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Prokaryotic biodiversity of halophilic microorganisms isolated from Sehline Sebkha Salt Lake (Tunisia)

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North of Tunisia consists of numerous ecosystems including extreme hypersaline environments in which the microbial diversity has been poorly studied. The Sehline Sebkha is an important source of salt for food. Due to its economical importance with regards to its salt value, a microbial survey has been conducted. The purpose of this research was to examine the phenotypic features as well as the physiological and biochemical characteristics of the microbial diversity of this extreme ecosystem, with the aim of screening for metabolites of industrial interest. Four samples were obtained from 4 saline sites for physico-chemical and microbiological analyses. All samples studied were hypersaline (NaCl concentration ranging from 150 to 260 g/L). A specific halophilic microbial community was recovered from each site and initial characterization of isolated microorganisms was performed by using both phenotypic and phylogenetic approaches. The 16S rRNA genes from 77 bacterial strains and two archaeal strains were isolated and phylogenetically analyzed and belonged to two phyla Firmicutes and gamma-proteobacteria of the domain *Bacteria.* The results show that the Sehline Lake harbored novel prokaryotic diversity, never reported before for solar salterns. In addition, diversity measurement indicated an increase of bacterial diversity with rising salinity gradient, which is probably due to competition between bacteria and others species.

Key words: Sebkha, bacteria, extremophiles, biodiversity, screening.

INTRODUCTION

Previous studies on the microbiology of hypersaline environments showed that halophilic members of the domain Archaea were dominant, whereas those of the domain Bacteria represented limited components (Litchfield and Gillevet, 2002; Ochsenreiter et al., 2002; Baati et al., 2008; Hedi et al., 2009). Nevertheless, other studies have demonstrated that members of the bacteria domain play an important role in hypersaline environments (Antoin et al., 2000). Such extremophiles were described by their ability to produce compounds of industrial interest and biotechnological products (biopolymers, exopolysaccharides, hydrolases, amylases, cellulases, proteases and lipases). In addition, halophilic organisms play important roles in fermenting fish sauces and in transforming and degrading waste and organic pollutants (Grant et al., 1998; Boone and Garrity, 2001). Although molecular diversity studies have been carried out in other hypersaline environments, this work represents the first one where such a

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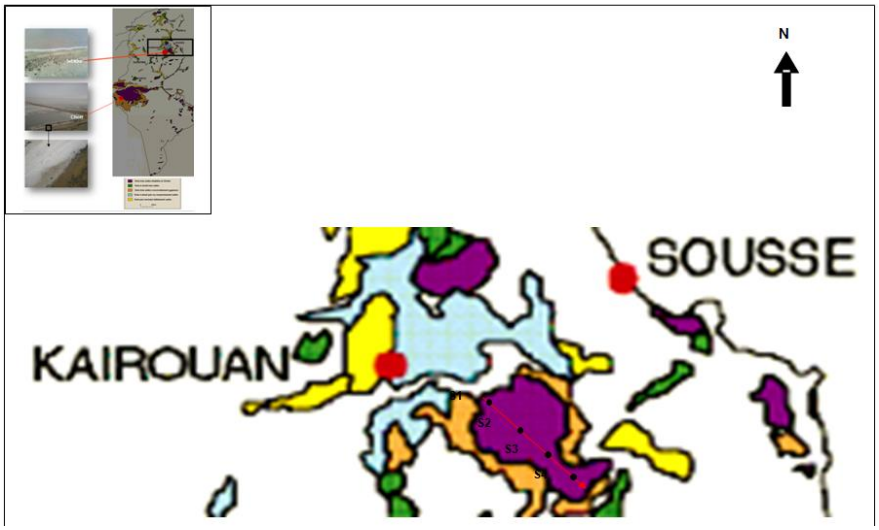

Figure 1. Location map of the Sebkha Sehline Lake (Tunisia) and sampling points.

study was performed on the anoxic sediment underlying microbial mats in Mediterranean salterns. North of Tunisia consists of numerous ecosystems including extreme (e.g. hypersaline) environments (Monastir, Tunisia) in which the microbial diversity has been poorly studied. The largest saline lake named Sehline Sebkha, located in North of Tunisia covers nearly 16 km^2.

Sehline Sebkha salt lake is a hypersaline environment in the north east part of Tunisia which is considered as a thalassohaline habitat, putting it in the same category as the Great Salt Lake or solar salterns (Rodriguez-Valera et al., 1988). Physico-chemical conditions of the Sebkha revealed that this extreme environment showed high salinity, high radiations (U-V) and changes in temperatures and dryness which make it relevant to be studied by microbiologists. The saltern lake is also an important source of salt for food, providing a wide set of ecological niches for halophilic microorganisms.

However, no study with regards to its microbial diversity has been undertaken so far. The purpose of this research was to chemically analyse salt and brine samples collected from the lake, to isolate novel extremely halophilic aerobic or facultative anaerobic microorganisms that develop in it, and to examine their phenotypic features and their physiological and biochemical characteristics with the aim to screen for metabolites of industrial interest produced by the novel halophilic isolates.

