***Nocardioides nitrophenolicus* sp. nov., a *p*-nitrophenol-degrading bacterium**

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A *p*-nitrophenol-degrading bacterial strain was isolated from industrial wastewater. This strain (NSP41^T) was identified as a member of the genus *Nocardioides* from chemotaxonomic characterizations and phylogenetic inference based on 16S rDNA sequence analysis. The G+C content is 71.4 mol%. The diamino acid in the cell-wall peptidoglycan is LL-diaminopimelic acid. The predominant menaquinone is MK-8(H₄). The cellular fatty acid profile is similar to those of *Nocardioides* species. 16S rDNA sequence analysis show that strain NSP41^T is the most related to *Nocardioides simplex* strains with a level of nucleotide similarity of 98.6%. The levels of 16S rDNA similarity between strain NSP41^T and other *Nocardioides* species ranged from 93.8 to 95.1%. This organism is distinguishable from some other *Nocardioides* species as well as *N. simplex* strains by DNA–DNA relatedness data. This organism is different from *N. simplex* strains in some phenotypic characteristics. Therefore, on the basis of the data presented, a new species of the genus *Nocardioides*, *Nocardioides nitrophenolicus*, is proposed. The type strain of the new species is strain NSP41^T (= KCTC 0457BP^T).

Keywords: *Nocardioides nitrophenolicus*, *p*-nitrophenol degradation

INTRODUCTION

Biotechnologically important *Nocardioides* species have been described previously (Arima *et al.*, 1969; Gundersen & Jensen, 1956; Yoon *et al.*, 1997), and recently we isolated a *Nocardioides*-like isolate (strain NSP41^T) which simultaneously degrades phenol and *p*-nitrophenol. From 16S rDNA sequence analysis (Yoon *et al.*, 1998a), a strain, NSP41^T, was shown to be the most related to *Nocardioides simplex* strains by its nucleotide similarity of 98.6%. However, all of the strains that have been identified as *N. simplex* showed identical 16S rDNA sequences. Recently, 16S to 23S rDNA internally transcribed spacer (16S–23S ITS) sequence analysis showed that strain NSP41^T was distinguished from *N. simplex* strains in length and sequence variability of 16S–23S ITS. The 16S–23S ITS of strain NSP41^T was 328 bp long, whereas the 16S–23S ITSs of *N. simplex* strains were 386 to 388 bp long. Strain NSP41^T exhibited levels of 16S–23S ITS

similarity of 81.8–84.6% and levels of 16S–23S ITS similarity with gaps included of 68.4–70.1% with *N. simplex*, whereas the levels of 16S–23S ITS similarity between *N. simplex* strains ranged from 97.7% to 100%. Strain NSP41^T's ability to degrade *p*-nitrophenol was not found in *N. simplex* strains. Therefore, it was necessary to determine the exact taxonomic position of strain NSP41^T through detailed taxonomic study. On the basis of data described below, we propose that this strain (NSP41^T) should be placed in the genus *Nocardioides* as a new species, *Nocardioides nitrophenolicus*.

METHODS

Bacterial strain and culture conditions. Strain NSP41^T was isolated from an industrial wastewater. The wastewater samples were inoculated in 50 ml minimal salts medium (Yoon *et al.*, 1997) containing (l⁻¹) 1 g K₂HPO₄, 0.5 g NaH₂PO₄·2H₂O, 0.25 g KCl, 0.25 g MgSO₄·7H₂O, 1 ml trace element solution (Lee *et al.*, 1991) supplemented with 100 mg *p*-nitrophenol l⁻¹ and 15 g agar (if needed). This medium was incubated at 30 °C on a horizontal shaker at 150 r.p.m. When the yellow colour of the medium disappeared, 5 ml of the suspension was transferred into 50 ml fresh medium. The suspension was plated on solid media

Abbreviations: FAME, fatty acid methyl ester; ITS, internally transcribed spacer.

