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RESEARCH PAPER

Isolation, molecular characterization and cloning of *ect* A gene from *Halomonas elongata*

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Abstract: Salinity, an everlasting threat to agricultural production worldwide, affects almost every aspect of the physiology of plants. The present situation calls for the development of salt tolerant transgenic lines to combat this abiotic stress. In this background, the investigation was conducted to isolate the *ect*A gene responsible for salt tolerance from *Halomonas elongata* at IABT, Dharwad, to develop transgenic tomato lines by cloning and transferring *ect*A gene, an ectoine derivative capable of enzymatic action for the production of acetyl-diaminobutyric acid, gene *ect*A is involved in maintaining the osmotic balance of plants. The bacterial strains AUDI series was isolated from marine samples along the West-Cost of India which showed salinity tolerance up to 20 per cent salt concentration. AUDI8 isolate from the PCR positive AUDI series strains was selected for the presence of *ect*A gene. A 579 bp PCR product containing *ect*A gene was amplified using *ect*A gene specific forward and reverse primers. The amplified product was cloned into pTZ57R/T and it was named as pDBJ26 and sequencing of this construct shows 99.66 per cent similarity with other isolates in NCBI (LC168837.1 and AF031489.1).

Key words: Ectoine, Osmotic balance, Salt tolerance, Transgenic lines

Introduction

Salinity is a soil condition characterized by a high concentration of soluble salts in the soil. Salinity is increasing in the regions where irrigation is an essential aid to agriculture (Flowers, 2004). Overall, 20 per cent of all the cultivated land and 33 per cent of irrigated lands are affected by salinity. Further, the salinized areas are increasing at the rate of 10 per cent every year and 50 per cent of the arable land would be salinized by the year 2050 (Jamil *et al.,* 2011). In India extent and distribution of salt affected area is about 6.5 million hectares among that, area under saline soils is about 1.7 million hectares, alkali soil is about 3.8 million hectares and that of coastal saline soil is about 12.5 million hectares.

Halophiles are salt cherishing microorganisms that develop over a wide range of salt concentration, (3-15% NaCl, w/v or more) and possess hyper saline environment (Shivananda and Mugeraya, 2011). A range of heterotrophic and methanogenic archaea, photosynthetic, lithotrophic and heterotrophic microbes, photosynthetic and heterotrophic eukaryotes have been recorded among Halophilic microorganisms. Being the reservoir of the salt tolerant species, marine environment is the promising source for salt tolerant genes (Rajan, 2010).

Ectoine is synthesized in three progressive enzymatic steps beginning from aspartic β-semialdehyde. The genes engaged with the biosynthesis are called *ect*A, *ect* B and *ect* C and they encode the enzymes L-2, 4-diaminobutyric acid acetyltransferase, L-2, 4-diaminobutyric acid transaminase and L-ectoine synthase, respectively. The next step, which is catalyzed by ectoine hydroxylase *ect* D, changes over ectoine into its S, S-hydroxy derivative. The relative ratio of hydroxy ectoine is commonly increased in *H. elongata* under salt and temperature stress and hence, accepted to exert prevalent adjustment properties.

Cloning and expression of individual genes encoding ectoine biosynthetic pathway were conducted by Afzal, 2014 *ect*B and *ect*A and Holck, 2014 *ect*C in the chloroplast of *Chlamydomonas reinhardtii.* Diaminobutyric acid (DABA) acetyl transferase (*ect*A) catalyzes the acetylation of diaminobutyric acid to acetyldiaminobutyric acid, which is involved in salt tolerance in plants.

Material and methods

Bacterial samples

The available marine bacterial isolates of AUDI series (Alone, 2012) were screened on the basis of percentage salt tolerance *viz*., 1, 2, 4, 8, 12, 16 and 20 per cent using sodium chloride in Luria Betani Agar media for growth. Sodium chloride was added at the rate of 1, 2, 4, 8, 12, 16 and 20 gm per 100 ml of LB media to get 1, 2, 4, 8, 12, 16 and 20 per cent, respectively. The bacterial cultures were screened on the basis of salt tolerance.

