Deletion of Glycine Betaine Transporter Gene Confers Increased Ectoine Accumulation in the Moderately Halophilic *Halomonas elongata*

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ABSTRACT

To survive in high-salinity environments, the moderately halophilic *Halomonas elongata* OUT30018 uses several mechanisms, including the biosynthesis and the uptake of compatible solutes, ectoine (Ect) and glycine betaine (GB), to accumulate as osmolytes inside the cell. This study aims to develop an *H. elongata* cell factory that can use a high-salinity medium derived from soy sauce dregs' hydrolysate (SH medium) as a substrate for efficient production of Ect, a high-value chemical used in the cosmetic and medical industries. However, soy sauce dregs and other waste biomass contain GB, and GB uptake and accumulation strongly suppress Ect production. To block the entry of GB into *H. elongata* cell, we deleted the *betH* gene, which encodes the GB transporter, from the genome of *H. elongata* OUT30018. Analysis of intracellular concentrations of Ect and GB in the recombinant *H, elongata* BN1 (Δ*betH*) cultured in SH medium containing 9% w/v NaCl shows that *H. elongata* BN1 accumulated higher level of Ect than the wild-type *H. elongata* OUT30018 cultures under the same conditions. As the *H. elongata* OUT30018 genome contains more than one GB transporter gene, subsequent deletion of more GB transporter genes from the *H. elongata* BN1 genome may further increase Ect accumulation.

Keyword: Ectoine/ Glycine betaine/ Osmolyte/ GB transporter/ High salinity/ *Halomonas elongata*

1. INTRODUCTION

In modern society, large-scale production technologies have improved and made our lives more comfortable. However, these technologies also generate large amounts of industrial waste, which has become a global problem. As a result, the concept of Sustainable Development Goals (SDGs) is gaining international momentum, although the advancement of science and technology to support SDGs is still in its early stages.

In our laboratory, we aim to establish the foundation for a recycling-oriented society by using genetic engineering approaches to develop cell factories for upcycling waste biomass from agricultural and fermentation industries into high-value chemicals.

In the project presented here, we focus on developing cell factories that can effectively produce Ect, which is a compound used in cosmetic, pharmaceutical, and medical industries [1, 2] using media derived from soy sauce dregs, which are the nutrient-rich high-salinity byproducts from the fermentation of soy sauce—an essential ingredient in Japanese food culture.

H. elongata OUT30018, a moderately halophilic bacterium isolated from salty soil in the Northeastern region of Thailand [3], was chosen as a host due to its ability to thrive in high-salinity media derived from biomass waste [4]. Although *H. elongata* OUT30018 can grow under salinity ranging from 0.3% to 21% w/v NaCl, the cell is subject to osmotic stress that causes various inhibitory effects on its biological functions in high-salinity environments. To protect cellular functions, *H. elongata* cell produces or imports compatible solutes such as Ect and GB (Fig. 1), accumulating them inside the cell as osmolytes. When the salinity level of the environment changes, these osmolytes can be released from the cell to maintain osmotic homeostasis with the environment. Interestingly, there is evidence that the uptake and accumulation of GB could decrease the ability of the cell to produce and accumulate Ect [5].

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As soy sauce dregs contain GB, we took a genetic engineering approach to delete a GB transporter gene (*betH* gene) from the genome of *H. elongata* 30018 in an attempt to minimize the negative effect of GB accumulation on Ect accumulation. Here, we report the effect of this deletion on the level of Ect and GB accumulation in the recombinant *H. elongata* BN1 (Δ*betH*) and discuss strategies to further increase Ect accumulation in *H. elongata* cells.

2. MATERIALS AND METHODS

2.1 Triparental mating method for transformation of H. elongata

The strains used in the triparental mating are the recipient *H. elongata* OUT30018 wild type, the helper *Escherichia coli*, and the donor *E. coli* harboring an engineered pk18mobsacB-Δ*betH* plasmid. These three strains were mixed and co-cultured on the PTFE membrane, and the *H. elongata* transformants with successful *betH* gene deletion were selected by culturing on selective media. Confirmation of the *betH* gene deletion was done by genomic PCR using specific primers, which bind to the 5' and 3' franking regions of the gene.

