New Species of the Genus *Actinoplanes*, *Actinoplanes ferrugineus*

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An extensive phenotypic characterization of an actinoplanete capable of producing the antimetabolite L-azetidine-2-carboxylic acid is presented. On the basis of the properties of this organism, it is concluded that it belongs to a new species, for which the name *Actinoplanes ferrugineus* is proposed. The type strain is X-14695 (= ATCC 29868).

In the course of screening for new antibiotics, I isolated an actinoplanete which produced L-azetidine-2-carboxylic acid, a proline antimetabolite (D. Pruess and J. Scannell, personal communication). Since this compound had never before been reported as being produced by a procaryotic organism (3), it was of interest to characterize the producing strain, X-14695. Colonies of this organism have a rusty, reddish-brown color when grown on starch-casein agar. This color and the production of azetidine-carboxylic acid suggested that strain X-14695 belongs to a new species of *Actinoplanes*. This report provides morphological and physiological support for the recognition of a new species. The isolation, characterization, and biosynthesis of L-azetidine-2-carboxylic by this organism will be reported elsewhere.

MATERIALS AND METHODS

Organisms. Strain X-14695 of our collection was isolated in our laboratory by means of a novel method (to be described in detail elsewhere) for the isolation of actinoplanetes. The source of the culture was a red soil sample collected by T. Daniels at Dorriggo Mountain, Australia. *Actinoplanes brasiliensis* ATCC 25844, *A. italicus* ATCC 27366, and *A. utahensis* ATCC 14539 were purchased from the American Type Culture Collection (ATCC), Rockville, Md.

Culture methods and nutritional screening. To determine the growth characteristics of, and color production by, this organism, it was grown in several of the culture media recommended by Shirling and Gottlieb (8) for streptomycetes. Other media used were nutrient agar (Difco Laboratories), the medium described by Hickey and Tresner (4), glucose-asparagine agar, potato-dextrose agar, and Czapek-glucose agar prepared by the method of Waksman (11). The medium routinely used for maintenance of the cultures was the starch-casein agar of Waksman (11), modified by the addition of 0.05% MgSO₄ (or 0.102% MgSO₄·7H₂O).

Growth on a large number (140) of low-molecular-weight organic compounds was determined by the method of Prosser and Palleroni (7). Individual carbon sources were added to the basal mineral agar to a final

concentration of 0.1%. Geraniol and naphthalene were not incorporated into the medium but instead were placed in the lids of the petri dishes; after inoculation, the plates were individually wrapped in plastic bags for incubation. A plate with no added carbon source was used as a control of background growth.

Screening for utilizable substrates (with the exception of geraniol and naphthalene) was also performed in liquid mineral medium dispensed in tubes (4 ml of medium in each test tube [20 by 150 mm]); the inoculated tubes were incubated in a water bath with rotary shaking (250 rpm) at 28°C.

