Nocardioides daphniae sp. nov., isolated from *Daphnia cucullata* (Crustacea: Cladocera)

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A Gram-positive, rod-shaped or coccoid, yellow-pigmented bacterial strain, D287^T, was isolated from the water flea *Daphnia cucullata* (Crustacea: Cladocera) collected from Lake Balaton in Hungary. Phylogenetic analysis on the basis of 16S rRNA gene sequence comparisons revealed that the strain represented a distinct lineage within the cluster of the genera *Nocardioides* and *Marmoricola*. The following characteristics were consistent with the affiliation of strain D287^T to the genus *Nocardioides*: peptidoglycan based on LL-2,6-diaminopimelic acid, MK-8(H₄) as the major menaquinone, iso-C_{16:0} as the predominant cellular fatty acid, the presence of phosphatidylglycerol and diphosphatidylglycerol and a DNA G + C content of 69.9 mol%. Owing to characteristic differences in physiological traits and levels of 16S rRNA gene sequence similarity to its phylogenetically closest neighbours that were below 97 %, strain D287^T is considered to represent a novel species of the genus *Nocardioides*, for which the name *Nocardioides daphniae* sp. nov. is proposed. The type strain is D287^T (=DSM 18664^T=CCM 7403^T).

Crustacean species, among them *Daphnia cucullata* (Crustacea: Cladocera) (G.-Tóth, 2000), are the most frequent members of the zooplankton of Lake Balaton (Hungary) and play a key role in the food web and energy circulation of the lake as a result of their filtration-type feeding. A detailed bacteriological investigation was performed with this crustacean species based on the isolation and identification of bacteria associated with it (Homonnay *et al.*, 2006). Among the organisms isolated, an actinobacterium (designated strain D287^T) was detected that could be affiliated to the genus *Nocardioides* on the basis of morphological, physiological, chemotaxonomic and phylogenetic studies. Species of the genus *Nocardioides* have hitherto been isolated from soils, sands or sediments as *Nocardioides albus* (Prauser, 1976), *N. luteus* (Prauser, 1984), *N. simplex* (O'Donnell *et al.*, 1982), *N. jensenii* (Collins *et al.*, 1989), *N. insulae* (Yoon *et al.*, 2007), *N. dubius* (Yoon *et al.*, 2005c), *N. alkalitolerans* (Yoon *et al.*, 2005a), *N. kribbensis* (Yoon *et al.*, 2005b), *N. kongjuensis* (Yoon *et al.*, 2006b), *N. lentus* (Yoon *et al.*, 2006a), *N. furvisabuli* (Lee, 2007), *N. ganghwensis* (Yi & Chun, 2004a) and *N. aestuarii* (Yi & Chun, 2004b), aquatic habitats as *N. aquiterrae* (Yoon *et al.*, 2004), *N. aquaticus* (Lawson *et al.*, 2000) and *N. marinus* (Choi *et al.*, 2007), meadow herbage

as *N. plantarum* (Collins *et al.*, 1994), as well as from polluted environments as *N. oleivorans* (Schipper *et al.*, 2005), *N. nitrophenolicus* (Yoon *et al.*, 1999), *N. pyridinolyticus* (Yoon *et al.*, 1997) and *N. aromaticivorans* (Kubota *et al.*, 2005). However, an association of members of the genus *Nocardioides* with other organisms as described herein for strain D287^T has not previously been reported. The aim of the present work was to determine the taxonomic position of strain D287^T and to describe the novel strain by means of a polyphasic approach.

Strain D287^T was isolated in August 2004 from a whole-body homogenate sample of *Daphnia cucullata* (Crustacea: Cladocera) collected from Lake Balaton at Tihany (46° 55' 20" N 17° 55' 39" E), Hungary: 30 adult individuals of *D. cucullata* were selected under a stereomicroscope (Leica), washed five times with 5 ml sterile water from the lake, homogenized with a sterile glass mortar and used as composite sample. Bacterial strain D287^T was isolated and later maintained on King B agar medium (King *et al.*, 1954).

Colony morphology was studied on King B agar medium by direct and stereomicroscopic observations of single colonies. Cell morphology and motility were studied by native preparations and by Gram staining according to Claus (1992).

