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Halobacterium Identification in Saltworks of Gran Canaria (Canary Islands, Spain)

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ARTICLE INFO	ABSTRACT
	This work analyzes bacterial communities present in evaporation ponds of solar salterns of Gran Canaria and reveals specific organisms through molecular techniques. Solar salterns are protected areas in Canary Islands where salt is produced from sea water by solarand windpowered evaporation. Salt was an important product for ancient islanders who used it for a broad field of purposes, but also has a great importance in recent time for its implications in the island economy. Based on amplifications with specific primers for 16S ribosomal DNA (16S rDNA) and subsequent nested-PCR approaches, different amplicons were obtained, and analyzed in silico. A taxonomic classification was carried out through phylogenetic trees. Results revealed different bacteria according to the evaporation grade of crystallizer ponds in saline works. It is worthstanding the presence of the genus *Halobacterium* in all crystallizer ponds. This opens an interesting framework for further studies and continuative molecular characterization approaches of bacterial communities in solar salterns of Gran Canaria.

1. Introduction

Traditional solar salterns still have a high socioeconomic impact due to their primary and secondary products. There are numerous ways of extracting salt from seawater, however this work is focusing on salt extraction from seawater using multipond solar salterns (salt works or salterns). This process involves the selective recovery of pure NaCl, whilst seawater is evaporated in artificial pond systems through natural evaporation driven by insolation and wind.

Multipond salterns create a gradient of salt concentrations which has an impact on the environment and their microbial population [1]. Ponds not only serve as refuge zones for migratory birds, but also host the halophilic unicellular green algae *Dunaliella* grown as a source of valuable chemicals [2]. There have been various studies regarding the microbial populations of crystallizer ponds around the world [3-5], the contribution of halobacterial pigments to the color of the water of crystallizer ponds [6,7] and the interrelationships between *Dunaliella* and halophilic prokaryotes in saltern crystallizer ponds [8], just to mention a few of them.

Furthermore the increase in salinity in different ponds is accompanied by a decrease in prokaryotic diversity, from the marine biota to the dense populations of halo-

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philic Archaea and bacteria [5]. High salinity and long-term selection pressure, as a result of evaporation, have resulted in specific metabolic mechanisms that render biologically active substances which are directly related to their halophilic behavior [9]. Halophilic bacteria and archaea are, for instance, useful biological sources of carotenoid pigments [10,11].

Evaporation of seawater also leads to crystallization of salts contained, process based on their varying solubility. The thin layer on the surface of the salt tide only forms in traditional salt works during the continuous evaporation and has to be harvested daily by hand. Provision of salt for consumption to the marketplace, "flower of salt" can be obtained from this thin layer. Even more, it is highly valuable and offers wealth and work to the local population.

Moreover, secondary salts, so called bitterns, which are used for chemical industry can be obtained and betacarotenes and glycerol can be extracted from microalgae and halobacteria inhabiting pond systems [2]. Red bacteria of the *Halobacterium – Haloferax – Haloarcula* group contribute to the red color of saltern crystallizer ponds which increases light absorption by the brine. This leads to an increase of temperature and enhances the salt production process so that the microbial composition is of interest for operating solar salterns. Even purely aesthetic considerations make it interesting to study the highly diverse communities of microorganisms in salterns, since the broad range of red shades beautify the landscape and attracts tourism [2,7].

No screening of the bacterial community in solar saltworks of Gran Canaria (Spain) has been conducted until now. The hypothesis of this work is that halophilic bacteria can be characterized in order to thereupon isolate and identify specific species with biotechnological methods. Since the source of water, the Atlantic Ocean, as well as the conditions of wind and temperature are nearly the same for both solar salterns, it is presumed that the organisms contained in the crystallizer ponds of Vargas and Tenefé are equal. The aim of this work is to screen bacteria from crystallizer ponds featuring different salinities, fatty layers, and dried salt in order to characterize specific bacteria.

