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ORIGINAL PAPER

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Marinococcus halophilus DSM 20408^T encodes two transporters for compatible solutes belonging to the betaine-carnitine-choline transporter family: identification and characterization of ectoine transporter EctM and glycine betaine transporter BetM

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Abstract In response to osmotic stress, the halophilic, Gram-positive bacterium *Marinococcus halophilus* accumulates compatible solutes either by de novo synthesis or by uptake from the medium. To characterize transport systems responsible for the uptake of compatible solutes, a plasmid-encoded gene bank of *M. halophilus* was transferred into the transport-deficient strain *Escherichia coli* MKH13, and two genes were cloned by functional complementation required for ectoine and glycine betaine transport. The ectoine transporter is encoded by an open reading frame of 1,578 bp named *ectM*. The gene *ectM* encodes a putative hydrophobic, 525-residue protein, which shares significant identity to betaine-carnetine-choline transporters (BCCTs). The transporter responsible for the uptake of glycine betaine in *M. halophilus* is encoded by an open reading frame of 1,482 bp called *betM*. The potential, hydrophobic BetM protein consists of 493 amino acid residues and belongs, like EctM, to the BCCT family. The affinity of whole cells of *E. coli* MKH13 for ectoine ($K_s = 1.6$ μM) and betaine ($K_s = 21.8$ μM) was determined, suggesting that EctM and BetM exhibit a high affinity for their substrates. An elevation of the salinity in the medium resulted in an increased uptake of ectoine via EctM and glycine betaine via BetM in *E. coli* MKH13 cells, demonstrating that both systems are osmoregulated.

Keywords Betaine-carnitine-choline transporter · Compatible solute · Ectoine · Glycine betaine · *Marinococcus halophilus* · Osmoregulation

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Introduction

The non-spore-forming, Gram-positive bacterium *Marinococcus halophilus* is a moderately halophilic microorganism phylogenetically related to the *Bacillus* group (Farrow et al. 1992) and can tolerate salt concentrations in the medium up to 5.5 M NaCl (Novitsky and Kushner 1976). To establish an osmotic equilibrium with the saline environment, *M. halophilus* synthesizes ectoine as its main compatible solute like many other chemoheterotrophic halophiles (Severin et al. 1992). The enzymes required for the production of ectoine are encoded by three genes, *ectABC*, which were isolated for the first time from *M. halophilus* (Louis and Galinski 1997). However, *M. halophilus* does not entirely rely on ectoine synthesis for osmoadaptation, but can also take up compatible solutes from the medium, which is a far more economical way to amass osmoprotectants in the cell (Oren 1999). To allow for the uptake of external solutes, *M. halophilus* must be equipped with osmoregulated transport systems, which have been mostly studied so far in non-halophilic bacteria such as *Escherichia coli* (MacMillan et al. 1999; Racher et al. 1999), *Bacillus subtilis* (Kappes and Bremer 1996; von Blohn et al. 1997), and *Corynebacterium glutamicum* (Peter et al. 1998; Rübenhagen et al. 2001). Until now, only one compatible solute transporter has been described from a halophilic bacterium, namely TeaABC, an osmoregulated ectoine transporter found in *Halomonas elongata* (Grammann et al. 2002). Mutagenesis experiments revealed that TeaABC is not only involved in the uptake of external solutes, but also serves as a recovery system salvaging endogenous ectoine leaking through the cell membrane. Due to the lack of information on transporters in other halophilic bacteria, it is not known whether the recycling of lost compatible solutes by a specific transporter is a common theme in ectoine-producing halophiles. To gain a deeper insight into compatible solute uptake in halophilic bacteria, which synthesize ectoine as their main osmolyte, this present

study focused on the isolation and characterization of genes coding for compatible solute transporters from *M. halophilus*. We identified and isolated, by functional complementation of the transport-deficient *E. coli* mutant MKH13, two structural genes encoding a betaine transporter named BetM that also accepts ectoine as an additional substrate, and a transport system specific for the uptake of ectoines named EctM. We then characterized the role of both transport proteins in solute accumulation.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The strains, vectors, and recombinant plasmids used for this study are listed in Table 1. For DNA isolation *M. halophilus* was grown in Luria Bertani (LB) medium containing 1.72 M sodium chloride (NaCl) at 37°C and harvested in the late exponential growth phase.

E. coli strains were grown aerobically at 37°C in either LB medium or MM63 medium (Larsen et al. 1987) at various NaCl concentrations. To support growth of *E. coli* in MM63 medium containing more than 680 mM NaCl, compatible solutes were supplied with the medium at concentrations up to 2 mM. If necessary, chloramphenicol (Cm) was added to the medium to a final concentration of 50 μg ml^{-1}.

Osmotic shock experiments

Overnight cultures of *E. coli* MKH13 expressing *ectM* or *betM* were inoculated into MM63 medium containing 170 mM NaCl and adjusted to an optical density (OD_{540}) of 0.8. To mid-exponential phase cultures, ectoine and glycine betaine, respectively, were added to a final concentration of 2 mM. Thirty minutes later, the salt concentration was increased to 680 mM by adding sterile NaCl. Before and after osmotic shock, samples were taken to determine the protein content and to analyze the compatible solute content of the cells.

Compatible solute limited growth of *E. coli* MKH13

To determine the affinity of recombinant *E. coli* MKH13 expressing *ectM* or *betM* for the compatible solutes ectoine and betaine, respectively, minimal medium (MM63 containing 680 mM NaCl) was prepared having ectoine or glycine betaine at final concentrations of 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 1 mM. One hundred milliliters of the medium was inoculated with a suspension of MKH13 cells and adjusted to an OD_{540} of ≈0.1. Cells were incubated for 36 h, and the growth rate was determined.

DNA isolation and manipulation

Genomic DNA from *M. halophilus* was isolated using Qiagen Genomic-tips 100/g (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Routine manipulation of DNA, plasmid isolation from *E. coli* strains, construction of recombinant plasmids, electrophoresis of DNA, DNA amplification by PCR, and transformation were carried out according to standard procedures. DNA sequencing, based on the method of Sanger et al. (1977), was carried out by SequiServe (Vaterstetten, Germany). Primers for PCR were synthesized by MWG Biotech AG (Ebersberg, Germany).

