Nocardioides hwasunensis sp. nov.

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Two novel actinomycete strains, designated HFW-18 and HFW-21^T, were isolated from a water sample around Hwasun Beach on the coast of Jeju Island, Republic of Korea. The cells of the organisms were aerobic, Gram-positive, non-motile rods. The temperature and initial pH ranges for growth were 4–37 °C and pH 5.1–9.1, respectively. They had LL-diaminopimelic acid in their cell-wall peptidoglycan, MK-8(H₄) as the major menaquinone and DNA G+C contents of 71.1–72.2 mol%. The cellular fatty acids consisted of straight-chain saturated, branched, monounsaturated and 10-methyl fatty acids, with a major component of iso-C_{16:0}. The polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and an unknown phospholipid. The isolates were identical in terms of their 16S rRNA gene sequences and BOX-PCR DNA fingerprints. Phylogenetic analyses based on 16S rRNA gene sequences showed that the isolates occupied distinct positions within the radiation of the genus *Nocardioides*. According to 16S rRNA gene sequence similarity, the closest relatives were *Nocardioides ganghwensis* JC2055^T (97.4%), *Nocardioides oleivorans* DSM 16090^T (97.3%) and *Nocardioides furvisabuli* SBS-26^T (97.0%). Levels of 16S rRNA gene sequence similarity between the isolates and other members of the genus *Nocardioides* were in the range 91.8–95.3%. On the basis of the phenotypic and molecular genetic data presented here, the isolates represent members of a novel species of the genus *Nocardioides*. The name *Nocardioides hwasunensis* sp. nov. is proposed, and the type strain is HFW-21^T (=KCTC 19197^T=DSM 18584^T).

The genus *Nocardioides* was proposed by Prauser (1976) with a single species, *Nocardioides albus*. At the time of writing, the genus now contains 20 species with validly published names, including the recently described species *Nocardioides furvisabuli* (Lee, 2007) and *Nocardioides insulae* (Yoon *et al.*, 2007). Among them, *Nocardioides nitrophenolicus* (Yoon *et al.*, 1999), *Nocardioides aquaticus* (Lawson *et al.*, 2000), *Nocardioides aquiterrae* (Yoon *et al.*, 2004) and *Nocardioides aromaticivorans* (Kubota *et al.*, 2005) were isolated from aquatic environments such as industrial wastewater, a hypersaline lake, groundwater and river water.

During a study of the diversity of marine bacteria on the coast of Jeju Island, Republic of Korea, two actinomycete

strains, HFW-18 and HFW-21^T, were isolated from a water sample collected in the area, where running water from a valley merges into seawater on Hwasun beach. Aliquots (100 µl) of the water sample were transferred directly onto SC-SW agar (Lee, 2007), supplemented with 60% (v/v) natural seawater. After incubating for 7 days at 30 °C, the colonies on the isolation plates were subcultured on marine agar no. 2216 (MA; Difco). Pure cultures were stored on MA at 4 °C, and in 20% (v/v) glycerol solution supplemented with 60% (v/v) natural seawater at –20 and –80 °C.

The isolates were grown on ISP medium 2 (Shirling & Gottlieb, 1966), nutrient agar (NA; Difco), trypticase soy agar (TSA; Difco) and MA. Plates were incubated for 5 days at 30 °C. The requirement of seawater for growth was tested as described previously (Lee, 2007). Cell morphology and motility were observed using phase-contrast microscopy, with cultures grown in marine broth (Difco) for 6, 15, 24 and 48 h at 30 °C. The presence of flagella was checked by using a 1200EXII transmission electron microscope (JEOL), for which the cells were negatively stained with 2% (w/v) phosphotungstic acid. Colonial morphology was observed visually and recorded using 5-day-old cultures on TSA at 30 °C. The temperature

Abbreviations: MA, marine agar; NA, nutrient agar; TSA, trypticase soy agar.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA sequences of strains HFW-18 and HFW-21^T are AM295257 and AM295258, respectively.

