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# Identification and Characterization of Ectoine Biosynthesis Genes and Heterologous Expression of the *ectABC* Gene Cluster from *Halomonas* sp. QHL1, a Moderately Halophilic Bacterium Isolated from Qinghai Lake

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**The moderately halophilic bacterium *Halomonas* sp. QHL1 was identified as a member of the genus *Halomonas* by 16S rRNA gene sequencing. HPLC analysis showed that strain QHL1 synthesizes ectoine in its cytoplasm. The genes involved in the ectoine biosynthesis pathway were identified on the chromosome in the order *ectABC*. Subsequently, the *ectB* gene from this strain was amplified by PCR, and the entire *ectABC* gene cluster (3,580 bp) was cloned using genome walking. Analysis showed that the *ectA* (579 bp), *ectB* (1269 bp), and *ectC* (390 bp) genes were organized in a single transcriptional unit and were predicted to encode three peptides of 21.2 kDa, 46.4 kDa, and 14.7 kDa, respectively. Two putative promoters, a $\delta^{70}$-dependent promoter and a $\delta^{38}$-controlled promoter, as well as several conserved motifs with unknown function were identified. Individual *ectA*, *ectB*, and *ectC* genes, and the entire *ectABC* gene cluster were inserted into the expression plasmid pET-28a(+) to generate the recombinant plasmids pET-28a(+)-*ectA*, pET-28a(+)-*ectB*, pET-28a(+)-*ectC* and pET-28a(+)-*ectABC*, respectively. Heterologous expression of these proteins in *Escherichia coli* BL21 (DE3) was confirmed by SDS-PAGE. The recombinant *E. coli* strain BL21 (pET-28a (+)-*ectABC*) displayed a higher salt tolerance than native *E. coli* cells but produced far less ectoine than the wild-type QHL1 strain.**

***Keywords***: *Halomonas*, compatible solutes, ectoine, *ectABC* gene cluster, cloning and expression

## Introduction

Halotolerant and halophilic microorganisms have evolved different strategies, the salt-in-cytoplasm strategy and the organic-osmolyte strategy, to maintain turgor pressure and to circumvent the detrimental consequences of water loss when exposed to increasing osmolality (Galinski and Trüper, 1994; Bohnert, 1995; Ventosa *et al*., 1998; Saum and Müller, 2008; Schwibbert *et al*., 2011). Moderately halophilic and the most halotolerant bacteria primarily employ the organic osmolyte strategy where small, highly water-soluble organic compounds (the so-called "compatible solutes") are accumulated (Brown, 1976). The compatible solute ectoine (1, 4, 5, 6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid), was first discovered in *Ectothiorhodospira halochloris* (Galinshi *et al*., 1985) and is one of the most commonly found osmolytes in nature.

Microorganisms synthesizing ectoine are widespread among α-, γ-Proteobacteria and Actinobacteridae, although some others have also been observed in a limited number of β-, δ-, and ε-Proteobacteria, Firmicutes, and one Plantomycete (Pastor *et al*., 2010). The genus *Halomonas* (family Halomonadaceae within the γ-Proteobacteria) is widely distributed in hyper-saline environments and is capable of synthetizing ectoine and/or hydroxyectoine in response to high ionic strength in environments. A number of species within *Halomonas* have been shown to produce compatible solutes, including *H. elongate* ATTC33173$^T$ (Wohlfart *et al*., 1990; Peters *et al*., 1990; Maskow *et al*., 2001), *H. halmophila* CCM2833$^T$ (Pastor *et al*., 2010), *H. halodenitrificans* DSM735, *H. variabilis* DSM 3051$^T$ (Severin *et al*., 1992), *H. boliviensis* (Guzmán *et al*., 2009), and *H. salina* DSM 5928$^T$ (Zhang *et al*., 2009). Ectoine is well known to serve as an osmoprotectant against increased external osmotic pressure. It functions to stabilize enzymes in the cell and has been suggested to be potentially useful commercially as an additive to cosmetics where it would function to maintain moisture levels (Graf *et al*., 2008; Pastor *et al*., 2010; Reshetnikov *et al*., 2011).

Ectoine is a highly soluble, low molecular weight and zwitterionic organic molecule that is often considered to be a heterocyclic amino acid or a partially hydrogenated pyrimidine derivative (Galinski *et al*., 1985). Most halophiles can synthesize ectoine via a pathway utilizing enzymes specified by three genes, *ectA*, *ectB*, and *ectC*, according to current knowledge (Cánovas *et al*., 1998). The genes coding for these enzymes are usually located in the *ectABC* or *ectABC-ask* gene cluster (Louis and Galinski, 1997; Kuhlmann and Bremer, 2002; Vargas *et al*., 2008; Lo *et al*., 2009; Schwibbert *et al*., 2011; Seip *et al*., 2011). An additional enzyme, encoded by *ectD*, is often found in ectoine producing species where

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it functions to convert ectoine to hydroxyectoine (Peters *et al.*, 1990). Meanwhile the degradation of ectoine in *H. elongata* proceeds via hydrolytic enzymes encoded by four genes: *doeA*, *doeB*, *doeC*, and *doeD* (Schwibbert *et al.*, 2011).