DNA isolation from *Halomonas elongata*

Isolation of total DNA from bacterial isolates was done using standardized protocol (Chougala, 2016). Bacterial inoculation into LB broth was done on the previous day of DNA isolation and was given an overnight shaking at 200 rpm at 37^0C for 12 hours.

A quantity of 5 ml of the overnight grown bacterial culture was transferred to 1.5 ml eppendorf tube and then centrifuged at 13,000 rpm for 1 min. The supernatant was discarded without disturbing the pellet. The pellet was resuspended in 600 µl lysis buffer and vortexed completely to resuspend cell pellet. It was incubated at 65°C for 10 mins. Equal volume of phenol: chloroform: isoamyl alcohol was added with mixing by inverting the tube until the phases are completely mixed. A spin at 13000 rpm for 5 mins was given at room temperature until the separation of the layer is clearly visible. The upper aqueous layer alone

was slowly pipetted out into a new 1.5 ml eppendorf tube. Equal volume of chloroform was added and was mixed well by inverting the tubes to remove phenol. The mixture was given a spin at maximum speed of 13000 rpm for 5 mins. The aqueous layer was removed to a new tube. Three volumes of cold isopropanol were added to precipitate DNA and were mixed gently. Incubation was given at -20^{0}C for half an hour and further it was given a maximum spin at 13000 rpm for 15 mins at 4^{0}C. The supernatant was discarded and the pellet was rinsed with 70 per cent ethanol stored at room temperature. Further, it was given a maximum spin at 13000 rpm for 2 mins. The supernatant was discarded and dried at 37^{0}C. Then, DNA was resuspended in TE buffer. The presence of DNA was checked using a gel run. The quantity and quality of the DNA was checked in a nano drop spectrophotometer.

Primer designing

The sequence coding for *ect*A gene was retrieved from NCBI database. Using vector NTI software gene specific primers were designed, the designed primers were synthesized at Sigma-Aldrich chemicals Pvt. Ltd., Bangalore. The designed sequence of primers is as follows.

*ect*A (forward) - 5'ATGAACGCAACCACAGAGCCCTTTACA 3'

*ect*A (reverse) - 5'CGGCGTCCGAGCTCAGATCTGGT 3'

PCR amplification of *ect*A using gene specific primers

Polymerase chain reaction (PCR) was conducted to amplify the *ect*A gene using gene specific primers. DNA was diluted to 100 ng using $T_{10}E_1$ and it was used as template for PCR. Reactions were incubated for initial denaturation of 5 mins at 95^{o}C and then run for 35 cycles at 95^{o}C for 1 min, 55.5^{o}C for 1 min and 72^{o}C for $1^{1/2}$ mins. Final extension of 72^{o}C for 8 mins and hold at 4^{o}C was done. The amplicons were separated electrophoretically on 1 per cent agarose gel.

***Pfu* polymerase based PCR**

The *ect*A gene amplification was done using the aforementioned protocol with *Pfu* polymerase instead of *Taq* polymerase mainly for proof reading activity.

PCR purification of the amplified products

The bulk amplified gene products were purified using QIAquick PCR purification kit (Cat.No.28104; Qiagen, Germany) as per the protocol provided.

A-Tailing of the PCR amplified product

A separate A-tailing was given to the gene amplified product using the following reaction components. PCR amplified DNA 30 µl, 10x buffers 5 µl, 1 mM dATP 10 µl, *Taq* DNA polymerase 0.2 µl, nuclease free water 4.8 µl and the final volume was made upto 50 µl then incubated at 72°C for 20 minutes. Then, the A-tailed product was run on 0.8 per cent agarose gel prepared in 1xTAE buffer for getting a confirmation with the gene size.

Gel elution and ligation of specific *ect*A gene fragment

The desired amplicon was purified from agarose gel by using QIA quick gel extraction kit (Cat.No.28704; Qiagen, Germany) according to the protocol made available. The gel purified 579 bp amplicon was ligated into pTZ57R/T (as per Fermentas Life Sciences, EU). The ligated products were used for transformation of *E. coli* DH5α. Transformants were screened by blue white colony assay. Then further, the clones were confirmed by colony PCR and also by restriction analysis using *Kpn*1 and *Bam*H1. Then the construct was named as pDBJ26.

