

Anaerobic Obligatory Xylitol Production in *Escherichia coli* Strains Devoid of Native Fermentation Pathways[∇]

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Anaerobic glucose oxidation was coupled to xylose reduction in a nonfermentative *Escherichia coli* strain expressing NADPH-dependent xylose reductase. Xylitol production serves as the primary means of NAD(P)⁺ regeneration, as glucose is converted primarily to acetate and CO₂. The membrane-bound transhydrogenase PntAB is required to achieve the maximum theoretical yield of four moles of xylitol per mole of glucose consumed.

Microorganisms are attractive hosts for NAD(P)H-dependent biocatalysis due to advantages that include the ability to regenerate cofactors *in vivo* via metabolism of abundant raw materials (e.g., glucose). However, challenges lie in properly constraining metabolism for implementing whole-cell transformations such that reduced cofactors are effectively utilized to drive the reactions of interest. We previously engineered *Escherichia coli* to produce xylitol from glucose-xylose mixtures by heterologous expression of NADPH-dependent xylose reductase from *Candida boidinii* (CbXR) (7). In this system, aerobic glucose catabolism to carbon dioxide provides NADPH for xylose reduction to xylitol. Similar to other reports on NAD(P)H-dependent biotransformations catalyzed by heterologous enzymes in *E. coli*, the quantity of reducing equivalents used for xylitol production is maximally ~60% of the theoretical maximum value under nongrowing conditions (3, 4, 6, 21). The disparity between the experimental and theoretical yields (yield is defined as moles of reduced product per mole of glucose consumed and denoted “Y_{RPG}”) has been suggested to result from various factors, including the inability of the heterologous enzyme and/or transhydrogenases to effectively compete with other cofactor-utilizing cellular reactions, such as those involved in aerobic respiration (3, 6). Decreasing aeration or deleting NADH dehydrogenase-encoding genes results in incomplete glucose oxidation and the secretion of pyruvate and fermentation products (6, 7).

Here, we describe an alternate, anaerobic strategy of coupling NADPH-dependent xylose reduction to cofactor regeneration via glucose oxidation (depicted in Fig. 1). Fermentation pathways were eliminated so that xylitol production serves as the sole means of regenerating NAD⁺ and maintaining redox balance. The expression of a variant of lipoamide dehydrogenase (LPD) conferring reduced sensitivity of the pyruvate dehydrogenase complex (PDH) to NADH (15) resulted in the reduction of ~4 moles of xylose to xylitol per mole of glucose metabolized to acetate. The deletion of *pntA*, encoding

a subunit of the membrane-bound transhydrogenase (PntAB), resulted in a more than 50% decrease in the xylitol yield.

The strains used in this study are listed in Table 1. PCRs and gene cloning were performed following standard protocols (16, 18) and as previously described (6; J. W. Chin and P. C. Cirino, submitted for publication). The *CbXR* gene was inserted into the *E. coli* chromosome at the phage HK022 attachment site as described previously (11; Chin and Cirino, submitted for publication). *E. coli* K-12 strains with single gene deletions carrying an FLP recombination target (FRT)-flanked kanamycin resistance cassette in place of the selected gene (*ldhA*, *adhE*, *fdxA*, *pntA*, or *sthA*) were obtained from the Keio collection (2). The multigene deletions $\Delta zwf-eda$ and $\Delta xylAB$ originated from strains JC68 (6) and JC82 (precursor for strain OA24 [1]), respectively. Subsequent strains were created via phage P1 transductions followed by flippase recombinase-mediated excision of the corresponding antibiotic resistance marker gene (8).

Cultivations and analytical methods. All cultivations were controlled by a Sixfors parallel fermentation system (Infors-HT). The fermentation vessels contained 500 ml minimal medium (prepared as described in reference 5) supplemented with 100 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) to induce expression of the *CbXR* gene (under the control of a *tac* promoter). An aerobic growth phase was first operated in fed-batch mode with an initial glucose concentration of 25 mM. Two additional 25 mM doses of glucose were added manually upon glucose depletion. Following depletion of all glucose during the aerobic phase (cellular optical density at 600 nm of ~6 to 9), cultures were switched to an anaerobic phase by sparging with 2 vvm nitrogen and operated in batch mode initially containing 14 mM glucose and 300 mM xylose. Cultures were performed in at least duplicates, and the data reported represent the averages of the results of at least two experiments. The concentrations of sugars, sugar alcohols, and organic acids in culture broth at the final time point of the anaerobic phase ($t = 20$ h) were measured by high-pressure liquid chromatography as previously described (1).

