Nocardia niwae sp. nov., isolated from human pulmonary sources

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Members of the genus *Nocardia* are responsible for cutaneous, pulmonary and disseminated human infections. From 2003 to 2008, four nocardioform strains (W8027, W8681, W9071 and W9241^T) were isolated from patients in the state of Florida, USA. Ribosomal gene sequencing analysis suggested that a novel species of the genus *Nocardia* had been isolated. These strains were subjected to a taxonomic analysis using a polyphasic approach. Phenotypic analyses included morphological examination, biochemical profiling and antimicrobial susceptibility testing. Molecular studies included 16S rRNA and DNA gyrase B subunit (*gyrB*) gene sequence analyses and DNA–DNA hybridization. Phylogenetic neighbours were determined through 16S rRNA and *gyrB* gene sequence analyses. Phenotypic characteristics that differentiated the novel isolates from phylogenetically related species were growth at 45 °C, and three of the four novel strains utilized L-rhamnose. The antimicrobial profiles could not reliably distinguish the novel species from related nocardiae. Analysis showed that the 16S rRNA gene sequences of the four novel isolates were identical. The BLAST analysis of the near full-length 16S rRNA gene showed 99.2 % sequence similarity to *Nocardia araoensis* DSM 44729^T, *Nocardia arthritidis* DSM 44731^T and *Nocardia beijingensis* JCM 10666^T, 98.7 % to *Nocardia amamiensis* DSM 45066^T, 98.2 % to *Nocardia pneumoniae* JCM 12119^T and 97.8 % to *Nocardia takedensis* JCM 13313^T. Analysis of partial *gyrB* gene sequences showed that the novel isolates had 95.4 % similarity to *N. arthritidis* DSM 44731^T, 95.3 % to *Nocardia gamkensis* DSM 44956^T, 94.4 % to *N. pneumoniae* JCM 12119^T, 93.8 % to *Nocardia asiatica* DSM 44668^T, 93.5 % to *N. amamiensis* DSM 45066^T, 93.4 % to *N. beijingensis* JCM 10666^T and 93.2 % to *N. araoensis* DSM 44729^T. The DNA–DNA relatedness values between the four novel strains were 86–89 %; the relatedness value for strain W9241^T compared with *N. beijingensis* JCM 10666^T was 47 % and 46 % with *N. araoensis* DSM 44729^T, 44 % with *N. arthritidis* DSM 44731^T, 32 % with *N. amamiensis* DSM 45066^T and 20 % with *N. asiatica* DSM 44668^T. The results of the taxonomic analysis suggested that the new isolates represent a novel species of the genus *Nocardia* for which the name *Nocardia niwae* sp. nov. is proposed. The type strain is W9241^T (=DSM 45340^T=CCUG 57756^T).

Species of the genus *Nocardia* are ubiquitous environmental pathogens that cause a variety of different

disease manifestations in humans and animals. Members of the genus *Nocardia* have been identified as the source of non-specific pulmonary, extrapulmonary, cutaneous and catheter-related infections in humans (Brown-Elliott *et al.*, 2006). Since members of the genus *Nocardia* do not present definitive clinical manifestations, nocardial infections associated with humans and animals are difficult to diagnose (Gordon *et al.*, 1974; Boiron *et al.*, 1992; Goodfellow, 1998) and *Nocardia* species are challenging to identify phenotypically (Conville & Witebsky, 2007). However, during the last ten years, with the advent of molecular techniques, the genus *Nocardia* has undergone

Abbreviation: MIC, minimum inhibitory concentrations.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences for *Nocardia niwae* sp. nov. isolates W8027, W8681, W9071 and W9241^T are FJ765053, FJ765054, FJ765055 and FJ765056, respectively. The accession numbers for the *gyrB* gene sequences for *N. niwae* isolates W8027, W8681, W9071 and W9241^T are FJ765057, FJ765058, FJ765059 and FJ765060, respectively.

Supplementary figures and supplementary tables are available with the online version of this paper.

many taxonomic changes (Roth *et al.*, 2003). Current methods of classification of species of the genus *Nocardia* investigate not only morphological, biochemical and chemotaxonomic characteristics, but also include phylogenetic analyses and DNA–DNA hybridization (Stackebrandt *et al.*, 2002).

