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Expression of galactose permease and pyruvate carboxylase in *Escherichia coli ptsG* mutant increases the growth rate and succinate yield under anaerobic conditions

Qingzhao Wang, Chanyuan Wu, Tao Chen, Xun Chen & Xueming Zhao*
Department of Biochemical Engineering, School of Chemical Engineering and Technology, Tianjin University, 300072, Tianjin, PR China
**Author for correspondence (Fax: +86-22-2740-6770; E-mail: xmzhao@tju.edu.cn)*

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Abstract

In *Escherichia coli*, disruption of *ptsG*, which encodes the glucose-specific permease of the phosphotransferase transport system (PTS) protein EIICB^{Glc}, is crucial for high succinate production. This mutation can, however, cause very slow growth and low glucose consumption rates. The *ptsG* mutant (TUQ2), from wild type *E. coli* W1485, and *E. coli galP* (encoding galactose permease) and *glk* (encoding glucose kinase) gene expression plasmids were constructed. TUQ2 increased the generation time to approximately 4 h and gave a higher final cell density of 0.5 g/l by expression of *galP*. However, *glk* expression had no effect on the mutant. After expression of pyruvate carboxylase (PYC) and galactose permease, the *ptsG* mutant showed higher succinate yield (1.2 mol/mol glucose) and the specific rate of glucose consumption from 0.33 to 0.6 g/1 h.

Introduction

Escherichia coli utilizes two phosphotransferse transport system (PTS) transporters for the uptake of glucose. The first transporter consists of two subunits, IIA^{Glc} and IICB^{Glc}. The second transporter consists of three subunits, IIAB^{Man}, IIC^{Man} and IID^{Man} (Erni *et al.* 1987, Gutlnecht *et al.* 1999). Besides the PTS, glucose can also be taken up by the constitutively expressed galactose transporters and then phosphorylated by glucokinase. The PTS has complex mechanisms and many regulatory functions (Tchieu *et al.* 2001, Erni 2002). The role of EIICB^{Glc} (*ptsG*) as a global regulator of PTS functions mainly by interaction of the *Mlc* gene, which encodes a repressor of several genes of the PTS. EIICB^{Glc} has also been proposed to have regulatory functions, including a direct effect on the expression of several genes, including its own regulatory gene, and possibly a role as a global sensor of glucose (Postma *et al.* 1996, Erni 2002). Inactivation of *ptsG* usually has a widespread effect on the metabolism of *E. coli* and its inactivation has been used to improve the production of succinate (Chatterjee *et al.* 2001).

Although inactivation of *ptsG* can improve the yield of succinate, especially combined with other gene manipulations for blocking formate and lactic acid fermentation, it also decreases the growth rate of the strain, resulting in a slower fermentation of glucose (Sanchez *et al.* 2005). With the purpose of determining the effect of *ptsG* knock out on the metabolic flux and eliminating the slow fermentation problem, we constructed the *ptsG* knock out mutant (TUQ2)

from the wild type *E. coli* W1485 and expression plasmids containing *E. coli* galactose permease and glucokinase, which can co-express these two enzymes in one strain. The effects of co-expression *galP* and *glk* in the mutant were studied. In order to simultaneously increase the glucose consumption rate and the succinate yield, one *pyc* expression plasmid (pQZ6) was constructed further. The effects of *pyc* and *galP* co-expression on this mutant were explored, including the glucose consumption kinetics and succinate productivity.

Materials and methods

Strains and plasmids

The strain genotypes and plasmids used are shown in Table 1. TOP10 was used for the propagation and amplification of the plasmids used in this work. W1485 is the wild type parent strain, which was donated by DP Clark (Bunch *et al*. 1997). The *ptsG* knock-out mutant (TUQ2) was obtained according to the phage λRed recombinase methods (Datsenko & Wanner 2000) with pTP801 as the template (Poteete *et al*. 1999). The *galp* and *glk* genes were amplified from W1485 by PCR and ligated into the expression plasmid pET28-a(+) with suitable enzyme excision to give plasmid pQZ3 and pQZ4. In order to co-express the *glk* and *galp*, the *ori* of plasmid pQZ3 was replaced with the *ori* of plasmid pACYC184 to give plasmid pQZ5. The *pyc* gene comes from *Bacillus subtilis*168 genome. It was amplified with PCR and also ligated into pET28-a(+) to give expression plasmid pQZ6.

