ORIGINAL ARTICLE

Nonomuraea thailandensis sp. nov. isolated from Thai soil

Paranee Sripreechasak¹, Somboon Tanasupawat¹, Khanit Suwanborirux², Yuki Inahashi³, Atsuko Matsumoto³, Kazuro Shiomi^{3,4} and Yoko Takahashi^{3,4}

A novel actinomycete, strain KC-061^T was isolated from a soil sample collected from Angthong Islands National Park, Surat Thani province, Thailand. Strain KC-061^T was identified as a member of the genus *Nonomuraea* through the use of a polyphasic approach. Phylogenetic analyses based on 16S rRNA gene sequencing, strain KC-061^T belongs to the genus *Nonomuraea* and was closely related to '*N. monospora*' PT708^T (99.3%), *N. rhizophila* YIM 67092^T (98.6%), *N. dietziae* DSM 44320^T (98.5%) and *N. rosea* GW 12687^T (98.3%). On the basis of phenotypic characteristics, DNA–DNA relatedness and phylogenetic distinctiveness, the novel isolate was identified as representing a novel species of the genus *Nonomuraea*, for which the name *Nonomuraea thailandensis* sp. nov. (type strain KC-061^T = JCM 18408^T = KCTC 29074^T = PCU 327^T) is proposed.

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INTRODUCTION

The genus *Nonomuraea* belongs to the family *Streptosporangiaceae*, which was first proposed by Zhang *et al.*¹ Members of the genus *Nonomuraea* are aerobic, Gram-positive, non-acid-fast, non-motile actinomycetes that can form extensively branched substrate and aerial mycelia. At the time of writing, 30 *Nonomuraea* species names and 2 recognized subspecies have been validly published.² In addition, some new species have been described in the past few years, namely *N. antimicrobica*,³ *N. candida*,⁴ *N. endophytica*,⁵ *N. maritima*,⁶ *N. rhizophila*,⁷ *N. rosea*,⁸ *N. wenchangensis*,⁹ *N. jiangxiensis*¹⁰ and *N. soli*.¹¹ It has been reported that the strains that belong to the genus *Nonomuraea* produce various types of secondary metabolites; for example, glycopeptide antibiotic A40926,^{12,13} myxochelin A,¹⁴ anthelmintic macrolactams, fluvirucin B0, Sch 38516/fluvirucin B1 and Sch 39185/fluvirucin B3,¹⁵ a novel cyclic tetrapeptide WSS2220¹⁶ and new cyclic tetrapeptides.¹⁷ The strain KC-061^T was isolated from the southern area of Thailand, which also produce madurahydroxylactone.^{18,19} In this paper, we describe the characterization and classification of the strain in the genus *Nonomuraea*.

RESULTS AND DISCUSSION

Morphological, cultural, physiological and biochemical characteristics

Strain KC-061^T grew well on ISP 2, 3, 4, 5 and 7 media, YS agar and nutrient agar. The aerial mycelia appeared white on ISP 2 medium,

whitish purple on ISP 3 medium and pale orange on ISP 5 and 7 media. No aerial mycelia were formed when the strain cultivated on ISP 4 and 6 media, YS agar and nutrient agar. The substrate mycelium branched extensively and the colors on various media were reddish brown to yellowish brown. Brown soluble pigments on ISP 2 and 3 media, YS agar and nutrient agar were produced (Table 1). The aerial hyphae produced long and spiraled spore chains, which had spiral bearing > 10 spores (Figure 1). The strain grew at 14 to 38°C (optimally at 20 to 30°C) and at pH 6.0 to 11.0 (optimally pH at pH 7 to 9) and tolerated up to 4% NaCl. Nitrate was reduced to nitrite. Casein and starch were hydrolyzed but gelatin was not liquefied. Milk was peptonized weakly and was not coagulated. The strain KC-061^T utilized D-glucose, myo-inositol, D-mannitol, melibiose, raffinose, L-rhamnose and D-xylose but did not utilize sucrose. Enzyme activities of the API ZYM system were positive for alkaline phosphatase, leucine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrazide, α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase and weakly positive for esterase (C4), esterase lipase (C8), valine arylamidase and α-mannosidase but negative for cystine arylamidase, trypsin, α-galactosidase and β-galactosidase (Table 2).

