UNIVERZITA KARLOVA

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

KATEDRA BIOLOGICKÝCH A LÉKAŘSKÝCH VĚD

UNIVERSITY OF SEVILLA

FACULTY OF PHARMACY

DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

DIPLOMOVÁ PRÁCA

DIPLOMA THESIS

FYLOGENETICKÁ DIVERZITA KULTIVOVATEĽNÝCH PROKARYOT Z HYPERSALINNÝCH PROSTREDÍ

PHYLOGENETIC DIVERSITY OF CULTURABLE PROKARYOTES FROM HYPERSALINE ENVIRONMENTS

Dáša Straková

Vedúci diplomovej práce/Supervisors:

Prof. Antonio Ventosa, Ph.D., Assoc. Prof. Cristina Sánchez-Porro, Ph.D.

PharmDr. Petr Jílek, CSc.

SEVILLA, 2019

I would like to thank especially to Ana Durán-Viseras for her guidance, kindness, patience, motivation and for supporting me in the laboratory. Her guidance helped me in all the time of research and writing of this thesis.

My sincere thanks also go to Prof. Antonio Ventosa and Prof. Cristina Sánchez-Porro, who provided me an opportunity to join their team. I am grateful for their guidance, reading and revising this work.

Furthermore, I would like to thank all the people in the laboratory for having fun together and creating pleasant and harmonious ambience.

Last but not least I must express my gratitude to my parents, sister and my friends for providing me with support and encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them.

„Prehlasujem, že táto práca je mojím pôvodným autorským dielom. Použitá literatúra aj všetky ďalšie zdroje, z ktorých som pri spracovaní čerpala, sú uvedené v zozname použitej literatúry a v práci riadne citované. Práca nebola využitá k získaniu iného alebo rovnakého titulu."

"I declare that this work is my original work in authorship. All the literature and other sources I used for writing the thesis are listed in the bibliography and cited in the work. The work has not been used to obtain any other or equal degree."

Hradec Králové, 2019 Dáša Straková

CO NTENTS

LIST OF ABBREVIATIONS 1

ABSTRAKT 2

ABSTRACT 3

1. OBJECTIVES 4

2. INTRODUCTION 5

2.1. Extreme environments 5

2.1.1. Hypersaline environments 5

2.2. Halophilic microorganisms 7

2.2.1. Classification 7

2.3. Haloarchaea 16

2.3.1. Classification 16

2.3.2. Characteristics 17

2.3.3. Osmoregulation 18

2.4. Applications of halophiles 19

2.4.1. Biotechnology 19

2.4.2. Astrobiology 20

2.5. Studied hypersaline environments 21

2.5.1. Salterns of Isla Cristina, Huelva 21

2.5.2. Salterns of Isla Bacuta, Huelva 21

2.5.3. Saline soils of Odiel Salt Marshes, Huelva 22

3. MATERIAL AND METHODS 18

3.1. Microorganisms 18

3.2. Culture media 18

3.3. DNA extraction, purification and amplification 20

3.3.1. Extraction of DNA..20

3.3.2. Agarose gel DNA electrophoresis 21

3.3.3. DNA amplification by PCR...22

3.3.4. Purification 25

3.4. Phylogenetic studies...26

3.4.1. Sequencing of the 16S rRNA gene and *rpoB'* gene 26

3.4.2. Phylogenetic analysis of 16S rRNA gene and *rpoB'* gene.........................27

4. RESULTS AND DISCUSSION 28

4.1. Strains studies...28

4.2. Sequencing 30

4.3. Phylogenetic analysis 31

4.3.1. Phylogenetic analysis based on the partial sequence of the 16S rRNA gene 31

4.3.2. Phylogenetic analysis based on the almost complete sequence of the 16S rRNA gene 36

4.3.3. Phylogenetic analysis based on the sequence of the *rpoB'* gene................40

5. CONCLUSIONS...44

6. LIST OF TABLES...45

7. LIST OF FIGURES 46

8. REFERENCES 48

LIST OF ABBREVIATIONS

bp	base pair
DDBJ	DNA Data Bank of Japan
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ed.	Editor
EDTA	ethylene diamine tetra acetic
EMBL	European Molecular Biology Laboratory
et al.	and others (from Latin “et alii”)
G+C content	guanine plus cytosine content
PCR	Polymerase chain reaction
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
rpoB’	gene encoding the β subunit of RNA polymerase
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate-EDTA solution
Taq	*Thermus aquaticus* DNA polymerase
U	unit (s) of enzyme

ABSTRAKT

Kandidát: Dáša Straková

Názov diplomovej práce: Fylogenetická diverzita kultivovateľných prokaryot z hypersalinných prostredí.

Univerzita Karlova, Farmaceutická fakulta v Hradci Králové

Katedra biologických a lékařských věd

University of Sevilla, Faculty of Pharmacy

Department of Microbiology and Parasitology

Študijný program: Farmácia

Hypersalinné prostredia sú široko distribuované biotopy charakterizované zvýšenými koncentráciami solí, ktoré predstavujú hlavne vodné systémy a hypersalinné pôdy. Ich štúdia umožnila v posledných desaťročiach izoláciu a charakterizáciu veľkého počtu halofilných mikroorganizmov, čo sú mikroorganizmy, ktoré vyžadujú k životu a rastu vysokú koncentráciu solí.

Cieľom tejto práce bolo pokračovať v počiatočnej charakterizácii halofilných archeí a baktérií, ktoré izolovala Ana Durán-Viseras v roku 2016 z niekoľkých vzoriek vody zo soľných polí z Isla Cristina a Isla Bacuta (Huelva, Španielsko) a z hypersalinnej pôdy nachádzajúcej sa v lokalite Odiel Saltmarshes (Huelva, Španielsko) a vykonať štúdiu biodiverzity týchto vzoriek. Ana Durán-Viseras začala tento projekt a ja som v ňom pokračovala počas môjho pobytu v Seville.

Ako súčasť charakterizácie sme sekvenovali čiastočný 16S rRNA gén izolovaných kmeňov. Pre tie, ktoré boli potenciálne zaujímavé, sme uskutočnili fylogenetickú štúdiu kompletného 16S rRNA génu a *rpoB'* génu. Na základe týchto výsledkov sme predpokladali, že by mohli predstavovať nové halofilné taxóny.

ABSTRACT

Candidate: Dáša Straková

Title of diploma thesis: Phylogenetic diversity of culturable prokaryotes from hypersaline environments.

Charles University, Faculty of Pharmacy

Department of Biological and Medical Sciences

University of Sevilla, Faculty of Pharmacy

Department of Microbiology and Parasitology

Study program: Pharmacy

Hypersaline environments are widely distributed habitats characterized by elevated concentrations of salts, mainly represented by aquatic systems and hypersaline soils. Their study has enabled the isolation and characterization of a large number of halophilic microorganisms during the last decades, which are microorganisms that require a high concentration of salts in order to be capable of life and growth.

The aim of this work was to continue the initial characterization of halophilic Archaea and Bacteria, previously isolated by Ana Durán-Viseras in 2016 from several water samples from Isla Cristina and Isla Bacuta solar salterns (Huelva, Spain) and from a hypersaline soil located in Odiel Saltmarshes (Huelva, Spain), and to carry out a biodiversity study of these samples. Ana Durán-Viseras started this project and I continued with it during my stay in Sevilla.

As a part of the characterization, we sequenced the partial 16S rRNA gene of the isolated strains. For those that were potentially interesting, we carried out a phylogenetic study of the complete 16S rRNA gene and of the *rpoB'* gene. Based on these results, we assumed that they could represent new halophilic taxa.

1. OBJECTIVES

Previous studies carried out in different hypersaline environments located in southwest of Spain, Isla Cristina and Isla Bacuta marine salterns and in a hypersaline soil from Odiel Saltmarshes, have permitted the isolation of 214 strains of halophilic Archaea and Bacteria.

The main objective of this work was the identification of those isolates and to perform a biodiversity study of these environments. More specifically we propose the following objectives:

1. Determination of the 16S rRNA gene partial sequence of the 78 strains.
2. Determination of the almost complete 16S rRNA gene sequence of selected strains which showed a percentage of similarity lower than 98% with known taxa.
3. Determination of the haloarchaeal *rpoB'* gene of selected strains.
4. Determination of the phylogenetic position of those strains based on both genes.

2. INTRODUCTION

2.1. Extreme environments

The word "extreme" comes from the Latin word "extremus," the superlative of "exter" (= on the outside). Whereas there is no general agreement on how to define an extreme environment, the term is commonly used for any setting that exhibits life conditions fatal to higher organisms with respect to its physicochemical properties (Thiel, 2011). Not so long ago it was thought that life could not occur under extreme conditions. In the 1960s, Professor Thomas D. Brock isolated and described the first organisms from Yellowstone National Park, USA. This organism, *Thermus aquaticus*, is capable of growing at temperatures higher than 70°C (Gomez, 2015). It is not easy to find a definition that is completely acceptable for all environments that are considered as extreme, but we observe that in some habitats environmental conditions such as pH, temperature, pressure, nutrients or saline concentrations are extremely high or low and that only limited numbers of species are well adapted to those conditions (Ventosa, 2006).

2.1.1. Hypersaline environments

Hypersaline environments are extreme habitats with limited microbial diversity due to the combined effects of several environmental factors, including high salt concentrations, temperatures and pH, low nutrient and oxygen availability, solar radiation, heavy metals and other toxic compounds (pesticides, chemicals), etc. (Ventosa et al., 2015). Hypersaline environments are represented by aquatic and terrestrial systems, as well as salted products, such as salted foods, hides, marine or rock salt, etc. (Ventosa et al., 2015).

Hypersaline waters, those with higher concentrations of salt than seawater, can be divided into *thalassohaline*, if derived from marine origin and have salt ratios similar to those of seawater, or *athalassohaline*, if their composition reflects the composition of the surrounding geology, topography and climatic conditions, often particularly influenced by the dissolution of mineral deposits; thus the composition of such waters varies widely (Rodriguez-Valera, 1988). Thalassohaline habitats are typified by solar salterns, which are constituted by a series of shallow ponds in which the seawater is

evaporated until the salts are precipitated and they are excellent models for the study of halophiles (Ventosa, 2006).

While extensive studies have been performed on aquatic hypersaline habitats, other saline environments such as **soil systems** have been much less thoroughly investigated. With an estimated extension of 397 million hectares, salt-affected soils comprise more than 3% of the world's land area (Vera-Gargallo and Ventosa, 2018). A soluble salts content exceeding 0.2% in soil is enough to classify it as saline (Ventosa and Arahal, 2002). According to Torsvik et al. (2002), soil environments are the most diverse environments on Earth, presumably due to a higher heterogeneity of the soil structure, and a higher disturbance rates. Soil environments are also more dynamic and heterogeneous than aquatic systems, especially in relation to water and nutrient content. Thus, metabolically versatile microbes, able to use different carbon and energy sources may be able to survive to the frequent changes of those factors in soils (Vera-Gargallo and Ventosa, 2018).

There are also higher plants that are halotolerant (**halophytes**), which are well adapted to growing in different levels of salinity and play important roles in different ecological nutrient cycles. The interactions between plants and microbes vary with different saline habitats (Imran et al., 2016), for example, communities of halophilic and halotolerant microorganisms inhabit the leaves of certain plants such as *Tamarix* tree and *Atriplex* spp. that grow in arid areas and excrete salt from salt glands on their leaves (Simon et al., 1994).

Also, **salted food** may represent a hypersaline habitat. In Thailand, *Halobacterium salinarum* and *Halococcus thailandensis* have been isolated from fish sauce samples: these are extremely halophilic archaea that grow optimally at 20–25 % NaCl (Namwong et al., 2007). Jeotgal is one of the representative traditional foods in Korea. It is prepared through blending of various kinds of seafoods, including seawater and other ingredients, and becomes palatable through subsequent preservation and fermentation. The study on the microbiota of jeotgal has shown that a variety of halotolerant and halophilic bacterial strains exist in it (Yoon et al., 2001).

2.2. Halophilic microorganisms

2.2.1. Classification

The relationships of different microorganisms with salt have been studied in detail and several classifications have been proposed. Halophilic microorganisms are classically categorized on the basis of their optimal growth in different salt concentrations (Ventosa et al., 2015). We will adopt the approach proposed by Kushner and Kamekura (1988) that is widely used by most scientists where microorganisms are divided into the following classes:

- **Non-halophilic**: grow best in media with less than 1% (0.2 M) NaCl. However, some of them are able to tolerate high NaCl concentrations and they are defined as **halotolerant** or extremely tolerant organisms.
- **Slight halophiles** (represented by most marine microorganisms): are able to grow optimally between 1 and 3% (0.2-0.5 M) NaCl
- **Moderate halophiles**: growing optimally in media with 3-15% (0.5- 2.5 M) NaCl
- **Extreme halophiles**: are able to grow optimally in media with 15-30% (2.5-52 M) NaCl

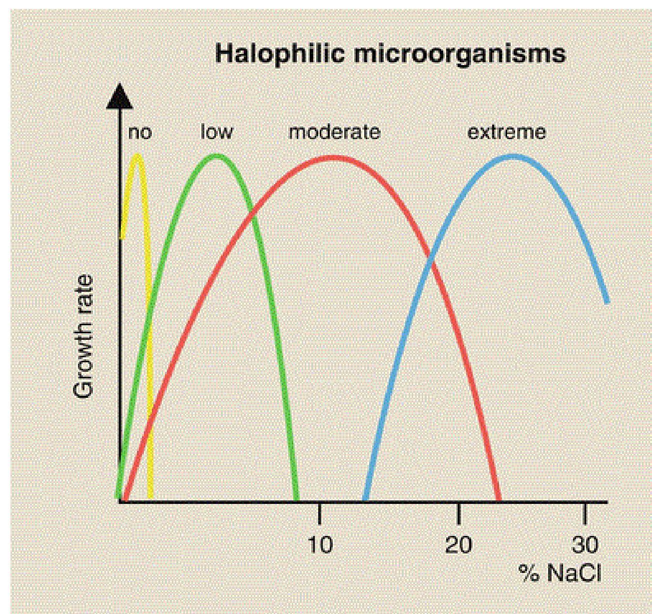


Figure 1. *Classification of prokaryotic microorganisms according to their behavior towards NaCl. Drawing: M.-J. Bodiou (Cayol et al., 2015)*

Halophiles can be found in all three domains of life: *Archaea, Bacteria,* and *Eukarya* (Cayol et al., 2015). Cellular life in hypersaline habitats is dominated by prokaryotes (Archaea and Bacteria), with a few microbial eukaryotes, such as photosynthetic and heterotrophic protists and fungi, and the crustacean *Artemia salina*. Also, viruses are a significant part of the community (Ventosa et al., 2015).

