New Genus of the *Actinomycetales*: *Actinosynnema* gen. nov.

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An aerobic species of a new genus, *Actinosynnema*, is described under the name *A. mirum* sp. nov. The type strain of *A. mirum* is strain Hasegawa 101 (= IMRU 3971). The new genus is characterized by the formation of synnemata originating from the substrate mycelium. At the tip of the synnemata, chains of zoospores are produced.

Synnemata, also called coremia, are compacted groups of erect hyphae which are often fused and which bear conidia at their apex only or at both their apex and on their sides. Sterile columnar hyphal structures are sometimes also called synnemata or coremia.

The formation of synnemata and related structures in *Actinomycetales* is well documented. These have been reported in the genus *Streptomyces* by Krasil'nikov (*Actinomyces*) (11), Waksman (21), Grein (6), and Grein and Spalla (7), and "spore dome colonies" have been observed by Willoughby (22) in unidentified isolates of actinomycetes.

The present paper reports the description of an aerobic actinomycete that was isolated from a grass blade and which forms synnemata with zoospores. The new organism differs significantly from previously described species of actinomycetes and cannot be accommodated in any of the previously described genera of the actinomycetes. It is proposed to include it in a new genus, *Actinosynnema*, the type species of which is *A. mirum*. A culture of the type strain, Hasegawa 101, has been deposited in the culture collection of the Waksman Institute of Microbiology, Rutgers, The State University, Piscataway, N.J., under the number IMRU 3971.

MATERIALS AND METHODS

Bacterial strain. Strain 101 was isolated in September 1976 from a grass blade which was collected from the Raritan River, New Jersey, placed on yeast extract agar (0.02% yeast extract [Difco], 1.5% agar), and incubated for 3 weeks at 28°C. After that period of incubation, the surface of the agar was covered with various types of microbial growth, but, on the grass blade itself, small synnemata could be observed with a stereoscopic microscope. With a flamed loop, some of the synnemata were transferred to a sterile plate of the same medium from which the strain was isolated.

Media. The following media were used in this study: Bennett agar (9), tyrosine agar (18), yeast-malt agar

(18), water agar (8), yeast-starch agar (1), Czapek agar (21), oatmeal agar (18), nutrient agar (Difco), thin Pablum agar (15), yeast-dextrose agar (21), glucose-asparagine agar (21), N-Z-amine glycerol agar (12), soil agar (12), inorganic salts-starch agar (18), thin potato-carrot agar (TPC) (8), and defined agar (12). The medium used for morphological observations was mainly TPC agar.

Microscopy. The study of whole mounts of sporophores by transmission electron microscope was done by the method of Lechevalier and Lechevalier (14) with a JEM-120 electron microscope operated at 80 kV. For observation of zoospores, drops of the suspension containing zoospores were placed on Formvar-coated, 300-mesh copper grids. The excess liquid was absorbed on filter paper and was replaced by a drop of 2.0% phosphotungstic acid adjusted to pH 6.2 with potassium hydroxide. The excess stain was removed with filter paper, and, after air drying, the preparations were promptly examined with the electron microscope.

For scanning electron microscopy, a TPC agar block with numerous synnemata was put on an aluminum stub and dried overnight in vacuo. The dried specimen was mounted with silver paint on the same stub and was then coated with gold. The coated specimen was examined in an Autoscan scanning electron microscope.

Other conditions of growth. Grass blades washed with sterile water were placed both in a moist chamber and on TPC agar. Spores of *A. mirum* were inoculated onto the surface of the grass blades and cultured for 1 week at 28°C.

For growth tests both in a 4% CO₂ atmosphere and under anaerobic conditions, the GasPak systems (BBL) were used.

Physiological tests. The tests employed were those of Gordon and Mihm (5), and Gordon (3, 4); phosphatase production was estimated by the method of Barber and Kuper (2).

Cell wall and whole-cell analyses. Cell walls were analyzed by the method of Šuput et al. (19), and whole cells were analyzed by the procedure of Lechevalier (13).

