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Molecular and physiological characterization of plant growth promoting rhizobacteria from rhizosphere soil in Al-Qassim, Saudi Arabia

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Abstract

Plant growth-promoting bacteria (PGPB) are associated with many plant species. The most widely studied group of PGPB are plant growth-promoting rhizobacteria (PGPR) colonizing the root surfaces. PGPR have shown promise as biofertilizers. Moreover, phosphorus solubilizing bacteria (PSB) are important for crop plants as they increase phosphorus uptake. The goal of this study was the identification of PGPR isolates by 16S rDNA sequencing, and the evaluation of their ability of phosphorus solubilizing and their physiological traits. Eighteen soil samples were collected from the rhizosphere of alfalfa plants in different areas of Al-Qassim region. The isolates were identified using 16S rDNA gene sequencing. Based on 16S rDNA gene sequencing, these bacterial strains were identified under several genera: *Enterobacter*, *Acinetobacter*, *Pantoea*, *Stenotrophomonas, Klebsiella* and *Serratia.* In a total of eighteen isolates from alfalfa, just 4 isolates (*Acinetobacter calcoaceticus*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Serratia marcescens*) showed high ability in phosphorus solubilizing. All the PGPR isolates were able to grow in NaCl up to 6% and temperatures between 35 and 40°C. QUSA 6, identified as *Acinetobacter lwoffii*, was found to grow even at 50°C. Therefore, these isolates may have a potential to act as plant-growth-promoting rhizobacteria and can enhance plant growth, particularly under stressed environmental conditions.

Key words: PGPR, 16S-rDNA**,** phosphorus solubilizing, salinity, heat stress.

Introduction

Rhizobia are symbiotic bacteria that have the ability to establish a N_2-fixing symbiosis with leguminous plants. These bacteria are taxonomically and physiologically diverse members of the a and b subclasses of the Proteobacteria, and mostly comprise the genera *Rhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium* and *Azorhizobium*. The establishment of the symbiosis requires coordination of plant and bacterial gene expression, which is regulated through the mutual change of molecular signals [12, 14, 18]. The recent completion of nucleotide sequence of seven rhizobial genomes and several earlier studies revealed that bacterial genes responsible for nodulation and nitrogen fixation are located on the mega plasmids (pSym) in Rhizobium and Sinorhizobium [12]. Nodulation genes have been classified into three categories:(i) the common nodulation genes *nodABC*, which are essential for nodulation and the mutations in which lead to Nod_ phenotype [4]; (ii) host- specificity genes (*nodFE, nodH, nodG, nodPQ* and others) that determine the host range of micro-symbionts and influence the rate and frequency of nodule formation [4, 10, 17] and (iii) a family of regulatory nodD genes [18]. Understanding of rhizobial genetic diversity should facilitate the selection of efficient native rhizobia from soils. PCR–RFLP analysis of the16S–23SrDNA intergenic transcribed spacer (ITS) has been demonstrated as a useful tool to study the genetic diversity of rhizobia belonging to different genera [11, 19].

In Saudi Arabia, commercialization of microbial biofertilizers and biocontrol are in its initial stages and no local PGPR bacteria are available in the market. To the best of our knowledge, no work has been performed to develop effective local microbial strains. Thus, most microbial biofertilizers and biocontrol in the market are imported from several countries. It has been shown that survivability of PGPR isolated from temperate climate rapidly decreases when added to arid soil 2. Isolation of native strains adapted to the arid environment may contribute to the formulation of inoculants suitable for use in local region crops. Native isolates may be preferred in the selection of bacteria for inoculation, as they are adapted to the environment and can be more competitive than the foreign bacteria [13]. The advantage of using natural soil isolates is the easiest way for adaptation and succession when inoculated into the plant rhizosphere [3]. Qassim, Central of Saudi Arabia, is situated in arid region and characterized by low rainfall, extreme temperatures and infertile and salt-affected soils. However, Qassim is known to have agricultural activities which depend on groundwater and the use of chemical fertilizers to enhance soil fertility and crop productivity. The use of excessive chemical fertilizers often results in unexpected harmful environmental effects, including accumulation of nitrate in plant tissues, leaching of nitrate into groundwater and surface runoff of phosphorus and nitrogen. Needless to say that integrated nutrient management

