

## Cloning and Characterization of the Genes Encoding a Glycine Betaine ABC-Type Transporter in *Halobacillus trueperi* DSM10404<sup>T</sup>

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**Abstract.** By using Southern blot hybridization and inverse polymerase chain reaction, a 5.5-kb DNA fragment was obtained from the genomic DNA of *Halobacillus trueperi* DSM10404<sup>T</sup>. Sequence analysis revealed that it contained a potential operon with high levels of sequence similarity to the *opuA* operon encoding glycine betaine transporter from *Bacillus subtilis*, which is a member of the ATP-binding cassette (ABC) substrate binding the protein-dependent transporter superfamily. The potential operon, designated as *qatA* (quaternary amine transporter), consists of three structural genes, which are predicted to encode an ATP-binding protein (QatAA), a membrane-associated protein (QatAB), and an extra-cellular substrate-binding protein (QatAC). Moreover, the putative promoter region of the operon was found with close homology to the  $\sigma^A$ -dependent promoter of *B. subtilis*. Reverse transcription (RT)–PCR analysis revealed that *qatAA*, *qatAB*, and *qatAC* genes were transcribed in cells of *H. trueperi*. Cells of *Escherichia coli* mutant MKH13 harboring *qatA* on pAY41 were able to grow on selective M9 salt medium containing glycine betaine and accumulated glycine betaine in the cytoplasm, showing that *qatAA*, *qatAB*, and *qatAC* genes together encode a functional glycine betaine transporter.

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 [19, 22]. During adaptation to salt stress, most of eubacteria and several archaeobacteria establish an osmotic equilibrium with the external environment by accumulating osmoprotective organic compounds called compatible solutes [3]. Glycine betaine, one kind of compatible solute, is accumulated by these prokaryotes in proportion to the salinity of the medium, which implies that this compound plays a major role in osmoregulation [17]. Most of the heterotrophic bacteria cannot synthesize glycine betaine *de novo*, but can

accumulate it from the growth medium by uptake through secondary transport systems and/or ATP-binding cassette (ABC) transport systems or synthesis from its precursor choline by oxidation [3].

*Halobacillus trueperi* DSM10404<sup>T</sup> (hereafter *H. trueperi*) is a spore-forming, Gram-positive moderately halophilic bacterium, which can grow in medium with salt concentrations up to 25% [13], and accumulates glycine betaine as its major compatible solute under this condition (data not shown). A secondary glycine betaine transport system, *betH* gene, in *H. trueperi* was characterized, suggesting that the transcription of *betH* might be regulated by a  $\sigma^B$ -like factor [14]. The osmoregulated ABC transport systems for quaternary compounds had been found in many Gram-positive and Gram-negative bacteria, including *Escherichia coli* (ProU) [4], *Bacillus subtilis* (OpuA) [10], and *Listeria monocytogenes* (Gbu) [11], among others. These transporters belong to the superfamily of ATP-dependent transport systems, consisting of three different protein

subunits: the ATP-binding component, the integral membrane component, and the periplasmic binding component [10]. The former two components form a membrane complex, and in Gram-negative bacteria, the substrate-binding protein resides in the periplasm as a soluble protein, whereas it is tethered to the cytoplasmic membrane in Gram-positive bacteria [10]. They constitute the main system to protect these organisms against hyperosmotic stress. Interestingly, no ABC transport system for quaternary ammonium compounds in the moderately halophilic bacteria has been reported until now. To gain a deeper insight into compatible solute uptake in halophilic bacteria, which accumulate glycine betaine as their main osmolyte, the genes encoding an ABC glycine betaine transporter in *H. trueperi* were cloned and characterized in this study.

## Materials and Methods

**Bacterial Strains and Culture Conditions.** *Halobacillus trueperi* DSM10404<sup>T</sup> from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) was grown at 37°C in SWYE medium and *E. coli* DH5 $\alpha$  was grown at 37°C in Luria–Bertani medium. The antibiotics used were 50  $\mu$ g/mL ampicillin and 50  $\mu$ g/mL kanamycin. pGEM-T easy vector (Promega, Madison, WI, USA) was used as a general vector for cloning and sequencing.

