A survey of halophilic aerobic prokaryotes associated to the leaf surface of *Avicennia germinans* at the Solar Salterns of Cabo Rojo, Puerto Rico

by

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Abstract

Mangroves represent the major component of coastal marine-lagoon ecosystems in the tropics. In the Caribbean, *Avicennia germinans* (black mangrove) is one of the dominant plant species within this ecosystem. In Puerto Rico, Cabo Rojo has one of the most important forest of *A. germinans,* a species threatened by anthropogenic activity. Mangroves are highly productive sites due to the microorganisms that inhabit their sediments and rhizosphere (Bashan, 2000). Few studies concerning the relationship between mangroves and the microbial community have been reported. The main objective of this research was to determine the prokaryotic diversity associated to the leaf surface of the black mangrove by culture-dependent and culture-independent techniques. Several prokaryotic groups were isolated from this extreme environment representing four bacterial genera: *Staphylococcus, Pontibacillus, Halomonas,* and *Halobacillus.* Two isolates were classified as new species; *Halomonas avicenniae* sp. nov. (MW2a^T) and *Halobacillus puertoriconensis* sp. nov. (MS10^T). Results from non-culture dependent methods such as 16S rDNA environmental clone libraries suggest that the leaf surface of *A. germinans* has high prokaryotic diversity. A total of 1712 clones were obtained and 95 clones were analyzed *in silico* to classify them into Operational Taxonomical Units (OTU). Most of the OTU's belonged to the bacteria domain having a close relationship to the Bacteroidetes and Proteobacteria (alpha, beta and gamma) groups. Several OTU's such as G58, G8 and G56 were also closely related to the extremely halophilic Archaea. The isolates obtained in this study will increase our understanding about plant-microbial interactions and about the relative contribution of their microenvironment. Furthermore, knowledge about the identity of those microorganisms, using the 16S rDNA technique,

will allow the potential development of physiological and biochemical applications in biotechnology and astrobiology. This study is the first attempt to study the prokaryotic diversity present on the leaf surface of *Avicennia germinans* from the Caribbean.

Resumen

Los mangles representan uno de los mayores componentes del ecosistema costero-marino en el trópico. En el Caribe, *Avicennia germinans* (mangle negro) es una de las especies de plantas dominantes dentro de este ecosistema. En Cabo Rojo, Puerto Rico existe uno de los bosques más importantes de *A. germinans,* especie amenazada por actividades antropogénicas. Los mangles son considerados uno de los ecosistemas mas productivos junto a los microorganismos que habitan en la rizoesfera y los sedimentos (Bashan, 2000). Pocos estudios se han enfocado en la relación de la comunidad microbiana con los mangles. El objetivo principal de este estudio era determinar la diversidad procariótica asociada a la superficie de la hoja de mangle negro utilizando técnicas dependientes e independientes de cultivo. Varios grupos procariotas fueron aislados de este ambiente extremo donde se cultivaron cuatro géneros del dominio Bacteria: *Staphylococcus, Pontibacillus, Halomonas* y *Halobacillus. Halomonas avicenniae* sp. nov. ($MW2a^T$) and *Halobacillus puertoriconensis* sp. nov. ($MS10^T$) fueron dos aislados clasificados como especies nuevas. La diversidad procariota asociada a la superficie de mangle negro también fue analizada por métodos independientes de cultivo como, por ejemplo, genotecas ambientales amplificando la región 16S rDNA. Un total de 1712 clones fueron obtenidos de los cuales 95 clones fueron analizados *in silico* para clasificarlos en Unidades Taxonómicas Operacionales (OTU). La mayoría de las secuencias de OTU's pertenecían al dominio Bacteria asociados a los grupos Bacteroidetes y Proteobacterias (alpha, beta y gamma). Varios OTU's tales como G58, G8 y G56 estaban estrechamente relacionados a los halofílicos extremos del dominio Archaea. Los aislados en este estudio son importantes para conocer su interacción con el

mangle y su contribución a este microambiente. Conociendo la identidad de estos microorganismos, mediante la técnica del 16S rDNA, podemos aumentar nuestro conocimiento acerca de las aplicaciones fisiológicas y bioquímicas en el campo de la biotecnología y astrobiología. Este estudio representa el primer esfuerzo en estudiar la diversidad procariota presente en la superficie de la hoja de *Avicennia germinans* en el Caribe.

Dedication

This thesis is dedicated to my husband and my little girl Gabriela del Mar for being a support in my educational career. Thanks for being part of my life and being there in the good and bad moments. They were an inspiration to reach all my goals and desires as a mother, wife, and student, and finally as a professional scientist in the biological sciences.

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Introduction

Mangrove forests are known to support a diverse community of invertebrates and vertebrate species. Its intermediate location, between land and sea, provides a variety of suitable habitats to sustain such diversity. They serve as feeding, spawning, and reproductive areas for numerous economically and ecologically important marine species. Mangroves have different growth forms, i.e. as a shrub and palm, and these types also share the ability to live in salt water. There are about 65 recognized species of mangroves worldwide and they belong to 20 families (Feller and Sitnik, 1996). The richest mangrove communities occur in tropical and sub-tropical areas where mountain altitudes are greater than 700m in proximity to the coast. In terms of its biological value, mangroves provide food and shelter for estuarine fauna, particularly for commercially important crustaceans such as *Uca* spp. and other crabs.

The ecological importance of mangrove forests includes protection of coastlines from erosion, storm damage, and wave action (Feller and Sitnik, 1996). They prevent shoreline erosion by acting as buffers and catching alluvial materials, therefore stabilizing land elevation by sediment accretion that balances sediment loss. Vital coral reefs and sea grass beds are also protected from damaging siltation.

Unfortunately, anthropogenic activities such as trampling, oil pollution, altered freshwater flow regimes, and excess nutrient inputs are known to disturb the natural processes within this ecosystem. One of the most disturbed mangrove ecosystems is the one dominated by *Avicennia germinans*. This mangrove is distributed along the American coastal areas, particularly in tidal areas with salty or brackish waters.

They grow in tropical and subtropical dry, moist, and wet life zones, with a broad range of precipitation regimes (i.e. 800 to 7,000 mm y^{-1}) (Jiménez and Lugo, 1985). The species is highly polymorphic in terms of both, ecological function and leaf morphology. Large variation such as leaf morphology exists among populations growing under different conditions. Leaf size is one of the most variable parameters and this variation has been attributed to soil salinity (Jiménez and Lugo, 1985).

In spite of their importance, there are few studies about the composition of microbial communities associated with mangroves. Prokaryotic diversity seems to be in the mangrove rhizosphere as a major component assuring mangrove productivity (Sherinan, 2001). However, the diversity associated to leaf surfaces is just being explored. Sherinan (2001) studied the cyanobacterial mat community on the trunk and branches of black mangrove and found that those communities have morphological and physiological adaptations to UV radiation. A study showed that the diazotrophic cyanobacterium *Microcoleus chthonoplastes* improved N_2-fixation and nitrogen incorporation in black mangrove seedlings (Bashan *et al*., 1998). Ananda and Shridhar (2002) found high diversity of endophytic fungi in the roots of mangrove species, suggesting that studying tropical areas such as India and Puerto Rico could increase our understanding about microbiological diversity associated to mangroves.

In Puerto Rico, there are few studies concerning the relationship between mangroves and the microbial community. Inventories of microbial diversity of microorganisms associated with mangroves could lead us to the discovery of new species, new ecological (symbiotic) processes to understand their metabolism, and how it

could be applied to biotechnology and microbial ecology. A halophilic community is expected to be associated to the leaf surface of black mangrove due the saline microenvironment present on the leaf surface. The present study leads us to determine the halophilic aerobic prokaryotic diversity associated to the leaf surface of *Avicennia germinans* and *Rhizophora mangle* in the Solar Salterns of Cabo Rojo, Puerto Rico by culture-dependent and culture-independent approaches. Species associated to the genera *Halobacillus, Pontibacilllus*, *Staphylococcus* and *Halomonas* were found. The halotolerant and halophilic nature of several strains suggests that they probably are components of the community of the leaf surface of *A. germinans*. By culture-independent methods, OTU's related to Bacteroidetes group, Alpha-Proteobacterias, Beta-proteobacterias, Gamma-Proteobacterias, and from the Archaea domain, Euryarchaeota group were found. This information will provide further insight about the potential prokaryotic communities involved in the metabolic processes in mangrove ecosystems.

Literature Review

I. Hypersaline Environments and their communities

A diversity of microorganisms can live and interact in hypersaline environments according to the ecophysicochemical conditions that a particular ecological niche can offer. Representatives of the three life domains: Archaea, Bacteria, and Eucarya can inhabit and coexist to accomplish their roles in such ecosystems. Most halophiles can inhabit in very extreme environments such as salt lakes and salt evaporation ponds. An example of such environments can be found in the two largest hypersaline lakes the Great Salt Lake and the Dead Sea. Other are small evaporation ponds located near coastal areas are the Solar Lake, Gavish Sabkha, Ras Muhammad Pool near the Red Sea coast, Guerrero Negro on the Baja California coast, Lake Sivash near Black Sea, and Sharks Bay in western Australia. Also hypersaline evaporation ponds are located in the Deep Lake, Organic Lake and Lake Suribati found in Antarctica. Soda brines include the Wadi Natrum lakes of Egypt, Lake Magadi in Kenya, and the Great Basin lakes as Mono Lake, Owens lake, Searles Lake and Big Soda Lake in the United States (DasSarma and Arora, 2001).

Eukaryotic green algae of the genus *Dunaliella* (*D. salina*, *D. parva*, and *D. viridis*) as well as other eukaryotic green algae such *Asteromonas gracilis* (Ben-Amotz and Grunwald, 1981; Wegmann *et al.*, 1980; Vreeland and Hochstein, 1993) have been found both in the Dead Sea and in the Great Salt Lake.

In addition to natural hypersaline lakes, numerous artificial solar salterns have been constructed worldwide such as the marine solar saltern in Korea, the solar salterns in Cabo Rojo, Puerto Rico, and Solar Lake, Sinai in Egypt. Other saline environments

occur in subterranean evaporated deposits and deep-sea basins. Those deep-sea basins have been found in the Red Sea and the Gulf of Mexico (MacDonald *et al*., 1990).

In such environments scientists have been studying numerous microorganisms that possess various inner mechanisms to tolerate high salt concentrations. Halophiles are distinguished by their requirement of hypersaline conditions for growth. They are classified as: slight halophiles growing optimally at 0.2-0.85molL^{-1} (2-5%) NaCl; such as *Methanosalsum zhilinae* (Boone and Baker 2001), moderate halophiles grow optimally at 0.85-3.4 molL^{-1} (5-20%) NaCl, such as *Halomonas almeriensis* isolated from Cabo de Gata, Almeria, south east Spain (Martínez-Checa *et al*., 2005). Extreme halophiles grow optimally above 3.4-5.1 molL^{-1} (20-30%) NaCl, such as *Halogeometricum borinquense* isolated from solar salterns in Cabo Rojo, Puerto Rico (Montalvo-Rodríguez *et al*., 1998). Many halotolerant microorganisms can grow in a wide range of salt. For example *Halobacillus yeomjeoni* was isolated from marine solar salterns in Korea can grow from 0.5% to 21%(w/v) NaCl (Yoon *et al*., 2005).

Most halophilic microorganisms are highly adapted, both externally and internally, to withstand the osmotic pressure of salt ions. Halophiles generally accumulate high solute concentrations within the cytoplasm to prevent loss of cellular water (Vreeland and Hochstein, 1993). They can occur at such high cell densities that they cause brines to turn red which further encourages evaporation by trapping solar radiation. The red color is caused by carotenoid pigments that protect cells from the harmful effects of ultraviolet light (UV) (McGenity *et al*., 2000). Such brines have been found in the Dead Sea and Great Salt Lake where a diversity of halophilic microorganisms live. Aside

from the green micro-algae (i.e., *Dunaliella salina*), archaea such as *Halogeometricum borinquense* (Montalvo-Rodríguez *et al*., 1998) and bacteria such as *Salinibacter ruber* (Antón *et al*., 2002), can inhabit water that is up to 10 times more saline than ordinary seawater (approximately 30 percent salt content). Such microorganisms have been isolated from the Great Salt Lake, from Owens Lake, from the Dead Sea, and from saltines (Grant and Larsen, 1989). Unicellular species *Aphanothece halophytica*, which was isolated by Brock (1976) and Yopp *et al* (1978), is a cyanobacterium reported from hypersaline environments such as the Great Salt Lake, Dead Sea, and Solar Lake. Teske *et al* (1998) studied the sulfate-reducing bacteria in the surface layer of the hypersaline cyanobacterial mat of the Solar Lake. They found in the oxic surface layer 10^6 and 10^7 cultivable sulfate- reducing bacteria ml^{-1} and in the in the mat layers below, the filamentous sulfate-reducing *Desulfonema* bacteria were found. Another sulfate-reducing bacterium *Desulfocella halophila* was isolated from sediments of the Great Salt Lake (Brandt *et al*., 1999). Phototrophic bacteria occur beneath the cyanobacterial layers in anaerobic but lighted zones in hypersaline microbial mats (Satyanarayana *et al*., 2005). Species of *Acinetobacter*, *Alteromonas*, *Deleya*, *Flavobacterium*, *Marinomonas*, *Pseudomonas* and *Vibrio*, aerobic gram-negative organotrophic bacterias, are abundant in brines of medium salinity. Also aerobic heterotrophs such as species of the genera *Marinococcus, Sporosarcina, Salinococcus* and *Bacillus* have been isolated from saline soils and salterns (Satyanarayana *et al*., 2005).

In Kebrit Deep, northern Red Sea, Eder and collegues (2001) found representatives of the halophilic genus *Halanaerobium* obtained from deep-sea, anaerobic brine pools. In alkaline brines of Lake Magadi and Lake Natron in the Rift Valley, and

Owens Lake in California communities of anoxygenic phototrophs belonging to the genera *Ectothiorhodospira* and *Halorhodospira* have been found (Grant *et al*., 1999; Jones *et al*., 1998). Numerous extreme alkaliphilic, moderate halophilic, benthic cyanobacteria were isolated from Lake Magadi such as *Synechocystis salina*, *Aphanothece stagnina*, *Chamaesiphon subglobosus*, *Rhabddoderma lineare*, *Synechococcus elongates*, *Phormidium ambiguum*, *Phormidium ambiguum*, *Phormidium foveolarum*, *Phormidium retzii*, *Oscillatoria splendida*, *Oscillatoria limnetica*, *Spirulina fusiformis,* and *S. laxissima* (Dubinin *et al*., 1995). Also, representatives of anoxygenic phototrophic purple bacteria of the genera *Ectothiorhodospira* and *Halorhodospira*, have been isolated from solar lakes, which oxidize hydrogen sulfide with intermediate extra-cellular sulfur deposition. A very low diversity have been reported for purple nonsulfur bacteria in such solar lakes, but the Lake Bongoria (Kenya) resides an alkaliphilic alpha-proteobacterium *Rhodobaca bogoriensis* which is a sole representative of purple nonsulfur bacteria described from soda lake environments (Milford *et al*., 2000). Also aerobic alkaliphiles such as members of the genus *Halomonas* (Duckworth *et al*., 2000) have been isolated from soda lakes as the Rift Valley. Other studies reveal the isolation of members of the genera *Pseudomonas* and *Stenotrophomonas*, typical aquatic bacterias such as *Aeromonas*, *Vibrio* and *Alteromonas* (Duckworth *et al*., 1996; Jones *et al*., 1998).

In the Siberian soda lakes and some lakes of the Kenyan Rift Valley aerobic sulfur-oxidizing bacteria of the genera *Thioalkalimicrobium* and *Thioalkalivibrio* have been reported (Sorokin *et al*., 2001). Alkaliphilic methane-oxidizing bacteria of the *Methylococcaceae* were isolated from moderately saline soda lakes in Tuva, Central Asia (Khmelenina *et al*., 1997). In the soda lake in the Kunkur steppe in Siberia and from Lake

Nakuru and Crater Lake in Kenya a diverse of lithotropic, nitritr-oxidizing bacteria (e.g. *Nitrobacter alkalicus*) of the alpha subdivision of Proteobacteria have been found (Sorokin *et al*., 1998). These bacteria oxidize reduced inorganic nitrogen to nitrate. As well as Gram-negative, aerobic Gram-positives with high G+C and low G+C have been found. Some representatives of the high G+C are the members of the genus *Dietzia* (Duckworth *et al*., 1998), and species of the genera *Arthrobacter* and *Terrabacter* (Duckworth *et al*., 1996). *Bacillus alcalophilus* is one of the low G+C content members species reported from the *Bacilli* taxon (Nielsen *et al*., 1994 and 1995).

In Lake Magadi, Lake Wadi Natrun, and Owens Lake, a diverse of haloalkaliphilic archaea of the family *Halobacteriaceae* have been reported (Tindall, 1984). Archaebacteria of the genus *Halobacterium, Haloferax* and *Haloarcula* are generally the dominant organisms in hypersaline brines and are also found in solar salterns.

Anaerobic microorganisms have been studied in such environments. Most of these anaerobic microorganisms perform biological process as sulfate reduction (sulfidogenesis). They are responsible for organic matter degradation and also generate alkaline conditions as a result of the transformation of sulfate to sulfide (Zavarzin *et al*., 1999). Zhilina *et al*. (1997) and Pikuta *et al*. (1997) isolated *Desulfonatronovibrio hydrogenovorans*, a member of the delta subclass of the Proteobacteria, in a drainage ditch at Lake Magadi and in the soda lakes of Tuva, respectively. Also, extremely alkaliphilic strains of methanogens were isolated from Lake Wado-el-Natrun, Egypt (Boone *et al*., 1986) and Lake Magadi (Zhilina and Zavarzin, 1994) and mixed samples from Tuva lakes (Zavarzin *et al*., 1999). Other methanotrophic methanogens were

isolated from several soda lakes related to the *Methanosarcinaceae* group. An example is *Methanohalophilus oregonense* isolated from anoxic aquifer near Alkali Lake from the south central Oregon in the United States (Liu *et al*., 1990).

A member of the *Halobacteroidaceae* within the Gram-positive *Natroniella acetigena* an obligately haloalkaliphilic acetogenic bacterium was detected in the Lake Magadi, Kenya (Zhilina *et al*., 1996). Also some acetogenic ammonifiers such as *Natronincola histidinovorans* (Zhilina *et al*., 1998) and the non-spore-forming bacterium *Tindallia magadiensis* (Kevbrin *et al*., 1998) were found in the Lake Magadi. Several bacterial isolates related to the genus *Pseudomonas* were reported from Maras salterns in the Peruvian Andes (Maturrano *et al*., 2006). Also, with culture-independent methods they found microorganisms related to the square archaeon *Haloquadra walsbyi* and others were related to *Halobacterium* sp. Within the Bacteria domain they found clones related to *Pseudomonas halophila*. A study conduced by Shailesh and Desai (2006) in the marine salterns near Bhavnagar, Gujarat, India reported strains related to the genera *Salinivibrio*, *Flavobacterium*, *Alcaligenes,* and *Chromohalobacter*. In another study in the Lake Chaka of China they employed culture-dependent and culture-independent techniques. Some isolates recovered were related to the genus *Halomonas* of the *Gammaproteobacteria* group, other were classified as *Firmicutes,* and another to *Actinobacteria* (Hongchen *et al*., 2006). Also, archea isolates were closely affiliated to the genus *Haloarcula*. The predominant clone sequences were affiliated with the *Bacteroidetes* and low G+C Gram-positive bacteria.

II. Mangroves and microbe interactions

Four species of mangroves can be found in the shores and adjacent islands of Puerto Rico: *Rhizophora mangle*, *Avicennia germinans*, *Laguncularia racemosa* and *Conocarpus erectus*. The Southwestern coast of Puerto Rico contains 996 ha of mangroves, representing 15.3% of the total mangrove area in Puerto Rico (Martínez *et al*., 1979). The original area of mangroves in Puerto Rico was estimated at 30,000 ha but by 1975, only half of that area remains. Unfortunately, the reduction of mangroves forest in Puerto Rico continues despite political resolutions and laws (Lugo, 1988). Paradoxically, natural disturbances such as the incoming waves on the offshore islands constitute one of the reasons of the mangrove reduction (Yoshioka, 1975).

Within the family *Avicenniaceae*, the genus *Avicennia* contains about 15 species that grow along tropical and subtropical shores. This genus grows in pure, dense stands on mud flats along the coast and estuaries, in brackish coastal swamps, and on river banks along lower brackish parts (Jiménez and Lugo, 1985). The tree sometimes reaches a height of 23.01 m, although normally only 8.0 m, with a 0.61 m of diameter. Black mangroves have long heavy roots that grow extensions known as pneumatophores that aid in gas exchange and coincidentally trap silt to extend the shoreline (Jiménez and Lugo, 1985). The tree has pale gray-green opposite leaves that are shiny above, and often found encrusted with salt. The leaves are larger than most mangroves (4.83 to 14.99 cm) and are oblong to lanceolate in shape (Jiménez and Lugo, 1985).

The red mangrove, *Rhizophora mangle*, is the dominant species on island reefs growing in brackish areas along creeks, bays, and lagoons. Typically grows along the

water's edge and then on higher elevations upland inhabits the black mangrove. It is a tall tree that reaches 21 meters (m) to 24 m in height in the tropics and is characterized by its numerous above ground roots called prop roots. The leaves are 7 cm to 12 cm long and 2.54 cm to 5.08 cm wide and do not excrete salt crystals. They possess pale yellow and white flowers and leathery fruits. Since red mangroves grow close together, their roots form an impenetrable tangled network which slows down the movement of water underneath the trees. This causes a deposition of sediment and traps an enormous amount of debris. This build up of sediment and debris under the right conditions can create a thick layer of organic peat (Nelson, 1994). Mangrove development is greatest in zones of intermediate wave energy. On the middle shelf zone, waves and currents are strong enough to maintain a constant flow of water, yet allow for accumulation of fine sediments. Consequently, red mangroves prevail at these middle shelf reefs. The inner shelf reefs are not subject to enough wave energy to maintain adequate flushing; consequently, these reefs normally have strong transverse salinity gradients. Salt builds up in the center of these islands and enables the succession of red mangroves by the more salt-tolerant black mangrove. Prolonged accumulation of salt eventually leads to the death of the black mangroves. Even though an excess of salt can lead to the death of black mangrove, this specie can tolerate soil salinities from 36 to 66% (Cintrón *et al*., 1978).

The species can grow on soils where salinities range from 0 to 100 parts per thousand. *Avicennia* sp. take up salt in the transpiration stream and excrete salt through glands in their leaves (Tomlinson, 1986). Through evaporation, the salt crystallizes on the leaf surface, where it then falls or washes off. In contrast, *Rhizophora* sp. filter salt from

the water as it passes into their roots, so that little salt passes into the plant and up to the leaves (Tomlinson, 1986).

Given that mangrove ecosystems are situated at the inner-phase between the terrestrial and marine environments several bacteria get nourished by detritus and in turn help the mangrove ecosystem in different ways (Holguin *et al*., 2001). Bacteria are directly involved in photosynthesis (Vethanayagam, 1991), nitrogen fixation (Toledo *et al*., 1995), methanogenesis (Mohanraju and Natarajan, 1992). Other studies showed mutualism between the root nodules of some mangroves and members of *Rhizobium*, a bacterium that forms nitrogen-fixing nodules almost exclusively on the roots of the *Leguminosae.*

One of the most studied is the relationship between the number and size of leaf salt glands and the number of bacteria growing in a desert plant *Antriplex halinus*, widespread in the Negev Desert, Israel and in other deserts environments. The leaves excrete salt through salt glands. The number and size of the gland depend on the amount of salt present during plant growth. Salt crystallizes on the leaves when the salt gland bladder burst stimulating up to 5 x 10^5 bacteria per cm^2 of the leaf surface (Simon *et al*; 1994). In spite of the high abundance of bacteria, they found that diversity of the cultivable bacteria was limited, with the dominant organism identified as *Pseudomonas* sp. This bacterium is known to grow from 0.05% to 20% of NaCl with an optimum at 5% and 30ºC (Simon *et al*., 1994). Similar to *Antriplex halinus,* the leaves of *Avicennia germinans* also excrete salt crystals, which may suggest that prokaryotic diversity may also inhabit leaves.

Indeed, fungi such as *Asteridiella sepulta* is known to be present on the black mangrove leaf surface in Los Morrillos, Puerto Rico (Nieves-Rivera and Darrah, 2002). Also other studies show the presence of two new ascomycetous species growing on mangrove host in the Caribbean Sea (Kohlmeyer and Schatz, 1985). A recent study concludes that salt excretion in leaves of some mangrove species may serve as an important defense against fungal attack by reducing the fungal high density on the leaves (Gilbert *et al*., 2002). Gilbert and colleagues performed a field survey of Caribbean mangrove forests in Panama. *Avicennia germinans* suffered much less damage from foliar diseases than *Laguncularia racemosa* or *Rhizophora mangle*. Similarly, *Avicennia* leaves supported the least superficial fungal growth, endophytic colonization, and diversity, followed by *Laguncularia* and *Rhizophora*.

III. Taxonomy

Polyphasic taxonomy arose 25 years ago and is aiming at the integration of different kinds of data and information such as phenotypic, genotypic, and phylogenetic on microorganism and indicates a consensus type of taxonomy (Vandamme *et al*., 1996). Murray *et al*. (1990) stated that polyphasic taxonomy is used for the delineation of taxa at all levels. All genotypic, phenotypic, and phylogenetic information may be incorporated in polyphasic taxonomy. Genotypic information is derived from the nucleic acids (DNA and RNA) present in the cell. Determinations of the moles percent G+C content and DNA-DNA hybridization studies became classic and were applied in taxonomic analyses of virtually all bacteria. Chemotaxonomic methods such as fatty acid analysis are fast methods, which allow us to compare and group large numbers of strains in a minimal

period (Vandamme *et al*., 1996). Techniques such as direct sequencing of parts or nearly entire 16S or 23S rDNA molecules by using the PCR technique and a selection of appropriate primers is a useful technique to complement the delineation of taxa. They provide a phylogenetic framework which serves as the backbone for modern microbial taxonomy (Vandamme *et al*., 1996).

Polyphasic taxonomy has been applied for the identification of a variety of microorganism such as the moderately halophilic prokaryotes. Moderately halophilic bacteria constitute a heterogeneous physiological group of microorganisms which belong to different genera (Ventosa *et al*., 1998). Some moderately halophilic bacterias are *Vibrio* (*Salinivibrio*) *costicola* (Mellado *et al*., 1996), *Micrococcus* (*Nesterenkonia*) *halobius* (Onishi and Kamekura, 1972), *Paracoccus* (*Halomonas*) *halodenitrificans* (Kocur, 1984), *Flavobacterium* (*Halomonas*) *halmephilum* (Elazari-Volcani, 1940), *Planococcus* (*Marinococcus*) *halophilus* (Novitsky and Kushner, 1976), and *Spirochaeta halophila* (Greenberg and Canale-Parola, 1976). Although some Gram-negative species were considered members of different genera (*Halomonas*, *Deleya*, *Volcaniella*, *Flavobacterium*, *Paracoccus*, *Pseudomonas*, *Halovibrio*, or *Chromobacterium*), phenotypic and phylogenetic data support their close relationship, and they are currently included in the family *Halomonadaceae* as members of two genera: *Halomonas* and *Chromohalobacter* (Dobson and Franzmann, 1996; Franzmann *et al*., 1989).

The family *Halomonadaceae* is a member of the gamma subclass of Proteobacteria (Franzmann *et al*., 1989). Members of this family are Gram-negative, straight or curved, rod-shaped, slightly or moderately halotolerant, possess ubiquinone-9 (H2) as the respiratory lipoquinone, and contain C18:1, cyclo-C19:0 and C16:0 as major

fatty acids. The family *Halomonadaceae* currently includes three genera (Arahal *et al*., 2002), namely *Halomonas* (Vreeland *et al*., 1980; Dobson and Franzmann, 1996), *Chromohalobacter* (Ventosa *et al*., 1989; Arahal *et al*., 2001), and *Zymobacter* (Okamoto *et al*., 1993). Today *Halomonas* includes 36 species of moderately halophilic bacteria mostly from saline environments (Arahal *et al*., 2002; Bouchotroch *et al*., 2001; Dobson and Franzmann, 1996; García *et al*., 2004; Lim *et al*., 2004; Lee *et al*., 2005; Martínez-Cánovas *et al*., 2004, 2005; Martínez-Checa *et al*., 2005; Mata *et al*., 2002; Mormile *et al*., 2000; Quillaguaman *et al*., 2004; Romanenko *et al*., 2002; Ventosa *et al*., 1998; Vreeland *et al*., 1980; Yoon *et al*., 2001; 2002; Cabrera *et al*., 2007). Recently other *Halomonas* species has been describe such as *Halomonas taeanensis* (Lee *et al*; 2005), *Halomonas almeriensis* (Martínez-Checa *et al*., 2005), and *Halomonas anticariensis* (Martínez-Canóvas *et al*; 2004). *Halomonas neptunia* sp. nov., *Halomonas sulfidaeris* sp. nov., *Halomonas axialensis* sp. nov. and *Halomonas hydrothermalis* (Kaye *et al*., 2004) have been isolated from deep hydrothermal vents and *Halomonas muralis* from dry mural paintings (Heyrman *et al*., 2002). Besides members of the family *Halomonadaceae*, several other Gram-negative strictly aerobic or facultatively anaerobic species have been described as moderate halophiles belonging to genera that include nonhalophilic species as well, such as *Pseudomonas*, *Flavobacterium*, or *Spirochaeta*, while others are placed in genera represented, at least until now, exclusively by halophilic species such as *Salinivibrio*, *Arhodomonas*, or *Dichotomicrobium* (Ventosa *et al*., 1998).

Studies show that *Halomonas*, *Deleya*, *Halovibrio*, and *Volcaniella*, as well as *Paracoccus halodenitrificans*, form a monophyletic group within the gamma subclass of

the *Proteobacteria* (Dobson and Franzmann, 1996; Dobson *et al*., 1993; Mellado *et al*., 1995; Miller *et al*., 1994; Romano *et al*., 1996).

The Gram-positive moderately halophilic aerobic bacteria, with the exception of two *Bacillus* species, belong to genera that include only species with halophilic requirements: *Halobacillus*, *Marinococcus*, *Salinicoccus*, *Nesterenkonia*, and *Tetragenococcus* (Ventosa *et al*., 1998). The genus *Halobacillus* was first described by Spring et al. (1996); at present, it comprises nine species with validly published names: *Halobacillus halophilus*, *H. litoralis* and *H. trueperi* (Spring *et al*., 1996), *H. salinus* (Yoon *et al*., 2003), *H. karajensis* (Amoozegar *et al*., 2003), *H. locisalis* (Yoon *et al*., 2004), *H. yeomjeoni* (Yoon et al., 2005), *H. dabanensis* and *H. aidingensis* (Liu *et al*., 2005).

The genus *Halobacillus* is clearly differentiated from other related genera in the cell-wall peptidoglycan type. Members of the genus have peptidoglycan based on L-Orn–D-Asp (Spring *et al*., 1996), whereas other related genera contain meso-diaminopimelic acid or L-lysine at position 3 of the cell-wall peptidoglycan (Shida *et al*., 1997; Wainø *et al*., 1999; Yoon *et al*., 2001(b)).

Moderate halophiles are represented in many of the major bacterial phyla: spirochetes, *Proteobacteria*, *Flavobacterium-Bacteroides*, and low-G+C and high-G+C Gram-positive organisms (Ventosa *et al*., 1998). The use of 16S rDNA alone to describe taxa is not enough and the combination of genotypic and phenotypic characteristics (polyphasic taxonomy) constitutes a practical solution to the delineation of taxa (Hezayen *et al*., 2002).

IV. Applications in biotechnology

Recently, interest in the mass cultivation of microorganisms from hypersaline environments has grown considerably, since they represent an innovative low technology approach for biotechnological exploitation (Nicolaus *et al*., 1999). One particular interest is the usage of halophilic microorganisms in environmental bioremediation. A recent study described an halophilic bacterium named *Halomonas campisalis* isolated from eastern Washington State that completely degraded 140 mg/L phenol as the only source of carbon and energy at pH's 8, 9, 10 and 11 in 10 % NaCl saline synthetic wastewater (Peyton and Apel, 2005.) Hypersaline habitats often harbor microorganism of considerable biotechnological interest (Bouchotroch *et al*., 2001). Some halophilic microorganisms like *Haloarcula* spp. are able to biosynthesize exo- and endopolymers such as polyhydroxyalkanoate and exopolysaccharides that can be isolate for further applications in biotechnology (Nicolaus *et al*., 1999). Three organisms have been used in physiological and biochemical studies dealing with the mechanism of haloadaptation: *Salinivibrio costicola, Halomonas elongata,* and *H. israelensis* (Ventosa *et al*., 1998). Also microbial communities are potential sources of industrial potent enzymes such as amylase, proteases and beta-galactosidase (Horikoshi, 1996). Other studies shows that several bacteria isolated from mangroves promoted the growth of *Salicornia bigelovii*, a potential oilseed crop that grows in semiarid mangrove areas (Bashan *et al*., 2000).

Part I. Sampling Procedure, Isolation and Characterization of Isolates

Material and Methods

I. Description of the black mangrove forest at the Solar Salterns in Cabo Rojo, Puerto Rico

The *Avicennia germinans* mangrove forest is located at the Cabo Rojo National Wildlife Refuge. The refuge lies along the coastal plain of Southwestern Puerto Rico. This land had been in agricultural use for at least two centuries prior to Wildlife Service ownership. At present, the refuge is approximately 65 % forest/shrub and 35 % grassland. The mangrove forest lies near the solar salterns and the area includes an estuary that is surrounded by natural mats (Figure 1.1).

II. Sample Processing and Isolation of Halophilic Bacteria

The Sehgal and Gibbons (SG) medium (1960) was selected for this study and it consist of yeast extract 10g/L, casamino acids 7.5 g/L; trisodium citrate 3.0 g/L; KCl 2.0 g/L; $MgSO_4 \bullet 7H_2O$ 20 g/L; NaCl 150 g/L; FeCl 2.3µg/L, (20g/L) pH 7.2-7.8. The pH was adjusted with 1M NaOH (Nicolaus *et al*; 1999).

During April 2005 a survey of four *A. germinans* trees growing near the solar salterns of Cabo Rojo, Puerto Rico was performed (Figure1.1). For microbial isolation, a total of 40 leaves were collected randomly from *A. germinans* trees. Two different strategies were designed to archive microbial isolation. Leaves were placed in sterile bags (Whirl Pak®) containing 1% phosphate buffer pH 7 with 15% (w/v) NaCl and mixed. This procedure allowed suspending the microorganisms present in the salt crystals and the surface of the leaves. Ten milliliters of the suspension were then obtained for serial

dilutions. The dilutions were poured into agar plates containing Seghal-Gibbons medium at 15% NaCl (w/v). Inoculated plates were incubated at 30ºC. After 3 days of incubation, colonies were selected and purified by the quadrant streak plate method. Pure cultures were transferred into SG medium for further analysis.

For the second isolation strategy, whole leaves from *Avicennia germinans* were obtained and printed against agar plates containing SG medium at 15% (w/v) NaCl. This procedure allowed the transfer of the microorganisms present in the salt crystals and the surface of the leaves to the growth medium. Plates were incubated at 30ºC for 3 days. The leaves were removed aseptically and colonies were then selected and purified by the quadrant streak plate method. Pure cultures were transferred into SG medium for further biochemical and molecular analysis.

For comparison purposes, the red mangrove (*Rhizophora mangle*) whose physiology does not produce salt crystals on the leaves was selected as a contrast group and the treatment described above was applied to its leaves for the determination of the prokaryotic community.

Figure 1.1 Location area of study (black mangrove forest) near the solar salterns at Cabo Rojo. Map by Roy Ruiz, PaSCoR laboratory.

III. Morphological and cultural characterization

Gram stains were performed using both heat-fixed smears and smears fixed in acetic acid 5% (Dussault, 1955). Macroscopic characteristics were documented using the classical characterization of colony appearance. The morphology of cells was examined by Nomarsky and scanning electron microscopy (SEM) at logarithmic phase of growth under optimal conditions. Electron microscopy procedures were performed as previously described (Díaz-Muñoz and Montalvo-Rodríguez, 2005). The strains were examined using JEOL JSM-541 OL SEM microscope at 15 kv.

IV. Physiological characterization

Optimal conditions for growth were determined by growing the strains in SG solid media supplemented with 0, 5, 10, 15, 20, 25 and 30% (w/v) NaCl at temperatures of 20, 25, 30, 35, and 40°C, respectively. Growth was monitored visually at 24h and 48h. The pH range of growth for the isolates was tested in SG medium at the strain optimal temperature and salinity adjusting the pH to the following values: 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. The cells were cultivated with constant agitation (150 rpm) and growth was monitored by measuring absorbance at 600 nm. The pH tolerance was tested in SG broth buffered with 20 mM MES (pH 5.0, 5.5), PIPES (pH 6.0, 6.5), tricine (pH 7.0, 7.5, 8.0) or TAPS (pH 8.0, 9.1) (Montalvo-Rodríguez *et al*., 2000). All conditions were performed in duplicates.

V. Biochemical tests

All biochemical tests were carried out at 30°C unless it is stated otherwise. Catalase activity was determined by adding a 1% (w/v) H_2O_2 solution to colonies on SG agar medium. Oxidase test was performed using the Dry Slide (Difco®) biochemical test (Montalvo-Rodríguez *et al*; 1998). Hydrolysis of starch, Tween 80, and aesculin were determined as described by Cowan and Steel (1965) with the addition of basal salts (5% NaCl, 2% $MgSO_4 \cdot 7H_2O$, and 0.2% KCl) to the medium. Hydrolysis of gelatin and production of urease was determined according to Cowan & Steel (1965). Citrate utilization was determined on Simmon's citrate medium supplemented with basal salts (Simmons, 1926). Acid production from carbohydrates was determined using phenol red base supplemented with 0.7% of the carbohydrate and basal salts. Motility, H_2S

production and indole was determined using SIM a multi-test medium comprising 3 tests: sulfide (H_2S gas), indole production, and motility supplemented with basal salts. Production of H_2S was determined using the API 20E system as previously described (Yoon *et al.*, 2001). Growth under anaerobic conditions was determined by incubating strains in an anaerobic chamber in SG medium with 5% NaCl. Nutritional features were determined using the Koser medium (1923) as modified by Ventosa *et al*. (1982), (%, w/v): NaCl, 5.0; KCl, 0.2; $MgSO_4.7H_2O$, 0.02; KNO_3, 0.1; $(NH_4)_2HPO_4$, 0.1; and KH_2PO_4, 0.05. The filter-sterilized substrate was added to the medium at a final concentration of 0.1% (w/v), with the exception of carbohydrates, which were used at a final concentration of 0.2% (w/v).

VI. Fatty acids analysis

Isolated strains were sent to the Belgian Coordinated Collections of Microorganisms, Laboratory of Microbiology of Gent (BCCM/LMG), Gent, Belgium (Germany) and to Dr. Aharon Oren's Laboratory at the University of Jerusalem for fatty acids analysis. Cells were cultured on SW10 medium (Ventosa *et al*., 1982) for 24h at pH 7.0, 30ºC. Fatty acids were analysed using GC (Miller, 1982; Kämpfer & Kroppenstedt, 1996).

VII. Isolation of genomic DNA

Strains were grown in SG with 15% NaCl and incubated at 30°C. DNA was extracted from cells using lysis buffer (40mM Tris-acetate pH 7.8-8.0, 20mM Sodium-acetate pH 8.0, 1.0mM EDTA pH 8.0 and 1% SDS) treated with lyzosyme followed by phenol/chloroform extraction and ethanol precipitation. The isolated DNA was

resuspended in 50 µl of molecular water and treated with RNAse (at a final concentration of 20 µg/µl) for 30 minutes at 37ºC. DNA quality was checked on 0.8% agarose gels after staining with ethidium bromide. All genomic DNAs were used as templates for subsequent PCR amplification.

VIII. Polymerase chain reaction (PCR) and gel electrophoresis

The gene encoding the 16S rRNA was amplified by PCR using the combination of forward primer Univ-519-F (5'-CAGCMGCCGCGGTAATWC) and with the reverse primer Univ-1392-R (5'-ACGGGCGGTGTGTRC). The reaction mixture consist of ddH_2O, buffer 1X, $MgCl_2$ 2.5mM, dNTP's 250mM, Primer F 1pmol, Primer R 1pmol, DNA (10 ng), and Taq polymerase 0.026U/µl. The following standard conditions were used for universal 16S rRNA gene amplification: initial denaturation at 95°C for 5 min; 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 50°C), and extension (3 min at 72 °C); and a final extension at 72°C for 10 min (Hezayen *et al.,* 2002). PCR amplicons were purified using the MinElute PCR purification kit (USA QIAGEN Inc.) according to manufacturer instructions and the concentration was determined using a spectrophotometer at 260 nm.

IX. Restriction fragment length polymorphism (RFLP)

A double digestion was performed on the amplicons using the restriction endonucleases *Msp I* and *HaeIII.* All digestions were performed for an hour (twice) at 37ºC in a final volume of 10 µl. RFLP patterns were verified on 3% low melting agarose gels after staining with ethidium bromide.

X. DNA Sequencing

Selected PCR products were sent to a DNA sequencing facility in Korea (Macrogen). Samples were prepared according to the facility instructions.

XI. Phylogeny analysis

Distance analysis of the resulting DNA sequences were performed using the PHYLIP program (version 3.63) (Felsenstein, 1993). A multiple-sequence alignment was made by using the Clustal W program with 16S rRNA gene sequences of closely related organisms (as determined by BLAST analysis) (Maidak *et al*., 1996). The 16S rRNA gene similarity values were calculated by pairwise comparison of the sequences within the alignment. Seqboot was used to generate 100 bootstrapped data sets. Distance matrices were calculated with dnadist. One hundred trees were inferred by using neighbor. Any bias introduced by the order of sequence addition was minimized by randomizing the input order. Consense was used to determine the most frequent branching order. Final trees were drawn using TREEVIEW (Page, 1996).

Results

Morphological characterization

Morphological characterization was performed using the classical macroscopic technique of color, margin, elevation, and form of pure colonies. Microscopic characterization was performed using the Gram reaction and cell shape after staining.

Table 1.1 shows the macroscopic and microscopic characteristics of isolates from the leaf surface of *Avicennia germinans*.

Table 1.1. Macroscopic and microscopic characteristics of the isolated strains from *Avicennia germinans*, Cabo Rojo, Puerto Rico.

Strain	Color	Morphology	Gram Stain	Morphology
A1	White	Circular, entire, raised	+	Rods
A2	Yellow	Circular, entire, flat	+	Cocci
A3	Cream	Circular, entire, flat	+	Thin short rods
A4	Cream	Circular, entire, flat	+	Long rods
A5	Cream	Circular, entire, raised	+	Thin rods
A6	Cream	Circular, entire, raised	+	Long rods
A7	Orange	Circular, entire, raised	-	Short rods
A8	Orange	Circular, entire, raised	-	Thin long rods
A9	Orange	Circular, entire, convex	-	Thin long rods
A10	Orange	Circular, entire, flat	-	Thin long rods
A11	Cream	Circular, entire, raised	+	Cocci
A12	Brown	Circular, entire, raised	+	Short rods
A13	Orange	Circular, entire, raised	-	Short rods
A14	Cream	Circular, entire, raised	+	Thin rods
A15	Yellow	Circular, entire, raised	+	Cocci
A16	Orange	Circular, entire, raised	-	Short rods (cocci-rods)
A17	Cream/brown	Circular, entire, raised	+	Short thick rods

Table 1.1 cont.

A18	Cream	Circular, entire, flat	-	Long rods
A19	Cream	Circular, entire, convex	+	Short rods
A20	Cream	Circular, entire, raised	+	Long rods
B1	Yellow/white	Circular, entire, raised	+	Cocci
B2	Cream	Circular, entire, flat	-	Thin long rods
B3	Orange	Circular, entire, raised	-	Short rods
B4	Orange	Circular, entire, raised	-	Short rods
B5	Orange	Circular, entire, flat	-	Short rods
B6	Orange	Circular, entire, raised	-	Short rods
B7	Orange	Irregular, entire	+	Long thin rods
B8	Orange	Circular, entire, flat	-	Thin rods
B9	Yellow	Circular, entire, raised	+	Cocci
B10	Orange	Circular, entire, flat	-	Cocci-rods
B11	White	Irregular, entire, raised	+	Cocci
B12	Yellow/cream	Circular, entire, raised	+	Cocci-rods
B13	Yellow	Circular, entire, raised	+	Cocci
B14	Orange	Circular, entire, raised	-	Rods
C1	Orange	Circular, entire, flat	-	Short rods
C2	Cream	Circular, entire, flat	-	Thick long rods
C3	Brown	Circular, entire, flat	+	Cocci-rods
C4	Orange	Circular, entire, raised	-	Short rods
C5	Orange	Circular, entire, flat	-	Short rods
C6	White	Circular, undulated, raised	+	Cocci
C7	White	Circular, entire, flat	+	Cocci
C8	Cream	Circular, entire, raised	+	Short thick rods
C9	White/yellow	Circular, entire, raised	+	Cocci
C10	White/yellow	Circular, undulated, raised	+	Cocci

Table 1.1 cont.

