A New Actinomycete Species, *Nocardiopsis lucentensis* sp. nov.

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A new species of the genus *Nocardiopsis*, for which we propose the name *Nocardiopsis lucentensis* sp. nov. (type strain, strain DSM 44048), was isolated from a salt marsh soil sample near Alicante, Spain. Whole-cell hydrolysates contain the *meso* isomer of diaminopimelic acid and no characteristic sugar; thus, the cell wall composition is type III. Menaquinone MK-10(H₈) is the major menaquinone, and the phospholipid type is type PIII (phosphatidylcholine present). Spore chains are rectiflexibiles, and in the early stages of sporulation zig-zag-shaped aerial hyphae are observed. This microorganism produces compatible solutes of the ectoine type and is characterized by a yellowish to yellowish brown substrate mycelium and a white aerial mycelium. This organism exhibits only 40 to 50% DNA relatedness to other *Nocardiopsis* spp.

In 1904 Brocq-Rousseau (4) created the name *Streptothrix dassonvillei* to designate a strain isolated from mildewed grain. Some years later, Liegard and Landrieu (33) isolated a microorganism from a case of conjunctivitis which they considered to be identical to *Streptothrix dassonvillei* but which they assigned to the genus *Nocardia*. Gordon and Horan (18) discovered that the macroscopic appearance and a number of the physiological characteristics of *Nocardia dassonvillei* were similar to those of *Streptomyces griseus*. Subsequently, Lechevalier and Lechevalier (30) described the genus *Actinomadura* to harbor *Nocardia madurae* (Vincent) Blanchard (the type species), *Nocardia pelletieri* (Laveran) Pinoy, and *Nocardia dassonvillei* (Brocq-Rousseau) Liegard and Landrieu. However, striking morphological differences of *Actinomadura dassonvillei* (e.g., the mode of sporulation of the aerial mycelium, the nature of the substrate mycelium, and the lack of madurose in whole-cell hydrolysates) led Meyer to transfer this species to a new genus, *Nocardiopsis*, as *Nocardiopsis dassonvillei* (35). The genus *Nocardiopsis* was described to comprise strains that exhibit fragmenting substrate and aerial mycelia and have cell wall chemotype III (*meso* isomer of diaminopimelic acid and no characteristic sugar in whole-cell hydrolysates). An earlier description of the genus included microorganisms with a type PIII phospholipid pattern sensu Lechevalier et al. (32) (phosphatidylcholine as a diagnostic phospholipid), as well as microorganisms with a type PIV phospholipid pattern (glucosamine-containing phospholipid). In recent studies (20, 21, 24, 28), strains which possess type PIV phospholipids, including *Nocardiopsis coeruleofusca* (41, 42), *Nocardiopsis flava* (13, 41, 42), *Nocardiopsis longispora* (41, 42), *Nocardiopsis mutabilis* (43), *Nocardiopsis mutabilis* subsp. *cryophilus* (45), and *Nocardiopsis syringae* (14), were found to contain rhamnose and large amounts of galactose in whole-cell hydrolysates (the sugar pattern characteristic of the genus *Saccharothrix* [29]) and therefore were reclassified in the genus *Saccharothrix*. Another species, *Nocardiopsis africana*, was found to belong to the genus *Microtetraspora* (25). Phenetic data indicated that the single remaining species in the genus, *Nocardiopsis antarctica* (1), is identical to the type strain of *Nocardiopsis dassonvillei* (36). To maintain the integrity of the genus *Nocardiopsis*, only those actinomycete isolates should be

included in the genus which possess *meso*-diaminopimelic acid as the cell wall diamino acid, have no characteristic sugar in whole-cell hydrolysates and no mycolic acids in whole-cell methanolysates (cell wall chemotype III), have phosphatidylcholine as a characteristic phospholipid (phospholipid type III), have fatty acid profiles which comprise saturated, unsaturated, iso, anteiso, and tuberculostearic acids, have menaquinones with 10 isoprene units with a high degree of hydrogenation as major menaquinones, and have DNAs with G+C contents of 60 to 70 mol%.

