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Halotolerant Spore-Forming Gram-Positive Bacterial Diversity Associated with *Blutaparon portulacoides* (St. Hill.) Mears, a Pioneer Species in Brazilian Coastal Dunes

BARBOSA, DEYVISON CLACINO, IRENE VON DER WEID, NATALIE VAISMAN, AND LUCY SELDIN*

Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Bloco I, Ilha do Fundão, CEP 21941-590, Rio de Janeiro, Brasil

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Abstract Halotolerant spore-forming Gram-positive bacteria were isolated from the root, rhizosphere, and non-rhizosphere soil of *Blutaparon portulacoides*. The different isolates were characterized genetically using an amplified ribosomal DNA restriction analysis (ARDRA), and phenotypically based on their colonial morphology, physiology, and nutritional requirements. Three different 16S rRNA gene-based genotypes were observed at a 100% similarity using the enzymes HinfI, MspI, and RsaI, and the phenotypic results also followed the ARDRA groupings. Selected strains, representing the different ARDRA groups, were analyzed by 16S rDNA sequencing, and members of the genera *Halobacillus*, *Virgibacillus*, and *Oceanobacillus* were found. Two isolates showed low 16S rDNA sequence similarities with the closest related species of *Halobacillus*, indicating the presence of new species among the isolates. The majority of the strains isolated in this study seemed to belong to the species *O. iheyensis* and were compared using an AP-PCR to determine whether they had a clonal origin or not. Different patterns allowed the grouping of the strains according to Pearson's coefficient, and the resulting dendrogram revealed the formation of two main clusters, denoted as A and B. All the strains isolated from the soil were grouped into cluster A, whereas cluster B was exclusively composed of the strains associated with the *B. portulacoides* roots. This is the first report on the isolation and characterization of halotolerant spore-forming Gram-positive bacteria that coexist with *B. portulacoides*. As such, these new strains may be a potential source for the discovery of bioactive compounds with industrial value.

Key words: *Blutaparon portulacoides*, halotolerant bacteria, spore-forming Gram-positive bacteria, coastal dunes, diversity

*Corresponding author
Phone: 55(21) 2562.6741 or 99897222; Fax: 55(21) 2560.8344;
E-mail: lseldin@micro.ufrj.br

Spore-forming Gram-positive bacteria are especially useful in industrial processes owing to their capability to produce secondary metabolites with diverse biological activities [18]. Thus, searching for novel spore-forming bacteria is essential for the discovery of natural product-based drugs and/or enzymes. In particular, halophilic and/or halotolerant spore-forming Gram-positive bacteria are very important because of the wide range of salinity in which this group of microorganisms can grow optimally, presenting haloadaptation mechanisms to grow and survive in harsh environments [28]. This group of bacteria is widely distributed in different saline habitats, such as hypersaline lakes, saltern ponds, water from the Dead Sea [2, 25, 29], the marine solar saltern of the Yellow sea [32], surface saline soils [1, 22], fermented seafood [30], desert iguanas [15], and mural paintings, causing biodeterioration [12].

When halophilic and halotolerant spore-forming Gram-positive bacteria were first studied, the majority of the isolates were assigned as members of the genus *Bacillus* [6]. However, the recent application of molecular methods and chemical analysis has aided in the recognition of a number of new species and genera harboring halophilic and halotolerant strains, as follows: *Gracilibacillus* [29], *Halobacillus* [25], *Virgibacillus* [11, 12], *Oceanobacillus* [16], *Filobacillus* [22], and *Jeotgalibacillus* [30]. Although the presence of members of these genera is acknowledged in different saline habitats, the characterization of halophilic/halotolerant bacteria associated with plants living in saline habitats is still basically lacking.

It has been previously demonstrated that bacterial adaptation to specific heterogeneous and fluctuating environments, such as the plant rhizosphere, is dependent on the diversity of the bacterial population [20]. Thus, to explore the Gram-positive spore-forming community associated with plants in tropical soils, the roots of *Blutaparon portulacoides* were sampled and investigated. *Blutaparon portulacoides*

(St. Hill.) Mears (Amaranthaceae) is a perennial rhizomatous herb, with succulent and frequently shed leaves, and colonizes the embryo dunes and backshores of the Southwestern Atlantic Ocean beaches [7]. *B. portulacoides* tolerates this salt-stressed zone and the high temperatures quite well, and is invariably exposed to storm tides [5]. This species is also considered of great interest because of its medical value, due to the presence of flavonol, irisone B, sitosteryl, vanillic acid, and the steroids stigmasterol, sitosterol, and campesterol [8].