Chemotaxonomy

Strain KC-061^T contained *meso*-diaminopimelic acid isomer as diagnostic diamino acid and galactose, mannose, madurose and

¹Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand; ²Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand; ³Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan and ⁴Graduate School of Infection Control Sciences, Kitasato University, Tokyo, Japan

Correspondence: Dr S Tanasupawat, Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand. E-mail: somboon.t@chula.ac.th

or Dr Y Takahashi, Kitasato Institute for Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan.

E-mail: ytakaha@iisci.kitasato-u.ac.jp

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Table 1 Cultural characteristics of strain KC-061^T and closely related type strains

Medium	KC-061 ^T	<i>N. monospora</i>	<i>N. rhizophila</i>	<i>N. dietziae</i>	<i>N. rosea</i>
<i>ISP medium 2</i>					
Growth	Good Dark wine (7pi)	Good Deep red brown (6 ^{1/2} pi)	Good Camel (3ie)	Good Light tan (3gc) to tile red (5ne)	Good Bamboo (2gc) and light rose brown (7lg)
Reverse	Red mahogany (6 ^{1/2} pi)	Wine (7 pg)	Bamboo (2gc)	Light tan (3gc) to rust tan (5le)	Bamboo (2gc) and light rose brown (7lg)
Aerial mycelium	Rare, white (a)	Abundance, orchid mist (10cb)	Abundance, white (a)	Moderate, light tan (3gc)	None
Soluble pigment	Terra cotta (5pe)	Bittersweet (5pc)	None	None	None
<i>ISP medium 3</i>					
Growth	Good Tile red (5ne) to brown mahogany (6pi)	Good Burnt orange (5nc)	Good Pearl (3ba) to mustard (2le)	Good Cinnamon (3le) to light brown (4ng)	Good Dark orchid taupe (9nl)
Reverse	Tile red (5ne)	Burnt orange (5nc)	Light ivory (2ca)	Cinnamon (3le)	Eggplant (9ni) to dark orchid taupe (9nl)
Aerial mycelium	Abundance, orchid mist (10cb)	Moderate, dawn pink (7dc)	Abundance, pearl (3ba)	Abundance, orchid tint (10ba)	Poor, ashes (5fe)
Soluble pigment	Light persimmon (5ic)	Light persimmon (5ic)	None	Camel (3ie)	Ashes (7fe)
<i>ISP medium 4</i>					
Growth	Good Golden brown (3pg)	Good Light antique gold (1 ^{1/2} ia)	Good Bamboo (2gc) to yellow maple (3ng)	Good Bamboo (2gc)	Good Bamboo (2gc) to yellow maple (3ng)
Reverse	Bisque (3ec)	Pearl (3ba)	Sand (3cb)	Bisque (3ec)	Bisque (3ec)
Aerial mycelium	None	None	None	None	None
Soluble pigment	None	None	None	None	None
<i>ISP medium 5</i>					
Growth	Good Honey gold (2ic) Light antique gold (1 ^{1/2} ia)	Good	Good	Good	Good
Reverse	Bisque (3ec)	Light ivory (2ca) to bamboo (2gc) Bamboo (2fb)	Light ivory (2ca) to bamboo (2gc) Light ivory (2ca) to bamboo (2gc)	Light ivory (2ca) to bamboo (2gc) Light ivory (2ca) to bamboo (2gc)	Light ivory (2ca) to bamboo (2gc) Light ivory (2ca) to bamboo (2gc)
Aerial mycelium	Poor, flesh pink (4ca)	Poor, white (a)	Abundance, pearl (3ba)	Abundance, white (a)	None
Soluble pigment	None	None	None	None	None
<i>ISP medium 6</i>					
Growth	Moderate Yellow maple (3ng)	Moderate Copper brown (5pi)	Good Golden brown (3pg)	Moderate Golden brown (3pg)	Moderate Yellow maple (3ng)
Reverse	Yellow maple (3ng)	Copper brown (5pi)	Golden brown (3pg)	Golden brown (3pg)	Yellow maple (3ng)
Aerial mycelium	None	None	None	None	None
Soluble pigment	None	None	None	None	None
<i>ISP medium 7</i>					
Growth	Good Honey gold (2ic) Light antique gold (1 ^{1/2} ia)	Good	Good	Good	Good
Reverse	Bisque (3ec)	Light ivory (2ca) to bamboo (2gc) Bamboo (2fb)	Light ivory (2ca) to bamboo (2gc) Light ivory (2ca) to bamboo (2gc)	Light ivory (2ca) to bamboo (2gc) Light ivory (2ca) to bamboo (2gc)	Light ivory (2ca) to bamboo (2gc) Light ivory (2ca) to bamboo (2gc)
Aerial mycelium	Poor, flesh pink (4ca)	Poor, white (a)	Abundance, pearl (3ba)	Abundance, white (a)	None
Soluble pigment	None	None	None	None	None
<i>YS agar</i>					
Growth	Good Deep red brown (6 ^{1/2} pi) to dark rose brown (7pn)	Good Redwood (6ne)	Good Bamboo (2gc)	Good Yellow maple (3ng) to brick red (5ng)	Good Light ivory (2ca) to rose mauve (7 ^{1/2} lg)
Reverse	Red mahogany (6 ^{1/2} pi)	Cinnamon (3le)	Bamboo (2gc)	Yellow maple (3ng)	Cream (1 ^{1/2} ca) to rose mauve (7 ^{1/2} lg)
Aerial mycelium	None	None	Moderate, pearl (3ba)	Abundance, white (a)	None
Soluble pigment	Terra cotta (5pe)	Light antique gold (1 ^{1/2} ia)	None	None	None
<i>Nutrient agar</i>					
Growth	Good Deep red brown (6 ^{1/2} pi) to deep red mahogany (6 ^{1/2} pg)	Good Bright cherry red (7pa)	Good Bamboo (2gc)	Good Light tan (3gc)	Good Cinnamon (3le) to cocoa brown (5ni)
Reverse	Red mahogany (6 ^{1/2} pi)	Tomato red (6 ^{1/2} pc)	Bamboo (2gc)	Light tan (3gc)	Cork tan (4ie) to cocoa brown (5ni)
Aerial mycelium	None	None	Poor, bamboo (2gc)	Poor, light tan (3gc)	None
Soluble pigment	Copper or persimmon (5ic)	Light persimmon (5ic)	None	None	None

