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Control of Acetate Production Rate in *Escherichia coli* by Regulating Expression of Single-Copy *pta* Using *lacI*^{Q} in Multicopy Plasmid

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A tightly regulated gene expression system composed of a single-copy target gene under the control of a *lac* promoter derivative and *lacI* gene in a multicopy plasmid is proposed, and its ability to control the flux of a metabolic pathway is demonstrated. A model system to control the flux of acetyl-CoA to acetyl phosphate was constructed by integrating *pta*, a gene encoding phosphotransacetylase, under a *tac* promoter into the chromosome of *E. coli* with a *pta*-negative background and transforming a multicopy plasmid containing the *lacI*^{Q} gene into the strain. The production rate of acetate was shown to be tightly controlled when varying the concentration of the inducer (IPTG) in the model system.

Keywords: Metabolic engineering, tight regulation, metabolic flux, *tac* promoter, *lacI*

Metabolic engineering has enabled the development of many industrially useful strains, as well as a deeper understanding of the metabolic physiology of microbes [1, 14, 18]. Essentially, metabolic engineering means controlling the metabolic flux through regulating the expression of genes encoding an enzyme in an interesting pathway [13, 20]. Thus, metabolic engineering requires a precisely controlled and tightly regulated gene expression system, as subtle changes in intracellular enzyme activity can be sufficient to cause large changes in the metabolic flux [21]. However, most gene expression systems have unfortunately been developed to overproduce proteins and are not suited to metabolic engineering [11], since they typically employ strong, inducible promoters in high-copy-number plasmids that are too leaky and unstable to permit precise control of the metabolic flux [2, 19]. Nonetheless, a few expression systems allowing for subtle changes in the expression of target genes have recently been developed, and most are based on the P_{BAD} system, the promoter for the *E. coli* arabinose catabolic genes *araBAD* that commands a very tight control of gene expression [3, 11]. It has also been reported that the P_{BAD} system in a low-copy-number plasmid can be efficiently utilized for metabolic engineering studies [10]. Yet, the drawback of the P_{BAD} system is that it requires additional host engineering, such as deletion of the arabinose transporter genes, to avoid the all-or-none phenomenon of induction [11].

Accordingly, this study presents a strategy for tightly regulating gene expression in *E. coli* that can be employed without any primary host engineering, and demonstrates its ability to control metabolic flux. The features of the proposed expression system are as follows: First, the copy number of the target gene is minimized to a single copy by integrating the target gene into the genome, thereby allowing a subtle change of enzyme activity from the target gene, along with high gene stability. Second, the expression of the target gene is controlled by a derivative of a *lac* promoter (P_{lac}) induced by IPTG, thereby avoiding the problem of all-or-none expression without any transporter engineering [11]. Third, the tightness of the P_{lac} derivative in the chromosome is accomplished by simply introducing a multicopy plasmid encoding a LacI repressor into the cell. Thus, the centerpiece of the devised system is a single-copy target gene under the control of a P_{lac} derivative and *lacI* in a multicopy plasmid, which allows a high ratio of the LacI repressor to the repressor-binding site in the DNA, a parameter that can determine the level of control in the absence of induction [8, 15].

Therefore, an expression system was constructed to control the acetate excretion pathway in *E. coli* as a model to demonstrate the feasibility of the strategy described above. In *E. coli*, acetate is produced mainly through the actions of phosphotransacetylase (encoded by *pta*) and acetate kinase (encoded by *ack*). The pathway for acetate production has often been a target in metabolic engineering, since the acetate production rate is related with cell growth and recombinant protein production [5, 12, 16, 17]. In

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addition, the pathway has recently been employed as a component in the design of artificial gene circuits that require an extremely tights regulated system [4, 6]. The model system was constructed using phosphotransacetylase as the target to control the acetate production rate. Specifically, *pta* under a *tac* promoter (P_{tac}) was integrated into the chromosome of *E. coli* with a *pta*-negative background, and the resulting strain was transformed with a multicopy plasmid containing the *lacI*^{Q} gene. The *pta* gene is responsible for the conversion of acetyl-CoA into acetyl phosphate, whereas *lacI*^{Q} is a mutant of *lacI* that can express ten times more of a LacI repressor than *lacI*, caused by a single point mutation of the promoter region of *lacI* [8].

