# Diversity of the Cabo Rojo Solar Salterns utilizing Culture-Dependent Methods and Description of Two Novel Species

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# Abstract

Archaea compose the third domain of life and are most notable for the ability of some to thrive in extreme environments, such environment is that of high salinity. This project focused on the culturable haloarchaeal diversity of the crystallizer ponds of Cabo Rojo, Puerto Rico. Different carbon sources (glycerol vs. pyruvate), water sources (artificial seawater vs crystallizer pond water) , and solidifying agents (agar vs. agarose) combinations were used in media for cultivation. Although no consistent significant difference for colony formation was observed in any combination of variables for the media, members of the genera *Haloarcula, Halorubrum, Haloferax,* and *Halogeometricum* were isolated. Phylogenetic analysis utilizing multilocus sequencing analysis was performed to establish relationships with related species. As a result, two novel species of the genera *Haloarcula* and *Halorubrum,* respectively, were isolated and subsequently described. The proposed species names are *Haloarcula rubripromontorii* and *Halorubrum tropicale.*

# Resumen

Las arqueas componen el tercer dominio de la vida y se reconocen principalmente por su habilidad de ser exitosas bajo condiciones extremas. Los ambientes hipersalinos son clasificados como uno de estos lugares extremos. Este projecto se enfocó en la diversidad cultivable de los cristalizadores de Cabo Rojo, PR. Diferentes fuentes de carbono, fuentes (glycerol vs. piruvato) agua (agua salada artificial vs. agua de las salinas) y agentes solidificantes (agar vs. agarosa) fueron utilizadas para los medios de cultivos. Aunque no se encontró alguna ventaja consistente y significativa en conteo de colonias para alguna combinación de variables en el medio, miembros de los géneros *Haloarcula, Halorubrim, Haloferax* y *Halogeometricum* fueron aislados y utilizando la técnica de MLSA. Se establecieron relaciones filogenéticas con especies dentro de estos géneros. En fin, dos especies nuevas del género *Haloarcula* y *Halorubrum* fueron aisladas y descritas. Los nombres propuestos son *Haloarcula rubripromontorii* y *Halorubrum tropicale.*

# Dedication

I would like to dedicate my thesis to the memory of my grandmother Miriam Cruz, or how we all knew her Mami Millan. Growing up all my family would meet at her house for special occasions, dinners, lunches, or just because we would rather be there than anywhere else. We would go to her house because we felt that it was home; sometimes, in my case, more home then where I actually lived. She was the glue that kept all of us together and to a certain extent she had a part in raising us all. I am certain that I wouldn't be the person I am today if it wasn't for her and the love she showed me throughout her entire life. I am very thankful for having her in my life and will always try my best to emulate her compassion and love.

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I would like to thank my graduate committee for their invaluable input and advice. My project reached a higher level to with the help and encouragement of Dr. Carlos Rodríguez, Dr. Carlos Santos, and Dr. R. Thane Papke. Most of all I'd like to thank the committee chairperson and my mentor: Dr. Rafael Montalvo. I am very thankful for the opportunity to work under his mentorship, both as an undergraduate student and as a graduate. Dr. Montalvo has shown me the importance of quality science and that it does not always come easily. Thanks to his guidance I am a better scientist, student, and professional.

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## Introduction

Extreme environments were once thought of as sterile and impossible for harboring life. Hot springs, thermal vents, acidic ponds, and hypersaline environments had gone unstudied for years due to this assumption. It is now known, however, that these environments harbor a plethora of lifeforms pertaining mostly to the microbial world. One such group, the archaea, which constitute the third domain of the tree of life, have since been viewed as the archetype for life in such extreme and harsh conditions. In hypersaline environments, high concentrations of salt inhibit growth of most organisms, yet a group of archaea, known as haloarchaea, along with few other groups thrive in environments where NaCl can be found at saturation. Among the group of haloarchaea, taxonomy and diversity has been a constant field of study and attention. As of 2014, there were a total of 47 genera and 165 described haloarchaea species, making these environments much more diverse than previously imagined (Oren, 2014). Recently, three orders were established: *Halobacteriales, Haloferacales,* and *Natrialbales* and families *Haloferacaceae* and *Natrialbaceae* by Gupta et al. (2015). In Cabo Rojo, Puerto Rico, the crystallizer ponds, which were made for commercial use, are not the exception when it comes to haloarchaeal taxonomy and diversity. The genus *Halogeometricum,* as well as the species *Haloterrigena thermotolerans,* were isolated from these salterns (Montalvo-Rodríguez et al., 2000; Montalvo-Rodríguez et al., 1998). However, studies are constantly being made on haloarchaeal taxonomy and new species are constantly being described; especially given the fact that some haloarchaea are known for high degrees of recombination which sometimes lead to speciation of populations (Papke et al., 2004).

Throughout the search for more species of haloarchaea, attempts were developed to create specific media for isolating certain organisms such as *Haloquadratum walsbyi*, which in culture-independent studies was regarded as the most abundant haloarchaea in most hypersaline environments, yet was never isolated by Burns et al (2007). Another example of a haloarchaeon with specific media needs is *Halosimplex carlsbadense,* which grows only on defined media containing either pyruvate or a combination of glycerol and acetate (Vreeland et al., 2002). Because the microbial diversity of the solar salterns in Cabo Rojo, PR have not yet been fully described, in terms of culture-dependent methods, and because certain haloarchaea have been known to require specific needs in terms of cultivation media, this project was developed with these objectives in mind. Specifically, this study utilized culture-dependent methods to assess the culturable diversity of the haloarchaea in the Cabo Rojo solar salterns. The media utilized had three variables: water source (brine vs. artificial seawater), carbon source (pyruvate vs. glycerol), and solidifying agent (agar vs. agarose). These variables were used in different combinations with the purpose of higher CFU/mL yield and to encourage cultivable diversity. Furthermore, isolated strains were analyzed phylogenetically utilizing multilocus sequencing analysis (MLSA), which has been proposed for haloarchaeal taxonomy by Papke et al. (2011).

# Literature Review

## Extremophiles

Extreme environments are defined as environments that do not meet the rules established as "normal". Such environments include those with high or low temperatures, high or low pH levels, high pressure, extreme desiccation, and high salinity. The organisms that thrive in these environments are known as extremophiles. Although it is true that what is considered extreme is an anthropocentric notion, these environments are few (at least in proportion to non-extreme environments) and are not considered ideal for biological molecules and reactions to occur in the best way possible (Rothschilde & Mancinelli, 2001). So, an extreme environment is therefore an environment that makes life difficult to thrive at a molecular level.

Biological molecules such as nucleic acids, proteins, and membranes have specific conditions because of their chemical compositions. This means that if conditions change, then these molecules begin to lose function and denature (Jaenicke & Böhm, 1998). The molecular level is truly where an organism is defined as extreme or not. At extreme conditions bonds begin to dissociate, molecules precipitate, and structures become undone. Yet, many organisms have found a way to thrive in such hostile environments. For the purposes of this study, the focus will be solely on halophiles, and so the adaptations necessary to life will be explored specifically for the challenge of high salt concentrations.

Halophiles

Life in hypersaline environments was first discovered by Benjamin Elazari-Volcani in the 1930's in the Dead Sea. Before this groundbreaking study, the Dead Sea (and presumably all other hypersaline environments) were thought to be sterile. The organism that was isolated by Elazari was published as *Halobacterium marismortui,* which was later named *Haloarcula marismortui* (Oren & Ventosa, 1999). *Haloferax volcanii* was named after him and is widely used as model for the study of halophiles and Archaea in general (Allers & Ngo, 2003; Kiljunen et al., 2014; Thompson et al., 1999). At this point in history, microbial taxonomy was based solely on morphological and biochemical characteristics, which means the organisms Elazari discovered were classified as Bacteria and not as Archaea. It wasn't until 1978 when Carl Woese and his colleagues, based on phylogenetic analysis of the small subunit ribosomal RNA, came to the conclusion that halophiles, along with methanogens and a various number of thermophiles, branched out into a separate domain and had nothing to do with Bacteria. The name Archaea was later proposed in 1990 as the name of this domain within the Tree of Life (Woese et al., 1978; Woese et al., 1990). Since then, many molecular studies have revealed that Archaea have their own metabolism which in some ways are alike to Eukaryotes and in some ways are alike to Bacteria. They have been noted to take on prokaryotic mechanisms with eukaryotic molecules and enzymes (Myllykallio et al., 2000; Keeling & Doolittle. 1995; Doolittle & Logsdon, 1998). This fact, and the fact that most are adapted to extreme environments, make Archaea an interesting and worthwhile subject to study.

Adaptations to extreme environments

In halophiles, adaptations to extreme environments focus on the fact that high salinity is directly proportional to low water activity and a high number of ions. The water in this environment is thus saturated with salts and would cause any organism with a semipermeable membrane to have its water content drawn out through osmosis (Litchfield, 1998). This means that halophiles must find a way to counteract the effect of osmosis, which they do by pumping $K^+$ ions into their cytoplasm. By doing this, halophiles create an internal pressure similar to that of the environment. This differs from what is done by other organisms that live at high salt concentrations, which produce organic solutes in order to balance out the amount of salts in the environment (Litchfield, 1998). Furthermore, it has been discovered that halophilic Archaea have a higher number of negatively charged or acidic amino acids, especially glutamate or glutamic acid (Jaenicke & Böhm, 1998). This is probably the reason why some media specific for halophilic Archaea, such as JCM 168, contain sodium glutamate. Halophilic proteins have approximately 12-20 mol% of negatively charged amino acids, whereas *Escherichia coli* ranges from 0.2-9 mol% (Lanyi, 1974). When the final protein conformation is obtained, these acidic residues are usually found on the outer part of the protein. This allows for these residues to form ionic bonds with the previously mentioned $K^+$ ions. This high acidity of the outer parts of proteins is necessitated, and is not simply a random characteristic of halophilic organisms. Proteins from organisms that do not need to survive at high salt concentrations (and as such do not have high levels of negatively charged residues) reach their final conformation in a much more aqueous, less saturated environment. If these proteins were suddenly found to be in an environment with a higher amount of salts (as is the case of our halophilic Archaea) then precipitation would occur, as these proteins have residues which need to interact with water in order to properly function and to have the correct

conformation. It is for this same reason that halophilic proteins need to have these acidic residues (Eisenberg et al., 1992). Without the negatively charged amino acids the final structure of these proteins would not be possible. It is therefore obvious that halophilic Archaea require certain specific parameters in order to grow optimally (Mevarech et al., 2000). This, as discussed, is especially the case of salinity but also for a number of other factors.

Cultivation and metabolism of Haloarchaea

Since such a large number of genera exist within the *Halobacteriaceae*, many efforts have been made in order to cultivate these organism as quickly and as numerous as possible. This is due to the fact that halophilic Archaea have different physiologies and thus require different conditions in order to be grown in artificial media for further studies. Mainly, halophiles are grown in media that contain a number of complex components such as yeast extract, casamino acids, and peptone (Tindall, 1991). Yet, in some instances more defined media have been used in order to obtain different results and isolate organisms that cannot be otherwise grown. This is the case of *Halosimplex carlbadense*, which cannot be grown in media that contain complex carbon sources like yeast extract or almost any other media for that matter (Vreeland et al., 2002). The only medium that supported growth for this species was those containing pyruvate as a sole carbon source or combinations of pyruvate, acetate, and glycerol (Vreeland et al., 2002). This leads to interesting implications in terms of what is necessary for the adequate growth of different organisms. In contrast to this, questions can be asked in terms of what is being added to media that has inhibited the growth of organisms like *H. carlbadense.* Perhaps studies made to this day have not been able to unlock the secret that will provide science with a cultivable diversity that is more representative of what is found in the place of sampling. It is widely known that most of what is out there, in terms of the microbial world, has not yet been isolated. In fact, over 99% of the

microbial diversity found on Earth has never been grown on plates (Rappé & Giovannoni, 2003). The problem with this is that to understand the entire ecology of an environment, profound knowledge needs to be gathered about a large representative sample of the organisms living in said environment. Knowledge on culture-dependent methods is necessary. It is true that methods to study diversity independent of culture have come a long way, and it is no longer necessary to isolate to have a sense of the diversity of an environment, yet these studies only provide an overview and not an in-depth look at specific organisms and their role in their environment. Therefore, even though culture dependent methods provide less throughput results, they are still essential for the study of diversity (Alain & Querellou, 2009).

To better understand what needs to be added to media to have a higher yield of diversity, it is necessary to understand the physiology and metabolism of the organisms. Each organism within an environment has a niche, and part of that niche must do with what the organism metabolizes (or prefers to) and what can cause enough stress to inhibit growth. *Halosimplex carlbadense* is a very good example of an organism that has occupied a niche that allows it to specifically digest certain carbon compounds such as glycerol, acetate, and pyruvate (Vreeland et al., 2002). Among the genera of *Halobacteriaceae*, these niches are unique. They are unique among what has been cultured, yet given the number of uncultured organisms, it is very likely that all other organisms have similar restrictions on carbon utilization. Likewise, there is a high possibility that there are other very specific metabolisms. Another example of a specialized carbon utilization is that of *Haloferax volcanii*, which can grow on glycerol and organic acids but does not utilize sugars like glucose and fructose (Falb et al., 2008; Kauri et al., 1990). The metabolism of halophiles is surprisingly diverse considering the ecological restrictions applied by the hypersaline environment. Among the most widely used non-hexose carbon sources are glycerol

and pyruvate (Falb et al., 2008). The preference for pyruvate is quite simple and straight forward. Pyruvate is an essential molecule within cell respiration. It is converted into acetyl-CoA by the enzyme pyruvate-ferredoxin oxidoreductase which is then fed into the tricarboxylic acid cycle (Rawal et al., 1988). This makes pyruvate an essential molecule, not just for halophiles, but also for all organisms that have a tricarboxylic cycle, which is necessary for cell respiration. Glycerol, on the other hand, is not necessary for the tricarboxylic cycle but is itself found within glycolysis (Falb et al., 2008). When the 6-carbon molecule is separated into 2 3-carbon molecules, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, the former turns into glycerol with glycerol 3-phosphate as an intermediate (Nishihara et al., 1999). Glycerol then goes on to form part of certain lipids. Glycerol can also be readily converted into glyceraldehyde 3-phospate and utilized in the main cell respiration metabolism (Nishihara et al., 1999). Glycerol uptake is thought to be common among halophilic Archaea given that glycerol is usually readily available in the environment. This is because *Dunaliella salina,* and possibly other organisms, produces organic solutes, glycerol included, intracellularly to equilibrate with the high salt solute concentration in the external environment (Phadwal & Singh, 2003). This contrasts with the accumulation of $K^+$ in the interior of the cell done by the Archaea.

Besides these relatively simple molecules that halophilic Archaea seem to prefer as part of their metabolism, these organisms seem to consume exogenous DNA (Chimileski et al., 2014). When it comes to various organisms, this is usually done to take advantage of new genes or more genetic variability in general. Contrary to this, haloarchaea have been known to utilize nucleic acid as a source of the most essential nutrients for any living thing: carbon, nitrogen, and phosphorous (Zerulla et al., 2014). In fact, environments with high salt concentrations have been found to be among the ones with most exogenous DNA (Dell'Anno & Corinaldesi, 2004). This creates an

extraordinary opportunity for any organism that can metabolize DNA; *Haloferax volcanii* is such an organism. A study performed my Chimileski et al., (2014) demonstrated that *H. volcanii* can, in fact metabolize DNA and that this exogenous DNA is mainly utilized as a phosphate source (Zerulla et al., 2014). This study also demonstrated that *H. volcanii* had a bias towards utilizing its own DNA as a phosphate source. When the media was supplemented with DNA from *E. coli*, *H. volcanii* did not demonstrate the ability to metabolize DNA. It was discovered that this is due to the methylation of the *E. coli* DNA. This study, performed on the model organism for haloarchaea, leads to the question of whether this is specific for *H. volcanii* or whether many other haloarchaea have the same ability. In an environment where exogenous DNA is abundant, it seems like an evolutionary advantage to be able to metabolize this DNA and use it for essential nutrients and would make sense for it to be a widespread ability among organisms occupying the same conditions. The authors of the study even propose that in the RNA world, DNA was first developed by primordial cells as source of nutrients, given its molecular composition (Chimileski et al., 2014). This is because some haloarchaea have demonstrated polyploidy or various copies of their chromosome, which is used as a storage for when the other sources of nutrients are depleted (Zerulla et al., 2014). Given the amount of organic molecules, including glycerol and DNA, found in hypersaline environments it stands to reason that there are a lot of missing ingredients that must form part of culture media in order to better replicate what is in that environment and think about which reagents are missing from culture media utilized today for haloarchaea cultivation.

To replicate an environment on a Petri dish is almost certainly an impossible task. Primarily because of the difficulty in knowing exactly what is the complete makeup of the water in these environment, but also because developing a culture-dependent methodology depends on media being sterile upon inoculation. Sterility, by definition, is not a natural condition for any

environment where life occurs. Even though getting around that sterility barrier is near impossible and might provide more problems than solutions, there is a way to get around the problem of replicating the ingredients of a natural environment. This can be done by, instead of using distilled water and adding salts, utilizing the natural hypersaline water where the sample was taken from. This technique has been used before, yet not for haloarchaea, but for algae. A study done by Green in 1977 demonstrated the differences between using saltwater made in the lab by adding salts to it and by natural water directly from the sampling site. In this study, it was found that using natural water increased the cell density of *Acetabularia* sp. in culture (Green, 1977). Experiments, like this one, which involve the effects that media and their ingredients are scarce. Primarily because biology as a whole is moving towards a more *in situ* and *in silico* focus and in doing so has left the aspects of conventional culture-dependent studies aside. That is why studies in cultivable diversity within the haloarchaea have been rare most recently. Notwithstanding, the fact that methods independent of culture have shed light on a vast number of questions in biology, being able to grow organisms in the lab is still an invaluable tool and optimizing this tool is still relevant.

## Haloarchaea Diversity

Due to numerous studies focused on diversity many species of the families *Haloferaceceae*, *Natrialbaceae*, and *Halobacteriaceae* and orders *Haloferacales* and *Natrialbales* (recently reclassified by Gupta et al., 2015) have been discovered and described. As of 2014, a total of 47 genera have been described and a total of 167 species (Oren, 2014). Of these, the genera with much of species are, in descending order, *Halorubrum*, *Haloferax,* and *Haloarcula* (www.bacterio.net). Besides these three, most genera have only a couple organisms each. The disparity between *Halorubrum, Haloferax*, *Haloarcula*, and all the other genera can be attributed to one of two realities: 1) these genera are the most abundant organisms in hypersaline environment or 2) there

exists a culture bias that enhances the chances of these specific genera being grown in plate and therefore isolated with more frequency. The first is highly improbable given that it has been widely regarded that *Haloquadratum walsbyi* and nanohaloarchaea are the most predominant organisms at higher concentrations of NaCl (Oh et al., 2010). It is clear then that some genera, such as *Halorubrum* are quicker to grow in media than other less representative groups.

Species of haloarchaea have been found all around the world. Members of the genus *Haloarcula* alone have been found in Spain, Japan, and Egypt etc. (Juez et al., 1986; Oren et al., 1999; Takashina et al., 1990). Diversity studies for haloarchaea have been performed not only on salterns, but also soda lakes, and food condiments such as fish sauce (Namwong et al., 2007; 2011; Xu, 2001). Soda Lakes, in particular, host alkaliphilic haloarchaea due to their high pH levels, typically ranging from 9-12 (Jones et al., 1998). Haloarchaea have been found in all continents, including Antarctica where *Halorubrum lacusprofundi* was discovered (Franzmann et al., 1988). In the solar salterns of Cabo Rojo, Puerto Rico, where the samples for the present project were taken from, extensive studies have been done in diversity, which include the description of a new genus, *Halogeometricum*, and the species *Haloterrigena thermotolerans* (Montalvo-Rodriguez et al., 1998; 2000). This wide distribution of diversity spanning the entire globe, yet all within an extreme environment, is a special trait that the haloarchaea possess.

It has been demonstrated that haloarchaea have a high rate of speciation (Papke et al., 2007). This is due to the fact that these organisms have a very high rate of recombination within populations. For example, *Halorubrum* populations have been found to be in near linkage equilibrium, meaning that alleles are almost completely randomly distributed (Papke et al., 2007). This trait has been studied more in-depth within the *Halorubrum* populations, yet it is possible that

other haloarchaea have similar rates of speciation. This trait makes haloarchaea ideal when studying population genetics and evolution overall in prokaryotes, as a model.

When it comes to animals, the answer is usually fairly simple: A species in a taxonomical group of organisms which can interbreed with each other and produce fertile offspring. The problem with this definition is that it only applies to animals. As it is widely known, prokaryotes do not have offspring, nor do they have sex for (at least not as we know it). Therefore, such a definition does not work, in any regards, to define a prokaryotic species. In fact, even defining a genus is difficult to establish. When deciding if two organisms belongs to different genera, the 16S rRNA gene is utilized as a genetic marker (Woese, 1987). The cutoff for what constitutes a same genus is usually around 96-97% (Stackebrandt & Goebel, 1994). The 16S rRNA gene, which constitutes the ribosomal RNA of the small subunit of the ribosome, was first selected by Carl Woese, which led to the phylogenetic tree of life as it is known today (Woese, 1990). This gene was chosen because it is ubiquitous among all prokaryotes (Eukaryotes possess an 18S rRNA gene), necessary for the cell to function and therefore is conserved, but also contains regions that allow for variation between organisms that are different enough (Case et al., 2007).

The 16S rRNA gene, however, is not without its drawbacks. Many microorganisms have multiple copies of this gene (Case et al., 2007). Not only do many bacteria and archaea have many copies of this gene, but many organisms have heterogeneous copies. Heterogeneous copies refer to copies that differ in sequence within the genome; differences of about 11% have been observed in some bacteria (Case et al., 2007). The group of haloarchaea is one example of microorganisms where intraspecific heterogeneity is found, specifically among *Haloarcula* and *Halomicrobium* (Boucher et al., 2004). It has been found that among these genera differences between their 16S genes is around 5% (Cui et al., 2009). This poses a problem when classifying prokaryotes by their 16S

rRNA gene given the consensus for difference between two genera is about 3%-4%. Intraspecific polymorphism of the 16S rRNA gene also presents a problem when amplifying by polymerase chain reaction (Boucher et al., 2004). Since the two 16S rRNA sequences are still closely related, primer annealing will occur for both copies, the problem is that this process will inevitably amplify both copies and form chimeras which, if utilized for phylogenetic purposes, will unavoidably present an inaccurate sequence. To date, every species of *Haloarcula* and *Halomicrobium* has at least two different 16S rRNA genes, making it a fundamental characteristic of the genera (Cui et al., 2009). Among other disadvantages of this gene is that the taxonomical resolution of this gene is only enough to differentiate at the genus level; at the species level other methods such as DNA-DNA hybridization are required (Oren, 1997). Another problem with the 16S rRNA gene, especially among haloarchaea, is that there exists high levels of lateral gene transfer among closely related taxa (Papke, 2007).

Given the disadvantages of the 16S rRNA gene, other genetic markers have been suggested in order to improve the phylogenetic analysis which would hopefully result in more resolute and congruent taxa. Among other markers, the *rpoB* gene has been proposed as an alternative to the 16S rRNA gene (Vos et al., 2011). This gene, which codes the B subunit of the RNA polymerase, makes for a highly conserved housekeeping gene. The *rpoB* gene is also a protein coding gene which means alignments and analysis could be done using both the DNA sequence and the amino acid sequence. Being a protein coding sequence, there is more selective pressure against base changes which might lead to changes in the amino acid and in turn to the protein structure as a whole. Most importantly, among the differences between the 16S rRNA gene and the *rpoB* gene is that usually only one copy is found within a genome, which would in turn eliminate the problem of intraspecific polymorphism. When comparisons were performed, it was found that *rpoB* has

better resolution at the lower taxa levels such as species (Case et al., 2007). However, this resolution was not too significant and in some cases both genes faired equally. Within the haloarchaea group, the same problem is observed among similar taxa: lateral gene transfer, leading to incongruent relationships when both genes are compared (Papke et al., 2004).

