Streptomyces costaricanus sp. nov., Isolated from Nematode-Suppressive Soil

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A new bacterial strain, strain CR-43T (T = type strain), which was isolated from tropical soil and was previously shown to have antinematodal and antibiotic properties, is described. The name Streptomyces costaricanus is proposed for this organism. The generic placement of strain CR-43T was based on its typical morphology, its production of LL-diaminopimelic acid, and its fatty acid composition. To clarify the taxonomic position of strain CR-43T, it was compared with the type strains of similar Streptomyces species. The results of a number of biochemical tests and a profile analysis of the hydrolyzable fatty acids indicated that CR-43T differs from previously described species. Strain CR-43 (ATCC 55274 = NRRL B-16897) is the type strain of S. costaricanus sp. nov.

Nematode-suppressive soils have been found in several locations throughout the world. In our laboratory, microorganisms with antinematodal or antifungal properties have been isolated from suppressive soils obtained from Mexico (12, 23) and Costa Rica (4). The biological basis of this suppressiveness was established by showing that heat sterilization abolishes the suppressive effect. One of the organisms isolated from suppressive soil obtained from Costa Rica exhibited both antinematodal and antifungal activities in laboratory, greenhouse, and field trials (4). A culture of this organism (designated strain CR-43T [T = type strain]) was submitted for identification to the American Type Culture Collection (ATCC), Rockville, Md., which concluded that the isolate was a member of the genus Streptomyces and could not be placed in a previously described species (1). Later, a University of Massachusetts research team reported (4) that this isolate specifically inhibited reproduction of the free-living nematode Caenorhabditis elegans in vitro under axenic conditions. Such an effect is referred to as antinematodal activity, in contrast to the nematocidal activity exhibited by organisms such as Streptomyces avermitilis.

On the basis of the results of successful field trials in which the control of several species of plant parasites was examined, an application for a patent for CR-43T as a biocontrol agent for nematodes was filed by Research Corporation Technologies, Tucson, Ariz., under exclusive license from the University of Massachusetts, Amherst. Cryopreserved subcultures of CR-43T have been deposited in the ATCC (as ATCC 55274T) and in the U.S. Department of Agriculture Agricultural Research Service Culture Collection (as NRRL B-16897T).

In this paper we describe CR-43T as a member of a new species, for which we propose the name Streptomyces costaricanus. This organism was compared with plant-pathogenic, anthelmintic, and other similar Streptomyces species.

MATERIALS AND METHODS

Microorganisms. Characteristics of nine previously described Streptomyces species were compared with characteristics of strain CR-43T, which was isolated from nematode-suppressive soil obtained from Costa Rica and stored at -80°C in cryopreservation buffer (2). S. avermitilis ATCC 31267T was obtained from the ATCC, while Streptomyces scabies ATCC 49172T and Streptomyces acidiscabies ATCC 49002T, two plant-parasitic organisms, were supplied by R. Loria, Cornell University, Ithaca, N.Y. Comparison with plant-parasitic streptomycetes was required by the Animal and Plant Health Inspection Service since CR-43T was being introduced as a biological control agent for plant nematodes. S. avermitilis is a known anthelmintic species and therefore was appropriate for comparative studies. Members of six other species and subspecies (Streptomyces hygroscopicus NRRL B-1865T, S. marinus NRRL B-2286T, S. griseoviride NRRL B-1315T, S. hygroscopicus subspp. decolorans NRRL ISP-5087T, S. rubiginosus NRRL B-3983T, and S. griseofuscus NRRL B-5429T) have characteristics similar to CR-43T characteristics and were obtained from David Labeda, U.S. Department of Agriculture, Peoria, Ill. All bacterial strains were stored at -80°C as described above. Initially, all strains were grown on International Streptomycetes Project (ISP) medium 2 (17) at 28°C. Data were supplemented or confirmed by using data for species characteristics published previously.