2. Methodology and Methods

2.1 Solar Salterns on Gran Canaria

The Canary Islands are located in the southeastern sector of the North Atlantic Ocean, approximately between $27°$ to $29°$N and $14°$ to $18°$W and are closed to the occidental African coast. They are highly influenced by the dominant northeast trade winds associated with the Azores High Pressure Area resulting in strong and frequent wind and a high number of hours of sunshine. Gran Canaria Island (Figure 1A), with its area of 1560 km^2, is located between 27.7°28.2°N and 15.3°15.9°W and features perfect conditions for solar salterns [12]. Samples processed in this work were taken on Gran Canaria southeast coast (Figure 1B) at the solar salterns of Vargas (Figure 1C) and Tenefé (Figure 1D).
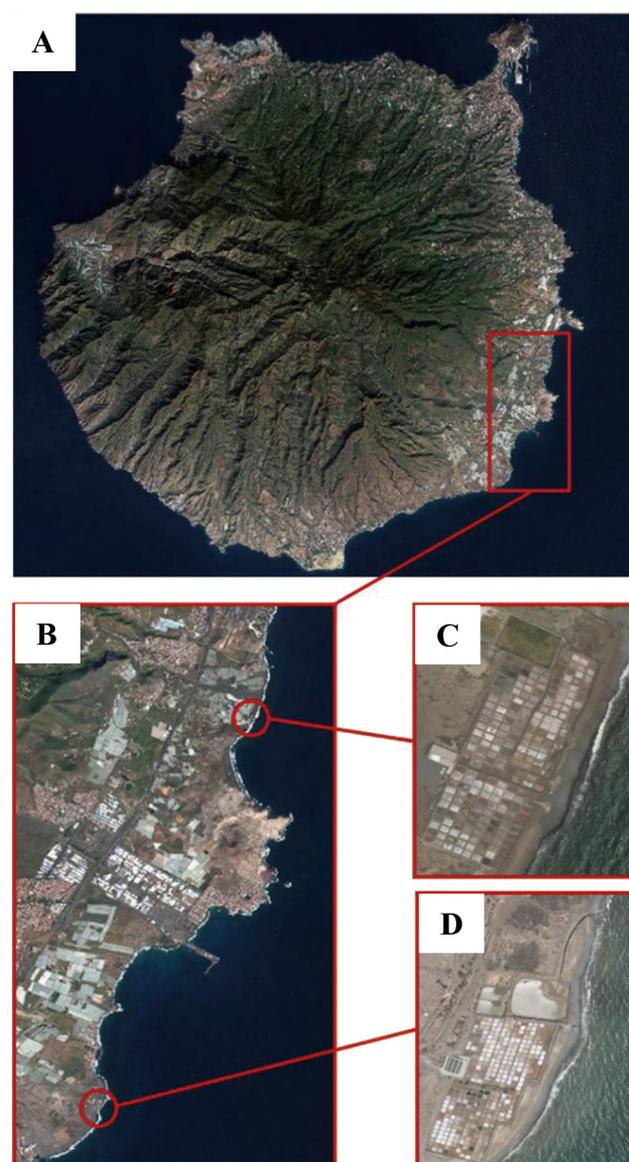

Figure 1. (A) Satellite images of Gran Canaria Island; (B) Southeast coast Gran Canaria Island; (C) The solar salterns of Vargas; (D) The solar salterns of Tenefé

2.2 Collection of Samples in Local Saltworks

Solar saltworks consist of a single series unconnected ponds. Ponds were selected according to their progress of

evaporation, representing habitats of different specificity due to the changing salt concentrations. The selection and classification were carried out through previous visualization and photographic recording. These were ordered by increasing salinity and denoted as station 1 (ST1; Supplementary Figure 1A), station 2 (ST2; Supplementary Figure 1B), and station 3 (ST3). The latter was subdivided into station 3.1 (ST3.1; Supplementary Figure 1C) representing lipid layers on the surface and station 3.2 (ST3.2; Supplementary Figure 1D) representing water and lipids from the crystallizer pond. Moreover dried salt was also analyzed (SAL2; Supplementary Figure 1E)

One liter of water was taken at each sampling site and subsequently centrifuged (BECKMAN Avanti™ J-25) at 20000 rpm for 10 min. Pellets were then pooled and two grams (c.a.) were put into a 5 ml screw cap tube, filled up with lysis buffer (50 mM TRIS HCl, 2 mM EDTA, 0.1% SDS and 1% TRITON X-100) and afterwards stored at -20 °C until use.

