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ORIGINAL PAPER

Streptomyces canchipurensis sp. nov., isolated from a limestone habitat

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Abstract Hundung Limestone habitat, Manipur, India is an unexplored site for microbial diversity studies. Using polyphasic taxonomy, a *Streptomyces* strain, MBRL 172^T, has been characterized. The strain was found to show highest 16S rRNA gene sequence similarity with *Streptomyces coeruleofuscus* NBRC 12757^T (99.2 %). The DNA relatedness between MBRL 172^T and *S. coeruleofuscus* NBRC 12757^T, and between MBRL 172^T and *Streptomyces nogalater* NBRC 13445^T, were 36.8 ± 4.4 and 52.5 ± 2.7 %, respectively. Strain MBRL 172^T was found to contain LL-diaminopimelic acid as the diagnostic diamino acid and glucose, mannose and xylose as the major sugars in whole cell hydrolysates. The polar lipids in the cell membrane were identified as diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositolmannoside. The predominant menaquinones detected were MK-9(H_6) and MK-9(H_8). The cellular fatty acids identified were mainly saturated fatty acids: anteiso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{15:0}$. Based on differences in the biochemical and molecular characteristics from its closest relatives, the strain can be proposed to represent a novel taxon in the genus *Streptomyces*, for which the name *Streptomyces canchipurensis* is proposed, with the type strain MBRL 172^T (=JCM 17575^T = KCTC 29105^T).

Keywords *Streptomyces canchipurensis* sp. nov. · Hundung · Limestone habitat

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Introduction

Limestone habitats usually have high deposits of $CaCO_3$ salts and therefore may be considered as unique habitats. Although microbes have been reported to play a major role in the formation of caves by dissolution of limestone and other calcareous rocks, and through extracellular precipitation of calcium carbonate (Danielli and Edington 1983; Engel et al. 2001; Riding 2000), relatively few studies have been performed on microbial diversity, including actinobacterial diversity, in limestone habitats (Groth et al. 1999a; Jurado et al. 2009; Kim et al. 1998; Nakaew et al. 2009; Niyomvong et al. 2012). To date, three new genera, *Beutenbergia, Hoyosella* and *Knoellia*, have been reported from limestone habitats and related limestone ecosystems, such as cave biofilms (Groth et al. 1999b, 2002; Jurado et al. 2009).

The genus *Streptomyces* is the currently most diverse known group of bacteria with more than 650 species reported (http://www.bacterio.net/streptomyesa.html; Parte 2014). *Streptomyces* species have been found to have diverse roles in nature, from being antibiotic producers to being pathogens (Keiser et al. 2000). The genus is characterized by high G+C content in DNA, formation of extensively branched substrate and aerial mycelia, presence of *LL*-diaminopimelic acid (*LL*-DAP) and absence of characteristic sugars in the cell wall (cell wall type I) (Kämpfer 2012). The present study reports the polyphasic characterization of a novel strain isolated from a limestone environment, MBRL 172^T, which is proposed as representative of a novel species of the genus *Streptomyces*.

Materials and methods

Strain and culture conditions

Strain MBRL 172^T was isolated from a soil sample collected from a limestone quarry site at Hundung, Manipur, India (25.05°N, 94.33°E). Isolation was performed on Gauze's Medium No. 1 using a procedure described earlier (Nimaichand et al. 2012). Strain MRBL 172^T was preserved as lyophilized spore suspensions in skim milk at room temperature and as glycerol suspensions (20 %, v/v) at −80 °C.

The related type strains *Streptomyces coeruleofuscus* NBRC 12757^T and *Streptomyces nogalater* NBRC 13445^T were obtained from Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Japan and cultured under comparable conditions as reference strains.

