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Diversity of hydrolase-producing halophilic bacteria and evaluation of their enzymatic activities in submerged cultures

Leila Batsoule Drissi Kaitouni[1], Jaouad Anissi[1,2], Khalid Sendide[3] and Mohammed El Hassouni[1*]

Abstract

Purpose: In this work, we assessed the diversity of culturable halophilic bacteria that produce hydrolytic enzymes from both natural and artificial hypersaline regions in the pre-Rif region of Morocco.

Methods: Bacteria were isolated from three hypersaline sites, in solid medium supplemented with various salt concentrations ranging from 0 to 330 g/L. Physical and chemical characteristics of samples from the isolation site were determined to suggest eventual correlations with the occurrence of the halophilic bacteria. Assays on enzymatic activities were performed in submerged cultures in the presence of various salt concentrations and appropriate substrates.

Results: Out of a collection of 227 halophilic bacteria, four halophilic groups were established as slightly halophilic, moderately halophilic, halotolerant, or extremely halophilic, with a predominance of halophilic bacteria in the natural hypersaline sites compared to the artificial one. Within this collection, 189 strains showed important hydrolytic activities in submerged cultures with enzymatic activities up to 76 U/mg. Strain characterization and identification was based on phenotypic and molecular traits and allowed the identification of at least 26 genera including *Bacillus*, *Chthonibacter*, *Mariniabilia*, *Halobacillus*, *Salinococcus*, *Cerasicoccus*, *Ulvibacter*, *Halorubrum*, *Jeatgalicoccus*, *Brevibacterium*, *Sanguibacter*, *Shewanella*, *Exiguobacterium*, *Gemella*, and *Planomicrobium*.

Conclusion: Data from this study give insights about the origin and the occurrence of halophilic bacteria in natural hypersaline environments compared to artificial hypersaline sites. The occurrence of halophilic hydrolase enzymes from halophilic bacteria gives insights to different applications in biotechnology, thanks to their ability to produce adaptive enzymes and survival strategies to overcome harsh conditions.

Keywords: Hypersaline environment, Halophilic bacteria, Hydrolase enzymes

Introduction

Hydrolases (EC 3 in Enzyme Commission classification) are ubiquitous in nature and are widely distributed among microorganisms, in particular within bacteria (Quax 2006) and fungi (Demain and Adrio 2008). These enzymes are critical for biomass and complex macromolecule modification and breakdown at both environmental level, where they contribute to most of the geochemical processes, and at the industrial level as critical ingredients in most of the food, agricultural, and pharmaceutical industries (Singh et al. 2016). The requirements and the modus operandi of the abovementioned industries made the urge to explore other sources of enzymes, among the extremophile microorganisms. Recently, the halophilic bacteria gain interest from their capacity to adapt to very high salt concentrations; a halophilic organism needs imperatively the presence of moderate and high salt concentrations, and the halotolerant have no need of salt for their growth but can grow in the presence of very high salt concentrations

* Correspondence: mohammed.elhassouni@usmba.ac.ma
[1]Faculty of Sciences Dhar El Mahraz, Laboratory of Biotechnology, Environment, Agri-Food and Health, Sidi Mohamed Ben Abdellah University, Fez, Morocco
Full list of author information is available at the end of the article

(Torregrosa-Crespo et al. 2017). Halophilic bacteria have been increasingly studied for their biotechnological potential to produce enzymes that are stable and active at alkaline pH, high temperature, and high salt concentrations (Di Donato et al. 2018). These multifaceted attributes are attractive in diverse industries (Delgado-Garcia et al. 2012), such as in fermented food, in textile, in pharmaceutical, in cosmetic, and in leather industries (De Lourdes et al. 2013). Such requirement of salts for growth is decidedly required for the functioning of most of the metabolic pathways orchestrated by enzymes and thus make them suitable for use in industrial processes that include high salts concentrations (Liu et al. 2018).

In Morocco, several hypersaline environments were described (Bouchotroch et al. 1999; Berrada et al. 2012). Among them, the pre-Rif region is characterized by the abundance of Triassic outcrops that form a lithologic complex of ophites and gypso-salt clay. The main areas are those located in the region of Fez and Taza, where Triassic diapirs are scattered, frequently associated with salt ponds and gypsum quarries of marine origin, and mined in traditional settling ponds (Michard et al. 2014). Hypersaline biotopes such as salt mines and saline lakes are typical examples of extreme environments that house an omnifarious biodiversity of halophilic bacteria. In this kind of extreme environments, studies generally reveal the predominance of genera of the domain bacteria, including *Bacillus*, *Halobacillus*, *Virgibacillus*, *Oceanobacillus*, *Staphylococcus*, *Pseudomonas*, *Idiomarina*, *Halomonas*, *Marinobacter*, *Thalassobacillus*, *Piscibacillus*, *Gracilibacillus*, *Salicola*, *Salinicoccus*, *Flavobacterium*, *Exiguobacterium*, *Paracoccus*, *Chromobacterium*, *Kushneria*, *Cobetia*, *Marinococcus*, *Nesterenkonia*, and *Tetragenococcus* (Babavalian et al. 2014; Al-Rubaye et al. 2017;Dumorné 2018).

In the present work, we assessed the diversity of cultivable halophilic bacteria with hydrolytic enzymes activity, namely amylase, pectinase, protease, inulinase, and cellulase, which were chosen based on their potential utilization in multiple industrial processes, as they are characterized by their stability at high salt concentrations (Lima and Porto 2016; Dumorné 2018). The halophilic and halotolerant bacteria were collected from three hypersaline sites: the Mount Zalagh salt mine (site 1) and, two salt marshes, Meknassa (site 2) and Bab Merzouka (site 3), located in the pre-Rif region of Morocco. Phenotypic/phylogenetics traits as well as data related to their hydrolytic activities were emphasized in order to get insight about bacterial occurrence in such hypersaline environments and to explore the hydrolytic activities of such bacteria.

Material and methods

Sampling, physicochemical analysis, and bacteria isolation

Aqueous and solid samples were collected from sites with the following GPS coordinates: 34° 7′ 34.95″ N 4° 54′5 1.67″ W, 34° 15′ 50.21″ N 4° 3′ 26.46″ W, and 34° 13′ 17.29″ N 4° 6′ 24.66" W, for site 1 (salt mine), site 2, and site 3 (salt marshes), respectively. A map and pictures of sampling sites are shown the Fig. S1 and S2 (supplementary material). Aqueous samples from all the three sites were collected from the settling pond; the mud represents the mixture of water and soil of the edge of these settling pond and the soil represents the dry part around the basin or settling ponds; bare soil with no vegetation is often covered with salt crystals; it is at 2 m of the settling pound that vegetation begin, as showed in the of the supplementary material. Samples were aseptically collected in sterile plastic containers and analyzed within the next 10 h. Physical and chemical parameters such as pH, T°C, and electrical conductivity (EC) were also recorded at the time of sampling. The mineral profile of each site was analyzed by Inductively coupled plasma atomic emission spectroscopy (ICP-AES). The organic matter, moisture, chemical oxygen demand (COD), biological oxygen demand (BOD_5), total dissolved salts, and total mineral matter were also measured (Rodier et al. 2009; Graf-Rosenfellner et al. 2016). Bacteria were isolated from aqueous and re-suspended solid samples using the serial dilution and plating methods, and cells were spread out on modified Luria Bertani (LB) agar medium (composition per liter: tryptone 10 g, yeast extract 5 g, Agar 20 g, pH 7.2) containing different NaCl concentrations (0.0 to 330 g/L). The plates were incubated at 37 °C, and the growth was monitored at 1-day intervals for 72 h. Independent bacterial colonies with different morphologies were isolated, purified, and then stored for further use.

Phenotypic and biochemical identification

Isolates phenotypic characteristics were assessed as described by Gerhardt et al. (1994). Tests include Gram staining, motility, sporulation, catalase and oxidase activities, carbon source utilization and fermentation, and the production of hydrolase enzymes (cellulases, pectinases, amylases, inulinases, and proteases). Isolates response to NaCl was determined at different concentrations of NaCl, ranging from 0.0 to 330 g/L at 30 g/L increment, and each strain was classified according to Ventosa and Arahal (2009) halophilic bacteria classification.

