A survey of the alkalithermophilic prokaryotic diversity from the hot spring waters in Coamo Puerto Rico

by

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Abstract

Thermal springs are high temperature aquatic environments. The prokaryotic diversity in these environments has been studied world wide. However, there are few studies on the prokaryotic diversity in thermal springs in Puerto Rico. The main objective of this research was to isolate and characterize alkalithermophilic bacteria from the main thermal spring in Coamo Puerto Rico. To determine the culturable diversity of these bacteria, thermal spring water samples were filtered, plated into *Thermus* medium (TM) and Alkaline Yeast Extract Malt Medium (AYEMM) and incubated at 70ºC. Selected isolates were characterized by standard microbiological techniques, by scanning electron microscopy, and by growing at optimal pH and different temperatures. Molecular analysis using Restriction Fragment Length Polymorphism (RFLP) patterns of a Polymerase Chain Reaction (PCR) amplicon containing the 16S rDNA region was also used to classify the isolates. Taxonomic results indicated that the isolates belonged to the *Bacteria* Domain. All the 185 strains recovered represented the spore forming genus *Geobacillus*. This genus has been reported from other hot springs and during this study it was recovered recurrently and was the most abundant in all the three sampling sites of the spring. Many of these isolates showed a wide range of growth at various temperatures and pH values. The ability of these isolates to grow at high temperatures might indicate that they represent the extremophilic diversity of the thermal springs. The Bacterial diversity was also analyzed by culture independent methods like 16S rDNA environmental clone libraries and Terminal Restriction Fragment Length Polymorphism (TRFLP). Clone libraries were very difficult to achieve due to the low diversity present in the community and chemical complexity of the sample. However, after protocol

optimization some clones were obtained and *in silico* analysis was performed. Environmental sequences belonging to the β-Proteobacteria were the most frequent Operational Taxonomic Units (OTUs), but some OTUs were closely related to members of the *Bacteroidetes* and γ-Proteobacteria. In addition, some cyanobacterial OTUs were obtained from this environment. TRFLP patterns were also used to study structure and diversity of the 16S rDNA microbial community. The profiles obtained with different enzymes were similar among all the springs, suggesting that the community structure was very homogeneous throughout the sampling area. The combination of morphological, physiological and molecular approaches was very useful to describe the bacterial community present at the thermal spring of Coamo Puerto Rico.

Resumen

Las aguas termales se caracterizan por ser ambientes acuáticos con altas temperaturas. La diversidad procariota en estos lugares ha sido estudiada alrededor del mundo. Sin embargo, existen pocos reportes enfocados en la diversidad bacteriana presente en este tipo de ambiente en Puerto Rico. El objetivo principal de este estudio lo fue el aislar y caracterizar bacterias alcalino-termofílicas presentes en las aguas termales de los Baños de Coamo en Puerto Rico. Para determinar la presencia de bacterias en estas aguas termales, las muestras fueron filtradas y colocadas en *Thermus* medium y Alkaline Yeast Extract Malt medium e incubadas a 70º C. Los aislados seleccionados se caracterizaron utilizando microscopía de luz, rastreo y algunas pruebas fisiológicas tales como: pruebas bioquímicas, pH óptimo y temperaturas de crecimiento. Un análisis molecular fue realizado utilizando patrones de RFLP de los productos de PCR conteniendo la región 16S del rDNA para poder clasificar los aislados. Los resultados taxonómicos indicaron que todos los aislados pertenecían al Dominio *Bacteria*. Los 185 aislados recuperados representaban a la bacteria formadora de esporas *Geobacillus*. Este género ha sido reportado en otros manantiales termales y fue recuperado con una alta frecuencia, siendo el más abundante en las tres áreas de muestreo de estas aguas. Muchos de los aislados demostraron una gran amplitud de crecimiento a distintas temperaturas y pH. La habilidad de estas cepas de crecer a altas temperaturas, podría indicar que representan la diversidad extremófila de las aguas termales. La diversidad bacteriana también fue analizada mediante técnicas independientes de cultivo, como las librerías genómicas utilizando la subunidad ribosomal 16S y TRFLP. Las librerías genómicas fueron difíciles de obtener debido a la baja diversidad de microorganismos presentes en

esta comunidad y la complejidad química de la muestra. Sin embargo, luego de la optimización experimental se obtuvieron algunos clones y estos fueron analizados *in silico*. Las secuencias ambientales pertenecientes a las β-proteobacterias. fueron UOTs más frecuentes, algunos estrechamente relacionados a miembros de los *Bacteroidetes* y algunas γ-proteobacterias. En adición a estos, otros UOTs relacionados a las cianobacterias fueron recuperados de este ambiente. Patrones de TRFLP fueron utilizados también para estudiar la estructura y diversidad del 16S rDNA de la comunidad de este ambiente. Los patrones obtenidos con las distintas enzimas fueron similares en casi todas las area de muestreo de las aguas termales, indicando que la estructura de la comunidad es muy homogénea a través del área de muestreo. La combinación de técnicas morfológicas, fisiológicas y moleculares fueron de gran utilidad para describir la comunidad bacteriana de las aguas termales de Coamo Puerto Rico.

Dedication

I dedicate this work to the people I love…

Thank you for been always there for me, in the good and bad moments. You helped me through this journey. You brought me serenity and support. Thanks for giving me your best and for putting your heart in all what you do. You believed in me more than I believe in myself. Because of you I was able to pursue my happiness and finish all this work.

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Introduction

Thermal springs are high temperature aquatic ecosystems. These water bodies are widely distributed throughout the world but are most numerous in areas that are volcanically active, such as hot springs (Saha, 1993). These environments are of special interest from an ecological and evolutionary point of view, as inhabiting microorganisms that have developed mechanisms to thrive at different temperature ranges according to their optimal growth requirements.

In general microorganisms have been grouped in different categories: psychrophiles, mesophiles, thermophiles and hyperthermophiles (Kristjansson and Stetter, 1992). In addition to these categories, they can be grouped as acidophiles and alkaliphiles according to their growth requirements at different pH ranges (Horikoshi, 1998).

Thermophiles have an optimum growth temperature of around 50ºC with a maximum around 70ºC and a minimum of about 20ºC (Stetter, 1998). On the other hand, alkaliphiles prefer high pH, and require high sodium ion concentrations for survival (Kitada *et al*., 2000). The optimum pH for most alkaliphiles is about 10 (Horikoshi, 1998). Many alkaline environments can be placed into several broad categories depending on the nature of the process generating alkalinity (Jones *et al*., 1998).

Many studies concentrate their efforts documenting diversity of thermophilic microorganisms from hot springs and hydrothermal systems around the world. One of the most studied springs are those at the Yellowstone National Park (Reysenbach *et al*., 2000). These hot springs have unique characteristics and can vary in temperature, flow rate and chemistry of the water (Brock, 1994). The most common prokaryotic group found in these environments is the *Aquificales* (Huber *et al*., 1986). On the other hand,

alkaliphiles are widely distributed throughout the world and have been isolated from a variety of biotopes, including mesophilic and neutrophilic soils and sediments (Wiegel & Kevbrin, 2004), but they can also be found growing at thermal waters where the temperature is very high. These microorganisms are named alkalithermophiles depending on their adaptation to grow in the presence of conditions of high pH and temperature (Wiegel & Kevbrin, 2004).

Alkalithermophiles have been studied recently because they are the subset of extremophiles that are adapted to two extreme conditions; a combination of alkaline and thermobiotic growth conditions. Their adaptation to both high pH and high temperature draws the attention not only because they are potential sources of industrially valuable enzymes but also because of their adaptive mechanisms to extreme environmental parameters. Thus, they could function as model organisms for extraterrestrial life in some environments and for theories on the origins of life (Wiegel & Kevbrin, 2004).

Reports on the microbial diversity concerning these organisms in the Caribbean are scarce. One of such studies dealt with the properties of the microbial communities present in the volcano of Montserrat Island. Atkinson *et al*., (2000) found a variety of heterotrophic and chemolithotrophic thermophilic bacteria at this place. Most of the bacteria recovered in pure culture were already known acidophiles and neutrophiles, but a novel iron-oxidizing species of *Sulfobacillus* was characterized. This bacterium was the first *Sulfobacillus* species having a maximum growth temperature of 65°C, which is highest, reported for this moderately thermophilic genus (Atkinson *et al*., 2000).

To our knowledge there are no published reports about the prokaryotic microbial diversity present at the Coamo thermal springs in Puerto Rico besides the presence of

cyanobacteria (Almodóvar, 1958). The study of the heterotrophic bacteria present in this interesting and unusual site by applying morphological and molecular analyses might provide important information about the prokaryotic diversity that inhabits this extreme environment. Therefore, the main goal of this research was to survey the alkalithermophilic prokaryotes isolated from the alkaliphilic thermal springs at Coamo, Puerto Rico, using traditional methods such as morphological and physiological studies combined with molecular techniques. Studying this type of habitat is of great interest because ancestral alkalithermophiles could have been one of the earliest forms of life (Kevbrin *et al*., 1999). Moreover, they can be regarded as a model organism for the study of possible extraterrestrial life (Kevbrin *et al*., 1999). The isolation and characterization of these microorganisms could provide crucial knowledge on the diversity of this group in this unexplored thermal environment that might also contain sources of products with biotechnological importance.

Literature Review

Life at Extreme Conditions

The majority of microbial populations that inhabit extreme environments belong to the domains *Archaea* and *Bacteria*. These habitats are distinguished by means of extreme conditions like salinity, temperature, pH, and oxygen concentrations (Stetter, 1998). Microorganisms not only growing under but requiring unusual environmental conditions for growth are grouped under the term extremophiles. Extremophiles have been defined based on the nature of the environments where they are found. For example, extremophiles that live under high pressure are called piedzophiles. Those that require cold temperatures for growth and reproduction are called psychrophiles, and those that have adapted to high temperatures are called thermophiles. Microorganisms associated to acidic environments are called acidophiles, whereas those found in highly alkaline conditions are alkaliphiles. The adaptation to thrive in these conditions draws the attention because of their mechanisms to flourish in environments with combined conditions such as pH and temperature (Wiegel & Kevbrin, 2004).

Thermal Environments

The most common thermal biotopes are volcanically and geothermally heated hydrothermal systems such as solfataric fields, neutral hot springs, and submarine saline hot vents. Hot solfataric fields consists of one upper layer that contains significant amounts of oxygen, which present an ochre color owing to the presence of the ferric ion, and a layer below that shows a blackish-blue color due to the presence of ferrous ion. Submarine thermal systems consist of hot fumaroles, springs, sediments, and hot vents with temperature up to 400°C (Stetter, 1998). Other submarine hydrothermal systems

usually contain high concentrations of sodium chloride and exhibit a slightly acidic to alkaline pH between 5-8 (Horikoshi, 1998).

Thermal environments differ in temperature, flow rate, and chemistry of the water (Brock, 1994). The Yellowstone National Park (Wyoming, USA) has one of the highest concentrations of hot springs in the world. Hot springs can be found also at Norris and Mud Vulcano in Italy, Kamchatka in Russia (Wiegel, 1990), along the west coast of India (Saha, 1993), Sao Michel in Azores, submarine hot springs in Iceland, the Mount Grillo at Baia Naples in Italy (Romano *et al*., 2004), and various places in the Caribbean.

Other examples of thermal environments are the hydrothermal vents at Guaymas Basin and the east Pacific Rise in México. The hydrothermal vents are located in shallow and abyssal depths (Stetter, 1998). These environments also have a unique chemical composition such as high content of sulfur and hydrogen sulfide.

In Puerto Rico there is at least one natural environment that can be considered alkalithermophilic. This habitat is the Coamo thermal springs located in the Municipality of Coamo (in south central Puerto Rico), which forms the east-early trending of the Cordillera Central (Glover, 1971). Most of this area lies on the flank of a much-faulted geanticlinal volcanic core that is partly overlapped by a sequence of carbonate rocks and sediments (Glover, 1971).

In this site, water resurfaces as a thermal spring having a constant temperature of 44°C. The chemical composition of the water has been determined previously and it contains carbonates, sulfates, calcium, magnesium, sodium, potassium, fluoride, manganese, and chloride. The analysis on these thermal springs has shown that they

contain high amounts of carbonates, causing the pH to be around 8.5-9 (Quiñones & Guzmán, 1983).

Biotic Communities in thermal environments: Bacteria, Archaea and Eukarya

A diversity of microorganisms can thrive in thermal environments. Microbial life in these habitats is represented by the three domains, in both high pH and high temperature conditions. In Yellowstone National Park, several taxonomic studies on the characterization of members of Bacteria, Archaea and Eukarya had been conducted (Ward, 1998). Most of the microbiological research at Yellowstone has involved studies on prokaryotes. These studies have been focused particularly on bacterial diversity, including cyanobacteria, anoxygenic phototrophs, and aerobic and anaerobic chemoorganotrophs (Hugenholtz *et al*., 1998; Ward, 1998). Munster *et al*. (1986) reported preliminary taxonomic studies on *Thermus* strains isolated from this habitat. There also have been reports on several unusual thermoacidophiles, such as *Sulfolobus acidocaldarius* (Ward, 1998).

Several taxonomic studies had been performed on diverse hot springs. Mono Lake in California, an alkaline, hypersaline and closed basin, was the site of a study performed by Gorlenko *et al*. (2004). A novel species, *Anaerobranca californiensis*, was then isolated from the lake's sediment. Another hot spring that has been studied is Kamchatka at Russia, where *Thermoproteus uzoniensis*, an extremely thermophilic bacterium was isolated (Bonch-Osmolovskaya *et al*., 1990). In Naples, Italy, there have also been reports of thermophilic slightly halophilic species from saline hot springs (Tenreiro *et al*., 1997).

A study of fresh water and terrestrial *Cyanophyta* from Puerto Rico was performed by Almodóvar (1958), including many aspects of this group in the Coamo thermal springs. Several species belonging to the genera *Nostoc*, *Fisherella*, *Hapalosiphon*, *Amphithrix*, *Cylindrospermum*, *Scytonema*, *Microcoleus*, *Schizothrix*, *Oscillatoria*, *Phormidium*, *Plectonema*, and *Symploca* were reported in this study. Representatives from the *Diatomaceae* can also be found in this environment. Hagelstein (1938), reported the presence of diatoms in the water that flows away from the cliff and the pools formed at the top and base, but there are no diatoms in the warm water.

Numerous thermophilic microorganisms have also been isolated from chimneys, sediments, and ambient water of hydrothermal vent fields (Reysenbach *et al*., 2000). *Thermotoga maritima* was described from marine thermal vents at Vulcano (Huber *et al*., 1986). A similar organism, *Thermotoga neapolitana* was isolated from a submarine thermal vent at Lucrino, Italy (Belkin *et al*., 1986; Jamnasch *et al*., 1988).

Another specific group of thermophiles, including some bacilli, have also been reported from natural and artificial high temperature biotopes (Caccamo *et al*., 2001; McMullan *et al*., 2004). These thermophilic bacilli belonging to *Bacillus* genetic group 5, have been reclassified as members of the recently named genus *Geobacillus* (Mcmullan *et al*., 2004). Most *Geobacillus* species are widely distributed and have been successfully isolated from continents where geothermal areas occur (McMullan *et al*., 2004; Sharp *et al*., 1992). Geobacilli are also isolated from shallow marine hot springs and from deep-sea hydrothermal vents. Maugeri *et al*. (2002) previously described the isolation of three novel halotolerant and thermophilic *Geobacillus* strains from three separate shallow marine vents of the Eolian Islands, Italy. High temperature oilfields

have also yielded strains of *Geobacillus*, where two novel species, *G. subterraneus* and *G. uzenensis*, were isolated from the Uzen oilfield in Kazakhstan (Nazina *et al*., 2001; 2004). In addition, *Geobacillus* species have also been recovered from temperate soils (McMullan *et al*., 2004) and artificial hot environments such as hot water pipelines, heat exchangers, waste treatment plants, burning coal refuse piles, and bioremediation biopiles (Maugeri *et al*., 2001; Obojska *et al*., 2002).

On the other hand, Archaea is the least understood in terms of its diversity, physiology, and ecological panorama of the three primary phylogenetic domains. Although many species of Crenarchaeota (Woese *et al.*, 1990) have been isolated, they constitute a relatively tight-knit cluster of lineages in phylogenetic analyses of rRNA sequences. It seemed possible that this limited diversity is merely apparent and reflects only a failure to culture the organisms, not their absence. This approach obviates the need for cultivation to identify organisms. The analyses documented the existence not only of species belonging to well-characterized crenarchaeal genera or families but also of crenarchaeal species for which no close relatives have so far been found. The large number of distinct archaeal sequence types retrieved from this single hot spring was unexpected and demonstrates that *Crenarchaeota* is a much more diverse group than previously suspected. These results have a strong impact on the concepts of the phylogenetic organization of Archaea.

The use of molecular phylogenetic approaches in microbial ecology has revolutionized the view of microbial diversity at high temperatures and has led to the proposal of a new kingdom within the Archaea, namely the "Korarchaeota" (Reysenbach *et al*., 2000). Their report consisted on the occurrence of another member of this archaeal

group and a deeply rooted bacterial sequence from a thermal spring in Yellowstone National Park. The phylotype is a lineage within the *Aquificales*. In situ hybridization with bacterial-specific and *Aquificales*-specific fluorescent oligonucleotide probes indicated that the bacterial populations dominated the community and most likely contributed significantly to biogeochemical cycling within the community (Reysenbach *et al*., 2000).

Representatives from the *Eukarya* domain can also be found in thermal environments. Eukaryotic microbial life may be found actively growing in almost any extreme condition where there is a sufficient energy source to sustain it, with the exception of high temperature (>70ºC). For most eukaryotes, therefore, a central requirement for growth in a habitat is sufficient energy flowing through the biosphere to support a second trophic level, as illustrated by the Simi and Nakuru soda lakes.

Colonization of extreme habitats is not normally restricted to a single taxonomic group, with the exception of xerophytic habitats which are only tolerated by fungi. Eukaryotic cells are exceedingly adaptable and not notably less adaptable than prokaryotes, although most habitats have not been sufficiently well explored for sound generalizations. The best studied high-temperature eukaryote is the acidophilic phototroph *Cyanidium caldarium* (Seckbach, 1994). Its exact taxonomic affiliations are still unclear, but it is generally grouped with the "red algae" (rhodophytes) since its chloroplasts possess chlorophyll-*a* and C-phycocyanin. Brock (1978) carefully examined the growth and ecology of this organism and determined its optimal growth temperature was 45ºC and the maximum temperature at which growth occurred was 57ºC. Earlier reports of growth at much higher temperatures, 75-80˚C (Copeland, 1936), were

attributed by Brock and his colleagues to either the measurement of temperature away from the organisms themselves, or to the temperature having increased and the organisms being observed in the process of dying. It is interesting to note that Brock isolated numerous strains of *C. caldarium* growing at various temperatures and found that they all had the same optimum growth temperature; they do not seem to have adapted to growth at higher temperatures. It is also interesting to note that the niche occupied by *Cyanidium*, of hot acid conditions, does not seem to have any competition for the available resources in that thermophilic cyanobacteria require alkaline conditions for growth. It is also noteworthy that at the time he was writing (Brock, 1994), all the hot, acid soils and waters in the world were colonized by *Cyanidium*, which seem identical, except for the acid springs in Hawaii, which seem to be devoid of life. Brock suggests that this might simply be because Hawaii is geologically recent and far distant from other hot springs, so that there has not yet been an opportunity for *Cyanidium* to colonize this particular habitat. There is a wide variety of other eukaryotes living at somewhat less extreme temperatures (Tansey & Brock, 1978).

Brock (1994), stressed the difficulties of estimating growth temperatures from ecological observations. There are many thermophilic fungi which have been isolated from compost and similar environments where temperatures can exceed 80ºC, but, until laboratory cultures demonstrate growth at these temperatures, the observations should be treated with caution. The upper limit for thermophilic fungi seems to be around 60ºC (Tansey & Broock, 1978).

The polychaete *Alvinella pompejana*, (Pompeii worm) lives in burrows on hydrothermal vent chimneys with a strong temperature gradient, which averages 68ºC but

has frequent growth at temperatures exceeding 81ºC (Cary *et al*., 1998), although they do leave their burrows to feed. It is possible that, like *Heteromita* (soil flagellates), the worm conducts temperature-sensitive biochemical processes during those times when the temperature is more modest.

Unlike the situation in *Cyanidium*, three species of flagellates have been raised to grow at 70ºC by incremental increases in cultivation temperature (Dallinger, 1887). This study reported more than 100 years ago has not been repeated, but Dallinger noted that increasing the temperature in too large steps, or before the cells had fully adapted to the current temperature, killed the cultures. This phenomenon of incremental adaptation to growth temperature has also been noted for psychrophiles (Lee & Fenchel, 1972).

There is a vigorous debate about whether it is possible for eukaryotic architecture to evolve as a true hyperthermophile (Forterre, 1995; Forterre *et al.*, 1995; Miller & Lazcano, 1995). The crux of the debate revolves around the ability of the central biochemical machinery, nucleic acid transcription, and translation, to operate at these high temperatures. Clearly the cell's membrane composition must change to retain the required degree of fluidity for proper function (Sprott *et al.*, 1991). Also, all hyperthermophiles contain reverse gyrases, which induces positive super-coiling of DNA that enhances its thermal stability (Bouthier de la Tour *et al.*, 1991; Forterre *et al.*, 1996; Forterre *et al.*, 1995). It seems clear that all the protective mechanisms operating, allowing cells like the archaeon *Pyrococcus* to grow above 100ºC are not well understood, and indeed what the actual upper limit for life might be (Erauso *et al.*, 1996; Stetter *et al.*, 1990). The half-life of unprotected RNA falls very rapidly with increasing temperature (Forterre *et al.*, 1995) and as direct consequence eukaryotes face two major

problems. First, following transcription, many eukaryotic genes are subjected to post-transcriptional modification, a process which takes a certain amount of time. However, a number of hyperthermophilic archaea post-transcriptionally modify the product of their tRNA genes in a manner which is reminiscent of the eukaryotes (Edmonds *et al.*, 1991). Furthermore, the essential organization of the transcriptional apparatus predates the divergence of the Archaea and the Eukarya (Ciaramella *et al.*, 1995). Second, the mRNA has to make its way out of the nuclear membrane in order to be translated by ribosomes. Any hyperthermophile would have to possess a mechanism to protect the mRNA from hydrolysis. From an evolutionary perspective, if the Archaea and the Eukarya are sisters taxa then the potential to colonize high-temperature environments presumably existed in their last common ancestor and there is therefore the potential in the eukaryotic lineage to do so too (Ciramella *et al*., 1995).

Two African soda lakes, with a pH of about 10, have been studied for their microbial populations: Lake Nakuru and Lake Simbi (Curds *et al.*, 1986; Finlay *et al.*, 1987). The former lake supports a very high population of flamingoes, counted in millions, feeding largely on cyanobacteria (Brown, 1975) (mostly *Spirulina*) growing in the lake. In this rich environment, there were at least 20 different heterotrophic species of protists, and three species of rotifers. Lake Simbi, on the other hand, was stratified with an extensive hypolimnion and there were far fewer flamingoes. The diversity and abundance of eukaryotic species was much lower than at Lake Nakuru. Samples from populations at Lake Nakuru, grew readily in the laboratory in a medium designed to mimic the ionic strength and pH of the lake, where several species not included in the above surveys were observed after enrichment. The diversity and abundance are in the

same range as similar studies on non-soda lakes in the same region of Africa (Curds *et al*., 1986).

Extrapolating from the observation that *Cyanidium* maintains its internal milieu at close to neutral pH, one might speculate that at pH 10 these cells were able to resist the influence of the external medium on their internal chemistry. Eukaryotes are now accepted as being the product of symbiotic events, which are normally thought of as between an eukaryote and a prokaryote (as in the mitochondrion or plastid, for instance). Many eukaryotes are themselves involved in a variety of intimate associations and there is some evidence that this close relationship is stressful, or in this context extreme (Douglas, 1996). There are many highly evolved groups of parasites which have adapted to these stresses and now cannot grow outside their hosts, and thus, can be considered to be living in an extreme habitat.

Adaptation of Thermophiles to Thermal Conditions

The modifications to protein structure for survival at extremes of temperature have been extensively reviewed (Fields, 2001; Jaenicke & Buhm, 1998), with most research into the field of thermophilic enzymes (thermozymes) (Adams, 1993; Burg *et al*., 1998). As thermophilic microorganisms cannot shield their internal environment from the external temperature, the cellular components have adapted to cope with elevated temperatures. Studies of extremophilic proteins have revealed no structural motifs, covalent modifications, or additional amino acids that would explain the ability of the proteins to function at extremes. Analyses of structural data have shown that a redistribution of the same forces that ensure stability in mesophilic environments, and changes in protein-solvent interaction, are sufficient to maintain structural integrity at

temperature extremes. This approach allows rapid modification of enzyme stability in response to environmental change by simply modifying the concentration of solutes, and allows the organism to adapt to new thermal niches (Fields, 2001).

High temperatures lead to an increase in the fluidity of the cellular membrane. Excessive membrane fluidity is proposed to lead to "leaky" membranes, and a corresponding loss of function of membrane proteins. In thermophilic bacteria, fatty acids are more saturated and longer than mesophilic fatty acids. Polar liquids are also enriched in carbohydrates and contain a greater proportion of methyl-branched fatty acid chains (Mermelstein & Zeikus, 1998). The archaeal cytoplasmic membrane contains unique ether lipids that are temperature and degradation resistant. They are also resistant to mechanical degradation and high salt concentrations, making these lipids more suitable for the membranes of extreme microorganisms than eubacterial ester-type lipids (Vossenberg *et al*., 1998).

Alkaliphiles keep their internal pH near neutral and require high concentrations of sodium ions. This indicates that the proteins on the cellular surface must be adapted to pH extremes. The maintenance of pH homeostasis is an important topic in extremophile research, and several mechanisms have been suggested to play a role (Kitada *et al*., 2000). Sodium dependent transport systems have been reported, which generate a sodium motive force via H^+/Na^+ antiport systems. Internal H^+ is exchanged with Na^+ by the cells, and Na^+ then accompanies substrates into the cells (Horikoshi, 1998).

The cell wall of extremely thermophilic archaea has been shown to be composed of an "S-layer" structure. This is a simple, regular two-dimensional lattice of glycoproteins that covers the cell surface, and leaves no periplasmic space. The

glycoprotein layers are highly resistant to mechanical and chemical degradation, and spontaneously reassemble due to strong subunit interactions (Herbert & Sharp, 1993).

The cell wall of an alkaliphilic *Bacillus* was recently shown to contain an acidic teichuronopeptide polymer, which serves as a barrier to ionic flux and plays a role in pH homeostasis (Kitada *et al*., 2000). Several alkaliphilic cell walls also contain a large amount of acidic amino acids. The acidic charges on these components may act as charged membranes, reducing the pH on the cell surface between 8 and 9 (Horikoshi, 1998) allowing the cell to maintain a neutral internal pH.

Part I. Isolation and characterization of the culturable thermophilic prokaryotic diversity present at the Coamo thermal springs

Material and Methods

I. Description of the thermal springs at Coamo, Puerto Rico

The Coamo area encompasses about 518 km^2 in south-central Puerto Rico, eastern most of the Greater Antilles (Glover, 1971) (Figure 1.1). It is approximately bounded on the north by the east-trending crest of the Cordillera Central and on the south by the shore of the Caribbean Sea (Glover, 1971). The thermal waters are located in this municipality, where they resurface as thermal springs (Figure 1.2). The origin of these thermal springs is not totally clear, however, there are some theories about the reasons of their existence (Giusti, 1971). The most accepted theory is based on a geological fault in this area and the natural thermal gradient that exists on the terrestrial cortex (Quiñones & Guzmán, 1983).

II. Sample processing and isolation of alkalithermophilic bacteria

The *Thermus* medium (TM) and Alkaline Yeast Extract Malt medium (AYEMM) were selected for this study. TM consisted of 8.0 g/L peptone; 4.0 g/L yeast extract and 2.0 g/L NaCl, pH 7.5, adjusted with 1M NaOH. AYEMM consisted of 10g/L malt extract; 4.0 g/L yeast extract; 4.0 g/L glucose, pH 9.0, adjusted with Na_2CO_3, 10% (w/v) solution. For solid media, 12g/L of gelrite and 1.62 g/L of $MgCl_2$ were added (Lin & Casida, 1984).

During August 2004, an assessment of three different sites of the thermal springs in Coamo, was performed (Figure1.2). For microbial isolation, five samples containing one liter (1L) of thermal water were collected (in triplicate) at the main water stream in

Coamo, from the three different sampling points and during different seasons of the year. The sampling was performed using sterile Whirl Pack® bags. One liter of each sample was filtered through 0.45 µm nitrocellulose membrane (Millipore ®) and transferred into Petri plates containing TM and AYEMM. Inoculated plates were incubated at 70˚C. After 3 weeks of incubation colonies were selected and purified by the quadrant streak plate method. Pure cultures were transferred into TM and AYEMM media for further biochemical and molecular analyses.

Figure 1.1. Aerial view of the thermal springs from Coamo, Puerto Rico. Map by Roy Ruiz, Puerto Rico Water Resources and Environmental Research Institute (PRWRERI) -UPRM.

Figure 1.2. Sampling areas of Coamo thermal springs: main stream (a), waterway (b), and outer watercourse (c).

III. Morphological and cultural characterization

Gram staining was performed using heat-fixed smears. Macroscopic characteristics were documented using the classical characterization of colony appearance. The morphology of cells was examined by Nomarsky technique and scanning electron microscopy (SEM) under optimal growth conditions. Electron microscopy procedures were performed with modifications as previously described (Díaz-Muñoz and Montalvo-Rodríguez, 2005). The strains were examined using a JEOL JSM-541 OL SEM microscope at 15 kv.

IV. Molecular and physiological Analysis

A. Isolation of genomic DNA

Isolated strains were grown in TM and AYEMM and incubated at 70°C for 24 hrs. 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) with lysozyme treatment followed by chloroform extraction and ethanol precipitation. The isolated genomic 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. The 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.

B. 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 the reverse primer Univ-1392-R (5'-ACGGGCGGTGTGTRC). The reaction mixture consisted of ddH_2O, buffer 1X, $MgCl_2$ 2.5mM, dNTP's 250mM, primer foward 1pmol, primer reverse 1pmol, DNA (10 ng), and *Taq* polymerase 0.026U/µl. PCR reaction consisted of 30 cycles with a denaturation period of 5 min at 94°C, 1 min at 50°C and polymerization for 3 min at 72 °C (Hezayen *et al.,* 2002). PCR amplicons were purified using the MinElute PCR purification kit (USA QIAGEN Inc.), according to the manufacturer instructions, and the product concentration was determined using a spectrophotometer at 260 nm.

C. Restriction fragment length polymorphism (RFLP)

A double digestion was performed on the amplicons using the restriction endonucleases *Hae* III, *Msp* I, and *Rsa* I. 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.

D. DNA sequencing

Selected PCR products were sent to a DNA sequencing facility in Korea (Macrogen ®) and UPR- Río Piedras Sequencing Facility. Samples were prepared according to the facility instructions.

E. Phylogenetic analysis

Distance analysis of the resulting DNA sequences were performed using the PHYLIP program (version 3.63) (Felsenstein, 1993). A multiple-sequence alignment with 16S rRNA gene sequences of closely related organisms (as determined by BLAST analysis) was made by using the Clustal W program (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 program. One hundred trees were inferred by using neighbor program. 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).

F. DNA base composition by HPLC

For G +C content, the strain CS_4 was selected as a representative of the isolated strains and was sent to DSMZ Germany to perform the analysis. Strains were subjected to DNA isolation. The cells were disrupted with French pressure cell. The DNA was purified on hydroxyapatit according to the procedure of Cashion *et al*., (1977). The DNA was degraded according to the method of Mesbah *et al.,* (1989). The resulting deoxyribonucleosides were analyzed by HPLC.

G. DNA-DNA hybridization

The DNA-DNA hybridization for strain CS4 was performed following De Ley e*t al*. (1970) with some modifications as described by Huss *et al*. (1983), using a model Cary 100 Bio UV/VIS- spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian®). The DNA-DNA percent similarity (in 2 X SSC + 5 % formamide at 68°C) was performed against *Geobacillus thermodenotrificans* DSM 465^T, *Geobacillus stearothermophilus* DSM 22^T, *Geobacillus subterraneus* DSM 13552^T, and *Geobacillus uzenensis* DSM 13351^T.

H. Physiological characterization

Optimal growth conditions were determined by cultivating the strains in TM and AYEMM solid media at temperatures of 40, 50, 60 and 70°C, respectively. Growth was monitored visually at 24h and 48h. The pH range of growth for the isolates was tested in TM and AYEMM media at the strain optimal temperature adjusting the pH to the following values: 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. The pH tolerance was tested in TM and

AYEMM plates 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 achieved in duplicates.

I. Biochemical tests

All biochemical tests were carried out at 60°C unless stated otherwise. Gelatin liquefaction, starch hydrolysis, indole production, Voges-Proskauer reaction, citrate utilization, hydrogen sulfide production, nitrate reduction, and acid and gas production from carbohydrate fermentations were performed as described elsewhere (Nazina *et al*., 2001).

Growth under anaerobic conditions was determined by incubating strains in an anaerobic chamber in TM and AYEMM media. Nutritional requirements were determined as previously described by Nazina *et al*. (2001). The filter-sterilized carbohydrates were added to the medium at a final concentration 0.2% (w/v).

J. Fatty acids analysis

Isolated strains were sent to Dr. Aharon Oren's Laboratory at the University of Jerusalem for fatty acids analysis (Miller, 1982; Kämpfer & Kroppenstedt, 1996). Cells were cultured on TM medium for 24 hours at pH 7.5, 70˚C to perform the analysis.

Results

A summary of the physical parameters of the thermal springs is presented in Table 1.1. Temperature and pH values varied among all the three sampling sites of the thermal waters.

Table 1.1. Summary of physical parameters from the Coamo alkaliphilic thermal waters

Sampling Site	Temperature ˚C	pH
Main stream	38.5	8.89
Waterway	38.5	8.15
Outer watercourse	44.0	9.36

Isolation of alkalithermophilic Bacteria

After 3 weeks of incubation, several colonies were observed growing on the inoculated membranes. The highest number of colony forming units (CFU's) was obtained in *Thermus* medium (Table 1.2). The number of CFU's obtained from the three samplings varied among seasons of the year. Appendix 1, show the significance difference of CFU's obtained per sampling on the different media. All the isolates were grouped in one morphotype, recorded as the most abundant and frequent recovered isolate.

Table 1.2 Numbers of CFU's obtained per sampling on the different media per liter of filtered water

Sampling	**TM**	**AYEMM**
1	48	16
2	20	12
3	85	4
Total	153	32

Morphological characterization of isolates

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

A total of 185 strains were isolated from the Coamo springs. Most of the isolates showed circular, entire, and flat macroscopical morphology. Colonies showed also white to cream pigmentation. Light microscopy of these strains revealed that all were gram-positive rods in a wide variety of arrangements such as diplobacilli, streptobacilli and single bacilli among others. Appendix 2 and figures 1.3, 1.4, 1.5, 1.6, 1.7, 1.8 and 1.9 show the macroscopical and microscopical characteristics of the isolated strains. Scanning Electron Microscopy (SEM) was very useful to examine the rod- shaped cells (Figure 1.10).

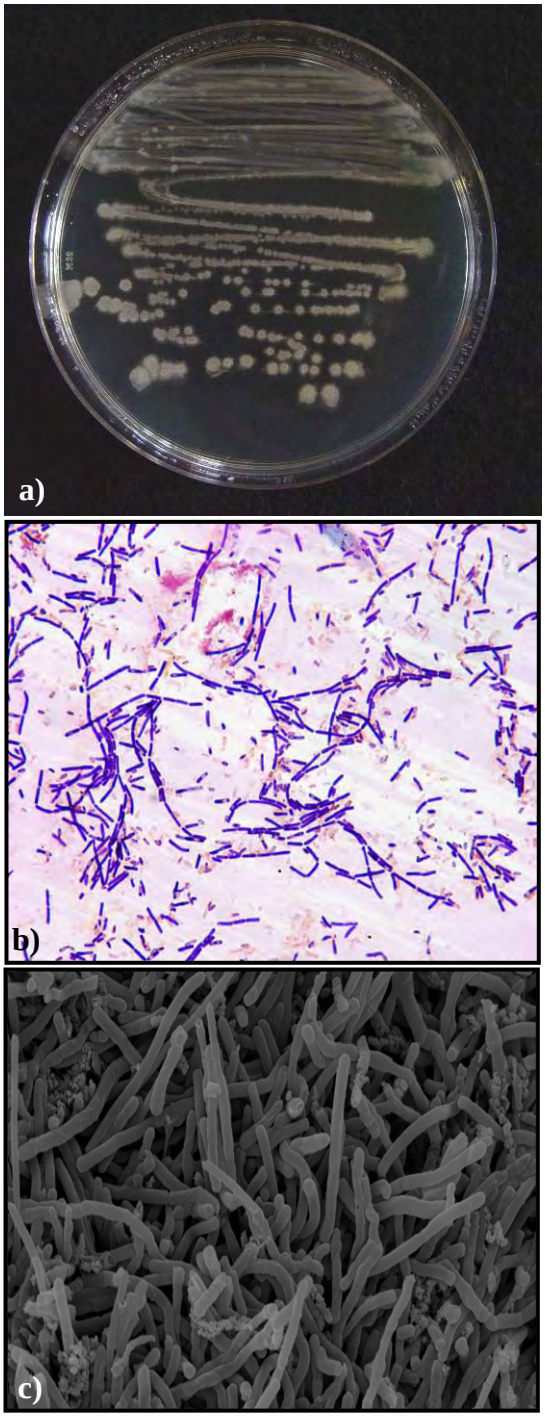


Figure 1.3 Macroscopical and microscopical characteristics of strain CS_4 isolated from Coamo springs: Strain CS_4 on TM (a), Gram positive stain on light microscopy (b), and SEM of rod cells (c).

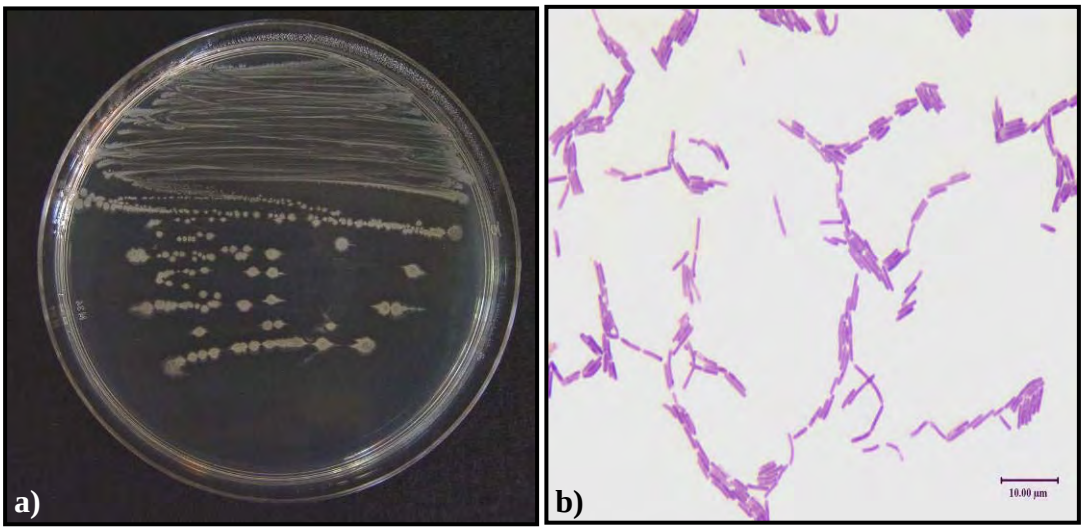


Figure 1.4 Macroscopical and microscopical characteristics of strain CS_{17} isolated from Coamo springs: Strain CS_{17} on TM (a), and Gram positive, rod shape on Bright Field microscopy (b).

