Nocardia paucivorans sp. nov.

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Chemotaxonomic and 16S rDNA sequence analyses of an isolate from the sputa and bronchial secretions of a patient with chronic lung disease clearly demonstrated that it belongs to the genus *Nocardia*. DNA–DNA hybridization data, as well as the biochemical characteristics of the isolate, indicate that it belongs to a new species that differs from previously described members of the genus *Nocardia*. The name *Nocardia paucivorans* sp. nov. is proposed for this isolate and is represented by strain IMMIB D-1632^T (= DSM 44386^T).

Keywords: *Nocardia paucivorans*, *Nocardia brevicatena*, chemotaxonomy, DNA–DNA hybridization, 16S rRNA gene sequence

INTRODUCTION

Members of the genus *Nocardia* form a complex group of organisms, of which *Nocardia asteroides*, *Nocardia farcinica*, *Nocardia brasiliensis* and *Nocardia otitidiscaviarum* are responsible for most cases of human nocardiosis. Nocardiae are morphologically characterized by the formation of extensively branched substrate hyphae which fragment into rod-shaped, non-motile elements. In addition, aerial hyphae are usually formed but are sometimes only visible microscopically (Gordon & Mihm, 1957, 1962; Goodfellow & Lechevalier, 1989). Nocardiae are also characterized by the presence of a number of chemical markers including meso-diaminopimelic acid, arabinose and galactose in whole-cell hydrolysates (i.e. they possess cell wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970), mycolic acids with 40–60 carbon atoms, phospholipid type PII *sensu* Lechevalier *et al.* (1977) (i.e. phosphatidylethanolamine is the characteristic phospholipid), hexahydrogenated menaquinones with eight isoprene units as the major

quinones and a DNA G+C content of 64–72 mol % (Goodfellow & Lechevalier, 1989; Goodfellow, 1992).

Current methods of recognition of nocardiae in the clinical laboratory include the following: microscopic and colonial morphology; hydrolysis of casein, tyrosine, xanthine, hypoxanthine and testosterone; the degradation of urea and aesculin; acid production from carbohydrates; utilization of various carbon compounds as simultaneous sources of carbon and energy and various nitrogen-containing compounds as sources of carbon, energy and nitrogen; decarboxylation of citrate; and production of nitrate reductase (Mishra *et al.*, 1980; Schaal, 1983). These tests differentiate species of medical importance such as *N. asteroides*, *N. brasiliensis*, *N. farcinica*, *N. otitidiscaviarum*, *Nocardia brevicatena* and *Nocardia transvalensis* as well as some additional mesophilic *Nocardia* species.

When subjected to chemotaxonomic methods used for the identification of clinical bacterial isolates the strain IMMIB D-1632^T was found to have chemotaxonomic characteristics which suggested its inclusion in the genus *Nocardia*. Further taxonomic and phylogenetic investigations indicated that it belongs to a new species that differs from previously described species of the

The GenBank/EMBL/DBJ accession number for the 16S rDNA sequence of strain IMMIB D-1632^T (= DSM 44386^T) is AF179865.

genus *Nocardia*. In this paper, we describe the morphological, chemotaxonomic, physiological and phylogenetic characteristics of this new species.

METHODS

Bacterial strains. Strain IMMIB D-1632^T was isolated more than once from the sputa and bronchial secretions of a 51-year-old patient with chronic lung disease. *N. asteroides* ATCC 19247^T and *Nocardia nova* ATCC 33726^T were obtained from the American Type Culture Collection (ATCC). *N. brasiliensis* DSM 43758^T, *N. farcinica* DSM 43665^T, *N. otitidiscaviarum* DSM 43242^T, *N. brevicatena* DSM 43024^T, *Nocardia carnea* DSM 43397^T and *Nocardia vaccinii* DSM 43285^T were obtained from the German Culture Collection of Microorganisms and Cell Cultures (DSMZ).

Morphology and pigmentation. Strain IMMIB D-1632^T was grown on brain–heart infusion (BHI) agar and was examined for pigmentation, for the production of aerial hyphae and for other morphological characteristics. Cultures were grown for 4 weeks and observed weekly. Air-dried smears from BHI agar cultures were stained by the Gram and Ziehl–Neelsen methods in order to determine the Gram reaction and acid-fastness, respectively.