A transmission electron micrograph of a cell of strain HFW-21^T and a neighbour-joining tree of representatives of the family *Nocardioidaceae*, and a supplementary table of cellular fatty acid composition are available with the online version of this paper.

for growth was tested on TSA at 4, 10, 20, 30, 37, 42 and 45 °C. The pH for growth was assessed on MA, which had been adjusted to an initial pH of 4.1–12.1 at intervals of 1.0 pH unit with 6 M HCl or 10 M NaOH before sterilization. NaCl tolerance during growth was determined on ISP medium 2, supplemented with 1–9 % (w/v) NaCl. Strains HFW-18 and HFW-21^T showed good growth on ISP medium 2, TSA and MA. In the case of media with natural seawater, good growth occurred on ISP medium 2, but poor growth on NA and TSA. The cells of both isolates were Gram-positive, aerobic, non-motile rods (see Supplementary Fig. S1, available with the online version of this paper).

Gram-reaction, oxidase and catalase activities, utilization of carbohydrates and decomposition of hypoxanthine, DL-tyrosine and xanthine were determined as described previously (Lee, 2007). Hydrolysis of starch and casein was investigated using starch agar (Difco) and ISP medium 2 supplemented with 1 % (w/v) skimmed milk, respectively. Other physiological and biochemical properties were tested with API 20NE and API ZYM strips (bioMérieux), according to the manufacturer's instructions. Data on cultural, physiological and biochemical characteristics are summarized in the species description and in Table 1.

The isomer of diaminopimelic acid in the cell wall, isoprenoid quinones and polar lipids were analysed as described previously (Lee, 2007). Cells of strains HFW-18 and HFW-21^T were grown in marine broth (Difco) at 30 °C for 7 days. The G+C content of the DNA was determined by using HPLC as described by Mesbah *et al.* (1989), for which extraction and purification of chromosomal DNA were performed using the method of Hopwood *et al.* (1985). For determination of cellular fatty acids, the test strains were grown on TSA at 30 °C for 3 days. Fatty acid methyl esters were prepared and analysed using the Sherlock Microbial Identification System (version 6.0; MIDI) according to the instructions of the manufacturer. The fatty acid profiles of strains HFW-18 and HFW-21^T were characterized by the presence of straight-chain saturated, branched, monounsaturated and 10-methyl fatty acids (see Supplementary Table S1, available with the online version of the paper). The major fatty acid was iso-C_{16:0} (27.8–30.0 %), followed by C_{16:0} (10.6–10.8 %), C_{18:0} (8.6–8.8 %), iso-C_{15:0} (7.5–8.3 %) and C_{18:1ω9c} (7.3–8.2 %). The polar lipid profiles contained diphosphatidylglycerol, phosphatidylinositol, phosphatidylglycerol and an unknown phospholipid. The DNA G+C contents of strains HFW-18 and HFW-21^T were 72.2 and 71.1 mol%, respectively.

Purification of chromosomal DNA was carried out by using the Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's instructions. Rep-PCR fingerprinting was performed on the isolates, using the BOX A1R primer (Versalovic *et al.*, 1994), as described by Rademaker & de Bruijn (1997). The reaction mixture contained 50 ng DNA, 0.3 mM dNTP, 1 µM BOX

Table 1. Differential characteristics of *Nocardioides hwasunensis* sp. nov. and its closest phylogenetic neighbours

Species: 1, *Nocardioides hwasunensis* sp. nov.; 2, *Nocardioides furvisabuli* SBS-26^T; 3, *Nocardioides ganghwensis* IMSNU 14028^T; 4, *Nocardioides oleivorans* DSM 16090^T. Data are from Yi & Chun (2004), Schippers *et al.* (2005), Lee (2007) and this study. +, Positive; –, negative; w, weak; v, variable. Data for the type strain are given in parentheses. All the strains utilized acetate, D-cellobiose, D-fructose, D-galactose, D-glucose, D-maltose, D-mannitol, D-mannose and trehalose as sole carbon and energy source, but not benzoate and D-ribose. Of the API ZYM tests, the results for alkaline phosphatase, esterase lipase (C8), leucine arylamidase and valine arylamidase were positive in all the strains, while lipase (C14), trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, N-acetyl-β-glucosamidase, β-glucuronidase, α-mannosidase and α-fucosidase were negative. Indole production, glucose fermentation, degradation of hypoxanthine, arginine dihydrolase and urease activities were not observed in any of the strains.