Construction of a plasmid-encoded genomic library of *M. halophilus* and complementation of transport-defective *E. coli* mutants

Genomic DNA of *M. halophilus* was partially hydrolyzed using restriction enzyme *Sau*3A. The chromosomal *Sau*3A fragments were ligated into low-copy plasmid pHSG575 (Takeshita et al. 1987), and the ligation products were transformed into *E. coli* XL1-blue (Bullock et al. 1987). The resulting colonies were pooled; plasmids were isolated from the XL1-blue cells, and transferred into *E. coli* mutant MKH13 defective in osmoregulated, compatible solute uptake (Haardt et al. 1995). *E. coli* MKH13 clones with the gene library of *M. halophilus* were selected for compatible solute uptake on MM63 mineral-salt medium containing 680 mM NaCl and either 1 mM ectoine or 1 mM glycine betaine at 37°C. *E. coli* strains able to grow on either one of the selection media were isolated and further analyzed.

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or description[a]	Source or reference
Marinococcus halophilus		
DSM 20408^T	Type strain	DSMZ[b]
Escherichia coli		
MKH13	Δ(*proP*)2, Δ(*proU*)608, Δ(*putPA*)101, Spc^r	Haardt et al. 1995
XL1-blue	*recA*1, *endA*1, *gyrA*96, *thi*, *hsd*R17, (rh^-, mk^+), *supE*44, *relA*1, λ^-, lac^-, [F', *proAB*, *lacI*^{q}, ZΔM15, Tn*10*(*tet*)]	Bullock et al. 1987
Plasmids		
pHSG575	Cm^r,*lacZ'*	Takeshita et al. 1987
pVVB1	pHSG575 with 4.7-kb *Sau*3A genomic DNA fragment carrying *betM* from *M. halophilus*	This study
pVVB11	pHSG575 with 3.6-kb fragment generated by ligation of *Eco*RI-hydrolyzed pVVB1	This study
pVVB12	pHSG575 with 1.3-kb fragment generated by ligation of *Pst*I-hydrolyzed pVVB1	This study
pVVB13	pHSG575 with 2.7-kb fragment carrying *betM;* generated by ligation of *Hin*dIII-hydrolyzed pVVB1	This study
pVVB2	pHSG575 with 6.4-kb *Sau*3A genomic DNA fragment carrying *ectM* from *M. halophilus*	This study
pVVB21	pHSG575with 4.5-kb fragment generated by ligation of *Eco*RI-hydrolyzed pVVB2	This study
pVVB22	pHSG575with 0.9-kb fragment generated by ligation of *Pst*I-hydrolyzed pVVB2	This study
pVVB23	pHSG575with 3.5-kb fragment carrying *ectM;* generated by ligation of *Hin*dIII-hydrolyzed pVVB2	This study
pVVB24	pHSG575 with 2.9-kb *Hin*dIII DNA fragment from pVVB2	This study
pVVB25	pHSG with 3.6-kb *Pst*I DNA fragment from pVVB2	This study

[a]Antibiotics: *Cm* chloramphenicol, *Spc* spectinomycin
[b]Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany

High-performance liquid chromatography

For identification and quantification of intracellular compatible solutes, cells were harvested and extracted with methanol/chloroform/water as described by Galinski and Herzog (1990). Cellular extracts were analyzed by isocratic high-performance liquid chromatography (HPLC) on an NH_2 column using a refractive index monitor (Frings et al. 1993).

Computer methods

Protein databases and translated nucleotide databases were screened to find similar proteins to EctM and BetM using the FASTA (Pearson and Lipman 1988) and BLAST (Altschul et al. 1990) programs. Multiple sequence alignments were constructed on an Apple Macintosh computer using the CLUSTAL X program (Thompson et al. 1997). Hydropathy profiles of proteins were constructed by the method of Kyte and Doolittle (1982). Potential transmembrane spanning units were identified by using the HMMTOP program (Tusnády and Simon 1998, 2001) available through the HMMTOP transmembrane prediction server (http://www.enzim.hu/hmmtop/) and the SOSUI program (Hirokawa et al. 1998; http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html). Free energies of RNA stem-loop structures were calculated using the Mfold program (Zucker 2003) available at http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1-2.3.cgi.

Nucleotide sequence accession number

The nucleotide sequences of *betM* and *ectM* were submitted to GenBank and assigned accession nos. AY326959 (*betM*) and AY327499 (*ectM*), respectively.

Results

Cloning genes from *M. halophilus* encoding compatible solute transporters

The genes coding for compatible solute transport systems from *M. halophilus* were identified and isolated by functional complementation of mutant *E. coli* MKH13. *E. coli* MKH13 is deficient in osmoregulated uptake of compatible solutes, which is needed to tolerate salinities of 680 mM NaCl and higher. MKH13 cells were transformed with the low-copy-number plasmid pHSG575 (Cm^r) harboring a gene library of partial *Sau*3A restriction fragments from chromosomal DNA of *M. halophilus*. The resulting transformants were selected for colonies that can grow on high-saline medium containing 680 mM NaCl and the compatible solute ectoine (1 mM) or glycine betaine (1 mM). Growth of such colonies indicated transport of ectoine and betaine, respectively, via a plasmid-encoded transporter from *M. halophilus*. Two strains that could grow under these selective conditions were found: one carrying plasmid pHSG575 with a 4.7-kb DNA fragment (pVVB1) allowing for growth in the presence of glycine betaine, and a second strain harboring pHSG575 with a 6.4-kb DNA fragment (pVVB2) that enabled growth in ectoine medium.

