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Switch on a more efficient pyruvate synthesis pathway based on transcriptome analysis and metabolic evolution

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Due to the decrease of intracellular NADH availability, gluconate metabolism is more conducive to pyruvate production than glucose. Transcriptome analysis revealed that the Entner–Doudoroff (ED) pathway was activated by gluconate in *Escherichia coli* YP211 (MG1655 Δ*ldhA* Δ*pflB* Δ*pta-ackA* Δ*poxB* Δ*ppc* Δ*frdBC*). To construct a new pyruvate producing strain with glucose metabolism via ED pathway, the genes *ppsA*, *ptsG*, *pgi* and *gnd* were deleted sequentially to reduce the demand for PEP and block the Embden–Meyerhor–Parnas pathway and Pentose-Phosphate pathway. After nearly 1000 generations of growth-based selection, the evolved strain YP404 was isolated and the ED pathway was proved to be activated as the primary glycolytic pathway. Comparing with YP211, the pyruvate concentration and yield increased by 59% and 10.1%, respectively. In fed-batch fermentation, the pyruvate concentration reached 83.5 g l^{-1} with a volumetric productivity of 2.3 g l^{-1} h^{-1}. This was the first time to produce pyruvate via ED pathway, and prove that this was a more effective way.

[**Keywords:** Pyruvate; Transcriptome; Entner–Doudoroff pathway; Metabolic evolution; Redox state]

In cells, pyruvate is a direct or indirect precursor of high-value-added products such as alanine (1), phenylpyruvate derivatives (2), isobutanol (3), and carotenoids (4). As a chemical, pyruvate is also used as a raw material for drug, agrochemical, chemical, and food industries (5,6). The commercial demand for pyruvate has been expanding. Therefore, a strain with a high tendency for accumulating pyruvate is desired for commercial production of pyruvate.

Pyruvate is at a key junction of metabolic networks. Accumulating large amounts of pyruvate extracellularly is usually difficult under normal condition (7). Through the alteration of the microbial metabolic pathways, the accumulation of pyruvate can be achieved in cells. Causey et al. (8) deleted the genes *focA-pflB*, *frdBC*, *ldhA*, *atpFH*, *adhE*, *sucA*, *poxB*, and *ackA* from *Escherichia coli* to obtain pyruvate producing strain. The pyruvate conversion rate reached 0.75 g/g glucose, and the maximum concentration of pyruvate reached 66 g l^{-1}. *E. coli* CGSC7916, constructed by Tomar et al. (9) by inactivating the *aceF* and *ppc* genes, had a pyruvate yield of 0.78 g/g glucose and a volumetric productivity of 1.2 g l^{-1} h^{-1}.

Although a variety of pyruvate-producing bacteria strains have been built in this area, over-accumulation of NADH has been a major problem that needs to be overcome, otherwise the glucose consumption and cell growth are both inhibited (10,11). Regulation of the redox state in the cells, specifically lowering the NADH/NAD^+ ratio, could provide a solution to the above problem. Liu et al. (12) used two strategies for increasing the availability of NAD^+. Addition of nicotinic acid as the precursor of NAD^+ and acetaldehyde as exterior electron acceptor of alcohol dehydrogenase in the medium resulted in a substantial increase in the glucose consumption rate and the pyruvate concentration in *T. glabrata*. Besides, oxidation of NADH to NAD^+ by NADH oxidase can reduce the ratio of NADH/NAD^+ (13). Glycolytic flux could be increased by 70% by introduction of water-forming NADH oxidase (NOX) from *Streptococcus pneumoniae* into *E. coli* (14). However, for pyruvate production, overexpressing NADH oxidase did not increase the yield of pyruvate, although it could decrease the intracellular NADH content and increase the consumption rate of glucose.

Using an appropriate carbohydrate in high oxidation state as carbon source was another method for reducing intracellular NADH (15). From our results, sodium gluconate has been proved to be a better carbon source than glucose, which brought a decrease of NADH production and a significant increase of pyruvate production in engineered strain YP211 (16). Because the price of sodium gluconate is higher than glucose, it is necessary to establish a new metabolic pathway with glucose as carbon source to imitate the conversion of gluconate to pyruvate.

