Pseudonocardia compacta sp. nov.

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The new mesophilic species *Pseudonocardia compacta* was isolated from soil. *P. compacta* resembles the type species of the genus *Pseudonocardia* in exhibiting acropetal budding of the hyphae, in having a two-layered hyphal wall, in cell wall composition (chemotype IV), in phospholipids (type III), in the lack of mycolic acid, and in the occurrence of yellow pigments (carotenoids). *P. compacta* is characterized by densely aggregated substrate and aerial hyphae that frequently bear apical and intercalary swellings, by thick-walled spores, by white aerial mycelium and by being mesophilic. The type strain is strain MB H-146 (= DSM 43592 = CBS 160.82).

Two strains of a mesophilic actinomycete isolated from soil proved to be a new species of the genus *Pseudonocardia*. The new species, named *Pseudonocardia compacta*, differs in some features of hyphal growth and spore formation from the type species *Pseudonocardia thermophila* Henssen and from *Pseudonocardia spinosa* Schäfer, the only two species included on the Approved Lists of Bacterial Names (23). Three other organisms have been assigned to the genus *Pseudonocardia*, "*Pseudonocardia fastidiosa*" (W. D. Celmer et al., U.S. patent 4,031,206, June, 1977), "*Pseudonocardia thermospinosa*" (15), and "*Pseudonocardia azurea*" (17). Preliminary studies of the type strains of "*P. azurea*" and "*P. fastidiosa*" performed by us (unpublished data) revealed that these two organisms deviate in hyphal growth and fine structure and should not be included in the genus *Pseudonocardia*.

The genus *Pseudonocardia* is characterized by acropetal budding of substrate and aerial mycelia, leading to segmented hyphae (6-8), as well as by a two-layered wall and interspace septa (9). In *P. thermophila* the segments may function directly as spores, or they may be divided secondarily by intercalary septa (9). Henssen and Schäfer claimed that *P. spinosa* has no cross walls (7), but recent studies of ultrathin sections have revealed septa in substrate and aerial hyphae (unpublished data).

MATERIALS AND METHODS

Bacterial strains. The two strains of *P. compacta* (strains MB H-146^T [T = type strain] and MB H-147) were isolated from garden soil collected in Wohra near Marburg, Hessen, Federal Republic of Germany in

1980. For isolation the simplified method described for the isolation of strains of *Streptosporangium* (2) was used.

Media. The following media were used: Casamino Acids-peptone Czapek agar (CPC agar) (7), Czapek agar (26), yeast extract-starch agar (E agar) (2), EGH agar (soil extract 250 ml; yeast extract, 0.5 g; glucose, 0.5 g; agar, 15 g; tap water 750 ml), yeast extract-malt extract agar (ISP no. 2 medium) (22), artificial soil extract-yeast extract agar (7), and peptone-beef extract agar (26).

Morphological studies. Budding and sporulation of hyphae were examined in hanging-drop cultures with a Leitz Ortholux microscope. For scanning electron microscopy with a Leitz model AMR 1200B instrument, sporulating colonies on E agar were fixed with osmium tetroxide vapor overnight (14). Small pieces were cut out from the plate and dried in a deep frozen condition in a closed plastic box with phosphorus pentoxide (H. A. v. Stosch, personal communication). The samples were attached to stubs and coated with a thin layer of gold by using a sputter coater (Balzers A/U. Balzers, Liechtenstein). Silhouettes of spores and hyphae were studied with a transmission electron microscope, using untreated impressions of aerial mycelium. For ultrathin sections small blocks were cut out from 4- to 15-day-old cultures on E or CPC agar. These blocks were fixed with formaldehyde-glutaraldehyde (10) and postfixed overnight with 1% osmium tetroxide in sodium cacodylate buffer (0.05 mol/liter; pH 7.0). The blocks were dehydrated in ethanol and embedded in epoxy resin (24). Thin sections were cut with a Reichert Ultracut 42 instrument and poststained with uranyl acetate (27) and lead citrate (19). Preparations were examined with a Siemens Elmiskop IA instrument.

Cell wall analysis. For chemical analysis of cell walls, substrate mycelium was harvested from submerged cultures incubated in 200-ml Erlenmeyer flasks in CPC liquid medium at room temperature on a rotary shaker. Sporulating aerial mycelium was scraped off CPC agar plates.

