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ARTICLE

Parallel experimental evolution reveals a novel repressive control of GalP on xylose fermentation in *Escherichia coli*

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Abstract

Efficient xylose utilization will facilitate microbial conversion of lignocellulosic sugar mixtures into valuable products. In *Escherichia coli*, xylose catabolism is controlled by carbon catabolite repression (CCR). However, in *E. coli* such as the succinate-producing strain KJ122 with disrupted CCR, xylose utilization is still inhibited under fermentative conditions. To probe the underlying genetic mechanisms inhibiting xylose utilization, we evolved KJ122 to enhance its xylose fermentation abilities in parallel and characterized the potential convergent genetic changes shared by multiple independently evolved strains. Whole-genome sequencing revealed that convergent mutations occurred in the galactose regulon during adaptive laboratory evolution potentially decreasing the transcriptional level or the activity of GalP, a galactose permease. We showed that deletion of *galP* increased xylose utilization in both KJ122 and wild-type *E. coli*, demonstrating a common repressive role of GalP for xylose fermentation. Concomitantly, induced expression of *galP* from a plasmid repressed xylose fermentation. Transcriptome analysis using RNA sequencing indicates that *galP* inactivation increases transcription levels of many catabolic genes for secondary sugars including xylose and arabinose. The repressive role of GalP for fermenting secondary sugars in *E. coli* suggests that utilization of GalP as a substitute glucose transporter is undesirable for conversion of lignocellulosic sugar mixtures.

KEYWORDS

adaptive laboratory evolution, GalP, lignocellulose, succinate, transport, xylose

1 | INTRODUCTION

Lignocellulose is a complex matrix present in the cell wall of plants that makes up more than half of the earth's total biomass, making it an abundant renewable feedstock (Mishra & Singh, 1993). Independent of source, lignocellulose usually contains cellulose and hemicellulose, which can be degraded to glucose and pentoses for use as a feedstock for microbial conversion (Garvey, Klose, Fischer, Lambertz, & Commandeur, 2013; Saha, 2003). Glucose is the only sugar monomer in cellulose and xylose is the major sugar component for common hemicellulose fractions (Girio et al., 2010; Saha, 2003). Simultaneous coutilization of both sugars is desired for an efficient microbial lignocellulose bioconversion (Kim, Block, & Mills, 2010; Nieves, Panyon, & Wang, 2015). However, cofermentation of mixed sugars often presents an obstacle to model microbial biocatalysts such as *Escherichia coli* due to a global regulatory mechanism known as carbon catabolite repression (CCR; Deutscher, 2008; Gorke & Stulke, 2008; Kim et al., 2010). The presence of glucose induces transcriptional repression of catabolic genes for secondary sugars due to the lack of functional global transcriptional regulator

cyclic-AMP receptor protein (CRP) caused by the low level of cyclic adenosine monophosphate (cAMP), the required ligand for CRP function. In the absence of glucose, the increased amount of cyclic-AMP will activate CRP, and both functional CRP and transcriptional regulators specific for secondary sugars, such as XylR (activated when bound by xylose), will coactivate catabolic operons for secondary sugars (Figure 1a; Deutscher, 2008; Gorke & Stulke, 2008).

As an important component of CCR, the phosphoenolpyruvate: sugar phosphotransferase system (PTS) is the main system in *E. coli* for glucose uptake and phosphorylation (Figure 1a; Escalante, Salinas Cervantes, Gosset, & Bolivar, 2012; Kim et al., 2010; Postma, Lengeler, & Jacobson, 1993). Disruption of the PTS system (deletion of *ptsG* or *ptsI*) is an effective approach to release CCR, but glucose uptake is significantly impaired (Gosset, 2005; Hernandez-Montalvo, Valle, Bolivar, & Gosset, 2001), which necessitates an alternative glucose transporter for optimal productivity (Hernandez-Montalvo et al., 2003; Lu et al., 2012; Zhang, Jantama, Moore et al., 2009). However, even with disrupted CCR, xylose utilization in these strains remain inefficient, limiting their usage as biocatalysts for lignocellulose conversion. For instance, a PTS deficient *E. coli* KJ122 has previously been engineered to produce succinate from glucose with high production metrics (a titer of 83 g/L, yield of 0.92 g/g, and productivity of 0.88 $g \cdot L^{-1} \cdot h^{-1}$; Jantama, Zhang et al., 2008), but the fermentative growth of this strain on xylose is very slow, accompanied with much lower production metrics (Wang et al., 2013). Adaptation of KJ122 for using xylose was previously observed, and two evolved strains XW055 and AS1600a were isolated (Sawisit et al., 2015; Wang et al., 2013). XW055 was isolated after approximately 40 generations, but the mechanism improving xylose fermentation remains unknown (Wang et al., 2013). For AS1600a, the causative mutation was identified as a point mutation in the coding region of galactose permease gene, *galP* (G236D).

