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ORIGINAL ARTICLE

A salt-induced *butA* gene of *Tetragenococcus halophilus* confers salt tolerance to *Escherichia coli* by heterologous expression of its dual copies

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Abstract Accumulating glycine betaine through the ButA transport system from an exogenous supply is a survival strategy employed by *Tetragenococcus halophilus*, a moderate halophilic lactic acid bacterium with crucial role in flavor formation of high-salt food fermentation, to achieve cellular protection. In this study, we firstly confirmed that *butA* expression was up-regulated under salt stress conditions by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Subsequently, we discovered that recombinant *Escherichia coli* MKH13 strains with single- and double-copy *butA* complete expression box(es) showed typical growth curves while they differed in their salt adaption and tolerance. Meanwhile, high-performance liquid chromatography (HPLC) experiments confirmed results obtained from growth curves. In summary, our results indicated that regulation of *butA* expression was salt-induced and double-copy *butA* cassettes entrusted a higher ability of salt adaption and tolerance to *E. coli* MKH13, which implied the potential of muti-copies of *butA* gene in the genetic modification of *T. halophilus* for improvement of salt tolerance and better industrial application.

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Introduction

The tetrad-forming, Gram-positive lactic acid bacterium (LAB) *Tetragenococcus halophilus* can grow optimally at a concentration of 1-M salt and can tolerate 26 % saturated NaCl solutions (Gürtler et al. 1998). It is widely used in high salt food fermentation (Collins et al. 1990). As the dominant microorganism in soy sauce fermentation, *T. halophilus* plays an active role in deepening the taste and emphasizing the aroma of soy sauce (Onda et al. 2002, 2003). *T. halophilus* is also associated with other food processes under reduced water activities, such as cured anchovies, where it become the dominant bacterium at the end of the curing process (Villar et al. 1985). This halophilic bacterium also appears as the dominant microorganism during storage of thick sugar juice (Justé et al. 2008). Furthermore, its immune modulating function was also investigated (Masuda et al. 2008).

Soy sauce fermentation is a dynamic process of stresses, including changing salinity, temperature, pH and osmotic pressure. As the major LAB contributing to the formation of soy sauce flavor, *T. halophilus* adjusts to various stresses produced by fermentation, mainly through molecular chaperones (Schlieker et al. 2004; Sugimoto and Sonomoto 2008; Hartl et al. 2011), protease systems (Udomsil et al. 2010) and compatible solutes (Kempf and Bremer 1998; Bhargava and Singh 2006). Compatible solutes are operationally defined as non-ionic, highly water-soluble organic compounds that can be accumulated to extremely high intracellular concentrations without inhibiting overall cellular functions, although they may modulate individual enzyme activities (Empadinhas and

da Costa 2008). These compatible solutes can be classified into three groups as follows: 1) sugars and polyols such as glycerol and trehalose; 2) amino acids such as glutamate and proline; and 3) amino acid derivatives such as betaines and ectoine (Kempf and Bremer 1998; Roberts 2005). *T. halophilus* exhibits a large spectrum of compatible solutes including glycine betaine, proline, L-carnitine, ectoine, dimethylsulfonioacetate and dimethylsulfoniopropionate (Baliarda et al. 2003a), and converts exogenously provided choline into glycine betaine (Robert et al. 2000). Among the most potent compatible solutes are the quaternary ammonium compounds glycine and carnitine, which are preferentially used by most prokaryotes and eukaryotes for osmoprotection (Ko et al. 1994; Pichereau et al. 1999). Accumulating glycine betaine is a strategy widely employed by *T. halophilus* to protect itself against deleterious hyper-osmotic injury. However, unlike other moderate halophiles, *T. halophilus* is not able to produce glycine betaine through de novo synthesis but can accumulate it from an exogenous supply under hyper-osmotic constraint. Available information about system functions in compatible solutes transportation of *T. halophilus* remains scarce. It has been reported that dimethylsulfonioacetate, choline, proline and L-carnitine can efficiently compete with glycine betaine transport, suggesting the existence of (a) common transporter(s) for these molecules (Baliarda et al. 2003a).

