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Characterization of Plant Growth-Promoting Rhizobacteria Associated with Wheat (*Triticum aestivum* L.) from Saline Soil

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) have the potential to enhance plant growth by various direct or indirect mechanisms. Eleven bacterial isolates were isolated from different salinized rhizospheric soils in which wheat (*Triticum aestivum* L.) cv. 'Giza-139' was growing, in Sahl-El-Tina, South Sinai, Egypt. These isolates, whose morphological and biochemical characteristics were studied, were screened *in vitro* for the production of indole-3-acetic acid (IAA), hydrogen cyanide (HCN), siderophores and Catalase (CAT). Results were compared with three reference strains (*Pseudomonas fluorescens*, *Bacillus polymyxa* and *Bacillus circulans*). Most (i.e., 8) of the tested isolates and all the reference strains (i.e., 3) could produce siderophores. IAA was detected in all isolates except for one isolate (HM9) and *B. circulans*. In addition, CAT activity was pronounced in isolate HM4, *P. fluorescens* and *B. circulans*. No CAT activity was detected in HM5, HM8 and HM11 isolates. The tested isolates and reference strains showed higher antagonistic activity against *Alternaria triticina* than against *Fusarium graminearum* and *Helminthosporium sativum*. All the isolates and reference strains were able to produce HCN. The tolerance of bacterial isolates and reference strains were evaluated against NaCl (the response of bacteria to different salt concentrations varied from one isolate to another), temperature (the highest growth was recorded at 30°C after 72 h incubation for HM1 and HM2 isolates and reference strains) and pH (highest growth of the different isolates and reference strains was recorded at pH 7 after 48 h incubation). The bacterial isolates were also characterized at a molecular level (SDS-PAGE and RAPD) in which 5 isolates (HM1, HM2, HM4, HM9, HM10) could be successfully differentiated from two reference strains (*P. fluorescens* and *B. polymyxa*).

Keywords: hydrogen cyanide, indole-3acetic acid, PGPR, siderophores, protein, SDS-PAGE
Abbreviations: IAA, indole-3acetic acid; **HCN**, hydrogen cyanide; **PGPR**, plant growth-promoting rhizobacteria; **NaCl**, sodium chloride; **N_2-ase**, Nitrogenase activity

INTRODUCTION

In Egypt, the average cultivated area with wheat in the last 10 years ranged from 1.0 to 1.3 million ha, producing about 8.27 million tons ha^{-1} $year^{-1}$ (Anonymous 2009).

Three wheat rust diseases are still the major biotic stress that limited the productivity and longevity of most wheat cultivars released. Leaf rust caused by *Puccinia triticina* is the most widespread rust in almost all wheat-growing areas in Egypt and Zambia and causes a considerable loss in grain yield, affecting ~23% of the susceptible wheat cultivars under suitable environmental conditions (Nazim *et al*. 2001). In Egypt, rust diseases are the most dangerous to wheat plants. Leaf rust, in particular, has eliminated several cultivars ('Giza-139', 'Mexpak-69', 'Super-x' and 'Chenap-70') because of their susceptibility. It is widespread on most wheat cultivars grown in Egypt (Kolmer *et al*. 1995).

Nearly 40% of the world's surface has salinity problems. Salinization of soil is a serious problem and is increasing gradually in many parts of the world, particularly in arid and semi-arid areas (Jadhav *et al*. 2010). At present, out of 1.5 billion ha of cultivated land around the world, about 77 million ha are affected by excess salt (Evelin *et al*. 2009). The percentage of cultivated land affected by salt is even greater, with 23% of the cultivated land being saline and 20% of the irrigated land suffering from secondary salinization. Egypt is one of the countries that suffers from severe salinity problems.

Rhizospheric microbes can improve the uptake of nutrients by plants. They also protect plant root surfaces to be colonized by pathogenic microbes through direct competitive and production of antimicrobial agents. Rhizospheric bacteria colonize plant roots and enhance plant growth, and are thus known as plant growth-promoting rhizobacteria, or PGPR (Kloepper *et al*. 1989).

PGPR, after colonizing a plant, trigger growth through their potential to synthesize plant hormones such as indole-3-acetic acid (IAA), cytokinins, and gibberellins and increase mineral and nitrogen availability in the soil (Sekar and Kandavel 2010).

The objective of this study was to isolate, purify, characterize and select PGPR isolates to use as biological agents to control the leaf rust pathogen *P. triticina* furthering future studies.

MATERIALS AND METHODS

Bacterial isolation

A number of 11 bacterial isolates were obtained from the rhizosphere of wheat seedlings cultivated in saline soil at Sahl-El-Tina, South Sinai, Egypt, by using a plate pour technique.

Ten grams of soil adhering to roots of each sample were suspended in 90 ml of sterile distilled water, shaken for 30 min on an orbital shaker (Stuart Scientific Orbital Shaker SOI UK) then serially diluted in sterile distilled water. Samples of 1 ml were taken

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from 10^{-5} and 10^{-6} dilutions and spread on nutrient agar medium (NAM; Dowson 1957). Petri dishes (9 cm diameter) were incubated at 28°C for 1-2 days. A single developing colony of different isolates was picked and streaked on agar slants.

Growth and cultural conditions

Isolates and reference rhizobacteria strains were maintained on NAM (Difco Manual 1985) containing (g l^{-1}): Beef extract, 3.0; Peptone, 5.0; pH 7; agar, 15.0; incubation at 30°C, under aerobic conditions.

Morphological characterization

The morphology of bacterial colonies was examined to observe cell morphology, Gram-stain (+ or-) and growth shape on NAM were studied.

Physiological characterization

The generation time under growth conditions was determined spectrophotometrically (Yelton *et al*. 1983) or by plate counting for all the tested isolates and reference strains (Somasegaran and Hoben 1994). For the spectrophotometric method, the isolates were grown in nutrient broth (Vincent 1970) at 30°C. Growth (turbidity) was monitored daily over 3 days by measuring optical density (OD) at 600 nm in a spectrophotometer (Spectromic Instruments Inc., NY, and USA).

The physiological characteristics were estimated based on the following features:

a. *Temperature tolerance*: To determine the highest temperature which the isolates and reference strains tolerated by incubating cultures at different temperatures (20, 30, and 40°C) for 1-2 days.

b. *NaCl tolerance*: Nutrient agar broth medium containing 0, 80, 175, 350 and 900 mM NaCl was inoculated with 1 ml per Petri dish of a culture of tested rhizobacteria and incubated at 30°C for 1-2 days.

c. *pH tolerance*: To determine the highest pH which the isolates and reference strains could tolerate, conical flasks containing 100 ml of nutrient broth with different pH values (4, 5, 6, 7, 8, 9 and 10) were inoculated with 1 ml of a culture of the tested isolates and reference strains, then incubated at 30°C for 1-2 days.

