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Cultivable Nitrogen Fixing Bacteria from Extremely Alkaline-Saline Soils

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Abstract

Three soils with different Electrolytic Conductivity (EC) from the former Lake Texcoco (soil with low EC 1.2 dS·m^{-1}, medium with EC 83.1 - 107.8 dS·m^{-1}, and high with EC 137.3 - 152.5 dS·m^{-1}) were used to isolate nitrogen-fixing bacterial strains through enrichment cultures in nitrogen-free media. The medium and high EC in the soil affected negatively the nitrogen-fixing activity, which was generally ten times lower compared to the activity in the soil with low EC. Twenty-one bacterial strains were isolated, identified and characterized for their nitrogen fixation capacity. The diazotrophic genetic potential of all isolates was confirmed by amplification and sequencing of partial *nif*H and *nif*D genes and diazotrophic activity quantified by the acetylene reduction assay. *Azospirillum brasilense*, and several species of *Paenibacillus* (*P. fujiensis*, *P. durus*, *P. borealis*, *P. graminis*, *P. massiliensis* and *P. wynnii*) were identified. Isolates belonging to the *Paenibacillus* genus were found in the three soils. *Paenibacillus fujiensis* and *P. durus* showed a high nitrogenase activity. The phylograms based on *nif*H and *nif*D gene sequences were consistent with 16S rRNA gene phylogeny.

Keywords

Diazotrophs, Extremophyles, Haloalkaline, Halotolerant, *Paenibacillus*

1. Introduction

Globally, saline soils or solonchaks cover between 260 million and 340 million ha [1] [2]. Antropogenic activi-

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ties, such as irrigation and excessive use of fertilizer, have increased salt contents in important agriculture production areas affecting soil processes and limiting crop production. Excessive amounts of salts often result in poor soil structure and affect chemical and biological processes [3]-[6]. Saline soils are of particular interest as they allow investigating the effect of high salt contents on microbial activity in a natural environment [6]-[9].

Biological nitrogen fixation controls soil fertility as it represents the most important input of nitrogen into an ecosystem. Activity of nitrogen fixating bacteria, however, is often inhibited by large concentrations of salts [10]-[13]. Nevertheless, Sorokin *et al*. [12] demonstrated that diazotrophs might still be active in soda soils with moderate salinity and alkalinity. Isolation of pure cultures of haloalkaliphilic diazotrophs from several locations in Central Asia and Egypt through micro-oxic enrichments of soils yielded the aerotolerant fermentative haloalkaliphilic bacterium *Amphibacillus tropicus* and the obligately anaerobic haloalkaliphile *Bacillus arsenicoselenatis*. Apparently, nitrogen fixing activity in alkaline saline soils can be attributed to free-living fermentative low- GC gram-positive bacteria [10] [13]-[15].

Most studies on nitrogen fixation activity in soils with high salinity have focused on *Rhizobium*-legume symbioses. Nodule formation on legumes is more sensitive to salt or osmotic stress than the rhizobia. Salt stress inhibits the initial steps of *Rhizobium*-legume symbioses or affects legume root-hair morphology [11]. Another group of diazotrophs well described in salt stressed environments are the haloalkaliphile cyanobacteria found to be responsible for nitrogen fixation in soda lakes and soils [16]-[18]. Only a few obligate anaerobes isolated from soda lakes, such as *Clostridium alkalicellum* [19] and *Geoalkalibacter ferrihydritucus* [20], have been described as diazotrophs.

The soil of the former Lake Texcoco located in the valley of Mexico City (Mexico) can be classified as soda solonchaks. Its pH ranges from 8.3 to 10.2, electrolytic conductivities (EC) in saturation extracts from 1.2 to 200 $dS \cdot m^{-1}$, exchangeable sodium percentages from 75% to 98% and sodium adsorption ratio from 103 to 1718 mM [21]. High evaporation rate and low precipitation (700 $mm \cdot y^{-1}$) increase the concentration of salts. Dynamics of C and N have been investigated intensively [22], and although the bacterial and archaeal communities have been studied [21] [23] [24], no potential diazotrophs have been identified. It can be speculated, however, that nitrogen fixation represents an important input of nitrogen into the environment so diazotrophs should be present. The aim of this work was to determine the nitrogen fixing activity in soils with different electrolytic conductivity, and to identify the cultivable diazotrophic community and to evaluate their potential of nitrogen fixing capacity.

2. Materials and Methods

2.1. Sampling and Sample Description

Three soils from the former Lake Texcoco (19˚30'N, 98˚59'W) with different electrolytic conductivity (EC) were sampled in triplicate. As such, nine soil samples were obtained. Soil was sampled by augering the 0 - 10 cm layer of a 1 m^2-delimited area 20 times while the 0 - 2 cm layer was discarded. Soil samples were transported to the laboratory in black polyethylene bags on ice, 5 mm sieved separately and stored at −20˚C pending analysis.

