ORIGINAL ARTICLE

Streptomyces hundungensis sp. nov., a novel actinomycete with antifungal activity and plant growth promoting traits

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A novel actinobacterium MBRL 251^T, isolated from a sample collected from a limestone quarry at Hundung, Manipur, India, was characterized using a polyphasic approach. The 16S ribosomal RNA gene sequence of strain MBRL 251^T showed closest similarities with *Streptomyces xanthochromogenes* NRRL B-5410^T (99.6%) and *Streptomyces michiganensis* NBRC 12797^T (99.6%). The DNA relatedness between MBRL 251^T and *S. xanthochromogenes* NBRC 12828^T, and *S. michiganensis* NBRC 12797^T was 46.6% and 40.7%, respectively. Strain MBRL 251^T contained LL-diaminopimelic acid as the diagnostic diamino acid, with glucose and xylose as the main cell wall sugars, whereas small amounts of galactose, mannose, rhamnose and ribose were also detected in the whole-cell wall hydrolysates. The major fatty acids identified were anteiso-C_{15:0} (35.1%), iso-C_{16:0} (21.1%) and anteiso-C_{17:1} (13.2%). The predominant menaquinones detected were MK-9(H₆) and MK-9(H₈), whereas the polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositolmannosides. The G + C content of the genomic DNA was 72.3%. The phenotypic and genotypic data showed that strain MBRL 251^T merits the recognition as a representative of a novel species of the genus *Streptomyces*. It is proposed that the isolate should be classified in the genus *Streptomyces* as a novel species, *Streptomyces hundungensis* sp. nov. The type strain is MBRL 251^T (= JCM 17577^T = KCTC 29124^T).

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INTRODUCTION

The genus *Streptomyces* is a major producer of diverse bioactive metabolites. Actinomycetes produce half of the known antibiotics from microbial sources, of which 75% are made by the streptomycetes.¹ Two of the common antifungal compounds produced by this genus are nystatin (*Streptomyces noursei*) and amphotericin B (*Streptomyces nodosus*), both of which belongs to the group of polyene antibiotics.^{2,3} Yuan and Crawford⁴ reported that a strain of *Streptomyces lydicus* was a potential biocontrol agent. Members of the genus have also been reported to have a role in plant growth promotion.^{5–7}

The genus is characterized by the presence of a high DNA G + C content, formation of extensively branched substrate and aerial mycelia, the presence of LL-diaminopimelic acid and absence of characteristic sugars in the cell wall (cell wall type I).^{8,9} At the time of

writing, there were 609 validly published *Streptomyces* species, containing 38 subspecies (<http://www.bacterio.cict.fr/s/streptomycesa.html>). This study presents the polyphasic characterization of a novel strain, MBRL 251^T, which is proposed to represent a novel species of the genus *Streptomyces*.

MATERIALS AND METHODS

Strain and culture conditions

Strain MBRL 251^T was isolated from a soil sample collected from a limestone quarry at Hundung, Manipur, India (25.05°N, 94.33°E) on starch casein nitrate agar¹⁰ adjusted to pH 8.5 as the selective isolation medium. Soil samples were air dried for a week, crushed and sieved. One gram of the sieved soil was suspended in 99 ml sterile distilled water and kept in an orbital shaker at 150 r.p.m. for 30 min. The suspension was centrifuged (1600 × g; 10 min) and 0.1 ml of the supernatant was serially diluted. 0.1 ml each of the diluted

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samples was spread on starch casein nitrate agar plates (pH 8.5) and incubated at 28 °C for 1 week. The isolates obtained were subcultured on the same medium to obtain pure cultures. Strain MBRL 251^T was preserved as lyophilized spore suspensions in skimmed milk at room temperature and as glycerol suspensions (20%, v/v) at –80 °C.

