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Quantification and analysis of metabolic characteristics of aerobic succinate-producing *Escherichia coli* under different aeration conditions

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ABSTRACT

The production of succinate by engineered *Escherichia coli* strains has been widely investigated. In this study, quantitative comparison of metabolic fluxes was carried out for the wild-type *E. coli* strain and a quintuple mutant strain QZ1111 that was designed for the production of succinate aerobically by knocking out five genes (*ptsG*, *poxB*, *pta*, *sdhA*, *iclR*) of the wild-type *E. coli* MG1655. Metabolic flux distributions of both strains were quantified by ^{13}C-labeling experiments, together with the determination of physiological parameters and the expression level of key genes. The experimental results indicated that under the same aeration condition the fraction of oxaloacetate molecules originating from phosphoenolpyruvate was increased in *E. coli* QZ1111 compared to that in the wild-type *E. coli* MG1655. The glyoxylate shunt was likely activated in *E. coli* QZ1111 only under high aeration condition but repressed in other conditions, indicating that the deletion of the *iclR* gene could not completely remove the repression of the glyoxylate shunt with limited oxygen supply. Our results also suggested further genetic manipulation strategies to enhance the production yield of succinate.

1. Introduction

Succinate is an important precursor for many medicines and industrial materials [1]. The synthesis of succinate mainly depends on traditional chemistry methods. However, this process would bring sever environmental pollution and be energy consuming. Nowadays more and more attempts were made to use biological method for the production of succinate, and big progress has been achieved. Some strains have the natural ability to produce succinate, such as *Actinobacillus succinogene* and *Anaerobiospirillum succiniciproducens* [1–6]. However, the growth of these strains largely depends on complex culture components and strict environmental conditions, therefore not suitable for large scale industry production. An alternative strain is the engineered *Escherichia coli* [7–15] because of its fast growth, simple culture demands and clear genetic background. Under anaerobic condition, *E. coli* produces succinate as a minor fermentation product normally. Succinate is not produced aerobically by the wild-type *E. coli* strain, where acetate is the main byproduct. A variety of strategies, such as blocking the byproducts formation pathways, activating the glyoxylate shunt, and increasing the flux of the reductive TCA pathway, to enhance succinate production in *E. coli* under anaerobic conditons have met with success [10,13–15]. Unfortunately, there are also some disadvantages of anaerobic fermentation that are difficult to surmount, such as poor cell growth, slow carbon throughput, and low succinate production rate. One solution for overcoming the anaerobic fermentation conditions is to design an aerobic succinate production system that allows *E. coli* to produce succinate completely under aerobic conditions. To this end, mutations in the TCA cycle (*sdhAB*, *icd*, *iclR*) and acetate pathway (*poxB*, *ackA-pta*) of *E. coli* were created to produce succinate under absolute aerobic condition. In aerobic batch reactor, succinate production rate of this strain reached 0.7 mol per mol glucose, and there was also 0.2 mol pyruvate and 0.25 mol acetate per mol glucose accumulation in the medium [7]. To make the aerobic succinate production more effective, the *icd* gene was not removed in order to decrease the production of by-products pyruvate and acetae. Moreover, the *ptsG* gene was inactivated to allow a more balanced glucose metabolism. The maximal theoretical yield of 1 mol succinate per mol glucose was obtained by overexpressing the *Sorghum pepc* gene in the strain of HL27659k (pKK313) [8]. Fed-batch fermentation of the strain HL27659k (pKK313) resulted in production of up to 494 mmol/L succinate with a succinate yiled of 0.85 mol per mol glucose [9].

Metabolic flux analysis (MFA) has been becoming a powerful tool to understand how nutrient sources are utilized by individual reaction pathways under specific conditions [16–22]. It overcomes the blindness of unidirectional genetic manipulation and gives