## Multilocus Sequencing Analysis

Given the high levels of recombination between haloarchaea populations, to the point where some populations have been found to be in near linkage equilibrium, it is necessary to look at more than one gene to draw significant conclusions. This is why multilocus sequencing analysis (MLSA) has been introduced into the field of haloarchaeal taxonomy (Papke et al., 2011). MLSA utilizes short fragments of different housekeeping genes, usually from 4-7 and is utilized specifically for descriptions of new species (Papke et al., 2011).

Multilocus sequencing analysis or typing, as is also widely used in literature, was primarily used in the field of epidemiology. Specifically, it was used to compare strains of *Neisseria meningitidis* in Maiden et al. (1998). In epidemiology, there are two situations which require typing of strains of a pathogen in light of an outbreak. These two situations are on a local level and on a global level (Maiden et al., 1998). When a local outbreak occurs, it is important to know if this outbreak was caused by a single strain or various strains. On a global scale, when outbreaks occur around the world, it is also important to note how these pathogens are related and thus how a spread could have occurred on a geographical scale (Maiden, 2006). Typically, these strain comparisons were done using different molecular methods that, at the end, would require gel electrophoresis in order to assess variance and make comparisons of amplified sequences, or fragments of DNA cut with restriction enzymes. Specifically, a method named multilocus enzyme electrophoresis (MLEE), which uses variances in enzymes, was very popular for these studies (Sullivan et al.,

2005). This method, although sufficiently accurate and effective, only really works when samples are run together and under the same circumstances. For example, gels run with the same types in two different laboratories can differ if certain aspects of the procedure are altered such as voltage or gel density. Thus, for two laboratories to compare typing results would necessitate a method that can be reliably shared between laboratories. Multilocus sequencing typing was developed for this precise reason; it is a way of comparing strains of pathogens that could be used between laboratories in an efficient and precise way. So far, this technique has been used within the clinical field with emphasis on organisms such as *Staphylococcus sp.*, *Campylobacter sp.,* and *Klebsiella sp.,* but also with other organisms such as fungi and archaea (Enright & Spratt, 1999).

Even though MLSA was primarily used as a method for differentiating between strains of pathogens it is an ideal method for quickly assessing relatedness between closely related species. This makes MLSA a tool that can be used more generally for taxonomy to decipher relatedness between species in a genus. As mentioned before, usually what is used in terms of sequence comparisons for taxonomical purposes is the 16S rRNA gene which is used mostly at the genus level. Because of the reasons explained above, the 16S gene is not a reliable source for discerning between closely related taxa. MLSA has been used as such a method for discerning phylogeny outside of the world of clinical microbiology. Nevertheless, other techniques for discerning phylogeny at the species level such as DNA-DNA hybridization (DDH) and average nucleotide identity (ANI) are typically recommended or required to validate a new species, MLSA compliments and provides further evidence for new taxa (Feil et al., 2004). Similarity percentage cut-offs such for the 16S, DDH, and ANI can even be made but within the appropriate measures, such as was done for species of *Vibrio* by Thompson et al. (2007). In terms of MLSA, a percentage

similarity can be established within a genus when all concatenated genes are the same and a comparison can then be established within the group.

For this study, three housekeeping genes were utilized: *rpoB, ppsA,* and *aptB.* These genes have been utilized before in studies of haloarchaea by Papke et al. (2011). These genes pertain, respectively, to the B subunit of the RNA polymerase, the phosphoenolpyruvate (PEP) synthase, and the B subunit of the ATP synthase. Because these genes are described as "housekeeping" it is important to recognize their function and importance within their systems and the cell in general.

*rpoB*

The *rpoB* gene encodes for the B subunit of the RNA polymerase, the holoenzyme which is is responsible for transcription in the cell. In prokaryotes, there is only one RNA polymerase, whereas in Eukaryotes there are known to be four RNA polymerases. The archaeal RNA polymerase, however, has been shown to be more alike to the RNA polymerase II of eukaryotes, they both typically consist of 12 sub-units, and are very similar in form (Hirata et al., 2008). Because of the similarities in structure, it has been hypothesized that the archaeal RNA polymerase is the precursor to eukaryotic RNA polymerases (Werner, 2007).

The archaeal RNA polymerase, much like the eukaryotic RNA polymerase, has a clamp and stalk formation. At the clamp segment of the holoenzyme are, primarily, the A and B subunits which are the largest subunits of the enzyme. The *rpoB* gene encodes the B subunit. The A and B subunits possess the active site and the main function of transcription which is RNA polymerization, in fact, the genes which encode these proteins form a multicistronic operon in almost all archaea (Werner, 2007). This is not to say that the rest of the subunits are not necessary for transcription. Thus, the B subunit of the RNA polymerase is a fundamental part of the entire

enzyme, which in turn makes this subunit an essential part of cell function and merits the term housekeeping gene. This gene in particular, among haloarchaea, possess ~1.8Kb and has been used by itself as a phylogenetic marker, in many cases to supplement 16S phylogenies. The advantage of this gene is that, as a protein coding gene, can be used as a protein sequence or as a nucleotide sequence for phylogenetic analysis, although it has been suggested to utilize the nucleotide sequence at lower taxonomical levels; the reason being that protein sequences might encode the same amino acids among closely related taxa, yet silent mutations might be present at the nucleotide level (Case et al., 2007).

*ppsA*

The ability to produce carbohydrates from other carbon sources in essential to any lifeform known today. The process of gluconeogenesis can be viewed as a reversal of the glycolysis pathway in that components that are broken down into simpler molecules, such as pyruvate, are converted into glucose-6-phosphate (Verhees et al., 2003). This is of particular importance to organisms such as halophilic archaea, which live in environments that are abundant in organic solutes such as glycerol and exogenous DNA (Phadwal & Singh, 2003; Chimileski et al., 2014). The gene *ppsA* encodes a protein essential to the gluconeogenesis pathway, namely the phophoenolpyruvate synthase (PPS) which is responsible for transforming pyruvate into phosphoenolpyruvate (PEP). Pyruvate kinase (PYK), in turn, does the reverse by synthesizing pyruvate from PEP. Studies done in archaea surrounding this enzyme are limited, yet a study done on *Thermococcus kodakarensis* has established that PPS, as well as PYK essential for a modified Embden-Meyerhof pathway, in other words, it seems that the reaction catalyzed by PPS can be reversed (Imanaka et al., 2006). As stated before, not many studies focus on this enzyme meaning that the property observed in *T. kodakarenisis* is not necessarily the case in haloarchaea or even

widespread in archaea in general. As for its status as a housekeeping gene, this fact is of little importance. The fact that gluconeogenesis is such an essential and ubiquitous process in biology is enough for this gene to have sfficient selective pressure as to avoid excessive variability.

*atpB*

One of the most essential parts of cell function is the ability to create ATP which is later to be utilized as an energy source for the cell. The ATP synthase is responsible for converting ADP and inorganic phosphate into ATP by utilizing a proton pump in what is called oxidative phosphorylation. The archaeal ATP synthase, also called A-ATP synthase or $A_1A_0$ ATP synthase, is unique in that it resembles the eukaryotic V-ATPase, which breaks down ATP, but functions like the bacterial F-ATP synthase, which creates ATP (Grüber et al., 2014). The archaeal ATP synthase is composed of 9 subunits which are A-F, H, *a* and *c.* Of these the B subunit, along with the A subunit, are responsible for nucleotide binding and forms the integral part of the ATP synthase complex (Schäfer et al., 2006). As for the gene, *atpB,* it seems to be included in an operon that also includes 4 other ATP synthase genes in the following order: *atpD, atpC, atpE, atpA,* and *atpB* (Grüber et al., 2014).

# Methodology

## Sampling

Three samples were taken from the solar crystallizer ponds in Cabo Rojo, Puerto Rico. These ponds are located at 17º52'12"N, 67º11'45"W at about 10m elevation from sea level. A sterile 2L glass bottle of water was used to collect 1L of saltern pond water. The salinity of the water was measured with a refractometer and then the sample was transported to the lab. For each sample a liter of water was then filtered through a 0.22μm filter utilizing the Millipore stainless steel pressurized filtration system in order to retain and concentrate cell mass. This filtering system uses two 142mm diameter disc filters of 5μm and 0.22μm pores diameter, respectively. After 1L of pond water was filtered, the 0.22 μm filter was placed in a sterile 50mL Corning tube with 25mL of sterile artificial seawater. This water contained (for 1L of artificial seawater): 200g of NaCl, 20g $MgCl_2$, 23g $MgSO_4$, 7g KCl, and 5mL of Tris buffer. The water was then adjusted to a pH of 7.2 with NaOH. This recipe is a modified version of the artificial seawater recipe found in the Halohandbook (Dyall-Smith, 2006). The tube was shaken for 10 minutes in order to suspend the cells in the saline water solution.

## Media and Plating

The media utilized in this project had 3 variables: 20g/L solidifying agent (agar or agarose), carbon source (5ml/L glycerol or 5g/L pyruvate equivalent to 0.07 moles and 0.05 moles, respectively) and water source (artificial seawater or natural saltern pond water). Media prepared for this project included a combination of these variables, for a total of 8 different media. Salinity in the water for both cases was adjusted to 20% total salt concentration and a pH to 7.4, Figure 5 shows the combinations and dilutions for plating. Additionally, all media had 5g/L of yeast extract and 1ml of ampicillin sodium salt solution (50μg/mL).

Plating was performed through serial dilutions ($10^{-1}$, $10^{-2}$, and $10^{-3}$) in an artificial water solution in which the filter was resuspended. Petri dishes were then incubated for one month at 40ºC, according to average temperatures at the sampling site. Each plate was inoculated in duplicate, for a total of 48 plates per liter of water sample.

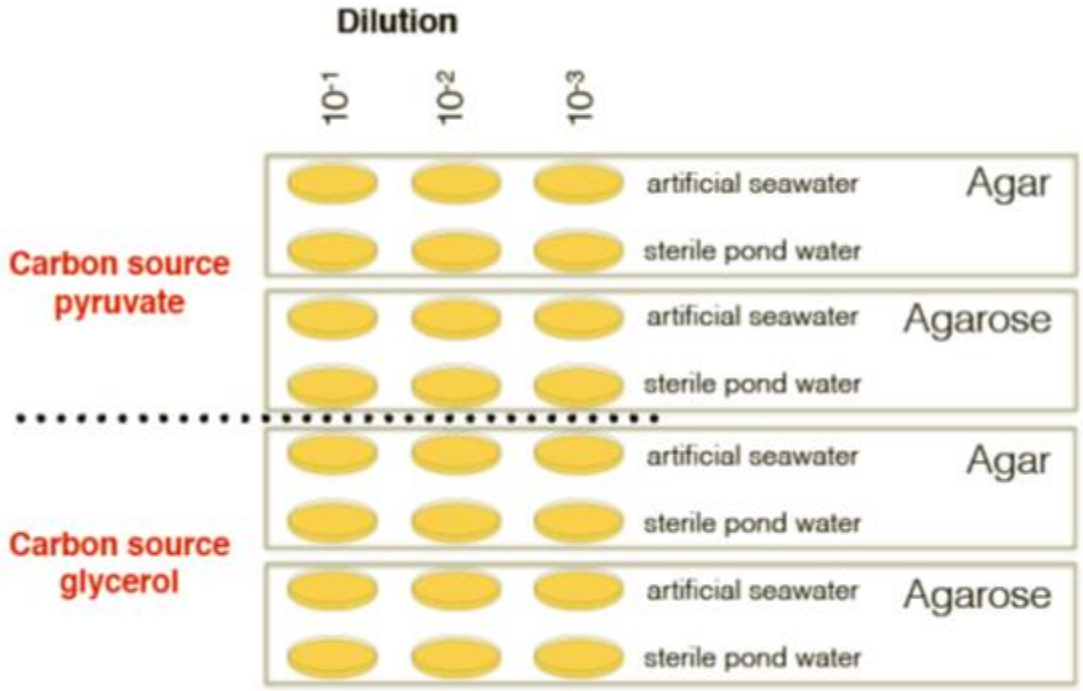


*Figure 1 Summary of the media variables and plating methods used in this project. This figure was rendered by Dr. Marc Facciotti for an HHMI-sponsored workshop pertaining to this project.*

**Isolation**

After a month of incubation at 40ºC colonies were counted and all single colonies were restreaked in plates in order to guarantee a pure isolate. The isolates were cryopreserved by flash freezing. Freezing was done by resuspending cells in 930µL of sterile saline water with 70µL of DMSO. This resuspension is done within a 1.5mL microtube and frozen by flash freezing and stored at -80ºC.

**DNA Extraction**

DNA extraction was performed by resuspending cells in sterile distilled and deionized water as described by the Halohandook (Dyall-Smith, 2006). Once the cells are resuspended in water, they were shaken vigorously for 10s and placed at 70ºC for 10min in order to denature proteins. Afterwards, tubes were centrifuged for 30s and the aqueous layer was transferred to a new sterile 1.5mL microtube. This method is fully described in The Halohandbook (Dyall-Smith, 2006).

**Gene amplification**

After DNA extraction, isolate DNA was subjected to polymerase chain reaction (PCR) for the amplification of the gene fragments used in subsequent analyses. The three genes amplified were *ppsA, atpB,* and *rpoB.* These genes are for the following proteins respectively: phosphoenolpyruvate synthase, B subunit of the ATP synthase, and the β subunit of the RNA polymerase. The primers for these genes were synthesized by IDT DNA Technologies© utilizing the sequences from Fullmer et al. (2014). The denaturing step of the PCR was performed for 1min at 95, annealing for *atpB, rpoB,* and *ppsA* was done at 57ºC, 58ºC, and 63ºC, respectively for 30sec, and extension was performed at 72ºC for 1min. These steps were done in 35 repetitive cycles. All reagents for DNA amplification were part of the GoTaq® Amplification kit by Promega™.

PCR Reagents and desired concentration:

*Table 1 PCR Reagents and concentrations*

| PCR Reagent | Initial concentration | Final Concentration for PCR |
|---|---|---|
| **Buffer** | 5x | 1x |
| **$MgCl_2$** | 25mM | 2.5mM |
| **Primers** | 50pM | 1pM |
| **BSA** | 100x | 4x |
| **dNTPs** | 10,000mM | 400mM |
| **Taq** | 5 units | 0.026 units |

After amplification, 1% agarose gel electrophoresis was performed in order to verify amplification of correct fragment length. 1Kb ladder was utilized on every agarose gel and the desired bands were ~500bp. Gel electrophoresis was used in order to verify amplification and PCR product quality.

Verified PCR products were purified in order to obtain pure DNA. Two volumes of cold absolute isopropyl alcohol were added to the PCR product and incubated for 45min at -80°C. The samples were then centrifuged for 15min at 15,000rpm and the supernatant was removed. The samples were then centrifuged for 5min at 15,000rpm and any remaining supernatant was removed. 500mL of cold 70% ethanol solution was added to the samples which are then again centrifuged for 15min at 15,000rpm and the supernatant was removed. The samples were then centrifuged for 5min at 15,000 rpm and all remaining supernatant removed. The samples were then left overnight to dry. Subsequently, DNA was rehydrated with distilled and deionized water. The DNA concentration is measured using the Nanodrop 1000. DNA samples of good quality and

concentration (at least 15ng/mL) were sent in 96-well full-skirted plates to Macrogen USA in Rockville, MD.

**Phylogenetic Tree construction**

Once DNA sequences were made available from the sequencing facility, each read was curated for quality and sufficient length. Given the size of each gene fragment, an estimated size of ~450bp was the ideal size for each fragment. If after curation, the size or the quality of the DNA sequences was not ideal, then the sequence was discarded and the process was redone for that sequence. Curation was performed using Bioedit (Hall, 1999).

After curation, the remaining DNA sequences were put into BLAST (Basic Local Alignment Search Tool) to find the most similar sequence and organism to each query sequence. Similar sequences were downloaded to be used as reference and comparison in a phylogenetic tree. The remaining reference sequences not found in GenBank were supplemented by MLSA Haloarchaea sequences provided by Dr. Thane Papke and his lab. This process was done for all three genes.

Once a sufficient amount of organisms were obtained for each gene, alignment for each individual gene was done in Bioedit. Concatenation of the three genes was also performed in Bioedit but the final alignment of the concatenation was done using MEGA 7 (Kum software), as well as the rendering of the phylogenetic tree. Both neighbor-joining and maximum likelihood methods were used. Trees were rendered by genera and by media variables to assess diversity among each genus isolated and diversity when comparing variables. The parameters used for the phylogeny are as follows in figures 6 and 7.

| Option | Selection |
|---|---|
| **Analysis** | Phylogeny Reconstruction |
| Scope | All Selected Taxa |
| Statistical Method | Neighbor-joining |
| **Phylogeny Test** | |
| Test of Phylogeny | Bootstrap method |
| *No. of Bootstrap Replications* | 2000 |
| **Substitution Model** | |
| Substitutions Type | Nucleotide |
| Model/Method | p-distance |
| Substitutions to Include | d: Transitions + Transversions |
| **Rates and Patterns** | |
| Rates among Sites | Uniform rates |
| *Gamma Parameter* | *Not Applicable* |
| Pattern among Lineages | Same (Homogeneous) |
| **Data Subset to Use** | |
| Gaps/Missing Data Treatment | Pairwise deletion |
| *Site Coverage Cutoff (%)* | *Not Applicable* |

*Figure 2 Neighbor-Joining parameters utilized in this project. Screenshot taken from Mega 7.*

Options Summary

| Option | Selection |
|---|---|
| **Analysis** | Phylogeny Reconstruction |
| Statistical Method | Maximum Likelihood |
| **Phylogeny Test** | |
| Test of Phylogeny | Bootstrap method |
| *No. of Bootstrap Replications* | 1000 |
| **Substitution Model** | |
| Substitutions Type | Nucleotide |
| Genetic Code Table | *Not Applicable* |
| Model/Method | Tamura-Nei model |
| **Rates and Patterns** | |
| Rates among Sites | Uniform rates |
| *No of Discrete Gamma Categories* | *Not Applicable* |
| **Data Subset to Use** | |
| Gaps/Missing Data Treatment | Complete deletion |
| *Site Coverage Cutoff (%)* | *Not Applicable* |
| Select Codon Positions | ☑ 1st ☑ 2nd ☑ 3rd ☑ Noncoding Sites |
| **Tree Inference Options** | |
| ML Heuristic Method | Nearest-Neighbor-Interchange (NNI) |
| Initial Tree for ML | Make initial tree automatically (Default - NJ/BioNJ) |
| *Initial Tree File* | *Not Applicable* |
| Branch Swap Filter | None |
| **System Resource Usage** | |
| Number of Threads | 1 |

*Figure 3 Maximum likelihood parameters utilized on Mega 7 (screenshot).*

## Species Description

After identification was done on all strains, it was noted that two species were candidates for new species of the genera *Haloarcula* and *Halorubrum*, respectively. A plethora of biochemical, genomic, and phenotypical tests were done according to Oren (1997). Subsequently, the draft genomes for these strains were published (Sanchez-Nieves et al, 2016a; 2016b). Given how the isolation of these strains spawn from the work done in this project, we have decided to include both species descriptions, which are soon to be published, and the genome announcements

which were published earlier this year, along with genomic comparisons done within each respective genus as a separate chapter.

# Results

## Media and Sampling

After a month of incubation, colonies for each sample processed were counted. In total three samplings of the salterns were taken. Many other samples were taken before or between these samples reported here, yet incubation and cell growth were, in many cases, not successful. Thus, these samples were taken when successful colony growth was acquired. Results for each sampling were viewed independently. Within each sample the variables are compared individually, followed by a comparison across all combinations.

A general linear model or a general factorial regression was utilized as a model in order to establish relationships between CFU/mL and the factors which were included in the media. To achieve the presumptions of a linear model the CFU/mLs were transformed utilizing Log10. The significance level utilized was 5% (0.05) when comparing the p-value and asserting significance. Dilution was also taken into account as an independent factor for these models.

### First Sampling

*Table 2 Colony Forming Units/mL (CFU/mL) for the first samples. Two numbers are present for each plate as they were done in duplicate.*

| | *ASW/Agar* | | *ASW/Agarose* | | *Pond/Agar* | | *Pond/Agarose* | |
|---|---|---|---|---|---|---|---|---|
| $10^{-1}$ | $1.23x10^4$ | $4.03x10^4$ | $1.24x10^4$ | $4.3x10^4$ | $5.4x10^4$ | $4.45x10^4$ | $3.02x10^4$ | $1.03x10^4$ |
| | $9.4x10^3$ | $2.54x10^4$ | $2.58x10^4$ | $5.63x10^4$ | $2.74x10^4$ | $4.84x10^4$ | $7.4x10^3$ | $1.08x10^4$ |
| $10^{-2}$ | $5.6x10^4$ | $2.65x10^5$ | $2.5x10^4$ | $1.88x10^5$ | $1.11x10^5$ | $9.4x10^4$ | $6.0x10^4$ | $4.9x10^4$ |
| | $6.8x10^4$ | $4.14x10^5$ | $2.9x10^4$ | $2.81x10^5$ | $1.21x10^5$ | $1.0x10^5$ | $6.6x10^4$ | $2.3x10^4$ |
| $10^{-3}$ | $6.0x10^4$ | $1.17x10^6$ | $2.0x10^4$ | $4.4x10^5$ | $1.1x10^5$ | $3.4x10^5$ | $2.0x10^4$ | $1.0x10^4$ |
| | $1.1x10^5$ | $6.7x10^5$ | $4.0x10^4$ | $3.9x10^5$ | $5.0x10^4$ | $3.2x10^5$ | $9.0x10^4$ | 0 |
| *Carbon Source* | Glycerol | Pyruvate | Glycerol | Pyruvate | Glycerol | Pyruvate | Glycerol | Pyruvate |

Table 2 demonstrates that the combination of agar, artificial seawater (ASW), and pyruvate yielded the highest CFU/mL at the $10^{-3}$ ($1.17x10^{6}$cfu/mL). Among the $10^{-1}$ and $10^{-2}$ dilutions differences in CFU/mL were subtle yet among $10^{-3}$ dilutions we can observe great differences among the variables.