16S rDNA sequencing and phylogenetic analysis

The DNA extraction and purification was performed as follows: cells from 3 ml cultures in LB medium were harvested at 10 000 g for 10 min. The pellet was resuspended in 500 μl of TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA and pH 8), and then, 10 g/L of SDS and 0.01 g/L of proteinase-K (Sigma-France) were added. The mixture was incubated at 50 °C for 5 min. Two volumes of ethanol were added and the mixture was

centrifuged at 10 000*g* for 10 min, at 4 °C. The pellet was then washed with 75% ethanol, harvested, dried, and then resuspended in 100 μL MilliQ water. 16S rDNA amplification was carried out using universal primers, 27F (AGAGTTTGATCCTGGCTCAG) and 1392R (GGTTACCTTGTTACGACTT). The reaction mix was prepared in a final volume of 20 μL containing 4 μL *Taq* buffer (5×), 1.2 μL of MgCl2 (25 mmol/L), 4 μL of dNTPs (1 mmol/L), 0.5 μL of each primer (10 μmol/L), 0.2 μL of *Taq* polymerase (5 U/μL), 6.6 μL of pure H_2O, and 2 μL of extracted DNA. Amplification was conducted as described by Turner et al. (1999). Sanger sequencing was performed at the Center of Innovation (USMBA, Fez-Morocco) using an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems). Preliminary identifications were performed based on sequence assembly and by search in the NCBI database. Strains were attributed to a particular genus when the sequence similarity with a strain type was at least 98% and to a given species when sequence similarity was at least 99.5%. To study the phylogenetic relationship among the isolates and other homologous species, we applied the neighbor-joining (NJ) criteria, using the MEGA X software (Kumar et al. 2018). Phylogeny tests were assessed by bootstrapping with 1000 replicates; the maximum likelihood composite was used as substitution model. The obtained 16S rDNA gene fragments sequences were deposited in the NCBI database under the accession numbers MK713683 – MK713732.

Qualitative and quantitative assessments of extracellular hydrolytic activities

The isolated strains were preliminarily characterized for their qualitative enzyme production and activity on agar plates on mineral medium M9 (composition per liter: 6 g Na_2HPO4, 3 g KH_2PO4, 1 g NH_4Cl, 0.5 g yeast extract, 0.1 mmol/l $CaCl_2$, 1 mmol/L $MgSO_4$, 18 g Agar-Agar) supplemented with a final substrate concentration of 5 g/L and different concentrations of NaCl ranging from 0.0 to 330 g/L. The plates were incubated at 37 °C for 48 h except the plates for the protease activity, which were monitored for 168 h. In such conditions, cellulase activity was determined on mineral medium M9 agar supplemented with 5 g carboxymethyl cellulose salt. After 2 days of incubation at 37 °C, CMCase activity was revealed by covering the surface of the agar plate with Congo red solution (1 g/L) for 10 min and then washing with a solution of 60 g/L NaCl: a yellowish surrounding zone against a red background confirmed the activity (Gohel et al. 2014). Amylase activity was qualitatively determined on minimal starch agar medium (M9 medium supplemented with 5 g/L starch). Plates were incubated at 37 °C for 2 days, and the activity was revealed using iodine reagent (0.01 mol/L KI solution). A positive assay occurs when a transparent zone forms around the strain colony, opposed to a dark purple background (Mishra and Behera 2008). Pectinolytic activity was determined on M9 minimal medium supplemented with 5 g/L polygalacturonic acid at a pH of 8.2. After incubation at 37 °C for 2 days, the medium surface was layered with a copper acetate solution (75 g/L) for 10–20 min, and then washed several times with distilled water. Positive pectinolytic activity was revealed by a clear zone around the growth colony opposed to a blue background. Inulinase activity was carried out on agar M9 minimal medium plates containing inulin at 5 g/L as a sole carbon source. Inulinase activity was determined by appearance of transparent zone around the colony when the plate was flooded with iodine reagent and after 2 days of incubation at 37 °C (Li et al. 2011). Protease activity was assayed using milk agar medium (M9 medium supplemented with 5 g/L powder milk). After 7 days of growth, clear zones around the colonies indicated positive proteolytic activity.

Extracellular hydrolytic enzymes production and activity measurements were assessed in submerged cultures (M9 mineral medium) supplemented with appropriate substrate at 5 g/L and different concentrations of NaCl ranging depending on each strain's salt tolerance category: 1 g/L for the slight halophilic and halotolerant strains, 60 g/L for the moderately halophilic strains, and 150 g/L for the extremely halophilic strains. The growth and enzymatic activities were monitored at 37 °C under orbital shaking at 140 rpm. After 24 h, the supernatants were collected after centrifugation at 10,000*g* for 10 min at 4 °C and used to carry out all the enzymatic tests.

The reducing units were determined for the CMCase, amylase, pectinase, and inulinase using the dinitrosalicylic acid method, 500 μl of supernatants, and 500 μl of the substrate buffer (sodium acetate at pH 4.8 and 20 g/L of the substrate). The substrates used were as follows: starch, CMC, pectin, and inulin respectively for amylase, cellulase, pectinase, and inulinase activities. The mixtures were incubated at 50 °C for 30 min, and after the addition of DNS reagent, the mixture was incubated at 100 °C for 5 min. Protease activity was determined by the method of Hagihara (Hagihara 1958), using casein buffer (NaOH-Borax pH 10.0 and 6 g/L casein). Protease assay was conducted at 37 °C, 20 min. The TCA was added, and the mixture was incubated at room temperature for 20 min. Total protein content was determined using Bradford reagent (Bradford 1976). Bacterial growth was taken by spectrophotometry at 600 nm. One unit of CMCase, amylase, pectinase, inulinase, and protease activities was defined as the amount of enzyme that releases 1 μmol of glucose, maltose, galacturonic acid, fructose, and tyrosine per minute, respectively.

Statistical analysis

Data were expressed as means ± standard deviation (SD) obtained from triplicate experiments, and all statistical

analyses and data plotting were performed under R program version 3.5.1.

Results and discussion

Physical and chemical analysis of the sampling sites

In this study, many samples from three hypersaline sites located in the pre-Rif region in Morocco (Fig S1) were prospected for halophilic bacteria producing highly active hydrolases. As shown in Table 1, the bacterial colony count ranged from $1.2.10^5$ to $3.3.10^6$ CFU/g and from $2.2.10^5$ to $2.25.10^6$ CFU/L in the salt mine and salt marshes, respectively. pH of the samples was neutral to slightly alkaline, ranging from 7.0 to 8.3. Measurements of EC of soil and mud samples showed values ranging from 5.4 to 13.9 dS/m. EC of the aqueous samples from the sites 1, 2, and 3 showed values of 9.3 dS/m, 10.6 dS/m, and 11.3 dS/m, respectively, indicating the occurrence of high salt concentrations in such samples. However, EC of the water source (WSS1) feeding site 1 showed a low value of 2.1 dS/m. Subsequently, samples were classified based on their EC values in salinity categories, distribution of soil samples studied according to the USSLS standards by Richards (1954), and water samples according to Rhoades et al. (1992) (see Table 1). The mineral profiling of the samples showed a high content of Na^+, Mg^{2+}, Ca^{2+}, and K^+. Soil and mud samples from the three sites showed the predominance of sodium and calcium ions, with concentrations ranging from 1.5 g/L to more than 1000 g/L. K^+ and Mg^{2+} ions follow at relatively lower concentration (see Table 2). Other ions such as Ni^{2+} and Cd^{2+} were present at concentrations less than 0.03 g/L in the three sites. Yet, Al^{3+}, Co^{2+}, Cr^{2+}, Cu^{2+}, and Fe^{2+} ions showed concentrations less than 0.01 g/L. Analysis of the bacterial load from the solid samples (mud and soil) showed a positive correlation with the concentration of mineral ions, mainly sodium, magnesium, and calcium ions. However, bacterial loads from aqueous samples showed a negative correlation with ions concentrations (Fig. 1). Strong correlations were obtained between bacterial load/halophilic bacteria frequency from solid samples (mud and soil) and salt concentrations of the quantified ionic entities. This situation was reversed in aqueous samples: the higher the salt concentration, the lower the bacterial load. Furthermore, depending on the isolation site, bacterial load responded differently to the different ions (Na, K, Mg, Mn, and Ca) at different concentrations, with a preference to the presence of Na^+ ion. These differences in bacterial loads from solid to aqueous samples may be explained by water activity as well as the ionic strength (Fox-Powell et al. 2016) and the availability of the ionic forms of the corresponding salts in the biotope (more free ions in the aqueous samples than in the solid ones). Also, it is worth mentioning that, in contrast to what is commonly accepted regarding salinity, such correlations indicate an increase in the bacterial load—mainly the increase in the frequency of halophilic bacteria—with an increase of salt concentration in solid samples, as opposed to the aqueous samples. On the other hand, organic matter content showed a relatively high percentage—ranging from 8 to 12% (w/w or w/v)—in both aqueous and solid samples, but the correlation with the bacterial load showed a low and a divergent response to organic matter. In solid samples, the water content showed a negative correlation with the microbial load. The BOD_5 of the aqueous samples showed values ranging from 0.54 to 1.6 (g O_2/L), and the values of total COD in the soil of sites 1 and 2 (SS1 and SS2) were higher compared to other samples (water and mud) in the same site. In contrast, site 3 showed high

