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Research Article

**Phosphatases and phosphate affect
the formation of glucose from pentoses
in *Escherichia coli***

Metabolically engineered *Escherichia coli* MEC143 with deletions of the *ptsG*, *manZ*, *glk*, *pfkA*, and *zwf* genes converts pentoses such as arabinose and xylose into glucose, with the dephosphorylation of glucose-6-phosphate serving as the final step. To determine which phosphatase mediates this conversion, we examined glucose formation from pentoses in strains containing knockouts of six different phosphatases singly and in combination. Deletions of single phosphatases and combinations of multiple phosphatases did not eliminate the accumulation of glucose from xylose or arabinose. Overexpression of one phosphatase, haloacid dehalogenase-like phosphatase 12 coded by the *ybiV* gene, increased glucose yield significantly from 0.26 to 0.30 g/g (xylose) and from 0.32 to 0.35 g/g (arabinose). Growing cells under phosphate-limited steady-state conditions increased the glucose yield to 0.39 g glucose/g xylose, but did not affect glucose yield from arabinose (0.31 g/g). No single phosphatase is exclusively responsible for the conversion of glucose-6-phosphate to glucose in *E. coli* MEC143. Phosphate-limited conditions are indeed able to enhance glucose formation in some cases, with this effect likely influenced by the different phosphate demands when *E. coli* metabolizes different carbon sources.

Keywords: Arabinose / Glucose / HAD phosphatases / Pentose phosphate pathway / Xylose

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1 Introduction

Metabolically engineered *Escherichia coli* MEC143 converts pentoses such as arabinose and xylose into glucose [1]. The transformation of five-carbon sugars to six-carbon glucose occurs because of the reversibility of transketolase and transaldolase, key enzymes of the pentose phosphate pathway (Fig. 1), which exchange monosaccharides of three to seven carbon length and ultimately lead to fructose-6-phosphate (fructose-6P) and glyceraldehyde-3P:

The quantity of ATP required for this conversion depends on the mechanism the cells use for pentose transport [1].

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Abbreviations: HAD12, haloacid dehalogenase-like phosphatase 12; LB, lysogeny broth

Escherichia coli MEC143 contains deletions in several genes involved in the initial catabolism of glucose (phosphotransferase enzymes capable of glucose uptake, *ptsG* and *manZ* genes, and glucokinase, *glk*), and in glucose-6P metabolism (6P-fructokinase I, *pfkA*, and glucose-6P 1-dehydrogenase, *zwf*). While the *ptsG manZ glk* gene deletions prevent consumption of glucose, the *pfkA zwf* knockouts prevent the reentry of fructose-6P formed within the pentose phosphate pathway (Eq. (1)) into central metabolism by the Embden–Meyerhof–Parnas and pentose phosphate pathways. After an equilibrium between fructose-6P and glucose-6P by phosphoglucose isomerase (*pgi* gene), the final step in the conversion of pentoses via fructose-6P to glucose is the dephosphorylation of glucose-6P [1]:

Previous studies with *agp* knockouts coding glucose-1-phosphatase demonstrated that glucose-6P, and not glucose-1P, is the primary phosphorylated source of glucose in *E. coli* *ptsG manZ glk pfkA zwf*, a result which is consistent with glucose-6P formation being favored in the equilibrium with glucose-1P mediated by phosphoglucomutase [1].

