Streptomyces torulosus sp. n., an Unusual Knobby-Spored Taxon

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Several strains of a streptomycete with unusual spore wall ornamentation were isolated from Iowa soils. They are further characterized by coiled chains of spores, gray aerial mycelium, melanin-like pigments, and the production of antibacterial and antifungal antibiotics. Spore wall ornamentations, as seen with the transmission electron microscope, do not correspond with any of the four generally recognized categories of spore wall ornamentation—smooth, warty, spiny, or hairy—but seem to represent an intermediate position between warty and spiny. This unusual ornamentation consists of blunt projections rising out of an otherwise smooth surface and is termed "knobby." To obtain further information on the nature of the knoblike projections and their possible status in representing a defined spore wall ornamentation category, scanning electron micrographs and transmission electron micrographs representing all sporewall ornamentation categories were compared. Differentiation between the several categories was more difficult with the scanning electron microscope than with the transmission electron microscope.

One of the chief characteristics used for the description and identification of streptomycetes is morphology of the spore surface. Four main categories have been used in describing the spore surface: smooth, warty, spiny, and hairy. However, during a recent reexamination of data on several thousand streptomycete spore surfaces, an unusual knobby type was found listed in only three instances. Three strains of streptomycetes having this character had been isolated in April and May 1953 from Iowa soil samples. Each strain is characterized by unusual knobby projections rising out of an otherwise smooth surface. We believe these strains represent a spore ornamentation category between "warty" and "spiny" and consider them a new species.

These three strains have been further characterized and compared by transmission and scanning electron microscopy.

MATERIALS AND METHODS

Strains. The streptomycetes studied are maintained in the Agricultural Research Service Culture Collection as yeast extract (YE)-agar slant cultures (7), as soil stock cultures, and as lyophilized preparations. Liquid inoculum for all media needed in a more complete characterization of the strains was obtained from tryptone-glucose-liver extract-yeast extract (TGLY) broth as described by Lyons and Pridham (4).

Nature of spore surfaces. The nature of the spore surface (transmission electron microscopy) was determined by methods outlined in Lyons and Pridham (4) and Tresner et al. (16).

In addition to these methods, scanning electron micrographs were made of the different spore types and compared to those of spores from the same culture as seen with the transmission electron microscope. Aluminum stubs were prepared by pressing their surfaces on 14-day-old sporulating petri dish cultures. The stubs were then placed in a high-vacuum evaporator (Denton DV-502) and coated with metallic aluminum. After coating, the stubs were placed in a scanning electron microscope (Cambridge Stereoscan) and micrographs were made at 10,000 and 20,000 X.

Further characterization of strains. All strains were characterized by methods outlined in Pridham (6), Pridham and Gottlieb (8), Pridham and Lyons (11, 12), and Lyons and Pridham (4, 5).

In addition to the criteria used in the methods cited above, antibiotic spectrum patterns were determined by the Waksman agar-streak method by using a medium modified from medium A-4h of Warren et al. (17). The agar medium used [soybean meal-yeast extract-glycerol (SYG) agar] consisted of soybean meal (Staley's 4-S), 1.5%; yeast extract (Difco), 0.1%; n-glucose (Difco), 1.5%; glycerol, 0.25% (w/v); NaCl, 0.5%; CaCO₃, 0.1%; agar (Difco), 1.5%; and distilled water; pH (after sterilization for 25 min at 121 C) 6.70 to 6.85. After sterilization, the medium was cooled to 45 C and dispensed into petri dishes. Before inoculation, the petri dishes were allowed to stand at room temperature for 24 hr to dry the surface.

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of the agar; on a wet surface, the inoculum tends to spread and gives an uneven band of growth.

For the agar-streak tests, TGLY inoculum was used. The surfaces of quadruplicate dishes of SYG agar were streaked in a single line ca. 0.25 inch (0.63 cm) in width to give a confluent streak of growth about 3 inches (7.5 cm) in length, approximately 1 inch (2.54 cm) from the edge of the dish. Cultures were incubated for 7 days at 28 to 30 C. At that time, the test organisms were streaked at right angles to the streptomycete growth, taking care not to touch the edge of the growth. The inoculated dishes were then incubated for an additional 3 days at 28 to 30 C after which lengths of inhibition zones were measured and other unusual characteristics were recorded.