MATERIALS AND METHODS

Sample collection

The strains were isolated aseptically from mixed water and sediments of the Sehline Sebkha (Figure 1). Taking into account the *in situ* physico-chemical conditions and level of wastewater pollutants, the Sebkha was divided into four experimental sites. Water and sediment samples were collected at the surface and at various depths (0.1, 0.2 and 0.3 m), within each site.

Physico-chemical analysis of the samples

The physico-chemical analysis of water and soil samples from the hypersaline Sehline Sebkha (Tunisia), performed by standard methods (Trussel et al., 1989); are reported in Table 1. Cl^- was quantified by titration with $AgNO_3$, Mg^{2+} was quantified by atomic absorption spectrophotometry, Na^+ was quantified by flame spectrophotometry, and Ca^{2+} was quantified by complexometry using EDTA. Temperature and pH were measured *in situ*.

Enrichment and isolation

Considering the importance of salinity within the Sebkha, we focused our isolation procedures particularly on extreme halophilic archaea and bacterial microbiota.

Enrichment cultures and isolation procedures to recover aerobic or facultative anaerobic extremely halophilic microorganisms were performed in medium containing (per liter): NaCl, 250 g; $MgCl_2.6H_2O$, 13 g; $MgSO_4.7H_2O$, 20 g; KCl, 4 g; $CaCl_2.2H_2O$, 1 g; NaBr, 0.5 g; $NaHCO_3$, 0.2 g; yeast extract, 5 g; tryptone, 8 g; and glucose, 1 g. This medium was selected because it contains the majority of components which can be utilized by halophilic microorganisms. The pH was adjusted to 7.2 and 8.2 with 10 M NaOH before autoclaving. Enrichment cultures were subcultured several times under the same conditions.

Strains were grown in 100 mL of medium in 250 mL Erlenmeyer flasks in a rotatory shaker under agitation at 150 rpm. The adequate temperature chosen for growth was 37°C (average temperature at the sampling sites for isolation at 12 h in the morning). Aliquots (100 µL) of 10^{-1} - 10^{-4} dilutions were plated onto agar medium.

After two weeks of incubation (at least) at 37°C (plates incubated in a humid steam room adding distilled water to avoid dryness), red, orange-red, pale-pink, yellowish, cream, white, and also transparent colonies were observed. Different colonies were picked and restreaked several times (three times at least) to obtain pure cultures.

Characterization and identification of isolates

The isolates that showed different phenotypic characteristics and phylogenetic signatures (Amplified rDNA Restriction Analysis; ARDRA, 16S rRNA gene sequences), were chosen for further characterization. Isolated strains were examined for colony and cell morphologies and cell motility. Colonial morphologies were described by using standard microbiological criteria such as pigmentation, colonial elevation, consistency and opacity. Gram-staining was carried out with the method described by Dussault (1955). The temperature for cultures growth was 37°C and NaCl concentration growth was limited to 25%. The pH tolerance of each isolate was tested in medium with pH values of 7.2 and 8.2.

Biochemical tests of bacteria

Chitinase, cellulase, xylanase, protease and curdlanase activities were analysed for each representative species of the bacteria using colonies of the strains. For each test, a mixture of 50% agar (4% agar dissolved in sodium acetate 0.1 M, pH 5) and 50% enzymatic substrate such CM-Curdlan-RBB, CM-Cellulose-RBB, CM-Xylan-RBB or CM-Chitine-RBV (LOEWE Biochemica GmBH Laboratory), was prepared in plate. Test protease of bacteria was examined in medium containing 50% nutrient agar and 50% half-skim milk. All the tests were supplemented with 10% NaCl. Microorganisms showing clearing zones after 48 h of incubation at 37°C were considered as enzymatic producers.

PCR amplification of 16S rDNA

The PCR amplification and restriction endonuclease digestions were performed as previously described (Hedi et al., 2009). The DNA from bacterial cultures was extracted using a Wizard Genomic DNA Purification Kit (Promega). The 16S rRNA gene of the isolated strain was amplified by adding 1 µL of extracted DNA to a thermocycler microtube containing 5 µL of 10 × taq buffer, 0.5 µL of each 50 mM Fd1 and Rd1 primers, 5 µL of 25 mM $MgCl_2.6H_2O$, 0.5 µL of 25 mM dNTPs, 0.5 µL of Taq polymerase (5 U μL^{-1}), and 38 µL of sterilized distilled water. Universal primers Fd1 and Rd1 (Fd1, 5-AGAGTTTGATCCTGGCTCAG-3 and Rd1, 5-AAGGAGGTGATCCAGCC-3) were used to obtain a PCR product of 1.5 kb corresponding to base positions 8 - 1542 based on *Escherichia coli* numbering of the 16S rRNA gene (Winker and Woese, 1991).