In this paper, the morphological, biochemical and chemotaxonomic characteristics along with analyses of the 16S rRNA and *gyrB* gene sequences and DNA–DNA hybridization are reported for four isolated strains of the genus *Nocardia*. As part of a taxonomic analysis based on a polyphasic approach, these four strains were compared by 16S rRNA gene sequence analysis with neighbouring reference strains of the genus *Nocardia* in order to characterize a novel species.

From 2003 to 2008, four clinical strains were sent to the Special Bacteriology Reference Laboratory (SBRL) at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, USA. The four strains examined (W8027, W8681, W9071 and W9241^T) were isolated from clinical pulmonary sources and were initially identified as a putative novel species of the genus *Nocardia* through 16S rRNA gene sequence analysis. Strains W8681 and W9071 were isolated from bronchial washings of a 37-year-old female and a 92-year-old female, respectively. The remaining two strains, W8027 and W9241^T, were isolated from a pleural mass aspirate of a 72-year-old female and a lung biopsy of a 60-year-old male, respectively. All four strains originated from individuals residing in the state of Florida, USA.

The morphological features of these *Nocardia* strains were studied from aerobic growth on heart infusion agar with rabbit blood (BBL) and Middlebrook 7H11 agar slants (Remel) for 7 days at 35 °C (Brown-Elliott *et al.*, 2006). The organisms were aerobic, Gram-positive and weakly acid-fast by modified Kinyoun stain (Berd, 1973). They formed colonies with white aerial and substrate hyphae on rabbit blood media and orange–coral-coloured hyphae on Middlebrook 7H11 media after 7 days at 35 °C; the reverse side was also orange–coral-coloured on Middlebrook agar. The phenotypic characteristics of the strain designated as the type strain, W9241^T, are given in the species description and were determined using methods described previously by Berd (1973).

Tests for the utilization of 22 carbohydrates, production of arylsulfatase, decomposition of adenine, aesculin, casein, hypoxanthine, tyrosine and xanthine, utilization of acetamide, citrate and urea, nitrate reduction, growth in lysozyme and growth at 25, 35 and 45 °C were conducted according to previously described methods (Berd, 1973; Conville & Witebsky, 2007). The phenotypic characteristics that could be used to differentiate strains W8027, W8681, W9071 and W9241^T and the type strains of phylogenetically related species of the genus *Nocardia* are presented in Table 1.

The minimum inhibitory concentrations (MIC) for ten antimicrobial agents: amikacin, amoxicillin/clavulanate,

Table 1. Differential phenotypic characteristics of strains W8027, W8681, W9071, W9241^T and some phylogenetically related type strains

Strains: 1, W8027; 2, W8681; 3, W9071; 4, W9241^T; 5, *N. amamiensis* DSM 45066^T; 6, *N. arthritidis* DSM 44731^T; 7, *N. asiatica* DSM 44668^T; 8, *N. beijingensis* JCM 10666^T. All strains utilized citrate, D-glucose, glycerol and trehalose and were resistant to lysozyme. Strains did not utilize adonitol, L-arabinose, *i*-erythritol, D-galactose, lactose, D-mannitol, D-sorbitol or D-xylose. All strains grew at 25 and 35 °C and were unable to hydrolyse adenine, hypoxanthine, tyrosine and xanthine. All strains lacked arylsulfatase activity. –, Negative; +, positive.

Characteristic	1	2	3	4	5	6	7	8
Utilization as carbon source:								
Cellobiose	–	–	–	–	–	+	–	–
Dulcitol	–	–	–	–	–	–	+	–
D-Fructose	–	–	–	–	+	+	–	+
<i>myo</i> -Inositol	–	–	–	–	+	+	–	–
Maltose	–	–	–	+	+	+	+	+
Mannose	–	–	–	–	+	+	–	–
Melibiose	–	–	–	–	–	+	–	–
Raffinose	–	–	–	–	–	+	–	–
L-Rhamnose	+	+	–	+	–	–	+	–
Salicin	–	–	–	–	–	–	–	–
Sucrose	+	+	–	+	–	+	–	+
Nitrate reduction	+	+	+	+	–	–	+	+
Utilization as carbon and nitrogen source:								
Acetamide	–	–	–	–	–	–	–	+
Hydrolysis of:								
Aesculin	–	–	–	–	–	–	+	–
Urea	+	+	–	+	+	+	+	–
Growth on heart infusion agar at 45 °C	+	+	+	+	–	+	–	–

