***Nocardiopsis kunsanensis* sp. nov., a moderately halophilic actinomycete isolated from a saltern**

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A moderately halophilic actinomycete, designated HA-9^T, was isolated from a saltern in Kunsan, Republic of Korea, and was the subject of polyphasic identification. Analysis of 16S rDNA indicated that the isolate belonged to the genus *Nocardiopsis*, but differed genetically from other *Nocardiopsis* species. Strain HA-9^T contained meso-diaminopimelic acid, no diagnostic sugars, hexa- or octa-hydrogenated menaquinones with 10 isoprene units, straight-chain saturated or monounsaturated, iso-, anteiso-, 10-methyl branched fatty acids with 13–18 carbons and type III phospholipids. All of these characters consistently assign the isolate to the genus *Nocardiopsis*. All of the validly described *Nocardiopsis* species, including moderately halophilic *Nocardiopsis halophila*, can be differentiated from the saltern isolate using morphological and physiological traits. On the basis of polyphasic evidence, the name *Nocardiopsis kunsanensis* sp. nov. is proposed for strain HA-9^T (= KCTC 9831^T), which is designated the type strain.

Keywords: *Nocardiopsis kunsanensis* sp. nov., polyphasic taxonomy, 16S rDNA sequencing

INTRODUCTION

The genus *Nocardiopsis* was created by Meyer (1976) to harbour *Actinomadura dassonvillei* on the basis of morphological and chemotaxonomic properties. The genus currently comprises seven validly described species, namely *Nocardiopsis alba*, *Nocardiopsis dassonvillei*, *Nocardiopsis halophila*, *Nocardiopsis listeri*, *Nocardiopsis lucentensis*, *Nocardiopsis prasina* and *Nocardiopsis synnemataformans* (Al-Tai & Ruan, 1994; Kroppenstedt, 1992; Yassin *et al.*, 1993, 1997). The members of *Nocardiopsis* are phylogenetically coherent and form a monophyletic clade that is equated with the family *Nocardiopsaceae* (Rainey *et al.*, 1996). None of the *Nocardiopsis* species to date have been reported as halophilic except *N. halophila*, which can grow best at 5–15% NaCl (Al-Tai & Ruan, 1994). In this study, we report the results of polyphasic identification of an actinomycete strain, designated HA-9^T, isolated from a saltern sample. The strain grew

optimally in medium containing 10% NaCl and showed properties consistent with its classification in the genus *Nocardiopsis* Meyer 1976 as a new species, of which the name *Nocardiopsis kunsanensis* sp. nov. is proposed.

METHODS

Bacterial strains. The saltern sample was collected from Kunsan, Republic of Korea. Bacterial strains were isolated by plating serially diluted samples onto Bennett medium based on seawater (2 g glucose, 1 g yeast extract, 1 g malt extract, 2 g peptone, 20 g agar, 1 l aged seawater, pH 7.2). After incubating at 30 °C for 2 weeks, a visible colony, designated HA-9^T, was transferred and subcultured until pure culture was obtained. Complex medium (CM; 7.5 g Casamino acids, 10 g yeast extract, 3 g sodium citrate, 10 g magnesium sulfate, 2 g potassium chloride, 1 ml 4.98% iron sulfate, 100 g NaCl, 1 l distilled water, pH 7.4) was used for growing strain HA-9^T and *Nocardiopsis halophila* KCTC 9825^T (= A.S.4.1195). The strain was maintained as a glycerol suspension (20%, w/v) at –80 °C.

Morphology and cultural characteristics. The cultures for morphology were prepared by incubating the test strain on CM agar at 37 °C for 7 d. The morphological properties of

The GenBank accession numbers for the 16S rDNA sequences of *Nocardiopsis kunsanensis* HA-9^T (= KCTC 9831^T) and *Nocardiopsis halophila* KCTC 9825^T are AF195412 and AF195411, respectively.

colonies, cells and spores were determined using light and scanning electron microscopes (Phillips model 515). For spore observation, the sample was fixed with glutaraldehyde, critical-point-dried, then gold-coated using the Bio-Rad SEM coating system (model E5550). Culture characteristics were observed using CM, tryptone-yeast extract, yeast extract-malt extract, peptone-yeast extract-iron, tyrosine agar, inorganic salts-starch, glycerol-asparagine (Shirling & Gottlieb, 1966), nutrient agar (Difco) and potato-dextrose agar (Difco). All media were supplemented with NaCl (final concentration 10%, w/v).