Character	1	2	3	4
Motility	–	+	–	–
Nitrate reduction	–	+	+	–
NaCl tolerance for growth (%)	0–4	0–6	0–8	0–2
Degradation of:				
Casein	–	+	+	+
Starch	+	+	+	–
Tyrosine	–	–	+	–
Enzyme activities (API ZYM):				
β-Galactosidase	–	+	+	w
α-Glucosidase	+	–	+	+
Utilization of carbon source:				
Adonitol	–	+	–	–
L-Arabinose	v(+)	+	+	–
Glycerol	–	+	+	–
Inulin	+	+	–	–
Lactose	–	+	+	+
Malate	+	+	–	+
Melibiose	–	–	+	+
Raffinose	–	+	+	–
D-Salicin	+	–	+	–
Sucrose	+	–	+	+
D-Xylose	+	+	+	–

A1R primer, 2.5 U DyNAzyme DNA polymerase (Finnzymes) and 1 × DyNAzyme buffer. 16S rRNA genes were amplified by PCR as described by Lee *et al.* (2000) and purified by Wizard PCR Preps (Promega) in accordance with the manufacturer's instructions. The resultant PCR products were subjected to direct sequence determination using an ABI PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 3730xl; Applied Biosystems). The 16S rRNA gene sequences of the isolates were aligned with the corresponding sequences retrieved from public databases by using the CLUSTAL_X program (Thompson *et al.*, 1997). Phylogenetic analyses were performed with the PHYLIP software package (Felsenstein, 1993) as described

previously (Lee, 2007). Bootstrap analysis (Felsenstein, 1985) was used for evaluating the tree topology.

Almost-complete 16S rRNA gene sequences for strains HFW-18 (1415 nt) and HFW-21^T (1415 nt) were identical to each other. The preliminary BLAST search of the 16S rRNA gene sequences of the isolates against the GenBank database showed that they were related to members of the genus *Nocardioide*s in the family *Nocardioideaceae*. The affiliation of the isolates to the genus *Nocardioide*s was also supported by chemotaxonomic characteristics typical for the genus *Nocardioide*s (O'Donnell *et al.*, 1982; Yi & Chun, 2004; Schippers *et al.*, 2005; Lee, 2007), in having LL-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan, MK-8(H₄) as the major menaquinone and iso-C_{16:0} as the major fatty acid.

For performing phylogenetic analyses, the 16S rRNA gene sequences of strains HFW-18 and HFW-21^T were compared with those of all members of the genera *Nocardioide*s and *Marmoricola*. A total of 1348 nt in unambiguously aligned positions were used for tree construction. The 16S rRNA gene sequence analyses (Fig. 1) indicated that the organisms belonged to the family *Nocardioideaceae*, and occupied distinct phylogenetic positions between *N. furvisabuli* and a *Nocardioide*s *oleivorans*–*Nocardioide*s *ganghwensis* cluster. These branching patterns were supported by a high bootstrap value (100%) and were also found in maximum-parsimony and maximum-likelihood trees. Based on 16S rRNA gene sequence similarity, the closest relatives of the isolates were *N. ganghwensis* JC2055^T (97.4%), *N. oleivorans* DSM 16090^T (97.3%) and *N. furvisabuli* SBS-26^T (97.0%). The isolates revealed relatively low levels of 16S rRNA gene sequence similarity (93.0–95.3%) to other members of the genera *Nocardioide*s and *Marmoricola*. DNA–DNA hybridization studies between the isolates and their closest phylogenetic neighbours were not performed, as it has been reported

within the evolutionary radiation that includes the two novel isolates that the type strains of *N. oleivorans* and *N. ganghwensis* share only 32% DNA–DNA relatedness, lower than the 70% cut-off point recommended for the delineation of genospecies (Wayne *et al.*, 1987), albeit with a much higher 16S rRNA gene pairwise similarity of 99% (Schippers *et al.*, 2005).

