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Heterologous expression of D-xylulokinase from *Pichia stipitis* enables high levels of xylitol production by engineered *Escherichia coli* growing on xylose

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ABSTRACT

Deletion of the *Escherichia coli* xylulokinase gene (*xylB*) is essential for achieving high xylitol titers from xylitol-producing *E. coli* strains growing on glucose in the presence of xylose. Our study suggests that this is due to XylB-catalyzed toxic synthesis of xylitol-phosphate. This activity prohibits the use of xylose as the sole carbon source during xylitol production by *E. coli*. To overcome this limitation we turned to the yeast *Pichia stipitis*, which naturally produces xylitol, as a source of xylulokinase (Xyl3). We examined the effects of plasmid-based expression of Xyl3 versus XylB on growth and xylitol production by engineered *E. coli* strains. Xylulokinase activity assays show similar levels of functional expression of both enzymes (determined as activity on xylulose), and reveal significantly more activity on xylitol by XylB compared to Xyl3. ^{31}P NMR confirms the production of xylitol-phosphate from *in vitro* reactions with XylB. Lastly, the replacement of *xylB* with *XYL3* results in drastically enhanced xylitol titers from *E. coli* strains co-expressing xylose reductase during growth on xylose.

1. Introduction

Xylitol is a natural sweetener with notable health-promoting properties. It is known to reduce dental caries (Edgar, 1998; Scheinin et al., 1974), serves as a low-calorie supplement in diabetic and obesity diets (Parajo et al., 1998; Winkelhausen and Kuzmanova, 1998), and is a potential ingredient in the treatment of osteoporosis (Mattila et al., 2002). Xylitol is a pentitol that is traditionally synthesized by reduction of D-xylose over Raney-nickel catalyst, although whole-cell and *in vitro* biocatalyst production routes have become attractive alternatives (Bae et al., 2004; Granstrom et al., 2007; Kim et al., 2004; Parajo et al., 1998; Silva et al., 2003). As depicted in Fig. 1, D-xylose-metabolizing yeasts such as *Pichia stipitis* naturally produce xylitol by the action of NAD(P)H-dependent xylose reductase (XR), as an intermediate in the synthesis of the pentose phosphate xylulose-5-phosphate (X-5-P).

We previously described two strategies of engineering *Escherichia coli* to produce xylitol by xylose reduction during growth on glucose (Cirino et al., 2006; Khankal et al., 2008). Both strategies employed heterologous expression of NADPH-dependent XR from *Candida boidinii* (CbXR). In one approach to overcome glucose repression of xylose transporter expression (Cirino et al., 2006), we replaced the native gene encoding cyclic AMP receptor protein (CRP) with a mutant CRP which has reduced sensitivity to cAMP (named "CRP*" (Eppler and Boos, 1999)). An alternate approach was to express either of the native xylose transporters (XylE or XylFGH) under control of a (CRP-independent) *tac* promoter (Khankal et al., 2008). Fig. 1 also depicts the first steps of D-xylose metabolism in *E. coli*. Xylose isomerase (XylA) converts xylose to xylulose, which is then phosphorylated to X-5-P by xylulokinase (XylB). *xylA* and *xylB* genes in *E. coli* are arranged in a single operon governed by a promoter that is co-regulated by CRP and the xylose-inducible regulator XylR (Song and Park, 1997). In our engineered strains, xylose assimilation was prevented by deletion of *xylB*. Thus, glucose served as the growth substrate and source of reducing equivalents for reduction of xylose to xylitol.

Xylulokinase plays a critical role in xylose utilization by all natural and engineered microorganisms. Pentulose kinases have documented relaxed substrate specificities (Leblanc and Mortlock, 1972; Mortlock, 1976; Neuberger et al., 1981). Kinetic studies with *E. coli* XylB revealed its ability to phosphorylate several other sugars and polyols (including xylitol) with low catalytic efficiencies (Di Luccio et al., 2007). D-xylulokinase from *Aerobactoer aerogenes* has also been shown to phosphorylate xylitol at a low rate (Simpson, 1966). This non-specific activity of xylulokinases results in the production and accumulation of potentially toxic phosphorylated compounds that are not further metabolized. A study aimed at constructing a xylose catabolic pathway in *Zymomonas mobilis* by heterologous expression of *xylAB* genes from *Klebsiella pneumoniae* revealed growth inhibition and

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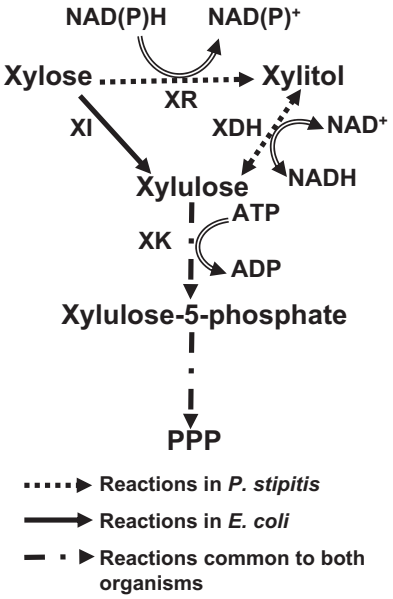

Fig. 1. Early steps of xylose catabolism in *P. stipitis* and *E. coli.* Similar reactions occur in many other yeasts and prokaryotes (respectively). *Abbreviations*: XR, D-xylose reductase; XDH, xylitol dehydrogenase; XI, xylose isomerase; XK, D-xylulokinase (XylB in *E. coli*, Xyl3 in *P. stipitis*); PPP, pentose phosphate pathway.

an intracellular accumulation of (putatively) xylitol-phosphate in the presence of xylose (Feldmann et al., 1992). This was attributed to non-specific xylose reduction followed by XylB-mediated xylitol phosphorylation. An analogous pattern of toxic synthesis has been shown in *E. coli* C mutants, where xylulokinase activity on D-arabitol led to the synthesis of arabitol-phosphate and the inability of strains to grow in the presence of D-arabitol (Scangos and Reiner, 1979). This toxicity was relieved by a xylulokinase gene mutation. Toxic synthesis in *E. coli* strains due to phosphotransferase activity on xylitol and arabitol has also been reported (Reiner, 1977).

