Genetic and phenotypic traits of streptomycetes used to characterize antibiotic activities of field-collected microbes

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Abstract: Although antibiotic production may contribute significantly to microbial fitness, there is limited information on the ecology of antibiotic-producing microbial populations in soil. Indeed, quantitative information on the variation in frequency and intensity of specific antibiotic inhibitory and resistance abilities within soil microbial communities is lacking. Among the streptomycetes, antibiotic production is highly variable and resistance to antibiotics is highly specific to individual microbial strains. The objective of this work was to genetically and phenotypically characterize a reference collection of streptomycetes for use in distinguishing inhibition and resistance phenotypes of field-collected microbes. Specifically, we examined inhibition and resistance abilities of all isolates in all possible pairwise combinations, genetic relatedness using BOX-PCR and 16S rDNA sequence analyses, nutrient utilization profiles, and antibiotic induction among all possible three-way combinations of isolates. Each streptomycete isolate possessed a unique set of phenotypic and genetic characteristics. However, there was little correspondence between phenotypic and genetic traits. This collection of reference isolates provides the potential for distinguishing 1024 inhibition and resistance phenotypes in field-collected microbes. Relationships between the genetic and phenotypic characteristics examined may provide preliminary insight into the distinct strategies that microbes use in optimizing their fitness in natural environments.

Key words: antibiotic inhibition, resistance, nutrient utilization, BOX-PCR, 16S rDNA.

Résumé : Bien que la production d'antibiotiques peut contribuer à la valeur adaptative des microbes, il existe peu d'information sur l'écologie des populations microbiennes productrices d'antibiotiques dans le sol. En effet, il existe une carence de données quantitatives sur les variations de la fréquence et de l'intensité des capacités d'inhibition ou de résistance spécifiques aux antibiotiques dans les communautés microbienne du sol. Parmi les streptomycètes, la production d'antibiotiques est hautement variable et la résistance aux antibiotiques est fortement spécifique aux souches microbiennes individuelles. L'objectif de ce projet fut de caractériser sur les plans génétiques et phénotypiques une collection de référence de streptomycètes afin de distinguer les phénotypes d'inhibition et de résistance chez les microbes recueillis sur le terrain. Nous avons évaluée plus spécifiquement les capacités d'inhibition et de résistance de tous le isolats pour toutes les paires possibles, les relations génétiques par BOX-PCR et analyses de la séquence de l'ADNr 16S, les profils de consommation de nutriments, et l'induction d'antibiotiques parmi toutes les combinaisons à triples facteurs des isolats. Chaque isolat de streptomycète possédait un ensemble unique de caractéristiques phénotypiques et génétiques. Cette collection d'isolats de référence permet de distinguer 1024 phénotypes d'inhibition et de résistance chez les microbes recueillis sur le terrain. Les relations entre les caractéristiques génétiques et phénotypiques analysées pourraient permettre de mieux comprendre les stratégies distinctes qu'empruntent les microbes afin d'optimiser leur adaptation dans des environnements naturels.

Mots clés : inhibition d'antibiotiques, résistance, consommation de nutriments, BOX-PCR, ADNr 16S.

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Introduction

Antibiotic-producing microorganisms are a common component of most soil communities (Williams and Vickers 1986; Vining 1990). It is hypothesized that antibiotic production confers a competitive advantage to microbes and thus may contribute significantly to microbial fitness in the soil (Williams et al. 1989*a*; Maplestone et al. 1992). However, there is limited information on the ecology of antibiotic-producing microbial populations in soil. In particular, there is little quantitative information on spatial or temporal variation in the frequency and intensity of specific antibiotic inhibitory and resistance abilities within soil microbial communities (but see Huddleston et al. 1997; Wiener et al. 1998; van Overbeek et al. 2002).

The majority (>70%) of known antibiotics are produced by streptomycetes (Tanaka and Omura 1990). Strepto-

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mycetes (Order Actinomycetales, Family Streptomycetaceae, referred to here as the streptomycetes; Williams et al. 1989*b*) are Gram-positive, filamentous bacteria that are ubiquitous in soil. Among the streptomycetes, antibiotic production is highly variable among individuals of the same species in terms of both the amounts and identities of antibiotics produced (Vining 1990; Hotta and Okami 1996). Likewise, resistance to antibiotics is highly specific to individual microbial strains. Many streptomycetes produce more than one antibiotic and also possess resistance to multiple antibiotics (Fujisawa and Weisblum 1981; Phillips et al. 1994). Antibiotics produced by streptomycetes have the capability to inhibit diverse soilborne microbes, including Gram-positive and Gram-negative bacteria as well as many fungi (e.g., Liu 1992; El-Abyad et al. 1993; Jones and Samac 1996; Liu et al. 1996; Doumbou et al. 2001; Xiao et al. 2002).

The diversity of antibiotic inhibitory and resistance abilities among individual streptomycete strains suggests that inhibitory interactions among streptomycetes in soil are likely to be highly variable, with the outcome of an interaction (inhibition or no inhibition) being specific to the particular pair of interacting isolates. This specificity in the outcome of the interaction is analogous to the specificity of interactions between many plant hosts and their microbial pathogens. Among hosts and their parasites, the presence or absence of disease is a function of the specific resistance and avirulence genes present in the host and parasite, respectively (i.e., gene-for-gene interactions; reviewed in Burdon 1987; Bogdanove 2002). Likewise, for streptomycetes in soil, the presence or absence of inhibition is determined by the specific antibiotic production and resistance abilities of the interacting isolates. Among plant hosts and parasites, collections of test organisms, with known resistance or virulence capacity, are used to identify the virulence or resistance of unknown parasites or hosts (i.e., differential sets; reviewed in Burdon 1987). Similarly, for streptomycetes, we propose that the quantification of the inhibition and resistance potential among a collection of unstudied field isolates be done based upon their interactions with a collection of test standards. Although there is a great deal of biochemical information on antibiotic production and resistance in the streptomycetes, our emphasis is on an ecological analysis of these interactions. Therefore, we focus on inhibition or resistance phenotypes (analogous to virulent pathogen and resistant plant phenotypes) rather than the specific antibiotics produced or resisted.