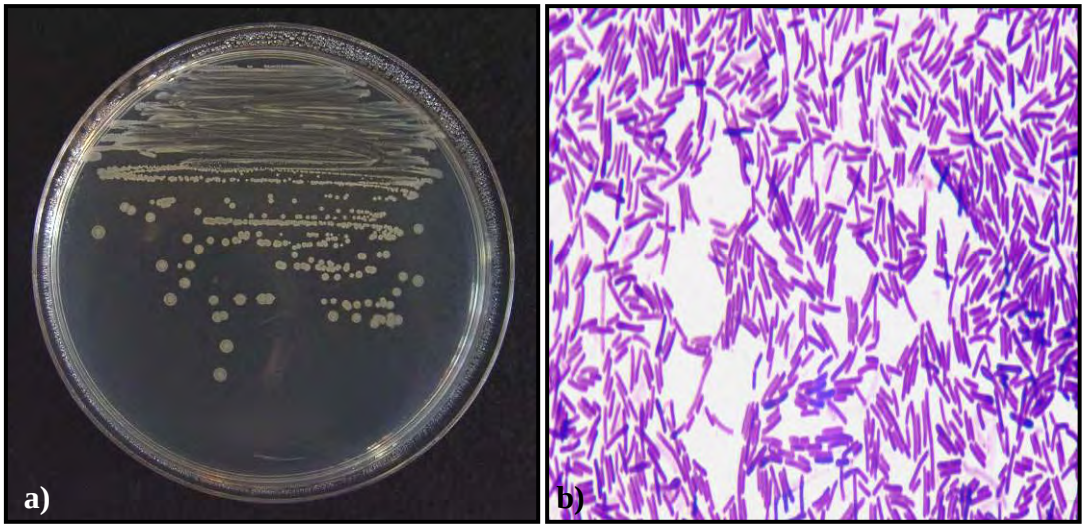


Figure 1.5 Macroscopical and microscopical characteristics of strains CS_{30} isolated Coamo springs: Strain CS_{30} on TM (a), and Gram positive, rod shape on Bright Field microscopy (b).

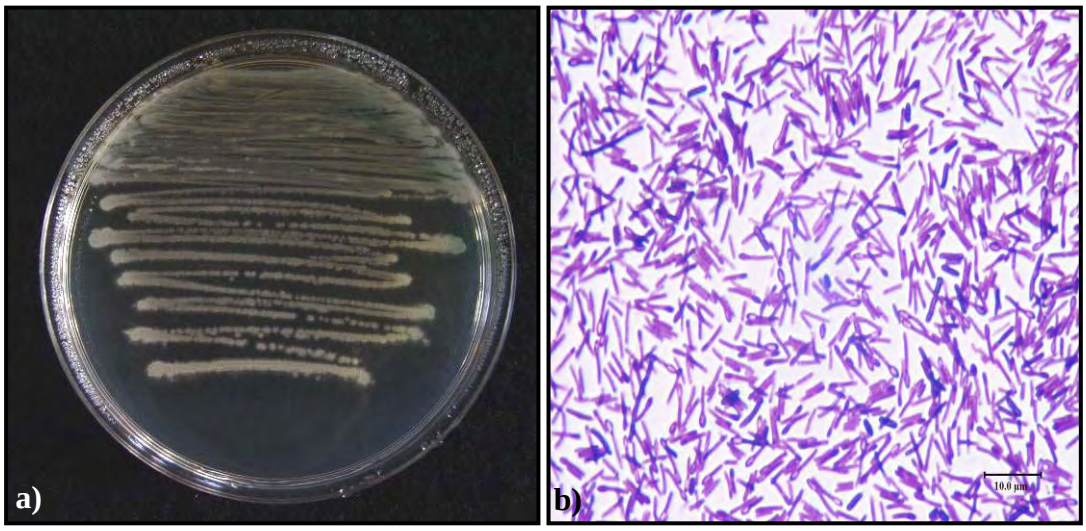


Figure 1.6 Macroscopical and microscopical characteristics of strains CS_{32} isolated from the Coamo springs: Strain CS_{32} on TM (a), and Gram positive, rod shape on Bright Field microscopy (b).

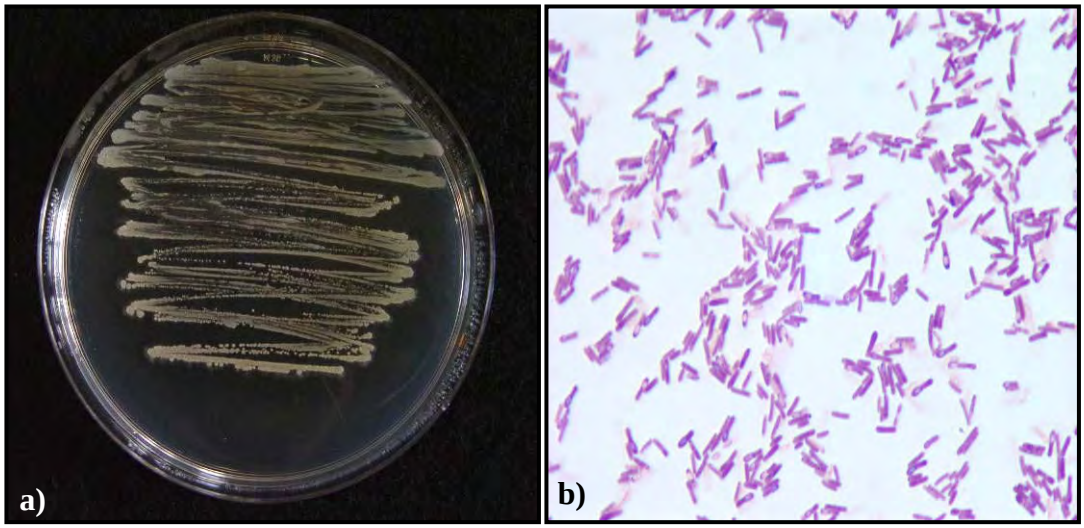


Figure 1.7 Macroscopical and microscopical characteristics of strains CS_{33}isolated from the Coamo springs: Strain CS_{33} on TM (a), and Gram positive, rod shape on Bright Field microscopy (b).

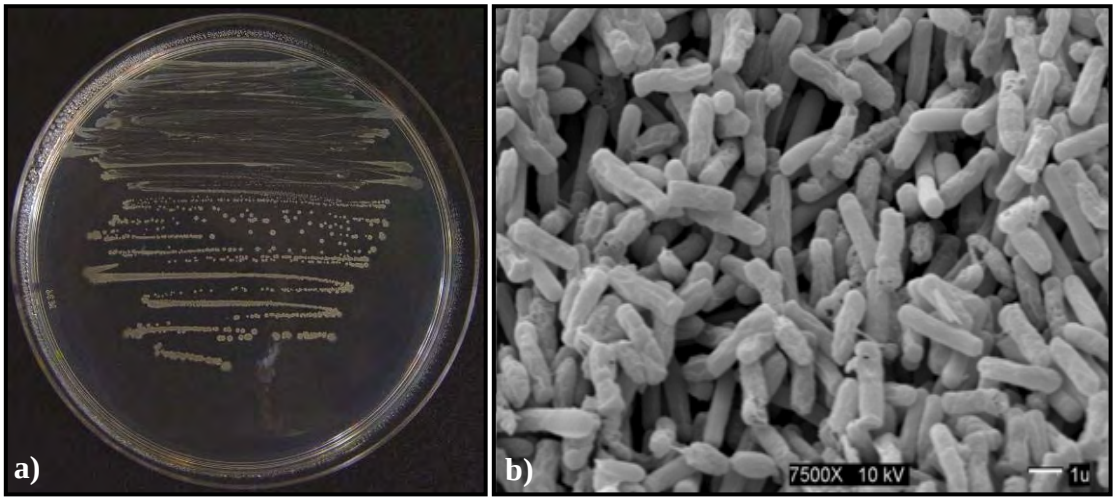


Figure 1.8 Macroscopical and microscopical characteristics of strain CS_{101} isolated from the Coamo springs: Strain CS_{17} on TM (a), and SEM of rod cells (b).

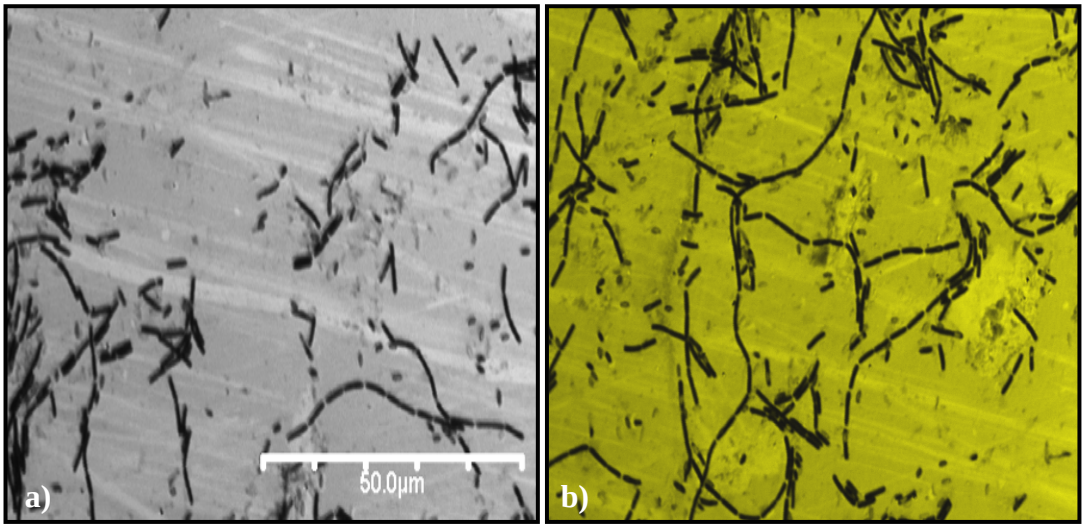


Figure 1.9 Nomarsky micrographs showing the prevailing Gram-positive rod-shape arrangement of some strains isolated from Coamo springs: Strain CS_4 (a), and strain CS_{36} (b).

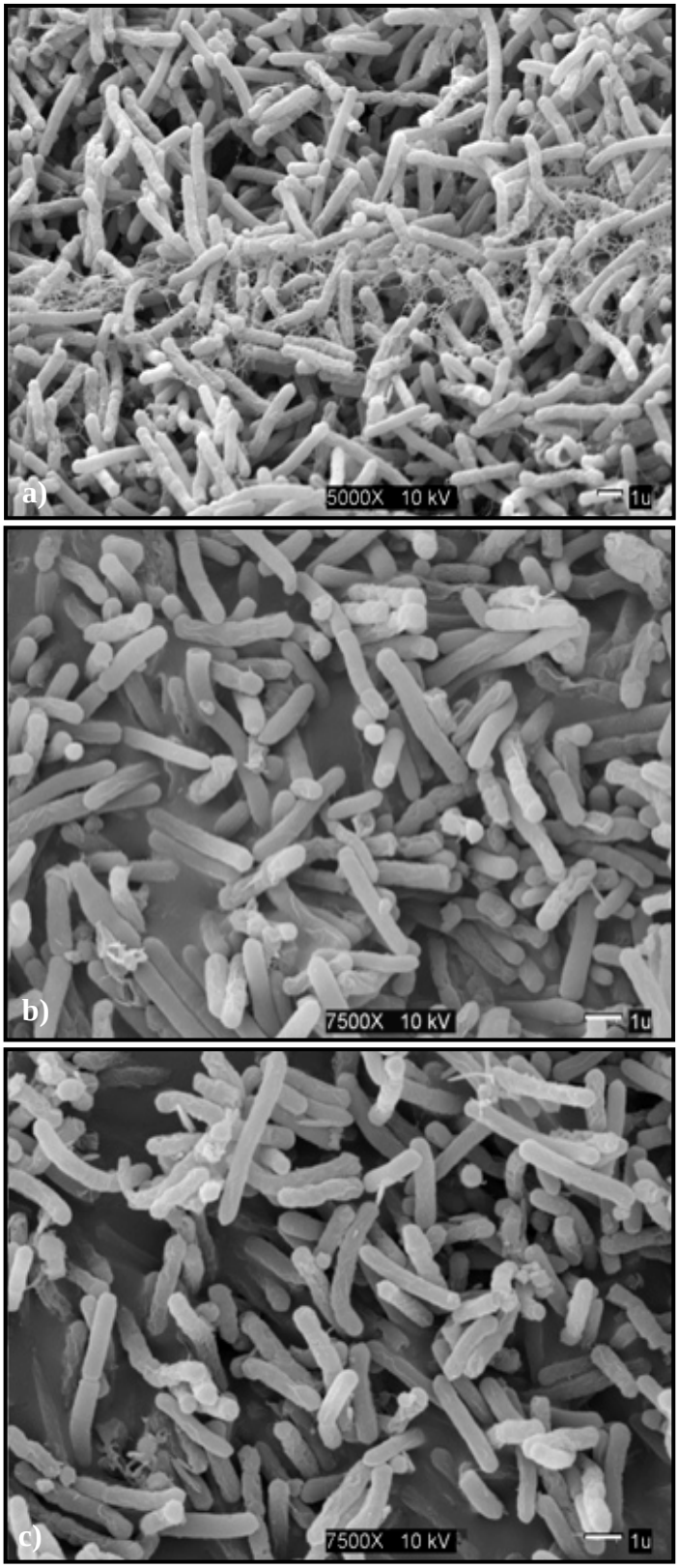


Figure 1.10 Scanning electron micrographs showing the prevailing Gram-positive rod-shape arrangement of some strains isolated from Coamo springs: Strain CS_{131} (a), strain CS_{130} (b), and strain CS_{8} (c).

Molecular and physiological analysis of isolates

After macroscopical and microscopical observations, the strains were further analyzed using molecular and physiological approaches. The 16S rDNA region was used for the molecular characterization. DNA from all strains were extracted using the phenol/chloroform technique. Figures 1.11, 1.12 and 1.13, show the quality of genomic DNA extractions from the isolated strains.

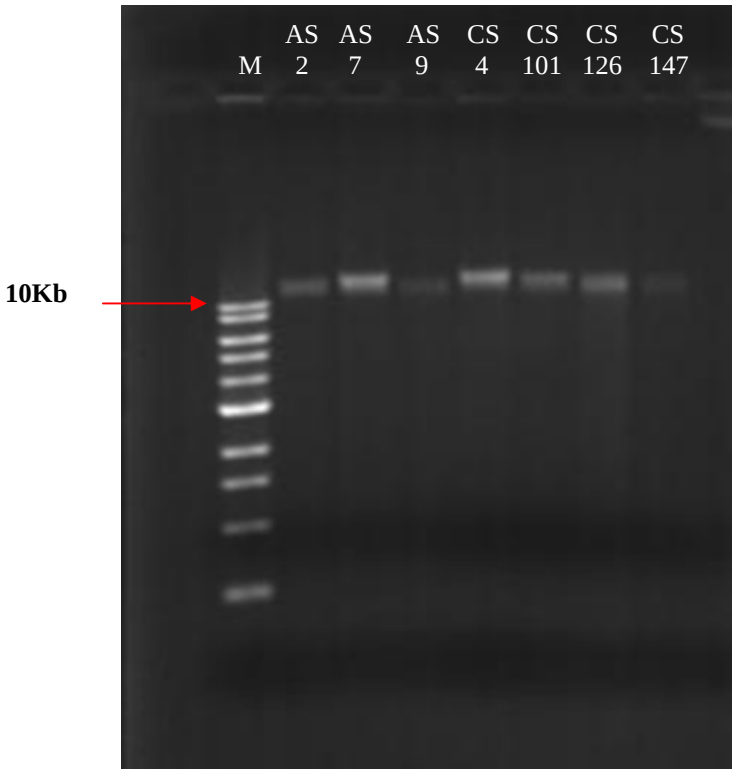


Figure 1.11 Genomic DNA extractions of alkalithermophilic strains isolated from Coamo springs

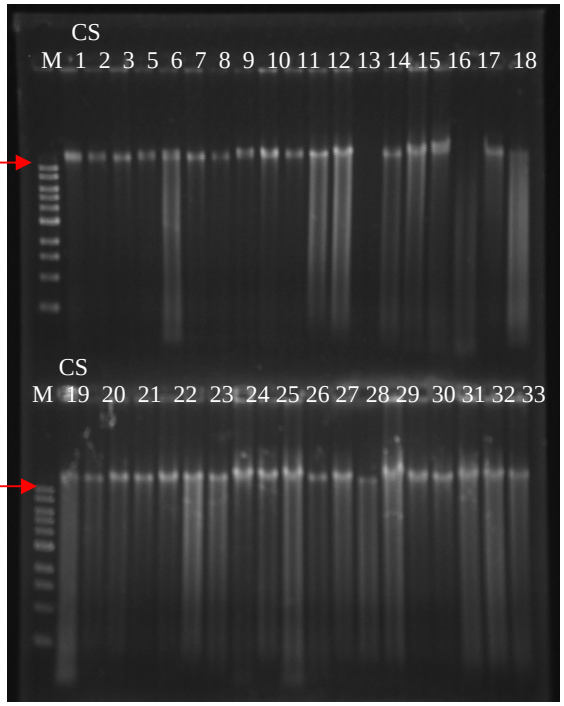


Figure 1.12 DNA extractions of alkalithermophilic strains isolated from Coamo springs

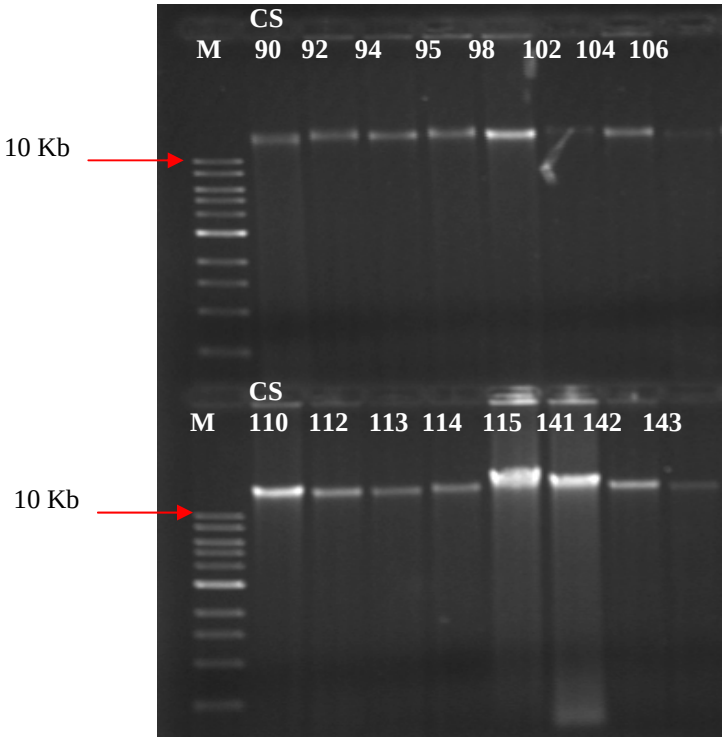


Figure 1.13 Extracted DNA of the alkalithermophilic strains isolated from Coamo springs

Genomic DNA's were used as templates for 16S rDNA amplification by PCR. The PCR amplicons obtained had a size of approximately 873 bp (Figures 1.14, 1.15, and 1.16). It was necessary to perform optimization of the PCR parameters for some strains in order to achieve optimal amplification.

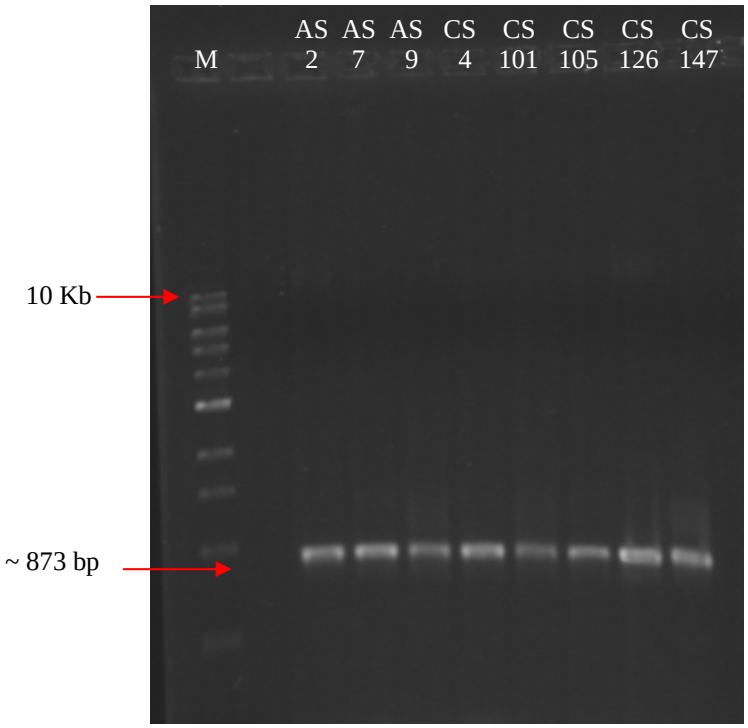


Figure 1.14. 16S rDNA amplification using universal primers 519-F and 1392-R, for strains isolated from the Coamo springs.

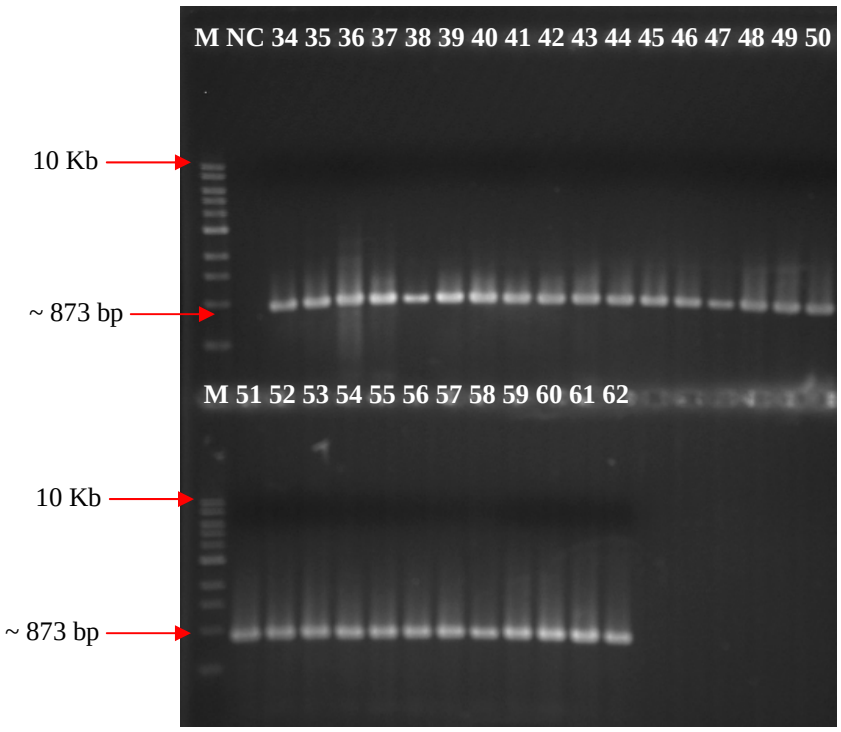


Figure 1.15 Amplification of the 16SrDNA using forward primers Univ-519-F and reverse primer Univ-1392-R

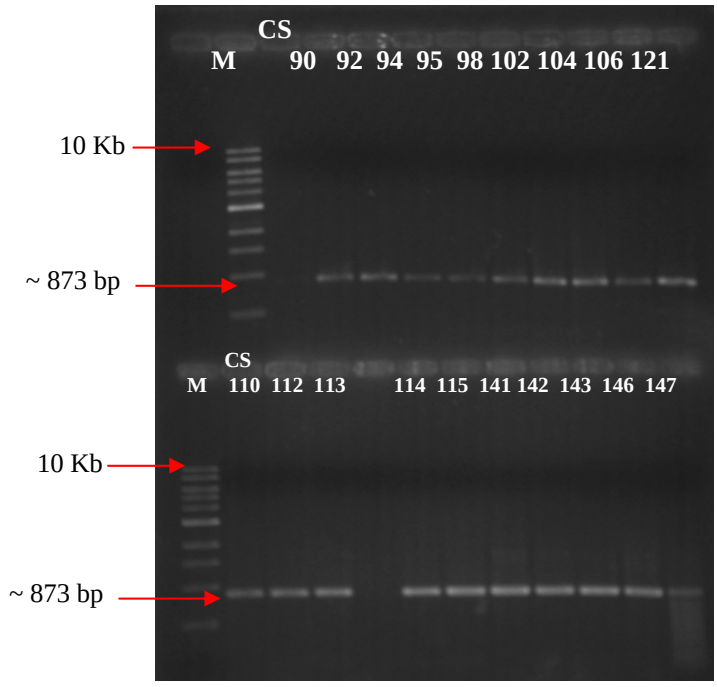


Figure 1.16 16S rDNA amplification using universal primers for strains isolated from Coamo springs.

RFLP analysis of the PCR products was performed to determine how many genotypes resulted from the digestion with different restriction enzymes. The restriction patterns were observed in 3% low melting agarose gel, allowing us to differentiate between strains and to classify them into groups based on restriction patterns. Figures 1.17, 1.18, and 1.19 show the restriction patterns using *Hae* III, *Msp* I and *Rsa* I for the alkalithermophilic strains recovered.

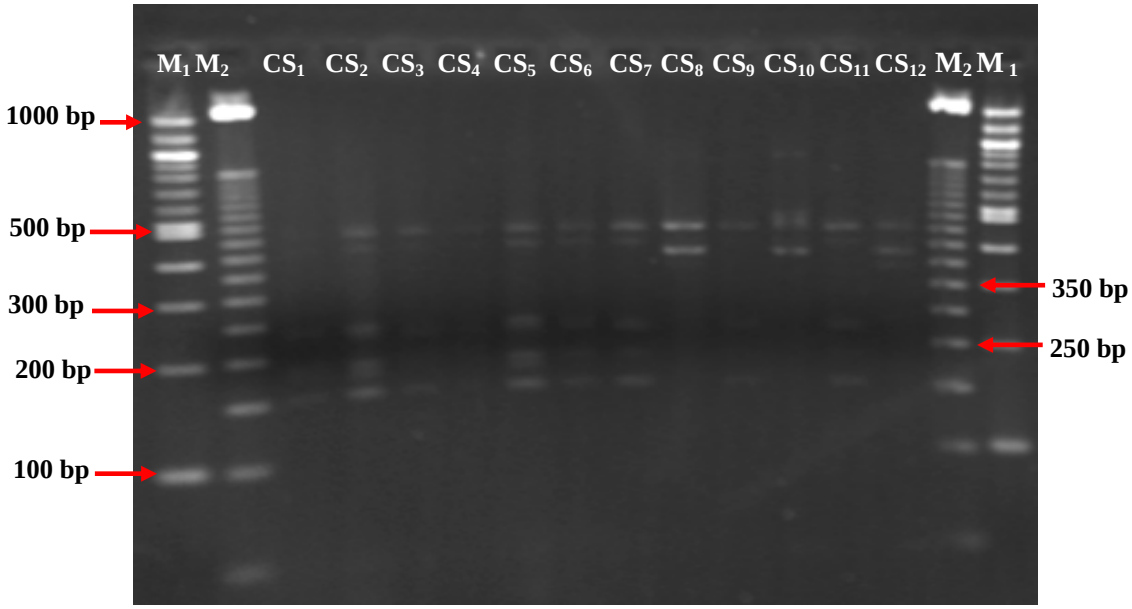


Figure 1.17. RFLP patterns of some strains isolated from Coamo springs using *Hae* III, and *Rsa* I enzymes in 3% low melting agarose.

Strains CS_1, CS_2, CS_3, CS_4, CS_5, CS_6, CS_7, CS_9, and CS_{11} from the thermal springs showed the same restriction patterns when digested with enzymes *Hae* III and *Rsa* I (Figure 1.17). Strains CS_8, CS_{10}, and CS_{12} formed only one type of RFLP profile when double digested with *Hae* III and *Rsa* I. Additional digestions were performed, using the enzyme *Msp* I (Figures 1.18 and 1.19) to improve resolution on the restriction patterns already obtained. Restriction analysis showed some significant differences for The isolated strains CS_{17}, CS_{30}, CS_{32}, CS_{33}, CS_{36}, CS_{42}, CS_{44}, CS_{48}, CS_{83}, CS_{92}, CS_{100},

CS_{101}, CS_{105}, CS_{106}, CS_{110}, CS_{117}, CS_{120}, CS_{121}, CS_{125}, CS_{126}, CS_{134}, CS_{136}, CS_{144}, CS_{146} and CS_{147} when triple digested with *Hae* III, *Msp* I, and *Rsa* I. In total, 5 groups were formed based on restriction patterns of the 16S rDNA amplicon.

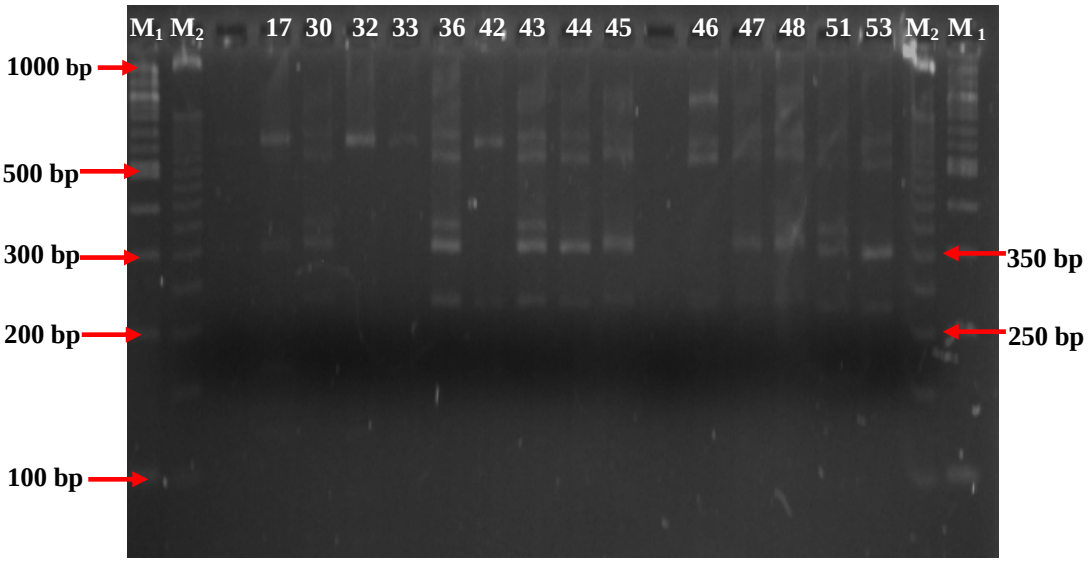


Figure 1.18. RFLP patterns of some strains isolated from Coamo springs using *Hae* III, *Msp* I, and *Rsa* I enzymes in 3% low melting agarose.

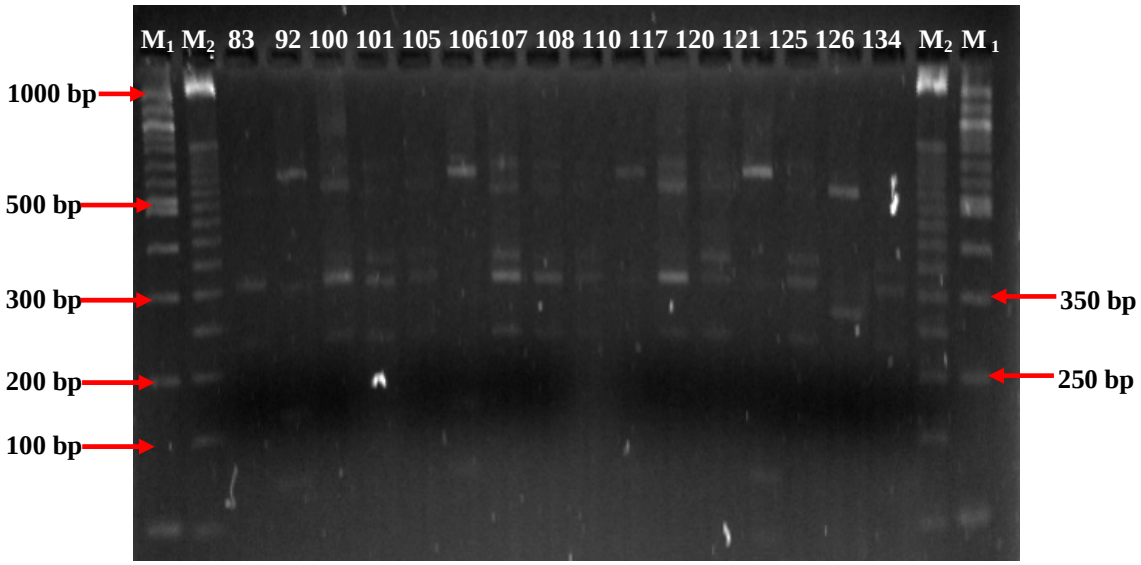


Figure 1.19 RFLP patterns of some strains isolated from Coamo thermal Springs using *Hae* III, *Msp* I, and *Rsa* I enzymes in 3% low melting agarose.

Strains representing different restriction patterns were selected for *in silico* analysis by sequencing. Additional strains with particular properties (morphological or physiological) were also selected for sequencing analysis.

Phylogenetic relationships of these sequences were determined using neighbor joining analysis. According to the results, all of the isolated strains are closely related to the group of the genus *Geobacillus,* a gram positive subdivision of the *Bacteria*. These strains were closely related to different *Geobacillus* species that had been isolated from other thermal environments (Nazina *et al*., 2001).

Strain CS_{87} was closely related to *G. lituanicus,* forming a branch in this cluster (Figure 1.20). Strain CS_{91}, was in the *G. vulcani* branch, while CS_{78} and CS_{93} were closely related to the branch that included strain CS_{91}. Strain CS_{79} was grouped with the branch that included *G. uzenensis* and *G. stearothermophilus*. This strain was more related to *G. stearothermophilus* (Figure 1.20). Strains CS_{74}, CS_{73}, CS_{77}, CS_{17}, CS_{83}, CS_{81}, CS_{75}, CS_{72}, and CS_{110} belonged to the *G. thermodenitrificans* cluster. Two of them (CS_{74} and CS_{73}) formed their own independent branch (Figure 1.20). CS_{71} was more closely related to *Geobacillus thermodenitrificans.* Strain CS_{121} and CS_{106} were clustered with *G. tobebii* (Figure 1.20).

Geobacillus strain CS_4 and *G. thermodenitrificans* are in the cluster of *G. subterraneus*. CS_4 is a strain of particular interest because it formed a distinctive branch closer to *G. thermodenitrificans* (Figure 1.21). Strain CS_{101} is related to the *G. toebii* cluster (Figure1.22). Another *Geobacillus* isolates, AS_7 and AS_9 were grouped into the branch that includes *G. caldoxyloxyliticus* (Figure 1.23). Although these strains were in this cluster, they formed a distinctive branch.

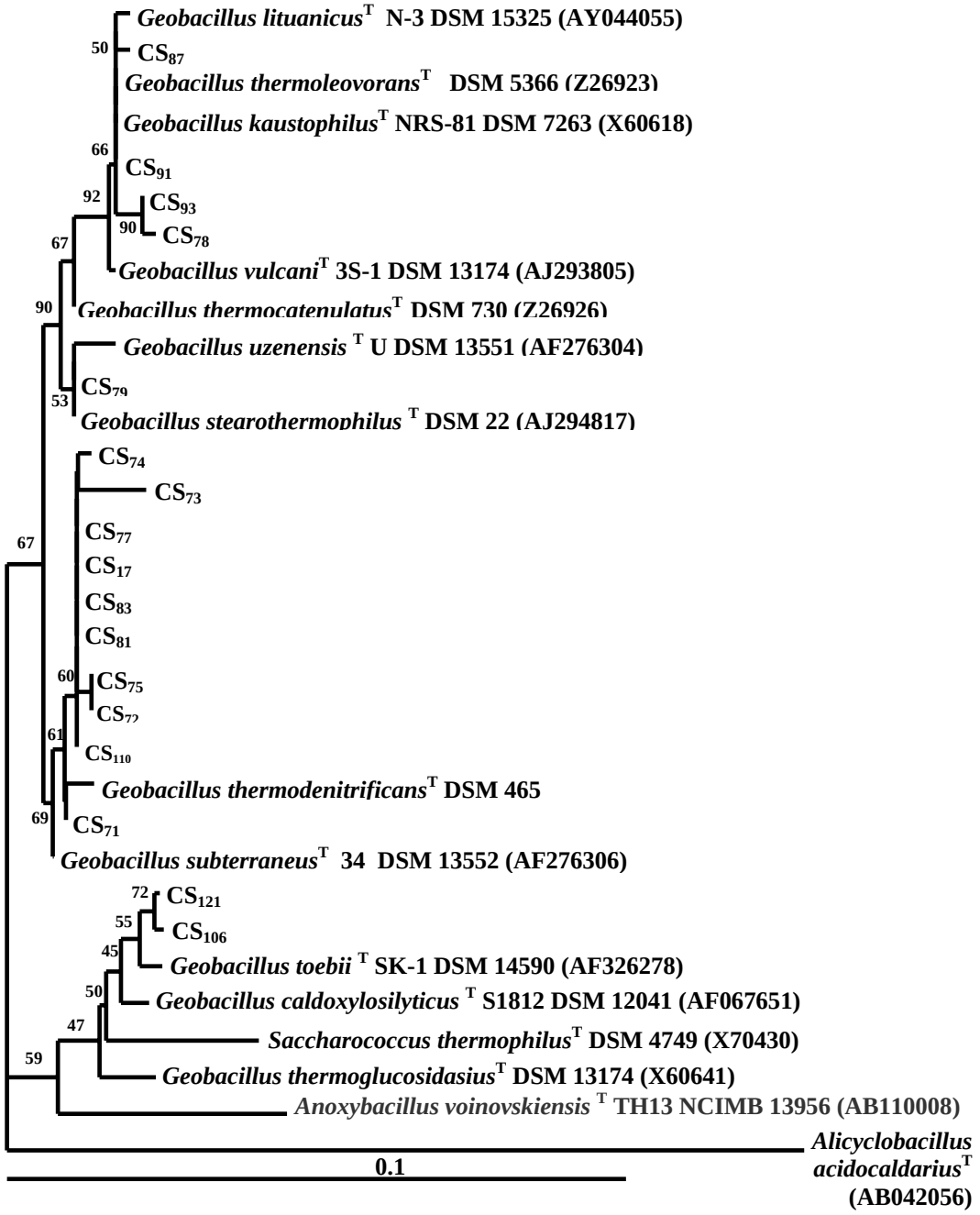


Figure 1.20 Neighbor-joining distance tree using the 16S rRNA sequences of *Geobacillus* strains isolated from Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown. ***Alicyclobacillus acidocaldarius*** ^{T} (AB042056)

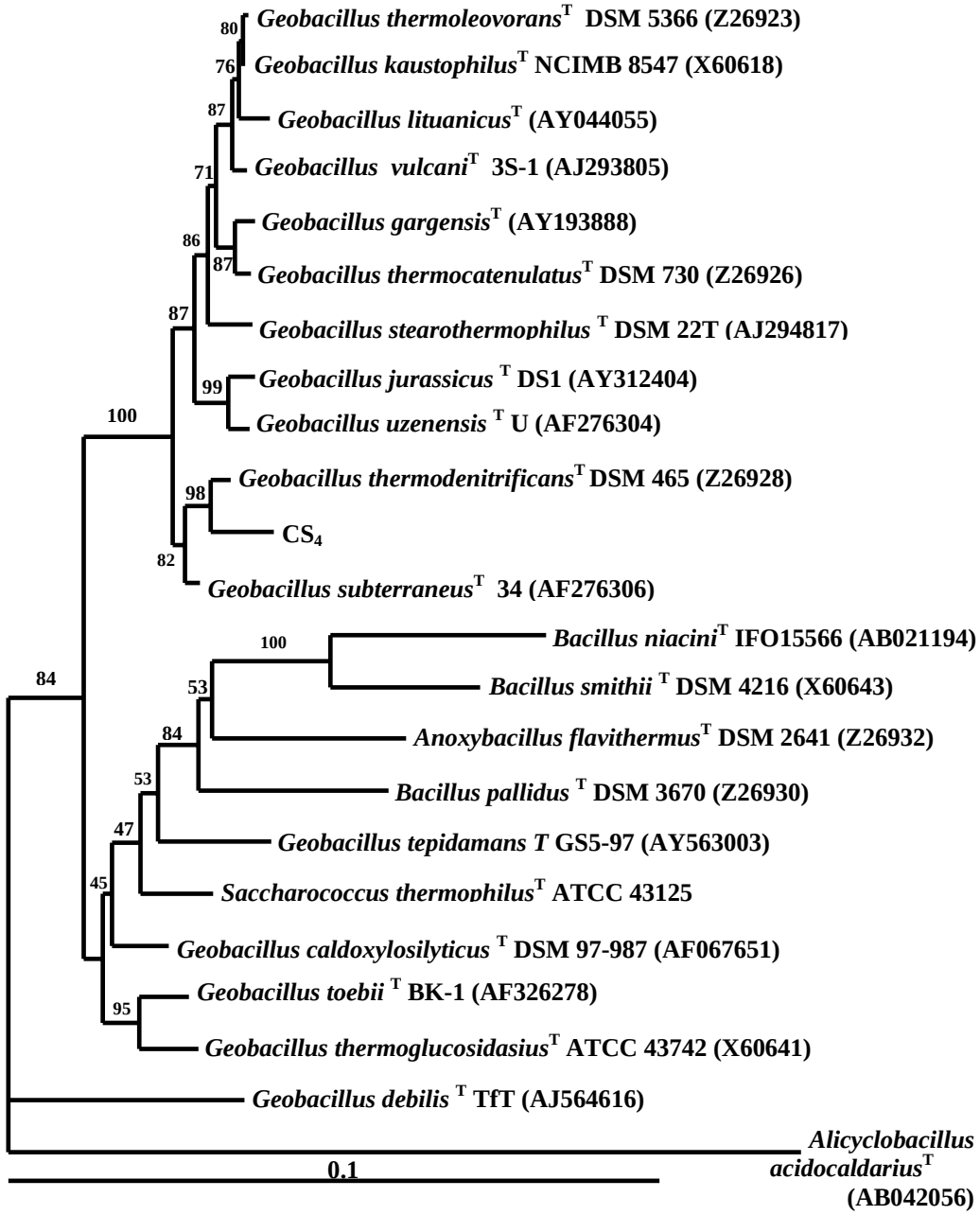


Figure 1.21 Neighbor-joining distance tree using the 16S rRNA sequences of *Geobacillus* strain CS_4 isolated from Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown. *Alicyclobacillus acidocaldarius*^{T} (AB042056).

