Cloning and characterization of the *Halobacillus trueperi* betH gene, encoding the transport system for the compatible solute glycine betaine

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Abstract

*Halobacillus trueperi* accumulates glycine betaine under condition of high osmolarity. A fragment of the glycine betaine transporter betH gene was obtained from the genome of *H. trueperi* with degenerate primers. Through Southern blot hybridization and inverse PCR, a 5.1 kb EcoRI fragment containing the complete betH gene was identified and subsequently sequenced. The betH gene was predicted to encode a 55.2 kDa protein (504 amino acid residues) with 12 transmembrane regions. BetH showed 56% identity to the OpuD of *Bacillus subtilis* which belongs to the betaine/carnitine/choline transporter (BCCT) family. Its putative promoter region was highly homologous to theβ-dependent promoter of *B. subtilis*. A 2.6 kb fragment containing the betH gene was cloned into pUC18 and transformed into the *Escherichia coli* MKH13. The accumulation of glycine betaine in transformed *E. coli* MKH13 bacteria was confirmed using 13C nuclear magnetic resonance spectroscopy.

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1. Introduction

Moderately halophilic bacteria which grow optimally between 0.5 and 2.5 M salt constitute a very heterogeneous physiological group containing bacteria from several different genera [1]. When growing in hypersaline environments, moderately halophilic bacteria do not possess a highly saline cytoplasm and salt-requiring proteins as can be found in some archaea. Most halophilic bacteria control cytoplasmic osmolarity by accumulating a limited number of metabolically inert, organic compounds (named compatible solutes) when extracellular osmolarity rises, and rapidly release these solutes into the environment when extracellular osmolarity declines [2]. Intracellular accumulation of compatible solutes in bacterial cells can be accomplished through biosynthesis and/or transport from the environment [2,3]. For energetic reasons, halophilic bacteria prefer uptake of the compatible solutes to *de novo* biosynthesis [4].

The mechanism of osmosensing and osmoregulation has been most intensively studied in *Escherichia coli*, *Salmonella typhimurium* [5], *Bacillus subtilis* [6], *Listeria monocytogenes* [7], *Sinorhizobium meliloti* [8] and Gram-negative moderately halophilic bacteria such as *Halomonas elongata* (DSM 2581T) [9,10] and *Chromohalobacter salexigens* DSM 3043 (formerly classified as a strain of *H. elongata*) [11–14]. In contrast, the situation in Gram-positive moderately halophilic bacteria, especially in spore-forming ones, is not as well investigated. A physiological study of *Halobacillus halophilus* revealed the existence of a chloride-dependent glycine betaine transporter [15], but its genetic background remains to be elucidated. It can be imagined that spore-forming bacteria can escape the environmental stress by...
producing a highly desiccation-resistant endospore. However, high osmolarity actually inhibits spore formation by impeding the signal transduction cascade that activates a number of transcription factors controlling the sporulation process [16].

The genome of Oceanobacillus iheyensis HTE831, an alkaliphilic and extremely halotolerant Bacillus-related species isolated from deep-sea sediment, has recently been published [17]. Comparative analysis of this genome with that of the neutrophilic and halotolerant Bacillus subtilis showed that there were no great differences in the genes of the glycine betaine and choline ABC (ATP binding cassette) transport systems; however, a difference was found in the glycine betaine secondary transporter genes [18]. This result led us to focus our research on the glycine betaine transporter in spore-forming moderately halophilic bacteria. Halobacillus trueperi is a spore-forming moderately halophilic, aerobic and heterotrophic bacterium, which was isolated from hypersaline sediments of the Great Salt Lake in Utah [19]. Glycine betaine ($N,N,N$-trimethylglycine) and glutamate are the predominant compatible solutes in H. trueperi grown on SWYE [20] (10% total salt) media (data not shown). In this work, we used H. trueperi (DSM 10404$^T$) as a model to study the mechanism of osmoregulation for glycine betaine.