Numbers and letters in parenthesis referred to the color based on the Color Harmony Manual.²⁰

Figure 1 Scanning electron micrograph of strain KC-061^T grown on International *Streptomyces* Project (ISP) 3 medium for 4 weeks at 27 °C.

ribose as diagnostic sugars in whole cells. The *N*-acyl type of muramic acid was acetyl. Mycolic acids were not detected (data not shown). The predominant menaquinone was MK-9 (H₄) (80%) and the minor menaquinones were MK-9 (H₆) (10%), MK-9 (H₂) (7%), MK-9 (H₀) (2%) and MK-10 (H₄) (1%). Strain KC-061^T contained the same predominant menaquinone of MK-9 (H₄) as strain '*N. monospora*' PT708^{T,21} however, the minor amounts of MK-9 (H₀) and MK-10 (H₄) was absent in strain '*N. monospora*' PT708^{T,21}. Diphosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, hydroxy-phosphatidylmonomethylethanolamine, hydroxy-phosphatidylethanolamine, phosphatidylglycerol, ninhydrin positive phosphoglycolipid and unknown phospholipid (PL1) were detected as phospholipids. Strain KC-061^T contained the same polar lipids as strain '*N. monospora*' PT708^{T,21} but the unknown phospholipid (PL2) was not detected as found in '*N. monospora*' PT708^T (Figure 2). The major fatty acids were iso-C_{16:0} (25.3%), 10-methyl-C_{17:0} (13.4%), C_{17:1} ω8c (7.7%), C_{16:1} ω7c/C_{16:1} ω6c (7.0%), C_{16:0} (6.9%), iso-C_{16:1} G (6.4%), iso-C_{15:0} (4.8%), 10-methyl-C_{16:0} (4.0%) and C_{16:0} 2-OH (3.8%). Strain KC-061^T contained the same cellular fatty acid profiles as *Nonomuraea* species but it showed different in the amount of fatty acids (Table 3). The G + C content of genomic DNA was 72.4 mol%. Chemotaxonomic analyses confirmed that strain KC-061^T exhibited typically chemical characteristics of members of the genus *Nonomuraea*.

