Nocardia elegans sp. nov., a member of the *Nocardia vaccinii* clade isolated from sputum

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Two bacterial isolates from the sputa of a patient with a pulmonary infection were subjected to a polyphasic taxonomic study. Chemotaxonomic investigations revealed the presence of cell-wall chemotype IV and mycolic acids consistent with the profile for the genus *Nocardia*. Comparative 16S rRNA gene sequencing showed that these isolates constitute a distinct subline within the genus *Nocardia*, displaying 99.6–95.5 % sequence similarities with established species. However, DNA–DNA hybridization studies demonstrated unambiguously that the isolates are genealogically distinct from closely related species, namely *Nocardia veterana* and *Nocardia africana*, which show high levels of 16S rRNA sequence similarity (99.2 and 99.6 % sequence similarity, respectively). On the basis of both phenotypic and phylogenetic evidence, it is proposed that these isolates be classified as a novel species of the genus *Nocardia*, for which the name *Nocardia elegans* sp. nov. is proposed. The type strain is IMMIB N-402^T (= CCUG 50200^T = CIP 108553^T).

The genus *Nocardia* represents a group of chemotaxonomically homogeneous, aerobic, Gram-positive, high-G + C-content organisms that form extensively branched substrate hyphae that fragment into rod-shaped to coccoid, non-motile elements and belong phylogenetically to the *Actinobacteria*. In the past, the recognition of novel *Nocardia* species was problematic because of limitations in the taxonomic tests (primarily morphological and biochemical) used to identify these organisms. However, in recent years, the implementation of molecular identification methods, particularly 16S rRNA gene sequencing (Ruimy *et al.*, 1994; Chun & Goodfellow, 1995; Rainey *et al.*, 1995), in parallel with improved phenotypic approaches has greatly facilitated the discovery of novel species and has resolved the taxonomy of isolates that were collectively assigned to the *Nocardia asteroides* complex (Yassin *et al.*, 2000b, 2001). The genus *Nocardia* has undergone considerable expansion in the past few years, and at the time of writing over 40 species are recognized. The vast majority of these species have been isolated from human clinical specimens (Hamid *et al.*, 2001; Yassin *et al.*, 2000a, b, 2001) and, to a lesser extent, from animal (Isik *et al.*, 1999a) and soil (Isik *et al.*, 1999b; Albuquerque de Barros *et al.*, 2003) sources. In the present study, the taxonomic status of two isolates, IMMIB N-402^T

and IMMIB N-403, isolated from the sputa of a patient with a pulmonary infection, was determined using polyphasic taxonomic approaches. On the basis of the genotypic and phenotypic data reported here, the two isolates should be recognized as a novel species of the genus *Nocardia* for which the name *Nocardia elegans* sp. nov. is proposed.

Strains IMMIB N-402^T and IMMIB N-403 were cultured on brain-heart infusion (Becton Dickinson) agar to determine their morphological properties. The strains were biochemically characterized by using tests for the hydrolysis of complex substrates, as described previously (Gordon, 1966, 1967; Gordon & Mihm, 1957), as well as tests to determine carbon-source utilization (according to Yassin *et al.*, 1995). The presence of the isomeric form of diaminopimelic acid was tested by using the methods of Becker *et al.* (1964), while whole-cell sugars were determined by using the method of Lechevalier (1968). Lipids were extracted using acid methanolysis. Mycolic acids were detected by TLC as described by Minnikin *et al.* (1980); pyrolysis GC of the mycolates was performed according to Yassin *et al.* (1993a). Non-hydroxylated fatty acids were purified, identified and quantified by GC as described by Yassin (1988). Menaquinones were extracted, purified and identified according to Collins *et al.* (1977). Phospholipids were extracted, purified and identified as described previously (Yassin *et al.*, 1993c).

DNA was isolated and purified as described previously (Yassin *et al.*, 2000a). DNA–DNA hybridization studies were carried out by using the thermal renaturation method (Yassin *et al.*, 1993b). Genomic DNA extraction,

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains IMMIB N-402^T (= CCUG 50200^T = CIP 108553^T) and IMMIB N-403 (= CCUG 50201 = CIP 108554) are AJ854057 and AJ854058, respectively.