2.2.1.1. Archaea

The halophilic *Archaea* are included on the phylum *Euryarchaeota*. They are represented by the extremely halophilic aerobic *Archaea*, also designated as haloarchaea, currently included within the class *Halobacteria*. However, some halophilic methanogenic species have been described from hypersaline environments. *Halobacteria* are considered as the prokaryotes best adapted to high salt concentrations. They can grow easily aerobically in media with 20-25% NaCl and will be described in more detail later. Methanogenic *Archaea* play an important role in hypersaline environments but few halophilic species have been characterized. Taxonomically the methanogenic *Archaea* are grouped within five orders, but only the order *Methanosarcinales* include halophilic species. They are strictly anaerobic and obtain energy by formation of methane (de la Haba et al., 2010).

2.2.1.2. Bacteria

Halophilic bacteria exist in various forms of colonies, ranging from pigmented to non-pigmented, according to the salt concentration in the media. They are slow growing compared to non-halophilic bacteria and the extremely halophilic bacteria grow extremely slowly (Imran et al., 2016).

The domain *Bacteria* is currently subdivided into 28 phyla. Moderately or extremely halophilic species with validly published names are included in the following phyla: *Proteobacteria, Firmicutes, Actinobacteria, Spirochaetes, Bacteroidetes, Thermotogae, Cyanobacteria* and *Tenericutes* (de la Haba et al., 2010).

2.2.1.3. Eukarya

Although prokaryotic organisms are dominant in hypersaline ecosystems, eukaryotic organisms may inhabit such ecosystems, especially in habitats with lower salinities. Species of the unicellular green algae genus *Dunaliella* are the main primary producers in saltern crystallizer ponds. Some species become orange red colored when grown under stress due to the massive accumulation of granules of β-carotene (Oren, 2016).

Rodriguez-Valera et al. (1985) reported high productivity values in saltern ponds between 10 and 30% salinity, with a maximum at around 25% salts, which corresponded to the highest densities of *Dunaliella*. In ponds of salterns and saline lakes with salinities higher than 10-15%, large organisms disappear and the brine shrimp *Artemia salina* and the larvae of the brine fly *Ephydra* are the only macroscopic organisms that are observed (Ventosa, 2006).

The contribution of salt-loving and salt-tolerant fungi to the biota of hypersaline environments became recognized only recently. Species of black yeasts, notably *Hortaea werneckii* and *Trimmatostroma salinum*, are commonly found in marine salterns, and they are well adapted to life at high-salt concentrations (Gostinčar et al., 2009). The most halophilic fungus known is *Wallemia ichthyophaga* (Zajc et al., 2013).

During recent decades, studies on extreme environments have introduced us to archaeal viruses and viruses infecting extremophilic bacteria or eukaryotes. Hypersaline environments are known to contain high numbers of viruses. Halophilic microorganisms inhabiting hypersaline environments have only a few cellular predators, indicating that the role of viruses is highly important in these ecosystems. Viruses thriving in high salt are called haloviruses and to date more than 100 such viruses have been described (Atanasova et al., 2016).

2.3. *Haloarchaea*

Extremely halophilic aerobic archaea, also referred to as haloarchaea, dominate hypersaline environments. To survive under such extreme conditions, haloarchaea and their enzymes have evolved to function optimally in environments with high salt concentrations and, sometimes, with extreme pH and temperatures (Amoozegar et al., 2017).

2.3.1. Classification

Because of the applied impact of haloarchaea and their specific ability to live in the presence of high salt concentrations, studies on their systematics have intensified in recent years, identifying many new genera and species (Amoozegar et al., 2017).

Currently, the haloarchaea are included in the class *Halobacteria*, within the phylum *Euryarchaeota*, which is divided into three orders: *Halobacteriales, Haloferacales* and *Natrialbales*. Fig. 2 shows the haloarchaeal classification from phylum to genus levels.

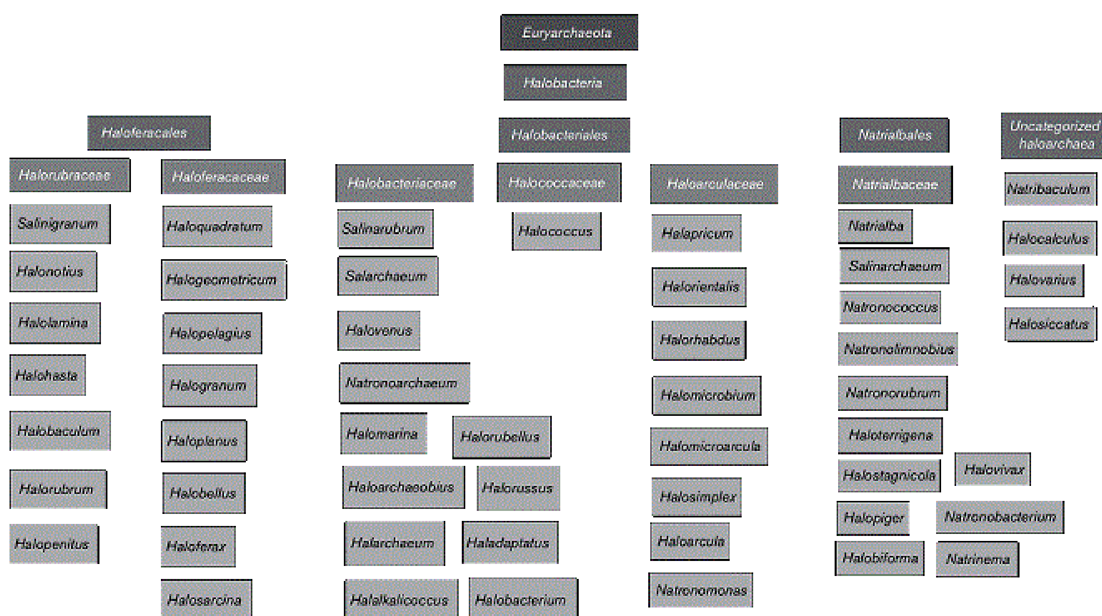


Figure 2. *Classification of haloarchaea from phylum to genus (Amoozegar et al., 2017)*

The current classification of *Bacteria* and *Archaea* is based on a polyphasic approach, comprised of phenotypic, chemotaxonomic and genotypic data, as well as phylogenetic information (Schleifer, 2009).

The 16S rRNA gene sequence is a universal phylogenetic marker within the prokaryotes, and therefore considered essential in taxonomic studies of haloarchaea. However, its highly conserved nature does not allow relevant discernible differentiation among closely related species (Ventosa et al., 2015). Additionally, many haloarchaeal genera have multiple divergent copies of rRNA genes with greater than 6% sequence dissimilarity - the divergence between copies in a single cell can be equal to that seen between different genera. To overcome some of these limitations, protein encoding housekeeping genes, for example *rpoB'* gene, have been suggested as alternative phylogenetic markers within the class *Halobacteria* (de la Haba et al., 2018).

2.3.2. Characteristics

Haloarchaeal cell shapes are very diverse and can be observed as simple forms, such as rods, cocci or disks, or as more unusual forms, such as triangles, squares or pleomorphic cells, especially when they are cultured at higher temperatures or the lower NaCl concentrations needed for their optimal growth (Amoozegar et al., 2017).

The most important feature of the haloarchaea is their absolute requirement for high concentrations of NaCl. They are considered as the prokaryotes best adapted to high salt concentrations (de la Haba et al., 2010).

They produce red- to pink-pigmented colonies due to the presence of bacterioruberins, 50-carbon carotenoids that are partially responsible for the typical coloration of many natural environments in which they may develop in large numbers. However, there are a few exceptions that are not pink- to red-pigmented, including species of the genus *Natrialba* (Ventosa, 2006).

Haloarchaea have typical archaeal characteristics such as the presence of ether-linked lipids that can be easily detected by thin-layer chromatography and they are used for the differentiation of taxa, especially at the genus level. All haloarchaea contain phytanyl ether analogues of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester. Many species also have phosphatidylglycerol sulfate and one or more glycolipids and sulfated glycolipids. Haloarchaea have diphytanyl ($C_{20}C_{20}$) glycerol ether core lipids but some species may have additional phytanylsesterterpanyl ($C_{20}C_{25}$) glycerol core lipids (Grant et al., 2001).

Haloarchaeal DNA is characterized by a high G+C content that stabilizes it in the face of the high cation concentrations in their cytoplasm. However, the genus *Haloquadratum* shows a relatively low G+C content (15-20% less than in other halophilic archaea) (Bolhuis et al., 2006). All studied haloarchaea have polyploid genomes and this increased copy number strategy imparts ecological and evolutionary benefits such as protection against the negative effect of DNA damage caused by irradiation (X-ray), desiccation or mutagens. Glycosylation of haloarchaeal proteins is another adaptation of haloarchaea to high salinity. It has been shown that glycosylation of the S-layer in haloarchaea enables them to grow in media containing high salt concentrations and protects them against proteases (Amoozegar et al., 2017).

Some halophilic archaea are capable of synthesizing internal gas vesicles - flotation devices (Walsby, 1994). Gas vesicles are filled by diffusion with gases dissolved in the environment; their function is apparently to provide buoyancy and enabling cells to regulate their position in the water column (Fendrihan et al., 2006).

2.3.3. Osmoregulation

Halophiles can live in hypersaline environments because they are able to sustain osmotic balance. They use two main strategies to withstand the adverse consequences of water loss when exposed to high salt concentrations. The first strategy used by extremely halophilic archaea (haloarchaea) and halophilic anaerobic bacteria such as members of *Halanaerobiales* is known as the **'salt-in-cytoplasm'** strategy, where salts such as NaCl or KCl are accumulated in the cytoplasm and balance extracellular concentrations (Ventosa et al., 1998).

Another mechanism to cope with high salt concentrations is the accumulation of organic molecules such as amino acids, sugars and polyols, known as compatible solutes or osmolytes. Organic solutes used for this purpose need to be small molecules, highly soluble in water, that do not negatively interfere with the activity of intracellular enzymes. As the intracellular concentrations of these osmotic solutes can be regulated in accordance with the outside salt concentrations, this **'salt-out'** strategy generally allows for a high degree of flexibility, so that cells can rapidly adapt to changes in the salinity of their medium (Galinski, 1995; Oren, 2008).

2.4. Applications of halophiles

2.4.1. Biotechnology

Halophiles are one of the most important groups of extremophiles, with potential for industrial and biotechnological applications. Haloenzymes, especially those from haloarchaea, are also very attractive. At high salt concentration conditions, halophilic enzymes may not need purification or sterilization for applied use, since unwanted contaminating proteins or other biomolecules are inactive under these conditions (van den Burg, 2003). Many halophilic enzymes can work under low water activity, in the presence of organic solvents, and in some cases, at high temperatures (Litchfield, 2011).

Liposomes are novel drug delivery systems with growing usage. In spite of the various applications of liposomes in medicine, novel liposomes composed of ether lipids derived from haloarchaeal cytoplasmic membranes have advantages compared to ester-lipid liposomes because of their stability over wide pH and temperature ranges, and their intrinsic resistance to enzymatic degradation, for example by esterases (Galinski and Tindall, 1992).

Bacteriorhodopsin is a light energy-transducing pigment. It can be used in different fields, including gene therapy and the production of artificial retinas and biosensors, because of its resistance to chemical and photochemical reactions, as well as against increased temperatures. Security-ink production, optical-data storage and a new expression vector comprising bacteriorhodopsin are other applications of this protein (Kapilesh et al., 2018).

Halocins are antimicrobial compounds produced by some haloarchaea and only affect archaea and halophilic bacteria. Halocins can inhibit unwanted haloarchaeal growth in textile industries processes with high salt concentrations. They can also be used in medical fields for the reduction of injury in organ transplantation (Charlesworth, 2015).

One of the most important applications of haloarchaea in biotechnology is the bioremediation of xenobiotics. Industrial processes such as oil or gas recovery produce saline wastewater. Due to the tolerance of halophilic microorganisms to salt and organic contaminants, they can be used to treat contaminated saline soils, toxic effluents and hydrocarbon-polluted environments (Amoozegar et al., 2017).

The biotechnological applications where moderate/extreme halophiles are involved include the fermentation of soy sauce by halotolerant/moderately halophilic bacteria and archaea which is used in the preparation in a Vietnamese fish dipping sauce called "nuoc mam" (Cayol et al., 2015). They are also involved in the preparation of other salted-food products obtained by fermentation (Amoozegar et al., 2017).