In the course of isolation of microorganisms from uncommon ecological niches, some unusual filamentous bacteria were isolated from salt marsh soils collected near Alicante, Spain. These organisms had chemotaxonomic and morphological characteristics which justify their placement in the genus *Nocardiopsis*. However, the physiological behavior of these microorganisms on different media, their inability to grow in basal International *Streptomyces* Project (ISP) medium 9, but only when it was supplemented with 5 to 10% NaCl, and their formation of aerial hyphae on different ISP media only when they were supplemented with 5 to 10% NaCl led us to propose the name *Nocardiopsis lucentensis* sp. nov. for strain A₅₋₁.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this study include *Nocardiopsis dassonvillei* DSM 43111^T (T = type strain) (2, 4, 30, 33, 35), *Nocardiopsis alborubida* DSM 40465^T (12, 21), *Nocardiopsis listeri* DSM 40297^T (10, 21, 48), *Nocardiopsis alba* subsp. *alba* DSM 43119 (2, 21, 35), and *Nocardiopsis alba* subsp. *prasina* DSM 43845^T (21, 40). Strains A₅₋₁^T and A₄₋₂ were isolated from a soil sample collected in a salt marsh area near Alicante, Spain. The strains were obtained from plates of mineral salts-starch medium containing the following ingredients: 0.5 g of K₂HPO₄, 0.1 g of MgSO₄ · 7H₂O, 3.0 g of NH₄HCO₃, 0.05 g of CaCl₂ · 2H₂O, 0.01 g of FeSO₄ · 7H₂O, 100 g of NaCl, 10.0 g of starch, and 1,000.0 ml of distilled water (pH 7.5). The plates were incubated at 30°C for 7 days, and the isolates were then subcultured on the same medium. Stock cultures were grown on inorganic salts-starch agar (ISP medium 4). The other media used in this study were those recommended for use by the ISP (44) and include yeast extract-malt extract agar (ISP medium 2), oatmeal agar (ISP medium 3), and glycerol-asparagine agar (ISP medium 5).

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Morphology and pigmentation. The organism was grown on ISP medium 2, ISP medium 3, ISP medium 4, and ISP medium 5, as described by Shirling and Gottlieb (44), and was examined for pigmentation, aerial mycelium, and other morphological features. Cultures were grown for 4 weeks, and observations were made at weekly intervals. Slide preparations from 4-, 7-, or 14-day-old cultures were examined for the presence of spore chains. Air-dried smears from ISP medium 4 were stained by Gram's method and a modification of the Ziehl-Neelsen method in order to determine the Gram reaction and acid fastness, respectively. The micromorphology was determined with a culture grown at 37°C for 10 days on ISP medium 4. Preparations of whole cells were stained with fuchsin and safranin and examined under a light microscope at a magnification of $\times 700$. Electron micrographs were made with a Leitz model MMR 1600 scanning electron microscope. Samples of 14-day-old colonial growth on ISP medium 4 supplemented with 10% NaCl were prepared by cutting agar blocks from the growth medium, fixing them with glutaraldehyde, and dehydrating them by using a graded methoxyethanol series and finally 100% acetone. The dehydrated sections were critical point dried, mounted on aluminum stubs, and then sputter coated with gold-palladium.

Physiological tests. The physiological tests used to characterize strains A_{5-1}^T and A_{4-2} were those of Gordon (15, 16) and Gordon and Mihm (19). The tests used to determine carbohydrate utilization and the test used to determine melanoid pigments were those of Shirling and Gottlieb (44). Catalase production was tested by mixing 1 loopful of a 1-week-old culture in glucose-nutrient agar with 1 drop of freshly prepared 5% hydrogen peroxide. Phosphatase activity was determined by the methods of Kurup and Schmitt (27). The release of *o*-nitrophenol from *o*-nitrophenyl- β -D-galactopyranoside and *p*-nitrophenyl- β -D-glucoside was used to assay β -galactosidase and β -glucosidase activities, respectively, by the method of Tsukamura (46). Degradation of elastin was determined by the disappearance of the substrate (0.3%, wt/vol) around the area of growth by using nutrient agar as a basal medium; the preparations were incubated for 10 days. Arbutin degradation was determined by the method of Kurup and Schmitt (27). To determine lysozyme susceptibility, a 0.05% (wt/vol) solution of lysozyme was sterilized by membrane filtration and added to autoclaved Czapek Dox-yeast extract-Casamino Acid broth (7) to give a final concentration of 0.0025% (wt/vol). Small tubes of medium with and without lysozyme were inoculated with 1 loopful of an old culture and examined for growth for up to 10 days. Strains which grew in both the control and test media were scored as positive for lysozyme resistance (17). Tolerance to salt was determined by growing the organisms on glucose-nutrient agar plates supplemented with 5 and 10% NaCl. The susceptibilities of the organisms to various antibiotics were studied on nutrient agar plates containing various concentrations of the drugs. The drugs were aseptically mixed with sterile molten agar, and the preparations were maintained at 45°C and poured into plates. After inoculation, the plates were incubated at 37°C for 1 week. Growth on the media was compared with growth of a control and was recorded as negative or positive (no growth or growth, respectively). Various concentrations (0.03 to 128 μ g/ml) of penicillin G, neomycin, lincomycin, gentamicin, rifampin, and streptomycin were tested.