The detailed procedure for the construction of the proposed expression system is as follows. The *pta* gene from *Bacillus subtilis* was amplified using the 5' primer GGATCCAGGAAACAGATTATGGCAGATTTATTTT-CAACA and 3' primer CTGCAGTTATTACAGTGCTTG-CGCCGCTGT, and then cloned into pJF118EH using the BamHI and PstI sites, resulting in pJF:pta (Fig. 1A). pJF118EH is a multicopy expression vector that has a pBR322 replication origin and can be used to produce a target protein under the control of a *tac* promoter controlled by the *lacI*^{Q} gene in the same plasmid [7]. The P_{tac}-pta sequence in pJF:pta was amplified using the 5' primer CTGCAGGCTGTGGTATGGCTGTGCAG and 3' primer TCTAGATTATTACAGTGCTTGCGCCG, and then cloned into pAH144 using the PstI and XbaI sites, resulting in pAH144:tac-pta (Fig. 1B). pAH144 is a CRIM plasmid that allows a target gene to be integrated into the *attB* site in the chromosome of *E. coli* [9]. The P_{tac}-pta sequence in pAH144:tac-pta was then integrated into the chromosome of the *pta* knockout mutant BW18793 [4], according to a previously reported procedure [9], resulting in BW18793/tac-pta (Fig. 1C). Finally, pJF118EH containing the *lacI*^{Q} gene was transformed into BW18793/tac-pta to form BW18793/tac-pta/pJF118EH (Fig. 1D).

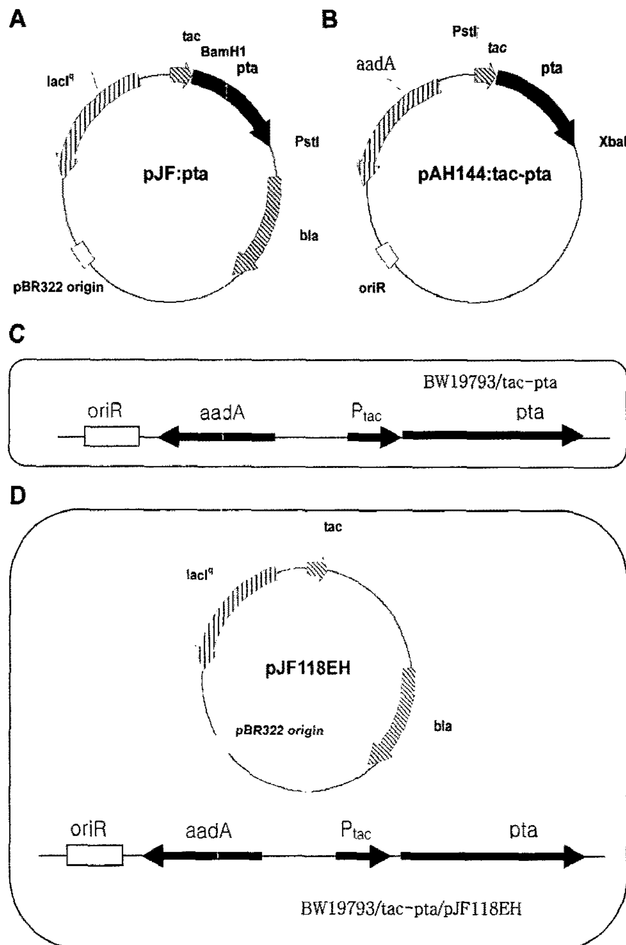

Fig. 1. Schematics of constructed plasmids and strains: (**A**) pJF:pta plasmid constructed by cloning the *pta* gene from *B. subtilis* into pJF118EH, (**B**) pAH144:tac-pta constructed by cloning the *tac-pta* gene from pJF:pta into pAH144, (**C**) BW18793/tac-pta constructed by integrating the *tac-pta* gene from pAH144:tac-pta into chromosomal DNA of BW18793, and (**D**) BW18793/tac-pta/pJF118EH, *pta*- strain containing a single copy of the *pta* gene under control of the *tac* promoter and *lacI* gene in a multicopy plasmid.