Numerous recent studies have focused on the organization/regulation of the ectoine biosynthetic pathway in various bacterial taxa. The *ectABC* genes and the proteins for ectoine synthesis are highly conserved among ectoine producing bacteria (Schwibbert *et al.*, 2011). Previous studies centered on transcriptional regulation revealed that the *ectABC* gene cluster is organized as an operon in almost all cases (Kuhlmann and Bremer, 2002; Saum and Müller, 2008). A total of five promoters regulating *ectABC* transcription were mapped in *Chromohalobacter salexigens*. Two $\delta^{70}$-dependent promoters, one $\delta^{s}$-controlled promoter, and a promoter of unknown specificity are located upstream of *ectA*, while a fifth promoter was found upstream of *ectB* (Calderón *et al.*, 2004). In *H. elongata*, two transcriptional initiation sites for the vegetative sigma factor $\delta^{70}$ and the factor $\delta^{38\text{-osmo}}$ could be pinpointed upstream of *ectA*, and one site for promoter controlled by $\delta^{54}$ was mapped immediately upstream of *ectC* (Schwibbert *et al.*, 2011). Although many bacteria can synthesize and accumulate ectoine, there are many differences in the evolution and gene organization of ectoine synthesis pathways. Here, we reported on the characterization of the ectoine biosynthesis genes in strain QHL1, a potential novel species isolated from Qinghai Lake. This study revealed that the evolution and organization of the *ect* genes in strain QHL1 was different from the *ectABC* operon in *Halomonas*. Furthermore, successful heterologous expression of the individual ectoine biosynthesis genes (*ectA*, *ectB*, and *ectC*) and the *ectABC* cluster from QHL1 in *E. coli* BL21 provides a framework for future genetic manipulation and gene regulation of ectoine biosynthesis pathways for use in industrial applications.

## Materials and Methods

### Bacterial strains, media, growth conditions, and plasmids

*Halomonas* sp. QHL1 was isolated from Qinghai Lake in northwest China. The QHL1 strain was a moderate halophile, according to the grouping of halophiles by Kushner and Kamekura (1988). Strain QHL1 was grown aerobically in improved Oesterhelt-Stoeckenius's medium (1974) containing 5% (w/v) NaCl, 0.97% (w/v) $MgSO_4$, 0.02% (w/v) $CaCl_2$, 0.2% (w/v) KCl, 0.3% (w/v) citric acid sodium, 1% (w/v) bacterial peptone, and 0.2% (w/v) yeast extract. The pH of medium was adjusted to 8.5 using 3 M NaOH. Cultures were incubated at 37°C. *E. coli* DH5α and *E. coli* BL21 (DE3) were grown in Luria-Bertani (LB) medium (Miller, 1992) at 37°C with shaking (150 rpm), and were used as the host strains for transformation of plasmid clones and expression experiments, respectively. Plasmid pMD18-T with an Ampicillin coding sequence (TaKaRa, China) and pET-28a with a Kanamycin resistant tag (TaKaRa) were used as cloning and expression vectors, respectively.

### Analysis of intracellular ectoine content

Extraction of intracellular ectoine was carried out with methanol as described previously with a slight modification (Ono *et al.*, 1998; Wei *et al.*, 2011). The accumulated concentration of intracellular ectoine was determined by HPLC analysis (Nagata *et al.*, 2002; Onraedt *et al.*, 2005), using an Agilent Technologies 1200 Series HPLC (Agilent Technologies, USA) system with an 150 mm × 4.6 mm Agilent ZORBAX Eclipse XDB-C18 5 μm column (Agilent Technologies). Chromatography was performed at a flow rate of 1 ml/min with acetonitrile/ultrapure water (4/1, v/v) as the mobile phase at 20°C. The presence of ectoine was monitored at 215 nm by a UV/VIS detector. The retention time

**Table 1. Oligonucleotide primer sequences used in this study**

| Primer[a] | Gene targeting | Sequence (5′→3′) | Cleavage site |
|---|---|---|---|
| 27F | 16S rRNA | AGAGTTTGATCCTGGCTCAG | |
| 1541R | 16S rRNA | AAGGAGGTGATCCAGCC | |
| *ectB* CSF[b] | *ectB* conserved sequence | ATGCAGACCCAGAYKCTYGAACGC | |
| *ectB* CSR[b] | *ectB* conserved sequence | CGCTMACRTCRCGCTCRAAGGTGTC | |
| SPF1 | *ectB* downstream | ATAACGGCACTTTCCGTGGTTTCAG | |
| SPF2 | *ectB* downstream | GCGTAAAGGACGCATTGTAGAAGAG | |
| SPF3 | *ectB* downstream | ATCGACGTAGTGTCTGGTGACATCG | |
| SPR1 | *ectB* upstream | CAGATGCACTTTGTAGTCAAGCCC | |
| SPR2 | *ectB* upstream | TGAACAACACCGTCAGTCGACAG | |
| SPR3 | *ectB* upstream | GAGGAAATCAATGTACTCACGGCC | |
| *ectA* F | *ectA* | CGGATCCATGAGCACGCCGACACACC | *Bam*HI |
| *ectA* R | *ectA* | GAAGCTTTTAGATGCGGTCTGTTTGGAAC | *Hin*dIII |
| *ectB* F | *ectB* | CTCGAATTCATGCAGACCCAAACGCTTGAAC | *Eco*RI |
| *ectB* R | *ectB* | AGTGAGCTCTCATTCCAGAATATCCAAGCCTTC | *Sac*I |
| *ectC* F | *ectC* | CGGATCCATGATCGTTCGTAATATTGAAG | *Bam*HI |
| *ectC* R | *ectC* | GAAGCTTCTATTCAGAAGGTGCGTATGAGC | *Hin*dIII |
| *ectABC* F | *ectABC* | GGGATCCATGAGCACGCCGACACAACC | *Bam*HI |
| *ectABC* R | *ectABC* | GGAGCTCCTATTCAGAAGGTGCGTATGAGCC | *Sac*I |

[a] All primers were designed by a Primer Premier software, and synthesized by GenScript Corporation (Nanjing) in China.
[b] The highlighted basepairs (in yellow) correspond to degenerate bases as follows: Y(C/T), K(G/T), M(A/C), R(A/G).

of ectoine was determined using commercially available authentic ectoine (purity more than 95%, Fluka Analytical, Germany). Intracellular ectoine content was calculated as milligrams per gram dry cells.

