Nocardia coubleae sp. nov., isolated from oil-contaminated Kuwaiti soil

Verónica Rodríguez-Nava,^{1,2,3,4} Z. U. Khan,⁵ Gabriele Pötter,⁶ Reiner M. Kroppenstedt,⁶ Patrick Boiron^{1,2,3,4} and Frédéric Laurent^{1,2,3,4}

Correspondence

Verónica Rodríguez-Nava
veronica.Rodriguez@sante.
univ-lyon1.fr

¹Research group on 'Bacterial Opportunistic Pathogens and Environment', Université de Lyon, F-69003 Lyon, France

²Université Lyon 1 and CNRS, UMR 5557, Ecologie Microbienne, F-69622 Villeurbanne, France

³Institut des Sciences Pharmaceutiques et Biologiques, F-69373 Lyon, France

⁴Observatoire Français des Nocardioses, F-69373 Lyon, France

⁵Department of Microbiology, Faculty of Medicine, Kuwait University, Kuwait

⁶Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany

Two bacterial isolates from Kuwaiti soil contaminated by crude oil were analysed by using a polyphasic taxonomic approach. The isolates, designated OFN N11 and OFN N12^T, were shown to have molecular, chemical and morphological properties typical of members of the genus *Nocardia*. Based on a multigenic approach that included 16S rRNA, *hsp65* and *sod* gene sequencing, these novel isolates formed a monophyletic clade within the genus *Nocardia*. The closest species was *Nocardia ignorata* (with 99.4 %, 99.5 %, 98.6 % gene sequence similarity to the 16S rRNA, *hsp65* and *sod* genes, respectively). The novel isolates could be distinguished phenotypically from the type strains of recognized species of the genus *Nocardia*. The novel isolates were not related to the type strain of *N. ignorata* in DNA–DNA hybridization experiments (26 % relatedness). On the basis of these genotypic and phenotypic data, the two isolates appear to represent a novel species, for which the name *Nocardia coubleae* sp. nov. is proposed. The type strain is OFN N12^T (= DSM 44960^T = CIP 108996^T).

Bacteria belonging to the genus *Nocardia* were first isolated in 1888 by the French veterinarian Edmond Nocard (Nocard, 1888). This genus is a member of the family *Nocardiaceae* and the order *Actinomycetales* (Stackebrandt *et al.*, 1997). During the last ten years, with the advent of molecular phylogenetic techniques, the genus *Nocardia* has undergone a taxonomic revolution (Roth *et al.*, 2003). At the time of writing, 40 species have so far been isolated from human and animal samples and 23 species have been isolated from environmental samples (<http://www.bacterio.cict.fr/n/nocardia.html>). During a taxonomic re-evaluation of environmental and human strains deposited in the culture collection of the French Nocardiosis Observatory

(OFN, Lyon, France), we comprehensively investigated the taxonomic position of two selected strains, OFN N11 and OFN N12^T. An integrated approach was used based on molecular, chemotaxonomic and phenotypic methods.

The two novel strains were isolated in 1997 from soil from the Ahmadi oilfield which had been contaminated with crude oil during the Gulf war. The paraffin bait technique used to isolate these micro-organisms was first described by Kurup & Schmitt (1971). The novel strains were initially identified as *Nocardia asteroides* by using basic physiological methods (Khan *et al.*, 1997). Following this initial identification, the isolates were sent to the OFN by the Microbiology Department of Kuwait University for accurate identification. The strains were maintained as glycerol suspensions (20 %, v/v) at –20 °C. Growth was achieved on Bennett's agar at 37 °C. On this agar, both isolates were able to grow at temperatures between 25 and 37 °C. The orange-coloured substrate mycelium was well-developed, with irregular branches penetrating the agar and bearing sparse white aerial hyphae. The cells were Gram-positive and acid-fast as determined by a modified Ziehl–Neelsen method (1 % acid decoloration).

Abbreviation: OFN, French Nocardiosis Laboratory.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *hsp65* and *sod* gene sequences of strains N11 and N12^T are DQ250024, DQ235689, DQ25025, DQ235688, DQ683744 and DQ683745, respectively.

Additional phylogenetic trees based on analysis of the 16S rRNA, *hsp65* and *sod* gene sequences are available as supplementary figures with the online version of this paper.

