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Original Research Article

Transcriptional profile in response to NaCl in a halophilic bacteria strain isolated from solar salterns in the southeast of Mexico

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Keywords

Halophilic bacteria, Hypersaline environment, Transcriptional profile, *Virgibacillus*, cDNA-AFLP

A B S T R A C T

Throughout the last decades it has been shown that microbial communities can subsist in adverse conditions such as hypersaline environments with sodium chloride concentrations close to saturation. Halophilic prokaryotes are adapted to thrive in extreme conditions of salinity. Analysis of distinct molecular, physiological and phenotypic characteristics of halophiles provides an insight into the factors responsible for their adaptation to these extreme environments. Here, we report the identification of a moderately halophilic bacterium designated strain COL III-59 that was isolated from a multi-pond solar saltern located in Las Cloroadas, Yucatan (Mexico). The isolate was phylogenetically characterized using 16S rRNA sequencing, showing the strain was affiliated with the phylum *Firmicutes*, genus *Virgibacillus* with a similarity of 99.78%. Transcriptional profiles show changes in response to two different NaCl conditions and a possible key role in the transcriptional regulation that confer the capability to adapt and survey in saline conditions to the bacteria *Virgibacillus sp.*

Introduction

Hypersaline environments are widely distributed on our planet and they are mainly represented by saline lakes, coastal lagoons, man-made salterns and various other water systems, as well as saline soils (Guang *et al.*, 2009; Oren, 2003). One of the most saline

environments that can be found are solar salterns, which consist of a series of shallow ponds in which seawater is evaporated during production of NaCl. The microbial diversity of this type of hypersaline environments has been extensively studied, focusing on the use of both molecular and cultivation-based methods (Benlloch *et al*., 2002). Such microorganisms are exposed to adverse conditions and must cope with its cytoplasmic water, which has higher water potential than the water of the surrounding environment (Van den Burg, 2003). Because of this, the accumulation of organic solutes to establish osmotic balance is the most used adaptive strategy by bacteria to achieve a high osmotic pressure in the cytoplasm (Oren, 1999).

Some halophilic microorganisms are economically important due to biotechnological applications and the amazing ecological traits that allow them to survive in these extreme environments (Oren, 2010). To this aim, we have chosen the most southerly solar salt production system on the Gulf of Mexico used by Industria Salinera de Yucatán S.A. de C.V. "Las Coloradas" solar salterns located in the municipality of Río Lagartos in the state of Yucatán. The solar salterns exhibit several interesting features, the salt production in that area dates from long before the Spanish, probably a few years after Maya colonization in Yucatan, about 475-731 AD (Echánove-Trujillo, 1945).

Nowadays, the multi-pond solar salterns "Las Coloradas" is one of the most important salt productions in the southeast of Mexico; they receive enough solar energy from March to September to reach a production of 500,000 ton per year (Industria Salinera de Yucatán S.A. de C.V.). Therefore the objective of this study was to characterize a halophilic microorganism from "Las Coloradas" solar saltern capable to survive on extremely salt conditions, to study their phenotypic characteristics, phylogenetic affiliation, and their macromolecular characteristics in order to develop a transcriptional profile of differentially expressed sequence by cDNA-AFLP in response to two different conditions of salinity, in the bacteria subsequently identified as *Virgibacillus sp.*

Materials and Methods

Saltern and sample collection

Water and sediment samples were collected from multi-pond solar salterns Las Coloradas (Yucatán). Samples taken along the salinity gradient from six ponds (COL I, COL II, COL III, COL IV, CHIII, CHIII_1), having different salt concentration (6.8–32.9%) were collected during July 2000. Water samples were placed in sterile containers and put on ice until further processing in the laboratory. The temperature and pH was determined for each pond. Isolation of halophilic strains was carried out on a saline basal medium with 5g l^{-1} yeast extract (Difco), 10g l^{-1} tryptone (Difco) (medium 1) and for each pond a specific media was prepared for bacterial isolation according to the pond's intrinsic salinity. Colonies growing on the plates were subjected to successive streak plating to ensure clone purity. All the isolated bacteria were preserved by the Enzyme and Microbial Biotechnology Research Group of the Technological Institute of Merida (ITM).