Phenotypic characterization

To observe its morphological characteristics, strain MBRL 172^T was cultivated aerobically in Gauze's Medium No. 1 (28 °C) for 2 weeks. Morphology of spores and mycelia was observed by light microscopy (Olympus BH2) and scanning electron microscopy (SEM) (Quanta 200, FEI) (Williams and Davies 1967). Growth on various International *Streptomyces* Project media (ISP, Shirling and Gottlieb 1966), Tryptic Soy Agar (TSA, Difco), Starch Casein Nitrate Agar (SCNA), Gauze's Medium No. 1, Czapek's Dox agar and Nutrient agar (NA) were observed. The colony colour was determined using the ISCC-NBS colour chart (Kelly 1964).

Utilization of sole carbon and nitrogen sources was determined as described by Shirling and Gottlieb (1966). Tests for decomposition of casein and acid production from carbohydrates were performed following the methods of Gordon et al. (1974). Hydrolysis of gelatin and starch was determined as described by Collins et al. (2004) and that of Tweens 20, 40, 60 and 80 according to Sierra (1957). Growth at different temperatures (5, 15, 20, 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) was determined on TSA as described by Goodfellow (1986). Catalase activity was observed by assessing bubble production in 3 % (v/v) H_2O_2 and oxidase activity was determined by using a 1 % (w/v) solution of tetramethyl-*p*-phenylenediamine (Kovacs 1956). Other biochemical tests including Voges-Proskauer, methyl red and indole tests were performed according to Goodfellow (1986).

Chemotaxonomy

The amino acid content of the cell wall was determined according to Staneck and Roberts (1974) and the sugars in the whole cell hydrolysates were analyzed as described by Tang et al. (2009). For other chemotaxonomic analyses, cell biomass from a two week old culture on Tryptic Soy Broth (TSB, 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. 1984). The extraction of menaquinones was performed according to Collins et al. (1977) and analyzed by HPLC (Tamaoka et al. 1983). Cellular fatty acids were extracted, methylated and analyzed by using Sherlock Microbial Identification System (MIDI) according to the method of Sasser (1990) and the manufacturer's instructions. Fatty acid methyl esters were analysed by GC (Agilent Technologies 7890A GC System) and identified using the Microbial Identification Software Package (Sherlock Version 6.1; MIDI database: TSBA6).

Molecular analysis

Genomic DNA extraction and PCR amplification of the 16S rRNA gene was performed as described by Li et al. (2007) with a slight modification. The strain was given a brief ultrasonic wave shock (53 kHz, 30 s) prior to lysozyme treatment. The almost complete 16S rRNA gene sequence (1,534 bp) of the strain was identified using the EzTaxon-e server database (Kim et al. 2012) and aligned with the 16S rRNA gene sequences of other *Streptomyces* species using CLUSTAL X version 2.1 (Larkin et al. 2007). Phylogenetic analyses were performed using the software package MEGA version 6.0 (Tamura et al. 2013). Phylogenetic distances were calculated with the Kimura two-parameter model (Kimura 1983) and tree topologies were inferred using the maximum-likelihood (Felsenstein 1981), maximum-parsimony (Fitch 1971) and neighbour-joining (Saitou and Nei 1987) methods. To determine the support of each clade, bootstrap analysis was performed with 1,000 resamplings (Felsenstein 1985).

The G + C content of the genomic DNA prepared by the method of Marmur (1961) was determined as described by Mesbah et al. (1989). DNA–DNA relatedness was studied by a fluorimetric method (Ezaki et al. 1988, 1989). One of the two DNA molecules for hybridization was labeled while the other was immobilized and vice versa. Six replications were done for each sample and the two extreme values (highest and lowest) for each sample were excluded. The relatedness values are expressed by calculating the means of the remaining four values and the DNA–DNA hybridization was taken from the two means of relatedness values.

