博士論文

Expansion of assimilable substrates in *Escherichia coli* for efficient fermentation and biocontainment applications

大腸菌における資化可能な基質の拡張の研究：
発酵生産の効率の向上と
生物学的封じ込め技術への応用

2017 年

広島大学大学院先端物質科学研究科
分子生命機能科学専攻

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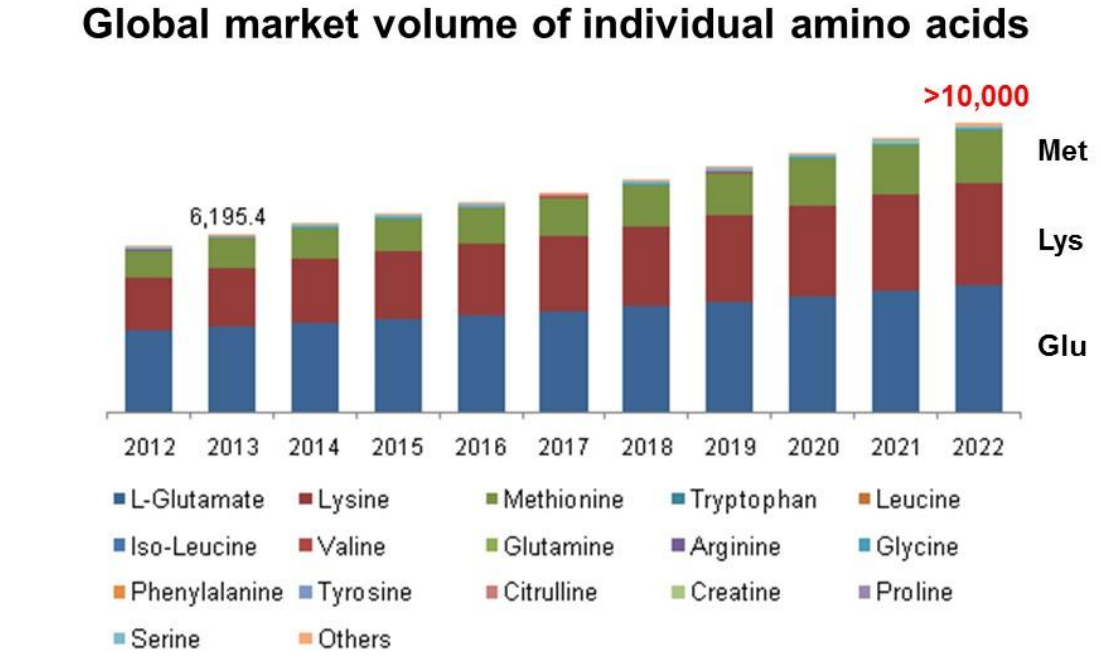
Chapter 1

General introduction

1-1 The use of glucose feedstock in the fermentation industry: a demand to be improved

Fermentative production of useful compounds, such as alcohols, gases, pharmaceutical ingredients, and organic/amino acids, is conducted worldwide. The market volume and value of the fermentation products reaches 55 million tons and 700 billion USD per year, respectively, in the United States alone. Amino acids are biologically, economically, and environmentally important compounds that are used as, for example, seasonings, feed additives, and sweeteners. The market volume of amino acids is expected to expand by over 10 million tons per year by 2020 (Fig. 1-1).

In general, genetically modified microorganisms (GMMs), which are highly engineered to yield a high productivity, are used in the commercial fermentative production of amino acids. *Escherichia coli* is the most useful bacterium for the production of valuable compounds, such as amino acids and organic acids, because *E. coli* cells grow quickly, convert substrates to products rapidly, and are genetically engineered readily[1,2]. For example, L-lysine, which is used as a feed additive worldwide, is produced on the scale of approximately 1,500,000 metric tons per year[3].



Ref.: http://www.grandviewresearch.com/industry-analysis/amino-acids-market

Fig. 1-1 Global market volume of individual amino acids.

On the other hand, in the industrial production of useful compounds by fermentation, glucose is one of the most frequently used carbon sources[4]. Industrial glucose feedstock is prepared from starch, a polysaccharide composed of glucose units linked together by α-1,4 and α-1,6 glycosidic bonds, by means of enzymatic hydrolysis[5,6]. Complete hydrolysis of starch into glucose adds significant cost; therefore, most commercially available glucose feedstock is processed incompletely[7]. Because of incomplete enzymatic hydrolysis and/or reverse reactions, the glucose feedstock contains significant amounts of maltose [4-O-α-D-Glucopyranosyl-D-glucopyranose] (1–2%), isomaltose [6-O-α-D-Glucopyranosyl-D-glucopyranose] (0.5–2%), and other oligosaccharides, such as panose [α-D-Glucopyranosyl-(1->6)-α-D-glucopyranosyl-(1->4)-D-glucopyranose] (1% or less)[6-12] (Fig. 1-2). If the microorganisms used in fermentation cannot metabolize these saccharides, then the valuable carbohydrates would be wasted.

Saccharides that contain α-1,6 bonds, such as isomaltose and panose, are not used up by *E. coli* during fermentation because *E. coli* cannot assimilate isomaltose or panose as carbon sources. Furthermore, these saccharides, which contain reducing sugar moieties, can react with free amino groups of amino acids during the purification step[13,14]; this so-called Maillard reaction decreases the yield of the final product and contaminates the reaction mixture with undesirable compounds. These problems must be overcome to increase the yield and productivity of fermentation when using glucose feedstock as a carbon source.

In this study, I investigated a sugar phosphotransferase system of *Bacillus subtilis* and found that the heterologous expression of the phosphotransferase system conferred *E. coli* cells with the ability to assimilate isomaltose. The recombinant bacterium also assimilated trisaccharides containing α-1,6 bonds, such as panose, as well as the alcoholic forms of these saccharides, such as isomaltitol. The expansion of assimilable sugars by *E. coli* would contribute to the yields of amino acids produced using glucose feedstock in industrial-scale fermentation.

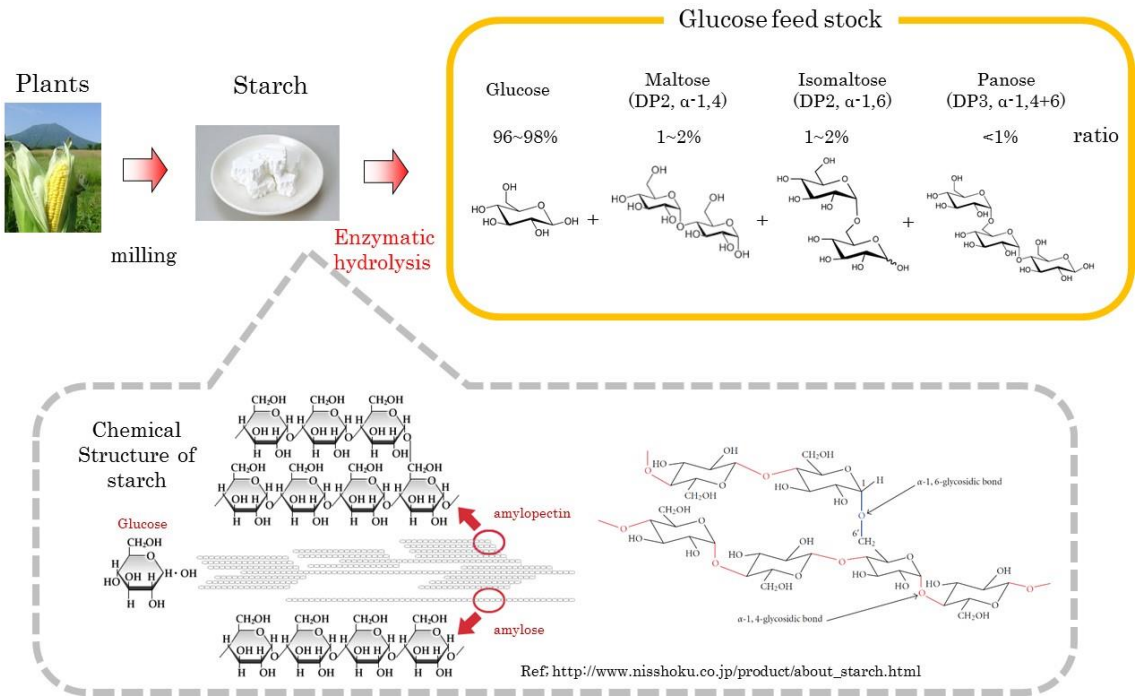


Fig. 1-2 Structure of starch and composition of glucose feedstock.

1-2 A novel biocontainment strategy for the fermentation industry

As I mentioned in the previous section, GMMs are typically used in commercial fermentative production of amino acids. The guidelines for safe handling of recombinant DNA molecules and GMMs, first outlined in 1975[15], state that biological and physical barriers adequate to contain newly created organisms should be employed. Although physical containment—physical barriers limiting the release of viable organisms into the environment—have been widely adopted in both laboratory and large-scale biotechnological applications in industry, intrinsic biocontainment—biological barriers limiting the spread and survival of microorganisms in the natural environment[16]—remains a challenge, especially in the application of GMMs in bioremediation, agriculture, bioenergy, therapeutics, etc.

Biocontainment is a methodology that involves the design of GMMs that are able to grow and survive only under particular conditions[15]. Current biocontainment strategies can be classified into active and passive forms. The former employs killing switches, such as toxin/antitoxin genes[17,18] and self-destructing DNA[19], while the latter creates a requirement for externally supplied molecules (auxotrophy) by eliminating essential gene functions[20,21]. Several auxotrophic strategies that render cells dependent on artificial compounds have recently been reported[16,22,23]. Two research groups have developed genomically recoded *Escherichia coli* strains, in which expanded codons for non-standard amino acids (NSAAs) were introduced into essential genes demonstrating that the resultant strains strictly rely on the availability

of NSAAs for their growth[16,22]. Lopez and Anderson[23] developed an alternative biocontainment strategy that introduces ligand-dependent essential genes into an *E. coli* BL-21 strain. Only the addition of the synthetic chemical benzothiazole in the culture medium rescues bacterial growth by restoring the activities of the essential gene products. Due to the absence of these synthetic chemicals in nature, such microbes should be unable to grow in the open environment. However, these synthetic materials, such as NSAA, are relatively rare and expensive than other commodity chemicals. Thus, due to cost concerns, it seems impractical to apply these synthetic materials to biocontainment in industrial fermentation.

Phosphite (H_3PO_3, Pt) and hypophosphite (H_3PO_2, HPt) are salts of phosphorous acid, where phosphorus (P) has an oxidation state of +3 and +1, respectively (Fig. 1-3). On Earth, virtually all known P deposits exist in the +5 oxidation state, phosphate (H_3PO_4, Pi). This is true for Pi-esters that play important roles in many biological reactions. Therefore, Pt has to be oxidized to Pi, prior to P metabolism. Although most organisms cannot assimilate Pt, it is known that several groups of bacteria possess an oxidization system for Pt and, less commonly, for HPt. The molecular basis of Pt/HPt-oxidation in bacteria was established using the soil bacterium *Pseudomonas stutzeri* WM88[24]. In this bacterium, HPt is oxidized to Pt by HPt dioxygenase (HtxA)[25], and the resultant Pt is oxidized to Pi by NAD-dependent Pt dehydrogenase (PtxD)[26].

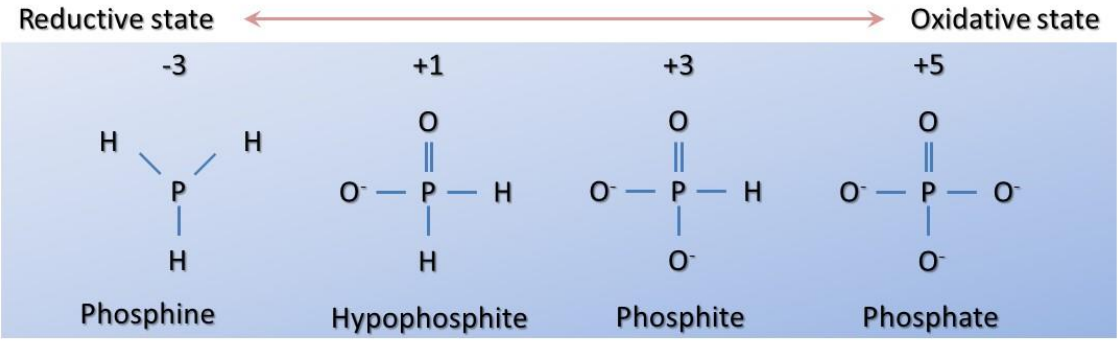


Fig. 1-3 Chemical structures of phosphate and its reduced forms.