Accordingly, this study attempted to determine the diversity of the Gram-positive spore-forming bacterial populations associated with *B. portulacoides* by their phenotypic and genetic characteristics. As such, the bacterial populations isolated from the root, rhizosphere, and non-root-associated soils were identified and compared. This approach was used to obtain an indication of the bacterial groups, group the isolates into clusters of similar strains, and help elucidate whether *B. portulacoides* selects specific halophilic/halotolerat spore-forming bacterial populations to coexist with it. Exploring the diversity of these populations may also represent a potential source for the discovery of novel strains and bioactive compounds.

MATERIALS AND METHODS

Sampling Site

The study area was located north of the Rio de Janeiro State in the "Parque Nacional of Restinga de Jurubatiba" (22°17'S and 41°41'W), which occupies 60 km of the Brazilian coastline [3]. The plants were found in the sandy soil parallel to the beach line.

Isolation and Maintenance of Bacterial Strains

The spore-forming bacteria were isolated from the surface and interior of the *B. portulacoides* roots using the method described by Seldin *et al.* [23]. The plants were harvested and the roots shaken to remove the loosely attached soil. One gram of roots together with the adhering soil was then mixed with 9 ml of distilled water and shaken for 10 min. Thereafter, the water was separated in a sterile Erlenmeyer flask and another 9 ml of water added to the roots. This procedure was repeated three times to wash the surface of the roots, and the collected water pasteurized for 10 min at 80°C to represent the plant rhizosphere and root surface. The washed roots were then macerated, mixed with 9 ml of distilled water, and pasteurized to represent the rhizoplane plus root interior. In addition, 1 g of soil (sampled far from the plants) was also treated in the same manner, resulting in three samples named L (washed roots), M (macerated roots), and S (non-root-associated soil). Two-fold serial dilutions of the three samples were then plated onto an LB agar (tryptone 1%, yeast extract 0.5%) supplemented with 10% NaCl and incubated for 3 days at 32°C. Thereafter, the bacterial cultures were stored at −80°C on an LB medium containing 20% glycerol.

Physiological Properties of Bacterial Strains

The isolates were tested for their capability to grow in the presence of different salt concentrations using an LB medium containing 0 to 25% (wt/vol) NaCl. The pH range for growth was determined by adjusting the final pH of the medium LB with 10% NaCl to values between 5.5 and 10 with either HCl or NaOH. Growth in an anaerobic atmosphere was observed after incubating the LB plates for 3 days in Gaspak jars with an atmosphere of 80% N_2, 10% CO_2, and 10% H_2. The biochemical tests (casein and starch hydrolyses, nitrate reductase, catalase, and Voges-Proskauer) were performed as described previously [9], always with the addition of 10% NaCl.

Preparation of Genomic DNA

Total DNA was extracted from all the isolates using a Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, U.S.A.). The DNA concentrations were determined spectrophotometrically using a GeneQuant apparatus (Amersham Pharmacia Biotech, New Jersey, U.S.A.). Agarose gel electrophoresis of the total DNA was performed in 0.8% agarose gels in a Tris-Borate-EDTA (TBE) buffer [21] at 70 V for 4 h at room temperature.

Amplified Ribosomal DNA (rDNA) Restriction Analysis (ARDRA)

To amplify the 16S rDNA fragments, the procedure described by Massol-Deya *et al.* [17] was employed for the PCR amplification of the 16S rRNA gene fragments using a pair of universal primers (pA and pH). The amplification conditions were 35 cycles at 92°C (1 min 10 s), 48°C (30 s), and 72°C (2 min 10 s). A hot start (2 min 10 s at 92°C) was applied to avoid any initial mispriming and enhance the specificity. A final extension step was run for 6 min 10 s at 72°C, and then the reaction tubes were cooled to 4°C. Negative controls (without DNA) were also run for all the amplifications, then the PCR products were visualized by 0.8% agarose gel electrophoresis in TBE at 80 V for 4 h at room temperature and stained with ethidium bromide. Next, samples (10 ml) of the 16S rRNA amplified products were digested with the endonucleases RsaI, MspI, TaqI, HinfI, and HaeIII (Invitrogen), for 16 h, according to the manufacturer's specifications. Agarose (2%) gel electrophoresis of the restricted DNA was then performed at 80 V for 4 h at room temperature.