Assessment enzymatic activities in growth media

1. Nitrogenase activity

To assess the ability of bacterial isolates to fix atmospheric nitrogen, Nitrogenase activity was assayed using the acetylene reduction technique (Soasegaran and Hoben 1985). Using 24 h-old cultures of selective medium in test tubes, cotton plugs were removed and replaced by serum stoppers. After inoculation, pure acetylene (C_2H_2) was injected into test tube to give an atmospheric concentration 10% (v/v) of acetylene. Test tubes were incubated for different periods (24, 48, 72 h), then 1.2 ml of gas samples were withdrawn for the determination of C_2H_2 by gas chromatography using a Hewlett Packard chromatograph (Hp 6890 GC) fitted with a dual flame detector and a 150 cm × 0.4 cm diameter stainless steel column fitted with a Propa XR 100-120 mesh. The data are expressed as $\mu mol/C_2H_4/h$.

Evaluation of PGPR activities

1. Siderophore production

8-hydroxyl quinoline (50 mg/l) was added to 500-ml conical flasks. Tryptone Soya Agar (TSA) medium were sterilized at 121°C for 15 min and TSA plates were inoculated with 1 ml of culture of the isolates under investigation. The inoculated Petri dishs were incubated at 28°C for 3 days. Bacterial isolates and reference strains which grew 1 mm on TSA medium were considered positive for the production of siderophoric compounds (Alexander and Zuberer 1991).

2. Indole-3-acetic acid (IAA) production

A modified method as described by Bric *et al*. (1991) was used to detect IAA and/or IAA analogs. TSA medium amended with 5 mM tryptophan was overland with a nitrocellulose membrane disk (82 mm diameter, Bio-Rad, CA, USA). Agar plates were inoculated with a loopful of the tested isolates and then incubated at 28°C for 3 days. The membranes were overlaid on a single sheet of Whatman No. 2 filter paper saturated with Salkowski reagent (aqueous $FeCl_3$ to which a slug of concentrated H_2SO_4 is added; it is specific for indoles) Organisms producing IAA and/or IAA analogs were distinguished from organisms producing other indoles (producing yellow to yellow brown pigment) by their characteristic pink to red color after 0.5 to 0.3 h.

3. Hydrogen cyanide production

TSA medium was used for detection of hydrogen cyanide (HCN) after supplemented with 4.4 g/l of glycine and sterilized at 121°C for 15 min. TSA plates were streaked with the test microorganisms and incubated at 28°C for 3 days. The bacterial cultures were transferred to individual agar plates containing the same medium and inverted after a piece of filter paper impregnated with 0.5% picric acid and 2% sodium carbonate had been placed in the lid of each Petri dish. The plates were incubated at 28°C for 3-5 days. A change in color from yellow to orange-brown on the filter paper indicated the production of HCN (Bakker and Schippers 1987).

4. Antifungal activity

Antifungal activity was assayed *in vitro* by inhibiting the growth of a phytopathogenic fungus (*Fusarium graminearum*, *Helminthosporium sativum* and *Alternaria triticina*) on potato dextrose agar (PDA) media (Altindag *et al*. 2006). The bacterial inoculum of the bacterial isolates (HM1 to HM11) was picked aseptically and streaked (using a loop) on the center of a Petri dish. Fungal inocula consisted of an agar (PDA) disc (1 cm in diameter) punched out with sterilized cork borer from the growing margin of colonies that was placed on either site of plates inoculated with bacteria. The Petri plates were incubated at 28°C for 5 days. The diameter of hyaline inhibition zones was measured and the experiment was repeated three times.

Molecular characterization

1. SDS-PAGE

Protein was electrophoretically detected by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to (Laemmli 1970). Bacterial protein extraction was carried out using yeast extract peptone medium (YPM). The protein content in the supernatant was estimated (Bradford 1976) by using bovine serum albumin as the standard protein. Protein content was adjusted to 2 mg/ml per sample. 30 µl of the extracted protein was mixed with 5 µl of sample buffer and boiled in a boiling water bath for 3 min to denature protein.

Electrophoresis was carried out using a Hoefer Vertical Slab gel unit, Model SE-400. The method of Weber and Osborne (1969) was used to determine the apparent (subunit) molecular weight of the extractable protein. Electrophoretic mobilities were calculated relative to the mobility of the pre-stained protein marker in a 12% (separating gel) and a 5% (stacking gel) polyacrylamide slab gel. The following proteins were used as molecular mass standards (in KDa; all Sigma-Aldrich, St. Louis, USA): β-galactosidase (117.0), bovine serum albumin (90.0), ovalbumin (49.0), carbonic anhydrase (35.0), β-lactoglobulin (26.0) and lysozyme (19.0). The RM (relative mobility) values were then plotted against the log of the molecular mass.

Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR)

DNA was extracted from 50 mg of bacterial culture using the Qiagen Kit (CA, USA) for DNA extraction. 30 ng from the extracted DNA was used for the amplification reaction. The polymerase