Hirota et al. demonstrated that PtxD can be used as a selectable marker in yeasts[27]. Since Pt cannot be metabolized without its oxidation to Pi, the introduction of *ptxD* into cells that are incapable of utilizing Pt, confers them with the ability to grow on medium containing Pt as the sole source of P. Pt and HPt are ecologically rare compounds not available in sufficient amounts to support bacterial growth[28]. In this study, based on this expanded Pt utilization ability of a host organism and given the scarcity of Pt in the environment, I conceived that engineered dependency on Pt could also be used as a strategy for biocontainment (Fig. 1-4a). Under laboratory conditions, microbial growth is maintained by using any P source, including Pt and HPt. Common microbial strains are able to grow both inside and outside the laboratory facilities by using Pi or organic Pi compounds as P sources. A Pt/HPt-dependent strain, which can utilize Pt or HPt but not Pi, is only able to grow under laboratory conditions where Pt/HPt is provided as a P source, but not in the absence of Pt/HPt (Fig. 1-4a). In this scenario, the Pt/HPt-

dependent strain expressing PtxD and a Pt transporter should be engineered to eliminate the ability to take up Pi or other Pi compounds commonly present in the environment. Following this strategy, a HPt transporter, HtxBCDE, that can take up Pt but not Pi was introduced into *E. coli* in which all endogenous Pi transporters (PitA, PitB, PstSCAB, and PhnCDE) and Pi-ester transporters (UhpT, UgpB, and GlpT) were disrupted (Fig. 1-4b). This genetically modified *E. coli* was not able to grow on media without Pt or HPt. Furthermore, I showed that this strain did not yield any escape mutants under non-permissive growth conditions (without Pt or HPt) with an assay detection limit of 1.9×10^{-13} per CFU, which is lower than that of any other reported biocontainment strategies. As the cost of Pt is extremely low compared to those of other commonly used selection chemicals for biocontainment, the Pt/HPt dependency of the engineered strain provides a cost-effective antibiotic-free cultivation method. These advantages, together with a strikingly low escape frequency, make Pt/HPt dependency a promising biocontainment strategy for practical applications.

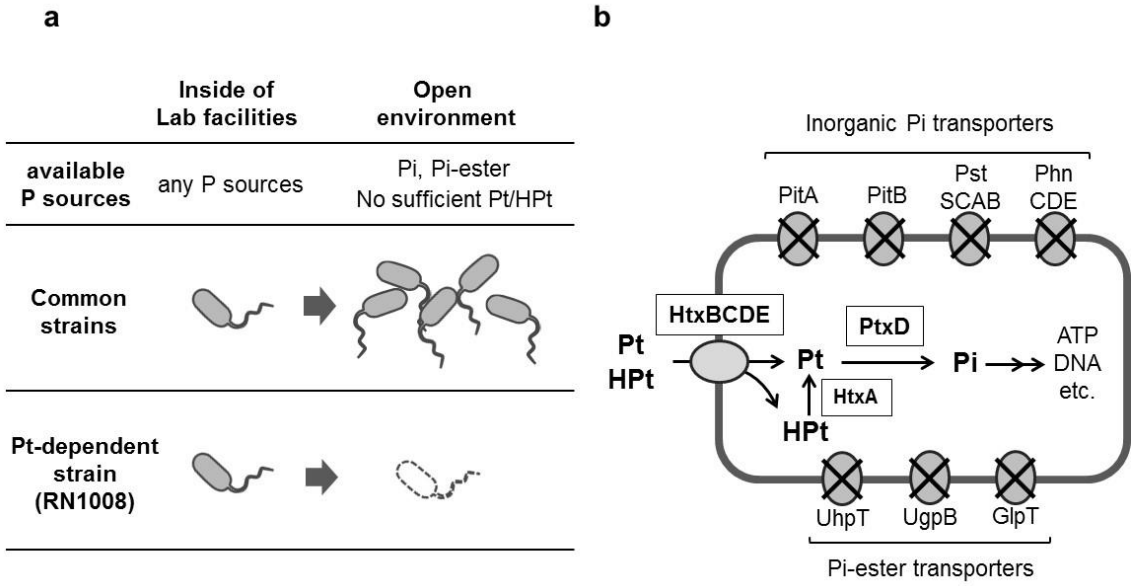


Fig. 1-4 (a) Biocontainment strategy using engineered dependency on Pt/HPt, and (b) schematic representation of the engineered P metabolic pathway for biocontainment.

1-3 Outline of the thesis

In this chapter, I have described several demands of the fermentation industry. One is related to sugar assimilation by *E. coli.* Since the glucose feedstock contains significant amounts of non-assimilable sugars, expansion of assimilable substrates for *E. coli* is important for efficient fermentation. The other is related to biocontainment technology. There is a growing demand for safeguarding against the spread and proliferation of GMMs in the environment. To meet these demands, I expanded assimilable substrates (sugars and phosphorus compounds) in *E. coli.* I briefly summarized how the expansion of assimilable substrates contributes to improve fermentation efficiency and to develop biocontainment technology.

In chapter 2, I would describe in detail about expansion of assimilable sugars to facilitate efficient utilization of isomaltose and panose contained in the glucose feedstock. In addition, I demonstrated an increase of yield in L-lysine production in modified *E. coli*.

In chapter 3, I would describe in detail about a new biocontainment strategy to make bacterial growth and survival dependent on phosphite. I would also show that the escape efficiency of this method was extremely low compared to that of other biocontainment methods.

In chapter 4, I would state a general conclusion and a summary of the results.

Chapter 2

Engineering of *Escherichia coli* to facilitate efficient utilization of isomaltose and panose in industrial glucose feedstock

2-1 Introduction

As I have discussed in chapter 1, industrial glucose feedstock contains significant amounts of maltose (1–2%), isomaltose (0.5–2%), and other oligosaccharides, such as panose (1% or less)[6-12], because of incomplete enzymatic hydrolysis of starch. *E. coli* cannot assimilate isomaltose or panose as carbon sources. To increase the yield and productivity of fermentation when using glucose feedstock as a carbon source, I intended to expand sugar assimilation in *E. coli*.

The phosphotransferase system (PTS) is responsible for the transport and phosphorylation of sugars. The multi-component PTS comprises a phosphohistidine carrier protein (HPr), an enzyme I (EI) component, and a membrane-bound enzyme complex (EII). The HPr and EI components transfer a phosphoryl group of phosphoenolpyruvate (PEP) to the sugar-specific enzymes, EIIA and EIIB. EIIC is an integral membrane protein permease that recognizes and transports the sugar, which is then phosphorylated by EIIB[29]. There are 21 different EII complexes encoded in the *E. coli* chromosome; these complexes are involved in the transport of approximately 20 different carbohydrates[30]. Pikis et al. reported that the heterologous expression of *Klebsiella pneumoniae aglA* (a single chain polypeptide of EIIC and EIIB that mediates the transport and phosphorylation of sucrose and various other α-linked glucosides) and *aglB* (a phospho-α-glucosidase) confers *E. coli* cells with the ability to utilize isomaltose[31,32]. Although *E. coli* K-12 strain has homologs of *aglA* and *aglB*[32],

these seemed to be cryptic or non-functional truncated proteins[33,34]. However, *Bacillus subtilis* strains, which are generally regarded as safe (GRAS) organisms by the Food and Drug Administration (FDA)[35-38], have *glvA* and *glvC*, the functional homologs of *aglB* and *aglA*, respectively[32]. GlvA and GlvC are known to be involved in maltose assimilation in *B. subtilis*[39,40]. Although a wide variety of phosphorylated α-linked aryl glucosides can be degraded by GlvA[41], there are no other reports describing its substrate specificity.

In this study, I found that the heterologous expression of *glvA* and *glvC* conferred *E. coli* cells with the ability to assimilate isomaltose. Unexpectedly, the recombinant also assimilated trisaccharides containing α-1,6 bonds, such as panose, as well as the alcoholic forms of these saccharides, such as isomaltitol. The results of this study may facilitate increased production yields using glucose feedstock in industrial-scale fermentation by *E. coli*.

2-2 Experimental section

2-2-1 Bacterial strains, plasmids, and primers.

All strains, plasmids, and primers used in this study are listed in Tables 2-1 and 2-2.

Table 2-1 Strains and plasmids used in this study.

Strain or plasmid	Description, genotype, or sequence	Reference, source
Strains		
E. coli K-12 MG1655	F- l- *ilvG rfb-50 rph-1*	CGSC collection
E. coli WC196LC	W3110 NTG mutant (S-aminoethyl-L-cysteine-resistant mutant) Δ*ldc* Δ*cadA*	42
Bacillus subtilis 168	*trpC2 ypqP*::SPβ	38
MG1655 (empty vector)	*E. coli* K-12 MG1655 harboring pTWV229 and pMW219-Δplac	this study
MG1655 (glvAC)	*E. coli* K-12 MG1655 harboring pTWV229-self-glvA-Fw and pMW219-ΔPlac-*tac-glvC	this study
WC196LC (pCABD2)	*E. coli* WC196LC harboring pCABD2	3, 42, 43
Plasmids		
pTWV229	Cloning vector, Ap^r	Takara Bio Inc, (Japan)
pMW219	Cloning vector, Km^r	Nippon gene.co.ltd (Japan)
pMW219-ΔPlac	pMW219 derivative lacking the *lac* promoter region	this study

pMW219-ΔPlac-tac-glvC-R2	pMW219-ΔPlac derivative harboring the Ptac-*glvC* gene	this study
pMW219-ΔPlac-Ptac4075-glvC-Rv	pMW219-ΔPlac derivative harboring the Ptac4075-*glvC* gene	this study
pTWV229-self-glvA-Fw	pTWV229 derivative harboring the Pself-*glvA* gene	this study
pMW219-ΔPlac-*tac-glvC	pMW219-ΔPlac-tac-glvC-R2 derivative harboring a mutation in the -10 region of the tac promoter (TATAAT to AATAAT)	this study
pCABD2	pRSF1010 harboring mutated *lysC*, mutated *dapA*, mutated *dapB*, and *C. glutamicum ddh*	43

Table 2-2 Primers used in this study.

Primer	Sequence
pMW119-F-Hind3	GCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGG
pMW119-R-Hind3	CCCAAGCTTGCTAACTCACATTAATTGCGTTG
pTWV229-F-Hind3	GCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGG
pTWV229-R-Hind3	CCCAAGCTTCACATTACTTGGCAGAACATATCC
glvC-F-tac	CAATTTCACACAAGGAGACTGCCATGATGCAAAAAATTCAGCG
glvC-R2	CCCAAGCTTCCCCTTTTTACTCGATTGTCTC
tac-Promoter-glvC-1	CGTATAATGTGTGGAATCGTGAGCGGATAACAATTTCACACAA GGAGACTGCCATGATGCAAAAAATTCAGCGCTTTGGA

Hind3-tac-Promoter	CCCAAGCTTCCTGTTGACAATTAATCATCGGCTCGTATAATGT GTGGAATCGTGAGCGGATAACAATTTCACACAAGGAG
tac-Promoter-glvC-2	CGAATAATGTGTGGAATCGTGAGCGGATAACAATTTCACACAA GGAGACTGCCATGATGCAAAAAATTCAGCGCTTTGGA
glvA-self-Fw1	AGAAATTTCCCGCTCTATGG
glvA-self-Rv1	TGTAGTGCTGATTGATCAGTTC

*HindIII recgnition site was underlined.

2-2-2 Construction of vectors.

The plasmid pMW219-ΔPlac was constructed by deleting the *lac* promoter from the vector plasmid pMW219 (Nippon Gene Co., Ltd., Tokyo, Japan) as follows. A DNA fragment was amplified using the primer set pMW119-F-Hind3 and pMW119-R-Hind3, and *Hin*dIII/*Pst*I-digested pMW219 was used as a template. The polymerase chain reaction (PCR)-amplified fragment was digested by *Hin*dIII and subsequently self-ligated by DNA-ligase. *E. coli* JM109 competent cells were transformed with the DNA, and transformants were selected on LB-agar medium containing kanamycin.

2-2-3 Construction of *glvC*-expressing plasmids.

A DNA fragment containing *glvC* was amplified by PCR with the primer set glvC-F-tac and glvC-R2 and with the *Bacillus subtilis* 168 genome as a template. In order to add a promoter sequence upstream of *glvC*, the

amplified DNA fragment containing *glvC* and synthetic single strand DNA (tac-Promoter-glvC-1) were mixed, and another PCR was then carried out using the primer set Hind3-tac-Promoter, glvC-R2. The amplified DNA fragment and *Sma*I-digested pMW219-ΔPlac were ligated by DNA ligase. The plasmid pMW219-ΔPlac-glvC-R2 was extracted from transformants, and its structure was confirmed. The plasmid pMW219-ΔPlac-Ptac4075-glvC-Rv containing *glvC* under the control of the *tac* promoter variant Ptac4075, which was a weaker promoter than *tac* promoter because of a mutation in the consensus sequence, was constructed in a similar manner using primers Hind3-tac-Promoter, glvC-R2 and synthetic DNA, tac-Promoter-glvC-2.

2-2-4 Construction of *glvA*-expressing plasmid.

