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Characterization of genes for the biosynthesis of the compatible solute ectoine from *Marinococcus halophilus* and osmoregulated expression in *Escherichia coli*

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The genes of the biosynthetic pathway of ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) from the Gram-positive moderate halophile *Marinococcus halophilus* were cloned by functional expression in *Escherichia coli*. These genes were not only expressed, but also osmoregulated in *E. coli*, as demonstrated by increasing cytoplasmic ectoine concentration in response to medium salinity. Sequencing of a 4·4 kb fragment revealed four major ORFs, which were designated *ectA*, *ectB*, *ectC* and *orfA*. The significance of three of these genes for ectoine synthesis was proved by sequence comparison with known proteins and by physiological experiments. Several deletion derivatives of the sequenced fragment were introduced into *E. coli* and the resulting clones were investigated for their ability to synthesize ectoine or one of the intermediates in its biosynthetic pathway. It was demonstrated that *ectA* codes for L-2,4-diaminobutyric acid acetyltransferase, *ectB* for L-2,4-diaminobutyric acid transaminase and *ectC* for L-ectoine synthase. A DNA region upstream of *ectA* was shown to be necessary for the regulated expression of ectoine synthesis in response to the osmolarity of the medium.

Keywords: *Marinococcus halophilus*, compatible solutes, ectoine genes, osmoregulation, salt stress

INTRODUCTION

Saline environments are characterized by high osmotic strength (low water potential). Most halophilic eubacteria cope with these conditions by accumulating small, highly water-soluble organic compounds, the so-called compatible solutes (Brown, 1976). These osmolytes enable organisms to adapt to a wide range of salt concentrations by adjusting the cytoplasmic solute pool to the osmolarity of the surrounding environment. Ectoines represent the predominant class of osmolytes in aerobic chemoheterotrophic eubacteria (Severin *et al.*, 1992; Frings *et al.*, 1993; Galinski, 1995). The biosynthetic pathway for ectoine has been elucidated at the enzymological level in Gram-negative eubacterial halophiles (Peters *et al.*, 1990; Tao *et al.*, 1992; Galinski & Trüper, 1994). It comprises three steps, the first being the conversion of aspartate semialdehyde, an intermediate in amino acid metabolism, to L-2,4-diaminobutyric acid. This is followed by acetylation to N^{γ}-acetyldiaminobutyric acid. The last step consists of a cyclic condensation reaction to form the tetrahydropyrimidine ectoine (Fig. 1).

Expression and regulation of genes involved in osmoadaptation has thus far been investigated almost exclusively in non-halophilic bacteria, especially *Escherichia coli* (for recent reviews see Csonka & Hanson, 1991; Lucht & Bremer, 1994; Galinski, 1995). This organism responds to increased salinity with the rapid accumulation of potassium and concomitant synthesis of glutamate as a counter anion. Subsequently, these charged solutes are partially replaced by endogenous trehalose or compatible solutes accumulated from the medium, if present (Dinnbier *et al.*, 1988). Most studies at the molecular level have so far focused on the various solute uptake systems of *E. coli* (Altendorf & Epstein, 1993; Mellies *et al.*, 1995; Gowrishankar & Manna, 1996). The only investigations concerned with the biosynthesis of compatible solutes covered choline oxidation and trehalose synthesis (Lamark *et al.*, 1996; Strøm & Kaasen, 1993). It has been proposed that relatively non-specific signals, such as intracellular

The GenBank accession number for the sequence reported in this paper is U66614

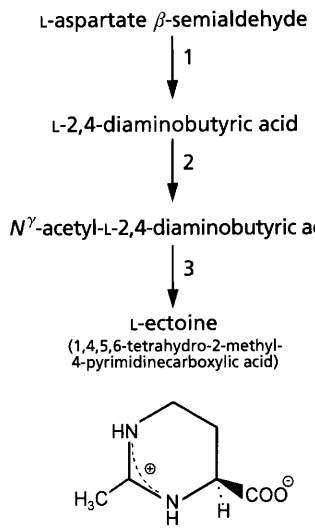

Fig. 1. Biosynthetic pathway for ectoine based on enzymological studies (Peters *et al.*, 1990; Tao *et al.*, 1992). 1, L-2,4-diaminobutyric acid transaminase; 2, L-2,4-diaminobutyric acid N^{γ}-acetyltransferase; 3, L-ectoine synthase.

potassium glutamate concentration and DNA supercoiling (Booth & Higgins, 1990), as well as specific regulatory mechanisms of the different genes investigated play a role in osmoregulation.

In this study, we report the identification and sequencing of the three genes responsible for the synthesis of the compatible solute ectoine from the Gram-positive halophile *Marinococcus halophilus* and their osmoregulated expression in *E. coli*. Our investigation provides, for the first time, an opportunity to compare the molecular organization of osmoregulatory elements from a true halophile with that of non-halophiles.