Table 1 Physical and chemical properties of the samples from the three sites

	Site 1, JZ					Site 2, M			Site 3, BM		
	WS[c]	WM[d]	SS	S[d]	M[d]	W[c]	S[d]	M[d]	W[c]	S[d]	M[d]
Samples classification	Moderately saline	Highly saline	Extremely saline	Saline	Slightly saline	Highly saline	Saline	Slightly saline	Highly saline	Saline	Slightly saline
pH[a]	7.0	7.0	NA	7.7	7.9	8.0	7.6	8.1	7.5	7.7	8.3
Conductivity (dS/m)[b]	2.1	9.3	13.9	10.5	5.4	10.6	9.4	4.9	11.3	9.2	5.4
Salinity (%)	4.5	19.5	29.2	22.0	12.0	22.4	19.7	10.4	23.7	19.3	11.2
CFU/g	ND	ND	$1.25.10^5$	$2.9.10^5$	$3.3.10^6$	ND	$2.85.10^6$	$2.85.10^6$	ND	$2.95.10^6$	$2.85.10^6$
CFU/L	$1.5.10^6$	$2.2.10^5$	ND	ND	ND	$2.25.10^6$	ND	ND	$2.85.10^5$	ND	ND
Number of halophilic	ND	17	13	71	65	17	14	8	6	4	12
Number of hydrolases-producing bacteria	ND	12	13	64	55	15	14	8	5	2	1
Total	166					39			22		

WS water of the source, *WM* water inside the mine, *SS* salt sediment, *W* water of the site, *S* soil, *M* Mud, *ND* not determined
[a]Minimal/maximal for the season
[b]Conductivity and salinity were measured by "water quality instrument, YSI Scientific"
[c]The water is saline if EC > 0.25 dS/m
[d]The soil is saline if EC > 4.0 dS/m

Table 2 Mineral profile and classification of each studied site

	Site 1, JZ					Site 2, M			Site 3, BM		
	WS[a]	WM[a]	SS[b]	S[b]	M[b]	W[a]	S[b]	M[b]	W[a]	S[b]	M[b]
Ca[a,b]	< 0.01	60.62	ND	0.151	3.09	82.81	93.52	> 1000	21.33	725.25	218.29
K[a,b]	< 0.01	7.00	ND	5.47	5.58	12.58	5.95	12.58	1.43	12.79	10.03
Mg[a,b]	< 0.01	18.32	ND	< 0.01	< 0.01	1.49	1.89	28.17	3.74	35.01	17.08
Mn[a,b]	0.035	0.18	ND	0.035	0.074	0.110	0.106	0.130	0.171	0.171	0.117
Na[a,b]	1.50	329.75	ND	> 1000	> 1000	340.29	881.23	> 1000	222.16	657.18	881.18
CDOt (g O_2/l)	3.43	3.63	ND	8.88	0.90	2.42	7.57	0.90	24.24	1.79	1.28
BOD_5 (g O_2/l)	1.53	1.24	ND	ND	ND	1.60	ND	ND	0.54	ND	ND
Water content (%)	ND	ND	ND	12	41	ND	15	30	ND	10	35
Organic matter (%)	8	11.2	ND	6	8	6.06	7.05	10	12.5	10	11.5
Total dissolve salt (%)	5.4	22.9	ND	24.6	12.8	23.0	12.1	15.7	26.3	16.9	14.3

WS water of the source, *WM* water of the mine, *SS* salt sediment, *W* water of the site, *S* soil, *M* mud, *ND* not determined
[a]Ion concentration in g/L
[b]Ion concentration in mg/g

values of the total COD from the aqueous sample (WS3). COD_t/BOD_5 ratio was < 2 for sites 1 and 2, indicating an easily biodegradable biotope. However, site 3 showed a ratio higher than 2, indicating a scarcely biodegradable biotope. When correlated, BOD_5 and the microbial load from aqueous samples showed positive values, but no correlation was shown between the microbial load and the total COD or BOD_5 from the solid samples.

Phenotypic and phylogenetic diversity analysis of the isolated bacteria

At first, a total of 227 pure strains were isolated based on their responses to different NaCl concentrations in the culture media. Moderate halophilic bacteria were the most represented category (100/227) with predominance in site 2 (56.4%), followed by site 1 (42.7%) and then site 3 (31.8%). Slight halophilic bacteria were more represented in site 3 (36.4%), followed by site 1 (34.3%) and site 2 (20.5%). Halotolerant bacteria were represented in the collection by 35/227 with a predominance in site 3 (31.8%) followed by site 2 (20.5%) and then site 1 (12.1%). Nineteen of 227 extremely halophilic bacteria have been isolated in this collection. This category was more represented in site 1 (10.8%) compared to site 2 (2.6%), and no extremely halophilic bacterium was isolated from site 3. Moreover, these frequencies appeared to be similar to the results obtained from marshes and salternes located in lower Loukkos in west of Morocco (8.6 %), and we noticed the same pattern with regards to the genera described in this study in comparison to those by Berrada et al. (2012). Eighty strains out of 227 are Gram-negative (with 41/80 being rods and 39/80 cocci), and 147/227 were Gram-positive (with 112/147 being rods and 35/147 cocci). Particularly, 189/227 strains have shown promising extracellular hydrolytic production. The strains of this sub-collection of 189 strains were characterized and positioned at genus level based on their phenotypic characteristics as described by Bergey's Manual of Systematic Bacteriology (Boone et al. 2001; De Vos et al. 2012; Whitman et al. 2012; Krieg et al. 2012). Figures 2a and 3a show the

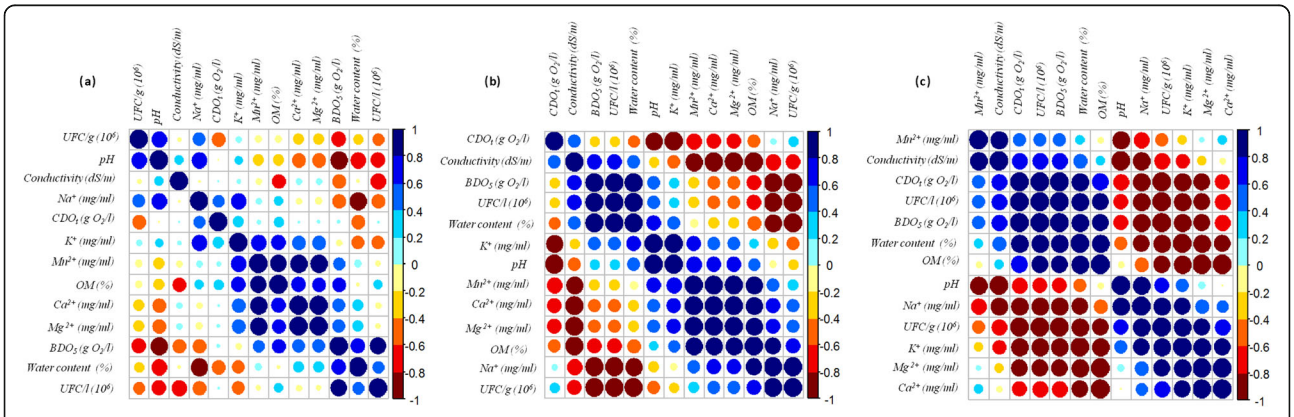

Fig. 1 Correlation matrix between the physicochemical properties and the bacterial load in the solid and aqueous samples in **a** site 1, **b** site 2, and **c** site 3