Restriction analysis of the 4.7-kb *M. halophilus* DNA from plasmid pVVB1 and nucleotide sequence of *betM*

To determine which part of the 4.7-kb DNA fragment from pVVB1 is coding for the potential transporter mediating the uptake of glycine betaine in *E. coli* MKH13, restriction analyses were carried out. Plasmid pVVB1 was hydrolyzed by restriction enzymes *Eco*RI, *Pst*I, and *Hin*dIII. Each of these enzymes has one restriction site in the multiple cloning site of plasmid pVVB1 next to the inserted DNA of *M. halophilus*. After hydrolysis, the remaining *M. halophilus* DNA still attached to the plasmid was religated, generating three new plasmids: pVVB11 carrying an *Eco*RI/*Sau*3A fragment, pVVB12 with a *Sau*3A/*Pst*I fragment, and pVVB13 containing a 2.7 kb *Sau*3A/*Hin*dIII fragment (Fig.1). Growth experiments in saline glycine betaine medium showed that only pVVB13 supported growth of *E. coli* MKH13. For DNA sequence analysis, the entire *Sau*3A/*Hin*dIII insert was sequenced. Inspection of the sequenced DNA revealed the presence of a 1,482-bp open reading frame, which we refer to as *betM*. The sequence immediately upstream of the start codon is complementary to the 3′ end of the 16S rRNA of *M. halophilus* and is likely to function as a ribosome-binding site. Downstream of the *betM* stop codon TAA, a putative stem loop structure was found [ΔG (25°C) = −107 kJ] followed by a stretch of six thymidine nucleotides, which together could serve as a factor-independent transcription termination signal for the *betM* transcript. PCR analysis using genomic DNA from *M. halophilus* as template amplified a 2,083-bp fragment comprising *betM* and adjacent DNA, which proved that *betM* is indeed *M. halophilus* DNA, and that the cloned *betM* locus is organized in the same way as on the chromosome of *M. halophilus*.

Putative BetM protein shows characteristics of a hydrophobic integral membrane protein

The *betM* gene encodes a 493-residue protein with a calculated molecular mass of 53.9 kDa. No obvious N terminal secretion signal sequence was found. The amino acid sequence composition of BetM reveals that the potential protein is very hydrophobic, consisting of 65.8% apolar residues (MIVLPGAFW). The hydropathy profile, according to the method of Kyte and Doolittle (1982), showed 12 particular hydrophobic regions, which are predicted to form membrane-spanning α-helices. Analysis of the BetM sequence suggests that both the amino terminus and carboxy terminus are facing the cytoplasm, a feature common in secondary transporters (Saier 1994). The N terminus is made of only eight amino acids comprising two positively charged lysine residues. The C terminus forms a short extension of 11 residues containing five charged amino acids (one aspartate, three lysine, and one arginine).

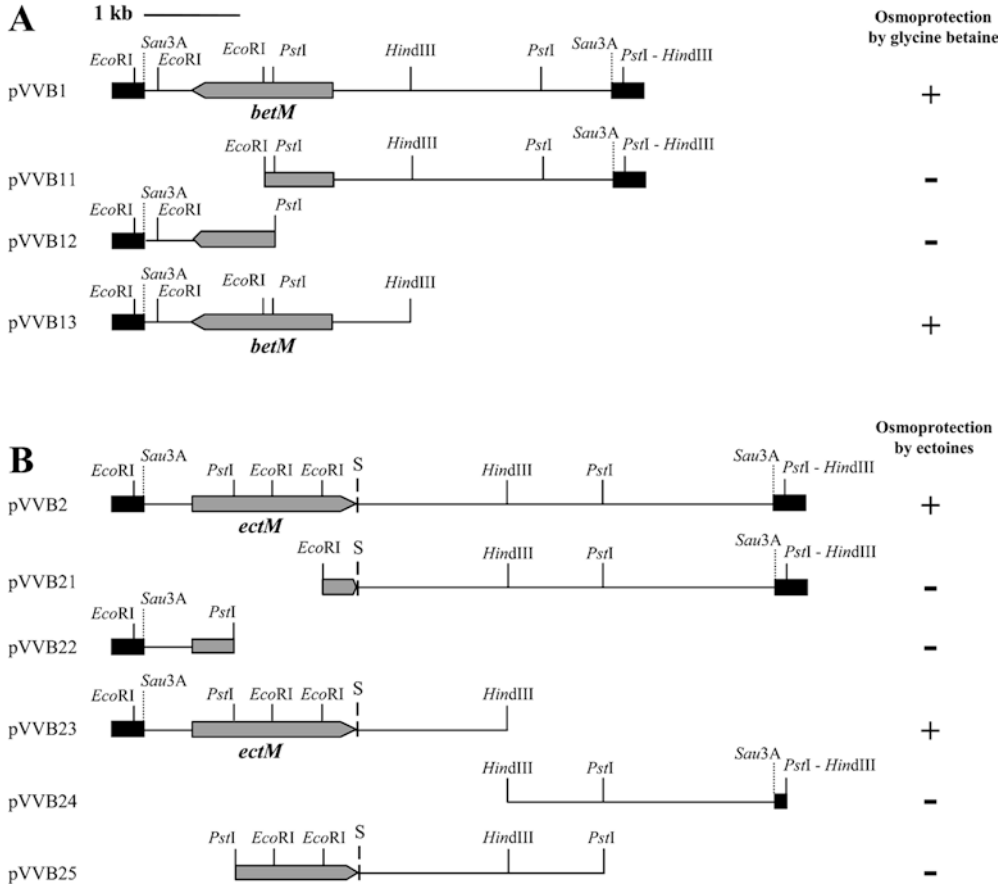

Fig. 1A,B Physical and genetic organization of the cloned *Marinococcus halophilus* DNA fragments carrying *betM* and *ectM* and subcloning experiments to prove the relevance of both genes for osmoprotection in *E. coli* MKH13. **A** A 4.7-kb DNA fragment from *M. halophilus* cloned into pHSG575 allowed transport deficient *Escherichia coli* MKH13 to accumulate glycine betaine in saline minimal medium. Hydrolysis of the 4.7-kb fragment by *Eco*RI, *Hin*dIII, and *Pst*I and subcloning of the resulting fragments showed the relevance of *betM* for the transport of glycine betaine. **B** A DNA fragment of 6.4 kb from *M. halophilus* mediated uptake of ectoine and hydroxyectoine in *E. coli* MKH13. The 6.4-kb fragment was hydrolyzed by *Eco*RI, *Hin*dIII, and *Pst*I, and the resulting fragments were subcloned. Only the DNA containing the open reading frame *ectM* allowed for the uptake of ectoine. The relevance of *betM* and *ectM* for osmoprotection was verified by growth experiments on saline minimal plates [MM63 with 0.77 M sodium chloride (NaCl)] in the presence of glycine-betaine (1 mM) and ectoine (1 mM), respectively. Growth (+) or no growth (–) of *E. coli* MKH13 containing the cloned *M. halophilus* DNA was scored after 3 days of incubation at 37°C

