RESEARCH ARTICLE

Nutrient overlap, genetic relatedness and spatial origin influence interaction-mediated shifts in inhibitory phenotype among *Streptomyces* spp.

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Abstract

Chemical communication among kin bacteria modulates diverse activities. Despite the general consensus that signaling among non-kin organisms is likely to influence microbial behavior, there is limited information on the potential for microbial interactions to alter microbial phenotypes in natural habitats. We explored patterns of interaction that alter inhibitory phenotypes among *Streptomyces* isolates from distinct communities. Shifts in inhibition in response to the presence of a partner were evaluated for 861 isolate combinations, and were considered in relation to nutrient use, 16S sequence, inhibition phenotype and community origin. The frequency of inhibition-shifting interactions was significantly higher among isolates from the same (0.40) than from different (0.33) communities, suggesting local selection for inhibition-shifting interactions. Communities varied in the frequency with which *Streptomyces* isolates responded to a partner but not in the frequency with which isolates induced changes in partners. *Streptomyces* isolates were more likely to exhibit increased inhibition of a target bacterium in response to isolates that compete for the same nutrients, are closely-related or are strongly inhibited by their antibiotics. This work documents a high frequency of interactions among *Streptomyces* that shift the capacity of *Streptomyces* to inhibit other microbes, and suggests significant potential for such interactions to shape microbial community dynamics.

Introduction

Microorganisms in natural habitats are immersed in an environment filled with diverse chemicals. Bacteria depend upon cues from their environment to modulate gene expression in order to increase their chances of survival. These cues include small molecules produced by potential competitors (Teplitski *et al.*, 2000; Camilli & Bassler, 2006; Straight *et al.*, 2006; López *et al.*, 2009). Chemical communication or signaling among kin organisms modulates activities as diverse as sporulation, antibiotic production, entrance to a DNA-receptive or -competent state, biofilm production, and expression of pathogenesis-related molecules (Weinrauch *et al.*, 1991; Williams, 2007). Among organisms of different species, genera, or even across kingdoms, signaling has been shown to alter phenotypes significantly (Wang *et al.*, 2004; Williams, 2007; Pérez *et al.*, 2011) and is believed to have a significant impact on microbial community dynamics in nature (Bassler & Greenberg, 1997; Slattery *et al.*, 2001). Despite the general consensus that interspecies communication is important in soil and is likely to influence microbial behavior, there is limited information on the dynamics of signaling interactions and their implications for the ecology and evolutionary biology of microbial communities in soil.

Signaling has been defined as an interaction in which both the signal and the receptor are produced or have evolved strictly for the specific purpose of communication (Maynard Smith & Harper, 2003; Keller & Surette, 2006; Diggle *et al.*, 2007), which implies a mutual benefit to both the producer and the receiver. However, this definition excludes many types of interactions that occur in complex communities. For instance, using this definition, eavesdropping (Duan *et al.*, 2003), a process in which signals that modulate activities among kin organisms are

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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 (Egland *et al.*, 2004; Keller & Surette, 2006), would also be excluded by a strict definition of signaling. Although these interactions do not fit such strict definitions, they can significantly alter the phenotypes of the interacting species and therefore are also likely to shape the structure and function of natural microbial communities. Consequently, it is critical to consider more broadly the complex dynamics of phenotype-altering chemical interactions among microorganisms such as those influenced by space, phenotype and phylogeny.

Bacteria of the genus *Streptomyces* are ubiquitous in soil, freshwater and marine habitats (Schrey & Tarkka, 2008; Schneemann *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 is expected to 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. furans, and γ-butyrolactones (GBL) of several types (e.g. A-factor, virginiae butanolide and IM2; Healy *et al.*, 2009; O'Rourke *et al.*, 2009; Nakano *et al.*, 2000; Takano *et al.*, 2000; Arakawa *et al.*, 2007; Kato *et al.*, 2007). Individual *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 with respect to structural families of molecules. Thus, 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). Previous work has suggested that GBLs are likely to play a role in interspecific interactions among *Streptomyces* spp. (Ueda *et al.*, 2000). Furthermore, *Streptomyces* spp. commonly possess more than one type of GBL receptor gene in their genomes and receptor genes have been proposed to be horizontally transferred (Nishida *et al.*, 2007). This may enable 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 fitness benefits of antibiotic production will depend upon the extent of nutrient competition among coexisting populations (Kinkel *et al.*, 2014). This suggests that species interactions that mediate antibiotic production should be influenced by patterns of nutrient use among competitors. Furthermore, if interactions that alter antibiotic production confer fitness benefits, then such interactions should be locally adapted, or should be more likely among sympatric (locally coexisting) than allopatric (having different spatial origins) microorganisms.

