PHYLOGENETIC CHARACTERIZATION OF A SOIL BACTERIUM SHOWING ANTAGONISTIC BEHAVIOUR

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Abstract: In the present study a soil bacterium was isolated from potato rhizosphere and characterized in terms of morphology, biochemistry and phylogeny. This bacterium showed antagonistic behavior to other soil bacterium and type strains. DNA from the pure culture was isolated and used in PCR amplification of 16S rRNA gene followed by DNA sequencing and phylogenetic analysis. Phenotypical, biochemical and 16S rRNA gene-based phylogenetic analysis revealed its closer affiliation to the genus Bacillus but it did not share any clad with type strains and hence representing a new novel isolate of genus Bacillus and named as Bacillus sp. EIKU1. Bacillus sp. EIKU1 showed antagonistic behaviour mostly against Gram-positive bacteria, including methicillin-resistant Staphylococcus aureus. This study paves the way for further characterisation of Bacillus sp. EIKU1 for its optimum utilisation as a source of antimicrobials.

Key words: Rhizosphere; Bacillus; Antagonistic behaviour; Phylogeny

I INTRODUCTION

Control of multidrug-resistant microorganisms, plant diseases, and reduction of food spoilage are the challenging tasks for public health concerns, agriculturalist, and food processing industry, respectively. To date, a wide range of antibiotics are available in the market, but the Infectious Disease Society of America reported that 70% of pathogenic bacteria are resistant to one or more available antibiotics (Hassan *et al.*, 2012). This prompted us to search improved alternatives antibacterial to fight antimicrobial resistance. Antimicrobials from a natural source are expected to have lesser chances for resistance development and lower side effects than that of chemical antibiotics (Sumi *et al.*, 2015).

Varieties of microbes from various environments have been reported to produce a range of antimicrobials of different chemical natures that includes peptides synthesized both ribosomally and non-ribosomally (NPKs) and polyketides (PKs) (Zhao, 2016). Antimicrobial peptides display both narrow and broad spectrum of inhibition, which cover pathogenic Gram-positive and Gram-negative bacteria. Bacteriocins or bacteriocin-like substances produced by *Bacillus* spp. act through inhibition of different microbial life processes (Pálffy *et al.*, 2009)**.** Rhizosphere is one of the best sources of diverse antimicrobial molecules as this is the hotspot of microbial assemblage. In this complex environment, microorganisms showed competitive as well as synergistic characteristics for survival and shared niche in a community. During the competitive interaction, multiple strains produce various antimicrobial molecules; many of them exhibit a selective spectrum of inhibition (Hassan *et al.*, 2012). Recently, Shafi *et al.* (2017) reviewed the bio-control potential of different *Bacillus* species in relation to their antagonizing attributes against plant pathogens. The frequently studied species of *Bacillus* includes *B. subtilis*, *B. cereus* and *B. thuringiensis* because of their importance as a potential pathogen or insecticidal activity (Yanglei and Oscar, 2017). Considering the enormous diversity of *Bacillus* species in the soil ecosystem it is very important to isolate and preserve the strains producing antimicrobial peptides followed by detailed characterisation (Baindara *et al.*, 2013).

In the present study, we have isolated two bacterial strains from the potato cultivating field and characterised, one of them produces antimicrobial molecule.

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II MATERIALS AND METHODS

Collection of soil

For the isolation of bacteria, soil from potato cultivated land was collected. Nearly 10 cm top soil was removed by sterile scoop; a lump of soil was withdrawn and placed into sterile plastic bag. The collected soil was brought to the laboratory placing in ice. One aliquot of the soil was used to isolates the bacteria.

Isolation of antimicrobial producing organism

During isolation of nitrogen-fixing microorganisms from the soil, collected from potato cultivating field using the nitrogen-free yeast extract mannitol agar medium (Wagh *et al.*, 2015), few colonies were observed with a clear zone of inhibition around them in the crowded spread plate. Both the colonies that showed a zone of clearance around them and the colonies that surround the clear zone were isolated and purified by repeated streaking on a similar agar medium to get pure cultures. The pure culture of the isolates that showed antagonistic activity is designated as EIKU1 to the growth of the sensitive organism, EIKU2. Pure cultures of the isolated bacteria were grown in rich medium and stored at –80°C in 15% glycerol for future use.

