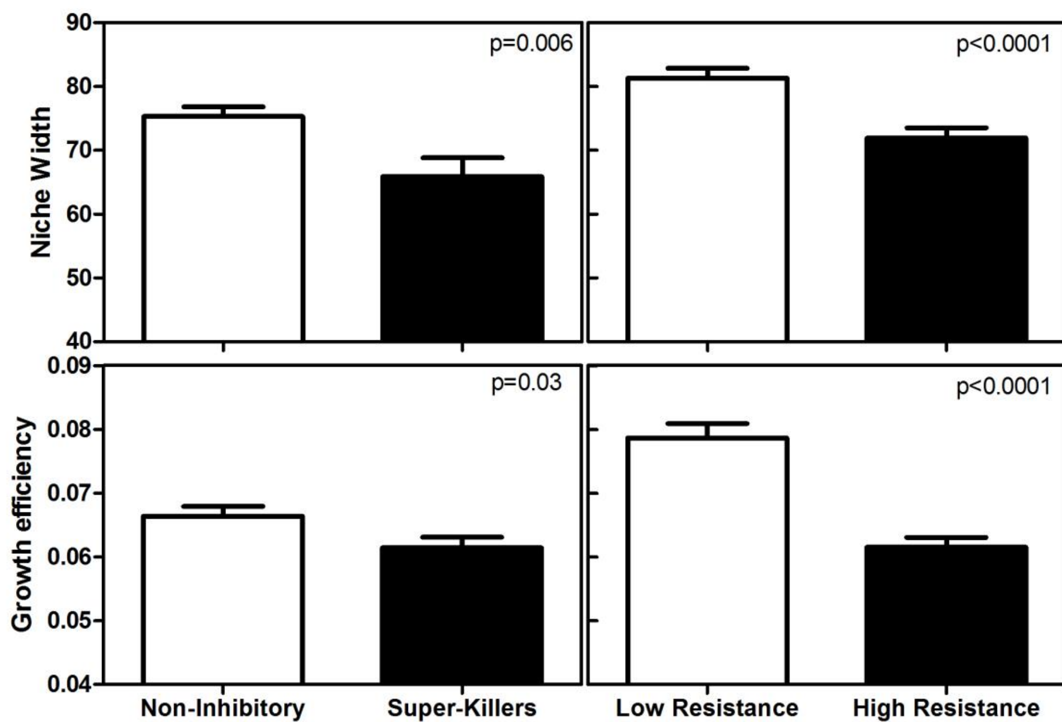

Figure 3. Niche width (±SEM; top panels) and growth efficiency (±SEM; bottom panels) among *Streptomyces* exhibiting distinct inhibition strategies (non-inhibitory vs super-killers; left panels) and resistance strategies (low resistance vs high resistance; right panels). P-values represent Student's t-tests between groups in each panel.

Surprisingly, the apparent cumulative 'costs' of high resistance in reduced growth efficiencies were greater than those for accumulated antibiotic inhibition. Specifically, the slope of the relationship between cumulative antibiotic resistance (number of standards resisted) and growth efficiency was significantly more negative than the slope between cumulative inhibition (number of standards inhibited) and growth efficiency (slope of inhibition = -0.0001 vs. resistance = -0.003; Fisher transformed $z=-3.729$, $p<0.0001$), suggesting that there are greater cumulative costs of multiple antibiotic resistance than cumulative antibiotic production phenotypes.

When considering *Streptomyces* communities, rather than strains, communities from distinct locations in soil (n=6 locations) varied in mean abilities to inhibit (ANOVA $F=5.50$, $p<0.0001$) and resist (ANOVA $F=8.77$, $p<0.0001$) standards. Among communities there was a significant positive correlation between the mean number of standards that *Streptomyces* inhibited and the mean number of standards that they resisted ($R=0.82$, $p=0.05$). Thus, highly inhibitory *Streptomyces* communities also harbored good resistors.

Culturable *Streptomyces* densities were not correlated with cumulative antibiotic inhibition or resistance capacities within *Streptomyces* communities (Table 1). In contrast, soil edaphic characteristics were correlated with antibiotic inhibition and resistance among *Streptomyces* communities. Specifically, the average number of standards that isolates could inhibit was significantly positively correlated with soil N and P among locations, but negatively positively correlated with soil pH (Table1). Soil P was

significantly correlated with the mean number of standards that *Streptomyces* could resist (Table 1). Thus, high N and P supported more inhibitory or more inhibitory and resistant *Streptomyces* communities, respectively. Total soil carbon was not significantly related to antibiotic inhibition or resistance among *Streptomyces* communities.

Table 1. Pearson correlation coefficients (p-value) between soil properties and mean *Streptomyces* inhibition and resistance capacities from different communities.

	C (%)	**N (ppm)**	**P (ppm)**	**K (ppm)**	**pH**	**LogStrep (cfu/g soil)**
Mean # Inhibited	0.40 (0.18)	0.93 (<0.01)	0.64 (0.06)	0.07 (0.62)	-0.88 (<0.01)	-0.04 (0.70)
Mean # Resisted	0.56 (0.09)	0.46 (0.14)	0.86 (<0.01)	0.02 (0.80)	-0.43 (0.16)	0.002 (0.93)

Discussion

Most *Streptomyces* were characterized by one of two distinct inhibition strategies and tended to be either non-inhibitory or highly inhibitory “super-killers”. Because antibiotic inhibitory interactions can be highly specific among *Streptomyces* isolates (Davelos et al., 2004a; Vetsigian et al., 2011), the benefits of producing a single antibiotic may vary across communities. Specifically, since genes encoding resistance to antibiotics are broadly distributed in natural soil habitats (Allen et al., 2009; Bhullar et al., 2012), competitors may rapidly acquire resistance to any given antibiotic and offset the benefits of antibiotic production (Kinkel et al., 2011). However, combinations of antibiotics, especially those with different modes of action, can substantially reduce the likelihood that any one competitor bears resistance to each antibiotic and reduce the

likelihood that resistance will evolve (Yeh et al., 2009). Indeed, selection for *Streptomyces* to produce antibiotic compounds that act synergistically is thought to play a significant role in the evolution of *Streptomyces* secondary metabolism (Challis and Hopwood, 2003). Thus, accumulating multiple synergistic or complementary pathways for antibiotic production within individual *Streptomyces* strains may confer substantial and potentially synergistic fitness benefits to producers across time and space. As a result, optimal strategies for an antagonistic lifestyle may tend towards relatively high accumulation of antibiotic phenotypes (super-killers).

Antibiotic phenotypes are assumed to impose a physiological cost of production (Garbeva et al., 2011) and non-producers are expected to have a competitive advantage over producers when there are low competitor densities (Czaran et al., 2002; Kinkel et al., 2011). Although we found only weak correlations between cumulative *Streptomyces* inhibitory phenotypes, niche width and growth efficiency, the substantial difference in growth efficiency and niche width among non-inhibitory and super-killer *Streptomyces* suggests significant growth-inhibition and niche-inhibition tradeoffs. However, our ability to detect physiological tradeoffs associated with antibiosis may have been limited by our experimental design. Since antibiotic compounds vary considerably in their chemical structures and require distinct pathways for synthesis, it is likely that different antibiotics vary in costs of production, thereby confounding any relationship between the number of antibiotics produced and growth efficiency. Antibiotic compounds also vary in their spectrum of activity, so that one antibiotic may inhibit multiple standards. As a result, inhibition of standards is likely to be imperfectly correlated with the number of

compounds produced by an inhibitor. Furthermore, genes encoding the production of many *Streptomyces* secondary metabolites are tightly regulated and may require specific growth conditions or inter-species signals in order to be expressed (Takano, 2006; Hsiao et al., 2009; Kitani et al., 2011). Thus, this work may have captured only a subset of the inhibitory potential of our isolates. In total, these factors may obscure tradeoffs of antibiotic production with niche width and growth efficiency.

In contrast to antibiotic inhibition, resistance was more evenly distributed among *Streptomyces*, and resistance to inhibition by an intermediate number of standards was the most common phenotype among isolates. *Streptomyces* in natural settings are commonly resistant to many antibiotics, even in environments with no history of antibiotic contamination from anthropogenic sources (D'Costa et al., 2006; Bhullar et al., 2012). This suggests that species interactions in natural populations contribute to the maintenance of multiple resistance phenotypes. In particular, strong selection for resistance is expected to result from the severe fitness consequences of being susceptible to inhibition by antibiotics produced by competitors. The significant reductions in niche width and growth efficiency with increasing resistance capacities among *Streptomyces* suggest that in natural communities there are typically significant growth-resistance and niche-resistance tradeoffs. As a result of these tradeoffs and the substantial cost of lacking resistance, rather than having very few or very many antibiotic resistances, stabilizing selection appears to favor *Streptomyces* with moderate resistance capacities as an evolutionarily stable strategy. Thus, growth-resistance and niche-resistance tradeoffs are especially important for structuring antibiotic resistance phenotypes.

Ongoing reciprocal selection between antibiotic inhibition and resistance among *Streptomyces* may be a crucial driver of the accumulation of antibiotic inhibitory or resistance phenotypes within individuals (e.g. coevolutionary escalation; Thompson et al., 2005; Kinkel et al., 2011) and generate *Streptomyces* with highly antagonistic or resistant life-history strategies. The strong positive relationship between cumulative antibiotic inhibition and resistance phenotypes among communities is consistent with an antagonistic arms race between inhibitory and resistance phenotypes. Specifically, communities that supported highly inhibitory *Streptomyces* also harbored *Streptomyces* with many resistances, suggesting that arms race coevolution between inhibitory and resistant *Streptomyces* selected for accumulation of inhibitory and resistant phenotypes among individuals in these communities. In contrast, *Streptomyces* communities in which individuals were able to inhibit fewer standards on average had less resistant isolates. These communities may be following an alternative coevolutionary trajectory towards niche differentiation (Kinkel et al., 2011).

Correlations between growth efficiency or niche width and cumulative antibiotic inhibition capacities were smaller than those between growth efficiency or niche width and cumulative resistances, suggesting that it may be more costly to accumulate greater resistance than to accumulate more inhibitory capacities. This is particularly surprising considering that the fitness costs of antibiotic resistance in clinical settings are often small (Sander et al., 2002; Gagneux et al., 2006) and that the pathways responsible for antibiotic biosynthesis require one or more large, multi-domain enzymes (polyketide synthases and non-ribosomal peptide synthases). However, it has been

suggested that the accumulation of resistances in bacteria have greater than additive effects on fitness (Ward et al., 2009). Differences in costs for the accumulation of antibiotic resistance and antibiotic production may contribute significantly to the dynamics of antibiotic inhibition and resistance in natural communities across the landscape (Zhang et al., 2013).

More frequent and intense competitive interactions among soil bacteria may occur in communities with higher versus lower population densities (Kinkel et al., 2012). However, we found no significant relationship between *Streptomyces* densities and accumulation of antibiotic inhibition or resistance phenotypes. Thus, *Streptomyces* densities alone are not an accurate proxy for the significance of competition or species interactions to fitness. Similarly, although soil C is suggested to play a central role in resource competition among soil microbes (Aldén et al., 2001; Kinkel et al., 2011), the relationship between soil C content and the accumulation of antagonistic or resistance phenotypes was not significant. Soil carbon occurs in many chemical forms that vary in availability and nutritional content for soil bacteria (Meier and Bowman, 2008). Thus, in addition to the amount of carbon, carbon type may also play a significant role in selection for competitive phenotypes among *Streptomyces* (Schlatter et al., 2009), suggesting that total soil C may be too crude a measure of resource limitation for soil microbes. Additionally, the absence of significant correlations of competitive phenotypes with *Streptomyces* densities and soil C may have been due to the limited sample size (n=6 communities) and the fact that *Streptomyces* densities and soil C among these communities fell within a very small range (10^4.6 cfu/g to 10^5.5 cfu/g and 1.4% to 2%

soil C, respectively). Further work in field and controlled settings is needed to disentangle the influences of microbial densities and soil carbon on selection for antibiotic inhibition and resistance among soil microbial populations and fitness tradeoffs between different life history strategies.

In contrast to carbon and *Streptomyces* densities, soil N, P, and pH were correlated with *Streptomyces* inhibitory phenotypes. Although not generally considered limiting resources for soil microbes, *Streptomyces* secondary metabolism is often regulated by the availability of nitrogen and phosphate and is sensitive to pH (Bibb, 2005; van Wezel and McDowall, 2011). A greater availability of N and P may reduce the costs associated with antibiotic production and favor selection for more inhibitory *Streptomyces*. In addition, these resources are all critical to mediating plant productivity and corresponding plant-derived inputs into soil, which have been shown to mediate antagonistic phenotypes among *Streptomyces* (Bakker et al., 2013b). Linkages between soil resources, plant growth, and microbial species interactions are likely to be critical to selection for tradeoffs among soil microbes.

Our findings suggest that tradeoffs between accumulating antibiotic inhibition or resistance capacities and growth efficiency or niche width are fundamental to structuring inhibition and resistance phenotypes among *Streptomyces* in nature. Specifically, the occurrence of tradeoffs is likely to preclude the evolution of ‘ultimate’ inhibitors and resistors. Moreover, greater apparent costs of accumulating multiple resistance versus inhibitory capacities appears to generate different distributions of these phenotypes among *Streptomyces*, reflecting distinct life history strategies. Further work exploring the

roles tradeoffs play in the evolution of *Streptomyces* will contribute to our understanding of the forces that generate and maintain the vast diversity of antibiotic inhibitory and resistance phenotypes in soil communities.

Chapter 3: *Streptomyces* inhibition and resource competition: Coevolutionary arms race and niche differentiation as alternative trajectories.

Background

Antibiotic-producing microbes are a common component of soil communities. Antibiotics have been traditionally perceived to be important to antagonistic species interactions and to confer a fitness benefit to producers in competitive habitats (Williams et al, 1989; Riley and Gordon, 1999; Martinez et al, 2009a). More recently, antibiotics have been suggested to function predominantly as signaling molecules that may mediate competitive, mutualistic, pathogenic, or commensal species interactions (Goh et al, 2002; Ng et al, 2003; Lin et al, 2005; Yim et al, 2007; Fajardo and Martinez, 2008; Martinez, 2008; Aminov, 2009; Romero et al, 2011). At sub-inhibitory concentrations, antibiotics modulate transcription of a wide variety of bacterial genes, including genes that influence nutrient acquisition, virulence, motility, antibiotic production, and biofilm formation (Bagge et al, 2004; Hoffman et al, 2005; Linares et al, 2006; Mitova et al, 2008; Ryan and Dow, 2008; Cummins et al, 2009; Kaplan et al, 2012). Though it has been argued that antibiotics are likely to be present predominantly at sub-inhibitory concentrations in natural habitats, and thus that antibiotics may act only rarely as `weapons', there is little empirical data on antibiotic concentrations in soil or aquatic environments.

While broad consensus supports the concept that antibiotics play diverse roles in natural communities, we have limited understanding of the natural history and evolutionary dynamics of antibiotic phenotypes. Globally, antibiotics are central to the

management of infectious diseases and scientists are challenged to discover novel antibiotics while managing the spread of antibiotic resistance in clinical populations. Unfortunately, our lack of understanding of the ecology and evolutionary biology of antibiotics in natural habitats significantly constrains our abilities to develop deliberate strategies for antibiotic exploration, manage indigenous soil microbial communities for enhanced inhibitory phenotypes, and minimize accumulation of antibiotic resistance in environmental microbes. Multiple models have been proposed for describing the competitive and coevolutionary dynamics of antibiotic-producing microbes (Czárán et al, 2002; Czárán and Hoekstra, 2003; Wloch-Salamon et al, 2008; Laskaris et al, 2010), and antagonistic interactions have been suggested to exhibit coevolutionary arms race or polymorphism dynamics, with reciprocal accumulation of inhibitory and resistance phenotypes in interacting populations over time. Though these models assume that antibiotics confer consistent fitness advantages to the producer while imposing significant fitness costs on non-producing organisms within local communities, we have limited experimental evidence to support or refute these assumptions. Further information on the dynamics of antibiotic phenotypes in microbial populations is needed to shed light on the roles of antibiotics in natural communities and on the evolutionary or coevolutionary trajectories contributing to the accumulation of antibiotic inhibitory and resistance phenotypes in the environment.

Our work explores the dynamics of antibiotic inhibitory and resistance phenotypes among soilborne *Streptomyces* in natural habitats. *Streptomyces* are notable as producers of the majority (>70%) of naturally-occurring antibiotics (Tanaka and

Omura, 1990; Watve et al, 2001; Challis and Hopwood, 2003; Baltz, 2007). *Streptomyces* are gram positive, filamentous bacteria that are excellent saprophytes, prolific producers of extracellular enzymes, and ubiquitous in soil and marine sediments (Gotang et al, 2007; Chater et al, 2010). *Streptomyces* are the source of many clinically significant antibiotics, and have been investigated in agricultural settings for their capacities to suppress plant pathogens (Bressan and Figueiredo, 2007; Hiltunen et al, 2009; Karimi et al, 2012; Kinkel et al, 2012; Meschke et al, 2012; Otto-Hanson et al, 2013). Because of their capabilities at producing bioactive compounds and in response to the tremendous therapeutic value of many of these compounds, antibiotic production in *Streptomyces* has been studied extensively in vitro. In general, antibiotic production is highly variable among individual *Streptomyces* isolates in terms of both the amounts and the identities of antibiotics produced, and most *Streptomyces* isolates produce multiple antibiotics (Omura et al, 2001; Bentley et al, 2002; Challis and Hopwood, 2003). Patterns of antibiotic resistance are likewise decidedly variable among *Streptomyces* isolates, and as a consequence, inhibitory interactions among *Streptomyces* isolates tend to be highly specific (Davelos et al, 2004a; Vetsigian et al, 2011).

In this work we evaluate the hypotheses that antibiotic inhibitory and resistance phenotypes confer local fitness benefits and that antibiotic inhibitory phenotypes mediate competitive and coevolutionary species interactions within local soil communities. Specifically, we evaluated inhibitory intensities, resistance frequencies, and relationships between nutrient use (niche) overlap and inhibition among sympatric and allopatric *Streptomyces* isolates from diverse locations. Differences in sympatric vs. allopatric

inhibition intensities and resistance frequencies provide insight into both the fitness benefits and potential roles of inhibitory interactions in mediating local species interactions. Furthermore, these data shed light on the potential for reciprocal selection of inhibitory and resistance phenotypes among locally-coexisting populations, consistent with coevolutionary arms race or polymorphisms dynamics. Finally, relationships between niche overlap and antibiotic inhibition among sympatric vs. allopatric populations provide crucial information on the significance of inhibitory interactions to mediating nutrient competition among coexisting *Streptomyces*, as well as possible alternatives to coevolutionary arms race dynamics.

Materials and Methods

Soil sample collection and processing:

We evaluated inhibitory intensities, resistance frequencies, and relationships between nutrient use (niche) overlap and inhibition among sympatric and allopatric Streptomyces isolates from 7 locations representing temperate and tropical habitats. The sampling sites encompassed diverse soil characteristics, microbial communities, and environmental conditions. Soil samples were collected at Cedar Creek Ecological Science Reserve (CCESR) in east-central Minnesota (MN1, MN3, MN5) and the Konza Prairie (KS), two National Science Foundation Long-Term Ecological Research (LTER) sites. In addition, soils were collected at Fort Sherman (PFS), Santa Clara (PSC) and Volcan Baru (PVB) in Panama. Soil corers (10 cm x 1 cm or 2.5 x 10 cm) were used to collect soil at each location. Samples were transported back to the laboratory and maintained at 12°C until processing. Soil from each sample was placed under two layers of sterile cheesecloth to

dry overnight and 5 g subsamples from each location were subsequently placed into 50 ml centrifuge tubes containing 10 ml of buffered phosphate solution (0.5 M K_2HPO_4, 0.4 M KH_2PO_4, pH 7.0). Tubes were shaken for 1 h on a reciprocal shaker (4°C, 250 rpm). Resulting soil suspensions were dilution plated onto oatmeal agar. Plates were incubated at 28°C for 7 d. Colonies exhibiting characteristic *Streptomyces* colony morphology were picked, purified, and spore suspensions of each isolate were maintained in 20% glycerol at -80°C. In total, from 9 – 10 *Streptomyces* isolates were collected randomly from each sample. Specifically, colonies exhibiting characteristic *Streptomyces* colony morphology were subcultured, purified, and spore suspensions of each isolate were maintained in 20% glycerol at -80°C. A total of 69 isolates from 7 locations (soil cores) were considered in this work.

Assaying inhibitory and resistance phenotypes:

Inhibitory interactions were evaluated for all possible pairwise isolate combinations for a total of 4692 interactions. To test for inhibition among isolate pairs, 10 ul of spore stock suspension (~10^8 cfu/ml) of each isolate was dotted onto starch-casein agar and grown for 3 d at 28°C. Dotted isolates were killed by inverting the uncovered petri plates over 4 ml of chloroform in a watch glass for 1 h. Watch glasses were removed, and plates were aerated in a flow hood for 30 min to permit evaporation of chloroform. Plates were subsequently overlaid with 15 ml of 1% water agar and inoculated with 100 ul of a test isolate (~10^8 cfu/ml) spread uniformly over the surface of the agar. Plates were incubated at 28°C for 3 d. The size of any zone of growth inhibition of the overlaid isolate

surrounding any dotted isolate was measured in millimeters from the edge of the dotted colony to the edge of the cleared zone; each inhibition zone was measured in two locations, at right angles to one another. Each isolate pair was replicated 3 times among Minnesota isolates, and 2 times among isolates from all other locations. Among global isolate pairs, inconsistent inhibition phenotypes (difference in zone size between measurements was greater than 2 mm) were repeated a third time. Inhibitory interactions were defined as interactions in which inhibition of one isolate by another was evident (zone size > 1.0 mm). Interactions were categorized as resistant in the absence of any inhibition zone.