METHODS

Bacterial strains, growth conditions and plasmid. *M. halophilus* DSM 20408^{T} was grown aerobically at 37 °C in LB medium (Miller, 1972) containing 0·3 % (w/v) artificial sea salt and 3·7 % (w/v) NaCl. *E. coli* XL1-Blue (Stratagene) was grown aerobically at 37 °C either in LB medium or in medium MM63 (Larsen *et al.*, 1987) with 1·5 ml vitamin solution l^{-1} (Imhoff & Trüper, 1977) and 1–5 % NaCl. To select for cells bearing plasmid pHSG575 and recombinant derivatives, the medium contained chloramphenicol at a final concentration of 25 μg ml^{-1}. For the selection of cells containing recombinant plasmids on agar plates, isopropyl β-D-thiogalactopyranoside (IPTG, 0·5 mM) and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal, 40 μg ml^{-1}) were added. For supplementation studies, MM63 with 2–3 % NaCl contained 2 mM diaminobutyrate or N^{γ}-acetyldiaminobutyrate. At the end of the exponential growth phase, cells were harvested (15000 *g*, 18 °C) and freeze-dried.

Plasmid pHSG575 (Takeshita *et al.*, 1987), a *lacZ* α-complementing low-copy-number cloning vector, was kindly provided by E. Bremer (Universität Marburg, Germany).

DNA techniques. Genomic DNA of *M. halophilus* was prepared using Qiagen genomic-tips as described by the manufacturer. Small- and large-scale plasmid preparations of *E. coli* XL1-Blue were performed using Qiaprep spin columns and the Plasmid Midi kit from Qiagen, respectively.

For the construction of a library, genomic DNA from *M. halophilus* was partially digested with the restriction endonuclease *Sau*3A and separated on an agarose gel. Fragments ranging from 5 to 15 kb were recovered by electroelution and ligated into the dephosphorylated *Bam*HI site of pHSG575 according to standard techniques (Ausubel *et al.*, 1991). *E. coli* XL1-Blue was transformed with the recombinant plasmids by the $CaCl_2$ method (Cohen *et al.*, 1972).

Southern hybridization analysis was performed on nitrocellulose membranes (Schleicher & Schuell) according to standard techniques (Ausubel *et al.*, 1991) using the Non-radioactive Digoxigenin DNA Labeling and Detection kit from Boehringer Mannheim for the preparation of DNA probes.

Plasmids pOSM11, pOSM12, pOSM13, pOSM14 and pOSM18 were constructed by deletion of defined restriction fragments from pOSM1 and religation after the creation of blunt ends with the Klenow fragment of DNA polymerase I. Restriction enzymes and the Klenow fragment were purchased from Boehringer Mannheim with the exception of *Ppu*MI, which was obtained from New England Biolabs.

Nested deletions for sequencing were prepared with the Double Stranded Nested Deletion kit (Pharmacia). Sequencing was performed with the AutoRead 1000 Sequencing kit and the automatic fluorescent sequencer ALF (Pharmacia) in one direction. Sequencing of the opposite strand was performed by Sequiserve (München, Germany).

DNA sequences were analysed with the programs GENEPRO, DNASIS and MACAW. Databank searches were carried out through the National Center for Biotechnology Information (NCBI) with the BLAST program (Altschul *et al.*, 1990) and current versions of the available databases (October 1996).

Analytical methods. For the identification of intracellular solutes, freeze-dried cells were extracted with methanol/chloroform/water (10:5:4) by a modification of the technique of Bligh & Dyer (1959), as described by Galinski & Herzog (1990). Extracts were analysed by isocratic and gradient HPLC methods as described previously (Galinski & Herzog, 1990; Kunte *et al.*, 1993).

RESULTS AND DISCUSSION

Cloning and osmotic expression of ectoine genes in *E. coli*

E. coli is able to grow at salt concentrations of up to 3 % NaCl in the minimal medium MM63 because of its intrinsic ability to adjust its cytoplasmic potassium glutamate and trehalose pools. At 5 % NaCl, more efficient compatible solutes, such as betaine or ectoine, are needed for growth. Our aim was to provide *E. coli* XL1-Blue with genes for the synthesis of the compatible solute ectoine from *M. halophilus*, and thus increase its osmotolerance. To achieve this, colonies of *E. coli* XL1-Blue carrying a genomic DNA library of *M. halophilus* were replica-plated onto medium MM63 containing