A DNA fragment containing *glvA* and its upstream region containing a promoter sequence was amplified by PCR with the primer set glvA-self-Fw1 and glvA-self-Rv1 and with the *Bacillus subtilis* 168 genome as a template. The PCR-amplified DNA and *Sma*I-digested pTWV229 were ligated by DNA ligase. In the resulting plasmid, pTWV229-self-glvA-Fw, *glvA* mRNA was transcribed via the *lac* promoter of pTWV229.

2-2-5 Assimilation test in M9 minimal medium.

M9 liquid minimal medium[44] supplemented with 2 g/L isomaltose or maltose was used for assimilation tests. *E. coli* strains were precultured overnight at 37 °C on LB medium. The cells were washed three times with cold saline and

adjusted to an OD_{620} of 7.0. The cell suspension (70 µL) was added to 5 mL M9 minimal medium in an L-shaped test tube and cultured at 37 °C with shaking at 70 rpm using a Bio-Photorecorder (TN-1506; Advantec, Inc., Tokyo, Japan). In all experiments, appropriate antibiotics were added to the medium. M9 solid minimal medium[44] supplemented with 2 g/L of various types of sugars and sugar alcohols was used for assimilation tests. *E. coli* strains were precultured overnight at 37 °C on LB medium. The cells were washed 3 times by cold saline and adjusted to OD_{620} of 5.0. The cell suspension (20 µL) was inoculated on M9 minimal medium plates containing various types of sugars and sugar alcohols and incubated at 37 °C for 48 h. Glucose and sucrose were purchased from Junsei Chemical Co. Ltd. (Tokyo, Japan). Maltose was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). α-Methyl-glucoside was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Isomaltulose, maltotriitol, isomaltitol, lactitol, and erlose were purchased from Hayashibara Co., Ltd. (Okayama, Japan). Isomaltose, panose, isomaltotriose, maltitol, trehalose, turanose, maltulose, galactinol, cellobiose, gentiobiose, lactose, melibiose, lactulose, maltotriose, maltotetraose, maltopentaose, and raffinose were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). For solid medium, 15 g/L Bacto agar (Becton Dickinson and Company, USA) was added.

2-2-6 Assimilation tests for maltose and isomaltose in the presence of glucose.

E. coli MG1655 harboring pTWV229-self-glvA-Fw and pMW219-ΔPlac-*tac-glvC (obtained by an unintended mutation, as described in the Results section) was inoculated into M9 minimal medium containing 1.0 g/L maltose or isomaltose combined with 1.0 g/L glucose. Cells were then cultured at 37 °C with shaking at 70 rpm using a Bio-Photorecorder (Advantec) and sugar concentrations were assayed.

2-2-7 L-Lysine production using glucose, maltose, isomaltose, and panose as carbon sources.

The L-lysine-producing strain WC196LC harboring pCABD2 [encoding *dapA24, lysC80, dapB,* and *ddh*[3,42,43]], was transformed with pMW219-ΔPlac-Ptac4075-glvC and pTWV229-self-glvA-Fw. The transformant was inoculated on an LB plate containing 20 mg/L streptomycin, 100 mg/L ampicillin, and 50 mg/L kanamycin and incubated at 37 °C for 24 h. Colonies were scratched off, suspended in saline, and adjusted to an OD_{620} of 15. Next, 250 µL of the cell suspension was added to 5 mL Lys production medium containing 16 g/L glucose, 1.6 g/L maltose and/or isomaltose and/or 1.6 g/L panose, 1 g/L $MgSO_4$ heptahydrate, 24 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 2 g/L yeast extract, 0.1 g/L isoleucine, 12 mg/L $FeSO_4$ heptahydrate, 9.6 mg/L $MnSO_4$ pentahydrate, 30 g/L $CaCO_3$ (as dry heat-sterilized powder), 20 mg/L streptomycin, 100 mg/L ampicillin, and 50 mg/L kanamycin (pH 7.0 with KOH). Cells were then cultivated at 37 °C for 41 h with reciprocal shaking at 120 rpm. Glucose and

L-lysine were assayed by a biotech analyzer (AS310; Sakura Si Co., Ltd., Tokyo, Japan). Maltose, isomaltose, and panose were measured using an ICS-3000 Ion Chromatography System with a CarboPac PA1 column (DIONEX, CA, USA).

2-3 Results

2-3-1 Evaluation of the functions of GlvA and GlvC from *B. subtilis* in *E. coli.*

GlvA and GlvC have been reported to be involved in the assimilation of maltose in *B. subtilis*[39,40]. I constructed the plasmids pTWV229-self-glvA-Fw and pMW219-ΔPlac-tac-glvC-R2 for the expression of *glvA* and *glvC* from *B. subtilis* in *E. coli.* The growth of the *E. coli* MG1655 recombinant harboring both of the plasmids on M9 medium containing isomaltose was severely limited (data not shown). However, after prolonged incubation (45 h) of the recombinant at 37 °C on the isomaltose medium, I found that some mutants started to form larger colonies. Because plasmids isolated from one of the mutants enabled *E. coli* to grow rapidly on medium containing isomaltose, I analyzed the DNA sequences of these plasmids. A mutation was found in the -10 region of the *tac* promoter[45] (TATAAT to AATAAT) upstream of *glvC*. This mutation is expected to reduce the expression level of *glvC* by affecting the binding affinity of RNA polymerase. I speculated that strong expression of GlvC, a membrane permease, may be toxic in *E. coli*. The mutant plasmid was renamed pMW219-ΔPlac-*tac-glvC. Next, I tested the growth of *E. coli* MG1655 harboring pTWV229-self-glvA-Fw and pMW219-ΔPlac-*tac-glvC plasmids in M9 liquid medium containing isomaltose as a sole carbon source. The recombinant could grow on isomaltose efficiently, whereas *E. coli* harboring the empty vector plasmids could grow only on glucose (Fig. 2-1).

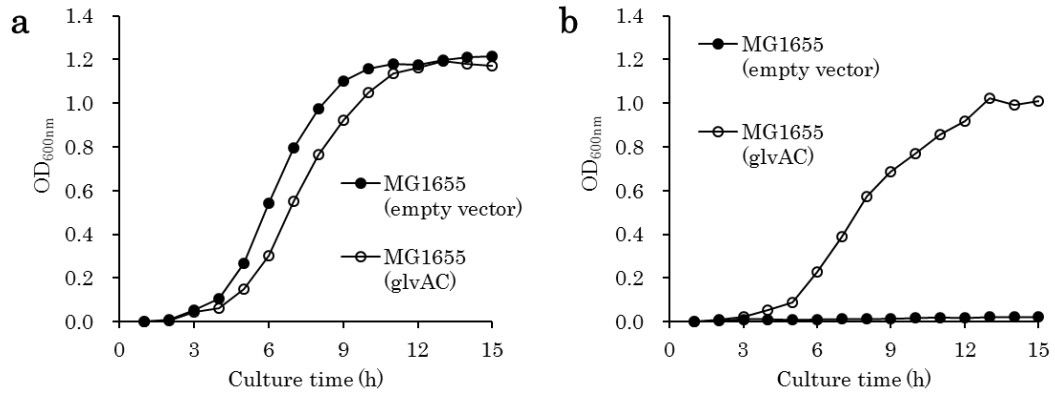


Fig. 2-1 Growth curves of *E. coli* strains on M9-glucose (a) and M9-isomaltose (b). Each point in the curve represents the mean of two independent experiments.

2-3-2 Simultaneous assimilation of glucose and isomaltose by *glvA*- and *glvC*-expressing *E. coli*.

To test whether the *E. coli* recombinant could assimilate isomaltose without catabolite repression, I used M9 medium containing an excess amount (1.8 g/L) of isomaltose or maltose supplemented with a small amount (0.2 g/L) of glucose[46]. In this medium, cell growth would stop temporarily at an OD_{600} of 0.1–0.2 if the strain consumed glucose first (showing diauxic growth). The *E. coli* recombinant showed relatively slow but smooth cell growth, without characteristics of diauxic growth (data not shown), suggesting that the *E. coli* recombinant could assimilate isomaltose without catabolite repression. Then, to demonstrate this phenomenon more clearly, I measured sugar

concentrations during cultivation on M9 minimal medium containing 1.0 g/L maltose and glucose or 1.0 g/L isomaltose and glucose. The *E. coli* carrying empty vector did not assimilate maltose or isomaltose in the presence of glucose (Fig. 2-2a, 2-2b), because *E. coli* assimilates maltose under the control of catabolite repression[47,48] and cannot assimilate isomaltose. On the other hand, the recombinant assimilated maltose or isomaltose in the presence of glucose (Fig. 2-2c, 2-2d). Surprisingly, the recombinant preferentially assimilated isomaltose over glucose (Fig. 2-2d). These results indicated that the heterologous expression of *glvA* and *glvC* conferred upon the cells the ability to assimilate maltose and isomaltose even in the presence of glucose.

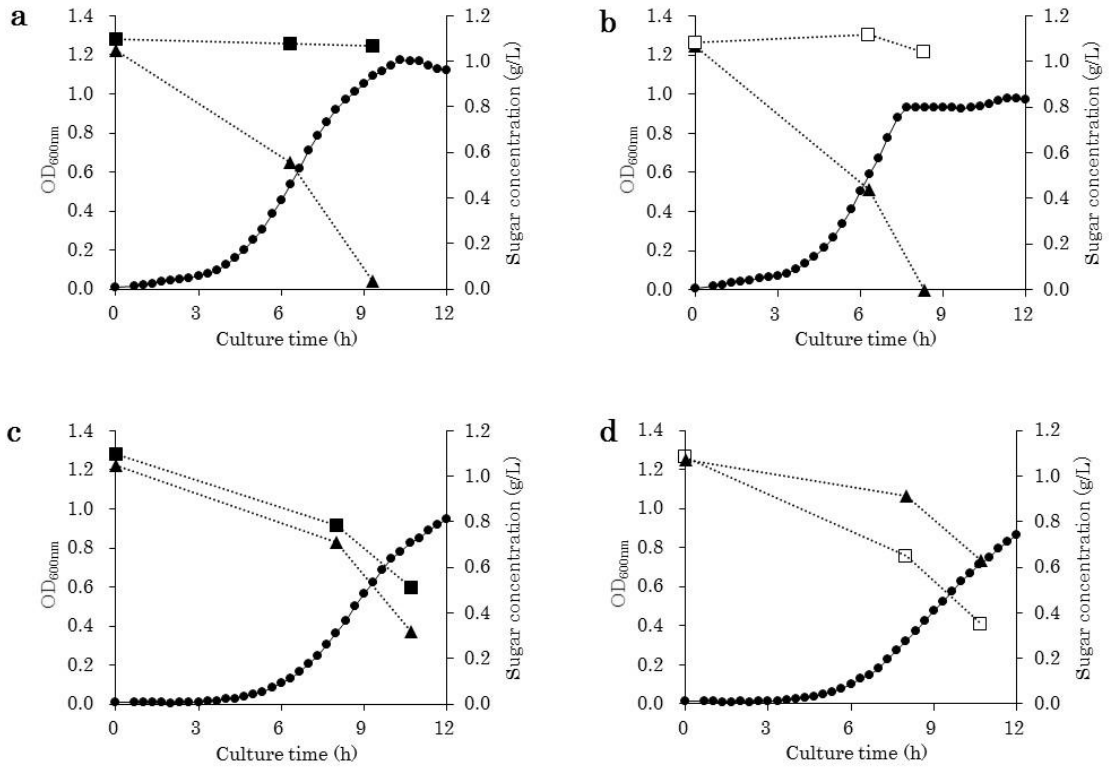


Fig. 2-2 Growth curves of *E. coli* recombinants on M9-glucose-maltose or M9-glucose-isomaltose. **a** MG1655 (empty vector) on M9-glucose-maltose. **b** MG1655 (empty vector) on M9-glucose-isomaltose. **c** MG1655 (glvAC) on M9-glucose-maltose. **d** MG1655 (glvAC) on M9-glucose-isomaltose. Closed circle; cell growth, closed triangle; glucose concentration, closed square; maltose concentration, open square; isomaltose concentration.

2-3-3 Evaluation of the substrate specificity of GlvA and GlvC in *E. coli*.