Cell chemistry. Analysis of whole-cell hydrolysates for characterization of amino acids and sugars was performed by the methods of Becker et al. (3) and Lechevalier (31).

Cellular fatty acid and mycolic acid methyl esters were prepared from whole-cell methanolysates by the method of Minnikin et al. (37, 38); the presence of both fatty acid and mycolic acid methyl esters was detected by thin-layer chromatography, and the fatty acid methyl ester profile was determined by gas-liquid chromatography. Menaquinones were extracted and purified by the methods of Collins et al. (6), and menaquinone composition was determined by using a Finnigan Mat 212 mass spectrometer. Phospholipids were extracted by the method of Minnikin et al. (39) and were determined by two-dimensional thin-layer chromatography, using chloroform-methanol-water (65:25:4, vol/vol) in the first dimension followed by chloroform-acetic acid-methanol-water (40:7.5:6:2, vol/vol) in the second dimension. The phospholipids were visualized by spraying the preparation with the phosphate stain of Dittmer and Lester (9) and were tentatively identified by using phospholipid standards. Phosphatidylcholine was identified with Dragendorf's reagent (47).

DNA isolation and characterization. DNA was isolated by chromatography on hydroxyapatite by using the method of Cashion et al. (5). The G+C contents were determined by high-performance liquid chromatography (34). DNA-DNA hybridizations were carried out by using the method of De Ley et al. (8) as modified by Huss et al. (22). The concentration of DNA was 45 μ g/ml. Because of the high G+C content of the DNAs, 20% (vol/vol) dimethyl sulfoxide (11) was added to 2.5 $\times$ standard saline citrate (1 $\times$ standard saline citrate is 0.15 M NaCl plus 0.015 M sodium citrate) to depress the melting point (T_m) of the DNA. The resulting buffer contained 2 $\times$ standard saline citrate and 20% dimethyl sulfoxide. The average T_m was determined from the DNA melting curves, and the optimal temperature of renaturation (TOR) was calculated as follows: TOR = $T_m - 25^\circ\text{C}$. Levels of DNA-DNA relatedness were determined from the renaturation rates, which were calculated by regression analysis of data obtained between 10 and 30 min after the start of the reaction, using the computer program TRANSFER.BAS (23). The DNA relatedness values (H) were calculated by the following formula: $H = [(4 \times VM) - VA - VB] / (2 \times \sqrt{VA \times VB})$, where V is the renaturation rate, A is DNA_a, B is DNA_b, and M is a 1:1 mixture of DNA_a and DNA_b.

RESULTS AND DISCUSSION

Strain A_{5-1}^T has microbiological properties consistent with its assignment to the genus *Nocardioopsis* (35, 36). It is an aerobic, gram-positive, non-acid-fast, nonmotile organism which forms an extremely branched substrate mycelium that fragments into rod and coccoid elements. The substrate mycelium bears the aerial hyphae, which frequently form chains of smooth spores (Fig. 1). During spore formation hyphae have a zig-zag appearance because of developing spore subtending angles of various degrees with respect to the neighboring spores.