ceftriaxone, ciprofloxacin, clarithromycin, imipenem, linezolid, minocycline, tigecycline and trimethoprim/sulfamethoxazole were determined using panels from PML Microbiologicals, Inc. following guidelines and breakpoints established for members of the genus *Nocardia* (NCCLS, 2003). The results of the antimicrobial susceptibility testing indicated that the four clinical strains were susceptible to amikacin, ceftriaxone, clarithromycin, linezolid, minocycline and trimethoprim/sulfamethoxazole. All strains showed resistance to amoxicillin/clavulanate and varied in their resistance to ciprofloxacin. The results for the four novel strains and the type strains of related species are presented in Supplementary Table S1 (available in IJSEM Online).

Analyses of diaminopimelic acid isomers were performed according to the methods described by Rhuland *et al.* (1955). Following the methods of Minnikin *et al.* (1984), a small-scale integrated procedure was used to extract and purify quinones and polar lipids. A 200 µl aliquot of 2-propanol was used to dissolve the preparations and 1–10 µl aliquots, without further purification, were separated

through HPLC and analysed as described by Kroppenstedt (1982, 1985). Extracted polar lipids were examined through two-dimensional TLC and identified according to Minnikin *et al.* (1977). The Microbial Identification System (MIDI Inc.) for GC analyses (Sasser, 1990) was utilized for the analysis of fatty acid methyl esters and mycolic acid trimethylsilyl esters prepared as described by Klatte *et al.* (1994). The properties of the novel strains were chemotaxonically consistent with those expected for members of the genus *Nocardia* (Kämpfer *et al.*, 2004). The fatty acid patterns of the novel strains are presented in Supplementary Table S2 (see IJSEM Online). Whole cell hydrolysates of strain W9241^T contained *meso*-diaminopimelic acid as the diagnostic amino acid of the cell-wall peptidoglycan. The major cell-wall sugars were arabinose, galactose, glucose and ribose; mannose, xylose and rhamnose were not present. MK-8 (H_{4cyc}) was the major quinone for this strain. The polar lipids present were phosphatidylinositol mannoside, phosphatidylinositol, phosphatidylethanolamine and diphosphatidylglycerol. These corresponded with lipids reported previously for members of the genus *Nocardia* by Minnikin *et al.* (1977). The fatty acid patterns of the four novel strains were of straight chain saturated and unsaturated fatty acids plus tuberculostearic acid. Mono- and di-unsaturated mycolic acids ranged from 50–60 carbon atoms, as consistent with chain length expected for members of the genus *Nocardia* (Klatte *et al.*, 1994; Baba *et al.*, 1997). The G + C content of the DNA of strain W9241^T was determined by HPLC as described by Mesbah *et al.* (1989). The G + C value of 68.5 mol% was within the range of the DNA base composition previously reported for members of the genus *Nocardia* (Goodfellow & Maldonado, 2006).

Almost complete 16S rRNA gene sequences (1441 bp) were determined for the four novel strains and amplification of the 16S rRNA gene was performed as described by Morey *et al.* (2006) except for the modification of primers FL1

(5'-CCGAATTCGTCGACA) and RL1 (5'-CCCGGGATC-CAAGCT). BLAST software (nucleotide–nucleotide BLAST: <https://www.ncbi.nlm.nih.gov/BLAST>) was used to identify related sequences in GenBank. Bootstrap analysis with 1000 replicates was performed using MEGA4 software to assess the support for each node of the phylogenetic tree (Tamura *et al.*, 2007). The 16S rRNA gene sequences for the four novel strains were identical. The phylogenetically related neighbours of the novel strains (Fig. 1) were *Nocardia araoensis* DSM 44729^T (99.2 % similarity), *N. arthritidis* DSM 44731^T (99.2 %) and *N. beijingensis* JCM 10666^T (99.2 %), *N. amamiensis* DSM 45066^T (98.7 %), *N. pneumoniae* JCM 12119^T (98.2 %) and *N. takedensis* JCM 13313^T (97.8 %).

