Nocardia puris sp. nov.

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A bacterial isolate (IMMIB R-145^T) from a human abscess was subjected to a polyphasic taxonomic study. Chemotaxonomic investigations revealed the isolate to have cell wall chemotype IV and mycolic acids consistent with characteristics of the genus *Nocardia*. Comparative 16S rRNA gene sequencing showed that the isolate constituted a distinct subline within the genus *Nocardia*, displaying greater than 1.7 % sequence divergence with established species. However, a DNA–DNA hybridization study demonstrated unambiguously that the isolate was genealogically distinct from closely related species, namely, *Nocardia abscessus*, *Nocardia cyriacigeorgica* and *Nocardia farcinica*, with which it showed high levels of 16S rDNA sequence similarity (97.8, 97.9 and 98.3 %, respectively). Based on phenotypic and phylogenetic data, it is proposed that this isolate be classified as a new species of the genus *Nocardia*, for which the name *Nocardia puris* (type strain IMMIB R-145^T = DSM 44599^T = NRRL B-24204^T) is proposed.

The application of chemotaxonomic methods (Collins *et al.*, 1977; Kroppenstedt, 1985; Lechevalier, 1976; Lechevalier *et al.*, 1977; Minnikin *et al.*, 1980; Schleifer & Kandler, 1972), in combination with a phylogenetic approach based on 16S rRNA gene sequence analyses (Chun & Goodfellow, 1995; Ruimy *et al.*, 1994; Rainey *et al.*, 1995; Stackebrandt & Goodfellow, 1991; Stackebrandt *et al.*, 1997; Stackebrandt & Goebel, 1994; Stackebrandt & Woese, 1981), has resulted in a much improved taxonomy of the genus *Nocardia* (Goodfellow *et al.*, 1999). The improved classification provides a sound framework for the recognition of additional species and for resolving the taxonomy of isolates which were collectively assigned to the *Nocardia asteroides* complex. Thus far, *Nocardia abscessus* (Yassin *et al.*, 2000b) and *Nocardia cyriacigeorgica* (Yassin *et al.*, 2001) equated to some strains originally classified as *Nocardia asteroides* subgroup IIIA by Schaal & Reutersberg (1978). The genus *Nocardia*, at the time of writing, encompasses 30 validly described species, which form a distinct phylogenetic line within the suborder *Corynebacterinae* (Stackebrandt *et al.*, 1997) that constitutes a home for the mycolic-acid-containing genera of the order *Actinomycetales*. This study describes a novel *Nocardia* species isolated from a human abscess. Based on phylogenetic and phenotypic data, it is proposed that this species (strain IMMIB R-145^T) be classified as *Nocardia puris*.

Strain IMMIB R-145^T was cultured on Brain–Heart Infusion (BHI) agar to determine its morphological properties. The

strain was biochemically characterized by using tests to determine 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 isomeric form of the diaminopimelic acid was determined by the methods of Becker *et al.* (1964) and whole-cell sugars were determined by the method of Lechevalier (1968). Lipids were extracted using acid methanolysis and mycolic acids were detected with TLC as described by Minnikin *et al.* (1980); pyrolysis gas chromatography of the mycolates was performed according to Yassin *et al.* (1993a). Non-hydroxylated fatty acids were purified, identified and quantified by gas chromatography 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, 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) 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 rDNA sequence of strain IMMIB R-145^T as well as those of the validly described species of the genus *Nocardia* retrieved from the

The GenBank accession number for the 16S rDNA sequence of *Nocardia puris* IMMIB R-145^T is AJ508748.

GenBank database were added to the ARB database (Ludwig & Strunk, 1996) and aligned using the respective tool of the ARB package. The resulting alignment was corrected manually and evolutionary trees were inferred using the maximum-parsimony (Kluge & Farris, 1969), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms. 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 phylogenetic analyses were carried out using the ARB package (Ludwig & Strunk, 1996).