2.3 Isolation of DNA

DNA extraction was performed following the previously described in Garcia-Jimenez et al. [13]. DNA from each of sampling site were separately isolated. This way, water was centrifuged and resulting pellet was homogenised in liquid nitrogen and then incubated in 800 µl of isolation solution containing 100 mM Tris-HCl (pH 8.2), 4 M NaCl, 20 mM EDTA, CTAB (2%, w/v), PVPP (0.1%, w/v), SDS (0.1%, w/v) and mercaptoethanol (2%) in a water bath at 65 ºC for 1 h. A volume of chloroform:isoamyl alcohol solution (24:1 v/v) was added and the samples were gently mixed by inversion at intervals of 20 s. The mixture was then centrifuged for 10 min at 3000 rpm in a Beckman Coulter Allegra X-22R centrifuge (Beckman Coulter Inc. Brea, CA USA). Successive washings with chloroform:isoamyl alcohol (24:1 v/v) solution were performed. The supernatant was then placed in a fresh tube and an equal volume of n-propanol (-20 °C) was added, mixed gently and centrifuged at 13000 g for 30 min. The resulting pellet, containing DNA, was washed with ethanol (80%, v/v, molecular grade), dried and suspended in sterile deionised water. DNA yield were assessed using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). All samples were in triplicate. Purity DNA was valued by smear absence migrating on a 0.8% (w/v) agarose gel.

2.4 Amplification with 16S rRNA and Specific Primers

DNA (90-95 ng) was amplified using oligonucleotide pairs, 16S rRNA-F as a forward primer, and 16S rRNA-R as a reverse primer (Table 1).

Table 1. Sequences of the forward (F) and reverse (R) primers, for 16S rRNA gene

Target	Direction	Primer Pair	Nucleotide Sequence 5' → 3'	Reference
Archaea	Forward	BF27	AGAGTTTGATCCTGGCTCAG	[14]
	Reverse	BR1462	TCCAGCCGCAGATTCCCCTAC	
Bacteria	Forward	BF27	AGAGTTTGATCCTGGCTCAG	[14]
	Reverse	B765R	CTGTTTGCTCCCCACGCTTTC	[15]
Archaea	Forward	B704F	GTAGCGGTGAAATGCGTAGA	[15]
	Reverse	BR1462	TCCAGCCGCAGATTCCCCTAC	[14]
Bacteria	Forward	B22F	ATTCCGGTTGATCCTGC	[15]
	Reverse	B1521R	AGGAGGTGATCCAGCCGCAG	
Archaea	Forward	BF8	TTGATCCTGCCGGAGGCCATTG	[14]
	Reverse	BR1462	TCCAGCCGCAGATTCCCCTAC	

Amplification was performed in a GenAmp 2400 thermal cycler (PerkinElmer Inc., USA) with 30 cycles consisting at 95 °C for 1 min, 48 °C for 1 min, and 72 °C for 1.5 min, followed by a final extension step of 5 min at 72 °C. Each PCR reaction mixture contained 0.5 U Takara Ex Taq DNA polymerase (TaKaRa Shuzo Co., Shiga, Japan), 2.5 mM dNTP, 10 µl Takara Ex Taq PCR buffer with MgCl2, 10 µM each forward and reverse primers and DNA template.

Amplification products corresponding to 16S rRNA were purified through QIAEX agarose gel extraction kit (Qiagen Inc., Hilden, Germany) according to manufacturer. Sequences were used for designing new primer pair for nested PCR. The specific primer pairs used and annealing temperatures, including putative bacterial species associated, are shown in Table 2.

