JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Strategy for Stable and High-Level Expression of Recombinant Trehalose Synthase in *Escherichia coli*

Po Ting Chen,[†] Chung-Jen Chiang,[‡] Yu-Ting Chen,[§] Hsien-Chung Lin,[∥] Cheng-Huan Liu,[∥] Yun-Peng Chao,*[,⊥] and Jei-Fu Shaw*[,#,∇]

[†]Department of Biotechnology, Southern Taiwan University, No. 1, Nan-Tai Street, Tainan 710, Taiwan

[‡]Department of Medical Laboratory Science and Biotechnology, China Medical University, No. 91, Hsueh-Shih Road, Taichung 40402, Taiwan

[§]Insititute of Genomics and Bioinformatics, National Chung Hsing University, Taichung 40227, Taiwan

[∥]Department of Food Science and Biotechnology, National Chung Hsing University, Taichung 402, Taiwan

[⊥]Department of Chemical Engineering, Feng Chia University, No. 100, Wenhwa Road, Taichung 40724, Taiwan

[#]Agricultural Biotechnology Research Center, Academia Sinica, Taipei 11529, Taiwan

[∇]Department of Biological Science and Technology, I-Shou University, Kaohsiung City 84001, Taiwan

ABSTRACT: Trehalose is a nonreducing disaccharide and has a wide range of applications in food and biorelated industry. This sugar can be synthesized from maltose in one step by trehalose synthase. In this study, we attempted to overproduce trehalose synthase from *Picrophilus torridus* (PTTS), a thermoacidophilic archaea, in *Escherichia coli*. However, overproduction of PTTS was hampered when the T7 promoter-driven PTTS gene (P_{T7}-PTTS) on a multicopy plasmid was employed in *E. coli*. The factors limiting PTTS production were identified in a systematic way, including the codon bias, plasmid instability, a redundant gene copy, a high basal level of PTTS, and metabolic burden resulting from the mutlicopy plasmid DNA and antibiotics. To overcome these difficulties, an *E. coli* strain was developed with insertion of P_{T7}-PTTS into the chromosome and enhanced expression of genomic *argU* tRNA and *ileX* tRNA genes. Without the selective pressure, the constructed producer strain was able to produce a stable and high-level production of recombinant PTTS. Overall, we proposed a simple and effective method to address the issue that is most commonly raised in overproduction of heterologous proteins by *E. coli*.

KEYWORDS: *trehalose synthase, recombinant protein production, T7 expression system*

INTRODUCTION

Trehalose consists of two D-glucose molecules that are joined via the 1,1-glycosidic linkage. This nonreducing disaccharide is widespread among a large variety of living organisms, including bacteria, yeast, fungi, insects, and plants.[1−4] Trehalose is recalcitrant to hydrolysis by acid and α-gluosidase,[5] and it exists in three isomers, the α,α form (α,α-trehalose), α,β form (neotrehalose), and β,β form (isotrehalose).[5,6] In nature, the α,α form prevails and plays a profound role in the cell physiology. For instance, this isomer form acts as a signaling molecule and provides living cells with a carbon source, energy, and the structural component as well.[7−11] In particular, trehalose can help prevent cells from drastic damage resulting from various environmental stresses. One well-known example is anhydrobiotic organisms that largely accumulate trehalose are able to survive years of dehydration.[12] This remarkable characteristic has broadened the application range of trehalose, which involves increasing the survival rate of electroporated cells,[13] protecting enzymes or cells from freeze-drying destruction, and enhancing the storage stability of vaccines at room temperature.[14,15] The growing interest in potential application of this chemically underactive sugar can be readily realized in the fields of food, pharmaceuticals, and cosmetics.[16,17]

Production of trehalose mainly depends on the biotransformation process. The enzymatic route currently available includes the one- and two-step conversion reaction.[16] The latter reaction scheme typically involves two sequential enzymatic reactions. A representative example is that maltose is converted to trehalose in yeast by coupling the reaction of maltose phosphorylase (EC 2.4.1.8) and trehalose phosphorylase (EC 2.4.1.64).[16] In addition, trehalose can be obtained from maltodextrin hydrolyzed by maltooligosyl trehalose synthase (EC 5.4.99.15) and maltooligosyl trehalose trehalohydrolase (EC 3.2.1.141).[18,19] In contrast, one-step conversion of maltose to trehalose is mediated by trehalose synthase (TSase) (EC 5.4.99.15) via the intramolecular transglucosylation reaction.[20−22] This TSase-based reaction for production of trehalose appears to be relatively simple. Moreover, maltose is an inexpensive and easily obtainable substrate, thereby making the one-step production of trehalose industrially appealing.