Strain IMMIB R-145^T has morphological properties

Table 1. Differential physiological characteristics of strain IMMIB R-145^T and other significant validly described *Nocardia* species

Strain: 1, IMMIB R-145^T; 2, *N. veterana* DSM 44445^T; 3, *N. africana* SD769^T; 4, *N. ignorata* DSM 44496^T; 5, *N. salmonicida* JCM 4826^T; 6, *N. cyriacigeorgica* DSM 44484^T; 7, *N. abscessus* DSM 44432^T; 8, *N. paucivorans* DSM 44386^T; 9, *N. asteroides* ATCC 19247^T; 10, *N. nova* ATCC 33726^T; 11, *N. brasiliensis* DSM 43758^T; 12, *N. farcinica* DSM 43665^T; 13, *N. otitidiscaviarum* DSM 43242^T; 14, *N. vaccinii* DSM 43285^T; 15, *N. transvalensis* NCTC 2392^T; 16, *N. beijingensis* JCM 10666^T (data from Wang *et al.*, 2001). –, Negative; +, positive; ND, not determined; w, weakly utilized after 3 weeks incubation; d, different, some strains are positive and some are negative.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Hydrolysis of:																
Casein	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
Elastin	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
Aesculin	+	+	–	+	+	+	–	–	+	–	+	+	+	–	–	+
Gelatin	–	–	–	+	+	–	–	–	–	–	–	–	–	–	+	ND
Hypoxanthine	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–
Testosterone	+	+	+	+	+	+	+	–	+	–	–	+	+	–	+	ND
Tyrosine	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
Xanthine	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+
Utilization as sole sources of carbon and energy:																
Acetate	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+
Citrate	+	–	–	–	–	–	–	–	–	–	–	–	–	+	+	ND
Gluconate	–	–	–	+	+	+	–	–	+	–	–	–	w	+	+	+
L-Arabinose	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+
Cellobiose	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+
Galactose	–	–	–	–	+	–	–	–	–	+	+	–	–	+	+	+
Glucose	+	+	+	+	+	–	+	–	+	+	+	+	+	+	+	+
Maltose	+	–	–	+	–	–	+	–	–	–	+	+	–	–	+	+
Raffinose	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+
L-Rhamnose	–	–	–	–	–	–	d	–	–	–	–	+	–	+	–	+
Sorbitol	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND
Sucrose	+	–	–	w	–	+	+	–	+	–	w	+	–	–	+	+
Trehalose	–	–	–	+	+	–	+	+	–	–	+	+	w	+	+	+
Xylose	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+
meso-Erythritol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
myo-Inositol	+	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–
Mannitol	+	–	–	+	+	–	–	–	–	–	w	–	+	+	+	ND
Isoamyl alcohol	–	–	–	–	+	–	–	–	–	+	w	+	–	–	+	–
2,3-Butanediol	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	ND
1,2-Propanediol	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	ND
m-Hydroxybenzoate	–	–	+	–	–	–	–	–	–	–	–	+	–	–	w	–
p-Hydroxybenzoate	–	–	–	–	–	–	–	–	–	–	–	–	–	w	–	+
Utilization as sole sources of carbon and nitrogen:																
Acetamide	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–
L-Alanine	+	–	+	–	–	–	–	–	–	–	+	–	+	–	–	w
Gelatin	–	–	–	w	–	–	–	–	–	–	+	–	–	–	–	–
Proline	–	–	–	+	+	–	–	–	+	–	+	–	–	–	+	+
Serine	–	–	–	–	+	–	–	–	–	–	w	–	w	–	+	ND

consistent with its assignment to the genus *Nocardia*. It is an aerobic organism which forms hyphae that are Gram-positive and slightly acid-alcohol-fast. The vegetative hyphae were 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 strain IMMIB R-145^T are given in the species description (see below). Differences in the biochemical characteristics of strain IMMIB R-145^T compared to those of some of the validly described species of the genus *Nocardia* examined in this study can be found in Table 1.