Although species interactions among *Streptomyces* have been documented previously in diverse contexts (Becker *et al.*, 1997; Davelos *et al.*, 2004a; Vetsigian *et al.*, 2011; Vaz Jauri *et al.*, 2013), little work has focused on the dynamics of signaling within *Streptomyces* communities. This work focuses on the patterns of interaction among *Streptomyces* from localized communities that alter antibiotic inhibitory phenotypes. Specifically, we examined the frequency and direction (increase or decrease) of changes in inhibitory phenotypes among sympatric and allopatric isolate pairs, and the relationships between the induced changes and nutrient overlap, genetic distance, and inhibition among isolates. Our results suggest that interactions among *Streptomyces* spp. play a significant role in structuring inhibitory phenotypes and consequently population dynamics within communities, and provide novel information on relationships between nutrient overlap, genetic relatedness, antagonistic interactions, spatial origin, and shifts in inhibitory capacity in the presence of a partner.

Materials and methods

Isolates

The *Streptomyces* used in this study were isolated from the Cedar Creek Ecosystem Science Reserve (CCESR) in Minnesota, USA (www.cedarcreek.umn.edu), a National Science Foundation Long-Term Ecological Research Site. Soil was collected in December 1999 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). All plots were <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} $CaCO_3$, 15 g 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 removed from crop production in 1934, and natural prairie vegetation had been allowed to recolonize the site.

Soil samples 10 cm deep by 1 cm diameter were obtained with small aluminum corers. Samples were dried overnight, suspended and agitated in phosphate buffer solution for 1 h, plated on oatmeal agar with antibiotics, and incubated at 28 °C for 7 days, as described by Davelos *et al.* (2004b). From each plot, 10 isolates from a single soil core were randomly selected for further study. In total, 30 *Streptomyces* spp. isolates from the three communities were used in the analyses. These isolates represent a subset of a larger set of isolates whose inhibitory and nutrient use phenotypes have been explored in previous work (Davelos *et al.*, 2004a, b; Schlatter *et al.*, 2013; Kinkel *et al.*, 2014), though this work represents the first study of their phenotype-shifting interactions. *Bacillus* isolates 51-U-1 and 41-D-2 were selected for phenotype-shifting assays based on their sensitivity to inhibition by these *Streptomyces*. Specifically, every one of the *Streptomyces* isolates studied here was able to inhibit at least one of the two *Bacillus* isolates. The *Bacillus* isolates were obtained from soil samples from the same experimental field (E001) at the Cedar Creek Ecosystem Science Reserve.

Phenotype-shifting assays

We quantified the effects of a partner *Streptomyces* isolate on the inhibitory phenotype of another isolate, regardless of the mechanism(s) by which the shifts in phenotype occur. Possible outcomes included no change, a significant increase in inhibition of a target in the presence of a partner, or a significant decrease in inhibition of a target in the presence of a partner. Interactions were evaluated among all pairwise sympatric and allopatric isolate combinations ($n = 861$), and inhibitory phenotypes were determined by the presence of zones of growth inhibition on *Bacillus* lawns overlaying the *Streptomyces*. Of the total 870 possible combinations of the 30 isolates with a partner, only nine could not be accounted for, either because one of the partners was inhibited by the presence of the other or because of extreme variability in the production of antibiotics by the isolates.

Assays were carried out by inoculating paired *Streptomyces* isolates 1 cm apart on plates containing 15 mL of the rich medium ISP2, which contains malt extract, yeast extract and dextrose as nutrient sources (Fig. 1; Schirling & 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 spread onto each plate. Briefly, a 12-h culture of the *Bacillus* targets (52-U-1 or 41-D-2) grown in Nutrient Broth (Difco) to an $OD_{600\ nm} = 0.800$ was diluted 1 : 10 in Nutrient Broth containing 0.8% agar and added as a 10-mL overlay on the plates. After 24 h 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 the 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.