Growth under anaerobic conditions was examined by using slant agar cultures on King B medium incubated in an

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain D287^T is AM398438.

anaerobic chamber (Forma Scientific). Oxidase activity was tested according to the method of Tarrand & Gröschel (1982). Catalase production and the Voges–Proskauer reaction were investigated by using the methods of Cowan & Steel (1974). Acid production from glucose was studied by the O/F test according to Hugh & Leifson (1953). Growth at different temperatures (4, 28, 38, 45 °C), NaCl concentration (0, 2.5, 5, 10 %, w/v) and pH (pH 4–12 in steps of 0.5 pH units) were determined by using King B agar or broth. Urease activity, nitrate reduction, starch hydrolysis, indole production from tryptophan, caseinase, gelatinase and phosphatase activities, hydrolysis of Tween 80 and H₂S production from cysteine were studied according to the methods of Smibert & Krieg (1994). Haemolysin production was tested on ox-blood agar plates (Elek & Levy, 1954). Chitinase activity was studied according to a modified version of the method of Holding & Collee (1971). Other enzyme activities were tested by using the API ZYM system (bioMérieux). Utilization of 95 different carbon sources as sole source of carbon was studied on Biolog GP2 microplates by using an ELISA reader (SUNRISE Remote; Tecan). System 3.5 was used to evaluate the results according to the manufacturer's instructions. For comparison, Biolog test results were evaluated with *N. alkalitolerans* DSM 16699^T, *N. dubius* DSM 19084^T and *N. jensenii* DSM 20641^T.

The cell-wall diamino acid of strain D287^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 according to Stead *et al.* (1992) and were analysed by GC (Groth *et al.*, 1996). Polar lipids were determined according to the method described by Minnikin *et al.* (1979). The DNA base composition was determined after disruption of bacterial cells by using a French press. After purification on hydroxyapatite following the procedure of Cashion *et al.* (1977), the DNA was degraded to nucleosides by using P1 nuclease and bovine intestinal mucosa alkaline phosphatase as described by Mesbah *et al.* (1989). The nucleosides were separated by reversed-phase HPLC (HP 9001) according to Tamaoka & Komagata (1984). The G+C content of the DNA was calculated from the ratio of deoxyguanosine to thymidine.

Extraction of genomic DNA and PCR-mediated amplification of the 16S rRNA gene of strain D287^T were carried out by using the methods of Rainey *et al.* (1996). The PCR product was purified by using a Prep-A-Gene kit (Bio-Rad). Cycle sequencing was performed with the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's protocol, and sequence analysis was performed in an Applied Biosystems model 310 Genetic Analyzer.

A BLAST (Altschul *et al.*, 1997) and GenBank database search (<http://www.ncbi.nlm.nih.gov/>) for 16S rRNA gene sequences similar to that of strain D287^T was performed. Pairwise similarity values to the phylogenetically closest type strains of recognized members of the genus *Nocardioides* were calculated by using the SimTable analysis of the EzTaxon server (<http://www.eztaxon.org>). The 16S rRNA gene sequence of strain D287^T was manually aligned by using the BioEdit program (Hall, 1999) against sequences available from EMBL. Evolutionary distances were calculated according to the Kimura two-parameter method (Kimura, 1980) and a phylogenetic dendrogram was constructed by using the neighbour-joining method (Saitou & Nei, 1987) within the CLUSTAL_X software package (Thompson *et al.*, 1997). Bootstrap analysis was based on 1000 resamplings (Felsenstein, 1985).

Morphological, cultural and physiological characteristics of strain D287^T are given in the species description below. Phenotypic characteristics that differentiate strain D287^T from its closest phylogenetic neighbours are given in Table 1.

The 16S rRNA gene sequence of strain D287^T comprised 1333 nt. The phylogenetic tree based on the neighbour-joining algorithm showed that strain D287^T represents a distinct lineage within the cluster formed by members of the genera *Nocardioides* and *Marmoricola* (Fig. 1). In addition, the BioEdit program was used for a maximum-likelihood analysis (Felsenstein, 1981), which resulted in almost the same clustering for closely related species as in the neighbour-joining tree (data not shown). Strain D287^T showed highest 16S rRNA gene sequence similarity to the type strains of *N. dubius* (96.6 %), *N. jensenii* (96.2 %), *N. pyridinolyticus* (96.0 %) and *N. kribbensis* (95.9 %). The 16S rRNA gene sequence of strain D287^T clustered together with those of the type strains of *N. dubius*, *N. jensenii* and *N. alkalitolerans* in a neighbour-joining tree (Fig. 1). The phylogenetically nearest neighbours of strain D287^T were selected on the basis of pairwise similarity calculations and clustering in the phylogenetic tree, and their phenotypic characteristics as compared with the novel strain are given in Table 1.

