Metabolic Engineering 47 (2018) 496–504

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/meteng

Engineering synergetic CO_2-fixing pathways for malate production

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ARTICLE INFO

Keywords:
CO_2 fixation
Malate production
ATP balance
Pathway engineering

ABSTRACT

Increasing the microbial CO_2-fixing efficiency often requires supplying sufficient ATP and redirecting carbon flux for the production of metabolites. However, addressing these two issues concurrently remains a challenge. Here, we present a combinational strategy based on a synergetic CO_2-fixing pathway that combines an ATP-generating carboxylation reaction in the central metabolic pathway with the ATP-consuming RuBisCO shunt in the carbon fixation pathway. This strategy provides enough ATP to improve the efficiency of CO_2 fixation and simultaneously rewires the CO_2-fixing pathway to the central metabolic pathway for the biosynthesis of chemicals. We demonstrate the application of this strategy by increasing the CO_2-fixing rate and malate production in the autotroph *Synechococcus elongatus* by 110% and to 260 μM respectively, as well as increasing these two factors in the heterotrophic CO_2-fixing *Escherichia coli* by 870% and to 387 mM respectively.

1. Introduction

Elevated atmospheric CO_2 concentrations have been caused by human activities globally. CO_2 emissions for 2007 were 28.8 Gt, which is expected to grow to 40.3 Gt by 2030 and to 50 Gt by 2050 (Venkata Mohan et al., 2016). To decrease atmospheric CO_2 concentrations, there is an urgent need to reduce CO_2 emissions and develop CO_2 sequestration strategies. Conventional methods for CO_2 sequestration, including CO_2 capture (Leung et al., 2014), CO_2 separation (Chou, 2013), and CO_2 storage (Boot-Handford et al., 2014), have obvious shortfalls, such as energy-intensive processes and high operational costs (Hicks et al., 2017). To overcome these disadvantages, biological CO_2-fixing techniques have garnered much attention, and such biorefinery approaches have been used to produce value-added chemicals (Liao et al., 2016). However, a remaining key issue is how to enhance biological CO_2-fixing efficiency by replenishing ATP deficiencies in CO_2-fixing pathways and engineering efficient biosynthetic pathways for value-added chemical production.

To explore more efficient CO_2-fixing pathways able to reduce the demand for ATP, several synthetic carbon fixation pathways, which combine existing metabolic building blocks from various organisms, were computationally identified based on pathway kinetics and thermodynamics (Bar-Even et al., 2010). Then, a synthetic pathway for CO_2 fixation in vitro was built and optimized using enzyme engineering and metabolic proofreading, in which an efficient ATP recycling system was devised to meet the need for ATP (Schwander et al., 2016). These pathways can realize the high efficiency of an ATP supply, but it is difficult to rewire them into central metabolic pathways in vivo (Gong and Li, 2016). To circumvent this difficulty, the power of natural CO_2-fixing pathways has been harnessed. First, several intermediate metabolites have been engineered as the branching points for value-added chemical production in autotrophic microbes, such as 2,3-butanediol from pyruvate (Kanno et al., 2017; McEwen et al., 2016; Oliver et al., 2013), lipid from acetyl-CoA (Ajjawi et al., 2017), and ethylene from oxoglutarate (Xiong et al., 2015). Second, since the genetic tools for autotrophic microbes remain limited, some natural CO_2-fixing pathways, such as the Calvin-Benson-Bassham (CBB) cycle and 3-hydroxypropionate bicycle, have been transplanted into heterotrophs to pave the way for the creation of completely synthetic autotrophs (Antonovsky et al., 2016; Kerfeld, 2016; Mattozzi et al., 2013). Although these CO_2-fixing pathways are more easily rewired into central metabolic pathways than synthetic pathways, they still exhibit ATP deficiencies. In addition, researchers have paid attentions to the Wood-Ljungdahl (WL) pathway, which is the most ATP-efficient among the six natural CO_2-fixing pathways. However, the genetic tools available for the microbes that harbor the WL pathway remain limited to date, and

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https://doi.org/10.1016/j.ymben.2018.05.007
Received 1 March 2018; Received in revised form 10 May 2018; Accepted 10 May 2018

