Short Communication

Detection of the *ectC* gene in *Halomonas* strains by polymerase chain reaction

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Abstract
1, 4, 5, 6-Tetrahydro-2-methyl-4-pyrimidine carboxylic acid (ectoine) is an excellent osmoprotectant. Ectoine and hydroxyl ectoines are of great significance to the biotechnology industry, thus the detection and isolation of ectoine producing bacteria is of great importance. Hence, this study involved the detection of the *ectC* gene (encoding ectoin synthase enzyme) using polymerase chain reaction (PCR) method. For isolation of moderately halophilic bacteria, environmental samples were collected from various sites of a tannery factory in Isfahan, and the Persian Gulf. A synthetic broth medium was used and the optimum concentration of salt (NaCl) was determined by the microtitre plate method. Based on the alignment of relevant *ectC* gene sequences available in the GenBank which included sequences from 24 validly described *Halomonas* species, putative genus-specific primers were designed. Primers were designed in such a way to amplify a 277bp region of the *ectC* gene in the putative *Halomonas* strains. PCR analysis showed that 75% (34/45) of the samples belong to the *Halomonas* genus capable of producing ectoine synthase. Ectoine primer pair was designed to amplify all *Halomonas* species capable of producing ectoine synthase.
Keywords: Halotolerant/halophilic; Ectoine; PCR; Salt; *Halomonas*.

Halotolerant or halophilic microorganisms, able to live in saline environments, offer a multitude of actual or potential applications in various fields of biotechnology (Wang *et al*., 2004; Margesin *et al*., 2001). These microorganisms are usually isolated from saline environments which are characterized by having high osmotic strength and low water activity. Most halophilic/halotolerant eubacteria cope with these conditions by accumulating small, highly water-soluble organic compounds, the so-called compatible solutes (Brandon, 2006; Dassarma and Arora 2001; Ciulla *et al*., 1997). Ectoines represent the predominant class of osmolytes in aerobic chemo-heterotrophic eubacteria of the family *Halomonadaceae* (Louis and Galinski, 1997; Wohlfarth *et al*., 1990). Members of the genus *Halomonas* tolerate high concentrations of sodium chloride (NaCl) as compared to other Gram-negative and rod-shaped bacteria (Brenner *et al*., 2005; Holt *et al*., 2005).

Ectoine is of vital importance to agriculture and the biotechnological and pharmaceutical industries. Infact ectoine and its derivatives are patented as moisturizers in cosmetics for the care of aged, dry, or irritated skin (Margesin and schinner, 2001; Wood *et al*., 2001). One of the most promising applications of ectoine is its use as a stabilizer in the polymerase chain reaction (PCR) and as an enzyme protectant against heat, freezing, and drying (Detkova and Boltyanskaya, 2006; Margesin and Schinner, 2001; Lippert and Galinski, 1992). Ectoine is biosynthesized from aspartic β-semialdehyde in three successive enzyme reactions. The genes responsible for these three enzymes are designated *ectA*, *ectB*, and *ectC*, which code for L-2,4-diaminobutyric acid acetyltransferase, L2-,4-diaminobutyric acid transaminase, and L-ectoine synthase respectively (Nakayama *et al*., 2000; Ono *et al*., 1999; Louis and Galinski, 1997; Peters *et al*., 1990).

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The aim of this study was to isolate ectoine-producing *Halomonas* strains. Thus, a pair of common primers was designed to detect the *ectC* gene (encoding ectoine synthase) in bacterial species that belong to the genus *Halomonas*. For isolation of moderately halophilic bacteria, environmental samples were collected from various sites of a tannery factory in Isfahan, and the Persian Gulf, where the salt concentration is high. Samples included salted sheepskin and sheep intestine, wastewater and salt water. Samples from salted sheep skin and intestine were transferred to defined broth for enrichment and isolation. Wastewater and salt water were collected from a depth of 10 cm in sterile 500-ml bottles and transported on ice to the laboratory for isolation on the same day (Brent, 2002). The synthetic defined broth medium used was based on mineral salts medium containing (g/l): Nutrient broth, 8; $MgSo_4$. $7H_2O$ 9.6; $MgCl_2$, 7; KCl, 2; $CaCl_2$. $2H_2O$, 0.36; $NaHCo_3$, 0.06; NaBr, 0.026 and NaCl, 50; pH 7.4 (Nieto *et al*., 1989). The synthetic defined agar medium was prepared as above but with the addition of 20 g/l of nutrient agar instead of nutrient broth. This medium was modified by addition of NaCl at a final concentration of 300 g/l. All chemicals were obtained from Merck, Germany. Media were sterilized at 121ºC for 15 min. Wastewater samples (5ml), saltwater samples (5ml), and swabs from salted sheep skin and salted sheep intestine were added to Erlenmeyer flasks containing 25 ml defined broth medium. Flasks were then incubated for 1 to 2 days at 28ºC on a rotary shaker (INFORS AG, Germany) operating at 150 rpm.