Recently, we have cloned and characterized TSase from *Picrophilus torridus* (PTTS).[23] PTTS is the only TSase studied by far that exhibits high stability at the acidic condition. In

Received: April 13, 2012
Revised: May 18, 2012
Accepted: May 21, 2012
Published: May 21, 2012

ACS Publications © 2012 American Chemical Society

Table 1. Strains, Plasmids, and Primers Used in This Study[a]

	relevant characteristics	source
	Strains	
DH5α	*deoR endA1 gyrA96 hsdR17 supE44 thi1 recA1 lacZM15*	laboratory collection
BL21(DE3)	F^- *ompT* $hsdS_B(r_B^-\ m_B^-)$ *gal dcm* (DE3)	Novagen
Rosetta(DE3)	as BL21(DE3) pRARE (cam^r)	Novagen
BT01	as BL21(DE3) but P_{EM7}-*argU*	this study
BT02	as BL21(DE3) but P_{EM7}-*ileX*	this study
BT03	as BL21(DE3) but P_{EM7}-*argU* P_{EM7}-*ileX*	this study
BT03-PTTS	as BT03 FRT-*kan*-FRT HK::P_{T7}-PTTS	this study
BT04-PTTS	as BT03-PTTS but Δ*treA* kan^-	this study
BT05-PTTS	as BT03-PTTS but Δ*treF* kan^-	this study
BT06-PTTS	as BT3-PTTS but Δ*treF* Δ*treA* kan^-	this study
	Primers	
KD01	gtgtaggctggagctgcttc	
KD02	ggatccgtcgacctgcagtt	
KD03	ttgtcgtattatactatgccga	
argU01 (A1)	gcgtgatcaaataggcaagc	
argU02 (A2)	tcggcatagtataatacgacaataacaccgtgcgtgttgact	
argU03 (B1)	aactgcaggtcgacggatcctaatcgcgtgaattatacggt	
argU04 (B2)	agataatcgatggttatgcggt	
ileX01 (A1)	atcagctgtatgtctgcgat	
ileX01 (A2)	tcggcatagtataatacgacaatggcccccttagctcagtggt	
ileX01 (B1)	aactgcaggtcgacggatcctggtagcggattataaagtaac	
ileX01 (B2)	tgcgtgtagctcctgctttg	
T701	tgcccgctgcagcgatcccgcgaaattaatacga	
T702	atatagggatcccttcagcaaaaaacccctcaag	
treA01 (A1)	atgaaatcccccgcaccttc	
treA02 (A2)	gaagcagctccagcctacactaataccggccaaagtccgt	
treA03 (B1)	aactgcaggtcgacggatccgccaaatggctgggcaccgt	
treA04 (B2)	ttaaggtgtgggttgtgcct	
treF01 (A1)	atgctcaatcagaaaattcaaaac	
treF02 (A2)	gaagcagctccagcctacaccagcgggtccattttaggtg	
treF03 (B1)	aactgcaggtcgacggatcctggcaagcgagtacgaaacc	
treF04 (B2)	ttatggttcgccgtacaaac	

[a]Abbreviations: cam^r, the chloramphenicol-resistant determinant; P_{EM7}, EM7 promoter; P_{T7}, T7 promoter; *kan*, the kanamycin-resistant determinant.

addition, it has a greater affinity and catalytic efficiency toward maltose than trehalose. Irrespective of the initial maltose concentration, the trehalose conversion yield by PTTS could reach around 70% at 20−30 °C and pH 6. To make the trehalose production scheme more practical, mass production of recombinant PTTS appears to be the first key step. Therefore, this study was initiated to achieve stable and high-level production of PTTS in *Escherichia coli*. In addition, production of trehalose using the crude recombinant PTTS was also investigated. The result reveals that the production scheme is feasible if a producer strain lacking *treF* is implemented.

MATERIALS AND METHODS

Bacterial Strains and Culture Condition. Bacterial strains and primers applied in this study are summarized in Table 1. All recombinant *E. coli* strains were derived from the BL21(DE3) strain. Bacterial strains were grown on Luria−Bertani (LB) medium, and cell densities were measured with a spectrophotometer at a wavelength of 550 nm (OD_{550}). For protein production, recombinant strains were cultivated overnight and then inoculated into shake flasks containing fresh LB medium (20 mL). With the initial cell density reaching 0.1 at OD_{550}, shake flask cultures were carried out in an orbital shaker set at 150 rpm and 30 °C. To induce protein production, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to bacterial cultures at OD_{550} reaching 0.3−05. The culturing was terminated upon entry into the stationary growth phase (approximately 8 h). Whenever necessary, ampicillin (50 μg/L), chloramphenicol (25 μg/L), and kanamycin (50 μg/L) were used for selection of recombinant strains.

