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DIPLOMOVÁ PRÁCE

DIPLOMA THESIS

TAXONOMICKÁ CHARAKTERIZACE NOVÝCH IZOLÁTŮ RODU *HALORUBRUM* Z HYPERSALINNÍCH PŮD

TAXONOMIC CHARACTERIZATION OF NEW *HALORUBRUM* ISOLATES FROM HYPERSALINE SOILS

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I would like to thank especially to Ana Durán Viseras for her guidance, patience, kindness and for helping me all the time.

My great thanks go also to Prof. Antonio Ventosa and Prof. Cristina Sánchez-Porro for their guidance, reading and revising this work.

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„I declare that this work is my author work. All literature and other resources I used to make this work can be found in references and they are cited properly“

In Hradec Králové Markéta Horáčková

ABSTRAKT

Kandidát: Markéta Horáčková

Název diplomové práce: Taxonomická charakterizace nových izolátů rodu *Halorubrum* z hypersalinních půd.

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Studijní program: Farmacie

Cílem této práce je pokračovat v charakterizaci nových *Archaeí* rodu *Halorubrum* izolovaných z hypersalinních půd lokalizovaných u města Huelva v jihozápadním Španělsku. Tato *Archaea* jsou mikroorganismy vyžadující ke svému životu vysokou koncentraci solí v okolním prostředí. S jejich charakterizací začala minulý rok Ana Durán Viseras a během svého pobytu jsem se podílela na pokračování této charakterizace.

Pro doplnění fylogenetické studie jsme amplifikovali gen *rpoB* jednotlivých *Archeí* pomocí MLSA a vytvořili fylogenetický strom. Na základě výsledků této studie jsme usoudili, že se jedná o nový druh a započali jsme s fenotypickou charakterizací. U izolované DNA jsme určili obsah guaninových a cytosinových bazí, jež je součástí taxonomických charakterizací nových druhů.

ABSTRACT

Candidate: Markéta Horáčková

Title of diploma thesis: Taxonomic characterization of new *Halorubrum* isolates from hypersaline soils.

Charles University, Faculty of Pharmacy in Hradec Králové

Department of Biological and Medicinal Sciences

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Department of Microbiology and Parasitology

Study program: Pharmacy

The aim of this work is to continue in taxonomic characterization of new strains of the genus *Halorubrum* isolated from hypersaline soils from Paraje Natural Marismas del Odiel, Huelva, Spain. These *Archaea* are micro-organisms that require high salt concentration in their environment. With the characterization started Ana Durán Viseras last year and I participated on continuation of this characterization.

To complete phylogenetic study we performed MLSA of *rpoB* gene of each sample to create phylogenetic trees. Based on results of the phylogenetic study we inferred that studied strains could constitute new species. We determined the guanine plus cytosine content in DNA and also carried out phenotypic studies as important part of the taxonomic characterization of the new species.

CONTENTS

LIST OF ABREAVIATIONS

DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ed.	editor
EDTA	ethylene diamine tetraacetic
et al.	et alii (and others)
G+C	guanine plus cytosine content
miliQ	ultrapure water of Type 1
MLSA	Multilocus sequence analysis
PCR	Polymerase chain reaction
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
TAE	Tris-acetate-EDTA solution
Taq	Thermus aquaticus DNA polymerase
U	unit (s) of enzyme

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Figure 7. Neighbour-joining phylogenetic tree based on the analysis of rpoB gene showing the position of strains A34, A37, B52 and B53 with respect to their most relative species. 39

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1. OBJECTIVES OF THIS WORK

The hypersaline habitats represent excellent models for studying the microbial diversity of extreme environments. However a big progress has been made in describing halophilic microorganisms in last decades but only small part of the organisms has been isolated and described yet. In recent years mainly the aquatic hypersaline habitats have been studied and but not much attention was payed to hypersaline soils, the other representative of hypersaline habitats.

The research group of Prof. Antonio Ventosa from the Faculty of Pharmacy of the University of Seville in Spain, has a long experience of studying halophilic micro-organisms. In recent years this group has been focused on the isolation and taxonomic characteristics of new halophilic bacteria and archaea from hypersaline soils. Recognition of these micro-organisms can help to understand their function and ecological role in hypersaline environments.

Nowadays, to obtain adequate classification system of prokaryotes, mainly in taxonomic ranges smaller than species and genus is necessary to realize a polyphasic approach. This approach combines phylogenetic, genotypic, phenotypic, and chemotaxonomic characterization (Vandamme et al., 1996; Goodfellow et al., 1997).

The objectives of this work is:

1. To perform a Multilocus sequence analysis (MLSA) of six *Halorubrum* strains isolated from hypersaline soils from Paraje Natural Marismas del Odiel, Huelva, SW Spain to complete previous genetic studies based on the 16S rRNA gene sequence comparison.
2. To carry out phenotypic characterization of these six new *Archaea.*
3. To determine the percentage of guanine + cytosine bases of their DNA, in order to carry out future DNA-DNA hybridization experiment among these six haloarchaea.

2. INTRODUCTION

2.1 Extreme environments

Extreme environments can be described as those habitats with one or more life conditions extremely different from the conditions that humans consider to be normal (Gorbushina and Krumbein, 1999). These conditions can be high or low pH values, temperature, concentration of salt or nutrients, radiation, pressure, heavy metals, toxins (Ventosa, 2006). But this is only one point of view, the human's one, it is very difficult to make a definition with versatile applications. It was thought that for extreme environments is typical low species diversity because only a limited number of species can adjust their morphology and physiology and metabolism to these hostile conditions (Brock, 1979) but in last years, several studies have shown that the biodiversity is much more higher than it was expected (Ventosa et al., 2015). Extreme environments can have a natural origin but they can also be caused by human activity.

For example a long history of mining activity in the area of river Río Tinto (SW Spain) turned the surrounding into a unique extreme environment (*Figure 1*) that is 100 km long. The main cause of this extremity is in the water of the river with constant high acidity (pH 2.3) and high level of heavy metals (Fe, Cu, Zn,...). Sulphidic minerals in contact with water and oxygen dissolve and oxidize. This process that products acid is supported by the activity of microorganisms. In acidic water is higher solubility of cationic metals therefore this river has large concentrations of metallic sulphides and other metals (Aguilera, 2013).

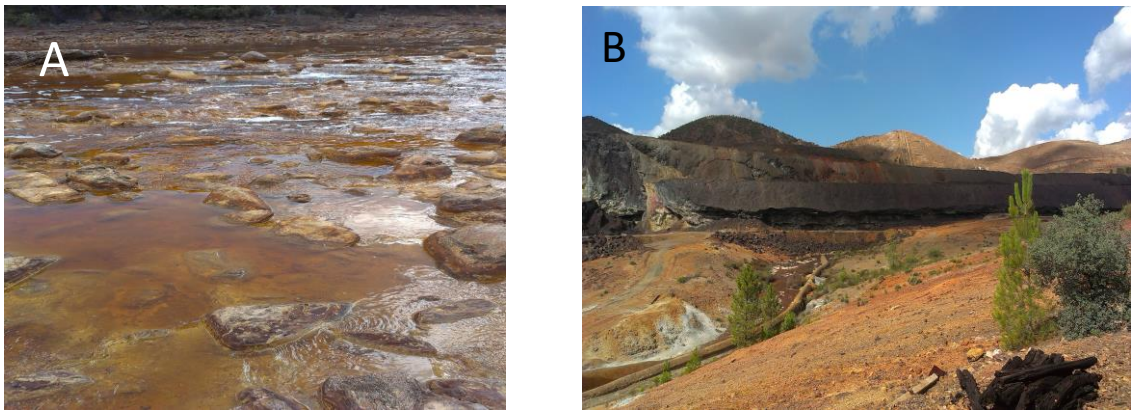

Figure 1. Extreme acidic environment in Río Tinto (SW Spain): (A) Río Tinto, mine area surrounding Río Tinto.

