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# Improvement of ectoine productivity by using sugar transporter-overexpressing *Halomonas elongata*

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ABSTRACT

We successfully enhanced the productivity of ectoine with *Halomonas elongata* by improvement of the transport of sugar. First, we carried out screening for sugar transporters capable of improving glucose and xylose consumption. We found two transporters: b3657 from *Escherichia coli*, which is capable of improving glucose consumption, and HEO_0208 from *H. elongata*, which is capable of improving xylose consumption. Using transporter-overexpressing strains, the productivity of ectoine was improved. These results indicate that sugar consumption is important for efficient ectoine production. As result of phenotypic analysis of a HEO_0208 deletion strain, we discovered that HEO_0208 is the major xylose transporter in *H. elongata*. This is the first report demonstrating improvement of ectoine productivity by enhancing the transport of sugar.

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## 1. Introduction

Many halophilic microorganisms accumulate ectoine (1,4,5,6-tetrahydro-2-methyl-4-primidinecarboxylicacid) in order to counteract saline environments. Ectoine is a cyclic amino acid derivative of aspartate and possesses excellent stabilizing effects on biological compounds (e.g. protein, DNA, whole cells) [1–3]. Therefore, ectoine protects biological compounds from environmental stresses, such as heat, freezing, drying, and high salinity. Owing to these properties, ectoine is used in cosmetic moisturizers and other skin care products. Furthermore, beneficial effects on atopic dermatitis have been suggested [4], and protective effects against certain other diseases have also been reported [5–7]. The increasing commercial demand for ectoine has led to a number of efforts to improve its production from bacteria [1,8,9]. To date, ectoine is produced on an industrial scale of many tons using *Halomonas elongata* [10].

*H. elongata* is a gram-negative bacterium first isolated from a solar saltern, and its complete genome sequence has been reported [10]. Therefore, metabolic engineering approaches based on genomic information for the enhancement of ectoine production have attracted attention. For example, the pathway that degrades ectoine into N-α-Acetyl-L-2,4-diaminobutyrate has been identified, and the deletion of this pathway improves ectoine production in *H. elongata* [10]. We also reported that ectoine production is increased by deletion of the pathway leading from ectoine to hydroxyectoine [11]. These efforts have made improvements downstream of the ectoine biosynthesis pathway; however, improvements upstream of the pathway, such as with sugar uptake, have not yet been considered.

Transport of sugars from the medium into cells is the first step in cellular metabolism and exerts great influence on total carbon flux [12]. Therefore, the transport of sugar is closely related to consumption of sugar by microbes. Sugars are transported by transporter proteins, such as ATP-binding cassette (ABC) transporters, the major facilitator superfamily (MFS), and the phosphotransferase system (PTS) [13,14]. The important role of transporter proteins in the consumption of sugar by microbes is demonstrated by the increase of pentose consumption by overexpressing sugar transporters in *Corynebacterium glutanicum* and *Saccharomyces cerevisiae* [15,16].

In our previous study, *H. elongata* consumed small amounts of sugars during the ectoine producing phase to maintain osmotic balance, and consumed most of the sugar during the growth phase

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after ectoine production. Improvement of sugar consumption in the ectoine producing phase should enhance the rate of ectoine production. Hence, the enhancement of sugar transport is a promising way to improve the productivity of ectoine; however, there are no studies concerning this in *H. elongata*.

In the current study, we screened for sugar transporters capable of improving the transport of glucose or xylose. As a result, we found two transporters that improved transport of glucose and xylose, respectively. We also demonstrated that the productivity of ectoine was improved by overexpressing these transporters in *H. elongata*. In addition, we revealed the major xylose transporter in *H. elongata*. This is the first report concerning the improvement of ectoine productivity by enhancing the transport of sugar.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The type strain *H. elongata* OUT30018 was used for all experiments [17]. Cells were grown in MM63 medium [19], which consisted of 100 mM $KH_2PO_4$, 15 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 3.9 μM $FeSO_4$, 3% or 15% NaCl, and a carbon source. The pH was adjusted to 7.2 with KOH. Glucose and xylose were used as carbon sources. For ectoine production, *H. elongata* was cultivated for 20 h or 24 h at 37 °C in MM63 medium containing 3% NaCl, after which cell density was adjusted to an initial $OD_{600}$ of 2.2, a 2-mL aliquot was collected, and the cells were incubated for a further 4 h at 37 °C. Cells were then collected by centrifugation at 3500 rpm for 15 min, and the resulting pellet was resuspended in distilled water (40 μL/mg fresh cell weight (FW)) to extract ectoine from inside the cells. The extracted solutions were then examined by HPLC.