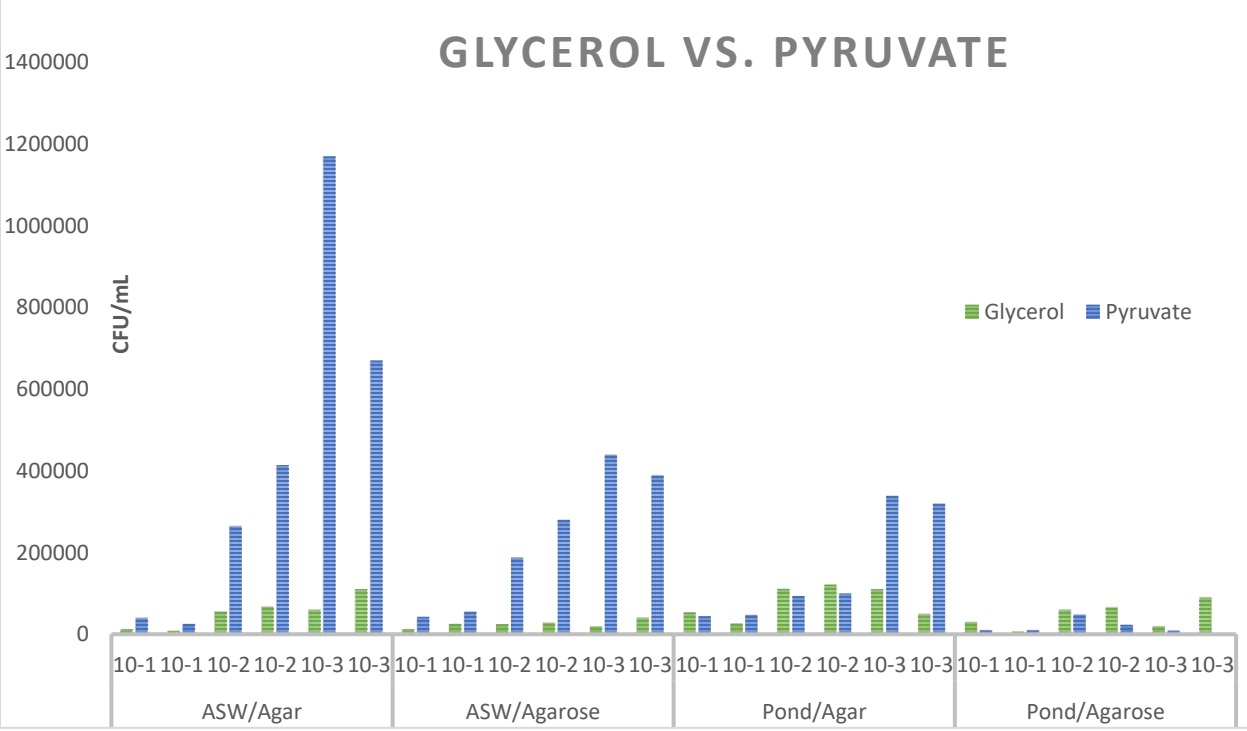


*Figure 4 Graph demonstrates the CFU/mL count of each plate in combination by comparing carbon sources independently.*

In Figure 8 pyruvate and glycerol were compared independently among all media. As mentioned above, the greater differences are made apparent in the $10^{-3}$ dilutions. In almost all cases, it is clear that pyruvate is present in media with higher CFUs. In fact, in all cases where CFU difference was greatest, pyruvate was incorporated into the media. The residuals for this sampling were transformed and the plots are as follow:

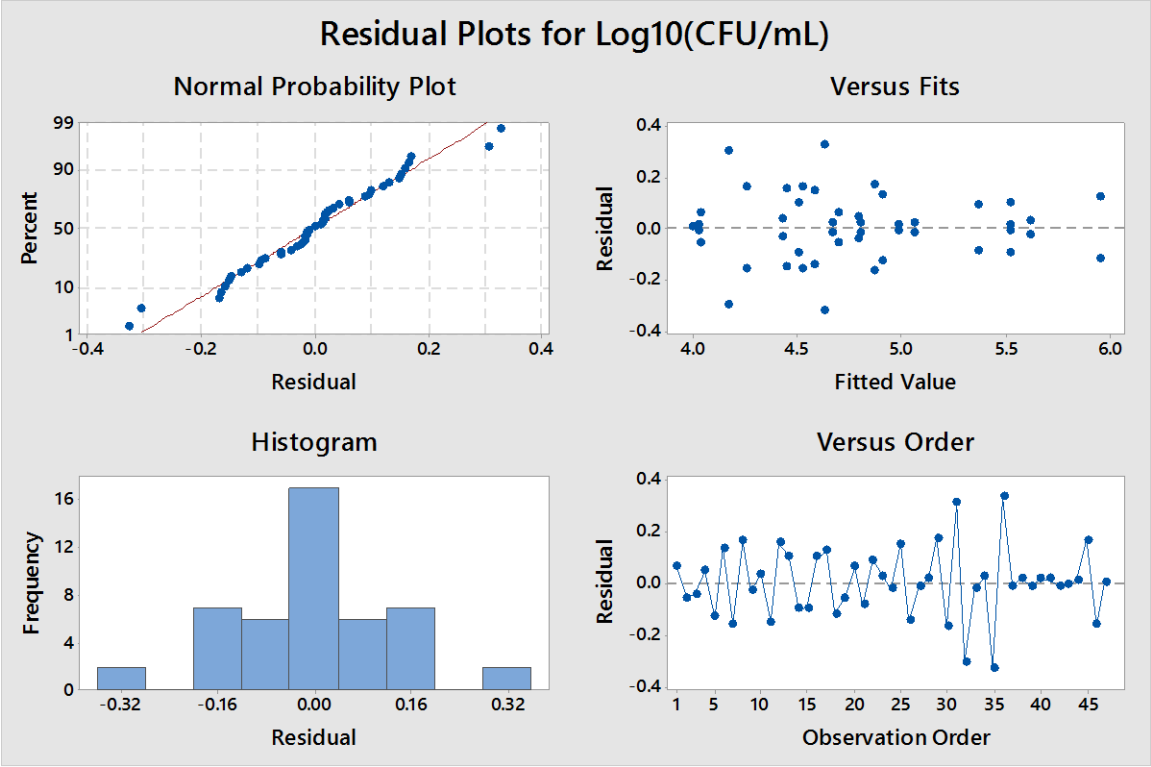


*Figure 5 These plots demonstrate and validate the presumptions for the general linear model.*

*Table 3 The p-values for the independent factors in the first sampling in the Analysis of Variance for the linear model..*

| Variable | p-value |
|---|---|
| **Carbon Source** | 0.000 |
| **Water Source** | 0.090 |
| **Solidifying Agent** | 0.003 |
| **Dilution** | 0.000 |

The general linear model demonstrated that the most statisitically significant factors for colony formation are carbon source and dilution, with water source being the least significant.

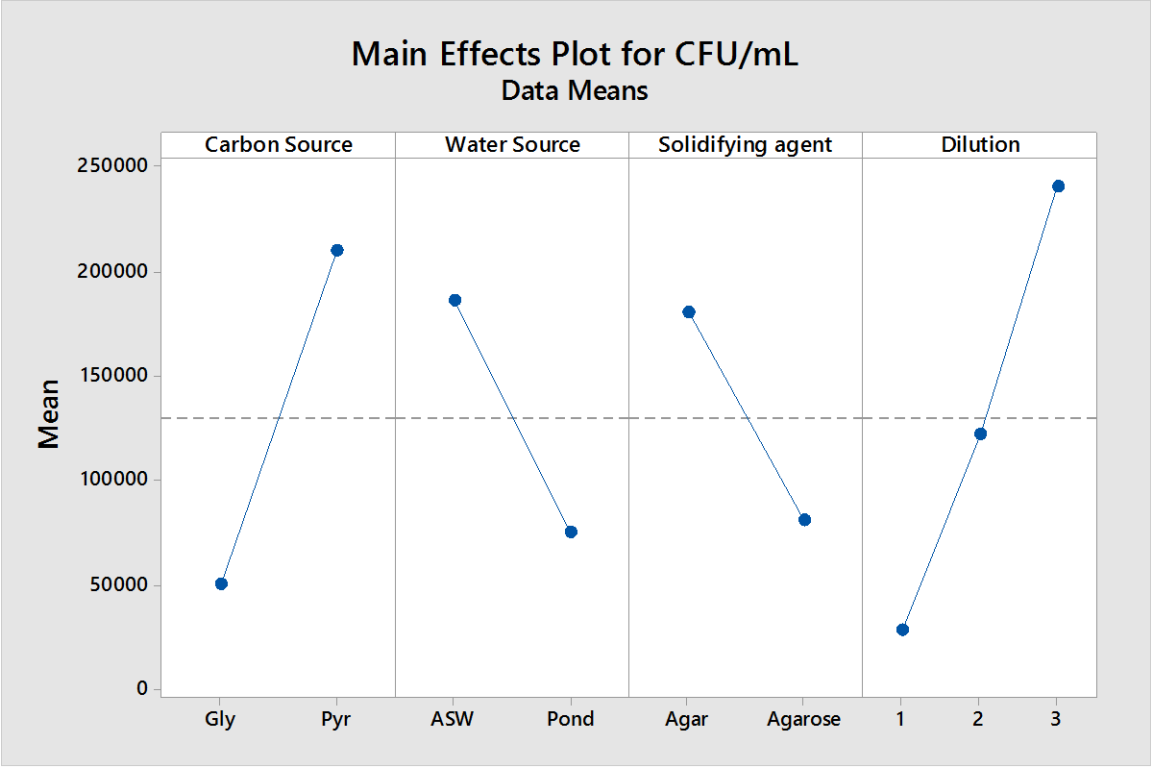


*Figure 6 CFU/mL means for each variable demonstrate which specific condition yields more colonies and the difference between them.*

Once the model demonstrated the statistical significance, a means graph (Figure 10) showed which condition for each factor yielded more colonies and the difference. It is clear that in the first sampling pyruvate performs better and these results are best seen in the $10^{-3}$ dilution.

## Second Sampling

*Table 4 Colony Forming Units/mL (CFU/mL) for the second samples. Two numbers are present for each plate as they were performed in duplicate.*

| | *ASW/Agar* | | *ASW/Agarose* | | *Pond/Agar* | | *Pond/Agarose* | |
|---|---|---|---|---|---|---|---|---|
| *$10^{-3}$* | $1.02x10^6$ | $1.95x10^6$ | $1.96x10^6$ | $5.0x10^5$ | $5.8x10^5$ | $7.5x10^5$ | $4.0x10^4$ | $1.37x10^6$ |
| | $6.9x10^5$ | $1.4x10^6$ | $1.2x10^6$ | $5.1x10^5$ | $4.9x10^5$ | $3.2x10^5$ | $1.2x10^5$ | $2.67x10^6$ |
| *Carbon Source* | Glycerol | Pyruvate | Glycerol | Pyruvate | Glycerol | Pyruvate | Glycerol | Pyruvate |

In Table 4 only the $10^{-3}$ dilution is shown due to difficulties in lesser dilutions ($10^{-1}$, $10^{-2}$). These dilutions were successful yet did not provide reliable or discernable colonies. Many colonies

in these plates were grouped together very closely, produced smears, and much of the cell mass was concentrated in the edges which led to much of the smearing and "clumped" appearances, thus these plates were regarded as too many to count. In the case of the second sampling; media containing saltern pond water, agarose, and pyruvate yielded the highest CFU/mL yet numbers in this round of samples are, on average, higher than that of the first sample. The CFU/mL for this second round of sampling is also a lot more uniform than the first sample. Comparisons directly between different variables are also a lot less clear (Figure 11).

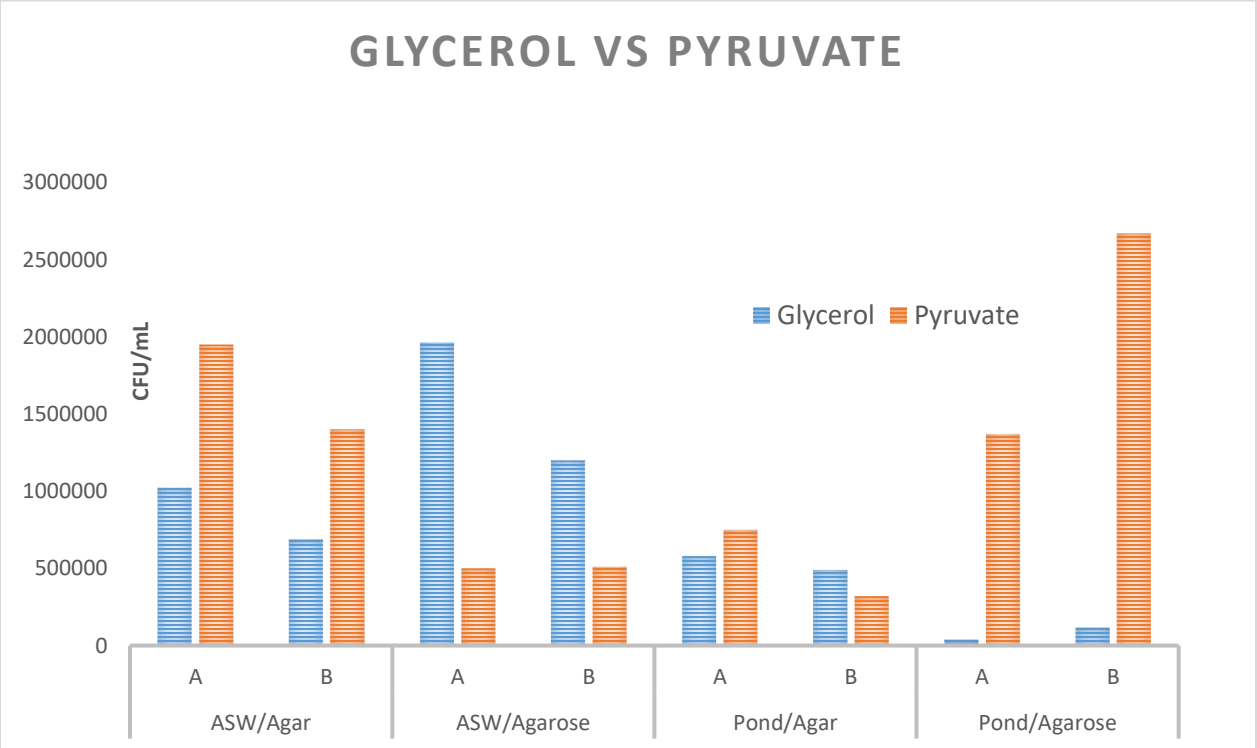


*Figure 7* Graph demonstrates differences among combinations and does a direct comparison for carbon sources to media in second round of sampling.

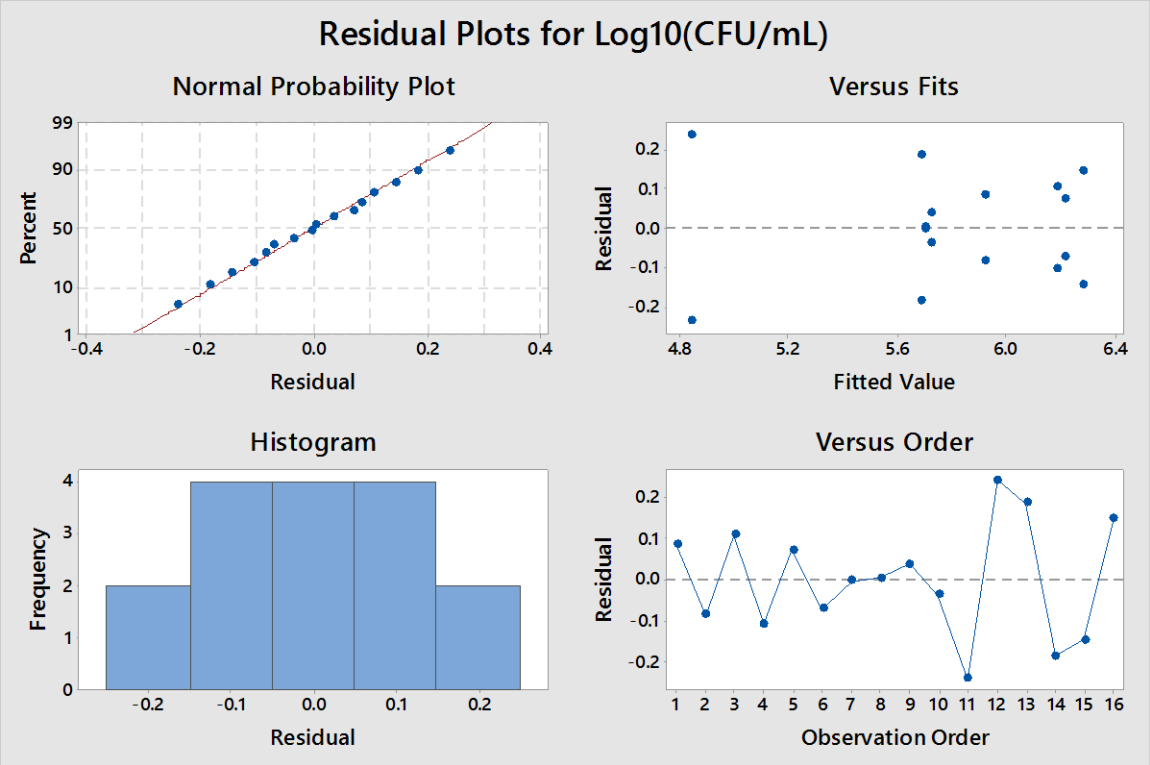


*Figure 8 These plots demonstrate and validate the presumptions for the general factorial regression model for the second sampling.*

To better explain the second sampling data, a general factorial regression was utilized because a linear model did not adequately fit the data. This model, instead demonstrates interactions between factors and demonstrates that the combination of water source and carbon source provides a statistical significance with a p-value of 0.003 (Table 5).

*Table 5 2-Way Interactions between factors and the corresponding p-values.*

| 2-Way Interactions | P-Value |
|---|---|
| **Carbon Source/Water Source** | 0.003 |
| **Carbon Source/Solidifying Agent** | 0.095 |
| **Water Source/ Solidifying Agent** | 0.913 |

When observing the main effects plot (Figure 13), it is clear that carbon source and water source are the most determining factors and that pyruvate and artificial seawater encourage more colony formation.

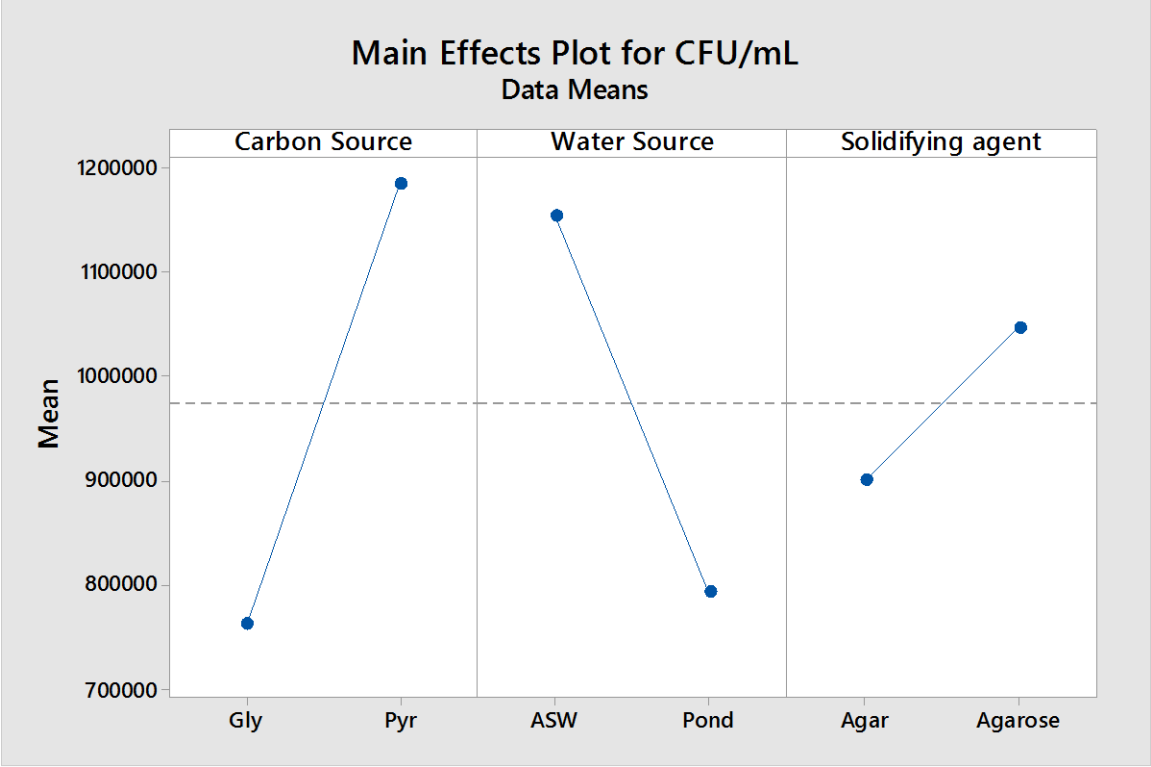


*Figure 9 Main effects plot for the second sampling compares CFU/mL means and the differences among the variables within each factor.*

## Third Sampling

*Table 6 Colony forming units per milliliter for different media inoculated on the third sampling. Each dilution is duplicated.*

| | *ASW/Agar* | | *ASW/Agarose* | | *Pond/Agar* | | *Pond/Agarose* | |
|---|---|---|---|---|---|---|---|---|
| $10^{-1}$ | $1.22x10^4$ | $1.47x10^4$ | $1.35x10^4$ | $1.57x10^4$ | $1.06x10^4$ | 0 | 0 | $1.32x10^4$ |
| | $9.4x10^3$ | $1.01x10^4$ | $1.16x10^4$ | $1.44x10^4$ | $1.14x10^4$ | 0 | 0 | $1.19x10^4$ |
| $10^{-2}$ | $8.3x10^4$ | $2.4x10^4$ | $4.1x10^4$ | $5.8x10^4$ | $9.0x10^3$ | 0 | 0 | $7.0x10^4$ |
| | $4.9x10^4$ | $1.1x10^4$ | $3.2x10^4$ | $5.7x10^4$ | $5.2x10^4$ | 0 | 0 | $4.2x10^4$ |
| $10^{-3}$ | $6.0x10^4$ | $6.0x10^4$ | $8.0x10^4$ | $1.2x10^5$ | $2.0x10^4$ | 0 | 0 | $3.0x10^4$ |
| | $4.0x10^4$ | $1.0x10^4$ | $3.0x10^4$ | $3.0x10^4$ | 0 | 0 | 0 | $9.0x10^4$ |
| *Carbon Source* | Glycerol | Pyruvate | Glycerol | Pyruvate | Glycerol | Pyruvate | Glycerol | Pyruvate |

The third round of sampling had an overall colony count much lower than previous rounds of sampling, media containing saltern pond water, agar and pyruvate did not show any growth nor did media containing saltern pond water, agarose, and glycerol.

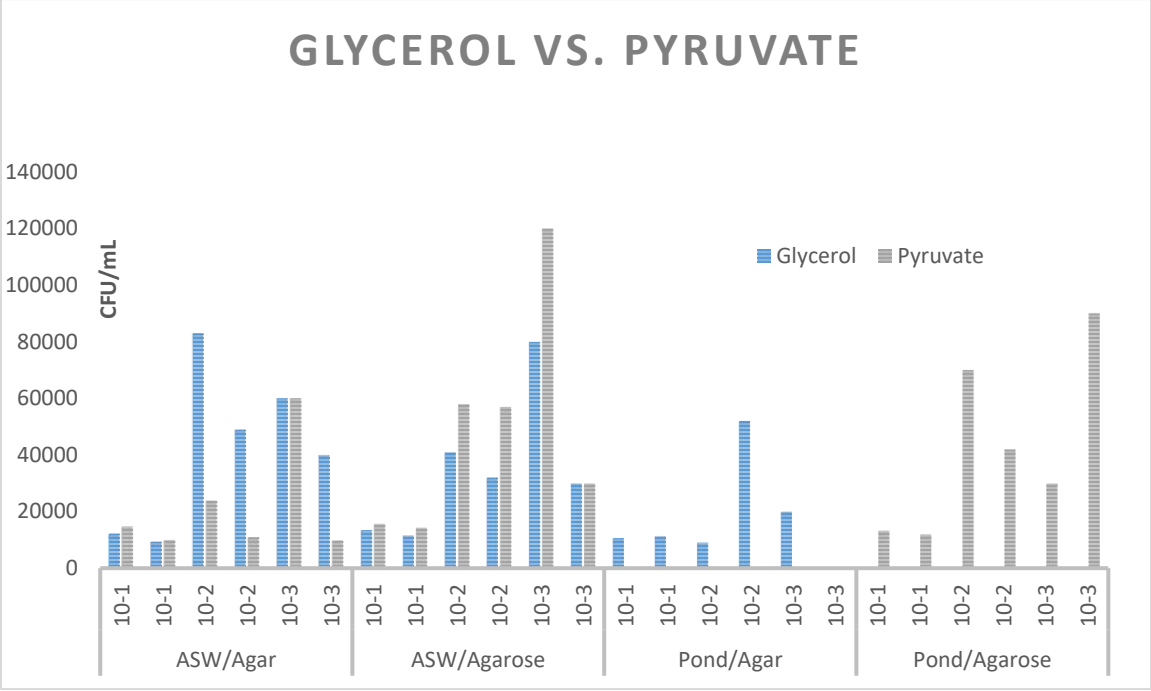


*Figure 10 Graph demonstrates the comparison of media combinations while directly comparing glycerol to in the third round of sampling.*

CFU/mL were varied across media when comparing glycerol to pyruvate. Even though pyruvate had a slightly higher average, the difference was not significant. In Figure 14 the graph demonstrates how there is no clear correlation of carbon source with CFU/mL. In some cases, glycerol achieves more growth when all other conditions are the same and in other cases pyruvate seems to correlate better to cell growth.