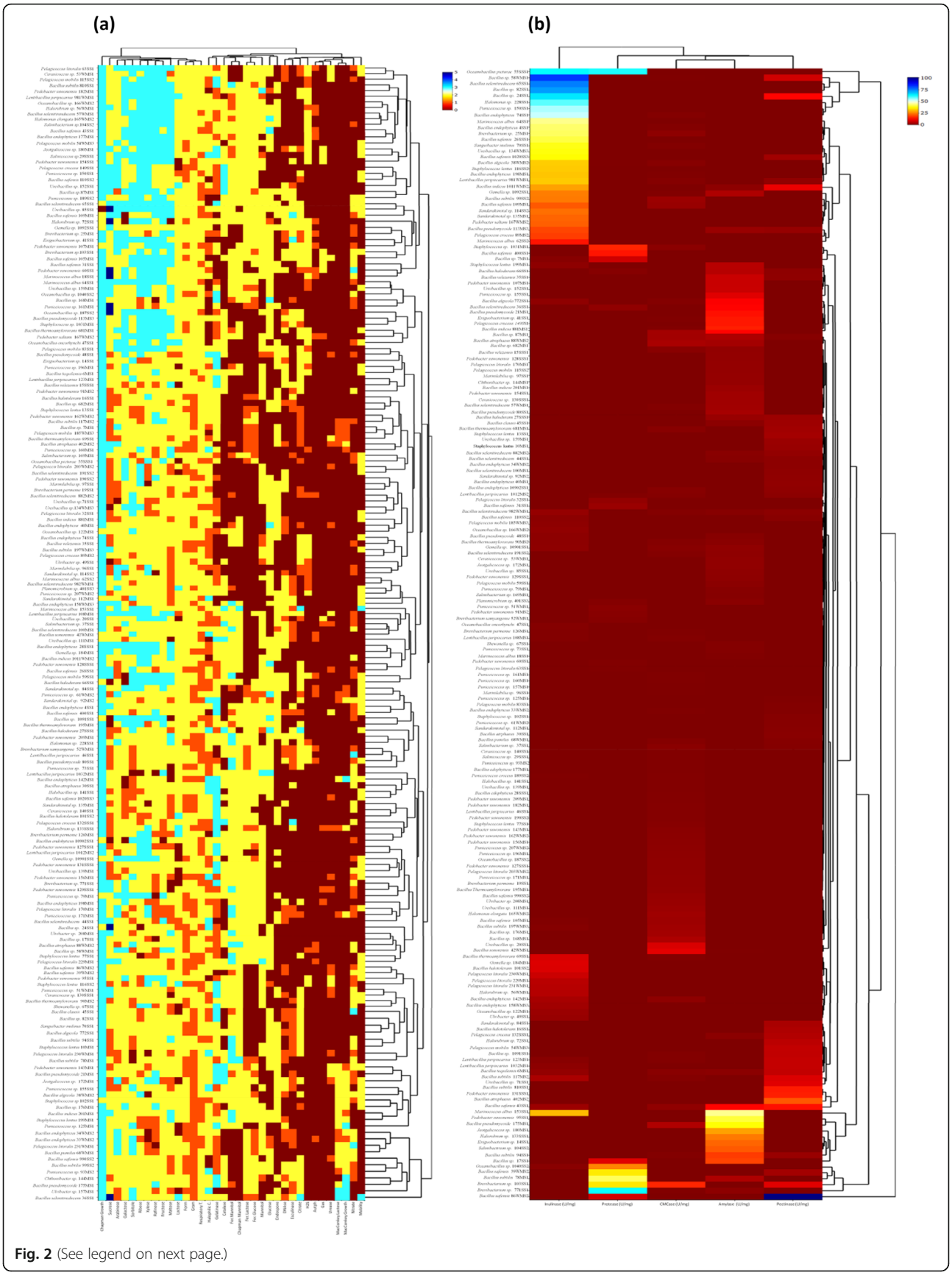

Fig. 2 (See legend on next page.)

(See figure on previous page.)
Fig. 2 Clustering of the 189 isolates based on their phenotypic characteristics (**a**) and based on their enzymatic activities values (**b**). Values in the scales for (**a**) indicate the magnitude of the response to the assay in a scale from 0 to 5, and values in the scales for (**b**) indicate the calculated enzymatic activities expressed in a scale from 0 to 100 U/mg

clustering of the 189 halophilic strains with hydrolytic activities, based on their phenotypic properties, and the clustering of the sub-collection of 56 strains, with high hydrolytic activities, respectively. The data analysis shows that the distribution is relatively redundant at the genus level along the three sites. *Bacillus* spp. were the most representative species in all three sites, in addition to *Chthonibacter* spp., *Mariniabilia* spp., *Halobacillus* spp., *Salinococcus* spp., *Cerasicoccus* spp., *Ulvibacter* spp., *Halorubrum* spp., *Jeatgalicoccus* spp., *Brevibacterium* spp., *Sanguibacter* spp., *Shewanella* spp., *Exiguobacterium* spp., and *Gemella* spp., which were obtained only from site 1. *Planomicrobium* spp. were present in site 3 only (Table 3). The analysis of the results displayed in Figs. 2 and 3 shows the predominance of bacteria belonging to the genera *Pedobacter*, *Pelagicoccus*, and *Puniceicoccus* as the representatives of the Gram-negative group. Gram-positive strains were dominated by the genera *Bacillus*, *Staphylococcus*, and *Lentibacillus*.

Phylogenetic analysis

Preliminary comparative phylogenetic relatedness of the selected halophilic bacteria with high hydrolytic enzyme production was carried out based on the 16S rDNA gene. 16S rDNA sequences corresponding to the size of 1420 bp from the studied strains, as well as from type strains (Table 4 and Table S1), were analyzed for their phylogenetic relatedness. As far as the sampling site is concerned, the data obtained from the phylogenetic analysis led us to classify the isolates in nine genera: *Bacillus*, 44.6% from site 1, 16.1% from site 2, and 3.6% from site 3; *Staphylococcus*, 14.3%; *Brevibacterium*, 5.35%; *Shewanella*, 1.8%; *Oceanobacillus*, 5.35%; *Planomicrobium*, 1.8%; *Exiguobacterium*, 3.6%; *Sanguibacter*, 1.8%; and *Halomonas*, 1.8%. We also found differences in the genera and species isolated (Fig. 2 and Fig. 3). For example, species of the genera *Brevibacterium*, *Exiguobacterium*, *Oceanobacillus*, *Sanguibacter*, and *Shewanella* were only found in site 1. The genus *Halomonas* was only found only in site 2, and *Planomicrobium* was only

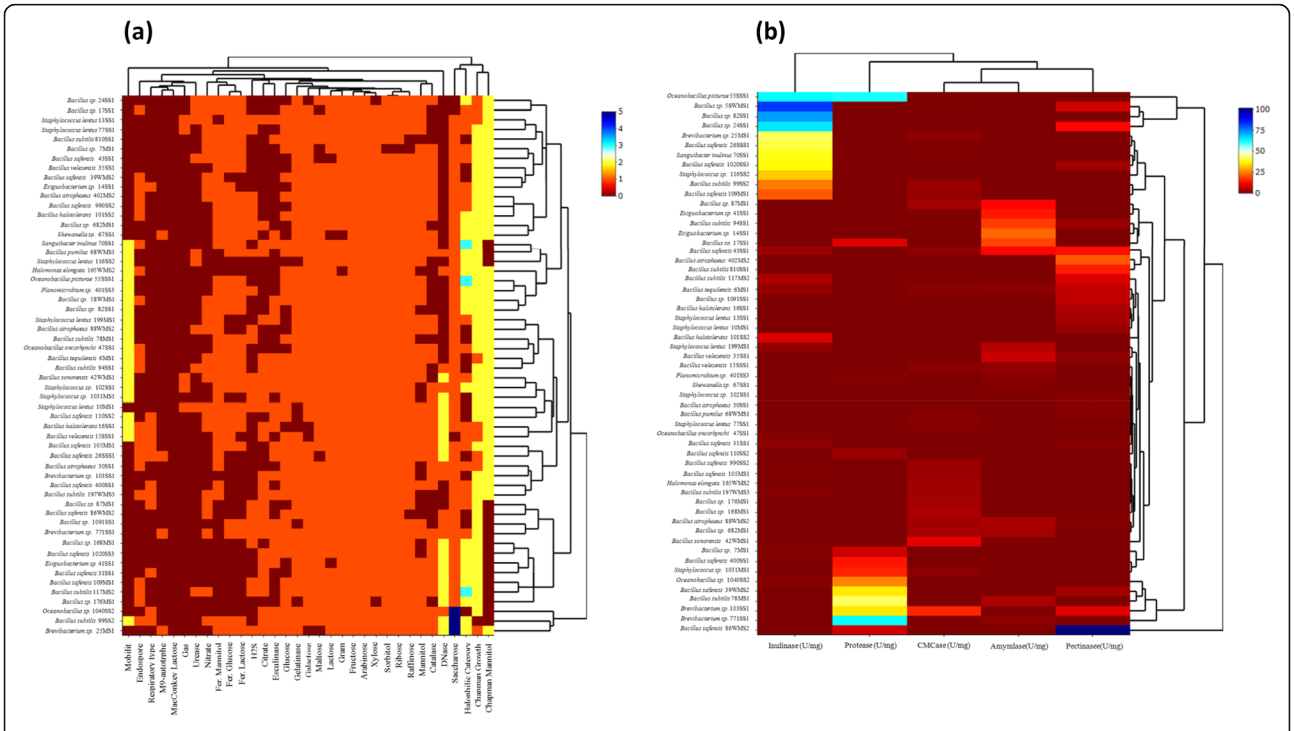

Fig. 3 Clustering of the 56 isolates based on their phenotypic characteristics (**a**) and on their enzymatic activities values (**b**). Values in the scales for (**a**) indicate the magnitude of the response to the assay in a scale from 0 to 5, and values in the scales for (**b**) indicate the calculated enzymatic activities expressed in U/mg

