Metabolic engineering of high-salinity induced biosynthesis of γ-aminobutyric acid improves salt-stress tolerance in a glutamic acid overproducing mutant of an ectoine-deficient *Halomonas elongata*

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Short running title

High-salinity induced GABA biosynthesis in *H. elongata*

Author Statement

Conflict of Interest

The authors declare no conflict of interest.

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ABSTRACT

A moderately halophilic eubacterium, *Halomonas elongata*, has been used as cell factory to produce fine chemical 1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid (ectoine), which function as a major osmolyte protecting the cells from high-salinity stress. To explore the possibility of using *H. elongata* to biosynthesize other valuable osmolytes, an ectoine-deficient salt-sensitive *H. elongata* deletion mutant strain KA1 (Δ*ectABC*), which only grows well in minimal medium containing up to 3% NaCl, was subjected to an adaptive mutagenesis screening in search of mutants with restored salt tolerance. Consequently, we obtained a mutant, which tolerates 6% NaCl in minimal medium by overproducing L-Glutamic acid (Glu). However, this Glu overproducing (GOP) strain has a lower tolerance level than the wild-type *H. elongata*, possibly because acidity of Glu interferes with pH homeostasis of the cell and hinders its own cellular accumulation. Enzymatic decarboxylation of Glu to γ-aminobutyric acid (GABA) by a Glu Decarboxylase (GAD) could restore cellular pH homeostasis, therefore, we introduced an engineered salt-inducible *HopgadBmut* gene, which encodes a wide pH-range GAD mutant, into the genome of the *H. elongata* GOP strain. We found that the resulting *H. elongata* GOP-Gad strain exhibits higher salt tolerance than the GOP strain by accumulating high concentration of GABA as an osmolyte in the cell (176.94 μmol/g cell dry weight in minimal medium containing 7% NaCl). With *H. elongata* OUT30018 genetic background, *H. elongata* GOP-Gad strain can utilize biomass-derived carbon and nitrogen compounds as its sole carbon and nitrogen sources, making it a good candidate for development of GABA-producing cell factories.

IMPORTANCE

While the wild-type moderately halophilic *H. elongata* can synthesize ectoine as a high-value osmolyte via the aspartic acid metabolic pathway, a mutant *H. elongata* GOP strain identified in this work opens doors for biosynthesis of alternative valuable osmolytes via glutamic acid metabolic pathway. Further metabolic engineering to install a GAD system into the *H. elongata* GOP strain successfully created a *H. elongata* GOP-Gad strain, which acquired higher tolerance to salt stress by accumulating GABA as a major osmolyte. With ability to assimilate biomass-derived carbon and nitrogen sources and thrive in high salinity environment, the *H. elongata* GOP-Gad strain can be used in the development of sustainable GABA-producing cell factories.

KEYWORDS

Halomonas elongata, compatible osmolyte, L-glutamic acid, metabolic engineering, γ-aminobutyric acid

INTRODUCTION

Halophilic eubacteria possess varieties of physiological functions to facilitate their adaption to extracellular osmotic changes, which happen dynamically in their habitats. To protect cell and cellular components such as membranes and proteins from hyperosmotic stress under high salinity, certain halophilic bacteria and *Archaea* maintain their intracellular osmotic homeostasis through biosynthesis, accumulation, and transportation of specific organic compatible solutes, which are small organic osmolytes with high water solubility that can be accumulated to a high concentration in the cells without interfering with cellular activities. Previous studies have shown that many bacteria including not only halophiles but also thermophiles as well as some *Archaea* including methanogens could efficiently biosynthesize and accumulate various compatible osmolytes including polyols, sugars, amino acids, and amino acid derivatives such as *N,N,N*-trimethyl glycine (glycine betaine) and 1,4,5,6-tetrahydro-2-methyl-4-primidinecarboxylic acid (ectoine) (1-4).

A moderately halophilic eubacterium, *Halomonas elongata*, can be found in various high-salinity environments. For example, *H. elongata* DSM 2581^T strain (formerly 1H9 strain) was isolated from a solar salt facility in the Netherlands (5) and *H. elongata* OUT30018 strain (formerly KS3 strain) was isolated from a high-salinity agricultural field in Khon Kaen, Thailand (6, 7). *H. elongata* DSM 2581^T and OUT30018 strains are known to adapt to dynamic range of salinity around 0.1-32.5% NaCl and 0.3-21% NaCl, respectively (6). When exposed to high-salinity environment with salt concentration higher than 3% NaCl, *H. elongata* cells biosynthesize and accumulate ectoine as a major osmolyte and when the cells are transferred to a low salinity medium (3% NaCl; osmotic down shock), the cells secrete ectoine rapidly into the environment to reestablish osmotic equilibrium (8). Formerly, applying this process of synthesis and secretion upon osmotic shock (the so-called bacterial milking process), *H. elongata* was used in ectoine production (8). Later on, engineered *H. elongata* strains have

been developed to improve ectoine yield, for example, *H.* elongata DSM 2581^T strain that overproduces and excretes ectoine into the culture medium have been successfully used for the production of ectoine at industrial level (9-11). The ability of *H. elongata* strains to assimilate various carbon and nitrogen sources including biomass-derived sugars, amino acids, and biogenetic amines together with its ability to thrive in high salinity environment (5, 12, 13) make them valuable as cell factory host for producing useful compounds from biomass waste. *Halomonas*-based cell factories were recently developed as the next-generation industrial biotechnology for producing various chemicals, demonstrating that *Halomonas* strains can be used as low-cost chassis for biomanufacturing with high-salinity seawater-based unsterile open fermentation (14).

Different from the *H. elongata* DSM 2581^T, *H. elongata* OUT30018 can utilize the major putrefactive non-volatile amines, which are histamine and tyramine, as its sole carbon and nitrogen sources (13). Therefore, *H. elongata* OUT30018 would be one of the most promising cell factories for production of fine chemicals such as ectoine from the putrefactive non-volatile amines in protein-rich bio-waste. Here, we explored the possibility of using *H. elongata* OUT30018 as cell factory to produce high value osmolyte, other than ectoine. In this study, an ectoine-deficient salt-sensitive *H. elongata* mutant strain KA1, which can grow well only in culture medium containing 3% NaCl due to the loss of the ectoine biosynthesis genes (Δ*ectABC*; FERM P-22094) (15), was subjected to an adaptive mutagenesis screening in search of spontaneous suppressor mutants with restored salt tolerance trait. Consequently, a spontaneous phenotypic suppressor mutant, which could grow on a medium containing 6% NaCl was obtained. HPLC analysis of major osmolytes in the spontaneous suppressor mutant cells revealed that the mutant became more salt tolerant than the KA1 strain by producing and accumulating L-Glutamic acid (Glu) at a higher level than the KA1 strain. Therefore, we named this mutant *H. elongata* Glu overproducing (GOP) strain. However, the acidic nature of Glu

has interfered with its own cellular accumulation and restricted the GOP strain from achieving tolerance to salt stress greater than 7% NaCl in the medium.

The Glutamic Acid Decarboxylase (GAD) system has been known to facilitate intracellular pH homoeostasis by consuming protons in a decarboxylation reaction that simultaneously produces an osmolyte γ-aminobutyric acid (GABA) from Glu (16). To establish the GAD system in the *H. elongata* GOP strain, a synthetic *H. elongata*'s codon-usage optimized (Hop) *GadB* mutant gene (*HopGadBmut*), which encodes a mutant GAD with activity under broader pH range than the wild-type GAD (17), was put under the control of a salt-inducible *ectA* promoter and introduced into the genome of the *H. elongata* GOP strain. HPLC analysis confirmed that the resulting *H. elongata* GOP-Gad strain produced and accumulated GABA in response to salt stress. Moreover, the *H. elongata* GOP-Gad strain also showed higher salt-stress tolerance than the GOP strain. We concluded that *de novo* biosynthesis and cellular accumulation of GABA attribute to higher salt tolerance trait of the engineered *H. elongata* GOP-Gad strain. We present here, the first report demonstrating that Glu and GABA could function as major osmolytes in *H. elongata* under high salinity growth condition, and that GABA is a better compatible osmolyte than Glu because it can be accumulated to a higher concentration in the cells without interfering with cytosolic pH homeostasis.

RESULTS

Adaptive mutagenesis screening identified a suppressor mutant of an ectoine-deficient salt-sensitive *H. elongata* KA1 strain

In search of compounds that can be accumulated in *H. elongata* as osmolytes instead of ectoine, an ectoine-deficient salt-sensitive *H. elongata* KA1 strain (Table 1), in which the ectoine biosynthesis operon harboring the *ectABC* genes was removed from the wild-type *H.*

158 *elongata* OUT30018 strain (6), were subjected to an adaptive mutagenesis screening to find
159 suppressor mutants with restored salt-tolerant trait. The *H. elongata* KA1 strain can grow well
160 in liquid M63 medium containing 3% NaCl and grow with slight growth suppression in the
161 M63 medium containing 4% NaCl, but not in the medium containing 6% NaCl. Therefore, in
162 this screening, the *H. elongata* KA1 strain was precultured for 2 days in liquid M63 medium
163 containing 3% NaCl before the cells were harvested and used as a 5% inoculum for a main
164 culture in liquid M63 medium containing 6% NaCl. After culturing for 7 days, suspension of
165 the cells harvested from the main culture were streaked on a solid M63 medium containing 6%
166 NaCl. As a result, a mutant, which contains a mutation that partially suppressed the salt-
167 sensitive phenotype of *H. elongata* KA1 strain was identified.
168 Glycerol is one of the important biomass derived carbon sources produced as the by-products
169 from oleochemical and biodiesel industries (18, 19). Therefore, glycerol is used instead of
170 glucose as a sole carbon source in the M63 medium used for growth test to determine the level
171 of salt tolerance of the newly isolated mutant. Growth of the mutant was compared to that of
172 the wild-type *H. elongata* OUT30018 and the salt-sensitive KA1 strains in liquid M63 medium
173 containing 4% glycerol with different concentration of NaCl. The three bacterial strains were
174 precultured in M63 medium containing 4% glycerol and 3% NaCl before they were used as
175 5% inoculums for the main cultures in M63 medium containing 4% glycerol and 3, 6, 7, or 8%
176 NaCl. As shown in Fig. 1, the mutant could tolerate growth condition with higher salt stress
177 than the *H. elongata* KA1 strain, *i.e.,* it could grow in M63 medium containing 6% NaCl, while
178 the *H. elongata* KA1 strain could not. However, the mutant is still less salt tolerant than the
179 wild-type *H. elongata* OUT30018 and fails to grow in the medium containing more than 7%
180 NaCl.
181

Identification of major osmolytes accumulated under high salinity growth conditions in the suppressor mutant of the salt-sensitive *H. elongata* KA1 strain

To identify compounds, which confer salt tolerance to the isolated mutant, amino acid profile of the mutant grown under no-stress or under salt-stress conditions was determined by HPLC analysis. In extraction step, weight of wet cell pellet was measured as cell fresh weight (CFW) and cytosolic amino acids accumulated as osmolytes were extracted from the cells by hypo-osmotic shock extraction with pure water mimicking the bacterial milking method (8, 20). As shown in Fig. 2A and 2B, both the ectoine-producing wild-type *H. elongata* OUT30018 strain and the ectoine-deficient *H. elongata* KA1 strain accumulate higher amount of Glu than L-alanine (Ala), however, Glu accumulated in the wild-type *H. elongata* OUT30018 strain stay essentially the same in the cells grown under salt-stress conditions (6 and 7% NaCl in culture medium) due to the accumulation of ectoine as a major osmolyte (data not shown). As shown in Fig. 2C, the suppressor mutant accumulated higher concentration of Glu and Ala in the cells when grown under high salt-stress condition, *i.e.* the mutant accumulated 25.58±1.94 μmol/g CFW of Glu and 11.32±2.94 μmol/g CFW of Ala when grown under no stress condition (grown in M63 medium containing 3% NaCl), while it accumulated 32.42±2.27 μmol/g CFW of Glu and 16.99±1.47 μmol/g CFW of Ala when grown in M63 medium containing 7% NaCl. Glu accumulated in the cells of the mutant notably increased with the degree of salt-stress ($p \leq 0.05$) and was 1.9-fold higher than that of Ala under salt-stress condition. Moreover, due to the lack of ectoine synthetic pathway in the KA1, GOP and the GOP-Gad strains, Ala concentration in these strains are considerably higher than that accumulated in the wild-type OUT30018 strain probably due to the metabolic shift that increase the amount of pyruvate pool for Ala synthesis (Fig. 10). These results suggested that Glu functions as a major osmolyte that confers higher salt-stress tolerance to the suppressor mutant, therefore, we named this mutant *H. elongata* Glu overproducing (GOP) strain.