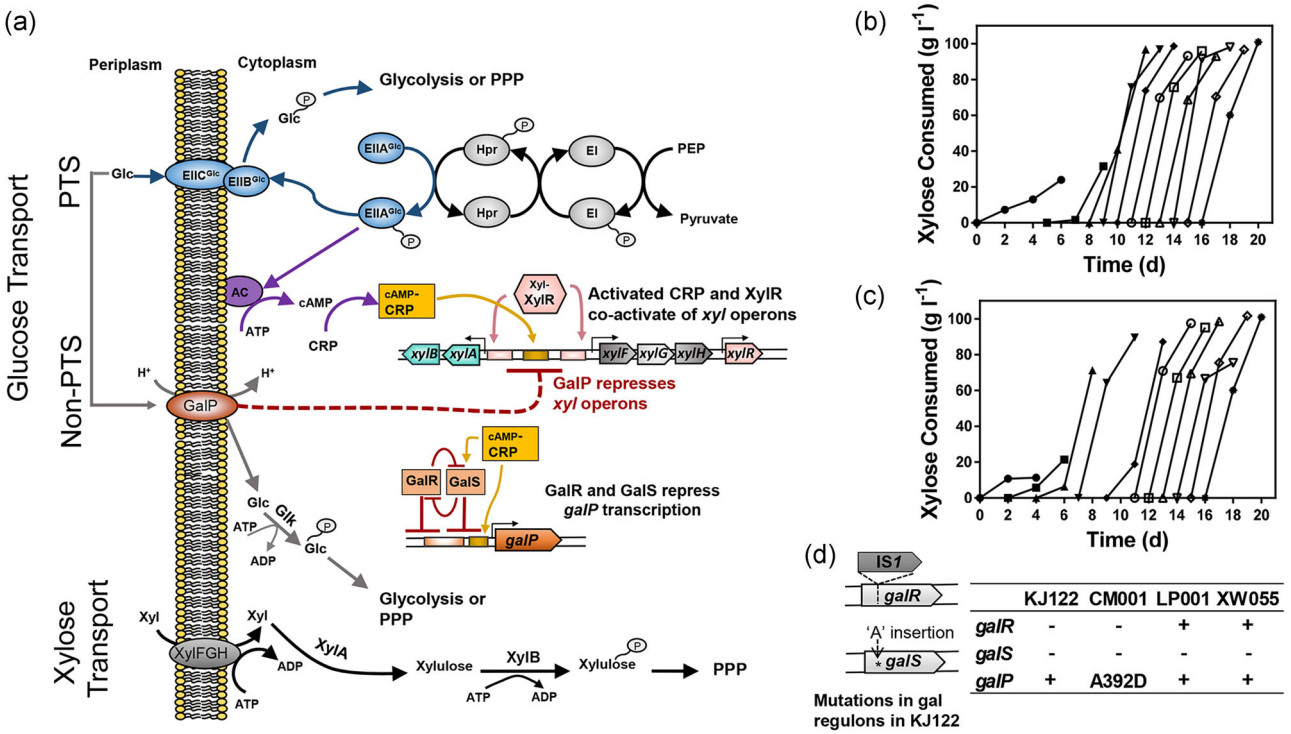

FIGURE 1 Regulatory and catabolic pathways for xylose metabolism and adaptive laboratory evolution of KJ122 to improve xylose fermentation. (a) In *Escherichia coli*, there are two main mechanisms for glucose transport including PTS and non-PTS transporters. EII^{Glc}-based PTS is the predominant mechanism for glucose transport, including components $EIIBC^{Glc}$, $EIIA^{Glc}$, Hpr, and EI (encoded by *ptsG*, *crr*, *ptsH*, and *ptsI*, respectively). The phosphoryl group of PEP is eventually transferred to glucose via these proteins. In the presence of abundant glucose, cAMP biosynthesis catalyzed by AC is inhibited by highly active PTS, leading to low levels of cAMP and nonfunctional CRP. Both activated CRP and XylR (activated when bound by xylose) are required to activate the xylose catabolic operons, *xylAB* and *xylFGH*. Phosphorylated sugar intermediates from glucose and xylose catabolism enter glycolysis or PPP pathways for full degradation. GalP can function as an alternative non-PTS glucose transporter, and this study indicated the presence of a novel GalP-induced transcriptional repression for xylose catabolism (dotted line). The *galP* expression is repressed by both GalR and GalS, which are isorepressors recognizing the same binding site. GalR and GalS also repress the expression for each other. Functional CRP activates the transcription of *galP* and *galS*. The given size of genes does not reflect real proportions of sequence length. (b) CM001 and (c) LP001 were isolated from the evolved cultures by serial transfers of cultures in AM1 medium supplemented with 10% xylose (w/v). Xylose consumption was determined for each transfer during fermentation. (d) In KJ122, *galR* was disrupted by an IS*1* element inserted at position 261 in the *galR* ORF, whereas *galS* was inactivated by an adenine insertion at position 231 in the *galS* ORF. Primary convergent mutations altering GalP levels or activities were characterized using genome sequencing for CM001, LP001, and XW055, a previously evolved strain (Wang et al., 2013). AC: adenylate cyclase; cAMP: cyclic adenosine monophosphate; CRP: cyclic-AMP receptor protein; Glc: glucose; PEP: phosphoenolpyruvate; PPP: the pentose phosphate pathway; PTS: phosphotransferase system; Xyl: xylose [Color figure can be viewed at wileyonlinelibrary.com]

However, the working mechanism remains elusive. It seems plausible that there are other inhibitory mechanisms repressing xylose utilization in KJ122 besides CCR.

In this study we discovered that GalP represses xylose utilization in *E. coli* through comprehensive characterization of the convergent genetic changes shared by multiple independently evolved strains. The GalP-induced repression is achieved by transcriptionally down-regulating many catabolic genes for secondary sugars and inactivation of GalP in *E. coli* enhances xylose fermentation. Our results suggest that using GalP as a substitute glucose transporter is undesired for conversion of lignocellulosic sugar mixtures due to its repressive role for the catabolism of secondary sugars.

2 | MATERIALS AND METHODS

2.1 | Strains, plasmids, and cultivation conditions

All strains and plasmids used in this study are listed in Table 1. The plasmid encoding *galP* was constructed by assembling a fragment containing the native ribosomal binding site, coding region, and terminator of *galP* with the backbone of pTrc99A at the multiple cloning site using the circular polymerase extension cloning method (Quan & Tian, 2011). Primers for plasmid construction are listed in Table S1. The constructed plasmid was verified using Sanger sequencing. For strain construction, all manipulations were done in Luria broth (LB;10 g/L difco tryptone, 5 g/L yeast extract, and 5 g/L NaCl) at 30°C, 37°C, or 39°C as needed with rotation at 180 rpm when in liquid culture. During genetic manipulations, 5% arabinose (w/v) was added to induce λ-red recombinase expression. 100 mg/L ampicillin and 50 mg/L chloramphenicol were supplemented as needed.

2.2 | Genetic methods

Deletion of genes was performed using a λ-red recombinase-mediated two-step method as previously described (Datsenko & Wanner, 2000; Jantama, Zhang et al., 2008; Sievert et al., 2017). Briefly, strains transformed with pKD46 were grown in LB media

TABLE 1 Strains and plasmids used in this study

Strain/ plasmid	Relevant characteristics	References
Strains		
KJ122	ATCC 8739, *pck*[a], *ptsI*[b], Δ*ldhA*, Δ*adhE*, Δ*ackA*, Δ(*focA-pflB*) Δ*mgsA*, Δ*poxB*, Δ*tdcDE*, Δ*citF*, Δ*aspC*, Δ*sfcA*	Jantama, Zhang et al., 2008
AG055	KJ122, Δ*galP*	This study
XW055	KJ122 isolate adapted for approximately 40 generations in 10% (w/v) xylose	Wang et al., 2013
CM001	KJ122 isolate adapted for approximately 60 generations in 10% (w/v) xylose	This study
LP001	KJ122 isolate adapted for approximately 60 generations in 10% (w/v) xylose	This study
XZ721	ATCC 8739, *pck*[a], ΔptsI, Δ*pflB*	Zhang, Jantama, Shanmugam et al., 2009
XW01	XZ721, Δ*ldhA*	This study
LP03	XW01, Δ*galR*	This study
LP05	XW01, Δ*galS*	This study
LP07	XW01, ΔgalR, Δ*galS*	This study
ATCC 9637	Wild-type *Escherichia coli* W	ATCC
GK501	ATCC 9637, Δ*galP*	This study
LY180	*frdBC*::(*Zm frg celY*$_{Ec}$) Δ*ldhA*::(*Zm frg casAB*$_{Ko}$) *adhE*::(*Zm frg estZ*$_{Pp}$ *FRT*) Δ*ackA*::FRT *rrlE*::(*pdc adhA adhB* FRT) Δ*mgsA*::FRT; evolved for ethanol production using xylose	Miller et al., 2009
TG114	Δ*pflB*, *frdBC*::FRT, *adhE*::FRT, *ackA*::FRT, *mgsA*::FRT; evolved for D-lactate production using glucose	Grabar et al., 2006
Top10F'	F [´*lacIq*, Tn10(TetR)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX*74 *recA*1 *ara*D139 Δ (*ara leu*) 7697 *galU galK rpsL* (StrR) *endA1 nupG*	Invitrogen™
Plasmids		
pKD46	*bla*, *γ β exo* (red recombinase)	Datsenko & Wanner, 2000
pXW1	The *cat-sacB* cassette with the *sacB* native terminator cloned into a modified vector pLOI4162	Sievert et al., 2017
pTrc99A	P$_{trc}$, *bla*, *lacI*^{q}	Lab collection
pGalP	*galP* in pTrc99A	This study