Soil physicochemical characteristics were determined as described previously [25]. The EC was measured in a saturated solution extract and pH was measured in 1:2.5 soil-H_2O suspensions using a glass electrode (**Table 1**). The first soil had a low EC of 1.2 $dS \cdot m^{-1}$ (considered the Tex-Low soil), the second soil had an EC ranging from 83.1 to 107.8 $dS \cdot m^{-1}$ (considered the Tex-Med soil), while the third sample had EC ranging from 137.3 to 152.5 $dS \cdot m^{-1}$ (considered the Tex-High soil). According to the FAO soil classification**,** a very strong saline soil has an electrolytic conductivity (EC) > 16 $dS \cdot m^{-1}$ [26].

2.2. Culture Enrichments and Isolation of Free Living Nitrogen-Fixing Bacterial Cultures

Diazotrophic enrichment cultures were obtained by adding subsamples of 0.5 g soil to 125 ml serum-flasks containing 100 ml N-free semisolid medium [27]. The medium contained ($g \cdot l^{-1}$): 0.1, $MgSO_4 \cdot 7H_2O$; 0.5, sodium thioglycolate; 0.008, ferric citrate; 0.008, $Na_2MoO_4 \cdot 2H_2O$, and agar 0.1% (w/v). The medium was autoclaved at 121˚C for 15 min. After sterilization, the medium was supplemented with 55 mM filter-sterilized glucose and phosphate buffer (36 mM K_2HPO_4 and 16 mM NaH_2PO_4). Serum flasks were stoppered with cotton plugs and incubated statically at 22˚C for several days. When turbidity appeared, the flasks were closed with sterile rubber stoppers, acetylene added and nitrogenase activity measured after one day. Positive cultures were stabilized by repeated transfers (1% inoculum) with an interval of two weeks.

Table 1. Nitrogen fixation activity in soils of the former Lake Texcoco with different electrolytic conductivity.

Soil	EC[a] ($dS \cdot m^{-1}$)	Acetylene reduction assay (nmol C_2H_4 $h^{-1} \cdot g^{-1}$ soil)		
		1	2	3
Tex-Low	1.2	172 (69)[b]	296 (55)	255 (57)
Tex-Med	83 - 108	47 (33)	16 (6)	16 (6)
Tex-High	137 - 153	30 (12)	8 (4)	30 (10)

[a]EC: Electrolytic conductivity. [b]Values between parenthesis are standard deviations of the mean.

Nitrogen fixing bacteria were isolated in solid N-free culture medium. Serial decimal dilutions of enrichment cultures in sterile water were done and 10^{-5} to 10^{-9} dilutions were spread onto N-free Bridges agar and incubated in an atmosphere enriched with GasPack® system at 28˚C for five days. Five representative colonies of each morphotype obtained were used to confirm nitrogenase activity by the acetylene reduction assay. The positives were conserved in 25% v/v glycerol at −70˚C.

2.3. Differentiation of Pure Cultures and 16S rRNA Gene Sequence Analysis

Random amplification polymorphic DNA (RAPD) fingerprints were obtained to distinguish morphologically similar strains. Briefly, genomic DNA from the isolates was obtained from 48 h bacterial cultures with a QIAamp DNA minikit (Qiagen Inc., Valencia, CA) on the QIAcube® apparatus (Qiagen). Extracted bacterial DNA was eluted from the columns with 200 µl elution buffer and stored at −20˚C.

The RAPD fingerprints were generated using the primer OPB01 (5'-GTT TCG CTC C-3') and the reaction conditions were used as previously reported [28]. The RAPD products were electrophoresed in 1.5% agarose gels in 1 × TAE (40 mM Tris, pH 8.3; 20 mM acetic acid; 1 mM EDTA) and stained with SYBR® Gold (Invitrogen Corporation, Carlsbad, CA).

At least two isolates of each group were selected for identification by a similitude and phylogenetic analysis of the 16S rRNA gene partial sequence. The amplification of 16S rRNA genes was done using the universal bacterial primer 8 forward and 1492 reverse [29]. The PCR products were purified using the QIAquick PCR purification kit according to manufacturer's instructions (Qiagen Inc., Valencia, CA). Sequencing was done by Macrogen Inc. (DNA Sequencing Service, Seoul, Korea).

