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Molecular Detection of Streptomycin-Producing Streptomycetes in Brazilian Soils

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Actinomycetes were isolated from soybean rhizosphere soil collected at two field sites in Brazil. All the isolates were identified as *Streptomyces* species and were screened for streptomycin production and the presence of two genes, *strA* and *strB1*, known to be involved in streptomycin biosynthesis in *Streptomyces griseus*. Antibiotic resistance profiles were determined for 53 isolates from cultivated and uncultivated sites, and approximately half the strains were streptomycin resistant. Clustering by the unweighted pair group method with averages indicated the presence of two major clusters, with the majority of resistant strains from cultivated sites being placed in cluster 1. Only representatives from this cluster contained *strA*. Streptomycetes containing *strA* and *strB1* were phenotypically diverse, and only half could be assigned to known species. Sequence comparison of 16S rRNA and *trpBA* (tryptophan synthetase) genes revealed that streptomycin-producing streptomycetes were phylogenetically diverse. It appeared that a population of streptomycetes had colonized the rhizosphere and that a proportion of these were capable of streptomycin production.

The ecological importance of antibiotic production by soil actinomycetes has been debated, but only limited evidence for production in natural environments was obtained (45). The production of antibiotics by streptomycetes was demonstrated with strains inoculated into sterile soil; the compounds detected included chloramphenicol (9), geldanamycin (29), and thiostrepton (41). Evidence for production under more natural conditions was difficult to obtain, possibly due to problems associated with adsorption of the antibiotics to clays and the very small amounts likely to be produced in oligotrophic environments such as soil (43). Streptomycetes have been recommended for the biocontrol of fungal root and seed pathogens (15, 37) but are better known as prolific producers of commercially important clinical antibiotics (22).

The control of soil-borne pathogens and use of bacteria as biocontrol agents in the rhizosphere has led to more recent evidence for the importance of antibiosis. Evidence for antibiosis via the control of soil-borne pathogens has been well documented and is reviewed by Weller and Thomashow (38). It appears that antibiotic and bioactive metabolites are produced where nutrient levels are enhanced in the rhizosphere, and detection is density dependant, requiring a high inoculum level.

The central highland region of Brazil is dominated by acidic, infertile savannah soil known as *Cerrados*. Liming is required to prevent aluminum toxicity after clearing and burning, before planting of leguminous crops. Yields of soybean can potentially be increased by up to 30% by using seed inoculation with efficient hydrogenase-positive (Hup^+) strains of *Bradyrhizobium japonicum*, when compared to the yields obtained with existing commercial inoculants (21). Establishment of these more efficient strains depends upon their ability to compete and nodulate under the prevailing environmental conditions. Studies have shown that bradyrhizobia reisolated after 2 years from limed soil plots grown with successive crops of *Phaseolus vulgaris* exhibited tolerance to a wide range of antibiotics when compared to bradyrhizobia isolated from unlimed acid soil (28). Scotti et al. (31) found that *B. japonicum* strains selected for ability to nodulate soybean in newly cleared Cerrado were highly resistant to streptomycin. Other researchers have also demonstrated high levels of intrinsic streptomycin and spectinomycin resistance among root nodule bacteria (3, 20, 30, 35).

Streptomycin and related compounds such as bluensomycin and hydroxystreptomycin are produced by several different *Streptomyces* species. The biosynthetic gene cluster in two species, *Streptomyces griseus* and *S. glaucescens*, has been well studied (6). Genes for resistance and biosynthesis have been cloned and characterized (27). The gene cluster is comprised of over 30 genes, including an aminoglycoside phosphotransferase gene, *strA*, for streptomycin resistance. The arrangement of genes within the clusters is conserved between *S. griseus* (streptomycin), *S. glaucescens* (hydroxystreptomycin), and *S. bluensis* (bluensomycin), but there is variable homology between specific genes. The biosynthetic gene *strB1*, which codes for an amidinotransferase, is the most highly conserved, with over 84% identity at the DNA level in *S. griseus*, *S. glaucescens*, and *S. bluensis*. The resistance gene *strA* is more variable, with *S. griseus* and *S. glaucescens* genes sharing 75% identity (27).

The aim of this study was to isolate and identify streptomycin producers from soybean rhizosphere soils in Brazil to establish the potential for antagonism against *Bradyrhizobium japonicum*. We wanted to determine the taxonomic diversity of these isolates to establish if the streptomycin-producing microorganisms were from one species only. Alternatively, this characteristic may have been distributed in a range of taxa colo-

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TABLE 1. Streptomycetes used in this investigation

No. investigated	Taxonomic identification[a]	Name
24	1	*S. griseus* DSM 40236[b], *S. griseus* DSM 40236,[c] *S. griseus* ATCC 12475; *S. griseus* N-2-3-11e, *S. ornatus* ATCC 25481, *S. ornatus* ISP 5307, *S. nitrosporeus* DSM 40023, *S. naraensis* DSM 40508, *S. flavovirens* ATCC 3320, *S. sindensis* ISP 5255, *S. willmorei* ISP 5459, *S. bikiniensis* ISP 5255, *S. coelicolor* ISP 5233, *S. globisporus* ISP 5199, *S. limosus* ISP 5131, *S. fellus* ISP 5130, *S. olivaceiscleroticus* ISP 5595, *S. canescens* ISP 5001, *S. rutgerensis* subsp. *rutgerensis* ISP 5077, *S. ororifer* ISP 5347, *S. gougerotii* ISP 5324, *S. albidoflavus* ISP 5455, *S. intermedius* ISP 5372
1	3	*S. scabies* ISP 5078
1	6	*S. violaceus* ISP 5082
1	10	*S. fulvissimus* ISP 5593
1	12	*S. griseorubens* ISP 5162
1	16	*S. albus* ISP 5313
1	18	*S. cyaneus* ISP 5085
2	19	*S. diastochromogenes* ISP 5745, *S. humidus* ATCC 12760
4	21	*S. lividans* ISP 5434, *S. lividans* TK23, *S. lividans* TK21, *S. coelicolor* A3(2)
2	28	*S. glaucescens* DSM 40716, *S. glaucescens* DSM 40155
2	32	*S. hygroscopicus* ATCC 14607, *S. hygroscopicus* ATCC 21705
1	42	*S. rimosus*
2	55[d]	*S. mashuensis* ATCC 23934, *S. hachijoensis* KCCS331
1	58[d]	*S. griseoverticullatum* S767
1	61	*S. subrutilus* ATCC 27467
1	61	*S. subrutilus* DSM 40446
1	64	*S. bikiniensis* ATCC 11062
1	68	*S. fradiae* ISP 4037
25		Santa Fé soil isolates
49		Brasilia soil isolates
10		Warwick culture collection soil isolates

[a] The cluster designation of type strains was that of Williams et al. (46). Specific Santa Fé and Brasilia soil isolates are listed in Table 3. Warwick Culture Collection isolates are characterized by Phillips (24).
[b] Strain obtained from DSM.
[c] Strains obtained from W. Piepersberg.
[d] Formerly *Streptoverticillium.*

nizing the rhizosphere. The population of highly streptomycin-resistant streptomycetes was not recovered from virgin soil prior to planting of soybeans. Comparisons were made with populations isolated from virgin and limed, previously cultivated nonrhizosphere soil to determine the enrichment effects of the rhizosphere.