Dephosphorylation is mediated by phosphatases, enzymes that typically act on multiple substrates. In *E. coli*, for example,

Figure 1. Metabolic pathways from D-xylose or L-arabinose to glucose in five or six respective enzymatic steps. Key reversible enzymes include transketolase and transaldolase. Metabolite abbreviations shown are D-xylulose-5-phosphate (Xu5P), D-ribulose-5-phosphate (Ru5P), D-ribose-5-phosphate (R5P), D-sedoheptulose-7-phosphate (S7P), D-erythrose-4P (E4P), D-fructose-6-phosphate (F6P), and D-glucose-6-phosphate (G6P). As shown in the figure, in addition to knockouts in *ptsG*, *manZ*, and *glk*, all strains used in this study have gene deletions in glucose-6P 1-dehydrogenase (first enzyme mediating entrance of G6P into pentose phosphate pathway coded by *zwf* gene) and 6-phosphofructokinase (first enzyme mediating conversion of F6P to Gly3P coded by *pfkA* gene).

glucose-6P has been shown to be dephosphorylated by alkaline phosphatase [2] and by several haloacid dehalogenase-like phosphatases [3]. The specific enzyme responsible for the final step (Eq. (2)) in the conversion of pentoses to glucose is unknown.

Regardless of the enzyme responsible for the *in vivo* dephosphorylation of glucose-6P, Eq. (2) also indicates that phosphate is a by-product of glucose production. This chemical equation therefore suggests, by simple mass action, that phosphate-limited conditions should favor the forward reaction forming glucose. Moreover, phosphate limitation is well known to induce expression of phosphatases [4]. Although batch culture of organisms provides cells with excess phosphate and other nutrients during the majority of growth, phosphate-limited conditions can readily be accomplished by growing cells under steady-state conditions in a medium in which phosphate is always limiting and the carbon source is in excess. Similarly, during carbon-limited (with excess phosphate) steady-state growth of chemotrophic cells such as *E. coli* would use the largest possible fraction of the carbon source for energy, likely resulting in the least amount of glucose accumulation.

The objectives of this study were to determine which enzymes mediate the dephosphorylation of glucose-6P to glucose during the formation of glucose from pentoses. Additionally, we sought to determine if a greater glucose yield could be obtained from xylose or arabinose by growing cells under phosphate-limited conditions.

2 Materials and methods

2.1 Bacterial strains and construction

Several strains of *E. coli* were constructed from MEC149 (MG1655 *ptsG manZ glk pfkA zwf*) as listed in Table 1. These strains were constructed by transduction of MEC149 and the corresponding Keio (FRT)Kan deletions strains using P1 bacteriophage [5], and if necessary, curing the Kan(R) using the pCP20 plasmid, which contains a temperature-inducible FLP recombinase as well as a temperature-sensitive replicon [6, 7]. PCRs were conducted to confirm each strain.

The gene *ybiV* coding the haloacid dehalogenase-like phosphatase 12 (HAD12) in *E. coli* [3, 8] was PCR amplified by the gene-specific primers 5'-GGGAAAGGTA CCATGAGCGTAAAAGTTATCGTCACAG-3' (forward) and 5'-GGGAAATCTAGATCAGCTGTTAAAAGGGGATGTG-3' (reverse) using genomic DNA of wild-type MG1655 as template. The PCR product (794 bp) was purified and digested with KpnI and XbaI and ligated into the expression vector pZE12, which was also digested with same endonuclease enzymes. This plasmid-gene cassette pZE12-*ybiV* was transformed into MEC143 resulting in *E. coli* MEC143/pZE12-*ybiV*.

2.2 Growth medium and culture conditions

The defined medium used for the shake flask experiments contained (per liter): 1.70 g citric acid, 13.30 g KH_2PO_4 , 4.00 g $(\text{NH}_4)_2\text{HPO}_4$, 1.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 13 mg $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, 1.5 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 15 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.5 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 3.0 mg H_3BO_3 , 2.5 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 100 mg Fe(III) citrate, 4.5 mg thiamine-HCl, 8.4 mg $\text{Na}_2(\text{EDTA}) \cdot 2\text{H}_2\text{O}$, and 5.0 g D-xylose or L-arabinose. The pH was adjusted to 7.0 with 30% w/v NaOH. Cells were routinely stored on lysogeny broth (LB) agar plates inoculated to 3 mL LB in 15 mL tube from which 1 mL was transferred to 20 mL defined medium in a 125 mL shake flask. From this flask 1 mL was transferred to the 50 mL defined medium in a 250 mL shake flask used for these studies. The flasks were incubated at 37°C with an agitation of 250 rpm and for the further analysis samples were stored at -20°C. Shake flask studies were replicated three or more times, and statistical analyses were completed using Student's *t*-test (two-tailed, equal variance), and $p < 0.05$ was considered the criterion for significance.