The GenBank accession number for the 16S rDNA sequence of strain NSP41^T is AF005024.

containing *p*-nitrophenol in order to isolate pure cultures. Single colonies were tested to investigate the ability to utilize *p*-nitrophenol as a sole carbon and energy sources for growth. Among isolates, one strain, NSP41^T, that showed high *p*-nitrophenol-degrading activity was selected for further studies.

For the investigation of morphological and physiological characteristics, strain NSP41^T was cultivated on nutrient agar (Difco) at 30 °C for 36–48 h. Cell mass for the analyses of cell wall and menaquinones was obtained from nutrient broth (Difco) culture at 30 °C. For fatty acids methyl ester (FAME) analysis, strain NSP41^T and reference strains also were grown for 4 d on nutrient agar (Difco). Reference strains for FAME analysis included *Nocardioideis albus* KCTC 9186^T (T = Type strain), *Nocardioideis jensenii* KCTC 9134^T, *Nocardioideis luteus* KCTC 9575^T, *Nocardioideis simplex* strains KCTC 9106^T, JCM 1366, JCM 1367, NCIMB 12919 and ATCC 15799, *Aeromicrobium erythreum* NRRL B-3381^T and *Aeromicrobium fastidiosum* KCTC 9576^T.

Morphological and physiological tests. The morphology of cells grown was examined by light microscopy. Motility was determined with an optical microscope using the hanging drop technique (Skerman, 1967). Flagellum type was examined with transmission electron microscopy using cells from the exponential phase of growth. The cells were negatively stained with 1% (w/v) phosphotungstic acid, and after air-drying the grids were examined by using a model CM-20 transmission electron microscope (Philips). Catalase activity was determined by bubble production in a 3% hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1% tetramethyl-*p*-phenylenediamine. Hydrolyses of casein and starch, and production of urease were determined as described previously (Cowan & Steel, 1965). Hydrolyses of aesculin and arbutin were determined according to the methods of Kurup & Fink (1975). Hydrolysis of elastin was determined according to the method of Williams *et al.* (1983). Tests for utilization of various substrates as sole carbon and energy sources were performed as described previously (Shirling & Gottlieb, 1966). Most of the substrates were tested at a concentration of 1% (w/v); the exception was glycerol (0.1%, w/v). The utilization results were checked over a period of 4 weeks.

Isolation of DNA. Chromosomal DNA was isolated and purified according to the method described previously (Yoon *et al.*, 1996).

Chemotaxonomic characterizations. The diamino acid of the peptidoglycan was determined by the method described previously (Komagata & Suzuki, 1987). Menaquinones were analysed as described previously (Komagata & Suzuki, 1987) using reversed-phase HPLC. Fatty acids were extracted and analysed according to the instructions of the Microbial Identification System (MIDI).

Determination of G+C content. The G+C content was determined by the method of Tamaoka & Komagata (1984). DNA was hydrolysed and dephosphorylated, and the resultant nucleosides were analysed by reversed-phase HPLC.

Phylogenetic analysis based on 16S rDNA sequences. The determination of 16S rDNA sequence was described previously (Yoon *et al.*, 1998a). The 16S rDNA sequence of strain NSP41^T was aligned with 16S rRNA gene sequences of *Nocardioideis* species and some other actinomycete taxa by using CLUSTAL W software (Thompson *et al.*, 1994). Other reference sequences were obtained from the GenBank database with the following accession numbers: *Nocar-*

