Nocardioides kongjuensis sp. nov., an *N*-acylhomoserine lactone-degrading bacterium

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A Gram-positive, rod- or coccoid-shaped and *N*-hexanoyl-L-homoserine lactone-degrading bacterial strain, A2-4^T, was isolated from a soil in Korea, and its taxonomic position was investigated by using a polyphasic approach. Strain A2-4^T grew optimally at pH 7.0–8.0 and 30 °C without NaCl. Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain A2-4^T is most closely related to members of the genus *Nocardioides*. Strain A2-4^T possessed chemotaxonomic properties indicative of members of the genus *Nocardioides*; the cell-wall peptidoglycan type was based on LL-diaminopimelic acid, MK-8(H₄) was the predominant menaquinone and iso-C_{16:0} was the predominant fatty acid. The DNA G + C content was 72.1 mol%. The 16S rRNA gene sequence of strain A2-4^T was 98.3–99.1 % similar to those of the type strains of *Nocardioides simplex*, *Nocardioides aromaticivorans* and *Nocardioides nitrophenolicus* and 93.8–96.3 % similar to those of the type strains of other *Nocardioides* species. Strain A2-4^T could be distinguished from the three phylogenetic relatives, *N. nitrophenolicus*, *N. aromaticivorans* and *N. simplex*, by DNA–DNA relatedness (25–42 %) and by differences in some phenotypic characteristics. On the basis of the phenotypic, phylogenetic and genetic data, the strain represents a novel species of the genus *Nocardioides*, for which the name *Nocardioides kongjuensis* sp. nov. is proposed. The type strain is A2-4^T (=KCTC 19054^T =JCM 12609^T).

Many bacteria, particularly Gram-negative bacteria, are known to control the expression of specific genes in response to cell density by means of a signalling mechanism referred to as quorum-sensing (Salmond *et al.*, 1995; Swift *et al.*, 1996; Fuqua & Greenberg, 1998). This type of signalling mechanism depends on small, diffusible signal molecules, *N*-acylhomoserine lactones (AHLs), all of which have an identical homoserine lactone ring, although the acyl side-chain length and substitutions in the side-chain differ. The quorum-sensing system plays an important role in the control of virulence-gene expression in pathogenic bacteria, including *Erwinia carotovora* (Jones *et al.*, 1993) and *Pseudomonas aeruginosa* (Pearson *et al.*, 1994, 1997). Enzymic degradation of AHL molecules was reported as a useful strategy for disrupting the quorum-sensing signal system, thereby interfering with infection in the case of many pathogenic bacteria. Some AHL-degrading micro-organisms and enzymes have been reported recently (Dong

et al., 2000; Leadbetter & Greenberg, 2000; Lee *et al.*, 2002; Flagan *et al.*, 2003; Park *et al.*, 2003; Uroz *et al.*, 2003). In this study, an AHL-degrading *Nocardioides*-like bacterial strain, A2-4^T, which was isolated from a soil sample, is described. To date, bacteria that are able to degrade AHLs have not been reported for the genus *Nocardioides*. Accordingly, the aim of the present study was to determine the exact taxonomic position of strain A2-4^T by using a polyphasic taxonomic approach.

Soil samples collected from Kongju, Korea, were used as the source for the isolation of AHL-degrading bacteria. The soil samples (5 mg) were inoculated in 2 ml modified minimal medium (Leadbetter & Greenberg, 2000), adjusted to pH 6.5, containing *N*-hexanoyl-L-homoserine lactone (HHL) as the carbon source and NH₄Cl as the nitrogen source. This medium was incubated at 30 °C on a horizontal shaker at 150 r.p.m. After incubation for 3 days, 0.1 ml suspension was transferred into 2 ml fresh medium and the medium was reincubated under the same conditions. After three successive transfers, the suspension was plated on medium solidified with 1.5 % (w/v) agar in order to isolate pure cultures. Of the isolates, strain A2-4^T showed high

Abbreviations: AHL, *N*-acylhomoserine lactone; HHL, *N*-hexanoyl-L-homoserine lactone.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A2-4^T is DQ218275.

levels of HHL-degrading activity and was selected for further study. The utilization of HHL as a sole carbon source was investigated in modified minimal medium (Leadbetter & Greenberg, 2000) containing 1 mM HHL. The amounts of residual HHL in the growth medium were measured as described by Park *et al.* (2003). *Nocardioideis simplex* KCTC 9106^T, *Nocardioideis nitrophenolicus* KCTC 0457BP^T and *Nocardioideis aromaticivorans* JCM 11674^T were used as reference strains for DNA–DNA hybridization and phenotypic characterization.