Frequent fluctuation of external osmolality is one of the most common stress situations that LAB encounters in their native surroundings and industrial processes. The ability to respond quickly to stress is essential for survival. It is well established that LAB evolves cross-protection and defense mechanisms to withstand harsh conditions and sudden environmental changes (van de Guchte et al. 2002). In the LAB family, the identification and the role of compatible solutes have been investigated in *Lactobacillus plantarum* (Glaasker et al. 1996), *Lactococcus lactis* (O'Callaghan and Condon 2000), *Oenococcus oeni* (Le Marrec et al. 2007), *Pediococcus pentosaceus* and *Tetragenococcus halophilus* (Baliarda et al. 2003a). Transporters for compatible solutes in LAB so far characterized at molecular level are mainly: 1) high-affinity ATP-binding cassette (ABC) system (Schneider and Hunke 1998), described as BusA and OpuA in *Lactococcus lactis* ssp. *cremoris* NCDO 763 and MG1363, respectively (Obis et al. 1999; Patzlaff et al. 2003); 2) betaine choline carnitine transporter (BCCT) family (Ziegler et al. 2010), including ButA in *T. halophilus* (Baliarda et al. 2003b). ButA is a secondary glycine betaine transport system which solely transports glycine betaine in *T. halophilus*, and in *Escherichia coli*, statement of ButA is mainly constitutive and maximal uptake activity may result from a weak osmotic induction (Baliarda et al. 2003b). However, regulation of *butA* expression in *T. halophilus* in response to osmotic pressure induced by NaCl stress and effects of multi-copy *butA* complete expression boxes on salt tolerance of strains remain unknown up to now. In the present study, we analyzed relative *butA* expression levels during salt tolerance by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Subsequently, we constructed plasmids with a single- and double-copy of *butA* cassette(s) and compared their effects on *Escherichia coli* MKH13, which is defective in glycine betaine transport, and then we found a double-copy of *butA* cassettes entrusted higher salt adaption and tolerance to *E. coli* MKH13. Furthermore, we also studied the hyperosmotic induction of glycine betaine transport in *E. coli* MKH13 by high-performance liquid chromatography (HPLC). Our work laid a solid foundation for further research and industrial application of *T. halophilus.*

Materials and methods

Bacterial strains and growth conditions

Tetragenococcus halophilus (CICC 10469), which had been preserved by the China Center of Industrial Culture Collection (CICC) with accession number CICC 10469, was isolated from Chinese-type soy sauce in our laboratory and was grown in de Man, Rogosa and Sharpe (MRS) broth aerobically without agitation at 30 °C (De Man et al. 1960). *T. halophilus* CICC 10469 optimally grew at 1-M salt and could bear 4-M saturated NaCl solutions (data not shown). *Escherichia coli* MKH13, which is defective in glycine betaine transport (Kempf and Bremer 1995), was kindly donated by Prof. Dr. Erhard Bremer (Laboratory for Microbiology, Department of Biology, Philipps-Universität, Marburg, Germany). *E. coli* MKH13 and its subsequent recombination strains were cultivated in minimal medium A (MMA; May et al. 1986). MMA medium contained 10.5 g/L of K_2HPO_4, 4.5 g/L of KH_2PO_4, 1 g/L of $(NH_4)_2SO_4$, 0.5 g/L of $Na_3C_6H_5O_7{\cdot}2H_2O$, and 0.1 g/L of $MgSO_4{\cdot}7H_2O$. Then 0.2 % glucose filtered through 0.22 μm polyamide membrane was added.

General DNA techniques

Genomic DNA was isolated from *T. halophilus* CICC 10469 using a Hipure bacterial DNA kit (Magen, Guangzhou, Guangdong, China) as instructed by the manufacturer. Plasmid DNA was isolated with the Hipure plasmid micro kit (Magen). Gel extraction was conducted followed the instructions of a gel extraction kit (OMEGA bio-tek, USA). Fast digest restriction enzymes were obtained from Thermo Scientific (Massachusetts, USA). The glycine betaine standard was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All other reagents used in HPLC were chromatographically

pure. Primers were synthesized by GENEray Biotechnology (Shanghai, China). *E. coli* was transformed by using standard methods (Hanahan 1985).

Gene expression analysis by quantitative RT-PCR

Tetragenococcus halophilus CICC 10469 was activated in MRS with 1 M NaCl to an exponential phase, and then was transferred to MRS with 0 M, 1 M, 2 M, 3 M NaCl at a 2 % inoculum size. 2 mL cells were collected after incubation for 12, 24, 36, 48 h, and corresponding ribonucleic acid (RNA) were extracted using an RNAiso TM Plus kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. We firstly treated the collected thallus with 200 μL lysozyme (10 mg/mL) in consideration of the Gram-positive features of *T. halophilus*. The concentration and quality of extracted RNA were assessed with a ultraviolet-visible light (UV–Vis) spectrophotometer (Nano-Drop ND-2000, USA). In addition, RNA degradation and contamination were monitored on 1.2 % agarose gel. Approximately 1 μg of RNA extracted was used for cDNA synthesis with a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa). The remaining genomic DNA contamination was eliminated with DNAse, and the absence of DNA contamination was assessed by normal PCR with RNA treated by DNAse as thetemplate and primers used in RT-PCR to eliminate genomic DNA. Gel electrophoresis confirmed that the quality of mRNA was good and there existed no DNA contamination. Then, reverse transcriptase reactions were carried out according to the specifications of the supplier. Primers used for RT-PCR assay are listed in Table 1. The 16S rRNA of *T. halophilus* growing in MRS with 1 M NaCl for 12, 24, 36, and 48 h were used as internal controls for quantification. qRT-PCR assay was carried out using the SYBR® Premix EX TaqTM Kit II (Tli RNaseH Plus; TaKaRa). The qRT-PCR amplification was performed in an ABI StepOneTM RT-PCR system using the double stranded DNA intercalating fluorescent agent SYBR Green for product detection. Each well contained 10 μL of 1× SYBR Green Master Mix, 200 nM of each primer, and 2 μL cDNA templates. PCR amplification was initiated at 95 °C for 30 s, followed by 40 cycles of 95 °C f or 5 s and 60 °C for 34 s. The expression levels of *butA* gene were normalized against the expression level of the internal control gene (16S rRNA of *T. halophilus* growing in MRS with 1-M NaCl for 12, 24, 36, and 48 h). All samples were performed in triplicate (three different RNA isolations from three different cultures). The generation of quantitative data by qRT-PCR was based on the number of cycles required for amplification-associated fluorescence to reach the detection threshold (CT). For each reaction, a CT value was obtained. The relative *butA* expression levels during different times and different salinities were calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Table 1 Primers used for qRT-PCR and plasmid construction

