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Effect of pyruvate kinase gene deletion on the physiology of *Corynebacterium glutamicum* ATCC13032 under biotin-sufficient non-glutamate-producing conditions: Enhanced biomass production

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ABSTRACT

The effect of pyruvate kinase gene (*pyk*) deletion on the physiology of *Corynebacterium glutamicum* ATCC13032 was investigated under biotin-sufficient, non-glutamate-producing conditions. In a complex medium containing 100 g/L glucose, a defined *pyk* deletion mutant, strain D1, exhibited 35% enhancement in glucose consumption rate, 37% increased growth and a 57% reduction in respiration rate compared to the wild-type parent. Significant upregulation of phosphoenolpyruvate (PEP) carboxylase and downregulation of PEP carboxykinase activities were observed in the D1 mutant, which may have prevented over-accumulation of PEP caused by the *pyk* deletion. Moreover, we found a dramatic 63% reduction in the activity of malate:quinone oxidoreductase (MQO) in the D1 mutant. MQO, a TCA cycle enzyme that converts malate to oxaloacetate (OAA), constitutes a major primary gate to the respiratory chain in *C. glutamicum*, thus explaining the reduced respiration rate in the mutant. Additionally, pyruvate carboxylase gene expression was downregulated in the mutant. These changes seemed to prevent OAA over-accumulation caused by the activity changes of PEP carboxylase/PEP carboxykinase. Intrinsically the same alterations were observed in the cultures conducted in a minimal medium containing 20 g/L glucose. Despite these responses in the mutant, metabolic distortion caused by *pyk* deletion under non-glutamate-producing conditions required amelioration by increased biomass production, as metabolome analysis revealed increased intracellular concentrations of several precursor metabolites for building block formation associated with *pyk* deletion. These fermentation profiles and metabolic alterations observed in the mutant reverted completely to the wild-type phenotypes in the *pyk*-complemented strain, suggesting the observed metabolic changes were caused by the *pyk* deletion. These results demonstrated multilateral strategies to overcome metabolic disturbance caused by *pyk* deletion in this bacterium.

1. Introduction

Corynebacterium glutamicum is a Gram-positive, non-pathogenic, rod-shaped and biotin-auxotrophic bacterium that shows abundant growth under aerobic conditions. *C. glutamicum* is used widely for industrial production of amino acids such as glutamate and lysine (Kimura, 2005; Kelle et al., 2005). Use of this microorganism in the fermentation industry is gaining in importance as *C. glutamicum* can produce a wide variety of metabolites, such as organic acids (Wieschalka et al., 2012), amino acid derivatives (Kind et al., 2011; Krause et al., 2010), alcohols (Blombach et al., 2011) and bioplastics (Jo et al., 2007).

To improve the ability of metabolite production in this bacterium, pyruvate kinase (PYK) has long attracted attention for strain improvement, especially for lysine production. PYK of *C. glutamicum* catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate (Fig. 1), generating ATP from ADP. As the activity of this enzyme is allosterically regulated negatively by ATP and positively by AMP (Jetten et al., 1994; Ozaki and Shiio, 1969), PYK is a key enzyme that regulates glycolytic flux in response to the intracellular energy level. Furthermore, PYK is involved in the metabolism of PEP, an important intermediate that is then converted into oxaloacetate (OAA) by PEP carboxylase (PEPC) to replenish the carbon source in the TCA cycle (Fig. 1). OAA is a precursor for glutamic acid and aspartic acid family amino acids including lysine. Therefore, changing PYK activity may contribute to efficient metabolite production by this bacterium.

Previously, we reported the effect of *pyk* deletion on glucose metabolism in wild-type *C. glutamicum* ATCC13032 under glutamate-producing conditions induced by biotin limitation

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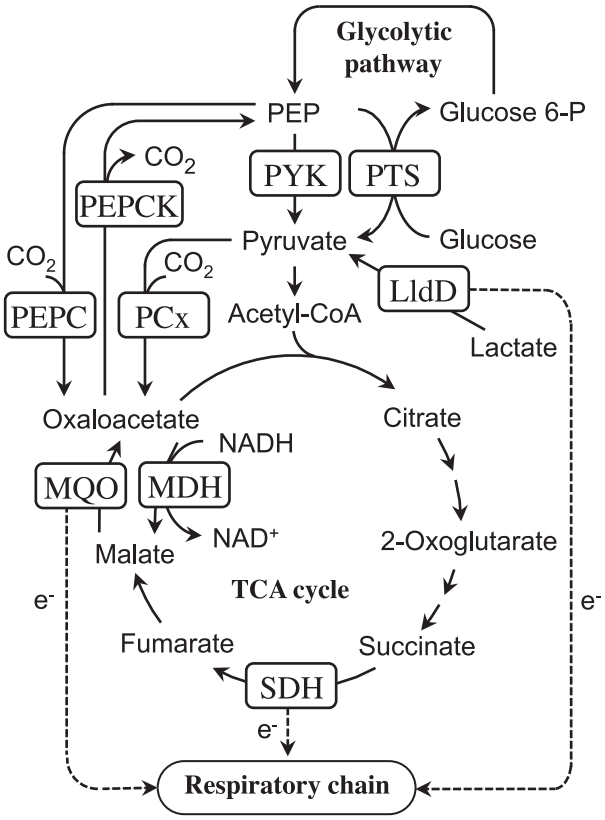