2.1.1 Hypersaline habitats

Example of these extreme environments are hypersaline habitats where can be found higher levels of salts than in seawater but also low oxygen concentration, different pH and temperature depending on the geographical location (Rodriguez-Valera, 1988). Hypersaline environments can be aquatic, terrestrial but also can be represented by rocks, salted food, or hides of animals (Ventosa et al., 1998). Water hypersaline systems are divided into two categories according to the origin of the salt. When the composition of minerals is similar to sea water it refers to marine origin and the system is called **thalassohaline**; a water system in which the composition is influenced by climate, geology or dissolving of minerals from surroundings is called **athalassohaline** (Rodriguez- Valera, 1988; Grant, 1990). The salt lakes and marine solar salterns show optimal conditions for studying halophilic micro-organisms. Hypersaline soils are very heterogeneous with wide range of salinities influenced by the weather conditions (Grant, 1990). In Figure 2 can be found examples of hypersaline habitats. Solar saltern Santa Pola represents thalassohaline system (Ventosa et al., 2014) and Dead Sea is example of very deeply-studied athalassohaline habitat (Volcani, 1940; Nissenbaum, 1975; Oren, 1983; 1988; 2006; 2007; Arahal et al., 1996; Arahal, 1997; Ventosa et. al. 1998; Bodaker et al., 2010)

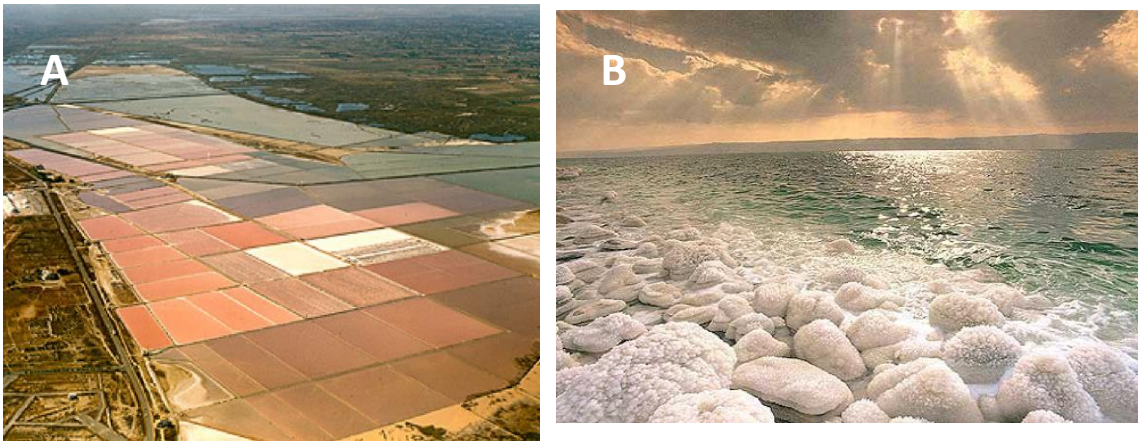

Figure 2. Examples of hypersaline habitats: (A) Santa Pola saltern, (B) Dead Sea.

Hypersaline soils

This habitat is much less studied than the aquatic hypersaline habitats, but recently is getting more attention. They are widely present on the Earth but not well defined, however according to Kaurichev (1980), as hypersaline soil can be considered soil with concentration of soluble salts over 0.2 % (w/v). The micro-organism isolated from hypersaline soils show wide range of salt requirement for growth. Most of them have optimum between 5 and 15% of NaCl but they are also able to grow at much lower salinities. This phenomenon can be explained by the fact that they live in a habitat with rapid changing of salinity due to raining and other climate conditions. In the soils can be found archaea as well as moderate halophilic bacteria (Ventosa et al., 2008).

Solar salterns

Solar salterns are formed by system of ponds where the NaCl is produced from seawater in the tropical and subtropical areas. One of the best-studied solar saltern world-wide is the Bras del Port saltern (Santa Pola, Alicante, Spain) (Ventosa et al., 2014). The water is pumped to the set of shallow ponds, it evaporates, and salt solution is getting concentrated. In this process different salts precipitate because of the different solubility, first precipitates $CaCO_3$, when the salinity is about twice of seawater, when the salinity is four times of the seawater $MgSO_4$. 2 H_20 precipitates and finally precipitates NaCl (Oren, 2002).

From biologist' point of view it can be divided into three areas. In the first one is salt concentration similar to seawater, so the inhabitants are similar to the sea ones. In the second area with higher salinity the colour of water is darker due to presence of algae *Dunaliella.* Also many species of moderate halophilic bacteria inhabit this environment. In the hypersaline area occur red archaea and bacteria, which is in a majority depends mainly on the temperature of the water. Archaea prefer higher temperatures than bacteria (Oren, 2002).

2.2 Extremophilic organisms

The name Extremophilic (*extremus*= extreme, *philia*= love) reflects that they require conditions for living that are extremely different from those found on the majority on the Earth. Mostly they are prokaryotic organisms (archaea, bacteria) because they are amazing in adapting to conditions that are impossible to survive for most eukaryotes (Gupta et al., 2014). This ability to live under severe conditions leads to the conclusion of some scientist that these organisms might be at the beginning of the life on the Earth, where the environment was very extreme (NASA, 2003). Other major importance and interest of studying these organisms is in using of their enzymes (extremozymes) that are specialized to extreme conditions. The extremozymes can be used in pharmaceutical, food, chemical industry and agriculture (Gupta et al., 2014). Categorization of extremophilic organisms is described in *Table 1*.

Table 1. Categories of extremophilic organisms and examples of their applications.

Category	Habitat	Application
Thermophiles	Temperature > 50°C	Thermostable enzymes for bioengineering, *Taq* polymerase (PCR),etc.
Psychrophiles	Temperature < 15°C	Maturation of cheese, brewery, detergents, etc.
Acidophiles	pH < 3	Recovery of valuable metals, desulfurization of coal, etc.
Alkaliphiles	pH > 8	Starch hydrolysis, food industry, etc.
Halophiles	Salinity > 2 M	Oil recovery, medical plastics, etc.
Piezophiles	Pressure up to 130 MPa	Protection of food, antibiotics against high pressure during sterilization, etc.
Radiophiles	Resistant to UV and ionizing radiation	Remediation of radioactive wastes
Toxicophiles	Resistance to toxic reagents	Reactions in non-aqueous conditions
Metallophiles	Resistance to high levels of heavy metals	Removing toxic heavy metals from soil
Micro-aeorophiles	Saturation of O_2 < 21%	Production of superoxide, peroxide

2.2.1 Halophilic organisms

As we can say from ethymology of the name Halophiles (*halos*= salt, *philiā*= love), they are organisms that need hypersaline environments for growth. They also developed ability to deal with the osmotic pressure and denaturing effect of the high salt levels. They are mostly prokaryotic micro-organisms, but representatives can be also found in the multicellular eukaryotes (Ventosa, 2006). This work is focused on the prokaryotic micro-organisms.

According to the optimal salt level for growth Kushner and Kamekura (1988) grouped micro-organisms into four categories (*Table 2*).

Table 2. Different categories of micro-organism according to their requirement of salt.