The objective of this work was to characterize a collection of streptomycetes developed for use as test standards. Specifically, we characterized (*i*) inhibition and resistance abilities of all isolates in all possible pairwise combinations, (*ii*) genetic relatedness using BOX-PCR and 16S rDNA sequence analyses, (*iii*) nutrient utilization profiles, (*iv*) and antibiotic induction among all possible three-way combinations of isolates. Our primary interest was in identifying variation in the abilities of our test isolates to distinguish inhibition and resistance phenotypes of other isolates. However, the relationships of antibiotic activities to other phenotypic or genetic characteristics of an isolate may provide important insight into the factors that are significant for generating diversity in antibiotic phenotypes in soil.

Materials and methods

Streptomycete isolates

To develop a collection of streptomycetes with a range of inhibitory and resistance capabilities, 25 isolates from the rhizosphere of potatoes grown in a naturally disease suppressive soil were selected at random from each of five locations in Grand Rapids, Minn. (Lorang et al. 1989). These 125 isolates were paired with a set of 25 field-collected isolates to determine inhibitory and resistance abilities (6250 interactions total), as described below. From this collection of 125 isolates, eight streptomycetes (LK2-12, LK4-2, LK4-16, LK4-20, LK4-21, LK4-24, LK6-14, LK10-3) were selected for study based on variation in inhibitory and resistance patterns (Kinkel 1997). Two remaining strains, DL87 and DL93, were described previously in Liu et al. (1996). DL87 is a plant pathogenic isolate of *Streptomyces scabies* and causes scab on susceptible potato cultivars. DL93 is a streptomycete isolate that can inhibit *S. scabies*, as well as other streptomycetes and fungal plant pathogens, in greenhouse, growth chamber, in vitro, and field trials (Liu et al. 1996; Xiao et al. 2002). Spore suspensions of each isolate were prepared by scraping spores from oatmeal agar (OA) plates (Xiao et al. 2002) into vials containing 20% glycerol that were then stored at –80 °C.

Inhibition and resistance

The ability of each isolate to inhibit and resist all others in the collection was evaluated on starch casein agar (SCA) using a modification of a double-layer agar method (Vidaver et al. 1972). Spore suspensions (approximately 10^8 spores/mL in 20% glycerol) of individual isolates were dotted (10 μL per spot) onto 15-mL SCA plates (100 mm × 15 mm), three dots per plate. Following incubation 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. Watch glasses were removed and plates were aerated in a fume hood for 30 min to permit evaporation of chloroform. Plates were subsequently overlaid with 15 mL of 1% water agar and inoculated with 100 μL of a test isolate (approximately 10^8 spores/mL) spread uniformly over the surface of the agar. Plates were incubated at 28 °C for 3 days. The size of any zone of growth inhibition of the overlaid isolate surrounding any dotted isolate was measured in millimetres from the edge of the dotted colony to the edge of the cleared zone. Each isolate was both dotted (to measure its inhibitory ability) and overlaid (to measure its resistance to inhibition) in all possible pairwise combinations with every other isolate. Both inhibitory and resistance interactions between every pair of strains were evaluated on three separate plates.

The frequency and intensity of antibiotic interactions among isolates were examined. For every isolate, the proportion of other isolates that were inhibited by that isolate (presence of inhibition zone) represents the inhibition frequency. Likewise, for each isolate the proportion of other isolates against which that isolate was resistant (absence of inhibition zone) represents the resistance frequency. Intensity is used to describe the quantitative degree of inhibition or resistance, as determined by the size of the inhibition zone. When quantifying the intensity of inhibition, which is a characteristic of the "dotted" isolate, a small zone size in-

dicates little ability to inhibit. In contrast, when quantifying resistance, a characteristic of the overlaid isolate, an absent or very small zone indicates a high ability of the isolate to resist inhibition. Differences between mean inhibition and resistance zone sizes and between the frequencies of isolates inhibited or resisted were evaluated with ANOVA (PROC GLM; SAS Institute Inc. 1988*b*). Frequency data were arcsine transformed prior to analysis (Sokal and Rohlf 1981).

To examine the association between inhibition and resistance abilities among isolates, the correlation between mean zone sizes for each isolate as an inhibitor and as a resistor was determined (PROC CORR; SAS Institute Inc. 1988*a*). A positive correlation indicates that isolates that are good inhibitors (large mean inhibition zone size) are poor resistors (large mean resistance zone size). Further, among all isolates, correlations between the number of strains that an isolate inhibited and the number of strains that an isolate could resist, between the number of strains that an isolate could resist and the mean inhibition zone size, and between the number of strains that an isolate could inhibit and the mean resistance zone size were examined.

Based on the presence or absence of the ability to inhibit or resist each of the isolates, similarity matrices were generated using simple matching criteria (Sokal and Michener 1958). The presence of a zone was defined as a mean zone size greater than 1 mm for the three trials. Cluster analyses were performed using UPGMA (unweighted pair-group method, arithmetic average; Sneath and Sokal 1973) and dendrograms were constructed (NT-SYS; Rohlf 1998). The correlation between the similarity matrices was examined using Mantel's test (NT-SYS; Rohlf 1998). A positive correlation indicates that clustering among isolates is similar for both inhibition and resistance. Significance of the correlation was determined as described in Lapointe and Legendre (1992).

Induction

The ability of each isolate to induce antibiotic activity in every other isolate was examined on OA using a triple-layer agar method (Becker 2001) modified from Vidaver et al. (1972). Inducing isolates were streaked onto OA plates (1.5% agar) and incubated at 28 °C for 3 days. A sterile cork borer (7 mm diameter) was used to remove an agar plug near the streaked isolates, avoiding any growing cells. Plugs were placed in wells created with a sterile cork borer in an OA test plate (1.5% agar), four plugs per plate. Test plates were overlaid with 15 mL of 1% OA and maintained at room temperature overnight. Spore suspensions (approximately 10^8 spores/mL in 20% glycerol) of strains to be induced (test strain) were dotted (10 μL per spot) directly over two of the agar plugs, with the remaining two plugs serving as negative controls. A dot of the test strain was also placed in the absence of a plug to examine inhibition by the test strain in the absence of an inducing isolate. After 3 days of incubation at 28 °C, isolates were killed over chloroform, as above, and plates were overlaid with 1% water agar. One hundred microlitres of an overlay isolate (approximately 10^8 spores/mL) was spread uniformly over the surface of the agar. Each of the 10 isolates was used as an inducing isolate, test isolate, and overlay isolate in all possible combinations. Each experiment was replicated three times for each combination of inducing isolate, test isolate, and overlay isolate.