Several molecular methods were used to characterize the two isolates. Their affiliation to the genus *Nocardia* was confirmed by genus-specific 16S rRNA gene amplification (Laurent *et al.*, 1999). *Hsp65* polymorphism analysis (Steingrube *et al.*, 1997) yielded a restriction pattern (*Bst*EII 320 and 80 bp, *Msp*I 130, 120, 115 and 70 bp and *Hinf*I 250 and 190 bp) that was identical to that found for *Nocardia ignorata* (Rodríguez-Nava *et al.*, 2005).

A multigene sequencing approach was then applied. Part of the 16S rRNA gene (around 1322 nucleotides, corresponding to positions 46–1400 of the *Escherichia coli* numbering system) of strains OFN N11 and OFN N12^T was amplified by using primers SQ1 (5'-AGAGTTTGATCMTGGCTC-AG-3') and SQ6 (5'-CGGTGTGTACAAGGCC-3') as described by Rodríguez-Nava *et al.* (2004). A partial *hsp65* gene sequence (65 kDa heat-shock protein, 441 nucleotides corresponding to positions 396–836 of *Mycobacterium tuberculosis* CIP 105795) was amplified by using primers TB11 (5'-ACCAACGATGGTGTGTCCAT-3') and TB12 (5'-CTTGTCGAACCGCATACCCT-3') as described by Rodríguez-Nava *et al.* (2006) and a partial *sod* gene sequence (superoxide dismutase, 442 nucleotides corresponding to positions 82–523 of *Nocardia farcinica* IFM 10152) was obtained with primers Z205 (5'-ACGTTCCACCACAGCA-AGCACCA-3') and Z212 (5'-TCGGCCAGTTCACGAC-GTT-3') for the two test strains and for ten *Nocardia* type strains with similar 16S rRNA genes (*N. ignorata*, *Nocardia soli*, *Nocardia cummidelens*, *Nocardia salmonicida*, *Nocardia fluminea*, *N. asteroides*, *Nocardia brasiliensis*, *Nocardia uniformis*, *Nocardia cyriacigeorgica* and *Nocardia tenerifensis*) as described by Devulder *et al.* (2005).

The PCR products were purified by using a Microspin Gel Extraction kit (Omega, Bio-tek) and sequenced on both strands by using the same primers, the *Taq* Dye Deoxy terminator cycle sequencing kit (Applied Biosystems) and a DNA sequencer (model 373A; Applied Biosystems). For phylogenetic analysis, the nucleotide sequences (excluding the primers) were aligned with the corresponding sequences of representative *Nocardia* species from the GenBank

database and the BIBI database (Devulder *et al.*, 2003) by using CLUSTAL_X (Thompson *et al.*, 1997).

The phylogenetic trees were constructed with the MEGA (Kumar *et al.*, 2004) and PHYLO_WIN (Galtier *et al.*, 1996) software packages with three treeing algorithms, namely the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) methods. The tree topology was determined by using 1000 bootstrapped datasets.

Strains OFN N12^T and OFN N11 shared identical sequences for all three of the genes examined. The closest relative of strains OFN N12^T and OFN N11 was *N. ignorata* DSM 44496^T, showing 99.4 % sequence similarity (8 nucleotide differences out of 1322 nt) for the 16S rRNA gene (Fig. 1 and Supplementary Fig. S1 in IJSEM Online), 99.5 % sequence similarity (2 differences out of 401 nt, excluding the primers) for the *hsp65* gene (see Supplementary Fig. S2 in IJSEM Online) and 98.5 % sequence similarity (6 differences out of 406 nt, excluding the primers) for the *sod* gene (see Supplementary Fig. S3 in IJSEM Online). Smaller sequence similarities were found with the other species of the genus *Nocardia*. On the basis of the 16S rRNA and *hsp65* gene sequences, phylogenetic analysis showed that the two novel isolates matched the *N. salmonicida* subclade (*N. cummidelens*, *N. fluminea*, *N. ignorata*, *N. salmonicida* and *N. soli*) (Maldonado *et al.*, 2000). In contrast, this specific subclade was not recognized when the *sod* gene sequences were analysed (see Supplementary Fig. S2). Regardless of which of the three genes was considered, *N. ignorata* was the closest species phylogenetically with bootstrap values of 98, 100 and 100, respectively, for the 16S rRNA, *hsp65* and *sod* genes (See Supplementary Figs S1–S3). These results demonstrate that strains OFN N12^T and OFN N11 form a distinct phyletic line within the genus *Nocardia*.