BetM is related to osmoregulated secondary transport proteins of the betaine-carnetine-choline transporter family

The deduced amino acid sequence of *betM* was compared to sequences available from databases. The putative BetM protein revealed a high degree of identity to proteins of the betaine-carnetine-choline transporter (BCCT) family, a group of secondary carriers that transport trimethylammonium compounds by sodium or proton symport. BetM displays 48% identical amino acids to the osmoregulated glycine betaine transporters BetL from *Listeria monocytogenes* (Sleator et al. 1999) and OpuD from *B. subtilis* (Kappes et al. 1996). BetM still shares 39% identical amino acids with BetP, a well-described BCCT found in *C. glutamicum*, which facilitates the uptake of glycine betaine as well. Like other BCCTs, BetM contains a highly conserved stretch of 32 amino acids residues corresponding to amino acid residues 351 to 382. This stretch forms the eighth transmembrane helix and the following cytoplasmic domain and contains the BCCT signature sequence: (GSDN)-WT-(LIVM)-X-(FY)-WXWW (Kappes et al. 1996; Boscari et al. 2002).

Restriction analysis of the 6.4-kb *M. halophilus* DNA from plasmid pVVB2 and nucleotide sequence of *ectM*

Plasmid pVVB2 was hydrolyzed by restriction enzymes *Eco*RI, *Pst*I, and *Hin*dIII to determine which part of the 6.4-kb DNA insert from *M. halophilus* enabled growth of *E. coli* MKH13 in saline medium containing ectoine or hydroxyectoine. Ligation of the hydrolyzed DNA with pHSG575 generated five different plasmids, of which only pVVB23 restored ectoine transport activity in *E. coli* MKH13 (Fig. 1). An approximately 3.5-kb *Sau*3A-*Hin*dIll fragment was inserted in plasmid pVVB23. The entire 3.5 kb was sequenced and revealed the presence of a 1,578-bp open reading frame, which was named *ectM*. The start codon ATG of *ectM* is

preceded by a potential ribosome-binding site (GAGGGGGAAGC). Downstream of the stop codon, an inverted repeat was detected [ΔG (25°C) = −117 kJ] followed by nine AT nucleotides, which together could function as the transcriptional termination signal for the *ectM* transcript. To prove that *ectM*, including the adjacent sequences, exists on the chromosome of *M. halophilus*, a 2,288-bp fragment was amplified by PCR using chromosomal DNA from *M. halophilus* as a template and two primers derived from the pVVB23 insert DNA upstream and downstream of *ectM*. Restriction analysis verified that the amplified DNA was identical to the cloned DNA fragment of pVVB23.

EctM is a potential hydrophobic transmembrane protein

The putative EctM protein has a molecular weight of 58.48 kDa, consisting of 525 amino acid residues. Signal sequences at the N terminal for protein export were not found. EctM is a hydrophobic protein having 62.9% apolar amino acids. The hydropathy plot, according to the method of Kyte and Doolittle (1982), revealed an alteration of 12 hydrophobic and hydrophilic segments that is characteristic for integral membrane proteins. As predicted by computer analysis, the 12 hydrophobic segments are potential transmembrane-spanning α-helices, and both the amino terminus and carboxy terminus are thought to be located in the cytoplasm. The N terminus is made of approximately 12 residues, while the C terminus has a length of about 30 amino acids. In contrast to the N terminus, the C terminus contains more than 50% charged amino acids, namely, seven glutamate, two aspartate, three lysine, and seven asparagine residues.

EctM is a member of the BCCT family

The deduced amino acid sequence of *ectM* was compared to amino acid sequences available from the databases. The EctM protein showed a high degree of identity to proteins of the BCCT family. EctM shares 41% identical amino acids with OpuD from *B. subtilis* and 35% identical amino acids with the *C. glutamicum* transporters BetP and EctP. EctP is an osmoregulated carrier for ectoine, proline, and glycine betaine (Peter et al. 1998). The best score was found for a hypothetical BCCT protein of *B. halodurans* (Takami et al. 2000) and the ectoine transporter EctT of *Virgibacillus pantothenticus* (GenBank accession no. AF421189), both having 49% residues identical with EctM. When compared with its counterpart BetM from *M. halophilus*, EctM exhibits 37% sequence identity. EctM contains a stretch of 32 highly conserved amino acid residues as described for BetM and other BCCT systems. Similar to BetM, this region comprises the eighth membrane helix and the connecting loop to helix nine. In EctM, the region contains a modified BCCT signature (GSDN)-W *P*-(LIVM)-X-(FY)-WXW *T*, having a proline residue instead of a threonine at position 3 and a threonine instead of a tryptophan at position 10 of the signature sequence (Fig. 2).