Staining procedures. The Gram stain for actinomycetes, in which aniline crystal violet is used as the primary stain, and the Kinyoun acid-fast stain modified for actinomycetes (16) were used.

Susceptibility to antibiotics and antimicrobial agents. The susceptibility of strain 101 to a few antibiotics and antimicrobial agents was examined by the

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following methods: (i) susceptibility disks (Difco) were placed onto Bennett agar and TPC agar seeded with a suspension of the test strain; after incubation for 1 week at 28°C, the diameters of the inhibition zones were measured; (ii) antibiotics were incorporated into nutrient agar (Difco), and the test organisms were streaked onto the surface; after 1 week of incubation, the surface growth was compared with that on a plate without antibiotics (control).

Antibiotic properties. Antibiotic properties were studied by the cross-streak test on TPC and Bennett agars (20) and by the agar-dilution method. The strain was grown in four liquid media: Pabulum (14), NYG-3 (8 g of nutrient broth [Difco], 10 ml of glycerol, 1 g of yeast extract, 1 g of NaNO₃, and 1,000 ml of tap water), yeast-dextrose agar (20), and N-Z-amine glycerol agar (12). After 4 and 7 days the broths were filtered through paper and assayed by dilution in nutrient agar against four test organisms: *Escherichia coli* IMRU 54, *Staphylococcus aureus* IMRU 15, *Pseudomonas* sp. LL-NC2, and *Candida albicans* LL-204.

RESULTS

Description of *Actinosynnema* gen. nov.

(From *actis*, *actinos* [Gr. noun], ray; *syn* [Gr. adv.], together; and *nema*, *nematos* [Gr. noun, neuter], thread.)

Fine hyphae (about 0.5 μ m in diameter) are produced which form a mycelium differentiated into: (i) a substrate mycelium that penetrates the agar and also grows into and forms synnemata or domelike bodies on the surface of agar; and (ii) an aerial mycelium that arises from the synnemata, domelike bodies, or flat colonies. The aerial hyphae bear chains of conidia capable of forming flagella in an aqueous environment. The cell wall is of type III, and the whole-cell sugar pattern is of type C.

Type species: *Actinosynnema mirum* sp. nov. (from *mirus*, -a, -um [Lat. adj.], marvelous).

Substrate mycelium. The hyphae are about 0.5 to 1.0 μ m in diameter. Long, branching filaments penetrate the agar medium and form synnemata or domelike bodies that are at first whitish on most media and then become yellowish or yellowish orange.

Aerial mycelium. The hyphae are about 0.5 to 1.0 μ m in diameter. Long, branching hyphae, white to pale yellow, are produced, but not abundantly, on most media. Aerial mycelium is formed at the ends of synnemata on such agar media as TPC, tyrosine agar, and water agar. On yeast-malt agar, yeast-dextrose agar, Bennett agar, thin Pabulum agar, and glucose-asparagine agar, aerial hyphae are formed both at the ends of synnemata or domelike bodies and on the surfaces of flat colonies. In young, growing cultures, the tips of the aerial hyphae often curl (Fig. 1).

Synnemata. Synnemata originate from the

substrate mycelium and are formed on complex and synthetic media. TPC agar is the best medium found so far for the production of synnemata and the formation of the aerial mycelium at its tip (Fig. 2). Also, synnemata are formed on the surfaces of grass blades placed on agar media or in a moistened atmosphere. The size of a typical synnema formed on the TPC is illustrated in Fig. 3.

General microscopic appearance. The surface of a sporulating colony of *A. mirum* viewed perpendicularly with a light microscope appears covered with tufts of aerial hyphae. If the surface of the agar is tilted, the synnemal nature of the tufts is revealed (Fig. 4).

