ORIGINAL ARTICLE

Characterization of *Yuhushiella* sp. TD-032 from the Thar Desert and its antimicrobial activity

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ABSTRACT

During a screening program for antimicrobial compounds from underexplored habitats, a Gram-positive bacterium TD-032, was isolated from arid soil, Thar Desert (India), and analyzed for its morphological, physicochemical, and antimicrobial properties. The 16S ribosomal DNA (rDNA) sequence of the isolate was further studied for the novelty of γ-hyper variable region. TD-032 was grown in large-scale culture, and aqueous and organic solvent extracts analyzed for antimicrobial activity. Culture characteristics showed a lack of diffusible and melanoid pigments. The morphological features were pale yellow aerial mycelium colony color with brownish yellow substrate mycelium and leathery texture. The isolate could grow at 1% concentration of sodium chloride, temperature of 40°C, and a wide range of pH (7.0–12.0). An evaluation for extracellular enzymatic activities showed secretion of gelatinase(s), cellulase(s), and lipase(s). The γ-hyper variable region of 16S rDNA sequence of TD-032 showed 98.33% relatedness to *Yuhushiella deserti*, indicating a potential new species. Aqueous and ethyl acetate extracts showed antimicrobial activity against Gram-positive and Gram-negative bacteria inclusive clinical isolates. Inhibition of both test bacteria suggests that TD-032 produces a broad spectrum of antimicrobial substances.

Key words: Actinomycetes, antimicrobial activity, hyper-variable regions/16S ribosomal DNA, Thar Desert

INTRODUCTION

Increasing multidrug resistance among pathogens has necessitated screening of microorganisms for the production of novel drugs. However, it has become very difficult to find commercially useful secondary metabolites from well-known actinomycetes; therefore, screening of microorganisms for antimicrobial compounds from under/unexplored habitat is required.[1]

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Actinomycetes account for over 45% of bioactive secondary metabolites,[2] used in medicine and agriculture with 80% being produced by one genus *Streptomyces*.[3] Among underexplored habitats, the Thar Desert in India is an arid desert with the ratio of mean annual rainfall to mean annual evaporation in the range of 0.05–0.07 and with varying temperature conditions.[4] Actinomycetes from the Atacama Desert have been reported to produce novel bioactive compounds such as Atacamycins A-C, chaxalactins, and chaxamycins from *Streptomyces leeuwenhoekii*, and chaxamycins A-D from *Streptomyces* sp. strain C34.[5-7]

During the course of screening the diversity of actinomycetes from the Thar Desert, isolate TD-032 was obtained from rocky soil. The objectives of this study were to

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(a) taxonomically characterize and identify isolate TD-032 and (b) investigate its antimicrobial activity.

MATERIALS AND METHODS

Sample collection and screening

Rocky soil of the Thar Desert was crushed, serially diluted, and plated on starch casein agar consisting of (in g/L: 10.0 starch, 0.3 casein, 2.0 KNO_3, 2.0 NaCl, 2.0 K_2HPO_4, 0.05 $MgSO_4.7H_2O$, 0.02 $CaCO_3$, 0.01 $FeSO_4.7H_2O$, and 15 agar). Among other isolates, TD-032 was obtained, after incubation for 2 weeks at 30°C.

Characterization of TD-032

The isolate TD-032 was characterized morphologically as per Shirling and Gottlieb.[8] Colony characterization was carried out on International *Streptomyces* Project (ISP) media series (ISP 2, ISP 3, ISP 5, and ISP 7), glucose yeast extract agar, and Bennett's media (BM) after incubation for 2 weeks at 30°C.[1] Temperature range for growth was performed at 4–50°C.[9] Growth at different pH (2.0–12.0) was checked in Bennett's agar after incubating for 1 week.[10] Effect of salinity (0.1–30% NaCl) was studied after incubation at 30°C for 14 days.[11] Resistance to antibiotics - cycloheximide, neomycin, tetracycline, and rifampicin (5–35 µg/mL) was also studied.[1] The nitrogen (DL-tyrosine, DL-threonine, L-cysteine, L-ornithine, L-alanine, L-tryptophane, L-proline, DL-aminobutyric acid, and hydroxyphenylalanine) and carbon (raffinose, D-lactose, mannitol, D-maltose, dextrose, D-fructose, and sucrose) sources utilized were tested.[12] Growth of the isolate was recorded as a positive result. Production of extracellular enzymes (protease, cellulase, catalase, amylase, urease, gelatinase, and lipase) was also studied.[13]