MATERIALS AND METHODS

Bacterial strains. All streptomycetes used throughout this study are given in Table 1. Type strains were obtained from either the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) or the American Type Culture Collection (ATCC). *S. lividans* TK21 and 23 were kindly supplied by T. Kieser, John Innes Institute, Norwich, England. Strains isolated from Brazilian Cerrado soils are prefixed by the letters AS_B (Brasilia) and AS_{SF} (Santa Fé) (Table 1) referring to the field sites where nodulation trials were conducted. All strains were maintained on tryptone soya agar (Oxoid) or oatmeal agar (44). The strains were incubated at 28 to 30°C for 5 to 7 days. Long-term storage was facilitated by the preparation of spore suspensions by the method of Hopwood et al. (11). Spore suspensions were then stored in 10% (vol/vol) glycerol at −20°C. *Salmonella typhimurium* (ATCC 15277 and ATCC 10249) was maintained on nutrient agar (Oxoid), and *Escherichia coli* 711 was maintained on nutrient agar with 35 μg of nalidixic acid per liter. These were cultured at 37°C in nutrient broth (Oxoid) for use in bioassays. *B. japonicum* strains were grown on yeast extract-mannitol agar (YMA) (1) for use in antagonism studies and stored on YMA slopes supplemented with 3% $CaCO_3$ at 4°C.

Soils. Soils were obtained from two sites in the Cerrado region of Brazil, Brasilia and Santa Fé. Details of the soils can be obtained directly from Empresa Brasileira de Pesquisa Agrophquaria (EMBRAPA). At the Brasilia site, samples were taken from virgin (not previously cultivated) and cultivated soils. Bulk samples were taken from the former, and rhizosphere samples were collected from the latter. At Santa Fé, only previously cultivated soil was available, so that the soil samples were from bulk unlimed previously cultivated soil and from the rhizosphere of limed cultivated soil.

Isolation and characterization of actinomycetes. Isolations were performed by the dilution plate procedure (2) with one-fourth-strength Ringer's solution and Waksman and Henrici or Thorntons medium. Isolations were also made by dilution spread plating (40) with one-fourth-strength Ringer's solution and reduced arginine starch salts agar (10) with and without the addition of streptomycin at 50, 25, 10, 5, or 1 μg/ml. All plates were incubated at 28°C for 7 days. Streptomycetes were identified by direct examination via bifocal microscopy (magnification, ×50). Selected streptomycetes (approximately 300) were subcultured onto oatmeal or tryptone soya agar. Soil isolates were identified by the methods of Williams et al. (47). Antibiotic resistance profiles were determined for 46 isolates from Brasilia (20 from virgin soil, 26 from limed rhizosphere soil), 7 Santa Fe (3 from unlimed soil, 4 from limed rhizosphere soil), and 18 soil isolates from the Warwick culture collection (which were included for comparison). 14 *Streptomyces* type strains, known to produce aminoglycoside antibiotics, were included to determine whether there was any correlation between resistance profile and the antibiotic produced. The Brazilian isolates included were selected randomly. The antibiotics used were streptomycin, thiostrepton, erythromycin, and oxytetracycline at 0, 2.5, 7.5, 15, 25, 37, and 50 μg/ml. Neomycin was also used at 10 μg/ml. Resistance was determined by observing whether any growth occurred on starch caesin medium (16) containing the specified concentration of antibiotic. The data was analyzed in binary form with the NTSYS-pc (Exeter Publishing Ltd.). Measures of similarity were determined for the isolates by using the simple matching coefficient (S_{SM}) (34), and clusters were resolved by using the unweighted pair group arithmetric average algorithm (UPGMA). The test error was calculated by determining the probability of obtaining an erroneous result (33) for each antibiotic. A test error of 5% or less was acceptable (33).

Streptomycin production was detected by bioassay and bioautography. For the bioassays, filter discs containing broth extracts were placed onto a prepoured nutrient agar bioassay plate. A seeded overlay of soft nutrient agar was poured over the filter discs. The overlay was seeded with *E. coli* 711 (streptomycin MIC, 0.05 mg/ml), *S. typhimurium* ATCC 15277 (streptomycin MIC, 0.00002 mg/ml), or *S. typhimurium* ATCC 10249 (streptomycin MIC, 3 mg/ml). The plates were then incubated at 37°C overnight, and the inhibition zone was measured. Streptomycin production was confirmed by bioautography by a method adapted from that of Phillips et al. (25). Six-day-old streptomycete broth cultures were dried in an oven at 80°C, and the residue was resuspended in 0.2 ml of methanol. This was then subjected to thin-layer chromatography by previously described methods (25) with a solvent system of butanol-acetic acid-water (3:1:1, vol/vol/vol). Duplicate plates were used; they were visualized by inverting the prerun thin-layer chromatography plate on a 200-ml base layer of nutrient agar in a Nunc bioassay dish. These were left for 30 min to allow the metabolites to diffuse into the medium. The plates were removed and discarded. The bioassay dishes were seeded with overlays containing 200 μl of either *S. typhimurium* ATCC 15277 or *S. typhimurium* ATCC 10249 and incubated at 37°C overnight. Streptomycin production was indicated by zones of inhibition with an R_f of 0.2 when *S. typhimurium* ATCC

TABLE 2. Distribution of streptomycin resistance in streptomycetes

Species or isolate	Strain	Cluster[a]	ARPG[b]	Resistance to streptomycin[c]	Production of streptomycin[d]
S. griseus	DSM 40236[e]	1	1i	+	+
S. griseus	DSM 40236[f]	1	1ii	+	+
S. griseus	ATCC 12475	1	1i	+	+
S. griseus	N-2-3-11[f]	1	1ii	+	+
S. ornatus	ATCC 25481	1	1iii	+	+
S. naraensis	DSM 40508	1	1i	+	−
S. fluorescens	ATCC 23907	1	1v	+	−
S. nitrosporeus	DSM 40023	1	2i	−	−
S. humidus	ATCC 12760	19	1i	+	+[g]
S. hygroscopicus	ATCC 14607	32	1v	+	+[h]
S. hygroscopicus	ATCC 21705	32	1iii	+	+[h]
S. hachijoensis	KCCS331	55	1v	+	−
S. subrutilus	DSM 40446	61	1iii	+	+[i]
S. bikiniensis	ATCC 11062	64	1i	+	+
Santa Fé soil isolates	7 AS_{SF} strains	Table 3[k]	1ii, 1iii and 2i	5	Table 3
Brasilia soil isolates	46 AS_B strains	Table 3	1i, 1iii, 1iv, 2i, 2ii and 2iii	21	Table 3
Warwick culture collection soil isolates	18 WCC strains[j]		1i, 1iii, 2i, 2ii and 2iii	10	2

[a] Cluster designations of type strains are those of Williams et al. (46).
[b] ARPG, antibiotic resistance profile group (see Fig. 1).
[c] Streptomycin resistance measured at 10 μg/ml.
[d] Streptomycin production was determined by bioassay.
[e] Strain obtained from DSM.
[f] Strain obtained from W. Piepersberg.
[g] Dihydrostreptomycin.
[h] Glebomycin.
[i] Hydroxystreptomycin.
[j] Warwick Culture Collection isolates characterized by Phillips (24).
[k] Table 3, refer to Table 3 for specific strains.