The reactions were put in a thermal reactor thermocycler (BIOMetra, Leusden, The Netherlands), denatured for 1 min at 95°C and subjected to 30 cycles for 20 s at 95°C, 30 s at 55°C, and 1 min and 30 s at 72°C. This was followed by a final elongation step for 5 min at 72°C. The PCR products were analyzed on 1% (w/v) agarose gels and sent to GATC (Germany) for sequencing using universal primers Fd1 and Rd1 described previously. Sequence data were imported into the BioEdit version 5.0.9 sequence editor (Hall, 1999); base-calling was examined, and a contiguous sequence was obtained. The full sequence was aligned using the RDP Sequence Aligner program (Maidak et al., 2001). The consensus sequence was manually adjusted to conform to the 16S rRNA gene secondary structure model (Winker and Woese, 1991). A nonredundant BLAST search (Altschul et al., 1997) identified its closest relatives. Sequences used in the phylogenetic analysis were obtained from the RDP (Maidak et al., 2001) and GenBank databases (Benson et al., 1999). Sequence positions and alignment ambiguities were eliminated and pairwise evolutionary distances were calculated using the method of Jukes and Cantor (1969). A dendrogram was constructed using the neighbour-joining method (Saitou and Nei, 1987). Confidence in tree topology was determined using 100-bootstrapped trees (Felsenstein, 1985).

Restriction endonuclease digestions

The PCR amplification and restriction endonuclease digestions were performed as previously described (Hedi et al., 2009). Enzymatic digestions were performed by incubating 5 µL of the PCR products with 10 U of each endonuclease and the corresponding enzyme buffer. Digestions were incubated for one hour at 37°C for *Alu*I, *Hae*III, and *Rsa*I and products were analyzed on 2% (w/v) agarose gels.

RESULTS

Physico-chemical analysis of the samples

The temperature at the sampling sites was 21°C at 8 h in the morning. The pH of sediment samples was between 8.6 and 9.1 and may be considered as weakly alkaline. The highest values of moisture and salt saturation content were found in the S2 sample. Sodium and chloride were the most abundant ions. Sulfate and magnesium content were found higher also in the four samples when compared with others ions (Table 1). The total salt composition of the S4 sampling site was higher than the other sampling sites (Table 1). Total ionic composition of the lake differed depending on the area sampled. Taking into account the mineral composition of the lake, with regard to its concentration in Na^+, K^+, Ca^{2+}, Mg^{2+}, SO_4^{2-} and Cl^-, it is clear that halophilic microorganisms should inhabit this lake, thus justifying studies on the microbial survey of it.

Microbiological analyses

After several dilutions and subculturing in the same liquid medium under aerobic conditions, colonies were isolated in the agar medium containing 25% NaCl. The total number of extremely halophilic bacteria in the salt samples of sites 1 and 3 (3.49×10^4 - 7.3×10^5 CFU/g, respectively) was higher than in the salt samples of sites 2 and 4 (4.1×10^3 - 3.2×10^4 CFU/g, respectively). The high concentration of salt limited the number of strains. The 126 strains isolated belong to 11 different genera within Bacteria and Archaea domains (Table 3). The total strains give an idea about the distribution of major microbial groups that inhabit the Sebkha, taking into account the salinity of the culture medium used for isolation. The number of genera found decreased specially in sites 2 and 4, because of increase of ecosystem salinity (Rodriguez-Valera, 1993).

On the basis of phenotypic characteristics (macro and microscopic analysis), physiological analysis (NaCl, pH), biochemical tests (chitinase, cellulase, curdlanase, xylanase and protease) and molecular approaches [16S analysis, ARDRA], only 79 isolates were selected for characterization and examined in greater details. These strains have been identified by analyzing sequences of genes encoding for the 16S rRNA (Figure 2). The others

Table 1. Physico-chemical characteristics of the sediment samples.

Characteristic	S1	S2	S3	S4	Average
Colour of sampling site	Cream	Dark-cream	Cream	Dark-cream	
pH	8.66	9.11	8.73	8.60	8.77
Moisture (%)	17.58	20.91	15.24	17.44	17.79
Saturation (g/l)	282. 5	300	250	230	265.6
Electric conductivity (ms/cm)	189.30	181.00	187.60	185.20	185.77
Anions (mg/g)					
Chloride	98.00	102.00	101.20	101.80	100.51
Carbonate	0 .52	0.08	0.33	0.09	0.25
Bicarbonate	0.06	0.24	0.19	0.20	0.17
Sulfate	34.42	46.03	40.51	39.90	40.21
Cations (mg/g)					
Sodium	98.00	104.00	10 1.00	101.00	101.00
Potassium	5.80	8.60	6.00	6.00	6.6
Calcium	0.93	1.14	1.04	1.04	1.04
Magnesium	32.27	30.61	30.19	33.95	31.75
Total	270	292.7	280.46	283.98	281.53

strains were a repeated isolates showing the same ARDRA profiles analyses.

Colonial and cell morphology

The dominant bacterial population in hypersaline environment comprised motile or non motile, Gram-positive microorganisms and most of them were spore-forming bacteria. Most colonies on agar media were 0.5 - 2 mm in diameter after three weeks of incubation. These colonies were smooth, circular, low-convex, transparent or translucent and entire. Cells of all strains isolated were short, long and swollen rods and occurred in singles, pairs or short chains. Colonial pigmentation from these samples ranged from blood-red to pale-pink. Optimum growth occured at, 25% (w/v) NaCl, 37°C, and two pH (7.2 and 8.2) (Table 2). No growth was observed at NaCl concentrations of less than 15% (w/v) for the majority of isolates, thus suggesting that these isolates should be considered as extremely halophilic according to the definition of Ventosa et al. (1998).