Primer	Oligonucleotide sequence (5'-3')
butA-Tha-RT-forward	TCGGCGGTAACGATGCTA
butA-Tha-RT-reverse	GATGGGTTCTGCGACACTCC
16S-Tha-RT-forward	GCTTTCTGGTCAGCTACCGT
16S-Tha-RT-reverse	GAATCTTCGGCAATGGACGC
*but*A-complete-(S)	CGCGGATCCACGTAACAACA GTATACCATAAT (*Bam*HI)
butA-complete-(A)	CCGCTCGAGACTTGGATAAG TCTTTTTGG (*Xho*I)
Double-*but*A-complete(S)	CATGCCATGGACGTAACAAC AGTATACCATAATG (*Nco*I)
Double-*butA*-complete(A)	CGCGGATCCACTTGGATAAG TCTTTTTGGTTTG (*Bam*HI)
PET-32a-T7(F)	TAATACGACTCACTATAGGG
PET-32a-T7(R)	TGCTAGTTATTGCTCAGCGG

Construction of plasmids

We cloned a single-copy *butA* cassette from *T. halophilus* CICC 10469 into expression vector pET-32a, using primer pairs *butA*-complete-(S)/*butA*-complete-(A) — with *Xho*I and *Bam*HI — and termed it pButA-S. Based on this, *Bam*HI and *Nco*I were chosen and another *butA* cassette was inserted into pButA-S, using primer pairs double-*butA*-complete(S)/double-*butA*-complete (A), constructing a plasmid named pButA-D. These constructed plasmids were then transformed into *E. coli* MKH13. The potential promoter sequence that governed *but*A expression was 5'-AGATCAAAACG TAACCCAGAACTTGAAT-3' (deviations underlined), which resembled the *xyl* promoter from *T. halophilus* 5'-TG GACA (17) TAGAAT-5' (Takeda et al. 1998; Baliarda et al. 2003b). In order to ensure successful expression of the *but*A gene, we cloned 360 bp upstream of the potential promoter and 240 bp downstream of the potential *rho*-independent transcription termination signal. Primers used for plasmid construction are listed in Table 1. Successful construction was confirmed by gel electrophoresis and sequencing (data not shown). All the operations in plasmid construction followed standard manipulation procedures.

Growth curves under salt stress

Osmolality of the liquid MMA medium was increased by addition of different concentrations of NaCl. Glycine betaine was added to the media as a sterilized osmoprotectant to a final concentration of 1 mM. Three *E. coli* MKH13 recombination strains were grown overnight in MMA medium

without salt and glycine betaine exogenous provided, and then transferred into fresh MMA medium without NaCl addition and with 0.8-M NaCl, along with or without glycine betaine added, to almost the same OD_{600} (0.05±0.01). Cells were then incubated aerobically at 37 °C at 220 rpm on a rotary shaker. Growth curves of *E. coli* MKH13 with pET-32a, pButA-S and pButA-D were measured at 600 nm using an UNICO UV-2102 spectrophotometer (Shanghai, China) every 2 h. The maximum concentration, 0.8 M, was modified from previous research (Yu et al. 2014). Each sampling and measurement was performed in triplicate.

HPLC

HPLC was used to detect intracellular and extracellular contents of glycine betaine. Each kind of cell, cultivated in 100 mL MMA with added 1 mM glycine betaine without NaCl addition and with 0.8 M NaCl, was collected at log phase (both without NaCl addition and with 0.8 M NaCl) and stationary phase (0.8 M NaCl only), washed and centrifugated twice with corresponding media, and then dried in a frozen dry machine. After the first centrifugation, supernatants collected were passed through a polyamide membrane with a pore size of 0.22 μm, and stored at 4 °C until analysis. Detected concentrations of glycine betaine from these samples represented extracellular contents of glycine betaine. Cells after being frozen dry were suspended in 10 mL of double distilled water followed by sonic disruption over 30 min at 40 % power (5 s on, 5 s off), and extracted overnight at 4 °C. Supernatants were collected after centrifugation at 13, 000*g*, for 30 min, and were put in a vacuum freeze dryer. Then, precipitates were diluted with ultrapure water, and were detected immediately. Concentrations of detected glycine betaine from these supernatants represented intracellular contents of glycine betaine. In order to be standard unified, intracellular and extracellular contents of glycine betaine were converted to use a mol/L culture broth as a unit of concentration. All samples were prepared in triplicate. A water extracting method was proved to be better than alcohol extraction (Bessieres et al. 1999).

