Nocardioides fonticola sp. nov., a novel actinomycete isolated from spring water

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A Gram-positive, rod-shaped, yellow-pigmented actinomycete, designated strain NAA-13^T, was isolated from a freshwater spring located in Kaoshiung, southern Taiwan. This strain was characterized using a polyphasic taxonomic approach. The organism had chemotaxonomic and morphological properties consistent with its classification in the genus *Nocardioides*. The strain contained LL-diaminopimelic acid as the principal diamino acid of the peptidoglycan, MK-8(H₄) as the major menaquinone, and iso-C_{16:0} and C_{17:0} as the predominant fatty acids. 16S rRNA gene sequence studies showed that the strain formed a monophyletic branch at the periphery of the evolutionary radiation occupied by the genus *Nocardioides*; its closest neighbours were *Nocardioides insulae* DS-51^T (94.9% similarity), *Nocardioides pyridinolyticus* KCTC 0074BP^T (96.5% similarity) and *Nocardioides aquiterrae* GW-9^T (96.6% similarity). The DNA G+C content of strain NAA-13^T was 71.8 mol%. On the basis of phenotypic, chemotaxonomic and phylogenetic data, strain NAA-13^T represents a novel species of the genus *Nocardioides*, for which the name *Nocardioides fonticola* sp. nov. is proposed. The type strain is NAA-13^T (=LMG 24213^T=BCRC 16874^T).

The genus *Nocardioides* (Prauser, 1976, 1989) belongs to the family *Nocardioideaceae*, a member of the suborder *Propionibacterineae* proposed by Stackebrandt *et al.* (1997). At the time of writing, this genus encompassed 23 species with validly published names (<http://www.bacterio.cict.fr/n/nocardioides.html>). *Nocardioides* species are widely distributed in soil and aquatic habitats, including marine sediments. All of these taxa have been isolated from natural and man-made habitats including soil, herbage, ground-water, river water, industrial wastewater, an oil-shale column, black sand, crude oil, a saline lake and a tidal flat (Yoon *et al.*, 1997, 2004, 2007a, b; Lee, 2007; Choi *et al.*, 2007).

The aim of the present study was to determine the taxonomic position of an actinomycete, strain NAA-13^T, that formed a pale yellow colony on an R2A agar (Difco) plate that had been incubated at 25 °C for 3 days following inoculation with a water sample collected from a

freshwater spring, Kaoshiung County, Taiwan. The organism was the subject of a polyphasic taxonomic study.

Subcultivation of this strain was performed on R2A agar at 25 °C for between 48 and 72 h. The strain was preserved by lyophilization with 20% (w/v) skimmed milk. Bacterial cells were observed by light and phase-contrast microscopy (DM2000; Leica) in the lag, exponential and stationary phases of growth to ascertain their morphology. Cell dimensions were measured with ocular (×10) and objective (×100/1.25 numerical aperture) lenses. Motility of cells was tested by using the hanging-drop method. The Spot Test flagella stain (BD Difco) was used to stain any flagella that might be present. A Gram stain set (BD Difco) and the Ryu non-staining KOH method (Powers, 1995) were used to ascertain the Gram reaction of strain NAA-13^T. Acid-fastness was determined by Ziehl–Neelsen staining (Hendrickson & Krenz, 1991). Poly-β-hydroxybutyrate granule accumulation was observed under light microscopy after staining cells with Sudan black (Smibert & Krieg, 1994). Colony morphology was examined using a stereoscopic microscope (SMZ800; Nikon). The pH range for growth was examined in nutrient broth (BD Difco) with the appropriate biological buffers (pH 4–10, at increments of 0.5 pH units) (Chung *et al.*, 1995). Tolerance to various NaCl concentrations was tested in

Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NAA-13^T is EF626689.

