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Phenotypic and Genotypic Characterization of Three Novel Halophilic *Bacillus* Strains from Jordanian Hot Springs

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Abstract

Three strains of facultatively anaerobic thermotolerant and moderately halophilic bacteria were isolated from two Jordanian hot spring locations. The novel *Bacillus* strains were short rods, Gram-positive, motile and spore formers. The strains were capable of anaerobic (but not aerobic) growth at 63°C, moderately halophilic, able to grow at NaCl concentration range of 12 - 15 % and at temperatures range of 37 -63°C. Moreover, the strains were resistant to aztreonam, bacitracin and oxacillin. The range of the guanine-plus-cytosine content of the strain's DNAs was 43 – 54 mol%. The three strains HAR7100^T, HAR720^T and HAR200^T, differ phenotypically from each other in terms of antibiotic resistance, compositions of polar lipids, presence and absence of several enzyme activities such as amylase, protease alkaline phosphatase, G6PDH and 6PGDH. Moreover, the novel *Bacillus* strains were shown to differ genotypically by random amplified polymorphic DNA fingerprinting and 16S rDNA amplification patterns. Phenotypic and genotypic variations may support establishment of new strains of the same genus *Paenibacillus*.

الملخص

ثلاث عترات من البكتيريا اللاهوائية المحبة للحرارة و للملوحة المتوسطة الموسومة ب HAR200^T HAR720^T HAR100^T تم عزلها من موقعين للينابيع الساخنة في الاردن . و تتصف هذه العترات بانها تنمو في وسط ملحي 12-15% من كلوريد الصوديوم ولها القدره على النمو لاهوائيا (وليس هوائيا) على درجات حراره 37-63 مئويه ؛ وهي عصيات قصيرة متحركة ومتجرثمة وتفاعلها ايجابي مع صبغة غرام. وجدت هذه الدراسه ان محتوى قواعد (الجوانين + السيتوسين) في الحامض النووي لهذه العترات يتراوح ما بين 43-54 % كما ان تواجد او غياب انزيمات الاميليز والبروتييز والفوسفاتيز القلويه وانزيمي جلوكوز 6 فوسفات و 6 فوسفو جلوكونيت النازعين للهيدروجين ومحتواها من الدهنيات القطبيه ومقومتها لبعض الادويه والبصمه الوراثيه لمحتوي دي.ان. اي. العشوائي وتحليل بصمة 16 sDNA تثبت ان هذه العترات الثلاثه تختلف اختلافا جذريا بالصفات الجينية والنوعية مما يدعم كونها عترات جديدة من الجنس *Paenibacillus* .

Keywords: Paenibacillus Hashemite; Paenibacillus rimawi; Paenibacillus zara; moderate halophilic; hot springs.;

1. Introduction

Organisms isolated from hot springs have received considerable interest in recent years. The thermotolerant microorganisms are members of the genus *Paenibacillus.* Bacteria of the genus *Paenibacillus* are facultative anaerobic, endospore-forming, Gram-positive rods. Representatives of this genus are widely distributed in soil, water, air, the plant rhizosphere, food, and diseased insect larvae (Daane et. al., 2002). The genus currently contains over 30 species and is phenotypically related to other genera belonging to the family Bacillaceae (Chung et. al., 2000).

Few publications are devoted to the study of thermotolerant halophilic *Paenibacillus* species isolated from the hot spring due to their ubiquity and capability to survive under adverse conditions (McDonald, 2001).

This study describes the isolation and characterization of a moderate halophile and thermotolerant bacteria. We have concluded based on detailed phenotypic, chemotypic, and genotypic investigations. Followed by numerical analysis that the three novel isolates represent a new *Paenibacillus* species, for which the names *P. hashemite sp.* nov. (HAR710^T), *P. rimawi sp*. nov. (HAR720^T) and *P. zara sp.* nov. (HAR200^T) are proposed.