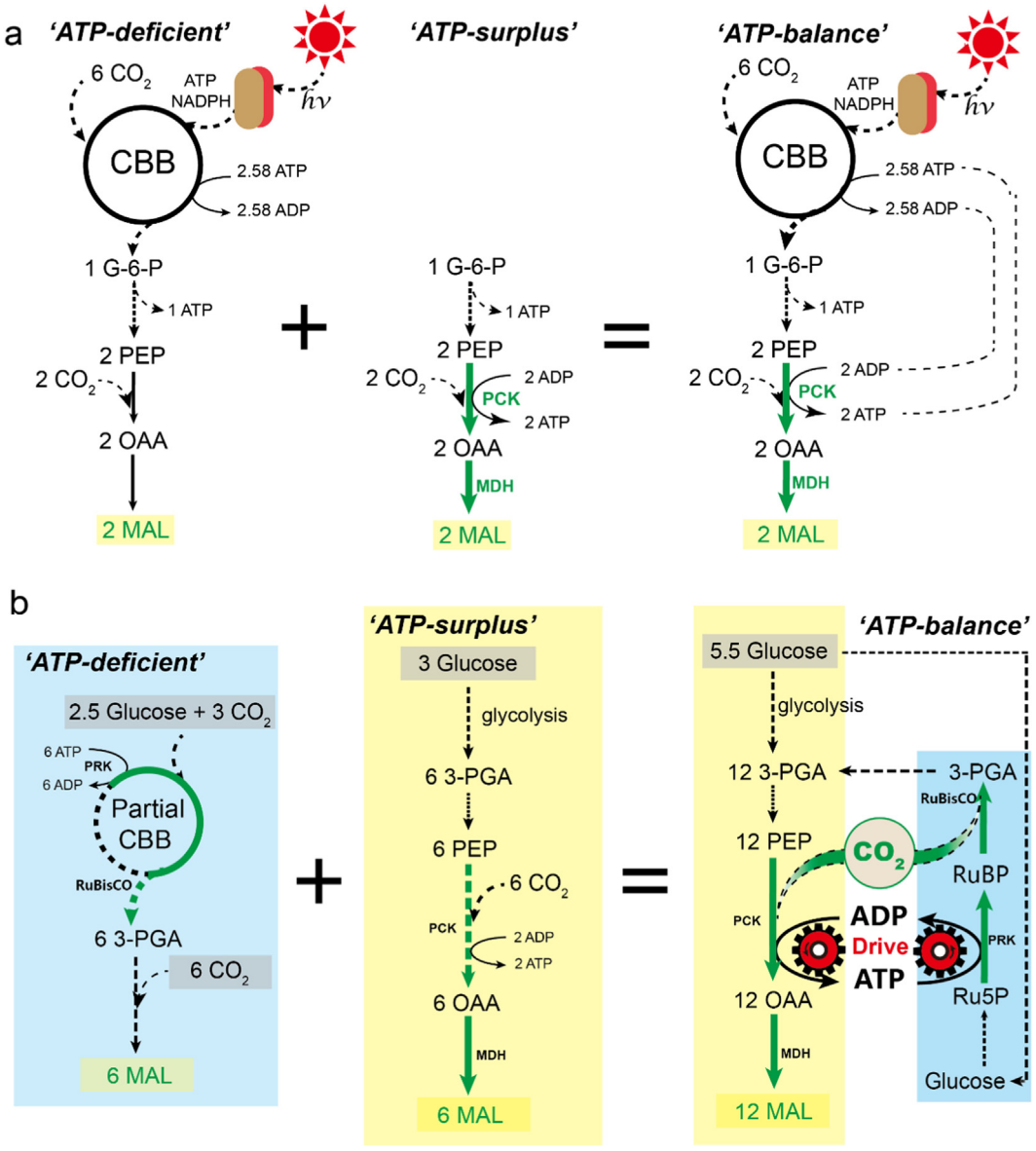

Fig. 1. Overview of ATP balance in CO_2-fixing pathways to improve malate production. ATP balance was achieved by introducing the PCK pathway. (a) Stoichiometric calculations of ATP balance for CO_2 fixation with malate production in *S. elongatus*. (b) Stoichiometric calculations of ATP balance for CO_2 fixation with malate production in *E. coli*. Abbreviations: hv, photon energy; G-6-P, glucose 6-phosphate; Ru5P, ribulose-5-phosphate; RuBP, ribulose-1,5-bisphosphate; 3-PGA, 3-phosphoglyceric acid; PEP, phosphoenolpyruvate; OAA, oxaloacetate; MAL, malate; PRK, phosphoribulokinase; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; PCK, phosphoenolpyruvate carboxykinase; MDH, malate dehydrogenase; CBB, Calvin-Benson-Bassham cycle.

this CO_2-fixing pathway is strictly anaerobic (Schuchmann and Muller, 2014). These findings indicate the importance of developing novel strategies to improve CO_2 biorefineries by considering the balance between efficient ATP supply and major metabolic pathway rewiring.

In this study, we provide a practical solution for this balance. Based on stoichiometric calculations, we first identified a synergetic CO_2-fixing pathway that combines the ATP-generating phosphoenolpyruvate carboxykinase (PCK) pathway with the ATP-consuming RuBisCO shunt (or the CBB cycle) (Fig. 1). Then, the enzymes for building the PCK pathway were screened using a cell-free system. We subsequently demonstrated the application of the synergetic CO_2-fixing pathway in *Synechococcus elongatus*, which resulted in a higher CO_2-fixing rate (R_{Cf}) and malate titer. Finally, the synergetic CO_2-fixing pathways were reconstructed in *Escherichia coli*, and the R_{Cf} and yield of malate form glucose were both significantly improved.

2. Materials and methods

2.1. Strains and plasmids

All strains and plasmids used in this study are listed in Supplementary Table 1 and Supplementary Table 2. *S. elongatus* UTEX 2973 and *E. coli* MG1655 were used as autotrophic and heterotrophic hosts, respectively, for investigating CO_2 fixation and malate production. *E. coli* DH5a was used to construct vectors for engineering *S. elongatus* and *E. coli*. The plasmid pEM (a modified pETM6 (Xu et al., 2012) plasmid formed by replacing the promoter and multiple cloning enzyme cleavage sites with those from pQE-80l-kan) was used to provide a platform for the combinatorial optimization of CA and RuBisCO in *E. coli*. During the construction of *S. elongatus* mutants, cultures were grown in BG-11 medium with 50 mM $NaHCO_3$ at 38 °C, and illumination intensity was controlled at 200 μmol photons/m^2/s. During the construction of *E. coli* mutants, cultures were grown aerobically at