15277 was used and no corresponding zone when *S. typhimurium* ATCC 10249 was used.

Phosphotransferase production was detected by assaying the enzyme activity in cell supernatants by the method of Shinkawa et al. (32). Streptomycetes were grown in GMP medium (32) at 28°C. Cell samples were taken at 24-h intervals and investigated for activity.

Antagonism by streptomycetes toward *B. japonicum* strains was determined by bioassay. YMA (300 ml) was used for the basal layer, which was inoculated with *B. japonicum* (25 ml of a 4-day yeast extract-mannitol broth culture). Filter discs containing streptomycete broth extracts were placed on the *B. japonicum* lawn, the plates were incubated at 25°C for 7 days, and antagonism was scored according to the zone of inhibition size: 0, no zone of inhibition; 1, zone of inhibition between 6 and 10 mm; 2, zone of inhibition between 11 and 14 mm and 3, zone of inhibition larger than 14 mm. Streptomycetes that scored 2 or 3 were considered to be causing antagonism. All bioassays were done in triplicate.

Chromosomal DNA extraction and purification from streptomycete isolates. Streptomycete DNA was isolated by the method of Fisher (procedure 4 in reference 11). DNA was resuspended in 50 μl of Tris-EDTA (TE) buffer and stored at 4°C until required.

Total community DNA. Total DNA was extracted from soil by a method based on the bead-beating technique described by Cresswell et al. (4, 5). Soil (10 g) was bead beaten in 20 ml of sodium phosphate buffer (pH 8) with glass beads with diameters of 0.10 to 0.11 mm. After 5 min, the suspension of beads and soil was recovered and centrifuged at 1,660 × *g* for 10 min. DNA was precipitated from the recovered supernatant by the addition of 1.0 volume of ethanol and 0.1 volume of 3 M sodium acetate. After being mixed, solution was left overnight at −20°C. The sample was then centrifuged at 2260 × *g* for 10 min, and the resultant pellet was resuspended in 5 ml of TE buffer. The DNA was purified by phenol-chloroform extraction and reprecipitated with ethanol by standard procedures (38); the final pellet was resuspended in 100 μl of TE buffer. Samples were stored at 4°C until required. The specific target (*strA*) was quantitated by most-probable-number PCR (26). Sterile Santa Fé soil samples (1 g) were inoculated with a dilutions series of *S. griseus* spores from 10^8 to 10^{-1}/g of soil. The DNA was extracted from these soil samples by the method described above. Sterile uninoculated soil was also included as a negative control. All samples were done in triplicate. The detection limit for *strA* in the soil was then determined by PCR with the specific *strA* PCR primers and Southern hybridization by the methods described below.

Amplification, cloning, and sequencing. Four sets of primers were selected for use in PCR amplification experiments. The first set selectively amplified the *strA* streptomycin phosphotransferase gene (17) to give a product of approximately 920 bp with *S. griseus* DSM 40236, ATCC 12475, or N-2-3-11 chromosomal DNA as the template. *strA* primers were 5′-ATG AGT TCG TCG GAC CAC AT-3′ (forward) and 5′-TCA GGG CTT CGC CAG CGC TT-3′ (reverse). The second set amplified *strB1*, a biosynthetic gene that codes for streptomycin amidinotransferase (6), 5′-TG AGC CTT GTA AGC GTC CAC-3′ (forward) and 5′-TT CAT GCC GTG CTT CTC CAG-3′ (reverse) to yield a 940-bp product with *S. griseus* DSM 40236, ATCC 12475, or N-2-3-11 and *S. glaucescens* DSM 40716 or DSM 40155. The 16S rRNA primers were 5′-GGG ATT AGT GGC GAA CGG GTG AGT AAC-3′ (forward) and 5′-CCT GAC GCG GCG ACG CTG CAT CAG GCT-3′ (reverse) to yield a 267-bp product that included the highly variable gamma region (36). The tryptophan synthase (*trpBA*) primers were 5′-G CGG TAC CCG ATC TCG GCC GGT CTG GAC TA-3′ (forward) and 5′-C CCT CGA GCA CCG GGT CGC TGT GCG GC-3′ (reverse) and amplified a 492-bp region that included the β subunit of *trpB* and the α subunit of *trpA*. The protocol for all sets of primers was the same except for different primer-annealing temperatures in the cycling protocol.

The reaction mixture for PCR amplification was prepared as follows: primer 1, 100 ng; primer 2, 100 ng; dimethyl sulfoxide, 5 μl; bovine serum albumin, 5 μl (10 mg/ml; Sigma); $MgCl_2$, 5 μl (25 mM; Perkin Elmer Cetus); 10 × PCR buffer, 5 μl (Perkin-Elmer Cetus, $MgCl_2$ free); deoxynucleoside triphosphates (dNTPs), 2 μl (10 mM each dNTP); H_2O, to 48.5 μl. DNA (1 μl) was added to the reaction mixture, at 1 to 10 ng of chromosomal DNA or 1 μl of 1:9-diluted purified soil DNA, as a template. After addition of the template, 50 μl of mineral oil was added as an overlay. The PCR cycling protocol used a hot start of 95°C for 10 min, after which the temperature was reduced to 80°C to allow the addition of 0.5 μl (2.5 U) of Perkin-Elmer Cetus Amplitaq. Cycling then continued with an annealing temperature of either 57°C (*strA*, 16S rRNA, and *trpBA*) or 55°C (*strB1*) for 1 min, an extension temperature of 72°C for 2 min, followed by a denaturation temperature of 94°C for 1 min. The number of cycles carried out was normally between 30 and 35. After amplification, the oil overlay was extracted by the addition of an equal volume of chloroform followed by mixing and centrifugation (11,600 × *g* for 2 min). The aqueous phase was recovered and stored at 4°C until required. PCRs were checked for product by standard electrophoresis procedures with 1.0% (wt/vol) agarose gels and TAE buffer (18). Fragments corresponding to the expected size were excised and purified with the Wizard miniprep kit (Promega). The fragments were cloned into the vector pGEM-T (Promega) by the methods recommended by the manufacturer. Plasmid sequencing was performed with the Sequenase 2.0 kit (United States Biochemical Corp.).