Table 3 Distribution of the different species showing hydrolytic activities according to their isolation site

Genus/species	Total number	Number per site		
		Site 1	Site 2	Site 3
Bacillus spp.	72	52	16	4
Pedobacter spp.	17	12	5	0
Pelagicoccus spp.	15	10	3	2
Puniceicoccus spp.	13	10	3	0
Ureibacillus spp.	8	7	0	1
Staphylococcus spp.	8	7	1	0
Lentibacillus spp.	6	5	1	0
Brevibacterium spp.	6	6	0	0
Oceanobacillus spp.	6	3	3	0
Sandarakinotalea spp.	5	3	2	0
Marinicoccus spp.	4	3	1	0
Gemella spp.	3	3	0	0
Salinibacterium spp.	3	2	1	0
Halorubrum spp.	3	3	0	0
Cerasicoccus spp.	3	3	0	0
Ulvibacter spp.	3	3	0	0
Halomonas spp.	2	1	1	0
Jeatgalicoccus spp.	2	2	0	0
Marinilabilia spp.	2	2	0	0
Exiguobacterium spp.	2	2	0	0
Salinicoccus spp.	1	1	0	0
Planomicrobium spp.	1	0	0	1
Chthonibacter spp.	1	1	0	0
Halobacillus spp.	1	1	0	0
Sanguibacter spp.	1	1	0	0
Shewanella spp.	1	1	0	0
Total	189	144	37	8

found in site 3. Nevertheless, genus *Staphylococcus* was isolated from sites 1 and 2. We applied the neighbor-joining criteria and obtained a phylogenetic tree, grouping the 56 sequences in eight groups (A–H) (Fig. 4). In the case of the phylogenetic tree constructed with the species of *Bacillus* (Fig. 5), we found 12 subgroups (A1–A12). The 16S rDNA gene sequences from *Bacillus* sp. strains strain 7MS1 (MK713705), *Bacillus* sp. strain 682MS1 (MK713707), and *Bacillus* sp. strain 176MS1 (MK713714), showed a relationship of less than 98% with other related type strains. Regarding the genus *Bacillus*, strains with closely similar 16S rDNA sequences displayed different biochemical characteristics, which is worth considering for future studies in order to resolve whether they constitute new species or subspecies within their related genera.

Group A, represented by strains of the genus *Bacillus*, was the dominant group (36) among the isolated strains. Figure 5 shows the neighbor-joining sub-tree of this group. *Bacillus safensis* was the major representative (27.77%) within this group, followed by *Bacillus subtilis* (16.66%), *Bacillus atrophaeus* (8.33%), and *Bacillus halotolerans* (5.33%). Species of *Bacillus velezensis* represented 5.55% and *Bacillus tequilensis* have been isolated from site 1 only. Group B was denoted by strains from the genus *Staphylococcus*, *as* the second dominant genus from the studied sites, represented mainly by *Staphylococcus lentus* but with a remarkable diversity in their biochemical characteristics, more specifically their hydrolase activities (Fig. 2 and Fig. 3). So far, species of the genus *Staphylococcus* have never been described as halophilic bacteria, but rather as halo-duric ones. Ten strains of *Staphylococcus* were largely halotolerant, tolerating NaCl concentrations from 0 to 300 g/L. Moreover, they showed themselves to be poly-halophilic, by being halotolerant, and also extremely halophilic, tolerating high salt concentrations ranging from 150 to 300 g/L of NaCl (Table 4). Group C is represented by three strains identified as genus *Oceanobacillus*. Strains *Oceanobacillus oncorhynchi* 47SS1 and *Oceanobacillus picturae* 55SSS1 were isolated from site 1 (salt mine) and showed different tolerance windows to salt concentrations, with a range of 10–210 g/L and 150–210 g/L, respectively. *Oceanobacillus* sp. 1040SS2, isolated from site 2, was characterized as a halotolerant bacterium with extended range of salt tolerance from 0 to 270 g/L.

One species, identified as a *Planomicrobium* sp. strain 401SS3 and isolated from site 3 (salt marsh), is the only representative of the group D. Strain 401SS3 showed a halophilic behavior with an optimal range of growth from 30–210 g/L. Genus *Planomicrobium* has been reported in specimens from marine environments (seafood, coastal sediments, Antarctic sea ice), with a tolerance range from 0 to 140 g/L (Yoon et al. 2015). Group E is represented by two species of the genus *Exiguobacterium.* This genus has been widely described as a diverse group found in different habitats, but less described in saline environments (Oren 2015; Ventosa et al. 2015; Remonsellez et al. 2018). Phylogenetic phenotypic analysis of *Exiguobacterium* sp. strain 14SS1 (MK713684) and *Exiguobacterium* sp. strain 41SS1 (MK713691) showed a relatively higher salt tolerance (10–180 g/L and 30–180 g/L, respectively), compared to reported strains from the same phylotype (Remonsellez et al. 2018). *Brevibacteria* have been isolated from several habitats, particularly from high salt concentration environments such as

Table 4 Accession numbers of the strains of the collection as well as their range of NaCl concentration for optimal growth and their enzymatic activities expressed in U/mg

Strain name	16S rDNA accession numbers	NaCl range tolerance for growth (g/L)	Cellulase (U/mg)	Amylase (U/mg)	Protease (U/mg)	Pectinase (U/mg)	Inulinase (U/mg)
Bacillus atrophaeus 30SS1	MK713688	10–60	0.00	0.00	0.00	0.97 ± 0.06	0.00
Bacillus atrophaeus 402SS2	MK713721	10–60	0.00	0.00	0.00	21.40 ± 3.02	0.00
Bacillus atrophaeus 88WMS2	MK713729	0–60	3.46 ± 0.17	1.73 ± 0.14	0.00	0.39 ± 0.04	0.00
Bacillus halotolerans 16SS1	MK713685	10–90	0.00	0.00	0.00	4.63 ± 0.13	0.00
Bacillus halotolerans 101SS2	MK713724	30–120	0.00	0.00	0.00	0.00	9.13 ± 1.46
Bacillus pumilus 68WMS1	MK713720	30–120	1.02 ± 0.20	0.00	0.00	0.39 ± 0.07	0.00
Bacillus safensis 105MS1	MK713711	10–60	2.96 ± 1.03	0.00	0.00	0.00	0.27 ± 0.12
Bacillus safensis 31SS1	MK713689	60–120	0.00	0.00	0.03 ± 0.01	0.00	0.00
Bacillus safensis 400SS1	ND	10–120	0.00	0.00	1.89 ± 0.25	0.00	0.00
Bacillus safensis 39WMS2	ND	0–120	0.00	0.00	0.83 ± 0.10	3.54 ± 0.34	0.00
Bacillus safensis 26SSS1	MK713716	0–180	0.00	0.00	0.00	0.00	41.28 ± 1.81
Bacillus safensis 110SS2	MK713725	0–210	0.00	0.00	0.00	0.88 ± 0.27	1.40 ± 0.22
Bacillus safensis 86WMS2	MK713728	0–60	0.00	0.00	0.03 ± 0.01	11.12 ± 0.49	0.00
Bacillus safensis 990SS2	MK713723	10–60	2.26 ± 0.84	0.00	0.00	0.00	0.00
Bacillus safensis 1020SS3	MK713731	30–120	0.00	0.00	0.00	3.40 ± 0.41	37.96 ± 6.97
Bacillus safensis 109MS1	MK713712	30–180	2.49 ± 0.18	0.00	0.00	0.00	21.44 ± 1.80
Bacillus sp. 24SS1	MK713687	30–210	0.09 ± 0.01	0.00	0.00	12.95 ± 0.06	65.37 ± 4.60
Bacillus sp. 17SS1	MK713686	10–60	0.00	3.23 ± 0.63	1.06 ± 0.13	0.00	0.00
Bacillus sp. 43SS1	MK713692	10–180	1.72 ± 0.54	12.98 ± 2.12	0.00	14.18 ± 2.30	2.86 ± 0.01
Bacillus sp. 82SS1	MK713699	30–150	0.00	0.00	0.00	0.00	72.89 ± 2.46
Bacillus sp. 682MS1	MK713707	30–120	0.90 ± 0.06	3.70 ± 1.23	0.00	0.00	0.00
Bacillus sp. 168MS1	MK713713	30–150	1.64 ± 0.21	0.00	0.00	0.00	0.00
Bacillus sp. 87MS1	MK713709	10–90	3.55 ± 1.45	12.98 ± 0.08	0.00	0.00	0.00
Bacillus sp. 7MS1	MK713705	10–150	0.00	0.00	0.46 ± 0.19	0.00	0.78 ± 0.01
Bacillus sp. 176MS1	MK713714	30–150	1.25 ± 0.06	0.00	0.00	0.00	0.00
Bacillus sp. 42WMS1	MK713718	10–120	10.02 ± 2.24	0.03 ± 0.01	0.00	0.00	0.00
Bacillus sp. 58WMS1	MK713719	30–120	0.07 ± 0.05	0.00	0.00	7.93 ± 1.34	66.51 ± 0.87
Bacillus subtilis 94SS1	MK713700	0–120	0.01 ± 0.00	0.90 ± 0.03	0.00	0.21 ± 0.07	0.00
Bacillus subtilis 78MS1	MK713708	0–120	0.00	0.77 ± 0.15	0.03 ± 0.15	0.00	0.00
Bacillus subtilis 99SS2	MK713722	60–200	2.75 ± 0.87	0.00	0.00	0.00	24.78 ± 7.05
Bacillus subtilis 810SS1	MK713698	0–120	0.00	0.00	0.00	7.16 ± 0.63	0.00
Bacillus subtilis 117MS2	MK713727	150–210	0.54 ± 0.15	0.00	0.00	9.13 ± 0.62	4.26 ± 1.01
Bacillus subtilis 197WMS3	MK713732	10–90	3.27 ± 0.62	0.00	0.00	0.00	0.27 ± 0.16
Bacillus tequilensis 6MS1	MK713704	0–270	0.96 ± 0.18	1.18 ± 0.66	0.00	6.69 ± 0.93	2.01 ± 0.11
Bacillus velezensis 35SS1	MK713690	0–90	0.00	2.91 ± 0.41	0.00	1.55 ± 0.09	0.00
Bacillus velezensis 15SSS1	ND	10–90	1.03 ± 0.17	1.84 ± 0.10	0.00	0.00	0.00
Brevibacterium sp. 103SS1	MK713702	0–120	16.33 ± 4.77	0.00	0.28 ± 0.05	9.71 ± 2.91	0.00
Brevibacterium sp. 771SS1	MK713697	10–90	0.31 ± 0.14	1.39 ± 0.29	0.35 ± 0.06	0.00	0.00
Brevibacterium sp. 25MS1	ND	30–210	0.09 ± 0.00	0.00	0.00	0.00	0.07 ± 0.11
Exiguobacterium sp. 14SS1	MK713684	10–180	0.00	0.09 ± 0.02	0.00	0.00	0.00
Exiguobacterium sp. 41SS1	MK713691	30–180	0.16 ± 0.05	14.93 ± 2.57	0.00	0.00	0.00
Halomonas elongata 165WMS2	ND	60–270	3.16 ± 0.34	0.00	0.00	0.00	0.00
Oceanobacillus oncorhynchi 47SS1	MK713693	10–210	0.00	0.00	0.00	0.00	0.44 ± 0.10