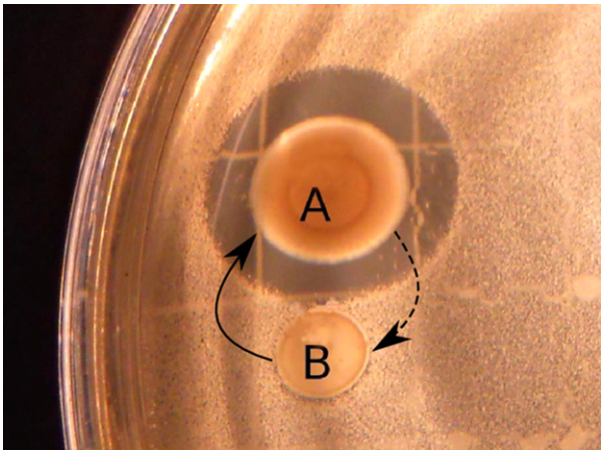

Fig. 1. Phenotype-shifting assays. *Streptomyces* isolates were spotted 1 cm apart and subsequent inhibition zones on *Bacillus* lawns were measured and compared with zones made by each of the isolates (a or b) growing alone. Four replicate isolate combinations were carried out per plate.

Spore suspensions of *Streptomyces* isolates were made by collecting spores of each isolate grown on Oatmeal Agar plates in 30% glycerol. Suspensions were filtered through cotton and the concentration of spores in each filtrate was quantified by dilution plating.

Genetic analyses

The 16S rRNA gene sequences of the isolates used in this work have been reported earlier (Davelos *et al.*, 2004b; Schlatter *et al.*, 2013); Genbank accession numbers for all isolates used are presented in Supporting Information, Table S1. 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 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; D.C. Schlatter, pers. commun.).

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.*, 2013). Nutrient use profiles for these isolates within a larger collection of isolates have been explored in depth in previously published work (Schlatter *et al.*, 2013); the work reported here focuses specifically on the relationships of isolate patterns of interaction to nutrient use phenotypes. Briefly, freshly grown spore suspensions of each isolate were adjusted to an $OD_{590\ nm} = 0.22$, diluted according to the manufacturer's instructions, and inoculated into Biolog plates. After 3 days of incubation at 28 °C, the $OD_{590\ nm}$ 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 to be 0. Readings above 0.005 following subtraction of the water control OD value 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 relative to the total number of nutrients used by the first isolate:

$$N.O.(A,B) = \frac{(nutrients\ used\ by\ A\ and\ B)}{(nutrients\ used\ by\ A)}$$

Using this definition of nutrient overlap, one nutrient overlap value was obtained for each isolate in the pair, and each isolate has a unique niche overlap value with respect to every other isolate in this work.

Antagonism among *Streptomyces* isolates

Previous studies have reported on the antagonistic characteristics of the isolates used here (Davelos *et al.*, 2004a; Kinkel *et al.*, 2014). Within this manuscript, we focus specifically on the relationships between these antagonistic capacities and species interactions that modify inhibitory phenotypes among isolates. Briefly, spore suspensions (approximately 1×10^8 spores mL^{-1}) 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^{-1}) 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 were 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 in all parwise intrctions). Interactions were replicated three times.

Analyses

For phenotype-shifting assays, significant differences in inhibition were calculated with *t*-tests using SAS 9.2 (PROC GLM; SAS Institute Inc., Cary, NC). Differences in the frequency of interactions were analyzed using chi-square tests (http://quantpsy.org). Nutrient overlap among isolates and significant differences in means of nutrient overlap and genetic distance among isolates (one-way ANOVAs and Tukey's least significant differences, LSD) were calculated using MATLAB STATISTICS TOOLBOX (MATLAB version 7.8.0. Natick, MA: The MathWorks Inc., 2009; www.mathworks.com).

Results

Inhibition of a target in the presence of a partner was screened among 861 pairwise *Streptomyces* isolate combinations from three locations in soil. Overall, there were significant changes in inhibition in the presence vs. absence of a partner in 35.4% of isolate combinations. *Streptomyces* from different locations varied significantly in their frequency of response or, specifically, the frequency with which their inhibition was shifted by the presence of a partner. 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 finally by isolates from plot 1 [Fig. 2a; χ^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 (Fig. 2b). Thus, the frequency of