2.4. Molecular Analyses of *nif*H and *nif*D Genes

The *nif*H and *nif*D genes fragments were amplified from chromosomal DNA samples using primers designed for this work. These primers were chosen from conserved regions detected in multiple sequence (nucleotidic and aminoacidic) alignments of a broad collection of *nif*H and *nif*D genes and design degenerate primer pairs. The designed primers correspond to N19-N38 (5'-TAY GGI AAR GGI GGI ATH GG-3') and N378-N398 (5'-GGI GAY GTI GTI TGY GGI GGI-3') of *nif*H *Methanocaldococcus jannaschii* MJ-4000-136 (DQ516852), and N181-N200 (5'-CGC GGC TGC GCC TAY GCM GG-3') and N1309-N1328 (5'-CCK TTC CGY CAG ATG CAY TC-3') of *nif*D *Klebsiella pneumoniae* (X13303). The PCR reactions were done with an initial denaturation step at 94˚C for 10 min, 35 cycles at 94˚C for 60s, at 55˚C for 60 s, and at 72˚C for 60 s and a final extension at 72˚C for 10 min. Reactions contained 20 ng template DNA, 1× reaction buffer, 50 mM $MgCl_2$, 0.25 mM of each dNTP, 10 pM of each primer and 1 U *Taq* polymerase, adjusted to 25 µl. Purified PCR products were sequenced by Macrogen Inc.

2.5. Phylogenetic Analysis

Taxonomic assignations were done with the RDP classifier 2.2 at an 80% confidence threshold [30] and based on the Greengenes reference database (version 1210). A collection of taxonomically related sequences obtained from the national center for biotechnology information (NCBI) taxonomy homepage (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/) and Ribosomal Database Project-II Release 10 (http://rdp.cme.msu.edu) were used for a multiple alignment analyses with CLUSTAL X [31]. Only common 16S rRNA gene regions were included in the phylogenetic analyses. Maximum likelihood analyses were done

using MEGA version 5 [32]. "Find best model" tool were used to evaluate the substitution models. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (*lnL*), and the number of parameters (including branch lengths) were calculated [33]. The confidence at each node was assessed by 500 bootstrap replicates [34]. The similitude percentages were obtained by subtracting p-distance from one.

2.6. Acetylene Reduction Assay

The nitrogen-fixation (NF) activity of the enrichment-cultures and pure cultures was examined by means of the acetylene reduction activity assay (ARA) as reported by Navarro-Noya *et al*. [28]. Briefly, strains were grown in 10 ml vials containing 5 ml N-free semisolid medium to determine their nitrogenase activity. A single colony of each bacterial strain was incubated at 30˚C for 72 h. The vials were sealed with rubber stoppers and acetylene was injected to a final concentration of 5% (v/v) by replacing an identical volume of air. The test tubes were incubated at 30˚C for 12 or 24 h. The produced ethylene was measured with an Agilent 4890-D GC-17 gas chromatograph (USA). The gas chromatograph was fitted with a 0.2 ml sample loop on the injector port, a 50/80 Porapak N column (182.88 cm by 3.175 mm) and a flame ionization detector. The temperature of the injector was 40˚C, the detector 250˚C and the oven 55˚C. Concentrations of ethylene were calculated every time samples were analysed by comparing peak areas against a standard curve prepared from a standard concentration of 250 ppm ethylene. Reference strain *Klebsiella variicola* ATCC BAA-830T was included as positive and *Escherichia coli* DH10b as negative control.

Total cell protein concentration in the semisolid medium used to determine ARA was measured by the coomassie brilliant blue assay [35]. Bovine serum albumin (sigma no. A5503, Grade V, 99% purity) was used as standard. Prior to the protein determination, the sample was incubated with 10% (w/v) trichloroacetic acid at 90˚C for 20 min, then cooled to room temperature and centrifuged at 8000× g for 15 min [36]. The insoluble protein was disolved in 0.1 M NaOH at 55˚C for 1 h. Semisolid medium without inoculum were processed as control.

2.7. Determination of Tolerance to NaCl

Tolerance to NaCl was determined by broth microdilution according to the CLSI document M27-A2 (2002). NaCl concentrations were varied from 0% to 25% with 1% increments. Briefly, an inoculum suspension was adjusted to a 0.5 McFarland standard and diluted first 1:50 and then 1:20 in R2A medium. Medium R2A contained ($g \cdot l^{-1}$): 0.5, yeast extract; 0.5, proteose peptone (Difco no. 3); 0.5, casamino acids; 0.5 g, glucose; 0.5, soluble starch; 0.3, sodium pyruvate; 0.3, K_2HPO_4; 0.05, $MgSO_4 \cdot 7H_2O$; pH 7.2 [37]. The minimum inhibitory concentration (MIC) microplates were incubated at 28˚C until growth in the medium without NaCl.

2.8. Sequence Accession Numbers

The sequence data reported in this paper have been deposited in the GenBank database, under accession numbers from JQ436863-JQ436919.

