Nocardioides pyridinolyticus sp. nov., a Pyridine-Degrading Bacterium Isolated from the Oxic Zone of an Oil Shale Column

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A bacterial strain which is able to degrade pyridine was previously isolated from the oxic zone of an oil shale column and described as *Pimelobacter* sp. strain OS4^T. However, *Pimelobacter* species have been transferred to the genera *Nocardioides* and *Terrabacter*. Strain OS4^T was identified as a member of the genus *Nocardioides* on the basis of chemotaxonomic analysis and phylogenetic inference based on 16S ribosomal DNA (rDNA) sequence analysis. The G+C content of strain OS4^T is 72.5 mol%. The cell wall peptidoglycan contains LL-diaminopimelic acid as the diamino acid. The predominant menaquinone is MK-8(H₄). The cellular fatty acid profile of strain OS4^T is similar to that of the genus *Nocardioides*. The 16S rDNA similarity of strain OS4^T with previously described *Nocardioides* species is 94.5% ± 0.7%, and a phylogenetic tree based on 16S rDNA sequences revealed a distinct lineage for strain OS4^T within the evolutionary radiation enclosed by the genus *Nocardioides*. Therefore, on the basis of our data, we propose that strain OS4^T should be placed in the genus *Nocardioides* as a member of a new species, *Nocardioides pyridinolyticus*. The type strain of the new species is strain OS4 (= KCTC 0074BP).

Pyridine is a toxic aromatic compound which poses serious problems for human health (5). A slight modification of ring substituents can dramatically increase the toxic activity of pyridine. Pyridine occurs in the environment as a result of industrial activities (17, 23) and, due to its high solubility in water, is thought to be highly mobile through the environment (9). A bacterial strain which is able to degrade pyridine was recently isolated from the oxic zone of an oil shale leachate column and was identified as *Pimelobacter* sp. (8, 15).

Although the genus *Pimelobacter* was proposed in 1983 by Suzuki and Komagata for *Arthrobacter* species containing LL-diaminopimelic acid in the peptidoglycan (24), species of the genus *Pimelobacter* have been transferred to the genus *Nocardioides* or the genus *Terrabacter* (3, 12). Three *Pimelobacter* species, namely, *Pimelobacter jensenii*, *Pimelobacter simplex*, and *Pimelobacter tumescens*, were recognized first. However, O'Donnell et al. proposed that *Arthrobacter simplex* should be assigned to the genus *Nocardioides* as *Nocardioides simplex* (12). *P. jensenii* was transferred to the genus *Nocardioides* as *Nocardioides jensenii*, and *P. tumescens* was reclassified in the new genus *Terrabacter* as *Terrabacter tumescens* by Collins et al. (3).

The genus *Nocardioides* was introduced with its type species, *Nocardioides albus*, in 1976 by Prauser (13) for gram-positive, non-acid-fast, aerobic, mesophilic nocardioform actinomycetes that form a mycelium which breaks up into irregular rod- to coccuslike fragments. The members of the genus characteristically contain LL-2,6-diaminopimelic acid in their cell walls (wall chemotype I) and lack mycolic acids. They have a complex cellular fatty acid profile containing iso-, anteiso-, straight-chain, unsaturated, and 10-methyl-branched acids. The major fatty acid characterizing the genus is iso-C_{16:0} (10, 12). The predominant isoprenoid quinone found in the genus *Nocardio-*

ides is a tetrahydrogenated menaquinone with eight isoprene units [MK-8(H₄)] (12, 27). There are currently five validly described *Nocardioides* species, namely, *N. albus* (13), *N. jensenii* (3), *Nocardioides luteus* (14), *Nocardioides plantarum* (4), and *N. simplex* (12). *Nocardioides fastidiosa* was created in 1989 by Collins and Stackebrandt (2) but was transferred to the genus *Aeromicrobium* (10) as *Aeromicrobium fastidiosum* in 1994 by Tamura and Yokota (27).