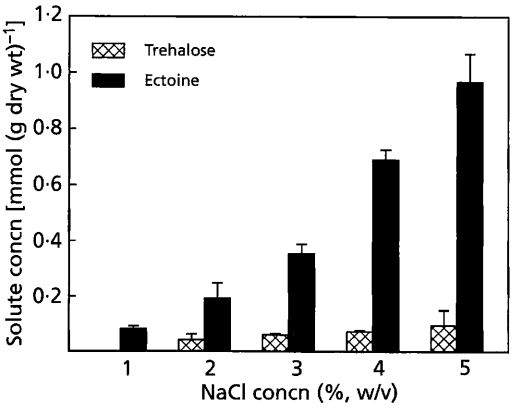

Fig. 2. Relationship between the intracellular solute concentration of *E. coli*(pOSM11) and medium salinity during growth in minimal medium MM63 as measured by isocratic HPLC. Mean values and standard deviations from three independent experiments are shown. Similar results were obtained with *E. coli*(pOSM1). Glutamate levels did not exceed 0·12 mmol (g dry wt)$^{-1}$ regardless of the salt concentration of the medium. The trehalose pool of *E. coli* XL1-Blue in the presence of 3 % NaCl was 0·35 mmol (g dry wt)$^{-1}$.

elevated salt concentrations and screened for clones with enhanced salt tolerance. One clone that was able to grow in the presence of 5 % NaCl was obtained. Plasmid preparations of this clone confirmed the presence of a recombinant plasmid, which we designated pOSM1.

To examine whether the enhanced salt tolerance of this clone was due to the synthesis of additional compatible solutes, the intracellular solute content was examined by HPLC. Besides trehalose, we detected the compatible solute ectoine in *E. coli*(pOSM1). Its cytoplasmic concentration increased with elevated salt concentrations to a maximum of 1 mmol (g dry wt)$^{-1}$ at 5 % NaCl (Fig. 2). At 3 % NaCl the cytoplasmic ectoine concentration was comparable to that gained through ectoine uptake in *E. coli* MC4100, as observed by Jebbar *et al.* (1992). Trehalose levels on the other hand were considerably lower in *E. coli*(pOSM1) [0·06 mmol (g dry wt)$^{-1}$] than in the ectoine-free host *E. coli* XL1-Blue [0·35 mmol (g dry wt)$^{-1}$]. As we could not detect ectoine extracellularly, we conclude that ectoine was synthezised in an osmoregulated manner rather than simply excreted by the cells. In the complex medium LB containing 5 % NaCl, the observed biosynthesis of ectoine was suppressed almost completely (not shown). Instead, betaine was accumulated from the medium, which conforms with the current view that uptake of compatible solutes is preferred over biosynthesis (Dinnbier *et al.*, 1988).

To confirm that the production of ectoine was dependent on plasmid pOSM1, we extracted it from the original isolate and retransformed it into *E. coli* XL1-Blue, which then acquired osmotolerance by means of ectoine synthesis. Hybridization studies with the recombinant fragment of pOSM1 confirmed the presence of this particular sequence in genomic DNA of *M. halophilus* (not shown). This proves that the cloned DNA originated from *M. halophilus*.

Sequencing of pOSM11

Restriction analyses of pOSM1 revealed a recombinant fragment of 5·9 kb. Following the deletion of a 1·5 kb *Eco*RI fragment, we were able to demonstrate that the resulting plasmid pOSM11, containing a 4·4 kb recombinant fragment, still displayed osmoregulated ectoine synthesis (Fig. 2).

The entire recombinant DNA fragment of pOSM11 was sequenced (Fig. 3). Four major ORFs, oriented in the same direction, were identified. They are predicted to encode proteins of 172, 427, 129 and 110 amino acids, with deduced molecular masses of 19385, 47192, 14796 and 13142 Da, respectively. All reading frames, which we designated *ectA*, *ectB*, *ectC* and *orfA*, are preceded by putative ribosome binding sites (Fig. 3). Downstream of *orfA* lies the 5′-end of another ORF (*orfB*). The *lacZ* promoter of the vector is oriented in the opposite direction, and thus transcription of these reading frames from the *lacZ* promoter can be excluded.

The gene for the ectoine synthase from *Halomonas* sp. had already been cloned by reverse translation and sequenced by Min-Yu *et al.* (1993). We compared their sequence with our ORFs and found 47 % identity to *ectC*. An alignment is shown in Fig. 4. Thus, *ectC* seems to encode the ectoine synthase from *M. halophilus*.

Databank searches using the BLAST algorithm (Altschul *et al.*, 1990) revealed a high similarity of ORF *ectB* to transaminases belonging to subgroup II (Mehta *et al.*, 1993). All of these transaminases aminate a terminal oxo group. The same holds true for L-2,4-diaminobutyric acid transaminase in the biosynthetic pathway for ectoine. The highest similarity was observed with γ-aminobutyrate-, ornithine- and acetylornithine transaminases. An alignment is shown in Fig. 5. Of four residues found in all transaminases examined previously (Mehta *et al.*, 1993), three were present in *ectB* (see ▼ in Fig. 5). The fourth, an arginine, which binds the α-carboxylate of the substrate, is replaced by a lysine in *ectB*. Studies with aspartate transaminase have shown that when arginine is replaced by lysine, which also carries a positive charge, enzyme activity is maintained, albeit at a strongly reduced rate (Inoue *et al.*, 1989). Considering that replacement of arginine with lysine is likely to have a greater steric effect on α-aminating enzymes like aspartate transaminase, this exchange may have less relevance for a γ-aminating transaminase, especially as this particular region is not very conserved (Fig. 5). From these sequence comparisons, we conclude that *ectB* is the first gene in the biosynthetic pathway of ectoine, the transaminase converting aspartate-β-semialdehyde to diaminobutyric acid.