Phylogenetic analysis

The 16S rRNA gene sequence analysis showed that strain KC-061^T belongs to the genus *Nonomuraea*. The 16S rRNA gene sequence similarity values between the strain KC-061^T and the type strains in the genus *Nonomuraea* ranged from 95.4 to 99.3%. The phylogenetic trees based on 16S rRNA gene sequences of strain KC-061^T and the type strains of members in the genus *Nonomuraea* revealed that strain KC-061^T constructed a cluster with '*N. monospora*' PT708^T, *N. rhizophila* YIM 67092^T and *N. rosea* GW 12687^T (Figure 3). The strain KC-061^T showed high 16S rRNA gene sequence similarity values to '*N. monospora*' PT708^{T,21} (99.3%), *N. rhizophila* DSM

Table 2 Differential characteristics of strain KC-061^T and closely related type strains

Characteristics	KC-061 ^T	<i>N. monospora</i>	<i>N. rhizophila</i>	<i>N. dietziae</i>	<i>N. rosea</i>
<i>Utilization of</i>					
L-Arabinose	±	–	–	+	+
D-Fructose	+	–	+	+	+
D-Mannitol	+	–	+	–	+
Melibiose	+	–	+	–	–
Raffinose	+	–	+	–	+
Sucrose	–	–	–	–	+
D-Xylose	+	+	–	+	+
Nitrate reduction	+	+	–	–	+
Degradation of casein	±	+	–	–	–
Gelatin liquefaction	–	+	–	–	–
Hydrolysis of starch	+	+	–	±	+
NaCl tolerance (%)	4	3	3	3	2
<i>Enzyme activity of</i>					
Cystine arylamidase	–	–	+	+	+
Esterase C4	±	±	+	+	+
Esterase lipase C8	±	–	±	+	±
α-Galactosidase	–	±	±	–	±
β-Galactosidase	–	+	+	+	+
β-Glucosidase	+	±	±	±	±
α-Mannosidase	±	+	±	±	±
Trypsin	–	+	+	+	+
Valine arylamidase	±	–	+	+	+

Symbols: +, positive; ±, weakly positive; –, negative.

45382^T (98.6%), *N. dietziae* NBRC 14039^T (98.5%) and *N. rosea* DSM 45177^T (98.3%). These results indicate that strain KC-061^T is the closest with '*N. monospora*' PT708^T. However, '*N. monospora*' PT708^T producing single spore is clearly distinguished from the strain KC-061^T, which produced spiral spore chains.²¹

DNA–DNA hybridization

DNA–DNA relatedness values between strain KC-061^T and strains '*N. monospora*' PT708^T, *N. rhizophila* DSM 45382^T, *N. dietziae* NBRC 14309^T and *N. rosea* DSM 45177^T were 47 ± 2.9%, 55 ± 2.5%, 48 ± 9.0% and 54 ± 8.4%, respectively and reciprocally, strain '*N. monospora*' PT708^T showed 54 ± 4.8%, 50 ± 2%, 39 ± 1.6% and 46 ± 4.8% DNA–DNA relatedness to KC-061^T, *N. rhizophila* DSM 45382^T, *N. dietziae* NBRC 14309^T and *N. rosea* DSM 45177^T, respectively. These values are obtained from three independent determinations and are below the 70% cutoff point recommended by Wayne *et al.*²² for assigning strains to the same species and confirms the separation of strain KC-061^T from its closely related phylogenetic neighbors. Therefore, strain KC-061^T clearly represents a novel species of the genus *Nonomuraea*.

