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DIPLOMOVÁ PRÁCE

DIPLOMA THESIS

FYLOGENETICKÁ A TAXONOMICKÁ CHARAKTERIZACE NOVÝCH HALOARCHEÍ BLÍZKÝCH RODU *NATRONOMONAS*

PHYLOGENETIC AND TAXONOMIC CHARACTERIZATION OF NEW HALOARCHAEA RELATED TO *NATRONOMONAS*

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My sincere thanks to Ana Durán-Viseras for supporting and leading me in the laboratory, for her patience, kindness and all her help without which this work would not be possible.

I would like to thank Prof. Antonio Ventosa and Prof. Cristina Sánchez-Porro for their guidance and for reading and revising this work, and to Assoc. Prof. PharmDr. Miloslav Hronek, PhD. for his support.

Finally, my thanks go to all people from the laboratory for being kind and helpful and to my family and friends for supporting me.

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„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, 2018

Anna Šantorová

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LIST OF ABBREVIATIONS

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ed.	editor
EDTA	ethylene diamine tetra acetic
Et al.	and others (from Latin "*et alii"*)
G+C content	guanine plus cytosine content
HPTLC	High Performance Thin Layer Chromatography
Milli-Q	ultrapure water type 1
MLSA	Multilocus sequence analysis
N. goms.	*Natronomonas gomsonensis*
N. mool.	*Natronomonas moolapensis*
N. phar.	*Natronomonas pharaonis*
PCR	Polymerase chain reaction
PG	Phosphatidylglycerol
PGP-Me	Phosphatidylglycerolphosphate methyl ester
PGS	Phosphatidylglycerol sulphate
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
TAE	Tris-acetate-EDTA solution
Taq	*Thermus aquaticus* DNA polymerase

ABSTRAKT

Kandidát: Anna Šantorová

Název diplomové práce: Fylogenetická a taxonomická charakterizace nových haloarcheí blízkých rodu *Natronomonas*.

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Tato práce pokračuje v charakterizaci nových *Archeí* blízkých rodu *Natronomonas* izolovaných z hypersalinních vod solných polí, lokalizovaných u města Huelva v jihozápadním Španělsku. Jedná se o mikroorganismy vyžadující k životu vysokou koncentraci solí v okolním prostředí. S jejich charakterizací začala Ana Durán-Viseras a během svého pobytu jsem se podílela na jejím pokračování.

Pro doplnění fylogenetické studie jsme amplifikovali gen *rpoB'* jednotlivých *Archeí* v rámci MLSA a vytvořili fylogenetický strom. Na základě těchto výsledků jsme usoudili, že se jedná o nový rod. Tento fakt byl dále potvrzen analýzou složení polárních lipidů pomocí HPTLC. U izolované DNA jsme určili obsah guaninových a cytosinových bazí, jenž je součástí taxonomických charakterizací nových druhů. Započali jsme s fenotypickou charakterizací.

ABSTRACT

Candidate: Anna Šantorová

Title of diploma thesis: Phylogenetic and taxonomic characterization of new haloarchaea related to *Natronomonas*.

Charles University, Faculty of Pharmacy

Department of Biological and Medicinal Sciences

University of Sevilla, Faculty of Pharmacy

Department of Microbiology and Parasitology

Study program: Pharmacy

This work continues with the characterization of new Archaea related to the genus *Natronomonas* isolated from hypersaline waters of solar salterns located near the city of Huelva in the southwest of Spain. These microorganisms need a high concentration of salts in the environment for their survival and growth. Ana Durán-Viseras started with their characterization and I was participating on the continuation of the project during my stay in Sevilla.

To complete the phylogenetic studies, we amplified the *rpoB'* gene of the potentially new species of *Archaea* as a part of MLSA and we created a phylogenetic tree. Based on these results we came to the conclusion that they represent new genus, which was later confirmed by the analysis of polar lipids profile by the HPTLC method. We have determined the guanine and cytosine content, which is part of the taxonomic characterization of new prokaryotic species. We have started the phenotypic characterization.

1. OBJECTIVES

The objectives of this work were to carry out a polyphasic characterization of two haloarchaeal strains, F17-44 and F12-1, isolated from the two listed locations, that could probably correspond to new taxa not described before. This work has specifically been focused on the study of biodiversity of haloarchaea and halophilic bacteria from two hypersaline environments: the solar salterns of Isla Cristina and Isla Bacuta, located in Huelva, southwestern Spain.

2. INTRODUCTION

2.1 Extreme environments

Although there is no simple definition of extreme environments, they could be defined as environments that are unfavourable or even lethal for a majority of living organisms (Rodríguez-Valera, 1993). The limiting conditions can be high or low pH values, temperature, pressure, radiations, presence of heavy metals or toxins or limited concentration of nutrients (Ventosa, 2006).

If we focus on the optimal physical and chemical life conditions for human beings and mammals, what we consider "extreme" could be considered as "normal" for certain microorganisms. The environments we consider extreme can be populated by well adapted microorganisms, called extremophiles, which not only tolerate these conditions without experiencing any stress, but find these conditions optimal and even require strictly these conditions for their growth (Gorbushina and Krumbein, 1999).

Thomas D. Brock (1979) defined extreme environments from the taxonomic point of view, characterized by their low species diversity and absence of certain taxonomic groups. Nowadays, thanks to the studies of the last decades, it is known that the biodiversity in these environments is much higher than had been expected (Ventosa, 2006).

2.1.1 Hypersaline environments

Hypersaline environments are characterized by elevated concentrations of salts (much higher than those in seawater) and they represent typical examples of extreme environments. However, high ion concentration is not the only environmental factor limiting the biodiversity of these environments. Additional factors, depending on the particular geographical area, can be high or low temperature or pH values, hydrostatic pressure, limited oxygen availability or nutrients accessibility, solar radiations and the presence of heavy metals or other toxic compounds (Rodríguez-Valera, 1988). They are represented mainly by aquatic systems and hypersaline soils, but we can also mention salt deposits, salt water from oil reservoirs or a variety of salty products, from fish or seasoned meat to salted fermented food (Grant *et al.*, 1998; Ventosa, 2006).

The **aquatic hypersaline environments** have been deeply studied. They are defined as environments with salts concentrations higher than those of the seawater, it means superior to approximately 3.5% (w/v) of total salts (Rodríguez-Valera, 1988). Depending on their origin and the composition of salts, aquatic hypersaline environments do or do not differ from the composition of seawater; based on this criterion, they are classified either as thalassohaline or athalassohaline.

On the other hand, there is no specific definition of saline or **hypersaline soil**. According to Kaurichev (1980), saline soils are those where the concentration of soluble salts is higher than 0.2% (w/v). They are widely distributed across our planet and unlike the aquatic hypersaline environments, just a few studies have been performed on the biodiversity of hypersaline soils. It is known that the microbiota of these environments can grow in a wide range of salinities (Quesada et al., 1982). It is thought that this phenomenon is due to the great heterogeneity and wide range of salinity resulting from changes caused by rain and environmental conditions (Ventosa and Arahal, 2002b). This heterogeneity can cause periodic elimination of excessively specialized organisms, which are replaced by euryhaline microorganisms, capable to adapt to a wide range of salinities (Ventosa et al., 2008).

Hypersaline environments represent excellent models of habitats for the determination of the microbiota of extreme environment and their activities. Their study has enabled the isolation and characterization of a large number of halophilic microorganisms during the last decades. However, a great number of groups of microorganisms have never been isolated until now, due to the difficulty of cultivation of these microorganisms under laboratory conditions.

The research group of Prof. Antonio Ventosa at the Faculty of Pharmacy of the University of Sevilla in Spain has been studying the halophilic microorganisms for a long time and they have focused on the isolation and characterization of new halophilic bacteria and archaea from solar salterns and hypersaline soils.

2.2 Extremophilic microorganisms

Depending on the nature of their adaptation, the extremophilic microorganisms are classified into different categories, which are listed in Table 1.

Table 1. *Categories of extremophilic and extremotolerant microorganisms according to the nature of their adaptation (Torsvik and Øvreås, 2008).*

Extreme environmental conditions		Type of extreme microorganism
Temperature	Low	Psychrophile/psychrotroph
	High	Thermophile/hyperthermophile
pH	Acidic	Acidophile
	Alkaline	Alkaliphile
Radiation		Radioresistant/radiotolerant
Heavy metals		Metal-resistant/metal-tolerant
Xenobiotic compounds		Degradator of xenobiotics
High hydrostatic pressure		Barophile or piezophile
Drought (low water activity)		Xerophile/xerotolerant
Salinity		Halophile/halotolerant

The suffix "-philic" is used for microorganisms that require extreme conditions for their optimal growth, while the suffixes "-troph" or "-tolerant" are used for microorganisms that can tolerate extreme conditions, but their growth is not optimal under such conditions. In general, the expression "extremophilic microorganism" describes an organism which is not only adapted, but even bound to live in extreme environments and requires these specific extreme conditions for its growth (Torsvik and Øvreås, 2008).

2.2.1 Halophilic microorganisms

2.2.1.1 Concept

The term *halophiles* (*halos* =salt, *philia = love*) can be translated as a "friend or lover of salt", in reference to the physiological need of salt in levels above normal values that this type of microorganism has. The problem of this definition is the fact that certain level of salt is normal in all organisms, and all of them need a certain quantity of salt to survive. The difference in halophilic microorganisms is that they need certain quantities of salts to grow and they not only survive, but even thrive in saline or hypersaline environments

(Ventosa and Arahal, 2002a). Likewise, halotolerant microorganisms tolerate elevated salt concentrations, but they grow better in its absence (Kushner, 1978).

Halophilic microorganisms are found in many saline environments, especially in saline lakes and soils. They can also be isolated from salt or saline deposits and from salty products such as fish, meat or fermented foods (Ventosa, 2006).

