The Journal of Microbiology, June 2008, p. 309-318 Vol. 46, No. 3
DOI 10.1007/s12275-008-0001-x
Copyright © 2008, The Microbiological Society of Korea

Cloning and Characterization of the Gene Cluster for Biosynthesis of Ectoine from *Nesterenkonia halobia* DSM 20541

Bo Zhang, Xin Bao, Lei Wang, and Su Sheng Yang*

Department of Microbiology and Immunology, College of Biological Sciences, China Agricultural University and Key Laboratory of Agro-Microbial Resource and Application, Ministry of Agriculture, Beijing 100193, P. R. China

(Received December 25, 2007 / Accepted March 26, 2008)

The *ectABC* genes encoding the biosynthesis of ectoine were identified from *Nesterenkonia halobia* DSM 20541. The intergenic regions of the *ectABC* genes from *N. halobia* DSM 20541 were more loosely spaced than those that had been reported before. The amino acid sequence deduced from *ectABC* of the strain was highly homologous to the EctABC of *Brevibacterium linens* BL2 (EctA 50%, EctB 70%, and EctC 68% identities). The osmoprotection of *ectABC* was studied in the *Escherichia coli* KNabc and *E. coli* XL1-Blue. The results revealed that *ectABC* could shorten the lag phase and enhance the final OD600 of *E. coli* XL1-Blue in MM63 medium containing 0.68 M NaCl, and could initiate KNabc growth in 0.2 M NaCl. Ectoine was proven to be accumulated in *E. coli* KNabc/pGEM-Nect using HPLC-UV, and validated by LC-MSD-Trap-VL.

Keywords: *ectABC* gene cluster, *Nesterenkonia halobia* DSM 20541, osmoprotection

Halotolerant and halophilic prokaryotes living in high salt environments have developed two principal mechanisms, the salt-in-cytoplasm mechanism and the organic-osmolyte mechanism, to avoid the loss of water from the cell and achieve a cytoplasm osmotic strength similar to that of the surrounding environments (Spring *et al.*, 1996; Sleator and Hill, 2001; Oren, 2002; Lu *et al.*, 2006). Moderate halophilic and the most halotolerant bacteria mainly adopt the organic-osmolyte mechanism. Ectoine, one of the best-investigated compatible solutes, is the most common osmolytes in aerobic moderate halophilic bacterium (Goller *et al.*, 1998; Ventosa *et al.*, 1998). The synthesis pathways of ectoine have been elucidated in many bacteria (Peters *et al.*, 1990; Reshetnikov *et al.*, 2006; Zhao *et al.*, 2006). The genes (*ectABC*) of the biosynthetic pathway of ectoine were first cloned from *Marinococcus halophilus* by function complementation in *E. coli* XL1-Blue, which could make XL1-Blue grow in the MM63 medium containing 0.86 M NaCl and cause osmoregulation in response to medium salinity (Louis and Galinski, 1997). Recently, more *ectABC* clusters have been cloned from *Halomonas elongata* (Goller *et al.*, 1998), *Chromohalobacter salexigens* (formerly *Halomonas elongata* DSM 3043) (Canovas *et al.*, 1998), *Bacillus pasteurii* (Kuhlmann and Bremer, 2002), *Halobacillus dabanensis* D-8^T (Zhao *et al.*, 2006) and *Methylomicrobium alcaliphilum* 20Z (Reshetnikov *et al.*, 2006), but they did not initiate *E. coli* XL1-Blue growth on MM63 medium containing 0.86 M NaCl (Louis and Galinski, 1997; Canovas *et al.*, 1998; Reshetnikov *et al.*, 2006; Zhao *et al.*, 2006).

The use of sodium proton antiporters is another strategy to induce high salinity tolerance, and they are universal secondary ion transporters in bacteria. Typically, they expel toxic Na^+ and Li^+ ions from the cytoplasm at the expense of the proton motive force, thus playing an important role in cytoplasmic Na^+ and pH homeostasis and providing energy for Na^+ symports. There exist four Na^+/H^+-antiporters (NhaA, NhaB, Cha, and Mdf) in *E. coli*. *E. coli* KNabc, the mutant of *NhaA*, *NhaB*, and *Cha*, does not grow in LBK medium containing 0.2 M NaCl (Ohyama *et al.*, 1994; Nakamura *et al.*, 1996). However, *E. coli* MKH13, the mutant of *proP* and *proU*, can not accumulate choline and glycine betaine by transportation into the cytoplasm and does not grow in minimal medium A containing 0.8 M NaCl (Haardt *et al.*, 1995). So the compatible solutes, such as trehalose, proline betaine and glycine betaine together with the Na^+/H^+ antiporters confer the ability of halotolerance on *E. coli* wild type strains, and the Na^+/H^+ antiporters are more important. Hence, the salt sensitive mutant *E. coli* KNabc can be used to study the osmoprotection of compatible solutes, because the halotolerance endowed by the Na^+/H^+ antiporters was almost completely attenuated, which makes the compatible solutes become more important.

N. halobia DSM 20541 (formerly designated as *Micrococcus halobia*; hereafter *N. halobia*), the type species of the genus of *Nesterenkonia*, is a Gram-positive, moderately halophilic, heterotrophic bacterium and its GC content is rich (about 72%). Optimum growth occurs on media containing 1 to 2 M NaCl, and moderate growth occurs in the presence of 4 M NaCl, but no growth occurs on media lacking NaCl or KCl. Ectoine and hydroxyectoine are the predominant compatible solutes in *N. halobia* (Ventosa *et al.*, 1998). The characteristics of this strain are quite different from other bacteria which contained similar pathway. It is a good material to study the halophilic mechanism, thus the *ectABC* gene cluster

* To whom correspondence should be addressed.
(Tel) 86-10-6273-7827; (Fax) 86-10-6273-1332
(E-mail) yangssh@cau.edu.cn

responsible for the synthesis of ectoine from *N. halobia* was cloned, and its osmoprotection in different *E. coli* strains was studied.

Materials and Methods

Bacterial strains, plasmids, media, and growth conditions

N. halobia from DSMZ was routinely propagated at 37°C in Gibson's medium containing 1.5 M NaCl or in DSMZ medium 229. *E. coli* XL1-Blue and *E. coli* KNabc were grown at 37°C in LBK (Goldberg *et al.*, 1987) or in MM63 medium (Miller, 1972). The antibiotic used was 100 μg/ml of ampicillin. The pGEM-T easy vector (Promega, USA) was used as general vector for cloning and sequencing. Plasmid pGEM-3zf (+) was kindly provided by T.A. Krulwich.

DNA manipulation and materials

Nucleic acid manipulation and general cloning procedures were performed with standard methods (Joseph and Davis, 2001). Competent cells of *E. coli* XL1-Blue and *E. coli* KNabc were prepared with the method described by Inoue *et al.* (1990). Restriction endonucleases, TaKaRa RNA PCR Kit (AMV, ver3.0), LA *Taq* DNA polymerase and T4 DNA ligase were purchased from TaKaRa Dalian Co. Phusion DNA polymerase for PCR amplification was obtained from New England Biolabs Inc.. Ectoine was purchased from Sigma, USA. Synthesis of oligonucleotide primers was conducted at Sangon Biotech (China). DNA sequencing was performed at TaKaRa Dalian Co. (China). Databank searches were performed through the National Center for Biotechnology Information (NCBI) using the BLAST program.

