Full Length Research Paper

**Microbacterium arborescens AGSB** sp. nov., isolated from the rhizosphere of sand dune plant, *Ipomoea pes caprae*

Aureen L Godinho* and Saroj Bhosle

Department of Microbiology, Goa University, Taleigao Plateau, Goa-403206, India.

Accepted 8 October, 2013

Phenotypic and phylogenetic studies were performed for the facultative alkalophile from the rhizosphere of *Ipomoea pes caprae*, a plant growing on coastal sand dunes. The isolate was Gram positive and showed optimum growth at pH 10.5. Chemotaxonomic analysis revealed that the isolate contained type B1 peptidoglycans with L-lysine as the diamino acid; rhamnose and galactose were the cell wall sugars and belonged unambiguously to the genus *Microbacterium*. The major menaquinones were MK-11 and MK-12. The 16S rDNA sequence of the *Microbacterium arborescens* isolate was deposited in the GenBank with an accession number DQ287961. The phylogenetic and phenotypic distinctiveness of the strain indicates it as a novel *Microbacterium* sp., named as *M. arborescens AGSB*.

**Key words**: facultative alkaliphile, *Microbacterium arborescens AGSB*, Coastal sand dune vegetation, *Ipomoea pes caprae*, 16S rRNA sequencing.

**INTRODUCTION**

The sand dune ecosystem is a stressed habitat with only certain type of vegetation surviving in this ecosystem, one such plant is *Ipomoea pes caprae* which is commonly found on coastal dunes. Although a nutrient limiting ecosystem, in the rhizosphere, plant litter contributes to humus and organic matter on which the microbiological communities survive. Few studies on the bacterial species present in coastal dunes have been published. The isolates obtained in this study have been identified as Acinetobacter, Pseudomonas, Paenibacillus, Microbacterium, Agrobacterium Chryseobacterium and Pseudomonas (Park et al., 2005, 2006; Leveau et al., 2009; Godinho and Bhosle, 2010; Muthezhilan et al., 2012; Gaonkar et al., 2012). Earlier studies on isolates from this ecosystem have shown their ability to produce exopolymers which aid in sand aggregation and stabilize the dunes (Godinho and Bhosle, 2009). A potent exopolymer producing isolate was selected for identifying it to genus level. We report here the characteristics of a predominant facultative alkaliphile from the rhizosphere of *Ipomoea pes caprae* that exhibited orange, pigmented colonies and gram positive, non sporing regular rods which has previously not been reported. Further, the isolate was identified using polyphasic taxonomic tools including chemotaxonomic and 16S rRNA sequencing. Based on the results, a new species is proposed as *Microbacterium arborescens AGSB*.

**MATERIALS AND METHODS**

**Strain, cultivation and maintenance**

The strain chosen for this study was a predominant isolate from coastal sand dunes; it was isolated from the rhizosphere of *Ipomoea pes caprae* sand dune vegetation by serial dilution method of the rhizosphere sand and then plating on polypeptide yeast extract glucose agar (PPYG) medium, pH 10.5. The plates were incubated for 2 days at 30°C on polypeptide yeast extract glucose agar (PPYG) medium containing (g/l): peptone, 5; yeast extract, 1.5;...
Biomass for chemotaxonomic analysis was obtained by growing the strain aerobically in PPYG without agar (Polypeptone yeast extract glucose broth) on an orbital shaker at approximately 160 rpm for 2 days before harvesting by centrifugation (10,000 rpm for 20 min). The cells were washed twice with sterile distilled water and freeze dried.

Morphological, physiological and biochemical characterization

Cell morphology was determined by phase contrast microscopy and electron microscopy (Figure 1), motility by the hanging drop method, biochemical characteristics were determined by the method described (Takeuchi and Yokota, 1994; Zhang et al., 2010; Shivakumar, 2012).

Chemotaxonomic methods

Preparation of cell walls and determination of peptidoglycan structure was carried out by the methods described by Schleifer and Kandler (1972). Menaquinones were extracted and analysed as described by Komagata and Suzuki (1987), Collins (1985) and Minnikin and Goodfellow (1985). Polar lipids were extracted and analyzed by Thin Layer Chromatography according to Komagata and Suzuki (1987).

DNA extraction, PCR amplification and sequencing

A single isolated colony of the selected bacterial cultures was taken from agar plate and suspended in 50 µl of colony lysis solution. The reaction mixture was incubated at 55°C for 15 min followed by proteinase K inactivation at 80°C for 10 min. The reaction mixture was centrifuged at 15,000 rpm at 4°C for 15 min. The supernatant containing genomic DNA was directly used as template in PCR reaction. PCR amplification of almost full length 16S rRNA gene was carried out with eubacteria specific primer set 16F27N and16R1525XP, in a 25 µl final reaction volume, containing about 10 ng of genomic DNA, 1X reaction buffer, 0.4 mM (each) dideoxynucleoside triphosphates (Invitrogen), 0.5 U of DNA Polymerase (New England Labs, UK) and the final volume was made 25 µl by adding sterile nuclease free water. The PCR was performed in an Automated Gene Amp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, USA) under the following conditions. The amplification conditions were as follows: 94°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 1.30 min (elongation), and 72°C for 10 min final elongation. Expected PCR product of around 1.5 kb was checked by electrophoresis of 5 µl of the PCR product on 1% agarose gel in 1X TBE buffer and stained with ethidium bromide (0.5 µg/ml). The PCR product was precipitated by PEG-NaCl (20%PEG in 2.5MNaCl) precipitation at 37°C for 30 min. The reaction mixture was centrifuged at 12,000 rpm for 30 min at room temperature. The supernatant was discarded and the pellet was washed twice with 70% ethanol. After drying the pellet, it was resuspended in 5 µl of sterile nuclease free water. One microliter (50 ng) of purified 16S rRNA PCR product was sequenced by 16S rRNA specific primer that is 16F27N, 530F and 16R1525XP.