16S ribosomal DNA hypervariable sequence analyses

Genomic DNA was isolated.[14] Quality of DNA was checked on 1% agarose gel followed by amplification of the 16S ribosomal RNA gene using universal primers (8-27F-5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R-5'-GGTTACCTTGTTACGACTTC-3') with initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 3 min. Then, the reaction mixture was kept at 72°C for 10 min, cooled to 4°C, and polymerase chain reaction product purified by gel elution and sequenced.[15] The 1367 nucleotide sequence was aligned using EzTaxon server: http://eztaxon-e.ezbiocloud.net.[16] The γ-hyper variable region (158–227 sequences of 120 base pair length) was selected and aligned in EzTaxon and 10 nearest match strains retrieved. The software Molecular Evolutionary and and Genetic Analysis (MEGA6) was used to construct the phylogeny tree neighbor joining treeing algorithm.[17]

Antimicrobial activity

Isolate TD-032 was grown in 200 mL of Bennett's broth and incubated for 14 days at 30°C and 180 rpm. It was subsequently centrifuged at 5000 rpm for 15 min. The supernatant was used as a neat aqueous extract. From other similar experimental setups, the supernatant was extracted with equal volumes of ethyl acetate (EA), petroleum ether (PE), dichloromethane (DCM), hexane (H), and butanol (B) mixed thoroughly for 1 h, organic layer evaporated to dryness and residue dissolved in methanol.[5] The aqueous and organic solvent extracts were tested for antimicrobial activity against a panel of Gram-positive (*Micrococcus luteus* [MTCC-106], *Staphylococcus epidermidis* [MTCC-435], *Brevibacterium linens* [MTCC-268], *Bacillus subtilis* [MTCC-1427]); and Gram-negative bacteria (*Pseudomonas fluorescens* [MTCC-2421], *Escherichia coli* [MTCC-1679]) as well as against clinical isolates *E. coli* (from pus, semen, urine, blood, and dialysis tip), *Klebsiella pneumoniae* (from urine), and *Pseudomonas aeruginosa* (from sputum).[18]

For further characterization, thin layer chromatography (TLC) was performed. The organic solvent (EA and n-butanol) and aqueous extracts were loaded onto the TLC plates (Silica gel 60 F_{254}, 20 cm × 20 cm; Merck) and run using EA and hexane (80:20) as the mobile phase. Subsequently, the plates were dried and observed under ultraviolet (UV) light. Since EA gave positive results against a maximum number of isolates, it was further analyzed for its UV-visible spectrum (190–500 nm).

RESULTS

Characterization of TD-032

Isolate TD-032 was characterized using morphological and biochemical tests. TD-032 was Gram-positive with filamentous morphology. Aerial mycelium was pale yellow and substrate mycelium brownish yellow with leathery texture in all the tested media. Melanoid and diffusible pigments were not observed [Table 1]. Isolate TD-032 grew well at temperatures 20–40°C and pH 7.0–12.0 and showed growth in up to 1% NaCl. It also showed resistance to four antibiotics (rifampicin -2 0 µg/mL, tetracycline - 32 µg/mL, neomycin - 4 µg/mL, and cycloheximide - 25 µg/mL).

TD-032 utilized all the nitrogen sources except glycine [Table 1]. The isolate utilized raffinose, dextrose, sucrose, and D-fructose, while lactose, maltose, and mannitol were not utilized. Cellulase, lipase, and gelatinase production was observed.

16S ribosomal DNA γ-hyper variable sequence analyses

The γ-hyper variable region (158–227 base pairs) of TD-032 formed a separate clade with *Yuhushiella deserti* and showed a close relationship with 98.33% relatedness value [Figure 1].

Antimicrobial activity

The aqueous and organic extracts (EA and B) of isolate TD-032 exhibited antimicrobial activity against target

Table 1: Characteristics of isolate TD-032 obtained from the Thar Desert with the nearest matching strain *Y. deserti*