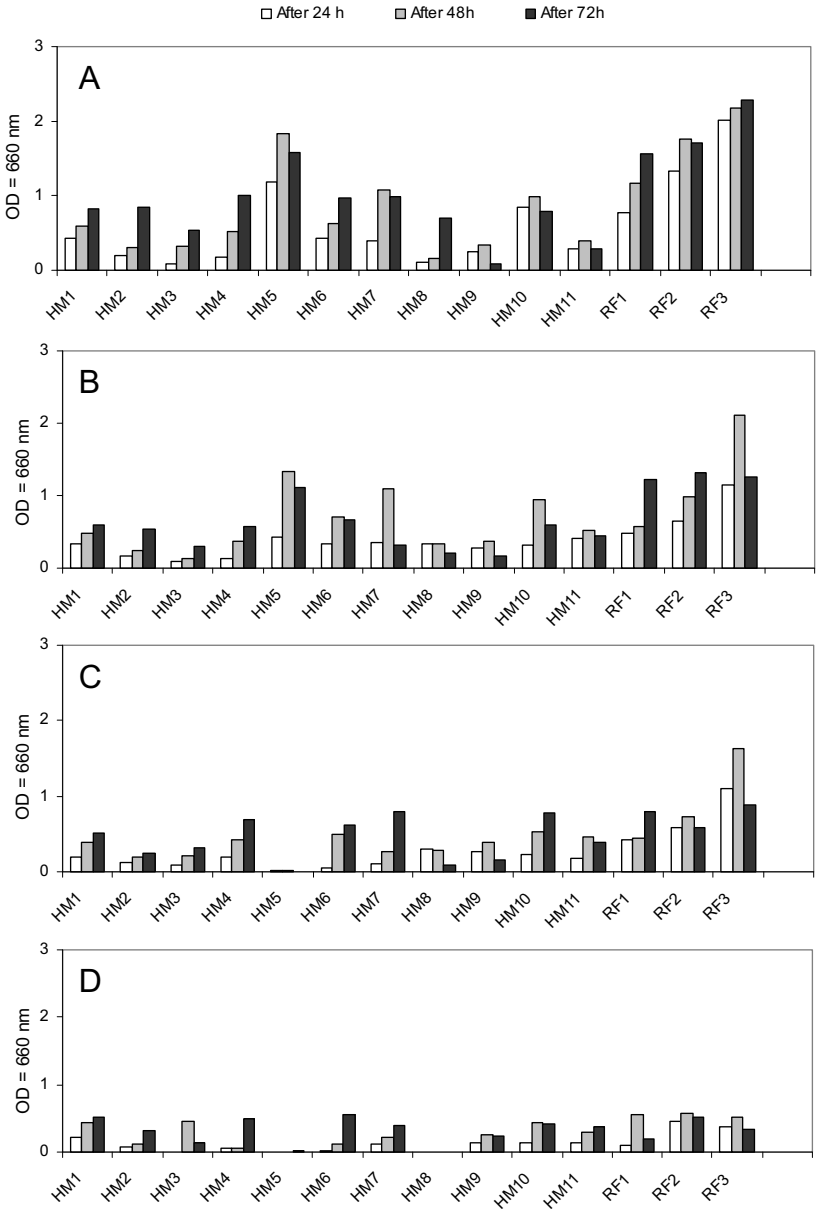

Fig. 1 Effect of different salt concentrations on growth (turbidity, 660 nm) of bacterial isolates (HM1 to HM11) and reference strains (RF1 to RF3) at different intervals. (**A**) 90 mM; (**B**) 180 mM; (**C**) 360 mM; (**D**) 720 mM.

chain reaction (PCR) mixture contained PCR beads tablet (manufactured by Amersham Pharmacia Biotech), which containing all of the necessary reagents except for the primer and the DNA that were added to the tablet. The Amersham Pharmacia Biotech kits also included the following 10-mer primers, 5 µl of which was added:

RAPD Analysis Primer 1:6-d 5′-GGTGCGGGAA-3′
RAPD Analysis Primer 2:6-d 5′-GTTTCGCTCC-3′
RAPD Analysis Primer 3:6-d 5′-GTAGACCCGT-3′
RAPD Analysis Primer 4:6-d 5′-AAGAGCCCGT-3′
RAPD Analysis Primer 5:6-d 5′-AACGCGCAAC-3′
RAPD Analysis Primer 6:6-d 5′-CCCGTCAGCA-3′

The total volume was made up to 25 µl using sterile distilled water. The amplification protocol was carried out as follows using PCR unit II Biometra: denaturation at 95°C for 5 min; 45 cycles each of the following steps [denaturation at 95°C for 1 min; annealing at 36°C for 1 min; extension at 72°C for 2 min]; final extension at 72°C for 5 min. 7 µl of 6X tracking buffer (Qiagen) were added to 25 µl of the amplification product.

Analysis of amplification products

15 µl of the amplified DNA for all samples was electrophoresed on an electrophoresis unit (WIDE mini-sub-cell GT; Bio-RAD) on a 1% agarose gel containing ethedium bromide (0.5 µg/ml), at constant 75 V and DNA bands were detected with a UV transilluminator. All gel types (protein and DNA) were scanned for band R_f using a gel documentation system (AAB Advanced American Biotechnology 1166E, Fullerton, CA). The MW of bands was visually referenced against a DNA marker (1 Kb Sigma Ladder (D-0428 0.5-10 Kb 11 fragments, 0.5 kb, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8 and 10 kb) and the protein marker MoBiTec P815MBB (66, 45, 35, 25, 18 and 14 kD, as shown in **Fig. 1**).

RESULTS

Isolation of PGPR from salinized soil

Data presented in **Table 1** shows the morphological characteristics of different isolates and reference strains (*Pseudomonas fluorescens*, *Bacillus polymyxa* and *Bacillus circulans*). Most isolates and reference strains are bacilli, aerobic, and Gram-positive. Colonies of isolates on NAM were circular, cream or white colored. Since they often spread over an entire plate within 1-2 days, they were considered to be fast-growing isolates.

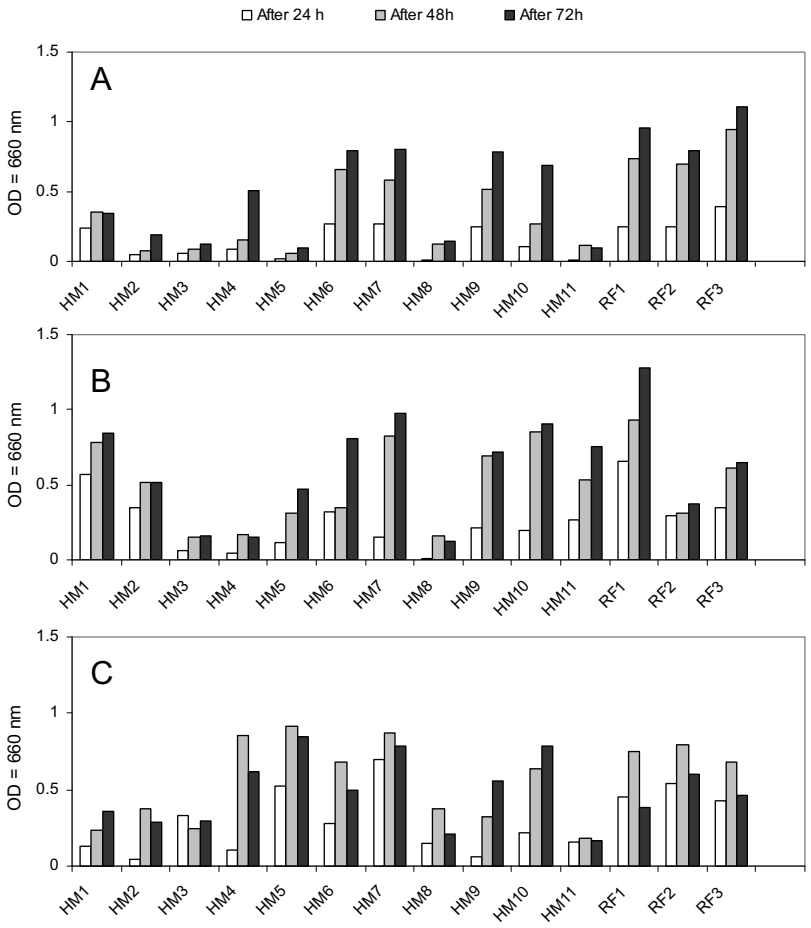

Fig. 2 Effect of different temperatures on growth (turbidity, 660 nm) of bacterial isolates (HM1 to HM11) and reference strains (RF1 to RF3) at different intervals. (**A**) 20°C; (**B**) 30°C; (**C**) 40°C.

Table 1 Morphological characteristics of some plant growth-promoting bacterial isolates (HM1 to HM11) compared with reference strains (RF1 to RF3).