Six test organisms were used to indicate the range of antibiotic activity of each strain. The test organisms were: Bacillus subtilis Cohn emend. Prazmowski, NRRL B-765; Sarcina lutea Schroeter, NRRL B-1018; Escherichia coli (Migula) Castellani and Chalmers, NRRL B-766; Saccharomyces pastorianus Hansen, NRRL Y-139; Candida albicans (Robin) Burk., NRRL Y-477; and Mucor ramannianus Moel., NRRL 1839. With this selection of test organisms, one could learn whether a strain, under the experimental conditions, produces antibiotic activity against gram-positive rods, gram-positive cocci, gram-negative rods, yeasts, and molds.

Inocula of the several test organisms used in the agar-streak tests were prepared as follows.

B. subtilis Cohn emend. Prazmowski, NRRL B-1018. Preparation. The inoculum was based on a method proposed by Abbey (1). The broth medium (TSMn) consisted of; Trypticase soy broth, 3.0%; MnSO4.4H2O, 0.0027% (10 μg of Mn+/ml); and distilled water; no pH adjustment was made. The medium was dispensed in 100-ml amounts into 500-ml Erlenmeyer flasks, stoppered with cotton, and sterilized for 20 min at 121 C.

Inoculate 100 ml of TSMn broth with one loopful of cells taken from a TGLY-agar slant culture of the organism held under refrigeration at 3 to 5 C and transferred periodically to fresh TGLY slants. Incubate for 4 days at 28 to 30 C on a Gump rotary shaker operating at 200 rev/min. Centrifuge in a 250-ml bottle, decant supernatant fluid, and resuspend packed cells and spores in 100 ml of sterilized saline (0.85%). Heat-shock for 30 min at 70 C to kill vegetative cells. The spore concentrate may be stored under refrigeration (3 to 5 C). For use in agar-streak tests, dilute portions of the concentrate with sterilized saline solution to give a Lumen colorimeter reading of 80% light transmission with the red filter (650 nm). The diluted suspension contains ca. 100 × 10⁶ viable spores per ml as determined by plating. Suspensions made as described are viable for at least 1 year when stored under refrigeration (3 to 5 C).

S. lutea Schroeter, NRRL B-1018; E. coli (Migula) Castellani and Chalmers, NRRL B-766; S. pastorianus Hansen, NRRL Y-139; C. albicans (Robin) Burk., NRRL Y-477. For each organism, inoculate 10 ml of TGLY broth in a test tube (25 by 150 mm) with one loopful of cells taken from a TGLY-agar slant culture of the organism held under refrigeration at 3 to 5 C and transferred periodically to fresh TGLY slants. Incubate at 28 to 30 C overnight (16 to 18 hr) on a Gump rotary shaker operating at 200 rev/min. Dilute the culture with sterilized saline to give a Lumen colorimeter reading of 50% light transmission for all organisms except S. pastorianus, which is diluted to give a reading of 40% with the red filter (650 nm). Use the diluted suspensions as inocula for agar-streak tests.

M. ramannianus Moel., NRRL 1839. Flood a large Macor-synthetic agar (MS) slant (reference 8; 25 by 150-mm test tube) of a well-sporulated culture with sterilized saline (5 to 10 ml). Incubate the surface of 100 ml of MS agar in a 500-ml Erlenmeyer flask by swirling 1 to 2 ml of the saline spore suspension over the surface. Incubate the flask agar culture at 28 to 30 C until a good web of the typical dull red aerial mycelium forms (5 to 7 days). Add 20 ml of sterilized saline and a few sterilized glass beads to the flask. Dislodge the spores with gentle agitation by moving and swirling the flask while it rests on the laboratory bench. Decant the suspension of spores and dilute with sterilized saline to give a reading of 65% light transmission with the Lumen colorimeter with the red filter (650 nm). Use the suspension as inoculum for agar streak tests. Suspensions made as above remain viable for at least 1 year when held under refrigeration (3 to 5 C).

Other test organisms and substrata, of course, can be used for work of this sort. We find the ones described to be especially suitable.

The test organisms were always streaked from left to right in the same order as they appear above. Their inhibitions were recorded in millimeters. Average inhibitions were calculated generally from triplicate readings. Although some strains showed only minimal inhibitions, they were considered to be positive if the inhibition was demonstrated on at least two tests. A Polaroid photograph was taken to provide a permanent record of the total inhibitory activity of each strain.