Table 4 Accession numbers of the strains of the collection as well as their range of NaCl concentration for optimal growth and their enzymatic activities expressed in U/mg *(Continued)*

Strain name	16S rDNA accession numbers	NaCl range tolerance for growth (g/L)	Cellulase (U/mg)	Amylase (U/mg)	Protease (U/mg)	Pectinase (U/mg)	Inulinase (U/mg)
Oceanobacillus picturae 55SSS1	MK713717	150–210	0.00	0.00	1.64 ± 0.18	0.00	31.13 ± 3.32
Oceanobacillus sp. 1040SS2	ND	0–270	0.00	0.00	0.49 ± 0.11	0.00	0.49 ± 0.11
Planomicrobium sp. 401SS3	MK713730	30–210	0.00	0.09 ± 0.03	0.01 ± 0.00	0.00	0.00
Sanguibacter inulinus 70SS1	MK713695	150–210	0.35 ± 0.23	0.00	0.00	0.00	25.74 ± 0.44
Shewanella sp. 67SS1	MK713694	30–210	0.00	0.01 ± 0.00	0.00	0.00	0.00
*Staphylococcus lentus*13SS1	MK713683	10–210	0.00	0.00	0.00	3.62 ± 0.12	0.00
*Staphylococcus lentus*77SS1	MK713696	10–150	0.05 ± 0.02	0.00	0.00	0.00	0.00
*Staphylococcus lentus*10MS1	MK713706	0–300	0.00	0.00	0.00	2.51 ± 0.24	0.00
*Staphylococcus lentus*199MS1	MK713715	10–150	0.00	5.56 ± 1.08	0.00	0.00	1.46 ± 0.06
Staphylococcus lentus 116SS2	MK713726	30–150	0.00	0.00	0.00	0.00	0.36 ± 0.14
Staphylococcus sp. 102SS1	MK713701	30–150	0.00	0.00	0.00	0.71 ± 0.05	0.00
Staphylococcus sp. 1031MS1	MK713710	0–150	1.18 ± 0.04	0.00	0.16 ± 0.01	0.00	0.00
Staphylococcus sp. 1091SS1	ND	0–180	0.44 ± 0.12	0.00	0.00	6.50 ± 1.39	0.00

ND not determined

sea water (Lee 2008) and sediment samples (Küster and Williams 1964). The genus *Brevibacterium* (group F) is represented by three species denoted *Brevibacterium* sp. strain 103SS1, *Brevibacterium* sp. strain 771SS1, and *Brevibacterium* sp. strain 25MS1 and were found in Mount Zalagh only. NaCl tolerance of these strains ranges 0–120 g/L, 10–90 g/L, and 30–210 g/L, respectively, as compared to reports on strains from the same genus ranging from 50 to 180 g/L (Trujillo and Goodfellow 2015). *Sanguibacter inulinus* strain 70SS1 as a sole representative of the group G shows a slightly high salt tolerance ranging from 150 to 210 g/L. Data on halophilic bacteria from this genus remains scarce, with some species of this genus being described as slightly halotolerant, within the range of 0 to 70 g/L of NaCl (Ramos et al. 2015). Group H was represented by *Shewanella* sp. strain 67SS1, isolated from site 1. It shows a broad range of NaCl tolerance from 30-210 g/L, in contrast to most species of this genus described as slightly halotolerant with average NaCl tolerance range from 0 to 60 g/L (Bowman 2015). Generally, species of *Shewanella* have been described to be strictly of marine origin, and several species of this genus *Shewanella* have been isolated from various saline habitats, including salt marshes (Rosselló-Mora et al. 1995), from marine algae (Simidu et al. 1990), seawater, and salted food products (Vogel et al. 1997). Phylogenetic and phenotypic data clustering showed divergent results, thus indicating remarkable diversity within the isolated strains in this work, in line with what has been discussed by other reports (Berrada et al. 2012).

Hydrolytic activities of halophilic bacteria

The qualitative test on agar plates allowed the determination of the 189 strains (out of the 227 of the collection) demonstrating at least one of the fives tested activities. As shown if Fig. 6, the isolated bacteria showed differences in the production and release of hydrolytic enzymes as function of NaCl concentrations. Actually, conventional assay for pectinases production in agar media using copper acetates failed to reveal the clear zone at high NaCl concentrations in the medium. Instead, layering the agar plate with iodine reagent has succeeded to reveal the clear zone that indicates the breakdown of PGA to their reducing sugars (Fig. 6d, d'). The results of the production and activity in submerged cultures confirmed the activities in the conditions tested. Figure 2b and Fig. 3b show the clustering of the 56 halophilic bacteria (collection with 16S rDNA sequenced) and of the 189 halophilic strains, respectively, based on quantitative assays for hydrolytic enzyme production in submerged cultures. All the strains were able to produce hydrolytic enzymes within their range of NaCl tolerance. Out of the 189 strains with hydrolytic activity, 42.85% were cellulase producers, 33.86% were amylase positive, 33.33% were inulinase positive, 38.04% were pectinase positive, and 12.16% were proteases positive. 93.75% of the Gram-negative strains showed hydrolytic activities as opposed to 77.55% for the Gram-positive ones.

