Ecology of interspecies signaling among *Streptomyces* and its relationship to pathogen suppression

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Abstract

Interspecies signaling may be defined as the induced change in phenotype of one species by another that is not due to the metabolism of the signal. Although suggested to be a relatively widespread phenomenon, the role of signaling in natural soil communities has not been thoroughly studied. Within *Streptomyces* communities in soil, understanding the impacts of interspecies signaling on species interactions, and especially on nutrient competition and antagonism, may be key to effective *Streptomyces*-based suppression of plant pathogens. I evaluated the frequency of signaling interactions and their effect on inhibitory phenotypes of *Streptomyces* isolated from natural prairies. Signaling among *Streptomyces* was frequent, and observed in 35% of all interactions. Isolates from the same location in soil were more likely to signal one another than isolates from different locations, suggesting local selection for signaling interactions. Signaling was similarly more frequent between isolates that had similar nutrient use profiles. Finally, closely-related isolates were more likely to increase inhibition towards one another via signaling than distantly-related isolates. In chapter 2, subinhibitory concentrations of antibiotics were studied as signals, specifically in relation to their capacities to shift nutrient use among *Streptomyces*. I found that some antibiotics altered nutrient use by *Streptomyces* in ways that could reduce nutrient competition among isolates. Finally, pathogen suppression and signaling were evaluated in soils with different cropping histories. Pathogen suppression by *Streptomyces* varied significantly among soils, and suppressive activity was positively correlated with bacterial density. Among *Streptomyces* from these plots, shifts in inhibitory phenotypes in response to signaling by another isolate were very frequent ($\approx 56\%$ of all interactions). Overall, signaling in *Streptomyces* is frequent and varies with spatial origin, nutrient overlap, antagonistic phenotype, and genetic relatedness among isolates, as well as soil cropping history. Moreover, some antibiotics have the potential to act as signals that

can significantly alter nutrient competition among *Streptomyces*. Variation in signaling has significant potential to mediate pathogen suppression in soil communities.

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Introduction

Microbial interactions in soil shape the behavior and activities of soil communities. Competitive interactions among pathogenic and non-pathogenic rhizospheric microorganisms may determine if plant disease will develop (Scheuring and Yu, 2012; Verholt, 2012). Symbiotic interactions among bacteria also occur such as metabolic cooperation in which one organism uses the by-product of another as an energy source (Little *et al.*, 2008) or among different individuals that cooperate in biofilm formation (Driscoll *et al.*, 2011). Recently, the focus of the study on microbial interspecies interactions has added another level of complexity: signaling or interspecies communication among genetically diverse microorganisms (Pierson *et al.*, 1998; Egland *et al.*, 2004; Shank and Kolter, 2009). Interspecies signaling has been defined as the induced change in phenotype of one species by another that is not due simply to the metabolism of the signal or compound (Straight and Kolter, 2009). Although originally explored among kin organisms (Sakuda *et al.*, 1992; Maddula *et al.*, 2006), signaling among non-kin organisms has been shown to have significant influence on microbial phenotypes and to occur among organisms of different species, genera, and even across kingdoms (Lyon and Muir, 2003).

Bacterial signal molecules with diverse chemical structures have been identified (Horinouchi and Beppo, 1994; Ansaldi and Dubnau, 2004; Dulla and Lindlow, 2009; Hsiao *et al.*, 2009; Corre *et al.*, 2010). Molecules known to act in quorum-sensing, such as acyl-homoserine lactones, that coordinate activities among gram-negative bacteria,

have been shown to induce phenotypic changes in organisms other than the producers (Pierson *et al.*, 1998). Similar dynamics are observed among *Streptomyces* spp., and their quorum-sensing molecules, the gamma-butyrolactones, and among *Bacillus* spp. and their peptidic quorum-sensing molecules (Slattery *et al.*, 2001; Stefanic *et al.*, 2009). Our understanding of interspecies signaling interactions continues to evolve, with the recent recognition that other compounds besides the well-known quorum-sensing molecules may induce phenotypic changes in target organisms (Yi *et al.*, 2007; Linares *et al.*, 2006). For example, it has been argued that antibiotics in natural settings may not serve as weapons, but rather as signals to mediate microbial gene expression (Davies, 2006). Yet, the scope of implications of microbial signaling interactions for microbial activities in natural communities remain poorly understood.

The use of the word "signal" has brought some debate among scientists. According to some authors, a molecule can only be considered a signal when its sole function in the producer organism is to be received by the target, and the sole use of the signal receptor (in the target organism) is to sense the presence of the signal (Keller and Surette, 2006). However, this strict definition excludes many relevant interactions among microorganisms in natural habitats. For instance, eavesdropping, a process in which an organism may detect quorum-sensing molecules produced by another species (Duan *et al.*, 2003), may alter the timing or quantity of antibiotic production by the eavesdropper, thus enhancing the fitness benefit of antibiotic production in the presence of a competitor (Chandler *et al.*, 2012). Chemical manipulation, which would also be excluded from the strict definition of signaling, has also been found among plants that produce compounds similar to bacterial quorum-sensing molecules (Teplitski *et al.*, 2000) or degrade quorum-sensing molecules produced by bacteria (Teplitski *et al.* 2011; Sánchez-Contreras *et al.*, 2007). These examples, which suggest complex interaction dynamics in natural communities argue for a less-restrictive definition of signaling. In this work, we follow the

definition proposed by Straight and Kolter (2009), considering signaling as an event in which one organism induces a phenotypic change in another that is not due to the metabolism of the signal.

Signaling among soil bacteria has not been explored as a factor that may significantly influence pathogen suppression in soil communities. Yet, signaling interactions among individuals of soil communities are likely to influence significantly the behavior of the community as a whole. Bacteria that were apparently non-antagonistic towards plant-pathogenic fungi were shown to significantly inhibit growth of fungal pathogens when grown together (De Boer *et al.*, 2007). Although this was shown for a small collection of species in a laboratory setting, competitive interactions among bacteria in natural settings are highly likely to influence inhibitory phenotypes through signaling interactions (Chandler *et al.*, 2012; Slattery *et al.*, 2001). This suggests that understanding the frequency and effects of signaling interactions in inhibitory phenotypes is critical to predicting disease suppression.

Bacteria of the genus *Streptomyces* are ubiquitous in bulk and rhizospheric soil (Kieser *et al.*, 2000; Challis and Hopwood, 2003). *Streptomyces* belong to the class Actinobacteria, which have been shown to vary significantly among soils under different management practices (Shange *et al.*, 2012; Ceja-Navarro *et al.*, 2010) and among soils with varying levels of suppressiveness (Becker *et al.*, 1997; Mendes *et al.*, 2011). *Streptomyces* are known for their antibiotic production; isolates of this genus vary in their inhibitory activity (Davelos *et al.*, 2004) and commonly posses several gene clusters associated with antibiotic biosynthesis (Challis and Hopwood, 2003). Their unmatched antibiotic activity and ability to colonize soils makes *Streptomyces* an outstanding subject to study soil-borne pathogen suppression.

In this thesis, diverse aspects of signaling among *Streptomyces* are explored. In

the first chapter, called "Nutrient overlap, genetic relatedness and spatial origin influence the effect of interspecies interactions on inhibition phenotypes among *Streptomyces* spp.", the frequency and effect of signaling on three *Streptomyces* communities are explored. A high frequency and diversity of signaling interactions among *Streptomyces* are reported. Most importantly, signaling interactions are found to be locally selected, which suggests a fundamental role of signaling for microbial communities in natural conditions. Interactions that shifted inhibitory phenotypes had varying frequency among communities, and isolate pairs were found to be more or less likely to signal each other depending on their nutrient overlap and phylogeny. These relationships show a biological context in which signaling occurs among *Streptomyces*.

In the second chapter, called "Subinhibitory antibiotic concentrations mediate nutrient use and competition among soil *Streptomyces*", the effects of subinhibitory concentrations of antibiotics on nutrient utilization by *Streptomyces* were evaluated. Antibiotics were found to vary in their effects on nutrient use. Antibiotics for which there are low levels of resistance, if any, among globally sampled *Streptomyces* (D'Costa *et al.*, 2011), reduced potential competition for nutrients, while the opposite was observed for antibiotics for which there is a high frequency of resistance in natural communities. This supports the hypothesis that some antibiotics may serve as signals in natural settings. This work was done in collaboration with Matthew G. Bakker, Christine E. Salomon and my adviser, Linda L. Kinkel and will have me as leading author.

The third and last chapter is called "Cropping history effects on pathogen suppressive activity and shifts in inhibition in *Streptomyces* communities". This chapter aimed to evaluate the effects of cropping history on pathogen suppression and to integrate information about signaling with management practices that promote pathogen suppressive communities. Briefly, soil samples from a rotation experiment established 13 years ago in Uruguay were evaluated in their bacterial and *Streptomyces* density as well

as in their suppressive activity and edaphic characteristics. Signaling characteristics were evaluated among isolates from selected samples in an attempt to find a relationship between management practices, microbial densities, and signaling characteristics of *Streptomyces* populations. Overall, cropping history was found to influence significantly pathogen suppression and a high frequency of signaling interactions was found among *Streptomyces* from the selected communities.

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Chapter 1

Nutrient overlap, genetic relatedness and spatial origin influence the effect of interspecies interactions on inhibition phenotypes among *Streptomyces* spp.

Chemical communication among kin organisms modulates diverse activities. Despite a general consensus that signaling among non-kin organisms is likely to influence significantly microbial behavior, there is limited information on the implications of interspecies signaling in soil microbial communities. In this work, we explored patterns of interactions that alter inhibitory phenotypes among *Streptomyces* isolates from three distinct communities. Nutrient utilization, ribosomal 16S sequences, pairwise inhibition and pairwise inhibition shifts were evaluated for all isolates. We found that the frequency of inhibition-shifting interactions was higher within than among communities, suggesting local adaptation of inhibition-shifting phenotypes. Communities varied in the frequency with which their isolates responded to a partner, but not in the frequency with which they induced changes in their partners. Isolates were more likely to increase their inhibition in response to isolates that are strong competitors for nutrients, to closely-related isolates and to isolates that are highly sensitive to their antibiotics. Overall, we document a high frequency and diversity of interactions among *Streptomyces* and a meaningful biological context for neighbor-induced shifts in inhibition, which add a layer of complexity to interspecies interactions. This work shows significant ways in which chemical communication may shape microbial community dynamics.

1.1 Introduction

Microorganisms in natural habitats are immersed in an environment filled with diverse chemicals. Bacteria depend upon cues from their environment in order to increase their chances of survival through the modulation of gene expression. The origin of these cues is diverse, ranging from the nutrients that are available in a particular setting to small biological molecules produced by potential competitors and other sympatric organisms from different kingdoms (Camilli and Bassler, 2006; Lopez *et al.*, 2009; Teplitsky *et al.*, 2000). Chemical communication among kin organisms modulates activities as diverse as sporulation, antibiotic production, entrance to a competent state, biofilm production and expression of pathogenesis-related molecules (Weinrauch et al., 1991;Williams, 2007). However, signaling among organisms of different species, genera or even across kingdoms can have a significant impact in microbial communities in natural conditions (Slattery *et al.*, 2001; Bassler and Greenberg, 1997). Despite a general consensus that interspecies communication is important in soil and is likely to influence microbial behavior, there is limited information on the implications of signaling interactions for the ecology and evolutionary biology of microbial communities in soil.

Some authors define signaling as an interaction in which both the signal and the receptor are produced strictly for the specific purpose of communication (Keller and Surette, 2006; Diggle *et al.*, 2007). However, this definition excludes many forms of communication that occur in complex communities. For instance, using this definition, eavesdropping (Duan *et al.*, 2003), a process in which signals that modulate activities within kin organisms are detected and induce a response by non-intended recipients, would not be considered signaling. Eavesdropping, however, may alter the timing or quantity of antibiotic production by the eavesdropper, potentially enhancing the fitness benefit of antibiotic production in the presence of a competitor (Chandler *et al.*, 2012). Chemical manipulation, a process in which one organism secretes chemicals that

modify the target population's metabolism, usually to the secretor's benefit, (Keller and Surette, 2006; Egland *et al.*, 2004) would also be excluded. While these interactions may influence the fitness of the interacting species, the sum of these and other chemical interactions are likely to shape the structure and function of natural microbial communities. Consequently, it is critical to explore the complex dynamics of chemical communication among microorganisms as influenced by space, phenotypes and phylogeny.

Bacteria of the genus *Streptomyces* are ubiquitous in soil, freshwater and marine habitats (Schrey and Tarkka, 2008; Schneeman et al., 2010). *Streptomyces* have been studied most extensively for their unmatched diversity in antibiotic production, producing 50-80% of all antibiotics of microbial origin (Kieser *et al.*, 2000). Antibiotic production in *Streptomyces* is tightly regulated (Bibb 2005) and usually requires complexes of enzymes (20-30) dedicated to that function (Kieser *et al.*, 2000). Thus, antibiotic production can be costly. Antibiotics are usually produced at the onset of the stationary growth phase when grown in liquid culture, and are often associated with aerial hyphae and spore formation on solid medium. Antibiotic production in *Streptomyces* is regulated by a wide array of small molecules, e.g. furanes, A-factor-, virginiae butanolide- and IM2-type γ-butyrolactones (GBL) (Healy *et al.* 2009; O'Rourke *et al.* 2009, Nakano *et al.*, 2000; Takano *et al.*, 2000; Kato *et al.*, 2007; Arakawa *et al.*, 2007). *Streptomyces* strains commonly produce several signal molecules of the same type that contain small variations in their acyl chains (Hsiao *et al.*, 2009). In addition, the recognition of these molecules is specific towards structural families of molecules so that a GBL receptor will bind strongly to some GBLs, weakly to GBLs of similar structure, and will not recognize GBLs of a different type (Hsiao *et al.*, 2009). *Streptomyces* spp. commonly possess more than one type of GBL receptor gene in their genomes and they are thought to be horizontally transferred (Nishida *et al.*, 2007). This potentially enables divergent *Streptomyces* species to recognize and react to similar signal molecules.

Nutrient competition among *Streptomyces* can play a significant role in selection for antibiotic inhibitory phenotypes (Schlatter *et al.*, 2009). Moreover, the benefits of antibiotics to fitness will depend upon the extent of nutrient competition among coexisting populations (Kinkel *et al.*, 2011). This suggests that both antibiotic production and species interactions that mediate antibiotic production should be influenced by patterns of nutrient use among competitors. Furthermore, if signaling interactions that alter antibiotic production confer fitness benefits, then signaling should be locally adapted, or more likely among sympatric (locally coexisting) than allopatric populations.

In this work, we study patterns of interaction among *Streptomyces* that alter inhibitory phenotypes. We examined the frequency and direction (increase or decrease) of changes in inhibitory phenotypes among isolates paired with a sympatric or allopatric partner and examined the relationships between the induced changes and nutrient overlap, genetic distance, and antagonism among isolate pairs. Our results suggest that chemical communication among *Streptomyces* spp. has a significant role in structuring inhibitory phenotypes among individuals within communities.

1.2 Materials and Methods

1.2.1 Isolates

The *Streptomyces* used in this study were isolated from Cedar Creek Ecosystem Science Reserve in MN, USA (www.cedarcreek.umn.edu), a National Science Foundation Long-Term Ecological Research Site. Soil was collected in December, 2000, from three randomly-selected locations within the central 1×1 m of each of three different plots (plots 08-A, 26-A, and 47-A, in E001, referred to here as plots 1, 3, and 5, respectively). Plots were all less than 25 m apart. The plots had been amended with a base nutrient treatment (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) applied twice a year (early May and late June) starting in 1982, 18 years prior to sampling. All plots had been abandoned from crop production in 1934, and natural prairie vegetation had been allowed to recolonize the site.