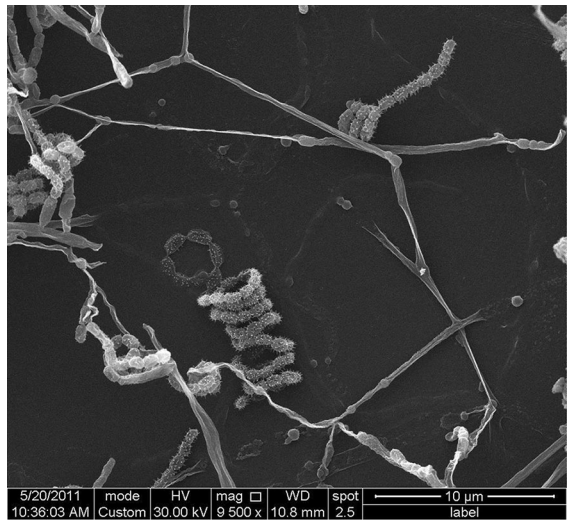

Fig. 1 Scanning electron micrograph for strain MBRL 172^T grown on Gauze's medium no. 1 for 2 weeks at 28 °C, *Bar* 10 µm

Table 1 Culture characteristics of strain MBRL172^T on different ISP and other media as observed using ISCC-NBS Colour Chart (Kelly 1964)

Medium	Colour of mycelium		Soluble pigment
	Aerial (spore mass)	Substrate	
ISP2	Black	Black	–
ISP3	White	Black	–
ISP4	White	Red brown	–
ISP5	White	Black	–
ISP6	Grey	Green brown	–
ISP7	Black white	Black	Brown
NA	Grey	Grey yellow brown	–
Czapek's dox	White	Brown	–
SCNA	White	Black	–
TSA	Green grey	Yellow brown	–
Gauze's medium no. 1	White	Blue	Blue

Results and discussion

Strain MBRL 172^T was observed to form extensive substrate and white coloured aerial mycelia on Gauze's Medium No. 1. The strain was observed to form *spiralis* spore chains with warty spores (approximately 50 spores per chain, each spore measuring ~600 × 900 nm in dimensions, Fig. 1). The strain

Table 2 Differential characteristics between strains MBRL 172^T, *S. coeruleofuscus* NBRC 12757^T and *S. nogalater* NBRC 13445^T

Characteristics	*S. canchipurensis* MBRL172^T	*S. coeruleofuscus* NBRC 12757^T	*S. nogalater* NBRC 13445^T
Temperature range for growth (°C)	20–37	20–37	28–50
NaCl tolerance for growth (%)	0–2	0–5	0–2
pH range	6–9	6–9	6–8
Optimum pH for growth	28	28	37
Hydrolysis of			
Casein	−	w	+
Starch	+	+	−
Acid production from			
Fructose	−	−	+
Galactose	−	−	+
Glucose	−	−	+
Maltose	+	−	+
Mannose	−	−	+
Utilization of sole C-sources			
Arabinose	−	−	+
Cellobiose	−	−	+
Dulcitol	+	+	−
Inositol	+	−	−
Inulin	+	+	−
Melibiose	+	+	−
Raffinose	+	+	−
Salicin	−	+	−
Sorbitol	+	+	−
Utilization of sole N-sources			
L-Alanine	+	+	−
L-Arginine	+	+	−
L-Asparagine	+	−	+
L-Hydroxyproline	+	−	−
L-Isoleucine	+	−	w
L-Leucine	+	+	−
L-Lysine	+	−	−
DL-Methionine	+	−	−
L-Tryptophan	+	−	−
L-Tyrosine	+	−	−
L-Valine	+	w	+
Major fatty acids (>5 %)			
Iso-$C_{14:0}$	−	5.3	34.4
Iso-$C_{15:0}$	14.9	26.0	32.3
Anteiso-$C_{15:0}$	19.7	15.7	−
Iso-$C_{16:1}$ H$^{\#}$	5.9	−	–
Iso-$C_{16:0}$	19.5	24.8	–
$C_{16:0}$	–	7.6	–
Summed Feature 9	6.5	–	–
Anteiso-$C_{17:1}\omega 9c$	5.8	–	–
Anteiso-$C_{17:0}$	8.5	–	14.9