Organisms and selection of experimental material

Five halophilic bacteria isolated from pond COL III of the solar salterns "Las Coloradas", (labeled as COL III-58, COL

III-59, COL III-60, COL III-61 and COL III-70) were used for an experimental design to determine the salt tolerance on the microorganisms' growth and to select a halophilic strain for this study. Each strain was grown in LB medium (Luria Broth) with three different concentrations of salt; basal saline media (1%w/v), moderate saline media (14.6%w/v) and high saline media (29.41%w/v). The initial optical density of the inoculum at 600nm was identical for all isolates. Each saline treatment was cultivated in three different pH values (6, 7 and 8) and three different temperatures (20, 30 and 40°C). All growth tests were done in continuous shaking at 175 rpm in an orbital shaker (MaxQ4000, Barnstead Lab-line). After 48 hours the absorbance of each culture was measured at 600 nm. These experiments were repeated three times with identical or similar results. The data set was submitted to a two-way analysis of covariance (ANCOVA) using temperature and pH as covariates using StatSoft, Inc. STATISTICA, v.8 computer package.

Nucleic acid extraction, cloning and sequencing

For DNA extraction, bacterial suspension was prepared in TELT buffer (50 mM Tris/HCL pH 7.5, 2.5 mM EDTA, Triton 100%, 2.5 M LiCl), and extracted by phenol-chloroform-isoamylalcohol (50:24:1, v/v/v), precipitated with isopropanol and washed with ethanol 70%. The pellet was dissolved in an appropriate volume of sterile deionized water. 16S rRNA PCR amplification was performed using 1.5u GoTaq-Flexi DNA Polymerase (PROMEGA, Cat. M829B) and primers 16ss (5'-AGAGTTTGATCCTGGCTC-3') and 16sr (5'-CGGGAACGTATTCACCG-3').

PCR product was purified by QIAprep Spin Miniprep Kit (QIAGEN, Cat. # 27106) and cloned using the pGem-T easy vector (Promega, Cat. # A1360) according to the manufacturer's instructions. Then, three clones were selected for complete sequencing. The amplicons were sequenced by Sanger (Cinvestav-Langenbio, Irapuato, Mexico).

Organism Identification and phylogenetic reconstruction

The 16s sequence (1402 bp) identity was calculated using the database analysis tool of EzTaxon server 2.1 (Chun *et al.,* 2007). Relevant sequences of 16S rRNA genes were obtained for comparison from the GenBank (http://www.ncbi.nlm.nih.gov/) and Ribosomal Database Project, RDP (http://rdp.cme.msu.edu/index.jsp). Putative chimeric sequences were recognized and omitted from further studies by using the DECIPHER program (Wright *et al.,* 2011). Evolutionary distance matrices were calculated by using the algorithm of Kimura two-parameter model (Kimura, 1980).

The neighbor-joining (Saitou and Nei, 1987) and maximum-likelihood (Felsenstein, 1981) phylogenetic trees were constructed using MEGA 5 (Tamura *et al.,* 2011). Phylogenetic trees were constructed using subsets of data that included outgroup reference sequences, as well as members of the genera *Ornithinibacillus*, *Oceanobacillus, Jeotgalibacillus* and *Marinibacillus*. The topology and stability of the phylogenetic tree was assessed by the bootstrap analysis based on 1000 replications (Felsenstein, 1985).

Nucleotide sequence accession number

The sequence reported in this study was named COL III-59 with the GenBank accession number KF170374.

Phenotypic and physiological characterization

Cellular morphology was determined by micrograph in Scanning Electron Microscopy (SEM) (Bozzola and Russell, 1992). On the other hand, to evaluate salt tolerance, strain COL III-59 was inoculated into liquid medium 1 with 1% of salt, subsequently serial dilutions were made (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) before plating in LB medium with three different concentrations of salt (1, 5.8 and 14.6%), then 1 µl of each dilution was plated and incubated for 48 h at 35°C. *Escherichia coli* DH5-α was used as test control.

Total RNA Extraction and cDNA-AFLP Analysis

Microorganism COL III-59 was grown in liquid LB medium with for 48 h at 35°C with shaking at 175 rpm. Subsequently we proceeded to collect the entire bacterial culture in two falcon tubes at 1557 x g (4000 rpm) (Sorvall Heraeus Primo/Primo R, Thermo Scientific). One bacterial pellet was resuspended in 16 ml of LB medium with 14.6%w/v NaCl (high salt treatment) and the other bacterial pellet was resuspended in 16 ml of medium 1 with 1%w/v (low salt treatment). Subsequently both bacterial cultures (high and low treatment) were inoculated separately in three assay tubes and then were incubated at 35ºC in three different time periods: 1 hour, 12 hours and 24 hours with shaking at 175 rpm. Total RNA was extracted from the different cultural treatments according to the method "Illustra, RNAspin Mini RNA isolation Kit", (GE *Healthcare,* Cat. # 25-0500-71) following the manufacturer's instructions.

