ORIGINAL ARTICLE

Nonomuraea indica sp. nov., novel actinomycetes isolated from lime-stone open pit mine, India

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A Gram-positive, aerobic, nonmotile actinomycete strain designated DRQ-2^T was isolated from the soil sample collected from lime-stone open pit mine from the Gulbarga region, Karnataka province, India. Strain DRQ-2^T was identified as a member of the genus *Nonomuraea* by a polyphasic approach. Strain DRQ-2^T could be differentiated from other members of the genus *Nonomuraea* on the basis of physiology and 16S rRNA gene sequence analysis. The 16S rRNA gene sequence similarity of strain DRQ-2^T showed highest sequence similarity to *Nonomuraea muscovyensis* DSM 45913^T (99.1%), *N. salmonea* DSM 43678^T (98.2%) and *N. maheshkhaliensis* JCM 13929^T with 98.0%, respectively. Chemotaxonomic properties showing predominant menaquinones of MK-9 (H₄), MK-9(H₂) and MK-9(H₆), major polar lipids comprised diphosphatidylglycerol, phosphatidylmono methyl ethanolamine (PME), phosphatidylethanolamine (PE), hydroxy-PME (OH-PME), hydroxy PE (OH-PEE), phosphatidylglycerol (PG), ninhydrin-positive phosphoglycolipid and unknown phospholipid, fatty acids with major amounts of i-C_{16:0}, ai-C_{15:0} and ai-C_{17:0} supported allocation of the strain to the genus *Nonomuraea*. Results of DNA–DNA hybridization and physiological tests allowed genotypic and phenotypic differentiation of strain DRQ-2^T from closely related species. The genomic DNA G+C content of the organism was 72.5 mol%. On the basis of phenotypic, chemotypic and molecular characteristics, strain DRQ-2^T represents a novel species of the genus *Nonomuraea*, for which the name *N. indica* sp. nov. is proposed, with type strain DRQ-2^T (= NCIM 5480^T = CCTCC AA 209050^T). *The Journal of Antibiotics* (2015) 68, 491–495; doi:10.1038/ja.2015.24; published online 18 March 2015

INTRODUCTION

The genus *Nonomuraea* was initially proposed in 1988 by Goodfellow, Stackebrandt and Kroppenstedt, but the name was not formally recognized in bacterial taxonomy. Zhang *et al.*¹ realized that the *Microtetraspora pusilla* group was phylogenetically distinct from other members of the genus *Microtetraspora* and established a new genus *Nonomuraea* to accommodate these species. Chiba *et al.*² subsequently corrected the spelling to *Nonomuraea*. The genus was grouped within the family *Streptosporangiaceae* by Quintana *et al.*³ Currently this genus harbors 36 validly described species and subspecies; including recently described valid members, like *N. fuscirosea*, *N. jabiensis*, *N. thailandensis* and *N. soli*.^{4–7} Members of this genus are believed to be widely distributed in soils, but are considered to be under speciated.³ *Nonomuraea* species can be separated from one another using a combination of phenotypic properties.^{2,8,9} The aim of the present study was to establish the taxonomic position of a lime-stone open pit strain DRQ-2^T within its classification in the genus *Nonomuraea*.

MATERIALS AND METHODS

Strain and culture collection

The soils of lime-stone open pit mine from the Gulbarga region, Karnataka province, India seems to be gold mine for the rich biodiversity of actinobacteria. *Streptomyces gulbargensis*, *S. tritolerans* and *S. deccanensis*^{10–12} have already been reported as novel isolates from these soils. The strain DRQ-2^T was isolated by standard serial dilution technique from the soil collected from lime-stone open pit mines. Aliquots of serial dilutions were spread onto starch–casein agar (1% soluble starch, 0.03% casein, 0.2% KNO₃, 0.2% NaCl, 0.2% KH₂PO₄, 0.002% CaCO₃, 0.005% MgSO₄·7H₂O, 0.001% FeSO₄·7H₂O and 1.8% agar; pH 7.2) and the plates were incubated at 45 °C for 2 weeks. A single colony was selected and further streaked on International Streptomyces Project (ISP) medium 2 as proposed by Shirling and Gottlieb,¹³ at least three times. Strain DRQ-2^T was grown on ISP medium 3 for 14 days at 45 °C for phenotypic and genetic comparison.