Fermentation mediums and growth conditions

All *E. coli* strains used in this study (Table 1) were routinely cultured in Luria-Bertani (LB) medium at 37 °C. The *galp*, *glk* and *pyc* expression were all induced by the addition of IPTG to 0.5 mM unless otherwise indicated. Fermentations were carried out in sealed serum tubes containing 10 ml LB medium supplemented with 0.3 g $MgCO_3$ (added to maintain the pH of the medium), the appropriate antibiotic(s), approximately 15 g glucose per liter and agitated at 250 rpm. The headspace in the sealed tubes was CO_2, established by means of a gassing manifold. A dual-phase culture technique was used to examine the fermentation product distributions (Vemuri *et al*. 2002).

Analytical methods

Substrate consumption and product formation during fermentation were quantified by HPLC using an Agilent Zorbax C18 column (4.6×250 mm) and a HP1100 chromatography

Table 1. Strains and plasmids used in this study.

Strains or plasmids	Relevant genotype or phenotype	Reference or source
Strains		
W1485	Wild type	Bunch *et al*. (1997)
TUQ2	*ptsG: Cam* of W1485	This work
*Bacillus subtilis*168	Providing *pyc* gene	BGSC
Plasmids		
pET28-a(+)	Expression vectors with T7 promoter	Novagen
pTP801	Used as the PCR template	Poteete *et al*. (1999)
pKD46	*AraBp-gam-bet-exo*, *bla (ApR)*, *repA101 (ts)*, *oriR101*	Datsenko & Wanner (2000)
pQZ1	*ptsG* gene cloned from *E. coli* into pUC18	This work
pQZ3	*galP* gene cloned from *E. coli* under the T7 promoter of pET28-a(+)	This work
pQZ4	*glk* gene cloned from *E. coli* under the T7 promoter of pET28-a(+)	This work
pQZ5	Replace the pQZ3 colE1 *ori* with P15A *ori* from pACYC184	This work
pQZ6	Pyruvate carboxylase gene from *B. subtilis* cloned into pET28-a(+)	This work

working station system equipped with UV absorbance and refractive index detectors. The glucose kinase and pyruvate carboxylase activities were tested according to the methods of Lessie & Wyk (1972) and Maeba & Sanwal (1969).

Results

Effect of galp and glk expression on TUQ2

The *ptsG* mutant can increase the *E. coli* growth rate and glucose consumption rate in LBG medium under aerobic conditions, which had been illustrated before. However, under anaerobic conditions, the mutation of *ptsG* can slow the glucose consumption and cell growth rates (Chatterjee *et al.* 2001). Although the *ptsG* mutation can significantly shift the metabolic fluxes to succinate production, this slow growth characteristic will result in a longer fermentation period. In order to eliminate this problem, the effects of *galP* and *glk* expression were studied. W1485 grew at a generation time of 1 h; TUQ2 grew at a generation time of 8 h. TUQ2 (pQZ3) increased the generation time to approx. 4 h and reached a higher final cell density after induction with IPTG. But it did not reach the level of the wild type. The glucose consumption rate also converged with the growth rate. TUQ2 (pQZ5) can consume up to 15 g glucose/l in 25 h; whereas TUQ2 needs 40 h and leaves about 2 g glucose/l unfermented. Expression of *glk* has no effect on the mutant strain in glucose consumption and growth rates. When the *galP* and *glk* are expressed together, the result is almost the same as the single expression of *galP*. The over-expressed glucose kinase activity was verified, but actual data are not shown here. The detailed growth kinetics is shown in Figure 1a. Based on the above, we conclude that *galP* expression can accelerate the glucose consumption in the *ptsG* mutant.

Regarding the effect on the fermentation products, *galp* expression has very little influence on the succinate, ethanol and formate yields compared with the *ptsG* mutant TUQ2. However, acetate yield is doubled and lactate yield is also increased sharply (Table 2). The fumarate accumulation at the end of the fermentation is also interesting.