Two biological replicates were performed at each time point. RNA was quantified by agarose gel electrophoresis and spectrophotometric reading (NanoDrop 1000 Spectrophotometer, Thermo Fisher Scientific, USA). First and second strand cDNA synthesis was carried out according SMART™ cDNA Library Construction Kit, Clontech. The first-strand cDNA was synthesized from 1µg of total RNA using 200 U of MMLV reverse transcriptase (New England Biolabs, Cat. # 639523) and 250 ng of random primers (PROMEGA, Cat #C118A). Double-strand cDNA was synthesized using 2µl of 50X *Advantage*® 2 polymerase *Mix* (Clontech, Cat. # 639201). cDNA-AFLP analysis was performed essentially as described previously Bachem *et al*., (1996) and Vos *et al*., (1995) with some modifications (Table 2). PCR reactions were carried out using combinations of the MseI-BstyI primers listed in Table S1 (Aquea and Arce-Johnson, 2008). Selective amplification products were separated in a 6% polyacrylamide sequencing gel. The bands were silver stained as previously reported by An *et al*., (2009).

Results and Discussion

Isolation, sample characteristics and halophilic strains selection

Six saline ponds were tested where the temperature growth range was between 25-30°C, the pH growth range was 7.4–7.84 and the salinity percentage range was 6.8%-32.9% w/v. Five halophilic strains from the pond COL III, with a percentage of salinity of 15.3% w/v (2.62 M NaCl) were named as COL III-58, COL III-59, COL III-60, COL III-61 and COL III -70, and reactivated in liquid medium for 48-72 h at 35°C.

The two-way ANCOVA analysis showed a statistically significant growth of the strain COL III-59, it grows better at 14.6% w/v NaCl than the other strains (Fig. 1) that did

not show a significant difference for the treatments. Temperature and pH covariates used in the experimental design did not show a statistically significant stake in the bacterial growth ($P > 0.05$ in both cases). Therefore strain COL III-59 was selected for further analysis.

Phylogenetic reconstruction

The 16S rRNA gene sequence of the strain COL III-59 was determined as a continuous stretch of 1402 bp. The strain COL III-59 belong to the class *Clostridia*, order *Clostridiales* and is affiliated to the genus *Virgibacillus*. A phylogenetic tree was constructed using the 16S rRNA gene sequence. COL III-59 was compared with all sequences currently available for members of the Genus *Virgibacillus*, and the tree was constructed based on the neighbor-joining method, formed a cluster with *V. salarius*, *V. marismortoui* and *V. olivae* (Fig. 2). The analysis reveals the strain COL III-59 closely related to *V. salarius* AB197851, with a sequence similarity of 99.78%. We used the 16s rRNA sequence of four different genera of the *Bacillaceae* family as outgroup, to frame the genus affiliation *Virgibacillus*. The topology was similar to those of the phylogenetic tree constructed using maximum-likelihood method (Data not shown).

Phenotypic features

The strain COL III-59 was rod-shaped and a borderline extreme halophilic (growing in media containing 1-14.6% salt). Colony formed was cream-slightly brown in color, with smooth edges and circular on basal nutrient agar medium after 2 days of incubation at 35°C. The strain can grow in nutrient medium containing a wide range of NaCl concentration (1-14.6% w/v) but the strain did not grow in the absence of NaCl (Fig. 3). Electron microscopy analysis from strain COL III-59 with an image enlargement of "12,000*X*" and "13,000*X*", showed a bacterial average length of 2 µm and an average diameter of 0.5 µm as show in Figure 4.

Transcriptional Analysis

To determinate transcriptional changes during the early events on stress treatment, RNA samples of *Virgibacillus sp.* COL III-59 strain were collected after inoculation in saline treatments (1, 12 and 24 h). We selected nine combinations of the MseI-BstyI primers as listed in Table S1 for reproducible banding patterns. Only the MseI-1/BstyI-1 combinations produce a transcriptional profile of saline conditions shown in Figure 5. We developed a matrix of Transcript Derived Fragments (TDF's) set from Figure 5, using the presence-absence methodology, assigning the value "1" for present and "0" for absent (Table S2).

A total of 164 differentially expressed TDF's and 18 constitutive TDF's were obtained in a 6% polyacrylamide gel (Table 1). For low saline treatment (1% w/v NaCl) 25 TDF's were increased in intensity (up-regulated) at 1 h post-inoculation, 40 TDF's were up-regulated at 12 h and 39 TDF's at 24 h. For the high saline treatment (14.7% w/v NaCl) 44 TDF's were up-regulated at 1 h post-inoculation, 48 TDF's were up-regulated at 12 h and 39 TDF's at 24 h post-inoculation. Thirty-tree TDF's were regulated in both treatments although differing in time and changes in signal intensity. Several studies have been conducted on the ecology, taxonomy, and phylogeny of moderately halophilic bacteria (Baati *et al.*, 2010). Salt-loving microorganisms that inhabit hypersaline environments need a specialized molecular machinery to cope with the challenges imposed by the osmotic pressure of the environment in order to withstand the

denaturing effects of salt (Sariyar-Akbulut *et al*., 2008).

