Molecular Detection of Streptomycin-Producing Streptomyces 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. Streptomyces 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 streptomyces 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). Streptomyces 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 antibiotics. Evidence for antibiotic production by soil actinomycetes has been well documented and is reviewed by Weller and Thomasow (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).

Streptomyces 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.
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 soil collected from the latter. At Santa Fe, only previously cultivated soil was available, so that the soils samples were from bulk unlimed previously cultivated soil (Table 1) referring to the field sites where nodulation trials were conducted. All strains were maintained on tryptone soya agar (Oxoid) or oatmeal agar (4). 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% CaCO₃ 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 Agropecuária (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 solution.

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 Thorrton’s 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 Fé (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 NTYSY-ps (Exeter Publishing Ltd.). Measures of similarity were determined for the isolates by using the simple matching coefficient (SXY) (34), and clusters were resolved by using the unweighted pair group arithmetic 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 prepared 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, 0.0002 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 adopted 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 prerin 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 Rf, of 0.2 when S. typhimurium ATCC

### 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 TK23 were kindly supplied by T. Kieser, John Innes Institute, Norwich, England. Strains isolated from Brazilian Cerrado soils are prefixed by the letters ASB (Brasilia) and ASF (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 (4). The strains were incubated at 28 to 30°C for 5 to 7 days.

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<table>
<thead>
<tr>
<th>No. investigated</th>
<th>Taxonomic identification</th>
<th>Name</th>
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<tr>
<td>24</td>
<td>1</td>
<td>S. griseus DSM 40236</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>S. griseus DSM 40236</td>
</tr>
<tr>
<td></td>
<td>1</td>
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<td>1</td>
<td>S. ornatus ATCC 5307</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>S. nitrosus DSM 40023</td>
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<tr>
<td></td>
<td>1</td>
<td>S. naraensis DSM 40508</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>S. flavovirens ATCC 3320</td>
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<tr>
<td></td>
<td>1</td>
<td>S. sindenis ISP 5255</td>
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<td></td>
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<td></td>
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<td>S. bikenii ISP 5255</td>
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<td>S. felst ISP 5130</td>
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<td>S. canescens ISP 5001</td>
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<td></td>
<td>1</td>
<td>S. abidaliavus ISP 5455</td>
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<tr>
<td></td>
<td>1</td>
<td>S. intermedius ISP 5372</td>
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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).

* Warwick culture collection soil isolates

**TABLE 1. Streptomycetes used in this investigation**
TABLE 2. Distribution of streptomycin resistance in streptomyces

<table>
<thead>
<tr>
<th>Species or isolate</th>
<th>Strain</th>
<th>Cluster</th>
<th>ARPG</th>
<th>Resistance to streptomycin</th>
<th>Production of streptomycin</th>
</tr>
</thead>
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<tr>
<td>S. griseus</td>
<td>DSM 40236&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>ii</td>
<td>+</td>
<td>+</td>
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<tr>
<td>S. griseus</td>
<td>DSM 4026&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>i</td>
<td>+</td>
<td>+</td>
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<td>S. griseus</td>
<td>ATCC 12475</td>
<td>1</td>
<td>i</td>
<td>+</td>
<td>+</td>
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<tr>
<td>S. griseus</td>
<td>N-2-3-11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
<td>iii</td>
<td>+</td>
<td>+</td>
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<tr>
<td>S. mutthus</td>
<td>ATCC 25481</td>
<td>1</td>
<td>ii</td>
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<td>+</td>
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<td>DSM 40508</td>
<td>1</td>
<td>i</td>
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<td>+</td>
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<td>i</td>
<td>+</td>
<td>+</td>
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<tr>
<td>S. nitrosoporeus</td>
<td>DSM 40023</td>
<td>1</td>
<td>i</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. humidus</td>
<td>ATCC 12760</td>
<td>19</td>
<td>i</td>
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<td>+</td>
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<td>S. hygroscopicus</td>
<td>ATCC 14607</td>
<td>32</td>
<td>i</td>
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<td>S. hygroscopicus</td>
<td>ATCC 21705</td>
<td>32</td>
<td>ii</td>
<td>+</td>
<td>+</td>
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<td>S. hachijoensis</td>
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<td>55</td>
<td>lv</td>
<td>+</td>
<td>+</td>
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<td>S. subtilis</td>
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<td>61</td>
<td>li</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. bikinensis</td>
<td>ATCC 11062</td>
<td>64</td>
<td>li</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Santa Fé soil isolates</td>
<td>7 ASSF strains Table 3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>li, iiii and 2i</td>
<td>5</td>
<td>Table 3</td>
<td></td>
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<tr>
<td>Brasilia soil isolates</td>
<td>46 ASH strains Table 3</td>
<td>li, lii, liiv, 2i and iiivi</td>
<td>21</td>
<td>Table 3</td>
<td></td>
</tr>
<tr>
<td>Warwick culture collection soil isolates</td>
<td>18 WCC strains&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1i, 2i and iiivi</td>
<td>10</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Cluster designations of type strains are those of Williams et al. (46).
<sup>b</sup> ARPG, antibiotic resistance profile group (see Fig. 1).
<sup>c</sup> Streptomycin resistance measured at 10 μg/ml.
<sup>d</sup> Streptomycin production was determined by bioassay.
<sup>e</sup> Strain obtained from DSM.
<sup>f</sup> Strain obtained from W. Piepersberg.
<sup>g</sup> Chloramphenicol.
<sup>h</sup> Glycine.
<sup>i</sup> Hydroxystreptomycin.
<sup>j</sup> Warwick Culture Collection isolates characterized by Phillips (24).