Genomic Insertion of DNA Cassettes and Gene Deletion. Strains with fusion of the EM7 promoter to genomic *argU* and *ileX* were constructed exactly following our reported method.[24] In brief, the PCR-amplified FRT-kan-FRT-EM7 template was first produced using plasmid pKD-EM7 with primers KD02 and KD03. Two megaprimers containing the upstream region and the 5′-end structural sequence of *argU* or *ileX* were then created by polymerase chain reaction (PCR) with the primer sets argU01−02/argU03−04 and ileX01−02/ileX03−04, respectively. Followed by overlapping PCR, two inserted DNA cassettes consisting of FRT-kan-FRT-EM7 flanked by two homologous DNAs of *argU* or *ileX* were obtained with the DNA template and each megaprimer pair. Finally, the inserted DNA cassettes were individually electroporated into the BL21(DE3) strain carrying the helper plasmid pKD46.

Furthermore, *treA* and *treF* genes in bacteria were deleted in a similar way. The FRT-kan-FRT template was synthesized from plasmid pKD-EM7 by PCR using primers KD01 and KD02. Oligomers that prime two ends of the DNA template were designed with two extension sequences homologous to *treA* and *treF*. On incorporation by PCR, the inserted DNA cassettes that contained the FRT-kan-FRT DNA flanked by two homologous extensions were obtained. Targeted deletion of genes was then carried out by electroporation of the inserted DNA cassettes into the plasmid pKD46-bearing strain.

By PCR, the passenger DNA containing the T7 promoter-driven PTTS gene (P_{T7}-PTTS) and the T7 terminator was amplified from

plasmid pET-23a(+)-PTTS with primers T701 and T702. After digestion with *Pst*I−*Bam*HI, the passenger DNA was incorporated into conditional-replication plasmid pHK-Km to give plasmid pHK-T7PTTS. The passenger DNA was then integrated into the prophage HK attachment site by transformation of plasmid pHK-T7PTTS into the plasmid pAH69-carrying strain according to our previous report.[25]

To remove the inserted marker flanked by FRT, the strains were transiently provided with Flp from the helper plasmid pCP20 as described previously.[26]

Analytical Methods. At the end of culturing, bacteria were harvested by centrifugation and cell pellets were processed for protein analyses by 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) as previously reported.[27] To quantify the soluble PTTS yield, the cell density of harvested bacteria was adjusted to reach 20 at OD_{550} in 1 mL. Followed by sonication, the cell-free extract (CFX) was recovered from the supernatant after centrifugation. The protein content of CFX was determined by the Bio-Rad dye reagent. Finally, samples containing 20 μg of proteins were loaded onto the SDS−PAGE syste,, and the concentration of the target protein spot was determined after resolution by an Image Analyzer (Alpha Innotech Co., San Leandro, CA).

The biotransformation reaction was carried out by adding 150 μg of PTTS in CFX into 500 μL of reaction solution containing 0.1 M sodium phosphate (pH 6.0) and 150 mM maltose. Subsequently, the reaction was initiated at 30 °C and terminated by heating at 100 °C for 15 min. The concentration of trehalose was measured by high-performance liquid chromatography (HPLC) with a carbohydrate analysis column (APS-2 HYPERSIL, Thermo Scientific, Waltham, MA). The mobile phase consisted of a mixture of 80% acetonitrile and 20% double-distilled water and was pumped at a rate of 1.0 mL/min.

Plasmid Stability. The stability of plasmid pET23a(+)-PTTS in *E. coli* strains was determined by the plating test according to a previous method.[28] In brief, at the end of culturing, plasmid-bearing strains were serially diluted and plated on LB agars without ampicillin. After incubation overnight, 100 cell-forming units (CFUs) appearing on agar plates were randomly picked and then pasted onto LB agar plates with ampicillin. The plasmid stability (%) was calculated by dividing the number of CFUs on the antibiotic-containing agar plates by 100.

■ RESULTS

Overcoming the Codon Bias. Plasmid pET23a(+)-PTTS carries the PTTS gene under the control of the T7 promoter. The production of recombinant PTTS was first investigated in two plasmid-bearing *E. coli* strains, BL21(DE3)/pET23a(+)-PTTS and Rosetta(DE3)/pET23a(+)-PTTS. Shake-flask cultures were carried out without IPTG induction and harvested for determination of the protein production. Consequently, the Rosetta(DE3) strain produced 4-fold more soluble PTTS than the BL21(DE3) strain (Figure 1). As recognized, the Rosetta(DE3) strain carries a plasmid containing extra copies of tRNAs that supplement six rare amino acid codons (Novagen Co.). Therefore, the result suggests that PTTS production is likely limited by the codon bias in *E. coli*. By analyzing the amino acid sequence of PTTS, multiple copies of two rare codons were revealed: AGG/AGA (encoding Arg) and AUA (encoding Ile).