Soil samples 10 cm deep were obtained with small corers made of aluminum tubes with a diameter of 1 cm. Samples were dried overnight, suspended and agitated in phosphate buffer solution for 1 hour, plated on oatmeal agar with antibiotics, and incubated at 28°C for 7 days, as described by Davelos *et al.*, (2004b). From each plot, ten isolates from a single soil core were randomly selected for further study. In total, thirty *Streptomyces* spp. isolates from the three communities were used in the analyses. *Bacillus* isolates 51-U-1 and 41-D-2 were selected for signaling assays based on their sensitivity to inhibition by the *Streptomyces*. Specifically, every one of the *Streptomyces* isolates studied here was able to inhibit at least one of the two *Bacillus* isolates. They were obtained from soil samples from the same experimental field (E001) at the Cedar Creek Ecosystem Science Reserve.

1.2.2 Signaling assays

We were interested in quantifying interactions in which one *Streptomyces* isolate modifies the inhibition phenotype of another isolate. We refer to such interactions as "signaling". Interactions were evaluated among all pairwise isolate combinations ($n = 861$), and inhibitory activity was determined by the presence of zones of growth inhibition on *Bacillus* lawns overlaying the *Streptomyces*.

Assays were carried out by inoculating paired *Streptomyces* isolates 1 cm apart on plates containing 15 ml of ISP2 medium (Figure 2.1; Shirling and Gottlieb, 1966), with four replicates of each pair per plate. Control plates were inoculated with each isolate individually, with four replicates per plate. Isolates were inoculated as 4 μl drops of spore suspensions containing approximately 5×10^7 spores. Plates were incubated at

28°C for 3 days, at which time *Bacillus* overlays were made onto each plate. Briefly, a 12-hour culture of the *Bacillus* targets (52-U-1 or 41-D-2), grown in Nutrient Broth to an $OD_{600} \approx 0.800$ was diluted 1:10 in Nutrient Broth (Difco) containing 0.8% agar and added as a 10 ml overlay on the plates. After 24 hours at 30°C, inhibition zones on the *Bacillus* lawns were measured. Two perpendicular measurements were made per zone from the edge of the *Streptomyces* colony to the end of the inhibition zone, away from the paired isolate, and their average was used for statistical analyses. Inhibition zones of paired *Streptomyces* were compared with zones generated by the *Streptomyces* isolates grown on ISP2 plates alone (controls). The effect of a paired isolate was evaluated by testing the significance and direction (increase or decrease of inhibition) of differences between the inhibition zones of isolates in the presence and the absence of a paired isolate.

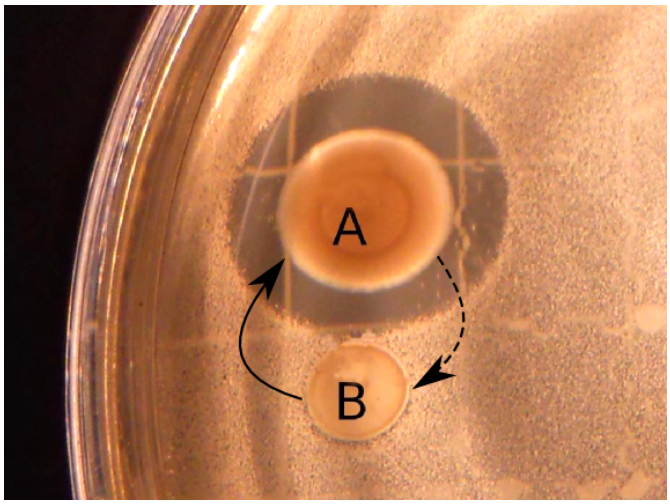

Figure 1.1: Isolates were spotted 1 cm apart and inhibition zones on *Bacillus* overlaying lawns were measured and compared to zones made by each of the isolates (A or B) growing alone. The arrows indicate the direction of the induction of change. Isolate A is responding to and inducing isolate B and vice-versa. Four replicate experiments were carried out per plate.

1.2.3 Genetic analyses

Ribosomal DNA sequences were obtained as described in Davelos *et al.* 2004a. Briefly, genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, WI). Genes of 16S rRNA were amplified almost to their full length using the universal bacterial primers 27F (pA; 5'-AGAGTTTGATCCTGGCTCAG-3') and 1541R (pH; 5'-AAGGAGGTGATCCAGCCGCA-3') (Edwards *et al.*, 1989) and PCR Supermix High Fidelity master mix (Invitrogen, Carlsbad, CA) with each primer at 10 pM and 100 ng of template DNA. The thermocycling protocol of Takeuchi *et al.* (1996) was followed. Amplicons were then sequenced at the University of Minnesota Biomedical Genomics Center (Saint Paul, MN). Sequences were edited manually using BioEdit (Hall, 1999) and 703 bp of high quality sequence were used for 16S distance calculations using Mothur (http://www.mothur.org/wiki/Dist.seqs; Schlatter, personal communication).

1.2.4 Nutrient use analyses and niche overlap

Nutrient utilization phenotypes were determined for each *Streptomyces* isolate on 95 nutrient sources using Biolog SF-P2 plates (Biolog, Inc. Hayward, CA; Schlatter *et al.*, 2012). Briefly, freshly grown spore suspensions of each isolate were adjusted to an OD_{590} = 0.22, diluted according to the manufacturer's instructions, and inoculated into Biolog plates. After 3 days of incubation at 28°C, the OD_{590} of each well was determined using a Multiskan EX microplate reader (Labsystems, Helsinki, Finland. For each plate, the OD value of the water control well was subtracted from that of all other wells before analysis, and nutrients for which the resulting well readings were below 0.005 were considered as 0. Readings above 0.005 were considered positive. For every individual isolate, nutrient overlap with each of the other isolates was calculated as the proportion of the nutrients used by both isolates with respect to the total number of nutrients

used by the first isolate. Using this definition of nutrient overlap, two values of nutrient overlap are obtained per isolate pair, and the potential influence of the presence of one isolate on another can be estimated.

1.2.5 Inhibition among *Streptomyces* isolates

Isolates were tested in all possible pairwise combinations for the ability to inhibit one another using a protocol modified from Davelos *et al.* (2004a). Briefly, spore suspensions (approximately 1×10^8 spores/ml) of individual isolates were dotted (10 μl per spot) onto 15-ml starch casein agar plates, three replicate dots per plate, and incubated at 28°C for 3 days. Dotted isolates were killed by inverting the uncovered petri plates over 4 ml of chloroform in a watch glass for 1 h. Plates were removed and aerated in a fume hood for 30 min to allow evaporation of chloroform. Plates were subsequently overlaid with 15 ml of 1% water agar and after solidified, inoculated with 100 μl of the test isolate (approximately 1×10^8 spores/ml) spread on the surface of the agar. Plates were incubated at 28°C for 3 days. The size of the zones of growth inhibition of the overlaid isolate on top of any dotted isolate was measured from the edge of the dotted colony to the edge of the cleared zone. Each isolate was both dotted (to measure inhibition) and overlaid (to measure resistance).

1.2.6 Analyses

For signaling assays, significant differences in inhibition were calculated using SAS 9.2 (PROC GLM; SAS Institute Inc., Cary, NC, USA). Differences in frequency of signaling were analyzed using χ^2 tests (http://quantpsy.org). Nutrient overlap among isolates and significant differences in means of nutrient overlap and genetic distance (1-way ANOVAS and Tukey's Least Significant Differences) were calculated using Matlab Statistics Toolbox (MATLAB version 7.8.0. Natick, MA: The MathWorks Inc, 2009).

1.3 Results

Inhibition of a target in the presence of a partner was screened among all possible pairwise *Streptomyces* isolate combinations ($n = 861$) from three locations in soil. Overall, there were significant changes in inhibition (signaling) in the presence vs. absence of a partner in 35.4% of isolate combinations. *Streptomyces* from different locations varied significantly in the frequency with which their inhibition was shifted by the presence of a partner, or the frequency of response. Specifically, among all isolate combinations, inhibition by isolates from plot 5 was most frequently altered by the presence of a partner, followed by isolates from plot 3, and lastly by isolates from plot 1 (Figure 2.2a; $\chi^2\ [2, n = 861] = 25.092$, $p < 0.0001$). In contrast, the frequency with which isolates induced changes in inhibition by a partner did not vary among isolates from different communities (Figure 2.2b). Thus, the frequency of response to presumptive signals, but not the frequency of induction of changes, varies among communities.

Among isolates, changes in the presence of a partner included both significant increases and decreases in inhibition. Among all combinations in which the change in inhibition was significant, 60% were increases and 40% were decreases ($n = 181$ and 122 of 303 isolate pairs, respectively). As receptors of presumptive signals, isolates from plot 5 differed significantly from isolates from both plots 1 and 3 in the direction of change in inhibition activity. Isolates from plot 5 most commonly exhibited decreases in inhibition in the presence of a partner, while isolates from plots 1 and 3 generally exhibited increased inhibition in the presence of a partner (Figure 2.3a; $\chi^2\ [2, n = 303] = 77.7$, $p < 0.00001$). However, there were no significant differences among the three plots in the capacities of the isolates to induce increases vs decreases in inhibition by others (Figure 2.3b; $\chi^2\ [2, n = 303] = 0.337$, $p = 0.845$). Thus, isolates from the three communities varied in the tendencies of their responses, but not in the responses they induced in others.

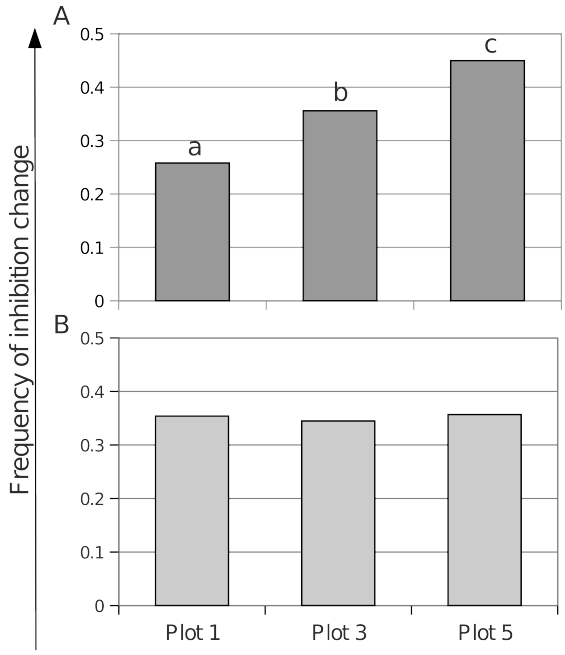

Figure 1.2: Frequency of changes in inhibition in each community when grown with a partner isolate, in all (sympatric and allopatric) pairwise interactions. Different letters indicate significant differences in frequencies. (A) Frequency of responses to the presence of a partner (χ^2 $[2, n = 861] = 25.092$, $p < 0.0001$). (B) Frequency of induction of changes in a partner (χ^2 $[2, n = 861] = 0.101$, $p = 0.95$).

Every isolate enhanced inhibition in some isolates and repressed inhibition in others, but not all isolates had their own inhibition both increased and decreased by the presence of a partner. Considering all pairwise interactions, isolates induced reductions in antibiotic inhibition in one to nine of the 29 isolates (mean = 4.1 isolates), and induced increases in three to 11 of the 29 isolates (mean = 6.0 isolates). Among sympatric interactions, only one isolate (Plot 1) was found that did not induce any change in inhibition by any of the paired isolates from the same community. Instead, when responding to the presence of a partner, although 17 of the 30 isolates showed both increases and decreases in inhibition, two isolates never had their inhibition increased, 10 isolates never exhibited decreased inhibition and 1 isolate showed no significant shifts in inhibition in

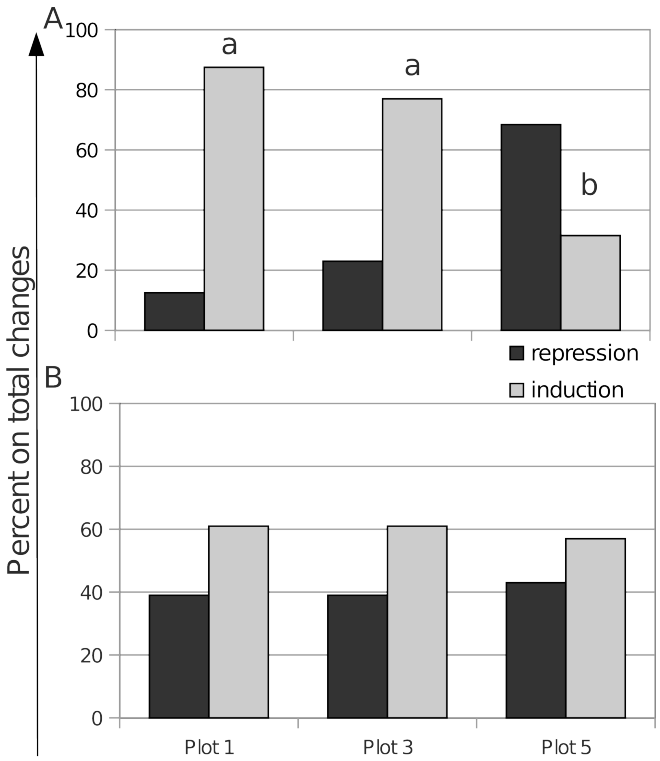

Figure 1.3: Inhibition may be increased or decreased in the presence of a partner in all pairwise interactions. Different letters indicate significant differences in proportions. (A) Percentage of increases/decreases of inhibition in each community (χ^2 $[2, n = 303] = 77.7$; $p = 0$). (B) Percentage of induction of increases/decreases of inhibition in each community (χ^2 $[2, n = 303] = 0.337$; $p > 0.8$).

response to a partner. Thus, it is likely that in natural communities the majority of isolates both alter their inhibition and have their inhibition altered by a neighbor.

1.3.1 Evidence for local selection

If inhibition mediates local interspecies interactions, then alterations in inhibition phenotypes are likely to shift fitness benefits for the isolates and be under local selection. To evaluate evidence for local selection for signaling interactions, the frequency of shifts in inhibition among sympatric and allopatric isolate pairs was compared. Sympatric

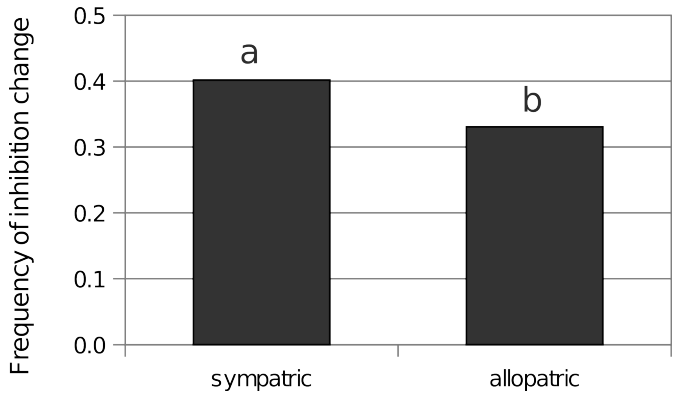

Figure 1.4: Frequency of shifts induced by sympatric and allopatric isolates (χ^2 $[1, n = 857] = 4.03$, $p = 0.045$). Different letters indicate significant differences in frequencies.

isolates were significantly more likely to exhibit changes in antibiotic inhibition in response to a partner than were allopatric isolates (Figure 2.4; χ^2 $[1, n = 857] = 4.03$, $p = 0.045$). Higher frequencies of signaling among sympatric than allopatric isolates suggest that there is local selection for the capacity to modulate antibiotic production in response to coexisting *Streptomyces* populations.