The following organic compounds were tested as single sources of carbon: D-ribose, D-xylose, D-arabinose, L-arabinose, D-fucose, L-fucose, L-rhamnose, D-glucose, D-mannose, D-galactose, D-fructose, L-sorbose, sucrose, trehalose, maltose, cellobiose, lactose, melibiose, salicin, raffinose, melezitose, starch, chitin, gluconate, 2-ketogluconate, saccharate, mucate, lactobionate, formate, acetate, propionate, butyrate, isobutyrate, valerate, caproate, heptanoate, caprylate, pelargonate, caprate, oxalate, succinate, malonate, maleate, fumarate, glutarate, adipate, pimelate, suberate, sebacate, tartronate, D-malate, L-malate, D-(–)-tartrate, L-(+)-tartrate, *m*-tartrate, beta-hydroxybutyrate, DL-lactate, glycolate, glycerate, citrate, alpha-ketoglutarate, pyruvate, aconitate, levulinate, itaconate, ethylene glycol, glycerol, propylene glycol, erythritol, 2,3-butylene glycol, D-arabitol, xylitol, dulcitol, adonitol, mannitol, sorbitol, *m*-inositol, methanol, ethanol, *n*-propanol, isopropanol, *n*-butanol, isobutanol, *sec*-butanol, geraniol, L-mandelate, benzoate, phenylacetate, naphthalene, phenol, quinate, glycine, L-alanine, D-alanine, beta-alanine, L-threonine, L-leucine, L-isoleucine, DL-norleucine, L-valine, L-aspartate, D-aspartate, L-glutamate, L-asparagine, L-glutamine, L-lysine, L-arginine, L-citrulline, L-orithine, alpha-aminobutyrate, gamma-aminobutyrate, alpha-aminovalerate, delta-aminovalerate, L-homoserine, L-methionine, L-histidine, L-proline, L-hydroxyproline, L-tyrosine, L-phenylalanine, L-tryptophan, kynurenate, anthranilate, methylamine, ethanolamine, putrescine, spermine, butylamine, *sec*-butylamine, *tert*-butylamine, alpha-amylamine, betaine, sarcosine, creatine, hippurate, pantothenate, acetamide, nicotinate, isonicotinate, and trigonelline. Sodium salts of the anionic substrates were prepared by neutralization of the free acids with sodium hydroxide. All compounds were reagent grade.

The inoculum for the various media was a washed suspension of spores. Good sporulation was obtained on mineral agar with 0.1% L-aspartate. Lawns of the organism on the surface of this medium were flooded with sterile 0.005 M phosphate buffer (pH 6.8). About 1 h later the suspension of motile spores was collected, and the spores were washed once with buffer of the same composition. The washed spores were finally suspended in buffer to a concentration of about 10^7 to 10^8 /ml. Small drops of this suspension were used to inoculate the plates or the liquid media.

Growth characteristics on the various carbon sources were determined after incubation for 14 days at 28°C. Colors were determined according to the *Color Harmony Manual* (4th ed., Container Corporation of America, Chicago, Ill., 1958). The plates were examined under a microscope with low magnification ($\times 100$ to $\times 200$) for the presence of spores in the patches.

Physiological tests. Gelatin hydrolysis was determined by the method in Skerman's *Guide* (9); the medium contained Difco *Thermoactinomyces* fermentation broth instead of nutrient broth. Casein hydrolysis was tested in this same medium supplemented with 0.2% casein. Temperature relationships were determined in *Thermoactinomyces* fermentation broth, and the cultures were incubated in a well-thermoregulated water bath with rotary shaking (250 rpm).

Analysis of carotenoid pigments. A pellet of cells of strain X-14695 was extracted with methanol under a nitrogen atmosphere, and the extract was scanned with a Gilford 250 recording spectrophotometer in the visible region of the spectrum between 400 and 600 nm.

Cell wall analyses. Cell wall analyses were performed on cell walls isolated by the method of Yamaguchi (12). Hydrolyzed cell walls were analyzed by the procedure of Becker et al. (1). Electron micrographs of platinum-shadowed spores were kindly made by Frank Jenkins of the Roche Institute of Molecular Biology. Chemotactic behavior of the spores was studied by the method of Palleroni (5, 6).

RESULTS AND DISCUSSION

Morphological properties. The characteristics of strain X-14695 are as follows. Growth occurred on many media currently used for actinomycetes. The colonies on many of these media were brown to dark red (mahogany) in color (see below). When observed under the microscope with low magnification, sporangia were visible in many cases (Fig. 1). These sporangia had diameters which ranged from 4 to 12 μ m. The formation of sporangia by strain X-14695 was far less abundant and reproducible in comparison with other actinoplanetes, and the sporangia were more difficult to observe. Thus, whereas *A. brasiliensis* ATCC 25844, *A. italicus* ATCC 27366, and *A. utahensis* ATCC 14539 formed sporangia very readily in various standard media, and the numerous sporangia on the upper surface of the colonies could be easily observed with a standard dissecting microscope,

the sporangia of strain X-14695 did not form in media allowing good growth, and, if they were formed in small numbers, their detection was usually obscured by the vegetative growth. When observed with reflected light, these sporangia appeared as small protuberances barely discernible from the growth mass. The best conditions for observing the sporangia were obtained with transmitted light in a compound microscope under magnifications between $\times 100$ and $\times 200$. Under these conditions, the sporangia formed on mineral agar with one of a number of carbon sources (see below) appeared as irregularly shaped bodies resembling sclerotia (Fig. 1). Detached sporangia had, however, a normal appearance in wet mounts (Fig. 2), where the release of motile spores was by the same mechanism as with other actinoplanetes.