RESULTS

Streptomyces torulosus sp. n. Type strain: S-124 = F-57 = NRRL B-3889 (by original designation by Lyons and Pridham herein). tor'ul'o'sus. L. n. torus any round swelling, a protuberance; L. dim. n. torulus a small protuberance; L. adj. suffix -osus full of, having; M.L. torulosus having small protuberances.

Spore surfaces. Transmission and scanning electron micrographs of representative cultures of the four recognized spore wall categories are shown in Fig. 1. Photomicrographs of the knobby spores of S. torulosus, as obtained from both the transmission and scanning electron microscopes, are shown in Fig. 2.

Morphology of spore chains. Spores are arranged in both dextrorse and sinistorse coiled chains, with three to five volutions per coil.

Color of aerial mycelium. The color of the aerial mycelium observed with inorganic salts-
starch-agar after 14 days at 28°C was placed in the gray series of both Pridham et al. (9) and Tresner and Backus (15).

**Ability to darken peptone-iron-agar.** A brown to black diffusible pigment is formed in peptone-iron-agar.

**Melanin-like chromogenicity.** Brown to black diffusible pigments are formed in TGLY broth.

**Ability to utilize various carbon sources for growth.** D-Glucose, D-xylose, L-arabinose, L-rhamnose, D-fructose, D-galactose, raffinose, D-mannitol, i-inositol are utilized; salicin is not.

**Ability to grow on Czapek's solution-sucrose-agar.** Growth is excellent on Czapek's solution-sucrose-agar.

**Whole-cell hydrolysates.** The L-form of diaminopimelic acid is found in whole-cell hydrolysates. Faint spots for mannose and arabinose also can be detected.

**Streptomycin sensitivity.** Strains are sensitive to streptomycin.

**Standard antibiotic spectrum patterns.** The type strain is able to inhibit *B. subtilis, S. lutea, E. coli,* and *M. ramannianus.* *S. pastorius*
was very slightly inhibited, and *C. albicans* was not inhibited.

**DISCUSSION**

Dietz and Mathews (2) recently proposed a fifth spore surface category which they term "rugose." Our strains of *S. torulosus* would not be categorized as rugose. Strains of streptomycetes with spores of the rugose kind were considered as *S. albus* (Rossi Doria, 1891) Waksman and Henrici 1943 in the key to species proposed by Pridham et al. (13) in 1965. We still consider them as strains of subspecies of *S. albus*. Dietz and Mathews comment that the well-defined spore chains they illustrate in their Fig. 5B are, in fact, smooth and that transmission electron microscopy never reveals this feature. We have noted a number of strains of the rugose kind in our transmission electron microscopy studies. As illustrated in Fig. 5D of Dietz and Mathews, transmission electron microscopy of such strains generally reveals a long roughened tube in which individual spores are difficult to discern. Despite the fact that a number of different specific and subspecific epithets are represented among the strains we know with spores of the rugose kind, most of these probably would be identified by specialists as strains of *S. hygroscopicus* (Jensen, 1931) Waksman and Henrici 1948 as originally characterized by Jensen (3) and further amplified by Tresner and Backus (14). Based on our concepts they would be *S. albus*. Our strains of *S. torulosus* have not exhibited hygroscopicity on any of the media we have used which ordinarily would allow development of this characteristic. Furthermore, transmission electron microscopy clearly shows individual spores in chains. One might speculate that the ill-defined spore chains exhibited by strains of the rugose kind, as shown by both scanning and transmission electron microscopy, are somehow related to hygroscopicity and its effect on the spore sheath.

After careful examination of photomicrographs of the spore types taken with the scanning electron microscope, we found it difficult to separate them into the spore wall ornamentation groups. By mounting our specimen directly on the aluminum stub, instead of using adhesives, we obtained better results. The spines observed with the transmission electron microscope, however, appeared as blunt knobs with the scanning microscope. Hairs appeared as thick or matted bristles. Smooth, rough, and warty spores were almost impossible to differentiate. Knobby spores, however, appeared to have round knobs with the scanning microscope but looked more like attached flakes of outer covering with the transmission microscope.

The projections rising out of the spore surface or spore sheath (spines, hairs, or knobs) seem to build up an excess of metal coating during processing for scanning microscopy. With our procedures, spores have no coatings when viewed with the transmission electron microscope and hence a truer picture of the spore outline is seen.

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