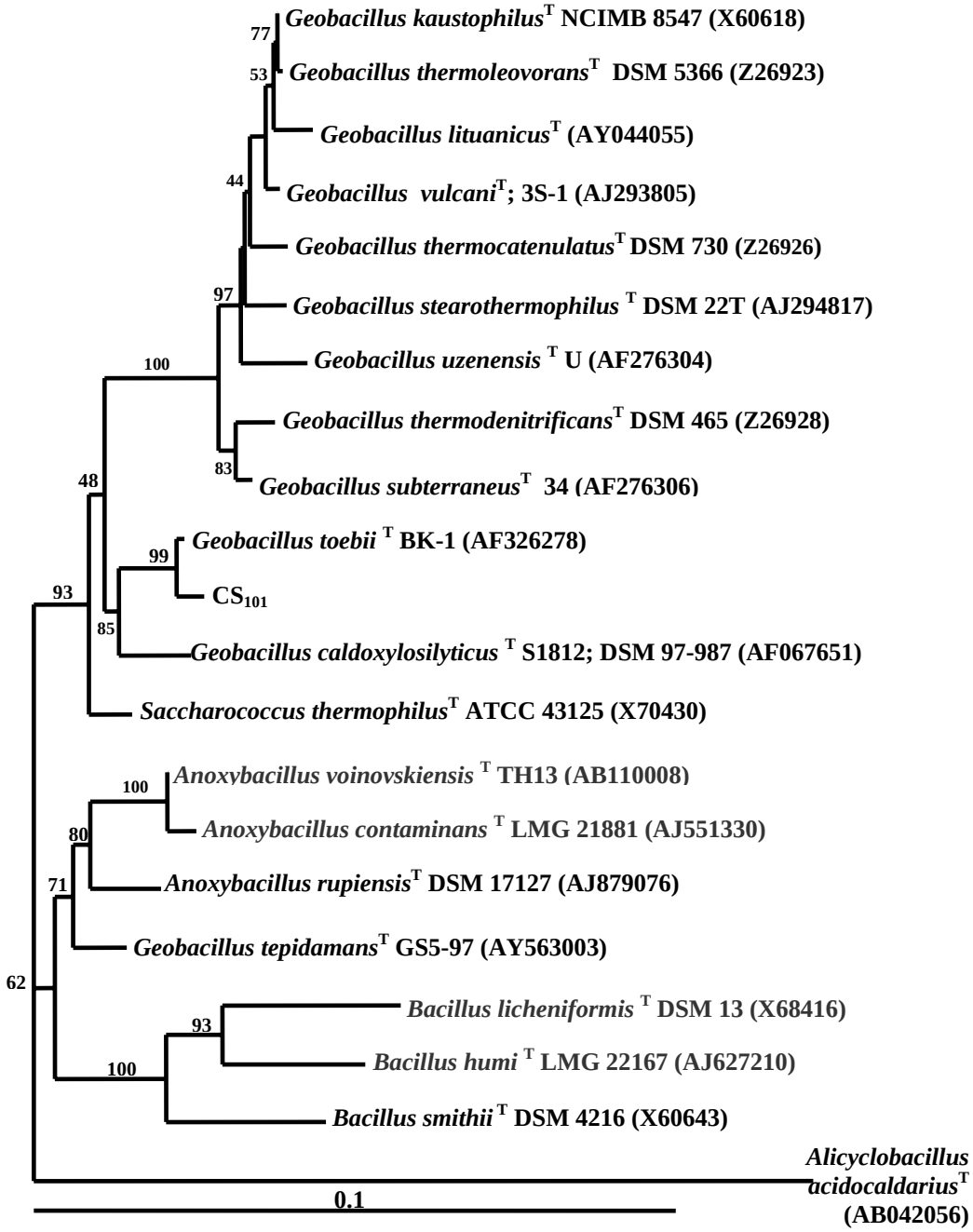


Figure 1.22 Neighbor-joining distance tree using the 16S rRNA sequences of *Geobacillus* strain CS_{101} isolated from Coamo thermal springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown. *Alicyclobacillus acidocaldarius*^{T} (AB042056).

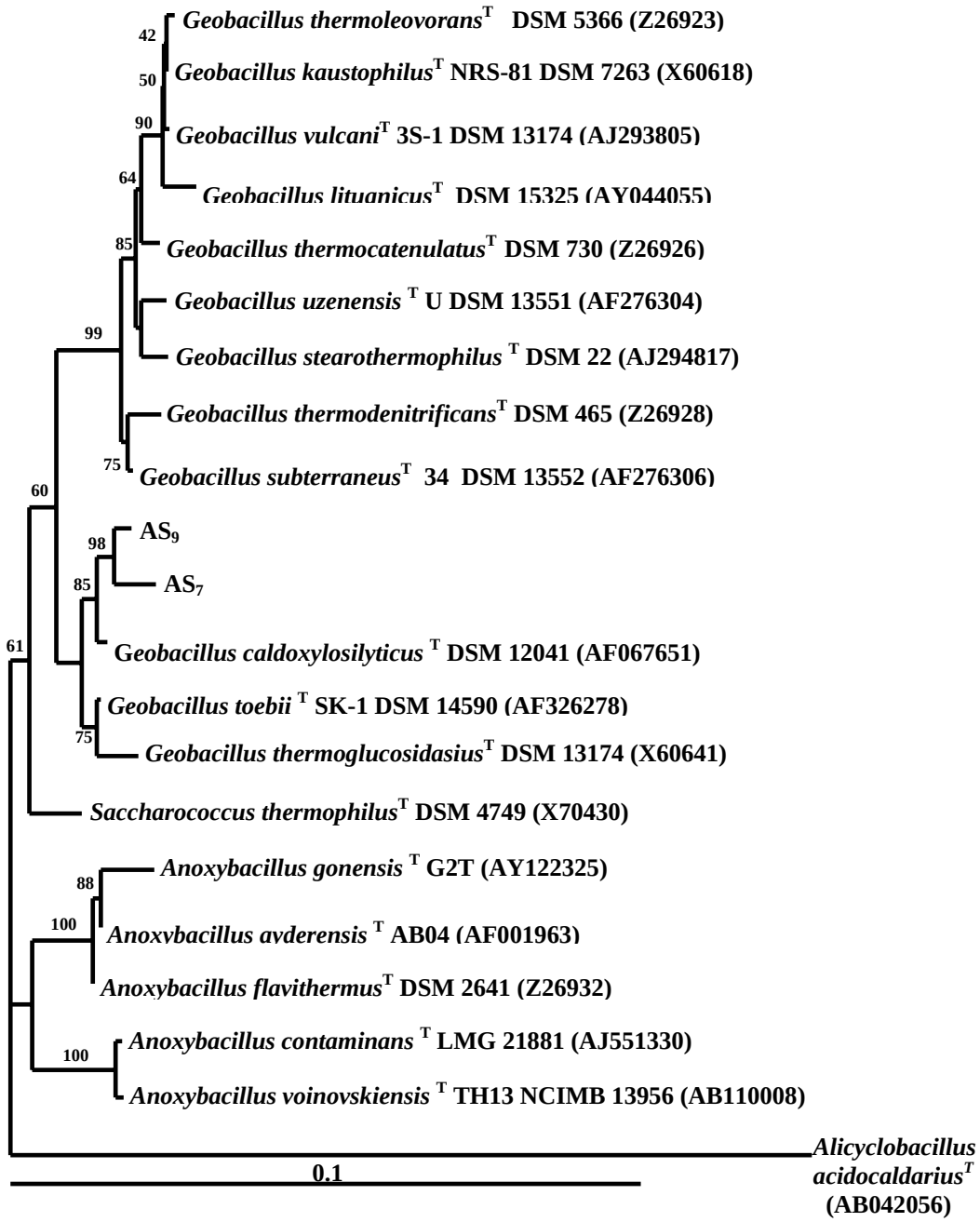


Figure1.23 Neighbor-joining distance tree using the 16S rRNA sequences of *Geobacillus* strains AS_7 and AS_9 isolated from Coamo thermal springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown. *Alicyclobacillus acidocaldarius*^{T} (AB042056).

G+C content and DNA-DNA similarities

DNA base composition analysis for strain CS_4 revealed a 49.4 mol % of G+C content, which is in agreement for members of the genus *Geobacillus*. The level of DNA-DNA similarities between strains CS_4 and *Geobacillus thermodenotrificans* DSM 465^T was 81.1%. The level of similarity of CS_4 against *Geobacillus stearothermophilus* DSM 22^T, *Geobacillus subterraneus* DSM 13552^T, and *Geobacillus uzenensis* DSM 13351^T were 36.5%, 52.7%, and 50.6%, respectively. Strain CS_4 seems to belong to the species *Geobacillus thermodenotrificans* DSM 465^T and does not belong to *Geobacillus stearothermophilus* DSM 22^T, nor to *Geobacillus subterraneus* DSM 13552^T or *Geobacillus uzenensis* DSM 13351^T.

Physiological characterization

After screening for different genotypic groups based on patterns in RFLP analysis and phylogenetic relationships, a total of twenty nine strains were selected as representatives of these groups for physiological characterization. The selected strains were CS_4, CS_{17}, CS_{30}, CS_{32}, CS_{33}, CS_{36}, CS_{42}, CS_{44}, CS_{48}, CS_{83}, CS_{92}, CS_{100}, CS_{101}, CS_{105}, CS_{106}, CS_{110}, CS_{117}, CS_{120}, CS_{121}, CS_{125}, CS_{126}, CS_{134}, CS_{136}, CS_{144}, CS_{146},CS_{147}, AS_2, AS_7, and AS_9 (Table 1.3). These isolates were transferred to different media in order to perform a better characterization for each strain. The strains that grew on AYEMM medium were very difficult to cultivate and only three of them (AS_2, AS_7, and AS_9) were viable after several culturing rounds.

Optimal growth conditions (temperature and pH) were determined in TM solid media for each strain after 24-48 hours of incubation. Table 1.3 shows the results obtained for the isolated strains tested.

Growth temperatures ranged between 40°C-70°C. It was observed that some of the isolates had more tolerance to high temperatures than others. Most of the isolated *Geobacillus* strains from Puerto Rico had an optimal temperature for growth between 50 -70°C. *Geobacillus* strains CS_{126} and CS_{146} were capable of growing at 40˚C. These results indicate that the isolated strains had a preference for temperatures between 50-70˚C as previously reported (Nazina *et al*; 2001). Therefore, the majority of these isolates can be considered thermophilic.

Optimal pH for the isolates tested ranged from 5 to 9 (Table 1.3). Some *Geobacillus* strains grew at pH 5 (CS_{36}, CS_{83}, CS_{100}, CS_{110}, CS_{117}, CS_{120}, CS_{121}, CS_{125}, CS_{126}, CS_{134}, CS_{136}, CS_{144}, and CS_{146}). Other strains (AS_2, AS_7, and $AS_{9)}$ were also tested at pH 10 but none of these strains was able to grow at this pH. They showed an optimal growth pH value of 9.0.

Most of *Geobacillus* strains had a preference for neutral pH values to slightly alkaliphilic pH values. These strains had optimal conditions to grow at a pH ranging from 6 to 9. The optimal growth condition found was around 8.0

Biochemical tests

All biochemical tests were carried out at 60˚C. The tests were performed in duplicates for best accuracy and validation of results. Appendix 3 shows the biochemical tests results for the twenty nine *Geobacillus* strains selected.

Table 1.3. Optimal growth conditions for Geobacillus strains of the isolates from thermal springs at Coamo, P.R.

Strain	Temperature range ˚C	Optimal Temperature ˚C	pH range	Optimal pH
CS_4	50-70	70	6-9	7.0
CS_{17}	50-70	70	6-9	7.5
CS_{30}	50-70	60	6-9	8.0
CS_{32}	50-70	50	7-9	8.0
CS_{33}	50-70	70	6-9	7.5
CS_{36}	50-70	70	5-9	7.0
CS_{42}	50-70	70	6-9	7.0
CS_{44}	50-70	50	6-9	7.5
CS_{48}	50-70	50	6-9	8.0
CS_{83}	50-70	60	5-9	7.0
CS_{92}	50-70	50	6-9	7.5
CS_{100}	50-70	60	5-9	7.5
CS_{101}	50-70	70	6-9	7.0
CS_{105}	50-70	60	6-9	7.5
CS_{106}	50-70	60	6-9	7.5
CS_{110}	50-70	70	5-9	7.5
CS_{117}	50-70	60	5-9	7.5
CS_{120}	50-70	50	5-9	8.0
CS_{121}	50-70	60	5-9	7.0
CS_{125}	50-70	50	5-9	8.0
CS_{126}	40-70	60	5-9	8.0
CS_{134}	50-70	60	5-9	8.0
CS_{136}	50-70	50	5-9	8.0
CS_{144}	50-70	60	5-9	7.5
CS_{146}	40-70	50	5-9	8.0
CS_{147}	50-70	60	6-9	8.0
AS_2	50-70	60	6-9	9.0
AS_7	50-70	60	6-9	9.0
AS_9	50-70	60	6-9	9.0

Physiologically, all these strains were facultative anaerobic. Negative results were documented for motility test in all strains. Voges-Proskauer and methyl red tests were negative for all the strains except for CS_4 and CS_{17}. The methyl red test was not determined for strain CS_{101}. All strains can use glucose, xylose, and sucrose as carbon sources and produced acid but no gas, with the exception of strain CS_{92}. None of the

strains can hydrolyze gelatin. Strains CS_4, CS_{30}, CS_{83}, CS_{100}, CS_{105}, CS_{110}, CS_{125}, CS_{126}, CS_{134}, CS_{136}, CS_{144}, CS_{146}, and CS_{147} have an extracellular amylase. Phenylalanine was not deaminated and H_2S and indole were not produced in any of the tested strains.

Fatty acids analysis

The fatty acid composition of *Geobacillus* strain CS_4 was determined. This strain was dominated by branched fatty acids, in agreement with previous reports (Nazina *et al*., 2001). The predominant fatty acids for *Geobacillus* strain CS_4 were iso-$C_{15:0}$, $C_{16:0}$, and iso-$C_{17:0}$ (Table 1.4). All the strains exhibited anteiso-$C_{15:0}$ and $C_{17:0}$ as minor components. However, a difference could be seen in the content of $C_{17:0}$, methyl- $C_{18:0}$ / iso- $C_{18:0}$ and $C_{18:0}$. None of the standards (type strains) had the high content of iso-$C_{15:0}$ and iso-$C_{17:0}$ seen in strain CS_4.

Table 1.4 Cellular fatty acid composition (% w/w) of the thermophilic *Geobacillus* strain CS_4 and the closely related type strains of thermophilic *Geobacilli*

Fatty acids	***Geobacillus subterraneus*^{T}** DSM 13552	***Geobacillus thermoglucosidasius*^{T}** DSM 2542	***Geobacillus kaustophilus*^{T}** DSM 7263	***Geobacillus uzenensis*^{T}** DSM 13551	**CS_4**
$C_{14:0}$ methyl / $C_{14:0}$ iso	0.64	0.54	1.52	1.16	NP
$C_{14:0}$	1.15	NP	2.03	0.93	0.96
$C_{15:0}$ iso	17.97	18.51	11.83	36.25	38.42
$C_{15:0}$ anteiso	4.42	2.95	6.08	6.46	3.09
$C_{15:0}$	1.69	1.93	2.26	0.45	0.99
$C_{16:0}$ methyl / $C_{16:0}$ iso	22.19	22.30	35.23	12.61	8.41
$C_{16:0}$	15.11	16.57	17.25	5.28	11.15
$C_{17:0}$ iso	15.94	17.64	6.61	19.67	25.91
$C_{17:0}$ anteiso	18.60	17.38	13.93	12.71	10.10
$C_{17:0}$	0.60	0.67	0.63	NP	NP
$C_{18:0}$ methyl/ $C_{18:0}$ iso	0.34	0.34	0.53	0.44	NP
$C_{18:1}$ cis- 9	0.36	0.29	0.55	0.55	NP
$C_{18:0}$	0.55	0.50	0.67	1.76	0.49

* Values are percentages of total fatty acids. NP means not produced.

Discussion

This study was focused on the isolation of alkalithermophilic prokaryotes present in the Coamo thermal springs, and it represents the first attempt to determine the heterotrophic prokaryotic biota at this site. Low numbers of isolates per liter of water sampled were obtained growing on the different media used in this study. The polyphasic taxonomical analysis performed on selected isolates led to the classification of a total of 185 isolates, which represented 29 species within the genus *Geobacillus*. Their characterization was based on morphological features, molecular analysis using 16S rDNA region, fatty acid profiles, and physiological properties.

Two different media were used in this study, *Thermus* medium (TM) and Alkaline Yeast Extract Malt Medium (AYEMM). They were used to increase the chances of proper isolation of prokaryotes able to grow at extreme conditions. They were modified by the addition of 1.2% of Gelrite and 0.162% of $MgCl_2$ in order to grow the isolates at 70˚C, since agar cannot be used as solidifying agent at this temperature (Lin & Casida, 1984). Also, the concentrations of gelling agent and gelling aid in media were optimized for the growth of thermophilic prokaryotes. TM is commonly used for the isolation of *Thermus* strains (Oshima & Imahori, 1974) and AYEMM is usually selected for the growth of alkaline microorganisms such as *Nocardiopsis* spp. However, these enriched media were used first in our preliminary samplings and showed good quality of bacterial growth and differences on the number of CFU"s obtained. The differences were attributed more to the spring characteristics than the media used for isolation. The most significant differences between the media used were found in the number of morphotypes obtained. TM medium sustained a highest

number of CFU's when compared to AYEMM. However, TM medium showed a low diversity within morphotypes recovered since most of the isolates represented the same strain. It is common to find a very low diversity of alkalithermophiles in hot environments. Most of the alkalithermophiles are distributed in many mesophilic environments, but others have been isolated only from one specific location (Wiegel, 1998). This group is comprised by physiologically Gram positive *Bacillus-Clostridim* phylogenetic sub-branch.

Preliminary observations on the strains revealed that aerobic, alkalithermophilic, spore forming and gram positive rod cells had the highest frequency of isolation obtained from the thermal springs. The morphological characteristics observed on these strains revealed features corresponding to those described in the literature for the genus *Geobacillus* (Nazina *et al*., 2001) and are consistent with its natural biotope, where geothermal areas occur, such as hot springs (McMullan *et al*., 2004).

The isolated strains (CS_{17}, CS_{30}, CS_{32}, CS_{33}, CS_{36}, CS_{42}, CS_{44}, CS_{48}, CS_{83}, CS_{92}, CS_{100}, CS_{101}, CS_{105}, CS_{106}, CS_{110}, CS_{117}, CS_{120}, CS_{121}, CS_{125}, CS_{126}, CS_{134}, CS_{136}, CS_{144}, and CS_{146}) from this study belonged to *Geobacillus*. In general, these strains show cream to brown pigments and long thin rods. Physiological analysis revealed that they do not grow at less than 50°C. Optimal growth conditions were around 50-70°C and a pH ranged from 5 to 9. One of the main features of the genus *Geobacillus* is the ability to produce ellipsoidal or cylindrical endospores at terminal or subterminal position in slightly swollen or no swollen sporangia (Nazina *et al*., 2001), which was observed for our isolates.

Restriction fragment length polymorphism analysis of a PCR amplicon containing the 16S rDNA was used to molecularly classify the strains into groups. The restriction pattern obtained with *Hae* III and *Rsa* I revealed that the first isolates $CS_{1,}$ CS_2, CS_3, CS_4, CS_5, CS_6, CS_7, CS_9, and CS_{11} could not be divided in more than two different groups and might represent the same *Geobacillus* species. Therefore, strain CS_4, was selected randomly as representative of the isolated *Geobacillus* strains for further analyses. The restriction pattern obtained with *Hae* III, *Msp* I and *Rsa* I revealed more differences and allow us to divide isolates in other groups when compared to the RFLP profiles first obtained.

Strains CS_{74}, CS_{73}, CS_{77}, CS_{17}, CS_{83}, CS_{81}, CS_{75}, CS_{72}, CS_{110}, and CS_{71} were closely related to *G. thermodenitrificans.* Strain CS_{87} was closely related to *G. lituanicus*, while strains CS_{91}, $CS_{78,}$ and CS_{93} were related to the *G. vulcani* branch. Strain CS_{79} was more related to strain *G. stearothermophilus*. Strains CS_{101}, CS_{121}, and CS_{106} were related to *G. tobebii*. Another *Geobacillus* isolates, AS_7 and AS_9, were grouped with *G. caldoxyloxyliticus*. Although these strains are related to most *Geobacillus* species, our isolates behave differently in the abilities to growth in presence of citrate.

In silico analysis of the 16S rDNA sequences revealed similarities to strains of *G. thermodenitrificans*, *G. toebii*, *G. lituanicus*, and *G. caldoxylosilyticus*. This information was used to construct a phylogenetic tree using the neighbor-joining method. The consensus distance tree places strain CS_4 in the *G. thermodenitrificans* cluster. On the basis of DNA base composition and DNA-DNA hybridization data, CS_4 showed 49.4 mol% of G+C 81.1% of similarity. All the results presented here strongly

suggest that isolate CS_4 is a strain of *G. thermodenitrificans.* Isolate CS_{101} is in the cluster of *G. toebii* and might be a strain of this species.

Various studies reveal that *Geobacillus* species have been successfully isolated from all continents, specifically where geothermal biotopes exist (McMullan *et al*., 2004). *Geoobacillus gargensis* (Nazina *et al*., 2004) was isolated from Garga hot spring, Transbaikal. Other species have been isolated from hydrothermal vents, hay compost, oilfields and cool soil environments (Kuisiene *et al*., 2004, McMullan *et al*., 2004; Nazina *et al*., 2001; 2004). The common abundance of this genus in hot environments, as thermal springs, together with the ability to produce spores could explain why those isolates were present in the alkalithermophilic waters at Coamo, Puerto Rico.

We found a low diversity of microorganisms in this habitat. Most alkalithermophiles are found frequently in non-alkaline environments and are as a group ubiquitous, although some species might be endemic to specific environments (Wiegel, 1998). One of the reasons that several of these alkalithermophilic bacteria are found in environments with conditions less suitable for their optimal growth may lay in the fact that they are all facultative alkaliphiles and that their doubling times are as short as ten minutes (Wiegel, 1998). Most of the alkalithermophilic bacteria belong to the Gram positive *Bacillus* subphylum, (Wiegel, 1998), currently known as *Geobacillus* (Nazina *et al*., 2001). Therefore, the fact that we mainly isolated *Geobacillus* species from the thermal waters of Coamo indicates that this genus prefers this type of extreme environment; representing the first report of the genus *Geobacillus* from Puerto Rico and the Caribbean. It remains possible that there are other type of extremophilic

prokaryotes present in this waters but the fact that we impose two extreme conditions at the same time (high pH and high temperature) was one factor that can explain the low diversity documented, mainly restricted to an spore forming genus.

Although the alkalithermophilic species that has been described previously (Wiegel and Kevbrin, 2004) neither represent the most thermophilic nor the most alkaliphilic microorganisms known. Our isolates represent the most alkaliphilic among the thermophiles and the most thermophilic among alkaliphiles. The combination of two extreme conditions of physico-chemical growth parameters restricts the range in which microorganisms can proliferate more than does one single growth condition. Most of the genera isolated from alkalithermophilic environments included the spore forming bacteria *Clostridium paradoxum* and *Thermoalkalibacter bogoria*. Other bacteria have been isolated from mesophilic environments such as *Clostridium thermoalkalophilum* (Wiegel, 1998).

Geobacillus strains described in this study had the physiological properties that allow their survival in extreme environments, like the thermal waters of the alkalithermophilic springs. Many of them probably can not survive for long terms. Spore production may contribute to their presence in the water. However, several strains isolated demonstrated to have alkaliphilic properties. They can grow at alkaline pH and high temperature for a long time. The results presented here may indicate a preference for thermal environments.

Part II. Diversity of prokaryotic communities present at the Coamo thermal springs by culture independent techniques

Material and Methods

I. Sample processing

Five samples containing one liter (1L) each of thermal water were collected (in triplicate) at the main water stream in Coamo from the three different sampling points during different seasons of the year. The samples were collected using sterile Whirl Pack® bags. One liter of each sample was filtered through 0.45 µm nitrocellulose membrane (Millipore ®). Membranes were stored at -20 ºC until DNA extraction was performed.

II. Extraction of total genomic DNA from environmental samples

Membranes containing microorganisms from the thermal springs 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 clean 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 clean 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 *et al*., 1995). The purity of the DNA was assessed by gel electrophoresis

(Øvrea°s *et al*., 2003). Total genomic DNA was 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 consisted 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 (Promega®) 0.026U/µl in storage buffer B (Promega®). Those primers (IDT®) correspond to universal primers for amplification of the prokaryotic 16S rRNA gene. PCR was performed for 30 cycles with denaturation periods of 5 min at 94°C, 1 min at 50°C, and polymerization for 3 min at 72 °C (Hezayen *et al.*, 2002). One Kb DNA Ladder (New England Biolabs®) was used as DNA marker. Samples and controls were observed by eletrophoresis 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 QIAGEN 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 (Güssow 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 achieve proper amplification of 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 observed by electrophoresis 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 QIAGEN 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, *Rsa* I, and *Hae* III. 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.

VII. Terminal restriction fragment length polymorphism (T-RFLP)

The gene encoding the 16S rRNA was amplified by PCR as described in the improved protocol for T-RFLP by capillary electrophoresis (Grüntzig *et al*., 2002) with some modifications. The primers used for the amplification were the labeled-forward primer 519F/ FAM (5'- /56-FAM/ CAGCMGCCGCGGTAATWC-3') and the reverse primer 1392R (5'- ACGGGCGGTGTGTACA-3'). PCR reactions were carried in a total volume of 50 µl. The reaction mixture consisted 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 ®). PCR was performed for 30 cycles with a denaturation periods of 5 min at 94°C, 1 min at 50°C, and polymerization for 3 min at 72 °C (Hezayen *et al.,* 2002). Samples and controls were observed by electrophoresis in agarose 1.0% and their quality were assessed 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.

A total of 200 ng of each labeled 16S rDNA product was used for restriction digestions separately with the following enzymes: *Hae* III, *Rsa* I, and *Msp* I (New England BioLabs®). Each digestion reaction consisted of 2.0 µl of 10X reaction buffer, 5 units of each restriction enzyme, and the ddH2O volume was adjusted by the amount of PCR product added for a total reaction volume of 20 µl. The digestions were incubated at 37°C in a the thermal cycler for 4 hours followed by 10 minutes at 65°C

to inactivate the enzymes. Samples were processed using the LI-COR Biosciences NEN®DNA Analyzer Model 4300 (LICOR Inc.).

VIII. DNA sequencing

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

IX. 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 by BLAST analysis) (Kamekura and Dyall- Smith, 1995; McGenity & 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. CONSENSE was used to determine the most frequent branching order. The final tree was drawn using TREEVIEW (Page, 1996).

X. Statistical analysis of clones libraries

Statistical analyses were performed as Stach and collegues (2003) 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

After performing several environmental DNA extractions for the spring samples, the Saano's protocol with some modifications was selected since it was the best to achieve optimal environmental genomic DNA extraction (Saano *et al*; 1995). Three environmental genomic DNA extractions were obtained and named as Main stream, Waterway, and Outer watercourse which corresponded to the samples of first, second and third survey accordingly (Figures 2.1, 2.2, and 2.3).

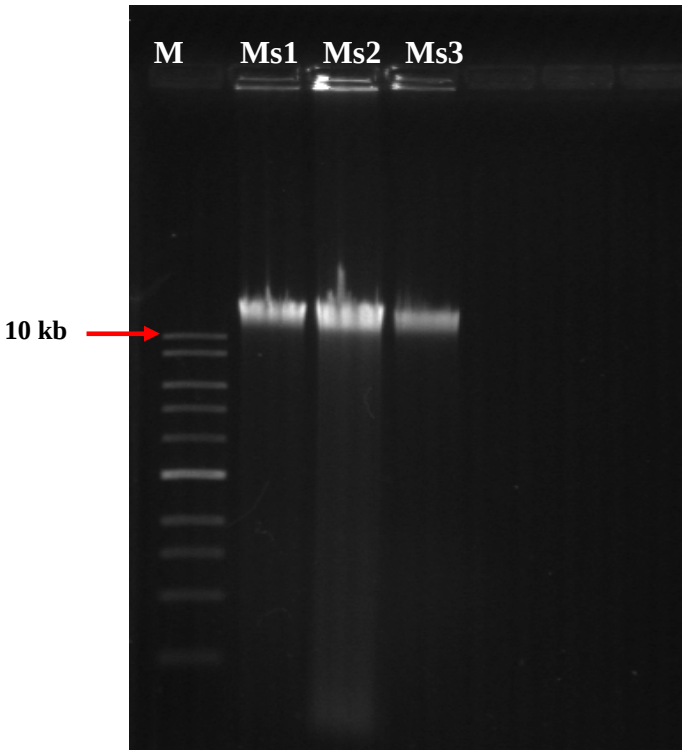


Figure 2.1 Total population genomic DNA extraction from the mainstream of the Coamo springs. Samples: mainstream first survey (Ms1), mainstream second survey (Ms2), and mainstream third survey (Ms3).

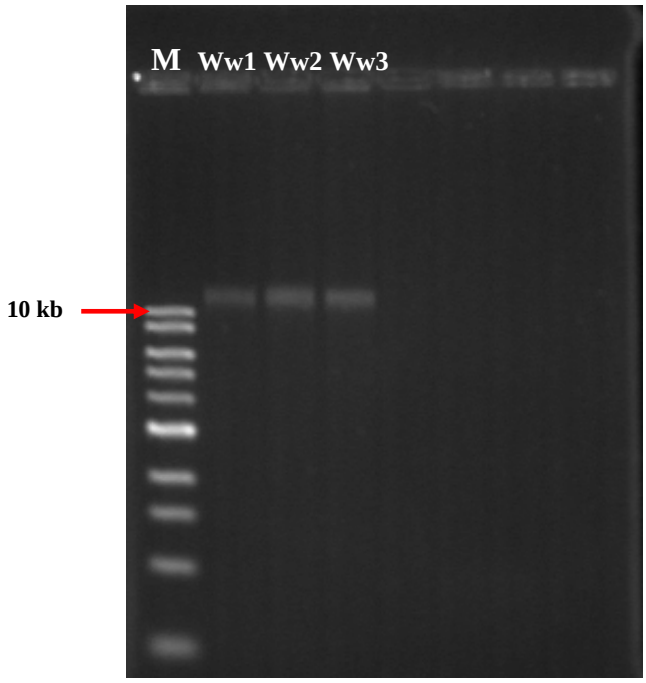


Figure 2.2 Total population genomic DNA extraction from the waterway of the Coamo springs. Samples: waterway first survey (Ww1), waterway second survey (Ww2), and waterway third survey (Ww3).

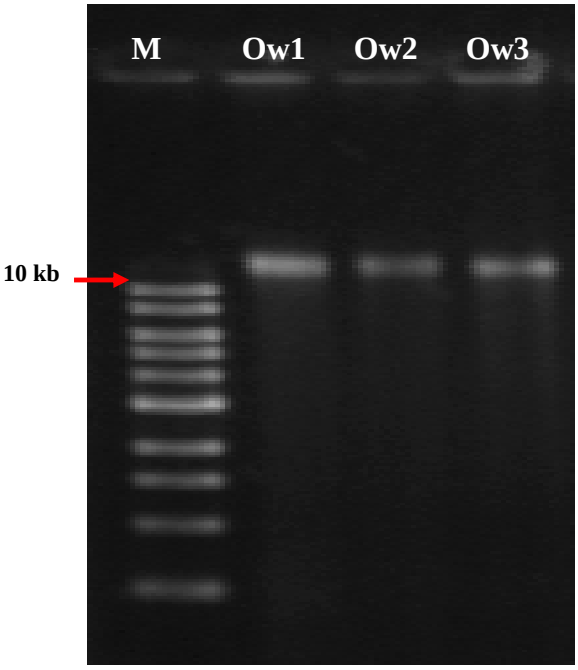


Figure 2.3 Total population genomic DNA extraction from the outer watercourse of the Coamo springs. Samples: outer watercourse first survey (Ow1), outer watercourse second survey (Ow2), and outer watercourse third survey (Ow3).

The total population genomic DNA obtained was of PCR quality (Figures 2.4, 2.5, and 2.6). These products 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 for PCR reactions. Amplicons had a size of approximately 873bp.

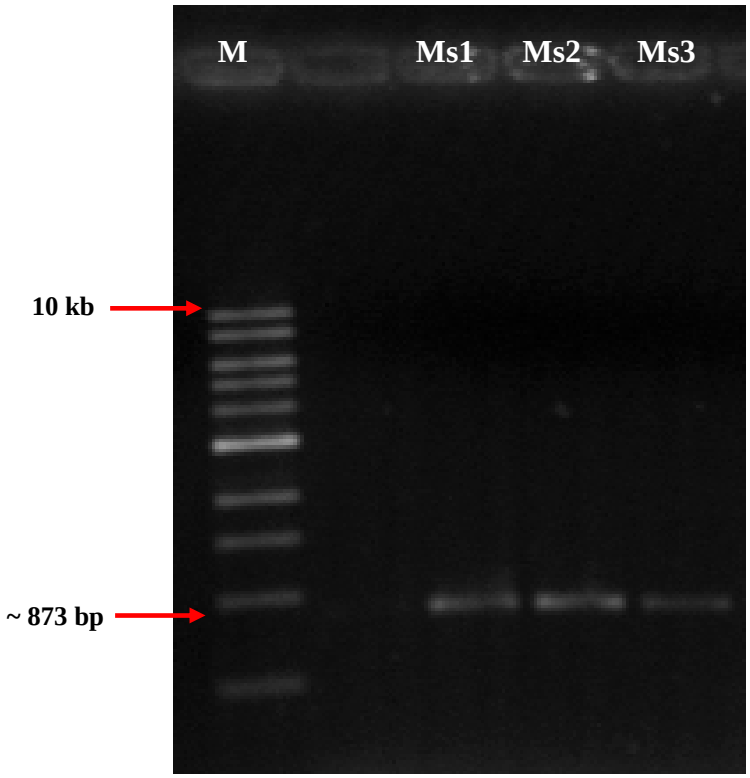


Figure 2.4 16S rDNA PCR product from the thermal waters of the Coamo springs using universal primers. Samples: mainstream first survey (Ms1), mainstream second survey (Ms2), and mainstream third survey (Ms3).