Probe construction and labeling

To isolate a fragment of the *ectABC* genes from *N. halobia*, a PCR strategy with degenerate primers was used. Forward primer; (ectB-up) 5'-GGWGARATHTTYGTBATWATGGG DCT-3' and reverse primer; (ectB-down) 5'-AWVCGHARB GCYTCRTCCAARTCATG-3' were used for PCR with chromosomal DNA of *N. halobia* as a template. The reaction was conducted using the following conditions: denaturation at 94°C for 50 sec, annealing at 52°C for 50 sec, extension at 72°C for 40 sec, 30 cycles. The PCR product was cloned into the pGEM-T easy vector and sequenced. The recombinant plasmid was digested completely with *Eco*RI, and then the DNA fragments of about 497 bp were purified and labeled as a probe to detect the entire *ectABC* genes in *N. halobia*. Labeling of the fragments was performed with the DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostic GmbH, Germany) as recommended by the manufacturer.

Southern blot hybridization

Genomic DNA of *N. halobia* was digested completely with *Aat*II, *Bam*HI, *Not*I, *Sac*I, and *Sal*I. The resulting fragments were fractionated by electrophoresis in 0.7% agarose gels, and transferred to a positive-charged nylon membrane (Hybond, USA) and hybridized with the above-mentioned probe. Southern blot hybridization and detection were performed according to standard techniques, using the DIG-High Prime DNA Labeling and Detection Starter Kit I as described previously.

Inverse PCR (IPCR)

To obtain the nucleotide sequence flanking the DNA fragment, inverse PCR (IPCR) was carried out. IPCR was performed essentially as described earlier (Ochman *et al.*, 1988). The genomic DNA was completely digested by *Bam*HI, and purified and self-ligated with T4 DNA ligase at 16°C for 48~72 h. Then the circular fragments were used as templates and one pair of primers: NIP1; 5'-GCCGCGAACCT GCCTCAGAA-3' and NIP2: 5'-TGACGCTGGGCTCGCTG TCGGT-3', derived from the nucleotide sequence of the PCR products were used for the IPCR.

Osmotic expression of *ectABC* gene cluster in *E. coli*

The forward primer; 5'-CGGAATTCTGAAGCGTCCCGTC CG-3': the *Eco*RI site is underlined and the reverse primer; 5'-GCTCTAGAGTGATGCTCGTCTGCCTGAT-3': the *Xba*I site is underlined, were used for PCR with the genomic DNA of *N. halobia* as a template to obtain a 3.444 kb fragment. The reaction was conducted using Phusion DNA polymerase as the following conditions: denaturation at 98°C for 10 sec, annealing at 52°C for 10 sec, extension at 72°C for 1 min 40 sec, 30 cycles. The amplified DNA was purified and digested with *Eco*RI and *Xba*I and cloned into pGEM-3zf (+) (also digested with *Eco*RI and *Xba*I), resulting in pGEM-Nect. The plasmid pGEM-Nect was transformed into *E. coli* KNabc and *E. coli* XL1-Blue. Then the salt tolerance and growth curves of the two *E. coli* strains with pGEM-Nect in different concentrations of NaCl were determined.

Identification and validation of ectoine accumulation in KNabc/pGEM-Nect

The extraction of ectoine was carried out according to Kuhl-

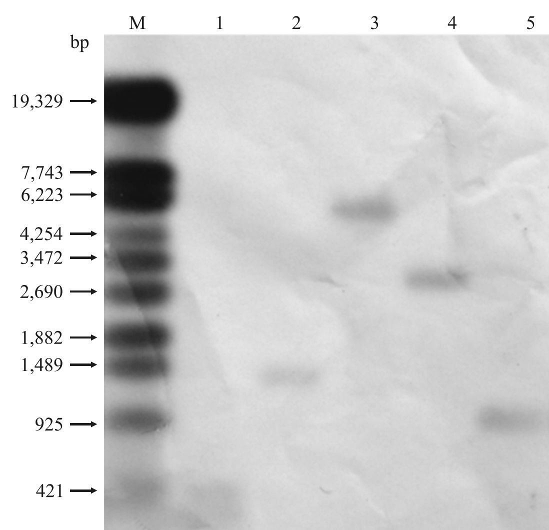

Fig. 1. Southern blot analysis of *Sal*I (1), *Sac*I (2), *Not*I (3), *Bam*HI (4) and *Aat*II (5) completely digested genomic DNA of *N. halobia*. The DIG-labeled 497 bp fragment DNA probe produced by PCR was used to detect the entire ORF of *ectABC* gene cluster. M, λ/EcoT14 DNA Marker.

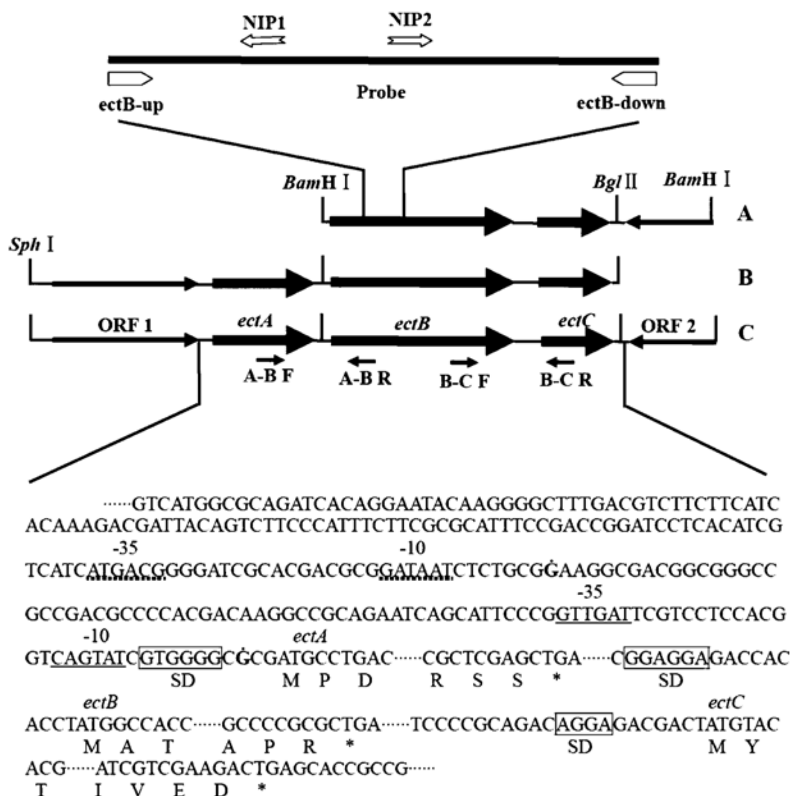

Fig. 2. Cloning strategy: a physical map of the 5.496 kb fragment from *N. halobia* is shown. A, the segment obtained by inverse PCR (IPCR); B, the segment obtained from a partial gene bank; C, the segment merged with A and B. Orientation of oligonucleotide primers and the probe are shown. The -10 and -35 regions of the putative σ^{70}-dependent promoter are marked with dotted lines, and the -10 and -35 regions of the putative σ^{B}-dependent promoter are underlined. The potential ribosome-binding sites (RBSs) are boxed. Stop codons are marked with asterisks. The degenerate primers ectB-up and ectB-down, the inverse PCR primers NIP1 and NIP2 and the RT-PCR primers A-B F, A-B R, B-C F and B-C R are also indicated.

mann (Kuhlmann and Bremer, 2002). HPLC-UV (Agilent, USA) was used to prove ectoine synthesis in *E. coli* KNabc/pGEM-Nect. The chromatographic conditions were as follows: the mobile phase was acetonitrile: water (80:20 v/v) at a flow rate of 1 ml/min. Detection was performed at 210 nm. Validation of the corresponding HPLC peak with ectoine was confirmed by LC-MSD-Trap-VL (Agilent, USA). The aim of the LC/MS analysis was to identify ectoine according to the mol peak of 142 g/mol.