The use of the halophilic osmolytes (such as betaine, ectoine, β-glutamine, etc.) at industrial scale is expanding. After reorganizing their potential, they are widely used for different biotechnological applications including stabilization of protein and DNA, as a cryoprotectant of microorganism during long-term storage, in cosmeceuticals (Cosmeceutical industries like RonaCare™, Merck KGaA, and Darmstadt) and pharmaceuticals, as PCR enhancers, and in generation of stress resistance in non-halotolerant plants (Roberts, 2005). Unique properties of osmolytes such as protective metabolic, antioxidant, and protecting macromolecules are now being explored in health care. Osmolytes such as glycerol and sucrose that belong to polyhydric alcohols are used to protect cells from the toxic effect of chemicals and plasma during cancer treatment (Kapilesh et al., 2018). Some osmolytes have excellent therapeutic potential; betaine is used in the initial stage of cirrhosis (Detkova and Boltyanskaya, 2007). Apart from this, it decreases the side effects of anti-inflammatory preparation, has anticoagulant property, prevents thrombus formation, and decreases the probability of heart attacks, infarctions, and strokes (Messadek, 2005).

2.4.2. Astrobiology

The study of extremophiles and their habitats increases our understanding of the limits of life and the nature of the first organisms on early Earth, and in addition gives us an insight into the possibilities of extraterrestrial life: one of the goals of the field of astrobiology (Seckbach et al., 2006).

Extremely halophilic archaea (haloarchaea) are of astrobiological interest since viable strains have been isolated from million-year-old deposits of halite, suggesting the possibility of long-term survival under desiccation. Extraterrestrial halite has been identified, for example, in Martian meteorites, in chloride-containing surface pools on Mars, and in the presumed salty ocean beneath the ice cover of Jupiter's moon

Europa. These discoveries make a consideration of the potential habitats for halophilic life in space intriguing. Recent data on the physical occurrence of liquid saline water on Mars have added another novel aspect to this notion (Stan-Lotter et al., 2012).

2.5. Studied hypersaline environments

Marine salterns are composed of interconnected ponds with increasing salt concentrations and they constitute excellent models for studying microbial populations growing along a salinity gradient (Fernández et al., 2014a).

For our research, we obtained samples from different ponds of salterns located in Isla Cristina and Isla Bacuta and from soils of Odiel Saltmarshes (southwest of Spain).

2.5.1. Salterns of Isla Cristina, Huelva

Isla Cristina saltern (Figure 3) is located at the marsh of the river Carreras in the village of Isla Cristina and it gets its water from the Atlantic Ocean (Moreno et al., 2009). Isla Cristina saltern is subjected to rainfall seasons, high solar radiation, and larger temperature fluctuations between day and night (Papke et al., 2015).

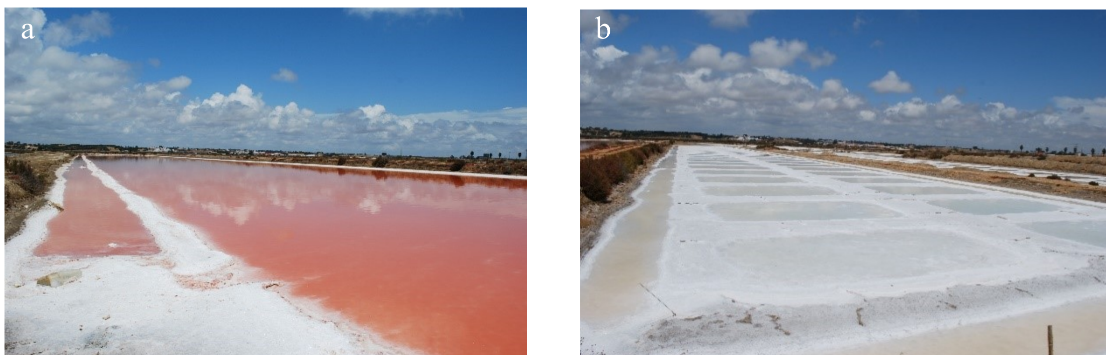


Figure 3. *(a) Isla Cristina salterns and (b) Isla Cristina salterns (once water is evaporated), Huelva, Spain. Author/Source: Antonio Ventosa*

2.5.2. Salterns of Isla Bacuta, Huelva

Isla Bacuta saltern (Figure 4) is located in the municipality of Punta Umbría within the Natural Reserve of Odiel Saltmarshes in Huelva (Infante-Domínguez, 2015). Both

salterns (Isla Cristina and Isla Bacuta) are composed by multipond systems and are closely located to food-processing industries (Moreno et al., 2009).

Figure 4. *Isla Bacuta salterns, Huelva, Spain. Author/Source: Antonio Ventosa*

2.5.3. Saline soils of Odiel Saltmarshes, Huelva

The Odiel Saltmarshes (Figure 5) are situated at the estuary of the Odiel and Tinto rivers. The area has been affected by the metal load transported by those rivers, which drain the Iberian Pyrite Belt, as well as by industrial effluents from the chemical pole located in Huelva (Vera-Gargallo et al., 2018).

Figure 5. *Saline soils of Odiel Saltmarshes, Huelva, Spain. Author/Source: Antonio Ventosa*

3. MATERIAL AND METHODS

3.1. Microorganisms

In this work, we carried out a biodiversity study from water samples from Isla Cristina and Isla Bacuta solar salterns (Huelva, Spain), and from soil samples from a hypersaline soil located in Odiel Saltmarshes (Huelva, Spain).

Halophilic archaea and bacteria strains were isolated by Ana Durán-Viseras in 2016 by plating diluted samples on different composition and salinity media. Plates were incubated at 37ºC up to 8 weeks. After incubation, colonies were selected according to its morphology and subculture until pure cultures were obtained. In total, we have worked with 214 isolates.

3.2. Culture media

In order to obtain the maximum representation of halophilic Archaea and Bacteria present in the samples, three different culture media were used at two different salinities percentages of 15 and 25%. The pH of all media was adjusted to 7.5 and they were sterilized in autoclave at 121ºC for 20 minutes.

Seawater solution 30% (SW30) (Subow, 1931): diluted volumes of this stock were added to the culture media to obtain the required salinity.

NaCl	234.0 g
$MgCl_2.6H_2O$	39.0 g
$MgSO_4$.7H2O	61.0 g
$CaCl_2$	1.0 g
KCl	6.0 g
$NaHCO_3$	0.2 g
NaBr	0.7 g
Distilled water	to 1000ml

This mixture of salts maintains the relative proportions of seawater, with a slight decrease in the concentrations of the salts $CaCl_2$ and $NHCO_3$ to avoid its precipitation. For the same reason, both salts are added separately and once dissolved to the solution.

The three different culture media used were: R2A medium, glycerol medium and pyruvate medium. The compositions are detailed below.

R2A medium (Reasoner and Geldreich, 1985)

Yeast extract	0.05 g
Proteose peptone No. 3	0.05 g
Casamino acids	0.05 g
Glucose	0.05 g
Soluble starch	0.05 g
Sodium pyruvate	0.03 g
Dipotassium phosphate	0.03 g
Magnesium sulphate	0.005 g
SW30	50 or 83.33 ml
Distilled water	to 100 ml

Glycerol medium

Yeast extract	0.01 g
Glycerol	0.099 g
SW30	50 or 83.33 ml
Distilled water	to 100 ml

Pyruvate medium

Casein digest	0,5 g
Sodium pyruvate	0.11 g
SW30	50 or 83.33 ml
Distilled water	to 100 ml

Purified agar was added when necessary, to a final concentration of 2%.

3.3. DNA extraction, purification and amplification

First, the pure cultures of the isolates were inoculated in 5 ml of liquid media (R2A medium, glycerol medium or pyruvate medium according to its isolation medium) and cultivated at 37ºC during around 7-10 days. After that the genomic DNA of the 214 isolates was extracted following the protocol described below.

3.3.1. Extraction of DNA

Genomic DNA was extracted following the method described by Marmur (1961) (slightly modified).

---

1. Centrifuge the cells for 5 minutes at 13000 rpm
2. Discard the supernatant and resuspend the pellet
3. Add 500 µL of saline EDTA (pH 8)
4. Incubation at 37ºC for 60 minutes
5. 50 µL of 25% SDS
6. Incubation at 60ºC for 10 minutes
7. Add 120 µL of sodium perchlorate
8. Add 800 µL of chloroform–isoamyl alcohol (24:1)
9. Keep 30 minutes of intense agitation (vortex)
10. Centrifugation at 13000 rpm for 10 minutes
11. Take the supernatant
12. Add 1.2 mL of cold 100% ethanol
13. Reverse the tube 3-4 times
14. Centrifugation at 13000 rpm for 15 minutes
15. Discard supernatant
16. Wash with 70% ethanol (500 µL)
17. Centrifugation at 13000 rpm for 5 minutes
18. Discard supernatant
19. Dry
20. Resuspension in 50 µL of sterile water

3.3.2. Agarose gel DNA electrophoresis

Agarose gel DNA electrophoresis is used to separate and identify fragments of DNA of different sizes. It allows to check the integrity of the DNA extraction or the PCR amplification.

DNA is a molecule that has a negative charge and therefore, when it is placed on an agarose gel and subjected to an electric field (electrophoresis), it migrates towards the anode (positive pole).

To carry out the electrophoresis, horizontal agarose gel (Seakem, LE Agarose) was prepared at a concentration of 1% (w/v), in 1X TAE buffer (TAE 50X buffer: glacial acetic acid, 57.1 ml; 50 mM EDTA, pH 8.0; tris-base, 242 g; distilled water, to 1000 ml) and immersed in the same buffer (Figure 6). DNA visualization was performed by staining the gel with RedSafe™ (iNtRON Biotechnology) Nucleic Acid Staining Solution and exposing to UV light (360 nm), using a transilluminator (TPF-M/WL). 1 kb DNA ladder (Invitrogen) was used as the molecular weight standard.

Figure 6. *DNA electrophoresis system*

Preparation of agarose gel DNA electrophoresis 1% (Figure 7).

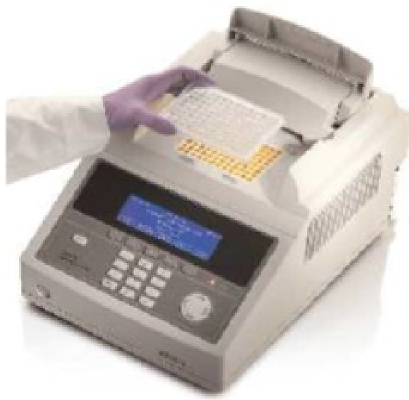
---

1. Add 0.4 g of agarose in 40 ml of TAE 1X electrophoresis buffer (prepared from TAE 50X buffer) and heat the mixture in microwave until complete dissolution.
2. After the gel cooled down, add 3 µl of RedSafe™ Nucleic Acid Staining Solution (20,000x) to the agarose solution. Swirl the flask gently to mix the solution and avoid forming bubbles.
3. Pour it into the gel tray until the comb teeth are immersed about 1/4~1/2 into the agarose. Let gel to solidify at room temperature for 15-30 minutes.
4. Once solidified, remove the combs and place the gel in the electrophoresis tank immersed in TAE 1X buffer, taking care to completely coat the surface of the gel.
5. Load samples on the gel and perform electrophoresis.
6. Detect the bands under UV illumination.

Figure 7. *Steps in performing a DNA electrophoresis in agarose gel (Corral Villa, 2014)*

3.3.3. DNA amplification by PCR

The Polymerase Chain Reaction (PCR) technique is a method that allows to amplify nucleic acids (Saiki et al., 1985, Mullis and Faloona, 1987). It is based on the natural process of DNA replication. Starting from a molecule of target DNA, a specific sequence contained in it is amplified using primers designed for that purpose. The primers used are two oligonucleotides of synthetic origin capable of binding to complementary DNA sequences that limit the region to be amplified. Each of them is a replica of one of the two strands of DNA (one is called a forward primer and the other reverse) and are designed in a way they are confronted by their 3´ ends after binding to the "target DNA." The distance between them will determine the length of the amplified DNA sequence in each case. For the amplification of the 16S rRNA gene, this PCR technique was used and carried out following the indications of Sambrook and Russell (2001), using the Mastercycler Ep (Eppendorf) and GeneAmp® PCR System 9700 (Applied Biosystems) (Figures 8 and 9).



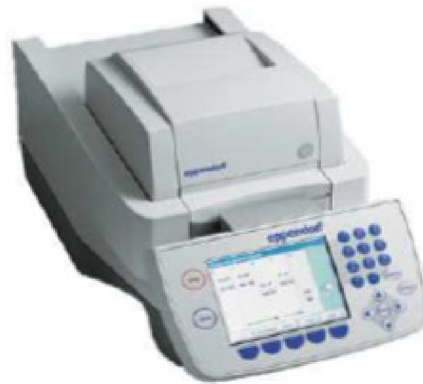


Figure 8. *GeneAmp® PCR System 9700 (Applied Biosystems)*

Figure 9. *Mastercycler Ep (Eppendorf)*

General scheme of the PCR reactions:

Reaction buffer 10X	5.0 µl
$MgCl_2$ (25 mM)	2.5 µl
dNTPs (1.25 mM each dNTP)	8.0 µl
Forward primer (12 µM)	2.5 µl
Reverse primer (12 µM)	2.5 µl
DNA	
Taq DNA polymerase (5 U/µl)	0.5 µl
Sterile water	to 50.0 µl

All the PCR reactions were performed in final volumes of 50 µl in microtubes of 0.2 ml, which were previously sterilized to eliminate the possible presence of nucleases in them.

The PCR reaction for the ***amplification of the 16S rRNA gene*** consists of the following steps:

• Denaturation of the template DNA: 5 minutes at 95°C.