2. Materials and methods

2.1. Bacterial strains and growth condition

Halobacillus trueperi (DSM 10404$^T$) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and grown on SWYE (10% total salts) medium or DSMZ medium 755 (http://www.dsmz.de/media/med755.htm) at 30°C. E. coli MKH13 [21] was grown at 37°C either in Luria–Bertani medium or in M9 minimal medium containing 0.5% d-glucose, 0.04% arginine, 0.04% isoleucine and 0.04% valine [22]. E. coli DH5$\alpha$ used for cloning experiments was maintained on LB agar and grown at 37°C. Plasmid pUC18 and pGEM-T easy vectors (Promega, Madison, USA) were used in cloning and sequencing experiments.

2.2. DNA manipulations and sequence analysis

Nucleic acid manipulations and general cloning procedures were performed with standard methods [22]. Competent cells of E. coli DH5$\alpha$ were prepared with the method described by Inoue et al. [23]. The PCR products were purified with the FZ Spin column DNA gel extraction kit (Bio Basic, Canada). Restriction enzymes, DNA Blunting Kit, T4 DNA ligase and Takara Ex Taq$^\text{TM}$ for PCR amplification were purchased from TaKaRa (Dalian, China). Glycine betaine was purchased from Sigma, USA. Synthesis of oligonucleotides and DNA sequencing were conducted at Sangon Biotech. (Sangon, Shanghai, China). Databank searches were performed through the National Center for Biotechnology Information (NCBI) using the BLAST program [24].

2.3. Probe construction and labeling

To isolate a fragment of the glycine betaine transporter gene from H. trueperi, a PCR strategy with degenerate primers was designed. Forward primer (trans1): 5′-GCDGGWATGGGDATWGGYTHGT-3′, and reverse primer (trans2): 5′-GCTGARTCHGCGWGANGTRATAAGAAG-3′ were used for PCR with chromosomal DNA of H. trueperi as a template. The reaction was conducted using the following conditions: denaturation 94°C for 30 s, annealing 60°C for 1 min, extension 72°C for 1 min, 30 cycles. The PCR products were cloned into the pGEM-T easy vector and sequenced. The recombinant plasmid was digested completely with EcoRI, then the DNA fragments of about 1 kb were purified and labeled as a probe to detect the entire betH gene in H. trueperi. Labeling of the fragments was performed with Dig high prime DNA labeling and detection starter kit I (Roche Diagnostic GmbH, Mannheim, Germany) as recommended by the manufacturer.

2.4. Southern blot hybridization

Total DNA of H. trueperi was digested completely with BamHI, EcoRI and HindIII, and the resulting fragments were fractionated by electrophoresis in 0.8% agarose gels. DNA fragments were transferred to a positive-charged nylon membrane (Roche Molecular Biochemicals, Mannheim, Germany), and then hybridized with the above-mentioned probe. Southern hybridization and detection were performed according to standard techniques [22], using the Dig high prime DNA labeling and detection starter kit I as described above.

2.5. Inverse PCR (IPCR)

IPCR was performed essentially as described in Ochman et al. [25]. Two pairs of primers were used in IPCR: the internal pair of primers (91R1-up: 5′-ACC-GAATCCAGGGGTCGTCG-3′, 91R1-down: 5′-TGTTGGAATTCATTTATTTGCTG-3′) and the external pair of primers (91R2-up: 5′-ACACGGCGATGACA-TCGACG-3′, 91R2-down: 5′-GGGACATTCTTTATTTATCACTACGACG-3′) were used for the first and nested PCR respectively with chromosomal DNA of H. trueperi as a template. The fragment generated by IPCR was cloned into the pGEM-T easy vector and sequenced.
2.6. Complementation of E. coli MKH13

The forward primer (5'-CGCGGATCCAAAGGTTGTCGGTAGG-3'; BamHI site is underlined) and the reverse primer (5'-CCGGAATTCACCTGAAATCCAATCCTTTG-3'; EcoRI site is underlined) were used for PCR with chromosomal DNA of H. trueperi as a template to obtain a 2.6 kb fragment. The amplified DNA was purified and digested with BamHI and EcoRI, then ligated into pUC18 also digested with BamHI and EcoRI. The ligation products were transformed into the E. coli MKH13 and the transformants were grown on the selective M9 minimal medium containing 0.8 M NaCl, 5 mM glycine betaine, 100 mg ampicillin per liter, D-glucose, 0.04% arginine, 0.04% isoleucine and 0.04% valine [26]. One of the clones was picked up and the extracted plasmid, designated as pUH25, was sequenced. As a control the plasmids pUH26, pUH27 and pUH28 were constructed by deletion of the defined restriction fragments from pUH25 and religation after the creation of blunt ends with a DNA Blunting Kit (TaKaRa).