207 The *H. elongata* GOP strain was, however, unable to thrive in the M63 medium containing
208 8% NaCl (Fig. 1), possibly due to the inability of the cell to accumulate the acidic amino acid,
209 Glu, to a concentration high enough to counter the effect of severe salt stress without interfering
210 with intracellular pH homeostasis.
211
212 **Introduction of custom-engineered salt-stress responsive Glutamic Acid Decarboxylase**
213 **(GAD) system into *H. elongata* GOP and *H. elongata* KA1 strains**
214 In acidic environments, the bacterial GAD system facilitates intracellular pH homeostasis
215 through a decarboxylation reaction that raises cellular pH by consuming protons in the reaction
216 that produces γ-aminobutyric acid (GABA) from Glu (16, 21). Although the enzyme GAD is
217 active only in acidic environment, double mutations in *E. coli*'s GadB at Glu89 and His465,
218 which involved in a cooperativity system of GAD, could generate a mutant GAD
219 (Glu89Gln/Δ452-466, named GadBmut), which is active even at a neutral pH (active from pH
220 4 to 8) (17, 22). We saw a potential of using the GadBmut as a tool to mitigate acid stress that
221 is caused by Glu accumulation in the *H. elongata* GOP strain. In addition to neutralizing the
222 intracellular pH, GadBmut would also generate GABA from Glu in the *H. elongata* GOP cells.
223 This can be beneficial for the *H. elongata* GOP cells because GABA was reported to function
224 as a compatible osmolyte in plant cells (23) and in other bacteria (24, 25).
225 To express GadBmut in *H. elongata* GOP strain, we designed and constructed an artificial
226 bicistronic *mCherry-HopGadBmut* operon by putting an *mCherry* reporter gene, which
227 encodes a red-florescent protein, and a *H. elongata*'s codon-usage optimized *GadBmut*
228 (*HopGadBmut*) gene under the control of an endogenous salt-stress inducible *ectA* promoter
229 which contains two putative biding sites for the vegetative sigma factor σ^{70} and the
230 hyperosmotic stress responsive sigma factor σ^{38} (9). Then, as shown in Fig. 3, the bicistronic
231 operon was introduced into the genome of *H. elongata* GOP and KA1 strains to generate *H.*

elongata GOP-Gad and KA1-Gad strains. Selection of the resulting recombinant *H. elongata* GOP-Gad and KA1-Gad strains and confirmation of the transgene expression were facilitated by visualization of the red fluorescence of the mCherry reporter protein. As shown in the upper panel of Fig. 4A, pellets of the recombinant *H. elongata* GOP-Gad and KA1-Gad cells cultured under optimal salt condition (M63 medium containing 3% NaCl) emitted red fluorescence, which were further intensified in the cells that were grown under salt-stress condition (M63 medium containing 6% NaCl, Fig. 4A, lower panel).

Fig. 4B shows immunodetection of the mCherry reporter protein in the crude proteins extracted from the recombinant *H. elongata* GOP-Gad and the KA1-Gad strains grown in liquid M63 media containing 3 or 6% NaCl. Corresponding to the increase in the fluorescent intensity shown in Fig. 4A, the amount of mCherry protein also increased when the cells were grown in medium with high salt content. These results suggest that expression of the artificial *mCherry-HopGadBmut* operon is induced by salt stress under the control of the *ectA* promoter in the recombinant *H. elongata* GOP-Gad and KA1-Gad strains.

Confirmation of GABA production in *H. elongata* GOP-Gad and *H. elongata* KA1-Gad strains

To confirm that the codon-optimized *HopGadBmut* gene was expressed in the recombinant *H. elongata* KA1-Gad and GOP-Gad strains, and that the HopGadBmut protein could function properly to synthesize GABA from Glu, major osmolytes in cells of the *H. elongata* KA1-Gad and GOP-Gad strains were profiled in comparison to that of the *H. elongata* KA1 and GOP strains. All strains were cultured in M63 medium containing 4% glycerol and 3% NaCl, in which the *H. elongata* KA1 and KA1-Gad strains could grow as well as the *H. elongata* GOP and GOP-Gad strains, and major osmolytes were extracted and analyzed by HPLC. As shown in Fig. 5, 25.47±4.99 and 24.99±5.12 μmol/g CFW of GABA were detected in the *H. elongata*

KA1-Gad and GOP-Gad strains, but not in the *H. elongata* KA1 and GOP strains. Notably, concentration of Glu accumulated in the *H. elongata* KA1-Gad and GOP-Gad strains were 7.17±0.88 and 7.47±2.19 μmol/g CFW, the amount much lower than 28.48±4.07 and 30.21±4.85 μmol/g CFW of Glu accumulated in the *H. elongata* KA1 and GOP strains.

This result showed that the engineered HopGadBmut in the recombinant *H. elongata* KA1-Gad and GOP-Gad strains could successfully convert Glu to GABA even in the absent of coenzyme pyridoxal-5'-phosphate (PLP) supplementation in the media. This is different from other bacterial strains currently used for developing GABA-producing cell factory (26-28). As growth of the recombinant *H. elongata* KA1-Gad strain was severely inhibited in M63 medium containing more than 6% NaCl (data not shown), this strain was not used in experiment hereafter.

Salt-inducible bioconversion of Glu to GABA improves salt tolerance of the recombinant *H. elongata* GOP-Gad strain

To examine the effect of GABA production on the level of salt tolerance of the recombinant *H. elongata* GOP-Gad strain, growth curves of the *H. elongata* GOP and GOP-Gad strains, cultured in M63 medium containing 4% glycerol with a series of increasing salt-stress level (3, 6, 7, and 8% NaCl), were plotted and compared. As shown in Fig. 6A and B, although both the GOP and the GOP-Gad strains could proliferate equally well in M63 medium containing 3% NaCl, a significant difference can be observed when they were cultured in M63 medium containing 6 or 7% NaCl. After 7 days of cultivation in M63 medium containing 6% NaCl, OD_{600} of *H. elongata* GOP-Gad culture was 0.47 while that of the *H. elongata* GOP culture was 0.15. When cultured in M63 medium containing 7% NaCl, OD_{600} at day 7 of *H. elongata* GOP-Gad culture reached 0.18 while *H. elongata* GOP strain was barely proliferated ($p \leq 0.05$) (Fig. 6C).

Notably, during routine subculture of the *H. elongata* GOP-Gad strain, we found substantial variation in growth rate among cultures. To determine the underlying cause of this growth variation, 30 test tubes of *H. elongata* GOP-Gad cultures were grown in M63 medium containing 4% glycerol and 7% NaCl and intracellular contents of the cells harvested at late log phase, when OD_{600} reached 0.80–1.11, were analyzed by HPLC. We found that GABA concentration of the cultures varied greatly from 3.51 to 34.78 μmol/g CFW (Fig. 7A), which result in a large standard deviation of GABA concentration among cultures as shown in Fig. 7A. After 7 days, evident difference in growth was observed among the cultures (OD_{600} varied from 0.51 to 1.11), 21 of the 30 cultures had OD_{600} of more than 0.80 (0.80 to 1.11) and were categorized as a fast-growing group, while the remaining 9 cultures had OD_{600} that was less than 0.80 (i.e., 0.51 to 0.75) and were categorized as a slow-growing group (Fig. 7B). Remarkably, when the amount of Glu and GABA accumulated in the cells of the two groups were compared (Fig. 7C), the average cellular concentration of GABA in the fast-growing group was 26.11±5.56 μmol/g CFW, a much higher concentration than the average 6.19±2.41 μmol/g CFW of the slow-growing group. Moreover, cellular concentration of Glu in the fast-growing group (11.11±3.71 μmol/g CFW) was lower than that found in the slow-growing group (16.16±1.80 μmol/g CFW). These results indicate that conversion of Glu to GABA was more efficient in the fast-growing group, and further confirm that efficient bioconversion of Glu to GABA positively improves growth vigor of the recombinant GOP-Gad strain grown under salt-stress condition.

To determine ratio of Glu to GABA bioconversion in the *H. elongata* GOP-Gad strain grown under different salt-stress conditions, the strain was cultured in M63 medium containing 3, 6, or 7% NaCl. The fast-growing cells were harvested when OD_{600} of the cultures of GOP-Gad strain reached around 0.8 at 2, 6, or 7 days in the medium containing 3, 6, or 7% NaCl, respectively. Then, major osmolytes were extracted from the cells and analyzed by HPLC. As

307 shown in Fig. 8A, Glu, Ala, and GABA were identified as the top 3 osmolytes in the *H.*
308 *elongata* GOP-Gad strain. When grown in M63 medium containing 3%NaCl (no salt stress),
309 Glu (18.30±3.27 μmol/g CFW), Ala (15.45±3.39 μmol/g CFW), and GABA (23.56±3.97
310 μmol/g CFW) were almost equally accumulated as major osmolytes. When grown in M63
311 medium containing 6% NaCl (salt stress), Glu (29.13±4.05 μmol/g CFW) was found as a major
312 osmolyte, at the concentration higher than that of Ala (15.89±3.96 μmol/g CFW) and GABA
313 (17.37±2.99 μmol/g CFW). GABA, however, became the major osmolyte of the *H. elongata*
314 GOP-Gad cells grown in the medium containing 7% NaCl (Fig. 8A). At 34.82±5.33 μmol/g
315 CFW, GABA was accumulated to a much higher concentration that that of Glu (11.97±2.04
316 μmol/g CFW) and Ala (19.17±2.40 μmol/g CFW). Result in Fig. 8B shows that intracellular
317 GABA/Glu molar ratio of the *H. elongata* GOP-Gad cells was at the highest when the cells
318 were grown in the medium containing 7% NaCl.
319
320 **Comparison of major osmolytes extraction methods: wet cells, hypo-osmotic extraction**
321 **method *vs.* freeze-dried cells, phase-separation extraction method**
322 In all free amino acids profiling experiments in this study, free amino acids were extracted
323 from wet pellets of *H. elongata* cells by a simple hypo-osmotic shock treatment with pure water,
324 which mimics bacteria milking process (8, 20). To make certain that this simple extraction
325 method could provide reliable estimation of free amino acid and major osmolytes in the cells,
326 a conventional phase-separation extraction (29) was done in parallel with the hypo-osmotic
327 extraction to prepare major osmolytes samples from the same batch of *H. elongata* GOP-Gad
328 culture. In this comparison experiment, the *H. elongata* GOP-Gad was cultured in M63 liquid
329 medium containing 3% NaCl until it reached mid log phase (OD_{600}=0.7-0.9). This mid log-
330 phase culture was used as a 5% inoculum for the main culture in 120 mL of M63 liquid medium
331 containing 7% NaCl in 300 mL flask, which was incubated at 37 °C with agitation until the

culture reached a late log phase (OD_{600}=1.0-1.2). Cell pellets from 50 mL of this late log-phase culture were harvested in duplicate and the weights of the wet cell pellets were recorded as cell fresh weight (CFW, average 75.33 mg CFW/50 mL culture, n=3). To extract major osmolytes by hypo-osmotic extraction method, pure water was added to one of the pellet samples for hypo-osmotic shock treatment, while the other pellet sample (also average 75.33 mg CFW/50 mL culture, n=3) was freeze dried for use in phase-separation extraction method. The weight of freeze-dried cell pellet was recorded as cell dry weight (CDW, average 19.0 mg CDW/50 mL culture, n=3), and major osmolytes in the cell pellets were extracted with methanol/chloroform/water (10:5:3.4, by vol.) following the method described by Galinski and Oren (29). The amount of GABA in the extracts were then determined by HPLC and the results are shown in Fig. 9. The average concentrations of Glu, Ala, and GABA in the extract derived from hypo-osmotic extraction method were 21.40±15.93 μmol/g CFW, 15.94±3.47 μmol/g CFW, and 42.71±21.13 μmol/g CFW, while that of the extracts obtained by the phase-separation extraction method were 61.30±26.31 μmol/g CDW, 41.16±17.84 μmol/g CDW, and 176.94±139.06 μmol/g CDW (Fig. 9A and B). After converting the unit of the yield back to be per their origin fresh weights, the average concentrations of Glu, Ala, and GABA in the extracts derived from the phase-separation extraction method became 15.54±6.91 μmol/g CFW, 10.18±3.74 μmol/g CFW, and 42.66±29.60 μmol/g CFW (Fig. 9C). To compare efficiency of the two extraction methods, ratios of major osmolytes in the extracts obtained by hypo-osmotic method (Fig. 9A) to those obtained by conventional phase-separation method (Fig. 9C) were calculated. As shown in Fig. 9D, the ratios are all equal to or more than 1 (1.38, 1.57, and 1.00 for Glu, Ala, and GABA respectively) suggest that the hypo-osmotic extraction method could extract more Glu and Ala from the cells than the phase-separation method, while both methods could extract about the same amount of GABA. This result suggests that hypo-osmotic

extraction (bacteria milking) method is a reliable extraction method for quantification of Glu, Ala, and GABA accumulated as major osmolytes in *H. elongata* cells.

DISCUSSION

The classification of microorganisms based on their preference to salt proposed by Oren (30) divided microorganisms into non-halophilic (grow best in medium containing less than 0.2 M or 1.2% NaCl), slight halophile (grow best in medium containing 0.2−0.5 M or 1.2−2.9% NaCl), moderate halophile (grow best in medium containing 0.5−2.5 M or 2.9−14.7% NaCl), borderline extreme halophile (grow best in medium containing 1.5−4.0 M or 8.8−23.5% NaCl), extreme halophile (grow best in medium containing more than 2.5−5.2 M or 14.7−30.6% NaCl), halotolerant microorganisms (non-halophile which can tolerate and grow in medium containing less than 2.5 M or 14.7% NaCl), and extremely halotolerant microorganism (non-halophile, which can tolerate and grow in medium containing above 2.5 M or 14.7% NaCl).

