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Original Research Article

Establishing a synergetic carbon utilization mechanism for non-catabolic use of glucose in microbial synthesis of trehalose

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ABSTRACT

In nature glucose is a common carbon and energy source for catabolic use and also a building unit of polysaccharides and glycosylated compounds. The presence of strong glucose catabolic pathways in microorganism rapidly decomposes glucose into smaller metabolites and challenges non-catabolic utilization of glucose as C6 building unit or precursor. To address this dilemma, we design a synergetic carbon utilization mechanism (SynCar), in which glucose catabolism is inactivated and a second carbon source (e.g. glycerol) is employed to maintain cell growth and rationally strengthen PEP driving force for glucose uptake and non-catabolic utilization. Remarkably, a trehalose biosynthesis model developed for proof-of-concept indicates that SynCar leads to 131% and 200% improvement in trehalose titer and yield, respectively. The conversion rate of glucose to trehalose reaches 91% of the theoretical maximum. This work demonstrates the broad applicability of SynCar in the biosynthesis of molecules derived from non-catabolic glucose.

1. Introduction

Microorganisms utilize simple carbon sources such as glucose to propagate and generate molecules that are essential to life, which forms the foundation of the fermentation industry. In microorganisms, the catabolism of glucose is initially realized through glycolysis and pentose phosphate pathway (PPP)(Munoz-Elias and McKinney, 2006), which not only provides energy, reducing agents, and small molecules for continuous glucose uptake, cell growth and other physiological behaviors but also supports anabolic activities. Such activities have been greatly harnessed for microbial synthesis by metabolic engineering efforts. For instance, pyruvate, acetyl-CoA, and other small molecules derived from glucose catabolism can be converted or reassembled into fuels, bulk chemicals, fine chemicals, and even structurally-complicated natural products through various biochemical reactions and biosynthetic mechanisms (Atsumi et al., 2008; Bhan et al., 2013; Farmer and Liao, 2000; Lin et al., 2013a, 2013b, 2014a, 2014b; Pandey et al., 2016; Peralta-Yahya et al., 2012; Santos et al., 2011; Stephanopoulos, 2007; Sun et al., 2013; Wang et al., 2016; Yuzawa et al., 2012).

In addition to the above conventional utilization of glucose, non-catabolic use of glucose as C6 building unit or precursor for the biosynthesis of glycosylated molecules and polysaccharides is also necessary to microorganisms and critical for microbial synthesis. For example, glycosylation of natural products such as anthocyanin and puerarin can greatly enhance their stability, bio-solubility and bioavailability (Lim et al., 2015; Pandey et al., 2016; Wang et al., 2014; Yan et al., 2005). During these processes, glucose needs to be activated into UDP-sugars to provide intact glycosyl groups, which poses a real conflict to its regular catabolism and creates a dilemma to engineer such biosynthesis. The extremely active catabolic pathways would dominate glucose utilization, rapidly decompose glucose to smaller metabolites, and direct more carbon flux into biomass. These challenges would dramatically reduce the utilization efficiency of glucose as C6 building unit or precursor. However, reducing or eliminating such catabolism competition by attenuating or blocking the glycolysis and PPP would disrupt glucose uptake and cellular metabolism, affect cell viability, and in turn result in low efficiency of glucose utilization for microbial synthesis.

To address this dilemma, pioneering explorations have been attempted recently (Pandey et al., 2013; Shiue et al., 2015). To solve the growth problem associated with blocking glycolysis and PPP, a second carbon source or enriched medium was used to support cell growth. Although these efforts reserved glucose as C6 building unit or precursor, the crosstalk or coupling between carbon sources has not

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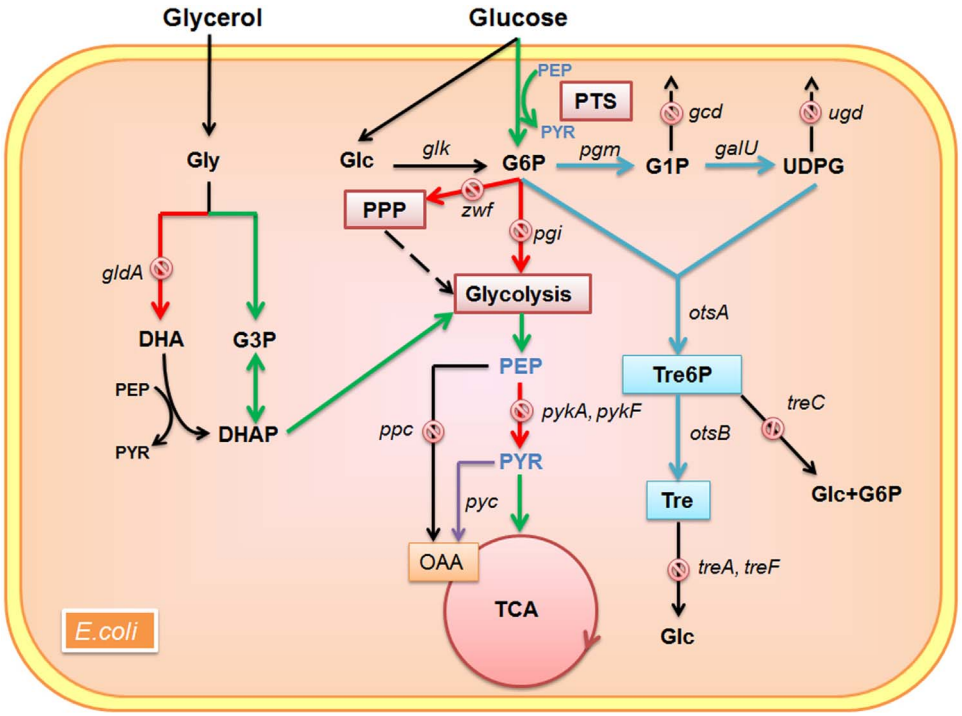