Pikis et al. reported that that expression of *aglA* and *aglB* from *K. pneumonia* in *E. coli* allowed the cells to assimilate α-methyl-glucoside, isomaltose,

trehalulose, turanose, maltulose, leucrose, and isomaltulose[31]. To investigate whether the heterologous expression of *glvA* and *glvC* enabled *E. coli* to assimilate other types of carbon sources, particularly panose, I performed growth tests using M9 solid medium supplemented with various types of sugars and sugar alcohols as a sole carbon source. I tested glucose, α-methyl-glucoside, sucrose, maltose, isomaltose, maltitol, trehalose, maltulose, isomaltulose, galactinol, cellobiose, gentiobiose, lactose, melibiose, lactulose, maltotriose, panose, isomaltotriose, maltotetraose, raffinose, maltotriitol, isomaltitol, lactitol, erlose, and maltopentaose (Fig. 2-3 and Table 2-3). My results showed that the recombinant could assimilate many types of sugars and sugar alcohols, including α-methyl-glucoside, isomaltose, turanose, maltulose, and isomaltulose, which had been reported to be assimilated by AglA and AglB[31]. In contrast, galactinol, maltotriitol, and erlose could not be assimilated by the recombinant. Interestingly, the recombinant was able to assimilate trisaccharides, such as panose and isomaltotriose. Moreover, ß-linked disaccharides such as sucrose and gentiobiose were also assimilated by the recombinant. It is an unexpected result because GlvA is classified as a 6-phospho-α-glucosidase[41].

The expression of either *glvA* or *glvC* alone is not sufficient to confer upon the recombinant the ability to assimilate any of the tested sugars and sugar alcohols except α-methyl-glucoside. It is known that α-methyl-glucoside is transported by PtsG of *E. coli* with concomitant phosphorylation[31]. GlvA

can hydrolyze the phosphorylated α-methyl-glucoside, conferring the ability to grow on the medium containing α-methyl-glucoside.

A common chemical structure among the substrates assimilated by the GlvA- and GlvC-expressing recombinant was the presence of glucose at one terminal of the sugar or sugar alcohol. Therefore, GlvC appeared to recognize the glucose unit of the sugar or sugar alcohol and transport the unit with concomitant phosphorylation of the glucose terminal. Moreover, GlvA may hydrolyze the 6'-phospho-sugars and sugar alcohols to release glucose 6-phosphate. This is the first report to show expansion of the sugar substrates of *E. coli* to trisaccharides by heterologous expression of 6-phospho-α-glucosidase and PTS components.

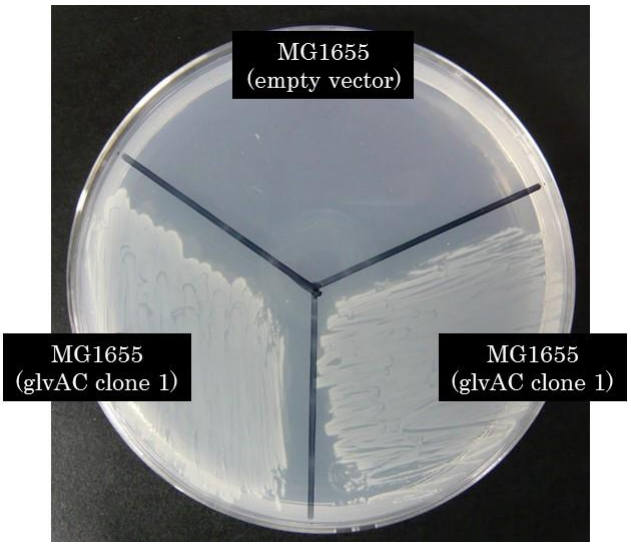


Fig. 2-3 Growth of *E. coli* recombinants on M9 solid medium containing panose as the sole carbon source. Two clones were tested in case of MG1655 (glvAC).

Table 2-3 Growth of *E. coli* recombinants on selected sugars.

Substrate	DP	Form	MG1655 (empty vector)	MG1655 (glvAC)	MG1655 (pAP2) (Ref. 31))
Glucose	1	Sugar	++	++	++
α-Methyl-glucoside	1	Sugar	NDG	++	++
Sucrose	2	Sugar	NDG	++	-
Maltose	2	Sugar	++	++	++
Isomaltose	2	Sugar	NDG	++	++
Maltitol	2	Sugar Alcohol	NDG	++	++
Trehalose	2	Sugar	++	++	++
Turanose	2	Sugar	+	++	++
Maltulose	2	Sugar	NDG	++	++
Isomaltulose	2	Sugar	NDG	++	++
Galactinol	2	Sugar Alcohol	NDG	NDG	No information
Cellobiose	2	Sugar	+	+	No information
Gentiobiose	2	Sugar	NDG	+	No information
Lactose	2	Sugar	++	++	No information
Melibiose	2	Sugar	++	++	No information
Lactulose	2	Sugar	++	++	No information
Maltotriose	3	Sugar	++	++	No information
Panose	3	Sugar	NDG	++	No information
Isomaltotriose	3	Sugar	NDG	++	No information
Maltotetraose	4	Sugar	++	++	No information

Raffinose	3	Sugar	+	++	No information
Maltotriitol	3	Sugar Alcohol	NDG	NDG	No information
Isomaltitol	3	Sugar Alcohol	NDG	++	No information
Lactitol	2	Sugar Alcohol	++	++	No information
Erlose	3	Sugar	NDG	NDG	No information
Maltopentaose	5	Sugar	++	++	No information

Data of MG1655 (pAP2) which express *aglA* and *aglB* from *K. pneumoniae* is described in the reference[31] and listed in this table for comparison to show what kinds of sugars and sugar alcohols were newly assimilated by heterologous expression of *glvA* and *glvC*.

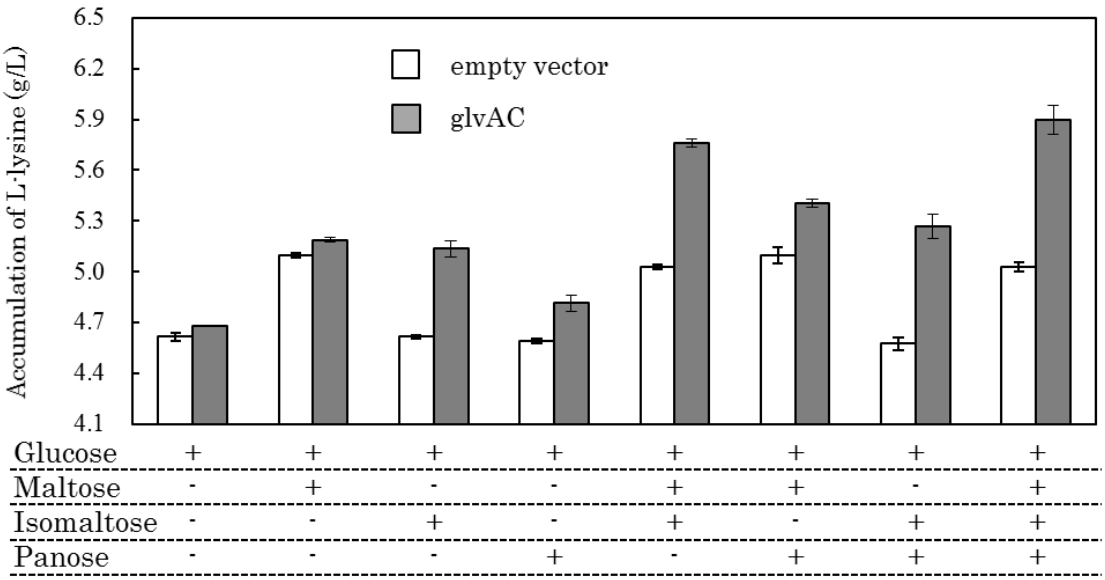
DP: degree of polymerization; NDG:no detectable growth; -: minimal growth; +: slight growth; ++: clear growth.

2-3-4 Utilization of isomaltose and panose using GlvA and GlvC in an L-lysine-producing model strain.

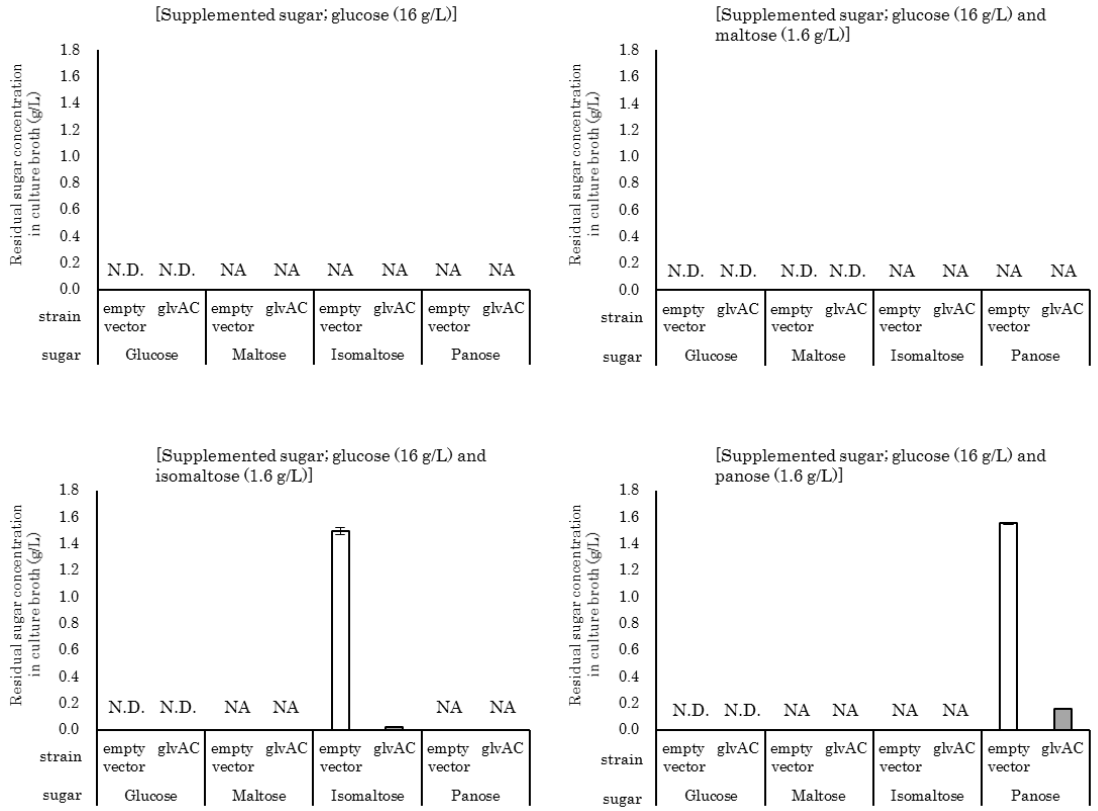
To evaluate the effects of isomaltose and panose utilization on fermentation efficiency, I introduced the plasmids to an L-lysine-producing *E. coli* strain WC196LC (pCABD2)[3,42,43]. The recombinant was cultivated on L-lysine-production medium supplemented with glucose or glucose combined with isomaltose, panose or maltose (as a control). Additionally, media containing different combinations of the above saccharides were also prepared and used

for L-lysine-production tests. L-Lysine accumulation in the culture broth of *E. coli* WC196LC (pCABD2) harboring the empty vector plasmids was increased by approximately 0.5 g/L only when maltose, which can be assimilated intrinsically by *E. coli*, was contained in the medium in addition to glucose (Fig. 2-4a). In contrast, the L-lysine production by the recombinant was increased when maltose, isomaltose, and panose were all contained in the medium. In the case of isomaltose utilization by the recombinant, L-lysine accumulation in the culture broth was increased by approximately 0.5 g/L; in contrast, in the case of panose, it was increased by approximately 0.2 g/L, showing lower utilization efficiency compared with that for maltose and isomaltose (Fig. 2-4a). Residual sugar analysis (Fig. 2-4b) indicated that about 98% of supplemented isomaltose and 90% of panose were consumed by the recombinant. Although small amounts of isomaltose and panose remained in the culture broth, the assimilation of isomaltose and panose was clearly enhanced by the introduction of *glvA* and *glvC.* These results showed that isomaltose and panose could be utilized as carbon sources and converted to L-lysine, suggesting that the heterologous expression of *glvA* and *glvC* could increase the efficiency of glucose feedstock utilization.

a


Accumulation of L-lysine (g/L)
6.5
6.2
5.9
5.6
5.3
5.0
4.7
4.4
4.1
empty vector
glvAC
Glucose + + + + + + + +
Maltose - + - - + + - +
Isomaltose - - + - + - + +
Panose - - - + - + + +

b


[Supplemented sugar; glucose (16 g/L)]
[Supplemented sugar; glucose (16 g/L) and maltose (1.6 g/L)]
[Supplemented sugar; glucose (16 g/L) and isomaltose (1.6 g/L)]
[Supplemented sugar; glucose (16 g/L) and panose (1.6 g/L)]
Residual sugar concentration in culture broth (g/L)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
N.D.
NA
strain
sugar
empty vector
glvAC
Glucose
Maltose
Isomaltose
Panose