All of the solutions were detected using a Waters 1525 series liquid chromatography machine equipped with a UV-visible diode array detector and an Agilent ZORBAX HILIC Plus column (100 mm×4.6, i.d. 3.5 μm). Conditions for HPLC analysis were as follows: mobile phase, acetonitrile:water=85:15; detection wavelength, 196 nm; injection volume, 5 μL; flow rate, 1 mL/min; column temperature, 25 °C.

Results

Relative *butA* expression levels were up-regulated in response to salt stress

Figure 1 shows comparison of relative gene expression at the RNA level of *butA* under different salinities and treatment times in *T. halophilus* CICC 10469. *T. halophilus* CICC 10469 was most fitted in MRS with 1-M NaCl, so gene expression level under 1 M NaCl for 12, 24, 36, and 48 h was used as controls to calculate relative gene expression level under different salinities. When *T. halophilus* was transferred to a new environment with especially adverse circumstance like hyperosmotic stress, it exhibited a prolonged lag phase, so

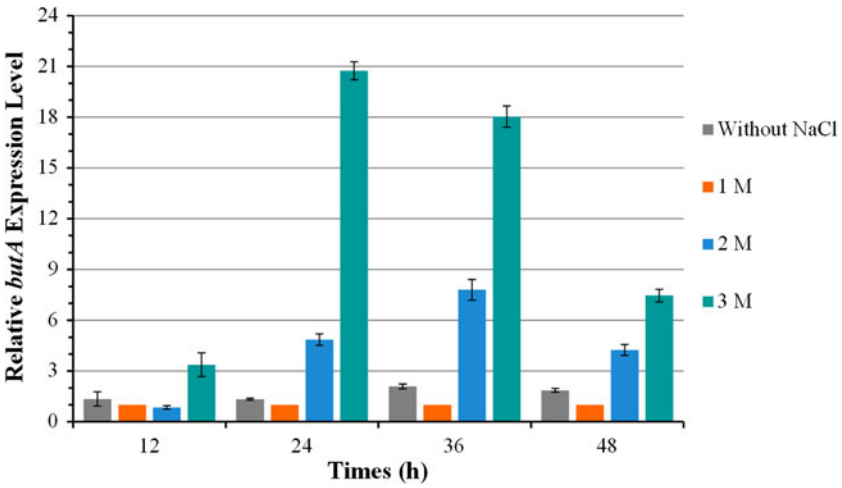

Fig. 1 Comparison of relative *butA* gene expression at the mRNA levels under different salinities and times in *T. halophilus* CICC 10469. The 16S rRNA of *T. halophilus* growing at MRS with 1 M NaCl for 12, 24, 36, and 48 h was used as calibrator for quantification. The *colorful bar* with the associated *standard error bar* represents relative *butA* expression level determined by q-PCR using the $2^{-\Delta\Delta Ct}$ method. Results represent mean standard deviations (SDs) of three biological replicates

expression of *butA* under different salinities at 12 h were nearly the same, with 3 M as an exception (Fig. 1). Gene expression level at 3 M was nearly three times as much than others. With respect to other salinities, 3-M NaCl was an extreme environment to *T. halophilus*; cells increased expression of *butA* quickly to cope with such hyperosmotic stress. When cells grew to the log phase (24 h), in which enough biomass and activity were obtained, *butA* expression levels were generally improved compared with 1 M, namely, without NaCl addition (≈1.3-fold), 2 M (≈4.7-fold), and 3 M (≈20-fold). 2 M and 3 M NaCl were high osmotic environments and, therefore, inhibited cell growth, which triggered more expression of the *butA* gene.

When cells reached a stationary phase (36 h), *butA* transcripts were also generally enhanced with respect to 1 M, namely, without NaCl addition (≈2.1-fold), 2 M (≈7.6-fold), and 3 M (≈4.3-fold). From log phase to stationary phase, differences of *butA* expression levels between conditions without NaCl addition and 1 M NaCl were enlarged, while those of 2 M and 3 M were narrowed, which indicated that a condition without NaCl addition was a continuous stress to *T. halophilus*, and cells needed more transmembrane proteins to counteract low water activity. The tendency of gene expression at 48 h was nearly the same as that at 36 h.

It was worth noting that *butA* expression under conditions without NaCl addition was slightly higher than the control (1 M) over time. *T. halophilus* is a moderately halophilic bacterium; an appropriate concentration of salt is essential for its normal growth. Conditions without NaCl addition could be regarded as a hostile environment (hypo-osmolality) and, therefore, inhibited cell growth (data not shown) through slight elevation of *butA* expression was observed (Fig. 2).

