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ORIGINAL ARTICLE

Allostreptomyces indica sp. nov., isolated from India

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A novel actinobacterium, designated strain YIM 75704^T, was isolated from a limestone quarry located at Gulbarga, Karnataka, India. The novel strain has showed typical morphological and chemotaxonomic characteristics of the family *Streptomycetaceae*. Comparison of 16S rRNA gene sequences indicated that this strain represents a novel member of the family *Streptomycetaceae* and exhibited 99.0% 16S rRNA gene sequence similarities with the type species of the recently described novel genus *Allostreptomyces*, that is, *Allostreptomyces psammosilenae*, whereas other species of *Streptomyces* were below 95% sequence similarity. The cell hydrolysates contained the LL-isomer of diaminopimelic acid and the predominant quinones were MK-9 (H_6, H_8 and H_4). The polar lipid profile consisted of diphosphatidylglycerol, phosphatidylinositolmannosides and three unknown phospholipids. The DNA G+C content was 75.0 mol%. A polyphasic study of the strain with morphological, phenotypic, phylogenetic and with DNA–DNA hybridization evidence with related members showed that this strain represents novel species of *Allostreptomyces* for which the name *Allostreptomyces indica* sp. nov., is proposed. The type strain is YIM 75704^T (= DSM 41985^T = CCTCC AA 209051^T = NCIM 5485^T).
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INTRODUCTION

Streptomycetaceae is the one of largest families within *Actinobacterium*, containing the genus *Streptomyces* as proposed by Waksman and Henrici,[1] and later emended to separate the genera *Streptomyces*, *Kitasatospora*[2] and *Streptacidiphilus*.[3] Members of the family *Streptomycetaceae* have attracted great attention because of their production of various natural products of considerable commercial value.[4] Streptomycetes are predominantly found in soil and decaying vegetation, most produce chains of spores and some are noted for their distinct 'earthy' odor that results from production of a volatile metabolite, geosmin. Up to now, there have been several reports on the physiology and energetics of alkaliphilic bacteria,[5,6] whereas there are very few reports on alkaliphilic actinobacteria. Thus, studies on the physiology of alkaliphilic actinobacteria are urgently required to exploit this microbial resource with great biotechnological potential. The soils from an open pit limestone mine in the Gulbarga region, Karnataka, India, seems to be great sources for a rich biodiversity of actinobacteria. *Streptomyces gulbargensis*,[7] *Streptomyces tritolerans*[8] and *Streptomyces deccanensis*[9] have been already reported as novel isolates from these soils. A study was undertaken to discover novel actinomycetes from this limestone quarry during which an actinobacterium strain, YIM 75704^T, was isolated and characterize by a polyphasic study designed to establish the taxonomic status of this strain. On the basis of the data obtained from this study, isolate YIM 75704^T represents a novel species of recently proposed genus *Allostreptomyces* of the family *Streptomycetaceae*, for which we propose the name *A. indica* sp. nov.,

MATERIALS AND METHODS

Organisms, maintenance and culture conditions

Strain YIM 75704^T was isolated by standard serial dilution technique from soil collected from an open pit limestone mine. Aliquots of serial dilutions were spread onto starch-casein agar (1% soluble starch, 0.03% casein, 0.2% KNO_3, 0.2% NaCl, 0.2% KH_2PO_4, 0.002% $CaCO_3$, 0.005% $MgSO_4{\cdot}7H_2O$, 0.001% $FeSO_4{\cdot}7H_2O$ and 1.8% agar; pH 7.2) and the plates were incubated at 28 °C for 2–3 weeks. A single colony was selected and further streaked on ISP 3 medium (Hi-media, Mumbai, India) at least three times to obtain a pure culture. The pure culture was maintained as agar slopes and as suspensions of hyphal fragments and spores in 20% (v/v) glycerol at − 80 °C. Further, for phenotypic and genetic comparison, strain YIM 75704^T was grown on ISP 3 medium for 5 days at 28 °C.

Morphological, physiological and biochemical characterization

The temperature and pH range for growth were determined at temperatures 4, 10, 20, 30, 37, 45, 50 and 55 °C and pH 4.0–12.0 (at intervals of pH 1.0 unit), respectively. NaCl tolerance for growth was tested on ISP 3 medium supplemented with 0–9% (w/v) NaCl. A range of phenotypic properties was examined using standard procedures,[10,11,12] Gram stain, oxidase and catalase activities, degradation ability and utilization of carbohydrates were determined using previously described methods.[13] In addition, acid production from carbohydrates was tested using the media and methods described by Gordon *et al.*[14] Other physiological and biochemical properties were tested using the API- 50CH, API-20E and API-ZYM kits (bioMérieux, New Delhi, India) according to the recommendations of the manufacturer. The arrangements of hyphae and spore chains were detected on ISP 3 after 14 days at 28 °C, using the coverslip technique of Kawato and Shinobu.[15,16] Spore chain arrangement and spore surface ornamentation were observed by examining gold coated