After enrichment, inoculum from the flasks was streaked out onto the defined solid medium and phenotypically varied colonies were purified on this medium. Phenotypically different colonies obtained from the plates were transferred to the defined solid medium containing various concentrations of NaCl (0-250 g/l). Most isolates were observed to grow in a wide range of NaCl concentrations. The optimum concentration of NaCl was determined by the microtitre plate method. Seed cultures were prepared by growing isolates in the defined broth medium for 24 h. A 50 µl sample of each seed culture was used to inoculate individual wells of a 96-well microtitre plate containing 200 µl of sterile synthetic broth medium with different concentrations of NaCl (0-250 g/l). The microtitre plates were then incubated at 28ºC for 24 h, followed by optical density measurements at 600 nm (OD_{600}) using an ELISA reader (Stat fax 2100, Germany) (Emtiazi *et al*., 2005).

In this study, 78 bacterial isolates were identified by Gram staining and the presence of catalase, cytochrome oxidase, and their tolerance to high NaCl concentrations were also tested (Vreeland and Hochstein, 1993). The above tests were performed according to Bergey's Manual of Determinative Bacteriology (Brenner *et al*., 2005; Holt *et al*., 1994). For the final identification of ectoine producing isolates, a 277 bp region located within the *ectC* gene was amplified using common primers (Cinagen, Iran).

The relevant *ectC* gene sequences available in the GenBank were aligned by using the MegAlign software package. These included sequences from 24 validly described *Halomonas* species. Based on this alignment, putative genus-specific primers were designed. Primers were designed in such a way to amplify a 277bp region of the *ectC* gene in the tentative *Halmonas* strains.

The primer were designed according to the methods available in http://www. Ncbi.NlmNih.Gov/entrez/query and http://www.ebi. ac.uk/cgi-bin/jobresults/clustalw/clustalw-20060710-515216.aln). The sequences for the forward and the reverse primers are as follows:

Forward: 5′-GGTAAYTGGGAYAGYACRC-3′
Reverse: 5′-GBGGHGTRAAKACRCADCC-3′

Y=C or T	R=A or G	B=C, G or T
H=A, C or T	K=G or T	D=A, G or T

DNA used for PCR was prepared as previously described (Sambrook and Russel, 2001). Multiple bands initially observed on the agarose gel were eliminated by changing the $MgCl_2$ and primer concentrations and altering the annealing temperature (McPherson and Moller, 2000) (Fig. 1). The PCR reaction was finally optimized as follows: A 25 µl volume of reaction mixture contained 2 mM $MgCl_2$, 10X PCR buffer (200 mM Tris, 500 mM KCl), 0.24 mM dNTPs, 60 pmol of each forward and reverse primer and one unit of *Taq* DNA polymerase and the final volume was adjusted to 25 µl by adding water. Amplification was carried out in a thermal cycler (Eppendorf AG 22331, Hamburg, Germany). After an initial denaturation at 94ºC for 5 min, 30 cycles of the three- step PCR amplification were completed, each consisting of denaturation at 98ºC for 20 sec, primer annealing at 57ºC for 1 min and primer extension at 72ºC for 1 min. The samples were incubated at 72ºC for 10 min at the end of amplification cycles to complete the extension reac-

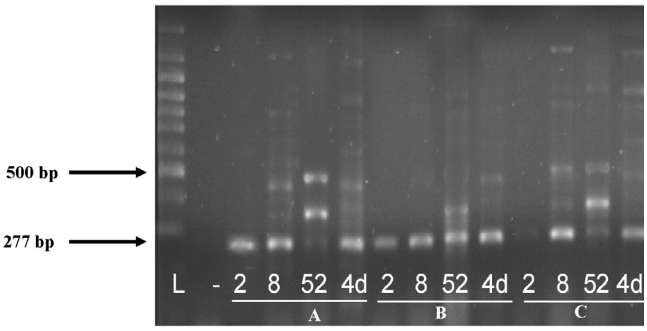

Figure 1. Gel electrophoresis of the PCR products from strains H2, H8, H52 and H4d. L: 100bp DNA ladder size marker (Cynagen, Iran), -: negative control (H_2O), 2: positive control (*Halomonas salina* ATCC 49509) 8, 52, 4d: strain H8, H52, H4d. A: Annealing temprature 68ºC, 30 cycles, primer pair: 80 Pmol, $MgCl_2$ 2.4 mM, B: Annealing temprature 57ºC, 30 cycles, primer pair: 60 Pmol, $MgCl_2$ 2 mM, C: Annealing temprature 68ºC, 25 cycles, primer pair: 60 Pmol, $MgCl_2$ 2.4 mM.

tion (Nakayama *et al*., 2000).