Continuous processes of 1 L volume were conducted as phosphate- or carbon-limited chemostats in a 2.5 L fermenter (Bioflo 2000, New Brunswick Scientific Co. Edison, NJ, USA) using MEC143 strain with xylose or arabinose. Carbon-limited chemostats used the medium described above. In the medium for phosphate-limited chemostats, 3.25 g $\text{NH}_4\text{Cl/L}$ (60.8 mM N) replaced $(\text{NH}_4)_2\text{HPO}_4$, and 0.12 g $\text{KH}_2\text{PO}_4/\text{L}$ (0.74 mM P) was used as the sole phosphate source. These processes were conducted at dilution rate of 0.15/h at 37°C with an air flowrate of 1.0 L/min, an agitation of 500 rpm and a pH of 7.0. When appropriate for the strain, 40 mg/L (LB) or 100 mg/L (defined medium) kanamycin and 100 mg/L (LB) or 50 mg/L (defined medium) ampicillin were used.

Table 1. *Escherichia coli* strains used in this study

Strain	Genotype	Reference
MEC143	MG1655 $\Delta ptsG763::(\text{FRT}) \Delta manZ743::(\text{FRT}) \Delta glk-726::(\text{FRT}) \Delta pfkA775::(\text{FRT}) \Delta zwf-777::\text{Kan}$	[1]
MEC149	MG1655 $\Delta ptsG763::(\text{FRT}) \Delta manZ743::(\text{FRT}) \Delta glk-726::(\text{FRT}) \Delta pfkA775::(\text{FRT}) \Delta zwf-777::(\text{FRT})$	This study
MEC149 <i>ybiV</i>	MEC149 $\Delta ybiV722::\text{Kan}$	This study
MEC149 <i>yigL</i>	MEC149 $\Delta yigL771::\text{Kan}$	This study
MEC149 <i>yfbT</i>	MEC149 $\Delta yfbT775::\text{Kan}$	This study
MEC149 <i>yniC</i>	MEC149 $\Delta yniC726::\text{Kan}$	This study
MEC149 <i>yidA</i>	MEC149 $\Delta yidA733::\text{Kan}$	This study
MEC149 <i>phoA</i>	MEC149 $\Delta phoA748::\text{Kan}$	This study
MEC149 <i>ybiV yidA</i>	MEC149 $\Delta ybiV722::(\text{FRT}) \Delta yidA733::\text{Kan}$	This study
MEC149 <i>ybiV yigL</i>	MEC149 $\Delta ybiV722::(\text{FRT}) \Delta yigL771::\text{Kan}$	This study
MEC149 <i>ybiV yigL yidA</i>	MEC149 $\Delta ybiV722::(\text{FRT}) \Delta yigL771::(\text{FRT}) \Delta yidA733::\text{Kan}$	This study

2.3 Analytical methods

The cell growth was monitored using OD at 600 nm (UV-650 spectrophotometer, Beckman Instruments, San Jose, CA). LC with a refractive index detector and a Coregel 64-H ion-exclusion column (Transgenomic Ltd., Glasgow, UK) using a mobile phase of 4 mM H_2SO_4 was used for analysis of sugars as described previously [9]. For dry cell weight (DCW) measurement, three 10.0 mL samples were centrifuged ($8400 \times g$, 10 min), the pellets were washed by vortex mixing with 30 mL deionized water three times with centrifugation and then dried at 60°C for 24 h. Phosphorus concentration was analyzed using the ascorbic acid reduction method [10, 11]. Growth rate was calculated from the slope of five to six measurements of OD (logarithm) versus time, while the specific glucose formation rate was calculated from three to four measurement of glucose (formation) and pentose (consumption) over the course of 2 h when the OD reached 1.0 divided by the log mean cell mass concentration.