2.2 Culturing condition and osmolytes qantification

Pre-cultures were grown in 5 mL of LB medium containing 3% w/v NaCl in a 37°C water-bath shaker until their OD_{600} reached 0.8. The main cultures were grown in a medium derived from soy sauce dregs hydrolysate (SH medium) containing 9% w/v NaCl. Osmolytes were extracted by dissolving cell pellets in MilliQ water for the cell to release osmolytes, including Ect and GB, into the supernatant, and the concentrations of Ect and GB were quantified by HPLC as mentioned previously [6, 7].

3. RESULTS AND DISCUSSION

3.1 Analysis of Intracellular Accumulation of Ect and GB in H. elongata OUT30018 grown in media containing GB

To determine the extent of interference the imported GB has on the cellular accumulation of Ect, we cultured the wild-type *H. elongata* OUT30018 in the LB medium containing 12% w/v NaCl. As shown in Fig. 1A, GB accumulation was much higher than the Ect accumulation in the cells cultured under this condition. These may result from the high GB content in the LB medium, making it easier for the cells to uptake GB and accumulate it as a major osmolyte than using cellular resources and energy in *de novo* biosynthesis of Ect. As the media derived from biomass waste usually contain high concentrations of GB, active GB accumulation would interfere with the ability of the cells to produce and accumulate Ect.

3.2 Generation of the Recombinant H. elongata BN1 *with Genomic Deletion of GB Transporter Gene*

As shown in Fig. 1, *H. elongata* OUT30018 prefers to use the less energy-demanding GB uptake pathway over the *de novo* biosynthesis of Ect to protect the cells from hyperosmotic stress. Therefore, we hypothesize that suppression of the GB uptake pathway may force the cells to increase the production and accumulation of Ect. To block the entry of GB into *H. elongata* cells, we deleted the *betH* gene, which encodes one of the *H. elongata* OUT30018's GB transporters, from its genome. The genomic structure of the *betH* locus on the genome of the resulting recombinant *H. elongata* BN1, in which the coding sequence (CDS) of the *betH* gene was deleted, is shown in Figure 2A. The result from genomic PCR with specific primers designed to amplify the DNA fragment between the 5'-upstream and the 3'-downstream regions of the *betH* CDS confirms the lack of the *betH* coding region in the genome of *H. elongata* BN1 (Figure 2B, lane #2).

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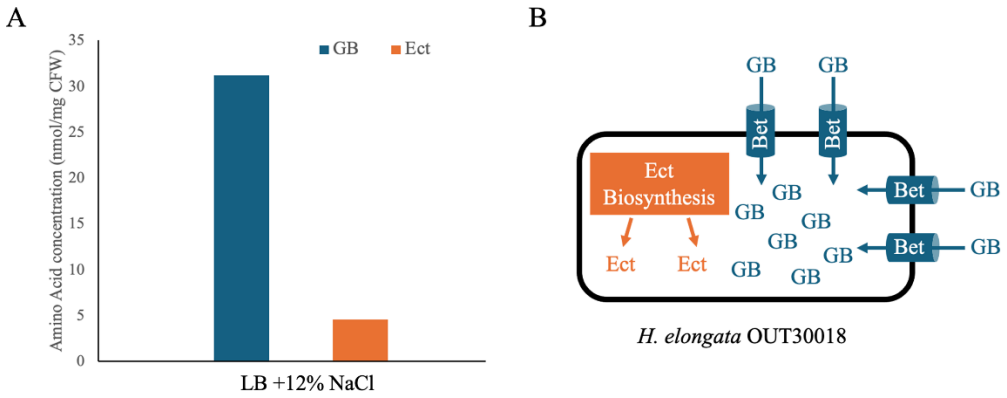