RNA isolation and RT-PCR

Total RNA and RT-PCR were prepared according to Lu (Lu *et al.*, 2006). Primers A-B F; 5'-ACCTGTTCACCGAAGACCTC-3', A-B R; 5'-TTGGTGAACGACAGGATGTG-3', B-C F; 5'-AGAAGGGCATCAAGGTCATC-3' and B-C R; 5'-GTGATCTCGTGGACCTCGTC-3' were designed from the *ectA*, *ectB*, and *ectC* sequences, respectively (Fig. 2). The absence of contaminating genomic DNA was controlled by non-RT-PCR performed under the same conditions, except that the avian myeloblastosis virus reverse transcriptase was replaced with DEPC-treated H_2O.

Nucleotide sequence accession number

The nucleotide sequence of the *N. halobia ectABC* genes and the flanking sequences have been submitted to GenBank and have been assigned accession number EU035983.

Results and Discussion

Cloning of the *ectABC* genes from *N. halobia*

Two stretches of amino acids, AGSLNYGHN (N-terminus) and BTVQGEGGIN (C-terminus) based on the well-conserved regions of *ectB*, were chosen to synthesize the degenerate primers called ectB-up and ectB-down. A 497 bp amplicon using the genomic DNA of *N. halobia* as the template was obtained and sequenced. BLAST search showed that the deduced amino acid sequence of this fragment was highly similar to the *ectB* gene of *Brevibacterium linens* BL2 (81% identity, 92% positive), so we concluded it might be the partial sequence of *ectB* from *N. halobia*. In order to obtain the entire ORF of the *ectABC* gene cluster, Southern blot hybridization experiments were carried out and the *ectABC* gene cluster was detected on a 2.87 kb *Bam*HI fragment (Fig. 1). A pair of primers, NIP1 and NIP2, derived from the 497 bp PCR product was used to amplify the entire ORF of *ectABC* from the 2.87 kb fragment by IPCR technique. The strategy for IPCR is shown in Fig. 2. However, computer analysis and database searches revealed

Table 1. Sequence identities (%) between the diaminobutyric acid acetyltransferase (EctA), diaminobutyric acid aminotransferase (EctB), ectoine synthase (EctC) proteins of '*Nesterenkonia halobia* DSM 20541' and those from the other bacteria

Bacteria	Proteins			The length of intergenic region between	
	EctA	EctB	EctC	*ectA* and *ectB*	*ectB* and *ectC*
Nesterenkonia halobia DSM 20541	100	100	100	169 bp	206 bp
Marinococcus halophilus DSM 2581	38	54	41	89 bp	104 bp
Sporosarcina pasteurii DSM 33^T	34	57	46	49 bp	158 bp
Halobacillus dabanesis D-8^T	37	56	48	15 bp	overlap
Chromohalobacillus salexigens DSM 3043	36	50	36	22 bp	115 bp
Methylobacter alcaliphilus 20Z	36	51	40	42 bp	50 bp
Brevibacterium linens BL2	50	70	68	167 bp	155 bp
Rhodococcus sp. RHA1	50	61	58	27 bp	19 bp
Nocardia farcinica IFM 10152	50	60	58	overlap	overlap
Streptomyces avermitilis	48	60	59	29 bp	57 bp
Streptomyces chrysomallus	46	60	56	158 bp	86 bp
Bacillus halodurans	37	59	42	106 bp	39 bp
Vibrio cholera	36	52	42	23 bp	35 bp
Oceanobacillus iheye HTE831	38	57	46	0 bp	15 bp

that the 2.87 kb IPCR product only contained the entire *ectB* and *ectC* but not the *ectA* gene.

Because of the high GC content of the genomic DNA, it was quite difficult to carry out a second inverse PCR. So after a few unsuccessful attempts, the second IPCR was abandoned. Then several Southern blot hybridizations (data not shown) were performed and revealed a *Sph*I site located in front of the 2.87 kb DNA segment with a distance of about 2.4 kb, which comprised the entire *ectA* (about 600 bp in length). Also, a *Bgl*II site was located in the 2.87 kb DNA segment at a distance of 2.6 kb from the 5' end (Fig. 2). These two sites (*Sph*I and *Bgl*II) would be good ones to construct the partial gene library. The genomic DNA was digested completely with *Sph*I and *Bgl*II, and the 4.5~5.5 kb fragments containing the entire *ectA* gene were obtained and purified using the EZ Spin Column DNA Gel Extraction Kit (BBI, Canada). The fragments were ligated into pUC18 digested with *Sph*I and *Bam*HI, and the ligation products were transformed into *E. coli* DH5α. The white colonies were all picked out, and the correct transformants were selected by colony PCR. After sequenced and merged with the 2.87 kb DNA fragment, a 5.496 kb fragment was obtained (Fig. 2).

Sequence analysis of the 5.496 kb fragment

DNA sequence analysis of the 5.496 kb fragment revealed the presence of four complete open reading frames (ORFs) oriented in the same direction (Fig. 2): ORF1, *ectA*, *ectB*, and *ectC*. They were predicted to encode proteins of 538, 175, 427, and 128 amino acids with deduced molecular masses of 57261, 19137, 46929, and 14696 Da, respectively. Also an incomplete ORF, ORF2, was in the 3' end of the 5.496 kb fragment and oriented in the reverse direction. In the *ectABC* gene cluster, each gene is preceded at an appropriate distance by a putative ribosome-binding site oriented in the same direction. The intergenic region between the *ectA* stop codon (TAG) and the *ectB* start codon (ATG) is 169 nucleotides in length, and the intergenic region between the *ectB* stop codon and the *ectC* ATG start codon is 206 nucleotides in length. Both are more loosely spaced than those of *H. dabanensis*, *C. salexigens*, *M. halophilus*, *B. halodurans*, and *Methylomicrobium alcaliphilum* 20Z etc. (Table 1). The transcription initiation site of the DNA sequence upstream of the *ectA* was predicted through the Internet (http://www.fruitfly.org/seq_tools/promoter.html) and revealed the presence of two putative promoters: σhttp://www.fruitfly.org/seq_tools/promoter: σ^{70} and σ^{B} (Fig. 1). The -35 and -10 sequences (ATGACG [16nt] GATAAT) of the putative σ^{70}-dependent promoter matched well with the one (TTGACA [16-18nt] TATAAT) of *E. coli*. However, the -35 and -10 sequences (GTTGAT [14nt] CAGTAT) of the putative σ^{B}-dependent promoter did not match well with the one (GTTTAA [12-14nt] GGGTAT) of *Bacillus subtilis*. Examination of the translation termination codon downstream of the *ectC* gene did not reveal the presence of a factor-independent transcription terminator signal with its typical inverted repeat structure and run of consecutive T residues.

Amino acid sequence homologies

Database searches with the BLAST network service showed a significant degree of sequence identity of the *N. halobia* EctABC proteins to enzymes known to be involved in ectoine biosynthesis in the moderate halophilic bacteria *M. halophilus*, *H. elongata*, *C. salexigens* (formerly *H. elongata* DSM 3043), *Sporosarcina pasteurii* (formerly *Bacillus pasteurii* DSM 33^T) and *Halobacillus dabanesis*. These database searches also revealed *ectABC* gene clusters in the finished genomes of *Rhodococcus* sp. RHA1, *Nocardia farcinica* IFM 10152, *Streptomyces avermitilis*, *Streptomyces chrysomallus*, *B. halodurans*, *Vibrio cholerae*, *Oceanobacillus iheye* HTE831

and in the incomplete genome sequence of *Brevibacterium linens* BL2 (Table 1 and Fig. 3). Generally, the EctABC proteins of *N. halobia* shared a much higher sequence identity with the taxonomically closely related species belonging to Actinobacteridae such as *B. linens* BL2, *Rhodococcus* sp. RHA1, *S. avermitilis*, and *S. chrysomallus*. *B. linens* BL2 showed the highest degree of amino acid sequence identity to *N. halobia* (EctA, 50%; EctB, 70%; EctC, 68%) (Fig. 3). ORF1 was predicted to encode di- and tri-carboxylate transporters showing a high level of amino acid sequence identity with *B. linens* BL2 (48%). The closest homologue of ORF2, the incomplete ORF of 622 bp, is a gene encoding a TctA subunit of the tripartite tricarboxylate transport (TTT) family from *Ralstonia eutropha* H16, with 57% derived amino acid identities or a gene encoding the large inner membrane subunit of the TRAP-T family transporter from *Rhodobacter sphaeroides* 2.4.1, with 56% derived amino acid identities. From the above-mentioned, the genes flanking the *ectABC*