Phylogenetic analysis

Purified double stranded PCR fragments were directly sequenced using BIG DYE Terminator cycle sequencing ready reaction kit (v3.1) in ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, USA). The sequences were compared with the 16S rDNA sequences available in the public databases from a BLAST search, and identified to the generic level.
Table 1. Significant characteristics of the isolate Microbacterium arborescens AGSB.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>M. arborescens AGSB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour of the colony</td>
<td>Orange</td>
</tr>
<tr>
<td>Motility</td>
<td>Positive</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>Positive</td>
</tr>
<tr>
<td>Starch</td>
<td>Negative</td>
</tr>
<tr>
<td>H₂S production</td>
<td>Positive</td>
</tr>
<tr>
<td>VP test</td>
<td>Negative</td>
</tr>
<tr>
<td>Arginine dihydrolase</td>
<td>Negative</td>
</tr>
<tr>
<td>Assimilation</td>
<td></td>
</tr>
<tr>
<td>Arabinose</td>
<td>Positive</td>
</tr>
<tr>
<td>N-acetylgulosamine</td>
<td>Positive</td>
</tr>
<tr>
<td>Malate</td>
<td>Positive</td>
</tr>
<tr>
<td>Citrate</td>
<td>Positive</td>
</tr>
<tr>
<td>Phenyl acetate</td>
<td>Negative</td>
</tr>
<tr>
<td>Fumarate</td>
<td>Positive</td>
</tr>
<tr>
<td>Propionate</td>
<td>Negative</td>
</tr>
<tr>
<td>Acid from</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>Positive</td>
</tr>
<tr>
<td>Cell wall diamino acid</td>
<td>Lysine</td>
</tr>
<tr>
<td>Major menaquinone acid</td>
<td>MK-11,12</td>
</tr>
</tbody>
</table>

The 16S rDNA sequences were aligned using CLUSTALX (ftp://ftp.ebi.ac.uk/pub/software/clustalw2) with Microbacterium nucleotide sequences derived from GenBank. The trees were constructed using the neighbour-joining method. The PHYLIP package (Felsenstein, 1993) was used to generate trees with the four algorithms and the trees were viewed using the TREEVIEW package (Page, 1996; Felsenstein, 1981, 1985). Tree topologies were evaluated by bootstrap analysis of the neighbour-joining tree using the original dataset and 1000 bootstrap datasets.

RESULTS AND DISCUSSION

The phenotypic characteristics of the strain are in agreement with the placement of the strain in the genus Microbacterium. The strain showed biochemical characteristics as described in Table 1. The specific characteristics which indicated that the isolate belongs to the genus Microbacterium are hydrolysis of gelatin, hydrogen sulphide production, assimilation of malate, citrate, N-acetylgulosamine, fumarate and arabinose. Chemo-taxonomic analysis of the isolate revealed the presence of the amino acid lysine and sugars rhamnose and galactose in the cell wall, unsaturated menaquinones MK-11 and MK-12 while polar lipids present were diphosphatidylglycerol and phosphatidylinositol. Based on these results and the identification in Bergey’s Manual of Systematic Bacteriology, this isolate has been designated to genus Microbacterium. The present isolate is a facultative alkaliphile and is present in highly stressed environment. There have been no reports so far of a facultative alkaliphile from coastal sand dune ecosystem.

In the present study, an attempt was made to sequence the 16S rDNA in order to study its relationship to other species of Microbacterium. The phylogenetic analysis of the 16S rDNA sequence was accompanied by PCR amplification of approximately 1500 base pairs using universal primers. The resulting PCR segments were sequenced and optimally aligned and phylogenies were constructed using algorithms available in the PHYLIP site of phylogenetic analysis programs. Phylogenetic tree was constructed by neighbour joining method (bootstrap method) as shown in Figure 2. The sequences were deposited in the GenBank and the extent of similarities with other Microbacterium strain were determined. The phylogenetic tree showed the strain to be 97% similar to Microbacterium sp.MSCB7. The genus Microbacterium was established to accommodate a diverse collection of gram positive non spore forming rods isolated during studies on lactic acid producing bacteria. Extensive phylogenetic studies have recently resulted in amalgamation of the genera Microbacterium and Aureobacterium into a redefined genus Microbacterium (Takeuchi and Hatano, 1998).

The isolate characterized in the study is a bacteria which can tolerate a high alkaline pH and survive in stressed conditions where the moisture holding capacity of the sand is minimal. Interestingly, this isolate was found to produce large quantities of exopolysaccharide
Figure 2. Unrooted tree showing the phylogenetic relationships of *Microbacterium arborescens* sp. nov. and members of the genus *Microbacterium* based on 16S rDNA sequences. The tree, constructed using the neighbour-joining method, bootstrap values, expressed as percentage of 1000 replications, are given at the branching points.

(Godinho and Bhosle, 2009) which perhaps supports its adherence and survival in this otherwise stressed ecosystem. Based on these results, we report the isolation and characterization of a new facultative alkaliphile *M. arborescens* AGSB.

**REFERENCES**