Good formation of sporangia was observed in many of the plates of mineral medium with various carbon sources, including the following: L-rhamnose, D-galactose, D-fructose, lactose, gluconate, pimelate, L-aspartate, D-aspartate, L-asparagine, L-histidine, L-tyrosine, L-phenylalanine, L-tryptophan, anthranilate, sarcosine, and acetamide. Copious formation of sporangia occurred in numerous instances in solid or liquid media with carbon sources which permitted very poor or no visible growth. Few sporangia were formed in the solid mineral medium not supplemented with a carbon source.

Release of motile spores from the sporangia is triggered by flooding the plates with water or buffer solutions. The globose spores measured 0.9 to 1.0 μ m in diameter and had a tuft of flagella inserted in a limited area of the cell (Fig. 3).

Analyses of the cell wall hydrolysates by paper chromatography demonstrated the presence of

FIG. 1. Formation of sporangia by strain X-14695 on mineral agar medium with 0.1% L-aspartate as the sole carbon source. Bar represents 20 μ m.

FIG. 2. Sporangia of strains S-14695. The sporangium at the right is releasing motile spores. Bar represents 10 μ m.

FIG. 3. Spores of strain X-14695. Bar represents 1 μ m.

an isomer of diaminopimelic acid (DAP) other than the LL form. Presumably, this compound is the *meso* isomer, but no further discrimination from the DD isomer was attempted. In addition to *meso*-DAP, the hydrolysates also contained a lowly moving component which had the same staining properties with ninhydrin as did DAP. This component appeared in a smaller quantity than did DAP, and it is probably one of the isomers of 3-hydroxy-DAP. The R_f of this com-

pound was identical to that of the hydroxy-DAP of a hydrolysate of *A. utahensis* ATCC 14539 cell walls (10, 12). In addition, it was found that *A. brasiliensis* ATCC 25844 also contained *meso*- and/or DD-DAP and 3-hydroxy-DAP.

The morphological and chemical characteristics of strain X-14695 permit a clear diagnosis of this organism as a member of the genus *Actinoplanes*.

Growth characteristics and physiological

TABLE 1. *Growth characteristics and color production by strain X-14695 on various agar media*

Medium	Type of vegetative growth	Color ^a	Sporangia	Diffusible pigments
ISP 2	Abundant growth; colonies with wrinkled surface, cracking with age	Varies from maple (4 <i>le</i>) to chocolate brown (4 <i>pn</i>)	Absent	Absent
ISP 3	Abundant growth; colonies flat, with surface somewhat irregular	Center of colonies turns deep brown (4 <i>pl</i>) with age; younger areas cinnamon (3 <i>le</i>)	Absent	Absent
ISP 4	Abundant growth; colonies of smooth surface, with elevated center; edges of colonies biting into the agar; mild starch hydrolysis	Deep red brown (6.5 <i>pl</i>)	Absent	Absent
ISP 5	Abundant growth; wrinkled colonies with edges biting into agar	From red mahogany (6.5 <i>pl</i>) to rust brown (5 <i>pg</i>)	Present; easy to observe during early growth; later obscured by excess vegetative growth	Absent
ISP 6	Fairly abundant growth	Same as for ISP 3	Absent	Absent
ISP 7	Abundant growth; colonies with wrinkled surface, edges biting into agar; occasionally the edge of colonies appears white due to the development of loose aerial hyphae	Well-defined areas of two different colors: one area is dark brown mahogany (6 <i>pn</i>), and the other is cocoa brown (5 <i>ni</i>)	Absent	Reddish-brown pigment
Hickey and Tresner medium	Moderate growth; circular to irregular colonies with elevated center	Light amber (3 <i>ic</i>)	Absent	Absent
Glucose-asparagine	Very scanty growth		Absent	Absent
Nutrient agar (Difco)	Moderate growth, colonies with flat edges	Various shades of pink color in sector-like outgrowths; some areas become very dark, wine color (6.5 <i>pg</i>)	Absent	Absent
Czapek-glucose	Abundant growth; colonies with smooth flat edges; often center raised as a ring or as a single heap	Color is darker towards the center of colonies; varies from light brown to red mahogany (6.5 <i>pl</i>)	Absent	Absent
Potato-dextrose	Abundant growth; irregularly shaped colonies with edges biting into agar	Color varies from maple (6 <i>le</i>) to oak brown (4 <i>pl</i>)	Absent	Absent