Bacterial Strains

In this study antagonistic behaviour of EIKU1 was tested against the following microorganisms: *Staphylococcus aureus* ATCC 29213, *S. aureus* MTCC 96, *S. aureus* MTCC 2488, *S. aureus* (MRSA) ATCC:BAA-1717, *Klebsiella pneumonia* ATCC33495, *Enterobacter aerogenes* ATCC 13048, *Pseudomonas aeruginosa* ATCC 15692, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Salmonella typhimurium* ATCC 14028, *Enterobacter aerogenes* ATCC 13048 and *Ochrobactrum* sp. EIKU2. These organisms were obtained from ATCC and MTCC, and EIKU2 was isolated from soil during this study.

Phenotypic and Biochemical Characterisation

The physiological, biochemical, and cultural characteristics of the antimicrobial producing isolate (EIKU1) were examined using the standard protocol and compared with Bergey's Manual of

Determinative Bacteriology (Bergey and Holt, 1994). Growth progress, colony morphology, pigmentation, and ability to form spore of the bacteria were performed using different standard media. Production of catalase, protease, lipase, amylase and gelatinase, utilisation of different carbon sources, and ability to grow at different pH and salinity were carried out according to the standard protocols.

Molecular characterisation of the isolates

Genomic DNA was isolated from the overnight grown culture of both the isolates using DNeasy tissue kit (QIAGEN). PCR amplification of 16S rRNA gene from genomic DNA was performed using primers 27F and 1492R and the reaction was carried out following the protocol as described by Chakraborty and Islam (2017). The purified DNA was sequenced and phylogenetic analysis was performed using MEGA 4.0 (Tamura *et al.*, 2007). The 16S rRNA sequences of the bacterium was deposited in the NCBI nucleotide database under the accession number MF574394.1.

Anti-bacterial activity

Cross streak method was used to ascertain the antagonistic nature of EIKU1 against different type cultures. EIKU1 was streaked along the diameter of the plate on agar medium. Test organism was streaked vertically along the side of EIKU1 at 9, 24, and 36h after the inoculation of producer organism.

III RESULT

Identification of bacterial stains

The initial observations for EIKU1 noted, it to be producing a yellowish pigment both in solid and liquid media with cream, an irregular edged colony of diameter 3-4 mm. The microscopic examinations revealed it as Gram-positive, rod-shaped, spore former, other biochemical & physiochemical characteristics are summarized in table 1. The bacterium grows optimally at neutral pH, at 37°C and can tolerate NaCl (2%), however, its growth was also observed in absence of NaCl. In the agar plate with sheep blood, after 48h of growth ß-haemolytic activity was observed.

Phylogenetic characterization

The BLAST analysis of 16S rRNA gene of EIKU1 reveals that it belongs to the genus *Bacillus.* BLAST analysis showed 99% sequence similarity with reference strains of *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. wiedmannii* and *B. toyonensis* available in the GenBank. Neighbour-joining phylogenetic analysis incorporating Jukes-Cantor distance model considering closely matched 16S rRNA gene sequences indicated its uniqueness from reported *Bacillus* species (Fig. 1). All the sequences considered for phylogenetic analysis distributed in two distinct clades, one includes *B. cereus*, *B. thuringiensis*, *B. wiedmannii*, *B. weihenstephanensis* and *B. toyonensis* and the other was of *B. mycoides.* But EIKU1 was found to distinctly branch out from these two clades with more closeness to *mycoides* group. The BLAST analysis of 16S rRNA gene of EIKU2 reveals that it belongs to the genus *Ochrobactrum.*

Antimicrobial activity

In the initial experiment, the culture supernatant of EIKU1 exhibited clear zone indicating antagonistic nature against the growth of *Ochrobactrum* (EIKU2). The over-night culture supernatant showed inhibition of different type strains of *S. aureus* but fails to restrict the growth of Gram-negative bacteria except modest inhibition for *E. coli*. In cross streak method, EIKU1 showed antagonistic behaviour against *S. aureus* and in each case the inhibition effect increased as the culture of EIKU1 became older i.e. there was no observable inhibition of growth when the indicator organism was streaked at 9h but effectively prevent the growth, when the indicator organism was inoculated after 24h and 36h growth of EIKU1 (Fig. 2).

