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Isolation and Characterization of Moderately Halophilic Bacteria from Tunisian Solar Saltern

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Abstract Bacterial screenings from solar saltern in Sfax (Tunisia) lead to the isolation of 40 moderately halophilic bacteria which were able to grow optimally in media with 5–15% of salt. These isolates were phylogenetically characterized using 16S rRNA gene sequencing. Two groups were identified including 36 strains of *Gamma-Proteobacteria* (90%) and 4 strains of *Firmicutes* (10%). The *Gamma-Proteobacteria* group consisted of several subgroups of the *Halomonadaceae* (52.5%), the *Vibrionaceae* (15%), the *Alteromonadaceae* (10%), the *Idiomarinaceae* (7.5%), and the *Alcanivoracaceae* (5%). Moreover, three novel species: 183ZD08, 191ZA02, and 191ZA09 were found, show <97% sequence similarity of the 16S rRNA sequences while compared to previously published cultivated species. Most of these strains (70%) were able to produce hydrolases: amylases, proteases, phosphatases, and DNAases. Over the isolates, 60% produced phosphatases, 15.0% proteases, 12.5% amylases and DNAases equally. This study showed that the solar saltern of Sfax is an optimal environment for halophilic bacterial growth, where diverse viable bacterial communities are available and may have many industrial applications.

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Introduction

Hypersaline environments are inhabited by abundant microbial communities adapted to these specific ecosystems. In such environments, the common phenomenon is the occurrence of salinity gradient, owing to the evaporation of seawater. This process leads to the selection of microbial species variety adapted to the different salinity ranges [5]. At the solar saltern, the salinity gradient increase has a noticeable effect on microbial population evolution and structuration, organized according to the progressive salt concentration. Among this biota, *Bacteria* and *Archaea* may be the dominant microorganisms, especially in saline and hypersaline environments [19]. Moderately halophilic bacteria are one of the most important bacterial groups adapted to the hypersaline environments. These may use adaptive strategies to achieve a high osmotic pressure in the cytoplasm by accumulation of organic solutes to establish osmotic balance [12]. They are attracting interest because this bacterial group has great biotechnological potential with compatible solutes or hydrolytic enzymes production [11, 14].

The multi-pond solar saltern of Sfax (Tunisia) includes a range of environments with different salinities, starting with that of seawater up to that of sodium chloride saturation ponds. In each evaporation pond, a very stable environment is established where extreme environment stresses exert pressure selection of microbial communities which include novel taxa representing remarkable survivability.

The objective of this study was to isolate and to investigate some moderately halophilic strains found in a Mediterranean solar saltern located in Sfax (Tunisia) for their phenotypic characteristics, phylogenetic affiliation, and their enzymatic activities. A comparative analysis of these isolates to previous studies in other solar saltern was undertaken.

Materials and Methods

Sample Collection

Water samples from 17 ponds of the solar saltern of Sfax (Tunisia), having different salinities (3.7–31%), were collected during September 2005.

Isolation of Halophilic Bacteria

Enrichments and isolation of halotolerant to moderately halophilic bacteria were performed in two media. The first medium was prepared using the brine sampled from the solar saltern enriched with 5 g l^{-1} yeast extract (Difco) and 10 g l^{-1} tryptone (Difco). For each pond, a specific media was prepared for bacterial isolation according to pond's intrinsic salinity. The second medium was synthetic, including (per liter): NaCl, 98 g; KCl, 2 g; $MgSO_4{\cdot}7H_2O$, 1 g; $CaCl_2{\cdot}2H_2O$, 0.36 g; $NaHCO_3$, 0.06 g; NaBr, 0.24 g; $FeCl_3{\cdot}6H_2O$, 1 g; bactotryptone (Difco), 10 g; glucose, 1 g, and agar, 20 g [4]. The pH was adjusted to 7.4. The pH of the medium was adjusted to 7.2. For all the media used, the plates were incubated at 37°C aerobically in a salt saturated atmosphere. Colonies growing on the plates were selected, based on morphological features, considering pigmentation and size. Each isolate was subjected to successive streak plating to ensure clone purity. The isolates were preserved in 50% glycerol at −80°C.

Bacterial DNA Preparation and 16S rRNA Amplification

Bacterial suspension from each isolate was prepared in 30 µl of sterile distilled water and heated at 94°C for 10 min to release the DNA. Cell debris was eliminated by centrifugation at 12,000×*g* for 15 min. The crude sample was used as template in a PCR amplification of 16S rRNA. Partial 16S rRNA sequences were amplified by PCR using TaKaRa Ex Taq™ (2.5 units, Promega) in 100 µl reaction buffer, containing 2.5 mM of each dNTP (dATP, dTTP, dGTP, dCTP), 0.5 mM of each primer, and 10 µl of 10× Ex *Taq* buffer™. The primers used were bacterial-specific forward 008F [7], combined with the universal reverse primer 1390R [20]. The PCR amplification was carried out according to the following program: initial denaturation at 94°C for 5 min and 24 cycles consisting of denaturation at 94°C for 1 min, primer annealing at 59°C for 1 min, and extension at 72°C for 1.5 min. The final elongation step was extended to 15 min. Under these conditions, a single PCR product of 1.4 kb was analyzed on 1% agarose gel stained with ethidium bromide (0.5 µg ml^{-1}) and visualized under ultra violet trans illumination.