Table 2. Specific Primer Pairs design in this study with correlating Bacterial Species

Target	Direction	Primer Pair	Tm [°C]	Ta [°C]	Sequence 5' - 3'
Halobacterium salinibacter	Forward	BACTF	60,5	52	GTCCGGGGTAGGAGTGAAAT
	Reverse	BACTR	56,4		CCCGCCAATTCCTTTAAGTT
Halobacterium cutirubrum	Forward	HCF	70,7	51	ATTCCGGTTGATCCTGCCGGAGGTC
	Reverse	HCR	64,6		GATCCAGCCGCAGATTCCCC
Halorubrum salinum	Forward	HSALIF	60,5	52	GGTAGTCCTGGCCGTAAACA
	Reverse	HSALIR	60,5		AGGTCATCAACCTGGTCGTC
Salinobacter	Forward	SALIBF	58,4	51	CAGGAATAAGCACCGGCTAA
	Reverse	SALIBR	56,1		ACATGCTCCACCGCTTGT

PCR products were visualised by agarose gel electro-phoresis. In addition, amplification products were obtained and purified using the QIAEX agarose gel extraction kit. The fragments were then ligated to the pGEM-T-easy cloning vector (Promega, Wisconsin, USA) and cloned in JM109 cells according to the manufacturer's instructions (Promega). Plasmids were isolated using a plasmid purification kit (Qiagen Inc.). The insert in the plasmid was checked by PCR using primers M13F and M13R (Promega). The insert was then sequenced on both strands using an ABI-310 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA) and BigDye Terminator v3.1. Nucleotide sequences were submitted to NCBI GenBank BLAST search and identified through similarity values. Alignment of sequences was performed with ClustalX v.1.7 [16] using the default settings and was further refined by visual inspection. The alignment output was used to generate a phylogenetic tree based on the Maximum Likelihood method and General Time Reversibility model [17] as implemented in MEGA X [18]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed [19]. Branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates collapse. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [19]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences between sites (2 categories (+G, parameter = 0.1000)).

3. Results and Discussion

The saline waters of the solar salterns in Gran Canaria have a marine origin with a gradient of salt concentrations according to evaporation. The appearance of salt produced in Vargas and Tenefé (Gran Canaria) ranged from a pink color gradient to strong intense pink in relation to the salinity increment in different ponds. It has also been observed those ponds, whose color intensity is high, brine become oily and viscous.

Despite diverse microbial groups inhabit multi-pond solar saltworks, in which a gradient of salinities ranges from seawater to NaCl precipitation, little is known about bacterial communities in saltworks. Thus an approach to characterize them via 16S rDNA gene sequencing method was conducted. In order to determine the bacteria population in different ponds, we identified bacteria through 16S DNA-molecular technique. It is worth be aiming that the objective was only a 16S DNA-bacteria screening. An approach to study structure of microbial communities and biodiversity cultivation is needed onwards.

Amplified 16S rDNA gene sequences with a molecular size of approximately 1500 bp serve as genetic marker and can be used to study bacterial phylogeny and taxonomy by further processing combined with biocomputing analysis [20]. Universal primers (Table 1) have been validated for high-salinity bacteria on isolated DNA derived from crystallizer ponds in different stages of evaporation. Different universal primers match different positions of the genome, identifying different areas of the 16S region [21-23]. Since a broad range of primer combinations was already tested, successful results were obtained with all primer pairs in different sampling stations. In general, bacteria biodiversity detected in ponds is quite similar although a bacteria genus is remarkable over others.

Furthermore internal-PCR amplifications were conducted as consequence of combinations of sequences obtained by universal PCR and different specie-specific primer pairs (Table 2). Twenty-nine sequences of internal-amplifications were obtained and 18 out of them allowed successfully identifying. 16S rDNA-internal sequences ranged from 133 to 223 bp were most closely annotated to three genera namely *Halobacterium sp.*, *Halorubrum sp.*, and *Salinobacter sp*. All of three genus have been described as extremely halophilic bacteria that grows in saturated sodium chloride [24].