C11	Orange	Circular, entire, raised	-	Long thin rods
C12	Yellow	Circular, entire, flat	+	Cocci
C13	Green	Circular, entire, raised	+	Long rods
C14	Yellow	Circular, undulated, flat	+	Cocci
C15	Yellow	Circular, undulated, flat	+	Cocci
C16	Orange	Circular, entire, raised	-	Thin long rods
C17	White	Circular, entire, raised	+	Cocci
C18	White/yellow	Circular, entire, flat	+	Cocci
C19	Cream	Circular, entire, flat	+	Short rods
C20	Yellow/orange	Circular, entire, flat	+	Long rods
C21	Orange	Circular, entire, flat	-	Short rods
C22	Orange	Circular, entire, flat	-	Long rods
D1	Cream	Circular, entire, flat	+	Thin short rods
D2	Cream	Circular, entire, raised	+	Short thick rods
D4	Cream	Circular, entire, raised	+	Long thick rods
D5	Orange	Circular, irregular, raised	-	Long thin rods
D6	White	Circular, entire, flat	+	Cocci
D7	Cream	Circular, entire, raised	+	Short thick rods
D8	White	Circular, entire, flat	+	Cocci
D9	Orange	Circular, entire, flat	-	Long thin rods
D10	Cream/yellow	Circular, entire, raised	+	Short thick rods
D11	Cream	Circular, entire, raised	+	Short rods
D12	Cream	Circular, entire, flat	+	Thin short rods
D13	Cream	Circular, entire, raised	-	Short rods
D14	Brown	Circular, entire, raised	+	Cocci-rods
D15	Brown	Circular, entire, raised	+	Short rods
D16	Brown	Circular, entire, raised	+	Short rods

Table 1.1 cont.

MW2a	Orange	Circular, entire, raised	-	Cocci-rods
MS10	Brown	Circular, entire, convex	+	Thick shorts rods
MW3	Cream	Circular, entire, raised	+	Long rods

A total of 73 strains were isolated from the leaf surface of *Avicennia germinans*. From these isolates, 17 (23%) were had a cocci shape while the remaining 56 (76%) were rod shaped. A total of 27 isolates were Gram-negative (37%) and 46 were Gram-positive (63%) having a frequency of 0.37 and 0.63, respectively. Most of the isolates showed circular, entire and flat macroscopical morphology. Colonies showed a diversity of colors like green, orange, cream, brown, white and yellow.

In contrast from the black mangrove, only 9 strains were isolated from the red mangrove using SG with 15% NaCl. All of the isolates were Gram-positive. Some strains had similar macroscopic morphology such as MR-6, MR-3-1 and MR-1. Colony appearance was circular, entire, and convex. From the nine strains isolated, four were cocci-shaped (44%), and the remaining five were rod shaped (55%). Table 1.2 shows the macroscopic and microscopic characteristics of the microorganisms isolated from the leaf surface of *Rhizophora mangle*.

Table 1.2 Macroscopic and microscopic characteristics strains isolated from *Rizophora mangle*, Cabo Rojo, Puerto Rico.

Strain	**Color**	**Macroscopic morphology**	**Gram Stain**	**Microscopic Morphology**
MR-1	Cream	Circular, entire, convex	+	Rods
MR-2-1	Yellow	Circular, entire, flat	+	Cocci
MR-3	Yellow	Circular, entire, flat	+	Cocci
MR3-1	Cream	Circular, entire, convex	+	Short thin rod
MR3-2	Cream	Circular, entire, flat	+	Thick rods
MR-4	White	Circular, entire, raised	+	Cocci
MR4-1	White/Yellow	Circular, entire, flat	+	Cocci
MR-5	Cream	Circular, irregular, flat	+	Thick rods
MR-6	Cream	Circular, entire, convex	+	Thick rod

Black mangrove isolates showed multiple forms and arrangements. The most predominant forms were the cocci-shaped and the rod-shaped. The most predominant arrangements were staphylococcus, diplococcus, and diplorods (Figure 1.2). Macroscopic characteristics showed a diversity of colors present on the isolated strains. A variety of cream, brown, and orange colors were observed. Only one strain, C13, presented a light green color. *Staphylococcus* spp. presented different pigmentations like yellow and white. Figure 1.3-1.8 shows a variety of macroscopic and microscopic characteristics of the isolated strains.

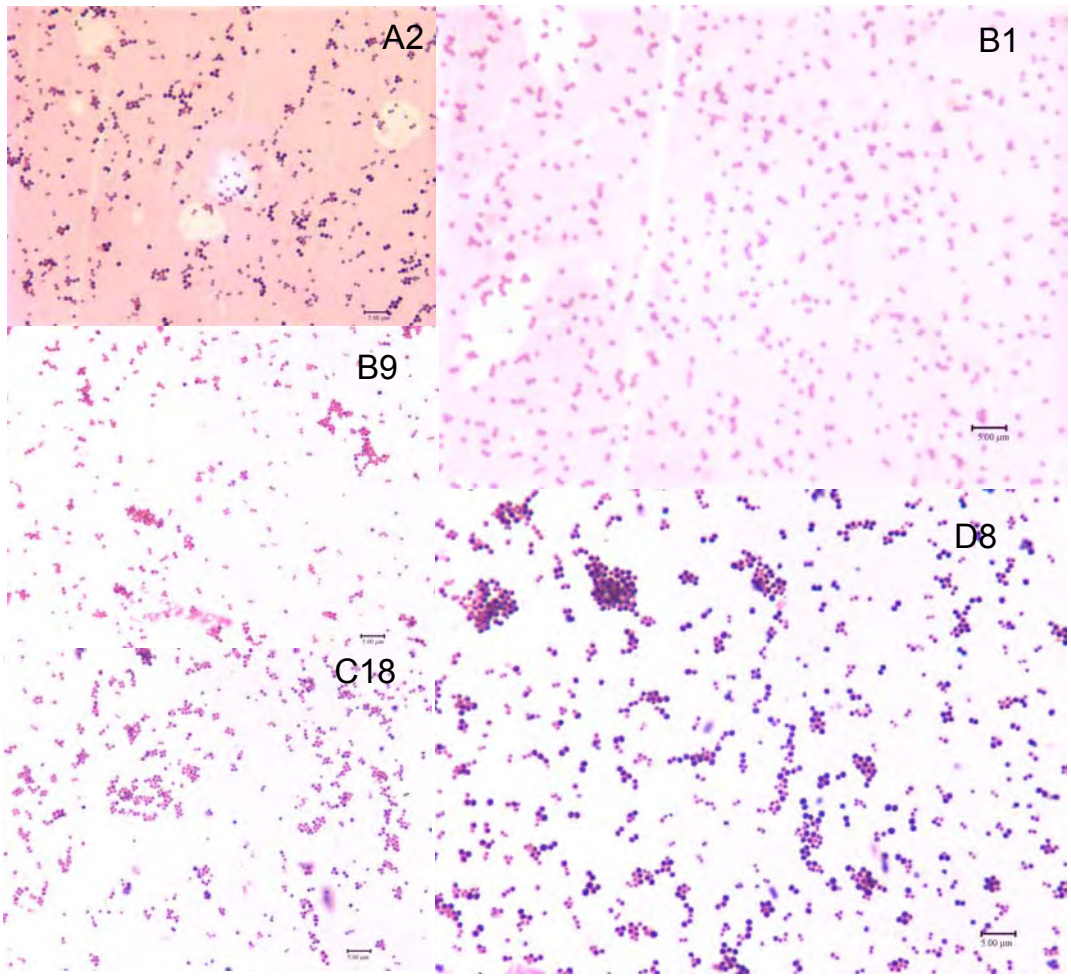

Figure 1.2 Nomarsky micrographs showing the prevailing Gram-positive cocci-shape arrangement found in *Avicennia germinans*, Cabo Rojo, Puerto Rico.

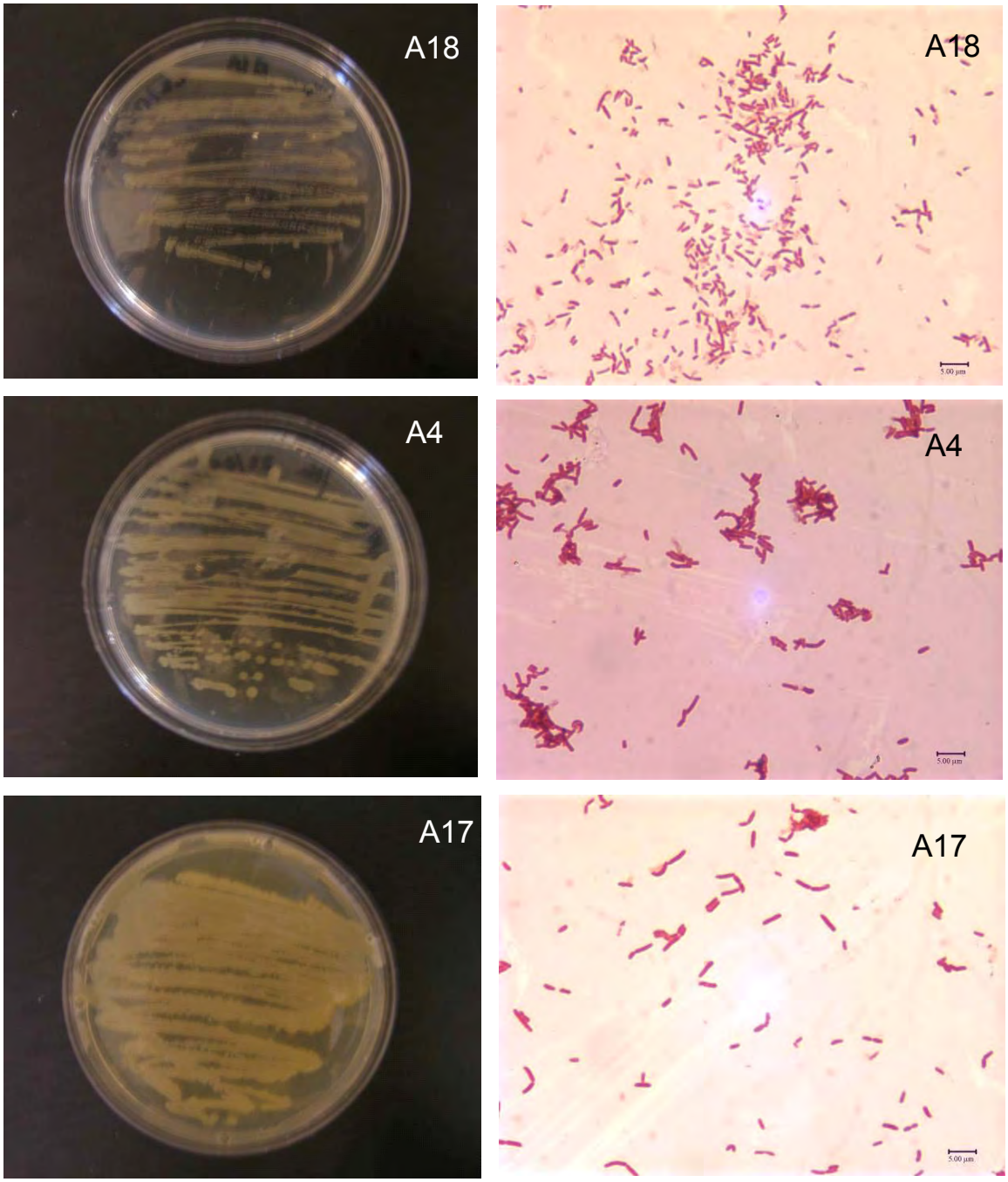

Figure 1.3 Macroscopical and microscopical characteristics of A18, A4 and A17 strains isolated from black mangrove, Cabo Rojo, Puerto Rico, using light microscope.

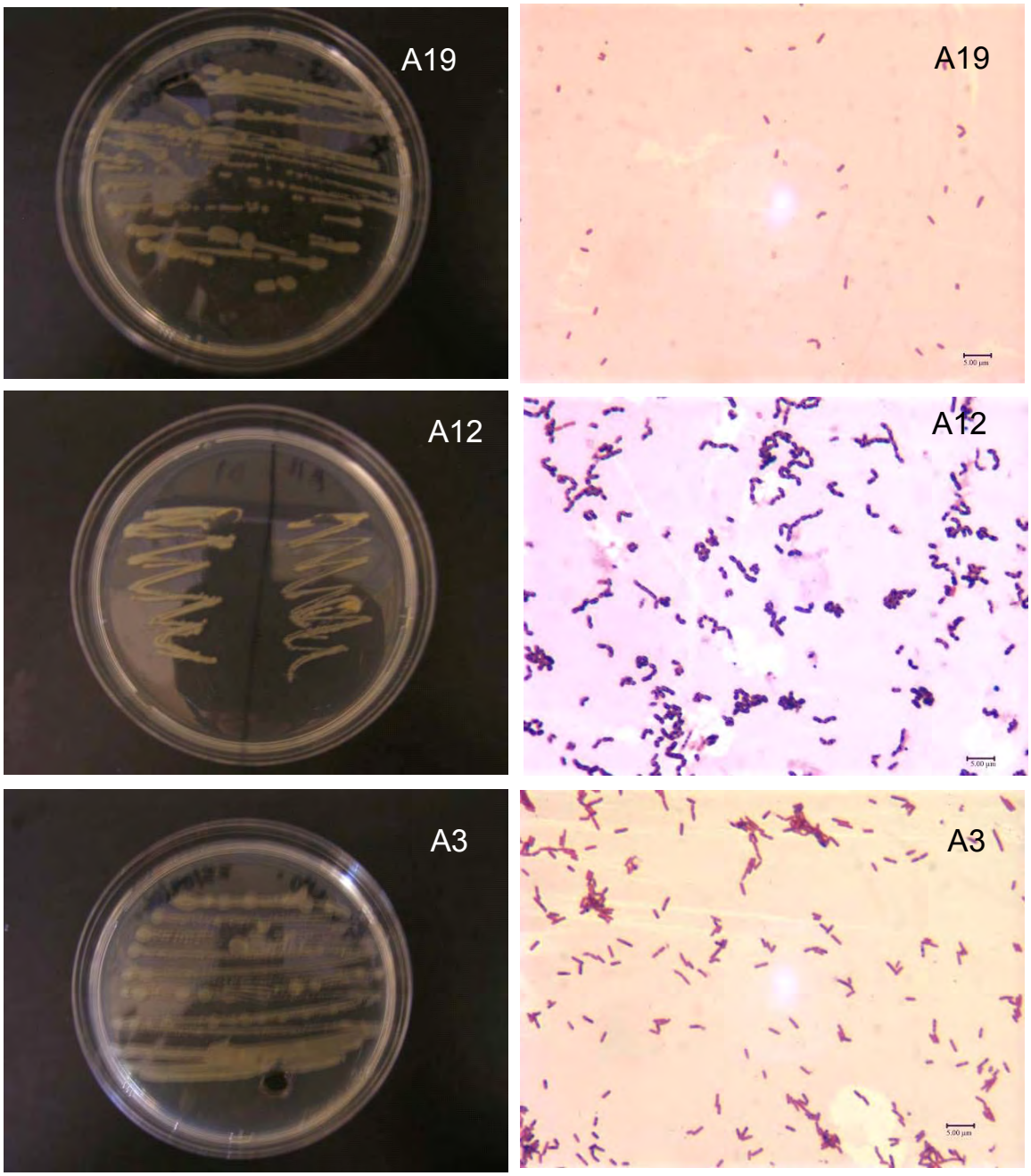

Figure 1.4 Macroscopical and microscopical characteristics of A19, A12 and A3 strains isolated from black mangrove, Cabo Rojo, Puerto Rico, using light microscope.

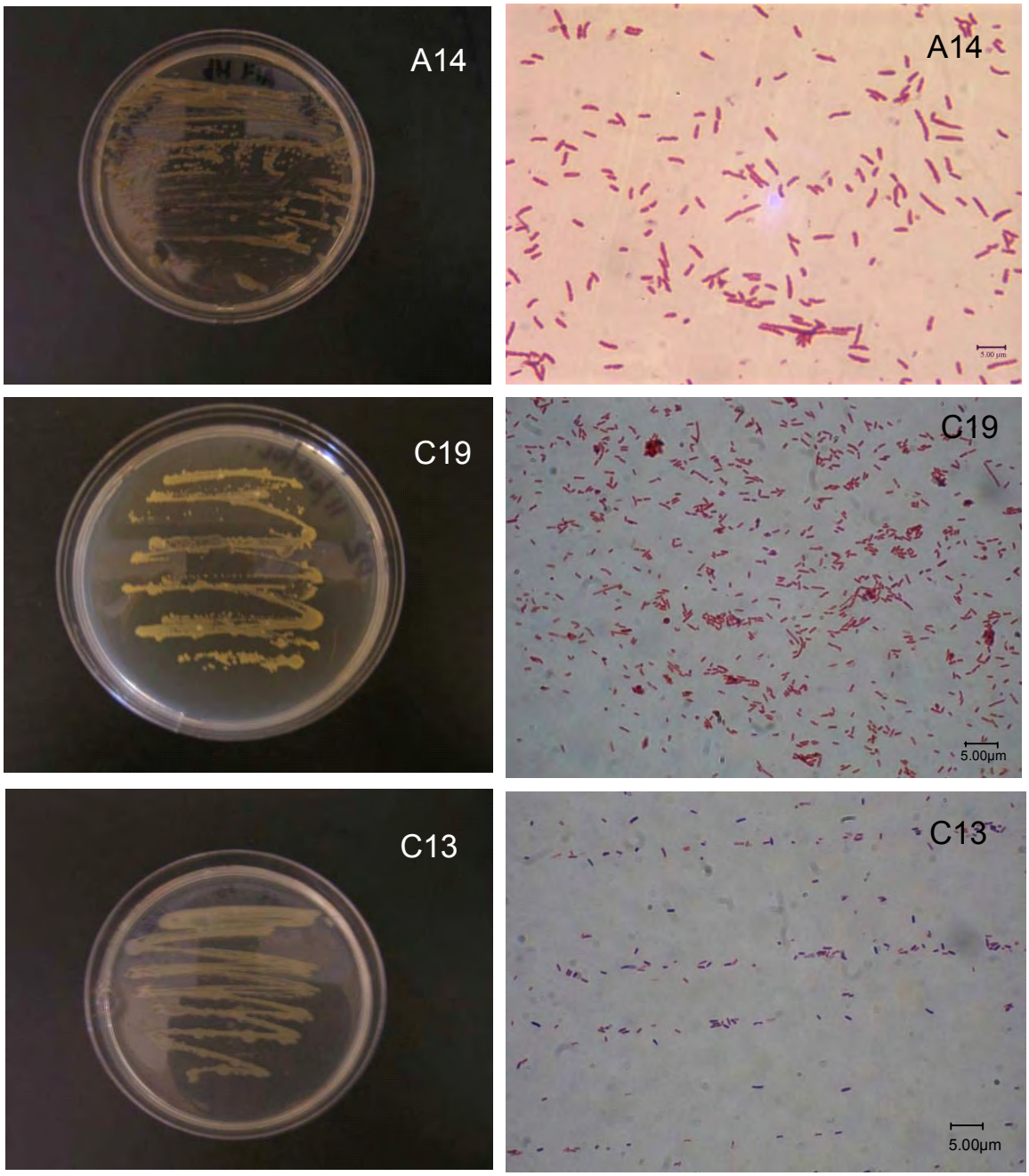

Figure1.5 Macroscopical and microscopical characteristics of A14, C19 and C13 strains isolated from black mangrove, Cabo Rojo, Puerto Rico, using light microscope.

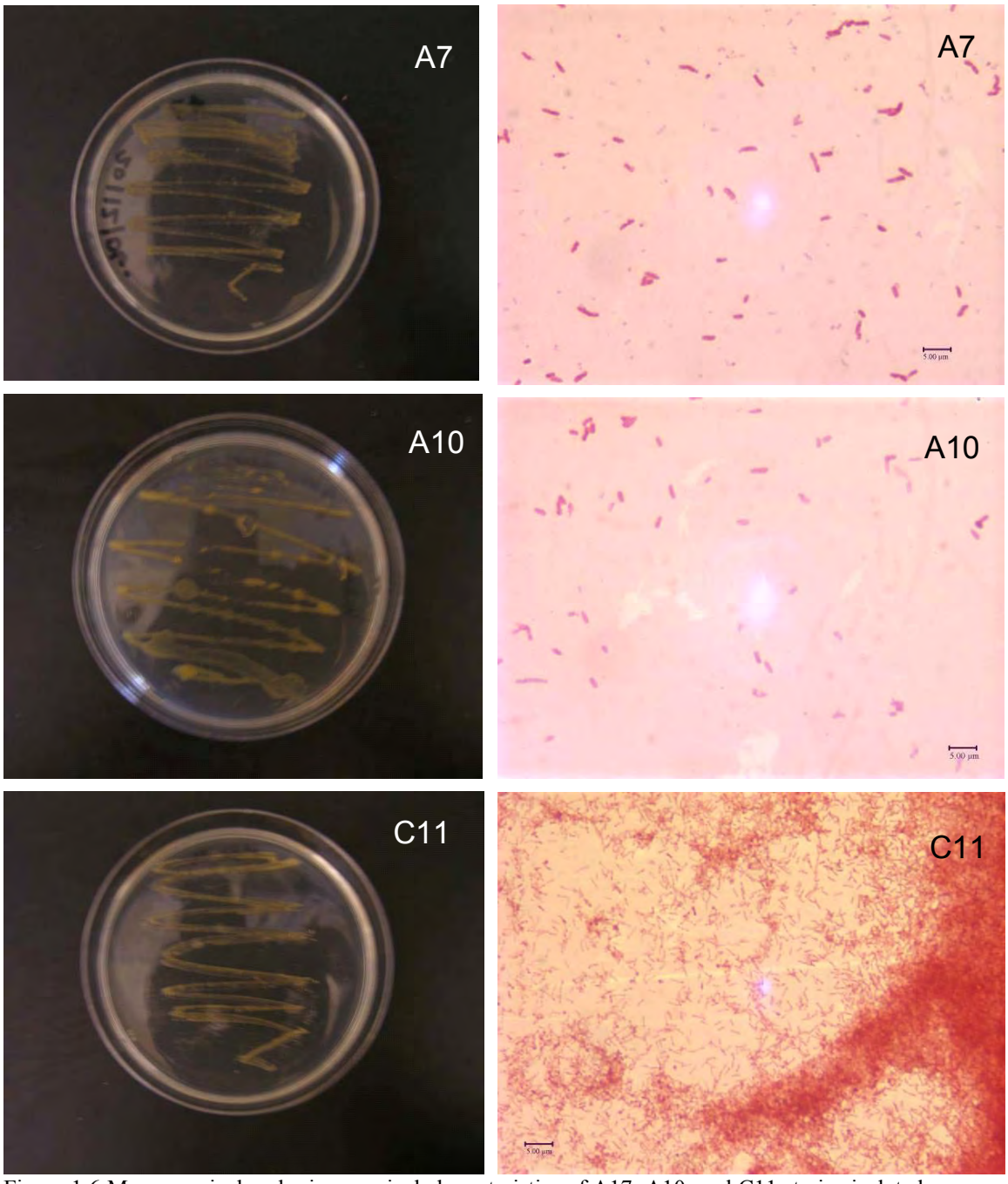

Figure 1.6 Macroscopical and microscopical characteristics of A17, A10, and C11 strains isolated from black mangrove, Cabo Rojo, Puerto Rico, using light microscope.

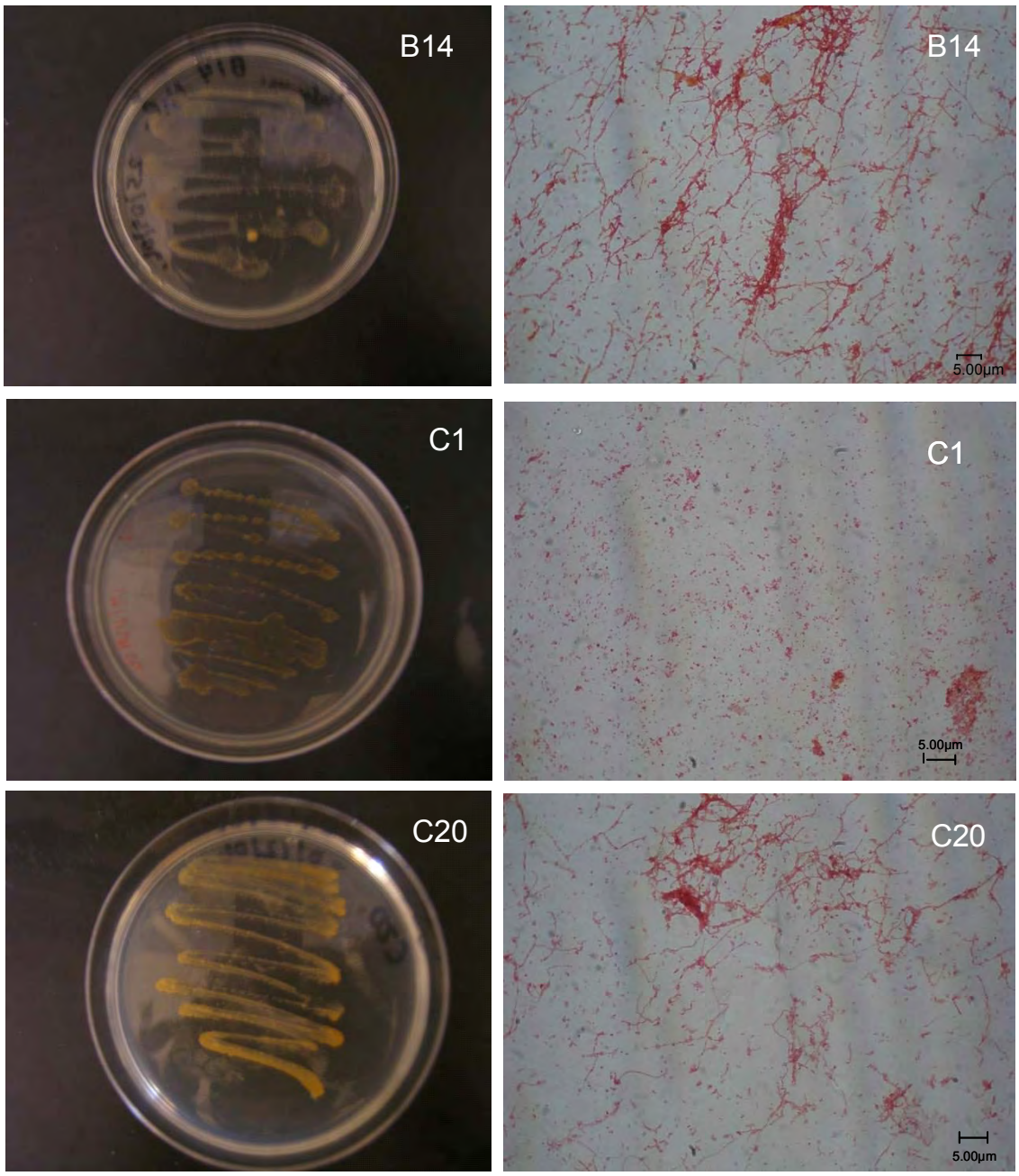

Figure 1.7 Macroscopical and microscopical characteristics of B14, C1 and C20 strains isolated from black mangrove, Cabo Rojo, Puerto Rico, using light microscope.

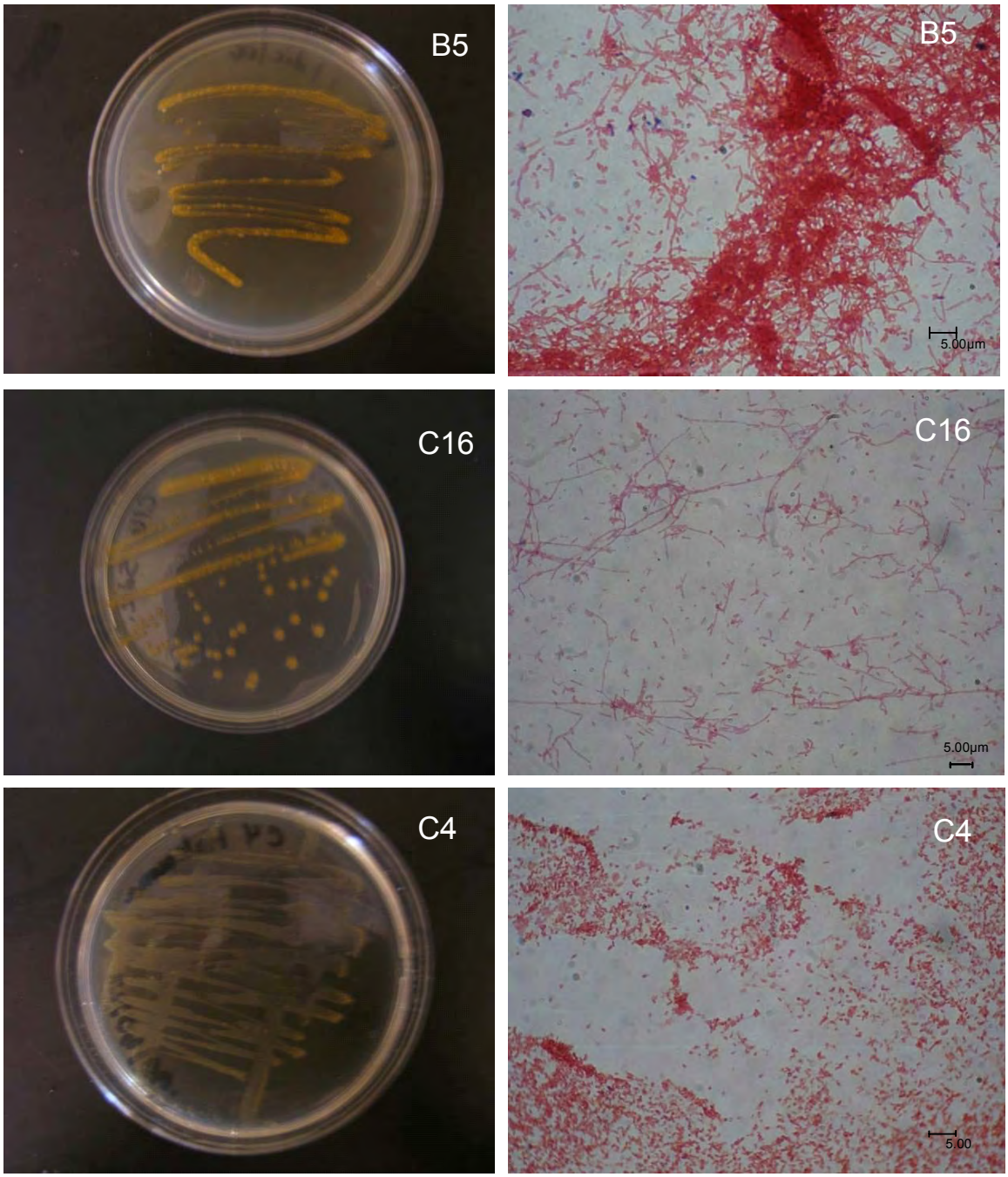

Figure 1.8 Macroscopical and microscopical characteristics of B5, C16 and C4 strains isolated from black mangrove, Cabo Rojo, Puerto Rico, using light microscope.

Only Gram-positive strains were isolated from the red mangrove. Figure 1.9 demonstrated the macroscopical and microscopical characteristics of these strains.

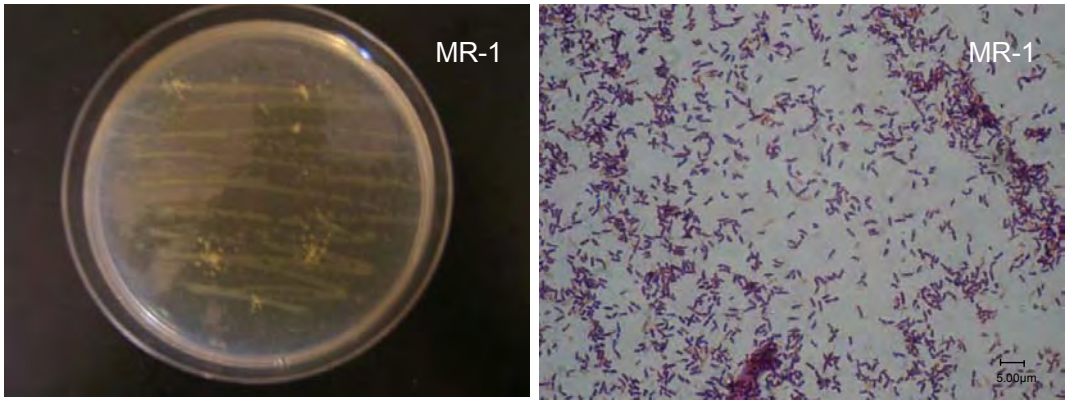

Figure 1.9 Macroscopical and microscopical characteristics of MR-1 strain isolated from red mangrove, Cabo Rojo, Puerto Rico, using light microscope.

Table 1.3 shows the size of several isolated strains from the black mangrove leaf surface. Usually *Halobacillus* strains are longer than the *Halomonas* strains.

Table 1.3 Bacterial size from different strains isolated from the black mangrove.

***Halobacillus* strains**	**Mean length (µm)**	**Mean width (µm)**
MS10	1.50± 0.50	0.75± 0.25
A18	2.00± 0.80	0.65± 0.25
A4	2.90± 0.70	0.90± 0.20
A17	3.25± 0.85	1.00± 0.20
A19	1.65± 0.45	0.80± 0.20
A12	2.50± 0.90	0.75± 0.35
A3	2.25± 0.65	0.45± 0.15
D2	1.80± 0.50	0.60± 0.3
A14	2.70± 0.81	0.74± 0.09
C19	1.83± 0.50	0.54± 0.17
D1	2.11± 0.52	0.70± 0.20
***Halomonas* strains**		
C4	2.30± 0.90	0.80± 0.30
B6	1.55± 0.35	0.75± 0.25
A7	2.30± 0.60	0.85± 0.15
A10	2.30± 0.80	0.85± 0.25
C11	1.75± 0.65	Nd
D5	2.45± 0.55	0.50± 0.16
C16	1.80± 0.70	0.50± 0.16
MW2a	2.50± 0.50	1.50± 0.50
B14	2.49± 0.90	0.64± 0.10
C20	2.00± 0.66	0.66± 0.14

Table 1.3 cont.

C1	1.55± 0.38	0.71± 0.17
***Staphylococcus* strains**		
A2	n/a	0.59± 0.28
B9	n/a	0.36± 0.10
D8	n/a	0.65± 0.21
C18	n/a	0.47± 0.11
B1	n/a	0.53± 0.24
***Pontibacillus* strain**		
C13	1.70± 0.31	0.62± 0.15

Physiological characterization and growth curves

Optimal growth conditions for black mangrove isolates were determined in SG solid media. Table 1.4 shows the results obtained for the isolated strains for optimal growth parameters.

Table 1.4 Optimal growth condition of prokaryotes isolated from *A. germinans*, Cabo Rojo, Puerto Rico.

***Halobacillus* spp**						
Strains	**Salinity range**	**Optimal Salinity**	**Temperature range**	**Optimal temperature**	**pH range**	**Optimal pH**
A4	0-15%	5%	25 – 50°C	30°C	(6-9)	8
A14	0-20%	5%	20 – 37°C	30°C	(6-9)	7-8
A19	0-20%	5%	12 – 42°C	30°C	(5-9)	7-8
C19	0-20%	5%	12 – 50°C	25°C	(5-9)	7-8
D2	0-15%	5%	20 – 37°C	37°C	(6-9)	7-8
A3	0-20%	10%	20 – 42°C	30°C	(7-9)	7
A12	0-20%	10%	25 – 50°C	30°C	(6-8)	7-8
A17	0-20%	10%	12 – 37°C	37°C	(7-8)	7-8
A18	0-20%	10%	25 – 42°C	30°C	(7-9)	7-8
MS10	5-20%	10%	15 – 42°C	33-35°C	(7-9)	7
D1	0-15%	10%	20 – 50°C	30°C	(6-9)	7-8

***Halomonas* spp**						
Strains	**Salinity range**	**Optimal Salinity**	**Temperature range**	**Optimal temperature**	**pH range**	**Optimal pH**
A7	5-20%	5%	25 – 37°C	30°C	(6-9)	7
A10	5-20%	5%	20 – 37°C	30°C	(6-8)	7
B5	0-15%	5%	20 – 42°C	25°C	(6-9)	6-7
B14	0-20%	5%	12 – 42°C	37°C	(5-8)	6-7
C1	0-20%	10%	20 – 42°C	25°C	(5-8)	7
C4	0-20%	10%	12 – 42°C	25°C	(7-8)	8
C16	0-20%	10%	12 – 42°C	25°C	(6-8)	6
MW2a	0-25%	5%	12 – 42°C	30-35°C	(6-9)	7-8
C20	0-20%	10%	20 – 42°C	25°C	(6-8)	8
C11	5-20%	5-10%	25 – 37°C	37°C	(6-8)	7-8
B7	5-20%	5-10%	20 – 37°C	30°C	(6-8)	7

Table 1.4 cont.

***Staphylococcus* spp**						
Strains	**Salinity range**	**Optimal Salinity**	**Temperature range**	**Optimal temperature**	**pH range**	**Optimal pH**
A2	0-15%	0%	12 – 50°C	30°C	(5-9)	8
B9	0-15%	0%	12 – 42°C	30°C	(5-9)	7
C18	0-15%	0%	12 – 42°C	30°C	(5-9)	8
D8	0-20%	0%	12 – 50°C	37°C	(5-9)	8
B1	0-20%	5%	25 – 50°C	37°C	(5-9)	8

***Pontibacillus* spp**						
Strains	**Salinity range**	**Optimal Salinity**	**Temperature range**	**Optimal temperature**	**pH range**	**Optimal pH**
C13	5-20%	10-15%	20 - 42°C	30 °C	(6-8)	7-8

Data from the tables shows the physiological behavior of various strains isolated from black mangrove leaf surface. Most of them can grow at salinity range from 0-20% NaCl and temperatures ranging from 20-42°C depending on the salinity. *Halomonas* strains A10, A7, C11 and B7 presented similar salinity ranges from 5 to 20% NaCl but can not grow at more than 37°C. The optimal pH was 7, salinity range 5%-10% NaCl, and temperature was 30°C. *Halomonas* strains B5, B14, C1, C4, C16, MW2a and C20 presented similar physiological patterns such as the temperature range for growth (12-42°C). *Halobacillus* strains showed a different physiological behavior. They do not grow at more than 50°C and 20% NaCl. *Staphylococcus* strains were halotolerant and most of them presented the same physiological growth conditions, such as salinity range (0 to 15%), optimal salinity (0% NaCl) and optimal temperature (30 to 37°C). The optimal pH for growth was 8.0. *Pontibacillus* strain salinity ranged from 5 to 20% NaCl, temperature range of 20 to 42°C, and pH range of 6 to 8. The optimal growth condition was 10-15% NaCl, 30°C and pH 7-8.

Biochemical tests

All biochemical tests were carried out at 30°C. The tests were performed in duplicates for best accuracy and validation of results. Tables 1.5 demonstrate the biochemical tests for the eleven *Halobacillus* strains.

Phenotipically, strains MS10, A18, A4, D2, C19, A3 were strictly aerobic and showed motility. All strains were strictly aerobic. Catalase and oxidase was produced by all strains except A17. Negative results were documented for aesculin except for C19. Negative results for indole, H_2S production, Voges-Proskauer, urease, Tween 80 for all *Halobacillus* strains were also recorded. Methyl red positive results for A17, A19 and A12. Gelatin was hydrolyzed by MS10, D1, A17, C19 and A19. Starch was hydrolyzed by MS10, D2, A12, A14, D1, A17 and A19. Citrate was utilized by strain A17. Nitrate was not reduced. MS10, A4, A12, A14, D1, C19 and A3 did not produce acid from glucose, sucrose, maltose, mannitol, xylose, fructose, mannose, D-melibiose, L-rhamnose, D-sorbitol, D-galactose, and inositol. Only D2, A17 and A19 produced acid from D-glucose, D-sucrose and D- fructose. Acid produced by strain A18 from fructose. Acid produced by A17 from maltose and D-cellobiose. A18 produced acid from D-xylose. Strains MS10, A12 and A19 utilize starch, D-glucose, D-sorbitol and succinate as a carbon and energy source. Strains A17 and A3 utilized D-glucose as a carbon source. C19 utilized succinate as energy source. Propionate used as an energy source by A14. Strains D2, A14, A17, C19 and A3 utilized D-sorbitol as energy source. D-cellobiose, D-glucose, starch and maltose were used by strains A18, A12, A14 and A19 as energy source. D-cellobiose was used as energy source by A4. D-cellobiose, D-glucose, starch and maltose were used as carbon and energy source by A3, A17, A14 and A19 strains.

Table 1.5 Phenotypic characteristics that distinguish *Halobacillus* strains isolated from *A. germinans* from Cabo Rojo, Puerto Rico from other type strains of related species of the genus *Halobacillus*.

Tests	1	2	3	4	5	MS 10	A18	A4	D2	A12	A14	D1	A17	C19	A3	A19
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Catalase	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Facultative anaerobe	Nd	Nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid production:																
D-Glucose	+	+	+	-	+	-	-	-	+	-	-	-	+	-	-	+
D-sucrose	+	+	-	-	+	-	-	-	+	-	-	w	+	-	-	+
Maltose	+	+	+	-	+	-	+	-	w	-	w	w	+	-	-	+
Mannitol	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-
D-Xylose	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-
D-Fructose	+	+	+	-	+	-	+	-	+	w	w	-	+	w	-	+
Simmon's citrate	Nd	Nd	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Motility	+	+	-	+	+	+	+	+	+	-	-	-	-	+	+	-
MR	Nd	Nd	-	Nd	Nd	-	-	-	-	+	-	-	+	-	-	+
Hydrolysis of:																
Gelatin	+	-	+	+	+	+	-	-	-	-	-	+	+	+	-	+
Starch	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+
Aesculin	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-
Growth on Succinate	Nd	Nd	Nd	Nd	Nd	+	-	-	-	+	-	-	-	+	-	+
Propionate	Nd	Nd	Nd	Nd	Nd	-	-	-	-	-	+	-	-	-	-	-
D-sorbitol	Nd	Nd	Nd	Nd	Nd	+	-	-	+	+	+	-	+	+	+	+
Carbohydrates																
D-cellobiose	Nd	Nd	Nd	Nd	Nd	-	+	+	-	+	+	-	+	-	-	+
D-glucose	Nd	Nd	Nd	Nd	Nd	+	+	-	-	+	+	-	+	-	+	+
Starch	Nd	Nd	Nd	Nd	Nd	+	+	-	-	+	+	-	-	-	-	+
Maltose	Nd	Nd	Nd	Nd	Nd	-	+	-	-	+	+	-	+	-	+	+

*Data from Amoozegar *et al*., 2003, Liu *et al*., 2005 and this study. +, positive; -, negative, nd, not determined. (1) *H. aidigensis*, (2) *H. dabanensis*, (3) *H. karajensis*, (3) *H. halophilus*, (4) *H. litoralis*.

Table 1.6 Phenotypic characteristics that distinguish of *Halomonas* strains isolated from *A. germinans*, Cabo Rojo. Puerto Rico, from other type strains of the genus *Halomonas*.

Test	1	2	3	4	5	6	7	MW2a	A7	B14	C1	C4	C16	C20	A10	B5	C11	B7
Anaerobic conditions	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-
Motility	+	+	-	+	+	+	-	+	+	+	+	-	-	+	+	+	-	-
Oxidase	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-
Acid prod.																		
D-Glucose	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	-	-
D-Sacarose	+	+	-	+	+	+	-	+	-	+	-	-	-	-	-	+	-	-
Maltose	+	+	-	-	+	-	-	w	-	-	-	-	-	-	-	-	-	-
Mannitol	+/-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
D-Xylose	+	-	Nd	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-
D-Fructose	+	-	-	+	+	+	-	+	-	+	-	-	+	-	-	+	+	+
Lactose	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
L-arabinose	+	+	-	-	+	-	-	+	+	+	+	+	+	+	-	+	-	-
Nitrate reduction	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Simmon citrate	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-
MR	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	-	w	+	w	+	+	-	+	-	-
Hydrolysis of:																		
Gelatin	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Starch	-	-	-	-	-	-	-	-	+	-	+	-	+	+	+	-	-	-
Urea	-	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Tween 80	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-

Aesculin	+	-	-	+	-	-	+	+	+	-	+	+	+	+	+	+	-	-
Table 1.6 cont. Growth on: Alcohols																		
Mannitol	Nd	Nd	Nd	Nd	Nd	Nd	Nd	-	-	-	-	-	-	-	-	-	+	-
Glycerol	Nd	Nd	Nd	Nd	Nd	Nd	Nd	+	+	+	+	+	+	+	-	+	+	+
D-sorbitol	Nd	Nd	Nd	Nd	Nd	Nd	Nd	-	-	-	-	-	-	-	-	-	-	-
carbohydrates																		
D-cellobiose	Nd	Nd	Nd	Nd	Nd	Nd	Nd	+	-	-	-	-	-	-	-	-	-	-
D-glucose	Nd	Nd	Nd	Nd	Nd	Nd	Nd	+	-	-	-	-	-	-	-	-	-	+
Fructose	Nd	Nd	Nd	Nd	Nd	Nd	Nd	+	-	+	+	+	+	+	-	+	-	-
Maltose	Nd	Nd	Nd	Nd	Nd	Nd	Nd	+	-	+	+	+	+	+	-	+	-	-
Starch	Nd	Nd	Nd	Nd	Nd	Nd	Nd	-	-	-	-	-	-	-	-	-	-	-

Strains: 1, *Halomonas marisflavi* KCCM 80003^T; 2, *Halomonas elongata* DSM 33173^T; 3, *Halomonas salina* ATTC 49509^T; 4, *Halomonas halodurans* DSM 5160^T; 5, *Halomonas halmophila* DSM 5349^T; 6, *Halomonas marina* DSM 4741^T; 7, *Halomonas eurihalina* DSM 5720^T. Data from Arahal *et al.* (2001; 2002), Baumann *et al.* (1983), Franzmann *et al.* (1989), Hebert & Vreeland (1987), Mata *et al.* (2002), Mellado *et al.* (1995), Yoon *et al.* (2001) and this study. +, Positive; -, negative, +/-, weak reaction; nd, no data.