Hybridization studies. Whole gene probes were made by using digoxigenin-11-dUTP–dTTP (3.5:6.5) incorporated into PCR mixes with *S. griseus* ATCC 12475 DNA as the template and *strA* or *strB1* PCR primers. The dNTP stock solution used for these reactions was from Boehringer Mannheim. A 5-μl volume of 10× dNTPs was used in place of 2 μl of 10 mM dNTP stocks in standard reactions. Probes were manufactured by 30 cycles of amplification and were

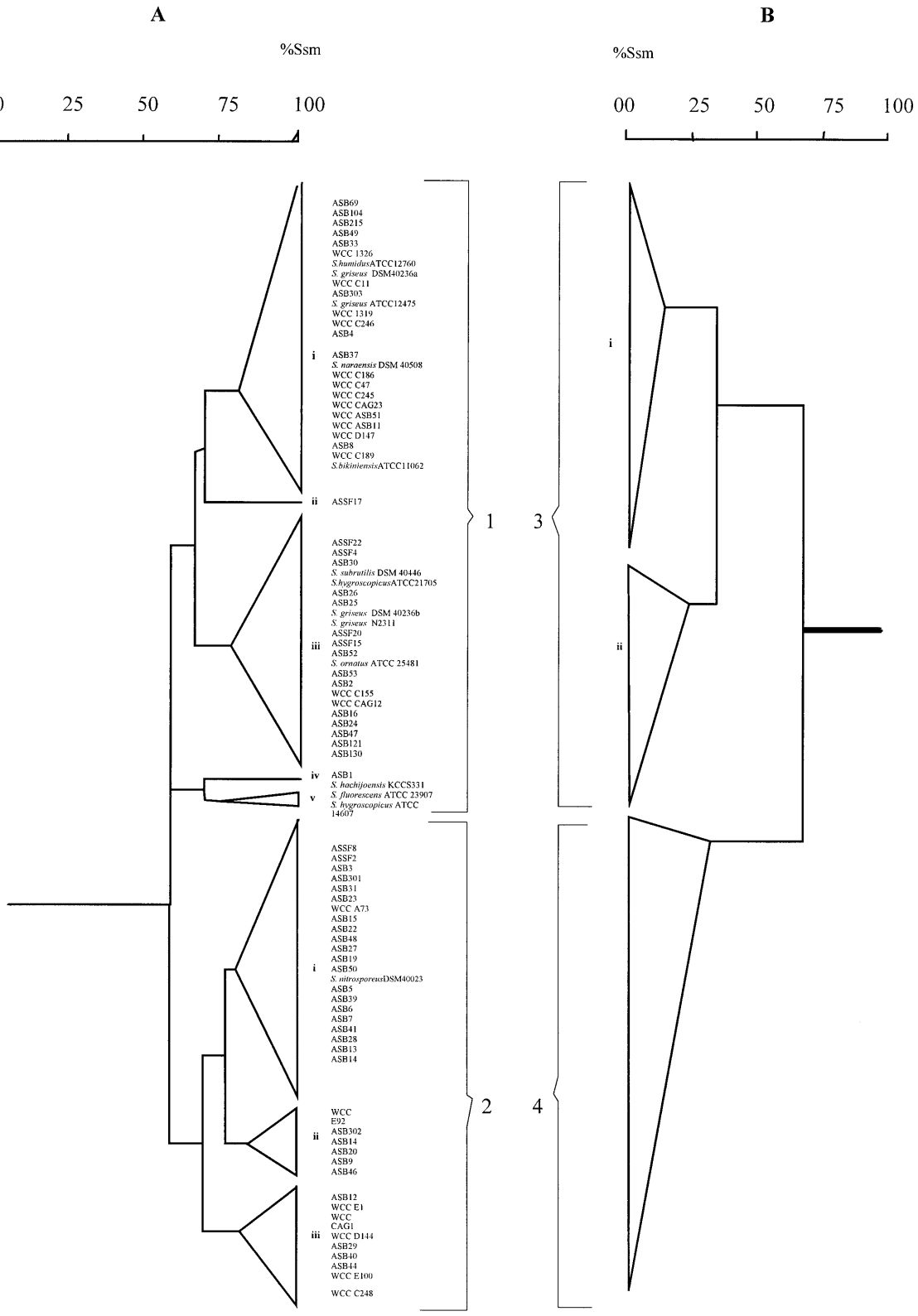

FIG. 1. Phenograms based on antibiotic resistance profiles patterns of 86 *Streptomyces* spp. Similarities were calculated by S_{SM} and clustered by using UPGMA. (A) Data from resistance profiles obtained with streptomycin, thiostrepton, neomycin, erythromycin, and oxytetracycline. Two groups were recovered at 60% S_{SM} (groups 1 and 2). (B) Data from streptomycin resistance profiles. Two groups were recovered at 30% S_{SM} (groups 3 and 4).

TABLE 3. Characterization of antibiotic-producing streptomycetes isolated from Cerrado rhizosphere soil

Strain	Strain source[a]	Identity[b]	Cluster[c]	Streptomycin production[d]	Phosphotransferase activity[e]	*strA* blot	*strB1* blot
DMS 40236		*S. griseus*[f]	1	+	+	+	+
ATCC 12475		*S. griseus*[f]	1	+	+	+	+
N2-3-11		*S. griseus*[f]	1	+	+	+	+
DSM 40716		*S. glaucescens*[f]	28	+	+	−	+
DSM 40155		*S. glaucescens*[f]	28	+	+	−	+
AS_B 49	R	*S. albidoflavus*	1	+	±	+	+
AS_{SF} 4	R	*S. albidoflavus*	1	+	±	+	+
AS_B 33	R	*S. violaceus*	6	+	±	+	+
AS_{SF} 17	R	*S. phaeochromogenes*	40	+	+	+	+
AS_{SF} 8	R	*S. phaeochromogenes*	40	−	−	−	−
AS_{SF} 22	R	Low score	1	±	±	+	−
AS_B 28	B	Low score	6	+	+	−	−
AS_{SF} 10	B	Low score	15	−	−	−	−
AS_{SF} 15	R	Low score	15	−	+	+	−
AS_B 37	R	Low score	20	+	+	+	+

[a] Indicates source of wild isolates from bulk (B) or rhizosphere (R) soil.
[b] Identification was by the method of Willcox et al. (42), where a low score is indicated for probabilities of <0.85.
[c] Cluster designation of Williams et al. (46).
[d] Streptomycin production was determined by bioassays and bioautography.
[e] ±, variable level of activity (see Materials and Methods for assay details).
[f] Streptomycin- and hydroxystreptomycin-producing type strains included as positive controls.

checked by the procedures outlined above. The *strA* and *strB1* digoxigenin-11-dUTP gene probes migrated slower than ordinary PCR product, due to the incorporation of the digoxigenin label. DNA samples required for hybridization were subjected to electrophoresis (18) with 1.0% (wt/vol) agarose gels before being Southern blotted. Blotting was carried out with Hybond-N nylon filters (Amersham International), and DNA was fixed by UV cross-linking.