To address the issue of biased codons, marker-free strains were constructed by fusion of the EM7 promoter to the genomic *argU* tRNA and *ileX* tRNA genes of the BL21(DE3) strain. Consequently, three strains, BT01 (enhancement of *argU* tRNA), BT02 (enhancement of *ileX* tRNA), and BT03 (enhancement of *argU* and *ileX* tRNAs), were obtained. After transformation with plasmid pET23a(+)-PTTS, these strains were cultured in shake flasks in a similar way. As analyzed by SDS−PAGE, the level of soluble PTTS in the BT01 and BT02 strains was comparable to that for BL21(DE3) (Figure 1). In contrast, the BT03 strain produced the highest amount of PTTS among all strains examined. Its PTTS yield was 50% more than that in strain Rosetta(DE3). This result clearly indicates that PTTS production is restricted by its rare codon content and the underlying problem can be solved by using the BT03 strain.

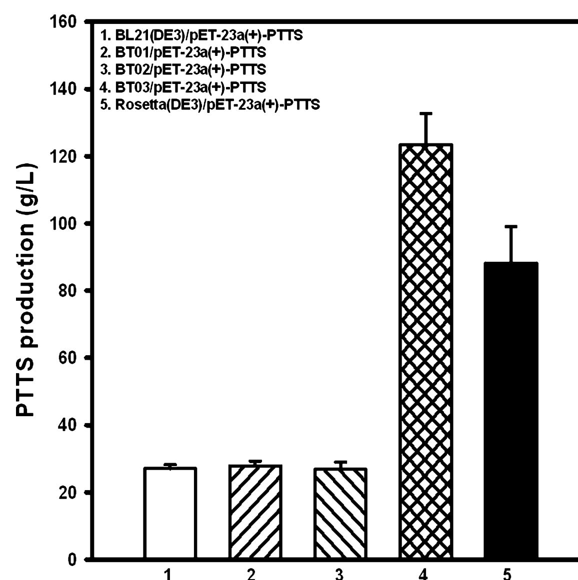


Figure 1. PTTS production in recombinant strains. Recombinant *E. coli* strains were grown in shake flasks containing LB medium plus antibiotics. At the end of culturing, bacteria were processed for determination of soluble PTTS by SDS−PAGE. The experiment was performed in triplicate.

Enhanced Production of Soluble PTTS. To further investigate its production performance, the BT03/pET23a(+)-PTTS strain was grown on the antibiotic-containing medium. As shown in Figure 2, a high level of soluble PTTS was obtained in this strain without induction. In comparison with its uninduced counterpart, this strain receiving IPTG induction exhibited a relatively poor growth and produced less soluble PTTS. Indeed, most of the PTTS that was expressed in this induced strain appeared in the form of insoluble aggregates (data not shown). The result indicates the redundancy of the mulitcopy plasmid for PTTS production.

Accordingly, the DNA containing P_{T7}-PTTS was integrated into the BT03 strain genome to obtain the BT03-PTTS strain. The PTTS production in the BT03-PTTS strain was then conducted in a similar manner. It was found that the basal level of PTTS in this strain was reduced by 50% relative to that for BT03/pET23a(+)-PTTS (Figure 2). Upon induction, this strain produced more soluble PTTS than the uninduced BT03/pET23a(+)-PTTS strain. In addition, the growth of the BT03-PTTS strain remained unaffected irrespective of IPTG.

Stable Production of PTTS. For practical application, it would be favorable to produce PTTS with recombinant strains in the absence of antibiotics. Therefore, producer strains were all grown on the antibiotic-free medium. As a consequence, the strain with a genomic copy of the PTTS gene (e.g., the BT03-PTTS strain) outperformed the counterpart carrying the PTTS gene-borne plasmid (e.g., the BT03/pET23a(+)-PTTS strain) in terms of soluble PTTS production and the final biomass (Figure 3A). The higher production of soluble PTTS in the BT03-PTTS strain was also confirmed by SDS−PAGE (Figure 3B). Notably, the PTTS production in the BT03/pET23a(+)-

