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

**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 \( \gamma \)-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 \( \gamma \)-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.\(^{[1]}\)

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 leeuanhoekii*, 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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**How to cite this article:** Ibeyaima A, Rana J, Dwivedi A, Gupta S, Sharma SK, Saini N, et al. Characterization of *Yuhushiella* sp. TD-032 from the Thar Desert and its antimicrobial activity. J Adv Pharm Technol Res 2016;7:32-6.
(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 KNO₂, 2.0 NaCl, 2.0 K₂HPO₄, 0.05 MgSO₄·7H₂O, 0.02 CaCO₃, 0.01 FeSO₄·7H₂O, 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.[10] 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.[11] 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, 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 assessed on 1% agarose gel followed by amplification of the 16S ribosomal DNA gene using universal primers (5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R-5'-GTTACCTTGTACGACCTC-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. The 16S nucleotide sequence was aligned using EzTaxon server: http://eztaxon-e.ezbiocloud.net.[16] The ϒ-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 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.[15] 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 F₂₅₄, 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 ϒ-hyper variable sequence analyses**

The ϒ-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...
Antimicrobial activity of Yuhushiella sp. TD-032 from the Thar Desert

Ibeyaima, et al.: Antimicrobial activity of Yuhushiella sp. TD-032 from the Thar Desert

Journal of Advanced Pharmaceutical Technology & Research | Apr-Jun 2016 | Vol 7 | Issue 2

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. 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). Actinomycetes from Qinghai Lake/saline soil (China) could tolerate up to 47% NaCl. Isolate TD-032 showed growth up to 3.5% NaCl. Okoro et al. reported actinomycetes that showed resistance to cycloheximide, tetracycline, rifampicin, and neomycin. 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.

Martin et al., documented the genomic sequence analysis of γ-hyper variable region of 16S ribosomal DNA for identifying Streptomyces albus. 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. It was reported that short nucleotide

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 |

[^9]: Present, −: Absent. Y. deserti: Yuhushiella deserti, ISP: International Streptomyces Project, BM: Bennett’s media

[^9]: Present, −: Absent. Y. deserti: Yuhushiella deserti, ISP: International Streptomyces Project, BM: Bennett’s media
sequences bearing γ-hyper variable region are useful for Streptomyces species identification. 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. 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. Recently, it was reported that metabolites from rare actinomycetes showed good inhibition of MDR Streptococcus pneumoniae. 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. This strain was suggested to produce either a broad-spectrum antimicrobial compound or several compounds with different activities. 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.

Acknowledgments
The authors are thankful to Jaypee Institute of Information Technology, Noida for providing the necessary facilities. A. Ibeyaima would like to thank Department of Science and Technology, Government of India for providing INSPIRE Fellowship (IF 120267). Authors thank Department of Microbiology, Pushpanjali Crosslay Hospital, Ghaziabad (India) for facilitating some of the experiments

Financial support and sponsorship
INSPIRE Fellowship (IF 120267) was provided to AI from Department of Science and Technology, Government of India.

Conflicts of interest
There are no conflicts of interest.

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