Conclusion

The results of phylogenetic and phenotypic characteristics of strain KC-061^T are consistent with its classification within the genus *Nonomuraea*. Furthermore, there were many cultural, physiological and biochemical features that differed between strain KC-061^T and the most closely related type strains, '*N. monospora*' PT708^T, *N. rhizophila*

Figure 2 Phospholipid compositions of strain KC-061^T (a) and *Nonomuraea monospora* TISTR 1910^T (b) (The TLC plates were sprayed with molybdatophosphoric acid). Diphenylphosphatidylglycerol (DPG), phosphatidylmonomethylethanolamine (PME), phosphatidylethanolamine (PE), hydroxyphosphatidylmonomethylethanolamine (OH-PME), hydroxyphosphatidylethanolamine (OH-PE), phosphatidylglycerol (PG), ninhydrin positive phosphoglycerolipid (NPG) and unknown phospholipids (PL1 and PL2). A full color version of this figure is available at *The Journal of Antibiotics* journal online.

DSM 45382^T, *N. dietziae* NBRC 14309^T and *N. rosea* DSM 45177^T, such as differences in production and color of aerial mycelium and soluble pigments, nitrate reduction, utilization of sole carbon sources and enzyme activities. The results from the DNA–DNA hybridization studies also support the classification of strain KC-061^T as a novel species of the genus *Nonomuraea* and the name, *Nonomuraea thailandensis* sp. nov., is proposed.

Table 3 Cellular fatty acid compositions (%) of KC-061^T and closely related type strains

Fatty acid	KC-061 ^T	<i>N. monospora</i>	<i>N. rhizophila</i>	<i>N. dietziae</i>	<i>N. rosea</i>
<i>Saturated straight-chain</i>					
C _{13:0}	0.6	0.2	1.2	0.6	1.1
C _{14:0}	3.3	3.3	4.2	5.9	3.6
C _{16:0}	6.9	11.0	8.2	23.4	9.5
C _{17:0}	1.7	1.2	3.4	8.7	4.0
<i>Unsaturated straight-chain</i>					
C _{15:1} ω6c	0.2	ND	0.3	ND	ND
C _{17:1} ω8c	7.7	6.7	8.4	9.9	8.3
C _{18:1} ω9c	2.8	3.5	0.6	5.3	0.9
<i>Saturated branched-chain</i>					
iso-C _{14:0}	1.3	0.7	3.1	1.3	2.5
iso-C _{15:0}	4.8	5.1	3.2	3.5	5.1
iso-C _{16:0}	25.3	23.5	27.7	9.6	18.9
iso-C _{17:0}	1.0	1.4	0.4	0.7	1.0
iso-C _{18:0}	0.6	0.4	ND	0.2	ND
anteiso-C _{15:0}	0.3	0.5	0.8	1.6	2.1
anteiso-C _{17:0}	0.7	1.1	0.4	0.7	1.4
10-methyl C _{16:0}	4.0	6.8	4.7	1.8	4.6
10-methyl C _{17:0}	13.4	13.2	13.1	6.2	14.6
10-methyl C _{18:0}	2.4	3.2	1.0	2.6	1.6
(TBSA)					
<i>Unsaturated branched-chain</i>					
iso-C _{15:1} G	0.3	ND	0.2	0.2	0.4
iso-C _{16:1} G	6.4	4.4	4.2	1.0	4.7
<i>Hydroxy</i>					
C _{13:0} 2-OH	ND	0.2	ND	0.1	ND
C _{15:0} 2-OH	0.7	0.2	2.9	1.1	3.0
C _{16:0} 2-OH	3.8	1.7	3.63	3.5	3.2
C _{17:0} 2-OH	0.5	0.2	0.5	0.6	0.5
<i>Summed feature^a 3</i>					
	7.0	7.1	4.1	6.1	3.1

Abbreviation: ND, not detected.

^aSummed feature 3 comprises C_{16:1} ω7c and/or C_{16:1} ω6c.

Description of *Nonomuraea thailandensis* sp. nov.

Nonomuraea thailandensis (thai.lan.den' sis. N.L. masc. adj. *thailandensis* pertaining to Thailand, where the strain was isolated).