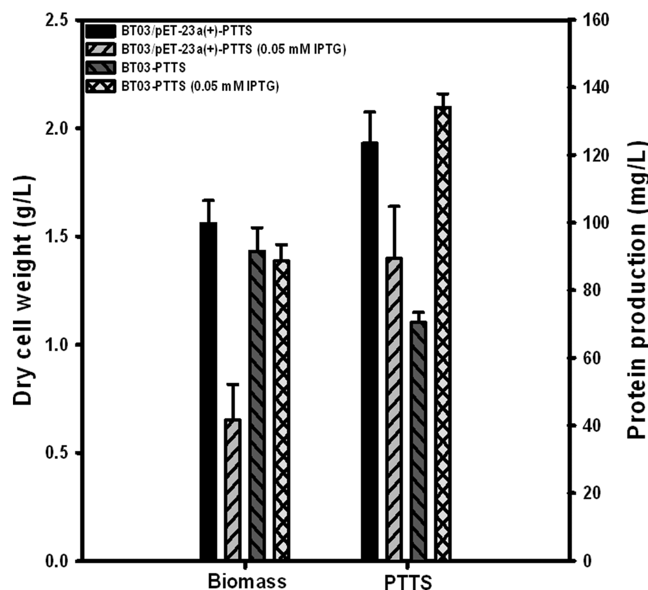
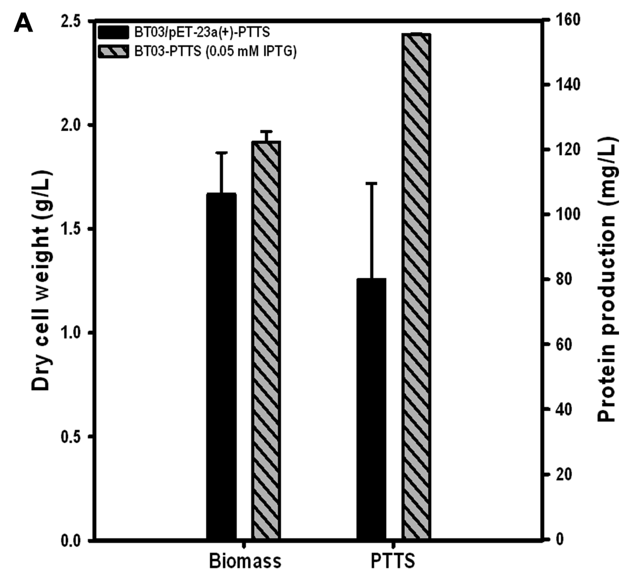


Figure 2. PTTS production and biomass yield of recombinant strains in the presence of antibiotics. Similarly, shake-flask cultures of recombinant *E. coli* strains were carried out in the antibiotic-containing LB medium. IPTG was added to bacterial cultures when required, and cultures were sampled for further analyses. In addition, the stability of plasmid pET23a(+)-PTTS in bacteria was assessed by the plating test. The experiment was performed in triplicate.



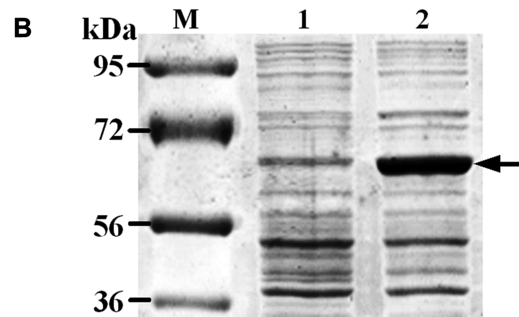


Figure 3. (A) PTTS production and biomass yield of recombinant strains in the absence of antibiotics. BT03-PTTS and BT03/pET23a(+)-PTTS strains were grown on the antibiotic-free medium. Production of PTTS in BT03-PTTS was induced by adding IPTG. The experiment was performed in triplicate. (B) SDS−PAGE analysis of proteins produced in recombinant *E. coli* strains. At the end of the experiment, bacterial cultures were processed for SDS−PAGE analyses. Key: lane M, protein marker; lane 1, soluble proteins of the uninduced BT03/pET23a(+)-PTTS strain; lane 2, soluble proteins of the IPTG-induced BT03-PTTS strain. The position of PTTS is indicated by an arrow.

PTTS strain without induction was reduced by 40% in the medium without antibiotics (Figures 2 and 3A). Further analyses by the plating test revealed that around 50% of the cell population became sensitive to the antibiotics, indicating the segregational instability nature of the plasmid. In contrast, the induced BT03-PTTS strain produced 2-fold more soluble PTTS and gained 25% more biomass in the antibiotic-free medium than in the antibiotic-containing medium (Figures 2 and 3A). The results indicate the fitness of the BT03-PTTS strain for high and stable production of PTTS.

Trehalose Production by recombinant PTTS. Finally, it was informative to learn the usefulness of recombinant PTTS for trehalose production. The BT03-PTTS strain was therefore grown on the antibiotic-free medium and induced for PTTS production. Subsequently, the biotransformation reaction was conducted with PTTS-containing CFX. As indicated in Figure 4, 93 mM trehalose was obtained after reaction for 5 h, whereas the product yield decreased in the prolonged reaction. The result implies that trehalose is likely decomposed in the reaction solution.