Spores. Spores are borne in chains. Septa are set down along the aerial hyphae and give rise to chains of spores. The septa are not apparent under the light microscope; however, in electron microscope preparations they appear as "swellings," which give the hyphae a bamboo-like appearance (Fig. 5 and 6). The spores are easily separated from each other. If the synnemata on TPC agar are placed on a slide and covered with brain heart infusion broth (Difco) and cover slips, motile spores can be observed after about 1 h at room temperature. The rod-shaped spores (0.4 to 1.0 μ m by 1.5 to 3.0 μ m) are peritrichous and bear 5 to 15 flagella (Fig. 7). Moreover, motile elements are observed in other media such as static cultures in actinomyces broth (Difco) or TPC broth. During germination, one to three germ tubes are formed.

Cell chemistry. The cells have cell walls of type III containing *meso*-diaminopimelic acid in addition to alanine, glutamic acid, and glucosamine. Galactose and mannose are present in whole-cell hydrolysates. Madurose is absent. The organism can thus be considered to have a type C whole-cell sugar pattern.

Diffusible pigments. This strain produces a pale yellowish-brown soluble pigment on tyrosine agar and a pale greenish one on oatmeal agar. It does not produce any diffusible pigments on other media.

Appearance on various media. All cultures were incubated for 2 weeks at 28°C. No growth took place on the defined medium.

Yeast-malt agar: Growth excellent, yellowish (color code Coo2a of Prauser [17]); synnemata moderate. Aerial mycelium poor, white.

Water agar: Growth moderate, whitish; synnemata numerous. Aerial mycelium moderate, white.

Yeast-starch agar: Growth moderate, yellowish (Coo3a); synnemata numerous. Aerial mycelium poor, white.

Tyrosine agar: Growth moderate, yellowish

FIG. 1. Scanning electron micrograph of aerial hyphae at the tip of a synnema. Bar represents 1 μ m. TPC agar, 5-day-old culture.

(Coo2a); synnemata numerous. Aerial mycelium moderate, white. Soluble pigment pale yellowish brown.

Czapek agar: Growth moderate, yellowish (Coo2a); dome-like bodies few. Aerial mycelium poor, white.

Bennett agar: Growth excellent, yellowish (Coo2a-Coo2r); synnemata and domelike bodies numerous. Aerial mycelium moderate, white.

Oatmeal agar: Growth excellent, yellowish (Coo4a-Coo2a); synnemata few. Aerial mycelium poor, white. Soluble pigment pale greenish yellow (Co4r).

Nutrient agar: Growth moderate, yellowish (Coo2a); synnemata few. No aerial mycelium.

Thin Pablum agar: Growth moderate, yellowish (Coo2a); synnemata numerous. Aerial mycelium moderate, white.

Yeast-dextrose agar: Growth moderate, yellowish (Coo2a); synnemata numerous. Aerial mycelium poor, white.

Glucose-asparagine agar: Growth moderate, yellowish (Coo2a); synnemata and domelike bodies moderate. Aerial mycelium moderate, white.

N-Z-amine glycerol agar: Growth moderate, orange-yellow; glistening, domelike bodies are moderate; synnemata few. Aerial mycelium poor, white.

Soil agar: Growth poor, whitish, thin; synnemata few. No aerial mycelium.

Inorganic salts-starch agar: Growth poor, whitish; synnemata few. Aerial mycelium poor, white.

TPC agar: Growth moderate, yellowish (Coo2a); synnemata numerous. Aerial mycelium moderate, white.

Staining characteristics. The hyphae and spores are mainly gram positive and are not acid fast.

Physiological and biochemical characteristics. No growth took place on any medium

FIG. 2. Scanning electron micrograph of a synnema. Bar represents 10 μm .

FIG. 3. Diagram representing the dimension of a synnema of *A. mirum* grown on TPC agar.

under anaerobic conditions, but growth was moderate under a CO_2 atmosphere. The temperature range for growth was 10 to 35°C. Starch, casein, tyrosine, and gelatin were hydrolyzed, but xanthine, hypoxanthine, adenine, and urea were not. Nitrate reductase and phosphatase were produced. Milk was peptonized. Growth was produced in lysozyme broth. No growth occurred at 37°C or on 5% NaCl. Tartrate, py-

ruvate, lactate, and malate were utilized. Benzoate, acetate, citrate, and succinate were not utilized. Acid was produced from fructose, lactose, maltose, D-mannitol, L-arabinose, D-melibiose, D-mannose, L-rhamnose, xylose, dextrin, galactose, glucose, trehalose, raffinose, soluble starch, sucrose, cellobiose, glycogen, and adonitol. No acid was produced from *D*-inositol, sorbitol, *D*-ribose, salicin, inulin, glycerol, dulcitol, erythritol, α -methyl-*D*-glucoside, or α -methyl-*D*-mannoside.

