Nocardioides exalbidus sp. nov., a novel actinomycete isolated from lichen in Izu-Oshima Island, Japan

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A short coccoid- or rod-shaped, non-motile actinomycete strain RC825^T was isolated from lichen in Izu-Oshima Island, Japan. A polyphasic study was undertaken to establish the taxonomic position of this strain. Phylogenetic analysis based on 16S rRNA gene sequence revealed that strain RC825^T forms an evolutionary lineage within the radiation of the genus *Nocardioides*. In particular, it forms a coherent cluster with *Nocardioides oleivorans* and *Nocardioides ganghwensis* with high sequence similarity of 98 and 97.5%, respectively. However, DNA–DNA hybridization experiment demonstrated that strain RC825^T is distinct from its closest phylogenetic neighbors, *N. oleivorans* and *N. ganghwensis*, with 44 and 39% relatedness, respectively. The cell-wall peptidoglycan of strain RC825^T contained LL-diaminopimelic acid, indicating wall chemotype I. The predominant menaquinone was MK-8(H₄). Strain RC825^T had a cellular fatty acid profile containing straight-chain, branched, unsaturated and 10-methyl fatty acids, and the major fatty acid was iso-C_{16:0}. The DNA G+C content of the strain was 74 mol%. On the basis of both phenotypic and phylogenetic evidence, the strain is separated from previously described *Nocardioides* species, and should be assigned to represent a novel species of the genus *Nocardioides*, for which the name *Nocardioides exalbidus* sp. nov. is proposed. The type strain is strain RC825^T (=IAM 15416^T=CCTCC AA206016^T).

INTRODUCTION

The genus *Nocardioides* was proposed by Prauser¹⁾ for nocardioform actinomycetes that form mycelium fragments into irregular rod to coccus-like elements. The organisms are Gram-positive, non-acid-fast, aerobic and mesophilic, and have a cell wall based on LL-diaminopimelic acid, the diagnostic diamino acid of wall chemotype I¹⁾. At the time of writing, the genus *Nocardioides* was composed of 26 species with validly published names, including the recently described species *N. kongjuensis*²⁾, *N. lentus*³⁾, *N. kribbensis*⁴⁾, and *N. oleivorans*⁵⁾. The aim of the present study was to determine the taxonomic position of isolate RC825^T using a combined polyphasic taxonomic approach. Based on the physiological properties, chemotaxonomic characteristics, and phylogenetic analysis, we propose that the isolate should be placed in the genus *Nocardioides* as a new species.

MATERIALS AND METHODS

During the course of the isolation of diazotrophs, strain RC825^T was isolated from lichen in Izu-Oshima Island, Japan, by plating it directly on IAM-A1 agar medium as follows: the lichen sample was washed five times with

sterilized water, and subsequently crushed in 1 ml of sterilized water. The effluent was streaked on IAM-A1 agar medium. After 2 weeks of incubation, white colonies developed on the medium were re-streaked and purified on Trypticase soy agar (TSA; BBL) and maintained on the same agar slant at 4 °C, and as a glycerol suspension (10%, v/v) at –80 °C. The cell morphology was observed by light microscopy (BX60; Olympus). For phenotypic properties, the growth range for temperature and pH were tested on TSA medium. Catalase activity was determined by bubble production in a 3% hydrogen peroxide solution. Oxidase was determined by cytochrome oxidase paper (Nissui Pharmaceutical). Salinity tolerance was determined by inoculating the strain into ISP2 (Difco) medium supplemented with 0–12% (w/v) NaCl at 1% increments. API 50CH, 20E, API ZYM strips (bioMérieux) were used according to the manufacturer's instructions to determine all physiological and biochemical characteristics of the strains.

Freeze-dried cells for chemotaxonomic studies were obtained upon incubation of the organism in trypticase soy broth (TSB; BBL) medium on a rotary shaker at 160 rpm at 27 °C for 4 days. For cellular fatty acid analysis, the strain was grown on TSA (BBL) at 27 °C for 3 days, and fatty acid methyl esters were prepared and identified using the

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The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain RC825^T is AB273624 (=IAM 15416^T=CCTCC AA206016^T).