Effect of galp and pyc Co-expression on TUQ2

The PYC is one of the key enzymes for succinate production (Vemuri *et al.* 2002). In order to simultaneously improve the succinate yields and increase the succinate productivity, the effects of co-expression *Bacillus subtilis pyc* gene and *E. coli galp* gene were explored. Under the induction of IPTG, the strain TUQ2 (pQZ5/pQZ6) consumes glucose quickly and consumes 15 g glucose/l in 20 h (Figure 1). *pyc* expression increased the succinate yield to 1.2 mol/mol glucose, while the acetate, formate and ethanol yields dropped to lower levels (See Table 2). These data showed that co-expression *galP* and *pyc* in TUQ2 could increase the succinate yield

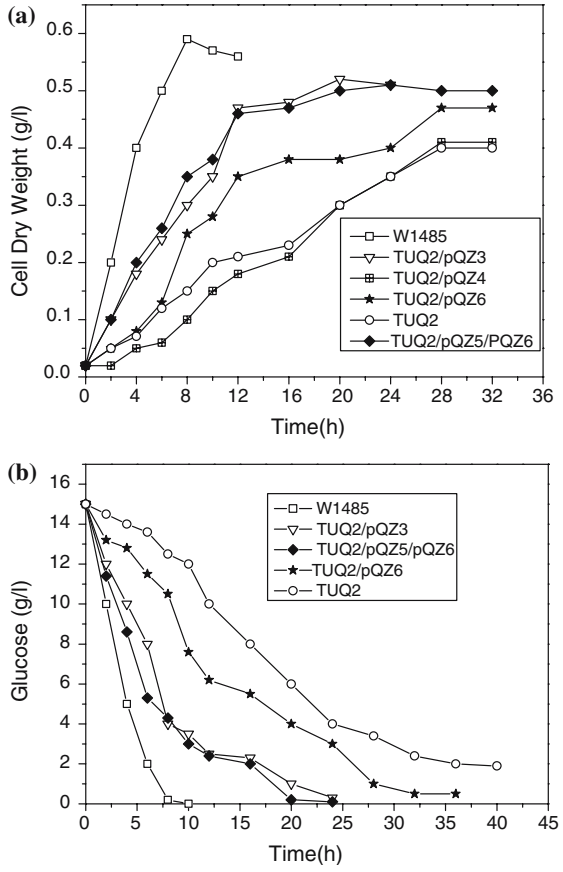

Fig. 1. Growth kinetics and glucose consumption curve under anaerobic conditions after the strains were transformed with pQZ3, pQZ4, pQZ5 and pQZ6, respectively (refer Table 1 for detailed information). The initial glucose content was 15 g/l. (a) cell growth kinetics; (b) glucose consumption curve.

Table 2. Fermentation product yields.

Strain	Products yields (±SD)[a]				
	Formate	Lactate	Acetate	Ethanol	Succinate
W1485	0.52(0.03)	0.07(0.03)	0.78(0.12)	0.82(0.13)	0.15(0.01)
TUQ2	0.15(0.06)	0.02(0.01)	0.55(0.07)	0.50(0.10)	0.75(0.07)
TUQ2/pQZ3	0.21(0.01)	0.13(0.01)	0.65(0.03)	0.25(0.02)	0.76(0.10)
TUQ2/pQZ5/pQZ6	0.15(0.04)	0.02(0.01)	0.34(0.05)	0.21(0.02)	1.20(0.09)

Results come from anaerobic experiments using LBG as the fermentation medium with initial glucose at 15 g/l after 48 h of culture (average of three independent experiments).
[a]Results are given as mol/mol of glucose consumed.
SD, standard deviations.

and simultaneously maintain a high growth rate and short fermentation period.

Discussion

The major finding of this study is that galactose permease is crucial to the increase of the *ptsG* mutant growth and glucose consumption rates, while the effect of glucose kinase is minimal. Expression of *pyc* from *B. subtilis* can increase the succinate yield. Although *pyc* had been identified to increase succinate production without affecting the glucose consumption rate (Gokarn *et al.* 1998, 2000), the effect of co-expression of *pyc* and *galp* in the *ptsG* mutant has not been studied before. Our results will have a positive impact on the future use of *E. coli* as an industrial strain to produce succinate.

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