Nocardioides marinus sp. nov.

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A Gram-positive, slightly halophilic, rod-shaped bacterium, designated CL-DD14^T, was isolated from seawater of the East Sea, Korea. Phylogenetic analyses based on 16S rRNA gene sequences indicated that strain CL-DD14^T belongs to the genus *Nocardioides*. Levels of 16S rRNA gene sequence similarity between strain CL-DD14^T and the type strains of recognized species of the genus *Nocardioides* were low (94.1–97.1 %). Strain CL-DD14^T grew over the pH range 6–9 and temperature range 10–40 °C. The strain grew at NaCl concentrations of 0.5–8 % (w/v) with optimum growth at 1–3 % and no growth was observed after 3 weeks on nutrient agar without any salts. It contained LL-diaminopimelic acid as the diamino acid in the cell wall. The major isoprenoid quinone was MK-8(H₄) and the major cellular fatty acids were iso-C_{16:0} and 10-methyl C_{17:0}. The DNA G + C content was 72.9 mol%. On the basis of phenotypic and phylogenetic data, strain CL-DD14^T is considered to represent a novel species of the genus *Nocardioides*, for which the name *Nocardioides marinus* sp. nov. is proposed. The type strain is CL-DD14^T (=KCCM 42321^T = DSM 18248^T).

Since the original description of the genus *Nocardioides* (Prauser, 1976), many species of the genus have been isolated from various environments including soil, herbage, groundwater, an oil-shale column, black sand, crude oil, a saline lake and a tidal flat (Yoon *et al.*, 2006a, b, 2007; Lee, 2007). Most of these species were isolated from non-saline environments, although species such as *Nocardioides aquaticus* (Lawson *et al.*, 2000), *Nocardioides aestuarii* (Yi & Chun, 2004b) and *Nocardioides ganghwensis* (Yi & Chun, 2004a) were isolated from marine environments. In the present study, a strain affiliated with the genus *Nocardioides* was isolated from seawater and subjected to a polyphasic taxonomic analysis.

Strain CL-DD14^T was isolated from seawater around ‘Dokdo’ island in the East Sea, Korea, using a selective S medium (10 g Na₂HPO₄, 3 g KH₂PO₄, 1 g K₂SO₄, 30 g NaCl, 0.2 g MgSO₄·7H₂O, 0.01 g CaCl₂, 0.001 g FeSO₄·7H₂O, 1 g Casamino acids, 1 g yeast extract, 20 g glucose and 20 g Bacto agar, per litre distilled water; Fialho *et al.*, 1999). Subsequently, the isolate was routinely cultivated on marine agar 2216 (MA; Difco) at 30 °C and maintained both on MA at 4 °C and in marine broth (MB; Difco) supplemented with 30 % (v/v) glycerol at –80 °C.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CL-DD14^T is DQ401093.

A table detailing the cellular fatty acid content of strain CL-DD14^T and other species of the genus *Nocardioides* is available as supplementary material in IJSEM Online.

Morphological, physiological and biochemical analyses were performed with MA as the basal medium at 30 °C. Gram staining was performed as described by Smibert & Krieg (1994). Cell morphology and motility were observed by phase-contrast microscopy. Transmission electron microscopy subsequent to negative staining was used to establish whether flagella were present. Anaerobic growth on MA was checked by using the GasPack anaerobic system (BBL). Catalase and oxidase activities and degradation of casein, hypoxanthine, xanthine and L-tyrosine were determined according to the protocols described by Smibert & Krieg (1994). Degradation of Tween 80 was examined as described by Hansen & Sørheim (1991).

The temperature range for growth was determined on the basis of formation of visible single colonies on MA incubated at 5–45 °C, at intervals of 5 °C for 1 month. The pH range (between pH 5 and 10 at intervals of one pH unit) for growth was determined by changes in OD₆₀₀ with time in MB. The final pH was adjusted using 1 M NaOH and 1 N HCl solutions. Tolerance of strain CL-DD14^T to NaCl was determined by using synthetic ZoBell broth (5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate, per litre distilled water) with various concentrations (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 %, w/v) of NaCl. Nitrate reduction, production of indole, arginine dihydrolase, urease, gelatinase and β-galactosidase activities, acid production from glucose, and hydrolysis of aesculin were tested by using the API 20NE kit (bioMérieux) according to the manufacturer’s instructions, except that cell suspension was prepared using

