Streptomyces antibioticalis, a Novel Species from a Sanitary Landfill Soil

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Abstract A novel isolate belonging to the genus *Streptomyces*, strain SL-4^T, was isolated from soil sample collected from a sanitary landfill, New Delhi, India. The taxonomic status of this isolate was studied by polyphasic approach including morphological, physiological and chemo-taxonomic characterization. Spore chains of SL-4^T were open loops, hooks or extended spirals of wide diameter (retinaculiperti). The cell wall peptidoglycan of the isolate SL-4^T contained L,L-diaminopimelic acid, suggesting that the strain has a cell wall of chemotype-I. The polar lipid profile of the isolate was of Type II, with phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides. The 16SrRNA gene sequence similarity between SL-4^T and its phylogenetic relatives *Streptomyces atrovirens* NRRLB 16357^T (DQ026672), *S. albogriseolus* NRRLB 1305^T (AJ494865), *S. viridodiataticus* NBRC 13106^T (AB184317), *S. caelestis* NRRL 2418^T (X80824), *S. flavoviridis* NBRC 12772^T (AB184842), *S. pilosus* NBRC 12807^T (AB184161) and *S. longispororuber* NBRC 13488^T (AB184440) was 99.65, 99.65, 99.64, 99.23, 99.15, 99.14 and 99.13 % respectively. Subsequent DNA–DNA hybridization experiments with the test strain and its clade members showed 55.27, 44.27, 36.86, and 15.65 % relatedness between SL-4^T and its relatives *S. atrovirens*, *S. albogriseolus*, *S. viridodiataticus* and *S. longispororuber* respectively. The genotypic and phenotypic data was analyzed to verify possibility of the isolate SL-4^T representing novel member of the genus *Streptomyces*, for which the name *S. antibioticalis* is being proposed. The type strain is SL-4^T (=CCM 7434^T=MTCC 8588^T).

Keywords *Streptomyces* · Sanitary landfill · Antimicrobial compound · Minimum inhibitory concentration · Polyphasic taxonomy

Introduction

Natural products from microbes are an important source of antibiotics. Bacteria are well known for production of valuable secondary metabolites [1, 2]. Actinomycetes comprise a major group of microbes biosynthesizing important secondary metabolites possessing broad spectrum activities [3]. The genus *Streptomyces* alone is responsible for production of large number of antimicrobial compounds [3, 4]. In the past decades, a large number of *Streptomyces* spp. have been isolated and characterized from terrestrial habitats [5]. These include aerobic, spore forming actinomycetes that have been classified into different species based on morphology and cell wall chemotaxonomic characteristics. *Streptomyces* spp. have distinct features, such as a high G + C content; the presence of L,L-diaminopimelic acid (LL-DAP); and the absence of characteristic sugars in the cell wall [5, 6]. They produce branched substrate and aerial mycelia [5]. During the screening for actinomycete isolates as potential producers of novel antibiotics, we have isolated a strain from soil sample collected from a sanitary landfill site, New Delhi, India. The present study was designed to determine the taxonomic status of this strain based on a judicious combination of genotypic and phenotypic features using polyphasic approach [7].

Materials and Methods

SL-4^T was isolated from soil sample collected from a sanitary landfill site (Sanjay Gandhi Nagar), New Delhi, India, using Starch-Casein (SC) agar. Morphological,

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physiological and biochemical characteristics of the strain SL-4^T were carried out following the standard protocols of the international *Streptomyces* project [8]. Color determinations were carried out by comparing the cultures with color chips from the ISSC-NBS color charts standard sample no. 2106. Microscopic observation of spores and mycelia of SL-4^T ISP2 medium was made by phase contrast (Nikon, E600) and scanning electron microscopy (Philips, Leo 435 VP 501 B). The novel strain was examined for a range of phenotypic properties. Tolerance to pH, temperature and salinity conditions was determined on modified ISP2 medium followed by incubation at 28 °C for 2–3 weeks. Aerial spore mass color, substrate mycelium pigmentation and coloration of the diffusible pigments of SL-4^T were recorded on different ISP media [8].

Polar lipids were examined and identified according to the method of Kates [9]. Isomers of DAP in the whole cell hydrolysates were analyzed by TLC [5, 10]. Fatty acid methyl ester (FAME) analysis was carried out at the Institute of Microbial Technology, Chandigarh, India, using the following procedure. Fatty acid methyl esters were analyzed from two to four loops of inoculum scraped from cultures grown on ISP2 medium for 7–10 days at 28–30 °C. The cells were subjected to saponification, methylation and extraction using the method of Miller [11] and Kuykendall et al. [12]. The FAME mixtures were separated using the Sherlock Microbial Identification System (MIDI, USA), which consisted of gas chromatograph (Agilent 6890) and FID detector. Identification and comparison were made using the Aerobe (TSBA, version 5) database of Sherlock Microbial Identification System.

