Nocardioides hungaricus sp. nov., isolated from a drinking water supply system

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Three Gram-positive, rod-shaped bacterial strains were isolated from the drinking water supply system of the Hungarian capital, Budapest. Phylogenetic analysis on the basis of 16S rRNA gene sequence comparison revealed that the isolates represented a distinct cluster within the clade of the genus *Nocardioides* and were most closely related to *Nocardioides pyridinolyticus* OS4^T, *Nocardioides aquiterrae* GW-9^T, *Nocardioides sediminis* MSL-01^T and *N. hankookensis* DS-30^T. The peptidoglycan based on LL-2,6-diaminopimelic acid, the major menaquinone MK-8(H₄), the cellular fatty acid profile with iso-C_{16:0} and anteiso-C_{17:0} as predominating components and the DNA G + C content of 71.4 mol% (strain 1RaM5-12^T) were consistent with the affiliation of the isolates to the genus *Nocardioides*. Because of differences in physiological characteristics, matrix-assisted laser-desorption/ionization time-of-flight mass spectra of protein extracts, PvuII RiboPrinter patterns and 96.1 % 16S rRNA gene sequence similarity between strain 1RaM5-12^T and its closest phylogenetic neighbour, *N. pyridinolyticus* OS4^T, a novel species, *Nocardioides hungaricus* sp. nov., is proposed. The type strain is 1RaM5-12^T (=DSM 21673^T =NCAIM 02330^T).

Drinking water systems represent distinct oligotrophic microbiological habitats, where the supplied water and different sites of the pipelines can be colonized by microbes, including bacteria. In these aquatic ecosystems, bacteria adapt to the extreme environment and are able to grow, often forming well-developed biofilms on the internal surfaces of pipelines (Garrett *et al.*, 2008). The drinking water of the Hungarian capital, Budapest, originates from riverbank filtration wells located on the River Danube. It is treated only by chlorination before its supply to users (Homonnay *et al.*, 2008).

The genus *Nocardioides* comprises 47 species at the time of writing, the type strains of which have been isolated from various sources, e.g. aquatic habitats [*Nocardioides aquiterrae* (Yoon *et al.*, 2004), *Nocardioides aquaticus* (Lawson *et al.*, 2000) and *Nocardioides marinus* (Choi *et al.*, 2007)], meadow [*Nocardioides plantarum* (Collins *et al.*, 1994)] and polluted environments [*Nocardioides oleivorans* (Schippers

et al., 2005), *Nocardioides nitrophenolicus* (Yoon *et al.*, 1999), *Nocardioides pyridinolyticus* (Yoon *et al.*, 1997) and *Nocardioides aromaticivorans* (Kubota *et al.*, 2005)]. The recently described species *Nocardioides daphniae* (Tóth *et al.*, 2008b) was isolated from the crustacean *Daphnia cucullata*, *Nocardioides hankookensis* (Yoon *et al.*, 2008), *Nocardioides tritolerans* (Dastager *et al.*, 2008b) and *Nocardioides islandensis* (Dastager *et al.*, 2008a) were isolated from soil and *Nocardioides sediminis* (Dastager *et al.*, 2009) was isolated from sediment.

A water sample of Tahi, Hungary (47° 45' N 19° 05' E), a collecting point of well water in the drinking water supply system of Budapest, was taken in 2007. Strains 1RaM5-12^T, 1RaM5-3 and 1RaM5-15 were isolated on R2A agar (Reasoner & Geldreich, 1985) after a standard spread-plate method and incubation at 28 °C for 1 week. The isolates were maintained on R2A agar. Colony morphology was studied on R2A agar by direct and stereomicroscopic observations of single colonies. Cell size, shape, arrangement and motility of the isolates were studied by native preparations and by Gram-staining according to Claus (1992). Endospore- and acid-fast-staining methods followed Murray *et al.* (1994).

Growth under anaerobic conditions was examined using R2A slant agar cultures incubated for 1 week in an

Abbreviation: MALDI-TOF, matrix-assisted laser-desorption/ionization time-of-flight.