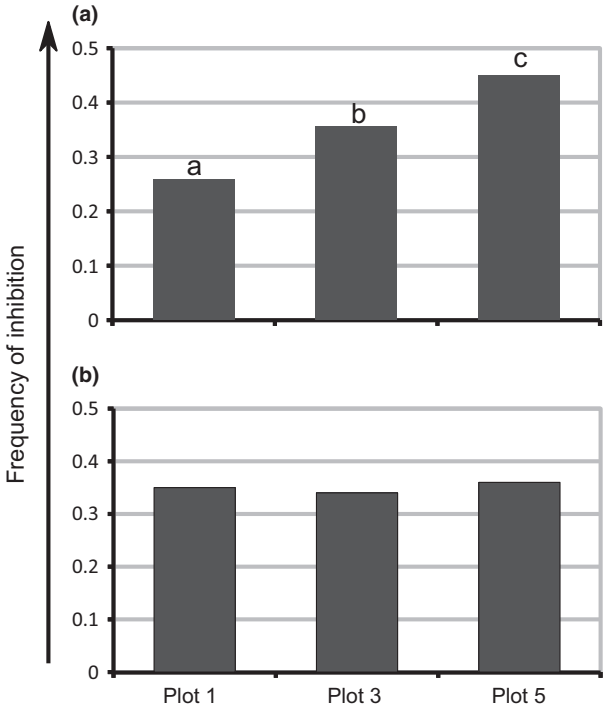

Fig. 2. Frequency of changes in inhibition. Mean frequency of changes in inhibition among isolates when grown with a partner isolate in all (sympatric and allopatric) pairwise interactions for each community (1, 3, 5). Different letters indicate significant differences in frequencies. (a) Frequency of 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].

response to presumptive signals, but not the frequency of induction of changes, varies among the three communities.

Among isolates, changes in the presence of a partner induced both significant increases and decreases in inhibition. Among all combinations in which the change in inhibition was significant, 60% of shifts 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, whereas isolates from plots 1 and 3 generally exhibited increased inhibition in the presence of a partner [Fig. 3; χ^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 (Supporting Information, Fig. S1). Therefore, although isolates from the three communities varied in their tendencies to increase or decrease inhibition in response to partners, they did not vary in the responses they induced in others.

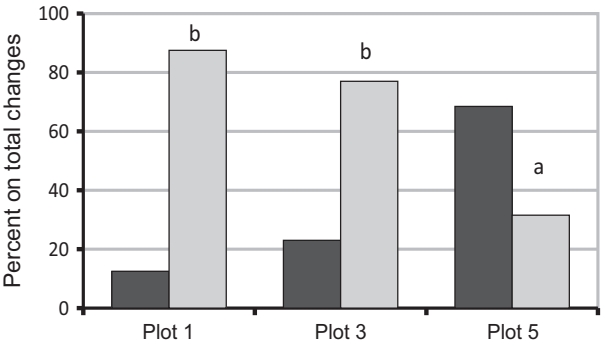

Fig. 3. Direction of changes in inhibition. Mean frequency of decreases (black bars) or increases (gray bars) in inhibition in response to a partner in all (sympatric and allopatric) pairwise interactions for each community (1, 3, 5). Different letters indicate significant differences in percentage of increases/decreases of inhibition in response to a partner in each community [χ^2 (2, n = 303) = 77.7; P < 0.00001).

Every isolate increased inhibition in some isolates and reduced inhibition in others. In contrast, not every isolate exhibited increased or decreased inhibition towards a target in 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 (from plot 1) was found that did not induce any change in inhibition by any of the paired isolates from the same community. When responding to the presence of a partner, 17 of the 30 isolates showed both increases and decreases in inhibition, two isolates showed only decreased inhibition, 10 isolates never exhibited decreased inhibition, and one isolate showed no significant shifts in inhibition in response to a partner. These numbers suggest that, in these communities, the majority of isolates both alter their inhibition and have their inhibition altered by a neighbor.

Evidence for local selection within communities

To evaluate evidence for local selection for inhibitory phenotype-shifting interactions, the frequencies of shifts in inhibition among sympatric and allopatric isolate pairs were compared. Isolates were significantly more likely to exhibit changes in antibiotic inhibition in response to a sympatric than an allopatric isolate [Fig. 4; χ^2 (1, n = 861) = 4.03, P = 0.045]. Higher frequencies of inhibition-shifiting interactions among sympatric than allopatric isolates suggest that there is local selection for the capacity to modulate antibiotic production in response to coexisting *Streptomyces* populations.