The results of the ARDRA were collected into a matrix indicating the presence or absence (scored as 1 or 0, respectively) of specific bands in the different restriction analyses. The simple matrixes were obtained by comparing pairs of strains using a simple matching (SM) coefficient,

while the final dendrogram was constructed using an unweighted pair group method based on the arithmetic mean (UPGMA). For these analyses, the NTSYS software package (version 2.02, Exeter Software, Setauket, New York, U.S.A.) was used.

Cloning and Sequencing of PCR Products

The PCR products of the 16S rDNA from the different ARDRA groups were cloned using a pGEM T-easy vector according to the instructions of the manufacturer (Promega, Wisconsin, U.S.A.). After the transformation into *E. coli* JM109 competent cells, certain clones were selected and sequenced using an ABI Prism 3100 automatic sequencer.

Sequence Alignment and Phylogenic Analysis

The 16S rDNA sequences of various halophilic/halotolerant Gram-positive bacteria were recovered from the GenBank database and aligned using the software Clustal-X [27]. Then, the 16S rDNA sequences obtained in this study from 10 isolates (M9, L4, L6, L9, L15, L18, S1, S4, S14, and S18) were aligned in the same way. Phylogenetic trees were constructed using the Neighbor-Joining (NJ) method, whereas the software MEGA 2.1 [14] was used to calculate the pair-to-pair p-distance values among the different species studied here for partial 16S rRNA gene sequences.

AP-PCR

Amplification reactions with an arbitrary primer (AP-PCR) were performed in a mix containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM $MgCl_2$, 250 µM of each deoxynucleoside triphosphate (dNTP), 0.01% of BSA, 0.7 µM of the primer OPAA-18 ($^{5'}$TGGTCCAGCC$^{3'}$) from Operon Technologies, Inc., 50 ng of the target DNA, and 1.25 U of *Taq* polymerase in a final volume of 25 µl. The cycle applied was 45× (1 min 94°C, 1 min 36°C, 2 min 72°C); 4°C. Image Quant (ver. 5.2) was used to analyze the AP-PCR profiles and bands were considered when the peak height relative to the total peak height exceeded 5% according to Iwamoto *et al.* [13]. The calculation of similarities was based on the Pearson (product-moment) correlation coefficient and resulted in a distance matrix. The clustering algorithm of Ward was then used to calculate the dendrogram using the software package Statistica (ver. 5.1, StatSoft).

RESULTS AND DISCUSSION

Thirty-eight isolates were obtained from the LB plates containing 10% NaCl previously inoculated with pasteurized samples of the *B. portulacoides* washed roots (n=16), macerated roots (n=9), and non-root-associated soil (n=13). The DNA from these 38 strains, as shown in Table 1, was amplified using universal primers (pA and pH) to obtain the 16S RNA gene sequences [17]. As expected, all strains produced a single band of about 1,500 bp and the PCR products were digested with RsaI, MspI, TaqI, HinfI, and HaeIII. Amplified rDNA restriction analyses have been shown to be useful tools for screening environmental bacterial isolates and/or clone libraries and assisting in distinguishing between taxonomic groups [19, 24]. When the enzymes TaqI and HaeIII were used, only one pattern was observed in all the strains tested. For the enzymes HinfI, MspI, and RsaI, two, two, and three restriction patterns were observed, respectively (Table 1 and Fig. 1). A similarity analysis was then performed based on UPGMA using 20 markers, which corresponded to all the different bands observed after digestion with the three endonucleases. Three different 16S rRNA gene-based genotypes were observed with a 100% similarity (Fig. 1). Group A was separated from groups B and C at a similarity of about 55%, where group A was composed of strains isolated from the washed roots and rhizosphere. Meanwhile, the strains in group B were only separated from the strains in group C at a similarity of 80%, where both groups were composed of representatives isolated from the root and

Table 1. Phenotypic and genetic characteristics of strains isolated in present study.