In *E. coli*, there are two genes, *treF* (cytoplasmic trehalase) and *treA* (periplasmic trehalase) that are involved in trehalose degradation.[29] Accordingly, the two genes in strain BT03-PTTS were individually deleted, thus giving strain BT04-PTTS (*treA* deletion), strain BT05-PTTS (*treF* deletion), and strain BT06-PTTS (*treA* and *treF* deletion). In a similar manner, CFXs from these resulting strains were employed for the trehalose production. With the exception of CFX from strain BT04-PTTS, 100 mM trehalose could be produced with PTTS from two other strains and the product yield remained unchanged in the extended reaction time (Figure 4). This result clearly indicates that *treF* is responsible for the degradation of produced trehalose in the reaction.

■ DISCUSSION

Expression of heterologous genes in *E. coli* is sometimes hampered by the problem of biased codons. To this end, the BL21(DE3) strain is equipped with plasmid pRARE (e.g, the Rosetta(DE3) strain) that carries tRNA genes for six rare codons of *E. coli*.[30] As illustrated in Figure 1, a high-level expression of PTTS was obtained in the Rosetta(DE3) strain instead of the BL21(DE3) strain, suggesting the bias of codon usage. Here, the problem of rare codons was approached by construction of the BT03 strain with fusion of a strong promoter to genomic *argU* and *ileX* tRNAs. The result illustrated that the ban in PTTS production could be lifted in the BT03 strain (Figure 1). Apparently, this new approach is not only useful to circumvent many problems associated with plasmids (see below) but also feasible to address the issue of protein production limited by rare codons.

Overproduction of recombinant proteins is commonly achieved by using multicopy plasmids that carry the encoding genes. The T7 expression system was first described in *E. coli*

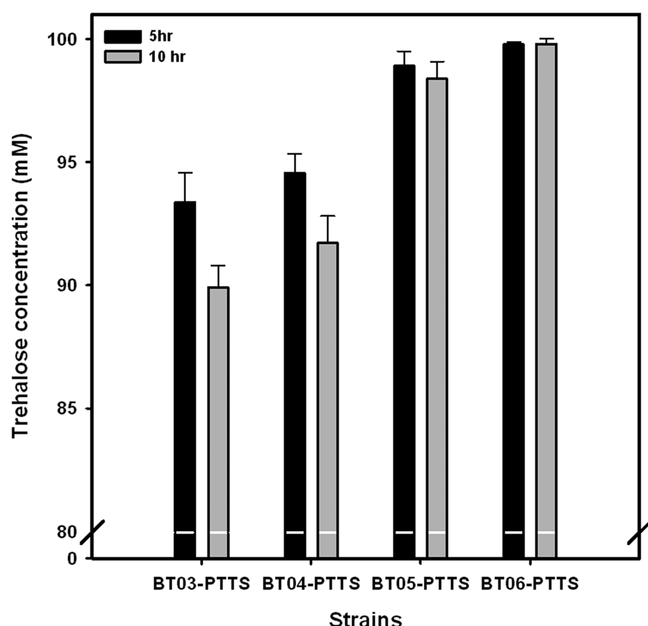


Figure 4. Production of trehalose by the PTTS-mediated biotransformation reaction. Shake-flask cultures of recombinant *E. coli* strains were conducted in the antibiotic-free medium and induced by adding 0.05 mM IPTG. At the end of culturing, bacterial cultures were processed for preparation of crude PTTS. The biotransformation reaction was carried out for either 5 or 10 h. The experiment was performed in triplicate.

and proven very efficient in the specific expression of genes driven by the T7 promoter on the plasmid.[31,32] To apply, the plasmid containing the T7 promoter-driven gene is first transformed into the BL21(DE3) strain that bears a genomic copy of T7 gene 1 under the control of the *lacUV5* promoter. Followed by adding IPTG, T7 RNA polymerase encoded by T7 gene 1 is produced in the strain and exclusively triggers the protein-synthesis machinery of the T7 promoter-regulated genes.[33] However, the frequently raised criticism of this expression system is induction of a high uninduced level of recombinant proteins due to the leaky nature of the *lacUV5* promoter.[33,34] This is particularly undesirable when the expressed proteins are toxic to the host cell. In this study, expression of PTTS with the T7 expression system in *E. coli* also encountered the same problem (Figure 2). Without IPTG induction, the strain harboring the plasmid-encoded PTTS gene (e.g., the BT03/pET23a(+)-PTTS strain) produced a high basal level of PTTS. This likely increases the physiological burden of bacteria, consequently leading to instability of plasmid pET23a(+)-PTTS when the selective pressure is absent. Moreover, a large amount of PTTS (most in the form of inclusion bodies) was produced in the BT03/pET23a(+)-PTTS strain receiving IPTG but at the expense of cell growth. This difficulty was overcome by genomic integration of P_{T7}-PTTS into the BT03 strain. As shown in Figure 2, the basal level of PTTS was greatly reduced in the strain bearing genomic PTTS (e.g., the BT03-PTTS strain) simply by decreasing the gene copy number. In the case that the selective pressure was absent, production of soluble PTTS in the BT03-PTTS strain could be further increased to reach 25% of total proteins without jeopardizing the cell growth (Figures 2 and 3A). This result implies that antibiotics additionally add stress to the PTTS-producing bacteria.