25 cycles in which three phases are repeated:

• Denaturation: 1 minute at 94°C

• Hybridization of the primers: 1 minute at 50°C

• Extension / Elongation: 2 minutes at 72°C

•Final elongation: 10 minutes at 72°C, with the aim of achieving complete elongation of the products of the reaction.

The PCR reaction for the ***amplification of the rpoB' gene*** consists of the following steps:

• Denaturation of the template DNA: 1 minute at 94°C.

35 cycles in which three phases are repeated:

• Denaturation: 1 minute at 94°C

• Hybridization of the primers: 1 minute at 61°C

• Extension / Elongation: 1 minute at 72°C

•Final elongation: 5 minutes at 72°C, with the aim of achieving complete elongation of the products of the reaction.

Finally, the samples are stored at 4°C.

The different primers that have been used in the PCR reactions in the present work, are detailed in Tables 1 and 2. The enzyme used was the modified DNA polymerase of *Thermus aquaticus* (Lawyer and col., 1989), called *Taq* DNA polymerase (Promega, Eppendorf and Dominion).

In order to verify the correct amplification of the DNA, we proceeded to visualize the fragments of DNA by submitting an aliquot of the PCR product to an agarose gel electrophoresis (see section 3.3.2.). Finally, the amplified DNA was purified to remove possible impurities, as a step prior to sequencing. For this, the commercial system MEGA quick-spin™ Plus Fragment DNA Purification kit was used following the manufacturer's instructions. (see 3.3.4. in Methods).

Table 1. *Primers used for the amplification of the 16S rRNA gene of haloarchaea and halophilic bacteria*

Primer	Specificity	Orientation	Sequence 5'→3'	Reference
21F	Archaea	Forward	TTC CGG TTG ATC CTG CCG GA	DeLong (1992)
1492R	Universal	Reverse	GGT TAC CTT GTT ACG ACT T	DeLong (1992)
F27	Bacteria	Forward	AGA GTT TGA TCM TGG CTC AG	Mellado et al. (1995)
R1488	Bacteria	Reverse	CGG TTA CCT TGT TAG GAC TTC ACC	Mellado et al. (1995)

Table 2. *Primers used for amplification of rpoB' gene*

Primer	Specificity	Orientation	Sequence 5'→3'	Reference
rpoBF	Archaea	Forward	TGT AAA ACG ACG GCC AGT TCG AAG AGC CGG ACG ACA TGG	Fullmer et al. (2014)
rpoBR	Archaea	Reverse	CAG GAA ACA GCT ATG ACC GGT CAG CAC CTG BAC CGG NCC	Fullmer et al. (2014)

3.3.4. Purification

To purify the DNA, we used MEGAquick-spin™ Plus Fragment DNA Purification Kit (iNtRON Biotechnology) following the manufacturer's instructions.

---

1. Transfer up to 100 µl of PCR product (excluding oil) to a microcentrifuge tube and add 5 volumes of BNL Buffer/Plus, mix well by vortexing.
2. Place a column into a collection tube.
3. Transfer the sample mixture to the column. Centrifuge at 11,000 x for 30 seconds, then discard the flow-through.
4. Add 750 µl of Washing Buffer / Plus to the column. Centrifuge at 11,000 x for 30 seconds, then discard the flow-through.
5. Centrifuge again at full speed (~18,000 x) for an additional 3 minutes to dry the column membrane.
6. Place the column to a new microcentrifuge tube.
7. Add 40 µl of Elution Buffer/Plus to the membrane centre of the column. Stand the column for 1 min.
8. Centrifuge at full speed (~18,000 x) for 1 min to elute the DNA.

3.4. *Phylogenetic studies*

3.4.1. Sequencing of the 16S rRNA gene and *rpoB'* gene

The fragments of PCR amplified in this work have been sequenced by the company STABVida (Oeiras, Portugal). Tables 3 and 4 include the primers used in the gene sequencing. The samples of PCR/DNA were sequenced following the Sanger method.

Table 3. *Primers used for sequencing of archaeal and bacterial 16S rRNA gene*

Primer	Specificity	Orientation	Sequence 5'→3'	Reference
21F	Archaea	Forward	TTC CGG TTG ATC CTG CCG GA	DeLong (1992)
1492R	Universal	Reverse	GGT TAC CTT GTT ACG ACT T	DeLong (1992)
D34	Archaea	Reverse	GGT CTC GCT CGT TGC CTG	Arahal et al. (1996)
B36	Archaea	Reverse	GGA CTA CCA GGG TAT CTA	Arahal et al. (1996)
F27	Bacteria	Forward	AGA GTT TGA TCM TGG CTC AG	Mellado et al. (1995)
R1488	Bacteria	Reverse	CGG TTA CCT TGT TAG GAC TTC ACC	Mellado et al. (1995)

Table 4. *Primers used for sequencing the archaeal rpoB' gene*

Primer	Specificity	Orientation	Sequence 5'→3'	Reference
rpoBF	Archaea	Forward	TGT AAA ACG ACG GCC AGT TCG AAG AGC CGG ACG ACA TGG	Fullmer et al. (2014)
rpoBR	Archaea	Reverse	CAG GAA ACA GCT ATG ACC GGT CAG CAC CTG BAC CGG NCC	Fullmer et al. (2014)

3.4.2. Phylogenetic analysis of 16S rRNA gene and *rpoB'* gene

Sequences were phylogenetically analyzed using different bioinformatic softwares:

ChromasPro

To obtain the complete sequence of the 16S rRNA gene, partial sequences were carefully checked for ambiguities and assembled with the help of the ChromasPro Version 1.5 program (Technelysium Pty Ltd).

BLAST and EzBioCloud

The 16S rRNA sequences were phylogenetically identified by comparing with the sequences available in the public databases (EMBL, GenBank and DDBJ), with the help of the BLAST program (Basic Local Alignment Search Tool) (Altschul et al., 1990) from the NCBI server (National Centre for Biotechnology Information) (https://blast.ncbi.nlm.nih.gov/BLAST/) and in EZBioCloud database (https://www.ezbiocloud.net/) (Yoon et al., 2017).

ARB

The ARB system (from the Latin "arbor" = tree) (Ludwig et al., 1998; 2004) is a set of free programs. It was used for phylogenetic analysis of 16S rRNA gene sequences.

BioEdit and ClustalX

BioEdit (Alzohairy, 2011) and ClustalX programs (Larkin et al., 2007) enabled us to obtain the complete alignment of the sequences corresponding to the *rpoB'* gene.

MEGA

For the construction of the phylogenetic trees of *rpoB'* gene, the MEGA (Molecular Evolutionary Genetics Analysis) program (Tamura, 2013) was used. These trees were constructed using the maximum-likelihood algorithm (Felsenstein, 1981).

4. RESULTS AND DISCUSSION

4.1. Strains studies

The isolation of the strains was carried out using different culture media with concentrations of salts of 15 and 25% and pH adjusted to 7.5 (see 3.2 in Material and Methods), in order to obtain the highest possible representation of haloarchaea and halophilic bacteria that could be present in viable state. The samples studied in this work were obtained from the solar salterns of Isla Cristina and Isla Bacuta and from a hypersaline soil from Odiel Saltmarshes (Table 5).

As a result of this previous study, a total of 214 strains were isolated in order to obtain their 16S rRNA sequence, however just 78 of them (50 from Isla Cristina, 17 from Isla Bacuta and 11 strains from Odiel Saltmarshes) were sequenced and analysed. Unfortunately, from the rest of the strains, the sequence could not be obtained, no matter how many attempts were made or conditions were tested. Figure 10 shows the percentage distribution of those 78 sequenced *Archaea* and *Bacteria* in our work.

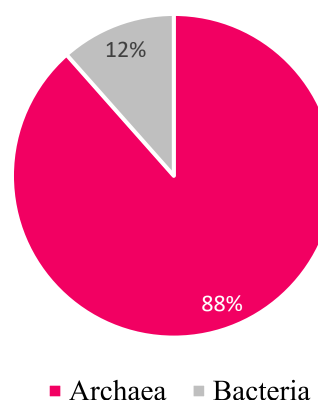


Figure 10. *Percentage distribution of the 78 sequenced Archaea and Bacteria*

Table 5. *Samples and data about their isolation places*

Sample	Salinity (%)	pH	Isolation date	Isolation place
F1	23	7.56	16/06/2016	Isla Cristina
F2	34	7.26	16/06/2016	Isla Cristina
F3	23	7.5	16/06/2016	Isla Cristina
F5	32	7.56	16/06/2016	Isla Cristina
F6	31	7.47	16/06/2016	Isla Cristina
F7	20	7.71	16/06/2016	Isla Bacuta
F8	32	7.46	16/06/2016	Isla Bacuta
F9	31	7.13	16/06/2016	Isla Cristina
F12	31	7.35	29/06/2016	Isla Cristina
F13	27	7.53	29/06/2016	Isla Cristina
F14	30	7.46	29/06/2016	Isla Cristina

F15	31	7.49	29/06/2016	Isla Cristina
F16	32	7.52	29/06/2016	Isla Cristina
F18	27	7.51	29/06/2016	Isla Bacuta
F19	19	7.82	29/06/2016	Isla Bacuta
F20	17	7.76	29/06/2016	Isla Bacuta
IB35	30	7.39	16/06/2016	Isla Bacuta
IC28	28	7.39	29/06/2016	Isla Cristina
IC30	29	7.38	29/06/2016	Isla Cristina
IC36	33	7.45	29/06/2016	Isla Cristina
S1	2	7.98	16/06/2016	Odiel Saltmarshes
S2	2	7.90	16/06/2016	Odiel Saltmarshes
S3	4	7.74	16/06/2016	Odiel Saltmarshes
S4	3	7.72	16/06/2016	Odiel Saltmarshes

4.2. Sequencing

As mentioned above, 78 strains of haloarchaea and halophilic bacteria were sequenced. In order to obtain their sequences, we carried out several steps such as DNA extraction and purification and amplification of the 16S rRNA and *rpoB'* genes (see Material and methods section 3.3). The DNA purification and PCR amplicons were examined by agarose gel electrophoresis (Figure 11) and sequenced by a commercial company STABVida (Oeiras, Portugal). Figure 11 shows all the sequencing steps.

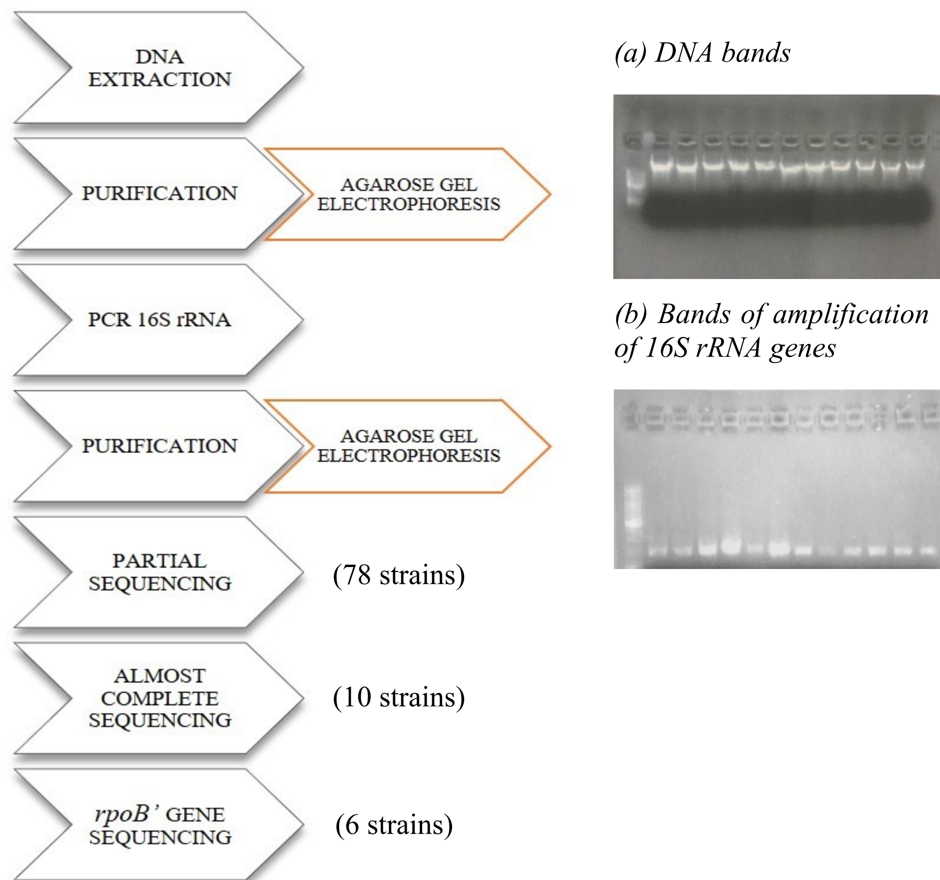


Figure 11. *Sequencing steps used in this study.*

4.3. Phylogenetic analysis

4.3.1. Phylogenetic analysis based on the partial sequence of the 16S rRNA gene

Firstly, to determine the phylogenetic position of each strain, partial sequencing of the 16S rRNA gene was performed. Using the PCR technique, the 16S rRNA gene was amplified and once the sequences of this gene were obtained, we analysed the sequence similarity by comparing them with the available sequences from the EzBioCloud and BLAST databases (Section 3.4.2 of Material and Methods).

Results from the phylogenetic study based on the comparison of the partial sequences of the gene 16S rRNA of each strain are shown in Tables 6, 7 and 8. The percentages of similarity obtained for the studied strains and their phylogenetically closest species, ranged between 92.49% and 99.68%.