Isolates[a] (n=38)	Phenotypic characteristics[b]						16S rRNA gene[c]	
	Starch	Casein	Nitrate	pH 5.7	0% NaCl	20% NaCl	Pattern	Genotype
L6, L7	+	-	-	-	-	-	ace	A
M9, L1, L2, L17	+	+	-	-	+	+	ace	A
L3, L9, L13, L15, M20, M21, S5, S10, S11, S13	-	-	+	+	-	+	adg	B
L4, L5, L11, L12, L16, L18, L20, M6, M11, M13, M14, M17, M18, S1, S4, S6, S9, S14, S15, S18, S19, S20	-	+	-	+	-	+	bdf	C

[a]L, washed roots; M, macerated roots; S, soil.

[b]Starch hydrolysis; casein hydrolysis; nitrate reduction; pH5.7, growth on LB adjusted to pH5.7; 0%, growth on LB broth without the addition of NaCl; 20% growth in LB broth with the addition of NaCl to reach 20% salt.

[c]The letters represent arbitrarily the different patterns obtained from the digestion of the PCR products (16S rRNA gene) with the endonucleases HinfI (a and b), MspI (c and d), and RsaI (e, f, and g), respectively. The different pattern combinations (letters) led to the formation of the genotypes.

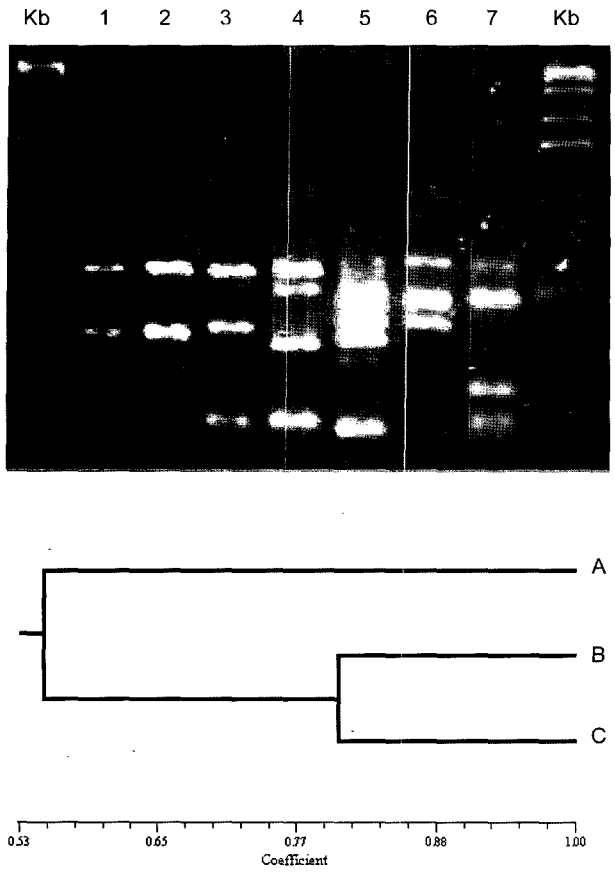

Fig. 1. Different patterns obtained by digestion of PCR products of the 16S rRNA gene (ARDRA) of strains studied (Table 1) using three restriction enzymes: Kb (1 kb ladder, Invitrogen); HinfI digestion, corresponding to patterns a (lane 1) and b (lane 2), MspI digestion, corresponding to patterns d (lane 3) and c (lane 4), and RsaI digestion, corresponding to patterns e, f, and g (lanes 5, 6, and 7, respectively). The dendrogram (UPGMA) was constructed using all the different bands obtained in the ARDRA profiles. The 16S rRNA gene-based genotypes (A, B, and C) are defined in Table 1.

non-root-associated soil. Therefore, no correlation between the origin of the isolates (washed roots, macerated roots, or soil) and the ARDRA groups B and C could be established, and the highest number of strains was in group C (57.9% of the isolates, Table 1). Furthermore, the formation of only three ARDRA groups indicated a relatively low diversity of the halotolerant or halophilic spore-forming bacterial species associated with *B. portulacoides*. One explanation for this observation could be the habitat where *B. potulacoides* is usually found in Brazil. The coastal dunes are submitted to frequent storm tides that result in constantly changing levels of salinity [4]. Therefore, the bacterial populations in these habitats have to adapt to a wide range of salt concentrations.