### Sequencing of 16S rRNA gene and phylogenetic analysis

Genomic DNA was isolated with a bacterial DNA extraction kit (Axygen Bioscience, China). The 16S rRNA gene was amplified using a PCR Mastercycler gradient thermocycler (Eppendorf, Germany) with the two universal primers 27F and 1541R (Table 1) (Woese *et al*., 1983). PCR reactions were carried out under conditions described previously (Springer *et al*., 1993). The PCR product was purified with a universal DNA purification kit (Axygen Bioscience, China) in accordance with the manufacturer's protocol. The 16S rRNA gene was sequenced by GenScript Corporation Ltd. (China). Alignment and analyses of sequences was completed with the Clustal W1.6 software (Thompson *et al*., 2007). Representative sequences of the family Halomonadaceae were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). Phylogenetic trees were constructed by the neighbour-joining method (Saitou and Nei, 1987) with the MEGA4.0 software (Felsenstein, 1981).

### Amplification of the highly conserved sequence of *ectB* gene

We designed a pair of degenerate primers *ectB*-CSF and *ectB*-CSR (Table 1) for amplifying the highly conserved sequence of the *ectB* gene. A reference *ectB* gene belonging to the genus *Halomonas* was obtained from the NCBI database. The reaction was performed in a 50 µl reaction volume containing 50 ng of genomic DNA, 0.5 µM of each primer, 200 µM each of dNTP, 1.25 U of TaKaRa Taq$^{TM}$, 5 µl 10× PCR buffer (0.1 M Tris-HCl, 0.5 M KCl) and 1.5 mM of $MgSO_4$. Amplification conditions were as follows: primary denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, annealing at 55°C for 40 sec and extension 72°C for 30 sec followed by a final extension at 72°C for 10 min. The PCR product of the conserved sequence of the *ectB* gene was ligated into the pMD18-T vector. The recombinant plasmid was transformed into *E. coli* DH5α and subsequently sequenced by GenScript Corporation Ltd. (China).

### Nested PCR amplification of *ectB* flanking sequences

To obtain the complete *ectABC* gene cluster, a nested PCR strategy was used as described by Wang *et al*. (2011). The method centered on a series of primers encompassing sequence-specific primers (designed based on regions of the known *ectB* gene) shown in Table 1. Primers SPR and primers SPF were used to amplify the upstream and downstream flanking sequences of *ectB*, respectively. Fusion primers (AP1/AP2/AP3/AP4), which contain an arbitrary degenerate section, were obtained from the Genome walking Kit D316 (TaKaRa). Nested PCR was performed in a Mastercycler gradient thermocycler (Eppendorf) according to the manufacturer's instructions. A 1.5 kb fragment upstream of *ectB* and a 1.25 kb fragment downstream from *ectB* were obtained using this approach and were subjected to sequencing. Alignment and informatics analyses of sequences were performed with the MEGA4.0 software.

### Construction of plasmids

The individual *ectA*, *ectB*, and *ectC* genes and the *ectABC* gene cluster were amplified from genomic DNA of QHL1 using TaKaRa LA Taq polymerase (TaKaRa) and custom-synthesized DNA primers containing restriction sites (underlined, Table 1). The reactions were performed in 50 µl reaction volumes as described previously. The annealing temperatures of *ectA*, *ectB*, *ectC*, and *ectABC* were 56°C, 55°C, 54°C, and 56°C, respectively. The resulting PCR fragments were ligated into the cloning vector pMD18-T (TaKaRa) and transformed into *E. coli* DH5α. The positive transformants were verified by a colony PCR assay. The resulting intermediate plasmids (pMD18-T-*ectA*, pMD18-T-*ectB*, pMD18-T-*ectC* and pMD18-T-*ectABC*) were re-cut by restriction enzymes (*Bam*HI/*Hin*dIII, *Eco*RI/*Sac*I, *Bam*HI/*Hin*dIII and *Bam*HI/*Sac*I, respectively) and directionally ligated into digested expression vector pET-28a(+) (TaKaRa), which yielded pET-28a(+)-*ectA*, pET-28a(+)-*ectB*, pET-28a(+)-*ectC*, and pET-28a(+)-*ectABC*, respectively. These plasmids were transformed into *E. coli* BL21. The positive transformants were confirmed by restriction enzyme digestion (Table 1) and sequencing.

### Gene expression for *ectA*, *ectB*, *ectC*, and the *ectABC* cluster

The *E. coli* transformants with either *ectA*, *ectB*, *ectC* or the *ectABC* cluster were grown at 37°C in 50 ml of LB broth with Kanamycin (50 µg/ml). When the optical density of the culture at 600 nm ($OD_{600}$) reached 0.6, isopropylthiogalactoside (IPTG) was added at 1 mM concentration and the incubation was continued at 30°C for 2, 4, 6, 8, 12, and 24 h. Proteins were separated by SDS-PAGE analysis as described by Laemmli (1970).

### Salt tolerance and intracellular ectoine of a recombinant *E. coli* strain

The recombinant *E. coli* strain harboring the *ectABC* gene cluster was grown aerobically in improved OS medium containing 0.2 M, 0.4 M, 0.6 M, 0.8 M, 1.0 M, and 1.2 M NaCl, and then cultivated in a rotary shaker at 37°C and 120 rpm for 12 h. Growth was determined by measuring $OD_{600}$ using a UV-VIS spectrophotometer (Shanghai Spectrum, China). Intracellular ectoine from the recombinant strain was extracted with 80% (v/v) ethanol and subjected to isocratic HPLC analysis for quantification of ectoine.

### Nucleotide sequence accession number

The nucleotide sequences of the ectoine biosynthesis genes from QHL1 (JN897385) have been deposited in the GenBank nucleotide sequence database (NCBI) under accession numbers JX312790, JX312791, and JX312792.