Table 1. Characteristics differentiating strain CL-DD14^T from related species of the genus *Nocardioides*

Taxa: 1, strain CL-DD14^T; 2, *N. aquiterrae*; 3, *N. pyridinolyticus*; 4, *N. albus*; 5, *N. luteus*; 6, *N. kribbensis*; 7, *N. aquaticus*; 8, *N. aestuarii*; 9, *N. ganghwensis*. Data are from Yoon *et al.* (1997, 2004, 2005, 2006a), Lawson *et al.* (2000) and Yi & Chun (2004a, b). +, Positive; −, negative; w, weakly positive; v, variable; ND, not determined. Strain CL-DD14^T was positive for α -glucosidase, esterase lipase (C8) and leucine arylamidase, but negative for β -galactosidase, β -glucosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -fucosidase and lipase (C14) according to API ZYM tests. Strain CL-DD14^T also utilized D-glucose, D-mannitol, D-trehalose and sucrose, but not acetamide, L-arginine or tartrate.

Characteristic	1	2	3	4	5	6	7	8	9
Cell morphology	Rods, cocci	Rods, cocci	Rods, cocci	Hyphae	Hyphae	Short rods, cocci	Cocci, short rods	Rods	Rods
Motility	−	+	+	−	−	−	−	−	−
Temperature range (°C) (optimum)	10–40 (25–30)	15–42 (30)	20–40 (35)	(28)	(28)	4–35 (30)	(16–26)	20–35 (30)	10–40 (30)
NaCl tolerance range (%) (optimum)	0.5–8 (1–3)	ND	ND	ND	ND	0–3	ND	0–8 (0–2)	0–8 (0–1)
pH range (optimum)	6–9 (7–8)	(6–7)	5–9 (8)	ND	ND	6–11 (9)	ND	6–10 (7)	6–10 (7)
Oxidase	−	+	−	−	−	+	−	−	−
Nitrate reduction	−	+	+	−	−	+	+	−	+
Gelatinase	−	+	+	+	+	+	+	+	+
Decomposition of:									
Starch	−	−*	+	+	+	−	w*	−	+
Tween 80	+	+	−	+	+	+	+	+	+
Tyrosine	−	−	+	+	+	+	+	−	+
Hypoxanthine	−	−	−	+	+	−	−	−	−
Xanthine	−	−*	+	+	−*	−	−	−	w
API ZYM test results:									
Alkaline phosphatase	+	−	+	+	v	+	v	w	+
Esterase (C4)	w	−	−	+	+	+	w	+	v
Valine arylamidase	w	−	w	−	−	−	w	+	+
Cystine arylamidase	−	w	−	−	−	−	v	−	w
Trypsin	+	+	+	+	+	−	w	+	−
α -Chymotrypsin	−	−	−	−	−	+	−	+	−
Acid phosphatase	w	+	+	−	−	+	+	w	w
Naphthol-AS-BI-phosphohydrolase	−	+	+	w	v	+	−	w	−
α -Galactosidase	−	−	−	−	−	−	−	−	+
α -Mannosidase	−	−	−	−	+	−	−	−	−
Carbon utilization:									
Benzoate	−	−	−	+	+	ND	−	−	−
Citrate	−	w	−	+	+	ND	−	+	+
D-Fructose	+	+	+	+	−*	−	+	+	+
D-Galactose	+	+	+	+	−	+	−*	+	+
D-Mannose	−	+	+	+	+	−	−	−	+
D-Raffinose	−	−	−	v	−	+	−	w	+
D-Ribose	−	w*	+	−	−	+	−	−	−
D-Xylose	+	+	+	+	+	−	w*	+	+

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9
Glycerol	-	-	-	W	-	ND	+	W	+
L-Arabinose	-	-	-	+	+	-	-	-	+
L-Asparagine	+	+	+	W	-	ND	-	-	+
L-Lysine	-	+	-	-	-	ND	-	W	-
L-Ornithine	-	+	-	W	-	ND	-	-	+
L-Rhamnose	+	W*	+	+	-	+	+	-	-
Lactose	-	+	-	-	-	-	-	W	+
myo-Inositol	-	-	+	-	-	-	W*	-	-
N-Acetylglucosamine	-	-	-	W	+	ND	-	-	+
Succinate	-	+	+	+	+	ND	+	+	+
DNA G + C content (mol%)	73	73	73	67	68	73-74	69	70	72
Isolation source	Seawater	Ground water	Oil-shale	Soil	Soil	Alkaline soil	Saline lake (Antarctica)	Tidal flat	Tidal flat