Detection of antibiotic producing potential of the isolate SL-4^T was done by using the following sensitive strains: *Escherichia coli* MTCC 433, *Staphylococcus aureus* MTCC 740, *Fusarium oxysporum* MTCC 284, *Candida albicans* MTCC 227 and *Bacillus cereus* MTCC 430. The cultures were streaked on ISP2 plates towards one end of the plate, growth was allowed to occur for 2–3 weeks. An agar plug was cut out from test plate and placed on sensitive strain seeded plates. Inhibition zones of sensitive strain around the plugs were observed after an overnight incubation [13]. Bioactive compounds were extracted using ethyl acetate [14]. Activity of the compound was quantified by determining minimum inhibitory concentration using microdilution method [15, 16].

Plasmid and Genomic DNA were isolated by methods standardized for actinomycetes [17]. PCR-mediated amplification of 16S rRNA gene and purification of amplicons from the strain were performed. PCR products were sequenced directly by using the Micro Seq^R 16S rRNA gene sequencing kit (Applied Biosystems, USA) and Applied Biosystems 3100 Avant^{TM} Genetic Analyzer Sequencer. Resultant sequence 1429 nt for SL-4^T (GenBank accession

no: EF424409) was aligned manually with corresponding sequences of available *Streptomyces* drawn from the Eztaxon [18]. A phylogenetic tree was constructed by neighbor-joining method [19] using Clustal_X version 1.81 [20] and MEGA version 4.1 [21]. The tree topology was also confirmed by using Maximum Parsimony method [22, 23]. Topology of the phylogenetic tree was evaluated by the bootstrap resampling method with 1,000 replicates [24, 25]. The evolutionary tree was rooted with *Actinomadura hibisca* JCM 9627^T (AF163115) as the out-group.

DNA–DNA hybridization experiment of strain SL-4^T with its relatives *S. atrovirens* NRRLB 16357^T, *S. albobovisporus* NRRLB 1305^T, *S. viridodiataticus* NBRC 13106^T and *S. longispororuber* NBRC 13488^T was performed according to the methods described by Bala et al. [26] and Tourova and Antonov [27]. DNA probes were labeled with random-primer labeling kit (Bangalore Genei). Hybridization was performed on nylon membranes (Hybond-N; Amersham Pharmacia Biotech).

Results and Discussion

Sanitary landfill was chosen for soil collection in this study as it is an unexplored site, rich in organic matter and with potential of harboring novel strains [28]. The strain SL-4^T was chosen among several isolates from this habitat because it showed antibacterial activity.

Morphological observations of the 2–3 weeks old culture of the isolate SL-4^T grown on nutrient medium agar and yeast extract-malt extract agar (ISP2) revealed that both the aerial and vegetative hyphae were abundant, well developed and not fragmented. Mature spore chains were extended spirals of wide diameter (retinaculiperti) and

Fig. 1 Spore chains and spore surface ornamentation of SL-4^T. Mature spore chains are predominantly open loops, hooks or extended spirals of wide diameter (retinaculiperti) and spore surface is smooth

Table 1 Biochemical characteristics of strain SL-4^T compared to its phylogenetic relatives

Characteristic	SL-4 ^T	<i>S. atroirens</i> NRRLB 16357 ^T	<i>S. albogriseolus</i> NRRLB 1305 ^T	<i>S. viridodiataticus</i> NBRC 13106 ^T	<i>S. caelestis</i> NRRL 2418 ^T	<i>S. flavoviridis</i> NBRC 12772 ^T	<i>S. pilosus</i> NBRC 12807 ^T	<i>S. longispororuber</i> NBRC 13488 ^T
Color of: Aerial mycelium substrate mycelium	Grey-white Grey-beige	White beige	White Beige	Grey-white brown	Green-white Green-beige	Grey-white Brown	White Beige-brown	Cream-white Grey-beige
Production of diffusible pigment	No	No	No	No	Brown diffusible pigment on ISP1, 6	Yellow diffusible pigment on ISP3, 7	No	Light brown diffusible pigment on ISP3
Spore chain	Retinaculiperti	Retinaculiperti	Rectiflexibiles	Rectiflexibiles	Hooked	Retinaculiperti	Rectiflexibiles	Rectiflexibiles
Adonitol	–	–	–	–	+	–	–	+
L(+)-Arabinose	+	+	+	+	–	+	+	+
Cellobiose	+	+	+	+	–	+	+	+
D(+)-Fructose	+	–	+	+	+	+	+	+
D(+)-Galactose	+	–	+	+	+	+	+	+
D(+)-Maltose	+	–	+	+	–	+	Weak	+
D(+)-Mannitol	+	+	+	+	–	+	+	+
D(+)-Mannose	+	Weak	+	+	–	+	+	–
D(+)-Xylose	+	Weak	+	+	+	+	+	+
Lactose	+	–	+	+	+	–	+	–
Melibiose	+	–	+	+	+	–	–	–
meso-inositol	+	–	+	+	+	+	+	–
Sucrose	–	–	–	–	+	–	–	–
Sodium formate	Weak	–	Weak	–	–	–	–	–
Sodium malonate	–	–	Weak	+	–	–	–	+
Sodium propionate	+	+	+	+	+	+	–	+
Allantoin	+	–	+	+	Weak	Weak	Weak	+
Casein	+	+	+	+	–	+	+	+
Gelatin	+	–	+	–	+	Weak	–	+
Starch	+	–	+	+	+	+	+	+
Urea	+	–	+	+	–	–	+	+
Nitrate reductase	+	+	+	+	+	+	–	–

