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Engineering and adaptive evolution of *Escherichia coli* for D-lactate fermentation reveals GatC as a xylose transporter

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ABSTRACT

Despite the abundance of xylose in nature, the production of chemicals from C5 sugars remains challenging in metabolic engineering. By deleting *xylFGH* genes and using adaptive evolution, an efficient *E. coli* strain capable of producing D-lactate from xylose was engineered. Quantitative proteomics and genome sequencing were used to understand the new phenotype and the metabolic limitations of xylose conversion to D-lactate. Proteomics identified major changes in enzyme concentration in the glycolytic and tricarboxylic acid pathways. Whole genome sequencing of the evolved strain identified a point mutation in the *gatC* gene, which resulted in a change from serine to leucine at position 184 of the GatC protein. The knockout of *gatC* in a number of strains and the insertion of the mutation in the non-evolved strain confirmed its activity as a xylose transporter and demonstrated that the mutation is responsible for the high xylose consumption phenotype in the evolved strain. The newly found xylose transporter is a candidate for future strain engineering for converting C5–C6 syrups into valuable chemicals.

1. Introduction

Although xylose is the major component of hemicellulose in plants, the production of fuels and chemicals from this pentose is a major challenge in metabolic engineering. Xylose is rarely present as a monomer, and most fermenting organisms are unable to utilize it as a carbon source (Hahn-Hägerdal et al., 2007). Therefore, the production of commodity chemicals, such as lactic acid, from xylose is a major challenge.

Polylactic acid is a biodegradable polymer used as an alternative to petrochemical-based polymers, such as polystyrene or polyethylene. Traditionally, lactic acid has been produced using lactic acid bacteria (LAB); however, most LAB are not able to use xylose as a carbon source and have complex and fastidious nitrogen requirements. In addition, few of them can ferment sugars to D-lactic acid, the isomer needed to attain stereo-complex thermal stable polylactic acid. Unlike LAB, *E. coli* is able to metabolize xylose under fermentative conditions to produce several products, including D-lactate, with the use of mineral media. Homolactic fermentation of hexoses and pentoses in *E. coli* is redox balanced by reducing pyruvate into D-lactate (Orencio-Trejo et al., 2010). Using glucose mineral medium engineered *E. coli* strains had achieved yields and productivities similar to LAB (90% of the maximum theoretical yield). The strains were obtained by deleting enzymes belonging to competing pathways (*pflB*, *adhE* and *frdA*) (Martinez et al., 2007; Utrilla et al., 2009; Zhou et al., 2003, 2005, 2006; Zhu and Shimizu, 2004, 2005). When *E. coli pfl* mutants grow on xylose, however, growth limitation caused by an ATP deficit is observed under anaerobic conditions (Hasona et al., 2004).

Xylose is known to be transported in *E. coli* through two different systems, an ABC transporter coded by the *xylF*, *xylG* and *xylH* genes and a proton/xylose symporter coded by *xylE* (Sumiya et al., 1995). Under some special conditions, the arabinose symporter (AraE) can also transport xylose (Hasona et al., 2004). Because the ABC transporter consumes one ATP to internalize a xylose molecule and other ATP is needed for phosphorylation of xylulose, low ATP/xylose molar yields (0.67) and no growth are observed in homolactic strains (Hasona et al., 2004). Despite numerous efforts to utilize complex sugar mixtures and avoid catabolite repression (Hernández-Montalvo et al., 2001), to the best of our knowledge, no prior studies have attempted to understand xylose metabolism limitations in *E. coli*.

In the present work, using metabolic engineering and growth- and production-coupled adaptive evolution, we obtained a strain capable of efficiently fermenting xylose into D-lactic acid. Using genome sequencing and quantitative proteomics, we were able to identify a modified GatC protein as a new xylose transporter that improves the growth and consumption rate of this sugar in the

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evolved strain. GatC had been reported as the IIC component of the galactitol PTS systems, its function as a xylose transporter was previously unknown.

2. Materials and methods

2.1. Materials

All the reagents were of the highest available purity and were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile (ACN) was purchased from ACI Labscan (RCI Labscan Ltd., Bangkok, Thailand).

2.2. Organisms and culture conditions

All the bacterial strains used in this study are derivatives of *E. coli* MG1655 and are listed in Table 1. Batch fermentations were performed with mineral AM1 medium (Martinez et al., 2007) supplemented with citric acid (100 mg/L) and xylose as the sole carbon source. The cells were cultured in mini-fermentors (300 mL fleakers; Beall et al., 1991) containing 200 ml media, without aeration at 37 °C, pH 7 and 100 rpm. The pH was kept constant with 2 N KOH automatic additions. All the fermentations were performed at least in triplicate (average and standard deviations are shown in the plots and tables).