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2. Materials and Methods

2.1. Isolation of bacteria

Samples of hot springs water were collected from hot springs located in Ma'in and Zara, Jordan. Water samples were transported without temperature control and analysed within 24 hr. Untreated, one hundred microliter samples of water, were spread directly onto Thermus agar plates (Williams and Da Costa, 1992) which were sealed in plastic bags and incubated at 53°C for 24-48 hr. Colonies were purified by serial transfers. All isolates were preserved in Thermus media containing 15% glycerol at –70°C. Three isolates designated HAR710^T, HAR720^T, and HAR200^T were chosen for in depth characterization.

2.2. Phenotypic characterization

Strains isolated in the present study were characterized by conventional microbiological methods, using API20E, morphology of vegetative cells and sporangia, shape and position of spores. In addition, the following characteristics were studied: utilization of citrate; hydrolysis of casein, starch and gelatine; catalase; oxidase; urease; motility; haemolytic type; aerobic growth at 37°C and 63°C; anaerobic growth at 37°C and 63°C; oxidation/fermentation; acid production from glucose, lactose, mannitol, inositol, sorbitol, rhabinose, saccharose, melebiose, amylase and arabinose; growth at (4°C, 10°C, 25°C, 30°C, 37°C, 43°C, 53°C, 63°C and 73°C); and NaCl requirement (0, 0.5, 1, 3, 7, 10, 12, 15 and 18%). Growth at different pH (2-12) was detected on the medium that contained (wt/vol): 0.2% Bacto-peptone, 0.2% casein hydrolysate, 0.2% yeast extract, 0.1% glucose, 0.02% KH_2PO_4, 0.005% $MgSO_4.7H_2O$, 1.5% Bacto-agar, 50% (vol/vol) of natural hot-spring water and 50% distilled water. The pH was adjusted with 10 M NaOH.

Cluster analysis was performed using SPSS 12.0.1 software for windows. An unweighted pair group average method was used for cluster analysis, and using a percentage disagreement method to drew a dendrogram.

2.3. DNA base composition and fatty acid analysis

DNA was isolated from the cells grown overnight in nutrient agar. The G + C contents of the DNA were determined by the method of (Marmur, and Doty, 1962). Fatty acid composition was essentially studied as described by (Reischl, 1998).

2.4. Antibiotic resistance

Resistance to antibiotic was determined by using the routine diffusion plate technique. Cultures were grown overnight on the nutrient medium at 37°C and were used to prepare suspensions with optical density of 0.5 McFarland Standard (1.5 x 10^8 cells per ml). A 0.1 ml portion of suspension was plated onto agar and disks containing antibiotics which were plated onto surface of the medium. After overnight incubation at 37°C, the diameters of zones of growth inhibition were measured. The following antibiotics were used (mcg/disk): azlocillin (75 mcg), amoxicillin (25 mcg), aztreonam (30 mcg), cefadroxil (30 mcg), bacitracin (10 U), carbenicillin (100 mcg), cefactor (30 mcg), ciprofloxacin (5 mcg), cefoxitin (30 mcg), imipenem (10 mcg), cephalothin (30 mcg), cefamandole (30 mcg), norfloxacin (10 mcg), piperacillin (100 mcg), streptomycin (10 mcg), risemycin (30 mcg), tobramycin (10 mcg) and oxacillin (1 mcg).

2.5. Polar lipid composition

The isolates were cultivated to late log phase, harvested by centrifugation at 8000 rpm for 20 min, then washed in distilled water and reharvested. Organisms were lyophilised and stored anhydrously as a fine powder until required.

Polar lipids were analysed by two-dimensional thin layer chromatography (TLC) using Silica Gel prepared plates (Merk HPTLC RP-18F 259). The chromatograms were developed using chloroform: methanol: water (65:25:4) as the first solvent system and chloroform: methanol: acetic acid: water (80:12:18:5) as the second solvent system. A variety of reagents was used to detect and partially identify the lipids. Ethanolic molybdophosphoric acid was used as a non-specific destructive detection reagent. Sprayed plates were charred in a forced draught at 180-190°C for 15 min. Lipids containing free amino groups were identified by using 0.2% w/v of ninhydrin solution in water saturated butanol. Sprayed chromatograms were heated at 100-105°C for 10 min. Lipids counting free amino groups were identified as a red-violet spots. Alpha naphthol reagent 10.5 ml of 15% (wt/v) alpha naphthol in 95% ethanol solution to 6.5 ml concentrated sulphuric acid, 405 ml of 95% ethanol and 4 ml water were added. The lightly sprayed chromatogram that was heated for 10 min at 120°C was used for detection of sugars that appeared as purple-blue spots.