In this study, transcriptome analysis was employed to compare the metabolic differences between gluconate and glucose. It was found that the activation of ED pathway was crucial in overcoming the NADH accumulation. Therefore, a new pathway to produce pyruvate from glucose was constructed based on the ED pathway in engineered strain *E. coli* YP211 (MG1655 Δ*ldhA* Δ*pflB* Δ*pta-ackA* Δ*poxB* Δ*ppc* Δ*frdBC*). In the desired engineered strain, the Embden–Meyerhor–Parnas (EMP) pathway and pentose-phosphate (PP) pathway were blocked by deletion of genes *pgi* and *gnd*, and

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genes *ppsA* and *ptsG*, which is responsible for the conversion of pyruvate to PEP and glucose transport, respectively, were knocked out to reduce the demands for PEP. Subsequently, metabolic evolution was carried out to achieve the redistribution of metabolic flow and increase the growth of strain. Finally, the performance of engineering strains was evaluated using fed-batch fermentation.

MATERIALS AND METHODS

Strains and plasmids Strains and plasmids constructed in this study are listed in Table 1. Restriction enzymes were purchased from Takara Biotechnology (Dalian, China). PCR purification kits, gel extraction kits were from Axygen (Union City, CA, USA). Uni-seamless cloning and assembly kits were from Transgen (Beijing, China). Primers were synthesized by Sangon Biotechnology (Shanghai, China).

Transcriptional analysis Strain YP211 was cultured in LB medium with glucose or gluconate as carbon source. Eight hours after inoculation, cells were harvested by quick centrifugation (at 10,000 ×*g*, 4 °C for 1 min) and then immediately frozen in liquid nitrogen. Total RNA was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) following lysozyme treatment. The total RNA in each sample was quantified with an Agilent 2100 Bioanalyzer (Agilent Technologies). Pair-end (PE) index libraries were constructed according to the manufacturer's protocol (NEBNext Ultra Directional RNA Library Prep Kit for Illumina). Sequencing was performed using a 2 × 100 PE configuration. Image analysis and base calling were conducted by the HiSeq Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina) in the HiSeq instrument.

From the quality analysis of sequencing data, the number of original Reads were 58,708,268 and 48,314,756 in the samples of glucose and sodium gluconate as the carbon source, respectively. After filtering based on the software Cutadapt (version 1.9.1), Reads numbers decreased to 58,430,000 and 47,950,720, respectively. Differentially expressed sequences between two samples were identified by the program DESeq (v1.18.0) from Bioconductor package. Genes with p-value and FDR (q-value) less than 0.05 and expression changing larger than 2 times were identified as significantly differentially expressed. The sequences were processed and analyzed by GENEWIZ (Suzhou, China).

Genetic manipulation A modified method based on tandem repeat primers and homologous recombination was developed for deletion of *E. coli* chromosomal genes (17). Primers were designed to amplify the *cat-sacB* cassette from plasmid pEASY-*cat-sacB* using PCR and Pfu polymerase (Table S1).

Media and fermentation During plasmid and strains construction, cultures were grown in LB broth or on LB plates. Assessments of the engineered strains were conducted in flask with M9 medium containing about 20 g l^{-1} glucose at 37 °C and 200 rpm. Two gram $CaCO_3$ was added to control the pH. Fed batch fermentation was conducted in a 5.0 L fermenter (BioFlo/Celli Gen115, New Brunswick Scientific) with M9 medium. During the experiments, glucose was added to about 40 g l^{-1} from a sterile 50% stock, when the remaining glucose was below 10 g l^{-1}. 20% NaOH were used to control pH. Samples were stored at −20 °C for subsequent analysis.

Metabolic evolution Metabolic evolution of strain YP304 was carried out by sequentially subculturing using pH controlled fermenters with total volume 100 ml. To increase the amount of gene mutations and accelerate the evolution process, a certain amount of sodium gluconate was added in the early stage of metabolic evolution. Selection was begun using 1% (w/v) glucose and 1% (w/v) gluconate with serial transfers at 48 h intervals. Subsequently, gluconate was no longer added and the glucose concentration was doubled and transfers continued. After nearly 1000 generations of growth-based selection, a clone was isolated from the last transfer and designated YP404.

Analytical methods Cell growth was monitored from the optical density at 600 nm (UV−Vis spectrophotometer, U2910, Hitachi), which is related to dry cell mass. Organic acids and glucose concentrations were determined by HPLC (1200 series, Agilent Technologies) equipped with a UV monitor (210 nm) and refractive index detector. Products were separated by using a Bio-Rad HPX-87H column using 5 mmol l^{-1} H_2SO_4 as the mobile phase (0.6 ml/min, 50 °C). NADH and NAD^+ concentrations were measured using NAD^+/NADH Quantitation Kit (Sigma−Aldrich).