Wall constituents were analyzed by the methods of Kroppenstedt (11) and Richter (20). Thin-layer chromatographic separation of sugars on cellulose layers was improved by using a new solvent system contain-

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ing 25 ml of boric acid (0.5 mol/liter), 35 ml of pyridine, and 100 ml of ethyl acetate, with three successive runs in the same direction.

Pigment production. Pigments of whole-organism extracts in methanol or chloroform were purified by thin-layer chromatography, using the following solvent system: petroleum ether (bp, 60 to 80°C)-diethyl ether (85:15, vol/vol) (1). Eluates of the pigments in hexane (18) were analyzed spectrophotometrically. Visible light spectra were recorded with a Zeiss model DMR 10 instrument.

Detection of phospholipids. Total lipids were extracted from simultaneously harvested and thoroughly washed mycelium by suspending mechanically homogenized lots of approximately 50 mg-equivalents (wet weight) in an extraction solution containing methanol and chloroform (2:1, vol/vol). The extraction vials were sealed with Parafilm and shaken in a water bath shaker at 30°C for 24 h. After the cellular debris was removed by centrifugation at 10,000 rpm, the solution was reduced under a vacuum to a final volume of 250 μ l.

Thin-layer chromatography was performed by the method of Vitiello and Zanetta (25), with the following alteration: HPTLC-precoated chromoplates (E. Merck AG, Darmstadt, Germany) were run once before sam-

ple application in methanol-chloroform (1:1, vol/vol) and dried for 30 min. The solvent system (25) was changed; we used ethyl acetate instead of methyl acetate and a slightly different amount of ethanol (25 ml of ethyl acetate, 25 ml of *n*-propanol, 25 ml of chloroform, 12 ml of methanol, 9 ml of 0.25% aqueous potassium chloride).

Phospholipids were visualized by spraying the plates with molybdenum blue reagent (Sigma, Munich, Germany). Standard phospholipids were obtained from Sigma. This method has the advantage of saving time and material in the purification of total lipid extracts; furthermore, it is highly reproducible.

Whole-cell methanolysis for the detection of mycolic acid was performed by the method of Minnikin et al. (16). Dry bacteria (approximately 50 mg) were mixed with a solution containing 5 ml of 100% methanol, 5 ml of redistilled toluene, and 0.2 ml of concentrated sulfuric acid in a tube with a polytetrafluoroethylene-lined screw cap. The contents were thoroughly mixed, and methanolysis was allowed to proceed for 15 h at 75°C. When the mixture cooled to room temperature, it was extracted with *n*-hexane, and the hexane extract was spotted onto thin-layer chromatographic plates: (Silica Gel 60_{F254}). The chromatogram was developed in petroleum ether (bp, 60 to 80°C)-

FIG. 1. Part of *P. compacta* colony incubated for 14 days on E agar. Scanning electron micrograph.

FIG. 2. *P. compacta* sporulating aerial hyphae on E agar. (A) Acropetal budding (B) Basipetal budding. Scanning electron micrograph. Bar = 5 μ m.

diethyl ether (85:15, vol/vol), and the spots were visualized by spraying the plates with 5% ethanolic molybdophosphoric acid and charring at 110°C.

Additional methods. Carbon utilization and cellulose degradation were determined by using Czapek solution containing 2.64 g of $(\text{NH}_4)_2\text{SO}_4$ per 1,000 ml as the basal medium; to this basal medium different types of sugars (sterilized by propylene oxide) or cellulose was added to a final concentration of 1%. Starch hydrolysis was tested with iodine on E agar (4). Temperature tolerance was determined on E agar and CPC agar. Tests for liquefaction of gelatin were carried out on 12% gelatin. Nitrate reduction medium containing 1% agar was used according to Waksman (26).

RESULTS AND DISCUSSION

Morphological characteristics. Strains MB H-146^T and MB H-147 form lumpy colonies on media which support production of aerial mycelium (Fig. 1). These colonies are composed of densely aggregated aerial hyphae, which frequently bear apical and intercalary swellings (Fig. 2 through 5). The straight or occasionally helically twisted hyphae are more or less regu-

larly segmented (Fig. 6). Substrate and aerial hyphae elongate by acropetal budding, and side branches arise from lateral buds (Fig. 2 through 5). The segments may function directly as spores, or they may be divided secondarily by intercalary septation during the process of sporulation (Fig. 7B and C). In addition, spores are formed basipetally in portions of unsegmented hyphae (Fig. 5).