^a See Materials and Methods.

properties. Growth of strain X-14695 was very good at 32°C and somewhat less rapid at 28°C. There was poor growth at 35°C and no growth at 37°C. Growth was extremely poor at 10°C.

Casein and gelatin were hydrolyzed. Growth was negative at NaCl concentrations of 5% or higher. Chemotactic experiments indicated a very moderate attraction of the spores toward chloride ions compared with the spores of *A. brasiliensis* ATCC 25844 (6).

Growth characteristics and color production in various media are given in Table 1.

No characteristic carotenoid spectrum was evident in a scan of a methanolic extract of cells. A major peak at 408 nm and a shoulder at 418 nm were the important characteristics of the absorption spectrum in the visible region.

Of 140 different carbon compounds, 29 supported good growth of strain X-14695. These compounds were D-xylose, L-arabinose, D-glucose, D-mannose, D-galactose, trehalose, maltose, cellobiose, lactose, starch, chitin, gluconate, acetate, succinate, fumarate, suberate, L-malate, pyruvate, glycerol, D-arabitol, mannitol, L-alanine, beta-alanine, L-threonine, L-aspartate, L-glutamate, L-asparagine, L-proline, and spermine. Growth was very slow on D-arabinose, D-fructose, salicin, lactobionate, propionate, sebacate, phenylacetate, and phenylalanine.

When compared with the nutritional spectra of *A. brasiliensis*, *A. italicus*, *A. utahensis*, *A. philippinensis*, *A. missouriensis*, and *A. armeniacus*, that of strain X-14695 appears to be quite restricted (B. Prosser and N. J. Palleroni, unpublished data). Strain X-14695 differs from strains of *A. italicus*, a red-pigmented species of the genus, in that the latter utilize many organic compounds as growth substrates. *A. italicus* grows on D-ribose, L-rhamnose, sucrose, beta-hydroxybutyrate, DL-lactate, citrate, alpha-ketoglutarate, *m*-inositol, quinate, D-alanine, L-arginine, L-glutamine, gamma-aminobutyrate, and L-histidine. These substrates are not used by strain X-14695. Strain X-14695 grows on D-arabitol and spermine, which are not utilized by *A. italicus*. Moreover, the pigment produced by strain X-14695 remains, in most cases, associated with the colonies, and no deep cherry-red diffusible pigment on medium ISP 4 (2) is apparent.

The morphological and physiological properties of strain X-14695 are different from those of

other members of the genus *Actinoplanes*. In addition, the organism has the capacity of producing the proline antimetabolite L-azetidine-2-carboxylic acid. This antimetabolite was found to be produced by this strain and not by many hundreds of other strains of the same genus (Pruess and Scannell, personal communication). Therefore, strain X-14695 appears to be a member of a new species of the genus *Actinoplanes*, for which the name *Actinoplanes ferrugineus* is proposed (*fer.ru.gi.ne.us*. L. adj. *ferrugineus* of the color of iron rust). Strain X-14695 is the type strain of *A. ferrugineus*. A culture of this strain has been deposited in the American Type Culture Collection under the number 29868.

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REPRINT REQUESTS

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