Sequencing and sequence based analysis

The confirmed clone was sent for sequencing to Eurofins Genomics India Pvt. Ltd, Bengaluru, India. Sequencing was done by using gene specific forward and reverse primers. Sequences were processed to remove the vector contamination by using BioEdit software. Further, the homology search was done with BLAST algorithm available at *http://www.ncbi.nih.gov.*

Results and discussion

During this investigation, previously recognized salt tolerant bacterial isolates AUDI8, AUDI18, AUDI34, AUDI144 from the marine water samples along the West-Cost of India were collected (Fig.1).

Total genomic DNA was extracted and isolated from salt tolerant bacterial strains which were of good quality based on nanodrop analysis. The OD proportion and concentration at 260/280 nm for all genomic DNA isolated from samples was ranged between 100-1000 ng/µl and 1.8-1.9. Further, quality of DNA present in the sample was confirmed by agarose gel electrophoresis (Fig.2).

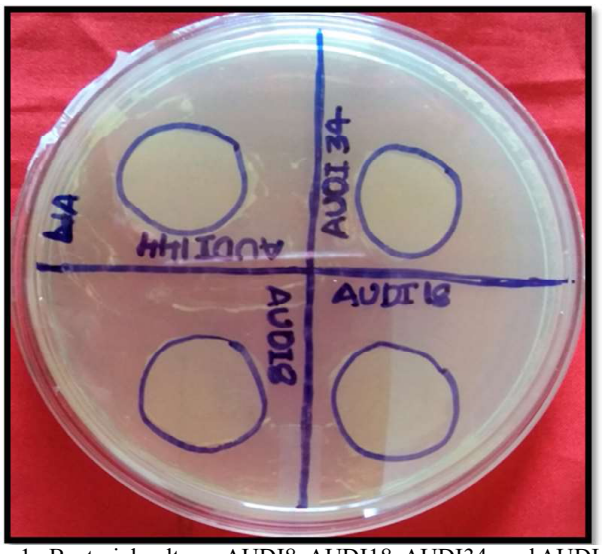

Fig. 1. Bacterial cultures AUDI8, AUDI18, AUDI34, and AUDI144

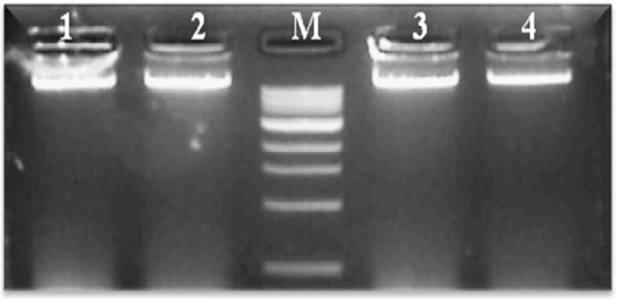

Fig. 2. Genomic DNA of bacterial cultures AUDI8, AUDI18, AUDI34, and AUDI144
(M: 1kb ladder, 1-4: Bacterial DNA samples)

The total DNA isolated from AUDI 8, 18, 34, 144 were used as template for the PCR amplification of *ect*A gene using gene specific forward and reverse primers which were designed using vector NTI software. The amplified product showed an expected amplicon size of 579 bp on 2 per cent agarose gel (Fig.3). It was then kept running on 0.8 per cent agarose gel and was further subjected to gel elution, which correspond to study done by Nethravathi, (2014).