Mean inhibition zone sizes between an isolate in the presence of an inducing isolate and in the absence of an inducing isolate were compared (PROC TTEST; SAS Institute Inc. 1988*b*) after 3 days of incubation at 28 °C. The mean effect of each inducing isolate on each test isolate was examined across all overlay isolates (n = 30 for each mean). Correlations among the number of isolates induced, the number of isolates inducing a given isolate, the number of isolates inhibited, and the number of isolates resisted were examined (PROC CORR). For subsequent analyses, induction was coded as 1 and noninduction was coded as 0. Similarity matrices and dendrograms were generated as described above. Correlations between the induction similarity matrices and the resistance and inhibition similarity matrices were examined as above.

Nutrient utilization

Nutrient (carbon source) utilization profiles were determined for every isolate using Biolog SFP2™ plates (Biolog, Inc., Hayward, Calif.). These 96-well plates contain 95 carbon sources plus a water control. Spore suspensions of each isolate were made in 0.2% carrageenan by harvesting an inoculated OA plate after 7–10 days growth at 28 °C. Spore concentration was adjusted to an optical density of 0.20–0.24 at 590 nm. The adjusted spore suspension (1.5 mL) was diluted in 13.5 mL of 0.2% carrageenan, and 100 μL of the resulting suspension was inoculated into each well of the Biolog SFP2™ plate, using an eight-channel repeating pipettor. Plates were incubated at 28 °C. At 72 h, plates were measured on a Multiskan EX microtiter reader (Labsystems, Helsinki, Finland). The absorbance of each well at an optical density of 590 nm was recorded. The test was repeated on three separate Biolog SFP2™ plates for each isolate.

To standardize nutrient data among strains, for every strain the absorbance of the water control well was subtracted from each substrate absorbance for each of the 95 carbon sources within a plate. Mean absorbance for each substrate was calculated over the three trials. All negative mean absorbance values were set to zero for subsequent analyses. Substrate richness, total activity, and utilized substrates (Vahjen et al. 1995; Zak et al. 1994) were examined for each isolate. Substrate richness is the total number of substrates with positive mean absorbance values after correction. Total activity is the sum of the positive mean absorbance values. Utilized substrates are those substrates with an absorbance greater than the average well color development (AWCD = total activity/95; Garland 1996) for a given isolate. For subsequent analyses, utilized substrates were coded as 1 and nonutilized substrates were coded as 0. A similarity matrix and dendrogram were generated as described above. Correlation between the nutrient utilization similarity matrix and the similarity matrices described previously was examined as above. In addition, correlations among the number of utilized substrates, substrate richness, and total activity were examined, as well as correlations between substrate richness and the number of utilized substrates and those described above (PROC CORR). Further, carbon sources were divided into the guilds (carbohydrates,

Table 1. Antibiotics, concentrations, and cellular targets used in antibiotic-resistance screening.

Antibiotic	Concns.	Cellular target
Erythromycin	1, 5, 10, 20 μg/mL	Protein synthesis (50S ribosome)
Spectinomycin	2, 10, 20, 40 μg/mL	Protein synthesis (30S ribosome)
Chloramphenicol	2, 10, 20, 40 μg/mL	Protein synthesis (50S ribosome)
Neomycin	0.5, 2.5, 5, 10 μg/mL	Protein synthesis (30S ribosome)
Novobiocin	0.5, 2.5, 5, 10 μg/mL	DNA
Gentamicin	0.1, 0.5, 1, 2 μg/mL	Protein synthesis (30S ribosome)
Streptomycin sulfate	5, 25, 50, 100 U/mL	Protein synthesis (30S ribosome)
Chlortetracycline	20, 100, 200, 400 U/mL	Protein synthesis (30S ribosome)
Penicillin	50, 250, 500, 1000 U/mL	Cell wall
Bacitracin	1, 5, 10, 20 U/mL	Cell wall

polymers, amino acids, carboxylic acids, amines, and miscellaneous) described by Zak et al. (1994). Carbon utilization guilds have been used to distinguish soil microbial communities among sites and among plant communities as well as among plant species (Zak et al. 1994; Campbell et al. 1997; Grayston et al. 1998). Differences in the distribution of guild utilization profiles (number of substrates utilized in each guild) among isolates were examined (PROC GENMOD; SAS Institute Inc. 1997). Proportions of carbon sources utilized within a guild were compared among isolates (PROC FREQ; SAS Institute Inc. 1988*a*).

Antibiotic resistance screening

The resistance of each strain to four concentrations of 10 antibiotics (Table 1) was examined by plating each isolate onto 1 mL of antibiotic-amended SCA in a 48-well tissue culture plate. Antibiotics with a range of cellular targets (e.g., cell wall, protein synthesis) were selected intentionally. Concentration ranges were determined based on literature values (Phillips et al. 1994; Ambaye et al. 1997; Tisa et al. 1999). A range approximate to these literature values, which enabled differences among isolates to be determined, was selected by trial and error. Antibiotics were dissolved in 30% ethanol except for neomycin, gentamicin, and penicillin stocks, which were dissolved in distilled water. One hundred microlitres of a spore suspension (approximately 10^8 spores/mL) of each isolate was pipetted onto the solidified agar in each well. Two wells of each plate containing only SCA with no antibiotics served as positive (inoculated) and negative (not inoculated) controls. Wells were scored qualitatively for growth (+ or –) after incubation at 28 °C for 7 days. The assay was repeated and growth ratings were consistent between the two trials (data not shown).

