Indian Journal of Agricultural Sciences **91** (4): 550–4, April 2021/Article
https://doi.org/10.56093/ijas.v91i4.112655

Morphological and molecular characterization of Actinomycetes isolates and their metabolite fingerprinting

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Received: 06 April 2020; Accepted: 22 December 2020

ABSTRACT

Two actinomycetes isolates RK-302 and RK-320 obtained from limestone rock of Dehradun, Uttarakhand were characterized morphologically, biochemically and by using 16S rDNA sequencing at Jaypee Institute of Information Technology, Noida during 2017–18. Differences in morphological/biochemical features showed that the isolates differed from each other as well as from the nearest matching strains in the GenBank database. Variation in dimension for spore size of both isolates was observed through transmission electron microscopy. Both isolates, RK-302 and RK-320 showed activity against phytopathogenic fungi, *Fusarium graminearum* and *Bipolaris maydis* using agar plugs *in vitro*. Metabolite fingerprinting with GC-MS revealed the presence of 37 and 31 compounds, respectively from RK-302 and RK-320. Combined analysis of phenotypic, biochemical and molecular studies showed that the isolates differed at strain level. From the metabolite fingerprint and antimicrobial activity data, it was concluded that the antimicrobial compounds identified in present study may be useful in developing novel bio control agents for plant pathogens.

Keywords: GC-MS, Limestone, Metabolite fingerprinting, Phytopathogens, *Streptomyces*

Emergence of fungicide resistance among plant pathogens has necessitated the discovery of new compounds to prevent loss of agricultural crops to pests and diseases. Plant pathogens and pest cause approximately 30% loss to agricultural products at pre- and post-harvest levels (Gu *et al.* 2019). Excessive use of chemicals in agriculture have been leading to loss of soil fertility, accumulation in soil over the years and threatening global health. Hence, alternate methods need to be employed for sustainable pathogen and pest management. Amongst eco-friendly options for managing plant diseases, biological control is an effective alternative (Zucchi *et al.* 2008). Antibiotics produced by the *Streptomyces* spp., are considered most important tools to control the soil-borne diseases (Berg *et al.* 2001). For instance, reveromycin A and B have been reported as wide antifungal metabolites produced by *Streptomyces* sp. (Lyu *et al.* 2017).

MATERIALS AND METHODS

Limestone rock samples (1 g) collected from Maldevta, Dehradun (30° 33' N and 78° 13' E), were studied for morphological and biochemical characteristics at Jaypee Institute of Information Technology, Noida during 2017–18. Both isolates were plated on Glucose Yeast Extract Agar (GYE) After incubation for a week at 30°C, isolates RK-302 and RK-320 were maintained on modified Bennett's agar (BM). Morphological characterization of both isolates was done as per Shirling and Gottlieb (1966). Scanning Electron Microscopy (JEM-1011, JEOL, Japan) was used to understand the morphology of both isolates. The isolates were characterized for their cultural characteristics on different media, GYE, International *Streptomyces* Project (ISP) 2, ISP 3, ISP 5, ISP 6 and ISP 7. Isolates RK-302 and RK-320 were assessed for growth at different temperatures (4, 10, 30, 40 and 50 °C), pH (4.0-12.0; at one-unit intervals) and sodium chloride [0.1, 0.5, 1, 2.5, 3, 3.5, 5, 7, 10, 20 and 30% (w/v)] on BM at 30 °C for 7 days. Both isolates were assessed for using sole carbon compounds (Pridham and Gottlieb 1948), enzymes, H_2S, indole and siderophore production (Schwyn and Neilands 1987, Meena *et al.* 2013), 2, 6 diaminopimelic (DAP-A_2pm) acid of cell wall was detected (Becker *et al.* 1965).