The theoretical Y_{RPG} values are based on the assumption that glucose metabolism occurs via the Embden-Meyerhof-Parnas (EMP) pathway (Fig. 2), which was later verified as described below. The maximum theoretical Y_{RPG} for glucose metabolism to acetate under anaerobic and nonrespiratory

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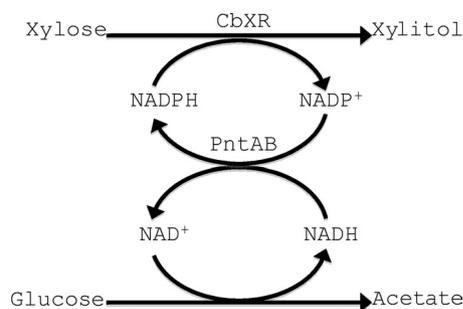


FIG. 1. Anaerobic xylitol production in engineered *E. coli*. Glucose conversion to acetate serves as the source of reducing power for xylose reduction to xylitol by heterologously expressed, NADPH-dependent xylose reductase (CbXR). Reducing equivalents are transferred from NADH to NADP⁺ in a reaction catalyzed by the native pyridine nucleotide transhydrogenase PntAB. Xylose reduction to xylitol serves as the primary means of NAD(P)H reoxidation and maintenance of intracellular redox balance.

conditions is therefore 4 (compared to the aerobic theoretical maximum Y_{RPG} of ~ 9.2 [6]).

Elimination of competing fermentation pathways. Strain JC11 carries a xylulokinase gene deletion ($\Delta xylB$) to prevent xylose metabolism, as well as a chromosomal copy of the CbXR gene (13). In the anaerobic production phase, strain JC11 undergoes mixed acid fermentation (as shown in Fig. 2 and Table 2), secreting a mixture of lactic acid, succinic acid, formic acid, acetic acid, and ethanol. A low level of xylitol production is also observed ($Y_{RPG} = \sim 1.4$).

Deletion of the native NADH-utilizing fermentation pathways in strain JC11 was expected to preserve reducing power and increase the xylitol yield from glucose/xylose mixtures. The stepwise deletion of the *ldhA*, *adhE*, and *frdA* genes (Fig. 2) that led to strain OA126 increased the xylitol yield during the anaerobic production phase by $\sim 57\%$ (compared to the production level in JC11), with pyruvate and acetate being the major end products of glucose metabolism (Table 2). A small amount of succinate was also produced. Eighty-five percent of the glucose carbon is accounted for in the products secreted by OA126, and no cell growth was observed in the anaerobic phase.

Expression of LPD mutant with reduced NADH sensitivity. LPD is a component of the multienzyme PDH complex (Fig. 2) (10). Sensitivity of the *E. coli* K-12 LPD to NADH renders the

native PDH complex essentially inactive under typical nonrespiratory catabolism conditions, in which the NADH/NAD⁺ ratio is elevated (9, 12). An LPD variant having reduced NADH sensitivity has been described [*lpd101*, encoding LPD(E354K)], and the expression of this variant in place of the native enzyme substantially increases PDH activity during anaerobic glucose catabolism (15). This allows for the production of an additional NADH during the conversion of pyruvate to acetyl coenzyme A (instead of formate which is otherwise produced by pyruvate formate lyase [PFL]). Strain OA87 carries the *lpd101* mutant and a chromosomal copy of *CbXR* and is devoid of the fermentative genes *ldhA*, *adhE*, *frdA*, and *pflB*. This strain produces ~ 4 moles of xylitol per mole of glucose consumed during the anaerobic cultivation phase (Table 2), corresponding to the theoretical maximum xylitol yield for the engineered strain. In addition to xylitol, acetate and small amounts of succinate are produced in strain OA87. It is unclear how succinate is produced in OA87, given that the experimental xylitol yield corresponds to the theoretical maximum yield. One possibility is succinate production due to unexpected flux through the glyoxylate shunt (17), which increases the calculated theoretical maximum xylitol yield. In the absence of xylose, OA87 consumes minimal amounts of glucose coupled to a low background level of glucose reduction to sorbitol (not shown).