The blots were hybridized with digoxigenin-labelled probes by the methods described for the Boehringer Mannheim nonradioactive DNA-labelling kit, with some minor modifications. All solutions with blocking reagent (vial 11) contained 1.5% (wt/vol) instead of 0.5%. Hybridizations were done at 70°C, not 68°C. Stringency washes were also carried out at this temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% (wt/vol) sodium dodecyl sulfate for two 15-min washes followed by 0.1× SSC–0.1% (wt/vol) sodium dodecyl sulfate for two 15-min washes. Color development to visualize hybridized probe was carried out by the Boehringer Mannheim protocol.

Sequence alignment and phylogenetic reconstruction. Sequence data was aligned in GCG (7a). The Fitch-Margoliash criterion (7) was then used to construct bootstrapped distance matrix trees for the 16S rRNA sequences and the *trpBA* sequences by using SEQBOOT, DNADIST, FITCH, and CONSENSE in the PHYLIP Package (7).

Statistical analysis. All the isolation data was subject to statistical analysis with the MINITAB statistical package (Minitab Statistical Software, State College, Pa.). All isolations were done in triplicate, and an analysis of variance was calculated for each data set. Minimum significant differences (MSD) between means were calculated from analysis of variance by the method of Peterson (23).

Nucleotide sequence accession numbers. The GenBank accession numbers of the sequences reported in this study are as follows. *strA* sequences for *Streptomyces* soil isolate AS_{SF} 17, U59006 and U59007. 16S rRNA sequences: *Streptomyces hygroscopicus* ATCC 14607, U72167; *Streptomyces griseus* N2-3-11, U72168; *Streptomyces humidus* ATCC 12760, U72169; *Streptomyces griseus* ATCC 12475, U72170; *Streptomyces flavovirens* ATCC 3320, U72171; *Streptomyces subrutilus* ATCC 27467, U72172; *Streptomyces mashuensis* ATCC 23934; U72173; *Streptomyces bikiniensis* ATCC 11062, U72174; *Streptomyces glaucescens* DSM 40716, U72175; *Streptomyces coelicolor* A3(2), U72176; *Streptomyces* soil isolate AS_B 37, U72177; *Streptomyces* soil isolate AS_{SF} 17, U72178; *Streptomyces lividans* TK21, U72179. *trpB/A* sequences: *Streptomyces hygroscopicus* ATCC 14607, U72180; *Streptomyces humidus* ATCC 12760, U72181; *Streptomyces griseus* ATCC 12475, U72182; *Streptomyces griseus*, N2-3-11, U72183; *Streptomyces* soil isolate AS_B 37, U72184; *Streptomyces coelicolor* A3(2), U72185; *Streptomyces bikiniensis* ATCC 11062, U72186; *Streptomyces flavovirens* ATCC 3320, U72187; *Streptomyces subrutilus* ATCC 27467, U72188; *Streptomyces mashuensis* ATCC 23934, U72189; *Streptomyces* soil isolate AS_{SF} 17, U72190.

RESULTS

Isolation studies. Actinomycetes were isolated from cultivated limed soybean rhizosphere and from virgin, unlimed, uncultivated bulk soil sites in Brasilia and from a cultivated site in Santa Fé that was sampled before liming and then 28 days after liming. Isolation media used either no selection (Brasilia, Santa Fé virgin, and Santa Fé limed) or streptomycin (25 and 50 μg/ml) selection (Santa Fé unlimed and limed). When no selection was used, 10^7 CFU of actinomycetes per g was detected in both Santa Fé soil types (MSDs, 0.35 and 0.47, respectively). A streptomycin concentration of 25 μg/ml in the isolation medium resulted in 1.3×10^4 CFU/g (MSD, 0.27) was detected from the unlimed Santa Fé soil and 1.37×10^6 CFU/g (MSD, 0.33) was detected from the limed Santa Fé soil. When the streptomycin concentration in the isolation medium was further increased to 50 μg/ml, no isolates were obtained from the virgin Santa Fé soil whereas 1.15×10^5 CFU/g (MSD, 0.61) was detected from the limed Santa Fé soil.

Antibiotic resistance profiles were determined for a selection of soil isolates. The test error for each antibiotic was calculated by using the formula of Sneath and Johnson (33). Thiostrepton, erythromycin, oxytetracycline, and neomycin all had values within the acceptable limit (<5%). Streptomycin resistance was significantly variable only at the highest concentration used (50 μg/ml).

The complete antibiotic resistance profiles were compared by using the simple matching coefficient (S_{SM}) and clustered by UPGMA (Fig. 1A). Two major clusters were recovered at the 60% S_{SM} level (1 and 2), and eight minor clusters were recovered at the 75% S_{SM} level; these were labelled 1i through to 1v and 2i through to 2iii. Members of cluster 1 were resistant to streptomycin (>2.5 μg/ml), and all the strains in cluster 2 were sensitive (>2.5 μg/ml). All the known streptomycin producers were recovered in cluster 1. The Brazilian soil isolates were distributed throughout the phenogram; 21 of the Brasilia rhizosphere isolates were found in cluster 1, and the remaining 5 were in cluster 2, along with the 20 Brasilia bulk isolates. Four Brasilia bulk isolates positioned in cluster 2 (AS_B 13, AS_B 19, AS_B 27, and AS_B 28) were resistant to 2.5 μg of streptomycin per ml and sensitive to the higher concentrations used. Five of the Santa Fé isolates were positioned in cluster 1 and were resistant to streptomycin (>2.5 μg/ml); four of these were from rhizosphere soil, and one (AS_{SF} 20) was from bulk soil. The two remaining Santa Fé isolates were positioned in cluster 2 and were sensitive to streptomycin. All Santa Fé rhizosphere