Characterizing nutrient utilization:

Nutrient use profiles (niche) were evaluated for each isolate for 95 single carbon sources using Biolog SF-P2 plates as described previously (Schlatter et al, 2009). Briefly, spore suspensions (OD_{590}=0.22) were used to inoculate Biolog plates following the manufacturer's instructions. Plates were incubated for 3 d at 28°C and the growth on each carbon source was evaluated by measuring the absorbance of each well at 590 nm using a Multiskan EX microplate reader. The absorbance of the water control well was subtracted from the reading for each well.

Data analyses:

Pairwise inhibitory interactions among isolates were used to determine the specific inhibition and resistance profiles for each isolate and the proportion of inhibitory

interactions among isolates from each location. Sympatric isolate pairs were defined as *Streptomyces* from the same soil core and allopatric isolates were different soil cores. Niche overlap for every isolate pair was calculated using the formula:

Niche Overlap= $\{(\sum$ Min absorbance a,b) / Total absorbance a + $(\sum$ Min absorbance a,b) / Total absorbance b $\} / 2$

where (Min absorbance a, b) is the minimum absorbance value for a pair of *Streptomyces* isolates (a and b) on a given nutrient; these values are summarized over all n = 95 nutrients. The Total absorbance for an isolate a or b is the sum of absorbance values for that isolate over all n = 95 nutrients. Thus, niche overlap for any isolate pair (a and b) is the mean proportion of total nutrient use (absorbance values) which overlaps between the two isolates. All statistical analyses were conducted in SAS (SAS Institute, Inc.) or R (R Core Development Team, 2011).

Results

Inhibitory interactions among sympatric and allopatric isolates

Frequencies of inhibition among sympatric isolates varied significantly among locations ($X^2 = 28.3$; $p < 0.001$), ranging from 10% (PVB and PSC) to 33% (MN3 and MN5), suggesting that the significance of inhibition to species interactions varies across the landscape (Figure 1). Among individual isolates there was an extraordinary diversity of distinct inhibitory phenotypes. Most isolates (86%) were unique with respect to the

specific profile of isolates they inhibited. Consistent with previous work, individual *Streptomyces* isolates resisted inhibition consistently better than they inhibited others (Davelos et al, 2004a; D'Costa et al, 2006), and on average were resistant to 83% of all other isolates.

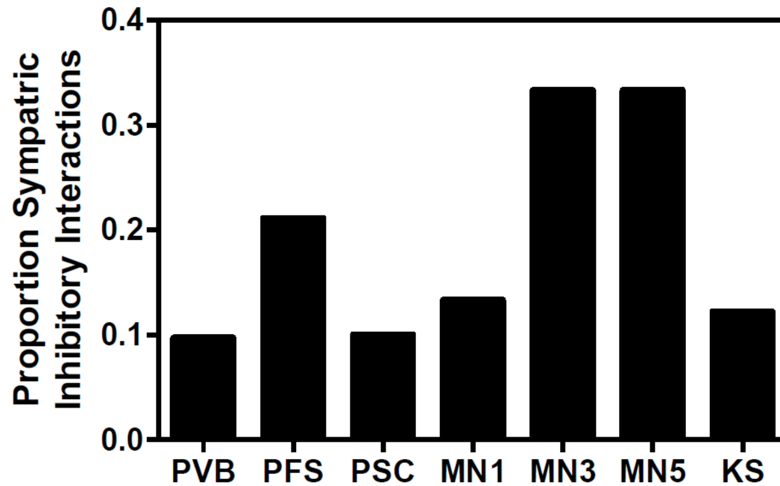

Figure 1. Frequency of inhibitory interactions among sympatric isolate pairs for each of the 7 sampling locations. Differences in the frequency of sympatric inhibitory interactions varied significantly among locations ($X^2 = 28.3$; $p < 0.001$). MN1, MN3, and MN5 = Minnesota 1, 3, and 5, respectively; KS = Konza Prairie; PVB = Panama Volcan Baru; PFS = Panama Fort Sherman, and PSC = Panama Santa Clara. There were n = 9-10 isolates from each location, resulting in a total of n = 72-90 sympatric interactions per location (n = 612 sympatric interactions total).

Sympatric and allopatric inhibitory interactions were evaluated to determine whether there is evidence for local selection of inhibitory phenotypes. Among all inhibitory interactions, the intensity of antibiotic inhibition was significantly greater among sympatric than among allopatric isolates (Figure 2; Welch two-sample t-test, $p < 0.0001$). Within individual locations, inhibition was also always greater against

sympatric than allopatric isolates (7 of 7 locations), though differences were statistically significant in only 5 of 7 locations (Figure 3; Welch two-sample t-test, $p < 0.05$). These data suggest that antibiotics that are strongly inhibitory towards coexisting isolates provide a local fitness benefit, and that antibiotic phenotypes are under local selection.

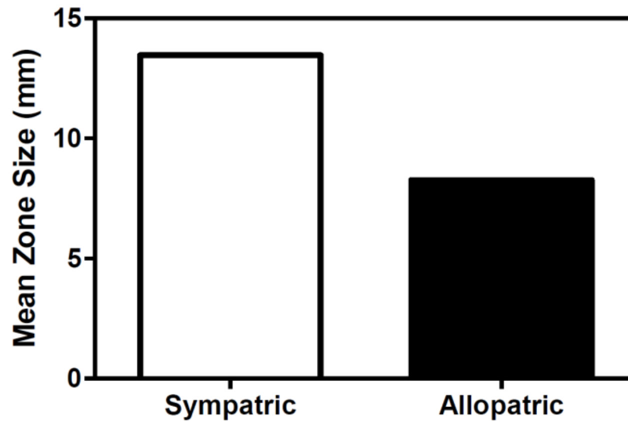

Figure 2: Intensity of antibiotic inhibition (mean zone size) among all inhibitory isolate pairs from the same (Sympatric, n = 118 interactions) and different (Allopatric, n = 667 interactions) locations. Mean zone size differed significantly among sympatric vs. allopatric isolate pairs ($p < 0.0001$, Welch two-sample t-test).

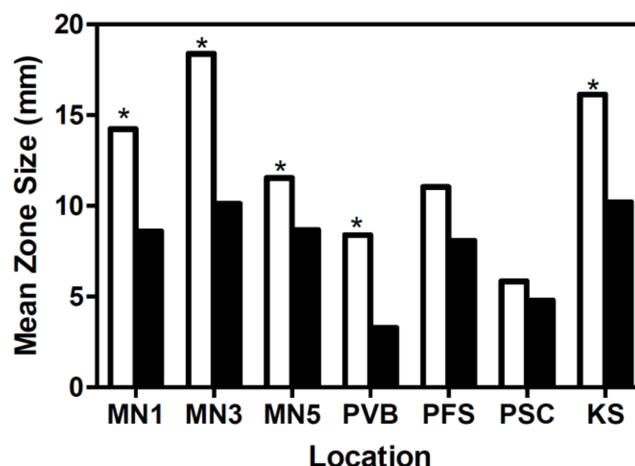

Figure 3: Intensity of antibiotic inhibition (mean zone size) among all inhibitory isolate pairs from the same (sympatric, open bars) and different (allopatric, solid bars) locations. There were n = 9-10 isolates from each location, and a total of n = 72-90 sympatric interactions and 540-590 allopatric interactions for each location. Pairs of bars from the same location marked with an asterisk differ significantly (Welch two-sample t-test $t = -6.5$; $p < 0.05$). MN1, MN3, and MN5 = Minnesota 1, 3, and 5, respectively; KS = Konza Prairie; PVB = Panama Volcan Baru; PFS = Panama Fort Sherman, and PSC = Panama Santa Clara.

Resistance interactions among sympatric and allopatric isolate pairs

In contrast to the inhibitory phenotypes, there was no consistent evidence among locations for accumulation of resistance to locally coexisting isolates. Specifically, comparing the frequency of resistance to antibiotics produced by sympatric vs. allopatric *Streptomyces*, there was greater resistance to sympatric than allopatric isolates in 4 of the 7 locations, and greater resistance to allopatric than sympatric isolates in 3 of the 7 locations, though differences were statistically significant in only two locations (data not shown). When data from all locations were combined, there was no significant

difference in the frequencies of resistance to allopatric v. sympatric isolates (Figure 4; $X^2 = 0.501$; $p = 0.48$).

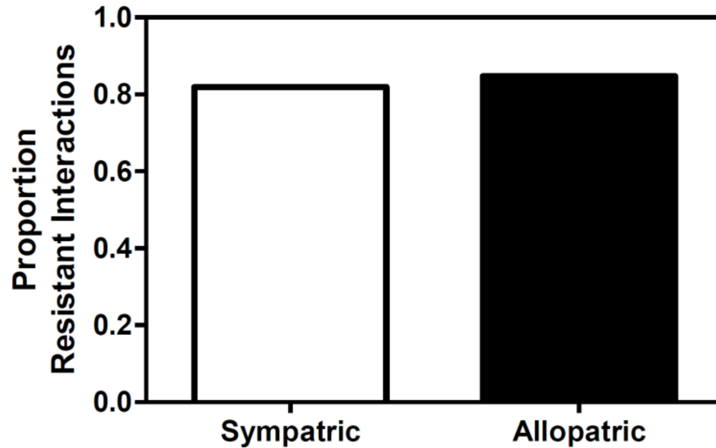

Figure 4: Frequency of non-inhibitory (resistant) interactions among all possible isolate combinations from the same (Sympatric, n = 612 interactions) and different (Allopatric, n = 4080 interactions) locations. The frequency of resistant interactions was not significantly different between sympatric vs. allopatric isolate pairs ($X^2 = 0.501$; $p = 0.48$).

Niche overlap and inhibition among sympatric and allopatric isolates

To explore the role of antibiotics in mediating competitive interactions among *Streptomyces*, we considered the relationships between niche (resource use) overlap and antibiotic inhibition among *Streptomyces*. Among inhibitory isolate pairs, there was a significant positive correlation between niche overlap and the intensity of antibiotic inhibition among sympatric ($R = 0.29$; $p = 0.0013$) but not allopatric isolate pairs ($R = 0.009$; $p = 0.478$). Thus, isolates from the same location were better at inhibiting isolates with which they had large niche overlap than isolates with which they exhibited little niche overlap. The strength of this relationship varied and was not always significant

within locations: niche overlap explained from 7 to 60% of the total variation in inhibition zone sizes for sympatric isolate pairs within individual locations (e.g. Figure 5). These data are consistent with the hypothesis that antibiotics mediate nutrient competition among *Streptomyces* populations within some local communities.

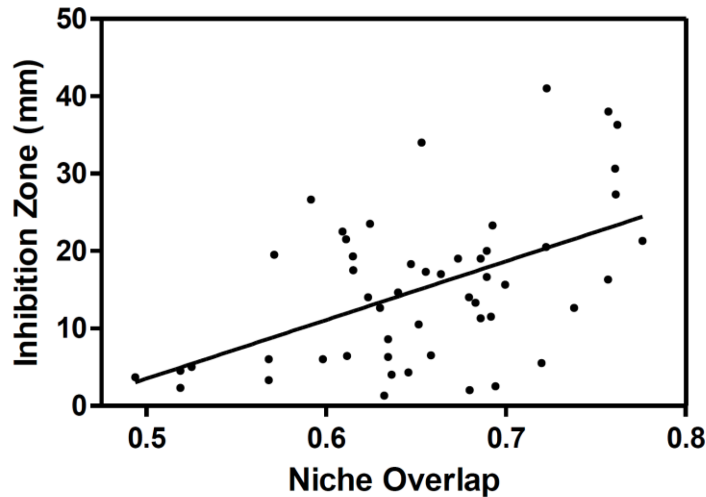

Figure 5: Relationships between mean niche overlap and mean inhibition zone size among sympatric inhibitory isolate combinations from three locations (PSC, MN1, and MN3; sympatric interactions only; R= 0.51, $p < 0.0001$; n = 51 independent isolate combinations).

Discussion

The greater accumulation of resistance versus inhibitory phenotypes suggests that there are consistently greater fitness benefits and/or smaller fitness costs for resistance than inhibitory phenotypes. For example, there is evidence from clinical settings that compensatory mutations may significantly reduce the costs to maintaining antibacterial resistance phenotypes, suggesting the possibility that, once acquired, there may be little loss of antibiotic resistance beyond genetic drift (Salyers and Amabile-Cuevas, 1997; Davies and Davies, 2010). Such `fossil resistance' may contribute significantly to the

total resistance profile of soil microbes. The greater frequency of resistance may also reflect a significant role for general resistance mechanisms that confer protection against multiple distinct antibiotics (e.g. efflux pumps) (Martinez et al, 2009b). Finally, resistance phenotypes may plausibly enhance microbial fitness in ways unrelated to protection against antibiotics (Martinez et al, 2009b; Allen et al, 2010; Davies and Davies, 2010), potentially contributing to the maintenance of resistance phenotypes in the absence of antibiotic selection. Regardless of the specific mechanisms leading to the accumulation of resistance capacities within individual isolates, the data are consistent with a substantial role for antibiotic resistance phenotypes in the fitness of *Streptomyces* across their geographic range.

The broad variation in antibiotic inhibitory and resistance phenotypes among *Streptomyces* isolates within and among locations suggests significant potential for selection. The greater intensity of antibiotic inhibition among sympatric versus allopatric *Streptomyces* provides evidence that the production of antibiotics that are highly effective against sympatric competitors confers a specific fitness benefit to the producer. Variation in the intensity of antibiotic inhibition among locations suggests shifts in the significance of competitive interactions and, presumably, antibiotics to *Streptomyces* fitness across the landscape. Populations having highly inhibitory phenotypes (e.g. location MN3) may experience the greatest fitness costs due to competitive interactions and, consequently, strongest selection for inhibitory phenotypes. In contrast, communities having less inhibitory local species interactions (e.g. location PSC) may be locations in which competitive interactions are less important to fitness, perhaps reflecting a significant

influence of the physical environment on fitness. For example, location PSC was located in a dunegrass stand on the edge of a hot, dry, sandy beach site along the Pacific coast in Panama. Alternatively, communities with less inhibitory interactions may be sites where antibiotics serve other functions (e.g. may act as signals rather than as weapons in mediating species interactions), and thus there is little production of antibiotics at inhibitory concentrations, or habitats where temperature and moisture stress, rather than species interactions, limit fitness, thus minimizing the benefits of inhibitory phenotypes. While variation in the dynamics of antibiotic-mediated species interactions and the significance of antibiotic inhibition to *Streptomyces* fitness may lead to diverse local coevolutionary dynamics across the landscape (Thrall and Burdon, 2003; Thompson, 2005; Nuismer and Thompson, 2006), overall the data provide compelling evidence for the significance of antibiotic inhibitory phenotypes to local species interactions and *Streptomyces* fitness in soil.

In contrast to inhibition, the apparent lack of locally-adapted resistance suggests a number of possibilities about the evolutionary or coevolutionary dynamics of antibiotic inhibitory and resistance phenotypes within soil *Streptomyces* communities. While one possible interpretation is that resistance does not confer a fitness benefit in local species interactions, this seems unreasonable given the accumulation of resistance among *Streptomyces* isolates from all locations and the substantial metabolic cost of resistance (Chapter 2). Instead, the lack of locally-adapted resistance may reflect the different cost:benefit ratios of antibiotic production (high cost, high benefit) vs. resistance (low cost, high benefit). Low fitness costs or a high frequency of compensatory mutations that

reduce costs of resistance may lead to retention of resistance in local habitats long after an antibiotic has been `lost' from the population (Bottger et al, 1998; Lenski and Riley, 2002; Luciani et al, 2009; Brandis et al, 2012; Dillon and Parti, 2012; Sousa et al, 2012). Specifically, while resistance phenotypes may be under strong local selection in the presence of a novel antibiotic, there may be a correspondingly rapid loss of antibiotic production capacity in the presence of high frequencies of resistance to that antibiotic. Novel antibiotic inhibitory phenotypes may be highly transient within populations (Cordero et al, 2012), reflecting the metabolic dexterity of *Streptomyces* (Tanaka and Omura, 1990; Challis and Hopwood, 2003), the high costs of antibiotic production (Williams and Vickers, 1986; though note Garbeva et al, 2011), and the frequencies of local resistance. This may generate an unbalanced coevolutionary dynamic in which local adaptation of inhibitory phenotypes is stronger than local adaptation of resistance, as resistance against antibiotics no longer produced by local competitors may be retained within the population. Consequently, evidence for local selection for resistance may be difficult to detect. General resistance mechanisms may also minimize the likelihood of locally-adapted resistance. More extensive, time-series analyses of antibiotic inhibitory and resistance phenotypes within coexisting populations under diverse cultural conditions are needed to determine the extent to which novel resistance phenotypes proliferate in response to new inhibitory phenotypes as predicted by coevolutionary models.

Resource competition is suggested to be a critical driver of antibiotic inhibitory interactions among *Streptomyces* (Schlatter et al., 2013). The positive correlation between niche overlap and the strength of inhibition among sympatric *Streptomyces*

supports this hypothesis. In contrast, there was no relationship between niche overlap and the intensity of inhibition among *Streptomyces* from different locations in soil, indicating that the niche - inhibitory phenotype relationship reflects local selection and not physiological tradeoffs. Thus, local selection for inhibitory phenotypes is significantly related to niche overlap and presumably the intensity of competition between isolates from the same location in soil. Although results suggest that antibiotic inhibitory interactions are broadly important for local resource competition, the data also suggest that the significance of antibiotics to competitive interactions vary across the landscape, with communities ranging from those in which competition is `managed' predominantly by niche differentiation to communities that are strongly antagonistic. This may reflect habitat variation (e.g. locations with little nutrient diversity and thus limited potential for niche differentiation may be most likely to support antagonistic species interactions), or the evolutionary potential of local populations. Perhaps most importantly, these data offer a distinct alternative to a coevolutionary arms race among interacting *Streptomyces*. Among isolates with little niche overlap, there is relatively less accumulation of inhibitory capacity. Thus, niche differentiation may significantly reduce the strength of selection for strong inhibitory phenotypes, and coevolutionary character displacement (Thompson, 2005) may minimize the potential for a coevolutionary arms race among sympatric *Streptomyces* populations in soil.

We conclude that antibiotic inhibitory phenotypes are under significant local selection and mediate nutrient competition in local soil communities. However, the relatively greater accumulation of resistance than inhibitory phenotypes among

Streptomyces isolates in natural habitats suggests that an escalating coevolutionary arms race may represent a cost-prohibitive trajectory for antibiotic-producers or that coevolutionary polymorphisms may be unbalanced at best. The significant positive association of inhibition with nutrient overlap among locally-coexisting *Streptomyces* suggests that antibiotics are likely to act as `weapons' against sympatric competitors, but that coevolutionary displacement may provide a significant means for minimizing fitness costs of interspecies resource conflicts and the likelihood of a coevolutionary arms race.

This work sheds light on roles of antibiotics in species interactions and coevolutionary dynamics within complex soil communities. However, because this study emphasizes inhibition and resistance among isolate pairs, these results are likely to underestimate both the complexity of *Streptomyces* coevolutionary dynamics and the potential outcomes of multi-species antibiotic inhibitory and resistance interactions in natural communities (Chait et al, 2012). Deeper understanding of the diverse roles of antibiotics in species interactions in soil, the dynamics of antibiotic inhibition in natural habitats, and especially the factors that may determine the potential for a coevolutionary arms race vs. coevolutionary differentiation are crucial for understanding the long-term trajectories of antibiotic-producing microbes in soil. Recent work suggests the potential for soil edaphic characteristics, nutrient availability or nutrient diversity in soil, physical environmental stress, and phylogeny to predict microbial inhibitory activities and coevolutionary interactions in soil populations (Schlatter et al, 2009; Bakker et al, 2010; Kinkel et al, 2011; Bailey and Kassen, 2012; Otto-Hanson et al, 2013). More detailed understanding of the precise roles of these factors in mediating microbial species

interactions and coevolution in soil will contribute significantly to the search for novel antibiotic biochemistries, to enhancing insight into the maintenance of antibiotic resistance genes in environmental microbes, and to managing disease suppressive activity of indigenous soil microbes (Kinkel et al, 2011; Kinkel et al, 2012; Martinez et al, 2011).

Chapter 4: Evolutionary biogeography of *Streptomyces* antibiotic inhibition, resistance, and resource use.

Background

Microbial biogeography, the study of the spatial distribution of microbial diversity, has engaged microbial ecologists for almost a century (Beijerinck, 1913; Baas-Becking, 1934). Recent studies of global patterns of soil microbial diversity have concluded that the taxonomic structure and diversity of soil microbial communities are variously organized by soil edaphic characteristics (eg. pH), soil history, climatic variables (moisture, temperature), and plant communities (Fierer et al., 2006; Griffiths et al., 2011; Garbeva et al., 2004; Garbeva et al., 2008; Lauber et al., 2009). Although valuable to our understanding of microbial community ecology and distribution, these studies have relied predominately on highly-conserved marker genes (eg. 16S rRNA genes) to infer bacterial community composition, structure, and diversity. As a result, these studies fail to capture important variation in bacterial phenotypes or functions that are critical to microbial populations. Many phenotypic traits that are critical to microbial fitness and expected to play important roles in structuring microbial communities are highly variable among closely related microbial taxa (Oda et al., 2002; Davelos et al., 2004a; Al Dahouk et al., 2012; Schlatter et al., 2013). Moreover, it has been argued that phenotypic traits, rather than phylogenetic units, are more accurately considered the units of microbial biodiversity (Green et al., 2008). However, there have been few efforts to explore the biogeography of microbial traits. A trait-based approach for studying microbial biogeography is likely to provide unique insight into how functional traits are

organized among distinct microbial populations and into the factors that structure the distribution of traits in space.