The peptidoglycan of strain D287^T was based on LL-2,6 diamminopimelic acid. The major menaquinone was MK-8(H₄). Phosphatidylglycerol, diphosphatidylglycerol and one unidentified phospholipid were found as polar lipids. The fatty acid profile (i.e. fatty acids representing >1 % of the total) of strain D287^T consisted of the following components: iso-C_{16:0} (42.71 %), C_{18:1ω9c} (9.88 %), C_{17:0} 10-methyl (9.67 %), iso-C_{17:1ω9c} (8.10 %), iso-C_{17:0} (8.09 %), C_{17:1ω8c} (3.55 %), iso-C_{16:1} (2.63 %), anteiso-C_{17:0} (2.44 %), iso-C_{18:0} (2.35 %), C_{16:1ω7c} (2.19 %), iso-C_{14:0} (1.72 %), iso-C_{15:0} (1.64 %) and C_{18:1ω7c} (1.15 %). This fatty acid profile, with a high proportion of iso- and anteiso-branched chain fatty acids, clearly differentiated strain D287^T from *Marmoricola aurantiacus*, which has a cellular fatty acid pattern with straight-chain saturated and monounsaturated components and 10-methyl octadecanoic (tuberculostearic) acid as the only branched-chain fatty acid

Table 1. Differential phenotypic characteristics of strain D287^T and related *Nocardioides* species

Taxa: 1, strain D287^T; 2, *N. alkalitolerans*; 3, *N. dubius*; 4, *N. jensenii*. Data for reference taxa are from Yoon *et al.* (2005a, c), Yi & Chun (2004a), Suzuki & Komagata (1983) and Collins *et al.* (1989). All carbon source utilization results are from the present study. +, Positive; –, negative; ND, not determined; w, weakly positive; v, variable; data in parentheses are for the type strain.

Characteristic	1	2	3	4
Cell size (µm)	0.8–1.0 × 1.2–2.2	0.8–1.0 × 1.5–2.0	0.8–1.0 × 1.5–2.5	0.6–0.8 × 3.0–7.0
Motility	–	–	+	–
Colony colour	Yellowish	Milky white	Yellowish white	Yellowish white
Optimal temperature for growth (°C)	28–38	25–30	30	ND
Optimal pH for growth	7.5–8.5	7.0–9.0	7.0–8.0	ND
Nitrate reduction	(+)	+	(–)	+
Hydrolysis of:				
Aesculin	(+)	–	(+)	–
Casein	(w)	+	ND	+
Gelatin	(+)	v (+)	(+)	+
Tween 80	(+)	+	(–)	+
Urea	(–)	–	(–)	+
Utilization of:				
D-Cellobiose	(–)	(+)	(–)	(–)
D-Fructose	(+)	(+)	(–)	(–)
D-Glucose	(+)	(+)	(–)	(–)
Maltose	(–)	(–)	(+)	(–)
D-Mannose	(+)	(+)	(–)	(–)
D-Ribose	(+)	(+)	(–)	(–)
Sucrose	(–)	(+)	(–)	(–)
Trehalose	(+)	(+)	(–)	(–)
Tween 40	(–)	(+)	(+)	(+)
Mannan	(–)	(+)	(–)	(–)
Tween 80	(–)	(–)	(+)	(+)
Maltotriose	(–)	(+)	(+)	(–)
3-Methyl glucose	(–)	(+)	(–)	(–)
Palatinose	(+)	(+)	(–)	(–)
D-Psicose	(+)	(+)	(–)	(–)
Pyruvic acid methyl ester	(+)	(–)	(+)	(+)
L-Lactic acid	(+)	(+)	(–)	(+)
Succinic acid monomethyl ester	(–)	(+)	(+)	(–)
Propionic acid	(–)	(+)	(–)	(+)
L-Alaninamide	(–)	(–)	(+)	(+)
L-Alanyl glycine	(–)	(–)	(+)	(–)
Inosine	(+)	(–)	(+)	(+)
API ZYM results:				
Alkaline phosphatase	(w)	+	(+)	(+)
Lipase (C14)	(w)	+	(–)	(–)
Leucine arylamidase	(+)	–	(+)	(+)
Valine arylamidase	(w)	–	–	v
Trypsin	(–)	–	–	w
α-Chymotrypsin	(–)	–	(–)	(–)
Acid phosphatase	(w)	+	+	v
Naphthol-AS-BI-phosphohydrolase	(w)	w	+	v
β-Galactosidase	(–)	–	(–)	(–)
α-Glucosidase	(–)	+	+	v
β-Glucosidase	(–)	–	+	–
Source of isolation	Water flea <i>Daphnia cucullata</i>	Alkaline soil	Alkaline soil	Soil
DNA G + C content (mol%)	69.9	72.4–73.6	70.6	69

Fig. 1. 16S rRNA gene sequence neighbour-joining tree (Saitou & Nei, 1987) showing the phylogenetic position of strain D287^T among members of the genera *Nocardioides* and *Marmoricola*. Bootstrap values (>50%) based on 1000 resamplings (Felsenstein, 1985) are indicated at nodes. *Terrabacter tumescens* KCTC 9133^T served as the root for the tree. Bar, 1 nucleotide substitution per 100 nucleotides.