Substrate specificity of transporter BetM

E. coli MKH13 cells expressing *betM* were selected on saline medium containing 1 mM glycine betaine. HPLC measurement of the cytoplasm of *E. coli* $betM^+$ showed that BetM is catalyzing the uptake and accumulation of glycine betaine (Fig. 4). To test whether BetM accepts other compatible solutes as substrates, growth experiments with *E. coli* MKH13 pVVB1 ($betM^+$) were carried out in the presence of 1 mM glycine betaine, ectoine, hydroxyectoine, proline, and carnitine, respectively. *E. coli* MKH13 pVVB1 was spread out on MM63 medium containing 765 mM NaCl with and without compatible solutes and incubated at 37°C. After 4 days, *E. coli* was only grown on medium with glycine betaine and ectoine proving that BetM can transport ectoine in addition to glycine betaine. Proline, carnitine, and also hydroxyectoine, which differs from ectoine only by having a hydroxy group at the C3 position, did not support growth.

Growth of *E. coli* MKH13 pVVB1 (*betM*) in glycine betaine and ectoine limited medium

Since growth of *E. coli* MKH13 pVVB1 ($betM^+$) in high-saline minimal medium is dependent on the presence of external glycine betaine or ectoine, lowering the compatible solute concentration in the medium caused a decrease in growth rate, which enabled the affinity of the cells for glycine betaine and ectoine, respectively, to be determined. The growth rates under glycine betaine-limited conditions were fitted by non-linear regression. The model that best described the correlation was the Monod model (Fig. 3). The plot of growth rate against growth rate/glycine betaine concentration (Eadie-Hofstee plot) showed that the cells had a saturation constant K_s of 21.8 μM (±8.45 SD), suggesting that BetM is a high-affinity transport system for glycine betaine.

In ectoine-limited medium, *E. coli* grew significantly only at concentrations above 0.5 mM ectoine, indicating that BetM has only a low affinity for ectoine.

Transport of glycine betaine via BetM is induced by osmotic upshock

To test whether the uptake of glycine betaine by BetM is enhanced by increasing osmolality, upshock experiments with *E. coli* MKH13 pVVB1were carried out. Exponentially growing *E. coli* cells were subjected to an osmotic upshift from 170 mM NaCl to 680 mM NaCl in the presence of 2 mM glycine betaine. HPLC measurement of the cytoplasmic solute content revealed that glycine betaine was already taken up in cells grown at a constant salinity of 170 mM NaCl, and betaine

Fig. 2 Amino acid sequences of ectoine transporter EctM and glycine betaine transporter BetM from *M. halophilus* DSM 20408^T are aligned with sequences of the compatible solute transporters OpuD from *Bacillus subtilis* and EctT from *Virgibacillus pantothenticus*. Amino acid residues, which are identical in at least three proteins out of four, are marked *black*. Conserved amino acids are shown in *gray*. Conservative replacements are defined as follows: G → A; I → L → V → M; S → T; R → K → H; D → E; N → Q; F → Y → W. The amino acid stretch containing the betaine-carnetine-choline transporter signature sequence is *underlined*

accumulated to over 1 μmol mg protein^{-1} (Fig. 4). After osmotic upshock, the transport via BetM was strongly induced, and glycine betaine was amassed to a maximum concentration of approximately 2.2 μmol mg protein^{-1}. In addition, the cytoplasmic concentration of the endogenous solute trehalose was briefly increased by de novo synthesis (Fig. 4). Cells of *E. coli* MKH13 without *betM* were unable to transport glycine betaine from the medium (data not shown).

Substrate specificity of transporter EctM

E. coli MKH13 cells expressing *ectM* were selected on saline medium containing 1 mM ectoine. To test whether EctM accepts compatible solutes other than ectoine as substrates, growth experiments with *E. coli* MKH13 pVVB2 (*ectM*$^+$) were carried out in the presence of 1 mM glycine betaine, ectoine, hydroxyectoine, proline, and carnitine, respectively. *E. coli* MKH13 pVVB2 was spread out on MM63 medium containing 765 mM NaCl with and without compatible solutes and incubated at 37°C. After 4 days, *E. coli* was grown on medium with ectoine and hydroxyectoine, but not on medium with proline, carnitine, and glycine betaine. Although further tests have to be carried out, the presented results indicate that EctM is a transporter for ectoines with narrow substrate specificity.

Growth of *E. coli* MKH13 pVVB2 in ectoine- and hydroxyectoine-limited medium

Since growth of *E. coli* MKH13 pVVB2 (*ectM*$^+$) in high-saline, minimal medium is dependent on the presence of external compatible solutes, lowering the ectoine and hydroxyectoine concentration in the medium caused a decrease in growth rate, which enabled the affinity of