[a]*pck** stands for a mutated form of *pck* (G to A at position −64 relative to the ATG start codon).
[b]*ptsI** stands for a mutated form of *ptsI* (single base deletion at position 1,673 causing a frameshift mutation in the carboxyl-terminal region).

supplemented with 5% arabinose (w/v) to an $OD_{550\,nm}$ at approximately 0.3 before being washed with ice-cold water and electroporated with a linear DNA fragment containing homology to the region of interest. The DNA fragment used in the first-step integration was made by amplifying the *cat-sacB* cassette from pXW1 (Sievert et al., 2017) with 50 bp of homology to the flanking regions of target gene. The second-step integration was performed using linear fragments generated using fusion PCR (Shevchuk et al., 2004) with 500 bp homology at each end. Chloramphenicol resistance and colony PCR were used to select the successful clones for the first-step integration. For the second-step integration, counter selection was performed in media supplemented with 10% (w/v) sucrose. Gene deletions were verified using colony PCR and Sanger sequencing if needed. Primers for generation of all cassettes are listed in Table S1.

2.3 | Fermentation

Preinoculum for fermentation was prepared by transferring cells freshly grown on AM1 mineral salts agar plates (Martinez et al., 2007) supplemented with 2% glucose (w/v) into a 250 ml flask containing 100 ml AM1 media supplemented with the appropriate sugar (2% w/v) and incubating for approximately 18 hr (37°C, 120 rpm). All batch fermentations were performed using AM1 mineral salts media supplemented with the appropriate concentrations of sugars in fermentation vessels with 300 ml working volume as previously described (Jantama, Zhang et al., 2008). 100 mM potassium bicarbonate was included in the fermentation medium for succinate fermentative production (Jantama, Zhang et al., 2008). Co-sugar fermentation was performed using 5% glucose and 5% xylose (w/v). An initial inoculum of 0.022 g cell dry weight (CDW) L^{-1} (0.05 as $OD_{550\,nm}$) was used for all fermentation tests (Sievert et al., 2017). A total of 100 mg/L ampicillin and 10 μM Isopropyl β-D-1-thiogalactopyranoside (IPTG) were included for fermentations of the strains containing plasmids. All fermentations were performed at 37°C and pH was controlled at 7.0 by automatic addition of base solutions (2.4 M potassium carbonate and 1.2 M potassium hydroxide, 2.0 M, and 6.0 M potassium hydroxide for succinate, ethanol, and lactate production, respectively) as previously described (Grabar, Zhou, Shanmugam, Yomano, & Ingram, 2006; Jantama, Zhang et al., 2008; Miller et al., 2009; Zhang, Jantama, Shanmugam, & Ingram, 2009). Experimental data represent average values of at least three measurements with standard deviations.

2.4 | Adaptive laboratory evolution

The strain KJ122 was consecutively transferred 10 times (approximately 60 generations) during the exponential growth phase or early stationary phase into new fermentation vessels with fresh AM1 medium (pH 7.0) supplemented with 10% xylose (w/v) and 100 mM potassium bicarbonate. An initial inoculum of 0.022 g CDW L^{-1} was used for all transfers. This was performed for two independent evolutionary trajectories until xylose utilization became stable (10 transfers for each trajectory). The final population was preserved in a cryogenic tube (−80°C) and individual isolates were refermented to confirm stability of the phenotype. CM001 and LP001 were selected from each evolved population for further investigation along with another previously evolved strain XW055 (Wang et al., 2013).

2.5 | Genome sequencing and variant calling

Genomic DNA was extracted from KJ122 and all evolved strains (CM001, LP001, and XW055) using the Promega Wizard genomic DNA purification kit (Madison, WI) according to manufacturer instructions. Purified DNA was fragmented to an average size of 500 bp and libraries were generated with a TruSeq DNA sample preparation kit (Illumina®; San Diego, CA). Sample preparation and paired-end sequencing was performed with technical duplicates using an Illumina MiSeq (2 × 300 bp) by the DNASU Sequencing Core at Arizona State University. Reads were trimmed with Trim Galore (https://github.com/FelixKrueger/TrimGalore), and aligned to *E. coli* ATCC 8739 reference genome using BWA (Li & Durbin, 2009). Sequencing duplicates were removed with Picard (https://github.com/broadinstitute/picard), and variants were called using GATK HaplotypeCaller (Van der Auwera et al., 2013). Duplications, deletions, and other sequence junctions were called using CNVnator (Abyzov, Urban, Snyder, & Gerstein, 2011) and breseq (Deatherage & Barrick, 2014).

2.6 | Transcriptomic profiling by RNA sequencing

Strains KJ122 and AG055 were grown to an $OD_{550\,nm}$ at approximately 0.7 under 10% xylose (w/v) fermentation conditions and had total RNA extracted using a Qiagen RNAeasy kit (Germantown, MD) according to the manufacturer instructions. Two pools (two biological replicates for each pool) of total RNA for each strain were prepared by combining equal amounts of RNA for each replicate. Samples were depleted of ribosomal RNA using a RiboZero kit (Illumina). Random hexamer priming was used to generate cDNA and libraries were prepared using a Nextera library prep kit (Illumina). Sample preparation and sequencing using an Illumina NextSeq (2 × 150 bp) was performed by the DNASU Sequencing Core at Arizona State University. Reads were trimmed with Trim Galore, aligned to *E. coli* ATCC 8739 reference using STAR (Dobin et al., 2013) and had differential gene expression analysis performed using edgeR (Robinson, McCarthy, & Smyth, 2010).

2.7 | Analyses

Sugars, ethanol, and organic acids in fermentation broth were measured by high-performance liquid chromatography, using an Aminex® HPX-87H column (Bio-Rad; Hercules, CA) and 4 mM sulfuric acid as the mobile phase as previously described (Sievert et al., 2017). Cell dry weight was calculated from the measured optical density at 550 nm using a Beckman DU730 spectrophotometer (Beckman Coulter; Brea, CA). A total of 0.44 g CDW L^{-1} per $OD_{550\,nm}$ was experimentally

obtained for fermentation cultures using standard cell dry weight measurement (Glazyrina et al., 2010).

3 | RESULTS

3.1 | Quick adaptation for xylose utilization in a succinate producing *E. coli* biocatalyst

To gain insights of the potential mechanism inhibiting xylose fermentation in KJ122, we hypothesized that characterization of independently evolved strains with improved xylose utilization would reveal the convergent causative genetic changes that mitigate this inhibition mechanism. To obtain more evolved strains derived from KJ122 facilitating the characterization of convergent genetic changes, we repeated the evolution of KJ122 in two independent experiments for enhanced xylose catabolism as previously described (Wang et al., 2013; Figure 1b,c). In both evolutionary trajectories, a rapid adaptation occurred even at the second or third transfer that simultaneously increased xylose catabolism and cell growth (Figure 1b,c; Figure S1). From these two evolved populations (approximately 60 generations), strains LP001 and CM001 were isolated and confirmed with increased xylose fermentation capabilities (approximately 15-fold increase in succinate titers relative to KJ122; Table 2).