Chemotaxonomically, strain IMMIB R-145^T contained chemical markers which support its assignment 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 methanolysates of strain IMMIB R-145^T revealed the presence of two lipid spots on the chromatogram: the lower one corresponded to mycolic acids as identified by its *R_F* value (0.47) and the higher one corresponded to the non-hydroxylated fatty acids. Pyrolysis gas chromatography of the purified mycolic acid methyl esters from strain IMMIB R-145^T released fatty acid methyl esters of C_{14:0} (4.4% of total cleavage products), C_{16:0} (61.5%), C_{17:0} (3.6%) and C_{18:0} (30.6%) as pyrolysis cleavage products. Gas chromatographic analyses of the non-hydroxylated fatty acid methyl esters revealed the presence of tetradecanoate (0.9% of total fatty acids), pentadecanoate (0.6%), *cis*-hexadecenoate (3.1%), hexadecanoate (32.7%), heptadecanoate (2.40%), octadecanoate (10.0%), octadecanoate (14.0%), tuberculostearic acid (10-methyl octadecanoate, 20.4%), nonadecanoate (0.2%), eicosenoate (6.0%), eicosanoate (4.7%), heneicosenoate (0.4%), heneicosanoate (0.3%), docosenoate (3.0%) and docosanoate (2.1%) as the major cellular fatty acid methyl esters. Polar lipid analysis showed that strain IMMIB R-145^T contains phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol 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 strain IMMIB R-145^T possesses 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 the validly described species of the genus *Nocardia* are supported by the high levels of 16S rRNA gene sequence similarity observed between isolate IMMIB R-145^T and members of the genus *Nocardia* (>95%).

To ascertain the phylogenetic position of strain IMMIB R-145^T, its almost-complete 16S rRNA gene sequence [1474 nt; 95.6% of the *Escherichia coli* sequence (Brosius *et al.*, 1978)] was determined in this study and subjected

to a comparative analysis. The 16S rRNA gene sequence comparison clearly showed that strain IMMIB R-145^T is a member of the family *Nocardiaceae* (Stackebrandt *et al.*, 1997) and that the determined sequence contains all the signature nucleotides designated for this lineage. The high values for the 16S rRNA gene sequence similarity to other previously described members of the genus *Nocardia* (95.5–98.3%) support the addition of strain IMMIB R-145^T to this genus. Significantly lower levels of similarity were shown with other taxa of the order *Actinomycetales* (data not shown). Highest sequence similarities were shown with *Nocardia abscessus* (97.8%), *Nocardia cyriacigeorgica* (97.9%) and *Nocardia farcinica* (98.3% sequence similarity). A tree, constructed using the neighbour-joining method, depicting the phylogenetic placement of strain IMMIB R-145^T within a subset of the genus *Nocardia* is shown in Fig. 1. It was evident from the tree that strain IMMIB R-145^T represents a distinct subline within the genus *Nocardia* that is associated with *Nocardia beijingensis*, *Nocardia brasiliensis*, *N. farcinica* and *Nocardia transvalensis*,

Fig. 1. Unrooted phylogenetic tree showing the position of strain IMMIB R-145^T within the radiation of species of the genus *Nocardia*. The tree, constructed using the neighbour-joining method, was based on a comparison of sequences that were at least 90% complete (with regard to *E. coli* sequence). Bar, 5.0% sequence divergence.

the strain displayed sequence similarity values of 97.6, 97.5, 98.3 and 97.6 %, respectively, with these species. These results suggest that strain IMMIB R-145^T belongs to a genetically distinct *Nocardia* species. However, the sequence similarity values are too high to allow the definition of a new species on the basis of 16S rDNA sequence alone, since values below 97 % sequence similarity and/or genomic DNA reassociation values below 70 % are considered relevant for the establishment of new bacterial species (Stackebrandt & Goebel, 1994). In view of the high levels of 16S rDNA sequence similarity between strain IMMIB R-145^T and some *Nocardia* species, chromosomal DNA–DNA hybridization studies were performed to establish whether strain IMMIB R-145^T represents a distinct species of the genus *Nocardia*. Strain IMMIB R-145^T displayed low levels of DNA–DNA reassociation with *N. abscessus* DSM 44432^T (20.6 %), *N. cyriacigeorgica* DSM 44484^T (37.6 %) and *N. farcinica* DSM 43665^T (42.3 %), results which are below the cut-off point recommended for the circumscription of bacterial genomic species by Wayne *et al.* (1987) and confirm the separation of strain IMMIB R-145^T from its nearest phylogenetic neighbours.