PCR-mediated amplification of 16S rRNA genes 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) as described in the manufacturer's protocol. An Applied Biosystems 310 DNA genetic analyser was used for the electrophoresis of the sequence reaction products. The 16S rRNA gene sequences of strains IMMIB N-402^T and IMMIB N-403, as well as those (retrieved from GenBank) of *Nocardia* species with validly published names, were added to the ARB database (Ludwig *et al.*, 2004) and aligned using the integrated aligner software in the ARB package. The resulting alignment was corrected manually; evolutionary trees were inferred using maximum parsimony (Kluge & Farris, 1969), neighbour joining (Saitou & Nei, 1987) and maximum likelihood (Felsenstein, 1981). An evolutionary distance matrix was calculated using the corrections of Jukes & Cantor (1969). The tree topology was evaluated according to the results of the neighbour-joining and maximum-likelihood analyses. The robustness of the phyletic lines was evaluated by using bootstrap analyses (Felsenstein, 1985) of neighbour-joining datasets for 1000 resamplings. The phylogenetic analyses were carried out using the ARB package.

Strains IMMIB N-402^T and IMMIB N-403 have morphological properties consistent with their assignment to the genus *Nocardia*. They are aerobic organisms that form hyphae that are Gram-positive and slightly acid-alcohol-fast. The vegetative hyphae are orange in colour, well developed, with irregular branches penetrating the agar and bearing white aerial hyphae. At a late stage of growth the hyphae fragment into rod-shaped elements characteristic of nocardiae. The physiological properties of isolates IMMIB N-402^T and IMMIB N-403 are cited in detail later, in the description of *Nocardia elegans* sp. nov. Biochemical differences between the isolates and some *Nocardia* species with validly published names (i.e. those examined in this study) are shown in Table 1.

Chemotaxonomically, isolates IMMIB N-402^T and IMMIB N-403 contained chemical markers that support assignment of these micro-organisms to the genus *Nocardia*. The cell wall contains *meso*-diaminopimelic acid as well as arabinose and galactose (i.e. wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970). One-dimensional TLC of whole-cell acid methanolsates of strain IMMIB N-402^T and strain IMMIB N-403 revealed the presence of two lipid spots, for each organism, with different flow rates on the chromatogram.

Table 1. Differential physiological characteristics of strains IMMIB N-402^T, IMMIB N-403 and other significant *Nocardia* species with validly published names

Taxa are identified as follows: 1, IMMIB N-402^T, IMMIB N-403; 2, *N. veterana* DSM 44445^T; 3, *N. africana* SD 769^T; 4, *N. cerradoensis* DSM 44546^T; 5, *N. vaccinii* DSM 43285^T; 6, *N. nova* ATCC 33726^T. All strains are positive for utilization of glucose as a carbon source and for hydrolysis of urea. All strains are negative for the following: hydrolysis of casein, elastin, gelatin, tyrosine, xanthine; utilization of cellobiose, raffinose, sorbitol, sucrose, *meso*-erythritol and *myo*-inositol as carbon sources; and utilization of acetamide, gelatin, proline and serine as simultaneous carbon and nitrogen sources. w, Weakly utilized after 3 weeks incubation.

Characteristic	1	2	3	4	5	6
Hydrolysis of:						
Aesculin	+	+	–	w	–	–
Hypoxanthine	–	–	–	+	–	–
Testosterone	–	+	+	+	–	–
Utilization as sole sources of carbon and energy						
Acetate	+	+	+	+	–	+
Citrate	–	–	–	–	+	–
Gluconate	–	–	–	–	+	–
L-Arabinose	–	–	–	–	+	–
Galactose	–	–	–	+	+	+
Maltose	–	–	–	+	–	–
L-Rhamnose	–	–	–	w	+	–
Trehalose	–	–	–	+	+	–
Xylose	–	–	–	–	+	–
Mannitol	–	–	–	–	+	–
iso-Amyl alcohol	–	–	–	–	–	+
2,3-Butanediol	–	+	–	–	–	–
1,2-Propanediol	–	+	–	w	–	–
<i>m</i> -Hydroxybenzoate	–	–	+	+	–	–
<i>p</i> -Hydroxybenzoate	–	–	–	–	w	–
Utilization of L-alanine as sole source of carbon and nitrogen	–	–	+	–	–	–