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

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

Antagonism by streptomyces 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 streptomyces 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: 0, no zone of inhibition; 1, zone of inhibition between 6 and 10 mm; 2, zone of inhibition between 11 and 14 mm; 3, zone of inhibition larger than 14 mm. Streptomyces that scored 2 or 3 were considered to be causing antagonism. All bioassays were done in triplicate.

Chromosomal DNA extraction and purification from streptomyces isolates. Streptomyces 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 g 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 repurificated 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<sup>-5</sup> to 10<sup>-8</sup>/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 streptomyces phosphotransferase gene (17) to give a product of approximately 920 bp, which was the search of 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′-TCG GGG CTT CTT CAG CCG TT-3′ (reverse). The second set amplified strB1, a biosynthetic gene that codes for streptomycin amidotransferase (6), 5′-TG ACC CTT GTA AGC GTC CAC-3′ (forward) and 5′-TT CAT GCC GTT 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 RNA primers were 5′-GGG ATT AGT GGC GAA CCG GTG AGT AAC-3′ (forward) and 5′-CCT GAC GCG GGC ACC CTG CAT CAG GCT-3′ (reverse) to yield a 267-bp product that included the highly variable gamma region (36). The trpC prophylase synthase (trpB1) primers were 5′-GG CGG TAC CCG ATC TCG GCT GGT CAG TAC-3′ (forward) and 5′-CCT CTA GCA GCA CGG GGT CGC GTG GC-3′ (reverse) and amplified a 492-bp region that included the β subunit of trpB and the subunit of trpA. The protocol for all sets of primers was the same except for different primer-amplification temperatures in the cycling protocols.

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<sub>2</sub>, 5 μl (25 mM; Perkin Elmer Cetus); 10× PCR buffer, 5 μl (Perkin-Elmer Cetus, MgCl<sub>2</sub>-free); deoxynucleoside triphosphates (dNTPs), 2 μl (10 mM each dNTP); H<sub>2</sub>O, 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 trpB1) 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% (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–dITTP (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 10X 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).
checked by the procedures outlined above. The \textit{strA} and \textit{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 non-radioactive 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. Stringent 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 \textit{trpB4} 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. \textit{str} sequences for \textit{Streptomycetes} soil isolate AS\textsc{sf} 17, U59006 and U59007. 16S rRNA sequences: \textit{Streptomycetes} hygroscopicus ATCC 14607, U72167; \textit{Streptomycyes griseus} N2-3-11, U72166; \textit{Streptomycyes humida} ATCC 12760, U72169; \textit{Streptomycyes griseus} ATCC 12475, U72170; \textit{Streptomycyes flavovirens} ATCC 3320, U72171; \textit{Streptomycyes substratulis} ATCC 27467, U72172; \textit{Streptomycyes mashuensis} ATCC 23934; U72173; \textit{Streptomycyes binkinis} ATCC 11062, U72174; \textit{Streptomycyes flavovirens} DSM 40716, U72175; \textit{Streptomycyes coelicolor} A3(2), U72176; \textit{Streptomycyes soil isolate AS\textsc{bf} 28, U72177; \textit{Streptomycyes soil isolate AS\textsc{bf} 17, U72178; \textit{Streptomycyes livida} TK21, U72179. \textit{trpB4} sequences: \textit{Streptomycyes hygroscopicus} ATCC 14607, U72180; \textit{Streptomycyes humida} ATCC 12760, U72181; \textit{Streptomycyes griseus} ATCC 12475, U72182; \textit{Streptomycyes griseus}, N2-3-11, U72183; \textit{Streptomycyes soil isolate AS\textsc{bf} 28, U72184; \textit{Streptomycyes coelicolor} A3(2), U72185; \textit{Streptomycyes binkinis} ATCC 11062, U72186; \textit{Streptomycyes flavovirens} ATCC 3320, U72187; \textit{Streptomycyes substratulis} ATCC 27467, U72188; \textit{Streptomycyes mashuensis} ATCC 23934, U72189; \textit{Streptomycyes soil isolate AS\textsc{bf} 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, 10⁷ 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 × 10⁴ CFU/g (MSD, 0.27) was detected from the unlimed Santa Fé soil and 1.37 × 10⁵ 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 × 10⁵ 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). Thioestrepton, 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 (\textit{S}_{SM}) and clustered by UPGMA (Fig. 1A). Two major clusters were recovered at the 60% \textit{S}_{SM} level (1 and 2), and eight minor clusters were recovered at the 75% \textit{S}_{SM} level; these were labelled i through to 1v and 2i through to 2iii. Members of cluster 1 were resistant to streptomycin (>2.5 μg/ml), 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\textsc{bf} 13, AS\textsc{bf} 19, AS\textsc{bf} 27, and AS\textsc{bf} 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\textsc{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
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* homologues.

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 ASF 4, 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
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, ASp37 and ASp17, 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 new 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/ml.

**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.
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 strB1 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), $10^4$ (lane 6), $10^5$ (lane 7), and $10^6$ (lane 8); 9, DNA extracted from limed Santa Fé soil; 10 DNA extracted from unlimed Santa Fé soil.

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$_{Sg}$ 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. bluenisi (bluenosmycin) 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$_{Sg}$ 22. Analysis of DNA extracted from Santa Fé indicated a population of 10$^6$ strains containing strA which correlated with the cultivable 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.

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