Strain MW2a, B14, C20, A7, C4, C16 and B5 were strictly aerobic. On the other hand, strains C1 and A10 were facultative anaerobes. No oxidase production was detected for these strains. Catalase was produced by MW2a, A7, B14, C1, C4, C16, C20 and B5. Gelatin was hydrolyzed by MW2a. Aesculine was hydrolyzed by MW2a, A7, C1, C4, C16, C20, A10 and B5. Negative results for indole, H_2S production, Tween 80 hydrolysis, urease, VP and nitrate reduction by any *Halomonas* strains. Mannitol was not fermented by any strain. Acid produced from D-glucose, xylose and L-arabinose by MW2a, A7, B14, C1, C4, C16, C20 and B5. Acid from xylose was generated by strains A10 and C11. Acid production reported from sacarose and fructose by MW2a, B14 and B5. Lactose is fermented only by A7. Strains MW2a, B14, C1, C4, C16, C20 and B5 utilized D-fructose, maltose, glycerol as carbon and energy sources. All the *Halomonas* strains showed negative results for mannitol (except C11), D-sorbitol and starch as energy and carbon source. MW2a used D-cellobiose and D-glucose as carbon and energy source.

Pontibacillus strain C13 produced catalase but not oxidase. Negative results for Simmon citrate, nitrate reduction, anaerobic conditions, and VP were documented. Gelatin, aesculine, and Tween 80 were not hydrolyzed. Urease activity was detected. Acid production from D-glucose, maltose, sucrose, and fructose was observed. The phenotypic characteristics of strain C13 are summarized and compared to the type strains of related *Pontibacillus* species in Table 1.7.

Table 1.7 Characteristics of strain C13 (*Pontibacillus* strain) and some other related species. Strains: *P. marinus* BH030004T; *P. chungwhensis* DSM 16287T. Data from Lim et al. (2005a), Lim et al. (2005b) and this study.

Characteristic	**C13**	***P. marinus***	***P. chungwhensis***
Pigmentation	Light green	Cream	Yellow
Gram reaction	+	+	+
Motility	+	+	+
Oxidase	-	+	-
Facultative anaerobe	-	-	-
Acid production from:			
D-Glucose	+	-	+
Maltose	+	+	+
Mannitol	-	-	-
D-Xylose	-	-	-
D-Fructose	+	+	-
Nitrate reduction	-	+	-
Hydrolysis of:			
Gelatin	-	-	-
Starch	-	-	+
Urea	+	-	-
Aesculin	-	+	-

Fatty acids analysis

Fatty acids were determined for various *Halobacillus* and *Halomonas* strains. All *Halobacillus* strains were dominated by branched fatty acids, in agreement with the literature (Monteoliva-Sánchez *et al*., 1989). The predominant fatty acids for *Halobacillus* strains were iso-$C_{15:0}$, anteiso-$C_{15:0,}$ $C_{16:0}$ and $C_{16:0}$ Methyl / iso-$C_{16:0}$ (Table 1.8). None of the standards (type strains) had the high content of $C_{18:1}$ cis 11 seen in all the isolates. Apparently there are some differences in the location of the double bond in the $C_{18:1}$ in the different *Halomonas* strains. Predominant fatty acids for *Halomonas* strains were $C_{16:0}$ and $C_{18:1}$ cis 11 and $C_{18:1}$ cis 9 (Table 1.9).

Table 1.8 Comparison of cellular fatty acid composition (%) of different isolated strains and the closely related *Halobacillus* species.

Fatty acids	***H. litoralis***	***H. karajensis***	**A12**	**A14**	**A17**	**A18**	**A19**	**A3**	**A4**	**C19**	**D1**	**D2**
$C_{14:0}$ methyl / iso												
$C_{14:0}$	1.12	0.56	1.75	2.42	1.98	2.38	2.58	3.01	2.90	2.03	2.07	1.98
$C_{14:0}$	NP	NP	0.72	0.66	0.57	NP	0.71	0.65	0.63	0.80	0.82	0.82
iso-$C_{15:0}$	8.16	6.80	10.3	10.42	8.15	9.16	9.40	10.6	10.29	11.71	11.77	12.49
anteiso-$C_{15:0}$	53.55	50.88	40.80	39.35	42.4	40.06	36.05	38.56	39.24	33.26	33.83	34.81
$C_{16:0}$ methyl / iso-												
$C_{16:0}$	5.18	5.03	10.6	13.1	11.98	16.36	14.29	15.45	15.36	8.54	8.53	7.65
$C_{16:0}$	5.61	5.27	12.49	10.65	9.87	9.09	10.31	10.39	10.32	14.06	13.55	14.22
iso-$C_{17:0}$	4.06	4.59	5.78	6.75	5.65	6.72	6.25	6.39	6.50	11.28	11.13	11.06
anteiso-$C_{17:0}$	22.32	26.86	17.11	16.23	19.41	16.22	15.20	14.51	14.76	16.73	15.76	15.09
$C_{18:0}$	NP	NP	0.46	0.42	NP	NP	1.61	0.44	NP	0.98	0.82	0.72

Values are percentages of total fatty acids. NP, not produced.

Table 1.9 Cellular fatty acid composition (%) of different isolated strains and the closely related *Halomonas* species.

Fatty acids	***C. maris-mortui***	***H. halmophila***	***H. eurihalina***	***H. halodurans***	**B14**	**B5**	**C1**	**C16**	**C20**	**C4**
$C_{10:0}$	1.06	NP	0.45	1.95	NP	1.24	NP	NP	NP	NP
$C_{12:0}$	4.79	2.02	2.03	2.18	2.44	2.70	1.99	1.98	1.71	2.28
$C_{14:0}$	0.91	0.94	NP	1.00	NP	NP	NP	NP	NP	NP
$C_{16:1}$ cis 7	NP	NP	NP	NP	3.03	3.18	NP	NP	NP	NP
$C_{16:1}$ cis 9	NP	23.57	10.50	26.74	NP	NP	3.95	3.68	4.38	4.63
$C_{16:0}$	48.41	46.54	42.03	48.09	54.78	54.48	53.27	52.80	47.56	55.56
$C_{17:1}$ cis 8	3.13	13.87	1.38	14.57	NP	0.58	NP	NP	NP	0.62
$C_{18:1}$ cis 9	NP	NP	0.44	NP	21.39	17.86	NP	NP	NP	NP
$C_{18:1}$ cis 11	NP	7.55	35.01	NP	NP	NP	23.88	22.71	34.02	25.49
$C_{18:0}$	1.58	0.81	1.07	0.83	NP	2.24	1.62	3.71	3.08	2.03
$C_{19:1}$ cis 11 /19: cy	10.49	1.00	2.99	1.34	5.87	5.76	5.48	3.16	5.12	5.28

Values are percentages of total fatty acids. NP, not produced.

Molecular characterization

A total of 73 DNA extractions were performed with the phenol/chloroform technique. Figures 1.10, 1.11 and 1.12 show examples of the quality of genomic DNA extractions.

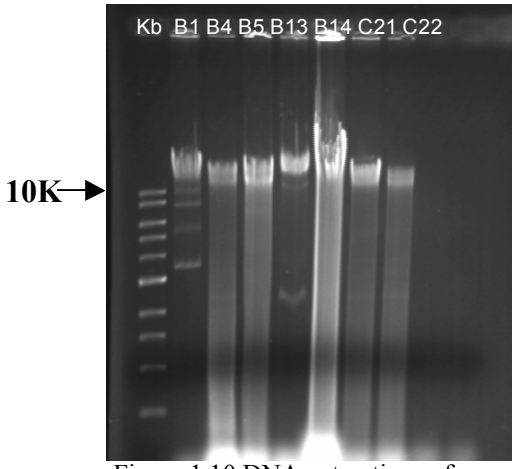

Figure 1.10 DNA extractions of prokaryotes isolated from *Avicennia germinans*.

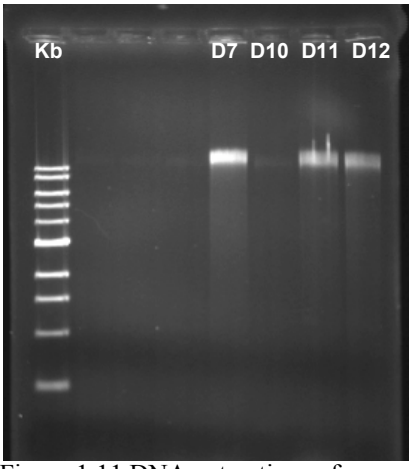

Figure 1.11 DNA extractions of prokaryotes isolated from *Avicennia germinans*.

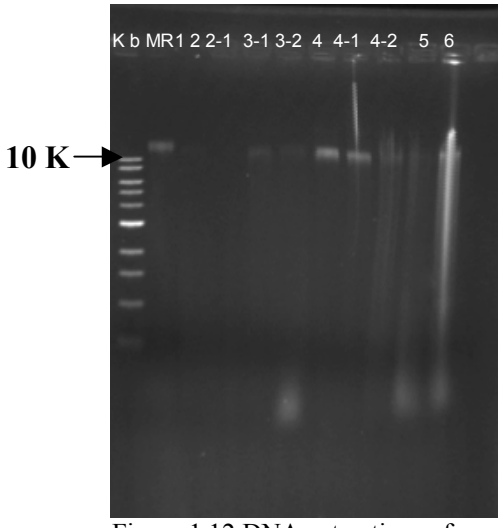

Figure 1.12 DNA extractions of prokaryotes isolated from red mangrove.

These genomic DNA's were used as templates for 16S rDNA amplification by PCR. This molecule is currently used for the molecular characterization of prokaryotic microorganisms. PCR amplicons were of approximately 873bp (Figures 1.13, 1.14, 1.15).

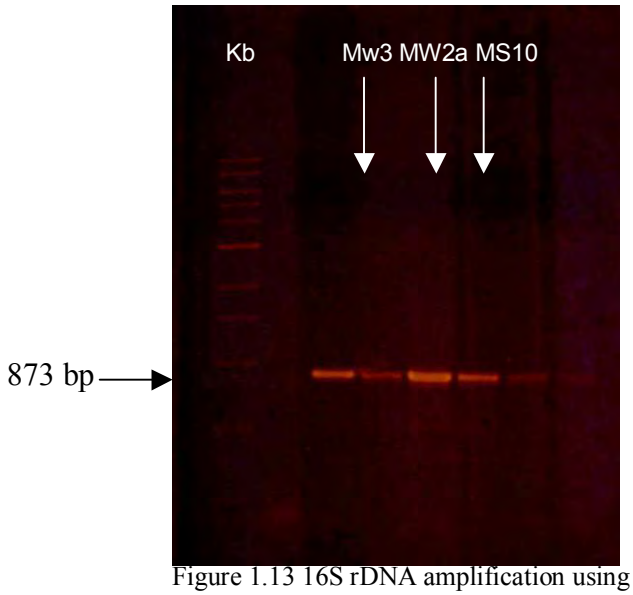

Figure 1.13 16S rDNA amplification using universal primers 1392-R, 519-F of strains isolated from *Avicennia germinans*.

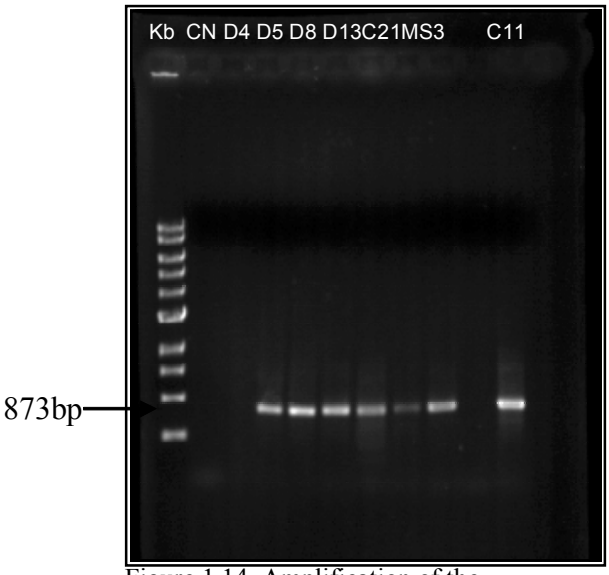

Figure 1.14. Amplification of the 16SrDNA using forward primers Univ-519-F and reverse primer Univ-1392-R.

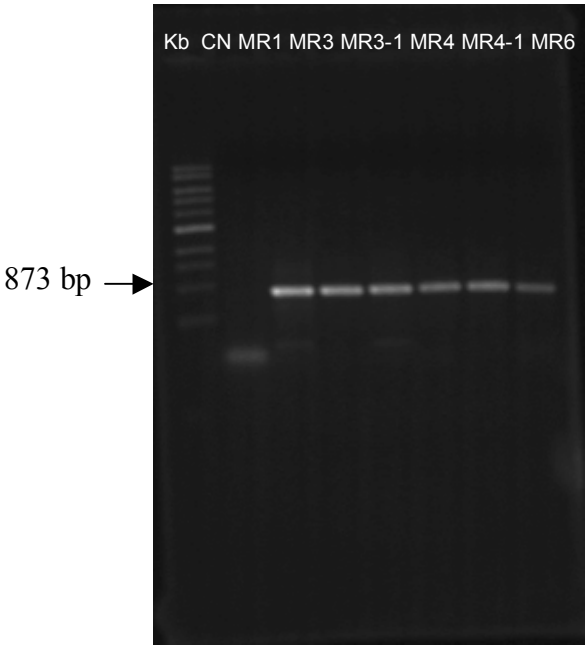

Figure 1.15 16S rDNA amplification using universal primers 1392-R, 519-F of strains isolated from red mangrove

RFLP was performed to analyze the variable lengths of DNA fragments that result from the digestion of genomic DNA with a restriction endonuclease. It recognizes a specific site within the DNA called the restriction endonuclease recognition site. The presence or absence of such sites in the DNA samples will generate variable lengths of DNA fragments, which will be separated using 3% low melting agarose gel. These allow us to differentiate between different strains. RFLP analysis was performed for all isolated strains from black mangrove and red mangrove. Figures 1.16, 1.17 and 1.18 show the restriction patterns using *Msp I* and *Hae III* for *Avicennia germinans* strains.

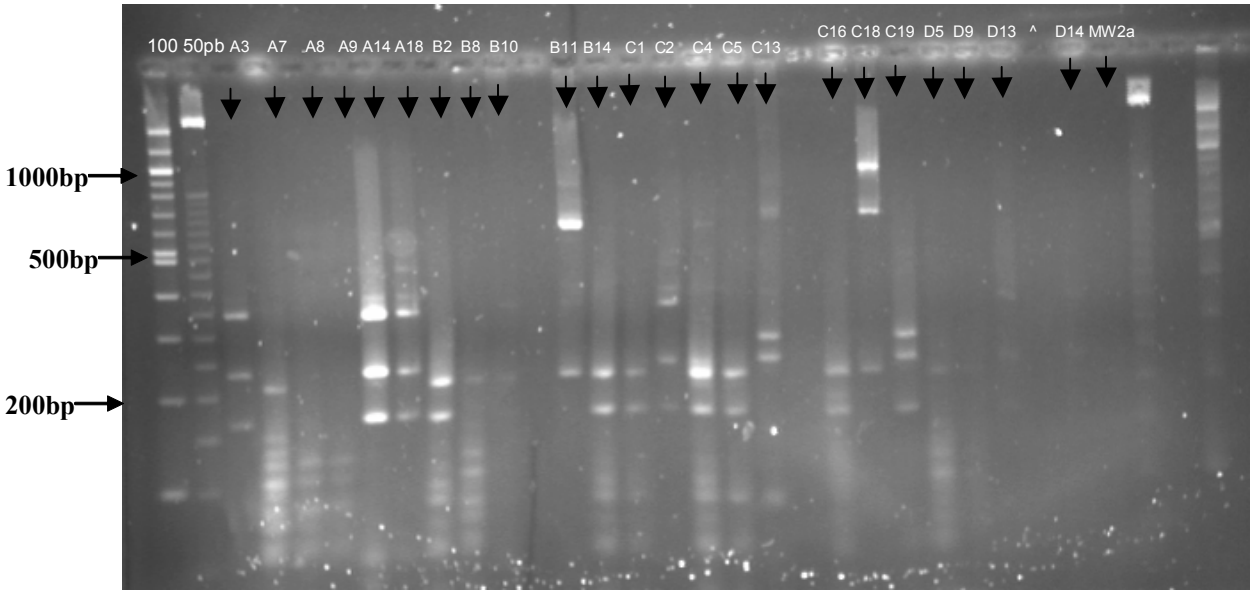

Figure 1.16 RFLP of some strains isolated from black mangrove using *Msp* I and *Hae* III enzymes in agarose low melting 3%.

Similar patterns restriction patterns were observed in Figure 1.17 for A3, C2, D13, and D14; A7, A8 and A9; D5, B8 and D9; B14 and B2; C1 and C5; B11 and C18. Only A14, A18, C4, C13, C16 and C19 presented different restriction patterns. The A3 group corresponded to *Halobacillus* strains. The A7, D5, B14 and C1 group

corresponded to the genus *Halomonas,* and the groups of strain C18 were representatives of the genus *Staphylococcus*.

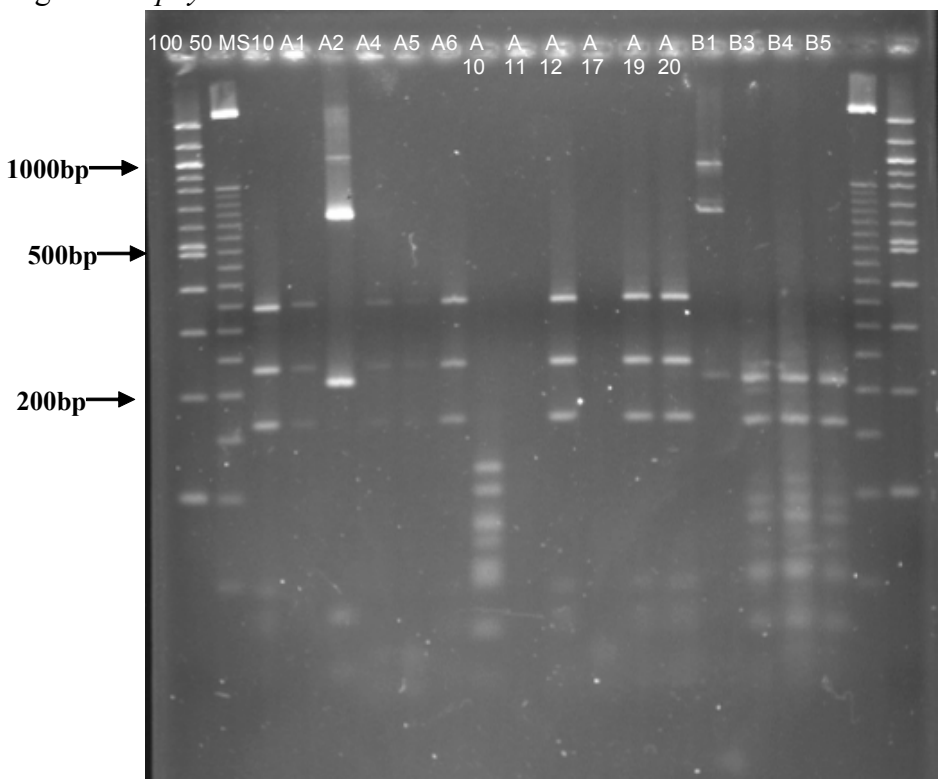

Figure 1.17 RFLP of some strains isolated from black mangrove using *Msp* I and *Hae* III enzymes in agarose low melting 3%.

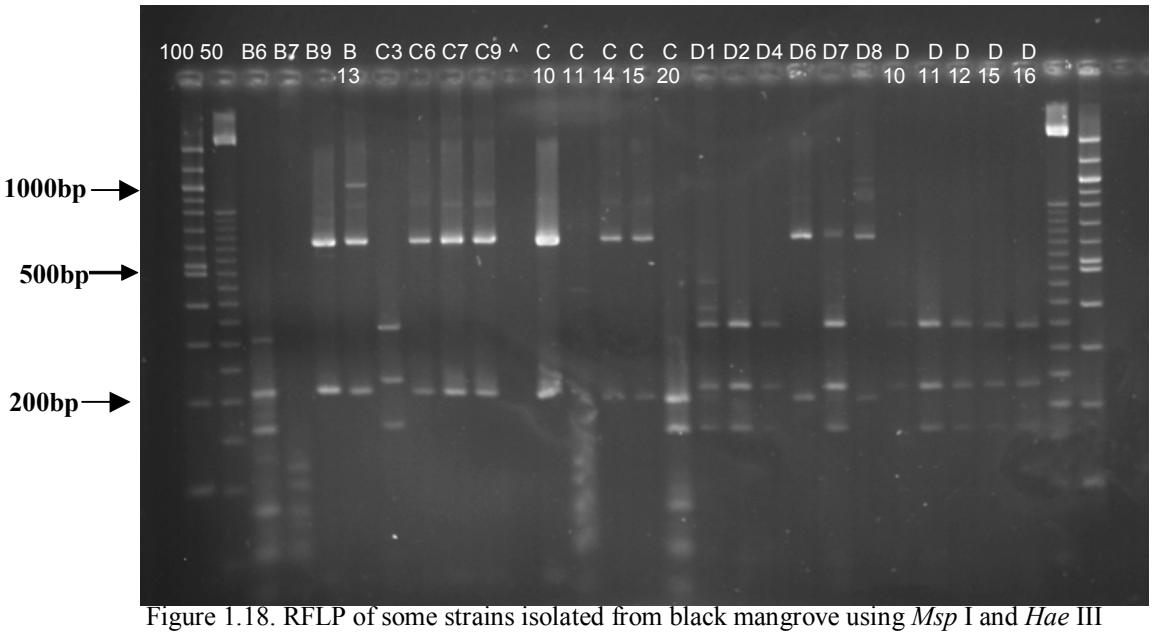

Figure 1.18. RFLP of some strains isolated from black mangrove using *Msp* I and *Hae* III enzymes in agarose low melting 3%.

Similar restriction patterns were observed in Figure 1.18 such as A1, A4 and A5; D2, C3, D4, D10, D11, D12, D15, and D16; MS10, A6, and A12. Other similar restriction patterns were observed in A2 and B13; A19 and A20; B3, B4, and B5; and D8, C6, C9, C7, C10, C14, C15, and D6. Only A10, B1, B6, B7, B9, C11, C20, and D1 show different patterns.

The A1, D2, MS10, and A19 groups corresponded to *Halobacillus* representative strains. On the other hand, the groups of D8 and A2 are *Staphylococcus* representative strains. The only *Halomonas* group was B5.

A total of 63 genomic DNA's from different strains were screened using *Msp* I and *Hae* III restriction enzymes. The *Halobacillus* genera represent 41% of the isolates. *Halomonas* showed a frequency of 0.30. The *Staphylococcus* group had a frequency of 0.25 and *Pontibacillus* showed a frequency of 0.03. Representatives from each of the restriction pattern groups were selected for sequencing.

A total of nine strains were isolated for the control group (red mangrove). A RFLP was performed for screening of the red mangrove isolates. Figure 1.19 shows the restriction patterns for red mangrove isolates using *Msp I* and *Hae III* restriction enzymes. Similar restriction patterns were observed for MR 1, MR3-1 and MR6; for MR2-1 and MR4-1; for MR3 and MR4; and for MR3-2 and MR 5. *In silico* analysis using BLAST showed that five of the sequences are related to *Halobacillus*. This genus had the highest frequency of isolation (55%). The other four sequences represented the genus *Staphylococcus* having a frequency of 44%. Representatives from each of the restriction pattern groups were selected for sequencing.

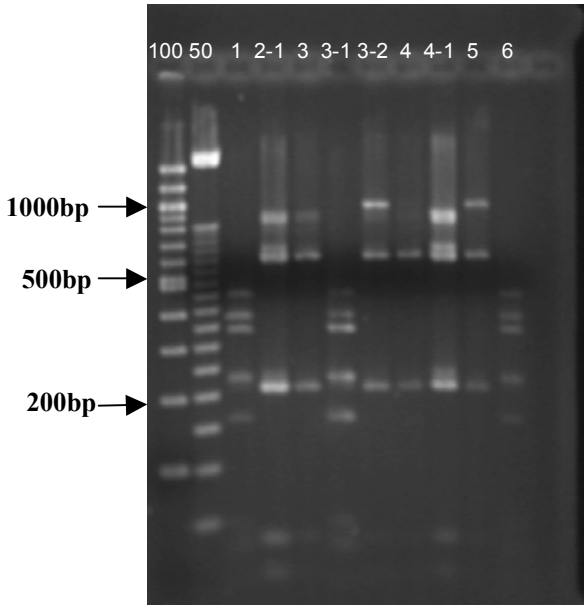

Figure 1.19 RFLP of prokaryotes isolated from the leaf of *R. mangle*. *Msp I* and *Hae III* enzymes using 3% low melting agarose.

Phylogenetic analysis

The phylogenetic relationship of these sequences was determined by using neighbor joining analysis. Phylogenetic analyses using the 16S rRNA gene revealed that strains A4, A19, A3, D2, A14, MS10, D1, A12, C19, and A18 are within the *Halobacillus* species phylogenetic branch, and the most closely relative was *Halobacillus aidingensis* JCM 12771^{T} (Figure 1.20).

Strains C1, C4, B5, B14, C20, C16, and MW2a were closely related to *Halomonas marisflavi* and *Halomonas indalinina* forming a branch in this cluster (Figure 1.21). Isolates C11, B7, A7, and A10 formed an independent branch distant to the other *Halomonas* species included in the analysis of the 16S rRNA regions.

The only *Pontibacillus* representative, strain C13, was closely related to *Pontibacillus chungwhensis* forming a branch in this cluster with a bootstrap value of 99% (Figure 1.22). Strains B13, A2, D8, and C18 were closely related to *Staphylococcus arlettae* forming a branch in this cluster (Figure 1.23).

Isolates from the red mangrove demonstrated low diversity. Phylogenetic analysis revealed that strain MR-1 formed an independent branch inside the *Halobacillus* cluster. When we compare MR-1 versus the *Halobacillus* strains isolated from the black mangrove, MR-1 is inside the cluster of these black mangrove isolates (Figure 1.24). Phylogenetic analysis revealed that the *Staphylococcus* strains isolated from red mangrove (MR 2-1, MR 3-2, and MR 4) are inside the cluster of the black mangrove strains and both closely related to *Staphylococcus arlettae* forming a branch in this cluster with a bootstrap value of 80% (Figure 1.25).

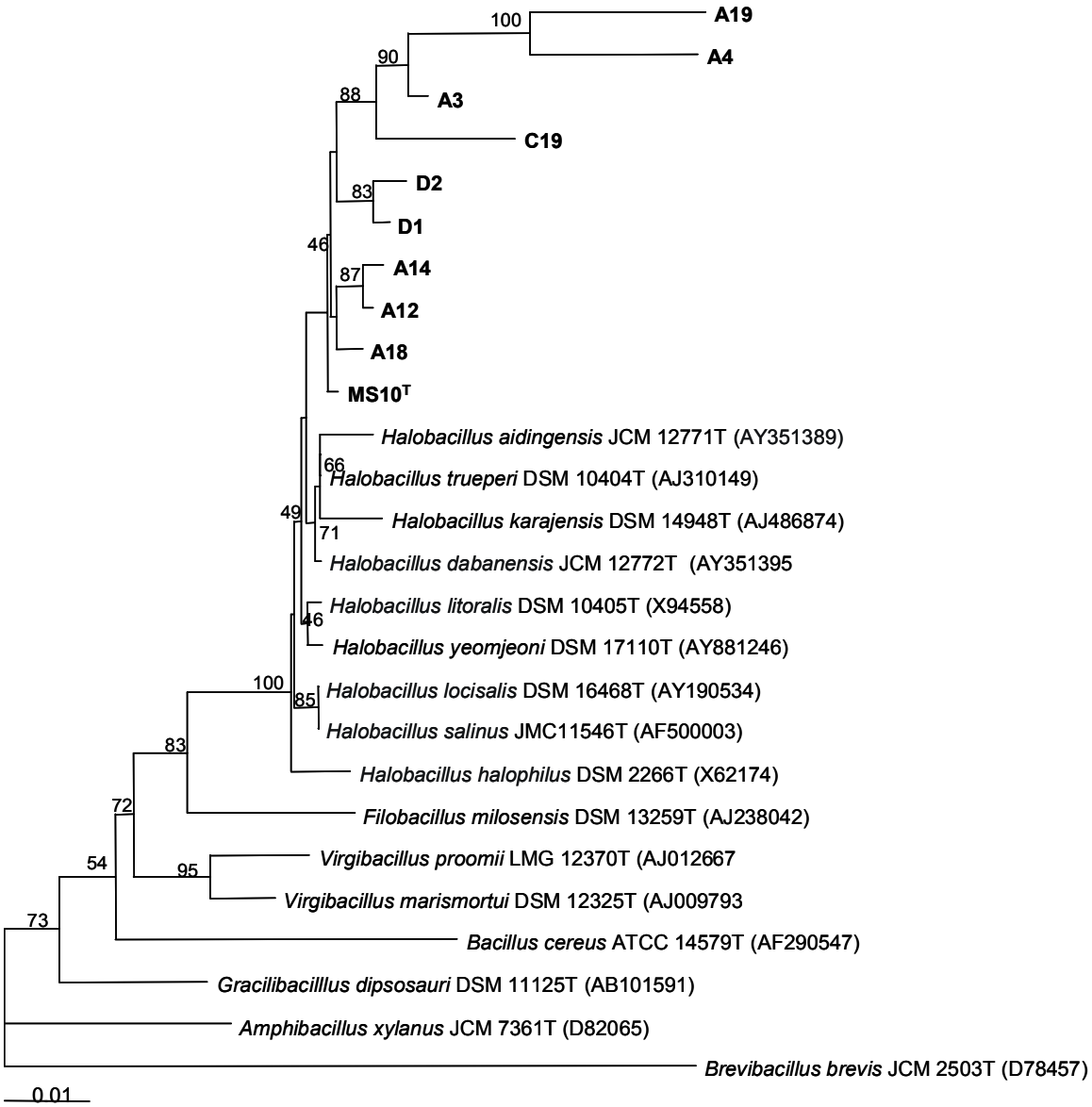

Figure 1.20 Neighbour-joining distance tree using the 16S rRNA sequences of *Halobacillus* strains isolated from black mangrove and closely related species. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown. *Brevibacillus brevis* JCM 2503T was used as the outgroup.

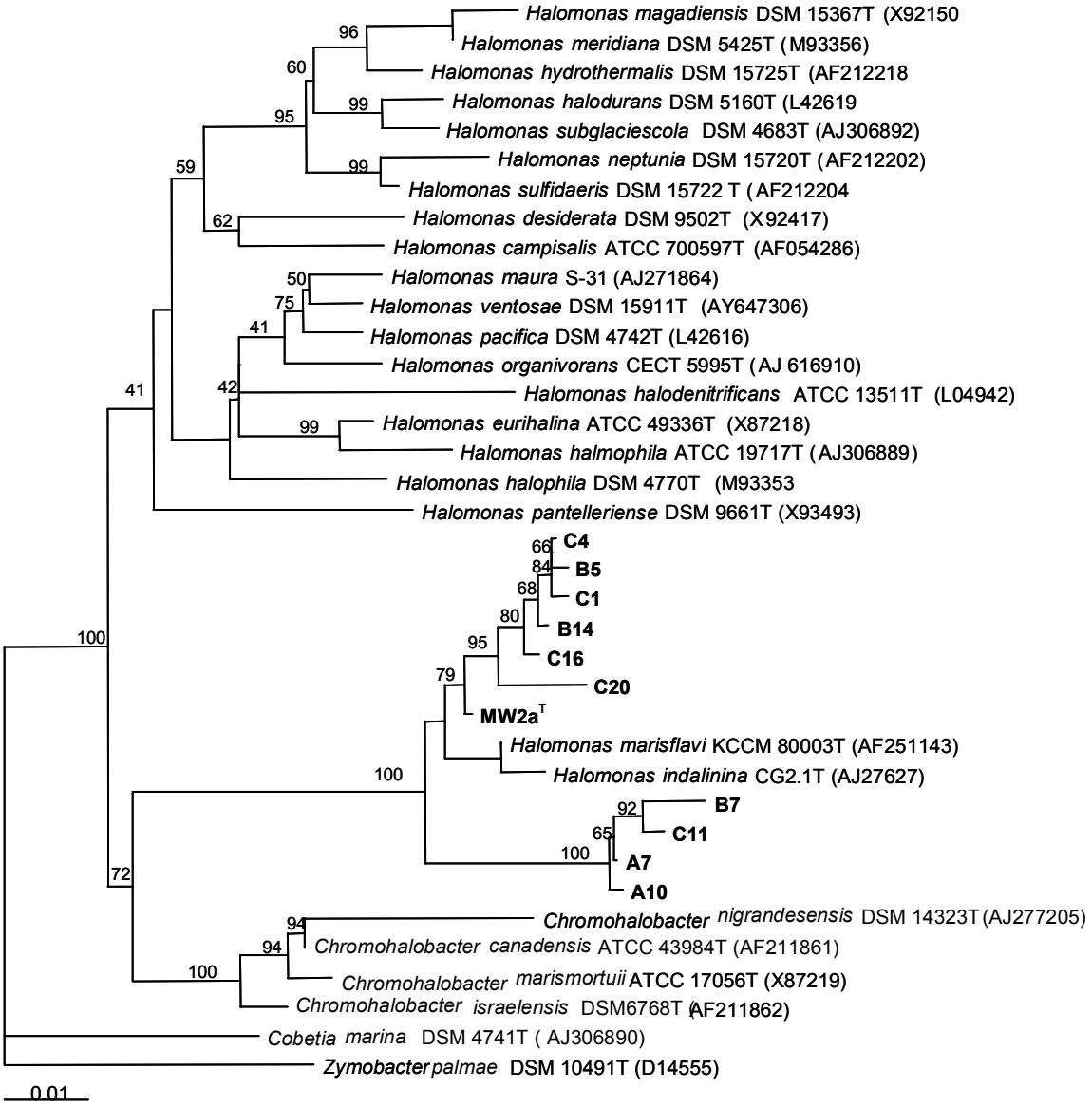

Figure 1.21 Neighbour-joining distance tree using the 16S rRNA sequences of *Halomonas* strains isolated from black mangrove and closely related species. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown *Zymobacter palmae* DSM 10491T was used as the outgroup.

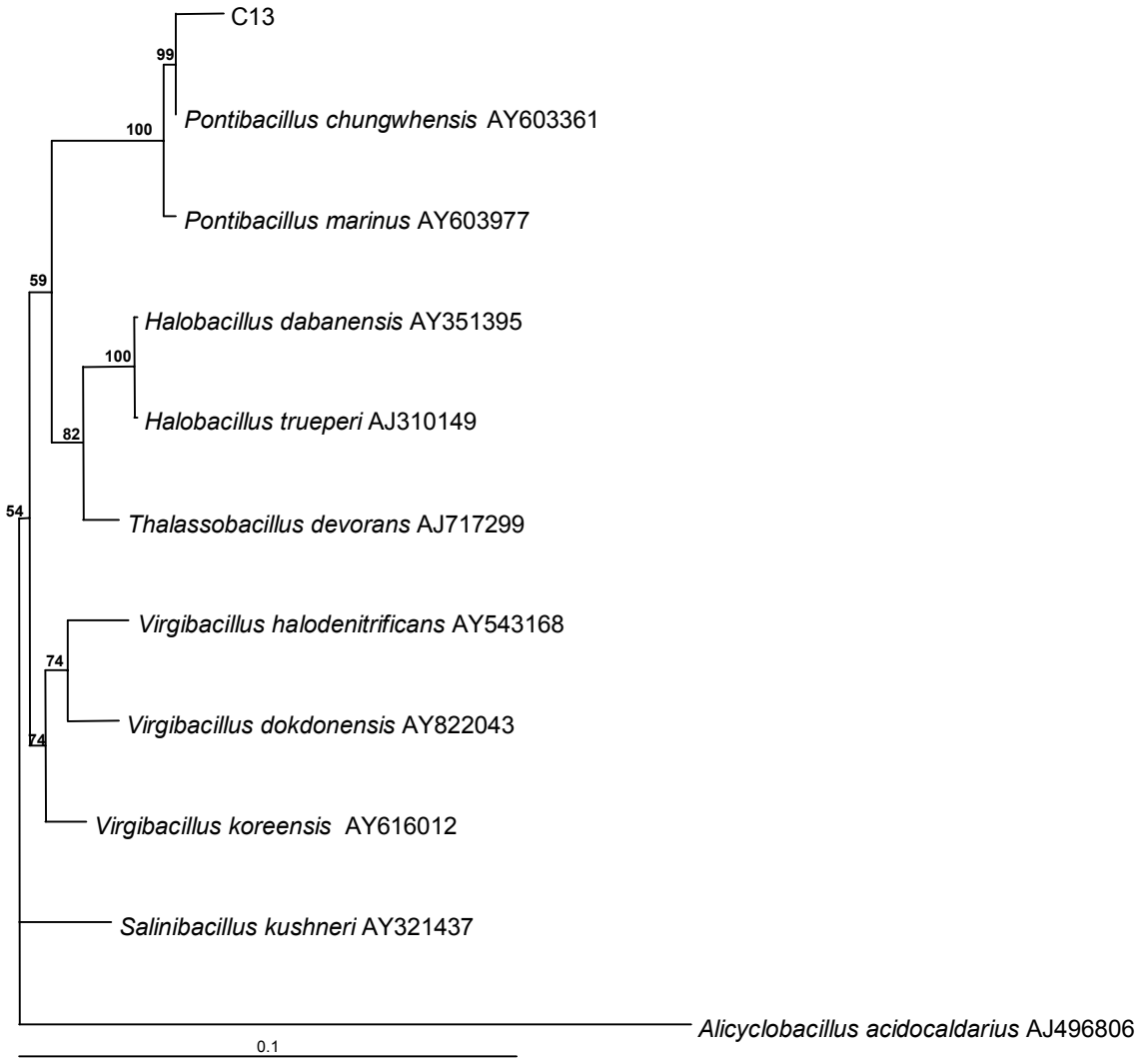

Figure 1.22 Neighbour-joining distance tree using the 16S rRNA sequences of *Pontibacillus* strains isolated from black mangrove and closely related species. Bar represents 1 substitution per 10 nucleotides. Bootstrap values higher than 40% are shown. *Alicyclobacillus acidocaldarius* AJ496806 was used as the outgroup.

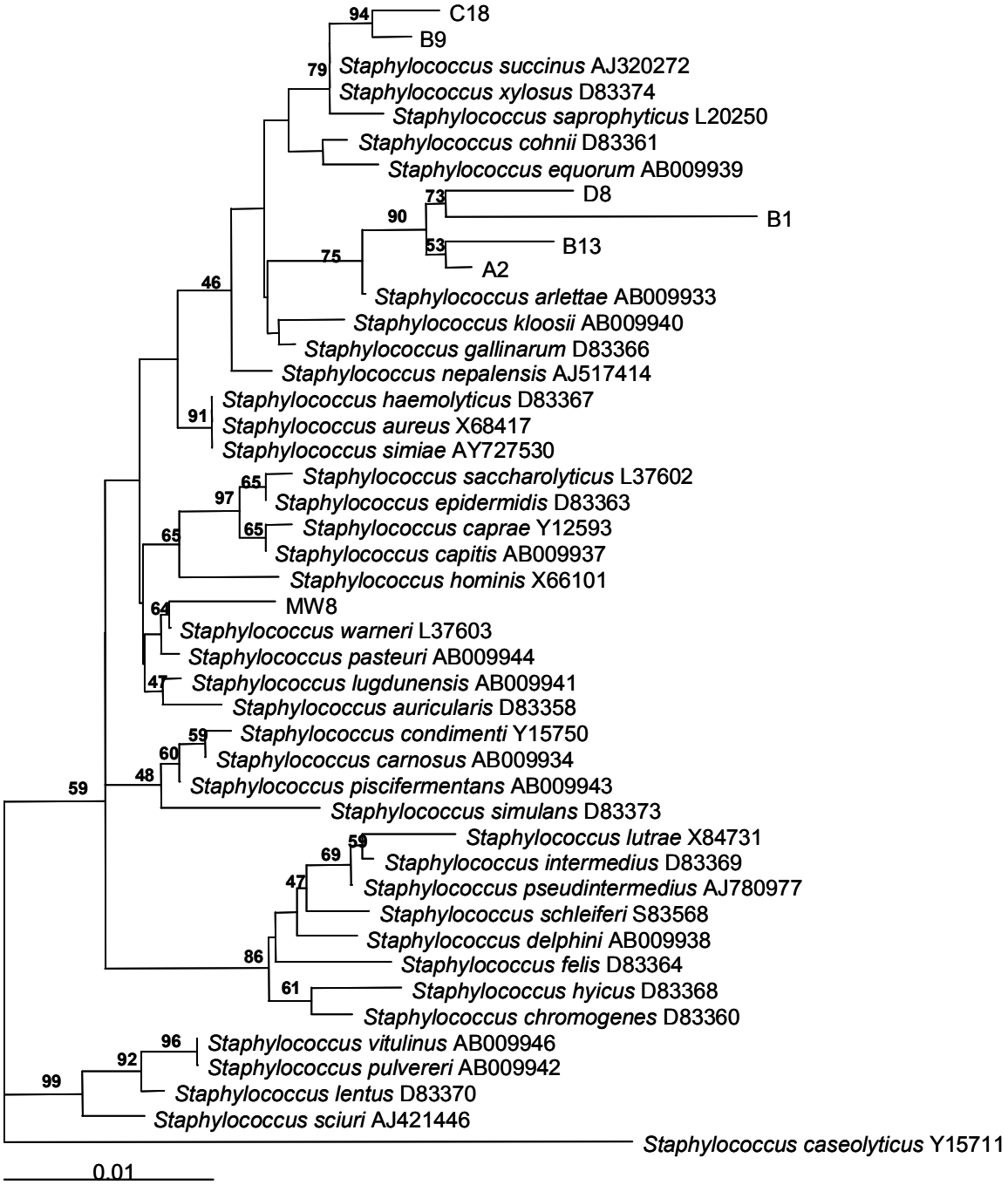

Figure 1.23 Neighbour-joining distance tree using the 16S rRNA sequences of *Staphylococcus* strains isolated from black mangrove and closely related species. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown. *Staphylococcus caseolyticus* Y15711 was used as the outgroup.

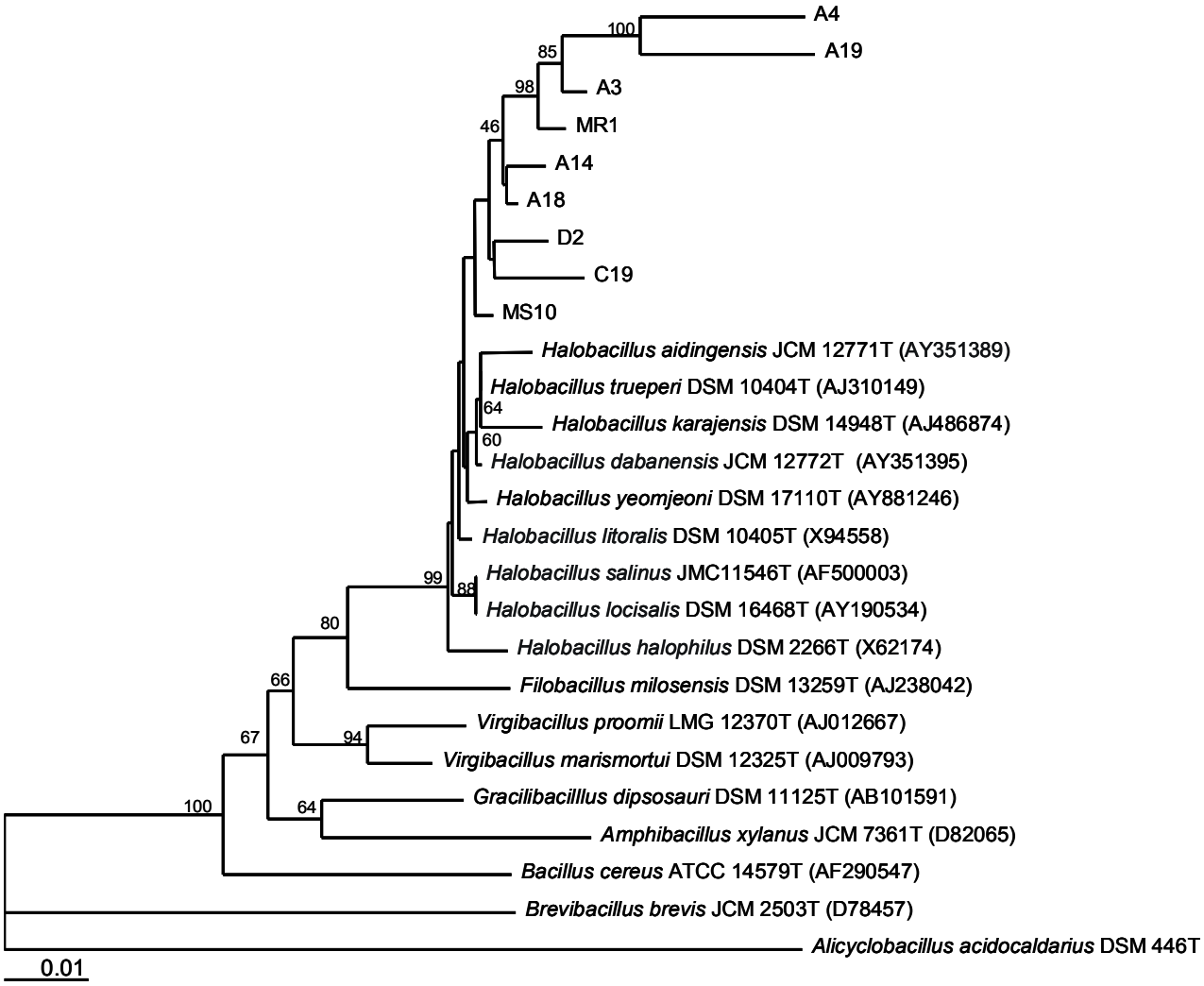

Figure 1.24 Neighbour-joining distance tree using the 16S rRNA sequences of *Halobacillus* strains isolated from red and black mangrove and closely related species. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown. *Alicyclobacillus acidocaldarius* DSM 446T was used as the outgroup.