A table showing the cellular fatty acid composition of strain NAA-13^T grown on R2A agar is available with the online version of this paper.

nutrient broth prepared according to the formula of the Difco medium, except that the NaCl concentration was altered as required (0, 0.5 and 1.0–10 %, w/v, at increments of 1.0 %). Growth at various temperatures (4–45 °C) was measured in nutrient broth. Cellular growth was determined by measuring the turbidity (OD₆₀₀) of cultures grown at various pH values, NaCl concentrations and temperatures. Anaerobic cultivation was performed on nutrient or R2A agar using the Oxoid AnaeroGen system.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Chen *et al.*, 2001). Sequence analysis was achieved using a DNA sequencer (ABI Prism 310; Applied Biosystems) and sequences were assembled by using the Fragment Assembly System program from the Wisconsin package version 9.1 (GCG, 1995). An almost-complete 16S rRNA gene sequence (1403 nt) of strain NAA-13^T was compared against 16S rRNA gene sequences available from the Ribosomal Database Project and GenBank. Multiple sequence alignment including strain NAA-13^T and its closest relatives was performed using BioEdit software (Hall, 1999) and MEGA version 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were inferred by using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) algorithms. An evolutionary distance matrix was generated for the neighbour-joining algorithm by using the Jukes & Cantor (1969) distance model; bootstrap analysis for the neighbour-joining tree was performed on the basis of 1000 resamplings. A comparison of the 16S rRNA gene sequence of strain NAA-13^T with those of members of genera listed under the family *Nocardioidaceae* showed that strain NAA-13^T fell within the evolutionary radiation occupied by the genus *Nocardioides* (Fig. 1). Strain NAA-13^T formed a distinct lineage within the genus *Nocardioides*, a phyletic line that was supported by all of the tree-making algorithms. The 16S rRNA sequence similarity between strain NAA-13^T and the type strains of the genus *Nocardioides* ranged from 93.6 to 96.6 %. It is also apparent from Fig. 1 that the isolate was loosely associated with the type strains of *Nocardioides insulae* DS-51^T (94.9 % similarity), *Nocardioides pyridinolyticus* KCTC 0074BP^T (96.5 % similarity) and *Nocardioides aquiterrae* GW-9^T (96.6 % similarity), although these relationships were not supported by a high bootstrap value (75 %). It is clear that the observed divergence values with respect to all recognized *Nocardioides* species (>3 %) are consistent with separate species status (Stackebrandt & Goebel, 1994). Further evidence for the separate species status of strain NAA-13^T comes from phenotypic data.

Chemosystematic studies were carried out to establish whether strain NAA-13^T had a chemical profile that was consistent with its assignment to the genus *Nocardioides*. Biomass of NAA-13^T was obtained after growing the strain on R2A at 25 °C for 3 days. Fatty acid methyl esters were prepared, separated and identified according to the instructions of the Microbial Identification system

(Microbial ID; MIDI) (Sasser, 1990). Purified cell-wall preparations were obtained by the method of Schleifer & Kandler (1972) and the isomer type of the diamino acid in the cell-wall peptidoglycan was analysed using TLC according to the method described by Schleifer (1985). Menaquinones were extracted and analysed by HPLC as described by Collins (1994) along with *N. pyridinolyticus* KCTC 0074BP^T as a reference strain. The DNA G+C content of strain NAA-13^T was estimated in duplicate as described by Mesbah *et al.* (1989). Strain NAA-13^T contained LL-diaminopimelic acid (LL-DAP) as the diagnostic diamino acid in the cell wall. The predominant isoprenoid quinone was a tetrahydrogenated menaquinone with eight isoprene units [MK-8(H₄)] and the isolate possessed straight-chain, branched, unsaturated and 10-methyl fatty acids (Supplementary Table S1, available in IJSEM Online). The major fatty acids were iso-C_{16:0} (19.93 %) and C_{17:0} (19.90 %). All of these properties, including fatty acid composition, are typical of *Nocardioides* species (Yoon *et al.*, 1997, 2004, 2007a, b; Lee, 2007). The DNA G+C content of strain NAA-13^T was 71.8 ± 1.0 mol%, which is in the range reported for *Nocardioides* species (67.0–74.8 mol%) (Yoon *et al.*, 2007a, b; Lee, 2007; Choi *et al.*, 2007).