**DNA Manipulation and Materials.** The genomic DNA from *H. trueperi* was extracted as previously described by Spring et al. [20]. The general recombinant DNA manipulation was performed by standard procedures [18]. Restriction endonucleases, DNA Blunting Kit, TaKaRa RNA PCR kit (AMV, ver. 3.0) (TaKaRa), T<sub>4</sub> DNA ligase, and *Pyrobest* DNA polymerase were purchased from TaKaRa Dalian Co. and New England Biolabs Inc. The synthesis of oligonucleotide primers and DNA sequencing were conducted at Sangon Biotech. Carnitine, glycine betaine, and ectoine were purchased from Sigma (St. Louis, MO). All other chemicals were of the highest purity commercially available.

**Cloning and Sequencing.** To isolate a fragment of the glycine betaine ABC transporter genes from *H. trueperi*, a polymerase chain reaction (PCR) strategy with degenerate primers was used. Two stretches of amino acids, GEIFVIMGL (N-terminus) and HDLDEALRL (C-terminus), based on the well-conserved regions of ATPase were chosen to synthesize the degenerate primers. A 539-bp amplicon with primers AA-up (5'-ggwgarathtygtbatwggdct-3') and AA-down (5'-awvcg harbgyctertccaartcatg-3') was obtained and sequenced. Similarity between the deduced amino acid sequence of this fragment and that present in databases showed that it was homologous to the *opuAA* gene of *B. subtilis*. To obtain the nucleotide sequence flanking the DNA fragment, inverse PCR (IPCR) was performed. The genomic DNA was completely digested by *EcoRI*, purified and self-ligated with T<sub>4</sub> DNA ligase at 16°C for 48 h. Then the circular fragments were used as templates and two sets of primers [up-144 (5'-actctgagcagcagcttcc-3') and down345 (5'-gatgcaacagcgtgtggtc-3'), up-111 (5'-gctgtccatcttag ccaaatcc-3') and down370 (5'-cgagccctgcaagatcc-3')] derived from the nucleotide sequence of the PCR products were used for the first and nested IPCR, respectively.

Another degenerate primer AC-down (5'-accwgcmcwgytcrath ccwgrat-3'), deduced from the well-conserved region (ITGIEPEAG) of glycine betaine-binding proteins for glycine betaine ABC transporter was designed. Primers AA-up and AC-down were used for PCR with genomic DNA of *H. trueperi* as a template. In the following IPCR experiment, *AflIII* was chosen to digest the genome of *H. trueperi* completely, and IPCR was performed as described earlier to obtain the nucleotide sequence flanking the DNA fragment. Primers [R1-up (5'-tggacttaccctctgctgatctcg-3') and R1-down (5'-cggtatggtccagggtgtt tgc-3'), R2-up (5'-gaagtggcagctcgcgcaaatgg-3') and R2-down (5'-tcggaactggttctggtcagtc-3')] were chosen for the first and nested IPCR, respectively. The 3.2-kb IPCR product was cloned and sequenced. To confirm that the assembled sequence was the same as on the chromosome of *H. trueperi*, PCR analysis with primers ABC-up (5'-ttcccgggttccgctgttgcattc ctct-3', *SmaI* site is underlined) and ABC-down (5'-aaccgggaagtggcagctcgcgcaaatg-3', *SmaI* site is underlined) using genomic DNA from *H. trueperi* as template amplified a 5525-bp fragment, which was digested by *SmaI* and cloned into the *SmaI* site of the pACYC177 vector to generate pAY55 and sequenced.

**Probe Labeling and Southern Blot Analysis.** A 2.1-kb DNA fragment from *H. trueperi* was amplified by PCR with degenerate primers AA-up and AC-down, purified on agarose gels, and labeled with digoxigenin-dUTP using Dig high prime DNA labeling and the detection starter kit I (Roche, Mannheim, Germany). For Southern blot analysis, *H. trueperi* genomic DNA was completely digested with six different restriction enzymes (*AflIII*, *ApaI*, *BglII*, *EcoRV*, *MluI*, and *SphI*), respectively. After electrophoresis, it was transferred onto a positive-charged membrane (Amersham) under vacuum and hybridized with the probe. The hybridization was carried out at 38°C overnight. Detection was carried out by color detection using an anti-digoxigenin-alkaline phosphatase conjugate and nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) according to the manufacturer's instructions.