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of strains 1RaM5-12^T, 1RaM5-3 and 1RaM5-15 are AM981198, AM981197 and AM981199, respectively.

A supplementary table and two supplementary figures are available with the online version of this paper.

anaerobic chamber (Forma Scientific) at 28 °C. Oxidase activity was tested by the method of Tarrand & Gröschel (1982). Catalase production and Voges–Proskauer reaction were demonstrated by the methods of Cowan & Steel (1974). Acid production from glucose was studied by the OF test according to Hugh & Leifson (1953). Growth at 4, 20, 28, 37 and 45 °C was determined on R2A agar and growth with 0, 2.5, 5 and 10 % (w/v) NaCl and at pH 4–12 (at intervals of one pH unit) was determined in R2A broth. Urease activity, nitrate reduction, starch hydrolysis, indole production from tryptophan, caseinase, gelatinase and phosphatase activity, hydrolysis of Tween 80 and H₂S production from cysteine were studied according to Smibert & Krieg (1994). Substrate utilization, other physiological properties and enzyme activities were tested using the API 20 NE, API 50 CH and API ZYM systems (bioMérieux), according to manufacturer's instructions.

The cell-wall diamino acid of strain 1RaM5-12^T was determined from whole-cell hydrolysates as described by Hasegawa *et al.* (1983). Isoprenoid quinones were extracted from cells cultivated in liquid Rich medium (Yamada & Komagata, 1972) according to the method of Collins *et al.* (1977) and the profile was analysed by HPLC (HP 9001; Hewlett Packard) (Groth *et al.*, 1997). Cellular fatty acids were extracted from cells cultivated on trypticase soy agar (Difco) at 28 °C for 48 h according to Stead *et al.* (1992) and analysed by gas chromatography (Groth *et al.*, 1996). Summed features were identified thereafter by GC/MS using a Singlequad 320 instrument (Varian). Polar lipids were determined according to the method described by Minnikin *et al.* (1979). Genomic DNA for analysis of the base composition was isolated after disruption of bacterial cells using a French pressure cell (Thermo Spectronic). After purification on hydroxyapatite according to the procedure of Cashion *et al.* (1977), the DNA was degraded to nucleosides using P1 nuclease and bovine intestinal mucosa alkaline phosphatase as described by Mesbah *et al.* (1989). The nucleosides were separated by reversed-phase HPLC (Shimadzu LC 20A) according to the method described by Tamaoka & Komagata (1984). The G+C content of the DNA was calculated from the ratio of deoxyguanosine to thymidine.

Extraction of DNA from strains 1RaM5-12^T, 1RaM5-3 and 1RaM5-15 and PCR-mediated amplification of the 16S rRNA gene were carried out by the method of Rainey *et al.* (1996). The PCR products were purified with a Prep-A-Gene kit (Bio-Rad). Cycle sequencing was performed with a Big Dye terminator cycle sequencing kit, according to the manufacturer's protocol, and sequence analysis was performed using a model 310 Genetic Analyzer (Applied Biosystems). The search for phylogenetic neighbours was initially carried out using BLAST (Altschul *et al.*, 1997) and FASTA (Pearson & Lipman, 1988) and a database of sequences of type strains with validly published prokaryotic names (Chun *et al.*, 2007). Pairwise 16S rRNA gene sequence similarities were calculated using the SimTable analysis of the EzTaxon server (<http://www.eztaxon.org>).

The 16S rRNA gene sequences of strains 1RaM5-12^T, 1RaM5-3 and 1RaM5-15 were aligned against sequences available from the GenBank database (<http://www.ncbi.nlm.nih.gov/>) and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al.*, 2007). Evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1980) and phylogenetic dendrograms were reconstructed using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1992) and maximum-parsimony (Fitch, 1971) methods. Bootstrap analysis was based on 1000 resamplings (Felsenstein, 1985).