Standard physiological and biochemical tests were performed as described previously (Smibert & Krieg, 1994). Additional biochemical tests were performed using the API ZYM, API 20NE and API Coryne (all bioMérieux) microtest systems according to the methods outlined by the manufacturer. Utilization of various substrates was also determined by inoculating API 50CH strips with cells suspended in AUX medium (bioMérieux). Hydrolysis of casein, starch, corn oil, and Tweens 20, 40, 60 and 80 was tested using R2A as the basal medium. DNase test agar (BD Difco) was used to assay DNase activity. Sensitivity to antibiotics was examined after spreading cells (0.5 McFarland standard) on R2A agar. The discs contained the following antibiotics: ampicillin (10 µg), ceftizoxime (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), novobiocin (30 µg), rifampicin (5 µg), penicillin G (10 µg), streptomycin (10 µg), tetracycline (30 µg) or sulfamethoxazole (23.75 µg) plus trimethoprim (1.25 µg). The effects of the antibiotics on cell growth were assessed after 3 days incubation and susceptibility was scored on the basis of the distance from the edge of the clear zone to the disc. Detailed phenotypic and biochemical analyses of strain NAA-13^T are provided in Table 1 and in the species description. Phenotypic characteristics that differentiate strain NAA-13^T from its closest neighbours are presented in Table 1.

From the above data, it is evident that isolate NAA-13^T exhibits an overall chemotaxonomic profile that is consistent with those of members of the genus *Nocardioides*. Phylogenetic analysis based on 16S rRNA gene sequencing further confirmed its provisional assignment and clearly demonstrated that isolate NAA-13^T

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences available from the Ribosomal Database Project and NCBI/GenBank (accession numbers are given in parentheses) constructed after multiple alignments of data showing the position of strain NAA-13^T in the genus *Nocardioideae*. Distances and clustering with the neighbour-joining method were determined by using the software package BioEdit. Numbers at the nodes are bootstrap values (%) based on 1000 resampled datasets; only values above 50 % are given. Bar, 0.01 substitutions per nucleotide position.

represents an unknown subline within the genus *Nocardioideae*. Biochemically, isolate NAA-13^T can be differentiated from the phylogenetically and biochemically most closely related species of the genus *Nocardioideae* (Table 1). On the basis of phenotypic, chemotaxonomic and phylogenetic evidence, it is apparent that strain NAA-13^T represents a novel species within the genus *Nocardioideae*, for which the name *Nocardioideae fonticola* sp. nov. is proposed.

Description of *Nocardioideae fonticola* sp. nov.

Nocardioideae fonticola [fon.ti.co'la. L. masc. n. *fontis*, *fontis* a spring, fountain; L. suff. *-cola* (from L. masc. or fem. n. *incola*) an inhabitant of a place, a resident; N.L. n. *fonticola* an inhabitant of a fountain or spring].

Gram-positive and strictly aerobic. Oxidase-negative, catalase-positive and non-acid-fast. No endospore formation. Cells are non-motile rods, approximately 0.8 µm wide and 2.0–9.0 µm long. Substrate or aerial mycelium is not observed. Colonies on R2A are pale yellowish in colour, circular and convex with entire edges. Colonies are approximately 0.9–1.0 mm in diameter after 3 days (on R2A at 25 °C) of incubation and reach a maximum of 2 mm after 5 days of growth. Growth occurs at 0.5–1.0 % (w/v) NaCl, pH 5–9 and 25–37 °C, with optimum growth at 0.5 % (w/v) NaCl, pH 7–8 and 30 °C. Starch, skimmed milk, and Tweens 20, 40, 60 and 80 are hydrolysed. DNase-positive and lipase (corn oil)-negative. The following characters are positive (API 20NE test) for the type strain: aesculin hydrolysis, gelatin hydrolysis, β-galactosidase and assimilation of glucose and mannitol. Negative for nitrate

Table 1. Differential phenotypic characteristics between *Nocardioides fonticola* sp. nov. strain NAA-13^T and closely related *Nocardioides* species