Morphological and cultural characterization. Light microscopy was used to study the aerial mycelium of strain CR-43T; the characteristics of this organism were then compared with the characteristics of the other type strains examined. Standards described by Stirling and Gottlieb (17) were used to describe spore chain morphology. Cultural characteristics of strain CR-43T and S. hygroscopicus subspp. decolorans were determined by using media recommended by the ISP (14, 17). The presence of soluble pigments was investigated on yeast extract-malt extract agar (ISP medium 2; 4 g of yeast extract [Difco] per liter, 10 g of malt extract [Difco] per liter, 4 g of glucose per liter; pH 7.3 before 20 g of agar per liter was added), oatmeal agar (ISP medium 3), inorganic salts-starch agar (ISP medium 4), glycerol-asparagine agar (ISP medium 5), and ATCC medium 172. Melanoid pigment production was studied on peptone-yeast extract-iron agar (ISP medium 6) and tyrosine agar (ISP medium 7).

Carbon source utilization. Each carbon source was added at a final concentration of 1% (wt/vol) and was prepared as a 10% solution in glass-distilled deionized water that was sterilized by passing it through a 0.22-μm-pore-size Acrodisc filter. The basal salt medium which we used has been described previously by Pridham and Gottlieb (14). The absence or presence of growth on each medium was recorded. The carbon source utilization data described above are data from this study. The data for different species shown in Table 1 were compared with previously published data (3, 9–11, 18–21). The following 10 carbon sources were tested: α-arabinose, β-fructose, β-glucose, ω-mannitol, α-fucose, α-rhamnose, α-sucrose, α-xylene, salicin, and galactose. Acid production by strain CR-43T was tested after growth in nocardia purple broth supplemented with cellulose, β-glucose, cellobiose, maltose, galactose, α-mannitol, α-xylene, α-arabinose, α-fructose, lactose, sucrose, ribitol, galactitol, erythritol, or l-ribose.

Antibiotics and other inhibitory compounds. Growth of the streptomycetes in the presence of streptomycin (20 μg/mL), penicillin (10 IU/mL), penol (0.1%), thallium acetate (10 μg/mL), and crystal violet (0.5 μg/mL) was tested in ISP medium 2.

NaCl tolerance. Sodium chloride tolerance was tested on yeast extract-malt extract agar (ISP medium 2) supplemented with 5, 6, or 7% (wt/vol) NaCl.

pH sensitivity. The minimum pH that allowed growth was determined on modified ISP medium 2. The pH was adjusted with 1 M HCl or 0.25 M KOH.

Cellular fatty acid profile analysis. S. costaricanus CR-43T, S. hygroscopicus, S. marinus, and S. griseoviride were quadrant streaked onto ISP medium 2 agar and grown aerobically in the dark for 96 h at 28 ± 0.1°C. In another experiment, S. costaricanus CR-43T, S. hygroscopicus subspp. decolorans, S. rubiginosus, and S. griseofuscus were grown at 27 to 28°C in ISP medium 2 broth with vigorous shaking for 72 h. A fatty acid analysis was performed (in duplicate) as described by Esnard et al. (5). A Hewlett-Packard model HP5890 series II Plus gas chromatography-mass spectrometry system coupled to a model HP5972 mass selective detector was used. The gas chromatograph was equipped with a type HP-5
### Table 1. Characteristics of S. costaricanus, S. avermitilis, S. scabies, S. acidiscabies, S. munitus, S. hygroscopicus, S. hygroscopicus subsp. decorticatus, S. griseoluteus, S. rubiginosus, and S. griseofuscus