The 38 strains were further characterized based on their colonial morphology, physiology, and nutritional requirements. With only a few exceptions, the results obtained also followed the ARDRA groupings. The colonies from the genotype A strains grown on LB supplemented with 10% salt were pigmented (orange), circular, opaque, and had entire margins. Growth was observed between pH 7 and 9.5, and no growth was observed at pH 5.7. The strains were all strictly aerobic, catalase positive, and Voges-Proskauer (VP) test negative as regards the reduction of nitrate to nitrite (Table 1). Strains L1, L2, L17, and M9 were able to hydrolyze both starch and casein, whereas strains L6 and L7 were only positive for starch degradation. Growth on the LB medium with 0 and 20% NaCl was observed when strains L1, L2, L17, and M9 were tested. Strains L6 and L7 were also able to grow on the LB medium containing 0.5 to 15% of NaCl. Meanwhile, the colonies from the genotype B strains were cream-colored and spread out with entire margins. The strains were all catalase positive, strictly aerobic, VP test negative, and nitrate positive. None of the strains was able to hydrolyze either starch or casein, yet all the strains tested were able to grow on the LB medium containing 0.5 to 20% NaCl, although not without the addition of salt. Growth was also observed within a wide pH range (5.7 to 9.5). Finally, the colonies from the genotype C strains were circular, creamy white, and slightly transparent. The isolates were all strictly aerobic, catalase positive, nitrate negative, VP negative, casein positive, and starch hydrolysis negative. The strains were able to grow on the LB medium containing 0.5 to 20% NaCl and at a pH varying from 5.7 to 9.5. It is also worth noting that all the strains studied here were shown to be facultatively alkaliphlilic and exhibited halotolerance, confirming the need for bacteria associated with *B. portulacoides* to be adaptable to a wide range of salt concentrations.

To help the phylogenetic placement of the strains described in this study, at least two representative isolates from each ARDRA group were investigated based on partial (500 bp) 16S rDNA sequencing. Goto *et al.* [10] have previously demonstrated that sequencing the first 400 nt of the 16S rRNA gene is sufficient for species differentiation among Gram-positive spore-forming bacteria. In the present analyses, it was found that strains L6 and M9 (ARDRA group A) were positioned within the genus *Halobacillus* (Fig. 2) with similarity values varying from 95.2 to 97.7% for strain M9 and from 95.4 to 98.5% for strain L6 when compared with the different species of *Halobacillus* (Table 2). Therefore, these two strains were considered as well characterized at the genus level. According to Stackebrandt and Goebel [26], two strains that show 16S rDNA sequence homologies of 97% or higher belong to the same species. In the present case, neither strain could be attributed to a particular species, since their sequences were related to those of two or more species with similar sequence homologies (Table 2). The closest species to L6 were *H. trueperi*, *H. karajaensis*, and