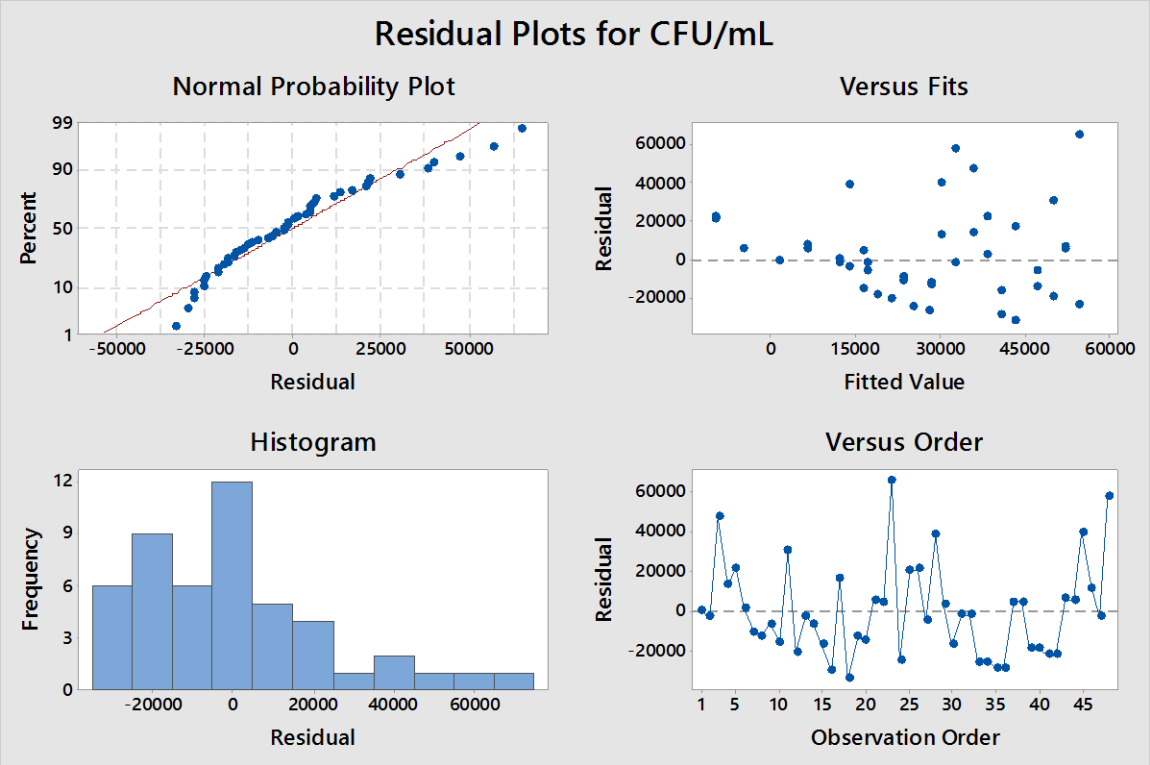


*Figure 11 These plots demonstrate and validate the presumptions for the general factorial regression model for the third sampling.*

*Table 7 This table demonstrates the variables and their respective p-value within the general linear model.*

| Variable | p-value |
|---|---|
| **Carbon Source** | 0.490 |
| **Water Source** | 0.003 |
| **Solidifying Agent** | 0.111 |
| **Dilution** | 0.006 |

Since many of the plates containing pond water showed no growth, it is clear that the water source was a lot more significant in this sampling. This is proven by table 7 which indicates that the water source was the most significant factor with a p-value of 0.003. It is also clear from Figure

16 that the main effect difference was shown in water source and that ASW encouraged more colony formation.

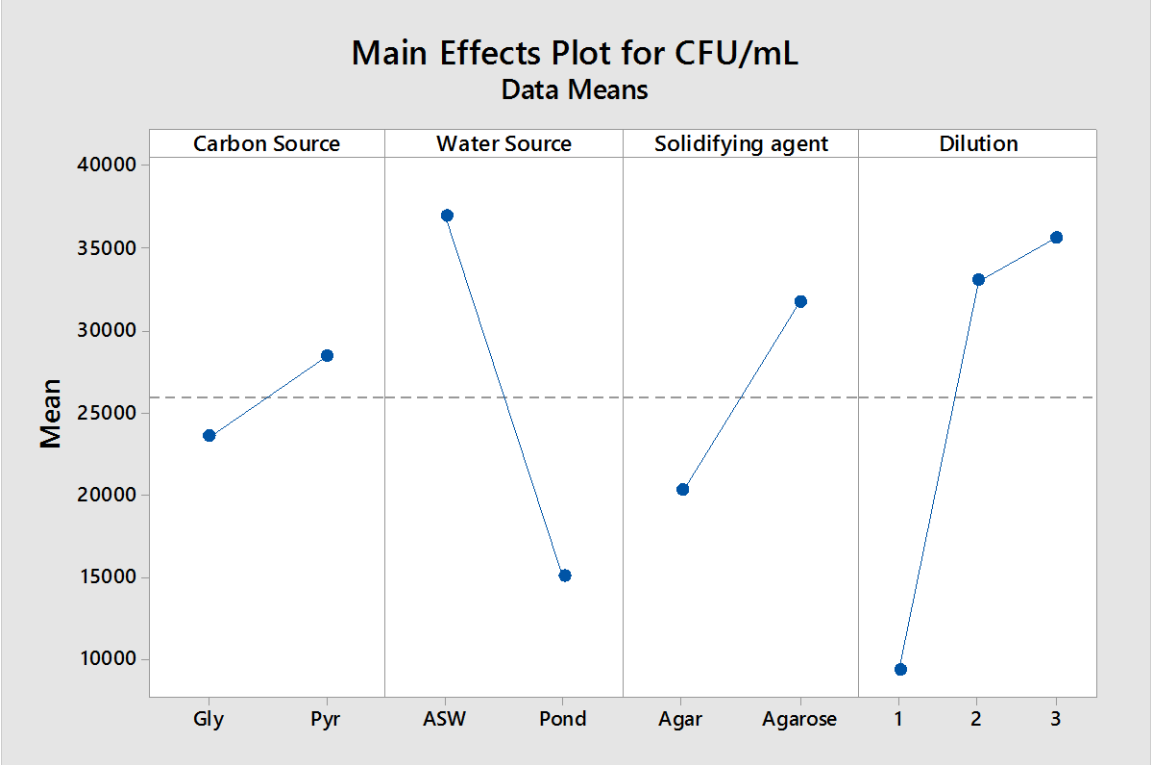


*Figure 12 Main effects plot for the third sampling compares CFU/mL means and the differences among the variables within each factor.*

When comparing all media combinations to one another, we can see that the media with the highest cfu/mL sum contained ASW and pyruvate. All the samplings were utilized in order to create a final general linear model in which only the $10^{-3}$ dilution was used.

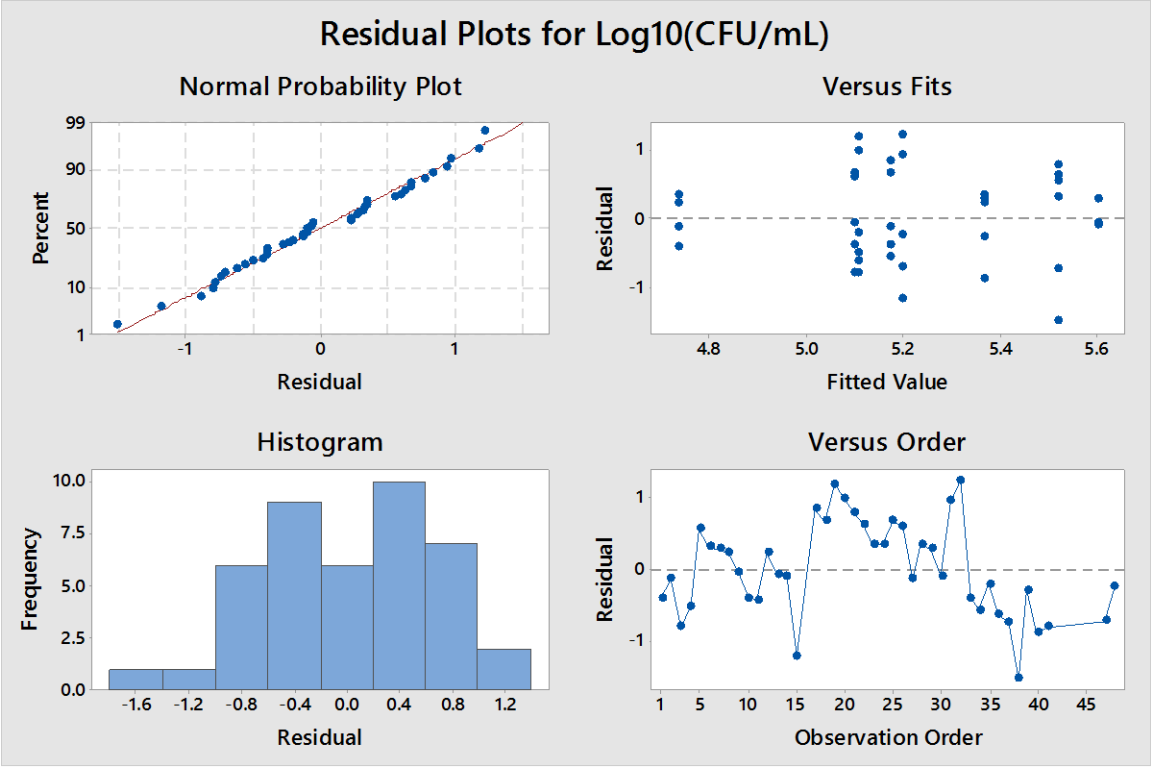


*Figure 13 Residual plots demonstrate the assumptions for the General lineal model of all samplings.*

*Table 8 Variables for the media in all samplings and their respective p-values*

| Variables | P-Value |
|---|---|
| **Carbon Source** | 0.006 |
| **Water Source** | 0.033 |
| **Solidifying Agent** | 0.148 |
| **Sampling** | 0.000 |

Table 8 demonstrates that the biggest difference was seen from sampling to sampling, yet the other contributing factors which also provide a statistical difference are carbon source and

water source, as seen in each sampling individually. Figure 18 demonstrates that overall pyruvate and ASW were consistently associated with colony formation.

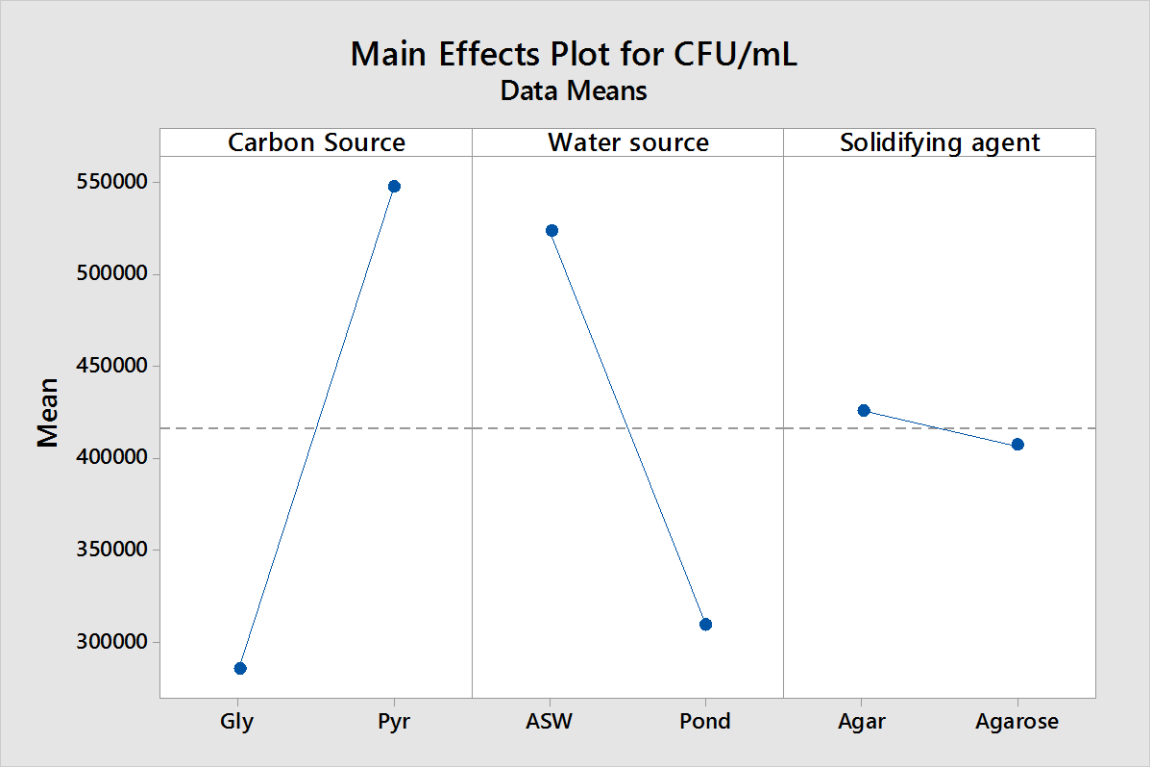


*Figure 14 Main effects plot demonstrates the differences of by means of conditions under each variable for the combination of all samplings.*

**Isolated Strains and Phylogeny**

A total of 72 strains were isolated and completely processed as to obtain all three gene sequences. Many more colonies were selected for isolation yet many did not grow or purify adequately. Many, although adequately purified, did not amplify nor sequence at an acceptable quality. Many others were very redundant and identical, in terms of the MLSA sequences, to other strains. In total isolates from four different genera were found: *Haloarcula, Halogeometricum, Haloferax,* and *Halorubrum.* The majority (55/72) of the strains completely processed were of the genus *Haloarcula,* 11 were of the genus *Halogeometricum,* 4 were of the genus *Haloferax,* and 2 were of the genus *Halorubrum.* No preferred media was detected among these groups, as all media yielded mostly strains most closely related to the genus *Haloarcula*. However, the two strains from the *Halorubrum* were only isolated from media containing pyruvate.

*Haloarcula sp.*

As can clearly be seen from Figs. 17 and 18, many strains isolated are very closely related and in many cases identical, demonstrating only small variations in some cases (Group 1a in Figure 18). This strain of *Haloarcula* does not, however, group into any described species of the genus. Figure 18 demonstrates that the relationship between described species of *Haloarcula* can be divided into two groups: 1) *H. japonica, H, vallismortis, H. argentinensis, H. salaria, H. amylolytica,* and *H. hispanica;* 2) *H. sinaiiensis, H. marismortui, H. californiae,* and *H. quadrata.* Figure 17, which provides a better description of molecular history, demonstrates that most isolates are more closely related to group 1 than to group 2. A few isolates, however, are a lot closer related to group 2. Nine isolates of the 55 do not form part of the clade which contains almost all the isolates and, of these nine, five were isolated from media containing agar, saltern pond water and pyruvate (III, IV, VI, VII, VIII), two were isolated from agar, artificial seawater, and glycerol

(HC17 and HC29), one from ASW, agar, and pyruvate (HC22), and one from saltern water, agar, and glycerol (HB6).

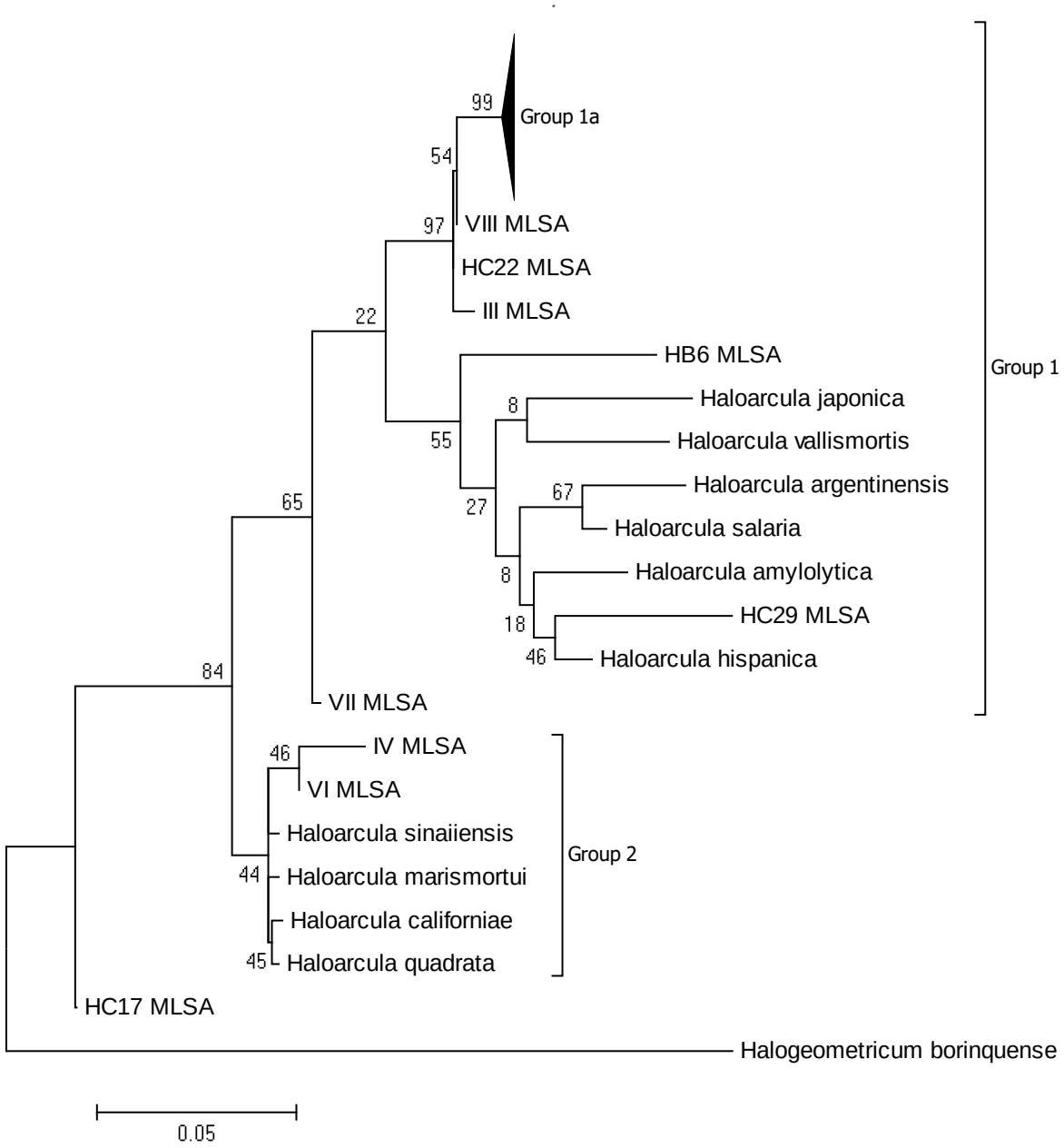


*Figure 15 Maximum likelihood phylogenetic tree with collapsed Group 1a.*

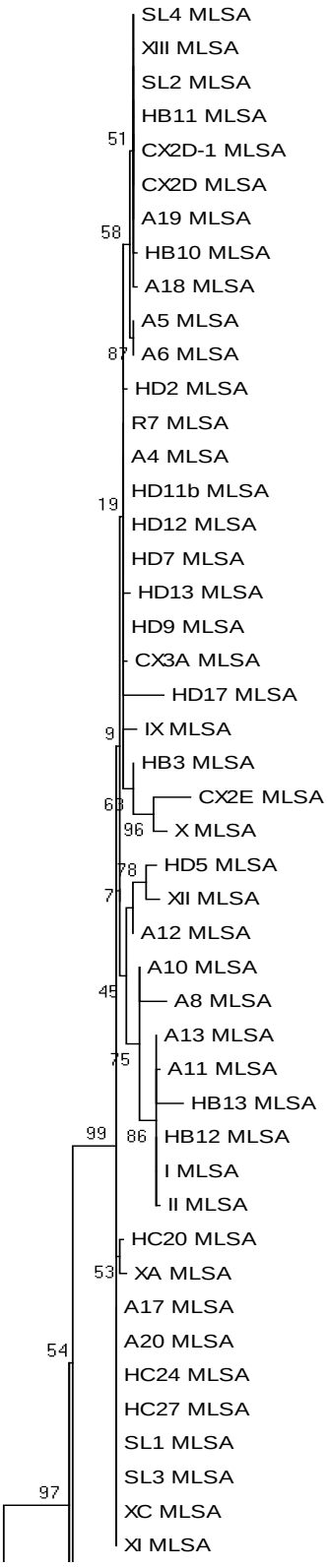


*Figure 16 Close-up of Maximum likelihood phylogenetic tree MLSA sequences of Group 1a, closely related to the genus Haloarcula.*

*Halogeometricum sp.*

A total of eleven isolates most closely related to *Halogeometricum* were fully processed. All the isolates, with the exception of one (HD4b), which was isolated from media containing agarose, saltern water and glycerol were isolated from media containing artificial seawater, agar, and either glycerol or pyruvate. Figure 19 and 20 show that all strains seem to be virtually identical to *Halogeometricum borinquense,* which was isolated from the same saltern ponds by Montalvo-Rodríguez et al. (2000).