Bacterial community fluctuated depending on the progress of evaporation and concomitant change in salinity and environmental conditions. Moreover, the increasing salinity leads to a progressive specification of species being uninhabitable for unspecified organisms [25]. *Halobacterium* genus was always reported in all crystallizer ponds and salt whilst *Halorubrum sp*. and *Salinobacter sp*. were unreported in solid salt. *Halobacterium sp*. species get benefits from increasing salinity.

As this *Halobacterium* species have a cell membrane red-color [26], presumably the red shade in ponds comes from cell membrane. Additionally, since the red color is correlated to the presence of these kind of halophilic bacteria, an increasing habitability and improvement of living conditions can be assumed [6].

Other authors have also shown that *Halobacterium* imparts red coloration to the brine and favor evaporation of brine [27]. Likewise viscosity levels promote the formation of larger salt crystals and thereby improve the salt quality [27,28].

The phylogenetic analysis positioned the *Halobacterium* sequences in a clade within *Halobacterium salinarium* and *Halobacterium sp*. (Figure 2). Evolutionary analysis
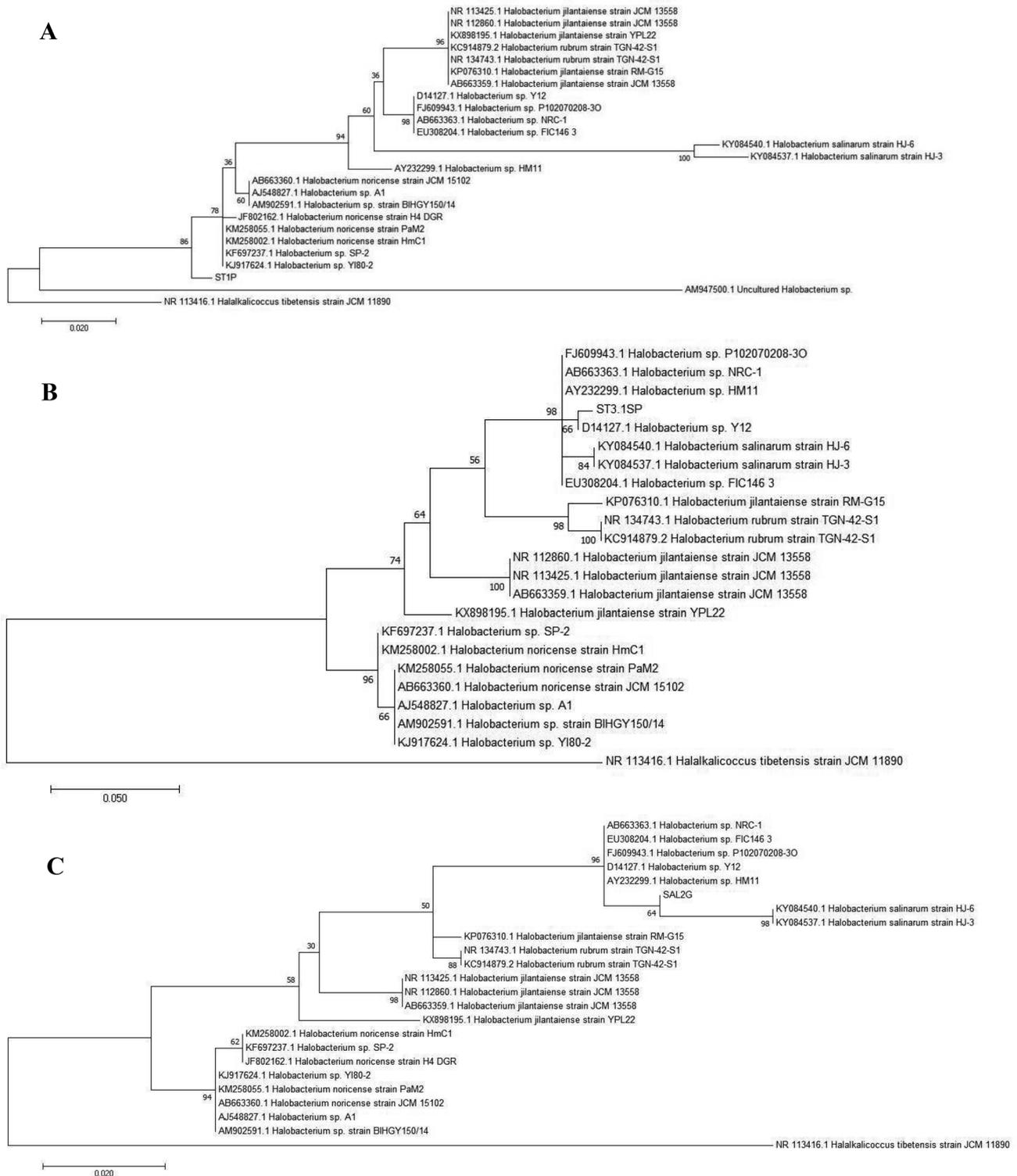
Figure 2. Phylogenetic trees created with MEGA X for nested PCR amplicon sequences. (A) ST1P; (B) ST3.1SP; (C) SAL2G