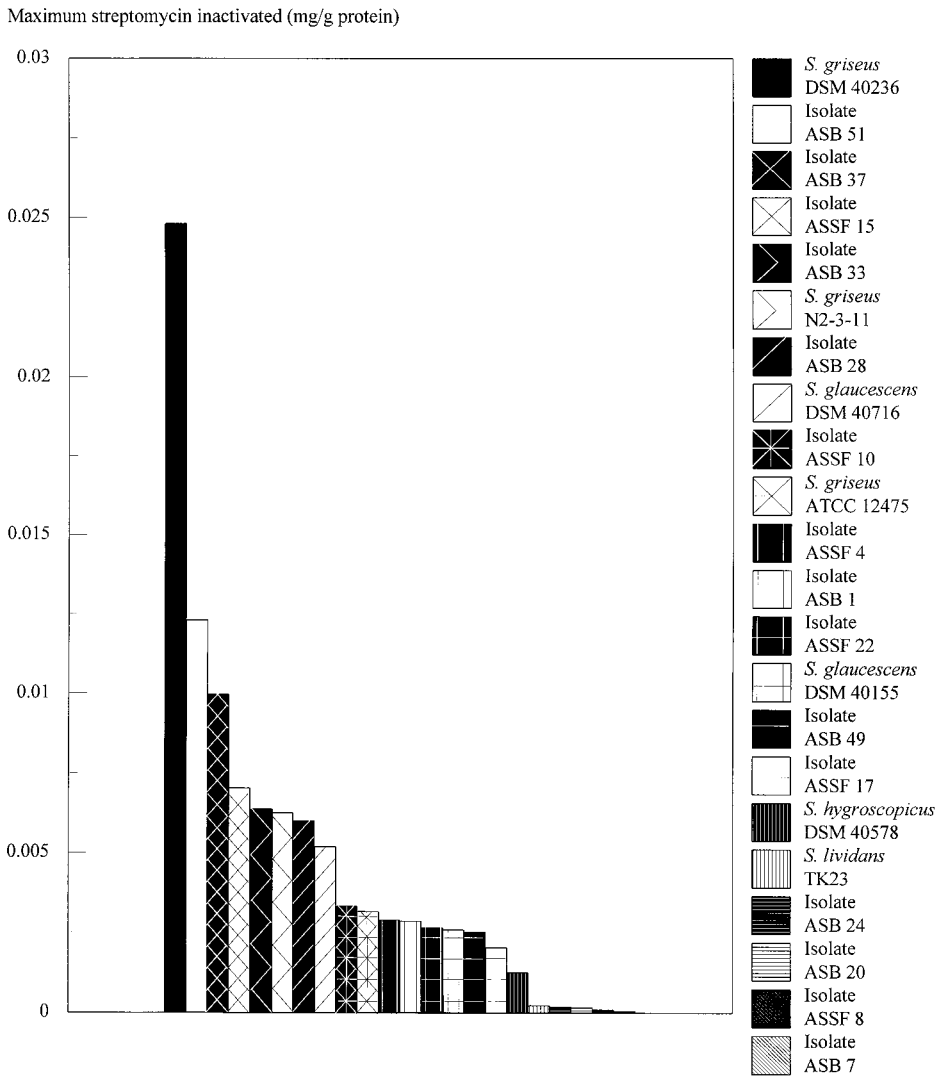

FIG. 2. Streptomycin 6-phosphotransferase activity detected in *Streptomyces* type strains and wild isolates.

isolates were resistant to streptomycin at 25 and 50 μg/ml. Clustering streptomycin resistance profiles gave a phenogram congruent with that obtained for the complete profiles (Fig. 1B). The clustering positions of the strains are given in Table 2.

Hybridization studies. Forty-seven *Streptomyces* type strains were screened for the presence of *strA*. Twelve of these strains produced streptomycin or related aminoglycosides. The positive controls (*S. griseus* DSM 40236, ATCC 12475, and N2-3-11) all gave the expected 920-bp product. No product was obtained from the negative controls, *S. lividans* TK21 and TK23. Products were obtained from two close relatives of *S. griseus*, *S. limosus* ISP 5131 and *S. bikiniensis* ISP 5235, and also from *S. glaucescens* DSM 40155 but not from *S. glaucescens* DSM 40716. Southern blotting with labelled *strA* PCR product indicated homologies only for *S. limosus* ISP 5131; *S. bikiniensis* ISP 5235 and *S. glaucescens* DSM 40155 products failed to hybridize. The *strA*-specific primers were therefore used to screen for the presence of *strA* homologes.

Thirty-five natural isolates were selected to represent the eight minor clusters (Fig. 1A) and were screened with the *strA* primers. Of these, 15 gave a positive result, and 13 of the 15 products hybridized with the specific probe. The isolates with *strA* were from cluster 1 (Fig. 1A). The PCR product obtained from isolate AS_{SF} 17 was cloned, and the sequences obtained corresponded to nucleotides (nt) 1 to 366 and nt 737 to 924 of the *strA* gene. Sequenced regions shared 98.5% identity at the DNA level and 97.7% identity at the amino acid level with *strA* from *S. griseus*. Chromosomal digests from isolates were screened with the *strA* probe, and the results corresponded to those obtained from the hybridizations with the PCR product. Screening for the biosynthetic gene, *strB1*, indicated that some strains possessed the resistance gene but not the biosynthetic cluster (Table 3). Presence of either or both *strA* and *strB1* positively correlated with streptomycin production, but there were some exceptions to this (Table 3).

Detection of antibiotic production and aminoglycoside phosphotransferase activity. Streptomycin production was detected by bioassay with streptomycin-sensitive strains of *E. coli* 711. This was further confirmed by bioautography with streptomycin-resistant and -sensitive strains of *S. typhimurium*. In vitro bioassays done with *B. japonicum* strains showed inhibition by all isolates recorded as streptomycin producers (data

A

	158 … 203
S. griseus ATCC 12475	UCUAAUACCGGAUAACACUCUGUCCCGCAUGGGACGGGGUU++AAA
S. griseus N2-3-11	---++---
S. hygroscopicus ATCC 14607	---++---
S. flavovirens ATCC 3320	---++---
S. humidus ATCC 12760	---++---
S. subrutilis ATCC 27467	--------------C-----CUGU-U-------CA------+G---
S. bikiniensis ATCC 11062	--------------CG--C-G-GAAG----CUUCUC----GG+---
S. mashuensis ATCC 23934	--------------CG--A-GCGA--------UC--C-UG-GG---
S. glaucescens DSM 40716	--------------CUGA-UGUCUUG-GCAUCCUU-AU-A-CG---
Streptomyces soil isolate AS$_{SF}$ 17	------------AU-AUACUGUG---CUCCU--GG-AC-C-UG---
Streptomyces soil isolate AS$_{B}$ 37	--------------CUGAC-CU-G-A-GCAUCUG--A----CG---
S. lividans TK21	--------------CUGAC-CUCG-A-GCAUCUG--A----CG---
S. coelicolor A3(2)	--------------CUGAC-CUCG-A-GCAUCUG--A----CG---

B

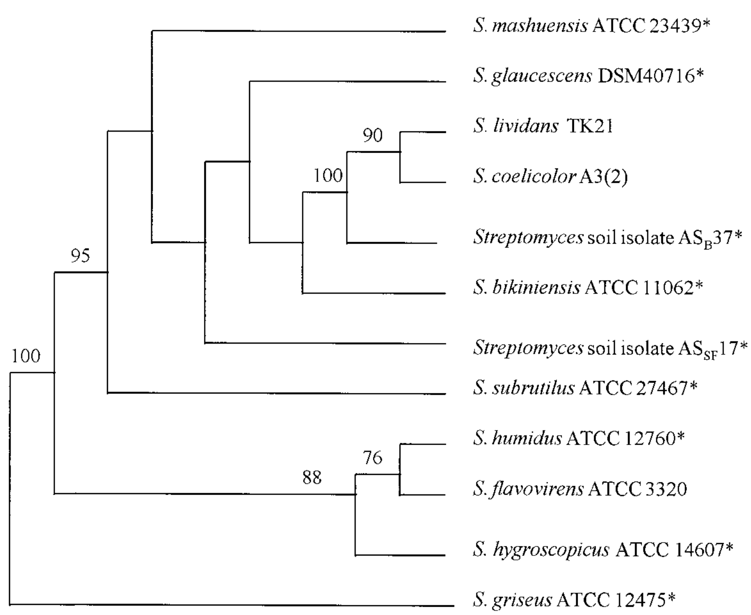

FIG. 3. Alignment (A) and comparison (B) of partial 16S rRNA nucleotide sequences from various streptomycetes, including only the highly variable gamma region (nt 158 to 203). (A) -, nucleotide sequence identical to the *S. griseus* ATCC12475 sequence; +, alignment gaps. (B) The phylogenies were compared in PHYLIP using the Fitch-Maroliash criterion and supported by at least 75% of bootstrap samples (the percent support is indicated on each branch). Branch lengths are arbitary. Asterisks indicate that the strains produce streptomycin or a related product.

not shown). All isolates found to possess *strA* had measurable phosphotransferase activity (Table 3; Fig. 2).