Physiological characteristics. The following properties were determined, for the above listed strains, as described previously: tests for the decomposition of adenine, guanine, hypoxanthine, xanthine, tyrosine, elastin, keratin and testosterone were performed using the method of Gordon (1967); aesculin decomposition was performed using the method of Gordon (1966); and casein- and gelatin-hydrolysis tests were performed using the method of Gordon & Mihm (1957). The urea-decomposition test was performed with urea agar base (catalogue no. CM 53; Oxoid) after the addition of 2.2% urea. To determine the utilization of a substrate as a carbon source or as a simultaneous carbon and nitrogen source, we used the media described by Yassin *et al.* (1995).

Cell chemistry. The strains studied were cultivated at 37 °C in shake flasks containing BHI (Difco) broth for 1 week. After being checked for purity at maximum growth, the organisms were killed with formaldehyde (1%, v/v), harvested by centrifugation, washed with distilled water and freeze-dried. Analyses of whole-cell hydrolysates for amino acids and sugars were performed using the methods of Becker *et al.* (1964) and Lechevalier (1968), respectively. Mycolic acids were detected using acid methanolysis and one-dimensional TLC; Py-GC was performed as previously described by Yassin *et al.* (1993a); the composition of the mycolic acids was determined using electron-impact mass spectrometry according to Collins *et al.* (1982). Non-hydroxylated fatty acids were purified with preparative TLC and then separated, identified and quantified by GC as described by Yassin (1988). Menaquinones were extracted and purified by the method of Collins *et al.* (1977) and were identified by using a Finnigan Mat 212 mass spectrometer. Phospholipids were extracted, purified and identified as described previously (Yassin *et al.*, 1993c).

DNA isolation and characterization. Cell mass for DNA–DNA hybridization was obtained as described above for cell chemistry determinations. DNA was isolated using the phenol method of Saito & Miura (1963), with some

modifications. The packed cells (6.0 g) were mixed with 20 mg lysozyme dissolved in 6 ml saline/EDTA (pH 9.0) and the mixture was kept at 37 °C for 30 min. When the cells were just beginning to lyse they were quickly frozen in an acetone/dry ice mixture and kept at –20 °C for 10 min. Then 20 ml Tris/SDS buffer (pH 9.0) was added to the frozen cells, which were suspended by stirring with a glass rod while being warmed in a water bath at 60 °C. When lysis was incomplete, the freezing and thawing processes were repeated. The cell suspension was then mixed with an equal volume of phenol saturated with Tris/SDS buffer (pH 9.0) and the mixture was shaken by hand for 20 min at 4 °C. The resulting emulsion was separated into two layers by centrifugation at 5000 r.p.m. for 15 min. The nucleic acids were precipitated by gently mixing the supernatant with 2 vols cold ethanol. The thread-like precipitate was collected and dissolved in MOPS buffer and then purified by chromatography on hydroxyapatite using the method of Cashion *et al.* (1977). The G+C content of DNA was determined by HPLC (Mesbah *et al.*, 1989). DNA–DNA hybridization studies were carried out by using the thermal renaturation method (Yassin *et al.*, 1993b).

16S rRNA gene sequence determination. Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and the purification of PCR products were carried out using procedures described previously (Rainey *et al.*, 1996). Purified PCR products were sequenced using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The Applied Biosystems 310 DNA Genetic Analyser was used for the electrophoresis of the sequence reaction products.