To investigate its morphological, physiological and biochemical characteristics, strain A2-4^T was routinely cultivated at 30 °C on nutrient agar (Difco) and in nutrient broth (Difco). Morphological, physiological, cultural and biochemical properties were examined as described by Yoon *et al.* (2005a). Growth at various NaCl concentrations (0–8 %, w/v, in increments of 1.0 %) was investigated in trypticase soy broth prepared according to the formula of the Difco medium except that no NaCl was used. The pH range for growth was determined in nutrient broth adjusted to various pH values (pH 4.0–10.5, in increments of 0.5 pH units). The pH was adjusted, prior to sterilization, to various levels by the addition of HCl and Na₂CO₃. Other physiological properties were tested by using the API 20E system (bioMérieux). Cell biomass for DNA extraction and for analyses of the cell wall and the isoprenoid quinones was obtained by cultivation for 3 days in nutrient broth at 30 °C. For fatty acid methyl ester analysis, cell mass of strain A2-4^T was harvested from nutrient agar after incubation for 6 days at 30 °C. Chemotaxonomic and molecular systematic studies were performed as described by Yoon *et al.* (2005a). The isomer type of the diamino acid in the cell-wall peptidoglycan was analysed using TLC according to the method described by Komagata & Suzuki (1987). DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded and the means of the remaining three values were quoted as the DNA–DNA relatedness values.

Strain A2-4^T grew with HHL as the sole carbon source. As the cell density increased, the residual amount of HHL in the culture medium decreased (Fig. 1). Most of the HHL in the culture medium was consumed after cultivation for 10 h (Fig. 1). No growth was observed in an HHL-depleted minimal medium. The details of HHL degradation by strain A2-4^T will be described elsewhere. The morphological, cultural, physiological and biochemical characteristics of strain A2-4^T are given in the species description (see below) or are shown in Table 1, together with those of *N. simplex* and *N. nitrophenolicus*. The almost complete 16S rRNA gene sequence of strain A2-4^T, comprising 1475 nt (approx. 96 % of the *Escherichia coli* 16S rRNA sequence), was determined in this study. Comparative 16S rRNA gene sequence analyses showed that strain A2-4^T fell within the phylogenetic

Fig. 1. Growth of strain A2-4^T in minimal medium containing HHL as the sole carbon source. Growth (OD₆₀₀) with HHL (■) and without HHL (●) was assessed. The residual HHL concentration (▲) in the culture medium was measured by using a bioassay (Park *et al.*, 2003).

radiation encompassed by the genus *Nocardioideis*, forming, in particular, a coherent cluster with *N. nitrophenolicus*, *N. aromaticivorans* and *N. simplex* (Fig. 2). In the neighbouring phylogenetic tree based on 16S rRNA gene sequences, strain A2-4^T clustered with *N. nitrophenolicus* with a high level of bootstrap confidence (91.2 %), and this cluster joined the phylogenetic lineage comprising *N. simplex* and *N. aromaticivorans* with a bootstrap resampling value of 100 % (Fig. 2). Strain A2-4^T exhibited 16S rRNA gene sequence similarity values of 99.1, 98.6 and 98.3 % with respect to *N. nitrophenolicus* KCTC 0457BP^T, *N. aromaticivorans* H-1^T and *N. simplex* KCTC 9106^T, respectively, and values of less than 96.3 % (the similarity with *Nocardioideis pyridinolyticus*) with respect to the type strains of the other *Nocardioideis* species. The values for sequence similarity with respect to other species used in the phylogenetic analysis were in the range 89.5–94.2 %.