2.2.1.2 Classification

According to the optimal salt level for growth, Kushner and Kamekura (1988) have grouped microorganisms into four categories:

- **Non-halophiles:** grow optimally in environments with less than 1% of NaCl (approx. 0.2 M). Some of them can tolerate elevated salt concentrations and they are called **halotolerant**. If they tolerate concentrations of NaCl higher than 15% (approx. 2.5 M), they are called extreme halotolerant.
- Slight **halophiles** (marine microorganisms): optimal growth concentration between 1 and 3% of NaCl (approx. 0.2 – 0.5 M).
- **Moderate halophiles:** grow optimally in environments with 3 – 15% of NaCl (approx. 0.5 – 2.5 M).
- **Extreme halophiles:** thrive in environments with concentrations between 15-31% of NaCl (approx. 2.5 – 5.2 M).

The two predominant groups of microorganisms in hypersaline environments are the moderately halophilic archaea and bacteria, which grow mainly at concentrations from 10 to 20% of NaCl, and the extremely halophilic archaea and bacteria, mainly present in salinities over 20% of NaCl. There is a range in which both categories of halophilic microorganisms coexist (Rodríguez-Valera, 1988).

2.2.2 Phylogeny and taxonomy

Living organisms are divided into five kingdoms, based on their structural characteristics. Moreover, living beings are branched to prokaryotic and eukaryotic organisms. During the 1970s, Carl Woese and his collaborators classified organisms into three domains:

Archaea, Bacteria and *Eukarya*, based on the use of small subunit of ribosomal RNA as a phylogenetic marker (Woese, 1987).

The Domain *Archaea* includes extremely halophilic archaea (called haloarchaea), belonging to the class *Halobacteria,* with the orders *Halobacteriales* (including the families *Halobacteriaceae, Halococcaceae* and *Haloarculaceae*)*, Natrialbales* (including the family *Natrialbaceae*) and *Haloferacales* (including the families *Halorubraceae* and *Haloferaceae)* (Grant *et al.*, 2001, Gupta *et al.*, 2015; 2016a; 2016b). Haloarchaea require at least 9% (1.5 M) of NaCl to grow, many species do optimally in media containing 20 to 25% (3.4 – 4.5 M) of NaCl (Ventosa, 2006) and some of them are even able to grow in the saturated concentration of 30% (5.2 M) of NaCl (Grant *et al.*, 2001) and can be found in a great variety of hypersaline ecosystems and in the crystallization ponds of salterns (Oren, 2006). These organisms are the main components of the microbial biomass which is found in the hypersaline environments (with a concentration higher than 20% of NaCl). Likewise, they are the main reason responsible for the pink-red coloration of these environments, because of carotenoids with 50 atoms of carbon (C_{50}), called bacterioruberins, and their derivates which are present in the membrane of almost all members of this family (Oren and Dubinski, 1994).

The Domain *Bacteria* includes halophilic and halotolerant microorganisms from various phylogenetic branches. They are mainly moderate halophiles growing at concentrations from 10 to 20% of NaCl (lower than archaea) and chemoorganotrophs, except for some specific phototrophic bacteria (Ventosa, 1988; 1993; 1994; Ventosa et al., 1998b, de la Haba *et al.*, 2010).

Living beings inhabiting the hypersaline environments are predominantly prokaryotic (archaea and bacteria), but some organisms of the Domain *Eukarya* can also constitute a part of the biota, especially in the environments with low concentrations of salt, which is the case of the green alga *Dunaliella* or the crustacean *Artemia salina*. Finally, viruses are also present in hypersaline environments, typically viruses of haloarchaea (called haloviruses) and bacteriophages of moderately halophilic bacteria.

2.3 Haloarchaea

2.3.1 Taxonomy and phylogeny

As has been mentioned above, the aerobic extremely halophilic archaea, called haloarchaea, belong to the class *Halobacteria*, with the orders *Halobacteriales* (families *Halobacteriaceae, Halococcaceae* and *Haloarculaceae*), *Natrialbales* (family *Natrialbaceae)* and *Haloferacales* (families *Halorubraceae* and *Haloferacaceae*) (Grant *et al.*, 2001, Gupta *et al.*, 2015; 2016a; 2016b). For a long time, they were simply differentiated microscopically as bacilli or cocci, which were respectively included into the genera *Halobacterium* or *Halococcus*, with a small number of species (Gibbons, 1974). Huge advances in molecular biology of the past years have resulted in an enormous increase of the number of species within this microbial group, including at present 49 genera and more than 190 species (de la Haba *et al.*, 2010; Parte, 2014).

The current taxonomic classification of haloarchaea is based on a polyphasic approach, which includes phylogenetic, genotypic, phenotypic and chemotaxonomic features. (Grant *et al.,* 2001).

The main phylogenetic marker used for prokaryotic classification is based on the comparison of the 16S rRNA gene; however, in many cases the 16S rRNA gene is not an adequate tool to establish the phylogenetic relations between very close species or strains and currently, an alternative use of other techniques is recommended, for example multilocus sequence analysis (MLSA), which is based on the comparison of a set of linked housekeeping genes with the objective to determine the genomic similarity between organisms with a better accuracy (Papke *et al.*, 2011).

2.3.2 Physiology

Aerobic extremely halophilic archaea represent a group of microorganisms which need at least 9% (1.5 M) of NaCl to grow, with an optimum level between 20 to 25% (3.4 – 4.5 M) of NaCl (Ventosa, 2006). Certain members of this group of extremely halophilic microorganisms are able to grow even at 30% (5.2 M) of NaCl. Pigmented colonies, pink or red, are often formed by this group of microorganisms. This phenomenon is caused by the presence of carotenoids of 50 atoms of carbon (C_{50}), more specifically bacterioruberin

and its derivatives. These compounds are present in the membrane of almost all members of this family (Grant *et al.*, 2001). Another interesting aspect of haloarchaea is the presence, in some of their species, of a compound called bacteriorhodopsin, which acts like a proton pump, transforming light into energy (Lanyi, 1995).

The haloarchaea are aerobic and heterotrophic prokaryotic microorganisms. The aerobic degradation of carbon sources is based on the cycle of tricarboxylic acids, and potentially also the glyoxylate cycle, and on respiratory transport of electrons (Oren, 2006).

The typical species of haloarchaea, *Halobacterium salinarum,* is unable to grow on sugars, but many other species can utilize different carbohydrates. The glucose degradation by *Halorubrum saccharovorum* and probably by other members of the group, follows the modified Entner-Doudoroff pathway, in which phosphorylation is postponed. Glucose is oxidized to 2-keto-3-deoxygluconate and then phosphorylated to 2-keto-3-deoxy-6-phosphogluconate which is split to pyruvate and glyceraldehyde 3-phosphate (Tomlinson *et al.*, 1974). The use of carbohydrates is commonly associated with the production of acids, as the oxidation of each substrate is incomplete (Hochstein, 1978).

2.3.2.1 Anaerobic metabolism

Oxygen can easily become a limiting factor for growth of haloarchaea due to its low solubility in hypersaline aquatic systems. Some members of haloarchaea can compensate the lack of oxygen by the production of gas vacuoles and therefore floating on the surface, on the interphase water-air (Oren, 2006). Moreover, many haloarchaea are capable of growing in anaerobiosis. Some known anaerobic growth forms of *Halobacteria* species include the use of alternative electron acceptors such as nitrate, dimethyl sulfoxide, trimethylamine N-oxide or fumarate and the fermentation of arginine (Oren, 2006).

2.3.2.2 Polar lipids

The presence of polar lipids with ether bonds in the cell membranes is one of the typical features of archaea, present also in haloarchaea. These compounds can be easily detected by thin layer chromatography (Ventosa, 2006). In these microorganisms, polar lipids are represented mainly by 2,3-di-O-phytanyl-*sn*-glycerol, abbreviated as $C_{20}C_{20}$ (Figure 1a), but some species contain also the asymetrical 2-O-sesterpanyl-3-O-phytanyl-*sn*-glycerol ($C_{20}C_{25}$) in various quantities (Grant *et al.*, 2001) (Figure 1b). Another polar lipid that can be present in haloarchaea is 2,3-di-O-sesterpanyl-*sn*-glycerol ($C_{25}C_{25}$) (Figure 1c).

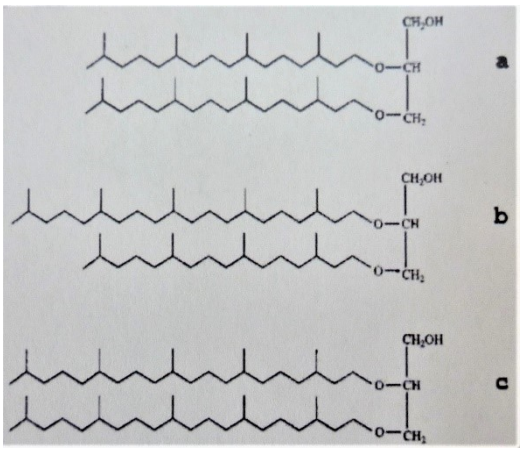


***Figure 1**. Main polar lipids in haloarchaea. (a) 2,3-di-O-phytanyl*-sn-*glycerol ($C_{20}C_{20}$); (b) 2-O-sesterpanyl-3-O-phytanyl*-sn-*glycerol ($C_{20}C_{25}$) and (c) 2,3-di-O-sesterpanyl*-sn-*glycerol ($C_{25}C_{25}$) (Grant et al., 2001).*

The hydrophobic chains are usually found highly saturated. The introduction of double bonds to carbon chains is important probably for the regulation of membrane fluidity in the species adapted to cold (Grant *et al.*, 2001).