Table 6. *Percentages of similarity between the studied strains isolated from Isla Cristina and their phylogenetically closest species, based on the comparison of their partial 16S rRNA gene sequences. Strains selected for further studies have been marked with different colour.*

Strain	Top hit taxon	Length (bp)	Similarity (%)	Completeness (%)
Archaea:				
IC36-41	*Haloarcula vallismortis*	733	93.07	51.7
F6-79	*Haloarcula salaria*	1130	98.58	79.2
F2-101	*Haloarcula marismortui*	1019	98.82	71.4
F2-159	*Haloarcula vallismortis*	1013	98.91	71.7
F13-50	*Haloarcula hispanica*	860	99.65	60.4
IC30-61	*Halomicroarcula limicola*	1125	96.53	94.2
F2-221B	*Halonotius pteroides*	943	95.33	70.3
F2-216C	*Halonotius pteroides*	976	98.98	70.2
F16-84	*Haloplanus rallus*	1090	96.51	75.5
F2-19	*Haloplanus salinarum*	1075	99.63	95.2
F16-72	*Haloplanus salinus*	946	99.68	68.2
F12-7	*Halorientalis persicus*	958	95.72	68.1
F3-333	*Halorubrum lipolyticum*	750	98.00	51.0

F3-313	*Halorubrum depositum*	1024	98.34	71.2
F9-202	*Halorubrum orientale*	1091	98.35	98.2
F13-101	*Halorubrum kocurii*	719	98.58	55.9
F14-107	*Halorubrum kocurii*	1090	98.60	98.0
IC30-37	*Halorubrum persicum*	1048	98.66	74.4
F14-76B	*Halorubrum halophilum*	981	98.67	70.4
IC28-63	*Halorubrum californiense*	714	98.88	67.7
IC36-37	*Halorubrum coriense*	1204	98.92	97.4
F9-200	*Halorubrum orientale*	965	98.96	69.4
F5-67	*Halorubrum chaoviator*	959	99.06	69.0
F1-107	*Halorubrum chaoviator*	996	99.10	71.3
F14-77	*Halorubrum sodomense*	1012	99.11	72.2
IC28-1	*Halorubrum chaoviator*	1020	99.12	72.7
F14-105	*Halorubrum coriense*	1086	99.17	98.0
F2-155A	*Halorubrum chaoviator*	1039	99.23	73.9
F15-19	*Halorubrum coriense*	1098	99.27	98.3
IC36-40	*Halorubrum rutilum*	1010	99.31	72.1
IC36-5	*Halorubrum chaoviator*	885	99.44	64.5
F14-81	*Halovenus aranensis*	1100	94.17	98.2
F2-12	*Natronomonas moolapensis*	719	97.64	51.4
F16-168	*Natronomonas moolapensis*	1056	98.86	97.3
F16-141A	*Natronomonas moolapensis*	723	99.03	51.6
F16-76	*Natronomonas moolapensis*	726	99.17	51.2
F16-169	*Natronomonas moolapensis*	723	99.17	49.2
F9-153	*Natronomonas moolapensis*	727	99.45	51.9
F2-206	*Natronomonas moolapensis*	751	99.47	53.6
F9-25	*Natronomonas moolapensis*	728	99.59	51.3
F9-149	*Natronomonas moolapensis*	1045	99.62	74.4
F16-32	*Natronomonas moolapensis*	806	99.63	54.9

F9-28	*Natronomonas moolapensis*	981	99.69	69.1
Bacteria:				
F6-104	*Salicola salis*	1113	99.64	76.7
F14-71	*Salinibacter ruber*	1039	97.59	71.4
F2-10	*Salinibacter ruber*	1028	97.76	71.4
F9-2	*Salinibacter ruber*	1024	97.85	70.0
F1-7	*Salinibacter ruber*	1023	97.95	69.9
F3-317	*Salinibacter ruber*	1015	99.11	69.4
F13-313	*Salinibacter ruber*	1029	99.51	70.4

Table 7. *Percentages of similarity between the studied strains isolated from Isla Bacuta salterns and their phylogenetically closest species, based on the comparison of their partial 16S rRNA gene sequences. Strain selected for further studies have been marked with different colour.*

Strain	Top hit taxon	Length (bp)	Similarity (%)	Completeness (%)
Archaea:				
F8-44	*Haloarcula marismortui*	916	98.14	87.2
F20-227B	*Haloarcula vallismortis*	929	98.17	65.1
IB35-35	*Haloarcula marismortui*	788	98.34	56.2
IB35-40	*Haloarcula marismortui*	910	98.90	64.7
IB35-36	*Haloarcula vallismortis*	920	99.24	65.4
IB35-39	*Haloarcula argentinensis*	870	99.54	61.8
F18-61	*Halobellus salinus*	903	99.33	63.8
F8-121	*Haloplanus natans*	1060	99.34	75.2
F8-48	*Halorubrum coriense*	1099	98.91	98.0
IB35-46	*Halorubrum orientale*	1115	99.10	97.9
F18-96	*Halorubrum chaoviator*	1030	99.32	73.3
F20-229	*Natronomonas moolapensis*	1034	92.84	70.1
F7-147	*Natronomonas moolapensis*	720	99.30	48.9
F18-118B	*Natronomonas moolapensis*	910	99.56	64.2
F18-70	*Natronomonas moolapensis*	897	99.67	64.0
Bacteria:				
F19-64	*Salicola salis*	1050	99.52	72.6
F7-160	*Salinibacter ruber*	1034	97.49	70.7

Table 8. *Percentages of similarity between the studied strains isolated from Odiel Saltmarshes (soil) and their phylogenetically closest species, based on the comparison of their partial 16S rRNA gene sequences. Strain selected for further studies have been marked with different colour.*

Strain	Top hit taxon	Length (bp)	Similarity (%)	Completeness (%)
Archaea:				
S4-6	*Haloarcula salaria*	1155	98.18	97.9
S2-9	*Haloarcula hispanica*	1000	98.20	71.0
S2-3	*Haloarcula marismortui*	1176	98.81	98.0
S4-39	*Haloarcula marismortui*	1115	98.83	75.9
S1-80	*Haloarcula marismortui*	956	99.16	66.5
S2-2	*Haloarcula marismortui*	1122	99.29	98.2
S4-4	*Haloarcula marismortui*	1118	99.37	76.0
S1-15	*Halobacterium litoreum*	1039	99.04	73.7
S1-172	*Halobellus inordinatus*	1065	96.15	75.6
S3-41	*Halonotius pteroides*	750	92.49	52.2
S2-8	*Haloplanus natans*	1063	98.78	75.5

From the phylogenetic point of view, of the total of 78 studied strains (whose percentages are shown in Figure 12), 22 were related to species of the genus *Halorubrum,* 18 to the genus *Haloarcula,* 15 to the genus *Natronomonas,* 5 to the genus *Haloplanus,* 3 to the genus *Halonotius,* 2 to the genus *Halobellus,* and 1 to the genera *Halobacterium, Halomicroarcula, Halorientalis* and *Haloven*us, respectively. Regarding bacterial strains, 7 strains were related to species of the genus *Salinibacter* and 2 to the genus *Salicola.*

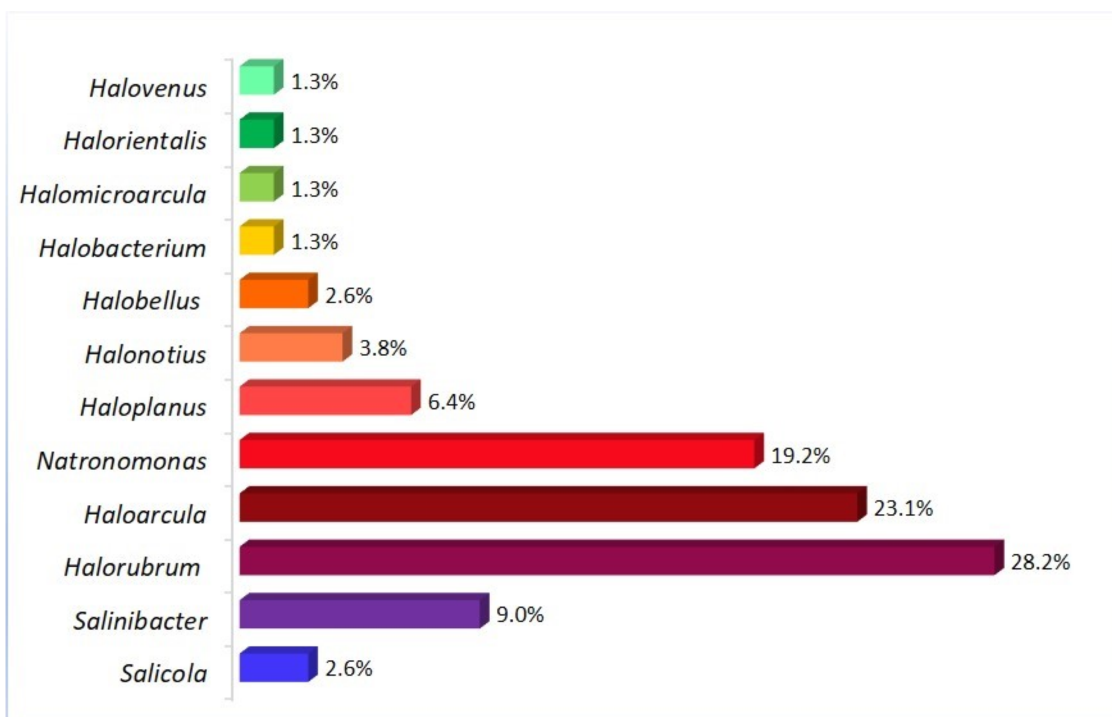


Figure 12. *Graphic representation of the percentage of isolated strains grouped by genus which were most closely related to.*

Additionally, Figure 13 shows the representation of the different genera regarding the isolation place. The predominant genus in this study from Isla Cristina solar salterns was *Halorubrum,* while from Isla Bacuta solar salterns and from the hypersaline soils from Odiel Saltmarshes, the most abundant genus was *Haloarcula* (almost 50%).

In the recent years, several metagenomic studies carried out on these hypersaline environments have permitted the determination of the microbial diversity of these habitats (Fernández et al., 2014b; Vera-Gargallo and Ventosa, 2018). These studies have brought to light that the most abundant genus in Isla Cristina solar saltern (Fernández et al., 2014b) was the genus *Halorubrum*, which also corresponds with the most abundant one according to the results obtained from this study. On the other hand, according to the metagenomic studies carried out in two hypersaline soils from Odiel Saltmarshes, indicates that one of the most abundant genus on that soils was the genus *Haloarcula* (Vera-Gargallo and Ventosa, 2018) which corresponds to the most abundant one obtained in this study. There is no metagenomic study from Isla Bacuta solar saltern, thus we compared the results with the studies from Bras del Port solar saltern (Santa Pola, Alicante), being probably the best known hypersaline environment in our planet and an excellent model for the study of the microbial diversity (Ventosa

et al., 2014). Metagenomic studies on this salterns have determined that the predominant genera in the crystallizer and concentrator ponds are *Haloquadratum*, *Halorubrum* and *Salinibacter* (Ventosa et al., 2014; 2015).

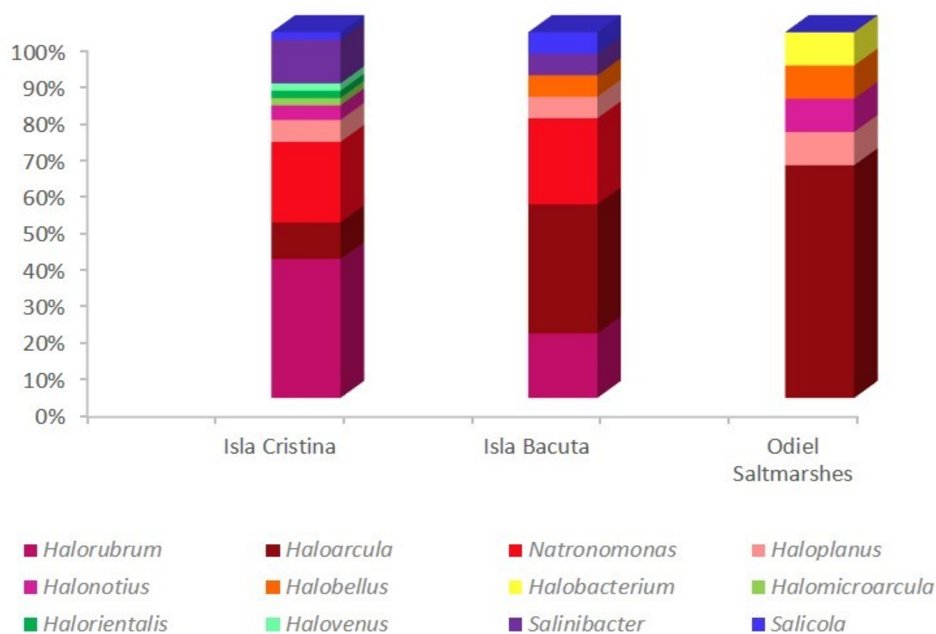


Figure 13. *Relative percentages of the different genera in the salternsof Isla Cristina, Isla Bacuta and in the hypersaline soil from Odiel Saltmarshes.*

4.3.2. Phylogenetic analysis based on the almost complete sequence of the 16S rRNA gene

For strains which showed a phylogenetic relationship with a percentage of similarity lower than 98% with closely related taxa based on the 16S rRNA partial sequences, 16S rRNA gene was completely sequenced. Thus, ten strains were selected to perform a more detailed phylogenetic study. For strains F1-7, F9-2 and F7-160, the sequence could not be obtained before departing Spain.