3. Results

3.1. Measurements of Nitrogen Fixation Activity in Enrichment Cultures

Nitrogen fixation rate in the Tex-Low soil varied from 172 to 296 nmol C_2H_4 $h^{-1} \cdot g^{-1}$ soil, while in the Tex-High soil from 7.4 to 30.4 nmol C_2H_4 $h^{-1} \cdot g^{-1}$ soil (**Table 1**). Stabilized cultures were obtained after six transfers in N-free medium. Nitrogenase activity in the stabilized cultures of the Tex-Low was 14.2 C_2H_4 $h^{-1} \cdot culture^{-1}$, in Tex-Med 4.3 C_2H_4 $h^{-1} \cdot culture^{-1}$ and in Tex-High 4.8 nmol C_2H_4 $h^{-1} \cdot culture^{-1}$ (data not shown). Gram-positive spore-forming bacilli dominated in all the enrichment cultures.

3.2. Isolation and Identification of Diazotrophic Strains

A total of 39 strains with positive AR activity were isolated from the enrichment cultures. However, after RAPD screening 21 different RAPD-profiles were found (Data not shown). Seven different profiles were found in the Tex-Low soil, 10 in the Tex-Med soil and four in the Tex-High soil.

Strains Tex01-S1, Tex01-S2, Tex01-S3, Tex01-S5, Tex22-S1, Tex02-S4, Tex03-P3, Med01-S2 and Hgh02-

P1 were isolated in consortium with negative NF isolates. Consortiums were more frequent in soil with low EC.

Approximately 1430 nucleotides of 16S rRNA gene sequence of each isolate were used for identification based on pair alignments with sequences from databases and relationships in a phylogenetic tree. A majority of the isolates belonged to the genus *Paenibacillus* (*P. durus*, *P. graminis*, *P. wynnii*, *P. massiliensis*, *P. borealis* and *P. fujiensis*), with similarity percentages of 99.1 - 100 (**Table 2**). Only three of the isolates were identified as *Azospirillum brasilense* (Tex03-P3, Tex02-S3 and Tex02-S3c) and were detected in the Tex-Low sample soil. *Paenibacillus durus* and *P. fujiensis* were isolated from the Tex-Low soil, *P. durus* and *P. graminis* from Tex-Med and *P. borealis*, *P. massiliensis* and *P. wynnii* from Tex-High soil.

Consortium members with negative NF activity were also analyzed for identification. Thus, *Agrobacterium tumefaciens*, *Celullomonas hominis*, *Bacillus circulans*, *Achromobacter xylosoxidans* and *Pantoea agglomerans* were found (**Table 2**).

3.3. Characterization of the Nitrogen-Fixing Isolates

Nitrogen fixation capacity of the isolates was investigated by PCR-amplification of *nif*H and *nif*D genes and quantified by ARA (**Table 3**). Except for the Hgh03-P4 isolate, *nif* genes were detected in all strains. In general, higher NF activities were found in isolates from the Tex-Low soil. Strains with a high activity (2811 - 4358 nmol C_2H_4 h^{-1}·mg·protein^{-1}) were Tex01-S5 and Tex02-S5 identified as *P. fujiensis* (first two) and Md01-S1 as *P. durus*. Strains Tex01-S4, Tex02-P4 and Tex02-S4 showed an NF activity of approximately 1500 nmol C_2H_4 h^{-1}·mg·protein^{-1}.

The isolates grew in medium containing 2% - 10% NaCl. Isolates with a tolerance to 10% NaCl were Hgh02-S1, Hgh02-S1 and Tex01-S5. There was no relationship between source of isolation (soil with low, medium or

Table 2. Phylogenetic affiliation of nitrogen-fixing isolates from soils of the former Lake Texcoco.

Soil	Bacterial strain (GenBank accession number)	Best match[a] (GenBank accession number)	Similarity[b] (%)
Tex-Low	Tex01-S4 (JQ436894); Tex02-S4 (JQ436895)	*Paenibacillus durus* (NR_037017)	100
	Tex01-S5 (JQ436893); Tex02-S5 (JQ436897); Tex02-P4 (JQ436896)	*Paenibacillus fujiensis* (AB092351)	100
	Tex03-P3 (JQ436891); Tex02-S3 (JQ436890); Tex02-S3c (JQ436892)	*Azospirillum brasilense* (EF634031)	99 - 99.2
	Tex03-P3c (JQ436912); Tex01-S2c (JQ436913); Tex01-S3c (JQ436914)	*Bacillus circulans* (JN644554)	99.1
	Tex01-S5c (JQ436888); Tex01-S3b (JQ436889)	*Agrobacterium tumefaciens* (AJ389909)	98.7
	Tex02-S1c (JQ436918)	*Pantoea agglomerans* (AM184264)	100
	Tex01-S2a (JQ436919)	*Achromobacter xylosoxidans* (FJ796451)	100
	Tex01-S1c (JQ436916)	*Celullomonas hominis* (AB480700)	99 - 100
Tex-Med	Md01-S1 (JQ436900); Md01-S4 (JQ436898); Md01-S5 (JQ436899)	*Paenibacillus durus* (NR_037017)	99.5 - 99.8
	Md02-S1 (JQ436905); Md02-S3 (JQ436901); Md03-S1 (JQ436902); Md03-S2 (JQ436907); Md03-S3 (JQ436904); Md03-S5 (JQ436903); Md03-S6 (JQ436906)	*Paenbacillus graminis* (AM745263)	99.6
	Med01-S2c (JQ436915)	*Bacillus circulans* (JN644554)	99.9
Tex-High	Hgh02-S4 (JQ436909); Hgh02-P4 (JQ436908)	*Paenibacillus borealis* (AB073364)	99.1 - 99.6
	Hgh02-S1 (JQ436911); Hgh02-S2 (JQ436910)	*Paenibacillus massiliensis* (AY912109)	100
	Hgh02-P1c (JQ436917)	*Celullomonas hominis* (AB480700)	97.5 - 99.8

