Journal of Biotechnology 258 (2017) 69–78

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Modular pathway engineering of *Corynebacterium glutamicum* to improve xylose utilization and succinate production☆

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A R T I C L E I N F O

Article history:
Received 6 October 2016
Received in revised form 24 January 2017
Accepted 27 January 2017
Available online 30 January 2017

Keywords:
Corynebacterium glutamicum
Synthetic biology
Metabolic engineering
Xylose utilization
Phosphoketolase

A B S T R A C T

Xylose-negative *Corynebacterium glutamicum* has been engineered to utilize xylose as the sole carbon source via either the xylose isomerase (XI) pathway or the Weimberg pathway. Heterologous expression of xylose isomerase and overexpression of a gene encoding for xylulose kinase enabled efficient xylose utilization. In this study, we show that two functionally-redundant transcriptional regulators (GntR1 and GntR2) present on xylose repress the pentose phosphate pathway genes. For efficient xylose utilization, pentose phosphate pathway genes and a phosphoketolase gene were overexpressed with the XI pathway in *C. glutamicum*. Overexpression of the genes encoding for transaldolase (Tal), 6-phosphogluconate dehydrogenase (Gnd), or phosphoketolase (XpkA) enhanced the growth and xylose consumption rates compared to the wild-type with the XI pathway alone. However, co-expression of these genes did not have a synergetic effect on xylose utilization. For the succinate production from xylose, overexpression of the *tal* gene with the XI pathway in a succinate-producing strain improved xylose utilization and increased the specific succinate production rate by 2.5-fold compared to wild-type with the XI pathway alone. Thus, overexpression of the *tal*, *gnd*, or *xpkA* gene could be helpful for engineering *C. glutamicum* toward production of value-added chemicals with efficient xylose utilization.
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1. Introduction

Microbial cell factories have been developed to produce value-added chemicals by utilizing sugars derived from cellulose and hemicellulose (Bokinsky et al., 2011; Choi et al., 2016). D-Xylose, the second most abundant sugar-derived hemicellulose, can be metabolized via the xylose isomerase (XI) pathway, the xylose reductase (XR)-xylitol dehydrogenase (XDH) pathway, the Weimberg (WMB) pathway (Weimberg, 1961), or the Dahms pathway (Dahms, 1974). Recently, a synthetic pathway (XI, xylulose-1-kinase, and xylulose-1-phosphate aldolase) for xylose assimilation has been proposed and applied to produce ethylene glycol and glycolic acid from xylose, comparing natural pathways (XI, XR-XDH, WMG, and Dahms) using a stoichiometric model (Cam et al., 2016).

Corynebacterium glutamicum is a predominantly aerobic, non-pathogenic, biotin-auxotrophic, Gram-positive bacterium that is used industrially for the production of amino acids, in particular the flavor enhancer L-glutamate and the feed additive L-lysine (Eggeling and Bott, 2015). Due to the lack of a xylose isomerase (XI) gene for xylose metabolism, *C. glutamicum* is not able to utilize xylose as a carbon source. Introduction of the *xylA* gene encoding xylose isomerase is sufficient to allow for xylose consumption by *C. glutamicum*, and its co-expression with the *xylB* gene encoding xylulose kinase enhances cell growth (Kang et al., 2014; Kawaguchi et al., 2006; Meiswinkel et al., 2013; Yim et al., 2016).

In addition to the xylose isomerase pathway, the heterologous Weimberg pathway (WMB), through which xylose is catabolized into α-ketoglutarate, has been introduced to minimize carbon loss during xylose utilization in *C. glutamicum* (Radek et al., 2014). However, lower growth rates were observed in *C. glutamicum* with the WMB pathway than in *C. glutamicum* with the XI pathway. A recent study of the WMB and XI pathways in *C. glutamicum* revealed that xylitol 5-phosphate, which inhibits the growth of *C. glutamicum* on xylose, and xylitol was accumulated as by-products via unspecific side-reactions (Radek et al., 2016). Further engineering of the WMB pathway may be necessary to circumvent the cytotoxicity of xylitol 5-phosphate.