To achieve osmotic adaption, these halophilic and halotolerant bacteria accumulate varieties of organic compatible solutes through *de novo* biosynthesis or by importing the compounds from their environment (31). There was evidence that non-halophilic, slightly halophilic, and halotolerant bacteria accumulate Glu, glutamine (Gln), Ala, proline (Pro), and GABA in their cells in response to increase NaCl in their environment (1, 4, 24, 30-34). Some bacteria, for example *Halobacillus halophilus*, copes with the effect of external salt by accumulating a cocktail of different osmolytes, such as Glu, Gln, Pro, Ala, and ectoine (35).

H. elongata is a moderate halophile, which synthesizes and accumulates ectoine as a major osmolyte when grown in high-salinity environment (3, 6). In this work, to search for novel compounds that could function as osmolytes under high-salinity condition in *H. elongata*, an ectoine-deficient and salt-sensitive *H. elongata* KA1 (15), which only grow well in the medium

381 containing 3% NaCl due to the lack of the 3 ectoine-synthesis genes (Δ*ectABC*) from the
382 genome of the wild-type *H. elongata* OUT30018, was subjected to an adaptive mutagenesis
383 screening in search of spontaneous suppressor mutants with restored or improved salt-tolerant
384 trait. As a result of prolonged culturing of the *H. elongata* KA1 in the medium containing 6%
385 NaCl, a spontaneous phenotypic suppressor mutant, *H. elongata* Glu overproducing (GOP)
386 strain, was obtained. As the name indicated, we found that the *H. elongata* GOP mutant could
387 grow on a medium containing 6% NaCl by producing and accumulating Glu as a major
388 osmolyte in the cells. Being the metabolic entry point for ammonia, Glu is an important amino
389 acid in the cell. In *H. elongata*, however, the importance of Glu is even more pronounced. For
390 wild-type *H. elongata,* both ectoine biosynthesis and Glu synthetase (GOGAT) pathways are
391 activated simultaneously in response to salt-stress and Glu provides amino groups for
392 production of ectoine (36). In ectoine-deficient *H. elongata* KA1 and GOP strains, Glu
393 produced through the GOGAT pathway could be accumulated and function as a major
394 osmolyte when grown in medium containing 3% and 6% NaCl (Fig. 2 and 10). Comparably,
395 an ectoine-deficient and salt-sensitive *H. elongata* SAA4 was found to accumulate Glu and
396 Gln inside the cells when grown in the medium containing 2 to 4% NaCl (37). *H. elongata*
397 SAA4 is a transposon insertion mutant of the *ectA* gene, therefore the enzyme L-2,4-
398 diaminobutylic acid (DABA)-transaminase (DAT) encoded by the *ectB* gene is still
399 functionally expressed in the cells growing in the medium containing 3 to 4% NaCl and
400 produce a small amount of DABA from Glu derived from Gln and α-ketoglutaric acid (α-KG)
401 via the GOGAT pathway (37). As both the *H. elongata* KA1 and the GOP strains no longer
402 have the *ectB* gene on their genome, metabolic homeostasis of Glu and Gln in these strains
403 would be different from that of the *H. elongata* SAA4. Glu also functions as an osmolyte in
404 response to salt stress in other microorganisms. For example, a marine bacterium, *Beneckea*
405 *harveyi*, accumulates increasing amount of Glu in the cell when cultured in medium containing

406 1 to 3% NaCl (38), and a *Rhizobium* sp. strain WR1001, isolated from desert area, accumulates
407 Glu in response to osmotic stress up to 2.9% NaCl (33).
408 Here, we found that Ala was accumulated together with Glu when the *H. elongata* GOP strain
409 was cultured in the medium containing 3 to 7% NaCl (Fig. 2). Ala was also accumulated in the
410 wild-type *H. elongata* OUT30018 and the ectoine-deficient *H. elongata* KA1 strains (Fig. 2B),
411 but not in the closely related *H. elongata* SAA4 strain, which accumulated Gln together with
412 Glu instead (37). As Gln is a precursor of Glu in most bacteria, accumulation of Gln in response
413 to osmotic stress may regulate pH homeostasis to avoid increased accumulation of Glu under
414 high salinity conditions (39). Although a gene encoding a Gln synthetase (GS) is present in the
415 genome of *H. elongata* OUT30018 strain, Gln was not accumulated in the *H. elongata*
416 OUT30018, KA1, and GOP strains under high salinity conditions. The basis of this difference
417 will be an interesting subject for further investigation.
418 We found that the *H. elongata* GOP strain, which accumulates Glu in response to salt stress,
419 has much lower level of salt tolerance than the wild-type *H. elongata* OUT30018. Growth of
420 the *H. elongata* GOP strain was suppressed when cultured in M63 medium containing 7%
421 NaCl (Fig. 1), while the wild-type *H. elongata* OUT30018 strain could grow well in M63
422 medium containing more than 15% NaCl. This probably is caused by inability of the *H.*
423 *elongata* GOP strain to accumulate the acidic Glu to a higher concentration without interfering
424 with intracellular pH homeostasis (39). This possibly is also a reason behind the synthesis and
425 accumulation of specific compatible solutes as osmolytes found in halophilic and moderately
426 halophilic bacteria, which are regularly exposed to severe osmotic stress (above 6% NaCl).
427 Among halophilic bacteria, the moderately halophilic bacteria are the most capable to grow
428 across wide salinity range, from 3 to 15% NaCl. To adapt to dynamic changes in salinity of
429 their environment, these bacteria accumulate not just one but rather two or more compatible
430 osmolytes. For example, *H. elongata* accumulates Glu and ectoine in response to mild salt

stress (4% NaCl), while it accumulates ectoine alone as a major osmolytes under severe salt-stress condition (above 6% NaCl) (3, 6, 39). Similarly, *H. halophilus* accumulates Glu, Gln, and small amount of Pro in response to mild salt stress (2.3% NaCl), while accumulating Pro as a major osmolyte under severe salt stress condition (17.5% NaCl) (40). This led us to find solution to mitigate the acidic stress caused by intracellular accumulation of Glu for improving salt-tolerant level of *H. elongata* GOP strain. In addition, during initial phase of osmotic adaptation to high salinity environments, potassium ion (K^+) may be accumulated as an inorganic compatible solute and as counterions coupled with Glu (1). Therefore, K^+ and Glu may function to balance excess positive or negative charges in the bacterial cells prior to the onset of further adaptive responses against high-salinity stress (1). It will be interesting to observe changes in potassium homeostasis in the *H. elongata* GOP and GOP-Gad cells during their adaptation to salinity stress.

Glutamate decarboxylase (GAD) system is one of the most effective acid responses and tolerance mechanisms in microorganisms (16). The GAD system facilitates intracellular pH homoeostasis by consuming protons during the enzymatic decarboxylation reaction that produces GABA from Glu (16). This system was found in various bacterial species such as *Lactobacillus plantarum*, *L. reuteri*, and *Lactococcus lactis* (41), *L. brevis* (21), *Shigella flexneri* (42), and *E. coli* (43, 44). Moreover, several non-halophilic bacteria including *E. coli*, *Clostridium sporogenes*, and *Streptococcus faecalis* use the GAD system to produce and accumulate GABA as a compatible osmolyte in response to increasing NaCl concentration (24, 25).

Here, using metabolic engineering approach, we installed the GAD system into the *H. elongata* GOP strain to mitigate acidic stress caused by Glu accumulation. A salt-stress responsive bicistronic operon harboring an *mCherry* reporter gene and a *H. elongata* codon-optimized *HopGadBmut* gene, which encode a wide pH range mutant GAD enzyme, was

456 engineered and introduced into the genome of the *H. elongata* GOP strain (Fig. 3). As shown
457 in Fig. 5 and 8, the resulting recombinant *H. elongata* GOP-Gad strain could convert Glu to
458 GABA, which was accumulated as the major osmolyte in the cells, especially when the cells
459 were grown under salt-stress condition. Most importantly, this accumulation of GABA
460 evidently improved salt-tolerance of the *H. elongata* GOP-Gad strain over that of the *H.*
461 *elongata* GOP strain when grown in M63 medium containing 6 and 7% NaCl (Fig. 6).
462 Intriguingly, we observed variation of growth vigor in some batches of the *H. elongata* GOP-
463 Gad cells cultured in M63 medium containing 7% NaCl. Further analysis of these cultures
464 revealed that the cells in the fast-growing cultures accumulated significantly more GABA than
465 that of the slow-growing cultures (Fig. 7C). Moreover, when compared with the slow-growing
466 *H. elongata* GOP-Gad cultures, the cells of the fast-growing cultures emitted stronger red
467 fluorescence of the reporter mCherry protein (data not shown). Because the recombinant
468 *HopGadBmut* gene was put under the control of the same bicistronic operon that control the
469 expression of the *mCherry* gene, higher expression of the *HopGadBmut* transgene could be
470 responsible for better growth vigor of the fast-growing cultures. Further investigation such as
471 western blot analysis to compare the amount of HopGadBmut enzyme produced in these
472 cultures could help clarify this hypothesis.
473 Difference in expression level of the *HopGadBmut* transgene as a result of different
474 activation level of the salt-inducible *ectA* promoter could also be the reason behind the increase
475 in GABA accumulation and Glu-to-GABA conversion efficiency observed when the *H.*
476 *elongata* GOP-Gad cells were grown in the medium with higher salinity (Fig. 8A and 8B).
477 Intracellular pH of *H. elongata* GOP-Gad cells cultured under 6% NaCl condition might not
478 be acidic enough for the HopGadBmut enzyme to perform at their optimal activity, while in
479 the cells cultured under higher salt stress (7% NaCl condition) might cause enough drop of the
480 intracellular pH to the level that could increase the activity of the HopGadBmut enzyme.

In this study, to increase throughput of free amino acids profiling in mutant and recombinant *H. elongata* cells, free amino acids were extracted from wet cells by hypo-osmotic shock extraction using pure water to mimic the bacterial milking method. To confirm that this simple method is reliable, a conventional method (29), which uses methanol/chloroform/water mixture to extract free amino acids from freeze-dried cells was also done in comparison. As shown in Fig. 9A and 9C, we found that the ratios of Glu, Ala, and GABA concentrations in the extract derived by hypo-osmotic extraction (bacterial milking) method (Fig. 9A) to those derived by the phase-separation extraction method (Fig. 9C) were 1.38, 1.57, and 1.00, respectively (Fig. 9D). This result shows that the amount of extracted GABA is similar by both methods, while the amount of Glu and Ala extracted by the hypo-osmotic method are slightly higher those extracted by the phase-separation method. These discrepancies may be caused by different solubility in methanol of the extracted amino acid osmolytes, which could be neutralized by K^+/Na^+ counterions by forming potassium/sodium salts to maintain cytosolic charge balance in the cells (45). Due to enhanced solubility in methanol, the neutralized amino acid salts may be trapped in the lower chloroform phase, which contains small amount of methanol. Therefore, our result suggests that hypo-osmotic extraction method is a reliable method for quantification of cellular content of Glu, Ala, and GABA in *H. elongata* cells.

Conclusion

In this work, we set out to find a new strain of *H. elongata* that can produce and accumulate valuable osmolytes other than ectoine during salt-stress by mutant screening. As a result, a mutant Glu overproducing *H. elongata* GOP strain, which accumulates Glu as its major osmolyte was obtained. At the start, acidic stress caused by Glu accumulation made the strain seems unsuitable for further use. However, in the end, our metabolic engineering approach to install the GAD system, which convert Glu to GABA, into the *H. elongata* GOP strain not only

506 successfully alleviated the problem of acidic Glu accumulation in the resulting *H. elongata*
507 GOP-Gad strain, but also increase salt tolerance of the strain through the accumulation of
508 GABA as a major osmolyte. Therefore, we successfully improved both pH homeostasis and
509 osmotic equilibrium of the *H. elongata* GOP strain by simply introducing a one-step enzyme
510 reaction of the GAD system.

511 *H. elongata* was isolated from brine used in meat fermentation (46), therefore, it is listed as
512 one of the microorganisms with beneficial use (47). With modern fermentation technology, *H.*
513 *elongata* has also been used as cell factory to produce ectoine at industry level (8-11). There
514 are evidence that *H. elongata* OUT30018 strain used in this work could assimilate various
515 biomass-derived carbon sources (glycerol, glucose, xylose, and arabinose) (6, 12) and nitrogen
516 sources (ammonium, amino acids, and their spoilage amines) (13). Other than working as a
517 compatible osmolyte that protect *H. elongata* GOP-Gad cells from salinity stress, GABA is
518 used in many applications. Among these, GABA is used as food supplements and feed
519 additives for poultry farming (48). GABA has also been used in chemical industry to produce
520 biobased 2-pyrrolidone (2-PRN) for synthesizing a biodegradable polymer, Polyamide 4 (PA4)
521 (49-51). Combination of both the versatility of *H. elongata* OUT30018 strain and the
522 usefulness of GABA in various industries has made the *H. elongata* GOP-Gad strain developed
523 in this work a good candidate for development of a biomass-based GABA-producing
524 *Halomonas* cell factory. In future, the GABA-producing *Halomonas* cell factory could be used
525 for upcycling nitrogen-rich high-salinity waste biomasses to produce GABA rich *H. elongata*
526 cells, which can be used as a whole-cell feed additive (named as a single-cell eco-feed). This
527 would contribute to sustainable development of livestock and aquaculture industries for
528 protection of our planetary health.