Dual-copy *butA* cassettes enhanced salinity adaptation and tolerance of *E. coli*

After successful construction of the plasmid (Fig. 2), we examined the effects of heterologous expression of single (pButA-S)- and dual (pButA-D)-copy *but*A cassette(s) from *T. halophilus* on the salinity adaptation and salt tolerance of *E. coli* MKH13 strain. Figure 3 shows the growth curves of recombinant *E. coli* strains and control under medium without NaCl addition and with 0.8 M NaCl. In the medium without NaCl addition, there was no distinct difference between three strains (Fig. 3a), which suggested that heterologous

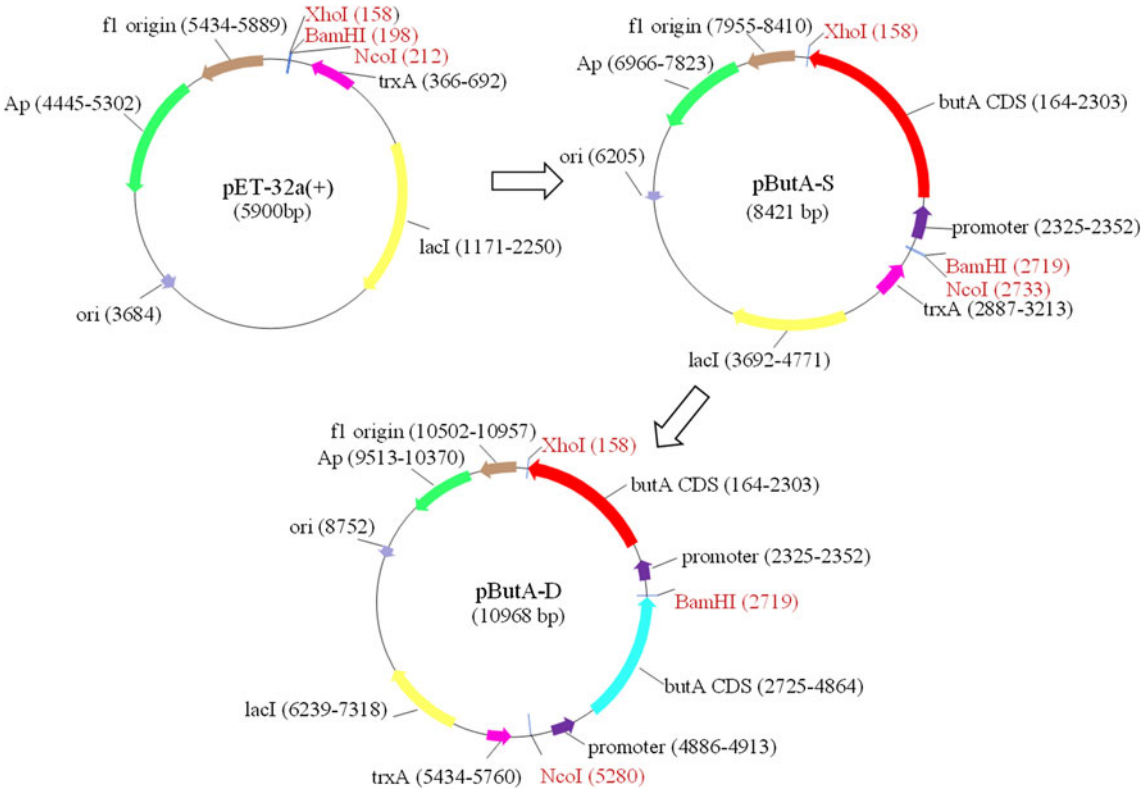

Fig. 2 Schematic presentation of plasmid construction. *Bam*HI – *Xho*I and *Bam*HI – *Nco*I were chosen to construct plasmid pButA-S and pButA-D, respectively. pButA-D, which harbored two copies of *butA* cassettes, was first generated by an in vitro method. The potential promoter sequence that governs *but*A expression was 5'-AGATCAAAACGTAACCCAGAACTTGAAT-3' (deviations underlined), which resembled the *xyl* promoter from *T. halophilus* 5'-TGGACA (17) TAGAAT-5' (Takeda et al. 1998; Baliarda et al. 2003b)