Strain A_{5-1}^T has chemotaxonomic characteristics which support its placement in the genus *Nocardioopsis*. The cell wall contains *meso*-diaminopimelic acid as the diamino acid and possesses no characteristic sugars (wall chemotype III); the organism lacks mycolic acids, contains major amounts of iso and anteiso branched-chain fatty acids, as well as substantial amounts of tuberculostearic acid, has menaquinones with 10 isoprene units, with the octahydrogenated form MK-10(H_8) as the major menaquinone, and has phospholipid type PIII *sensu* Lechevalier et al. (32) (phosphatidylcholine as the characteristic phospholipid).

FIG. 1. Scanning electron micrograph of strain $A_{5.1}^T$, showing zig-zag hyphae and spore chain with a smooth surface. The culture was grown on ISP medium 4 supplemented with 10% NaCl for 14 days at 35°C. Bar = 1.0 μm .

Identity of species. A total of five strains of *Nocardiopsis* spp. were grown along with strain $A_{5.1}^T$ on different media to study 56 physiological characteristics (Tables 1 and 2). Although these strains share many characteristics, they can be separated into two groups on the basis of the ability to grow in ISP medium 9 and utilize different carbon sources. The first group includes strain $A_{5.1}^T$, *Nocardiopsis alborubida* DSM 40465^T, *Nocardiopsis listeri* DSM 40297^T, and *Nocardiopsis alba* subsp. *prasina* DSM 43845^T, which were not able to grow in ISP medium 9 and to utilize carbon sources unless the medium was supplemented with 5% NaCl (Table 2). The second group includes *Nocardiopsis dassonvillei* DSM 43111^T and *Nocardiopsis alba* subsp. *alba* DSM 43119, which were able to grow and to utilize carbon sources in the presence or absence of NaCl (Table 2). Tables 1 and 2 list the differences between strain $A_{5.1}^T$ and other members of the two groups.

The G+C content of the DNA of strain $A_{5.1}^T$ was 71.0 mol%. Table 3 shows the levels of DNA relatedness between strain $A_{5.1}^T$ and the other strains, as determined by the optical method. The DNA of strain $A_{5.1}^T$ exhibits levels of relatedness ranging from 40 to 50% with the DNAs of other *Nocardiopsis* species. These values indicate the homogeneity of the genus *Nocardiopsis*. This is in contrast to the previous description of Meyer (36), which was based on an old description of the genus.

Together, these differences suggest that strain $A_{5.1}^T$ is unlikely to be a previously described *Nocardiopsis* species. Thus, we propose for it the name *Nocardiopsis lucentensis*. A description is given below.

Nocardiopsis lucentensis sp. nov. *Nocardiopsis lucentensis* (lu. cen. ten'sis. M. L. adj. *lucentensis*, referring to Lucen-tum, the ancient Latin name of Alicante, a city in Spain,

where the type strain was isolated). Aerobic, gram-positive, non-acid-fast, nonmotile, filamentous actinomycete.

Cultural characteristics. Cultures of strain $A_{5.1}^T$ grow well on both complex and defined media. Aerial hyphae are initiated when NaCl at a concentration of up to 10% is added to the growth medium. The aerial spore mass color is predominantly white. The reverse side of culture growth is yellow to yellowish brown. No distinctive pigmentation is present.

Morphological characteristics. (i) **Aerial mycelium.** On ISP medium 2, ISP medium 3, and ISP medium 4, all supplemented with 5 to 10% NaCl, well-developed branched aerial hyphae are present. The aerial hyphae are white, long, and at the beginning of sporulation more or less zig-zag shaped (Fig. 1). The zig-zag-shaped hyphae subdivide into smaller spores (Fig. 1). Spores are elongated and have smooth surfaces (Fig. 1).

(ii) **Substrate mycelium.** Strain $A_{5.1}^T$ produces an extensive substrate mycelium that fragments. It first appears to be yellowish and then becomes yellowish brown on ISP medium 2, ISP medium 3, and ISP medium 4.

Physiological characteristics. Strain $A_{5.1}^T$ produces acid from the following carbohydrates: glucose, inositol, mannitol, raffinose, and rhamnose. Acid is not produced from adonitol, dulcitol, erythritol, lactose, melibiose, α -methyl-D-glucoside, glucitol, and xylose.