Category	Optimal concentration of NaCl for growth [M]
Non-halophiles	Up to 0.2
Slight halophiles	0.2 - 0.5
Moderate halophiles	0.5 – 2.5
Extreme halophiles	2.5 – 5.2

Some of non-halophiles can be halotolerant, they can grow in both high salt concentrations or without salt (DasSarma S and Arora P, 2001). These categories are based on data from laboratory conditions, in ideal case it should be based on the conditions of the natural habitat (Ventosa et al., 2015).

Predominant inhabitants of hypersaline habitats are moderately halophilic bacteria, extremely halophilic bacteria and archaea (Ventosa, 2006).

2.2.1.1 Haloarchaea

The extremely halophilic archaea belong to the class Halobacteria with orders: *Halobacteriales,* Haloferaces and Natrialbales. According to *List of procaryotic names with standing nomenclature* currently includes about 40 genera (*Halorubrum, Haloarcula, Haloquadratum etc.*) and many species. They inhabit many diverse hypersaline habitats such as salterns, salt lakes, salty food, salted soils, etc. Although Haloarchaea that grow at salinity of seawater have been described, optimum for the most of Haloarchaea is between 3.5 - 4.5 M NaCl. Some of Haloarchaea are also alkaliphiles and they occur in soda lakes (Grant et al., 2001).

Because of pigments bacterioruberin and C_{50} carotenoids, Haloarchaea have red coloured colonies that colour their natural habitats. The exception represents genus *Natrialba* that is colourless. Also bacteriorhodopsin, that works as a proton pump may occur in some species (Ventosa, 2006).

Composition of archaeal lipid membrane is very different from the composition of bacterial and eukaryotic membrane. The non-polar hydrocarbon chain is created by isoprenoid moieties linked to glycerol backbone by ether bond in comparison to bacteria and eukaryotes where is ester bonding (Jain et al., 2014). Lipids typical for Haloarchaea are phytanyl ether analogues of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester that can be found in all of them. They can also contain phosphatidylglycerol sulphate. Diphytanyl($C_{20}C_{20}$)glycerol ether core lipids occur in all Haloarchaea, some of them can have also phytanyl-sesterterpanyl($C_{20}C_{25}$)glycerol core lipids (Grant et al., 2001; Kates, 1993).

Some of them also produce gas vesicles which turn colour of colonies to pink and opaque. Gas vesicles are proteinaceous nanocompartments, are encoded by fourteen gas vesicle protein (*gvp*) genes and their synthesis depends also on light, oxygen, temperature and salt concentration. Gas-filled vesicles help cells to migrate in vertical direction to region with better life conditions. (Pfeifer, 2015).

The main mechanism of dealing with high osmolarity of their environment is in accumulating of KCl to at least the same concentration as NaCl (Soppa, 2006). This strategy is called salt-in and will be described below.

Haloarchaea are used as a model organism to carry out many studies, because they can be grown more easily in complex media under aerobic conditions than other archaeal extremophiles. They are heterotrophic and mainly facultative anaerobes. Bacteriorhodopsin from Haloarchaea is used for optical sensors, switches and bio-chips. The polyhydroxyalkanoates, polyesters from Haloarchaea can be used for example in the medicine for their biodegrability. Polymers can also recover residual oil and other pollution in nature, proteins were tested as cancer markers and some of their enzymes are widely used in cosmetics (Ventosa and Nieto, 1995).

Genus Halorubrum

This genus belonging to the family *Haloferacaceae* was first proposed in 1995 by McGenity and Grant and currently it contains 31 species according to the List of Prokaryotic names with Standing in Nomenclature. The name *Halorubrum* comes from the Latin words *halos*= salt and *rubrum*= red, so it can describe this genus as salt-requiring and red coloured. The members inhabit diverse hypersaline environments,

natural as well as artificial. They are rod-shaped or pleomorphic, some of them motile. The colonies of most *Halorubrum* species contain carotenoid pigments (bacterioruberin) which give them orange-red colouration, the species which poses gas vesicles are bright pink. They are strictly aerobic, chemo-organotrophic, oxidase and catalase positive organisms. They are capable of growth in the range of temperature between 4 and 58 °C and in the range of salinity from 1.0 to 5.2 M of NaCl. Members of this genus are neutrophilic and alkaliphilic with optimal pH 9-10 but some are able to grow up to pH 10.5. The DNA G+C content ranges between 61.7 and 71.2 mol % (McGenity and Grant, 1995, Oren et al., 2009).

2.2.2 Moderately halophilic bacteria

This heterogeneous group of micro-organisms contains many species that belong to many genera. But the majority of species are Gram-positive or Gram-negative with aerobic or facultative anaerobic metabolism. Morphologically it is also a very diverse group, counting rods, cocci, long cells, spirals, etc. (Ventosa, 2006).

Optimum concentration for growing is from 0.5 to 2.5 M NaCl, but halophilic bacteria can handle with very diverse salinity. Other important growing factor is temperature, bacteria prefer lower temperature than archaea, and nutrients composition. With increasing temperature is higher demand for salt. These micro-organisms inhabit wide range of habitats counting aquatic systems, soils, salt food and they even occur on the salt glands of animals and plants (Ventosa et al., 1998).

Most species of this group balance high osmolarity by strategy of compatible-solutes, but also some species use strategy salt-in (Ma et al., 2010).

Application of these micro-organisms is very diverse, from use of ectoine and β - hydroxyectoine in cosmetic industry to using them for recovery of saline wastes from industry (Gupta et al., 2014).

2.2.3 Other halophilic organisms

Archaea and halophilic bacteria can be also attacked by **viruses** but their role is being studied, probably they regulate the number of prokaryotes in the environment (Ventosa et al. 1998).

Representative of **algae** is unicellular green *Dunaliella* which is used as a model organism for studying adaptation of algae to hypersaline environments. It also produce β -carotene in large volume which is used commercially (Oren, 2005).

There are also described **fungi** isolated from hypersaline habitats such as waters of Dead sea and soils in Slovenia. Mostly they are halotolerant but some of them are halophilic (Ventosa, 2006).

2.2.4 Maintaining osmotic balance

Salt-in strategy uses cells accumulating salts (mainly KCl) in intracellular space to balance osmotic pressure. This strategy needs that intracellular structures and enzymes are adapted to high salinity. Also it is important for them to have continuous access to the salts in their environment. This strategy can be found in *Archaea* and anaerobic bacteria of the order *Haloanaerobiales* (Oren, 1999).

Compatible-solutes strategy uses accumulation of small organic compatible compounds that can be synthesized (ectoine or β-hydroxyectoine) or taken up from medium (betaines or choline). This strategy does not require special enzymes or adapted cell structures because compatible solutes even at high concentrations allow normal function of conventional enzymes and cell structures. This strategy is used by the majority of moderate halophilic and halotolerant *Bacteria* (Oren, 1999).

3. MATERIALS AND METHODS

3.1 Archaeal strains

In this work we studied samples that were obtained from saline soils located in Paraje Natural Marismas del Odiel (Huelva, Spain) in previous years. The samples were taken under sterile conditions.

The isolation of bacterial strains was performed by Ana Durán Viseras previously. The media we used were prepared from a 30 % (w/v) salt water stock solution (SW30) enriched with pyruvate (10 mM) or glycerol (10 mM), with the final salt concentration of 15 or 25 % and agar type I (Intron biotechnology) for solidifying of medium at a concentration of 2 %. Plates with these media were inoculated with the samples of saline soil diluted in different concentrations of stack salt water solution and incubated for 20 days at 37°C. After the incubation the colonies that seemed to have different morphology were selected and inoculated on new media with these colonies. These plates were also cultured at 37°C.