Further analyses considered the frequency and direction of changes in inhibitory phenotypes among sympatric isolates in different communities. Among the three communities there were substantial differences in the frequencies of within-community shifts in inhibition phenotypes. Specifically, significant shifts in inhibition were twice as frequent among isolates in plot 5 as in plot 1, and intermediate in plot 3 (signaling frequencies = 0.53, 0.40 and 0.27, respectively) (χ^2 $[2, n = 270] = 13.543$, $p = 0.0001$). The direction of changes in inhibition among sympatric pairs were similar to the changes observed in allopatric isolate pairs. Among all sympatric pairs inhibition was increased in 58.5% and decreased in 41.5% of signaling combinations ($n = 63$ and 43, respectively of 106 interactions), and in allopatric pairs inhibition shifts were 60.2% increases and 39.8% reductions ($n = 118$ and 78, respectively of 196 interactions).

When considered individually, the three communities varied in the proportions of sympatric enhancement and repression of inhibition (χ^2 $[2, n = 106] = 28.506$, $p < 0.0001$). Isolates from plots 1 and 3 tended to increase their inhibition in the presence of a partner (96 and 70% increases, respectively), while isolates from plot 5 tended to decrease inhibition in the presence of a partner (67% reduction). This suggests that interspecies interactions may have fundamentally different effects on inhibitory phenotypes in different communities.

1.3.2 Relationships among inhibitory and signaling phenotypes

We explored the relationships between shifts in inhibition induced by a partner and antagonism in sympatric isolate pairs. Isolates that had their inhibition enhanced by a partner were generally more inhibitory towards that partner. Specifically, *Streptomyces* isolates that showed increased inhibition of a *Bacillus* target in the presence of a partner produced on average, significantly larger inhibition zones against the partner than isolates that showed no increases in inhibition of the *Bacillus* target (mean zone size = 19.47, 13.89 and 13.90 mm for increased, reduced or unchanged inhibition; ANOVA, $F[2, 134] = 3.847$, $p = 0.0237$; Tukey's LSD, $p < 0.05$) (Figure 2.5). This suggests that in sympatric isolates signaling serves to increase the capacity of the receiving isolate to inhibit competitors.

1.3.3 Relationships among nutrient overlap and signaling

In this work, nutrient overlap is defined as a non-symmetrical measurement of how much one isolate influences the other. For example, isolate A may metabolize 45 different nutrients, isolate B may metabolize 90, and they share 15 nutrients. Niche overlap will be 33% for isolate A with isolate B but only 17% for isolate B with isolate A. The nutrient overlap of A with B is larger than the nutrient overlap of B with A, showing

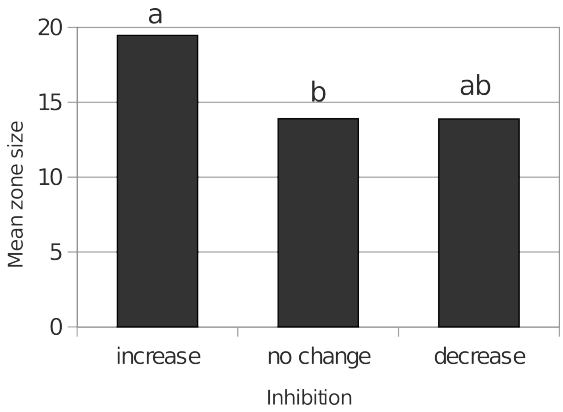

Figure 1.5: Mean inhibition zone sizes on sympatric partners that have inhibition increased, unchanged or decreased (ANOVA, $F[2, 134] = 3.847, p = 0.0237$; Tukey's LSD, $p < 0.05$). Different letters indicate significant differences in mean sizes.

that isolates need not be equally challenged by the presence of each other.

Induction of shifts in inhibition were related to nutrient overlap of the inducer with the responding isolate among sympatric but not allopatric isolate pairs. Among allopatric isolate pairs no significant differences were observed between mean nutrient overlap among isolates that significantly altered inhibitory activity in a partner and isolates that did not modify inhibition (ANOVA, $F[1, 598] = 0.08$, $p = 0.774$; Figure 2.6A). However, among sympatric pairs, isolates that altered inhibition by a partner (signalers) had a slightly smaller nutrient overlap with their partner than isolates that induced no changes (nutrient overlap = 0.809 and 0.845, respectively; ANOVA, $F[1, 268] = 3.613$, $p = 0.0584$; Figure 2.6C). Thus, isolates tended to alter inhibition phenotypes on other isolates that were weaker competitors for nutrients.

In contrast, *Streptomyces* were most responsive to isolates that were potentially more challenging competitors for nutrients. Specifically, among allopatric isolate pairs, isolates that responded to the presence of a partner by modifying inhibition of a target (recipients) had higher average nutrient overlap with their partners than isolates that

showed no significant change in inhibition (nutrient overlap = 0.844 and 0.805, respectively; ANOVA $F[1, 598] = 7.823$, $p = 0.0053$; Figure 2.6B). This difference was even greater in sympatric interactions (nutrient overlap with responsive isolates = 0.872, non-responsive = 0.801; ANOVA $F[1, 268] = 12.495$, $p = 0.0005$; Figure 2.6D). Thus, isolates are more likely to respond to the presence of a strong competitor for nutrients (Figure 2.7).

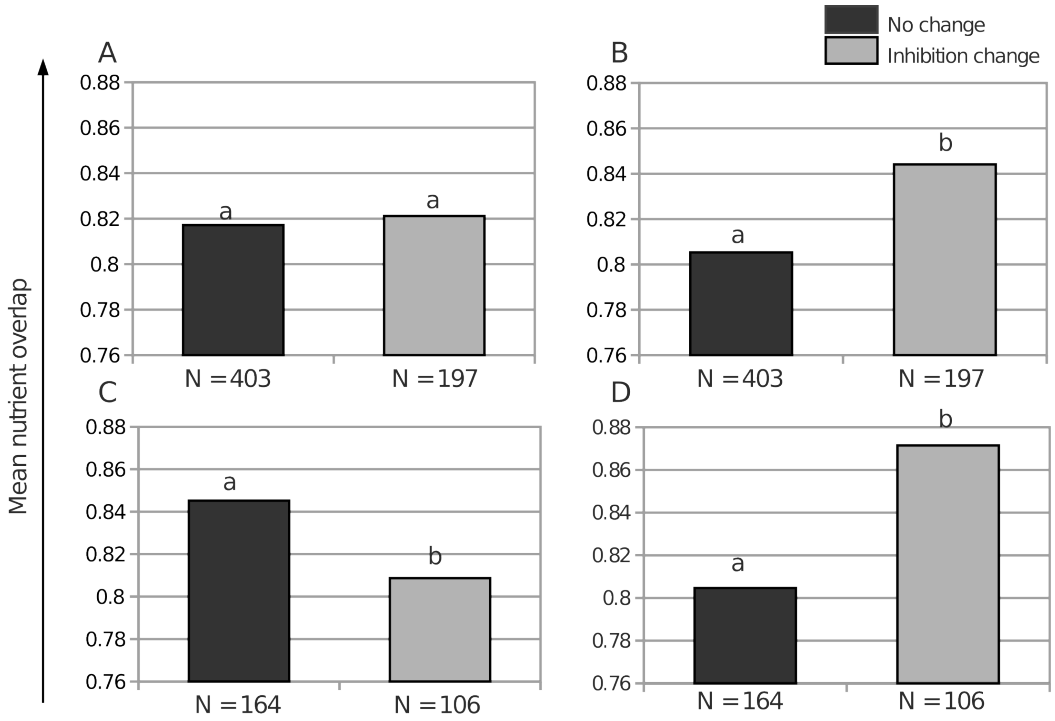

Figure 1.6: Nutrient overlap varies with frequency of shifts in inhibition. Different letters indicate significant differences in nutrient overlap. (A) Allopatric inductions (ANOVA, $F[1, 598] = 0.08$, $p = 0.774$). (B) Allopatric responses (ANOVA $F[1, 868] = 14.980$, $p = 0.0001$). (C) Sympatric inductions (ANOVA $F[1, 268] = 12.495$, $p = 0.0005$). (D) Sympatric responses (ANOVA $F[2, 267] = 9.865$, $p = 0.00007$).

Nutrient overlap of isolates with their partners was also a good predictor of the specific response in the presence of that partner. Thus, among both sympatric and allopatric isolate pairs, nutrient overlap with a partner was higher for isolates that

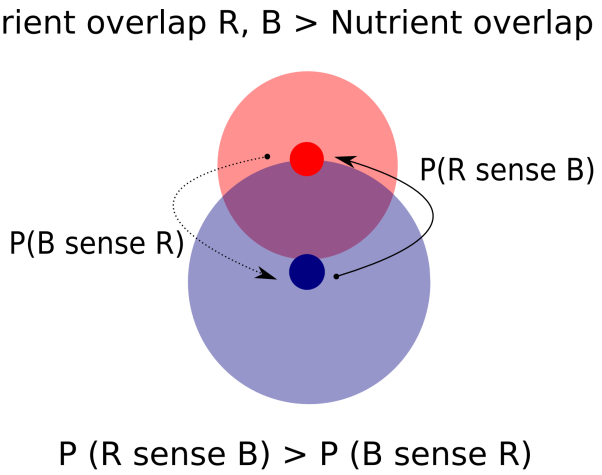

Figure 1.7: Schematic representation of the relationship between nutrient overlap and the proclivity of an isolate to respond or be sensed. Isolate Red has a smaller niche width, represented by a light red circle, therefore the potential competition with isolate Blue has a higher influence on its fitness, and vice versa. Isolates like Red are more likely to respond to the presence of isolate Blue than vice versa in both sympatric and allopatric interactions. In sympatric interactions, but not in allopatric interactions isolates like Red are less likely to induce changes in isolates like Blue (Blue is less likely to respond to Red).

increased their inhibition of a target in the presence of that partner as compared to isolates that decreased or did not change inhibition with their partners (n = 181, 122 and 567; mean nutrient overlap = 0.894, 0.787 and 0.8072, respectively; ANOVA, $F[2, 867] = 24.591$, $p < 0.00001$; Tukey's LSD < 0.05; Figure 2.8A). Similarly, among sympatric interactions, isolates that increased their inhibition had higher mean niche overlap with their partners (n = 62; mean nutrient overlap = 0.904) than isolates that either repressed or did not change inhibition of a target in the presence of a partner (n = 44 and 164; nutrient overlap = 0.826 and 0.805, respectively; ANOVA $F[2, 267] = 9.865$, $p = 0.00007$; Figure 2.8C).

Differences in mean nutrient overlap of isolates with their partners were observed between isolates that increased, decreased or did not change their inhibition in the presence of that partner in sympatric but not in allopatric interactions (Figures 2.8D

and 2.8B, respectively). In sympatric interactions, isolates that induced increases in inhibition towards a target isolate in their partners showed a significantly smaller mean niche overlap with their partners than isolates that did not modify inhibitory activity and their partners (mean nutrient overlap = 0.798 and 0.845, respectively; Tukey's LSD < 0.05). Isolates that induced decreases in inhibition in a partner did not differ from isolates that increased or did not change inhibition in their partners in their mean nutrient overlap with their partners (Figure 2.8D). Thus, in local interactions but not overall, isolates induced increases in inhibition in weaker competitors.

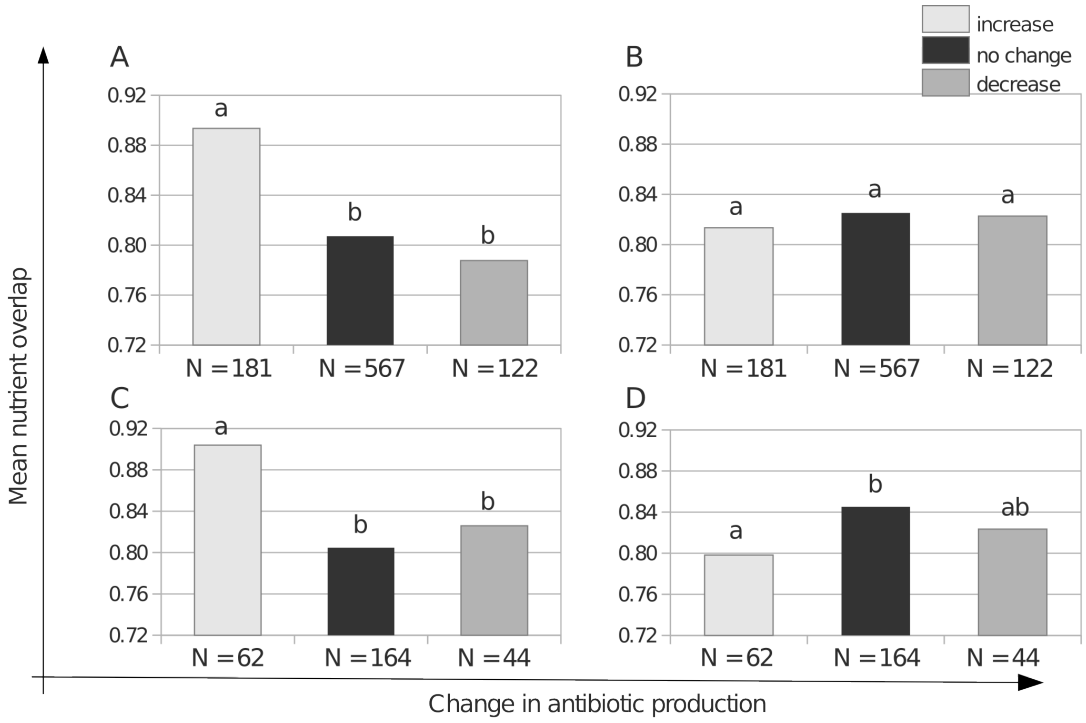

Figure 1.8: Nutrient overlap varies with the direction of change in inhibition. Different letters indicate significant differences in nutrient overlap. (A) Allopatric and sympatric responses (ANOVA, $F[2, 867] = 24.591$, $p = 4x10 - 11$; Tukey's LSD < 0.05). (B) Allopatric and sympatric inductions (ANOVA, $F[2, 867] = 0.3705$, $p = 0.691$). C: Sympatric responses (ANOVA $F[2, 267] = 9.865$, $p = 0.00007$). D: Sympatric inductions (ANOVA, $F[2, 267] = 2.151$, $p = 0.118$) Tukey's LSD < 0.05).

1.3.4 Relationships among genetic relatedness and signaling

Sympatric and allopatric isolate pairs in which at least one of the isolates modified inhibition in the partner were significantly less-closely related than isolate pairs in which inhibition was not altered in either partner ($n = 303$ and 565; mean genetic distances = 0.0380 and 0.0351, respectively; ANOVA $F[1, 868] = 6.334$, $p = 0.012$). Among only sympatric isolates, significant differences in mean genetic distance were also observed among inhibition-shifting and non-shifting isolate pairs ($n = 106$ and 164; mean genetic distances = 0.0356 and 0.0304, respectively; ANOVA $F[1, 268] = 5.395$; $p = 0.021$). Therefore, more distantly-related *Streptomyces* isolates were more likely to alter inhibitory phenotypes in one another than more closely-related isolates.