TABLE 1. Sources and characteristics of strains and plasmids used in this study.

Strain/Plasmid	Relevant characteristics	Source
Strain		
YP211	*E. coli* K12 MG1655 Δ*ldhA* Δ*pflB* Δ*pta-ackA* Δ*poxB* Δ*ppc* Δ*frdBC*	Lab collection
YP301	YP211 Δ*ppsA*	This study
YP302	YP211 Δ*ppsA* Δ*ptsG*	This study
YP303	YP211 Δ*ppsA* Δ*ptsG* Δ*pgi*	This study
YP304	YP211 Δ*ppsA* Δ*ptsG* Δ*pgi* Δ*gnd*	This study
Plasmid		
pKD46	Red recombinase, temperature-conditional replicon	Lab collection
pEASY-*cat-sacB*	T-easy vector with *cat-sacB* cassette	Lab collection

RESULTS AND DISCUSSION

Transcriptome analysis In *E. coli*, the over-accumulation of NADH is known to be the main cause of limited pyruvate production (18). This problem can be alleviated by using sodium gluconate as carbon source. In order to explore the metabolic advantages of gluconate, transcriptome analysis was employed to compare the metabolic differences between gluconate and glucose. The strain used in this study was YP211 (*E. coli* K12 MG1655 Δ*ldhA* Δ*pflB* Δ*pta-ackA* Δ*poxB* Δ*ppc* Δ*frdBC*). According to the results of DESeq, the most significant transcriptional differences were observed for genes related to the gluconate transport and ED pathway, as shown in Fig. 1. Of these genes, the transcription of the *gntU*, *gntK*, and *gntT* genes, which are responsible for the intracellular transport and phosphorylation of gluconate, was found to have increased by factors of 78.0, 58.1, and 49.5, respectively. The transcription of gene *edd* and *eda*, which are responsible for encoding the 6-phosphogluconate dehydratase and 2-dehydro-3-deoxy-phosphogluconate aldolase,

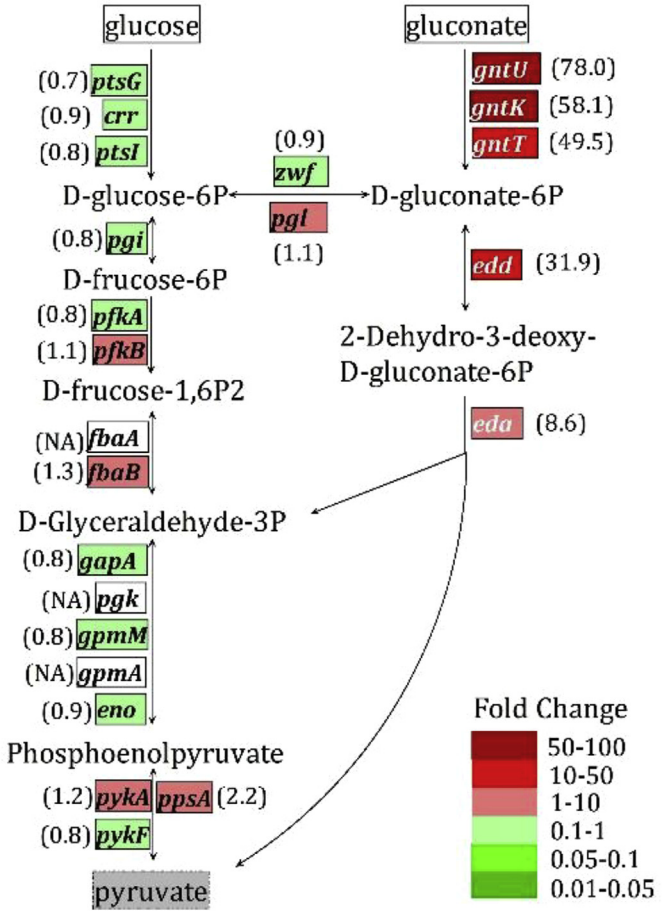

FIG. 1. Transcriptional differences of genes related to EMP and ED pathway between glucose and gluconate metabolism. The number in parentheses is the ratio of the average value of the mRNA fragments of a gene in the gluconate metabolism to in the glucose metabolism. Red means transcription enhancement, and green means transcription weaken. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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