PCR products were separated by gel electrophoresis using a horizontal 1% (w/v) agarose gel (Sigma, St. Louis, MO, USA) in Tris-base-Ethylene diamine tetraacetic acid (TBE) buffer (80 volts for 1 h and 20 min). Gels were then stained in a solution of ethidium bromide and visualized with a UV transilluminator (UVP Inc., San Gabriel, CAS, USA).

The primary tests, i.e. Gram staining, oxidase and catalase tests and range of tolerance to NaCl (%), showed that out of the 78 isolates, 45 were Gram-negative rods, oxidase and catalase positive and all were able to tolerate a wide range of NaCl concentrations (Table 1). The microtiter plate method showed that from the 45 isolates, only those strains originating from the tannery factory showed the greatest growth (as OD_{600}) at NaCl concentrations of 100-150 g/l (10-15%), where as the isolates from the Persian Gulf showed the greatest growth at NaCl concentrations of 50 g/l (5%). Such a difference is to be expected since the amount of NaCl in sea and lake water is approximatly 3-5% (Ventosa *et al*. 1998) but that in the tannery factory is much higher, being saturated in the salted intestine (Verma *et al*., 2001).

The results also showed that the growth of all isolates was at a minimum and almost equal, at a NaCl concentration of 250 g/l (25%). From the data obtained, it was deduced that these strains were closely related to the genus *Halomonas*.

Based on alignment of the *ectC* sequences belonging to the *Halomonas* species available in the GenBank, a primer pair (named ectione) was designed. The ectoine primer pair intended to amplify all *Halomonas* species capable of producing ectoine synthase. Out of the 78 isolates, 45 were identified by primary and biochemical tests as tentative *Halomonas* strains. The 45 isolates were then further investigated by applying the new PCR assay. PCR analysis showed that 34 of the 45 (75%) of the isolates belonged to the genus *Halomonas*, capable of producing ectoine synthase (Fig. 2). The specificity of the putative genus-specific PCR assays was determined by testing some of the bacteria previously identified as non-*Halomonas* strains and non-halophilic bacteria (Fig. 3). The novel genus-specific PCR assays indicated that several of the 45 isolates had been misidentified by the primary morphological and biochemical tests.

In this study, the *Halomonas* strains were easily and quickly differentiated from other genera by their ability to survive exposure to, and grow at very high (20%) NaCl concentration. All species tested were catalase positive, and most of them were also oxidase positive.

Table 1. Some of the preliminary tests for identification of bacterial strains and results of PCR.

Morphology	Reaction of the Gram stain	Range of tolerance to NaCl (%)	Numbers of isolates	PCR test
Rod-shaped	Negative	32-2	1	+
		32-0.5	3	+
		32-0	2	+
		0.5-30	4	+*
		30-0	1	+
		25-0	33	+**
		0.5-25	1	+

*:From 4 strains 3 were positive for PCR
**:From 33 strains 23 were positive for PCR

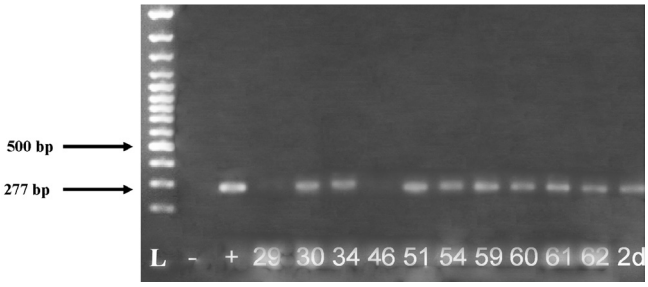

Figure 2. Gel electrophoresis of the PCR products from strains H29, H30, H34, H46, H51, H54, H59, H60, H61, H62, and H2d. L: 100bp DNA ladder size marker, -: negative control (H_2O), +: positive control (*Halomonas salina* ATCC 49509).