Strains HFW-18 and HFW-21^T showed almost identical phenotypic features to each other. Different results were only obtained in gelatin liquefaction and utilization of L-arabinose as sole carbon and energy source. The isolates also showed the same banding patterns in each DNA fingerprint (data not shown), revealing that strains HFW-18 and HFW-21^T belong to the same genospecies. Differential phenotypic characteristics between the isolates and the closest phylogenetic neighbours are given in Table 1.

On the basis of the phenotypic and molecular genetic data, it is suggested that strains HFW-18 and HFW-21^T represent members of a novel species of the genus *Nocardioide*s, for which the name *Nocardioide*s *hwasunensis* sp. nov. is proposed.

Description of *Nocardioide*s *hwasunensis* sp. nov.

*Nocardioide*s *hwasunensis* (hwa.sun.en'sis. N.L. adj. *hwasunensis* of Hwasun, the place where the type strain was isolated).

Gram-positive, catalase-positive, oxidase-negative and non-spore-forming. Cells are non-motile rods (0.4–0.7 µm wide and 1.0–1.7 µm long) and occur singly, in pairs or in aggregates. Older cultures are composed of oval or short rods. Colonies are opaque, smooth, convex and circular with an entire margin. The colonial pigment is yellowish cream-coloured on MA, but light yellow on ISP

Fig. 1. An abbreviated neighbour-joining tree (Saitou & Nei, 1987) showing phylogenetic positions of strains HFW-18 and HFW-21^T within the radiation encompassing representatives of the family *Nocardioideaceae*. (The full version is available as Fig. S2, available with the online version of this paper.) Evolutionary distances were calculated by using the method of Jukes & Cantor (1969). *Streptomyces griseus* KCTC 9080^T (M76388) was used as an outgroup. Asterisks at the corresponding branches are those also found in both maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) trees. Numbers at the nodes are percentages of bootstrap support (>50%), based on a neighbour-joining analysis of 1000 resamplings. Bar, 1 substitution per 100 nt positions.

medium 2 and TSA. The temperature range for growth is 4–37 °C, with good growth at 30 °C. No growth is observed at 42 °C. Growth occurs over a pH range of 5.1–9.1 and in the presence of up to 4% (w/v) NaCl. Growth at or above 5% (w/v) NaCl does not occur. Aesculin and xanthine are not degraded. Acid is produced from glucose. API ZYM tests are weakly positive for esterase (C4), acid phosphatase and α -glucosidase. Cystine arylamidase and β -galactosidase are negative. Utilizes citrate, dextran, L-rhamnose, D-sorbitol, succinate and tartrate as sole carbon and energy source, but not D-arabinose, 2,3-butanediol, D-dulcitol, meso-erythritol, meso-inositol, melezitose, methyl- β -D-glucoside, methyl- β -D-mannoside, 1,2-propanediol, L-sorbose or D-xylitol. Utilization of formate is weakly positive. Gelatin liquefaction and utilization of L-arabinose are variable depending on the strain. The data on other physiological and biochemical characteristics are given in Table 1. The polar lipid profile contains diphosphatidylglycerol, phosphatidylinositol, phosphatidylglycerol and an unknown phospholipid. LL-Diaminopimelic acid is the diagnostic diamino acid in the cell-wall peptidoglycan. The major menaquinone is MK-8(H₄). The major cellular fatty acid is iso-C_{16:0} (27.8–30.0%). The G+C content of the DNA is in the range 71.1–72.2 mol%.

The type strain, HFW-21^T (=KCTC 19197^T=DSM 18584^T), was isolated from Hwasun beach on the coast of Jeju island, Republic of Korea.

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