Taxonomic and phylogenetic diversity of streptomycin-producing isolates and type strains. The identifications obtained for the Brazilian isolates are given in Table 3. All actinomycetes were identified to the *Streptomyces* genus by use of traditional taxonomic criteria (39). A collection of 10 morphologically distinct isolates selected from the 35 strains used in hybridization studies were identified to species level. Of the 10, 5 could not be matched to existing species (Table 3). Two of the 10 isolates, AS$_{B}$ 37 and AS$_{SF}$ 17, were included in phylogenetic analysis with the 16S rRNA and *trpBA* genes to investigate the evolutionary relatedness of streptomycin producers. Partial-sequence alignments demonstrated that the two isolates were taxonomically distinct (Fig. 3A and 4A and B). Unrooted trees were obtained from comparison of partial sequences for isolates and selected type strains (Fig. 3B and 4C). In both trees, the antibiotic producers were recovered in different clades and did not form a distinct group. Both soil isolates were recovered in groups distinct from *S. griseus* and were more closely related to the non-streptomycin-producing species *S. coelicolor* and *S. lividans.*

Total-community analysis. DNA was extracted from Santa Fé bulk unlimed previously cultivated soil and from the rhizosphere of limed cultivated soil and used for the detection of *strA* with the specific PCR primers. Products were obtained from both samples, and these hybridized with the *strA* probe; however, the limed cultivated soil DNA gave a stronger signal. The detection limit for *strA* in sterile soil seeded with *S. griseus* spores was 10^6 spores per g (Fig. 5) This indicated a possible target population of 10^6 bacteria per g of Santa Fé limed rhizosphere soil.

DISCUSSION

Analysis of the actinomycete population in Santa Fé rhizosphere soil samples revealed a culturable streptomycete population of approximately 10^7/g of soil. A recent extensive iso-

A

```
                                   1                                                            60
S.griseus ATCC12475                PGIGPEHSYL KDSGRGEYRA VTDDAAMQAL RLLSRTEGII PAIESAHALA GALDLGKELG
S. griseus N2-3-11                 ---------- --V------- ---------- ---------- ---------- ----------
S. humidus ATCC 12760              ---------- ---------- ---------- ---------- ---------- ----------
S. hygroscopicus ATCC14607         -------A-- --V------- ---------- ---------- ---------- ----------
Streptomyces soil isolate ASSF17   -------A-- ---------- ----D----- L--------- ---------- ---EV-SD--
S. bikiniensis ATCC 11062          -------A-- ---------- ---------- ---------- ---------- --IEV-----
S. coelicolor A3(2)                -------A-- ---------- ---------- ---------- ---------- ---EV-R---
Streptomyces soil isolate ASB37    -------A-- ---------- ---------- ---------- ---------- ----I-----
S. subrutilus ATCC 27467           ---------- ---------- ----P----- ---------- ---------- ----------
S. flavovirens ATCC 3320           -------A-- --V------- -------R-- ---------- ---------- ---EV-----
S. mashuensis ATCC 23934           ---------- --T--A---- ---------- ---------- ---------- ---EV-R---
CONSENSUS                          PGIGPEH YL KD GR EYRA VTDD AA AL  LLSRTEGII PAIESAHALA GA   G  LG

                                   61                                          110
S.griseus ATCC12475                KDGLILVNLS GRGDKDMDTA ARYFGLYDAD AAVEADAESD +RAEIEGDA+ ++K
S. griseus N2-3-11                 ---------- ---------- ---------- -------D-- ---------- ---
S. humidus ATCC 12760              ---------- ---------- ---------- ----G--D-- ---------- ---
S. hygroscopicus ATCC14607         ------I--- ---------- --------T- ----+--D-- ---------- ---
Streptomyces soil isolate ASSF17   R----V---- ---------- --------++ ++++++++++ -+++++-ESA ---
S. bikiniensis ATCC 11062          P----V---- ---------- --------T- -E-A---GA- DT-------- ---
S. coelicolor A3(2)                R---LV---- ---------- --------T- -E-A--GTGA -+-------- ---
Streptomyces soil isolate ASB37    P----V---- ---------- ----R---T- -E-A---AA+ -T---A---- ---
S. subrutilus ATCC 27467           R---VV---- ---------- --------V- ++++++++++ -+++++-E+- -T-
S. flavovirens ATCC 3320           T--------- ---------- --------G+ -DAVVE-DV+ -+++AD--VE VT-
S. mashuensis ATCC 23934           P----V---- ---------- --------+- --++++++++ -+++++--+- --Q
CONSENSUS                           DGL  VNLS GRGDKDMDTA ARYF LYD                    G
```

B

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                                   1                                                       56
S.griseus ATCC12475                VS+GNIELLN TTLAAAKAAD RAALIAYLPA GFPTVDGGIE AVKAVIAGGA DVVESG
S. griseus N2-3-11                 ---------- ---------- ---------G ---------- ---------- ----I-
S. humidus ATCC 12760              ---------- ---------- ---------- -------S-- ---------- ----I-
S. hygroscopicus ATCC14607         ---------- -----TR--- ---------- -----N---- ------G--- -I--I-
Streptomyces soil isolate ASSF17   ------Q--S D---T--NEG ---------- -----D---D -----FD--- ----V-
S. bikiniensis ATCC 11062          ------Q--- D-----K-E- ----V----- -----D---A -I---FD--- ----V-
S. coelicolor A3(2)                -----VQ--- D-----KS-G ---------- -----T---- ----ALD--- ----V-
Streptomyces soil isolate ASB37    ------K--T D-------EG -------H-- -----T---- -I---FD--- ----V-
S. subrutilus ATCC 27467           --T-T-Q--N D---R---E- ----V----- -------AV- -V-------- ----I-
S. flavovirens ATCC 3320           -----R---S ----K-AFE- ---------G ---------A ----AV---- --I-V-
S. mashuensis ATCC 23934           -MA------- SV------E- ----VG---- -----TT-VR -IT-MLD--C ----V-
CONSENSUS                          V  G   LL    LA       RAAL  Y P  GFPTV      A  A   GG  D--E-G
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C