The hyphal wall is composed of two layers, an inner evenly thick layer and an outer more electron-dense irregularly shaped layer to which the fibrous sheath is closely attached in aerial hyphae (Fig. 7A through D and 8A through C). The septa are interspace septa (9), and the cross walls are type 2 of Williams et al. (28). The cross walls are formed by the inward growth of a double annulus with separate tips (Fig. 7B) or united tips (Fig. 7C). Each double cross wall starts with a triangular thickening from the inner wall, which remains visible in mature spores (Fig. 7B and C, arrows). The spores separate by autolysis (Fig. 7A, C, and D).

FIG. 3. *P. compacta* aerial mycelium formed in a hanging-drop culture in E solution after 12 days (A) and 9 days (B) of incubation. Light micrograph. Bar = 10 μ m.

FIG. 4. Development of *P. compacta* aerial hyphae after 6 days (A and A'), 7 days (B and B'), and 8 days (C and C') in a hanging-drop culture in CPC solution. The arrows indicate acropetal budding, and the arrowheads indicate formation of swelling. Light microscope time-lapse photographs and corresponding drawings. Bar = 10 μ m.

Lateral budding starts below a septum. When a bud enlarges, the inner hyphal wall becomes gradually thinner toward the tip, and wall material is deposited at the base of the bud (Fig. 8A). The formation of an apical bud is also accompanied by a basal increase in the inner wall layer (Fig. 8B). Later, the septum is formed not at this point but further below at a distance (Fig. 8C); the same has been observed when intercalary swellings are separated by septa.

In general, the growth morphology and ultrastructure of *P. compacta* correspond well with the morphology and ultrastructure of *P. thermophila*, the type species of the genus. This holds for the acropetal budding that gives rise to segmented hyphae, as well as for the basipetal formation of spores (designated sporulation type 2 in *P. thermophila*) (6). The zigzag growth characteristic of other species of *Pseudonocardia* (5, 7, 8) is less distinct in *P. compacta*; helically twisted hyphae have been observed so far only in *P. compacta*. The short aerial hyphae with frequent branching, as well as the apical and intercalary swellings, are characteristic fea-

tures of the new species. Irregular swellings in the substrate mycelium have been observed in *P. spinosa* (7).

The ultrastructure of hyphae and the process of sporulation have been studied in *P. thermophila* (9). In this species the layers of the hyphal wall are much thinner and usually separated by a less electron-dense zone; the spores also remain thin walled.

FIG. 5. Development of *P. compacta* aerial hyphae in a hanging-drop culture in CPC solution after 10 days (A), 12 days (B), 13 days (C), 14 days (D), and 19 days (E) of incubation. The arrows indicate basipetal production of spores, and the arrowheads indicate lateral budding, terminating in apical swelling (E, left side). Drawings of time-lapse photographs.

FIG. 6. *P. compacta* aerial hyphae: silhouettes of whole mounts. (A) Segmented hypha with apical bud. (B) Spore chain having bamboo-like appearance. (C) Helically twisted hypha. Transmission electron micrographs. Bar = 1 μ m.

Details of the budding process documented in ultrathin sections are first presented in this paper. The formation of septa not adjacent to but at a distance from apical and intercalary swellings seems to be a characteristic of *P. compacta*.

Chemical composition of cell wall. A chemical analysis of sporulating aerial mycelium and submerged growing substrate mycelium proved the presence of *m*-diaminopimelic acid, arabinose, and galactose, the constituents of chemotype IV (12), corresponding to *P. thermophila* and *P. spinosa* (7). We found no differences when we analyzed whole-organism hydrolysates and enriched cell wall fractions.

Diagnostic phospholipids. The phospholipids identified in *P. compacta* were phosphatidylinositol, phosphatidylcholine, and phosphatidylglycerol. Two additional spots were tentatively identified as phosphatidylethanolamine (traces) and disphosphatidylglycerol (traces in strain MB H-146^T, absent in strain MB H-147); this pattern corresponds to type III, which has been reported to be characteristic for the genus *Pseudonocardia* (13).

Mycolic acid. Using the method of Minnikin et

al. (16), we found no mycolic acid in either strain of *P. compacta*, corresponding to results obtained for *P. thermophila* (3).