[a]The best match was selected using the closest sequence from the phylogenetic tree. [b]Similarity percentage was estimated by considering the number of nucleotide-substitutions between a pair of sequences divided by the total number of compared bases ×100.

Table 3. Acetylene reduction activity and NaCl tolerance in nitrogen-fixing isolates from soil of the former Lake Texcoco.

Soil	Bacterial strain	Acetylene reduction activity (nmol C_2H_4/h/mg protein)	NaCl tolerance[a] (%)
Tex-Low	Tex01-S4	1960 ± 56	2
	Tex01-S5	4358 ± 143	10
	Tex02-S2	429 ± 78	2
	Tex02-S3	484 ± 46	3
	Tex02-S3c	97 ± 4	3
	Tex02-S4	1722 ± 76	3
	Tex02-S5	2811 ± 190	3
	Tex02-P4	1835 ± 24	3
	Tex03-P3	124 ± 7	3
Tex-Med	Md01-S1	2982 ± 699	7
	Md01-S5	574 ± 44	4
	Md02-S1	435 ± 36	2
	Md02-S3	399 ± 29	2
Tex-High	Hgh02-P4	638 ± 75	3
	Hgh02-S1	234 ± 53	10
	Hgh02-S2	324 ± 78	10
	Hgh02-S4	175 ± 12	4
	Hgh03-P2	184 ± 56	3

[a]NaCl tolerance was determined in R2A medium amended with NaCl.

high EC) and NaCl tolerance.

3.4. Phylogenetic Analyses

Maximum-likelihood phylogenetic trees of 16S rRNA, *nif*H, and *nif*D genes were constructed to determine relationships between the sequences of the isolates and related organisms from the GenBank database. Phylogenetic trees obtained with 16S rRNA sequences of the diazotrophic isolates and negative NF co-isolates showed that nitrogen-fixing species of the genus *Paenibacillus* were grouped in three different clusters (**Figure 1**). Cluster A grouped Tex-Low and Tex-Med isolates, cluster B grouped Tex-Med and Tex-High isolates and cluster C grouped Tex-High isolates. The 16S rRNA phylogram also included sequences belonging to the *Proteobacteria* and the *Actinobacteria* phyla, which corresponded to ribosomal sequences of the negative NF co-isolates.

Topology of the Maximum-Likelihood *nif*H tree displays a similar tendency with isolate sequences grouped in three phylogenetic clusters (**Figure 2**). Cluster A was formed by 7 *nif*H gene sequences of isolates from the Tex-Med soil and was related to *P. graminis nif*H gene (84% of identity). Cluster B grouped 3 *nif*H gene sequences of isolates from the Tex-High soil and were related to *P. wynnii nif*H (Hgh02-S4 and Hgh02-P4) and *P. massiliensis nif*H (Hgh02-S2). Cluster C comprised five isolate sequences from the Tex-Low soil and two from the Tex-Med soil. These *nif*H sequences were related to a compressed group formed by *P. forsythia*, *P. sabinae*, *P. durus*, *P. zanthoxyli* and *P. fujiensis*.

4. Discussion

Nitrogen fixation is a key process that is, in part, directly correlated with the primary production of many environments [38] [39]. Together with other processes of the N cycle (e.g. ammonification, nitrification and denitrification), nitrogen fixation is affected by environmental factors, such as temperature, pH, oxygen, heavy metals and mineral nutrients [40]-[42]. Our study documents nitrogen-fixing cultivable bacteria obtained by enrichment