systems are needed to maintain agricultural productivity and protect the environment. As mentioned above, microbial inoculants are promising components for integrated solutions to agro-environmental problems It is, therefore, necessary to develop effective biofertilizers containing native PGPR strains that can well adapt to the arid environment [13]. Rhizospheres of perennial plants are perhaps the best source of native microbial isolates which are well adapted to the local environment. Thus, it is essential to validate the soil beneficial bacteria based upon molecular tagging (DNA sequence) and use as biofertilizers for sustainable crop production. The objective of this study was to isolate and identify PGPR using 16SrRNA gene sequencing. It was also aimed to evaluate the potential of these PGPR strains.

Material and Methods

Isolation of PGPR: Eighteen soil samples (QUSA-1 to QUSA-18) were collected from the rhizosphere of alfalfa plants in different areas of Al-Qassim region. PGPR strains were isolated by serial dilution agar plate method and 0.1 mL of serially diluted soil suspensions from 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions were spread on the plates of nutrient agar medium. Plates were incubated for 3 days at 28°C to observe the colonies of bacteria.

Media and growth of bacteria: PGPR grown in nutrient broth (peptone 5 g/L, meat extract 1 g/L, yeast extract 2 g/L, sodium chloride 5 g/L) or nutrient broth plus agar 15 g/L, *Rhizobium* spp. were grown in TY- broth (5 g/L tryptone, 0.4 g/l $CaCl_2$ and 3 g/L yeast extract or TY- broth plus agar 15 g/L. All the microbial strains were incubated at 30°C .

Storage of bacterial strains: Bacteria were grown in the liquid medium overnight until the optical density (OD600) of 1 was achieved. Of bacterial culture 100 µL was mixed with 120 µL of 87 % (v/v) glycerol and stored at -80°C.

Identification of PGPR isolates through DNA sequencing: Bacteria isolates were grown overnight in nutrient broth and genomic DNA was extracted by the hexadecyltrimethyl-ammonium bromide (CTAB) method [5]. The bacterial 16S rDNA primer Ps-for (5'GGTCTGAGAGGATGATCAGT3') and Ps-rev (5'TTAGCTCCA CCTCGCGGG3') were used to amplify of the 16S rDNA gene region. Polymerase chain reaction (PCR) was done using a DNA Engine DYADTM Peltier Thermal Cycler. Thermal cycling was performed as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min and final extension at 72°C for 8 min. The fragments obtained were analyzed by gel electrophoresis (24 cm × 12 cm) with 2% agarose, and carried out at 80 V for 2 h. A 1-kb gene ladder load on the left lane of the gel was used as a molecular size marker. The gel was stained with ethidium bromide and digital picture of amplified gene was taken under UV light. The PCR products were sent to Macrogen Company in Korea for sequencing. Sequence search for the alignment of nucleotides was performed using the BLAST (Basic Local Alignment Search Tool) web-based program.

Determination of phosphate solubilization: The potential of PGPR isolates for solubilization of insoluble phosphates was checked on the Pikovskaya's medium [15]. Isolates having the potential to solubilize insoluble phosphates on the Pikovskaya's medium formed halos. The growth and solubilization diameter were determined after incubation at 28°C for seven days. On the bases of diameter of clearing halo zones, solubilization efficiency (SE) and solubilization index (SI) were calculated using the following formulas:

$$SE = \frac{\text{Solubilization diameter}}{\text{Growth diameter}} \times 100$$

$$SI = \frac{\text{Colony diameter + Halozone diameter}}{\text{Colony diameter}}$$

Evaluation of PGPR isolates for their physiological traits:

Salinity: PGPR isolates were streaked on Luria Bertani (LB) agar with various concentrations of NaCl ranging from 2% to 12% at the interval of 2% and incubated at 28°C for 72 h.