2.6. Enzymatic activity

The following condition was used to analyse enzyme activity at 53°C, pH 6.5 and different NaCl concentrations (0.5, 1, 5, 7 and 10%).

Protease enzyme activity was detected by measuring the diameter of the clear zone produced in peptone yeast extract agar plates in the presence of 2% skim milk (casein) as substrate (Al Baker et al, 2000). A positive result was indicated by a clear zone around a streak inoculation after flooding with 10% (wt/v) $HgCl_2$.

Amylase enzyme activity was detected by measuring the diameter of the clear zone produced in peptone yeast extract agar plates in the presence of 1% starch as substrate (Al Baker et al, 2000). A positive result was indicated by a clear zone around a streak inoculation after flooding with 1% (wt/v) Lugol's iodine solution.

Alkaline phosphatase activity was detected by measuring the yellow diameter zone produced in peptone yeast extract agar plates in the presence of 5 mM p-nitrophenyl phosphate as substrate in 50 mM glycine/NaOH buffer, pH 9, containing 10 mm $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $ZnCl_2$.

Glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) activity were measured at 43°C using the method of (Hohorsts, 1965)

with slight modification, which depends on the reduction of $NADP^+$ to NADPH by G6PDH and 6PGDH. The activity measurement was made by monitoring the increase in absorbance at 340 nm, and the calculation of enzyme unit per litre (U/L) was done by assuming a molar extinction coefficient of 6270 U/L/mole for NADPH as described by (Wei-Ying and Tang, 1999).

2.7. Random amplified polymorphic DNA fingerprinting (RAPD) of the new isolates

PCR was performed in a volume of 50 µl containing 1 µl bacterial genomic DNA solution, 5 µl of 10X PCR reaction buffer, 200 µM of each nucleotide, 2.5 mM of $MgCl_2$, 1 µM of primer RAP-1 (5'-CAGCGACAAG-3'), 1 U of Taq polymerase and endonuclease free water up to 50 µl. All PCR materials were obtained from Promega (USA). The temperature profile was as follows: 5 cycles consisting of 94°C for 1 min, 31°C for 45 sec and 72°C for 2 min, and 25 cycles consisting of 92°C for 1 min, 40°C for 45 sec and 72°C for 45 sec; the final cycle was followed by an additional 7 min at 72°C. After amplification, 20 µl of the PCR product was electrophoresed in 1.5% agarose gel in TAE buffer and photographed by Digi-Doc camera (Vilber Lourmat).

2.8. 16S rDNA-based phylogenetic analysis

A DNA 16S-23S spacer region polymorphism analysis was performed in a volume of 25 µl containing: 1 µl of bacterial genomic DNA solution obtained as described by (Mora et al.; 1998), 2.5 µl of 10 x PCR reaction buffer, 200 µM of each dNTP, 2.5 mM of $MgCl_2$. 0.3 µM of each primer and 1.3 U of Taq polymerase. Primer set C (forward primer 5-GTCGTAACAAGGTAGCCGTA-3' and reverse primer 5'-CAAGGCATCCACCGT-3') was used to amplify the IIS region between the 16S and the 23S rDNA genes. The temperature profile was the following: 5 cycles consisting of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, and 30 cycles consisting of 92°C for 45 sec, 60°C for 45 sec, 72°C for 2 min and the final cycle was followed by an additional 7 min at 72°C. After amplification, 8 µl of product was electrophoresed in agarose gel.