### 2.2. HPLC analysis of ectoine extract

The ectoine derivatizing solution was made by dissolving 2.5 mmol of 18-crown-6 and 50 mmol of 4-bromophenacyl bromide in acetonitrile. To 30 μL of ectoine extract sample was added 30 μL of 100 mmol/L $KH_2PO_4$. After the solution was vortexed, 540 μL of derivatizing solution was added. The tubes were vortexed and heated to 80 °C for 60 min [20]. The derivatized products were analyzed by HPLC as described below. The concentration of ectoine was determined using a Shimadzu HPLC system (Kyoto, Japan) equipped with a SUPELCOSIL™ LC-SCX column (Sigma-Aldrich Corp. St. Louis, MO, USA). The analyses were carried out at 28 °C using 22 mM choline chloride dissolved in 90% acetonitrile as the mobile phase. The column was eluted at a flow rate of 1.5 mL/min, and the eluate was analyzed using an ultraviolet absorbance detector set at 254 nm.

### 2.3. Construction of sugar transporter-overexpressing H. elongata

The pHS15 [21] derivative pHS15 N [22] was employed as a shuttle vector for overexpression of the sugar transporter genes. The b0045, b1769, b3657, and b3877 genes were amplified using the genome of *Escherichia coli*, and the HEO_0208, HEO_0361, and HEO_2569 genes were amplified using the genome of *H. elongata*. Each amplified fragment was digested and ligated into the *Spe*I/*Nsi*I restriction site of pHS15N. The resulting plasmids were designated pHS15N-b0045, pHS15N-b1769, pHS15N-b3657, pHS15N-b3877, pHS15N-HEO_0208, pHS15N-HEO_0361, and pHS15N-HEO_2569, respectively. The primers used in this study are listed in Supplementary Table S1 in the online version at DOI: 10.1016/j.enzmictec.2016.03.006.

All plasmids were introduced into *H. elongata* using *E. coli* HB101/pRK2013-mediated conjugation [23]. To eliminate *E. coli* cells after plasmid transfer, a streptomycin-resistant strain of *H. elongata* was isolated by serial plating on LB medium containing streptomycin (500 mg/L) and 6% NaCl with incubation at 37 °C. The resulting strains were designated *H. elongata*/pHS15N, *H. elongata*/pHS15N-b0045,*H. elongata*/pHS15N-b1769, *H. elongata*/pHS15N-b3657, *H. elongata*/pHS15N-b3877, *H. elongata*/pHS15 N-HEO_0208, *H. elongata*/pHS15N-HEO_0361, and *H. elongata*/pHS15N-HEO_2569, respectively.

### 2.4. Western blotting

b3657HA and HEO_0208HA were amplified and ligated into pHS15 N with HA-tags fused at their C-termini as per above. The resulting plasmids were designated pHS15N-b3657HA and pHS15 N-HE0208HA, respectively. These were introduced into *H. elongata* as described above to obtain *H. elongata*/pHS15N-b3657HA and *H. elongata*/pHS15N-HE0208HA. *H. elongata*/pHS15N, *H. elongata*/pHS15N-b3657HA, and *H. elongata*/pHS15N-HE0208HA were cultured at 37 °C for 24 h in 3 ml LB medium containing streptomycin (500 mg/L) and 6% NaCl. After cultivation, cells were centrifuged at 21,880*g* for 5 min. Cells were resuspended in 1 mL phosphate-buffered saline. Resuspended cell samples were mixed and homogenized in the presence of 2% sodium

**Table 1**
Strains, plasmids, and primers used in this study.

| Strain or plasmid | Relevant phenotype, description, or sequence (5′-3′) | Source or reference |
|---|---|---|
| Strains | | |
| *Escherichia coli* | | |
| DH5α | *F*⁻ ϕ*H5αlacZDM15*Δ*(lacZYA-argF)U169 recA1 hsdR17*($r_K^-$ $m_K^+$) *supE44* Δ⁻*thi-1 gyrA relA1* | Lab stock |
| HB101 | *supE*44, Δ(*mcrC-mrr*), *recA*13, *ara*-14, *proA*2, *lacY*1, *galK*2, *rpsL*20, *xyl*-5, *mtl*-1, *leuB*6, *thi*-1 | Lab stock |
| *Halomonas elongata* | | |
| OUT30018 | Wild-type (Osaka University type culture), formerly designated strain KS3 | [17] |
| Plasmids | | |
| pRK2013 | ColE1 $Km^r$ $Tra^+$ helper plasmid for mobilization | [18] |
| pK18*mobsacB* | $Km^r$ *mob sacB* | [24] |
| pHS15N | *E. coli-H. elongata* shuttle vector | [22] |
| pHS15N-b0045 | pHS15 N containing b0045 from *E. coli* | This study |
| pHS15N-b1769 | pHS15 N containing b1769 from *E. coli* | This study |
| pHS15N-b3657 | pHS15 N containing b3657 from *E. coli* | This study |
| pHS15N-b3877 | pHS15 N containing b3877 from *E. coli* | This study |
| pHS15 N-HEO_0208 | pHS15 N containing HEO_0208 from *H. elongata* | This study |
| pHS15 N-HEO_0361 | pHS15 N containing HEO_0361 from *H. elongata* | This study |
| pHS15 N-HEO_2569 | pHS15 N containing HEO_2569 from *H. elongata* | This study |
| pHS15N-b3657HA | pHS15 N containing b3657HA from *E. coli* | This study |
| pHS15 N-HEO_0208HA | pHS15 N containing HEO_0208HA from *H. elongata* | This study |

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