Figure 1. Ect biosynthesis and GB uptake are primary mechanisms for maintaining osmotic homeostasis within the cells of *H. elongata* OUT30018 grown in a high-salinity LB medium. A. The amount of Ect and GB accumulated in *H. elongata* OUT30018 cells cultured in LB medium containing 12% w/v NaCl. As the LB medium also contains GB, it was imported and accumulated in the cells as one of the major osmolytes. B. Schematic diagram showing the mechanisms used by *H. elongata* OUT30018 to maintain osmotic balance with its environment. The high salinity of the medium activates Ect biosynthesis and GB uptake. Ect, ectoine; GB, glycine betaine; Bet, GB transporter.

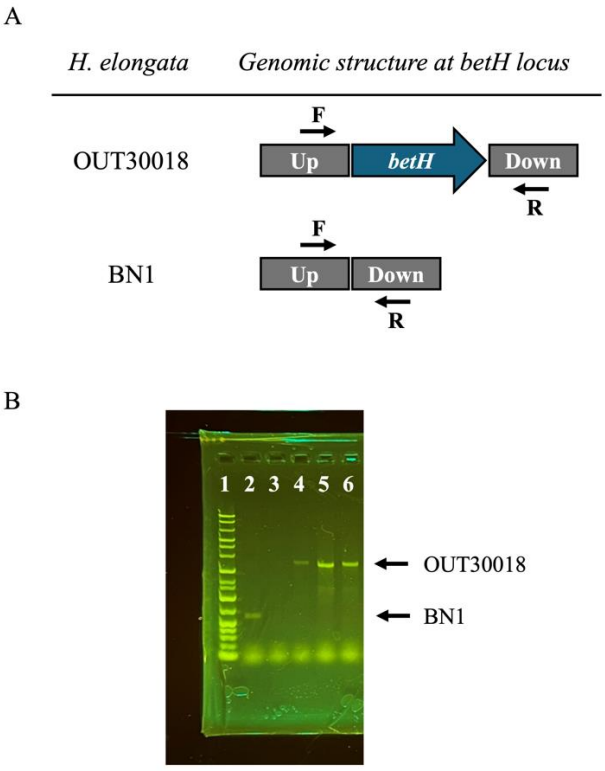

Figure 2. Genomic PCR is used to verify the absence of the *betH* coding region from the *betH* locus in the recombinant *H. elongata* BN1. A. Schematic diagram showing genomic structures of the *betH* loci on the genomes of *H. elongata* OUT30018 and *H. elongata* BN1. Up, 5' flanking region of *betH* CDS; Down, 3'-franking region of the *betH* CDS; F and R, forward and reverse primers used in the genomic PCR reactions. Arrows indicate the annealing positions and directions of the primers. B. Agarose gel electrophoresis of the products of genomic PCR using specific F and R primers shown in A. Lane 1, Size marker; Lane 2, amplified fragments from PCR reaction using genomic DNA of *H. elongata* BN1 as a template; Lanes 4 to 6, amplified fragments from PCR reaction using genomic DNA of *H. elongata* OUT30018 as a template. Arrows indicate the amplified products with the correct sizes.

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3.2 Analysis of Intracellular Accumulation of Ect and GB in H. elongata OUT30018 and BN1 cultured in high-salinity SH medium

To determine the effect of *betH* gene deletion on the production and accumulation of Ect and GB, *H. elongata* OUT30018 and BN1 strains were cultured in SH medium containing 9% w/v NaCl. When the optical density at 600 nm (OD_{600}) of the cultured reached 0.8, the amounts of Ect and GB accumulated in their cells were analyzed by HPLC. As shown in Figure 3, the recombinant *H. elongata* BN1 accumulated a much higher amount of Ect than GB, which is the opposite of the result obtained for the wild-type *H.* elongata 30018 cultured in the high-salinity LB medium (Fig. 1A). However, the level of GB accumulated in the *H. elongata* BN1 cells was not significantly different from the level accumulated in *H. elongata* OUT30018 cultivated under the same conditions. This result indicates that, although *betH* gene deletion confers enhanced Ect accumulation in *H. elongata* BN1, this deletion alone was insufficient to suppress the GB transporting activity of *H. elongata* BN1 cells. Interestingly, different from the cell cultured in LB medium containing 15% w/v NaCl (Fig. 1A), *H. elongata* OUT30018 cultured in SH medium containing 9% w/v NaCl did not accumulate Ect in the cells (Fig. 3B). This result implies the existence of a strong suppression mechanism on Ect production or accumulation in the cell of *H. elongata* OUT30018 cultured in high-salinity SH medium.