The lower spots correspond to mycolic acids, as identified by their R_F value (0.47), and the higher ones correspond to the non-hydroxylated fatty acids. Pyrolysis GC of the purified mycolic acid methyl esters from isolates IMMIB N-402^T and IMMIB N-403 released fatty acid methyl esters of C_{16:0} (33.2 % of total cleavage products of the mycolates from strain IMMIB N-402^T), C_{18:1} (27.8 %) and C_{18:0} (39.0 %) as pyrolysis cleavage products. GC analyses of the non-hydroxylated fatty acid methyl esters revealed the presence of tetradecanoate (0.26 % of total fatty acids from isolate IMMIB N-402^T), pentadecanoate (0.52 %), *cis*-hexadecenoate (0.89 %), hexadecanoate (30.98 %), 10-methylhexadecanoate (0.1 %), heptadecanoate (1.86 %), 10-methylheptadecanoate (0.39 %), octadecenoate (3.85 %), octadecanoate (18.15 %), tuberculostearic acid (10-methyl octadecanoate, 38.91 %), nonadecanoate (0.14 %), eicosenoate (0.95 %), eicosanoate (1.4 %), heneicosenoate (0.61 %) and heneicosanoate (1.0 %) as the major cellular fatty acid methyl esters. Polar lipid analysis showed that strains IMMIB N-402^T and IMMIB N-403 contain phosphatidylethanolamine, phosphatidylinositol, phosphatidyl-inositol mannoside and diphosphatidylglycerol as the characteristic phospholipids (i.e. phospholipid type PII *sensu* Lechevalier *et al.*, 1977). Mass-spectral analysis of the respiratory quinones showed that isolate IMMIB N-402^T and isolate IMMIB N-403 possess hexahydrogenated menaquinones with eight isoprene units in which the two terminal isoprene moieties are cyclized. The main component corresponds to MK-8(H₆) and the minor component corresponds to 2,3-epoxy-MK-8(H₆). These chemotaxonomic similarities to *Nocardia* species with validly published names are supported by the high levels of 16S rRNA gene sequence similarity observed between isolates IMMIB N-402^T and IMMIB N-403 and members of the genus *Nocardia*.

To ascertain the phylogenetic position of strains IMMIB N-402^T and IMMIB N-403, their almost-complete 16S rRNA gene sequences [1483 nt; 96.1 % of the *Escherichia coli* sequence (Brosius *et al.*, 1978)] were determined in this study and subjected to a comparative analysis. The 16S rRNA gene sequence comparison clearly shows that strains IMMIB N-402^T and IMMIB N-403 are members of the family *Nocardiaceae* (Stackebrandt *et al.*, 1997). The high values for the 16S rRNA gene sequence similarities to other previously described members of the genus *Nocardia* (95.5–99.6 %) support the addition of strains IMMIB N-402^T and IMMIB N-403 to this genus. There were significantly lower levels of similarity with other *Actinomycetales* taxa (data not shown). The highest sequence similarities were shown with *Nocardia nova*, *Nocardia vaccinii*, *Nocardia cerradoensis*, *Nocardia veterana* and *Nocardia africana* (98.3, 98.5, 98.7, 99.2 and 99.6 % sequence similarity, respectively). A tree constructed using the neighbour-joining method, depicting the phylogenetic placement of strains IMMIB N-402^T and IMMIB N-403 within a subset of the genus *Nocardia*, is shown in Fig. 1. It is evident from the tree that the two isolates represent a distinct subline within the

genus *Nocardia* that is associated with *N. africana*, *N. veterana*, *N. cerradoensis* and *N. vaccinii*. These results suggest that strains IMMIB N-402^T and IMMIB N-403 belong to a genetically distinct *Nocardia* species showing close relatedness with *N. africana*, *N. veterana*, *N. vaccinii* and *N. cerradoensis*. These sequence similarities are too high to allow the definition of a novel species, since values below 97 % and/or genomic DNA reassociation values below 70 % are considered necessary for the establishment of a novel bacterial species (Stackebrandt & Goebel, 1994). In view of the high levels of 16S rRNA sequence similarities between isolates IMMIB N-402^T and IMMIB N-403 and some *Nocardia* species, chromosomal DNA–DNA hybridization studies were performed to establish whether strains IMMIB N-402^T and IMMIB N-403 represent distinct species. Strains IMMIB N-402^T and IMMIB N-403 displayed low levels of DNA–DNA reassociation with the type strains of *N. africana* SD 769^T (30.2 and 33.6 %, respectively) and *N. veterana* DSM 44445^T (33.6 % and 27.5 %, respectively), results which are below the cut-off point recommended by Wayne *et al.* (1987) for the circumscription of bacterial genomic species, and confirm the separation of isolates IMMIB N-402^T and IMMIB N-403 from their nearest phylogenetic neighbours.