Morphological, physiological and biochemical characterization

Strain DRQ-2^T grew well on ISP2, 3, 4, 5 and 6 media, nutrient Agar, Czapek dox agar, glucose–asparagine agar and starch casein agar. The aerial mycelia

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Received 18 September 2014; revised 20 January 2015; accepted 16 February 2015; published online 18 March 2015

that was white to light pink appeared on all the media tested. Substrate mycelium was branched extensively and the colors on various media were pink to reddish pink-brownish yellows. No soluble pigments were observed on any media (Table 1). The aerial hyphae produced long and spiral spore chains, which had a spiral structure bearing 10 spores (Figure 1). Unless specified

Table 1 Cultural characteristics of the strains of DRQ-2^T on different growth media

Medium	Aerial mycelium	Substrate mycelium	Soluble pigment
ISP2	Light Pink	Reddish pink	None
ISP3	Light Pink	Reddish pink	None
ISP4	Pink	Dark pink	None
ISP5 (glycerol–asparagine agar)	White	Reddish yellow	None
ISP6	Pinkish white	Pink	None
Nutrient Agar	White	Light red	None
Czapeks Dox agar	White	Pinkish orange	None
Glucose–asparagine agar	White	Light brownish yellow	None
Starch casein agar	Light pink	Dark pink	None

otherwise, the following morphological and physiological characteristics were investigated using ISP2 as the basal medium. The temperature and pH for the growth were determined at 4, 10, 20, 30, 37, 45, 50 and 55 °C and at an initial pH 4.0–12.0 (at intervals of pH 1.0 unit), respectively. NaCl tolerance for growth was tested on ISP 4 medium supplemented with 1–9% (w/v) NaCl. Gram staining, oxidase activity was tested by spreading cell pellets on oxidase test paper (Hi-Media, Mumbai, India) and catalase activity was tested by using a 3% H₂O₂ solution, degradation ability and utilization of carbohydrates were determined using previously described methods of Lee and Lee.¹⁴ Other physiological and biochemical properties were tested using the API- 50CH, API-20E and API-ZYM kits (bioMérieux, Marcy l'Etoile, France) according to the recommendations of the manufacturer. Colonial morphology and pigmentation of the cells were observed and recorded after an incubation of 14 days at 45 °C. Cell morphology and motility were observed by using phase contrast and scanning electron microscopy. For scanning electron microscopy examination, 1-ml samples were fixed overnight at 4 °C by adding formaldehyde to a final concentration of 7%. Nine milliliter phosphate-buffered saline (130 mM NaCl, 10 mM sodium phosphate, pH 7.0 ± 0.2) was added to the samples, which were then filtered through 0.2- µm Millipore filters (Merck, Bengaluru, India) and washed with phosphate-buffered saline. The filters were then serially dehydrated in 25, 50, 70 and 100% ethanol solutions (three times for 10 min at each stage), critical-point dried, mounted on scanning electron microscope stubs, sputter coated with gold and viewed on Philips XL30 (FEI Company, Hillsboro, OR, USA); ESEM-TMP.

Figure 1 Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing the relationship between the strain DRQ-2^T and all the validly published species of the genus *Nonomuraea*. Asterisks indicate phyletic lines that were also recovered using the minimum- and maximum-likelihood algorithm. Bootstrap values were expressed as a percentage of 1000 replications. Only bootstrap values of > 50 % are shown. Scale bar, 0.005 substitutions per site.

16S rRNA gene sequencing and phylogenetic analyses

Extraction of genomic DNA and PCR amplification of 16S rRNA gene were done as described by Li *et al.*¹⁵ Multiple alignments with sequences of the most closely related *Nonomuraea* and the calculations of levels of sequence similarity were carried out using <http://eztaxon-e.ezbiocloud.net> of Kim *et al.*¹⁶ Phylogenetic analyses were performed using three tree-making algorithms that were the neighbour-joining,¹⁷ maximum-likelihood¹⁸ and maximum-parsimony¹⁹ methods. A phylogenetic tree was constructed using the neighbor-joining method of Saitou and Nei¹⁷ from Knuc values²⁰ using MEGA version 6.0.²¹ The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein¹⁸ with 1000 replicates. The genomic DNA of strain DRQ-2^T for the determination of G+C content was prepared according to the method of Marmur.²² The G+C content of the DNA was determined by reverse-phase HPLC of nucleosides according to Mesbah *et al.*²³ NA was hydrolyzed and the resultant nucleotides were analyzed by reversed phase HPLC.²³ DNA–DNA hybridization was carried out based on the principles and equations described by De Ley *et al.*²⁴ under the consideration of the modifications carried out by Huss *et al.*²⁵ and the optimized fluorimetric procedure by Loveland-Curtze *et al.*²⁶ was evaluated by using a Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) fitted with 96-well thermal cycling blocks. DNA dissolved in the 2 × SSC is used for the

analysis in three independent samples. The reassociations were carried out at an optimum renaturation temperature of 65 °C.^{27,28}

Chemotaxonomy

Chemotaxonomic characteristics were investigated as described by Lee and Lee,¹⁴ with cell biomass obtained from the culture grown in yeast malt glucose broth (0.4% yeast extract, 1.0% malt extract and 0.4% glucose, pH 7.2) for 5 days at 45 °C for the isomer of diaminopimelic acid in cell wall,²⁹ respiratory quinones,^{30,31} polar lipids³² and DNA G+C content.²³ Strain DRQ-2^T was grown on tryptone soy broth agar for 5 days at 45 °C for fatty acid analysis. The cellular fatty acid methyl esters were prepared and analyzed according to the standard protocol of the Microbial Identification System (version 6; MIDI, Newark, DE, USA).