Morphological characteristics		
Shape	**Color**	**Gram stain**
Bacilli	Cream	+
Bacilli	Cream	+
Cocci	Cream	+
Short rods	Cream	-
Short rods	Cream	-
Bacilli	White	+
Bacilli	Cream	+
Bacilli	White	+
Cocci	White	+
Short rods	Cream	-
Short rods	Cream	-
Reference strains		
Rods	Cream	-
Bacilli	Cream	+
Bacilli	Cream	+

+ = Gram positive, - = Gram negative, RF1 = *Pseudomonas fluorescens*, RF2 = *Bacillus polymyxa*, RF3 = *Bacillus circulans*

Physiological characterization

1. NaCl tolerance

The growth of bacterial isolates from HM1 to HM11 and reference strains *P. fluorescens*, *B. polymyxa* and *B. circulans* decreased in the interaction between bacterial isolates and reference strains, different salt concentrations and interval periods (**Fig. 1**). In each interval period, the growth of each isolate and reference strain decreased as a result of growing bacteria on different salt concentrations compared to the growth of the reference strains, in most cases.

Data revealed that the response of bacteria to different salt concentrations varied from one isolate to another. Isolate HM5 was unable to grow up to 180 mM NaCl and HM6 and HM8 isolates could not survive and grow at 720 mM NaCl. In most cases, the highest growth of each isolate and reference strains was recorded after incubation for 48 h. On the other hand, isolates HM1, HM2 and reference strains survived with a longer incubation period of 72 h. Moreover, at each interval period, bacterial growth was possible at 360 mM NaCl (**Fig. 1**).

2. Temperature tolerance

Data in **Fig. 2** indicates that the growth of bacterial isolates and reference strains was affected by the different temperatures and periods of growth. The growth of each isolate and reference strain increased as temperature increased up to 30°C and at different interval periods; however, the growth of isolates HM4 and HM5 increased up to 40°C. As temperature increased, growth decreased in all interval periods.

Generally, best growth was recorded with HM1 and HM2 isolates and reference strains at different interval periods.

3. pH tolerance

The growth of bacterial isolates and reference strains was affected by the interaction between different pH values and interval periods (**Fig. 3**). The isolates HM8, HM9 and HM10 grew weakly at pH 4 and 5. The growth of tested isolates and reference strains increased with an increase in pH values until pH 7 and then decreased with an increase in pH values until pH 10. At each pH value, the growth of each isolate and reference strain increased until 72 h relative to growth values recorded at both 24 and 48 h incubation.

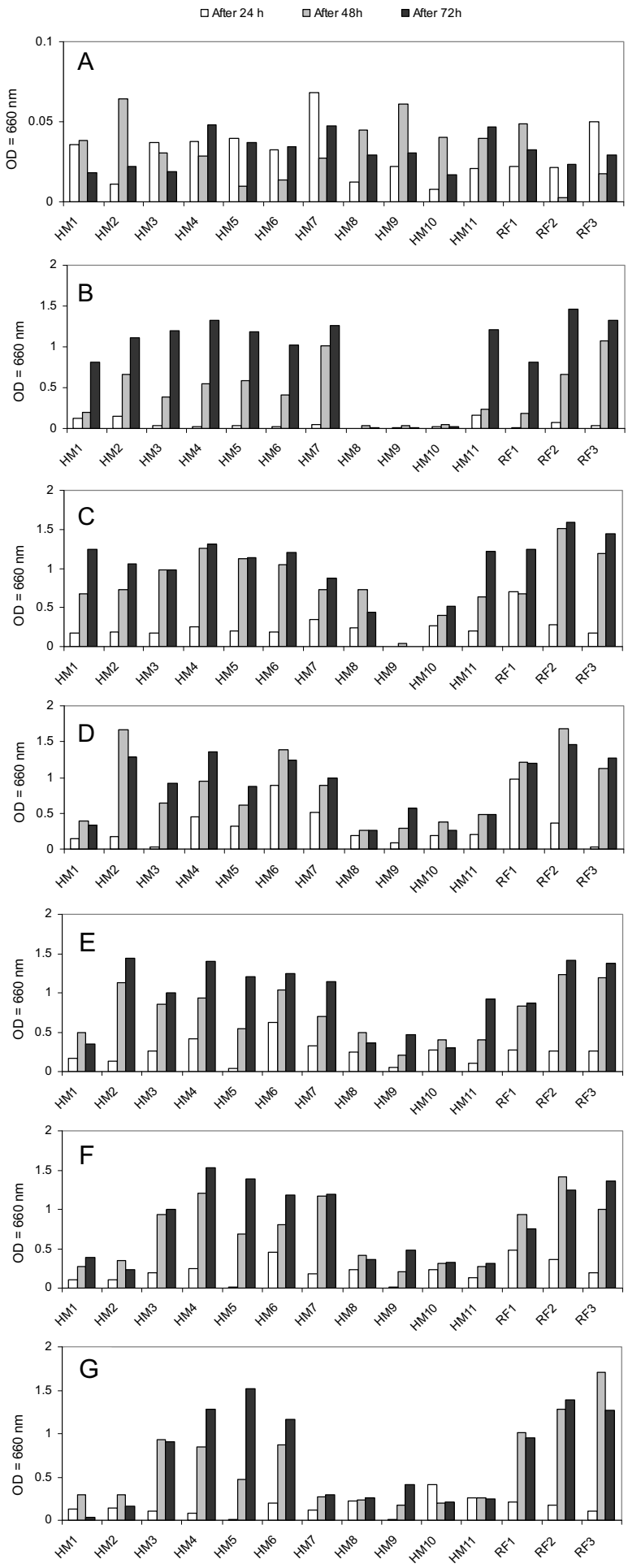

Fig. 3 Effect of different pH on growth (turbidity, 660 nm) of bacterial isolates (HM1 to HM11) and reference strains (RF1 to RF3) at different intervals. (**A**) pH = 4; (**B**) pH = 5; (**C**) pH = 6; (**D**) pH = 7; (**E**) pH = 8; (**F**) pH = 9; (**G**) pH = 10.

Table 2 Nitrogenase activity of the isolates and reference strains.

Bacterial isolates	Nitrogenase activity (μmoles/C_2H_4/h)
HM1	0.1879
HM2	0.1005
HM3	0.2647
HM4	0.0464
HM5	0.0616
Reference strains	
RF2	0.1419
RF3	0.1902

RF1 = *Pseudomonas fluorescens*, RF2 = *Bacillus polymyxa*, RF3 = *Bacillus circulans*

Generally, the highest growth of the tested isolates and reference strains was recorded at pH 7 after 48 h incubation.

Assessment of PGPR activities

1. Nitrogenase activity

Five isolates and two reference strains (*B. polymyxa* and *Bacillus circulans*) had pronounced Nitrogenase activity (**Table 2**). All other isolates showed no activity.

2. Antagonistic activities and production of plant growth-promoting substances

All the tested isolates were tested *in vitro* for their potential to antagonize fungal pathogens (*Fusarium graminearum*, *Helminthosporium sativum* and *Alternaria triticina*), as well as to estimate their ability to produce IAA, catalase (CAT), siderophores and HCN suppressing plant pathogens.