Physiological characteristics. All tests were done at 37 °C, unless otherwise specified. The NaCl requirement for growth was determined using CM supplemented with different concentrations of NaCl (0, 1, 3, 5, 10, 15, 20, 25, 30%). The temperature and pH ranges for growth were determined using CM. Degradation of adenine, casein, starch, hypoxanthine, tyrosine and xanthine was tested using CM as basal medium, as described by Gordon & Smith (1955) and Gordon *et al.* (1974). Liquefaction of gelatin was tested using CM supplemented with gelatin (12%, w/v). Hydrolysis of urease was detected using basal medium (1 g glucose, 1 g casein-peptone, 1.98 g disodium hydroxyphosphate, 1.51 g monobasic potassium phosphate, 0.5 g magnesium sulfate, 0.012 g phenol red, pH 9.0, 1 l distilled water). Urea was filter-sterilized and added to a final concentration of 2% (w/v). Hydrolysis of aesculin was determined using Bile aesculin agar (Difco). Growth in the presence of phenol (0.1%), potassium tellurite (0.0001%), sodium azide (0.01%) and crystal violet (0.0001%) was examined using CM as basal medium. Catalase production was assayed by using 0.3% hydrogen peroxide with colonies taken from CM plates. Production of H₂S was determined using Triple-sugar iron agar (Difco). Utilization of carbohydrates as sole carbon source was examined using basal medium (1 g ammonium sulfate, 7 g dibasic potassium phosphate, 2 g monobasic potassium phosphate, 0.1 g magnesium sulfate, 100 g NaCl, 20 g agar, 1 l distilled water) in triplicate. Carbon compounds were sterilized by filtration and added to a final concentration of 1% (w/v). Similarly, basal medium without ammonium sulfate was used for testing utilization of substrates as sole carbon and nitrogen sources.

Chemotaxonomy. Freeze-dried biomass was prepared from CM broth grown at 37 °C for 7 d using a shaking incubator. Analyses for diaminopimelic acid and major sugars were carried out using the procedures described by Stanek & Roberts (1974) and Lechevalier & Lechevalier (1970), respectively. Menaquinones were extracted from dried biomass (ca 50 mg), using the small-scale method of Minnikin *et al.* (1984), and purified preparations were examined by electron-impact MS, as described previously (Chun *et al.*, 1997). Fatty acid methyl esters were prepared and analysed using the MIDI system (Hewlett Packard; Sasser, 1990). The presence of mycolic acids was checked by the acid methanolysis method described by Minnikin *et al.* (1980). Phospholipids were extracted and detected by two-dimensional TLC according to Embley & Wait (1994).

Determination of DNA base composition. DNA was prepared according to Chun & Goodfellow (1995). The G+C contents of the resultant preparations were determined using the thermal denaturation method (Mandel & Marmur, 1968).

16S rDNA analysis. The nearly complete nucleotide sequence of 16S rDNA was determined as described previously (Chun & Goodfellow, 1995). The resultant sequence of strain

HA-9^T was manually aligned with representatives of the genus *Nocardopsis* and related taxa using known 16S rRNA secondary structure information. Phylogenetic trees were inferred by using the neighbour-joining (Saitou & Nei, 1987), Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1972) methods. Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes & Cantor (1969). The trees were rooted using *Actinomadura madurae* (accession number X97889) as outgroup. The PHYLIP package (Felsenstein, 1993) was used for all analyses. The resultant unrooted tree topology was evaluated in bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings.

RESULTS AND DISCUSSION

An almost complete sequence of 16S rDNA was determined for isolate HA-9^T (1449 bp). Preliminary comparison of the sequence against the GenBank database indicated that the isolate was closely related to the members of the genus *Nocardopsis*. The 16S rDNA of *N. halophila* KCTC 9825^T was examined, as it was not reported in the original publication (Al-Tai & Ruan, 1994) and GenBank database. Two sequences generated in this study were manually aligned with representatives of *Nocardopsis* and related taxa and the resultant phylogenetic tree is given in Fig. 1. All *Nocardopsis* species, including *N. halophila* and strain HA-9^T, formed a monophyletic clade that was recovered in four treeing algorithms and supported by a 99% bootstrap value. *N. halophila* formed the deepest branch within the clade and the mean sequence similarity values to other *Nocardopsis* species was 95.6±0.5%. Strain HA-9^T formed an independent phyletic line within a monophyletic subclade containing *Nocardopsis* species except *N. halophila*. The 16S rDNA similarity values of the isolate to all of the *Nocardopsis* species ranged from 94.6 (*N. halophila*) to 97.0% (*N. alba*, *N. lucentensis*). It is clear from phylogenetic analyses based on nearly complete 16S rDNA sequences that our isolate belongs to the genus *Nocardopsis* and represents a distinct phyletic line that can be equated with genomic species (Stackebrandt & Goebel, 1994).