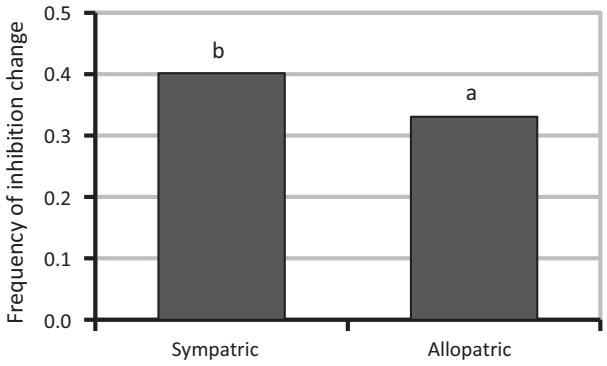

Fig. 4. Frequency of shifts in inhibition among sympatric and allopatric isolate pairs. Frequency of shifts in inhibition in the presence of an isolate varied significantly between sympatric and allopatric parners [χ^2 (1, n = 861) = 4.03, P = 0.045).

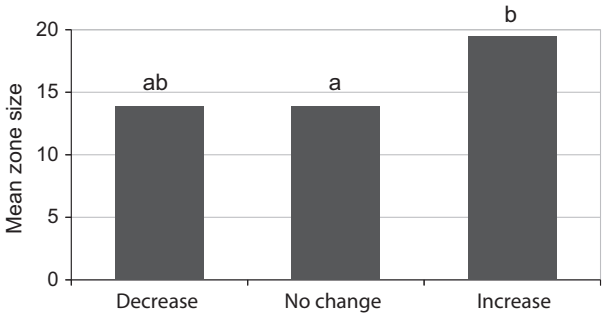

Fig. 5. Intensity of antagonism and shifts in inhibition towards a target. Mean inhibition zones on a partner (intensity of antagonism was greater for isolates that induced their inhibition towards a target in the presence of that partner (ANOVA, $F_{2,134}$ = 3.847, P = 0.0237; Tukey's LSD, $P < 0.05$). Different letters indicate significant differences in mean inhibition zone sizes.

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 almost twice as frequent among isolates in plot 5 as in plot 1, and intermediate in plot 3 (Fig. S2). Furthermore, the three communities varied in the proportions of sympatric increases and decreases in inhibition (Fig. S3). Isolates from plots 1 and 3 tended to increase their inhibition in the presence of a sympatric partner (96% and 70% of interactions were increases in inhibition, respectively), whereas isolates from plot 5 tended to decrease inhibition in the presence of a sympatric partner (67% of interactions were reductions in inhibition). Such drastic differences suggest that interspecies interactions may have fundamentally different cumulative effects on inhibitory phenotypes in different communities.

Relationships between antagonism and inhibition-shifiting phenotypes

Further analyses explored the relationships between shifts in inhibition induced by a partner and antagonism of that partner in sympatric isolate pairs: are isolates more likely to increase inhibitory activity in response to a susceptible or a resistant partner? 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, $P < 0.05$) (Fig. 5). This suggests that among sympatric isolates, species interactions may generally serve to increase the capacity of the antibiotic-producing signal recipient to inhibit its competitors. However, there were some sympatric isolate pairs in which antibiotic inhibition was enhanced by the presence of a resistant partner.

Relationships between nutrient overlap and inhibitory phenotype-shifting interactions

Among sympatric isolate pairs, shifts in inhibition were very modestly related to nutrient overlap of the inducer of change with the responding isolate. Sympatric isolates that altered inhibition by a partner had a slightly smaller nutrient overlap with their partner than isolates that induced no changes (nutrient overlap = 0.809 and 0.845, respectively; ANOVA, $F_{1,264}$ = 3.613, P = 0.0584). Thus, isolates tended to alter inhibition phenotypes of other isolates that were weaker competitors for nutrients.

Nutrient overlap of an isolate with a partner was a good predictor of the specific response to the presence of that partner. Among sympatric isolate pairs, isolates that increased their inhibition in response to a partner had higher mean niche overlap with that partner (n = 63; mean nutrient overlap = 0.904) than with partners that either repressed or did not change inhibition by that isolate (n = 43 and 158; nutrient overlap = 0.826 and 0.805, respectively; ANOVA, $F_{2,264}$ = 9.865, P = 0.00007; Fig. 6). Similarly, among all interactions (sympatric and allopatric), nutrient overlap with a partner was higher for isolates that increased their inhibition of a target in the presence of that partner as compared with isolates that decreased or did not change inhibition with a partner (Fig. S4). Thus, increases in inhibition in the presence of a strong competitor may be advantageous to soil *Streptomyces*,