Gram-positive and aerobic actinomycete. Substrate mycelia are branched and not fragmented. Depending on the cultivation medium, the color of aerial mycelia is white to pale orange. No aerial mycelia are produced on ISP 4 and 6 media, YS agar and nutrient agar and the substrate mycelia appear pale orange to dark reddish brown. A light brown to deep orange soluble pigment is produced on ISP 2 and 3 media, YS agar and nutrient agar. Growth occurs at 14–38 °C (optimum 20–30 °C) and pH 6 to 11 (optimum pH 7 to 9). The NaCl tolerance for growth is up to 4% (w/v). Utilizes D-glucose, myo-inositol, D-mannitol, melibiose, raffinose, L-rhamnose and D-xylose as the sole carbon sources, but does not utilize sucrose. Reduces nitrate, hydrolyzes casein and starch but does not liquefy gelatin. According to the API ZYM system, the strain shows alkaline phosphatase, leucine arylamidase, α-chymotrypsin, acid phosphatase,

Figure 3 Neighbor-joining tree based on 16S rRNA gene sequences showing relationship between KC-061^T and members of the genus *Nonomuraea*. Only bootstrap values above 50% (percentages of 1000 replications) are indicated. •, branch also recovered in the maximum-parsimony tree; *, branch also recovered in the maximum-likelihood tree; Bar, 0.01 nucleotide substitutions per site.

naphthol-AS-BI-phosphohydrazide and α -glucosidase, β -glucosidase and *N*-acetyl- β -glucosaminidase activities. Esterase (C4), esterase lipase (C8), valine arylamidase and α -mannosidase of strain are weak activities, and strain shows no activities on cystine arylamidase, trypsin, α -galactosidase and β -galactosidase. The diagnostic amino acid within peptidoglycan is *meso*-diaminopimelic acid. Whole cell hydrolysates contain galactose, mannose, madurose and ribose. The *N*-acyl type of muramic acid is acetyl. Diposphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, hydroxy-phosphatidylmonomethylethanolamine, hydroxy-phosphatidylethanolamine, phosphatidylglycerol, ninhydrin positive phosphoglycolipid and unknown phospholipid (PL) are present. The predominant menaquinone is MK-9 (H₄) and MK-9 (H₀), MK-9 (H₂), MK-9 (H₆) and MK-10 (H₄) are also present. Major fatty acids are iso-C_{16:0}, 10-methyl-C_{17:0}, C_{17:1} ω 8c, C_{16:1} ω 7c/C_{16:1} ω 6c, C_{16:0}, iso-C_{16:1} G, iso-C_{15:0}, 10-methyl-C_{16:0} and C_{16:0} 2-OH. The genomic DNA G + C content is 72.4 mol%. The type strain is KC-061^T (= JCM 18408^T = KCTC 29074^T = PCU 327^T), which was isolated from soil in Angthong Islands National Park, Surat Thani, Thailand.

METHODS

Strain KC-061^T was isolated from soil sample collected from Angthong Islands National Park, Surat Thani province, Thailand. The soil sample (1 g) was suspended in distilled water (9 ml) and heated at 55 °C for 5 min followed by a serial dilution method for the isolation using potato starch-glycerol agar (potato starch 1.0%, glycerol 1.0%, K₂HPO₄ 0.2%, (NH₄)₂SO₄ 0.2%, MgSO₄·7H₂O 0.1%, NaCl 0.1%, CaCO₃ 0.2%, agar 1.2%, pH 7.0)²³ supplemented with nystatin (25 mg l⁻¹) and novobiocin (50 mg l⁻¹). After 2 weeks of incubation at 27 °C, the strain was transferred and purified on yeast extract-malt extract medium (International *Streptomyces* Project medium 2, ISP 2 medium).²⁴ The strain was maintained on Starch-Yeast extract-Meat extract (SYM agar) (starch 1.0%, N-Z amine 0.3%, yeast extract 0.1%, meat