Phenotypic characterization

To observe its morphological characteristics, strain MBRL 251^T was cultivated aerobically on starch casein nitrate agar (28 °C) for 2 weeks. Morphology of spores and mycelia was observed by light microscopy (Olympus BH2, Tokyo, Japan) and scanning electron microscopy (Quanta 200, FEI, Hillsboro, OR, USA). Growth on various International *Streptomyces* Project¹¹ media, tryptic soy agar (Difco, Sparks, MD, USA), starch casein nitrate agar, Czapek's dox agar (HiMedia, Mumbai, India) and Nutrient agar (HiMedia) were observed (28 °C, 7 days). The colony colour was determined using the Inter-Society Colour Council-National Bureau of Standard (ISCC-NBS) colour chart.¹² Utilization of sole carbon and nitrogen sources was determined as described by Shirling and Gottlieb.¹¹ Tests for the decomposition of casein and tyrosine, and acid production from carbohydrates were performed following the methods of Gordon *et al.*¹³ Hydrolysis of starch, gelatin and Tweens 20, 40, 60 and 80 was determined as described by Collins *et al.*¹⁴ Nitrate reduction was monitored as described by Lanyi.¹⁵ Growth at different temperatures (5, 15, 28, 37, 42, 50 and 60 °C), pH (4, 5, 6, 7, 8, 9 and 10) and NaCl concentrations (0, 2, 5, 7 and 10% w/v) were determined on tryptic soy agar as described by Goodfellow.¹⁶ Catalase activity was observed by assessing bubble production in 3% (v/v) H₂O₂. Other biochemical tests including Voges–Proskauer, methyl red and the production of indole were performed as described by Goodfellow.¹⁶

Chemotaxonomy

The amino-acid content of the cell wall was determined according to Stanek and Robert¹⁷ and the sugars of the whole-cell wall hydrolysates were analyzed as described by Tang *et al.*¹⁸ For other chemotaxonomic analyses, cell biomass from a 1-week-old culture in tryptic soy broth (Difco) was harvested by centrifugation, washed with distilled water and lyophilized. Polar lipids were extracted and analyzed by two-dimensional TLC as described by Minnikin *et al.*¹⁹ The extraction of menaquinones was performed as described by Collins *et al.*²⁰ and analyzed by HPLC.²¹ Cellular fatty acids were extracted, methylated and analyzed by using the Sherlock Microbial Identification System according to the method of Sasser²² and the manufacturer's instructions. The fatty acid methyl esters were then analyzed by GC (Agilent Technologies 7890A GC System, Wilmington, DE, USA) by using the Microbial Identification software package (Sherlock Version 6.1; MIDI database: TSBA6, MIDI Inc, Newark, DE, USA).

Molecular analysis

Genomic DNA extraction and PCR amplification of the 16S ribosomal RNA (rRNA) gene was performed as described by Li *et al.*²³ The almost complete

16S rRNA gene sequence (1529bp) of the strain was identified using the EzTaxon-e server database²⁴ (<http://eztaxon-e.ezbiocloud.net/>) and aligned with the 16S rRNA gene sequences of other *Streptomyces* species using CLUSTAL X version 2.1.²⁵ Phylogenetic analyses were performed using the software package MEGA version 5.0.²⁶ Distances (using distance options according to Kimura's two-parameter model²⁷) were calculated and clustering was performed with the neighbour-joining method.²⁸ To determine the support of each clade, bootstrap analysis was performed with 1000 resamplings.²⁹ The validity of the neighbour-joining tree was evaluated with the maximum parsimony tree³⁰ drawn using MEGA 5.0. The G + C content of the genomic DNA was determined according to the method described by Mesbah *et al.*³¹ DNA–DNA relatedness was studied in triplicate by the thermal renaturation method³² using Lambda 35 UV/Vis Spectrophotometer (Perkin Elmer, Waltham, MA, USA) equipped with PTP 6+6 Peltier Temperature Programmer (Perkin Elmer) and BG-chiller E15 (Baygene Biotech, Beijing, China).

The GenBank accession number for the 16S rRNA gene sequence of strain MBRL 251^T is JN560157.