Phylogenetic analyses of 16S rRNA gene sequence data. The ae2 editor (Maidak *et al.*, 1996) was used to align the 16S rRNA gene sequence of strain IMMIB D-1632^T against the 16S rRNA gene sequences of the validly described species of the genus *Nocardia* available from the public databases. The strain designations and nucleotide sequence accession numbers of the analysed sequences are as follows: *N. asteroides* ATCC 19247^T, X84850; *N. brasiliensis* DSM 43758^T, X80608; *N. brevicatena* ATCC 15333^T, X80600; *N. carnea* DSM 43397^T, X80607; *Nocardia crassostreae* ATCC 700418^T, Z37989; *N. farcinica* ATCC 3318^T, X80595; *Nocardia flavorosea* JCM 3332^T, Z46754; *N. nova* ATCC 33726^T, X80593; *N. otitidiscaviarum* ATCC 14629^T, X80599; *Nocardia pseudobrasiliensis* ATCC 51512^T, X84857; *Nocardia salmonicida* JCM 4826^T, Z46750; *Nocardia seriolae* ATCC 43993^T, X80592; *N. transvalensis* DSM 43405^T, X80609; *Nocardia uniformis* JCM 3224^T, Z46752; and *N. vaccinii* DSM 43285^T, Z36927. The alignment used in the phylogenetic analyses comprised 1379 nucleotide positions between positions 38 and 1449 (*Escherichia coli* numbering; Brosius *et al.*, 1978). The programs of the PHYLIP package including DNADIST and NEIGHBOR were used for the phylogenetic analyses (Felsenstein, 1993). The tree topology was reanalysed using 1000 bootstrapped data sets and the programs SEQBOOT, DNADIST and CONSENSE of the PHYLIP package (Felsenstein, 1993).

RESULTS

Micromorphology

The hyphae of strain IMMIB D-1632^T were Gram-positive and slightly acid- and alcohol-fast. The vegetative hyphae were well developed with irregular

Table 1. Physiological characteristics of strain IMMIB D-1632^T and some validly described *Nocardia* species

The following tests gave the same results (in parentheses) for all of the micro-organisms listed: hydrolysis of adenine (–), gelatin (–), guanine (–), urea (+) and keratin (–); utilization of adipate (–), benzoate (–), lactate (–), cellobiose (–), lactose (–), D-melezitose (–), raffinose (–), adonitol (–), *meso*-erythritol (–) and paraffin (+) as sole sources of carbon and energy. w, Weakly utilized after incubation for 3 weeks.

Characteristic	IMMIB D-1632 ^T	<i>N. brevicatena</i> DSM 43024 ^T	<i>N. carnea</i> DSM 43397 ^T	<i>N. vaccinii</i> DSM 43285 ^T	<i>N. asteroides</i> ATCC 19247 ^T	<i>N. nova</i> ATCC 33726 ^T	<i>N. brasiliensis</i> DSM 43758 ^T	<i>N. farcinica</i> DSM 43665 ^T	<i>N. otitidiscaevium</i> DSM 43242 ^T
Hydrolysis of:									
Aesculin	–	+	+	–	+	–	+	+	+
Casein	–	–	–	–	–	–	+	–	–
Elastin	–	–	–	–	–	–	+	–	–
Hypoxanthine	–	–	–	–	–	–	+	–	+
Testosterone	–	–	–	–	+	–	–	+	+
Tyrosine	–	–	–	–	–	–	+	–	–
Xanthine	–	–	–	–	–	–	–	–	+
Utilization as sole sources of carbon and energy:									
Acetate	+	+	+	–	+	+	+	+	+
Citrate	–	–	–	+	–	–	+	–	–
Gluconate	–	–	–	+	+	–	–	–	w
L-Arabinose	–	–	–	+	–	–	–	–	–
Galactose	–	–	+	+	–	+	+	–	–
Glucose	–	–	+	+	+	+	+	+	+
Maltose	–	–	–	–	–	–	+	+	–
L-Rhamnose	–	–	–	+	–	–	–	+	–
Sucrose	–	–	–	–	+	–	w	+	–
Trehalose	+	+	+	+	+	–	+	+	w
Xylose	–	–	–	+	–	–	–	–	–
<i>myo</i> -Inositol	–	–	–	–	–	–	+	–	+
Mannitol	–	–	–	+	–	–	w	–	+
D-Sorbitol	–	–	w	–	–	–	–	–	–
Isoamyl alcohol	–	+	–	–	–	+	w	+	–
2,3-Butanediol	–	–	–	–	–	–	–	+	–
1,2-Propanediol	–	+	–	–	–	–	–	+	–
<i>m</i> -Hydroxybenzoate	–	–	–	–	–	–	–	+	–
<i>p</i> -Hydroxybenzoate	–	–	w	w	–	–	–	–	–
Utilization as sole sources of carbon and nitrogen									
Acetamide	–	–	–	–	–	–	–	+	–
L-Alanine	–	–	–	–	–	–	+	–	+
Gelatin	–	–	–	–	–	–	+	–	–
Proline	–	–	–	–	+	–	+	–	–
Serine	–	–	–	–	–	–	w	–	w

branches penetrating the agar and bearing white aerial hyphae. At a late stage of growth the hyphae fragment into rod-shaped elements.