## Results

### Characterization and accumulation of ectoine in QHL1

Strain QHL1 could grow in a range of NaCl concentrations from 0.04 to 2.74 M and was found to grow optimally at 0.86 M NaCl. The 16S rRNA gene from strain QHL1 was

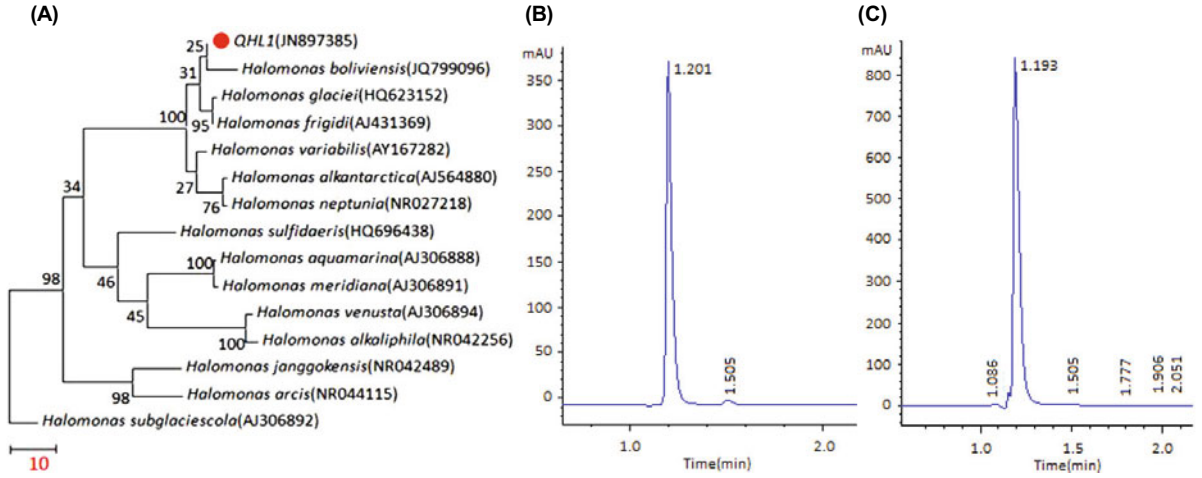


**Fig. 1. Neighbour-joining phylogenetic tree and HPLC map.** (A) Neighbour-joining phylogenetic tree based on 16S rDNA. The bootstrap consensus tree is based on 1,000 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The accession numbers of the respective peptides are given in brackets. (B) HPLC map showing the spectra of authentic ectoine at 39.06 μg/ml. (C) The HPLC map of intracellular ectoine extracted from strain QHL1. QHL1 was cultivated at 1.0 M of NaCl in improved Oesterhelt-Stoeckenius medium.

99% identical to that from *H. boliviensis* (JQ799096), *H. frigidi* (AY167282), *H. glaciei* (HQ623152), *H. variabilis* (DQ520887), *H. neptunia* (NR027218), and *H. alkantarctica* (AJ564880), and very low scores were obtained with other type strains. Phylogenetic analysis showed that strain QHL1 was most closely related to the genus *Halomonas*, belonging to the family Halomonadaceae (Fig. 1A).

The majority of species in *Halomonas* are able to grow at high salt concentrations by counteracting the salt stress through intracellular accumulation of ectoine. To ensure the detection of ectoine, we compared our extracts with HPLC chromatograms of authentic ectoine (Fig. 1B). Ectoine was detected in extracts of QHL1 cells (Fig. 1C). The retention time of authentic ectoine was from 1.188 to 1.205 min, depending on the concentration. Under the same conditions, the retention time of ectoine from QHL1 (cultivated at 1.0 M NaCl) was detected from 1.189 to 1.195 min and the same peak shape was observed. The concentration of ectoine in extracts of QHL1 was closely related to the NaCl concentration at which the cultures were grown. The optimal concentration of NaCl for producing ectoine was 1.0 M, with yields of 167.1 mg/g cell dry weight when grown under these conditions for 12 h.

## Amplification of conserved sequences and flanking sequences of *ectB* in QHL1

A PCR and genome walking approach was employed to identify the genes conferring the ability of strain QHL1 to synthesize ectoine. NCBI BLAST analysis revealed the presence of *ectABC* genes in a wide variety of halophilic and halotolerant bacteria. The *ectABC* genes for ectoine synthesis were typically co-localized on the genome and their primary sequences were highly conserved (Fig. 2A), particularly for the *ectB* gene (more than 80% similarity in *Halomonas*). Based on the similarity of *ectB* genes in the different species of *Halomonas*, we designed degenerate primers for this gene and obtained 977 bp of the *ectB* sequence of QHL1. Upon analysis with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST), it was found that the *ectB* fragment from QHL1 was homologous to *ectB* of several eubacteria: 87% identity with *Halomonas* sp. NJ223 (DQ886907); 79% identity with *Halomonas* sp. BYS-1 (DQ017757) and 98% identity with *Halomonas* sp. TNB I20 (AB119507).

To obtain the entire *ectABC* operon, genome waking was employed using a nested PCR approach as shown in Fig. 2B. A 1.5 kb fragment upstream of *ectB* was obtained with the fusion primer AP1 and specific primers SPR1/SPR2/SPR3 (Fig. 2C). Likewise, a 1.25 kb fragment downstream from *ectB* was obtained with fusion primer AP4 and specific primers SPF1/SPF2/SPF3 and sequenced (Fig. 2D). Sequence analysis revealed that the total 3.58 kb DNA fragment completely overlapped the *ectABC* operon. The *ectA*, *ectB*, and *ectC* genes encode L-2, 4-diaminobutyric acid acetyltransferase (DAA), L-2, 4-diaminobutyric acid transaminase (DAT), and L-ectoine synthase (ES), respectively.