The genus *Nocardia* contains a number of species for which numerous distinctive characters have been described that fully justify their classification in separate species but that exhibit only limited 16S rDNA divergence. For instance, the 16S rDNA of *N. beijingensis* is 97.6 and 97.5 % similar to that of *N. brasiliensis* and *N. farcinica*, respectively, though the distinction between *N. beijingensis* and the two species has been convincingly illustrated by different characters and did not require the determination of DNA hybridization values (Wang *et al.*, 2001). Similarly, *Nocardia vaccinii* and *Nocardia africana* share 98.5–98.7 % 16S rDNA sequence similarity (Hamid *et al.*, 2001). More critical examples are *Nocardia veterana* and *N. africana*, the 16S rDNA of which are 99.3 % similar, whereas the total labelled genomic DNA of the two species exhibits only 28.8 % homology. Similar situations were found for *Nocardia brevicatena* and *Nocardia paucivorans*, which have a 16S rDNA 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). The 97 % limit is thus not always fulfilled in the genus *Nocardia* and additional distinctive characteristics must be determined to allow the definition of a new species. Given the 2.4–2.5 % sequence divergence values of strain IMMIB R-145^T and its closest relatives *N. transvalensis*, *N. beijingensis* and *N. brasiliensis* on the one hand and the biochemical tests presented in Table 1 to discriminate strain IMMIB R-145^T from the last three species on the other hand, we feel that it is reasonable to define a new species of the genus *Nocardia*. The chemical (and morphological) data clearly indicate that strain IMMIB R-145^T belongs to the genus *Nocardia*, and the 16S rDNA sequencing, DNA–DNA pairing and biochemical results indicate that it forms a new species when compared with the type strains

of validly described species of the genus *Nocardia*. Thus, on the basis of the results of the reported polyphasic taxonomic study, we consider that strain IMMIB R-145^T merits classification as a new species of the genus *Nocardia*, for which the name *Nocardia puris* is proposed.

Description of *Nocardia puris* sp. nov.

Nocardia puris (pu'ris. L. gen. neut. n. *puris* of corrupt matter, pus, pertaining to the abscess from which the type strain was isolated).

Hyphae are Gram-positive and partially acid–alcohol-fast. Vegetative hyphae are well developed with irregular branches penetrating the agar; they also bear white aerial hyphae. At a late stage of growth, the hyphae fragment into rod-shaped elements. Grows between 22 and 45 °C. Contains the salient chemotaxonomic characteristics of nocardiae. Mycolic acids cleaved on pyrolysis to release fatty acids of C_{14:0}, C_{16:0}, C_{17:0} and C_{18:0}, with C_{16:0} as the major cleavage product. Hydrolyses aesculin, testosterone and urea but not adenine, casein, elastin, gelatin, guanine, hypoxanthine, tyrosine or xanthine. Assimilates acetate, citrate, glucose, *myo*-inositol, maltose, mannitol, sorbitol and sucrose as carbon sources but not adonitol, adipate, isoamyl alcohol, arabinose, 2,3-butanediol, cellobiase, *meso*-erythritol, galactose, gluconate, *m*-hydroxybenzoate, *p*-hydroxybenzoate, lactate, lactose, melezitose, 1,2-propanediol, raffinose, rhamnose, trehalose or xylose. Utilizes alanine but not acetamide, arginine, gelatin, ornithine, proline or serine as simultaneous carbon and nitrogen sources.

The type strain is IMMIB R-145^T (= DSM 44599^T = NRRL B-24204^T). Isolated from a human abscess.

Note added in proof

Since this work was submitted for publication, two more species of *Nocardia* have been described, *Nocardia cerra-doensis* (Albuquerque de Barros *et al.*, 2003) and *Nocardia caishijiensis* (Zhang *et al.*, 2003).

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