Nocardioides dokdonensis sp. nov., an actinomycete isolated from sand sediment

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A strictly aerobic, non-motile, short rod-shaped actinomycete, designated strain FR1436^T, was isolated from sand sediment from a beach on Dokdo Island, Republic of Korea. The major menaquinone was MK-8(H₄), the predominant cellular fatty acid was iso-C_{16:0} (40.4 %) and the G+C content of the DNA was 69.1 mol%. A phylogenetic tree based on 16S rRNA gene sequences showed that strain FR1436^T formed a lineage within the genus *Nocardioides* and was closely related to *Nocardioides marinisabuli* SBS-12^T (97.8 % sequence similarity) and *Nocardioides salarius* CL-Z59^T (97.4 %). Phenotypic characteristics and DNA–DNA relatedness data served to distinguish strain FR1436^T from *N. marinisabuli*. On the basis of the evidence presented in this study, strain FR1436^T represents a novel species of the genus *Nocardioides*, for which the name *Nocardioides dokdonensis* sp. nov. is proposed. The type strain is FR1436^T (=KCTC 19309^T =JCM 14815^T).

The genus *Nocardioides* is characterized chemotaxonomically by the combination of LL-diaminopimelic acid in the peptidoglycan and MK-8(H₄) as the major menaquinone (O'Donnell *et al.*, 1982; Prauser, 1976). Since the original description by Prauser (1976), the number of *Nocardioides* species described has increased rapidly because of improved classification resulting from the polyphasic approach. At the time of writing, there are more than 20 *Nocardioides* species with validly published names. Species of the genus have been isolated from various environments including soil, herbage, groundwater, an oil-shale column, black sand and beach sand, crude oil, a saline lake, a tidal flat, seawater and a water flea (Yoon *et al.*, 2005, 2006a, b, 2007, 2008; Lee, 2007; Lee *et al.*, 2007, 2008; Tóth *et al.*, 2008). Of these, *Nocardioides ganghwensis* (Yi & Chun, 2004a), *Nocardioides aestuarii* (Yi & Chun, 2004b) and *Nocardioides marinus* (Choi *et al.*, 2007) were isolated recently from tidal flat sediments.

Recently, during the course of a study on the cultivable aerobic bacterial community in sand sediment, a Gram-positive bacterium that showed high levels of 16S rRNA gene sequence similarity to members of the genus *Nocardioides* was isolated from Dokdo Island, Korea (37°

05' N 131° 13' E). The sediment isolate, designated strain FR1436^T, was the subject of this study.

Strain FR1436^T was isolated from a sediment sample during February 2005, using the standard dilution plating technique. Isolation was achieved using MR2A [R2A (Difco) supplemented with 3.5 % artificial sea salts (Sigma)]. The isolate was routinely cultured on tryptic soy agar (TSA; Difco) at 25 °C and maintained as a glycerol suspension (20 %, w/v) at –80 °C.

Bacterial DNA preparation, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Chun & Goodfellow, 1995). The resultant sequence of strain FR1436^T (1385 nt) was aligned manually against sequences obtained from the GenBank database. Phylogenetic trees were inferred from the regions available in all sequences (positions 39–1447; *Escherichia coli* numbering system) by using the Fitch–Margoliash (Fitch & Margoliash, 1967) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices were generated as described by Jukes & Cantor (1969). The resultant neighbour-joining tree topology was evaluated by means of bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Alignment and phylogenetic analyses were carried out using the jPHYDIT program (available at <http://plaza.snu.ac.kr/~jchun/jphydit/>) and PAUP 4.0 (Swofford, 1998), as described previously (Chun *et al.*, 2000).

Preliminary sequence comparisons with 16S rRNA gene sequences held in GenBank indicated that strain FR1436^T

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain FR1436^T is EF633986.