Fully automated ribotyping of *Pvu*II-digested DNA of strains 1RaM5-12^T, 1RaM5-3 and 1RaM5-15 was performed with the RiboPrinter system (DuPont Qualicon) as described by Bruce (1996). Matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was carried out as described previously (Tóth *et al.*, 2008a).

The detailed morphological, cultural and physiological characteristics of strains 1RaM5-12^T, 1RaM5-3 and 1RaM5-15 are given in the species description and in Table 1. The chemotaxonomic analyses confirmed that the isolates belonged to the genus *Nocardioide*s in that they had LL-2,6-diaminopimelic acid in their cell walls, the major isoprenoid quinone was MK8(H₄) and the only polar lipid was diphosphatidylglycerol. The fatty acid profiles of strains 1RaM5-12^T, 1RaM5-3 and 1RaM5-15 are given in Supplementary Table S1 (available in IJSEM Online). The fatty acid profiles of the three isolates were similar qualitatively in the major components and were in this respect similar to those reported for *N. pyridinolyticus* and *N. aquiterrae* (Yoon *et al.*, 2004). However, the fatty acid profiles were different from those reported for *N. sediminis* and *N. hankookensis* (Dastager *et al.*, 2009; Yoon *et al.*, 2008) by having remarkably lower amounts of C_{17:1ω8c}, which was most probably because of differences in cultivation and extraction conditions. Other phenotypic characters that differentiate our strains from their closest relatives are given in Table 1. The DNA G+C content of strain 1RaM5-12^T was 71.4 mol%.

The neighbour-joining phylogenetic tree (Fig. 1) based on nearly complete (1462 bp) 16S rRNA gene sequences showed that strains 1RaM5-12^T, 1RaM5-3 and 1RaM5-15 formed a distinct group within the genus *Nocardioide*s. The phylogenetic trees recreated using the minimum-evolution and maximum-parsimony algorithms resulted in similar tree topologies (Fig. 1). The closest relatives of the isolates were *N. pyridinolyticus* OS4^T, *N. aquiterrae* GW-9^T, *N. sediminis* MSL-01^T and *N. hankookensis* DS-30^T: 16S rRNA gene sequence similarities between strain 1RaM5-12^T and these type strains were 96.1, 95.7, 95.4 and 95.4 %, respectively. 16S rRNA gene sequence similarities between the isolates and all other members of the genus *Nocardioide*s were lower than 95.2 %.

The three isolates were shown to be very closely related. 16S rRNA gene sequence similarities with each other were: 99.2 %

Table 1. Differential phenotypic characteristics of strains 1RaM5-12^T, 1RaM5-3 and 1RaM5-15 and their closest phylogenetic neighbours

Strains: 1, *Nocardioides hungaricus* sp. nov. 1RaM5-12^T; 2, *N. hungaricus* sp. nov. 1RaM5-3; 3, *N. hungaricus* sp. nov. 1RaM5-15; 4, *N. pyridinolyticus* OS4^T; 5, *N. aquiterrae* GW-9^T; 6, *N. hankookensis* DS-301^T. Data were taken from Yoon *et al.* (2008) and this study. c, Coccus; r, rod; +, positive; w, weakly positive; –, negative; ND, not determined.

