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Research article

ISOLATION AND CHARACTERIZATION OF HALOPHILIC BACTERIAL STRAINS FROM SALINE WATERS OF KHEWRA SALT MINES ON THE BASIS OF 16S rRNA GENE SEQUENCE

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ABSTRACT: Halophiles are salt loving microbes optimally growing at high concentrations of salt. Khewra salt mines of Pakistan provide extreme saline conditions where enormous halophilic microbial biota thrives. The present study aimed at isolation and molecular identification of bacterial strains from saline waters of Khewra salt mines. Using halophilic media, nine halophilic bacterial strains from saline water bodies were cultured and studied under optimized growth conditions (NaCl, pH and temperature). Bacterial growth at different NaCl concentrations was measured at 600nm wavelength, showing optimal growth at 1.5M NaCl. 769bp size 16S rRNA gene was amplified for molecular identification of bacterial strains. The amplified genes of the strains FA2.2 and FA3.3 were sequenced and their homology with other bacterial strains was analyzed. The results showed FA2.2 shared maximum homology with *Bacillus anthracis* strain while FA3.3 showed close resemblance with *Staphylococcus saprophyticus* subsp. *bovis.* Isolated halophilic bacterial strains possess potential for various biotechnological applications. They could be manipulated for synthesizing transgenic crops tolerating high salinity boosting the agricultural yield. Moreover extremozymes of these bacteria holds great industrial importance.

Keywords: Halophile, 16S rRNA gene, Molecular identification, Khewra salt mine

INTRODUCTION

Halotolerance is the protoplasmic component of resistance to salt stress. It involves the degree to which the protoplasm, can tolerate the ionic imbalance associated with salt stress, and the osmotic and toxic effects of increased ion concentrations (Larcher, 2001). Microorganisms growing optimally at salt concentrations higher than 100 g/L fall under the category of halophiles. Halophiles are found in all three domains of life i.e., *Archaea*, Bacteria and Eukarya, thriving in widely varying concentrations of NaCl ranging from (0.2 – 5.1M or 2 – 30%). On the basis of this varying NaCl concentration, halophiles are categorized as slight, moderate and extreme halophiles (DasSarma, 2006). The largest family of halophilic *Archaea* is represented by *Halobacteriaceae* comprising of 36 genera with 129 species. Moreover, *Halobacteriaceae* holds the most salt requiring strains (Oren, 2012). In hypersaline environment cell is challenged with reduced water availability and high contents of inorganic ions. During evolution, halophilic bacteria have adopted mechanisms to tolerate high salt concentration. The basic mechanisms involve the extrusion of toxic inorganic ions and accumulation of compatible solutes (Hagemann, 2011). Cell membrane of certain halophiles contains a protein chromoprotein, bacteriorhodopsin which acts as a trans membrane proton pump (Hampp et al., 2000). These bacteria produces large quantity of compatible solutes, gas vesicles, intracellular and extracellular enzymes, salt resistant proteins and high contents of acidic to basic amino acids which acts as adaptive mode to tolerate high salt concentrations (Saum and Muller, 2007).

Halophilic bacteria have the potential to be utilized in biotechnology. They have been used in the production of certain useful products such as β-cartenoids, enzymes, polymers and compatible solutes. Halotolerant bacteria are effective in the treatment of waste water from tannery industry or pickle industry (Kubo et al., 2001). Recently it has been indicated that halotolerant microorganisms have potential to degrade organic and inorganic pollutants (Oren, 2010). These bacteria can be very helpful in genetic engineering as their genetic studies have revealed that they contain large dynamic plasmids having multiple transposable insertion sequences (DasSarma et al., 2001). When the morphological and phenotypic tests for bacterial identification fail to identify the isolates, 16S rRNA based molecular identification achieves the goal because it is universally distributed in bacteria and has species specific variations. This study is based on isolation and molecular identification of halophilic bacterial strains thriving in saline waters using PCR amplification of 16S rRNA gene.

MATERIALS AND METHODS

Sampling

Halophilic bacterial strains were isolated from saline water bodies of Khewra salt mines (Latitude: 32°38'57.69"N and Longitude: 73° 0'32.90"E). Nine water samples were collected in sterile plastic vials from different sites and were taken to molecular biology laboratory for storage at 4°C. Description of samples is given below in Table-1.