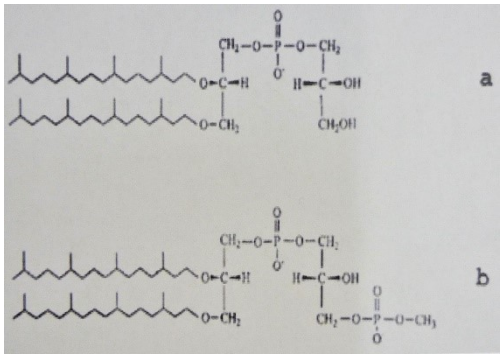


***Figure 2.** Structure of isoprenoid diether analogs of (a) phosphatidylglycerol (PG); (b) phosphatidylglycerolphosphate methyl ester (PGP-Me) (Grant et al., 2001).*

Diverse structures of polar lipids are derived from the central lipids $C_{20}C_{20}$ or $C_{20}C_{25}$ (Figure 1a and 1b). Thus, haloarchaea contain analogues of phosphatidyglycerol (PG)

(Figure 2a) and phosphatidylglycerolphosphate methyl ester (PGP-Me) (Figure 2b) (Grant *et al.*, 2001), the proportion between them can depend on the growth conditions.

Comparative studies of polar lipids composition in non-alkaliphilic haloarchaea and alkaliphilic species have proven to be a useful chemotaxonomic tool for classification of these types of archaea (Torreblanca *et al.*, 1986; Grant and Larsen, 1989; Kates, 1993). The glycolipids became a valid taxonomic marker for classification of haloarchaea, as the type of present glycolipid is constant in most of the species. Phosphatidylglycerolsulphate (PGS) is found in many neutrophilic species (Hancock and Kates, 1973).

2.3.2.3 Nonpolar lipids

They represent around 10% of total lipids in haloarchaea and are almost entirely derived from isoprenoids (Kates, 1993). They are classified into the following groups: a) isoprenoid lipids of 20 atoms of carbon, b) neutral phytanyl ethers of glycerol, c) isoprenoid compounds of 30 atoms of carbon: squalene, dihydroxysqualene, tetrahydroxysqualene and dehydroxysqualene (Figure 3) (Grant *et al.*, 2001).

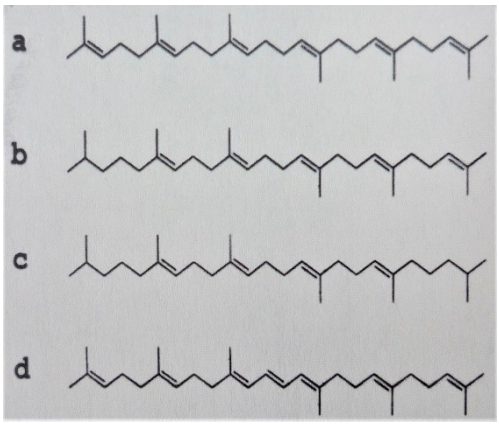


Figure 3. *Certain non-polar lipids present in haloarchaea. (a) squalene; (b) dihydroxysqualene; (c) tetrahydroxysqualene and (d) dehydroxysqualene (Grant et al., 2001).*

Carotenoid pigments are responsible for the red-orange coloration of members of the *Halobacteria* class (Grant et al., 2001). Most of the carotenoids have linear chains of 50 atoms of carbon (C_{50}), derived from α-bacterioruberins. The most frequent are α-bacterioruberin, monoanhydroruberin and bis-anhydrobacterioruberin (Kelly *et al.*, 1970; Kushawaha *et al.*, 1975). On the other hand, the carotenoids of 40 atoms of carbon (C_{40}) are found only in small quantities, represented mainly by lycopene and β-carotene (Kushawaha *et al.*, 1982a; Tindall 1992).

There are two main types of **respiratory quinones** present in species of the haloarchaea, both are menaquinones with eight isoprenoid units: 2-methyl-3-octaprenyl-1,4-naphthoquinone (menaquinone 8, MK-8) and 2-methyl-3-VIII-dihydroxy-octaprenyl-1,4-naphthoquinone [dihydroxymenaquinone 8, MK-8 (VIII-H_2)] (Collins and Jones, 1981; Tindall and Collins, 1986). These compounds are present in large quantities and represent approximately 9% of the total amount of neutral lipids in the cell (Kamekura and Kates, 1988a).

2.3.2.4 Biotechnological applications

The biotechnological potential of halophilic microorganisms is enormous, though they are not yet so frequently used compared to other groups of extremophiles. Haloarchaea were at first studied for their enzymatic activities. Other uses of haloarchaea could be the production of bacteriorhodopsin, extracellular enzymes, carotenoids, production of bioplastics or antimicrobial compounds (halocins) and potentially they could even be used for diagnostics of certain types of cancer (Ventosa and Arahal, 2002a). Halobacteria have a great biotechnological potential thanks to their simple cultivation in culture media with high salt concentration, avoiding the possibility of contamination, as well as their easy genetic manipulation (Ventosa and Nieto, 1995; Ventosa, 2006).

2.3.3 The genus *Natronomonas*

The genus *Natronomonas* was described by Kamekura *et al.* (1997). The species of this genus are Gram-negative rods, producing red-pigmented colonies, due to the presence of C_{50}-carotenoids, chemoorganotrophic and aerobic, halophilic, with a minimum salt requirement of 2 M NaCl. They are alkaliphilic or non-alkaliphilic. Alkaliphilic species grow at pH values from 7 to 10, optimum at 8.5, and non-alkaliphilic species grow at pH values from 5.5 to 8.5, with the optimum between 7 and 7.5. The DNA G+C content is 63-64 mol%. Present polar lipids are glyceroldiether analogues of phosphatidylglycerol (PG) (Figure 2a), phosphatidylglycerophosphate methyl ester (PGP-Me) (Figure 2b) and phosphatidic acid (PA). Phytanyl-sesterterpanyl moieties ($C_{20}C_{25}$) are present (Burns *et al.*, 2010).

At present, this genus includes only three species. The type species is *Natronomonas pharaonis,* described by Soliman and Trüper (1982), being the first haloalkaliphilic

extreme halophile described, with pleomorphic motile rods, isolated from the alkaline brines of eutrophic desert lakes of Wadi Natrun, Egypt. The two remaining species from the genus *Natronomonas* are the non-alkaliphilic species *Natronomonas moolapensis* (Burns *et al.*, 2010) and *Natronomonas gomsonensis* (Kim *et al.*, 2013).

2.4 Studied hypersaline environments

During this work, we focused on the study of the biodiversity from several saline water samples from different ponds located in Isla Cristina and Isla Bacuta solar salterns (southwest of Spain). The locations of these places are marked on the map (Figure 4).

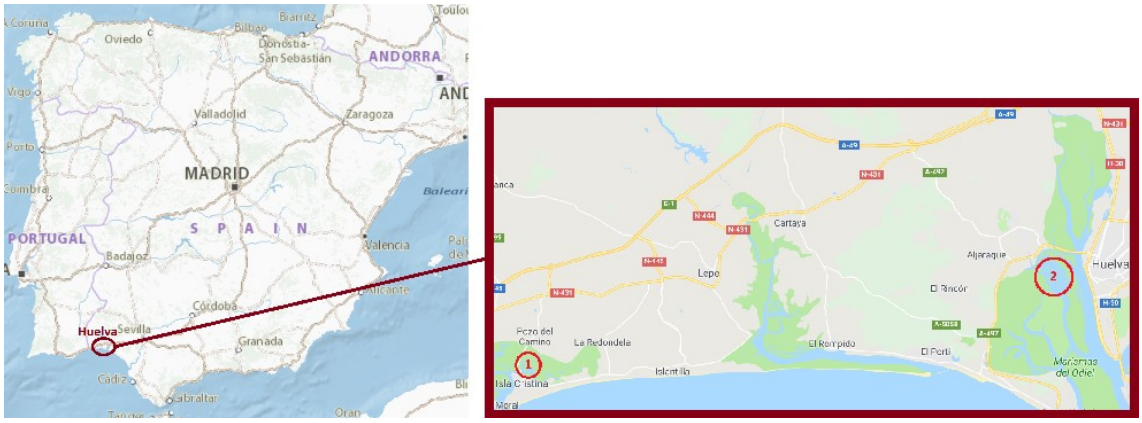


Figure 4. *Location of the two studied places: (1) Salterns of Isla Cristina; (2) Salterns of Isla Bacuta. Source: Google maps; https://www.viamichelin.co.uk/web/Maps*

2.4.1 Salterns of Isla Cristina, Huelva

The salterns of Isla Cristina (Figure 5a), located in Huelva in the southwest of Spain (37°13' N – 7°19'E) were built in 1955 over the Carreras river marshes close to the food industries. The region is characterized by rainy seasons, high solar radiation and high temperature changes between the day and night (Moreno *et al.*, 2010).

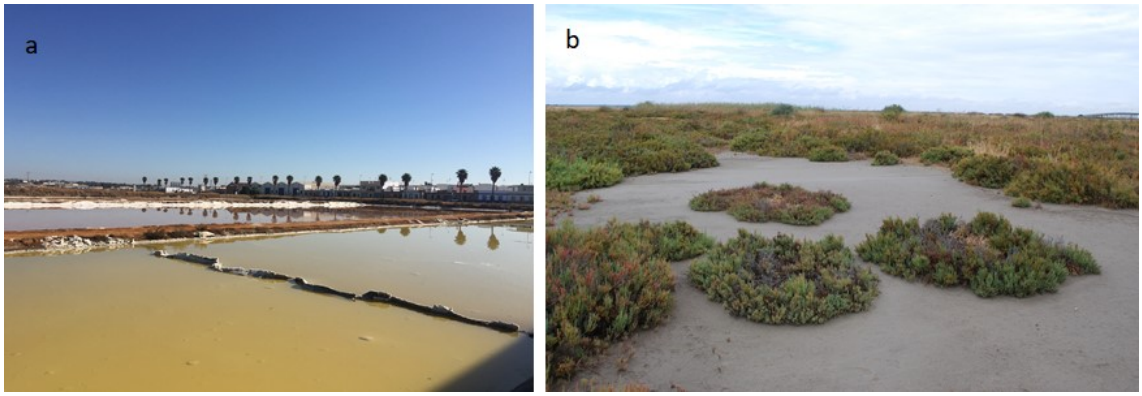


Figure 5. *(a) Isla Cristina solar salterns and (b) Isla Bacuta solar salterns, Huelva, Spain. Author/Source: (a) Antonio Ventosa, (b) http://huelvabuenasnoticias.com*

2.4.2 Salterns of Isla Bacuta, Huelva

The salterns of Isla Bacuta (Figure 5b) are located in the Odiel Natural Marshes Park in Huelva (Spain), occupying both sides of the first kilometres of the road that runs around the sand containment dam Prince Juan Carlos Ist. These coastal salterns, a result of a salt marsh transformation by man, are used for salt extraction from seawater; they comprise 20 ponds. Water coming from Las Madres Channel passes in function of the amount of water evaporation through different evaporation and concentration ponds connected by small channels until it comes to the last ponds where the salts precipitate (Infante-Domínguez, 2015).