3.2 | Identification and characterization of convergent genetic changes occurring at the galactose regulon in the ancestor and evolved strains

The genomic DNA of the ancestor KJ122 as well as three evolved strains LP001, CM001, and XW055 was extracted and sequenced using Illumina paired-end sequencing. Interestingly, all three evolved strains have genetic differences in the *gal* regulons including *galR*, *galS*, and *galP*, compared with the ancestor KJ122 (Figure 1d), suggesting a result of convergent evolution to relieve the inhibitory mechanism for xylose catabolism in the ancestor strain. We hypothesized that these genetic changes in *gal* regulon are responsible for the increased xylose fermentation.

KJ122 has an IS*1* insertion sequence at position 261 in the *galR* ORF and an adenine insertion at position 231 in the *galS* ORF causing a frameshift mutation, which likely inactivate both proteins (Figure 1d). Both GalR and GalS are known to be repressors of genes needed for the transport and utilization of galactose, including *galP*, *galETKM*, and *mglBAC* (Weickert & Adhya, 1993; Figure 1a). It was reported that expression of *galP* was increased to compensate for the PTS deficiency in the precursor strain of KJ122 due to adaptive evolution for increased glucose to succinate bioconversion. However, the exact genetic mechanism remains uncharacterized (Zhang, Jantama, Moore et al., 2009). We hypothesized that these two mutations are responsible for enhanced *galP* expression that leads to higher glucose–succinate conversion. To test this, we reconstructed a succinate producing *E. coli* XW01 derived from wild-type *E. coli* ATCC 8739 with only four defined mutations (Δ*ptsI* Δ*pflB pck*::*pck** Δ*ldhA*; Zhang, Jantama, Shanmugam et al., 2009) and then tested effect of the deletion of *galR*, *galS*, or both on succinate production. In the strain XW01, *pflB*, and *ldhA* were deleted to eliminate competing fermentation pathways for ethanol and D-lactate, respectively. The *ptsI* gene was deleted to disrupt PTS for increased levels of phosphoenolpyruvate (PEP), a precursor for succinate (Zhang, Jantama, Shanmugam et al., 2009). In addition, *pck* expression was enhanced with an upstream mutation (*pck*::*pck**; G to A at position −64 relative to the ATG start codon) to increase adenosine triphosphate (ATP) yield (Zhang, Jantama, Moore et al., 2009). Fermentation of XW01 in glucose resulted in succinate titers of 28 g/L at 96 hr and 62 g/L at 144 hr with 34 and 65 g/L glucose consumed, respectively (Figure 2a,b). Single deletion of *galR* decreased the succinate titers to 24 g/L at 96 hr and 41 g/L at 144 hr. Similarly, deletion of *galS* lowered the succinate titers to 9.3 g/L at 96 hr and 21 g/L at 144 hr (Figure 2b). Interestingly, deletion of both *galR* and *galS* enhanced succinate production, increasing the titer from 28 to 46 g/L after 96 hr and from 62 to 71 g/L after 144 hr (Figure 2b). Overall productivity, specific growth rate, and titer were all increased when both *galR* and *galS* were deleted, but all decreased by inactivation of either *galR* or *galS* (Figure 2). This result supports that inactivation of both *galR* and *galS* is the causative mechanism leading to increased *galP* expression in KJ122. For the evolved strains, both XW055 and LP001 reverted *galR* back to the wild-type sequence during adaptive evolution while CM001 gained a nonsynonymous mutation in *galP* (A392D; Figure 1d). Given the results of single deletions of *galR* and *galS* regarding their effect on succinate production (Figure 2), the restored *galR* in LP001 and XW055 will potentially decrease *galP* levels, which is supported by the defective performance of glucose fermentation (Table 2). Similarly, CM001 showed decreased glucose utilization and succinate production, suggesting that this point mutation negatively influences GalP function (Table 2). It is plausible that decreased levels or activities of GalP are the underlying mechanism for enhanced xylose utilization by adaptive evolution.

3.3 | Confirmation of the inhibiting role of *galp* on xylose fermentation in succinate-producing *E. coli* biocatalysts

To test if GalP represses xylose fermentation and also to directly prove that the inactivation of GalP is the causative mechanism for xylose adaption in KJ122, we deleted *galP* in KJ122 and compared the resulting strain AG055 to KJ122, CM001, and LP001 in terms of their fermentation performance using mono-sugar and co-sugar conditions (Table 2). With xylose as the only sugar substrate, AG055 produced 70 g/L succinate after 96 hr, 14-fold that of KJ122 (Table 2). This was comparable to the titers of the evolved strains XW055, CM001, and LP001 in the same period of time, producing 76, 80, and 77 g/L, respectively (Table 2). This demonstrated that inactivation/repression of *galP* was primarily responsible for the observed phenotype in the evolved strain. With a mixture of glucose

TABLE 2 Succinate production metrics using different sugar sources in AM1 mineral salts medium for 96 hr fermentation

Strains	Carbon source	Biomass (g/L)	Succinate titer (g/L)	Yield (g/g)	Productivity ($g \cdot L^{-1} \cdot h^{-1}$)
KJ122	10% Glc	2.9 ± 0.3	87 ± 3	0.91 ± 0.02	0.91 ± 0.03
	10% Xyl	0.2 ± 0.1	5 ± 2	0.41 ± 0.15	0.05 ± 0.02
	Co-sugar[a]	2.2 ± 0.1	61 ± 3	0.86 ± 0.04	0.64 ± 0.03
AG055	10% Glc	2.9 ± 0.1	45 ± 1	0.93 ± 0.01	0.47 ± 0.01
	10% Xyl	2.0 ± 0.2	70 ± 5	0.90 ± 0.05	0.73 ± 0.05
	Co-sugar	1.9 ± 0.1	76 ± 3	0.94 ± 0.05	0.80 ± 0.03
CM001	10% Glc	2.5 ± 0.1	66 ± 2	0.93 ± 0.04	0.68 ± 0.02
	10% Xyl	2.3 ± 0.1	80 ± 3	0.85 ± 0.03	0.80 ± 0.01
XW055	10% Glc	3.2 ± 0.2	62 ± 3	0.81 ± 0.07	0.64 ± 0.05
	10% Xyl	2.2 ± 0.1	76 ± 1	0.91 ± 0.01	0.83 ± 0.03
LP001	10% Glc	2.3 ± 0.2	45 ± 5	1.07 ± 0.03	0.46 ± 0.02
	10% Xyl	2.5 ± 0.2	77 ± 6	0.93 ± 0.09	0.79 ± 0.04

Abbreviations: Glc: glucose; Xyl: xylose.
[a]Co-sugar indicates the fermentation of a glucose–xylose mixture (5% w/v for each sugar).

and xylose as sugar substrates, titer and productivity increased by approximately 25% in AG055 compared with KJ122 (Table 2). However, similar to evolved strains, when using glucose as the sole carbon source, AG055 had decreased succinate production (Table 2). This is consistent with the finding that GalP is a major glucose transporter in KJ122 which has a disrupted PTS (Zhang, Jantama, Moore et al., 2009). To directly test the repressive effect of *galP* on xylose fermentation, wild-type *galP* was cloned into a plasmid pTrc99A and *galP* expression was induced by 10 μM IPTG in AG055 to test the effect on xylose fermentation. Induced expression of *galP* resulted in a 3-day lag for AG055 before any succinate production and a threefold decrease in succinate titer after 96 hr (Figure 3).