The aim of this study was to determine the exact taxonomic position of a bacterial strain previously described as *Pimelobacter* sp. strain OS4^T (8, 15) that is capable of degrading pyridine. We describe morphological, physiological, and biochemical characteristics of this organism and its phylogenetic position on the basis of its 16S ribosomal DNA (rDNA) sequence. On the basis of data described below, we propose that this organism should be placed in the genus *Nocardioides* as a new species, *Nocardioides pyridinolyticus*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The isolation of strain OS4^T was described in a previous study (8). Strain OS4^T was cultivated on nutrient agar (Difco) and minimal salts medium supplemented with 0.05% (wt/vol) yeast extract and 0.05% (wt/vol) tryptone. The minimal salts medium contained (per liter) 1.0 g of KH₂PO₄, 0.25 g of KCl, 0.25 g of MgSO₄ · 7H₂O, 1 ml of trace element solution (8), and 15 g of agar (if needed). Strain OS4^T and some reference strains were also grown at 30°C for 4 days on nutrient agar (Difco) for fatty acid methyl ester analysis. The following reference strains were used: *N. albus* KCTC 9186^T, *N. jensenii* KCTC 9134^T, *N. luteus* KCTC 9575^T, *N. simplex* KCTC 9106^T, *Aeromicrobium erythreum* NRRL B-3381^T, *Aeromicrobium fastidiosum* KCTC 9576^T, and *T. tumescens* KCTC 9133^T.

Morphological and physiological tests. The morphology of cells grown on minimal salts medium supplemented with 0.05% (wt/vol) yeast extract and 0.05% (wt/vol) tryptone was examined by phase-contrast microscopy. Motility was determined with an optical microscope by using the hanging drop technique (18). Flagellum type was examined with transmission electron microscopy by using cells from the late exponential phase of growth. The cells were negatively stained with 1% ammonium molybdate, and after air drying the grids were examined by using a model H-800 transmission electron microscope (Hitachi, Tokyo, Japan).

Catalase activity was determined by bubble production in a 3% hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1% tetramethyl-*p*-phenylenediamine on filter paper. Tests for utilization of various organic substrates as sole carbon and energy sources were performed as described previously (22). Most of the substrates were tested at a concentration of 1%

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(wt/vol); the exceptions were glycerol (0.1%, wt/vol), pyridine (0.01%, wt/vol), and phenol (0.01%, wt/vol). Other phenotypic tests were performed with the API 20NE system (BioMerieux, Marcy-l'Etoile, France).

Isolation of DNA. Chromosomal DNA was isolated and purified by the method described previously (33).

Determination of G+C content. The guanine-plus-cytosine (G+C) content was determined by the method of Tamaoka and Komagata (25). DNA was hydrolyzed and dephosphorylated, and the resultant nucleosides were analyzed by isocratic reverse-phase high-performance liquid chromatography (HPLC) by using an instrument equipped with a Novapak C₁₈ column (Millipore, Waters, Bedford, Mass.).

Analysis of isoprenoid quinones. Menaquinones were extracted and purified as described by Komagata and Suzuki (7) except for the use of an alternative solvent system for thin-layer chromatography; this solvent system was petroleum ether-diethyl ether (85:15, vol/vol). The purified menaquinones were dissolved in acetone and separated by isocratic reverse-phase HPLC by using methanol-isopropyl alcohol (50:50, vol/vol) as the eluting solvent.

Fatty acid methyl ester analysis. Bacterial cultures were harvested from nutrient agar plates for total fatty acid analysis. Fatty acids were extracted and analyzed by using the instructions of the Microbial Identification System (MIDI; Microbial ID, Inc., Newark, Del.).

Cell wall analysis. The diamino acid of the peptidoglycan was determined by the method described previously (7). The cell wall sugar analysis was performed at the whole-cell level by the method described previously (7).

16S rDNA sequence analysis. Two primers described previously (20), primers 9F (5'-GAGTTTGATCCTGGCTCAG-3'; positions 9 to 27 [*Escherichia coli* 16S rRNA numbering]) and 1542R (5'-AGAAAGGAGGTGATCCAGCC-3'; positions 1542 to 1525 [*E. coli* 16S rRNA numbering]), were used for amplification of the 16S rRNA gene. The 5' ends of the two primers were phosphorylated by using T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, Mass.) according to the instructions included with a Strandase ssDNA preparation kit (Novagen, Inc., Madison, Wis.). The 16S rRNA gene was amplified as described previously (34) by using phosphorylated primer 9F plus nonphosphorylated primer 1542R and phosphorylated primer 1542R plus nonphosphorylated primer 9F. The PCR products were precipitated with 1 µl of 1% (wt/vol) glycogen, 9 µl of 3 M sodium acetate (pH 5.2), and 70 µl of isopropanol and were resuspended in 10 µl of distilled water.