3. MATERIAL AND METHODS

3.1 Samples and strains

In this work, we studied water and soil samples that were obtained from Isla Cristina and Isla Bacuta (Huelva, Spain) solar salterns and from hypersaline soils located in Odiel Salt Marshes (Huelva, Spain) during the year 2016. The samples were taken under sterile conditions.

The isolation of haloarchaeal and bacterial strains was performed by Ana Durán-Viseras previously, using different isolation media (see 3.2 in Material and Methods). Plates were incubated for more than 2 months at 37°C. After incubation, the colonies that seemed to have different morphology were selected and subcultured on new media. These plates were also incubated at 37°C.

3.2 Culture media

With the aim to obtain the major biodiversity of haloarchaea and halophilic bacteria present on the different water and soil samples, a series of culture media with two different salt concentrations were used.

Seawater Solution 30% (SW30). Consists of a 30% solution of salts that possesses relative proportions similar to the ones of the seawater (Subow, 1931). It is used as the basis to prepare different culture media with the addition of diverse nutrients.

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

* $CaCl_2$ and $NaHCO_3$ are dissolved separately to eliminate the formation of insoluble complexes of $CaCO_3$. They are added at the end, when the rest of the components is perfectly dissolved.

R2A media (Reasoner and Geldreich, 1979) used for the isolation and cultivation of strains, with the following composition and prepared with the final salt concentration of 15% or 25% (w/v):

Yeast extract	0.05 g
Protease peptone No. 3	0.05 g
Casamino acids	0.05 g
Dextrose	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
Adjust pH to 7.5	

Glycerol media used for the isolation and cultivation of strains, with the following composition and final concentrations of 15% or 25% (w/v):

Yeast extract	0.01 g
Glycerol	0.099 g
SW30	50 or 83.33 ml
Distilled water	to 100 ml
Adjust pH to 7.5	

Pyruvate media used for the isolation and cultivation of strains, with the following composition and final concentration of 15% or 25% (w/v):

Casein digest	0.5 g
Sodium pyruvate	0.11 g
SW30	50 or 83.33 ml
Distilled water	to 100 ml
Adjust pH to 7.5	

3.3 DNA Extraction, purification and manipulation

3.3.1 Extraction of genomic DNA

The extraction of genomic DNA of the strains was carried out by different methods depending on the objective of its use.

For the 16S rRNA gene amplification, the following method was used: a small aliquot of the culture was taken using a sterile stick and resuspended in 40 µl of Milli-Q water in an Eppendorf tube. The hypoosmotic environment causes plasmolysis and DNA liberation. In order to completely finish the lysis, closed tubes with extracted cells were boiled in water for thirty seconds.

For the determination of the Guanine plus Cytosine (G+C) content of the DNA, large quantities of genomic DNA are needed and thus we used the method described by Marmur (1961).

For the MLSA study, the DNA was obtained using the G-spin Total DNA Extraction Kit (iNtRON Biotechnology, Inc.), following the recommendations of the manufacturer.

3.3.2 Agarose gel DNA electrophoresis

Agarose gel electrophoresis was used to confirm that the DNA extraction had been done correctly or if the DNA fragment of determinate size had been amplified by PCR.

To carry out the electrophoresis, 1% (w/v) horizontal agarose gel was prepared (Seakem LE Agarose), using TAE 1x buffer and RedSafe^{TM} (iNtRON Biotechnology) as the stain. The visualization of the DNA fragments was done by exposing the gel to UV light (360 nm), using a transilluminator (TFP-M/WL). 1 kb DNA ladder (Invitrogen) was used to determine different molecular weights.

3.3.3 DNA amplification by PCR

The 16S rRNA gene and *rpoB'* gene amplification was carried out following the indications by Sambrook and Russell (2001), using Mastercycler Ep (Eppendorf) as the thermocycler.

All the PCR reactions were carried out in final volumes of 50 µl in 0.2 ml microtubes, which were previously sterilized to eliminate possible presence of nucleases. The enzyme used was a modified DNA polymerase of *Thermus aquaticus* (Lawyer *et al.*, 1989), called *Taq* DNA polymerase (Promega, Eppendorf and Dominion).

General schema for PCR:

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 (50 ng/µl)	5.0 µl
Taq DNA polymerase (5 U/µl)	0.5 µl
Sterile water	to 50.0 µl

Primers used in this work are detailed in Tables 2 and 3.

Table 2. *Primers used for the amplification of archaeal 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)

Table 3. *Primers used for amplification of 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)

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

- DNA denaturation: 5 minutes at 95°C.

And 25-35 cycles in which these three phases are repeated:

- Denaturation: 30-60 seconds at 94°C.
- Hybridization of the primers: 1 minute at 50°C.
- Extension/Elongation: 2 minutes at 72°C.

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

- DNA denaturation: 5 minutes at 95°C.

And 40 cycles in which these three phases are repeated:

- Denaturation: 30 seconds at 98°C.
- Hybridization of the primers: 5 seconds at 61°C.
- Extension/Elongation: 1 minute at 72°C.

The right amplification was verified by visualizing the DNA fragments by electrophoresis and finally, the PCR product was purified using the FavorPrep (Favorgen Biotech Corp.) kit.

3.4 Phylogenetic studies

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

The amplified PCR products were sequenced by the company STABVida (Portugal). The primers used for sequencing were those shown in Tables 4 and 5.

Table 4. *Primers used for sequencing the archaeal 16S rRNA gene.*

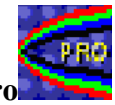
Primer	Specificity	Orientation	Sequence 5'→ 3'	Reference
D34	Archaea	Reverse	GGT CTC GCT CGT TGC CTG	Arahal *et al.* (1996)
B36	Archaea	Reverse	GGA CTA CCA GGG TAT CTA	Arahal *et al.* (1996)

Table 5. *Primers used for sequencing of 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

Once the sequence data were obtained, the phylogenetic analysis was carried out using diverse bioinformatic programs.

ChromasPro 

The partial or indeterminable sequences were corrected manually with ChromasPro Version 1.5 (Technelysium Pty Ltd), obtaining the complete sequence of 16S rRNA gene.

BLAST and EzBiocloud 

Each of the obtained sequences was compared with sequences available in public databases (EMBL, GenBank and DDBJ), using BLAST program (Basic Local Alignment Search Tool) (Altschul et al., 1990) from NCBI server (National Centre for Biotechnology Information) (http://www.ncbi.nlm.nih.gov/BLAST/) and the tools available on EzBiocloud (https://www.ezbiocloud.net), Yoon *et al.*, 2017.

ARB 

Using the obtained results as a starting base, the program ARB (Ludwig *et al.*, 1998; Ludwig *et al.*, 2004) was used for phylogenetic analysis of 16S rRNA gene sequences.

ClustalX and BioEdit

The complete alignment of sequences corresponding to the *rpoB'* gene was carried out with ClustalX and BioEdit program (Larkin *et al.*, 2007). All the alignments were checked and corrected manually.

MEGA

The program MEGA (Molecular Evolutionary Genetics Analysis) (Tamura, 2013) was used for the construction of phylogenetic trees based on the comparison of the *rpoB'* gene sequences.

3.5 Genotypic characterization

3.5.1 Determination of DNA Guanine plus Cytosine (G+C) content

3.5.1.1 Determination of the average denaturalization temperature (T_m)

The Guanine plus Cytosine (G + C) content of the DNA was determined from the average temperature of denaturation (T_m).

Procedure:

DNA samples were heated from 50 to 100°C with a rate of 1°C/min using the thermostat Perkin-Elmer PTP-1. The changes at optical density during the heating were measured at wavelength of 260nm in a Lambda 20 spectrophotometer and recorded by using the PETemplab program.

Calculation of the G+C content:

The melting temperature (T_m) was determined by the graphic method described by Ferragut and Lecrerc (1976) and based on this value, the G+C content was determined using the formula described by Owen and Hill (1979) for the DNA dissolved at 0.1% SSC:

$$\%G+C = (\%G+C)_{mr} + 2.08 \times (T_{mp} - T_{mr})$$

Where:

$(\%G+C)_{mr}$ =% of G+C DNA of the reference strain
T_{mr} = denaturalization temperature of DNA of the reference strain
T_{mp} = denaturalization temperature of DNA of the tested strain

As reference strain, we used *Escherichia coli* NCTC 9001, whose DNA contains 50.9 molar% of G+C and the T_m value in 0.1% SSC is 74.6°C (Owen and Pitcher, 1985).

3.6 Phenotypic characterization

3.6.1 Morphological characteristics

3.6.1.1 Macroscopic characteristics

The macroscopic properties were determined by the classical characterization of appearance of the colony, considering the size, shape, pigmentation, consistence, edge type, surface and its elevation.

3.6.1.2 Morphology and motility

To carry out this test, a few drops of sterile 20% solution of salts are added to an inclined agar culture of the microorganism. After a few minutes, motile haloarchaea move to the saline solution and are observed under phase-contrast microscopy. At the same time, the morphology and typical groupings of each strain are observed. An Olympus BX41 microscope provided with a digital camera DP70 (Olympus) was used.

