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Isolation of Cultivable Halophilic *Bacillus* sp. from the Makgadikgadi Salt Pans in Botswana

Bakang Baloi • Maitshwarelo Ignatius Matsheka* • Berhanu Abegaz Gashe

Department of Biological Sciences, University of Botswana, Pr Bag UB00704, Gaborone, Botswana

Corresponding author: * matsheka@mopipi.ub.bw

ABSTRACT

Halophilic bacteria from the Makgadikgadi salt pans in north central Botswana were isolated using culture-dependent methods. Polymerase chain reaction (PCR) amplification of the 16S rRNA gene and phylogenetic analysis were used to identify the strains. Culturing was done aerobically in six different complex salt media. Salt concentrations used were 15, 20, 25 and 30% (2.6, 3.4, 4.3 and 5.1 M, respectively) NaCl, at pH 7.2 to pH 8.0. Four colony morphology types were isolated in axenic cultures comprising Gram-positive cells. Universal bacterial primers were used to amplify 16S rDNA from chromosomal DNA isolated from three of the four distinct colony groups. Restriction enzyme digests analysis of the 16S rDNA revealed seven RFLP types. Five of the RFLP types were subjected to sequencing. Comparison of the 16S rDNA sequence alignment to reference sequence data bases showed samples S2012A3, S2012B2 and S2012B3 to have between 95 and 99% homology to *Bacillus* sp. BH 164 and *Bacillus* sp. HS 136^T, a novel species recently described as *Bacillus persepolensis*. Isolate S4102D4 showed 95 to 99% homology to *Thalassobacillus* sp. JY0201 and *Thalassobacillus* sp. FIB228 and *Halobacillus* sp. MO56 species. All five isolates had at least 95% similarity to published sequences implying they could be species within the described genera. A sub-tree drawn to compare the isolates indicated two phyletic lines with S4102D4 being an outlying strain and S2012A3, S2012B2 and S2012B3 being a closely related clonal group all of which branch from *Bacillus* sp. BH 164. Pending conclusive culture, biochemical and polar lipid composition data these microorganisms are regarded as previously un-described and therefore novel species of halophilic bacteria.

Keywords: 16S rRNA, halophiles, Makgadikgadi pans, novel species, *Thalassobacillus*

INTRODUCTION

The Makgadikgadi salt pan complex comprising Ntwetwe and Sua pans is located in the north-central part of Botswana. The pans are remnants of a Quaternary super lake that covered between 60 000 and 80 000 km^2 of present day Botswana (Cooke 1980). As an ecological unit the pans cover an area of up to 37 000 km^2, making them some of the largest ephemeral wetlands in the world. They are athalassohaline pans; salt found in the pans is not of marine origin, but rather a result of different geological and hydrological processes (Kerkar 2005).

Sua (20, 55°S: 26, 28°E) is the smaller of the two pans with a surface area of 3400 km^2 and forms the eastern part of the pan complex (Eckardt *et al*. 2008). When it rains, water collects on the shallow pans making a dilute saline solution in which different autotrophic and heterotrophic microbiota flourish. As the year progresses water evaporates and the brine solution becomes increasingly concentrated. Recorded water salinity on different parts of the pans varies seasonally and ranges between 258 mg/l to 31 200 mg/l TDS (total dissolved salts), while the TDS for brines is in the range 90 000-190 000 mg/l (Eckardt *et al*. 2008). In addition to sodium (Na^+) and chloride (Cl^-), other major ions include carbonate (CO_3^{2-}) and hydrogen carbonate (HCO_3^-) with low amounts of sulphate (SO_4^{2-}) or sulphite (SO_3^{2-}), magnesium (Mg^{2+}) and calcium (Ca^{2+}) ions (McCulloch 2003). Increase in salinity from brackish to extremely saline selects for highly salt adapted microorganisms and creates a unique possibility for microbial endemism. Therefore, it is expected that microorganisms that colonize these areas would display unique physiological and biochemical characteristics.