3. Results

Differentiation between novel isolates and other related *Paenibacillus* species:

3.1. Phenotypic characteristics.

Morphological, biochemical, physiological and genetic composition tests of the novel isolates and other selected *Paenibacilli* are shown in Table-1. Cells of the novel isolates were Gram-positive, motile, spore forming rods of approximately 1.0-3.0 x 0.5-1.5 µm size. The new isolates produced spherical endospores in swollen sporangia in the central or subterminal region of the cell (Figure-1). Novel isolates were facultatively anaerobic, able to grow at 18% NaCl, catalase and oxidase positive and grew well on sheep blood and chocolate agar but not on MacConkey and xylose lysine desoxycholate agar. On sheep blood agar, the beta hemolytic colonies were grayish white with a convex elevation and regular margins.

Growth was found to be optimal at 43°C, 52°C and 50°C, with a range from 37°C to 63°C. In contrast, the optimal growth temperature for the majority of *Paenibacillus* species were 28 to 30°C (Shida et. al.; 1997) and 42°C (Bosshard et. al.; 2002). Contrary to almost all other *Paenibacillus* species, novel isolates were negative for Voges-Proskauer test, lactose, xylose, sucrose, melebiose and growth in 0.001% lysozymes. They were able to grow at 63°C and at pH 4 with optimum NaCl concentration of 15% (unpublished data). The new isolates had low G+C DNA content compared with other known *Paenibacillus* species (Table-1).

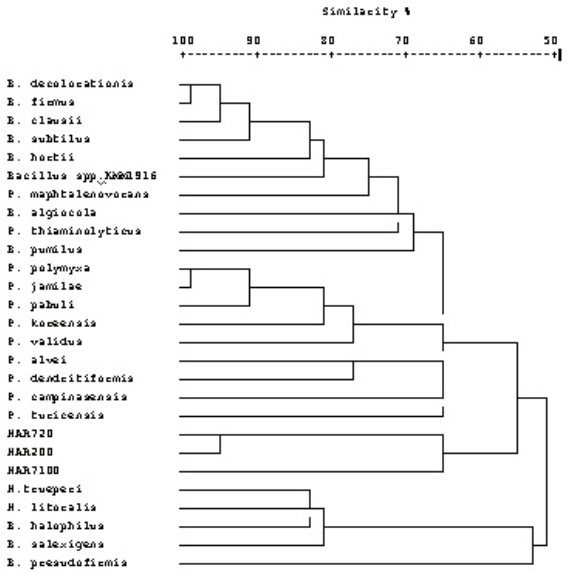

Figure-1. Dendrogram of relationship based upon similarity of average linkage clustring based on data obtained from this study; data obtained from Bosshard et al. (2002); data obtained from Bosshard et al. (2002), Daane et al. (2002), Shida et al. (1997a, b); data obtained from Bosshard et al. (2002) and Arguilera et al. (2001); data obtained from Enright et al. (2003) and Elo et al. (2001); data obtained from Chung et al. (2000); data obtained from (Velázquez et al. 2004).

3.2. Chemotaxonomy.

Cellular fatty acids analysis is shown in Table-2. The predominant fatty acids in whole-cell methanolysate of novel species were anteiso-15:00 (59.7, 38.6 and 29.1%, respectively), 16:00 (20.5, 15.2 and 8.9%, respectively), 17:00 (15.4, 20.1 and 9.0%, respectively) and iso-16:00 (20.1, 14.5 and 15.1%, respectively).

Table-1. Phenotypic characteristics of novel isolates and other related *Paenibacillus* species; (1) *P. hashemite*; (2) *P. rimawi*; (3) *P. zara*; (4) *P. turicensis*; (5) *P. alvei*; (6) *P. pabuli*; (7) *P. validus*; (8) *P. jamilae*; (9) *P. polymyxa*; (10) *P. macerans*; (11) *P. koreensis*; (12) *P. favisporus*; C= Central; St= Subterminal; T= Terminal; Sph= Spherical; Elp= Ellepsiodal; LTk= Long thick rods; SR= Short rods; β= Beta hemolysis; γ= Gamma hemolysis (nonhemolytic); WFRF= White, filamenteous, raised and filamanteous; WCUU= White, circular, umbonate and undulate; WFRU= White, filamentous; raised and undulant; GCRE= Green, circular, raised and entire; GCUU= Green, circular, umbonate and undulant; WCRU= White, circular, raised and undulant; GCUE= Green, circular, umbonate and erose; WCCT= White, circular, convex and translucent; LAS= layer at surface only. NR = not reported; $ Data obtained from this study; # Data obtained from (Bosshard et al.; 2002); & Data obtained from (Daane et al 2002; Shida et al.; 1997, and Bosshard et al.; 2002); * Data obtained from (Bosshard et al.;2002 and Aguilera et al,2001); ^ Data obtained from Enright et al.; 2003 and Elo et al.; 2001); Data obtained from (Chung et al.; 2000); ~ Data obtained from (Velázquez, et al.; 2004)