3.6.2 Physiological characteristics

3.6.2.1 Growth at different salt concentrations

The growth of each strain was determined in R2A medium using a range of gradually increasing concentration of salts. The tested concentrations were 0.9, 3, 5, 10, 15, 20, 25 and 30%, prepared from the 30% solution of salts (SW30). The media were inoculated from a liquid culture in logarithmic phase of growth and then incubated at 37°C. The plates were observed daily during 14 days and each time there was evident growth, the result was considered positive.

3.6.2.2 Growth at different pH values

This test was carried out using the R2A medium, adjusting the pH from 5.0 to 10.0. After inoculating the media, they were incubated at 37°C and the cultures were read daily during 14 days, noting as a positive result every case in which growth was observed.

3.6.2.3 Growth at different temperatures

Plates with R2A medium were prepared and their pH was adjusted between 7.2-7.4. The plates were inoculated and incubated at the following temperatures: 4, 10, 20, 25, 30, 37, 40, 45, 50, 55 and 60°C. The periodic reading of the plates was done as in the previous test and considered positive when any growth was observed.

3.6.2.4 Anaerobic growth

The anaerobic growth was determined in the presence of nitrate or dimethyl sulfoxide (DMSO) as an electron acceptor, or arginine which can be fermented by certain haloarchaea (Oren *et al.*, 1997). The media used for this test were prepared with 3% of KNO_3, 10% of DMSO or 4% of L-arginine. The cultures were incubated for 14 days at 37°C in an anaerobiosis jar.

3.6.3 Biochemical tests

3.6.3.1 Oxidase test

This test determines if the microorganism produces the enzyme cytochrome C oxidase. A young culture is spread with a sterile toothpick on a filtration paper impregnated with 1% aqueous solution of tetramethyl-p-phenylendiamine. The reaction is considered positive when an intensive purple colour appears within 10 seconds (Kovacs, 1956).

3.6.3.2 Catalase test

This test determines the presence of the enzyme catalase, which is produced by a majority of aerobic haloarchaea. The test is conducted by adding a few drops of 3% H_2O_2 (v/v) to a young culture of the microorganism. If bubbles appear immediately, it is a consequence of the reaction of catalase, decomposing the H_2O_2 on water and oxygen and the result of the test is considered positive (Cowan and Steel, 1982).

3.6.3.3 Production of indole

This test determines if the microorganism possesses the enzyme tryptophanase able to produce indole and alanine from tryptophan. The medium containing tryptone and yeast

extract was inoculated and incubated for 10 days at 37°C. To detect the presence of indole, a few drops of Kovacs's reagent were added (Kovacs, 1928), agitated and after one minute the colour was observed. The test is considered positive when a red ring appears, and negative when the ring has yellow colour.

3.6.3.4 Methyl red test

This test is carried out to determine if the microorganism produces strong acids by mixed acid fermentation of glucose, which leads to a considerable decrease of the pH of the medium. The change of the pH value is detected by adding the methyl red indicator. The medium containing glucose was inoculated from a liquid culture and incubated at 37°C for 10 days. For reading the results, a few drops of red methyl solution were added, agitated and a possible change of colour was observed: red indicates a positive result, orange dubious and yellow negative (Cowan and Steel, 1982).

3.6.3.5 Voges-Proskauer test

It is intended to determine if the microorganism utilizes glucose by the butanediol fermentation pathway, producing acids and a big quantity of butanediol. An intermediary product of this pathway is acetoin, which, in presence of oxygen and reagents α-naphthol and 40% KOH, is transformed to diacetyl, producing a red coloured complex. The medium and conditions are the same as for the methyl red test. For the reading of results, a few drops of 0.5% α-naphthol in ethanol and 40% aqueous solution of KOH are added. The colour is observed 20 minutes after adding the reagents, intensive red colour indicates a positive reaction (Barrit, 1936).

3.6.3.6 Nitrate and nitrite reduction test

The microorganisms can utilize the nitrate by three different pathways (Holding *et al.*, 1971). The medium was prepared following the methodology described by Skerman (1967); the medium contained 0.2% of potassium nitrate. The Durham tube was used for the detection of gas. The medium was inoculated and after 10 days of incubation in 37°C, the possible apparition of gas in the Durham tube was observed, indicating that the nitrate was reduced to nitrite and then to gas. A few drops of the reagent A (0.8% solution of sulphanilic acid in 5M acetic acid) and the reagent B (0.5% solution of α-naphthylamine

in 5M acetic acid) were added to the tubes where the formation of gas was not observed. The red coloration indicates the presence of nitrites, considering the nitrate reduction test as positive. In the tubes where the result was not positive within 5 minutes, zinc powder was added (approximately 5 mg/ml). The apparition of red colour in a few seconds indicates the presence of nitrate in the media. On the other hand, the absence of colour indicated the reduction of both nitrate and nitrite.

3.6.3.7 Simmons' citrate test

This test determines the ability of the microorganism to use sodium citrate as the only source of carbon. The technique of Koser (1923) was followed. The tubes were inoculated and incubated at 37°C for 10 days. If the colour of the medium turned intensively blue, the result is positive, remaining green indicates a negative result (Harrigan and McCance, 1979).

3.6.3.8 Hydrogen sulfide production

The medium used for this test contained yeast extract and cysteine. To detect the presence of H_2S, a strip impregnated with lead acetate was used (Clarke, 1953). The medium was incubated at 37°C for 10 days and then the colour of the strip was observed. Intensive black colour indicates that there was a reaction between the liberated hydrogen sulfide and the lead acetate and thus the test result is positive.

3.6.3.9 Decarboxylase test

The medium used for this test contained the amino acids arginine, lysine or ornithine, and the colour was adjusted to orange-yellow using phenol red as pH indicator. The inoculation of the microorganism was carried out and the tubes were covered with a 1 cm high layer of liquid paraffin. The incubation was done at 37°C for 10 days, and after this period, the presence of red colour indicated a positive reaction, due to the liberation of amines by decarboxylation (Koneman *et al.*, 1983).

3.6.3.10 Urease test

It is intended to determine if the microorganism possesses the enzyme urease, which through urea hydrolysis produces ammonia and carbon dioxide, that create sodium

carbonate and produce alkalinisation of the medium. The medium described by Christensen (1946) was used. The tubes were inoculated by a young culture and incubated at 37°C for 10 days. An intensive red colour at the end of this period indicates positive result.

3.6.3.11 Production of acid from carbohydrates

The production of acid was determined using the carbohydrates from Table 6. These carbohydrates were added at the concentration of 1% to the media containing phenol red as indicator; the pH was adjusted to approximately 7.5. The inoculated media were incubated at 37°C for 14 days; the production of acid was considered positive when the indicator turned to yellow colour (Cowan and Steel, 1982).

Table 6. *Carbohydrates used as substrates for the test on production of acid.*

Carbohydrates	D-amygdalin, D-arabinose, arbutin, L-citrulline, dulcitol, D,L-ethionine, D-fructose, D-galactose, glycerol, D-glucose, inulin, lactose, D-maltose, D-mannitol, D-melezitose, D-raffinose, sorbitol, sucrose, D-trehalose, L-xylitol and D-xylose

3.6.3.12 Starch hydrolysis

The strains were inoculated in the medium containing starch and incubated at 37°C for 10 days. In order to determine the result, the plates were covered with Lugol's solution, which gives a dark blue colour in contact with starch. The test is positive when a transparent halo occurs around the zone of growth, which indicates the starch hydrolysis (Cowan and Steel, 1982).

3.6.3.13 Gelatin hydrolysis

The method by Frazier (1926) was followed. The medium was prepared according to the recommendations of Sneath and Collins (1974). After inoculating the media and 10 days of incubation at 37°C, the results were detected by Frazier reagent, which reacts with gelatin creating a whitish opaque complex. The apparition of a transparent halo in an opaque background indicates that the test was positive.

3.6.3.14 DNA hydrolysis

DNAse agar media (Scharlau) was used. The inoculation and incubation were done under the same conditions as the starch and gelatin hydrolyses. To see the result, the plates were put on a dark background and a 1 N solution of HCl was added. In these conditions, the DNA precipitates, giving a whitish colour. The result is considered positive when a clear halo is observed around the zone of growth (Jeffries *et al.*, 1957).

3.6.3.15 Esculin hydrolysis

To study the esculin hydrolysis, a medium with 0.1% esculin and 0.5% ferric citrate was used. The pH was adjusted to 7.6; the media was sterilized, inoculated and incubated under the same conditions as for the casein hydrolysis. The result is positive when a brown-black halo occurs around the area of growth, due to the esculin hydrolysis to esculetin, which forms a black coloured complex with the Fe^{3+} ions (Koneman *et al.*, 1983).

3.6.3.16 Tween 80 hydrolysis

To study the lipolytic activity, 1% of Tween 80 was added to the medium. Strains were inoculated and incubated at 37°C for 10 days. If the strain possesses a lipolytic activity, a precipitate is observed around the area of growth, as a consequence of the reaction between $CaCl_2$ present in the solution of salts in the media and the fatty acids liberated by the Tween 80 hydrolysis (Cowan and Steel, 1982).

3.6.4 Nutritional tests

3.6.4.1 Utilization of carbohydrates, alcohols and organic acids as the sole source of carbon and energy

This test is to determine the ability of the microorganisms to use a large number of substrates (Table 7) as the sole source of carbon and energy. The test was carried out following the work of Stanier *et al.*, (1966). After inoculating the media, they were incubated at 37°C and periodic readings were performed. In all cases, a negative control without any substrate was used. A positive result was considered when a more intensive

growth than on the control plate was observed; in the opposite case, the result was considered negative.