The *ectA* gene revealed no sequence similarity to known proteins, whereas *orfA* and the truncated *orfB* shared

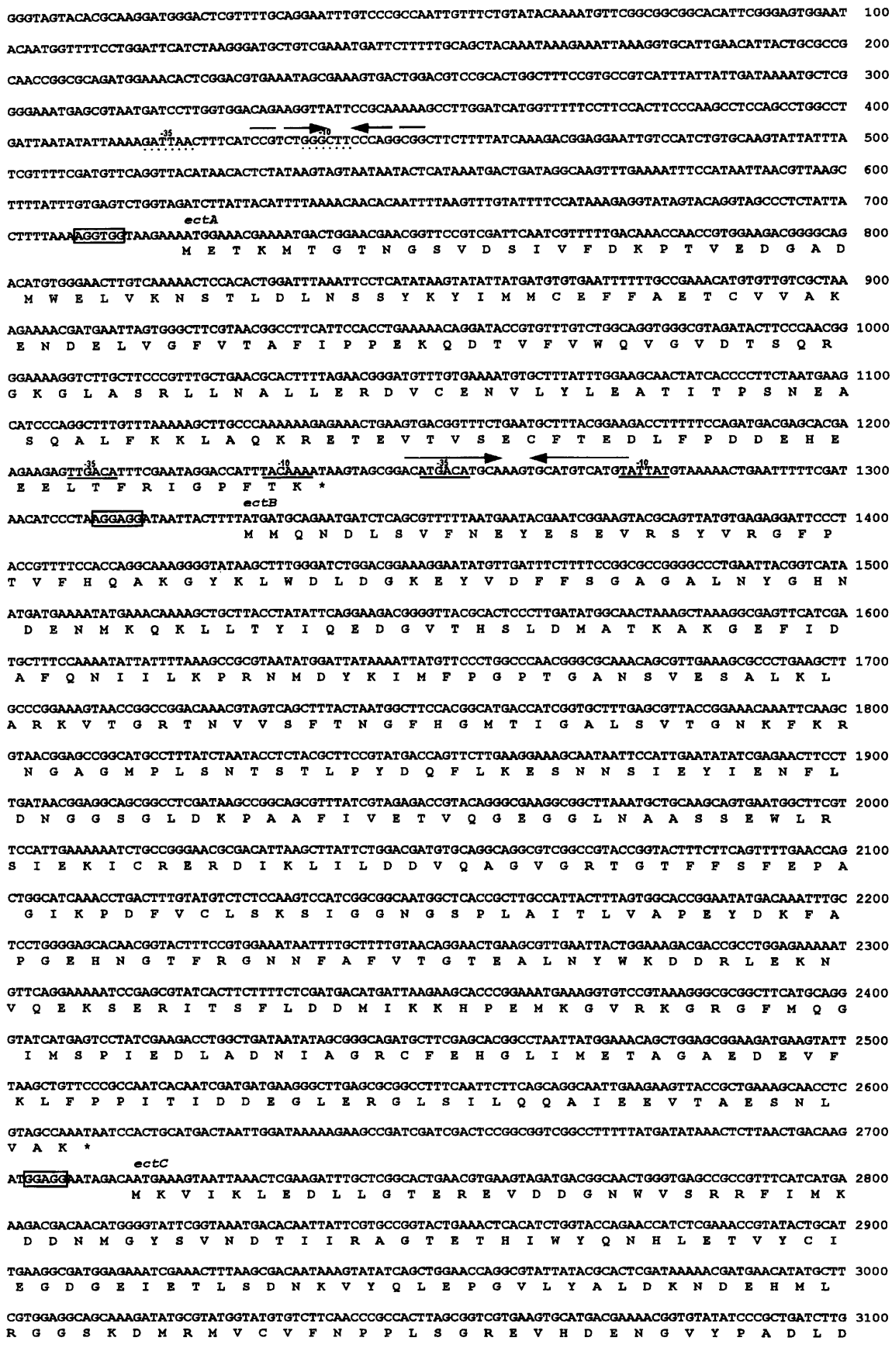

Fig. 3. For legend see facing page.