Sequencing and Phylogenetic Analysis of 16S rRNA Sequences

PCR products of all colonies were sequenced using automated sequencer as described by Artiguenave et al. [1]. The 16S rRNA gene sequences were compared to those of the GenBank and EMBL databases by advanced BLAST searches from the National Center for Biotechnology Information. Sequences were analyzed by using the ARB software package (http://www.arb-home.de, 2005 version).

Nucleotide Sequence Accession Numbers

Sequences reported in this study have been submitted to EMBL, GenBank databases under Accession Numbers AM945650 to AM945689.

Isolates Hydrolytic Activities

Proteolytic activity of each isolate was screened qualitatively as described in previous study [16]. Starch hydrolysis was tested according to Barrow and Feltham [2]. DNAase activity was revealed as described by Jeffries et al. [9]. Phosphatase activity was revealed according to the methods described by Sangeeta and Chandra [15].

Results

Isolation and 16S rRNA Gene Sequences Identification of the Strains

In order to screen useful microorganisms inhabiting the solar saltern of Sfax, 40 strains were isolated. The majority of isolates grow optimally in media with 5–15% of salt at 37°C and pH 7. The strains were characterized phylogenetically by sequencing PCR-amplified 16S rRNA gene of all isolates. The sequenced genes were compared to those deposited in public database using BLAST program. Phylogenetic analysis using 16S rRNA revealed that the 40 strains could be further affiliated with two major groups, 36 strains of *Gamma-Proteobacteria* (90%) and 4 strains of

Firmicutes (10%). The phylogenetic relationships between the isolated 16S rRNA gene sequences compared to those of representative species were illustrated in Fig. 1a and b.

The *Gamma-Proteobacteria* group consisted of several subgroups. The group of the *Halomonadaceae* representing 52.5% of the strains included three genera. The first genus