A. triticina was more resistant to tested isolates and reference strains than *F. graminearum* or *H. sativum* (**Table 3**). Most of the tested isolates and reference strains had the ability to produce siderophores. IAA was detected in all the tested isolates except for HM9 and *B. circulans*. CAT activity was high by isolate HM4, *P. fluorescens* and *B. circulans* but no CAT activity was observed with isolates HM5, HM8 and HM11. All the tested isolates and reference strains could produce HCN.

Molecular characterization

1. Protein pattern

SDS-PAGE extractable protein of the tested bacterial isolates and reference strains are illustrated in **Table 4** and **Fig. 4**.

SDS-PAGE detected 10 polypeptide bands (55, 51, 40, 38, 35, 32, 29, 23, 18 and 14 KDa) in all reference strains and in most of the tested isolates. Several polypeptide bands

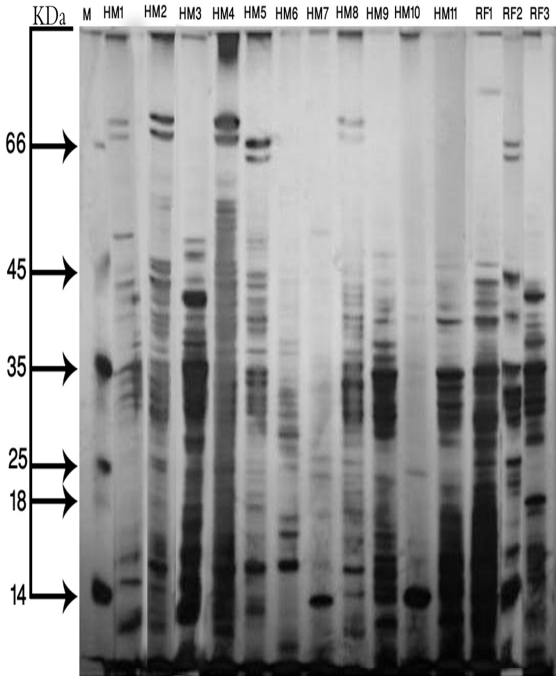

Fig. 4 SDS-PAGE protein extract of bacterial isolates (HM1 to HM11) and reference strains. M, marker protein; Bacterial isolates from HM1 to HM11; reference isolates: RF1 = *Pseudomonas fluorescens*, RF2 = *Bacillus polymyxa*, RF3 = *Bacillus circulans*.

(56, 53, 50, 48, 46, 43, 41, 36 and 12 KDa) were absent in reference strains but were present in all the tested isolates.

There were three major groups in the dendrogram (**Fig. 5**) consisting of 5 isolates (HM1, HM2, HM4, HM9, HM10), and two reference strains, *P. fluorescens* and *B. polymyxa*.

2. DNA profile

Genomic DNA isolated from accessions of 5 isolates and two reference strains (*P. fluorescens* and *B. polymyxa*) was successfully amplified following optimization of the amount of template DNA, *Taq* DNA polymerase and $MgCl_2$ concentrations, and also the thermal profile of the amplification reaction. DNA amplification was performed using six random primers of 10 nucleotides. All primers resulted in the appearance of PCR products with a variable number of DNA fragments (**Fig. 6A-F**; **Table 5A-F**). In addition, PCR product could be detected in the tested bacterial isolates using all primers. However, four primers (1, 2, 3 and 6) had a variable number of DNA fragments with the tested

Table 3 Some properties of isolates (HM1 to HM11) and reference strains (RF1 to RF3) to antagonize *F. graminearum*, *H. sativum* and *A. triticina* and their ability to produce siderophores, indole-3-acetic acid (IAA), catalase (CAT) and hydrogen cyanide (HC).

Bacterial isolates	Antagonistic to			Siderophore production	Indole-3-acetic acid production	Catalase production	Hydrogen cyanide production
	F. graminearum	*H. sativum*	*A. triticina*				
HM 1	++	++	-ve	+ve	+ve	+ve	+ve
HM 2	+++	++	+++	+ve	+ve	+ve	+ve
HM 3	+++	+	++	+ve	+ve	+ve	+ve
HM 4	+++	++	++	+ve	+ve	+ve	+ve
HM 5	++++	++++	++	-ve	+ve	-ve	+ve
HM 6	+++	++++	-ve	+ve	+ve	+ve	+ve
HM 7	+++	+++	-ve	+ve	+ve	+ve	+ve
HM 8	+++++	++	++	-ve	+ve	-ve	+ve
HM 9	++++	++++	-ve	+ve	-ve	+ve	+ve
HM 10	+++	+	+	+ve	+ve	+ve	+ve
HM 11	+++++	++	++	-ve	-ve	-ve	+ve
Reference strains							
RF 1	+++++	++	+++	+ve	+ve	+ve	+ve
RF 2	+++++	++	+	+ve	-ve	+ve	+ve
RF 3	++++	++	+	+ve	+ve	+ve	+ve

Very high activity = +++++; High activity = ++++; Medium activity = +++; Low activity = ++ Very low activity = +; No activity = -ve
RF1 = *Pseudomonas fluorescens*, RF2 = *Bacillus polymyxa*, RF3 = *Bacillus circulans*

Table 4 SDS-PAGE protein extract of bacterial isolates (HM1 to HM11) and reference strains (RF1 to RF3).

Serial	MM	Bacterial isolates											Reference strains		
no.	(kDa)	HM1	HM2	HM3	HM4	HM5	HM6	HM7	HM8	HM9	HM10	HM11	RF1	RF2	RF3
1	78	+	+	-	+	-	-	-	+	-	-	-	-	-	-
2	68	+	+	-	+	+	-	-	+	-	-	-	-	+	-
3	59	-	-	-	-	+	-	-	-	-	-	-	-	+	-
4	56	-	_+	-	+	-	-	-	-	-	-	-	-	-	-
5	55	-	+	-	--	-	-	-	+	+	-	-	+	+	+
6	53	-	+	-	+	-	-	-	-	-	-	-	-	-	-
7	52	-	+	-	+	-	-	-	-	-	-	-	-	-	-
8	51	-	+	-	-	+	-	+	-	-	-	+	+	+	+
9	50	-	-	-	+	-	-	-	-	-	-	-	-	-	-
10	49	-	+	-	+	-	-	-	-	-	-	-	-	-	-
11	48	+	-	+	+	+	-	-	-	-	-	-	-	-	-
12	47	-	-	-	+	-	-	-	-	-	-	-	-	-	-
13	46	+	-	+	-	-	-	-	-	+	-	+	-	-	-
14	44	+	+	-	+	-	-	-	-	-	-	-	+	-	-
15	43	+	+	-	+	+	-	-	-	-	-	-	-	-	-
16	41	-	+	+	+	+	+	-	+	+	-	-	-	-	-
17	38	-	+	+	-	+	-	-	+	+	-	-	+	+	+
18	36	+	+	+	+	+	+	-	+	+	-	-	-	-	-
19	32	+	+	+	-	+	+	+	+	+	-	+	+	+	+
20	29	-	-	-	-	-	+	+	+	+	-	+	+	+	+
21	23	-	-	+	+	+	-	-	+	+	-	-	+	+	+
22	18	-	+	+	-	+	-	+	-	-	-	+	+	+	+
23	17	+	+	+	+	+	+	+	+	+	+	+	-	+	+
24	16	+	+	+	+	-	+	+	+	+	+	+	+	+	-
25	15	+	+	+	+	+	+	+	+	+	+	+	+	--	+
26	14	+	+	+	+	+	+	+	+	+	+	-	+	+	+
27	13	+	-	+	+	+	+	+	+	+	+	+	-	-	+
28	12	+	+	-	+	-	+	+	+	+	+	-	-	-	-