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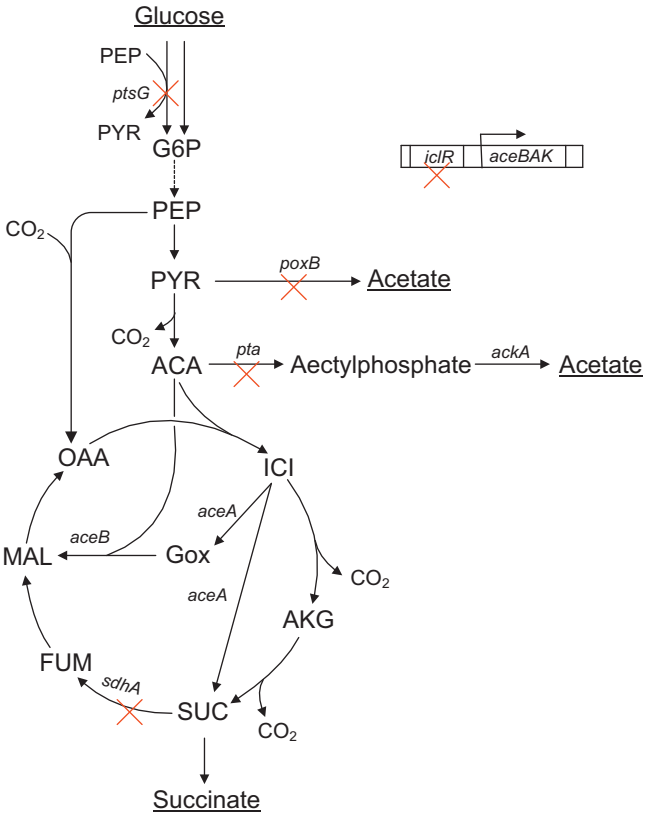

Fig. 1. Metabolic engineering of glycolysis, TCA cycle, and glyoxylate shunt in the construction of the aerobic succinate fermentation platform. Crosses represent for the blocked pathways of which the key enzymes were deleted. Extracellular metabolites are underlined. *ptsG*, glucose phosphotransferase system; *iclR*, isocitrate lyase regulator; *poxB*, pyruvate oxidase; *pta*, phosphotransacetylase; *sdhA*, succinate dehydrogenase.

advice on further modifications on metabolic engineering. Compared with traditional flux analysis methods that rely on the simplified metabolic reaction model and several biological assumptions, MFA using stable isotope tracers takes advantage of relative pathway contributions and thus provides more elaborate metabolic information [23]. ^{13}C-metabolic flux analysis method for determination of metabolic reaction fluxes has been shown to be extremely useful in understanding cell physiology [24].

In our precious study, an aerobic succinate fermentation platform was constructed with a quintuple mutant of *E. coli* (QZ1111) that is deficient in the *sdhA*, *iclR*, *pta*, *poxB*, and *ptsG* genes (Fig. 1). Succinate accumulation might be increased with deficient succinate dehydrogenase (*sdhA*) activity and the activation of the glyoxylate shunt by deletion of the *iclR* gene encoding isocitrate lyase regulator. Both phosphotransacetylase (*pta*) and pyruvate oxidase (*poxB*) were deficient in the constructed strain to reduce acetate overflow and channel more carbon flux toward the oxidative branch of the TCA cycle. In addition, the glucose phosphotransferase system was inactivated by knocking out the *ptsG* gene to increase phosphoenolpyruvate pool. Results of flask cultivation showed that the *E. coli* QZ1111 strain could accumulate succinate with a concentration of 26.4 g/L under aerobic conditions [25]. The aerobic conditions have a great effect on the cell physiology and metabolite production [26]. In this study, we also found that the succinate production rates of the engineered quintuple mutant varied markedly with aeration level. In order to understand the underlying metabolic and regulatory mechanisms and further optimize the aerobic succinate fermentation platform, we performed detailed quantitative study of cell metabolism based on the ^{13}C-labeling of metabolites as well as gene expression data for the strain grown under various oxygen supply conditions.