All the test strains were positive for hydrolysis of Tweens 20, 40, 60, 80; catalase, citrate utilization, nitrate reduction and oxidase and starch hydrolysis tests and acid production from lactose and sucrose. All of them utilized galactose, lactose, maltose, mannose, sucrose and L-glutamine as sole C and N sources. They showed negative results for gelatin liquefaction, indole production, MR, VP and urea hydrolysis tests. None of the strains could utilize L-cysteine as sole N source

+ Positive, − negative, *w* weakly positive, # indicates that the position and configuration of the double bond is not known; Summed feature 9 consisted of iso-$C_{17:1}\omega 9c$ and/or 10 methyl $C_{16:0}$; all the data are from this study

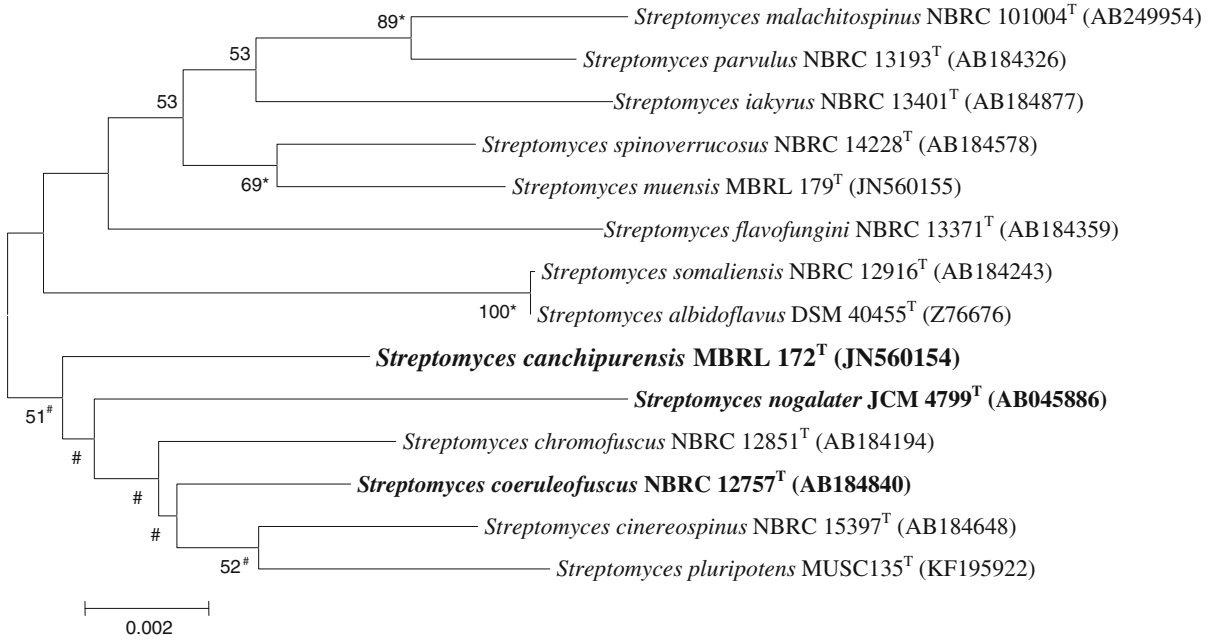

Fig. 2 Neighbour-joining tree, based on 16S rRNA gene sequences, showing the relationships between strain MBRL 172^T and other type strains of the genus *Streptomyces*. *Hash* and *asterisks* indicate the branches that were also recovered in the maximum-likelihood analysis, and both maximum-likelihood and maximum parsimony trees, respectively. Numbers at nodes are levels of bootstrap support (%) for branch points (1,000 resamplings). *Bar* 0.002 substitutions per nucleotide position

Table 3 DNA relatedness between strains MBRL 172^T, *S. coeruleofuscus* NBRC 12757^T and *S. nogalater* NBRC 13445^T as determined by the fluorimetric method