(A)

```
Nesterenkonia halobia         ..................MPDSADDPAHSGEEIELRAPVLSDGAALWRIAEGTGVLDVNT   42
Bacillus halodurans           .MQVQCNEKAFKGGFIINSQIATAPPKTLDTTITIGKPTVEDGAAMWELVNKS.TLDTNS   58
Brevibacterium linens         ......................................MEDGQHMWRLAKDSAVLDLNS   21
Chromohalobacter salexigens   MDMTPTTENFTPSADLARPSVADTVIGSAKKTLFIRKPTTDDGWGIYELVKACPPLDVNS   60
Rhodococcus sp.               ................MTPVQKSAIPTTAPDAVLFRSPEITDGVRLWEIARNTEVLDLNS   44
Streptomyces avermitilis      ..............MTAAHADLQAEFLEMPEGLRIDRPDVADGSALWRIAKDSKTLDLNS   46
Vibrio cholerae               ..................MIYPQIMHKPALPWVFRRPTQEDGLSIHELIAQCAPLDQNS   41

Nesterenkonia halobia         PYAYLLWTRDFATTSVIALVD.GRPAGFISGYLRPSDPQTLFIWQVAVDSEFRGRRLASR  101
Bacillus halodurans           PYKYIMMCEYFAETCVVAKEN.ERLVGFVTAFIPPEHQDVIFVWQIGVDSSQRGKGLASK  117
Brevibacterium linens         SYSYILWCRDFSATSTIARIG.GEPAGFVTGYTRPDRPNTLMIWQVAVSSDFRGHGLAKT   80
Chromohalobacter salexigens   GYAYLLLATQFRDTCAVATDEEGEIVGFVSGYVKRNAPDTYFLWQVAVGEKARGTGLARR  120
Rhodococcus sp.               SYAYLLWCRDFSRSSVVAVVD.ERVVGFVSGFIRPESPATLFVWQVAVDADQRGKGIAGR  103
Streptomyces avermitilis      SYSYLLWCRDFAGTTAVARAADGTPVGFITAYVRPERPHTLLVWQVAVDAAYRGRGLAAR  106
Vibrio cholerae               AYCNFLQSSHFQTTCLMAEQQE.LLVGFVSAYRKPEQQNELFIWQVAVHPSARGKGLAYQ  100

Nesterenkonia halobia         MLADVVARS....GARRLETTITADNTASIALFTGLARDHDAEITRSDLFTEDLFPAQQE  157
Bacillus halodurans           LLQELISRDICS.NVNYVEATVTPSNKASQALFQKLAREYNTQCEVSECFSEDLFPGDDH  176
Brevibacterium linens         MLNELADRT....NALRLETTITDDNDASNRLFQSFAEQRDANCERSALITPDLYPDGHD  136
Chromohalobacter salexigens   LVEAVLMRPGMG.DVRHLETTITPDNEASWGLFKRLADRWQAPLNSREYFSTG.QLGGEH  178
Rhodococcus sp.               MLSALLDRLAP.EGITHLETTISPDNEASIALFTALARRRDTAINKQELFSPNDFPDGHE  162
Streptomyces avermitilis      MLDGLTARVTDEYGVTGIETTISPGNTASERLFTSYAQRHGADLEREVLFEAGLFPDAPH  166
Vibrio cholerae               MLKHLLAREDLA.DITVLETTITRSNQASWRLFQKLDREQGEQGSVSTFLDETCHFEGEH  159

Nesterenkonia halobia         TGEHHAAEDLYTVEPLRSS.                                         176
Bacillus halodurans           EA.....ELTFRIGPLHP..                                         189
Brevibacterium linens         T......EYLYEIAPL....                                         146
Chromohalobacter salexigens   DP.....ENLVRIGPFEPQQ                                         193
Rhodococcus sp.               A......EDLYTIG......                                         170
Streptomyces avermitilis      DP.....EVLYRIGPLSH..                                         179
Vibrio cholerae               DT.....EYLYRI.PLQSSN                                         173
```

(B)

```
Nesterenkonia halobia         MYTLHIDDLNDGERDIRDAD..WRSRRMVLGREKVGFSLHETTIYAGSTHSFWYANHIEA   58
Bacillus halodurans           MKVVKLEDVIGTEQEVKGEN..WTSRRLLLKKDGMGYSVHDTIIKAGTETHIWYQNHLEA   58
Brevibacterium linens         MYVVNRDDLNDTDRDIKSET..WRSRRMVLGKERVGFSLHDTVIYAGTTSTFHYQNHVEA   58
Chromohalobacter salexigens   MIVRNLEECRKTERFVEAENGNWDSTRLVLADDNVGFSFNITRIHPGTETHIHYKHHFEA   60
Rhodococcus sp.               MIVRTTAEITDTDRDITSEDGNWRSKRIILGGDKVGFSFHETTIKAGSVNEFHYANHVEA   60
Streptomyces avermitilis      MIVRSFKDIEGTDRHVKAASGTWESKRIVLAKEKVGFSLHETVLYAGTETSMWYANHIEA   60
Vibrio cholerae               MIVRTLEECRQSERRVVAEN..WESVRMLLKDDHMGFSFHITTIYANTQTHIHYRNHLES   58

Nesterenkonia halobia         VYCVGGKGRLTNLETDEVHEITDGFLYLLDGHEKHQVEADE.ELRLVCVFNPPVTGKEIH  117
Bacillus halodurans           VYCIEGEGEVETVKDGKVWPIKANEIYALDEHDEHLLRAKT.DMRMVCVFNPPITGKETH  117
Brevibacterium linens         VYLVQGKGTLTDHETGETYPLSDGTMYLLDGHEKHTVVAEE.ELRMACVFNPPVTGRETH  117
Chromohalobacter salexigens   VFCYEGEGEVETLADGKIHPIKAGDMYLLDQHDEHLLRGKEKGMTVACVFNPALTGREVH  120
Rhodococcus sp.               VWLVEGTGKLIDLDNDKVYELGPGSMYLLNGHERHRVEPET.EMRMLCVFNPPVTGREVH  119
Streptomyces avermitilis      VLCVEGEAELTDDETGEKHWITPGTMYLLDGHERHTMRPKT.DFRCVCVFNPPVTGREDH  119
Vibrio cholerae               VYCMSGEGEIEVVG.GKTYPIQPGTLYILDQHDEHYLRAFSSEMVMACVFNPPLTGHEIH  117

Nesterenkonia halobia         DENGVYPLIVED.........                                        129
Bacillus halodurans           DENGVYPLVDDE.........                                        129
Brevibacterium linens         DENGVYPLIVEED........                                        130
Chromohalobacter salexigens   REDGSYAPVD...........                                        130
Rhodococcus sp.               DENGVYPLVEVPA........                                        132
Streptomyces avermitilis      DENGVYPLLTEPEEV......                                        134
Vibrio cholerae               DAEGVY.PLDKSELISQCHKE                                        137
```