The genus *Virgibacillus* was proposed by Heyndrickx *et al*., (1998) and the genus includes 28 validly named species. The members of genus *Virgibacillus* have been isolated from different samples around the world such as saline lakes, hypersaline sediments, mangrove soil, sea water, and solar salterns among others and one of the characteristics that distinguish the genus *Virgibacillus* is their dependence to salt concentration (Zhang *et al*., 2012; Amziane *et al*., 2013).The isolate we obtained from a solar saltern of Yucatán (México) was a moderately halophilic strain capable of significantly growth with respect to other isolated bacteria from the same site at a concentration of 14.6% w/v NaCl. The isolate was identified as *Virgibacillus sp.* strain COL III-59. Strain COL III-59 is easily cultivated in minimal medium with a wide range of salt concentration, similar to that reported by Hua *et al*. (2008) which mentions that *V. salarius* strain SA-Vb1 can grow at 0.5-25% w/v NaCl, with small differences with *V. marismortui* that optimally grow at 3-20% w/v NaCl and is not capable to grow more than 20% w/v NaCl (Arahal *et al*., 1999).

The physiological characterization of strain COL III-59 (Fig. 3) shows growth dependence to salt, similar to that reported by Hua *et al*., (2008). The morphology of *Virgibacillus sp.* strain COL III-59 culture was examined by Scanning electron microscopy (SEM) and displayed an ellipsoidal shape, sometimes nearly spherical after 24 h growth on LB 5.8% NaCl where the colonies are 0.5–0.8 µm in diameter, low-convex with slightly irregular margins. The 16S rRNA gene sequence of COL III-59 strain was grouped in the class *Clostridia*, order *Clostridiales* and is affiliated to the genus *Virgibacillus*. The sequence was grouped with *V. salarius* AB197851, *V. marismortui* AJ009793 and *V. olivae* DQ139839 (99.78, 99.78 and 99.5% of identity). The transcriptional profile seen in Figure 5 show different TDF's sizes that ranged from 900-250 bp, similar to the report by Scippa *et* al., (2006) and Dellagi *et al*., (2000). Transcriptional Profiles after 1 and 12 h of growth in high-saline medium were found to have a high similarity with a greater number of TDF's than low-saline treatments. At 24 h the profile in high-saline treatment showed a reduction in a TDF's number, but an equal number of TDF's in low-saline profiles at 12 and 24 h in comparison with 1 h low-saline profile. Clearly the increase in TDF in the high-saline treatments, and the significant increase in the number of TDF's from 1 to 12 h in low-saline profiles indicates that there might be differences in the uptake, production and secretion rates during the treatments therefore causing differences in transcript profiles (Sariyar-Akbulut *et al*., 2008). We can hypothesize the action of regulated late genes to increased stress in accordance with Jurkowski and Reid (2007).

Kuhlmann *et* al., (2011) demonstrated that *Virgibacillus pantothenticus* synthetize the compatible solute ectoine in response to high salinity and carries out the transcription of ectoine biosynthetic enzymes (EctA, EctB, EctC) typically encoded by a gene cluster, ectABC, and genes for ectoine/hydroxyectoine transporters engaged in osmostress protection, whose transcription is up-regulated in response to high salinity and induced in response to increases in the external osmolarity. Sandip *et al*., (2008) reported that molecular signatures for environmental adaptation of extreme saltloving organisms, advocates the convergent evolution of halophilic species towards specific genome and amino acid composition. The adapted features of

halophiles seem to be related to physical principles governing DNA and protein stability, in response to the extreme environmental conditions under which they thrive. They concluded that like proteome composition, halophilic adaptation is also associated with a specific genome signature (Sandip *et al*., 2008).

The present results allowed us to hypothesize about the key role of the saline conditions in the transcriptional regulation that confer *Virgibacillus sp.* COL III-59 the capability to adapt at extreme saline conditions. On the other hand, the data presented here allowed us to conclude that cDNA-AFLP offers the possibility of a quick and reliable analysis of transcription profiles in bacteria in accordance with Decorosi *et al*., (2005) and can also be applied to different microorganisms of different species simultaneously (Sariyar-Akbulut *et al*., 2008). This study proved that the solar saltern of "Las Coloradas" (México) may contain a diversity of halophilic bacteria which could be used for many industrial applications.