Antifungal screening

The antifungal bioassay was done by Dual Culture Technique⁶ against the rice fungal pathogens viz, *Bipolaris oryzae* (Microbial Type Culture Collection (MTCC) 3717, Brown spot disease), *Curvularia oryzae* (MTCC 2605, Leaf spot disease), *Fusarium oxysporum* (MTCC 287, Root rot disease), *Pyricularia oryzae* (MTCC 1477, Blast disease), *Rhizoctonia solani* (MTCC 4633, Sheath blight disease) and *Rhizoctonia oryzae-sativae* (MTCC 2162, Aggregate sheath blight disease) procured from the MTCC, Institute of Microbial Technology, Chandigarh, India. Plate containing only the fungal disc was used as the control. The mycelial growth inhibition was calculated using the formula given below:

$$\text{Percentage of mycelia growth inhibition} = \frac{C - T}{C} \times 100 \%$$

where, C = radial growth size of the test pathogen in the control plate
T = radial growth size of the test pathogen in the test plate.

Screening for plant growth promotion activity

Siderophore, indole acetic acid and ammonia production was screened according to the method of You *et al.*,³³ Bano and Musarrat,³⁴ and Cappuccino and Sherman.³⁵ Phosphate solubilization was screened using modified Pikovskaya medium containing bromophenol blue.³⁶

RESULTS AND DISCUSSION

Strain MBRL 251^T formed extensive substrate and aerial mycelia with a long spore chain (approximately 50 spores). At maturity, the strain formed *rectiflexibile* spore chains. A scanning electron micrograph demonstrating the aerial mycelia for strain MBRL 251^T is shown in Figure 1. The strain grew well on all the media tested with no pigment

Figure 1 Scanning electron micrographs for strain MBRL 251^T grown on starch casein nitrate agar (SCNA) medium for 2 weeks at 28 °C, bar 5 and 10 µm.

Table 1 Cultural characteristics of MBRL 251^T on different ISP and other selective media as observed using ISCC-NBS colour chart (Kelly¹²)

Medium	Colour of mycelium	
	Aerial (spore mass)	Substrate
ISP2	Grey brown	Brown
ISP3	Grey brown	Grey
ISP4	White	Yellow white
ISP5	Yellow brown	Grey yellow brown
ISP6	Grey	ND ^a
ISP7	Yellow pink	Brown
NA	Grey	Orange yellow
Czapek's	Yellow pink	Grey yellow brown
SCNA	Reddish brown	Orange yellow
TSA	Yellow brown	Yellow brown

Abbreviations: ISP, International *Streptomyces* Project; NA, nutrient agar; ND, not determined; SCNA, starch casein nitrate agar; TSA, tryptic soy agar.

^aThe result was not determined because of deep colour from media.

production was detected on any medium (Table 1). The strain was found to be able to hydrolyze tyrosine, gelatin and Tweens 20, 40, 60 and 80 but not casein and starch. The strain was positive for catalase, methyl red and indole production and nitrate reduction tests but negative for Voges–Proskauer and citrate utilization tests. The differentiating properties of strain MBRL 251^T from the related type strains *Streptomyces xanthochromogenes* NBRC 12828^T and *Streptomyces michiganensis* NBRC 12797^T are listed in Table 2 and other phenotypic characteristics are mentioned in the species description.

Strain MBRL 251^T had LL-diaminopimelic acid as the diagnostic cell wall diamino acid, glucose and xylose were the main sugars detected in the whole-cell wall hydrolysates along with small amounts of galactose, mannose, rhamnose and ribose. The major polar lipids detected were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol-mannoside, with other unknown phospholipids, aminophospholipid and lipids (see Supplementary Figure S1). MK-9(H₆) (72.4%) and MK-9(H₈) (27.6%) were the predominant menaquinones detected. The fatty acid methyl ester profile (>1%) contained anteiso-C_{15:0} (35.1%), iso-C_{16:0} (21.1%), anteiso-C_{17:1} (13.2%), iso-C_{14:0} (6.5%), C_{16:0} (6.1%), iso-C_{15:0} (3.8%), iso-C_{17:0} (2.9%), cyclo-C_{17:0} (2.8%) and iso-C_{16:1} H (1.7%).