*Different results were reported by Yi & Chun (2004b).

half-strength artificial seawater (ASW; 24 g NaCl, 5.1 g MgCl₂, 4 g Na₂SO₄, 1.1 g CaCl₂, 0.7 g KCl, 0.2 g NaHCO₃, 0.1 g KBr, 0.027 g H₃BO₃, 0.024 g SrCl₂, 0.003 g NaF, per litre distilled water; Lyman & Fleming, 1940) as the suspension medium. Other enzymic activities were determined with the API ZYM kit (bioMérieux) and half-strength ASW as the suspension medium. Carbon utilization was tested by using a modified basal medium (11.8 g NaCl, 0.32 g KCl, 2.26 g MgCl₂·6H₂O, 2.97 g MgSO₄·7H₂O, 0.65 g CaCl₂·2H₂O, 0.2 g NaNO₃, 0.2 g NH₄Cl, per litre distilled water) supplemented with yeast extract (0.05 g l⁻¹) based on changes in OD₆₀₀ with time for 1 month. Concentrations of added carbon source were 0.2% (final concentration). A test tube without any carbon source was used as a negative control. The results of morphological, physiological and biochemical tests are given in Table 1 and in the species description below.

Isoprenoid quinones were isolated according to the method of Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985) at the Korean Culture Center of Microorganisms (KCCM, Seoul, Republic of Korea). Cell-wall diamino acids were determined as described by Stanek & Roberts (1974). The whole-cell fatty acid methyl esters of strain CL-DD14^T, grown on MA plates at 30 °C for 1 day, were analysed by GC according to the instructions of the Microbial Identification System (MIDI) at KCCM. Strain CL-DD14^T contained tetrahydrogenated menaquinone with eight isoprene units [MK-8(H₄)] as the major menaquinone and LL-diaminopimelic acid as the cell-wall diamino acid. The cellular fatty acid composition of strain CL-DD14^T was as follows: branched fatty acids iso-C_{16:0} (71.5%), iso-C_{18:0} (2.1%) and iso-C_{14:0} (2.0%); 10-methyl fatty acids C_{17:0} (5.1%), C_{18:0} (1.7%) and C_{16:0} (1.4%); unsaturated fatty acids C_{18:1ω9c} (4.4%), C_{17:1ω8c} (3.8%) and C_{18:1ω7c} (1.6%); saturated fatty acid C_{16:0} (1.3%), and summed feature 3, comprising iso-C_{15:0} 2-OH and/or C_{16:1ω7c} (1.3%) (see Supplementary Table S1 available in IJSEM Online). Predominant fatty acids were iso-C_{16:0} and the 10-methyl fatty acid C_{17:0}, which are typical characteristics of the genus *Nocardioides*.

The 16S rRNA gene was amplified by PCR with *Taq* DNA polymerase (Bioneer) and primers 27F and 1492R (Lane, 1991). The PCR product was purified by using the AccuPrep PCR purification kit (Bioneer) and cloned by using the pGEM Easy TA vector (Promega). Sequencing of the 16S rRNA gene was performed with an Applied Biosystems automatic sequencer (ABI3730XL) at Macrogen Corp., Seoul, Republic of Korea. The almost-complete 16S rRNA gene sequence of strain CL-DD14^T (1440 bp) was obtained. The sequence was compared with those available in GenBank using BLASTN searches (Altschul *et al.*, 1990). The 16S rRNA gene sequence of strain CL-DD14^T was manually aligned with those of the type strains of other species belonging to the genus *Nocardioides* and phylogenetically related genera obtained from GenBank by using known 16S rRNA gene secondary structure information.

Phylogenetic trees were obtained by the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. An evolutionary distance matrix for the neighbour-joining method was generated according to the model of Jukes & Cantor (1969). The robustness of tree topologies was assessed by bootstrap analyses based on 1000 replications for the neighbour-joining and maximum-parsimony methods and 100 replications for the maximum-likelihood method. Alignment was carried out via the jPHYDIT program (Jeon *et al.*, 2005) and phylogenetic analyses were performed with MEGA 3 (Kumar *et al.*, 2004) and PAUP* 4.0 (Swofford, 1998). Likelihood parameters were estimated by using the hierarchical ratio tests in MODELTEST version 3.04 (Posada & Crandall, 1998). The DNA G + C content was determined by HPLC analysis (Tamaoka & Komagata, 1984) at KCCM.