3.2 List of equipment

1. Autoclave PRESOCLAVE SELECTA
2. Automatic pipettes Gilson
3. DNA extraction kit iNtRON Biotechnology The G-spin™ Total DNA Extraction Kit
4. DNA purification kit iNtRON Biotechnology MEGA-spin TM Kit
5. Microwave oven LG
6. Centrifuge Eppendorf 5424
7. Centrifuge Eppendorf 5810 R
8. Bain-marie Grant QBD2
9. Bain-marie Cole Parmer
10. Mastercycler Eppendorf
11. Thermostat PerkinElmer PTP-1

12. Gel logic 100 imaging system
13. pH-meter Crison Basic 20
14. Power suply Bio-Rad PowerPac 300
15. PerkinElmer UV/VIS spectrometer Lambda 20
16. PETempLab PC program
17. Cell Bio-Rad Mini-Sub Cell GT

3.3 Growing media

For growing archaeal strains we used different media prepared from the SW30 salt solution by adding different nutrients and diluting to proper salt concentration. The pH of the media was measured by pH-meter Crison Basic 20 and adjusted with 0.1 M KOH or 0.1 M HCl. Media were autoclaved for 20 minutes at 121 °C at the autoclave PRESOCLAVE SELECTA. If the medium was supposed to be solid, it contained 2 % of agar type I (Intron Biotechnology) and it was poured to Petri dishes right after autoclaving in sterile conditions. We inoculated the media under sterile conditions and kept them for 10 days at 37°C.

Concentrated salt water (SW) stock solution 30% (w/v) described by Rodriguez-Valera et al. (1980) has similar proportions as the seawater but with higher concentration of total salts:

NaCl	234.0 g
$MgCl_2$. 6 H_2O	39.0 g
$MgSO_4$ .7 H_2O	6.0 g
$CaCl_2$	1.0 g
KCl	6.0 g
$NaHCO_3$	0.2 g
NaBr	0.86 g
Distilled water	to 1000 ml

We dissolved $CaCl_2$ and $NaHCO_3$ in a small amount of distilled water and we added in the end to the solution to avoid precipitation of ions. Finally we filtrated the solution.

Gly medium used for the isolation and cultivation of strains. Final pH was adjusted to 7.2-7.4. With a salinity of 15 % (w/v) it has the following composition:

Glycerol	0.921 g
SW30	500 ml
Distilled water	to 1000 ml
Agar (for solid)	20.0 g

Modified growth medium (MGM) for haloarchaea (Rodriguez-Valera et al., 1980) was used for cultivation of strains with final pH 7.2-7.4. with a salinity of 20 % (w/v) it has following composition:

Peptone	10.0 g
Yeast extract	2.0 g
SW30	670 ml
Distilled water	to 1000 ml
Agar (for solid)	20.0 g

Pyr medium used for the isolation as well as cultivation of strains. Final pH was adjusted to 7.2-7.4. With 25 % (w/v) of salinity it has the following composition:

Piruvic acid	1.1 g
SW30	830 ml
Distilled water	to 1000 ml
Agar (for solid)	20.0 g

R2A agar medium (Reasoner and Geldreich, 1979) used for the cultivation of strains in the plates, with the following composition and a salinity of 25 % (w/v):

Yeast extract	0.5 g
Peptone protease	0.5 g
Casaminoacids	0.5 g
Dextrose	0.5 g
Soluble starch	0.5 g
Sodium pyruvate	0.3 g
K_2HPO_4	0.3 g
Mg $SO_4 \cdot 7H_2O$	0.05 g
SW30	833.3 ml
Distilled water	to 1000 ml
Agar	20.0 g

SSL medium used for growing the strains with a final pH 7.5. the composition for salinity of 15 % (w/v) is:

Casein digest	5.0 g
Sodium pyruvate	1.1 g
SW30	500 ml
Distilled water	to 1000 ml
Agar (for solid)	20.0 g

SW medium (Rodriguez-Valera et al. 1980) was used for cultivating strains with a final pH adjusted to 7.5-7.8. Composition for 20 % (w/v) salinity is:

Yeast extract	1.0 g
Casamino acids	0.1 g
SW30	666 ml
Distilled water	to 1000 ml
Agar (for solid)	20.0 g

3.4 Solutions

Cloroform-isoamyl alcohol mixture used for Marmur method (1961):

Cloroform	960 ml
Isoamyl alcohol	40 ml

EDTA disodium salt 0.1 M solution (pH 8) used for Marmur method (1961):

Na_2EDTA	33.62 g
NaCl	8.7 g
Distilled water	to 1000 ml

RNAse solution used for Marmur method (1961):

RNAse	2 mg
NaCl	16 mg
Distilled water	to 10 ml

Heat to 100°C during 10 minutes to eliminate possible presence of DNAse. Keep at 4°C or -20°C.

Saline-citrate (SSC) 20 % (w/v) solution used for Marmur method (1961):

NaCl	175.3 g
Sodium-citrate	88.2 g
Distilled water	to 1000 ml

Sodium dodecyl sulphate (SDS) 25 % (w/v) solution used for Marmur method (1961):

SDS	250.0 g
Distilled water	to 1000.0 ml

Sodium perchlorate 5 M solution used for Marmur method (1961):

$NaClO_4$	702.3 g
Distilled water	to 1000 ml

TAE 50X (Tris-Acetate-EDTA) used for the preparation of TAE 1X:

Glacial acetic acid	57.1 ml
EDTA 50mM	100 ml
Tris-Base	242 g
Distilled water	to 1000 ml

TAE 1X used for the preparation of agarose gel for electrophoresis and as the buffer:

TAE 50X	20 ml
Distilled water	980 ml

3.5 Extraction, amplification and purification of DNA

3.5.1 Extraction of DNA

Marmur method (1961)

This method was used for the extraction of DNA used for the determination of the DNA G+C content.

Protocol:

1. Centrifuge 1 litre of the culture for 10 minutes, 7,000 rpm.
2. Resuspend cells in 100 ml of 0.1 M Na_2EDTA, 0.15 M NaCl solution.
3. Lysis of cells: add 10 ml of 25 % sodium dodecyl sulphate (SDS) solution and incubate during 10 minutes at 60°C, after cooling to room temperature add 20 ml of 5 M $NaClO_4$ solution.
4. Deproteinization: add 130 ml of cloroform-isoamyl alcohol mixture, shake in the well stoppered flask and leave for 30 minutes.
5. Take the supernatant into centrifugation tube and centrifuge for 10 minutes, 6,000 rpm.
6. Precipitation: take the supernatant with the pasteur pipette into clean breaker, add 2 volumes of cold 95 % ethanol and stir the mixture with the clean glass rod, gelatinous DNA will appear on the rod (*Figure 3*)

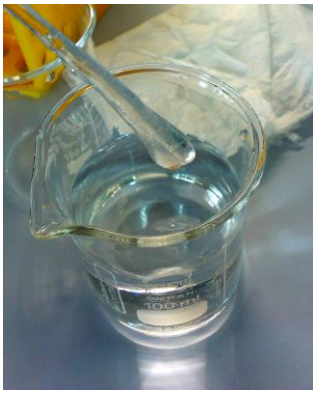

Figure 3. Precipitation of DNA on the glass rod.

7. Transfer DNA to clean test tube filled with 0.1 % (prepared from 20 % SSC).
8. RNAse treatment: incubate DNA at 37°C with RNAse solution for 30 minutes, shake well with proper volume of the cloroform-isoamyl alcohol mixture and leave for 30 minutes, repeat process of precipitation with ethanol, transfer DNA to a clean test tube with 0.1 % saline citrate.
9. Repeat the RNAse treatment again.
10. Store DNA in the test tubes with 0.1 % SSC at 4°C for a short term and in –20°C for long period.