Descriptions of microbiota and meso-fauna species found in the Makgadikgadi include Cyanobacteria, Chlorophyta, Bacillariophyta, Fungi and different types of arthropods (McCulloch, pers. comm.). Brendonck and Riddoch (1997) carried out comprehensive studies for identification, distribution and endemicity of anostracans in different parts of Botswana including the Makgadikgadi. A study by Hulsmans *et al.* (2006) described the Makgadikgadi pans as the only place south of the Sahara where the anostracan *Phallocryptus spinosa* has been recorded. Several genera of salt adapted fungi including *Aspergillus*, *Dendryphiopsis*, *Chaetomium*, *Curvularia*, *Dreshlera* and *Epicoccum* have been described from the Makgadikgadi (Lebogang 2004).

In Africa extensive research has been conducted in the Rift Valley lakes of Magadi, Nakuru, Bogoria and Natron to isolate and identify halophiles for biotechnological applications (Grant *et al*. 1998; Duckworth *et al*. 2000; Yakimov *et al*. 2001; Grant 2004). The Rift Valley contains several soda lakes that represent the most alkaline naturally occurring environments on Earth, with salinities ranging from 5% salt to saturation (Yakimov *et al*. 2001). The majority of these isolates were novel species that had not been isolated before. To our knowledge halophilic bacteria and archaea of Makgadikgadi salt pans have not been identified. No records of bacterial isolation and identification in the Makgadikgadi were found. As such description of halophilic bacteria and archaea in the Makgadikgadi is expected to give an indication of the microbiota in the area. A deliberate effort was made to isolate cultivable species instead of just determining the bacterial diversity, in a bid to obtain viable cultures in which future studies could possibly exploit their potential economic utilisation through biotechnology.

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Original Research Paper

MATERIALS AND METHODS

Sample collection

Soil samples were collected from Sua pan. The preferred sites were those with high recorded salinities. Four soil samples were obtained from three random locations using sterile augers. The first sample (S1) was obtained from loose dry soil (20° 38.78 S: 026° 06.93E), the second (S2) sample was from damp dark grey clayey soil (20° 40.90S: 026° 07.08E). The third and fourth (S3 and S4 respectively) samples were obtained from the same location (20° 38.49S: 026° 06.18E). S3 was sampled from the surface while S4 was from the damp, dark grey and clayey subsurface at a depth of about 10 cm. Soil samples were put into sterile sample bags and transported at ambient temperature to the University of Botswana in Gaborone. On arrival at the University, samples were stored at 4°C for short-term storage or -20°C for longer periods.

Culturing

5 g of each soil sample was inoculated into 45 ml sterile halophile medium (HM) broth (composition (g/l), NaCl (depending on % total salt), $MgCl_2$. $7H_2O$ 20 g, K_2HPO_4 5 g, $CaCl_2$. $2H_2O$ 0.1 g and yeast 5 g extract), and modified halophile medium (MHM) broth (medium composition as for HM, but with the following additional ingredients: Peptone (Biolab) 1 g/l, Tryptone (Bacto™ Tryptone) 1 g/l and 1-5 ml/l glycerol) and incubated at 37°C in an incubator-shaker (Innova™ 4000 Incubator Shaker, New Brunswick Scientific), or a rotor shaker (Orbit Shaker, Lab Line®) at 150 to 200 rpm. Total salt concentrations used were 15, 20, 25 and 30% (w/v) with sodium chloride being the dominant salt. After 7 days' incubation 5 ml of the culture was transferred into 45 ml fresh medium and incubated at 37°C with shaking. From each of the enrichment broth flasks (7 days old) 1ml of culture was aseptically transferred into 9 ml sterile phosphate buffered saline (PBS) to make serial dilutions which were plated onto halophile medium (HM) agar and modified halophile medium (MHM) agar or agarose (15 to 25% NaCl w/v) plates. The plates were incubated aerobically for up to 12 weeks at 37°C.

Light microscopy

At seven day intervals a loopful of broth culture was placed onto microscope slides to make wet mounts. Simple stains and Gram stains were performed using standard procedures on culture smears prepared by the method of Dussault (1955). These were observed by light microscopy at magnification of 1000X using a Carl Zeiss Axiolab and Axioskop2 Plus microscope and photographed using a Carl Zeiss, Axiocam MRC camera.