Biolog GP2 MicroPlate results and the fatty acid composition for strain FR1436^T are available as supplementary material with the online version of this paper.

was closely related to the genus *Nocardioideis*. The newly determined sequence was then aligned manually against representatives of the genus *Nocardioideis*. Strain FR1436^T showed the highest 16S rRNA gene sequence similarity with respect to *Nocardioideis marinisabuli* SBS-12^T (97.8 %) and *Nocardioideis salarius* CL-Z59^T (97.4 %); the sequence similarities with respect to all other species of the genus *Nocardioideis* were below 96.6 %. Also, strain FR1436^T occupied a distinct position in the neighbour-joining tree, clustering only with *N. marinisabuli* and *N. salarius* (Fig. 1). The tree based on the Fitch–Margoliash method showed essentially similar topology (data not shown).

In view of the results of the 16S rRNA gene sequence analysis and the subsequent phylogenetic analysis, DNA–DNA hybridization between FR1436^T and the type strain of *N. marinisabuli* was the only test needed to define the strain as a novel species, because the sequence similarities (<97 %) between strain FR1436^T and all *Nocardioideis* species other than *N. marinisabuli* and *N. salarius* were below the level indicative of relatedness at the species level (Stackebrandt & Goebel, 1994). DNA–DNA hybridization was performed using a membrane filter technique according to the method described by Baik *et al.* (2006). The DNA–DNA relatedness between strain FR1436^T and *N. marinisabuli* KCCM 42681^T was 23.1 ± 5.6 %. It is clear from the 16S rRNA gene sequence and DNA–DNA

hybridization data that strain FR1436^T represents a novel species in the genus *Nocardioideis* (Wayne *et al.*, 1987).

Bacterial growth was tested on nutrient agar (Difco), TSA, R2A agar, glucose-yeast extract agar (Gordon & Mihm, 1962) and marine agar 2216 (Difco). Formation of mycelium and spores and production of pigment and melanin were examined for 3 weeks on several culture media, namely ISP 3, ISP 4 (Difco), ISP 5 and ISP 6 (Williams *et al.*, 1983). The following features were determined as reported previously (Park *et al.*, 2008): Gram stain, motility, oxidase and catalase activities, temperature, pH and NaCl tolerance for growth, acid production from sugars and hydrolysis of casein, L-tyrosine and starch. Acid-fastness was determined by means of Ziehl–Neelsen staining (Hendrickson & Krenz, 1991). Degradation of urea (0.15 %, w/v) and allantoin (0.15 %, w/v) was tested using the medium of Gordon *et al.* (1974) containing 1 % KH₂PO₄, 0.95 % Na₂HPO₄, 0.1 % yeast extract and 0.04 % phenol red. Degradation of arbutin (0.1 %, w/v) was tested using the medium of Williams *et al.* (1983) containing 0.3 % yeast extract, 0.05 % ferric ammonium citrate and 1.5 % agar. Hydrolysis of Tween 20 (1 %, w/v) and Tween 80 (1 %, w/v) was tested using TSA as the basal medium (Barrow & Feltham, 1993). Hydrolysis of hippurate (1 %, w/v) was tested using hippurate agar (Williams *et al.*, 1983). DNase test agar

Fig. 1. Neighbour-joining phylogenetic tree, based on almost-complete 16S rRNA gene sequences, showing relationships between strain FR1436^T and members of the genus *Nocardioideis*. Numbers at nodes are bootstrap percentages (based on 1000 resampled datasets). The sequence of *Nocardia asteroides* ATCC 19247^T was used as an outgroup. Bar, 0.01 nucleotide substitutions per position.

(Difco) was used to assay DNase activity. Decomposition of elastin (0.3 %, w/v), guanine (0.3 %, w/v), xanthine (0.4 %, w/v) and xylan (0.4 %, w/v) was checked using modified Bennett's agar as described by Gordon *et al.* (1974). Utilization of compounds as sole carbon sources was examined on Stevenson medium (Stevenson, 1967) suitably supplemented with each carbon source, and growth was recorded after 10 days incubation at 30 °C. Utilization of compounds as sole nitrogen sources was

examined on Tsukamura's medium (Tsukamura, 1975) suitably supplemented with each nitrogen source, and growth was recorded after 10 days incubation at 30 °C. Other biochemical features and enzyme activities were determined using API 20NE, API 50 CH and API ZYM kits (all from bioMérieux). Growth in the presence of various chemicals was determined on appropriately supplemented TSA after 10 days incubation at 30 °C. Antibiotic resistance was determined using the disc diffusion method with