2.7. Extraction and determination of intracellular solutes by 13C NMR spectroscopy

Compatible solutes of E. coli MKH13 growing in the selective M9 minimal medium were extracted twice with boiling 80% ethanol described by Reed et al. [27], and 4 μl of dimethyl sulfoxide (DMSO) was added as internal standard. 13C nuclear magnetic resonance (NMR) spectra were recorded with a Bruker Advance DPX 300MHz spectrometer. Chemical shifts were referenced to the resonance of external DMSO, designated at 40.6 ppm. The identity of osmolytes was determined by comparison with known standards.

2.8. Nucleotide sequence accession number

The nucleotide sequence data reported in this paper has been submitted to GenBank and assigned Accession No. AY496459.

3. Results and discussion

3.1. Cloning of the betH gene from H. trueperi by IPCR

To design the degenerate primers, the amino acid sequences of glycine betaine transporters from OpuD of Bacillus subtilis (Accession No. GI 1524397), BetL of L. monocytogenes (Accession No. GI 11279740), the putative glycine betaine transporter of O. iheyensis (Accession No. GI 22776940) and Bacillus halodurans (Accession No. GI 10175266) were aligned to identify well-conserved amino acid residues. Two stretches of amino acids, AGMGIGLVF (N-terminus) and FFVTSSSADSA (C-terminus) were chosen to synthesize degenerate primers called tran1 and trans2. PCR amplification with these primers using total genomic DNA of H. trueperi as the template resulted in a 970-bp amplified fragment. Analysis of the translated sequence showed great similarity to OpuD of B. subtilis (58% identity), BetL of L. monocytogenes (57% identity) and the putative glycine betaine transporter of O. iheyensis (53% identity), so we named its entire ORF as betH gene. In order to obtain the entire ORF of betH gene, Southern hybridization experiments were carried out and the betH gene was detected on a 5.1 kb EcoRI fragment (Fig. 1, lane 3). Two sets of primers derived from the 970-bp PCR product were used to amplify the entire ORF of betH from the 5.1 kb fragment by IPCR technique. The strategy for IPCR was shown in Fig. 2.

3.2. Sequence analysis of the 5.1 kb fragment

As shown in Fig. 2, protein sequence analysis of the 5.1 kb EcoRI fragment showed the presence of four ORFs: BetH (1512 bp) showed high level sequence similarity with other glycine betaine secondary transporters: OpuD of B. subtilis (56% identity), BetL of L. monocytogenes (55% identity), the putative glycine betaine
secondary transporter of *O. iheyensis* (54% identity), and BetM of *Marinococcus halophilus* (Accession No. GI 34398345) (44% identity). All possessed 12 putative transmembrane α-helical spanners. Alignment of amino acid sequences was performed with Clustal W 1.74 (http://www.ch.embnet.org/software/ClustalW. html#) and shown in Fig. 3. ORF1 (534 bp), was predicted to encode an acetyltransferase belonging to GNAT family and closely related to a conserved hypothetical protein from *Thermotoga maritima* (35% identity). ORF3 (804 bp) was predicted to encode a membrane protein showing a high level of amino acid sequence homology to a conserved protein from *O. iheyensis* (31% identity), with unknown function. ORF4 (651 bp) showed high level sequence similarity with a pyridine nucleotide-disulfide oxidoreductase family protein of *Geobacter sulfurreducens* (30% identity).