Table 9 shows the almost complete 16S rRNA gene sequences similarity of the selected strains with the most related taxa. Identity values ranged from 93.40 to 97.90% and closest related members were *Halobellus inordinatus, Halomicroarcula limicola, Halonotius pteroides, Haloplanus vescus, Halorientalis brevis, Halovenus aranensis,*

Natronomonas moolapensis and *Salinibacter ruber.* According to Stackebrandt et al. (2002), considering a 97% 16S rRNA gene species cutoff, sequence data suggest that some of the isolates of this study might represent new species or even new genera.

Table 9. *Percentages of similarity between the studied strains and their phylogenetically closest species, based on the comparison of their almost complete 16S rRNA gene sequences.*

Strain	Top hit taxon	Length (bp)	Similarity (%)
S1-172	*Halobellus inordinatus*	1042	95.57
IC30-61	*Halomicroarcula limicola*	1385	96.66
F2-221B	*Halonotius pteroides*	1378	96.21
F16-84	*Haloplanus vescus*	1392	96.55
F12-7	*Halorientalis brevis*	1381	95.93
F14-81	*Halovenus aranensis*	1398	94.10
F2-12	*Natronomonas moolapensis*	1380	97.90
F20-229	*Natronomonas moolapensis*	1393	93.40
F14-71	*Salinibacter ruber*	1101	97.50
F2-10	*Salinibacter ruber*	1091	97.48

A phylogenetic study based on the comparison of 16S rRNA gene sequence was performed by constructing phylogenetic trees using the maximum-parsimony and maximum-likelihood algorithms integrated in the ARB program (see Material and methods section 3.4.2).

The phylogenetic tree based on the 16S rRNA gene shows that strains F2-12, F12-7, F14-81 and IC30-61 cluster in the branches of species of the genus *Natronomonas*, *Halorientalis*, *Halovenus* and *Halomicroarcula*, respectively, confirming they belong to those genera. Besides, they cluster in a different branch from the species of these genera, suggesting that they could constitute new species within their most related genera (Figure 14).

On the other hand, strain F20-229, which was most closely related to *Natronomonas moolapensis* with a percentage of similarity of 93.4%, formed an independent phylogenetic branch, well separated and distant enough from the other *Natronomonas* species, which indicates that it might represent a new genus.

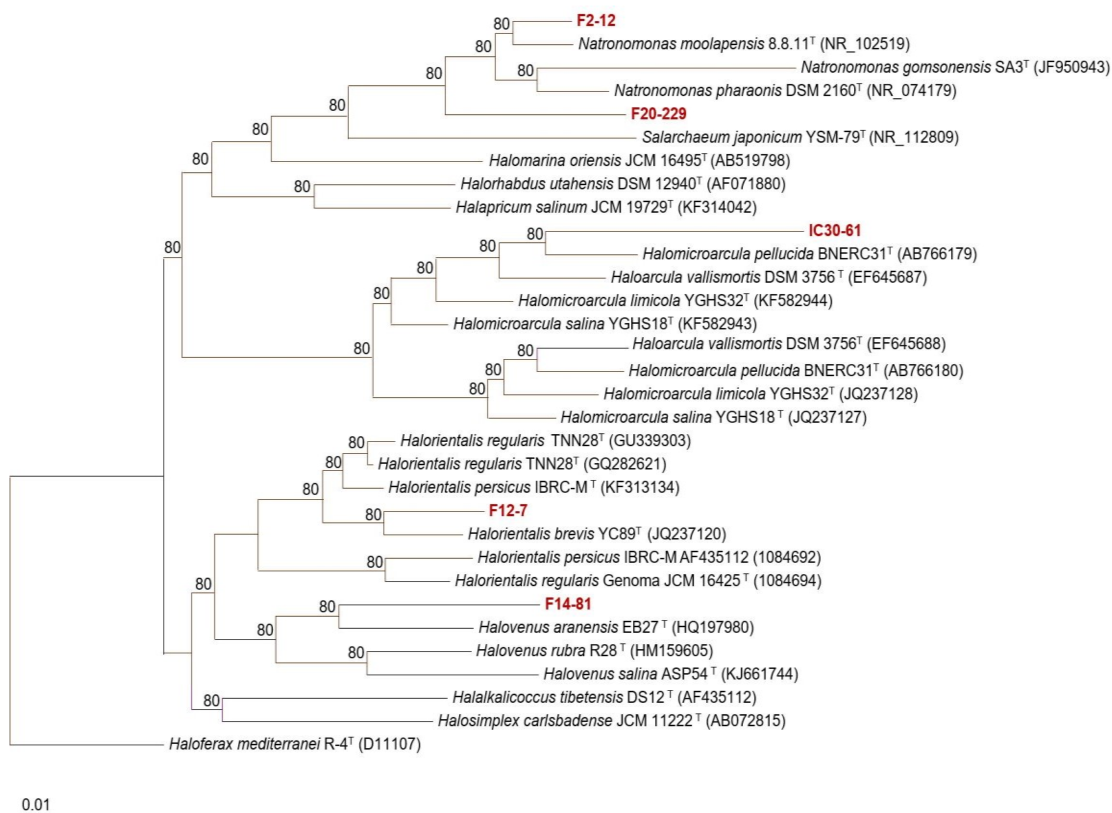


Figure 14. *Maximum-parsimony tree based on 16S rRNA gene sequence showing the phylogenetic position of strains F2-12, F20-229, F12-7, F14-81 and IC30-61 with respect to their most closely related species belonging to the order Halobacteriales. Bootstrap values >70% are indicated. The species Haloferax mediterranei R-4^T was used as an outgroup. The scale bar represents 0.01 substitutions per nucleotide position.*

The 16S rRNA phylogenetic tree of strains regarding the order *Haloferacales*, showed that strains S1-172 and F2-221B, clustered in a single branch with the species of the genera *Halobellus* and *Halonotius*, respectively, but formed separated phylogenetic branches, indicating that they might constitute new species within these genera (Figure 15). On the other hand, strain F16-84 formed an independent and defined branch, clearly separated from the other species of the genus *Haloplanus*, suggesting that it could constitute a new genus (Figure 15).

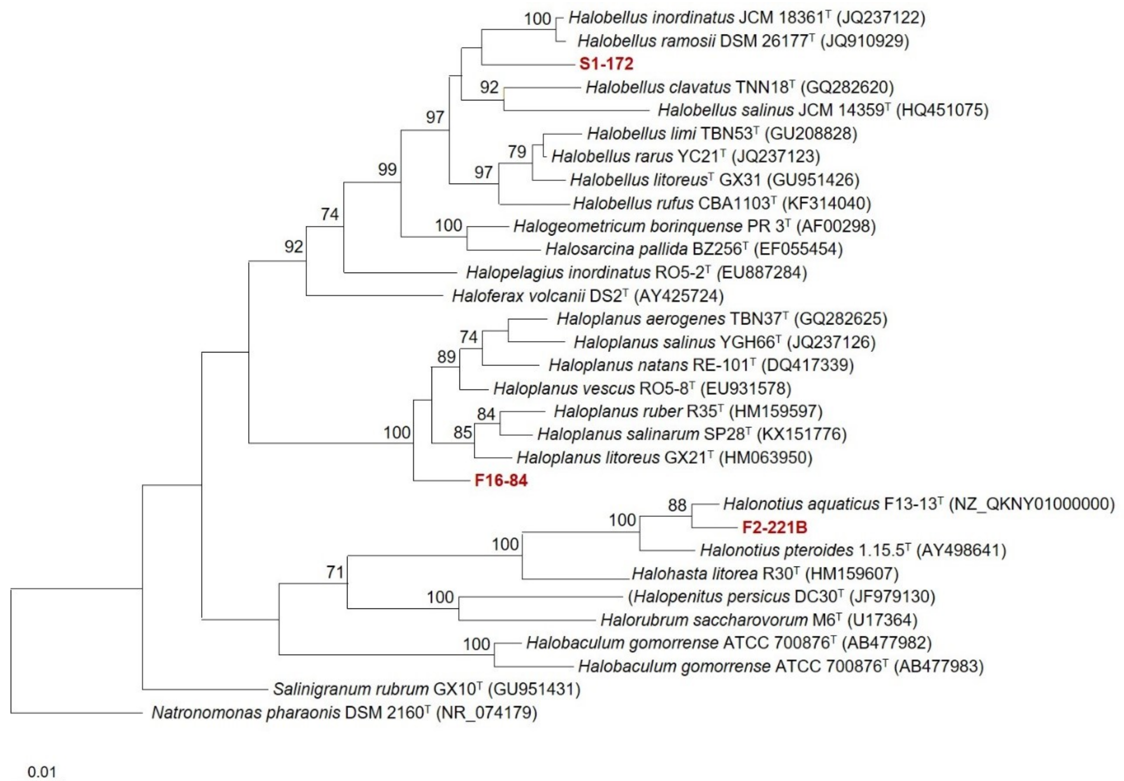


Figure 15. *Maximum-likelihood tree based on 16S rRNA gene sequence showing the phylogenetic position of strains S1-172, F16-84 and F2-221B with respect to their most closely related species belonging to order Haloferacales. Bootstrap values >70% are indicated. The species Natronomonas pharaonis DSM 2160^{T} was used as an outgroup. The scale bar represents 0.01 substitutions per nucleotide position.*

Bacterial strains, F14-71 and F2-10, were grouped together in a single branch, within the genus *Salinibacter*, but forming an independent branch well separated from the described species, suggesting that they could constitute a new species within this genus (Figure 16).

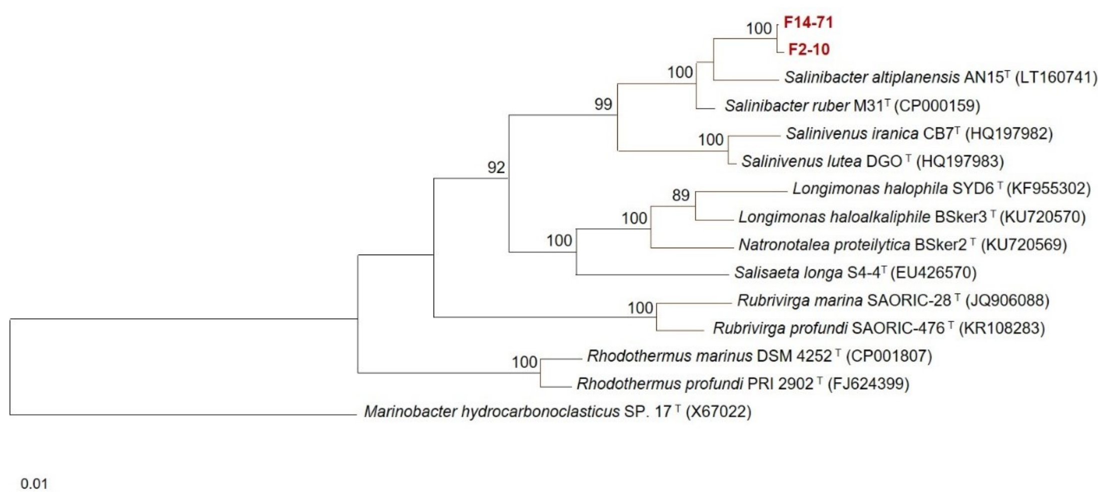


Figure 16. *Maximum-likelihood tree based on 16S rRNA gene sequence showing the phylogenetic position of strains F14-71 and F2-10 with respect to their most closely related species belonging to domain Bacteria. Bootstrap values >70% are indicated. The species Marinobacter hydrocarbonoclasticus SP. 17^T was used as an outgroup. The scale bar represents 0.01 substitutions per nucleotide position.*

4.3.3. Phylogenetic analysis based on the sequence of the *rpoB'* gene

Whereas the 16S rRNA gene has some limitations in haloarchaea phylogeny and several of the prokaryotic species present two or more different copies of the rRNA genes that do not allow to distinguish correctly between closely related species (de la Haba et al., 2018), the *rpoB'* gene of the six haloarchaeal selected strains was also sequenced (described in parts 3.3. and 3.4. of Material and Methods). The *rpoB'* phylogenetic trees were created using the maximum-likelihood algorithm integrated in the MEGA software (see Materials and methods section 3.4.2). Table 10 corresponds to the percentages of similarity based on *rpoB'* gene sequences between studied strains and their closest related species.

According to the results obtained for the 16S rRNA gene, phylogenetic analysis based on *rpoB'* gene also showed that strains F12-7, F2-12 and IC30-61 clustered with the species of the genera *Halorientalis, Natronomonas* and *Halomicroarcula*, respectively, but in different branches, suggesting their condition of different species within these genera (Figure 17).

In accordance to the 16S rRNA phylogenetic analysis of strain F20-229, which was closely related to species of the genus *Natronomonas* but clustered in a different branch well separated from them, this phylogeny is maintained on the *rpoB´* tree (Figure 17).

However, strain F14-81, which according to the 16S rRNA phylogenetic tree was within the genus *Halovenus*, in this case it appears in a different branch most related to species of the genus *Halorientalis* (Figure 18) even though, the sequence similarity according to the *rpoB´*gene indicates their closest similarity to *Halovenus salina* (Table 10). Thus, more analyses need to be carried out. For the rest of haloarchaeal strains, we were not able to amplify the *rpoB'* gene, that is why data are not included.

Regarding the only strain of the class *Haloferacales* for which the *rpoB´*gene could be obtained (F16-84), results are in concordance with those obtained for 16S rRNA phylogeny. Strain F16-84 clustered within the genus *Haloplanus* but in a different branch well separated from the other species which suggests that it could constitute a new species within this genus.