(C)

```
Nesterenkonia halobia         .......MATDIFETFESQVRSYCMNWPAVFEKASGSYQYTEDGSRYLDFFSGAGALNYG     53
Bacillus halodurans           ....MSQTDMNVFEQLESEVRSYCRSFPTVFTKAKGYKMWDEAGKEYIDFFSGAGALNYG     56
Brevibacterium linens         MTENSKTTKPDIFETRESEVRGYSRSWPATFAKAQGAKQWGEDGKEFIDFFSGAGALNYG     60
Chromohalobacter salexigens   .......MQTQILERMESEVRTYSRSFPTVFTEAKGARLHAEDGNQYIDFLAGAGTLNYG     53
Rhodococcus sp.               ....MTNFDTNIFDNLESEVRSYSRGWPAVFESASGSWIRDENGRDYLDFFAGAGSLNYG     56
Streptomyces avermitilis      ..MTITQPDLSVFETVESEVRSYCRGWPTVFDRAQGSRMYDEDGHAYLDFFAGAGSLNYG     58
Vibrio cholerae               .........MDIFKHHESQVQSYANHFPVLFGTAKGSWLYSQQGDAYLDFLSGAGALNYG     51

Nesterenkonia halobia         HNHPELRDLLVDYVANDGVTHSLDMKTPSKRRFLETFERVILEPRNMEYKVMFPGPTGTN    113
Bacillus halodurans           HNDEKMKKALVDYIMDDGITHSLDMATTPKGKFLQKFHDVILKPRNLDYKVMFPGPTGTN    116
Brevibacterium linens         HNNPVVMNPLIEYLQSGAVLHSLDMKTPAKREFLETFQDLILKPRGLDYTVMFPGPTGTN    120
Chromohalobacter salexigens   HNHPKLKQALADYIASDGIVHGLDMWSAAKRDYLETLEEVILKPRGLDYKVHLPGPTGTN    113
Rhodococcus sp.               HNNPVLKSALVDYIVSDGITHGLDMSTVAKRDLLQTFQDKILKPRGLDYKVQFPGPTGTN    116
Streptomyces avermitilis      HNNPVLKRALIDYLERDGVTHGLDMSTAAKRAFLESFQNLILRPRDLPYKVMFPGPTGTN    118
Vibrio cholerae               HNNAVLKQALLEYIERDGLTHGLDMHSEAKAHFIQALQTHILEPRGLNYKLQFTGPTGTN    111

Nesterenkonia halobia         SVEAALKLARKVTGRQHILSFTNAFHGMTLGSLSVTGNSMKRRGAG.IPLTNSSKIPYDD    172
Bacillus halodurans           TVESALKLARKVTGRTDIISFTNGFHGMTIGSLSVTGNSFKRKGAG.IPLTNVVTMPYDN    175
Brevibacterium linens         TVEAALKLARKVTGRQHMLSFTNAFHGMTLGSLSVTGNSMKREGAG.IPLTNSSKIPYDD    179
Chromohalobacter salexigens   AVEAAIRLARNAKGRHNIVTFTNGFHGVTMGALATTGNRKFREATGGIPTQGASFMPFDG    173
Rhodococcus sp.               TVEAALKLARKVTGRSSIINFTNAFHGMTLGALSVTGNSMKRAGAG.IPLVHATPMPFDN    175
Streptomyces avermitilis      AVESALKLARKVKGREAIVSFTNAFHGMSLGSLAVTGNAFKRAGAG.IPLVHGTPMPFDN    177
Vibrio cholerae               AVEAALKLARKVTGRHNVVTFTNGFHGCSLGALAATGNQHHRQGAG.LALSGVYRVPYDG    170

Nesterenkonia halobia         YFDGNIPDFIWLEKVLQDSGSGVDKPAAVIVETVQGEGGLNAARMEWLKELSALLRRHKI    232
Bacillus halodurans           FVSESLDTLDYLERFLEDGGSGVEIPAAMILETVQGEGGINAARTEWLQRVEKICKRWGI    235
Brevibacterium linens         YFDGEIPDFLWLEKVLEDSGSGVDKPAAVIVETVQGEGGLRAARAEWLRALSELTKKHDI    239
Chromohalobacter salexigens   YMGEGVDTLSYFEKLLGDNSGGLDVPAAVIIETVQGEGGINPAGIPWLQRLEKICRDHDM    233
Rhodococcus sp.               YFDGVTEDFHWFRRVLDDSGSGLNRPAAVIVETVQGEGGVNVARAEWLRALADLCAEREI    235
Streptomyces avermitilis      YFDGKVPDFLWFERLLEDQGSGLNKPAAVIVETVQGEGGINVARPEWLRALAELCKRQDM    237
Vibrio cholerae               YA..GVDGLTLFETMLQDNSSGLDKPAAVLLETVQGEGGLNVASDAWLQRVQAICRAQQI    228

Nesterenkonia halobia         LLIIDDVQAGCGRTGTFFSFEEAGITPDIICMSKSISGYGLPMAITLFKPELDVWEGGEH    292
Bacillus halodurans           LLIIDDVQAGVGRTGTFFSFEDAGITPDIVCLSKSIGGFGLPLAITLFRPELDIWAPGEH    295
Brevibacterium linens         LLIVDDVQAGCGRTGSFFSFEEAGIEPDIVCLSKSISGSGLPMALTLFRPELDVWEPGEH    299
Chromohalobacter salexigens   LLIVDDIQAGCGRTGKFFSFEHAGITPDIVTNSKSLSGFGLPFAHVLMRPELDIWKPGQY    293
Rhodococcus sp.               LLIVDDVQMGCGRTGPFFSFEVAGITPDIVCLSKSISGYGLPMALTLFKRELDVWGPGEH    295
Streptomyces avermitilis      LLIVDDIQMGCGRTGAFFSFEEAGVTPDIVTVSKSISGYGLPMSLCLFKPELDIWEPGEH    297
Vibrio cholerae               LLIVDDIQAGCGRTGTFFSFEPSGIEPDMVTLSKSLSGYGLPMALVLFKPEWDQWKPGEH    288

Nesterenkonia halobia         NGTFRGNNLGFITGARALELFWSD.DSFQKQLAAKIETLREGLEDIAQH..VKGAT...L    346
Bacillus halodurans           NGTFRGNNHAFVTATEALS.YWED.DSFEKDIQEKSATISDFLVKLVTEYPEIKGE...V    350
Brevibacterium linens         NGTFRGNNPAFVTATAAIKNFWAD.NTFQNELADTIAALHQRLDSIVEK..AEGAS...I    353
Chromohalobacter salexigens   NGTFRGFNLAFVTAAAAMRHFWSD.DTFERDVQRKG.RVVEDRFQKLASFMTEKGHPASE    351
Rhodococcus sp.               NGTFRGNNPAFVTSKVALDHYWSD.DTLHKSTLTKGEKIHQAFTDLANQ.FDGSVS...T    350
Streptomyces avermitilis      NGTFRGNNPAFVTAAAALQTYWADGSAMEKQTLARGEQVEQALISITEENLADVKE...Y    354
Vibrio cholerae               NGTFRGNNHAFVTATRALEAYWAN.QDFQTHIAARSEQVTQALLQCLSRYPTLFSG...L    344

Nesterenkonia halobia         RGRGFLTGICFPDADTAGKVAAESYKRNLLLETSGPEDEVIKVMPPLTIEDDDLQKGIKV    406
Bacillus halodurans           KGKGFMVGIASDVEGFASKVTEEAFSRGLIMETSGPNDEVFKLFPPLTIDDEGLEKGLAI    410
Brevibacterium linens         RGRGLLAGLHFADDEVAGKVAAEAFENGLLLETSGPKDEVTKIMPPLTISSHDLEQGLDI    413
Chromohalobacter salexigens   RGRGLMRGLDVGDGDMADKITAQAFKNGLIIETSGHSGQVIKCLCPLTITDEDLVGGLDI    411
Rhodococcus sp.               RGRGLVQGLVFDEPENAGKVCKLAFDEGLLAETSGPSDEVVKLLPALTITDEELDHGLAI    410
Streptomyces avermitilis      RGRGLVWGIEFKDKDRAGRIAQRAFELGLLIETSGPESEVVKLLPALTITPEELDEGLRT    414
Vibrio cholerae               KGRGLMQGLACHNGDIARDIAALCFQKGLIIETAGAEDEVLKVFCPLTITEADLAHGLTI    404

Nesterenkonia halobia         IEDAGLAAT.GQMSEPSRLRAP                                          427
Bacillus halodurans           IEESIKALVETKELVMQ.....                                          427
Brevibacterium linens         IEAAVMKFAPATNAEPAAV...                                          432
Chromohalobacter salexigens   LEQSVKEVFGQA..........                                          423
Rhodococcus sp.               LADATGKVCS............                                          420
Streptomyces avermitilis      LARAVRETA.............                                          423
Vibrio cholerae               IERVLLE...............                                          411
```