529

530 **MATERIALS AND METHODS**

Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1 and 2, respectively. *Halomonas elongata* KA1 strain was derived from the wild-type *H. elongata* OUT30018 by deletion of the *ect* operon harboring the *ectABC* genes, which is responsible for ectoine biosynthesis (15). The ectoine-deficient *H. elongata* KA1, which has enhanced salt sensitivity than the wild-type *H. elongata* OUT30018, was used as the initial strain for spontaneous suppressor mutant screening. The *H. elongata* glutamic acid overproducing (GOP) strain was obtained as a suppressor mutant of the salt-sensitive *H. elongata* KA1 strain.

Codon-optimized *HopGadBmut* gene (DDBJ accession number LC649950) encoding *E. coli*'s Glutamic Acid Decarboxylase (GAD) mutant (Glu89Gln/Δ452-466) (17) was designed for expression in *H, elongata* and introduced into *H. elongata* KA1 and GOP strains to generate γ-aminobutyric acid (GABA) producing *H. elongata* KA1-Gad and GOP-Gad strains, respectively.

For routine bacterial cultures, Luria-Bertani (LB) medium (58) was used. LB medium contains 10 g/L Bacto-tryptone, 5 g/L Bacto-yeast extract, and either 10, 20, or 60 g/L NaCl to generate a series of salt-stress media. LB medium containing 1% NaCl was used for routine culturing of *E. coli*. LB medium containing 2% NaCl was used for triparental mating to generate *H. elongata* KA1-Gad and GOP-Gad strains. LB medium containing 6% NaCl was used for routine culturing of *H. elongata* strains. Solid LB medium was supplemented with 15 g/L Agar. The antibiotics Kanamycin (Kan) or Ampicillin (Amp) were added to culture media as selection markers and for maintaining of the plasmids containing the marker genes in recombinant *E. coli* (50 mg/L Kan or 100 mg/L Amp) and in *H. elongata* (100 mg/L Kan) strains. For selection of counter-selectable marker gene (*sacB*) in *H. elongata*, 150 g/L sucrose was added to solid LB medium containing 6% NaCl. *E. coli* and *H. elongata* strains were

555 cultured in the liquid or on the solid media for 17 to 24 hours at 37 °C. Liquid cultures were
556 aerated by shaking in water bath at the speed of 120 rpm.
557 For mutant screening, growth tests, and osmolyte analyses, *H. elongata* strains were cultured
558 in M63 minimal medium (59), which consisted of 100 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 1 mM
559 $MgSO_4$, 3.9 μM $FeSO_4$, supplemented with 3 or 6% NaCl and 2% glucose as the sole carbon
560 source in adaptive mutagenesis screening or with 3, 6, 7 or 8% NaCl and 4% glycerol as the
561 sole carbon source for growth analysis and osmolytes profiling. Glycerol was selected as the
562 sole carbon source because it can be found in biomass derived waste from global biodiesel fuel
563 production including the production established in Nagasaki prefecture, Japan. Initial pH of the
564 media was adjusted to 7.2 with 1 M KOH solution. *H. elongata* strains were precultured in
565 M63 medium supplemented with 3% NaCl and 4% glycerol before being used as 5% (vol/vol)
566 inoculums for the main cultures.
567
568 **Adaptive mutagenesis screening for spontaneous mutant of *H. elongata* KA1 strain with**
569 **increase salt tolerance**
570 The ectoine-deficient *H. elongata* KA1 strain (Δ*ectABC*) pre-cultured for 2 days in M63
571 medium containing 3% NaCl with 2% glucose as sole carbon source was used as a 5%
572 inoculum for the main culture in M63 medium containing 3 or 6% NaCl with 2% glucose as
573 sole carbon source. After 7 days, an aliquot of 100 μL of the main culture was spread onto solid
574 M63 medium containing 6% NaCl to screen for spontaneous mutant with the enhanced salt
575 tolerance.
576
577 **Recombinant DNA construction**
578 Primers used in this study are synthesized by Eurofins Genomics K. K., Tokyo, Japan and
579 sequence of the primers are listed in Table 3. PCR reactions were performed with the

580 primeSTAR® HS DNA Polymerase (Takara Bio Inc., Shiga, Japan) or Quick Taq™ HS
581 DyeMix (Toyobo, Osaka, Japan).
582 For construction of pBSK-*mCherry* to be used as a tool for sub-cloning of DNA fragments,
583 *mCherry* fragment with ribosome binding sequence (*mCherry*-RBS) was PCR amplified with
584 *mCherry*-F and *mCherry*-R primer pair using the pmCherry plasmid (Clontech/Takara Bio Inc.,
585 Shiga, Japan) as a template. The amplified *mCherry*-RBS (692 bp) was digested with
586 restriction enzymes *Xba*I and *Sma*I then inserted into a *Xba*I-*Sma*I gap of pBluescript II SK (-)
587 to create pBSK-*mCherry* (Table 2), which expresses both red fluorescent protein mCherry and
588 lacZ' proteins for red-white and blue-white colony selection.
589 For construction of a salt-inducible bicistronic *mCherry-HopGadBmut* operon, the 5' and 3'
590 flanking regions of *ect* operon (*ectABC*) in the genome of *H. elongata*, upstream fragment of
591 *ectA* gene (U_{ectA}) and downstream fragment of *ectC* gene (D_{ectC}), were PCR amplified from
592 genomic DNA of *H. elongata* OUT30018 using U_{ectA}-F+U_{ectA}-R and D_{ectC}-F+D_{ectC}-R primer
593 pairs. PCR cycles were initial denaturation at 98 °C for 2 min, followed by 30 cycles of
594 denaturation at 98 °C for 10 sec, and annealing/extension at 68 °C for 1 min 30 sec. The
595 amplified D_{ectC} fragment (1,227 bp) was digested with restriction enzymes *Bam*HI and *Hin*dIII
596 then inserted into a *Bam*HI-*Hin*dIII gap of pBSK-*mCherry* to create pBSK-*mCherry*-D_{ectC},
597 while the amplified U_{ectA} fragment (1,203 bp) was digested with restriction enzymes *Xba*I and
598 *Bgl*II then inserted into a *Xba*I-*Bgl*II gap of the pBSK-*mCherry*-D_{ectC} to create pBSK-U_{ectA}-
599 *mCherry*-D_{ectC}. A synthetic *H. elongata*'s codon-usage optimized *HopGadBmut* gene was
600 designed using the Gene Designer software (60) to encode a mutant version of Glutamic Acid
601 Decarboxylase (GAD), which is active under broader pH range than the wild-type GAD (17).
602 The *Nde*I-*HopGadBmut*-*Bam*HI fragment was subcloned from pUC57-*HopGadBmut* (Table
603 3) using the pET-*Lipop5*-*HA* as a sub-cloning vector to generate pET-*HopGadBmut* plasmid.
604 Then the *Nde*I-*HopGadBmut*-*Bam*HI fragment from the pET-*HopGadBmut* was inserted into

605 a *Nde*I-*Bam*HI gap of the pBSK-U_{ectA}-*mCherry*-D_{ectC} plasmid to generate pBSK-U_{ectA}-
606 *mCherry-HopGadBmut*-D_{ectC}. Finally, the *Xba*I-*UectA-mCherry-HopGadBmut-DectC-*
607 *Hin*dIII fragment from pBSK-*UectA-mCherry-HopGadBmut-DectC* plasmid was inserted into
608 a XbaI-*Hin*dIII gap of the pK18*mobsacB* to generate the pK18*mobsacB*-U_{ectA}-*mCherry-*
609 *HopGadBmut*-D_{ectC}. The *mCherry* and *HopGadBmut* genes were engineered to express as an
610 artificial bicistronic operon under the control of the *ectA* promoter in *H. elongata*. The *mCherry*
611 gene was used as a reporter to confirm expression of the artificial bicistronic operon in *H.*
612 *elongata*.
613
614 **Generation of the recombinant *H. elongata* KA1-Gad and GOP-Gad strains**
615 The U_{ectA}-*mCherry-HopGadBmut*-D_{ectC} fragment in pK18*mobsacB*-U_{ectA}-*mCherry-*
616 *HopGadBmut*-D_{ectC} plasmid, which was maintained in *E. coli* DH5α strain, was introduced into
617 the genome of *H. elongata* KA1 and GOP strains by a 2-step homologous recombination using
618 *E. coli* HB101/pRK2013-mediated tri-parental conjugation method (61). Three bacterial strains,
619 the donor *E. coli* DH5α harboring the pK18*mobsacB*-U_{ectA}-*mCherry-HopGadBmut*-D_{ectC}
620 plasmid, the helper *E. coli* HB101 harboring the pRK2013 conjugative plasmid, and the
621 recipient *H. elongata* KA1 or GOP strains were co-cultured for triparental mating on
622 Omnipore^{TM} membrane (Merck Millipore, Darmstadt, Germany), which was placed on 2%
623 NaCl solid LB medium. After triparental conjugation, cells were cultured on 6% NaCl solid
624 LB medium containing 100 mg/L Kan for selection of single-crossover strains. Subsequently,
625 Kan-resistant recombinant cells were selected and cultured on 6% NaCl LB solid medium
626 supplemented with 15% sucrose for the selection of the second single-crossover strains with
627 sucrose-tolerant phenotype, in which 50% of the cells would be revertants (reverting back to
628 the original *H. elongata* KA1 or GOP strains), and another 50% would be recombinants (KA1-
629 Gad or GOP-Gad strains). The *H. elongata* KA1-Gad or GOP-Gad strains were identified by

genomic PCR using a *HopGadBmut*-specific primer pair (*HopGadBmut*-F and *HopGadBmut*-R, Table 3), which amplify a partial 987 bp fragment of the *HopGadBmut* gene. PCR cycles were initial denaturation at 94 °C for 2 min, follow by 35 cycles of denaturation at 94 °C for 30 sec, and annealing/extension at 68 °C for 1 min 20 sec.

Crude protein extraction

Production of the mCherry reporter protein was used to indirectly verify the expression of the *HopGadBmut* gene. The wild-type *H. elongata* OUT30018 strain (negative control), *H. elongata* KA1-Gad strain, and *H. elongata* GOP-Gad strain, cultured in liquid M63 medium containing 3 or 6% NaCl to optical density at 600 nm (OD_{600}) of around 0.8, was used as a 5% inoculum for the main culture in the same salinity medium. When OD_{600} of the main cultures reached 1, the cells were collected by centrifugation at 13,000 rpm for 3 min, resuspended in 200 µL PBS (phosphate-buffered saline), and disrupted by 3 sets of 30-sec sonication to yield cell lysates, which contain crude protein extract. 15 µL aliquot of lysates from each strain were mixed with 15 µL Ez-Apply dye (Tris-HCl buffer, 1% SDS, 10% sucrose, BPB, and 50 mM DTT; AE-1430, ATTO Corporation, Tokyo, Japan), homogenized, and heated at 100 °C for 3 min to prepared for electrophoresis separation on SDS-polyacrylamide gel.

Western blot analysis

Identical set of crude protein samples were separated by electrophoresis in two 5–20% gradient SDS-polyacrylamide gels (E-R520L, ATTO Corporation, Tokyo, Japan) in Ez-Run (25 mM Tris, 192 mM glycine and 0.1% SDS; AE-1410, ATTO Corporation, Tokyo, Japan) buffer. Separated proteins on one of the gels were electrophoretically transferred onto PVDF membranes (WSE-4050, ATTO Corporation, Tokyo, Japan) using western blotting transfer buffer EzBlot (AE-1460, ATTO Corporation, Tokyo, Japan). Nonspecific binding sites were

655 blocked using TBS-T solution containing 0.1% EzTween20 (WSE-7235, ATTO Corporation,
656 Tokyo, Japan) in EzTBS buffer (25 mM Tris, 150 mM NaCl; WSE-7230, ATTO Corporation,
657 Tokyo, Japan) with 5% skim milk for 1 hr at room temperature. The membrane with transferred
658 protein was washed once with TBS-T and incubated with 5 μL of primary antibody (RFP-tag
659 Rat monoclonal 5F8, 1:1000, ChromoTek, Planegg-Martinsried, Germany) in 20 mL TBS-T
660 with 5% skim milk for 2 hr at room temperature. After 3 washes in TBS-T with 5% skim milk,
661 the membrane was incubated with 5 μL of second antibody (Goat anti-Rat IgG/IgM(H+L) HRP,
662 1:1000, Novus Biologicals USA) in 20 mL TBS-T with 5% skim milk for 1 hr at room
663 temperature. After 3 washes with TBS-T, immunoreactive bands were visualized by incubating
664 the membrane in reaction solution EzWestlumi (AE-1495, ATTO Corporation, Tokyo, Japan).
665 The other SDS-polyacrylamide gel was dyed with EzStain AQUA (Coomassie brilliant Blue
666 (CBB); AE-1340, ATTO Corporation, Tokyo, Japan) at room temperature for 1 hr for
667 visualization of total proteins separated in each lane.
668
669 **Hypo-osmotic extraction (bacterial milking) of free amino acids from *H. elongata* cells**
670 *H. elongata* strains were cultured in liquid M63 medium supplemented with 4% glycerol
671 with different level of salinity until OD_{600} reached around 0.8 (log phase), then the cells were
672 harvested by centrifugation at 13,000 rpm for 3 min and the weight of the cell pellet was
673 recorded as cell fresh weight (CFW). Mimicking the bacteria milking process (8, 20), the cell
674 pellets were suspended in of pure water (20 μL per 1 mg cell fresh weight) for hypo-osmotic
675 extraction of free-amino acids from the cells. After centrifugation at 13,000 rpm for 3 min, the
676 supernatant containing the free amino acids was collected as major osmolytes' sample.
677
678
679

Phase-separation extraction of free amino acids from *H. elongata* cells

H. elongata was cultured, harvested, and fresh weight of the cell pellets (CFW) were determined in the same way as mentioned in the Hypo-osmotic extraction (bacterial milking) method. However, this method (29) required the cells to be freeze dried before the extraction, therefore, the cell pellets were frozen overnight in -80 °C freeze before they were freeze dried overnight in a Freeze dryer (FDL-1000, Shanghai EYELA CO. LTD, Shanghai, China) and the weight of the freeze-dried pellets were recorded as cell dry weight (CDW). The extraction was done by vigorously mixing the freeze dried cells overnight with the extraction solution, which contains 10:5:3.4 by vol. mixture of methanol:chloroform:water (18.4 mL extraction solution/mg CDW). After centrifugation (5000×g) to remove cell debris, the supernatant was re-extracted in 1:1 by vol. mixture of chloroform:water (10 mL chloroform:distilled water/mg CDW). After vigorous mixing and centrifugation (5000×g), the top hydrophilic phase was collected as major osmolytes' sample.