dioides albus KCTC 9186^T (AF004988), *Nocardioideis jensenii* KCTC 9134^T (AF005006), *Nocardioideis luteus* KCTC 9575^T (AF005007), *Nocardioideis plantarum* KCTC 9577^T (AF005008), *Nocardioideis simplex* KCTC 9106^T (AF005009), *Aeromicrobium erythreum* NRRL B-3381^T (AF005021), *Aeromicrobium fastidiosum* KCTC 9576^T (AF005022), *Terrabacter tumescens* KCTC 9133^T (AF005023), *Microtholunatus phosphovorus* DSM 10555^T (Z78207), *Luteococcus japonicus* DSM 10546^T (Z78208), *Friedmanniella antarctica* DSM 11053^T (Z78206), *Intrasporangium calvum* IFO 12989^T (D85486), *Kineosporia aurantiaca* ATCC 28727^T (X87110), *Terracoccus luteus* DSM 44267^T (Y11928), *Sporichthya polymorpha* DSM 46113 (X72377), *Propionibacterium freudenreichii* DSM 20271^T (X53217), *Propionibacterium acnes* DSM 1897^T (X53218), *Streptomyces albus* DSM 40313^T (X53163), *Arthrobacter globiformis* DSM 20124^T (M23411), *Micrococcus luteus* ATCC 381 (M38242), *Rhodococcus rhodochrous* strain 116 (Karlsen *et al.*, 1993) (X70295), *Nocardia asteroides* ATCC 19247^T (Z36934) and *Sphaerobacter thermophilus* DSM 20745^T (X53210). The 16S rRNA sequence of *Propionifera innocua* NCTC 11082^T was obtained from the study of Pitcher & Collins (1991). The 16S rDNA similarity values were calculated from the alignment and the evolutionary distances were calculated by using Kimura two-parameter correction with CLUSTAL W software. A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) on the basis of distance matrix data. A bootstrap analysis with 1000 replications for evaluating the topology of phylogenetic tree was performed with CLUSTAL W.

DNA–DNA relatedness test. *N. albus* KCTC 9186^T, *N. jensenii* KCTC 9134^T, *N. luteus* KCTC 9575^T, *N. simplex* strains KCTC 9106^T, JCM 1366, JCM 1367, NCIMB 12919 and ATCC 15799 were used as reference strains for DNA–DNA hybridization. DNA–DNA hybridization to determine genomic relatedness was performed by dot-blot hybridization method. Labelling of probe and visualization of hybridized DNA were performed by using a non-radioactive ECL direct nucleic acid labelling and detection system (Amersham International). Resultant quantitative DNA–DNA relatedness was determined by using a densitometer (Bio-Rad).

RESULTS

Morphological and physiological characteristics

Strain NSP41^T is aerobic, non-spore-forming, non-acid-fast and motile, and it is Gram-positive but Gram-variable in old cultures. After 2 d cultivation on nutrient agar, single cells are rod-shaped that are 0.5–0.8 µm wide by 1.0–3.0 µm long (Fig. 1). Single cells bear a single polar flagellum (Fig. 1), whereas *N. simplex* strains KCTC 9106^T, JCM 1366 and JCM 1367 are known to bear peritrichous flagella (Suzuki & Komagata, 1983). Single cells show a coccoid form in old cultures. The cells exhibit rod-coccus morphogenesis from the early-exponential phase to the stationary phase. The colonial morphology is nearly identical with that of type strain of *N. simplex* on nutrient agar. Colonies are smooth, glossy and yellowish white with irregular edges on nutrient agar. Neither substrate mycelium nor primary mycelium is formed. Strain NSP 41^T grew at pH 6 and 10, but it did not grow at pH 5. Strain NSP 41^T grew at 20 and 40 °C

Fig. 1. Transmission electron micrograph of strain NSP41^T grown on nutrient agar for 48 h at 30 °C. Bar, 0.5 µm.

and weakly at 15 °C, but it did not grow at 45 °C. The optimal pH and temperature for growth were 8 and 30 °C, respectively. Some other physiological properties of strain NSP41^T are represented in Table 1, together with those of *N. simplex* strains.

Chemotaxonomic characteristics and DNA base composition

Strain NSP41^T had cell-wall peptidoglycan based on LL-diaminopimelic acid as the diamino acid. Tetrahydrogenated menaquinone with eight isoprenoid units [MK-8(H₄)] was predominant in strain NSP41^T. The major fatty acids were iso-C_{16:0} and 10-methyl-branched fatty acids, which are characteristically found as major components in the genus *Nocardioides*

(Table 2). Strain NSP41^T also had relatively high proportions of iso-C_{17:0} and anteiso-C_{17:0} when compared to *N. simplex* strains and other *Nocardioides* species (Table 2).