Microbial Identification System as described by Xie and Yokota⁶⁾. Amino acid composition and isomers of diaminopimelic acid (DAP) in the cell walls were examined by two-dimensional TLC (Tokyo Kasei Co., Ltd., Tokyo, Japan) as described by Harper and Davis⁷⁾ and by HPLC according to the procedures of Yokota *et al.*⁸⁾. Phospholipids were extracted and identified using two-dimensional TLC, followed by spraying with appropriate detection reagents according to the method of Yokota *et al.*⁹⁾. Respiratory quinone was extracted and analyzed by the method of Komagata and Suzuki¹⁰⁾.

DNA was prepared according to the method of Marmur¹¹⁾ from cells grown on TSB medium, and the G+C content of DNA was determined using the HPLC method of Mesbah *et al.*¹²⁾. An approximately 1500-bp fragment of the 16S rRNA gene was amplified from extracted DNA using bacterial universal primers specific to the 16S rRNA gene, primer 8F, 5'-AGAGTTTGATCCTGGCTCAG-3' (positions 8–27, according to the *Escherichia coli* numbering system of Brosius *et al.*¹³⁾), and primer 1510R, 5'-GGCTACCTTGTTACGTA-3' (positions 1510–1527). PCR products were purified by the PEG precipitation method¹⁴⁾. Amplicons were directly sequenced using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). We compared the 16S rRNA gene sequence of RC825^T with sequences obtained from GenBank (National Center for Biotechnology Information). These sequences were aligned with the CLUSTAL X software package¹⁵⁾, and evolutionary distances and the *Knuc* value¹⁶⁾ were generated. Alignment gaps and ambiguous bases were not taken into consideration in the calculations. A phylogenetic tree based on a comparison of 1336 bases was constructed using the neighbor-joining method¹⁷⁾. The topology of the tree was evaluated using the bootstrap resampling method of Felsenstein¹⁸⁾ with 1000 replicates, whereas similarity values were calculated using MEGA3¹⁹⁾. DNA–DNA hybridization was performed by photobiotin-labelled probes in microplate wells as described by Ezaki *et al.*²⁰⁾, using a multi-well plate reader (CytoFluoR; PerSeptive Biosystems).

RESULTS AND DISCUSSION

The morphological and chemotaxonomic characteristics of strain RC825^T are consistent with its assignment to the genus *Nocardioidea*. The cells are Gram-positive, non-endospore-forming, non-motile, irregular coccoid- to rod-shaped, and about 0.5 µm wide and up to 2.0 µm long. Substrate or aerial mycelia were not observed (Fig. 1). The strain is positive for catalase activity and negative for oxidase activity. Growth occurs at 15–35 °C, pH 6–9, with the optimum being at 30 °C and pH 7. The results of API 50CH, 20E and API ZYM kits and carbon source utilization are given in Table 1.

Chemotaxonomic characteristics: cell wall analysis by HPLC and TLC indicated that the strain contained LL-

Fig. 1. Light micrograph of strain RC825^T cells grown on ISP2 agar medium for 48 h at 30 °C.

DAP in cell wall peptidoglycan, characteristic of cell wall chemotype I *sensu* Lechevalier & Lechevalier²¹⁾. The predominant menaquinone was MK-8(H₄). Diphosphatidylglycerol and phosphatidylinositol were present as major polar lipids, but no phosphatidylcholine was found. The DNA G+C content of the strain was 74 mol%. 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 *Nocardioidea*²²⁾. The strain also had relatively high proportions of iso-C_{17:0} and anteiso-C_{17:0} (Table 2).

Phylogenetic analysis: an almost complete 16S rRNA gene sequence (1453 nt) of RC825^T was determined and has been deposited in the DDBJ database. Phylogenetic analysis of the 16S rRNA gene sequence revealed that the isolate fell within the genus *Nocardioidea* (Fig. 2) with 99% bootstrap support. To ascertain the phylogenetic position of strain RC825^T, we aligned its 16S rRNA gene sequence (1336 nt) with those of type strains of species of the genus *Nocardioidea*. The strain showed the highest similarities to the sequences of *N. oleivorans* IAM 15341^T (98%) and *N. ganhwensis* IAM 15306^T (97.5%). However, a DNA–DNA hybridization experiment demonstrated that the strain was distinct from *N. oleivorans* IAM 15341^T and *N. ganhwensis* IAM 15306^T with 44% and 39% relatedness, respectively, which were well below the 70% cut-off point recommended for the delineation of bacterial species²³⁾. These results indicate that strain RC825^T represents a separate species in the genus *Nocardioidea*.