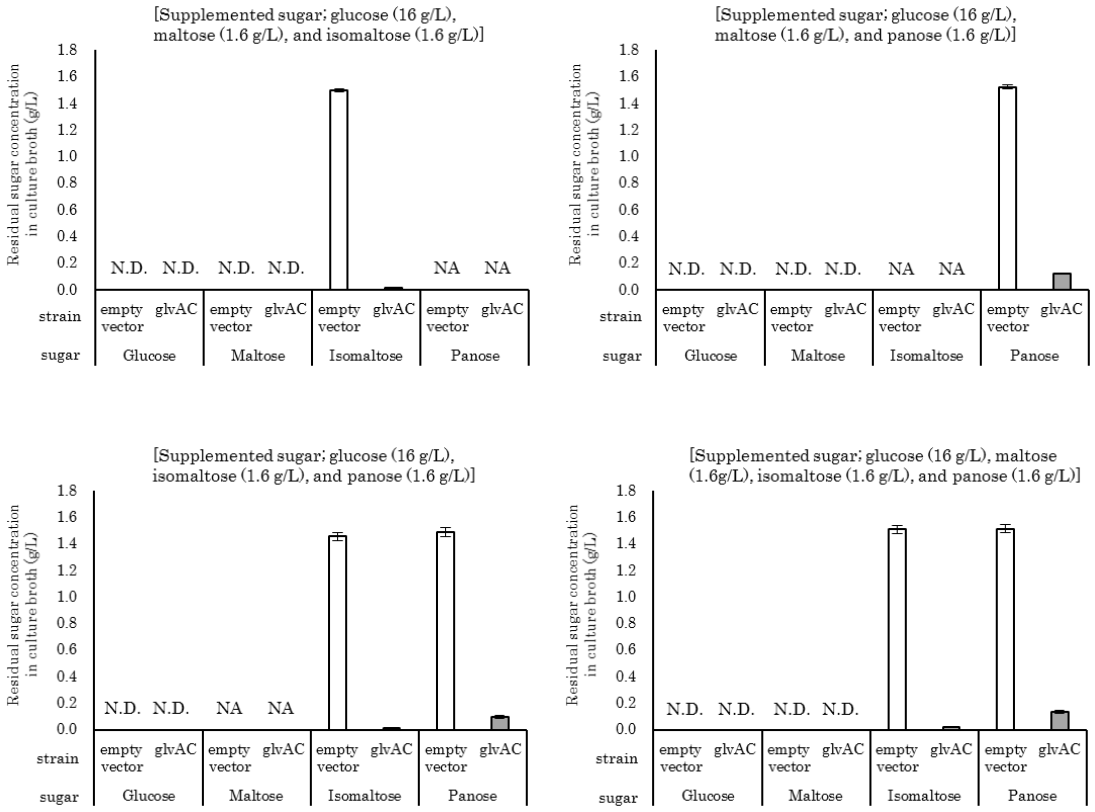


Fig. 2-4 Utilization of maltose, isomaltose, and panose in the L-lysine-producing model strain. **a** Accumulation of L-lysine in the L-lysine-production medium supplemented with glucose or glucose combined with maltose, isomaltose, and panose at the end of fermentation. **b** Residual maltose, isomaltose, and panose in the culture broth at the end of fermentation. Values are the means of more than three independent samples. SE bars represent the standard error of the mean calculated with Excel software. Empty vector: WC196LC (pCABD2) harboring the empty vector plasmids, pTVW229 and pMW219-Δplac; glvAC: WC196LC (pCABD2) harboring the *glvA*- and *glvC*-

expressing plasmids, pTWV229-self-glvA-Fw and pMW219-ΔPlac-Ptac4075-glvC; N.D.: not detected; NA: no addition.

2-4 Discussion

The PTS is composed of the phosphohistidine carrier protein (HPr), the enzyme I (EI) component, and the enzymes EIIA, EIIB, and EIIC. Although heterologous expression of *glvA* (encoding phospho-α-glucosidase) and *glvC* (encoding EIICB) conferred upon *E. coli* the ability to assimilate isomaltose, panose, and various sugars and sugar alcohols, the combination of GlvA and GlvC did not provide all the components needed to produce PTS activity, functioning only as an EIICB enzyme. HPr and EI are not specific to particular sugars and EIIAs also do not have strict substrate selectivity for each sugar and EIICB. For example, $EIIA^{Glc}$ can interact with glucose-PTS and trehalose-PTS[29]. Pikis et al. reported that AglA, a homolog of GlvC, interacts with $EIIA^{Glc}$, which is encoded by the endogenous *crr* gene in *E. coli*[31]. Therefore, GlvC is also likely to interact with $EIIA^{Glc}$ of *E. coli.* I disrupted the *crr* gene and tested whether the heterologous expression of *glvA* and *glvC* allowed the mutant to assimilate isomaltose. The *crr* mutant harboring the *glvA* and *glvC* plasmids could not grow on M9 medium containing isomaltose as a sole carbon source (data not shown). My results suggested that GlvC (a single chain polypeptide of EIIB and EIIC) derived from the Gram-positive bacterium *B. subtilis* could associate with $EIIA^{Glc}$ of *E. coli*, similar to AglA of *K. pneumoniae*.

In industrial production of valuable compounds with *E. coli*, purified sugars are rarely used due to high cultivation cost and hence various sugar mixtures are used as carbon sources[7,49]. However, assimilation of many

sugars starts sequentially after consumption of glucose with lag phase, resulting in the extension of culture time and decrease of productivity[49,50] due to carbon catabolite repression[51]. In order to overcome this problem, several researchers have attempted to confer upon *E. coli* the ability to assimilate arabinose[52], xylose[52,53], and maltose[54] even in the presence of glucose. For example, Hernández-Montalvo used a mutant devoid of the phosphotransferase system to escape catabolite repression. In this study, I demonstrated that the heterologous expression of *glvA* and *glvC* under constitutive promoter allows *E. coli* to assimilate maltose and isomaltose in the presence of glucose. Surprisingly, the recombinant could also assimilate various other sugars and sugar alcohols, including several trisaccharides. This genetic engineering expanded the assimilable sugars of *E. coli* and could increase product yield when using glucose feedstock. I demonstrated that an L-lysine-producing *E. coli* harboring *glvA* and *glvC* converted isomaltose and panose to L-lysine efficiently. This approach should increase the efficiency of industrial fermentation using *E. coli* and would facilitate full utilization of valuable carbohydrate resources.

Chapter 3

A novel biocontainment strategy makes bacterial growth and survival dependent on phosphite

3-1 Introduction

GMMs play a vital role in the chemical, pharmaceutical, and food industries. Owing to recent developments in the fields of synthetic biology[55,56] and genomics[57,58], there is a growing demand for functionalized GMMs that can be safely used outside the enclosed laboratory facilities. Novel applications of GMMs include the production of biofuels, bioremediation[17], and clinical treatments[59]. One of the biggest concerns for the practical use of GMMs in the open environment is the possibility of uncontrolled proliferation that, in a worst-case scenario, could endanger public health or biodiversity. Therefore, biocontainment strategies for safeguarding against the spread and proliferation of GMMs in the environment must be developed to realize their practical applications.

P is a component of nucleic acids, lipids, and various cellular metabolites, and is an essential nutrient for all living organisms. Nearly all biologically available P in the environment is in the form of Pi and its esters, in which the oxidation state of P is +5. Several groups of bacteria also possess an oxidization system for reducing P compounds, such as Pt and, less commonly, HPt. The molecular basis of Pt/HPt-oxidation in bacteria was established using the soil bacterium *Pseudomonas stutzeri* WM88[24]. In this bacterium, HPt is oxidized to Pt by HPt dioxygenase (HtxA)[25], and the resultant Pt is oxidized to Pi by NAD-dependent Pt dehydrogenase (PtxD)[26]. However, Pt and HPt are ecologically rare compounds not available in sufficient amounts to support bacterial growth[28].

The mechanisms involved in the metabolism of reduced P compounds are potentially useful for developing novel biotechnological applications[60,61]. Hirota et al. demonstrated that PtxD can be used as a selectable marker in yeasts[27]. Since Pt cannot be metabolized without its oxidation to Pi, the introduction of *ptxD* into cells that are incapable of utilizing Pt confers the ability to grow on a medium containing Pt as the sole source of P. Based on this expanded Pt utilization ability of a host organism and given the scarcity of Pt in the environment, I conceived that engineered dependency on Pt could also be used as a strategy for biocontainment. In this scenario, if a strain expressing PtxD and a Pt transporter were engineered to be unable to take up Pi or other Pi compounds commonly present in the environment, it would be unable to grow without exogenous Pt (Fig. 1-4a). This strategy, however, would require a Pt-specific transporter that is unable to transport Pi.

In this study, I found that the HPt transporter, HtxBCDE, of *P. stutzeri* WM88, exclusively takes up Pt and HPt but not Pi. This finding enabled me to develop a biocontainment strategy utilizing Pt or HPt as a required nutrient. Considering the extremely low escape frequency and the simplicity of required genetic modifications, this strategy may contribute to the development of a reliable and cost-effective biocontainment system for practical applications. This is the first report on the development of a biocontainment strategy based on the metabolism of P, a linchpin molecule for cellular metabolism.

3-2 Experimental section

3-2-1 Bacteria and media.

E. coli strains used in this study are listed in Table 3-1. Routine culture of *E. coli* strains was conducted in 2×yeast extract-tryptone (2xYT) medium. Morpholinepropanesulfonic acid-glucose synthetic (MOPS) medium[61] was used as a minimal medium. MOPS media containing Pi, Pt, HPt, and glycerol 3-phosphate (G3Pi) are designated as MOPS-Pi, MOPS-Pt, MOPS-HPt, and MOPS-G3Pi, respectively. P-free MOPS medium is designated as MOPS-0. The P concentration of MOPS media was 1.0 mM unless otherwise stated. Pt and HPt stock solutions (1.0 M) were prepared by dissolving phosphorous acid (Nakarai Tesque, Kyoto, Japan) or sodium hypophosphite in distilled water, respectively. These solutions were neutralized with sodium hydroxide, filtered, aliquoted, and stored at −20 °C until use. Soil extract was prepared from leaf mold soils purchased at a local market. Briefly, 0.1 kg air-dried soil was mixed with 0.3 L of tap water and autoclaved for 30 min. The extract was serially filtered with Whatman 3MM paper and a 0.45-μm pore-sized filter membrane to remove insoluble matter. The Pi and total P concentrations of soil extracts were determined by the method described previously[62]. Sheep blood agar plates (Kohjin BIO, Saitama, Japan), 2xYT, Terrific Broth (BD Biosciences, Franklin Lakes, NJ, USA), and chocolate agar plates (BD Biosciences) were used for challenges with naturally available P sources. The concentrations of antibiotics used were 50 mg/L for ampicillin or kanamycin and 25 mg/L for chloramphenicol.

Table 3-1. Bacterial strains used in this study.

Strain		Description	Reference or source
E. coli			
	DH5α	Cloning host strain	Toyobo Co. Ltd.
	MG1655	Wild-type strain; F⁻ *arcA-1655 fnr-1655*	Laboratory stock
	BW25113	*rrnB3* Δ*lacZ4787* *hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)568 *rph-1*	63
	BW17335	DE3(*lac*)X74 Δ(*pstSCAB-phoU*)*560*::Km^r	64
	JW2234	BW25113 Δ*glpT*::Km^r	NBRP
	JW3418	BW25113 Δ*ugpB*::Km^r	NBRP
	JW3641	BW25113 Δ*uhpT*::Km^r	NBRP
	MT2010	MG1655 Δ*pitA*::*frt* Δ*pitB*::*frt* Δ*phnC*::*frt* Δ*phoA*::*frt*	65
	MT2012	MG1655 Δ*pitA*::*frt* Δ*pitB*::*frt* Δ*phnC*::*frt* *phoA*::*frt* *pstSCABphoU*::*kan*	65
	MT2012-*ptxD*	MT2012 harboring ptxD/pSTV	This study
	RN1002	MG1655 Δ*pitA*::*frt* Δ*pitB*::*frt* Δ*phnC*::*frt* *phoA*::*frt* Δ*glpT*::*frt*	This study
	RN1004	MG1655 Δ*pitA*::*frt* Δ*pitB*::*frt* Δ*phnC*::*frt* *phoA*::*frt* Δ*glpT*::*frt* Δ*ugpB*::*frt*	This study
	RN1006	MG1655 Δ*pitA*::*frt* Δ*pitB*::*frt* Δ*phnC*::*frt* *phoA*::*frt* Δ*glpT*::*frt* Δ*ugpB*::*frt* Δ*uhpT*::*frt*	This study
	RN1007	MG1655 Δ*pitA*::*frt* Δ*pitB*::*frt* Δ*phnC*::*frt* *phoA*::*frt* Δ*glpT*::*frt* Δ*ugpB*::*frt* Δ*uhpT*::*frt* ptxD/pTWV229, htxABCDE/pSTV28	This study
	RN1008	MG1655 Δ*pitA*::*frt* Δ*pitB*::*frt* Δ*phnC*::*frt* *phoA*::*frt* Δ*glpT*::*frt* Δ*ugpB*::*frt* Δ*uhpT*::*frt* Δ(*pstSCAB-phoU*)*560*::Km^r, ptxD/pTWV229, htxABCDE/pSTV28	This study

Ralstonia sp. 4506	Pt-oxidizer, harboring ptxABCD gene	66
Pseudomonas stutzeri WM88	HPt- and Pt-oxidizer, harboring *htxABCDE* gene	24

3-2-2 Plasmid construction.