Growth does not occur in ISP medium 9 supplemented with different carbon sources. However, growth occurs in ISP medium 9 which is supplemented with 5 to 10% NaCl and contains mannose, glucose, rhamnose, maltose, mannitol, D-fructose, sucrose, raffinose, glycerol, trehalose, and *myo*-inositol as sole sources of carbon but not in medium containing L-arabinose, D-xylose, D-galactose, and lactose.

TABLE 1. Differential physiological characteristics of strain A₅₋₁ and other *Nocardiopsis* species

Characteristic	Strain A ₅₋₁ ^T	<i>Nocardiopsis dassonvillei</i> DSM 43111 ^T	<i>Nocardiopsis alborubida</i> DSM 40465 ^T	<i>Nocardiopsis listeri</i> DSM 40297 ^T	<i>Nocardiopsis alba</i> subsp. <i>alba</i> DSM 43119	<i>Nocardiopsis alba</i> subsp. <i>prasina</i> DSM 43845 ^T
Decomposition of:						
Adenine	+	ND ^a	+	+	+	+
Hypoxanthine	+	+	+	+	+	+
Tyrosine	+	+	+	+	+	+
Xanthine	+	+	+	+	+	+
Casein	+	+	+	+	+	ND
Urea	—	—	—	—	+	—
Allantoin	—	—	—	—	+	—
Hydrolysis of:						
Starch	+	ND	+	+	+	+
Esculin	+	+	+	—	+	—
Gelatin	+	—	+	+	+	+
Elastin	ND	+	+	+	+	+
Arbutin	—	+	—	—	—	—
Decarboxylation of:						
Citrate	+	+	+	+	+	+
Malate	+	+	+	+	+	+
Succinate	+	+	+	+	+	+
Acetate	+	+	+	+	+	+
Pyruvate	+	+	+	+	+	+
Propionate	+	—	+	+	+	+
Lactate	—	—	+	+	+	+
Oxalate	—	—	+	—	—	+
Acid production from:						
Adonitol	—	—	—	—	—	—
Dulcitol	—	—	—	—	—	—
Erythritol	—	—	—	—	—	—
Glucose	+	+	+	—	+	+
Inositol	+	—	—	—	—	—
Lactose	—	—	—	—	—	—
Mannitol	+	—	+	—	—	+
Melibiose	—	—	—	—	—	—
α-Methyl-D-glucoside	—	—	—	—	—	—
Raffinose	+	—	—	—	—	—
Rhamnose	+	+	+	+	—	—
Sorbitol	—	—	—	—	—	—
Xylose	—	+	+	+	+	+
Resistance to lysozyme	—	—	—	—	—	—
Production of:						
Phosphatase	+	+	+	+	+	+
β-Galactosidase	—	+	+	+	+	+
β-Glucosidase	—	+	+	+	+	+
Growth in the presence of:						
5.0% NaCl	+	+	+	+	+	+
10.0% NaCl	+	—	—	—	+	+

^a ND, not determined.

Citrate, malate, succinate, acetate, pyruvate, and propionate are decarboxylated, but lactate and oxalate are not. Strain A₅₋₁^T decomposes adenine, hypoxanthine, tyrosine, and xanthine and hydrolyzes casein, starch, gelatin, and esculin but not urea, allantoin, and arbutin. It produces catalase and phosphatase but neither β-galactosidase nor β-glucosidase. Melanin pigments are not produced on either ISP medium 6 or ISP medium 7. The organism reduces nitrates to nitrites.

Strain A₅₋₁^T is resistant to lincomycin (128 μg/ml), penicillin G (128 μg/ml), gentamicin (64 μg/ml), streptomycin (64

μg/ml), and neomycin (4 μg/ml) but not rifampin (128 μg/ml). The organism is susceptible to lysozyme and tolerates NaCl at levels up to and including 10%.