Biogeographic patterns in microbial traits may be generated by multiple processes. Environmental or niche filtering may occur where environmental parameters 'select' for taxa with traits that are best adapted to a given habitat (Langenheder and Székely, 2011). Alternatively, taxa may rapidly adapt to maximize fitness in local conditions via adaptive radiation (MacLean and Bell, 2003; Brockhurst et al., 2007), potentially resulting in similar traits among phylogenetically distinct taxa in the same locations. Although biogeographic patterns may emerge from niche filtering, local adaptation of traits, or a combination thereof, the role of local adaptation in the diversification and organization of microbial traits across space and evolutionary time in natural habitats is especially poorly understood. Efforts to link functional traits of microbial taxa from distinct geographic locations with phylogeny are needed to shed light on the role of functional trait adaptation in generating geographic patterns in microbial diversity.

Streptomyces are Gram-positive, filamentous bacteria that are ubiquitous in soils and sediments across the globe (Seipke et al. 2011; Kinkel et al., 2012). As a genus, *Streptomyces* harbors a remarkable diversity of traits important for human health and ecosystem function. Specifically, *Streptomyces* are well-known for their production of a large number of diverse antibiotic compounds, with an estimated 5-10% of a typical *Streptomyces* genome encoding antibiotic biosynthetic pathways (Omura, 2001; Watve et al., 2001; Bentley et al., 2002; Challis and Hopwood, 2003). Additionally, *Streptomyces*

are a significant reservoir of antibiotic resistance genes in soil, and may carry resistance to clinical antibiotics even in the absence of anthropogenic contamination (D'Costa et al., 2006; Bhullar et al., 2012). Finally, *Streptomyces* are capable of utilizing a diverse array of carbon compounds, including cellulose, chitin, and lignin, as resources for growth (Williamson et al., 2000; Schrempf et al., 2011; Schlatter et al., 2013), thus contributing to nutrient cycling in the environment. Insight into how variation in antibiotic inhibition, resistance, and resource use traits among *Streptomyces* populations is organized across diverse geographic locations will begin to shed light on the functional biogeography of this important genus.

Antibiotic inhibition, resistance, and resource use are believed to be crucial for competitive interactions among *Streptomyces* (Williams and Vickers, 1986; Davelos et al., 2004a; Hibbing et al., 2010; Schlatter et al., 2013). Because *Streptomyces* have linear chromosomes that are prone to recombination in regions that contain genes for antibiotic production and resistance (Egan et al., 1998; Wiener et al., 1998; Hopwood, 2007), as well as multiple plasmids, *Streptomyces* have the potential to evolve rapidly in response to selection, and specifically to interactions with coexisting community members. In particular, natural selection is expected to favor those antibiotic inhibitory traits that effectively inhibit resource competitors and resistance traits that protect individuals from inhibition (Kinkel et al., accepted). Thus, local selection for antibiotic inhibition, resistance, and resource use traits will depend on specific interactions that occur within the context of the microbial community. Variation in species interactions, and thus selection on traits involved in species interactions, across different communities may

generate biogeographic patterns in these traits and thus contribute to the extensive global diversity of antibiotic inhibition, resistance, and resource use traits among *Streptomyces*.

The objectives of this work were to 1) characterize a global collection of *Streptomyces* for antibiotic inhibition, resistance, and resource use traits; 2) explore variation in *Streptomyces* antibiotic inhibition, resistance, and resource use traits among distinct geographic locations; and 3) determine relationships between *Streptomyces* traits and phylogeny among locations. These data shed light on the biogeography of antibiotic inhibition, resistance, and resource use traits among *Streptomyces* and the role of adaptation in structuring trait characteristics among distinct geographic locations.

Methods

Streptomyces isolation

Soil samples from 15 different locations around the globe were collected using a standard soil corer (10 cm x 2.5 cm). Sampling locations and their abbreviations are described in Table 1. In Minnesota (MN), soil sampling was performed differently, and three adjacent small (30 cm x 1 cm) soil cores were taken from 3 locations in MN as described in Davelos et al. (2004a). Soil samples were homogenized and subsamples of each soil (1g) were shaken in 10 ml sterile deionized H_2O in an orbital shaker at 4 C for 1 h. Samples were dilution plated on starch-casein agar (SCA) and after 5-7 days of growth at 28 C, colonies exhibiting characteristic *Streptomyces* morphology were isolated and streaked to obtain pure cultures. A subset of isolates (n=9-12) from each location was

randomly selected for in-depth phenotypic and genetic characterization for a total of n=153 isolates.

Table 1. Sites from which soil was sampled for *Streptomyces* isolation.

Location of Origin	**Abbreviation**	**Number of Isolates**	**Habitat Type**
Antarctica	Ant	n=10	Antarctic peninsula
La Cima, California, USA	CALC	n=10	Hot desert
Cevennes National Park, France	Cev	n=10	Temperate forest
Hawaii, USA	Haw	n=10	Montane forest
Kansas, USA	KS	n=10	Native tallgrass prairie
Morocco	MC	n=10	Agricultural field
Minnesota, USA	MN1	n=10	Native tallgrass prairie
Minnesota, USA	MN3	n=10	Native tallgrass prairie
Minnesota, USA	MN5	n=10	Native tallgrass prairie
Montseny, Spain	Mont	n=11	Montane forest
New Zealand	NZ	n=10	Temperate beech forest
Fort Sherman, Panama	PanFS	n=10	Tropical rain forest
Santa Clara, Panama	PanSC	n=10	Tropical sand beach
Volcan Baru, Panama	PanVB	n=10	Tropical cloud forest
Witzenhausen, Germany	Witz	n=12	Temperate forest

Antibiotic inhibition

Antibiotic inhibitory traits of *Streptomyces* isolates were evaluated against 5 standard *Streptomyces* isolates that vary in their antibiotic resistances (DL87, 4-2, 2-12, 4-16, and 6-14; Davelos et al., 2004b) using an agar-overlay method as described in Davelos et al. (2004a). Briefly, 10 ul of spore suspension of each isolate (~10^8 CFU/ml) was dotted onto 15 ml SCA, grown for 3 days, and killed by inverting plates over 4 ml chloroform in a watchglass. After residual chloroform was left to evaporate in a laminar flow hood for 30 min, 15 ml of 1% water agar was pipetted onto each plate and 100 ul of

spore stock of each test standard was overlayed by spread plating. After 3 days of growth at 28 C, inhibition zone sizes were measured as the radius from the edge of the colony growth to the edge of any clear inhibition zone. Each isolate-standard interaction was replicated 2-3 times and mean inhibition zone sizes were determined. Interactions were considered inhibitory where inhibition zones were >2 mm.

Antibiotic resistance

Streptomyces isolates were characterized for resistance to common, *Streptomyces*-produced, clinical antibiotics using a disc-diffusion assay as described in Otto-Hanson et al. (2013). Briefly, filter paper disks containing streptomycin (10 ug), chloramphenicol (30 ug), erythromycin (15 ug), vancomycin (30 ug), rifampicin (10 ug), amoxicillin/calvulanic acid (30 ug), and tetracycline (30 ug) (BD BBL Sensi-Disc; Becton, Dickinson and Company, Sparks, MD) were placed on SCA plates immediately after 100 ul of spore suspension of an isolate was spread-plated and dried. After 3 days of growth at 28 C, inhibition zones were measured as described above. Isolate-antibiotic interactions where inhibition zones were <2 mm were considered resistant.

Resource use characterization

Resource use was evaluated on 95 sole carbon sources for each isolate using Biolog SF-P2 plates (Biolog, Inc. Hayward, CA) as described previously (Schlatter et al., 2009). Briefly, fresh spore suspensions of each *Streptomyces* isolate were adjusted to an optical density of 0.22 at 590 nm, diluted according to the manufacturer's instructions (1.5 mL spore suspension in 13.5 mL 0.2% carrageenan), and inoculated into 96-well Biolog plates. The absorbance (au) of each well was determined after 3 days of incubation at 28

C using a Multiskan EX microplate reader (Labsystems, Helsinki, Finland) at 590 nm. For each plate, the absorbance of the water control well was subtracted from the absorbance of all other wells before analysis. We considered used resources to be those on which a *Streptomyces* isolate grew to an absorbance greater than 0.005 above the water control well. Using this definition, niche width, resource use efficiency, and efficiency on preferred resources were determined for each isolate. We defined the niche width of an isolate as the number of used resources, total growth as the sum of absorbances across all resource, and resource use efficiency as the mean absorbance value for used resources. Niche overlap among isolates was calculated as described previously (Kinkel et al., in press).

16S rRNA gene sequencing

Sequences for 16S rRNA genes were obtained as described previously (Davelos et al., 2004c). Briefly, genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega. Madison, WI) according to the manufacturer's instructions with minor modification and 16S rRNA genes were amplified using the universal bacterial primers 27F (5'- AGAGTTTGATCCTGGCTCAG-3') and 1541R (5'- AAGGAGGTGATCCAGCCGCA-3') in a 50ul reaction volume using PCR Supermix High Fidelity master mix (Invitrogen, Carlsbad, CA) with 10 pM each primer and 100 ng template DNA following the thermocycling protocol of Takeuchi et al., (1996). Amplicons were sequenced using the forward primer (27F) at the University of Minnesota Biomedical Genomics Center (Saint Paul, MN). Sequences were edited manually, aligned, and trimmed to 685 bp of good quality sequence using MEGA (v5.2;

Tamura et al., 2011). A neighbor-joining phylogenetic tree was constructed in MEGA using 1000 bootstraps to assess confidence in branch points (Supplemental Figure 1). A matrix of pairwise distances was constructed and sequences were binned into operational taxonomic units (OTUs) based on a 99% similarity cutoff in mothur (v1.31; Schloss et al., 2009). Finally, sequences were classified using the Ribosomal Database Project Naïve Bayesian Classifier (Wang et al., 2007).

Statistical Analyses

All statistical tests assessing phenotypic differences among locations and OTUs were performed in R (R Development Core Team, 2011) unless noted otherwise. Similarity in antibiotic inhibition, resistance, and resource use profiles among isolate pairs was calculated as the Euclidean distance among quantitative profiles (inhibition and resistance zone sizes, resource use absorbance) using the vegan package in R (Oksanen et al., 2011). PERMANOVA was used to partition variance in similarity among locations using the Adonis function of vegan with 999 matrix permutations. Relationships between 16S distance and antibiotic inhibition, resistance, and resource use patterns were assessed using Mantel tests with 999 permutations. Finally, AMOVA and unifrac analyses of 16S rRNA sequences were performed in mothur to test for differences in community structure among locations.

Results

Diversity of antibiotic inhibition, resistance, and resource use among a global collection of *Streptomyces*

Among all isolates (n=153) there were 19 distinct profiles (+/-) of antibiotic inhibition of the 5 test standards out of a total of n=32 possible profiles. On average, isolates inhibited 0.9 standards although most *Streptomyces* did not inhibit any standards (63%, or 97/153; Figure 1). Of these, 42.9% (25/56 inhibitory isolates) inhibited only one standard, while 23.2% (13/56 inhibitory isolates) inhibited all 5 standards. This indicates a bimodal distribution of inhibitory capacities among *Streptomyces* isolates.

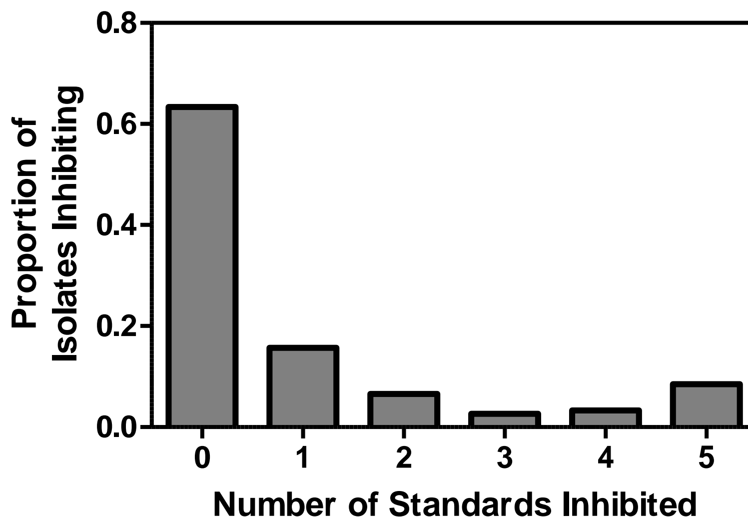

Figure 1. Distribution of antibiotic inhibitory capacities against 5 standards among a global collection of *Streptomyces*.

When all *Streptomyces* isolates were screened for resistance against 7 antibiotics, there were 27 unique resistance profiles out of n=128 possible profiles. Although some

isolates (21.6%, or 33/153) did not resist any antibiotics, most isolates (78.4%, or 120/153) resisted at least one of the antibiotics tested. The proportion of resistant *Streptomyces* varied among antibiotics (χ^2=216, df=6, $p<0.0001$; Figure 2). Resistance to tetracycline or rifampicin were most common (49% of isolates were resistant to one or both of these antibiotics), whereas resistance to streptomycin and vancomycin was rare (2.6% and 3.9% of isolates were resistant, respectively). Thus, although antibiotic resistance is common among *Streptomyces*, the likelihood of resistance varies among antibiotics.

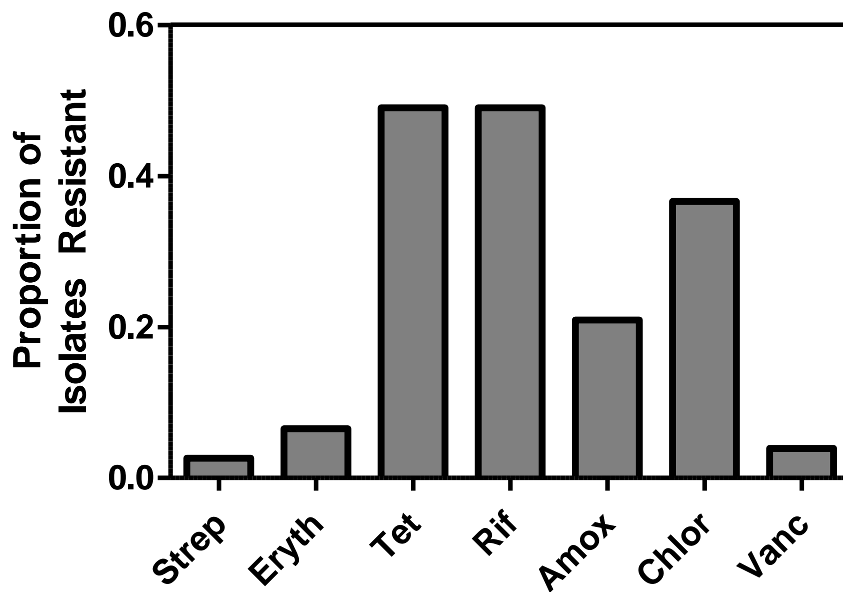

Figure 2. Proportion of all *Streptomyces* resistant to clinically relevant, *Streptomyces*-produced antibiotic compounds. Compounds include streptomycin (Strep), erythromycin (Eryth), tetracycline (Tet), rifampicin (Rif), amoxicillin (Amox), chloramphenicol (Chlor), and vancomycin (Vanc).

Resource use patterns among the global collection of *Streptomyces* were diverse. Considering patterns of utilized resources (+/-) there were 148 unique patterns among the 153 *Streptomyces* isolates. On average, individual *Streptomyces* isolates used 63.8

resources, though use ranged from 9 to 95 of 95 possible resources among all isolates. Total growth of *Streptomyces* on all resources ranged from 0.39 to 33.23 au with an average of 7.32 au. Growth efficiency, or mean growth on utilized resources, ranged from 0.033 to 0.44 au with an average of 0.11 au. Thus, *Streptomyces* isolates exhibited considerable variation in the numbers and identities of compounds on which they grew as well as growth efficiency.

Streptomyces communities vary among locations in antibiotic inhibition, resistance, and resource use.

The breadth and intensity of antibiotic inhibitory capacities differed among *Streptomyces* from distinct locations. Specifically, *Streptomyces* isolates from different locations varied in the mean number of standards that they inhibited (Figure 3A; ANOVA, df=14, F=2.39, p=0.005). On average, *Streptomyces* from PanFS and MN5 inhibited the most standards (2.1 and 2.0 standards, respectively), whereas those from Cev and CALC inhibited the fewest (0 and 0.2, respectively). Thus, some locations supported *Streptomyces* with broader antibiotic inhibitory capacities than others. In contrast, mean inhibition zone size among *Streptomyces* from different locations varied only marginally (Table 1; ANOVA; df=14, F=1.69, p=0.064). However, when considering *Streptomyces* inhibition of individual standards, mean zone sizes varied significantly among locations for 3 of the 5 standards (Supplemental Table 1). Thus, although the overall intensity of inhibition against all standards varied only marginally among *Streptomyces* from different locations, *Streptomyces* from some locations were

significantly more effective at inhibiting specific standards than those from other locations.

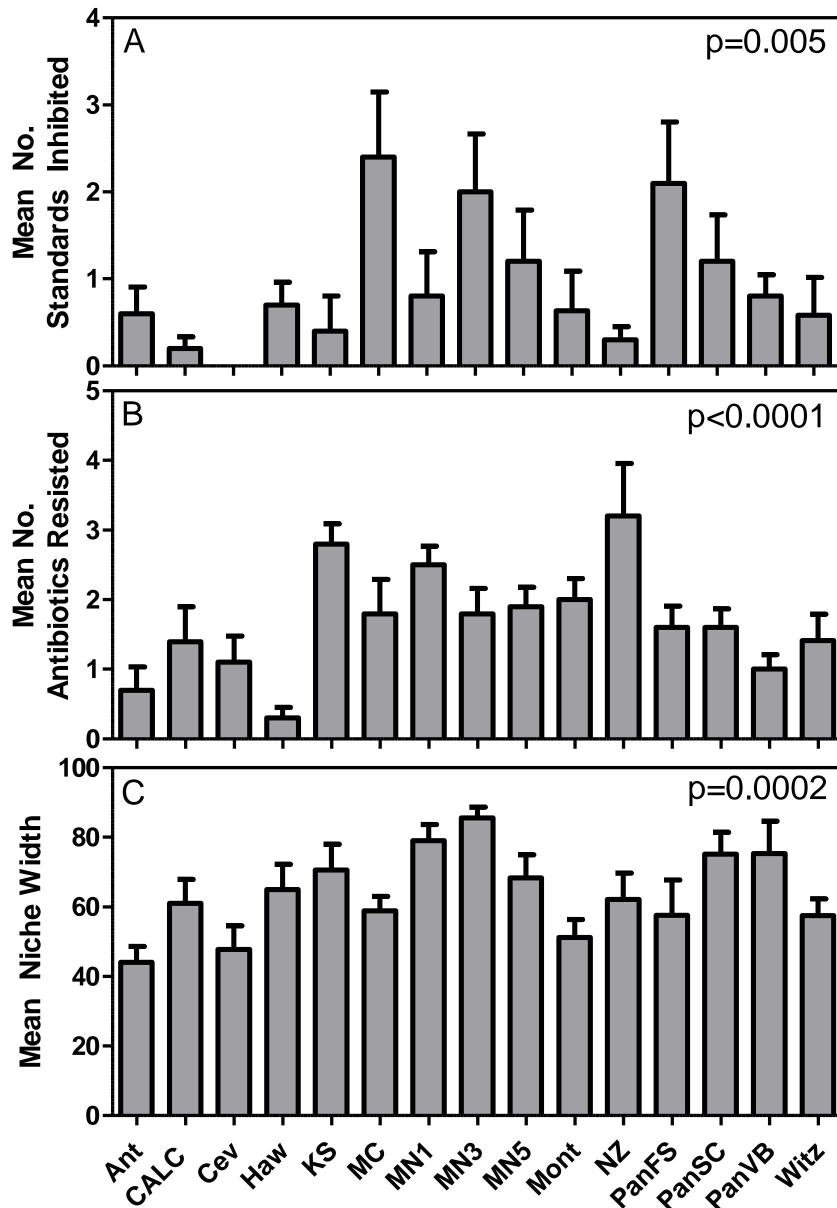

Figure 3. Variation in the mean number of standards inhibited (A), antibiotics resisted (B), and niche width (C) among *Streptomyces* populations from different locations. P-values of ANOVAs are presented.

Antibiotic resistance also varied among *Streptomyces* from different locations. Isolates from different locations varied in the mean number of antibiotics that they resisted (Figure 3B; ANOVA; F=4.06, p<0.0001). Populations from NZ and KS were the most resistant on average (resisting 3.2 and 2.8 of 7 antibiotics, respectively) whereas those from Haw and Ant were the least resistant (0.3 and 0.7 resistances, respectively). Thus, some geographic locations were more likely to harbor *Streptomyces* populations possessing resistance to a broad suite of antibiotics. *Streptomyces* also varied among locations in their susceptibility to each of the individual antibiotics tested (Table 2); populations from some locations had greater susceptibility/resistance to inhibition by specific antibiotics.

Table 2. Mean susceptibility (mm zone size) of *Streptomyces* to antibiotics (ug/disk) among locations and ANOVAs assessing significant variation in resistance among locations.