Strain RC825^T could also be distinguished from other species of the genus *Nocardioidea* by different physiological and biochemical characteristics of this new isolate as shown in Tables 1 and 2. In particular, strain RC825^T differed from the phylogenetically closest species *N. ganhwensis* and *N. oleivorans* in terms of utilization of D-cellobiose, D-galactose and *N*-acetyl-glucosamine as well as the activity of naphthol-AS-BI-phosphohydrolase. Other characteristics such as citrate utilization, activity of arginine dihydrolase, tryptophan deaminase, lysine decar-

Table 1. Characteristics that distinguish strain RC825^T from the closely related species of the genus *Nocardioidea* strains: 1, strain RC825^T; 2, *N. ganghwensis*; 3, *N. oleivorans*; 4, *N. albus*; 5, *N. aquaticus*; 6, *N. aquiterrae*; 7, *N. jensenii*; 8, *N. luteus*; 9, *N. nitrophenolicus*; 10, *N. plantarum*; 11, *N. pyridinolyticus*; 12, *N. simplex*. Characteristics are scored as follows: +, Positive reaction; –, negative reaction; W, weakly positive; V, variable. All *Nocardioidea* strains are positive for Gram reaction, catalase, esterase lipase (C8) and leucine arylamidase; negative for *N*-acetyl- β -glucosaminidase, β -glucuronidase and α -fucosidase. Data are from Schippers *et al.*⁵⁾, Yi & Chun²⁴⁾ and Yoon *et al.*³⁾.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Motility	–	–	–	–	–	+	–	–	+	–	+	+
Mycelium and spore formation	–	–	–	+	–	–	–	+	–	–	–	–
Nitrate reduction	–	+	ND	–	+	+	+	–	–	–	+	–
Urease	–	–	ND	–	–	–	+	–	–	–	–	–
Hydrolysis of aesculin	–	W	ND	W	–	+	–	+	W	W	+	+
Hydrolysis of starch	–	+	ND	+	–	+	–	+	–	–	+	W
Carbon source utilization:												
Citrate	–	+	–	+	–	W	+	+	+	+	–	+
D-Cellobiose	–	+	+	+	–	+	–	+	–	+	+	–
D-Fructose	+	+	+	+	+	+	–	–	+	+	+	–
D-Galactose	–	+	+	+	+	+	–	–	–	–	+	–
D-Glucose	+	+	+	+	+	+	+	+	+	–	+	+
D-Mannitol	+	+	+	+	+	+	–	+	–	–	–	–
D-Mannose	–	+	+	+	–	–	–	+	–	–	–	–
D-Raffinose	–	+	ND	–	–	–	–	–	–	–	–	–
D-Ribose	–	–	–	–	–	–	–	–	+	–	+	–
D-Salicin	–	+	–	W	–	–	–	–	–	+	–	–
D-Xylose	–	+	–	+	+	+	–	+	+	+	+	–
Glycerol	–	+	ND	W	+	–	W	–	–	+	–	–
Inositol	–	–	–	–	–	–	–	–	–	–	+	–
Lactose	–	+	ND	–	–	–	–	–	–	–	–	–
L-Arabinose	–	+	–	+	–	–	–	+	–	–	–	–
L-Rhamnose	+	–	+	+	W	–	+	–	+	+	+	–
<i>N</i> -Acetyl-glucosamine	–	+	+	W	–	–	–	+	–	–	–	–
Sucrose	+	+	+	–	+	+	W	–	+	+	+	+
API ZYM:												
Alkaline phosphatase	+	+	+	+	V	–	W	V	+	–	+	+
Esterase (C4)	W	V	–	+	W	–	W	+	–	+	–	–
Lipase (C4)	–	–	–	–	–	–	–	–	–	W	–	–
Valine arylamidase	+	+	+	–	W	–	V	–	+	W	W	W
Cystine arylamidase	W	W	W	–	V	W	–	–	W	+	–	W
Trypsin	–	–	–	+	W	+	W	+	+	–	+	+
Acid phosphatase	W	W	–	–	+	+	V	–	+	W	+	W
Naphthol-AS-BI-phosphohydrolase	+	–	W	W	–	+	V	V	+	+	+	–
α -Galactosidase	+	+	W	–	–	–	–	–	–	–	–	–
β -Galactosidase	+	+	+	V	–	–	–	V	–	–	–	–
α -Glucosidase	+	+	+	+	+	+	V	V	+	+	+	+
β -Glucosidase	W	–	–	W	–	W	–	–	W	+	–	W
α -Mannosidase	–	–	–	–	–	–	–	+	–	–	–	–