Table.1 Number of expressed TDF's in two different saline treatments in *Virgibacillus sp.* strain COL III-59

	1% w/v NaCl	14.6% w/v NaCl
1 h	25 TDF's	44 TDF's
12 h	40 TDF's	48 TDF's
24 h	39 TDF's	39 TDF's

Table.2 Primers used in cDNA-AFLP analysis

Primer	Core sequence	SN[a]
MseI-1	5'- GAT GAG TCC TGA GTA	AT
MseI-2	5'- GAT GAG TCC TGA GTA	CG
MseI-3	5'- GAT GAG TCC TGA GTA	CA
BstyI-1	5'-GAC TGC GTA GTG ATC	TAT
BstyI-2	5'-GAC TGC GTA GTG ATC	CAC
BstyI-3	5'-GAC TGC GTA GTG ATC	CAT

[a] Selective nucleotides

Figure.1 Statistical analysis of the growth of five halophilic bacteria strains from COL III pond on three different NaCl treatments (1%, 14.6%, and 29.4% w/v) for 48 h in medium 1

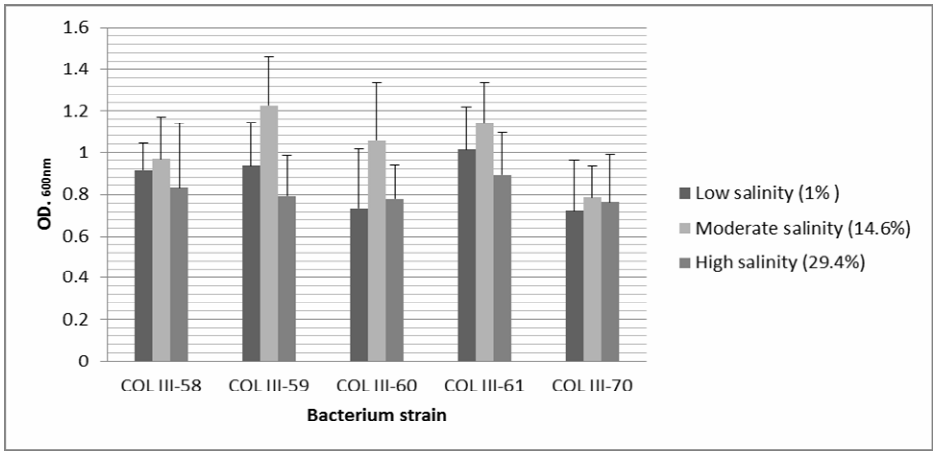

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD. 600nm
COL III-58
COL III-59
COL III-60
COL III-61
COL III-70
Bacterium strain
Low salinity (1%)
Moderate salinity (14.6%)
High salinity (29.4%)

Table.2 Comparative data matrix of TDF's profile constructed from transcriptional profile in Figure 5

		1 h		12 h		24 h	
		14.7% NaCl	1% NaCl	14.7% NaCl	1% NaCl	14.7% NaCl	1% NaCl
		1	2	3	4	5	6
***MWM**	**TDF's**	**44**	**25**	**48**	**40**	**39**	**39**
	1	0	0	1	0	0	0
900pb	2	0	0	1	0	0	0
	3	0	1	1	0	0	0
	4	0	1	0	1	0	1
	5	1	0	0	0	0	0
	6	0	0	0	1	0	0
	7	0	1	0	0	0	0
	8	0	0	1	1	0	0
	9	1	0	0	0	0	0
	10	1	1	0	1	1	1
800pb	11	0	0	1	0	0	0
	12	1	1	0	0	1	0
	13	0	0	0	0	1	0
	14	0	0	1	1	1	0
	15	0	1	1	1	0	0
	16	0	0	0	0	1	0
	17	1	0	0	1	0	1
	18	1	1	1	1	1	1
	19	1	0	0	0	1	0
	20	1	1	1	1	0	0
700pb	21	1	0	0	1	0	0
	22	1	0	1	0	0	0
	23	0	0	0	1	1	1
	24	0	0	1	0	0	0
	25	0	1	0	0	0	0
	26	1	0	0	0	1	1
	27	0	0	1	0	0	1
	28	1	0	0	0	0	0
	29	0	0	1	1	1	0
	30	0	0	0	0	0	1
	31	0	0	0	0	1	1
	32	0	1	0	1	1	1
	33	0	0	1	0	0	0
	34	0	0	0	0	1	1
600pb	35	1	0	0	1	0	0
	36	0	0	1	0	0	0
	37	0	0	0	0	0	1
	38	0	1	0	0	1	0
	39	0	0	1	0	1	1
	40	1	0	0	0	1	0
	41	0	1	0	0	0	1
	42	0	0	0	1	0	0
	43	0	0	0	1	1	0
	44	0	0	1	0	0	0
	45	1	0	1	1	0	0
	46	1	1	0	0	0	0
	47	1	1	1	0	0	0
	48	0	0	0	1	0	0
	49	0	0	1	0	0	0
	50	1	1	0	0	1	1
	51	0	0	0	1	0	0
	52	0	0	0	1	0	1
500pb	53	1	0	0	1	0	0
	54	0	0	0	0	0	1
	55	1	0	0	0	1	0
	56	0	0	1	0	0	0