Verification of pathway for glucose oxidation in engineered strains. As stated, theoretical maximum Y_{RPG} values are based on the assumption that glucose oxidation occurs via the EMP pathway (Fig. 2). To test whether flux through the pentose phosphate (PP) or Entner-Doudoroff (ED) pathway plays a role in achieving the Y_{RPG} obtained by strain OA87, the genes *zwf*, *edd*, and *eda*, encoding glucose-6-phosphate-1-dehydrogenase, phosphogluconate dehydratase, and 2-keto-3-deoxygluconate-6-phosphate aldolase, respectively (the reactions catalyzed are depicted in Fig. 2), were deleted from OA87, resulting in strain OA176. The Y_{RPG} obtained for OA176 was 3.9, which is essentially identical to that obtained for strain OA87 ($Y_{RPG} = 4.0$). This suggests that the PP and ED pathways did not contribute significantly to the supply of reducing equivalents for xylose reduction.

Role of transhydrogenases in xylitol production. Glucose oxidation via the EMP pathway produces NADH, while CbXR utilizes only NADPH for xylose reduction. It is therefore likely that reducing equivalents are transferred from NADH to

TABLE 1. Strains used in this study

<i>E. coli</i> strain	Relevant characteristics	Source or reference
W3110	Wild type	ATCC 27325
JC11	W3110, $\Delta xylB::FRT$ HK022::(<i>CbXR</i> -FRT)	Chin and Cirino, submitted
OA126	JC11, $\Delta ldhA::FRT \Delta adhE::FRT \Delta frdA::FRT$	This study
YK87 ^a	W3110, $\Delta ldhA \Delta (focA-pflB)::FRT \Delta adhE$ FRT-kan-FRT <i>lpd101</i> (E354K)	14
OA87	YK87 $\Delta xylAB::FRT \Delta frdA::FRT$ HK022::(<i>CbXR</i> -FRT)	This study
OA176 ^b	OA87, $\Delta (zwf-eda)::FRT$	This study
OA163	OA87, $\Delta sthA::FRT$	This study
OA120	OA87, $\Delta pntA::FRT$	This study
OA207	OA120, $\Delta sthA::FRT$	This study
OA205	OA176, $\Delta pntA::FRT$	This study

^a Strain YK87 was a gift from K. T. Shanmugam (University of Florida).

^b The *zwf*, *edd*, and *eda* genes were deleted in strain OA176.