Biochemical tests

To identify and characterize the enzymatic capabilities of the isolated strains, some biochemical tests were conducted. Large zones of clearing around the growing bacteria were observed. Others strains were also unable to form clearing zones. These results suggested that enzymes may be secreted by the strains into the culture medium. Results reveal also that many isolates were able to produce chitinase and curdlanase (Table 4). Moreover, most of them exhibited cellulase activity. A few number of the strains chosen were able to produce protease and xylanase. In addition, all isolates showed variability in degradation of enzymes that reflect inter- and intra-specific polymorphism.

Phylogenetic analysis

Based on the enzymatic digestion profiles obtained, 79 representatives bacteria of the 126 isolates were chosen for taxonomic and phylogenetic studies. To determine their phylogenetic position, the 16S rRNA gene sequence of each strain was analyzed and phylogenetic trees were constructed (Figure 2). Phylogenetic trees are presented as two clades of bacteria, Archaea are presented by two genus (*Haloferax* and *Natrinema*). The 16S rRNA gene sequences of strains have been deposited in the GenBank database (as the accession numbers ranged from KC142043 to KC142106). The phylogenetic analysis indicated that the first clade is made of up a wider variety of genera including *Halomonas* (*Halomonas koreensis*, *Halomonas salina*, *Halomonas halmophila*, *Halomonas eurihalina*, *Halomonas elongata* and *Halomonas sinaiensis*), *Pseudomonas*, *Halovibrio* and is dominated by isolates related to genera *Salicola* (Figure 2). The second clade is a group of isolates related to two genera (*Marinococcus* and *Bacillus*), including *Halobacillus karajensis*, *Halobacillus trueperi*, *Halobacillus yeomnijeoni*, *H. salinus*, *Marinococcus halophilus*,

Table 2. Phenotypic features of the 24 representatives strains studied.

Sampling sites (S)/ depth (cm)/ characteristics	**Strain (7SPE)**							
	2421 S2/40	**411 S4/10**	**121 S1/20**	**1132 S1/10**	**2412 S2/40**	**105 S1/0**	**4212 S4/20**	**323 S3/20**
Taxonomical status	*Halobacillus* sp.	*Halobacillus* sp.	*Marinococcus* sp.	*Bacillus* sp.	*Pontibacillus* sp.	*Marinococcus* sp.	*Bacillus* sp.	*Halovibrio* sp.
Colonial morphology	Circular	Circular	irregular and spreading	Circular	Circular	Circular	Circular	Circular
Colony size	0.5 mm	1 mm	1-2 mm	2 mm	1 mm	1mm	0.5 mm	0.3 mm
Colony	convex	flat	flat	convex	flat	convex	flat	convex
Colony density	Opaque matt	Opaque matt	Transparent glistening	Opaque matt	Opaque matt	Opaque matt	Opaque matt	Translucent matt
Pigmentation	Cream	Cream	Cream	White	Cream	Reddish pleomorphic	Cream	White
Cell shape	Pleomorphic	Pleomorphic	Pleomorphic	Pleomorphic	Pleomorphic		Pleomorphic	Pleomorphic
Cell arrangement	Rods long chains	Cells single and paired cells	Rods single and paired cells	Cells single and paired cells	Rods long chains	Rods single and paired cells	Rods single and paired cells	Cells single and paired cells
Chains	+	-	-	-	+	-	-	-
Motile	-	-	+	-	+	-	+	-
Cell size; length and width (µm)	2-5 X 1.5	3-7.5 X 1.5	1-4 X 1	1-3 X 0.5	1-4 X 1	1-5 X 2	1-4 X 1	1-4 X 0.5
Growth at 37°C, pH 7.2								
0% NaCl	-	-	-	-	-	-	-	-
2% NaCl	-	-	-	-	-	-	-	-
5% NaCl	+	+	+	-	+	+	+	+
8% NaCl	+	+	-	+	+	+	+	+
10% NaCl	+	+	+	+	+	+	+	+
15% NaCl	+	+	+	+	+	+	+	+
25% NaCl	+	+	+	+	+	+	+	+
30% NaCl	-	-	-	-	-	-	-	-
Growth at 37°C								
pH 4.5	-	-	-	-	-	-	-	-
pH 6	+	+	+	+	+	+	+	+
pH 7	+	+	+	+	+	+	+	+
pH 7.5	+	+	+	+	+	+	+	+
pH 8	-	-	-	-	-	-	-	-

Table 2. Contd.