Molecular identification: The genomic DNA of RK-302 and RK-320 was extracted as described (Srivastava *et al.* 2019). 16S rDNA of isolate RK-302 was amplified with primers, PA 5'-AGAGTTTGATCCTGGCTCAG-3' (forward) and PH 5'-AAGGAGGTGATCCAGCCGCA-3' (reverse). For RK-320, primers 5'–AGHGTBTGHTCMTGNCTCAS-3' (forward) and 5'– TRCGGYTMCCTTGTWHCGACTH–3' (reverse) were used. DNA amplification was done at 95°C

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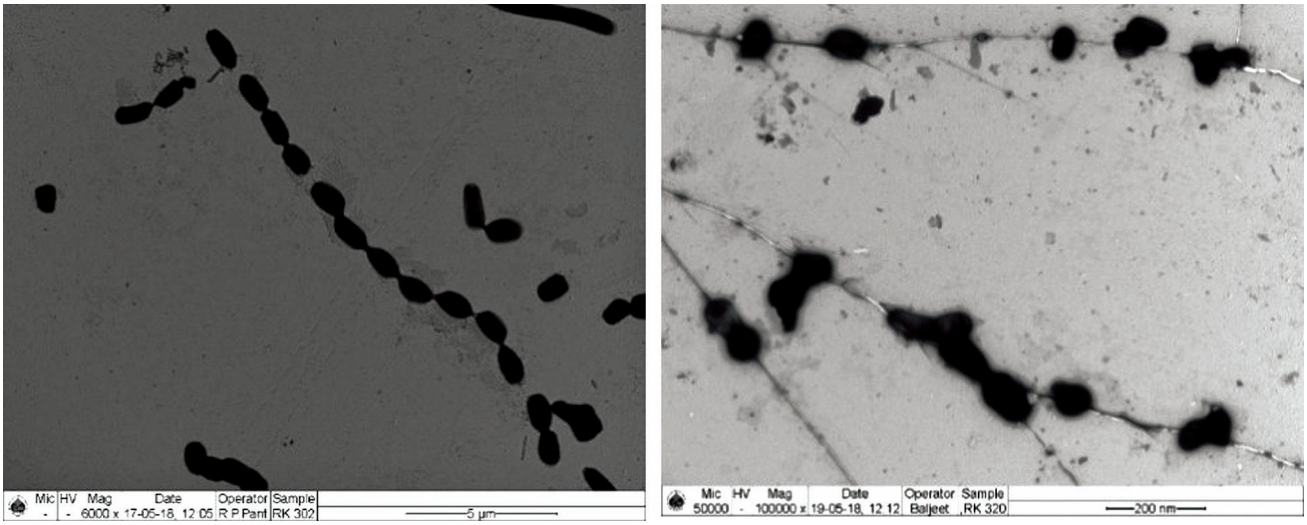

Fig 1 Scanning Electron Microscopy of RK-302 (left) and RK-320 (right).

for initial denaturation (5 min). For RK-302, the same temperature was used to amplify DNA in 35 cycles for 30 sec while it was 94°C for RK-320. Annealing temperatures were 53°C for 1 min and 50°C for 30 sec respectively for RK-302 and RK-320. Extension temperature was 72 °C (at 1 min for RK-302 and 1.3 min for RK-320). Subsequently, 72 °C temperature was used as holding temperature (5 min and 7 min for RK-302 and RK-320, respectively), followed by cooling at 4 °C. The amplified product was sequenced using above primers including 518F (5′-CCAGCAGCCGCGGTAATA CG-3′) as internal primer (on Applied Biosystems 3130 Genetic Analyzer). Contigs of RK-302 and RK-320 were formed by deleting overlaps, and quality checked for chimera formation using DECIPHER version 3.0. EzTaxon server software was used to blast the sequences of both isolates with type strains (Kim *et al.* 2012). Phylogenetic tree was prepared using MEGA 6.0 software and evolutionary distances, were computed (Jukes and Cantor 1969, Tamara *et al.* 2013). 16SrDNA sequences of both isolates were submitted in NCBI (GenBank).