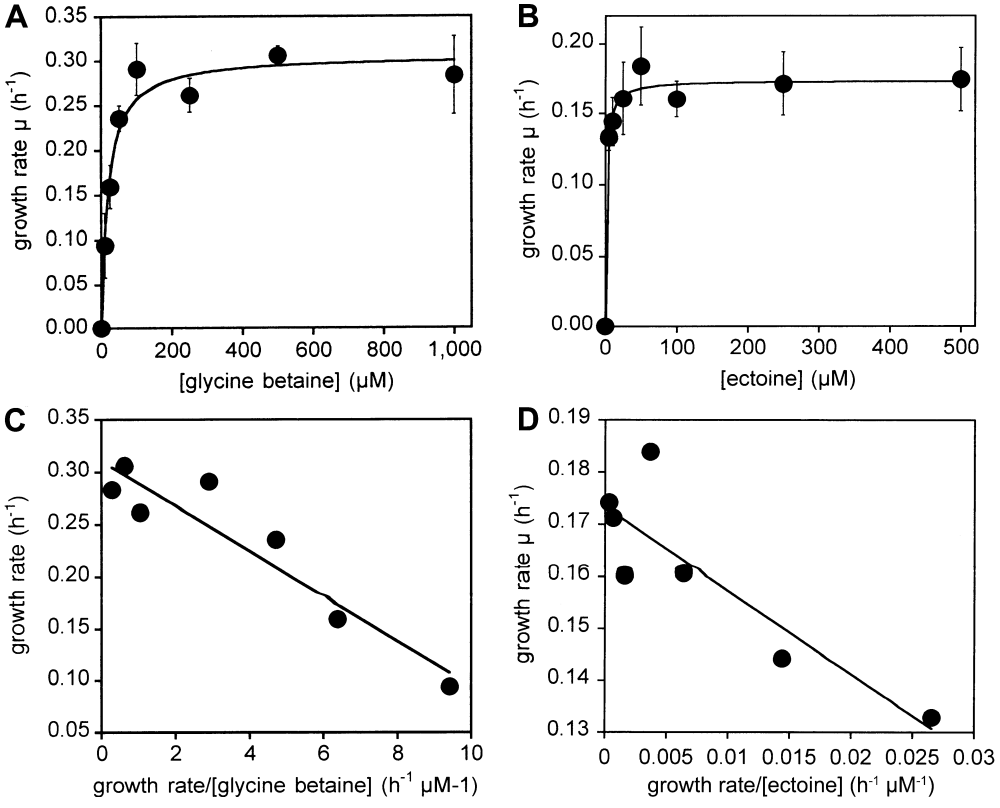

Fig 3A–D Affinity of whole cells for glycine betaine and ectoine was determined by growth experiments with *E. coli* MKH13 pVVB1 (*betM*$^+$) and *E. coli* MKH13 pVVB2 (*ectM*$^+$). Specific growth rates were determined from exponential phase growth of betaine-limited cultures of *E. coli* MKH13 containing *betM* (**A**) and ectoine-limited cultures of MKH13 containing *ectM* (**B**). Lowering the glycine betaine concentration from 1,000 μM to 10 μM (**A**) and the ectoine concentration from 500 μM to 5 μM (**B**) led to a decreasing growth rate of *E. coli* MKH13. No growth was detected in the absence of betaine and ectoine, respectively. The growth rate data of *E. coli* MKH13 pVVB1 (*betM*$^+$) and *E. coli* MKH13 pVVB2 (*ectM*$^+$) were fitted by nonlinear regression and described best by the Monod model (**A**, **B**). The plot of growth rate against growth rate/compatible solute concentration (Eadie-Hofstee plot) showed that cells containing *betM* have saturation constant (K_s) of 21.8 μM (**C**) and cells expressing *ectM* have a K_s value of 1.6 μM (**D**)

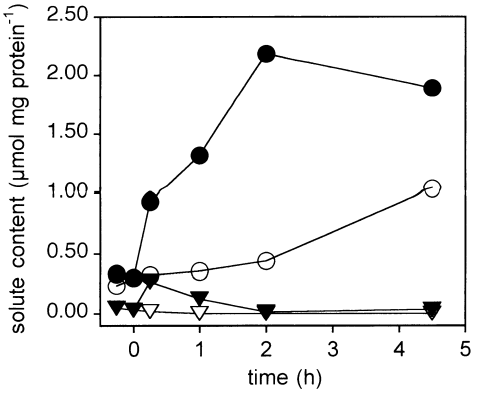

Fig. 4 Osmoregulated uptake of glycine betaine in *E. coli* by transporter BetM from *M. halophilus*. Cells of *E. coli* MKH13 pVVB1 (*betM*$^+$) were grown in minimal medium (MM63) containing 170 mM NaCl and 2 mM glycine betaine. At time zero, cells were exposed to osmotic stress by increasing the salt concentration to 680 mM NaCl, and changes in the cytoplasmic solute content were measured by high-performance liquid chromatography, HPLC (● glycine betaine, ▼ trehalose). Solute content of unshocked cells growing at a constant salinity of 170 mM NaCl is shown as a control (○ glycine betaine, ∇ trehalose)

the cells for ectoines to be determined. The growth rates under ectoine-limited conditions were fitted by non-linear regression. The model that best described the correlation was the Monod model (Fig. 3). The plot of growth rate against growth rate/ectoine concentration (Eadie-Hofstee plot) showed that the cells had a saturation constant K_s of 1.6 μM (±0.55 SD) for ectoine. The half-maximal growth rate under hydroxyectoine-limited conditions was determined to be 2.5 μM (data not shown), suggesting that EctM is a high-affinity transport system for both ectoine and hydroxyectoine. However, the maximum growth rate of *E. coli* cells accumulating ectoine was significantly lower than the growth rate of *E. coli* transporting betaine by BetM. This might be caused by the less effective uptake of ectoine via EctM compared to the betaine transport via BetM (Fig. 3).

Transport of ectoine via EctM is enhanced after osmotic upshock

To determine whether EctM is an osmoregulatory system for ectoine accumulation, osmotic upshock experiments similar to BetM were carried out. Mid-exponential phase cultures of *E. coli* MKH13 pVVB2 were exposed to osmotic stress by increasing the salt concentration from 170 mM NaCl to 680 mM NaCl in

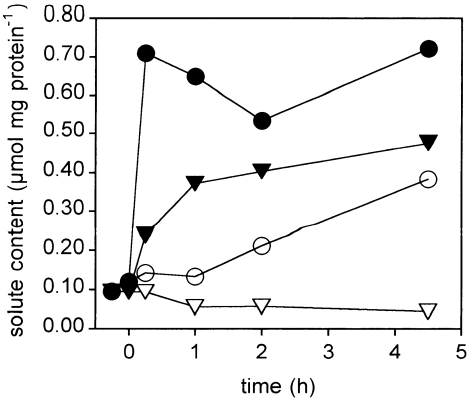

Fig. 5 Osmoregulated uptake of glycine betaine in *E. coli* by transporter EctM from *M. halophilus*. Cells of *E. coli* MKH13 pVVB2 (*ectM*$^+$) were grown in minimal medium (MM63) containing 170 mM NaCl and 2 mM ectoine. At time zero, cells were exposed to osmotic stress by increasing the salt concentration to 680 mM NaCl and changes in the cytoplasmic solute content were measured by HPLC (● ectoine, ▼ trehalose). Solute content of cells growing at a constant salinity of 170 mM NaCl is shown as a control (○ ectoine, ∇ trehalose)

the presence of 2 mM ectoine (Fig. 5). Ectoine uptake via EctM was enhanced in response to osmotic stress, and ectoine was accumulated to its final concentration immediately. However, the maximum reached was a concentration of 0.70 µmol ectoine mg protein^{-1} after 4.5 h; a value approximately three times lower than the concentration of betaine accumulated by the BetM system. Simultaneous with the transport of ectoine, trehalose was synthesized and accumulated as compatible solute (Fig. 5). However, in contrast to the situation with BetM, trehalose levels did not decline. The final compatible solute content (ectoine plus trehalose) after osmotic shock in cells expressing *ectM* was almost 50% lower, reaching only 1.2 µmol mg protein^{-1} compared to the solute content (betaine plus trehalose) of *E. coli* expressing *betM* having 2.2 µmol mg protein^{-1} (Fig. 4). The control cells of *E. coli* MKH13 without *ectM* failed to accumulate ectoine after osmotic shock (data not shown).