Fig. 1. Schematic representation of synergetic carbon utilization mechanism and trehalose biosynthesis model in *E. coli*. Black-colored arrows indicate native metabolic pathways in *E. coli*; black-colored arrows with dash line indicate several steps in the pathway; blue-colored arrows indicate the trehalose biosynthesis model. Red-colored arrows indicate the critical blocked steps for the synergetic carbon utilization mechanism. Green-colored arrows indicate the main metabolic pathways of carbon sources in the synergetic carbon utilization mechanism. Purple-colored arrow indicates the overexpression of the heterologous pathway from *Lactococcus lactis*. Gly, glycerol; Glc, glucose; G6P, glucose 6-phosphate; G1P, glucose 1-phosphate; UDPG, UDP-glucose; DHA, dihydroxyacetone; DHAP, glycerone phosphate; G3P, glycerol 3-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; Tre6P, trehalose 6-phosphate; Tre, trehalose; OAA, oxaloacetate; PPP, pentose phosphate pathway; PTS, phosphotransferase system. *pgi*, encoding phosphoglucose isomerase (E.C. 5.3.1.9); *zwf*, encoding glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49); *pgm*, encoding phosphoglucomutase (E.C. 5.4.2.2); *galU*, encoding glucose-1-phosphate uridylyltransferase (E.C. 2.7.7.9); *glk*, encoding glucokinase (E.C. 2.7.1.2); *pykA*, encoding pyruvate kinase II (E.C. 2.7.1.40); *pykF*, encoding pyruvate kinase I (E.C. 2.7.1.40); *gldA*, encoding glycerol dehydrogenase (E.C. 1.1.1.6); *ppc*, encoding phosphoenolpyruvate carboxylase (E.C. 4.1.1.31); *ugd*, encoding UDP-glucose 6-dehydrogenase (E.C. 1.1.1.22); *gcd*, encoding glucose dehydrogenase (E.C. 1.1.5.2); *otsA*, encoding trehalose-6-phosphate synthase (E.C. 2.4.1.15); *otsB*, encoding trehalose-6-phosphate phosphatase (E.C. 3.1.3.12); *treA*, encoding periplasmic trehalase (E.C. 3.2.1.28); *treC*, encoding trehalose-6-phosphate hydrolase (E.C. 3.2.1.93); *treF*, encoding cytoplasmic trehalase (E.C. 3.2.1.28); *pyc*, encoding pyruvate carboxylase (E.C. 6.4.1.1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

been explored. Those works simply employed the second carbon source for cell growth with little direct contribution to product formation, which led to low carbon yield. To address this problem, we design a synergetic carbon utilization mechanism (SynCar) by rewiring microbial native metabolic routes. More specifically, when the glucose catabolism is inactivated, the catabolic pathway of a second carbon source (e.g. glycerol) is rationally modified to accumulate PEP as a driving force to strengthen glucose transport into cells and subsequent utilization as building unit. As rationale, glucose enters cells as glucose-6-phosphate mainly through the PEP-dependent phosphotransferase system (PTS)(Hernandez-Montalvo et al., 2003). The system associates with glycolysis and PPP to realize the regeneration of PEP that supports continuous glucose uptake and normal cellular function (Fig. 1). When the glucose catabolic pathways are blocked and PEP cannot be regenerated from glucose, directing glycerol assimilation to enhance PEP generation as the driving force for glucose uptake would achieve the synergetic unitization of both carbon sources and increase the utilization efficiency of both carbon sources.

To validate this mechanism and examine the applicability of this mechanism in microbial synthesis, we selected trehalose as the target product and established a glucose-based trehalose biosynthesis model in *Escherichia coli*. Trehalose is a non-reducing disaccharide with very stable characteristics. It has a wide range of applications in the food and pharmaceutical industries, due to its protective function on biological molecules under oxidative or extreme conditions (Kidd and Devorak, 1994; Ohtake and Wang, 2011; Schiraldi et al., 2002). For instance, trehalose can be used to stabilize vaccines and preserve organs (Kim et al., 2010; Patist and Zoerb, 2005). More recently, it has been reported that trehalose could find the applications in the treatment of fatty liver disease as well as diabetes and Alzheimer's disease by triggering autophagy (Torrice, 2016). Its annual market value was estimated to be 206.41 million US dollars in year 2015 (Global Trehalose Market Size, 2016). So far, its industrial production completely relies on the enzymatic conversion of starch or maltose, which still suffers from side product formation (Kobayashi et al., 1997; Koh et al., 1998, 2003; Mukai et al., 1997; Yoshida et al., 1997). In this study, we first develop a glucose-based trehalose biosynthesis model in *E. coli*. With the employment of SynCar, the titers of trehalose increased from 1.59 g/L to 3.67 g/L at the end of 48 h, while the yield increased from 0.21 to 0.63 g trehalose/g glucose, equivalent to an enhancement by 131% and 200%, respectively. Remarkably, extended cultivation period allowed the conversion rate of glucose to trehalose to reach 91% of the theoretical maximum with the highest titer of 8.2 g/L in shake flasks. Overall, our results suggest that the SynCar has general applicability in microbial synthesis involving glucose as C6 building unit or precursor. In addition, this study demonstrates a novel microbial approach for trehalose production and has great scale-up potential.

2. Methods

2.1. Experimental materials

E. coli strain XL1-Blue was used for gene cloning and preparation of plasmids. *E. coli* strain BW25113 was used as parent strain for generating knockout strains. Keio knockout strains were purchased