3 Results and discussion

3.1 Enzymes that dephosphorylate glucose-6-phosphate

Escherichia coli MEC143 has deletions in the genes which provide for glucose conversion to glucose-6P (*ptsG*, *manZ*, *glk*), and in key genes involved in the metabolism of glucose-6P and fructose-6P (*zwf*, *pfkA*). MEC143 has been established to accumulate glucose by the dephosphorylation of glucose-6P when grown on either xylose or L-arabinose [1]. Mass glucose yields of 0.260 g glucose/g xylose and 0.316 g glucose/g L-arabinose were observed in shake flask studies using 5 g/L of either pentose (Fig. 2). Since several phosphatases that act on glucose-6P have been identified in *E. coli* including alkaline phosphatase [2] and various HAD phosphatases [3], we hypothesized that one or more of these enzymes were responsible for glucose formation in MEC143. We therefore first examined whether a deletion in any one of these genes alone would affect glucose formation from xylose or arabinose. We also determined specific growth rate and specific glucose formation rate for each of these strains.

Figure 2. Mass glucose yield from xylose or arabinose (g/g) during batch growth of various phosphatase knockout strains of *Escherichia coli*. Standard errors are indicated by error bars. An asterisk above a bar indicates a statistically significant lower yield compared to MEC143 for that substrate, while an x above a bar indicates a statistically significant greater yield ($p < 0.05$).

In all cases of growing these strains with deletions in a single phosphatase, the pentose was exhausted and glucose accumulated. Compared to MEC143, deletions in either the *phoA*, *ybiV*, *yfbT*, or *yniC* genes did not significantly reduce the glucose yield from xylose or arabinose (Fig. 2). A deletion in either *yidA* or *yigL* reduced the yields slightly, but significantly to approximately 0.22 g glucose/g xylose and 0.27–0.29 g glucose/g L-arabinose. Nevertheless, a deletion in any one gene alone was insufficient to prevent glucose formation from either xylose or arabinose. Thus, these results suggest that no one phosphatase is exclusively responsible for the conversion of glucose-6P to glucose in MEC143. Compared to MEC143, these single phosphatase deletions did not significantly affect specific growth rates for any strain on arabinose, although modest reductions of 10–20% in growth rates were observed on xylose for a *phoA*, *yidA*, *yfbT*, or *yniC* deletion (Fig. 3). The measurement of specific glucose formation rate was subject to greater variance than either yield or

Figure 3. Specific growth rate from xylose or arabinose (h^{-1}) during batch growth of various phosphatase knockout strains of *Escherichia coli*. SEs are indicated by error bars, and an asterisk above a bar indicates a statistically significant difference in growth rate compared to MEC143 for that substrate ($p < 0.05$).

Figure 4. Specific glucose formation rate from xylose or arabinose (g/g h) during batch growth of various phosphatase knockout strains of *Escherichia coli*. SEs are indicated by error bars. An asterisk above a bar indicates a statistically significant lower glucose formation rate compared to MEC143 for that substrate, while an x above a bar indicates a statistically significant greater rate ($p < 0.05$).

growth rate measurements, and the *ybiV* or *yniC* deletion strains growing on xylose were the only single-gene deletion cases in which glucose formation was significantly lower than MEC143 (Fig. 4).