Genomic DNA extraction and amplification of partial 16SrDNA

Genomic DNA was isolated from pure colonies using the guanidium thiocyanate method (Pitcher *et al*. 1989) and subjected to PCR using Universal Bacterial and archaeal primers. The primers used in the study are bacterial universal forward primer Eubact 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) based on the studies by DeLong (1992): Echigo *et al*. (2005) and Maturrano *et al*. (2006) and reverse primers 1492 R (5′-CGG TTA CCT TGT TAC GAC TT-3′) adapted from studies of Jiang *et al*. (2006) and Maturrano *et al*. (2006). Specific archaeal primers used were forward primer Ar3F (5′-TTC CGG TTG ATC CTG CCG GA-3′) (Maturrano *et al*. 2006) and archaeal reverse primer (5′-AGG AGG TGA TCC AGC CGC AG-3′) (Xu *et al*. 2006). All PCR reactions were done in a 50 or 25 µl reaction volumes, containing dNTP mix to a final concentration of 0.2 mM for each dNTP, primers to a final concentration 0.2 µM each, 1 unit of *Taq* polymerase enzyme and gDNA at a concentration of 25 ng. Promega and Roche PCR kits were used according to the manufacturers' instructions. PCR reactions were done on the Applied Biosystem GeneAmp® PCR System 2400 following a modified protocol described by Maturrano *et al*. (2006). PCR products were resolved by electrophoresis 1% ethidium bromide-stained agarose gel in 1X TAE buffer at 90V for 1 hour. The agarose gels were viewed and photographed using a Syngene® GeneGenius BioImaging System.

Purification and cloning of 16SrDNA PCR products

PCR products from the agarose gels were purified using the Zymoclean gel DNA recovery kit™, according to the manufacturer's instruction. Competent DH5α and *E. coli* JM107 #M109 (Fermentas) cells were prepared using the Z-Competent™ *E.coli* transformation kit and buffer set (Zymo Research). Cloning of the PCR fragment into the pTZ57R/T cloning vector using the InsTAclone™ PCR Cloning kit # K1214 (Fermentas). 100 µl of the transformed cells were platted out in duplicate onto LB medium without ampicillin and with 40 mg/ml X-gal and ampicillin (100 µg/ml). The plates were incubated overnight at 37°C.

Restriction fragment length analysis of the 16S rDNA

Selected recombinant clones containing the partial 16S rDNA fragments were subjected to restriction fragment length analysis using the following restriction endonucleases *Alu*I (Fermentas), *Bam*HI and *Hind*III (Roche). Plasmid isolation was performed using a modified method of Birnboim and Doly (1979) and the Zippy™ Plasmid Miniprep kit (Zymo Research), according to the manufacturer's instruction. The plasmid DNA from overnight colonies was isolated with a Qiagen QIAprep™ Spin miniprep kit for sequencing.

DNA sequencing and analysis

Sequencing was done at Inqaba Biotec laboratories in Pretoria using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA). DNA sequences were subjected to a BLAST nucleotide search on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST) search to compare with published sequences.

Phylogenetic analysis of rDNA sequences

Sample sequences and related sequences retrieved from GenBank were aligned (pair wise and multiple sequence alignments) and analysed using PAUP* version 4.0b10 (Swofford 1998) for MacIntosh (PPC/Altivec). Phylograms of the isolates and described halophilic bacteria were constructed using the Neighbour Joining (Saitou and Nei 1987), Maximum Likelyhood (Felsenstein 1981), Maximum Parsimony (Eck and Dayhoff 1966 cited by Saitou and Imanishi 1989), and UPMGA (Michener and Sokal 1957) algorithms.

RESULTS AND DISCUSSION

Turbidity, signifying microbial growth, was observed in broth cultures within 3-6 days of incubation. Growth in the broth cultures was observed microscopically. Predominant cells were spirals and flagellated rods at the early stages of incubation. These were later replaced by cocci and concave shaped cells and endospores during prolonged incubation. Volumes of broth cultures diluted to 10^{-4} in PBS were among the first to yield colonies on solid media. Direct soil plating also yielded colonies with relative ease compared to culturing from enriched cultures.