## DNA sequence analysis of the ectoine operon in QHL1

The ectoine operon of *Halomonas* sp. QHL1 was cloned and sequenced. The operon was 3,580 bp long consisting of the *ectA*, *ectB* and *ectC* genes, which were 579, 1,269 and 390 bp, respectively, encoding putative proteins of 192, 422, and 129 amino acids. The identities of genes *ectA*, *ectB*, and *ectC* from QHL1 with the type strains in *Halomonas* were 74–80%, 78–91%, and 72–96%, and the identities of the corresponding proteins EctA, EctB, and EctC were 58–98%, 85-98%, and 75–96%. Molecular masses of EctA, EctB, and EctC were predicted to be 21,178, 46,413, and 14,739 Da, respectively, based on *in silico* estimates. Isoelectric points were predicted to be 4.78, 5.98, and 5.30, respectively. The lengths and molecular masses of these predicted polypeptides were similar among the *Halomonas* strains studied. Based on multi-sequence alignments, the intergenic regions between *ectA* and *ectB* consisted of 109 bp (gap1) and that between *ectB* and *ectC* was 91 bp (gap2) in QHL1 (Fig. 3). Gap1 was

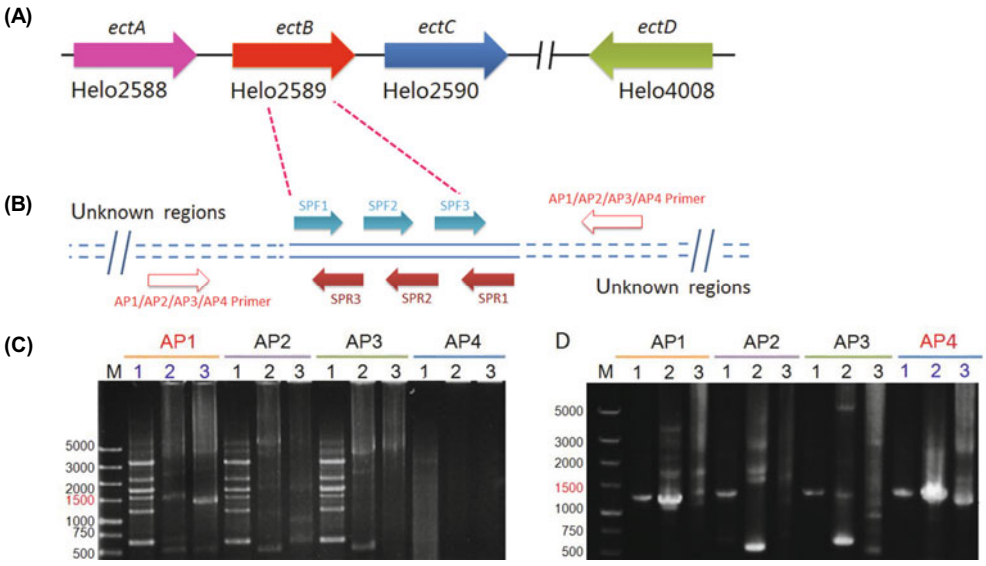


**Fig. 2. Strategy of nested PCR and amplification of flanking sequence from conserved *ectB* sequence in QHL1**. (A) The gene composition and organization of the *ect*-cluster in *H. elongate* DSM 2581$^{T}$ (http://www.ncbi. nlm.nih.gov/genome/3125). (B) Strategy of amplification of the flanking sequence by nested PCR. The arrows indicate the direction of the PCR amplification. Solid lines in the center represent 977 bp known conservative sequence of *ectB*. Primers AP1/AP2/AP3/AP4 were provided by the Genome Walking Kit D316 (TaKaRa Dalian, China). Specific primers SPR1/SPR2/SPR3 and primers SPF1/SPF2/SPF3 were used to amplify the upstream and downstream sequences, respectively. (C) The resulting upstream DNA fragments were separated on a 0.8% agarose gel. M means DNA marker. Lane 1 was the product of the first nested-PCR with SPR1 primer and random primer. Lane 2 was the second nested-PCR with SPR2 primer and random primer. Lane 3 was the third nested-PCR with SPR3 primer and random primer. (D) The resulting upstream DNA fragments were separated on a 0.8% agarose gel. Lane 1, 2 and 3 were shown to the first nested-PCR with SPF1 primer and random primer, the second nested-PCR with SPF2 primer and random primer and the third nested-PCR with SPF3 primer and random primer, respectively.