The genomic DNA G+C content of strain NSP41^T was 71.4 mol%. *Nocardioides* species are known to have genomic DNA G+C content ranging from 66.5 mol% (*N. albus*) to 73.0 mol% (*N. simplex*).

Phylogenetic analysis

The phylogenetic inference based on 16S rDNA sequence of strain NSP41^T were performed in a previous study. Strain NSP41^T formed a phylogenetic lineage within the cluster enclosed by the genus *Nocardioides*, and is phylogenetically related to *N. simplex*. The cluster of strain NSP41^T and *N. simplex* was supported by 100% in bootstrap analysis (Fig. 2). Strain NSP41^T exhibited levels of 16S rDNA similarity of 98.6% to *N. simplex* strains and levels of 16S rDNA similarity of 93.8 to 95.1% to the type strains of other validly described *Nocardioides* species except *N. simplex*.

DNA–DNA relatedness test

Strain NSP41^T exhibited levels of DNA–DNA relatedness of 41% to the type strain of *N. simplex* and of 35–43% to other *N. simplex* strains, whereas the type strain of *N. simplex* exhibited levels of DNA–DNA relatedness of 85–103% to other strains of *N. simplex*. Strain NSP41^T also exhibited levels of DNA–DNA relatedness of 27, 38 and 36%, to *N. albus* KCTC 9186^T, *N. jensenii* KCTC 9134^T and *N. plantarum* NCIMB 12834^T, respectively.

Table 1. Physiological characteristics of *N. simplex* strains and strain NSP41^T

+, Positive reaction; –, negative reaction; w, weakly positive reaction. All strains were positive for oxidase, catalase, hydrolysis of aesculin, starch and Tween 80, utilization of (+)-D-glucose, sucrose, (–)-D-trehalose, disodium fumarate and disodium succinate as sole carbon and energy source. All strains were negative for hydrolysis of elastin, utilization of L-arabinose, (+)-D-galactose, (+)-D-cellobiose, lactose, maltose, melezitose, α-D-melibiose, (+)-D-raffinose, adonitol, myo-inositol, D-mannitol, D-sorbitol, inulin, glycerol, CM-cellulose, trisodium citrate and sodium citrate as sole carbon and energy source.

Character	KCTC 9106 ^T	JCM 1366	JCM 1367	NCIMB 12919	ATCC 15799	NSP41 ^T
Hydrolysis of:						
Arbutin	–	–	–	w	–	w
Casein	+	+	+	w	+	+
Urea	–	–	–	–	–	+
Utilization of as sole carbon and energy source:						
(–)-D-Ribose	w	–	–	–	w	+
(+)-D-Xylose	–	–	–	–	–	+
(–)-D-Fructose	–	–	–	–	–	+
(+)-D-Mannose	–	–	–	–	–	w
α-(+)-L-Rhamnose	–	–	–	–	–	+
Sodium acetate	+	+	+	–	–	+
p-Nitrophenol	–	–	–	–	–	+

Table 2. Cellular fatty acid profiles of representatives of the genus *Nocardioides*, *Nocardioides* sp. strain NSP41^T and *Aeromicrobium* species

Strains: 1, *Nocardioides albus* KCTC 9186^T; 2, *Nocardioides jensenii* KCTC 9134^T; 3, *Nocardioides luteus* KCTC 9575^T; 4, *Nocardioides simplex* strains KCTC 9106^T; 5, JCM 1366; 6, JCM 1367; 7, NCIMB 12919; 8, ATCC 15799; 9, *Nocardioides* sp. NSP41; 10, *Aeromicrobium erythreum* NRRL B-3381^T; 11, *Aeromicrobium fastidiosum* KCTC 9576^T.