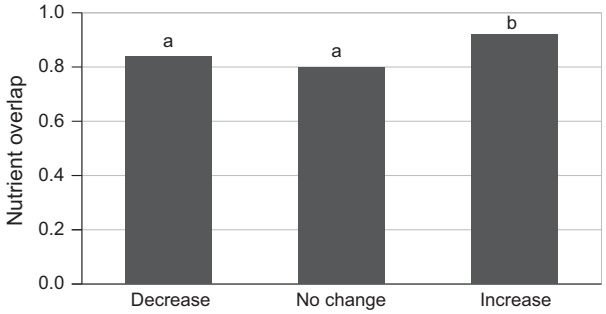

Fig. 6. Nutrient overlap and the direction (decrease, no change or increase) of shifts in inhibition of a target in the presence of a sympatric inducer. Mean nutrient overlap of the responding isolate with the inducer varies significantly among cases in which inhibition of a target increases, decreases or exhibits no change. Different letters indicate significant differences in nutrient overlap (ANOVA, $F_{2,267} = 9.865$, $P = 0.00007$).

which could suggest the existence of an arms-race dynamic among some individuals.

Relationships between genetic relatedness and inhibitory phenotype-shifting interactions

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 (Fig. S5A), and the same trend was observed when only sympatric pairs were considered (Fig. S5B). Overall, more distantly related *Streptomyces* isolates were more likely to alter inhibitory phenotypes in one another than were 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 inhibition was increased 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 non-shifting pairs). Isolate pairs in which at least one of the isolates decreased its inhibition in the presence of a partner were the least closely related (12% less than non-shifting pairs) (ANOVA, $F_{2,861} = 8.12$, $P = 0.0003$; Tukey's LSD, $P < 0.05$) (Fig. 7). Among sympatric isolates, pairs in which at least one of the isolates decreased its inhibition in the presence of the other were significantly more distantly related than pairs in which one isolate showed increased or unchanged inhibition (mean genetic distance = 0.0403, 0.0323 and 0.0304, respectively; ANOVA, $F_{2,264} = 5.234$; $P = 0.006$). Thus, *Streptomyces* were not only more likely to respond to distantly related isolates but also were more likely to show decreased inhibition by the presence of distantly related than more closely related isolates.

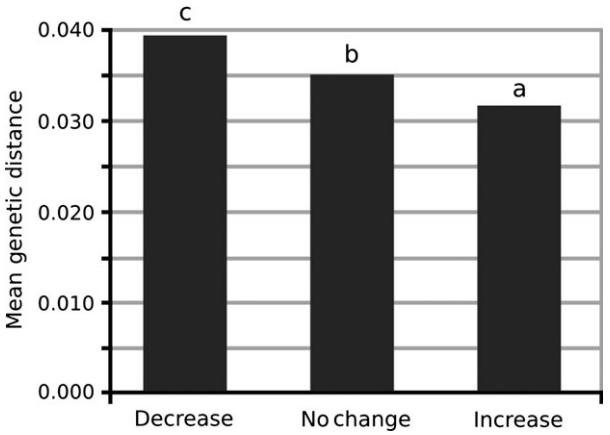

Fig. 7. Genetic relatedness and shifts in inhibition among isolate pairs. The direction of inhibition changes among sympatric and allopatric partners varied with genetic relatedness (ANOVA, $F_{2,861} = 8.12$, $P = 0.0003$; Tukey's LSD, $P < 0.05$).

Discussion

Species interactions commonly shifted inhibition phenotypes among diverse *Streptomyces*. Interactions among 35% of *Streptomyces* isolate combinations led to significant changes in the capacity of individual isolates to inhibit a target, suggesting that such interactions are important in mediating antibiotic phenotypes among soil microbes. Previous work on interspecies interactions among *Streptomyces* reported increased antibiotic production by isolates in the presence of a partner was relatively common (~ 20%, Ueda *et al.*, 2000). However, these isolates were from a culture collection without evidence for coevolution. Work on signaling among non-*Streptomyces* bacteria has reported similar frequencies of increased inhibition in co-culture experiments (~ 23%, Slattery *et al.*, 2001). Pierson *et al.* (1998) reported that 8% of isolates pairs exhibited interspecies signaling, but that work focused on interactions mediated by a very specific signal molecule. The present work is unique in its focus on interactions that increase and decrease inhibitory phenotypes among soil *Streptomyces* from localized soil communities. However, our data are consistent with these previous studies in suggesting that a high frequency of phenotype-shifting interactions, including signaling, occurs in soil communities, with significant potential consequences for microbial inhibitory dynamics.