Isolation of DNA with the G-spin Genomic DNA Extraction Kit

We followed this protocol for the isolation of DNA which we used for MLSA:

1. Take 1.5 ml of liquid culture into 2 ml Eppendorf tube.
2. Centrifuge for 1 minute at 13,000 rpm and discard the supernatant. Vortex to resuspend cells in the remnant liquid.
3. Add 200 µl of Buffer CL, 20 µl of Proteinase K, 5 µl of RNAse A and vortex.
4. Cultivate at 56°C in heat block or water bath for 30 minutes with gently inverting every 3 minutes.
5. Add 200 µl of buffer BL and mix well and incubate at 70°C for 5 minutes.
6. Centrifuge for 5 minutes at 13,000 rpm and transfer only the supernatant to new 1.5 ml Eppendorf tube carefully.
7. Add 200 µl of absolute ethanol and mix gently by pipetting.
8. Transfer all volume to Spin Column and centrifuge for 1 minute at 13,000 rpm.
9. Discard the liquid that flowed through, add 700 µl of Buffer WA to column and centrifuge at 13,000 rpm for 1 minute.
10. Discard the liquid and add 700 µl of Buffer WB to column and centrifuge for 1 minute at 13,000 rpm. Then centrifuge again 1 minute at 13,000 rpm to dry membrane.

11. Place the column into new 1.5 ml Eppendorf tube, add 50 µl of elution Buffer CE and let incubate at room temperature for 1 minute.
12. Centrifuge at 13,000 rpm for 1 minute.

We controlled the result of extraction using electrophoresis in 1.5 % agarose gel.

3.5.2 Electrophoresis on the agarose gel

This method was described by Sambrook and Russell (2001). It was used to control the DNA isolation as well as for the identification of the amplified DNA after Polymerase Chain Reaction (PCR).

Preparation of the gel:

1. Dissolve 0.6 g of agarose (Seakem, LE agarose) in 40 ml of TAE 1X using microwave oven, avoid boiling of liquid.
2. Add 2 µl of ethidium bromide 0.5 mg/ml (Promega) storage solution after the liquid cooled down.
3. Pour it slowly to avoid presence of bubbles into clean chamber with tray, gates and the comb.
4. After 20 minutes put the prepared gel out of the chamber to the cell, covered it with TAE 1X buffer and put the samples into holes.
5. The first hole is for 5 µl of 1 kb ladder (Intron), into the followings we placed mixture of 2 µl of bromophenol buffer and 5 µl of the sample from DNA extraction.
6. Cover box with the safety lid with the cables and plug the cables to the source of voltage, set to 70 V and 400 mA.
7. When blue bands come to the ¾ of the gel shut down the voltage.
8. Put the gel under the UV light (360 nm) with camera and take a picture using program, in the picture should be visible bands representing DNA, ladder is for recognition of approximate DNA molecule size (Figure 4).

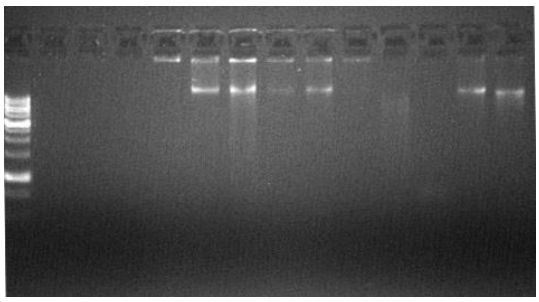

Figure 4. Bands of DNA in the agarose gel.

3.5.3 Amplification of DNA

We performed the amplification of the *rpoB* gene how as described Sambrook and Russell (2001), that completes previous analysis of 16S rRNA gene. *Rpo*B gene is a universal housekeeping gene in prokaryotes that encodes the β-subunit of RNA polymerase (Adékambi et al., 2008). Sequencing of this gene is used as complementation of 16S rRNA for describing new archaeal species. In the genus *Halorubrum* very often occurs recombination of genetic traits and promiscuous exchange of genetic information comparable to sexual population is present. These facts lead to need of *rpoB* analysis because 16S rRNA analysis cannot differentiate close relatives at the species level (Papke et al., 2011).

Principe:

1. Denaturation: in this step double-strain molecule of DNA separates to single strains.
2. Annealing: during this step primers can bind their complementary part of DNA strain.
3. Elongation: DNA polymerase elongates DNA strain by adding complementary dNTPs to template strain.

Schema for PCR:

Buffer (Reaction buffer Intron Biotechnology 10X)	5.0 µl
$MgCl_2$ (Intron Biotechnology 25 mM)	2.5 µl
dNTPs (Intron Biotechnology 1.25 mM)	8.0 µl
Primer (F) (12 µM)	2.5 µl
Primer (R) (12 µM)	2.5 µl
DNA sample (50 ng/µl)	5.0 µl
Maxime Taq polymerase (Intron Biotechnology 5 U/µl)	0.5 µl
Water milliQ	to 50 µl

Primers used for the PCR are described in *Table 3*.

Table 3. Primers used for the amplification of rpoB gene by PCR.

Primer	Orientation	Sequence 5' → 3'
Arch F	Forward	TGT AAA ACG ACG GCC AGT TCG AAG AGC C
Arch R	Reverse	CAG GAA ACA GCT ATG ACC GGT CAG CAC C

Programs:

For amplification of *rpoB* gene:

Initial denaturation at 98°C for 30 seconds, followed by 40 cycles of denaturation also at 98°C during 30 seconds, 5 seconds at 59°C of annealing and extension at 72°C for 15 seconds. Final elongation for 1 minute at 72°C.

Procedure

1. Add chemicals following the list above into sterile 0.2 ml Eppendorf tubes using pipettes with clean tips.
2. Place well-closed tubes into Mastercycler Eppendorf and switched on the appropriate program.
3. When the thermocycler finished the procedure, run the final product on the agarose gel using ladder to identify the molecule of DNA.

4. The amplified DNA can be stored for a short time period at 4°C, for long time period at – 20°C.

3.5.4 Purification of DNA

For purification we used DNA purification kit iNtRON Biotechnology MEGA-spin, following this protocol:

1. Load and run the whole volume of the product of PCR on the 1.5 % agarose gel.
2. After visualization under the UV light cut the DNA fragment with a clean razor blade and put into pre-weigh 1.5 ml Eppendorf tube.
3. Weigh the sample and add 3 volumes of BNL buffer to 1 volume of gel.
4. Incubate for 10 minutes at 55°C to melt down the gel slice, then transfer to MEGAquick-spin column.
5. Binding of DNA: centrifuge 1 minute at 13,000 rpm and discard the flow-through.
6. Add 700 µl of Washing Buffer and discard the liquid that flow though, then centrifuge again to dry membrane.
7. Place MEGAquick-spin column to clean 1.5 ml Eppendorf tube, apply 40 µl of Elution Buffer let 1 minute incubate at room temperature and centrifuge for 1 minute at 13,000 rpm.

We controlled the result of the purification on the 1.5 % agarose gel with first two positions occupied by lambda DNA yields used to approximate mass of DNA in bands. In the last position there is ladder (*Figure 5*). Then purified DNA was stored at -20°C.