Fatty acid	Strain										
	1	2	3	4	5	6	7	8	9	10	11
Saturated fatty acids											
C _{14:0}										0.7	0.6
C _{15:0}	0.7	0.3								1.7	1.0
C _{16:0}	0.7	1.3	0.6	1.1	1.4	2.0	2.0	1.1	2.3	20.0	10.1
C _{17:0}	1.5	2.7	0.5		1.1	0.9	0.6		0.9	2.4	2.1
C _{18:0}		2.0		1.3	2.6	2.9	3.0	0.9		6.1	9.7
C _{19:0}											
C _{20:0}											
Unsaturated fatty acids											
C _{15:1} ω6c		0.3									
C _{16:1} ω9c										0.8	1.4
C _{17:1} ω6c	15.6	5.2	15.9	13.1	14.5	11.9	13.4	14.8	10.8	0.6	
C _{17:1} ω8c	2.8	3.4	1.1	2.3	2.7	2.3	1.3	3.6	1.4	0.8	
C _{17:1} ω9c											0.9
C _{18:1} ω9c		3.3		7.3	12.0	9.2	8.5	7.9	4.5	25.6	25.1
C _{19:1} iso I*				1.6				1.1			
Branched-chain fatty acids											
iso-C _{13:0}					0.3						
iso-C _{14:0}	2.2	1.1	0.7	1.6	0.4	0.6	0.6	1.4	0.4		
iso-C _{14:0} 3-OH			0.6								
iso-C _{15:0}	3.1	3.7	3.7	1.5	1.6	1.7	2.1	1.6	3.1		
anteiso-C _{15:0}									0.5		
iso-C _{16:1} H*	1.1	14.0	2.1	5.4	2.8	2.8	3.3	6.6	1.3		
iso-C _{16:0}	53.6	45.5	49.1	33.7	29.0	36.6	32.3	30.5	28.1	1.4	
iso-C _{17:0}	1.3	1.7	1.0	2.2	6.1	6.4	5.4	2.2	12.3		
iso-C _{17:1} ω9c										0.4	0.7
anteiso-C _{17:1} ω9c											
anteiso-C _{17:0}	0.7	0.7	1.4	0.5	0.8	1.5	1.0		5.8		
iso-C _{18:0}	0.9	2.7		0.9	4.0	3.4	3.8	0.8	1.6	0.5	
10-Methyl fatty acids											
C _{16:0}	1.5	2.5	5.0	4.0	1.1	1.2	1.3	4.2	3.6		
C _{17:0}	12.2	5.4	15.0	5.4	3.6	2.6	2.4	6.3	5.1	0.3	
C _{18:0}	2.3	2.4	2.9	9.3	12.7	10.0	15.3	10.3	13.9	24.2	32.9
Hydroxy fatty acids											
C _{15:0} 2-OH										0.6	0.6
C _{16:0} 2-OH				0.7	0.5	0.4	0.5	1.0	0.7	11.3	8.9
C _{17:0} 2-OH									0.4	0.9	1.5
C _{18:0} 2-OH										1.1	3.7
Summed features†											
4		1.2		5.1	1.6	1.9	2.0	3.7	1.8		0.9
5											
6				0.7		0.3	0.3	0.5	0.4		
7				2.4	0.8	1.2	0.9	1.7	0.9		
8					0.5	0.3			0.4		

* The double bond position indicated by an upper-case letter is unknown.

† Summed features represent groups of two or three fatty acids which could not be separated by GLC with the MIDI system. Summed feature 4 contained one or more of following fatty acids: 15:0 iso 2-OH and/or 16:1 ω7c. Summed feature 6 contained one or more of following fatty acids: 18:0 anteiso and/or 18:2 ω6,9c. Summed feature 7 contained one or more of following fatty acids: 18:1 ω7c, 18:1 ω9t and/or 18:1 ω12t. Summed feature 8 contained one or more of following fatty acids: unknown 18:756 and/or 19:1 ω11c.

Fig. 2. Phylogenetic tree based on 16S rDNA sequences showing the position of strain NSP41^T, other representatives of the genus *Nocardioides* and some other actinomycete taxa.