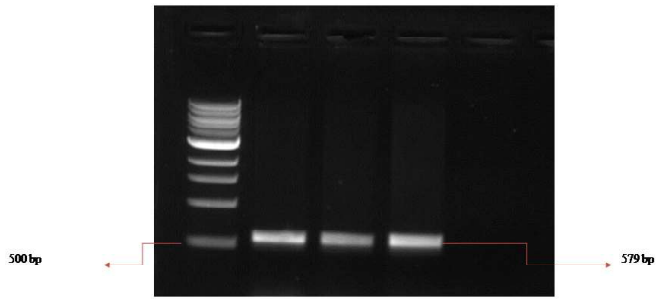

Fig. 3. PCR amplification of *ect*A gene
(M: 1 kb Ladder, 1, 2, 3: Bacterial sample, 4: Blank)

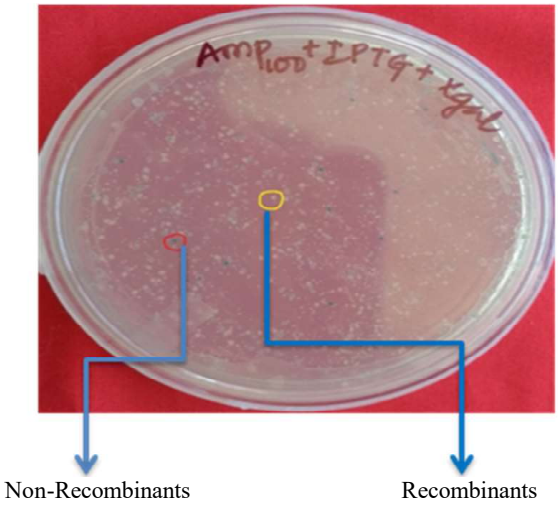

Fig. 4. Blue white colony assay
(White colony; Recombinants, Blue colony; Non-recombinants)

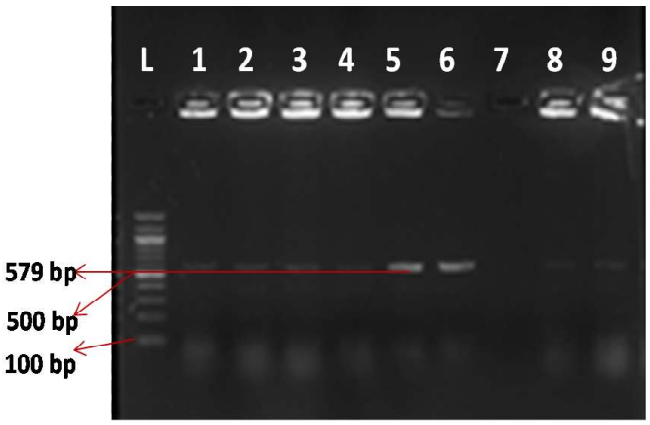

Fig. 5. Colony PCR confirmation of *ect*A gene in pTZ57R/T
(M: 100 bp ladder, 1 to 9: PCR amplification of *ect*A gene from recombinant clones)

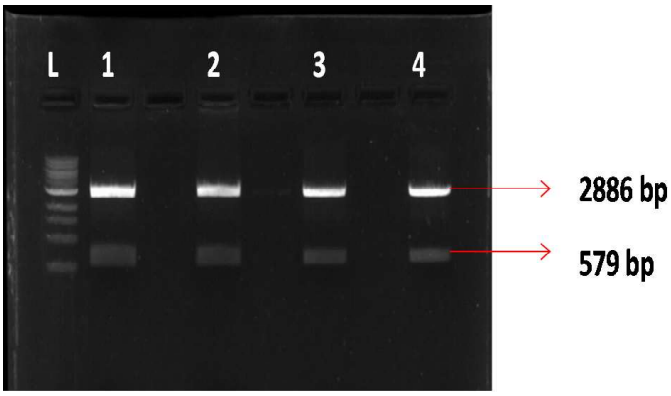

Fig. 6. Restriction confirmation of *ect*A clones in pTZ57R/T with *Kpn*I and *Hind*III
(M: 1kb ladder, 1-4: Plasmid cut with *Kpn*I and *Hind*III restriction enzymes)

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1    ATG AAC GCA ACC ACA GAG CCC TTT ACA CCC TCC GCC GAC CTG GCC   45
1    Met Asn Ala Thr Thr Glu Pro Phe Thr Pro Ser Ala Asp Leu Ala   15