The type of responses of the isolates to the presence of a competitor also varied with genetic relatedness. Sympatric and allopatric isolate pairs in which inhibitory activity was enhanced in at least one of the isolates were significantly more closely related than isolates in which there were no shifts in inhibition (10% more than no signaling pairs). Isolate pairs in which at least one of the partners decreased its inhibition were the least closely related (12% less than no signaling pairs) (ANOVA, $F[2, 897] = 8.12$, $p = 0.0003$; Tukey's LSD, $p < 0.05$) (Figure 2.9). Among sympatric isolates, pairs in which at least one of the isolates decreased its inhibition in the presence of the other had significantly higher mean genetic distance than pairs in which one isolate showed increased or unchanged inhibition (mean genetic distance = 0.0403, 0.0323 and 0.0304, respectively; ANOVA $[2, 267] = 5.234$; $p = 0.006$). Thus, *Streptomyces* were not only more likely to respond to distantly-related isolates, but also they were more likely to show decreased inhibition by the presence of distantly-related isolates than more closely-related isolates.

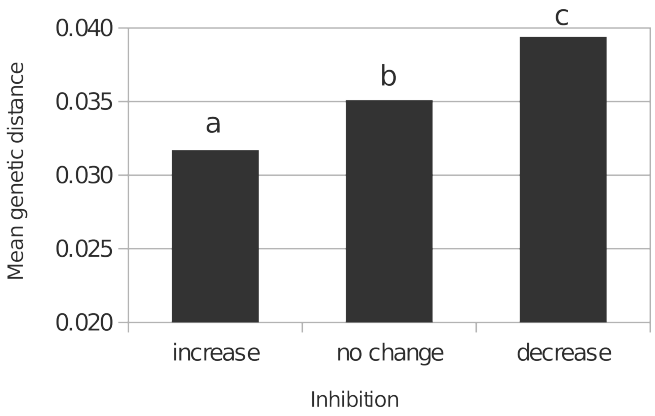

Figure 1.9: Genetic relatedness and direction of inhibition changes among sympatric and allopatric partners (ANOVA, $F[2, 897] = 8.12$, $p = 0.0003$; Tukey's LSD, $p < 0.05$).

1.4 Discussion

Species interactions have a significant impact on inhibition phenotypes in *Streptomyces* spp. A relatively high proportion of interactions (35%) among sympatric and allopatric *Streptomyces* led to significant changes in the capacity of individual isolates to inhibit a target. Previous work on interspecies signaling in bacteria has reported somewhat lower frequencies ($\approx$ 23%, Slattery *et al.*, 2001; 8%, Pierson *et al.*, 1998), but considered different targets. For example, Pierson *et al.* searched specifically for isolates that produced AHLs capable of restoring the production of the antibiotic phenazine in an AHL-deficient *Pseudomonas aureofasciens* mutant. In contrast, Slattery *et al.* looked for antibiotic increases and decreases in a single *Streptomyces tenjimarensis* isolate in response to a diverse collection of marine bacteria. Our worked focused on interactions among soil *Streptomyces* spp. from localized soil communities, and our data suggest a high frequency of signaling interactions are likely to occur in soil communities in natural conditions, with significant consequences for inhibitory phenotypes.

Given the strict regulation of antibiotic synthesis, and the number of factors that

influence their production, it is likely that the frequencies we present are an underestimation of the total interactions that could alter inhibition. Specifically, if our experiments had been carried out in other conditions (media, incubation times, temperature), it is likely that our results would be different. Because the differences we observed were consistent and statistically significant, it is unlikely that we have a considerable number of false positive cases of signaling. Instead, we cannot rule out that we have false negative cases of signaling, which would have been detected under different experimental conditions.

Our results show that interactions are highly specific among isolates. The effect of one isolate on inhibition by another was not consistent among isolates, so that the same isolate may decrease inhibition by some *Streptomyces* and increase inhibition by others. Similarly, the same *Streptomyces* may respond to one isolate by increasing inhibition while decreasing inhibition in response to a different isolate. This suggests that there are a diverse array of signaling molecules (Takano 2006, Lyon and Novick, 2004; Davies *et al.*, 2006) and receptors (Nishida *et al.*, 2007; Xu *et al.*, 2010; Wang *et al.*, 2011) that mediate complex interspecies interactions in natural communities.

One limitation of our work lies in the lack of understanding of the causes of the observed results. For example, increases in inhibition could be due to small molecules that directly increase antibiotic biosynthesis by the responding isolate, as has been observed among *Bacillus* (Stefanic and Manic-Mulec, 2009) and among *Pseudomonas savastanoi* pv. savastanoi in the induction of virulence factors (Hosni *et al.*, 2011). Alternatively, increased inhibition could reflect synergistic interactions among antibiotic molecules produced by the interacting isolates (Challis and Hopwood, 2003). Decreases in inhibition could similarly reflect several underlying causes. For example, an inducer may release small molecules that bind receptors in the responding isolate, thus downregulating antibiotic production. A homolog to GBL receptors (SCBR2) has been found in

S. coelicolor that represses GBL production (Wang *et al.*, 2011). Alternatively, the inducer of a change may release antibiotic-degrading enzymes, thus decreasing inhibition (Wright, 2005). A third explanation could be that reductions are mediated by quorum quenching, a process in which quorum sensing is repressed, which could in turn have several mechanisms. Quorum quenching may be accomplished by interference with the receptor proteins, as was observed by Ji *et al.* (1997) in *Staphylococcus* isolates, or by hydrolysis of signaling molecules (Dong *et al.*, 2007). Enzymes that degrade quorum-sensing molecules of gram-negative bacteria (acyl-homoserine lactones) are produced by a wide range of bacteria, including a *Streptomyces* sp. (Dong *et al.*, 2002). They are highly conserved and usually not very specific for one type of molecule, but rather cleave long or short acyl-chain molecules (Dong *et al.*, 2002, Chong *et al.*, 2012). Assuming that enzymes that degrade signal molecules from gram-positive bacteria behave in a similar fashion, if decreases in antibiotic inhibition were due mostly to quorum quenching, isolates from plot 5, for example, which had the greatest frequencies of reduction in antibiotic inhibition in response to a partner, could have signals that can be easily degraded or receptors that could be easily blocked by their partners. No specific mechanism for the observed changes in inhibition can be ruled out. However, regardless of the underlying cause, the outcome is that the antibiotic inhibitory phenotype is modified. It was not the intention of this work to determine the specific mechanisms by which inhibitory phenotypes are changed, but rather to document the frequency and effect of the interactions among *Streptomyces* spp. that have a significant influence on their behavior.

The higher frequency of sympatric than allopatric alterations in inhibition suggests local selection for phenotypes that modulate inhibition within communities. For selection to occur on these interactions, at least one of the interacting isolates must therefore

obtain a fitness benefit from the shift in inhibition, and our data suggest that both induction and response may confer a fitness benefit at times. Isolates have a tendency to increase their inhibition in the presence of highly challenging competitors, which could be beneficial to the responding isolate. It is thus likely that isolates are selected to respond to significant competitors by increasing antibiotic production. On the other hand, isolates are likely to not induce inhibition changes in neighbors that compete strongly for the nutrients they use. This anonymity could be beneficial to the non-inducing isolate, avoiding exposure to potentially damaging antibiotics. While the outcomes may seem contradictory, recall that niche overlap between any two isolates differs for each isolate. So, for example, A may have much greater niche overlap with B than B has with A. In this case A is likely to be selected to detect and respond to the presence of B increasing its inhibition. In contrast, A is less likely to induce antibiotic production in B. Such interactions are likely to generate diverse coevolutionary trajectories among coexisting isolates in which selection for antibiotic inhibition and resistance phenotypes interact with selection for signal emission or signal reception to produce complex coevolutionary dynamics among communities (Kinkel *et al.*, 2011).

Consistent with this prediction, communities varied widely in the frequency and pattern of increases and decreases in inhibition in response to others. It has been suggested that signaling among genetically diverse bacteria increase with both density and relatedness (Brown and Johnstone, 2001). This is in accordance with our results, in which the community from plot 1 had the lowest frequency of inhibition shifts and the highest mean relatedness. Nutrient availability is further likely to influence the necessity of individuals to compete through inhibitory compounds (Hibbing *et al.*, 2010). The community from plot 5 had a tendency to decrease inhibition, which was significantly different from the other two communities. This community had the lowest frequency

of antagonistic interactions and a low average nutrient overlap. In contrast, the community from plot 3 had the highest frequency of inhibitory interactions and average nutrient overlap, and isolates had a tendency to be increased in inhibition by others. These results may reflect different evolutionary trajectories of the communities, with community from plot 5 leaning toward niche displacement and the community from plot 3 engaging in a coevolutionary escalation (Kinkel *et al.*, 2011).

Isolates varied substantially in the proportion of increases and decreases in inhibition when responding to another isolate, but induced similar proportions of increases and decreases on other isolates. The smaller variation in the role of the isolates as the inducers of change and their marked differences in response frequency underscore the difference the potential benefits to each isolate of inducing others and responding to others. This result is in accordance with the work from Nishida *et al.*(2007), in which several receptor genes and only one GBL signal synthase gene were usually found within individual *Streptomyces* genomes.

Incentives for signaling vary between the presumptive signal producer and receiver. There are at least three possible fitness outcomes of increased inhibition of one isolate by the presence of a partner. In the first case, an isolate may sense the presence of an antibiotic sensitive competitor, for example through eavesdropping (Chandler *et al.*, 2012; Ji *et al.*, 1997), increase its antibiotic production, and thus increase the fitness of the antibiotic producing isolate. In within-community (sympatric) interactions, isolates that increased inhibition in the presence of a partner were also more effective at inhibiting that partner, suggesting that in most interactions in which the outcome is increased inhibition, the responding isolate may benefit from the interaction. This result is in agreement with the work of Chandler *et al.* (2012), which suggests that early eavesdropping on a susceptible competitor may increase the competitiveness of the antibiotic producer. As a second alternative, an isolate could be induced by an antibiotic

resistant neighbor. The increased antibiotic production by the responding isolate will likely inhibit other neighbors, while being harmless to the inducer. In this case the inducing isolate could take advantage of a shared good at the receiver's expense in what is considered chemical manipulation (Keller and Surette, 2006). In a third case, an isolate may receive a signal from a potentially synergistic neighbor (Hosni *et al.*, 2011; Challis and Hopwood, 2003), in which case both parts may benefit from the interaction.

When inhibition by an isolate is decreased by the presence of a partner, relative benefits to each isolate may also vary. If the "inducer" is sensitive to the antibiotics produced by the receiver, the decrease in inhibition could be a manipulation by the inducer to reduce its likelihood of being inhibited. Similar interactions have been observed in *Staphylococcus aureus* isolates that interfere with secretion of virulence factors in one another (Ji *et al.*, 1997). Conversely, if the inducer is not sensitive to the antibiotics produced by the inducer, the receiver may benefit from reduced investment in production of ineffective antibiotics. Overall, it is likely that conditions exist that select for enhanced responsiveness and signal production, contributing to complex interactions (Ji *et al.*,1997; Dulla and Lindlow, 2009).

Shifts in inhibition phenotypes are more likely in isolates that are exposed to challenging nutrient competitors. In contrast, within communities, isolates are less likely to promote shifts in inhibition, and particularly increases in inhibition, in strong competitors, suggesting that isolates may evolve to avoid notifying strong competitors of their presence. Nutrient competition is one of the forces driving interactions within soil microbial communities (Ghorbani *et al.*, 2008; Otto-Hanson *et al.*, 2013) and thus it is reasonable to expect that competition will shape inhibitory and signaling interactions among sympatric populations.

Closely-related *Streptomyces* had a tendency to increase inhibition in one another

but distantly-related *Streptomyces* tended to decrease inhibition in the other. Closely-related isolates tend to show more similar nutrient use phenotypes (Schlatter, personal communication), and are thus stronger competitors for nutrient resources. In addition, closely-related isolates are likely to share similar quorum-sensing molecules, which could be non-specifically recognized by one another.

We document a high frequency and diversity in interactions among *Streptomyces* spp., which add a significant layer of complexity to interspecies interactions. Interactions that shift inhibition are locally selected for and signaling phenotypes are coevolving among *Streptomyces* spp. within communities. Furthermore, there is a significant relationship between signaling frequencies and genetic relatedness, nutrient overlap and antagonism, which delineate a biological context for neighbor-induced shifts in inhibition. This work illustrates significant ways in which signaling among *Streptomyces* can influence interspecies interactions and suggests that a broad diversity of signals and receptors play fundamental roles in shaping microbial community dynamics.

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Chapter 2

Subinhibitory antibiotic concentrations mediate nutrient use and competition among soil *Streptomyces*

Recent studies document a role for antibiotics as signaling compounds rather than weapons, but shed little light on potential impacts of antibiotic signals on microbial species interactions. Such information is crucial for understanding the ecology and evolutionary biology of antibiotic-producing populations and identifying factors that influence selection for antibiotic inhibitory and resistance phenotypes. We evaluated effects of subinhibitory concentrations of antibiotics (SICA) on growth of *Streptomyces* isolates on 95 nutrients, and on niche overlap and escape from competition among isolate pairs. SICA-induced shifts in nutrient use resulted in increased competition-free growth for 33% of 360 isolate pair-antibiotic combinations, suggesting that SICA can mediate nutrient use and reduce competition among *Streptomyces*. Antibiotics varied in their effects on nutrient use, suggesting the potential for diverse antibiotic-mediated competitive and coevolutionary trajectories among coexisting populations in soil. We hypothesize that antibiotics that reduce competition at subinhibitory concentrations may enhance coevolutionary differentiation among competitors and minimize selection for antibiotic resistance.

One Sentence Summary At subinhibitory concentrations, antibiotics induce shifts in nutrient use that can increase competition-free growth and enhance niche differentiation among microbial competitors.

Antibiotic-producing bacteria are common in natural habitats, yet the roles of antibiotics in the ecology and evolutionary biology of soil populations remain poorly understood. While traditionally perceived as weapons mediating competitive interspecies interactions (Hibbing *et al.*, 2010), recent work emphasizes a role for antibiotics as signals (Miao and Davies, 2010; Yim *et al.*, 2006). It has been argued that antibiotics are rarely present at inhibitory concentrations in the environment, and thus that their role must be mediated primarily by whatever functions they accomplish at subinhibitory concentrations. However, despite significant evidence that subinhibitory concentrations of antibiotics (SICA) can alter bacterial gene expression in vitro, thereby acting as signals (Goh *et al.*, 2002; Bagge *et al.*, 2004; Subtr *et al.*, 2011; Cummins *et al.*, 2009; Linares *et al.*, 2006; Mesak and Davies, 2009), there is little experimental support from natural habitats for a predominant role for antibiotics as either signals or weapons. Our lack of understanding of the specific roles of antibiotics in natural populations constrains our abilities to identify habitats or selective conditions most likely to generate novel antibiotic phenotypes and to predict the dynamics of antibiotic resistance in environmental microbes.

As weapons, antibiotics are assumed to mediate antagonistic interactions in soil (Fischbach, 2009), and antibiotic-producing microbes have been suggested to exhibit coevolutionary arms race dynamics (Kinkel *et al.*, 2011). The arms race model predicts reciprocal accumulation of matching antibiotic inhibitory and resistance capacities in interacting populations over time, with ongoing selection for resistance in the presence of antibiotic-producers. In contrast, antibiotics as signals have been hypothesized to mediate neutral, or even cooperative species interactions (Linares *et al.*, 2009; Fajardo and Martinez, 2008; Romero *et al.*, 2011). If antibiotics represent signaling interactions, there may be little reason to expect ongoing selection for resistance, suggesting the

potential for fundamentally different coevolutionary dynamics. However, specific mechanisms by which antibiotic signals may facilitate non-antagonistic interactions remain predominantly hypothetical.

This work explores the effects of SICA on nutrient use, niche overlap, and escape from competition among a collection of antibiotic-producing *Streptomyces*. Because nutrient competition is a primary driver of species interactions in soil (Little *et al.*, 2008), this work considers the hypothesis that SICA play a significant role in mediating nutrient competition among soil *Streptomyces*. Recent work shows that antibiotic inhibition is greatest among sympatric *Streptomyces* that have large niche or nutrient use overlap (Schlatter, personal communication), supporting a role for antibiotics as weapons in nutrient competition. However, does the role of antibiotics in mediating species interactions change when antibiotics are present at subinhibitory concentrations?