An isolate was considered to be resistant to a particular antibiotic if it could grow at the highest concentration tested. Resistance was given a score of 1 and susceptibility was scored as 0. An antibiotic resistance similarity matrix was generated as described above. Correlations between this antibiotic resistance matrix and the resistance matrix generated from pairing among isolates, the inhibition matrix, the utilized substrate matrix, and the induction matrix were examined as described above.

Pathogenicity

Pathogenicity of each isolate was evaluated on minitubers of potato *Solanum tuberosum* 'Snowden' (susceptible to *S. scabies*), 'Kennebec' (moderately susceptible), and 'Norchip' (moderately resistant). Minitubers were generated following the leaf-bud cutting method of Lauer (1977). Pathogenicity of each strain was determined essentially as described in Liu et al. (1996). Briefly, potato plants were grown in a greenhouse in 3-gal (1 gal (Am.) = 3.79 L) plastic pots. After 8 weeks, leaf-bud cuttings were made and planted in clay pots filled with water-saturated sterile sand. After 6 weeks, two to five minitubers of each cultivar were placed in clay pots filled with sterile sand. Each minituber was inoculated with 500 μL of a spore suspension (approximately 10^8 spores/mL) of each isolate. Minitubers were evaluated for the presence or absence of scab lesions after 10 days.

16S rDNA sequences

DNA from each isolate was extracted using the Wizard Genomic DNA Purification kit (Promega Corp., Madison, Wis.), following the manufacturer's instructions. Universal bacterial primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′) that amplify nearly full-length (approximately 1.5 kb) 16S rDNA products were used in all PCR reactions (Edwards et al. 1989). PCR reaction mixtures (50 μL) consisted of 100 ng of DNA, 10 pmol of each primer, and PCR SuperMix High Fidelity (Invitrogen Corp., Carlsbad, Calif.), as described by the manufacturer. PCR was performed on a PTC-200 DNA Engine (MJ Research Inc., Waltham, Mass.), following the protocol of Takeuchi et al. (1996). Amplified PCR products were purified with the QIAquick PCR Purification kit, following manufacturer's instructions (Qiagen Inc., Valencia, Calif.). Partial 16S sequences for each isolate were obtained by automated DNA sequencing from the Advanced Genetic Analysis Center, University of Minnesota (St. Paul, Minn.), using pA as the primer.

16S rDNA sequences were compared with sequences in the GenBank database by using the Basic Alignment Search Tool (BLAST) (Altschul et al. 1990). 16S rDNA sequences were edited, aligned, and analyzed using the Wisconsin Package Version 10.2 (Genetics Computer Group (GCG), Madison, Wis.). A matrix of Jukes–Cantor distances (Jukes and Cantor 1969) was used to construct unrooted phylogenetic trees using the neighbor-joining method of Saito and Nei (1987). A bootstrap analysis was conducted to test the reliability of the tree (Felsenstein 1985). The distance matrix was compared with the similarity matrices for inhibition, re-

sistance (antibiotic and isolate pairing), induction, and utilized substrates, as described above.

BOX-PCR genomic DNA fingerprints

DNA was extracted from each isolate as described above. The BOXA1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′), which amplifies repetitive sequences within the bacterial genome, was used (Versalovic et al. 1994) in each 50-µL PCR reaction, following the protocol of Rademaker et al. (1998). PCR was performed on a PTC-200 DNA Engine (MJ Research Inc.) using conditions described in Rademaker et al. (1998). Genetic differentiation among isolates was explored using fluorophore-enhanced rep-PCR (FERP) (Rademaker et al. 1998), as modified for agarose gels by Johnson et al. (2003).

The image of the fingerprints was captured using a Typhoon 8600 Multi-Mode Imager (Amersham Pharmacia Biotech, Piscataway, N.J.) and analyzed using BioNumerics (Version 2.5; Applied Maths, Kortrijk, Belgium). Pearson product–moment correlations (Häne et al. 1993) were calculated for the array of densitometric values formed by the fingerprints using NT-SYS (Rohlf 1998). Cluster analysis was performed using UPGMA (Sneath and Sokal 1973) and a dendrogram was constructed (NT-SYS, Rohlf 1998). The correlation matrix was compared with similarity matrices for inhibition, resistance (antibiotic and isolate pairing), utilized substrates, induction, and the16S rDNA sequence distance matrix, as described above.

Results

Inhibition and resistance

Isolates differed significantly in the frequency of isolates inhibited ($F_{[9,20]} = 41.83$, $P < 0.0001$; Table 2) and mean inhibition zone size ($F_{[9,290]} = 16.35$, $P < 0.0001$; Fig. 1A). Isolates LK2-12, LK4-21, and LK4-24 inhibited a significantly higher frequency of other isolates and had significantly greater mean zone sizes than other isolates. Although isolates LK4-2, LK4-16, and DL93 each inhibited other isolates (Table 2), the mean zone size for these isolates was not significantly different from zero (Fig. 1A).

Isolates differed significantly in the frequency of isolates resisted ($F_{[9,20]} = 37.99$, $P < 0.0001$; Table 2) and mean resistance zone size ($F_{[9,290]} = 8.17$, $P < 0.0001$; Fig. 1B). Isolates LK4-20 and LK4-21 resisted significantly more isolates than others in the collection and, when inhibited, had smaller inhibition zones than other isolates.

The number of other isolates that a strain could inhibit and the number of other isolates resisted were positively correlated ($r = 0.62$, $n = 10$, $P < 0.06$) and mean inhibition and resistance zone sizes were negatively correlated ($r = –0.47$, $n = 10$, $P < 0.17$), indicating that good inhibitors tended to be good resistors. The negative relationship between mean resistance zone size and number of isolates inhibited ($r = –0.54$, $n = 10$, $P < 0.11$) and the positive relationship between mean inhibition zone size and the number of isolates resisted ($r = 0.58$, $n = 10$, $P < 0.08$) further support this finding.

Isolates with similar inhibition profiles did not have similar resistance profiles: similarity matrices for inhibition and resistance patterns were not significantly correlated (Man-

Table 2. Phenotypic characteristics of 10 streptomycete isolates.