Antimicrobial activity and metabolite fingerprinting: The antimicrobial activity of the isolates RK-302 and RK-320 was checked against the phytopathogenic fungi (*Fusarium graminearum* and *Bipolaris maydis*) using agar plugs. The plates were incubated for seven days at 25°C and mycelial growth inhibition was calculated (Bekker *et al.* 2006). To prepare EA extracts, both isolates were inoculated in Bennett's broth (250 ml) and incubated at 37°C for seven days at 100 rpm. Culture supernatants were obtained by centrifugation (5000 rpm/15 min) and mixed with equal volume of EA for 2 h. After evaporating the organic solvent layer, the remaining residue was dissolved in methanol to give 1 mg/ml final concentration. Column prepared with silica gel G slurry (60-120 mesh) was used to chromatograph (partial purification) EA extracts of both isolates. Mobile phase composed of ethyl acetate: ethanol (2:1) was used to collect fractions. Subsequently, antimicrobial activity of the obtained fractions was checked against the same panel of target organisms. MS [QP2010Ultra (SHIMADZU)] of the pooled fractions (showing activity) were proceeded individually (Nielsen *et al.* 2014). The specifications and conditions of GC-MS were the same as described in Srivastava *et al.* (2019). The mass spectrum data obtained after GC-MS was interpreted based on retention time, by observing differences of similarity index of obtained compound with the compounds reported in databases, mass ion spectra of the compounds, and by comparing the observed retention indices (National Institute of Standards and Technology) and calculated retention indices. RI of obtained compounds was calculated (Babushok *et al.* 2011) .

RESULTS AND DISCUSSION

Morphological and molecular characterization of actinomycete isolates: The aerial and substrate mycelia of RK-302 were grey and brown respectively. The colony harboured grey coloured spores. The colonies were rounded, rough, leathery, velvety, raised and around 5-7 mm in diameter with grey-coloured spores and produced brown diffusible pigment. However, the isolate RK-320 had white and yellow aerial and substrate mycelia respectively and colonies became grey on onset of sporulation. The colonies were rounded, rough, leathery slightly raised around 4-5 mm in diameter and did not produce any pigment. Dimension for spore size through transmission electron microscopy was 956.49-1357.60 nm × 634.77 nm and 1064.12-1357.60 nm × 790.63-962.20, for RK-302 and RK-320, respectively (Fig 1).

Phenotypic/physiological and biochemical characters of both isolates with the nearest type strains (*S. enissocaesilis, S. rochei* and *S. plicatus*) has been shown in Table 1. Colony characteristics of both isolates varied in different media. *Streptomyces enissocaesilis* showed grey/greyish yellow aerial/ substrate mycelium on ISP2, ISP3 and ISP5. It was grey/yellowish brown on ISP6 and ISP7. It has not been reported for diffusible pigment and melanin production. Aerial mycelium of *S. rochei* was poorly developed on

Table 1 Phenotypic properties that distinguished *Streptomyces* sp. RK-320 and RK-320 from the type strains of phylogenetically related species, *S. enissocaesilis, S. rochei* and *S. plicatus*

Characteristics	RK-320	RK-302	Near Type Strains (Goodfellow *et al.* 2012)		
			S. enissocaesilis	*S. rochei*	*S. plicatus*
ISP 2 (Yeast extract – malt extract agar)					
Aerial	Grey	White	Grey	ND	Grey/greyish yellowish pink
Substrate	Brown	Yellow	Greyish yellow	ND	Pale greyish yellow
ISP 3 (Oatmeal agar)					
Aerial	Grey	Whitish grey	Grey	Poorly developed	Grey
Substrate	Yellowish brown	Yellow	Greyish yellow	Brown	Yellowish brown
Diffusible pigment	Brown	-	-	Brown	-
ISP 5 (Glycerol-asparagine agar)					
Aerial	Grey	Grey	Grey	Whitish	Grey
Substrate	Brown	Yellowish brown	Greyish yellow	Black greyish brown	Dark greyish
ISP 6 (Peptone-yeast extract iron agar)					
Aerial	Grey	Whitish grey	Grey	ND	ND
Substrate	Brown	Yellowish brown	Yellowish brown	ND	ND
ISP 7 (Tyrosine agar)					
Aerial hyphae	Grey	White	Grey	ND	ND
Substrate	Brown	Brown	Yellow brown	ND	ND
Glucose yeast extract agar					
Aerial	Grey	White	ND	ND	ND
Substrate	Brown	Yellow	ND	ND	ND
Diffusible pigment	Dark brown	-	ND	ND	ND

ND- not determined, - absence.