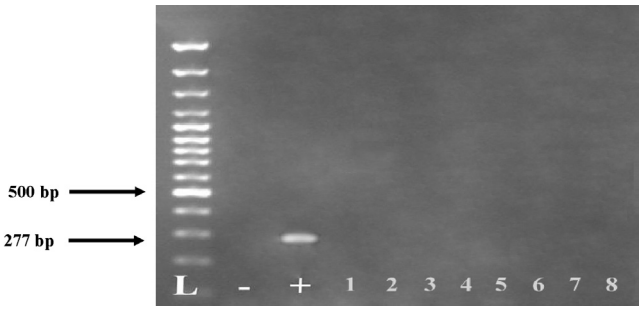

Figure 3. Gel electrophoresis demonstrating the specificity of PCR. L: 100bp DNA ladder size marker, -: negative control (H_2O), +: positive control (*Halomonas salina* ATCC 49509), Lane 1: *Escherichia coli*, Lane 2: *Klebsiella pneamoniae*, Lane 3: *Pseudomonas aeruginosa*, Lane 4: *Bacillus licheniformis*, Lane 5: *Bacillus polymyxa*, Lane6: *Micrococcus loteus*, Lane 7: *Listeria monocytogenes*, Lane 8: *Proteus mirabilis*.

Hence, we detected tentatively 45 isolates which belong to the *Halomonas* genus capable of producing ectoine synthase, but the specificity of the putative genus-specific anslysis showed that 34 of the 45 strains belonged to the genus *Halomonas*.

The types of osmotic solutes present in the cells depend greatly on the growth conditions such as, temperature, salinity, medium composition (Lamosa *et al*., 1998; Ventosa *et al*., 1998). Ectoine is found at high concentrations in different *Halomonas* species and is the dominant solute in cells grown in defined medium lacking glycine betaine, or its precursor choline, but in the presence of high NaCl concentrations (Ventosa *et al*., 1998; Wohlfarth *et al*., 1990). In this study, the synthetic defined solid medium was also devoid of glycine betaine but with high NaCl concentrations. This together with the PCR results suggest that the 34 isolates mentioned above are capable of tolerating high concentrations of NaCl by possibly producing ectoine.

Previous studies have used species specific primers, incapable of detecting the range of isolates demonstrated in this study. Nakayama *et al*. (2000) recognized *ect* genes in *Halomonas elongata* by special primers. They took a transgenic approach to investigate the function of ectoine as a compatible solute in plant cells. Louis *et al*. (1997) recognized *ect* genes in *Marinococcus halophilus* by special primers and cloned a 4.1-kb DNA fragment into *Escherichia coli*. The resulting clones exhibited accumulation of ectione and increased salt tolerance. The *ectA*, *ectB*, and *ectC*, were found to be located in the 4.1-kb DNA fragment. Jabber *et al*. (1992) and Min-Yu *et al*. (1993) while investigating on *Halomonas* strains, cloned the *ect* genes into *E coli*. The resulting clones exhibited tolerance and resistance to saline environments. The use of universal primers was not demonstrated in any of the above mentioned investigations. In this study, universal primers were designed for recognition of the *ectC* gene in the *Halomonas* species.

Ectoine is the dominant compatible solute in the *Halomonadaceae*, when grown at very high salt concnetrations. However, we found isolates with negative PCR results, proving that they do not belong to the *Halomonas* genus. Therefore, it is worthwhile mentioning that there also exists an alternative biosynthetic pathway for ectoine, isolates with glutamate (Ventosa *et al*., 1998; Louis and Galinski 1997). Some of the moderately halophilic bacteria also use different osmolytes to provide osmotic balance with the external medium (Ventosa *et al*., 1998).

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References

Brent P (2002). Biotransformation of toxic organic contaminants by halophilic bacteria from soap lake, WA. Reaserch & extension regional water quality conferance. Washington state university. PP: 26-28.

Brandon R, Litzner T, Caton M, schneegurt A (2006). carbon substrate utilization, antibiotic sensitivity and numerical taxonomy of bacterial isolates from the Great salt ploains of Oklahoma. *Arch Microbial*. 185: 286-296.

Brenner J, Krieg R, Staley T (2005). *Bergey's Manual of Systematic Bacteriology*. Sec edition. Vol 2. Part B. Springer, PP. 283-321.

Ciulla RA, Diaz MR, Taylor BF, Roberts MF (1997). Organic

osmolytes in aerobic bacteria from mone lake, an alkaline, moderately hypersaline environment. *Appl Environ Microbiol*. 25: 220-226.