Isolate	No. inhibited*	No. resisted†	No. induced‡	No. induced by§
LK2-12	5	9	0	2
LK4-2	1	7	2	4¶
LK4-16	2	8	1	1¶
LK4-20	0	10	3	0
LK4-21	8	10	1	5¶
LK4-24	6	9	1	1
LK6-14	0	5	1	0
LK10-3	0	6	2	0
DL87	0	4	0	0
DL93	3	7	2	0

*Number of isolates (out of 10) that the isolate inhibited.
†Number of isolates (out of 10) that the isolate was resistant to.
‡Number of isolates (out of 10) in which the isolate induced antibiotic production.
§Number of isolates (out of 10) that induced the isolate to produce antibiotics.
¶Includes auto-induction.

Fig. 1. Mean inhibition (A) and resistance (B) zone size for 10 streptomycete isolates. Significant differences among means are indicated by different letters (least significant difference; $P < 0.05$). A large inhibition zone size indicates that an isolate is a good inhibitor; a small resistance zone size indicates that an isolate is a good resistor.

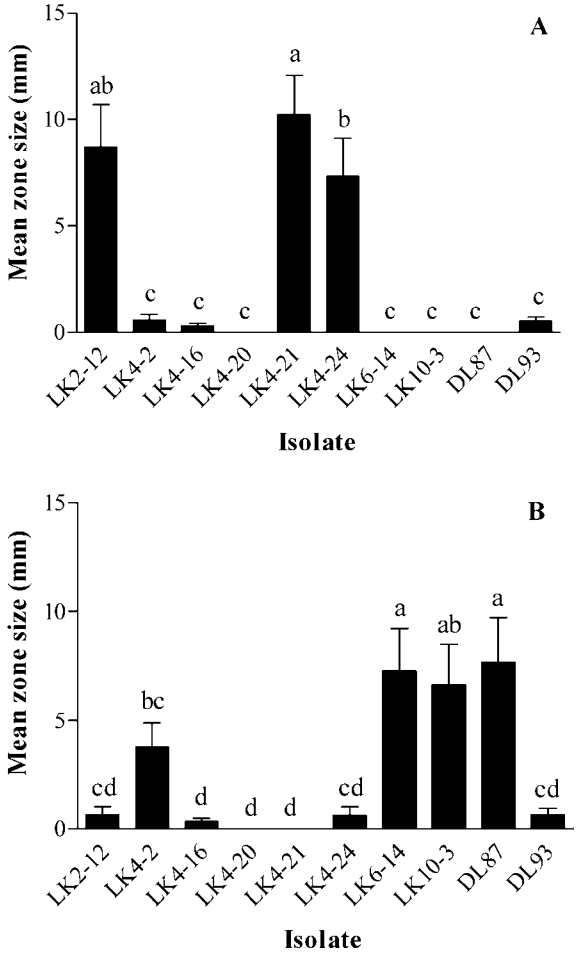


Table 3. Induction of antibiotic activity among 10 streptomycete isolates.

	Inducing isolate									
Isolate	LK2-12	LK4-2	LK4-16	LK4-20	LK4-21	LK4-24	LK6-14	LK10-3	DL87	DL93
LK2-12	0	+	0	0	0	0	0	0	0	+
LK4-2	0	+	0	+	0	0	+	+	0	0
LK4-16	0	0	+	0	0	0	0	0	0	0
LK4-20	0	0	0	0	0	0	0	0	0	0
LK4-21	0	0	0	+	+	+	0	+	0	+
LK4-24	0	0	0	+	0	0	0	0	0	0
LK6-14	0	0	0	0	0	0	0	0	0	0
LK10-3	0	0	0	0	0	0	0	0	0	0
DL87	0	0	0	0	0	0	0	0	0	0
DL93	0	0	0	0	0	0	0	0	0	0

Note: + indicates significant ($P < 0.05$) increase in mean inhibition zone size versus control ($n = 30$).

tel's test: $r = 0.25$, $P < 0.07$). Based on inhibition profiles, isolates clustered into two groups at 45% similarity: one group consisted of good inhibitors (LK2-12, LK4-21, LK4-24) and the other group included the remaining moderate to poor inhibitors. The dendrogram based on resistance profiles revealed that isolates again clustered into two groups, similar at 66%. One group consisted of isolates that resisted 8–10 other isolates; the other group contained isolates that resisted seven or fewer isolates. When inhibition and resistance profiles were combined, each of the 10 isolates examined had a unique inhibition/resistance profile. For example, although strains LK4-20 and LK4-21 were resistant to all other isolates, strain LK4-20 could not inhibit any other isolate, while strain LK4-21 was able to inhibit eight other isolates.

Induction

Five of the 10 isolates showed increased antibiotic activity in the presence of another isolate (Table 2). Isolate LK4-21 was most sensitive to the presence of other isolates: five isolates induced antibiotic activity in this strain. Isolates LK4-2, LK4-16, and LK4-21 were able to induce themselves. Only isolates LK2-12 and DL87 did not affect the antibiotic activity of at least one other isolate. Isolate LK4-20 induced antibiotic activity in three other isolates but was not inducible by isolates evaluated here. Only isolate DL87 could neither induce antibiotic activity in another isolate nor be induced by isolates considered in this work. The number of other isolates that an isolate could induce was not significantly correlated with either the number of other isolates inhibited ($r = –0.29$, $n = 10$, $P < 0.42$) or the number of other isolates resisted ($r = 0.18$, $n = 10$, $P < 0.63$). However, the number of isolates that a given isolate could be induced by was positively correlated with both the number of isolates inhibited ($r = 0.74$, $n = 10$, $P < 0.02$) and the number of isolates resisted ($r = 0.53$, $n = 10$, $P < 0.12$), indicating that good inhibitors and good resistors tended to have increased antibiotic activity in the presence of other isolates. The similarity matrix for ability to induce other isolates and inhibition (Mantel's test: $r = –0.10$, $P < 0.72$) and resistance ($r = –0.12$, $P < 0.77$) were poorly correlated. In addition, the similarity matrix for ability to be induced by other isolates was not significantly correlated with the resistance similarity matrix (Mantel's test: $r = –0.12$, $P < 0.74$). However, the similarity matrices for ability to be induced and for inhibition were significantly correlated (Mantel's test: $r = 0.43$, $P < 0.03$). Finally, the similarity matrices for ability to induce antibiotic activity in other isolates and for ability to be induced were poorly correlated with each other (Mantel's test: $r = –0.11$, $P < 0.70$).