☆ Special issues: On the occasion of the 40th anniversary of Biotechnology in Jülich.
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http://dx.doi.org/10.1016/j.jbiotec.2017.01.015
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The phosphoketolase (PHK) pathway (i.e., the bifid-shunt, named for a pathway used by *Bifidobacterium* species) produces acetate and lactate by converting xylulose 5-phosphate to glyceraldehyde 3-phosphate and acetyl phosphate (Fushinobu, 2010; Henard et al., 2015). Recent studies on overexpressed PHK genes have shown improved specific growth of *Aspergillus nidulans* on xylose (Panagiotou et al., 2008) and improved ethanol yield of *S. cerevisiae* on xylose without affecting the xylose utilization rate (Sonderegger et al., 2004). Moreover, 40% of the xylose catabolic fluxes used in the PHK pathway in *Clostridium acetobutylicum* have been mapped using ^{13}C metabolic flux analysis (Liu et al., 2012). In addition, the PHK pathway offers an opportunity to enhance carbon yields by by-passing the pyruvate decarboxylation reaction and also has a higher ATP production yield (1.25-fold more effective) than the EMP pathway (2 ATP/glucose) (Bogorad et al., 2013; Henard et al., 2015).

In this study, we metabolically-engineered *C. glutamicum* ATCC 13032 to improve xylose consumption by analyzing the GntR1/2 transcriptional regulator-lacking mutants and overexpressing a pentose phosphate pathway (PPP) gene or/and a heterologous PHK. Additionally, the subsequent xylose-utilizing *C. glutamicum* were used for succinate production.

2. Materials and method

2.1. Bacterial strains and growth condition

E. coli DH5α (Hanahan, 1983) and *C. glutamicum* ATCC 13032 (wild-type) were used in this study (Table 1). *E. coli* strains were grown in Lysogeny Broth (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) at 37 °C on a rotary shaker at 200 rpm. When appropriate, the medium was supplemented with 25 μg/mL chloramphenicol, 50 μg/mL kanamycin, or both antibiotics. *C. glutamicum* ATCC 13032 and its derivatives were cultivated in BHIS medium (Eggeling and Bott, 2005) at 30 °C on a rotary shaker at 120 rpm. When appropriate, the medium was supplemented with 7.5 μg/mL chloramphenicol, 15 μg/mL kanamycin, or both antibiotics. For the utilization of xylose, cells were pre-cultivated in BHIS medium overnight and then incubated aerobically in CgXII defined medium (50 mL media in a 250 mL baffled Erlenmeyer flask) containing 1% (wt/vol) xylose as the sole carbon source at 30 °C on a rotary shaker at 120 rpm (Kang et al., 2014).

For cell cultivations with varying concentrations of xylose, automated cell cultivations were carried out in 48-well FlowerPlates (m2p-labs GmbH, Baesweiler, Germany) incubated in a BioLector (m2p-labs GmbH). The cells were cultivated at 900 rpm, 85% relative humidity, and 30 °C in 1000 μL of fresh CgXII medium (starting inoculation of $OD_{600} = 1$). The FlowerPlates were covered with a gas-permeable sealing foil (m2p-labs GmbH) to prevent contamination and to allow for uniform gas exchange. Depending on the experiment, the online signals for biomass (backscatter; gain 15), dissolved oxygen (pO_2; gain 30), and pH (gain 21) were measured at 30-min intervals. The corresponding backscatter (BS) measurements were recalculated to cell dry weight (CDW) data using the calibration model CDW [g/L] = 0.048 × BS g/L − 0.78 g/L (Rohe et al., 2012).