Characteristics	Isolate TD-032	*Y. deserti*[9]
Colony characteristics		
Aerial mycelium	Pale yellow on BM, ISP 2, ISP 3, ISP 5 media	Pale yellow to light yellow on BM, ISP 2 and Gause's asparagine agar
Substrate mycelium	Brownish yellow on BM and ISP 2 media	Not determined
Colony texture	Leathery	Not determined
Colony color	Pale yellow	Yellow
Melanin	−	−
Diffusible pigment	−	Brown
Temperature tolerance (°C)	20–40	20–45
NaCl tolerance	Up to 1% NaCl	Up to 3.5% NaCl
pH tolerance	Up to 7.0–12.0	Only at 9
Utilization of nitrogen sources		
Tyrosine	+	+
Threonine	+	Not determined
Cysteine	+	Not determined
L-ornithine	+	Not determined
L-alanine	+	Not determined
Phenylalanine	+	Not determined
Tryptophan	+	Not determined
L-proline	+	Not determined
DL-aminobutyric acid	+	Not determined
Hydroxyphenylalanine	+	Not determined
Glycine	−	Not determined
L-histidine	+	Not determined
Leucine	+	Not determined
Utilization of carbon sources		
Raffinose	−	Not determined
D-lactose	−	+
Mannitol	−	Not determined
D-maltose	+	Not determined
Dextrose	+	+
D-fructose	+	Not determined
Sucrose	+	Not determined
Extracellular enzyme production		
Amylase	−	Not determined
Cellulase	+	Not determined
Lipase	+	Not determined
Gelatinase	+	Not determined
Protease	−	+
Urease	−	+
Catalase	−	Not determined

+: Present, −: Absent, *Y. deserti*: *Yuhushiella deserti*, ISP: International *Streptomyces* Project, BM: Bennett's media

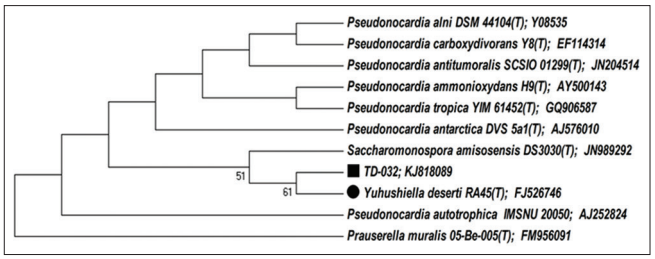

Figure 1: Neighbor-joining tree showing the relationship between the γ-hyper-variable regions of nearest match strains to TD-032. Unrooted phylogenetic tree was constructed by the neighbor-joining algorithm. Bootstrap values more than 50% are indicated at the nodes (1000 replications)

bacteria *S. epidermidis*, and *M. luteus* [Table 2] while DCM, H, and PE extracts showed no activity. Among the clinical isolates tested, the EA extract showed activity against *K. pneumoniae* (from urine). The activity against both Gram-positive and Gram-negative bacteria suggest that there may be more than one compound present in the extract that is active against different test organisms.

In TLC, the butanol extract showed three spots and EA extract two spots [Figure 2a]. The EA extract was further analyzed for its UV-visible spectrum. Two clear peaks were observed at 340 nm and 380 nm, matching the two bands seen under TLC [Figure 2b].

DISCUSSION

Mao *et al*. 2011 reported *Y. deserti* isolated from barren desert of China which was pale yellow to light yellow on BM, ISP 2, and Gause's asparagine agar while producing brown diffusible pigment.[9] This was reported to grow at 37–45°C and only at pH 9.0. Other reports show *Nocardiopsis alkaliphila* from the Egyptian desert to grow well at relatively broader temperature range (10–45°C) and pH (7.0–12.0).[19] Actinomycetes from Qinghai Lake/saline soil (China) could tolerate up to 47% NaCl.[11] It was reported, *Y. deserti* (nearest match to TD-032) showed growth up to 3.5% NaCl.[9] Isolate TD-032 showed growth at concentrations up to 1% NaCl (w/v). Okoro *et al*. reported actinomycetes that showed resistance to cycloheximide, tetracycline, rifampicin, and neomycin.[1] TD-032 differed from *Y. deserti* is not producing diffusible pigment, growth at broader pH range (0.7–12.0), and growth at a relatively lower 1% NaCl. TD-032 was negative for protease and urease, unlike *Y. deserti*.

Martín *et al*., documented the genomic sequence analysis of γ-hyper variable region of 16S ribosomal DNA for identifying *Streptomyces albus*.[20] Analysis of the γ-hyper variable region has been shown to be a useful tool to resolve the diversity reported in *Streptomyces* isolated from soil in Germany.[21] It was reported that short nucleotide

Table 2: Antimicrobial activities of isolate TD-032 extracted with different organic solvents on test bacteria