Chemotaxonomy. Hydrolyzed whole cells contain meso-diaminopimelic acid. Glucose and arabinose are detected in whole-cell extracts. Thus, strain A₅₋₁^T has a type III cell wall and a variant of the type C sugar pattern. Mycolic acids are not detected. Phospholipid determinations performed with whole cells indicate that phosphatidylcholine, phosphatidylmethylethanolamine, phosphatidylglycerol, and diphos-

TABLE 2. Carbon assimilation by strain A₅₋₁^T and other *Nocardiopsis* spp. on ISP medium 9 supplemented with 5% NaCl

Carbon source	Strain A ₅₋₁ ^T	<i>Nocardiopsis alba</i> subsp. <i>prasina</i> DSM 43845 ^T	<i>Nocardiopsis</i> <i>alborubida</i> DSM 40465 ^T	<i>Nocardiopsis</i> <i>listeri</i> DSM 40297 ^T	<i>Nocardiopsis</i> <i>dassonvillei</i> DSM 43111 ^T	<i>Nocardiopsis alba</i> subsp. <i>alba</i> DSM 43119
L-Arabinose	—	+	+	+	—	—
D-Xylose	—	+	+	—	+	—
D-Mannose	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+
L-Rhamnose	+	—	+	+	+	—
D-Mannitol	+	+	+	+	+	+
D-Fructose	+	+	+	—	+	+
D-Galactose	—	+	+	+	+	—
Maltose	+	+	+	+	+	+
Sucrose	+	+	+	—	+	+
Lactose	—	+	—	—	—	—
Raffinose	+	—	—	—	—	—
Glycerol	+	+	+	—	+	+
Trehalose	+	+	+	—	—	+
myo-Inositol	+	—	—	—	—	—

phatidylglycerol are present. Therefore, strain A₅₋₁^T has a type PIII phospholipid pattern. The menaquinones detected in strain A₅₋₁ are octahydrogenated menaquinones with 10 isoprene units [MK-10(H₈)]. Minor amounts of tetra-, hexa-, and decahydrogenated menaquinones with 10 isoprene units [MK-10(H₄), MK-10(H₆), MK-10(H₁₀)], as well as tetra-, hexa-, octa-, and decahydrogenated menaquinones with nine isoprene units [MK-9(H₄), MK-9(H₆), MK-9(H₈), MK-9(H₁₀)], are also present. The fatty acid profile of strain A₅₋₁^T consists of major amounts of saturated branched-chain fatty acids of the iso and anteiso types (iso-C_{16:0}, 32%; anteiso-C_{17:0}, 14.5%; iso-C_{18:0}, 9.4%), straight-chain saturated fatty acids (*n*-C_{16:0}, 3.9%; *n*-C_{17:0}, 1.5%; *n*-C_{18:0}, 9.9%), and 10-methyl branched-chain fatty acids represented by tuberculostearic acid (10-methyl C_{18:0}, 15.4%).

DNA-DNA hybridization. The renaturation rates as determined by the optical method reveal binding values that range from 40 to 46% between strain A₅₋₁^T and the other *Nocardiopsis* species (Table 3).

Type strain. The type strain is strain A₅₋₁ (= DSM 44048). It was isolated from soil collected in Alicante, Spain. The species description is based on a single strain and thus serves as the type strain description.

Another variant, strain A₄₋₂, which was found to be phenotypically identical to A₅₋₁^T, exhibited a DNA relatedness level of 39% to A₅₋₁^T (Table 3). However, we hesitate to suggest a new species for this strain because it is recommended that "a distinct genospecies that cannot be differentiated from another genospecies on the basis of any known

phenotypic property shall not be named until they can be differentiated by some phenotypic property" (49).

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TABLE 3. Degrees of DNA binding between A₅₋₁^T and other *Nocardiopsis* species

Organism	Degree of binding (%) to:	
	A ₅₋₁ ^T	A ₄₋₂
A ₅₋₁ ^T	100	39 ± 3 ^a
A ₄₋₂	39 ± 3	100
<i>Nocardiopsis dassonvillei</i>	44 ± 1	65 ± 4
<i>Nocardiopsis alborubida</i>	40 ± 1	63 ± 1
<i>Nocardiopsis listeri</i>	46	34 ± 1
<i>Nocardiopsis alba</i> subsp. <i>alba</i>	41	31 ± 1
<i>Nocardiopsis alba</i> subsp. <i>prasina</i>	42 ± 1	44 ± 1

^a Mean ± standard deviation.

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