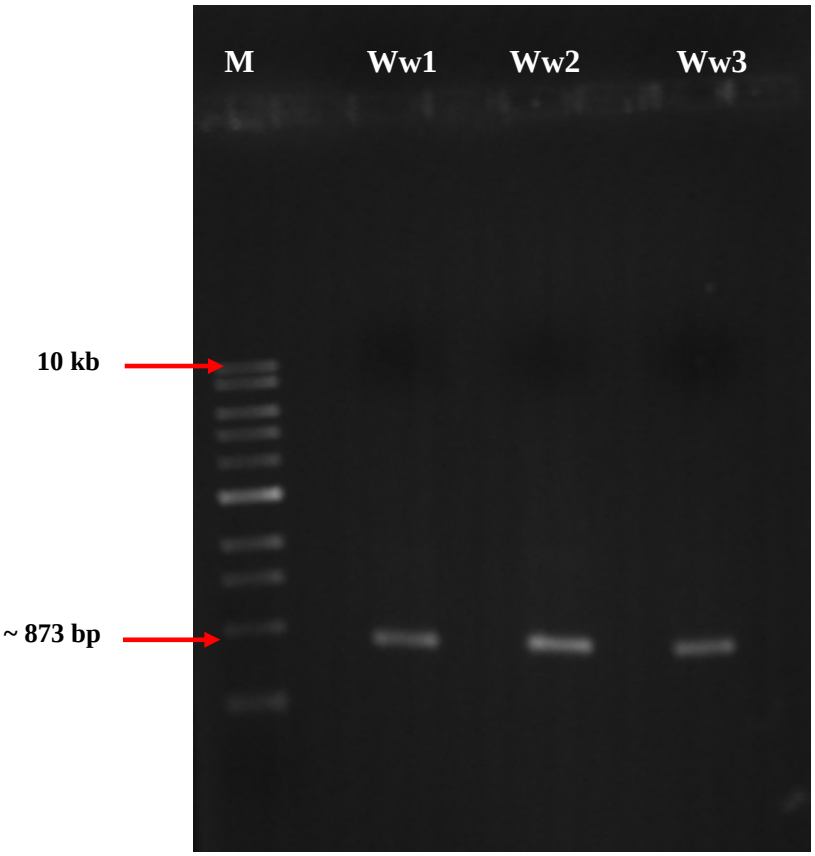


Figure 2.5 16S rDNA PCR product from the thermal waters of the Coamo springs using universal primers. Samples: waterway first survey (Ww1), waterway second survey (Ww2), and waterway third survey (Ww3).

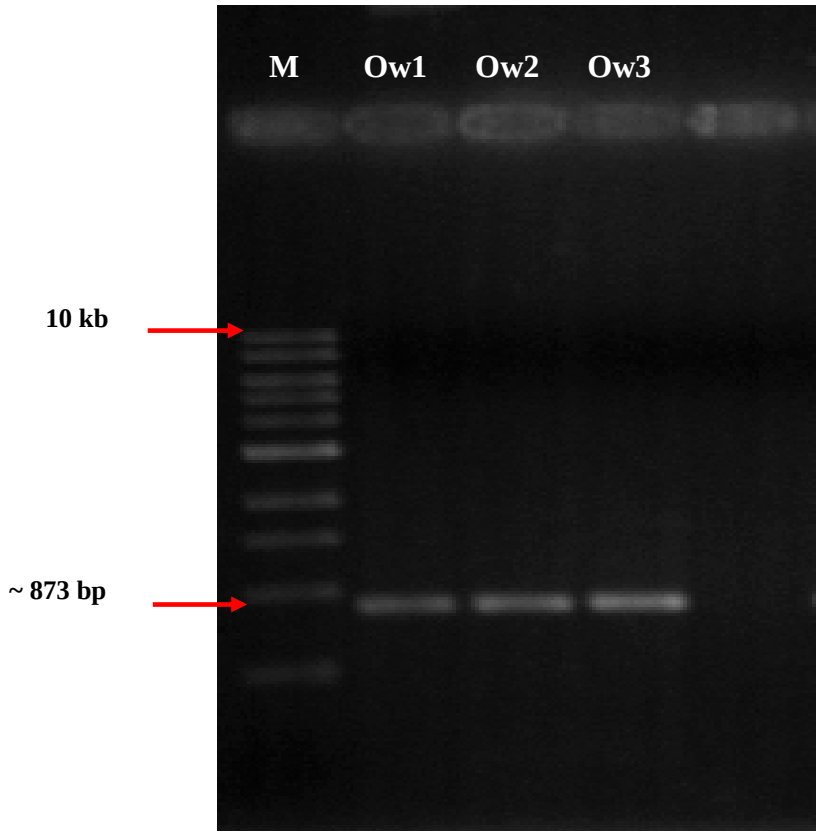


Figure 2.6 16S rDNA PCR product from the thermal waters of the Coamo springs using universal primers. Samples: outer watercourse first survey (Ow1), outer watercourse second survey (Ow2), and outer watercourse third survey (Ow3).

Generation of environmental 16SrDNA clone libraries from total genomic DNA population

Genomic libraries were constructed with the PCR purified products described above. A total of 132 clones were obtained. Colony PCR revealed that a total of 81 clones had inserts of the expected size. Amplicons with the expected size were submitted to RFLP analysis for classification into groups. Clones having different restriction patterns were classified as Operational Taxonomical Units (OTU's) and were selected for sequencing. Figure 2.7, 2.8 and 2.9 show some PCR products from clones from the mainstreams, waterway and outer watercourse genomic group library. The amplification product was approximately 1034 bp.

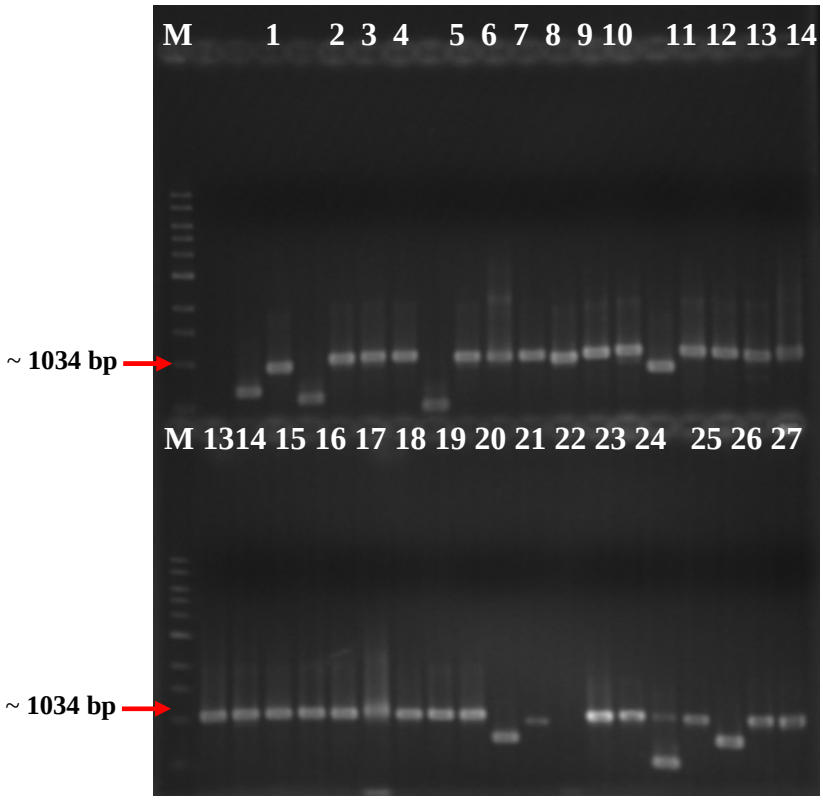


Figure 2.7 PCR product of colony PCR using SP6 and T7 Promoter primers of the total genomic DNA from the mainstream samples. Bands show a PCR product of 1034 bp.

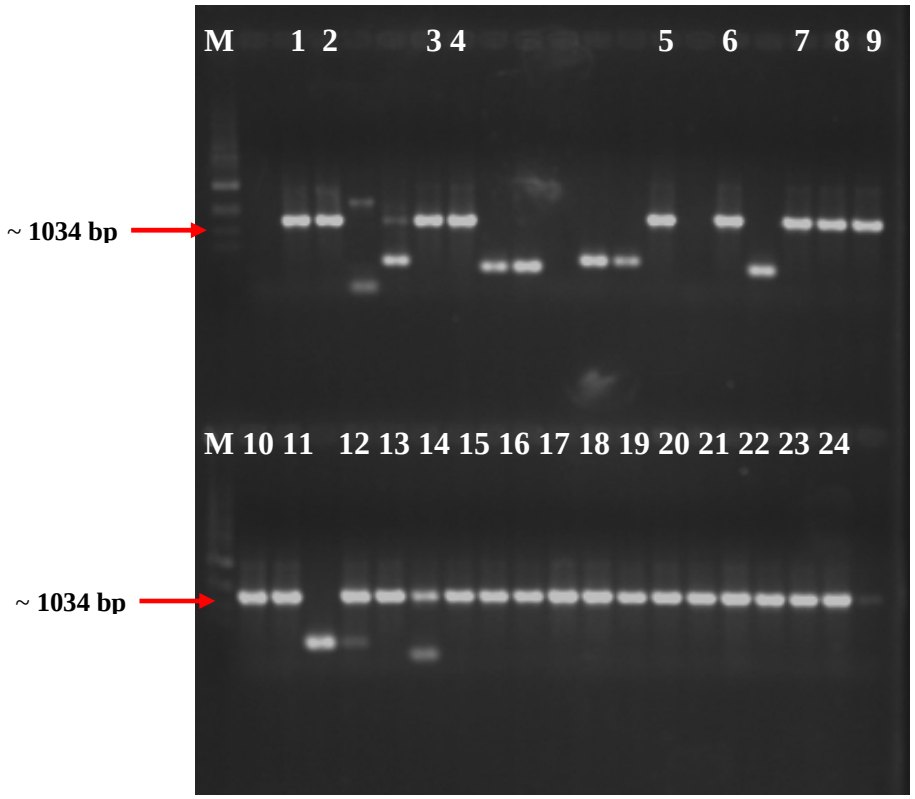


Figure 2.8 PCR product of colony PCR using SP6 and T7 Promoter primers of the total genomic DNA from the waterway samples. Bands show a PCR product of 1034 bp.

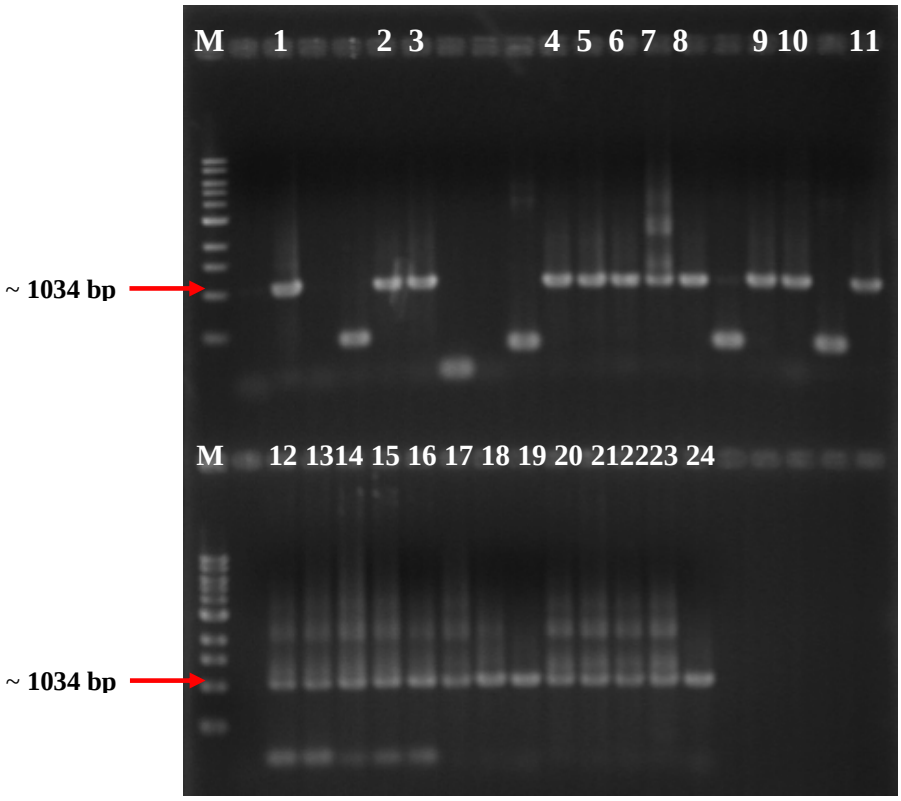


Figure 2.9 PCR product of colony PCR using SP6 and T7 Promoter primers of the total genomic DNA from the water outercourse samples. Bands show a PCR product of 1034 bp.

Figures 2.10, 2.11 and 2.12 show the screening of some clones from the three samplings sites using *Hae* III and *Msp* I restriction enzymes in 3% low melting agarose. A total of 0.5-0.7 μg of template was used for enzymatic digestion. The DNA markers were the100 bp and 50 bp ladders.

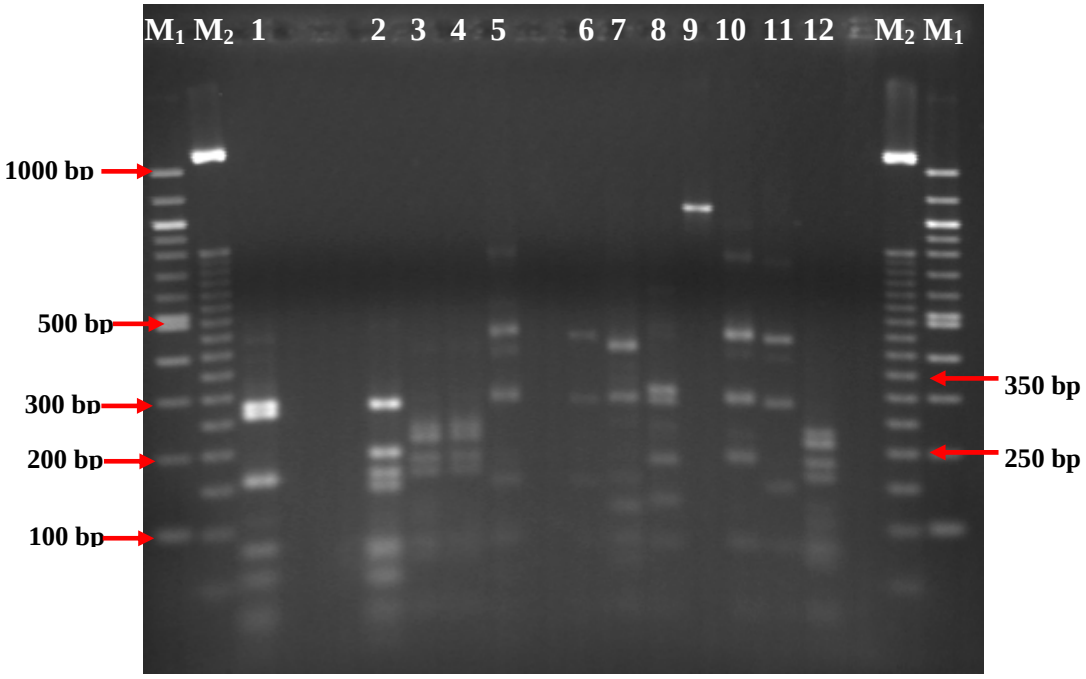


Figure 2.10 RFLP analysis of mainstream clones group using *Hae* III and *Msp* I restriction enzymes in 3% low melting agarose.

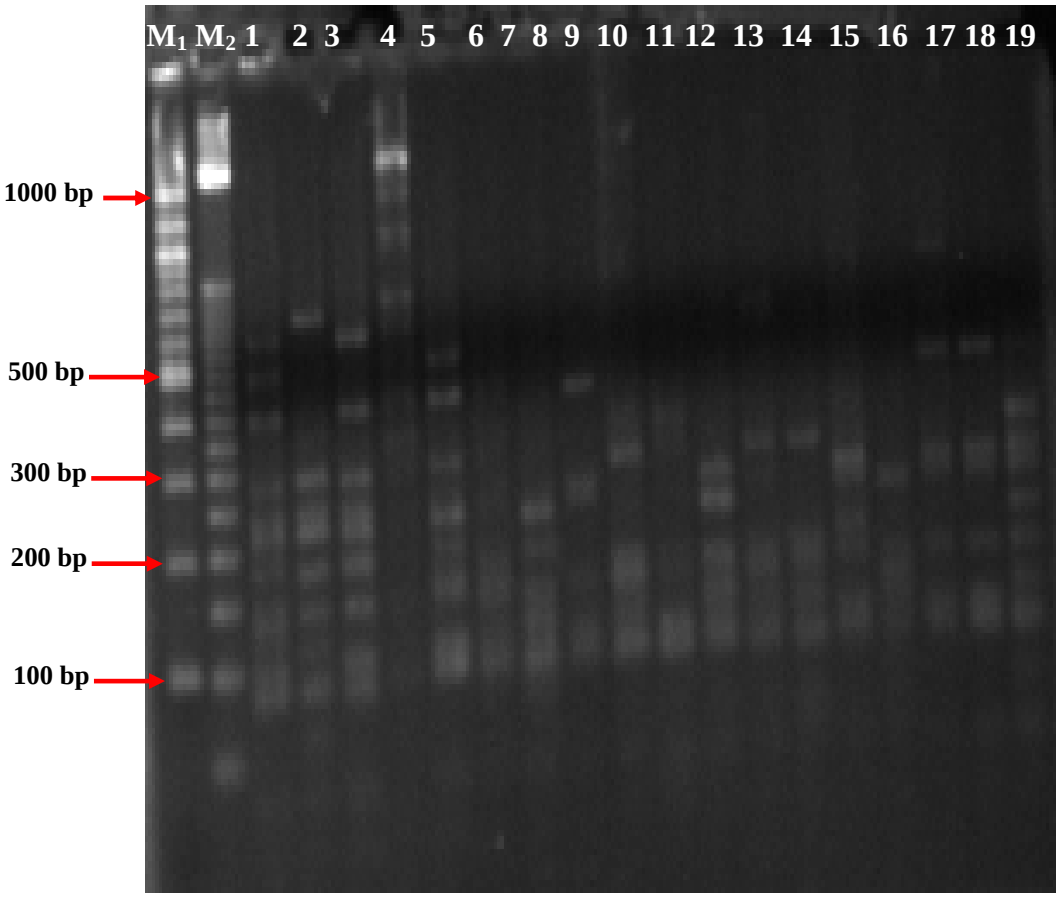


Figure 2.11 RFLP analysis of waterway clones group using *Hae* III and *Msp* I restriction enzymes in 3% low melting agarose.

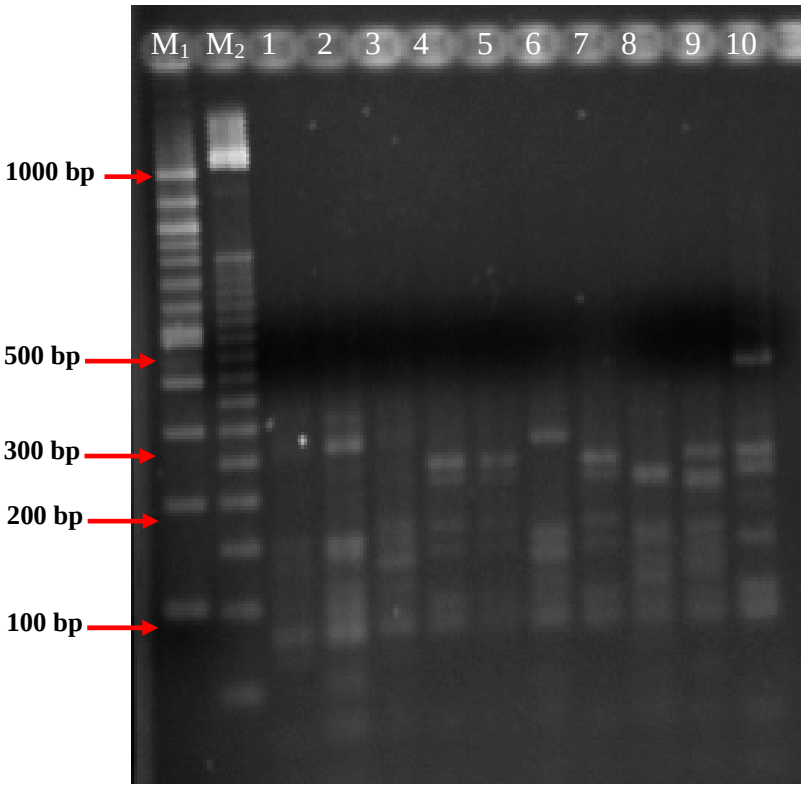


Figure 2.12 RFLP analysis of outer watercourse clones group using *Hae* III and *Msp* I restriction enzymes in 3% low melting agarose.

Restriction analysis showed a low diversity of OTU's among all the clone libraries constructed. Similar restriction patterns were observed and only a few OTUs showed different banding patterns. *Hae* III, and *Msp* I restriction enzymes were an efficient way to screen among different OTU's.

A total of 81 OTUs (F1-F32, C1-C24, and S1-S25) were selected after sequence comparison using *in silico* analysis. Sequences having a 97% similarity were considered the same OTU's. selected. OTU's accession numbers are listed in Table 2.1. The closest relatives of representative OTUs, identified by searching in the GenBank database, are given in Table 2.2.

Table 2.1 GenBank accession numbers for the environmental 16S rDNA clone libraries from Coamo, Puerto Rico

OTU	Accession No.
F1	EF660467
F2	EF660468
F3	EF660469
F4	EF660470
F5	EF660471
F6	EF660472
F7	EF660473
F8	EF660474
F9	EF660475
F10	EF660476
F11	EF660477
F12	EF660478
F13	EF660479
F14	EF660480
F15	EF660481
F16	EF660482
F17	EF660483

Table 2.1 Continuation

OTU	Accession No.
F18	EF660484
F19	EF660485
F20	EF660486
F21	EF660487
F22	EF660488
F23	EF660489
F24	EF660490
F25	EF660491
F26	EF660492
F27	EF660493
F28	EF660494
F29	EF660495
F30	EF660496
F31	EF660497
F32	EF660498
C1	EF584771
C2	EF584772

Table 2.1 Continuation

OTU	Accession No.
C3	EF584773
C4	EF584774
C5	EF584775
C6	EF584776
C7	EF584777
C8	EF584778
C9	EF584779
C10	EF584780
C11	EF584781
C12	EF584782
C13	EF584783
C14	EF584784
C15	EF584785
C16	EF584786
C17	EF584787
C18	EF584788
C19	EF584789

Table 2.1 Continuation

OTU	Accession No.
C20	EF584790
C21	EF584791
C22	EF584792
C23	EF584793
C24	EF584794
S1	EF584795
S2	EF584796
S3	EF584797
S4	EF584798
S5	EF584799
S6	EF584800
S7	EF584801
S8	EF584802
S9	EF584803
S10	EF584804
S11	EF584805
S12	EF584806
S13	EF584807
S14	EF584808

Table 2.1 Continutaion

OTU	Accession No.
S15	EF584809
S16	EF584810
S17	EF584811
S18	EF584812
S19	EF584813
S20	EF584814
S21	EF584815
S22	EF584816
S23	EF584817
S24	EF584818
S25	EF584819

Table 2.2. 16S rDNA sequences identified from the thermal springs, clone libraries from Coamo, Puerto Rico

OTU	Accession No.	Closest relative	Accession No.	% Identity
F1	EF660467	*Hydrgenophaga bisanensis*	EF532793	97
F2	EF660468	*Citrobacter* sp.	AF530068	94
F3	EF660469	Uncultured bacterium clone	DQ980877	93
F4	EF660470	Uncultured bacterium clone	EF584792	98
F5	EF660471	Uncultured β-proteobacterium	AB113609	98
F6	EF660472	Uncultured bacterium clone	EF584788	98
F7	EF660473	*Pseudomonas* sp.	AJ007005	99
F8	EF660474	Uncultured bacterium clone	DQ302443	99
F9	EF660475	*Hydrogenophaga* sp.	EF179863	98
F10	EF660476	Uncultured bacterium clone	EF584777	99
F11	EF660477	Uncultured bacterium clone	EF584777	99
F12	EF660478	Uncultured candidate bacterium	EF032775	93
F13	EF660479	Uncultured β-proteobacterium	DQ230946	99
F14	EF660480	*Microleus* sp.	AY768403	98
F15	EF660481	Uncultured β-proteobacterium	DQ230943	99
F16	EF660482	Uncultured bacterium clone	EF584792	100
F17	EF660483	Uncultured bacterium clone	EF584783	99
F18	EF660484	Uncultured bacterium clone	EF584783	100
F19	EF660485	Uncultured β-proteobacterium	DQ839333	95
F20	EF660486	Uncultured bacterium clone	EF017757	93

Table 2.2. Continuation

OTU	Accession No.	Closest relative	Accession No.	% Identity
F21	EF660487	Uncultured bacterium clone	EF584792	99
F22	EF660488	Uncultured bacterium clone	AB186827	99
F23	EF660489	Uncultured bacterium clone	AB186827	99
F24	EF660490	Uncultured bacterium clone	EF584777	99
F25	EF660491	Uncultured bacterium clone	AJ306778	98
F26	EF660492	Uncultured β-proteobacterium	DQ230943	99
F27	EF660493	Uncultured bacterium clone	DQ256357	100
F28	EF660494	*Microleus* sp.	AY768403	98
F29	EF660495	Uncultured bacterium clone	DQ302443	99
F30	EF660496	Uncultured bacterium clone	DQ202200	99
F31	EF660497	Uncultured β-proteobacterium	DQ230946	99
F32	EF660498	Uncultured bacterium clone	DQ302443	99
C1	EF584771	Uncultured *Comamonas* sp.	DQ2340	77
C2	EF584772	Uncultured β-proteobacterium	DQ230943	99
C3	EF584773	Uncultured β-proteobacterium	AB113609	98
C4	EF584774	Uncultured β-proteobacterium	AB113609	98
C5	EF584775	Uncultured soil bacterium	AF507682	86
C6	EF584776	Uncultured β-proteobacterium	AB113609	98
C7	EF584777	Bacterium strain 82348	AF227863	98
C8	EF584778	Uncultured soil bacterium	AF507682	86

Table 2.2. Continuation

OTU	Accession No.	Closest relative	Accession No.	% Identity
C9	EF584779	Uncultured cyanobacterium	DQ514215	93
C10	EF584780	Uncultured β-proteobacterium	DQ230943	99
C11	EF584781	Bacterium strain 82348	AF227863	98
C12	EF584782	Uncultured bacterium clone	DQ227863	99
C13	EF584783	Uncultured β-proteobacterium	DQ230943	99
C14	EF584784	Uncultured β-proteobacterium	AB113609	98
C15	EF584785	Uncultured bacterium clone	DQ988325	98
C16	EF584786	Uncultured soil bacterium	AF507682	86
C17	EF584787	Bacterium strain 82348	AF227863	98
C18	EF584788	Bacterium strain 82348	AF227863	98
C19	EF584789	Bacterium strain 82348	AF227863	98
C20	EF584790	Bacterium SRMC-277	DQ104947	93
C21	EF584791	Bacterium strain 82348	AF227863	98
C22	EF584792	Bacterium strain 82348	AF227863	98
C23	EF584793	Uncultured β-proteobacterium	AB113609	98
C24	EF584794	Uncultured bacterium clone	DQ337072	98
S1	EF584795	Uncultured bacterium clone	EF121342	99
S2	EF584796	*Pantoea* sp.	AM491458	100
S3	EF584797	*Pantoea* sp.	AM491458	99
S4	EF584798	Bacterium CCBAU	DQ988944	99

Table 2.2 Continuation

OTU	Accession No.	Closest relative	Accession No.	% Identity
S5	EF584799	Bacterium CCBAU	DQ988944	99
S6	EF584800	Bacterium CCBAU	DQ988944	99
S7	EF584801	Uncultured bacterium clone	EF153297	100
S8	EF584802	Uncultured bacterium clone	EF375730	77
S9	EF584803	Uncultured bacterium clone	DQ817705	99
S10	EF584804	*Enterobacter* sp.	EF489448	96
S11	EF584805	*Enterobacter* sp.	EF489448	96
S12	EF584806	Uncultured bacterium clone	EF153297	100
S13	EF584807	*Enterobacter* sp.	AY946283	99
S14	EF584808	*Pantoea* sp.	AM491458	99
S15	EF584809	*Pantoea* sp.	AM491458	99
S16	EF584810	*Pantoea* sp.	AM491458	99
S17	EF584811	*Pantoea* sp.	AM491458	99
S18	EF584812	*Pantoea* sp.	AM491458	99
S19	EF584813	*Pantoea* sp.	AM491458	99
S20	EF584814	Uncultured bacterium clone	EF205552	99
S21	EF584815	Uncultured bacterium clone	DQ817705	99
S22	EF584816	Uncultured bacterium clone	EF121342	99
S23	EF584817	Uncultured bacterium clone	EF121342	99
S24	EF584818	Uncultured bacterium clone	EF121342	99
S25	EF584819	Uncultured bacterium clone	EF121342	99

T-RFLP with different restriction enzymes

Total community 16S rDNA was also amplified using the labeled primer pair 519F/ FAM and 1392R (Figure 2.13) for T-RFLP analysis. This technique is able to study community patterns and allows comparison between samples to determine possible changes in structure. T-RFLP fingerprinting was applied using three different restriction enzymes. PCR amplicons were digested separately with *Hae* III, *Msp* I, and *Rsa* I (Figure 2.14, 2.15, and 2.16) and the community T-RFLP patterns were analyzed in a gel based DNA sequencer.

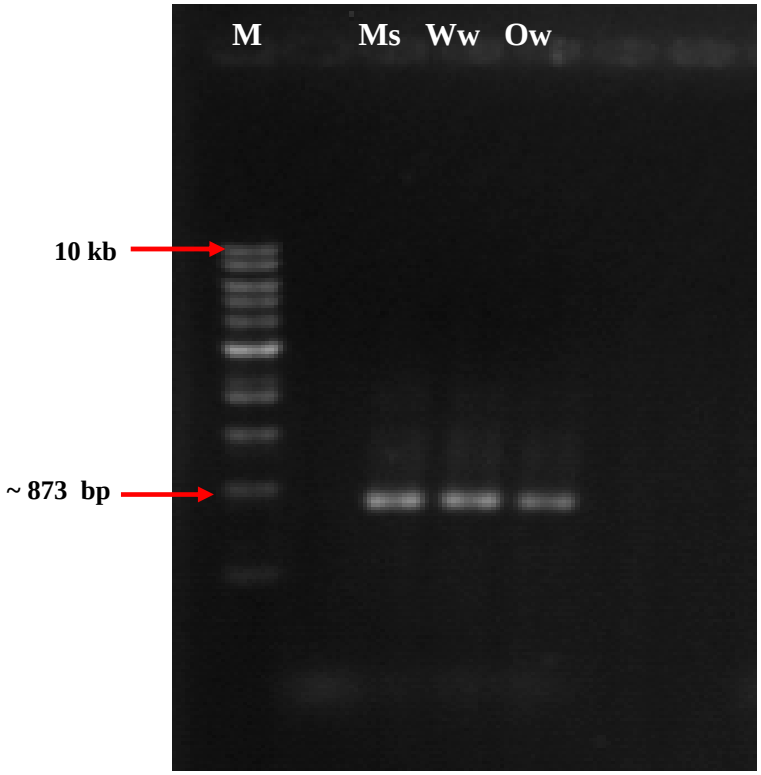


Figure 2.13 16S rDNA PCR product from the thermal waters of the Coamo springs using universal pair labeled 519F/ FAM and 1392R. Samples: mainstream (Ms), waterway (Ww), and outer watercourse (Ow).

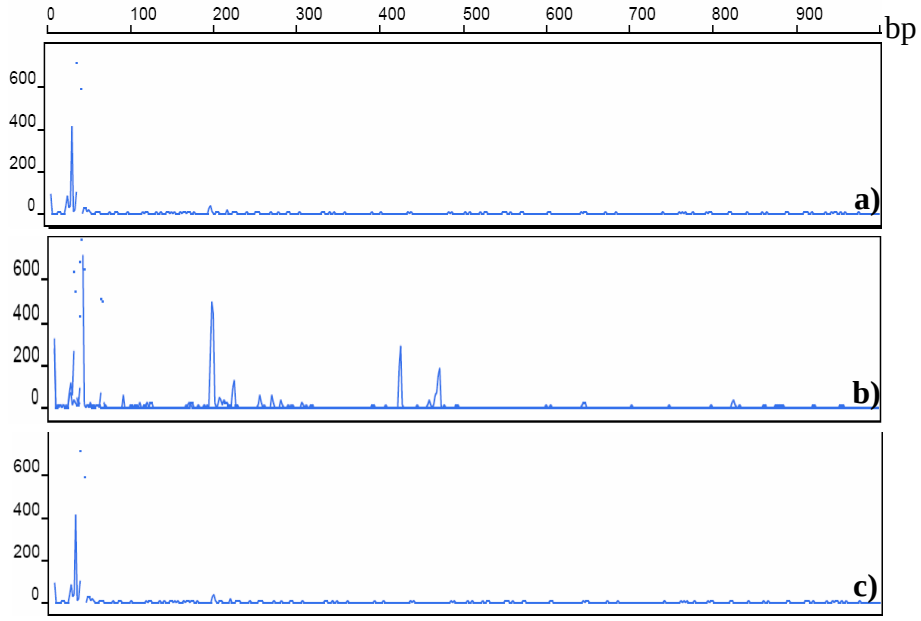


Figure 2.14 T-RFLP patterns of 16S rDNAs from Coamo springs digested with *Hae* III restriction enzyme. Samples: Main stream (a), waterway (b), and outer watercourse (c).

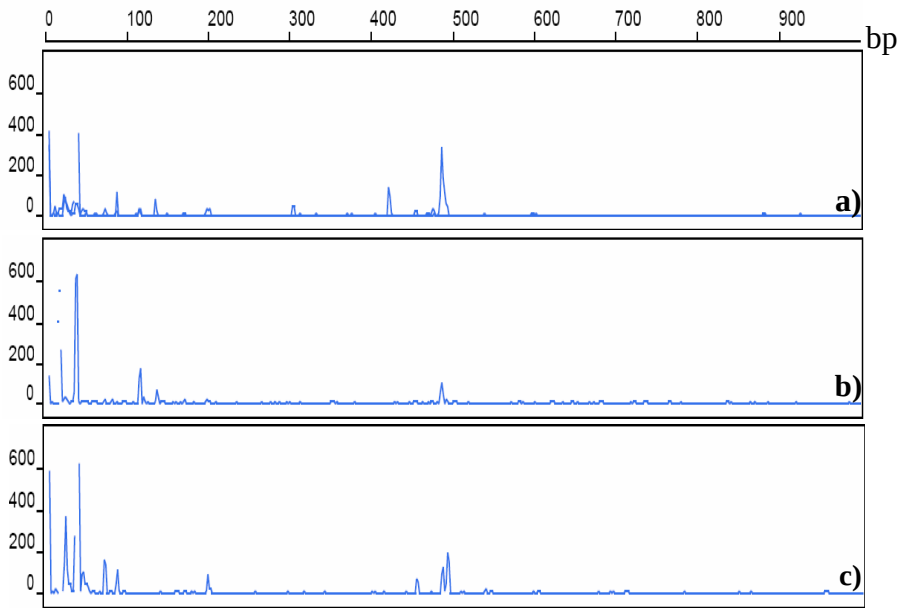


Figure 2.15 T-RFLP patterns of 16S rDNAs from Coamo springs digested with *Msp* I restriction enzyme. Samples: Main stream (a), waterway (b), and outer watercourse (c).

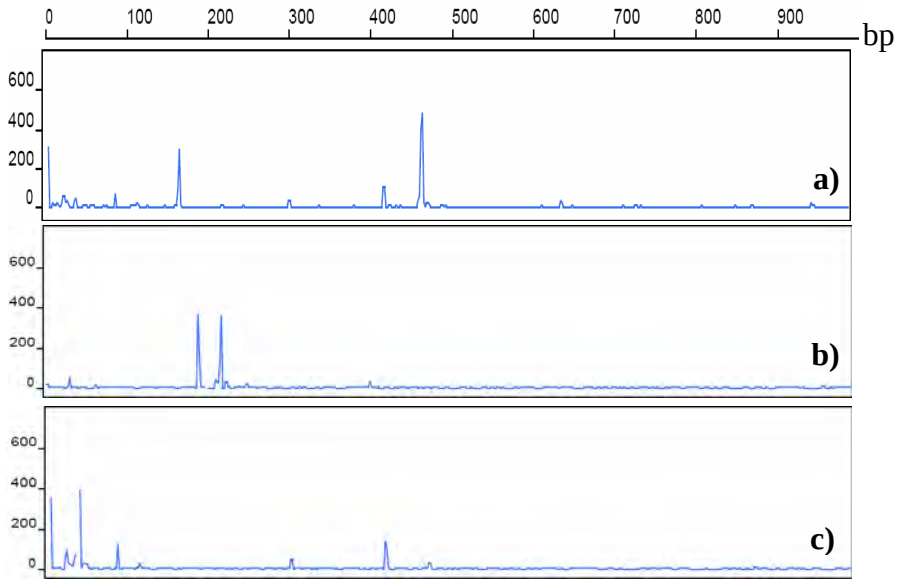


Figure 2.16 T-RFLP patterns of 16S rDNAs from Coamo springs digested with *Rsa* I restriction enzyme. Samples: Main stream (a), waterway (b), and outer watercourse (c).

Among the restriction endonucleases tested, *Msp* I generated the most T-RFs. The majority of the *Msp* I digested T-RFs had a size of less than 500 bp (Figure 2.15). *Hae* III also generated a large numbers of T-RFs with a broad size range of up to 900 bp (Figure 2.14). *Rsa* I generated somewhat smaller numbers of T-RFs than the other enzymes used, and most of the *Rsa* I- digested fragments had a size between 400 to 500 bp (Figure 2.16).

The T-RFLP patterns generated with each enzyme revealed a very low prokaryotic diversity present at the thermal waters of Coamo. Analysis of T-RFs patterns with *Msp* I indicated that there are some community changes among sampling sites and also gave different profiles for all samples in comparison with the digestion profiles of the other enzymes. The restriction profile for enzyme *Rsa* I revealed shared prokaryotic

populations among all sampling sites. The restriction analysis for enzyme *Hae* III also showed similarities among community samples.

Phylogenetic analysis

The phylogenetic relationship among a total of 81 clones was analyzed with the PHYLIP program package (version 3.5.1) using neighbor joining analysis. An analysis for the possibility of chimeric sequences was performed. This analysis indicated that no chimeric sequences were present in these clones except for OTU C25 from the waterway group. Therefore it was removed from the analysis.

Phylogenetic analysis using the neighbor joining algorithm revealed that several clones from the mainstream site represented the same operational taxonomic unit. The 32 clones analyzed from this site of the spring were clustered in 15 different OTUs. The OTUs obtained from this site of the spring were similar to the clones obtained from the waterway site (Figures 2.17-2.31). The 24 clones analyzed from the waterway site of the spring were clustered in 11 different OTU's. Clones C7, C11, C17, C18, C19, C21, and C22 corresponded to OTU one. This cluster is closely related to *Vogesella indigofera* (Figure 2.32). The second OTU is represented by C8 and C16 and formed a cluster in an independent branch near the branch of *Alishewanella fetalis* **(**Figure 2.33). C5 represents the third OTU and it is near the cluster of the uncultured bacterium SC-NB03 (Figure 2.34). C12 was closely related to an uncultured bacterium, this clone corresponded to OTU four (Figure 2.35). The clone C14, C23, C6, C4, and C3 represent OTU five and are related to the branch of *Hydrogenophaga flava* (Figure 2.36). In this cluster C14 and C23 formed a cluster in and independent branch. C15 corresponded to OTU six forming

an independent branch of the cluster of uncultured bacterium (Figure 2.37). The seventh OTU is represented by C20 and is clustered with bacterium SRMC-27-7 (Figure 2.38). OTU C1 formed an independent branch within the cluster of *Comamonas testosteroni* (Figure 2.39). OTU nine corresponded to C2, C10, and C13. These are clustered in an independent branch, within the branch of *Vogesella indigofera* and *Chromobacterium violaceum* (Figure 2.40). The tenth OTU is represented by C24 that is clustered with an uncultured soil bacterium (Figure 2.41). OTU eleven, C9, is closely related to an uncultured *Arthrospira* sp. clone (Figure 2.42).