boxylase, and ornithine decarboxylase, production of hydrogen sulfide and indole, and Voges-Proskauer test are negative. Positive results are for the activity of *o*-nitrophenyl- β -D-galactosidase (ONPG) and gelatin hydrolysis. On the basis of phenotypic, chemotaxonomic, and phylogenetic characteristics, we propose that strain RC825^T be assigned to the genus *Nocardioidea* as *Nocardioidea exalbidus* sp. nov.

DESCRIPTION OF *NOCARDIOIDES EXALBIDUS* SP. NOV.

Nocardioidea exalbidus ([ex.al.bi'dus]. L. adj. *exalbidus* whitish, i.e., white-colored).

The cells are Gram-positive, non-endospore-forming, non-motile, irregular rods of about 0.5 μ m wide and up to 2.0 μ m long. Grows well over a pH range of 6.0–9.0 on

Table 2. Whole-cell fatty acid composition (%) of strain RC825^T(1) and of the phylogenetically closest related type strains *N. ganghwensis* IAM 15306^T(2) and *N. oleivorans* IAM 15341^T(3). Data were taken from Yi & Chun²⁴), Schippers *et al.*⁵) and this study.

Fatty acids	1	2	3
Saturated fatty acids			
C _{17:0}	1.8	1.1	2.1
C _{18:0}	5.8	7.5	0.2
Unsaturated fatty acids			
C _{17:1ω6c}	4.7	5.7	1.6
C _{17:1ω8c}	1.3	2.2	25.7
C _{18:1ω9c}	6.0	27.5	5.7
Branched fatty acids			
iso-C _{15:0}	1.4	2.3	2.7
iso-C _{16:0}	28.0	18.6	30.4
iso-C _{17:0}	9.1	6.6	2.4
iso-C _{17:1ω9c}	2.2	2.0	1.6
anteiso-C _{17:0}	6.9	4.7	0.6
iso-C _{18:0}	5.3	1.8	1.2
10-methyl-fatty acids			
C _{17:0}	7.7	3.1	3.9
C _{18:0}	18.2	9.0	0.2

TSA medium; the optimal pH for growth is around 7.0. The temperature of growth was 15–35 °C; the optimal temperature for growth is 30 °C. Catalase activity is positive, whereas oxidase activity is negative. Positive results were recorded for the activity of alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, α -glucosidase, and β -glucosidase. The strain utilizes D-glucose, L-rhamnose, D-mannitol, amygdalin, melibiose, 2-keto-gluconate, and 5-keto-gluconate as sole carbon and energy sources. Glycerol, erythritol, L-arabinose, D-xylose, adonitol, methyl β -D-xyloside, D-galactose, D-mannose, sorbose, dulcitol, methyl α -D-mannoside, methyl α -D-glucoside, *N*-acetyl-glucosamine, arbutin, D-cellobiose, maltose, lactose, trehalose, sorbitol, inulin, melezitose, D-raffinose, starch, glycogen, gentiobiose, xylitol, D-arabitol, gluconate, D-turanose, D-lyxose, D-tagatose, and L-arabitol are not utilized. Diamino acid in the peptidoglycan is LL-diaminopimelic acid (wall type I). The predominant menaquinone was MK-8(H₄). Diphosphatidylglycerol and phosphatidylinositol were present as major polar lipids, but no phosphatidylcholine was found. The predominant fatty acids were iso-C_{16:0}, 10-methyl C_{18:0}, 10-

Fig. 2. The 16S rRNA gene sequence phylogenetic tree generated using the neighbor-joining method shows relationships between strain RC825^T and representatives of the genus *Nocardioides*. Numbers at the nodes indicate percentages of occurrence in 1000 bootstrapped trees; only values greater than 50% are shown.

methyl C_{17:0}, iso-C_{17:0} and anteiso-C_{17:0}. The DNA G+C content of the strain was 74 mol%.

The type strain, strain RC825^T (=IAM 15416^T=CCTCC AA206016^T), was isolated from the lichen in Izu-Oshima Island, Japan.

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