DISCUSSION

The chemotaxonomic properties of strain NSP41^T were compared with those of LL-diaminopimelic acid-containing actinomycete taxa. The comparison showed that strain NSP41^T has characteristics that are consistent with the genus *Nocardioides* in menaquinone composition and fatty acid profiles (Tamura & Yokota, 1994). Therefore, strain NSP41^T was considered to be a member of the genus *Nocardioides*. From phylogenetic inference based on 16S rDNA sequences, strain NSP41^T was considered as being related to *N. simplex* (Fig. 2). However, *N. simplex* did not exhibit intraspecific variation in 16S rDNA sequence, that is, *N. simplex* strains KCTC 9106^T, JCM 1366, JCM 1367, NCIMB 12919 and ATCC 39419 were found to have identical 16S rDNA sequences, whereas strain NSP41^T exhibited a level of 16S rDNA similarity of 98.6% to *N. simplex* strains (Yoon *et al.*, 1998a). The 16S–23S ITS sequence analysis also encouraged us to investigate the taxonomic position of strain NSP41^T in detail (Yoon *et al.*, 1998b).

Strain NSP41^T formed a cluster with *N. simplex* by 100% recovery in the bootstrap analysis (Fig. 2). Therefore, strain NSP41^T was mainly compared with *N. simplex* strains in some taxonomic properties. Some physiological characteristics and flagellum type of strain NSP41^T differed from those of *N. simplex* strains (Table 1). In particular, strain NSP41^T has the ability to degrade *p*-nitrophenol which is not found in *N.*

simplex strains. Strain NSP41^T was found to degrade up to 200 mg *p*-nitrophenol l⁻¹, whereas *N. simplex* strains could not degrade even minimum concentrations of *p*-nitrophenol. The fatty acid profile of strain NSP41^T showed differences from other *Nocardioides* species as well as *N. simplex* strains in the composition of iso-C_{17:0} and anteiso-C_{17:0} (Table 2). It has been already pointed out that strain NSP41^T is distinguished from *N. simplex* strains in length and sequence variability of the 16S–23S ITS region (Yoon *et al.*, 1998b). DNA–DNA relatedness provides final evidence that strain NSP41^T is a new species of the genus *Nocardioides*, since it has been recognized as an important criterion for defining species in current bacteriology (Wayne *et al.*, 1987). There is general agreement that a level of DNA–DNA relatedness of 70% is the threshold value for defining species.

On the basis of the data described above, we propose a new species, *Nocardioides nitrophenolicus*, for strain NSP41^T. The properties of the species are summarized below.

Description of *Nocardioides nitrophenolicus* sp. nov.

Nocardioides nitrophenolicus (ni.tro.phen.o'li.cus. L. masc. adj. *nitro* containing nitrogen; N.L. n. *pheno* phenol; L. fem. adj. *nitrophenolicus* relating to nitrophenols).

Cells are aerobic, non-acid-fast, non-spore-forming

rods that are 0.5–0.8 µm wide and 1.0–3.0 µm long in 2 d culture on nutrient agar. Cells exhibit coccoid morphology in the stationary phase of growth. Gram-positive but Gram-variable in old cultures. Motile by means of a single polar flagellum. Colonies are smooth, glossy and yellowish white with irregular edges on nutrient agar. Neither substrate mycelium nor primary mycelium is formed. Growth occurs at pH 6 and 10 and at 15 and 40 °C. Optimal pH and temperature for growth are 8 and 30 °C, respectively. Catalase- and oxidase-positive. Casein, aesculin, starch, Tween 80 and urea are hydrolysed. Arbutin is weakly hydrolysed. Elastin is not hydrolysed. D-Ribose, D-xylose, D-fructose, D-glucose, D-mannose, L-rhamnose, sucrose, D-trehalose, disodium fumarate, disodium succinate and sodium acetate are utilized as sole carbon and energy sources for growth. L-Arabinose, D-galactose, D-mannose, D-cellobiose, lactose, maltose, melzitose, D-melibiose, D-raffinose, adonitol, *myo*-inositol, D-mannitol, D-sorbitol, inulin, glycerol, carboxymethyl cellulose, trisodium citrate and sodium benzoate are not utilized. Degrades phenol and *p*-nitrophenol. The G + C content is 71.4 mol % (as determined by HPLC). Isolated from industrial wastewater in Cheong-ju, Korea. The type strain is strain NSP41^T, which has been deposited in the Korean Collection for Type Cultures as KCTC 0457BP^T.

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