Among antibiotic-producing microbes, the *Streptomyces* (Order Actinomycetales, Family Streptomycetaceae) are notable as prolific producers of antibiotics (Kieser *et al.*, 2000; Challis and Hopwood, 2003). *Streptomyces* are gram-positive, filamentous bacteria, excellent saprophytes, and ubiquitous in soil (Gontang *et al.*, 2007). Nine *Streptomyces* isolates having diverse nutrient use, phylogenetic, and resistance characteristics were selected for this study (Table 1.1). Five antibiotics varying in structure and mode of action and originating from *Streptomyces* (Kieser *et al.*, 2000) were evaluated (Table A.1). The minimum inhibitory concentration (MIC) of each antibiotic was determined for each *Streptomyces* isolate. The subinhibitory concentration of each antibiotic (SICA) was defined as 10% of the MIC for each antibiotic-isolate combination. For every isolate-antibiotic combination, nutrient use was evaluated in the presence of the specific SICA using Biolog SF-P2 plates containing 95 nutrients; control plates were non-amended. Every isolate-SICA combination was repeated on three replicate plates.

SICA had significant effects on nutrient use among *Streptomyces*. Shifts in nutrient

Table 2.1: Origin, total growth on all nutrients, niche width (number of nutrients used), and changes in niche width in the presence and absence of SICA. Values for total growth and niche width with SICA are the means for each isolate among all five antibiotic treatments. Changes in niche width are reported as percent change in total growth in the presence vs. the absence of each antibiotic. Significant differences in total growth or niche width in the presence and absence of antibiotics are indicated by (*) (t-test, $p < 0.05$). O.D.: optical density; N. nut.: number of nutrients; Chlor: chloramphenicol; Tet: tetracycline; Strep: streptomycin; Rif: rifampicin; Vanc: vancomycin.

ISOLATE ISOLATE	ORIGIN ORIGIN	TOTAL GROWTH (O.D.)		NICHE WIDTH (N. nut.)		CHANGES IN NICHE WIDTH WITH SICA (% change)				
		W/O SICA	WITH SICA	W/O SICA	WITH SICA	CHLOR	TET	STREP	RIF	VANC
1232-2	Minnesota, USA	6.86	3.30 (*)	81	41 (*)	-75.4 (*)	-37.7	-56.6 (*)	-62.3 (*)	-16.0
3211-5	Minnesota, USA	4.97	4.01	59	66	-16.5	12.5	19.9	8.0	37.5
5111-5	Minnesota, USA	3.41	1.74	41	37	-82.3 (*)	-33.1	17.7	45.1	0.8
Cev2-10	Cevennees, France	8.28	6.63	80	65 (*)	-20.0 (*)	-29.6 (*)	-13.3	-6.3	-24.2 (*)
Lub2-11b	Luberon, France	2.98	2.49	69	64	-17.4	1.0	-25.6	-15.9	24.2
Mont3-8	Montseny, Spain	10.87	6.85 (*)	70	67	-65.9	129.4	34.1	63.5	34.1
NZ816-12	New Zealand	0.89	1.18	28	39	-0.5	-11.4	-0.5	-8.6	-2.9
PanFS14	Fort Sherman, Panama	2.63	1.69	48	37	-14.5	-60.7	-46.9	-2.8	4.1
Witz25	Witzenhausen, Germany	4.84	3.48	50	45	-30.0	2.7	12.1	-2.0	-30.2

use in response to SICA were consistent among replicates for each isolate-antibiotic combination, but varied widely among isolates and antibiotics (Figure 1.1). Among isolates, total growth (growth summed over all nutrients) was significantly reduced in the presence of SICA in one-third of isolate-antibiotic combinations (Table 1.1; ANOVA; $p < 0.05$). Among antibiotics, chloramphenicol and tetracycline had the most consistent negative impacts on total growth. Despite the lack of evidence for negative impacts of SICA on growth on complex media, SICA had targeted impacts on growth on specific nutrients, including both increases and decreases.

Tetracycline, vancomycin and rifampicin induced the highest frequency of significant increases in growth on particular nutrients in the presence versus the absence of

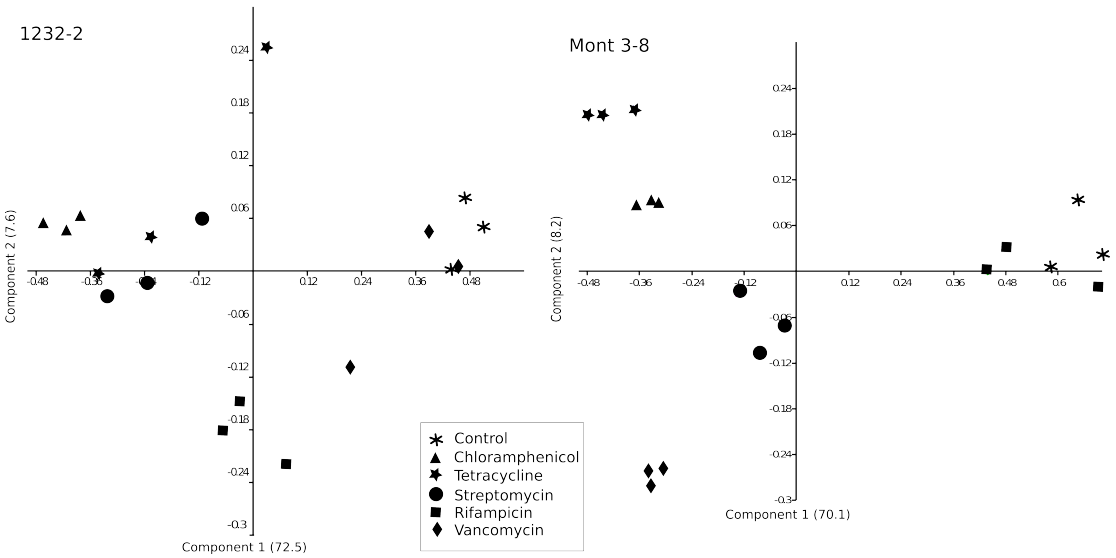

Figure 2.1: Principal components analysis of nutrient use profiles for *Streptomyces* isolates 1232-2 and Mont3-8 in the presence and absence of SICA. Growth was evaluated on Biolog SF-P2 plates; each treatment was replicated three times. The percent variation in nutrient use explained by each axis is shown in parentheses.

antibiotics, increasing growth in 2% (tetracycline) and 1.4% (vancomycin, rifampicin) of 855 nutrient-isolate combinations (data not shown). Considering both increases and decreases in growth, chloramphenicol had the greatest impact on nutrient use, shifting growth significantly on 22% of 95 nutrients on average among isolates. In contrast, rifampicin had relatively little effect on nutrient use, with significant impacts on use of 8.7% nutrients.

Different isolates varied in response to the same antibiotic. For example, streptomycin increased growth of isolate Cev2-10 on N-acetyl-D-glucosamine, but decreased growth of isolates 1232-2 and 5111-5 on the same nutrient. For some isolates, growth on particular nutrients was especially sensitive to antibiotics. For instance, growth of 1232-2 on putrescine was reduced significantly by subinhibitory concentrations of all antibiotics, but growth on putrescine was unchanged for all other isolates in the presence

vs. absence of SICA. Perhaps most surprisingly, some isolates grew on specific nutrients only when antibiotics were present. For example, Lub2-11b was able to grow on both N-acetyl-β-D-mannosamine and α-D-lactose in the presence of subinhibitory concentrations of vancomycin and tetracycline but not in the antibiotic-free control (t-test, $p < 0.05$).

Because SICA both eliminated growth on some nutrients and induced growth on others, SICA modified *Streptomyces* niche width, the total number of nutrients utilized by an isolate (Table 1.1). Niche widths of individual isolates were significantly reduced in the presence vs. absence of SICA in only seven isolate-antibiotic combinations (ANOVA; $p < 0.05$; Table 1.1). Six of these involved the same two isolates (1232-2 and Cev2-10) which appear to be especially sensitive to SICA.

Isolate-specific shifts in nutrient use and niche width suggest the potential for SICA to influence nutrient competition among *Streptomyces*. Consequently, we calculated niche overlap (NO) among all pairwise isolate combinations in the presence and absence of SICA. Antibiotics reduced NO relative to the control in 56% of the 360 possible combinations, with reductions ranging from 0.01% to 91% (mean = 28%). However, because total growth was sometimes concurrently reduced by SICA, reductions in NO may be of limited benefit to *Streptomyces* fitness overall. As an alternative for characterizing effects of SICA on nutrient competition, we calculated the total competition-free growth for each isolate, or cumulative growth for an isolate on all nutrients that were not utilized by a paired isolate, for all pairwise isolate combinations on each antibiotic (Figure 1.2). Competition-free growth provides a measure of escape from competition, and may be either increased or decreased by SICA. These increases or decreases can be indexed by an 'escape ratio', or the ratio of the total competition-free growth in the presence vs. the absence of SICA. Escape ratios greater than one characterize isolate-antibiotic combinations in which there are increases in total growth that are competition-free in

the presence of SICA. Escape ratios greater than one suggest SICA-isolate combinations in which isolates have undergone specific shifts in nutrient utilization in response to SICA that reduce the impacts of nutrient competition on total growth. In this case, SICA may act to enhance the fitness of interacting isolates.

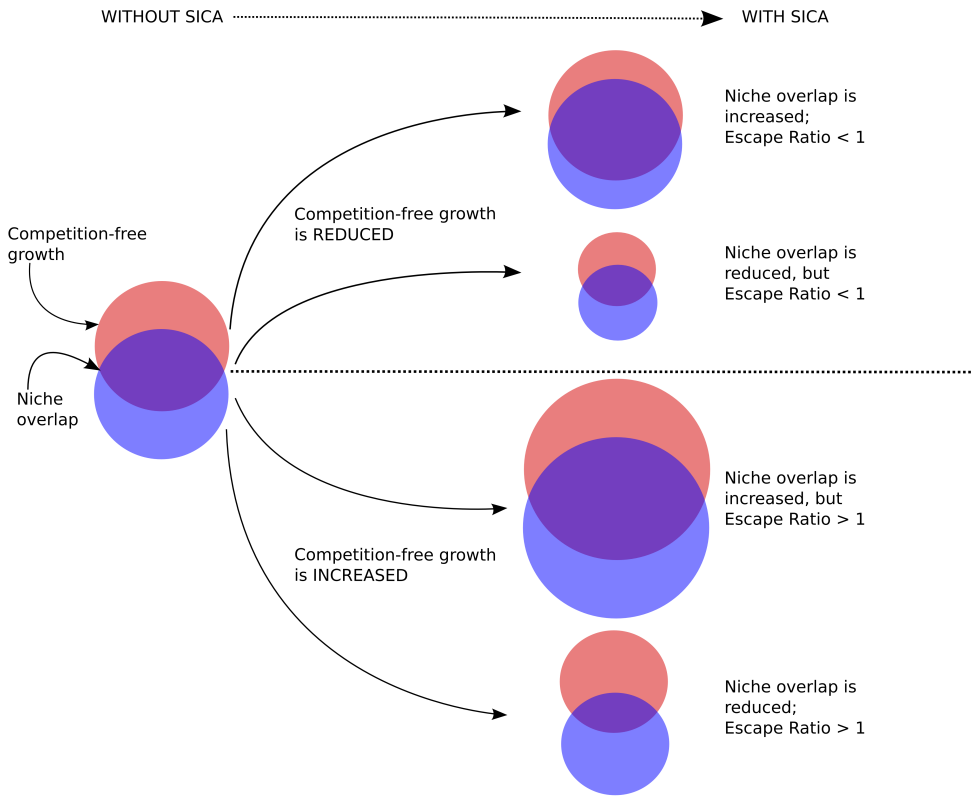

Figure 2.2: Characterizing escape ratios (ER) among competing microbes. The niche of each microbe is denoted by a shaded circle, which represents the cumulative growth of an isolate on the total suite of nutrients used by that isolate. Niche overlap is the area of intersection between the two circles, and quantifies the total growth for each isolate on nutrients used by both isolates. Competition-free growth is the non-intersecting area for each circle, or total growth for an isolate on nutrients that the competing isolate does not use. The escape ratio (ER) is the ratio of competition-free growth in the presence vs. the absence of SICA. When the escape ratio is greater than one, we hypothesize benefits to an isolate from the presence of SICA.

Among isolates, the frequency of escape ratios > 1 ranged from 5% to 68%. The

frequency of escape ratios > 1 also varied among antibiotics (Figure 1.3). While subinhibitory concentrations of chloramphenicol resulted in escape ratios > 1 in only 19% of isolate combinations, subinhibitory concentrations of vancomycin produced escape ratios > 1 in 43% of isolate combinations (Figure 1.3). This suggests that at subinhibitory concentrations, vancomycin commonly provides a potential fitness benefit to *Streptomyces* by reducing the significance of nutrient competition to growth. In contrast, chloramphenicol may reduce nutrient competition only rarely. Overall, streptomycin, rifampicin and vancomycin were significantly more likely (χ^2, $p < 0.05$) to enhance competition-free growth at subinhibitory concentrations than tetracycline or chloramphenicol.

The prospect that some antibiotics when present at subinhibitory concentrations can reduce the potential costs of nutrient competition to *Streptomyces* fitness has significant implications for the dynamics of antibiotic inhibitory and resistance phenotypes in soil. Specifically, if SICA can enhance fitness among coexisting competitors by reducing the costs of resource competition, there is likely to be little selection for resistance to that antibiotic. Thus, we hypothesize that there should be relatively less resistance in naturally-occurring *Streptomyces* populations to vancomycin, streptomycin and rifampicin, which reduce the influence of nutrient competition on growth in a high percentage of isolate combinations (Figure 1.3), than to chloramphenicol and tetracycline. Recent data (D'Costa *et al.*, 2006) support this hypothesis. Within a global collection of naturally-occurring *Streptomyces* isolates, high frequencies of resistance to chloramphenicol and tetracycline were found ($\approx 25\%$ and 60%), while frequencies of resistance to vancomycin, rifampicin, and streptomycin, were extremely low ($< 5\%$) (Figure 1.3). The high frequencies of resistance and correspondingly low frequencies of escape ratios > 1 are consistent with the hypothesis that chloramphenicol and tetracycline act commonly as weapons to mediate antagonistic species interactions in natural habitats.

In contrast, the limited frequencies of resistance in natural populations and the high frequencies of increased competition-free growth for *Streptomyces* in the presence of subinihibitory concentrations of vancomycin, streptomycin and rifampicin suggest that these antibiotics may act predominantly as signals to mediate neutral or beneficial species interactions in the environment.

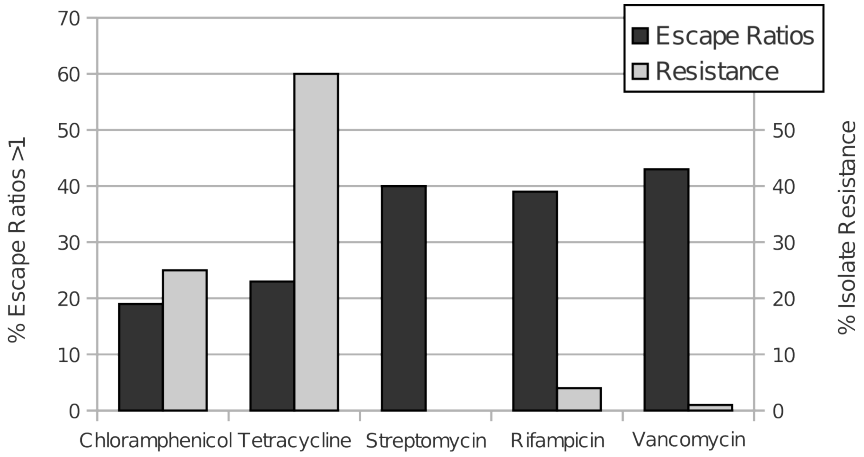

Figure 2.3: Frequencies of escape ratios greater than one among all possible isolate pair-antibiotic combinations ($n = 360$) and frequencies of resistance to the same antibiotics among a global collection of naturally-occurring soil *Streptomyces* (18).