Location	**Strep (10ug)**	**Eryth (15ug)**	**Tet (30ug)**	**Rif (10ug)**	**Amox (30ug)**	**Chlor (30ug)**	**Vanc (30ug)**
Ant	17.4	16.0	10.9	5.3	8.3	10.5	12.8
CALC	21.2	12.6	5.0	4.6	9.8	10.1	16.2
Cev	14.2	10.9	5.4	10.5	5.6	2.0	15.1
Haw	10.9	14.6	6.2	7.1	11.9	8.6	12.1
KS	14.5	9.7	1.4	1.3	4.0	2.8	9.6
MC	12.8	5.9	5.9	2.9	7.5	5.8	11.3
MN1	9.2	10.7	0.4	1.9	4.0	4.3	8.5
MN3	15.3	12.0	1.0	3.0	10.1	7.8	12.8
MN5	17.0	13.5	2.1	2.3	5.9	7.2	11.8
Mont	12.1	9.8	3.2	1.8	6.7	7.4	10.6
NZ	15.9	6.7	2.2	2.3	3.7	4.0	6.2
PanFS	12.7	8.0	3.8	4.3	5.7	7.4	13.0
PanSC	17.3	15.2	2.5	3.2	10.9	10.8	11.9
PanVB	9.1	18.0	6.7	5.1	13.6	3.7	10.8
Witz	9.4	5.4	5.5	5.5	5.4	6.7	8.6
df	14	14	14	14	14	14	14
F	5.06	4.07	5.1	4.56	3.27	2.68	4.99
p-val	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	0.002	<0.0001

In addition to antibiotic inhibition and resistance, *Streptomyces* from different locations also varied in their capacities to utilize resources. Specifically, total growth, mean efficiency, and niche widths of *Streptomyces* differed among locations (Figure 3C; Supplemental Table 2). *Streptomyces* populations from Mont and CA-LC had the highest total growth overall and on average grew more efficiently on used resources than those from other locations. In contrast, populations from MN1 and Cev had the lowest total growth and grew less efficiently than other locations. Similarly, *Streptomyces* populations from some locations tended to be niche generalists and had large resource use niches (eg. *Streptomyces* from MN1 and MN3 used an average of 79 and 85.6 of 95 possible resources, respectively) compared to populations from other locations that tended to support niche specialists (eg. *Streptomyces* from Ant and Cev used on average 44 and 47.8 of 95 possible resources, respectively).

Patterns of *Streptomyces* trait variation among locations

In addition to the breadth and intensity of inhibition, similarity in the strength and specific pattern of *Streptomyces* inhibition phenotypes against standards was significantly related to location of origin (Adonis; df=15, F=1.79, r^2=0.16, p=0.04). However, *Streptomyces* inhibition phenotypes were only marginally more similar among isolates from the same versus different locations (Euclidean distance = 7.76 and 8.43, respectively; Welch's t-test, t=1.71, p=0.09). When considering individual locations, *Streptomyces* from 6 of 15 locations (CALC, Cev, Haw, NZ, PanVB, and Witz) had

significantly more similar inhibitory phenotypes within versus among locations. One location (PanFS) had significantly less similar inhibitory phenotypes among isolates within versus among locations (Euclidean distance =18.81 and 14.42, respectively; Welch's t-test, t=2.18, p=0.035). Among the remaining locations, there was no significant difference in similarity in inhibitory phenotypes among isolates from within or among locations. More similar antibiotic inhibitory phenotypes within vs. among locations may reflect local spread of antibiotic genes within communities. However, the lack of significant difference in similarity in inhibitory phenotypes within versus among communities for many locations suggests that horizontal transfer of antibiotic production genes is often limited, or that there is little selection for specific antibiotic inhibitory phenotypes within geographically distinct *Streptomyces* communities.

Location of origin explained a significant portion of variation in patterns of antibiotic resistance among *Streptomyces* isolates (Adonis, df=15, F=3.95, r^2=0.30, p=0.001). *Streptomyces* from the same location had more similar resistance phenotypes than those from different locations overall (Euclidean distance =16.13 and 19.18, respectively; Welch's t-test, t=10.39, p<0.0001). Differences were significant in 10 of the 15 locations (data not shown). This suggests that, in general, selection for antibiotic resistance among locations favors similar antibiotic resistance traits within *Streptomyces* populations from the same location, which may reflect high frequencies antibiotic resistance gene exchange within *Streptomyces* communities.

Streptomyces isolates from the same locations in soil tended to utilize similar resources compared to those from different locations. Location of origin explained 32.2%

of similarity in resource use patterns among *Streptomyces* (Adonis; r^2=0.32 , F=4.23, p=0.001). *Streptomyces* resource use patterns were also more similar among isolates from the same location versus different locations (mean Euclidean distance = 1.08 and 1.26, respectively; Welch's t-test, t=6.32, p<0.0001). This pattern was significant for 10 of the 15 locations (data not shown), suggesting local selection for resource use preferences in many locations.

In addition to resource use patterns, average niche overlap among *Streptomyces* pairs was greater among isolates from the same versus different locations (mean niche overlap=0.60 and 0.52, respectively; Welch's t-test, t=15.24, p<0.0001). However, within-location niche overlap differed among locations (Figure 4; ANOVA; df=14, F=11.38, p<0.0001). Together these data suggest that resource competition is greater between coexisting *Streptomyces* but that the strength of resource competition may vary among *Streptomyces* communities. Moreover, within-location niche overlap was positively correlated with mean inhibitory capacity (number of standards inhibited) among *Streptomyces* populations (R=0.47, p=0.07), suggesting that locations with stronger resource competition support more inhibitory *Streptomyces*. However, mean resistance capacity (number of antibiotics resisted) was not significantly related to niche overlap (R=0.25, p=0.37).

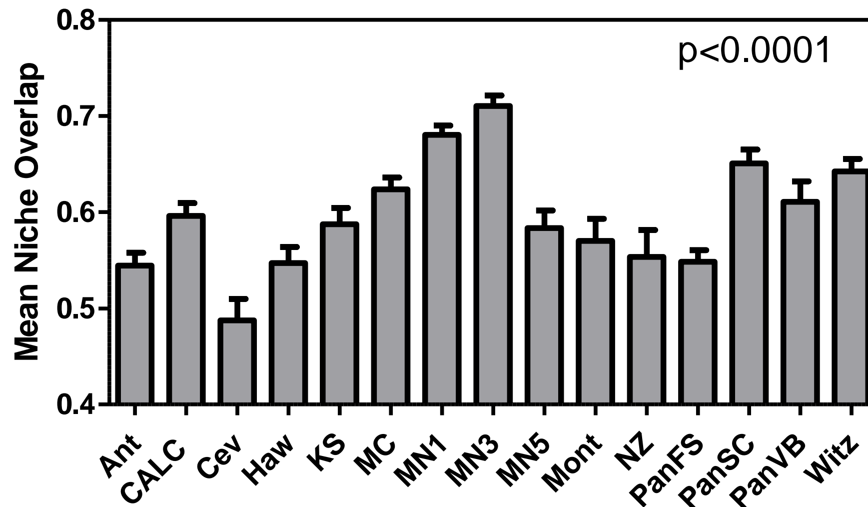

Figure 4. Mean niche overlap within *Streptomyces* communities among different locations. The P-value from an ANOVA across locations is presented.

Streptomyces phylogeny

All 153 isolates were identified as *Streptomyces* or closely related species (*Kitasatospora* and *Streptacidiphilus;* Supplemental Table 3). Isolates clustered into 60 OTUs at 99% similarity. Two-thirds (20/30) of non-unique OTUs were found in multiple locations whereas the remaining one-third (10/30 OTUs) of OTUs with multiple representatives clustered to a single geographic location. Although we evaluated only a small number of isolates from each location, *Streptomyces* populations were phylogenetically more similar within versus among locations (AMOVA; Fs=4.48, $p<0.001$; unweighted unifrac =0.53, $p<0.001$). These data suggest that although most OTUs are globally distributed, some *Streptomyces* may be more common within or endemic to specific geographic areas.

Relationships between *Streptomyces* phylogeny and phenotype

Streptomyces belonging to the largest OTUs (18 OTUs with n=3-20 isolates) varied in aggregate antibiotic inhibition, resistance, and resource use phenotypes. While *Streptomyces* OTUs did not vary significantly in the number of standard isolates that they could inhibit (ANOVA; F=1.41, p=0.15), there were differences among OTUs in mean inhibition zone sizes (ANOVA; F=2.03, p=0.019). Further, OTUs varied in intensity of inhibition against 2 of the 5 individual standards (data not shown). Overall, this suggests that some OTUs tend to be stronger inhibitors than others. Finally, *Streptomyces* OTUs also varied in the number of antibiotics that they could resist (ANOVA; F=5.79, p<0.0001) and their susceptibility (mean inhibition zone size) to antibiotics (ANOVA; F=5.01, p<0.0001). OTUs also differed in their susceptibility to each individual antibiotic tested (Supplemental table). Thus, *Streptomyces* OTUs differed in aggregate antibiotic resistance traits and resistance to specific antibiotics, suggesting that antibiotic resistance traits may be conserved among *Streptomyces* phylogenetic groups. Considering resource use, *Streptomyces* OTUs differed significantly in total growth and growth efficiency (ANOVA; F=3.20 and 4.29, p=0.0002 and <0.0001, respectively), but not niche width (ANOVA; F=1.34, p=0.19). Thus, some OTUs were better at utilizing resources for growth than others. In total, these data demonstrate that although antibiotic inhibitory phenotypes are highly diverse among isolates from the same OTU, *Streptomyces* OTUs have distinct strategies for resisting inhibition by antibiotics and for utilizing resources.

Despite some significant trait variation among *Streptomyces* OTUs, relationships between 16S rRNA gene sequences and specific inhibition, resistance, and resource use

phenotypes were modest. Similarity in 16S sequence and inhibition profiles were marginally related (Mantel r=0.10, p=0.05). There was a significant correlation between 16S sequences and antibiotic resistance profiles among *Streptomyces* isolates (Mantel r=0.16, p<0.001), though it explained only a small proportion of total variation in resistance profiles. Among all isolates there was no significant correlation between 16S similarity and resource use profile (Mantel r=-0.03, p=0.66) or niche overlap (Mantel r=-0.17, p=0.99) among isolates. Thus, 16S rRNA gene sequences have a limited capacity to inform specific inhibition, resistance, and resource use phenotypes among *Streptomyces*. This suggests that local adaptation and horizontal gene transfer may generate substantial trait diversity within closely related phylogenetic groups and thereby limit the utility of phylogenetic markers for understanding ecological and evolutionary dynamics of *Streptomyces* traits within communities.

<u>Trait adaptation within phylogenetic groups.</u>

When comparing *Streptomyces* belonging to the same OTUs, those that originated from distinct locations sometimes differed in antibiotic inhibition, resistance, and resource use. Two OTUs (OTU1 and OTU2) had at least 3 isolates from different locations, though sample sizes from individual locations were small (n=3-6). Among locations (MN1, n=6; PanFS, n=3; Ant, n=5), *Streptomyces* in OTU1 varied significantly in the number of standards that they could inhibit (Figure 5; ANOVA; F=5.851, p=0.019) and in mean inhibition zone sizes (ANOVA; F=4.40, p=0.04). Specifically, OTU1 *Streptomyces* from PanFS inhibited significantly more standards and had larger inhibition

zone sizes than those from MN1 or Ant (TukeyHSD, $p<0.03$ and $p<0.06$ among each comparison). Further, these isolates varied significantly among locations in inhibition zone sizes against 4 of the 5 individual standards (data not shown). However, isolates from OTU2 (MN5, n=3; Haw, n=3; Mont, n=3) originating from Haw and Mont did not differ significantly in number of standards inhibited (t-test; $t=1.34$, $p=0.25$), mean inhibition zone sizes (t-test; $t=0.36$, $p=0.74$), or in their inhibition zones against individual standards (data not shown). Although *Streptomyces* belonging to OTU2 originating from MN5 did not inhibit any standards, we were unable to statistically differentiate these from Haw or Mont isolates due to the lack of phenotypic variation. In total these data suggest that local selection for antibiotic inhibitory traits varies among OTUs.

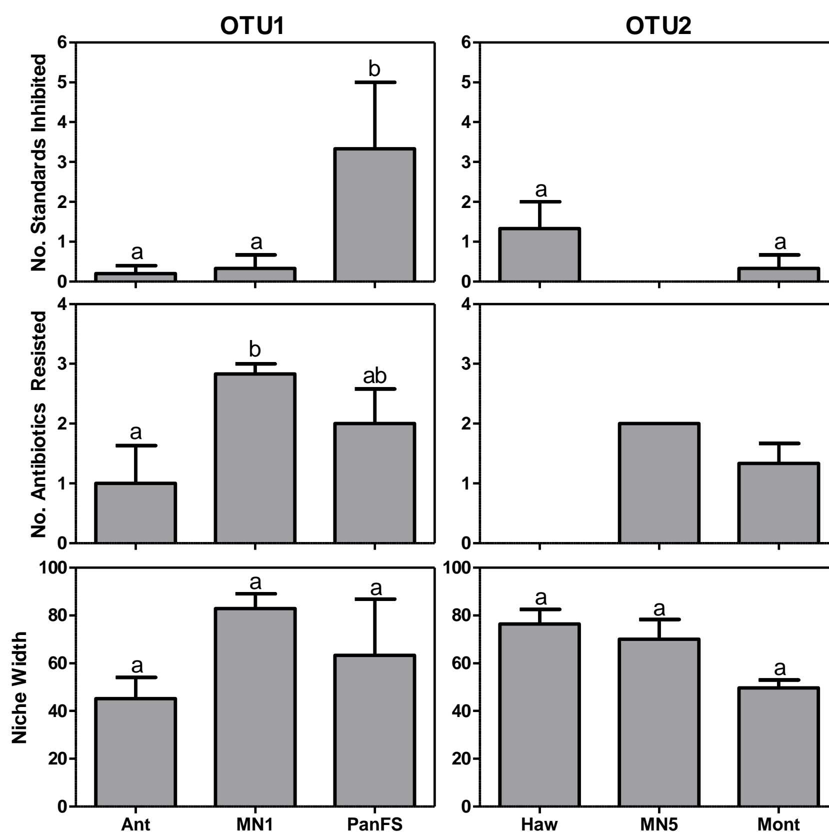

Figure 5. Variation in the number of standards inhibited (top row), number of antibiotic resisted (middle row), and niche width (bottom row) among *Streptomyces* isolates from the same OTU but originating from different locations. Error bars represent standard error. The same letters above bars indicates no statistical difference among bars, as determined by Tukey's post-hoc honest significance tests. For OTU2, MN5 was excluded from analyses of the number of standards inhibited among locations and t-test were used instead of ANOVA. Due to lack of variation in the number of antibiotics resisted among isolates within locations for OTU2, we were unable to test for statistical differences.

Streptomyces isolates from OTU1 but originating from different locations also differed in the number of antibiotics they were able to resist (ANOVA; F=4.65, p=0.03). Isolates from MN1 resisted a significantly greater number of antibiotics on average than

those from PanFS or Ant (mean of 2.8 vs 2 and 1 antibiotics resisted, respectively). Isolates from MN1 were significantly more resistant to streptomycin and vancomycin than those from Ant (TukeyHSD, p=0.04) and PanFS (TukeyHSD, p=0.02). OTU2 *Streptomyces* from Haw did not resist any antibiotics, whereas those from MN5 and Mont resisted 2 and 1.3 antibiotic on average, respectively. *Streptomyces* from Haw were significantly more susceptible to rifampicin than those from MN5 (TukeyHSD, p=0.02) and Mont (TukeyHSD, p=0.01).

Within OTU1, *Streptomyces* from different locations did not vary significantly in total growth or growth efficiency (ANOVA; F=0.57 and 1.94, p=0.58 and 0.19, respectively), and variation in niche width was marginally significant (ANOVA; F=3.55, p=0.06). Similarly, *Streptomyces* belonging to OTU2 but from different locations varied in niche width (Mont<HW and MN5; ANOVA; F=4.93, p=0.05), but not total growth or mean growth efficiency (ANOVA; F= 0.40 and 2.72, p=0.69 and 0.14, respectively). These data suggest that although OTU1 and OTU2 *Streptomyces* from different locations have comparable growth efficiencies, niche widths are locally adapted.

Discussion

Recent studies of soil microbial biogeography typically highlight the importance of soil physical or chemical characteristics, climatic variables, or plant communities for determining the phylogenetic structure and biodiversity of soil communities (Fierer et al., 2007; Griffiths et al., 2011; Garbeva et al., 2004; Garbeva et al., 2008). However, a trait-based view of microbial biogeography can offer insight into patterns in functional traits

crucial to microbial fitness and the ecological and evolutionary forces that structure and generate functional diversity in soil communities (Green et al., 2008). Trait-based microbial biogeography may also shed light on roles microbial species interactions in structuring microbial populations. As a first step towards a trait-based understanding of *Streptomyces* biogeography, we document here geographic patterns in antibiotic inhibitory, resistance, and resource use capacities among *Streptomyces* populations from distinct locations on multiple continents. Although, immigration/dispersal dynamics, environmental filtering, and environmental gradients are all likely to be important in structuring microbial traits (Martiny et al., 2006; Green et al., 2008), competitive species interactions are argued to be important drivers of antibiotic inhibition, resistance, and resource use among *Streptomyces* (Wiener et al., 1998; Davelos et al., 2004a; Schlatter et al., 2013; Kinkel et al, accepted). The great diversity of *Streptomyces* inhibitory, resistance, and resource use phenotypes observed here suggests that the selective forces that shape *Streptomyces* phenotypes vary substantially across locations and that *Streptomyces* phenotypes are highly plastic in response to selection. This suggests that variation in the significance of competitive interactions for *Streptomyces* fitness among distinct communities is likely to be crucial in generating biogeographic patterns in these traits among distinct locations in soil.

Streptomyces populations varied in their antibiotic inhibitory capacities among locations. Locations that support *Streptomyces* with broad and highly potent inhibitory phenotypes may be competitive ‘hotspots’ that have selected for *Streptomyces* populations that are effective inhibitors of resource competitors. In contrast, resource

competition may be less important to *Streptomyces* fitness within locations where populations have little inhibitory capacity. These populations may be niche differentiated (Kinkel et al., 2011), have alternative mechanisms for mediating competition such as signaling (Yim et al., 2007; Vaz Jauri et al., accepted), or be under strong selection by forces unrelated to resource competition (eg. abiotic stress, parasitism, or predation; Weekers et al., 1993; Ashelford et al., 2003). The positive relationship between mean niche overlap, a proxy for resource competition, and the mean number of standards inhibited among *Streptomyces* populations from different locations supports the idea that resource competition imposes significant selection for antibiotic inhibitory phenotypes within local *Streptomyces* populations. Because competitive dynamics and subsequent selection will vary among locations (Thompson, 2005; Kinkel et al., in press), resource competition is likely to contribute to the large diversity of *Streptomyces* antibiotic phenotypes at a global scale (Czárán et al., 2002; Kinkel et al., accepted).

The poor correspondence between phylogeny and inhibitory phenotype and the high variability of inhibitory phenotypes within and among locations suggests that geographic patterns of inhibitory phenotypes are not likely to be structured by the colonization of specific OTUs. Rather, adaptation of inhibitory phenotypes to inhibit locally coexisting resource competitors may play a major role in structuring *Streptomyces* inhibition (Kinkel et al., accepted). Indeed, OTU 1 *Streptomyces* from PanFS were better inhibitors than those from other locations, suggesting that *Streptomyces* in this phylogenetic group have been selected for stronger antagonistic capacities at PanFS. Alternatively, OTU1 *Streptomyces* may have lost antagonistic capacities in other

locations (MN1 and Ant) if antagonistic phenotypes incur a net fitness cost rather than a benefit in those locations (Schlatter and Kinkel, in review). In contrast, *Streptomyces* belonging to OTU2 did not differ in inhibitory phenotypes among locations, suggesting that not all phylogenetic groups are locally adapted, perhaps reflecting a smaller role of antibiotic inhibition in the competitive strategy for this OTU. Alternatively, limitations of our antibiotic assay or small sample size (n=3 isolates/location) may have inhibited our ability detect a signal of adaptation.

Consistent with previous work (D'Costa et al., 2006), we found that *Streptomyces* were commonly resistant to many clinical antibiotics. Selection for antibiotic resistance in natural communities is thought to be imposed by interactions with antibiotic-producing competitors (Weiner et al., 1998; D'Costa et al., 2007b; Kinkel et al., accepted). Thus, variation in resistance to antibiotics among *Streptomyces* from different locations may reflect a history of production of specific antibiotics within communities. Alternatively, antibiotic resistance may also be due to 'intrinsic' resistance mutations or the activity of broad-spectrum efflux pumps (D'Costa et al., 2006; Martinez et al., 2009). The lack of significant correlation between antibiotic resistance capacity and niche overlap among locations suggests that the dynamics of antibiotic resistance among *Streptomyces* populations are distinct from those of resource competition. The significant phylogenetic signal in resistance phenotypes suggests that phylogenetic constraints may limit the acquisition of resistance or that, once acquired, resistance is retained in lineages despite the absence strong selection. Thus, immigration/colonization of resistant OTUs may play an important role in structuring antibiotic resistance phenotypes within *Streptomyces*

communities in soil. Despite the phylogenetic signal of antibiotic resistance, greater similarity in resistance phenotypes among *Streptomyces* from the same versus different locations suggests that there may be local spread of antibiotic resistance genes within *Streptomyces* communities, likely facilitated by horizontal gene transfer (Wiener et al., 1998). Indeed, *Streptomyces* belonging to OTU1 originating from MN1 were more resistant to rifampicin and streptomycin than those from other locations (Ant and PanFS), suggesting distinct selective pressures for resistance among these locations. Adaptation of taxa from the same phylogenetic groups in response to distinct selection pressures across the landscape (adaptive radiation) may help generate substantial diversity in antibiotic resistance phenotypes.

Soilborne *Streptomyces* are saprotrophs that acquire carbon by degrading soil organic matter (Schrempf et al., 2011). As a result of their saprotrophic lifestyle, variation in resource use among *Streptomyces* populations from different geographic locations is expected to reflect adaptation to local organic carbon pools (Antony-Babu et al., 2008; Schlatter et al., 2009; Schlatter et al., 2013). Indeed, *Streptomyces* from the same locations had more similar resources and greater niche overlap than those from different locations, consistent with local selection for resource use phenotypes. Considering aggregate resource use phenotypes, rather than specific patterns, locations that supported *Streptomyces* with larger niche widths (resource generalists), such as MN1 and MN3, may have more diverse pools of available carbon compounds than locations that support *Streptomyces* with narrower niche widths (resource specialists), such as Ant and Cev. Although conditions that support *Streptomyces* with faster or more efficient

growth are less clear, they are likely to depend on resource availability (Schlatter et al., 2013). The availability and diversity of soil resources is thought to be strongly linked to plant community composition and diversity as well as soil type, age, and history (Batjes, 1996; Tilman et al., 1997; Conant et al., 2001; Tilman et al., 2001). Thus, the nested factors of geological history, management history, and plant cover are likely to be broad drivers of *Streptomyces* resource use phenotypes in soil.