2. Materials and methods

2.1. Strains and culture conditions

The strains used in this study were the wild-type *E. coli* strain K12 MG1655, and its quintuple deletion mutant *E. coli* QZ1111 (*E. coli* MG1655 Δ*ptsG*Δ*poxB*Δ*pta*Δ*iclR*Δ*sdhA*) constructed and stored in our lab [25]. All *E. coli* strains were cultured in M9 minimal medium containing (per liter): 3 g of glucose, 1.0 g of NH_4Cl, 2.7 g of $(NH_4)_2SO_4$, 6.8 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 0.6 g of NaCl, 0.2 g of $MgSO_4{\cdot}7H_2O$, 1.0 μg of thiamine HCl, and 10 mL of concentrated trace element solution (per liter): 0.55 g of $CaCl_2{\cdot}2H_2O$, 1.67 g of $FeCl_3{\cdot}6H_2O$, 0.10 g of $MnCl_2{\cdot}4H_2O$, 0.17 g of $ZnCl_2$, 0.04 g of $CuCl_2{\cdot}2H_2O$, 0.06 g of $CoCl_2{\cdot}6H_2O$, and 0.06 g of $Na_2MoO_4{\cdot}4H_2O$. Cultures were carried out in 250-mL shake flasks with 50 mL of culture medium. Both *E. coli* MG1655 and *E. coli* QZ1111 strains were incubated at 37 °C in the shaker with a rotational speed of 50 rpm, 220 rpm or 300 rpm, respectively. Preliminary experimental data showed that dissolved oxygen concentration in the shake flask increased with increasing rotational speed (data not shown). A mixture of 20% (wt/wt) uniformly ^{13}C-labeled ([U-^{13}C], >99% ^{13}C, Isotec Inc., Miamisburg, OH) and 80% (wt/wt) [1-^{13}C]-labeled glucose (>99%, Cambridge Isotope Laboratories, Andover, MA) was used for all labeling experiments. Each culture was inoculated from a preculture with a starting optical density at 600 nm (OD_{600}) of less than 0.005. Cell samples were taken at mid-exponential growth phase for labeling analysis of intracellular fluxes.

2.2. Determination of physiological parameters

Cell growth was monitored by measuring the optical density (OD_{600}). Dry cell weight was determined from cell pellets of 100-mL culture aliquots that were centrifuged for 10 min at 4 °C and 8000 × *g*, washed once with distilled water, and dried at 85 °C until constant weight.

For extracellular metabolite analysis, culture samples were centrifuged for 5 min at 4 °C and 20,000 × *g* to remove the cells. The depletion of glucose was determined using commercial enzymatic assay kit (Shanghai Kexin Biotechnology Research Institute, Shanghai, China). The secretion of succinate, acetate, lactate and other metabolites in the medium were detected by high-pressure liquid chromatography (Shimazu LC-20AD, Kyoto, Japan) equipped with a C18 column (Agilent Technologies, Santa Clara, CA), and a UV detector (Shimadzu SPD-20A). Standards were prepared for succinate, lactate and acetate for UV detection at 210 nm, and calibration curves were created.

Based on the correlation factor for dry cell weight and the above concentration change data, physiological parameters of biomass yield ($Y_{X/S}$), specific growth rate (μ), product yields ($Y_{P/S}$), specific glucose uptake rate (q_S), and specific product secretion rates (q_P) were determined for the exponentially growing cells [16].

2.3. GC–MS sample preparation and analysis

The subsequent sample preparation for GC–MS analysis was similar to that described in Hua et al. [27] with minor modifications. The cell pellet was washed once with 1 mL distilled water and then hydrolyzed in 200 μL of 6 M HCl at 105 °C for 16 h. The hydrolysate was dried in a heating block at 60 °C for about 8 h and proteinogenic amino acids were derivatized at 85 °C for 1 h in 100 μL N,N-dimethylformamide and 50 μL N-methyl-N-tert-butyldimethylsilyl trifluoroacetamide (Sigma–Aldrich, Saint Louis, MO). After filtration, 1 μL of derivatized sample was injected into the Agilent 6890/5975 GC–MS system (Agilent Technologies). The HP-5MS column (30 m × 0.25 mm × 0.25 μm) (Agilent Technologies) was used for GC–MS analysis. GC oven temperature was programmed from 100 °C (2 min) to 180 °C at 5 °C/min, then to 260 °C at 10 °C/min, and flow rate of carrier gas was set at 1 mL/min. Other settings were as follows: 1:100 of split ratio, 250 °C of interface temperature, 230 °C of ion source temperature, and electron impact ionization (EI) at −70 eV with a full scan ranging from 70 to 560 *m*/*z* and a solvent delay of 12 min.

2.4. Flux ratio and flux determination

Intracellular flux states of the *E. coli* strains were studied based on a model consisting of all major central carbon metabolic pathways comprising 29 reactions and 24 metabolites (see Appendix A). The model is underdetermined when only material balance constraints are conducted for 24 metabolites in the model network. The relative contributions of two or more converging pathways to the formation of certain target metabolites, i.e. metabolic flux ratios, were therefore quantified based on the principles developed by Sauer's group [28–30]. These ratios were employed to provide additional useful constraints for carbon flux determination.