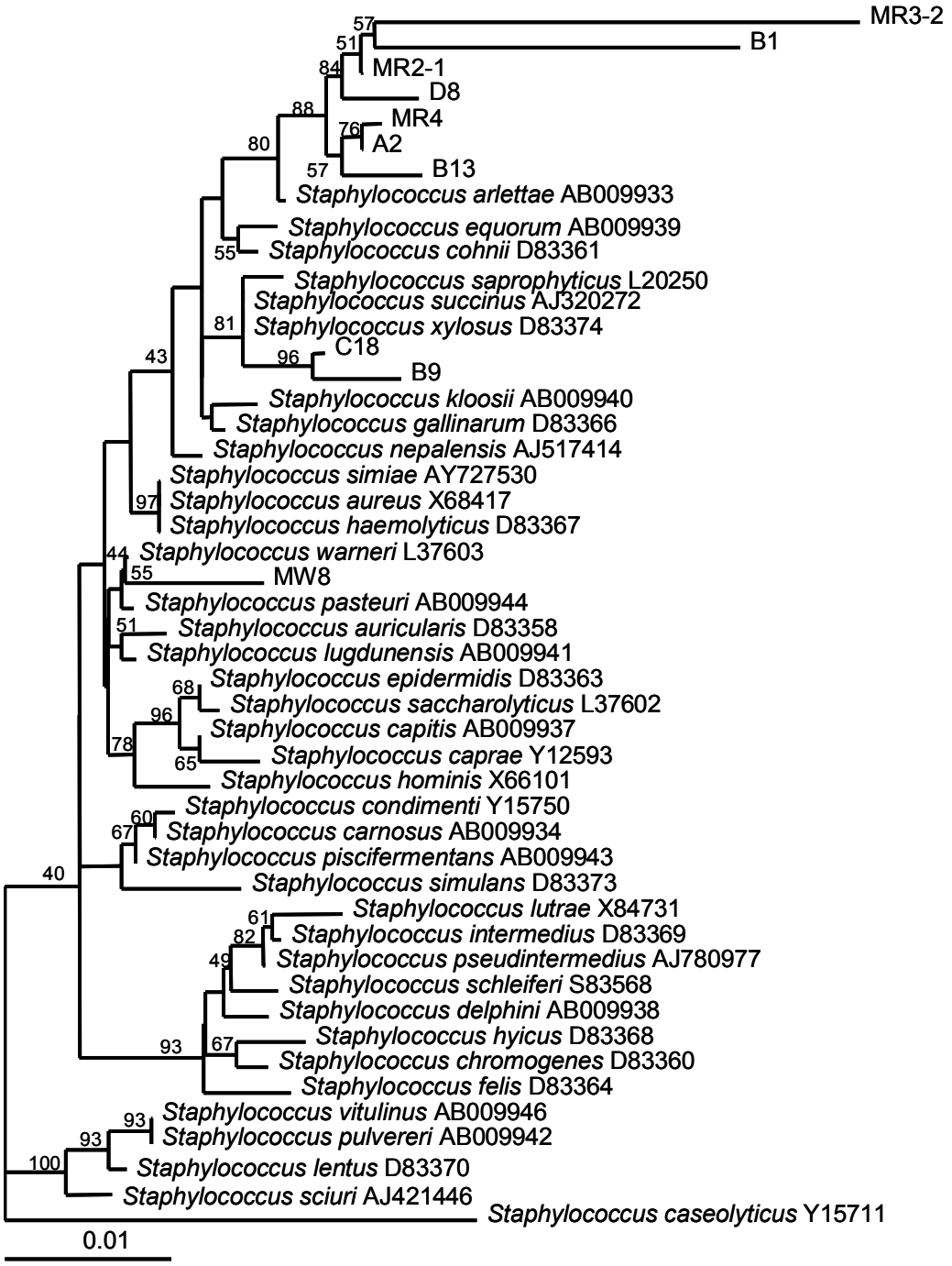

Figure 1.25 Neighbour-joining distance tree using the 16S rRNA sequences of *Staphylococcus* strains isolated from black and red mangrove and closely related species. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown. *Staphylococcus caseolyticus Y15711*was used as the outgroup.

Discussion

The objective of this part of the research was focused on the isolation of the prokaryotic halophilic microorganisms from the leaf surface of the black and red mangroves. This study represents the first attempt to determine the prokaryotic flora present in mangrove leaves especially in the Caribbean. Since the temperature range of the mangrove forests at the moment of the survey were 30-37°C, we selected 30°C as the initial incubation temperature. The Seghal and Gibbons medium was selected for the isolation of halophilic microorganisms because it is commonly used for the isolation of halophilic prokaryotes (Montalvo-Rodríguez *et al*., 1998). The original medium uses NaCl at 25% (w/v) but it was modified to 15% NaCl for the purpose of this study.

A large number of isolates were obtained growing in SG 15% NaCl medium. Based on macroscopical characteristics, most colonies showed circular, entire and flat macroscopical morphology. Based on microscopical characteristics most of the isolates were Gram-positives rods. The isolates were distributed among five genera: *Halomonas, Halobacillus, Pontibacillus, Bacillus,* and *Staphylococcus.* Their characterization was based on polyphasic taxonomy that included physiological, biochemical and molecular characterization. The molecular characterization was performed using the 16S rRNA gene.

It was found that strains belonging to *Halobacillus* were the most diverse and frequent (0.41). They were also isolated in high frequencies (0.55) from the red mangrove, which was used as a negative control due to the absence of salt crystals on the surface of the leaves. Typically, these strains show cream-brown pigments and long thin rods. Physiological analysis revealed that they do not grow at more than 50°C or above

20% NaCl. Optimal growth conditions were around 5-10% NaCl, 30-37°C and a pH range from 7 to 8. Besides the ability to produce ellipsoidal or spherical endospores at central or subterminal position, one of the main features of the genus *Halobacillus* is the cell-wall peptidoglycan type based on L-Orn-D-Asp, which is different from the meso-diaminopimelic acid or L-lysine type cell-wall peptidoglycan present on related genera (Shida *et al*., 1997; Yoon *et al*., 2003). Strain MS10 (a representative of the isolated *Halobacillus* strains) presented this type of cell-wall characteristic (see next chapter of this work). Phylogenetic analysis using the 16S rRNA gene revealed that strains A4, A19, A3, A14, A18, D2, C19, MS10, A12, D1, and A17 are within the *Halobacillus* cluster, and the most closely relative is the *Halobacillus aidingensis* JCM 12771T (Figure 1.20).

Various studies demonstrate that *Halobacillus* species can be isolated from solar salterns and salt lakes around the world. *Halobacillus dabanensis* and *Halobacillus aidingensis* (Liu *et al*., 2005) were isolated from salt lakes in Xinjiang, China. Other species were isolated from salt lakes East Sea in Korea (Yoon *et al*., 2005), in Yellow Sea in Korea (Yoon *et al*., 2004), and in marine solar saltern in Korea (Yoon *et al*., 2005b). Other studies have demonstrated the presence of *Halobacillus* species from saline soils in the Karaj region, Iran (Amoozegar *et al*., 2003). The common abundance of this genus in saline environments (like solar salterns) together with the ability to produce spores could explain why those isolates were present in the leaf surface of *Avicennia germinans* and *Rhizophora mangle* as well. Wind, birds, insects, and rain could be possible vectors that can transfer these strains from the soil or the salterns to the leave surfaces. However, there might be species of *Halobacillus* that are part of the halophilic community present on the leaf surface of black mangrove due the halophilic

microenvironment that the leaf can offer. Additional experimental strategies are needed to determine if these halophilic strains are unique of this microenvironment.

The second most frequent genus found in this study was *Halomonas* (0.30). Typically, these strains showed orange pigments and are short rods. However, the morphological characteristics of the isolated strains are different from those previously described (Yoon *et al*., 2001; Bouchotroch *et al.,* 2001; Garcia *et al.,* 2004; Lim *et al.,* 2004; Lee *et al.,* 2005; Martínez-Cánovas *et al.,* 2004, 2005; Martínez-Checa *et al.,* 2005; Mata *et al.,* 2002; Mormile *et al.,* 2000; Ventosa *et al.,* 1998; Vreeland *et al.,* 1980; Yoon *et al.,* 2001; 2002). They showed a lot of similarity to *Chromohalobacter marsmortui,* which is a closely related to the genus *Halomonas.* Physiological analysis revealed similar responses for the isolated strains such as salinity range (5 to 20% NaCl) and their inability to grow at more than 37°C. For most strains, the optimal pH condition was around 7 to 8, salinity 5-10% NaCl and temperature from 25-37°C. However, restriction analysis of 16S rDNA amplicons among *Halomonas* strains revealed high heterogeneity among isolated strains. (Figure 1.16). Furthermore, fatty acid analysis showed a high content of $C_{18:1}$ cis 11 in all *Halomonas* isolates, a characteristic that has not been reported for type strains. Phylogenetic analysis using the 16S rRNA gene revealed that these isolated strains form two distinguishable branches inside one of the *Halomonas* clusters and their most closely relative is *Halomonas marisflavi* KCCM 80003T (Figure 1.21).

Halomonas species have been isolated mostly from saline or hypersaline environments such as Cabo de Gata (Almeria Spain), Fuente de Piedra, a saline-wetland wildfowl reserve in Málaga (Southern Spain), soil around a Bolivian hypersaline lake,

mineral pool of Campania Region (Italy), Antarctic saline lakes, solar salterns in Korea, and a soda lake at the East African Rift Valley (Arahal *et al*., 2002; Bouchotroch *et al*., 2001; Dobson and Franzmann, 1996; Duckworth *et al*., 2000; García *et al*., 2004; James *et al*., 1990; Lim *et al*., 2004; Lee *et al*., 2005; Martínez-Cánovas *et al*., 2004, 2005; Martínez-Checa *et al*., 2005; Mata *et al*., 2002; Mormile *et al*., 2000; Quillaguaman *et al*., 2004; Romanenko *et al*., 2002; Ventosa *et al*., 1998; Vreeland *et al*., 1980; Yoon *et al*., 2001; 2002) or from unusual habitats like dry mural paintings (Heyrman *et al*., 2002), deep-sea hydrothermal vents (Kaye *et al*., 2004), and from a municipal sewage works (Berendes *et al*., 1997). Phylogenetic analysis using the 16S and 23S rRNA sequences and phenotypic studies demonstrated that this genus is very heterogeneous (Arahal *et al*., 2002; Mata *et al*., 2002). The *Halomonas* strains isolated in this study were only associated to the leaf surface of *Avicennia germinans.* This genus was not obtained from samplings of red mangrove. This might indicate the preference of this genus to saline or hypersaline environments. Also, all the *Halomonas* strains isolated in this study seems to have a more closely phylogenetical relationship between each other than other species of the genus (Figure 1.21). This result suggests the possibility of the development of unique mechanisms of adaptation to be part of the halophilic microenvironment that the leaf surface of *A. germinans* provides.

Different potential new species within the genera *Halomonas* and *Halobacillus* were obtained as a result from this study. For this reason, a collaborative effort was established with Dr. Antonio Ventosa at the University of Sevilla in Spain and Dr. Aharon Oren at the University of Jerusalem in Israel to perform essential analysis

required for the determination of the identity of the isolates. These analyses are discussed in more detail in the next chapter of this work.

The third most frequent (0.25) genus found was *Staphylococcus*. Typically the macroscopical and microscopical characteristics observed were a diversity of yellow tones and white pigments Gram-positive cocci-shaped. Physiological analyses revealed that *Staphylococcus* strains are halotolerant, mesophilic and an alkaline optimal pH. RFLP analysis among *Staphylococcus* strains revealed a high variety of restriction patterns. Phylogenetic analysis using the 16S rDNA revealed that *Staphylococcus* strains were closely related to *Staphylococcus arlettae* forming a branch in this cluster (Figure 1.23).

Several studies revealed that *Staphylococcus* representatives have been isolated from marine fish in Japan as the cause of disease in red sea bream *(Chrysophrys major)* and yellowtail *(Seriola quinqueradiata)* (Kusuda and Sugiyama, 1981). Since 1997, several species of staphylococci have been isolated either from sea bass or sea bream grown at different farming locations in Greece. The most frequent were *Staphylococcus epidermidis, S. xylosus, S. lentus, S. capitis, S. lugdunensis, S. hominis, S. warneri, S. cohnii, S. chromogenes,* and *S. aureus*.

Because most *Staphylococcus* species are related to human and animal tissue associated to aquatic ecosystems such as fishes or aqueous animals, strains of this genus could be present in the decomposition of those marine animals that could be present around these wetlands and mangrove forests.

Only one *Pontibacillus* strain (C13) was isolated from the tissue sampled. This strain showed light green pigments and were Gram-positive rod. Physiological analysis

revealed that the *Pontibacillus* strain is halophilic, mesophilic and neutral to alkaline pH. Phylogenetic analysis based in 16S rDNA revealed that *Pontibacillus* strain C13 was closely related to *Pontibacillus chungwhensis* forming a branch in this cluster with a bootstrap value of 99%. At the moment, only two species have been described for this genus. Both of them were isolated from solar salterns in Korea (Lim *et al*., 2005a; Lim *et al*., 2005b). The presence of C13 in the black mangrove leaf surface could become from the saline soil surface of the mangrove forest. Other possible explanation is that *Pontibacillus* representatives are spore-forming and some of those spores could land on the leaf surface of *A. germinans*.

A low diversity was found on the leaves of the red mangrove using SG at 15% NaCl. A total of nine strains were isolated using this medium. Several strains showed entire, circular and flat macroscopic morphology. Microscopic characteristic revealed that all nine strains were Gram-positive and the majority was rod-shaped. RFLP analysis using *Msp I* and *Hae III* restriction enzymes demonstrated only four different restriction patterns. BLAST results showed that five strains were related to *Halobacillus* (0.55) and four strains were *Staphylococcus* (0.44). Phylogenetic analysis revealed that the *Halobacillus* strains formed an independent branch inside the *Halobacillus* cluster (Figure 1.24). A phylogenetic comparison between the isolated *Halobacillus* strains from red mangrove versus black mangrove demonstrated that red mangrove strains are inside the cluster of the black mangrove isolates. Phylogenetic analysis revealed that those *Staphylococcus* strains isolated from red mangrove are inside the cluster of the black mangrove strains and both closely related to *Staphylococcus arlettae* forming a branch in this cluster with a bootstrap value of 80%.

A possible explanation why these microorganisms were found on the leaf surface of *Rhizophora mangle* is related to because the roots trap mud and silt that flows with the tide, thus gradually increasing the soil around them. They are found closer to the water than the other mangroves in the community. The common abundance of this genus in saline environments (like solar salterns) together with the ability to produce spores could explain why those isolates were present in the leaf surface of *R. mangle*. Wind, birds, insects, and rain could be possible vectors that can transfer these strains from the soil or the salterns to the leave surfaces. *Rhizophora mangle* excludes the salt in seawater at the root-substratum interface. On the other hand the black mangrove and the white mangrove (*Laguncularia racemosa*) are able to take up seawater through their roots, but they excrete excess salt through pores, or salt glands, located on the surface of leaves.

Phylogenetic analysis revealed that all strains isolated from black mangrove could represent a unique community from this microenvironment. Halophilic and halotolerant microorganism such as *Halobacillus*, *Halomonas, Staphylococcus* and *Pontibacillus* representatives were isolated. This study about prokaryotic diversity in the leaf surface of black mangrove is the first approach to survey the community present in such microenvironment. The tools applied in this research were phenotypic, genotypic, and chemotaxonomic techniques. It guides us to analyze and identify the identity of those microorganisms. The polyphasic taxonomy applied in this study was suitable to reach our objectives of determine the prokaryotic ecological diversity in such ecological niches.

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Part II. Characterization of two new species *Halobacillus puertoriconensis* (MS10^T) and *Halomonas avicenniae* (MW2a^T) from the leaf surface of *Avicennia germinans*

Material and Methods

I. Sample processing and isolation of halophilic bacteria

Strains MS10^T and MW2a ^{T} were isolated from the surface of black mangrove leaves. Leaves (n = 48) from *Avicennia germinans* trees (n = 4) growing near the solar salterns of Cabo Rojo, Puerto Rico were obtained and printed against agar plates containing Seghal-Gibbons medium at 15% (w/v) NaCl. This procedure allowed the transfer of the microorganisms present in the salt crystals and the surface of the leaves to the growth medium. Plates were incubated at 30ºC. After 3 days of incubation, the leaves were removed aseptically and colonies were then selected and purified by the quadrant streak plate method. Pure cultures were transferred into SG medium for further biochemical and molecular analysis.

II. Morphological and cultural characterization

The morphology of cells was examined by Nomarsky technique and scanning electron microscopy (SEM) at logarithmic phase of growth under optimal conditions. Electron microscopy procedures were performed as previously described (Díaz-Muñoz and Montalvo-Rodríguez, 2005). Gram stains were performed using both heat-fixed smears and smears fixed in 5% acetic acid (Dussault, 1955). Macroscopic properties were documented using the classical characterization of colony appearance.

III. Physiological characterization and growth curves

Optimal conditions for growth were determined by growing MW2a^T and MS10^T in SG broth supplemented with 0, 5, 10, 15, 20, 25 and 30% (w/v) NaCl at temperatures of 20, 25, 30, 35, and 40°C, respectively. The pH range for the isolate was tested in SG medium with 5% NaCl adjusted to the following pH values: 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. The cells were cultivated with constant agitation (150 rpm) and growth was monitored by measuring absorbance at 600 nm. The pH tolerance was tested in SG broth buffered with 20 mM MES (pH 5.0, 5.5), PIPES (pH 6.0, 6.5), tricine (pH 7.0, 7.5, 8.0) or TAPS (pH 8.0, 9.1) (Montalvo-Rodríguez *et al*., 2000). All conditions were recreated in duplicates.

IV. Biochemical tests

All biochemical tests were carried out at 30°C. Catalase activity was determined by adding a 1% (w/v) H_2O_2 solution to colonies on SG agar medium. Oxidase test was performed using the Dry Slide (Difco) biochemical test (Montalvo-Rodríguez *et al*., 1998). Hydrolysis of starch, Tween 80, and aesculin were determined as described by Cowan and Steel (1965) with the addition of basal salts to the medium. Hydrolysis of gelatin and production of urease were determined according to Cowan and Steel (1965). Citrate utilization was determined on Simmon's citrate medium supplemented with basal salts. Acid production from carbohydrates was determined using phenol red base supplemented with 0.7% of the carbohydrate and basal salts. Motility, H2S production, and indole were determined using SIM medium supplemented with basals salts. Production of H_2S was determined using the API 20E system as previously described

(Yoon *et al*., 2001). Growth under anaerobic conditions was determined by incubating strains in an anaerobic chamber in SG medium with 5% NaCl. Tests for sugar fermentation and enzymes (qualitative) were carry out using API 20 NE and API ID32E (bioMérieux) using the inoculated fluid at 5% of NaCl and incubated at 30ºC. Nutritional features were determined using the Koser medium (1923) as modified by Ventosa *et al*. (1982), (%, w/v): NaCl, 5.0; KCl, 0.2; $MgSO_4.7H_2O$, 0.02; KNO_3 , 0.1; $(NH_4)_2HPO_4$, 0.1; and KH_2PO_4, 0.05. The filter-sterilized substrate was added to this medium at a final concentration of 0.1% (w/v), with the exception of carbohydrates, which were used at a final concentration of 0.2% (w/v).

V. Antibiotic profile

Antibiotic susceptibility was determined according to the conventional Kirby-Bauer method (Bauer *et al*., 1966) by Dr. Antonio Ventosa.

VI. Fatty acids analysis

Fatty acids were analysed using GC at the Belgian Coordinated Collections of Microorganisms, Laboratory of Microbiology of Gent (BCCM/LMG), Gent, Belgium (Miller, 1982; Kämpfer & Kroppenstedt, 1996) or at Dr. Aharon Oren's Laboratory at the University of Jerusalem in Israel. Cells were cultured in SG or SW10 medium (Ventosa *et al*., 1982) for 24 hours at pH 7.0 at 30ºC.

VII. DNA-DNA hybridization

DNA-DNA hybridization studies were performed by the competition procedure of the membrane method (Johnson, 1994), described in detail by Mormile *et al*. (1999) by Dr. Antonio Ventosa at the University of Seville in Spain. The hybridization temperature was within the limit of validity for the filter method (De Ley and Titjgat, 1970) and the percentage of hybridization was calculated according to Johnson (1994). The experiments were carried out in triplicates.

VIII. G+C content

Samples were sent to Dr. AntonioVentosa at the University of Sevilla in Spain to analyze the G+C content of genomic DNA. This measurement was determined from the mid-point value (Tm) of the thermal denaturation profile (Marmur and Doty, 1962) using the equation of Owen and Hill (1979), as previously described in detail by Ventosa *et al*. (1999).

IX. Isolation of genomic DNA

Strains were grown in SG 15% NaCl and incubated at 30°C. DNA was extracted from cells using lysis buffer(40mM Tris-acetate pH 7.8-8.0, 20mM sodium-acetate pH 8.0, 1.0mM EDTA pH 8.0 and 1% SDS) treated with lysozyme followed by phenol/chloroform extraction and ethanol precipitation. The isolated DNA was resuspended in 50 µl of molecular water and treated with RNAse (final concentration of 20 µg/µl) for 30 minutes at 37ºC. The DNA quality was checked on 0.8% agarose gels

after staining with ethidium bromide. All DNAs was used as templates for subsequent PCR amplification.

X. Polymerase chain reaction (PCR) and gel electrophoresis

The gene encoding the 16S rRNA was amplified by PCR with the forward primer Univ-519-F (5'-CAGCMGCCGCGGTAATWC), and with the reverse primer Univ-1392-R (5'-ACGGGCGGTGTGTRC). The reaction mixture consist of ddH_2O, buffer 1X, $MgCl_2$ 2.5mM, dNTP's 250mM, Primer F 1pmol, Primer R 1pmol, DNA (10 ng of template)1.0µl and Taq polymerase 0.026U/µl. The following standard conditions were used for universal 16S rRNA gene amplification: initial denaturation at 95°C for 5 min; 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 50°C), and extension (3 min at 72 °C); and a final extension at 72°C for 10 min (Hezayen *et al.,* 2002). An electrophoresis was performed in agarose 0.8% after staining with ethidium bromide. Fragments of DNA were purified using the MinElute PCR purification kit (USA QIAGEN Inc.) according to the manufacturer instructions. PCR product concentration was determined by using a spectrophotometer at 260 nm.

XI. DNA sequencing

Selected PCR products were sent to a DNA sequencing facility in Korea (Macrogen). Samples were prepared according to the facility instructions.

XII. Phylogeny analysis

Distance analysis of the resulting DNA sequences were performed using the PHYLIP program (version 3.63) (Felsenstein, 1993). A multiple-sequence alignment was made by

using the Clustal W program with 16S rRNA gene sequences of closely related organisms (as determined by BLAST analysis) (Maidak *et al*., 1996). The 16S rRNA gene similarity values were calculated by pairwise comparison of the sequences within the alignment. Seqboot was used to generate 100 bootstrapped data sets. Distance matrices were calculated with dnadist. One hundred trees were inferred by using neighbor. Any bias introduced by the order of sequence addition was minimized by randomizing the input order. Consense was used to determine the most frequent branching order. The final tree was drawn using TREEVIEW (Page, 1996).

Results

Morphological characterization

Strain MW2a^T was a Gram-negative, nonspore-forming short rod or oval. Cells were 2- 2.6 μm long and 1-2 μm wide at logarithmic phase of growth in SG medium with 5% NaCl at 30ºC. On SG medium with 5% NaCl colonies were orange, smooth, circular/slightly irregular, and convex with an entire margin.

Strain MS10^T was a Gram-positive, spore-forming short rod. Cells were 1.0-2.0 μm long and 0.5-1.0 μm wide at logarithmic phase of growth in SG medium with 10% NaCl at 30ºC. Cells were motile. On SG medium with 10% NaCl colonies were cream-brown, entire, circular, convex and elevated.

Figure 2.1 demonstrate the macroscopical and microscopical characteristics of MS10^T and MW2a^T strains.

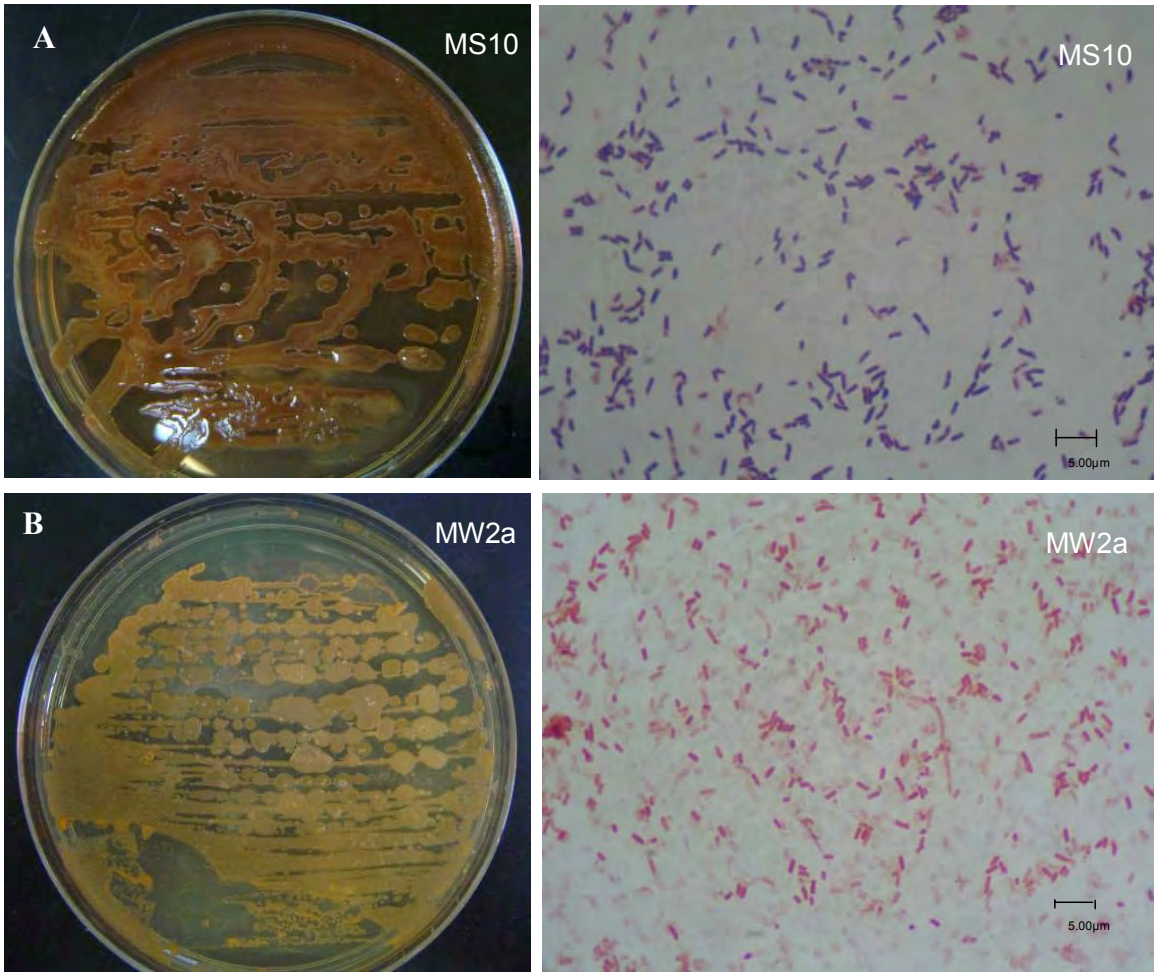


Figure 2.1 MW2a and MS10 macroscopical and microscopical characteristics at SG 15%NaCl medium sampled from *A. germinans*, Cabo Rojo, Puerto Rico. A. Gram-positive rod shape MS10^T strain is described as *Halobacillus puertoriconensis.* B. Gram-negative rod shape MW2a^T strain is described as *Halomonas avicenniae*.

Physiological characterization and growth curves

The optimal growth conditions of strains MS10 and MW2a were determined using growth curves at different temperatures, salinities and pH values. Strain MW2a grew in media containing 0-25% (w/v) NaCl, optimum growth occurred on media containing 5% (w/v) NaCl. It does not grow above 25% (w/v) of NaCl. It grows more easily at 5 and 10% of NaCl. Table 2.1 demonstrates the generation time at different temperatures and salinities. Figure 2.2 demonstrates the optimal growth condition for MW2a with the lowest generation time. The lowest generation time was between 30 -

35°C. Growth was observed at temperatures from 12-40ºC and pH range from 6.0-9.0. The optimal growth conditions for MW2a occurs in SG medium at 5% (w/v) NaCl at 30-35ºC and a pH of 7.0-8.0. This combination of conditions has the shortest generation time (1.42 hours). Figure 2.3 shows a generation time of MW2a at 30ºC and 5%NaCl at different pH. It demonstrates that the strain optimal growth was between pH values of 7-8.

Table 2.1. Generation time (h) of MW2a at different salinities and temperatures on SG medium.

Temperature (°C)	**0% NaCl**	**5% NaCl**	**10% NaCl**	**15% NaCl**	**20% NaCl**	**25% NaCl**	**30% NaCl**
26	2.50	2.03	2.69	3.03	3.08	NG	NG
30	2.25	1.42	1.83	2.22	3.00	12 h	NG
35	NG	1.58	2.19	2.42	4.92	NG	NG
40	NG	NG	NG	NG	NG	NG	NG

* NG = No growth

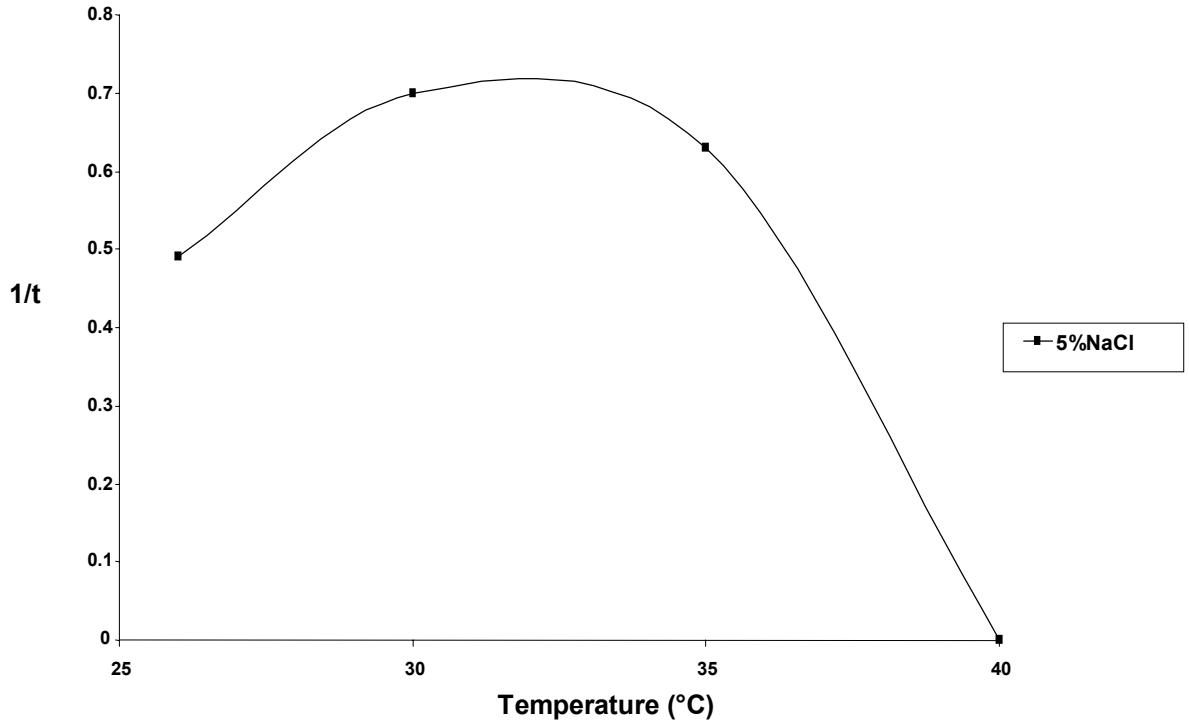


Figure 2.2 Optimal growth condition for MW2a isolated from A. germinans leaves, Cabo Rojo, Puerto Rico at different temperatures that shows the lowest generation time showing an optimal temperature from 30-33°C.

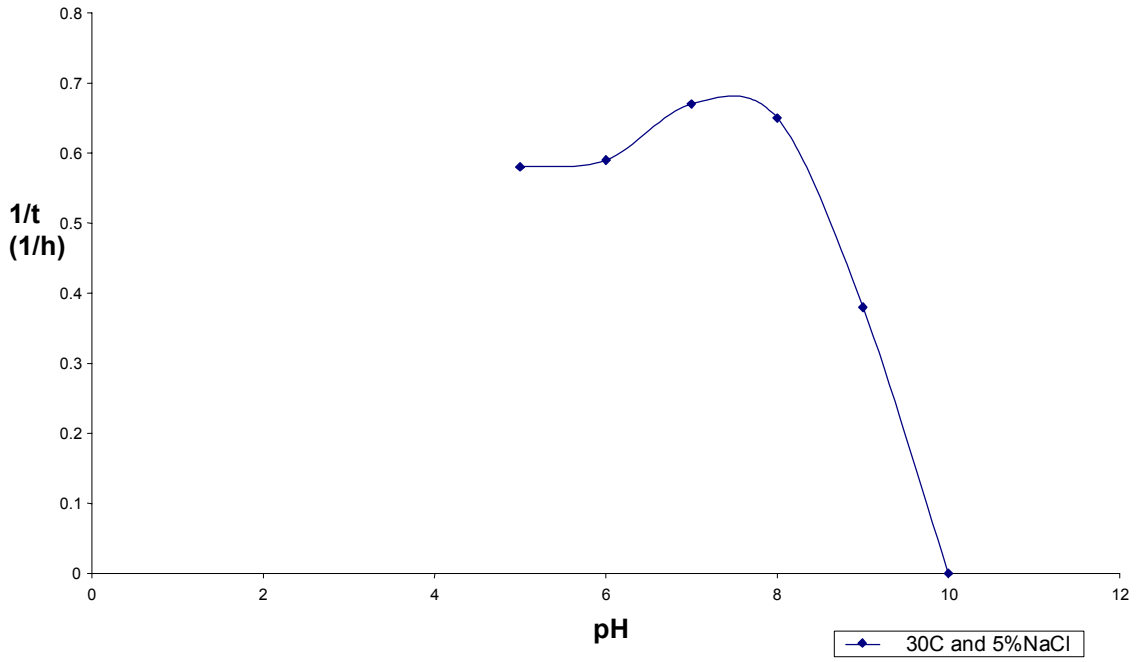


Figure 2.3. Generation time of MW2a isolated from A. germinans leaves, Cabo Rojo, Puerto Rico at 30ºC and 5%NaCl at different pHs showing an optimal pH of 7-8.

Strain MS10^{T} grew at NaCl concentrations in the range of 5-20% (w/v) in SG medium. It does not grow above 20% (w/v) of NaCl. Growth was observed at temperatures from 10-50ºC and pH range from 7.0-9.0. The optimal growth conditions occurred in SG medium at 10% (w/v) NaCl at 33-35ºC and a pH of 7.0-8.0. This combination of conditions had the shortest generation time (0.52 h). Table 2.2 resumes the generation time at different salinities. With this table it was demonstrated that MS10 can tolerate from 5 to 20% of NaCl in some cases. Figure 2.4 shows the generation time at 10% NaCl. The lower generation time was between 33°C - 35°C.

Table. 2.2 Generation time (h) of MS10^T at different NaCl percentages and temperatures using SG medium.

Temperature (°C)	0% NaCl	5% NaCl	10% NaCl	15% NaCl	20% NaCl	25% NaCl	30% NaCl
26	NG	1.56	1.52	3.15	NG	NG	NG
30	NG	2.15	1.08	3.20	NG	NG	NG
35	NG	1.30	0.52	1.15	4.92	NG	NG
40	NG	NG	NG	NG	NG	NG	NG

* NG= No growth

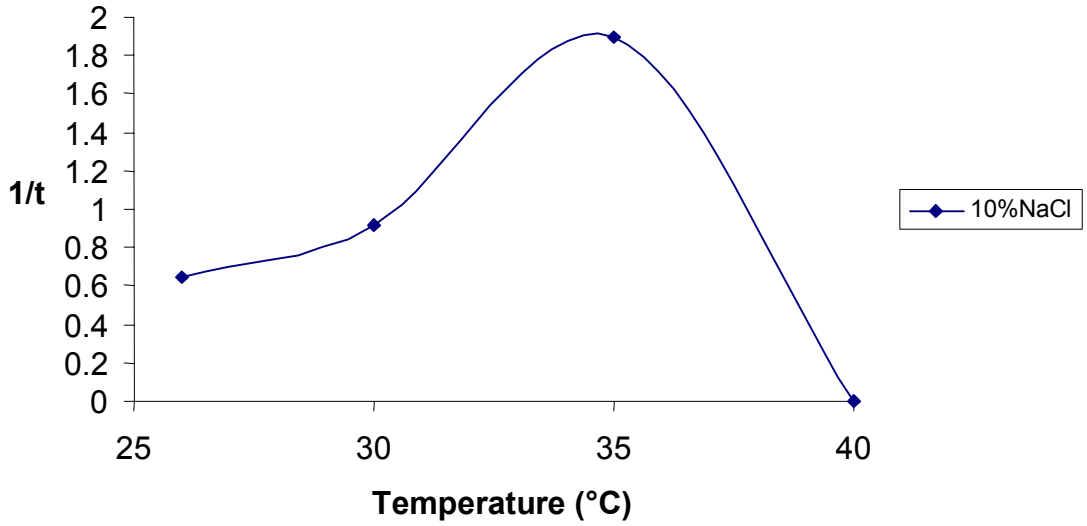


Figure 2.4 Strain MS10 isolated from *A. germinans*, Cabo Rojo, Puerto Rico, inverse generation time versus temperature at 10% NaCl showing an optimal temperature of 35°C.

Biochemical tests

All biochemical tests were carried out at 30°C. These tests were performed in duplicates for best accuracy and validation of results. Table 2.3 summarizes the results for the biochemical tests for strain MW2a.

Strain MW2a^T was able to use L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannose, and glycerol as carbon and energy sources. MW2a^T was strictly aerobic. Catalase was produced, gelatin and aesculin were hydrolyzed. Negative results for indole, H_2S production, starch, DNA and Tween 80 hydrolysis, urease, and oxidase. Nitrate was not reduced. Acid was produced from D-glucose, sucrose, xylose, D-fructose, and L-arabinose, and weakly produced from maltose. Negative reactions for arginine dihydrolase, ornithine decarboxylase and lysine decarboxylase. β-galactosidase, β-glucosidase, and α-glucosidase activities were observed. Also, negative reactions for β-glucuronidase, N-acetyl-β-glucosaminidase, α-galactosidase, α-maltosidase, and L-aspartic acid arylamidase were documented.

Table 2.3 Phenotypic characteristics that distinguish MW2a^T from other type strains of the genus *Halomonas*.

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Cell morphology	rods or ovals	rods or ovals	short rod	short rod	rod	short rod	Rod	rod
Pigmentation	Orange	yellow	white	Cream	none	cream	Cream	cream
Beta-galactosidase	+	-	+	+	-	-	+	+
Motility	+	+	+	-	+	+	+	-
Exopolysaccharide production	-	-	-	-	-	-	+	+
Oxidase	-	-	-	+	+	+	-	-
Catalase	+	+	+	+	+	+	+	+
Facultative anaerobe	-	+	+	-	-	-	-	-
Acid production from:								
Glucose	+	+	+	-	+	+	+	-
Sucrose	+	+	+	-	+	+	-	-
Maltose	+/-	+	+	-	-	+	-	-
Mannitol	+	+/-	+	-	+	+	+	-
Xylose	+	+	-	Nd	-	-	-	+
Fructose	+	+	-	-	+	+	+	-
Lactose	-	+	+	-	-	+	-	-
L-arabinose	+	+	+	-	-	+	-	-
Nitrate reduction	-	-	+	+	-	-	-	+
Simmon's citrate	+	+	+	+	+	-	-	+
Hydrolysis of:								
Gelatin	+	+	-	-	-	-	-	+
Starch	-	-	-	-	-	-	-	-

Table 2.3 cont.

Urea	-	-	+	+	+	-	+	+
Tween 80	-	-	-	-	+	-	+	+
Aesculin	+	+	-	-	+	-	-	+
DNA G+C content (mol%)	61.5	59.0	60.5	60.7-64.2	63.2	63.0	62.8	59.1-65.7

Strains: 1, MW2a^T; 2, *Halomonas marisflavi* KCCM 80003^T; 3, *Halomonas elongata* DSM 33173^T; 4, *Halomonas salina* ATTC 49509^T; 5, *Halomonas halodurans* DSM 5160^T; 6, *Halomonas halmophila* DSM 5349^T; 7, *Halomonas marina* DSM 4741^T; 8, *Halomonas eurihalina* DSM 5720^T. Data from Arahal *et al.* (2001; 2002), Baumann *et al.* (1983), Franzmann *et al.* (1989), Hebert & Vreeland (1987), Mata *et al.* (2002), Mellado *et al.* (1995), Yoon *et al.* (2001) and this study. +, Positive; -, negative, +/-, weak reaction; nd, no data.

All biochemical tests of strain MS10 was carried out at 30° C. Table 2.4 demonstrate the biochemical tests for MS10 strain.

Table 2.4 Phenotypic characteristics that distinguish strain MS10^T from other type strains of related species of the genus *Halobacillus*.

Characteristic	**Strain MS10^T**	***Halobacillus aidingensis***	***Halobacillus dabanensis***	***Halobacillus karajensis***	***Halobacillus halophilus***	***Halobacillus litoralis***
Cell morphology	rods	clubs	rods	rods	cocci or oval	Rods
Motility	+	+	+	-	+	+
Pigmentation	cream	Orange	orange	White	orange	Orange
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Facultative anaerobe	-	nd	nd	-	-	-
Acid production from:						
D-Fructose	-	+	+	+	-	+
D-Glucose	-	+	+	+	-	+
D-Galactose	-	-	-	-	-	-
D-Mannitol	-	+	+	+	-	+
Sucrose	-	+	+	-	-	+
Xylose	-	-	+	-	-	+
Nitrate reduction	-	-	-	-	-	-
Simmon's citrate	-	nd	nd	-	-	-
H_2S production	-	nd	nd	-	nd	nd
Indole	-	nd	nd	-	-	-
Methyl red	-	nd	nd	-	nd	nd
Voges Proskauer	-	-	-	-	-	-
Hydrolysis of:						
Aesculin	-	-	-	+	-	-
Gelatin	+	+	-	+	+	+
Starch	+	+	+	+	+	-
Tween 80	-	-	-	-	-	-
Urea	-	-	-	-	-	-

Data from Amoozegar *et al*., 2003, Liu *et al*., 2005 and this study. +, positive; -, negative, nd, not determined.

MS10 was strictly aerobic. Catalase and oxidase were produced. Negative results for aesculin, indole, H_2S production, methyl red, Voges-Proskauer, and urease Tween 80 were documented. Gelatin and starch were hydrolyzed. Nitrate was not reduced. No acid production from glucose, sucrose, maltose, mannitol, xylose, fructose, mannose, D-melibiose, L-rhamnose, D-sorbitol, D-galactose, inositol, sorbitol, adonitol, L-arabitol, D-arabitol, L-arabinose, palatinose, trehalose, cellobiose, galacturonic acid, potassium 5-ketogluconate, and sodium pyruvate was observed. Starch, D-glucose, D-sorbitol, and succinate were used as carbon and energy sources.

Antibiotic profile

Antibiotic susceptibility for strains MS10 and MW2a was carried out by Dr. Antonio Ventosa in Sevilla, Spain. Strain MS10 was resistant (μg per disc) to polymixin (300 units), neomycin (30), streptomycin (10), sulfamethoxazole/trimethoprim (23.75/1.25). Intermediate resistance to (μg per disc) erythromycin (15), nalidixic acid (30) and tetracycline (30). The strain was sensitive (μg per disc) to kanamycin (30), rifampicin (5), bacitracin (10 units), vancomycin (30), novobiocin (30), cephalothin (30), penicillin (10 units), and chloranphenicol (30).

Strain MW2a demonstrated intermediate resistance to (μg per disc): erythromycin (15), and resistance (μg per disc) to penicillin (10 units), bacitracin (10 units), cephalothin (30), tetracycline (30), rifampicin (5), streptomycin (10), neomycin (30), kanamycin (30), vancomycin (30), nalidixic acid (30), novobiocin (30) and chloranphenicol (30).

Fatty acids analysis

Fatty acids composition was determined for MW2a and MS10 strains. Cells were cultured on SW10 medium (Ventosa *et al*., 1982) for 24 h at pH 7.0, 30ºC. For MW2a the major fatty acids were $C_{16:0}$, $C_{19:0}$ cyclo w8c, $C_{18:1}$ w7c and $C_{12:0}$ 3OH. This composition was very similar to those described for other *Halomonas* species (Table 2.5).

Table 2.5 Comparison of the cellular fatty acid composition (%) of strain MW2a^T, the closely related *Halomonas* species, *H. marisflavi,* and the type species of the genus *Halomonas*, *H. elongata*.