When examining specific patterns of induction of antibiotic activity between isolates (Table 3), there was a trend for poor inhibitors to induce good or moderate inhibitors. Good inhibitors (LK2-12, LK4-21, LK4-24) induced at most one isolate. LK4-21 induced itself, while LK4-24 induced LK4-21. The antibiotic activity of poor inhibitors (LK4-20, LK6-14, LK10-3, DL87) was not increased by any of the isolates examined. Good resistors (LK2-12, LK4-16, LK4-20, LK4-21, LK4-24) tended to induce at most one other isolate but were inducible by one to five other isolates. The exception to this pattern was LK4-20, a good resistor, which induced three other isolates but was not inducible.

Nutrient utilization

Substrate richness (number of positive mean absorbance values) generally fell within a narrow range (89–94), with the exceptions of LK6-14 (78) and LK4-21 (62) (Table 4). In contrast, total activity (= ∑positive mean absorbance values) varied widely (Table 4), with LK4-2 having the greatest activity and LK4-21 having the least. When utilized substrates (mean absorbance > AWCD) were examined, each isolate had a unique nutrient utilization profile (Table 4; Fig. 2). Eight nutrients were utilized by all 10 isolates, and half of these nutrients were carbohydrates (α-D-glucose, maltotriose, turanose, *N*-acetyl-D-glucosamine, D-mannose). Glycerol, dextrin, and L-glutamic acid were also used by all isolates tested. Sixteen nutrients were not utilized by any isolate; the majority of these were carboxylic acids and carbohydrates.

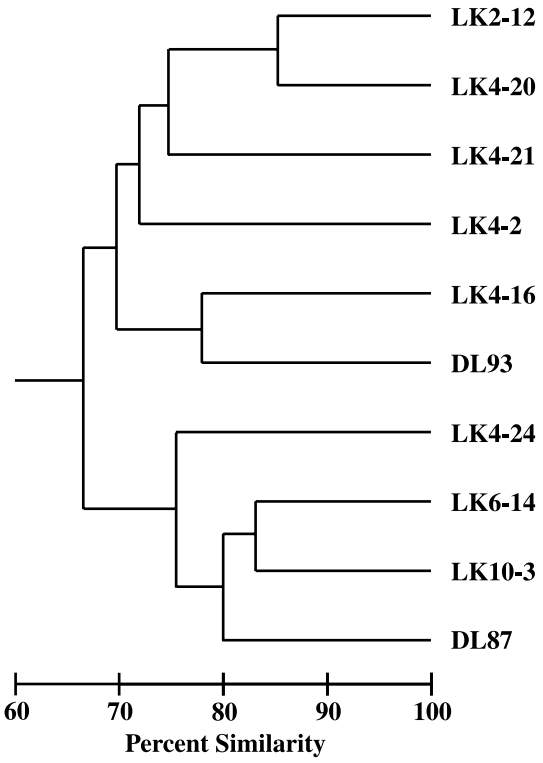
Examining correlations among nutrient utilization characteristics revealed that substrate richness and activity were significantly positively correlated ($r = 0.83$, $n = 10$, $P < 0.003$). In contrast, the number of utilized substrates was not related to either substrate richness ($r = –0.36$, $n = 10$, $P < 0.30$) or activity ($r = –0.30$, $n = 10$, $P < 0.41$). Further, the number of utilized substrates was not significantly correlated with any other phenotypic characteristic described above (results not presented). However, substrate richness was nega-

Table 4. Nutrient utilization characteristics of 10 streptomycete isolates.

Isolate	Substrate richness*	Total activity†	Utilized substrates‡
LK2-12	92	4.742	40
LK4-2	94	9.676	41
LK4-16	89	3.795	37
LK4-20	94	6.238	36
LK4-21	62	3.336	41
LK4-24	92	5.398	41
LK6-14	78	4.512	42
LK10-3	94	7.953	38
DL87	93	6.457	37
DL93	93	4.269	42

*Number of positive mean absorbance values.
†∑ positive mean absorbance values.
‡Number of mean absorbance values ≥total activity/95.

Fig. 2. Substrate utilization dendrogram based on simple matching and UPGMA (unweighted pair-group method, arithmetic average) clustering.



tively related to the number of isolates a strain could inhibit (r = –0.53, n = 10, P < 0.12), indicating that good inhibitors tended to use a narrower range of carbon sources. In addition, substrate richness and the number of isolates induced were positively related (r = 0.61, n = 10, P < 0.07); isolates capable of utilizing a wide variety of carbon sources were more likely to induce antibiotic activity in a greater number of isolates. The similarity matrix for utilized substrates was not significantly correlated with inhibition (Mantel's test: r = –0.02, P < 0.66) and resistance (r = 0.23, P < 0.07) similarity matrices. Further, the similarity matrix for utilized substrates was poorly correlated with similarity matrices related to induction of antibiotic activity (results not presented).

Based on utilized substrates, isolates clustered into two groups at 67% similarity (Fig. 2). One group (LK4-24, LK6-14, LK10-3, DL87) utilized a relatively high proportion of polymers and carbohydrates and a relatively low proportion of carboxylic acids and miscellaneous carbon sources. The other group utilized a relatively higher proportion of carboxylic acids and miscellaneous carbon sources. No significant differences among isolates were found in the number of substrates utilized in each guild (χ^2 = 3.35, df = 9, P < 0.95). Further, there was no difference among isolates in the proportion of substrates utilized within each of the six guilds (results not presented).