Sampling sites (S)/ depth (cm)/ characteristics	Strain (7SPE)							
	3212 S3/20	**116 S1/10**	**1211 S1/20**	**2224 S2/20**	**1131 S1/10**	**2215 S2/20**	**3128 S3/10**	**3030 S3/0**
Taxonomical status	*Salicola* sp.	*Halomonas* sp.	*Halomonas* sp.	*Halomonas sp*.	*Halomonas* sp.	*Halomonas* sp.	*Halomonas* sp.	*Halomonas* sp.
Colonial morphology	Irregular and spreading	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Colony size	0.5 mm	1 mm	1-2 mm	2 mm	1 mm	2mm	1 mm	1-2 mm
Colony	Convex	Flat	Flat	Convex	Flat	Convex	Flat	Slightly raised
Colony density	Opaque matt	Opaque matt	Opaque matt	Opaque matt	Opaque matt	Opaque matt	Opaque matt	Translucent
Pigmentation	Reddish	Cream	Cream	White	Cream	Cream	Cream	Glistening white
Cell shape	Pleomorphic Cells	Pleomorphic cells	Pleomorphic cells	Pleomorphic cells	Pleomorphic cells	Pleomorphic cells	Pleomorphic rods	Pleomorphic Cells
Cell arrangement	Single and paired cells	Long chains	Single and paired cells	Single and paired cells	Long chains	Long chains	Paired cells and long chains	Single and paired cells
Chains	-	+	-	+	+	+	+	-
Motile	-	-	-	-	-	-	+	-
Cell size; length and width (µm)	2-5 X 0.3	1-5 X 1	1-4 X 0.5	1-3 X 1.5	1-4 X 1.5	1-5 X 1	2-5 X 1	1-4 X 1
Growth at 37°C, pH 7.2								
0% NaCl	-	-	-	-	-	-	-	-
2% NaCl	-	-	-	-	-	-	+	-
5% NaCl	+	+	-	-	+	+	+	-
8% NaCl	+	+	+	+	+	+	+	-
10% NaCl	+	+	+	+	+	+	+	+
15% NaCl	+	+	+	+	+	+	+	+
25% NaCl	+	+	+	+	+	+	+	+
30% NaCl	-	-	-	-	-	-	-	-
Growth at 37°C								
pH 4.5	-	-	-	-	-	-	-	-
pH 6	+	+	+	+	+	+	+	+
pH 7	+	+	+	+	+	+	+	+
pH 7.5	+	+	+	+	+	+	+	+
pH 8	-	-	-	-	-	-	-	-

Table 2. Contd.

Sampling sites (S)/ depth (cm)/ characteristics	Strain (8SPE)					
	1262 S1/20	**236 S2/30**	**1359 S1/30**	**422 S4/20**	**607 S6/0**	**338 S3/30**
Taxonomical status	*Halobacillus* sp.	*Halobacillus* sp.	*Halobacillus* sp.	*Gracilibacillus* sp.	*Halovibrio* sp.	*Salicola sp*.
Colonial morphology	Irregular and spreading	Circular	Irregular and spreading	Circular	Circular	Circular
Colony size	0.5 mm	1 mm	1-2 mm	2 mm	1 mm	2 mm
Colony	convex	flat	flat	convex	flat	convex
Colony density	opaque matt	opaque matt	opaque matt	opaque matt	opaque matt	opaque matt
Pigmentation	reddish	cream	reddish	white	cream	cream
Cell shape	pleomorphic	pleomorphic	pleomorphic	pleomorphic	pleomorphic	pleomorphic
Cell arrangement	Cells single and paired cells	Cells single and paired cells	Cells paired and long chains	Cells single and paired cells	Cells single and paired cells	Cells paired cells
Chains	-	-	+	+	-	-
Motile	-	+	+	-	-	-
Cell size; length and width (µm)	2-5 X 0.3	1-5 X 1	1-4 X 1	1-3 X 0.5	1-4 X 1	1-5 X 0.5
Growth at 37°C, pH 7.2						
0% NaCl	-	-	-	-	-	-
2% NaCl	-	-	-	-	-	-
5% NaCl	+	+	+	+	+	+
8% NaCl	+	+	+	+	+	+
10% NaCl	+	+	+	+	+	+
15% NaCl	+	+	+	+	+	+
25% NaCl	+	+	+	+	+	+
30% NaCl	-	-	-	-	-	-
Growth at 37°C						
pH 4.5	-	-	-	-	-	-
pH 6	+	+	+	+	+	+
pH 7	+	+	+	+	+	+
pH 7.5	+	+	+	+	+	+
pH 8	-	-	-	-	-	-

Table 3. Distribution and taxonomic characteristics of micro-organisms isolated from the 4 sites sampled in Sehline Sebkha Lake.

Organisms	Number of strains / site				Number of strains
	S1	S2	S3	S4	
Halobacillus	4	2	0	4	10
Marinococcus	2	0	0	0	2
Pontibacillus	0	5	0	1	6
Bacillus	1	0	2	1	4
Salicola	8	3	16	7	34
Yeomjeonicoccus	4	0	0	0	4
Halomonas	4	8	2	0	14
Gracibacillus	0	0	0	1	1
Halovibrio	0	0	1	0	1
Pseudomonas	0	0	0	1	1
Others (*Archaea*)[a]	0	1	1	0	2
Total of strains	23	19	22	15	79

[a]Data not shown.

Marinococcus halotolerans, *Gracilibacillus halophilus*, *Pontibacillus chungwhensis*, *Pontibacillus marinus*, *Bacillus halophilus* and *Bacillus quingdaonensis* (Figure 2). The largest clusters are dominated by bacteria related to *Salicola* species, particularly *Salicola marasensis* and *Salicola salis*. The first group of bacteria are members of the Class Gammaproteobacteria, the second one are related to the Class Bacilli.