Strains: 1, *N. fonticola* sp. nov. NAA-13^T; 2, *N. insulae* DS-51^T (Yoon *et al.*, 2007a); 3, *N. pyridinoliticus* KCTC 0074BP^T (Yoon *et al.*, 1997); 4, *N. aquiterrae* GW-9^T (Yoon *et al.*, 2004); 5, *N. terrigena* DS-17^T (Yoon *et al.*, 2007b). +, Positive; –, negative; w, weakly positive. All strains listed are Gram-positive and positive for catalase, hydrolysis of casein and gelatin, utilization of glucose, cellobiose and D-xylose, and esterase lipase (C8). All strains are negative for hydrolysis of urea, lipase (C14), α -chymotrypsin, α -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase.

Characteristic	1	2	3	4	5
Cell morphology	Rods	Rods, cocci	Rods, cocci	Rods, cocci	Rods, cocci
Cell size (μm)	0.8 $\times$ 2.0–9.0	0.6–1.0 $\times$ 1.3–6.0	0.5–0.6 $\times$ 1.2–1.6	0.8–1.0 $\times$ 1.7–2.0	0.4–0.7 $\times$ 0.7–2.0
Motility	–	–	+	+	–
Colony colour	Yellow	Ivory	Cream	Cream	Ivory
Optimal temperature ($^{\circ}\text{C}$)	30	30	35	30	30
Nitrate reduction	–	+	+	+	+
Hydrolysis of:					
Aesculin	+	–	+	+	–
Starch	+	–	+	–	w
Tween 80	+	+	–	+	w
Utilization as sole carbon and energy source:					
L-Arabinose	+	–	–	–	–
D-Fructose	+	–	+	+	+
D-Galactose	+	+	–	+	+
Maltose	–	w	+	+	+
D-Mannose	+	w	–	+	–
Sucrose	–	–	+	+	+
Trehalose	–	+	+	+	+
API ZYM:					
Alkaline phosphatase	+	+	+	–	+
Esterase (C4)	+	+	–	–	+
Leucine arylamidase	+	–	+	+	+
Trypsin	–	–	+	+	–
Naphthol-AS-BI-phosphohydrolase	+	–	+	+	+
β -Galactosidase	+	–	–	–	+
α -Glucosidase	+	–	+	+	+
β -Glucosidase	+	–	–	w	–
DNA G + C content (mol%)	71.8	71.0	73.0	73.0	71.5
Isolation source	Freshwater	Soil	Oil-shale column	Groundwater	Soil

reduction, indole production, glucose fermentation, arginine dihydrolase, urease, and assimilation of *N*-acetylglucosamine, gluconate, malate, caprate, adipate, citrate and phenylacetate. Strain NAA-13^T is positive (API ZYM test) for alkaline phosphatase, C4 esterase, C8 lipase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, α -glucosidase and β -glucosidase, but is negative for C14 lipase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Positive (API Coryne test) for pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, β -galactosidase, α -glucosidase, and aesculin and gelatin hydrolysis, but negative for nitrate reduction, β -glucuronidase, *N*-acetyl- β -glucosaminidase, urease, and acid production from D-glucose, D-xylose, D-mannitol, maltose, D-lactose, sucrose, D-ribose and glycogen.

Resistant to nalidixic acid, ceftizoxime and sulfamethoxazole/trimethoprim antibiotics and sensitive to ampicillin, chloramphenicol, gentamicin, kanamycin, novobiocin, rifampicin, penicillin G, streptomycin and tetracycline. Major menaquinone is MK-8(H₄). Cell wall diamino acid is LL-DAP. Major fatty acids are iso-C_{16:0} (19.9%), C_{17:0} (19.9%), C_{17:1 ω 8c} (13.3%) and C_{18:1 ω 9c} (11.7%). The DNA G + C content of the type strain is 71.8 mol%.

The type strain, NAA-13^T (=LMG 24213^T=BCRC 16874^T), was isolated from spring water, southern Taiwan.

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