Characteristic	1	2	3	4	5	6
Colony colour	White	White	White	Cream	Cream	White
Cell characteristics						
Shape	R	R	R	R, C	R, C	R
Width (µm)	0.4–0.6	0.5–0.6	0.5–0.6	0.5–0.6	0.8–1.0	0.4–0.8
Length (µm)	0.9–1.7	0.9–1.6	1.0–1.7	1.2–1.6	1.7–2.0	1.5–10.0
Rod–coccus cycle	–	–	–	+	+	–
Motility	–	–	–	+	+	–
Optimal growth conditions						
Temperature (°C)	20–28	20–28	20–28	35	30	25
pH	6–7	6–7	6–7	8	6–7	6–7
NaCl (%)	0–2.5	0–2.5	0–2.5	ND	ND	0–0.5
Oxidase	–	–	–	–	+	+
Nitrate reduction to nitrite	+	+	+	+	+	–
Hugh and Leifson test	–	–	–	–	ND	ND
H ₂ S production	–	–	–	ND	ND	–
Hydrolysis of:						
Aesculin	+	+	+	+	+	ND
Casein	–	–	–	ND	+	ND
Gelatin	+	–	–	+	ND	ND
Tween 80	+	+	+	–	+	W
Starch	–	–	–	+	–	+
Enzyme activities						
Alkaline phosphatase	+	+	+	+	–	+
Esterase (C4)	+	+	+	–	–	+
Lipase (C14)	w	+	+	–	–	–
β-Galactosidase	+	w	+	–	–	–
Valine arylamidase	w	–	–	w	–	–
Cystine arylamidase	–	–	–	+	+	–
Trypsin	–	–	–	+	+	–
α-Glucosidase	+	+	+	+	+	–
β-Glucosidase	+	+	+	–	w	–
DNA G + C content (mol%)	71.4	ND	ND	73	73	71.3

between strains 1RaM5-12^T and 1RaM5-15; 99.6 % between strains 1RaM5-12^T and 1RaM5-3; and 99.7 % between strains 1RaM5-15 and 1RaM5-3. Their close relationship was also confirmed by the RiboPrinter results (Supplementary Fig. S1) and MALDI-TOF analysis (Supplementary Fig. S2) as well as many other phenotypic characteristics (Table 1).

Based on the distinctive genotypic, chemotaxonomic and phenotypic characteristics, strains 1RaM5-12^T, 1RaM5-3 and 1RaM5-15 are considered to represent a novel species within the genus *Nocardioides*, for which the name *Nocardioides hungaricus* sp. nov. is proposed.

Description of *Nocardioides hungaricus* sp. nov.

Nocardioides hungaricus (hun.ga'ri.cus. M.L. masc. adj. *hungaricus* Hungary, the name of the country where the species was first isolated).

Gram-positive (Gram-variable in old cultures), acid-fast, non-endospore-forming bacterium. Cells are non-motile and exhibit aerobic respiratory metabolism. Catalase-positive and oxidase-negative. Cells do not show a rod–coccus cycle from the early exponential phase to the stationary phase. Neither substrate nor primary mycelium is formed. Colonies on R2A agar are white, smooth, circular and convex after 7 days at 28 °C. Cells are 0.4–0.6 × 0.9–1.7 µm after 1 day on R2A agar at 28 °C. Growth occurs at 20–37 °C (optimum 20–28 °C), at pH 5–9 (optimum pH 6–7) and with 0.0–2.5 % NaCl. Glucose is not degraded under oxidative-fermentative conditions. Urease and phosphatase activity, Voges–Proskauer and methyl-red reactions, and indole and H₂S production are negative. Nitrate is reduced to nitrite. With API 50 CH, hydrolyses aesculin; all other test results are negative. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine

Fig. 1. 16S rRNA gene sequence neighbour-joining tree showing the phylogenetic positions of strains 1RaM5-12^T, 1RaM5-3 and 1RaM5-15 among closely related members of the genus *Nocardioideae*. Bootstrap values (>50%) based on 1000 resamplings (Felsenstein, 1985) are shown at branch nodes. Filled circles indicate that the corresponding nodes were recovered in the minimum-evolution and maximum-parsimony trees. Bar, 1 substitution per 100 nucleotide positions.

arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, α -glucosidase and β -glucosidase. The diagnostic diamino acid in the cell-wall peptidoglycan is LL-2,6-diaminopimelic acid. The predominant menaquinone is MK-8(H₄). The major fatty acids (>5%) are iso-C_{16:0} and anteiso-C_{17:0}. The DNA G+C content of the type strain is 71.4 mol%.

The type strain is 1RaM5-12^T (=DSM 21673^T =NCAIM 02330^T), isolated from the drinking water network of Budapest, Hungary.

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