All strains shared more than 97% identity with their closest phylogenetic relative (Table 3) thus suggesting that they may be considered at the same species level until the results of DNA/DNA hybridization studies will be performed to validate or not their affiliation (work under progress).

Only two strain representatives of domain *Archaea* were identified as *Haloferax* sp. and *Natrinema sp.*, but these microorganisms have not been further characterized

DISCUSSION

In recent years, a number of extremophilic micro-organisms ranging from aerobes to anaerobes have been isolated. Research on microorganisms from extreme environments also intensified with the recognition of a third domain of life (*Archaea*) by Woese and Fox (1977). Investigations on the microbial ecology of various hypersaline environments have been largely extended during the last decades. In contrast to halotolerant micro-organism which do not require NaCl for growth but can grow under saline conditions, halophiles must have NaCl for growth (Ventosa et al., 1998). Both molecular and microbiological studies revealed the presence of moderately to extremely halophilic microorganisms in a wide range of these saline environments (Cayol et al., 1994; Oren, 2002a, b; Demergasso et al., 2004; Ventosa, 2006).

In the present study, we described microbial diversity among Bacteria and Archaea domains within four sites of two pH media (7.2 and 8.2) and high salinity (25%). The microbial communities in the four studied sites were different in terms of diversity and phylogenetic distribution of the 16S rRNA sequences. The differences between the samples indicated that microbial diversity may be strongly influenced by physical and chemical parameters in the four sites, particularly differences in salt concentration and in ions specification. Analysis of soil samples from the four sites studied is reported in Table 1. They differ from those of the other hypersaline environments studied so far (Hedi et al., 2009). Sodium and chloride concentrations in the four sites are higher than those of the Dead Sea in Israel, in particular (Oren, 1993). Waters of the Dead Sea and the Great Salt Lake in the USA, are slightly acidic (pH between 6 and 7), but the pH of the four sites studied are up to 8, and should be therefore considered as weakly alkaline (Oren, 1993). On the other side, the pH of Lakes Wadi Natrun and Magadi (in Kenya) are considered as highly alkaline environments (pH 11) (Jeon et al., 2005).

A total of 126 extremely halophilic strains have been isolated. Among them, 79 strains (77 *Bacteria*, 2 *Archaea*) with different pigmentations (cream, white, yellowish, and reddish-orange) as observed with colonies on agar plates have been further characterized. All bacterial strains were found as Gram-positive rods. The phylogenetic analysis indicate that all isolates were members of ten genera of the domain *Bacteria* including *Salicola*, *Pontibacillus*, *Halomonas*, *Marinococcus*, *Bacillus*, *Gracibacillus*, *Halobacillus*, *Yeomjeonicoccus*,