3.4 | Transcriptomic changes caused by *galp* inactivation

To understand the potential transcriptomic changes upon *galP* inactivation, we isolated total RNA for both KJ122 and AG055 (KJ122 Δ*galP*) in early exponential growth during xylose fermentation and used RNA sequencing to quantify transcriptomic differences. There were 17 genes downregulated and 75 genes upregulated at least twofold in AG055 compared to KJ122 (Table 3 and complete raw data in Table S2). First, it is noticeable that many catabolic genes, especially for secondary sugars such as xylose, arabinose, ribose, and galactose are upregulated upon *galP* inactivation (Table 3). The xylose (*xylAB*, *xylFGH*, *xylE*, and *xylR*), arabinose (*araBAD*, *araFGH*, and *araC*), ribose (*rbsDACB*, *rbsK*, and *rbsR*), and galactose (*mglBAC* and *galETKM*) catabolic genes were upregulated to reach approximately 2.2-, 2.0-, 5.5-, and 2.4-fold on average estimated by relative ratio of transcripts per million (TPM; Figure 4 and Table S3). Second, many transcriptional changes facilitating succinate fermentative production were observed including genes involved in the anaplerotic reaction, the reductive branch of tricarboxylic acid cycle (TCA) cycle, succinate transport and high-sugar osmotic stress tolerance (Figure 4). In particular, there was a 2.5-fold increase in the expression of *pck* in AG055 that encodes PEP

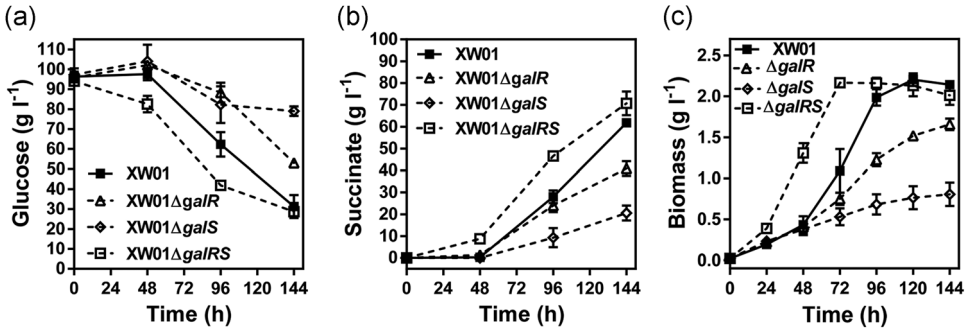

FIGURE 2 Inactivation of both *galR* and *galS* improves fermentative production of succinate from glucose. Fermentations of a rationally designed succinate producer XW01 (solid square) and its derivatives with inactivation of *galR* (open triangle), *galS* (open diamond), and *galRS* (open square) were performed in AM1 medium supplemented with 10% glucose (w/v). (a) Glucose, (b) succinate, and (c) biomass were measured at indicated time intervals

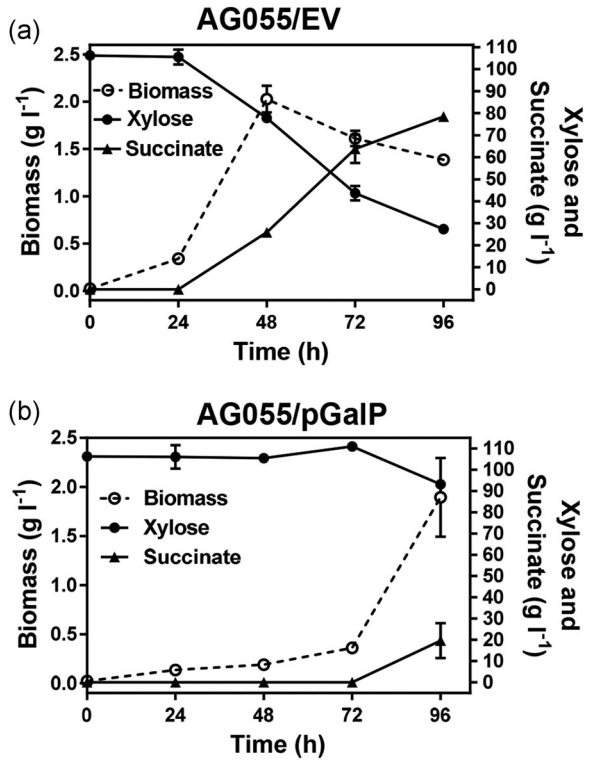

FIGURE 3 Effect of *galP* overexpression on xylose fermentation of AG055 (KJ122 Δ*galP*). Cells transformed with (a) an empty vector pTrc99A (EV), or (b) a plasmid encoding GalP were fermented in AM1 medium containing 10% (w/v) xylose and 10 μM IPTG

carboxykinase known to be crucial for conserving energy in succinate production (Zhang, Jantama, Moore et al., 2009), while the transcripts for competing carboxylation enzyme, *ppc*, were only 57% relative to the transcript levels of KJ122. This resulted in an increase in the ratio of TPM of *pck* over *ppc* from 1.5 to 8.5 in AG055. The transcripts of fumarate reductase, catalyzing the conversion of fumarate to succinate production (encoded by *frdABCD*), was 2.5-fold of those in KJ122 as well. These changes may benefit xylose to succinate fermentative conversion by enhancing xylose catabolism and carbon flux to succinate through the TCA cycle (Figure 4).

3.5 | GalP represses xylose catabolism in wild-type *E. coli* and other production strains

To test if the repressive effect of *galP* is a common scenario not only specific to succinate producers or its wild-type precursor *E. coli* ATCC8739, we deleted *galP* in wild-type *E. coli* W (ATCC 9637) and compared the resulting strain GK501 to wild-type strain for their xylose fermentation performance. Inactivation of *galP* enhanced the initial xylose consumption rate (0–24 hr) by 140% (Figure 5a). Cell growth of GK501 was also increased compared to wild-type with 66% more biomass at 24 hr (Figure 5a). As a complementary test, *galP* expression was induced from a plasmid in wild-type *E. coli* W to test the effect on xylose fermentation. Induced expression of *galP* resulted in much slower growth and xylose utilization with only approximately <10% biomass accumulated for 24 hr compared with the empty vector control (Figure 5b). To further test if GalP also represses xylose utilization in other *E. coli* production strains, *galP* expression was induced in an ethanol producer LY180 (Miller et al., 2009) and a D-lactate producer TG114 (Grabar et al., 2006) and the effect on fermentative production was investigated (Figure 6). Induced *galP* expression dramatically disrupted xylose fermentation and ethanol production in LY180 (Figure 6a,b), leading to essentially no cell growth for 96 hr (Figure 6c). Similarly, GalP-induced repression on xylose fermentation was observed in TG114 with approximately 48 hr growth delay upon IPTG induction (Figure 6f). For 96 hr fermentation, xylose utilization and lactate production were decreased by half compared to TG114 with empty vector (Figure 6d,e). These results collectively suggest that the repressive effect of *galP* is commonly present in *E. coli*.