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ATTAATAATAAAAAAATCCCCTGATGCTTTTTAGAGTGACCCCATAATATGAGACAGGAAAAAACACCCCCAACGTCGTATGTAGTATTAACGATGATCT 3200
 •
                     orfA
GGAGGTGTTTTTTTGATGGGAACCAATCGATTTTATCCGGAAGAAGTAAAGCGCGAAGTCATTCGTTTGAAATTAGAGGAGCAAACCTCCAACGAGGAAC 3300
                     M  G  T  N  R  F  Y  P  E  E  V  K  R  E  V  I  R  L  K  L  E  E  Q  T  S  N  E  E  L

TCATGAAACAATTTGGGATTAAGAATAAATCGCAGATTAAAACATGGGTCCGGTGGTATAAACAAGGAGCCTCCCATCGTCTGGCCCAGCCACCGGGAAA 3400
  M  K  Q  F  G  I  K  N  K  S  Q  I  K  T  W  V  R  W  Y  K  Q  G  A  S  H  R  L  A  Q  P  P  G  K

ACAGTATACCTTCGGTAAAGGACCCGAAGAGGACACGGAAATCGAGCAGCTGCGAAAGAAAGTCGCATATTACGAAATGAGAGATGATCTGATGGGAAAG 3500
 Q  Y  T  F  G  K  G  P  E  E  D  T  E  I  E  Q  L  R  K  K  V  A  Y  Y  E  M  R  D  D  L  M  G  K
                                                                                        orfB
GCGAAGGCCATCGAAAGGAGGTGGTCCCGGAAGTCATCGTCGAAGTGATAGAGGCGAACAGACATGCCTATGGCGTCCAGGAGATGTGTCATGCCTTCGG 3600
A  K  A  I  E  R  R  W  S  R  K  S  S  S  K  *                                          M  C  H  A  F  G

CATTCCCCGTTCCACCTACTATCGGTGGAAACAACGTTCCAACGAAATTCCAGCCCTGCACCGGACAATTATGGACCTCTGCCGCCGGCACCGATATTGG 3700
 I  P  R  S  T  Y  Y  R  W  K  Q  R  S  N  E  I  P  A  L  H  R  T  I  M  D  L  C  R  R  H  R  Y  W

CTGGGCCACCGCAAGGTGGCGGCCCTTCTGCGCAAAGACCACGGGATCAGAATCAATCGAAAAACCGCCCAGCGCGTGATGCAGAAGTATCAGCTCCAAT 3800
L  G  H  R  K  V  A  A  L  L  R  K  D  H  G  I  R  I  N  R  K  T  A  Q  R  V  M  Q  K  Y  Q  L  Q  C

GCCGGGTCAAGCCGAAGAAACAGGTCCAGCCGACGGGAGAAACCCATCAGACGGTGCCTAATCTCCTTCAGCAGGAATTTACGGCGGACCGTCCCAACCA 3900
  R  V  K  P  K  K  Q  V  Q  P  T  G  E  T  H  Q  T  V  P  N  L  L  Q  Q  E  F  T  A  D  R  P  N  Q

AAAATGGGTTACGGACATTACGTACCTGCCGTATGGAGAGAAAATGTGGTATTTATCTACGATCATGGATGTATACAATAATGAATTAGTGGCGTATCAG 4000
 K  W  V  T  D  I  T  Y  L  P  Y  G  E  K  M  W  Y  L  S  T  I  M  D  V  Y  N  N  E  L  V  A  Y  Q

CTACGGGATACGCAAGAAACGAGTCTGGTGCTTGATACGCTGGAAGCGGCCTGTCGAGGCCGGGAGACCTACGGTCTCCTCCTGCACAGTGATCAGGGCT 4100
L  R  D  T  Q  E  T  S  L  V  L  D  T  L  E  A  A  C  R  G  R  E  T  Y  G  L  L  L  H  S  D  Q  G  S

CCCAGTATACCTCGCGCGCGTTTCAGCAGGCAGCCAAAGAAAAAGGCATTATCACAAGCATGTCCCGGAAAGGAAACTGCTTGGATAATGCCATGATCGA 4200
  Q  Y  T  S  R  A  F  Q  Q  A  A  K  E  K  G  I  I  T  S  M  S  R  K  G  N  C  L  D  N  A  M  I  E

ATCCTTCCATTCCTCGCTAAAGTCGGAAGCATTCAGCATCCGCGTACGGATGCCCCTTACATCTTCTATTATAGTCGAAAAAGTCGATTCGTACATGTAT 4300
 S  F  H  S  S  L  K  S  E  A  F  S  I  R  V  R  M  P  L  T  S  S  I  I  V  E  K  V  D  S  Y  M  Y

TATTACAATTACATACGACCATTTACCAAATTAAACGACCACAGTCCGGTG                                                4351
Y  Y  N  Y  I  R  P  F  T  K  L  N  D  H  S  P  V
```

Fig. 3. Nucleotide sequence of the recombinant fragment of pOSM11. Deduced amino acid sequences of ORFs designated *ectA*, *ectB*, *ectC*, *orfA* and *orfB* (5′-end) are shown below the sequence. Stop codons are marked with asterisks. Putative ribosome-binding sites are boxed. Consensus sequences for two putative σ^{70}-dependent promoters (consensus TTGACA-N_{17}-TATAAT) are underlined. Putative consensus sequences for a σ^{B}-dependent promoter (consensus GTTTAA-N_{12-14}-GGGTAT) are marked with dotted lines. Palindromic sequences are marked by arrows.