Physiological characteristics

The physiological properties of strain IMMIB D-1632^T and some of the validly described species of the genus *Nocardia* as determined in this study are shown in Table 1. Strain IMMIB D-1632^T hydrolysed urea but not adenine, aesculin, casein, elastin, gelatin, guanine, hypoxanthine, keratin, testosterone, tyrosine or xanthine. It utilized acetate, trehalose and paraffin as carbon sources but did not utilize adipate, benzoate, citrate, gluconate, lactate, arabinose, cellobiose, galactose, glucose, lactose, maltose, melezitose, raffinose, rhamnose, sucrose, xylose, adonitol, *meso*-erythritol, *myo*-inositol, mannitol, sorbitol, isoamyl alcohol, 2,3-butanediol, 1,2-propanediol, *m*-hydroxybenzoate or *p*-hydroxybenzoate. Strain IMMIB D-1632^T did not utilize acetamide, alanine, gelatin, proline or serine as simultaneous carbon and nitrogen sources.

Lipid analysis

One-dimensional TLC of whole-cell acid methanolysates of strain IMMIB D-1632^T revealed the presence of two lipid spots on the chromatogram. The lower one corresponds to mycolic acids, as identified by its R_F value (0.47) and the higher spot corresponds to the non-hydroxylated fatty acids.

Py-GC of the pure mycolic acid methyl esters (isolated by preparative TLC) from strain IMMIB D-1632^T released fatty acid methyl esters of C14:0 (17.47%) and C16:0 (82.53%) as pyrolysis cleavage products. Mass spectral analysis of the pure mycolate revealed the presence of mycolic acids with 48–54 carbon atoms with 2–4 double bonds. Gas chromatographic analyses of the non-hydroxylated fatty acid methyl esters revealed the presence of hexadecanoate (33.4% of total fatty acids), hexadecanoate (2.3%), octadecanoate (5.8%), octadecanoate (2.9%) and tuberculostearic acid (10-methyl octadecanoate, 32.5%) as the major cellular fatty acid methyl esters.

Polar lipid analysis showed that strain IMMIB D-

Fig. 1. Unrooted phylogenetic tree showing the position of strain IMMIB D-1632^T (= DSM 44386^T) within the radiation of species of the genus *Nocardia*. Bootstrap values, expressed as a percentage of 1000 replications, are shown at the branching points. Bar, 2 inferred nucleotide substitutions per 100 nucleotides.

1632^T contains phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and diphosphatidylglycerol as the characteristic phospholipids.

The composition of the respiratory quinones of strains IMMIB D-1632^T was examined. On reverse-phase TLC using RP-18, the lipid extract from this strain gave two separate bands. Mass spectral analysis of the main component showed, in the high-mass region, a strong peak at m/z 720 attributable to M^+ as well as peaks of considerable intensity at m/z values of 582 and 594. These correspond to a hexahydrogenated menaquinone with eight isoprene units in which the two terminal isoprene moieties are cyclized (Howarth *et al.*, 1986). The second band shows, in the high-mass region, a strong peak at m/z 736 attributable to M^+ , with a second intense peak at m/z 720 (corresponding to loss of oxygen from M^+), while in the low-mass region it shows a peak of medium intensity at m/z 241 (indicative of an additional oxygen in the naphtho-

quinone ring system). This mass spectrum of the second band corresponds to a hexahydrogenated epoxymenaquinone with eight isoprene units in which the last two isoprene moieties are cyclized (Collins *et al.*, 1987).