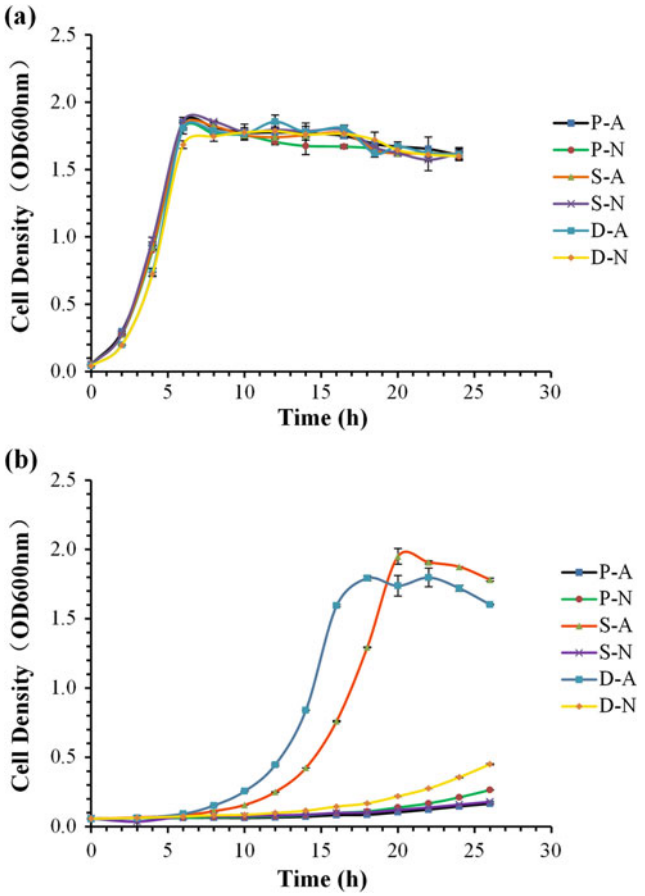

Fig. 3 Growth curves of recombinant strains and control in MMA medium without NaCl addition and with 0.8-M NaCl, with or without glycine betaine added. *E. coli* MKH13 strains with pET-32a, pButA-S and pButA-D were incubated at 37 °C. 1 mL of culture was taken and measured at 600 nm using an UNICO UV-2102 spectrophotometer (Shanghai, China) every 2 h. The optical density (OD_{600}) of cultures at 0 h was approximately 0.05 (±0.01). (**a**) Growth curves of recombinant strains and control under medium without NaCl addition; (**b**) Growth curves of recombinant strains and control under 0.8 M NaCl. "A" represents glycine betaine added, "N" represents no glycine betaine added. "P", "S" and "D" represent strains with pET-32a, pButA-S and pButA-D, respectively. *Error bars* indicate standard deviations (n=3)

expression of single (pButA-S)- and dual (pButA-D)-copy *but*A cassette(s) had neither a positive nor negative effect on the growth of *E. coli* MKH13 under a non-salt condition. Therefore, glycine betaine was not an essential element for *E. coli* MKH13 and its subsequent combination strains under conditions without NaCl addition. In the 0.8 M NaCl medium without exogenous glycine betaine supply (Fig. 3b), growth of all strains were almost slowed down; nevertheless, when glycine betaine was added, the *E. coli* recombinant strains with single- (S-A)- and double-copy *butA* cassettes (D-A) showed typical growth profiles, while growth of control (P-A and P-N) still stagnated (Fig. 3b). Moreover, *E. coli* MKH13 with pButA-D (dual-copy *butA* cassettes) showed a shorter lag phase and a higher growth rate at log phase than the strain with pButA-S (single-copy *butA* cassette). However, it exhibited lower maximum growth than the latter at the stationary phase. This suggested that overexpression of dual-copy *butA* cassettes (pButA-D) enabled the host cell to rapidly adapt to the salt stress condition, causing a shortened lag phase, while its overexpression might represent a tremendous genetic and metabolic burden to the host cell in the stationary phase, resulting in suppressed maximum growth level, as Fig. 3b indicated. On the contrary, the single-copy *butA* cassette (pButA-S) played a continuous and vital role during later stages of growth, resulting in a higher maximum growth level at the stationary phase.

Intracellular and extracellular contents of glycine betaine in *E. coli* MKH13 recombinant strains

Intracellular and extracellular contents of glycine betaine in *E. coli* MKH13 recombinant strains are demonstrated in Fig. 4. No glycine betaine was detected in all intracellular samples under medium without NaCl addition (Fig. 4b), which confirmed that *E. coli* MKH13 (0-P-L) and its transformants (0-S-L and 0-D-L) could not de novo synthesize and needed not to transport glycine betaine under hypo-osmotic stress. This result was consistent with the result that all the strains used under conditions without NaCl addition showed a coincident growth tendency with or without glycine betaine added (Fig. 3a).

E. coli MKH13 cells with pET-32a did not absorb any glycine betaine from medium neither at log phase (0-P-L) nor at stationary phase (0.8-P-L and 0.8-P-S'; Fig. 4b), while *E. coli* MKH13 transformants with pButA-S (single-copy *butA*) and pButA-D (dual-copy *butA*) exhibited strong glycine betaine transport ability to various degrees. Furthermore, intercellular glycine betaine contents of *E. coli* transformant with pButA-D (0.8-D-L) were higher than that of *E. coli* transformant with pButA-S (0.8-S-L), when cells grew to the log phase. These results indicated that the plasmid pET-32a without the *butA* coding sequence could not afford glycine betaine transport, while pButA-S and pButA-D entrusted *E. coli* MKH13 with the ability to transport glycine betaine, and *E. coli* transformant with dual copies of *butA* showed stronger ability to transport glycine betaine at log phase. However, the glycine betaine contents in supernatant of *E. coli* transformant with pButA-S at log phase (0.8-S-L) was higher than that of *E. coli* transformant with pButA-D (0.8-D-L), while no glycine betaine was detected in this transformant at the stationary phase (Fig. 4a). These results were consistent with the growth curves under high salinity (Fig. 3b), which further proved that dual-copy *butA* cassettes entrusted rapid salt adaptation ability to *E. coli* MKH13.