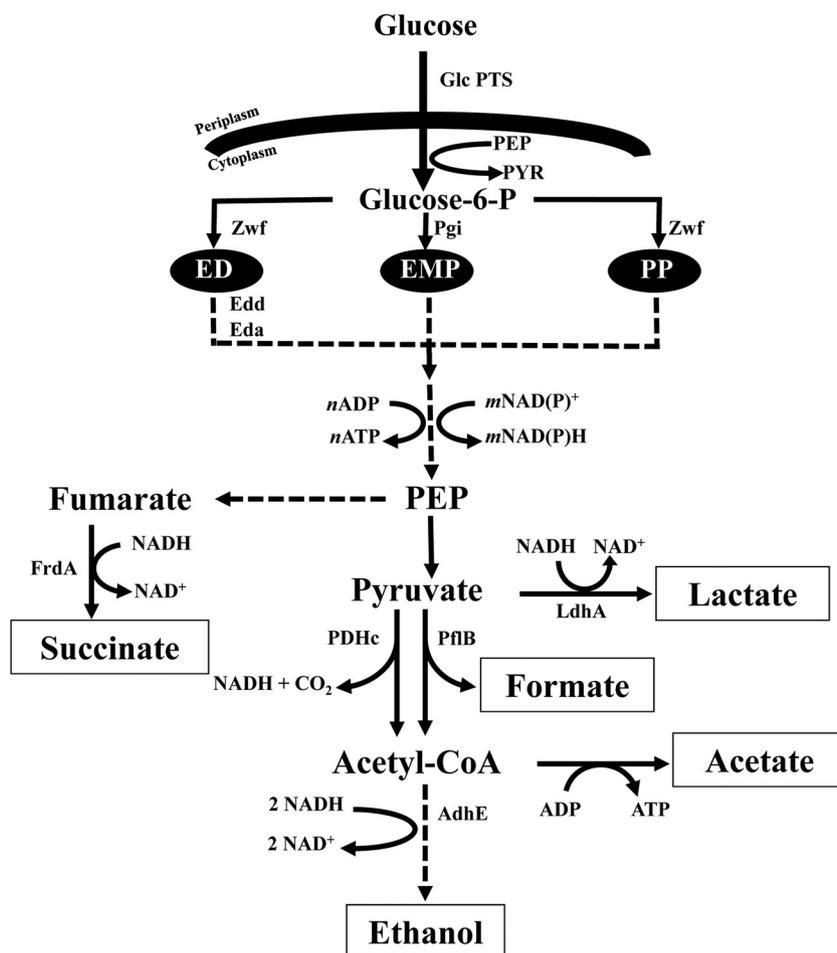


FIG. 2. *E. coli* central pathways and fermentation products. The Embden-Meyerhof-Parnas (EMP), pentose phosphate (PP), and Entner-Doudoroff (ED) pathways convert glucose-6-phosphate ultimately to pyruvate. The PP and ED pathways branch from glucose-6-phosphate and re-enter glycolysis at different locations. The molar yields of ATP (denoted as n) and NAD(P)H (denoted as m) per glucose depend on the pathway used in glucose oxidation. The EMP pathway generates the most ATP ($n_{\text{EMP}} = 2$ for conversion of 1 mole of glucose to 2 moles of pyruvate), with a corresponding m_{EMP} of 2 (16). Major fermentation products obtained from the central pathways are shown in boxes. Dashed arrows indicate multistep reactions. Abbreviations: glucose-6-P, glucose-6-phosphate; Glc-PTS, glucose phosphotransferase system; PEP, phosphoenolpyruvate; PYR, pyruvate; Zwf, glucose-6-phosphate dehydrogenase; Pgi, phosphoglucoisomerase; Edd, 6-phosphogluconate dehydratase; Eda, 2-keto-3-deoxy-6-phosphogluconate aldolase; FrdA, flavoprotein of the fumarate reductase enzyme complex; LdhA, lactate dehydrogenase; PflB, subunit of the pyruvate-formate lyase; PDHc, pyruvate dehydrogenase enzyme complex; AdhE, alcohol dehydrogenase; CoA, coenzyme A.

NADP⁺ in a reaction catalyzed by either of *E. coli*'s native pyridine nucleotide transhydrogenase enzymes: the membrane-bound and proton-translocating PntAB or the soluble SthA. The physiological role of PntAB is assumed to be the transfer of hydride from NADH to NADP⁺ to generate NADPH for use in reductive biosynthetic reactions, while SthA is deemed to function primarily in the direction of NADH synthesis (20). We previously showed that transhydrogenase activity does not contribute to the supply of NADPH for aerobic xylitol-producing biotransformations (6). Deletion of *sthA* in strain OA87 (resulting in strain OA163) did not significantly affect the experimental xylitol yield (Table 2). However, deletion of *pntA* in strain OA87 (resulting in strain OA120) resulted in an ~50% reduction in the experimental xylitol yield. Sorbitol (resulting from uncharacterized, nonspecific glucose reduction) was produced as a side product by OA120, and glucose consumption in this strain was incomplete (~40% less than that of strain OA87).