Plasmids and primers used in this study are listed in Table 3-2. The gene coding for PtxABC of *Ralstonia* sp. 4506[66] was amplified by PCR from genomic DNA using primer pair EcoPtxA(-186)_fw/BamPtxC(+13)_rv. The resultant PCR fragment was inserted into the *Eco*RI/*Bam*HI cloning site of pMW118. The DNA fragment containing *htxABCDE* of *P. stutzeri* WM88 was amplified from genomic DNA using primer pair htxA-14_fw2/htxE_rv2 and was inserted into the *Bam*HI sites of pMW118 and pSTV28 by using the In-Fusion HD cloning kit (Takara Bio, Inc., Tokyo, Japan). For construction of ptxD/pSTV28, a DNA fragment containing *ptxD* was amplified from *Ralstonia* sp. 4506 genomic DNA using primer pair EcoPtxD(-157)_fw/BamHI-ptxD(+24)_rv and was introduced into the *Eco*RI/*Bam*HI site of pSTV28. For construction of ptxD/pTWV229, primer pair ptxD_fw/ptxD_rv was used to amplify the *ptxD* gene from *Ralstonia* sp. 4506. The amplified DNA fragment was inserted into the *Sma*I site of pTWV229ΔPlac-Ptac4071[67] by the In-Fusion HD cloning method. The linearized vector was amplified from the plasmid by PCR using primer pair Ptac4071-fw/Ptac4071-rv. All DNA fragments cloned into vector plasmids were verified by sequencing.

Table 3-2. Primers and plasmids used in this study.

Name	Sequence (5'-3') or description	Source
Primers		
EcoPtxA(-186)_fw	aagaattcTAGCAGGCGTCTATATTTGGCATAG	-
BamPtxC(+13)_rv	aaatctagaGCTTTGGGAGTTATTTGAACTTGCG	-
EcoPtxD(-157)_fw	aagaattcAATCGGGTTCGAGCTGATGGGCTC	-
BamPtxD(+24)_rv	*aaatctagaTCGCCACACGCTCCAGATCTATCAC*	-
htxA-14_fw2	cggtacccggggatcCTAGGAGCATCACCATGTTTGCAGAGC	-
htxE_rv2	cgactctagaggatcTCAGATCAGCTTGGCGCGGATGCGCGCCTG	-
Ptac4071-fw	GCCCGCCATAAACTGCCAGGCATC	-
Ptac4071-rv	GGCAGTCTCCTTGTGTGAAATTGTTATCCG	-
ptxD-fw	cacaaggagactgccATGAAGCCCAAAGTCGTCCTC	-
ptxD-rv	cagtttatggcgggcTCACGCCGCCTTTACTCCCGG	-
pitA_chkx1-fw	CGTTGCGCTCCTCTTAGAAAA	-
pitA_chkx3-rv	GTGTTAACTGATTGGCAGCG	-
pitB_chkx1-fw	TTAACCAGTGGAATACCTGTG	-
pitB_chkx3-rv	CTCAGAATATCCGTTCAACC	-
phnC_chkx1-fw	AACTGTTCCGACGCGATTGC	-
phnC_chkx2-rv	ATCAACACGCTCCAGTAACC	-
pstSCABphoU_chk-fw	GACGTCGAAATCGCCTCTGAATTCC	-
pstSCABphoU_chk-rv	ACTTCAGATGTGTAACCAGTCGCTG	-
phoA_chkx1-fw	GAGTCGAAAGAACTGTGTGC	-
phoA_chkx2-rv	GAGGAGTTAAAGGAGGTTCC	-
glpT_chk-fw	TCATAAATAAGACCACGGGC	-
glpT _chkx3-rv	ACCAGTTTATTCTGCTGAGC	-
ugpB_chk-fw	CGCATTCGGTACAACAAGAG	-

ugpB _chkx3-rv	GAAGAACAGGAAGTTGTAGC	-
uhpT_chk-fw	ACAATGCATGCCTCACGCAG	-
uhpT _chkx3-rv	CATATGGCAACACCATTGCC	-

Plasmids

pMW118	Cloning vector; Ampr, a pSC101 derivative low-copy-number vector	Nippon Gene Co. Ltd.
ptxABC/pMW118	PtxABC expression plasmid	This study
htxABCDE/pMW118	HtxABCDE expression plasmid	This study
pSTV28	Cloning vector; Cm^r, a pACYC184 derivative medium-copy-number vector	Takara Bio Inc.
ptxD/pSTV28	PtxD expression plasmid	This study
htxABCDE/pSTV28	HtxABCDE expression plasmid	This study
pTWV229ΔPlac-Ptac	Cloning vector; a pTWV229 derivative medium-copy-number vector containing tac promoter in the multi-cloning site	67
ptxD/pTWV229	pTWV229ΔPlac-Ptac4071 containing ptxD	This study
pCP20	FLP expression plasmid; Ampr, temperature-sensitive replication and FLP synthesis	63

3-2-3 Characterization of P transporters.

The pMW118-derivative plasmids carrying genes for *ptxABC* or *htxABCDE* were individually introduced into the Pi transporter-null mutant MT2012[65]

expressing PtxD (MT2012-*ptxD*). For the growth assay, cells grown on MOPS-G3Pi were collected by centrifugation, washed three times with MOPS-0, and resuspended to an optical density at 600 nm (OD_{600}) of 1.0. Next, 50 μL of each cell suspension was used to inoculate glass test tubes containing 5 mL MOPS-Pi or MOPS-Pt, and these cultures were incubated at 37 °C with constant shaking (160 rpm). The turbidities of the cultures were monitored using an OD monitoring instrument (OD-Monitor C&T; Taitec Co. Ltd., Saitama, Japan).

3-2-4 Pi incorporation analysis.

[^{32}P]-labeled Pi ([^{32}P]Pi) uptake by *E. coli* cells was carried out under conditions in which Pi uptake was linear over time. Cells grown to mid-log phase in MOPS-G3Pi medium at 37 °C were collected by centrifugation, washed two times in MOPS-0, and resuspended in MOPS-0 to an OD_{600} of 0.2. Cells were kept at 37 °C until the assay was performed. Each assay was started by adding 200 μL of cell suspension to 1 mL of pre-warmed MOPS medium containing 20 μM Pi and [^{32}P]Pi (MP Biomedicals, Santa Ana, CA, USA). After the addition of the [^{32}P]Pi solution, a 200-μL aliquot was removed at various times, filtered, and the filter was washed twice with 5 mL of an ice-cold 10 mM LiCl solution. The filter was then transferred to a scintillation vial containing 5 mL of scintillation cocktail (GE Healthcare, Marlborough, MA, USA), and the amount of radioactivity taken up by cells was measured with a liquid scintillation counter.

3-2-5 Engineering of P metabolic pathway.

Fig. 1-4b illustrates the strategy used to construct an *E. coli* recombinant whose growth is strictly dependent on Pt/HPt. To construct a mutant deficient in Pi and organic Pi transport, three organic Pi transporters (*glpT*, *ugpB*, and *uhpT*) were disrupted in addition to four Pi transporters (*pitA*, *pitB*, *phnCDE*, and *pstSCAB*)[68]. The gene for *phoA* was also disrupted because *E. coli* alkaline phosphatase exhibits weak Pt-oxidizing activity[69] which could result in oxidization of Pt in the periplasmic space, reducing the amount of Pt taken up. At the beginning of the construction scheme, deletion of the organic Pi transporter *glpT* from the *E. coli* Keio collection[70] (National BioResource Project, National Institute of Genetics, Shizuoka, Japan) was transferred to the *E. coli* mutant MT2010 (Δ*pitA*, Δ*pitB*, Δ*phnC,* and Δ*phoA*)[65] by P1 transduction. A kanamycin resistance cassette in the chromosome of the resultant strain was eliminated by flippase (FLP)-mediated recombination using the pCP20 plasmid[63]. After plasmid clearance of the kanamycin-sensitive clone, which I designated RN1002, *ugpB* and *uhpT* were sequentially disrupted in the same manner. The resultant strain, lacking all P transporters except PstSCAB, was designated RN1006. Before disruption of *pstSCAB*, htxABCDE/pSTV28 and ptxD/pTWV229 were simultaneously introduced into RN1006 to support the uptake and subsequent oxidation of Pt, resulting in strain RN1007. Disruption of *pstSCAB* was completed last because the growth of *E. coli* using Pi as a P source is faster than that of cells using organic Pi compounds, facilitating cell culture during the series of gene

disruption experiments. *phoU*, the last gene in the *pst* operon, was deleted along with *pstSCAB* because inactivation of the *pst* gene results in severe defects in cell growth and generates various back mutants, probably due to the unknown function of the sensor protein PhoU[64,71]. P1 prepared from BW17355 was transduced into RN1007, and the resultant strain, RN1008, lacking all seven endogenous P transporters, was selected on MOPS-Pt plates containing ampicillin, chloramphenicol, and kanamycin. RN1008 could also be propagated on MOPS-Pt plates without antibiotics due to the selective pressure of Pt availability. Gene disruption was confirmed by PCR.

3-2-6 Environmental challenges and escape assay.

To determine whether RN1008 was able to grow on other Pi sources, a spot assay was performed in which 1 mL of RN1008 culture grown on MOPS-Pt at late log phase was centrifuged, washed once with an equal volume of MOPS-0, and diluted using a 10-fold series by 10^7-fold. Next, 10 μL of each diluted aliquot was spotted onto various culture media plates including Luria-Bertani (LB), 2xYT, Terrific Broth, sheep blood agar and chocolate agar plates, which were incubated at 37 °C, and the growth was monitored for 7 days. In order to investigate escape mutant generation, 5-L of RN1008 culture was grown to late log phase, pelleted, washed once with 100 mL MOPS-0, and resuspended in 10 mL MOPS-0. This cell suspension was plated on 50 large, square dish plates (245 mm × 245 mm × 25 mm; ThermoFisher Scientific, Kanagawa, Japan) containing 2xYT agar medium, and colony formation was

monitored for 21 days. Aliquots of the culture were diluted, spread onto MOPS-Pt plates, and CFU were counted after 48 h of incubation at 37 °C in order to determine total cell numbers used for the assay. The detection limit was calculated as one per the total CFU plated. For the viability assay, 1 mL of an overnight culture of RN1008 in MOPS-Pt medium was pelleted by centrifugation, washed once with MOPS-0, and resuspended in MOPS-0 to an OD_{600} of approximately 1.0. Then, 1 mL of the cell suspension was added to a 500-mL Erlenmeyer flask containing 100 mL of MOPS-Pt or 2xYT medium. At each sampling point, 0.5 mL of culture was collected from each flask, and its OD_{600} was measured. It was then diluted with MOPS-0 and spread onto 2xYT or MOPS-Pt agar plates. Dilution rates for spreading onto (permissive) MOPS-Pt plates ranged from 10^3-fold to 10^6-fold; for spreading onto (non-permissive) 2xYT plates, 0.1 mL of undiluted culture was used directly. Data were collected for 14 days following inoculation.

3-3 Results

3-3-1 The HPt transporter HtxBCDE takes up Pt but not Pi.

The key requirement for re-engineering the P metabolic system is a transporter that takes up Pt but not Pi (Fig. 1-4b). I expected that the Pt transporter, PtxABC, would satisfy this requirement. Therefore, I first investigated the substrate specificity of PtxABC. The MT2012[65], an *E. coli* strain lacking all indigenous Pi transporters (PitA, PitB, PstSCAB, and PhnCDE), can be used as a host strain to investigate Pi transport abilities of heterologously expressed transporters. Since MT2012 is also unable to grow on Pt (Fig. 3-1a), I constructed MT2012-*ptxD* to evaluate both Pi- and Pt-specific activities of PtxABC, and found that the resultant strain could grow on both MOPS-Pi and MOPS-Pt. This result indicated that, unfortunately, PtxABC can transport both Pi and Pt (Fig. 3-1a). I then tested HtxBCDE, a transporter for a more reduced form of inorganic P, HPt. The expression of HtxABCDE from *P. stutzeri* WM88[24] enabled MT2012-*ptxD* to grow on MOPS-Pt but not on MOPS-Pi (Fig. 3-1a), indicating that HtxBCDE transports only the reduced form of inorganic P.