**Functional Complementation of the Mutant *E. coli* MKH13.** To determine which part of the 5.5-kb DNA fragment from pAY55 is coding for the potential transporter mediating the uptake of glycine betaine in *E. coli* MKH13 [5], a number of deletion derivatives of pAY55 (Fig. 5) were constructed. Plasmid pAY55 was hydrolyzed by restriction enzymes *KpnI*, *BclI*, *XcmI*, *BsrBI* and *ApaI*, respectively, and relegated after the creation of blunt ends with the DNA Blunting Kit (TaKaRa). Plasmids pAY52 and pAY49 derived from pAY55 were constructed by *SmaI* digestion, followed by either *ApaI* (pAY52) or *BsrBI* (pAY49). Each of these plasmids was introduced into *E. coli* MKH13 by transformation. The transformants were selected on selective M9 minimal medium plates containing 100  $\mu$ g/mL ampicillin. The ability of the *qatA* encoding transporter to promote growth of *E. coli* MKH13 at elevated salt concentrations was tested on M9 mineral salt medium containing 0.8 M NaCl and 5 mM glycine betaine.

**Extraction of Intracellular Solutes and <sup>13</sup>C NMR Analysis.** *Escherichia coli* MKH13 transformed with pAY41 was grown at 37°C on the selective M9 minimal medium. Cells were harvested by centrifugation and extracted twice with 80% ethanol [16]. Freeze-dried extracts were dissolved in 0.5 mL D<sub>2</sub>O and analyzed with a Bruker Advance DPX 300MHz [14].

**RNA Isolation and RT-PCR.** Exponentially growing cells of *H. trueperi* were harvested by centrifugation (4°C, at 10,000g for 6 min), and the pellet was stored in liquid nitrogen. Total RNA was prepared with the Trizol reagent (Invitrogen) according to the manufacturer's

instruction, and RNA was dissolved in diethylpyrocarbonate (DEPC)-treated H<sub>2</sub>O. Residual DNA was removed from the RNA preparation by DNase I (Promega, China) digestion, followed by a phenol-acid extraction (at 65°C). A TaKaRa RNA PCR kit was used for reverse transcription (RT)-PCR. Primers Rta1 (5'-aatcgacactcgtccggtg-3') and Rta2 (5'-ctcggcacatccgtacgaag-3'), Rtb1 (5'-ttgccaaccaggagttatggg-3') and Rtb2 (5'-agcacgctgaagtcggtaa-3'), Rtc1 (5'-ggtgatgagaccgcttcgaa-3') and Rtc2 (5'-ccattccgtcagcttcacca-3') were designed from the *qatAA*, *qatAB*, and *qatAC* sequences. 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<sub>2</sub>O.

**Computer Analysis.** The sequences were assembled and analyzed using the DNAMAN version 3.0 software (Lynnon BioSoft). Predicted amino acid sequences homology searches were carried out at the National Center for Biotechnology Information by using the BLAST programs (<http://www.ncbi.nlm.nih.gov/BLAST>) [1]. Protein alignments were performed by using the Clustal W program (<http://www.ch.embnet.org/software/ClustalW.html#>). Potential transmembrane spanning fragments were identified by using the SOSUI program (<http://sosui.proteome.bio.tuat.ac.jp/sosui/frame0.html>) [8]. Free energies of the stem-loop structures were calculated using the Mfold program (<http://www.bioinfo.rpi.edu/applications/mfold>).

**Nucleotide Sequence Accession Number.** The nucleotide sequences of *H. trueperi qatAA*, *qatAB*, and *qatAC* and its flanking sequences have been deposited in GenBank under No. AY786324.

## Results and Discussion

**Cloning Genes Encoding Glycine Betaine ABC Transporter from *H. trueperi* 10404<sup>T</sup>.** By using degenerated primers AA-up and AA-down, a 539-bp DNA fragment encoding for a putative ATPase showing amino acid similarity with OpuAA from *B. subtilis* was obtained. A 2144-bp amplicon flanking the fragment was obtained by IPCR. After cloning and sequencing, the amino acid sequence deduced from the open reading frame (ORF) present in the fragment showed amino acid homology with the N-terminal and C-terminal sequences of OpuAA and with the N-terminal sequence of OpuAB from *B. subtilis*. Using primers AA-up and AC-down generated a second, somewhat larger PCR fragment of 2196 bp. The deduced amino acid sequence of the 2196-bp fragment showed significant similarity to the C-terminal sequence of OpuAA, the whole OpuAB, and the N-terminal sequence of OpuAC from *B. subtilis*. To obtain the complete sequence of genes encoding the putative glycine betaine ABC transporter QatA, a Southern hybridization experiment using the 2196-bp fragment as probe and another IPCR experiment were performed. Southern analysis indicated that the 3' end of the genes was located on a 5.3-kb *Afl*III fragment (Fig. 1). In all of the samples digested with restriction enzymes, only one strong band was observed, indicating that the chromosome of *H. trueperi* harbors only one copy of the *qat* gene cluster. The still missing sequences