### 3.3. Features of the betH-encoded gene product

The *betH* gene encoded a 504-residue protein (BetH) with a calculated molecular mass of 55.2 kDa. A hydrophobicity plot [28,29] of BetH revealed an alteration of hydrophobic and hydrophilic segments characteristic for integral membrane proteins, and suggested the presence of 12 transmembrane-spanning segments (Fig. 3). It belongs to the BCCT (betaine/carnitine/choline transporter) family (TC 2.A.15) [30]. A common functional feature of this family is the uptake of quaternary ammonium compounds coupled to the proton motive force. Based on homology data, an ATG start codon was identified as the most likely initiation codon (Fig. 2). Upstream of this ATG start codon, a putative ribosome binding site (AAGGAG) was detected at the position of 7 bp upstream of the start codon, a distance reported to be optimal for efficient translation in *B. subtilis* and *E. coli*. Potential −10 and −35 regions (GGGTAT [14 nt] ATTGTATA) have considerable homology with the *B. subtilis* σB-dependent consensus promoter (GGGTAT [14 nt] RGGXTRTA) [31]. It has been shown that σB participates in the general stress response, and that σB-dependent transcription is activated when *B. subtilis* is subjected to environmental stress such as high salt, O2 limitation and heat shock, σB is released from RsbW (an anti-σ factor protein) under this condition [31]. The presence of putative σB-dependent promoter binding sites suggests that BetH-mediated uptake of glycine betaine may be regulated in *H. trueperi* by a σB-like factor at the level of transcription. In contrast, the *opaD* gene of *B. subtilis* does not possess a σB-dependent promoter element like that of *betH* in *H. trueperi*. A long inverted repeat (ΔG [25 °C] = −28.84 kJ) which could form a stem-loop structure was identified at the position of 4 bp downstream from the stop codon of *betH* gene. It probably functions as a rho-dependent transcriptional terminator.

### 3.4. Functional complementation of *E. coli* **MKH13**

Since *E. coli* MKH13 lacks the transport systems *PutP*, *ProP* and *ProU*, it is unable to grow in high-osmolality (0.8 M NaCl) minimal medium containing glycine betaine. Plasmid pUH25 contains a 2.6 kb fragment with the whole ORF of the *betH* gene, 995 bp upstream of its 5' end and 150 bp downstream of its 3' end. After pUH25 was transformed into the *E. coli* MKH13, colonies appeared on the high-osmolarity minimal medium after 48 h at 37 °C. *E. coli* MKH13 harboring pUC18, pUH26, pUH27 or pUH28 did not grow on this medium (Fig. 4). Since no colonies appeared for the control experiment, uptake of glycine betaine could be considered due to the 2.6 kb fragment on pUH25. Given that the cloned gene was expressed, it was assumed that BetH was expressed under the control of its putative σB-dependent promoter or another still unknown promoter in *E. coli*. A similar phenomenon has also been found for BetL of *L. monocytogenes* [32].
Fig. 3. Alignment of the predicted amino acid sequence of *H. trueperi* BetH with OpuD from *B. subtilis*, BetL from *L. monocytogenes*, the putative glycine betaine transporter from *O. iheyensis*, and BetM from *M. halophilus*. Identical amino acids in all aligned proteins are indicated by asterisks. The positions of 12 predicted transmembrane helices are underlined.

Fig. 4. Map of the sequenced 2.6 kb DNA fragment of *H. trueperi* (pUH25) and deletion derivatives. pUH26, pUH27 and pUH28 were digestion of pUH25 with restriction enzymes as indicated and religation after the creation of blunt ends with the DNA Blunting Kit (TaKaRa). Numbers indicated the terminal positions (bp) of the subclones relative to pUH25. Osmoprotection by glycine betaine was assayed by monitoring the growth of *E. coli* MKH13 harboring the pUH25-derived plasmids on high-osmolarity minimal plates containing 0.8 M NaCl and 5 mM glycine betaine. Growth of the strains was scored after 3 days of incubation at 37 °C (+, growth; −, no growth).

Osmoprotection by glycine betaine

<table>
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<tr>
<th>Plasmid</th>
<th>Band (bp)</th>
<th>HpaI</th>
<th>NdeI</th>
<th>NcoI</th>
<th>EcoRI</th>
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3.5. Accumulation of osmolytes in *E. coli* MKH13

NMR has been shown to be a valuable technique for the rapid detection of compatible solutes [33]. The ethanol-soluble extract of MKH13 (pUH25) was examined by $^{13}$C NMR for the presence of compatible solutes (Fig. 5). Glycine betaine (resonances at 53.5, 66.4 and 169.3 ppm) and glutamate (resonances at 27.0, 33.5, 54.7, 174.6 and 181.2 ppm) were readily identified in the NMR spectra by comparison with authentic samples. This result showed that the accumulation of glycine betaine in cytosol of *E. coli* MKH13 was due to transforming of plasmid pUH25.

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