MM = molecular mass, + = present band, - = absent band.
Bacterial isolates from HM1 to HM11; RF1 = *Pseudomonas fluorescens*, RF2 = *Bacillus polymyxa*, RF3= *Bacillus circulans*

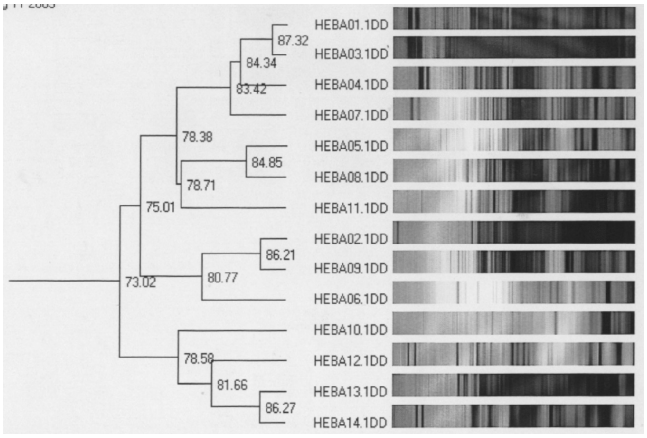

Fig. 5 Dendogram showing the relation among 11 bacterial isolates and 3 reference strains by protein data based on UPGMA clustering using Simple Band Match (tolerance: 3.20%). Bacterial isolates from HM1 to HM11; reference isolates: RF1 = *Pseudomonas fluorescens*, RF2 = *Bacillus polymyxa*, RF3 = *Bacillus circulans*.

isolates and gave a negative result with the reference strains; therefore, only two primers could be successfully used to differentiate tested isolates from reference strains (**Table 5D-E**). Primer 4 resulted in a 475-bp DNA fragment in *B. polymyxa* and HM2 and HM9 isolates while primer 5 amplified 450- and 220-bp DNA fragments in *B. polymyxa* and HM1 HM2 and HM4 isolates, respectively. A DNA fragment 440-bp long was found in *P. fluorescens* and isolate HM9.

DISCUSSION

Salinity adversely affects plant growth and development. Nearly 40% of the world's surface has salinity problems. Salinization of soil is a serious problem and is increasing gradually in many parts of the world, particularly in arid and semi-arid areas (Jadhav *et al*. 2010). Seed germination, seedling growth, vegetative growth, flowering and fruit set are adversely affected by high salt concentration, ultimately diminishing economic yield and product quality (Sairam and Tyagi 2004). PGPR have been used in agriculture because they can stimulate plant growth in different ways, including the production of plant growth regulators, nitrogen fixation or antagonism against phytopathogenic microorganisms through the production of siderophores (Scher and Baker 1982; Figueiredo *et al*. 2010), β-1,3-glucanase, chitinases (Renwick *et al*. 1991), or antibiotics and cyanide (de Freitas *et al*. 1997). The plant rhizosphere is a preferred ecological niche for soil microorganisms due to its rich nutrient availability. The growth of each isolate under investigation and reference strains increased significantly as incubation temperature increased up to 30°C, including the interval period. Similar trends were observed by others as salt, heat and drought tolerance of some rhizobial strains (Hafeez *et al*. 1991; Baldani and Weaver 1992; Surange *et al.* 1997; Hashem *et al*. 1998). Habish and Khairi (1970) and Zerhari *et al*. (2000) observed that tropical rhizobial strains isolated from *Acacia* sp. could grow at 30-35°C. Similar observations were obtained with a diversity of rhizobia isolated from the root nodules of leguminous trees (Zhang *et al*. 1991). Plant growth-promoting rhizobacteria could also be obtained from soils of stressed ecosystems (Zahran *et al*. 1994; Zahran 1999; Nautiyal 2003).

Mostly of the tested isolates produced siderophores. Siderophores chelate iron and other metals and contribute to disease suppression by conferring a competitive advantage to biocontrol agents for the limited supply of essential trace minerals in natural habitats (Hofte *et al.* 1992; Loper and Henkels 1997). Siderophores may directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to bacteria (Joseph *et al.* 2007). Antibiotics and siderophores may further function as stress factors or signals including local and systematic host resistance (Joseph *et al.* 2007). Promotion of plant growth by rhizobacteria through the suppression of disease might involve competition, production of siderophores and