Accumulating evidence suggests that antibiotics play diverse roles in natural habitats (Dietrich *et al.*, 2008; Price-Whelan *et al.*, 2006; Aminov, 2009; Shank and Kolter, 2009; Watve *et al.*, 2001). Understanding the specific roles played by different antibiotics and how they vary among ecological settings has significant implications for understanding the dynamics of selection for antibiotic inhibitory and resistance phenotypes. Our data lend support to the concept of hormesis (Davies, 2006), and specifically that the roles of antibiotics in mediating species interactions vary significantly depending upon whether the antibiotic is present at high vs. low concentrations in the environment. Biosynthetic pathways for antibiotic production are tightly regulated (Bibb, 2005; Stratigopoulos *et*

al., 2004), often by the presence of signaling molecules–including SICA. While a simple arms race model suggests that antibiotics as weapons will impose strong selection for resistance in target populations, this work suggests that smaller concentrations of antibiotics, or antibiotics as signals, may induce a distinct coevolutionary pathway. If SICA induce shifts in nutrient use, they may enhance the likelihood of niche differentiation as a viable coevolutionary trajectory for interacting species, rather than an antibiotic arms race. This may contribute to the ability of *Streptomyces* to produce a diverse array of highly-regulated antibiotics that serve distinct roles–sometimes weapons, sometimes signals– in interactions with different species, and whose production levels vary depending upon the habitat and coexisting populations. Rather than a dichotomy between weapon or signal, antibiotics appear to play a central role in mediating complex interactions within microbial communities, and the specific roles of a single antibiotic may vary across the landscape. Developing a more nuanced understanding of antibiotic-mediated species interactions in natural populations will help us effectively search for novel antibiotics, manage natural habitats to enhance inhibitory or resistant phenotypes, and develop predictive models for antibiotic-mediated coevolutionary dynamics in microbial populations.

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Chapter 3

Cropping history effects on pathogen suppressive activity and shifts in inhibition in *Streptomyces* communities

Diseases remain a yield-limiting factor for crops in spite of the availability of several control measures. Indigenous soil microorganisms naturally protect plants from pathogens in soils through various mechanisms. Soil bacterial communities are influenced by biotic and abiotic factors and agronomic practices attempting to promote pathogen-suppressive communities have been investigated. *Streptomyces* are associated with suppression of several diseases, mostly due to antibiotic production. However, pathogen-suppressive activity of *Streptomyces* communities may depend on bacterial density of the communities and coevolution of inhibitory phenotypes driven by competition among indigenous bacteria. We sought to find a relationship between bacterial density and pathogen-suppressive activity and evaluated bacterial,*Streptomyces* and inhibitory densities in soils from a crop rotation experiment. Inhibition shifts in pairwise interactions among *Streptomyces* were tested in three communities. Soils from longer rotations had larger microbial densities and were more inhibitory than soils from shorter rotations. In addition, inhibitory activity was shifted more frequently among isolates from denser populations. Our work suggests that practices that increase total bacterial densities will increase pathogen suppression. Signaling should be considered when evaluating the inhibitory potential of a *Streptomyces* community.

3.1 Introduction

Diseases of crop plants are a significant yield-limiting factor. Many organisms may harm crop production, ranging from viroids to bacteria, fungi, nematodes, insects, and more (Gordon and Leveau, 2010). There are diverse control measures to limit the spread and damage of crop diseases, including the use of crop rotations, development of resistant varieties, and pesticides (Panuwet *et al.*, 2012; Lew *et al.*, 2012). However, despite these options, disease management remains a persistent challenge.

Antagonistic soil microbes have the potential to protect plants from plant pathogens, and have been used as inoculants to control several diseases (Perneel *et al.*, 2008; Xiao *et al.*, 2002; Lumsden and Knauss, 2007; Janmaat, 2007). Suppressive soils offer another alternative for managing soil-borne pathogens. Suppressive soils are soils in which a pathogen may persist, but either causes little or no damage to crops. Naturally suppressive soils have been reported in many systems (Cook, 2007; Landa *et al.*, 2002; Keel *et al.*, 1996; Liu *et al.*, 1995). Disease suppression in such soils is usually associated with the presence of microorganisms that limit the survival, growth or infection of plant pathogens (Mendes *et al.*, 2011; Kinkel *et al.*, 2011, 2012). The mechanisms through which disease is reduced in suppressive soils are varied and include triggering of induced systemic resistance in the host plant (van Loon *et al.*, 1998; Mandeel and Baker, 1991; Larking *et al.*, 1996), niche competition (Cugudda and Garibaldi, 1981; Larkin *et al.*, 1996), or antibiotics (Keel *et al.*, 1992; 1996) and enzyme production (Hjort *et al.*, 2010).

Bacterial community composition, richness and diversity in soil are influenced by biotic and abiotic factors (Salles *et al.*, 2004; Shange *et al.*, 2012; Schmalenberger and Tebbe, 2002; Andrew *et al.*, 2012). Soil type (Araujo da Silva *et al.*, 2003; Lauber *et al.*, 2008; Birkhofer *et al.*, 2012), nutrient availability (Schlatter *et al.*, 2009; Veresoglou *et al.*, 2012), plant host (Bakker *et al.*, 2010) and management practices (Larkin *et al.*, 2010; Azziz *et al.*, 2011; Ceja-Navarro *et al.*, 2010) can all significantly influence

bacterial communities. However, although agronomic practices that attempt to favor pathogen-suppressive microbial populations have been widely investigated (Wiggins and Kinkel, 2005; Mazzola 2007; Pérez *et al.*, 2007; Sakuma *et al.*, 2011; Tamm *et al.*, 2010; Ryan *et al.*, 2009), relatively little effort has been made to systematically characterize effects of cultural practices on natural disease suppression potential of soil microbes.

Streptomyces is a genus of over 500 species that are ubiquitous in soil (Kinkel *et al.*, 2012). Organisms of the class Actinobacteria, to which *Streptomyces* belong, have been shown to shift with management practices, but most importantly, between some suppressive and conducive soils (Becker and Kinkel, 1997; Millard and Taylor, 1927; Mendes *et al.*, 2011; Kinkel *et al.*, 2012; Rosenzweig *et al.*, 2012). Organisms of this genus are prolific antibiotic producers, which is hypothesized to be the primary basis for their suppressive activity in soil (Kinkel *et al.*, 2012; Wiggins and Kinkel, 2005). The density of inhibiting *Streptomyces* and the intensity of their inhibition of pathogens was found to be higher in soils with better suppression of *Phytophtora* root rot of alfalfa (Wiggins and Kinkel, (2005).

It has been proposed that pathogen suppression by *Streptomyces* is an outcome of coevolution among competing soil bacteria (Kinkel *et al.*, 2011). Competition is likely to increase with bacterial density, and thus it is intuitive that soils with high bacterial densities may be more suppressive to pathogens.

In this work, we sought to explore the effects of cropping history on pathogen suppression characteristics of soil microbial communities. We evaluated bacterial and *Streptomyces* densities, pathogen suppressive activity and edaphic characteristics of soils with different cropping histories from a no-till rotation experiment established in 1999 in Paysandú, Uruguay. We selected three distinct plots to analyze signaling that shifts inhibition in pairwise interactions. *Streptomyces* were isolated from each of the three samples and signaling within communities was evaluated.

3.2 Materials and Methods

3.2.1 Soil sampling

Samples were collected from a no-till rotation experiment located at "Potrero número 36" (32° 21' 58" S; 58° 03' 56" O) at the Dr. Mario A. Cassinoni experimental station (http://www.eemac.edu.uy/sobre-la-eemac/ubicacion), in Paysandú, Uruguay. The experiment was established in 1999 and has 10 treatments in four crop sequences with varying rotation lengths. In each sowing season all crops of all rotations are planted, so that there is one treatment for the 1-year rotation, two treatments for the 2-year rotation, three treatments for the 3-year rotation and four treatments for the 4-year rotation (Table 3.1). Each of the 10 treatments are replicated in three blocks. The plots have not received any fungicide treatment since the beginning of the experiment and had been fertilized with 18-46 kg/ha of N (depending on the year) and 46 kg/ha of P twice a year. Samples were collected in March 2012, at the end of the southern summer, while the summer crops were still standing. Ten soil cores 2.5×10 cm were collected from five randomly-selected locations within the central rows of each plot. Soil cores were pooled for further analyses. In addition, small cores (1 x 10 cm) were collected at three of the five locations within each plot; these cores were kept separate for *Streptomyces* isolation and signaling assays.

Samples from non-cropped soils were collected in a similar fashion from a nearby non-cultivated woodland (treatment 11) and an non-cultivated prairie next to the cropped plots (treatment 12). All soil samples were kept at 4°C until processed.

Soil samples were tested for edaphic characteristics using standard methods at the University of Minnesota Soil Testing Labs (http://soiltest.cfans.umn.edu/).

Table 3.1: Description of treatments. Four rotations were implemented in three randomized blocks. All crops were planted at each time, making 2 treatments for the 2-year rotation, one with each of the summer crops, 3 treatments with the 3-year rotations, and 4 treatments with the 4-year rotations, respectively. Crops in bold were planted at the treatments with the corresponding numbers. Non-cropped soils were collected from below the fence (prairie) and from a nearby woodland.(*)*Lotus corniculatus*

TREATMENT	ROTATION	CROP/PLANT HOST
1	1-year	Wheat, **soybean**, wheat
2, 3	2-year	Barley, **sunflower**, wheat, **soybean**
4, 5, 6	3-year	Barley, **sorghum**, oat, **maize**, wheat, **soybean**
7, 8, 9, 10	4-year	Barley, **sunflower**, oat/**lotus***, **lotus***, wheat, **soybean**
11	-	Natural (woodland)
12	-	Natural (prairie)

3.2.2 Characterizing inhibition activity and microbial densities

Inhibitory activity in each community was evaluated following a modified version of Herr's assay (Pérez *et al.*, 2007). Briefly, from each sample, approximately 5 g soil were suspended in 50 mL deionized sterile water and agitated at 175 rpm for 1 hour at 4°C. Serial dilutions were made in deionized sterile water and 100 μL were plated onto petri dishes containing 15 mL starch-casein-agar (SCA). Plates were subsequently overlaid with a thin layer (5 mL) of SCA, and incubated at 28°C. After 3 days, bacteria and *Streptomyces* colonies were counted. Next, 10 mL of SCA were added to each plate and, after drying, one of two target *Streptomyces* (4-21 or 1324-2; Davelos *et al.*, 2004c) were spread from a concentrated spore suspension ($\approx 5\times10^8$ spores/ml). The two target isolates had been shown previously to have different sensitivity to antibiotics (Davelos *et al.*, 2004c), and inhibition to these targets was indicative of pathogen suppressive activity (Wiggins and Kinkel, 2005). Plates were incubated at 28°C for 3 days, at which time numbers of inhibitory *Streptomyces* were determined. For every inhibitor, the average of two perpendicular measurements of each inhibition zone was used to index inhibition. Inhibition towards each target was replicated three times per dilution, so for every plot there were three replicates for inhibition of each target and six replicates for

bacterial and *Streptomcyes* density determinations.

3.2.3 Evaluation of *Streptomyces* signaling interactions

Three plots were selected to isolate *Streptomyces* for signaling assays. Plots were selected based on their characteristics of bacterial, *Streptomyces* and inhibitors densities, and average inhibition zone size. One community had high microbial density and inhibition frequency, one low and one was intermediate. From each of these plots, ten isolates from a single small soil core were randomly selected. In total, thirty *Streptomyces* isolates from the three communities were evaluated. We define signaling as events in which one *Streptomyces* isolate modifies the inhibition phenotype of another isolate. Interactions were evaluated among all pairwise isolate combinations within each community ($n = 10\,\text{isolates} \times 9\,\text{partners} \times 3\,\text{communities} = 270\,\text{interactions}$).

For *Streptomyces* isolation, soil samples were suspended and diluted in sterile deionized water, plated on oatmeal agar (20 g/L oatmeal, 1 g/L casamino acids and 15 g/L agar) and incubated at 28°C for 3–7 days. Colonies displaying characteristic *Streptomyces* morphology were randomly selected and re-isolated. All isolates were kept as spore suspensions in 20% glycerol at −20°C and −80°C. In order to observe a change in inhibition, targets in which to measure inhibition changes were selected to be sensitive to each of the test isolates. *Streptomyces* targets were selected among the 30 evaluated isolates. Cases in which test isolates did not inhibit the target in control conditions could arise from two situations: a) the test pair was tested more than once due to the need to use different targets for each isolate, or b) no suitable target (an isolate with sensitivity to the test isolate when growing alone) could be found to the test isolate.

Assays were carried out as described previously (Vaz and Kinkel, 2012), with some modifications. Briefly, *Streptomyces* isolates were inoculated in pairs 1 cm apart on

plates containing 15 ml of ISP2 medium (Shirling and Gottlieb, 1966), with four replicates of each pair per plate. Control plates were inoculated with each isolate individually, with four replicates per plate. Isolates were inoculated as 4 μl drops from spore suspensions to a final number of $\approx 5\times10^7$ spores and plates were incubated at 28°C. After 3 days, 10 mL of ISP2 were overlaid on top of the colonies and were allowed to dry. After drying, the corresponding target *Streptomyces* was spread from a concentrated spore suspension ($\approx 5\times10^8$ spores/ml) and the plates were incubated at 28°C. After 3 days, inhibition zones were measured on the target *Streptomyces* lawns. Two perpendicular measurements were made per zone from the edge of the test *Streptomyces* colony to the end of the inhibition zone, away from the paired isolate, and their average was used for statistical analyses. Inhibition zones of paired *Streptomyces* were compared with zones generated by each test *Streptomyces* isolate grown on ISP2 plates alone (controls). The effect of a paired isolate was evaluated by testing the significance and direction (increase or decrease of inhibition) of differences between the inhibition zones of isolates in the presence and the absence of a paired isolate.

3.2.4 Analyses

Correlation analyses were carried out with Matlab Statistics Toolbox (MATLAB version 7.8.0. Natick, MA: The MathWorks Inc, 2009). In inhibition and signaling assays, significant differences in inhibition (1-way ANOVAS and Tukey's Least Significant Differences) were calculated using Matlab Statistics Toolbox. Differences in frequency of overall signaling and frequency of increases or decreases of inhibition were analyzed using the χ^2 test (http://www.quantpsy.org/chisq/chisq.htm).

3.3 Results

Bacterial densities varied significantly among soils with different cropping histories, differing up to two-fold among soils (Figure 3.1A; ANOVA, $F[11, 204] = 6.748$, $p < 0.00001$; Tukey's LSD < 0.05). Bacterial population densities also differed significantly between the two non-cropped soils tested (treatments 11 and 12). Bacterial densities also varied significantly depending upon the most recent plant host (Figure 3.1B). Plots planted most recently with maize harbored significantly higher bacterial densities than plots with any other plants. Plots planted most recently with soybean or sorghum had bacterial densities comparable to those in non-cropped prairie and woodland soils, and soils planted with soybean had significantly lower densities of bacteria than plots with sunflower or lotus (ANOVA, $F[5, 210] = 4.283$, $p < 0.001$; Tukey's LSD < 0.05).