Table 7. *Carbohydrates, alcohols and organic acids studied to determinate their use as the only source of carbon and energy.*

Carbohydrates	L(+)Arabinose, cellobiose, esculin, fructose, glucose, lactose, maltose, D-mannose, D-melezitose, D-melibiose, D-raffinose, salicin, starch, sucrose, D-trehalose, D-xylose
Alcohols	Buthanol, dulcitol, ethanol, glycerol, D-mannitol, methanol, propanol, D-sorbitol, xylitol
Organic acids	Benzoate, citrate, formate, fumarate, glutamate, malate, propionate, pyruvate, succinate, tartrate, valerate

3.6.4.2 Utilization of amino acids as the sole source of carbon, nitrogen and energy

The technique and methods were the same as in the previous test, the substrates (Table 8) were added by bacterial filtration, in concentration of 0.1%.

Table 8. *Amino acids and derivates studied to determinate their use as the only source of carbon, nitrogen and energy.*

Amino acids	Alanine, arginine, asparagine, aspartate, L-cystein, L-glycine, glutamine, isoleucine, L-lysine, L-methionine, L-ornithine, phenylalanine, L-serine, L-threonine, tryptophan, valine

3.6.5 Antibiotic susceptibility

The study of susceptibility to antibiotics was carried out following the technique by Bauer-Kirby (Bauer *et al.*, 1966). Commercial disks (Difco) of 6 mm diameter were used, containing the following antibiotics of detailed concentrations: nalidixic acid (30 µg/ml), ampicillin (10 µg/ml), chloramphenicol (30 µg/ml), gentamycin (10 µg/ml), neomycin (10 µg/ml), rifampicin (30 µg/ml) and sulphamethoxazole-trimethoprim (23.8/1.25 µg/ml). The plates were inoculated with a sterile cotton swab, wetted in a liquid culture

and 4 or 5 disks were placed per plate and incubated at 37°C. During 10 days, the halo of inhibition around each disk was measured and the susceptibility or resistance to antibiotics were determined.

3.7 Chemotaxonomic characterization

3.7.1 Extraction of polar lipids

The extraction of polar lipids of the strains was carried out following the method by Bligh & Dyer (1959), modified for haloarchaea by Corcelli *et al.*, (2000).

3.7.2 Analysis and identification of polar lipids by HPTLC

The identification of polar lipids was carried out by High Performance Thin Layer Chromatography (HPTLC), using Merck HPTLC silica gel 60 (Art. 5641) plates with maximum purity and glass support.

The plates were washed twice with chloroform/methanol (1:1, v/v) and activated at 180°C before use. The application of polar lipids was performed using 10 µl syringes Hamilton series 700, after applying the sample, the plates were eluted in a solvents system chloroform: 90% acetic acid: methanol (65:30:4, v/v).

The plates were revealed using 5% sulfuric acid in water, universal stain for visualization of all lipids, followed by a mild heating at 160°C.

4. RESULTS AND DISCUSSION

4.1 Isolation and selection of the strains and their phylogenetic analysis

This work constitutes part of a larger study in which water samples, obtained from Isla Cristina and Isla Bacuta (Huelva, Spain) solar salterns during the year 2016, were characterized. The isolation of haloarchaeal and bacterial strains was performed previously by Ana Durán-Viseras, using different isolation media with salt concentrations of 15% and 25% (see 3.2 in Material and Methods), with the objective to obtain the most possible biodiversity of haloarchaea and halophilic bacteria, that can be present in viable state in the studied habitats.

The selection of the strains was carried out randomly in function of the colony pigmentation, morphology and size and the strains were obtained in pure culture after successive growth in the same isolation media. A total of 110 haloarchaea and 21 halophilic bacteria were selected for further study.

The phylogenetic analyses of these 131 selected strains based on the comparison of their partial 16S rRNA gene sequences showed that they are phylogenetically related to the genera *Pseudomonas* (9 strains), *Halomonas* (4 strains), *Halovibrio* (2 strains), *Marinobacter* (2 strains), *Rhodovibrio* (2 strains), *Salicola* (1 strain) and *Talminaduibacter* (1 strain) for bacteria, and *Halorubrum* (73 strains), *Natronomonas* (22 strains), *Haloarcula* (5 strains), *Halobellus* (3 strains), *Halonotius* (3 strains) *Halogeometricum* (1 strain), *Halomicrobium* (1 strain) and *Halorientalis* (1 strain) for haloarchaea.

For this work we selected two of the most interesting strains, designated as strains F17-44 and F12-1, phylogenetically related to the genus *Natronomonas,* with percentages of similarity of their partial 16S rRNA gene sequences with their most closely related species lower than 92%, which probably might constitute a new genus within the haloarchaea. The rest of the strains remain for further detailed polyphasic studies in order to determine whether or not they could represent new taxa.

4.2 Taxonomic characterization of the selected strains

The objective of this work was the polyphasic characterization of the two most promising isolated strains. The current taxonomic classification of haloarchaea is based on a polyphasic approach, which includes a complete phylogenetic, genotypic, phenotypic and chemotaxonomic characterization (Grant *et al.,* 2001).

Here we present the results of the polyphasic characterization of the two strains, that were isolated from Isla Bacuta (strain F17-44) and Isla Cristina (strain F12-1) solar salterns, both located in Huelva, southwestern Spain; both strains were phylogenetically most closely related to the genus *Natronomonas*.

4.2.1 Phylogenetic analysis

First of all, in order to determine the exact phylogenetic position of the strains F17-44 and F12-1, the PCR amplification of 16S rRNA gene was repeated and the gene was completely sequenced. The comparison of these complete sequences (approximately 1400 bp) with all the 16S rRNA gene sequences available in the databases confirmed that the strains are phylogenetically related to the genus *Natronomonas*, followed by the genera *Halomarina, Salarchaeum* and *Halobacterium,* with percentages lower than 95% (Table 9). The cut-off of 95% is considered by Ludwig *et al.* (1998) as the reference percentage for differentiation at the genus level, therefore it can be expected that they could represent a new genus of haloarchaea.

Table 9. *Percentages of similarity between the selected strains (F17-44 and F12-1) and their phylogenetically closest species* Natronomonas moolapensis *8.8.11^T, based on the comparison of their complete 16S rRNA gene sequences.*

Strain	Sequence length (bp)	Completeness (%)	Closest related species	Similarity (%)
F17-44	1,366	93.2	*Natronomonas moolapensis* 8.8.11^T	93.9
F12-1	1,362	92.9	*Natronomonas moolapensis* 8.8.11^T	93.7

Figure 6 corresponds to the 16S rRNA phylogenetic tree obtained by *maximum-parsimony* algorithm, in which the phylogenetic position of the studied strains F17-44 and F12-1 is determined, in relation to the phylogenetically related type strains of species of *Natronomonas* and other haloarchaea. These two strains formed an independent phylogenetic branch, well separated and distant enough from other *Natronomonas* species, which seems to indicate that both of the studied strains form one group and they represent a new taxon, at least a new species or even a new genus, as the percentage of similarity showed previously. The bootstrap values, indicated at each branch, represent the percentage of reliability of the obtained results. For example, if the bootstrap value is 80, it means that in 80 cases out of 100 the branch resulted the way as it is shown on the phylogenetic tree.

The distance between the two strains F17-44 and F12-1 is very small in the case of this phylogenetic tree (Figure 6), which makes us wonder if the two strains could represent the same species. The percentage of similarity between these two strains, based on the comparison of their complete 16S rRNA sequences was 99.78%, this value was obtained by introducing the sequences to the Arb program, described in Material and Methods (section 3.4.2) and supports the hypothesis that the two strains could be members of the same species.

Similar results as in Figure 6 were obtained when we used other algorithms (*neighbour-joining* and *maximum-likelihood*) for the construction of the phylogenetic trees (data not shown).

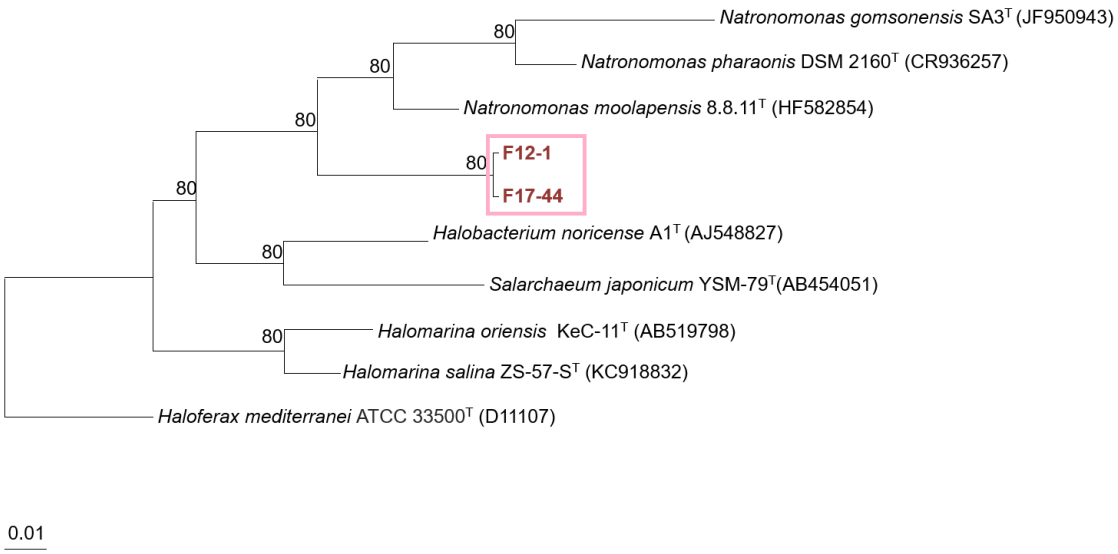


Figure 6. *Phylogenetic tree based on the complete 16S rRNA gene sequence comparisons, obtained by* maximum-parsimony *algorithm, in which the phylogenetic position of the strains F17-44 and F12-1 is shown, in relation to the type strains of species phylogenetically related and other haloarchaea. The 16S rRNA gene sequences are shown in parenthesis. Bootstrap values are indicated at each branch. The scale bar indicates the phylogenetic distance.*

The use of the 16S rRNA gene as a single conserved gene for phylogenetic analyses has some limitations and thus the phylogenetic study of haloarchaea requires other approximations such as the study of the *rpoB'* gene sequence, which codifies for RNA polymerase B' subunit, and represents one of the essential genes called housekeeping. The study of this gene allows to determine the genomic similarity between microorganisms and solve the genealogic structure of a taxon with high accuracy.