The G + C content of the genomic DNA was 72.3%. EzTaxon-e sequence similarity results showed that the strain MBRL 251^T shares close (>97%) 16S rRNA gene sequence homologies with 205 *Streptomyces* type strains. Combining EzTaxon-e analysis and phylogenetic neighbour-joining tree (Figure 2, expanded neighbour-joining tree is shown in Supplementary Figure S2) results indicated that strain MBRL 251^T is closely related to *S. xanthochromogenes* NRRL B-5410^T (99.66%) and *S. michiganensis* NBRC 12797^T (99.66%) and these phylogenetic relationships were also supported in the tree generated according to the maximum parsimony algorithm with higher bootstrap values (see Supplementary Figure S3). Given that *Streptomyces* strains sharing >99.5% 16S rRNA gene sequence similarities have been reported among novel species,^{37–40} the two closest relatives, *S. xanthochromogenes* NBRC 12828^T and *S. michiganensis* NBRC 12797^T, were selected for DNA–DNA hybridization studies. The experiments showed that strain MBRL 251^T displayed low DNA–DNA reassociation values with *S. xanthochromogenes* NBRC 12828^T (46.6 ± 8.9%) and

Table 2 Differential characteristics between strain MBRL 251^T and *S. xanthochromogenes* NBRC 12828^T and *S. michiganensis* NBRC 12797^T

Characteristics	<i>S. hundungensis</i> MBRL 251 ^T	<i>S. xanthochromogenes</i> NBRC 12828 ^T	<i>S. michiganensis</i> NBRC 12797 ^T
pH range	5–10	6–9	6–9
Optimum pH for growth	8	7	7
NaCl tolerance (% w/v)	7	2	2
<i>Hydrolysis of</i>			
Casein	–	–	+
Gelatin	+	–	+
Tyrosine	+	–	–
Catalase	w	+	+
Methyl red	+	–	+
Indole production	+	–	+
<i>Utilization of sole C-sources</i>			
L-Arabinose	+	–	+
D-Cellobiose	+	–	+
Maltose	+	–	+
Mannitol	–	–	+
Raffinose	–	–	+
Sodium malate	+	–	–
L-Sorbose	–	–	+
Trehalose	–	–	+
<i>Utilization of sole N-sources</i>			
L-arginine	+	–	+
Potassium nitrate	–	+	+
<i>Major fatty acids (> 5%)</i>			
iso-C _{14:0}	6.5	–	–
iso-C _{15:0}	–	5.7	5.2
anteiso-C _{15:0}	35.1	44.2	40.6
iso-C _{16:0}	21.1	12.9	13.2
C _{16:0}	6.1	–	8.1
Anteiso-C _{17:1ω9c}	–	5.1	–
Anteiso-C _{17:1}	13.2	12.9	19.2
G + C mol %	72.3	72.3	71.9

Abbreviations: +, positive; –, negative; w, weakly positive; all the data were from this study. All the test strains are positive for hydrolysis of Tweens 20, 40, 60, 80; catalase and nitrate reduction tests, and acid production from fructose and glucose. All of them utilize fructose, D-galactose, D-mannose, succinic acid, D-xylose, L-alanine, glycine, L-histidine, L-hydroxyproline, D-methionine, L-ornithine, L-phenylalanine, proline, L-serine and L-valine as sole C and N sources. They showed negative results for starch hydrolysis, Voges–Proskauer and citrate utilization tests, and acid production from lactose, maltose, mannitol and sucrose. All the above strains could not utilize dulcitol, meso-inositol, lactose, rhamnose and xylitol as sole C sources. They grew in the temperature range 15–37 °C with optimum growth at 28 °C.