4 | DISCUSSION

Characterization of microbial control mechanisms for sugar preference will help engineering biocatalysts for efficient lignocellulose conversion. In this study, we discovered a novel mechanism that represses xylose fermentation through galactose permease, GalP. The transcriptomic analysis suggests that the GalP-induced repression is likely due to the decreased expression of the genes important for secondary sugar catabolism.

Succinate, a C_4-dicarboxylic acid with a multibillion dollar market, can be used to make many commodity chemicals in plastics and solvents (McKinlay, Vieille, & Zeikus, 2007). Many efforts have been made to both isolate and engineer biocatalysts for succinate production with varying success (Ahn, Jang, & Lee, 2016). A series of efficient *E. coli* strains, such as KJ73 and KJ122, have been engineered using a combination of metabolic evolutions and chromosomal deletions of competing pathways, ultimately making succinate with high production metrics from glucose (Jantama, Haupt et al., 2008; Jantama, Zhang et al., 2008). In these strains, GalP was found to be more abundant (a 20-fold increase at transcriptional levels) compared with wild-type strain and serve as the dominant glucose transporter in evolved strains (Zhang, Jantama, Moore et al., 2009). However, the mutations increasing *galP* expression remain elusive until this study. Here we proved that inactivation of both *galR* and *galS* is required for increased GalP activity that serves an effective glucose uptake system in the PTS deficient background (Figures 1 and 2). Six defined chromosomal modifications (Δ*ptsI* Δ*pflB pck*::*pck** Δ*ldhA* Δ*galR* Δ*galS*) in wild-type *E. coli* ATCC8739 without any experimental adaptation yielded efficient succinate production with a titer at 71 g/L, a yield at 1.0 g/g, and a productivity at 0.50 $g \cdot L^{-1} \cdot h^{-1}$ for 6-day simple batch fermentations (Figure 2).

GalR and GalS are homologous dimeric isorepressors of the galactose regulons in *E. coli* (Geanacopoulos & Adhya, 1997). Genes encoding the ATP-dependent galactose transport system *mglBAC*, the Leloir pathway operon *galETKM*, and the galactose:proton symporter *galP* are repressed by GalR and GalS, and meanwhile activated by the

TABLE 3 Genes with transcriptional changes more than twofold in AG055 relative to KJ122 when fermenting xylose[a]

Functional categories	Downregulated genes			Upregulated genes			
Carbon metabolic genes							
				pck	*ygcE*	*ydjI*	
	maa	*dmlA*		*lpxL*	*agp*	*sucD*	*ykgEFG*
	glgC			*frdA,B,D*	*aspA*	*rbsK*	*rbsD*
Regulator for carbon utilization							
				cstA	*araC*	*rbsR*	
Sugar and acid transporters							
				xylH	*yqcE*	*glpT*	*yedE*
				rbsABC	*mglBC*	*btsT*	*dcuA*
Amino acid metabolism							
	nepI	*lysA*		*ilvN*	*mtr*	*ansB*	pepE
	asd			*fucO*	*trpE*	*oppB*	
Regulatory function unrelated to carbon metabolism							
	ada	*yihI*		*yrbL*	*ygiM*	*nuoHM*	*rpoE*
				ychH	*caiF*		
Other unrelated functions							
	mutM	*truC*		*proVWX* (betaine uptake)			
	yqcC	*alkB*	*alkA*	*ssuABCDE pspE*			
	phoE (porins)			(sulfonate-sulfur utilization)			
				abgA,ybcF, ylbF, fdrA, allCD			
				(allantoin/nitrogen catabolism)			
				ompW, ompF (porins)			*uspF*
				aslA	*cutC*	*pstA*	*ydeM*
Unclassified							
	ydjO	*yigI*		*ucpA*	*PreAT*	*ydeP*	*ybhG*
	gspG			*ybhF*	*ybhR*	*ybhQ*	*ylbE*
				ybaE			

[a]Shown genes are sorted by functional categories using EcoCyc with at least twofold changes in counts per million and false discovery rates lower than 0.1.

presence of CRP bound with cAMP (Semsey, Krishna, Sneppen, & Adhya, 2007; Weickert & Adhya, 1993). GalR and GalS have overlapping specificity for the operator sequences present in these *gal* regulons, and the repression caused by GalR and GalS can occur independently (Geanacopoulos & Adhya, 1997; Weickert & Adhya, 1993). Our data showed that succinate production was decreased in the single mutant of Δ*galR* or Δ*galS* (Figure 2b). One possible reason is that *galR* and *galS* can act as transcriptional repressors for each other in the presence of functional CRP (Semsey et al., 2007). In a *galR* mutant, the *galS* transcripts will increase, and vice versa. Increased repressor levels may lead to a lower expression of *galP* and thus lower fermentative production metrics. Alternatively, it has been shown that GalR and GalS can form heterodimers (El Qaidi, Allemand, Oberto, & Plumbridge, 2009). It is possible that homodimer and heterodimer of these two isorepressors have differential affinities to operator sequences and repression activity. Deletion of either *galR* or *galS* will prevent formation of GalR-GalS heterodimers, potentially altering the repression effect on *galP* expression. Only in the absence of both GalR and GalS, is repression of *galP* dramatically released, thus leading to increased succinate production (Figure 2). Our data suggest the presence of either GalR or GalS was sufficient to repress *galP* at some degree, which agrees with the evolved mechanism to restore only one copy of repressors, *galR*, in LP001 and XW055 (Figure 1d). The restored *galR* is due to the removal of IS*1* element which was strongly selected for higher fitness during xylose adaptation. In general, the high mobility of IS elements increases genome dynamics and cause genome rearrangements, which could be beneficial for cells to adapt to new environments (Schneider & Lenski, 2004).

A GalP mutation (G236D) was previously identified using a similar approach, but the exact working mechanism is unclear (Sawisit et al., 2015). Although it is possible that *galP* G239D is a