It is worth noting that, in Fig. 4, extracellular glycine betaine contents of strains with plasmid pET-32a (0-P-L, 0.8-P-L, 0.8-P-S') differed (between 0-P-L and 0.8-P-L) or

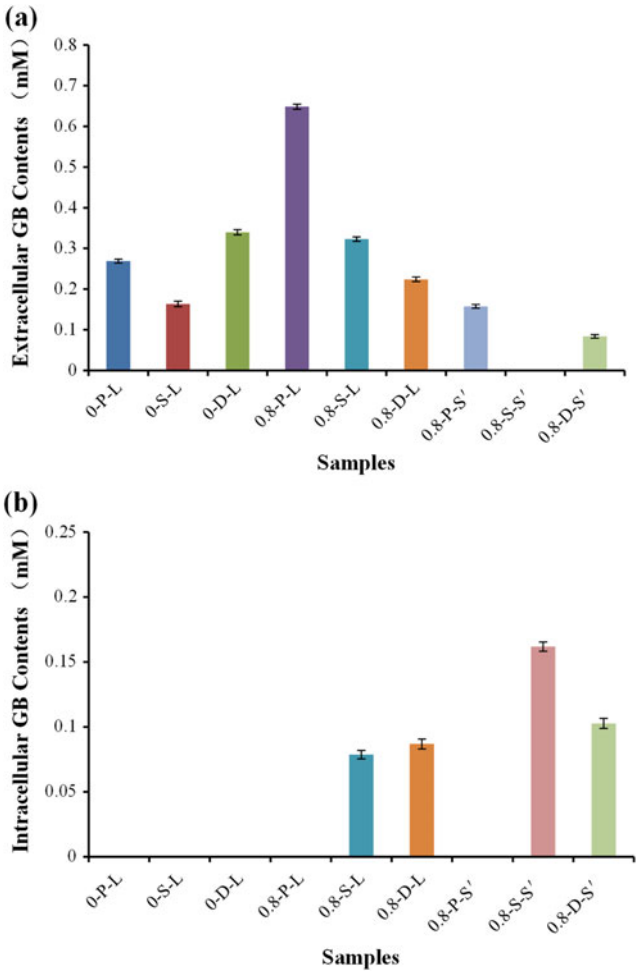

Fig. 4 Extracellular and intracellular contents of glycine betaine in recombinant *E. coli* MKH13strains. Each kind of cell, cultivated in MMA with 1 mM glycine betaine, without NaCl addition and with 0.8-M NaCl, was collected by centrifugation at the log phase (both without NaCl addition and with 0.8 M NaCl) and stationary phase (0.8 M NaCl only), followed by centrifugation and frozen dry. Contents of glycine betaine of supernatants after the first centrifugation represented extracellular contents of glycine betaine. After being frozen dry, cells were sonically disrupted and extracted overnight at 4 °C. Detected concentrations of glycine betaine from supernatants obtained via centrifugation and frozen dry represented intracellular contents of glycine betaine. "mM" in the Y axis means mol/L culture broth. (**a**) Extracellular contents of glycine betaine; (**b**) Intracellular contents of glycine betaine; "P", "S" and "D" represent strains with pET-32a, pButA-S and pButA-D, respectively. "0" and "0.8" represent conditions without NaCl addition and with 0.8-M NaCl addition, respectively. "L" and "S'" stand for log phase and stationary phase, respectively. *Error bars* indicate standard deviations (n=3)

changed over time (from 0.8-P-L to 0.8-P-S'). As a control, empty plasmid pET-32a should have no influence on extracellular levels of glycine betaine. In medium without NaCl addition, all the strains grew well, which meant they underwent vigorous metabolism. Complex metabolic products might cause degradation of glycine betaine during growth. In 0.8 M NaCl, strains grew slowly, which meant they had a suppressed metabolism. So, contents of glycine betaine under 0.8 M NaCl (0.8-P-L) were higher than those without NaCl addition (0-P-L).

For "0.8-P-L", we collected the *E. coli* MKH13 with PET-32a (the control) cells when they were cultivated for about 16 h (corresponding to log phase of *E. coli* MKH13 with single- and double-copy *butA* complete expression box(es), see Fig. 3). Thus, contents of glycine betaine were relatively high (≈0.7 mmol/L). However, for "0.8-P-S", we collected the control cells when they were cultivated for about 25 h (corresponding to the stationary phase of *E. coli* MKH13 with single- and double-copy *butA* complete expression box(es), see Fig. 3), at which it was supposed to lyse, and lysate might cause degradation of glycine betaine. Thus, contents of glycine betaine were relatively low (≈0.2 mmol/L). So, differences of glycine betaine contents between 0-P-L and 0.8-P-L might be due to degradation of glycine betaine by metabolism products during growth and differences between sample groups. Changes of glycine betaine contents over time from 0.8-P-L to 0.8-P-S' might be due to degradation of glycine betaine by lysate. The fact that standard glycine betaine stored in 4 °C over one week could not be detected by the C18 column (data not shown) supported this statement indirectly.