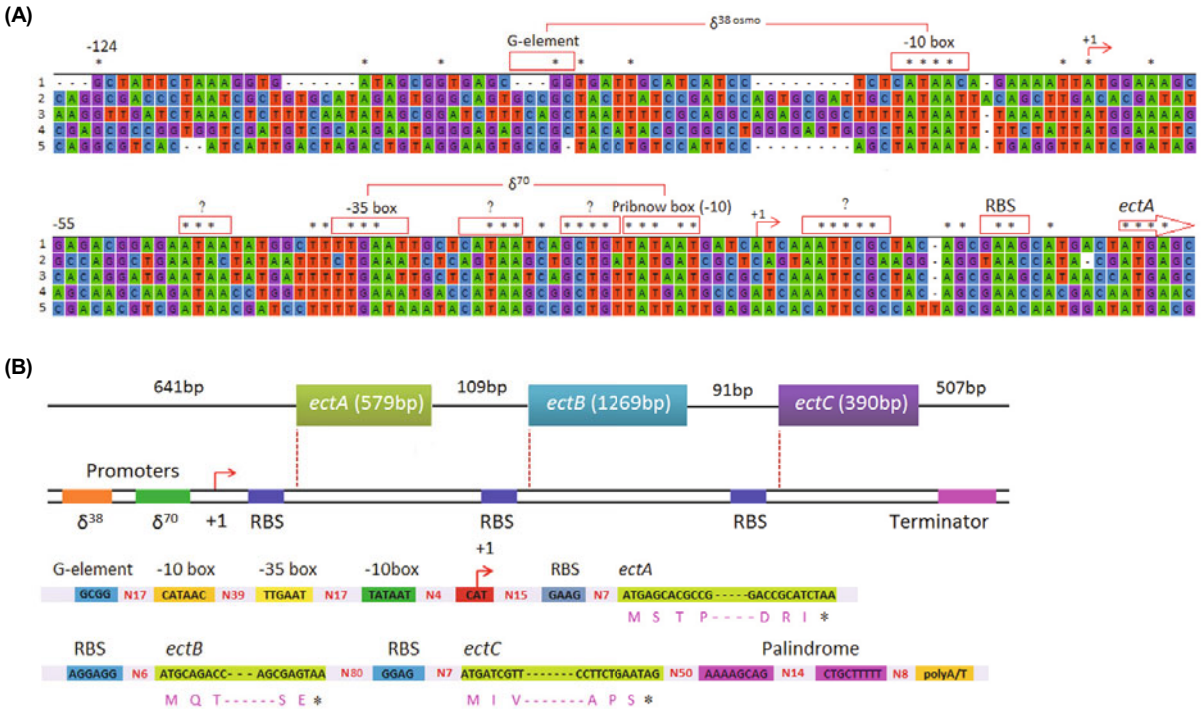


**Fig. 3. Sequence alignment of upstream *ectA* and putative promoter of the ectoine synthesis genes *ectABC* in QHL1**. (A) The upstream *ectA* promoter region was shown based on the sequence alignment by MEGA4.0 software. The numbers of 1, 2, 3, 4 and 5 indicated QHL1 in this study, *Halomonas* sp. Nj223 (DQ886907), *Halomonas* sp. BYS-1(DQ017757), *H. elongata* DSM2581 (FN869568), *H. elongata* DSM3043 (AJ011103), respectively. RBS was the ribosome-binding site, and arrows indicated the transcription initiation sites (+1). The -35 and -10 sequences of the $\delta^{70}$ like *E. coli* and $\delta^{38}$ promoters upstream of *ectA* were denoted with a red box. (B) Putative structural features of the promoter and terminator was predicted in *ectABC* genes cluster of QHL1. G-elements were characteristic for osmotically induced $\delta^{38}$ promoters. The putative terminator composing of the palindromic structure and A/T-rich region was located at the downstream sequence of *ectC*.

81–123 nucleotides in length, and gap2 was 84–114 nucleotides in length in the *Halomonas* strains examined. In the *ectABC* gene cluster, each gene was preceded at an appropriate distance by a putative ribosome-binding site (RBS) oriented in the same direction. The three putative RBS for *ectA*, *ectB*, and *ectC* were located at 7 bp (GAAG), 6 bp (AGGAGG) and 7 bp (GGAG), respectively, upstream from the start codons.

To gain further information on the transcriptional regulation of the *ectABC* gene-cluster, we predicted the transcriptional initiation sites (+1) in strain QHL1 using the online Neural Network Promoter Prediction tool (http://www.fruitfly.org/seq-tools/promoter.html). Inspection of the DNA sequence upstream of the *ectA* initiation site with the MEGA4.0 software revealed the presence of two putative promoters (Fig. 3A), a $\delta^{70}$-dependent promoter and a $\delta^{38}$-dependent promoter. These were also found in *Halomonas* sp. Nj223 (DQ886907), *Halomonas* sp. BYS-1(DQ017757), *H. elongata* DSM2581 (FN869568), and *H. elongata* DSM3043 (AJ011103). The -35 and -10 sequences (TTGAAT [17nt] TATAAT) of the putative $\delta^{70}$-dependent promoter matched well with the one (TTGACA [16-18nt] TATAAT) of *E. coli*. Generally, the distance between -10 and -35 was 17 bp, a typical spacing for promoter controlled by $\delta^{70}$ (Fig. 3B). Furthermore, the initiation site of the putative $\delta^{38}$ promoter was located at 91 bp upstream from the *ectA* start codon. The -10 DNA sequence (CATAAC) and -35 G-elements (GCCGC) identified in strain QHL1 resembles the $\delta^{38}$-controlled promoter in *H. elongata* DSM 2581$^{T}$, previously reported by Schwibbert *et al.* (2011). G-elements were characteristic for an osmotically induced $\delta^{38}$ promoter (Lee and Gralla, 2004). Additionally, some conserved motifs with unknown function were highly conserved in the upstream region of the *ectA* gene (Fig. 3B), including a 7 nt sequence (AATTCGC) located between the transcription initiation site and RBS, a 5 nt sequence (GCTGT) close to the -10 position, 5 nt sequence (CATAA) and 6 nt sequence (AATAAT) located on the flanks of the -35 position. Examination of the translation termination codon downstream of the *ectC* gene revealed that a typical inverted repeat structure (AAA AGCAG-$N_{14}$-CTGCTTTT) and the consecutive A/T-rich region were a putative termination signal without the presence of a factor-independent transcription terminator.

## Heterologous expression of ectoine biosynthesis genes from QHL1

The *ectA*, *ectB*, and *ectC* genes and the *ectABC* gene cluster were amplified from genomic DNA and cloned into the high-copy-number vector pMD18-T. Subsequently, the recombinant plasmids pET-28a (+)-*ectA*, pET-28a (+)-*ectB*, pET-28a (+)-*ectC* and pET-28a (+)-*ectABC* were successfully constructed by a subcloning strategy (Fig. 4A and 4B). The *E. coli* BL21 transformants harboring recombinant plasmids were induced with IPTG. SDS-PAGE electrophoresis