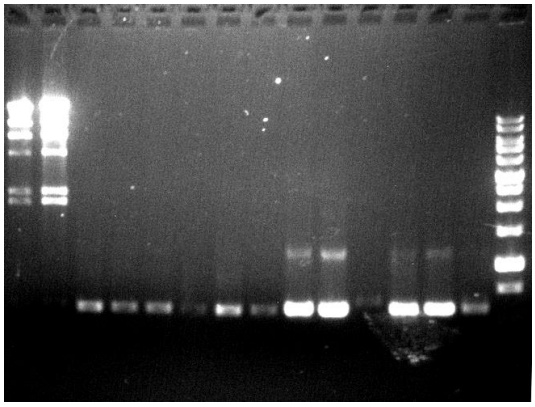

Figure 5. Bands of purified rpoB gene in agarose gel.

3.6 Sequencing and phylogenetic studies

For the realization of phylogenetic studies was amplified *rpoB* gene of the studied strains.

The extraction of DNA was performed as described in part 3.5.1 using the G-spin Genomic DNA Extraction Kit.

For the amplification of *rpoB* gene the technique PCR was used described in part 3.5.3. The conditions of the PCR reaction as well as primers used for this technique are described in part 3.5.3.

The procedure used for purification of amplified *rpoB* gene are described in part 3.5.4.

The purified DNA samples were sent for sequencing to the company NBT-NewBiotechnic in Seville. For the sequencing were used primers described previously in Table 3.

The sequences were identified using the EzTaxon (http://www.ezbiocloud.net/eztaxon; Kim et al., 2012) on the basis of 16S rRNA sequence data.

The ARB Software was used for constructing phylogenetic trees (Ludwig et. al, 2004). Two different approaches were used: *maximum-parsimony* (Kluge and Farris, 1969) and *neighbour-joining* algorithm (Saitou and Nei, 1987).

3.7 Genotypic characterization

Determination of Guanine plus Cytosine (G+C) content:

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

Procedure:

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

Calculation of the G+C content:

The melting temperature (Tm) was determined by the graphic method described by Ferragut and Leclerc (1976) and from this value was determined the G+C content with the formula described by Owen and Hill (1979) for the DNA dissolved at 0.1 % SSC:

$$\% GC = (\%G+C)mr + 2.08 \times (Tmp-Tmr)$$

(%G+C)mr = % of the G+C of reference strain

Tm = denaturation temperature of unknown DNA

Tmr= denaturation temperature of reference strain

As reference strain we used *Escherichia coli* NCTC 9001 which DNA has 50,9 % of G+C and the Tm in 0.1 % SSC is 74.6 °C (Owen and Pitcher, 1985).

3.8 Phenotypic studies

3.8.1 Morphological characteristics

For the observation of the cellular morphology was used phase-contrast microscopy with the Olympus BX41 microscope and the digital camera DP70. The shape of the micro-organism and motility was observed.

The pigmentation of colonies was observed macroscopically.

3.8.2 Physiological tests

3.8.2.1 Growth at different salt concentration

The strains were grown in media prepared from salt water stock solution with the following final salt concentrations: 0.9, 3, 5, 10, 15, 20, 25 and 30 %. Media were enriched with yeast extract at a final concentration at 0.5 %, the pH was adjusted to 7.2-7.4 and agar was added for solid media at a concentration of 2 %. The plates were observed daily during 14 days.

3.8.2.2 Growth at different pH

The strains were grown in media prepared from stack water solution with 20 % of salts and 0.5 % of yeast extract; the pH had was adjusted from 5.0 to 10.0. Inoculated media were incubated at 37°C for 14 days with daily observation of results.

3.8.3 Biochemical tests

3.8.3.1 Oxidase

This test shows if the enzyme cytochrome C oxidase is present or not in the tested strain. On the filter paper impregnated with 1% solution of tetramethyl-p-phenylenediamine (Difco) were put young bacterial colonies with a sterile toothpick. The reaction is positive if purple colour appears in 10 seconds (Kovacs, 1956).

3.8.3.2 Catalase

This test determines the presence of the enzyme catalase. A few drops of H_2O_2 at a concentration of 3% (v/v) were added to young colonies of the micro-organism. If the bubble appears immediately the result of the test is positive (Cowan and Steel, 1974).

3.8.3.3 Urease

This test determines if the micro-organism possess the enzyme that hydrolyse urea. It takes places in agar urea medium (Christensen, 1946) with a final pH 6.8. After autoclaving (20 minutes, 121°C) the medium was distributed to sterile test-tubes. Solidified medium was inoculated and incubated for 10 days at 37°C. It was observed every day if the positive reaction appears in form of intense rose colour.

3.8.3.4 Production of acids from carbohydrates

The carbohydrates teste were trehalose, D-xylose, D-rafinose, D-ribose, sorbitol, xylitol.

The basal medium contained:

yeast extract	5.0 g
Phenol red	0.01 g
SW30	833.3 ml
Distilled water (final volume)	1000 ml

We dissolved the phenol red in 2 ml of ethanol, then we added it to the medium. When the medium had proper pH (7.5), it had red colour. After autoclaving at 121°C for 20 minutes medium was transferred into sterile test tubes. Final concentration of carbohydrates was 1 %. Each carbohydrate solution was transferred to a test-tube through filter. Then the medium was inoculated. During the incubation at 37°C for 10 days it was observed daily. The positive reaction is represented by a change from red colour to yellow (Cowan and Steel, 1974).

4. RESULTS AND DISCUSSION

4.1 Phylogenetic analysis

The strains A29, A34, A34A, A37, B52 and B53 were isolated from hypersaline soils from Paraje Natural Marismas del Odiel, Huelva, Spain. Based on the previous analysis of gene encoding 16S rRNA they were phylogenetically most related to the genus *Halorubrum*. The most closely related species with the percentages of similarity are shown *Table 4.*

Table 4. Percentage of similarity between strains isolated from hypersaline soils from Paraje Natural Marismas del Odiel, Huelva, Spain and the most closely related species according to the comparison of complete sequences of the gene encoding 16S rRNA.

Strain	Most related species	% of similarity
A29	*Halorubrum kocurii*	98.7
A34	*Halorubrum kocurii*	98.7
A34A	*Halorubrum kocurii*	98.7
A37	*Halorubrum aidingense*	98.5
B52	*Halorubrum lipolyticum*	98.3
B53	*Halorubrum kocurii*	98.5

The phylogenetic tree based on the analysis of gene encoding 16S rRNA was created using the Maximum-parsimony algorithm (*Figure 6*). This phylogenetic tree reflects the relationship of studied strains and their most closely related species. It shows that studied strains are grouped in a separated phylogenetic branch with strain B52 separated from the rest of strains.