DNA extraction, amplification of a ~1245-bp *gyrB* gene fragment, primers and sequencing followed methods previously described by Shen *et al.* (2006). The MEGA4 program was used for phylogenetic assessment (Tamura *et al.*, 2007). The *gyrB* gene sequences for the four novel strains were identical. The closest phylogenetic neighbours were revealed (Fig. 2) as *N. arthritidis* DSM 44731^T (95.4 % similarity), *N. gamkensis* DSM 44956^T (95.3 %), *N. pneumoniae* JCM 12119^T (94.4 %), *N. asiatica* DSM 44668^T (93.8 %), *N. amamiensis* DSM 45066^T (93.5 %), *N. beijingensis* JCM 10666^T (93.4 %) and *N. araoensis* DSM 44729^T (93.2 %).

DNA was extracted from the four novel strains and from five closely related phylogenetic neighbours as described by Loeffelholz & Scholl (1989) and DNA–DNA hybridization studies were performed in the Special Bacteriology Reference Laboratory at CDC following the protocol by Brenner *et al.* (1982). DNA–DNA hybridization studies were performed between DNA samples from strains W8027, W8681, W9071 and W9241^T as well as between the DNA of strain W9241^T and five of the most closely phylogenetically related members of the genus *Nocardia*.

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships of strain W9241^T and a few closely related *Nocardia* species. The tree was constructed using the neighbour-joining method and was based on a comparison of ~1441 nt. The tree was rooted using *Mycobacterium tuberculosis* ATCC 27294^T as the outgroup (not shown). Bootstrap percentages were expressed as percentages of 1000 replicates and only values >50 % are presented. Bar, 0.005 substitutions per nucleotide position. The full tree from which this area is taken is available as Supplementary Fig. S1 in IJSEM Online.

Fig. 2. Phylogenetic tree, based on *gyrB* gene sequences, showing the relationships of strain W9241^T and a few closely related *Nocardia* species. For construction details, see legend of Fig. 1. Bar, 0.01 substitutions per nucleotide position. The full tree from which this area is taken is available as Supplementary Fig. S2 in IJSEM Online.

The mean DNA–DNA relatedness values between strains W8027, W8681, W9071 and W9241^T ranged from 86 to 89 %. The levels of DNA–DNA relatedness between strain W9241^T and *N. beijingensis* JCM 10666^T, *N. araoensis* DSM 44729^T, *N. arthritidis* DSM 44731^T, *N. amamiensis* DSM 45066^T and *N. asiatica* DSM 44668^T were 47 %, 46 %, 44 %, 32 % and 20 %, respectively. These values were below the 70 % cut-off recommended by Wayne *et al.* (1987) for species delineation.

As a result of the polyphasic approach applied to our taxonomic investigation, we found that the four strains isolated from pulmonary sources represented a novel species of the genus *Nocardia*. Phenotypic analysis showed that the new strains showed differential characteristics of growth at 45 °C and L-rhamnose utilization (except for strain W9071) from related species of the genus *Nocardia*. The results of the phylogenetic analysis of 16S rRNA and *gyrB* gene sequences and DNA–DNA hybridization demonstrated that these strains represented a distinct entity for which we propose the name *Nocardia niwae* sp. nov.

Description of *Nocardia niwae* sp. nov.

Nocardia niwae (ni'wa.e. N.L. gen. n. *niwae* of Niwa, named in honour of Dr Hidekazu Niwa, our Japanese colleague; a veterinarian and microbiologist).

Aerobic, Gram-positive, weakly acid-fast, non-motile actinomycete, forming orange–coral-coloured aerial and substrate hyphae on Middlebrook 7H11 media. Utilizes and produces acid from D-glucose, glycerol, L-rhamnose, sucrose and trehalose, but does not utilize adonitol, L-arabinose, cellobiose, dulcitol, i-erythritol, D-fructose, D-galactose, myo-inositol, lactose, maltose, D-mannitol, mannose, melibiose, raffinose, salicin, D-sorbitol or D-xylose. Utilizes citrate as sole carbon source, but not acetamide as a carbon and nitrogen source. Grows in the presence of lysozyme, reduces nitrate, but has no arylsulfatase activity. Hydrolyses urea, but does not hydrolyse adenine, aesculin, casein, hypoxanthine, tyrosine or xanthine. Grows at 25, 35 and 45 °C.

The type strain, W9241^T (=DSM 45340^T=CCUG 57756^T), was isolated from the lung tissue of a 60-year-old male in the state of Florida, USA. The G + C value of the DNA for the type strain is 68.5 mol%.

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