We show that a *xylB* gene deletion is essential for obtaining high xylitol titers in xylitol-producing *E. coli* strains. Deleting *xylB* alone or in combination with *xylA* results in significantly elevated xylitol titers (during growth on glucose), while deleting *xylA* but not *xylB* eliminates xylose metabolism but does not improve xylitol production. In addition, growth of strains is inhibited by the presence of xylitol when growing on xylose but not on glucose. We propose that xylitol-phosphate produced by XylB inhibits xylose uptake, which in turn prevents the use of xylose as the sole carbon source during xylitol production by engineered *E. coli. In vitro* studies have shown that the D-xylulokinase from *P. stipitis* strain NCYC 1451 does not use xylitol as a substrate (Flanagan and Waites, 1992). In our study, expression of xylulokinase from *P. stipitis* strain CBS 6054 (Xyl3) in *E. coli* functionally replaces XylB for growth on xylose. Xylulokinase assays reveal significant activity on xylitol by XylB but not Xyl3, and ^{31}P NMR confirms the production of xylitol-phosphate by cell lysates containing XylB. Finally, replacement of XylB with Xyl3 results in significantly improved xylitol titers from *E. coli* strains co-expressing CbXR during growth on xylose minimal medium.

2. Materials and methods

2.1. General

Wild-type *E. coli* strains W3110 (ATCC 27325) and its derivatives were maintained on agar plates containing Luria-Bertani (LB) medium (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, and 15 g agar). Kanamycin monosulfate (50 μg mL^{-1}) was added to media for plasmid maintenance when required. Restriction enzymes, *Taq* DNA polymerase and bovine serum albumin were from New England Biolabs (Beverly, MA, USA). iProof^{TM} high-fidelity polymerase was obtained from Bio-Rad laboratories (Hercules, CA, USA). D-Xylulose, ATP, phosphoenolpyruvate, NADH, lactate dehydrogenase (LDH), and pyruvate kinase (PK) enzymes from rabbit muscle (1000 U mL^{-1} LDH, 700 U mL^{-1} PK), dithiothrietol (DTT), phenylmethanesulphonyl fluoride (PMSF), potassium fluoride (KF), and potassium cyanide (KCN) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Primers for PCR and sequencing were synthesized by Invitrogen (Carlsbad, CA, USA).

2.2. Culture medium and cultivation conditions

All cultures were performed in at least duplicates and reported data represent the average of at least two experiments. Standard errors that are greater than 10% of the average value are reported for all data. Shake-flask cultures for xylitol production were carried out in 250 mL baffled flasks containing 50 mL of medium and were grown at 30 °C and 250 rpm. LB medium or minimal medium (per liter: 3.5 g KH_2PO_4; 5.0 g K_2HPO_4; 3.5 g $(NH_4)_2HPO_4$, 0.25 g $MgSO_4{:}7H_2O$, 15 mg $CaCl_2{:}2H_2O$, 0.5 mg thiamine, and 1 mL of trace metal stock prepared as described (Causey et al., 2003)) was used and supplemented with glucose and xylose at concentrations indicated in text.

Shake-flask cultures were inoculated to an initial cell density (OD_{600}) of 0.1. For LB cultures, overnight seed cultures were prepared by inoculating 3 mL of LB medium (in a 13 × 100 mm tube) with few colonies from a fresh LB plate. Overnight cultures were grown at 37 °C to an OD of 1–2.5 and then used to inoculate shake-flask cultures. Cells were conditioned to growth in minimal medium prior to culturing the seed as follows: overnight cultures were prepared as described above (in LB) and used to inoculate 10 mL of glucose (27.8 mM) minimal medium to an initial OD of 0.1. These cultures were grown at 37 °C, harvested in exponential phase and used to inoculate another 10 mL minimal medium culture (27.8 mM glucose or mixture of 27.8 mM glucose and 33.4 mM xylose). These seed cultures were grown at 30 °C harvested in exponential phase and used to inoculate the shake-flask cultures. Isopropyl-β-D thiogalactopyranoside (IPTG, 100 μM) was used in shake-flask cultures to induce expression of plasmid genes under the control of a *tac* promoter. 4-Morpholinopropanesulfonic acid (MOPS) was added to all cultures for pH control (50 mM, pH 7.4).

2.3. Analytical methods

Cell culture optical density was measured at 600 nm using a SPECTRAmax PLUS^{384} spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) and was used to estimate cell mass (1 OD_{600} unit = 0.3 g [cell dry weight] L^{-1}). For measuring secreted metabolites, cultures were centrifuged and supernatants were filtered through a 0.45 μm filter for high performance liquid chromatography (HPLC) analysis. Xylitol, xylose, xylulose, glucose, and organic acid concentrations in culture broth were measured using a Shimadzu LC-10AD HPLC (Columbia, MD, USA) equipped with a UV monitor (210 nm) and refractive index detector (RID). Separation of products was achieved using an Aminex HPX-87H column (Bio-Rad Laboratories) with 4 mM H_2SO_4 as the mobile phase (flow rate: 0.5 mL min^{-1}, column operated at 45 °C).