Nocardia iowensis sp. nov., an organism rich in biocatalytically important enzymes and nitric oxide synthase

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Nocardia strain NRRL 5646^T, isolated from a garden soil sample in Osceola, Iowa, USA, was initially of interest as an antibiotic producer. It contained biocatalytically important enzymes and represented the first described nitric oxide synthase enzyme system in bacteria. The present polyphasic taxonomic study was undertaken to differentiate strain NRRL 5646^T from related species of the genus *Nocardia*. Chemotaxonomic analyses included determinations of the fatty acid methyl ester profile (C_{16:1}ω6c/C_{16:1}ω7c, C_{16:0}, C_{18:1}ω9c and C_{18:0} 10-methyl as major components), quinone [cyclo MK-8(H₄) as the major component], polar lipid (diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannoside as major components) and mycolic acid. These results supported its placement within the genus *Nocardia*. Biochemical testing and 16S rRNA, 65-kDa heat-shock protein (*hsp65*) and preprotein translocase (*secA1*) gene sequence analyses differentiated strain NRRL 5646^T from recognized *Nocardia* species. Previous studies have demonstrated that other genetic sequences (carboxylic acid reductase, *Nocardia* phosphopantetheinyl transferase and GTP cyclohydrolase I) from strain NRRL 5646^T can also be used to substantiate its uniqueness. The level of 16S rRNA gene sequence similarity between strain NRRL 5646^T and the type strains of *Nocardia tenerifensis* and *Nocardia brasiliensis* was 98.8%. However, strain NRRL 5646^T could be clearly distinguished from these *Nocardia* species based on DNA–DNA hybridization data. Consequently, strain NRRL 5646^T is considered to represent a novel species of the genus *Nocardia*, for which the name *Nocardia iowensis* sp. nov. is proposed. The type strain is NRRL 5646^T (=UI 122540^T=NRRL B-24671^T=DSM 45197^T).

Strain NRRL 5646^T was initially identified as an antibiotic-producing bacterium isolated from a garden soil sample in Osceola, Iowa, USA (Hlavka & Bitha, 1977; Martin *et al.*, 1977). This strain showed an extraordinary diversity of biotransforming enzymes and the first bacterial nitric oxide synthase (NOS) system with possible physiological importance, making its identification to the species level important. Strain NRRL 5646^T converts many natural and synthetic organic compounds into valuable products,

including flavonoids (Herath *et al.*, 2006; Maatooq & Rosazza, 2005), vanillic acid (Dhar *et al.*, 2007) and 4-vinylphenol (Lee & Rosazza, 2002). A novel ATP/NADPH-dependent carboxylic acid reductase was isolated from strain NRRL 5646^T (Li & Rosazza, 1997) and subsequently cloned (*car*; GenBank accession number AY495697; He *et al.*, 2004a). The reduction system required a phosphopantetheinyl transferase (*npt*, GenBank accession number DQ904035; Venkitasubramanian *et al.*, 2007). Strain NRRL 5646^T contains the first NOS system characterized in prokaryotes (Chen & Rosazza, 1995). As part of the NOS system, strain NRRL 5646^T contains guanylate cyclase for cyclic GMP synthesis (Son & Rosazza, 2000), and GTP cyclohydrolase I (*gch*; GenBank accession number

Abbreviation: NOS, nitric oxide synthase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *secA1* and *hsp65* gene sequences of strain NRRL 5646^T are DQ925490, EU650382 and EU650383, respectively.

AY128670; He *et al.*, 2004b) for tetrahydrobiopterin biosynthesis.

The present polyphasic taxonomic study was used to identify strain NRRL 5646^T to the species level. Phenotypic, biochemical, chemotaxonomic (fatty acid methyl esters, quinones, polar lipids, mycolic acids) and genotypic [16S rRNA, 65-kDa heat-shock protein (*hsp65*) and preprotein translocase (*secA1*) gene sequence analyses, DNA–DNA hybridization] characteristics were used to show that strain NRRL 5646^T represents a novel species of the genus *Nocardia*.