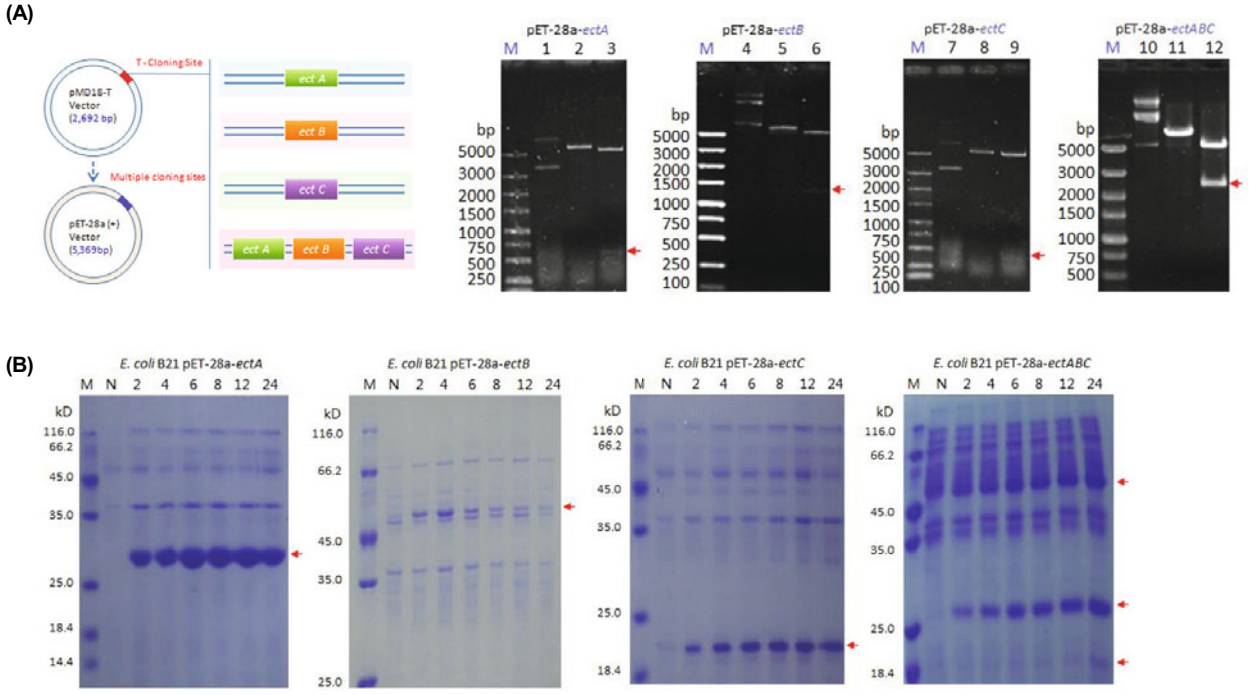


**Fig. 4. Subcloning strategy and heterologous expression of the ectoine biosynthesis genes.** (A) Subcloning strategy. The pMD18-T vector was an efficient TA cloning vector with an Ampicillin resistance tag. The pET-28a vector was a expression vector with a Kanamycin resistant tag. (B) Gel electrophoresis of the *ectA*, *ectB*, *ectC* gene and *ectABC* gene clusters were cloned to the vector pET-28a. M was the DNA molecular weight marker. Lanes 1, 4, 7 and 10 represented the recombinant plasmids. Lanes 2 (*Hin*dIII), 5 (*Eco*RI), 8 (*Hin*dIII) and 11 (*Bam*HI) were shown by mono-restriction endonuclease enzyme. Lanes 3 (*Bam*HI/*Hin*dIII), 6 (*Eco*RI/*Sac*I), 9 (*Bam*HI/*Hin*dIII) and 12 (*Bam*HI/*Sac*I) were shown by two restriction enzymes. Red arrows indicated the target gene. (C) SDS-PAGE analysis of the expressed *ectA*, *ectB*, *ectC* and *ectABC* in *E. coli* B21. M was the protein molecular weight marker. N indicated that the recombination strain was not induced. The numbers of 2, 4, 6, 8, 12, and 24 corresponds to the induction time at 37°C using 1 mM IPTG.

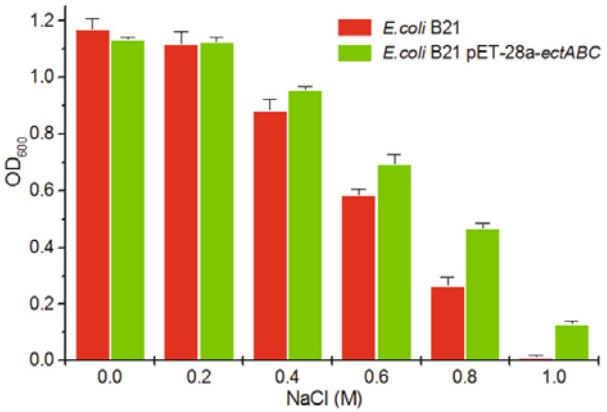


**Fig. 5. Salt tolerance of the native *E. coli* and *E. coli* engineered to express the *ectABC* gene cluster from *Halomonas* sp. QHL1.** Growth was checked by measuring the $OD_{600}$ after 12 h incubation at 37°C. Statistical analyses were performed using the Origin 5.0 software (OriginLab, USA). Analysis of variance was used to analyze experimental data. Results were expressed as means±standard deviation (n=3). Statistical significance was set at $P<0.05$.

of lysates of induced cells revealed proteins with molecular masses of 27.2 kDa, 52.5 kDa and 20.8 kDa, which correspond closely to the predicted size of EctA, EctB, and EctC, respectively (Fig. 4C). The pET-28a (+) vectors carried a 6.1 kDa N-terminal His-tag as part of the fusion protein. Therefore, the expressed bands were slightly more than the estimates *in silico*. To prove that the *ectABC* gene cluster from QHL1 was sufficient for ectoine production, we also constructed a recombinant strain of *E. coli* BL21 carrying the plasmid pET-28a (+)-*ectABC*. The results of heterologous expression indicated that the three genes could be simultaneously translated to proteins EctA, EctB, and EctC, as demonstrated using SDS-PAGE. However, the expression level of the *ectC* gene was significantly lower than that for the *ectA* and *ectB* genes, which may have been due to ectoine synthase degradation by *E. coli* proteases or a possible transcriptional arrest after *ectA* and *ectB*.