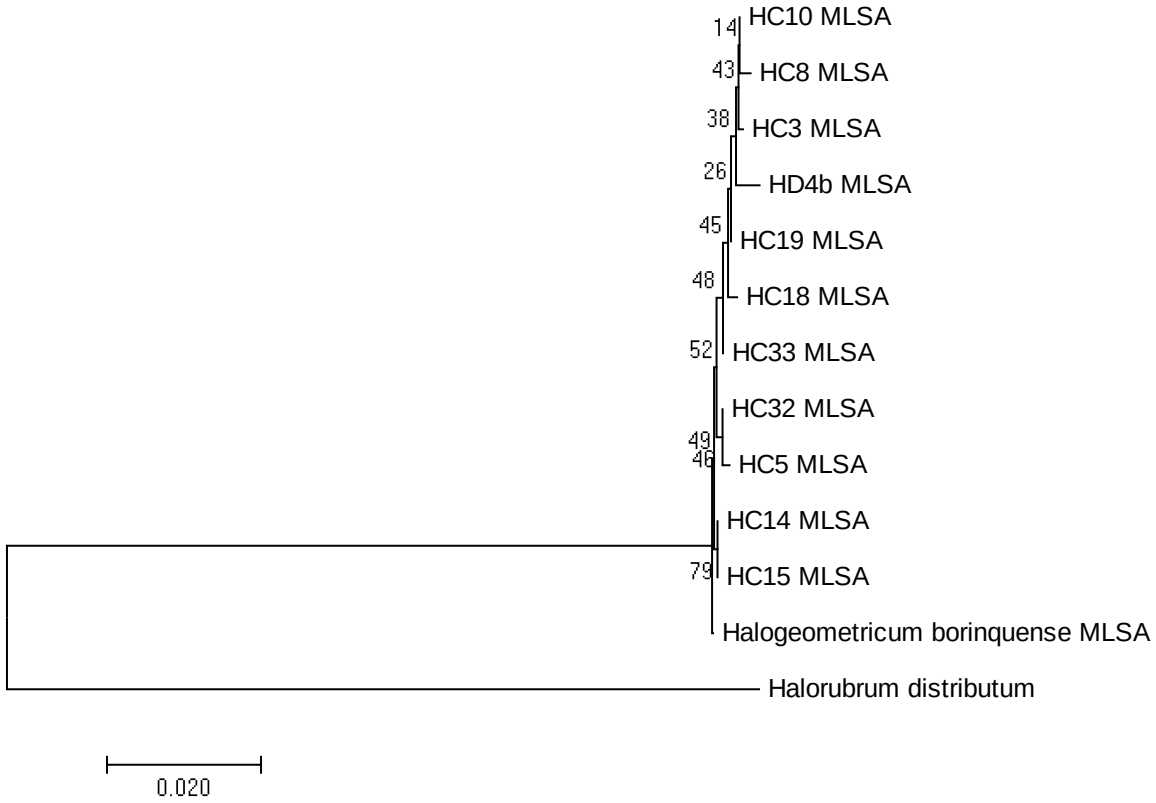


*Figure 17 Neighbor-joining phylogenetic tree of MLSA sequences of isolates most closely related to the genus Halogeometricum.*

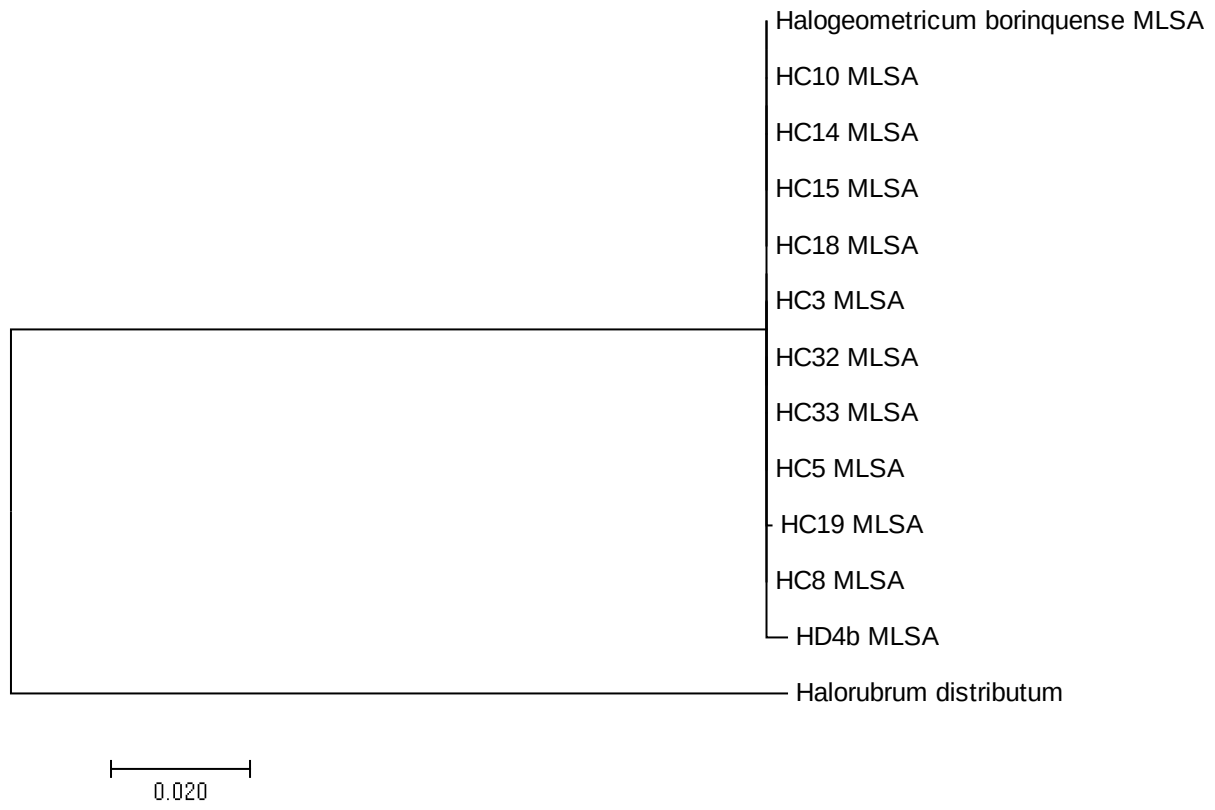


*Figure 18 Maximum likelihood phylogenetic tree of the MLSA sequences of isolates most closely related to the genus Halogeometricum.*

*Halorubrum sp.*

Two isolates were recovered most closely related to the genus *Halorubrum*. V was isolated from saltern water, agar and pyruvate, while HC25 was isolated from ASW, agar, and pyruvate. Both these isolates seem to be more closely related to each other than to any other described species (Figure 17 and Figure 18).

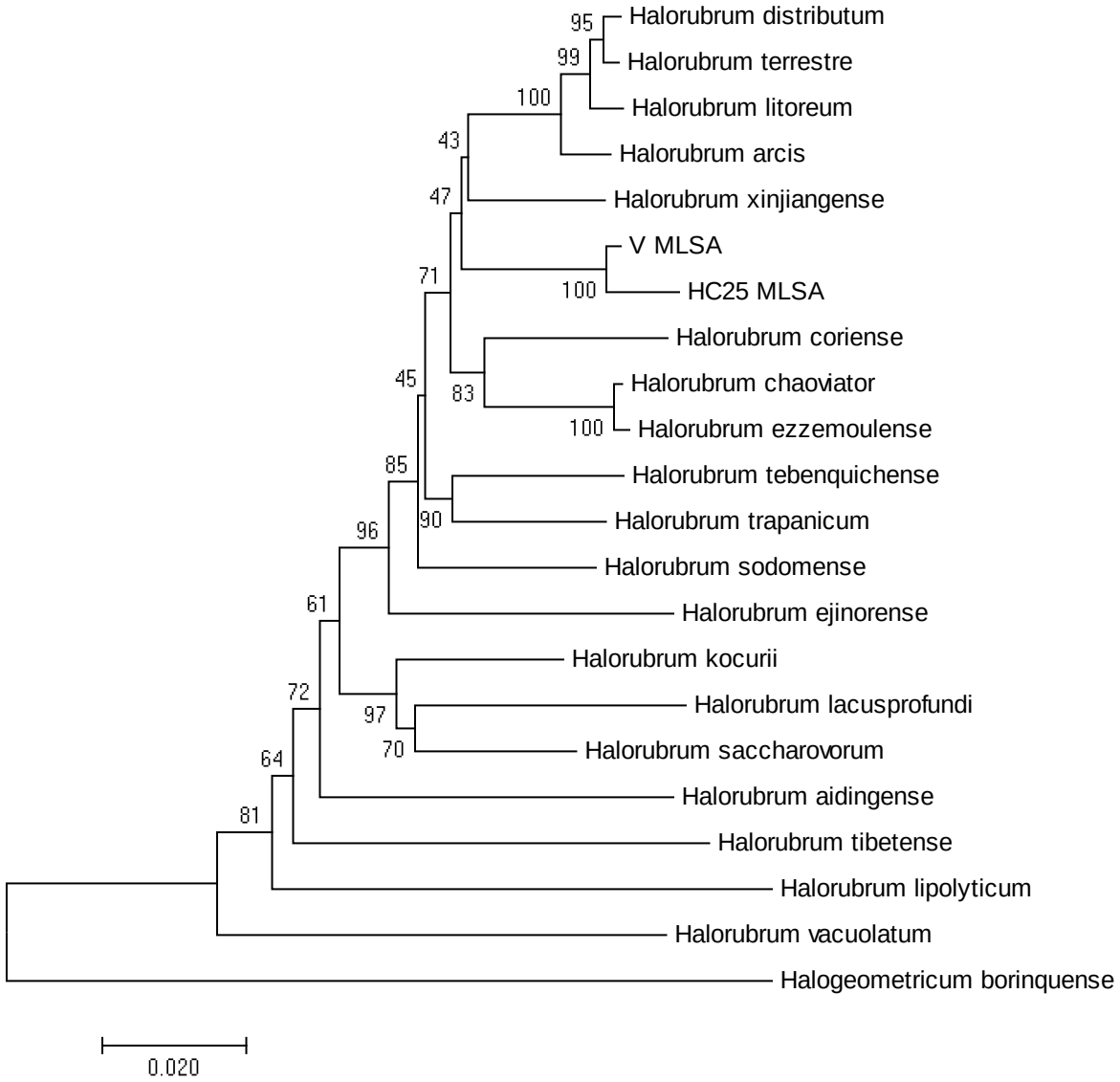


*Figure 19 Neighbor-joining phylogenetic tree of MLSA sequences of isolates more closely related to the genus Halorubrum.*

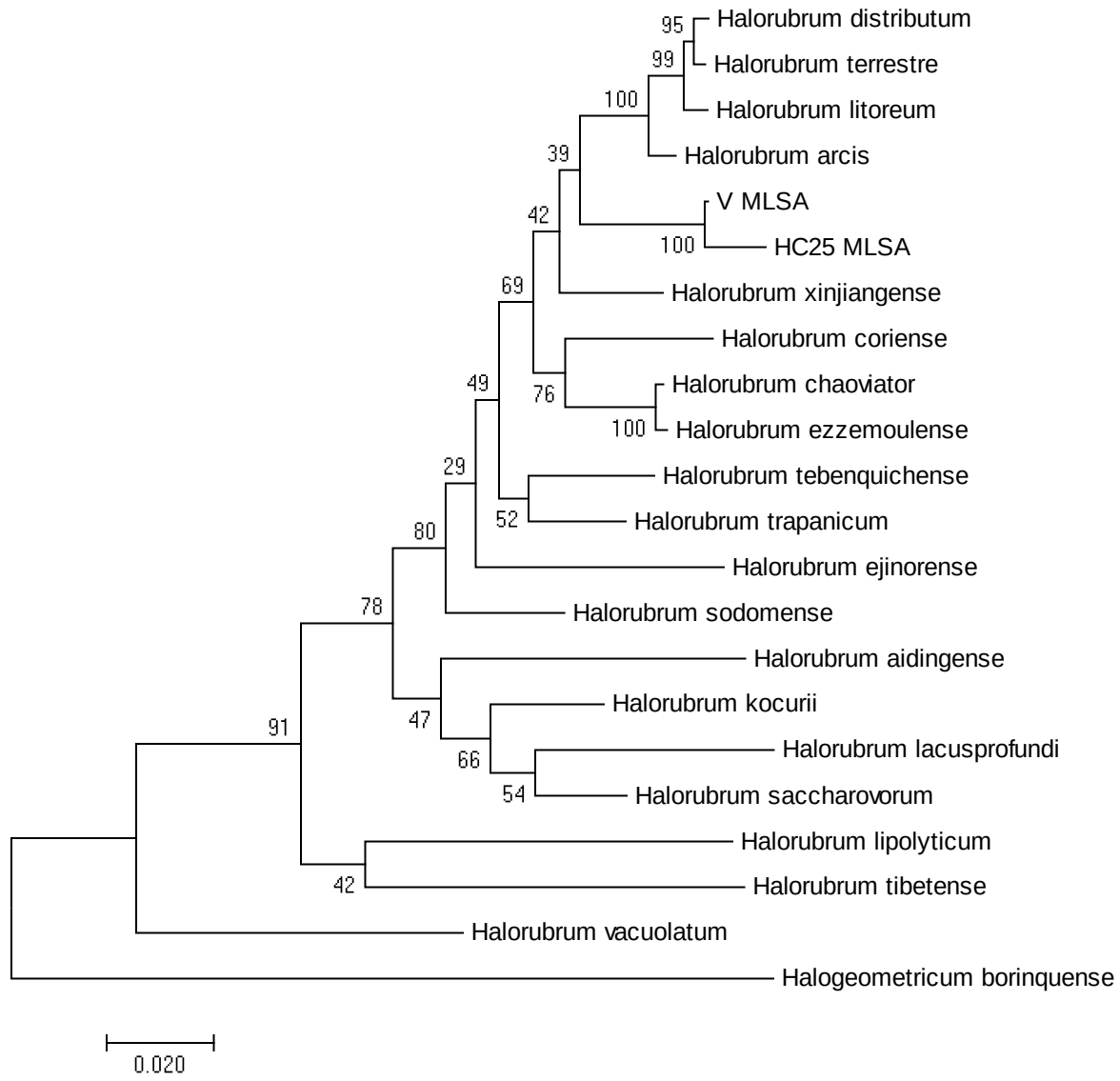


*Figure 20 Maximum likelihood phylogenetic tree of MLSA sequences of isolates most closely related to the genus Halorubrum.*

*Haloferax sp.*

The isolates more closely related with the genus *Haloferax* were difficult to process due to the fact that the *rpoB* gene fragment did not amplify successfully under varying amplification protocols. In order to have some degree of taxonomical estimation and representation, the 16S gene for these isolates was amplified and sequenced. Thus, Figure 23 and 24 do not demonstrate

MLSA phylogenetic trees but instead shows the 16S genes for these isolates. In total 4, isolates were recovered. Two isolates were recovered from ASW, agar, and glycerol (HC1 and HC7), one from agarose, saltern water, and glycerol (HD16), and one from ASW, agarose, and pyruvate (CX1C). HD16, HC1, and HC7 seem to be very closely related to *Haloferax larsenii* and CX1C appears to be very closely related to *Haloferax alexandrinus.* The 16S gene, as previously mentioned, does not have sufficient resolution at the species level and further analysis is required in order to truly assess the relationship of these isolates to the genus *Haloferax.*

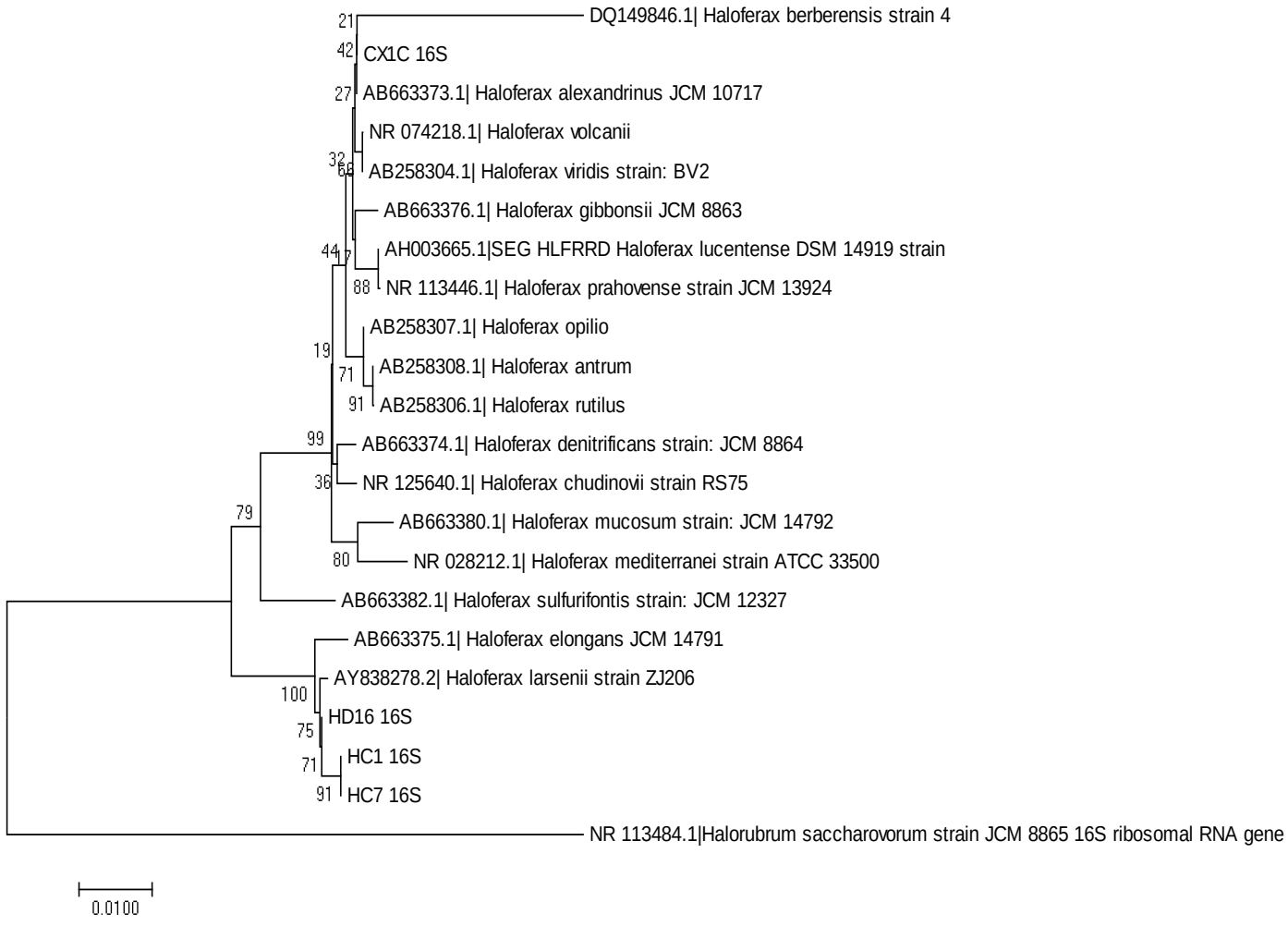


*Figure 21 Neighbor-joining phylogenetic tree of the 16 genes of isolates most closely related to the genus Haloferax.*

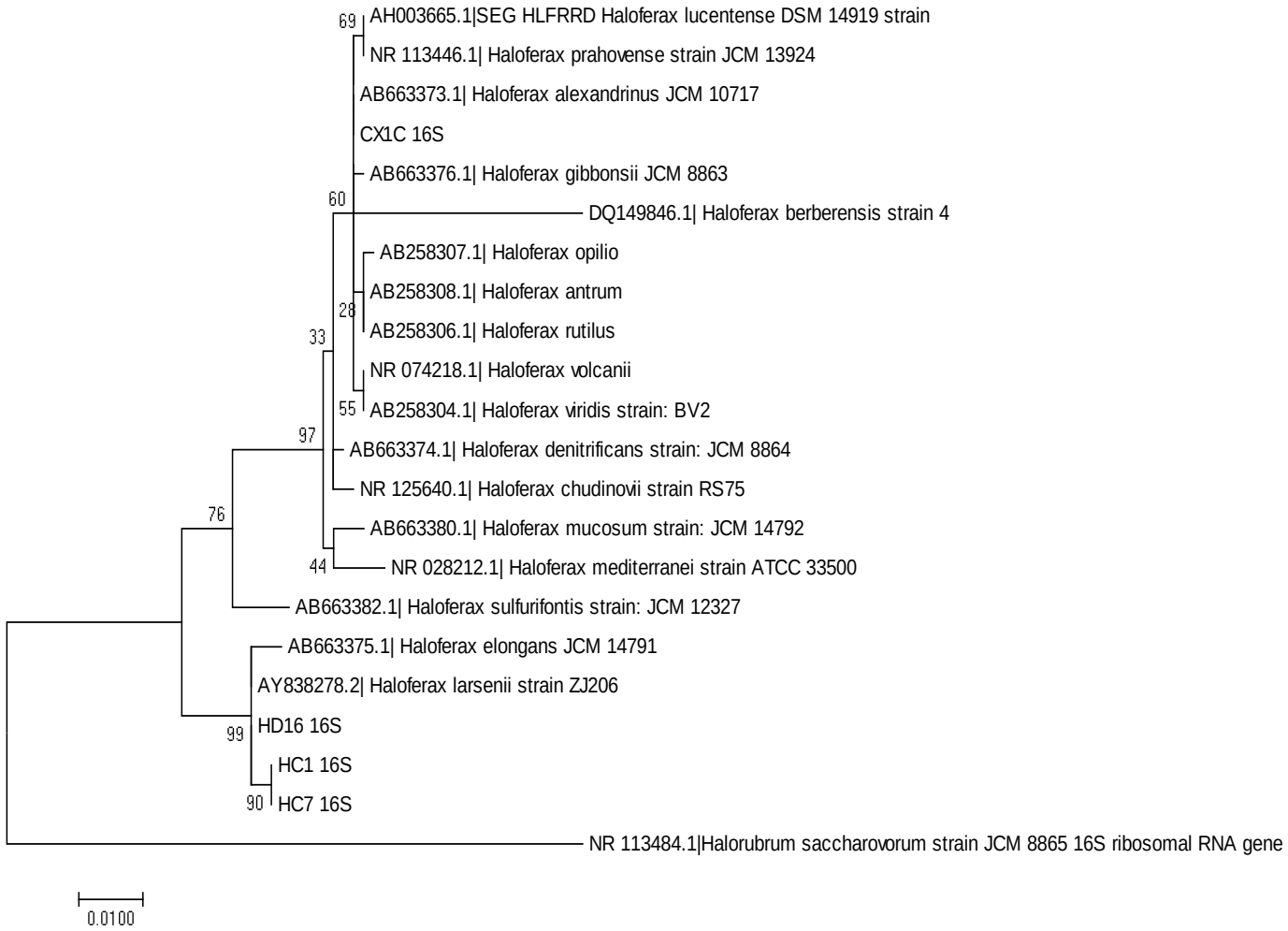


*Figure 22 Maximum likelihood phylogenetic tree of the 16S gene of isolates most closely related to the genus Haloferax.*

# Discussion

## Media and Sampling

In general, the three sampling rounds produced different results in terms of which media combination yielded more cell growth. Because populations of archaea may fluctuate over time in the saltern ponds and the samples taken over varying periods of time, the cell growth yielded from these samples will be viewed and analyzed as by themselves in terms of specific values. Nonetheless, general tendencies were examined throughout all sampling. The Crystallizer ponds in Cabo Rojo, Puerto Rico are drained and refilled in lapses of time that are not very predictable. This in turn leads to fluctuating salinities which affect haloarchaeal populations (Casamayor et al., 2002). If a certain variable is advantageous for cell growth, then this advantage should demonstrate tendencies throughout all media combinations that contain this variable and throughout all sampling rounds.

The ANOVA analysis performed when comparing each variable individually demonstrated in most cases that there is no significant preference between the two groups compared. Significant differences were found in only two instances: in the first sampling round for carbon source and in the third sampling round for water source as demonstrated in Table 3 and Table 14, respectively. In both cases there was no other significant difference viewed in all sampling rounds with regards to these variables. Among all other comparisons no statistically significant difference could be found. Nonetheless, when comparing the averages and sums of CFU/mL of the variables, in all cases, pyruvate and artificial seawater were associated with higher overall CFU/mL, and in two out of the three sampling rounds agarose was also associated with greater cell growth. These associations, however, are not statistically significant due to variability within groups. In all sampling rounds, there was a much variation between groups when the media was compared by

combinations, as opposed to comparing variables with their counterparts. In each sampling round, media combinations were observed to have a large amount of growth and in some cases, as shown in table 11, no growth could be found. However, when comparing which media combinations were those which more growth and those with less growth, a clear advantageous variable or group of variables were not always consistent.

In the first sampling, many plates had overall similar growth; the exception being the precise combination of pyruvate, artificial seawater and agar. This large difference in turn suggests that these variables were preferred for growth, even though this pattern was not seen in the other sampling rounds. In the first sampling, Table 2 suggests a significant difference between pyruvate and glycerol. The two other sampling rounds did not have such a distinct statistical difference between carbon sources, yet when compared directly, in most cases pyruvate yielded greater cell growth than glycerol. In the third sampling round (Table 14), there was a significant difference between water from the salterns and artificial seawater. In this case, half of the media containing water from the salterns showed no growth, even though growth was comparable to artificial seawater in media where it was observed. In other samplings, significant statistical differences were not observed but all sampling groups demonstrated, with more frequency, that artificial seawater was more associated with growth. When comparing solidifying agents, however, neither agar or agarose seemed to have a consistently better CFU/mL. When comparing carbon source and water source statistical significance were not observed in all cases but a tendency was observed throughout all samples for preference towards carbon source, this was not the case when looking at Figure10, 13, and 16.

Given that the two variables that affected growth to certain degree are water source and carbon source, it is important to view them in terms of the environment and with regards to what

has been studied involving pyruvate, glycerol, and saltern water or brine. Glycerol and pyruvate are both regularly used as carbon sources for taxonomical descriptions of species of haloarchaea (Oren, 2015; 2016). The reasoning behind this for glycerol is the fact that glycerol is very abundant in hypersaline environments, given that it is produced by *Dunaliella* (Bardavid et al., 2008) Pyruvate, in turn, has been used for isolating fastidious species such as *Halosimplex carlbadense* and *Haloquadratum walsby* (Vreeland, 2002; Burns et al., 2007). Many organisms that have been described recently have demonstrated growth utilizing these simple carbon source (Oren, 2015). Because of these reasons, it would not be uncommon to observe robust cell growth in media containing these carbon sources. Therefore, the presence of growth is not what is being scrutinized but whether or not there is a legitimate reason behind pyruvate being to some extent preferred to glycerol.