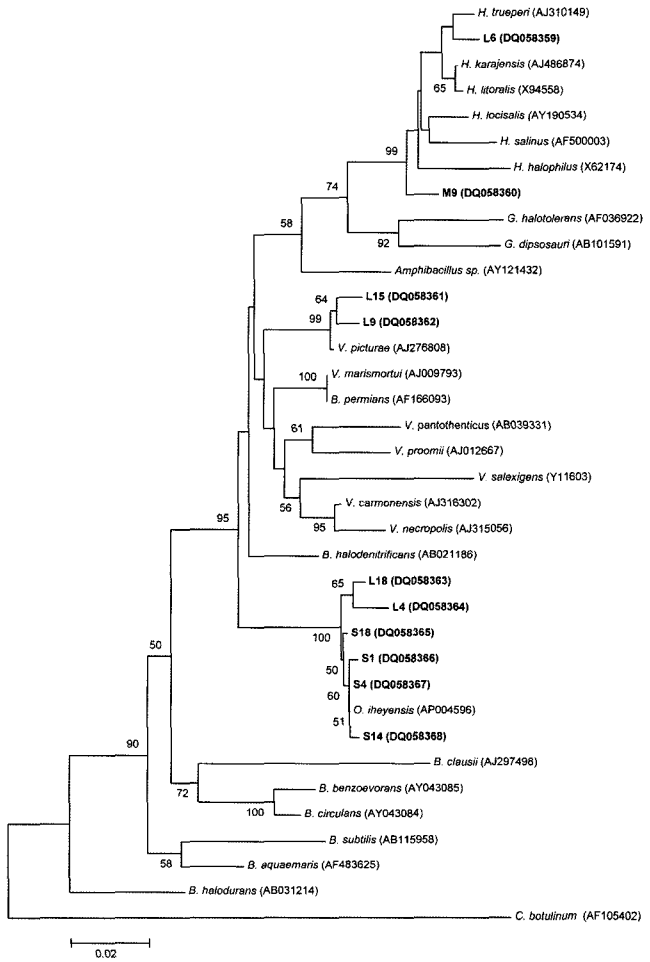

Fig. 2. Phylogenetic trees based on 16S rRNA gene sequences showing relationships of 10 representative isolates and different halotolerant/halophilic spore-forming bacteria. The tree was constructed using the neighbor-joining method and bootstrap results (1,000 replications) greater than 50% are represented at the branch points.

H. litoralis (Fig. 2 and Table 2), yet this strain exhibited different phenotypic characteristics to those described for the type strains of these species. For instance, in contrast to strain L6, both *H. litoralis* SL-4^T and *H. trueperi* SL-5^T are unable to hydrolyze starch [25], whereas colonies of *H. karajaensis* MA-2^T are white or cream colored [1], and strain L6 produced orange-pigmented colonies. Thus, when considering the phylogenetic and phenotypic characteristics of isolate M9, it was concluded that it belonged to the genus *Halobacillus*, although it showed differences when compared with other known species of this genus [1, 25, 31, 32]. Therefore, evidence was presented that the isolates from ARDRA group A may belong to a new species. However, additional phenotypic characterization and DNA-DNA hybridization experiments are still necessary to infer the correct taxonomic position.

Strains L9 and L15 (ARDRA group B) were positioned close to *Virgibacillus picturae* (Fig. 2) with a 96.9% and 97.5% similarity, respectively, to this species (Table 2). Strains of *V. picturae* were first isolated from deteriorated paintings [12] and the present strains shared many of the phenotypic characteristics presented by *V. picturae*, although the habitat they were isolated from was quite different. Therefore, it would appear that the strains of the species *V. picturae*, which means pertaining or belonging to a painting, are also ubiquitous in the environment and found to be associated with *B. portulacoides*.

Strains L4, L18, S1, S4, S14, and S18 (ARDRA group C) were all positioned close to *Oceanobacillus iheyensis* (Fig. 2), with similarity values varying from 95.3 to 99.4% (Table 2). This deep-sea species is described as extremely halotolerant and alkaliphilic, and was originally isolated from the Iheya Ridge at a depth of 1,050 m [16]. The isolates from *B. portulacoides* shared most of the phenotypic characteristics described for *O. iheyensis*, although growth at high pressures was not determined for the present isolates. *O. iheyensis* strain HTE831 grows at pressures of up to 30 Mpa, corresponding to the pressure at a depth of 3,000 m. Since it is believed that the present strains could belong to this species, the fact that they were isolated from the soil (10 cm upper layer), rhizosphere, and rhizoplane must be taken into account when attempting to establish a relationship between the species and the possible pressure at which it can grow.

Considering that only one strain of *O. iheyensis* has been described so far [16], and that the majority of the strains isolated in this study seemed to belong to this species, it was decided to genetically compare the present isolates using an AP-PCR to determine whether they had a clonal origin or not. Different patterns were obtained with primer OPA-A18 (data not shown) and similar fingerprints enabled the grouping of the strains according to Pearson's coefficient. The resulting dendrogram is shown in Fig. 3. Two main clusters, denoted as A and B, were observed. All the strains isolated from the soil were grouped into cluster A together with six strains isolated from the washed or macerated roots, whereas cluster B was exclusively made up of strains associated to the *B. portulacoides* roots. Although the dendrogram did not show the phylogenetic relationships, it did define the population considered to be genetically closely related, being originated from a common ancestor or co-isolate. As a result, it was concluded that the isolates obtained in this study were not co-isolates, with the exception of strains S6–S9 and S15–S19 that presented a 100% similarity. Therefore, it is possible that the species *O. iheyensis* is constituted by more than one strain.