The two DNAs for DNA–DNA hybridization	DNA as probe	DNA immobilized	Values of four replicates (%)				Average (%)	The result of DNA–DNA hybridization (%)
			1	2	3	4		
A and B	A	B	52.1693	47.3961	52.7408	56.5065	52.2 ± 3.74	52.53 ± 2.74
	B	A	50.2613	53.4918	53.2077	54.4474	52.85 ± 1.81	
A and C	A	C	33.5550	33.7654	34.4676	41.6141	35.85 ± 3.86	36.83 ± 4.42
	C	A	40.7762	30.4068	37.7062	42.3515	37.81 ± 5.3	

A Streptomyces canchipurensis MBRL 172^T, *B S. nogalater* NBRC 13445^T, *C S. coeruleofuscus* NBRC 12757^T

was found to grow well in all the media tested and formed brown and blue diffusible pigments on ISP 7 and Gauze's Medium No. 1, respectively (Table 1). The differentiating properties of strain MBRL 172^T relative to the related type strains *Streptomyces coeruleofuscus* NBRC 12757^T and *Streptomyces nogalater* NBRC 13445^T are listed in Table 2 and the detailed phenotypic characteristics are mentioned in the species description.

Strain MBRL 172^T was found to have *LL*-DAP as the diagnostic cell wall diamino acid and xylose (61 %), glucose (23 %) and mannose (13 %) were detected as the major sugars in whole cell hydrolysates, along with small amounts of ribose (4 %). The major polar lipids detected were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositolmannoside, along with other unknown phospholipids and

lipids (see Supplementary Fig. S1). MK-9(H_8) (47.5 %) and MK-9(H_6) (41.2 %) were identified as the predominant menaquinones, along with small amounts of MK-9(H_4) (9.8 %) and MK-9(H_2) (1.5 %). The fatty acid methyl ester profile (>1 %) was found to contain anteiso-$C_{15:0}$ (19.7 %), iso-$C_{16:0}$ (19.5 %), iso-$C_{15:0}$ (14.9 %), anteiso-$C_{17:0}$ (8.5 %), Summed Feature 9 (as defined by MIDI) containing iso-$C_{17:1}\omega9c$ and/or 10-methyl $C_{16:0}$ (6.5 %), iso-$C_{16:1}$- H (5.9 %), anteiso-$C_{17:1}\omega9c$ (5.8 %), $C_{16:0}$ (4.8 %), iso-$C_{17:0}$ (4.2 %), Summed Feature 3 containing $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$ (3.6 %) and iso-$C_{14:0}$ (3.1 %). The major fatty acids in *S. coeruleofuscus* NBRC 12757^T are unsaturated fatty acids iso-$C_{15:0}$ (26.0 %), iso-$C_{16:0}$ (24.8 %) and anteiso-$C_{15:0}$ (15.7 %), and in the case of *S. nogalater* NBRC 13445^T are iso-$C_{15:0}$ (34.4 %), anteiso-$C_{15:0}$ (32.3 %) and anteiso-$C_{17:0}$ (14.9 %). The fatty acids $C_{16:0}$ (7.6 %) and iso-$C_{14:0}$ (5.3 %) in *S. coeruleofuscus* NBRC 12757^T were not detected or detected in lesser quantities (<5 %) in the other two strains, indicating the variability in the fatty acids profile (Table 2).

The G + C content of the genomic DNA of strain MBRL 172^T was found to be 69.4 %. Based on the 16S rRNA gene sequence similarities and the neighbour-joining tree (Fig. 2), the strain forms a clade with *S. coeruleofuscus* NBRC 12757^T (99.2 %; 12/1462 nucleotide differences), *Streptomyces chromofuscus* NBRC 12851^T (99.0 %; 15/1460), *Streptomyces cinereospinus* NBRC 15397^T (98.8 %; 17/1462), *S. nogalater* JCM 4799^T (98.6 %; 21/1469) and *Streptomyces pluripotens* MUSC 135^T (98.5 %; 22/1483). This indicated that the strain belongs to clade 114 as defined by Labeda et al. (2012). The maximum-likelihood tree and maximum-parsimony tree are shown in Supplementary Fig. S2 and Fig. S3, respectively. As *Streptomyces* strains sharing more than 99.5 % 16S rRNA gene sequence similarities with their closest homologues have been reported among novel species (Bouchek-Mechiche et al. 2000; Dastager et al. 2007; Goodfellow et al. 2007; Nimaichand et al. 2012, 2013), strains *S. coeruleofuscus* NBRC 12757^T and *S. nogalater* NBRC 13445^T were selected as the representative strains to perform DNA–DNA hybridization studies. The experiments showed that DNA–DNA relatedness values with *S. coeruleofuscus* NBRC 12757^T and *S. nogalater* NBRC 13445^T (Table 3) are well below the 70 % cut off point for species identification (Wayne et al. 1987).