Many species of Haloarchaea do not grow under certain conditions such as low pH levels given that the pH of hypersaline environments is around 7.0-7.4. It is of critical importance that pH levels typically remain roughly neutral for most Haloarchaea. However, certain carbon sources are known to produce acid when catabolized by certain members of Haloarchaea. Glycerol is one such carbon source which is routinely tested for utilization and acid production, and many strains have been confirmed to exhibit acid production from glycerol. Such examples are *Haloarcula japonica*, *Haloarcula tradensis,* and *Haloferax alexandrinus* (Takashina et al., 1990; Namwong et al., 2011; Asker & Ohta, 2002). Pyruvate, in turn, is not as utilized as glycerol in characterization tests, though when utilized does not produce acid as often (Oren, 2015; Han & Cui, 2014; Juez et al., 1986). During this study, two strains, SL3 and V5, were characterized and are currently tentative novel species of the genus *Haloarcula* and *Halorubrum,* respectively. Glycerol and pyruvate were both utilized in characterizing these strains and both substances were utilized as

carbon sources. However, acid production was only observed in glycerol and not pyruvate. When isolating strains from media, it was very apparent that isolates closely related to SL3 were the most dominant strains in any media, by far with 55 out of the 72 isolates fully processed (Figure 17 & Figure 18). Thus, acid production is likely a contributing factor to a decline in cell growth in media containing glycerol. It is also important to note that SL3 has a pH range of 6 to 9, which would allow growth, although hindered, to continue in media which has suffered a pH drop.

Given that halophilic environments such as crystallizer ponds harbor populations of *Dunaliella*, which in turn produce glycerol that becomes part of the environment, it a reasonable assumption that media containing brine from saltern ponds contains some level of glycerol (Oren, 2016). Glycerol in brine would contribute to acidification of media along with other factors and components that are not accounted for as it would be in media utilizing water with salts added.

**Isolated Strains and Phylogeny**

As mentioned above, most isolates were more closely related to the SL3 strain which is partially characterized and forms part of group 1a in Figure 18. A total of 55 of the 72 isolates seen here are of this group. These strains were isolated from all eight media combinations and were in most cases the dominant strain, the only strain which, in some cases, dominated over *Haloarcula* in media were those related to *Halogeometricum borinquense* which has been isolated consistently from these ponds utilizing Sehgal and Gibbon's media (Montalvo-Rodriguez et al., 1998). *H. borinquense* usually grew faster when present in media and thus hindered other isolates from producing sufficient growth. Beside the *Haloarcula* isolates of group A1 and the *Halogeometricum* isolates, there were 16 strains isolated from the genera: *Haloarcula, Halorubrum,* and *Haloferax.* Isolates belonging to these genera are the most isolated groups of all the haloarchaea. In fact, when culture-dependent studies are performed, mostly what is isolated are strains belonging to these

genera, which would imply a bias in favor of these taxa (Oren, 2014). From the isolates gathered from the Cabo Rojo, Puerto Rico crystallizer ponds, this appears to be the general tendency for these salterns as well.

As mentioned above, most isolates were more closely related to the genus *Haloarcula.* Of these, 46 are part of a single clade and the rest (9), would seem to show varying degrees of divergence. Figure 17 divides the genus into 2 groups, group 1 being the one with the most isolates. Isolates in group 2, however, seem to be more closely related to species isolated in other parts of the world. Further phylogenetic studies need to be performed on these isolates, yet Figure 17 would suggest that strains from two evolutionarily distinct populations of *Haloarcula* were isolated.

Two strains related to the genus *Halorubrum* were isolated, from different media combinations: V and HC25. These strains seem to be more closely related to each other than any other species of the genus, yet are more distinct than other species are to each other: *H. ezzemoulense* and *H. chaoviator*. This difference between these isolates could signify that divergence has occurred to the degree where speciation was achieved, given that *Halorubrum* has been known for its high mutational and speciation rate (Papke et al., 2004). Nevertheless, a more robust phylogenetic and biochemical analysis is required in order to designate HC25 and V as different species.

Four isolates were analyzed and attributed to the genus *Haloferax.* Three isolates seem to be very closely related to each other and the other is more closely related to *Haloferax alexandrinus*. Not much information can be drawn at the species level for these isolates, given that amplification of loci proved to be troublesome, particularly with the *rpoB* gene. Thus, a 16S tree was developed in lieu of a proper MLSA phylogenetic tree. What can be said is that there are at least two possible distinct strains of the genus in these salterns which have been isolated. Further analysis would

clarify if these strains belong to an existent species or if they are part of a novel species found in Puerto Rico.

**Conclusion and Final Remarks**

In general, although tendencies in growth with specific media variables (artificial seawater and pyruvate) were observed, no clear and concise link between growth and these variables was proven. The samplings were not consistent in the number of CFU/mL cultivated, in some instances growth was observed in high numbers for a certain combination of variables, but do to the variation between samplings these numbers could not be adequately compared with the same combination in other sampling. When colony numbers were low, assessing differences between media combinations could not be accomplished, yet high overall number of colonies made differences between media combinations more apparent and, thus, more quantifiable. Factors, which are more difficult to control and observe, are most likely in part responsible for differences in cell growth: brine conditions. The water in the ponds, as mentioned above, is irregularly drained and refilled with seawater, thus changing the environment and composition of the environment. This factor could potentially affect growth in two ways: 1) the 1L of water processed and filtered for each sampling; 2) brine utilized for media. The liter used for each sampling would have to possess the overall same amount of microbial density every time. The water used for media, although always diluted to 20% NaCl, would have to always possess the same amount of nutrients and other salts. In general, differences among media combinations and variables are expected to be more apparent once growth in unhindered by this factor.

As for isolates, no media combination proved more adequate for displaying a higher diversity, as most isolates were part of the group 1a clade in all media combinations. Culture-dependent techniques, no matter how refined and varied, are always an underrepresentation of

what the environment truly holds (Alain & Querellou, 2009), the same is true for this project. Additionally, a project such as this, which contains every step of the process from sampling to establishing phylogenetic analysis, is very labor intensive and thus is subject to many biases including growth rate of isolate, if isolates can be adequately transferred and frozen, successful DNA extraction, loci amplification, and sequencing. Many colonies which are at first transferred for isolation do not reach the final step and are thus not included in the final analysis. As has been observed in other hypersaline environments, the genera most often isolated are *Haloarcula, Halorubum,* and *Haloferax* (Oren, 2014). Nevertheless, even within the constraints of culture-dependent media and bias for certain groups, the solar saltern in Cabo Rojo, rendered two novel species, *Halorubrum tropicale* and *Haloarcula rubripromontorii,* and at least one other possible new species for every genus mentioned above.

# *Chapter 2: Haloarcula rubripromontorii sp. nov.,* a halophilic Archaea isolated from the Solar Salterns in Cabo Rojo, Puerto Rico.

**Summary**

An extremely halophilic, pleomorphic Archaea was isolated from the solar salterns of Cabo Rojo, Puerto Rico. This coral-red pigmented Archaea was designated $SL3^T$ and was subjected to biochemical, morphological, genotypic, and phylogenetic taxonomical studies. Strain $SL3^T$ is an extremely halophilic Archaea which needs at least 15% NaCl for growth (20% optimum), 0% $Mg^+$ (2% optimum), has a pH range of 6.0-9.0 (7.0 optimum), and grows at temperatures of 30ºC-50ºC (optimum 38ºC). Phylogenetic analysis revealed that $SL3^T$ has two divergent 16S genes, one was most closely related to *Haloarcula salaria* HST01-2R, and the other to *Haloarcula hispanica* N60 (99% similarity each). The entire genome was sequenced and presented a GC content of 61.9%. Average Nucleotide Identity demonstrated 89% similarity to *Haloarcula vallismortui* ATCC29715, below the 94%-95% margin. Overall, phenotypic, biochemical, genotypic and phylogenetic analysis presented in this study demonstrate that strain $SL3^T$ should be placed as a new species of the genus *Haloarcula*. The name *Haloarcula rubripromontorii* sp. nov. is proposed for this novel species, with $SL3^T$ (CECT 9001) as the type strain.

The genus *Haloarcula* was first described by Torreblanca et al (1986). This genus was proposed, along with the genus *Haloferax*, after an extensive study which sampled many hypersaline ponds in Spain. As of today, the genus has 10 described species which have been found in geographically distant locations such as Spain, Japan, and Egypt (Juez et al., 1986; Oren et al., 1999; Takashina et al., 1990). It is one of the most ubiquitous genera in terms of how widespread its populations are across the world but also in terms of the number of species which have been discovered. *Haloarcula* is among the genera of Halobacteriaceae with most described species (10)

(www.bacterio.net). Besides having an elevated number of species, the genus is also known for having multiple copies of the 16S gene (Boucher et al., 2004). This characteristic, although common among many prokaryotic species, within halophilic Archaea is most commonly encountered within all species of the genera *Haloarcula* and *Halomicrobium* (Mylvaganam & Dennis, 1992). This is complemented by the fact that these intragenomic 16S genes are polymorphic, which is to say that they can demonstrate a divergence of ~5% (Cui et al., 2009). It has been hypothesized that these divergent 16S genes, which lead to differentiated ribosomes, have certain advantages for growth and adaptability to fluctuating conditions (Amann et al., 2000).

The $SL3^T$ strain was isolated from the solar salterns of Cabo Rojo, Puerto Rico. These salterns are manmade crystallizer ponds created from seawater that is then evaporate for the production and commercial use of sea salt. $SL3^T$ strain was isolated through filtering of the saltern pond water through a 0.2μm filter. The filter was subsequently submerged and shaken in 25mL of artificial seawater: 150g/L NaCl, 23g/L $MgSO_4$, 20g/L $MgCl_2$, 7g/L KCl. This recipe is a modification to the original recipe which can be found in the Halohandbook (Dyall-Smith, 2006). The artificial seawater solution with resuspended cells was then added to agar plates that contained natural saltern pond water diluted to a 20% (w/v) NaCl concentration, 5g/L of glycerol, 5g/L of yeast extract, 5mL of 1M Tris-Cl per liter of medium, and 20g/L of agar. The medium was adjusted to a pH of 7.4 using NaOH. The plates were incubated at 40ºC for 2 weeks. Colonies were transferred and subsequently isolated using quadrant streak method to undergo further biochemical, morphological, and phylogenetic analyses.

Cell morphology was determined utilizing Gram staining and phase contrast. Gram stains were performed with smears fixed in 5% acetic acid (Dussault, 1955). Optimal growth tests for $SL3^T$ were done using the mediu containing: the artificial seawater recipe mentioned above, 5g/L

of yeast extract and 5g/L of sodium pyruvate. For the NaCl growth range media was supplemented with 0%, 5%, 10%, 12%, 15%, 18%, 20%, 23%, 25%, 28%, 30% (w/v). Optimum temperature was tested at 10ºC, 20ºC, 25ºC, 30ºC, 38ºC, 40ºC, 45ºC, 50ºC, 55ºC, and 60 ºC. Optimum $Mg^{2+}$ range media was supplemented with 0%, 0.5%, 1%, 2%, 4%, 6%, 8%, 10% (w/v). Optimum pH was tested at 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0. Strain $SL3^T$ is a gram-stain positive, pleomorphic coccus. Colonies are coral-red, smooth and translucent. Growth was observed at 15%-30% NaCl (18%-20% optimal), 30 ºC-50ºC (38 ºC optimal), 0%-10% $Mg^{2+}$ (2% optimal), and at a pH of 6-9 (7-7.5 optimal). These conditions were used for subsequent tests.

Biochemical tests were performed utilizing the requirements for descriptions of new taxa published by Oren (1997). Presence of catalase and oxidase were done by adding 2-3 drops of 3% hydrogen peroxide solution and by oxidase reagent, respectively. Utilization of single carbon source and acid production were performed by adding 5g/L of the following: xylose, sorbitol, pyruvate, mannitol, glycerol, glucose, galactose, lysine, ribose, and raffinose. Media was also supplemented with artificial seawater (20% total salts) 0.01% (w/v) of yeast extract, 0.5g/L of $NH_4Cl$, and 0.05g/L of $KH_2PO_4$ (Tomlinson & Hochstein, 1976). Hydrolysis of Tween 80 was tested by adding 0.1% (w/v) of $CaCl_2$ and 1% (w/v) of Tween 80 to solid media; hydrolysis was verified by halos surrounding colonies (Gutiérrez & González, 1972). Gelatin hydrolysis was performed in media containing 12% (w/v) gelatin (Gutiérrez & González, 1972). Starch hydrolysis was tested adding 10g/L of potato starch in solid medium, hydrolysis was verified by adding iodine to the plates (Nicolaus et al., 1999). Skim milk was added to medium to test for casein hydrolysis, hydrolysis was verified by clear halos around colonies. Indole formation was determined using 1% tryptone in medium, formation of indole was determined using Kovac's reagent (Montalvo-Rodríguez et al., 1998). Nitrate reduction and gas production was performed in an anaerobic

chamber (Mancinelli & Hochstein, 1986). Anaerobic growth was determined with 5g/L of L-arginine, this test was also done within an anaerobic chamber (Hartmann. et al., 1980). The following antibiotic discs were placed on culture media to determine resistance/susceptibility: bacitracin, penicillin, novobiocin, erythromycin, chloramphenicol, sulfamethoxazole/trimethoprim, vancomycin, ampicillin, tetracycline, and rifampin. A summary and comparison with other *Haloarcula* type species of these tests is demonstrated in Table 17.

Phylogenetic analysis was done for the 16S gene, *rpoB* gene and by multilocus sequencing analysis (MLSA) (Minegishi. et al., 2010; Papke et al., 2011). DNA extraction was performed by adding cells to sterile deionized water, followed by heating to 70ºC for 10min; this protocol is described by Dyall-Smith (2006). Multilocus sequencing analysis was performed using 3 genes: *ppsA*, *rpoB*, and *atpB*. These genes were chosen and amplified according to Fullmer et al. (2014). Because of the intraspecific polymorphism of the 16S gene in *Haloarcula* sp, after amplification the gene was cloned in *E. coli* in order to avoid chimeric sequences (Boucher et al., 2004). The purified PCR products were sent to Macrogen USA for sequencing. Sequences were curated using Bioedit, alignments were performed using Clustal W and neighbor-joining trees were performed with Mega (Hall, 1999; Tamura et al., 2011). Preferences for the trees were the following: 2000 bootstraps, p-distance model, and pairwise deletion. Sequences for related taxa from the genus *Haloarcula* were taken from Genbank, and initial relatedness was determined by BLAST (Basic Local Alignment Search Tool). The whole genome was sequenced, assembled, and annotated by MRDNA Lab facilities. Average nucleotide identity was then performed in order to verify similarity between species of *Haloarcula* (http://www.ezbiocloud.net/ezgenome/ani).

In silico analyses, using BLAST, were performed for the both 16S genes (1472bp each) and for the *rpoB* gene (1827bp). In all cases, strain SL3$^T$ was closely related to the genus

*Haloarcula.* For the 16S genes, strain $SL3^T$ was most closely related to *Haloarcula hispanica* and *Haloarcula salaria,* respectively (99% each). As for the *rpoB* gene, the most related organism was *Haloarcula hispanica* (95%). In all cases, *Haloarcula japonica* and *Haloarcula amylolytica* were also closely related, at the same percentages. Phylogenetic analysis for both genes using the neighbor-joining algorithm demonstrated that strain $SL3^T$ is very closely related to other *Haloarcula* species. Much like what was demonstrated by BLAST, relatedness of strain $SL3^T$ to other species was very similar through most of the genus. Relatedness was confirmed by neighbor joining and maximum parsimony algorithms. The average nucleotide identity was determined by comparing all other available *Haloarcula* genomes with strain $SL3^T$. The proposed cut-off for species boundary is at 95-96% (Richter & Rosselló-Mora, 2009). All species of *Haloarcula* were between 88%-89% similar; the most similar species was *H. vallismortis* with 89%.

Overall the phenotypic, biochemical, and phylogenetic analysis have determined that strain SL3 is a new species within the genus *Haloarcula.* The name *Haloarcula rubripromontorii sp nov.* is proposed for this novel species.

**Description of *Haloarcula rubripromontorii*** (ru.bri.pro.mon.to'ri.i. L. adj. ruber red; L. neut. n. promontorium cape, promontory; N.L. gen. neut. n rubripromontorii of the red cape, pertaining of Cabo Rojo, PR)

Cells are Gram-stain positive, motile, facultative anaerobes. pleomorphic cocci. Cells present a coral-red pigmentation. Colonies are translucent and full. Cells lyse in distilled water. A minimum of 15% (w/v) NaCl is required in order to observe growth. Growth in concentrations of NaCl ranged from 15% -30% (Optimum 20%). Concentrations of $Mg^{2+}$ for growth ranged from 0%-

10% (optimum 2%), growth was observed at temperatures ranging from 30-50ºC (optimum 38ºC) and at pH ranging from 6.5-9 (optimum 7.5). Strain SL3$^T$ was catalase and oxidase positive. Sorbitol, pyruvate, mannitol, glycerol, glucose, galactose, and raffinose were utilized by SL3$^T$ as a carbon source. Acid was produced from galactose, glycerol, and glucose. Tween hydrolysis was observed, although casein, starch, gelatin hydrolyses were not observed. Indole did not form from tryptophan. SL3$^T$ was able to grow anaerobically with L-arginine and nitrate, gas production was observed. SL3$^T$ was shown to have susceptiblity to novobiocin and bacitracin but was resistant to penicillin, erythromycin, chloramphenicol, sulfamethoxazole/trimethoprim, vancomycin, ampicillin, tetracycline, and rifampin. The DNA GC content was 61.9%. The SL3$^T$ type strain was deposited at CECT 9001.

*Table 9* Summary of biochemical and phenotypic characteristics within the *genus Haloarcula*. Species included: 1) SL3$^T$; 2) *H. hispanica;* 3) *H. salaria;* 4) *H. japonica;* 5) *H. amylolytica;* 6) *H. vallismortis;* 7) *H. tradensis.*

| **Characteristic** | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
|---|---|---|---|---|---|---|---|
| **Cell shape** | Pleomorphic | Short rods | Pleomorphic | triangular | Short rods | rods | Pleomorphic |
| **Motility** | + | + | - | + | + | + | - |
| **Range for Growth:** | | | | | | | |
| **NaCl (%. w/v)** | 15-30 | 15-30 | 15-30 | 15-30 | 15-30 | 15-30 | 15-30 |
| **$Mg^{2+}$ (%, w/v)** | 0-10 | 0-10 | 2-10 | 1-10 | 0-10 | 1-10 | 2-10 |
| **Temperature (°C)** | 30-50 | 25-50 | 15-45 | 24-45 | 20-52 | 30-55 | 15-45 |
| **Utilization of** | | | | | | | |
| **Sorbitol** | + | + | - | + | + | + | + |
| **Raffinose** | + | - | + | ND | - | - | + |
| **Glycerol** | + | + | + | + | - | - | + |
| **D-Galactose** | + | + | - | + | + | - | + |
| **D-Glucose** | + | + | + | + | + | - | + |
| **Lysine** | - | + | - | ND | + | - | + |
| **Nitrate Reduction** | + | - | - | + | - | + | - |
| **Hydrolysis of** | | | | | | | |
| **Starch** | - | + | + | - | + | - | W |
| **Tween 80** | + | + | + | ND | + | - | + |
| **Gelatin** | - | + | - | - | + | - | - |

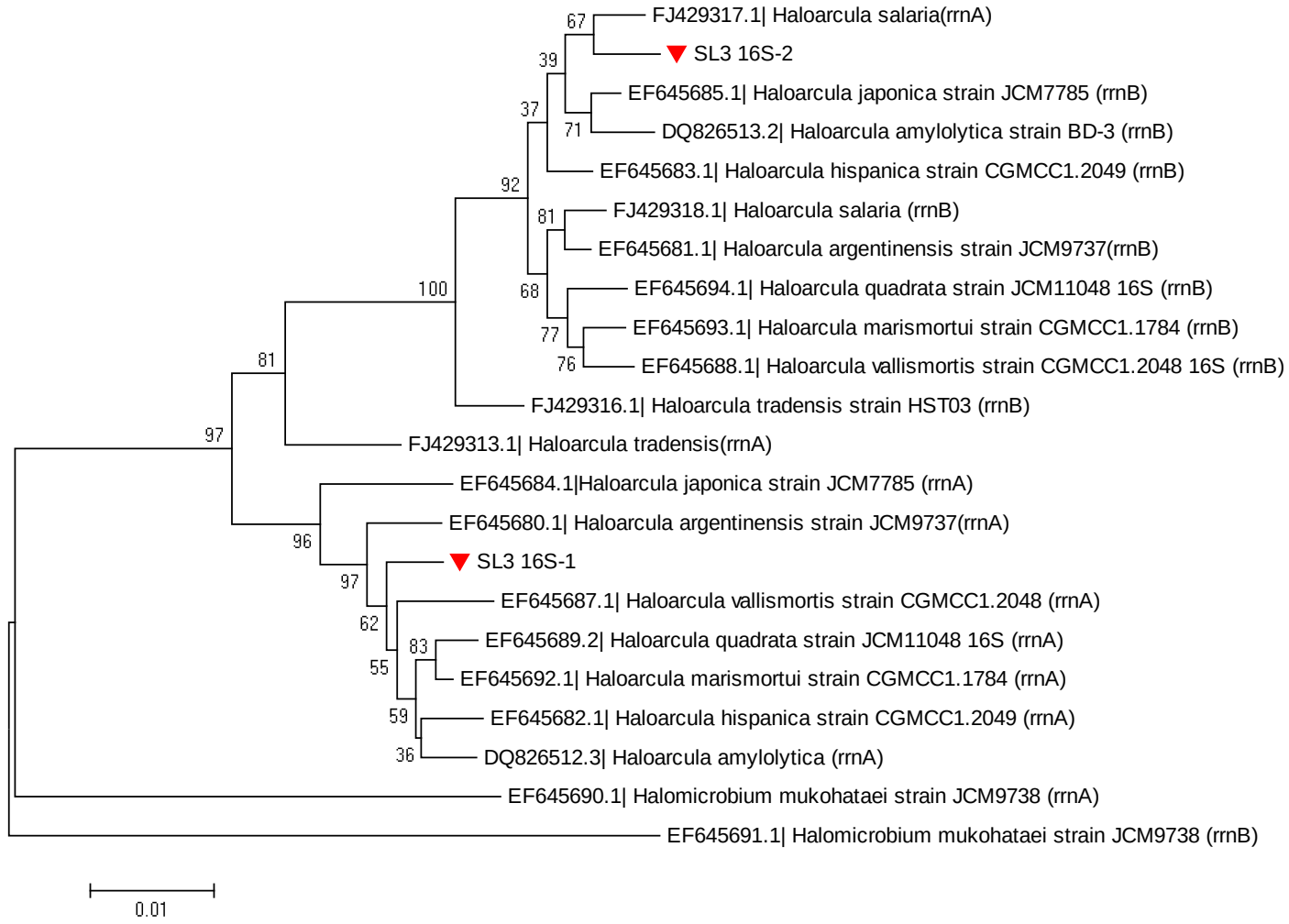


*Figure 23* Neighbor-joining tree utilizing the bootstrap method of the 16S ribosomal RNA gene for the genus *Haloarcula*. Both polymorphic operons found in the SL3$^{T}$ strain were utilized for the rendering of the tree.