To evaluate the tightness of the constructed single-copy *pta* expression system shown in Fig. 1D, it was compared with a *pta* expression system constructed in a multicopy plasmid. First, the tightness of the multicopy *pta* expression system was investigated using BW18793/pJF:pta, which was constructed by transforming the plasmid pJF:pta into BW18793. In addition, BW18793/pJF118EH, constructed by transforming the plasmid pJF118EH into BW18793, was utilized as the control strain for *pta* expression. The

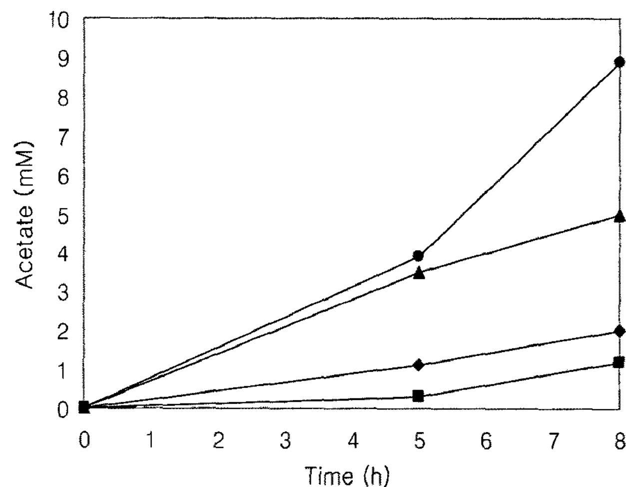

Fig. 2. Production of acetate controlled by expression of the *pta* gene under a *tac* promoter in a multicopy plasmid.

BW18793 transformants harboring pJF118EH or pJF:pta were induced with 1 mM IPTG or not induced, and the concentrations of acetate secreted into the medium were measured at the indicated times. (◆) BW18793/pJF118EF without induction, (■) BW18793/pJF118EF induced with 1 mM IPTG, (▲) BW18793/pJF:pta without induction, (●) BW18793/pJF:pta induced with 1 mM IPTG.

two strains, BW18793/pJF:pta and BW18793/pJF118EH, were inoculated into an M9 minimal medium containing 1% (w/v) glucose at an OD_{600} of 0.2, with or without IPTG, and their growth rates and acetate production rates monitored. Although the growth rates were almost identical (data not shown), the *pta*- strain, BW18793/pJF118EH, produced 1–2 mM acetate after 8 h, as shown in Fig. 2, which was presumed to be caused by amino acid metabolism [4]. However, the acetate production rate of BW189793/pJF:pta induced with 1 mM IPTG induction was much faster than that of the *pta*- strain, resulting in about 9 mM acetate after 8 h, which suggests that the expression of *pta* in pJF:pta properly triggered the pathway of acetyl-CoA to acetyl phosphate. In contrast, the acetate production rate of BW189793/pJF:pta without IPTG induction was somewhat reduced compared with that of the strain with IPTG induction, resulting in about 5 mM acetate after 8 h, which indicates that the expression of *pta* in pJF:pta was repressed by the LacI protein from *lacI*^{Q} in the plasmid, yet the expression system was so leaky that the basal level of *pta* expression in the multicopy expression system was sufficient to turn on the acetate production valve. Therefore, these results confirm the difficulty of metabolic flux control when expressing a target gene using a multicopy expression system.

Next, the tightness of the single-copy *pta* system shown in Fig. 1D was evaluated by examining the effect of the IPTG concentration on the production rate of acetate by BW18793/tac-pta/pJF118EH. The BW18793/tac-pta/pJF118EH strains were inoculated into an M9 minimal medium containing 1% (w/v) glucose at an OD_{600} of 0.4 with 0, 0.01, 0.1, and 1 mM concentrations of IPTG, and the growth and acetate production rates monitored. As shown in Fig. 3A, the growth rates were almost identical, indicating that the IPTG concentration had no influence on the growth rate of the strains in this experiment. Fig. 3B shows the acetate production rates, which were almost identical for the single-copy *pta* system without the addition of IPTG and the *pta*-negative strain, shown in Fig. 2, indicating that the system was very tight and the basal expression level of *pta* almost negligible. Meanwhile, the strain induced with 0.01 mM IPTG produced almost the same amount of acetate as the case with no induction, suggesting that 0.01 mM IPTG was insufficient to induce the expression of *pta* in the expression system. However, the addition of 0.1 mM and 1 mM IPTG did induce the production of acetate, resulting in approximately 2 mM and 4 mM acetate after 4 h and 8 mM and 10 mM acetate after 8 h, respectively, showing that the production rate of acetate in the single-copy system could be controlled by varying the concentration of the inducer, and indirectly indicating that the change in the intracellular concentration of *pta* in the system was subtle enough to permit control of the flux of acetyl-CoA to acetyl phosphate.