Dassarma S, Arora P (2001). Halophiles. *Encyclopedia of life sciences*. Nature publishing group/ www.els.net. University of Massachusetts, Amherst, Massachusetts, USA.

Detkova N, Boltyanskaya Yu (2006). Relationships between the osmoadaptation strategy, amine acid composition of bulk protein, and properties of certain enzymes of haloalkaliphilic bacteria. *Microbiology* 75: 259-265.

Emtiazi G, Hassanshahyan M, Golbang N (2005). Development of a microtitre plate method for determination of phenol utilization, biofilm formation and respiratory activity by environmental bacterial isolates. *Int Biodeterior Biodegradation* 56: 231-235.

Galinski EA (1995). Osmoadaptation in bacteria. *Adv Microb Physiol*. 37: 273-328.

Holt Jg, Krieg NR, Sneath PH, staley JT, Williams ST (2005). *Bergey's Manual of Determinative Bacteriology*. 9th edition. Williams & wilrins. Maryland, PP: 529-542.

Jebbar M, Talibart R, Gloux K, Bernard T, Blance C (1992). Osmoprotection of *Escherichia coli* by ectoine: uptake and accumulation characteristict. *J Bacteriol*. 174: 5027-5035.

Lamosa P, Martins LO, Dacosta M, Santos H (1998). Effects of temprerature, Salinity, and medium compositon on compatible solute accumulation by *Thermococcus* spp. *Appl Environ Microbiol*. 64: 3591-3598.

Lippert K, Galinski EA (1992). Enzyme stabilization by ectoine-type compatible solutes: protection against healting, freezing and drying. *Appl Microbiol Biotechnol*. 37: 61-65.

Louis P, Galinski EA (1997). Characterization of genes for the biosynthesis of the compatible solute ectoine from Marinococcus halophilus. and osmoregulated expression in *Escherichia coli*. *Microbiology* 143: 1141-1149.

Margesin R, Schinner F (2001). Potential of halotolerant and halophilic Microorgnisms for biotechnology. *Extremophiles* 5: 73-83.

Mc pherson MJ, Moller SG (2000). *PCR*. Bios scientific publisher. New York. PP. 46-58.

Min-Yu L, Ono H, Takan M (1993). Gene cloning of ectoine synthase from *Halomonas* sp. *Annu Rep Int cent Coop Res Biotechnol JPN*. 16: 193-200.

Nakayama H, Yoshia Ono KH, Murooka Y, Shinmyo A (2000). Ection the compatible solute of Halomonas elongata confers hyperosmotic tolerance in cultured Tobacco cells. *Plant Physiol*. 122: 1239-1247.

Nieto JJ, Fernandez- Castillo R, Marquez MC, Ventosa A, Quesada E, Ruiz- Berraquero F (1989). Survey of metal tolerance in moderately halophilic eubacteria. *Appl Environ Microbiol*. 55: 2385-2390.

Ono H, Sawada K, Khunajakr N, Morooka Y (1999). Characterization of biosynthetic enzymes for ectoine as a compatible solute in a moderately halophilic eubacterium, Halomonas elongata. *J Bacteriol*. 25: 91-99.

Peters P, Glanski EA, Truper HG (1990). The biosynthesis of ectoin. *FEMS Microbiol Lett*. 71: 157-162.

Sambrook J, Russel DW (2001). *Molecular cloning*. Cold Spring Harbor. New York. PP: 56-87.

Ventosa A, Nieto JJ, Oren A (1998). Biology of moderately holophilic aerobic bacteria Microbiol. *Mol Biol Rev*. 504-544.

Verma T, Srinath T, Gadpayle RU, Hans RK, Gars SK (2001). Chromate tolerant bacteria isolated from tannery effluent. *Bioresource Technol*. 78: 31-35.

Vreeland RH, Hochstein LI (1993). *The biology of holophilic bacteria*. Boca Raton, FL: CRC Press. PP. 97-114.

Wang J, Jiang X, Mou H, Guan H (2004). Anti-oxidation of agar oligosaccharides produced by agarase from a marine bacterium. *J Appl Phycol*. 16: 333-340.

Wohlfarth A, Severin J, Galinski EA (1990). The spectrum of compatible solute in heterotrophic halophilic eubacteria of the famili Halomonadaceae. *J General Microbiol*. 136: 705-712.

Wood M, Bremer E, Csonka N, Kraemer R, Smith L (2001). Osmosensing and osmoregulatory compatible solute accumulation by bacteria. Comparative. *Biochem Physiol*. 130: 437-460.