The genus *Nocardia* contains a number of species for which numerous distinctive characteristics have been described that fully justify their classification in separate species but that exhibit only limited 16S rRNA divergence. For instance, the 16S rRNA of *N. vaccinii* is 98.5–98.7 % similar to that of *N. africana*, though the distinction between the two species has been convincingly illustrated by virtue of different characteristics and did not require the determination of DNA hybridization values (Hamid *et al.*, 2001). More critical examples are *N. veterana* and *N. africana*, the 16S rRNA gene sequences of which are 99.3 % similar, whereas the total labelled genomic DNA of the two species exhibited only 28.8 % homology. Similar situations were found for *Nocardia brevicatena* and *Nocardia paucivorans*, which have a 16S rRNA similarity value of 99.6 % but 61.9 % DNA–DNA relatedness (Yassin *et al.*, 2000a), and for *Nocardia carnea* and *Nocardia flavorosea*, which have a 16S rRNA similarity value of 99.2 % but 5.0 % DNA–DNA relatedness (Chun *et al.*, 1998). Given the 1.4, 1.5 and 1.8 % sequence divergence between isolates IMMIB N-402^T and IMMIB N-403 and their closest relatives, *N. cerradoensis*, *N. vaccinii* and *N. nova*, respectively, and the biochemical tests (Table 1) differentiating isolates IMMIB N-402^T and IMMIB N-403 from the latter three species, it is reasonable to define a novel species. The chemical (and morphological) data clearly indicate that the isolates belong to the genus *Nocardia*. The 16S rRNA gene sequencing, the DNA–DNA pairing and the biochemical results indicate that the two isolates are similar and represent a novel species when compared with the type strains of *Nocardia* species with validly published names. Thus, on the basis of the results of the polyphasic taxonomic study reported here, we consider that isolates IMMIB N-402^T and IMMIB N-403 represent

Fig. 1. Neighbour-joining tree showing the position of the type strain of *Nocardia elegans*, strain IMMIB N-402^T (=CCUG 50200^T=CIP 108553^T), within the radiation of species of the genus *Nocardia*. The tree was based on a comparison of sequences that were at least 90% complete (with regard to the *E. coli* sequence). Bar, 10.0% sequence divergence.

one species and merit classification as a novel species of the genus *Nocardia*, for which the name *Nocardia elegans* sp. nov. is proposed, with IMMIB N-402^T as the type strain.

Description of *Nocardia elegans* sp. nov.

Nocardia elegans (e'le.gans. L. adj. *elegans* fastidious – with respect to utilization of nutrients).

The hyphae are Gram-positive and partially acid-alcohol-fast. Vegetative hyphae are orange in colour, 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. Grows at temperatures in the range 22–42 °C. The organism possesses the salient chemotaxonomic characteristics of the genus *Nocardia*. Its mycolic acids are cleaved, upon pyrolysis, releasing fatty acids C_{16:0}, C_{18:1} and C_{18:0} with C_{18:0} as the major cleavage product. Hydrolyses aesculin, testosterone and urea but not adenine, casein, elastin, gelatin, guanine, hypoxanthine, tyrosine or xanthine. Assimilates acetate and glucose as carbon sources, but not adonitol, adipate, iso-amyl alcohol, arabinose, 2,3-butanediol, cellobiose, citrate, *meso*-erythritol, galactose, gluconate, *m*-hydroxybenzoate, *p*-hydroxybenzoate, *myo*-inositol, lactate, lactose, maltose, mannitol, melezitose, 1,2-propanediol, raffinose, rhamnose, sorbitol, sucrose, trehalose or xylose. Does not utilize acetamide, arginine, gelatin,

ornithine, proline or serine as simultaneous carbon and nitrogen sources.

The type strain of *Nocardia elegans* is strain IMMIB N-402^T (=CCUG 50200^T=CIP 108553^T).

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