Characteristics	1[$]	2[$]	3[$]	4[#]	5[&]	6[&]	7[&]	8[*]	9[&]	10[^]	11[!]	12[~]
Microscopic examination:												
Spore position	C/St	C	C/St	T/St	C/T/St	NR	NR	NR	C/St	T/St	T	St
Spore morphology	Sph	Sph	Sph	Elp	Sph	NR	NR	NR	Sph	Sph	Elp	Sph
Cell morphology	LTk	SR	SR	SR	LTk	NR	NR	NR	LTk	LTk	SR	SR
Culture examination:												
Colony morphology	WFRF	WCUU	WFRU	GCRE	GCUU	NR	NR	NR	WCRU	GCUE	WCUU	WCCT
Broth growth	LAS	LAS	LAS	LAS	LAS	NR	NR	NR	LAS	LAS	LAS	LAS
Type of hemolysis	β	β	β	γ	γ	NR	NR	NR	β	β	NR	NR
Temperature growth (ºC):												
Minimum	28	37	37	15	15	10	15	30	28	NR	10	28
Optimum	43	52	50	42	28	30	30	30	30	NR	40	37
Maximum	63	63	63	48	43	48	53	40	42	NR	50	43
pH growth:												
Minimum	4	4	4	5.5	6.5	5.6	NR	5	4	4	NR	NR
Optimum	6.5	8	9	7	7	7.5	NR	7	6.5	6.5	NR	5
Maximum	7	11	11	9.5	8.5	8	NR	12	8	7.5	NR	7
NaCl growth:												
Minimum	12	12	12	3	4	3	NR	NR	NR	NR	NR	NR
Optimum	15	15	15	5	4	3	NR	NR	NR	NR	NR	NR
Maximum	18	18	18	5	5	4	NR	NR	3	NR	NR	5
Utilization of citrate	-	-	-	-	-	-	-	-	-	NR	-	-
Hydrolysis of:												
Casein	+	-	-	-	-	-	-	-	+	-	+	-
Starch	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin	+	-	-	-	+	-	-	+	+	-/+	NR	+
VP test	-	-	-	+	+	-	-	+	+	-	NR	+
Nitrate reduction	-	+	+	-	-	-/+	-/+	+	+	+	-	+
N2 gas	-	+	+	NR	NR	NR	NR	NR	NR	NR	+	NR
Indole	+	+	-	-	+	-	-	NR	-	NR	-	-
Catalase	+	+	+	-	+	+	+	+	+	NR	+	+
Oxidase	+	+	+	-	+	-	-	-	-	NR	+	+
Urease	-	-	-	-	+	-	+	NR	-	NR	-	+
TDA	-	-	+	-	-	-	-	NR	-	NR	+	-
ODC	-	+	+	-	-	-	-	NR	-	NR	-	NR
LDC	+	-	-	-	-	-	-	NR	-	NR	NR	-
ONPG	+	+	-	+	+	+	-	-	+	+	+	+
LV	+	-	-	NR	NR	NR	NR	NR	NR	NR	NR	NR
Growth in 0.001% lysozyme	-	-	-	+	+	-	NR	+	+	NR	+	NR
Anaerobic growth	+	+	+	+	+	-/+	-	+	+	+	+	+
% G+C	50	54	43	NR	46	49	54	NR	44	52	54	53
Carbohydrates, acid from:												
Glucose	+	+	+	+	-	+	+	+	+	NR	+	+
Lactose	-	-	-	+	-	+	-	+	+	+	+	+
Arabinose	-	-	-	-	-	+	-	-	+	+	+	-
Mannitol	-	-	+	-	-	+	+	+	+	+	+	+
Xylose	-	-	-	+	-	+	+	+	+	+	-/+	+
Sucrose	-	-	-	+	+	+	+	+	+	+	+	+
Amylase	-	-	-	-	-	-	-	-	-	-	-	+
Melebiose	-	-	-	-	+	+	+	+	+	+	NR	+
Inositol	-	-	-	-	-	-	+	-	-	-/+	+	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	+	-