(Urzi *et al.*, 2000). The G + C value of the genomic DNA of strain D287^T was 69.9 mol%. The chemotaxonomic characteristics of strain D287^T are in accordance with its affiliation to the genus *Nocardioides* (Yoon & Park, 2006).

Strain D287^T showed 16S rRNA gene sequence similarity values below 97% to the type strains of all recognized species of the genus *Nocardioides*, the value generally accepted for the delineation of separate genomic species (Stackebrandt & Goebel, 1994; Stackebrandt & Ebers, 2006). It could additionally be differentiated from its phylogenetically nearest neighbours on the basis of several phenotypic characteristics (Table 1). It is therefore concluded that strain D287^T represents a novel species of the genus *Nocardioides*, for which the name *Nocardioides daphniae* sp. nov. is proposed.

Description of *Nocardioides daphniae* sp. nov.

Nocardioides daphniae (daph'ni.æ. N.L. gen. n. *daphniae* pertaining to the water flea genus *Daphnia*, as the type strain was isolated from *Daphnia cucullata*).

Gram-positive, aerobic, oxidase-negative, catalase-positive bacterium. Cells are non-motile, short rods or coccoids (0.8–1.0 × 1.2–2.2 µm). Colonies on King B agar medium are circular, convex, smooth, glistening and of yellowish

colour. Spores and mycelia are not formed. The temperature range for growth is 4–38 °C, with optimum growth at 28 °C. The pH range for growth is pH 5.5–10.5, with optimum growth between pH 7 and 9. Growth occurs in the presence of 0–5% NaCl. Good growth occurs on King B and nutrient agar. H₂S and indole are not produced. Negative for the Voges–Proskauer and α-glucosidase reactions. Acid is not produced from glucose. Haemolysin is not detected. Positive for production of chitinase. Weakly positive for caseinase. D-Fructose, α-D-glucose, D-mannose, trehalose, D-ribose, methyl β-D-glucoside, palatinose, psicose, sorbitol, stachyose, acetic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketovaleic acid, L-lactic acid, D-malic acid, methyl-pyruvate, glycyl L-glutamic acid, pyruvic acid, 2,3-butanediol, adenosine, 2-deoxyadenosine, inosine, thymidine, uridine, adenosine 5'-monophosphate and uridine 5'-monophosphate are utilized as sole carbon source. α-Cyclodextrin, β-cyclodextrin, dextrin, glycogen, inulin, mannan, Tweens 40 and 80, N-acetylglucosamine, L-arabinose, arabinol, arbutin, D-cellobiose, D-galactose, myo-inositol, maltose, D-melezitose, D-melibiose, D-mannitol, D-raffinose, L-rhamnose, sucrose, D-xylose, L-pyroglyutamic acid, glycerol, D-galacturonic acid, gentiobiose, D-gluconic acid, lactulose, maltotriose, methyl α-D-galactoside, methyl β-D-galactoside, methyl α-D-mannoside, salicin,

sedoheptulosan, tagatose, xylitol, α -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, lactamide, D-lactic acid methyl ester, methyl α -D-glucoside, α -D-lactose, L-malic acid, monomethyl succinate, succinamic acid, succinic acid, N-acetyl-L-glutamic acid, alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-serine, putrescine, N-acetyl-D-mannosamine, amygdalin, fucose, 3-methyl glucose, turanose, α -ketoglutaric acid, propionic acid, L-glutamic acid, thymidine 5'-monophosphate, DL- α -glycerol phosphate, D-glucose 6-phosphate, D-fructose 6-phosphate, and glucose 1-phosphate are not utilized. The diagnostic diamino acid of the peptidoglycan is LL-2,6 diaminopimelic acid. The major menaquinone is MK-8(H₄). Phosphatidylglycerol and diphosphatidylglycerol occur as polar lipids. The predominant cellular fatty acid is iso-C_{16:0}. The DNA G + C content is 69.9 mol%. Other phenotypic characteristics are given in Table 1.

The type strain, D287^T (=DSM 18664^T=CCM 7403^T), was isolated from the water flea *Daphnia cucullata* originating from Lake Balaton in Hungary.

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