Fig. 3. Alignments of the predicted amino acid sequence of EctABC of *N. halobia* with those of *Bacillus halodurans*, *Brevibacterium linens*, *Chromohalobacter salexigens*, *Rhodococcus* sp., *Streptomyces avermitilis*, and *Vibrio cholerae*. Identical amino acids in all aligned proteins are highlighted in black and similar amino acids are shown in grey. Gaps introduced to maximize alignment are indicated by dashes. (A) the 2,4-diaminobutyrate aminotransferase (EctA), (B) the ectoine synthase (EctC), (C) the 2,4-diaminobutyrate acetyltransferase (EctB); Arrows indicate the four invariant amino acid residue conserved in EctB.

cluster were both related to the transportation of tricarboxylates, the substrates in the tricarboxylic acid cycle, so the synthesis of ectoine in *N. halobia* might be closely related with energy. Also, EctB has a high similarity to aminotransferase (Louis and Galinski, 1997; Canovas *et al*., 1998). There exist four invariant amino-acid residues (such as Gly-217, Asp-243, Lys-272, and Arg-393) of the highly conserved regions of all transaminases, which play a specific structural or functional role. The first three were present in EctB, and the fourth residue, an arginine binding the α-carboxylate group of the substrate, was replaced by lysine (Lys-393) in all EctB. As expected, the four amino-acid residues (Gly-211, Asp-237, Lys-266, and Lys-388) were all present in the EctB of *N. halobia*, and were consistent with those found in EctB reported previously (Fig. 3C).

RT-PCR analysis

The *ectABC* gene cluster has been proven to be transcribed as an operon in *M. halophilus*, *S. pasteurii*, and *M. alcaliphilum* 20Z, but *N. halobia* had much longer intergenic regions among the *ectABC* genes. The more loose physical organizations prompted the question of whether the *ectABC* genes were transcribed as an operon, so RT-PCR was carried out using primers as described in Fig. 2. The RT-PCR products of the correct size and sequence were obtained across each pair of the genes in the entire *ectABC* gene cluster, and controls for DNA contamination of the RNA preparations using direct PCR without the RT step were negative (Fig. 4). These results demonstrated that *ectA*, *ectB*, and *ectC* are organized in one operon and are transcribed in one common transcript. Moreover, the DNA sequences upstream of *ectB* and *ectC* were also analyzed through the Internet (http://www.fruitfly.org/seq_tools/promoter.html) and no putative promoter was found. Additionally, database searches showed that *B. linens* BL2, the taxonomically closely related species, has the same characteristics as *N. halobia*. So the intergenic regions of the *ectABC* genes might be different among the various species, in spite of the constitutive *ectABC* genes.

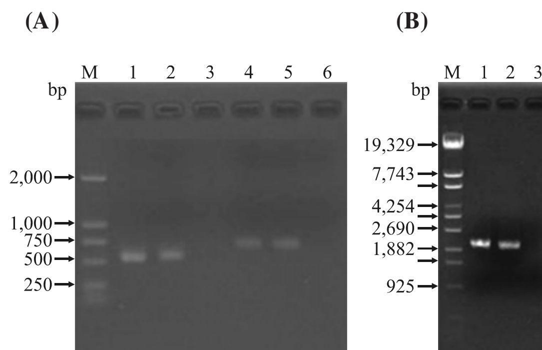

Fig. 4. Gel electrophoresis analysis of the RT-PCR products. (A) Products obtained with primers B-C F and B-C R (lanes 1 and 2), A-B F and A-B R (lanes 4 and 5) (B) Products obtained with primers A-B F and B-C R (lanes 1 and 2). Positions of molecular size markers (in base pairs) are indicated on the left. Negative controls containing the same amounts of RNA, primers and LA Taq polymerase, but not reverse transcriptase, were included in this assay [lanes 3 and 6 in (A) and lane 3 in (B), respectively]. M, DNA molecular marker.

Osmotic expression of *ectABC* genes in *E. coli* strains

To verify whether *ectABC* genes from *N. halobia* enhance

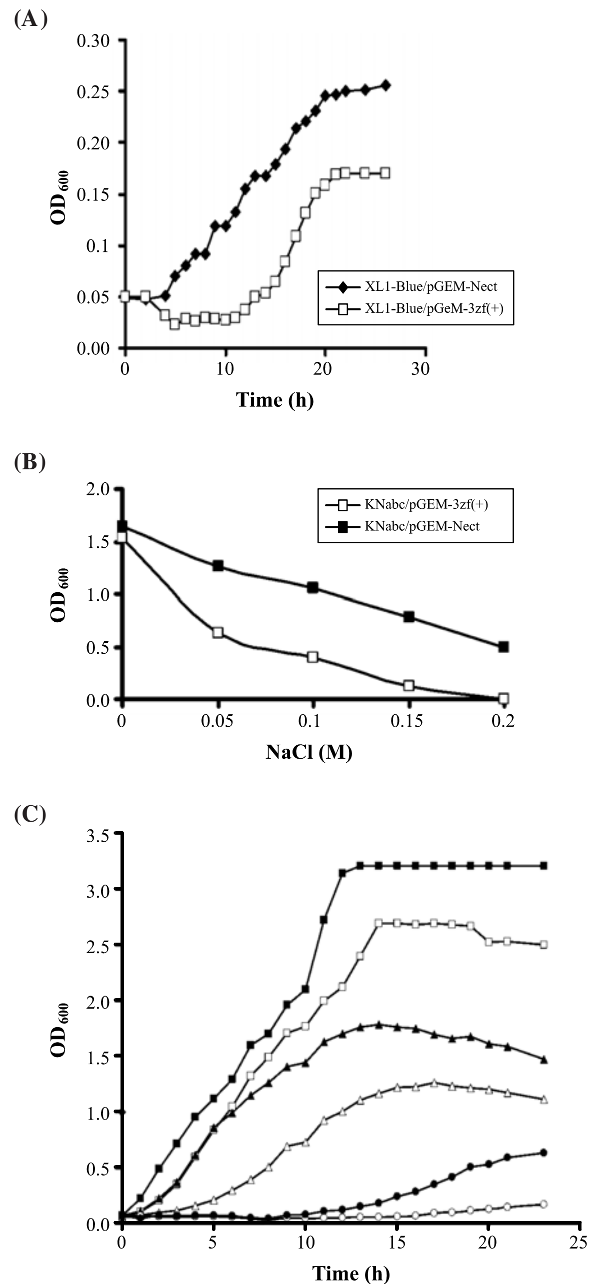

Fig. 5. Osmoprotection of the *ectABC* cluster from *N. halobia* in *E. coli*. (A) Growth curves of *E. coli* XL1-Blue transformants in MM63 medium with 0.68 M NaCl, (B) Growth of cells of *E. coli* transformant KNabc/pGEM-3zf (+) and KNabc/pGEM-Nect were grown in LBK medium in the presence of NaCl from 0 to 0.2 M in 48 h. (C) Growth curve of *E. coli* KNabc transformants in LBK with different concentrations of NaCl. Cells were grown in LBK medium containing 0 M NaCl [■, KNabc/pGEM-Nect; □, KNabc/pGEM-3zf(+)], 0.05 M NaCl [▲, KNabc/pGEM-Nect; △, KNabc/pGEM-3zf(+)] and 0.1 M NaCl [●, KNabc/pGEM-Nect; ○, KNabc/pGEM-3zf(+)].