Antibiotic resistance screening

Each isolate had a unique resistance profile against the 10 antibiotics (Table 5). No isolate was susceptible or resistant to all concentrations examined of the 10 antibiotics. No isolate was resistant to neomycin at the highest concentration investigated. Isolate DL87 was susceptible to 9 out of 10 antibiotics and was the only isolate susceptible to novobiocin and chlortetracycline.

Similarity matrices from antibiotic resistance screening and resistance in isolate pairings were significantly correlated (Mantel's test: r = 0.45, P < 0.005). However, there was poor correlation between matrices from antibiotic resistance screening and inhibition (Mantel's test: r = –0.03, P < 0.57), utilized substrates (r = 0.22, P < 0.08), ability to induce other isolates (r = 0.11, P < 0.29), or ability to be induced (r = –0.32, P < 0.90).

Pathogenicity

Only isolate DL87 produced lesions on 'Snowden' and 'Kennebec' minitubers. All minitubers of these cultivars showed symptoms of scab following inoculation with isolate DL87. Only 20% of 'Norchip' minitubers tested showed scab symptoms when inoculated with isolate DL87. No other strain produced scab lesions on any cultivar.

16S rDNA sequences

Partial 16S rDNA sequences (885–1004 bp) for each of the 10 isolates were compared with sequences in GenBank using BLAST, and the closest bacterial relative was determined (Table 6). The sequences for 6 out of the 10 isolates were at least 96% identical to *S. lavendulae* 16S rDNA sequences. Examining the phylogenetic relatedness of the isolates based on partial 16S rDNA gene sequences (335 bp) revealed four clusters of isolates (Fig. 3A), each of which represented a different species group. The region within the 16S gene used in this analysis included the γ region (Stackebrandt et al. 1991; Anderson and Wellington 2001), which has been shown to be useful in resolving relationships within the streptomycetes (Kataoka et al. 1997; Anderson and Wellington 2001).

The distance matrix based on 16S rDNA sequences and the similarity matrices for inhibition (Mantel's test: r = 0.14, P < 0.25), resistance (r = –0.50, P < 0.999), antibiotic resistance screening (r = –0.68, P < 0.999), utilized substrates (r = –0.21, P < 0.92), ability to induce other isolates (r =

Table 5. Highest concentration of 10 antibiotics (see Table 1) that streptomycete isolates were resistant to after 7 days of growth.

Antibiotic	LK2-12	LK4-2	LK4-16	LK4-20	LK4-21	LK4-24	LK6-14	LK10-3	DL87	DL93
Erythromycin (μg/mL)	5	0	1	10	0	5	20*	20*	1	1
Spectinomycin (μg/mL)	2	10	2	2	20	10	20	40*	2	20
Chloramphenicol (μg/mL)	20	40*	10	40*	20	40*	40*	40*	2	10
Neomycin (μg/mL)	5	0.5	2.5	5	2.5	5	0.5	0.5	0	2.5
Novobiocin (μg/mL)	10*	10*	10*	10*	10*	10*	10*	10*	2.5	10*
Gentamicin (μg/mL)	2*	0.1	2*	2*	2*	2*	0	0	0	2*
Streptomycin sulfate (U/mL)	50	25	0	100*	5	25	5	100*	5	5
Chlortetracycline (U/mL)	400*	400*	400*	400*	400*	400*	400*	400*	200	400*
Penicillin (U/mL)	1000*	250	1000*	1000*	1000*	1000*	500	500	1000*	1000*
Bacitracin (U/mL)	20*	5	20*	20*	20*	20*	5	20*	1	20*

Note: 0 indicates that the isolate was susceptible to the antibiotic at all concentrations tested.
*Indicates highest concentration tested.

Table 6. Closest bacterial relative of the 10 isolates based on BLAST search.

Isolate	Species	% identity	GenBank acc. No.
LK2-12	*Streptomyces lavendulae*	98	AY277375
LK4-2	*Streptomyces platensis*	99	AY277376
LK4-16	*Streptomyces lavendulae*	98	AY277377
LK4-20	*Streptomyces lavendulae*	96	AY277381
LK4-21	*Streptomyces lavendulae*	96	AY277382
LK4-24	*Streptomyces lavendulae*	99	AY277378
LK6-14	*Streptomyces flavogriseus*	95	AY277384
LK10-3	*Streptomyces flavogriseus*	98	AY277379
DL87	*Streptomyces scabies*	97	AY277383
DL93	*Streptomyces lavendulae*	96	AY277380

0.06, $P < 0.40$), and ability to be induced ($r = 0.11$, $P < 0.37$) were not significantly correlated.

The 16S rDNA gene sequences of isolates in this reference collection have been deposited in the GenBank database under accession numbers AY277375–AY277384 (Table 6).

BOX-PCR genomic DNA fingerprints

Based on BOX-PCR fingerprints (300–3000 bp), the 10 isolates generally had very distinct genotypes (Fig. 3B). Using this method, repeated samples of the same streptomycete isolate varied by an r value of less than 0.95 (Xiao and Kinkel 2003). Among this collection of isolates, if fingerprint patterns having an r value greater than 0.95 were considered to be the same genotype, each isolate had a unique genotype.

The correlation matrix for genomic DNA fingerprints was poorly correlated with similarity matrices for inhibition (Mantel's test: $r = 0.06$, $P < 0.36$), resistance ($r = 0.22$, $P < 0.07$), antibiotic resistance screening ($r = 0.03$, $P < 0.43$), ability to induce ($r = –0.09$, $P < 0.74$), ability to be induced ($r = –0.15$, $P < 0.85$), and utilized nutrients ($r = 0.02$, $P < 0.44$). Further, there was no significant correlation between the correlation matrix and the distance matrix on the basis of 16S rDNA sequences (Mantel's test: $r = –0.17$, $P < 0.88$).