```
ectC    MKVIKLEDLLGTEREV--DDGNWVSRRFIMKDDNMGYSVNDTIIRAGTETHIWYQNHLET   58
HECTS   MIVRNLEEARQTDRLVTAENGNWDSTRLSLAEDGGNCSFHITRIFEGTETHIHYKHHFEA   60

ectC    VYCIEGDGEIETLSDNKVYQLEPGVLYALDKNDEHMLRGGSKDMRMVCVFNPPLSGREVH  118
HECTS   CYCIEGEGEVETLADGKIWPIKPGDIYILDQHDEHLLRA-SKTMHLACVFTPGLTGNEVH  119

ectC    DENGVY-PADLD                                                  129
HECTS   REDGSYAPADEADDQKPE                                            137
```

Fig. 4. Alignment of the sequences of reading frame *ectC* with the sequence of the ectoine synthase of a *Halomonas* sp. (HECTS) (Min-Yu *et al.*, 1993). Identical positions are shown against a dark background and conservative replacements are boxed. The following were regarded as conservative replacements: R-K-H, D-E, N-Q, S-T, G-A, F-Y-W, I-L-V-M.

similarities with ORFs of the same name believed to encode transposases of IS elements.

Ectoine synthesizing capacity of deletion derivatives of pOSM11

In order to confirm the gene assignments based on sequence similarities and to determine the potential role of *ectA* and *orfA* in ectoine synthesis, we constructed several deletion derivatives of pOSM11 and examined them physiologically (Fig. 6). Analysis of pOSM12, which was deficient in the sequences 300 bp downstream of *ectC* and only retained a part of *orfA*, revealed the same osmoregulated pattern of ectoine synthesis as pOSM11 in *E. coli* XL1-Blue. This suggests that *orfA* is not involved in the ectoine biosynthetic pathway. pOSM13 carried a deletion within *ectC* but still contained *ectA* and *ectB* and the sequences upstream of *ectA*. No ectoine synthesis was observed; instead the precursor N^{γ}-acetyldiaminobutyric acid was accumu-

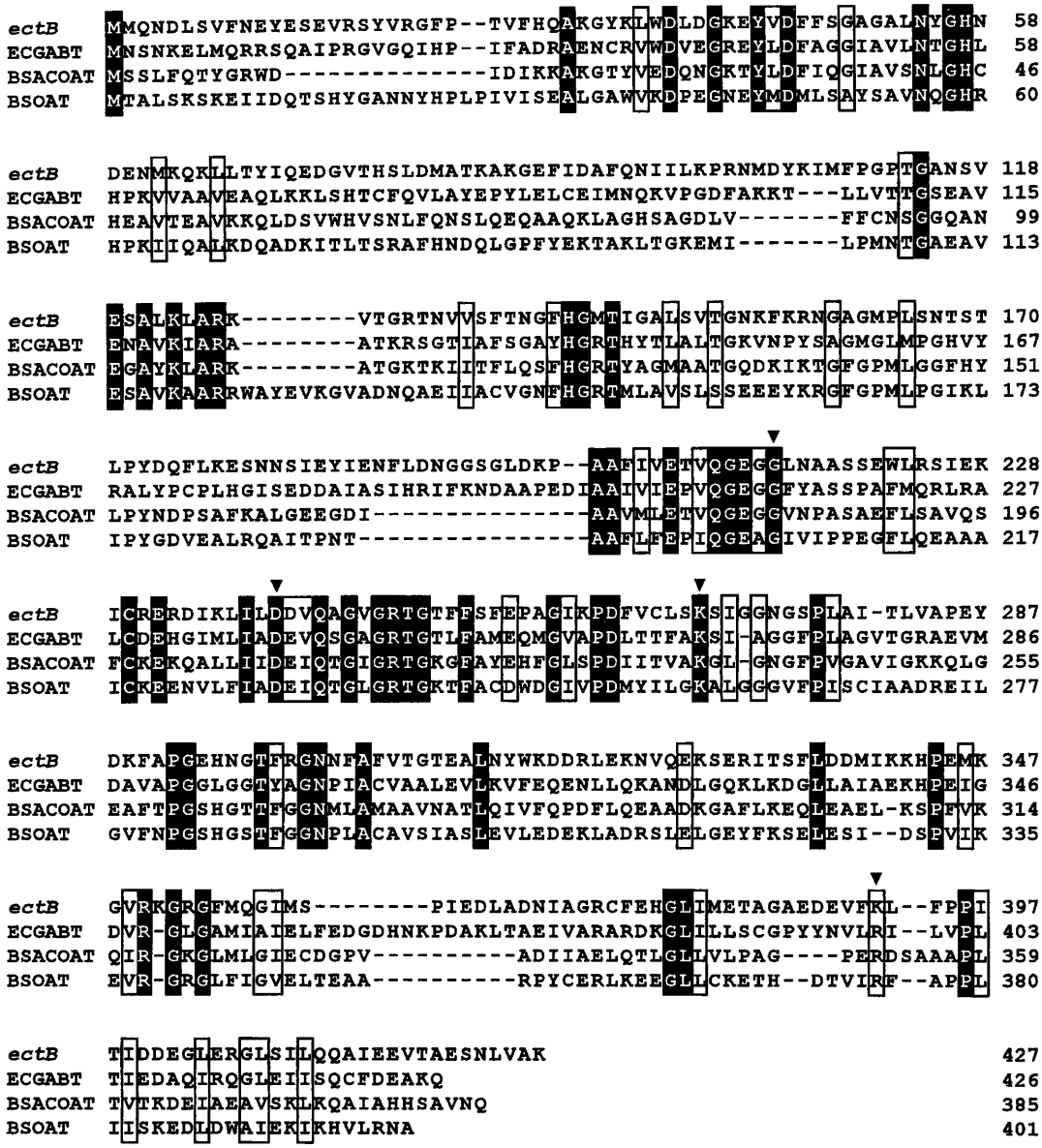


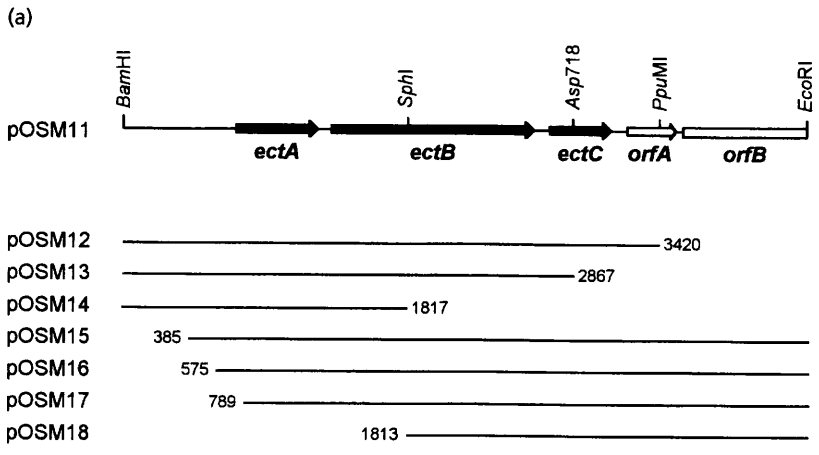
Fig. 5. Alignment of the sequences of reading frame *ectB* with transaminases. ECGABT, 4-aminobutyric acid transaminase from *E. coli* (Bartsch *et al.*, 1990); BSACOAT, acetylornithine transaminase from *B. subtilis* (O'Reilly & Devine, 1994); BSOAT, ornithine transaminase from *B. subtilis* (Gardan *et al.*, 1995). Identical positions are shown against a dark background and conservative replacements are boxed. Conservative replacements are defined in the legend to Fig. 4. ▼, Residues identical in all transaminases examined so far according to Mehta *et al.* (1993).

lated. This confirms that *ectC* encodes ectoine synthase, which converts N^{γ}-acetyldiaminobutyric acid to ectoine.