The usefulness of recombinant PTTS was also illustrated for the biotransformation of maltose to trehalose. The conversion yield reached 67% with crude PTTS free from contamination of TreF (Figure 4). Accordingly, it is conceived as a good practice to produce recombinant PTTS using the *treF*-deficient *E. coli* strain. In conclusion, production of recombinant PTTS in *E. coli* is mainly restricted by the problem of rare codon usage and the plasmid. The latter difficulty includes plasmid instability, a redundant gene copy, a high basal level of PTTS, and metabolic burden caused by the mutlicopy plasmid DNA as well as antibiotics. All the problems that restrict PTTS overproduction were surmounted by genomic insertion of PTTS and enhanced expression of genomic tRNAs in *E. coli*. This new approach provides a simple and appealing method to address the issue that is most commonly raised in production of heterologous proteins by *E. coli*.

■ AUTHOR INFORMATION

Funding

This work was supported by the National Science Council of Taiwan (Grant NSC 99-2218-E-218-002) and Ministry of Economic Affairs (Grant 99-EC-17-A-10-S1-156).

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Elbein, A. D.; Pan, Y. T.; Pastuszak, I.; Carroll, D. New insights on trehalose: a multifunctional molecule. *Glycobiology* **2003**, *13* (4), 17R−27.

(2) Augier, J. The biochemistry of a North American algae, *Tuomeyafluviatilis*. *Compt. Rend.* **1954**, *239*, 87−89.

(3) Nwaka, S.; Holzer, H. Molecular biology of trehalose and trehalases in the yeast, *Saccharomyces cerevcesiae*. *Prog. Nucleic Acid Res. Mol. Biol.* **1998**, *58*, 107−237.

(4) Trevelyan, W. E.; Harrison, J. S. Studies on yeast metabolism. 5. The trehalose content of Baker's yeast during anaerobic fermentation. *Biochem. J.* **1956**, *62*, 177−182.

(5) Birch, G. G. Trehaloses. *Advances in Carbohydrate Chemistry*; Academic Press: New York, 1963; Vol. *18*, pp 201−225.

(6) Elbein, A. D. The metabolism of α,α-trehalose. *Advances in Carbohydrate Chemistry and Biochemistry*; Academic Press: New York, 1974; Vol. *30*, pp 227−256.

(7) Richards, A. B.; Krakowka, S.; Dexter, L. B.; Schmid, H.; Wolterbeek, A. P.; Waalkens-Berendsen, D. H.; Shigoyuki, A; Kurimoto, M. Trehalose: a review of properties, history of use and human tolerance, and results of multiple safety studies. *Food Chem. Toxicol.* **2002**, *40*, 871−898.

(8) Crowe, J. H.; Crowe, L. M.; Chapman, D. Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science* **1984**, *223*, 701−703.

(9) Larsen, P. I.; Sydnes, L. K.; Landfald, B.; Strøm, A. R. Osmoregulation in *Escherichia coli* by accumulation of organic osmolytes: betaines, glutamic acid, and trehalose. *Arch. Microbiol.* **1987**, *147*, 1−7.

(10) C. Coutinho, C.; Bernardes, E.; Félix, D.; Panek, A. D. Trehalose as cryoprotectant for preservation of yeast strains. *J. Biotechnol.* **1988**, *7*, 23−32.

(11) Garg, A. K.; Kim, J. K.; Owens, T. G.; Ranwala, A. P.; Choi, Y. D.; Kochian, L. V.; Wu, R. J. Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15898−15903.

(12) Adams, R. P.; Kendall, E.; Kartha, K. K. Comparison of free sugars in growing and desiccated plants of *Selaginella lepidophylla*. *Biochem. Syst. Ecol.* **1990**, *18*, 107−110.

(13) Canatella, P. J.; Karr, J. F.; Petros, J. A.; Prausnitz, M. R. Quantitative study of electroporation-mediated molecular uptake and cell viability. *Biophys. J.* **2001**, *80*, 755−764.

(14) Guo, N.; Puhlev, I.; Brown, D. R.; Mansbridge, J.; Levine, F. Trehalose expression confers desiccation tolerance on human cells. *Nat. Biotechnol.* **2000**, *18*, 168−171.