Pigment. As in *P. thermophila* and *P. spinosa* (7), a yellow fat-soluble pigment is produced in the mycelium, which is stored inside the cells and accumulates during aging. The absorption spectrum produced by *P. compacta* type strain MB H-146 had shoulders at 424.5 and 472.5 nm and a maximum at 446.5 nm, a spectrum characteristic for carotenoids. The pigment of strain MB H-147 was characterized by an absorption spectrum with two shoulders at 423 and 473 nm and a maximum at 446.5 nm, almost exactly the same as the spectrum for strain MB H-146^T. Smaller deviations between the two spectra, as well as insignificant differences from the spectra of *P. thermophila* and *P. spinosa* (7), are most likely due to the degree of purification.

Cultural and physiological properties. The optimum temperature for growth is between 20 and 30°C; at 10 and 15°C growth is retarded, and no growth occurs at 35°C. On media suitable for growth the substrate mycelium penetrates deeply into the agar and has a yellow to brown color;

FIG. 7. *P. compacta*. (A through C) Sporulating aerial hyphae. (A) Chain of cylindrical spores. (B) Inwardly growing double cross wall with separate tips in hypha after the apical spore was broken off. The arrows indicate peripheral thickenings of the inner wall. (C) Double cross wall with united tips and spores separating by autolysis. (D) Sporulating substrate hypha. Transmission electron micrographs.

the aerial mycelium is white, and soluble pigments are not produced. Formation of melanin, a tryptosinase reaction, liquefaction of gelatin, and hydrolysis of starch could not be demonstrated; however, nitrate reduction was positive. The following carbon compounds are utilized: L-arabinose, D-fructose, D-glucose, *myo*-inositol, D-mannitol, L-rhamnose, D-raffinose, sucrose, and D-glucose. No growth occurs on cellulose.

On CPC agar: growth good, substrate mycelium well developed and yellow, and sporulating aerial mycelium abundant.

On Czapek agar: growth moderate, substrate mycelium light brown, and aerial mycelium lacking.

On E agar: growth good, substrate mycelium ochre, and aerial mycelium scarcely developed.

On EGH agar: growth good, substrate mycelium brown, and sporulating aerial mycelium abundant.

On ISP no. 2 medium: growth moderate,

substrate mycelium ochre, and aerial mycelium lacking.

On artificial soil extract-yeast extract agar: growth moderate to good, substrate mycelium almost lacking, and aerial mycelium abundant.

On peptone-beef extract agar: growth moderate, substrate mycelium yellow, and aerial mycelium almost lacking.

Pseudonocardia compacta sp. nov. (L. adj. fem. *compacta* compact, referring to the growth form of the colonies). Gram-positive. Substrate mycelium yellow to brown, forming a compact mass of septate hyphae 0.6 to 2.4 μ m thick. Aerial mycelium white, composed of densely aggregated hyphae. Apical and intercalary swellings abundant in substrate and aerial mycelia. Spores smooth, up to 3 μ m long; swellings up to 2.5 μ m wide. Elongation of hyphae by acropetal budding; formation of branches by lateral budding. Hyphal wall composed of two layers; interspace septa formed. Cell wall chemotype

FIG. 8. *P. compacta* aerial hyphae. (A) Formation of lateral bud. (B) Apical bud, with the cell wall considerably increased at the base. (C) Septum formed at a distance below the apical swelling. Transmission electron micrographs. Bar = 0.5 μ m.

IV, phospholipids type III, no mycolic acid. The type strain is strain MB H-146 (= DSM 43592 = CBS 160.82).

P. compacta differs from *P. spinosa* in having smooth spores and from *P. thermophila* in being mesophilic and having richly branched hyphae with apical and intercalary swellings.