Discussion

Uptake of compatible solutes from the surrounding by osmoregulated transporter is the most economic way of accumulating compatible solutes and therefore preferred over de novo synthesis (Oren 1999). Recent studies on osmoregulated transport in the halophilic bacterium *H. elongata* revealed an additional role of compatible solute transporters as a recovery system for ectoine, which leaks through the cytoplasmic membrane (Grammann et al. 2002). The data presented here show that *M. halophilus* carries the genes *ectM* and *betM*, which can express osmoregulated, compatible solute transporters for ectoine and betaine, respectively. Analysis of the deduced amino acid sequences of the genes revealed that both transporters belong to the BCCT family. BCCTs are energized by pmf-driven or smf-driven proton or sodium ion symport catalyzing the uptake of trimethylammonium compounds. Carriers of this family are widespread in members of the Bacteria and Archaea domain. Many of these uptake systems are involved in osmoregulated transport of compatible solutes in Gram-positive bacteria [e.g., glycine betaine transporters BetP ($K_m = 8.6$ µM), Peter et al. 1996; OpuD ($K_m = 13$ µM), Kappes et al. 1996; BetL ($K_m = 7.9$ µM), Sleator et al. 1999) and Gram-negative bacteria (BetS, $K_m = 16$ µM for glycine betaine; Boscari et al. 2002]. BetP from *B. subtilis* was characterized as a transporter for glycine betaine that can respond to osmotic stress by regulating its own activity. The C terminal cytoplasmic extension of BetP was proven to play an important role in measuring the cytoplasmic potassium concentration and thereby sensing changes in osmolality, which will activate or deactivate the transport of solutes by BetP (Rübenhagen et al. 2000, 2001). Both EctM and BetM lack extensive cytoplasmic termini. However, similar to BetP and OpuD, both EctM and BetM posses a carboxy terminus with a majority of charged amino acids. Like BetP and OpuD, the carboxy end of BetM consists mainly of positively charged amino acids, while the terminus of EctM has a nearly balanced number of negative (nine) and positive (ten) amino acid residues.

Like all other BCCTs, EctM is characterized by a conserved stretch of amino acid residues, which also contains the so-called signature sequence of BCCTs. However, the signature sequence of EctM is modified by two amino acid replacements at position 3 and at the end of the signature. So far, there are only two other transporters of the BCCT family in Bacteria, namely EctP of *C. glutamicum* (K_m for ectoine = 63 µM) and EctT of *V. pantothenticus* that have modified signature sequences as well. In EctP, the last position of that specific sequence carries a threonine instead threonine of tryptophan. In EctT, the same tryptophan is replaced by serine. It was argued by Kappes et al. (1996) that the conserved stretch might be involved in substrate binding and translocation across the membrane. Since EctM, EctT, and EctP are the only BCCTs accepting ectoine as substrate, it is tempting to speculate whether the observed sequence alterations are the cause for the different substrate specificities of these three carriers.

Both newly identified carriers EctM and BetM from *M. halophilus* led to an enhanced accumulation of their corresponding substrate in *E. coli* after osmotic upshock. Whether BetM and EctM function as osmoregulatory transporters in *M. halophilus* could not be tested because genetic methods for *M. halophilus* are not yet established. However, it was shown for other compatible solute transporters that they basically function in the same way in their parental strain as they do in *E. coli*. Therefore, it can be assumed that EctM and BetM function as osmoregulated transporters in *M. halophilus* as well. Although osmoregulated transporters posses the same basic functions in host cells, it was shown for BetP

of *C. glutamicum* that there are significant differences in the transport characteristics of BetP when expressed as a recombinant protein in *E. coli* MKH13 (Peter et al. 1996). The NaCl concentration in the medium needed for maximal activation of BetP by osmotic shift dropped from 625 mM NaCl in *C. glutamicum* to 200 mM NaCl when embedded in the *E. coli* membrane. While the affinity to the substrate glycine betaine remained similar, the V_{max} of BetP was remarkably lower in *E. coli*, even when *betP* was expressed under the control of a strong promoter. These findings help to explain why EctM accumulated only low amounts of compatible solute when compared to BetM following osmotic upshock in *E. coli*. This could be caused by a different membrane composition in *E. coli*, leading to low activity of the EctM protein or, by low expression of *ectM* under the control of a Gram-positive promoter resulting in insufficient production of protein in a Gram-negative cell. To characterize EctM and BetM in more depth, it is necessary to analyze these carriers and their genes in *M. halophilus* directly. This would also help clarify whether EctM is involved in the recovery of newly synthesized ectoine and hydroxyectoine leaking into the medium, as it was described for TeaABC of *H. elongata*. According to the presented data, EctM is the first BCCT unable to transport trimethylammonium compounds (glycine betaine, carnitine), and it seems to be a specific ectoine and hydroxyectoine transporter. This makes EctM a good candidate as a recovery system for ectoine. Its specificity for one type of substrate and its high affinity for the substrate are characteristics of other salvage systems described so far (Hagemann et al. 1997; Kempf and Bremer 1998; Grammann et al. 2002), both which are apparently required to take over such a role.