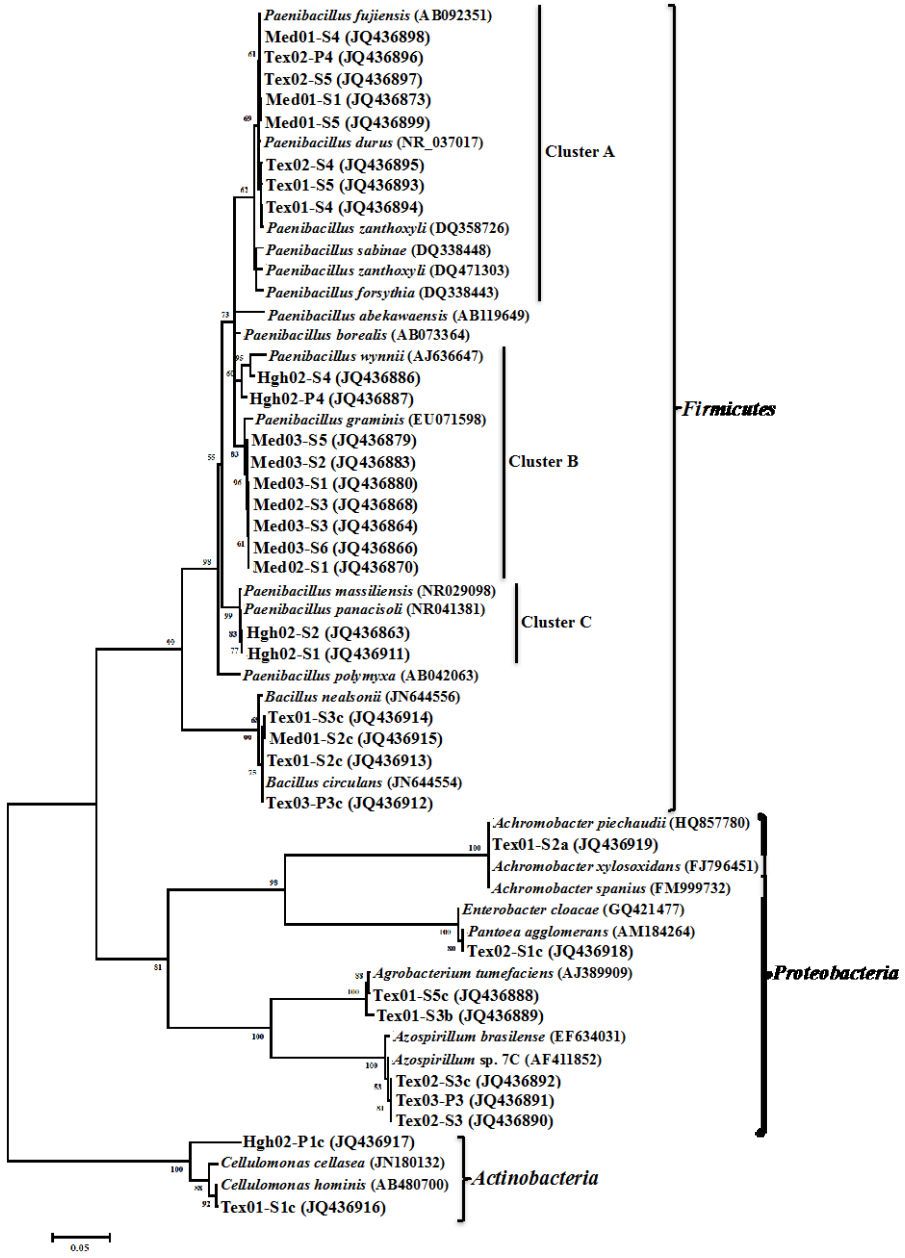

Figure 1. Maximum-likelihood phylogenetic tree showing the position of the 16S rRNA gene fragments amplified from the nitrogen-fixing isolates and consortium coisolates from soils of the former Lake Texcoco. The scale bars indicate the nucleotide substitutions per site. Numbers at the branches indicate the bootstrap values of 500 resamplings. Only values above 50% are shown. Actinobacteria phylum served as outgroup.

cultures in nitrogen-free media occurring in soils with a different degree of salinity.

N_2 fixation rates were obtained (296 nmol C_2H_4 $h^{-1}{\cdot}g^{-1}$ dry soil or 48.5 μg·N·$day^{-1}{\cdot}g^{-1}$ soil), similar to those found in other terrestrial environments, such as in soda solonchak soils and the rhizosphere of some plants [12] [14] [40]. In this work, the high EC, however, affected negatively the nitrogen fixation activity, as it was ten-times lower in Tex-High compared to the Tex-Low soil (**Table 1**). Different effects of salinity on N_2 fixing have been reported. Some studies have found that concentrations of NaCl as low as 75 mM had a negative effect on nitrogenase activity in symbiotic nitrogen fixers [41]. In mangroves, nitrogen fixation was also reduced when pH and salinity increased [43]. However, others have reported no negative effects of high salinity on nitrogen