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>S. costaricanus</th>
<th>S. avermitilis</th>
<th>S. scabies</th>
<th>S. acidiscabies</th>
<th>S. munitus</th>
<th>S. hygroscopicus</th>
<th>S. hygroscopicus subsp. decorticatus</th>
<th>S. griseoluteus</th>
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<td>BW/BG</td>
<td>G/G</td>
<td>W/B</td>
<td>G-B</td>
<td>G/CY</td>
<td>R'/Mo</td>
<td>G'/z</td>
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<td>R'G</td>
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<td>G/G</td>
<td>W/B</td>
<td>G-B</td>
<td>W-CG/B</td>
<td>R/G</td>
<td>G'/z</td>
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<td>R'G</td>
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<td>G/G</td>
<td>W/B</td>
<td>G-B</td>
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<td>W-CG/B</td>
<td>R/G</td>
<td>G'/z</td>
<td>G</td>
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<td>BG/GY</td>
<td>G/G</td>
<td>W/B</td>
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<td>G/B</td>
<td>G/CY</td>
<td>R'/Mo</td>
<td>G'/z</td>
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<td>G/B</td>
<td>W/B</td>
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<td>G/CY</td>
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<td>Y/R</td>
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<td>Minimum pH for growth</td>
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<td>3.5</td>
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<td>4.2</td>
<td>4.9</td>
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"Data from this study.
"Data from reference 3 unless indicated otherwise.
"Data from references 10 and 11 unless indicated otherwise.
"Data from references 10 and 11 unless indicated otherwise.
"Data from references 9 and 15 unless indicated otherwise.
"Data from references 13 and 15 unless indicated otherwise.
"Data from reference 14.
"ND, not determined.
"Color on acid media or alkaline media.
"+, positive reaction; -, negative reaction; tr, trace.

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The method of Becker et al. (1a) was slightly modified to determine the presence of the susceptible form of diaminopimelic acid in the cell wall of strain CR-43. Cells of CR-43 (test strain) and S. hygroscopicus (positive control) were grown for 72 h in yeast extract-malt extract-glucose medium at 28°C. Hydrolysates were prepared by autoclaving the biomass in 6 M HCl.
RESULTS AND DISCUSSION

Antinematodal strain CR-43\(^T\) was found to be distinctly different from the related Streptomyces type strains used for comparison. Morphological, cultural, and physiological characteristics of the organisms which we studied are summarized in Table 1.

Morphological characteristics. The spore-bearing aerial hyphae of strain CR-43\(^T\) were simple (nonverticillate), and tightly coiled spiral spore chains were formed on standard ISP agar media (ISP media 2 through 5). Each spore chain consisted of 10 to 50 smooth spores. No sclerotium-like bodies, sporangia, or flagellated or conidium-like spores were observed in the aerial or submerged mycelium. Fragmentation of the substrate mycelium was not observed.

Pigmentation. The aerial mycelial mass after 14 days was reddish gray to brown-gray on yeast extract-malt extract agar, oatmeal agar, inorganic salts-starch agar, or glycerol-asparagine agar. A yellow color was produced on ATCC medium 172 (N-Z amine supplemented with soluble starch and glucose). On ISP media 2 and 5 the substrate mycelia were light yellow and yellow to yellowish brown, respectively (golden on ATCC medium 172); no distinctive pigments were produced on ISP medium 3 or 4 agar, although a brown or yellow color was observed. A yellow diffusible pigment was produced on ISP medium 2, ISP medium 5, and ATCC medium 172, and this pigment was not sensitive to pH changes resulting from additions of HCl or NaOH. No melanoid pigments were observed on tyrosine agar and peptone-yeast extract-iron agar.

Physiological characteristics. On basal medium (14), CR-43\(^T\) utilized D-fructose, D-glucose, D-mannitol, D-xylose, salicin, and galactose but not L-arabinose, raffinose, L-rhamnose, and sucrose as sole carbon sources. This pattern of carbon source utilization was different from the patterns of all of the strains tested in each comparison by using MSTAT-C software (Michigan State University, East Lansing). All paired comparisons for species grown on solid or liquid medium yielded highly significant differences \((P < 0.001)\). The organism that was most similar to CR-43\(^T\) culturally and physiologically when the bacteria were grown on solid agar, S. murrayi NRRL B-2286\(^T\), was easily differentiated from the new strain by comparing the ratios of i-14:0 to 14:0, a-15:0 to 16:0, and 16:0 to a-17:0 in the two organisms. When broth cultures were examined, S. hygroscopicus subsp. decoyicus ISP-5087\(^T\) had the fatty acid profile that was most similar to the CR-43\(^T\) profile. The ratio of i-17:0 (peak 21) to i-15:0 (peak 9), i-16:0 (peak 15), a-17:0 (peak 22), or i-18:0 (peak 29) (which was <1 in CR-43\(^T\) but >1 in S. hygroscopicus subsp. decoyicus) clearly differentiated the two species.