To confirm that HtxBCDE is really unable to take up Pi, I performed Pi uptake analysis using [^{32}P]Pi. Consistent with the results of the growth assay, almost no Pi uptake was observed in MT2012-*ptxD* expressing HtxABCDE as well as in the control strain, confirming that HtxBCDE is not able to import Pi. In contrast, MT2012-*ptxD* expressing PtxABC took up Pi at the rate of approximately 5.4 ± 1.1 nmol Pi/min/mg cell (dry cell weight) (n=3,

mean ± s.d.) (Fig. 3-1b). It should be noted that the prolonged incubation (60 min) of MT2012-*ptxD* and MT2012-*ptxD* expressing HtxABCDE resulted in increased radioactivities (approximately 0.2 nmol Pi/min/mg cell). This may be due to the incorporation of trace amounts of Pi via organic Pi transporters (GlpT, UgpG, and UhpT), although such an unspecific Pi uptake is insufficient to support cell growth[72]. Thus, these results together indicated that HtxBCDE has the required characteristics for engineering a reduced-P based metabolic system. A novel combination of HPt transporter and Pt dehydrogenase (PtxD) was therefore used instead of PtxABC in my biocontainment strategy (Fig. 1-4). Since MT2012-*ptxD* expressing HtxABCDE was also able to grow on MOPS-HPt (see below), co-expression of HtxA additionally confers HPt-oxidation activities. Therefore, HPt can also be used as a required nutrient.

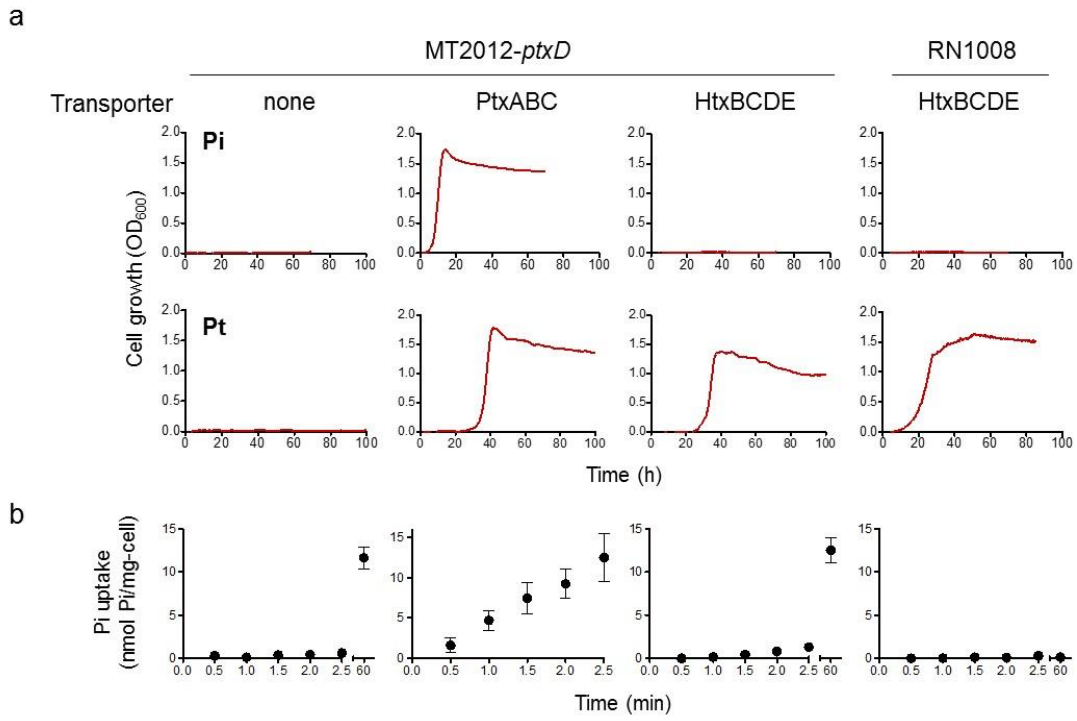


Fig. 3-1. Characterization of the Pi and Pt transport abilities of Ptx and Htx transporters. (a) Growth of MT2012-*ptxD* expressing PtxABC or HtxABCDE on MOPS-Pi (top) or MOPS-Pt (bottom). MT2012-*ptxD* was transformed with a pMW118-based transporter expression plasmid. Cell growth was monitored every hour by measuring cell turbidity at 600 nm using an OD monitor. Data are representative of two independent experiments with essentially the same results. The data are representative of two independent experiments with essentially the same results. (b) [^{32}P]Pi uptake of strains. The values expressed as mean ± s.d. of three biological replicates.

3-3-2 Engineering of an *E. coli* strain dependent on Pt/HPt for growth.

As expected, MT2012-*ptxD* expressing HtxABCDE was unable to grow on MOPS-Pi (Fig. 3-1). However, since this strain still possesses three transporters for organic Pi, including GlpT, UgpB (for G3Pi), and UhpT (for hexose phosphate), it can grow in the presence of organic Pi (Fig. 3-2, open squares). In order to completely eliminate organic Pi transport and make the strain Pt/HPt-dependent, I created strain RN1008, which lacks the four transporter genes for Pi (*pitA, pitB, phnC, and pstSCAB*) and those for organic Pi (*glpT, ugpB, and uhpT*) and harbors ptxD/pTWV and htxABCDE/pSTV (Fig. 1-4). This strain also lacks *phoA*, the gene for alkaline phosphatase, which can oxidize Pt in the periplasmic space. As expected, RN1008 was only able to grow on MOPS-Pt or MOPS-HPt media, and not on MOPS-Pi or MOPS-G3Pi (Fig. 3-1a, Fig. 3-2 open circles). Pi uptake analysis also showed that almost no Pi was taken up by RN1008, even after 60 min of incubation (Fig. 3-1b), confirming that the incorporation of trace amounts of Pi in MT2012-*ptxD* was via the organic Pi transporters. The growth rates of RN1008 on MOPS-Pt and MOPS-HPt were 0.40 ± 0.02/h and 0.31 ± 0.03/h (n=3, mean ± s.d.), respectively, which are 93 % and 71 %, respectively, of that of wild-type (MG1655) cultured in MOPS-Pi medium. The slower growth in MOPS-HPt medium could be due to the requirement for 2-oxoglutarate as a cofactor in HPt oxidation by HtxA[25]. Thus, Pt is the preferred selective nutrient for growing RN1008.

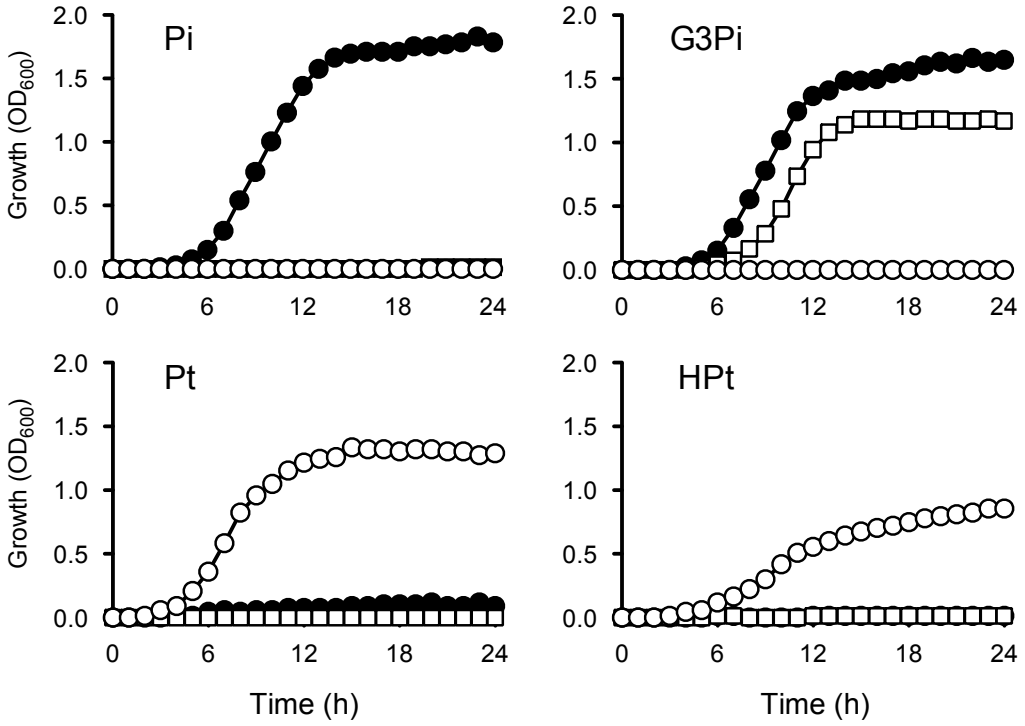


Fig. 3-2. Growth of *E. coli* strains on MOPS media containing four different P sources. Growth of *E. coli* MG1655 (closed circles), MT2012 (open squares), and RN1008 (open circles) on MOPS media containing 1.0 mM Pi, G3Pi, Pt, or HPt. Optical densities at 600 nm were measured every hour. The data are representative of two independent experiments with essentially the same results.

3-3-3 Growth and environmental challenge of the Pt/HPt-dependent strain.

To investigate the possibility that RN1008 is able to grow using other Pi compounds, cells were challenged to diverse types of media. A spot assay was

carried out with various types of solid media plates. This revealed that RN1008 was unable to form colonies on LB, 2xYT, and Terrific Broth plates for at least seven days (Fig. 3-3). Two types of enriched blood agar media, including sheep blood and chocolate agar media, also failed to support the growth of RN1008, suggesting that none of the biological P compounds in these media were able to support the growth of RN1008 (Fig. 3-3). To investigate the effect of P compounds present in the environment, I prepared two MOPS media containing soil extract (MOPS-SE) in which approximately 19% (corresponding to 0.39 mM, MOPS-SE[A]) and 86% (0.36 mM, MOPS-SE[B]) of P were present in the form other than Pi. The soil extract media also failed to support the growth of RN1008 (Fig. 3-3), suggesting that environmentally available P compounds cannot support the growth of RN1008. Phosphonate, which has a carbon-phosphorus (C-P) bond in its structure, is the only known biogenic reduced P compound produced by environmental microorganisms[73]. I examined the growth of RN1008 in MOPS medium containing 1.0 mM methylphosphonate, ethylphosphonate, or aminoethylphosphonate and found that none of these were able to serve as a P source for RN1008 (data not shown). These results strongly suggest that RN1008 is unable to grow in the open environment; therefore, the engineered Pt/HPt dependency represents a novel strategy for biocontainment.

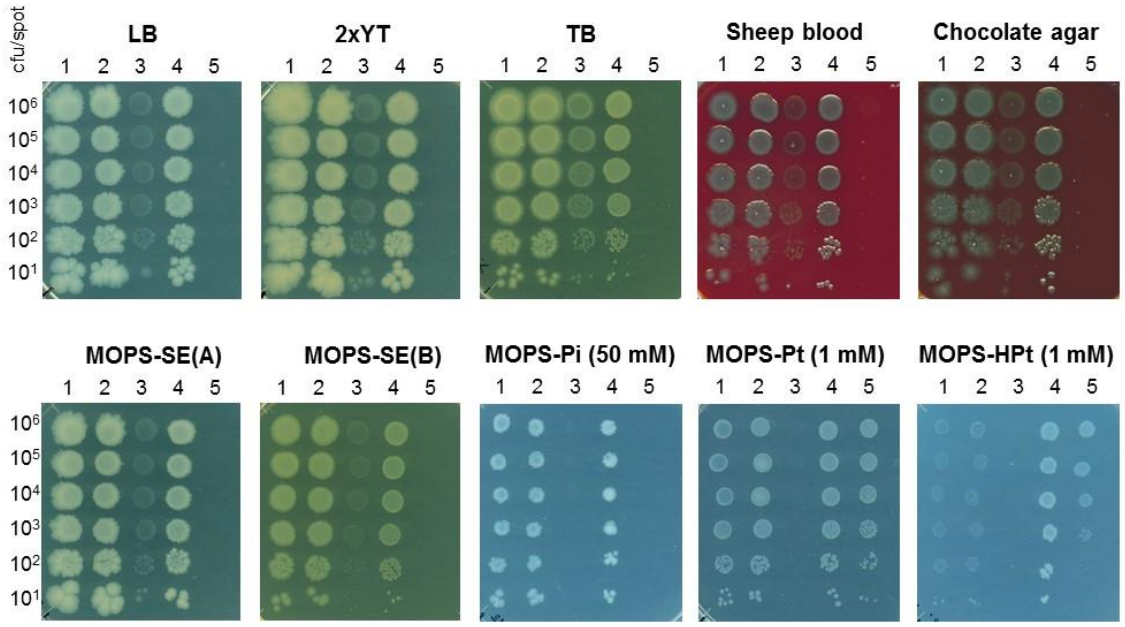


Fig. 3-3. Growth of RN1008 on diverse types of media plates. Spot assay to assess growth of RN1008 and related strains on various types of solid media. MOPS-SE were prepared from two types of leaf mold soils to create MOPS-SE[A] and MOPS-SE[B]. Pictures were taken at 48 h after incubation. Strain RN1008 did not form colonies, even after seven days of incubation (data not shown). Strains used (left to right): MG1655, MG1655 harboring ptxD/pTWV, MT2012, RN1007, and RN1008.