Table-1: Sample names in accordance with sample sites and pH

Sr.#	Sample name	Sample site	pH
1	FA 1.1	Minar	7.3
2	FA 1.2	Pond A	7.6
3	FA 1.3	Pond B	7.4
4	FA 2.1	Pond C	7.2
5	FA 2.2	Pond D	7.4
6	FA 2.3	Roof drips water	7.2
7	FA 3.1	Canal	7.3
8	FA 3.2	Near mosque	7.6
9	FA 3.3	Main entrance	7.5

Physico-chemical analysis of water samples:

All water samples were taken to Water and Soil Testing Laboratory, District Gujrat, for physico-chemical analysis. The analysis report is shown below in Table-2.

Table-2: Physico-chemical analysis of water samples

S. No	Sample name	Ece.dSm-1	CO_3^- meq/L	HCO_3^- meq/L	Cl^- mg/L	SO_4^{-2} mg/L	Na^+ ppm
1	FA 1.1	204	-	6.0	150000	7900	22700
2	FA 1.2	211	1.1	4.7	185000	7200	70900
3	FA 1.3	188	0.7	8.3	226000	7700	65500
4	FA 2.1	201	0.3	4.9	199000	7200	100100
5	FA 2.2	220	-	5.5	205000	7500	90100
6	FA 2.3	194	0.4	7.0	207000	7900	82100
7	FA 3.1	203	1.3	7.2	200000	7700	84100
8	FA 3.2	209	0.1	6.3	194000	7300	60900
9	FA 3.3	213	0.9	5.8	195000	7900	66500

Bacterial isolation and enrichment

For the recovery of moderately halophilic bacteria, enrichment and isolation procedures were performed. Serial dilutions for all samples were prepared and appropriately 5% of each sample by volume was inoculated on 3M halophilic media containing (g/L): NaCl, 250; KCl, 2; Peptone, 5; $MgSO_4$, 20; $FeCl_2$, 0.023; tri-Na-citrate, 3; yeast extract, 10; agar, 20. Sub-culturing and colony streaking of isolated strains was carried out to obtain pure cultures. Liquid media cultures were also prepared and inoculated with isolated pure strains. Turbidity in liquid media cultures represented bacterial growth. Further characterization and permanent storage of pure strains at -80°C was carried out later.

Morphological studies of isolates:

Characterization of bacterial colonies for color, form, margin, elevation etc was carried out. Gram staining and cell wall morphological studies under microscope were also performed.

Optimization of growth conditions:

For measuring optimal growth at varying NaCl concentrations (1M, 1.5M, 2M, 2.5M, 3M, 3.5M, 4M, 4.5M, 5M, 5.5M), optical density at 600 nm wavelength was measured using spectrophotometer.

PCR amplification and sequencing of 16S rRNA gene

For amplification of 16S rRNA gene, Genomic DNA was extracted by using TE buffer. Pure bacterial cultures were suspended in centrifuging tube containing buffer solution. After heating at 95°C for 10 minutes and centrifuging at 10,000 rpm for 5 minutes, supernatant was used as template DNA for 16S rRNA amplification. Following primers were used for amplification of genes.

Table-3: Primer set used for 16S rRNA gene amplification

S. No	Primer Name	5'-3' Sequence	Length (bases)	Gene product
1	UPRS	CTCCTACGGGAGGCAGCAGTAG	25	729 bp
2	UPRAS	GCTCGTTGCGGGACTTAACCCAACA	25	729 bp

Phylogenetic Analysis

The purified PCR products were sequenced through Nucleotide Sequencer. The sequencing results were further studied by using Chromas Lite software which resulted in the form of chromatogram. These sequences were then used to find out their phylogenetic relationship by using MEGA-5 tool. Later, phylogenetic trees were constructed showing evolutionary relationships.