Strain NRRL 5646^T was grown and maintained on sporulating agar slants (Li & Rosazza, 1997) at room temperature for 5 days and was maintained at 4 °C. Strain NRRL 5646^T was grown in shaken flask cultures at 29 °C to provide cell pellets for analysis as described by Li & Rosazza (1997). The strain initially grew as a whitish mycelium on sporulating agar at 29 °C and after several days showed whitish aerial hyphae. Smears were prepared from the growth on sporulating agar and were Gram stained according to Gerhardt *et al.* (1994). Smears were also stained via the modified Ziehl-Neelsen method of Gordon (1967) to determine the degree of acid-fastness. Cells of strain NRRL 5646^T stained as Gram-positive rods with branching characteristic of *Nocardia* species. The acid-fastness of strain NRRL 5646^T was confirmed by using modified Kinyoun acid-fast staining (Brown-Elliott *et al.*, 2006). For scanning electron microscopy analysis at the Central Electron Microscopy Research Facility at the University of Iowa, an agar block containing substrate and aerial mycelium was fixed with 25 % glutaraldehyde and subsequently stained with 1 % osmium tetroxide. The sample was rinsed with 0.1 M cacodylate buffer, then water and then progressively dehydrated with increasing concentrations of ethanol. The sample was critical-point dried, mounted on stubs and sputter coated, and scanning electron micrographs were taken with a Hitachi S-4800 scanning electron microscope. Micrographs revealed branching aerial hyphae.

The cultural, morphological and phenotypic properties of strain NRRL 5646^T were originally described by Martin *et al.* (1977) and Hlavka & Bitha (1977). Additional biochemical tests were done by using *Nocardia* ID QUAD plates (BD Microbiology Systems), bile aesculin agar and urea agar slants (BD Microbiology Systems). Hydrolysis of hypoxanthine and uric acid was determined as described by Isik *et al.* (1999). Duplicate plates containing the relevant substrates were inoculated from a glycerol stock and incubated at 29 °C for 2–4 weeks. Results of the physiological characterization are given in the species description below.

Fatty acid and mycolic acid profiles of strain NRRL 5646^T were determined by Microbial ID, Inc., Newark, Delaware, USA (<http://www.microbialid.com>), by using the Sherlock Microbial Identification System software (MIDI Inc.) following standard protocols (Miller, 1982; Sasser, 1990;

Butler *et al.*, 1986). Bacterial samples were grown at 29 °C on freshly prepared yeast-malt extract agar (Difco) prior to analysis. The major fatty acids of strain NRRL 5646^T were C_{16:0} (28.2 % of the total), C_{18:1ω9c} (27.9 %) and C_{18:0} 10-methyl (18.4 %). A mixture of C_{16:1ω6c} and C_{16:1ω7c} fatty acids was also detected as a significant component (19.2 %). This profile most closely matches those of members of the genus *Nocardia*. Table 1 provides a comparison of the fatty acid profiles of strain NRRL 5646^T and the type strains of closely related *Nocardia* species.

Mycolic acid analysis also confirmed strain NRRL 5646^T as a member of the genus *Nocardia*. MIS (Microbial Identification System) library comparisons gave low similarity indices of 0.346, 0.313 and 0.262 for strain

Table 1. Fatty acid methyl ester profiles of strain NRRL 5646^T and the type strains of related *Nocardia* species

Strains: 1, NRRL 5646^T (*N. iowensis* sp. nov.); 2, *N. tenerifensis* DSM 44704^T; 3, *N. transvalensis* DSM 43405^T; 4, *N. brasiliensis* DSM 43758^T; 5, *N. farcinica* DSM 43665^T; 6, *N. beijingensis* DSM 44636^T. Values are percentages of the total fatty acids. For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain; *cis* and *trans* isomers are indicated by the suffixes *c* and *t*, respectively. Data for reference strains were taken from Kämpfer *et al.* (2004). TSBA, Tuberculostearic acid. –, Not detected.