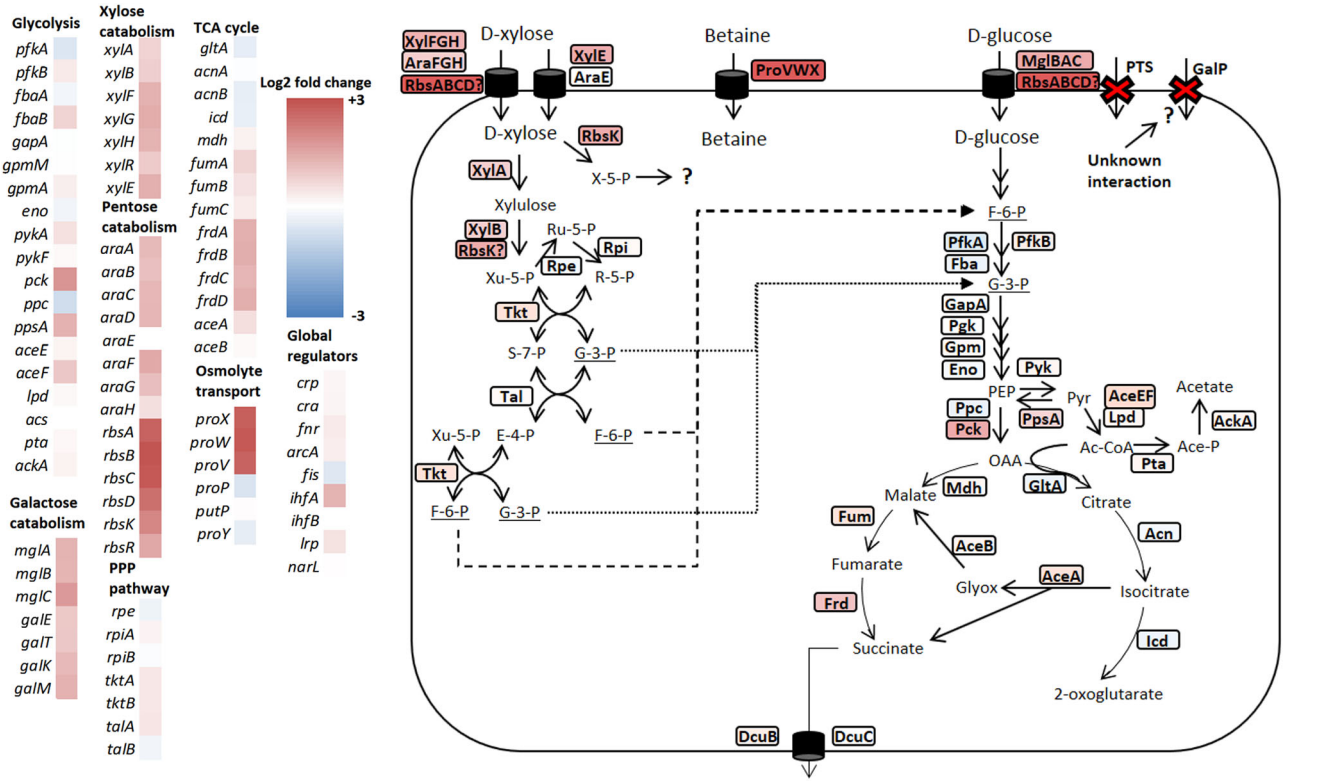

FIGURE 4 Deletion of *galP* leads to differential expression of the pathways relevant to succinate production. The genome-wide expression of AG055 is compared to that of KJ122 and relative fold change is expressed using a Log_2 scale with different colors. Ac-CoA: acetyl-CoA; Ace-P: acetyl phosphate; E-4-P: erythrose-4-phosphate; F-6-P: β-D-fructose-6-phosphate; G-3-P: D-glyceraldehyde-3-phosphate; OAA: oxaloacetate; PEP: phosphoenolpyruvate; PTS: phosphotransferase system; Pyr: pyruvate; R-5-P: ribose-5-phosphate; Ru-5-P: ribulose-5-phosphate; S-7-P: sedoheptulose-7-phosphate; Suc-CoA: succinyl-CoA; X-5-P: xylose-5-phosphate; Xu-5-P: xylulose-5-phosphate [Color figure can be viewed at wileyonlinelibrary.com]

"gain of function" mutation making GalP an efficient xylose transporter, the strain with a complete *galP* deletion in the same background (KJ122) showed very similar fermentation performance compared with the strain with *galP* (G236D) mutation (Table 2; Sawisit et al., 2015). In addition, the glucose transport function of GalP (G236D) is also disrupted (Sawisit et al., 2015), which is similar to the strain with the *galP* deletion and all other three evolved strains (Table 2). In this study, characterization of the convergent genetic basis for three independently evolved strains from the same ancestor KJ122 suggests that the "loss-of-function" of *galP* is the convergent mechanism for this quick xylose adaptation. Similarly, the GalP mutation (G236D) is likely also a "loss-of-function" mutation that disrupts GalP normal function. This study demonstrates that investigating multiple parallel evolutionary trajectories is an effective method to understand underlying molecular mechanisms for improved phenotypes.

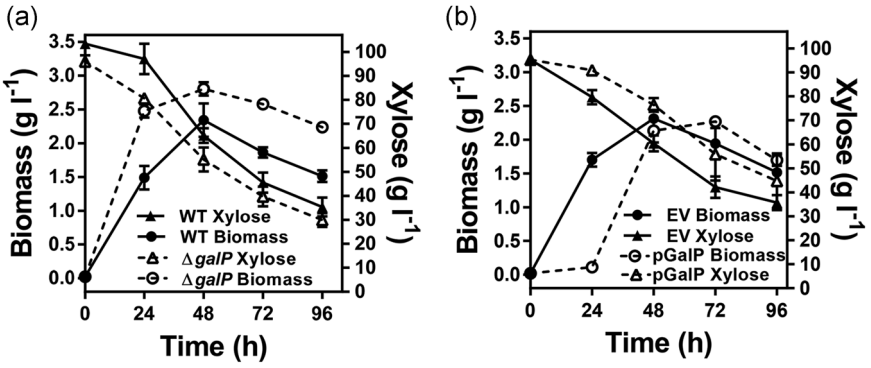

FIGURE 5 GalP represses xylose catabolism in wild-type *Escherichia coli*. Effect of *galP* in wild-type background on xylose fermentation was measured using AM1 medium supplemented with 10% xylose (w/v). (a) Wild-type *E. coli* W (solid lines) and GK501 (*E. coli* W Δ*galP*; dotted lines). (b) *E. coli* W with empty vector pTrc99A (solid lines) and with a plasmid encoding GalP (dotted line). A total of 10 μM IPTG was used to induce *galP* expression