Phylogenetic analysis

The almost complete 16S rRNA gene sequence of strain IMMIB D-1632^T comprising 1459 nucleotides (>95% of the *E. coli* sequence; Brosius *et al.*, 1978) was determined in this study. The unrooted phylogenetic tree (shown in Fig. 1) was constructed from evolutionary distances by the neighbour-joining method. A total of 1379 nucleotides present in all strains between positions 38 and 1449 (*E. coli* positions) was used for these analyses and compared with the sequences of the type strains of the 15 validly described *Nocardia* species. The phylogenetic tree, based on the 16S rRNA gene sequence, shown in Fig. 1 indicates the position of strain IMMIB D-1632^T

within the radiation of species of the genus *Nocardia*. The 16S rDNA sequence of strain IMMIB D-1632^T contains all of the 16S rDNA signature nucleotides defined for the family *Nocardiaceae* (Stackebrandt *et al.*, 1997). The 16S rRNA gene sequence similarity values between strain IMMIB D-1632^T and the other *Nocardia* species included in the tree (Fig. 1) show strain IMMIB D-1632^T to be closely related at the 16S rRNA gene sequence level to *Nocardia brevicatena* (99.6%). The 16S rRNA gene sequence similarity values for the sequence of strain IMMIB D-1632^T relative to other *Nocardia* species are in the range 97.0–98.3% (data not shown). These values and the results shown in the phylogenetic tree (Fig. 1) indicate that strain IMMIB D-1632^T is a member of the genus *Nocardia* and shows a high degree of relatedness to *N. brevicatena*.

DNA characteristics

The results of triplicate determinations of the G + C content of the DNA of strain IMMIB D-1632^T was 65.9 mol %. The levels of relatedness (binding rates) of triplicate measurements between DNAs of this strain and the DNA of *N. brevicatena* DSM 43024^T is 61.9%.

DISCUSSION

The 16S rDNA sequence comparison clearly shows that strain IMMIB D-1632^T is a member of the family *Nocardiaceae* (Stackebrandt *et al.*, 1997), since the determined sequence contains all of the signature nucleotides designated for this lineage. The high values for 16S rRNA gene sequence similarity to other previously described members of the genus *Nocardia* (97.0–99.6%) support the addition of strain IMMIB D-1632^T to this genus.

The intergeneric relationships of the *Nocardia* species (Fig. 1) based on 16S rRNA gene sequence comparison shows strain IMMIB D-1632^T to cluster with *N. brevicatena*. Even though the 16S rRNA gene sequences of these two taxa share 99.6% sequence similarity, the results of DNA–DNA homology studies (61.9%, which is clearly lower than the cut-off point of species as specified by Wayne *et al.*, 1987) and phenotypic testing show them to represent distinct species. A comparable case was found for *N. flavorosea* and *N. carnea* (Chun *et al.*, 1998).

The phenotypic characteristics of strain IMMIB D-1632^T were compared with those of validly described *Nocardia* species. Chemotaxonomically, strain IMMIB D-1632^T shows the characteristics of members of the genus *Nocardia*. All of them contain galactose and arabinose as characteristic whole-cell sugars in addition to *meso*-diaminopimelic acid as the wall diamino acid (i.e. they are wall chemotype IV organisms); all contain nocardomycolic acid which, upon pyrolysis, releases fatty acids that range from C14:0 to C18:0 carbon atoms as cleavage products; and their fatty acid profiles consist of saturated,

unsaturated and 10-methyl-branched-chain fatty acids. All of them contain hexahydrogenated menaquinones MK-8(H₆), in which the two terminal isoprene moieties are cyclized, as the major isoprenoid quinones and all of them possess phospholipid type PII with phosphatidylethanolamine as the characteristic phospholipid. These chemotaxonomic similarities are supported by the high levels of 16S rRNA gene sequence similarity (97–99.6%) observed between isolate IMMIB D-1632^T and members of the genus *Nocardia*.