Fatty acid	1	2	3	4	5	6
C _{10:0}	0.2	0.3	0.1	0.1	0.1	0.02
C _{11:0}	–	–	–	–	0.02	–
C _{12:0}	–	–	0.5	0.2	0.3	–
C _{14:0}	0.5	0.7	1.8	1.4	1.6	1.1
C _{15:0}	–	0.4	0.5	1.2	–	2.0
C _{15:1ω5c}	0.2	–	–	–	–	0.2
C _{16:1ω9c}	0.2	0.3	0.9	0.3	0.2	0.2
C _{16:1ω6c} and C _{16:1ω7c}	19.2	–	–	–	–	–
C _{16:0}	28.2	44.5	39.7	40.0	37.7	37.9
C _{17:1ω9c}	–	–	–	–	1.3	–
C _{17:1ω8c}	2.1	–	0.6	0.6	–	1.4
C _{17:1ω5c}	–	0.8	–	–	–	–
C _{17:0}	1.4	0.3	0.7	1.0	0.6	2.8
C _{18:3ω6,9,12c}	–	0.3	–	–	–	–
C _{17:0} 10-methyl	0.6	–	0.3	–	0.3	0.5
C _{18:1ω7c}	–	–	0.8	–	–	–
C _{18:1ω9c}	27.9	15.0	22.9	24.0	24.8	18.6
C _{18:0}	0.6	4.3	4.6	7.9	13.6	6.5
C _{18:0} 10-methyl (TSBA)	18.4	20.3	15.4	12.9	10.8	9.9
C _{19:0}	–	–	–	–	–	0.7
C _{19:1ω9c} and C _{19:1ω11c}	0.2	0.5	0.4	0.6	–	0.9
C _{20:0}	–	–	–	–	0.6	–
C _{20:4ω6,9,12,15c}	–	–	–	–	–	0.4
C _{20:2ω6,9c}	–	0.5	–	–	–	–
C _{20:1ω9c}	0.5	–	1.3	–	1.4	0.3

NRRL 5646^T versus the type strains of *Nocardia carnea*, *Nocardia brasiliensis* and *Nocardia asteroides*, respectively. Thus, although strain NRRL 5646^T was not present in the Microbial ID, Inc. databases, it has a mycolic acid composition similar to those of recognized members of the genus *Nocardia*.

Polar lipids and menaquinone analyses of strain NRRL 5646^T (performed by the DSMZ, Braunschweig, Germany; <http://www.dsmz.de>), important classification tools for the identification of members of the genus *Nocardia* (Kämpfer *et al.*, 2004), revealed the presence of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannoside as major polar lipids, and cyclo MK-8(H₄) as the major quinone. These data also supported the affiliation of strain NRRL 5646^T to the genus *Nocardia*.

Near-full-length 16S rRNA gene sequences are considered to provide reliable criteria for identification of members of the genus *Nocardia* (Brown-Elliott *et al.*, 2006; Roth *et al.*, 2003; Wauters *et al.*, 2005). Moreover, a variable region that exists within the first 500 bp of the gene is considered to be the basis for a reliable method for species differentiation among members of the genus (Conville *et al.*, 2000). The nearly full-length 16S rRNA gene sequence of strain NRRL 5646^T was determined by using eubacteria primers 27f and 1492R (Lane, 1991). A 1.4-kb fragment of the 16S rRNA gene from strain NRRL 5646^T was amplified (Lee *et al.*, 2007). Analysis of this sequence via the BLAST program (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>; Altschul *et al.*, 1997) showed that it shared 98.8 % similarity to *Nocardia tenerifensis* DSM 44704^T and 98.8 % to *N. brasiliensis* DSM 43758^T [along with several other

Fig. 1. Unrooted neighbour-joining tree (Saitou & Nei, 1987), based on nearly complete 16S rRNA gene sequences, showing the position of strain NRRL 5646^T among representatives of closely related *Nocardia* species. Phylogenetic data were recovered by using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) tree-making algorithms, respectively. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values >50 % are given. Asterisks indicate branches that were also found in trees constructed based on *hsp65* and *secA1* gene sequences (see Figs 2 and 3). Bar, 0.01 changes per nucleotide position.

N. brasiliensis strains (GenBank accession numbers Z36935, X80608, AY245543, DQ659902 and X80591)]. This confirmed the designation of strain NRRL 5646^T to the genus *Nocardia* but not to any recognized species as at least 99.2 % 16S rRNA gene sequence similarity is required for this level of classification (Convillie *et al.*, 2000). The 16S rRNA gene sequence of strain NRRL 5646^T was aligned with corresponding nucleotide sequences of the type strains of recognized *Nocardia* species, retrieved from the GenBank/EMBL/DDBJ databases, by using CLUSTAL_X software (Thompson *et al.*, 1997). Phylogenetic trees were inferred by using the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) algorithms from the PHYLIP suite of programs (Felsenstein, 1993). Evolutionary distance matrices were generated as described by Kimura (1980). The resultant unrooted tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) of the