Amino acids dabsylation

Amino acids dabsylation was performed based on a previous method (62) with a slight modification. Ten µL aliquot of free amino acids extracted from *H. elongata* cells or standard amino acids was mixed with 2 µL of 2.5 mM internal standard norvaline and 8 µL of 1 M $NaHCO_3$ pH adjustment solution. Then, the sample was mixed with 40 µL of dabsylation reagents containing 2 mg/L dabsyl chloride dissolved in acetonitrile and incubated at 70 °C for 15 min. After the incubation, 440 µL of 250 mM $NaHCO_3$ solution was added and the samples were centrifuged at 13,000 rpm for 3 min. The supernatant was collected and filtered through a filter vial with 0.2 µm pore-size PTFE membrane (SEPARA® Syringeless filter, GVS Japan K.K., Tokyo, Japan) prior to HPLC analysis.

705 **HPLC gradient system for determination of dabsyl amino acids**

706 The determination of dabsyl amino acids (63) derived from *H. elongata* cells was carried out
707 using high-performance liquid chromatography (HPLC) system (Shimazdu, Kyoto, Japan)
708 equipped with an UV/VIS detector (SPD-10 A VP), an auto sampler (SIL-10 AD VP), two
709 pumps (LC-10 AD VP), degasser (DGU-14A), system controller (SCL-10A Vp), and column
710 oven (CTO-10AC VP). The LabSolutions LC software (Shimadzu, Kyoto, Japan) was used for
711 the system control and data acquisition. Chromatographic separation of dabsyl amino acids
712 was achieved through an analytical C18 column (Poroshell 120 2.7 μm, EC-C18, 4.6 × 75 mm,
713 Agilent Technologies Inc.) with C18 guard column (Poroshell 120 2.7 μm Fast Guard, EC-C18,
714 4.6 × 5 mm, Agilent Technologies Inc.) using a mobile phase gradient system consisting of
715 15% acetonitrile in 20 mM sodium acetate (pH 6.0) (mobile phase A) and 100% acetonitrile
716 (mobile phase B). Dabsyl amino acids were determined by the UV/VIS detector at 468 nm.
717 The injection volume was 10 μL, the flowrate was 0.5 mL/min, and the column temperature
718 was maintained at 25 °C. The eluent gradient is listed in Table 4.
719
720
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1032 **FIGURES**

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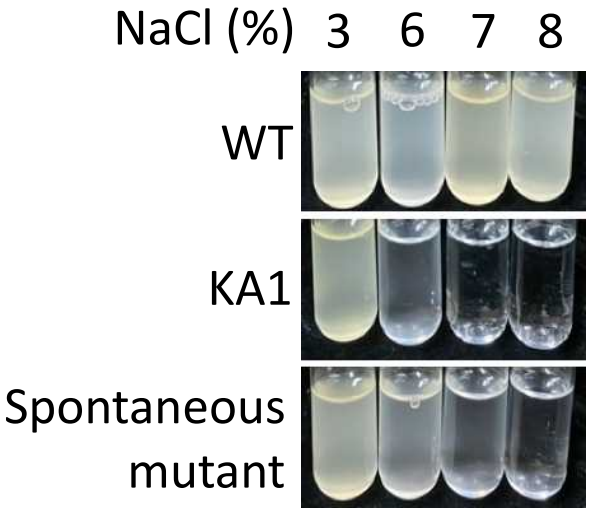

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1036 **Fig. 1. Growth of wild-type *H. elongata* OUT30018 (WT), ectoine-deficient salt-sensitive**
1037 **KA1, and KA1-derived spontaneous mutant strains under different salt-stress conditions.**
1038 *H. elongata* strains were precultured in M63 medium supplemented with 4% glycerol and 3%
1039 NaCl until Optical Density at 600 nm (OD_{600}) reached around 0.8 and used as 5% inoculum
1040 for main cultures in M63 medium containing 4% glycerol with different NaCl concentrations
1041 (3, 6, 7, or 8% NaCl). Photos of the cultures were taken after 48 hrs of incubation to show
1042 differences in cell density.
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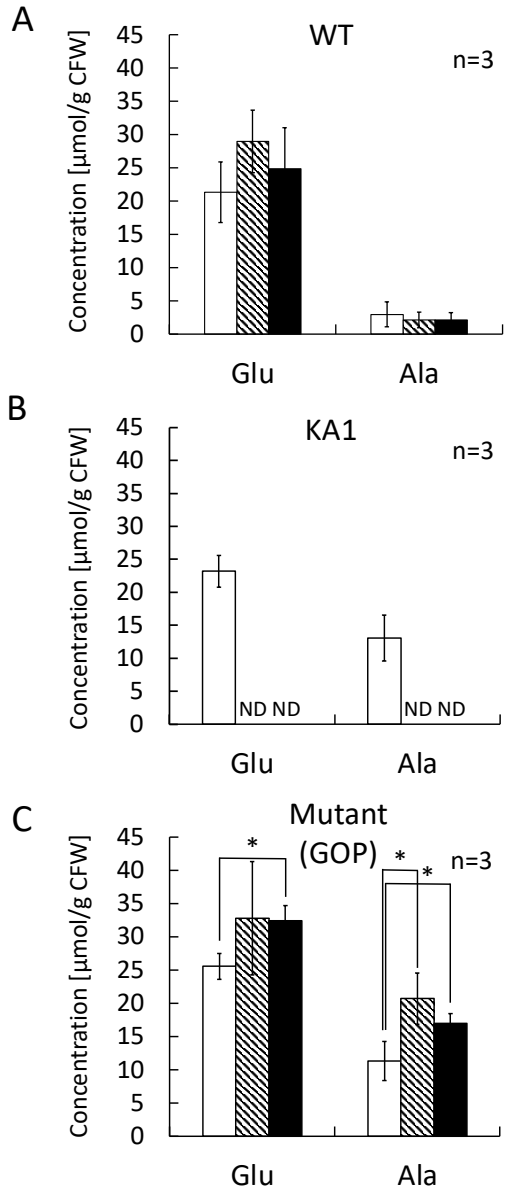

Fig. 2. Profiles of L-glutamic acid (Glu) and L-alanine (Ala) in the cells of wild-type (WT) *H. elongata* OUT30018, ectoine-deficient salt-sensitive KA1, and KA1-derived spontaneous mutant glutamic acid overproducing (GOP) strains culturing in M63 medium containing 4% glycerol with 3 (open column), 6 (hatched column), or 7% (filled column) NaCl.

H. elongata strains were pre-cultured in M63 medium containing 4% glycerol with 3, 6, or 7% NaCl until OD_{600} reached more than 0.80 and used as a 5% inoculum for the main cultures in same salinity (3, 6, or 7% NaCl) medium. When OD_{600} of the main cultures reached more than 0.80, free amino acids including Glu and Ala were extracted from the cells by dissolving cell pellets in pure water [20 µL pure water per 1 mg cell fresh weight (CFW)] and the extracts were analyzed by HPLC. Data were normalized with internal standard norvaline. Values are mean ± standard deviation (n=3). * $p \leq 0.05$. ND: no data; because KA1 strain is unable to grow in M63 medium containing 6 or 7% NaCl.

A. Profile of Glu and Ala in WT *H. elongata* OUT30018 cells.

B. Profile of Glu and Ala in *H. elongata* KA1 cells.

C. Profile of Glu and Ala in *H. elongata* GOP cells.

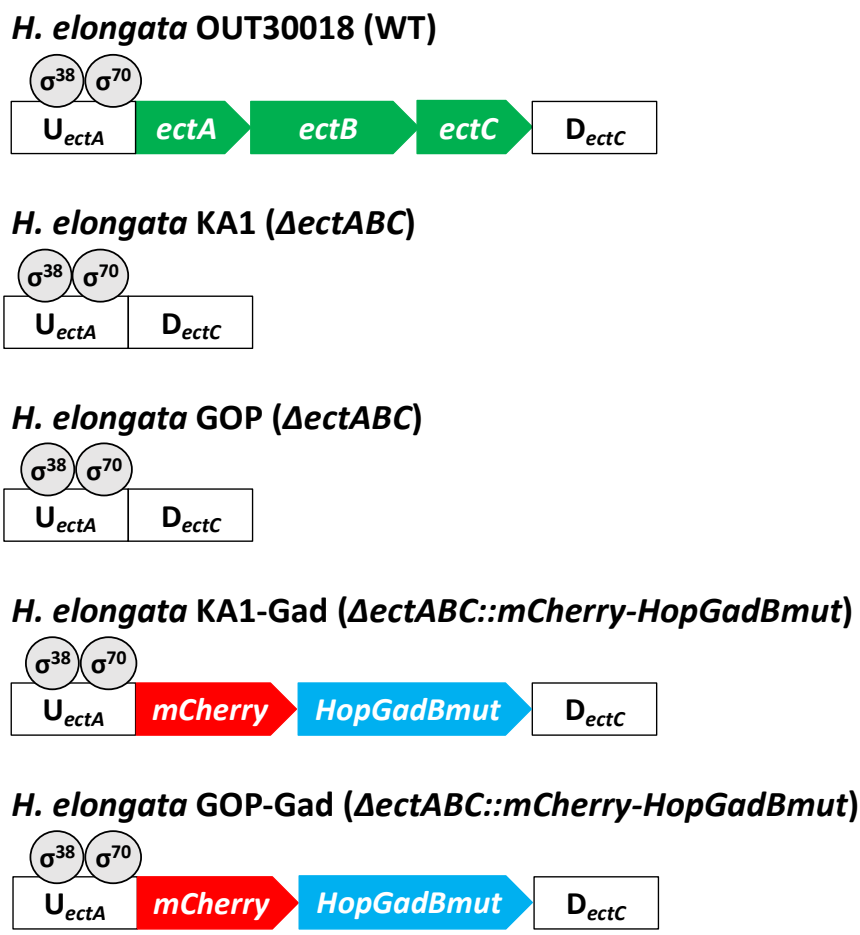

Fig. 3. Schematic diagram of genomic structure at *ectABC* locus in *H. elongata* OUT30018, KA1, GOP, KA1-Gad, and GOP-Gad strains.
U_{ectA}: 1-kb upstream region of the *ectA* gene, which contains an *ectA* promoter with putative binding sites for the osmotically induced sigma factor σ38, and the vegetative sigma factor σ70. This region was used as a target for homologus recombination at the *ectABC* locus.
D_{ectC}: 1-kb downstream region of the *ectC* gene , which contains an *ectC* termniator. This region was used as a target for homologus recombination at the *ectABC* locus.
ectA: gene, which encodes an L-2,4-diaminobutyric acid (DABA) acetyltransferase (DAA)
ectB: gene, which encodes a DABA transaminase (DAT).
ectC: gene, which encodes an ectoine synthase (ES).
mCherry: gene, which encodes a red fluorescent reporter protein mCherry.
HopGadBmut: synthetic *H. elongata's* codon-usage optimized (Hop) *GadB* mutant gene (*HopGadBmut*), which encodes a mutant Glutamate decarboxylase (GAD) with activity across broader pH range than the wild-type GAD.