Continuation of **Table.2**

*MWM	TDF's	1 h		12 h		24 h	
		14.7% NaCl	1% NaCl	14.7% NaCl	1% NaCl	14.7% NaCl	1% NaCl
	57	0	0	1	0	1	1
	58	0	0	0	0	0	1
	59	1	1	1	1	0	0
	60	1	0	0	1	0	1
	61	1	0	0	0	0	0
	62	0	0	0	1	0	1
	63	0	0	1	0	1	0
	64	1	0	0	1	0	0
	65	1	1	1	1	1	1
	66	0	0	1	0	1	0
	67	0	0	0	0	0	1
	68	0	1	1	0	1	1
	69	0	0	0	1	0	0
	70	1	0	1	0	1	0
	71	0	0	1	0	0	0
	72	1	0	1	1	0	0
	73	1	1	0	1	0	1
	74	1	1	**1**	**1**	**1**	**1**
400pb	75	1	0	**1**	**1**	1	1
	76	0	0	1	1	1	1
	77	0	0	0	0	1	0
	78	0	0	1	1	**1**	**1**
	79	0	0	0	0	1	1
	80	0	0	1	0	0	0
	81	0	0	0	0	0	1
	82	1	0	1	1	1	0
	83	0	1	0	0	0	1
	84	1	0	0	0	0	0
	85	1	0	1	0	0	0
	86	0	0	0	1	0	0
	87	1	0	0	1	0	0
	88	0	0	1	1	1	1
	89	0	0	1	1	1	0
	90	1	0	1	0	0	0
	91	0	0	1	1	0	0
	92	**1**	**1**	**1**	**1**	**1**	**1**
	93	1	0	0	0	0	0
	94	1	0	0	0	1	1
	95	1	0	1	0	1	0
	96	0	0	1	0	1	0
	97	0	0	1	0	0	1
	98	1	1	1	0	0	0
	99	1	0	1	1	0	1
	100	1	0	1	1	0	1
	101	0	1	1	0	0	0
	102	0	0	1	0	0	0
	103	0	0	0	0	0	1
	104	0	0	1	1	0	0
	105	0	0	1	0	0	0
	106	0	1	0	0	0	0
	107	0	0	0	0	1	0
	108	0	0	0	0	1	0
	109	1	1	1	1	1	1
	110	0	0	1	0	0	1
	111	0	0	0	0	0	1

9	Differential TDF's between treatments (always present in one treatment and always absent in the other treatment)
122	Differential TDF's on the time (absent in one treatment and present the other one at specific time)
18	Constitutive TDF's (always present with same band intensity)
33	Regulated TDF's. Present in both treatments but with different band intensity. Red numbers.

*MWM. Molecular wiegh marker

Figure.2 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence showing the relationship of strain COL III-59 with other known halophilic bacteria of *Virgibacillus* genus. Numbers at branching nodes are bootstrap values (percentages of 1000 replications); only the values greater than 50% are indicated. Bar, 0.01 substitutions per nucleotide position