Fatty acid composition	**Strain MW2a^T**	***H. marisflavi* KCCM 80003^T**	***H. elongate* ATCC 33173^T**
$C_{10:0}$	1.0	2.4	4.7
$C_{10:0}$ 3OH	-	-	3.6
$C_{12:0}$	0.8	-	4.7
$C_{12:0}$ 2OH	3.2	3.2	-
$C_{12:0}$ 3OH	11.3	11.0	15.3
$C_{14:0}$	0.4	0.5	-
$C_{16:1}$ ω7c*	2.6	3.5	4.5
$C_{16:0}$	37.4	33.4	25.6
$C_{16:0}$ 3OH	-	-	1.0
C_{17} cyclo	3.2	2.6	1.6
$C_{17:0}$	-	-	0.5
$C_{18:1}$ ω7c	16.6	25.7	25.6
$C_{18:0}$	1.2	2.1	0.6
C_{19}cyclo ω8c	22.3	15.7	10.6
$C_{19:0}$10 methyl	-	-	0.5

For MS10 the cells were cultured on Marine Agar supplemented with 8.1% NaCl for 24-48 h at 35ºC. Cell wall composition analysis was carried out by the Identification Service of DSMZ Braunschweig (Germany). Predominant fatty acids of strain MS10^T

were iso-$C_{16:0}$, anteiso-$C_{15:0,}$ iso-$C_{14:0}$ and iso-$C_{15:0}$ (Table 2.6). The cell wall peptidoglycan is of the L-Orn-D-Asp type.

Table 2.6 Cellular fatty acid composition (%) of strain MS10a^T and the closely related *Hallobacillus* species.

Fatty acid	**MS10**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
$C_{15:0}$	ND	ND	0.26	1.1	ND	1.6	1.1	1.5
$C_{16:0}$	1.53	2.34	2.28	0.6	0.9	1.0	0.9	0.9
anteiso-$C_{13:0}$	ND	ND	0.08	0.4	ND	ND	0.5	0.4
anteiso- $C_{15:0}$	22.81	49.92	42.04	57.0	25.3	31.7	42.0	47.3
anteiso- $C_{17:0}$	4.46	19.28	15.68	8.2	6.5	6.2	13.0	11.9
iso- $C_{14:0}$	17.13	1.87	2.34	6.4	21.7	9.4	11.2	12.2
iso- $C_{15:0}$	14.79	7.47	13.47	15.8	7.7	26.3	8.4	7.5
iso- $C_{16:0}$	32.18	14.23	7.47	5.4	31.5	15.7	15.9	15.2
iso- $C_{17:0}$	4.44	4.40	4.36	1.5	2.1	4.2	1.4	1.2
$C_{16:1}$ ω7c alcohol	2.65	0.48	3.01	2.0	3.8	4.0	5.7	1.8
Iso-$C_{17:1}$ ω10c	ND	ND	1.42	0.2	ND	ND	ND	ND
$C_{17:1}$ ω11c	ND	ND	0.67	ND	ND	ND	ND	ND

Species: 1, Strain MS10^T (data from this study); 2, *H. dabanensis* (data from this study); 3, *H. aidingensis* (data from Liu *et al.,* 2005), 4, *H. litoralis* (data from Yoon *et al.,* 2003); 5, *H. trueperi* (data from Yoon *et al.,* 2003); 6, *H. salinus* (data from Yoon *et al.,* 2003); 7, *H. locisalis* (data from Yoon *et al.,* 2004); 8, *H. halophilus* (data from Yoon *et al.*, 2003). All strains were grown on marine agar supplemented with 8.1 % NaCl (final NaCl concentration of 10%) according to Yoon *et al.* (2003). Values are percentages of total fatty acids. ND, not detected.

G+C content and DNA-DNA hybridization

The G+C content and DNA-DNA hybridization was performed for strains MS10 and MW2a. The DNA G + C content for MW2a is 61.5 mol%, which is in the range for species belonging to the genus *Halomonas* (Franzmann *et al*., 1989; Arahal and Ventosa, 2005). The percentage of DNA-DNA hybridization between strain MW2a^T and *Halomonas marisflavi* KCCM 80003^T was 42%, while with respect to *Halomonas elongata* ATCC 33173^{T}and *Halomonas salina* DSM 5928^{T}were 23% and 13%, respectively. These levels of DNA-DNA hybridization are low enough to classify strain

MW2a^T as a genotypically distinct species within the genus *Halomonas* (Wayne et al., 1987; Stackebrandt & Goebel, 1994).

The DNA G+C content of strain MS10^T is 45.7 mol %. The percentage of DNA-DNA hybridization between strain MS10^T and *H. dabanensis* JCM 12772^T was 29%, and percentages below 70% were also obtained with respect to other related *Halobacillus* species (Table 2.7). These levels of DNA-DNA hybridization are low enough to classify strain MS10^T as a genotypically distinct species within the genus *Halobacillus* (Wayne *et al*., 1987; Stackebrandt and Goebel, 1994).

Table 2.7 DNA G+C content and levels of DNA-DNA hybridization between strain MS10^T and species from the genus *Halobacillus.*

Source of unlabelled DNA	**G+C content (mol %)**	**Relatedness to ^{3}H-labelled DNA from MS10^T (%)**
Strain MS10^T	45.7	100
H. dabanensis JCM 12772^T	41.4*	29
H. locisalis DSM 16468^T	44.0*	44
H. trueperi CCM 4593^T	43.0*	32
H aidingensis JCM 12771^T	42.2*	49
H. litoralis DSM 10405^T	42.0*	36
H. karajensis DSM 14948^T	41.3*	51
H. yeomjeoni DSM 17110^T	42.9*	44
H. salinus JMC 11546^T	45.0*	36

*Data from Amoozegar *et al.* (2003), Claus *et al.* (1983), Liu *et al.* (2005), Spring *et al.* (1996),.Yoon *et al.* (2003; 2004; 2005).

Phylogenetic analysis

A phylogenetic study of these sequences was performed using neighbor joining analysis. Phylogenetic analysis using the 16S rRNA gene revealed that it is within the *Halobacillus* species phylogenetic branch, and the most closely relative is *Halobacillus dabanensis* JCM 12772^T having a 99.2% 16S rRNA gene sequence similarity (Figure 2.5). The phylogenetic position of strain MS10^T was also confirmed in a tree generated with the maximum-parsimony algorithm.

Phylogenetical analysis using the neighbor joining algorithm revealed that strain MW2a^T was closely related to *Halomonas marisflavi* forming a branch in this cluster with a bootstrap value of 100% (Figure 2.6). The phylogenetic position of strain MW2a^T was also confirmed in a tree generated with the maximum-parsimony algorithm.

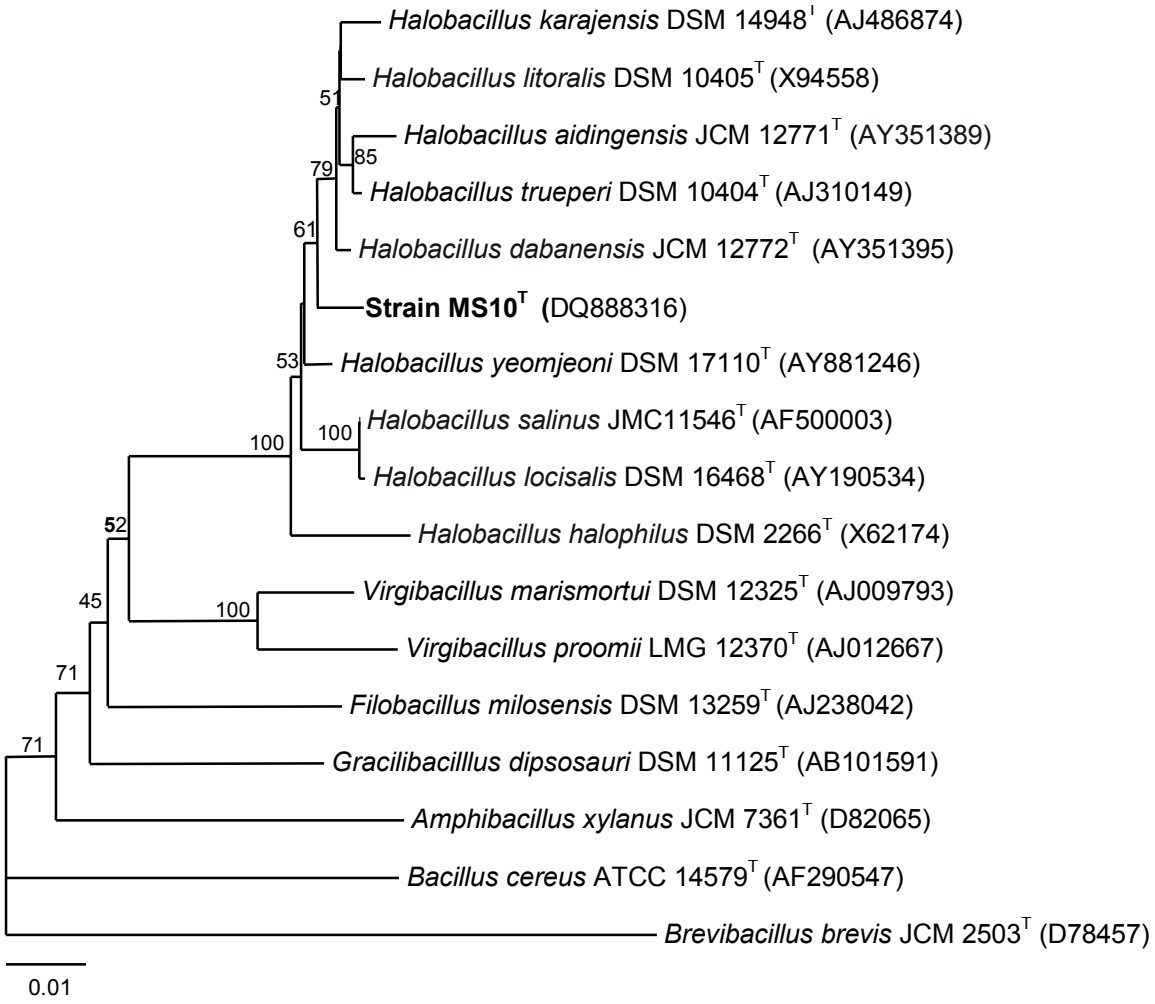


Figure 2.5 Neighbour-joining distance tree using the 16Sr RNA sequences of strain MS10^T and closely related species. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown. *Brevibacillus brevis* JCM 2503^T was used as the outgroup.

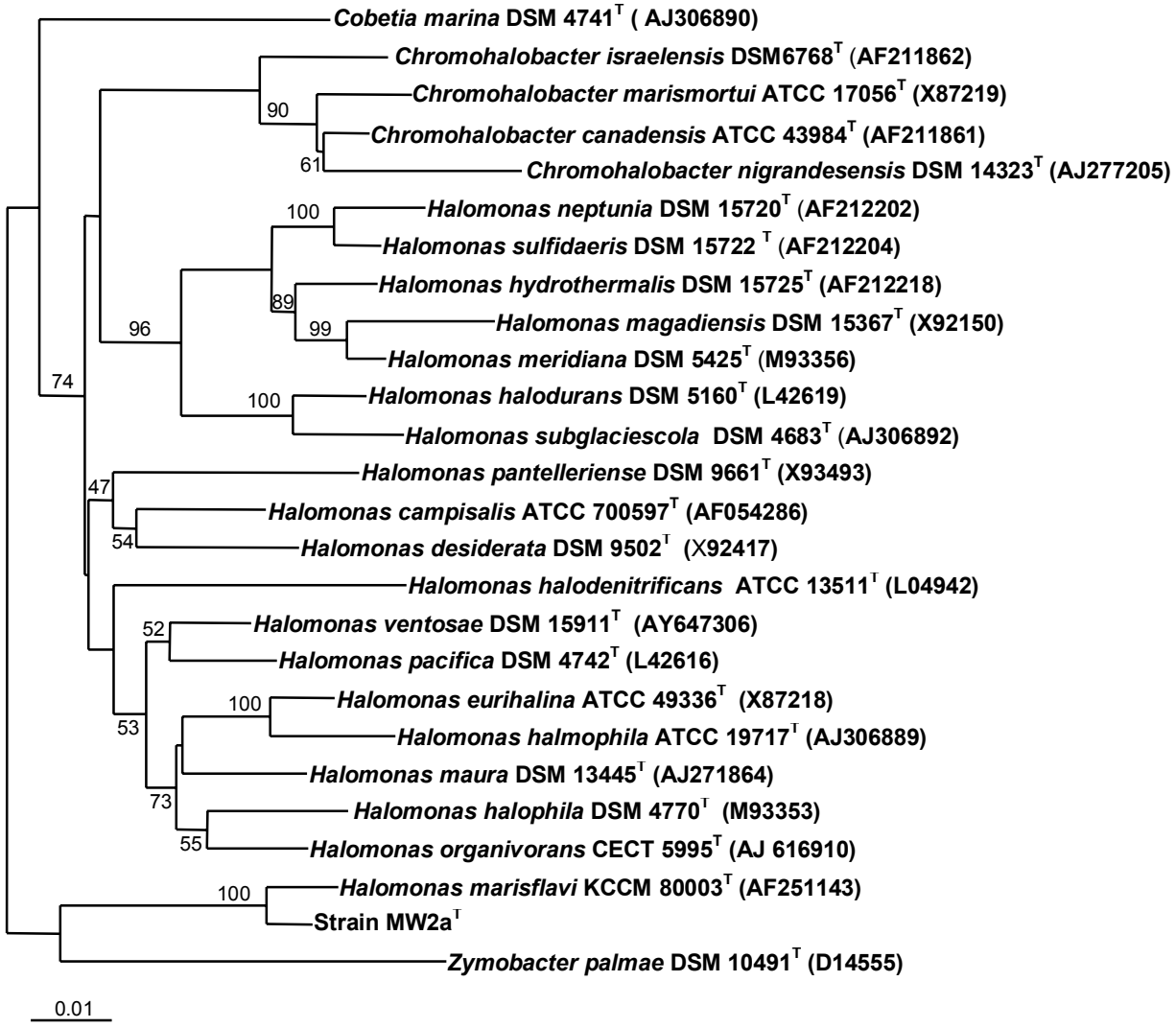


Figure 2.6 Neighbour-joining distance tree using the 16S rRNA sequences of MW2a^T and closely related species. Bar represents 1 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown. *Zymobacter palmae* DSM 10491^T was used as the outgroup

Description of MS10^T and MW2a^T

Description of *Halomonas avicenniae* sp. nov. [a.vi.cen.ni'ae. N.L. n. Avicennia, scientific name of a botanical genus which encompasses *Avicennia germinas* (the black mangrove); N.L. gen. n. avicenniae, of *Avicennia*, isolated from leaves of *Avicennia germinans*].

Gram-negative nonspore-forming short rod or oval, 2- 2.6 μm long and 1-2 μm wide. Cells are motile. Colonies are orange, smooth, circular/slightly irregular, and convex with an entire margin. Moderately halophilic, growing at NaCl concentrations in the range 0-25% (w/v) with an optimum at 5% (w/v) NaCl. Growth occurs at temperatures from 12-40ºC (optimal 30-35 ºC) and pH range from 5.0-9.0 (optimal 7.0-8.0). Strictly aerobic. Catalase is produced, gelatin and aesculin are hydrolyzed. Negative results for indole, H_2S production, starch, DNA and Tween 80 hydrolysis, urease, and oxidase. Nitrate was not reduced. Acid produced from D-glucose, sucrose, xylose, D-fructose, and L-arabinose and weakly produced from maltose. Negative for arginine dihydrolase, ornithine decarboxylase, and lysine decarboxylase. β-galactosidase, β-glucosidase and α-glucosidase activities are present. Negative for β-glucuronidase, N-acetyl-β-glucosaminidase, α-galactosidase, α-maltosidase and L-aspartic acid arylamidase. L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannose, and glycerol are used as carbon and energy sources. Intermediate resistance to (μg per disc): erythromycin (15). Resistant (μg per disc) to penicillin (10 units), bacitracin (10 units), cephalothin (30), tetracycline (30), rifampicin (5), streptomycin (10), neomycin (30), kanamycin (30), vancomycin (30), nalidixic acid (30), novobiocin (30), and chloranphenicol (30).

The major fatty acids are $C_{16:0}$, $C_{19:0}$ cyclo w8c, $C_{18:1}$ w7c and $C_{12:0}$ 3OH. The DNA G + C content is 61.5 mol%. Isolated from the leaf surface of *Avicennia germinans* (black mangrove) in Cabo Rojo, Puerto Rico. The type strain is MW2a^T (= CECT 7193^T = CCM 7396^T).

Description of *Halobacillus puertoriconensis* (pu.er.to.ri.co.nen'sis. N.L. masc. adj. *puertoriconensis*, pertaining to the Island of Puerto Rico).

Gram-positive spore-forming short rod, 1-2 μm long and 0.5-1 μm wide. Cells are motile. Colonies are cream-brown, entire, circular, convex, and elevated. Moderately halophilic growing at NaCl concentrations in the range of 5-20% (w/v) with an optimum of 10% (w/v) NaCl. Growth occurs at temperatures from 10-50ºC (optimal 33-35 ºC) and pH range from 5.0-9.0 (optimal 7.0-8.0). Strictly aerobic. Catalase and oxidase are produced. Negative results for aesculin, indole, H_2S production, methyl red, Voges-Proskauer, and urease Tween 80. Gelatin and starch were hydrolyzed. Nitrate was not reduced. No acid production from glucose, sucrose, maltose, mannitol, xylose, fructose, mannose, D-melibiose, L-rhamnose, D-sorbitol, D-galactose, inositol, sorbitol, adonitol, L-arabitol, D-arabitol, L-arabinose, palatinose, trehalose, cellobiose, galacturonic acid, potassium 5-ketogluconate, and sodium pyruvate. Starch, D-glucose, D-sorbitol, and succinate are used as carbon and energy sources. Negative for arginine dihydrolase, ornithine decarboxylase and lipase (C14). β-galactosidase, α-glucosidase, α-galactosidase, and α-mannosidase activities are present. Negative for lysine decarboxilase, β-glucosidase and β-glucuronidase, N-acetyl-β-glucosaminidase, and L-aspartic acid arylamidase.

Resistant (μg per disc) to polymixin (300 units), neomycin (30), streptomycin (10), and sulfamethoxazole/trimethoprim (23.75/1.25). Intermediate resistance to (μg per disc): erythromycin (15), nalidixic acid (30), and tetracycline (30). Sensitive (μg per disc) to kanamycin (30), rifampicin (5), bacitracin (10 units), vancomycin (30), novobiocin (30), cephalothin (30), penicillin (10 units), and chloranphenicol (30).

The cell wall peptidoglycan is of the L-Orn-D-Asp type. The major fatty acids are iso-$C_{16:0}$, anteiso-$C_{15:0,}$ iso-$C_{14:0,}$ and iso-$C_{15:0}$. The DNA G + C content was 45.7 mol%. Isolated from the leaf surface of *Avicennia germinans* (black mangrove), Cabo Rojo, Puerto Rico. The type strain is MS10^T which has been deposited at the CECT 7206^T and the CCM 7397^T.

Part III. Microbial diversity of the leaf surface of *Avicennia germinans* determined by environmental 16SrDNA clone libraries

Materials and Methods

I. Sample processing

A total of 40 leaves were collected randomly from four *A. germinans* trees growing near the solar salterns of Cabo Rojo, Puerto Rico. Leaves were placed in sterile bags (Whirl Pak®) containing 1% phosphate buffer pH 7 with 15% (w/v) NaCl and mixed. This procedure allowed the suspension of the microorganisms present in the salt crystals and the surface of the leaves. Ten milliliters of the suspension were then obtained for serial dilutions. The remaining volume (90) mL was filtered through a nitrocellulose membrane of 0.45 µm (Millipore). Membranes were stored at -20 ºC until DNA extraction was performed.

II. DNA extraction from environmental samples

Membranes containing the microorganisms from the leaf surface of *A. germinans* were suspended in 5 mL lysis buffer (1 mg/ml lysozyme, 40 mM EDTA, 50 mM Tris-HCl, 0.75M sucrose, pH 8.0) and incubated at 37ºC for 30min. Proteinase K (0.5 mg/ml) and SDS (1% w/v) were added to the suspension and incubated at 55ºC for 2h. The lysate was placed into a fresh tube. The lysate was then rinsed with an additional 2ml of lysis buffer and incubated at 55ºC for 10 min before pooling the lysates. To the pooled solution, 5M NaCl (final concentration 0.7 M) and hexadecyltrimethyl ammonium bromide were added and incubated at 65ºC for 20 min before extraction with chloroform-isoamyl alcohol (24:1). The upper aqueous-DNA phase was removed and placed into a fresh tube and DNA was precipitated after the addition of 0.6 volumes of isopropanol. The pellet was washed with 70% (w/v) ethanol, dried, and dissolved in 50 µl ultra pure

water (Saano, A. *et al*., 1995). The DNA purity was assessed by gel electrophoresis (Øvrea°s *et al*., 2003). Total genomic DNAs were used as templates for subsequent PCR amplification.

III. PCR amplification and gel electrophoresis

The gene encoding the 16S rRNA was amplified by PCR using the forward primer Univ-519-F (5'-CAGCMGCCGCGGTAATWC-3') and with the reverse primer Univ-1392-R (5' –ACG GGC GGT GTG TAC-3'). The reaction mixture consist of ddH_2O, buffer 1X, $MgCl_2$ 2.5mM, dNTP's 250mM, Primer F 1pmol, Primer R 1pmol, BSA 100ng/µl, DNA 1.0µl (≈ 10 ng of template), and Taq polymerase 0.026U/µl in storage Buffer B (Promega ®). Those primers correspond to universal primers (SIGMA GENOSYS). The polymerase and buffer is from the manufacture Promega. The following standard conditions were used for universal 16S rRNA gene amplification: initial denaturation at 95°C for 5 min; 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 50°C), and extension (3 min at 72 °C); and a final extension at 72°C for 10 min (Hezayen *et al.,* 2002). Samples and controls were electrophoresized in agarose 0.8% and their quality were checked after staining with ethidium bromide. Fragments of DNA were purified using the MinElute PCR purification kit according to the manufacturer instructions (USA QUIAGEN Inc.). The resulting amplicon concentration was determined measuring absorbance at 260nm.

IV. Cloning of PCR products

Environmental PCR products were ligated and transformed using the pGEM T Vector System II (Promega ®). The products were ligated into pGEM®-T cloning vector, and transformed into JM 109 high efficiency competent cells as described by the manufacturer's protocol. Transformed cells were plated in Luria-Bertani (LB) agar plates with ampicillin (100 µg/ml).

V. Screening and purification

The colony PCR technique (Gussow and Clackson, 1989) was selected to determine the nature of the insert present on the putative clones. The primers selected for this procedure were T7 promoter and SP6 promoter (Promega ®) as recommended by the manufacturer to amplify the insert present in the construct. The reaction mixture consisted of ddH_2O, buffer 1X, $MgCl_2$ 2.5mM, dNTP's 250mM, T7 promoter (5' TAA TAC GAC TCA CTA TAGGG 3') 1pmol, SP6 promoter (5' ATT TAG GTG ACA CTA TAG AA 3') 1pmol, DNA 2.0µl and Taq Polymerase 0.026U/µl. Samples and controls were electrophoresized in agarose 0.8% after staining with ethidium bromide. Fragments of DNA were purified using the MinElute PCR purification kit according to the manufacturer instructions (USA QUIAGEN Inc.). The resulting amplicon concentrations were determined measuring absorbance at 260nm.

VI. Restriction fragment length polymorphism (RFLP)

A double digestion was performed on the amplified PCR products using the restriction endonucleases *Msp I* and *HaeIII*. All digestions were performed for an hour

(twice) at 37ºC in a final volume of 10 µl. Restriction patterns were verified on 3% low melting agarose gels after staining with ethidium bromide.

VI. DNA Sequencing

Selected PCR products were sent to a DNA sequence facility in Korea (Macrogen). Samples were prepared according to the facilities instructions.

VII. Phylogenetic analysis

To check for possible chimeric sequences all clones were analyzed using the Chimera Check program from the RDP database (Cole *et al*., 2003) version 2.7 (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU). Distance analysis of the resulting DNA sequences was performed using the PHYLIP program (version 3.5.1) (Felsenstein, 1993). A multiple-sequence alignment was made by using the Clustal W program with 16S rRNA gene sequences of close related organisms (as determined BLAST analysis) (Kamekura and Dyall-Smith, 1995; McGenity and Grant, 1995; Oren *et al*., 1995; Maidak *et al*., 1996; Montalvo-Rodríguez *et al*., 1998; 2000). The 16S rRNA gene similarity values were calculated by pairwise comparison of the sequences within the alignment. SEQBOOT was used to generate 100 bootstrapped data sets. Distance matrices were calculated with DNADIST. One hundred trees were inferred by using NEIGHBOR. Randomizing the input order was minimize any bias introduced by the order of sequence addition. CONSENSE was used to determine the most frequent branching order. The final tree was drawn using TREEVIEW (Page, 1996).

VIII. Statistical analysis of clones libraries

Statistical analyses were performed as Stach and collegues (2003) were proposed. Analyses and index values using the Jaccard, SAce, and SChao richness index were calculated in order to corroborate richness between samples. To measure how well the sample represents the larger environment, the Good Coverage Index was calculated using the program ASLO (www.aslo.org/methods/free/2004/0114a.html). In order to evaluate a level of differences among clone libraries, a p-value was calculated using Web-LIBSHUFF program (http://libshuff.mib.uga.edu).

Results

Leaves from three black mangrove trees were used for total genomic DNA extraction. These samples were divided according to the time of sampling. Two of the samples corresponded to the second survey performed in August, 2005 and one sample represented the sampling of March, 2005. Several environmental DNA extraction methods were tested to achieve optimal DNA extraction. It was determined that the best extraction method was the Saano protocol with some modifications (Saano, 1995). Three DNA extractions were obtained and named as MS1, MS2 and MS3 which corresponded to the samples second survey, second survey and first survey accordingly (Figures 3.1, 3.2, 3.3).

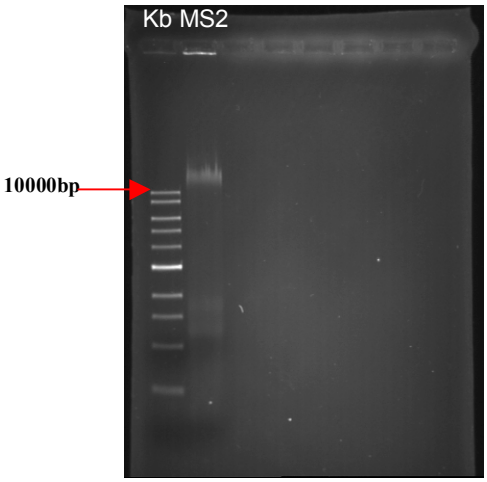


Figure 3.1 Total genomic DNA extraction (MS2) of *A. germinans* leaf surface community.

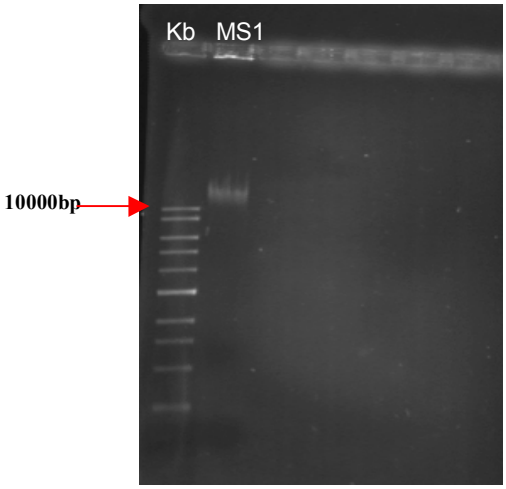


Figure 3.2 Total genomic DNA extraction (MS1) of *A. germinans* leaf surface community.

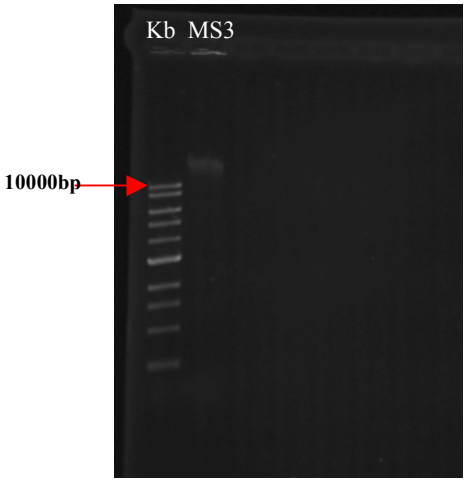


Figure 3.3 Total genomic DNA extraction (MS3) of *A. germinans* leaf surface community.

These total environmental DNAs were used as a template for PCR amplification of the 16S rDNA region. Several optimizations were performed to achieve optimal amplification of the desire products. Universal primers and Taq DNA polymerase (Promega ®) were used to amplify the desired product. PCR product had a size of 873bp (Figures 3.4, 3.5, 3.6).

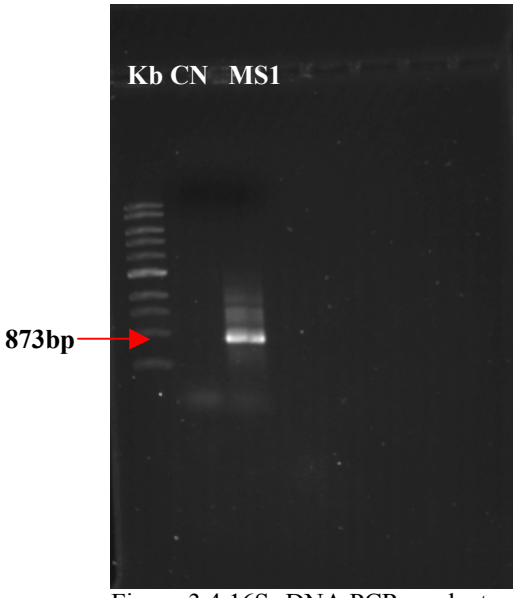


Figure 3.4 16S rDNA PCR product for MS1 using universal primers.

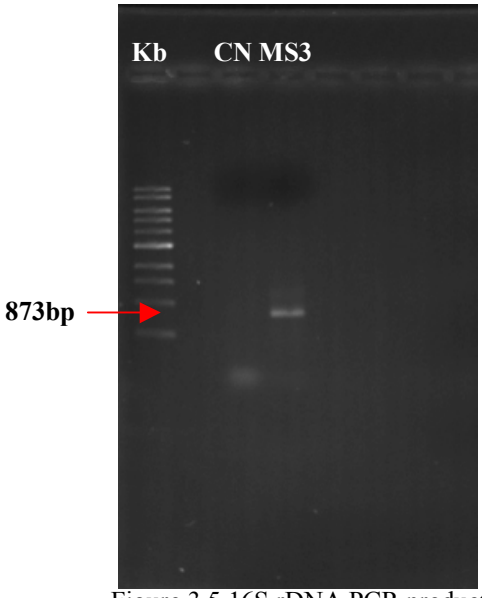


Figure 3.5 16S rDNA PCR product for MS3 using universal primers.

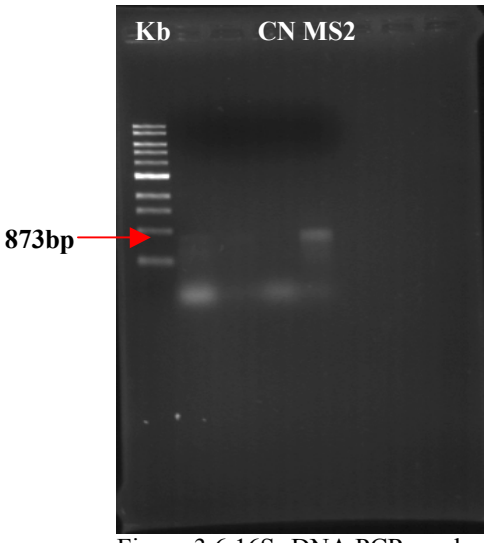


Figure 3.6 16S rDNA PCR product for MS2 using universal primers.

Various clone libraries were constructed with these purified PCR products. A total of two genomic libraries were obtained using the MS 2 total DNA product. From this library, approximately 500 clones were obtained. One clone library was obtained for MS1 and a total of 712 clones were obtained. The other clone library constructed was MS3. MS3 DNA corresponded to the first survey performed in March, 2005. From this environmental library 500 clones were obtained. Thirty percent of each clone library was screening using the RFLP technique, and 60 clones from each clone library were selected randomly for sequencing purposes.

Figure 3.7 shows some PCR products using clones from the MS3 library group I. The amplification product is approximately 1034bp.

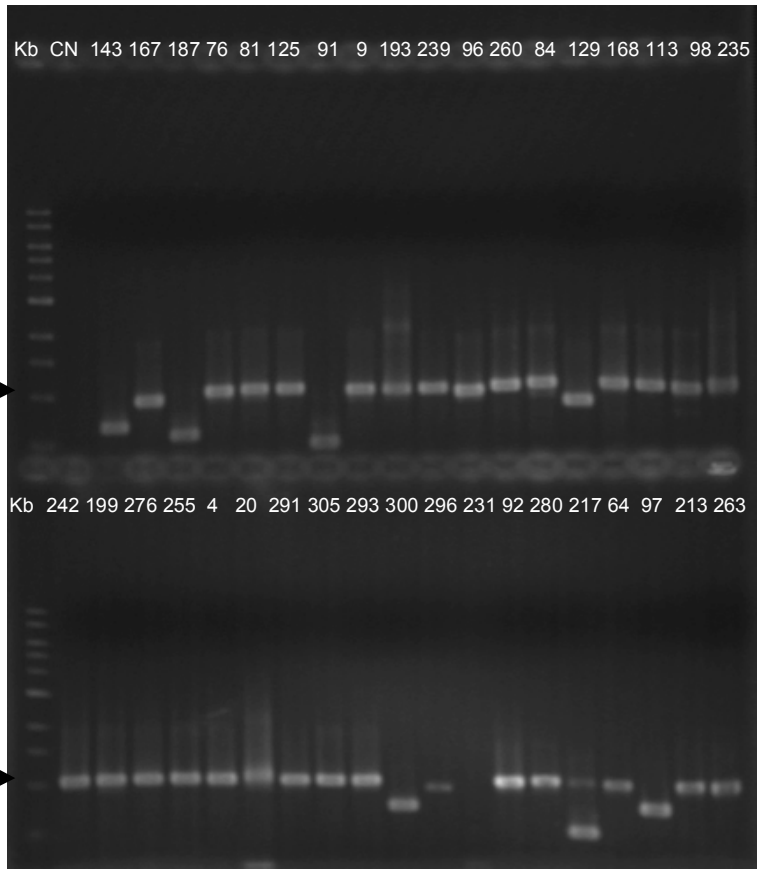


Figure 3.7 PCR product of colony PCR using SP6 and T7 Promoter primers of the total genomic DNA MS3. Bands show a PCR product of 1034bp.

Figure 3.8 demonstrates some PCR products using clones from the MS2 group G library. The amplification product is approximately 1034pb. Figure 3.9 demonstrates some PCR products using clones from the MS1 group H library. The amplification product is approximately 1034bp.

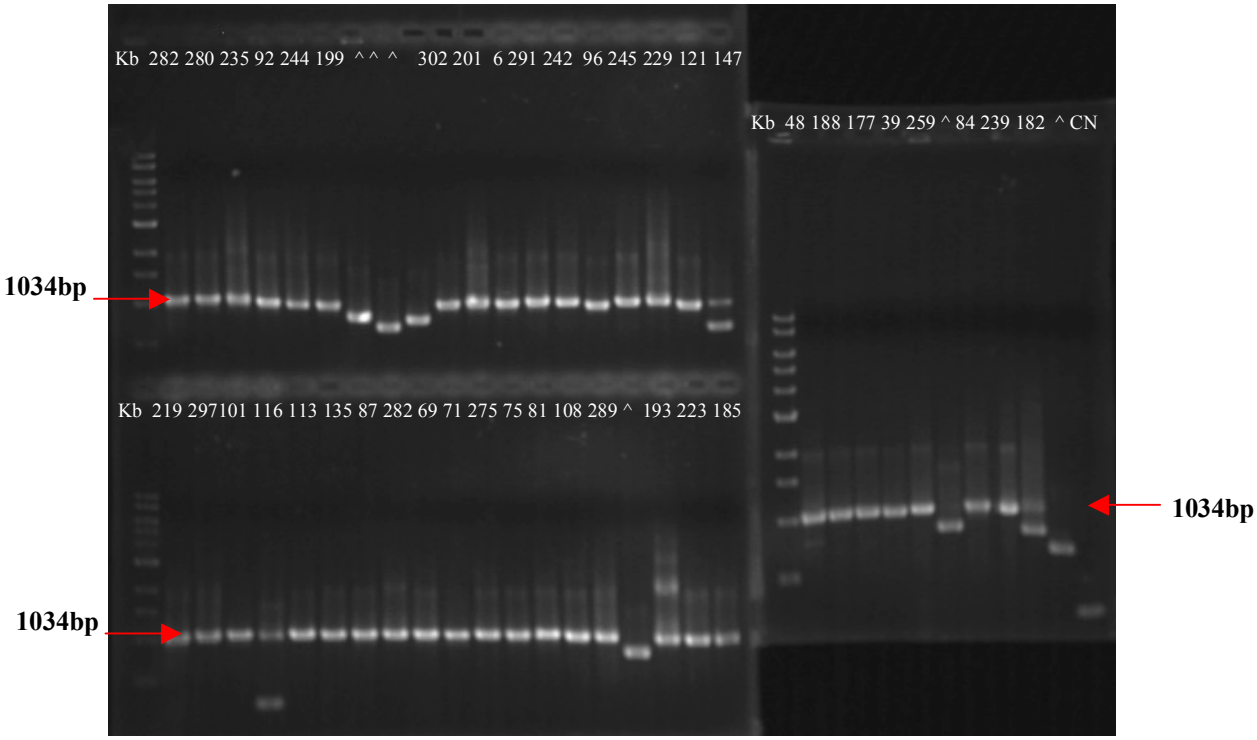


Figure 3.8 PCR product of colony PCR using SP6 and T7 Promoter primers of the total genomic DNA MS2.

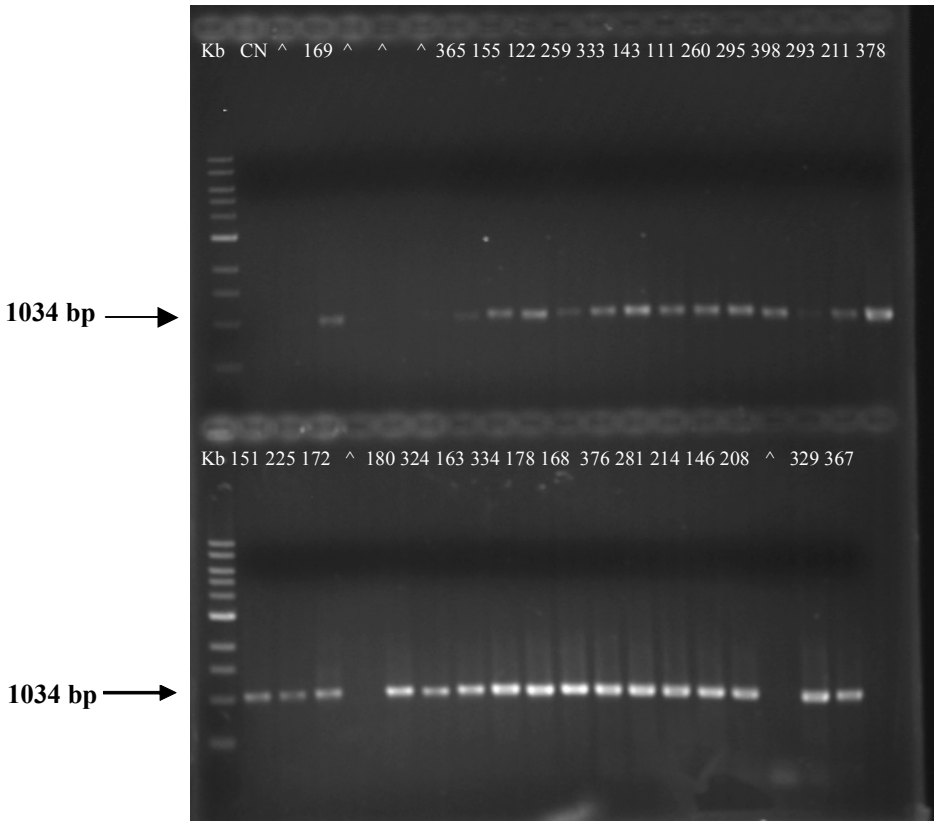


Figure 3.9 PCR product of colony PCR using SP6 and T7 Promoter primers of the total genomic DNA MS1.

Figures 3.10 and 3.11 show the screening of the first survey (MS3) named as group I using *Msp*I and *Hae* III restriction enzymes in 3% low melting agarose. A total of 0.75µg of template was used for enzyme digestion. The DNA markers were the100bp and 50bp ladders.

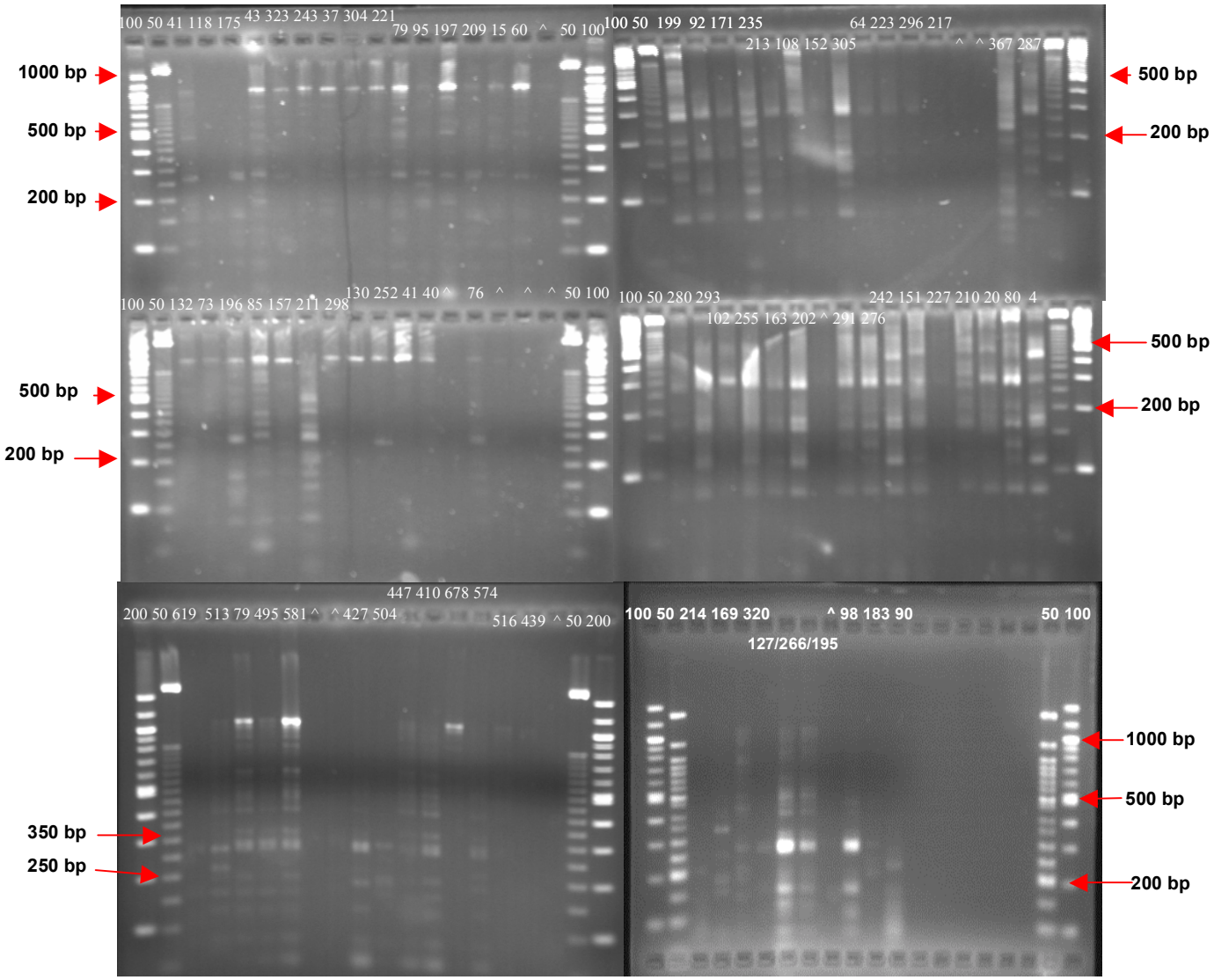


Figure 3.10. RFLP analysis of group I using *Hae* III and *Msp* I restriction enzymes in 3% low melting agarose.