Table 10. *Percentages of similarity between the studied strains and their phylogenetically closest species, based on the comparison of their rpoB' gene sequences.*

Strain	Closest related species	Similarity (%)
IC30-61	*Halomicroarcula salina*	91.65
F16-84	*Haloplanus salinus*	91.75
F12-7	*Halorientalis brevis*	83.33
F14-81	*Halovenus salina*	85.68
F20-229	*Natronomonas moolapensis*	84.64
F2-12	*Natronomonas moolapensis*	89.70

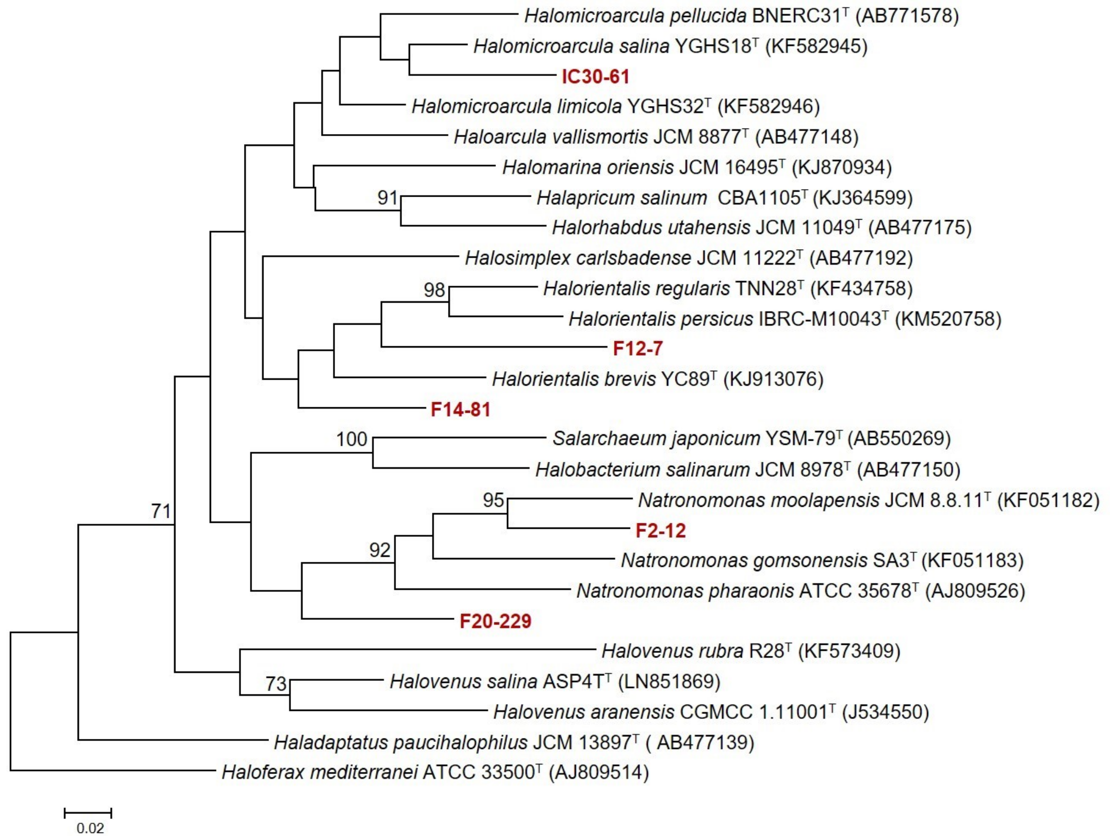


Figure 17. *Maximum-likelihood tree based on rpoB' gene sequence showing the phylogenetic position of strains IC30-61, F12-7, F14-81, F2-12, and F20-229 with respect to their most closely related species belonging to the order Halobacteriales. Bootstrap values >70% are indicated. The species Haloferax mediterranei ATCC 33500^T was used as an outgroup. The scale bar represents 0.02 substitutions per nucleotide position.*

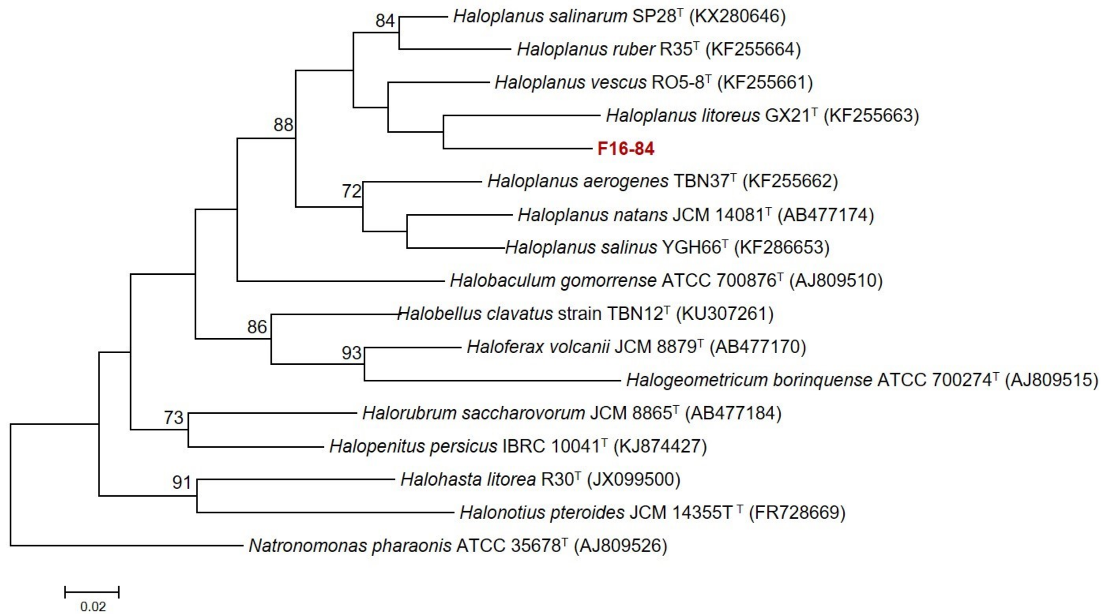


Figure 18. *Maximum-likelihood tree based on rpoB' gene sequence showing the phylogenetic position of strain F16-84 with respect to their most closely related species belonging to the order Haloferacales. Bootstrap values >70% are indicated. The species Natronomonas pharaonis ATCC 35678^T was used as an outgroup. The scale bar represents 0.02 substitutions per nucleotide position.*

5. CONCLUSIONS

The aim of this work was the identification of different halophilic bacteria and archaea isolated in previous studies from different hypersaline environments located in the southwest of Spain. We also carried out a biodiversity study of these samples. Strains of interest were also phylogenetically studied. The main conclusions of this study are the following:

1. The most abundant isolates were representatives of the genera *Halorubrum*, followed by *Haloarcula, Natronomonas* and *Haloplanus*.
2. Regarding the isolation place, the dominant genus in the salterns of Isla Cristina was *Halorubrum,* while in the salterns of Isla Bacuta and soils of Odiel Saltmarshes, the most abundant one was *Haloarcula.*
3. A total of 78 strains were analysed based on the partial sequences of the 16S rRNA gene. Ten of them were selected according to the percentage of similarity lower than 98%.
4. 16S rRNA gene was completely sequenced. We constructed phylogenetic trees which indicated the phylogenetic position of studied strains in relation to their most closely related species. Results indicated that ten strains might represent new taxa.
5. The analysis of the *rpoB'* gene supported the results previously suggested by 16S rRNA gene proposing that studied strains might constitute new species or even new genera.

This study could be the first step to determine if these ten strains might represent new taxa. To confirm it, the strains must be studied in more detail and further research should be included:

A. Determination of polar lipids by chromatographic methods, which is a very important feature for haloarchaea characterization.
B. A complete phenotypic characterization, which includes morphological, physiological, biochemical and nutritional characteristics.
C. Genome sequencing and comparison with the closely related taxa.

6. LIST OF TABLES

Table 1. Primers used for the amplification of the 16S rRNA gene of haloarchaea and halophilic bacteria....25
Table 2. Primers used for amplification of *rpoB'* gene 25
Table 3. Primers used for sequencing of archaeal and bacterial 16S rRNA gene 26
Table 4. Primers used for sequencing the archaeal *rpoB'* gene....26
Table 5. Samples and data about their isolation places 28
Table 6. Percentages of similarity between the studied strains isolated from Isla Cristina and their phylogenetically closest species, based on the comparison of their partial 16S rRNA gene sequences. Strains selected for further studies have been marked with different colour....31
Table 7. Percentages of similarity between the studied strains isolated from Isla Bacuta salterns and their phylogenetically closest species, based on the comparison of their partial 16S rRNA gene sequences. Strain selected for further studies have been marked with different colour. 33
Table 8. Percentages of similarity between the studied strains isolated from Odiel Saltmarshes and their phylogenetically closest species, based on the comparison of their partial 16S rRNA gene sequences. Strain selected for further studies have been marked with different colour. 34
Table 9. Percentages of similarity between the studied strains and their phylogenetically closest species, based on the comparison of their almost complete 16S rRNA gene sequences 37
Table 10. Percentages of similarity between the studied strains and their phylogenetically closest species, based on the comparison of their *rpoB'* gene sequences 41

7. LIST OF FIGURES

Figure 1. Classification of prokaryotic microorganisms according to their behavior towards NaCl. Drawing: M.-J. Bodiou (Cayol et al., 2015) 7
Figure 2. Classification of haloarchaea from phylum to genus (Amoozegar et al., 2017) 16
Figure 3. (a) Isla Cristina salterns and (b) Isla Cristina salterns (once water is evaporated), Huelva, Spain. Author/Source: Antonio Ventosa 21
Figure 4. Isla Bacuta salterns, Huelva, Spain. Author/Source: Antonio Ventosa 22
Figure 5. Saline soils of Odiel Saltmarshes, Huelva, Spain. Author/Source: Antonio Ventosa 22
Figure 6. DNA electrophoresis system (Corral Villa, 2014) 21
Figure 7. Steps in performing a DNA electrophoresis in agarose gel (Corral Villa, 2014) 22
Figure 8. GeneAmp® PCR System 9700 (Corral Villa, 2014) 28
Figure 9. Mastercycler Ep (Eppendorf) (Corral Villa, 2014) 23
Figure 10. Percentage distribution of 78 sequenced *Archaea* and *Bacteria* 28
Figure 11.Sequencing steps used in this study (a) DNA bands, (b) Bands of amplification of 16S rRNA genes 30
Figure 12. Graphic representation of the percentage of isolated strains grouped by genus which were most closely related to 35
Figure 13. Relative percentages of the different genera in the salterns of Isla Cristina, Isla Bacuta and in the hypersaline soil from Odiel Saltmarshes 36
Figure 14. Maximum-parsimony tree based on 16S rRNA gene sequence showing the phylogenetic position of strains F2-12, F20-229, F12-7, F14-81 and IC30-61 with respect to their most closely related species belonging to the order *Halobacteriales*. Bootstrap values >70% are indicated. The species *Haloferax mediterranei* R-4^T was used as an outgroup. The scale bar represents 0.01 substitutions per nucleotide position. 38
Figure 15. Maximum-likelihood tree based on 16S rRNA gene sequence showing the phylogenetic position of strains S1-172, F16-84 and F2-221B with respect to their most closely related species belonging to order *Haloferacales*. Bootstrap values >70% are

indicated. The species *Natronomonas pharaonis* DSM 2160^T was used as an outgroup. The scale bar represents 0.01 substitutions per nucleotide position 39
Figure 16. Maximum-likelihood tree based on 16S rRNA gene sequence showing the phylogenetic position of strains F14-71 and F2-10 with respect to their most closely related species belonging to domain *Bacteria*. Bootstrap values >70% are indicated. The species *Marinobacter hydrocarbonoclasticus* SP. 17^T was used as an outgroup. The scale bar represents 0.01 substitutions per nucleotide position 40
Figure 17. Maximum-likelihood tree based on *rpoB'* gene sequence showing the phylogenetic position of strains IC30-61, F12-7, F14-81, F2-12, and F20-229 with respect to their most closely related species belonging to the order *Halobacteriales*. Bootstrap values >70% are indicated. The species Haloferax mediterranei ATCC 33500^T was used as an outgroup. The scale bar represents 0.02 substitutions per nucleotide position 42
Figure 18. Maximum-likelihood tree based on *rpoB'* gene sequence showing the phylogenetic position of strain F16-84 with respect to their most closely related species belonging to the order *Haloferacales*. Bootstrap values >70% are indicated. The species *Natronomonas pharaonic* ATCC 35678^T was used as an outgroup. The scale bar represents 0.02 substitutions per nucleotide position 43

8. REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403-410.

Alzohairy, A. M. (2011). BioEdit: An important software for molecular biology. *GERF Bull Biosci* **2**: 60-61.

Amoozegar, M. A., Siroosi M., Atashgahi S., Smidt H. and Ventosa A. (2017). Systematics of haloarchaea and biotechnological potential of their hydrolytic enzymes. *Microbiology* **163**: 623–645.

Arahal, D. R., Dewhirst, F. E., Paster, B. J., Ventosa, A. and Volcani, B. E. (1996). Phylogenetic analysis of some extremely halophilic archaea isolated from Dead Sea water, determined on the basis of their 16S rRNA sequences. *Appl Environ Microbiol* **62**: 3779-3786.

Atanasova, N. S., Bamford D. H. and **Oksanen, H. M. (2016).** Virus-host interplay in high salt environments. *Environ Microbiol Rep* **8**: 431-444.

Bolhuis, H., Palm, P., Wende, A., Falb, M., Rampp, M., Rodriguez-Valera, F., and Oesterhelt, D. (2006). The genome of the square archaeon *Haloquadratum walsbyi*: life at the limits of water activity. *BMC Genomics* **7**: 169.