The membership of the isolate at the genus level was also confirmed by cell chemistry. The cell wall of isolate HA-9^T contained *meso*-diaminopimelic acid and no diagnostic sugars. The predominant menaquinone from the test strain was MK-10(H₈), though minor amounts of MK-10(H₆), MK-9(H₈) and MK-9(H₆) were also present. Major fatty acids found in whole-cell preparations were iso-C15:0, anteiso-C16:0 and 10-methyl-C18:0. Straight-chain saturated or monounsaturated, iso- or anteiso-branched fatty acids with 13–18 carbons were also found in minor quantities. Mycolic acid was absent. The phospholipid type was type III *sensu* Lechevalier *et al.* (1977); phosphatidylcholine, phosphatidylglycerol and diphosphatidylglycerol were found, but not phosphatidylethanolamine. All of these characters consistently

Fig. 1. Rooted neighbour-joining tree based on nearly complete 16S rDNA sequences showing relationships between strain HA-9^T and members of the genus *Nocardiopsis*. The numbers at the nodes indicate the levels of bootstrap support based on neighbour-joining analyses of 1000 resampled data sets; only values over 50% are given. The asterisks represent the clades that were also recovered in the Fitch–Margoliash, maximum-parsimony and maximum-likelihood trees. The scale bar indicates 0.01 nt substitutions per nucleotide position.

place the isolate in the genus *Nocardiopsis* (Al-Tai & Ruan, 1994; Grund & Kroppenstedt, 1990; Kroppenstedt, 1992; Yassin *et al.*, 1993, 1997). In addition, the G + C content of the DNA was 71 mol %, which lies within the range for the genus.

The isolate was Gram-positive, non-acid-fast and catalase-positive. It formed circular colonies that had yellow substrate mycelia and white aerial mycelia on CM agar containing 10% NaCl. Fragmentation of mycelia was not observed. The aerial mycelia contained chains of smooth spores, which were flexible and frequently formed open loops. No pigment was produced. The test strain was able to grow in tryptone-yeast extract, yeast extract-malt extract, peptone-yeast extract-iron, nutrient (Difco) and potato-dextrose (Difco) agar, and the colour of spores was invariably white. The isolate grew on CM with NaCl concentrations between 3 and 20%; no or rare growth was detected on CM supplemented 0 or 25% NaCl. Strain HA-9^T grew optimally at 37 °C and at pH 9 with 10% NaCl. No growth was observed at pH 5. The organism utilized D-glucose and sucrose as sole carbon source, and L-alanine as sole carbon and nitrogen source. Adenine, casein, starch, hypoxanthine, tyrosine, xanthine, gelatin and urea were degraded by the test organism. However, aesculin was not hydrolysed. It is evident from these phenotypic traits that saltern isolate HA-9^T has morphological features matching the description of the genus *Nocardiopsis* and exhibits a distinctive physiological pattern that differentiates it from all of valid species of the genus (Table 1).

Halophilic micro-organisms can be conveniently grouped according to NaCl requirements for growth (Ventosa *et al.*, 1998). Larsen (1986) defined moderate halophiles as organisms growing optimally between 5

and 20% NaCl. Extreme halophiles are able to grow in saturated NaCl and unable to grow in the presence of NaCl concentrations less than 12%. The occurrence of actinomycetes in highly saline environments is not rare (Gottlieb, 1973; Tresner *et al.*, 1968). Members of the genus *Actinopolyspora* are either extreme or moderate halophiles (Gochnauer *et al.*, 1975; Ruan *et al.*, 1994; Yoshida *et al.*, 1991). Among *Nocardiopsis* species, *N. halophila* is the only species that grows optimally in the presence of 5–15% NaCl; the trait defines the organism as a moderate halophile (Al-Tai & Ruan, 1994). Our isolate showed similar NaCl requirements for growth. However, it did not grow without NaCl in medium, and was phylogenetically distant and exhibited different physiological characteristics from *N. halophila* and other species in the genus *Nocardiopsis*. While this report was being reviewed, the descriptions of two new species in the genus *Nocardiopsis* were published (Evtushenko *et al.*, 2000). Both species, namely *Nocardiopsis trehalosi* and *Nocardiopsis tropica*, are not halophilic and differ from our isolate in 16S rDNA sequences (94.7 and 96.3% similarity, respectively). Several phenotypic properties, including growth at 20% NaCl, can be used to separate strain HA-9^T from these species. On the basis of molecular, chemical and phenotypic evidence, we propose isolate HA-9^T be classified in the genus *Nocardiopsis* as *Nocardiopsis kunsanensis* sp. nov.