Fig. 1. Central metabolic pathway around the phosphoenolpyruvate–oxaloacetate node in *Corynebacterium glutamicum*. Abbreviations: Glucose 6-P, glucose 6-phosphate; PEP, phosphoenolpyruvate; PTS, PEP: carbohydrate phosphotransferase system; PYK, pyruvate kinase; PEPC, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; PCx, pyruvate carboxylase; LldD, quinone dependent L-lactate dehydrogenase; MQO, malate:quinone oxidoreductase; MDH, malate dehydrogenase; SDH, succinate dehydrogenase; e^-, electron.

(Sawada et al., 2010). A defined *pyk*-deleted mutant (strain D1), derived using the double-crossover chromosome replacement technique, produced 25% more glutamate than the wild-type strain by altering the enzyme activities of the anaplerotic pathway to reduce PEP over-accumulation. This report was the first to demonstrate the primary effects of *pyk* deletion under biotin-limited conditions.

To our knowledge, no studies have investigated glucose metabolism in the simple *pyk*-deleted mutant under biotin-sufficient conditions. However, several reports have determined the effects of *pyk* deletion on lysine production under biotin-sufficient conditions using mutants derived by repeated random mutation and selection (Ozaki and Shiio, 1983; Shiio et al., 1987) or defined *pyk*-defective mutants (Becker et al., 2008; Gubler et al., 1994; Park et al., 1997). These reports provided insights into the effects of *pyk* deletion on glucose metabolism under biotin-sufficient lysine-producing conditions. However, it had been difficult to determine the primary effects of *pyk* deletion in a straightforward manner.

In this study, the primary effects of *pyk* deletion on glucose metabolism in *C. glutamicum* under biotin-sufficient conditions were evaluated using strain D1, a *C. glutamicum pyk*-deleted mutant (Sawada et al., 2010). The purpose of this study is not only to clarify metabolic alterations as the fundamental knowledge, but also to assess potentials of *pyk* deletion for metabolite production. In contrast to our previous study conducted under biotin-limited conditions (Sawada et al., 2010), strain D1 under biotin-sufficient conditions showed significantly increased biomass production and reduced respiration rate without glutamate production. Analyses of the metabolic alterations revealed enhanced anaplerotic activity that decreased the PEP over-accumulation caused by the *pyk* deletion. In addition, fine tuning activity of malate:quinone oxidoreductase (MQO), a respiratory chain component in *C. glutamicum* (Molenaar et al., 2000; Nantapong et al., 2004), seemed to preclude OAA over-accumulation. Enhanced biomass production may function to accommodate the precursor metabolites for building block formation formed differently in strain D1 due to *pyk* deletion during glucose metabolism. The results demonstrated unique strategies that relieve metabolic distortion caused by *pyk* deletion and highlighted the important roles played by MQO as a regulatory module for maintaining homeostasis in the central metabolism in *C. glutamicum*.

2. Materials and methods

2.1. Bacterial strains and media

C. glutamicum strains used in this study included the wild-type strain (ATCC 13032), a *pyk*-deleted mutant strain (D1), and a *pyk*-complemented strain (C1). The constructions of strains D1 and C1 were described previously (Sawada et al., 2010). For fermentation analysis, Medium 7 complete medium was used to refresh cultures (Sekine et al., 2001). To cultivate jar fermentor cultures in a complex medium, Medium S2 complex medium (Sekine et al., 2001) was used as a seed medium. Medium F4 complex medium (Sawada et al., 2010) containing 3 μg/L biotin or Medium F4 containing 60 μg/L biotin (termed Medium F5) was used as the main culture medium for biotin-limited and biotin-sufficient conditions, respectively. Medium F5 contained (per liter) 100 g glucose, 1 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 4$–$5H_2O$, 200 μg thiamine · HCl, 60 μg biotin, and 27.7 mL soybean-meal hydrolysate (total nitrogen, 35.0 g/L). Kanamycin (20 mg/L) was added to Medium 7 and Medium S2 when culturing strain C1 for preculture. To cultivate jar fermentor cultures in a minimal medium, Medium 7 was used as a first preculture medium, and optimized CGXII minimal medium (Keilhauer et al., 1993) containing (per liter) 40 g glucose, 20 g $(NH_4)_2SO_4$, 5 g