In contrast to the chemotaxonomic similarities of strain IMMIB D-1632^T and other members of the genus *Nocardia*, the results of our physiological tests (Table 1) revealed clear differences between them. Isolate IMMIB D-1632^T can be differentiated from *N. brevicatena* DSM 43024^T by its inability to hydrolyse aesculin as well as its inability to utilize isoamyl alcohol and 1,2-propanediol as sources of carbon and energy. Strain IMMIB D-1632^T cannot be differentiated from *N. vaccinii* DSM 43285^T and *N. nova* ATCC 33726^T on the basis of substrate-hydrolysis tests; all three strains are only able to hydrolyse urea and are unable to hydrolyse adenine, casein, elastin, gelatin, guanine, hypoxanthine, keratin, testosterone, tyrosine and xanthine. However, strain IMMIB D-1632^T can be differentiated from *N. vaccinii* DSM 43285^T and *Nocardia nova* ATCC 33726^T on the basis of carbon-assimilation tests (Table 1). Furthermore, strain IMMIB D-1632^T cannot be differentiated from *N. carnea* DSM 43397^T and *N. nova* ATCC 33726^T on the basis of utilization of a substrate as a simultaneous carbon and nitrogen source; all three species are unable to utilize acetamide, alanine, gelatin, proline or serine as simultaneous carbon and nitrogen sources. Strain IMMIB D-1632^T, however, can be differentiated from *N. carnea* DSM 43397^T on the basis of tests of substrate hydrolysis as well as a test of carbon source assimilation. Strain IMMIB D-1632^T can be differentiated from *N. asteroides* ATCC 19247^T, *N. brasiliensis* DSM 43758^T, *N. farcinica* DSM 43665^T and *N. otitidiscaeviarum* DSM 43242^T by tests of substrate hydrolysis, carbon assimilation and/or utilization of a substrate as a simultaneous source of carbon and nitrogen (Table 1).

We therefore propose that strain IMMIB D-1632^T should be described as a new species of the genus *Nocardia*. It represents the type species of this new species and has been deposited in the German Collection of Microorganisms and Cell Cultures as strain DSM 44386^T. The type strain was isolated more than once from the sputa and broncho-alveolar secretions of a 51-year-old man suffering from a lung disease. The description of this species is given below.

Nocardia paucivorans sp. nov.

Nocardia paucivorans (pau.ci.vo'rans. L. adj. *paucus* little; L. v. *vorare* to eat, to devour; L. pres. part. *vorans* eating; M.L. adj. *paucivorans* eating little,

referring to the few compounds that are utilized as sole sources of carbon and energy).

The hyphae are Gram-positive and slightly acid- and alcohol-fast. The vegetative hyphae are well developed with irregular branches penetrating the agar and bear white aerial hyphae. At a late stage of growth the hyphae fragment into rod-shaped elements. The organism contains *meso*-diaminopimelic acid as the wall diamino acid in addition to galactose and arabinose in whole-cell hydrolysates (i.e. the cell wall chemotype is chemotype IV). It has mycolic acids with 48–54 carbon atoms and 2–4 double bonds ($C_{48:3}$, $C_{50:4}$, $C_{52:2}$, $C_{52:4}$, $C_{54:2}$ and $C_{54:3}$) that are cleaved upon pyrolysis to release fatty acids with $C_{14:0}$ and $C_{16:0}$ carbon atoms, with $C_{16:0}$ as the major cleavage product; this *N. paucivorans* has a cellular fatty acid profile that comprises saturated, unsaturated and 10-methyl-branched fatty acids. It possesses type PII phospholipids with significant amounts of phosphatidylethanolamine. The menaquinone system consists of partially saturated menaquinones with eight isoprene units in which the two terminal isoprene moieties are cyclized; MK-8(H_6) is the major menaquinone and minor amounts of 2,3-epoxy-MK-8(H_6) are also present. It hydrolyses urea but not adenine, aesculin, casein, elastin, gelatin, guanine, hypoxanthine, keratin, testosterone, tyrosine or xanthine. It assimilates acetate, trehalose and paraffin as carbon sources but does not assimilate adipate, benzoate, citrate, gluconate, lactate, arabinose, cellobiose, galactose, glucose, lactose, maltose, melezitose, raffinose, rhamnose, sucrose, xylose, adonitol, *meso*-erythritol, *myo*-inositol, mannitol, sorbitol, isoamyl alcohol, 2,3-butanediol, 1,2-propanediol, *m*-hydroxybenzoate or *p*-hydroxybenzoate. It does not utilize acetamide, alanine, gelatin, proline or serine as simultaneous carbon and nitrogen sources. The G+C content of the DNA is 65.9 mol %. The type strain of *Nocardia paucivorans* is strain IMMIB D-1632^T (= DSM 44386^T).

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