extract 0.1%, CaCO₃ 0.3%, agar 1.2%, pH 7.0). '*N. monospora*' PT708^T (= TISTR 1910^T), *N. rhizophila* DSM 45382^T (= YIM 67092^T), *N. dietziae* (= DSM 44320^T) and *N. rosea* DSM 45177^T (= GW 12687^T) were used for comparative purposes in the study. In order to observe cultural characteristics, the strain KC-061^T, '*N. monospora*' PT708^T, *N. rhizophila* DSM 45382^T, *N. dietziae* NBRC 14309^T and *N. rosea* DSM 45177^T were cultured at 27 °C for 3 weeks on ISP 2, 3, 4, 5, 6 and 7 media,²⁴ YS agar (yeast extract 2.0, starch 1.0, agar 1.5%, pH 7.0) and nutrient agar (Difco). The Color Harmony Manual²⁰ was used to determine the color of aerial and substrate mycelia and soluble pigment. The morphological characteristics were observed by light microscopy and scanning electron microscopy (model JSM-5600, JEOL, Tokyo, Japan), after cultivation on ISP 3 medium at 27 °C for 4 weeks. For scanning electron microscopy investigation, the cultures were fixed with 4% osmium tetroxide vapor *in situ* for 16 h at room temperature and then dried at room temperature.²⁵ Physiological characteristics, the temperature range, pH range and NaCl tolerance for growth were determined on ISP 2 medium. Utilization of carbohydrates as sole carbon sources were tested by using ISP 9 medium.²⁶ ISP 4 medium was used for starch hydrolysis test. Nitrate medium (beef extract 0.3%, peptone 0.5%, KNO₃ 0.1%, pH 7.0) was used for nitrate reduction. Glucose-Peptone-Gelatin medium (glucose 2.0%, peptone 0.5%, gelatin 20%, pH 7.0) was used to test for gelatin liquefaction. Skimmed milk (10%) was used for coagulation and peptonization of milk. The enzyme activities were determined using the API ZYM system (bioMérieux, Lyon, France), according to the manufacturer's instructions. Biomass for the genotypic study and the chemotaxonomic studies was obtained after cultivation in Yeast Glucose broth (yeast extract 1.0%, glucose 1.0%, pH 7.0) on a rotary shaker at 27 °C for 1 week. Diaminopimelic acid isomers in whole cells were determined by TLC using whole-cell hydrolysis.²⁷ Whole-cell sugar composition was analyzed according to the method of Becker *et al.*²⁷ Isoprenoid quinones were extracted according to the method of Collins *et al.*²⁸ and were analyzed by LC/MS (JMS-T 100LP, JEOL) using a CAPCELL PAK C18 UG120 (Shiseido, Tokyo, Japan) with methanol-2-propanol (7:3) as the mobile phase. The *N*-acyl types of muramic acid were determined by the method of Uchida and Aida.²⁹ Phospholipids in cells were extracted and identified by the method of Minnikin *et al.*³⁰ The presence of mycolic acids was examined by TLC following Tomiyasu.³¹ The cellular fatty acid composition analysis was determined by GLC according to the instructions of the Microbial Identification System (MIDI) Sherlock version 6.0 with the RTSBA6 MIDI database as described by Sasser.³²

For DNA base composition analysis, chromosomal DNA was prepared following the procedure of Saito and Miura³³ and the DNA G + C content was determined by HPLC according to Tamaoka and Komagata.³⁴ DNA-DNA hybridization was performed by the photobiotin-labelling method of Ezaki *et al.*³⁵ The 16S rRNA gene was amplified using the primers described by Takahashi *et al.*³⁶ Amplifications were performed in a TaKaRa thermal cycler (Takara Bio Inc., Shiga, Japan) with an initial incubation of 1 min at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min at 50 °C and 1.5 min at 72 °C, followed by 2 min final extension at 72 °C. The PCR products were sequenced on a DNA sequencer (Applied Biosystems 3130 Genetic Analyzer) using a BigDye Terminator v3.1 cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The ClustalW2 program was used for multiple alignments with selected sequences for calculating evolutionary distances³⁷ by SeaView version 4.2.³⁸ The phylogenetic trees were constructed based on the neighbor-joining method,³⁹ the maximum-likelihood⁴⁰ and the maximum-parsimony methods.⁴¹ Data were resampled with 1000 bootstrap replications.⁴² The values for sequence similarities among the most closely related strains were determined using the EzTaxon server.⁴³

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