neighbour-joining method based on 1000 resamplings, by using the SEQBOOT and CONSENSE options within the PHYLIP package. Strain NRRL 5646^T formed a distinct phyletic lineage among other species of aerobic actinomycetes (Fig. 1). Within one important variable region of the 16S rRNA gene sequence, strain NRRL 5646^T (146–TTCCAGT–152) differed significantly from that of *N. tenerifensis* DSM 44704^T (172–TCATGTC–178) and showed one base difference from *N. brasiliensis* DSM 43758^T (149–TTTCAGT–155). (Nucleotide position numbers above refer to the original GenBank 16S rRNA gene sequences whereas comparisons were made based on CLUSTAL_X alignment.) This signature sequence also distinguished strain NRRL 5646^T from the type strains of *N. asteroides*, *Nocardia farcinica*, *Nocardia nova*, *Nocardia otitidiscaviarum*, *Nocardia pseudobrasiliensis* and *Nocardia transvalensis* (Convillie *et al.*, 2006). On this basis, strain NRRL 5646^T probably represents a novel *Nocardia* species. Strain NRRL 5646^T differed from all other recognized

Fig. 2. Unrooted neighbour-joining tree (Saitou & Nei, 1987), based on complete *hsp65* gene sequences, showing the position of strain NRRL 5646^T among representatives of closely related *Nocardia* species. Phylogenetic data were recovered by using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) tree-making algorithms, respectively. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values >50 % are given. Asterisks indicate branches that were also found in trees constructed based on 16S rRNA and *secA* gene sequences (see Figs 1 and 3). Bar, 0.01 changes per nucleotide position.

species of the genus with regard to this variable region of the 16S rRNA gene.

The use of *hsp65* gene sequences (but not *hsp65* PCR-restriction enzyme pattern analysis) containing sufficient polymorphism was found to be as reliable as 16S rRNA gene sequence analysis for comprehensive *Nocardia* species identification (Rodriguez-Nava *et al.*, 2006). Additionally, *secA1* gene sequences have been shown to be useful in this regard (Conville *et al.*, 2006). As a result, public sequence repositories such as GenBank have a growing number of both *hsp65* and *secA1* gene sequences.

The *hsp65* and *secA1* genes of strain NRRL 5646^T were amplified and sequenced according to Conville *et al.* (2000, 2006), respectively. Levels of *hsp65* and *secA1* gene sequence analysis were again determined by performing BLAST searches. Evaluation of *hsp65* gene sequence multiple

alignment data placed the novel strain in the genus *Nocardia* with closest matches to the type strains of *Nocardia ninae* (95.9 % similarity), *N. tenerifensis* (95.7 %), *Nocardia arthritidis* (94.9 %) and *N. brasiliensis* (94.6 %). The neighbour-joining phylogenetic tree based on *hsp65* gene sequences is shown in Fig. 2. The *secA1* gene sequence alignment data also placed strain NRRL 5646^T within the genus *Nocardia* with closest matches to the type strains of *Nocardia pneumoniae* (95.5 % similarity), *N. brasiliensis* (95.3 %), *Nocardia beijingensis* (95.3 %) and *N. asteroides* (95.3 %). The neighbour-joining phylogenetic tree based on *secA1* gene sequences is shown in Fig. 3.

DNA–DNA hybridization experiments (performed by the DSMZ) between strain NRRL 5646^T and itself, *N. tenerifensis* DSM 44704^T and *N. brasiliensis* DSM 43758^T revealed levels of relatedness of 92.1 (94.9), 5.4 (7.4) and 19.9 % (15.8 %), respectively (values in parentheses are the

Fig. 3. Unrooted neighbour-joining tree (Saitou & Nei, 1987), based on complete *secA1* gene sequences, showing the position of strain NRRL 5646^T among representatives of closely related *Nocardia* species. Phylogenetic data were recovered by using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) tree-making algorithms, respectively. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values >50 % are given. Asterisks indicate branches that were also found in trees constructed based on 16S rRNA and *hsp65* gene sequences (see Figs 1 and 2). Bar, 0.01 changes per nucleotide position.

results of duplicate measurements). Based on the recommended threshold value of 70 % DNA–DNA relatedness for species delineation (Wayne *et al.*, 1987), strain NRRL 5646^T represents a species distinct from *N. tenerifensis* and *N. brasiliensis*.