the growth of *E. coli* in high NaCl content, osmotic expression experiments were carried out with *E. coli* XL1-Blue and *E. coli* KNabc lacking three Na^+/H^+ antiporters of *NhaA*, *NhaB*, and *Cha*. Expression of the *ectABC* gene cluster from *N. halobia* in *E. coli* XL1-Blue did not enable it to grow in MM63 medium containing 0.86 M NaCl, but the expression in *E. coli* XL1-Blue could shorten the lag phase and raise the final OD_{600} in MM63 medium with 0.68 M NaCl (Fig. 5A). The growth of *E. coli* KNabc/pGEM-Nect in liquid LBK medium in the presence of NaCl was monitored (Fig. 5B). Cells of strain KNabc/pGEM-Nect could grow in the presence of 0.2 M NaCl, whereas cells of strain KNabc/pGEM-3zf(+) did not. As shown in Fig. 5C, *ectABC* gene cluster could enhance the final OD_{600} and shorten the lag phase of strain KNabc. To confirm ectoine accumulation in strain KNabc/pGEM-Nect, the cell extracts were analyzed using HPLC-UV. In the UV chromatogram, the peak of ectoine (at 4.430 min) was shown both in the standard of ectoine (Fig. 6A) and the extract of the KNabc/pGEM-Nect (Fig. 6B), but not in the extract of KNabc/pGEM-3zf(+) (Fig. 6C), which showed that ectoine was synthesized in the strain KNabc/pGEM-Nect, but not in KNabc/pGEM-3zf(+). To be more convinced, the peak of 4.430 min in the UV chromatogram was further examined by LC-MS, obtaining the characteristic peak of ectoine: $[M+H]^+$ at *m/z* 143.1 in the mass spectrum of the standard (Fig. 7A) and the extracts from the strain KNabc/pGEM-Nect (Fig. 7B). The mass spectrometry results confirmed that the substance detected by HPLC analysis of the extracts from the strain KNabc/pGEM-Nect was ectoine.

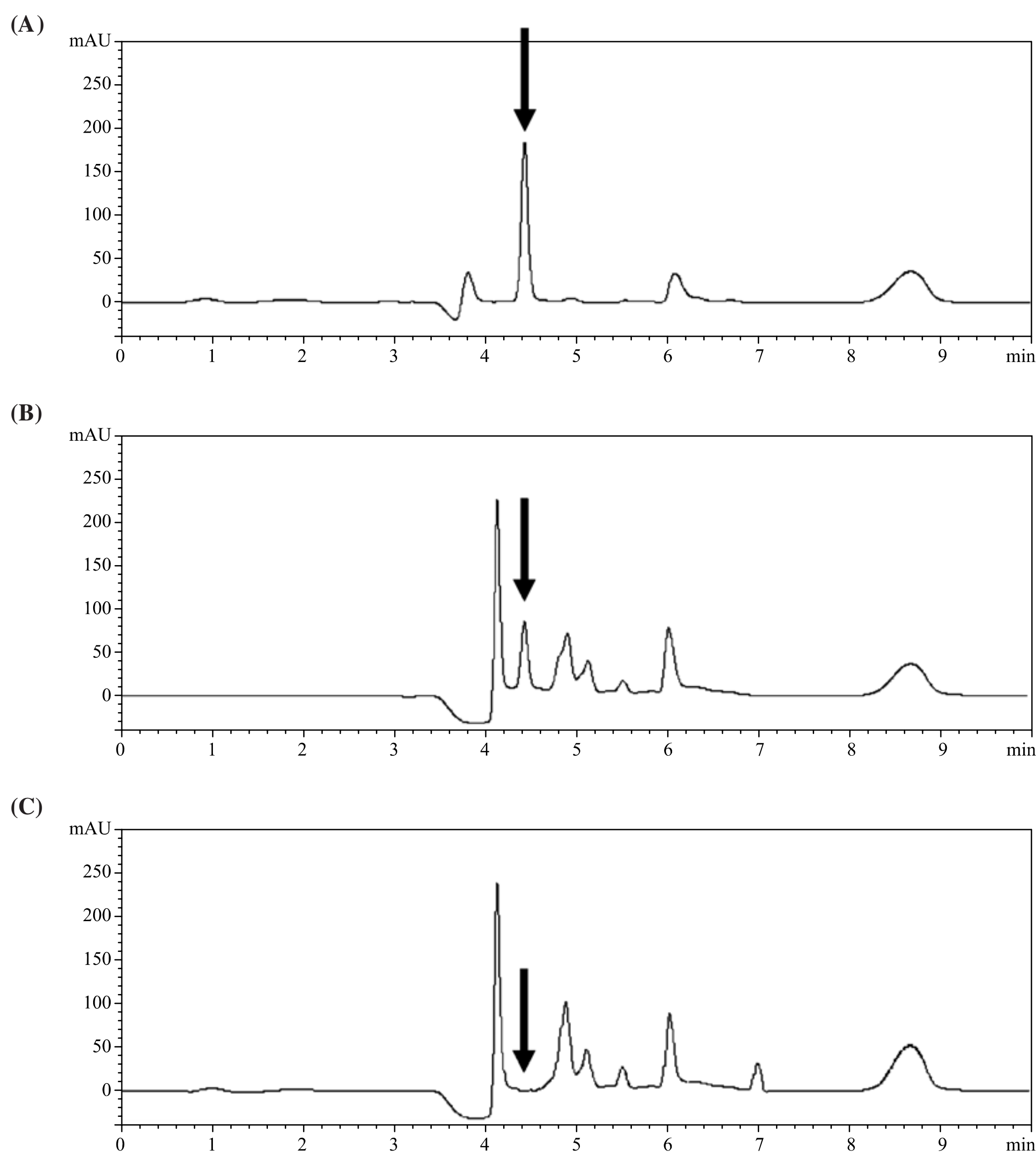

Fig. 6. Detection of ectoine accumulation in KNabc/pGEM-Nect cells using HPLC with UV detector. (A) UV spectra of the standard of ectoine, (B) UV spectra of the extracts from the strains KNabc/pGEM-Nect, and (C) UV spectra of the extracts from the strains KNabc/pGEM-3zf(+). The arrows indicate the ectoine peak.