Four distinct colonies types from the soil samples were isolated. Two of the colonies types (S201S301) and (S410) were highly mucoid implying production of bio-polymers. All the isolates were cultured in at least 20% salt concentration implying that they are indeed halophiles. Some of the colonies failed to grow during repeated purification steps. An important consideration for this failure may be that pure colony isolation disrupts the nature of intricate ecological interactions within these populations. Complex mutualistic, synergistic or otherwise beneficial associations which cannot be mimicked by laboratory conditions are known to exist in microbial communities (DeLong and Pace 2001). Failure to transfer cells that were growing in broth cultures to solid media in our study could be attributed to

Table 1 Description of isolates and growth conditions.

Strain number	Accession number	Colony characteristics	Growth media	Growth in % NaCl concentration		
				15	20	25
S2012A3	GU111724	Pale white, highly mucoid	Oligotrophic, HM, MHMOGLY	+	+	-
S2012B2	GU111725	Pale white, highly mucoid	Oligotrophic HM, 20MHMOGLY	+	+	-
S2012B3	GU111726	Pale white, highly mucoid	Oligotrophic HM, MHMOGLY	+	+	-
S4102D4	GU111727	Buttery, mucoid, convex	20HM, 20MHM, 20MHMOGLY, 25MHM	+	+	+

+ growth; - no growth.

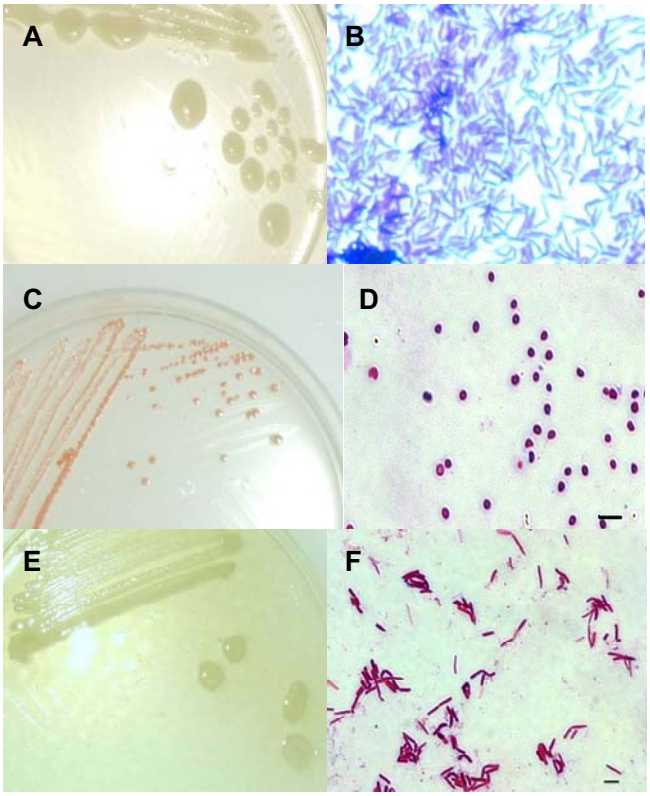

Fig. 1 Purified colonies isolated on 20% MHM and cellular morphologies. A and **B** are S201, **C** and **D** are S203 and **E** and **F** are S401.