ISP3 and ISP5. Its substrate mycelium was brown and black greyish brown on ISP3 and ISP5 respectively (Goodfellow *et al.* 2012). *Streptomyces plicatus* showed grey/greyish yellowish pink/pale greyish yellow, grey/yellowish brown, grey/ dark greyish aerial/substrate mycelium (Goodfellow *et al.* 2012). RK-302 differed from isolate RK-320 and other nearest matches in utilizing sucrose. RK-302 and RK-320 utilized raffinose while the nearest matches did not. RK-302 differed from RK-320 in utilizing arabinose and producing cellulose. The *p*H range for optimum growth of RK-302 and RK-320 was 6-12 and 6-10, respectively. RK-302 growth was observed up to 10% NaCl while RK-320 growth was only up to 7.5% NaCl. Cell wall composition showed the presence of LLA_2pm indicating it belongs to *Streptomyces* sp. In an earlier study, Ibeyama *et al.* (2016) isolated *Yuhushiella* TD-032 which showed growth at concentrations up to 1% NaCl (w/v).TD-032 differed from *Y. deserti* as TD-032 did not produce diffusible pigment, growth at broader pH range (0.7–12.0), and growth at a relatively lower 1% NaCl. EzTaxon server analysis showed that isolate RK-302 showed 100% similarity to *S. rochei*, *S. enissocaesilis* and *S. plicatus* without any nucleotide differences, while RK-320 showed 99.76% similarity to the same with 3 nucleotide difference out of 1240 bp. The phylogenetic tree of RK-302 and RK-320 is shown in Fig 2. Combined analyses of phenotypic, biochemical, chemotaxonomic and molecular studies showed that the isolates differ at strain level. The isolates were assigned the accession numbers: MH605515 (RK-302) and MH511607 (RK-320) by NCBI.

Antimicrobial activity of actinomycete isolates and metabolite fingerprinting: Both RK-302 and RK-320 showed activity against the selected phytopathogenic fungi. It is clear (Table 2) that best antifungal activity was exhibited by RK-320 which showed 88% inhibition against *B. maydis* and 57% against *F. graminearum.* RK-302 showed 18% inhibition against *B. maydis* and 25.62% against *F. graminearum.* Metabolite fingerprinting GC-MS data of isolate RK-302 showed the presence of 37 compounds which belonged to eight major categories (alcohols, aldehydes, alkaloids, amide, ketone, fatty acids, hydrocarbons and quinones). Among these 23 were hydrocarbons, five alcohols, four ketones and one aldehyde, alkaloid, amide, fatty acids and quinone each. Similarly, 31 compounds were identified from isolate RK-320 of which 19 belonged