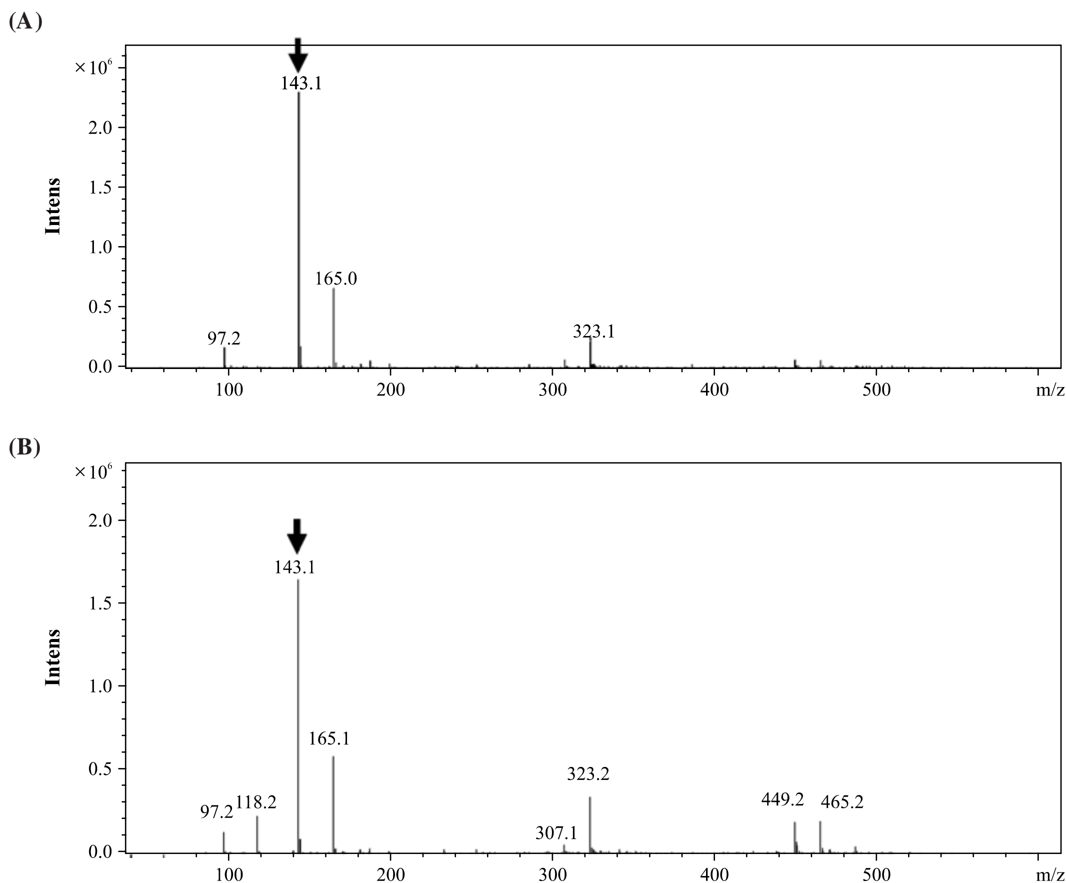

Fig. 7. Validation of the peak at 4.430 min by LC-MSD-Trap-VL. (A) Mass spectra at 4.430 min of the standard ectoine. (B) Mass spectra at 4.430 min of the cells extracts from the strain KNabc/pGEM-Nect. The arrows indicate the ectoine peak.

To our knowledge, it is the first time that the *ectABC* gene cluster was cloned from the GC-rich and the representative of halophilic actinomycetes. For the first time, the osmoprotection of the *ectABC* gene cluster was studied in the *E. coli* mutant of Na^+/H^+ antiporter. The salt sensitive *E. coli* mutant KNabc is a better object to study the osmoprotection of ectoine, when *ectABC* gene cluster was transferred to the mutant, the transformant strain KNabc/pGEM-Nect can grow in presence of 0.2 M NaCl, but did not grow in more than 0.2 M NaCl condition. It is meant that the ectoine could complement the *E. coli* strains lacking Na^+/H^+ antiporter in salt condition and hence the fact showed that the relationship between the compatible solutes and the Na^+/H^+ antiporters in halotolerant mechanism of moderate halophiles.

Acknowledgements

This work was supported by the China International Cooperation Program for Science and Technology (2006DFA31060).

References

Canovas, D., C. Vargas, M.I. Calderon, A. Ventosa, and J.J. Nieto. 1998. Characterization of the genes for the biosynthesis of the compatible solute ectoine in the moderately halophilic bacterium *Halomonas elongata* DSM 3043. *Syst. Appl. Microbiol.* 21, 487-497.

Goldberg, E.B., T. Arbel, J. Chen, R. Karpel, A.M. George, S. Shimon, and P. Etana. 1987. Characterization of a Na^+/H^+ antiporter gene of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 84, 2615-2619.

Goller, K., A. Ofer, and E.A. Galinski. 1998. Construction and characterization of a NaCl-sensitive mutant of *Halomonas elongata* impaired in ectoine biosynthesis. *FEMS Microbiol. Lett.* 161, 293-300.

Haardt, M., B. Kempf, E. Faatz, and E. Bremer. 1995. The osmoprotectant proline betaine is a major substrate for the binding-protein-dependent transport system ProU of *Escherichia coli* K-12. *Mol. Gen. Genet.* 246, 783-786.

Inoue, H., H. Nojima, and H. Okayama. 1990. High efficiency transformation of *Escherichia coli* with plasmids. *Gene.* 96, 23-28.

Joseph, S. and W.R. Davis. 2001. Molecular cloning: A laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, NY, USA.

Kuhlmann, A.U. and E. Bremer. 2002. Osmotically regulated synthesis of the compatible solute ectoine in *Bacillus pasteurii* and related *Bacillus* spp.. *Appl. Environ. Microbiol.* 68, 772-783.

Louis, P. and E.A. Galinski. 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.

Lu, W.D., B. Zhang, B.S. Zhao, D.Q. Feng, L.F. Yang, L. Wang, and S.S. Yang. 2006. Cloning and characterization of the genes encoding a glycine betaine ABC-Type transporter in *Halobacillus trueperi* DSM10404^T. *Curr. Microbiol.* 54, 124-130.

Miller, J.H. 1972. Experiments in molecular genetics, p. 352-355. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, NY, USA.

Nakamura, T., H. Enomoto, and T. Unemoto. 1996. Cloning and sequencing of the *nhaB* gene encoding an Na^+/H^+ antiporter from *Vibrio alginolyticus*. *Biochim. Biophys. Acta.* 1275, 157-160.

Ochman, H., A.S. Gerber, and D.L. Hartl. 1988. Genetic applications of an inverse polymerase chain reaction. *Genetics* 120, 621-623.

Ohyama, T., K. Igarashi, and H. Kobayashi. 1994. Physiological role of the *chaA* gene in sodium and calcium circulations at a high pH in *Escherichia coli*. *J. Bacteriol.* 176, 4311-4315.

Oren, A. 2002. Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. *J. Ind. Microbiol. Biotechnol.* 28, 56-63.

Peters, P., E.A. Galinski, and H.G. Trüper. 1990. The biosynthesis of ectoine. *FEMS Microbiol. Lett.* 71, 157-162.

Reshetnikov, A.S., V.N. Khmelenina, and Y.A. Trotsenko. 2006. Characterization of the ectoine biosynthesis genes of haloalkalotolerant obligate methanotroph "*Methylomicrobium alcaliphilum* 20Z". *Arch. Microbiol.* 184, 286-297.

Sleator, R.D. and C. Hill. 2001. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. *FEMS Microbiol. Rev.* 26, 49-71.

Spring, S., W. Ludwig, M.C. Marquez, A. Ventosa, and K.H. Schleifer. 1996. *Halobacillus* gen. nov. with description of *Halobacillus litoralis* sp. nov. and *Halobacillus trueperi* sp. nov., and transfer of *Sporosarcina halophila* to *Halobacillus halophilus* comb. nov. *Int. J. Syst. Bacteriol.* 46, 492-496.

Ventosa, A., J.J. Nieto, and A. Oren. 1998. Biology of moderately halophilic aerobic bacteria. *Microbiol. Mol. Biol. Rev.* 62, 504-544.

Zhao, B.S., W.D. Lu, L.F. Yang, B. Zhang, L. Wang, and S.S. Yang. 2006. Cloning and characterization of the genes for biosynthesis of the compatible solute ectoine in the moderately halophilic bacterium *Halobacillus dabanensis* D-8^T. *Curr. Microbiol.* 53, 183-188.