The strands containing phosphorylated primer from PCR products were selectively digested by using λ exonuclease according to the instructions included with the Strandase ssDNA preparation kit (Novagen, Inc.). The single-stranded DNA templates produced were directly used for the sequencing reaction. Sequencing was performed as described previously (6) by using α-³⁵S-labelled dATP and a DNA sequencing kit (U.S. Biochemicals, Cleveland, Ohio). The sequencing primers were derived from conserved regions of the 16S rRNA gene.

Phylogenetic analysis. The 16S rDNA sequence of strain OS4^T was aligned with the 16S rRNA and rDNA sequences of representatives of the genus *Nocardioide*s and some other actinomycete taxa by using CLUSTAL W software (28). Gaps at the 5' end and 3' end of the alignment were omitted from further analysis. Therefore, the region compared and used to calculate sequence similarity values and evolutionary distances corresponds to the region between positions 61 and 1484 (*E. coli* 16S rRNA numbering). Reference sequences were obtained from the EMBL and GenBank databases, where the nucleotide sequences are available under the following accession numbers: X53211 (*N. albus*), X53214 (*N. jensenii*), X53212 (*N. luteus*), X69973 (*N. plantarum*), M37200 (*Aeromicrobium erythreum*), X53189 (*Aeromicrobium fastidiosum*), M23411 (*Arthrobacter globiformis*), M38242 (*Micrococcus luteus*), X72377 (*Sporichthya polymorpha*), M76388 (*Streptomyces griseus* subsp. *griseus*), Y00484 ("Streptomyces lividans"), and X53215 (*T. tumescens*). The 16S rRNA sequence of *N. simplex* was obtained from the study of Collins et al. (3). 16S rDNA similarity values were calculated from the alignment, and evolutionary distances were calculated by using the Kimura two-parameter correction with the CLUSTAL W package (28). A phylogenetic tree was constructed by using the neighbor-joining method (16) on the basis of distance matrix data.

Nucleotide sequence accession number. The 16S rRNA gene sequence of strain OS4^T has been deposited in the GenBank database under accession no. U61298.

RESULTS

Morphological and physiological characteristics. Strain OS4^T is an aerobic, non-spore-forming, motile, gram-positive organism that is gram variable in old cultures. The colonies are smooth, round, raised, and cream colored on nutrient agar (Difco). They are approximately 1.0 to 1.5 mm in diameter after 5 days at 35°C on nutrient agar (Difco). This strain grew slowly on complex media such as Luria-Bertani medium and nutrient agar (Difco) but grew well on minimal salts medium supplemented with 0.05% (wt/vol) yeast extract and 0.05% (wt/vol) tryptone; a developed vegetative mycelium was not

formed. Single cells are rod shaped in the exponential phase of growth and bear a single polar flagellum (Fig. 1). The cells are 0.5 to 0.6 µm in diameter and 1.2 to 1.6 µm long. The cells show rod-to-coccus morphogenesis from the early exponential phase to the stationary phase and produce V-forms, indicating that postfission movement occurs during vegetative growth.

Strain OS4^T had catalase activity but no oxidase activity. Acid was not produced from glucose. Nitrate was reduced to nitrite. Strain OS4^T grew at pH 5 and 9 and at 20 and 40°C. The optimal temperature and pH were 35°C and 8.0, respectively.

Chemotaxonomic characteristics and DNA base composition. Strain OS4^T has the LL form of diaminopimelic acid as the diamino acid in its cell wall, indicating that the wall chemotype is type I. The major sugar found in the cell wall is galactose, and small amounts of glucose and ribose are also present.

The major menaquinone of strain OS4^T is a tetrahydrogenated menaquinone with eight isoprenoid units [MK-8(H₄)], and in addition, minor amounts of MK-7(H₄) and MK-8(H₂) are present.