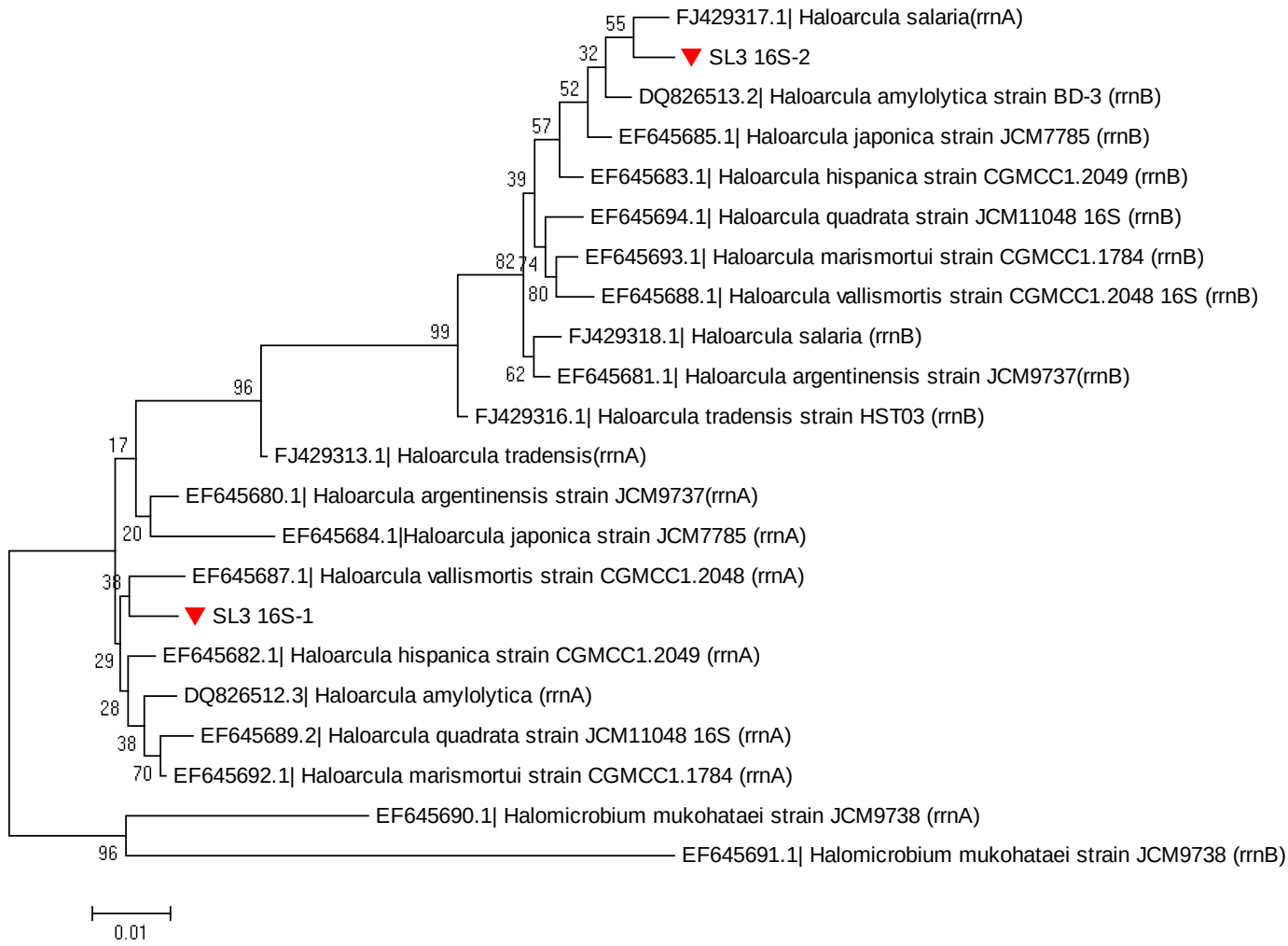


*Figure 24 Maximum likelihood tree utilizing the bootstrap method of the 16S ribosomal RNA gene for the genus Haloarcula. Both polymorphic operons found in the SL3$^T$ strain wee utilized for the rendering of the tree.*

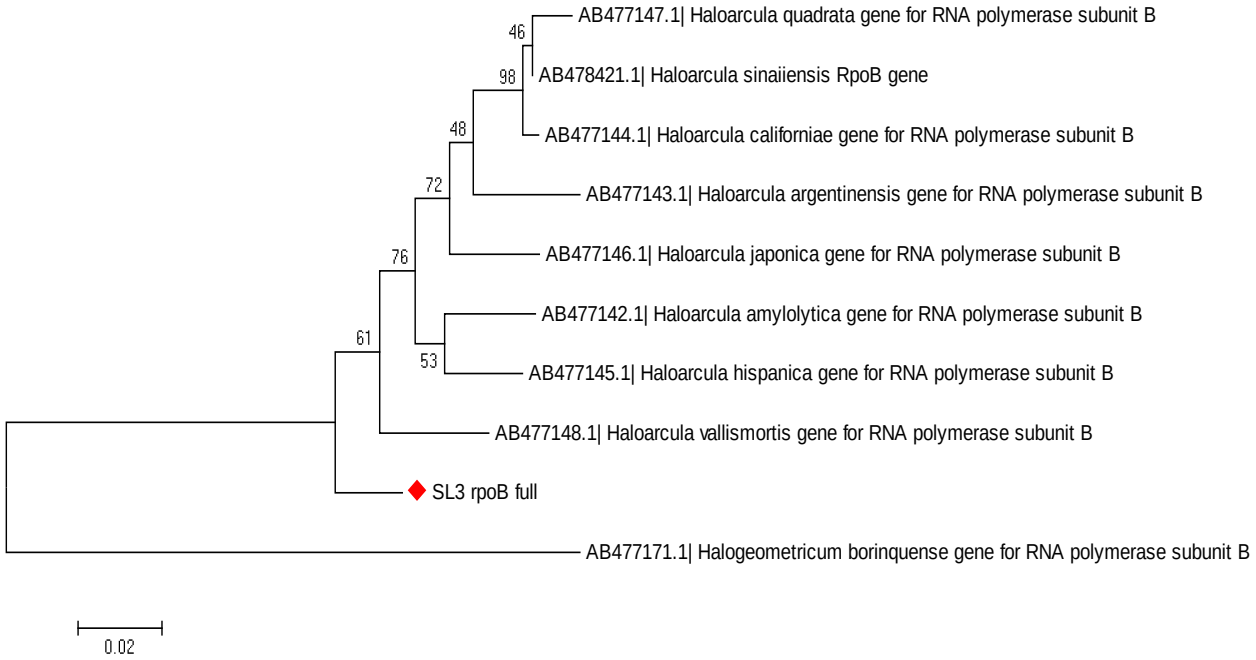


*Figure 25 Maximum likelihood phylogenetic tree of the genus Haloarcula using the bootstrap method to compare the full gene of the B subunit of the RNA polymerase.*

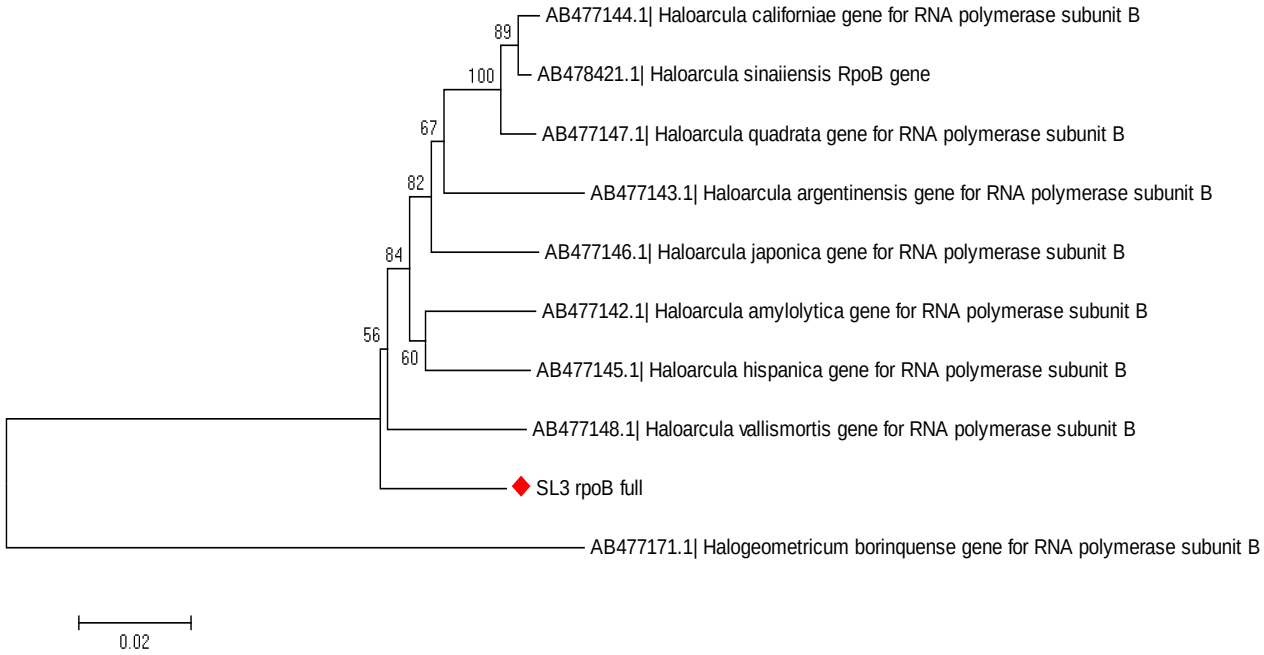


*Figure 26* Neighbor-joining phylogenetic tree of the *genus Haloarcula* using the bootstrap method to compare the full gene of the B subunit of the RNA polymerase.

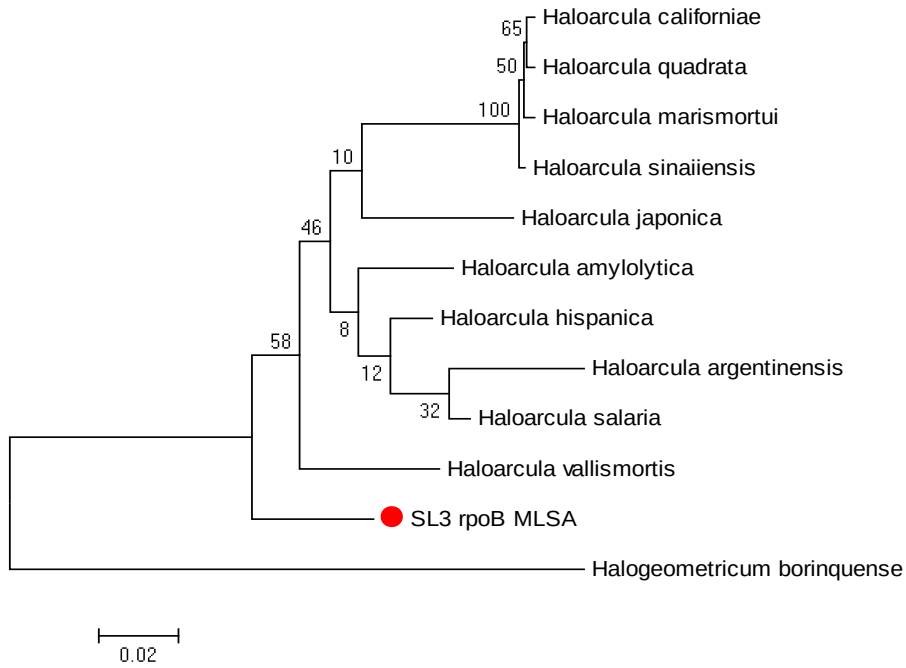


*Figure 27 Maximum likelihood phylogenetic tree of the genus Haloarcula using the bootstrap method to compare MLSA sequences which contain concatenated sections of the rpoB, ppsA, and atpB genes, respectively.*

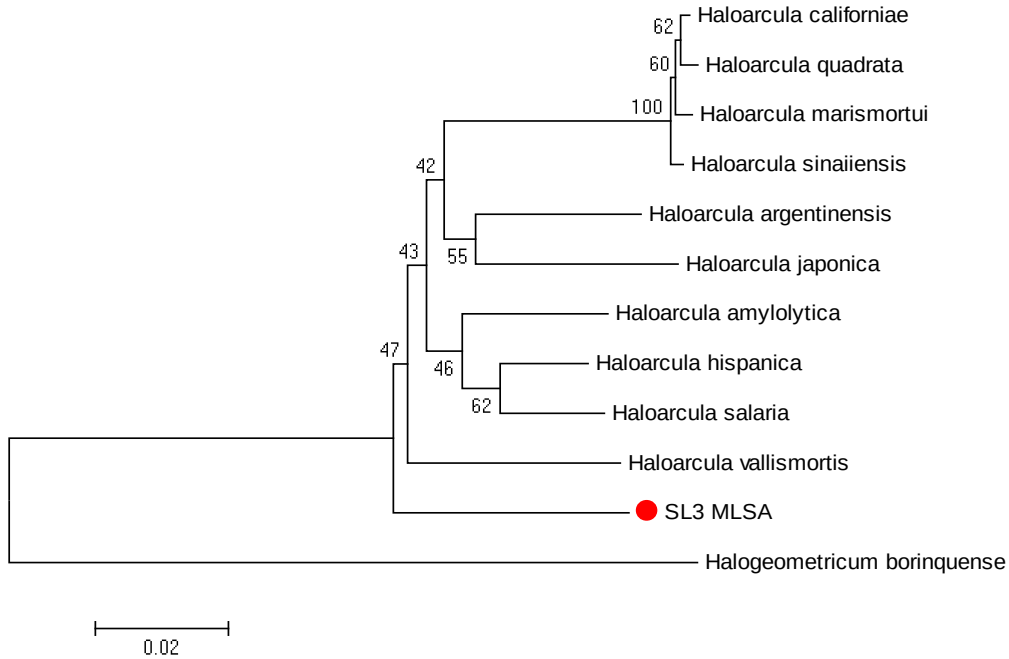


*Figure 28* Neighbor-joining phylogenetic tree of the *genus Haloarcula* using the bootstrap method to compare MLSA sequences which contain concatenated sections of the *rpoB, ppsA,* and *atpB* genes, respectively.

# *Chapter 3: Halorubrum tropicale sp. nov.*, a halophilic Archaea isolated from the Solar Salterns in Cabo Rojo, Puerto Rico.

**Summary**

An extremely halophilic, pleomorphic Archaea was isolated from the solar salterns of Cabo Rojo, Puerto Rico. This bright red pigmented Archaea was designated $V5^T$ and was subjected to biochemical, morphological, genotypic, and phylogenetic taxonomical studies. Strain $V5^T$ is an extremely halophilic Archaea which needs at least 15% NaCl for growth (20-25% optimum), 2% $Mg^+$ (4% optimum), has a pH range of 6.5-9.0 (7.5 optimum), and grows at temperatures of 37-55ºC (optimum 45-50 ºC). Phylogenetic analysis of the 16S gene revealed that $V5^T$ was most closely related to *Halorubrum distributum* and *Halorubrum terrestre* (99% similarity each). The entire genome was sequenced and presented a GC content of 67.6%. ANI demonstrated 88.36% similarity to *Halorubrum litoreum*, below the 94%-95% margin. Overall, phenotypic, biochemical, genotypic and phylogenetic analysis presented in this study demonstrate that strain $V5^T$ should be placed as a new species of the genus *Halorubrum*. The name *Halorubrum tropicale* sp. nov. is proposed for this novel species, with $V5^T$ (CECT 9000) as the type strain.

The solar salterns of Puerto Rico in Cabo Rojo have been subjected to many studies in diversity, yielding the isolation of many previously unknown organisms, from eukaryotes such as yeast to prokaryotes such as archaea and bacteria (Díaz-Muñoz & Montalvo-Rodríguez, 2005; Montalvo – Rodríguez et al, 1998; Soto-Ramírez et al, 2008). Recently, a new study on diversity has been performed which utilized glycerol and pyruvate and sole energy sources. Many isolates were obtained as a part of this study, one of these isolates belonging to the genus *Halorubrum.* Members of this genus were first thought to belong to the genus *Halobacterium* until phylogenetic analysis of the 16S rRNA gene was constructed and the genus *Halorubrum* was created (McGenity & Grant, 1995). This genus contains the highest number of species in the family *Halobacteriaceae*,

with a total of 31 species at the time of publication (Parte, 2014). This genus has also been studied due to the high rate of lateral gene transfer it undergoes, making populations of *Halorubrum* behave like populations of sexual organisms in terms of the frequency with which alleles recombine (Papke et al, 2004). These populations of *Halorubrum* have been found in very different saline environments, including Antarctica, fermented foods, soda lakes in China, Californian sea salt, and, as of now, the Caribbean (Feng et al, 2005; Franzmann et al, 1988; Yim et al, 2014).

The $V5^T$ strain was isolated from the solar salterns of Cabo Rojo, PR. These salterns are manmade crystallizer ponds that pump seawater then evaporate it for the production and commercial use of sea salt. $V5^T$ strain was isolated through filtering of the saltern pond water through a 0.2μm filter. The filter was subsequently submerged and shaken in 25mL of artificial seawater: 150g/L NaCl, 23g/L $MgSO_4$, 20g/L $MgCl_2$, 7g/L KCl. This recipe is a modification to the original recipe which can be found in the Halohandbook (Dyall-Smith, 2006). The artificial seawater solution with resuspended cells was then added to agar plates that contained natural saltern pond water diluted to a 20% (w/v) NaCl concentration, 5g/L of sodium pyruvate, 5g/L of yeast extract, 5mL of 1M Tris-Cl per liter of medium, and 20g/L of agar. The medium was adjusted to a pH of 7.4 using NaOH. The plates were incubated at 40ºC for 2 weeks. Colonies were transferred and subsequently isolated using quadrant streak method to undergo further biochemical, morphological, and phylogenetic analyses.

Cell morphology was determined utilizing Gram staining and phase contrast. Gram stains were performed with smears fixed in 5% acetic acid (Dussault, 1955). Optimal growth tests for $V5^T$ were done using the media containing the artificial seawater recipe mentioned above, 5g/L of yeast extract and 5g/L of sodium pyruvate. For the NaCl growth range medium was supplemented with 0%, 5%, 10%, 12%, 15%, 18%, 20%, 23%, 25%, 28%, 30% (w/v). Optimum temperature

was tested at 10ºC, 20ºC, 25ºC, 30ºC, 38ºC, 40ºC, 45ºC, 50ºC, 55ºC, and 60 ºC. Optimum $Mg^{2+}$ range media was supplemented with 0%, 0.5%, 1%, 2%, 4%, 6%, 8%, 10% (w/v). Optimum pH was tested at 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0. Strain $V5^T$ is a gram-stain positive, pleomorphic coccus. Colonies are bright red, smooth and translucent. Growth was observed at 15-30% NaCl (20-25% optimal), 37-55ºC (45-50°C optimal), 2-10% $Mg^{2+}$ (4% optimal), and at a pH of 6.5-9.0 (7.5 optimal). These conditions were used for subsequent tests.

Biochemical tests were performed utilizing the requirements for descriptions of new taxa published by Oren (1997). Presence of catalase and oxidase were done by adding 2-3 drops of 3% hydrogen peroxide solution and by oxidase reagent, respectively. Utilization of single carbon source and acid production were performed by adding 5g/L of the following: xylose, sorbitol, pyruvate, mannitol, glycerol, glucose, galactose, lysine, ribose, and raffinose. Media was also supplemented with artificial seawater (20% total salts) 0.01% (w/v) of yeast extract, 0.5g/L of $NH_4Cl$, and 0.05g/L of $KH_2PO_4$ (Tomlinson & Hochstein, 1976). Hydrolysis of Tween 80 was tested by adding 0.1% (w/v) of $CaCl_2$ and 1% (w/v) of Tween 80 to solid media, hydrolysis was verified by halos surrounding colonies (Gutierrez & Gonzalez, 1972). Gelatin hydrolysis was performed in media containing 12% (w/v) gelatin (Gutiérrez & González, 1972). Starch hydrolysis was tested adding 10g/L of potato starch in solid medium, hydrolysis was verified by adding iodine to the plates (Nicolaus et al, 1999). Skim milk was added to medium to test for casein hydrolysis, hydrolysis was verified by clear halos around colonies. Indole formation was determined using 1% tryptone in medium, formation of indole was determined using Kovac's reagent (Montalvo-Rodríguez et al, 1998). Nitrate reduction and gas production was performed in anaerobic chamber (Mancinelli & Hochstein, 1986). Anaerobic growth was determined with 5g/L of L-arginine, this test was also done within an anaerobic chamber (Hartmann et al, 1980). The following antibiotic

discs were placed on culture media to determine resistance/susceptibility: bacitracin, penicillin, novobiocin, erythromycin, chloramphenicol, sulfamethoxazole/trimethoprim, vancomycin, ampicillin, tetracycline, and rifampin. A summary and comparison with other *Halorubrum* type species of these tests is demonstrated in Table 18.

Phylogenetic analysis was done for the 16S gene, *rpoB* gene and by multilocus sequencing analysis (MLSA) (Minegishi et al, 2010; Papke et al, 2011). DNA extraction was performed by adding cells to sterile deionized water, followed by heating to 70ºC for 10min; this protocol is described by Dyall-Smith (2006). Multilocus sequencing analysis was performed using 3 genes: *ppsA, rpoB,* and *atpB*. These genes were chosen and amplified according to Fullmer et al (2014). The purified PCR products were sent to Macrogen USA for sequencing. Sequences were curated using Bioedit, alignments were performed using Clustal W and neighbor-joining trees were performed with Mega (Hall, 1999; Tamura et al, 2011). Preferences for the trees were the following: 2000 bootstraps, p-distance model, and pairwise deletion. Sequences for related taxa from the genus *Halorubrum* were taken from Genbank, and initial relatedness was determined by BLAST (Basic Local Alignment Search Tool). The whole genome was sequenced, assembled, and annotated by MRDNA Lab facilities. Average nucleotide identity was then performed in order to verify similarity between species of *Halorubrum* (http://www.ezbiocloud.net/ezgenome/ani).

In silico analyses, using BLAST, were performed for the both 16S genes (1449bp each) and for the *rpoB* gene (1830bp). In all cases, strain V5$^T$ was closely related to the genus *Halorubrum.* For the 16S genes, strain V5$^T$ was most closely related to *Halorubrum terrestre* and *Halorubrum distributum,* respectively (99%). As for the *rpoB* gene, the most related organism was *Halorubrum xinjiangense* (97%). Phylogenetic analysis for both genes using the neighbor-joining algorithm demonstrated that strain V5$^T$ is very closely related to other *Halorubrum* species. Much

like what was demonstrates by BLAST, relatedness of strain V5$^T$ to other species was very similar through most of the genus. Relatedness was confirmed by neighbor joining and maximum parsimony algorithm. The average nucleotide identity was determined by comparing all other available *Halorubrum* genomes with strain V5$^T$. The proposed cut-off for species boundary was at 95-96% (Richter & Rosselló-Mora, 2009). All species of *Halorubrum* were between 81-88% similar, the most similar species was *H. litoruem* (88.36%).

Due to the overall results of phenotypic, biochemical and phylogenetic analyses, it was determined that strain V5$^T$ is a new species within the genus *Halorubrum*. The name *Halorubrum tropicale sp. nov.* is proposed for this novel species.

**Description of *Halorubrum tropicale***

Cells are Gram-stain positive, motile, facultative, anaerobes, pleomorphic cocci. Cells present a bright red pigmentation. Colonies are translucent and full. Cells lyse in distilled water. A minimum of 15% (w/v) NaCl is required in order to observe growth. Concentrations of NaCl allowing growth ranged from 15 -30% (Optimum 20%). Concentrations for growth of $Mg^{2+}$ ranged from 2%-10% (optimum 4%), growth at was observed at temperatures ranging from 37ºC-55ºC (optimum 45ºC) and at pH ranging from 6.5-9 (optimum 7.5). Strain V5$^T$ was catalase and oxidase positive. Acetate, glucose, maltose, xylose, mannitol, pyruvate, sorbitol, and glycerol were all utilized by strain V5$^T$ as sole carbon sources. Acid was produced from maltose, xylose, and glycerol. Starch hydrolysis was observed, although casein, gelatin, and tween hydrolyses was not observed. Tryptophan did not induce indole formation. V5$^T$ was able to grow anaerobically with L-arginine and nitrate, although gas production was not observed. V5$^T$ was shown to have susceptible to

novobiocin, sulfamethoxazole/trimethoprim and bacitracin but was resistant to penicillin, erythromycin, chloramphenicol, vancomycin, ampicillin, tetracycline, and rifampin. The DNA GC content was 67.6%. The $V5^T$ type strain was deposited at CECT 9000.