46   AAG CCC AGC GTG GCC GAT GCC GTG GTC GGC CAT GAG GCC TCA CCG   90
16   Lys Pro Ser Val Ala Asp Ala Val Val Gly His Glu Ala Ser Pro   30

91   CTC TTC ATC CGC AAG CCA AGC CCC GAT GAC GGC TGG GGC ATC TAC   135
31   Leu Phe Ile Arg Lys Pro Ser Pro Asp Asp Gly Trp Gly Ile Tyr   45

136  GAG CTG GTC AAG TCC TGT CCG CCT CTC GAC GTC AAT TCC GCC TAC   180
46   Glu Leu Val Lys Ser Cys Pro Pro Leu Asp Val Asn Ser Ala Tyr   60

181  GCC TAT CTG TTG CTG GCC ACC CAG TTC CGC GAT AGC TGC GCC GTG   225
61   Ala Tyr Leu Leu Leu Ala Thr Gln Phe Arg Asp Ser Cys Ala Val   75

226  GCG ACC AAC GAA GAG GGC GAG ATC GTC GGC TTC GTT TCC GGC TAC   270
76   Ala Thr Asn Glu Glu Gly Glu Ile Val Gly Phe Val Ser Gly Tyr   90

271  GTG AAG AGC AAC GCC CCC GAT ACC TAT TTC CTC TGG CAG GTT GCC   315
91   Val Lys Ser Asn Ala Pro Asp Thr Tyr Phe Leu Trp Gln Val Ala   105

316  GTG GGC GAG AAG GCA CGT GGC ACC GGC CTG GCC CGT CGT CTG GTG   360
106  Val Gly Glu Lys Ala Arg Gly Thr Gly Leu Ala Arg Arg Leu Val   120

361  GAA GCC GTG ATG ACA CGC CCG GAA ATG GCC GAG GTC CAC CAT CTC   405
121  Glu Ala Val Met Thr Arg Pro Glu Met Ala Glu Val His His Leu   135

406  GAG ACC ACT ATC ACG CCC GAC AAC CAG GCG TCC TGG GGC TTG TTC   450
136  Glu Thr Thr Ile Thr Pro Asp Asn Gln Ala Ser Trp Gly Leu Phe   150

451  CGC CGT CTC GCC GAT CGC TGG CAG GCG CCG TTG AAC AGC CGC GAA   495
151  Arg Arg Leu Ala Asp Arg Trp Gln Ala Pro Leu Asn Ser Arg Glu   165

496  TAC TTC TCC ACC GAT CAA CTC GGC GGT GAG CAT GAC CCG GAA AAC   540
166  Tyr Phe Ser Thr Asp Gln Leu Gly Gly Glu His Asp Pro Glu Asn   180

541  CTC GTT CGC ATC GGC CCG TTC CAG ACC GAC CAG ATC TGA GCT CGG   585
181  Leu Val Arg Ile Gly Pro Phe Gln Thr Asp Gln Ile End Ala Arg   195

586  ACG CCG  591
196  Thr Pro
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Fig. 7. Complete nucleotide and amino acid sequence of *ect*A from pRHNM26

The eluted *ect*A gene was kept for A-tailing by using *Taq* polymerase enzyme by incubating it in water bath at 72^{0}C for 20 minutes. The A-tailed 579 bp *ect*A amplicon was then cloned into T/A cloning vector pTZ57R/T and it was named as pDBJ26. The ligated product was then transferred to *E. coli* DH5α and the recombinants were confirmed through blue-white screening (Fig.4), then with colony PCR (Fig.5), plasmid isolation and restriction digestion of the plasmid pDBJ26 (Fig.6) by using *Kpn*1 and *Bam*HI restriction enzymes. The restriction digested products were separated by running on agarose gel electrophoresis, resulted in forming two bands of different size, one of that *ect*A gene which is of 579 bp and another one is

pTZ57R/T of 2886 bp respectively and the results obtained were in accordance with the results obtained by Chougala, (2016). BLASTn and BLASTp analysis results indicated 94 per cent homology with synthetic construct Sumo-ScFv-9R mRNA, complete cds and further the BLASTp analysis shown that 95 per cent homology with anti-ivermectin antibody synthetic construct. The developed antibodies were characterized and validated with other virus samples. The result had indicated that genes in pPRSVNA1 and pPRSVNA33 bind to the same epitopes of the antigen.