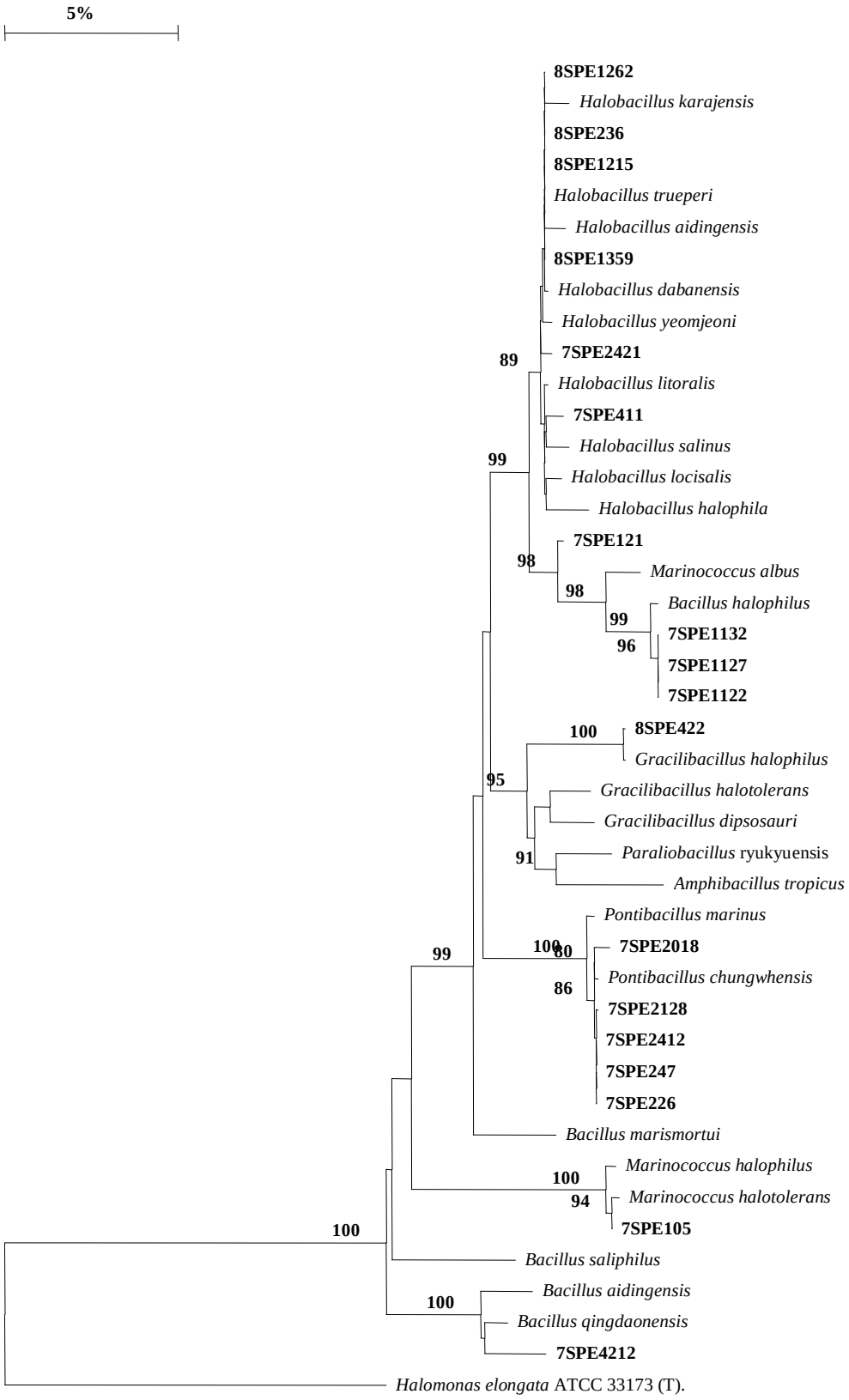

Figure 2. 16S rRNA gene-based phylogenetic trees of the bacterial domain, including the 16S rDNA sequences from sediments sample of Sehline Sebkha. The topologies of phylogenetic trees build using the maximum-likehood and maximum-parsimony algorithms were similar to those of the tree constructed by neighbour-joining analyses.

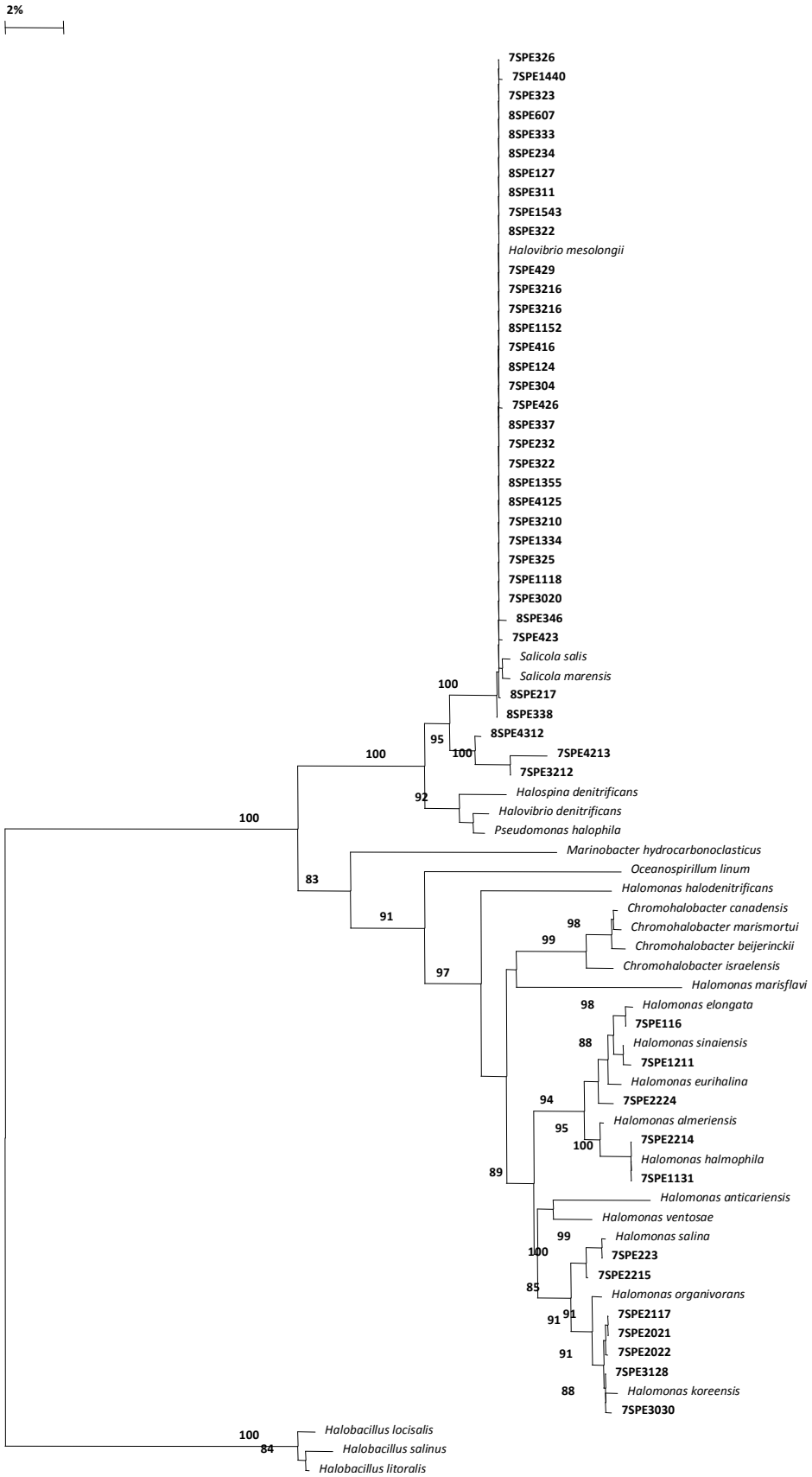

Figure 2. Contd**.**

Table 4. Enzymatic tests.