The chemotaxonomic data and the phylogenetic analysis clearly indicates the affiliation of strain MBRL 172^T to the genus *Streptomyces*. The genotypic and phenotypic features suggest that strain MBRL 172^T could be clearly distinguished from its closest phylogenetic relatives. Besides low DNA–DNA relatedness values, the strain is also distinguished from *S. coeruleofuscus* NBRC 12757^T and *S. nogalater* NBRC 13445^T by several phenotypic properties as listed in Table 2. Therefore, strain MBRL 172^T, isolated from Hundung, is considered to represent a new species of the genus *Streptomyces,* for which the name *Streptomyces canchipurensis* sp. nov. is proposed.

Description of *Streptomyces canchipurensis* sp. nov

Streptomyces canchipurensis (can.chi.pur.eńsis. N.L. masc. adj. *canchipurensis* of or belonging to Canchipur, Manipur University, India, where the MBRL research group which isolated the type strain is located).

Gram-stain positive, aerobic, with *Spiralis* spore chains containing up to 50 spores per chain. Each spore on maturity measures ~600 × 900 nm. Growth occurs at 20–37 °C and pH 6–9, with optimum growth at 28 °C and pH 7. Growth occurs in presence of up to 2 % NaCl. Able to utilise dulcitol, galactose, inositol, inulin, lactose, maltose, mannose, melibiose, raffinose, sorbitol and sucrose as sole carbon sources; and L-alanine, L-arginine, L-asparagine, L-glutamine, L-hydroxyproline, L-leucine, L-lysine, DL-methionine, L-tryptophan, L-tyrosine and L-valine as sole nitrogen sources. Does not utilise arabinose, cellobiose, salicin and L-cysteine as either sole carbon or nitrogen sources. Acid is produced from lactose, maltose and sucrose but not from mannose. Hydrolyzes starch and Tweens 20, 40, 60 and 80 but not casein, gelatin and urea. Positive in catalase, oxidase and citrate utilization tests but negative in methyl red, Voges-Proskauer and indole production tests. Contains *LL*-diaminopimelic acid, xylose, glucose and mannose with small amount of ribose in the whole cell hydrolysates. MK-9(H_6) and MK-9(H_8) are the predominant menaquinones, while the polar lipids consist of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositolmannoside along with other unknown

phospholipids and lipids. The fatty acid profile (>1 %) contains anteiso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{15:0}$, anteiso-$C_{17:0}$, Summed Feature 9 containing iso-$C_{17:1}\omega9c$ and/or 10-methyl $C_{16:0}$; iso-$C_{16:1}$- H, anteiso-$C_{17:1}\omega9c$, $C_{16:0}$, iso-$C_{17:0}$, Summed Feature 3 containing $C_{16:1}$-$\omega7c$ and/or $C_{16:1}\omega6c$ and iso-$C_{14:0}$. The G + C content of the genomic DNA of the type strain is 69.4 %.

The type strain MBRL 172^T (=JCM 17575^T - = KCTC 29105^T) was isolated from a limestone quarry site at Hundung, Manipur, India. The 16S rRNA gene sequence of strain MBRL 172^T has been deposited in GenBank under the accession number JN560154. The strain belongs to clade 114 of the genus *Streptomyces* as defined by Labeda et al. (2012).

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