Table 1 Morphological, physiological and biochemical characteristics of *Bacillus* sp. EIKU1 isolate

Parameters	Response of EIKU1
Morphology	
Colony	Round, Raised Margin, Cream
Pigmentation	Yellow, released in the media
Cell	Rods, single or chain
Size	3 µm × 1µm
Physiology	
Spore	Present, single & multiple
O_2 requirement	Facultative
Motility	+
Enzymatic Activity	
Catalase	++
Gelatinase	+++
Amylase	+++
Lipase	++
Casein Hydrolysis	+++
Carbon Source Utilization	
L-Arabinose	++
D-Manitol	+++
Lactose	++
D-Glucose	+++A
Starch	+++A
Citrate	-
Voges-Proskauer	++
Egg Yolk Reaction	+++
Growth at different pH	
pH 5	-
pH 6	++
pH 7	+++
pH 8	+++
pH 9	++
pH 10	-
Growth at different NaCl concentration	
0%	++
1%	+++
2%	+++
5%	-
10%	-
Growth with 0.001 % lysozyme	+++
Haemolysis	+

-, negative; +, weakly positive; ++, moderately positive; +++, highly positive; A, with acid formation

IV DISCUSSION

Soil is a very complex habitat, challenges the dwelling microorganisms for nutrient and space. During the competition for common resources, many organisms produce different mediator molecules which benefit the producing organism by limiting the growth of other microbes (Baindara *et al.*, 2013). Production of such antimicrobial molecules by *Bacillus* spp. has been well documented and considered for their uses in food processing and therapeutic industry (Yang *et al.*,2014; Abriouel *et al.*, 2011). In the present study, we report a novel isolate *Bacillus* sp. EIKU1, identified during screening process of nitrogen-fixing soil bacteria which

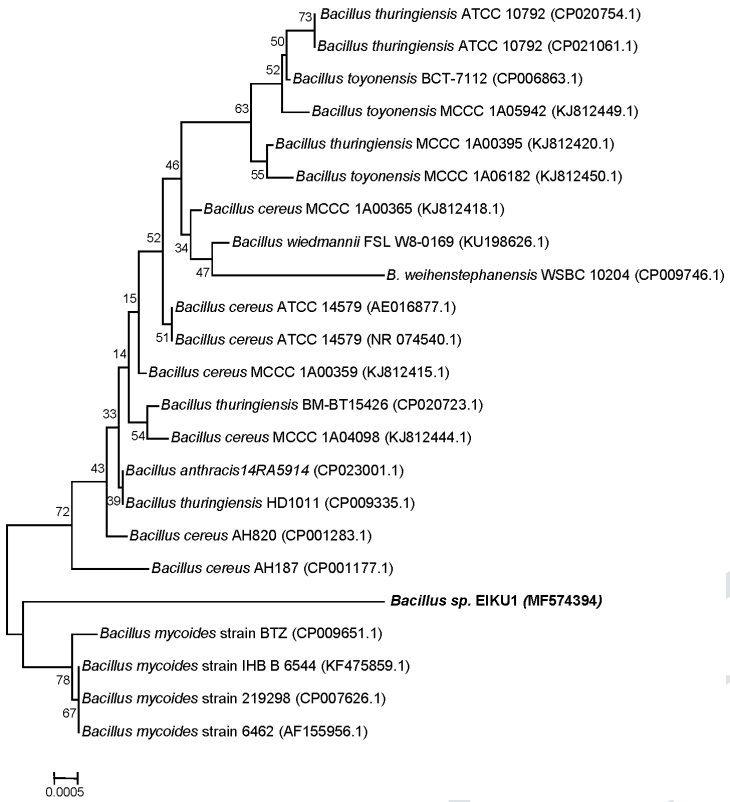

Fig. 1. Distance phylogram based on 16SrRNA gene sequences from isolate EIKU1and related type strains (accession numbers are shown in parentheses).