Strain A2-4^T contained LL-2,6-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan, which is characteristic of wall chemotype I *sensu* Lechevalier & Lechevalier (1970). The predominant isoprenoid quinone detected in strain A2-4^T was a tetrahydrogenated menaquinone with eight isoprene units [MK-8(H₄)]. The cellular fatty acids of strain A2-4^T (i.e. those fatty acids constituting >1 % of total fatty acids) were as follows: branched fatty acids iso-C_{16:0} (27.1 %), iso-C_{17:0} (6.3 %), anteiso-C_{17:0} (3.0 %), iso-C_{15:0} (2.2 %), iso-C_{18:0} (1.4 %) and iso-C_{16:1} (1.3 %); unsaturated fatty acids C_{17:1}ω6c (11.2 %), C_{18:1}ω9c (5.0 %) and C_{17:1}ω8c (2.9 %); straight-chain fatty acids C_{16:0} (3.9 %), C_{18:0} (3.4 %) and C_{17:0} (1.4 %); 10-methyl fatty acids C_{18:0} (14.4 %), C_{17:0} (6.0 %) and C_{16:0} (3.2 %); summed feature 3, comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH (2.3 %); and hydroxy fatty acid C_{16:0} 2-OH (1.5 %). This fatty acid profile was similar to those of *Nocardioideis* species, particularly those of *N. simplex* and *N. nitrophenolicus* in that the major fatty acids (i.e. those which

Table 1. Differential phenotypic characteristics of strain A2-4^T and three related *Nocardioides* species

Taxa: 1, strain A2-4^T; 2, *N. aromaticivorans* (unless indicated, data from Kubota *et al.*, 2005); 3, *N. nitrophenolicus* (Yoon *et al.*, 1999; Yi & Chun, 2004a); 4, *N. simplex* (O'Donnell *et al.*, 1982; Suzuki & Komagata, 1983; Yi & Chun, 2004a). +, Positive; –, negative; ND, not determined; w, weakly positive; v, variable. Data in parentheses are for the type strain. All taxa are positive for Gram-stain, catalase*, hydrolysis of casein and gelatin (not determined for *N. aromaticivorans*), alkaline phosphatase, esterase lipase (C8), leucine arylamidase, α -glucosidase and utilization of D-glucose, sucrose and succinate. All taxa are negative for nitrate reduction*†, hydrolysis of xanthine*†, esterase (C4), lipase (C14), α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase and for utilization of D-galactose* and benzoate* (not determined for *N. aromaticivorans*).

Characteristic	1	2	3	4
Cell morphology	Rods, cocci	Rods	Rods, cocci	Rods, cocci
Cell size (μm)	0.4–0.7 $\times$ 0.8–3.0	0.5–0.7 $\times$ 1.0–2.0	0.5–0.8 $\times$ 1.0–3.0	1.0–1.2 $\times$ 1.5–6.0
Motility	–	–	+	+
Colony colour	Yellowish white	Milky white	Yellowish white	Yellowish white
Optimal temperature for growth ($^{\circ}\text{C}$)	30–37	30	30	26–37
Oxidase	+	–	+	(+)
Hydrolysis of:				
Aesculin	–	+	+	(+)
Hypoxanthine	–	(+)†	–	(–)
Starch	–	–	+	(w)
Tween 80	+	–	+	(+)
Urea	–	+	+	–
L-Tyrosine	–	(–)†	+	(+)
Utilization of:				
Acetate	–	ND	+	+
Citrate	–	–	–	v (+)
L-Arabinose	–	+ or w	–	–
D-Cellobiose	–	+	–	(–)
D-Fructose	–	+	+	(–)
Maltose	–	+ or w	–	ND
D-Mannose	w	w	–	(–)
D-Ribose	–	+	+	(–)
D-Xylose	–	+	+	–
API ZYM tests				
Esterase (C4)	–	(+)†	–	(–)*
Valine arylamidase	–	(–)†	+	(w)*
Cystine arylamidase	–	(–)†	w	(w)*
Trypsin	–	(–)†	+	(+)*
Acid phosphatase	+	(–)†	+	(w)*
Naphthol-AS-BI-phosphohydrolase	w	(–)†	+	(–)*
β -Glucosidase	–	(–)†	w	(w)*
DNA G+C content (mol%)	72.1	72.0–72.4	71.4	72–74
Isolation source	Soil	River water, sediment, soil	Industrial wastewater	Soil

*Data for the type strain of *N. simplex* from Yi & Chun (2004a).