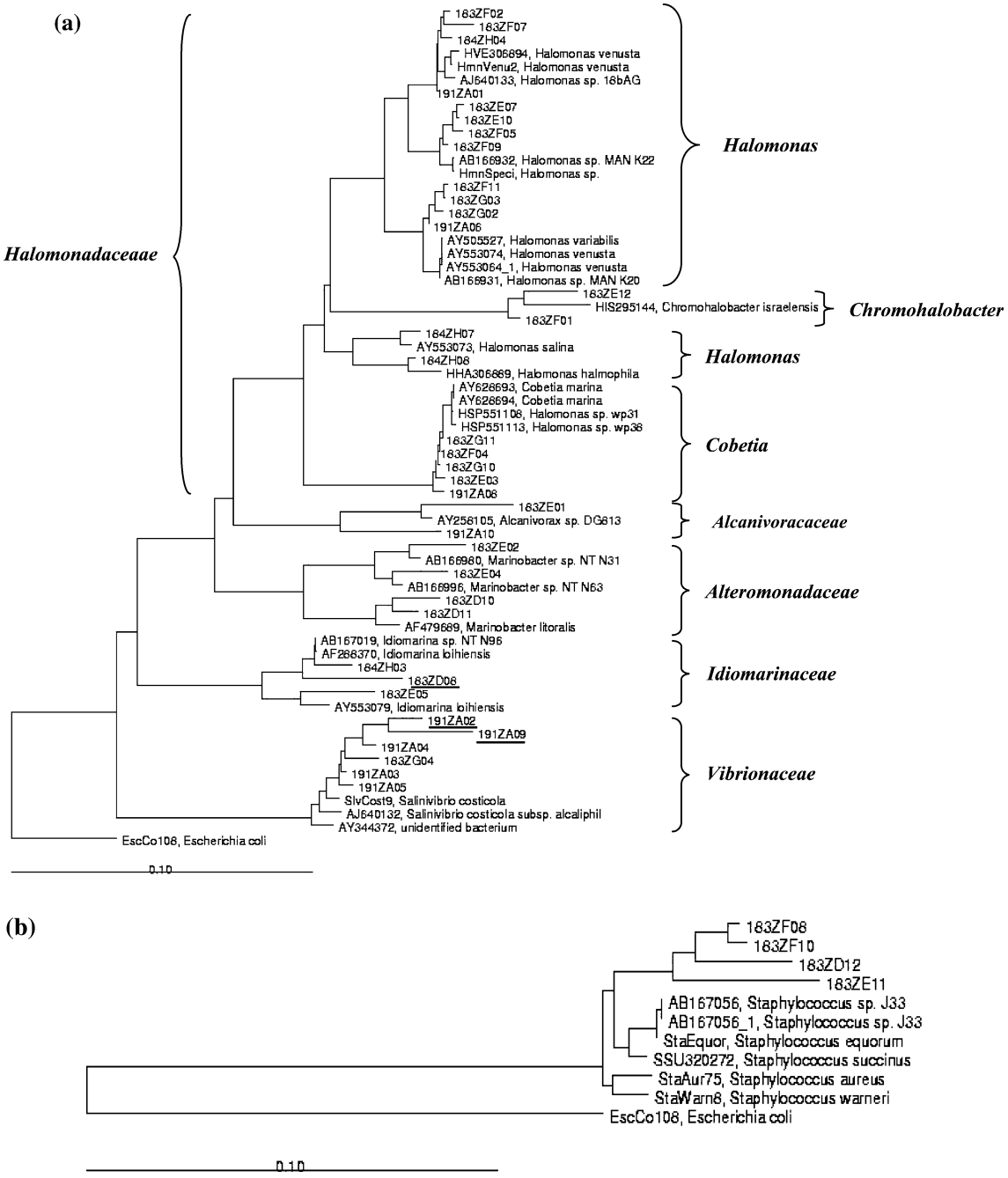

Fig. 1 **a** Phylogenetic relationships between the 16S rRNA sequences of the isolates affiliated with *Proteobacteria* group and other related bacterial sequences previously published in the databases. The phylogenetic tree was built by Neighbor-joining method using the ARB software package. The *underlined* sequences represent the sequence of novel isolates. The scale bar corresponds to a 10% estimated difference in nucleotide sequence positions. *Escherichia coli* was used as an outgroup. **b** Phylogenetic relationships between the 16S rRNA sequences of the isolates affiliated with *Firmicutes* group and other related bacterial sequences previously published in the databases. The phylogenetic tree was built by Neighbor-joining method using the ARB software package. The scale bar corresponds to a 10% estimated difference in nucleotide sequence positions. *Escherichia coli* was used as an outgroup

was *Halomonas*, the most abundant, representing 35% of the isolates and exhibiting 99–99.84% of similarity to published species. The second genus was *Cobetia*, representing 12.5% of the strains and showing 99.61–99.92% of similarity. The third genus was *Chromohalobacter*, representing only 5% of the strains. Among the *Gamma-Proteobacteria* group, the *Vibrionaceae* represented 15% of the strains which exhibited 95.93–98.69% of homology with *Salinivibrio costicola*, a moderately halophilic bacterium, previously isolated from salted foods [8].

The group of the *Alteromonadaceae* was represented by 10% of the strains affiliated to the genus *Marinobacter,* exhibiting 97.25–99.84% of similarity. Finally the groups of the *Idiomarinaceae* and the *Alcanivoracaceae* were represented by 7.5 and 5% of the strains affiliated, respectively, to the genus *Idiomarina* and *Alcanivorax*.

The *Firmicutes* group related to the *Bacillaceae* family was represented by only four strains exhibited 99.67–99.85% of sequence identity with the genus *Staphylococcus*. Similar results were recorded by Ghozlan et al. [6] in their study in hypersaline habitats in Alexandria, Egypt. Among the isolates, 36 strains showed ≥97% similarity of the 16S rRNA sequences while compared to previously published cultivated species, and three strains exhibited a percentage of 16S rRNA sequences similarity <97% with a database sequence and would represent novel species. These belonged to the *Idiomarinaceae* and *Vibrionaceae* families.

Hydrolytic Activities

The 40 isolated bacteria were screened for protease, amylase, DNAase, and phosphatase activities (Table 1). Phosphatase was produced by 60% of these strains, and *Halomonas* species was the most efficient producer, while 15% produced proteases. Only 12.5% produced DNAases or amylases, respectively. The relatively high rate of strains having phosphatase activities would be related to the abundance of this substrate in the selected ponds. The determination of the total phosphorus content in these ponds showed an increase of phosphorus with salt concentration (the value ranged between 0.20 and 10 mg/l). Other phenomena can explain this fact like chemical speciation and the concentrating effect which resulted from water evaporation in the ponds. Several combined activities were showed by some of these isolates. Twenty percent produced two hydrolytic activities, while 5% of the strains were able to produce three hydrolytic activities. Two hydrolytic enzyme activities were achieved by *Idiomarina loihiensis, Marinobacter* sp. NT, *Salinivibrio costicola* and *Halomonas* (sp. 18BAG, *halmophila*, and sp. K22) and *Cobetia marina*. Three hydrolytic enzyme activities were achieved by *Salinivibrio costicola* and *Halomonas variabilis*. Most of the isolates producing enzymes are affiliated with *Salinivibrio* or *Halomonas*, two genera widely distributed in hypersaline environments [17]. Two novels strains 191ZA09 and 183ZD08 related to *Salinivibrio costicola* and *Idiomarina* sp. NTN96, respectively, produced protease and phosphatase activities. No study of phosphatase from *Salinivibrio* has been carried out previously.