*Table 10* Summary of biochemical and phenotypic characteristics within the genus *Halorubrum*. Species included: 1) $V5^T$; 2) *H. terrestre;* 3) *H. californiense;* 4) *H. xinjiangense;* 5) *H. coriense;* 6) *H. aidingense;* 7) *H. laminariae.*

| **Characteristic** | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
|---|---|---|---|---|---|---|---|
| **Cell shape** | Pleomorphic | Pleomorphic | Rods | Rods | Pleomorphic rods | Rods | Rods |
| **Motility** | + | + | + | + | + | + | + |
| **Range for Growth:** | | | | | | | |
| **NaCl (%. w/v)** | 15-30 | 15-30 | 15-30 | 12-30 | 12-30 | 10-25 | 10-28 |
| **$Mg^{2+}$ requirement** | + | ND | + | - | + | - | - |
| **Temperature Optimum (°C)** | 45 | 37-45 | 37 | 40 | 50 | 40-42 | 30 |
| **Utilization of** | | | | | | | |
| **Sorbitol** | + | + | ND | - | ND | - | + |
| **Acetate** | + | ND | - | ND | ND | + | + |
| **Glycerol** | + | + | ND | - | + | + | + |
| **Fructose** | - | + | - | + | + | + | - |
| **D-Glucose** | + | + | + | + | + | + | + |
| **Nitrate Reduction** | + | - | + | ND | - | + | - |
| **Hydrolysis of** | | | | | | | |
| **Starch** | + | - | - | - | + | - | - |
| **Tween 80** | - | ND | - | - | ND | + | - |
| **Gelatin** | - | - | - | ND | - | - | - |

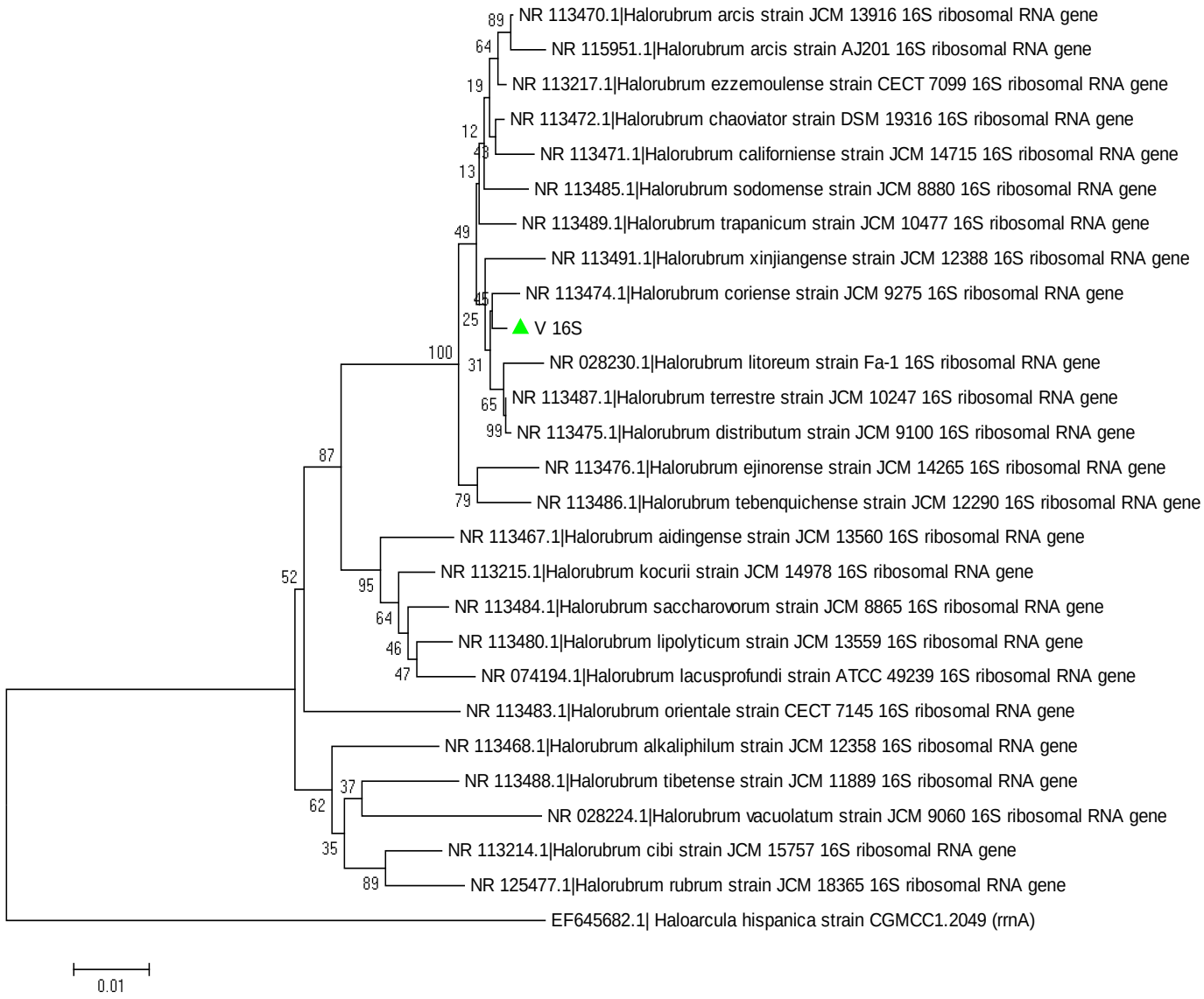


*Figure 29* Neighbor-joining phylogenetic tree of the 16S small subunit ribosomal RNA gene of the *genus Halorubrum*.

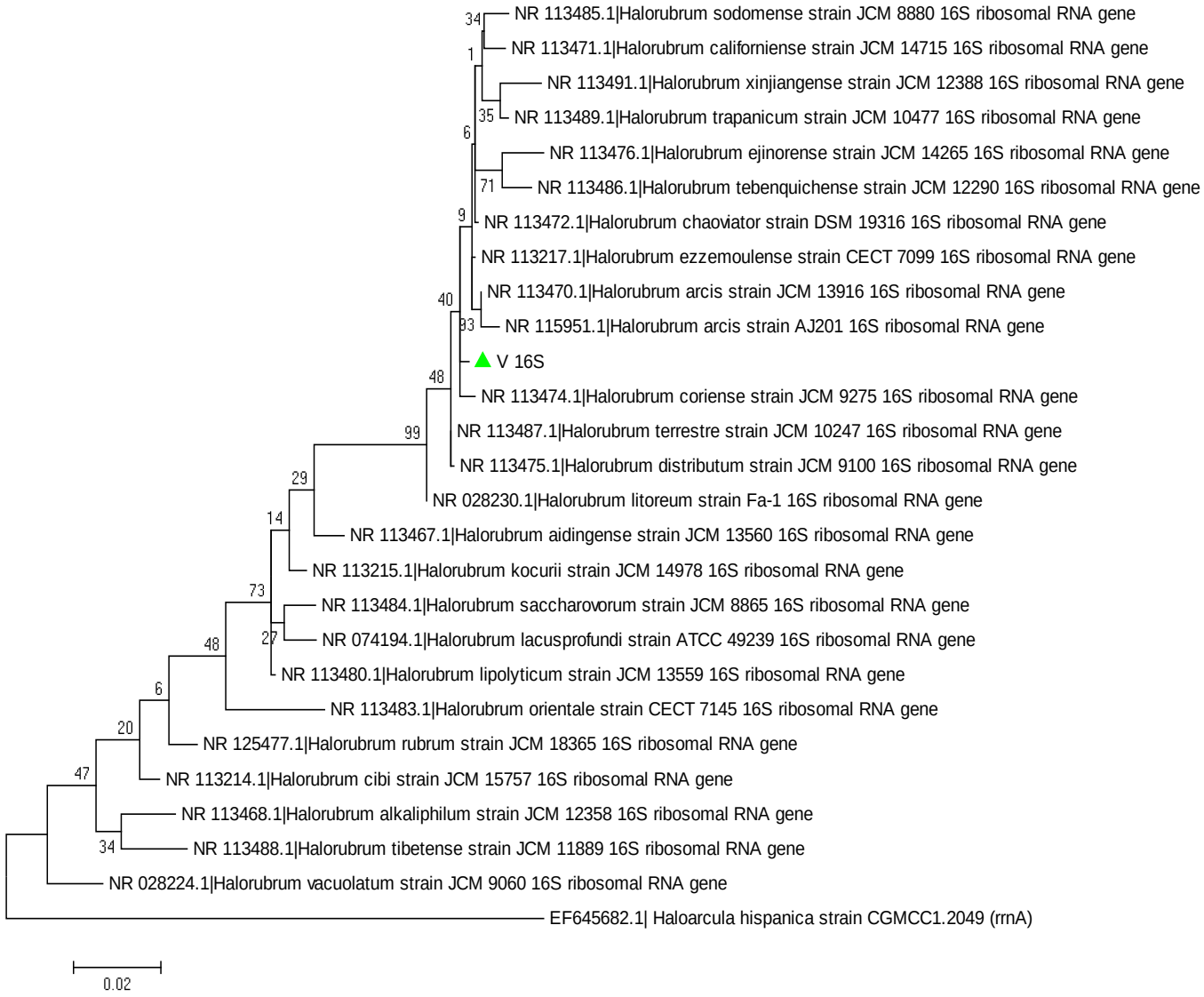


*Figure 30 Maximum likelihood phylogenetic tree of the 16S small subunit ribosomal RNA gene of the genus Halorubrum.*

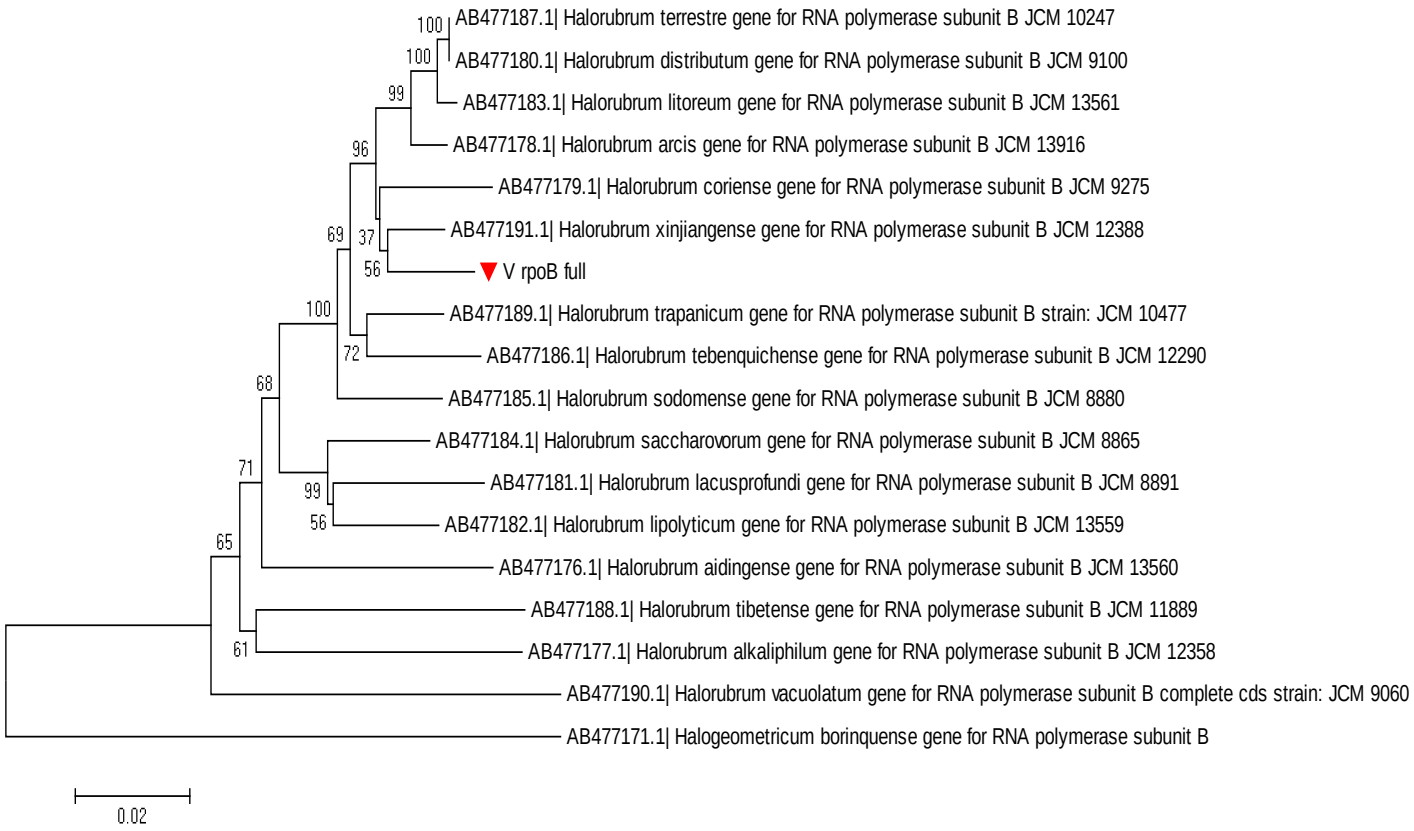


*Figure 31* Neighbor-joining phylogenetic tree of the full B subunit of the RNA polymerase gene for the genus *Halorubrum.*

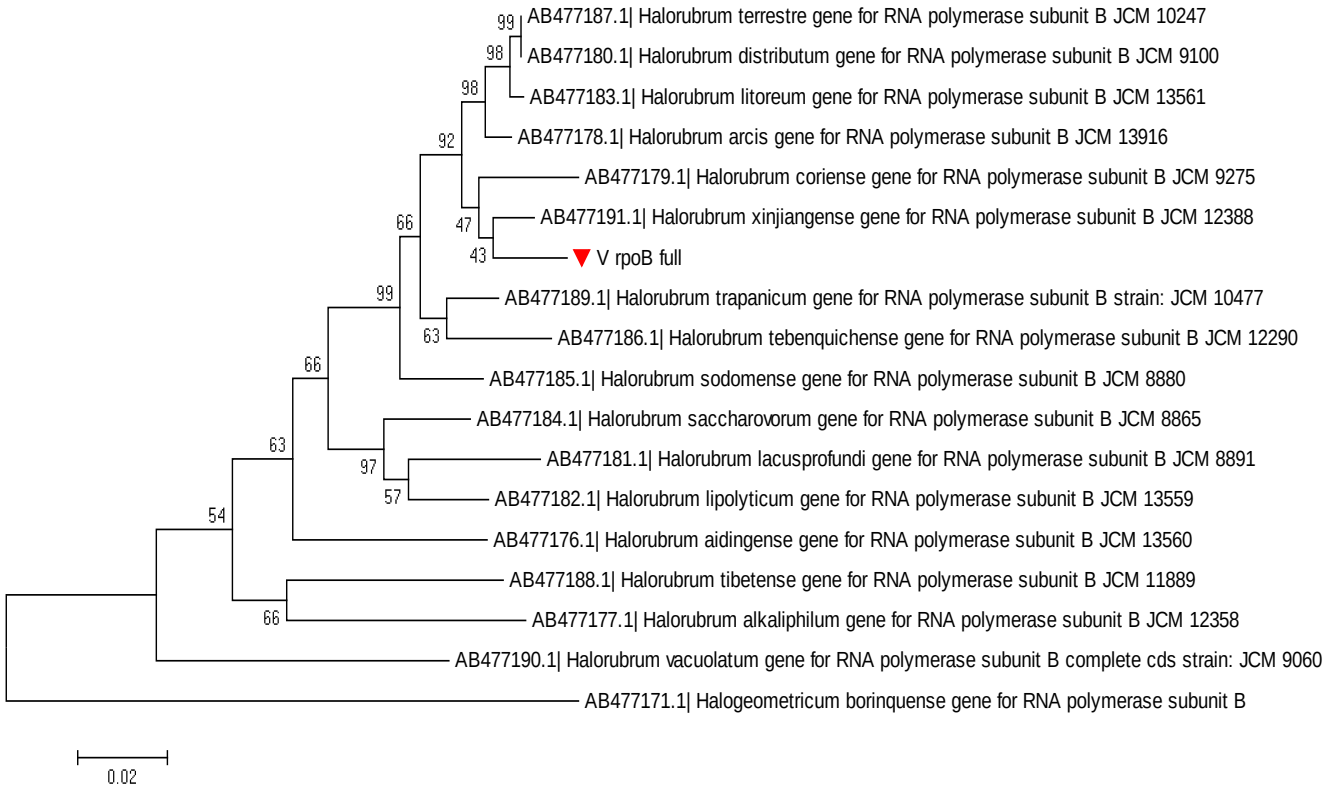


*Figure 32 Maximum likelihood phylogenetic tree of the full B subunit of the RNA polymerase gene for the genus Halorubrum.*

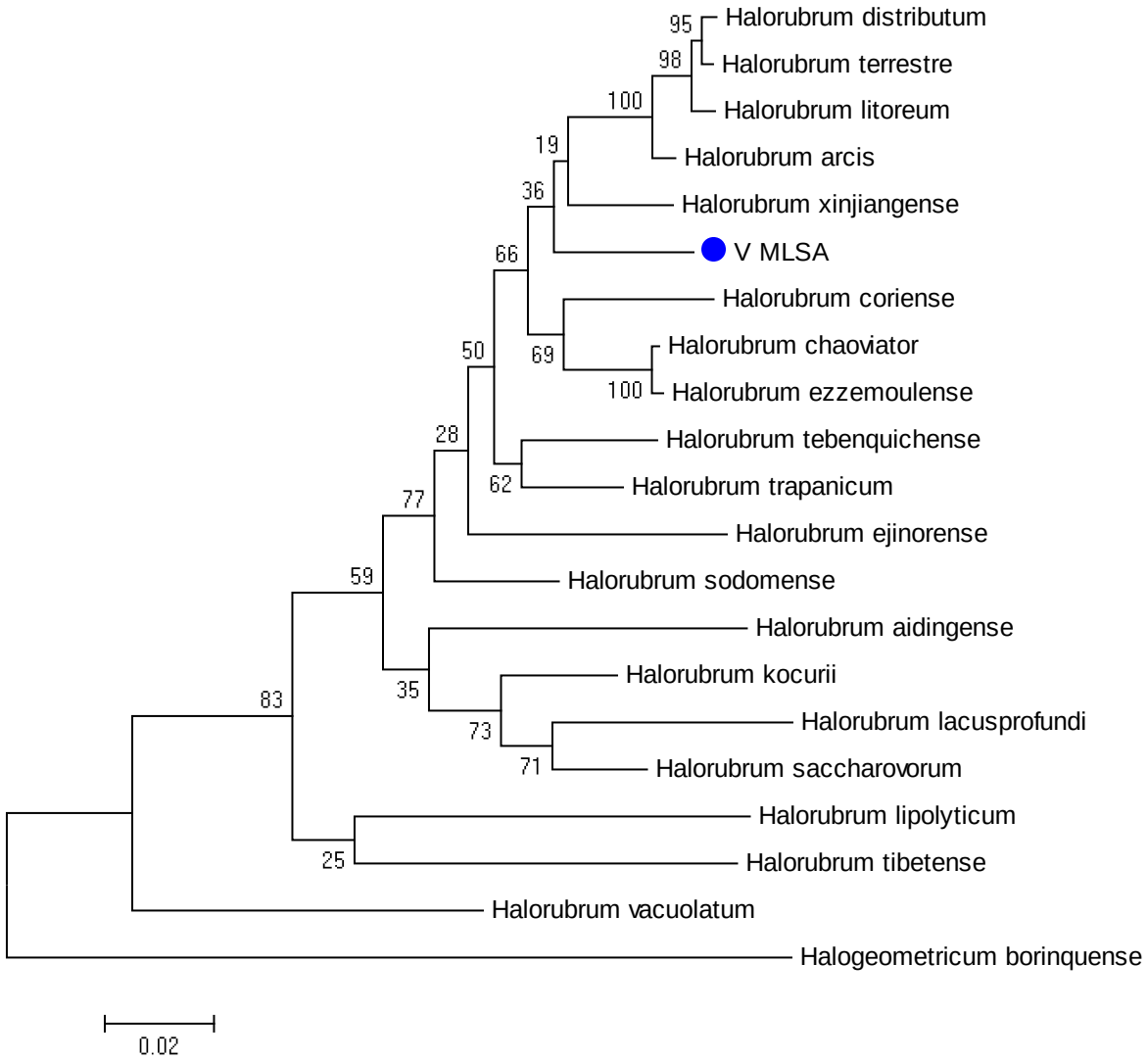


*Figure 33 Maximum likelihood phylogenetic MLSA tree containing concatenated sections of the rpoB, ppsA, and atpB genes, respectively for the genus Halorubrum*

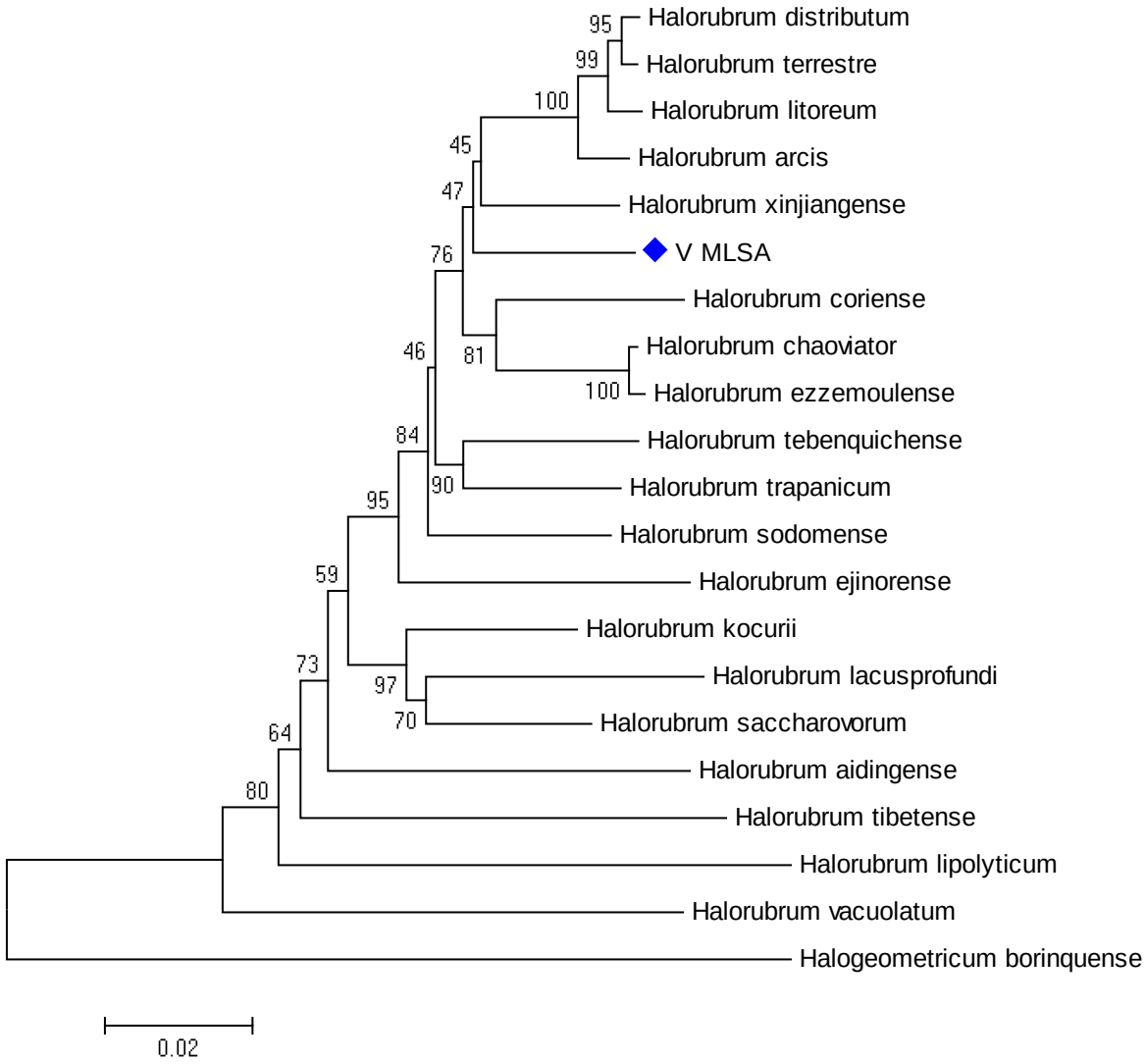


*Figure 34* Neighbor-joining phylogenetic MLSA tree containing concatenated sections of the *rpoB, ppsA,* and *atpB* genes, respectively for the *genus Halorubrum*.

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