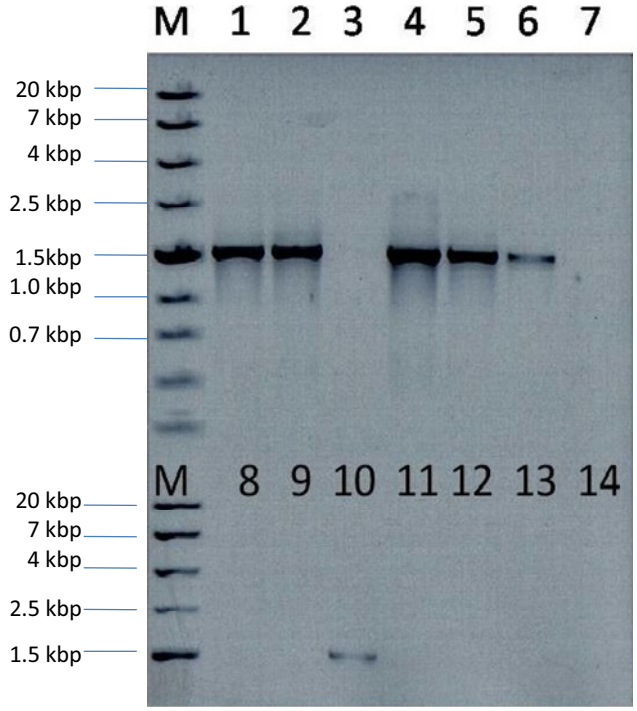

Fig. 2 PCR products of 16S rDNA from genomic DNA extracted from pure colonies. Lane 1, ZipRuler ™ Express DNA Ladder 2 (Fermentas). Lanes 1-6 contain amplicons from samples S201/1, S201/2, S201/3, S232/1, S232/2 and S301/1 respectively. Lanes 8-14 contain samples S203/1, S203/2, S103/1, S203/3, S203/4 and S203/5.

this phenomenon.

Three distinct colony types were eventually isolated and purified. One of the purified colonies was a highly mucoid form that ranged in size from pinhead size to 7 mm in diameter. Two colony representatives recognized (S201 and S301) were isolated from sample site S2 and S3 respectively (**Fig. 1A**). This colony morphology grew on HM, MHM and MHMOGLY, and at salt concentrations between 15 and 25%, showing optimum growth at 20% (**Table 1**). The cell morphology of this colony was mainly Gram negative disk-shaped cells and short rod shaped cells with variable Gram staining characteristics. Endopsores were observed in old cultures. Rods were also observed at higher salt concentrations (20 and 25% salt) (**Fig. 1B**). Pleiomorphology was a phenomenon observed in the isolates and is documented as a common trait for halophiles in culture (Kamekura *et al*. 1988: DasSarma and Aurora 2001: Castillo *et al*. 2006; Gutierréz *et al*. 2007).

The second colony morphology exhibited by pure culture S203, was an orange-red colony pigmentation with a diameter of 2 mm (**Fig. 1C**). This colony was capable of growing in all media and salt concentrations (between 15 and 30% total salt (w/v)) used. When transferred to fresh media for more than three times, the red colour was gradually lost. Slide preparations from broth cultures and pure colonies showed mainly cocci and rod shapes cells with endospores (**Fig. 1D**). The orange-red colour pigment production by S203 is possibly due to carotenoids and shows potential for some biotechnological application. Finally there was a flat, sometimes mucoid or buttery colony (S410)

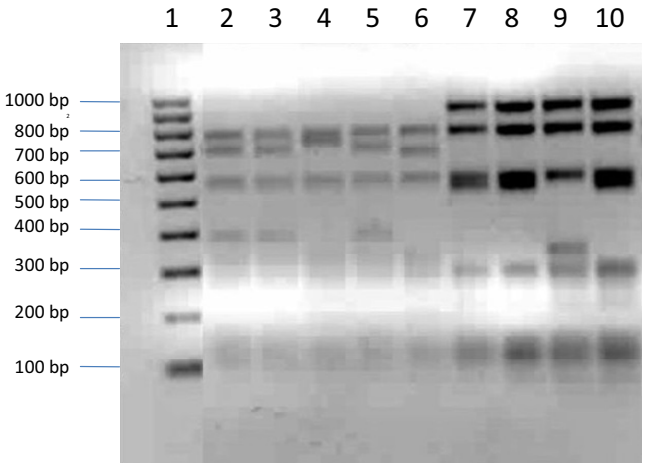

Fig. 3 Restriction fragment length polymorphism of the 16S rDNA of selected recombinant clones digested with *Alu*I. Lane 1, Biorad EZ load 100bp molecular weight marker. Digested plasmid DNA: lane 2 (3011C3), lanes 2 to 6 (S2012A1, S2012A2, S2012A3, S2012B2, S2012B3) lanes 7 to 10 (S4102D2, S4102D4, S4102D5, S4102D7).

with a diameter of about 3 mm (**Fig. 1E**). The colony was isolated from soil sample S4 and grew in media listed in **Table 1**. This colony had cells which appeared as predominantly Gram-positive rods (**Fig. 1F**).