Temperature: PGPR isolates were streaked on LB agar and incubated at 35, 40, 45°C, and 50°C for 72 h. At the end of 72 h incubation, the intensity of growth was measured at 600 nm in a spectrophotometer.

Results and Discussion

PGPR identification: The 18 bacterial isolates were identified via sequencing of its 16s rDNA gene. As shown in Table 1, BLAST searches against the NCBI nucleotide database revealed close relationship to known plant-associated bacteria, including genera *Enterobacter*, *Pantoea*, *Stenotrophomonas, Klebsiella, Serratia, Kluyveram, Enterobacter* and *Acinetobacter*. These isolates were also found from rhizospheric soil of Himachal Pradesh, India, by Kumar *et al*.[8] and used as biofertilizer formulation. BLAST search results through NCBI showed highest (99%) similarity of QUSA-12 with *Stenotrophomonas maltophilia*. Similarly, the highest sequence similarity of QUSA-16 matched with *Klebsiella pneumoniae* (99%), and *Serratia marcescens* (99%) for QUSA-18. Hayat *et al*.[9] identified nine Gram-positive bacterial strains from legumes rhizospheric soil using 16S rRNA gene sequencing. These strains showed highest similarity (97.9–99.8%) to the genus *Bacillus* and were characterized for plant growth promoting (PGP) activities in legume and cereal crops.

Analysis of phosphorus solubilizing activity by isolates: The phosphorus solubilizing activity was evaluated (Table 2). Out of the 18 isolates tested for phosphate solubilizing activity, four bacterial isolates (QUSA3, QUSA-5, QUSA-16 and QUSA-18) showed the highest phosphate solubilizition efficiency (36.8, 42.1, 29.4 and 25%, respectively). Based on sequence comparison, these isolates were confirmed to be *Acinetobacter calcoaceticus*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Serratia marcescens.* Two isolates (QUSA-3 and QUSA-5) had the highest phosphate solubilization index compared to the other isolates. Rokhbakhsh-Zamin *et al.*[16] reported that *Acinetobacter* strains have the potential to act as plant-growth-promoting rhizobacteria and can enhance the growth of pearl millet, particularly under stressed environmental conditions where the iron, phosphate or zinc sources may be limited. Phosphate solubilization was most frequently encountered by *Bacillus* isolates (80%), followed by *Azotobacter, Pseudomonas* and least by other isolates [1].

Table 1. Identification of bacterial isolates and their highest similarity of 16S-rDNA gene sequence.

PGPR Code	Geographical origin	Blast match	Highest similarity of 16SrDNA gene sequence (%)
QUSA1	Ashihiyah	*Enterobacter hormaeche*	93
QUSA 2	Ashihiyah	*Acinetobacter calcoaceticus*	97
QUSA 3	Albdaia	*Acinetobacter calcoaceticus*	97
QUSA 4	Unaizah	*Acinetobacter calcoaceticus*	93
QUSA 5	Unaizah	*Acinetobacter baumannii*	92
QUSA 6	Unaizah	*Acinetobacter lwoffii*	97
QUSA 7	Burydah	*Pantoea ananatis*	97
QUSA 8	Burydah	*Pantoea ananatis*	98
QUSA 9	Unaizah	*Pantoea* sp.	97
QUSA 10	Burydah	*Pantoea* sp.	95
QUSA 11	Unaizah	*Enterobacteriaceae bacterium*	98
QUSA 12	Ashihiyah	*Stenotrophomonas maltophilia*	99
QUSA 13	Ashihiyah	*Enterobacter cancerogenus*	98
QUSA 14	Ashihiyah	*Klebsiella pneumoniae* subsp. *pneumonia*	98
QUSA 15	Ashihiyah	*Klebsiella pneumoniae*	98
QUSA 16	Unaizah	*Klebsiella pneumoniae*	99
QUSA 17	Ashihiyah	*Klebsiella pneumoniae.*	99
QUSA 18	Unaizah	*Serratia marcescens*	99