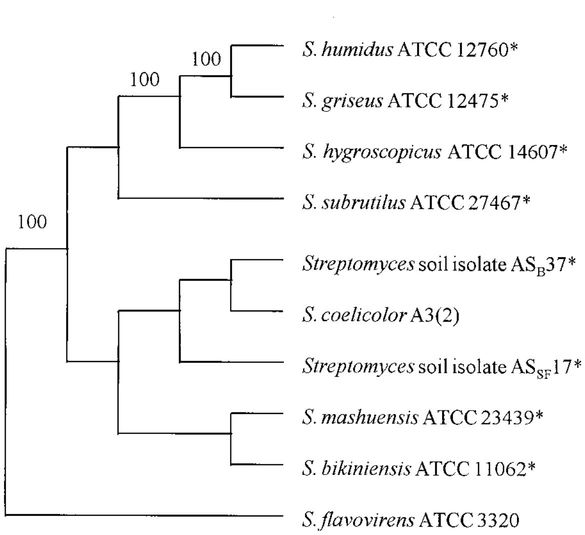

FIG. 4. Alignments of amino acid sequences (A and B) and dendrogram (C) obtained by comparing the tryptophan synthase gene sequences from streptomycin-producing streptomycetes. (A) Amino acid alignment of the *trpB* gene fragment. (B) Amino acid alignment of the *trpA* gene fragment. (C) The phylogenies of the *trpBA* fragment were compared in PHYLIP by using Fitch and supported by at least 75% of bootstrap samples (percent support is indicated on each branch). Branch lengths are arbitrary. Asterisks indicate that the strains produce streptomycin or a related product.

lation study of Chinese soils demonstrated the ubiquity of streptomycetes in soil and indicated that streptomycetes were the most numerous actinomycete group (48). In this study we report a change in phenotypic characteristics of a streptomycete population in soil following liming and planting with soybeans. The rhizosphere population had increased streptomycin resistance.

Streptomycetes resistant to high levels of streptomycin are candidates for production of this antibiotic (12). Antibiotic resistance profiles indicated that a group of limed rhizosphere soil isolates clustered with streptomycin-producing type strains. For the Santa Fé soils, all rhizosphere isolates recovered were highly resistant (>25 μg/ml) to streptomycin, as were the strains recovered from the soybean rhizosphere at the Brasilia site. Generally, strains growing with streptomycin at 25 μg/ml were also able to withstand concentrations of 50 and 100 μg/

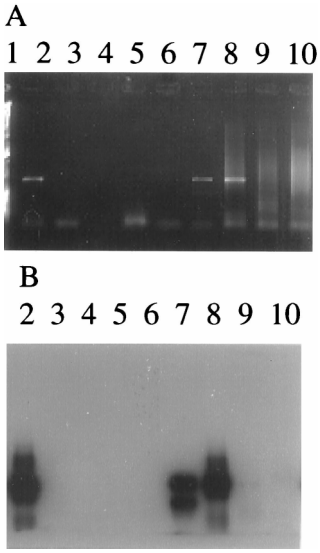

FIG. 5. PCR detection and quantification of *strA* in Santa Fé soil. Total-community DNA was extracted from limed and unlimed Santa Fé soil and then screened for *strA* with specific *strA* PCR primers (A) and hybridized (B) with the *strA* probe derived from *S. griseus*. Quantification was achieved by comparison with DNA extracted from sterile Santa Fé soil seeded with *S. griseus* spores and amplified by PCR with the *strA* primers. Lanes: 1, 1-kb lambda ladder; 2 to 10 PCR products obtained with the *strA* PCR primers; 2, *S. griseus* DSM 40236 DNA; 3, no DNA; 4, sterile Santa Fé soil DNA; 5 to 8, DNA from sterile Santa Fé soil inoculated with *S. griseus* spores at levels of 10^3 (lane 5), 10^5 (lane 6), 10^6 (lane 7), and 10^8 (lane 8); 9, DNA extracted from limed Santa Fé soil; 10 DNA extracted from unlimed Santa Fé soil.

ml. Such high levels of resistance are not common among streptomycetes, since Williams et al. (46) found only 24% of the griseus group able to grow at 100 μg/ml. Only 2 of the remaining 17 *Streptomyces* species groups showed any significant resistance. These two groups represented collections of antibiotic producers and were made up of *S. fulvissimus* and *S. rimosus.*

The pronounced difference in phenotype of the resistant rhizosphere isolates may be correlated with production. Studies of selected representatives from clusters 1 and 2 of the resistance phenogram indicated that the majority of rhizosphere strains were indeed streptomycin producers and contained the marker *strA* resistance gene. However, total-community analysis of DNA extracted from the soils sampled did show presence of the marker *strA* resistance gene, indicating that potential streptomycin producers were present but in smaller numbers and with a streptomycin-sensitive phenotype and no streptomycin production. Previous studies by Phillips et al. (25) had already indicated the usefulness of antibiotic resistance profiles for selection of antibiotic-producing strains.

Detailed analysis of isolates representing major clusters in the resistance phenogram showed that they were taxonomically heterogeneous and therefore did not represent a single species. The majority of streptomycin producers contained *strA* and *strB1*; however, there were some exceptions, possibly related to the stringent hybridization conditions used. AS_{SF} 22 failed to hybridize with the *strB1* probe but was a streptomycin producer and inhibited *B. japonicum*. Stringent conditions were used for detection because *strB1* was more highly conserved between known streptomycin producers and was also present in *S. bluensis* (bluensomycin) and *S. glaucescens* (hydroxystreptomycin) (19). Neither of these type strains hybridized with the *strA* probe (13). It is probable that the stringent hybridization conditions used were responsible for the failure to detect *strB1* in AS_{SF} 22. Analysis of DNA extracted from Santa Fé indicated a population of 10^6 strains containing *strA* which correlated with the culturable streptomycin-resistant population. This agreed with earlier studies reporting the detection of *strA* in DNA extracted from Brasilia soil samples (41). The data suggests that there is a resident population of streptomycetes with the capacity for streptomycin resistance in bulk and rhizosphere soil. The observed increase in the level of streptomycin resistance among Santa Fé rhizosphere isolates may be related to the onset of streptomycin production to avoid autoinhibition.

Several of the streptomycin-producing strains isolated could not be matched to existing species. Goodfellow and Dickenson (8) observed that many organisms from natural habitats do not form tight clusters with recognized reference strains. The taxonomic diversity was further confirmed by low-frequency restriction fragment analysis (14). Analysis of 16S rRNA and *trpAB* genes supported the separation of two isolates from known streptomycin producers and provided further evidence supporting the observation that streptomycin production was randomly distributed in diverse species. In this study, it has been possible to demonstrate the potential for antagonism against *B. japonicum*. Further studies are needed to demonstrate production in situ.

ACKNOWLEDGMENTS

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