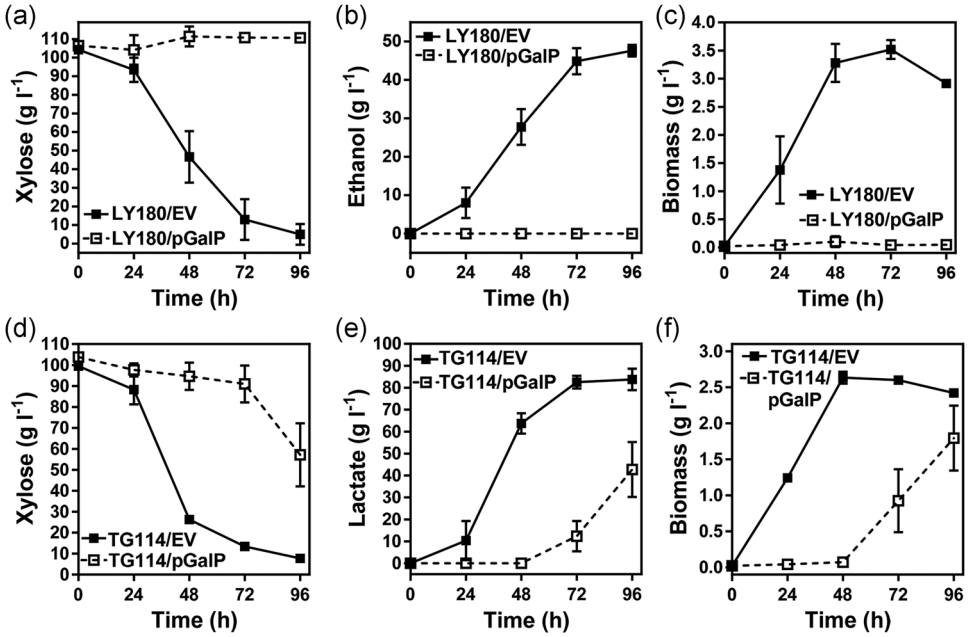

FIGURE 6 GalP represses xylose fermentation in *Escherichia coli* production strains. The effect of *galP* on xylose fermentation was tested in LY180 and TG114, *E. coli* strains previously engineered for ethanol and D-lactate production, respectively. (a) Xylose, (b) ethanol, and (c) biomass of LY180 with empty vector (solid square) or a plasmid encoding GalP (open square) were measured for fermentation cultures using AM1 medium supplemented with 10% xylose (w/v). (d) Xylose, (e) lactate, and (f) biomass of TG114 with empty vector (solid square) or a plasmid encoding GalP (open square) were similarly measured at indicated time intervals. A total of 10 μM IPTG was used to induce *galP* expression

Besides CCR occurring at the transcriptional level, the activities of some sugar transporters can be biochemically regulated through protein–protein interactions to achieve the control of sugar utilization preference (Dean, Reizer, Nikaido, & Saier, 1990; Deutscher, 2008; Osumi & Saier, 1982; Stulke & Hillen, 1999). Different from this type of biochemical regulation, the *galP*-induced repression seems to modulate global transcriptional regulation of many catabolic genes for secondary sugars as characterized by RNA sequencing (Figure 4). There are at least two possible mechanisms. First, GalP may directly participate in signaling pathways to transcriptionally downregulate catabolic genes for secondary sugars. A C4-dicarboxylate/succinate antiporter in *E. coli*, DcuB, is such an example which directly interacts with the two-component system (DcuS-DcuR in DcuB-induced regulation) for C4-dicarboxylate metabolism (Kleefeld, Ackermann, Bauer, Kramer, & Unden, 2009). Second, GalP may indirectly influence transcriptional regulation by blocking or sequestering a global transcriptional regulator for sugar catabolism. There are a few examples of bacterial transporters sequestering transcriptional regulators to alter expression levels of target genes (Coutts, Thomas, Blakey, & Merrick, 2002; Lee, Boos, Bouche, & Plumbridge, 2000; Lopian, Nussbaum-Shochat, O'Day-Kerstein, Wright, & Amster-Choder, 2003; Richet, Davidson, & Joly, 2012; Tanaka, Kimata, & Aiba, 2000). Transcriptional regulation by sequestration of transcription factors has previously been observed in sugar transporters from PTS (Tanaka et al., 2000) and ATP-binding cassette (ABC) family (Richet et al., 2012), although it has never been demonstrated to our knowledge in GalP or any other transporter from the major facilitator superfamily.

Transcriptomic data also suggest that there are beneficial changes for succinate production from xylose when *galP* is deleted. First, expression differences were observed in *ppc* and *pck* which encode two anaplerotic enzymes catalyzing carboxylation of PEP to form oxaloacetate (Figure 4). Compared with Ppc, Pck was found to conserve energy (generating one net ATP) and increased *pck* expression enhanced succinate production in *E. coli* (Zhang, Jantama, Moore et al., 2009; Zhang, Jantama, Shanmugam et al., 2009). The relative ratio of *pck* to *ppc* transcripts between KJ122 and its *galP* deletion mutant was changed from 1.5:1 to 8.5:1, presumably conserving more energy during the conversion of PEP to oxaloacetate. Modulating the relative abundance of Pck and Ppc has been shown to enhance glucose to succinate conversion (Tan, Zhu, Chen, Li, & Zhang, 2013). Similarly, overexpression of *pck* in combination with a *ppc* inactivation was needed to effectively convert xylose to succinate (Liu et al., 2012; Singh, Cher Soh, Hatzimanikatis, & Gill, 2011). The net energy gained from utilization of xylose is less than that of glucose (Gonzalez, Long, & Antoniewicz, 2017). Thus, the distribution of metabolic flux between these two enzymes to maximize energy conservation could be important for xylose to succinate conversion. The need for energy conservation during xylose to succinate conversion is further supported by a recent finding that inactivation of *xylFGH* (an energy intensive xylose transporter) enhances xylose to succinate conversion (Khunnonkwao, Jantama, Kanchanatawee, & Jantama, 2018). Second, the

expression of fumarase and fumarate reductase genes was increased upon *galP* inactivation, which would enhance carbon flux in the reductive branch of TCA cycle. Third, upregulation of a betaine ABC transporter, *proVWX*, was also observed upon *galP* inactivation. Osmotic stress is present in fermentation with 10% (w/v) sugar and previous evidence has shown that addition of the osmolyte betaine to AM1 mineral salts media can significantly increase production metrics (Zhou, Grabar, Shanmugam, & Ingram, 2006). Thus, enhanced import of betaine could also play a role in enhancing productivity in AM1 mineral salts medium during succinate production by reducing osmotic stress.

Inactivation of the PTS has been widely used as an engineering strategy in bacteria to release the control of CCR and increase metabolic flux downstream of PEP for a variety of products, such as C_4-dicarboxylates using the reductive branch of the TCA cycle (Zhang, Jantama, Moore et al., 2009; Zhang, Jantama, Shanmugam et al., 2009), and aromatic compounds derived from the shikimate pathway (Flores, Xiao, Berry, Bolivar, & Valle, 1996; Yi, Draths, Li, & Frost, 2003). To compensate for the deficiency in glucose uptake caused by PTS inactivation, GalP has been often used as an alternative glucose uptake system in PTS deficient strains (Hernandez-Montalvo et al., 2003; Lu et al., 2012; McDonald, Walmsley, & Henderson, 1997; Yi et al., 2003). However, these strains are not tested for xylose or glucose–xylose fermentation yet, and reported strains may be sub-optimally designed for conversion of sugar mixtures derived from lignocellulose because of the repressive effect of *galP*, especially under a *galP* overexpression scenario. Although *galP* inactivation can increase xylose and co-sugar utilization, glucose consumption remains low in these strains due to the defective PTS. Thus, further work may look into the use of heterologous glucose transporters presumably without repressive effect on secondary sugars, such as Glf from *Zymomonas mobilis*.

5 | CONCLUSIONS

We discovered that a commonly used substitute glucose transporter, GalP, represses the utilization of secondary sugars, such as xylose, in *E. coli* through a novel mechanism by transcriptionally downregulating many catabolic genes for secondary sugars. Therefore, using GalP as an alternative glucose transporter in PTS defective strains may hinder xylose conversion, an important consideration in engineering strains for bioconversion of lignocellulosic sugar mixtures to products that require the precursor PEP, such as C_4-dicarboxylates and aromatics derived from the shikimate pathway. Concomitantly, deletion of *galP* can be used as an effective strategy to enhance xylose fermentation in *E. coli*.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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