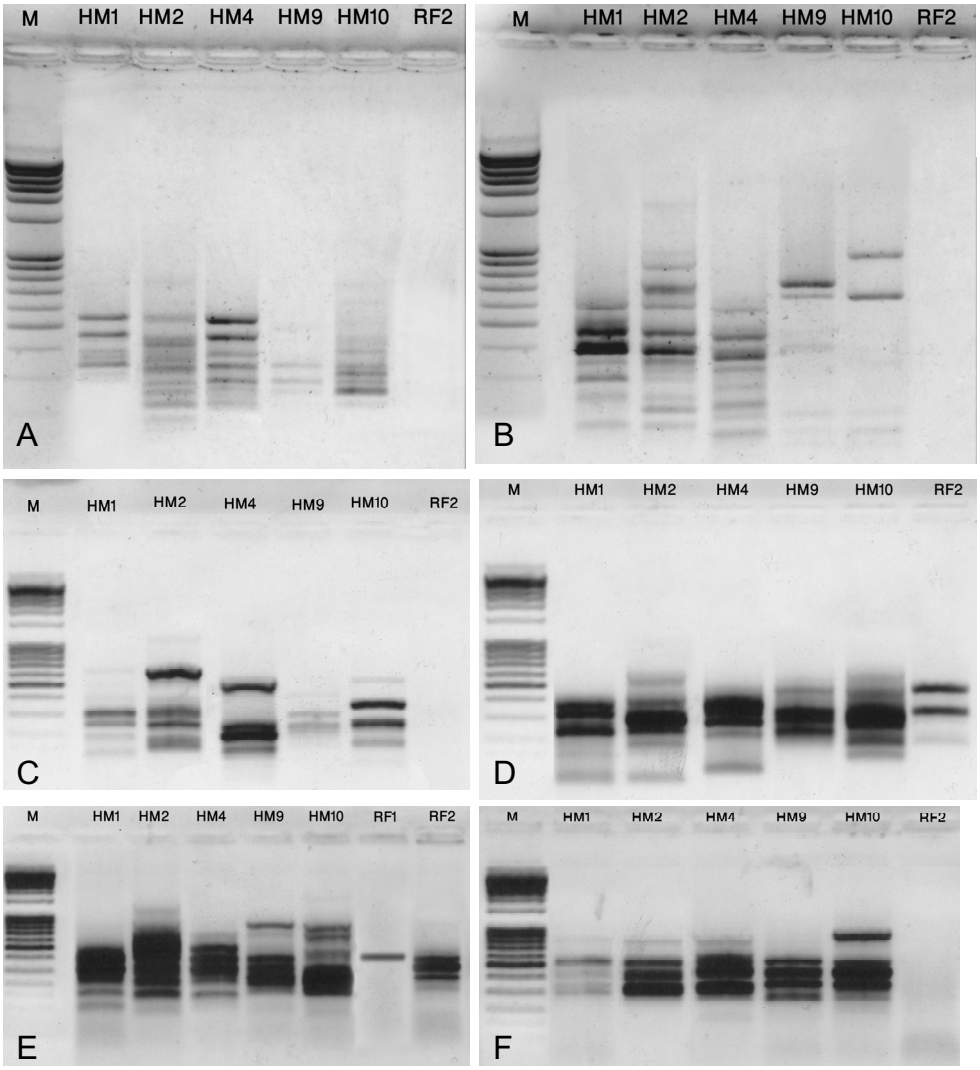

Fig. 6 (A-F) RAPD illustrating profiles of some bacterial isolates and reference strains. A = primer 1; B = primer 2; C = primer 3; D = primer 4; E = primer 5; F = primer 6.

HCN (Zdor and Anderson 1992; Saharan and Nehra 2011). All tested isolates had the ability to produce HCN, which has the potential to enhance plant defense mechanisms (Voisard *et al.* 1989; Dekkers *et al.* 2000; Lugtenberg *et al.* 2001). For example, microbial production of HCN is an important antifungal trait to control root-infecting fungi (Ramette *et al.* 2003).

In our study, most of the bacterial strains produced IAA. Similarly, Leinhos and Vacek (1994) reported the production of 1.6 to 3.3 mg auxin ml^{-1} filtrate by rhizospheric bacteria isolated from the rhizosphere of wheat. Furthermore, Prikryl *et al*. (1985) reported the production of IAA in liquid culture of *Pseudomonas cepacia* and *Pseudomonas fluorescens* isolated from maize (*Zea mays*) and bean (*Vicia faba*) rhizosphere, respectively. The production of IAA by *Bacillus polymyxa* was suggested to be a growth stimulant of crested wheat grass (*Agropyron cristatum* L.) (Holl *et al*. 1988). Phytohormones, including auxins (and as a subset IAA), are known to play a key role in plant growth regulation, promoting seeds germination, root elongation, and stimulation of leaf expansion (e.g., Boddy *et al*. 1995).

Most of the bacterial isolates in the current study were able to produce CAT. The mechanism of microbial resistance to oxidative stress is induced by photolysis of hydrogen peroxide (H_2O_2) in relation to microbial catalase activity. Some of the tested bacterial isolates were antagonistic against three pathogens: *Fusarium graminearum*, *Helminthosporium sativum* and *Alternaria triticina*.

A screening experiment of eleven different bacterial isolates and three reference strains (*Pseudomonas fluorescens*, *Bacillus polymyxa* and *Bacillus circulanc*) not only distinguishable based on biochemical tests; but also genotyping was performed by SDS-PAGE protein and RAPD-PCR analysis.

The SDS-PAGE proteins of studied bacterial isolates compared with reference strains and their dendrogram similarity have resulted in selection of five isolates representing all isolates. Genomic DNA isolated from accessions of five isolates and two reference strains was used for RAPD technique The RAPD bands which appeared consistently were evaluated. Biochemical and molecular characterization results showed that HM1 and HM2, *P. fluorescens* and *B. polymyxa* was the most salt tolerant strain. Expression of stress-related proteins is an important adaptive strategy of environmental stress tolerance. They are highly water soluble and heat stable, are associate with cytoplasmic membranes and organelles and act as molecular chaperones.

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Table 5A RAPD profiles of some bacterial isolates and reference strains using primer 1:6-d.

Primer	FS (bp)	Bacterial isolates					Reference strains	
		HM 1	HM 2	HM 4	HM 9	HM 10	RF1	RF2
Primer 1:6-d (5′-GGTGCGGGAA)-3′	978	-	+	-	-	-	-	-
	956	-	-	-	-	+	-	-
	875	-	+	-	-	-	-	-
	709	-	+	-	+	-	-	-
	677	-	+	-	-	-	-	-
	638	+	-	-	-	-	-	-
	631	-	-	-	-	+	-	-
	623	-	-	-	+	-	-	-
	600	+	+	-	-	-	-	-
	557	-	-	+	-	-	-	-
	507	+	-	-	-	-	-	-
	463	+	+	+	-	-	-	-
	437	-	-	+	-	-	-	-
	405	+	+	-	-	-	-	-
	384	-	+	-	-	-	-	-
	374	+	-	+	-	-	-	-
	358	-	-	+	-	-	-	-
	337	+	+	-	-	-	-	-
	295	+	-	+	-	-	-	-
	268	-	+	-	-	-	-	-
	263	+	-	-	-	-	-	-
	226	-	-	+	-	-	-	-
	195	-	+	-	-	-	-	-
	189	+	-	-	-	-	-	-
	163	-	-	+	-	-	-	-
	147	-	-	-	-	-	-	-
	74	-	+	-	-	-	-	-
	53	-	-	+	-	-	-	-

RF1 = *Pseudomonas fluorescens*; RF2 = *Bacillus polymyxa*, FS = fragment size, + = present, - = absent.

Table 5B RAPD illustrating profiles of some bacterial isolates using primer 2:6-d.

Primer	FS (bp)	Bacterial isolates					Reference strains	
		HM 1	HM 2	HM 4	HM 9	HM10	RF1	RF2
Primer 2:6-d (GTTTCGCTCC)-3	543	+	+	+	-	-	-	-
	463	+	-	-	-	-	-	-
	458	-	+	-	-	-	-	-
	453	-	-	+	-	-	-	-
	442	-	-	-	-	+	-	-
	432	-	+	-	-	-	-	-
	395	+	-	-	-	+	-	-
	368	+	-	-	-	-	-	-
	358	-	+	-	-	-	-	-
	342	-	-	-	+	-	-	-
	337	+	-	-	-	-	--	-
	332	-	+	+	-	+	-	-
	316	-	-	-	-	+	-	-
	268	-	+	+	-	-	-	-
	263	-	-	-	+	+	-	-
	226	-	-	-	-	+	-	-
	221	-	+	-	-	-	-	-
	174	-	+	-	-	-	-	-

RF1 = *Pseudomonas fluorescens*; RF2 = *Bacillus polymyxa*, FS = fragment size, + = present, - = absent.