Inhibition-shifting interactions were 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*, increase inhibition by others, and have no effect on others. Similarly, the same *Streptomyces* may respond to one isolate by increasing inhibition while decreasing

inhibition in response to a different isolate. The specificity of interactions among *Streptomyces* spp. has also been suggested in the work by Traxler *et al.* (2013), which showed variability in the metabolites produced by *Streptomyces coelicolor* depending on the interacting partner. Together, these suggest that a diverse array of potential signaling molecules (Lyon & Novick, 2004; Yamanaka *et al.*, 2005; Davies *et al.*, 2006; Takano, 2006) and/or receptors (Nishida *et al.*, 2007; Xu *et al.*, 2010; Wang *et al.*, 2011), as well as diverse potential mechanisms for mediating species interactions exist in natural communities. The existence of highly specific signaling or inhibitory phenotype-shifting interactions among soil bacteria suggests that such signaling or phenotype-shifting interactions give flexibility to microorganisms to alter phenotypes to potentially optimize their fitness depending on their biological environment. These data emphasize that *Streptomyces* populations behave differently depending on the biological context or community in which they live.

Isolates varied widely in the number of isolates to which they responded by shifting inhibition, but not in the number of isolates in which they induced changes in inhibitio. The smaller variation among isolates as inducers of change (which ranged from 6 to 14 out of 29) and their marked differences in response frequency (which ranged from 0 to 26 out of 29) suggests a greater variability in the number or types of receptor molecules than in the numbers or types of signals produced. 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. However, one limitation of our work lies in its inability to determine the specific mechanism(s) by which inhibition is changed in the presence of a partner. That is, increases in inhibition could be due to a small signal molecule directly increasing antibiotic biosynthesis by the responding isolate. Small signals produced by neighboring bacteria have been shown to influence *Bacillus* phenotypes (Stefanic & Mandic-Mulec, 2009) as well as induce the expression of virulence factors in the plant pathogen *Pseudomonas savastanoi* pv. savastanoi (Hosni *et al.*, 2011). Alternatively, increased inhibition could reflect synergistic interactions among antibiotic molecules produced by the interacting isolates (Challis & Hopwood, 2003). Decreases in inhibition could similarly reflect several possible underlying causes. For example, an inducer may release small molecules that bind receptors in the responding isolate, thus downregulating antibiotic production. This was described for *S. coelicolor*, where a homolog to GBL receptors (SCBR2) 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 in inhibition are mediated by quorum quenching, a process in which quorum sensing is repressed, which in turn could have several mechanisms. Quorum quenching may be accomplished by interference with the receptor proteins (Ji *et al.*, 1997) 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 *Streptomyces* sp. (Dong *et al.*, 2002). These enzymes 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). No specific mechanism for the observed changes in inhibition can be ruled out. However, regardless of the underlying causes, the outcome is that the antibiotic inhibitory phenotype is modified, with important implications for species interactions and fitness among interacting partners. 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 of interactions among *Streptomyces* spp. that alter inhibitory phenotypes.

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 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 competitors that utilize the same nutrients. This suggests that isolates are selected to detect and respond to significant competitive threats by increasing antibiotic production. On the other hand, isolates tended to avoid increasing inhibition in neighbors that compete strongly for the same nutrients. This anonymity may be beneficial to the noninducing competitor, minimizing its exposure to potentially damaging antibiotics. Although these outcomes may seem contradictory, if we consider isolates A and B, isolate A may have much greater niche overlap with isolate B than B has with A. In this case A is likely to be selected to detect and respond to the presence of B by increasing its inhibition. In contrast, A is less likely to induce an increase in antibiotic production in B. Such interactions are likely to generate diverse ecological outcomes among coexisting isolates in which selection for antibiotic inhibition and resistance phenotypes interacts with selection for signal emission or signal reception to produce complex coevolutionary dynamics among communities (Kinkel *et al.*, 2011). Consistent with this prediction, *Streptomyces* communities from different locations in soil varied widely in the frequency and pattern of increases and decreases in inhibition in response to others.