Isolates	Chitinase	Cellulase	Curdlanase	Protease	Xylanase
7SPE 1132	-	+	+	-	-
7SPE 3216	-	-	+	-	-
7SPE 2128	+	-	-	-	-
7SPE 429	+	+	+	+	-
7SPE 323	+	+	+	-	+
7SPE 423	-	-	+	+	-
7SPE 1440	+	+	+	-	-
7SPE 2421	-	+	+	-	-
7SPE 2214	-	+	+	-	-
7SPE 416	-	-	+	-	-
7SPE 2224	-	-	+	-	-
7SPE 326	+	-	-	-	-
7SPE 411	-	-	-	+	-
7SPE 325	-	-	+	-	-
8SPE 322	+	+	+	-	-
8SPE 337	-	+	-	-	-
8SPE 4214	-	+	-	-	-
8SPE 217	-	+	-	-	-
8SPE 338	-	+	-	-	-
8SPE 333	-	+	-	+	-
8SPE 1215	+	-	-	-	-
8SPE 346	-	-	-	+	-
8SPE 311	-	-	+	-	-
8SPE 1131	+	+	+	-	+

(+) Positive activity; (-) negative activity.

Halovibrio and *Chromohalobacter*. Members of the genera *Salicola*, *Pontibacillus, Marinococcus* and *Halobacillus* are considered as aerobic microorganisms, whereas those of genus *Halomonas* are considered as facultative anaerobes having the possibility to use nitrate as terminal electron acceptor under anaerobic conditions (Martínez-Cánovas et al., 2004). All these micro-organisms may use various organic compounds including sugars as substrates and should be considered as chemoorganotrophs. Almost all these isolates were detected on the surface of sediments (0.1 - 0.2 m) of each biotope.

The distribution of the microflora that inhabits the lake is reported in Table 3. *Salicola* species were distributed in all the 4 sites studied and represented the major strains isolated, especially in site 3. The large number of the genus *Halomonas* and *Salicola* may be due to the culture media used, which may have favoured the species growth and thus do not reflect their real distribution within the lake. Members of this genus together with those of genera *Halomonas*, *Gracibacillus*, *Bacillus*, *Halovibrio*, *Chromohalobacter*, *Yeomjeonicoccus*, *Pontibacillus*, *Marinococcus*, and *Halobacillus*, have also been isolated from other saline environments including athalassohaline and thalassohaline lakes and marine waters (Javor, 1989; Ventosa et al., 1998; Grant et al., 2001; Arahal and Ventosa, 2005).

With regards to the Archaea population, it could be noticed that all the 16S rRNA gene sequences obtained were affiliated with the Halobacteriales order of the Euryarchaeota. Among the halophilic micro-organisms isolated, only two originated from sites 2 and 3 pertaining to the domain *Archaea* (data not shown). Due to the industrial and economic importance of halophilic enzymes, the chitinase, cellulase, curdlanase, protease and xylanase activities of 24 extremely halophilic bacteria were screened. Ten of them produced two or more enzymes. They belong to genera *Halomonas*, *Salicola*, *Halobacillus* and *Bacillus*. Among the strains tested, only six strains were unable to produce any of the above enzymes. They are members of genera *Salicola* and *Marinococcus*. The others strains produced only one enzyme and they are members of genera *Salicola*, *Halomonas*, *Halobacillus* and *Pontibacillus*. Industrial enzymes obtained from halophiles might be used for improving garments during textile processing. Proteolytic enzymes from *Halobacterium* also play an important role in the brine fermentation of one type of traditional fish sauce (Quesada et al., 1990, 1993).

During an extensive search on different hypersaline

habitats in Spain and Marocco focused on the screening of new exopolysaccharide (EPS)-producing bacteria, several strains were isolated from saline soils and described as new species belonging to the genus *Halomonas* (Thongthai et al., 1992; Bouchotroch et al., 2001; Jones, 2001; Martínez-Cánovas et al., 2004). Similar to what we observed in our experiments, a minority of these micro-organisms isolated were identified as members of genera *Gracibacillus*, *Halovibrio*, *Marinococcus* and *Pseudomonas*. Several other aerobic or facultative anaerobic, moderately halophilic bacteria have been classified within genera related to the order *Bacillales* (Spring et al., 1996). The use of these microorganisms has been underlined (production of compatible solutes, halophilic enzymes, biopolymers and bioremediation processes) and reviewed in detail (Ventosa et al., 1998; Margesin and Schinner, 2001; Mellado and Ventosa, 2003). The potential industrial use of this collection of halophiles that we have got will be screened for molecules of industrial interest than enzymes (e.g. EPS and PHA, etc) (work in progress).

Finally, we believe that studies on these bacteria should be emphasized as they constitute a source of halostable enzymes (Table 4) which may be used in different pharmaco-chemical industries (Jones, 2004; Quesada et al., 2004).

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