Bacterial density in soil also varied significantly with rotation period (Figure 3.1C; ANOVA, $F[4, 211] = 7.305, p < 0.00002$; Tukey's LSD < 0.05). In general, soils from longer rotations harbored higher bacterial densities than soils from shorter rotations, and non-cropped soils had intermediate bacterial densities. Agricultural soils were also characterized based on the number of different plant species grown during the preceding 4-years. Rotations of 3- and 4-years length had the same number of species during the 4-year period (see Table 3.1). Soils cropped to a different number of plant species in each 4-year period had highly significant differences in bacterial densities (Figure 3.1D; ANOVA $F[2, 117] = 16.677, p < 0.00001$; Tukey's LSD < 0.05). Specifically, bacterial densities increased with higher host diversity; bacterial densities were significantly greater in plots planted to six than to two plant species in the previous 4 years.

Soils with different cropping histories also varied significantly in *Streptomyces* density (Figure 3.2A; ANOVA, $F[11, 204] = 2.998, p = 0.001$) and *Streptomyces* densities were positively correlated with bacterial densities among plots (Pearson's $r = 0.686, p <$

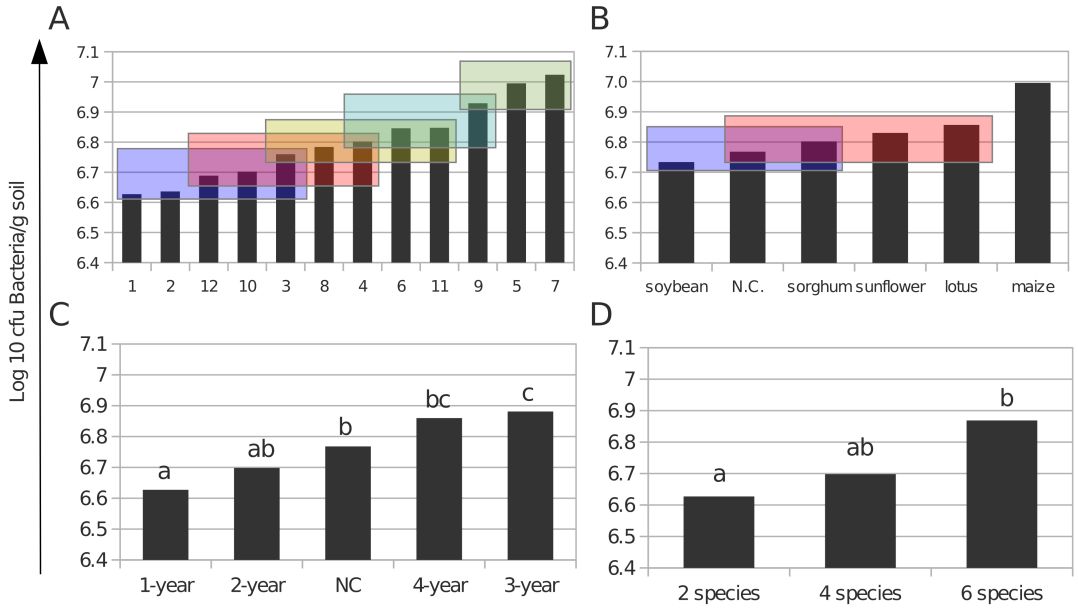

Figure 3.1: Bacterial densities varied among (A) treatments (ANOVA, $p < 0.000001$; Tukey's LSD < 0.05), (B) plant hosts (ANOVA $p < 0.0001$; Tukey's LSD < 0.05), (C) rotation periods (ANOVA, $p < 0.00001$; Tukey's LSD < 0.05) and (D) the number of species planted over the 4-year period (ANOVA $p < 0.00002$; Tukey's LSD < 0.05). In A and B samples within each rectangle are not significantly different from each other. In C and D different letters indicate significant differences. N.C. = Non-Cropped.

0.0001). However, in contrast with total bacterial populations, *Streptomyces* densities did not differ significantly among soils planted with different hosts, though soils planted most recently with lotus had lower *Streptomyces* densities than soils planted with sorghum.

Consistent with total bacterial populations, *Streptomyces* densities in soil also varied significantly among rotations (Figure 3.2C; ANOVA, $F[4, 211] = 3.614, p = 0.0071$; Tukey's LSD < 0.05). Soils from the 2-year rotations had significantly lower *Streptomyces* densities than soils from all other treatments, including non-cropped soils. Finally, in contrast to bacterial populations, *Streptomyces* populations did not increase

consistently with the number of plant hosts to which they are planted in each 4-year period (Figure 3.2D; ANOVA, $F[2, 177] = 6.582, p = 0.0017$). Soils planted to four species in the preceding 4-year period (which corresponds to the 2-year rotation) had a significantly lower streptomcyetes density (Tukey's LSD < 0.05) than soils planted to two (1-year rotation) or six species (3- and 4-year rotation).

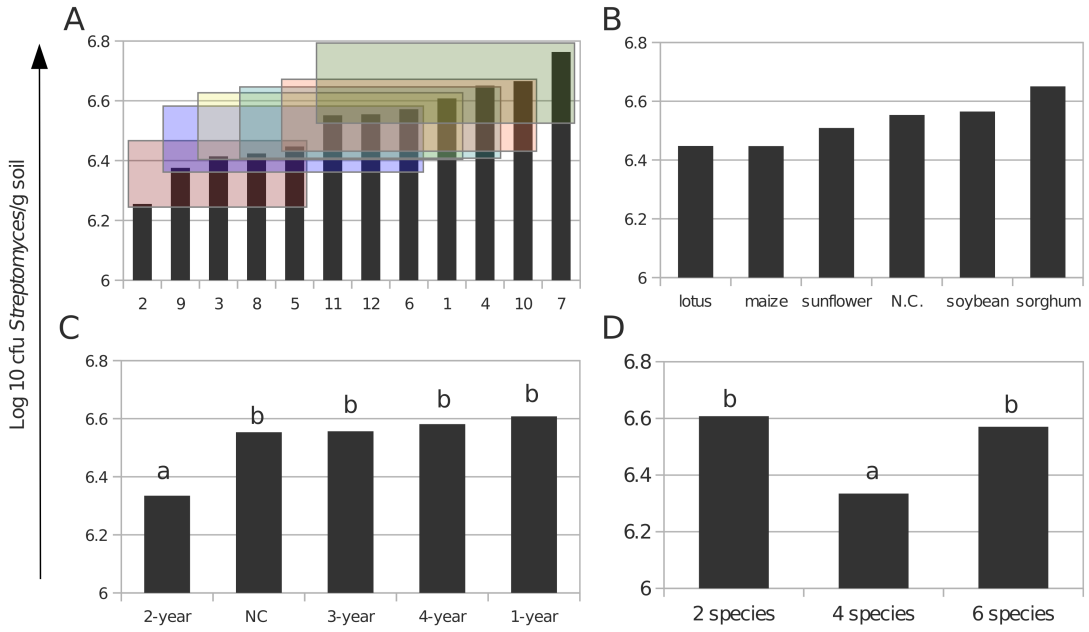

Figure 3.2: *Streptomyces* densities varied among (A) treatments (ANOVA, $F[11, 204] = 2.998, p = 0.001$; Tukey's LSD < 0.05), (B) plant hosts (ANOVA, $F(5, 210) = 1.203, p = 0.308$), (C) rotation periods (ANOVA, $F[4, 211] = 3.614, p = 0.0071$; Tukey's LSD < 0.05) and (D) the number of species planted over the 4-year period (D; ANOVA, $F[2, 177] = 6.582, p = 0.0017$; Tukey's LSD < 0.05). Treatments within each rectangle are not significantly different from each other. N.C. = Non-Cropped.

Pathogen suppression was evaluated in all soils as the density of *Streptomyces* inhibitory against two targets and the intensity of inhibition (size of the inhibition zones) against each target. The density of inhibitors against the two targets was positively correlated among soils (Spearman's $\rho = 0.4085, p = 0.0148$). Thus, soil communities that were good at inhibiting one target tended to be good at inhibiting the other target.

The density of inhibitors and average size of inhibition zones were significantly positively correlated among soils for target 4-21 (Pearson's $r = 0.3928$, $p = 0.0196$), but not for target 1324-2. Thus, in soils with higher densities of inhibitory *Streptomyces* against 4-21, inhibitors are more effective than in soils with lower densities of inhibitors. In addition, the mean zone size on target 1324-2 was positively correlated with bacterial density (Pearson's $r = 0.4323$, $p = 0.0095$), suggesting that soils with higher bacterial densities may have higher pathogen suppression.

Average density of inhibitors of the two targets was significantly different among soils with different cropping histories (Figure 3.3A ANOVA, $F[11, 180] = 2.355p = 0.0098$; Tukey's LSD $p < 0.05$). Density of inhibitors varied among soils with different plant host, though differences were not significant (Figure 3.3B; ANOVA, $F[5, 183] = 1.724, p = 0.1312$).

The density of inhibitors varied among rotations of varying length, though differences were not statistically significant (Figure 3.3C; ANOVA: $F[4, 187] = 2.1196$, $p = 0.08$). Pairwise comparisons among rotations (Tukey's LSD) showed the 3- and 4-year rotations had significantly higher inhibitors densities than the non-cropped soils. This situation contrasts with the density of total bacteria, which in non-cropped soils was intermediate. Soils in 1- and 2-year rotations had intermediate densities of inhibitors and were not significantly different from the others. Numbers of plant species in the preceding 4 years did not significantly influence inhibitor density (Figure 3.3D; ANOVA, $F(2, 162) = 1.2887$, $p = 0.2784$). However, when only inhibitors of *Streptomyces* target 4-21 were considered, soils planted with six different crop species in each 4-year period had significantly higher densities of inhibitors than soils planted to two species (ANOVA, $F[2, 76] = 2.456$, $p = 0.0366$; Tukey's LSD < 0.05).

Mean zone sizes of inhibition did not vary significantly among soils from different plant hosts, rotations or number of plant species planted in the previous 4 years (ANOVA

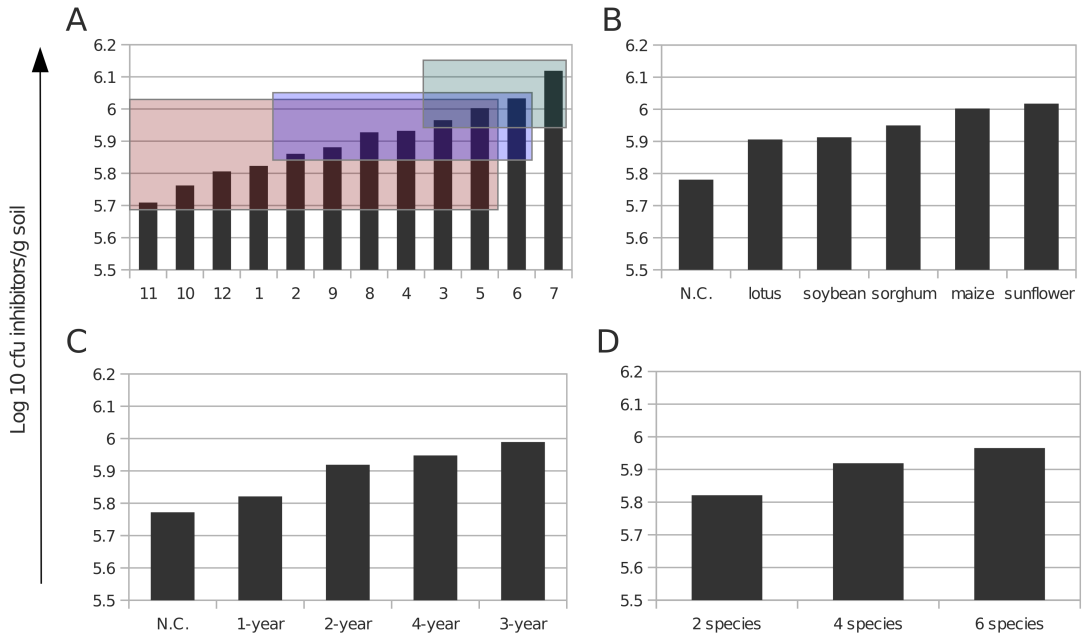

Figure 3.3: Average density of inhibitors of both targets varied among (A) treatments (ANOVA, $F[11, 180] = 2.355p = 0.0098$; Tukey's LSD $p < 0.05$) (B) plant hosts (ANOVA, $F[5, 183] = 1.724, p = 0.1312$),(C) rotations (ANOVA, $F[4, 187] = 2.1196, p = 0.08$) and (D) number of plant species over the 4-year period(ANOVA, $F[2, 162] = 1.2887, p = 0.2784$). N.C. = Non-Cropped.

$F[11, 136] = 1.671$, $p = 0.0862$; $F[5, 143] = 1.340$, $p = 0.251$; $F[4, 143] = 1.358$, $p = 0.251$, respectively for target 4-21). Differences in mean zone sizes on target 1324-2 among different soils were smaller than for target 4-21.

The soils sampled had a wide range of nitrate, organic matter, phosphorous and potassium concentrations and pH (Table 3.2). Nitrate content varied among soils with different cropping histories (ANOVA, $F[11, 23] = 16.65, p < 0.00001$), with nitrate content in non-cropped woodland soils being almost three times higher than in soils from 1-, 2- and 3-year rotations (Table 3.2). Differences in nitrate content among

soils cropped to different plant hosts or subjected to different rotations were not significant (ANOVA, $F[4, 25] = 2.474, p = 0.0702$ and $F[3, 26] = 2.475, p = 0.0838$, respectively). In addition, nitrate content in soil was positively correlated with *Streptomyces* and bacterial densities (Pearson's $r = 0.3935$ and $0.4255, p = 0.0194$ and 0.0108, respectively) and with mean zones of inhibition against target *Streptomyces* 4-21 (Spearman's $\rho = 0.40, p = 0.018$).Thus, soils with higher nitrate content supported higher bacterial and *Streptomyces* densities and better inhibitors. No significant differences were observed in organic matter and soluble potassium content among soils with different cropping histories. However, organic matter and soluble potassium were significantly positively correlated with inhibition zone sizes against target *Streptomyces* 1324-2 (Spearman's $\rho = 0.52$ and $0.4785, p = 0.0014$ and 0.0036, respectively). In addition, pH did not vary significantly among soils from different treatments, but was also positively correlated with the density of inhibitors of 1324-2(Spearman's $\rho = 0.3757; p = 0.0262$).Overall, edaphic properties are correlated with multiple biotic characteristics of the soil.

Soil samples from plots 21, 27 and 30 (see Table 3.2), which had diverse microbial densities and inhibition characteristics were selected to evaluate the tendencies of isolates within each community to modify their inhibition phenotypes in response to one another. Among all three communities, inhibition phenotypes were shifted in 56.4% of all pairwise within-community interactions. The two communities from 4-year rotations had higher frequencies of shifts in inhibition than the community from the 1-year rotation, though differences were not statistically significant (Figure 3.4).

Isolate interactions resulted in both increases and decreases in inhibition. Of the total shifts in inhibition, 50% were increases and 50% were decreases. Communities varied in the proportion of increases and decreases in inhibition, though differences were not significant (Figure 3.5). The proportion of increases in inhibition was 56% among

Table 3.2: Edaphic properties of all soils sampled. Agronomic soils were in three blocks, and had treatments 1-10. Three samples from a nearby wood from a non-cultivated (N.C.) prairie were taken, which are treatments 11 and 12, respectively.