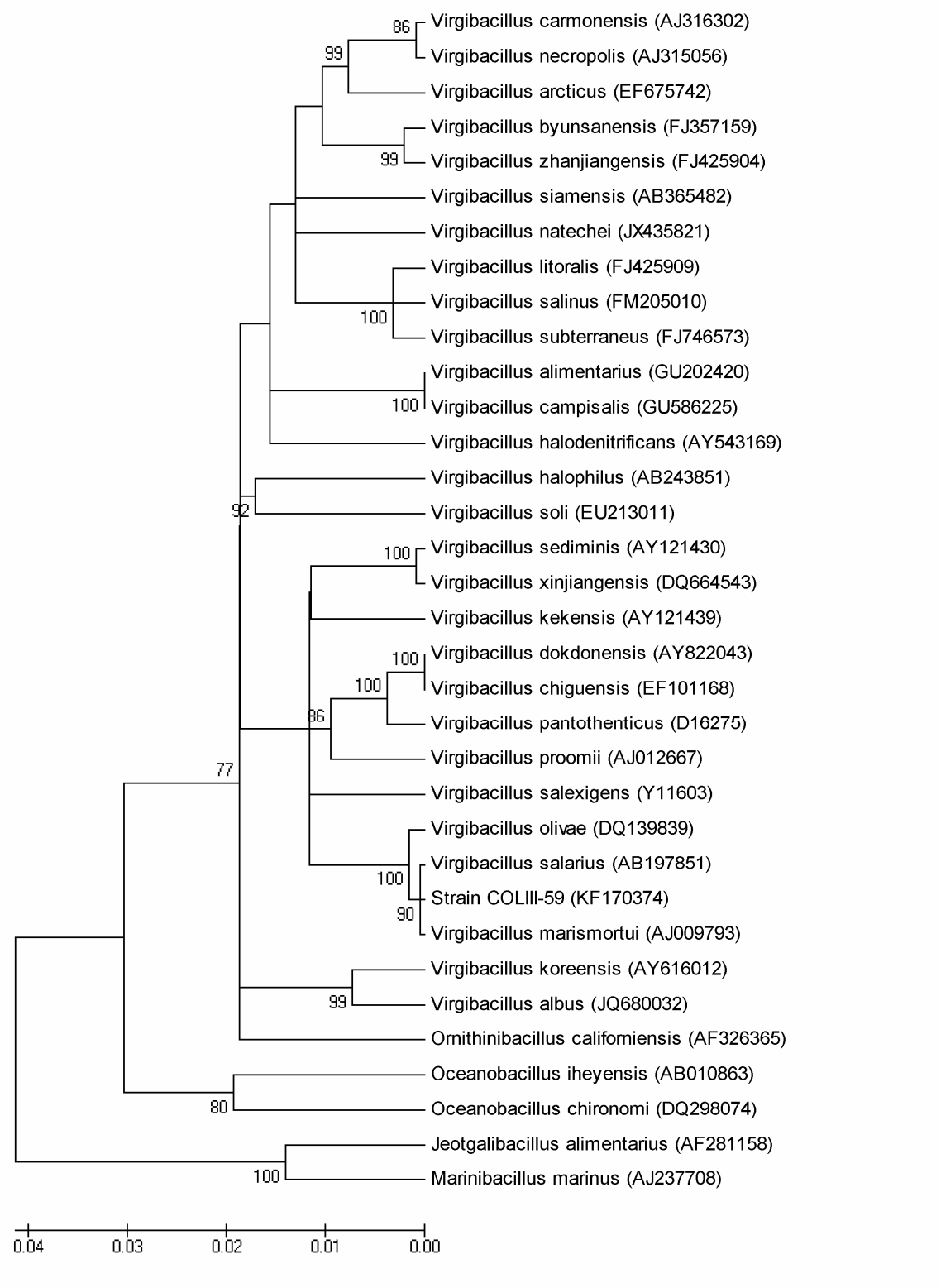


Figure.3 Test for the physiological dependence at saline conditions of *Virgibacillus sp.* (Col III-59) growing in different NaCl concentrations (1, 5.8 and 14.6% w/v). U = Undiluted medium, 10-x = Dilutions. *E. coli* (DH5-α) was used like negative control of saline conditions.