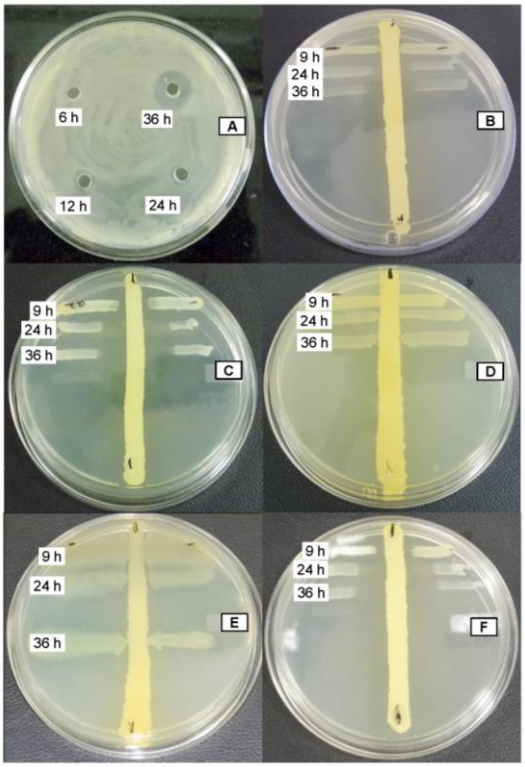

Fig. 2. Antagonistic effect of *Bacillus* sp. EIKU1 on growth of *Ochrobactrum* EIKU2 and reference strains, Inhibition zone of EIKU2 at different growth stages of *Bacillus* sp. EIKU1 (A), Cross-steak assay for *Bacillus* sp. EIKU1 against *Escherichia coli* ATCC 25922 (B), *Staphylococcus aureus* ATCC 29213 (C), *Enterobacter aerogenes* ATCC 13048 (D), *Pseudomonas aeruginosa* ATCC15692 (E) and *Staphylcoccus aureus* MTCC96 (F).

produced antimicrobial. The strain, EIKU1 showed highest 16S rRNA gene sequence similarity with the type strain *B. cereus* MCCC1A 00359 (99%) while general BLAST search indicated its maximum similarity to other *Bacillus* strains such as *Bacillus sp.* IHBB9471 and *B. mycoid* BTZ. The study showed *B. cereus* and *B. mycoid* are very closely related in respect to their 16S rRNA gene sequence and even many researchers considered the *mycoid* group as the variety of *cereus* group (Nakamura and Jackson, 1995). In general, the rhizoidal colony having non-motile behavior is considered as the distinguishable characteristics of *mycoid* group but in many cases, the ability to form rhizoidal colony may get lost (Bergey and Holt, 1994; Di Franco *et al.*, 2002). On the other hand, like *B. cereus*, *Bacillus* sp. EIKU1 was also found to produce yellow pigment and showed ß-haemolytic activity but differed in motility. In addition, the *Bacillus* EIKU1 showed aniline acetylation activity that takes it closer to *cereus* group (Nakamura and Jackson, 1995). Conversely, phylogenetic analysis of 16S rRNA genes showed that the *Bacillus* sp. EIKU1did not shares any existing clade of reported *Bacillus* species. Therefore, comparing the phenotypical, biochemical and molecular analysis, the isolate EIKU1 was assigned to be another strain of genus *Bacillus* and indicates it as a novel isolate.

Bacillus species are well known to secrete various metabolites that include lipopeptides, bacteriocin as well as several volatile compounds with different degrees of antimicrobial activities (Zhao, 2016). Like other reported *Bacillus* species *Bacillus* sp. EIKU1 produced the active molecule during late log phase of its batch growth. Similar observations have been reported for other *Bacillus* species like *B. subtilis* (Avci *et al.*, 2017) and *B. licheniformis* (Kayalvazhi and Gunasekaran, 2008). The active molecule in the culture supernatant of *Bacillus* sp. EIKU1 showed activity against Gram-positive bacteria and less active against Gram-negative bacteria. But the ability to restrict the growth of methicillin-resistant *Staphylococcus aureus* is noteworthy. *S. aureus* is a common opportunistic pathogen showed maximum sensitivity to the antimicrobial compound produced by *Bacillus* sp. EIKU1 and hence was used as reference strains in the antimicrobial study. Thus further research on the antimicrobial molecule(s) produced by EIKU1 may lead us to isolation a new molecule(s) and can be a useful tool to fight antibiotic resistance.

V CONCLUSIONS

Two novel bacterial strains were isolated from potato-growing rhizospheric soil and identified as *Bacillus* sp. EIKU1 and *Ochrobactrum* sp. EIKU2. *Bacillus* sp. EIKU1 was found to produce antimicrobial that could effectively restrict the growth of Gram negative *Ochrobactrum* sp. EIKU2 and Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*. Further research is required to purify and determine the structure of the antimicrobial compound produced by EIKU1.

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