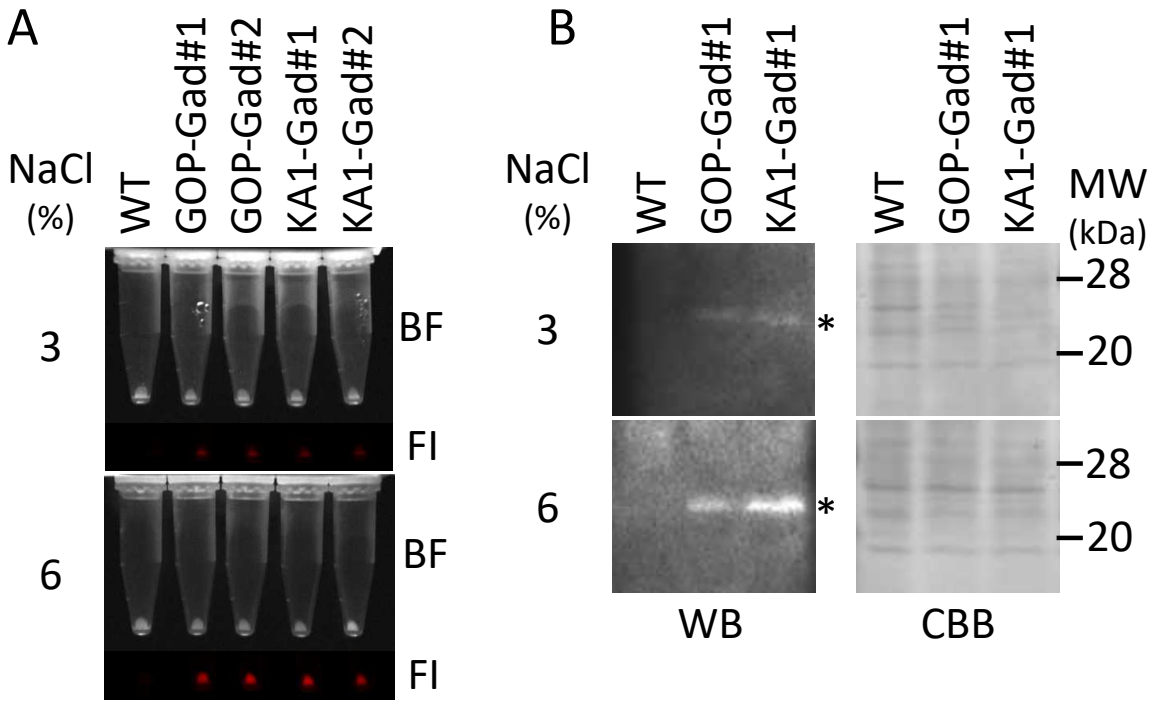

Fig. 4. Salt-inducible production of mCherry reporter protein in recombinant *H. elongata* KA1-Gad and GOP-Gad strains.

H. elongata OUT30018 (WT), GOP-Gad, and KA1-Gad strains cultured in M63 medium containing 4% glycerol with 3 or 6% NaCl until OD_{600} reached more than 1.00 were used as a 5% inoculum for the main cultures in same salinity (3 or 6% NaCl) M63 medium containing 4% glycerol. When OD_{600} of the main cultures reached more than 1.00, the cells were pelleted and subjected to tests.

A. Visualization of the salt-inducible production of the mCherry fluorescent reporter protein in the *H. elongata* GOP-Gad and KA1-Gad strains as shown by mCherry fluorescence of the cell pellets under fluorescent light (Fl) in comparison with Bright-field (BF) images. Cell pellet of *H. elongata* OUT30018 (WT) was used as a negative control.

B. Detection of mCherry protein produced in the *H. elongata* GOP-Gad and KA1-Gad strains by western blot analysis. Proteins extracted from the cell pellets shown in A. were electrophoresed in 2 identical 5–20% gradient SDS-Polyacrylamide gels. One gel was stained with Coomassie Brilliant Blue (CBB; right panels) for visualization of total protein separated on each lane, while proteins on the other gel were transferred to PVDF membrane and probed with antibody to Red Fluorescent Protein (RFP) in western blot analysis (WB; left panels). Protein extracted from *H. elongata* OUT30018 (WT) was used as a negative control. Rat anti-RFP tag was used as a primary antibody and Goat anti-Rat IgG/IgM(H+L) HRP was used as a secondary antibody. mCherry protein bands were detected at 26 kDa (*).

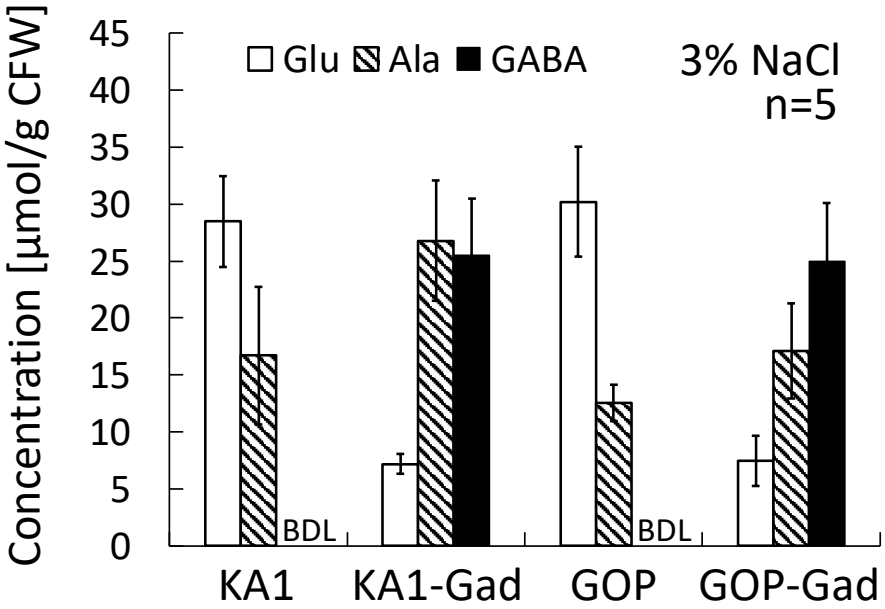

Fig. 5. Profiles of major osmolytes in *H. elongata* KA1, KA1-Gad, GOP, and GOP-Gad cells grown in M63 medium containing 3% NaCl.

Intracellular concentration of major osmolytes, Glu (open columns), Ala (hatched columns), and GABA (filled columns) in *H. elongata* KA1, KA1-Gad, GOP, and GOP-Gad cells cultured in M63 medium containing 4% glycerol with 3% NaCl were profiled. Precultures were grown in M63 medium containing 4% glycerol with 3% NaCl to the OD_{600} of more than 1.00 and used as a 5% inoculum for a main culture in fresh M63 medium containing 4% glycerol with 3% NaCl. When OD_{600} of the main cultures were 0.5 to 0.8 during exponential growth phase, osmolytes were extracted from the cells and analyzed by HPLC. Data was normalized with internal standard norvaline. Values are mean ± standard deviation (n = 5). BDL: below detection limit.

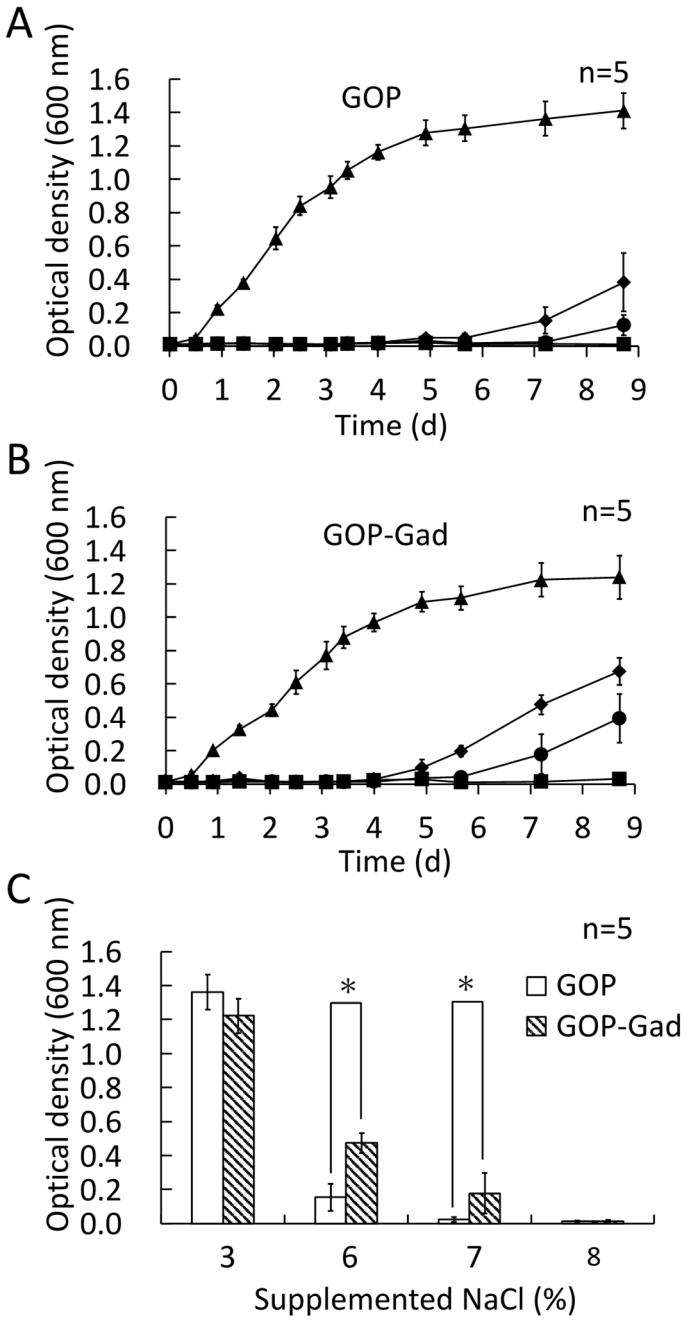

Fig. 6. Effect of medium salinity on growths of *H. elongata* GOP and GOP-Gad strains. *H. elongata* GOP and GOP-Gad strains were precultured in M63 medium containing 4% glycerol with 3% NaCl until OD_{600} reached 1.00 before used as an inoculum for the main cultures in M63 medium containing 4% glycerol with 3(▲), 6(◆), 7(●), or 8% (■) NaCl. The starting OD_{600} of all main cultures were adjusted to 0.01 and OD_{600} of each cell culture was measured at different time points. Values are mean ± standard deviation (n = 5).

A. Growth curve of *H. elongata* GOP strain.
B. Growth curve of *H. elongata* GOP-Gad strain.
C. Growth comparison between GOP (open columns) and GOP-Gad (hatched columns) strains cultured in M63 medium containing 4% glycerol with 3, 6, 7, or 8% NaCl for 7 days. * $p \leq 0.05$.

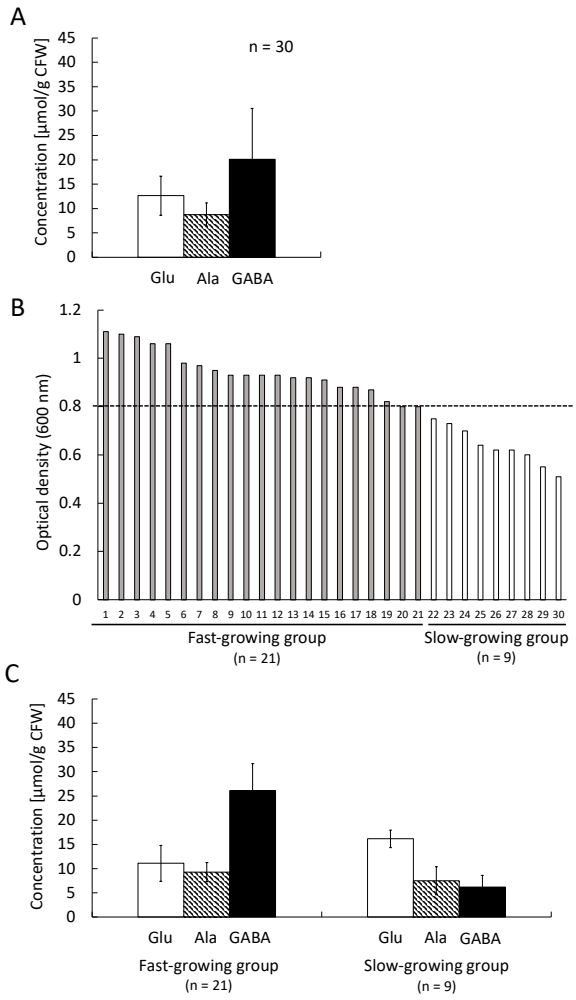

Fig. 7. Differences in major osmolytes composition among *H. elongata* GOP-Gad cultures, which are growing at different rates in high-salinity medium.

H. elongata GOP-Gad cells, cultured in M63 medium containing 4% glycerol and 3% NaCl to OD_{600} of more than 0.80, was used as a 5% inoculum for 30 main cultures in M63 medium containing 4% glycerol with 7% NaCl. When OD_{600} of the main cultures reached 0.80–1.11, intracellular osmolytes were extracted from the cells and analyzed by HPLC.