The fatty acid profiles of S. hygroscopicus subsp. decoyicus and S. murrayi were most similar to the CR-43\(^T\) profiles (Table 2). Phenotypically, CR-43\(^T\) differs from strain NRRL ISP-5087\(^T\) in that it consistently produces grey-brown spore masses in ISP media 2 through 5, whereas strain NRRL ISP-5087\(^T\) produces grey spore masses on ISP media 2 and 5, white to carbon grey spore masses on ISP medium 3, and grey to brown spore masses on ISP medium 4 after 14 days. Also, CR-43\(^T\) can clearly differentiate the two species.

The fatty acid profiles of S. hygroscopicus subsp. decoyicus and S. murrayi were most similar to the CR-43\(^T\) profiles (Table 2). Phenotypically, CR-43\(^T\) differs from strain NRRL ISP-5087\(^T\) in that it consistently produces grey-brown spore masses in ISP media 2 through 5, whereas strain NRRL ISP-5087\(^T\) produces grey spore masses on ISP media 2 and 5, white to carbon grey spore masses on ISP medium 3, and grey to brown spore masses on ISP medium 4 after 14 days. Also, CR-43\(^T\) can utilize salicin (1 mg/ml) as a sole carbon source and can grow in the presence of crystal violet (1 \(\mu\)g/ml), whereas strain NRRL ISP-5087\(^T\) does not grow under these conditions.

On the basis of morphological, cultural, and whole-cell chemical characteristics, CR-43\(^T\) is a member of a new Streptomyces species, for which we propose the name Streptomyces costaricanus Esnard, Potter, and Zuckerman. Strain CR-43, the type strain of S. costaricanus, has been deposited in the ATCC and U.S. Department of Agriculture Agricultural Research Service Culture Collection as strains ATCC 55274 and NRRL B-16897, respectively.

**Description of Streptomyces costaricanus sp. nov.** *Streptomyces costaricanus* (cos.ta.ri.can'us. L. adj. costaricanus, referring with workers conversant with Streptomyces taxonomy, we found that strain CR-43\(^T\) was most similar to the following six species and subspecies: S. hygroscopicus, S. murrayi, S. griseoluteus, S. hygroscopicus subsp. decoyicus, S. rubiginosus, and S. griseofuscus. However, CR-43\(^T\) differs from these taxa in its total physiological and cultural test reactions, fatty acid profile, and/or gross morphology (Table 1). Culturally, CR-43\(^T\) differs from the organism that has the most similar fatty acid profile in agar cultures, the type strain of *S. murrayi*, by not producing a soluble pigment when it is grown on oatmeal agar (ISP medium 3) or inorganic salts-starch agar (ISP medium 4). In addition, the *S. murrayi* spore mass is red on ISP medium 2, red or gray on ISP medium 3 or 5, and gray on ISP medium 4, while CR-43\(^T\) consistently produces a distinct gray-brown color when it sporulates on ISP medium 2, 3, 4, or 5 after 14 days. The substrate mycelium of CR-43\(^T\) is brown on oatmeal agar and yellow on inorganic salts-starch agar, while the substrate mycelium of the type strain of *S. murrayi* is grayish yellow on oatmeal agar and reddish on inorganic salts-starch agar. CR-43\(^T\), but not *S. rubiginosus* or *S. griseofuscus*, produces soluble pigments when it is grown on ISP medium 2 or 5. In addition, *S. rubiginosus* produces spiny spores and utilizes L-rhamnose and sucrose as sole carbon sources; CR-43\(^T\) does not utilize L-arabinose, while *S. griseofuscus* does utilize this compound.
TABLE 2. Fatty acid compositions of S. costaricanus CR-43* grown on ISP medium 2 agar and in ISP medium 2 broth