S. michiganensis NBRC 12797^T (40.7 ± 3.7%), thereby indicating that the whole genomic DNA relatedness values are well below the delineating 70% cutoff point for species identification.⁴¹ Based on its morphological characteristics and 16S rRNA gene sequence analysis results, the new species should be put in the cluster 29, which was identified by Labeda *et al.*⁴²

The genotypic and phenotypic features described above suggest that strain MBRL 251^T could be clearly distinguished from its closest phylogenetic relatives. Besides low DNA–DNA relatedness with the closest phylogenetic neighbours, the strain is also distinguished from them by several phenotypic properties as listed in Table 2. Therefore, the Hundung strain MBRL 251^T is considered to represent a new species of the genus *Streptomyces*, for which the name *Streptomyces hundungensis* sp. nov. is proposed.

The strain MBRL 251^T showed antifungal activities against *Bipolaris oryzae* MTCC 3717 (66% mycelial growth inhibition),

Figure 2 Neighbour-joining tree, based on 16S ribosomal RNA (rRNA) gene sequences, showing the relationships between strain MBRL 251^T and other *Streptomyces* type strains. Asterisks indicate branches that were also recovered using the maximum parsimony tree. Numbers at nodes are levels of bootstrap support (%) for branch points (1000 resamplings). Bar, 0.002 substitutions per nucleotide position.

Curvularia oryzae MTCC 2605 (57%), *Fusarium oxysporum* MTCC 287 (55%), *Pyricularia oryzae* MTCC 1477 (59%), *Rhizoctonia solani* MTCC 4633 (50%) and *Rhizoctonia oryzae-sativae* MTCC 2162 (60%). It also showed positive result for siderophore (see Supplementary Figure S4), indole acetic acid, ammonia production and phosphate solubilization.

Description of *Streptomyces hundungensis* sp. nov.

Streptomyces hundungensis (hun.dung.en'sis. NL masc. adj. *hundungensis* belonging to Hundung in Ukhrul, a hill district in Manipur, India from where the type strain has been isolated).

Gram positive, aerobic and spore chain containing up to 50 spores. *Rectiflexibile* spore chains, and each spore on maturity measures 0.6–1 µm in diameter. Growth occurs at 15–37 °C and pH 5–10, with optimum growth at 28 °C and pH 8. Growth occurs in presence of up to 7% NaCl. Utilizes L-arabinose, D-cellobiose, fructose, D-galactose, maltose, D-mannose, sodium malate, succinic acid and D-xylose as sole carbon sources; and L-alanine, L-arginine, glycine, L-histidine, L-hydroxyproline, DL-methionine, L-ornithine, L-phenylalanine, proline, L-serine and L-valine as sole nitrogen sources. Does not utilize dulcitol, meso-inositol, lactose, mannitol, raffinose, rhamnose, L-sorbose, trehalose or potassium nitrate as either sole carbon or nitrogen sources. Acid production from fructose and glucose, but not from lactose, maltose, mannitol or sucrose. Hydrolyzes gelatin, Tweens 20, 40, 60 and 80, and tyrosine but not casein and starch. Positive in catalase, methyl red, indole production and nitrate reduction tests but negative in Voges–Proskauer and citrate utilization tests. Contains LL-diaminopimelic acid, glucose and xylose with small amounts of galactose, mannose, rhamnose and ribose in the cell wall hydrolysates. MK-9(H₆) and MK-9(H₈) are the menaquinones present, whereas the polar lipids consist of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositolmannoside, with other unknown phospholipids, aminophospholipid and lipids. The fatty acid profile (>1%) is as follows: anteiso-C_{15:0}, iso-C_{16:0}, anteiso-C_{17:1}, iso-C_{14:0}, C_{16:0}, iso-C_{15:0}, iso-C_{17:0}, cyclo-C_{17:0} and iso-C_{16:1} H. Shows antifungal activities against *Bipolaris oryzae* MTCC 3717, *Curvularia*

oryzae MTCC, *Fusarium oxysporum* MTCC 287, *Pyricularia oryzae* MTCC 1477, *Rhizoctonia solani* MTCC 4633 and *Rhizoctonia oryzae-sativae* MTCC 2162. Positive for siderophore, indole acetic acid, ammonia production and phosphate solubilization.

The type strain, MBRL 251^T (= JCM 17577^T = KCTC 29125^T), was isolated from a limestone quarry at Hundung, Manipur, India.

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