RESULTS AND DISCUSSION

Gram staining and other morphological studies of isolates:

Table-4: Morphological characteristics of bacterial isolates

Sr.#	Bacterial isolates	Form	Color	Margin	Elevation	Gram stain
1	FA 1.1	Irregular	Orange	Entire	Raised	+
2	FA 1.2	Irregular	Cream	Entire	Raised	+
3	FA 1.3	Circular	White	Undulate	Flat	+
4	FA 2.1	Circular	Red	Entire	Raised	+
5	FA 2.2	Circular	White	Entire	Flat	-
6	FA 2.3	Irregular	Orange	Entire	Raised	+
7	FA 3.1	Circular	Cream	Undulate	Raised	+
8	FA 3.2	Irregular	Yellow	Lobate	Flat	-
9	FA 3.3	Irregular	White	Undulate	Raised	+

Measurement of optical density at 600nm for optimum growth check:

Strain FA 2.2 was analyzed for optimum growth check at varying salt concentrations. The results calculated by spectrophotometer are shown in Figure 1. According to figure results, FA 2.2 showed optimum growth at 1.5M NaCl concentration. Increase in NaCl concentration resulted in decline in bacterial growth.

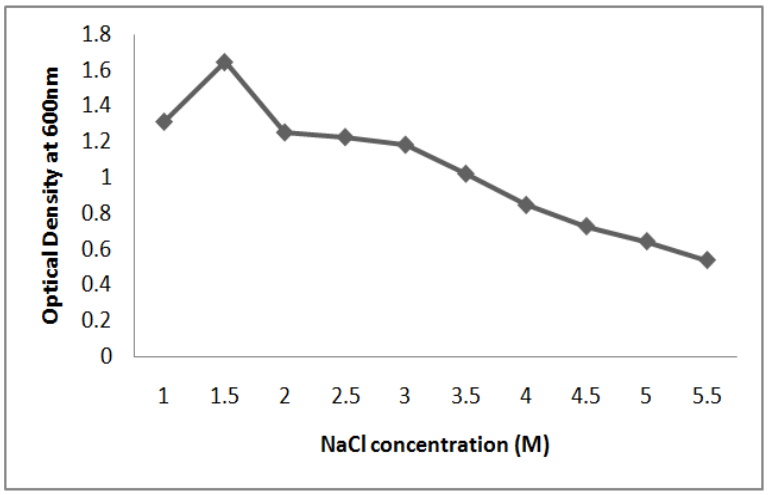

Fig. 1: Growth pattern of FA 2.2

PCR amplification of 16S rRNA gene:

Total 9 bacterial strains were investigated for 16S rRNA gene amplification. FA 1.2 and FA 1.3 did not show bands at all. Whereas FA 3.1 showed a very light band while rest of the samples showed bright bands indicating amplification of gene.

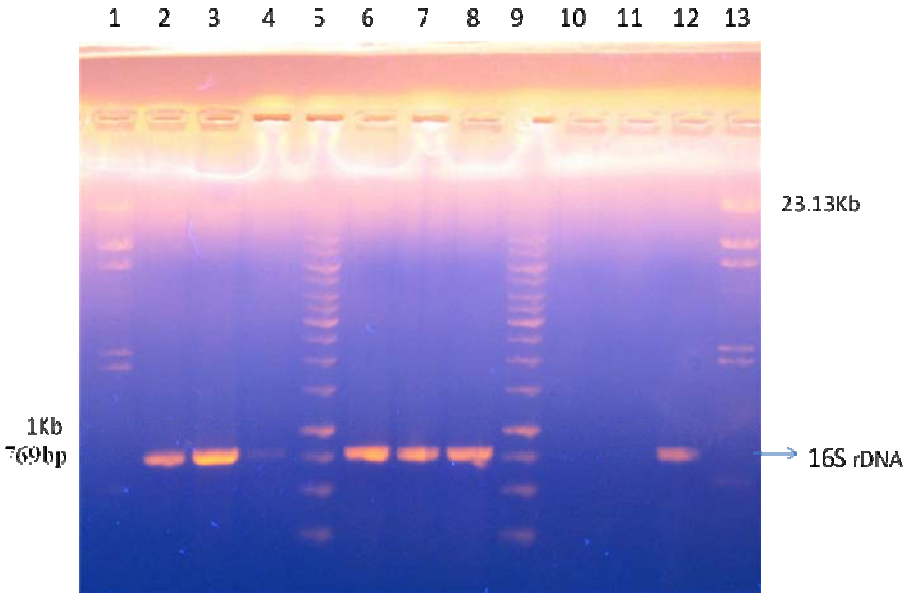

Fig. 2: 16S rRNA gene (769 bp) amplification of bacterial strains. Lane 1 & 13: lambda DNA Hind III marker, Lane 5 & 9: 1kb DNA marker; Lane 2-4: FA 3.3, FA3.2 and FA3.1 respectively; Lane 6-8: FA 2.3, FA 2.2 and FA 2.1 respectively; Lane 10-12: FA 1.3, FA 1.2 and FA 1.1 respectively.