The clones obtained for the group of outer watercourse were represented in six OTUs. The first OTU was represented by clones S1, S4, S5, S6, S7, S12, S15, S16, S17, S18, S19, S23, S24, and S25, forming an independent cluster. (Figure 2.43). The second OTU, S14, was related to *Averyella dalhousiensis* (Figure 2.44). The clones S10 and S11 represent the third OTU. These OTUs formed an independent branch within the branch of uncultured bacterium DQ011253 and *Pantoea aglomerans* (Figure 2.45). OTU four is represented by S8, forming an independent branch within the clustered proteobacteria (Figure 2.46). Figures 2.47 and 2.48 demonstrated how OTUs five and six are related to an uncultured *Pantoea* sp.

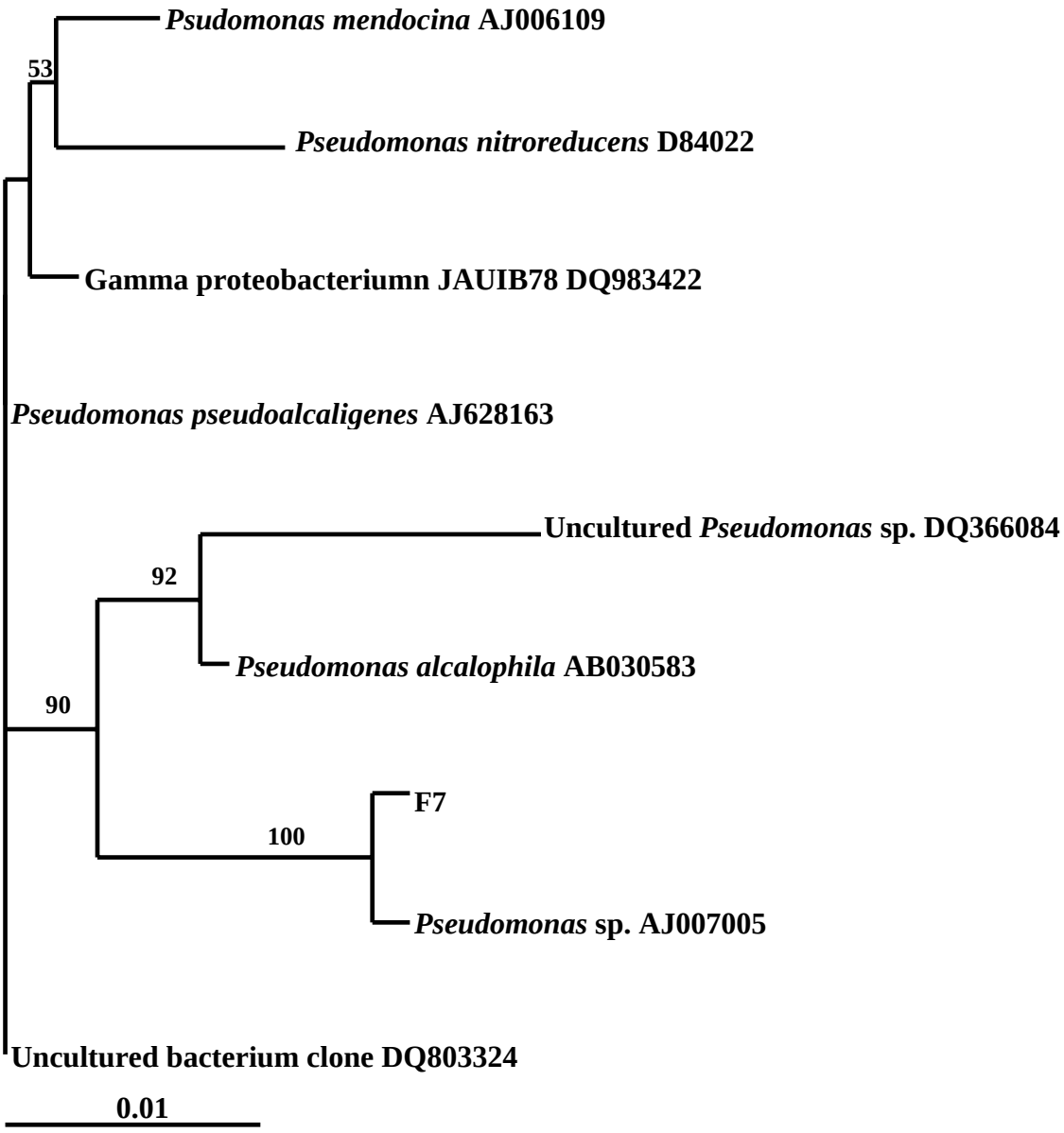


Figure 2.17. Neighbor-joining distance tree of partial 16SrDNA sequences for OTU F7, from the mainstream site, at the Coamo springs. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

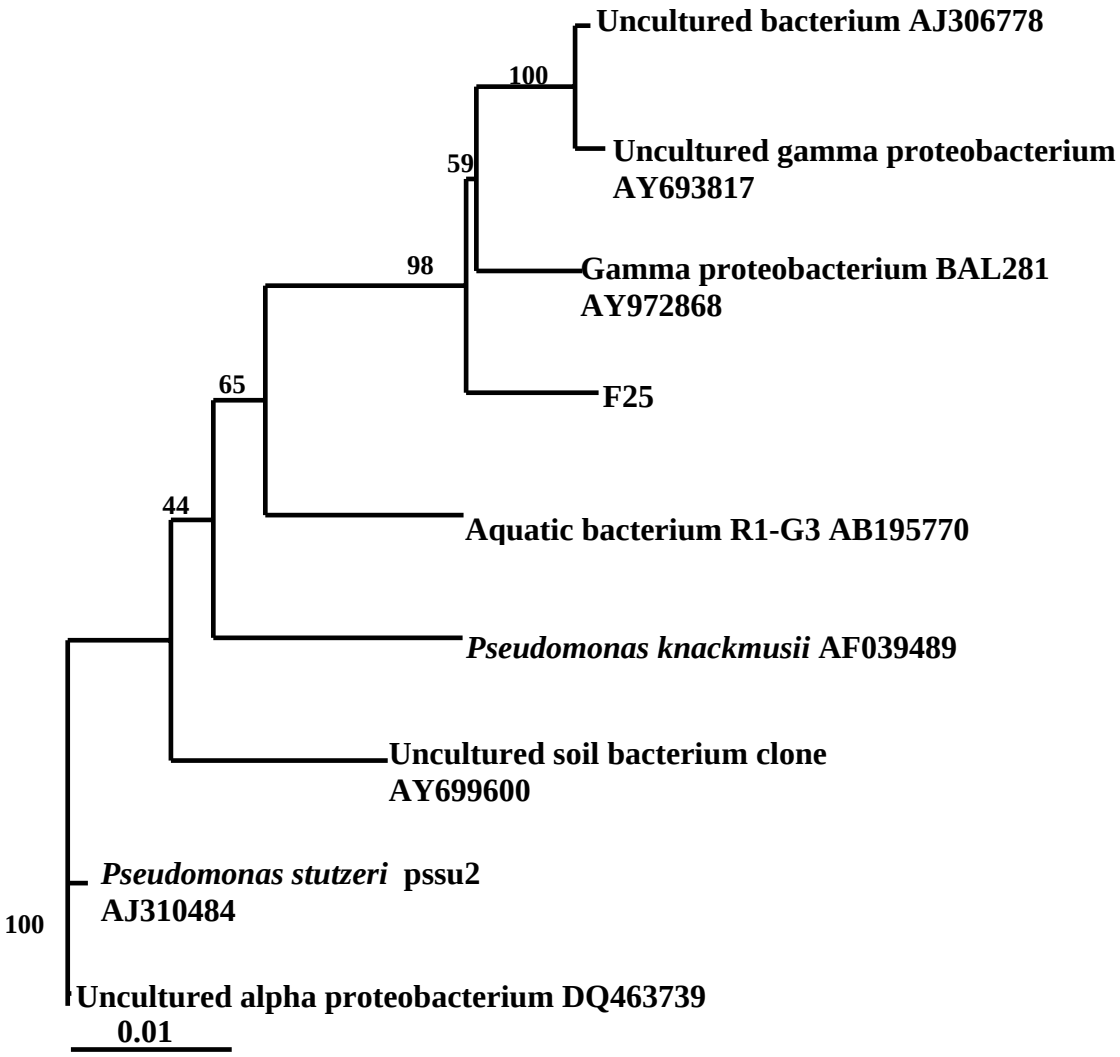


Figure 2.18 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU F25, from the mainstream site, at the Coamo springs. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

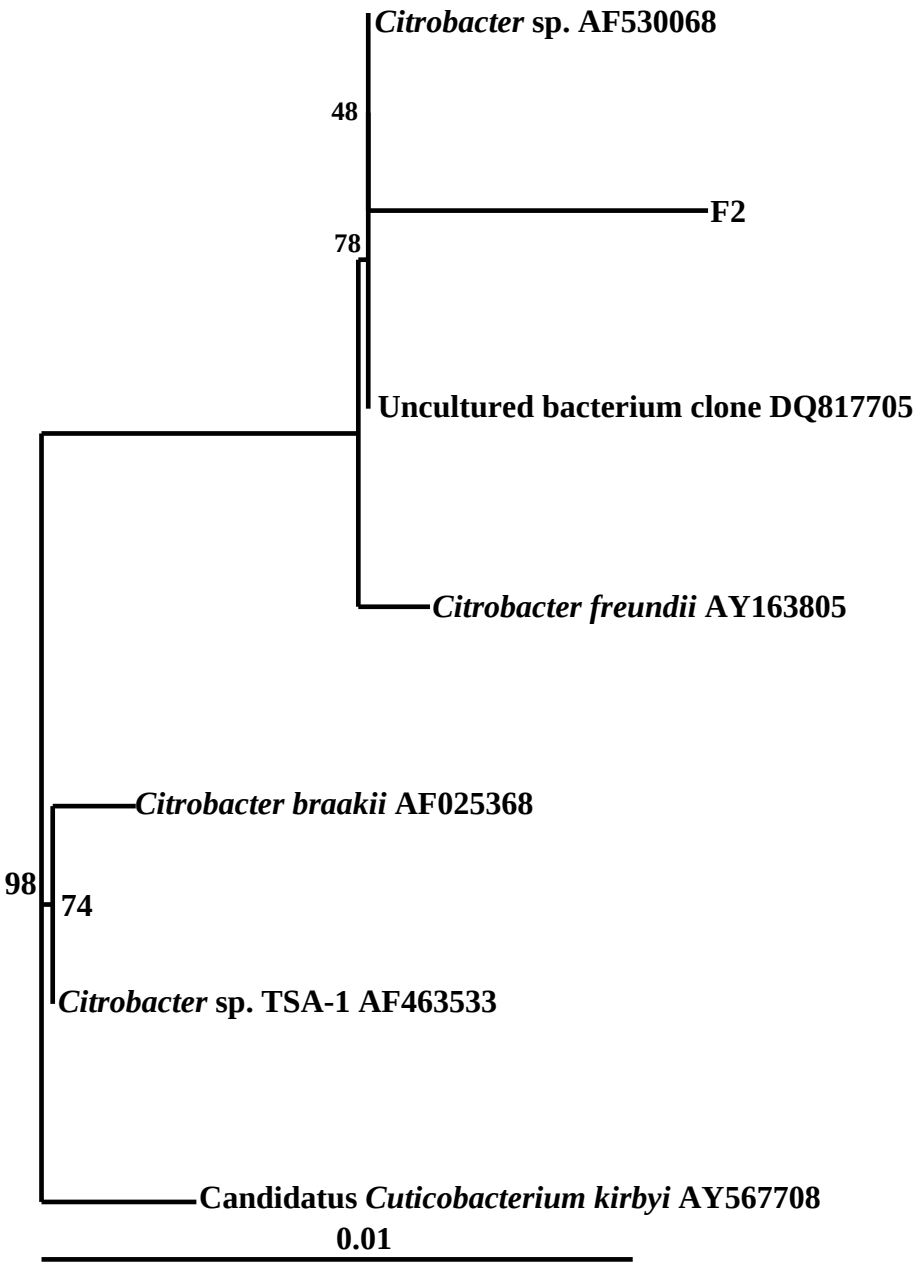


Figure 2.19 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU F2, from the mainstream site, at the Coamo springs. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

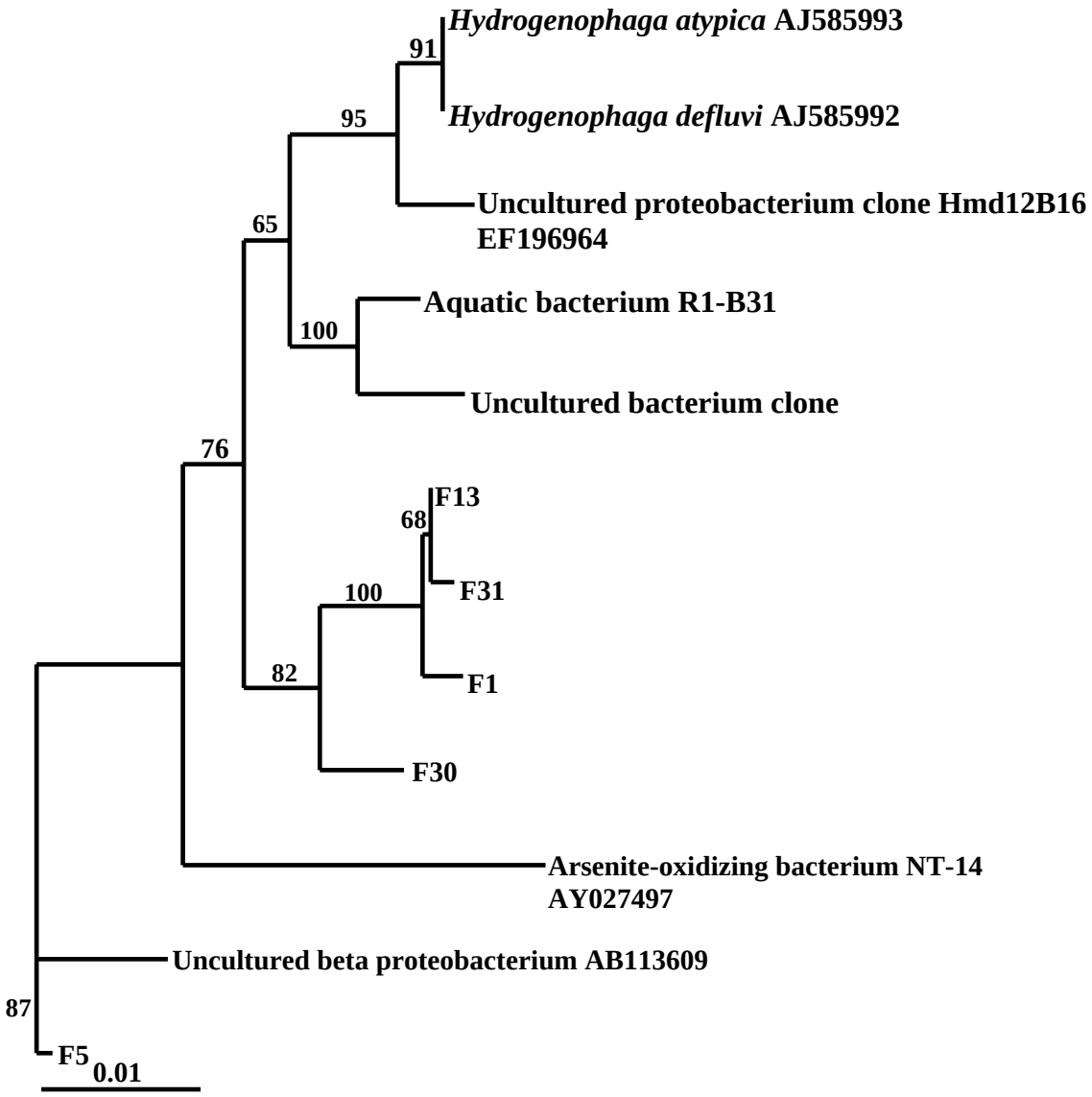


Figure 2.20 Neighbor-joining distance tree of partial 16SrDNA sequences for OTUs F13, F31, F1, F30, and F5 from the mainstream site, at the Coamo springs. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

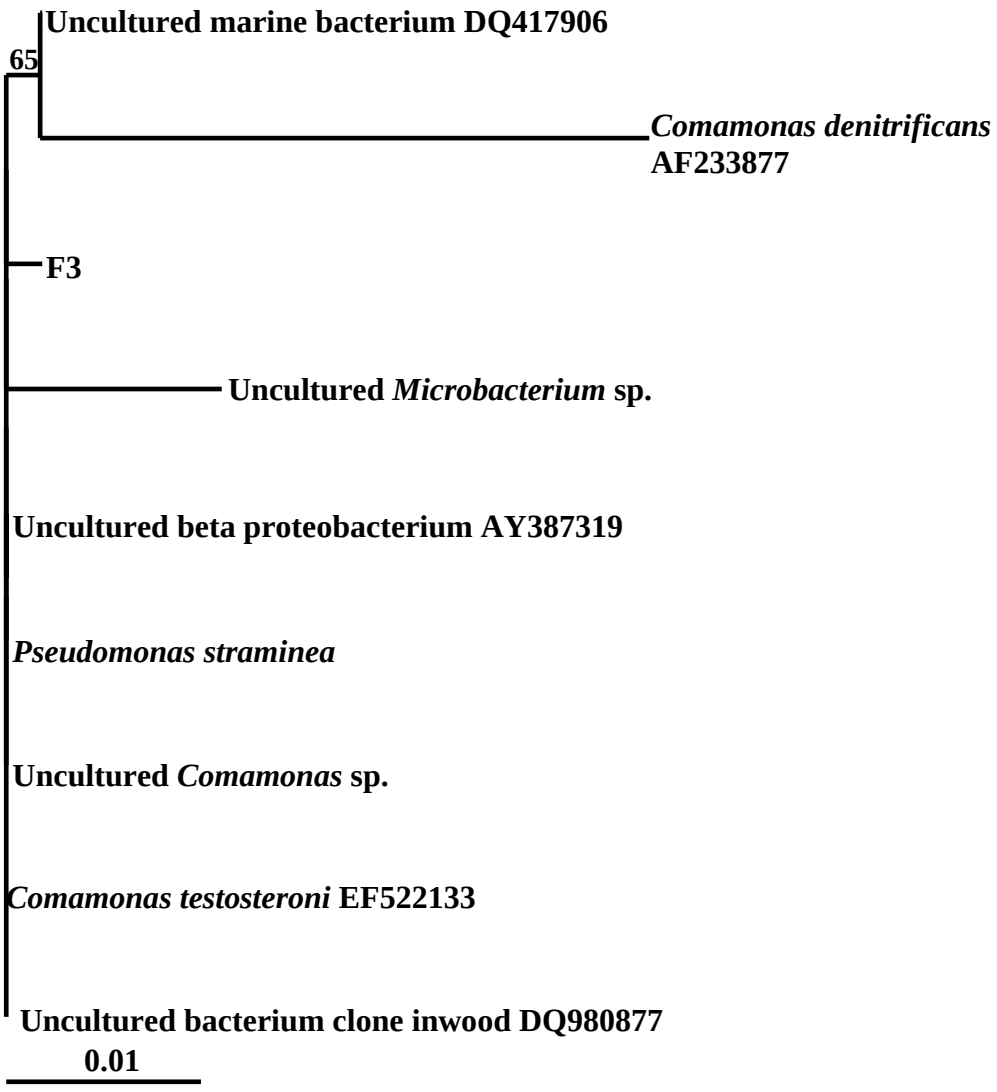


Figure 2.21 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU F3 from the mainstream site, at the Coamo springs. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

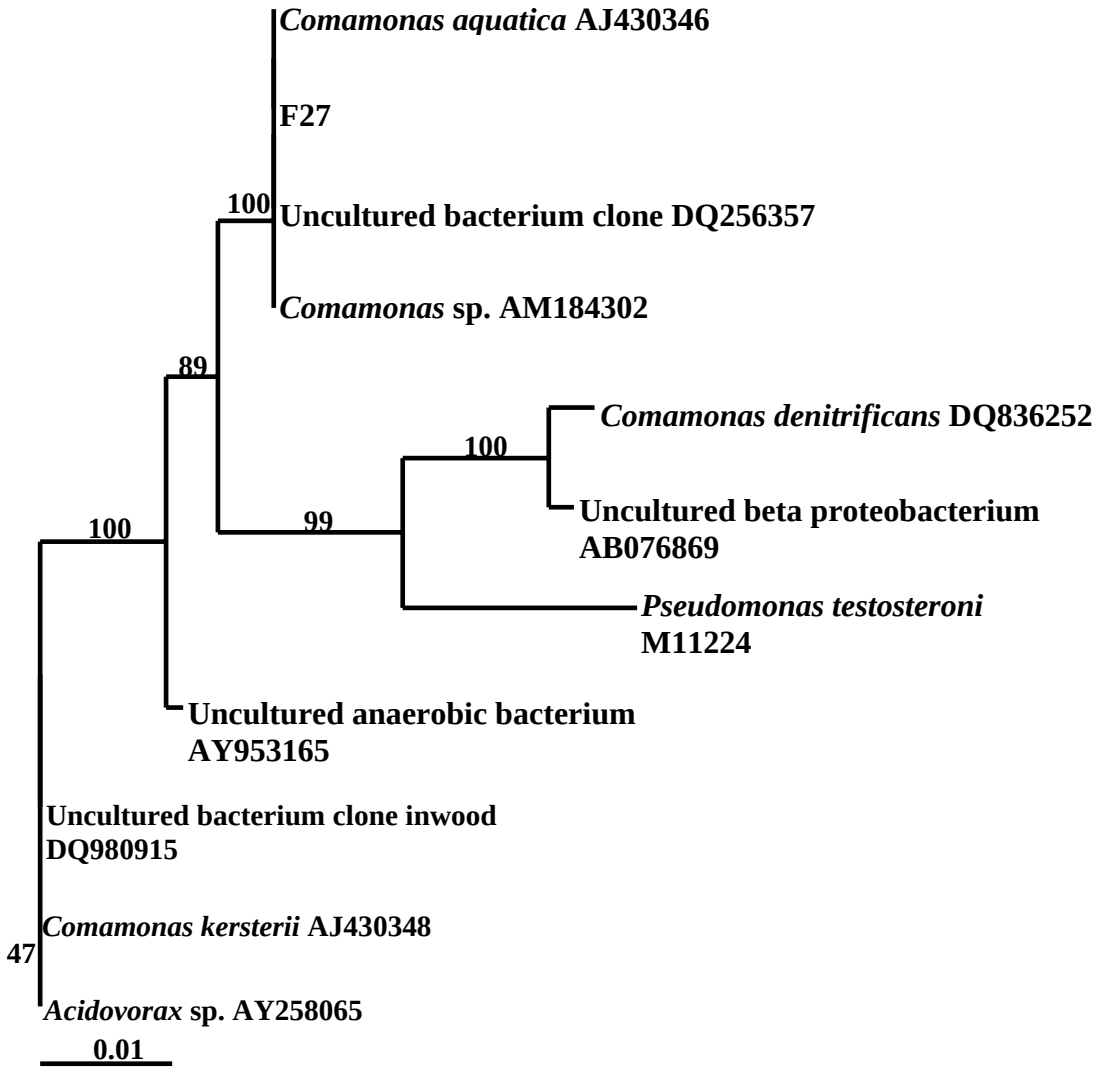


Figure 2.22 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU F27 from the mainstream site, at the Coamo springs. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

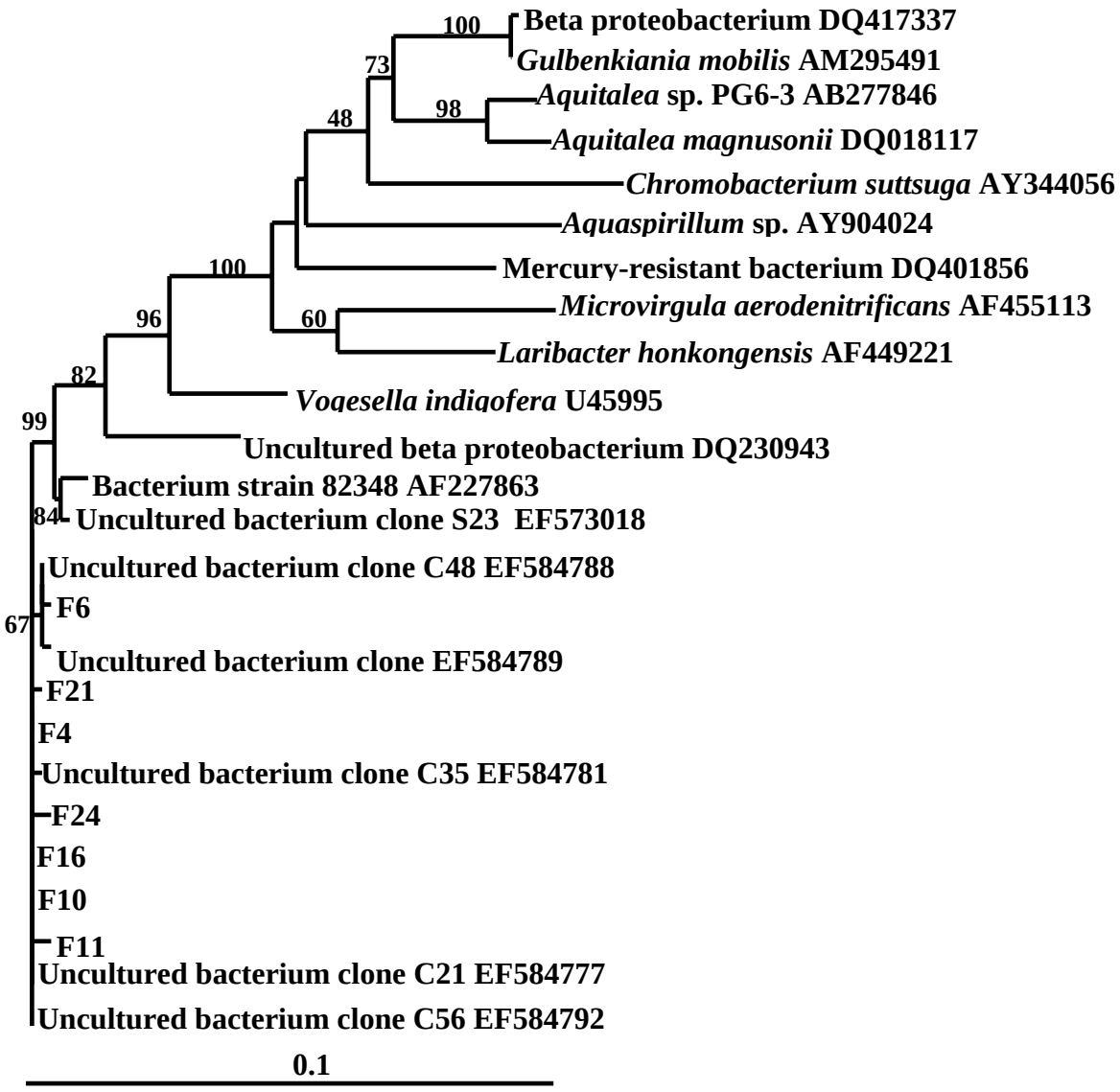


Figure 2.23 Neighbor-joining distance tree of partial 16SrDNA sequences for OTUs F6, F21, F4, F24, F16, F10, and F11 from the mainstream site, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

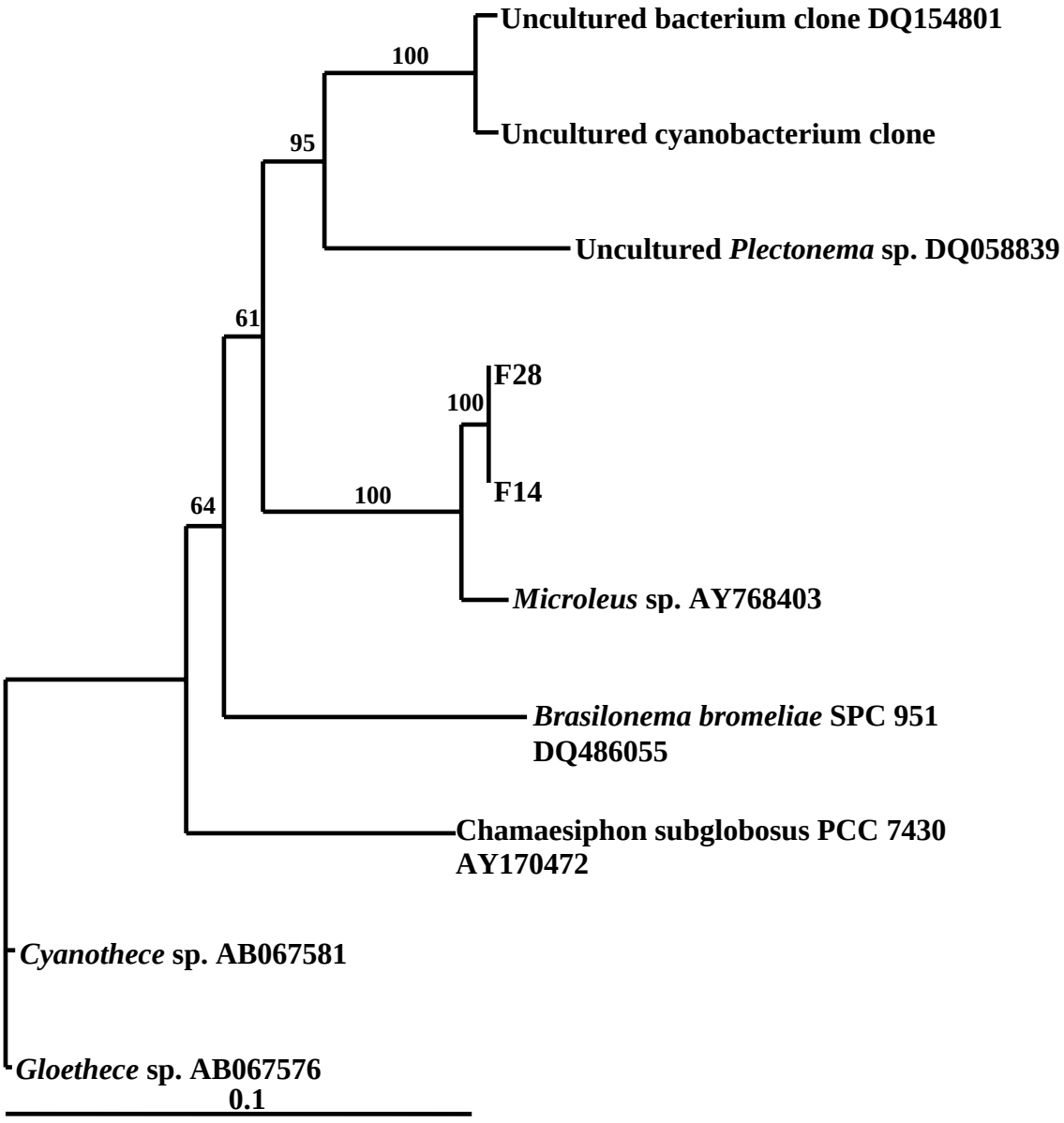


Figure 2.24 Neighbor-joining distance tree of partial 16SrDNA sequences for OTUs F28, and F14 from the mainstream site, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

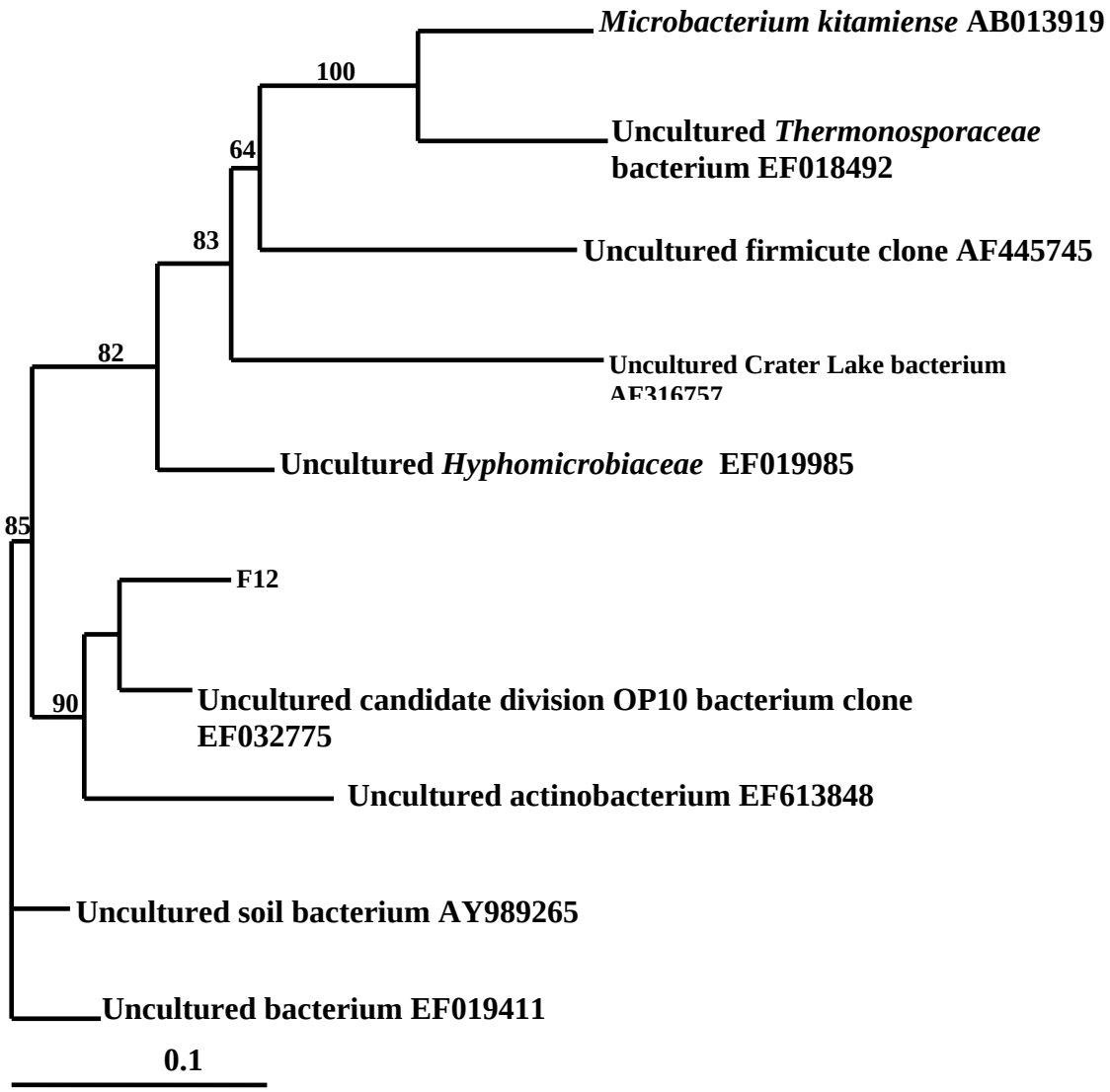


Figure 2.25 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU F12 from the mainstream site, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

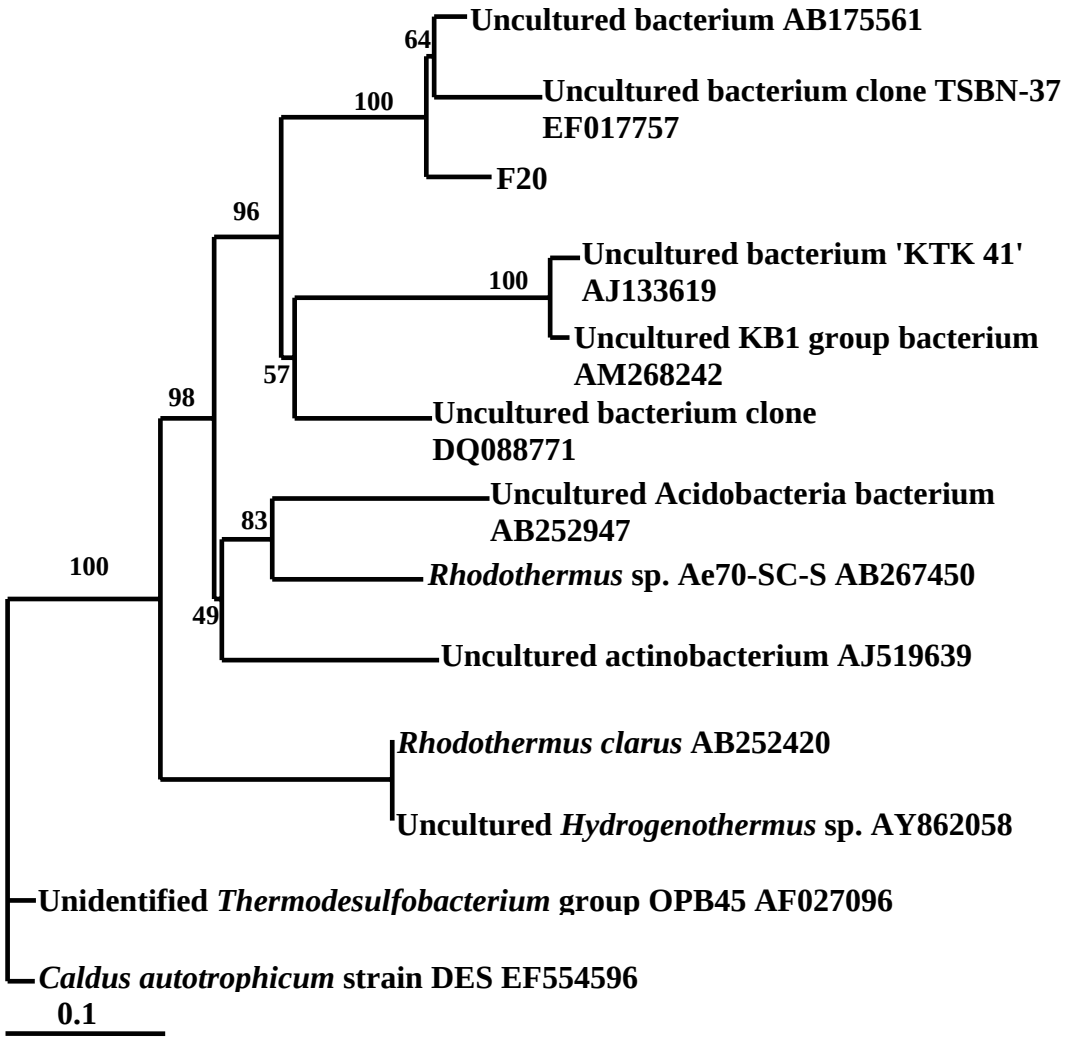


Figure 2.26 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU F20 from the mainstream site, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