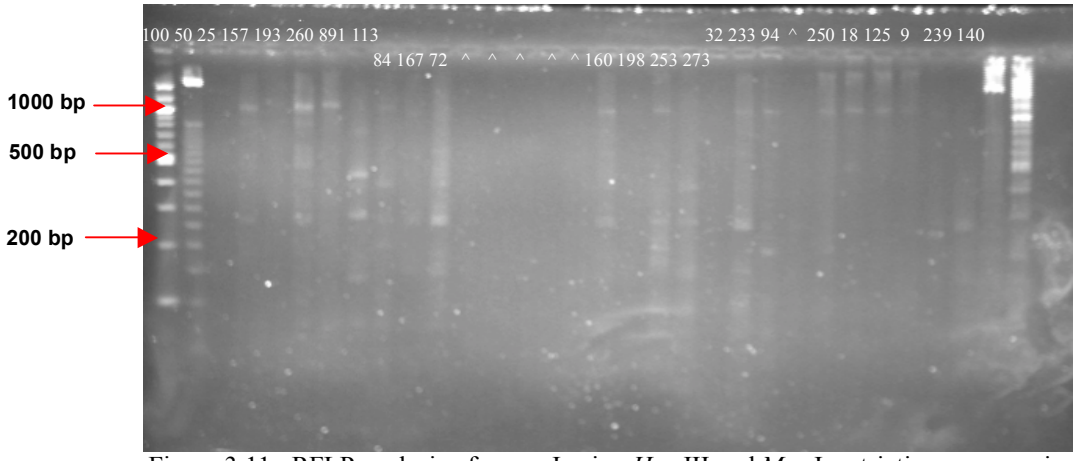
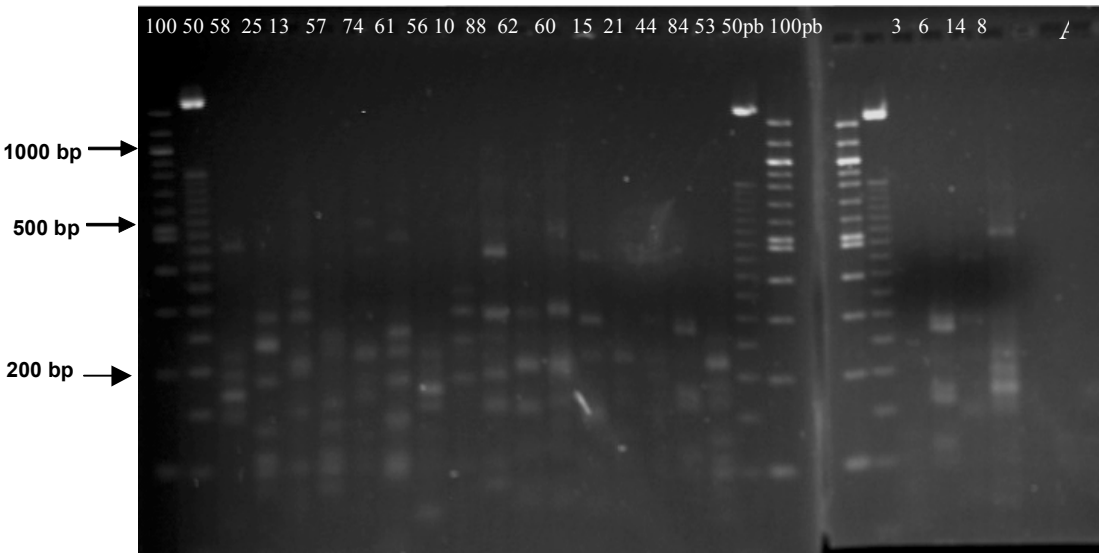


Figure 3.11. RFLP analysis of group I using *Hae* III and *Msp* I restriction enzymes in 3% low melting agarose.

Figures 3.12 and 3.13 demonstrate the screening of the second survey (MS2) named as group G using *Msp*I and *Hae* III restriction enzymes in 3% low melting agarose. A total of 0.75µg of template was used for enzyme digestion. The DNA markers were the100bp and 50bp ladders.



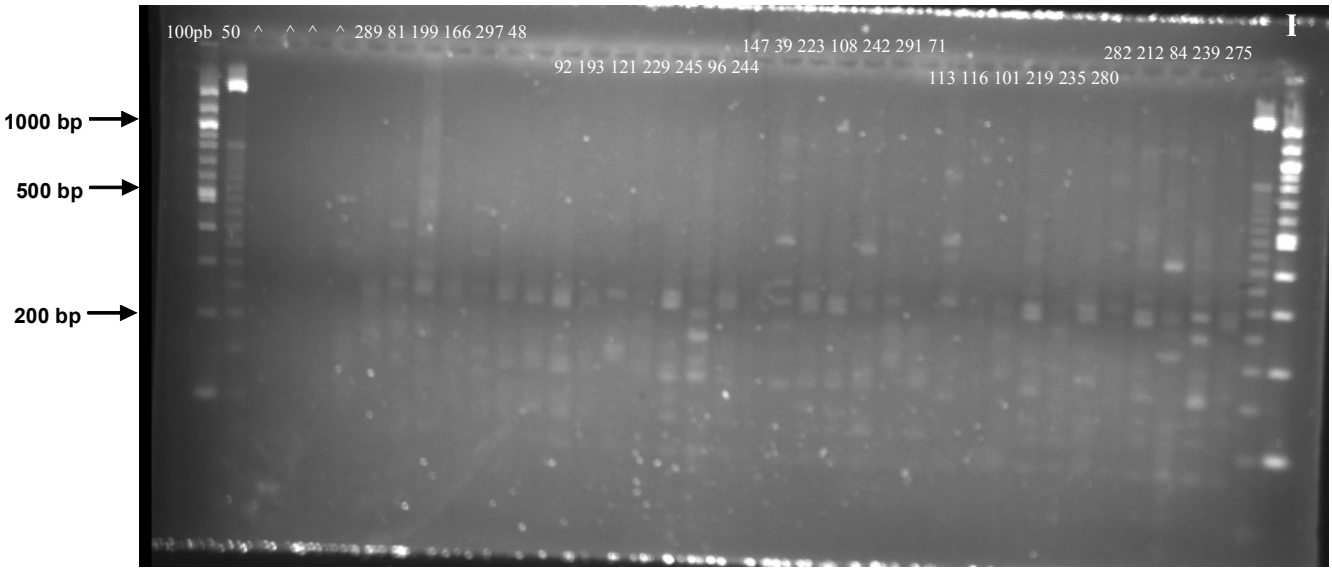


Figure 3.12 RFLP (A, B) analysis of group G using *Hae* III and *Msp* I restriction enzymes in 3% low melting agarose.

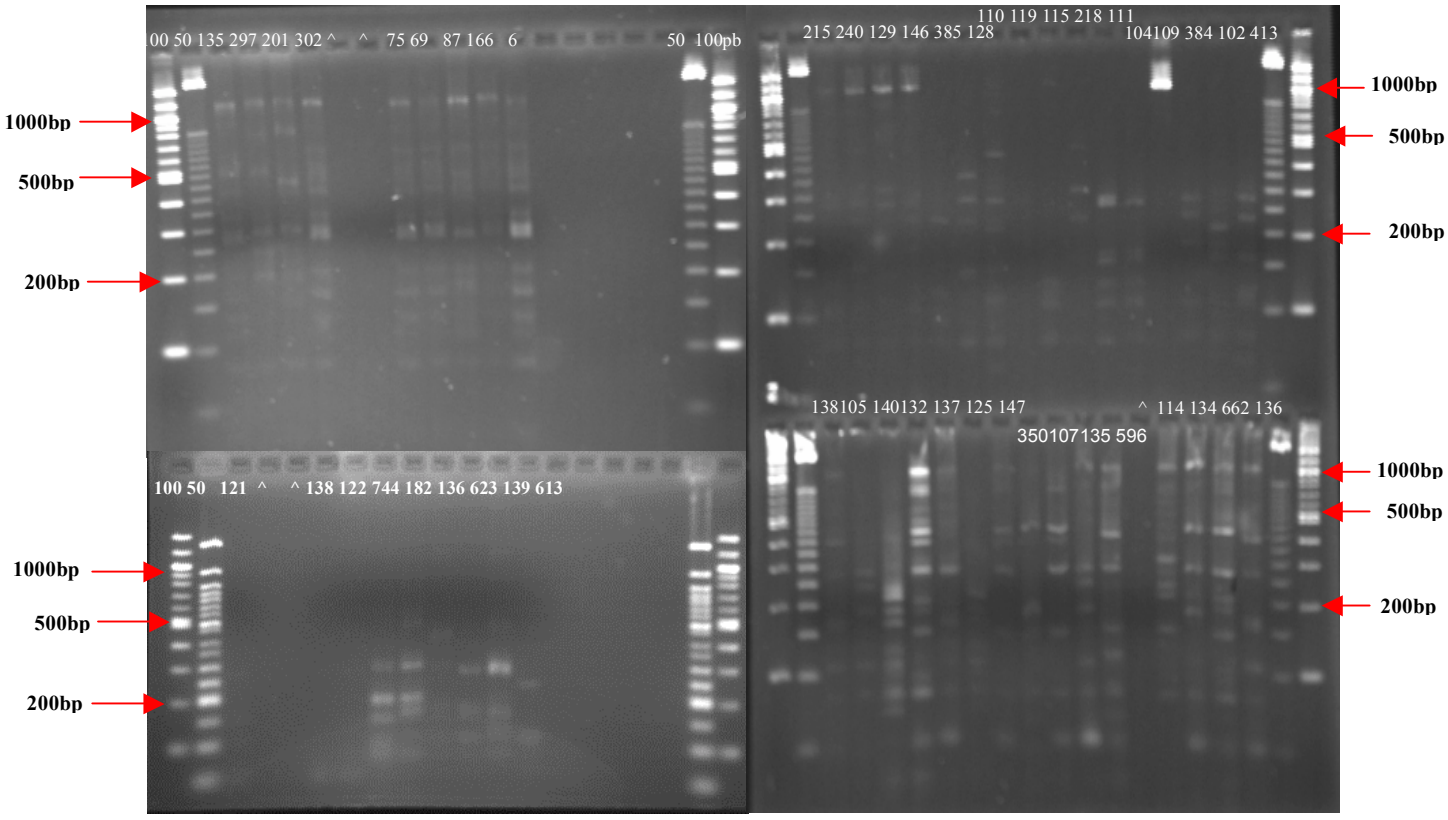


Figure 3.13 RFLP analysis of group G using *Hae* III and *Msp* I restriction enzymes in 3% low melting agarose.

Figures 3.14 and 3.15 demonstrate the screening of the second survey (MS1) named as group H using *Msp*I and *Hae* III restriction enzymes in 3% low melting agarose. A total of 0.75µg of template was used for enzyme digestion. The DNA markers were the 100bp and 50bp ladders.

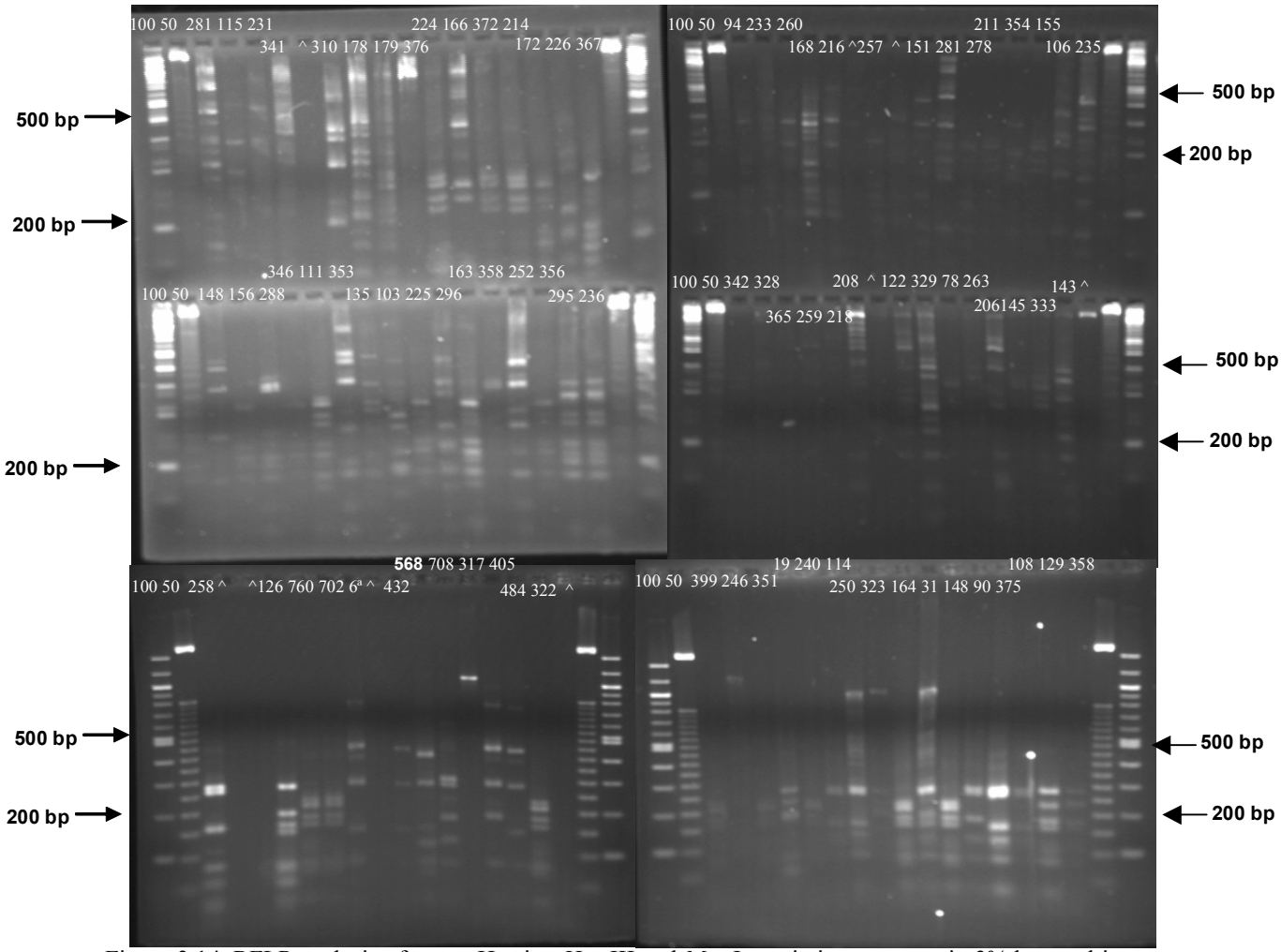


Figure 3.14 RFLP analysis of group H using *Hae* III and *Msp* I restriction enzymes in 3% low melting agarose.

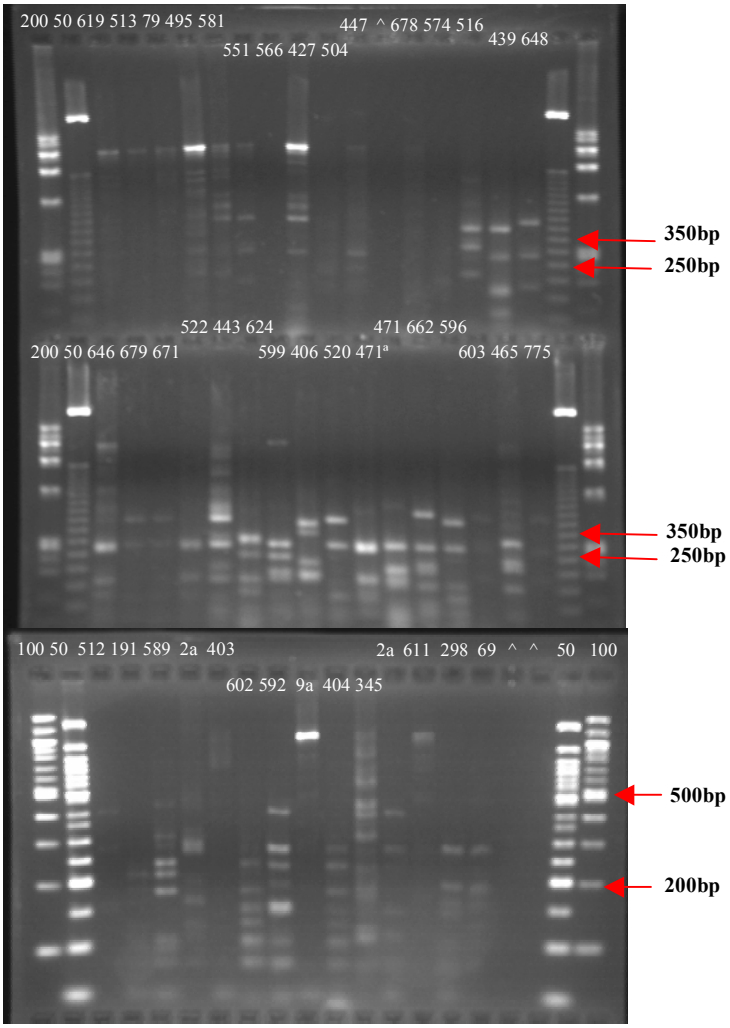


Figure 3.15 RFLP analysis of group H using *Hae* III and *Msp* I restriction enzymes in 3% low melting agarose.

Restriction analysis demonstrated a wide diversity of Operational Taxonomical Units (OTUs) among all the clone libraries constructed. Many different restriction patterns were observed and only a few OTU's showed similar banding patterns. *Hae* III and *Msp* I restriction enzymes were an efficient way to screen among different OTU's.

A total of 93 OTU's were selected after sequence comparison using *in silico* analysis. Sequences having a 97% similarity were considered the same OTU. The accession numbers of prokaryotic clones are listed in Table 3.1. The closest relatives of

representative OTUs, identified by searching in the GenBank database, are given in Table 3.2.

Table 3.1 Gen Bank accession numbers of environmental 16S rDNA clones.

Clone Number	Accession Number
G3	EF375729
G53	EF375730
G88	EF375731
G102	EF375732
G109	EF375733
G115	EF375734
G125	EF375735
G140	EF375736
E39	EF375737
E41	EF375738
G13	EF375739
G14	EF375740
G15	EF375741
G54	EF375742
G62	EF375743
G107	EF375744
G128	EF375745
G129	EF375746
G147a	EF375747
E40	EF375748
E42	EF375749
E85	EF375750
G74	EF375751
G84	EF375752
G9	EF375753
G111	EF375754
G135	EF375755
G8	EF375756
G58	EF375757
G56	EF375758
i157a	EF375759
i250	EF375760
i252	EF375761
i130	EF375762
i140	EF375763
i157	EF375764
i190	EF375765
i196	EF375766
i197	EF375767
i1	EF375768
i211	EF375769
i233	EF375770
i234	EF375771
i239	EF375772
i260	EF375773
i273	EF375774
i304	EF375775

Table 3.1 cont.

i309	EF375776
i40	EF375777
i41	EF375778
i4	EF375779
i52	EF375780
i60	EF375781
i72	EF375782
i94	EF375783
i298	EF375784
i43	EF375785
i73	EF375786
i85	EF375787
H143	EF375788
H263	EF375789
H178	EF375790
H208	EF375791
H179	EF375792
H206	EF375793
H211	EF375794
H214	EF375795
H224	EF375796
H235	EF375797
H255	EF375798
H278	EF375799
H333	EF375800
H295	EF375801
H406	EF375802
H443	EF375803
H495	EF375804
H115	EF375805
H148	EF375806
H310	EF375807
H513	EF375808
H329	EF375809
H471a	EF375810
H341	EF375811
H439	EF375812
H520	EF375813
H603	EF375814
H646	EF375815
H648	EF375816
H376	EF375817
H678	EF375818
H679	EF375819
H465	EF375820

Table 3.2 16S rDNA sequences identified in the black mangrove leaf surface from Cabo Rojo, Puerto Rico in the three clone libraries

OTU's	Accession no.	Closest relative	Accession no.	% Identity
G3	EF375729	*Halomonas avicenniae* strain MW2a 16S	DQ888315	96
G53	EF375730	Uncultured gamma proteobacterium clone LKC3_125.60	AY510238	100
G88	EF375731	Gamma proteobacterium KT0813 from North Sea	AF235130	97
G102	EF375732	Uncultured bacterium clone aaa69b07	DQ817187	99
G109	EF375733	Gamma proteobacterium KT0813 16S	AF235130	97
G115	EF375734	*Halomonas avicenniae* strain MW2a	DQ888315	96
G125	EF375735	*Pantoea agglomerans*	AM184272	98
G140	EF375736	*Pantoea agglomerans*	AM184272	99
E39	EF375737	Uncultured Bacteroidetes bacterium clone HPIRR1B05	AY852132	91
E41	EF375738	Uncultured Bacteroidetes bacterium clone HPIRR1H05	AY852135	91
G13	EF375739	Uncultured Bacteroidetes bacterium clone HPIRR1H05	AY852135	94
G14	EF375740	Uncultured Bacteroidetes bacterium clone HPIRR1H05	AY852135	96
G15	EF375741	Uncultured Bacteroidetes bacterium clone HPIRR1H05	AY852135	96
G54	EF375742	Uncultured Bacteroidetes bacterium clone HPIRR1B05	AY852132	92
G62	EF375743	*Salinibacter rubber*	AF323503	89
G107	EF375744	*Chryseobacterium meningosepticum*	AY468482	99
G128	EF375745	Uncultured bacterium microbial community in sponges	AJ347046	90
G129	EF375746	Uncultured Bacteroidetes bacterium clone HPIRR1B07	AY852133	97
G147a	EF375747	*Chryseobacterium meningosepticum* strain LDVH	AY468482	99
E40	EF375748	Uncultured bacterium clone E4bD01 hypersaline microbial mat	DQ103624	94
E42	EF375749	*Palleronia marisminoris*	AY926462	95
E85	EF375750	Marine bacterium B5-6 clone	AF076897	93
G74	EF375751	Marine bacterium B5-6 clone	AF076897	95
G84	EF375752	*Rhodovulum* sp. C2B-PN-R4	AM180479	95
G9	EF375753	Uncultured bacterium clone E4bD01 hypersaline microbial mat	DQ103624	95
G111	EF375754	*Rhodovulum* sp. C2B-PN-R4	AM180479	95
G135	EF375755	Uncultured proteobacterium clone 072DZ18	DQ330987	96
G8	EF375756	Uncultured euryarchaeote clone HPIRR2G10	AY852176	98
G58	EF375757	Uncultured euryarchaeote clone HPIRR2G10	AY852176	98
G56	EF375758	*Halococcus hamelinii*	DQ017835	93

Table 3.2
cont.

i157a	EF375759	Uncultured bacterium clone s4w18-5	DQ068915	99
i250	EF375760	Uncultured gamma proteobacterium clone LKC3	AY510236	99
i252	EF375761	Uncultured Alphaproteobacteria bacterium clone HPIRR1H10	AY852208	96
i130	EF375762	Uncultured bacterium clone E4bD01 hypersaline microbial mat	DQ103624	94
i140	EF375763	Uncultured bacterium clone E4bD01	DQ103624	94
i157	EF375764	Uncultured bacterium clone E4bD01	DQ103624	94
i190	EF375765	*Rhodovulum strictum*	D16419	94
i196	EF375766	Uncultured bacterium clone E4bD01	DQ103624	95
i197	EF375767	Uncultured bacterium clone E4bD01 hypersaline microbial mat	DQ103624	95
i1	EF375768	*Rhodovulum strictum*	D16419	95
i211	EF375769	*Rhodovulum strictum*	D16419	94
i233	EF375770	*Rhodovulum strictum*	D16419	95
i234	EF375771	Uncultured bacterium clone ELB25-004; lake Bonney Antarctica	DQ015780	94
i239	EF375772	Uncultured bacterium clone E4bD01 hypersaline microbial mat	DQ103624	93
i260	EF375773	Uncultured bacterium clone E4bD01 hypersaline microbial mat	DQ103624	95
i273	EF375774	Uncultured proteobacterium clone 06N224B from guerrero negro bay	DQ330982	96
i304	EF375775	Uncultured Rhodovulum sp. clone Nubeena257	AY499906	95
i309	EF375776	Uncultured Rhodovulum sp. clone Nubeena257	AY499906	95
i40	EF375777	Uncultured bacterium clone E4bD01 hypersaline microbial mat		95
i41	EF375778	Uncultured bacterium clone E4bD01 hypersaline microbial mat	DQ103624	95
i4	EF375779	*Rhodovulum strictum*	D16419	95
i52	EF375780	Uncultured Rhodovulum sp. clone Nubeena257	AY499906	94
i60	EF375781	*Rhodovulum strictum*	D16419	95
i72	EF375782	*Rhodovulum strictum*	D16419	95
i94	EF375783	Uncultured Rhodovulum sp. clone Nubeena257	AY499906	95
i298	EF375784	*Salinibacter rubber*	AF323503	90

Table 3.2
cont

i43	EF375785	Uncultured Bacteroidetes bacterium clone HPIRR2B09	AY852136	91
i73	EF375786	Uncultured Bacteroidetes bacterium clone HPDOMI1H12	AY851786	89
i85	EF375787	*Gramella echinicola* strain KMM 6050	AY608409	94
H143	EF375788	*Salinisphaera shabanense*	AJ421425	95
H263	EF375789	*Salinisphaera shabanense*	AJ421425	95
H178	EF375790	*Comamonas testosteroni* SB2	AY050494	97
H208	EF375791	*Comamonas testosteroni* SB2	AY050494	99
H179	EF375792	*Enterobacter ludwigii*	AJ853891	99
H206	EF375793	Marine bacterium B5-6 clone D	AF076898	95
H211	EF375794	*Pantoea* sp	AJ002811	99
H214	EF375795	*Averyella dalhousiensis*	DQ158206	99
H224	EF375796	*Averyella dalhousiensis*	DQ158206	99
H235	EF375797	*Aurantimonas coralicida*	AY065627	94
H255	EF375798	*Fulvimarina pelagi*	AY178862	95
H278	EF375799	gamma proteobacterium clone LKC3_125.60	AY510238	99
H333	EF375800	gamma proteobacterium clone LKC3_125.60	AY510238	99
H295	EF375801	*Morganella morganii*	DQ358146	99
H406	EF375802	proteobacterium clone 01D2Y70 from guerrero negro	DQ330828	91
H443	EF375803	proteobacterium clone 072DZ18 grom guerrero negro microbial mat	DQ330987	95
H495	EF375804	*Averyella dalhousiensis*	DQ158206	99
H115	EF375805	Uncultured Bacteroidetes bacterium clone HPDOMI2B11	AY851787	91
H148	EF375806	*Chryseobacterium* sp	AY468471	99
H310	EF375807	*Chryseobacterium* sp	AY468471	99
H513	EF375808	*Stenothermobacter spongiae*	DQ064789	91
H329	EF375809	Uncultured bacterium clone E4bD01 hypersaline microbial mat	DQ103624	93
H471a	EF375810	Uncultured bacterium clone E4bD01 hypersaline microbial mat	DQ103624	94
H341	EF375811	Marine bacterium B5-6 clone D	AF076898	95
H439	EF375812	proteobacterium clone 072DZ18	DQ330987	95
H520	EF375813	*Aurantimonas coralicida*	AY065627	94
H603	EF375814	*Pseudaminobacter* sp. mp-1	AY331575	94
H646	EF375815	Uncultured bacterium clone E4bD01 hypersaline microbial mat	DQ103624	95

Table 3.2
cont.

H648	EF375816	*Pseudaminobacter* sp. mp-1	AY331575	95
H376	EF375817	Uncultured bacterium clone E4aG09	DQ103651	88
H678	EF375818	*Stenothermobacter spongiae*	DQ064789	90
H679	EF375819	*Stenothermobacter spongiae*	DQ064789	90
H465	EF375820	*Comamonas testosterone*	AF532871	99

Phylogenetic trees

The evolutionary relationship among a total of 94 clones were analyzed using the PHYLIP program package (version 3.5.1). A phylogenetic study of these sequences was performed using neighbor joining analysis. An analysis for the possibility of chimeric sequences was performed. This analysis suggested that no chimeric sequences were present in these clones.

Phylogenetical analysis using the neighbor joining algorithm revealed that several clones G58, G8, G56 are related to the genus *Halococcus* from the domain Archaea (Figure 3.16). It was also found that a group of clones was closely related to the group of clones HPDOM and HRIPR from a stromatolite microbial community of Hamelian Pool in Shark Bay, Western Australia forming a branch in this cluster with a bootstrap value of 100%.

Several of the clones obtained for the group G library (second survey) were closely related to the Bacteroidetes group of the Bacteria domain. Phylogenetic analysis revealed that clones partial 16SrDNA sequences for OTUs E39, G62, G14, G129, G54, G128, G13, E41 and G15 formed a cluster in an independent branch which are closely related to a group of clones HPDOM and HRIPR from a stromatolite microbial community of Hamelian Pool in Shark Bay, western Australia and to clones from sponges from different oceans (Figure 3.17). Operational taxonomic units G147a and G107 are closely related to *Leeuwenhoekiella marinoflava* ATCC 19326; AF203475 and *Leeuwenhoekiella blandensis* MED217 forming a branch in this cluster with a bootstrap value of 100%.

Some of the obtained OTU's from the G group belonged to the gamma proteobacteria. Phylogenetic analysis revealed that OTU's G53, G102, G125, and G140 were closely related to *Pantoea agglomerans* WAB1870; AM184212, forming a branch in this cluster with a bootstrap value of 100%. These OTU's were also related to the *Enterobacter ludwigii* WAB1894 (Hoffmann *et al*., 2005) AM184235, and to a clone (LKC3) from a microbial mat cave sulfidic springs (Figure 3.18). Phylogenetic analyses revealed that OTU's G3, G115, G88 and G109 form an independent branch and were related to *Halomonas marisflavi* and *Halomonas avicenniae* forming a branch in this cluster with a bootstrap value of 60%.

Figure 3.19 shows the phylogenetic tree analysis for OTU G135 which was closely related to clone ACE-16 from a meromictic marine basin Antarctica and clone MPD-20 from a hypersaline evaporation pond forming a branch in this cluster with a bootstrap value of 64%. OTU's G84, G111, and G9 form an independent distant branch within the alpha-proteobacteria group.

Phylogenetic analyses revealed OTU's E42, E40, G74, and E85 form an independent branch and were closely related to a clone ACE-16 from a meromictic marine basin in Antarctica and to a clone MPD-20 from a hypersaline evaporation pond (Figure 3.20).

OTU's obtained from the library corresponding to group H (second survey) were related to the Bacteroidetes group. OTUs H679, H678, and H513 formed an independent distant branch which was related to *Stenothermobacter spongiae* (Figure 3.21). Phylogenetic analyses revealed that OTU's H310 and H148 form an independent cluster branch and were closely related to *Chryseobacterium ginsengisoli* forming a branch in

this group with a bootstrap value of 100%. OTU's H115 and H376 form a distant independent branch and were related to *Rhodothermus marinus* JCM 9785T.

The alpha-proteobacteria group was also found in the group H. Phylogenetic analysis revealed that OTU's H443 and H439 formed an independent branch and is related to *Paracoccus solventivorans* and *Paracoccus koreensis* (Figure 3.22). OTU H341 forms an independent branch and is closely related to a marine clone B5-6. OTU H646 is closely related to clone E4bD01 from a hypersaline endoevaporitic microbial mat. Phylogenetic analysis revealed that OTUs H603 and H648 form an independent branch and are related to *Pseudaminobacter salicylatoxidans* BN12. On the other hand OTU's H520 and H235 are closely related to *Aurantimonas coralicida* WP1 isolated from coral reefs. OTU H406 is related to clone 01D2Y70 from Guerrero Negro hypersaline microbial mat and OTU H465 presented a distant independent branch not closely related with any environmental sequence or genera but belongs to the alpha-proteobacteria group.

The group H also has OTU's related to the gamma-proteobacteria group. Phylogenetic analysis suggests that OTUs H78, H278, H211, H619, H214, H372, H224, and H495 form an independent branch and were related to the genus *Enterobacter asburiae* and also to some clones isolated from a rice plant and from anaerobic nitrogen-fixing consortia from plants (Figure 3.23). OTU H295 form a independent branch and was closely related to *Morganella psychrotolerans* a histamine-producing bacterium isolated from various seafoods, forming a branch in this cluster with a bootstrap value of 98%. OTU H79 was closely related to *Stenotrophomonas maltophilia* SB5, to a clone from a lagoon in south western Atlantic Ocean and to a *Xanthomonas* group from the

Hawaiian Archipielago forming a branch in this cluster with a bootstrap value of 99%. There are clones (H143 and H263) closely related to *Salinisphaera shabanensis* E1L3A isolated from the Red Sea. Phylogenic analysis revealed that OTU H255 was closely related to *Aquamicrobium defluvii* DSM 11603T and to *Nitratireductor aquibiodomus* NL21T forming a branch in this cluster with a bootstrap value of 100%. Clones H235 and H443 are closely related to *Aquamicrobium defluvii* DSM 11603T and to *Nitratireductor aquibiodomus* NL21T forming a branch in this cluster with a bootstrap value of 56%. On the other hand, clone H406 form an independent branch in the gamma-proteobacteria group (Figure 3.24). Representatives of the beta proteobacteria was also found in the clone library group H. OTU's H208 and H465 were closely related to *Pseudomonas straminea* forming a branch in the cluster of beta-proteobacterias with a bootstrap of 51% (Figure 3.25).

The clone library which was named group I represents the first sampling. Phylogenetic analysis showed that OTU I85 from the Bacteroidetes group was closely related to *Sufflavibacter litoralis* IMCC 1001 forming a branch in this cluster with a bootstrap value of 89%. OTUs i298, i73, and i43 form an independent branch and were related to *Salinibacter rubber* M1 (Figure 3.26). There are also representatives related to the alpha-proteobacteria group. Phylogenetic analysis revealed for OTU's i157, i211, i41, i40, i197, i260, i239, and i234 form a distant independent branch in this alpha-proteobacteria group (Figure 3.27). Also OTU's i52, i190, i4, i140, i130, i309, i1, i233, i72, i196, i94, i304, i60, and i273 form a independent branch in the alpha-proteobacteria group and could be related to clones from the guerrero negro hypersaline microbial mat (Figure 3.28). From the gamma-proteobacteria group, several clones were obtained.

Phylogenetic analysis revealed that OTU's i252 and i250b are related to the clone SZB65 from mangrove sediment (Figure 3.29). OTU i157a form an independent branch and could be related to members of the family *Enterobacteriaceae* such as *Pantoea agglomerans* WAB1870 and *Enterobacter hormaechei* (Figure 3.30).

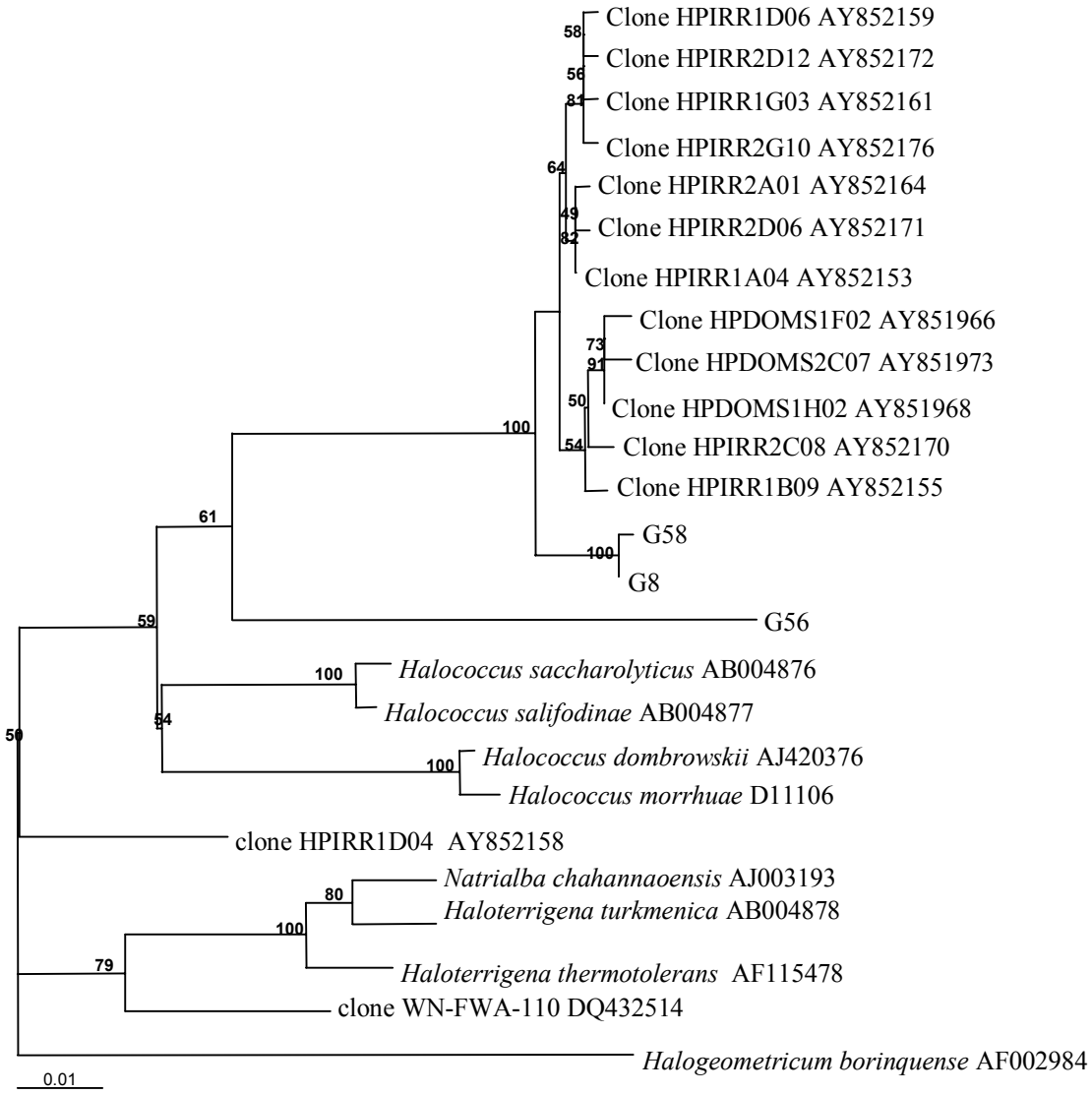


Figure 3.16 Neighbour-joining distance tree of partial 16SrDNA sequences for OTUs G8, G56 and G58 derived from *A. germinans* leaf surface, Cabo Rojo salterns, Puerto Rico. Bar represents 1 substitutions per 100 nucleotides. Bootstrap values higher than 45% are shown. *Halogeometricum borinquense* AF002984 was used as the outgroup.

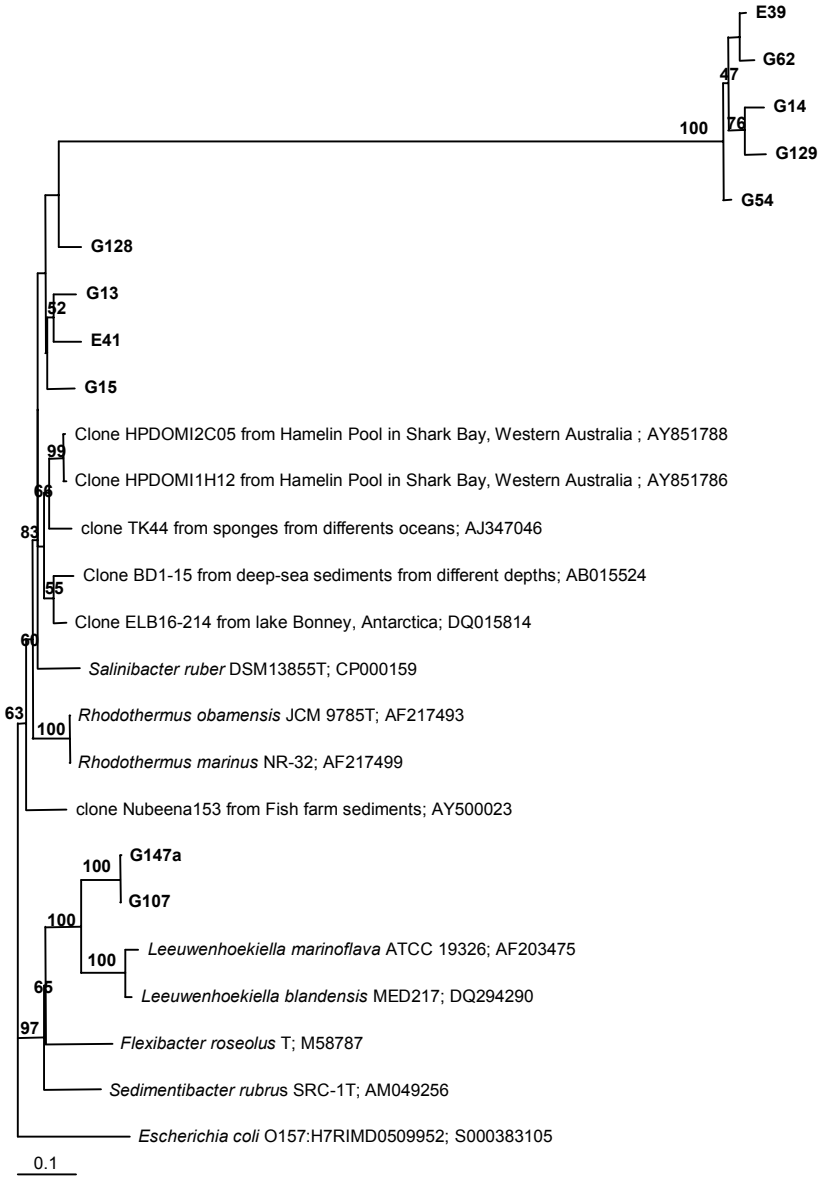


Figure 3.17. Neighbour-joining distance tree of partial 16SrDNA sequences for OTUs E39, G62, G14, G129, G54, G128, G13, E41, G15, G147a and G107 from *A. germinans* leaf surface, Cabo Rojo salterns, Puerto Rico. Bar represents 1 substitution per 10 nucleotides. Bootstrap values higher than 45% are shown. *Escherichia coli* O157:H7RIMD0509952 was used as the outgroup. Bacteroidetes group.

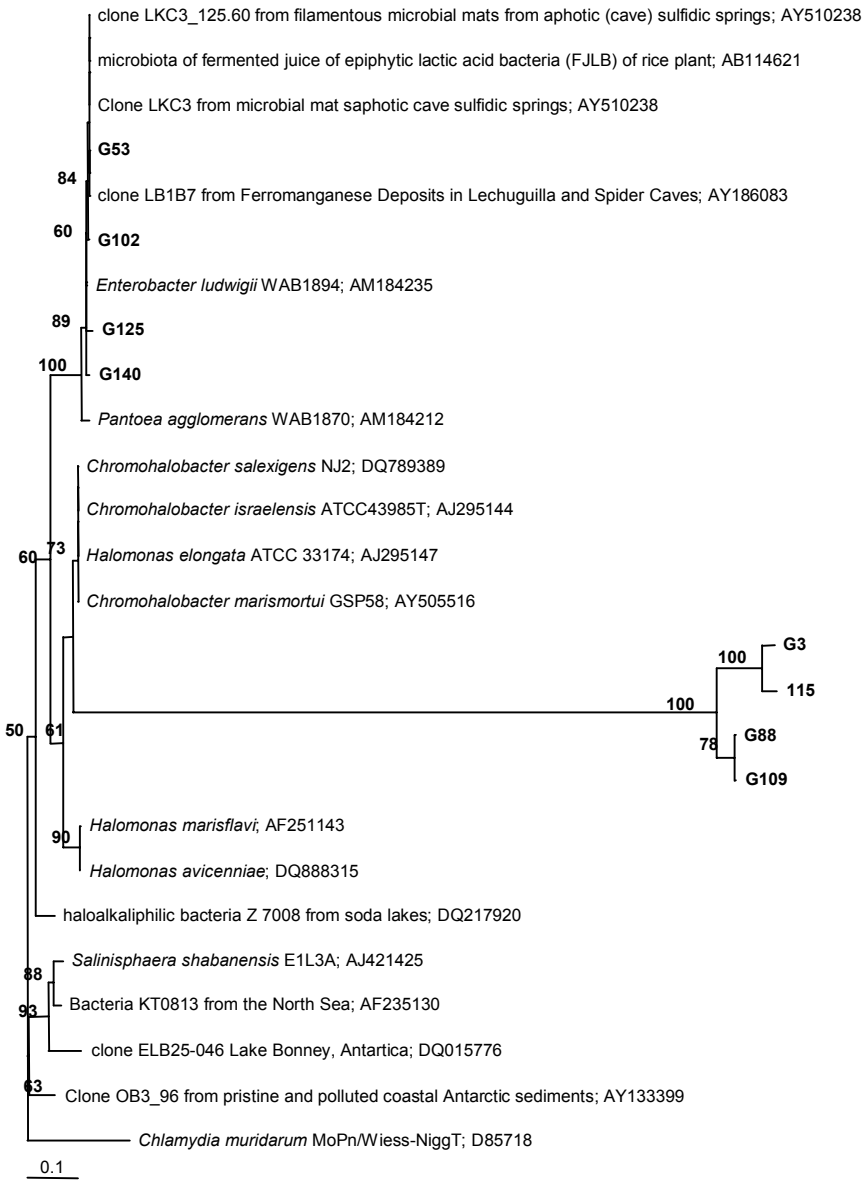


Figure 3.18 Neighbour-joining distance tree of partial 16SrDNA sequences for OTUs G53, G102, G125, G140, G3, G115, G88 and G109 from *A. germinans* leaf surface, Cabo Rojo salterns, Puerto Rico. Bar represents 1 substitution per 10 nucleotides. Bootstrap values higher than 45% are shown. *Chlamydia muridarum* MoPn/Wiess-NiggT; D85718 was used as the outgroup. Gamma Proteobacteria group.