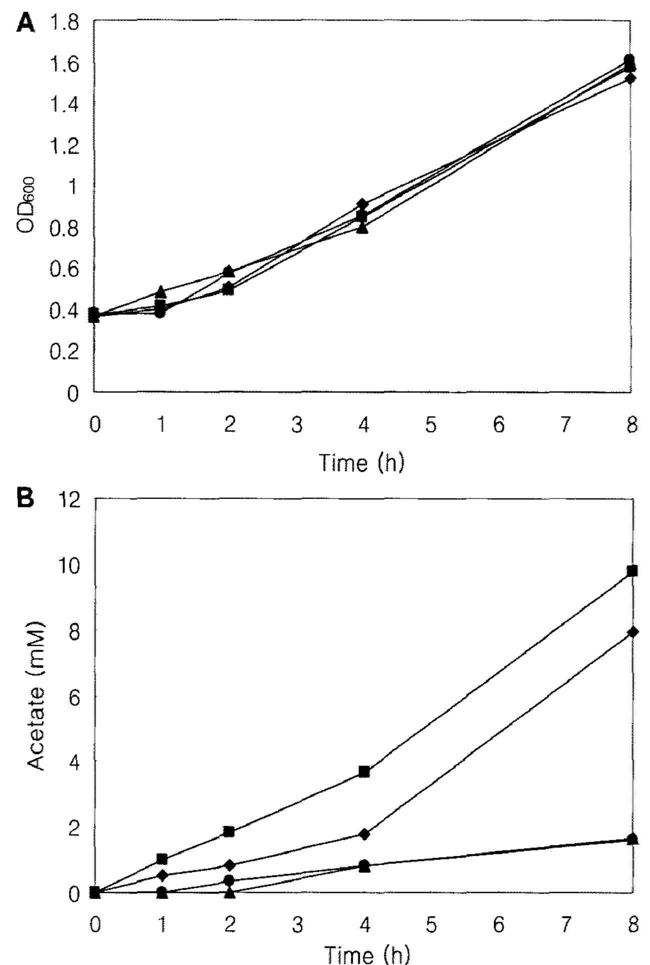

Fig. 3. Production of acetate, controlled by expression of the *pta* gene under a *tac* promoter in a single-copy plasmid.
BW18793/tac-pta/pJF118EH was induced with various concentrations of IPTG and the concentrations of acetate secreted into the medium were measured at the indicated times. **A.** Cell growth. **B.** Concentration of acetate. (▲) BW18793/tac-pta/pJF118EH without induction, (●) BW18793/tac-pta/pJF118EH induced with 0.01 mM IPTG, (◆) BW18793/tac-pta/pJF118EH induced with 0.1 mM IPTG, (■) BW18793/tac-pta/pJF118EH induced with 1 mM IPTG.

The *tac* promoter system used in this study is known to be about three-fold stronger, yet rather leaky compared with a *lac* promoter system [8]. Nonetheless, the present results demonstrated that even the leaky *tac* promoter system could still be utilized to control the metabolic flux when using the proposed approach. The present results also confirm the importance of the ratio of [LacI] to [*lac* operator] in the regulation of the *lac* promoter derivative. When assuming that a plasmid containing a pBR322 replication origin is present in approximately 50 copies per cell and that cells containing *lacI*^{Q} contain 100 molecules per cell [8], the ratio of [LacI] to [*lac* operator] for the multicopy *pta* expression system, BW18793/pJF:pta, and single-copy *pta* expression system, BW18793/tac-pta/pJF118EH, were estimated at approximately 100 and 5,000, respectively.

One advantage of the proposed expression system is that it includes a combination of well-characterized methodologies. The CRIM plasmid-host system used to integrate a target gene into a chromosome is a simple method that enables single-copy integration into specific *attB* sites in the *E. coli* chromosome [9], and can also be applied to various host strains. In terms of the *lacI* gene in a multicopy plasmid, various vectors containing the *lacI* gene are currently available, although they have been developed to control the promoters in the same vectors.

In conclusion, tight metabolic pathway regulation was achieved through employing a single-copy target gene under the control of a P_{lac} derivative and a multicopy *lacI* gene in a plasmid. It was also demonstrated that the metabolic pathway for acetate could be tightly controlled by extracellular IPTG. Therefore, the proposed system would appear to be suitable for metabolic engineering, which requires precise control of the metabolic flux compared with conventional multicopy inducible systems that are generally too leaky to control metabolic fluxes tightly. In addition, the proposed system employs a derivative of the *lac* promoter (P_{lac}) induced by IPTG instead of the P_{BAD} system, which has been generally utilized for tight control of gene expression, thereby avoiding the primary host engineering steps required to solve the problem of all-or-none expression in a P_{BAD} system. Thus, it is expected that the proposed expression system will facilitate further metabolic engineering studies.

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