Nucleotide sequence accession number

The 16S rRNA gene sequence of strain DRQ-2^T determined in this study has been deposited in GenBank under the accession number KM522835.

RESULTS AND DISCUSSION

An almost complete 16S rRNA gene sequence (1426 nt) of strain DRQ-2^T determined in this study was compared with those of the

Figure 2 Scanning electron micrograph of *N.onomuraea indica* sp. nov., DRQ-2^T grown on ISP 4 at 28 °C for 7 days. Scale bar, 2 μm. Arrow marks indicates the mycelium structure and spores arrangement.

representative species of the genus *Nonomuraea*. The results of 16S rRNA gene sequence comparison clearly demonstrated that strain DRQ-2^T is a member of the genus *Nonomuraea*. Strain DRQ-2^T was showing highest sequence similarity to *N. muscovyensis* DSM 45913^T (99.1%), *N. salmonea* DSM 43678^T (98.2%) and *N. maheshkhaliensis* JCM 13929^T (98.0%) with 12, 25 and 28 nt differences, respectively. In the phylogenetic tree based on the neighbour-joining algorithm, strain DRQ-2^T formed a separate clade with *N. muscovyensis* DSM 45913^T in the same branch with a higher bootstrap value of 99% (Figure 1). Topologies of phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms were similar to those of the tree constructed by the neighbour-joining analysis (Supplementary Figures S1 and S2). The 16S rRNA gene sequence similarities between strain DRQ-2^T and its closest neighbor, *N. muscovyensis* DSM 45913^T (99.1%), *N. salmonea* DSM 43678^T (98.2%) and *N. maheshkhaliensis* JCM 13929^T (98.0%) were further subjected for DNA–DNA hybridization. The DNA–DNA hybridization have a key role in microbial species discrimination in cases when 16S rRNA gene sequence, depending on the investigated taxonomic group, the threshold value has been increased to between 98.2 and 99.0% and it appears reasonable as recommended by Stackebrandt and Ebers³³ and Meier-Kolthoff et al.,³⁴ we have included two strains with above 99% similarity to two type strains with ≤ recommended threshold value. The G+C content of the DNA was 72.5 mol%. The determined DNA–DNA relatedness value between strain DRQ-2^T and its closest neighbor, *N. muscovyensis* DSM 45913^T, *N. salmonea* DSM 43678^T and *N. maheshkhaliensis* JCM 13929^T were 39.5 ± 3.2%, 48 ± 2.0% and 41.7 ± 2.5%, respectively (with s.d. in triplicate). DNA hybridization results indicated that, values of hybridizations were well below the

70% cutoff point for the recognition of novel genomic species,³⁵ thus suggesting that the strain DRQ-2^T should be considered as a new species of the genus *Nonomuraea*.

Cells of strain DRQ-2^T were aerobic, Gram-stain positive, non-motile (0.6–1.0 µm in diameter) that were present singly, in pairs or in clusters (Figure 2). Matured spore was not observed. Colonies of the cells were light pinkish, circular, convex with entire margins and reached 0.1–0.2 mm in diameter after an incubation for 5 days. Data for the other physiological and biochemical characteristics, and the species description are given in Table 2. Strain DRQ-2^T contained *meso* diaminopimelic acid and also showed the presence of galactose, glucose, mannose, ribose and madurose as whole-cell sugars. The predominant menaquinone was MK-9(H₄). The polar lipids comprised of diphosphatidylglycerol, phosphatidylmono methyl ethanolamine (PME), phosphatidylethanolamine (PE), hydroxy-PME (OH-PME), hydroxy phosphatidylethanolamine (OH-PEE), phosphatidylglycerol (PG), ninhydrin-positive phosphoglycolipid and unknown phospholipid were detected as phospholipids (Supplementary Figure S3). The DNA G+C content of strain DRQ-2^T was 72.5 mol%. The fatty acid profile of strain DRQ-2^T was characterized by large amounts of saturated and unsaturated fatty acids. The major fatty acids were i-C_{16:0} (37.8), C_{17:0} 10-methyl (16.8), iso-C_{16:0} 2OH (11.2), ai-C_{17:0} (9.4) and i-C_{15:0} (6.1) and the minor fatty acids (<5%) were C_{17:0} (4.2), i-C_{17:0} 2OH (2.1), iso-C_{17:0} 2OH (2.1), i-C_{16:1} (1.7), i-C_{17:0} (1.5), i-C_{14:0} (1.1) and C_{15:0} (1.0). Some of the fatty acids were absent compared with its nearest phylogenetic neighbors like, C_{14:0}, summed feature 4, whereas some fatty acids are higher in percentage compared with its neighbor. Detailed comparison of the fatty acids has been given in Supplementary Material in