The phylogenetic analysis of the *rpoB'* gene sequences helps to confirm the results already obtained from the phylogenetic tree based on the 16S rRNA gene analysis. The PCR amplification of the *rpoB'* gene and its sequencing were carried out as described in the methods (sections 3.3 and 3.4). Table 10 shows the percentages of similarity between each couple of the compared strains, F17-44, F12-1 and their most similar species *Natronomonas pharaonis, Natronomonas moolapensis* and *Natronomonas gomsonensis*. The comparison is based on their *rpoB'* gene sequences. As we can see, the percentage of similarity between the strains F17-44 and F12-1 is 91.4%, which is low enough to consider these strains as two different species of the same genus. Similarity between the type strains of the three *Natronomona*s species and the strain F17-44 is in all cases lower than 85%; for the strain F12-1 the percentages of similarity with the type strains of the *Natronomonas* species are even lower than 81%.

Table 10. *Percentages of similarity between the new strains F17-44 and F12-1 and the type strains* Natronomonas pharaonis *,* Natronomonas moolapensis *8.8.11^T and* Natronomonas gomsonensis*, based on the comparison of their* rpoB‘ *gene sequences.* N. phar., Natronomonas pharaonis DSM 2160^T; N. mool., Natronomonas moolapensis *8.8.11^T*; N. goms., Natronomonas gomsonensis SA3^T.

	F17-44	**F12-1**	***N. phar.***	***N. mool.***	***N. goms.***
F17-44	-	91.4	84.5	83.1	83.1
F12-1		-	80.8	80.4	80.1
N. phar.			-	85.3	86.8
N. mool.				-	85.7

Figure 7 shows the phylogenetic tree, obtained using the *neighbour-joining* algorithm and based on the comparison of the *rpoB'* gene sequences of the two new isolates with respect to the related species of haloarchaea. The two studied strains form a group united in one branch and well differentiated from the other species of the genus *Natronomonas* and far enough to constitute a different genus. In this tree, we can observe that the distance between the strains F17-44 and F12-1 is large enough to consider them as two different species.

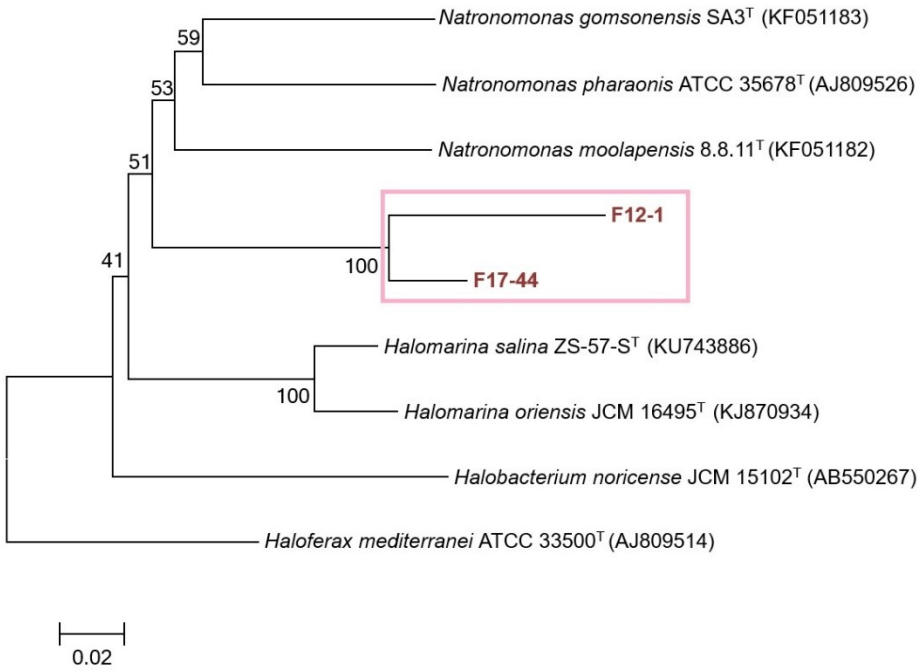


Figure 7. *Phylogenetic tree based on the study of* rpoB‘ *gene of the strains F17-44 and F12-1, in relation to the type strains of phylogenetically related species and other haloarchaea. The* rpoB‘ *gene sequences are shown in parenthesis. Bootstrap values are indicated at each branch. The scale bar indicates the phylogenetic distance.*

4.2.2 Genotypic characterization

The content of G+C in the DNA can be useful from the taxonomic point of view, because any big difference between two strains can be indicative of clear taxonomic differences between them (Goodfellow *et al.*, 1997). Nevertheless, in the cases where the values are similar, it should be considered as rather a confirmative information of taxonomic similarity, because the existence of different taxons with a high similarity of G+C content cannot be excluded. The values of G+C content for the new isolates in comparison with the two reference strains are shown in Table 11. The DNA G+C contents of the new strains differed by 2.1%, the strain F17-44 contained 66.3% of DNA G+C and the strain F12-1 only 64.2% and this result again indicates that the two strains F17-44 and F12-1 might probably represent two different species. The DNA G+C contents also differed from the values measured in the type strains of their most closely related species *Natronomonas moolapensis* CECT 7526^T, where the DNA G+C content was 63.4%, and *Natronomonas pharaonis* CECT 4578^T, where the DNA G+C content was 64.3%. This

result represents another confirmation that the two strains F17-44 and F12-1 are not the same species as the already known species of the genus *Natronomonas*.

Table 11. *DNA G+C content of the studied strains F17-44 and F12-1 compared with those from the reference species* Natronomonas pharaonis *CECT 4578^T and* Natronomonas moolapensis *CECT 7526^T.* N. mool., Natronomonas moolapensis *CECT 7526^T*; N. phar., Natronomonas pharaonis *CECT 4578^T*.

	Strain F17-44	**Strain F12-1**	***N. mool.***	***N. phar.***
DNA G+C content [mol%]	66.3	64.2	63.4	64.3

4.2.3 Phenotypic characterization

In order to carry out the phenotypic characterization of strains F17-44 and F12-1, various characteristics were studied, including morphological and physiological tests (Table 12), biochemical features (Table 13), production of acids from various substrates (Table 14) nutritional tests (Table 15) and their susceptibility to several antibiotics. The new strains F17-44 and F12-1 were compared with their phylogenetically closest species *Natronomonas moolapensis* CECT 4578^T and with the type species of the genus *Natronomonas*, *Natronomonas pharaonis* CECT 7526^T. All the tests were carried out under standardized conditions, with the optimal pH for non-alkaliphilic species, and that is the reason why the alkaliphilic species *Natronomonas pharaonis* CECT 7526^T was not able to grow optimally in these conditions and why in many tests we did not determine any results for this species.

The results of the morphological and physiological characterization are shown in Table 12. All the strains were rods, producing red or pink-pigmented colonies with a colony size of 0.5 mm. Some differences were observed between the new strains and their most phylogenetically related *Natronomonas* strains. The optimal concentration of NaCl of the studied strains was 25%, which is a 5% higher than the optimum concentration of NaCl of the reference strains. On the other hand, the optimal temperature of the studied strains was 37°C, which is 8°C lower than the optimum of *Natronomonas pharaonis* CECT 7526^T. The pH optimum of the new strains was lower than the pH optimum of the alkaliphilic species *Natronomonas pharaonis* CECT 7526^T, but similar

to *Natronomonas moolapensis* CECT 4578^{T} with the value 7.5; we can deduce that both new strains are non-alkaliphilic. The tested strains were not capable of anaerobic growth with neither L-arginine, nor potassium nitrate and nor dimethyl sulfoxide.

Table 12. *Morphological and physiological features of the strains: F17-44 and F12-1 compared with the species* Natronomonas moolapensis *CECT 7526^{T} and* Natronomonas pharaonis *CECT 4578^{T}. Symbols: +, positive; -, negative, ND, no data.* N. mool., Natronomonas moolapensis *CECT 7526^{T}*; N. phar., Natronomonas pharaonis *CECT 4578^{T}*.

Characteristics	**F17-44**	**F12-1**	***N. mool.***	***N. phar.***
Morphology	Rods	Rods	Rods	Rods
Motility	-	-	+	+
Colony pigmentation	Red	Red	Red	Pink
Colony size (mm)	0.5	0.5	0.5	ND
Gram stain	-	-	-	ND
NaCl requirement (%)				
Range	15-30	15-25	14-36	12-30
Optimum	25	25	18-20	20
Mg^{2+} requirement (%)	-	-	-	ND
Temperature requirement (°C)				
Range	30-50	25-45	25-45	20-55
Optimum	37	37	ND	45
pH requirement				
Range	6.5-8.5	6-8.5	5.5-8.5	8-11
Optimum	7.5	7.5	7-7.5	8.5
Anaerobic growth with				
L-Arginine	-	-	-	-
Potassium nitrate	-	-	-	-
Dimethyl sulfoxide	-	-	-	-

Concerning the biochemical tests shown in Table 13, some differences were also observed. The catalase test and nitrate reduction test were positive in both new strains F17-44 and F12-1 and negative in *Natronomonas moolapensis* CECT 7526^{T}. Oxidase, nitrite reduction, indole production, methyl red, Voges-Proskauer, Simmons' citrate and H_2S production were negative in both new strains, the difference with *Natronomonas moolapensis* CECT 7526^{T} was in the methyl red test. Urease test was negative in all tested strains. All the decarboxylases tests with L-arginine, L-lysine and ornithine were negative in the new strains F17-44 and F12-1, but positive for *Natronomonas moolapensis* CECT 7526^{T}. The strain F17-44 hydrolysed Tween 80, the rest of the hydrolysis tests (starch, gelatin, DNA and esculin) were negative for all strains including the reference species *Natronomonas moolapensis* CECT 7526^{T}.