Table-2. Fatty acid composition (% of total) of novel isolates and several other species of the genus *Paenibacillus* (Data were taken from (Daane et al 2002; Bosshard et al.; 2002 and Enright et al.; 2003).

Paenibacillus species	Saturated acids							
	Straight-chain			Iso-branched		Antesio-branched		
	$C_{14:00}$	$C_{15:00}$	$C_{16:00}$	$C_{15:00}$	$C_{16:00}$	$C_{15:00}$	$C_{16:00}$	$C_{17:00}$
P. hashemite	6.2	1.5	15.6	12.1	20.1	59.7	20.5	15.4
P. rimawi	4.1	0.5	8.9	10.6	14.5	38.6	15.2	20.1
P. zara	3.5	2.1	1.5	6.1	15.1	29.1	8.9	9.0
P. turicensis	8.9	ND	25.4	5.8	4.1	41.1	ND	ND
P. macerans	3.7	0.5	17.9	2.6	17.1	34.5	ND	16.1
P. korensis	ND	ND	ND	ND	20.6	51.1	28.3	ND
Other *Paenibacillus* species	0.2-3.7	ND-3.5	1.5-24.7	0.2-27.1	0.2-7.1	34.5-81	ND	1-30.2

ND, not detected.

3.3. Numerical analysis.

The numerical study of the selected species and the reference species using Jaccard coefficient (Sj), and clusters of species were obtained by average linkage (UPGMA) analysis. Clustered by the unweighted average linkage methods, gave the dendrogram shown in Figure 1. Phenotypic and chemotaxonomic characters resulted in dendrogram divided into three areas at similarity level 50%.

Area I divided into two clusters at similarity level 53%, cluster I contains one species *B. presudofirmis* in class I while class II contains four species *B. salexigens*, *B. halophilus, H. litoralis* and *H. trueperi* showing 81-83% intrasimilarity level.

Area II contains twelve species and divided into two subareas. Subarea I contains three species divided into two clusters at similarity level 65.0%. It contains one species *P. hashemite* sp. nov., while cluster II contains two species *P. rimawi* sp. nov. and *P. zara* sp. nov. at similarity level 96%. On the other hand, Subarea II contains nine species clustered into three clusters. Cluster I contains two species *P. turicensis* and *P. campinasensis* at intrasimilarity level 65%. Cluster II contains two species *P. dendritiformis* and *P. alvei* at intrasimilarity level 76%. Cluster III is divided into four subclusters at similarity level 91%. Subcluster I contains species *P. validus* at overall similarity level 76%; subcluster II contains species *P. koreensis* at overall similarity level 81%; subcluster III contains species *P. pabuli* at overall similarity level 91% and subcluster IV contains two species *P. jamilae* and *P. polymyxa* at intrasimilarity level 99%.

Area III is divided into nine clusters, 1-9 contains species *B. pumilus* (69%), *P.* thiaminolyticus (71%), *B. algiocola* (71%), *P. maphtalenovorans* (75%), *Bacillus spp.* KMM1916 (81%), *B. hortii* (83%), *B. subtilus* (91%), *B. clausii* (96%), *B. firmus* and *B. decolorationis* (99%) similarity level, respectively.

3.3.1. Differentiation of the three novel isolates

The phenotypic variability between the three novel species, were further discriminated using antibiotics resistance, polar lipid composition, enzymatic activity, RAPD , 16s rDNA and advanced PCR with specific markers for *Bacillus* species.

3.3.2. Resistance to antibiotics by the novel isolates.

The resistance of the new isolates to several antibiotics is shown in Table-3. They were resistant to aztreonam, bacitracin and oxacillin. Whereas, *P. zara* sp. nov. was resistant to streptomycin and *P. hashemite* sp. nov. was resistant to cefadroxil. On the other hand, both *P. zara* sp. nov. and *P. rimawi* sp. nov. were resistant to tobramycin.