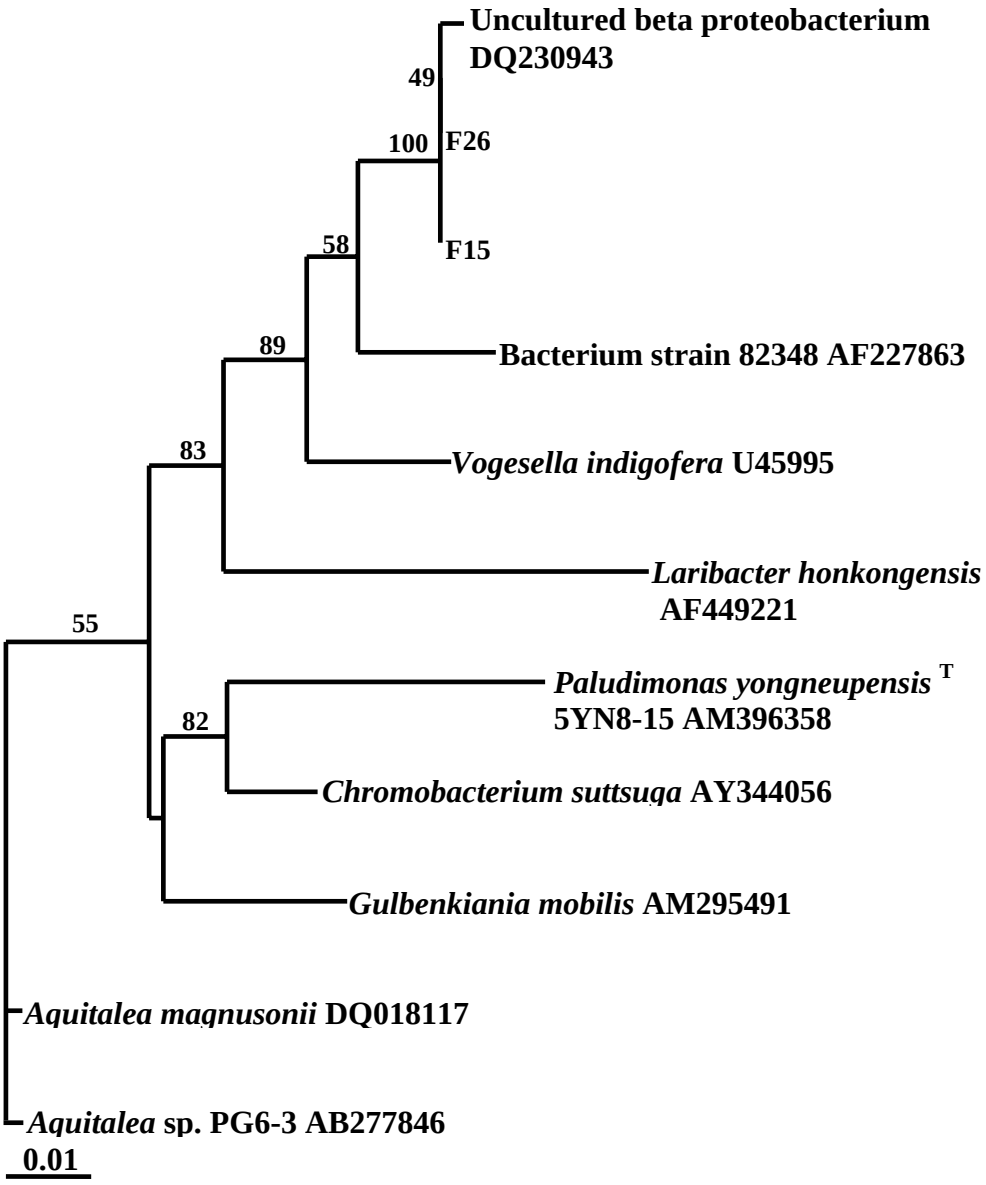


Figure 2.27 Neighbor-joining distance tree of partial 16SrDNA sequences for OTUs F26, and F15 from the mainstream site, at the Coamo springs. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

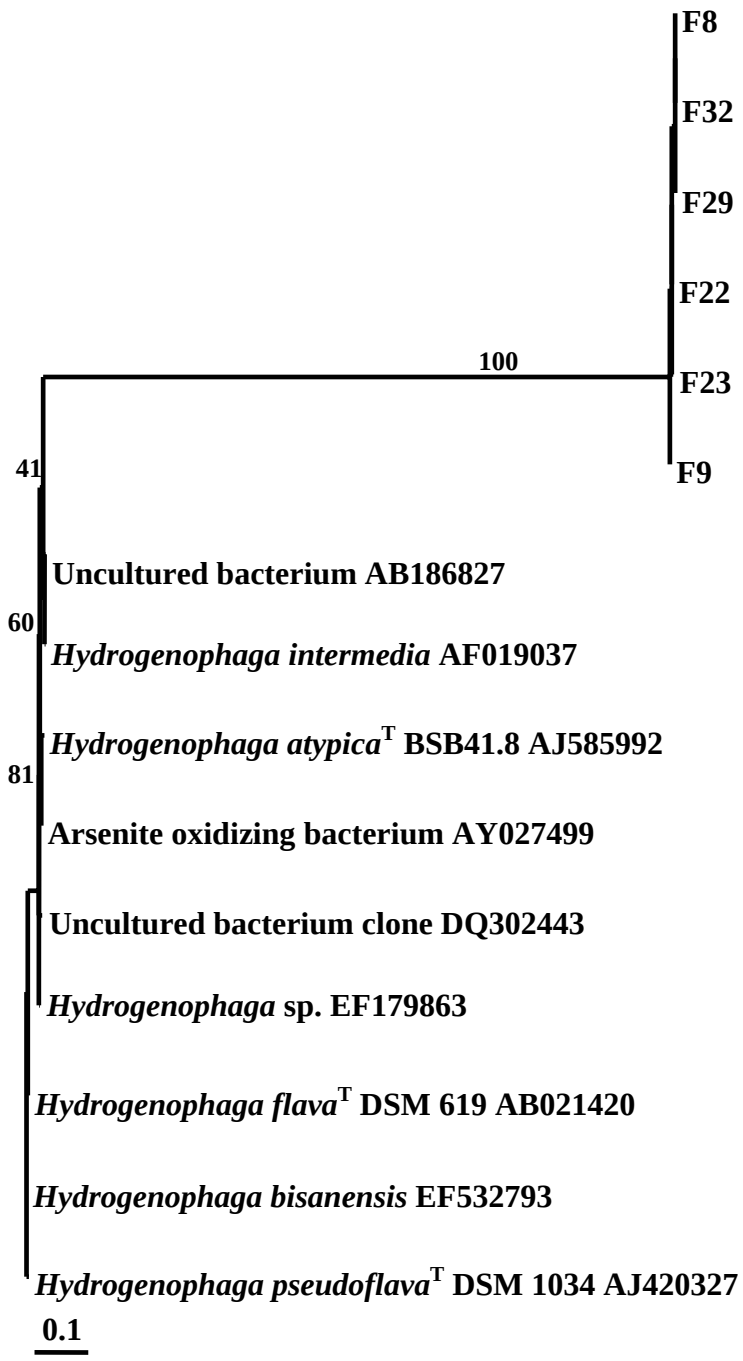


Figure 2.28 Neighbor-joining distance tree of partial 16SrDNA sequences for OTUs F8, F32, F29, F22, F23, and F9 from the mainstream site, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

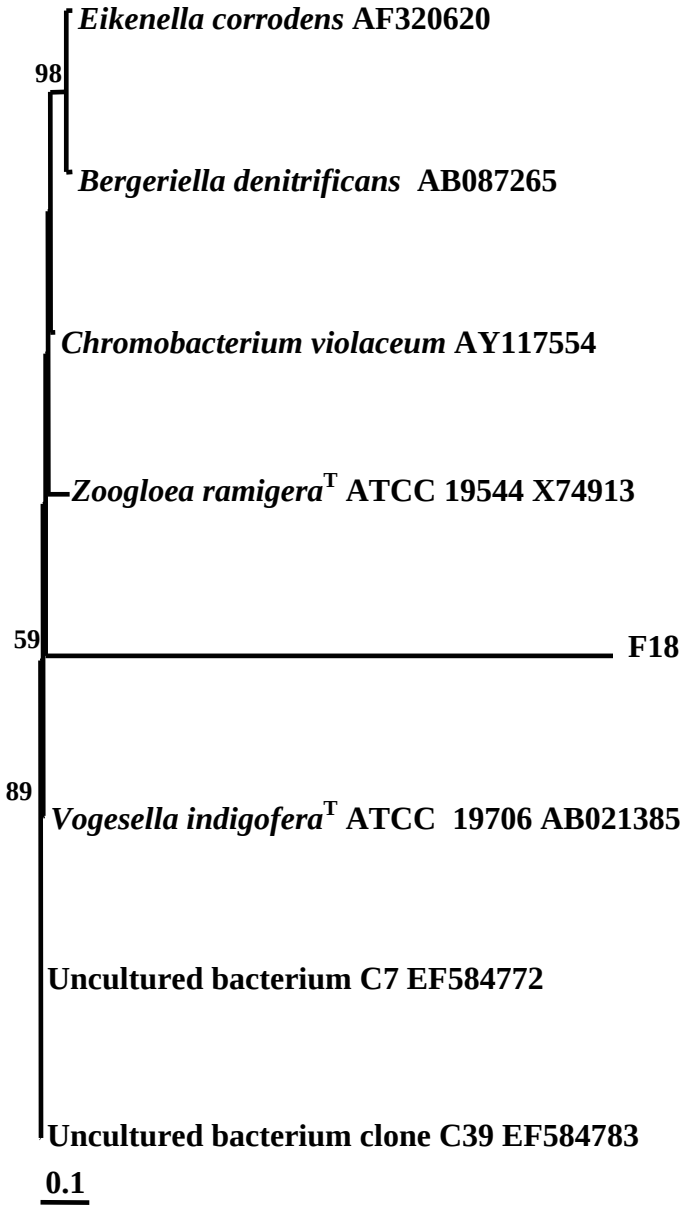


Figure 2.29 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU F18 from the mainstream site, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

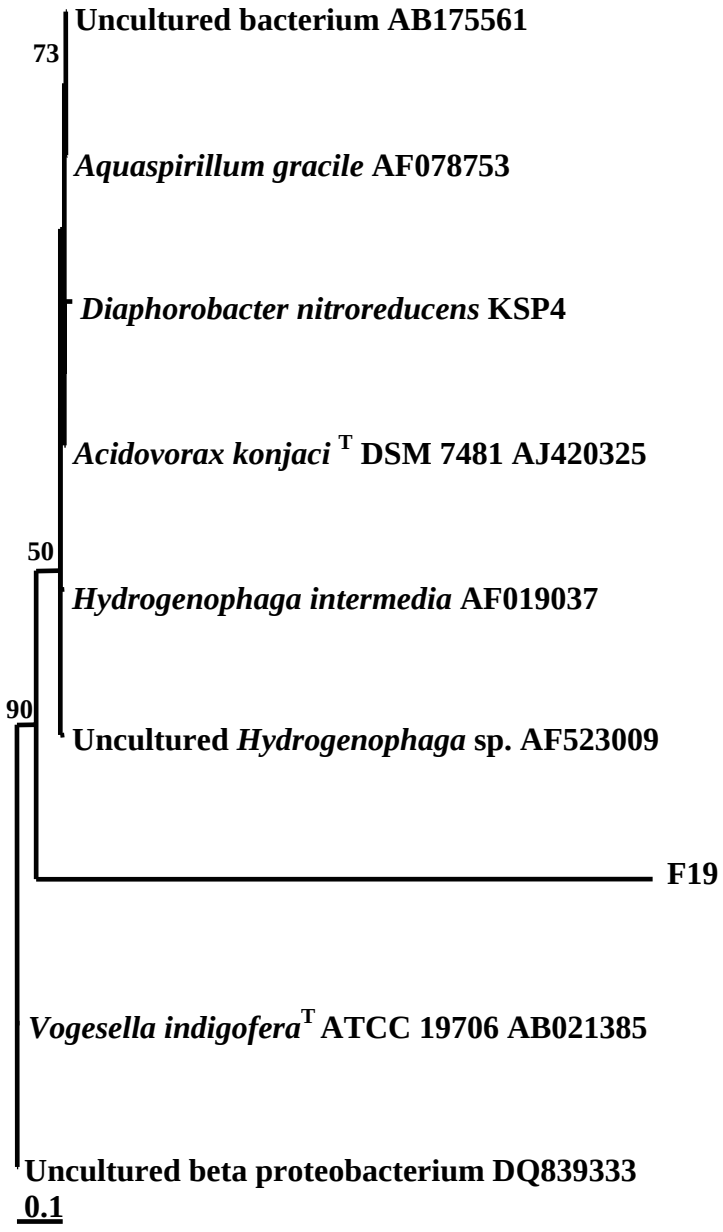


Figure 2.30 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU F19 from the mainstream site, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

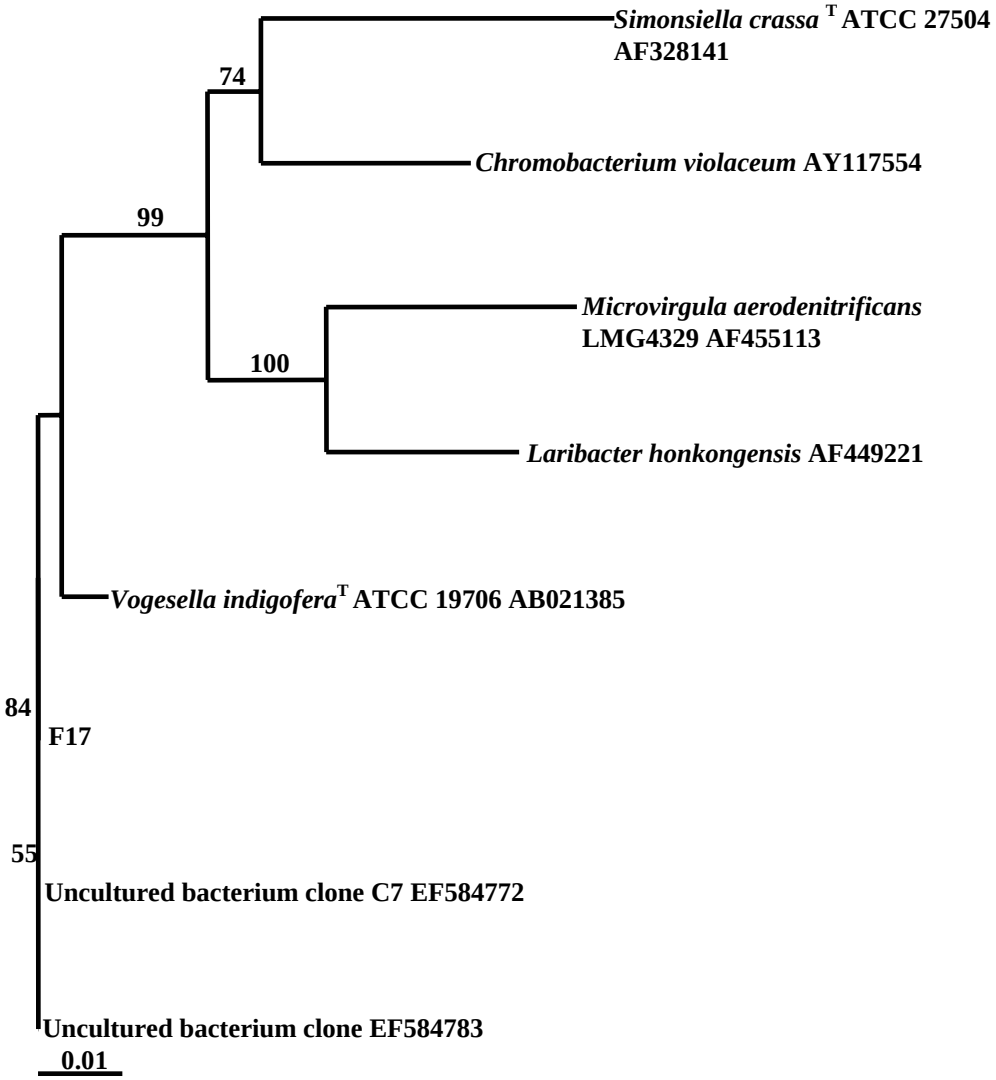


Figure 2.31 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU C17 from the waterway site, at the Coamo springs. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

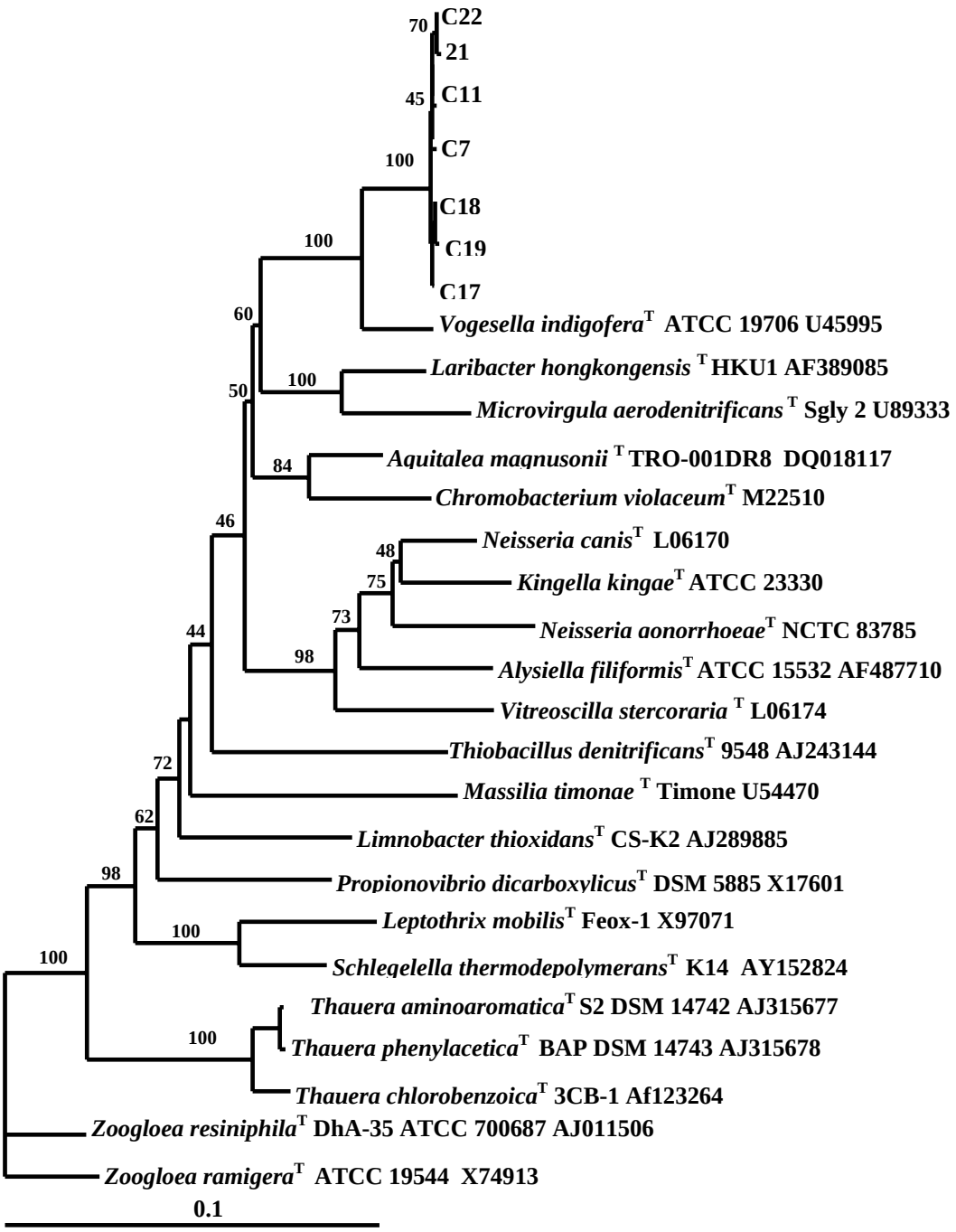


Figure 2.32 Neighbor-joining distance tree of partial 16SrDNA sequences for OTUs C7, C11, C17, C18, C19, C21, and C22 from the waterway site, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

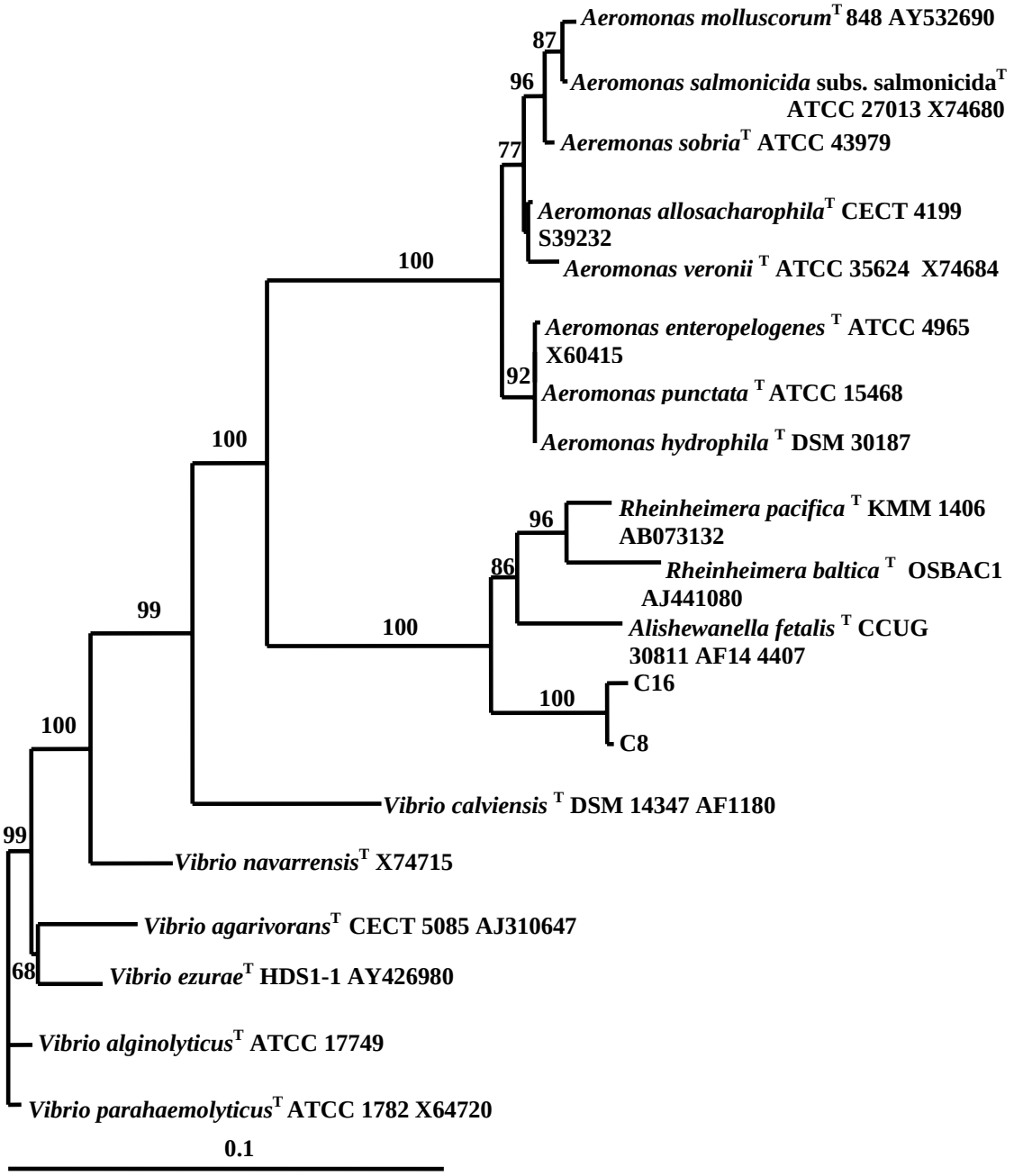


Figure 2.33 Neighbor-joining distance tree of partial 16SrDNA sequences for OTUs C8 and C16 from the waterway site, of the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

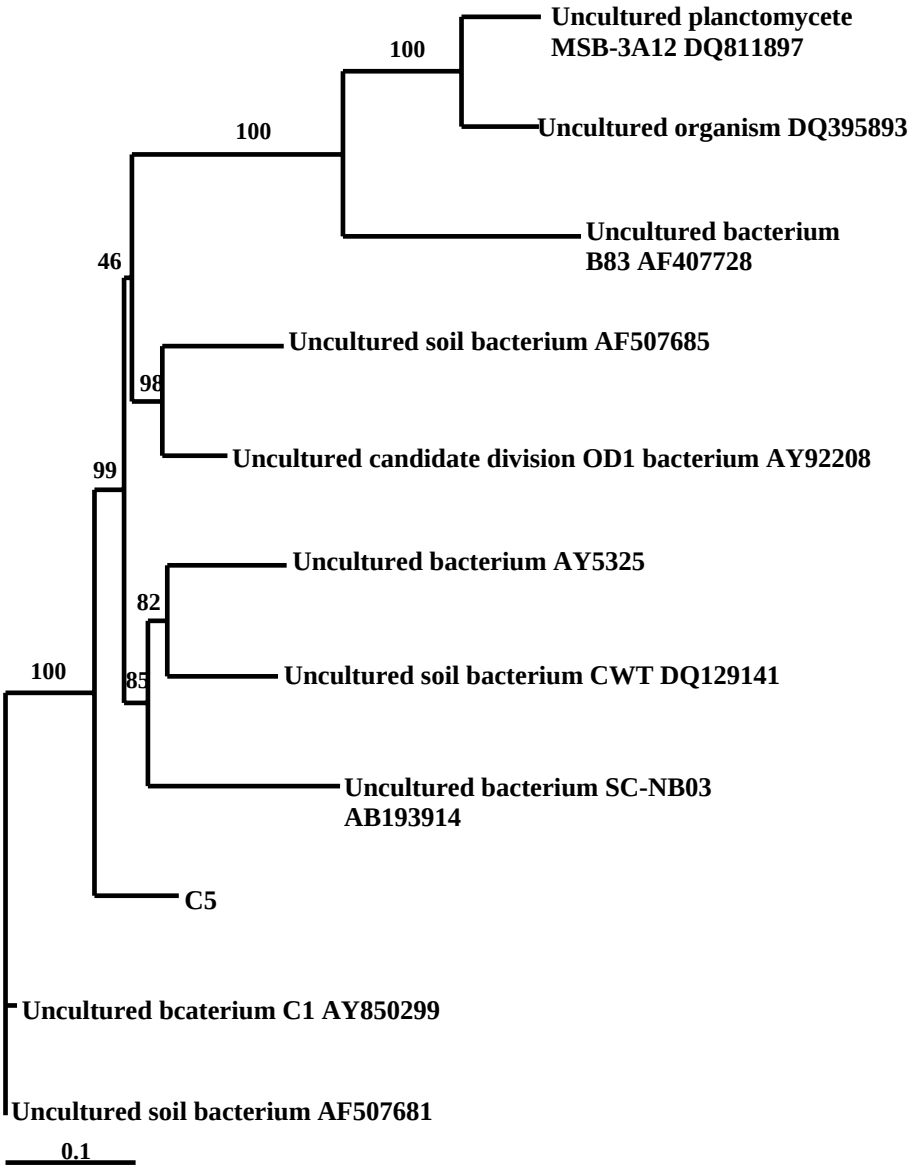


Figure 2.34 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU C5 from the waterway site, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

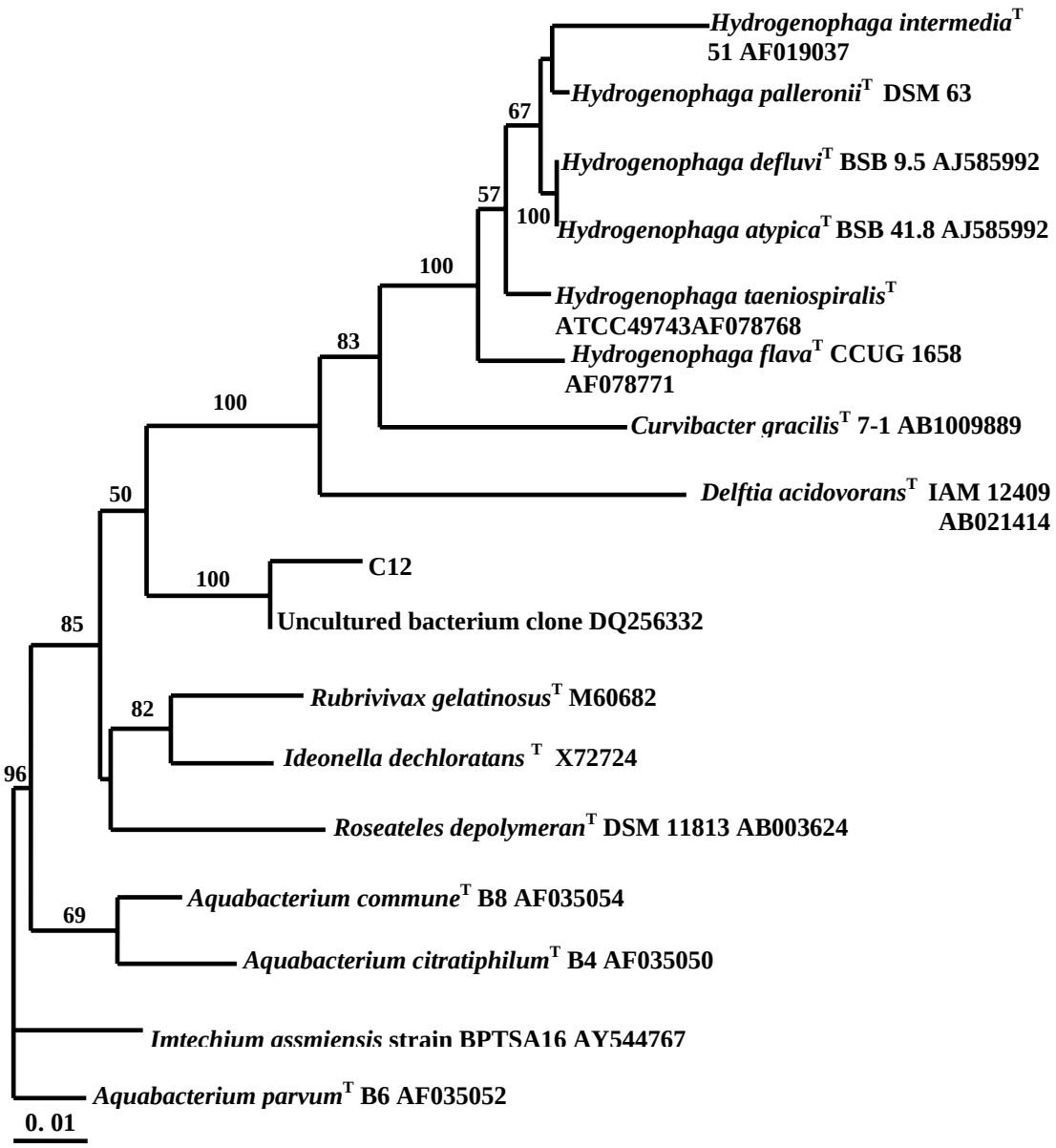


Figure 2.35 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU C12 from the waterway site, at the Coamo springs. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

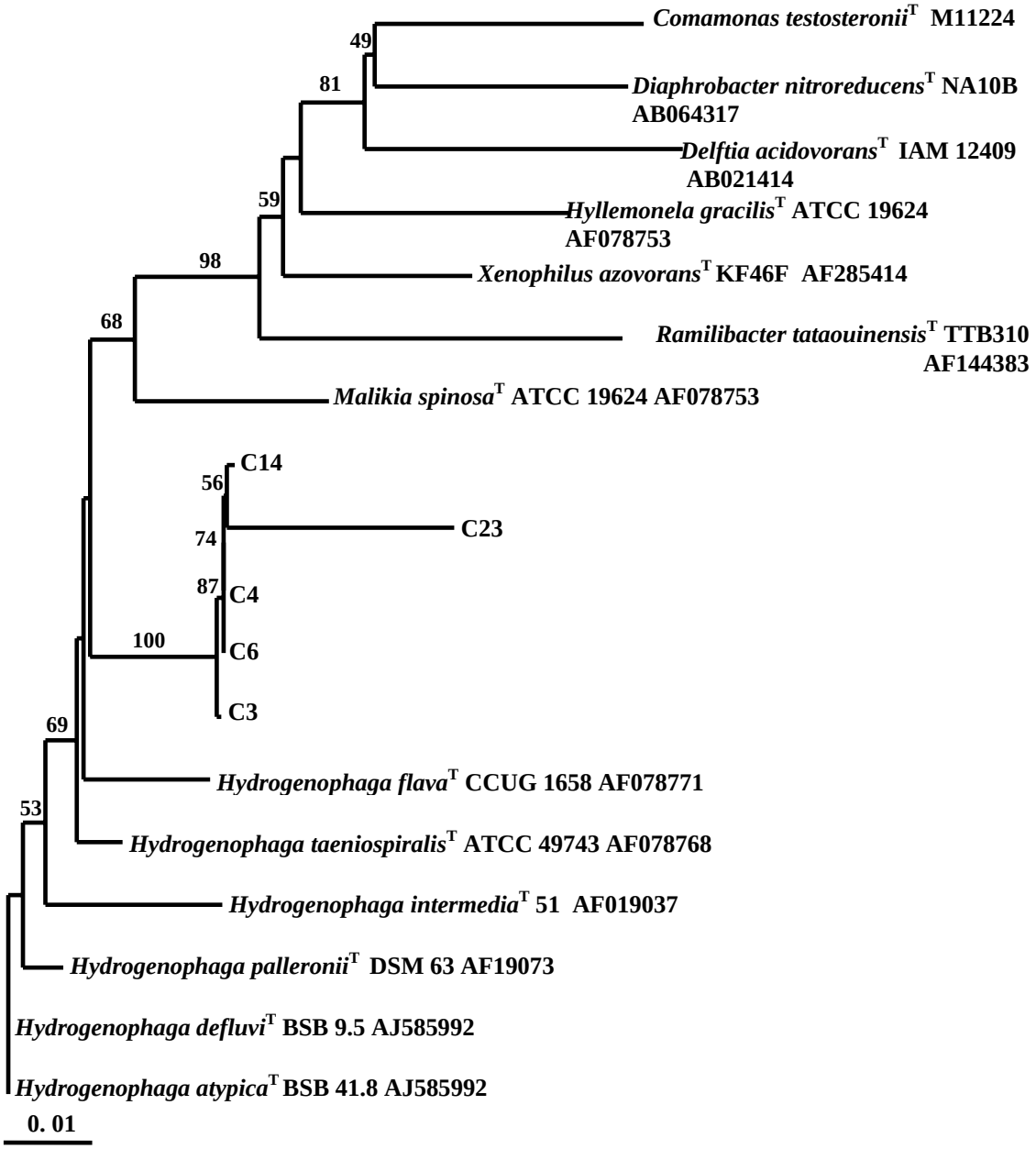


Figure 2.36 Neighbor-joining distance tree of partial 16SrDNA sequences for OTUs C14, C23, C4, C6, and C3 from the waterway site, at the Coamo springs. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

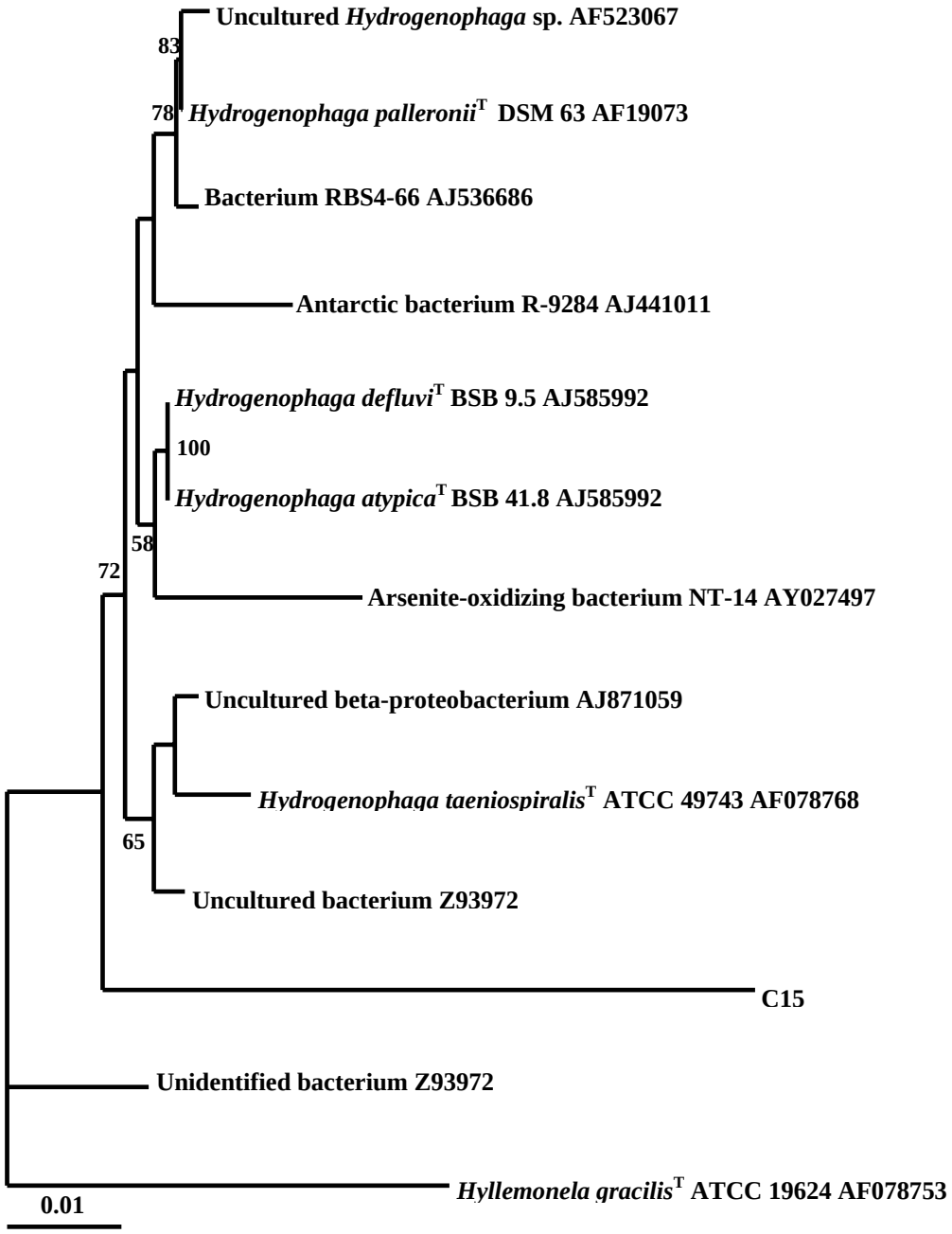


Figure 2.37 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU C15 from the waterway site, at the Coamo springs. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