Evaluation of PGPR isolates for their physiological traits: All the PGPR isolates grew up to 4% and none grew at 10 and 12% of NaCl conditions, but the concentrations of 6% and 8% were critical as the isolates showed discriminatory performances in these NaCl concentrations. At 6% NaCl, all isolates exhibited good growth except the isolate *Acinetobacter calcoaceticus*. At 8% NaCl, the isolates *Acinetobacter calcoaceticus*, *Acinetobacter baumannii*, and *Stenotrophomonas maltophilia* did not show any growth whereas the others showed good growth (Table 3). Temperatures of 35 and 40°C were found optimum for the growth of all the isolates while at 40°C all but four isolates (QUSA 5, QUSA 6, QUSA 16, and QUSA 17) exhibited good growth. At 50°C, a single isolate (QUSA 6) showed a good growth while others did not grow (Table 4). In this study, all the PGPR isolates were able to grow in NaCl up to 6% and temperatures between 35 and 40°C. QUSA 6, identified as *Acinetobacter lwoffii*, was found to grow even at 50°C, probably because

Table 2. Solubilization efficiency and solubilization index of bacterial isolates.

Isolates	Solubilization efficiency (SE %)	Solubilization index (SI)
QUSA1	18.2	1.2
QUSA 2	12.5	1.1
QUSA 3	36.8	1.4
QUSA 4	5.9	1.1
QUSA 5	42.1	1.4
QUSA 6	0	0
QUSA 7	0	0
QUSA 8	0	0
QUSA 9	22.2	1.2
QUSA 10	7.4	1.1
QUSA 11	21.1	1.2
QUSA 12	8.7	1.1
QUSA 13	12.5	1.1
QUSA 14	16.7	1.2
QUSA 15	21.1	1.2
QUSA 16	29.4	1.3
QUSA 17	8.7	1.1
QUSA 18	25.0	1.3

Table 3. Effect of NaCl conditions (%) on the growth of bacterial isolates.

Isolates	2 %	4 %	6 %	8 %	10 %	12 %
QUSA1	+	+	+	+	-	-
QUSA 2	+	+	+	+	-	-
QUSA 3	+	+	-	-	-	-
QUSA 4	+	+	+	+	-	-
QUSA 5	+	+	+	-	-	-
QUSA 6	+	+	+	+	-	-
QUSA 7	+	+	+	+	-	-
QUSA 8	+	+	+	+	-	-
QUSA 9	+	+	+	+	-	-
QUSA 10	+	+	+	+	-	-
QUSA 11	+	+	+	+	-	-
QUSA 12	+	+	+	-	-	-
QUSA 13	+	+	+	+	-	-
QUSA 14	+	+	+	+	-	-
QUSA 15	+	+	+	+	-	-
QUSA 16	+	+	+	+	-	-
QUSA 17	+	+	+	+	-	-
QUSA 18	+	+	+	+	-	-

Table 4. Effect of temperature on the growth of bacterial isolates.

Isolates	35°C	40°C	45 °C	50 °C
QUSA1	+	+	-	-
QUSA 2	+	+	-	-
QUSA 3	+	+	-	-
QUSA 4	+	+	-	-
QUSA 5	+	+	+	-
QUSA 6	+	+	+	+
QUSA 7	+	-	-	-
QUSA 8	+	+	-	-
QUSA 9	+	+	-	-
QUSA 10	+	+	-	-
QUSA 11	+	+	-	-
QUSA 12	+	+	-	-
QUSA 13	+	+	-	-
QUSA 14	+	+	-	-
QUSA 15	+	+	-	-
QUSA 16	+	+	+	-
QUSA 17	+	+	+	-
QUSA 18	+	+	-	-

of its spore-forming nature [6, 7]. Hence, it can be concluded that these isolates may have the ability to survive in the harsh environments such as saline and temperature.

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