Variation in total growth and growth efficiency among *Streptomyces* OTUs suggests that some OTUs tend to be r- or K- selected and may have physiologies that are 'hard-wired' to grow optimally in high or low resource environments (Fierer et al., 2007). Due to these physiological constraints, total growth and growth efficiency among *Streptomyces* may be less responsive to selection. In contrast, the large variation in niche width within and among OTUs suggest that niche widths may be more likely to adapt to local resource pools than the overall growth strategy of a streptomycete. Consistent with this idea, *Streptomyces* belonging to the same OTU but originating from different locations did not differ in total growth or growth efficiency while niche widths for individuals within the same OTU often varied widely among locations, suggesting that niche widths are more adaptable than growth strategy among *Streptomyces*.

This work explores the biogeography of *Streptomyces* species interaction traits. Variation in antibiotic inhibition, resistance, and resource use traits among locations demonstrates significant geographic differences in phenotypes that are critical to *Streptomyces* species interactions and fitness. Further, results suggest that local adaptation of *Streptomyces* traits is crucial to geographic differences in *Streptomyces*

antibiotic inhibition, resistance, and resources use. Moreover, local adaptation of species interaction phenotypes to unique selection pressures within distinct microbial communities and abiotic environments across the landscape is likely to play an important role in generating trait diversity within *Streptomyces* and other microbial groups. Further study of the geographic structure of microbial species interactions will be essential to understand the ecological and evolutionary forces that structure and generate microbial functional diversity.

Chapter 5: Bacterial communities respond to plant species, plant community richness, soil characteristics, and microbial interactions in soil.

Background

Soil bacterial communities are major contributors to soil health and to critical ecosystem functions including decomposition, nutrient cycling, and plant disease suppression (Garbeva et al., 2004; Singh et al., 2004; Berg and Smalla, 2009). Variation in bacterial community composition, diversity, and function is associated with a wide variety of abiotic and biotic variables including pH, soil type, soil resource availability, plant-microbe, and microbe-microbe interactions (Garbeva et al., 2004; Marschner et al., 2004; Lauber et al., 2009; Berg and Smalla, 2009; Campbell et al., 2010; Szekely et al., 2013). However, these variables are often structured on vastly different spatial scales relative to how bacterial communities are organized in soil. For example, microbe-microbe interactions may occur across micrometers, whereas plant communities may impact soil communities across hundreds or thousands of meters. A more complete understanding of the scales at which distinct drivers influence soil communities will shed light on the significance of these drivers in structuring microbial communities across the landscape. However, developing a unified understanding of drivers of soil communities at disparate scales remains a significant challenge for microbial ecologists. Here, we explore microbial interactions, plant species, plant community richness, and landscape variation as correlates of bacterial community composition, structure, and diversity in a single field experiment.

Microbes in soil exist in complex communities in which species interactions, including competition, antagonism, and signaling are hypothesized to be critical to fitness (Ryan and Dow, 2008; Hibbing et al., 2010). As a result, interactions among soil bacteria are likely to play a significant role in the assembly and dynamics of soil communities. However, interactions among bacteria vary across small spatial scales (Davelos et al., 2004a; Vetsigian et al, 2011) and are likely to be embedded within larger-scale drivers of microbial communities. In particular, since species often interact during resource competition, plant-produced compounds, which fuel saprophytic soil foodwebs, are likely to mediate competitive species interactions within soil communities (Schlatter et al., 2009; Kinkel et al., 2011).

Most studies on the specific effects of plant species on soil communities have focused on interactions between a single plant and its microbial consortia in the rhizosphere. However, in nature plants most often exist in complex, multi-species communities. While plant community diversity has been suggested to influence soil microbial communities (Kowalchuck et al., 2002; Zak et al., 2003; Chung et al., 2007; Lamb et al., 2011; Latz et al., 2012), studies typically do not distinguish additive effects of different plant species from interactive effects of plant community diversity (Bakker et al., 2013a). Additive effects of plant diversity may occur when each plant species supports a characteristic assemblage, such that the microbial community at a given site consists of a simple aggregation of various plant species-specific assemblages. In contrast, interactive effects are possible where growth inmulti-species communities alters critical plant phenotypes, such as nutrient acquisition, allocation and deposition, root

architecture and exudation, or secondary metabolite production (Gersani et al., 2001; Murphy and Dudley, 2009; Broz et al., 2010). Variation in these phenotypes may induce changes in soil microbial community structure and function that are distinct from additive effects of increasing plant diversity.

Although plants are viewed to be critical drivers of soil communities in the rhizosphere, plant community diversity and composition may also influence bacterial communities at broader spatial scales (Yannarell et al., 2011). Plant communities vary in diversity, productivity, and their effects on local microclimate and soil resource pools (Spehn et al., 2000; Tilman et al, 2001; Zak et al., 2003). Local environments associated with plant communities provide an environmental context within which plant-microbe and microbe-microbe interactions take place. Moreover, historical factors (eg. immigration, colonization, or disturbance events) or gradients in soil moisture or nutrient content also impact soil communities across broad spatial scales. Thus, patchiness in plant community composition and diversity and landscape-scale variation in the soil environment are likely to be important contributors to patterns in microbial community structure, diversity, and function.

In this work we explore soil bacterial community composition, structure, diversity, and antagonistic activity in soils associated with different prairie plant species growing in communities varying in plant species richness. Specifically, we examine relationships between microbial antagonistic interactions, plant species identity, plant community diversity, and landscape variation in soil edaphic characteristics with bacterial community composition, structure, and diversity. We hypothesize that bacterial

communities are influenced by all of these factors, and that interactions among these factors are critical for soil community composition and structure.

Materials and Methods

Experimental setup and soil sampling

Samples were collected at the University of Minnesota Cedar Creek Ecosystem Science Reserve (CCESR) in July 2009 from the long-term biodiversity experiment E120 (www.cedarcreek.umn.edu) as described in (Bakker et al., 2013a). Briefly, in 1994 plots were established with 1, 4, 8, and 16 plant species, each drawn from a pool of 16 native prairie plant species (Tilman et al., 2001). We collected soil cores (5cm x 30cm) from the base of 4 target plant species, two C4 grasses, *Andropogon gerardii* (Ag) and *Schizachyrium scoparium* (Ss), and two legumes, *Lespedeza capitata* (Lc) and *Lupinus perennis* (Lp). Within individual plots, four cores from different individuals of target plant species were collected. Soil cores from each plot were bulked for each plant species-plant richness combination, which was replicated across 3 plots for a total of 48 soil samples (4 species X 4 plant richness treatments X 3 plot-level replicates). Soil samples were stored at 4 C until processing.

Soil DNA extraction, PCR, and 454 pyrosequencing

DNA was extracted from soil samples using the PowerSoil DNA Kit (MO BIO; Carlsbad, CA USA) as described in Bakker et al. (2013). Barcoded 454 primers with universal bacterial template-specific sequences (Primer B-27F; (27f: 5'-AGAGTTTGATCCTGGCTCAG-3') and Primer A-MID-338R; (338R: 5'-

TGCTGCCTCCCGTAGGAGT-3')) were used to amplify bacterial 16S rRNA gene fragments from soil DNA. PCRs consisted of 45ul Accuprime Pfx Supermix (Invitrogen), 1ul (10pM) of each primer, 2ul (20ng) DNA, and 1ul H_2O. PCR conditions followed the protocol of Fierer et al. (2008) with an initial denaturation step of 94 C for 3 minutes followed by 35 cycles of 94 C for 45s, 50 C for 30s, and 72 C for 90s, and a final extension of 72 C for 10 min. PCR products were checked on a 2% agarose gel and purified using the Qiaquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Purified amplicons were quantified using fluorometry (Qubit dsDNA HS assay, Invitrogen) and 20ng of each sample was combined into a single pool. Pooled DNA was run on a 1% agarose gel, re-purified from the band of expected product size with the Qiaquick kit, and quantified as described above. The purified pool was submitted to the University of Minnesota Biomedical Genomics Center for sequencing using the 454 GS FLX+ pyrosequencing platform.

454 data processing

Raw flowgrams were denoised using the AmpliconNoise V1.24 algorithm (Quince et al., 2011). Next, barcodes were trimmed, reads were truncated to 315 bp, and the Seqnoise and Perseus algorithms were used to correct PCR errors and remove expected chimeras in the AmpliconNoise pipeline. The resulting FASTA file was further processed in mothur v1.22.2 (Schloss et al., 2009) using the Schloss SOPs as a guideline (Schloss et al., 2011). The reverse complements of unique sequences were aligned to the SILVA database using the Needleman-Wunsch algorithm with a kmer size of 8. To retain only the highest quality sequences in the alignment, the alignment was optimized to keep

the longest 85% of overlapping sequences from the start of the sequencing read (338R primer). The alignment was filtered to remove gaps common to all sequences and sequences were classified using the Ribosomal Database Project naïve Bayesian classifier (Wang et al., 2007). After sequences classified as chloroplasts were removed, sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using the average neighbor method. The consensus taxonomy was determined for each OTU. In order to equalize the depth of sampling for community structure and diversity analyses, 6837 sequences were randomly sub-sampled without replacement from each biological replicate. The sub-sampled OTU table was used to determine the observed and estimated (Chao1) OTU richness and the inverse Simpson's (1/D) and Shannon indices of diversity for each sample. Finally, the Yue & Clayton index of similarity (ThetaYC) was used to explore community structure among samples. Sequencing data are available in the NCBI Sequence Read Archive under accession SRR786944.

Soil characteristics and culturable bacterial densities

Soil pH, carbon (C), nitrogen (NO_3), phosphorus (P), potassium (K), and organic matter (OM) for each sample was determined at the University of Minnesota soil Research Analytical Lab (http://ral.cfans.umn.edu/) as reported elsewhere (Bakker et al., 2013a). Above-ground biomass and plant cover assessments for each plot were acquired from the CCESR database (http://www.cedarcreek.umn.edu/research/data/). Each soil sample was evaluated for culturable bacterial densities, *Streptomyces* densities, and antagonist densities as described in Bakker et al. (2013). *Streptomyces* were targeted in evaluating antagonists because they are ubiquitous in soil, produce a great diversity of

antagonistic compounds, and are associated with plant disease suppression (Davelos et al., 2004a; Kinkel et al., 2012). Briefly, for each sample soil suspensions were spread on 15ml of 1% water agar, dried, then overlaid with 5 ml of cooled starch-casein agar (Wiggins and Kinkel, 2005) and incubated at 28C. After 3 days bacterial and *Streptomyces* densities were counted and each plate was covered with a second layer (15ml) of starch-casein agar. Plates were then overlayed with each of three indicator *Streptomyces* strains having different antibiotic resistance profiles (Davelos et al., 2004b). After an additional 3 days of incubation at 28C, zones of inhibition around colonies, indicating the ability of colonies to produce antagonistic compounds, were measured for each plate. Antagonist densities and proportions were averaged across indicator strains for each soil sample. Bacterial and *Streptomyces* densities were log-transformed prior to statistical analysis.

Statistical analyses

All statistical analyses were conducted in R (R Core Development Team, 2011) unless noted otherwise. Abundances of the most common phyla and OTUs, bacterial richness, and bacterial diversity were compared among plant species and richness treatments using ANOVA with Tukey's post-hoc test. The impacts of plant species and plant richness on bacterial community structure were assessed with AMOVA and unweighted unifrac tests in mothur. Moreover, similarity in bacterial community structure was compared among community pairs from the same and different plant hosts or richness treatments using Student's t-tests. Similarities in plant community composition by percent cover, soil community antagonistic capacities (against 3 *Streptomyces*

indicator strains), and soil edaphic characteristics were calculated as the Euclidean distances among all possible plot pairs. Spatial distances among plots were estimated as the Euclidean distances among all plot pairs using the CCESR plot map (http://www.cedarcreek.umn.edu/research/data/) as a grid. Samples from the same plot were considered to have a distance of 0. Mantel tests were used to test for relationships among similarity matrices using 10,000 permutations to determine significance. Indicator species analyses, used to identify bacterial OTUs preferentially associated with each plant species, were performed using the labdsv package of R (Roberts, 2012) using 10,000 iterations to determine significance. Non-metric multidimensional scaling plots were constructed in mothur and used to visualize bacterial community similarity. Biplots with soil edaphic characteristics, above-ground plant biomass, and culturable bacterial characteristics were generated in the vegan package of R using 1000 permutations to determine significant relationships with NMDS axes (Oksanan et al., 2012). Finally, co-occurrence networks were constructed for taxa present in at least half (n=24) of all samples using custom R scripts (S. Bates, personal communication). Positively or negatively co-occurring taxa were defined as OTU pairs whose abundances were strongly correlated (Pearson, R>0.6 or R<-0.6, respectively, p<0.05 after correction for false discovery). The software Gephi (Gephi.org; Bastian et al., 2009) was used to visualize networks of co-occurring taxa, and to decompose the networks into modules, with randomization.

Results

454 pyrosequencing

We obtained 476,573 high quality sequences with an average length of 291bp after processing. Seventy-five percent of sequences were classified to 16 known phyla (supplemental table 1). Actinobacteria was the most abundant phylum represented in our data (32% of sequences), followed by unclassified sequences (25%) and Proteobacteria (25%). Sequences that could not be confidently classified may represent unexplored bacterial lineages, or simply limitations of classifying relatively short sequence reads. When sequences were binned at 97% similarity they formed 26,153 distinct OTUs. Thus, prairie soils at Cedar Creek Ecosystem Science Reserve harbored considerable bacterial diversity. Bacterial community composition and diversity were significantly associated with plant species identity, plant community richness, soil edaphic characteristics, and antagonistic microbial interactions.

Plant species impacts on soil bacterial communities

The relative abundances of bacterial phyla were generally consistent across plant species, though Lp supported significantly more Bacteroidetes than Ss (2% vs 1.5%; ANOVA, F=2.92; TukeyHSD, p=0.028). The abundances of phyla did not vary significantly among plant hosts from the same plant richness treatment (data not shown). The most abundant OTUs overall were found in association with every plant species and represent predominantly taxa from Actinobacteria, Proteobacteria, and unclassified phyla (supplemental table 2). Among the largest OTUs, only the second largest taxon

(unclassified phylum) varied in abundance among plant species, being significantly less abundant among samples from Lp than among other plant species (ANOVA, F=5.54, p=0.003).

Only 12.9% of OTUs (n=2394) were found associated with all plant species, but these ubiquitous OTUs represented 79.3% of all sequences, suggesting that a small subset of OTUs form an abundant, core community of plant-associated soil bacteria. In contrast to the most abundant OTUs, the majority of OTUs (60.6%) were found in association with only a single plant species, but these were rare (85% of these OTUs were singletons or doubletons) and represented only 5.8% of all sequences. When singletons were excluded, 38.1% of remaining OTUs (3.8% of sequences) were associated with a single plant species. Thus, plant-specific OTUs were relatively rare. However, indicator species analyses identified 280 OTUs that were significantly associated with a particular plant species (supplemental table 3). A larger number of significant indicators were found for Ag and Lp (71 and 123, respectively) than for Lc or Ss (43 and 43, respectively). Consistent with previous analyses, indicator OTUs were present in relatively low abundance. The 10 best indicators for each plant species accounted for only 0.4% (Lc) to 1.8% (Lp) of bacterial sequences from that species. Together, these data suggest specific effects of plant hosts on the presence or absence of soil bacterial taxa are limited to a small number of relatively infrequent OTUs, and that populations of most soil bacteria are not structured by tightly linked plant-microbe interactions.

Plant host species had small effects on bacterial community structure. On average, bacterial communities from the same plant species were not more similar (smaller

ThetaYC) to each other than those from the different plants (t-test; $t=1.347$, $p=0.19$). Similarly, bacterial communities did not cluster significantly by plant host species (Figure 1; unweighted unifrac $p>0.07$ for all pairwise comparisons). However, AMOVA revealed a significant influence of plant host on microbial community similarity (AMOVA, $Fs=1.55$, $p=0.021$), though when compared individually only bacterial communities associated with Lp differed from those associated with Ag and Ss (AMOVA, $Fs=2.80$, $p=0.003$ and $Fs=1.95$, $p=0.032$, respectively). When considering only samples from monoculture plots, where we might expect to see the strongest signatures of plant species, there was no significant difference in community similarity among communities from the same vs different plant species (t-test; $t=-1.299$, $p=0.21$) and no effect of plant species on bacterial community structure (AMOVA, $Fs=1.48$, $p=0.07$).

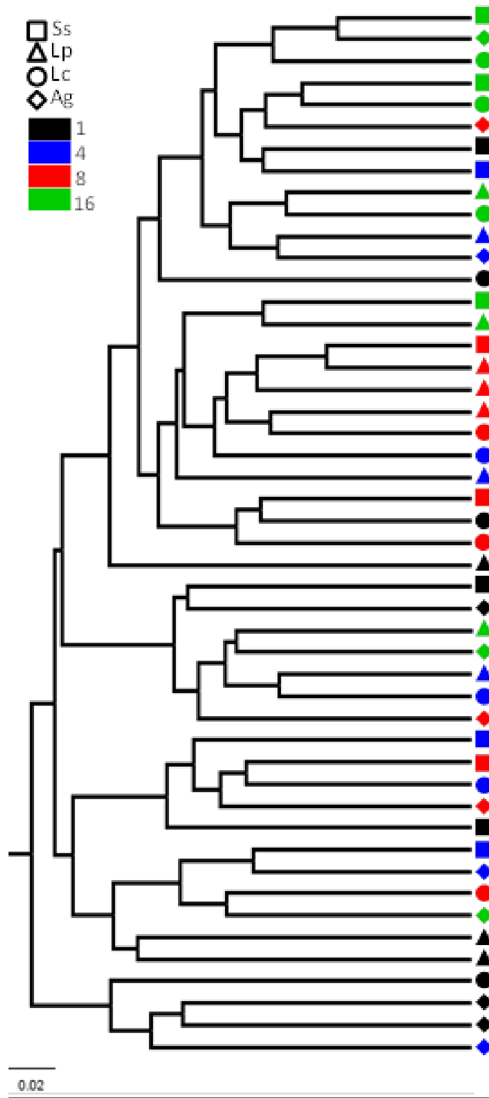

Figure 1: Dendrogram of soil bacterial communities using ThetaYC dissimilarity among samples. Communities are colored by plant richness treatment (Black=1spp, Blue=4spp, Red=8spp, and Green=16spp) and symbols represent different plant hosts (square= Ss, triangle=Lp, circle=Lc, diamond=Ag).

Among all samples, bacterial community richness and diversity were not strongly influenced by host plant species. Bacterial community richness (observed richness and Chao estimate; ANOVA: F= 0.422, p=0.738 and F=0.669, p=0.576, respectively) and

diversity (Shannon and 1/D; ANOVA: $F= 0.158$, $p=0.924$ and $F=1.127$, $p= 0.348$, respectively) did not vary among plant species. However, when only samples from monocultures were considered, plant species varied significantly in observed bacterial richness (ANOVA: $F=4.47$, $p=0.04$) but not estimated richness or diversity (data not shown). Specifically, bacterial communities associated with Lc supported soil communities with a significantly greater number of OTUs than Ag (average of 1547 vs. 1408 OTUs, respectively; Tukey's HSD $p=0.03$). Bacterial richness and diversity did not vary significantly among plant host species considering only 4-, 8-, or 16-species richness treatments (data not shown).

The diversity of OTUs classified to individual bacterial phyla sometimes varied among plant hosts. Ag harbored less diverse Actinobacterial communities than Lc (Shannon index; TukeyHSD, $p=0.03$), but more diverse Firmicutes communities than Lp (Shannon and 1/D; TukeyHSD, $p=0.005$). Ag soil communities also had more diverse populations of Proteobacteria than Ss (1/D; TukeyHSD, $p=0.02$). Moreover, when only monocultures were considered, Ss harbored less diverse Proteobacteria than Lc and Lp (Shannon; TukeyHSD, $p<0.05$). Thus, though plant host species have few significant impacts on bacterial diversity overall, they sometimes have targeted effects on the diversity OTUs belonging to individual phyla.

Plant richness impacts on soil bacterial communities

The abundances of some bacterial phyla and taxa varied among plant richness treatments. Communities with 16 plant species supported higher frequencies of

Proteobacteria than those with 1, 4, or 8 plant species (16 spp, 26.4%; 1 sp., 22.9%; 4 spp., 23.6%; 8 spp., 23.6%; ANOVA, $p<0.05$) and had significantly fewer unclassified bacteria than monocultures (16 spp., 22.8%; 1 sp., 26.0%; 4 spp., 24.6%; 8 spp., 24.1%; ANOVA, $p<0.05$). There were occasionally significant differences in abundances of phyla among plant richness treatments from soil communities associated with the same plant host, though patterns were inconsistent. The largest taxon (OTU18611; Phylum Proteobacteria, Family Bradyrhizobiacaea) was significantly more abundant among 8- and 16- species communities than 1- or 4- species communities (ANOVA, $F=6.78$, $p=0.0007$), but the 8th largest taxon (OTU14036; unclassified phylum) was more abundant in monocultures, regardless of the plant host species, than in 8- or 16- species plots (ANOVA, $F=8.06$, $p=0.0002$). These data illustrate that populations of some taxa are sensitive to plant community richness.