Based on colony morphological properties and micros-

copic cellular characteristics, S201 and S301 were determined to be similar and S201 was chosen as the representative strain for this group. To determine the identity of the three morpho-varieties described above, the representative isolates (S201, S203 and S410) were subjected to PCR using universal 16S rDNA bacterial and archaeal primers. Archaeal primers did not amplify genomic DNA from any of the pure cultures. Sample S203 did not amplify with either bacterial or archaeal primers, even after PCR was repeated several times and with the annealing temperature decreased by 5ºC below the thermal melting point.

Amplification of the 16S rDNA using bacterial primers was obtained for S201 and S410. The expected approximate 1.5 kb amplicons obtained from the genomic DNA of the purified colonies using universal 16S bacterial primers is shown in **Fig. 2**. Lanes 1 and 2 PCR bands of the expected size for S201 strain 1 (S2011) and strain 2 (S2012). No PCR product was obtained for S203 strains (Lanes 11 to 14, 8 and 9) The 16S rDNA amplicons were purified and ligated into the InstaClone TM pTZ57R/T cloning vector for sequencing purposes. However, due to the variable cellular morphologies that were observed in the colonies that were presumed to be pure, RFLP analysis was performed on the recombinant DNA plasmids obtained from ligating the 16S rDNA amplicon and pTZ57R/T cloning vector. This was carried to get an idea of the different types of recombinant DNA molecules yielded by the amplified 16SrDNA from the pure isolates and to establish if indeed the colonies existed as pure entities or a tightly associated conglomeration of symbiotic species. As a result, transformants derived from each of the pure colonies (S2012 and S4102) were subjected to RFLP analysis.

The restriction enzyme *Alu*I was ideal for restriction fragment analysis because it yielded several fragments. A total of five restriction fragment length polymorphism profiles were identified. Clones derived from S2012 gave three different restriction fragment profiles while S4102 transformants gave two profiles. Strains S2012A1, S2012A2 S2012B2 had the same RFLP profile (**Fig. 3**, Lanes 2, 3 and 5), while strains S2012A3 and S2012B3 had unique profiles. The strains S4102D2, S4102D4 and S4102D7 also formed a common profile group (**Fig. 3**, Lanes 7, 8 and 10), while S4102D5 had a unique profile. These different RFLP profiles could possibly represent different phylotypes and representative clones from each of the restriction profile groups namely S2012A3, S2012B2, S2012B3, S4102D4 and S4102D5 were selected and sequenced. Satisfactory sequenced data was generated for strains S2012A3, S2012B2, S2012B3 and S4102D4 and were deposited in GenBank. The accession numbers are given in **Table 1**.

Alignments and sequence comparison was done to assign the isolates to particular taxonomic groups. According to 16S rDNA sequence similarity samples S2012A3, S2012B2 and S2012B3 show high homology (95 to 99%) to *Bacillus* sp. BH 164, *Bacillus* sp. HS 136, *Bacillus* sp. SFB and to an uncultivable halophilic and alkaliphilic bacterium HN24 gene for 16S rRNA. Sample S4102D4 was found to be closely related (98-99%) to *Thalasobacillus* sp. JY0201, *Thalassobacillus* sp. FIB228 and the type strain *Th. Devorans* G-19.1 T, *Halobacillus* sp. MO56 and to *Halobacillus* sp. G19.1. *Bacillus* sp. BH 164 was isolated from hypersaline environment in Korea (Lee and Kim 2005, unpublished) while *Bacillus* sp. HS 136 was isolated from a hypersaline lake in Iran and has recently been described as *Bacillus persepolensis*, a novel species (Amoozegar *et al*. 2009). All of the isolates had at least 95% similarity, and up to 99% similarity, to described sequences within the phyllum Firmicutes, implying that the isolates even though closely related (possibly to genus level), belong to different species.