Table 4 shows the enzymatic activities (as U/mg of total proteins from the cell free medium) of the 56 isolates. The strains showed the capacity to produce, in

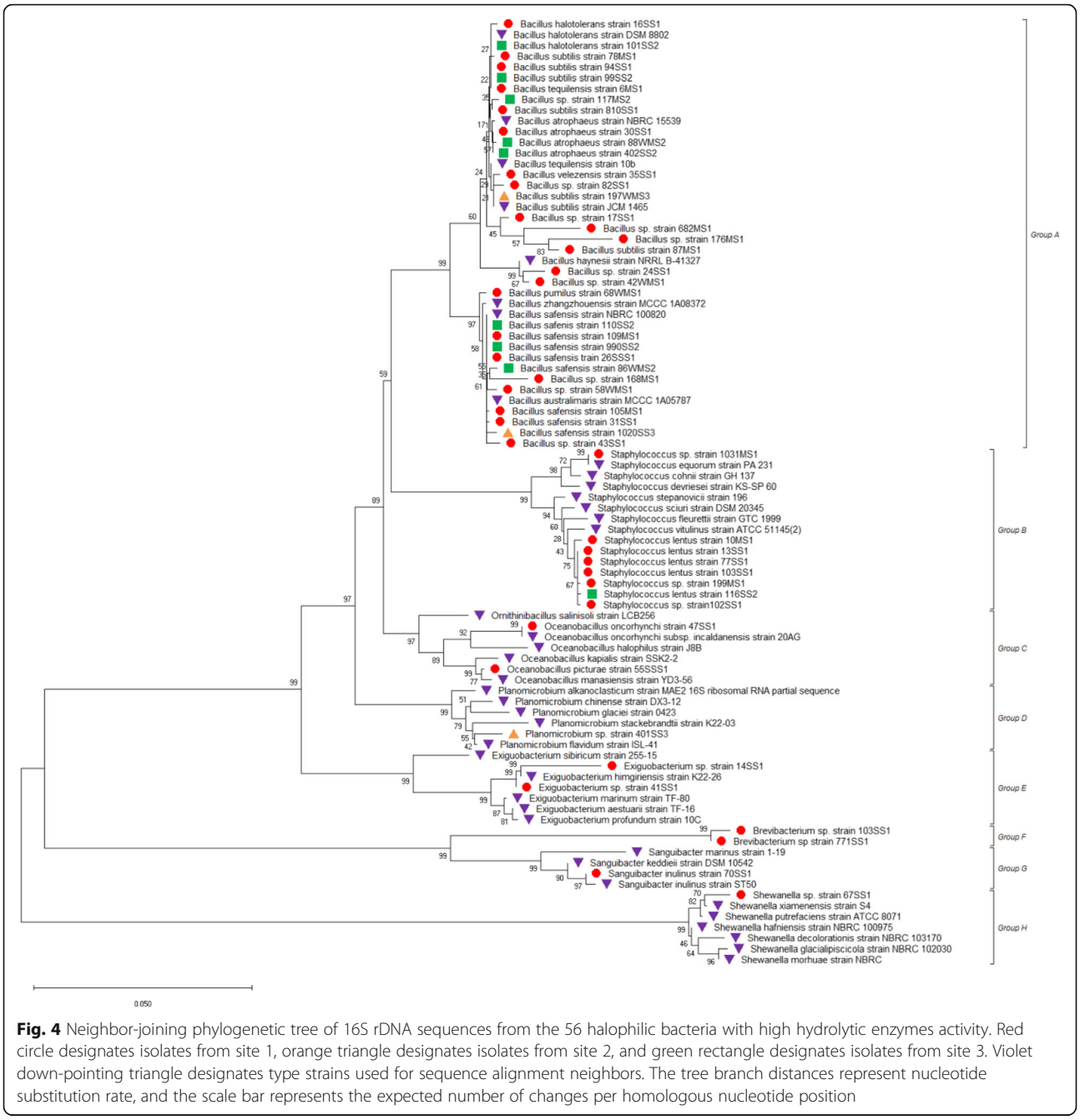

Fig. 4 Neighbor-joining phylogenetic tree of 16S rDNA sequences from the 56 halophilic bacteria with high hydrolytic enzymes activity. Red circle designates isolates from site 1, orange triangle designates isolates from site 2, and green rectangle designates isolates from site 3. Violet down-pointing triangle designates type strains used for sequence alignment neighbors. The tree branch distances represent nucleotide substitution rate, and the scale bar represents the expected number of changes per homologous nucleotide position

some cases, more than one activity, which can be a way of adaptation to such harsh conditions. Some bacteria develop diverse ability to cope with the low nutriment availability, like motility to acquire nutriments rapidly or the ability to use different carbon and energy sources (Vera-Gargallo and Ventosa 2018). The moderately and the slightly halophilic bacteria showed remarkable hydrolytic activities compared to the extremely halophilic strains. The genera *Bacillus*, *Pedobacter*, *Pelagicoccus*, *Puniceicoccus*, *Salinibacterium*, and *Brevibacterium* isolates presented more than one hydrolytic activity pattern. Inulinase, cellulase, pectinase, amylase, and protease activities were expressed by numerous strains belonging to 18, 17, 16, 16, and 10 different genera respectively (Table 4 and Figs. 2b and 3b).

As shown in Table 4, data related to the enzymatic performances of the isolated species in this study from different samples exhibited differences in their enzymatic behaviors as well as their tolerance to salt concentration (Ortega et al. 2011). Such data could be linked to the

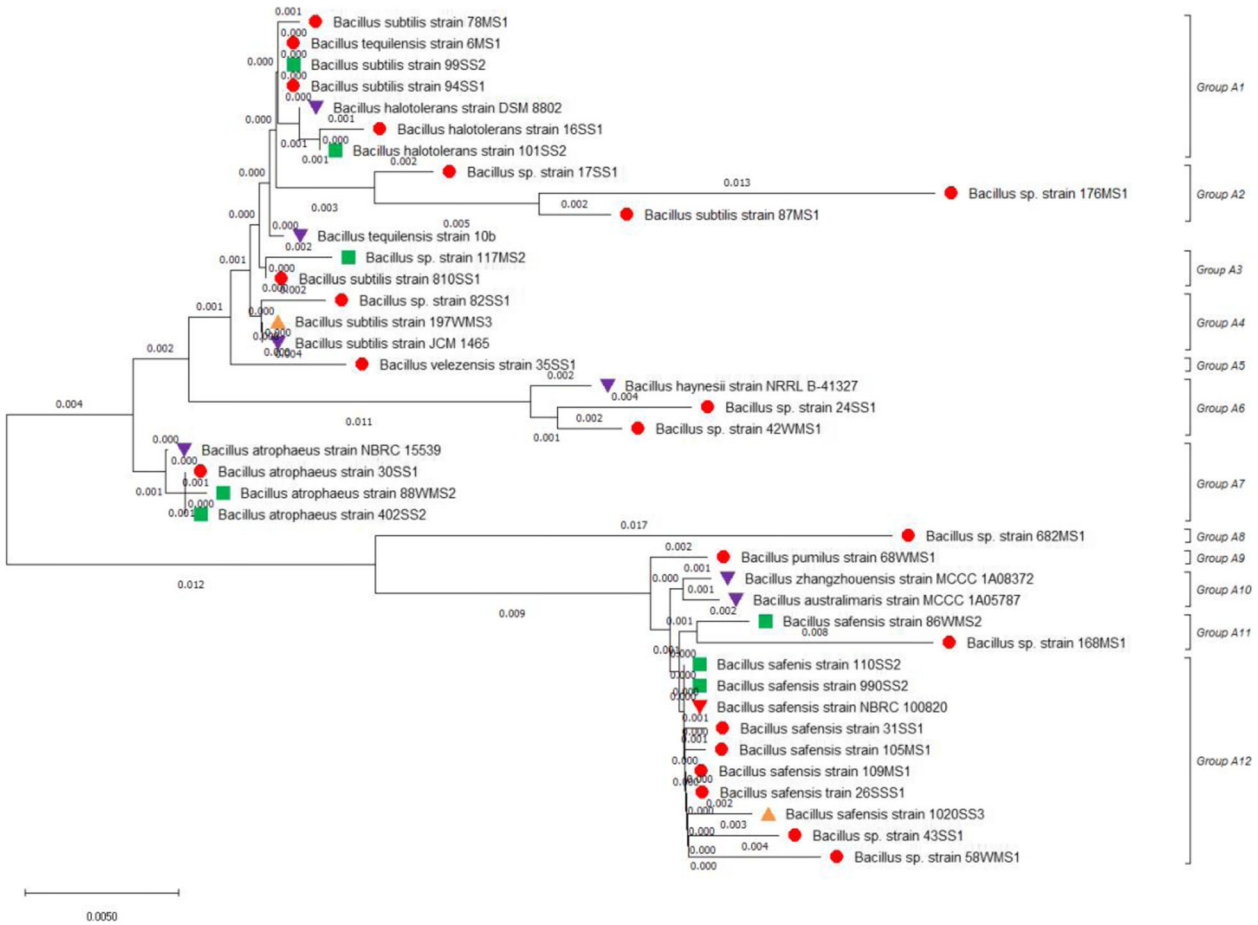

Fig. 5 Neighbor-joining phylogenetic tree of 16S rDNA sequences from diverse halophilic bacteria from group A. Red circle designates isolates from site 1, orange triangle designates isolates from site 2, and green rectangle designates isolates from site 3. Violet down-pointing triangle designates type strains used for sequence alignment neighbors. The tree branch distances represent nucleotide substitution rate, and the scale bar represents the expected number of changes per homologous nucleotide position