Across all plant species, microbial community structure was significantly influenced by plant community richness (AMOVA; $Fs=1.85$, $p=0.003$). Comparing individual richness treatments, differences were significant only between monoculture and 8 and 16 species communities (AMOVA, $Fs=1.77$, $p=0.037$ and $Fs=3.98$, $p<0.001$, respectively). Among all treatments, bacterial communities associated with 16 species communities clustered separately from other communities (Figure 1; unweighted unifrac $p<0.038$). However, bacterial community structure typically did not vary significantly among plant richness treatments for individual plant species (unweighted unifrac $p>0.05$, for Ag, Lp, Lc, and Ss; AMOVA, $p>0.05$ for Ag, Lp, and Ss). Lc-associated communities were an exception and the structure of these communities varied among diversity

treatments (AMOVA; Fs= 1.67, p=0.016). In total, bacterial communities from more species-rich plant communities were distinct from those from lower richness communities, but the impacts of plant community richness on soil bacterial community structure also depend on plant species, suggesting that the effects of plant hosts on soil communities are nested within the plant community context.

Plant richness treatments did not vary in observed or estimated bacterial OTU richness (ANOVA, F=0.274, p=0.844 and F=0.231, p=0.874, respectively). However, there were significant differences in bacterial diversity (1/D) among communities from different plant richness treatments (Figure 2; ANOVA, F= 7.235, p=0.0005). Specifically, 16 species plant communities harbored less diverse bacterial communities than 1, 4, or 8 species plant communities (Tukey's HSD, p≤0.02 for each comparison). The negative relationship between plant community richness and bacterial diversity was consistent among plant species (data not shown). However, there was no significant difference in bacterial diversity among plant richness treatments when using the Shannon index of diversity (ANOVA, F=1.618, p=0.20), suggesting that communities from 16-species plant communities are more uneven than 1-, 4-, or 8- species communities. Indeed, differences in bacterial diversity (1/D) among plant communities of varying richness could be largely explained by the abundance of the largest OTU (OTU18611, supplemental table 2), which was strongly negatively correlated with bacterial diversity (1/D; R=-0.895, p<0.0001). When this OTU was excluded from the analysis, there were no significant differences in bacterial diversity among plant species or plant diversity (ANOVA; F=1.627, p=0.265 and F=0.501, p=0.683). Thus, a single OTU was more

dominant in 16 species plant communities, resulting in substantially lower bacterial community evenness and diversity in soil.

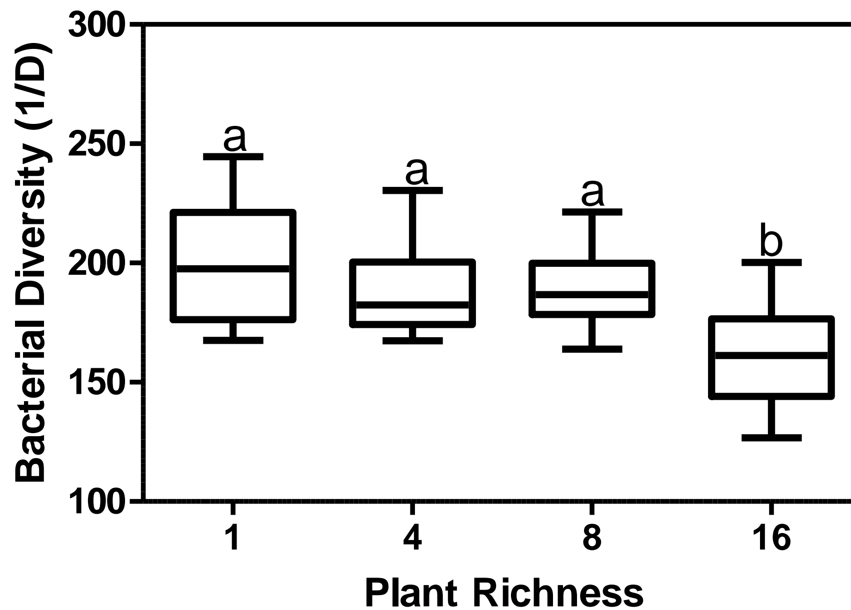

Figure 2. Bacterial diversity (1/D± standard error) among plant richness treatments. Different letters above bars represent significant differences among treatments.

Among OTUs classified to individual phyla, Bacteroidetes OTUs were more diverse among 16 species communities than among monocultures (1/D; TukeyHSD, p=0.01). In contrast, Gemmatimonadetes OTUs were more diverse in monocultures than in 16 species communities (Shannon and 1/D; TukeyHSD, p=0.04). Proteobacteria OTUs were less diverse among 4 (Shannon) and 16 species (1/D) communities than among monocultures (TukeyHSD, p=0.02 and 0.01, respectively).

Plant species x plant diversity interactions influence soil bacterial communities

The effects of plant host species on bacterial community structure varied among plant diversity treatments, though this variation was nuanced. Similarity among bacterial communities from different plant hosts varied significantly when communities within plant richness treatments were compared (ANOVA $p<0.0001$, $F=21.2$; Figure 3A). Specifically, bacterial communities from monocultures of different plant hosts were significantly more dissimilar than those from polycultures and community dissimilarity was lowest (most similar) among the 8- and 16-plant species treatments (Figure 3A). This suggests that plant host-specific impacts on bacterial community structure may be less significant in diverse plant communities or that plant species richness has a homogenizing effect on bacterial community structure. However, when communities from the same plant species and diversity treatment were considered together there were no significant differences in bacterial community similarity among plant richness treatments (ANOVA; $F=2.62$, $p=0.063$; Figure 3B), suggesting that impacts of individual plant hosts on bacterial community structure are consistent across plant richness treatments.

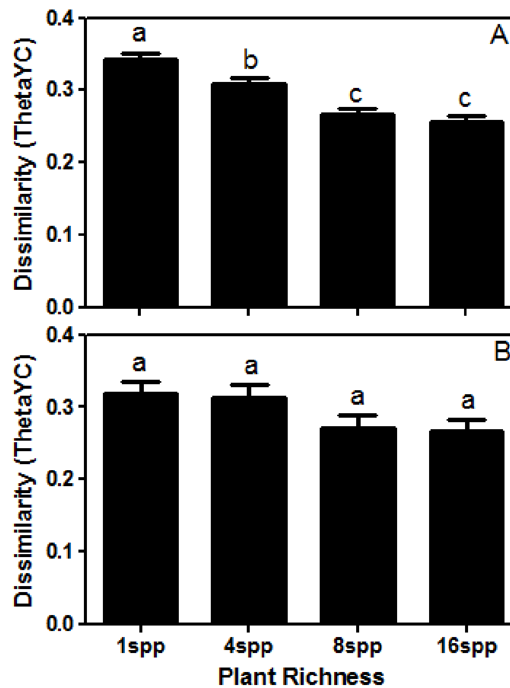

Figure 3: Mean dissimilarity (ThetaYC ± standard error) among bacterial communities from different (A) and the same (B) plant host species among plant richness treatments. Different letters above bars represent significant differences among samples.

Landscape variation and bacterial communities

Considering the composition of the plant community as a whole, there was a significant relationship between the similarity of plant community composition and bacterial community similarity (Mantel test, R=0.38, p<0.0001). Thus, soil bacterial communities were more similar among plots with more similar plant communities. In addition, similarity among bacterial communities was significantly correlated with the spatial distance between sampling plots (Mantel test, R=0.33, p<0.0001). Soils that were physically closer to each other tended to have more similar bacterial communities than

soils farther apart. Thus, despite the small spatial scale at which bacterial communities are organized, variation in plant community composition at the landscape-scale and differences in soil characteristics may contribute to variation in soil bacterial communities.

Plant productivity and soil edaphic characteristics impact soil bacterial communities

In order to shed further light on potential drivers of bacterial community composition, we used NMDS to evaluate patterns of similarity among bacterial communities, and correlated plant and bacterial community properties and soil edaphic characteristics (Figure 4). Plant richness, plant cover, soil pH, K, P, and bacterial diversity, densities, and antagonist densities were all significantly correlated ($p<0.05$) along NMDS axes (Table 1). Moreover, bacterial communities were more similar among soils with more similar edaphic characteristics (pH, N, P, K, C, and OM; Mantel, $R=0.22$, $p=0.007$). The trend between similarity in soil edaphic characteristics and bacterial community similarity was consistent among Ag- ($R=0.40$, $p=0.01$), Lp- ($R=0.34$, $p=0.03$), and Ss-associated ($R=0.37$, $p=0.02$) bacterial communities, but not Lc-associated communities ($R=0.05$, $p=0.30$). Thus, though soil characteristics were significantly related to bacterial community composition among most plant species, they were not a significant predictor of community composition among soil communities associated with Lc. Further, among individual plant richness treatments, there were significant correlations between similarity in soil edaphic characteristics and bacterial community similarity for communities from monocultures and 4 species treatments

(Mantel R=0.31, p=0.03; r=0.6, p=0.002, respectively) but not 8 or 16 species treatments (Mantel R=0.09, p=0.35, r=0.07, p=0.34, respectively). Overall, these data suggest that soil edaphic characteristics are frequently, but not always, important determinants of bacterial community structure across the landscape and that plant community diversity and soil edaphic factors interact in structuring soil bacterial community composition.

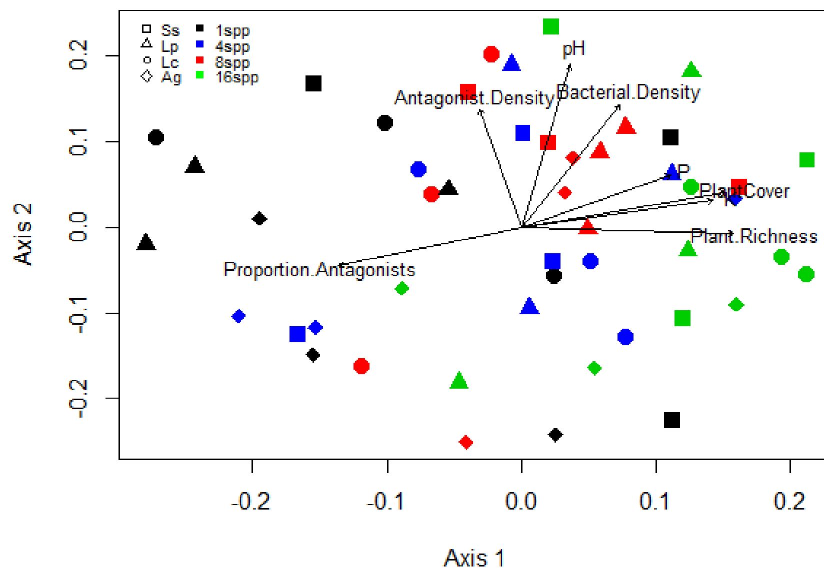

Figure 4: NMDS of bacterial community similarity (ThetaYC; R=0.88, stress=0.19). Different symbols represent different plant host species (square=Ss, triangle=Lp, circle=Lc, diamond=Ag) and different colors reflect plant richness treatments (Black=1 spp, Blue= 4 spp, Red= 8 spp, Green=16 spp). Environmental factors and community characteristics that were significantly correlated with NMDS axes were fitted to vectors along the direction of correlation. The length of the line representing each vector reflects the strength of the correlation (Table 1).

Table 1. Significant correlations of factors with NMDS axes

Variable	r^2	p-val
pH	0.44	0.001
Bacterial density (LogCFU/g)	0.31	0.002
Plant Richness	0.3	0.001
Plant Cover (%)	0.29	0.001
K (ppm)	0.25	0.002
Proportion Antagonists	0.24	0.005
Antagonist density (LogCFU/g)	0.24	0.004
P (ppm)	0.19	0.01

Soil bacterial communities with relatively high resource availability tended to have fewer OTUs with more uneven distributions. In general, soil resources (C, K, OM, N, P) and plant biomass were negatively correlated with bacterial richness and diversity measurements (Table 2). However, concentrations of soil resources were often positively correlated with one another and with plant richness (supplemental table 4), limiting our ability to discriminate the distinct roles of soil resources and plant diversity as drivers of bacterial diversity. Interestingly, relationships between soil resources and bacterial diversity differed among plant community diversity treatments. Among monocultures, soil K and aboveground plant biomass were positively correlated with bacterial diversity (1/D) (R=0.70, p=0.01 and R=0.68, p=0.015, respectively). In contrast, among more diverse plant communities (4-, 8-, and 16- species treatments) there were negative relationships between bacterial diversity (1/D) and both soil resources and plant biomass,

though these were only significant for K among 4- species plant communities (R=-0.61, p=0.035). Soil resource concentrations (C, N, P, K, and OM) and above-ground biomass were not significantly different among 1-, 4-, and 8-species plots, but 16-species plots had significantly greater concentrations of soil resources (C, N, P, K, and OM and above-ground biomass) than lower-richness plots. Moreover, across all plant richness treatments, Shannon diversity was negatively correlated with soil C (R=-0.58, p=0<0.0001), N (R=-0.49, p=0.0004), and OM (R=-0.60, p<0.0001), suggesting that there is a general negative trend between soil resource availability and bacterial diversity.

Table 2. Pearson correlations between soil edaphic characteristics, plant biomass, soil antagonists and bacterial richness and diversity (*p<0.05, **p<0.01, ***p<0.001).

Soil Characteristic	**Observed Richness**	**Chao1**	**1/D**	**Shannon**
C (ppm)	-0.48***	-0.27	-0.27	-0.58***
OM (%)	-0.46**	-0.22	-0.30*	-0.60***
N (ppm)	-0.43**	-0.23	-0.20	-0.49***
P (ppm)	-0.05	-0.19	-0.22	-0.01
K (ppm)	0.02	0.04	-0.42**	-0.13
pH	0.08	-0.06	-0.01	0.21
Above-ground Biomass (g/m^2)	-0.29*	-0.14	-0.14	-0.35*
Antagonist density (LogCFU/g)	0.09	-0.06	0.22	0.28
Proportion Antagonists	0.3	0.01	0.37**	0.14
Bacterial densities (LogCFU/g)	0.05	0.03	-0.13	0.1

Microbial co-occurrence networks

Across all samples, correlation network analysis identified 148 strong positive correlations among 136 OTUs ($R > 0.6$, $p \leq 1.04 \times 10^{-11}$) and 10 strong negative correlations among 16 OTUs ($R < -0.6$, $p \leq 1.92 \times 10^{-15}$). The network of positive correlations was divided into 23 distinct modules of co-occurring taxa (Figure 5). Eight large modules with ≥7 OTUs represented 16.5% of sequences sampled. These modules varied in composition (Supplemental table 5); some modules were dominated by a single phylum (eg. module 2 was dominated by Actinobacteria) whereas others were composed of many different phyla (eg. module 20 was composed of five different phyla, each representing 14-32% of sequences in that module). The phylogenetic composition of modules was unrelated to their relationships with soil characteristics and community antagonism; modules with high proportions of a given phylum (eg. modules 2, 9, 15, and 17 are largely Actinobacteria) varied in their relationships with soil edaphic characteristics and antagonistic activity of bacterial communities. This suggests that taxa from the same phylum are likely to respond differently to soil resources and antagonistic interactions, reflecting an enormous functional diversity of taxa within individual phyla.

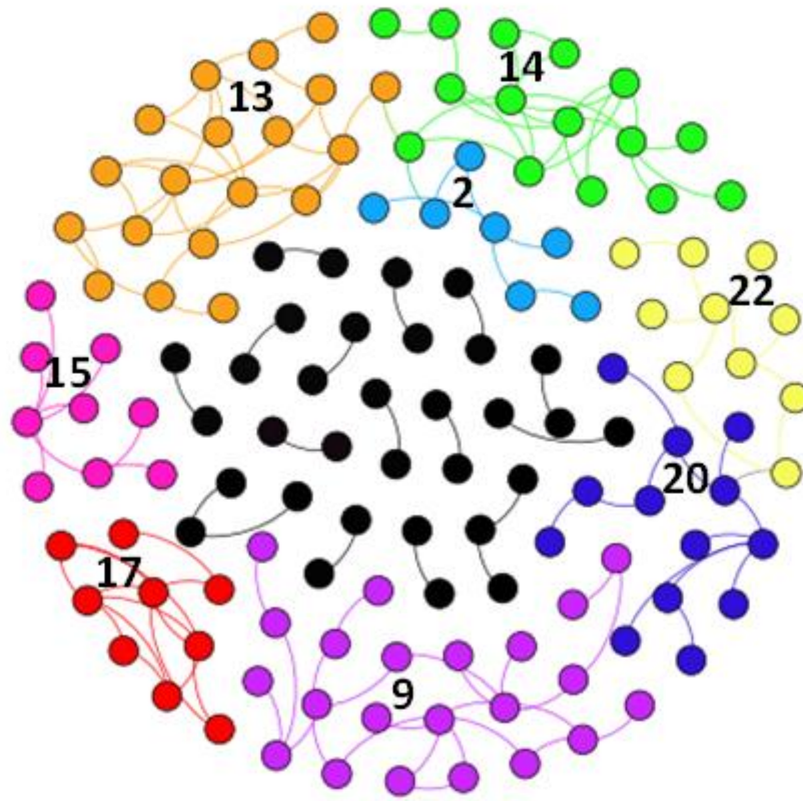

Figure 5. Correlation network (positive correlations only; n=148 edges) among bacterial OTUs across samples. Nodes (OTUs; n=136) belonging to large modules (n≥7 OTUs) are colored by module assignment. Large modules (n≥7 OTUs) are labeled.

There was not a single best predictor of the abundances of modules. Some modules of OTUs tended to be more common in soils that had higher quantities of resources (modules 9 and 17), where others tended to be more abundant in soils with fewer resources (modules 2 and 15) (Figure 6). Soil pH was a consistent significant predictor of the abundances of large modules, but was the best predictor among all soil characteristics only for module 13. Thus, variation in soil resources and differences in soil pH preferentially support some modules. In contrast, the abundances of some

modules were more strongly correlated (negatively and positively) with antagonist densities or proportions of antagonists (modules 20 and 22). This suggests that while some groups of co-occurring taxa respond to soil resource status, others may be highly sensitive to competitive species interactions in soil.

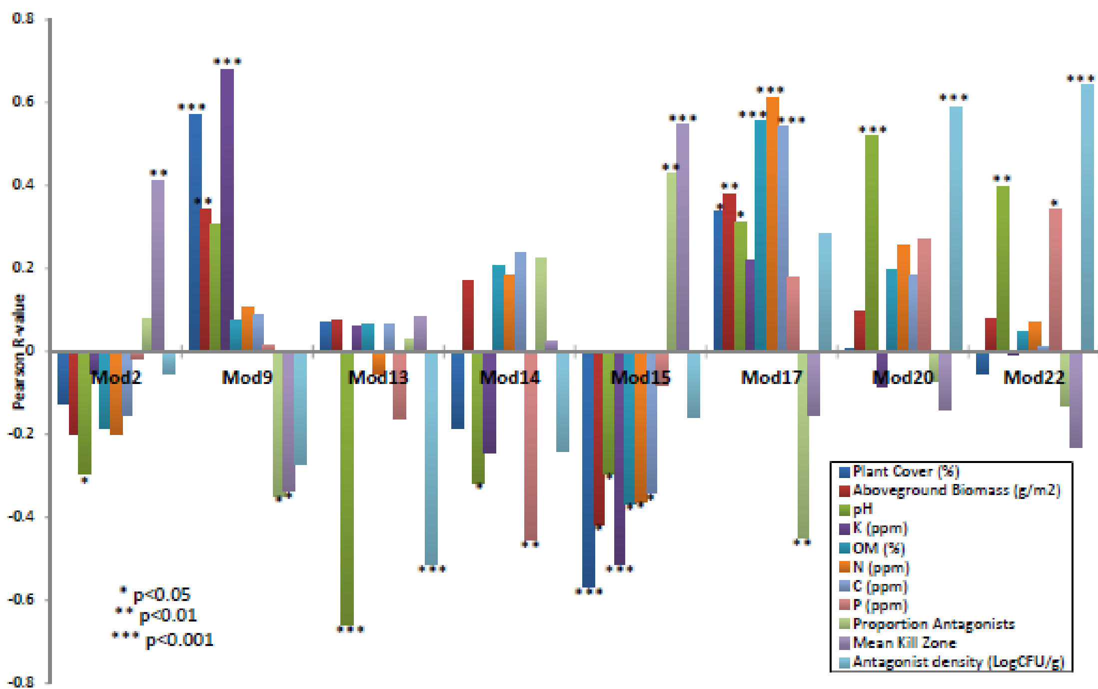

Figure 6. Correlations of the summed abundance of OTUs belonging to large modules (n≥7 OTUs) with soil edaphic characteristics, plant biomass, and antagonists.

Microbial interactions and bacterial community structure, composition, and diversity

Antagonistic activities of *Streptomyces* communities were significantly related to bacterial community structure, composition, and diversity. Among all pairwise community contrasts, there was a small but significant positive correlation between similarity in antagonistic activities and bacterial community similarity (Mantel test

R=0.17, p=0.036). This suggests that antagonistic communities have distinct compositions, perhaps reflecting the impacts of antagonistic populations on soil community structure. Indeed, frequencies of four of the ten most abundant OTUs and two of the five most abundant phyla were significantly correlated with culturable densities and proportions of antagonists (supplemental table 6a). Frequencies of Gemmatimonadetes were positively related to antagonist density and proportions of antagonists. In contrast, frequencies of Proteobacteria were negatively correlated with the densities and proportions of inhibitors. However, among individual taxa OTUs 18611 and 17875, both classified as Proteobacteria, had contrasting relationships with the proportion of inhibitors (supplemental table 6b; R=-0.45, p=0.001 and R=0.46, p=0.001, respectively). This suggests that taxa from other phyla may have different capacities to compete in antagonistic environments. Further, frequencies of Actinobacteria or large OTUs classified Actinobacteria were not significantly correlated with culturable densities or proportions of antagonists (supplemental table 6), suggesting that 16S gene sequencing does not provide direct insight into antagonistic population densities.

Bacterial communities with greater proportions of antagonists tended to be more diverse (1/D; R=0.37, p=0.009, Figure 7). However, this trend depended on plant diversity and was present only among 16 species plant communities (R=0.68, p=0.015). Relationships between antagonists and bacterial diversity based on the Shannon index were not significant (data not shown). These data suggest that antagonistic interactions play a significant role in generating or maintaining bacterial diversity within soil

communities, but that the significance of antagonistic interactions may vary depending on the plant community context and the diversity index used to evaluate communities.