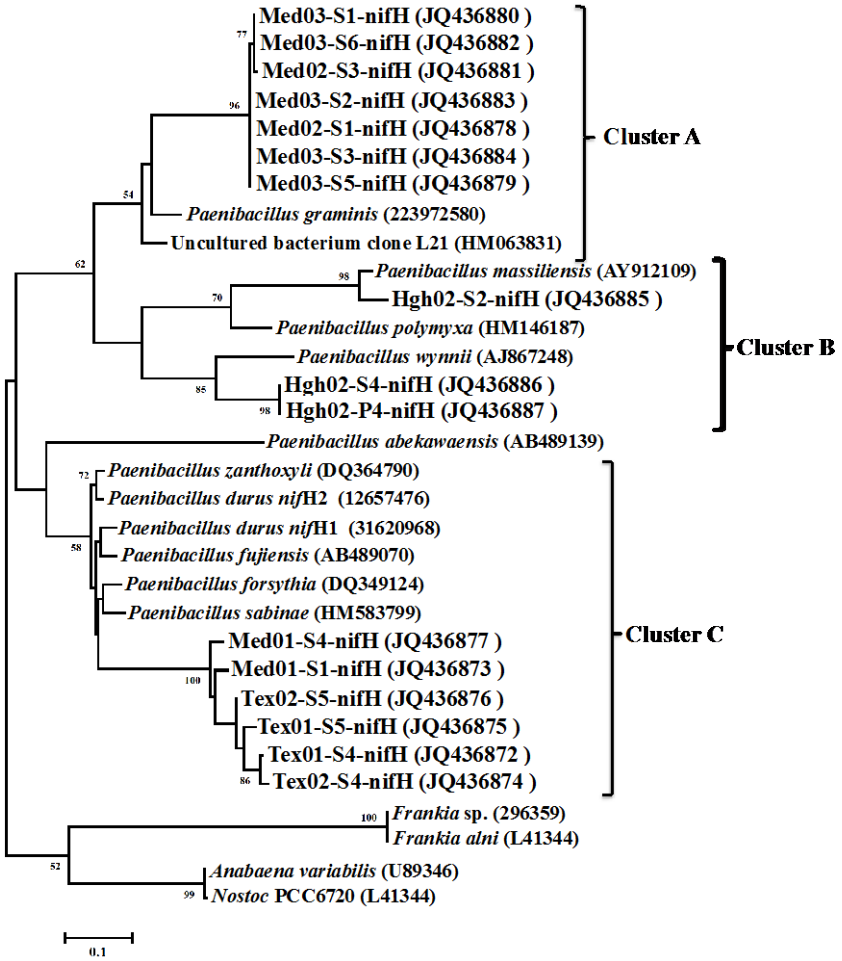

Figure 2. Maximum-likelihood phylogenetic tree showing the position of the *nif*H gene fragments amplified from the nitrogen-fixing isolates from soils of the former Lake Texcoco. The scale bars indicate the nucleotide substitutions per site. Numbers at the branches indicate the bootstrap values of 500 resamplings. Only values above 50% are shown. *nif*H of *Cyanobacteria* group served as outgroup.

fixation activity [44].

pH is also an important factor that affects nitrogen fixation [12] [43] reported that in soda solonchak soils maximum nitrogen fixation activity was obtained at pH 9.8, but at pH 7.3 and 10.5 it decreased 80%. Microorganisms involved in key processes of important biochemical cycles are probably well adapted to extreme conditions. It is possible that the decrease in nitrogen fixation activity in Tex-Med and Tex-High was a synergistic effect of a high salinity and pH.

Isolation of nitrogen-fixing bacteria through enrichment in nitrogen-free media allowed us to identify different species of the *Paenibacillus* genus and *Azospirillum brasilense*. *Paenibacillus* spp. are ubiquitous, and frequently isolated from the rhizosphere of maize, wheat, sugarcane, and pioneer plants growing on mine tailings [28] [45]-[47]. Nitrogen fixing species are also characterized by their capacity to produce phytohormones, solubilize phosphorous and control the access of phytopathogens in plants [45] [46] [48] [49]. In other salt stressed environments, some genera phylogenetically related with *Paenibacillus* have been isolated, e.g. *Amphibacillus tropicus*, *Bacillus arseniciselenatis* in solonchak soils and *Bacillus* spp. in coastal arable saline soils [12] [15]. In mangrove ecosystem other N_2 fixing bacteria have been isolated, e.g. *Azospirillum*, *Azotobacter*, *Rhizobium*, *Clostridium*, *Klebsiella*, *Vibrio*, and *Phyllobacterium* [50]. This is probably due to the specific physicochemical characteristics of this environment. *nif*H genes related with *P. durus* were also detected in solochak soils [12]. Particularly, *A. brasilense* was found only in the soil with low EC indicating its sensitivity to high salt concen-

trations. Nitrogen fixation activity of isolates Tex01-S4, Tex01-S5, Tex02-P4, Tex02-S4, and Med01-S1 identified as *P. durus* and *P. fujiensis* was high and similar to that of other free-living diazotrophs [51].