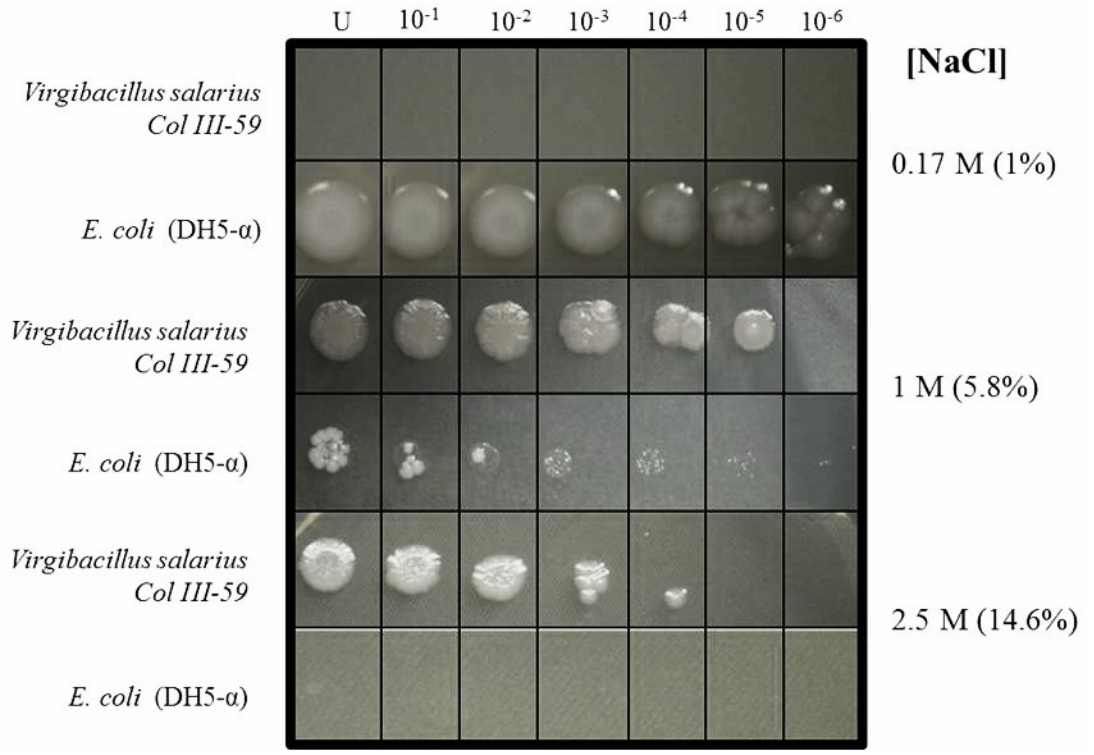


Figure.4 Electron micrographs of *Virgibacillus sp*. under SEM. (Bar, 1.0 µm). a) 12000x b)13000x

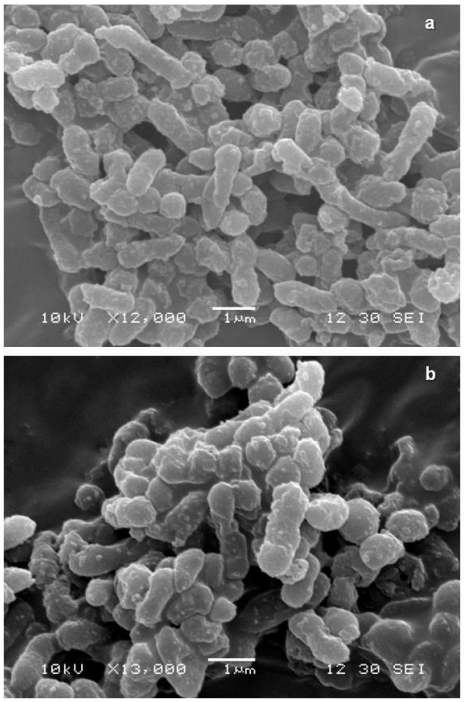
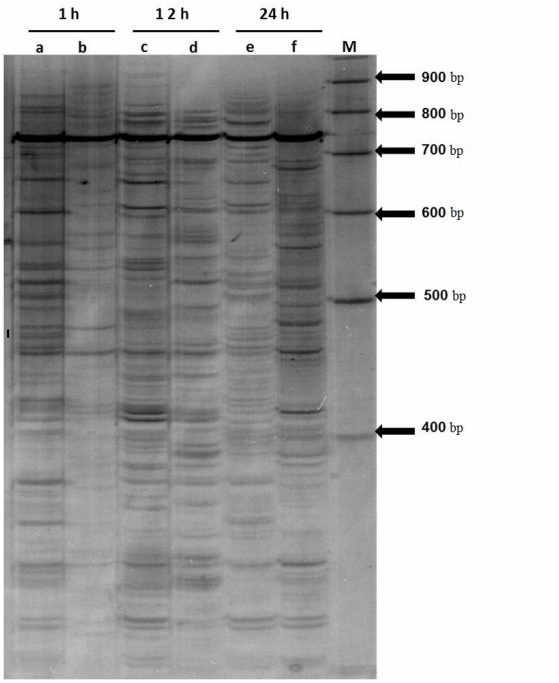


Figure.5 Transcriptional profile. *Virgibacillus sp.* strain COL III-59 cDNA-AFLP were separated on a gel (6% polyacrylamide) showing differentially expressed Transcript Derived Fragments (TDF's) obtained with selective primer combination MseI-1/BstyI-1. (a, c, e) under 14.6% w/v NaCl after 1, 12 and 24 h respectively, (b, d, f) and at 1% w/v NaCl for 1, 12 and 24 h respectively, (M) Molecular Weight Marker 100 bp.



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