Extracts	Zone of inhibition mean±SD (mm)											
	Biosafety level I strains					Clinical isolates						
	B. linens	*M. luteus*	*B. subtilis*	*P. fluorescens*	*S. epidermidis*	*E. coli* (blood)	*E. coli* (urine)	*K. pneumoniae* (urine)	*E. coli* (urine)	*E. coli* (semen)	*E. coli* (dialysis tip)	*P. aeruginosa* (sputum)
Aqueous extract	0	10±0.5	0	0	12±0.5	0	0	0	0	0	0	0
EA extract	0	30±0.5	0	0	30±0.5	0	0	10±0.5	0	0	0	0
Butanol extract	0	30±0.5	0	0	0	0	0	0	0	0	0	0
Butanol (negative control)	0	0	0	0	0	0	0	0	0	0	0	0
EA (negative control)	0	0	0	0	0	0	0	0	0	0	0	0
Methanol (negative control)	0	0	0	0	0	0	0	0	0	0	0	0
Positive control (tetracycline)	38±0.5	40±0.5	35±0.5	37±0.5	40±0.5	0	0	0	0	0	0	36±0.5

0: No activity, SD: Standard deviation, *B. linens*: *Brevibacterium linens*, *M. luteus*: *Micrococcus luteus*, *B. subtilis*: *Bacillus subtilis*, *P. fluorescens*: *Pseudomonas fluorescens*, *S. epidermidis*: *Staphylococcus epidermidis*, *E. coli*: *Escherichia coli*, *K. pneumoniae*: *Klebsiella pneumoniae*, *P. aeruginosa*: *Pseudomonas aeruginosa*, EA: Ethyl acetate

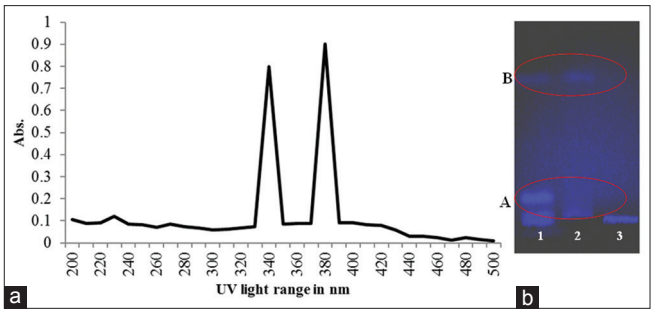

Figure 2: Analysis of ethyl acetate extract of *Yuhushiella* sp. TD-032. (a) Ultraviolet-visible spectrum of TD-032 ethyl acetate extract. (b) Thin layer chromatography of butanol extract (Lane 1), ethyl acetate extract (Lane 2), and aqueous extract (Lane 3) under ultraviolet light

sequences bearing γ-hyper variable region are useful for *Streptomyces* species identification.[22] Here, we show that this region can be useful for phylogeny analyses in other actinomycetes too. Based on the sequence data, and other morphological and physiological parameters, we suggest that isolate TD-032 may be a novel species of *Yuhushiella* genus in actinomycetes.

To our knowledge, *Y. deserti* has not been documented for its antimicrobial property.[9] Selvameenal *et al.* reported activity of yellowish antibiotic pigment produced by Streptomyces hygroscopicus subsp. ossamyceticus from Thar Desert against *Klebsiella* sp. and vancomycin-resistant *Staphylococcus aureus* and Mycobacterium tuberculosis.[23] Recently, it was reported that metabolites from rare actinomycetes showed good inhibition of MDR *Streptococcus pneumoniae*.[24] Our study shows that the most potent extract (EA) of TD-032 contains at least two compounds as evidenced by TLC and UV-visible spectrum. Parthasarathi *et al.* had reported that EA extract from fermented broth of *S. hygroscopicus* BDUS 49 showed absorption peaks at 210 and 225 nm corresponding to bioactive regions on TLC plate.[25] This strain was suggested to produce either a broad-spectrum antimicrobial compound or several compounds with different activities.[25] Our results indicate possibly different compound(s) since the absorption peaks are at 340 and 380 nm. Activity against both Gram-positive and Gram-negative bacteria further support the observations. The nature of the compound has to be substantiated by Fourier transform infrared spectroscopy or gas chromatography-mass spectrometry studies. However, antibacterial activity against clinical isolate (*K. pneumoniae*) is noteworthy from the point of view of TD-032 being a potential new species.

CONCLUSIONS

From the present investigation, it was concluded that isolate TD-032 could potentially be a new species of *Yuhushiella*. It was also observed that *Yuhushiella* sp. TD-032 inhibited both test bacteria inclusive clinical isolates which suggest that the isolate produces a broad spectrum of antimicrobial

substances. Further studies can confirm the identity of the antimicrobial compounds.

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Conflicts of interest

There are no conflicts of interest.

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