We constructed a subclone containing the complete ORF *ectA* and the upstream sequences (pOSM14). No accumulation of diaminobutyric acid or N^{γ}-acetyldiaminobutyric acid was observed, which is a further indication that *ectB* encodes the diaminobutyrate transaminase, the first enzyme in the ectoine pathway. When this subclone was supplemented with diaminobutyric acid, it synthesized N^{γ}-acetyldiaminobutyric acid, whereas *E. coli* XL1-Blue, containing only the vector pHGS575, was not able to acetylate diaminobutyric acid but simply accumulated it from the medium. This confirms our assumption that *ectA* is responsible for the acetylation step. In summary, our subcloning studies revealed that *ectA* encodes diaminobutyric acid acetyltransferase, *ectB* diaminobutyric acid transaminase and *ectC* ectoine synthase required for ectoine biosynthesis.

Osmoregulation and genetic organization of ectoine genes

Further deletion derivatives of pOSM11 were constructed to determine the approximate position of osmoregulating sequences in the promoter region. A



(b)

	Supplementation of culture	Ectoine biosynthesis	Accumulation of precursors inside cells
pOSM11	–	R	–
pOSM12	–	R	–
pOSM13	–	–	NAcDABA
pOSM14	DABA	–	NAcDABA
pOSM15	–	R	–
pOSM16	–	U	–
pOSM17	–	–	–
pOSM18	NAcDABA	–	NAcDABA

Fig. 6. (a) Map of the sequenced DNA fragment of *M. halophilus*(pOSM11) and deletion derivatives. pOSM12, pOSM13, pOSM14 and pOSM18 were constructed using the restriction sites indicated. pOSM15, pOSM16, and pOSM17 were generated with the Double Stranded Nested Deletion kit (Pharmacia). Numbers indicate the terminal positions (bp) of the subclones relative to pOSM11. Position of the ORFs in bp: *ectA*, 722–1240; *ectB*, 1329–2612; *ectC*, 2716–3105; *orfA*, 3216–3548; *orfB*, 3584–4351 (truncated). (b) Behaviour of the different subclones in physiological experiments. DABA, L-2,4-diaminobutyric acid; NAcDABA, N^{γ}-acetyldiaminobutyric acid; R, osmoregulated; U, unregulated; –, none.

deletion of the first 384 bp of the insert upstream of *ectA* (pOSM15, Fig. 6) had no effect on ectoine synthesis. Deleting a further 190 bp, however, apparently destroyed regulating sequences (pOSM16, Fig. 6), as the latter subclone showed constitutive ectoine synthesis. The cytoplasmic concentration ranged between 0·5 and 0·6 mmol (g dry wt)$^{-1}$, independent of the salt concentration of the medium. It can therefore be concluded that osmoregulating sequences are located at least 150 bp upstream of the first ORF.

Two possible consensus sequences for σ^{70}-dependent promoters were found upstream of *ectB* (Fig. 3). A search for consensus sequences of the osmoresponsive promoters for the compatible solute transport systems *proU*, *proP* and *opuA* (Mellies *et al.*, 1994, 1995; Kempf & Bremer, 1995) revealed no matches. As several osmoregulated genes in non-halophiles, including the genes for the biosynthesis of trehalose, are known to be under the control of σ^{S}-dependent promoters (Strøm & Kaasen, 1993; Gordia & Gutierrez, 1996; Manna & Gowrishankar, 1994; Mellies *et al.