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References

Altschul SF, Madden TL, Schäfer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Blohn C von, Kempf B, Kappes RM, Bremer E (1997) Osmostress response in *Bacillus subtilis*: characterization of a proline uptake system (OpuE) regulated by high osmolarity and the alternative transcription factor sigma B. Mol Microbiol 25:175–187

Boscari A, Mandon KM, Dupont L, Poggi M-C, Le Rudulier D (2002) BetS is a major glycine betaine/proline betaine transporter required for early osmotic adjustment in *Sinorhizobium meliloti*. J Bacteriol 184:2654–2663

Bullock WO, Fernandez JM, Short JM (1987) XL1 blue: a high-efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. Biotechniques 5:376–378

Farrow JA, Ash C, Wallbanks S, Collins MD (1992) Phylogenetic analysis of the genera *Planococcus*, *Marinococcus* and *Sporosarcina* and their relationships to members of the genus *Bacillus*. FEMS Microbiol Lett 72:167–172

Frings E, Kunte HJ, Galinski EA (1993) Compatible solutes in representatives of the genera *Brevibacterium* and *Corynebacterium*: Occurrence of tetrahydropyrimidine and glutamine. FEMS Microbiol Lett 109:25–32

Galinski EA, Herzog RM (1990) The role of trehalose as a substitute for nitrogen-containing compatible solutes (*Ectothiorhodospira halochloris*). Arch Microbiol 153:607–613

Grammann K, Volke A, Kunte HJ (2002) New type of osmoregulated solute transporter identified in halophilic members of the *Bacteria* domain: TRAP transporter TeaABC mediates the uptake of ectoine and hydroxyectoine in *Halomonas elongata* DSM 2581^T. J Bacteriol 184:3078–3085

Haardt M, Kempf B, Faatz E, Bremer E (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

Hagemann M, Richter S, Mikkat S (1997) The *ggtA* gene encodes a subunit of the transport system for the osmoprotective compound glucosylglycerol in *Synechocystis* sp. strain PCC 6803. J Bacteriol 179:714–720

Hirokawa T, Boon-Chieng S, Mitaku S (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics 414:378–379

Kappes RM, Kempf B, Bremer E (1996) Three transport systems for the osmoprotectant glycine betaine operate in *Bacillus subtilis*: characterization of OpuD. J Bacteriol 178:5071–5079

Kempf B, Bremer E (1998) Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. Arch Microbiol 170:319–330

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105–132

Larsen PI, Sydne LK, Landfald B, Strøm AR (1987) Osmoregulation in *Escherichia coli* by accumulation of organic osmolytes: betaines, glutamic acid, and trehalose. Arch Microbiol 147:1–7

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

MacMillan SV, Alexander DA, Culham DE, Kunte HJ, Marshall EV, Rochon D, Wood JM (1999) The ion coupling and organic substrate specificities of osmoregulatory transporter ProP in *Escherichia coli*. Biochim Biophys Acta 1420:30–44

Novitsky TJ, Kushner DJ (1976) *Planococcus halophilus* sp. nov., a faculatively halophilic coccus. Int J Syst Bacteriol 26:53–57

Oren A (1999) Bioenergetic aspects of halophilism. Microbiol Mol Biol Rev 63:334–348

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85:2444–2448

Peter H, Burkovski A, Krämer R (1996) Isolation, characterization, and expression of the *Corynebacterium glutamicum betP* gene, encoding the transport system for the compatible solute glycine betaine. J Bacteriol 178:5229–5234

Peter H, Weil B, Burkovski A, Krämer R, Morbach S (1998) *Corynebacterium glutamicum* is equipped with four secondary carriers for compatible solutes: identification, sequencing, and characterization of the proline/ectoine uptake system, ProP, and the ectoine/proline/glycine betaine carrier, EctP. J Bacteriol 180:6005–6012

Racher KI, Voegele RT, Marshall EV, Culham DE, Wood JM, Jung H, Bacon M, Cairns MT, Ferguson SM, Liang W-J, Henderson PJF, White G, Hallett FR (1999) Purification and reconstituation of an osmosensor: transporter ProP of *Escherichia coli* senses and responds to osmotic shifts. Biochemistry 39:1676–1684

Rübenhagen R, Rönsch H, Jung H, Krämer R, Morbach S (2000) Osmosensor and osmoregulator properties of the betaine carrier BetP from *Corynebacterium glutamicum* in proteoliposomes. J Biol Chem 275:735–741

Rübenhagen R, Morbach S, Krämer R (2001) The osmoreactive betaine carrier BetP from *Corynebacterium glutamicum* is a sensor for cytoplasmic K^+. EMBO J 20:5412–5420

Saier MH Jr (1994) Computer-aided analysis of transport protein sequences: gleaning evidence concerning function, structure, biogenesis, and evolution. Microbiol Rev 58:71–93

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Severin J, Wohlfarth A, Galinski E A (1992) The predominant role of the recently discovered tetrahydropyrimidines for the osmoadaptation of halophilic eubacteria. J Gen Microbiol 138:1629–1638

Sleator RD, Gahan CG, Abee T, Hill C (1999) Identification and disruption of BetL, a secondary glycine betaine transport system linked to the salt tolerance of *Listeria monocytogenes* LO28. Appl Environ Microbiol 65:2078–2083

Takami H, Nakasone K, Takaki Y, Maeno G, Sasaki Y, Masui N, Fuji F, Hirama C, Nakamura Y, Ogasawara N, Kuhara S, Horikoshi K (2000) Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. Nucleic Acids Res 28:4317–4331

Takeshita S, Sato M, Tabo M, Massahashi W, Hashimoto-Gotoh T (1987) High-copy-number and low-copy-number plasmid vectors for *lacZ* α-complementation and chloramphenicol- or kanamycin-resistance selection. Gene 61:63–74

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Tusnády GE, Simon I (1998) Principles governing amino acid composition of integral membrane proteins: application to topology prediction. J Mol Biol 283:489–506

Tusnády GE, Simon I (2001) The HMMTOP transmembrane topology prediction server. Bioinformatics 17:849–850

Zucker M (2003) Mfold Web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31:1–10