The major cellular fatty acids are iso-C_{16:0} and 10-methyl-branched fatty acids which are characteristic of the genus *Nocardioide*s. Strain OS4^T also has significant amounts of anteiso-C_{17:0}, which is not found in other *Nocardioide*s species (Table 1).

The genomic DNA G+C content of strain OS4^T is 72.5 mol%, indicating that this strain belongs to the high-G+C-content group of gram-positive bacteria.

Phylogenetic analysis. The nearly complete 16S rRNA gene of OS4^T was amplified by PCR by using universal primers described previously (20) and was directly sequenced. The sequence of the 16S rRNA gene determined was 1,482 bp long and corresponded to the region between positions 28 and 1524 by comparison with the 16S rDNA of *E. coli*. The 16S rDNA sequence of strain OS4^T was compared with the 16S rDNA sequences of *Nocardioide*s species and some other actinomycete taxa, and the nucleotide similarity values and evolutionary distances were calculated from a 1,430-nucleotide region. Figure 2 shows a phylogenetic tree constructed by the neighbor-joining method on the basis of the evolutionary distance matrix. Isolate OS4^T forms a distinct lineage within the evolutionary radiation enclosed by the genus *Nocardioide*s.

DISCUSSION

The results of chemotaxonomic analyses and phylogenetic inferences based on 16S rDNA sequences indicate that strain OS4^T belongs to the genus *Nocardioide*s. It is a fact that morphological traits cause confusion when workers attempt to assign a new isolate to the genus *Nocardioide*s. Previous inclusion of *Pimelobacter* species in the genus *Nocardioide*s rendered the genus morphologically heterogeneous (24). *N. albus* (13) and *N. luteus* (14) produce a well-developed mycelium, whereas *N. jensenii* (3), *N. plantarum* (4), and *N. simplex* (12) form neither substrate mycelium nor primary mycelium. Consequently, morphological traits, as well as physiological properties, are not sufficient for definitive identification of strain OS4^T. Additionally, chemotaxonomic and phylogenetic analyses are necessary for exact identification of strain OS4^T.

Our cell wall analysis showed that strain OS4^T contained LL-diaminopimelic acid, which is the diagnostic diamino acid of wall chemotype I. Representative taxa containing LL-diaminopimelic acid in the cell wall include the genera *Intrasporangium*, *Kineosporia*, *Nocardioide*s, *Sporichthya*, *Streptomyces*, and *Terrabacter* (29), as well as the genera *Aeromicrobium* (10), *Luteococcus* (26), *Microlunatus* (11), *Propionibacterium* (1),

FIG. 1. Transmission electron micrograph of strain OS4^T from the exponential growth phase.

and *Propionifelix* (32). The menaquinone type is important in distinguishing the genus *Nocardioides* from other genera containing LL-diaminopimelic acid in the cell wall. Among the genera containing LL-diaminopimelic acid in the cell wall, only the genera *Nocardioides* and *Terrabacter* contain a tetrahydrogenated menaquinone with eight isoprenoid units [MK-8(H₄)] (12, 27). Although the genus *Terrabacter* has the same menaquinone type as the genus *Nocardioides*, it has been found to be phylogenetically distantly related to the genus *Nocardioides* (3). Previously, *Nocardioides* species have been shown to have MK-8(H₄) or MK-9(H₄) as their predominant menaquinone. However, *N. fastidiosa*, which has MK-9(H₄) as its predominant menaquinone, has recently been transferred to the genus *Aeromicrobium* as *Aeromicrobium fastidiosum* (27). Although the genus *Aeromicrobium* is phylogenetically related to the genus *Nocardioides* (10), it possesses a tetrahydrogenated menaquinone with nine isoprenoid units [MK-9(H₄)] as its predominant menaquinone (10, 27).

Complex fatty acid types have been found in some actinomycetes, including the genus *Nocardioides*. Fatty acid profiles also show that strain OS4^T is a member of the genus *Nocardioides*. The major fatty acids of strain OS4^T are iso-C_{16:0} and 10-methyl branched acids (Table 1). This is consistent with the fatty acid patterns of other *Nocardioides* species but different from the fatty acid patterns of *Aeromicrobium* species (10, 27). The genus *Aeromicrobium* has significant amounts of C_{16:0}, C_{18:1}, and 10-methyl-C_{18:0} as its major fatty acids (10). Therefore, the diagnostic fatty acids for distinction between the genera *Nocardioides* and *Aeromicrobium* are iso-C_{16:0}, C_{16:0}, C_{18:1}, and 10-methyl-C_{18:0} (Table 1).