Table-3. Resistance to antibiotics by the novel isolates.

Antibiotics	*P. hashemite*	*P. rimawi*	*P. zara*
Azlocillin (75 mcg)	N	N	N
Amoxicillin (25 mcg)	N	N	N
Aztreonam (30 mcg)	P	P	P
Cefadroxil (30 mcg)	P	N	N
Bacitracin (10 U)	P	P	P
Carbenicillin (100 mcg)	N	N	N
Cefactor (30 mcg)	N	N	N
Ciprofloxacin (5 mcg)	N	N	N
Cefoxitin (30 mcg)	N	N	N
Imipenem (10 mcg)	N	N	N
Cephalothin (30 mcg)	N	N	P
Cefamandole (30 mcg)	N	N	P
Norfloxacin (10 mcg)	N	N	N
Piperacillin (100 mcg)	N	P	N
Streptomycin (10 mcg)	N	N	P
Risemycin (30 mcg)	N	N	N
Tobramycin (10 mcg)	N	P	P
Oxacillin (1 mcg)	P	P	P

N: Negative P: Positive

3.3.3. Polar-lipid composition of the novel isolates.

The polar lipid compositions of the novel isolates are shown in Table-4. All tested organisms had phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC) and phosphatidylinositol (PI); on the other hand, phosphatidylserine (PS) and phosphoglycolipid (PGL1, PGL2 and PGL3) were absent.

Simple lipid (SL) was present in *P. hashemite* sp. nov. and *P. rimawi* sp. nov. but was absent from *P. zara* sp. nov.. Moreover, biphosphatidylglycerol (DBG) and sulfoquinovosyldiacylglycerol (SQDG) were absent in *P. hashemite* sp. nov.. On the other hand, digalactosyldiacylglycerol (DGDG) was present in *P. rimawi* sp. Nov. only.

3.3.4. Enzymatic activity.

The enzymatic activities of the novel isolates are shown in Table-5. All enzymes were tested at 53°C. All tested isolates showed activity in the presence of high concentration of NaCl up to 7%. The exception was for alkaline phosphatase activity, from *P. rimawi* sp. nov. that showed activity only in the presence of 7% NaCl; whereas, *P. zara* sp. nov. had activity in NaCl concentrations of up to 5%, but no activity observed for *P. hashemite* sp. nov.

Table-4. Polar lipid composition of the novel isolates.

Polar lipid composition	*P. hashemite*	*P. rimawi*	*P. zara*
Simple lipid	P	P	A
Glycolipid	P	A	P
Monogalactosyldiacylglycerol	P	A	P
Phosphatidylethanolamine	P	P	P
Biphosphatidylglycerol	A	P	P
Phosphatidylglycerol	P	P	P
Phosphatidylcholine	P	P	P
Digalactosyldiacylglycerol	A	P	A
Sulfoquinovosyldiacylglycerol	A	P	P
Phosphatidylinositol	P	P	P
Phosphatidylserine	A	A	A
Phosphoglycolipid III	A	A	A
Phosphoglycolipid II	A	A	A
Phosphoglycolipid I	A	A	A

A: Absent P: Present

3.3.5. Genotypic characterization.

Polymorphism of these organisms is highlighted by both RAPD test and 16S rDNA. Genetic variability studies by RAPD test are presented in (Figure-2a); while the 16S rDNA studies are shown in (Figure-2b). Genetic variability among these novel strains was clearly demonstrated.

4. Discussion

In this paper we describe the characterization of a group of moderate halophilic spore forming bacteria, including strains HAR7100^T, HAR720^T and HAR200^T, isolated from two Jordanian hot springs located in different geographical areas. The novel isolates appear to resemble species within the genus *Peanibacillus* based on their phenotypic, chemosystematic standard and phylogenetic properties.