Table 1. Characteristics that serve to differentiate strain FR1436^T from closely related species

Strains: 1, FR1436^T; 2, *N. aquaticus* DSM 11439^T; 3, *N. kribbensis* KCTC 19038^T; 4, *N. lentus* KCTC 19039^T; 5, *N. marinisabuli* KCCM 42681^T; 6, *N. salarius* CL-Z59^T. Data for reference strains are from Lawson *et al.* (2000), Lee *et al.* (2007), Kim *et al.* (2008) and Yoon *et al.* (2005, 2006a). All of the strains are positive for Gram stain, catalase, esterase lipase (C8)* and leucine arylamidase* and negative for xanthine hydrolysis and production of lipase (C14)*, α -galactosidase*, β -glucuronidase*, *N*-acetyl- β -glucosaminidase*, α -mannosidase* and α -fucosidase*. Mannitol and trehalose were utilized by all of the strains. +, Positive; –, negative; v, variable; w, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6
Nitrate reduction	+	+	+	+	–	–
Oxidase	–	+	+	w	–	–
Hydrolysis of:						
Aesculin	–	–	+	–	+	+
Casein	–	+	+	+	+	+
Gelatin	–	+	+	+	+	+
Starch	–	w	–	–	w	w
Tween 80	+	+	+	+	–	+
Tyrosine	–	+	+	+	–	+
Urea	+	–	–	–	–	–
Utilization of cellobiose (API 50 CH)	–	–	+	+	–	+
Assimilation of maltose (API 20NE)	–	w	+	+	–	ND
Utilization of carbon sources						
L-Arabinose	–	–	–	w	–	–
D-Fructose	+	+	–	–	+	–
D-Galactose	–	–	+	+	+	–
Glucose	–	+	+	+	–	+
D-Lactose	–	–	–	+	–	–
Mannose	+	–	–	–	–	–
Raffinose	–	–	+	+	–	–
L-Rhamnose	–	+	+	+	–	–
Sucrose	–	+	+	+	–	+
D-Xylose	w	w	–	+	+	–
Enzyme activity (API ZYM)						
Acid phosphatase	+	+	+	+	–	+
Alkaline phosphatase	+	v*	+	+	+	+
α -Chymotrypsin	+	–*	+	–	–	+
Cystine arylamidase	–	v*	–	–	–	+
Esterase (C4)	+	+	+	+	–	+
β -Galactosidase	–	–*	w	–	–	–
α -Glucosidase	+	+	+	–	+	+
β -Glucosidase	–	–*	–	+	–	–
Naphthol-AS-BI-phosphohydrolase	+	–*	+	+	–	+
Trypsin	+	w*	–	–	–	+
Valine arylamidase	w	w*	–	–	–	+
DNA G + C content (mol%)	69.1	69	73–74†	74.6–74.8†	73.1	73

*Data for strain *N. aquaticus* DSM 11439^T from Yoon *et al.* (2006a).

†Ranges of values for three strains, including the type strain, in each case.

commercial antibiotic-impregnated discs (BBL Becton Dickinson). The results were interpreted according to the guidelines set forth by the NCCLS (2003). Antibiosis against micro-organisms was checked using an overlay technique (Williams *et al.*, 1983) on TSA and growth inhibition of target micro-organisms was measured after 24 h incubation at 30 °C.

Cells of strain FR1436^T grown on TSA for 2 days at 25 °C were prepared and analysed for fatty acids by GLC according to the instructions of the Microbial Identification System (MIDI, 1999). The diaminopimelic acid isomer in the cell wall was determined as described by Seong *et al.* (1999). Isoprenoid quinones were isolated according to the method of Minnikin *et al.* (1984) and were analysed using HPLC as described by Collins (1985). The G+C content of the DNA was determined using the thermal denaturation method of Marmur & Doty (1962).