Phylogenetic analysis based on 16S rDNA sequences is currently one of the most effective methods for estimating relationships between bacteria (21). A comparison of the 16S

rDNA sequence of strain OS4^T with the 16S rDNA sequences of previously described *Nocardioides* species and other actinomycete taxa showed that strain OS4^T belongs to the genus *Nocardioides* (Fig. 2). This phylogenetic inference is consistent with results obtained in the chemotaxonomic analyses. Strain OS4^T showed 16S rRNA and 16S rDNA similarity values of 94.5% ± 0.7% for a 1,430-nucleotide region with previously described *Nocardioides* species. A threshold value for 16S rRNA and 16S rDNA similarity values for species definition has been recognized (19). The phylogenetic definition of a species states that "strains with approximately 70% or greater DNA-DNA relatedness" are members of the same species (30, 31). According to Stackebrandt and Goebel (19), "organisms that have less than 97% sequence homology will not reassociate to more than 60%, no matter which hybridization method is applied." Therefore, strain OS4^T should be given status as a member of a new species in the genus *Nocardioides* on the basis of the 16S rDNA sequence analysis. The tree based on 16S rDNA sequences shows the phylogenetic position of strain OS4^T as a new species in the genus *Nocardioides* (Fig. 2).

On the basis of the data described above, we propose a new species, *Nocardioides pyridinolyticus*, for strain OS4^T. The properties of the species are summarized below.

Description of *Nocardioides pyridinolyticus* sp. nov. *Nocardioides pyridinolyticus* (py.ri.di.no.ly'ti.cus. M. L. n. pyridin, pyridine; M. L. adj. lyticus, dissolving; M. L. adj. pyridinolyticus, pyridine dissolving). Cells are aerobic, non-spore-forming rods that are 0.5 to 0.6 μm wide and 1.2 to 1.6 μm long in the exponential phase of growth. Cells exhibit rod-to-coccus morphogenesis from the early exponential phase to the stationary phase. Gram positive and gram variable in old cultures. Motile by means of a single polar flagellum. Colonies are smooth,

TABLE 1. Cellular fatty acid profiles of *N. pyridinolyticus* OS4^T, representatives of the genera *Nocardioide*s and *Aeromicrobium*, and *T. tumescens*