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LITERATURE CITED

- Alshamaony, L., M. Goodfellow, D. E. Minnikin, G. H. Bowden, and J. M. Hardie. 1977. Fatty acid composition of *Bacterionema matruchotii* and related organisms. *J. Gen. Microbiol.* **98**:205–213.
- Emerson, R. 1958. Mycological organization. *Mycologia* **50**:589–621.
- Goodfellow, M., and D. E. Minnikin. 1981. Classification of nocardioform bacteria. *Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Suppl.* **11**:7–16.
- Gordon, R. E., and M. M. Smith. 1955. Proposed group of characters for the separation of *Streptomyces* and *Nocardia*. *J. Bacteriol.* **69**:147–150.
- Henssen, A. 1957. Beiträge zur Morphologie und Systematik der thermophilen Actinomyceten. *Arch. Mikrobiol.* **26**:373–414.
- Henssen, A. 1974. *Pseudonocardia*, p. 746–747. In R. E. Buchanan and N. E. Gibbons (ed.), *Bergey's manual of determinative bacteriology*, 8th ed. The Williams & Wilkins Co., Baltimore.
- Henssen, A., and D. Schäfer. 1971. Emended description of the genus *Pseudonocardia* Henssen and description of a new species, *Pseudonocardia spinosa* Schäfer. *Int. J. Syst. Bacteriol.* **21**:29–34.
- Henssen, A., and E. Schnepf. 1967. Zur Kenntnis thermophiler Actinomyceten. *Arch. Mikrobiol.* **57**:214–231.
- Henssen, A., E. Weise, G. Vobis, and B. Renner. 1981. Ultrastructure of sporogenesis in actinomycetes forming spores in chains. *Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Suppl.* **11**:137–146.
- Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* **27**:137A.
- Kroppenstedt, R. M. 1979. Chromatographische Identifizierung von Mikroorganismen, dargestellt am Beispiel der Actinomyceten. *Kontakte (Merck)* **79**:12–21.
- Lechevalier, M. P., and H. A. Lechevalier. 1970. Chemical composition as a criterion in the classification of aerobic actinomycetes. *Int. J. Syst. Bacteriol.* **20**:435–444.
- Lechevalier, M. P., E. A. Stern, and H. A. Lechevalier. 1981. Phospholipids in the taxonomy of actinomycetes. *Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Suppl.* **11**:111–116.
- Locci, R. 1978. Micromorphological development of *Actinomyces* and related genera, p. 173–180. In M. Mordarski, W. Kurylowicz, and J. Jęlaszewicz (ed.), *Nocardia* and *Streptomyces*. Fischer, Stuttgart.
- Lu, Y., and X. Yan. 1978. Studies on the classification of thermophilic actinomycetes. II. Determination of *Pseudonocardia*. *Acta Microbiol. Sinica* **18**:8–10.
- Minnikin, D. E., L. Alshamaony, and M. Goodfellow. 1975. Differentiation of *Mycobacterium*, *Nocardia*, and related taxa by thin-layer chromatographic analysis of

- whole-organism methanolysates. J. Gen. Microbiol. **88**:200-204.
17. Omura, S., H. Tanaka, Y. Tanaka, P. Spiri-Nakagawa, R. Oiwa, Y. Takahashi, K. Matsuyama, and Y. Iwai. 1979. Studies on bacterial cell wall inhibitors. VII. Azureomycins A and B, new antibiotics produced by *Pseudonocardia azurea* nov. sp. J. Antibiot. **32**:985-994.
 18. Renner, B., and E. Gerstner. 1978. Dünnschichtchromatographische Isolierung von Flechtenstoffen und deren Identifizierung. Z. Naturforsch. Teil C **33**:340-345.
 19. Reynolds, E. S. 1963. The use of lead citrate on high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**:208-212.
 20. Richter, G. 1977. Routine use of thin-layer chromatography for cell wall analysis of aerobic actinomycetes, including two strains from sediments of the North Sea. Veroeff. Inst. Meeresforsch. Bremerhaven **16**:365-373.
 21. Schäfer, D. 1969. Eine neue *Streptosporangium*-Art aus türkischer Steppenerde. Arch. Mikrobiol. **66**:365-373.
 22. Shirling, E. B., and D. Gottlieb. 1966. Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. **16**:313-340.
 23. Skerman, V. B. D., V. McGowan, and P. H. A. Sneath (ed.). 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. **30**:225-420.
 24. Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**:31-43.
 25. Vitiello, F., and J. P. Zanetta. 1978. Thin-layer chromatography of phospholipids. J. Chromatogr. **166**:637-640.
 26. Waksman, S. A. 1961. The actinomycetes, vol. 2. The Williams & Wilkins Co., Baltimore.
 27. Watson, M. L. 1958. Staining of tissue sections for electron microscopy with heavy metals. J. Biophys. Biochem. Cytol. **4**:475-478.
 28. Williams, S. T., G. P. Sharples, and R. M. Bradshaw. 1973. The fine structure of the actinomycetales. Soc. Appl. Bacteriol. Symp. Ser. **2**:113-130.