A. Profiles of major osmolytes of *H. elongata* GOP-Gad strain cultured in M63 medium containing 4% glycerol and 7% NaCl. Concentration of Glu (open columns), Ala (hatched columns), and GABA (filled columns) were normalized with internal standard norvaline. Values are mean ± standard deviation (n = 30).
B. Growth profiles of *H. elongata* GOP-Gad cultures growing at different rate. The cultures were sorted by their OD_{600} at day-7 of cultivation into 2 groups. Out of 30 cultures, 21 cultures with OD_{600} that was equal to or more than 0.80 (OD_{600} = 0.80–1.11) were categorized as fast-growing cultures (filled columns), and 9 cultures with OD_{600} that was less than 0.80 (OD_{600} = 0.51–0.75) were categorized as slow-growing cultures (open columns).
C. Profiles of major osmolytes in the cells of the fast and the slow growing GOP-Gad cultures. Because intracellular osmolytes were extracted from the cultured with OD_{600} between 0.80–1.11, intracellular osmolytes of the fast-growing cultures (n = 21 of 30) were extracted after 7 days of cultivation, while those of the slow-growing cultures (n = 9 of 30) were extracted after 8 or 9 days of cultivation.

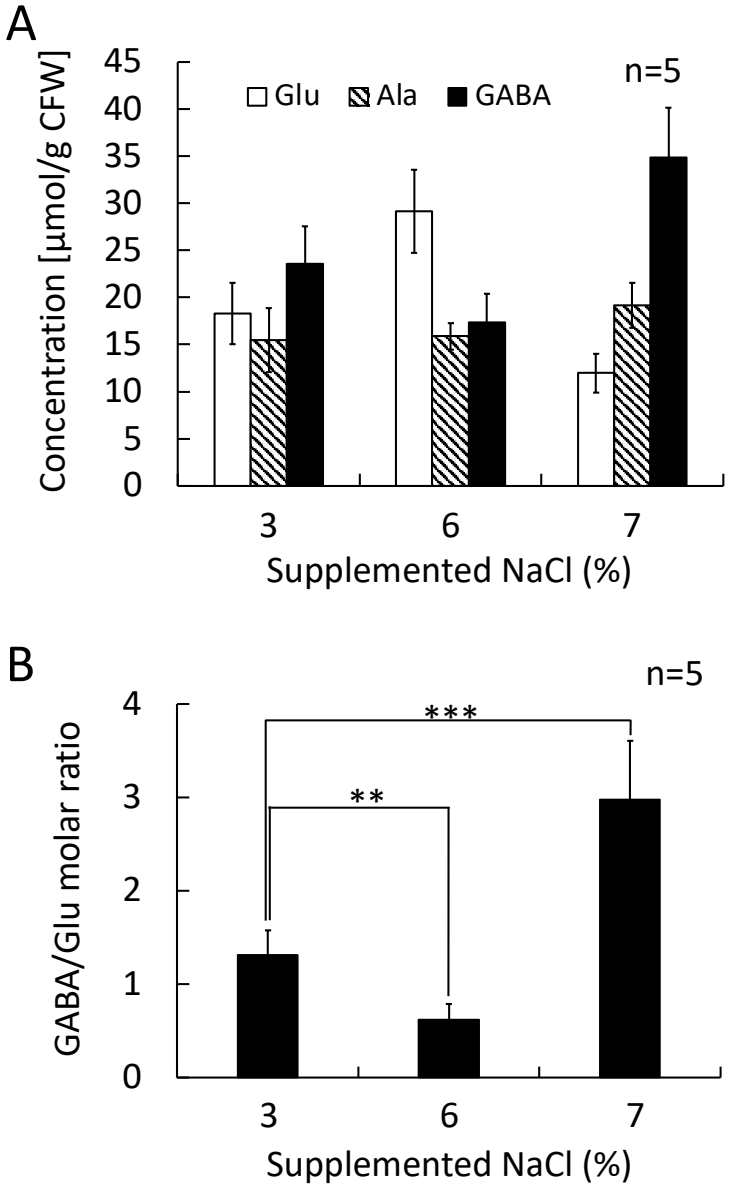

Fig. 8. Effect of medium salinity on GABA accumulation in the *H. elongata* GOP-Gad strain.

A. Profiles of major osmolytes in the cells of *H. elongata* GOP-Gad strain cultured in M63 medium containing 4% glycerol with 3, 6, or 7% NaCl. The strain was precultured in M63 medium containing 3% NaCl to OD_{600} of more than 0.8 and used as a 5% inoculum for the main culture in M63 medium containing 4% glycerol with 3, 6, or 7% NaCl. After 2 subcultures, osmolytes of the cells from the third culture were extracted when OD_{600} reached 0.9 to 1.2 and were analyzed by HPLC. Concentration of Glu, Ala, and GABA were normalized with internal standard norvaline. Values are mean ± standard deviation (n=5). Glu (open columns), Ala (hatched columns), GABA (filled columns).

B. Molar ratio of GABA to Glu (GABA/Glu) in the cellular extracts of *H. elongata* GOP-Gad strain cultured in M63 medium containing 4% glycerol with 3, 6, or 7% NaCl. Ratio were calculated from data shown in Fig. 8A. Values are mean ± standard deviation (n=5). ** $p \leq 0.01$, *** $p \leq 0.001$.

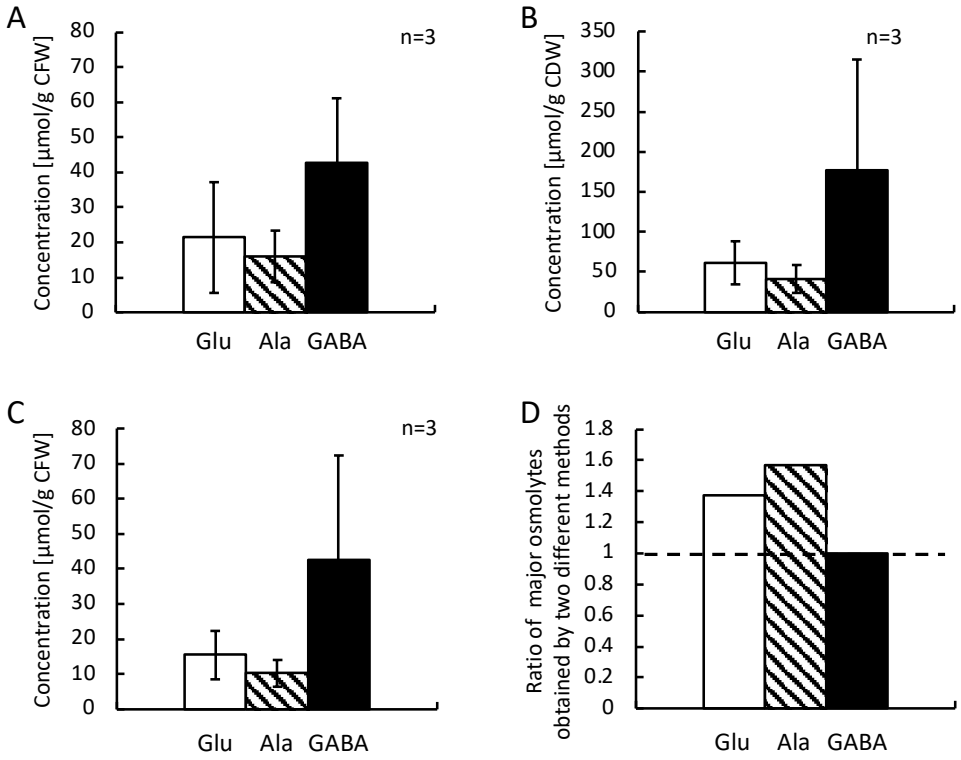

Fig. 9. Comparison of major osmolytes extraction methods: wet-cells, hypo-osmotic extraction (bacterial milking) method *vs.* freeze-dried cells, conventional phase-separation method.

H. elongata GOP-Gad strain was precultured in M63 medium containing 4% glycerol and 3% NaCl untill OD_{600} was more than 0.8 and used as a 5% inoculum for the main culture in 120 mL M63 medium containing 4% glycerol and 7% NaCl (n=3). When the culture reached late log phase (OD_{600}=1.0-1.2), cells were harvested from 50 mL of the culture in duplicate and the weights of the wet cell pellets were recorded as cell fresh weight (CFW). Pure water was added to one of the pellet samples to extract the major osmolytes by hypo-osmotic bacterial milking method, while the other pellet sample was freeze dried and the weight of the dried cell pellet was recorded as cell dry weight (CDW). Then the major osmolytes were extracted from the dried pellet by adding methanol/chloroform/water (10:5:3.4, by vol.) in the conventional phase-separation method. The amount of Glu, Ala and GABA in the extracts were determined by HPLC. Concentration of Glu (open columns), Ala (hatched columns), and GABA (filled columns) were normalized with internal standard norvaline.

A. Major osmolyte profile of *H. elongata* GOP-Gad strain growing under high salinity-stress condition derived from sample extracted by wet-cells, hypo-osmotic extraction (bacterial milking) method.

B. Major osmolyte profile of *H. elongata* GOP-Gad strain growing under high salinity-stress condition derived from sample extracted by freeze-dried cells, conventional phase-separation method.

C. A unit conversion of the data shown in B from μmol/g CDW to μmol/g CFW by calculating with original CFW of each sample measured before the freeze-drying process.

D. Ratios of the major osmolytes in the extract derived by wet-cells, hypo-osmotic extraction (bacterial milking) method (Fig. 9A) to those derived by freeze-dried cells, conventional phase-separation method (Fig. 9C).

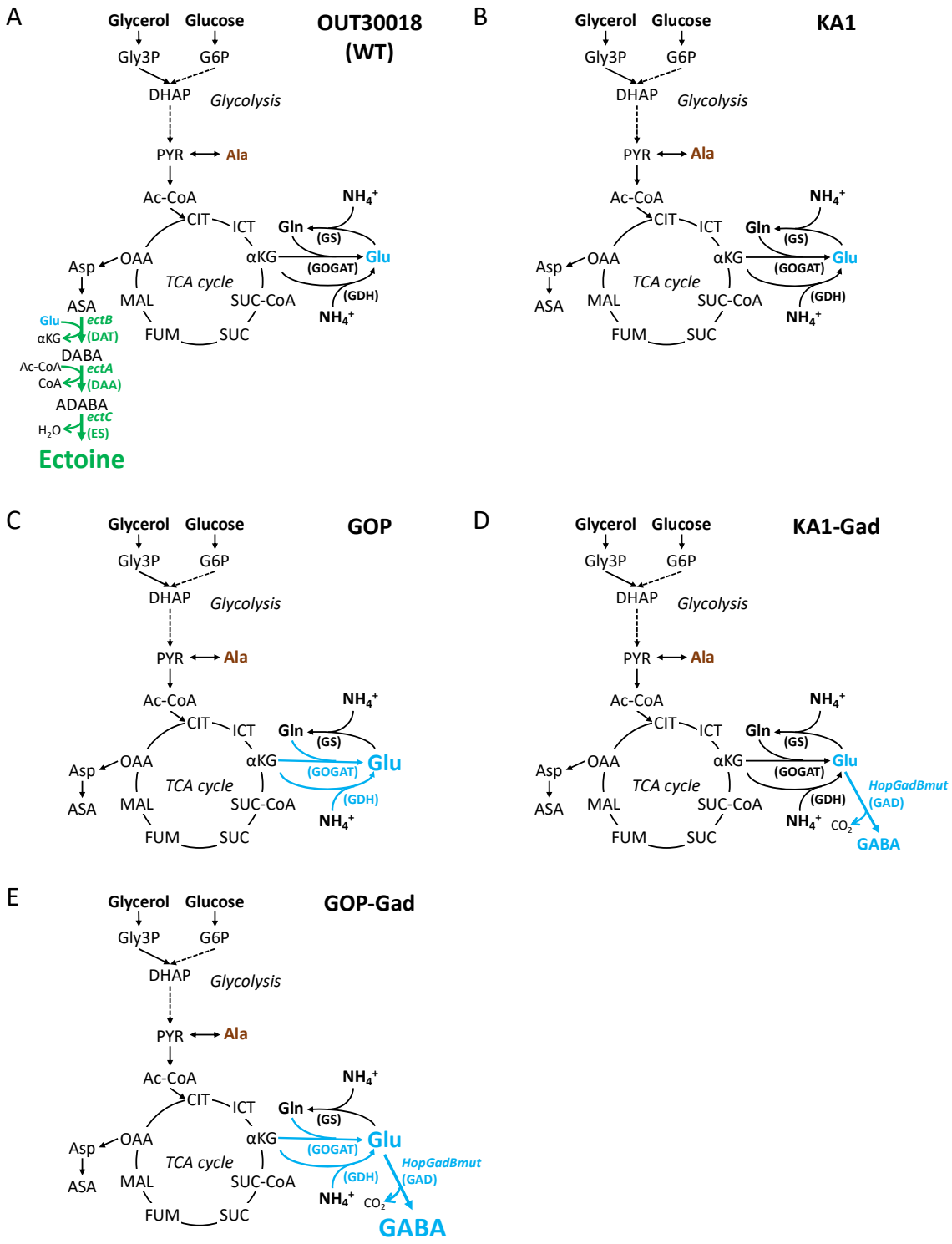

Fig. 10. Schematic diagram of major-osmolyte biosynthetic pathways operating in *H. elongata* OUT30018, KA1, GOP, KA1-Gad, and GOP-Gad strains.

A. *H. elongata* OUT30018 accumulates ectoine as a result of an expression of the salt-inducible *ectABC* operon, which contains genes that encode the 3 enzymes of the ectoine biosynthesis pathway; L-2,4-diaminobutyric acid (DABA) transaminase (DAT) encodedd by *ectB* gene, DABA acetyltransferase (DAA) encoded by *ectA* gene, and ectoine synthase (ES) encoded by *ectC* gene.