Phylogenetic analysis was carried out to infer evolutionary relationships between the isolates. Phylogenetic inferences made on the basis of 16S rDNA data indicated that the four isolates may be previously un-described halophilic or halotolerant bacteria. Interestingly sub-trees drawn using different algorithms implied that the isolates may belong to

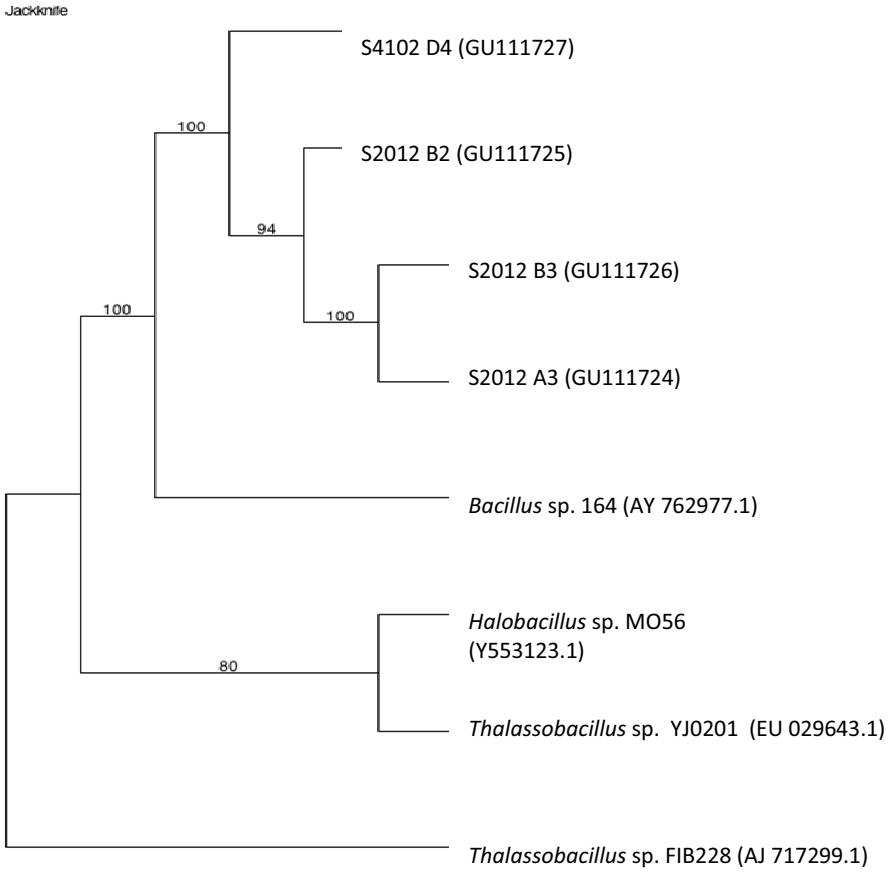

Fig. 4 Unrooted Neighbor-Joining Tree of isolates and described halophiles. Isolates S2012 A3 and S2012 B3 show strong branch support, while isolate S4102 D4 seems to be in a genus by itself.

three different genera within the Bacillacea. The trees showed that isolate S2012A3 and S2012B3 are more closely related than other isolates, with a strong branch support (100% of 1000 replicates) while S2012B2 is related to S4102D4 (75% of 1000 replicates). Other phylogenetic reconstructions show S2011A3 to be in a genus by itself, while isolates S2012B2 and S2012B3 are closely related Isolates S4102D4 is in a genus by itself (**Fig. 4**).

CONCLUSIONS

Therefore regarding all the data presented in this study these microorganisms are regarded as novel species within the phyllum Firmicutes and family Bacillaceae that have not been previously described, pending conclusive culture, biochemical and polar lipid composition data. Although only three of the cultivable bacterial strains were tentatively identified in this study, the Makgadikgadi salt pans seem to have diverse microbiota as numerous bacterial isolates displaying different cultural morphologies where also observed. In conclusion, the Makgadikgadi salt pans still remain a virgin environment in terms of potential bacterial diversity and more studies are required to elucidate the ecological roles and physiological capacity of microbiota inhabiting this unique environment and their prospective applications in biotechnology.

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