Strain OFN N12^T was characterized chemotaxonomically and the results supported its assignment to the genus *Nocardia*. Cell-wall composition was investigated using TLC as described by Boiron *et al.* (1993). For strain OFN N12^T, the cell-wall peptidoglycan contained *meso*-diaminopimelic

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequences showing the relationships between *N. coubleae* sp. nov. strains OFN N12^T and OFN N11 and species belonging to the genus *Nocardia*. The tree was constructed by using the neighbour-joining method and was based on a comparison of 1322 nucleotides. Bootstrap values were expressed as a percentage of 1000 replications. Bar, 0.005 substitutions per nucleotide position. An extended version of this tree including a wider selection of reference strains is available as a supplementary figure (see Supplementary Fig. S1 available in IJSEM Online).

acid as the diagnostic diamino acid. Analysis of whole-cell hydrolysates yielded arabinose, galactose, glucose and ribose.

Standard analyses of fatty acids, mycolic acids and isoprenoid quinones were performed by means of HPLC and were interpreted as described by Kroppenstedt with the standard Microbial Identification System (MIDI) for automated GC analysis (Kroppenstedt, 1982, 1985; Minnikin *et al.*, 1975, 1984). The analyses revealed the presence of mycolic acids with a chain length of 52–58 carbon atoms (the principal mycolic acids have a chain length of 56 carbon atoms) as expected for members of the genus *Nocardia* (C50–C62). The fatty acid content was C_{18:0} (16.0 %), C_{18:1} (6.5 %), C_{17:0} (3.8 %), C_{15:0} (1.7 %), C_{17:1} (1.5 %), C_{14:0} (1.1 %), C_{15:1} (0.3 %) and C_{10:0} (0.1 %) and the main fatty acids were palmitic acid (C_{16:0}, 27.2 %), palmitoleic acid (C_{16:1}, 20.22 %) and tuberculostearic acid (10 methyl-branched C_{18:0}, 19.8 %). The main menaquinone (74.2 %) was MK-8(H_{4cycl}) and small amounts of MK-8(H₂) (5.6 %) and MK-8(H₄) (8 %) were also detected.

The two novel isolates were also characterized phenotypically. The methods used for biochemical tests have been

described previously (Boiron *et al.*, 1993; Goodfellow, 1992, 1998). The ability to grow on various carbon sources was tested according to the methods of Goodfellow & Lechevalier, 1989 and Boiron *et al.*, 1993 and by using the API 50 CH (bioMérieux) system according to the manufacturer's instructions. The API 50 CH strips were utilized for only the type strain of *N. ignorata* and strains OFN N11 and OFN N12^T. Inocula were standardized to obtain a final concentration of 1 × 10⁴–1 × 10⁵ c.f.u. and were then suspended in API 50 CHB broth (bioMérieux). Tests were read daily, with a final reading taken at 4 days. Incubation beyond 4 days gave weak reactions in many wells that were interpreted as non-specific. Only the assimilation results were considered for the API 50 CH system as oxidation and fermentation reactions were weak and difficult to interpret. Sugar assimilations in the API 50 CH system were read as positive when heavy growth in the wells obscured the underlying stripes. These results showed that strains OFN N11 and OFN N12^T could be distinguished from the type strain of the closest phylogenetic species, *N. ignorata*, by their growth on media containing glycerol, raffinose and sucrose as the sole carbon sources by using conventional methods and the API 50CH system. Some other biochemical