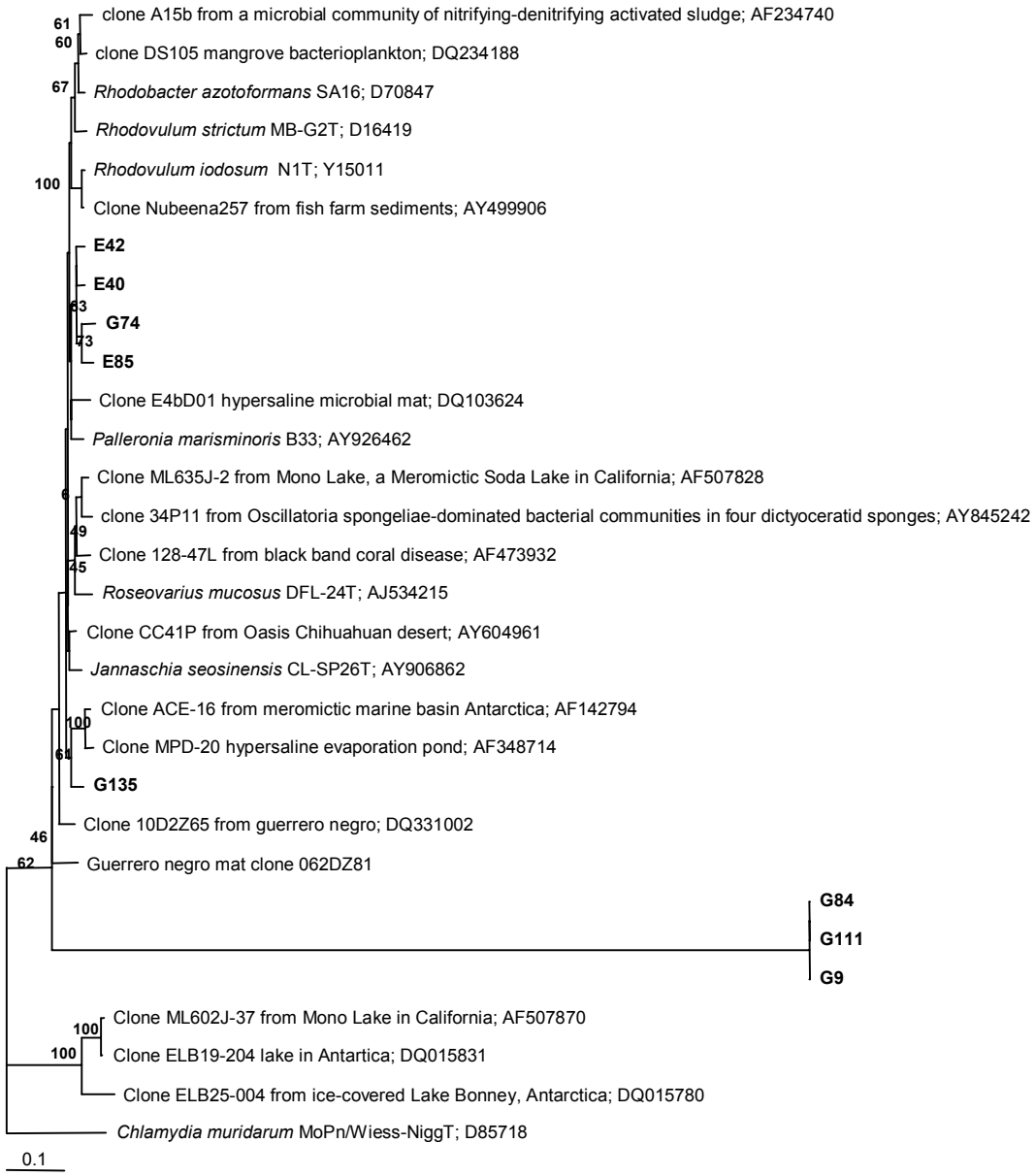


Figure 3.19 Neighbour-joining distance tree of partial 16SrDNA sequences for OTUs E42, E40, G74, E85, G135, G84, G111 and G9 from *A. germinans* leaf surface, Cabo Rojo salterns, Puerto Rico. Bar represents 1 substitution per 10 nucleotides. Bootstrap values higher than 45% are shown. *Chlamydia muridarum* MoPn/Wiess-NiggT; D85718 was used as the outgroup. Alpha Proteobacteria group.

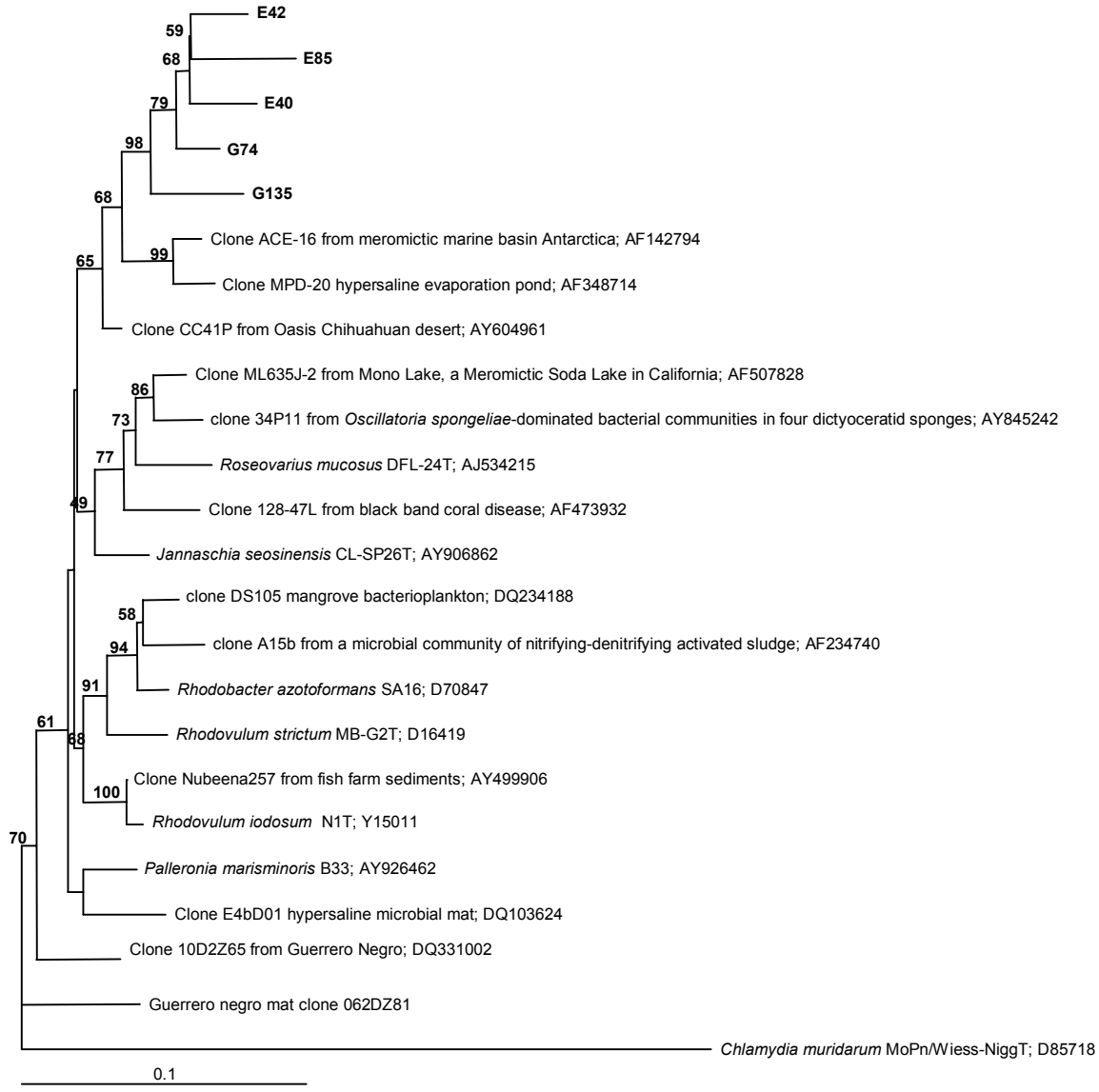


Figure 3.20 Neighbour-joining distance tree of partial 16SrDNA sequences for OTUs E42, E40, G74, E85 and G135 from *A. germinans* leaf surface, Cabo Rojo salterns, Puerto Rico. Bar represents 1 substitution per 10 nucleotides. Bootstrap values higher than 45% are shown. *Chlamydia muridarum* MoPn/Wiess-NiggT; D85718 was used as the outgroup. Alpha Proteobacteria group.

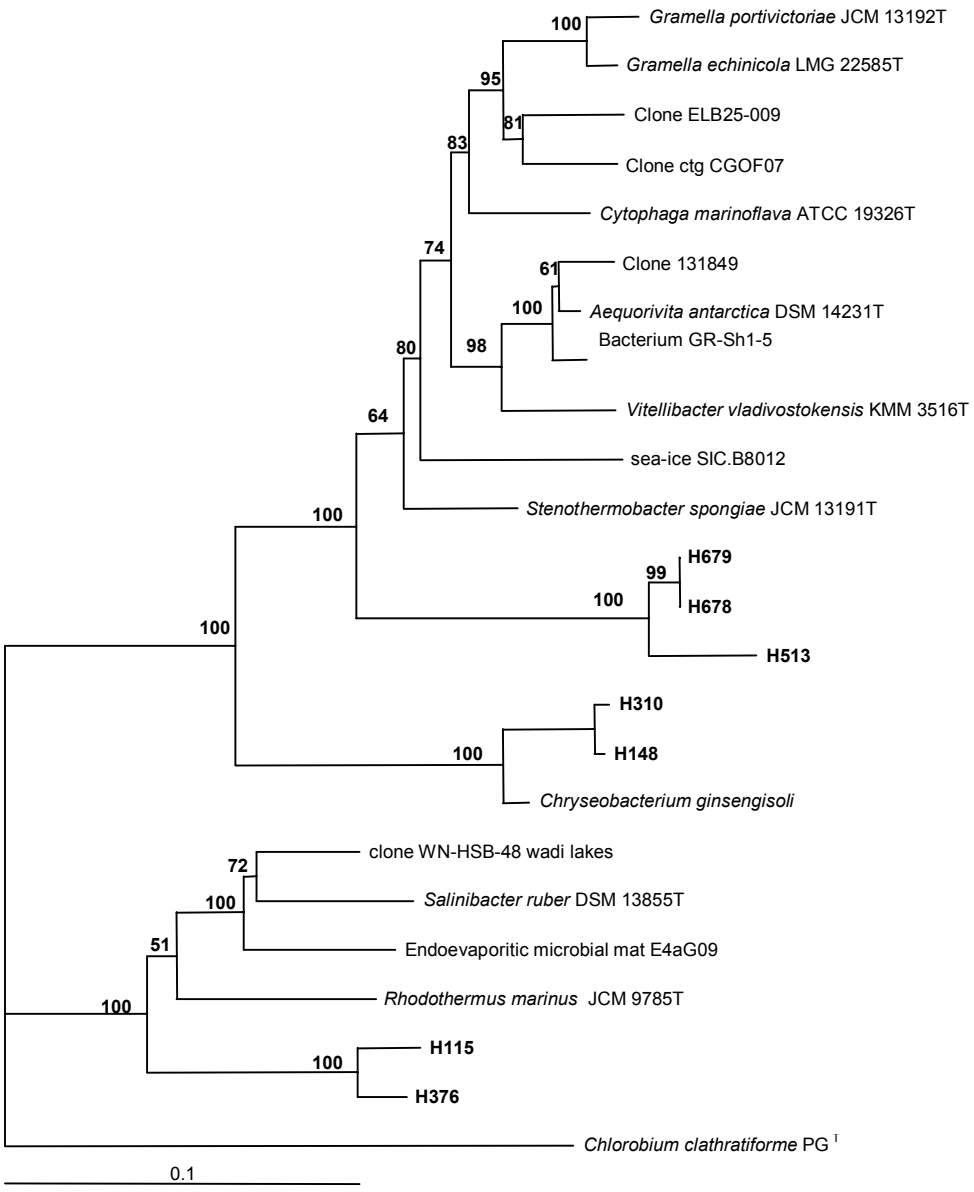


Figure 3.21 Neighbour-joining distance tree of partial 16SrDNA sequences for OTUs H679, H678, H513, H310, H148, H115 and H376 from *A. germinans* leaf surface, Cabo Rojo salterns, Puerto Rico. Bar represents 1 substitution per 10 nucleotides. Bootstrap values higher than 45% are shown. *Chlorobium clathratiforme* PG^T was used as the outgroup. Bacteroidetes group.

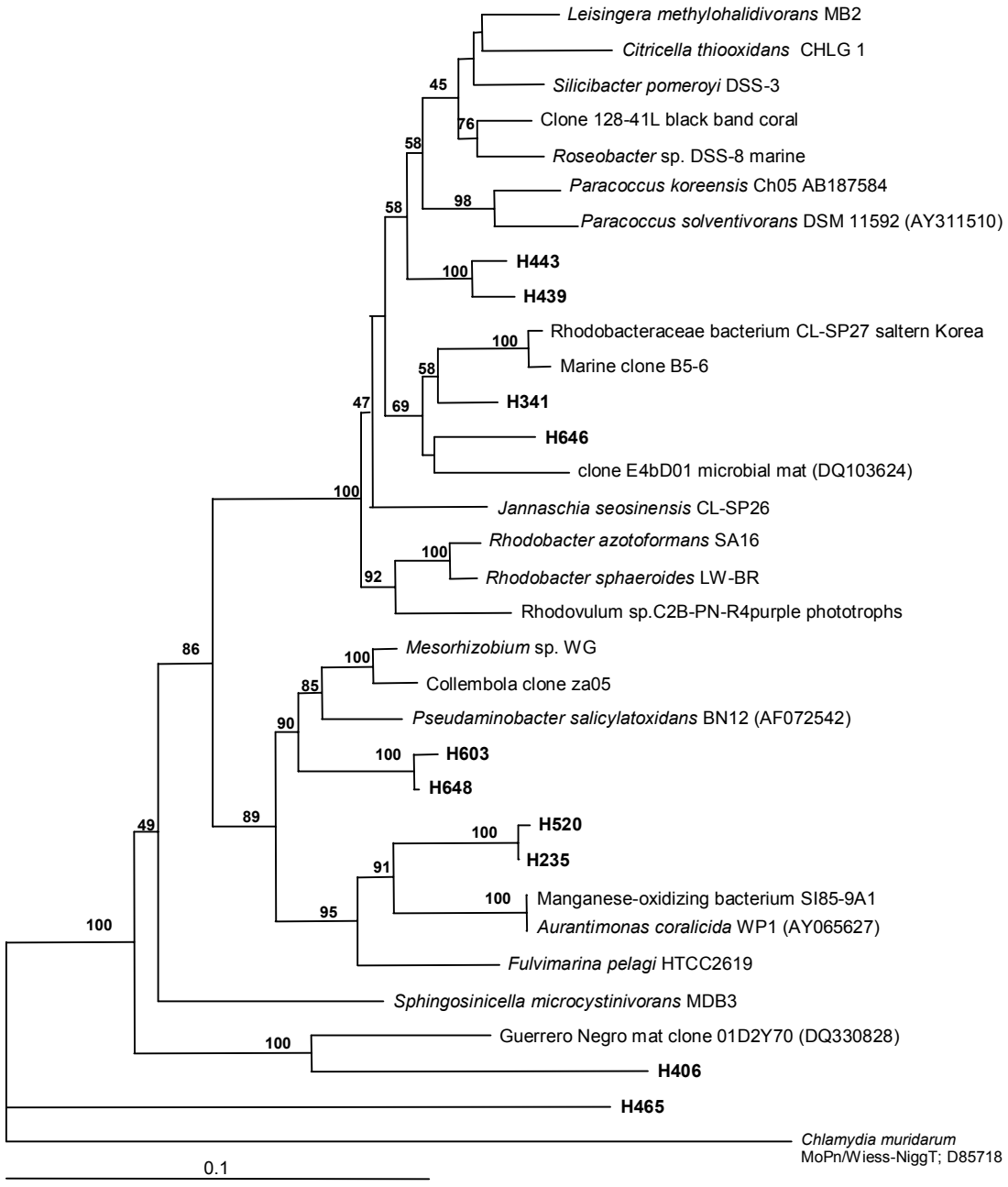


Figure 3.22 Neighbour-joining distance tree of partial 16SrDNA sequences for OTUs H443, H439, H341, H646, H603, H648, H520, H235, H406 and H465 from *A. germinans* leaf surface, Cabo Rojo salterns, Puerto Rico. Bar represents 1 substitution per 10 nucleotides. Bootstrap values higher than 45% are shown. *Chlamydia muridarum* MoPn/Wiess-NiggT; D85718 was used as the outgroup. Alpha-Proteobacteria group.

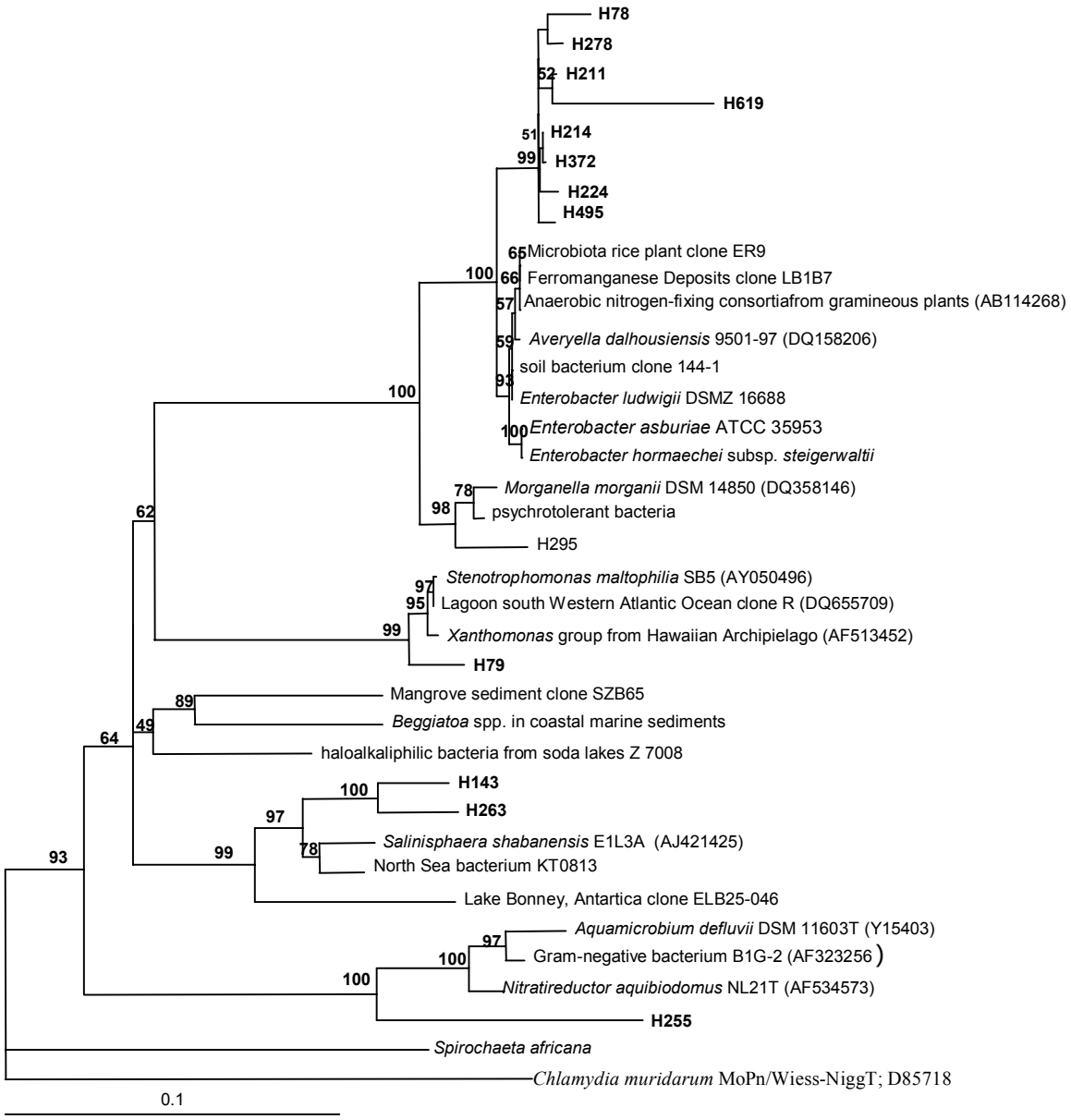


Figure 3.23 Neighbour-joining distance tree of partial 16SrDNA sequences for OTUs H78, H278, H211, H619, H214, H372, H224, H495, H295, H79, H143, H263 and H255 from *A. germinans* leaf surface, Cabo Rojo salterns, Puerto Rico. Bar represents 1 substitution per 10 nucleotides. Bootstrap values higher than 45% are shown. *Chlamydia muridarum* MoPn/Wiess-NiggT; D85718 was used as the outgroup. Gamma-Proteobacteria group.

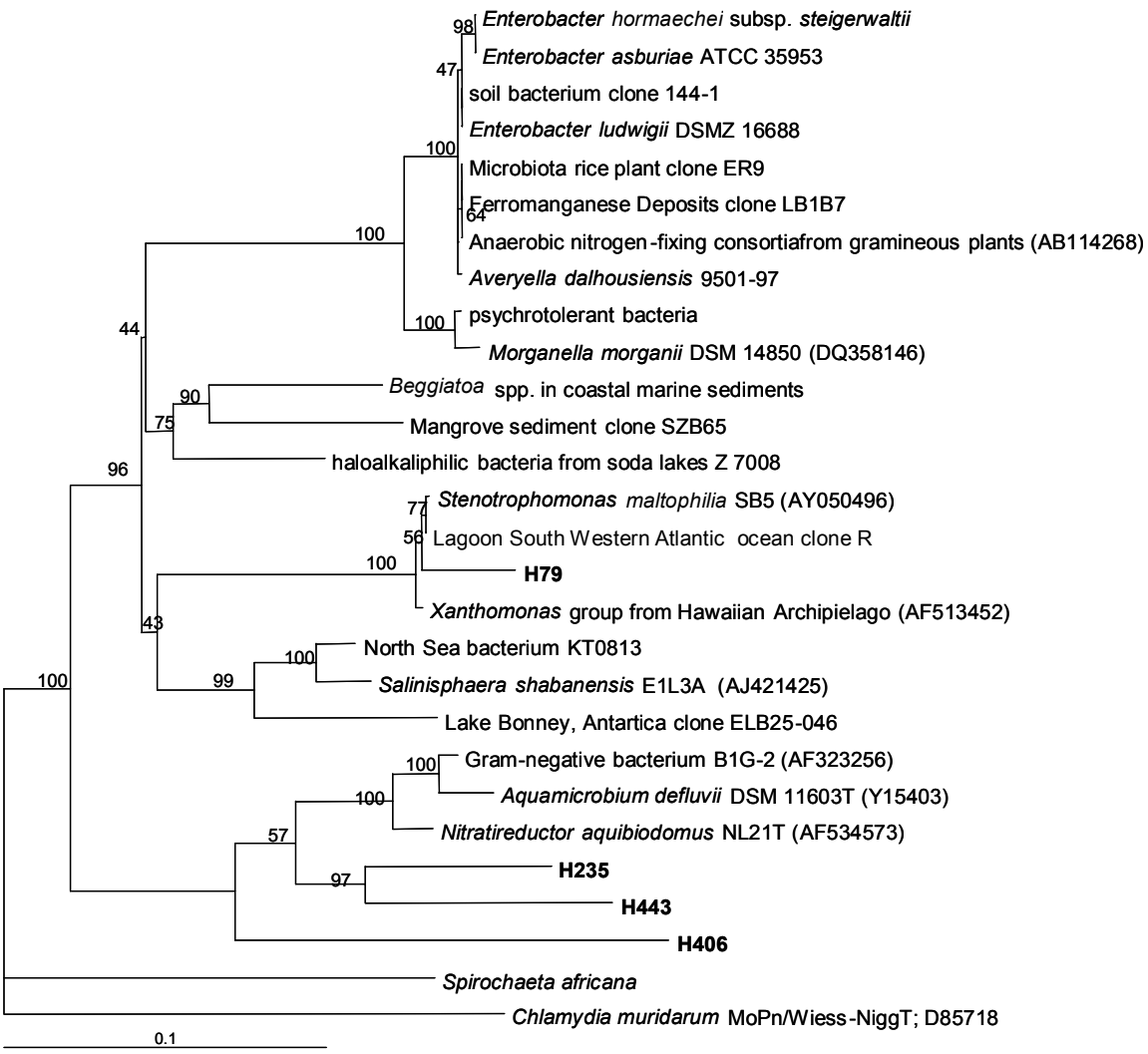


Figure 3.24. Neighbour-joining distance tree of partial 16SrDNA sequences for OTUs H79, H235, H443 and H406 from *A. germinans* leaf surface, Cabo Rojo salterns, Puerto Rico. Bar represents 1 substitution per 10 nucleotides. Bootstrap values higher than 45% are shown. *Chlamydia muridarum* MoPn/Wiess-NiggT; D85718 was used as the outgroup. Gamma-Proteobacteria group.

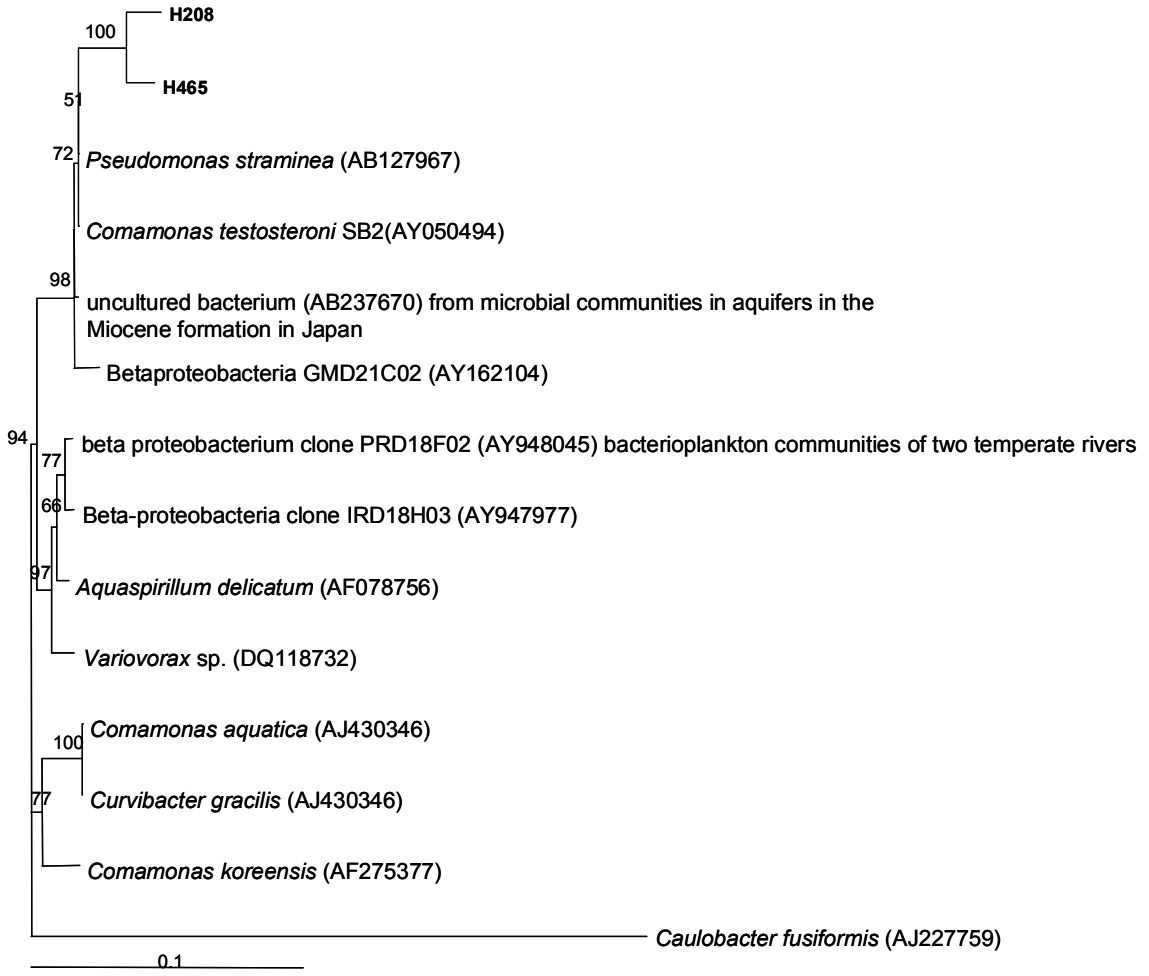


Figure 3.25 Neighbour-joining distance tree of partial 16SrDNA sequences for OTUs H208 and H465 from *A. germinans* leaf surface, Cabo Rojo salterns, Puerto Rico. Bar represents 1 substitution per 10 nucleotides. Bootstrap values higher than 45% are shown. *Caulobacter fusiformis*; AJ227759 was used as the outgroup. Beta-Proteobacteria group

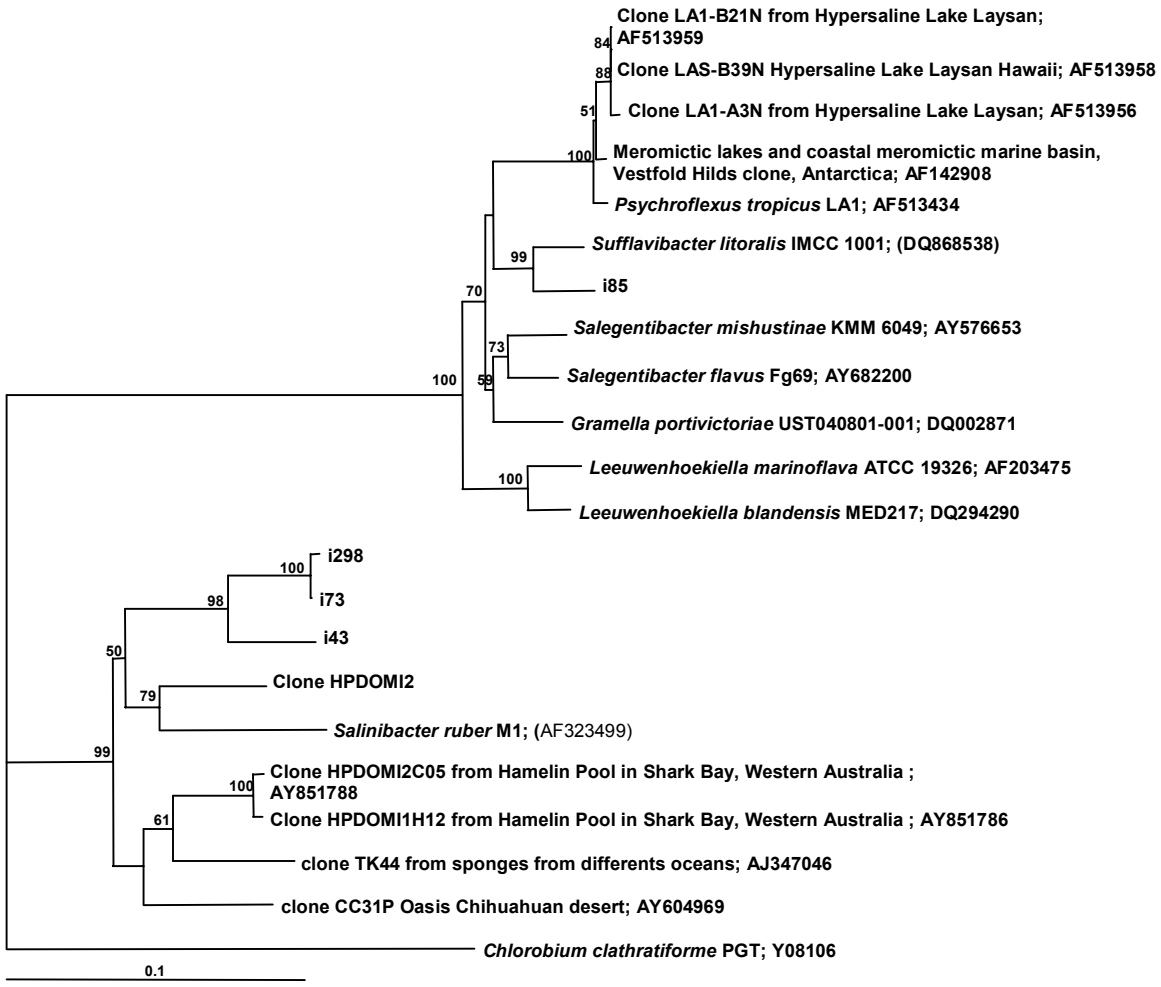


Figure 3.26 Neighbour-joining distance tree of partial 16SrDNA sequences for OTUs i85, i298, i73, and i43 from *A. germinans* leaf surface, Cabo Rojo salterns, Puerto Rico. Bar represents 1 substitution per 10 nucleotides. Bootstrap values higher than 45% are shown. *Chlorobium clathratiforme* PGT; Y08106 was used as the outgroup. Bacteroidetes group.

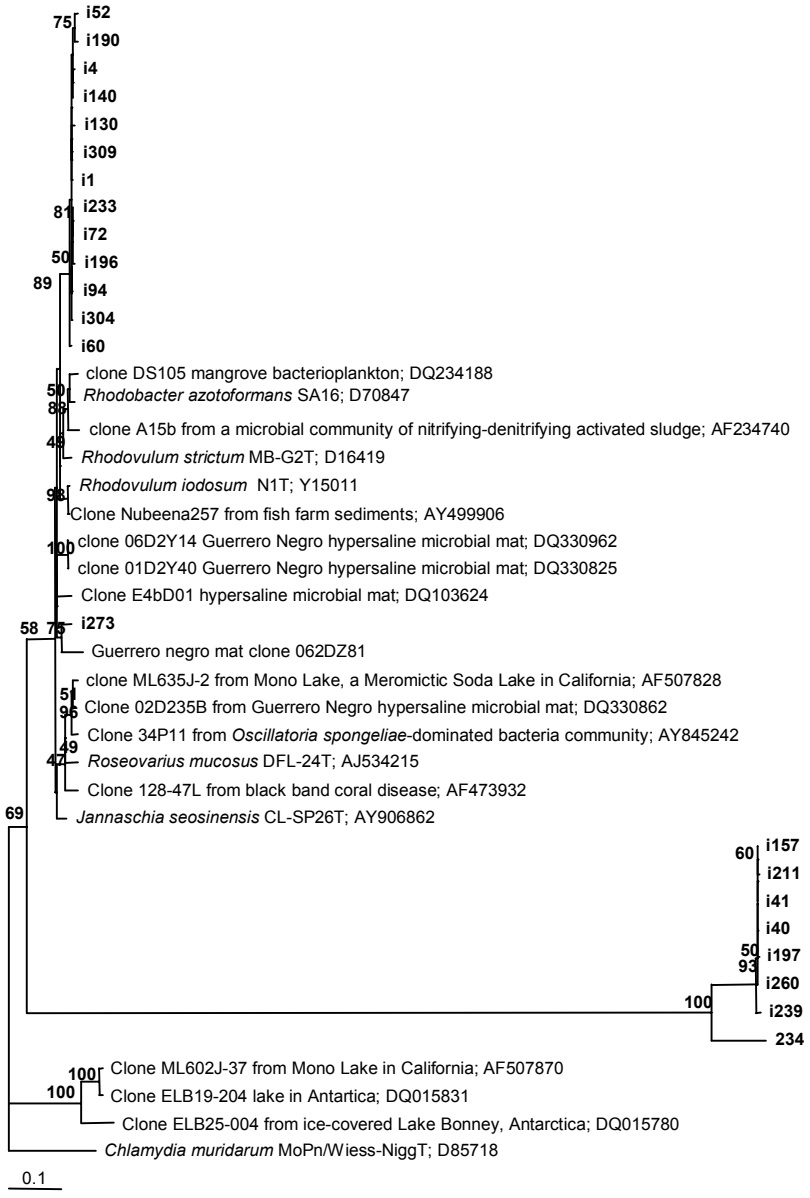


Figure 3.27 Neighbour-joining distance tree of partial 16SrDNA sequences for OTUs i52, i190, i4, i140, i130, i309, i1, i233, i72, i196, i94, i304, i60, i273, i157, i211, i41, i40, i197, i260, i239 and i234 from *A. germinans* leaf surface, Cabo Rojo salterns, Puerto Rico. Bar represents 1 substitution per 10 nucleotides. Bootstrap values higher than 45% are shown. *Chlamydia muridarum* MoPn/Wiess-NiggT; D85718 was used as the outgroup. Alpha-Proteobacteria group.

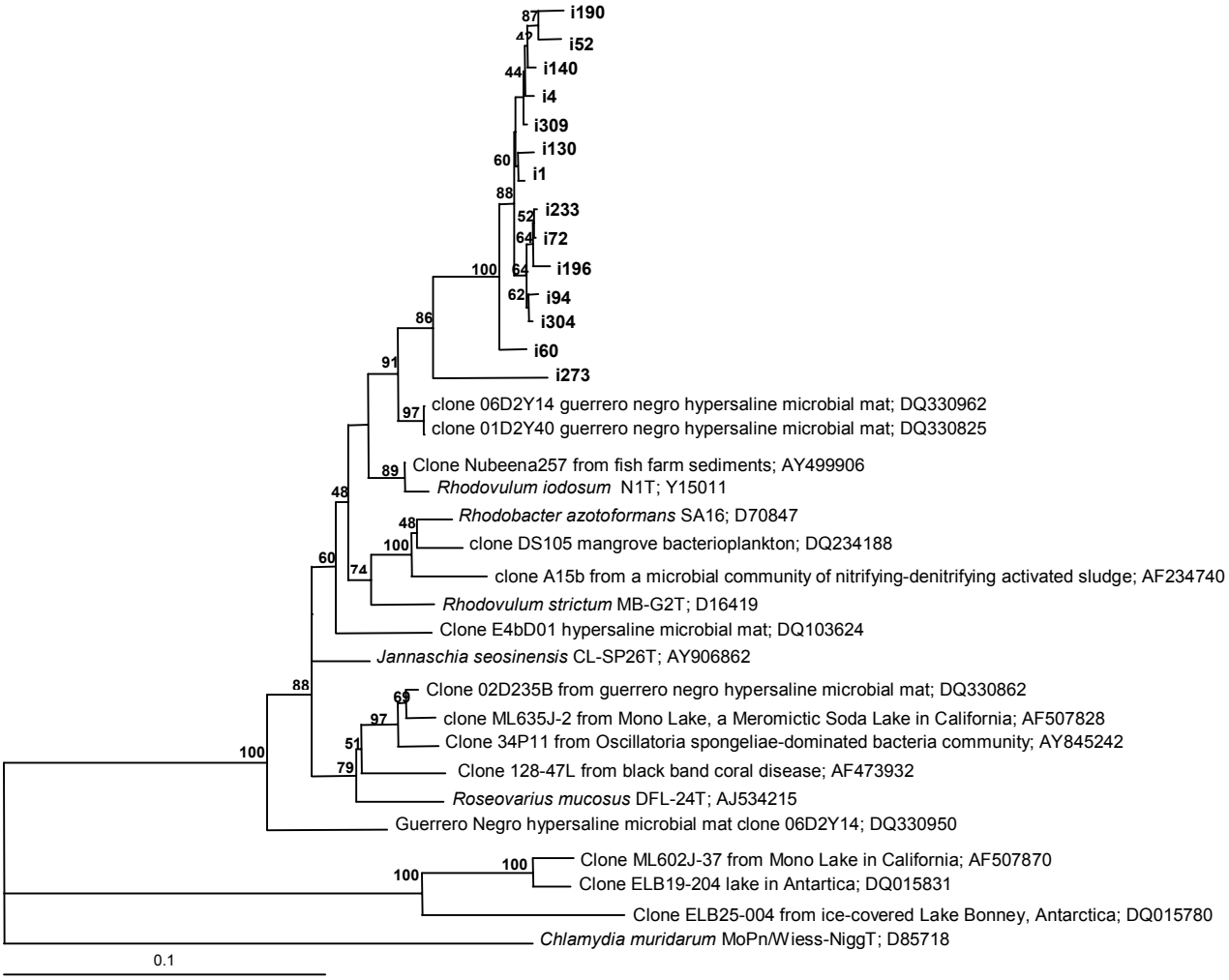


Figure 3.28 Neighbour-joining distance tree of partial 16SrDNA sequences for OTUs i52, i190, i4, i140, i130, i309, i1, i233, i72, i196, i94, i304, i60 and i273 from *A. germinans* leaf surface, Cabo Rojo salterns, Puerto Rico. Bar represents 1 substitution per 10 nucleotides. Bootstrap values higher than 45% are shown. *Chlamydia muridarum* MoPn/Wiess-NiggT; D85718 was used as the outgroup. Alpha-Proteobacteria group.

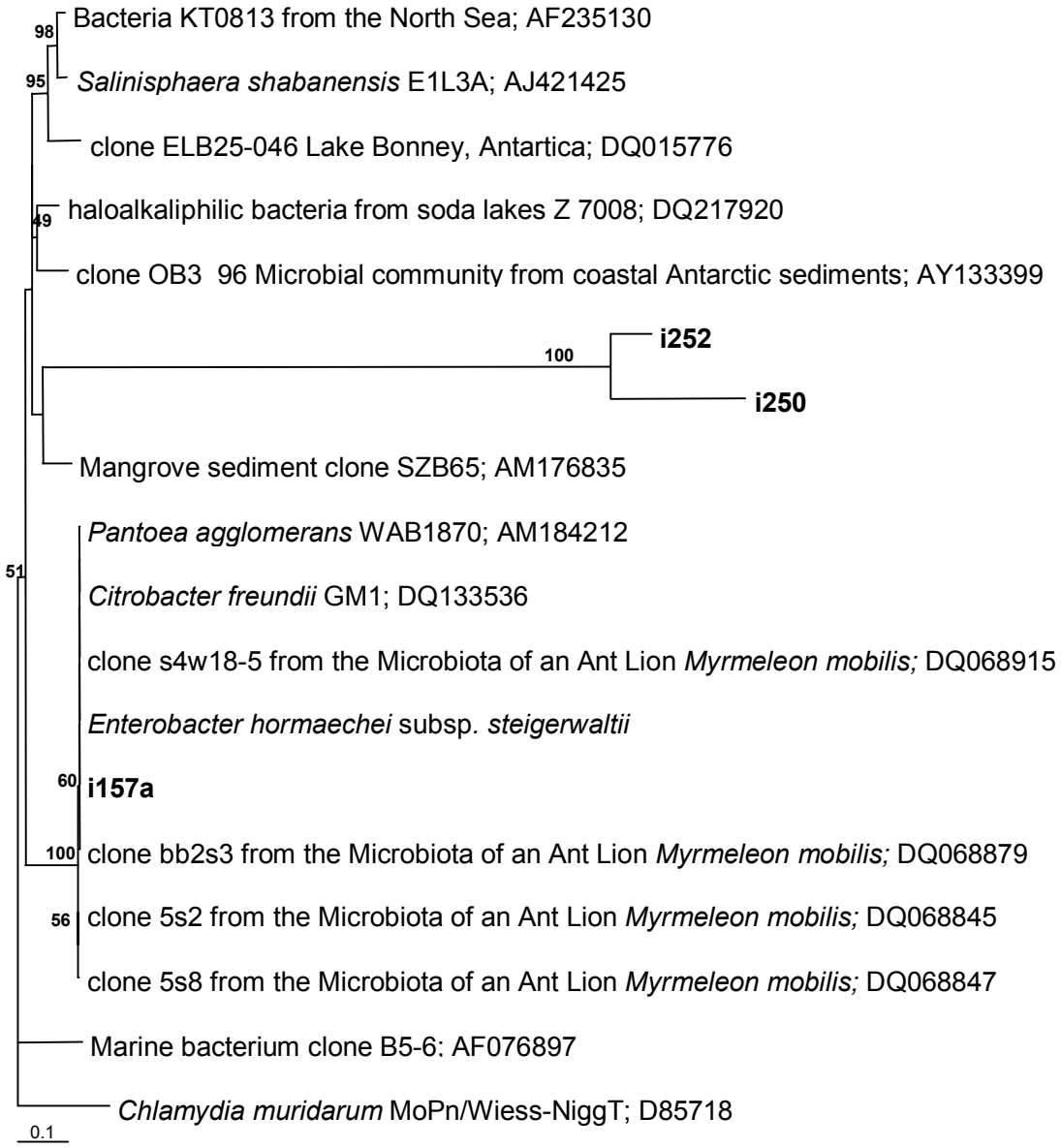


Figure 3.29 Neighbour-joining distance tree of partial 16SrDNA sequences for OTUs i252, i250b and i157a from *A. germinans* leaf surface, Cabo Rojo salterns, Puerto Rico. Bar represents 1 substitution per 10

nucleotides. Bootstrap values higher than 45% are shown. *Chlamydia muridarum* MoPn/Wiess-NiggT; D85718 was used as the outgroup. Gamma-Proteobacteria group.

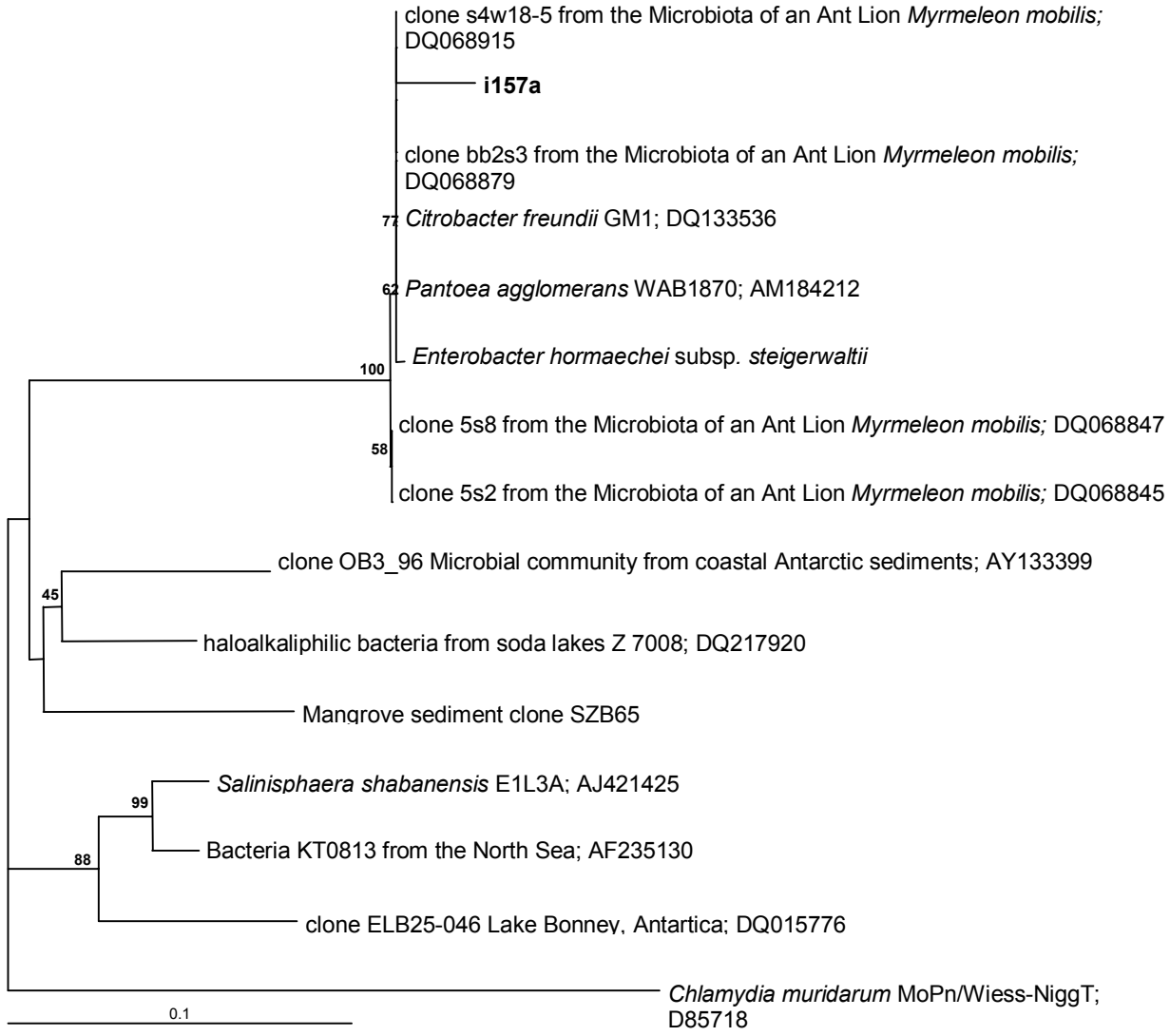


Figure 3.30 Neighbour-joining distance tree of partial 16SrDNA sequences for OTU i157a from *A. germinans* leaf surface, Cabo Rojo salterns, Puerto Rico. Bar represents 1 substitution per 10 nucleotides. Bootstrap values higher than 45% are shown. *Chlamydia muridarum* MoPn/Wiess-NiggT; D85718 was used as the outgroup. Gamma-Proteobacteria group.