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<th>Peak no.</th>
<th>Compound*</th>
<th>%&lt;sup&gt;1&lt;/sup&gt; on:</th>
<th>Agar medium</th>
<th>Broth medium</th>
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<td>Benzylic acid*</td>
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<td>2</td>
<td>i-12:0</td>
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<td>ND</td>
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<tr>
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<tr>
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* Identification of compounds was based on a combination of comparisons with authentic standards, mass spectral interpretation and database searches, and relative gas chromatography retention times. br, branched; cy, cyclo.

<sup>1</sup> The relative retention time of benzylic acid was 1.00 min.

<sup>2</sup> ND, not detected.

The values in parentheses are the molecular weights of methyl esters based on chemical ionization mass spectra.

cellobose, d-glucose, glycerol, maltose, galactose, d-mannitol, and d-xylose but not from l-arabinose, d-fructose, lactose, and sucrose. No growth occurs in the presence of ribitol, galactitol, erythritol, and i-inositol; 7% NaCl is inhibitory. Table 1 shows the tolerance of S. costaricanus to other toxic compounds.

Cell walls contain L,L-diaminopimelic acid. The most abundant hydrolyzable fatty acids are a-15:0, 16:0, a-17:0, i-15:0, i-16:0, and i-17:0 in cells grown on ISP medium 2 agar. The concentration of octadeconic acid is ninefold higher in ISP medium 2 broth (Table 2). Isolated from a tropical soil. Exhibits antinematodal activity against C. elegans and antibiotic activity against R. solani and Pythium aphanidermatum. The type strain is strain CR-43 (= ATCC 55274 = NRRL B-16897).

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We thank D. Labeda (U.S. Department of Agriculture Agricultural Research Service) for expert advice. D. Labeda and R. Loria (Cornell University) kindly supplied strains for comparison. We acknowledge the advice of the late Irving Fagerson and Tom Carpenter concerning the chemical analyses and the technical assistance of B. Kelly, G. Coles, R. Wick, and B. Dicklow in some stages of this investigation. This work was supported by a grant to B. M. Zuckerman from the Corporation for the Technological Development of Tropical Resources, Commonwealth of Puerto Rico.

REFERENCES


to Costa Rica, the geographic origin of the organism). Mature spore chains are tightly coiled spirals with 10 to 50 spores per chain. The spores are smooth and gray-brown in mature colonies.

The aerial mycelial mass is grayish brown on yeast extract-malt extract agar, oatmeal agar, inorganic salts-starch agar, and glycerol asparagine agar and yellow on inorganic salts-starch agar. A yellow pH-insensitive diffusible pigment is produced on yeast extract-malt extract agar and glycerol-asparagine agar. The pigment color is orange-yellow on ATCC medium 172. The spores are smooth and gray-brown in mature colonies.

The aerial mycelial mass is grayish brown on yeast extract-malt extract agar, oatmeal agar, inorganic salts-starch agar, and glycerol asparagine agar and yellow on N-Z amine medium containing soluble starch and glucose (ATCC medium 172). The substrate mycelium is light yellow on yeast extract-malt extract agar and glycerol-asparagine agar, golden on ATCC medium 172, brown on oatmeal agar, and yellow on inorganic salts-starch agar. A yellow pH-insensitive diffusible pigment is produced on yeast extract-malt extract agar and glycerol-asparagine agar. The pigment color is orange-yellow on ATCC medium 172. No melanoid pigment is produced on peptone-yeast extract-iron agar or tyrosine agar. The color of the reverse side of the colonies is also not sensitive to pH.

d-Fructose, d-glucose, d-mannitol, d-xylose, salicin, and galactose are utilized for growth, but L-arabinose, raffinose, rhamnose, and sucrose are not utilized. Acid is produced from