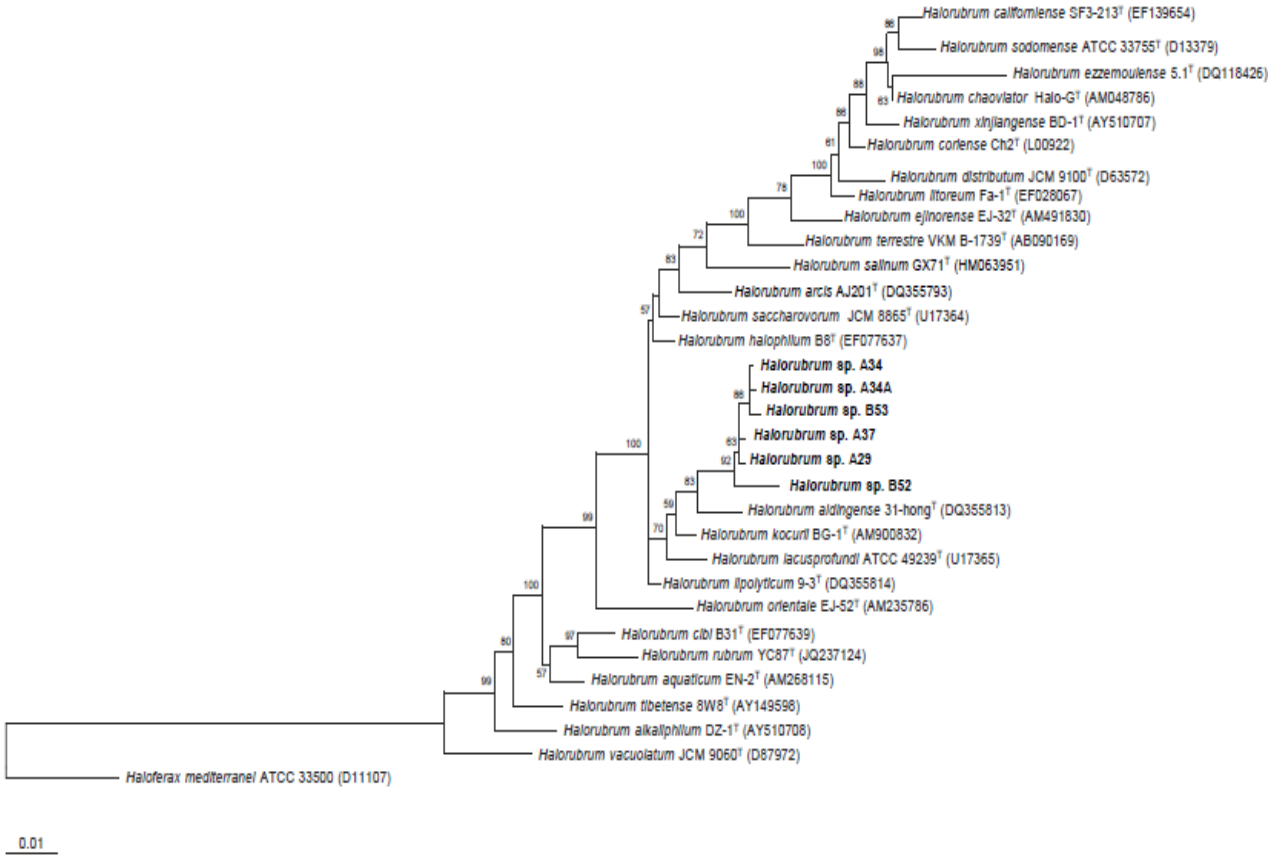

Figure 6. Maximum-parsimony phylogenetic tree based on the analysis of gene encoding 16S rRNA showing the position of strains A29, A34, A34A, A37, B52 and B53 with respect to their most relative species.

As it was mentioned previously in the genus *Halorubrum* there is a frequent gene transfer and homologous recombination of 16S rRNA gene. Therefore it is recommended a phylogenetic analysis of gene that has smaller recombination in order to determine clearly differences between close relatives. We performed Multilocus sequence analysis (MLSA) of the *rpoB* housekeeping gene which is more conservative and sensitive to differentiate closely related strains (Papke et al., 2011; Fullmer et al., 2014). The procedure of treatment of the strains was described before in part 3.6 of Material and methods. We were unsuccessful with amplifying *rpoB* gene of strains A29 and A34A, and for this reason they are not included in this part of results of phylogenetic analysis.

Table 5 shows the percentages of similarity based on *rpoB* gene sequences between studied strains and their most related species and *Table 6* shows the percentages of similarity based on *rpoB* gene sequences between the studied strains.

A recent study on the genus *Halorubrum* that compared values of MLSA and percentage of DNA-DNA hybridization observed that 97% is the limit value for delineation of new species in this genus (de la Haba, not published). Studied strains show lower similarity with all referenced species. Between strains each another there can be seen higher similarity between A34, A37 and B53 but strain B52 shows similarity slightly above 97%. This fact probably indicate that strains A34, A37 and B53 constitute one new species and strain B52 another second new species in the genus *Halorubrum*.

Table 5. Percentages of similarity between studied strains and the most related species based on the comparison of rpoB gene sequences.

Strain	*Hrr. kocurii* (%)	*Hrr. aidingense* (%)	*Hrr. lipolyticum* (%)	*Hrr. saccharovorum* (%)
Hrr. sp. A34	96.5	91.6	96.5	96.5
Hrr. sp. A37	96.7	91.8	96.3	96.7
Hrr .sp. B52	95.7	92.8	95.5	95.9
Hrr. sp. B53	96.7	91.4	96.7	96.7

Table 6. Percentages of similarity between studied strains based on the comparison of rpoB gene sequences.

Strain	*Hrr. sp.* A34	*Hrr. sp.* A37	*Hrr. sp.* B52
Hrr. sp. A34			
Hrr. sp. A37	99.4		
Hrr. sp. B52	97.3	97.5	
Hrr. sp. B53	99.4	99.6	97.1

Based on the data obtained from the analysis of *rpoB* gene we obtained phylogenetic tree using *Neighbour-joining* algorithm (*Figure 7*). This phylogenetic tree

demonstrates that the strains constitute a new separated branch, divided into two smaller branches. One branch contains strains A34, A37 and B53, while the strain B52 is separated and constitutes a second branch. This support the previous results that strains A34, A37, B53 constitute a new species and strain B52 another new species within the genus *Halorubrum*.