The fatty acid profile of strain FR1436^T is shown in Supplementary Table S1 (available in IJSEM Online). The predominant fatty acid was iso-C_{16:0} (40.4%); 10-methyl C_{17:0} (1.4%), which is characteristic of the genus *Nocardioide*s, was present. Considerable amounts of C_{18:1ω9c} (11.2%), C_{16:0} (7.7%) and C_{18:0} (7.2%) were detected. The major menaquinone was MK-8(H₄), as is the case for all *Nocardioide*s species. The DNA G+C content of strain FR1436^T was 69.1 mol%, which is similar to the value for the type strain of *Nocardioide*s *aquaticus* and lower than those of the type strains of *Nocardioide*s *kribbensis*, *Nocardioide*s *lentus* and *N. marinisabuli*.

A number of phenotypic characteristics clearly distinguish strain FR1436^T from other phylogenetically related *Nocardioide*s species (Table 1). Therefore, strain FR1436^T represents a novel species within the genus *Nocardioide*s, for which the name *Nocardioide*s *dokdonensis* sp. nov. is proposed.

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

*Nocardioide*s *dokdonensis* (dok.do.nen'sis. N.L. masc. adj. *dokdonensis* pertaining to Dokdo, the Korean island from where the type strain was isolated).

Gram-positive and strictly aerobic bacterium. Oxidase-negative, catalase-positive and not acid-fast. Spores are not formed. Cells are non-motile short rods (0.6–0.9 × 1.2–1.8 µm) in the exponential phase of growth. Substrate and aerial mycelia are not observed. Colonies on TSA are cream, circular, convex, entire, glistening and opaque. Colonies are approximately 1.0–2.0 mm in diameter after 3 days (on TSA at 25 °C) and reach a maximum diameter of 3 mm after 7 days. Growth occurs in 0–7% (w/v) NaCl (optimum, 0–3%), at pH 5–10 (optimum, pH 7) and at 4–30 °C (optimum, 25 °C). Diffusible pigment and melanin are not produced. Acid is not produced from 0.5% (w/v) D-glucose or maltose. Does not produce H₂S or indole. Arginine dihydrolase is absent. Allantoin, guanine, Tween 20 and xylan are hydrolysed, but arbutin, DNA, elastin and hippurate are not hydrolysed. The following substrates are

utilized as sole carbon and energy sources: adonitol, glycerol, melezitose, melibiose, ribose, sodium acetate, sodium citrate, sodium propionate and sodium pyruvate. Does not utilize *N*-acetylglucosamine, adipate, caprate, dextran, gentiobiose, *myo*-inositol, DL-malate, phenyl acetate, salicin or xylitol. The following substrates are utilized as sole nitrogen sources: L-cysteine, L-hydroxyproline, L-phenylalanine, L-threonine, L-valine and potassium nitrate. Does not utilize DL-α-amino-n-butyric acid or L-histidine. Cells are sensitive to the following antibiotics (µg per disc, unless otherwise indicated): amikacin (30), ampicillin (10), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), nalidixic acid (30), penicillin (10 U), polymyxin B (300 U), streptomycin (10), tetracycline (30) and vancomycin (30). The major menaquinone is MK-8(H₄). The cell-wall diamino acid is LL-diaminopimelic acid. The major fatty acids are iso-C_{16:0}, C_{18:1ω9c}, C_{16:0} and C_{18:0}. The fatty acid 10-methyl C_{17:0} is present, but tuberculostearic acid is absent. The DNA G+C content of the type strain is 69.1 mol%. Other physiological and biochemical characteristics are given in Table 1 and Supplementary Tables S1 and S2.

The type strain, FR1436^T (=KCTC 19309^T =JCM 14815^T), was isolated from sand sediment from a beach on Dokdo Island, Republic of Korea.

Acknowledgements

This work was supported by the BK21 programme (Ministry of Education and Human Resources Development), Republic of Korea.

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