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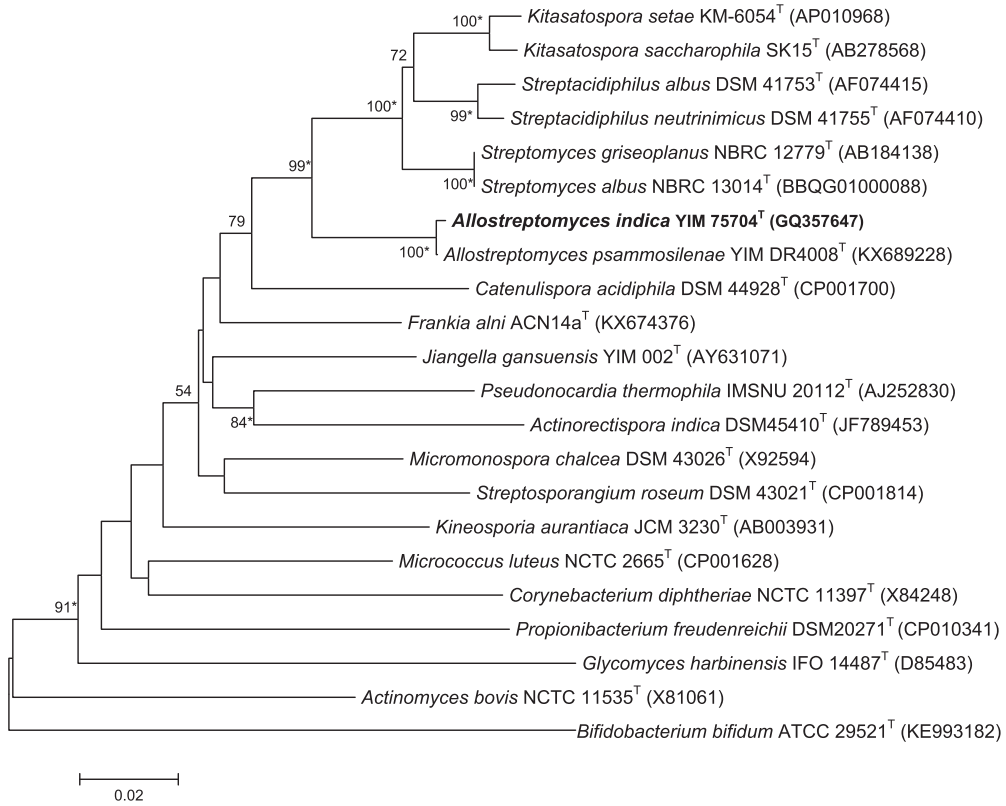

Figure 1 Neighbor-joining tree based on 16S rRNA gene sequences, showing the relationships between strain YIM 75704^T and other type strains of *Streptomyces* species. *Bifidobacterium bifidum* ATCC 29521^T (KE993182) was used as the out-group. Asterisks indicate branches that were also recovered using the maximum parsimony and maximum likelihood tree. Numbers at nodes are levels of bootstrap support (>50%) for branch points (1000 resampling's). Scale bar = 0.02 substitutions per nucleotide position.

dehydrated specimens, taken from the ISP 3 plate and under a scanning electron microscope (Philips XL30; ESEM-TMP, MD Eindhoven, The Netherlands) as described by O'Donnell *et al.*[17] Cultural characteristics were determined using ISP media after incubation at 28 °C for 14 days.

Chemotaxonomy and morphology

The isolate was examined for chemotaxonomic properties typical of the genus *Streptomyces*[18] with cell biomass obtained from culture grown in ISP 3 broth for 3 days at 28 °C for the isomer of diaminopimelic acid in cell wall, respiratory quinones, polar lipids and DNA G+C content according to standard procedure.[19–22] Strain YIM 75704^T was grown on tryptone soy broth agar for 5 days at 28 °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, Hewlett-Packard Co., Palo Alto, CA, USA) ACTINO database.[23]