Antibiotic properties. Zones of inhibition by cross-streak test were produced with gram-positive bacteria (*Bacillus megaterium* LL-CW 4A, 10 mm; *Sarcina lutea* IMRU 14, 23 mm), a mycobacterium (*M. smegmatis* ATCC 607, 8 mm), filamentous fungi (*Aspergillus niger* LL-13, 25 mm [incomplete inhibition]; *Penicillium notatum* LL-40, 25 mm [incomplete inhibition]) and yeasts (*Candida tropicalis* LL-206, 10 mm; *Saccharomyces cerevisiae* LL-216, 10 mm). Shake-flask cultures showed no activity except for a slight activity against *C. albicans* in the

FIG. 4. Micrograph of a synnema growing on a plate of TPC agar. The surface of the agar was slanted to visualize the stem of the synnema. Bright-field microscopy. Bar represents 10 μ m.

FIG. 5. Electron micrograph of spore chains from the tip of a synnema. TPC agar, 7-day-old culture. Bar represents 1 μ m.

case of Pablum broth (10 dilution units per ml).

Susceptibility to antibiotics and antimicrobial agents. On nutrient agar, this organism grew in the presence of 50 μg of candidin per ml and 100 μg of nystatin per ml. Dihydrostreptomycin at 10 $\mu\text{g}/\text{ml}$ completely inhibited growth as did 1 μg of tetracycline per ml. By the diffusion method, growth was inhibited strongly by disks impregnated with tetracycline and its derivatives (30 μg), chloramphenicol (30 μg), novobiocin (30 μg), and bacitracin (10 μg).

Type strain. The type strain is Hasegawa 101 (= IMRU 3971). Because the species de-

scription given above is based on a single strain, the type strain, it also serves as the description of the type strain.

DISCUSSION

In a recent review, Kalakoutsii and Agre (10) noted that synnemata are known to be produced by many actinomycetes but that no signs of special transformations of hyphae in these structures have been reported so far. We propose that organisms that form synnemata bearing, at their tips, chains of spores capable of forming flagella (when in an aqueous environment) be placed in the genus *Actinosynnema*.

It is difficult to place the genus *Actinosynnema* in any of the currently recognized families of the order *Actinomycetales*. Aerial chains of motile spores are formed in the genus *Sporichthya*, but the two genera can be differentiated on the basis of formation of substrate mycelium and synnemata in the genus *Actinosynnema* and

FIG. 6. Probable mode of spore formation in *Actinosynnema mirum*.

FIG. 7. Electron micrograph of a zoospore of *A. mirum*. Bar represents 0.1 μm .

their absence in the genus *Sporichthya*. In addition, members of these two genera are very different in their cell wall compositions (type I for *Sporichthya*, type III for *Actinosynnema*).

A number of actinomycetes with a type III cell wall are known to produce motile elements. These include the sporangia-forming, madurose-containing members of the genera *Planomonospora*, *Planobispora*, and *Spirillospora*. Actinomycetes of this spore wall type with motile elements which do not form sporangia include the madurose-containing members of the genus *Dermatophilus* and their relatives in the genus *Geodermatophilus* which lack madurose. However, there is no morphological similarity between the *Dermatophilaceae*, which do not form aerial hyphae but which produce masses of motile cocci by the division of hyphae, and *A. mirum* with its aerial chains of motile, rod-shaped spores.

Pending the isolation of more strains of actinomycetes forming synnemata with chains of spores at the tip of these structures, we feel that the genus *Actinosynnema* should be considered one of the genera of the order *Actinomycetales* which are of uncertain familial placement.

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REPRINT REQUESTS

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