The chemotaxonomic data, i.e. G+C content of DNA (range from 43 to 50) and the antesio-15:00 fatty acid as the major cellular fatty acid (range from 59.7 to 29.1%) also falls within the ranges exhibited by *Paenibacillus* species (Shida et al., 1997; Yoon et al., 1998; Chung et al., 2000). A comparison of the phenotypic characteristics of strains HAR7100^T, HAR720^T and HAR200^T with those of phylogenetically related *Paenibacillus* species such as: *P. turicensis, P. alvei, P. pobuli, P. validus, P. jamilae, P. polymyxa, P. macerans, P. koreensis* and *P. favisporus*, revealed that these novel strains were very different. The novel strains were negative for VP test, able to grow at 63^{o}C at pH 4 with optimum growth at NaCl concentration of 15%. Lactose, xylose sucrose and melebiose were not fermented by the isolates. The isolates were also able to grow anaerobically but not aerobically at 63^{o}C.

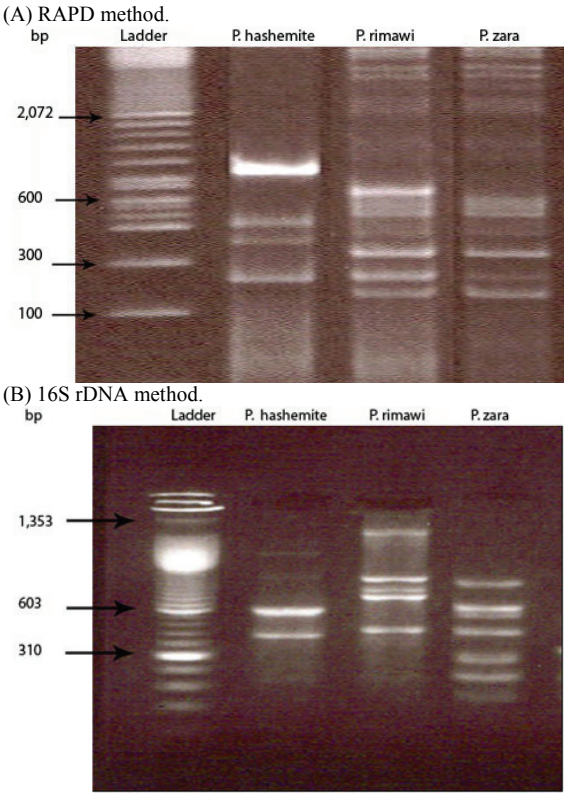

Figure -2. Comparison between patterns of the novel isolates by (A) RAPD and (B) 16S rDNA methods using PCR technique.

The three novel *Paenibacillus* strains have slight different phenotypic characteristics among each other in terms of growth conditions, resistance to antibiotics, and composition of polar lipid, enzymatic activity and fatty acid composition as demonstrated by this study.

DNA finger printings analysis, showed distinctive amplification patterns that attain a useful clustering at the genus level. RAPD and 16S rDNA amplification patterns explain polymorphism present among the three novel strains that belong to the same genus. The DNA analysis provides a more stable determination for the identification of the new isolates (Wisotzkey, et al.; 1992, Aguilera et al.; 2001).

Further DNA genetic tests such as DNA sequencing, 16s rRNA and DNA-homology are needed for precise characterization of these new different isolates.

Table-5. Some enzymatic activity at different NaCl concentrations, 53^{o}C and pH 6.5 of the novel isolates.

Bacillus	***P. hashemite***					***P. rimawi***					***P. zara***				
NaCl (%)	0.5	1	5	7	10	0.5	1	5	7	10	0.5	1	5	7	10
Amylase	P	P	P	N	N	P	P	P	N	N	P	P	P	N	N
Protease	P	P	P	P	N	P	P	P	N	N	P	P	P	N	N
Alkaline phosphatase	N	N	N	N	N	N	N	N	P	N	P	P	P	N	N
G6PDH	P	P	P	P	P	P	P	P	P	N	P	P	P	P	N
6PGDH	P	P	P	P	P	P	P	P	P	N	P	P	P	P	N

G6PDH= Glucose-6-phosphate dehydrogenase; 6PGDH= 6 Phosphogluconate dehydrogenase; P= Positive; N= Negative.

Aknowlegment

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