16S rRNA gene sequencing, phylogenetic analyses and DNA–DNA hybridization

Extraction of genomic DNA and PCR amplification of 16S rRNA gene followed the methods of Li *et al.*[24] Comparisons with sequences of most closely related *Streptomycetaceae* and calculations of levels of sequence similarity were carried out using MEGA 6.0 (www.megasoftware.net) with sequences downloaded from EzTaxon server 2.0.[25] Phylogenetic analyses were performed using the neighbour-joining,[26] maximum-likelihood[27] and maximum-parsimony[28] methods. A phylogenetic tree was constructed using the neighbor-joining method of Saitou and Nei[26] from *Knuc* values,[29] using MEGA version 6.0.[30] The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein[31] with 1,000 replicates. The genomic DNA of strain YIM 75704^T for the determination of G+C content was prepared according to the method of Marmur.[32] The G+C content of the DNA was determined by reverse-phase HPLC of nucleosides according to Mesbah *et al.*[22] For DNA–DNA hybridization experiments, genomic DNA was extracted and purified according to the method of Marmur.[32] Hybridization was carried out based on the principles and equations described by De Ley *et al.*[33] under the consideration of modifications carried out by Huss *et al.*[34] and the optimized fluorimetric procedure Loveland-Curtze *et al.*[35] was evaluated by using a Step One Plus Real-Time PCR system (Applied Biosystems, Thermo-fisher Scientific, Carlsbad CA, USA) fitted with 96 well thermal cycling blocks. DNA suspended in the 2× saline sodium citrate (SSC) is used for the analysis in three independent samples. The reassociation was carried out at an optimum renaturation temperature of 75 °C.[36,37]

Nucleotide sequence accession number

The 16S rRNA gene sequence of strain YIM 75704^T determined in this study has been deposited in GenBank under the accession number GQ357647.

RESULTS AND DISCUSSION

An almost complete 16S rRNA gene sequence (1430 nt) of strain YIM 75704^T determined in this study was compared with those of representatives species of the family *Streptomycetaceae*. The results of 16S rRNA gene sequence comparison clearly demonstrated that strain YIM 75704^T is a novel member of the genus *Allostreptomyces*, with 99.0% similarity with *A. psammosilenae* DSM 42178^T. All other type strains of the genus *Streptomyces* were below 95% sequence similarity. In the phylogenetic tree based on the neighbour-joining algorithm, strain YIM 75704^T formed a separate clade with the *Allostreptomyces* species with a higher bootstrap value of 100% (Figure 1). Topologies of phylogenetic trees built using the maximum-likelihood and

Table 1 Growth and cultural characteristics of strain YIM 75704^T on different growth media after incubation for 2 weeks at 28 °C

Medium	*Growth*	*Aerial mycelium*	*Substrate mycelium*	*Diffusible pigment*
Yeast extract-malt extract agar (ISP 2)	Good	Light yellow	Brown	None
Oatmeal agar (ISP 3)	Good	Olive green	Brown	None
Inorganic salts-starch agar (ISP 4)	Moderate	Light yellow	Slight yellow	None
Glycerol-asparagine agar (ISP 5)	Good	Light yellow	Slight yellow	None
Czapek solution agar (Difco)	Good	Light yellow	yellow pink	None
Nutrient agar (Difco)	Good	Cream	None	None
Tryptic soy agar (Difco)	Good	Olive yellow	Brown	None
Potato dextrose agar (Difco)	Poor	Cream	Medium gray	None

maximum-parsimony algorithms were similar to those of the tree constructed by neighbour-joining analysis. The determined DNA–DNA relatedness values between strain YIM 75704^T and *A. psammosilenae* DSM 42178^T, which is the only strain shared above 99% 16S rRNA gene sequence similarity, was 54.5% (3.0) (reading are the mean of triplicates), thereby indicating that the whole-genome DNA–DNA relatedness values with the isolate's closest phylogenetic neighbors are well below the delineating 70% cutoff point for species identification,[38] thus suggesting that the strain YIM 75704^T should be compared to the members of the genus *Allostreptomyces*. The genomic DNA G+C content of strain YIM 75704^T was 75.0 mol%, which was similar to related reference species of *Allostreptomyces* genera.

The spore surface appears to be smooth. The strain grew well in all the media tested (Table 1). Cells of strain YIM 75704^T were aerobic, Gram-positive, filamentous, non-motile (0.6-1.0 μm in diameter), spores appears as pairs and in clusters. Spores appears similar as compared to other *Streptomyces* species (Supplementary Figure S1). Colonies of the cells have brown to yellow substrate mycelia and olive green aerial hyphae, circular, convex with entire margins and reached 1.0–3.0 mm in diameter after incubation for 5 days at 28 °C. Strain YIM 75704^T was positive for gelatin liquefaction, milk coagulation and peptonization activity. The strain was positive for starch hydrolysis, arginine decarboxylase activity, negative for nitrate reduction, gas production from nitrate, growth on cellulose and H_2S production. Growth occurred at pH 6.0–10.0 (optimum, pH 7.2) and with 0–10% NaCl (optimum, 0% NaCl). Aesculin, citrate, cellobiose, glucose, inositol, mannose, melibiose, sorbitol, starch and sucrose were utilized, urease and ornithine decarboxylase activity were negative and acids were not produced from any of these carbon sources tested. Complete carbon utilization were mentioned in the species description as well as in Table 2.