2.3. Genetic methods

Deletions of the *xylFGH, xylE, and gatC* genes and a 27.3 kb region were made using the chromosomal gene inactivation method using PCR products (Datsenko and Wanner, 2000). The primers were designed to amplify FRT-Kan-FRT from pKD4 or FRT-cat-FRT from pKD3 with 40–50 nucleotides of homology to the chromosome sequence to inactivate target genes (supplementary Table 1). Introduction of the *gatCS184L* into the JU01 chromosome was performed in several steps. First, the complete *gatYZABCD* transcription unit from JU15 was cloned into a pCR®2.1-TOPO® vector (Invitrogen, Carlsbad, CA, USA). Second, a tetracycline (Tc) cassette from pBSL184 vector (Alexeyev et al., 1995) was introduced upstream to the cloned region. Third, the Tc cassette and a part of the transcription unit, including the complete *gatCS184L* gene, were PCR amplified. The PCR was done using a forward primer, which had a priming site from the pCR®2.1-TOPO® vector in the 3' end and a 50 nt homology to the intergenic region in the 5' end (supplementary Table 1). The homology region in the forward primer was the intergenic region between *gatYZABCD* transcription unit and *fbaB* gene (from 2,175,433 to 2,175,383 in *E. coli* genome). The reverse primer amplifies from the middle of the *gatG* gene in the cloned *gatYZACBD* transcription unit. Finally, 100 ng of the PCR product (approximately 5 kb) were electroporated to the strain JU01 *ΔgatC*, transformed with the vector expressing the lambda red system (Datsenko and Wanner, 2000) and strains were selected with the tetracycline resistance. PCR mapping and sequencing of the resulting strains were performed confirming the changes.

Table 1
Strains used in this study.

Strain	Relevant genotype	Reference
MG1655	Wild type	Laboratory stock
CL3	MG1655 *ΔpflB ΔadhE ΔfrdA*	Utrilla et al., 2009
JU01	CL3 *ΔxylFGH*	This work
JU15	JU01 evolved: *Δreg* 27.3 kb *gatCS184L*	This work
JU01 *Δreg* 27.3	JU01: 27.3 kb region deleted	This work
JU01 *ΔgatC*	JU01 *ΔgatC*	This work
JU15 *ΔgatCS184L*	JU15 *ΔgatC S184L*	This work
JU01 *gatCS184L* Tc^R	JU01 *gatC* replaced with *gatCS184L* Tc^R	This work
JU01 *ΔxylE*	JU01 *ΔxylE*	This work
JU15 *ΔxylE*	JU15 *ΔxylE*	This work
JU01 *ΔgatC ΔxylE*	JU01 *ΔgatC ΔxylE*	This work
JU15 *ΔgatCS184L ΔxylE*	JU15 *ΔgatCS184L Δ xylE*	This work

2.4. Adaptive evolution

Adaptive evolution was performed by serial transfers in pH-controlled mini-fermentors as described elsewhere (Lenski et al., 1991; Fong et al., 2006; Zhou et al., 2005). Briefly, samples from the mid-exponential growth phase, ($OD_{600}=\sim1$) were serially diluted to $OD_{600}=0.01$ in mini-fermentors containing mineral media with xylose as the sole carbon source. Initially, 40 g/L of xylose was used, which was increased to 120 g/L after the 9th transfer. Serial transfers were performed until no further improvement in the growth rate was observed. A strain collection was created by freezing 1 mL of culture and isolating four colonies from each passage. The selected colonies from each collection were isolated and characterized. A strain named JU15 was isolated from the 15th transfer. JU15 showed the highest growth rate and highest lactate productivity.

2.5. Genome sequencing and mutation validation

Whole genome sequencing was performed by comparative genome sequencing (Roche Nimblegen Inc, Madison, WI, USA) using JU01 as the reference strain. All the genomic changes reported by the company were PCR amplified and verified by capillary electrophoresis sequencing.

2.6. Analytical procedures

Growth was spectrophotometrically monitored at 600 nm (DU-70, Beckman Instruments, Inc. Fullerton, CA, USA) and converted to dry cell weight (DCW) per liter using the following equation: 1 optical density = 0.37 g_{DCW}/l. The samples were centrifuged, and the cell-free culture broth was frozen for subsequent analysis. Xylose, ethanol, acetate, formate, succinate, lactate, and pyruvic acids were determined by HPLC (Waters U6K, Millipore Co., Milford, MA, USA) using an Aminex HPX-87H ion exclusion column (300 × 7.8 mm; Bio-Rad Laboratories, Hercules, CA), 5.0 mM H_2SO_4 solution as the mobile phase (0.5 ml/min) at 45 °C, a photodiode array detector at 210 nm (Model 996, Waters, Millipore Co) and a refractive index detector (Model 2410, Waters, Millipore Co., Milford, MA, USA).

2.7. Proteomics

2.7.1. Protein extraction and trypsin digestion

Cells from the late exponential growth phase were harvested from mini-fermentors, centrifuged (4 °C, 4750 × *g*), washed with buffer and resuspended in lysis buffer, as described elsewhere (Macek et al., 2008). A 10 mg of dried proteins were resuspended in 6M urea, 2M thiourea, 2% CHAPS for protein denaturation and incubated for 45 min with 1 mM DTT. Subsequently, proteins were treated with 2.5 mM iodoacetamide in the dark for 45 min. The protein digests were then diluted with 25 mM Ambic to a final concentration of urea < 800 mM. Trypsin (Promega Gold, MS grade, Madison, WI, USA) was used at a ratio of 1/100 (trypsin/protein) and incubated for 12 h at 37 °C. Digested peptide mixtures were cleaned using a C18 cartridges (Sep-Pak tC18,