The reduction in xylitol yield for *pntA* deletion strain OA120 indicates that PntAB plays a major role in the NADPH supply for xylitol production in the engineered strains. However, the fact that xylitol is still produced in strain OA120 suggests that in the absence of PntA, another route is utilized for NADPH production. One possibility is that in the *pntA* mutant (OA120), SthA-mediated transfer of reducing equivalents from NADH to NADP⁺ is promoted due to elevated intracellular NADH and NADP⁺ concentrations (similar to a report by Sanchez et al. [19]). However, the deletion of *sthA* from OA120 (yielding strain OA207) actually caused a small increase in the xylitol yield, and glucose consumption was again incomplete (~50% less than that of strain OA87).

Another possibility is that in the *pntA* deletion strain, flux through the PP and/or ED pathways becomes elevated as a means of providing cellular NADPH. The experimental xylitol yield obtained during anaerobic cultivation of OA205 (OA120 Δ zwf Δ edd Δ eda) was about 80% less than that of strain

TABLE 2. Molar yields of products from anaerobic-phase cultivations initially containing 14 mM glucose and 300 mM xylose

Strain	Characteristics	Glucose remaining (mM)	Moles of product per mole of glucose consumed (\pm SD) ^a					
			Xylitol	Acetate	Pyruvate	Lactate	Succinate	Sorbitol
JC11 ^b	W3110, Δ <i>xyfB</i> HK022::(<i>CbXR-FRT</i>)	0	1.4	1.5	0	0.35	0.17	0
OA126 ^c	JC11, Δ <i>ldhA</i> Δ <i>adhE</i> Δ <i>frdA</i>	0	2.2	0.56	0.76	0.080	0.25	0
OA87	W3110, Δ <i>xyfAB</i> Δ <i>ldhA</i> Δ (<i>focA-pflB</i>) Δ <i>adhE</i> Δ <i>frdA</i> <i>lpd101</i> HK022::(<i>CbXR-FRT</i>)	0	4.0	1.8	0	0	0.15 \pm 0.03	0
OA176	OA87, Δ (<i>zwf-eda</i>)	0	3.9	1.6	0	0	0.17 \pm 0.03	0
OA163	OA87, Δ <i>sthA</i>	0	3.9 \pm 0.49	1.6	0	0	0.25 \pm 0.07	0
OA120	OA87, Δ <i>pntA</i>	5.4	1.8	0.26	0	0.15	0.15	0.57
OA207	OA120, Δ <i>sthA</i>	9.3	2.1 \pm 0.25	0.29	0.024	0	0.32 \pm 0.11	0.62
OA205	OA176, Δ <i>pntA</i>	4.0	0.36	0.025	0.24	0	0.090	0.46

^a Molar yields were determined after 20 h of cultivation. All calculated standard deviations are less than 10% of the mean except where indicated.

^b Formate (1.32 moles) was also produced in strain JC11.

^c Formate (0.47 moles) was also produced by strain OA126.

OA120, suggesting that in the context of the *pntA* deletion, flux through the NADPH-producing PP and/or ED pathways is elevated. Glucose consumption in strain OA205 was \sim 30% less than that observed for strain OA87, and the major by-products secreted were sorbitol and pyruvate. It is likely that reoxidation of NADH produced from glucose oxidation to pyruvate in OA205 occurs through glucose reduction to sorbitol.

Conclusions. Although there has been considerable success with engineering microorganisms to overproduce native fermentation products, such as ethanol, succinate, and lactic acid, at a high yield during anaerobic fermentations, less attention has been given to coupling anaerobic glucose oxidation to the production of nonnative and nonfermentative redox products. In this study, we have developed an anaerobic platform for xylitol production in *E. coli* from glucose-xylose mixtures, in which xylitol production serves as the main route for anaerobic NAD(P)H reoxidation. In contrast to other biotransformation systems, the metabolic behavior of strains described here is neither fermentative (as electrons are transferred to an external electron acceptor) nor respiratory (as the electron transport chain is not functional and energy is not generated via oxidative phosphorylation). Our results set the stage for achieving efficient trafficking of reducing power toward biotransformations of interest in *E. coli* strains engineered to more completely oxidize glucose under anaerobic conditions. While our study focused on improving coupling and yield, understanding and engineering factors controlling specific productivity must be addressed in future work.

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