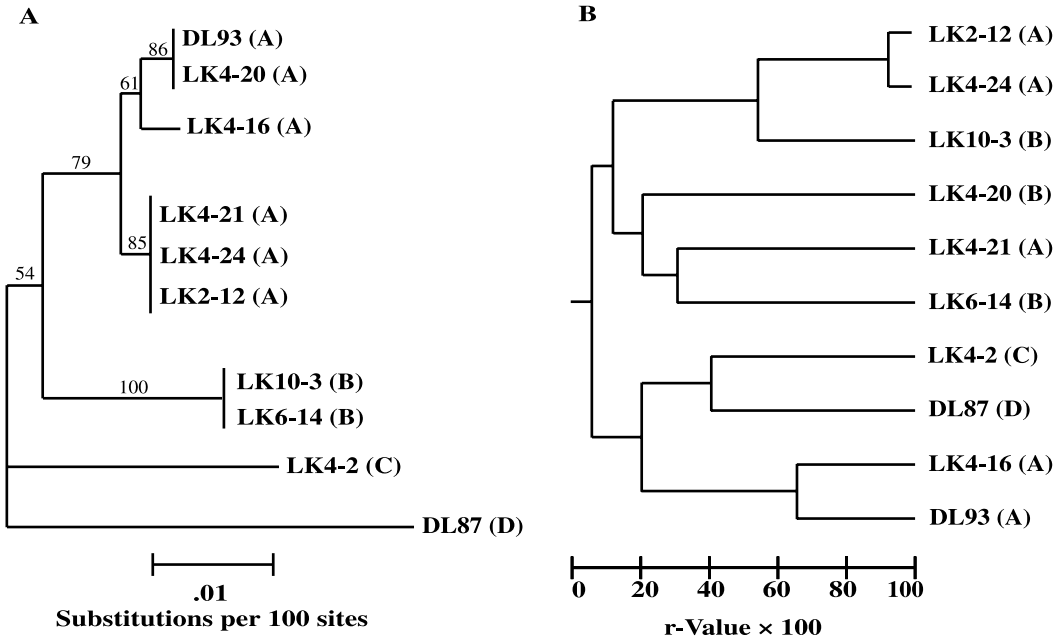
Discussion

Although the range of species represented in this collection was limited, each streptomycete isolate examined in this study possessed a unique set of phenotypic characteristics. Hypothetically, because any unknown field-collected isolate could inhibit or resist any of the 10 test isolates, this collection of test isolates is capable of distinguishing 1024 (2^{10}) different inhibition and 1024 resistance phenotypes (McVey and Leonard 1990). This range of phenotypes will enable field-collected microbes with a wide variety of antibiotic inhibition and resistance capabilities to be distinguished. In an analogous fashion, in recent studies investigating virulence in a variety of pathogens, differential sets of host lines that were similar in size to our collection of test standards enabled pathogen races to be distinguished (Chakraborty et al. 2002; Pietrek and Zinkernagel 2002; Wicker et al. 2003).

There was little correspondence between the phenotypic and genetic traits: isolates that were similar genetically were not more likely to be similar in nutrient utilization or inhibition/resistance profiles than were isolates that were genetically distinct. This result is consistent with other studies comparing genetic and phenotypic traits in soil bacteria (Brønstad et al. 1996; Bramwell et al. 1998; Lottmann and Berg 2001). In particular, the lack of consistency between phenotypic characters and taxonomic relationships has been suggested to reveal the problematic nature of the systematics within the streptomycetes (Bramwell et al. 1998). The lack of correlation between inhibition and resistance phenotypes, indicating that isolates having similar inhibitory phenotypes did not have similar resistance phenotypes, suggests that selection for specific inhibition and resistance abilities may be independent. In contrast, the significant correlation between the resistance to known antibiotics and resistance interactions among isolates confirms that the resistance evaluations performed using the pairwise interaction matrix provide substantial information on the capacity of an isolate to resist antibiotic inhibition.

Although the sample size was small, among isolates there were intriguing trends in relationships among phenotypic traits that suggest potential strategies by which microbes coexist within the soil community. The sole isolate that was pathogenic to potato in the collection (DL87) also was a poor inhibitor, a poor resistor, could not induce antibiotic ac-

Fig. 3. Phylogenetic and genetic relationships of 10 streptomycete isolates based on 16S rDNA gene sequences (335 bp) (A) and BOX-PCR genomic DNA fingerprints (B). Bootstrap values (based on 100 replicates) are given at branch points. Cluster designations (A–D) based on 16S rDNA gene sequences are shown in parentheses.



tivity in other isolates, and utilized relatively few nutrients. This may indicate that there is a trade-off in adopting a pathogenic strategy versus a competitive saprophytic strategy. Furthermore, among all isolates, good inhibitors tended to use fewer carbon sources. This suggests a potential trade-off between various competitive strategies, with individual isolates very effective in either interference competition (antibiotic production) or in sustaining a broad resource niche (nutrient utilization), but not both. Certainly, strong conclusions about the ecological relevance of these potential trade-offs cannot be drawn from our limited data set. However, these results indicate that rigorous examination of a wide variety of isolates should be conducted to determine the extent of these trade-offs in a broad range of ecological settings.

Isolates that were good inhibitors (LK2-12, LK4-21, LK4-24) were generally also good resistors. This may reflect the fact that habitats in which enhanced antibiotic inhibitory activity confers a fitness advantage are apt to be habitats in which resistance to inhibition is also critical to fitness. The good inhibitors or resistors were also most likely to produce more intense inhibition in the presence than in the absence of another isolate (i.e., be induced). Considering that antibiotics can be metabolically costly (Maplestone et al. 1992), but can also confer an advantage to the producer (Williams et al. 1989*a*; Maplestone et al. 1992), it seems reasonable that those organisms that possess significant inhibitory capability may be most likely to respond to environmental cues to minimize the costs and maximize the benefits of antibiotic production.

This phenotypically diverse collection of reference isolates provides the potential for distinguishing a wide variety of inhibitory, resistance, and induction capabilities among unknown, field-collected microbes. Future work will explore the use of this reference collection in characterizing microbial communities in time and space. In addition, relationships among phenotypic characteristics in this collection of isolates provide preliminary insight into the distinct strategies that microbes may use in optimizing their fitness in natural environments and on the factors that may be significant to the generation of antibiotic inhibitory and resistance activities in field populations. Clearly, more detailed experiments are required to investigate these strategies and their relative importance to the fitness of microbes in the environment.

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