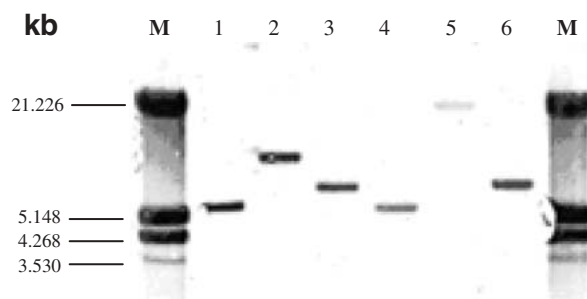


Fig. 1. Southern hybridization of a DIG-labeled 2196-bp PCR fragment to *Afl*III (1), *Apa*I (2), *Bgl*III (3), *Eco*RV (4), *Mlu*I (5), and *Sph*I (6) digests of genomic DNA from *H. trueperi* genomic. M:  $\lambda$ DNA/*Eco*RI+*Hind*III.

of the *qat* gene cluster were amplified by IPCR. The resulting 3.2-kb IPCR product was analyzed and the deduced amino acid sequence showed significant similarities to the N-terminal sequence of OpuAB and the C-terminal sequence of OpuAC from *B. subtilis*. An overview of the cloning strategy is shown in Fig. 2.

**Analysis of the 5.5-kb Fragment from *H. trueperi* 10404<sup>T</sup>.** Inspection of the sequenced 5.5-kb DNA fragment revealed the presence of four complete ORFs and one incomplete ORF. ORF2, ORF3, and ORF4 are each preceded at an appropriate distance by a putative ribosome-binding site and are in the same orientation, whereas ORF1 and ORF5 are divergent from the others (Fig. 2). The deduced amino acid sequences ORF2, ORF3, and ORF4 were subjected to BLASTP searches and showed similarity to the amino acid sequence of two known glycine betaine ABC transporters: OpuA from *B. subtilis* [10] and ProU from *E. coli* [4]. Both are glycine betaine transport systems belonging to the quaternary amine uptake transporter (QAT) family (3.A.1.12) of the ABC superfamily [15]. Therefore, we named the newly discovered ORFs as *qatAA*, *qatAB*, and *qatAC* (quaternary amine transporter), respectively. The end of the *qatAA* gene overlaps the beginning of the *qatAB* gene by 17 bp, and the intergenic distance between *qatAB* and *qatAC* is 70 bp. Interestingly, *proU* from *E. coli* [4], *gbu* from *L. monocytogenes* [11], and *opuA* from *B. subtilis* [12] also show similar overlaps between their first and second ORFs. The intergenic region (247 bp) between *qatAA* and ORF1 is highly A·T rich, which is also found in *gbsAB* genes of *B. subtilis* [2]. Inspection of the DNA sequence upstream of the *qatAA* gene revealed the presence of a potential ribosome-binding site (AGGTGG) and the putative -35 and -10 sequences (TTGAAC[17nt]TTACAT), which showed considerable similarity to the *B. subtilis*  $\sigma^A$ -dependent consensus promoter [6]. In contrast, the

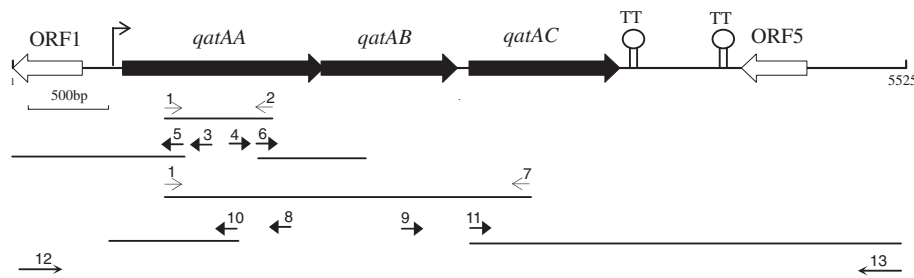


Fig. 2. Schematic diagram of the 5.5-kb cloned genomic DNA fragment containing the *qatA* operon in *H. trueperi*. The locations of the ORFs and their transcription orientation are shown as arrows. The positions for the potential transcriptional terminator are indicated (TT). The potential  $\sigma$ A-dependent promoter is indicated by an angled arrow. Primers used: 1, AA-up; 2, AA-down; 3, up-144; 4, down345; 5, up-111; 6, down370; 7, AC-down; 8, R1-up; 9, R1-down; 10, R2-up; 11, R2-down; 12, ABC-up; 13, ABC-down.