(15) Roser, B. Trehalose drying: a novel replacement for freeze-drying. *Biopharm. Int.* **1991**, *4*, 47−53.

(16) Schiraldi, C.; Lernia, I. D.; Rosa, M. D. Trehalose production: exploiting novel approaches. *Trends Biotechnol.* **2002**, *20* (10), 420−425.

(17) Higashiyama, T. Novel functions and applications of trehalose. *Pure Appl. Chem.* **2002**, *74*, 1263−1269.

(18) Maruta, K.; Nakada, T.; Kubota, M.; Chaen, H.; Sugimoto, T.; Kurimoto, M.; Tsujisaka, Y. Formation of trehalose from maltooligosaccharides by a novel enzymatic system. *Biosci., Biotechnol., Biochem.* **1995**, *59*, 1829−1834.

(19) Nakada, T.; Maruta, K.; Tsusaki, K.; Kubota, M.; Chaen, H.; Sugimoto, T.; Kurimoto, M.; Tsujisaka, Y. Purification and properties of a novel enzyme, maltooligosyl trehalose synthase, from *Arthrobacter* sp. Q36. *Biosci., Biotechnol., Biochem.* **1995**, *59*, 2210−2214.

(20) Nishimoto, T.; Nakano, M.; Ikegami, S.; Chaen, H.; Fukuda, S.; Sugimoto, T.; Kurimoto, M.; Tsujisaka, Y. Existence of a novel enzyme converting maltose to trehalose. *Biosci., Biotechnol., Biochem.* **1995**, *59*, 2189−2190.

(21) Nishimoto, T.; Nakano, M.; Nakada, T.; Chaen, H.; Fukuda, S.; Sugimoto, T.; Kurimoto, M.; Tsujisaka, Y. Purification and properties of a novel enzyme, trehalose synthase, from *Pimelobacter* sp. R48. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 640−644.

(22) Kim, T. K.; Jang, J. H.; Cho, H. Y.; Lee, H. S.; Kim, Y. W. Gene cloning and characterization of a trehalose synthase from *Corynebacterium glutamicum* ATCC13032. *Food Sci. Biotechnol.* **2010**, *19*, 565−569.

(23) Chen, Y.-S.; Lee, G. C.; Shaw, J. F. Gene cloning, expression, and biochemical characterization of a recombinant trehalose synthase from *Picrophilus torridus* in *Escherichia coli*. *J. Agric. Food Chem.* **2006**, *54*, 7098−7104.

(24) Wang, Z. W.; Lai, C. B.; Chang, C. H.; Chiang, C. J.; Chao, Y. P. A glucose-insensitive T7 expression system for fully-induced expression of proteins at a subsaturating level of L-arabinose. *J. Agric. Food Chem.* **2011**, *59*, 6534−6542.

(25) Chiang, C. J.; Chen, P. T.; Chao, Y. P. Replicon-free and markerless methods for genomic insertion of DNAs in phage attachment sites and controlled expression of chromosomal genes in *Escherichia coli*. *Biotechnol. Bioeng.* **2008**, *101*, 985−995.

(26) Datsenko, K. A.; Wanner, B. L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 6640−6645.

(27) Chiang, C. J.; Chern, J. T.; Wang, J. Y.; Chao, Y. P. Facile immobilization of evolved *Agrobacterium radiobacter* carbamoylase with high thermal and oxidative stability. *J. Agric. Food Chem.* **2008**, *56*, 6348−6354.

(28) Chen, P. T.; Chiang, C. J.; Chao, Y. P. Strategy to approach stable production of recombinant nattokinase in *Bacillus subtilis*. *Biotechnol. Prog.* **2007**, *23*, 808−813.

(29) Purvis, J. E.; Yomano, L. P.; Ingram, L. O. Enhanced trehalose production improves growth of *Escherichia coli* under osmotic stress. *Appl. Environ. Microbiol.* **2005**, *71* (7), 3761−3769.

(30) Novy, R.; Drott, D.; Yaeger, K.; Mierendorf, R. Overcoming the codon bias of *E. coli* for enhanced protein expression. *inNovations* **2001**, *12*, 1−3.

(31) Tabor, S.; Richardson, C. C. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82* (4), 1074−1078.

(32) Studier, F. W.; Moffatt, B. A. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **1986**, *189* (1), 113−130.

(33) Dubendorf, J. W.; Studier, F. W. Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with *lac* repressor. *J. Mol. Biol.* **1991**, *219* (1), 45−59.

(34) Chao, Y. P.; Chiang, C. J.; Hung, W. B. Stringent regulation and high-level expression of heterologous genes in *Escherichia coli* using T7 system controllable by the *araBAD* promoter. *Biotechnol. Prog.* **2002**, *18*, 394−400.