The construct pDBJ26 containing *ect*A gene was sequenced using gene specific forward and reverse primers. The complete sequence of *ect*A gene after sequencing (Fig.7) and the complete nucleotide sequence of the cloned gene *ect*A was analysed using BLAST algorithm available at *http:// www.ncbi.nih.gov*. It was observed that there was 99.66 per cent of homology with *ect*A gene of *Halomonas elongata*. This confirmed that amplification of *ect*A gene. These results are contradictory with the results of Zhao *et al.* (2006) and Zhang *et al.* (2008). Zhao *et al.* (2006) cloned the entire *ect*ABC cluster from *Halobacillus dabanensis* D-8^T and their results indicated that amino-acid sequence deduced from *ect*ABC was highly similar to that from *Virgibacillus pantethenticus* (*ect*A 52%, *ect*B 60%, *ect*C 67%, respectively). Zhang *et al.* (2008) cloned the entire *ect*ABC cluster from *N. halobia* DSM 20541 and the amino acid deduced from *ect*ABC of the strain was highly similar to the *ect*ABC of *Brevibacterium linens* BL2 (*ect*A 60%, *ect*B 70%, *ect*C 68% identities).

Conclusion

Intact ectoine purification is more challenging in case of *Halomonas elongata*. As the phage display technology needs purified antigen to produce scFv monoclonal antibodies recombinant DNA approach was used in this study to get purified antigen. Further, this purified *ect*A protein can be used as an antigen to develop monoclonal antibodies using phage display technology.

References

Afzal N B, 2014, Expression of bacterial *ect*B gene in the chloroplast of *Chlamydomonas reinhardtii. M. Sc. Thesis,* University of Oslo, Oslo (Norway).

Alone A G, 2013, Functional and molecular characterization of marine and freshwater microorganisms. *International Journal of Applied and Pure Agriculture,* 6(3): 98-116.

Chougala A S, 2019, Selection of single chain fragment variable monoclonal antibodies against papaya ringspot virus coat protein. *Journal of Plant Biochemistry and Biotechnology,* 12(3):54-59.

Flowers T J, 2004, Improving crop salt tolerance. *Journal of Experimental Botany*, 55(396): 307-319.

Holck V T, 2014, Expression of the bacterial *ect*C gene in the chloroplast of *Chlamydomonas reinhardtii. M. Sc. Thesis,* University of Oslo, Oslo (Norway).

Jamil A, Riaz S, Ashraf M. and Foolad M R, 2011, Gene expression profiling of plants under salt stress. *Critical Reviews in Plant Sciences,* 30(8): 435-458.

Nethravathi A H, 2016, Isolation and molecular characterization of salt tolerant genes from marine bacteria. *International Journal of Applied and Pure Agriculture,* 10(1):113-118.

Rajan A, 2010, Identification and characterization of bacterial genes imparting osmotolerance in *Bacillus halodurans* and *Escherichia coli* isolated from salted fish. *Ph. D. Thesis,* Central Institute of Fisheries Technology, Cochin, (India).

Shivanand P. and Mugeraya G, 2011, Halophilic bacteria and their compatible solutes-osmoregulation and potential applications. *Current Sciences.,* 100(10): 1516-1521.

Zhang B, Bao X Wang L. and Yang S S, 2008, Cloning and characterization of the gene cluster for biosynthesis of ectoine from *Nesterenkonia halobia* DSM 20541. *Journal of Microbiology,* 46(3): 309-318.

Zhao B, Lu W, Yang L, Zhang B, Wang L. and Yang S S, 2006. Cloning and characterization of the genes for biosynthesis of the compatible solute ectoine in the moderately halophilic bacterium *Halobacillus dabanensis* D-8T, *Current Microbiology*, 53(3): 183-188.