Table 2 Comparison of the phenotypic properties of DRQ-2^T with those species of the genus *Nonomuraea* with validly published names

Characteristic	DRQ-2 ^T	<i>N. muscovyensis</i> DSM 45913 ^T	<i>N. salmonea</i> DSM 43678 ^T	<i>N. maheshkhaliensis</i> DSM 45163 ^T
Growth on ISP3 medium				
Aerial mycelium	Light pink	Yellowish brown	Pinkish white	White
Substrate mycelium	Reddish pink	Brownish orange	Moderate red	Light wheat
Soluble pigments	None	None	None	None
Biochemical Tests				
Aesculin hydrolysis	+	+	+	+
Nitrate Reductase	+	+	+	+
Degradation of				
Casein	+	–	–	–
Gelatin	+	–	–	–
Hypoxanthine	+	–	–	–
Starch	+	–	–	–
Tyrosine	–	–	+	+
Xanthine	+	–	–	–
Utilization of				
L-Arabinose	+	–	–	+
D-Fructose	+	–	–	+
myo-Inositol	–	–	–	–
D-Mannose	–	–	–	+
L-Rhamnose	+	+	+	+
Sucrose	+	+	+	+
D-Xylose	+	+	–	+
Gelatinase	+	+	–	–
Urease	–	–	–	–

All data were obtained in this study. Symbols: +, positive; –, negative.

Supplementary Table S1. Besides the genotypic evidence above, the strain DRQ-2^T could also be distinguished from their related species by phenotypic characteristics (Table 2). It is evident from the phenotypic, chemotaxonomic and phylogenetic data that strain DRQ-2^T should be given a novel species status in the genus *Nonomuraea*, for which we propose the name *N. indica* sp. nov.

Description of *Nonomuraea indica* sp. nov.

Nonomuraea indica (in'di.ca. L. fem. adj. indica pertaining to India, Indian.).

Cells are aerobic, Gram positive, oxidase negative, catalase positive, nonmotile (0.6–1.0 µm in diameter), that are present singly, in pairs or as cluster. Colonies are light pink, circular, convex with entire margins and reached 0.1–0.2 mm in diameter after an incubation of 14 days. Growth is observed at pH 6.0–12.0 (optimum at pH 9.0), 5–55 °C (optimum at 45 °C) and 0–10% (w/v) NaCl (optimum at 4% NaCl). The cells were positive for amylase, asparaginase, cellulase, gelatinase, glutaminase and protease; and negative for citrate utilization, and urease hydrolysis. H₂S production was not observed. D-glucose, L-rhamnose, D-sucrose, D-melibiose and L-arabinose are utilized as sole carbon and energy source but D-mannitol, inositol, D-sorbitol and amygdalin are not. Data for the other physiological and biochemical properties are given in Table 1. *Meso*-diaminopimelic acid is the diagnostic diamino acid in cell-wall peptidoglycan. The polar lipids comprised of diphosphatidylglycerol, PME, phosphatidylethanolamine (PE), hydroxy-PME (OH-PME), hydroxy phosphatidylethanolamine (OH-PEE), phosphatidylglycerol (PG), ninhydrin-positive phosphoglycerolipid and unknown phospholipid were detected as phospholipids. The predominant menaquinones were MK-7, MK-9, MK-9(H₂), MK-9(H₄) and MK-9(H₆) in the ratio of 13.1:4.6: 12.6: 49.3:10.7 (%). The major fatty acids are i-C_{16:0}, i-C_{16:0} 2OH, C_{17:0} 10-methyl and i-C_{15:0}. The type strain DRQ-2^T (NCIM 5480^T = CCTCC AA 209050^T) was isolated as DRQ-2^T from lime-stone open pit of the Gulbarga region of Karnataka province, India. The DNA G+C content of the type strain is 72.5 mol%.

ACKNOWLEDGEMENTS

This work was supported by the National Basic Research Program of China (no. 2010CB833801) and the National Natural Science Foundation of China (no. 31070007) and the International Cooperation Research Program of Yunnan Province (no. 2009AC017). W-JL was supported by the Program for the New Century Excellent Talents in University. W-JL was also supported by the Guangdong Province Higher Vocational Colleges & Schools Pearl River Scholar Funded Scheme (2014), SGD was supported by the Council of Scientific and Industrial Research (CSIR) and CSIR-NCL, India. The GenBank accession number of the 16S rRNA gene sequence of *Nonomuraea indica* DRQ^T-is KM522835.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)