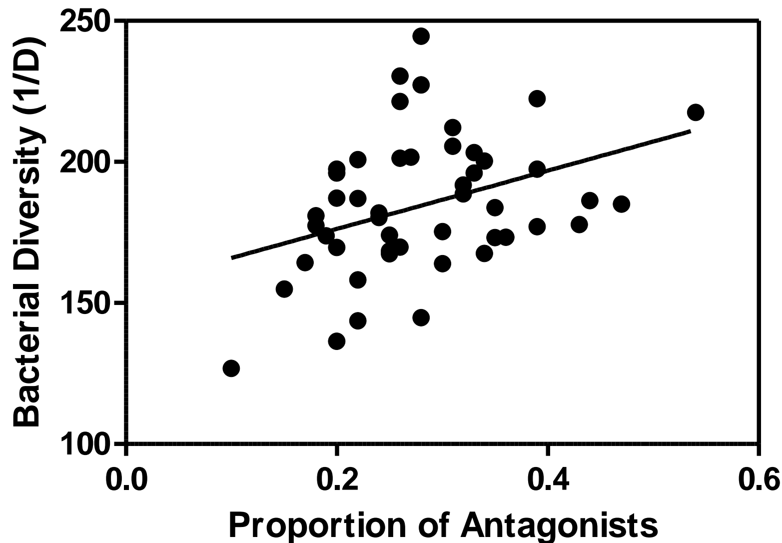

Figure 7: Pearson correlation (R=0.37, p=0.009) among the proportions of antagonists and bacterial diversity (1/D).

Discussion

Plant species, plant diversity, plant community composition, soil edaphic characteristics, and microbial species interactions can influence soil bacterial community structure, composition, and diversity (Garbeva et al., 2004; Marschner et al., 2004; Lauber et al., 2009; Berg and Smalla, 2009; Campbell et al., 2010; Szekely et al., 2013). However, these drivers affect communities at very different spatial scales and may interact in significant ways. Our data explore the impacts and interactions between diverse drivers of bacterial communities.

Though plants can harbor distinct rhizosphere bacterial communities (Kowalchuck et al., 2002; Marschner et al., 2004; Costa et al., 2005; Garbeva et al., 2008; Berg and Smalla, 2009), we found considerable variation in bacterial community structure among soils associated with the same plant hosts, even from monocultures maintained for 13 years. Though most bacterial OTUs were found in soils from a single plant species, most sequences belonged to OTUs that were ubiquitous among plant species. This suggests that there is an abundant, core soil bacterial microbiome found in association with prairie plants that is common among plant species. Members of this core microbiome may play an especially important role in plant-associated soil functions, such as decomposition, nutrient cycling, and plant disease suppression. Moreover, despite the huge diversity of soil bacterial communities that is often emphasized in studies using high-throughput sequencing efforts (Roesch et al., 2007), variation in the abundances of most community members, which were relatively rare, may represent 'noise' that has little relation to plant-associated community function as a whole. However, though plant-specific taxa were rare, the relative abundances of large OTUs sometimes varied among plant species. Thus, even though significant associations between plant species and soil bacteria are infrequent, plants may vary in the suitability of the habitat that they create in soil for abundant bacterial taxa.

Plant community richness may have distinct impacts on soil communities resulting from additive effects of individual plant species or from interactive effects due to interactions among plant species (Bakker et al., 2013a). In this work, plant community richness appeared to be more strongly associated with variation in soil microbial

community structure and diversity than plant species, and interactions among plant richness and plant species were nuanced. This suggests that plant richness effects on soil communities are largely due to additive rather than interactive effects. However, since sampling in this study did not specifically target root-adhered soil we may not have been able to detect fine-scale rhizosphere effects of plant species. Moreover, because species-rich plant communities were strongly associated with greater resource availability (Bakker et al., 2013a), we were unable to differentiate the effects of plant community richness from those of high resource availability.

Although additive effects of plant species as plant community richness increased appeared stronger than interactive effects, there were significant differences in bacterial community similarity when comparing communities associated with monocultures to those from polycultures, suggesting that plant-host specific impacts on soil bacterial communities vary depending on plant community richness. Specifically, plant species impacts on soil bacterial community similarity were less apparent among the high plant richness treatments. Thus, habitats associated with a particular plant species that support characteristic bacterial communities may be 'diluted' by effects of the plant community as plant diversity increases. Alternatively, plants in species-rich communities may allocate fewer resources below-ground to support soil communities, thereby weakening plant-specific signatures among soil bacterial communities. In either case, weaker plant-specific effects on soil communities among species-rich plant communities may reduce the accumulation of plant-specific beneficial or pathogenic soil organisms (van der Putten et al., 2013). As a result, strongly positive and negative plant-soil feedbacks may be less

significant for plant community composition among diverse plant communities. In particular, though plant-soil feedbacks are often important for plant community diversity, the sign and strength of feedbacks can change over time (Kardol et al., 2006; Hawkes, et al., 2012; van der Putten et al., 2013). Future studies linking detailed information on soil community composition with plant-soil feedbacks may offer insight into the specific mechanisms and organisms involved in positive and negative plant-microbe associations.

Consistent with previous work (Kowalchuck et al., 2002; Zak et al., 2003; Chung et al., 2007; Lamb et al. 2011), plant community richness significantly influenced soil bacterial community composition and structure. However, in contrast to other studies, we found that the least diverse bacterial communities were supported by plant communities with the highest species richness and that the most diverse bacterial communities were supported by monocultures. We hypothesize that this trend can be explained by an indirect effect of plant community richness on bacterial diversity resulting from bacterial competition for plant-derived resources in soil. Specifically, competitive interactions among soil bacteria are likely to be a function of the availability of plant-derived resources (Kinkel et al., 2011). In order to inhibit competitors, a wide variety of bacteria produce antagonistic compounds, such as antibiotics, degradative enzymes, and bacteriocins. Production of such compounds has been suggested to be critical for maintaining diversity in bacterial communities (Czárán et al., 2002). Since low-diversity plant communities are likely to provide more limited quantities and diversities of resources to soil than high-diversity plant communities, they may favor highly antagonistic bacteria that are better able to acquire resources in competitive environments

(ie. low resource concentrations). In contrast, soils associated with high-diversity plant communities are likely to have higher quantities and diversities of plant-derived resources and may generate a less competitive environment favoring non-antagonistic bacteria (Kinkel et al., 2011).

Previous work with the soils used here found that the proportions of antagonists were greatest among monoculture plant communities (Bakker et al., submitted), suggesting that antagonistic interactions contribute to the greater bacterial diversity observed among monocultures. Additionally, the positive correlation between proportions of antagonists and bacterial diversity indicates that higher relative frequencies of antibiotic producers in soil tend to support more diverse bacterial communities. However, relationships depended on the plant richness treatment, suggesting that plant communities may modulate the significance of antagonistic interactions to bacterial diversity in soil. In total, these data provide empirical support for predictions that antagonistic interactions among soil microbes can enhance the generation or maintenance of bacterial diversity (Czárán et al., 2002) and suggest that the plant community context is a crucial modulator of antagonistic interactions.

In addition to relationships between antagonists and bacterial diversity, we found that antagonistic phenotypes were related to the abundances of some bacterial phyla and bacterial community structure. This finding supports a role for antagonistic species interactions in structuring bacterial communities in soil and suggests that antibiotic-producing bacteria represent a key soil functional group that can influence community membership. Since production of antagonistic compounds and resistance to antagonism is

common among soil microbes (Davelos et al., 2005a; Hibbing et al., 2010; Vetsigian et al., 2011), it is likely that the complement of antagonistic bacteria and their interactions in soil are critical to community assembly. Indeed, some modules of co-occuring taxa were associated with more or less antagonistic communities, suggesting that these modules represent taxa that vary in their ability to compete in antagonistic environments.

Plant community composition has been hypothesized to explain variation among soil microbial communities (Wardle et al., 2004). Consistent with this idea, we found that plant community composition was significantly related to soil bacterial community structure across prairie plant communities. This provides further confirmation that plant community composition contributes to bacterial community structure across the landscape. Additionally, soil resources, which are a crucial trophic link between plant and soil ecosystems (Wardle et al., 2004), appeared to be critical for soil bacterial communities, as highlighted by significant relationships of resource concentrations with community similarity. In particular, the relative abundances of abundant phyla and the largest OTUs correlated significantly with soil resources. Moreover, modules of co-associated OTUs were consistently positively or negatively correlated with soil resources, suggesting that resource preferences of particular microbial community members play a significant role in determining soil community composition (Fierer et al., 2007).

These findings contribute to our understanding of interactions among the complex drivers of soil bacterial communities and especially the roles that plant species and plant community richness may play in moderating bacterial community composition, diversity, and species interactions in soil. In particular, our data suggest that plants, through

resource inputs to soil, set the context within which competitive interactions among soil microbes occur. Thus, considering both macro- and micro-scale drivers of microbial communities, including interactions among plant species and among soil microbial community members, will provide novel insight into soil ecosystems and enhance our knowledge of linkages between soil and above-ground communities.

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Appendix: Supplemental material.

Chapter 1 Supplemental Material.

Supplemental Table 1 Origin and Genbank accession numbers of isolates for which 16S rRNA gene sequences were obtained.

Isolate	Plot of Origin	N-treatment	GenBank accession no.
1111_1	1	non-amended	AY465184.1
1111_2	1	non-amended	AY465185.1
1111_3	1	non-amended	AY465186.1
1111_4	1	non-amended	AY465187.1
1111_5	1	non-amended	AY465188.1
1112_1	1	non-amended	AY465189.1
1112_2	1	non-amended	AY465190.1
1112_3	1	non-amended	AY465191.1
1112_4	1	non-amended	AY465192.1
1113_1	1	non-amended	AY465194.1
1113_3	1	non-amended	AY465195.1
1113_4	1	non-amended	AY465196.1
1113_5	1	non-amended	AY465197.1
1114_2	1	non-amended	AY465198.1
1114_3	1	non-amended	AY465199.1
1114_4	1	non-amended	AY465200.1
1114_5	1	non-amended	AY465201.1
1121_1	1	non-amended	AY465202.1
1121_2	1	non-amended	AY465203.1
1121_3	1	non-amended	AY465204.1
1121_4	1	non-amended	AY465205.1
1121_5	1	non-amended	AY465206.1
1122_4	1	non-amended	AY465209.1
1123_2	1	non-amended	AY465210.1
1123_4	1	non-amended	AY465211.1
1124_1	1	non-amended	AY465212.1
1124_2	1	non-amended	AY465213.1
1124_4	1	non-amended	AY465215.1

1131_1	1	non-amended	AY465216.1
1131_2	1	non-amended	AY465217.1
1131_3	1	non-amended	AY465218.1
1131_4	1	non-amended	AY465219.1
1131_5	1	non-amended	AY465220.1
1132_1	1	non-amended	AY465221.1
1132_2	1	non-amended	AY465222.1
1132_3	1	non-amended	AY465223.1
1132_4	1	non-amended	AY465224.1
1133_1	1	non-amended	AY465225.1
1133_2	1	non-amended	AY465226.1
1133_4	1	non-amended	AY465227.1
1133_5	1	non-amended	AY465228.1
1134_1	1	non-amended	AY465229.1
1134_4	1	non-amended	AY465231.1
1134_5	1	non-amended	AY465232.1
1211_1	1	non-amended	AY465233.1
1211_2	1	non-amended	AY465234.1
1211_3	1	non-amended	AY465235.1
1211_5	1	non-amended	AY465236.1
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1212_2	1	non-amended	AY465238.1
1212_3	1	non-amended	AY465239.1
1212_4	1	non-amended	AY465240.1
1212_5	1	non-amended	AY465241.1
1213_1	1	non-amended	AY465242.1
1213_2	1	non-amended	AY465243.1
1213_3	1	non-amended	AY465244.1
1213_4	1	non-amended	AY465245.1
1213_5	1	non-amended	AY465246.1
1214_1	1	non-amended	AY465247.1
1214_4	1	non-amended	AY465248.1
1214_5	1	non-amended	AY465249.1
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1221_3	1	non-amended	AY465252.1
1221_4	1	non-amended	AY465253.1
1221_5	1	non-amended	AY465254.1
1222_1	1	non-amended	AY465255.1
1222_2	1	non-amended	AY465256.1
1222_3	1	non-amended	AY465257.1
1222_4	1	non-amended	AY465258.1
1222_5	1	non-amended	AY465259.1
1223_1	1	non-amended	AY465260.1
1223_2	1	non-amended	AY465261.1
1223_3	1	non-amended	AY465262.1
1223_4	1	non-amended	AY465263.1
1223_5	1	non-amended	AY465264.1

1224_1	1	non-amended	AY465265.1
1224_2	1	non-amended	AY465266.1
1224_3	1	non-amended	AY465267.1
1224_4	1	non-amended	AY465268.1
1231_1	1	non-amended	AY465270.1
1231_3	1	non-amended	AY465271.1
1231_4	1	non-amended	AY465272.1
1231_5	1	non-amended	AY465273.1
1231_6	1	non-amended	AY465274.1
1232_2	1	non-amended	AY465275.1
1232_3	1	non-amended	AY465276.1
1232_4	1	non-amended	AY465277.1
1232_5	1	non-amended	AY465278.1
1233_1	1	non-amended	AY465279.1
1233_2	1	non-amended	AY465280.1
1233_3	1	non-amended	AY465281.1
1233_4	1	non-amended	AY465282.1
1233_5	1	non-amended	AY465283.1
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1234_6	1	non-amended	AY465287.1
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1311_4	1	non-amended	AY465291.1
1311_5	1	non-amended	AY465292.1
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1312_5	1	non-amended	AY465296.1
1312_6	1	non-amended	AY465297.1
1313_1	1	non-amended	AY465298.1
1313_2	1	non-amended	AY465299.1
1313_3	1	non-amended	AY465300.1
1314_5	1	non-amended	AY465301.1
1321_1	1	non-amended	AY465302.1
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1321_3	1	non-amended	AY465304.1
1321_4	1	non-amended	AY465305.1
1321_5	1	non-amended	AY465306.1
1322_1	1	non-amended	AY465307.1
1322_2	1	non-amended	AY465308.1
1322_3	1	non-amended	AY465309.1
1322_4	1	non-amended	AY465310.1
1322_5	1	non-amended	AY465311.1
1323_3	1	non-amended	AY465312.1
1323_5	1	non-amended	AY465313.1

1324_2	1	non-amended	AY465315.1
1324_3	1	non-amended	AY465316.1
1324_4	1	non-amended	AY465317.1
1324_5	1	non-amended	AY465318.1
1331_1	1	non-amended	AY465319.1
1331_2	1	non-amended	AY465320.1
1331_3	1	non-amended	AY465321.1
1331_4	1	non-amended	AY465322.1
1331_5	1	non-amended	AY465323.1
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1334_2	1	non-amended	AY465334.1
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1334_5	1	non-amended	AY465336.1
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2112_1	2	N-amended	EU262527.1
2112_2	2	N-amended	EU262535.1
2112_3	2	N-amended	EU262543.1
2112_4	2	N-amended	EU262485.1
2112_5	2	N-amended	EU262496.1
2113_5	2	N-amended	EU262511.1
2114_1	2	N-amended	EU262520.1
2114_2	2	N-amended	EU262528.1
2114_4	2	N-amended	EU262536.1
2114_5	2	N-amended	KC121143
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2133_3	2	N-amended	EU262497.1
2133_5	2	N-amended	EU262513.1
2221_1	2	N-amended	EU262529.1
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2221_3	2	N-amended	KC121144
2221_5	2	N-amended	EU262488.1
2224_1	2	N-amended	EU262530.1
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2231_2	2	N-amended	EU262489.1
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2234_5	2	N-amended	EU262514.1
2234_6	2	N-amended	EU262522.1
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2321_3	2	N-amended	EU262549.1
2321_5	2	N-amended	EU262491.1
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2322_1	2	N-amended	EU262506.1
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2322_4	2	N-amended	EU262523.1
2322_5	2	N-amended	EU262533.1
2323_1	2	N-amended	EU262540.1
2323_2	2	N-amended	KC121146
2333_5	2	N-amended	EU262494.1
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2334_2	2	N-amended	EU262509.1
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2334_5	2	N-amended	EU262526.1
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3113_1	3	non-amended	KC121148
3114_2	3	non-amended	KC121149
3121_2	3	non-amended	KC121150
3121_4	3	non-amended	KC121151
3122_1	3	non-amended	KC121152
3122_4	3	non-amended	KC121153
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3134_1	3	non-amended	KC121156
3134_3	3	non-amended	KC121157
3134_4	3	non-amended	KC121158
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3211_2	3	non-amended	KC121160
3211_3	3	non-amended	KC121161
3211_5	3	non-amended	KC121162
3211_6	3	non-amended	KC121163
3212_1	3	non-amended	KC121164
3212_2	3	non-amended	KC121165
3212_3	3	non-amended	KC121166
3212_4	3	non-amended	KC121167
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3213_1	3	non-amended	KC121169

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3311_6	3	non-amended	KC121174
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3312_5	3	non-amended	KC121176
3313_5	3	non-amended	KC121177
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3314_4	3	non-amended	KC121179
3321_3	3	non-amended	KC121180
3323_1	3	non-amended	KC121181
3323_2	3	non-amended	KC121182
3323_3	3	non-amended	KC121183
3331_3	3	non-amended	KC121184
3331_4	3	non-amended	KC121185
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4121_4	4	N-amended	KC121187
4121_5	4	N-amended	KC121188
4121_6	4	N-amended	KC121189
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4231_1	4	N-amended	KC121193
4231_3	4	N-amended	KC121194
4231_4	4	N-amended	KC121195
4231_5	4	N-amended	KC121196
4232_2	4	N-amended	KC121197
4232_3	4	N-amended	KC121198
4232_4	4	N-amended	KC121199
4233_2	4	N-amended	KC121200
4233_6	4	N-amended	KC121201
4234_6	4	N-amended	KC121202
4311_2	4	N-amended	KC121203
4311_5	4	N-amended	KC121204
4311_6	4	N-amended	KC121205
4312_5	4	N-amended	KC121206
4313_2	4	N-amended	KC121207
4313_3	4	N-amended	KC121208
4313_4	4	N-amended	KC121209
4313_5	4	N-amended	KC121210
4314_4	4	N-amended	KC121211
4314_5	4	N-amended	KC121212
5111_1	5	non-amended	KC121213
5111_3	5	non-amended	KC121214
5111_4	5	non-amended	KC121215
5111_5	5	non-amended	KC121216
5111_6	5	non-amended	KC121217

5112_2	5	non-amended	KC121218
5112_3	5	non-amended	KC121219
5112_4	5	non-amended	KC121220
5112_5	5	non-amended	KC121221
5112_6	5	non-amended	KC121222
5113_1	5	non-amended	KC121223
5113_2	5	non-amended	KC121224
5113_3	5	non-amended	KC121225
5113_4	5	non-amended	KC121226
5113_5	5	non-amended	KC121227
5114_1	5	non-amended	KC121228
5114_2	5	non-amended	KC121229
5114_4	5	non-amended	KC121230
5114_5	5	non-amended	KC121231
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5211_1	5	non-amended	KC121233
5212_1	5	non-amended	KC121234
5221_4	5	non-amended	KC121235
5221_6	5	non-amended	KC121236
5222_5	5	non-amended	KC121237
5223_2	5	non-amended	KC121238
5224_1	5	non-amended	KC121239
5231_1	5	non-amended	KC121240
5232_1	5	non-amended	KC121241
5232_4	5	non-amended	KC121242
5232_6	5	non-amended	KC121243
5234_4	5	non-amended	KC121244
5311_4	5	non-amended	KC121245
5311_6	5	non-amended	KC121246
5313_1	5	non-amended	KC121247
5313_6	5	non-amended	KC121248
5314_4	5	non-amended	KC121249
5323_4	5	non-amended	KC121250
5324_2	5	non-amended	KC121251
5331_2	5	non-amended	KC121252
5332_6	5	non-amended	KC121253
5334_2	5	non-amended	KC121254
6111_4	6	N-amended	KC121255
6112_1	6	N-amended	KC121256
6112_2	6	N-amended	KC121257
6112_5	6	N-amended	KC121258
6113_6	6	N-amended	KC121259
6221_4	6	N-amended	KC121260
6221_5	6	N-amended	KC121261
6222_1	6	N-amended	KC121262
6222_2	6	N-amended	KC121263
6222_3	6	N-amended	KC121264
6223_1	6	N-amended	KC121265

6224_1	6	N-amended	KC121266
6224_2	6	N-amended	KC121267
6331_3	6	N-amended	KC121268
6332_2	6	N-amended	KC121269
6332_3	6	N-amended	KC121270
6333_2	6	N-amended	KC121271
6334_6	6	N-amended	KC121272

Supplemental Table 2: Proportion of *Streptomyces* Using 20 Most Commonly Used Resources

Rank	Nutrient	% Isolates Using
1	Glycerol	98
2	a-D-Glucose	96
3	D-Gluconic Acid	95.7
4	Inosine	95.3
5	Adenosine	95
6	Tween 40	94.3
6	D-Trehalose	94.3
6	Putrescine	94.3
9	Maltotriose	93.8
10	Dextrin	93.1
10	L-Glutamic Acid	93.1
12	D-Tagatose	92.6
13	L-Alanine	92.1
14	Glycogen	91.8
15	L-Malic Acid	90.6
15	Succinic Acid	90.6
17	Adenosine-5-Monophosphate	90.3
18	N-Acetyl-L-Glutamic Acid	89.6
19	Lactamide	88.8
20	L-Asparagine	88.6

Supplemental Table 3: Mean Resource Use Efficiency of Top 20 Best Used Resources

Rank	Nutrient	Mean Efficiency
1	Tween 40	0.23
2	L-Malic Acid	0.23
3	Glycerol	0.14
4	Dextrin	0.13
5	D-Trehalose	0.12
6	Glycogen	0.12
7	L-Glutamic Acid	0.12
8	a-D-Glucose	0.11
9	Adenosine	0.11
10	D-Gluconic Acid	0.11
11	Maltotriose	0.11
12	D-Cellobiose	0.10
13	N-Acetyl-D-Glucosamine	0.10
14	Putrescine	0.10
15	Turanose	0.10
16	Succinic Acid	0.09
17	D-Galactose	0.09
18	D-Arabitol	0.09
19	D-Mannitol	0.09
20	Stachyose	0.08

Supplemental Table 4. Soil edaphic characteristics and *Streptomyces* community densities among plots. Different letters indicate significant differences (ANOVA, $p<0.05$) among plots.