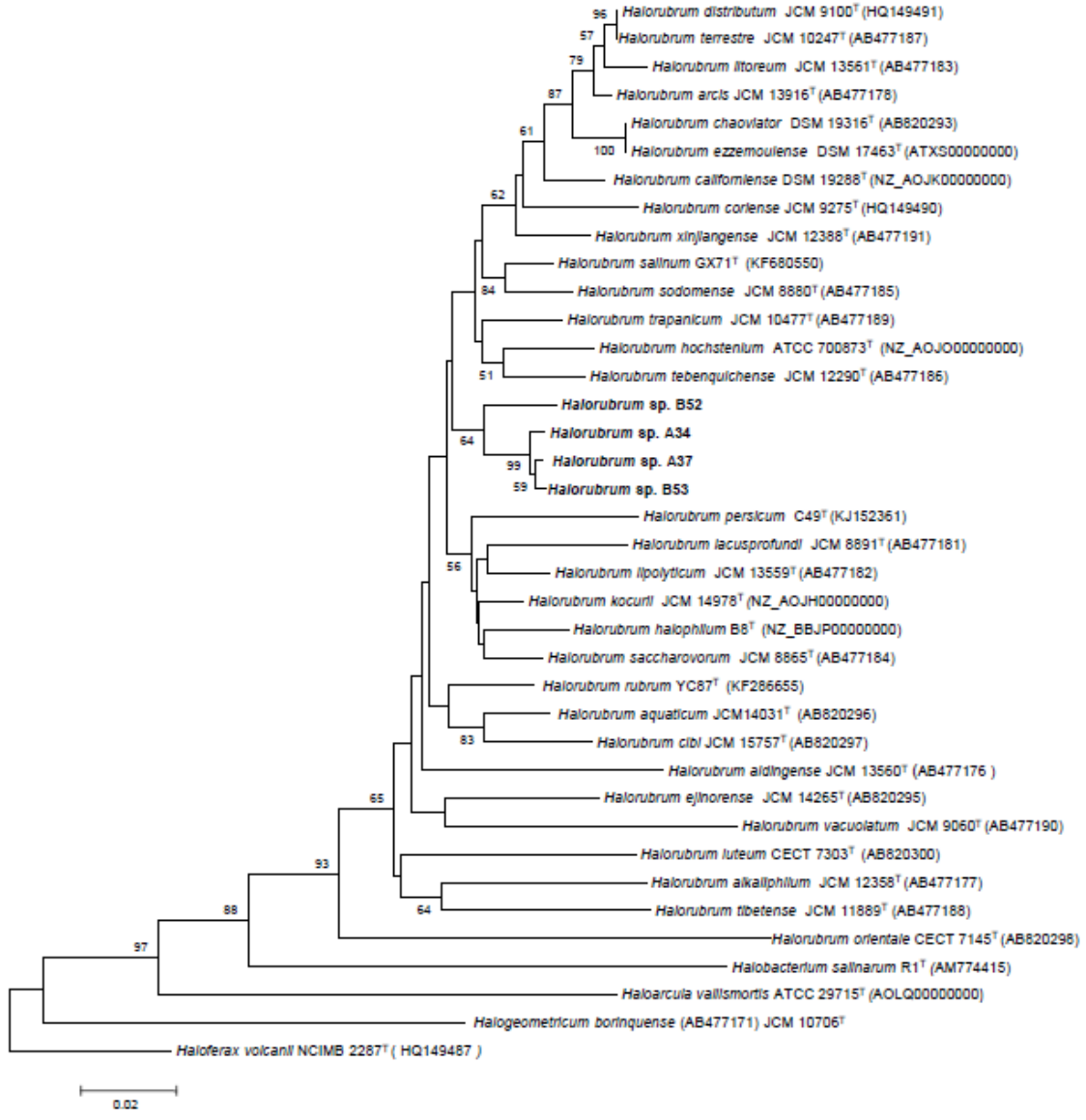

Figure 7. Neighbour-joining phylogenetic tree based on the analysis of rpoB gene showing the position of strains A34, A37, B52 and B53 with respect to their most relative species.

4.2 Genotypic analysis

The guanine plus cytosine content of the DNA can help to show differences between the bacterial strains when the difference between two strains are high (Goodfellow et al., 1997). The denaturation temperature of the DNA (Tm) that served for the computation of content of G+C of DNA in the studied strains and the G+C contents is shown in shows *Table 7.*

Table 7. The denaturation temperature of the DNA (Tm) and guanine plus cytosine content of the DNA of studied strains.

	A29	A34	A34A	A37	B52	B53
Tm [°C]	79.5	85.5	80.0	79.6	80.5	81.0
G+C content [%]	61.1	73.6	62.1	61.3	63.2	64.2

The G+C content of the DNA of strains A29, A34A, A37, B52 and B53 was in the range described for the genus *Halorubrum* (McGenity and Grant, 1995). The G+C content of the DNA of strain A34 was slightly above this range but we assume that the data are correct and that strain A34 belongs to the genus *Halorubrum*. The G+C content of the DNA is an important feature in taxonomic characterization of new species but for identification of new species is profitable the use of DNA-DNA hybridization to distinguished close species. Therefore next step in the genotypic analysis will be to carry out DNA-DNA hybridization for the determination if these strains belong to one or two species. In the time of writing this work the data are not available.

4.3 Phenotypic characterization

The strains A29, A34, A34A, A37, B52 and B53 have been tested phenotypically including morphological, physiological and biochemical tests. The results are shown in *Table 8, Table 9* and *Table 10*. Some of the physiological, biochemical or nutritional features and their sensibility to antimicrobial compounds need to be completed.

Table 8. Morphological characterization of the strains: 1. A29, 2. A34, 3. A34A, 4. A37, 5. B52 and 6. B53 compared to the most relative strains of Halorubrum, 7. Hrr. aidingense, 8. Hrr. kocurii, 9. Hrr. lipolyticum, 10. Hrr. saccharovorum. Symbols: +, positive; -, negative.

Feature	1	2	3	4	5	6	7	8	9	10
Morphology	Pleomorphic	Rods	Pleomorphic	Rods	Pleomorphic	Rods	Rods	Rods	Rods	Rods
Motility	+	-	+	+	+	+	+	-	+	+
Pigmentation	Red	Red	Red	Red	Light-rose	Red	Red	Red	Red	Red

All the most related strains were rod-shaped, strains A29, A34A and B52 were observed as pleomorphic. Strain B52 was also different in the pigmentation of its colonies, since it was the only strain showing light-rose coloration; all reference strains as well as the rest of studied strains were red pigmented.

Table 9. Physiological characterization of the strains: 1. A29, 2. A34, 3. A34A, 4. A37, 5. B52 and 6. B53 compared to the most relative strains of Halorubrum, 7. Hrr. aidingense, 8. Hrr. kocurii, 9. Hrr. lipolyticum, 10. Hrr. saccharovorum. Symbols: +, positive; -, negative.

Feature	1	2	3	4	5	6	7	8	9	10
Range of NaCl [%]	15-30	15-30	15-30	15-30	15-30	15-30	10-25	15-30	10-30	12-30
Optimal NaCl [%]	20	20	20	20	20	20	15	20	15	20
Range of pH	6-8	6-8	6-8	6-10	6-8	6-8	7-9	6-9	7-9	7-8.5
Optimal pH	7.5-8	7.5-8	7.5-8	7.5-8	7.5-8	7.5-8	7.5	7.5	7.5	7.4

The six strains studied showed the same physiological features with the respect to the range and optimal concentration of NaCl and pH for their living but some minor differences were observed with respect to the most related species.

Table 10. Biochemical characterization of the strains: 1. A29, 2. A34, 3. A34A, 4. A37, 5. B52 and 6. B53 compared to most relative strains of Halorubrum, 7. Hrr. aidingense, 8. Hrr. kocurii, 9. Hrr. lipolyticum, 10. Hrr. saccharovorum. Symbols: +, positive; -, negative.

Feature	1	2	3	4	5	6	7	8	9	10
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+
Production of acids from carbohydrates:										
Trehalose	-	-	-	-	-	-	-	-	-	-
D-xylose	+	+	+	+	+	+	+	+	+	+
D-raffinose	-	-	-	-	-	-	-	-	-	-
D-ribose	+	+	+	+	+	+	+	+	+	-
Sorbitol	-	-	-	+	-	+	-	-	-	-
L-Xylitol	-	-	-	-	-	-	-	-	+	-

In the production of enzymes catalase and oxidase all studied strains showed the same features as referenced strains. Some differences could be observed in the production acids from carbohydrates: all referenced strains were negative for the production of acids from sorbitol but strains A37 and B53 reacted positively.

5. CONCLUSIONS

The aim of this work was to follow in taxonomic characterization of new *Archaea* isolated previously from hypersaline soils in Paraje Natural Marismas del Odiel, Huelva, Spain. The main conclusions of this study are following:

1. Multilocus sequence analysis (MLSA) of four *Halorubrum* strains isolated from hypersaline soils from Paraje Natural Marismas del Odiel, Huelva, SW Spain showed that all studied strains had low percentage of similarity of sequence of *rpoB* gene with their most related species which indicates that they could constitute new species.
2. Strain B52 showed a low similarity of *rpoB* gene sequence in comparison with the other new strains, which can significate that it constitutes a separate species.
3. Phylogenetic tree based on the analysis of gene *rpoB* shows that studied strains constitute a new branch. Within this new branch are visible two separated branches, one branch is formed by strains A34, A37 and B53, and the second branch is represented by strain B52.
4. Phenotypic analysis showed several differences between strain B52 and the other studied strains as well as with respect to the most related strains.
5. Analysis of the percentage of guanine + cytosine of studied strains DNA confirmed their placement within the genus *Halorubrum* and it completed the polyphasic approach of the taxonomic characterization of newly isolated *Archaea*.

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