Table 2 Morphotypic and phenotypic properties of *A. indica* YIM 75704^T

Characteristic	A. indica *YIM 75704^T*	A. psammosilenae *YIM DR4008^T.*
Morphology		
Spore chains	Spiral	Spiral
Diffusible pigment o InSP 3	None	Brilliant orange
pH range (Optimum)	6.0–10.0 (7.2)	5.0–11.0 (7.0)
Temperature range (°C)	10–45	10–50
NaCl tolerance (w/v,%)	0–10.0	0–4.0
Nitrate reduction	–	+
β-Galactosidase	+	–
Citrate utilization	–	+
Sugar utilization		
D-Glucose	+	–
Mannitol	+	–
Maltose	–	+
Inositol	+	–
Sorbitol	+	–
Rhamnose	+	–
Sucrose	+	–
G+C (mol %)	75.0	75.3

Abbreviations: +, positive; –, negative.
Both strain are positive for gelatin liquefaction, acetoin production, aesculin production and utilization of arabinose, mannose and melibiose. Both strains showed negative for ornithine decarboxylase, adipose utilization, H_2S, indole and urease production.

Strain YIM 75704^T contained LL-isomer of diaminopimelic acid as the diagnostic diamino acid in the cell wall peptidoglycan and the major quinones were MK-9 (H_6 and H_8) with 43.5% and 29.3%, respectively, whereas minor amount of MK-9 (H_4) was also present at 4.0%. The polar lipids comprised diphosphatidylglycerol, phosphatidylinositolmannosides and three unknown phospholipids (Supplementary Figure S2). The fatty acid profile of strain YIM 75704^T was characterized by large amounts of saturated and unsaturated fatty acids. The major fatty acids (>5% of the total) were i-$C_{15:0}$ (35.2), ai-$C_{15:0}$ (31.5), ai-$C_{17:0}$ (12.0) and i-$C_{17:0}$ (9.2); complete fatty acid profile has been given in Supplementary Table 1. The DNA G+C content of the type strain is 75.0 mol%. It is evident from the phenotypic, chemotaxonomic and phylogenetic data that strain YIM 75704^T should be given novel species status in recently described novel genus *Allostreptomyces* of the family *Streptomycetaceae*, for which we propose the name *A. indica* sp. nov.

Description of *A. indica sp. nov.*

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

Gram-positive, aerobic actinobacteria forms brown to yellow substrate mycelia and olive green aerial hyphae, which differentiate into straight to spiral spore chains arranged in linear chains. The spores are 1–2 μm with smooth to rough surface. This organism grows well on medium ISP 2, ISP 3, ISP 4, ISP 5, Czapeks solution agar and Nutrient agar (Difco, BD, Franklin Lakes, NJ, USA) at 28 °C, whereas aerial mycelia are not easily observed on Potato agar. Soluble pigments are not produced. Aesculin, arabinose, cellobiose, D-glucose, inositol, D-sucrose, D-mannose, mannitol, D-sorbitol, L-rhamnose, melibiose and citrate are utilized as sole carbon source for growth, but not adipose, D-maltose, xylitol and D-raffinose. Positive for hydrolysis of starch, urea, oxidase activity, gelatin liquefaction, Tween 80, milk coagulation and peptonization, weak positive for hydrolysis of cellulose, but negative for nitrate reduction, catalase reaction, H_2S and melanin production. Acid is not produced from any of the tested carbon sources. Temperature, pH value and NaCl concentration (w/v) for growth are 10–45 °C, 6.0–10.0% and 0–10%, respectively, with the optimal ranges of 28 °C –37 °C, 7.2 and without any NaCl

concentration (w/v). Sensitive to erythromycin (15 μg per disk), gentamicin (10 μg per disk), vancomycin (30 μg per disk), novobiocin (30 μg per disk), rifampicin (5 μg per disk), netilmicin (30 μg per disk), ciprofloxacin (5 μg per disk), amikacin (30 μg per disk), tobramycin (10 μg per disk), chloramphenicol (30 μg per disk) and norfloxacin (10 μg per disk). The organism is resistance to penicillin G (10 μg per disk) and ampicillin (10 μg per disk). The major fatty acids (>5%) are i-$C_{15:0}$ (35.2%), ai-$C_{15:0}$ (31.5%), ai-$C_{17:0}$ (12.0%) and i-$C_{17:0}$ (9.2%). The G+C content of genomic DNA of type strain is 68.0 mol%. The type strain, YIM 75704^T (DSM 41985^T = CCTCC AA 209051^T = NCIM 5485^T), was isolated from lime stone open pit of Gulbarga region of Karnataka, India.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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