Phylogenetic analysis of strains

For sequencing, two strains FA 2.2 and FA 3.3 were selected. By studying the sequencing results through Chromas Lite software, it was revealed that the gene for FA2.2 was read accurately up to 300 base pairs while gene for FA3.3 was read up to 250 base pairs. The Nucleotide sequences and BLAST results for both strains are given below.

16S rRNA Gene sequence for FA 2.2:

AAGGGCGCAACGCGCGTGAGTATATGCAGGCTTTCGGTTCGTAAAACTCTGTTGTTAGGGA
AGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGC
GTAAAGGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGTCAACCGTGGAGGG
TCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTG
AAATGCGTAGAGATATGGAGGAACA

Fig. 3: 16S rRNA gene sequence of FA2.2 strain

BLAST results of FA2.2:

Table-5: BLAST results of FA2.2

Accession No	Description	Max score	Total score	Query coverage	E value	Max identity
NR_041248.1	*Bacillus anthracis* strain ATCC 14578 16S ribosomal RNA, partial sequence	562	562	98%	2e-160	98%
NR_024697.1	*Bacillus weihenstephanensis* strain DSM11821 16S ribosomal RNA, partial sequence	562	562	98%	2e-160	98%
NR_036880.1	*Bacillus mycoides* strain 273 16S ribosomal RNA, partial sequence	562	562	98%	2e-160	98%
NR_043403.1	*Bacillus thuringiensis* strain IAM 12077 16S ribosomal RNA, partial sequence	562	562	98%	2e-160	98%
NR_043774.1	*Bacillus acidiceler* strain CBD 119 16S ribosomal RNA, partial sequence	507	507	98%	7e-144	95%
NR_041248.1	*Bacillus anthracis* strain ATCC 14578 16S ribosomal RNA, partial sequence	562	562	98%	2e-160	98%
NR_024697.1	*Bacillus weihenstephanensis* strain DSM11821 16S ribosomal RNA, partial sequence	562	562	98%	2e-160	98%

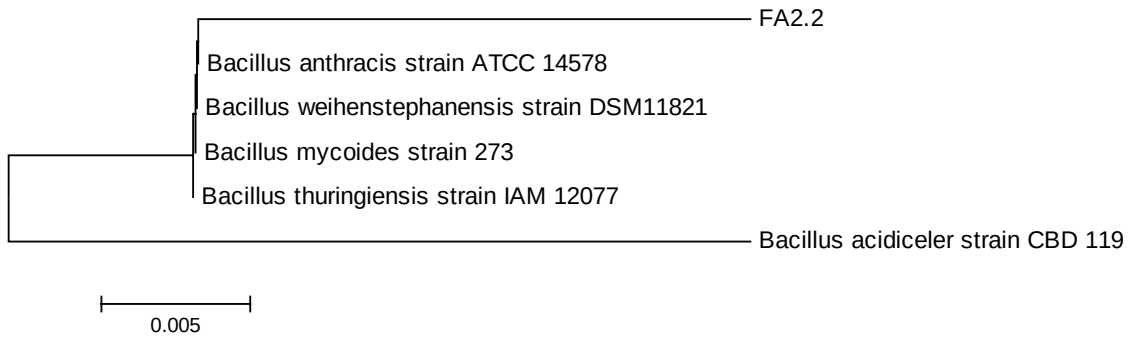

Fig. 4: Evolutionary relationships of taxa with reference to FA 2.2

The evolutionary history was inferred using Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.04956839 is shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using Maximum Composite Likelihood method (Tamura et al., 2004) and were in the units of number of base substitutions per site. The analysis involved six nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. A total of 329 positions were in the final dataset. Evolutionary analyses were conducted in MEGA5 software (Tamura et al., 2011)

16S rRNA Gene sequence for FA 3.3:

CTCCGGCACGAGCTGCGAGAACTATGCACGGCCTGTCGCTTTGTACCCTTTTGTATTAGTGAAGAACA
AGCGGTAAGGTAATGTTCCCCTTTTGATTGTCCTGATCCGAAAGCCCCGGGTTACTACGGGCCAGCCG
GCCCGGTAATACGTAGGTGGGAAGGGTTTTCCGGTATTATTGGGGGTAAAGCCCCCTAGGGGGGTTT
TTTAGTTTTATGTGGAAGCCCACGGGTTAACC