2.2. Plasmid construction and transformation

For CoryneBrick vectors, the *xpkA* gene from *A. nidulans* (Papini et al., 2012) and the *fxpk* gene from *Bifidobacterium adolescentis* (Bogorad et al., 2013) were synthesized (Genscript, USA) with codon-optimization for *C. glutamicum*. Each gene was then inserted into either pXylA or pXylAB (Kang et al., 2014) using the standard BglBrick cloning method, and the target gene was inserted at the *Eco*RI and *Xho*I sites. For plasmids harboring the pentose phosphate pathway genes, each target gene was cloned from a genomic DNA of *C. glutamicum* using oligo primers (Supplementary Table S1). All cloned genes were verified by DNA sequencing. Plasmids used in this work are listed in Table 1. For the transformation of *C. glutamicum*, competent cell preparation and electroporation were performed as previously described (Kang et al., 2014; Van der Rest et al., 1999).

2.3. HPLC analysis for xylose and succinate quantification

Xylose and succinate in the supernatant were quantified by HPLC as described previously (Kang et al., 2014; Lee et al., 2016). Briefly, after centrifugation at 10,000 × *g* for 10 min, the culture supernatant was passed through a syringe filter (pore size 0.2 μm). The concentrations of xylose and succinate were determined by a high-performance liquid chromatograph (HPLC, Agilent 1260, Waldbronn, Germany) equipped with a refractive index detector (RID), UV detector, and an Aminex HPX-87H Ion Exclusion Column (300 mm by 7.8 mm, Bio-Rad, Hercules, CA, USA) under the following conditions: sample volume of 20 μL, mobile phase of 5 mM H_2SO_4, flow rate of 0.6 mL/min, and column temperature of 65 °C.

2.4. Reverse transcription-PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR) analyses

RT-PCR analysis was performed as described previously (Kim et al., 2015). For total RNA purification, *C. glutamicum* strains were cultured in CgXII defined medium (50 mL media in a 250 mL baffled Erlenmeyer flask) containing 10 g/L xylose at 30 °C on a rotary shaker at 120 rpm, and cells were harvested at 6 h after inoculation. RNA isolation was performed with TRIzol (Ambion, Auckland, New Zealand), and then the samples were treated with DNaseI solution (Invitrogen, CA, USA) at 37 °C for 30 min. After heat inactivation and chloroform extraction, the RNA was precipitated with 100% EtOH containing 100 mM sodium acetate. After denaturation for 5 min at 70 °C, reverse transcription of 1 μg RNA was performed using the GoScript Reverse Transcription System (Promega, WI, USA) according to the manufacturer's instructions. Subsequently, cDNAs were amplified using the primers listed in Supplementary Table S2.

After total RNA purification, quantitative real-time PCR (qRT-PCR) was performed using TagMan Gene expression master mix (Applied Biosystems, CA, USA) and the Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific, MA, USA) in accordance with the manufacturer's instructions. The primers used for qRT-PCR are listed in Table S2. Expression levels for each gene were calculated relative to that of a control using the ΔΔCt method (Livak and Schmittgen, 2001).

3. Results and discussion

3.1. Xylose utilization in ΔgntR1ΔgntR2 mutant C. glutamicum

Previous studies have shown the role of the global regulators GntR1 and GntR2 of *C. glutamicum* in repressing the pentose phosphate pathway genes in the *tkt-tal-zwf-opcA-devB* cluster and the *gnd* gene (encoding for 6-phosphogluconate dehydrogenase) and controlling the genes for gluconate catabolism (Frunzke et al., 2008; Tanaka et al., 2014) (Fig. 1). Therefore, *gntR1/2* mutants were used to investigate whether the mutants harboring the xylose isomerase gene exhibit improved cell growth and xylose consumption rates (Table 1). Single mutants (Δ*gntR1* or Δ*gntR2*) harboring a pXylAB plasmid showed cell growth and xylose utilization similar to the WT pXylAB, but the double mutant harboring a pXylAB plasmid showed a slower growth rate and xylose consumption than WT pXylAB (Fig. 2 and Table 2). This phenotype could be due to

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