Cayol J.-L., Ollivier, B., Alazard, D., Amils, R., Godfroy A., Piette F. and Prieur D. (2015). The extreme conditions of life on the planet and exobiology**.** In: Bertrand, J. C., Caumette, P., Lebaron, P., Matheron, R., Normand, P. and Sime-Ngando, T. (eds.) Environmental Microbiology: Fundamentals and Applications. Springer, Dordrecht, pp. 353-394.

Charlesworth, J. C. and Burns, B. P. (2015). Untapped resources: biotechnological potential of peptides and secondary metabolites in archaea. *Archaea* **2015**: 1-7.

Corral Villa, M. P., (2014). Caracterización de arqueas halófilas basada en el estudio de la composición de lípidos polares y otros métodos taxonómicos. University of Sevilla, Sevilla. pp. 74-76.

de la Haba, R. R., Sánchez-Porro, C., Márquez, M. C. and Ventosa, A. (2010). Taxonomy of halophiles. En: K. Horikoshi, G. Antranikian, A. Bull, F. Robb and K. Stetter (eds.). Extremophiles Handbook. Springer-Verlag, Heidelberg, pp. 256-260.

de la Haba, R. R., Corral P., Sánchez-Porro C., Infante-Domínguez, C., Makkay A.M., Amoozegar, M. A., Ventosa, A. and Papke R. T. (2018). Genotypic and lipid analyses of strains from the archaeal genus *Halorubrum* reveal insights into their taxonomy, divergence, and population structure. *Front Microbiol* **9**: 512.

DeLong, E. F. (1992). Archaea in coastal marine environments. *Proc Natl Acad Sci USA* **89**: 5685-5689.

Detkova, E. N., Boltyanskaya, Y. V. (2007). Osmoadaptation of haloalkaliphilic bacteria: role of osmoregulators and their possible practical application. *Microbiology* **76**: 511–522.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**: 368-376.

Fendrihan, S., Legat, A., Pfaffenhuemer, M., Gruber, C., Weidler, G., Gerbl, F., and Stan-Lotter, H. (2006). Extremely halophilic archaea and the issue of long-term microbial survival. *Rev Environ Sci Biotechnol* **5**: 203–218.

Fernández, A. B., José León, M., Vera, B., Sánchez-Porro, C. and Ventosa, A. (2014a). Metagenomic sequence of prokaryotic microbiota from an intermediate salinity pond of a saltern in Isla Cristina, Spain. *Genome Announc* **2**: e00045–14.

Fernández, A. B., Vera-Gargallo, B., Sánchez-Porro, C., Ghai, R., Papke, R. T., Rodriguez-Valera, F. and Ventosa, A. (2014b). Comparison of prokaryotic community structure from Mediterranean and Atlantic saltern concentrator ponds by a metagenomic approach. *Front Microbiol* **5**: 1-12.

Fullmer, M. S., Soucy, S. M., Swithers, K. S., Makkay, A. M., Wheeler, R., Ventosa, A., Gogarten, J. P. and Papke, R. T. (2014). Population and genomic analysis of the genus *Halorubrum. Front Microbiol* **5**: 140.

Galinski, E. A. (1995). Osmoadaptation in bacteria. *Adv Microb Physiol* **37**: 273–328.

Galinski, E. A. and Tindall, B. J. (1992). Biotechnological prospects for halophiles and halotolerant micro-organisms. Glasgow: Blackie & Sons, pp. 76–114.

Gomez, F. (2015). Extreme environment. In: Gargaud, M., Irvine, W. M., Amils, R., Cleaves, H. J., Pinti, D. L., Quintanilla, J. C., Rouan, D., Spohn, T., Tirard, S. and Viso, M. (eds.) Encyclopedia of Astrobiology. Springer, Berlin, Heidelberg, pp. 50.

Gostinčar, C., Grube, M., de Hoog G. S., Zalar, P. and Gunde-Cimerman, N. (2009). Extremotolerance in fungi: evolution on the edge. *FEMS Microbiol Ecol* **71**: 2–11.

Grant, W. D., Kamekura, M., McGenity, T. J. and Ventosa, A. (2001). Class III. Halobacteria class. nov. In: Boone, D. R., Castenholz, R. W. and Garrity, G. M. (eds.). Bergey's Manual of Systematic Bacteriology, 2nd edition, vol. 1. Springer, New York, pp. 294–301.

Imran, A., Prasongsuk, S., Akbar, A., Aslam, M., Lotrakul, P., Punnapayak, H. and Rakshit, S. K. (2016). Hypersaline habitats and halophilic microorganisms. *Maejo Int J Sci Technol* **10**: 330-345.

Infante-Domínguez, C. (2015). Nuevos grupos de arqueas y bacterias halófilas: Filogenómica y taxonomía molecular. University of Sevilla, Sevilla. pp. 34.

Kapilesh, J., Bijayendra, K. and Indrani, J., (2018). Insight into compatible solutes from halophiles: exploring significant applications in biotechnology. In: Singh, J., Sharma, D., Kumar, G., Sharma, N. (eds.) Microbial Bioprospecting for Sustainable Development. Springer, Singapore, pp. 291-307.

Kushner, D. J. and Kamekura, M. (1988). Physiology of halophilic eubacteria. In: Rodríguez-Valera, F. (ed.). Halophilic Bacteria. CRC Press, Boca Raton, pp. 109-138.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. and Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947-2948.

Lawyer, F. C., Stoffel, S., Saiki, R. K., Myambo, K. D., Drummond, R. and Gelfand, D. H. (1989). Isolation, characterization, and expression in *E. coli* of the DNA polymerase gene from the extreme thermophile, *Thermus aquaticus*. *J Biol Chem* **264**: 6427-6437.

Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenernegger, M., Neumaier, J., Bachleitner, M. and Schleifer, K.-H. (1998). Bacterial phylogeny based on comparative sequences analysis. *Electrophoresis* **19**: 554-568.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, A., Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A. W., Gross, O., Grumann, S., Hermann, S., José, R., König, A., Liss, T., Lüβmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A. and Schleifer K.-H. (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363-1371.

Litchfield, C. D. (2011). Potential for industrial products from the halophilic archaea. *J Ind Microbiol Biotechnol* **38**: 1635–1647.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J Mol Biol* **3**: 208-218.

Mellado, E., Moore, E. R. B., Nieto, J. J. and Ventosa, A. (1995). Phylogenetic inferences and taxonomic consequences of 16S ribosomal DNA sequence comparison of *Chromohalobacter marismortui, Volcaniella eurihalina,* and *Deleya salina* and reclassification of *V. eurihalina* as a *Halomonas eurihalina* comb. nov. *Int J Syst Bacteriol* **45**: 712-716.

Messadek, J. (2005). Glycine betaine and its use. US Patent, 6855734.

Moreno, M. de L., García, M. T., Ventosa, A. and Mellado, E. (2009). FEMS Microbiol Ecol **68:** 59–71.

Mullis, K. B. and Faloona, F. A. (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Meth Enzymol* **155**: 335-350.

Namwong, S., Tanasupawat, S., Visessanguan, W., Kudo, T. and Takashi, I. (2007). *Halococcus thailandensis* sp. nov., from fish sauce in Thailand. *Int J Syst Evol Microbiol* **57**: 2199–2203.

Oren, A. (2008). Microbial life at high salt concentrations: phylogenetic and metabolic diversity**.** *Saline Syst* **4**: 2.

Oren A. (2016). Life in hypersaline environments. In: Hurst, C. (ed.) Their World: A diversity of microbial environments. Springer, Cham**,** pp. 301-339.

Papke, R. T., Oren, A., Ventosa, A., Dillon, J. (2015). The Proceedings from Halophiles 2013, the International Congress on Halophilic Microorganisms**.** *Frontiers Media,* Lausanne, pp. 125.

Reasoner, D. J. and Geldreich, E. (1985). A new medium for the enumeration and subculture of bacteria from potable water. *Appl Environ Microbiol* **49**: 1-7.

Roberts, M. F. (2005). Organic compatible solutes of halotolerant and halophilic microorganisms. *Saline Syst* **1**: 5.

Rodriguez-Valera, F., Ventosa, A., Juez, G. and Imhoff, J. F. (1985). Variation of environmental features and microbial populations with salt concentrations in a multi-pond saltern. *Microb Ecol* **11:** 107–115.

Rodríguez-Valera, F. (1988). Characteristics and microbial ecology of hypersaline environments. In: Rodríguez-Valera, F. (ed.). Halophilic Bacteria. CRC Press, Boca Raton, pp. 3–30.

Saiki, R. K., Scharf, S. J., Faloona, F., Mullis, K. B., Hom, G. T., Erlich, H. A. and Amheim, N. (1985). Enzymatic amplification of β-globin genomic sequence and restriction site analysis for diagnosis of sickle cell anaemia. *Science* **230**: 1350-1354.

Sambrook, J. and Rusell, D. W. (2001). Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Schleifer, K. H. (2009). Classification of *Bacteria* and *Archaea*: Past, present and future. *Syst Appl Microbiol* **32**: 533-542.

Seckbach, J., Oren, A., Chapman, R. L., Waters, D. A., Ross, I. K., Bonch-Osmolovskaya, B., Gunde-Cimerman, N. and Tamburini, C. (2006). Biodiversity and Extremophiles. In: Seckbach J. (ed.) Life as we know it. Springer, Dordrecht.

Simon, R. D., Abeliovich, A. and Belkin, S. (1994). A novel terrestrial halophilic environment: the phylloplane of *Atriplex halimus*, a salt-excreting plant. *FEMS Microbiol Ecol* **14**: 99-110.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A, Kämpfer, P., Maiden, M. C., Nesme, X., Rosselló-Mora, R., Swings, J., Trüper, H. G., Vauterin, L., Ward, A. C., Whitman, W. B. (2002). Report of the ad hoc committee for the reevaluation of the species definition in bacteriology. *Int J Sys Evol Microbiol* **52**: 1043–1047.

Stan-Lotter, H., Fendrihan, S., Dorn-Mayr-Pfaffenhuemer, M., Polacsek, T. K., Legat, A., Holzinger, A. K., Grösbacher, M. and Weigl, A. (2012). The likelihood of halophilic life in the universe. In: Hanslmeier A., Kempe S., Seckbach J. (eds.) Life on Earth and other Planetary Bodies. Cellular Origin, Life in Extreme Habitats and Astrobiology, vol 24. Springer, Dordrecht, pp. 345-365.

Subow, N. N. (1931). Oceanographical tables. Commissariat of agriculture of USSR. Hydro-Meteorological Committee of USSR. Oceanographical Institute of USSR, Moscow.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol*. **30**, 2725-2729.

Torsvik, V., Øvreås, L. and Thingstad, T. F. (2002). Prokaryotic diversity-magnitude, dynamics, and controlling factors. *Science* **296**: 1064–1066.

Thiel, V. (2011). Extreme Environments. In: Reitner, J. and Thiel, V. (eds.). Encyclopedia of Geobiology. Encyclopedia of Earth Sciences Series. Springer, Dordrecht, pp. 362.

van den Burg, B. (2003). Extremophiles as a source for novel enzymes. *Curr Opin Microbiol* **6**: 213–218.

Ventosa, A. (2006). Unusual micro-organisms from unusual habitats: hypersaline environments. In: Prokaryotic Diversity: Mechanism and Significance. Logan, N. A., Lappin-Scott, H. M. and Oyston, P. C. F. (eds.). Cambridge University Press, Cambridge, pp. 223-253.

Ventosa, A., Nieto, J. J. and Oren, A. (1998). Biology of moderately halophilic aerobic bacteria. *Microbiol Mol Biol Rev* **62**: 504–544.

Ventosa, A. and Arahal, D. R. (2002). Physico-chemical characteristics of hypersaline environments and their biodiversity. In: Gerday, C. and Glansdorff, N. (eds.). Encyclopedia of life support systems (EOLSS). UNESCO Publishing-Eolss Publishers, Oxford, pp. 10020–10031.

Ventosa, A., Fernández, A. B., León, M. J., Sánchez-Porro, C. and Rodriguez-Valera, F. (2014). The Santa Pola saltern as a model for studying the microbiota of hypersaline environments. *Extremophiles* **18**: 811–824.

Ventosa, A., de la Haba, R. R., Sánchez-Porro, C. and Papke, R. T. (2015). Microbial diversity of hypersaline environments: a metagenomic approach. *Curr Op Microbiol* **25**: 80–87.

Vera-Gargallo, B. and Ventosa, A. (2018). Metagenomic insights into the phylogenetic and metabolic diversity of the prokaryotic community dwelling in hypersaline soils from the Odiel Saltmarshes (SW Spain). *Genes* **9**: 152.

Vera-Gargallo, B., Navarro-Sampedro, L., Carballo, M. and Ventosa, A. (2018). Metagenome sequencing of prokaryotic microbiota from two hypersaline soils of the Odiel Salt Marshes in Huelva, Southwestern Spain. *Genome Announc* **6**: e00140-18.

Walsby, A. E. (1994). Gas vesicles. *Microbiol Rev* **58**: 94–144.

Yoon, S. H., Ha, S. M., Kwon, S., Lim, J., Kim, Y., Seo, H. and Chun, J. (2017). Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int J Syst Evol Microbiol* **67**: 1613-1617.

Yoon, J. H., Kang, S. S., Lee, K. C., Kho, Y. H., Choi, S. H., Kang, K. H. and Park, Y. H. (2001). *Bacillus jeotgali* sp. nov., isolated from jeotgal, Korean traditional fermented seafood. *Int J Syst and Evol Microbiol* **51**: 1087-1092.

Zajc, J., Liu, Y., Dai, W., Yang, Z., Hu J., Gostinčar, C. and Gunde-Cimerman, N. (2013). Genome and transcriptome sequencing of the halophilic fungus *Wallemia ichthyophaga*: haloadaptations present and absent. *BMC Genomics* **14**: 617.