Nutrient availability is further likely to influence microbial competitive dynamics in soil (Ghorbani *et al.*, 2008; Hibbing *et al.*, 2010; Otto-Hanson *et al.*, 2013). Communities that compete strongly for nutrients are hypothesized to be more likely to engage in antagonistic interactions through antibiotic production (Kinkel *et al.*, 2011). Isolates from plot 5 had the lowest frequency of antagonistic interactions and an average nutrient overlap significantly lower than that of isolates from plot 3. In the presence of a partner, isolates from plot 5 tended to decrease inhibition of a target, which differed significantly from the response of isolates from the other two communities. In contrast, *Streptomyces* from plot 3 had the highest frequency of inhibitory interactions, highest average nutrient overlap, and tended to increase inhibition in the presence of a sympatric partner. These results may reflect distinct coevolutionary trajectories among communities. For example, *Streptomyces* from plot 5 may reflect coevolutionary character or niche displacement, in which *Streptomyces* evolve to reduce nutrient competition by utilizing different nutrients. In contrast, *Streptomyces* from plot 3 may reflect coevolutionary escalation, where reciprocal selection for better antibiotic inhibition promotes communities with high frequencies and intensities of inhibition (Kinkel *et al.*, 2011).

Nutrient use is tightly linked to phylogeny (Schlatter *et al.*, 2013), and closely related isolates are more likely to have larger nutrient overlap than distantly related pairs. Thus, the observed tendency of closely related isolates to increase inhibition in each other may be linked to the nutrient use preferences of their partners. On the other hand, inhibition shifts among isolates that are closely related could also be a consequence of similarities in the chemistry of their signals and receptors, which would increase the likelihood of interference among them. Our results are unable to distinguish these two possibilities.

Incentives for signaling or inhibitory-phenotype shifting vary between the presumptive signal producer and receiver. There are at least three possible fitness outcomes of increased inhibition by one isolate in the presence of a partner. In the first case, an antibiotic-producing isolate may sense the presence of an antibiotic-sensitive competitor, for example through eavesdropping (Ji *et al.*, 1997; Chandler *et al.*, 2012), increase its antibiotic production, and thus increase its fitness. Among within-community (sympatric) interactions in this study, isolates that increased inhibition in the presence of a partner were also more effective at inhibiting that partner, suggesting that benefits to the responding isolate (antibiotic producer) are common. 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, which is an example of chemical manipulation (Keller & Surette, 2006) of the responding isolate by the inducer. Finally, as a third possibility, an isolate may receive a signal from a neighbor whose antibiotics are chemically synergistic with those produced by the isolate (Challis & Hopwood, 2003; Hosni *et al.*, 2011), so that both partners may benefit from the interaction by enhanced antagonism of other community members. Our results suggest the likelihood that all of these interaction types occur within *Streptomyces* communities.

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 (Ji *et al.*, 1997). Conversely, if the inducer is not sensitive to the antibiotics produced by the responding isolate, the responding isolate may benefit from reduced investment in production of ineffective antibiotics (Ji *et al.*,1997; Dulla & Lindow, 2009). Nutrient use and community competition are keys to determining roles of signaling or phenotype-shifting in mediating species interactions in complex communities. More comprehensive data from intact communities are needed to shed light on the ecology and evolutionary biology of these interactions in microbial communities.

This work provides novel insight into the frequency and diversity in inhibitory phenotype-altering interactions among *Streptomyces* spp, and adds a significant layer of complexity to our understanding of interspecies interactions in soil. Interactions that shift inhibition are locally selected, suggesting that such interactions play a significant role in *Streptomyces* fitness. Furthermore, there is a significant relationship between phenotype-shifting interaction frequencies and genetic relatedness, nutrient overlap and antagonism, which delineate a biological context for neighbor-induced shifts in inhibition. This work demonstrates that chemical interactions among *Streptomyces* have significant impacts on inhibitory phenotypes and suggests that a complex array of species interactions modulate microbial community dynamics in natural habitats.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Direction of changes in inhibition. Mean frequency of decreases (black bars) or increases (grey bars) in inhibition induced in a partner among communities.

Fig. S2. Frequencies of within-community shifts in inhibition phenotypes.

Fig. S3. Direction of changes in inhibition. Mean frequency of decreases (black bars) or increases (grey bars) in inhibition in response to a partner amongcommunities.

Fig. S4. Nutrient overlap and shifts in inhibition among all isolate pairs.

Fig. S5. Genetic relatedness and shifts in inhibition among all (A) and sympatric (B) isolate pairs.

Table S1. List of the isolates used in this study. Genebank accession numbers for each isolate are provided.