### Growth and ectoine production of genetically engineered *E. coli* BL21

To verify whether *ectABC* genes from QHL1 enhance the growth of *E. coli* BL21 in medium with high NaCl concentrations, osmotic expression experiments were carried out in the presence of different salinities ranging from 0 to 1.2 M NaCl. Based on the comparative analysis of growth (Fig. 5), the recombinant strain of *E. coli* BL21 containing the pET-28a (+)-*ectABC* plasmid could be inoculated in liquid OS medium containing 1.0 M NaCl, a salt concentration that is inhibitory for *E. coli* BL21. Control cultures of *E. coli* BL21 could be cultivated in a salinity range of 0 to 0.8 M NaCl, but growth decreased dramatically at salinities above 0.8 M NaCl. The recombinant strain, with a higher salt tolerance than *E. coli* BL21, showed a significant growth advantage over *E. coli* BL21 at high salinity. The accumulation of ectoine in lysate of the recombinant strain was a maximum of 25.05 mg/g cell dry weight at 0.8 M NaCl, in comparison to cell extracts of *E. coli* BL21, where no ectoine was detected. Hence, these results suggest that the accumulated ectoine functioned to improve the tolerance of *E. coli* cells to hyperosmotic shock.

## Discussion

Until now, *Halomonas* was comprised of 82 phylogenetically heterogeneous species according to the published literature (http://www.bacterio.cict.fr/h/halomonas.html). Ectoine synthesis was also examined in some of the species belonging to *Halomonas*, such as *H. salina* DSM 5928$^T$, *H. pacifica* DSM 4742$^T$, *H. ventosae* DSM 15911$^T$, *H. halodenitrificans* DSM 735$^T$ (Zhu *et al.*, 2007); *H. elongata* KS3 (Ono *et al.*, 1998) and *H. boliviensis* LC1$^T$ (Guzmán *et al.*, 2009). Some of the strains have been recognized for their potential application in biotechnology. In this study, we obtained a moderately halophilic bacterium identified as *Halomonas* sp. QHL1. It could accumulate ectoine as an osmoprotectant against increased external osmotic pressure.

The *ectABC* genes from QHL1 are similar to *ectABC* in other ectoine producing bacteria. Schwibbert and co-workers (2011) described the complete genome sequence of *H. elongata* DSM 2581$^T$ (4,061,296 bp) and the ectoine degradation pathway for the first time. In the present study, we obtained the complete *ectABC* gene sequence of *Halomonas* sp. Nj223 (DQ886907), *Halomonas* sp. BYS-1(DQ017757), *H. elongata* DSM2581 (FN869568), *H. elongata* DSM3043 (AJ011103), and *H. elongata* (AF031489 and D88359.1) provided by NCBI. Multiple sequence alignments indicated that the putative $\delta^{70}$-dependent promoter with characterized -35 and -10 sequences has the highest identity to the $\delta^{70}$-dependent consensus sequence of *E. coli* (Cánovas *et al.*, 1998; Schwibbert *et al.*, 2011), while the putative $\delta^{38}$-controlled promoter was identical to the osmotically induced $\delta^{38}$ promoter of *E. coli* (Lee and Gralla, 2004). Upstream of the initiation site of *ectC*, a putative $\delta^{54}$-controlled promoter involved in transcription of nitrogen regulated genes was found in *H. elongata* DSM2581 (Bordo *et al.*, 1998; Schwibbert *et al.*, 2011), while it was not present in other *Halomonas* species examined, based on the multiple sequence alignment of intergenic regions. It is noteworthy that, the putative $\delta^{70}$ and $\delta^{38}$ promoters are widespread in the genus *Halomonas*. In this study, some conserved motifs with unknown function were also highly conserved in the upstream region of the *ectA* gene. The presence of highly homologous conserved motifs in *Halomonas* might indicate a common regulatory mechanism providing osmoprotection among these species.

The *ectABC* gene cluster from QHL1 was heterologously expressed in *E. coli* BL21. The growth and amount of intracellular ectoine in recombinant strain BL21 increased with the salinity of the growth medium. This indicated that *E. coli* BL21 harboring the *ectABC* gene cluster had slightly improved salt tolerance, but could not acclimate to higher salinities (more than 1.0 M). When compared to the *E. coli* BL21, the recombinant strain displayed a higher salt tolerance. When compared to the wild-type QHL1 strain, the amount of ectoine produced was markedly lower, which is likely due to differences in transcriptional and translational machinery in the heterologous host bacteria. The culture

grown in the absence of IPTG did not produce any measurable amounts of ectoine, whereas IPTG induced expression of the T7 promoter of the *ectABC* gene cluster resulting in the production of ectoine. This is consistent with the result of Zhao *et al*. (2006) who cloned and characterized the *ect* genes from *Halobacillus dabanensis* D-8$^{T}$.

Very recently, Shikuma *et al.* (2012) identified a transcriptional regulator named CosR for compatible solute regulator in *Vibrio cholerae*. The expression of *cosR* was regulated by ionic strength, not by osmolarity, which regulated ectoine biosynthesis and compatible solute transport in a salinity-dependent manner. In addition, EctR, a MarR-type regulator from *Methylomicrobium alcaliphilum*, was also shown to regulate the transcription of the *ect*-operon (Mustakhimov *et al*., 2010). In *Halomonas*, there were two putative promoters that might be recognized by $\delta^{70}$ and $\delta^{38}$ factors, and the conserved motifs with unknown function may also play an important role in the regulation of ectoine biosynthesis. Hence, identifying and characterizing the regulation of conserved motifs in QHL1will be one of our main experimental aims in the future.

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