†Data for the type strain of *N. aromaticivorans* from this study.

each constituted >10 % of total fatty acids) are iso-C_{16:0}, 10-methyl C_{18:0} and C_{17:1 ω 6c}, although there are differences in the proportions of some fatty acids, perhaps because of differences in extraction and cultivation conditions (Yoon *et al.*, 1997, 1999, 2004, 2005a, b; Lawson *et al.*, 2000; Yi & Chun, 2004a, b; Kubota *et al.*, 2005). The DNA G+C content of strain A2-4^T was 72.1 mol%. These chemotaxonomic properties were in agreement with the result of the phylogenetic analysis, showing the affiliation of

strain A2-4^T with the genus *Nocardioides* (Tamura & Yokota, 1994; Yoon *et al.*, 1997; Lawson *et al.*, 2000; Urzì *et al.*, 2000; Wang *et al.*, 2001).

The levels of DNA–DNA relatedness between strain A2-4^T and the type strains of three phylogenetically related *Nocardioides* species, namely *N. nitrophenolicus* KCTC 0457BP^T, *N. simplex* KCTC 9106^T and *N. aromaticivorans* JCM 11674^T, were 38, 28 and 22 %, respectively, when their

Fig. 2. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strain A2-4^T and some related taxa. Bootstrap values (expressed as percentages of 1000 replications) above 50% are shown at branch points. Bar, 0.01 substitutions per nucleotide position.

DNA probes were used individually as labelled DNA probes. The corresponding relative binding values for the reciprocal experiment in which the DNA of strain A2-4^T was labelled were 46, 21 and 36%, respectively. Strain A2-4^T was differentiated from its closest phylogenetic neighbours *N. simplex*, *N. nitrophenolicus* and *N. aromaticivorans* by the phenotypic properties listed in Table 1. The phylogenetic distinctiveness of the novel strain, the differential phenotypic properties and the DNA–DNA relatedness data were sufficient to categorize strain A2-4^T as a species that is distinct from previously recognized *Nocardioideae* species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Therefore, on the basis of the data presented, strain A2-4^T should be classified in the genus *Nocardioideae* as a member of a novel species, for which the name *Nocardioideae kongjuensis* sp. nov. is proposed.

Description of *Nocardioideae kongjuensis* sp. nov.

Nocardioideae kongjuensis (kong.ju.en'sis. N.L. masc. adj. *kongjuensis* of Kongju, Korea, from where the type strain was isolated).

Cells are aerobic, non-spore-forming rods (0.4–0.7 × 0.8–3.0 µm) in the exponential phase of growth. Cells show rod-to-coccus morphogenesis from the early exponential phase to the stationary phase. Gram-positive. Colonies are irregular, smooth, flat, yellowish white in colour and 3.0–5.0 mm in diameter after 3 days incubation on nutrient agar at 30 °C. Neither substrate mycelium nor aerial mycelium is formed. Growth occurs at 10 and 40 °C, but not at 4 or 41 °C. Optimal pH for growth is 7.0–8.0; growth occurs at pH 5.5, but not at pH 5.0. Growth occurs in the presence of 0–5% (w/v) NaCl; optimum growth occurs in the absence of NaCl. Negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase. H₂S and indole are not produced. Tweens 20, 40 and 60 are hydrolysed. D-Trehalose, L-malate and

pyruvate are utilized, but formate and salicin are not. The diagnostic diamino acid in the cell-wall peptidoglycan is LL-2,6-diaminopimelic acid. The predominant menaquinone is MK-8(H₄). The major fatty acids (>10% of total fatty acids) are iso-C_{16:0}, 10-methyl C_{18:0} and C_{17:1}ω6c. The DNA G + C content is 72.1 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, A2-4^T (= KCTC 19054^T = JCM 12609^T), was isolated from a soil in Kongju, Korea.

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