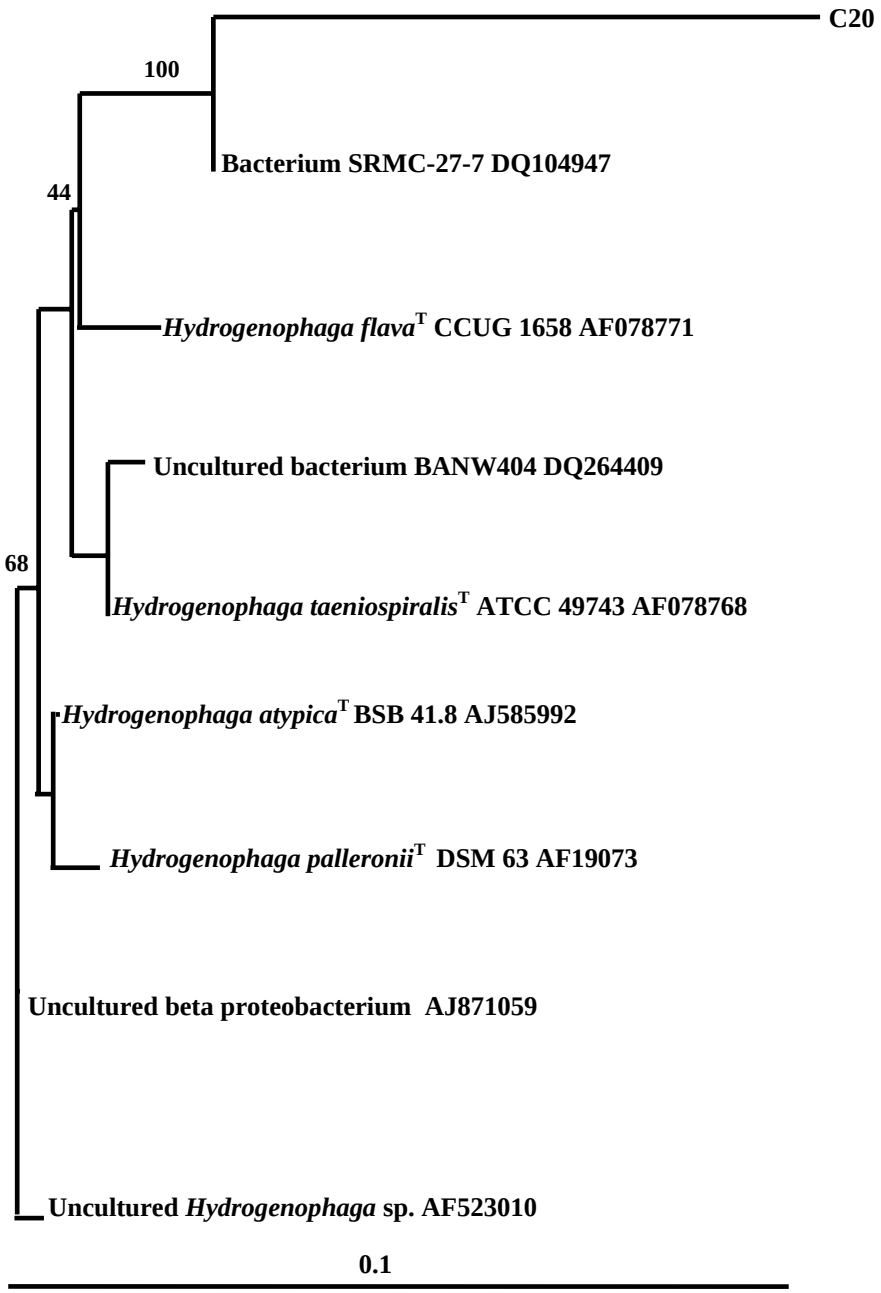


Figure 2.38 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU C20 from the waterway site, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

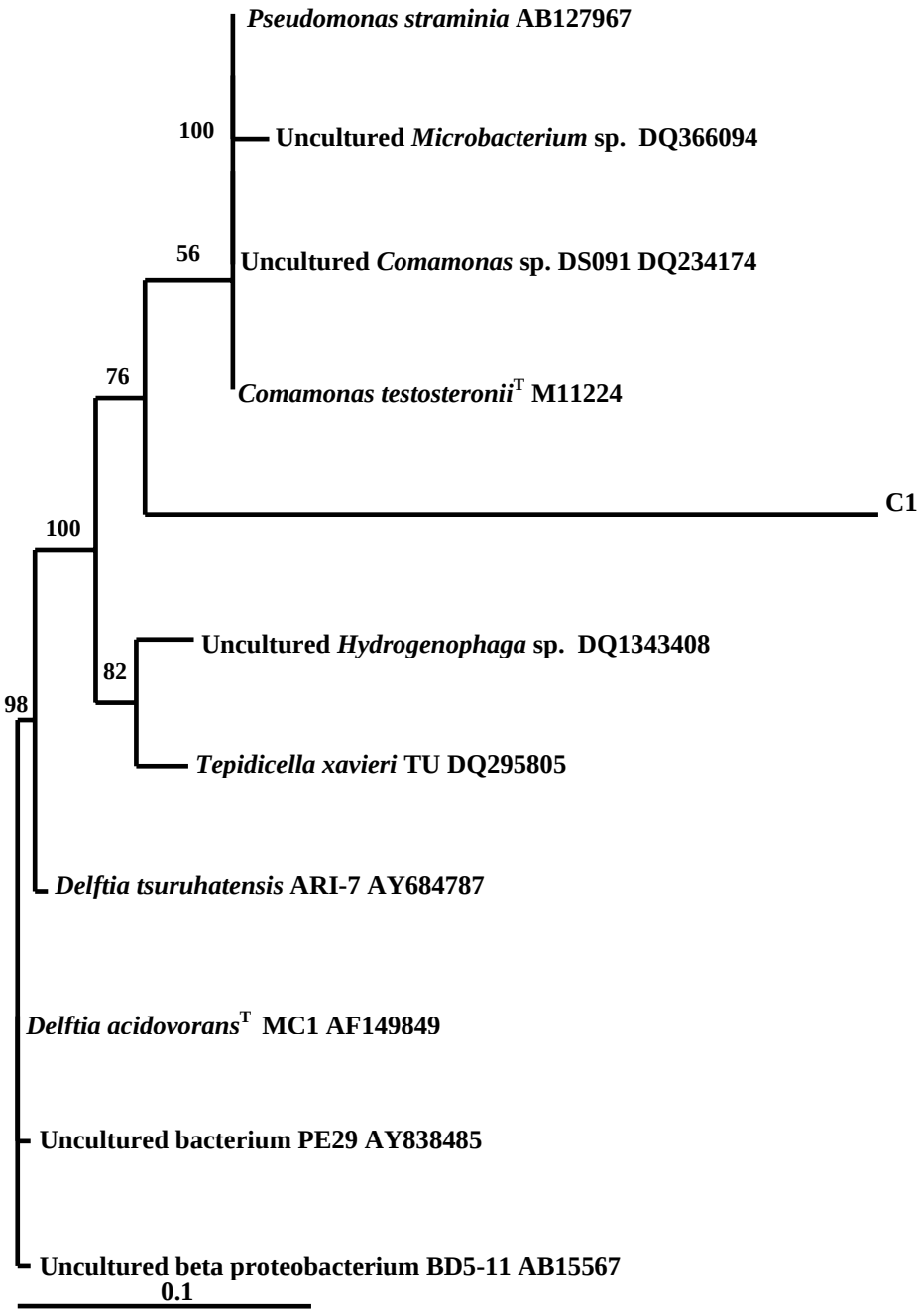


Figure 2.39 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU C1 from the waterway site, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

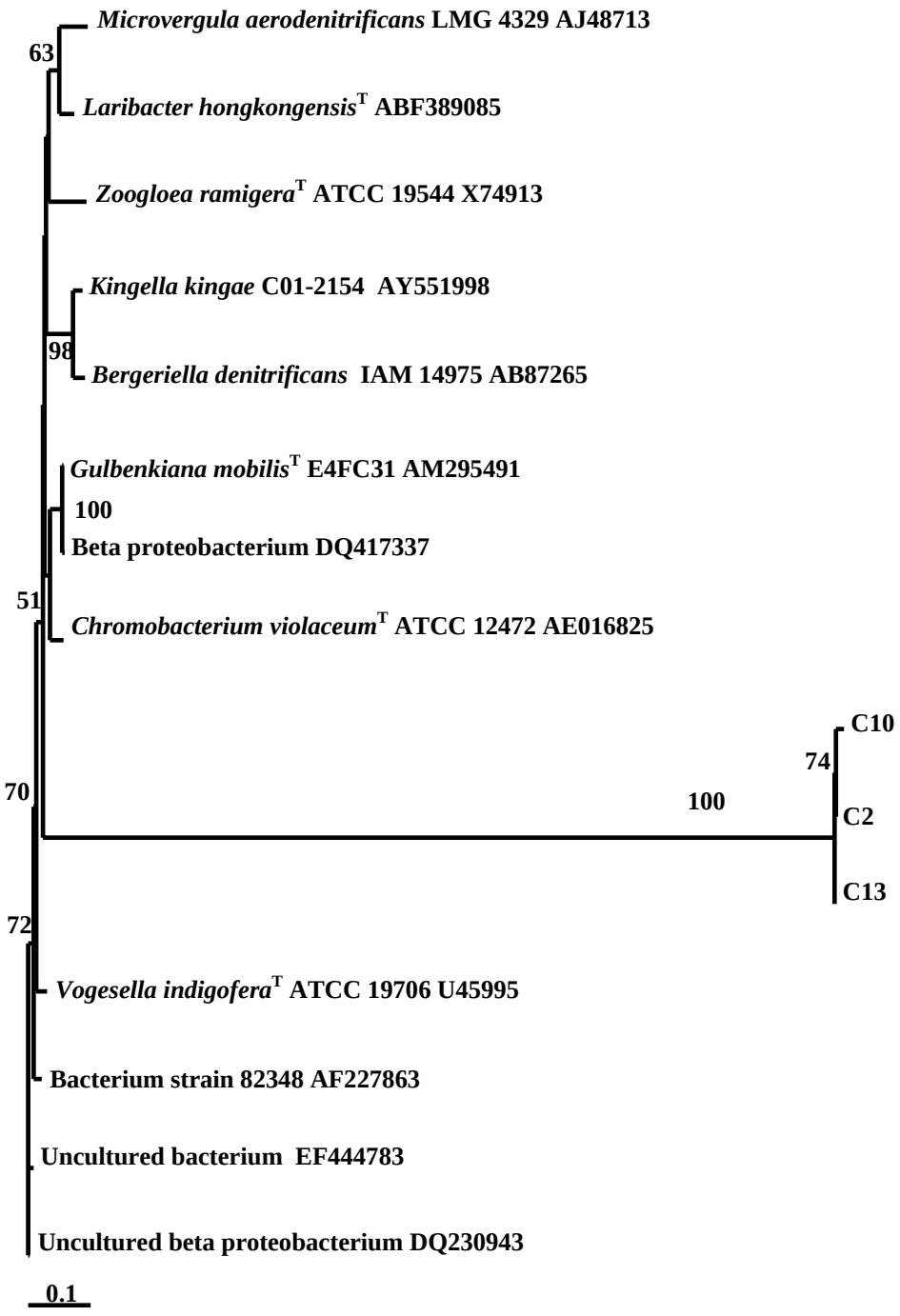


Figure 2.40 Neighbor-joining distance tree of partial 16SrDNA sequences for OTUs C10, C2, and C13 from the waterway site, of the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

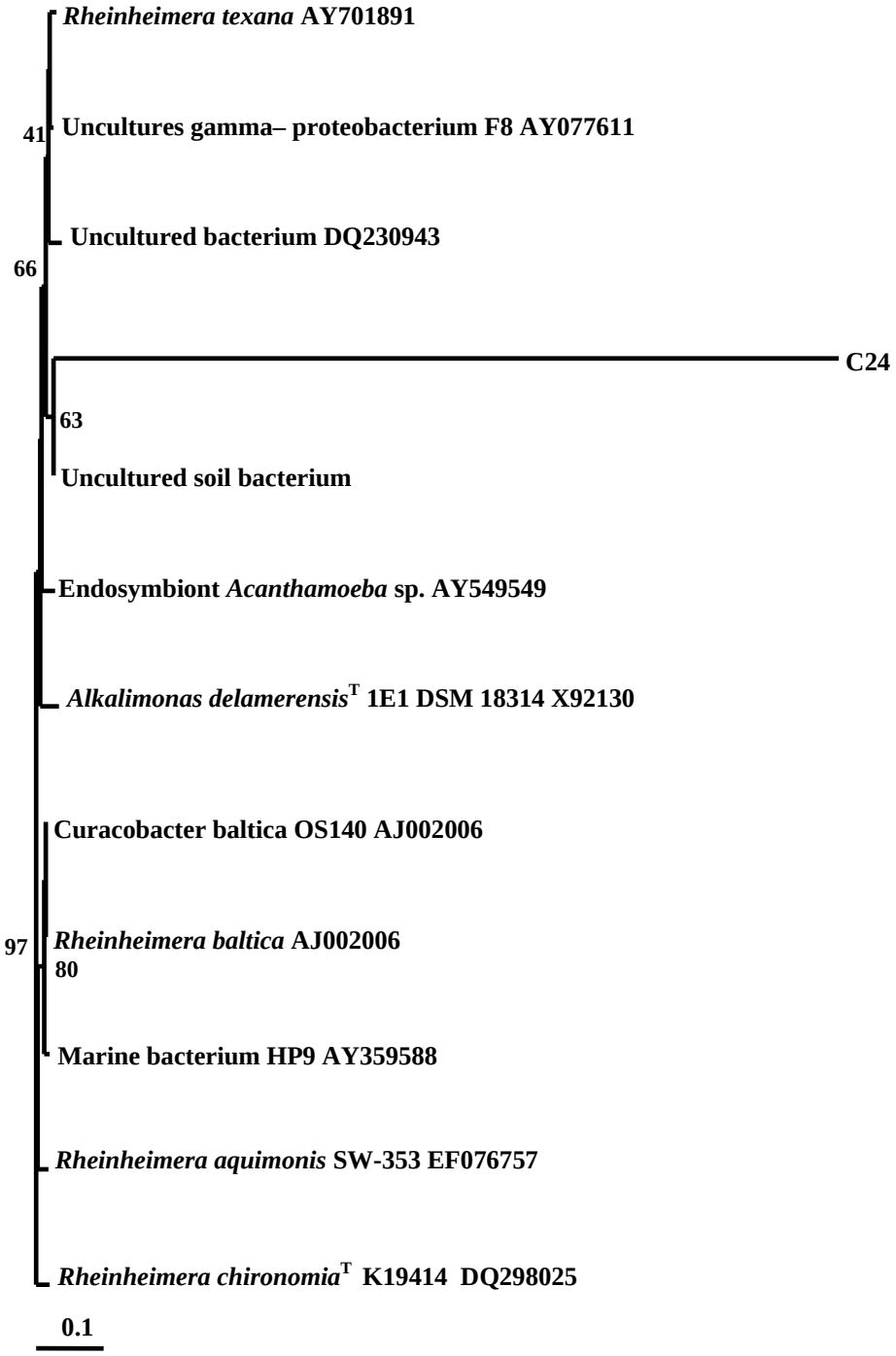


Figure 2.41 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU C24 from the waterway site, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

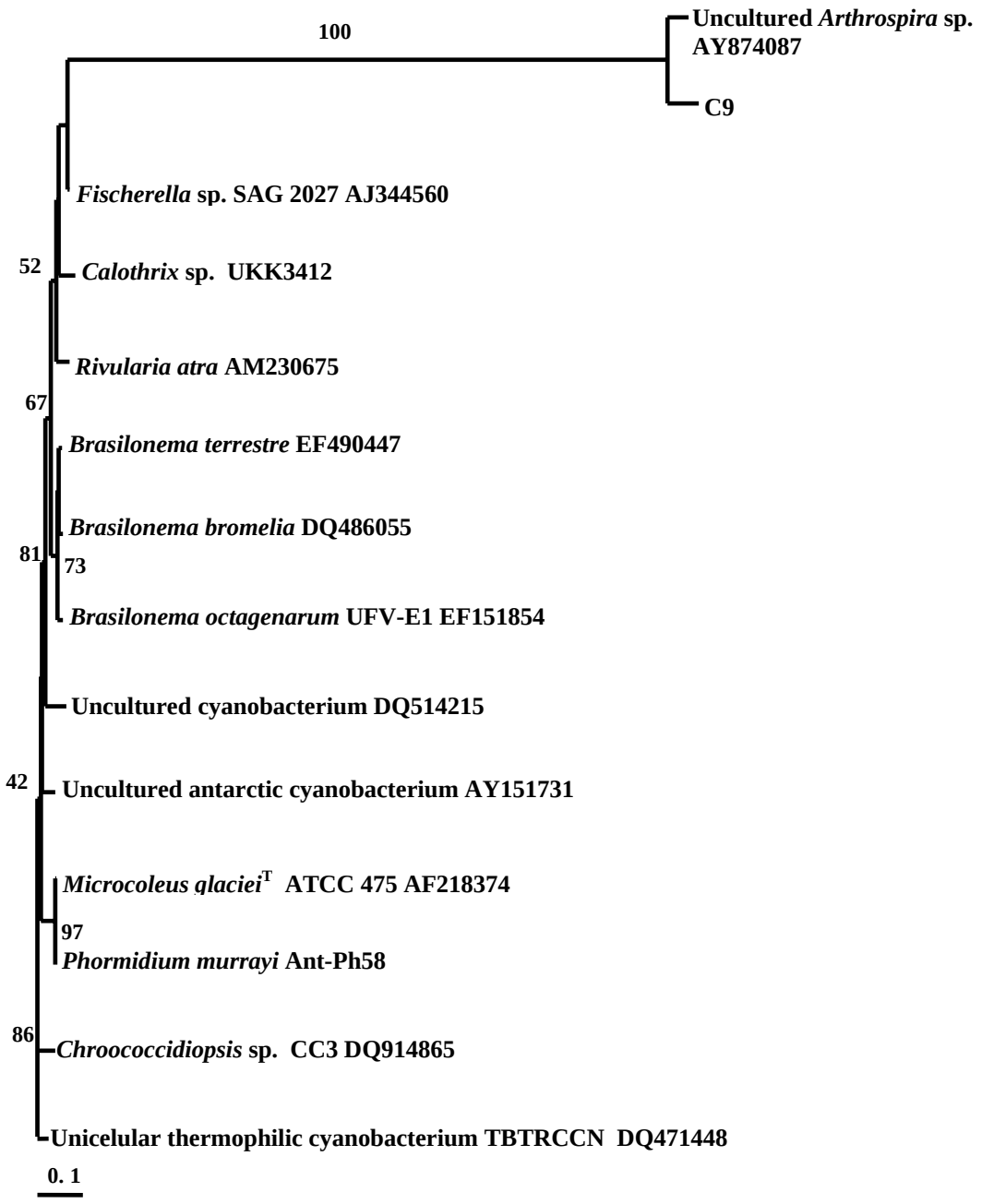


Figure 2.42 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU C9 from the waterway site, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

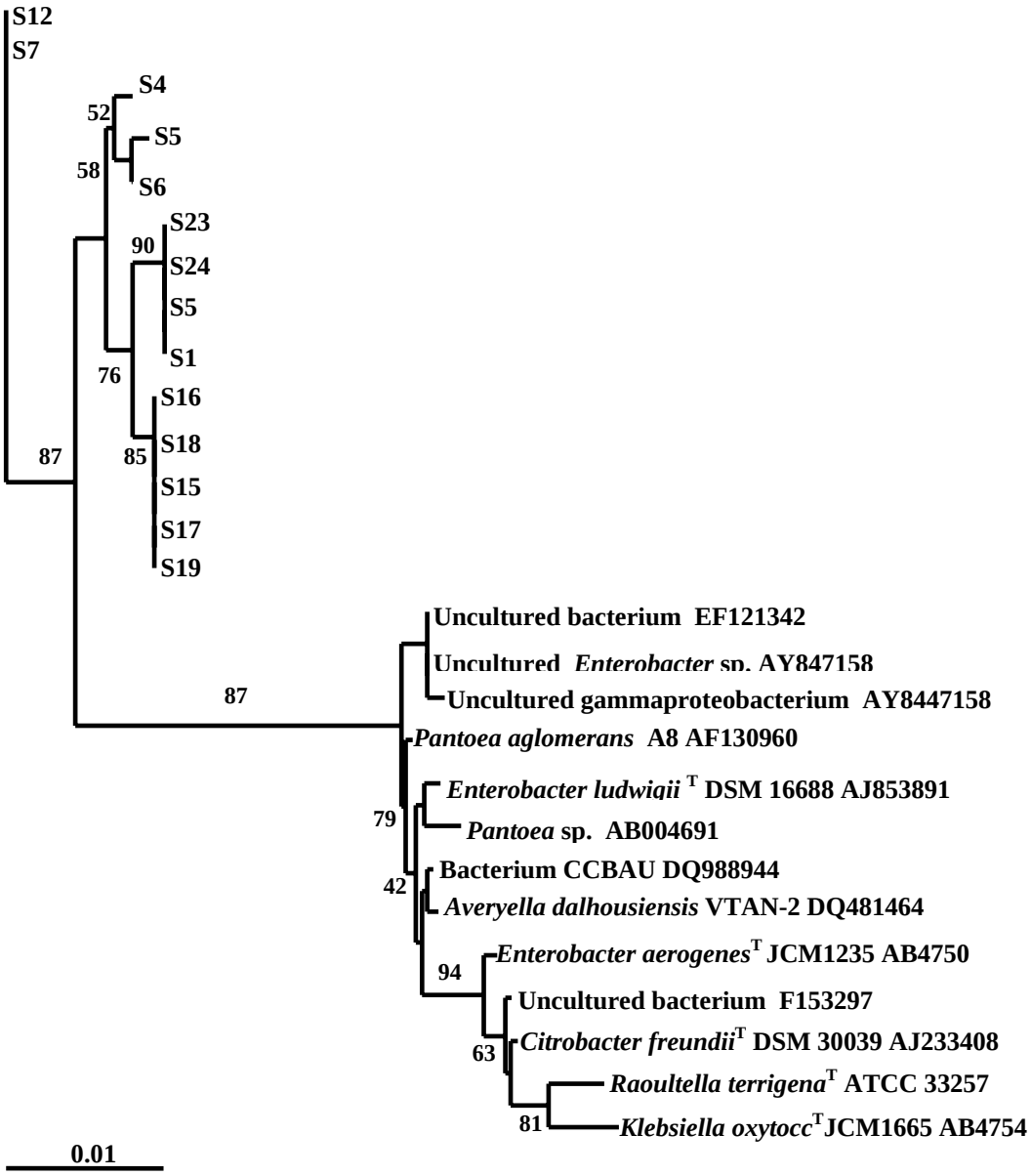


Figure 2.43 Neighbor-joining distance tree of partial 16SrDNA sequences for OTUs S12, S7, S4, S5, S6, S23, S24, S25, S1, S16, S18, S15, S17, and S19 from the outer watercourse site, at the Coamo springs. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

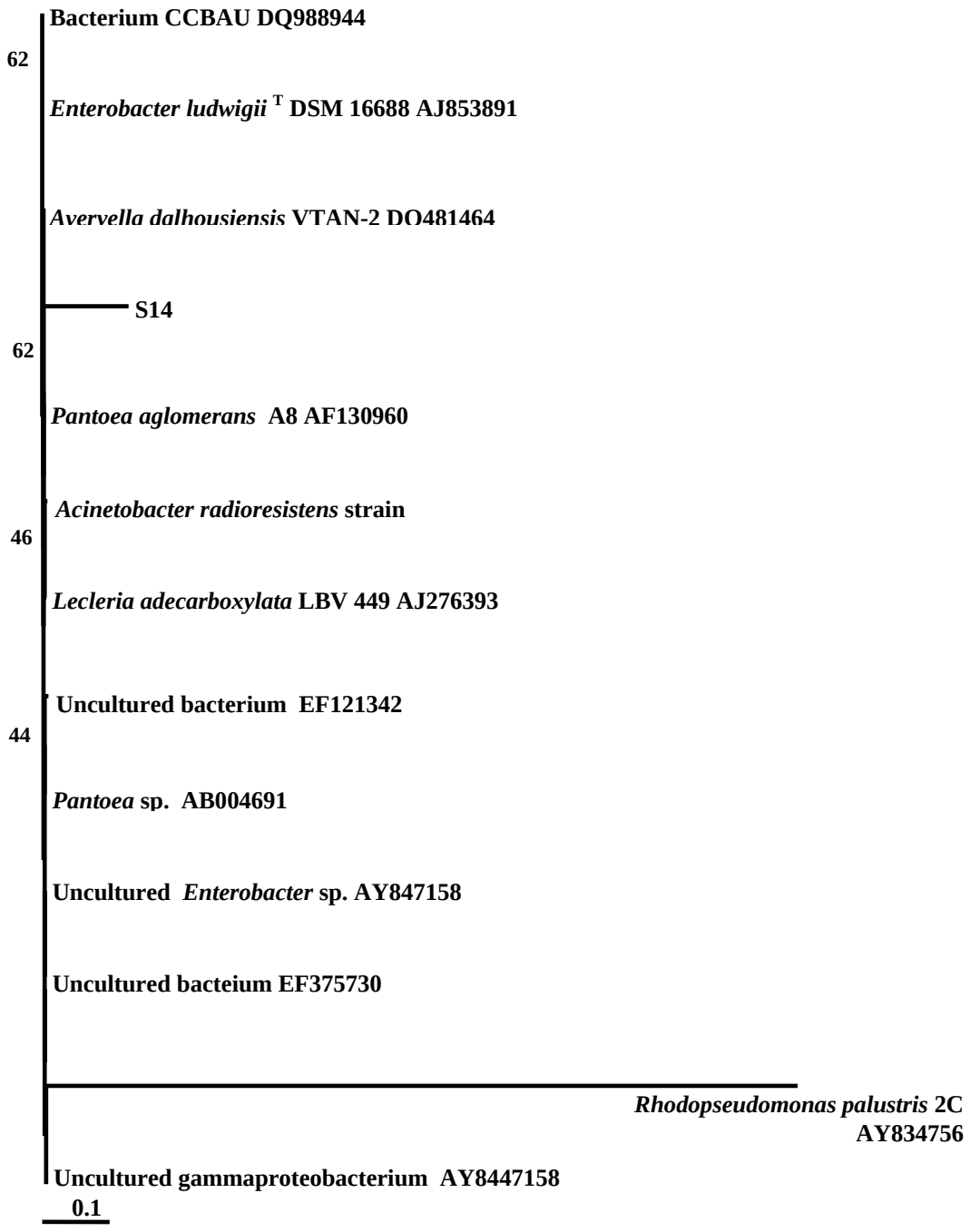


Figure 2.44 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU S14 from the outer watercourse, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

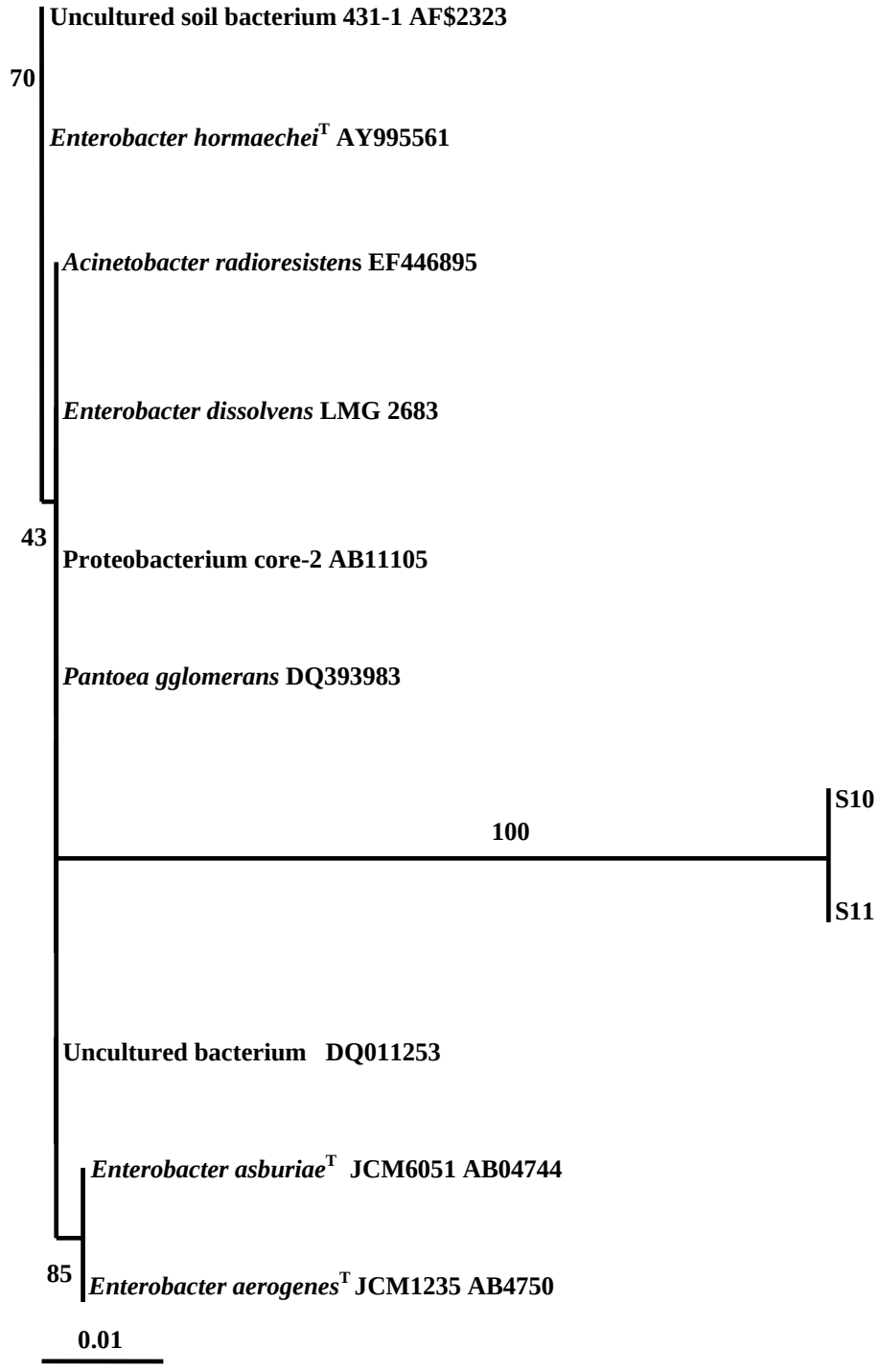


Figure 2.45 Neighbor-joining distance tree of partial 16SrDNA sequences for OTUs, S10, and S11 from the outer watercourse site, at the Coamo springs. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

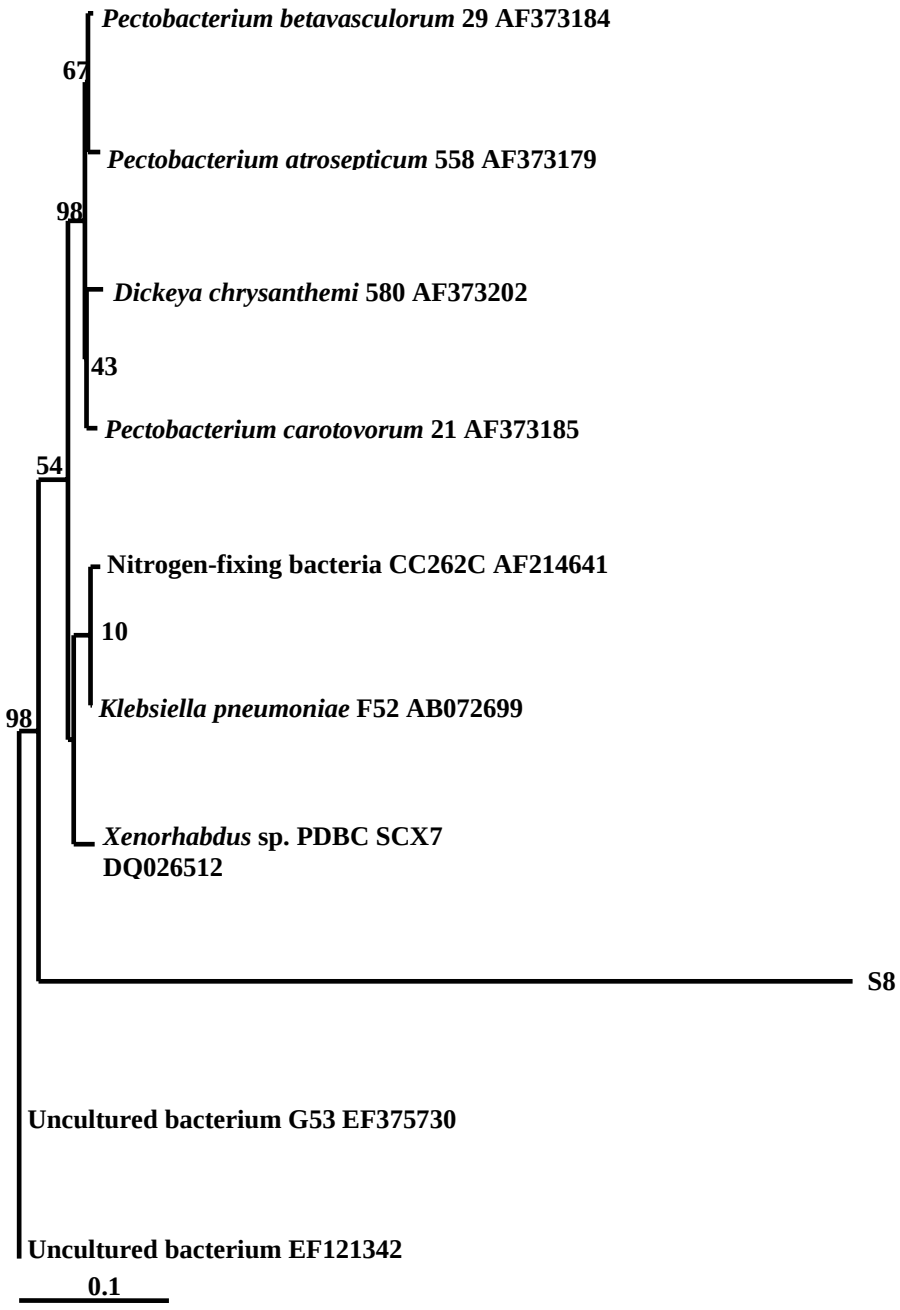


Figure 2.46 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU S8 from the outer watercourse site, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

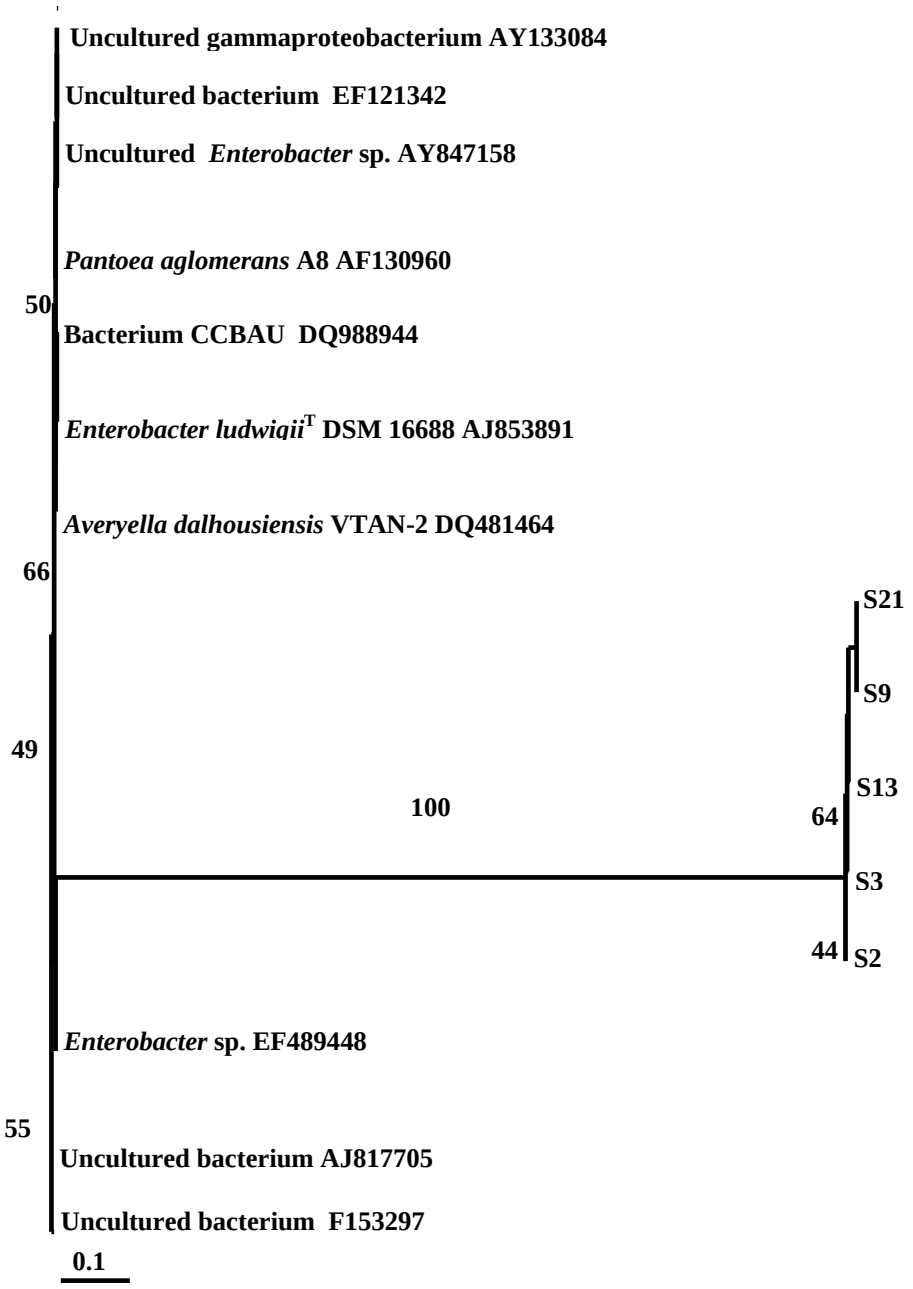


Figure 2.47 Neighbor-joining distance tree of partial 16SrDNA sequences for OTUs S21, S9, S13, S3, and S2 from the outer watercourse site, at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

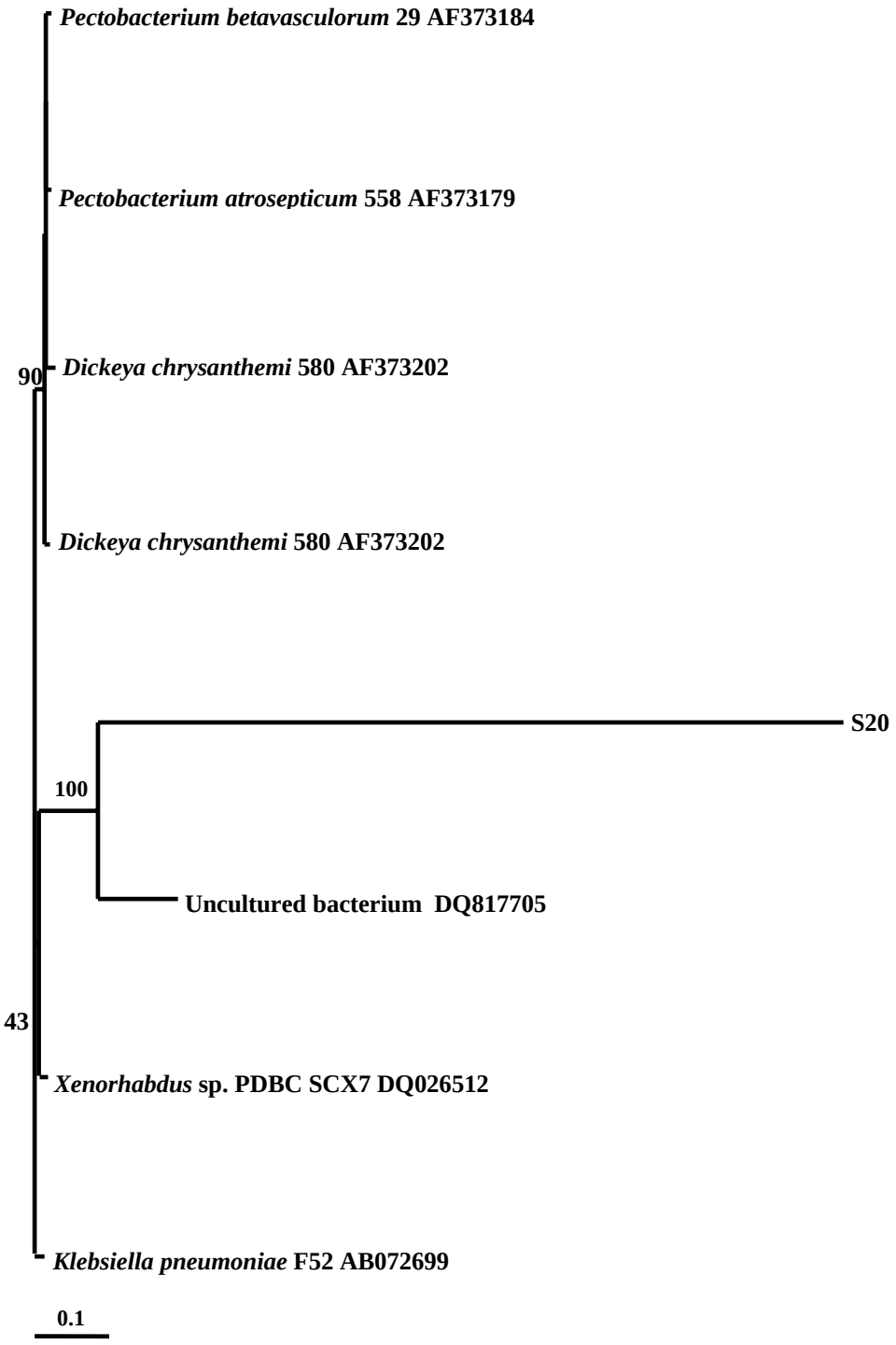


Figure 2.48 Neighbor-joining distance tree of partial 16SrDNA sequences for OTU S20 from the outer watercourse at the Coamo springs. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 40% are shown.