Fatty acid	% in:							
	<i>Nocardioide</i> s <i>albus</i> KCTC 9186 ^T	<i>Nocardioide</i> s <i>jensenii</i> KCTC 9134 ^T	<i>Nocardioide</i> s <i>luteus</i> KCTC 9575 ^T	<i>Nocardioide</i> s <i>simplex</i> KCTC 9106 ^T	<i>Nocardioide</i> s <i>pyridinolyticus</i> OS4 ^T	<i>Aeromicrobium</i> <i>erythreum</i> NRRL B-3381 ^T	<i>Aeromicrobium</i> <i>fastidiosum</i> KCTC 9576 ^T	<i>Terrabacter</i> <i>tumescens</i> KCTC 9133 ^T
Saturated fatty acids								
C _{14:0}						0.7	0.6	7.0
C _{15:0}	0.7	0.3			0.4	1.7	1.0	4.9
C _{16:0}	0.7	1.3	0.6	1.1	1.2	20.0	10.1	12.5
C _{17:0}	1.5	2.7	0.5		1.2	2.4	2.1	5.3
C _{18:0}		2.0		1.3	1.0	6.1	9.7	7.8
C _{19:0}								1.6
Unsaturated fatty acids								
C _{15:1} ω6c		0.3						0.9
C _{16:1} ω9c						0.8	1.4	0.3
C _{17:1} ω6c	15.6	5.2	15.9	13.1		0.6		
C _{17:1} ω8c	2.8	3.4	1.1	2.3	1.7	0.8		5.8
C _{17:1} ω9c							0.9	
C _{18:1} ω9c		3.3		7.3	2.0	25.6	25.1	13.9
C _{19:1} iso I				1.6				
Branched fatty acids								
iso-C _{14:0}	2.2	1.1	0.7	1.6	0.5			4.3
iso-C _{15:0}	3.1	3.7	3.7	1.5	5.1			8.3
anteiso-C _{15:0}					1.4			2.1
iso-C _{14:0} 3-OH			0.6					
iso-C _{16:1} H	1.1	14.0	2.1	5.4	4.2			1.0
iso-C _{16:0}	53.6	45.5	49.1	33.7	47.3	1.4		5.6
iso-C _{17:0}	1.3	1.7	1.0	2.2	1.7			
iso-C _{17:1} ω9c						0.4	0.7	
anteiso-C _{17:1} ω9c					2.12			
anteiso-C _{17:0}	0.7	0.7	1.4	0.5	14.2			1.1
iso-C _{18:0}	0.9	2.7		0.9	0.5	0.5		
10-Methyl-fatty acids								
C _{16:0}	1.5	2.5	5.0	4.0	5.0			2.8
C _{17:0}	12.2	5.4	15.0	5.4	5.5	0.3		1.2
C _{18:0}	2.3	2.4	2.9	9.3	3.4	24.2	32.9	1.0
Hydroxy fatty acids								
C _{15:0} 2-OH						0.6	0.6	
C _{16:0} 2-OH				0.7		11.3	8.9	
C _{17:0} 2-OH						0.9	1.5	
C _{18:0} 2-OH						1.1	3.7	
Summed features ^a								
Summed feature 4		1.2		5.1	0.8		0.9	8.1
Summed feature 6				0.7				1.7
Summed feature 7				2.4				1.0
Summed feature 8					0.5			1.9

^a Summed features represent groups of two or three fatty acids which could not be separated by gas-liquid chromatography with the MIDI system. Summed feature 4 contained one or more of the following fatty acids: iso-C_{15:0} 2-OH and C_{16:1} ω7c. Summed feature 6 contained one or more of the following fatty acids: anteiso-C_{18:0} and C_{18:2} ω6,9c. Summed feature 7 contained one or more of the following fatty acids: C_{18:1} ω7c, C_{18:1} ω9t, and C_{18:1} ω12t. Summed feature 8 contained one or more of the following fatty acids: unknown 18.756 and C_{19:1} ω11c.

round, and cream colored on nutrient agar. They are approximately 1.0 to 1.5 mm in diameter after 5 days at 35°C. Neither substrate mycelium nor primary mycelium is formed. Strain OS4^T grows at pH 5 and 9 and at 20 and 40°C. The optimal growth temperature and pH for strain OS4^T are 35°C and 8.0, respectively. Catalase positive. Oxidase negative. Acid is not produced from glucose. Esculin and gelatin are hydrolyzed. Indole is not produced. Nitrate is reduced to nitrite. D-Glucose, D-ribose, D-xylose, D-fructose, D-galactose, D-mannose, L-rhamnose, D-cellobiose, lactose, maltose, sucrose, D-treha-

lose, *myo*-inositol, D-mannitol, pyridine, and phenol are utilized as sole carbon and energy sources for growth. L-Arabinose, D-melibiose, D-raffinose, adonitol, D-sorbitol, inulin, and glycerol are not utilized.

The cell wall peptidoglycan contains LL-diaminopimelic acid as the diamino acid (wall chemotype I). The predominant menaquinone is MK-8(H₄), and minor amounts of MK-7(H₄) and MK-8(H₂) are also present. The cellular fatty acids are composed of saturated, unsaturated, iso-, anteiso- and 10-methyl-branched types, and the major fatty acids are iso-C_{16:0}

FIG. 2. Unrooted phylogenetic tree showing the positions of *N. pyridinolyticus* OS4^T, other representatives of the genus *Nocardioides*, and some other actinomycete taxa.

and 10-methyl-branched acids. The G+C content of the DNA is 72.5 mol% (as determined by HPLC). Isolated from the oxic zone of an oil shale column. The type strain is OS4, which has been deposited in the Korean Collection for Type Cultures as KCTC 0074BP.

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