Statistical analysis

Statistical indices for the gene libraries are shown in Table 3.3. The unique distance to define an OTU for both libraries was 0.03 or 97% of sequence similarity (Dunbar, 2002; Singleton *et al*., 2001; Stout and Nusslein, 2005) between sampled clones. Numbers in parenthesis indicate the number of 16S rDNA clones used in the analyses. Richness is the number of phylotypes observed. Each phylotype consisted of either unique clone or a group of clones that has sequence similarities of over 97%. Richness analysis showed greater diversity for community library G (second survey) with 22 different operational taxonomic units (OTU's). The Shannon and Simpson's diversity indices were calculated, and indexes showed that clone library G had higher diversity than H and I. The Jaccard, SAce, and SChao richness index values were calculated in order to corroborate richness between samples. All indexes indicated that library G had the highest level of richness when compared to clone library H and I.

To measure how well the samples represent the larger environment, the Good Coverage Index was calculated (Figure 3.32) using the program ASLO (www.aslo.org/methods/free/2004/0114a.html). The coverage was 43.33, 57.14 and 72.41% for libraries G, H and I respectively. Rarefaction curves were done using the program DOTUR (http://www.plantpath.wisc.edu/fac/joh/dotur.html). This is a method used to compare observed richness among environments that have been unequally sampled (Hughes and Bohannan, 2004). After 100 repeated randomizations of the samples, the results demonstrated an increase in richness for clone library G and H

(Figure 3.31). The graphic curves revealed that if clone library I had more clones screened, the distribution would be constant. In contrast, for clone library G and H additional clone screening will be necessary to reach a continuous tendency in the graphic pattern correspondent to coverage data.

In order to evaluate the level of differences among clone libraries, a p-value was calculated using Web-LIBSHUFF program (http://libshuff.mib.uga.edu). The following formula was used to calculate the standard p-value of two libraries: p=1-(1-a)k (k-1). A confidence percent had been established at p=0.05 with k being the number of clone libraries to be studied. The standard p-value for two clone libraries was 0.0253. The Web-LIBSHUFF results revealed a p-value minor of the minimum expected p-value of 0.001 indicating significant differed between the two 16S rDNA clone libraries. This was performed using all the possible combinations between libraries (Figure 3.33).

LIBSHUFF analysis of homologous and heterologous coverage curves indicated that the I community significantly different from both the G and H communities (P = 0.001[Figure 3.33A and C]). Comparison of the G community indicated a significant difference from both H and I communities (P = 0.001[Figure 3.33B and D]). Comparison of the H community indicated a significant difference from both G and I communities (P = 0.001[Figure 3.33E and F]). A test of multiple contrasts among I, G and H community was obtained by examination of the distribution of $(C_x\text{-}C_{xy})^2$ with Evolutionary Distance (D). During the calculation of ΔC, results suggests that the group I library differs greatly from the other two libraries at high levels of genetic distance and shares all deep taxa (D>0.10) (Figure 3.33A and C). Also G and H clone libraries differs greatly from each

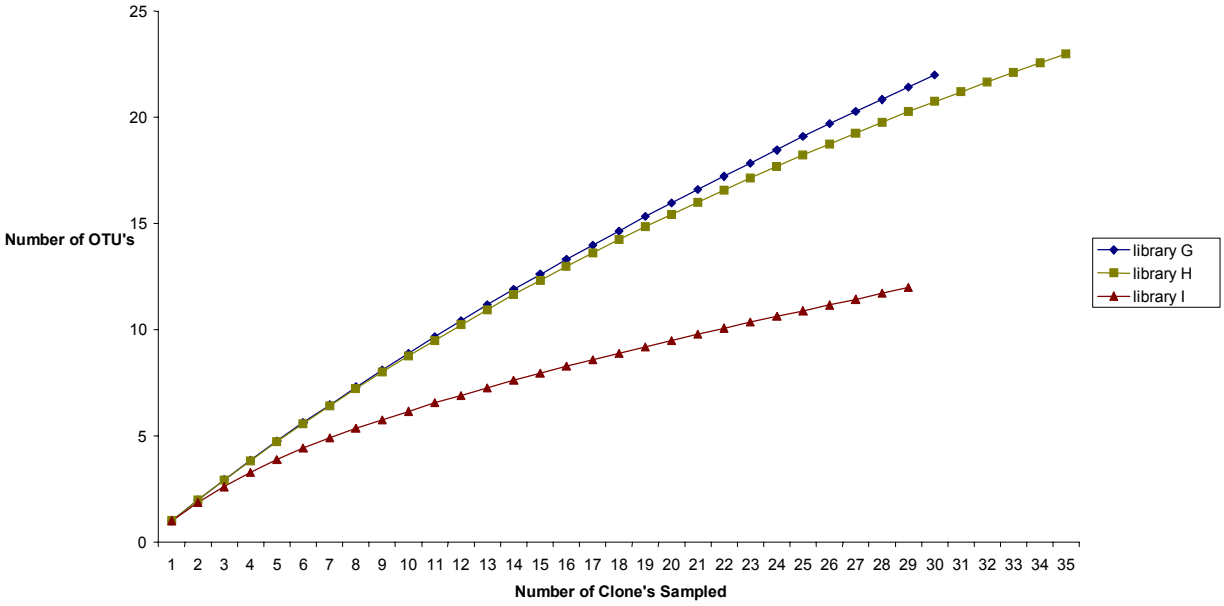
other and from group I community at high levels of genetic distance and shares all deep taxa (D>0.10) (Figure 3.33B, D, E and F).

In this study the computer program SONS was also used, since it implements nonparametric estimators for the fraction and richness of OTUs shared between two communities. SONS was used to characterize the differences between the three communities. After comparing the $OTU_{0.03}$ richness of each clone library (G, H, I) (Figure 3.34) it is shown that (i) the community I shared 41% and 25 % of its membership with those community H and G, respectively, (ii) the community H shared 26% and 21 % of its membership with those community G and I, respectively and (iii) the community G shared 13% and 27 % of its membership with those community I and H, respectively. An abundance-based Jaccard (J_{abund}) coefficient was determined for the three communities to measure the overlap/similarity, which is defined as the probability that a randomly selected OTU is found in both communities, given that it is in at least one of the communities (Schoss and Handelsman, 2006). The J_{abund} value between communities H and I was 0.33 (standard error [SE] = 0.2), which was significantly different from 1.0. The J_{abund} values between communities G and I (0.18, SE = 0.11) and G and H (0.49, SE = 0.26) were significantly different from 1.0. Therefore, the most abundant members were not shared between H and I, G and I and G and H. To compare community structure among G, H and I from the three clone libraries at specific OTU definition was calculated the community similarity index, θ. Using $OTU_{0.03}$ definition, the θ values for the comparison between H and I, G and I, and G and H were 0.08(SE=0.05), 0.16(SE=0.09), and 0.29(SE=0.12), respectively. With these results was concluded that the community structures were not identical or similar.

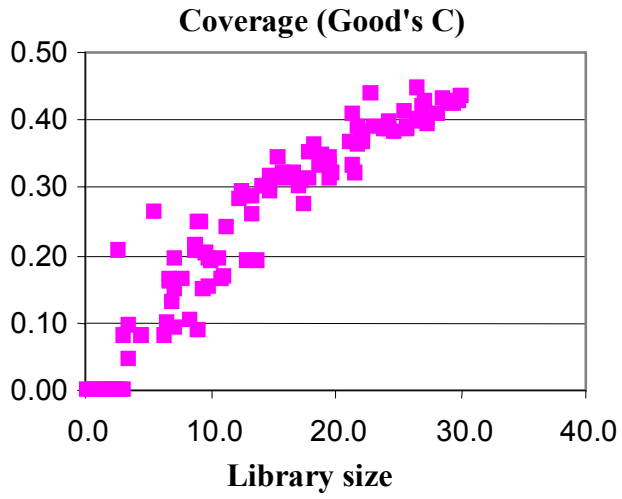
Table 3.3 Statistical Indexes for 16s rDNA clone libraries from *A. germinans*, Cabo Rojo salterns, Puerto Rico.

Clone Library	*Shannon (H)*	*Simpson's (1/D)*	*Richness Observed*	*Jaccard (%)*	*Sace (%)*	*SChao (%)*	*Coverage %*
G	2.96787	0.027586	22(30)	55.775	66.4824	56	43.33
H	2.99361	0.031933	23(32)	38.2668	47.739	38	57.14
I	2.09766	0.133005	12(29)	26.2722	29.8737	26	72.41

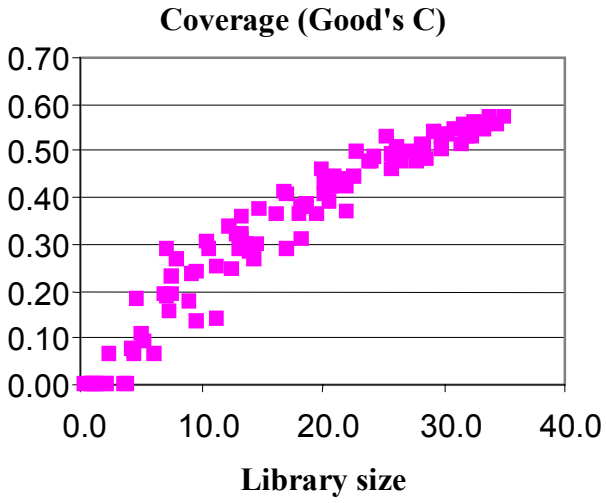
*Numbers in parenthesis indicate the number of 16S rDNA clones used in the analyses. Richness is the number of phylotypes observed. Each phylotype consisted of either unique clone or a group of clones that has sequence similarities of over 97%.



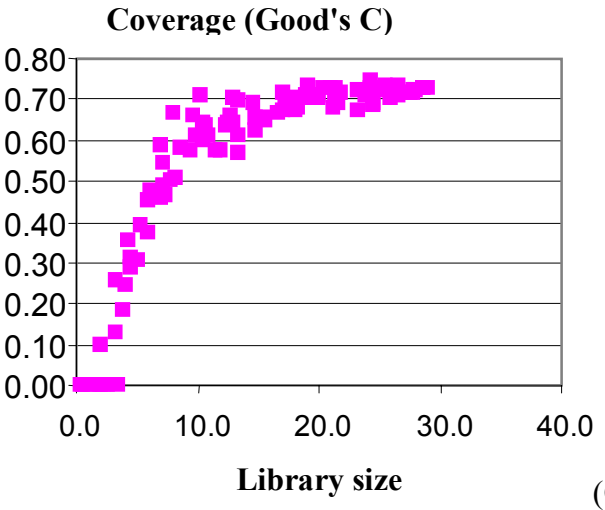
Figures 3.31 Rarefaction curves for three clones libraries sampled from the leaves of A. germinans. OTU's were determined by ≥97% similarity.



(A)



(B)



(C)

Figures 3.32 Good Coverage curves for 16S rRNA clone libraries (A) Library G (B) library H (C) library I.

(A)

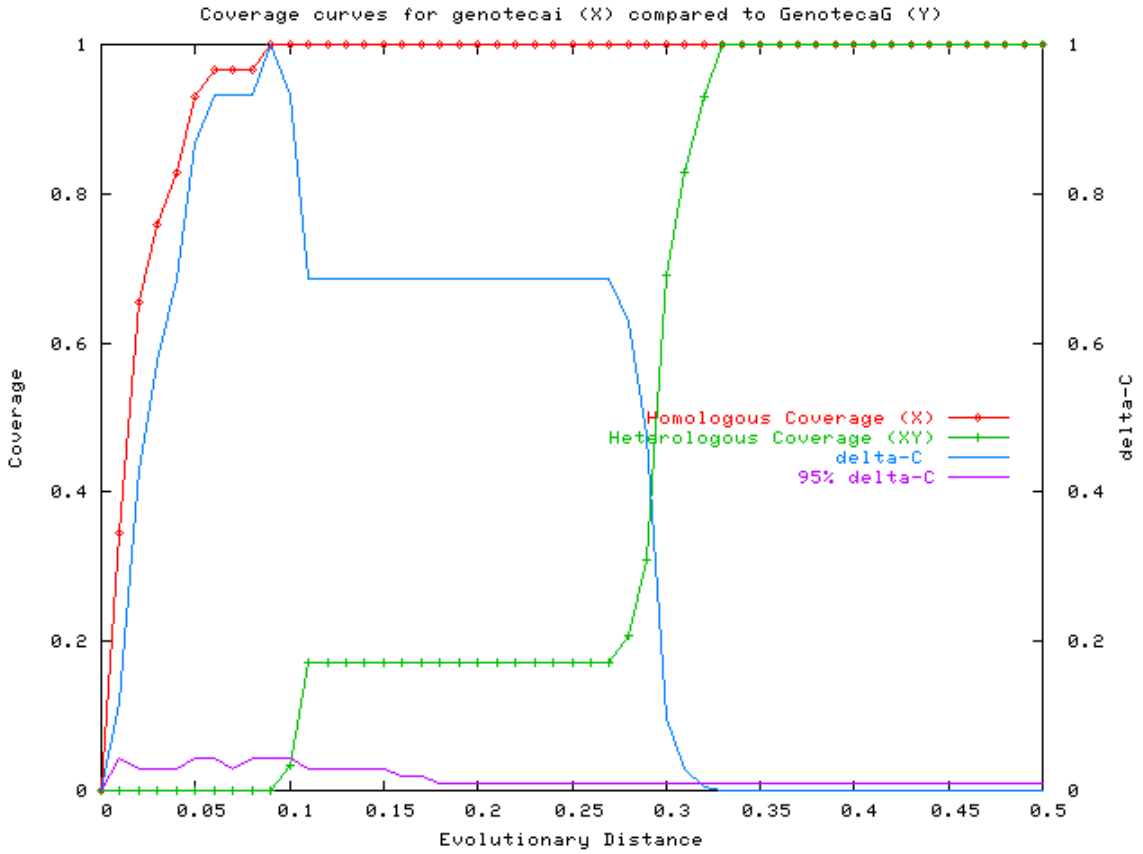


Figure 3.33a LIBSHUFF comparison of the two clones libraries. Blue lines indicate the value of $(C_x-C_{xy})^2$ for samples at each value of Evolutionary Distance (D). D is equal to the Jukes-Cantor evolutionary distance determined by the DNADIST program of PHYLIP. Purple lines indicate the P=0.05 value of $(C_x-C_{xy})^2$ for the randomized samples. (A) Comparison of the clone library I (red [homologous]) with clone library G (green [heterologous]).

(B)

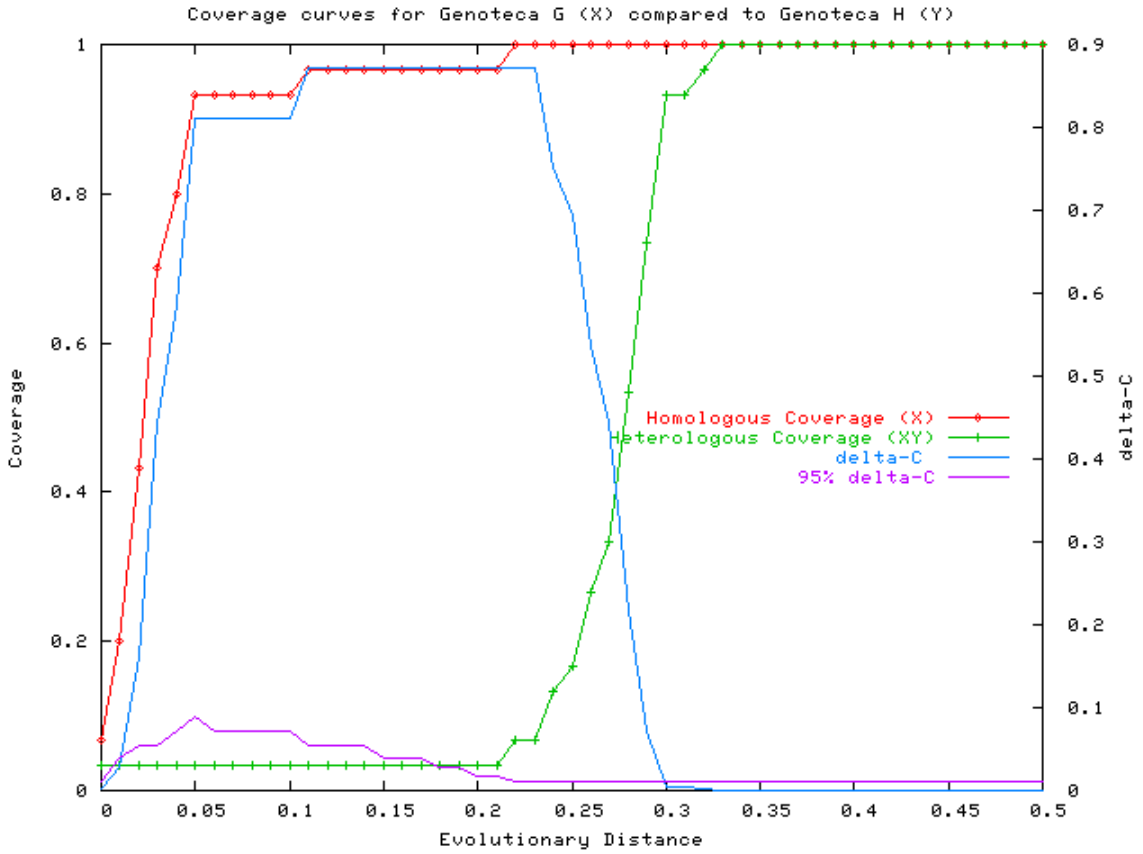


Figure 3.33b LIBSHUFF comparison of the two clones libraries. Blue lines indicate the value of $(C_x-C_{xy})^2$ for samples at each value of Evolutionary Distance (D). D is equal to the Jukes-Cantor evolutionary distance determined by the DNADIST program of PHYLIP. Purple lines indicate the P=0.05 value of $(C_x-C_{xy})^2$ for the randomized samples. (B)Comparison of the clone library G (red [homologous]) with clone library H (green [heterologous]).

(C)

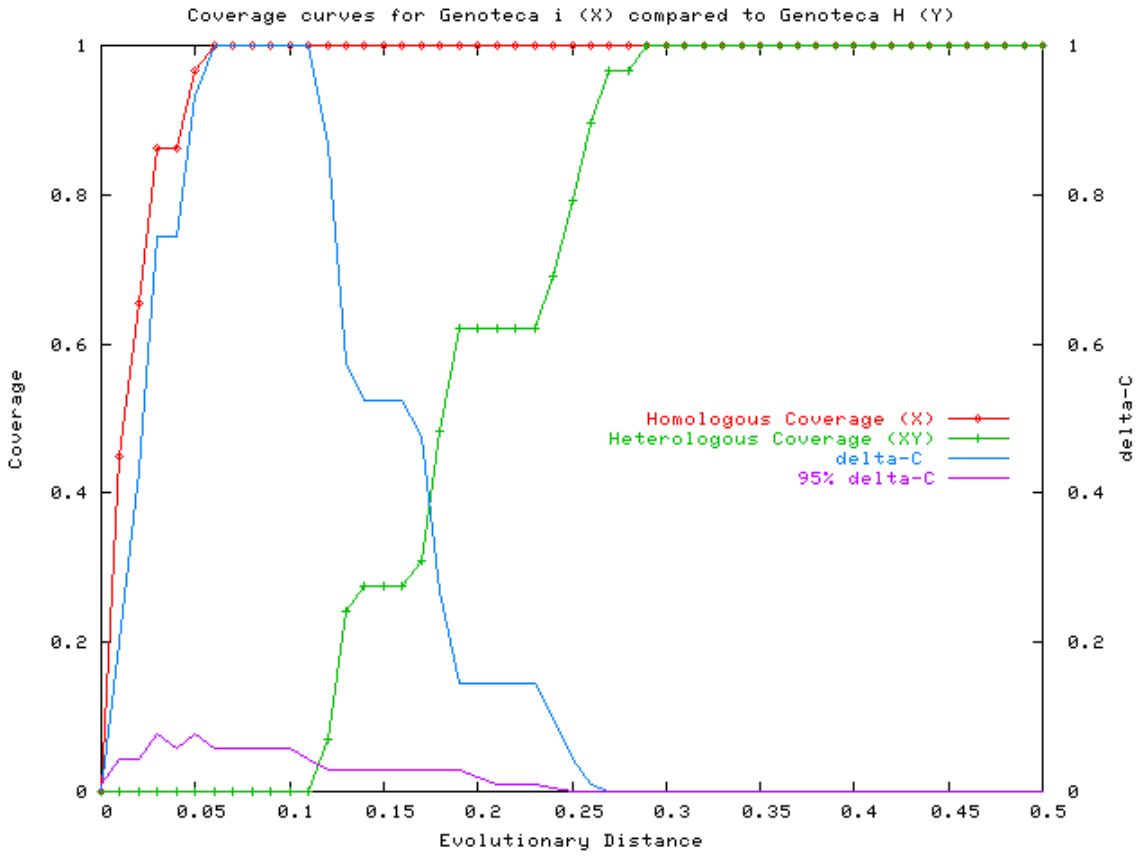


Figure 3.33c LIBSHUFF comparison of the two clones libraries. Blue lines indicate the value of $(C_x-C_{xy})^2$ for samples at each value of Evolutionary Distance (D). D is equal to the Jukes-Cantor evolutionary distance determined by the DNADIST program of PHYLIP. Purple lines indicate the P=0.05 value of $(C_x-C_{xy})^2$ for the randomized samples. (C) Comparison of the clone library I (red [homologous]) with clone library H (green [heterologous]).

(D)

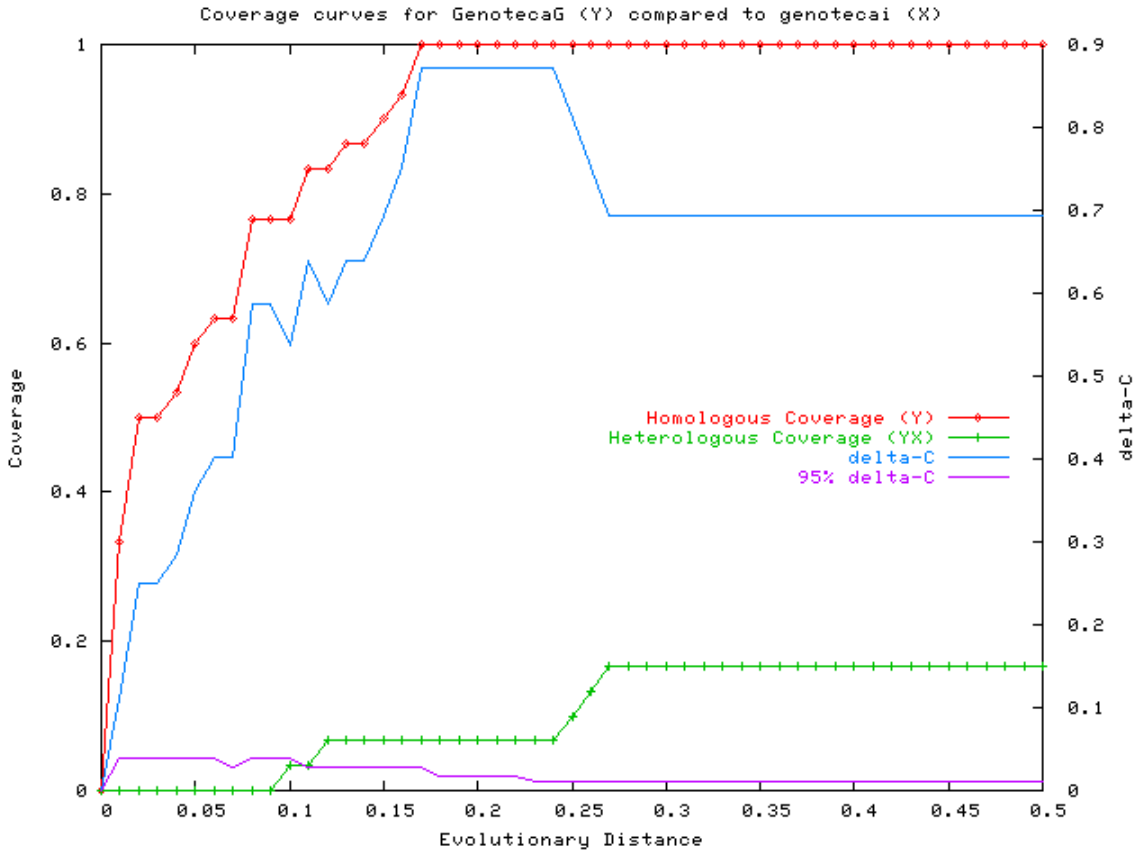


Figure 3.33d LIBSHUFF comparison of the two clones libraries. Blue lines indicate the value of $(C_x-C_{xy})^2$ for samples at each value of Evolutionary Distance (D). D is equal to the Jukes-Cantor evolutionary distance determined by the DNADIST program of PHYLIP. Purple lines indicate the P=0.05 value of $(C_x-C_{xy})^2$ for the randomized samples. (D) Comparison of the clone library G (red [homologous]) with clone library I (green [heterologous]).

(E)

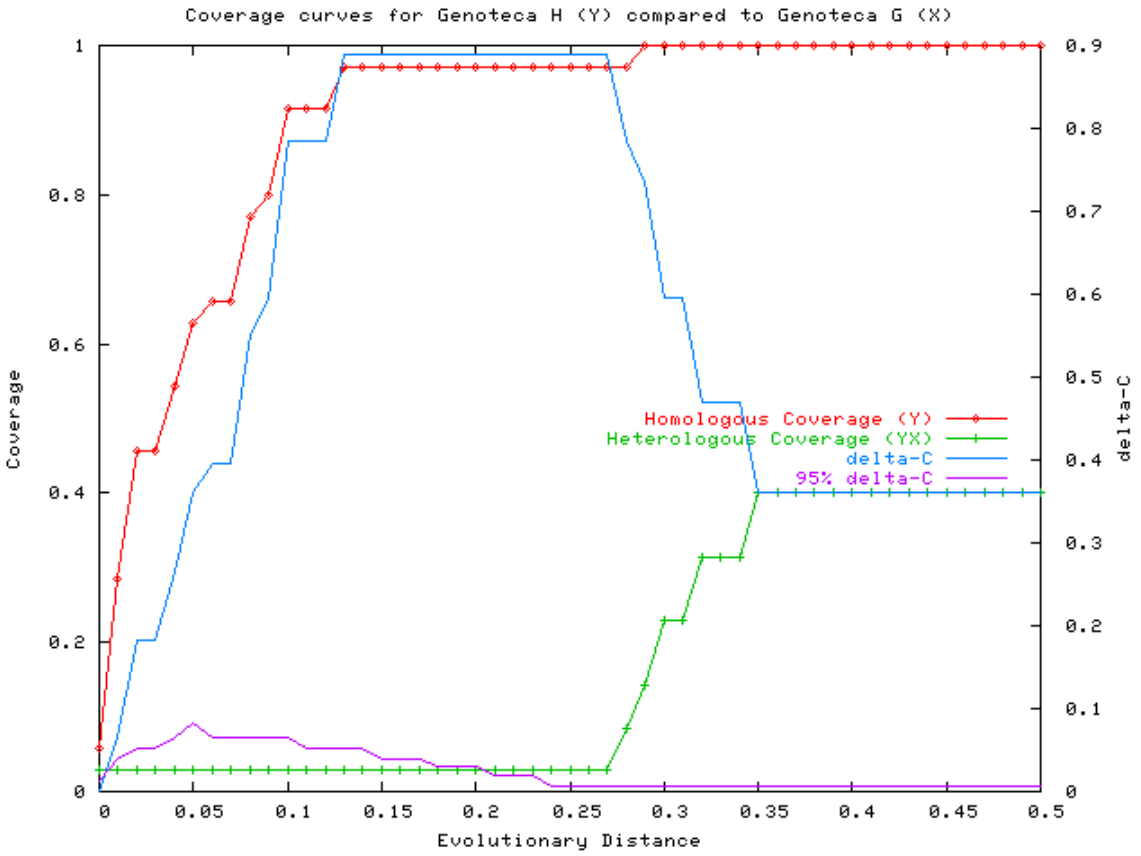


Figure 3.33e LIBSHUFF comparison of the two clones libraries. Blue lines indicate the value of $(C_x-C_{xy})^2$ for samples at each value of Evolutionary Distance (D). D is equal to the Jukes-Cantor evolutionary distance determined by the DNADIST program of PHYLIP. Purple lines indicate the P=0.05 value of $(C_x-C_{xy})^2$ for the randomized samples. (E) Comparison of the clone library H (red [homologous]) with clone library G (green [heterologous]).

(F)

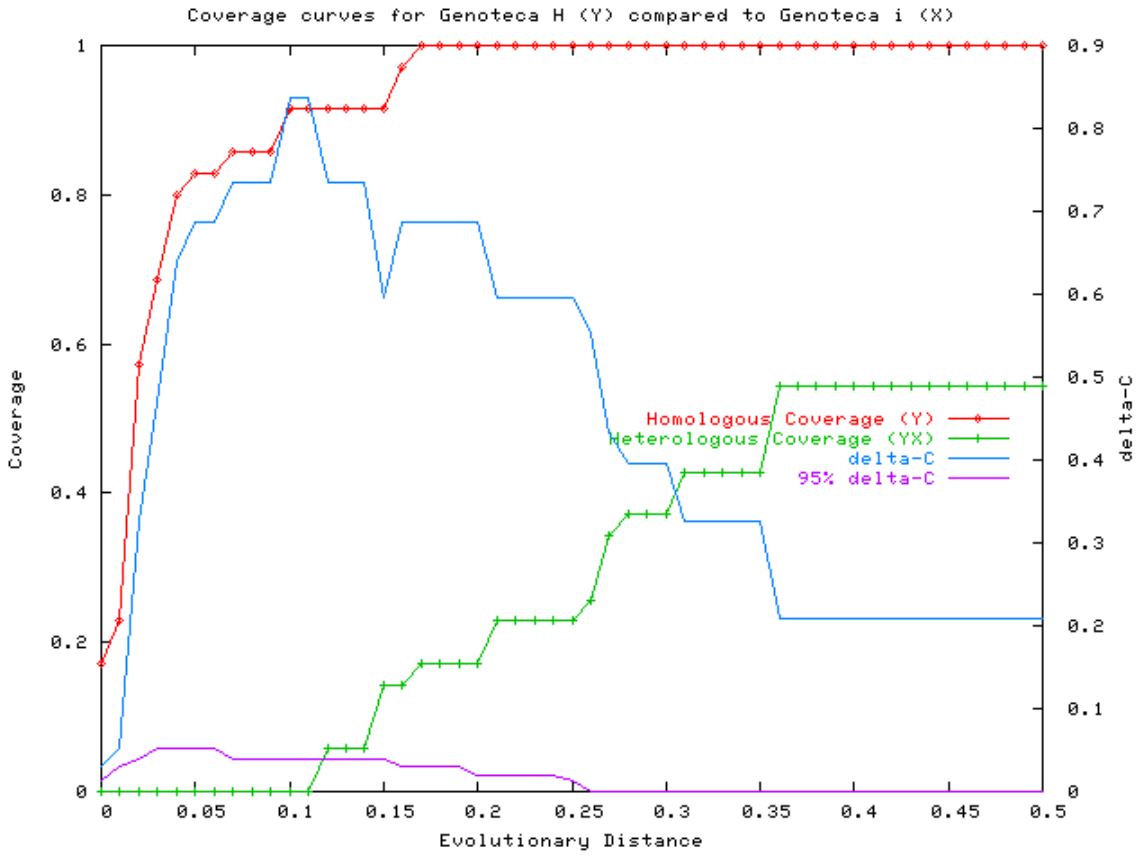


Figure 3.33f LIBSHUFF comparison of the two clones libraries. Blue lines indicate the value of $(C_x-C_{xy})^2$ for samples at each value of Evolutionary Distance (D). D is equal to the Jukes-Cantor evolutionary distance determined by the DNADIST program of PHYLIP. Purple lines indicate the P=0.05 value of $(C_x-C_{xy})^2$ for the randomized samples. (F) Comparison of the clone library H (red [homologous]) with clone library I (green [heterologous]).

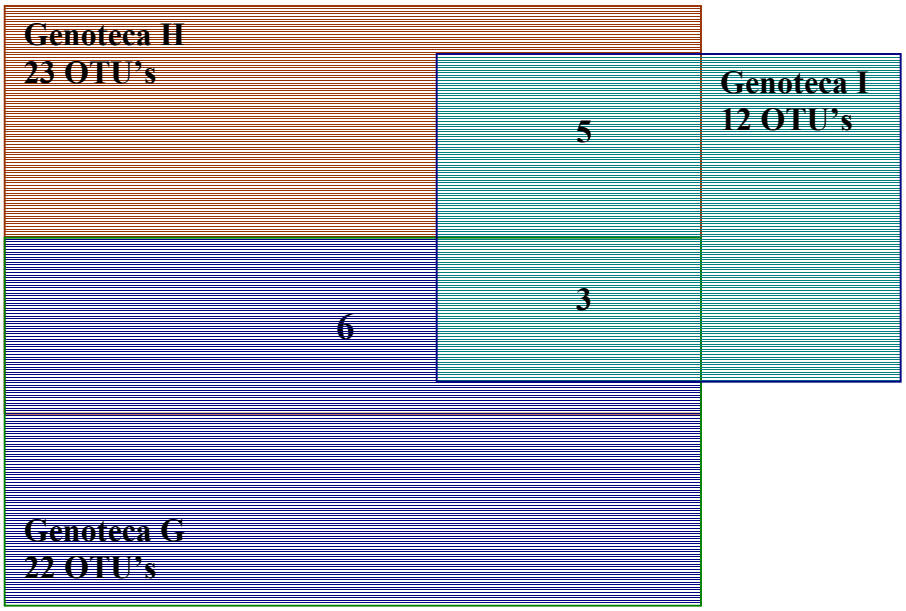


Figure 3.34 Venn diagram comparing the $OTU_{0.03}$ memberships found in the black mangrove leave surface of three mangrove trees (Group I[n = 29], group H[n = 32], and group G[n = 30]). The richness of the overlapping regions based on the pairwise $S_{1,2\ Chao}$ richness estimates shared by the three communities (right side of the figure).

Discussion

The second objective of this research was to determine the prokaryotic community structure from the *Avicennia germinans* leaf surface based on 16SrDNA clone libraries. Saahno DNA extraction protocol (Saahno *et al*., 1995) with some modification proved to be very efficient in extracting environmental genomic DNA from the resuspension of *A. germinans* leaves in phosphate buffer 15%NaCl. Other methods were previously used but they were not as effective to extract prokaryotic DNA. PCR reactions were optimized in order to archive the best amplification product. The polymerase used was the Taq polymerase in storage buffer B (Promega®). Universal primers such as Univ 519F and Univ 1392R were used. Three clone libraries were constructed to study the diversity present in those *Avicennia germinans* leaves selected. A total of 1,712 clones were obtained from the three genomic libraries. 30% of each clone library was screened using the RFLP technique. RFLP analysis from each clone library constructed revealed a variety of restriction patterns indicating a wide diversity among all the clone libraries constructed.

Approximately 10% of the clone libraries were sequenced. Clones were selected randomly to avoid possible biases. Phylogenetic analyses revealed that most of the OTU's are related to marine, soil, and plant environments, representing the Bacteria and Archaea domains. A diversity of clone sequences reflects association to Bacteroidetes, alpha-proteobacteria, beta-proteobacteria, and gamma-proteobacteria groups. In the Archea domain, only a small group was related to the genus *Halococcus*. OTU's belonging to a particular phylogenetic group were expected to have properties common to the group.

A total of 95 clones were phylogenetically analyzed. The Archaea domain represented only 3% of the clone library, meanwhile the Bacteria domain represented the other 97%. The most frequent OTU's belonged to the alpha-proteobacteria group with a frequency of 0.42. OTU's from the alpha-proteobacteria group were related to meromictic marine basin in Antarctica (Bowman *et al*., 2000), from marine environments, from hypersaline endoevaporitic microbial mats (Sorensen *et al*., 2005) and from a hypersaline evaporation pond (de Souza *et al*., 2001). Also several clones were related to species such as *Paracoccus solventivorans* (Siller *et al*., 1996) and *Paracoccus koreensis* (La *et al*., 2005) isolated from soils. Other clones from the alpha-proteobacteria group were closely related to *Pseudaminobacter salicylatoxidans* (Kampfer *et al*., 1999) isolated from River Elbe, Germany and to *Aurantimonas coralicida* WP1 (Denner *et al*., 2003) isolated from coral reefs.

The second most abundant OTU's belonged to the gamma-proteobacteria group with a frequency of 0.28. This group of clones were related to species such as *Pantoea agglomerans* and *Enterobacter ludwigii* (Hoffman *et al*., 2005) isolated from Tiete River downstream of Sao Paulo in Brazil. Some OTU's from the gamma-proteobacteria group were closely related to microbial communities isolated from microbial mat saphotic cave in sulfidic springs (Engel *et al*., 2004), from rice plant (Hiraoka *et al*., 2006) from a lagoon in south western Atlantic Ocean (Piccini *et al*., 2006), from mangrove sediment and from anaerobic nitrogen-fixing consortia from gramineous plants (Minamisawa *et al*., 2004). Some of the OTU's were related to *Halomonas marisflavi* (Yoon *et al*., 2001) isolated from Yellow Sea in Boryung City, Korea and *Halomonas avicenniae* (Soto-Ramírez *et al*., 2006 in press) isolated from the leaf surface *Avicennia germinans* in Cabo

Rojo, Puerto Rico (this study). OTU's pertaining to *Enterobacter asburiae* isolated from nodules of white legume and *Morganella psychrotolerans* (Emborg *et al*., 2006) isolated from a histamine-producing bacterium isolated from various seafoods were also found at the community of black mangrove leaf surface. Other species related to the prokaryotic community of the black mangrove leaves were *Stenotrophomonas maltophilia* SB5 (Radianingtyas *et al*., 2003) isolated from soil, *Xanthomonas* group from Hawaiian Archipielago (Donachie *et al*., 2004), *Salinisphaera shabanensis* E1L3A (Antunes *et al*., 2003) isolated from the Red Sea, *Aquamicrobium defluvii* DSM 11603T (Bambauer *et al*., 1998) isolated from sewage sludge, and *Nitratireductor aquibiodomus* NL21T (Labbe *et al*., 2003) isolated from a marine denitrification system of the Montreal Biodome (Canada).

Representatives OTU's were also related to the Bacteroidetes group. It represented 23% of the clone libraries. Operational taxonomic units were related to a group of clones HPDOM and HRIPR from a stromatolite microbial community of Hamelian Pool in Shark Bay, Western Australia (Papineau *et al*., 2005) and from sponges from different oceans (Lau *et al*., 2006). Clones were related to some species such as *Leeuwenhoekiella marinoflava* (Nedashkovskaya *et al*., 2005) isolated from Antarctic sea water and *Leeuwenhoekiella blandensis* (Pinhassi *et al*., 2006) isolated from seawater sample collected in the Bay of Blanes in the north-western Mediterranean Sea. This two species belonged to the family *Flavobacteriaceae* which were increasingly recognized to play important roles in the degradation of organic matter during and following algal blooms. The microbial community also was associated to *Stenothermobacter spongiae* (Lau *et al*., 2006) isolated from a marine sponge in the Bahamas and *Rhodothermus*

marinus (Hjorleifsdottir *et al*., 1988) a thermophilic, halophilic bacterium from submarine hot springs in Iceland. Other species related to isolated OTU's were *Sufflavibacter litoralis* an extremely halophilic member of the Bacteria from saltern crystallizer and *Salinibacter ruber* (Anton *et al*., 2002) from saltern crystallizer ponds.

From the Bacteria domain the less frequent group found was beta-proteobacteria. It represented only the 3% of the OTU's sequences. OTU's were related with the genera *Pseudomonas* sp., *Comanmonas* sp. and other uncultured beta-Proteobacteria. *Pseudomonas* sp. has been found associated to soils and *Comamonas* sp. was found from wetlands in Korea (Chang *et al*., 2002).

The less frequent group was from the Archaea domain with a percentage of 3%. Only three clones were related to the genus *Halococcus* where some of them were isolated from stromatolites in Shark Bay, Australia and from an Austrian salt mine. *Halobacillus*, *Staphylococcus* and *Pontibacillus* representatives were detected in isolated strains from *Avicennia germinans* leaf surface. Surprisingly, *Halobacillus, Staphylococcus* and *Pontibacillus* clones were not detected. The bias might be related to the primers used or the DNA extraction procedure. Eventhough there were no representatives OTU's of those genera, a high diversity of prokaryotic organisms were obtained. A broader diversity can be obtained amplifying more than one DNA product instead of one amplified DNA product. However, we obtain a wide variety and diversity of Bacteria domain representatives in our genomic libraries. Statistical analysis indexes, (Jaccard, SAce, and SChao richness) indicate that library G had the highest level of richness when compared with clone library H and I (Table 3.3). The LIBSHUFF program is a test of overlap, since it considers the distribution of pairwise differences (Stach *et al*.,

2003) Results from this program are dependent on sample size; the minimum number of sequences necessary to distinguish between two dissimilar libraries increases with library complexity and decrease with the magnitude of dissimilarity (Singleton *et al*., 2001). The Web-LIBSHUFF results revealed a p-value minor of the minimum expected p-value of 0.001 indicating significance differences among the 16S rDNA clone libraries. SONS was used to characterize the differences between the three communities. Estimating the $OTU_{0.03}$ richness of each clone library and the richness shared between groups (G, H, I) revealed a low similarity among communities and J_{abund} values between the three communities revealed that most abundant members were not shared. Community similarity index, θ revealed that the community structures were not identical or similar. These analyses describe the relative similarities of the memberships and structures of these communities for a specific OTU definition.

This is the first attempt to study the prokaryotic diversity on the leaf surface of *Avicennia germinans* mangrove from the Caribbean using molecular approaches like genomic libraries. This study demonstrates an unexpected high halophilic prokaryotic diversity associated to the leaf surface of *A. germinans* that might provide further insight about the potential prokaryotic communities involved in the metabolic processes in mangrove ecosystems. Knowledge about the identity of those microorganisms will allow the potential development of biochemical applications in biotechnology and astrobiology.

Conclusions

- A great variety of halotolerant and halophilic prokaryotic microorganisms can be isolated from the leaf surface of *Avicennia germinans* from the solar salterns of Cabo Rojo, Puerto Rico. In contrast, a low diversity of prokaryotes were recovered using SG with 15% NaCl from red mangrove leaves. The halotolerant and halophilic nature of several strains suggests that they probably are part of the community of the leaf surface of *A. germinans*.

- The isolated microorganisms in this study belonged mainly to 4 genera which have halophilic or halotolerant properties. These include *Halobacillus, Staphylococcus, Pontibacillus* (division *Firmicutes)* and *Halomonas* (gamma-proteobacteria).

- The genus *Halobacillus* showed the highest frequency of isolation in this research.

- Strain MS10^T was characterized as a new species within the genus *Halobacillus*. The name *Halobacillus puertoriconensis* sp. nov. is proposed for this novel species . Strain MW2a^T was also characterized as a new species within the genus *Halomonas*. The name *Halomonas avicenniae* sp. nov. was proposed for this novel species. In addition, several potential new species within the *Halomonas* (10) and *Halobacillus* (10) genera were isolated.

- Culture independent techniques revealed a very high prokaryotic diversity present at the leaf surface of the black mangrove. The most frequent OTU's belonged to the alpha-proteobacteria group. Also OTU's associated with the Archaea domain were obtained. This constitutes the first report of possible archaea organisms associated to mangroves.

- Statistical analysis (Jaccard, SAce, and SChao richness index) revealed that library G had the highest level of richness. Significant differences ($P = 0.001$) among OTU's from the three clone libraries were obtained.

- This study is the first attempt to study the prokaryotic diversity present on the leaf surface of *Avicennia germinans* from the Caribbean.

Recommendations

- To design different sampling methods and media that allow the isolation of different genera.

- To perform studies that compares the prokaryotic diversity present from *Avicennia germinans* mangrove versus *Laguncularia racemosa*.

- Design a temporal study to compare the prokaryotic diversity in different seasons of the year.

- Further analysis to complete the characterization of the *Halomonas* and *Halobacillus* putative new species.

- In order to reach and cover most of the prokaryotic diversity from the black mangrove leaf surface, more clones must be analyzed.

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