effect of the environment on the rearrangement of genetic information to induce/improve the performances of hydrolytic enzymes needed for the breakdown of complex polymeric substrates (He et al. 2010). The qualitative enzymatic assays showed that the categories of slightly halophilic and halotolerant bacteria were highly represented among hydrolytic halophilic bacteria (47.4%, 39.6%, and 41;2% for CMCase activity; 38.15%, 33.3%, and 23.5% for amylase activity; 35.5%, 22.9%, and 23.5% for pectinase activity; and 13.15%, 12.5%, and 5.9% for protease activity, respectively). Extremely halophilic bacteria showed the highest percentage for the inulinase activity, with 52.9% versus 26.04% for the moderate halophilic and 38.15% for the slightly halophilic and halotolerant. This result could suggest an adaptation of such halophilic bacteria to harsh environments where hardly degradable substrates such as inulin are most dominants rather than easily metabolized substrates. Furthermore, we noticed an increase in the number of isolates having extracellular enzymatic activities in high NaCl concentration (see Table 3). When assayed in liquid culture media, all isolates reproduced the same activities as agar media, nevertheless, is not quite sufficient to compare between the strains, but it gives qualitative insights toward the expressed enzymatic activity. The difference in activity seen within the same genera, and sometimes species, can be explained by the inoculation that was made by a colony. Halotolerant and slightly halotolerant isolates showed the highest enzymatic activities compared to the moderately and extremely halophilic bacteria. Actually, the main enzymatic activities for the slightly halophilic and halotolerant groups were for cellulase and amylase assays. In contrast, most of the moderately halophilic strains showed moderate cellulase enzyme production and high inulinase,

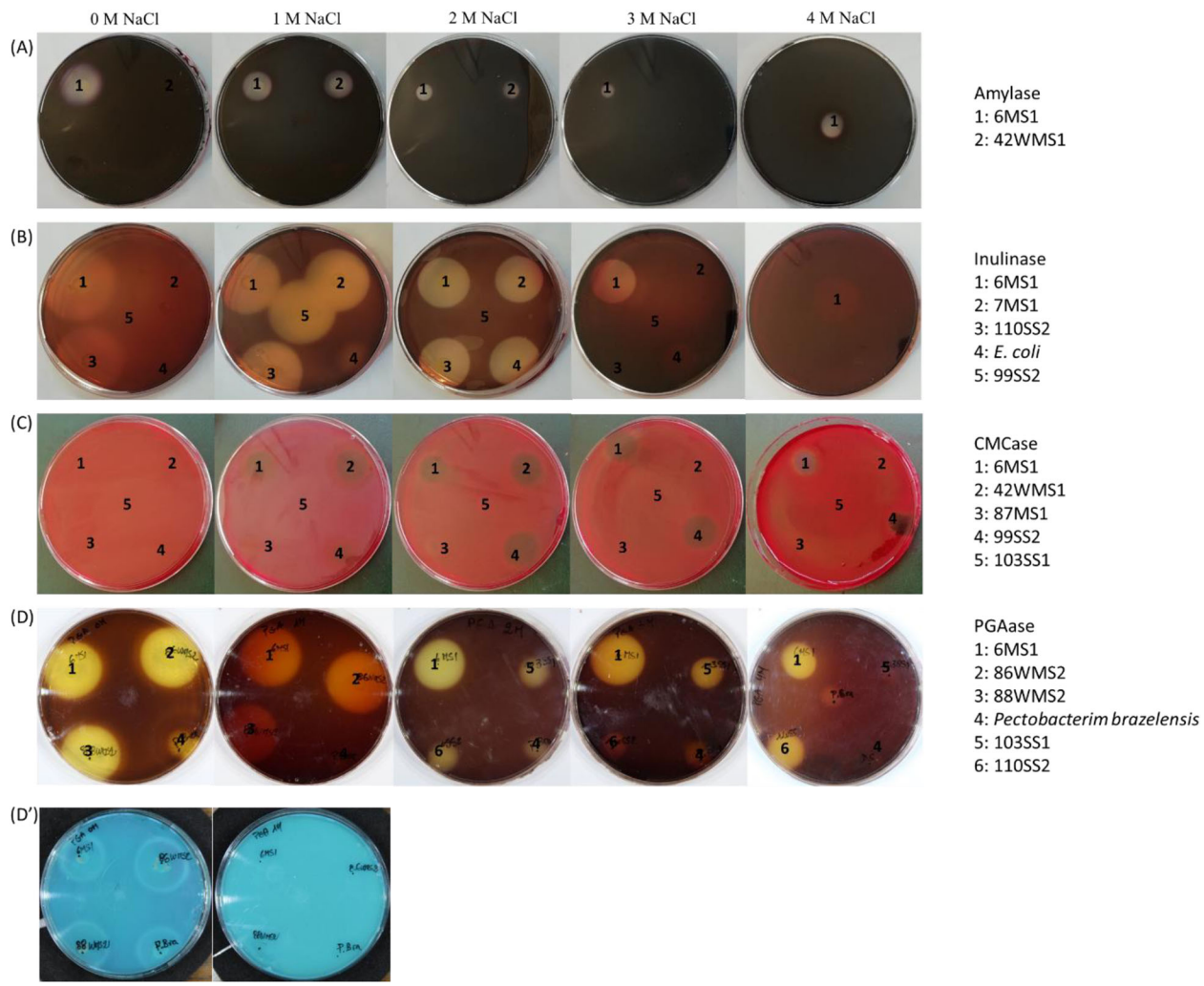

Fig. 6 Monitoring of hydrolytic enzymes production by typical halophilic bacteria from this study as function of NaCl concentration. **a** amylase activity, **b** inulinase activity, **c** CMCase activity, **d** PGA activity revealed with copper acetate solution, and **d'** PGA activity revealed with iodine reagent. A zone of clearance surrounding the colonies is indicative of the indicated activity

amylase, pectinase, and protease production. Extremely halophilic strains showed low cellulase production, moderate pectinase and protease, and high inulinase-degrading enzymes. The variation of enzyme activities for the same genera and species, especially for *Bacillus* and *Staphylococcus*, can also be explained by the wide diversity of physiological abilities (De Vos et al. 2012) and some minor factors like the evaporation from liquid cultures in media with high salinity that can lead to changes in salt concentrations with time.

Conclusion

This study allowed the isolation of a bacterial collection ranging from moderately to extremely halophilic bacteria. The subset of a collection from hypersaline environments in the pre-Rif region, Morocco, exhibiting hydrolase activity, was clustered into 26 genera. Our results relied mainly on varying NaCl composition within the isolation medium, but we believe that other isolation media might potentially generate different results. Data analysis and correlation from this study indicates the following:

1. Hypersaline environments harbor an important number of halophilic bacterial consortia, sharing different NaCl tolerance;
2. A large number of hydrolytic enzyme producers could be isolated frequently from hypersaline environments; and
3. The hydrolytic activities and efficiency correlate with the NaCl concentrations.

Moreover, this study gives insight on the relationship(s) between hypersaline environments and culturable bacteria that they contain. This reveals a wide diversity of halophilic bacteria from terrestrial hypersaline environments, fortunate enough to correlate with data from marine microbiology, thanks to the occurrence of several species frequently isolated from marine environments. The promising enzymatic activities displayed by these strains in the experimental conditions are often correlated to salt concentrations allowing such strains to operate under harsh conditions.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13213-020-01570-z.

Additional file 1: Figure S1. Location of the sampling region. **Figure S2.** Images of the sampling settling pond of the three sites. **Table S1.** Accession numbers of the designations type strains used as the closest neighbors as well as their range of NaCl concentration for optimal growth. ND: Not Determined.

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Authors' contributions
LBDK carried out the microbiological, chemical and biochemical studies, participated in the phylogenetic studies and drafted the manuscript. JA carried the microbiological, chemical and biochemical studies, carried out the phylogenetic studies, performed the statistical analysis, drafted and proof read the manuscript. MEH participated in the manuscript drafting and its proof reading. KS participated in the manuscript drafting and its proof reading. All authors read and approved the final manuscript.

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Competing interests
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Author details
[1]Faculty of Sciences Dhar El Mahraz, Laboratory of Biotechnology, Environment, Agri-Food and Health, Sidi Mohamed Ben Abdellah University, Fez, Morocco. [2]Pole of health, Euromed School of Engineering, Euro-Mediterranean University of Fez, Fez, Morocco. [3]School of Science and Engineering, Laboratory of Biotechnology, Al Akhawayn University in Ifrane, Ifrane, Morocco.

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