Discussion

Osmotic stress is a major challenge in the habitat of LAB, which can induce transcription of a related gene and regulate the activity of compatible solute transporters (Van Der Heide and Poolman 2000; Steil et al. 2003; Culham et al. 2003) . Our qRT-PCR results (Fig. 1) suggested that the relative *butA* expression level under hyperosmotic stress was up-regulated during saline adaptation, which implied that a complete cassette of *butA* might contain important regulating elements that could regulate glycine betaine accumulation of *T. halophilus*. The composition and fluidity of the cytoplasmic membrane are key factors in maintaining cell viability and metabolic function (Mykytczuk et al. 2007; Rodríguez-Vargas et al. 2007). ButA protein was predicted to be an integral membrane-bound protein containing 12 putative transmembrane segments (TMSs). In particular, the eighth segment and the connecting loop to the ninth segment were highly conserved. It harbored the signature sequence WTLFYWAWW, which is proposed to be involved in the binding of the trimethylammonium substrate and translocation across the membrane (Saier et al. 1999; Baliarda et al. 2003b). Therefore, we might hypothesize that membrane-spanning protein ButA was synthesized abundantly to transport more glycine betaine from outside when encountered with osmotic stress.

HPLC results (Fig. 4) showed that no glycine betaine could be detected in all intracellular samples of *E. coli* under conditions without NaCl addition. However, in a high-salt

environment (0.8 M), transportation of glycine betaine occurred (Fig. 4), which indicated that expression product of *butA* cassette (ButA protein) could afford glycine betaine transport and this transport ability could be regulated. These observations were different from Robert et al. (Robert et al. 2000; Baliarda et al. 2003b), who assumed that the uptake of osmotic protective substance was not induced by high osmotic pressure and that the statement of ButA was mainly constitutive in *E. coli* and that maximal uptake activity may result from weak osmotic induction. This difference may be caused by different sample treatments (in this present study, cells were continuously faced with an elevated salt environment instead of salt shock) and conservation within *E. coli* and *T. halophilus* in osmoregulation signals and effectors. Since there is no effect of chloramphenicol on the global transport of glycine betaine elicited by osmotic stressing *T. halophilus*, while a slight activation of the transport of glycine betaine is suggested (Robert et al. 2000), we assume that the ButA protein may function not only as a carrier but also as a sensor of osmolality. Nevertheless, how the environmental signal "salinity" is sensed and transmitted to various output modules at the level of the gene, enzyme or transporter activation are completely obscure. Further works should be conducted to elucidate how *butA* senses osmolality and how ButA protein detects osmotic shifts directly. These works are complex, since changes in transporter activity (itself subject to changes in membrane structure and the solution environment) are currently the sole indicators of osmo-sensing (Wood et al. 2001).

Tetragenococcus is resistant to electroporation or other transformation procedures. Transforming of exogenous plasmids into *T. halophilus* has been impossible until now, so has obtaining mutants defective in compatible solutes transport. We also have tried various electroporation methods but failed. It is difficult to evaluate the number and the structural diversity of systems involved in accumulation of glycine betaine, and then to assess the individual contribution of each system to *T. halophilus* (Averhoff and Müller 2010). Thus, we turn to in vitro study. On the basis of the probable halophilic mechanism described above, we successfully constructed a plasmid with dual-copy *butA* complete expression boxes, and proved its enhancement of saline adaptation and tolerance to *E. coli* MKH13 via results obtained from growth curves and HPLC (Figs. 3 and 4). Thus, double-copy *butA* cassettes provide an attractive prospect in salt adaptation and tolerance to strains. Since construction of multi-copy gene recombinant expression plasmids can usually increase the expression level of proteins and the central point of this approach is the construction of a multi-copy search model (Vagin and Teplyakov 2000), consequent effects of more copies of *butA* cassettes on *E. coli* should be further elucidated.

Many other similar works improved salt tolerance of *E. coli* by gene transfer (Sugimoto et al. 2003; Yu et al. 2014), yet they usually analyzed influences of isopropyl β-D-1-thiogalactopyranoside (IPTG) induced overexpressed protein on *E. coli*, these were different from our work. In plasmid construction, we cloned a *but*A complete expression box that contained a core promoter of *T. halophilus* itself. Our experimental data proved that the promoter of *T. halophilus but*A could actually function well in *E. coli*, and that different expression levels of ButA controlled by its own promoter had differential effects on the ability of this organism to grow at conditions of high salinity. A double-copy of *but*A cassettes provides an attractive prospect in salt tolerance for strains. Hence, using a *but*A promoter of its own was our distinct difference and innovation point from previous works. We abandoned the efforts to clone *but*A under control of an inducible promoter and to control (and quantify) expression levels by induction, because *Tetragenococcus* was characterized as being resistant to electroporation or other transformation procedures; thus, we had to manipulate it in vitro to study its function in improving salt tolerance of *E. coli* by muti-copies or IPTG to induce ButA. Our ultimate goal is to operate on *T. halophilus* and improve its industrial application; however, the influence of IPTG on growth of *T. halophilus* was unclear, and the activity of purified ButA needs to be validated.

Therefore, the results of the present study suggest that the expression of the induced *butA* gene and the accumulation of glycine betaine are two of the molecular mechanisms by which *T. halophilus* copes with harsh environments. Our work implied the potential of muti-copies of *but*A gene to genetically modify *T. halophilus* for improvement of salt tolerance, and further its industrial application. At the same time, we should continue our efforts to explore new transformation methods, which are the keys to carrying out genetic modification of *T. halophilus* and to illuminate mechanisms of its stress response, which finally contribute to its better industrial application.

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Conflict of interest The authors declare no conflicts of interest.

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