Fig. 2 Diaminopimelic acid profile of SL-4 From Left to Right: Lane 1 shows standard sample containing a mixture of LL-DAP and meso-DAP; Lane 2 whole cell hydrolysates of SL-4

Fig. 3 Phospholipid profile of *Streptomyces* sp. SL-4 Polar lipid profile of isolate is of Type II, containing phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides

spore surface was smooth (Fig. 1). The isolate SL-4^T has an aerial mass color in the white-grey series on YM. The substrate mycelium of SL-4^T shows grey-yellow color. However, strain does not produce any diffusible melanin pigment on YM agar or on any of the ISP media. The

Fig. 4 Zones of inhibition of SL-4 ethyl acetate extract against *Bacillus cereus*

isolate was also observed to grow well on a variety of ISP media. Strain SL-4^T grows well between pH 7.0–10.0. The culture showed extensive growth between 28 and 37 °C. The isolate showed a better salinity tolerance, as it grew well in the range of 1–3 % NaCl, showed moderate growth between 4 and 7 % NaCl but scant or no growth at 8, 9 and 10 % salinity. A comparative analysis of the morphological and biochemical characteristics of strain SL-4^T with respect to its phylogenetic relatives is shown in Table 1. A detailed account of the morphological and biochemical features of SL-4^T has been given in the species description.

The cell wall peptidoglycan of isolate SL-4^T contained L,L-diaminopimelic acid (DAP), indicating that the strain has a chemotype cell wall Type I (Fig. 2). The polar lipid profile of strain was of Type II, containing diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides (Fig. 3). The predominant cellular fatty acids of SL-4^T were iso-C_{16:0} (30.12 %), anteiso-C_{15:0} (17.69 %), and anteiso-C_{17:0} (11.28 %) Thus a study of all the above morphological and chemotaxonomic characteristics shows that features of the isolate are consistent with those of the genus *Streptomyces*.

SL-4^T was found to show antibacterial activity against *B. cereus*, but not against *E. coli*, *Staphylococcus aureus*, *C. albicans* and *F. oxysporum*. MIC of ethyl acetate extract against *B. cereus* was 1.5 mg/ml and zones of inhibition of pathogen produced by the extract at different concentrations were analyzed by the agar-plug method and have been depicted in Fig. 4.

During the isolation of genomic DNA, strain SL-4^T revealed the presence of a large, circular plasmid. The plasmid DNA had restriction sites for the enzymes *Bam* HI, *Kpn* I, and *Bcl* I and was found to have a size exceeding

Fig. 5 Restriction digestion of plasmid from SL-4^T. From left to right, lane 1 lambda DNA double digested *EcoRI* / *HindIII* (5 µl); lane 2 undigested plasmid DNA (20 µl); lane 3 *Hind III* digested (30 µl); lane 4 *Bam HI* digested (30 µl); lane 5 *EcoR I* digested (30 µl); lane 6 *Bcl I* digested (30 µl) lane 7 *Kpn I* digested (30 µl); lane 8 *Bgl II* digested (30 µl)

20 kb. Restriction enzyme digestion profile of the plasmid can be viewed in Fig. 5.

Comparison of the partial 16SrRNA gene sequence of strain SL-4^T (1429nt, GenBank accession no: EF424409) with sequences of close *Streptomyces* species deposited in databases indicated that this isolate is related phylogenetically to members of the genus *Streptomyces*. The rooted phylogenetic tree (Fig. 6) based on neighbor joining

method indicated that the strain SL-4^T was included in a distinct clade and formed a phyletic branch with the type strains of *S. atrovirens* NRRLB 16357^T, *S. albogriseolus* NRRLB 1305^T, *S. viridodiataticus* NBRC 13106^T and *S. longispororuber* NBRC 13488^T with which SL-4^T exhibited percentage similarities of 99.65 %, 99.65 %, 99.64 % and 99.13 % respectively. Levels of DNA–DNA relatedness between strain SL-4^T and its clade members *S. atrovirens* NRRLB 16357^T, *S. albogriseolus* NRRLB 1305^T (AJ494865), *S. viridodiataticus* NBRC 13106^T and *S. longispororuber* NBRC 13488^T was 55.27, 44.27, 36.86 and 15.65 % respectively. DNA–DNA relatedness values below 80 % have been recommended for the recognition of novel genomic species of *Streptomyces* [29, 30]. The observed levels of 16SrRNA similarities of the isolate with its phylogenetic relatives and the associated DNA–DNA relatedness values have been demonstrated in Table 2.