Plot	pH	P (ppm)	K (ppm)	NO_3-N (ppm)	Total C (%)	LogStrep
1	6.65a	120.5a	150.8a	5.87ab	1.54ab	5.08a
2	5.83b	190.5a	169.7a	32.68bc	1.76a	5.05a
3	6.46ab	149.8a	141.2a	1.43a	1.06b	4.73a
4	6.35ab	191.3a	250.5a	4.37ac	1.89a	4.66a
5	6.35ab	192.2a	230.5a	35.3bc	1.41ab	4.78a
6	6.1ab	191.7a	162.2a	16.33abc	1.49a	4.85a

Supplemental Figure 1. This figure is too large to include in the text of this dissertation, but is available online at http://link.springer.com/article/10.1007/s00248-013-0280-6.

Chapter 4 Supplemental Material.

Supplemental Table 1. Mean inhibition strength (mm zone size) of *Streptomyces* among locations against 5 individual test standards. ANOVAs among locations are presented.

Location	87	4-2	2-12	4-16	6-14
Ant	0.4	1.3	3.4	1.0	0.0
CALC	0.5	0.7	0.0	0.0	0.0
Cev	0.0	0.0	0.0	0.0	0.0
Haw	0.1	0.0	2.4	0.7	0.8
KS	1.8	1.1	1.3	0.2	1.5
MC	3.7	4.5	2.8	2.7	1.9
MN1	1.9	1.1	1.1	1.1	3.7
MN3	4.4	4.1	4.7	1.4	5.1
MN5	4.2	2.8	3.0	1.8	4.0
Mont	0.6	1.0	1.8	1.5	0.8
NZ	0.0	0.0	0.5	0.0	1.2
PanFS	6.4	4.5	3.3	2.7	8.2
PanSC	1.8	4.4	2.7	0.8	3.9
PanVB	0.0	2.8	0.0	0.8	0.0
Witz	0.4	1.3	0.3	0.5	1.3
df	14	14	14	14	14
F	2.34	1.85	1.55	1.03	2.16
p-val	0.006	0.037	0.101	0.424	0.012

Supplemental Table 2. Mean resource use phenotypes (total growth, growth efficiency, and niche width) of *Streptomyces* among locations. ANOVAs among locations are presented.

Location	Total Growth	Growth Efficiency	Niche Width
Ant	8.39	0.17	44.00
CALC	11.62	0.17	61.00
Cev	4.06	0.07	47.80
Haw	6.07	0.09	65.00
KS	6.94	0.11	70.60
MC	7.42	0.13	58.90
MN1	5.06	0.06	79.00
MN3	7.07	0.08	85.60
MN5	7.10	0.10	68.40
Mont	11.30	0.21	51.18
NZ	6.66	0.10	62.10
PanFS	8.60	0.14	57.60
PanSC	9.49	0.12	75.10
PanVB	5.18	0.07	75.40
Witz	4.97	0.09	57.50
df	14	14	14
F	2.2	6.26	3.25
p-val	0.011	<0.0001	0.0002

Supplemental Table 3. Classification of isolates used in this work as determined using the RDP Naïve Bayesian Classifier.

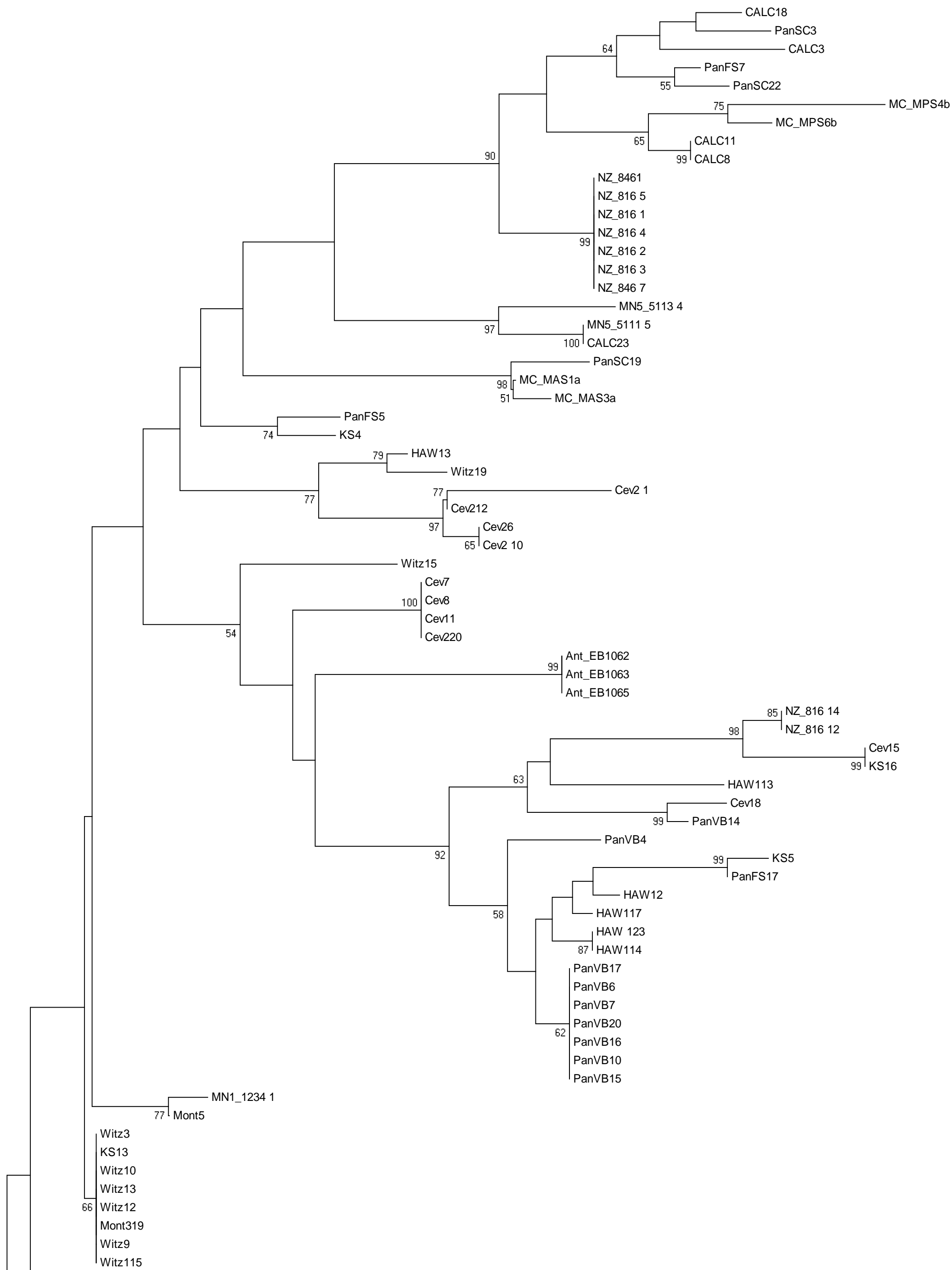
Isolate ID	Location of Origin	Classification	Confidence Threshold
DI-123-2	Ant	Streptomyces	100%
EB-106_2	Ant	Streptomyces	98%
EB-106_3	Ant	Streptomyces	97%
EB-106_5	Ant	Streptomyces	99%
HB-12_1	Ant	Streptomyces	100%
HB-12_2	Ant	Streptomyces	100%
HB-12_4	Ant	Streptomyces	100%
HB-12_5	Ant	Streptomyces	100%
HB-12_6	Ant	Streptomyces	100%
VP-09_3	Ant	Streptomyces	100%
CA_LC_11	CALC	Streptomyces	100%
CA_LC_13	CALC	Streptomyces	100%
CA_LC_17	CALC	Streptomyces	100%
CA_LC_18	CALC	Streptomyces	100%
CA_LC_23	CALC	Streptomyces	100%
CA_LC_27	CALC	Streptomyces	100%
CA_LC_3	CALC	Streptomyces	100%
CA_LC_4	CALC	Streptomyces	100%
CA_LC_8	CALC	Streptomyces	100%
CA_LC_9	CALC	Streptomyces	100%
CV11	Cev	Streptomyces	91%
CV15	Cev	Streptacidiphilus	100%
CV18	Cev	Kitasatospora	97%
CV2-1	Cev	Streptomyces	94%
CV2-10	Cev	Streptomyces	100%
CV2-12	Cev	Streptomyces	100%
CV2-20	Cev	Streptomyces	97%
CV2-6	Cev	Streptomyces	100%
CV7	Cev	Streptomyces	98%
CV8	Cev	Streptomyces	93%
HW_1-11	Haw	Streptomyces	100%
HW_1-13	Haw	Streptacidiphilus	100%
HW_1-14	Haw	Kitasatospora	98%
HW_1-17	Haw	Kitasatospora	93%
HW_1-2	Haw	Kitasatospora	100%
HW_1-20	Haw	Streptomyces	100%
HW_1-21	Haw	Streptomyces	100%
HW_1-23	Haw	Kitasatospora	100%

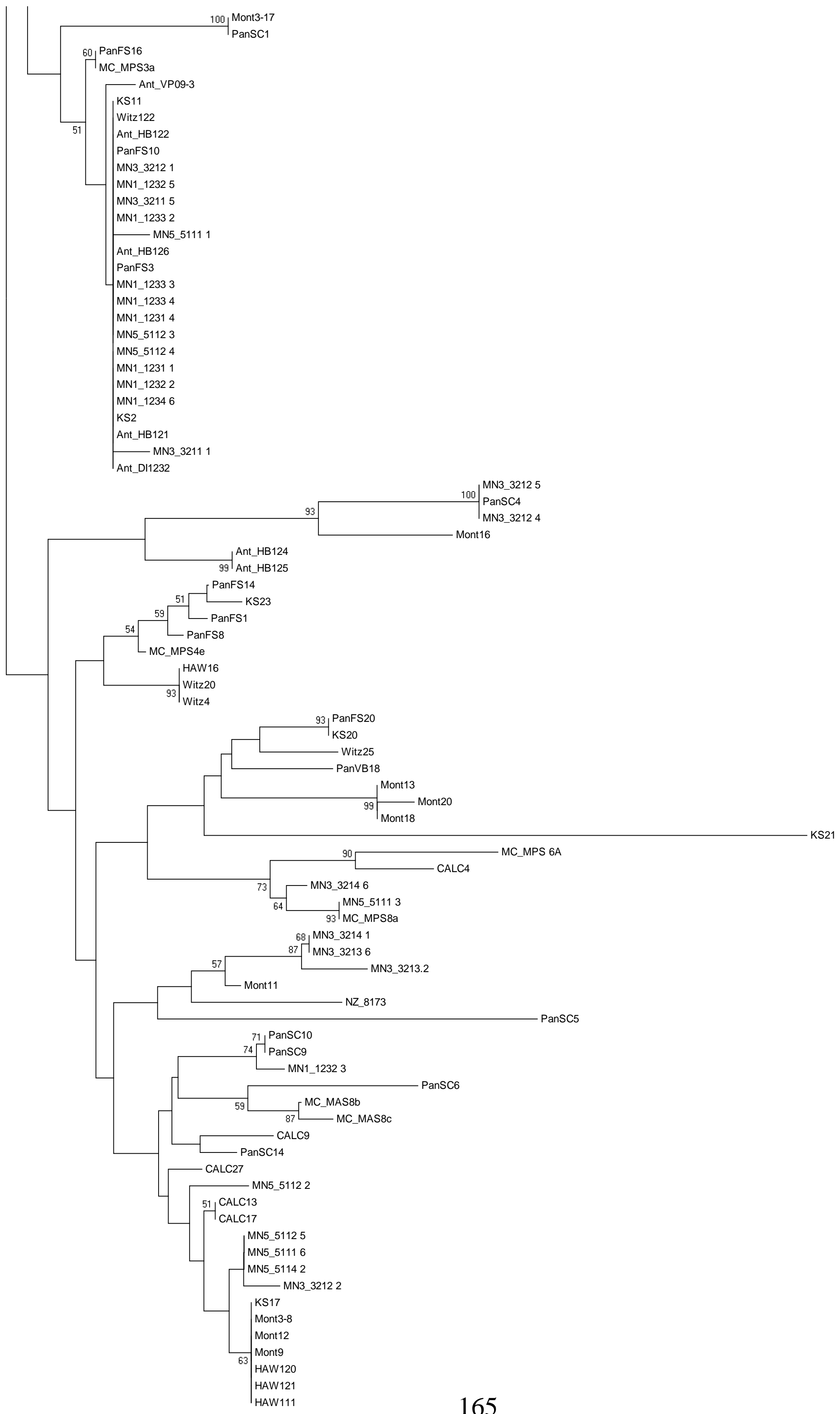
HW_1-3	Haw	Streptomyces	100%
HW_1-6	Haw	Streptomyces	100%
KS11	KS	Streptomyces	100%
KS13	KS	Streptomyces	100%
KS16	KS	Streptacidiphilus	100%
KS17	KS	Streptomyces	100%
KS2	KS	Streptomyces	100%
KS20	KS	Streptomyces	100%
KS21	KS	unclassified Actinomycetales	100%
KS23	KS	Streptomyces	100%
KS4	KS	Streptomyces	100%
KS5	KS	Kitasatospora	98%
MC_MAS_1a	MC	Streptomyces	100%
MC_MAS_3a	MC	Streptomyces	100%
MC_MAS_8b	MC	Streptomyces	100%
MC_MAS_8c	MC	Streptomyces	100%
MC_MPS_3a	MC	Streptomyces	100%
MC_MPS_4b	MC	Streptomyces	100%
MC_MPS_4e	MC	Streptomyces	100%
MC_MPS_6a	MC	Streptomyces	100%
MC_MPS_6b	MC	Streptomyces	100%
MC_MPS_8a	MC	Streptomyces	100%
1231.1	MN1	Streptomyces	100%
1231.4	MN1	Streptomyces	100%
1232.2	MN1	Streptomyces	100%
1232.3	MN1	Streptomyces	100%
1232.5	MN1	Streptomyces	100%
1233.2	MN1	Streptomyces	100%
1233.3	MN1	Streptomyces	100%
1233.4	MN1	Streptomyces	100%
1234.1	MN1	Streptomyces	100%
1234.6	MN1	Streptomyces	100%
3211.1	MN3	Streptomyces	100%
3211.5	MN3	Streptomyces	100%
3212.1	MN3	Streptomyces	100%
3212.2	MN3	Streptomyces	100%
3212.4	MN3	Streptomyces	100%
3212.5	MN3	Streptomyces	100%
3213.2	MN3	Streptomyces	99%
3213.6	MN3	Streptomyces	99%
3214.1	MN3	Streptomyces	99%

3214.6	MN3	Streptomyces	100%
5111.1	MN5	Streptomyces	100%
5111.3	MN5	Streptomyces	100%
5111.5	MN5	Streptomyces	100%
5111.6	MN5	Streptomyces	100%
5112.2	MN5	Streptomyces	100%
5112.3	MN5	Streptomyces	100%
5112.4	MN5	Streptomyces	100%
5112.5	MN5	Streptomyces	100%
5113.4	MN5	Streptomyces	100%
5114.2	MN5	Streptomyces	100%
M11	Mont	Streptomyces	100%
M12	Mont	Streptomyces	100%
M13	Mont	Streptomyces	100%
M16	Mont	Streptomyces	100%
M18	Mont	Streptomyces	100%
M20	Mont	Streptomyces	100%
M3-17	Mont	Streptomyces	100%
M3-19	Mont	Streptomyces	100%
M3-8	Mont	Streptomyces	100%
M5	Mont	Streptomyces	100%
M9	Mont	Streptomyces	100%
816-1	NZ	Streptomyces	100%
816-12	NZ	Streptacidiphilus	100%
816-14	NZ	Streptacidiphilus	100%
816-2	NZ	Streptomyces	100%
816-3	NZ	Streptomyces	100%
816-4	NZ	Streptomyces	100%
816-5	NZ	Streptomyces	100%
817-3	NZ	Streptomyces	100%
846-1	NZ	Streptomyces	100%
846-7	NZ	Streptomyces	100%
PanFS1	PanFS	Streptomyces	100%
PanFS10	PanFS	Streptomyces	100%
PanFS14	PanFS	Streptomyces	100%
PanFS16	PanFS	Streptomyces	100%
PanFS17	PanFS	Kitasatospora	98%
PanFS20	PanFS	Streptomyces	100%
PanFS3	PanFS	Streptomyces	100%
PanFS5	PanFS	Streptomyces	100%
PanFS7	PanFS	Streptomyces	100%

PanFS8	PanFS	Streptomyces	100%
PanSC1	PanSC	Streptomyces	100%
PanSC10	PanSC	Streptomyces	100%
PanSC14	PanSC	Streptomyces	100%
PanSC19	PanSC	Streptomyces	100%
PanSC22	PanSC	Streptomyces	100%
PanSC3	PanSC	Streptomyces	100%
PanSC4	PanSC	Streptomyces	100%
PanSC5	PanSC	Streptomyces	91%
PanSC6	PanSC	Streptomyces	100%
PanSC9	PanSC	Streptomyces	100%
PanVB10	PanVB	Kitasatospora	98%
PanVB14	PanVB	Kitasatospora	99%
PanVB15	PanVB	Kitasatospora	100%
PanVB16	PanVB	Kitasatospora	99%
PanVB17	PanVB	Kitasatospora	100%
PanVB18	PanVB	Streptomyces	100%
PanVB20	PanVB	Kitasatospora	99%
PanVB4	PanVB	Kitasatospora	67%
PanVB6	PanVB	Kitasatospora	100%
PanVB7	PanVB	Kitasatospora	100%
W10	Witz	Streptomyces	100%
W1-2	Witz	Streptomyces	100%
W1-22	Witz	Streptomyces	100%
W13	Witz	Streptomyces	100%
W15	Witz	Streptomyces	100%
W1-5	Witz	Streptomyces	100%
W19	Witz	Streptomyces	100%
W20	Witz	Streptomyces	100%
W25	Witz	Streptomyces	100%
W3	Witz	Streptomyces	100%
W4	Witz	Streptomyces	100%
W9	Witz	Streptomyces	100%

Supplemental Figure 1. Phylogenetic tree of *Streptomyces* used in Chapter 4. Tree was constructed with the neighbor-joining method using 1000 bootstraps. Support of nodes represented in >50% of bootstraps are presented as node labels. Isolate (leaf) labels begin with the abbreviation of the location from which each isolate originated.




Mont3-17
PanSC1
PanFS16
MC_MPS3a
Ant_VP09-3
KS11
Witz122
Ant_HB122
PanFS10
MN3_3212 1
MN1_1232 5
MN3_3211 5
MN1_1233 2
MN5_5111 1
Ant_HB126
PanFS3
MN1_1233 3
MN1_1233 4
MN1_1231 4
MN5_5112 3
MN5_5112 4
MN1_1231 1
MN1_1232 2
MN1_1234 6
KS2
Ant_HB121
MN3_3211 1
Ant_DI1232
MN3_3212 5
PanSC4
MN3_3212 4
Mont16
Ant_HB124
Ant_HB125
PanFS14
KS23
PanFS1
PanFS8
MC_MPS4e
HAW16
Witz20
Witz4
PanFS20
KS20
Witz25
PanVB18
Mont13
Mont20
Mont18
KS21
MC_MPS 6A
CALC4
MN3_3214 6
MN5_5111 3
MC_MPS8a
MN3_3214 1
MN3_3213 6
MN3_3213.2
Mont11
NZ_8173
PanSC5
PanSC10
PanSC9
MN1_1232 3
PanSC6
MC_MAS8b
MC_MAS8c
CALC9
PanSC14
CALC27
MN5_5112 2
CALC13
CALC17
MN5_5112 5
MN5_5111 6
MN5_5114 2
MN3_3212 2
KS17
Mont3-8
Mont12
Mont9
HAW120
HAW121
HAW111
0.002

Chapter 5 Supplemental Material.

Supplemental Table 1: Relative abundance of sequences classified to bacterial phyla in CCESR soil (476,573 total sequences).

Phylum	Proportion of Sequences (%)
Actinobacteria	32.1
unclassified	25.1
Proteobacteria	24.8
Acidobacteria	11.4
Gemmatimonadetes	3.3
Bacteroidetes	1.8
Firmicutes	<1
TM7	<1
Bacteria_incertae_sedis	<1
Nitrospira	<1
Planctomycetes	<1
Deinococcus-Thermus	<1
Chloroflexi	<1
WS3	<1
BRC1	<1
OP10	<1
Verrucomicrobia	<1

Supplemental Tables 2-6 are a series of tables that are too large to fit the formatting requirements of this thesis and will be made available online.

Supplemental Table 2: Abundances and classification of major OTUs.

Supplemental Table 3: Abundances and classification of the 10 best indicator OTUs for each plant species.

Supplemental Table 4: Correlations among plant richness, aboveground biomass, and soil edaphic characteristics.

Supplemental Table 5: Proportion of modules (by sequence abundance) belonging to bacterial phyla.

Supplemental Table 6: (a) Pearson correlations (p-values) betwen the relative abundance of major bacterial phyla with primary production, soil, and bacterial community characteristics. (b) Pearson correlations (p-values) betwen the abundance of major OTUs with primary production, soil, and bacterial community characteristics.