Table 1 Hydrolytic activities of the different isolates

Strains	Hydrolytic activities				Total of activities
	Protease	Amylase	Phosphatase	DNAase	
183ZD08	−	−	+	−	1
184ZH03	−	−	+	−	1
183ZE05	−	+	+	−	2
183ZD10	−	−	+	−	1
183ZD11	−	−	−	−	0
183ZE02	−	−	+	−	1
183ZE04	−	+	+	−	2
191ZA10	−	−	+	−	1
183ZE01	−	−	−	−	0
191ZA09	+	−	+	−	2
191ZA02	−	−	+	−	1
191ZA03	+	−	+	+	3
191ZA04	−	−	−	−	0
191ZA05	−	−	−	−	0
183ZG04	−	−	+	−	1
183ZF02	+	−	−	−	1
183ZF07	−	−	+	−	1
184ZH04	−	−	+	−	1
191ZA01	−	+	+	−	2
183ZF11	+	−	+	+	3
191ZA06	−	−	+	−	1
183ZG02	−	−	+	−	1
183ZG03	−	−	−	−	0
183ZE07	−	−	+	−	1
183ZE10	+	−	+	−	2
183ZF05	−	−	+	−	1
183ZF09	−	+	−	+	2
184ZH07	−	−	+	−	1
184ZH08	+	−	+	−	2
183ZE03	−	−	+	−	1
183ZF04	−	−	−	−	0
191ZA08	−	−	−	−	0
183ZG10	−	−	+	+	2
183ZG11	−	−	−	−	0
183ZE12	−	−	−	−	0
183ZF01	−	+	−	−	1
183ZD12	−	−	−	−	0
183ZF08	−	−	−	−	0
183ZE11	−	−	−	+	1
183ZF10	−	−	−	−	0

Discussion

Several studies have been conducted on the ecology, taxonomy, and phylogeny of moderately halophilic bacteria as well as their biotechnological applications [10, 13]. Among the isolated stains collection, the *Gamma-Proteobacteria* were dominant including organisms of various members of the genus *Halomonas*. These seemed to be more dominant in saline environments than the *Firmicutes*. The abundance of Gram-negative bacteria could be explained by the difference in the cell wall composition which protects the cell membrane by its rigidity from osmotic or mechanical rupture [3]. However, the *Alpha-Proteobacteria* and *Bacteroidetes* groups, which were repeatedly found in thalassohaline environments, were not isolated in this study.

The isolates obtained from a coastal saltern south of Alicante (Spain) were generally halotolerant and the predominant genera detected were: *Vibrio*, *Flavobacterium*, *Alcaligenes*, *Alteromonas*, and *Chromobacterium* [16]. In a study of an other Spanish saltern ponds of intermediate salinity (between 15 and 30% sea salts), the dominant types of isolate developing on agar plates were assigned by numerical taxonomy to the genera of *Salinivibrio*, *Acinetobacter*, *Flavobacterium*, and the *Pseudomonas–Alteromonas–Alcaligenes* group [13]. Recently, in Alexandria (Egypt), taxonomic analyses of moderately halophilic bacteria from hypersalines habitats showed that the Gram-negative bacteria represent 84.5% of the total isolates. They are affiliated with five genera including *Pseudoalteromonas*, *Flavobacterium*, *Chromohalobacter*, *Halomonas*, and *Salegentibacter*. The Gram-positive bacteria (15.5% of the total) are represented by the genera *Halobacillus*, *Salinicoccus*, *Staphylococcus*, and *Tetragenococcus* [6]. In Taean-Gun (Korea), similar study on moderately halophilic bacteria from solar salterns ponds indicated that the isolates collection was dominated by Gram-negative bacteria (70.3% of the total) affiliated to several subgroups of the *Gamma-proteobacteria*: *Vibrionaceae* (37.5%), *Pseudoalteromonadaceae* (10.9%), *Halomonadaceae* (7.8%), *Alteromonadaceae* (7.8%), and *Idiomarinaceae* (6.3%). The Gram-positive bacteria (29.7% of the total) are represented by *Bacillus*, *Halobacillus*, *Jeotgalibacillus*, and *Pontibacillus* genera [18].

This study proved that the solar saltern of Sfax (Tunisia) supported the growth of a diversity of moderately halophilic bacteria exhibiting various enzymatic activities (protease, amylase, DNAase, and phosphatase) which could be used for many industrial applications.

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