Table 1. Physiological characteristics of strains OFN N12^T and OFN N11

Strains/species: 1, strain OFN N12^T; 2, strain OFN N11; 3, *N. ignorata* DSM 44496^T; 4, *N. soli* DSM 44488^T; 5, *N. cummideiens* DSM 44490^T; 6, *N. salmonicida* JCM 4826^T; 7, *N. fluminea* DSM 44489^T; 8, *N. asteroides* ATCC 19247^T; 9, *N. farcinica* DSM 43578^T; 10, *N. brasiliensis* ATCC 19296^T; 11, *N. pseudobrasiliensis* DSM 44290^T. Data are taken from Isik *et al.* (1999). All strains were positive in tests for growth on D-glucose (1.0 %, w/v) and for growth on Bennett's agar at 25 °C. –, Negative; +, positive; w, weakly positive; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Growth on carbon sources (% w/v):											
L-Arabinose (1.0)	–	–	–	–	–	ND	ND	–	–	+	+
D-Fructose (1.0)	+	+	+	+	+	ND	ND	+	+	+	+
D-Galactose (1.0)	+	w	w	–	–	+	ND	–	+	+	+
Glycerol (1.0)	+	+	–	ND	ND	ND	ND	ND	ND	ND	ND
Maltose (1.0)	+	+	+	–	–	–	ND	–	+	+	+
D-Mannitol (1.0)	+	+	w	–	–	ND	–	–	–	w	+
Mannose (1.0)	+	+	+	+	+	ND	ND	+	+	+	+
Raffinose (1.0)	+	+	–	–	–	+	ND	–	–	–	–
L-Rhamnose (1.0)	–	–	–	–	+	–	+	–	+	–	–
D-Ribose (1.0)	+	+	+	+	+	ND	ND	+	–	+	+
Sucrose (1.0)	+	+	–	+	–	ND	ND	+	+	+	+
Sorbitol (1.0)	–	–	–	–	–	+	–	–	–	–	–
Growth on Bennett's agar at:											
37 °C	+	+	+	+	+	+	ND	+	+	+	+
45 °C	–	+	–	–	–	–	–	–	+	–	–
Decomposition of (% w/v):											
Adenine (0.4)	–	–	–	–	–	–	–	–	–	–	+
Casein (1.0)	–	–	–	–	–	–	–	–	–	–	+
Hypoxanthine (0.4)	–	–	–	–	–	–	–	–	–	+	+
Testosterone (0.1)	+	+	+	–	–	+	+	+	–	–	–
Tyrosine (0.5)	–	–	–	–	–	+	+	–	–	+	+
Uric acid (0.5)	–	–	–	–	+	–	–	–	–	–	–

properties useful for identifying and differentiating strain OFN N12^T and the type strains of other species of the genus *Nocardia* are presented in Table 1 and the species description.

To definitively establish the taxonomic status of the novel strains, the genetic relatedness of strain OFN N12^T to *N. ignorata* DSM 44496^T (taxonomically the closest species based on 16S rRNA sequence similarity) was determined by DNA–DNA hybridization on nylon membranes as described by De Ley *et al.* (1970), with modifications as described by Huß *et al.* (1983) and Escara & Hutton (1980). DNA was isolated by using a French pressure cell and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). Renaturation rates were computed with the TRANSFER.BAS program (Jahnke & Bahnweg, 1986; Jahnke, 1992). The DNA–DNA relatedness of strain OFN N12^T and *N. ignorata* DSM 44496^T was low (26 %) and well below the 70 % cut-off for species delineation as recommended by Wayne *et al.* (1987), confirming that strain OFN N12^T and *N. ignorata* are not related at the species level.

On the basis of the genotypic and phenotypic data described above, it is clear that strains OFN N11 and OFN N12^T represent a novel *Nocardia* species. The name *Nocardia coubleae* sp. nov. is proposed and isolate OFN N12^T (= CIP 108996^T = DSM 44960^T) is designated as the type strain.

The results obtained in this study confirm the utility of this multigenic approach (16S rRNA, *hsp65*, and *sod* gene sequencing) for the description of *Nocardia coubleae* sp. nov. and for the description of other novel species belonging to the genus *Nocardia* in the future.

Description of *Nocardia coubleae* sp. nov.

Nocardia coubleae (cou'ble.ae. N.L. gen. n. *coubleae* of Couble, named after Andrée Couble, in recognition of her contribution to the French Nocardiosis Observatory, Lyon, France).

Cells are Gram-positive, acid–alcohol-fast and strictly aerobic. Colonies on Bennett's agar are rough, 1–2 mm in diameter and include an orange to white substrate mycelium bearing sparse to abundant white aerial hyphae. Grows at 25 °C and 37 °C (optimum at 37 °C) and is able to utilize D-glucose, glycerol, raffinose and sucrose as sole carbon sources. Testosterone is decomposed, but adenine, casein, hypoxanthine, tyrosine, uric acid and xanthine are not decomposed. The most abundant fatty acids are palmitic acid (C_{16:0}), 27.2 % and tuberculostearic acid, 19.8 %. Mycolic acids are 52–58 carbon atoms long. The cell-wall chemotype is IV. The main menaquinone is MK-8(H_{4cycl}).

The type strain, strain OFN N12^T (= DSM 44960^T = CIP 108996^T), was isolated from Kuwaiti soil.

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