Since no single deletion in a gene coding a known glucose-6P phosphatase was sufficient to prevent glucose accumulation, we therefore next examined whether knocking out multiple

Figure 5. Comparison of mass glucose yield from xylose or arabinose (g/g) using *Escherichia coli* MEC143 during carbon-limited, batch, or phosphate-limited growth.

phosphatases would further reduce glucose formation. In particular, we examined combinations of knockouts of those genes that showed a small but significant reduction in glucose yield when knocked out singly (Fig. 2). For the three strains examined, the pentoses were exhausted within 40 h, and glucose still accumulated, though to a lower yield than MEC143. For example, the double-phosphatase knockout *ybiV yidA* resulted in a yield of 0.177 g glucose/g xylose and 0.232 g glucose/g L-arabinose, while the triple-phosphatase knockout *ybiV yidA yigL* resulted in a yield of 0.063 g glucose/g xylose and 0.117 g glucose/g L-arabinose (Fig. 2). While single phosphatase knockouts had a limited effect on growth rates on pentoses compared to MEC143, all three strains with multiple phosphatase knockouts increased the lag phase and reduced the growth rate for *E. coli* on either xylose and arabinose, generally by a factor of about two (Fig. 3). The two double-phosphatase knockouts also showed significantly lower specific glucose formation when growing on arabinose, while the *ybiV yidA yigL* strain showed lower glucose formation when growing on either xylose or arabinose (Fig. 4). Even though the observed reduced glucose yield could be partly attributed to reduced growth rate, the results do indicate that these phosphatases (YbiV, YidA, and YigL) are responsible for the conversion of glucose-6P to glucose, which leads to glucose formation in *E. coli* MEC143. Moreover, the results support the conclusion that multiple phosphatases are responsible for the in vivo dephosphorylation of glucose-6P. The reduction in growth rate with multiple phosphatase knockouts comes as no surprise since the phosphatases are known to act on many other organic phosphates [2, 3], and they likely mediate other important conversions that makes them collectively essential for cell health. Finally, in all shake flask studies less than 100 mg mannose/L also accumulated similar to previous reports [1]. These mannose yields were invariably low (about 0.02 g/g), and mannose formation was similarly not eliminated by the deletion of any one phosphatase or multiple phosphatases.

Since one or more HAD phosphatases appear to be involved in the dephosphorylation of glucose-6P to glucose in MEC143 derivatives, we also examined whether overexpression of a phosphatase would conversely increase the glucose formation from pentoses. We selected HAD12 coded by *ybiV* because high observed values for both the k_{cat} (22/s) and the pseudo first order

rate constant (k_{cat}/K_M) of $6900 \text{ M}^{-1}\text{s}^{-1}$ have been reported for this enzyme [3]. In shake flask studies using MEC143/pZE12-*ybiV*, we obtained yields of 0.304 g glucose/g xylose and 0.353 g glucose/g L-arabinose, 17 and 11% greater than the yields observed with MEC143. This increase in yield is particularly noteworthy since the overexpression of HAD12 reduced the specific growth rate on either xylose or arabinose (Fig. 3), and increased the specific glucose formation rate on either substrate (Fig. 4). As a control, the strain containing the empty plasmid MEC143/pZE12 compared to MEC143 had a lower glucose yield on arabinose (Fig. 2), a lower specific growth rate for either substrate, and a lower glucose formation rate on arabinose (Fig. 4). Thus, one method to increase the yield of glucose from pentoses is to enhance the final dephosphorylation of glucose-6P (Eq. (2)).