Description of *Nocardiopsis kunsanensis* sp. nov.

Nocardiopsis kunsanensis (kun.sa.nen'sis. M.L. adj. *kunsanensis* pertaining to Kunsan, a location in Korea where the species was isolated).

Gram-positive, non-acid-fast, filamentous, catalase-positive and strictly aerobic. Substrate mycelia are

Table 1. Differential phenotypic characteristics of strain HA-9^T and other members of the genus *Nocardiopsis*

w, Weakly positive; v, variably utilized.

Character	Strain HA-9 ^T	<i>N. alba</i> DSM 44048 ^{T*}	<i>N. dassonvillei</i> DSM 43111 ^{T*}	<i>N. halophila</i> KCTC 9825 ^T	<i>N. listeri</i> DSM 40297 ^{T*}	<i>N. lucentensis</i> DSM 44048 ^{T*}	<i>N. prasina</i> DSM 43845 ^{T*}	<i>N. synnemata-</i> <i>formans</i> DSM 44143 ^{T*}
Hydrolysis of:								
Adenine	+	+	+	+	+	+	—	+
Hypoxanthine	+	+	+	+	+	+	—	+
Xanthine	+	+	+	+	+	—	+	+
Utilization as sole carbon source:								
Acetate	—	—	—	—	—	+	—	—
Cellobiose	—	+	+	w	—	+	—	+
D-Galactose	—	+	v	+	—	—	—	+
D-Glucose	+	+	+	w	—	+	—	+
Gluconate	—	+	+	+	—	+	—	+
<i>p</i> -Hydroxybenzoate	—	—	—	—	—	+	—	—
<i>myo</i> -Inositol	—	—	—	+	—	+	—	—
Maltose	—	+	—	+	—	+	—	+
Mannitol	—	—	+	v	—	+	—	+
Paraffin	—	—	—	w	—	+	—	—
Raffinose	—	—	—	+	—	+	—	—
L-Rhamnose	—	—	w	w	—	+	—	+
Sucrose	+	—	+	+	—	+	—	—
Trehalose	—	+	+	+	—	+	—	—
D-Xylose	v	—	+	+	—	—	—	+
Utilization as sole carbon and nitrogen sources								
L-Alanine	+	—	+	—	—	+	+	+
Gelatin	—	+	+	v	+	—	+	+
Proline	—	+	w	+	+	+	—	+
Serine	—	—	w	—	—	+	—	—

* Data from this and earlier studies (Yassin *et al.*, 1997).

yellow and bear white aerial mycelia, which fragment into elongated non-motile spores with smooth surfaces. Fragmentation of substrate mycelia does not occur. No diffusible pigment is formed. Grows optimally at 37 °C and at pH 9 with 10 % NaCl. Does not grow on CM with 0 or 25 % NaCl, nor at pH 5. Unable to reduce nitrate to nitrite. H₂S is not produced. Utilizes D-glucose and sucrose as sole carbon source, but not acetate, cellobiose, D-galactose, gluconate, *p*-hydroxybenzoate, *myo*-inositol, maltose, mannitol, paraffin, raffinose, L-rhamnose or trehalose. Utilizes L-alanine, but not gelatin, proline or serine as sole carbon and nitrogen sources. Degrades adenine, casein, starch, hypoxanthine, tyrosine, xanthine, gelatin and urea, but not aesculin. Resistant to sodium azide (0.01 %), crystal violet (0.0001 %) and potassium tellurite (0.0001 %), but not phenol (0.1 %). Contains *meso*-diaminopimelic acid, no diagnostic sugars, hexa- or octa-hydrogenated menaquinones with 10 isoprene units, straight-chain saturated or monounsaturated, iso-, anteiso-, 10-methyl branched fatty acids with 13–18 carbons, and phosphatidylcholine, phospho-

tidylglycerol and diphosphatidylglycerol (phospholipid type III). The G+C content of the DNA is 71 mol %. The species was isolated from a saltern sample collected from Kunsan, Republic of Korea. The type and only strain of *Nocardiopsis kunsanensis* is strain HA-9^T (= KCTC 9831^T = JCM 10721^T).

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