Table 13. *Biochemical features of strains F17-44 and F12-1 compared with the species* Natronomonas moolapensis *CECT 7526^T and* Natronomonas pharaonis *CECT 4578^T. Symbols: +, positive; -, negative, ND, no data.* N. mool., Natronomonas moolapensis *CECT 7526^T*; N. phar., Natronomonas pharaonis *CECT 4578^T*.

Biochemical feature	F17-44	F12-1	*N. mool.*	*N. phar.*
Catalase	+	+	-	+
Oxidase	-	-	-	+
Nitrate reduction	+	+	+	-
Nitrite reduction	-	-	-	-
Indole production	-	-	-	+
Methyl red test	-	-	+	ND
Voges-Proskauer	-	-	-	ND
Simmons' citrate	-	-	-	ND
H_2S production	-	-	-	+
Urease	-	-	-	ND
Decarboxylases tests				
L-Arginine	-	-	+	ND
L-Lysine	-	-	+	ND
L-Ornithine	-	-	+	ND
Hydrolysis of:				
Starch	-	-	-	-
Gelatin	-	-	-	+
DNA	-	-	-	ND
Esculin	-	-	-	ND
Tween 80	+	-	-	-

Table 14 shows the results for the production of acid from different carbohydrates. The two strains F17-44 and F12-1 are compared with their closest related species *Natronomonas moolapensis* CECT 7526^T. In this case, the type species *Natronomonas pharaonis* is not shown in the table for the reason of no obtained results. Both new strains produced acid only in the presence of D,L-arabinose and D-xylose. The reference species *Natronomonas moolapensis* CECT 7526^T produced acid from D,L-arabinose, D-fructose, glycerol and D-xylose.

Table 14. *Production of acids from different carbohydrates by strains F17-44 and F12-1, compared with the species* Natronomonas moolapensis *CECT 7526^T. Symbols: +, positive; -, negative; +/-, unclear;* N. mool., Natronomonas moolapensis *CECT 7526^T*.

Production of acids from:	F17-44	F12-1	*N. mool.*
D-Amygdalin	-	-	-
D,L-Arabinose	+	+	+/-
Arbutin	-	-	-
L-Citrulline	-	-	-

Dulcitol	-	-	-
D,L-Ethionine	-	-	-
D-Fructose	-	-	+/-
D-Galactose	-	-	-
Glycerol	-	-	+/-
D-Glucose	-	-	-
Inulin	-	-	-
Lactose	-	-	-
D-Maltose	-	-	-
D-Mannitol	-	-	-
D-Melezitose	-	-	-
D-Raffinose	-	-	-
Sorbitol	-	-	-
D-Sucrose	-	-	-
D-Trehalose	-	-	-
L-Xylitol	-	-	-
D-Xylose	+	+	+/-

Table 15 shows the results of nutritional tests, the studied strains F17-44 and F12-1 were again compared with the type strain of the closest related species *Natronomonas moolapensis* CECT 7526^T. The type species *Natronomonas pharaonis* is again not present in the table due to no obtained results. Four different groups of substrates were tested: sugars, alcohols, amino acids and organic acids. Neither sugars nor alcohols were utilized by either of the tested strains, as opposed to *Natronomonas moolapensis* CECT 7526^T, which used glucose and salicin from the group of sugars, and butanol, ethanol, glycerol and propanol from the group of alcohols. Three amino acids were positive in both strains and *Natronomonas moolapensis* CECT 7526^T, specifically ornithine, L-lysine and valine. The strain F17-44 utilized also L-alanine, L-glycine and isoleucine, these amino acids were also utilized by *Natronomonas moolapensis* CECT 7526^T, which additionally used also L-threonine. Concerning the organic acids, all the results except one were negative in the tested strains, only the strain F17-44 grew on a medium with fumarate. The reference strain *Natronomonas moolapensis* CECT 7526^T grew also on a medium with propionate, pyruvate and tartrate.

Table 15. *Nutritional characteristics of strains F17-44 and F12-1 compared with their most closely related species* Natronomonas moolapensis *CECT 7526^T. Symbols: +, positive; -, negative.* N. mool., Natronomonas moolapensis *CECT 7526^T.*

SUGARS	F17-44	F12-1	*N. mool.*
Starch	-	-	-
D-Arabinose	-	-	-

D-Cellobiose	-	-	-
Fructose	-	-	-
Glucose	-	-	+
Lactose	-	-	-
Maltose	-	-	-
D-Mannose	-	-	-
D-Melibiose	-	-	-
D-Raffinose	-	-	-
Ribose	-	-	-
Sucrose	-	-	-
D-Trehalose	-	-	-
D-Xylose	-	-	-
Esculin	-	-	-
D-Melezitose	-	-	-
Salicin	-	-	+
ALCOHOLS	F17-44	F12-1	*N. mool.*
Butanol	-	-	+
Dulcitol	-	-	-
Ethanol	-	-	+
Glycerol	-	-	+
D-Mannitol	-	-	-
Propanol	-	-	+
D-Sorbitol	-	-	-
Xylitol	-	-	-
Methanol	-	-	-
AMINO ACIDS	F17-44	F12-1	*N. mool.*
L-Alanine	+	-	-
L-Arginine	-	-	-
Asparagine	-	-	-
Aspartate	-	-	-
L-Cysteine	-	-	-
Phenylalanine	-	-	-
Glutamine	-	-	-
L-Methionine	-	-	-
L-Ornithine	+	+	+
L-Serine	-	-	-
L-Glycine	+	-	+
Tryptophan	-	-	-
L-Lysine	+	+	-
Isoleucine	+	-	+
L-Threonine	-	-	+
Valine	+	+	+
ORGANIC ACIDS	F17-44	F12-1	*N. mool.*
Benzoate	-	-	-
Citrate	-	-	-
Formate	-	-	-
Glutamate	-	-	-
Fumarate	+	-	-
Propionate	-	-	+

Succinate	-	-	-
Valerate	-	-	-
Malate	-	-	-
Pyruvate	-	-	+
Tartrate	-	-	+

The antibiotic sensitivity was carried out using plates inoculated with a single strain on their whole surface, placing antibiotic sensitivity disks after inoculation. The method is described in the section 3.6.5 of material and methods.

Both new strains F17-44 and F12-1 were resistant to the tested antibiotics (nalidixic acid, ampicillin, chloramphenicol, erythromycin, gentamicin, neomycin, novobiocin, rifampicin and sulfamethoxazole-trimethoprim).

4.2.4 Chemotaxonomic characterization

The chemotaxonomic characterization in haloarchaea includes the study of the polar lipids composition and the respiratory quinones. In this work we show the results from the polar lipids analysis. The lipid profiles allow to differentiate between taxonomic ranges at the level of species and especially at the genus level, where the difference is usually more evident. For this reason, the study of polar lipids represents an important taxonomic tool for the characterization of haloarchaea.

The polar lipids were extracted using the modified method for haloarchaea described by Corcelli *et al.* (2000). The lipids were then separated by High Performance Thin Layer Chromatography (HPTLC) and detected using the methods described in Material and Methods (section 3.7.2). The expression of certain cellular components can differ according to the environmental conditions. For this reason, the culture conditions were rigorously standardized to obtain the most reliable approximation of the studied lipid profiles.

The results of the High Performance Thin Layer Chromatography (HPTLC) of the polar lipids indicated that the strains F17-44 and F12-1 do not belong to the genus *Natronomonas* because they do not show the same lipid pattern as the species of this genus.

To specify the differences, there was presence of sulfated acid galactosyl mannosyl glucosyl phosphatidic diether (S-TGD-1-PA) and sulfated tetraglycosyl diether (S-TeGD) in the studied strains; these compounds are not present in species of the genus *Natronomonas*. Moreover, an unidentified phospholipid between phosphatidylglycerophosphate methyl ester (PGP-Me) and phosphatidylglycerol sulfate (PGS) was also present in both studied strains, which was not detected on the reference strains used. All these differences support the hypothesis that these two strains could constitute a new genus.

5. CONCLUSIONS

We obtained results from all four parts of the polyphasic characterization. The main conclusions of the study are the following:

1. The complete 16S rRNA gene sequences of the two studied strains F12-1 and F17-44 were analysed and compared with the sequences from the databases, leading to the determination of the phylogenetic position of both strains. Based on their 16S rRNA gene, they were found to be phylogenetically related to the species of genus *Natronomonas*, followed by genera *Halomarina*, *Salarchaeum* and *Halobacterium*.
2. The phylogenetic tree based on the analysis of *rpoB'* gene sequence confirmed the results obtained previously by the study of 16S rRNA gene sequence and showed that the two strains are grouped together to form an individual phylogenetic branch separated from the genus *Natronomonas*. Their phylogenetic distance might indicate that they could constitute two separate species.
3. The genotypic characterization supported these results, the G+C DNA content of both studied strains differed from the content of their closest related species. There was also a significant difference in G+C DNA content between both new strains.
4. The phenotypic characterization revealed some differences between the studied strains and their closest, already known species *Natronomonas moolapensis* CECT 7526^{T} and *Natronomonas pharaonis* CECT 4578^{T}.
5. Chemotaxonomic characterization by the High Performance Thin Layer Chromatography (HPTLC) revealed that the two strains do not belong to the genus *Natronomonas*, because they show a different polar lipid profiles from those of the species of this genus.

Our studies indicate that both new strains F17-44 and F12-1 might represent two different species of a new genus of haloarchaea. Further studies based on the comparison of their genomes are in progress and will clarify this point.

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8. REFERENCES

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