3.2 Effect of phosphate or carbon limitation

The shake flask studies operated as batch reactors, wherein all nutrients required for cell growth were supplied in excess at the onset of cell growth. The shake flask results therefore represent cellular responses under maximal growth conditions under conditions of excess phosphorus and carbon. The final step in the overall conversion of pentoses to glucose is the dephosphorylation of glucose-6P accompanied by the formation of inorganic phosphate (Eq. (2)). Although a batch operation does not allow for continued nutrient limitation, a chemostat operated at steady state allows cell growth at a defined growth rate under a predetermined nutrient limitation. We hypothesized that phosphate limitation should increase glucose yield from pentose by MEC143. In contrast, under carbon-limited conditions, the glucose yield will be reduced compared to carbon-excess conditions of a batch culture, since cells must maximally metabolize that carbon for energy and biomass formation. To examine glucose formation under these two contrasting conditions, we completed several steady-state experiments under both phosphate limitation and carbon limitation (Fig. 3). As expected, carbon limitation significantly reduced the glucose yield from either pentose to 0.05 – 0.08 g/g, while phosphate limitation increased the glucose yield to 0.39 g glucose/g xylose, a 50% increase in glucose formation compared to batch conditions. Interestingly, we observed no significant change in glucose yield from arabinose (Fig. 3). Under phosphate-limited conditions, the observed yield coefficient ($Y_{X/P}$) of *E. coli* cells was 58.3 g DCW/g P. Considering that both pentoses use the final dephosphorylation of glucose-6P to generate glucose (Eq. (2)), we cannot find an explanation for the absence of an increase in glucose yield from arabinose (in contrast to results from xylose) under phosphate-limited conditions. Since in all shake flask studies the yield of glucose from arabinose was greater than the yield of glucose from xylose (Fig. 2), it may be that a difference exists in the cellular phosphate budget between xylose and arabinose, and the dephosphorylation step is not limiting glucose yield from arabinose. This possibility is supported by the different routes by which the two pentoses are transported and enter the PP pathway in *E. coli*. For xylose, an ATP-binding dependent system [12] appears to predominate under normal growth conditions [13], indicating that D-xylose uptake generally demands energy (ATP) directly.

In contrast, a low affinity, ATP-independent system appears to predominate for arabinose [14]. Thus, arabinose uptake is less directly affected by phosphate than xylose uptake, and cells may respond differently to phosphate limitation when the demand for ATP is low for the initial uptake and phosphorylation of the carbon/energy source. Our results nevertheless demonstrate that for xylose a phosphate-limited process is able to increase the yield of glucose and potentially other products derived from glucose-6P. This same strategy could potentially be employed to increase the yield of other products whenever the final biochemical step involves dephosphorylation.

Relatively little research has been completed using phosphate-limited steady-state growth (as opposed to phosphate depletion in batch culture), particularly using arabinose or xylose as carbon sources. Nevertheless, phosphate-limited conditions have been long-understood to upregulate *phoA* transcription strongly [4]. A recent study has furthermore shown that the HAD phosphatases coded by *yigL*, *yniC*, or *yidA* are upregulated twofold to 3.5-fold under phosphate-limited conditions compared to carbon (glucose) limited conditions [15]. Thus, phosphate-limited conditions tend to induce the phosphatases that encourage more glucose formation in the strains used in this present study.

4 Concluding remarks

An *E. coli* strain with gene deletions preventing the cells from consuming glucose is able to convert arabinose or xylose to glucose. The final enzymatic step is the dephosphorylation of glucose-6P to glucose (Eq. (2)), and it is shared by several general phosphatases. Additionally, the formation of glucose can be increased by growing cells in a phosphate-limited environment. Thus, our results shed light on conversions between monosaccharides in the upper metabolism of *E. coli* and also provide guidance on operational conditions to influence these conversions.

Practical application

Understanding the interconversion of hexoses and pentoses is important in designing microbial processes to generate many biochemical products. The highlights of this work include *E. coli* with deletions in glucose uptake and metabolic genes will accumulate glucose from the pentoses, arabinose, and xylose, glucose accumulation from pentoses results from the dephosphorylation of glucose-6-phosphate by multiple phosphatases expressed in *E. coli*, overexpression of haloacid dehalogenase like phosphatase 12 phosphatase increases glucose yield from pentoses, compared to batch growth, growth under phosphate-limited conditions increases glucose yield from xylose, while growth under carbon-limited conditions decreases glucose yield from either pentose.

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The authors have declared no conflicts of interest.

5 References

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