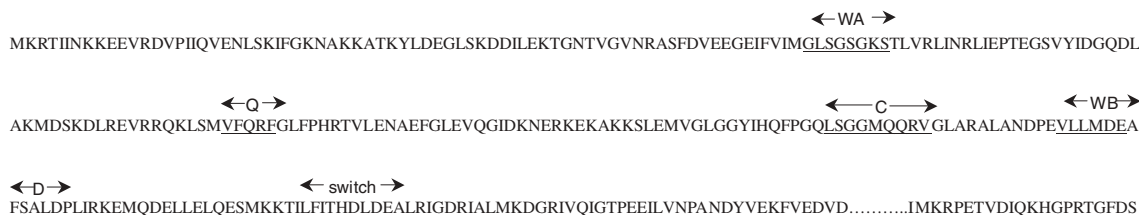


Fig. 3. Conserved sequence motifs of *H. trueperi* QatAA are underlined and arrowed: WA (the Walker A motif), Q (Q-loop), C (signature or C motif), WB (the Walker B motif), D (D-loop), and switch (the switch region).

promoters of *opuA* from *B. subtilis*, *gbu* from *L. monocytogenes*, and *proU* from *E. coli* do not contain consensus sequences recognized by any known sigma factor from Gram-positive and Gram-negative bacteria. Furthermore, an inverted repeat from 9 to 45 bp downstream of the *qatAC* stop codon TAA that potentially could form a stem-loop structure [ $\Delta G(25^\circ\text{C}) = -97.1$  kJ/mol] was found, which might function as a *rho*-independent transcription termination signal, followed by a stretch of five thymidine nucleotides. All of these suggested that the three genes might genetically be closely arranged in an operon.

Divergently oriented with respect to the *qatA* operon is an incomplete ORF (ORF1), which is highly similar (39% identity) to the transcriptional regulator from *Desulfotomaculum reducens* MI-1 (GI:88945602). Another ORF (ORF5) was found downstream of *qatAC*, encoding a potential 135-residue protein, which showed 53% and 41% amino acid identities with the protein of unknown function from *O. iheyensis* (GI:22776833) and *B. subtilis* (GI:2633336), respectively. It was preceded by a potential ribosome-binding site and was followed by a possible stem-loop structure, which was likely to function as a transcriptional terminator (data not shown).

**Features of the *qatA* Genes.** The ORF *qatAA* is predicted to encode a highly hydrophilic protein with a predicted molecular mass of 46 kDa, consisting of 414

amino acid residues. Inspection of the deduced amino acid sequence of *opuAA* revealed 63% and 53% identities to *OpuAA* of *B. subtilis* and *ProV* of *E. coli*, respectively. Both proteins have been proposed to form the ATPase subunit of *OpuA* and *ProU*, respectively. Most of the ATPase of glycine betaine ABC transporters usually contain two conserved sequences: the Walker A motif and the Walker B motif. The Walker A motif is also known as the P-loop and has a consensus sequence of GxxGxGKST, in which x represents any amino acid. The Walker B motif has four aliphatic residues followed by two negatively charged residues, generally aspartate followed by glutamate [21]. The putative QatAA amino acid sequence contained the Walker A and Walker B motifs, the highly conserved sequences of the ATPase subunit of these transporters, providing further evidence that QatAA is the ATPase subunit (Fig. 3). A highly conserved glycine-glutamine-rich sequence L-S-G-G-Q-Q-Q, named the linker peptide, was located between the membrane-spanning helical domain and the nucleotide-binding pocket in ABC transporters [9].

The ORF *qatAB* encodes a 281-residue hydrophobic protein with a predicted molecular mass of 30.78 kDa. The deduced amino acid sequence is similar to *OpuAB* (48% identity) from *B. subtilis* and *ProW* (48% identity) from *E. coli*, which are the transmembrane protein components of their respective transporters. Analysis of the hydrophobic characteristics of the amino acid se-