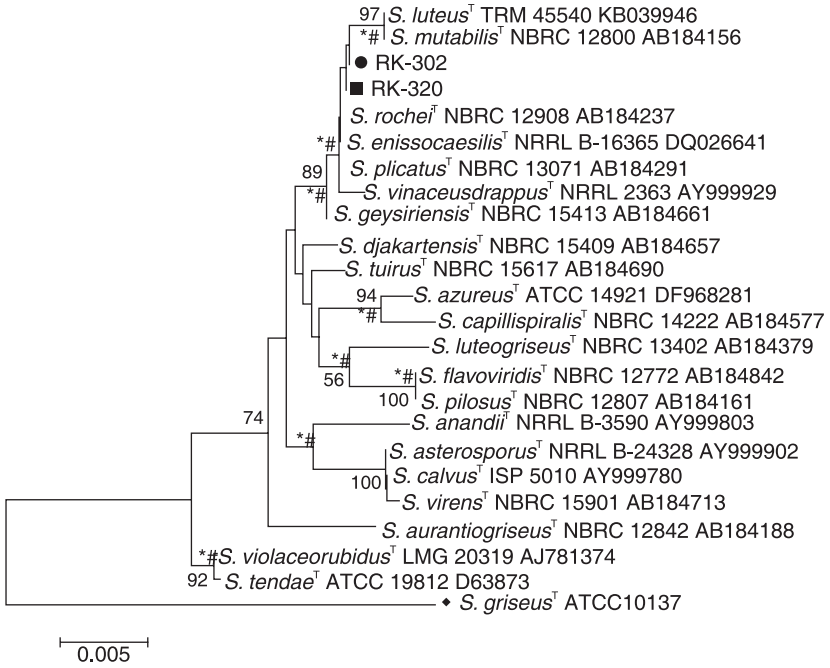

Fig 2 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between strain RK-302 and RK-320 and the type strains of Streptomyces and those of selected type strains of related genera.

to hydrocarbons, five to alcoholic group, three to ketone and one to aldehyde, alkaloid, fatty acids, and fatty acid ester each. These compounds were identified as per the (NIST11), WILEY8 libraries. Amongst these 16 compounds (1-Hexanol, 2-Ethyl-; 1-Tridecanol; Bicyclo[3.1.0] Hexane, 4-Methylene-1-(1-Methylethyl)-; 3,5-Cyclohexadiene-1,2-Dione,3,5-Bis(1,1-Dimethylethyl)-;7,9-Di-Tert-Butyl-1-xaspiro (4,5) Deca-,9-Diene-2,8-Dione; Decane; Undecane; Dodecane; 2,6,10-Trimethyltridecane; Tetradecane; Eicosane; 1-Hexadecane; Heptadecane; 1-Nonadecene; 1,8,11-Heptadecatriene, (Z, Z)- and Tetracontane were found common to both isolates.

Table 2 Antifungal activity of *Streptomyces* isolates against phytopathogenic fungi

Treatment	Average mycelial growth (sq mm)	Percent mycelia growth inhibition
Bipolaris maydis-control	3240.00	-
Bipolaris maydis with RK-320	382.91	88.17
Bipolaris maydis with RK-302	2650	18.21
Fusarium graminearum-control	6400.00	-
Fusarium graminearum with RK-320	2745.00	57.11
Fusarium graminearum with RK-302	4760.00	25.62

Control-without RK-302 and RK-320.

Approach described by Babushok *et al*. (2011) was followed for the tentative identification of compounds that do not have standard reference compounds. From RK-302, maximum similarity indices (SI) value of 97% was found for compounds (1-Hexanol, 2-Ethyl- and Dodecane). Calculated RI for the compound 1-Hexanol, 2-Ethyl- was 940.73 and reported RI was 1010-1064 and 1453-1520. For compound Dodecane calculated and reported RI were 1020.22 and 1200, respectively. From RK-320, maximum SI value of 98% was found for the compound- 1-Hexanol, 2-Ethyl- with calculated RI 940.71 and reported RI of 1010-1064 and 1453-1520. Antibiotics produced by the *Streptomyces* spp., are considered most important tools to control the soil-borne diseases (Berg *et al.* 2001). We observed that these compounds had differences in their calculated and reported RI and are potentially novel as substantiated from previous studies (Ibeyaima *et al.* 2017). Similar results were found with other compounds also. Twenty-four compounds obtained from RK-302 had SI values between 90-96%. While 21 compounds from RK-320 had SI values between 90-97%. Varying values of their reported and calculated RI further affirmed their novelty. In present study, among the tentatively identified compounds, many compounds have been reported to show phytopathogenic activity. From the metabolite fingerprint data, we concluded that the antimicrobial compounds identified in present study may be useful in developing novel bio control agents for plant pathogens.

ACKNOWLEDGEMENTS

Facilities provided by Jaypee Institute of Information Technology, Noida for research work is acknowledged. Thanks to AIRF, JNU, New Delhi, for GC-MS analyses and Dr R P Pant, Principal Scientist-Plant Pathology, IARI, New Delhi for electron microscopy.

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