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Table 5C RAPD illustrating profiles of some bacterial isolates and reference strains using primer 3:6-d.

Primer	FS (bp)	Bacterial isolates					Reference strains	
		HM 1	HM 2	HM 4	HM 9	HM 10	RF 1	RF 2
Primer 3:6-d (GTAGACCCGT)-3	755	+	-	-	-	-	-	-
	750	-	+	-	-	+	-	-
	700	-	-	+	-	-	-	-
	643	-	+	-	-	-	-	-
	629	-	-	+	-	-	-	-
	600	+	-	-	-	-	-	-
	575	-	-	+	-	-	-	-
	562	-	-	-	+	-	-	-
	500	-	-	-	-	+	-	-
	487	-	+	-	-	-	-	-
	475	-	-	+	-	-	-	-
	462	+	+	-	-	-	-	-
	412	-	-	-	+	-	-	-
	400	-	+	+	-	-	-	-
	379	-	-	-	-	+	-	-
	371	-	+	-	-	-	-	-
	364	+	-	+	-	-	-	-
	343	-	-	-	+	+	-	-
	314	+	+	+	-	-	-	-
	250	-	-	-	-	+	-	-
	230	+	-	-	-	-	-	-
	210	-	+	+	-	-	-	-
	180	-	+	-	-	-	-	-
	110	+	-	-	-	--	-	-

RF1 = *Pseudomonas fluorescens*; RF2 = *Bacillus polymyxa*, FS = fragment size, + = present, - = absent.

Table 5D RAPD illustrating profiles of some bacterial isolates and reference strains using primer 4:6-d.

primer	FS (bp)	Bacterial isolates					Reference strains	
		HM1	HM2	HM4	HM9	HM10	RF 1	RF 2
Primer 4:6-d (AAGAGCCCGT)-3	750	-	+	-	-	-	-	-
	657	-	+	-	-	-	-	-
	614	-	-	-	-	+	-	-
	538	-	-	-	-	+	-	-
	513	-	-	+	-	-	-	-
	500	-	-	-	-	-	-	-
	475	-	+	-	+	-	-	+
	462	-	-	-	-	+	-	-
	437	+	-	+	-	-	-	-
	400	-	-	-	+	-	-	-
	393	+	+	-	-	+	-	-
	386	-	-	+	-	-	-	-
	357	-	-	+	-	-	-	-
	350	-	-	-	+	-	-	-
	343	-	+	-	-	-	-	-
	336	-	-	-	-	-	-	+
	329	+	-	-	-	+	-	-
	314	-	-	-	-	-	-	-
	307	-	-	-	+	-	-	-
	300	-	-	+	-	-	-	-
	290	+	-	-	-	-	-	-
	280	-	+	-	-	-	-	-
	230	-	-	+	+	-	-	-
	200	-	-	-	-	+	-	-
	190	-	+	-	-	-	-	-
	170	-	-	-	-	+	-	-
	140	-	-	-	-	-	-	+
	70	-	-	-	-	+	-	-
	10	-	-	+	-	-	-	-

RF1 = *Pseudomonas fluorescens*; RF2 = *Bacillus polymyxa*, FS = fragment size, + = present, - = absent.

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Table 5E RAPD illustrating profiles of some bacterial isolates and reference strains using primer 5:6-d.

Primer	FS (bp)	Bacterial isolates					Reference strains	
		HM1	HM2	HM4	HM9	HM10	RF 1	RF 2
Primer 5:6-d (AACGCGCAAC)-3	1441	-	+	-	-	-	-	-
	1000	-	-	-	+	-	-	-
	925	-	+	-	-	+	-	-
	829	-	-	+	-	-	-	-
	800	-	-	-	+	-	-	-
	780	-	+	-	-	+	-	-
	667	-	+	-	-	-	-	-
	650	-	-	+	-	+	-	--
	600	+	-	-	-	-	-	-
	580	-	+	-	-	-	-	-
	570	+	-	+	-	-	-	-
	540	-	-	-	-	+	-	-
	520	-	-	+	-	-	-	-
	510	+	+	-	-	+	-	-
	500	-	-	-	-	-	-	+
	480	-	-	-	+	-	-	-
	470	-	-	+	-	-	-	-
	460	-	+	-	-	-	-	-
	450	+	-	-	-	-	-	+
	440	-	-	-	+	-	+	-
	410	-	+	-	-	-	-	-
	390	-	-	+	-	-	-	-
	380	-	-	-	+	-	-	-
	360	-	+	-	-	+	-	-
	350	-	-	+	+	-	-	-
	340	-	-	-	-	-	-	+
	330	+	-	-	-	-	-	-
	320	-	-	-	-	+	-	-
	300	-	+	+	--	-	-	-
	250	-	-	-	+	-	-	-
	240	+	-	-	-	-	-	-
	230	-	-	-	-	+	-	-
	220	-	+	+	-	-	-	+
	190	-	+	-	-	-	-	-
	180	+	-	-	+	-	-	-
	170	-	-	-	-	+	-	-
	120	-	-	-	-	+	-	-
	90	-	-	-	-	+	-	-
	80	-	-	-	+	-	-	-
	70	-	-	-	-	-	-	+
	60	-	-	+	-	-	-	-
	50	-	+	-	-	-	-	-
	40	+	-	-	-	-	-	-

RF1 = *Pseudomonas fluorescens*; RF2 = *Bacillus polymyxa*, FS = fragment size, + = present, - = absent.

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Table 5F RAPD illustrating profiles of some bacterial isolates and reference strains using primer 6:6-d.

Primer	FS (bp)	Bacterial isolates					Reference strains	
		HM1	HM2	HM 4	HM 9	HM 10	RF 1	RF 2
Primer 6:6-d (CCCGTCAGCA)-3	814	-	-	-	-	+	-	-
	720	-	-	+	-	-	-	-
	667	-	+	-	-	-	-	-
	633	-	-	-	-	+	-	-
	550	-	-	+	-	-	-	-
	490	-	-	+	-	-	-	-
	480	-	+	-	-	+	-	-
	470	+	-	-	+	-	-	-
	410	-	-	+	-	-	-	-
	390	--	+	-	+	-	-	-
	380	-	-	-	-	+	-	-
	370	-	-	-	+	-	-	-
	360	-	-	+	--	-	-	-
	320	-	+	-	-	-	-	-
	290	-	-	+	-	-	-	-
	280	-	-	-	-	+	-	-
	260	-	-	-	+	-	-	-
	250	+	-	-	-	+	-	-
	220	-	+	-	-	-	-	-
	210	-	-	+	-	-	-	-
	170	+	-	-	-	-	-	-
	160	-	-	-	+	-	-	-
	120	-	-	-	-	+	-	-
	110	-	-	-	+	-	-	-

RF1 = *Pseudomonas fluorescens*; RF2 = *Bacillus polymyxa*, FS = fragment size, + = present, - = absent.

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