B. *H. elongata* KA1 strain does not accumulate ectoine due to the lack of the *ectABC* gene cluster. This strain can only grow well in the medium contaning 3% NaCl.

C. *H. elongata* GOP strain does not accumulate ectoine due to the lack of the *ectABC* gene cluster, however, spontaneous mutation in its genome made this strain produces and accumulates higher Glu than the KA1 strain, possiblly, due to enhanced activity of either glutamate synthetase (GOGAT) or glutamate dehydrogenase (GDH). As a result, this strain has higher salt tolerance than the KA1 strain and can grow in the medium containing 6 and 7% NaCl.

D. *H. elongata* KA1-Gad strain is engineered to contain a salt-inducible artificial bicistronic *mCherry-HopGadBmut* operon encoding a red fluorescent reporter protein (mCherry) and a wide pH-range mutant of an L-glutamic acid decarboxylase (GAD), which converts Glu to GABA. This strain does not accumulate ectoine due to the lack of the *ectABC* gene and does not accumulate Glu to the concentration that is high enough to support GABA accumulation under high salinty conditions. Therefore, this strain cannot grow in the medium contaning more than 4% NaCl.

E. *H. elongata* GOP-Gad strain is engineered to contain a salt-inducible artificial bicistronic *mCherry-HopGadBmut* operon encoding an mCherry and a wide pH-range GAD mutant, which converts Glu accumulated in this spontaneous mutant into GABA. This strain grows better than the GOP strain in medium containing 6 and 7% NaCl due to its ability to accummulate GABA as a major osmolyte.

Gly: glycerol; DHAP: dihydroxyacetone phosphate; PYR: pyruvate; AcCoA: acetyl coenzyme A; Ac-P: acetyl phosphate; A-AMP: acetyl-AMP; CIT, citrate; ICT: isocitrate; α-KG: α-ketoglutarate; SUC-CoA: succinyl-coenzyme A; SUC: succinate; FUM: fumarate; MAL: malate; OAA: oxaloacetate; Asp: aspartate; ASA: aspartic β-semialdehyde; DABA: L-2,4-diaminobutyric acid; DAT: DABA transaminase; DAA: DABA acetyltransferase; ADABA: N-γ-acetyl-L-2,4-diaminobutyric acid; ES: ectoine synthase; GDH: glutamate dehydrogenase; GS: glutamine synthetase; GOGAT: glutamate synthetase; GAD: glutamate decarboxylase.

TABLES

Table 1. Bacterial strains used in this study.

Strains	Phenotypes and Descriptions	References
Halomonas elongata		
OUT30018	Wild type strain (Osaka University Type Culture, formerly designated as KS3). Salt-tolerant phenotype due to ability to produce and accumulate ectoine as a major osmolyte.	(6)
KA1	Deletion mutant of ectoine biosynthesis genes (*ΔectABC*) derived from the OUT30018 strain. Deposited as FERM P-22094 strain. Salt-sensitive phenotype due to inability to produce ectoine.	(15)
GOP	Spontaneous suppressor mutant of the salt-sensitive KA1 strain. Improved salt-tolerant phenotype due to ability to overproduce glutamate.	This study
KA1-Gad	Recombinant KA1 strain harboring an artificial bicistronic *mCherry-HopGadmut* operon, which regulate salt-inducible expression of a red-fluorescent mCherry reporter protein and a mutant Glutamate Decarboxylase (gadB), which is active at a wide pH range to convert Glutamate to GABA. Salt-sensitive phenotype due to the lack of ability to produce GABA to a concentration high enough to function as osmolyte.	This study
GOP-Gad	Recombinant GOP strain harboring an artificial bicistronic *mCherry-HopGadmut* operon, which regulate salt-inducible expression of a red-fluorescent mCherry reporter protein and a mutant Glutamate Decarboxylase B (gadB), which is active at a wide pH range to convert Glutamate to GABA. Improved salt-tolerant phenotype due to ability to produce and accumulate GABA as a major osmolyte.	This study
Escherichia coli		
DH5α	Used as host for pUC57-Kan-based, pBluescript II SK (-)-based, pET-based and pK18*mobsacB*-based plasmids, F^-, Φ80d*lacZ*⊿M15, ⊿(*lacZYA-argF*) U169, *hsdR*17(r_k^- m_k^+), *recA*1, *endA*1, *relA*, *deoR*, *supE*44, *thi*-1, *gyrA*96, *λ*-	(52)
HB101	Used as host for pRK2013 plasmids, F^-, *hsd S*20(rB-, mB-), *recA*13, *ara*-14, *proA*2, *lacY*1, *galK*2, *rpsL*20 (str), *xyl*-5, *mtl*-1, *supE*44, *leuB*6, *thi*-1	(53)

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1244 **Table 2.** Plasmids used in this study**.**

Plasmids	Descriptions	References
pBluescript II SK (-)	Standard cloning vector, for blue-white colony selection; Amp^r	(54)
pBSK-*mCherry*	pBluescriptII SK(-) containing an *Xba*I-*Bgl*II-*mCherry*-*Spe*I-RBS-*Bam*HI-*Sma*I insertion fragment. Contains bicistronic operon producing both red fluorescent protein mCherry and lacZ' proteins, designed for red/blue-white colony selection; Amp^r.	This study
pBSK-*mCherry*-D_{ectC}	pBSK-*mCherry* containing a *Bam*HI-*H. elongata* downstream region of *ectC* (D_{ectC})- *Hin*dIII insertion fragment; Amp^r.	This study
pBSK-U_{ectA}-*mCherry*-D_{ectC}	pBSK-*mCherry*-D_{ectC} containing an *Xba*I-*H. elongata* upstream region of *ectA* (U_{ectA})-*Bgl*II insertion fragment; Amp^r.	This study
pBSK-U_{ectA}-D_{ectC}	pBSK-U_{ectA}-*mCherry*-D_{ectC} digested with *Bgl*II-*Bam*HI, then self-ligated to remove *Bgl*II-*mCherry*-*Spe*I-RBS-*Bam*HI fragment; Amp^r.	This study
pUC57-*HopGadBmut*	pUC57-Kan containing a *Xho*I-*Spe*I-*Nde*I-*HopGadBmut*-*Nhe*I-*Bam*HI insertion fragment; *HopGadBmut* gene encodes an *H. elongata* codon-usage optimized mutant (Glu89Gln/Δ452-466) of *E. coli*'s Glutamate Decarboxylase B (gadB); Kan^r.	This study
pET-*Lipop5-HA*	pET15b derivative containing an *Nco*I-*Nde*I-*HeLipop5-HA*-*Bam*HI insertion fragment; used for sub-cloning of the synthetic *HopGadBmut* gene; Amp^r.	(55)
pET-*HopGadBmut*	pET-*Lipop5-HA* containing an *Nde*I-*HopGadBmut*-*Bam*HI insertion fragment; Amp^r.	This study
pBSK-U_{ectA}-*mCherry*-*HopGadBmut*-D_{ectC}	pBSK-U_{ectA}-*mCherry*-D_{ectC} digested with *Spe*I and *Bam*HI, then ligated with an *Xba*I-*HopGadBmut*-*Bam*HI fragment from pET-*HopGadBmut*; containing a bicistronic operon that encode a red fluorescent mCherry protein and a mutant gadB protein; Amp^r.	This study
pK18*mobsacB*	Suicide vector containing *sacB* gene, which allows for direct selection of transformants with double-crossover homologous recombination on medium containing 10% sucrose; Kan^r, *mob*, *sacB.*	(56)
pK18*mobsacB*-U_{ectA}-D_{ectC}	pK18*mobsacB* containing *Xba*I-U_{ectA}-D_{ectC}-*Hin*dIII insertion fragment used in homologous recombination to delete *ectABC* gene cluster in *H. elongata* to obtain *H. elongata* KA1strain; Kan^r.	This study
pK18*mobsacB*-U_{ectA}-*mCherry*-*HopGadBmut*-D_{ectC}	pK18*mobsacB* containing *Xba*I-U_{ectA}-*mCherry*-*HopGadBmut*-D_{ectC}-*Hin*dIII fragment used in homologous recombination to introduce a *HopGadBmut* gene into the *ectABC* locus of *H. elongata* GOP strain and KA1 strain. *mCherry* was used as a reporter for monitoring salt-induced expression of transgene under the control of an *ectA* promoter.	This study
pRK2013	Used as a mobilizing helper plasmid in triparental conjugation; Kan^r.	(57)

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1247 **Table 3.** Sequences of Oligonucleotide Primers

Primers	Sequence (5'-3')*	Descriptions	Restriction enzyme sites
U_{ectA}-F	TCTAGATTCGATCTCGATGACTTCCCGCAGC	Forward primer for amplification of an upstream region of *H. elongata*'s *ectA* (U_{ectA}).	*Xba*I
U_{ectA}-R	ACTAGTAGATCTTACGTACATTGTCGTGGTTCGCTGTAGCGAATTTG	Reverse primer for amplification of U_{ectA}.	*Spe*I, *Bgl*II, *Sna*BI
D_{ectC}-F	ACTAGTGGATCCTAACCCGGCGCAGTATTCTGCCG	Forward primer for amplification of a downstream region of *H. elongata*'s *ectC* (D_{ectC}).	*Spe*I, *Bam*HI
D_{ectC}-R	AAGCTTGGGCATGGTGCATTGTCGAGGGAG	Reverse primer for amplification of D_{ectC}.	*Hin*dIII
mCherry-F	AAATCTAGAAAGATCTGAGGAGGATAACATGGCCATCATCAAGGA	Forward primer for amplification of an *mCherry* gene.	*Xba*I, *Bgl*II
mCherry-R	AAACCCGGGGATCCCATGGTATATCTCCTTCTTAAACTAGTCAGTCCATGCCGCCGGTGGAG	Reverse primer for amplification of an *mCherry* gene.	*Sma*I, *Bam*HI, *Spe*I
HopGadBmut-F	GCGCCAAGAGCATCAGCACCATC	Forward primer for amplification of a partial *HopGadBmut* fragment.	
HopGadBmut-R	GGTAGCTGGCATTCTGCACCTTGG	Reverse primer for amplification of *HopGadBmut* fragment.	

1248 *Restriction endonuclease sites incorporated into primer sequences are underlined.
1249 Start codon, stop codon, or ribosome-binding site (RBS) are double underlined.
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Table 4. HPLC gradient condition.

Time (min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)	Mode
0.0	0.5	88.0	12.0	Injection
0.5	0.5	88.0	12.0	Isocratic
2.0	0.5	82.0	18.0	Linear gradient
2.5	0.5	82.0	18.0	Isocratic
3.0	0.5	80.0	20.0	Linear gradient
3.5	0.5	80.0	20.0	Isocratic
4.0	0.5	79.5	20.5	Linear gradient
4.5	0.5	79.5	20.5	Isocratic
5.0	0.5	79.0	21.0	Linear gradient
5.5	0.5	79.0	21.0	Isocratic
6.0	0.5	78.5	21.5	Linear gradient
6.5	0.5	78.5	21.5	Isocratic
7.0	0.5	78.0	22.0	Linear gradient
7.5	0.5	78.0	22.0	Isocratic
8.0	0.5	77.5	22.5	Linear gradient
8.5	0.5	77.5	22.5	Isocratic
9.0	0.5	77.0	23.0	Linear gradient
9.5	0.5	77.0	23.0	Isocratic
10.5	0.5	76.9	23.1	Linear gradient
11.0	0.5	76.9	23.1	Isocratic
11.5	0.5	76.8	23.2	Linear gradient
12.0	0.5	76.8	23.2	Isocratic
12.5	0.5	76.7	23.3	Linear gradient
13.0	0.5	76.7	23.3	Isocratic
14.0	0.5	76.5	23.5	Linear gradient
14.5	0.5	76.5	23.5	Isocratic
15.0	0.5	76.4	23.6	Linear gradient
15.5	0.5	76.4	23.6	Isocratic
16.0	0.5	76.3	23.7	Linear gradient
16.5	0.5	76.3	23.7	Isocratic
17.0	0.5	76.2	23.8	Linear gradient
17.5	0.5	76.2	23.8	Isocratic
18.0	0.5	76.1	23.9	Linear gradient
18.5	0.5	76.1	23.9	Isocratic
19.0	0.5	76.0	24.0	Linear gradient
19.5	0.5	76.0	24.0	Isocratic
20.5	0.5	72.0	28.0	Linear gradient
21.0	0.5	72.0	28.0	Isocratic
21.5	0.5	70.0	30.0	Linear gradient
22.0	0.5	70.0	30.0	Isocratic
22.5	0.5	20.0	80.0	Linear gradient
23.0	0.5	20.0	80.0	Isocratic
23.5	0.5	10.0	90.0	Linear gradient
24.0	0.5	10.0	90.0	Isocratic
24.5	0.5	88.0	12.0	Linear gradient
35.0	0.5	88.0	12.0	Isocratic

Mobile phase A: 20 mM sodium acetate buffer (pH 6)/Acetonitrile (85:15, by vol.)
Mobile phase B: Acetonitrile.