In addition, BLAST searches and analysis of *car*, *npt* and *gch* gene sequences of strain NRRL 5646^T revealed closest matches to *N. farcinica* IFM 10152, with maximum similarity values of 74, 84 and 81 %, respectively. However, *N. farcinica* IFM 10152 is the only *Nocardia* taxon for which the entire genome sequence has been established (Ishikawa *et al.*, 2004).

Based on the above physiological, biochemical and chemotaxonomic analyses, strain NRRL 5646^T is considered to represent a novel species of the genus *Nocardia*, for which the name *Nocardia iowensis* sp. nov. is proposed.

Description of *Nocardia iowensis* sp. nov.

Nocardia iowensis (i.o.wen'sis. N.L. fem. adj. *iowensis* after the state of Iowa, from where the type strain was isolated).

Initial growth on sporulating agar at 29 °C is as a whitish mycelium followed by whitish aerial hyphae after incubation for 5 days. Cells are Gram-positive branching rods that are acid-fast. Moderate growth occurs on yeast extract, asparagine glucose, Benedict's, Bennett's potato starch and Weinstein agars; light growth occurs on Hickey and Tresner's, tomato paste and oatmeal agars; and only trace growth occurs on inorganic salts-starch, Kustner's oatflake, Czapek's solution and rice agars. No growth occurs with L-rhamnose, adonitol, L-arabinose, melibiose or D-xylose. Predominant fatty acids are C_{16:0}, C_{18:1}ω9c and C_{18:0} 10-methyl, plus a mixture of C_{16:1}ω6c and C_{16:1}ω7c. The major menaquinone is cyclo MK-8(H₄). Polar lipids present are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannoside. No soluble pigments are produced; no liquefaction of gelatin. Nitrates are reduced to nitrites in 7 days. Can tolerate >4 but <7 % NaCl in yeast extract agar. Catalase-positive. Hydrolyses aesculin, urea and starch. Degrades xanthine, tyrosine, hypoxanthine and uric acid but not casein. Optimum temperature for growth is 29 °C. Utilization of carbon sources and additional phenotypic properties are given in Table 2.

The type strain, NRRL 5646^T (=UI 122540^T=NRRL B-24671^T=DSM 45197^T), was isolated from soil in Osceola, Iowa, USA.

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Table 2. Differential physiological characteristics of strain NRRL 5646^T and the type strains of *Nocardia* species grouped in the same cluster on the basis of 16S rRNA gene sequence analysis

Strains: 1, NRRL 5646^T (*N. iowensis* sp. nov.); 2, *N. tenerifensis* DSM 44704^T; 3, *N. brasiliensis* DSM 43758^T; 4, *N. beijingensis* DSM 44636^T; 5, *N. takedensis* DSM 44801^T. +, Positive; (+), weakly positive; –, negative; ND, not determined. Data for reference strains were taken from Kämpfer *et al.* (2004), Wang *et al.* (2001) and Yamamura *et al.* (2005). Data for growth on carbon sources for strain NRRL 5646^T were taken from Martin *et al.* (1977) and Hlavka & Bitha (1977).

Characteristic	1	2	3	4	5
Aesculin hydrolysis	+	+	+	+	–
Decomposition of:					
Casein	–	+	+	–	–
Tyrosine	+	+	+	–	–
Xanthine	+	–	–	+	–
Hypoxanthine	+	+	+	–	–
Uric acid	+	ND	–	–	+
Growth on sole carbon sources					
<i>i</i> -Inositol	+	+	+	–	ND
D-Mannitol	–	+	(+)	(+)	–
Trehalose	+	+	+	–	–
D-Fructose	(+)	–	–	–	–
DL-Lactic acid	–	–	–	–	ND
D-Glucose	+	+	+	+	+
Maltose	(+)	–	–	–	ND
Sucrose	–	–	–	–	+
Salicin	+	–	–	–	ND

necessarily reflect the views of the US Department of Agriculture. In addition, this research was supported in part by the Intramural Research Program of the NIH Warren G. Magnuson Clinical Center. The views expressed herein are those of the authors and should not be construed as those of the US Department of Health and Human Services.

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