Treatment	Sample	Plant host	NO_3-N (ppm)	OM (%)	Bray P (ppm)	NH_4OAc-K (ppm)	pH
1	1	soybean	21.5	3.5	26	247	5.8
	11		30.7	5.2	15	306	7.5
	21		26.3	4.7	29	253	5.3
2	2	sunflower	30.1	4.9	9	234	7.50
	12		25.8	4.9	17	211	7.10
	22		21.6	5.3	27	188	6.30
3	3	soybean	31.3	5.1	22	291	7.4
	13		24.2	4.6	22	199	5.6
	23		28.0	5.6	22	229	7.2
4	4	sorghum	18.3	3.9	10	159	5.70
	14		30.9	5.1	10	246	7.50
	24		25.7	5.05	18	153	5.45
5	5	maize	28.4	3.7	21	288	6.2
	15		16.6	5.2	12	264	7.5
	25		34.8	5.0	29	209	5.9
6	6	soybean	25.1	5.4	23	342	6.20
	16		31.8	5.1	13	270	6.40
	26		23.9	4.9	23	170	5.40
7	7	sunflower	33.7	4.8	21	217	7.5
	17		23.9	4.9	22	197	5.3
	27		24.1	5.5	28	243	6.4
8	8	lotus	27.9	3.9	17	195	5.60
	18		34.8	5.1	30	245	5.50
	28		29.9	4.9	19	219	6.10
9	9	lotus	33.5	5.2	14	214	5.6
	19		41.5	5.6	20	306	7.4
	29		35.5	4.8	24	208	5.9
10	10	soybean	33.1	5.1	13	200	5.50
	20		30.1	4.6	12	209	6.00
	30		30.4	5.2	24	225	6.30
11	31	N.C.	89.7	9.3	6	348	6.0
	32		77.6	5.6	25	240	5.6
	33		63.9	4.6	3	205	5.4
12	34	N.C.	35.3	3.9	4	268	5.9
	35		34.0	4.3	4	249	5.8
	36		37.3	5.1	8	354	6.1

isolates from plot 27 (4-year rotation, sunflower), 44% among isolates from plot 21 (1-year rotation, soybean) and 50% among isolates from plot 30 (4-year rotation, soybean). In addition, differences in the intensity of change in inhibition was not significantly

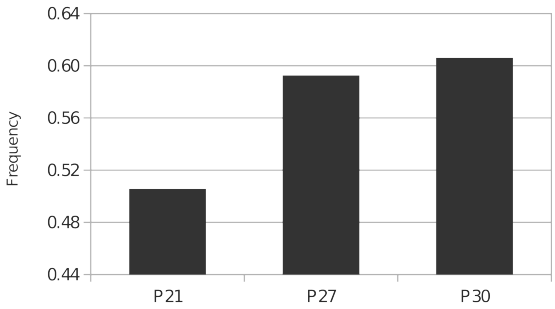

Figure 3.4: Differences in frequency of inhibition change among communities were not statistically significant ($\chi^2, p < 0.5$). P21, P27 and P30: communities from plots 21, 27 and 30, respectively.

different among isolates from different soils. Inhibition in isolates from plot 30 was increased, in average, in 279% with respect to the controls, while in plots 21 and 27 increases were 132% and 114%, respectively. These differences, although large, failed to be significant (Figure 3.6; ANOVA, $F[2, 49] = 2.586$, $p = 0.0855$).

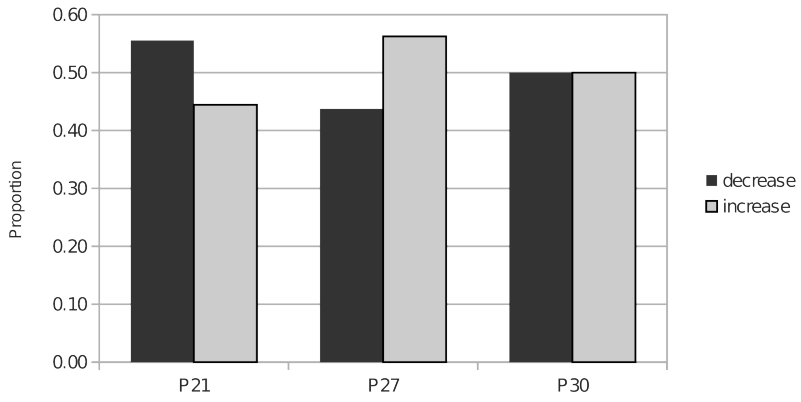

Figure 3.5: Differences in proportion of increases and decreases of inhibition among communities were not statistically significant ($\chi^2, p < 0.5$).

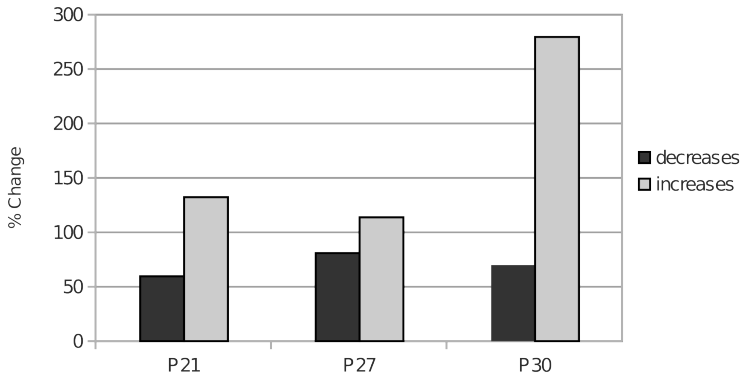

Figure 3.6: Differences in mean percent increase and decrease in inhibition were not significant (ANOVA, $F[2, 49] = 2.586, p = 0.0855$, $F[2, 65] = 2.111, p = 0.1293$, respectively).

3.4 Discussion

Consistent with previous work (Pérez *et al.*, 2007; Xuan *et al.*, 2011; Ceja-Navarro *et al.*, 2010), we found that bacterial populations vary significantly among soils with different cropping histories. The number of years in a rotation, most recent plant host, and the number of plant species in each rotation were all correlated with significant differences in bacterial density. *Streptomyces* densities were also significantly influenced by soil history. Soils with different cropping histories and rotation periods differed in *Streptomyces* density, consistent with previous work (Wiggins and Kinkel, 2005; Xuan *et al.*, 2011; Ceja-Navarro *et al.*, 2010). Soils with higher nitrate content had both higher bacterial and *Streptomyces* densities. *Streptomyces* communities have been shown to be affected by nitrogen abundance in soil in previous work (Ramirez *et al.*, 2012). Like total bacteria populations, *Streptomyces* populations are highly sensitive to soil management and nitrogen content.

In general, soils with higher bacterial densities showed higher density of inhibitors, as had been described before (Davelos *et al.*, 2004-a; Kinkel *et al.*, 2011). In addition, pathogen suppression activity was significantly influenced by soil history, consistently

with previous work (Peréz *et al.*, 2007; Wiggins *et al.*, 2005). However, non-cropped soils had lower densities of inhibitors despite having high densities of bacteria. Although it has been proposed that high bacterial densities are key to achieving high levels of suppressiveness in soil, not all high-density bacterial communities were highly antagonistic. Communities in conditions of diverse nutrient sources, such as non-cropped polyculture plant communities, may follow a coevolutionary trajectory towards niche displacement rather than other trajectories that may lead to highly suppressive communities (Craig McLean *et al.*, 2005; Kinkel *et al.*, 2011).

In addition to biotic factors, edaphic properties influence pathogen suppression. Higher nitrate, organic matter and potassium were positively correlated with greater zones of inhibition. Soils with higher nutrient content have been shown in some cases to have higher bacterial densities and higher inhibition frequencies and intensities (Schlatter *et al.*, 2009). Although this suggests that high nutrient content in soils may promote highly inhibitory communities though increased bacterial densities, the diversity of the nutrient sources deposited in soil from plant roots exudates may constrain simple relationships between nutrient availability and pathogen suppression in field settings (Salles *et al.*, 2004; Bakker *et al.*, 2012; Kinkel *et al.*, 2012).

Interspecies signaling that modifies inhibition among *Streptomyces* has been shown to vary among communities (Vaz and Kinkel, 2012). In this work we show that communities from soils with different cropping histories varied in their signaling characteristics. Overall, the frequency of interactions that shift inhibition phenotypes (signaling interactions) was higher in the agricultural soil communities studied here than in the native prairie soil communities described previously (Vaz and Kinkel, 2012; Slattery *et al.*, 2001). Although not statistically significant, differences in frequency of signaling interactions among the three communities were higher in soils from longer rotations. In

addition, within the two communities from soils of longer rotations there were interactions that induced inhibition in isolates that were not inhibitory when grown alone, suggesting interspecies signaling had a higher significance in these communities. These soils had higher bacterial, *Streptomyces* and inhibitors densities. Our results suggest that crop management practices such as longer rotation periods, which increase overall microbial densities, may contribute to maintenance of a higher frequency of interspecies signaling. Signaling may be more significant to fitness in high diversity communities in which interspecies interactions are more common than in low density communities. Signaling that increases inhibitory capacities may contribute significantly to enhanced disease suppression potential.

It would be desirable to predict the likelihood that a soil community will be suppressive of a newly-arrived soil pathogen. In general, communities that contain important antibiotic producers have been linked with suppressive soils (Keel *et al.*, 1996), and thus, communities with high densities of inhibitors are likely to be more pathogen suppressive (Wiggins and Kinkel, 2005). However, antibiotic production in *Streptomyces* is highly influenced by the presence of nearby isolates, therefore the combined information of inhibitors density with signaling properties of the communities will be more informative than the inhibition density information alone. These data suggest that both pathogen suppression and signaling are responsive to crop management practices. Targeting rotation lengths and plant hosts to maximize diversity and maintenance of a highly suppressive soil community could contribute significantly to more sustainable disease control.

3.5 References

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Appendix A

Supplementary material

A.1 Materials and methods for Chapter 2

Bacterial isolates. The *Streptomyces* isolates used were collected from diverse areas: 1232.2, 3211.5, and 5111.5 were from the central USA, Cev2-10 from southwestern France, Lub2-11b from south-central France, Mont3-8 from eastern Spain, NZ816-12 from the south island of New Zealand, Pan FS14 from central Panama, and Witz25 from western Germany. These isolates were selected from a global collection based on their diversity in nutrient utilization patterns (Schlatter, personal communication). Analysis of 16S sequences documented that all isolates are members of the genus Streptomyces (data not shown). Antibiotic treatments. Five antibiotics produced by or derived from *Streptomyces* were used in this work, including rifampin, tetracycline, vancomycin, streptomycin and chloramphenicol (Kieser *et al.*, 2000). Minimum inhibitory concentrations (MIC) were determined for each isolate-antibiotic combination on solid ISP2 medium (Shirling and Gottlieb, 1968) using filter-sterilized antibiotic stocks (Clinical Laboratory Standards Institute, 2006). Antibiotics were incorporated into ISP2 medium at a range of concentrations, and multiple individual 4 μl drops of suspensions of 1×10^8 spores/ml of the same isolate were spotted onto each plate. Plates were incubated at 28°C and presence/absence of growth was observed after 3 days. The MIC was determined as the lowest concentration that prevented any detectable growth among replicate

spots. The subinhibitory concentration of each antibiotic (SICA) was subsequently defined as 10% of the MIC for each antibiotic-isolate combination. Growth of each isolate on the subinhibitory concentration for each antibiotic was confirmed and compared to growth on non-amended ISP2 medium. For every isolate-antibiotic combination, we confirmed the presence of vigorous growth on solid ISP2 medium amended with the subinhibitory concentration of each antibiotic.

Nutrient Use. Biolog SF-P2 plates (Biolog, Inc. Hayward, CA) were inoculated as indicated by the manufacturer (http://www.biolog.com). Briefly, solutions with the corresponding SICA were mixed with spore suspensions of each isolate immediately prior to inoculation of Biolog plates to reach a final OD 590 of 0.22. Following inoculation, plates were incubated for 3 days at 28°C, and growth on each of the $n = 95$ nutrients was determined as OD 590 of each well using a Multiskan EX microplate reader (Labsystems, Helsinki, Finland). For each plate, the O.D. of the water control well was subtracted from that of all other wells prior to analysis, and differenced O.D. values below 0.005 were considered as no growth. Nutrient use was evaluated on three replicate plates for every isolate-antibiotic combination. Among all possible isolate-antibiotic combinations, nutrient overlap (NO) was calculated using the formula:

$$\mathrm{NO} = \frac{\text{number of nutrients used by both A and B}}{(\text{number of nutrients used by A } + \text{ number of nutrients used by B})/2}$$

The escape ratio (ER) was similarly determined for every isolate in combination with every other isolate in the presence and absence of antibiotics using the formula:

$$\mathrm{ER} = \frac{\text{competition–free growth with SICA}}{\text{competiton–free growth without SICA}}$$

where competition-free growth is the proportion of total growth that occurred on

nutrients that were not utilized by the paired isolate. Data analyses. Principal components analysis (PCA) was performed using the Past software (Hammer *et al.*, 2001). Student t-tests, ANOVAs and Tukey's LSD tests were carried out using Matlab Statistics Toolbox (MATLAB version 7.8.0. Natick, MA: The MathWorks Inc., 2009). Chi-square tests were carried out using an online interactive calculation tool for chi-square goodness of fit (http://quantpsy.org).

A.2 Supplementary figures and tables for Chapter 2

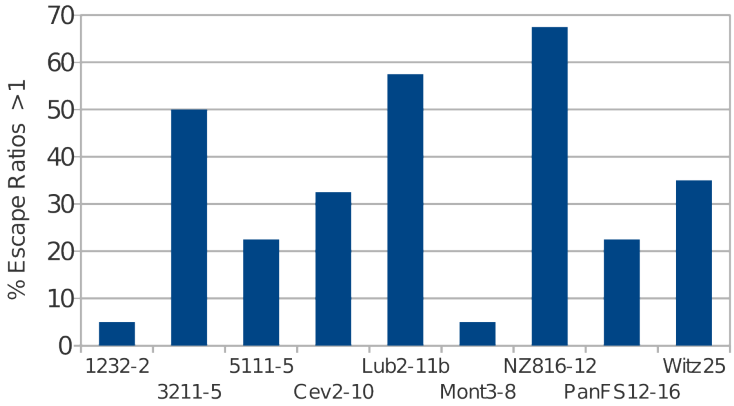

Figure A.1: Percentage of escape ratios greater than one among *Streptomyces* isolates. There were $n = 40$ isolate pair-antibiotic combinations per isolate. The frequency of escape ratios > 1 varied widely among isolates (Chi Square$[8, 360] = 69.99, p = 0$).

Table A.1: Concentrations of antibiotics (μg/ml) used for each isolate-antibiotic combination. Minimum inhibitory concentrations (MIC) were determined for each isolate-antibiotic combination on solid ISP2 medium. The concentrations defined as subinhibitory were 10% of the MIC. Growth on ISP2 containing the final subinhibitory concentration was confirmed for all isolate-antibiotic combinations.

ANTIBIOTIC	1232-2	3211-5	5111-5	Cev 2-10	Lub2-11b	Mont 3-8	NZ816-12	Pan FS14	Witz 25
CHLORAMPHENICOL	2	4	4	8	1	1	0.5	8	4
TETRACYCLINE	2	2	4	1	1	1	4	2	4
STREPTOMYCIN	2	1	16	0.5	1	0.5	0.05	16	0.2
RIFAMPICIN	0.2	0.5	0.5	0.05	0.05	0.2	0.05	2	0.5
VANCOMYCIN	0.05	0.2	0.5	0.03	0.05	0.2	0.03	0.1	0.1