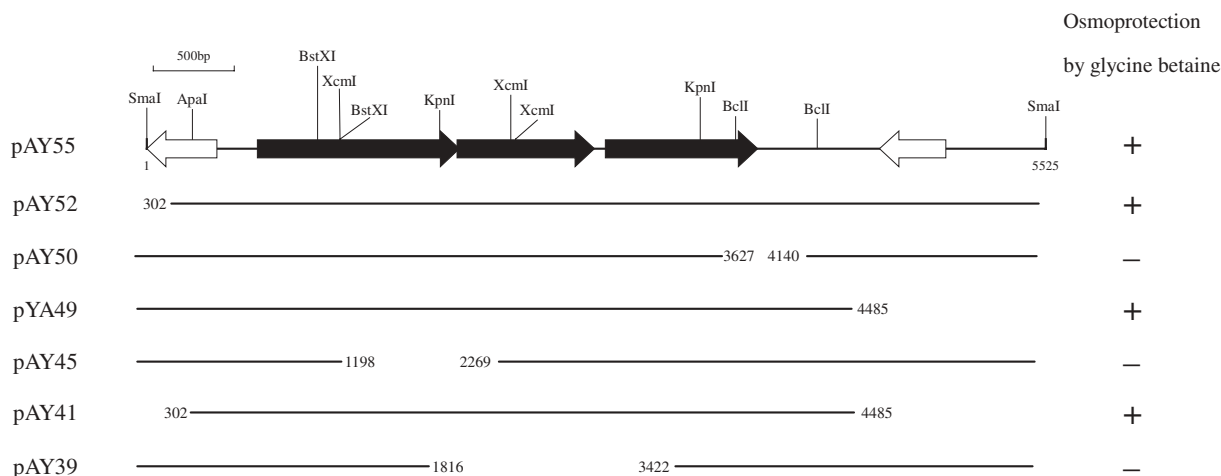


Fig. 5. Genetic map of the 5525-bp DNA fragment from *H. trueperi* (pAY55) and its deletion derivatives. The extent of the DNA segments present in the various deletion derivatives of plasmid pAY55 are indicated by the lines. Numbers indicate the terminal positions (bp) of the subclones relative to pAY55. Osmoprotection by glycine betaine was assayed by monitoring the growth of *E. coli* mutant strain MKH13 with various pAY55-derived plasmids on high-osmolarity minimal plates. Growth of the strains

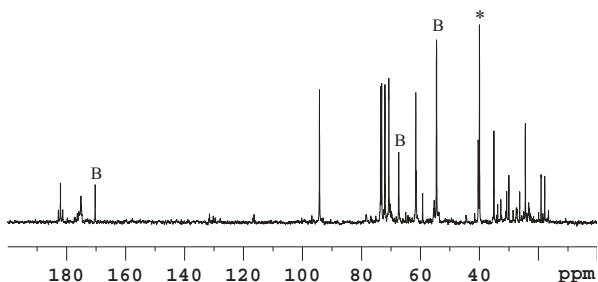


Fig. 6.  $^{13}\text{C}$  NMR spectrum of *E. coli* MKH13 (pAY41) ethanolic cell extracts. Resonances from glycine betaine (B) and the standard dimethyl sulfoxide (\*) were indicated.

cytoplasm of *E. coli* MKH13 harboring the *qat* gene cluster on plasmid pAY41. These results proved that *qatAA*, *qatAB*, and *qatAC* together encode a functional uptake system for glycine betaine.

**RT-PCR.** We had intended to detect *qatA* transcripts by Northern blots, but it was unsuccessful. This might be due to their instability or the low-level transcription *in vivo*. We thus tried to detect these transcripts by reverse PCR. To test whether the *qatA* genes were expressed *in vivo*, total RNA from *H. trueperi* was subjected to RT-PCR. Using primers as described earlier, three of the expected size DNA fragments (bands of 840 bp, 496 bp, and 666 bp for the *qatAA*, *qatAB*, and *qatAC* transcripts, respectively) were amplified (Fig. 7), which proved that the *qatA* genes are transcribed in cells of *H. trueperi*.

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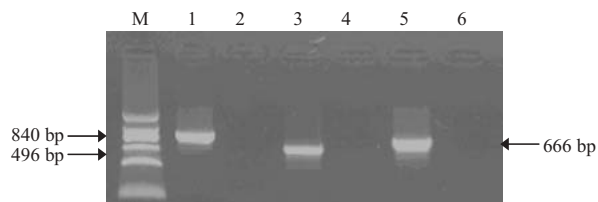


Fig. 7. RT-PCR fragments amplified from total RNA of *H. trueperi* for the *qatAA* transcript with the primers Rta1 and Rta2 (lanes 1 and 2, respectively); the *qatAB* transcript with the primers Rtb1 and Rtb2 (lanes 3 and 4, respectively), and the *qatAC* transcript with the primers Rtc1 and Rtc2 (lanes 5 and 6, respectively). Negative control was performed without reverse transcriptase (lanes 2, 4, and 6). M: DNA molecular marker.

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