Bacillus circulans was co-isolated in consortium with several strains. However, to our knowledge, no reports exist of this species as being a diazotroph or any of the other coisolates *Achromobacter*, *Cellulomonas* and *Patoea*. In this study, no nitrogen fixation activity of these bacteria was observed under the conditions tested.

Halotolerant strains of *Bacillus* and *Paenibacillus* were previously isolated from salt mining soils of Russia. However, their nitrogen fixation ability was not tested [52]. Most strains isolated in this work were not able to grow in high or moderate concentrations of NaCl. Isolates Tex01-S5 and Hgh02-S1 identified as *P. fujiensis* and *P. massiliensis*, however, tolerated up to 10% NaCl.

Phylogeny of the 16S rRNA and *nif*H genes showed a clear effect of EC on the distribution of the *Paenibacillus* spp. Apparently there is a gradient along soils with low, moderate and high EC. Thus, *A. brasilense*, *P. fujiensis*, *P. durus*, *P. graminis*, *P. wynnii*, *P. borealis*, and *P. massiliensis* were isolated in that order in the soils from low to high EC. However, their NaCl tolerance profile did not match this observation. It is possible that other characteristics than salinity affected the bacterial community, especially in the nitrogen fixing guild.

Phylogeny of the 16S rRNA gene showed a clear relationship between sequences from the databases and sequences of the isolates, with percentages of similitude ranging from 99% to 100%. *nif*H and *nif*D phylogenies agreed with the ribosomal relationships. Nevertheless, similarity and identity were in the range of 84% - 95% and 86% - 99%, respectively. Ribosomal phylogram showed a close relationship between the cluster formed by *P. fujiensis* and *P. durus* sequences from the database and sequences of the isolates (**Figure 1**). However, *nif*H and *nif*D phylograms showed a close-fitting group formed by *P. zanthoxyli*, *P. forsythia*, *P. durus* and *P. fujiensis*, and relatively distant related isolates (**Figure 2**, **Figure 3**). This suggests that they might be different ecotypes of this species and selected by the extreme conditions of this soil.

5. Conclusion

The results of this investigation concluded that despite extreme salinity and pH conditions, an active bacterial population with a nitrogen fixation potential was found in the soil of the former Lake Texcoco. The cultivable nitrogen-fixing guild in this environment was mainly represented by *Paenibacillus* spp.

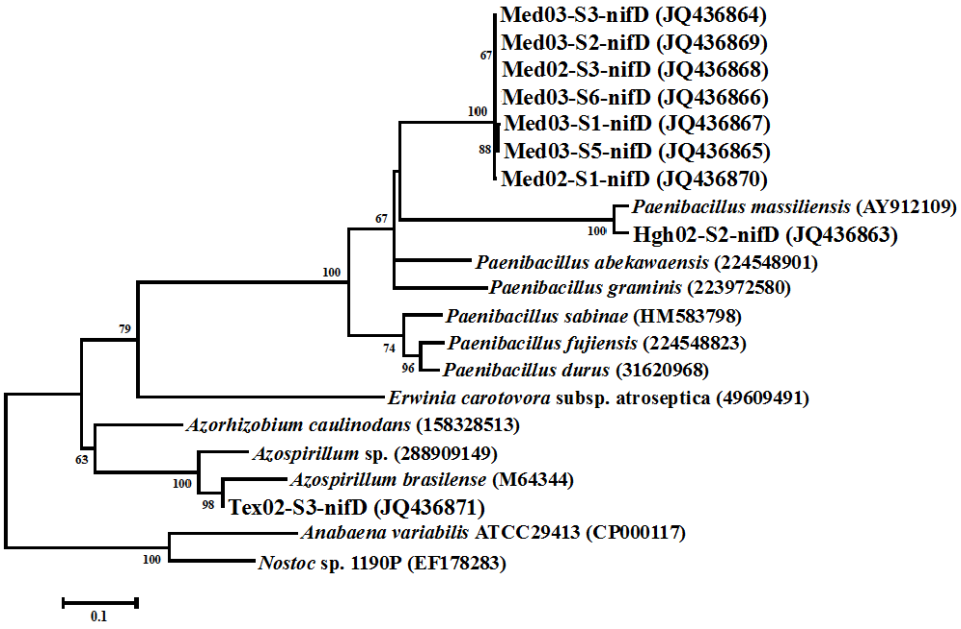

Figure 3. Maximum-likelihood phylogenetic tree showing the position of the *nif*D gene fragments amplified from the nitrogen-fixing isolates from soils of the former Lake Texcoco. The scale bars indicate the nucleotide substitutions per site. Numbers at the branches indicate the bootstrap values of 500 resamplings. Only values above 50% are shown. *nif*D of *Cyanobacteria* group served as outgroup.

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