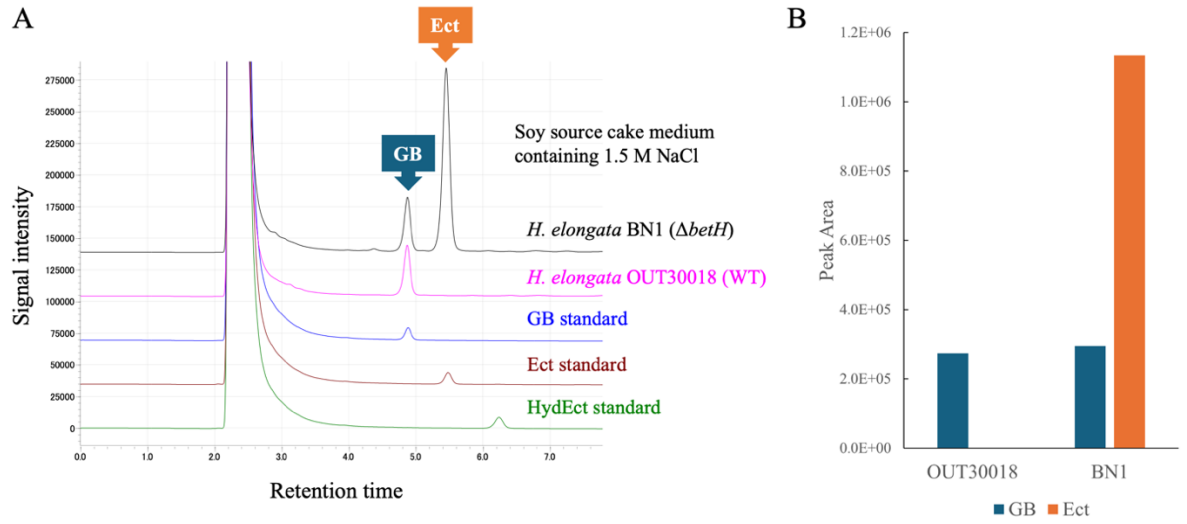

Figure 3. The amount of Ect and GB accumulated in the cells of *H. elongata* OUT30018 and BN1 strains cultured in SH medium containing 9% w/v (1.5 M) NaCl. GB, glycine betaine; Ect, ectoine.

4. CONCLUSIONS AND PROSPECTS

Based on the results obtained in this study, we conclude that the deletion of the *betH* gene positively affected the production and accumulation of Ect *in H.* elongata BN1 grown in high-salinity medium that contains GB. As the concentration of GB was still relatively high in the cells of *H. elongata* BN1, we plan to delete more GB transporter genes from the *H. elongata* BN1 genome to increase Ect accumulation further. To our surprise, *H. elongata* OUT30018 cells cultured in a high-salinity SH medium did not accumulate Ect inside their cells (Fig. 3B). This may be the result of severe suppression of Ect biosynthesis by high concentration of GB as shown in the case of *H. elongata* sp SBS 10 [5]. Other explanations include the influence of unknown amino acids or amino acid derivatives that can be imported from the SH medium and accumulated as intracellular osmolytes. Further experiments to obtain a comprehensive amino acid profile of the *H. elongata* OUT30018 and BN1 strains cultured in the high-salinity SH medium may help explain this result.

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