On the basis of the results of this investigation, different halotolerant spore-forming bacteria isolated from *B. portulacoides* usually found on Southwestern Atlantic Ocean beaches became available for further study. As such,

Table 2. 16S rRNA similarities based on comparison of 500 nucleotide bases of new isolates and eight reference strains of halotolerant/halophilic Gram-positive species (access number shown in Fig. 2).

	% Similarity of partial (500 bp) 16S rDNA sequences																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. *H. trueperi*	100																	
2. *H. locisalis*	97.5	100																
3. *H. salinus*	96.4	96.8	100															
4. *H. karajensis*	99.1	97.3	96.4	100														
5. *H. halophilus*	95.7	95.0	95.0	96.1	100													
6. *H. litoralis*	99.3	97.1	96.2	99.8	95.9	100												
7. M9	97.7	97.7	96.4	97.4	95.2	97.2	100											
8. L6	98.5	97.1	95.8	98.3	95.4	98.2	98.3	100										
9. *V. picturae*	92.9	92.7	92.4	92.9	91.7	92.7	93.1	92.3	100									
10. L9	90.7	90.8	90.0	90.7	89.9	90.7	91.4	90.3	96.9	100								
11. L15	90.7	90.8	90.0	90.7	90.1	90.7	91.4	90.3	97.5	98.5	100							
12. O. *iheyensis*	87.6	88.4	88.5	88.5	88.0	88.6	88.6	87.6	92.7	89.9	90.3	100						
13. S1	88.2	88.5	88.7	88.9	88.3	89.1	88.7	87.8	92.9	90.5	90.9	98.7	100					
14. S14	88.2	88.8	88.8	89.2	88.8	89.2	89.0	88.1	93.1	90.8	91.2	98.7	99.4	100				
15. S18	88.3	89.1	88.9	89.3	89.1	89.3	89.3	88.2	93.1	91.1	91.4	98.2	99.1	99.4	100			
16. S4	87.6	88.2	88.4	88.5	87.9	88.5	88.5	87.6	92.5	90.1	90.5	99.4	99.3	99.3	98.7	100		
17. L4	85.9	86.6	86.4	86.8	86.6	86.9	86.9	85.7	90.3	91.9	91.5	95.3	95.7	96.4	96.6	95.3	100	
18. L18	87.2	87.8	88.0	88.1	88.6	88.2	88.2	87.2	91.4	91.3	91.0	98.1	98.3	98.3	97.8	98.7	96.8	100

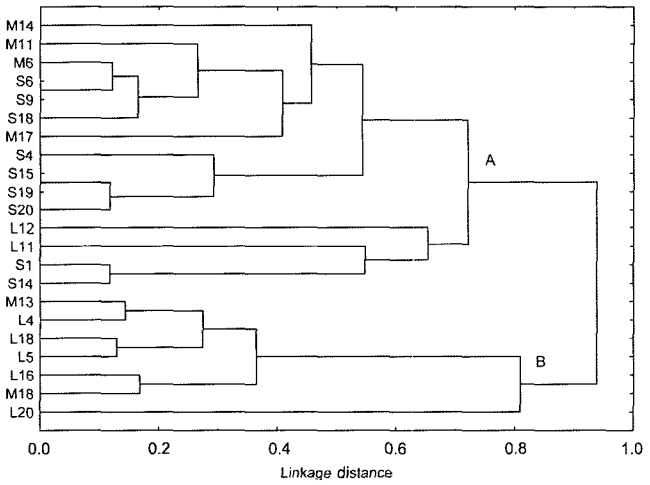

Fig. 3. Dendrogram obtained after cluster analysis using Pearson's correlation coefficient comparing different AP-PCR fingerprintings (primer OPA-A18) of strains identified as *O. iheyensis*.

this would appear to be the first report on the diversity of halotolerant spore-forming bacteria associated with plants, especially in a tropical environment. Therefore, the strains presented here may offer a potential source for the discovery of bioactive compounds with industrial value.

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