Fig. 5: 16S rRNA gene sequence of FA3.3

BLAST results of FA3.3

Table-6: BLAST results of FA3.3

Accession No	Description	Max score	Total score	Query coverage	E value	Max identity
NR_041323.1	*Staphylococcus hominis* subsp. novobiosepticus strain GTC 1228 16S ribosomal RNA, partial sequence	152	152	77%	2e-37	79%
NR_024669.1	*Staphylococcus pasteuri* strain ATCC51129 16S ribosomal RNA, partial sequence	152	152	77%	2e-37	79%
NR_024668.1	*Staphylococcus lugdunensis* strain ATCC 43809 16S ribosomal RNA, partial sequence	152	152	77%	2e-37	79%
NR_036956.1	*Staphylococcus hominis* subsp. hominis strain DM 122 16S ribosomal RNA, complete sequence	152	152	77%	2e-37	79%
NR_041324.1	*Staphylococcus saprophyticus* subsp. bovis strain GTC 843 16S ribosomal RNA, partial sequence	147	147	77%	1e-35	79%
NR_041323.1	*Staphylococcus hominis* subsp. novobiosepticus strain GTC 1228 16S ribosomal RNA, partial sequence	152	152	77%	2e-37	79%
NR_024669.1	*Staphylococcus pasteuri* strain ATCC51129 16S ribosomal RNA, partial sequence	152	152	77%	2e-37	79%

The evolutionary history was inferred using Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch lengths = 0.34787080 is shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using Maximum Composite Likelihood method (Tamura et al., 2004) and were in units of number of base substitutions per site. The analysis involved six nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. A total of 234 positions were in the final dataset. Evolutionary analyses were conducted in MEGA5 software (Tamura et al., 2011).

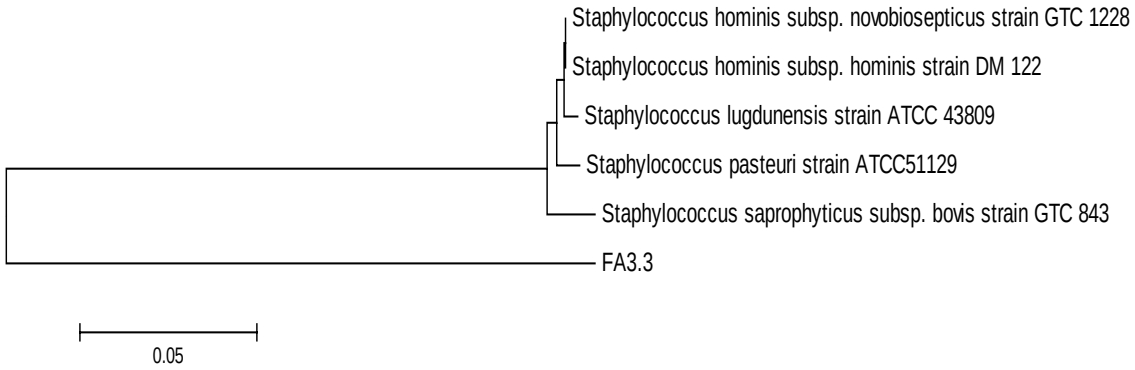

Fig. 6: Evolutionary relationships of taxa with reference to FA 3.3

The evolutionary tree of FA2.2 showed that the strain shows close resemblance with *Bacillus anthracis.* There is 98% homology of FA 2.2 with this strain. It indicates that strain FA2.2 belongs to genus Bacillus. Hence, it is designated as Bacillus spp.FA2.2. While the evolutionary tree of FA3.3 showed its close resemblance with *Staphylococcus saprophytics* with maximum 77% homology. It reveals that the strain FA3.3 belongs to genus Staphylococcus and it is designated as Staphylococcus spp.FA3.3. These two strains are halotolerant and possess adaptive mechanisms to tolerate the hypersaline conditions.

CONCLUSION

The present study successfully identified isolated bacterial strains from Khewra Salt Mine, Pakistan and determined their phylogenetic relationship with other bacterial species. Manipulation of these strains could lead to the synthesis of salt tolerant transgenic crops, degradation of organic and inorganic waste, pigment formation and other potential industrial uses.

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