Statistical analysis

Statistical indices for the environmental 16S rDNA clone libraries, for mainstream (M), waterway (W) and outer watercourse (O) are shown in Table 2.3. The unique distance to define an OTU for the three 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 communities mainstream and waterway libraries with 15 and 11 OTU's respectively. The Shannon and Simpson's diversity indices were calculated and showed that M and W clone libraries had higher diversity than O library. The Jaccard, SAce, and SChao richness index values were calculated in order to corroborate richness between samples. All indexes indicated that mainstream and waterway libraries had the highest level of richness when compared to outer watercourse clone library.

To measure how well the samples represent the larger environment, the Good Coverage Index was calculated (Figure 2.50) using the ASLO program (www.aslo.org/methods/free/2004/0114a.html). The coverage was 68% , 75% and 84% for libraries M, W and O respectively. Rarefraction 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 libraries M and W (Figure 2.49).

The graphic curves revealed that if clone library O had more clones screened, the distribution would be constant. In contrast, for clone libraries M and W more clones 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 the clone libraries was 0.0256. The Web-LIBSHUFF results revealed a p-value minor of the minimum expected p-value of 0.001 indicating significant differences between the three 16S rDNA clone libraries. This was performed using all the possible combinations between libraries.

LIBSHUFF analysis of homologous and heterologous coverage curves indicated that the M and W communities are significantly different from O community (P = 0.001) (Figure 2.51.). Comparison of the O community indicated a significant difference from M and W communities (Figure 2.52). A test of multiple contrasts among M, W and O communities were obtained by examination of the distribution of $(C_x-C_{xy})^2$ with evolutionary distance (D). During the calculation of ΔC, results suggests that the group M and W libraries differ from the other library at high levels of genetic distance and shares all deep taxa (D>0.10) (Figure 2.53).

Table 2.3 Statistical Indexes for 16s rDNA clone libraries from Coamo thermal springs Puerto Rico.

Clone Library	Shannon (H)	Simpsons (1/D)	Richness Observed	Jaccard %	Sace %	Schao %	Coverege %
M	2.4	0.10	15(32)	28	37.5	31.4	68
W	2.1	0.12	11(24)	17	21.1	15	75
O	1.8	0.19	6(25)	13	14.4	10.4	84

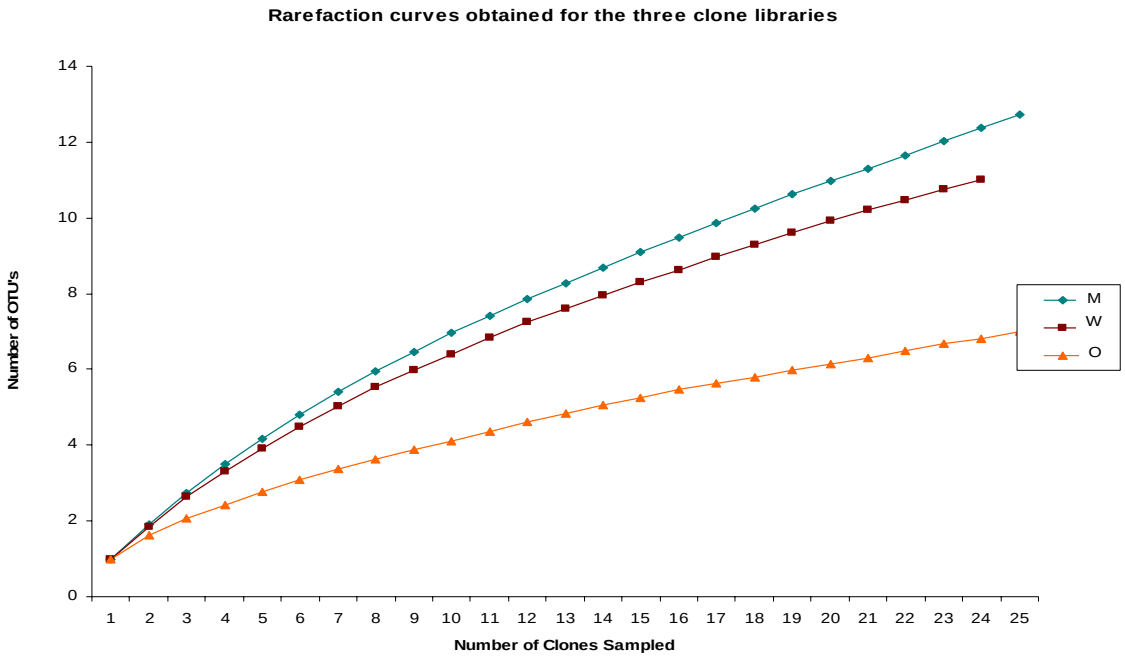
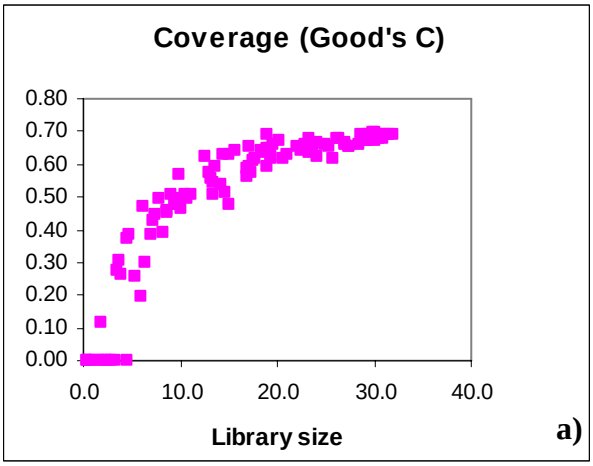
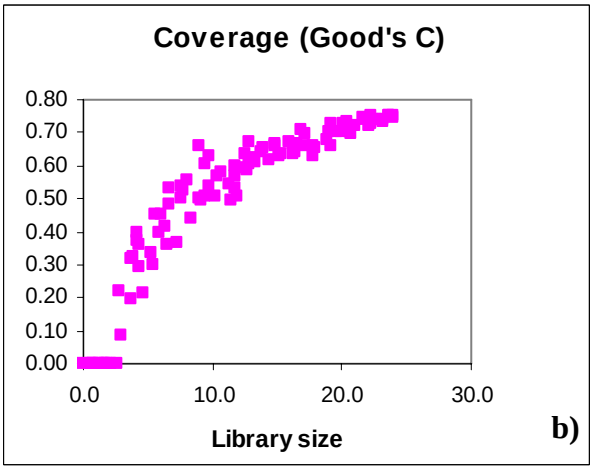


Figure 2.49 Rarefaction curves for the three clone libraries sampled from the Coamo thermal springs. OTU's were determined by ≥97% similarity.


Coverage (Good's C)
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.0
10.0
20.0
30.0
40.0
Library size
a)


Coverage (Good's C)
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.0
10.0
20.0
30.0
Library size
b)

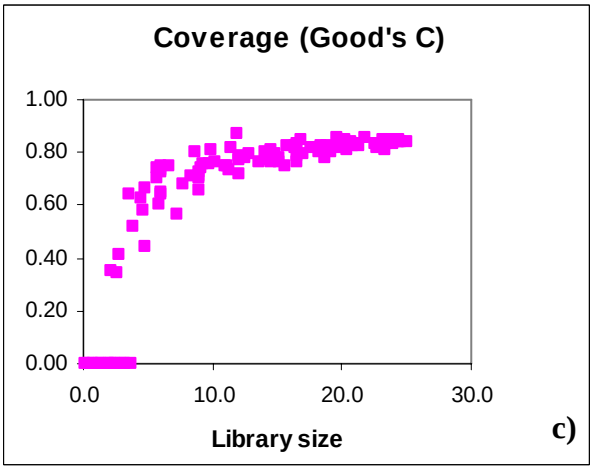

Coverage (Good's C)
1.00
0.80
0.60
0.40
0.20
0.00
0.0
10.0
20.0
30.0
Library size
c)

Figure 2.50 Good Coverage curves for 16S rRNA clone libraries: mainstream clone library (a), waterway clone library (b), and outer watercourse clone library (c).

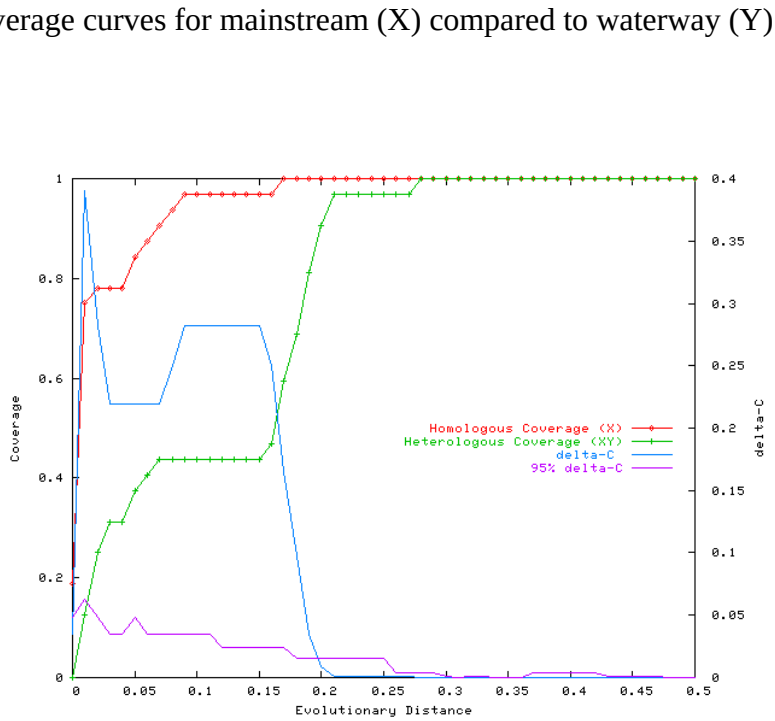


Figure 2.51 LIBSHUFF comparisons of the two clone libraries. Blue lines indicate the value of $(C_x\text{-}C_{xy})^2$ for samples at each value of evolutionary distance (D). D is equal to the Jukes-Cantor evolutionary distance determined by the DNADIST program of PHYLIP. Purple lines indicate the P=0.05 value of $(C_x\text{-}C_{xy})^2$ for the randomized samples. Comparison of the clone library O (red/homologous) with clone library W (green/ heterologous).

Coverage curves for mainstream (X) compared to outer watercourse (Y)

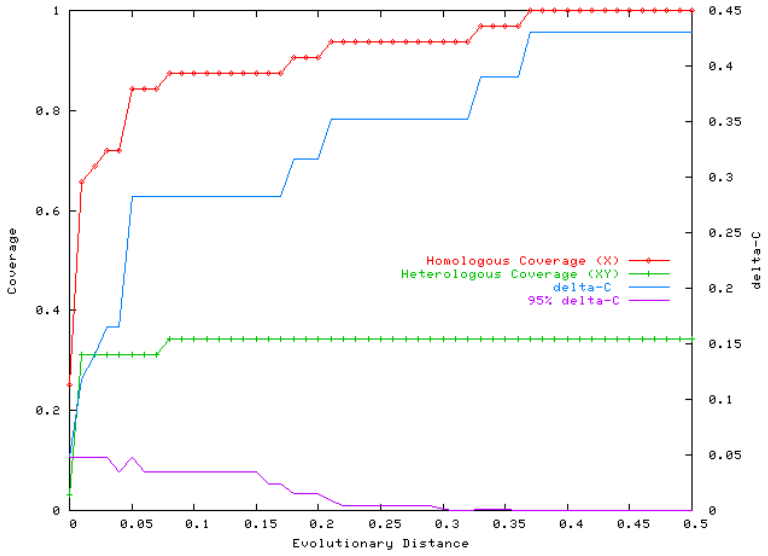


Figure 2.52 LIBSHUFF comparison of the two clones libraries. Blue lines indicate the value of $(C_x-C_{xy})^2$ for samples at each value of evolutionary Distance (D). D is equal to the Jukes-Cantor evolutionary distance determined by the DNADIST program of PHYLIP. Purple lines indicate the P=0.05 value of $(C_x-C_{xy})^2$ for the randomized samples. Comparison of the clone library W (red/homologous) with clone library O (green/heterologous).

Coverage curves for waterway (X) compared to outer watercourse (Y)

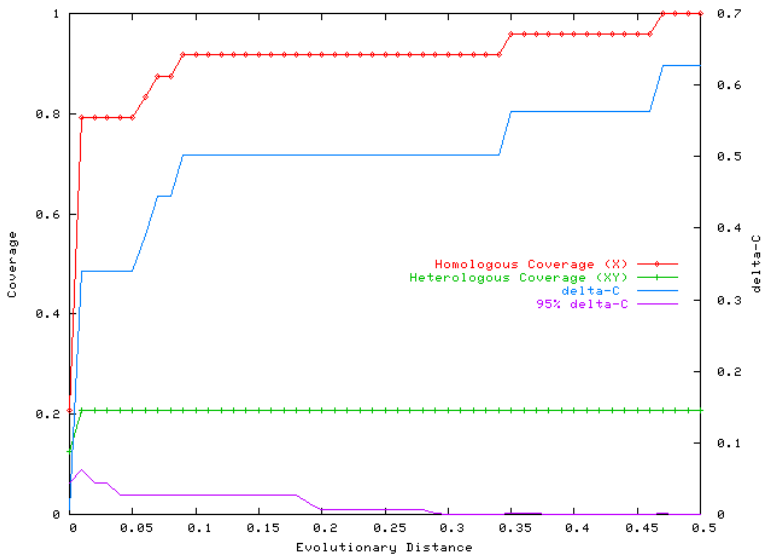


Figure 2.53 LIBSHUFF comparison of the two clones libraries. Blue lines indicate the value of $(C_x\text{-}C_{xy})^2$ for samples at each value of evolutionary Distance (D). D is equal to the Jukes-Cantor evolutionary distance determined by the DNADIST program of PHYLIP. Purple lines indicate the P=0.05 value of $(C_x\text{-}C_{xy})^2$ for the randomized samples. Comparison of the clone library W (red/homologous) with clone library O (green/heterologous).

Discussion

The principal goal of this part of the study was to determine the prokaryotic community composition from the alkaliphilic thermal springs at Coamo, Puerto Rico based on 16SrDNA environmental clone libraries. A total of 172 clones were obtained from the three genomic libraries. Each clone library was screened using the RFLP technique that revealed a small variety of restriction patterns indicating a low diversity among all the clone libraries constructed.

Approximately 25 OTUs for each library (M, W and O) were sequenced for a total of 81 OTUs analyzed *in silico*. Clones were selected randomly to avoid possible biases. The Bacteria domain represented the 100% of the clone library and no archaeal clones were found. Phylogenetic analyses revealed that most of the OTU's are related to bacterial groups associated with marine, soil, and plant environments. The majority of the sequences analyzed showed close relationship to the Bacteroidetes, beta-proteobacteria, and gamma-proteobacteria groups. The most frequent OTU's belonged to the beta-proteobacteria from humic lakes, activated sludge, hot springs, mangrove soils, and deep subsurface environments. OTU's were related with the genera *Pseudomonas* sp., *Hydrogenophaga* sp., *Comamonas* 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 second most abundant OTU's belonged to the gamma-proteobacteria group. These groups 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 a saprophytic microbial mat cave in sulfidic springs (Engel *et al*., 2004), and from rice plant (Hiraoka *et al*., 2006)

In this study, Microbial communities were also analyzed by T-RFLP. T-RFs patterns generated with each enzyme showed some similarities among the three sampling sites tested. T-RFLP profiles revealed that only a few changes in the community structure occurred in the three samplings. It is important to notice the presence of unique T-RFs among samples, the absence of some T-RFs fragments in some samples and changes in the T-RFs intensities demonstrating the possibility of changes in the abundance of some bacterial populations at a given stage and the appearance of new ones.

Geobacillus representatives were detected in isolated strains from the alkaliphilic thermal springs. Surprisingly, OTUs associated to the genus *Geobacillus* were not detected or found using non culture dependent method. One possible explanation for this might rely on the water physical factors. The temperature of the water at the time of samplings varied from 38.5-44˚C and the *Geobacillus* strains isolated in this study can not grow below 50˚C. This suggests that the isolated microorganisms were at the spore stage at the time of the samplings. Another explanation could be the bias related to the primers used or the DNA extraction procedure. A broader diversity might be obtained amplifying more than one DNA product instead of one amplified DNA product. However, the results from T-RFLP analysis and the genomic libraries agree in terms of the low prokaryotic diversity that these alkaliphilic thermal waters might contain which has been the case for similar environments around the world (Wiegel, 1998).

Statistical analysis indexes, (Jaccard, SAce, and SChao richness) indicated that library W had the highest level of richness when compared with clone library O (Table 2.2). 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 two communities. Estimating the $OTU_{0.03}$ richness of each clone library and the richness shared between groups (W and O) revealed no similarity among communities and J_{abund} values between the two 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 at alkaliphilic thermal waters of Coamo Springs using culture-independent techniques like genomic libraries and T-RFLP. This study showed that there is a low prokaryotic diversity associated to the thermal springs in Coamo. Knowledge about the identity of these microorganisms will allow the potential development of biochemical applications in biotechnology and astrobiology.

Conclusions

- Slightly alkalithermophilic bacterial strains can be isolated from the thermal springs at Coamo Puerto Rico. Most of the isolates obtained were gram-positive rods that grow well at temperatures above 40ºC. The average optimum temperature for growth was 60ºC and the maximum around 70˚C.

- The combination of morphological, physiological, and molecular approaches was very useful in the characterization of the isolates.

- A low diversity of prokaryotes was recovered using *Thermus* medium and Alkaline Yeast Extract Malt Medium.

- The isolated microorganisms in this study belonged mainly to the genus *Geobacillus*, which have alkaliphilic and thermophilic properties. This genus showed the highest frequency of isolation in this research using culture dependent approaches.

- Isolate CS4 is a strain of *Geobacillus thermodenitrificans* and CS 101 could be a strain of *Geobacillus toebii*.

- Culture independent techniques revealed a low prokaryotic diversity present at the Caomo springs. The most frequent OTUs belonged to the alpha-proteobacteria group.
- This study is the first attempt to study the prokaryotic diversity present at the thermal springs at Coamo Puerto Rico.

Recommendations

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

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

- Further analysis to complete the characterization of isolated strains belonging to the genus *Geobacillus*.

- In order to reach and cover most of the prokaryotic diversity from the alkaliphilic thermal waters at Coamo Puerto Rico, more clones must be analyzed.

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Appendix 1

Independent tests results for the numbers of CFU's obtained per sampling on the different media per liter of filtered water

Independent Test Shapiro-Wilks

Culture Media	Variable	n(Sampling)	Mean	S.D.	W*	p(one tail)
AYEMM	CFU's	3	10.67	6.11	0.96	0.6394
TM	CFU's	3	51	32.6	0.99	0.8483

Independent T Test

Culture Media	Variable	n(Sampling)	Mean	S.D.	LI(95)	LS	T	P(Bilateral)
AYEMM	CFU's	3	10.67	6.11	-4.51	25.84	3.02	0.0942
TM	CFU's	3	51	32.6	-29.99	131.99	2.71	0.1135

Appendix 2

Macroscopic and microscopic characteristic of isolated strains from Coamo springs. All strains were Gram- positive.

Strain	Colony Color	Colony Morphology	Morphology	Arrangement	Average size (µm)
CS_1	cream	irregular, undulate, umbonate	long rods	streptobacilli	3.0 x 1.0
CS_2	cream	irregular, undulate, flat	long rods	streptobacilli	3.5 x 0.6
CS_3	cream	irregular, undulate, flat	long rods	streptobacilli	3.5 x 0.8
CS_4	cream	circular, undulate, flat	long rods	streptobacilli	3.8 x 1.0
CS_5	cream	circular, undulate, flat	short rods	diplobacilli	1.3 x 0.5
CS_6	cream	irregular, undulate, flat	long rods	streptobacilli	3.5 x 1.2
CS_7	cream	circular, undulate, flat	short rods	diplobacilli	1.5 x 0.5
CS_8	cream	circular, undulate, flat	long rods	streptobacilli	3.8 x 1.0
CS_9	cream	circular, undulate, flat	long rods	streptobacilli	3.5 x 0.8
CS_{10}	cream	circular, undulate, flat	long rods	streptobacilli	3.5 x 0.4
CS_{11}	cream	irregular, undulate, flat	long rods	streptobacilli	3.1 x 0.7
CS_{12}	cream	circular, serrate, flat	short rods	diplobacilli	1.3 x 0.5

Appendix 2. Continuation

Strain	Colony Color	Colony Morphology	Morphology	Arrangement	Average size (µm)
CS_{13}	white	circular, undulate, flat	long rods	streptobacilli	3.4 x 0.6
CS_{14}	cream	circular, undulate, flat	short rods	streptobacilli	1.3 x 0.5
CS_{15}	cream	circular, undulate, flat	short rods	streptobacilli	1.3 x 0.5
CS_{16}	cream	circular, undulate, flat	short rods	diplobacilli	1.3 x 0.5
CS_{17}	cream	irregular, entire, flat	long rods	diplobacilli	1.3 x 0.6
CS_{18}	white	circular, entire, flat	long rods	streptobacilli	3.1 x 0.4
CS_{19}	white	circular, entire, flat	long rods	streptobacilli	3.2 x 0.5
CS_{20}	white	circular, entire, flat	long rods	streptobacilli	3.1 x 0.7
CS_{21}	cream	circular, entire, flat	long rods	streptobacilli	3.1 x 0.6
CS_{22}	cream	irregular, entire, flat	long rods	streptobacilli	3.5 x 0.8
CS_{23}	cream	irregular, entire, flat	long rods	streptobacilli	3.1 x 0.7
CS_{24}	cream	irregular, entire, flat	short rods	single bacilli	1.0 x 0.5
CS_{25}	cream	circular, undulate, flat	short rods	single bacilli	1.5 x 0.6

Appendix 2. Continuation

Strain	Colony Color	Colony Morphology	Morphology	Arrangement	Average size (µm)
CS_{26}	cream	circular, entire, flat	short rods	single bacilli	1.0 x 0.8
CS_{27}	cream	circular, entire, flat	long rods	diplobacilli	3.5 x 0.6
CS_{28}	cream	circular, entire, flat	long rods	single bacilli	3.5 x 0.8
CS_{29}	cream	circular, undulate, flat	long rods	single bacilli	3.1 x 0.6
CS_{30}	cream	circular, entire, flat	long rods	single bacilli	3.1 x 0.6
CS_{31}	cream	circular, entire, flat	long rods	single bacilli	3.1 x 0.6
CS_{32}	cream	circular, entire, flat	long rods	diplobacilli	3.1 x 0.6
CS_{33}	cream	circular, entire, flat	long rods	single bacilli	3.2 x 0.6
CS_{34}	cream	circular, entire, flat	long rods	single bacilli	3.5 x 0.7
CS_{35}	cream	circular, entire, flat	long rods	single bacilli	3. 0 x 0.4
CS_{36}	cream	circular, entire, flat	short rods	single bacilli	1.0 x 0.5
CS_{37}	cream	circular, entire, flat	short rods	single bacilli	1.0 x 0.5
CS_{38}	cream	circular, entire, flat	short rods	single bacilli	1.0 x 0.3

Appendix 2. Continuation

Strain	Colony Color	Colony Morphology	Morphology	Arrangement	Average size (µm)
CS_{39}	cream	circular, entire, flat	short rods	single bacilli	1.0 x 0.3
CS_{40}	cream	circular, entire, flat	short rods	single bacilli	0.9 x 0.5
CS_{41}	cream	circular, entire, flat	short rods	single bacilli	1.2 x 0.3
CS_{42}	cream	circular, entire, flat	short rods	single bacilli	1.1 x 0.3
CS_{43}	cream	circular, entire, flat	short rods	single bacilli	1.0 x 0.3
CS_{44}	cream	circular, undulate, flat	short rods	single bacilli	1.0 x 0.3
CS_{45}	cream	circular, undulate, flat	short rods	single bacilli	1.0 x 0.3
CS_{46}	cream	circular, entire, flat	short rods	single bacilli	1.1 x 0.3
CS_{47}	cream	circular, entire, flat	short rods	single bacilli	0.9 x 0.5
CS_{48}	white	circular, undulate, flat	short rods	single bacilli	1.0 x 0.3
CS_{49}	cream	circular, undulate, flat	short rods	single bacilli	1.0 x 0.5
CS_{50}	white	circular, entire, flat	short rods	single bacilli	1.0 x 0.5
CS_{51}	cream	circular, entire, flat	short rods	single bacilli	1.0 x 0.3

Appendix 2. Continuation

Strain	Colony Color	Colony Morphology	Morphology	Arrangement	Average size (µm)
CS_{52}	cream	circular, entire, flat	short rods	single bacilli	1.0 x 0.3
CS_{53}	cream	circular, entire, flat	short rods	single bacilli	1.2 x 0.3
CS_{54}	cream	circular, entire, flat	short rods	single bacilli	1.1 x 0.3
CS_{55}	cream	circular, entire, flat	short rods	single bacilli	1.0 x 0.3
CS_{56}	cream	circular, entire, flat	short rods	single bacilli	1.2 x 0.3
CS_{57}	cream	irregular, entire, flat	long rods	streptobacilli	3.0 x 1.0
CS_{58}	cream	irregular, entire, flat	long rods	streptobacilli	3.5 x 0.6
CS_{59}	cream	irregular, entire, flat	long rods	streptobacilli	3.5 x 0.8
CS_{60}	cream	irregular, entire, flat	long rods	streptobacilli	3.8 x 1.0
CS_{61}	cream	irregular, entire, flat	short rods	diplobacilli	1.3 x 0.5
CS_{62}	cream	irregular, entire, flat	long rods	streptobacilli	3.5 x 1.2
CS_{63}	cream	irregular, entire, flat	long rods	diplobacilli	1.5 x 0.5
CS_{64}	cream	irregular, entire, flat	long rods	streptobacilli	3.8 x 1.0

Appendix 2. Continuation

Strain	Colony Color	Colony Morphology	Morphology	Arrangement	Average size (µm)
CS_{65}	cream	circular, entire, flat	long rods	single bacilli	3.2 x 0.6
CS_{66}	white	circular, lobate, flat	long rods	streptobacilli	3.5 x 0.7
CS_{67}	cream	irregular, filamentous, flat	long rods	streptobacilli	3. 0 x 0.4
CS_{68}	white	circular, entire, flat	long rods	streptobacilli	3.0 x 0.5
CS_{69}	cream	irregular, lobate, flat	short rods	streptobacilli	1.0 x 0.5
CS_{70}	cream	irregular, lobate, flat	long rods	streptobacilli	3.4 x 0.3
CS_{71}	cream	irregular, filamentous, flat	short rods	streptobacilli	1.0 x 0.3
CS_{72}	cream	irregular, undulate, flat	long rods	streptobacilli	3.7 x 0.6
CS_{73}	cream	irregular, filamentous, flat	long rods	streptobacilli	3.1 x 0.6
CS_{74}	cream	irregular, filamentous, flat	long rods	streptobacilli	3.1 x 0.4
CS_{75}	white	irregular, filamentous, flat	long rods	streptobacilli	3.2 x 0.5
CS_{76}	white	circular, entire, flat	long rods	diplobacilli	3.1 x 0.7
CS_{77}	white	irregular, entire, flat	long rods	streptobacilli	3.1 x 0.6

Appendix 2. Continuation

Strain	Colony Color	Colony Morphology	Morphology	Arrangement	Average size (µm)
CS_{78}	white	circular, undulate, flat	long rods	streptobacilli	3.5 x 0.8
CS_{79}	cream	irregular, undulate, flat	long rods	streptobacilli	3.1 x 0.7
CS_{80}	cream	circular, lobate, flat	long rods	streptobacilli	3.5 x 0.8
CS_{81}	cream	irregular, filamentous, flat	long rods	streptobacilli	3.5 x 0.4
CS_{82}	cream	irregular, filamentous, flat	long rods	streptobacilli	3.1 x 0.7
CS_{83}	cream	irregular, filamentous, flat	long rods	streptobacilli	3.5 x 0.6
CS_{84}	cream	irregular, filamentous, flat	long rods	streptobacilli	3.5 x 0.8
CS_{85}	cream	irregular, undulate, flat	long rods	streptobacilli	3.5 x 0.5
CS_{86}	white	circular, entire, flat	long rods	diplobacilli	3.1 x 0.6
CS_{87}	cream	irregular, lobate, flat	long rods	streptobacilli	3.5 x 0.7
CS_{88}	white	irregular, entire, flat	long rods	streptobacilli	3.1 x 0.6
CS_{89}	white	circular, entire, flat	long rods	streptobacilli	3.5 x 0.4
CS_{90}	white	circular, entire, flat	long rods	streptobacilli	3.2 x 0.6

Appendix 2. Continuation

Strain	Colony Color	Colony Morphology	Morphology	Arrangement	Average size (µm)
CS_{91}	white	irregular, entire, flat	long rods	streptobacilli	3.5 x 0.7
CS_{92}	cream	irregular, filamentous, flat	long rods	streptobacilli	3. 0 x 0.4
CS_{93}	white	irregular, entire, flat	long rods	streptobacilli	3.0 x 0.5
CS_{94}	cream	irregular, filamentous, flat	long rods	streptobacilli	1.0 x 0.5
CS_{95}	cream	irregular, undulate, flat	long rods	streptobacilli	3.4 x 0.3
CS_{96}	white	irregular, filamentous, flat	long rods	streptobacilli	1.0 x 0.3
CS_{97}	cream	irregular, undulate, flat	long rods	streptobacilli	3.7 x 0.6
CS_{98}	cream	circular, entire, flat	long rods	diplobacilli	3.2 x 0.6
CS_{99}	cream	circular, entire, flat	long rods	single bacilli	3.0 x 1.0
CS_{100}	cream	circular, filamentous, flat	long rods	streptobacilli	3.5 x 0.6
CS_{101}	white	circular, entire, flat	long rods	single bacilli	3.5 x 0.8
CS_{102}	cream	circular, lobate, flat	long rods	single bacilli	3.8 x 1.0
CS_{103}	cream	circular, entire, flat	long rods	single bacilli	3.3 x 0.5

Appendix 2. Continuation

Strain	Colony Color	Colony Morphology	Morphology	Arrangement	Average size (µm)
CS_{104}	cream	irregular, lobate, flat	long rods	streptobacilli	3.5 x 1.2
CS_{105}	cream	irregular, undulate, flat	long rods	streptobacilli	3.5 x 0.5
CS_{106}	white	circular, entire, flat	long rods	single bacilli	3.5 x 1.0
CS_{107}	cream	irregular, undulate, flat	long rods	streptobacilli	3.0 x 1.0
CS_{108}	cream	irregular, undulate, flat	long rods	streptobacilli	3.4 x 0.8
CS_{109}	cream	irregular, undulate, flat	long rods	streptobacilli	3.0 x 0.6
CS_{110}	cream	irregular, lobate, flat	long rods	streptobacilli	3.3 x 0.8
CS_{111}	cream	irregular, filamentous, flat	long rods	streptobacilli	3.5 x 0.4
CS_{112}	cream	irregular, lobate, flat	long rods	streptobacilli	3.1 x 0.6
CS_{113}	cream	irregular, lobate, flat	long rods	streptobacilli	3.5 x 0.4
CS_{114}	cream	irregular, filamentous, flat	long rods	streptobacilli	3.1 x 0.8
CS_{115}	white	circular, entire, flat	long rods	single bacilli	3.5 x 0.8
CS_{116}	cream	irregular, lobate, flat	long rods	streptobacilli	3.5 x 0.4

Appendix 2. Continuation

Strain	Colony Color	Colony Morphology	Morphology	Arrangement	Average size (µm)
CS_{117}	cream	irregular, entire, flat	long rods	streptobacilli	3.0 x 1.0
CS_{118}	cream	irregular, lobate, flat	long rods	streptobacilli	3.5 x 0.6
CS_{119}	cream	irregular, lobate, flat	long rods	streptobacilli	3.5 x 0.8
CS_{120}	cream	irregular, filamentous, flat	long rods	streptobacilli	3.8 x 1.0
CS_{121}	cream	irregular, entire, flat	long rods	streptobacilli	1.3 x 0.5
CS_{122}	cream	circular, entire, flat	short rods	diplobacilli	0.9 x 0.3
CS_{123}	cream	circular, entire, flat	long rods	streptobacilli	3.0 x 0.3
CS_{124}	white	circular, entire, flat	long rods	streptobacilli	3.8 x 1.0
CS_{125}	cream	irregular, lobate, flat	long rods	streptobacilli	3.0 x 1.0
CS_{126}	white	circular, entire, flat	long rods	streptobacilli	3.2 x 0.6
CS_{127}	cream	irregular, lobate, flat	long rods	streptobacilli	3.5 x 0.7
CS_{128}	cream	irregular, lobate, flat	long rods	streptobacilli	3. 0 x 0.4
CS_{129}	cream	irregular, lobate, flat	long rods	streptobacilli	3.0 x 0.5

Appendix 2. Continuation

Strain	Colony Color	Colony Morphology	Morphology	Arrangement	Average size (µm)
CS_{130}	cream	circular, entire, flat	long rods	single bacilli	3.1 x 0.5
CS_{131}	white	circular, entire, flat	long rods	diplobacilli	3.4 x 0.3
CS_{132}	cream	circular, entire, flat	long rods	diplobacilli	3.5 x 0.8
CS_{133}	cream	circular, entire, flat	long rods	diplobacilli	3.7 x 0.6
CS_{134}	white	circular, entire, flat	long rods	diplobacilli	3.2 x 0.6
CS_{135}	cream	circular, lobate, flat	long rods	streptobacilli	3.0 x 1.0
CS_{136}	cream	circular, lobate, flat	long rods	streptobacilli	3.5 x 0.6
CS_{137}	cream	circular, lobate, flat	long rods	streptobacilli	3.5 x 0.8
CS_{138}	cream	circular, entire, flat	long rods	streptobacilli	3.8 x 1.0
CS_{139}	cream	circular, entire, flat	short rods	streptobacilli	1.3 x 0.5
CS_{140}	cream	circular, entire, flat	long rods	streptobacilli	3.5 x 1.2
CS_{141}	cream	circular, entire, flat	short rods	streptobacilli	1.5 x 0.5
CS_{142}	cream	circular, entire, flat	long rods	streptobacilli	3.8 x 1.0

Appendix 2. Continuation

Strain	Colony Color	Colony Morphology	Morphology	Arrangement	Average size (µm)
CS_{143}	cream	circular, entire, flat	long rods	streptobacilli	3.0 x 1.0
CS_{144}	cream	circular, lobate, flat	long rods	streptobacilli	3.0 x 1.0
CS_{145}	cream	circular, lobate, flat	long rods	streptobacilli	3.5 x 0.6
CS_{146}	cream	circular, lobate, flat	long rods	streptobacilli	3.5 x 0.8
CS_{147}	cream	circular, entire, flat	long rods	streptobacilli	3.8 x 1.0
CS_{148}	cream	circular, entire, flat	short rods	diplobacilli	1.3 x 0.5
CS_{149}	cream	circular, entire, flat	long rods	streptobacilli	3.5 x 1.2
CS_{150}	cream	circular, entire, flat	short rods	diplobacilli	1.5 x 0.5
CS_{151}	cream	circular, entire, flat	long rods	streptobacilli	3.0 x 1.0
CS_{152}	cream	circular, entire, flat	long rods	streptobacilli	3.5 x 0.6
CS_{153}	cream	circular, entire, flat	long rods	streptobacilli	3.5 x 0.8
AS_{2}	brown	circular, entire, flat	short rods	diplobacilli	1.2 x 0.5
AS_{7}	brown	circular, entire, flat	short rods	diplobacilli	1.0 x 0.3

Appendix 2. Continuation

Strain	Colony Color	Colony Morphology	Morphology	Arrangement	Average size (µm)
AS_9	brown	circular, entire, flat	short rods	diplobacilli	1.0 x 0.3

Appendix 3

Physiological characteristics that differentiate the alkalithermophilic bacilli from Coamo thermal springs from other *Geobacillus* type strains. Taxa are identified as: 1) *G. uzenensis*^{T} DSM 13551 , 2) *G. vulcani*^{T} DSM 13174, 3) *G. kaustophilus*^{T} DSM 7263, 4) *G. thermoleovorans*^{T} DSM 5366, 5) *G. subterraneus*^{T} DSM 13552, and 6) *G. thermoglucosidasius*^{T} DSM 2542

Characteristic	1	2	3	4	5	6	CS_4	CS_{17}	CS_{30}	CS_{32}	CS_{33}	CS_{36}	CS_{42}	CS_{48}	CS_{83}	CS_{92}	CS_{100}	CS_{101}	CS_{105}	CS_{106}
Amylase	+	+	+	-	+	+	+	-	+	-	-	-	-	-	+	-	+	-	+	-
VP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MR	+	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	ND	-	-
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Galactose	+	+	+	-	-	-	+	+	+	+	+	+	-	-	+	+	-	-	-	-
Lactose	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Gelatin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate	-	-	+	+	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-
Simmons Citrate	-	-	ND	+	-	+	-	-	ND	ND	ND	ND	ND	ND	-	-	ND	ND	-	ND

Appendix 3. Continuation

Characteristic	1	2	3	4	5	6	CS_{110}	CS_{117}	CS_{120}	CS_{121}	CS_{125}	CS_{126}	CS_{134}	CS_{136}	CS_{144}	CS_{146}	CS_{147}	AS_2	AS_7	AS_9
Amylase	+	+	+	-	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	-
VP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MR	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Simmons Citrate	-	-	ND	+	-	+	-	-	ND	ND	ND	ND	ND	ND	-	-	ND	ND	-	ND