*, 1995), we also conducted a search for consensus sequences of these (Strøm & Kaasen, 1993), but were unable to find any matches. Instead, we found a sequence similar to the consensus for σ^{B} of *Bacillus subtilis* (Fig. 3) known to transcribe the so-called 'general stress proteins', which are induced by various stimuli, such as salt stress, ethanol treatment and starvation (Hecker *et al.*, 1996). An imperfect palindrome lies within the probable −10 region.

A perfect palindrome downstream of *ectA* overlapping with one of the putative σ^{70} promoter sequences (Fig. 3) may represent a Rho-dependent terminator. Therefore, the question arises as to whether the three genes are transcribed separately or as a single operon. The subclone *E. coli*(pOSM17), with a deletion of both the upstream sequences and part of *ectA* (Fig. 6), contained the whole reading frame *ectB* as well as the possible σ^{70} consensus sequences and should therefore produce diaminobutyrate provided that *ectB* is transcribed from these putative promoters. However, we were not able to detect this compound inside the cells. Furthermore, another subclone containing pOSM18 (Fig. 6), which retained only the complete reading frame *ectC*, was also unable to produce ectoine when supplemented with N^{γ}-acetyldiaminobutyrate. These findings indicate that *ectB* and *ectC* are not transcribed independently. However, in order to ascertain that the ectoine genes are transcribed as a single operon, further studies at the RNA level are necessary.

In this study we have, for the first time, identified genes for the *de novo* biosynthesis of a compatible solute (ectoine) in addition to sequences necessary for its osmoregulated expression from a truly halophilic eubacterium. We were able to demonstrate that the osmoregulatory sequences of *M. halophilus* also function in *E. coli*. Earlier work on osmoregulated transport systems has also shown the potential of gene transfer. An osmoprotectant transporter from *Erwinia chrysanthemi*, encoded by the gene *ousA* and analogous to *proP* from *E. coli*, displayed osmoregulated expression in *E. coli*, although no DNA homology upstream of the coding sequences of *proP* and *ousA* was identified (Gouesbet *et al.*, 1996). In addition, the betaine uptake system *opuA* from *B. subtilis* (Kempf & Bremer, 1995) was also shown to be under osmotic control in *E. coli*. In this case, promoter consensus sequences resembling those of *proP* and *proU* were identified. These observations support the view that there are features in osmoregulation that are shared between different, even phylogenetically distant, bacteria. Further studies re-

garding the osmoregulation of the ectoine genes from *M. halophilus* will provide a deeper insight into these mechanisms.

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