Thus, based on results of the phenotypic and genotypic analyses, SL-4^T represents a novel species of the genus *Streptomyces*, for which we propose the name *Streptomyces antibioticalis* sp. nov.

Description of *Streptomyces antibioticalis* sp. nov.

Streptomyces antibioticalis [an.ti.bio.ti.ca'lis. N.L. n. antibioticum, antibiotic; L. suff. -alis, suffix used with the sense of pertaining to, related to; N.L. masc. adj. antibioticalis, related to antibiotics]

Aerobic, Gram-positive, forms a substrate mycelium of white-grey color and aerial mass color in the grey-yellow series on yeast extract-malt extract (ISP2) medium. Sporulates well on most ISP media. No diffusible pigment is produced on any of the test media. No growth at acidic pH (<pH 5.0) but grows well at pH 6.0–10.0. Grows scantily

Fig. 6 Rooted phylogenetic tree based on 16SrRNA gene sequences, showing the relationship between strain SL-4^T and related representative species of the genus *Streptomyces*. The sequence of the 16S rRNA gene of *Actinomadura hibisca* JCM 9627^T (AF163115) was

used as an outgroup. The tree was generated using the neighbor-joining method (Clustal_X version 1.81 and MEGA version 4.1) and includes bootstrap percentages based on an analysis of 1,000 resampled datasets

Table 2 DNA relatedness and 16S rRNA similarity between SL-4^T and its phylogenetic relatives

Phylogenetic neighbors of SL-4 ^T	16S rRNA similarity with SL-4 ^T (%)	Mean DNA–DNA relatedness with SL-4 ^T (%)
<i>Streptomyces atrovirens</i> NRRLB 16357 ^T	99.65	55.27
<i>Streptomyces albogriseolus</i> NRRLB 1305 ^T	99.65	44.27
<i>Streptomyces viridodiataticus</i> NBRC 13106 ^T	99.64	36.86
<i>Streptomyces caelestis</i> NRRL 2418 ^T	99.23	^a
<i>Streptomyces flavoviridis</i> NBRC 12772 ^T	99.15	^a
<i>Streptomyces pilosus</i> NBRC 12807 ^T	99.14	^a
<i>Streptomyces longispororuber</i> NBRC 13488 ^T	99.13	15.65

^a Relatives in the clade of SL-4^T only were chosen for DNA–DNA hybridization studies

between 16 and 18 °C, moderately between 18 and 20 °C, extensively from 28 to 37 °C but shows only a moderate growth from 40 to 45 °C. Grows well in the range of 1–3 % NaCl, shows moderate growth between 4 and 7 % NaCl but does not grow from 8 and 10 % salinity levels. Mature spore chains are predominantly open loops, hooks or extended spirals of wide diameter (retinaculiperti). The spore surface is smooth. Cell wall contains L,L-diaminopimelic acid (cell-wall type I). Polar lipid profile is of type II containing diphosphatidylglycerol, phosphotidylethanolamine, phosphotidylinositol and phosphotidylinositol mannosides. The predominant cellular fatty acids are iso-C_{16:0} (30.12 %), anteiso-C_{15:0} (17.69 %), and anteiso-C_{17:0} (11.28 %). Shows antibacterial activity against *B. cereus* but no antibiosis against *F. oxysporum*, *C. albicans*, *E. coli* and *Staphylococcus aureus*. Utilizes glucose, mannose, meso-inositol, xylose, galactose, fructose, lactose, ribose, maltose, melibiose, rhamnose, cellobiose, mannitol, trehalose, sodium pyruvate, sodium succinate, sodium acetate, sodium citrate and sodium propionate but sucrose, raffinose, adonitol, sorbitol, sodium malonate, sodium salicylate, sodium oxalate, sodium formate and sodium benzoate are not utilized. Possesses moderate catalase, gelatinase and caseinase activity. Degrades Tween 80, and aesculin but degrades starch weakly. Shows strong metabolism of hypoxanthine and urea. Nitrate is reduced to nitrite weakly.

The type strain SL-4^T (=CCM 7434^T=MTCC 8588^T) was isolated from a soil sample collected from a sanitary landfill, New Delhi, India.

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