Note: The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model, with the percentage of trees in which the associated taxa clustered together shown next to the branches [17]. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site.

using Maximum Likelihood method involved 24 nucleotide sequences (Table 3).

Table 3. Sequences retrieved from NCBI database to design phylogeny tree of *Halobacterium*

Species	Strain	Accesion Number
Halalkalicoccus tibetensis	JCM 11890	NR 113416.1
Halobacterium jilan-taiense	YPL22	KX898195.1
	RM-G15	KP076310.1
	JCM 13558	NR 113425.1
	JCM 13558	NR 112860.1
	JCM 13558	AB663359.1
Halobacterium noricense	JCM 15102	AB663360.1
	HmC1	KM258002.1
	PaM2	KM258055.1
	H4 DGR	JF802162.1
Halobacterium rubrum	TGN-42-S1	NR 134743.1
	TGN-42-S1	KC914879.2
Halobacterium salinarum	HJ-6	KY084540.1
	HJ-3	KY084537.1
Halobacterium sp.	Uncultured	AM947500.1
	NRC-1	AB663363.1
	BIHGY150/14	AM902591.1
	A1	AJ548827.1
	FIC146 3	EU308204.1
	HM11	AY232299.1
	P102070208-3O	FJ609943.1
	SP-2	KF697237.1
	Y12	D14127.1
	YI80-2	KJ917624.1

All in all, assuming the criterion for differentiating bacteria with a 16S rDNA gene sequence similarity value of over 85% [29], the phylogenetic trees of *Halobacterium* were consistent with the molecular characterization and their affiliations to the respective genus.

In conclusion, Vargas and Tenefé saltworks are only such biotope in Gran Canaria, which is done only for salt production, but there is unlimited scope for commercial exploitation of salts.

Nonetheless knowledge acquired in this work opens up an interesting framework for further biotechnological studies concerning revealed bacteria and the composition of the bacterial community, as well as for studies investigating hypersaline habitats of crystallizer ponds from Gran Canaria solar salterns. The commercial exploitation of putative bacterial rhodopsin could lead to value added by product.

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Author's contributions

P.G.J. conceived, designed and wrote the manuscript. M.C.A. conducted the phylogeny analysis. S.H. carried out microbiological assays. All the authors read and approved the manuscript.

Supplementary Figure

A

Pink color of the liquid phase
Salt in suspension

B

Orange/red color of the liquid phase
Beginning of salt precipitation without fats

C

Fatty supernatant on the surface

Fatty supernatant including water of the crystallizer

Dry salt from the corner of the crystallizer
Pink color

Supplementary Figure 1. Sampling sites order by increasing salinity and their properties

Note: A) Station 1 (ST1); Station 2 (ST2); Station 3 with fatty supernatant on the surface (ST3.1); Station 3 with fatty supernatant including water of the crystallizer (ST3.2); Dry salt (SAL2).

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