3-3-4 Pt/HPt-dependent strain does not yield escape mutants under non-permissive growth conditions.

Next, in order to assess the reliability of this containment strategy, I investigated the frequency of escape mutant generation under non-

permissive growth conditions. Approximately 5.2×10^{12} cells of RN1008 were incubated on non-permissive (2xYT) growth plates and did not form any colonies for 21 days, indicating that RN1008 does not yield any escape mutants with an assay detection limit of less than 1.9×10^{-13} per CFU. To investigate the viability of RN1008 during cultivation, approximately 10^8 cells were added to 0.1 L of 2xYT (non-permissive medium) or MOPS-Pt (permissive medium) and cultured for 14 days. RN1008 was unable to form colonies on non-permissive medium plates (data not shown), demonstrating that RN1008 did not yield any escape mutants during long-term liquid culture. In terms of the viability of RN1008, CFU number quickly declined during cultivation in 2xYT liquid medium, dropping to less than 100 CFU ($<1.0\times10^{-4}$ % of initial inoculum) at seven days and below the detection limit at 14 days after inoculation (Fig. 3-4). In contrast, the viability of RN1008 under permissive culture condition (MOPS-Pt) did not significantly decrease over 14 days (Fig. 3-4). These results indicate that the viability of RN1008 rapidly decreases in non-permissive culture conditions.

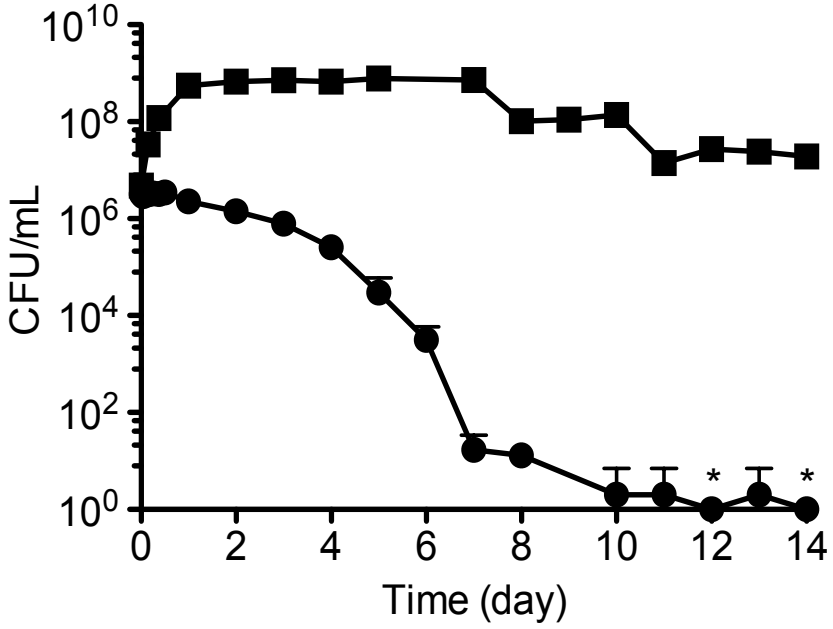


Fig. 3-4. Long-term stability of RN1008 in liquid media. A culture of approximately 10^8 RN1008 cells grown in MOPS-Pt medium was challenged over 14 days by growth on either permissive (MOPS-Pt, square symbols) or non-permissive (2xYT, circle symbols) media. Asterisks denote no CFU were observed. The values are expressed as mean + s.d. of three technical replicates. The data are representative of two biological replicates with essentially the same results.

3-4 Discussion

In this work, I developed a novel biocontainment strategy by engineering an *E. coli* strain that is dependent on Pt/HPt as required P sources. Engineering of Pt/HPt dependency required disruption of all endogenous P transporters supplemented by exogenous expression of PtxD and a P transport system that takes up Pt/HPt but not Pi. Initially, I expected that the Pt transporter, PtxABC, could be used as such a P transport system. However, I found that PtxABC also transports Pi. The finding that the HPt transporter HtxBCDE also takes up Pt but not Pi, enabled me to create an engineered Pt/HPt metabolic pathway for biocontainment. HtxBCDE, which is a crucial component of this biocontainment strategy, is a binding protein-dependent HPt transporter belonging to the ATP-binding cassette (ABC) transporter superfamily[74]. Within the HtxBCDE protein complex, discrimination between Pi and Pt/HPt is due to HtxB, a periplasmic substrate-binding protein (SBP). The molecular mechanism of Pi sequestration by SBPs has been elucidated in Pi binding protein PstS[75,76]. A Pi ion binds to PstS via 12 hydrogen bonds formed between the four oxygen atoms of a Pi molecule and -OH and -NH groups of the PstS protein. Eight conserved amino acid residues[75] located at the substrate-binding cleft of the PstS protein are responsible for hydrogen bond formation. Currently, there is no definitive information regarding the substrate recognition mechanism of HtxB. However, considering the difference in the number of oxygen atoms between the Pi and HPt molecules, a difference in the number of amino acids responsible for hydrogen bond

formation may account for the failure of HtxB to capture Pi. A deeper understanding of the structural basis of HtxB would help to reveal its mechanism of discriminating between Pi and Pt/HPt, as well as the minimum number of amino acid substitutions required to generate escape mutants.

As expected, RN1008, a strain lacking all endogenous P transporters and expressing PtxD and HtxABCDE, was not able to grow on media without Pt or HPt. Furthermore, I showed that this strain did not yield any escape mutants under non-permissive growth conditions with an assay detection limit of 1.9×10^{-13} per CFU, which is lower than that of any other reported biocontainment strategies. For the practical application of GMMs, the escape frequency should be low enough to ensure biosafety. Recent works showed that deliveries of 10^6 to 10^9 living GMMs into mice were effective for vaccination[77,78] or oncolytic therapies[79]. The use of GMM-based vaccinations or therapeutics in humans, however, would require development of a secure biocontainment system that can exclude generation of escape mutants. Considering the escape frequency of my strategy, it could be used in applications that involve the release of a large population of cells, such as therapeutics or bioremediation. I also note that this containment efficacy was achieved using a single strategy without any additional genetic modifications for preventing the generation of escape mutants. The extremely low escape frequency of my biocontainment strategy may be due to the fundamental role of Pi in cellular metabolism. Pi is required for numerous cellular metabolic events, such as ATP synthesis, nucleic acid synthesis, and signal transduction.

In particular, nucleic acid synthesis, which is involved in the generation of most types of adaptive mutations[80], requires a considerable amount of Pi. Thus, depletion of cellular Pi may result in the stalling of most metabolic events, including the induction of genetic mutations.

The growth of RN1008 strictly depends on the ecologically rare compounds, Pt or HPt. Although both P compounds are generally only present in quantities below the detection level in most environments, two environmental processes have been suggested that could possibly result in quantities of Pt and HPt entering the biosphere. First, some earlier reports proposed that microorganisms that are present in anoxic environments such as paddy fields may reduce Pi to generate Pt and/or HPt. However, these reports have never been confirmed. The reduction potential of the Pi/Pt couple is -650 mV, which far exceeds that of the NAD/NADH couple (-320 mV)[81]. Therefore, biological reduction of Pi is highly improbable in the present environment. Indeed, no reliable detection of biologically produced Pt or HPt has been documented[82]. Second, several geochemical reactions such as lightning strikes[83] and volcanic eruptions[81] may reduce Pi or release reduced P compounds. Although amounts of Pt/HPt produced by these geochemical reactions have not been determined, they are presumably not sufficient to enable cells survival and growth. In the absence of Pt, the viability of RN1008 cells was reduced by approximately 1.0×10^{-4} % after seven days of culture, and no cells were able to survive for more than 14 days (Fig. 3-4). Therefore,

it is unlikely that the engineered strain would be able to survive very long without externally supplied Pt or HPt.

Another possible risk for this strategy would be the acquisition of genes conferring Pi transport ability by the Pt/HPt-dependent strain via horizontal gene transfer (HGT). Although I did not evaluate the probability of mutant escape via HGT, the integration of a single gene with Pi transport function would be sufficient to generate escape strains. In order to reduce the above risks, the developed strategy should be used in combination with other containment strategies. The requirements for creating the containment strain are the disruption of eight genes involved in Pi uptake and introduction of the genes *ptxD* and *htxABCDE*. In contrast to more complicated biocontainment strategies that involve hundreds of genetic modifications, the developed strategy could be easily combined with other containment methodologies and applied to established host strains.

In addition to technical convenience, my system offers the ease of mass cultivation of the engineered microbe. As the cost of Pt is extremely low compared to those of other commonly used selection chemicals for biocontainment, the Pt/HPt dependency of the engineered strain provides a cost-effective, antibiotic-free cultivation methods[27,84,85]. These advantages, together with its strikingly low escape frequency, make Pt/HPt dependency a promising biocontainment strategy for practical applications.

Chapter 4

General conclusion

Fermentative production of useful compounds with GMMs is a powerful tool that enables cost-competitive production of bulk items such as alcohols and amino acids as well as stereochemically defined molecules whose chemical synthesis is difficult. In recent years, much effort has been devoted to creating new GMMs that produce items such as biofuels, proteins, and pharmaceuticals using synthetic biology techniques. A strong trend has also emerged to expand the usage of GMMs to fields such as bioremediation, agriculture, and healthcare applications, with development of adequate biocontainment strategies. I believes that fermentation and GMMs could make a substantial contribution to the quality of human life.

In the work described here, I developed a new method for engineering *E. coli* to facilitate efficient utilization of isomaltose and panose in industrial glucose feedstock as well as a new biocontainment methodology that employs phosphite.

Chapter 2 describes the new method for engineering *E. coli* to facilitate efficient utilization of isomaltose and panose in industrial glucose feedstock. Industrial glucose feedstock prepared by enzymatic digestion of starch typically contains significant amounts of disaccharides such as maltose and isomaltose, and trisaccharides such as maltotriose and panose. Maltose and maltosaccharides can be utilized in *Escherichia coli* fermentation using industrial glucose feedstock because there is an intrinsic assimilation pathway for these sugars. However, saccharides that contain α-1,6 bonds, such as isomaltose and panose, are still present after fermentation because

there is no metabolic pathway for these sugars. To facilitate more efficient utilization of isomaltose and panose contained in glucose feedstock, I introduced *glvA*, which encodes phospho-α-glucosidase, and *glvC*, which encodes a subunit of the phosphoenolpyruvate-dependent maltose phosphotransferase system (PTS) of *Bacillus subtilis*, into *E. coli.* The heterologous expression of *glvA* and *glvC* conferred upon the recombinant the ability to assimilate isomaltose and panose. The recombinant *E. coli* assimilated not only other disaccharides but also trisaccharides, including alcoholic forms of these saccharides, such as isomaltitol. To the best of my knowledge, this is the first report to show the involvement of the microbial PTS in the assimilation of trisaccharides. Furthermore, the I demonstrated that an L-lysine-producing *E. coli* harboring *glvA* and *glvC* converted isomaltose and panose to L-lysine efficiently. These findings are expected to be beneficial for industrial fermentation.

Chapter 3 describes a new biocontainment methodology using phosphite. There is a growing demand to develop biocontainment strategies that prevent unintended proliferation of genetically modified organisms in the open environment. I found that the hypophosphite (H_3PO_2, HPt) transporter HtxBCDE from *P. stutzeri* WM88 was also capable of transporting phosphite (H_3PO_3, Pt) but not phosphate (H_3PO_4, Pi), suggesting the potential for engineering a Pt/HPt-dependent bacterial strain as a biocontainment strategy. I disrupted all Pi and organic Pi transporters in an *E. coli* strain expressing HtxABCDE and a Pt dehydrogenase, leaving Pt/HPt

uptake and oxidation as the only means to obtain Pi. Challenge on non-permissive growth medium revealed that no escape mutants appeared for at least 21 days with a detection limit of 1.94×10^{-13} per colony forming unit, which is the lowest escape frequency among reported strategies. Since Pt/HPt are ecologically rare and not available in amounts sufficient for bacterial growth, this strategy offers a reliable and practical method for biocontainment.

I hopes that the work described here will contribute to the fermentative production of useful compounds and the realization of impregnable/practicable biocontainment systems.

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Acknowledgements

I would like to express my sincere gratitude to Doctor Akio Kuroda, Professor in the Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, for his kind instruction throughout this study.

I would like to express my deep appreciation to Doctor Ryuichi Hirota, Assistant Professor in the Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, for his instruction and continuous encouragement throughout this study, as well as experimental support.

I appreciate the help of Alexandrov Maxym, Postdoctoral Researcher in the Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, with proofreading the English of the manuscript.

I am grateful to Doctor Hisao Ito, Doctor Ryo Takeshita, and Doctor Yuri Nagai, Process Development Laboratories, Research Institute for Bioscience Products & Fine Chemicals, Ajinomoto Co., Inc. for providing experimental ideas and technical advice, as well as valuable support.

Finally, I would like to thank my wife and children, for their encouragement and devoted support.