The DNA G + C content of strain CL-DD14^T was 72.9 mol%. 16S rRNA gene sequence analysis indicated that the closest relatives of strain CL-DD14^T were the type strains of *Nocardioideis aquiterrae* (97.1 % similarity), *Nocardioideis pyridinolyticus* (96.6 %) and *N. aestuarii* (96.4 %). Other members of the genus *Nocardioideis* showed 16S rRNA gene sequence similarities of 94.1–96.3 % to the novel strain. Phylogenetic analyses based on 16S rRNA gene sequences indicated that strain CL-DD14^T formed an evolutionary lineage within the radiation enclosing most species of the genus *Nocardioideis*. Similarity between strain CL-DD14^T and *N. aquiterrae* was close to the recommended guideline (97 %) for species designation at the 16S rRNA gene level (Stackebrandt & Goebel, 1994). However, a dendrogram of

16S rRNA gene sequence relationships showed that strain CL-DD14^T formed a distinct branch from *N. aquiterrae* and was recovered as a sister group to the subclade containing *Nocardioideis albus* and *Nocardioideis luteus* that exhibited low 16S rRNA gene sequence similarity values (96.0–96.1 %) (Fig. 1). Furthermore, phenotypic features (NaCl requirement, temperature and pH ranges for growth, absence of motility, negative reactions for oxidase, gelatinase, nitrate reductase and naphthol-AS-BI-phosphohydrolase, presence of alkaline phosphatase, and carbon utilization pattern) clearly differentiated strain CL-DD14^T from *N. aquiterrae* (Table 1). In addition, strain CL-DD14^T could be differentiated from *N. albus* and *N. luteus* based on phenotypic characteristics (absence of hyphae, negative in tests for gelatin, tyrosine, hypoxanthine and starch hydrolysis, and carbon utilization patterns) (Table 1). In conclusion, phenotypic features and phylogenetic analyses based on 16S rRNA gene sequences suggest that strain CL-DD14^T represents a novel species of the genus *Nocardioideis*, for which the name *Nocardioideis marinus* sp. nov. is proposed.

Description of *Nocardioideis marinus* sp. nov.

Nocardioideis marinus (ma.ri'nus. L. masc. adj. *marinus* referring to the sea, from where the type strain was isolated).

Cells are rods (about 1.0–1.8 µm long and 0.4–0.6 µm wide) in the exponential phase and coccus forms are found in the stationary phase. Cells are non-motile and strictly aerobic. Gram-positive, oxidase-negative and weakly catalase-positive. Colonies on MA are circular, smooth,

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between strain CL-DD14^T and members of the genus *Nocardioideis*. Only bootstrap values (expressed as percentage of 1000 replications) greater than 70 % are shown at nodes. Solid circles indicate that the corresponding nodes were also recovered in the maximum-likelihood and maximum-parsimony trees. *Terrabacter tumescens* KCTC 9133^T (AF005023) was used as an outgroup (not shown). Bar, 0.01 nucleotide substitutions per site.

convex with an entire margin and creamy white. Substrate or aerial mycelium is not observed. Growth occurs at 10–40 °C (optimum about 25–30 °C) and at pH values of 6–9 (optimum pH 7–8). Growth occurs at NaCl concentrations of 0.5–8 % (w/v) (optimum 1–3 %). Negative in tests for nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, urease and gelatinase, and positive for aesculin hydrolysis and β -galactosidase in API 20NE tests. Casein and Tween 80 are hydrolysed, but hypoxanthine, xanthine, L-tyrosine and starch are not. Major menaquinone is MK-8(H₄). Cell-wall diamino acid is LL-diaminopimelic acid. The major fatty acids are iso-C_{16:0} (71.5 %) and 10-methyl C_{17:0} (5.1 %). Results of API ZYM and carbon source utilization tests are given in Table 1. The DNA G+C content is 72.9 mol%.

The type strain, CL-DD14^T (=KCCM 42321^T=DSM 18248^T), was isolated from seawater of the East Sea, Korea.

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