Metabolic and Regulatory Rearrangements Underlying Efficient D-Xylose Utilization in Engineered Pseudomonas putida S12*

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Received for publication, December 28, 2011, and in revised form, March 6, 2012 Published, JBC Papers in Press, March 13, 2012, DOI 10.1074/jbc.M111.337501

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Background: Metabolic changes associated with an improved d-xylose utilization phenotype were unknown.

Results: Metabolic and regulatory changes of the primary carbon metabolism are responsible for improved d-xylose utilization.

Conclusion: Valuable insight into system-wide rearrangements establishing efficient catabolism of non-natural carbon sources was obtained.

Significance: Multiple targets to rationally engineer and efficiently utilize non-natural carbon sources in industrial microorganisms were identified.

Previously, an efficient d-xylose utilizing Pseudomonas putida S12 strain was obtained by introducing the d-xylose isomerase pathway from Escherichia coli, followed by evolutionary selection. In the present study, systemic changes associated with the evolved phenotype were identified by transcriptomics, enzyme activity analysis, and inverse engineering. A key element in improving the initially poor d-xylose utilization was the redistribution of 6-phospho-D-glucuronate (6-PG) between the Entner-Doudoroff pathway and the oxidative pentose phosphate (PP) pathway. This redistribution increased the availability of 6-PG for oxidative decarboxylation to d-ribose-5-phosphate, which is essential for the utilization of d-xylose via the nonoxidative PP pathway. The metabolic redistribution of 6-PG was procured by modified HexR regulation, which in addition appeared to control periplasmic sugar oxidation. Because the absence of periplasmic d-xylonate formation was previously demonstrated to be essential for achieving a high biomass yield on d-xylose, the aberrant HexR control appeared to underlie both the improved growth rate and biomass yield of the evolved d-xylose utilizing P. putida strain. The increased oxidative PP pathway activity furthermore resulted in an elevated NADH/NAD+ ratio that caused the metabolic flux to be redirected from the TCA cycle to the glyoxylate shunt, which was also activated transcriptionally. Clearly, these findings may serve as an important case in point to engineer and improve the utilization of non-natural carbon sources in a wide range of industrial microorganisms.

The utilization of pentose sugars such as d-xylose and L-arabinose by industrial microorganisms is a major issue to be addressed in the quest for efficient bio-based production of fuels and chemicals (1–4). For this reason, much effort has been put into introducing this capacity into industrial production hosts like Saccharomyces cerevisiae, Zymomonas mobilis, and Corynebacterium glutamicum (5–12). In many cases, problems like redox imbalance and partly functional pentose phosphate (PP)3 pathways are encountered (2, 11–13). These problems illustrate the challenges associated with the pursuit of efficient utilization of non-natural carbon sources, which often requires extensive metabolic and/or regulatory rearrangements. Different approaches may be followed to achieve such systemic adjustments. The “rational approach” involves the introduction of targeted changes based on a design that presupposes detailed knowledge of the microbial system in terms of genetics, physiology, and metabolic networks (14). Alternatively, (parts of) metabolic pathways may be introduced after which the microbial host is subjected to evolutionary selection. This gives the system the opportunity to establish a new stable and optimized state after the perturbation caused by introducing foreign enzyme activities or pathways (10, 15, 16).

Such a semi-targeted approach was employed to obtain an efficient d-xylose utilizing strain of the solvent-tolerant bacterium Pseudomonas putida S12. This organism can be employed as a platform host for the production of aromatic compounds from renewable carbon sources like d-glucose and glycerol (17–22). However, since P. putida S12 lacks a d-xylose dissimilation
pathway, it is not able to produce these compounds from \( \text{D-xylose} \) and \( \text{L-arabinose} \) (23, 24). Because \( \text{D-xylose} \) is the second most abundant sugar in lignocellulosic materials, we previously introduced the \( \text{D-xylose} \) isomerase pathway from \textit{Escherichia coli}, which resulted in a strain that metabolized \( \text{D-xylose} \) via the PP pathway. Subsequent evolutionary selection resulted in substantial improvement of both growth rate and biomass-to-substrate yield (23). It was established that the absence of active \( \text{D-glucose dehydrogenase} \) accounted for most of the improved biomass yield on \( \text{D-xylose} \) (23). The molecular basis for the improved growth rate was not clarified, although the strongly improved growth rate on \( \text{D-xylose} \) indicated that the normally anabolic PP pathway had been transformed into an efficient catabolic route.

Due to the nature of the optimization procedure (by evolutionary selection), the molecular background of the improved phenotype was nonetheless largely obscure. Therefore, the evolved strain \textit{P. putida} S12xylAB2 was analyzed at the transcriptome level to gain more insight into the systemic changes associated with the improved \( \text{D-xylose} \) utilizing phenotype. Transcriptional changes revealed various important metabolic and regulatory rearrangements associated with the improved \( \text{D-xylose} \) utilization phenotype, which were verified by an inverse engineering approach. With these results we obtained further understanding of the effects brought about by the evolutionary selection. This may facilitate the design of effective artificial metabolic networks for the utilization of non-natural carbon sources in industrial microorganisms.

**EXPERIMENTAL PROCEDURES**

\textbf{Culture Conditions—}The strains and plasmids used in this study are shown in \textit{supplemental Table S1}. \textit{P. putida} S12xylAB1 was an engineered strain expressing the \( \text{D-xylose} \) isomerase pathway from \textit{E. coli} and was additionally optimized for enhanced \( \text{D-xylose} \) utilization by evolutionary selection (23). \textit{P. putida} S12xylIXAD is a transformant strain that expresses part of the oxidative \( \text{D-xylose} \) metabolic route from \textit{Caulobacter crescentus} (24). The media used were Luria broth (25) and a phosphate-buffered mineral salts medium, as described previously (26). In the mineral salts medium, 10 mM \( \text{D-glucose} \) (MMG) or 12 mM \( \text{D-xylose} \) (MMX) were used as sole carbon sources, unless stated otherwise. Biotin was added to a final concentration of 20 mg/liter for cultivation of S12xylIXAD. For expression of genes under control of the \textit{nagAa} promoter (like \textit{gtsA*} and \textit{gtsA*BCD}), 0.1 mM sodium salicylate was added as inducer. Antibiotics were added as required, in the following concentrations: gentamicin, 10 \( \mu \text{g/ml} \) for mineral salts medium, 30 \( \mu \text{g/ml} \) for Luria broth; kanamycin, 50 \( \mu \text{g/ml} \); tetracycline, 30 \( \mu \text{g/ml} \) for \textit{P. putida} S12, 10 \( \mu \text{g/ml} \) for \textit{E. coli}. Shaker flask experiments were performed in Boston flasks containing 20 ml of mineral salts medium in a horizontally shaking incubator at 30 °C

For chemostat cultivation, 1-liter fermentors were employed with a BioFlo110 controller (New Brunswick Scientific) containing MMG or MMX. The working volume of the cultures was kept constant at 0.7 liter by continuously removing culture broth. The pH was maintained at 7.0 by automatic addition of 2 N \( \text{NaOH} \) and the temperature was set at 30 °C. Dissolved oxygen concentrations were kept at 15% air saturation by automatically adjusting the agitation speed. As an inoculum, 35 ml of a late log-phase preculture in MMG or MMX was used. The dilution rate (D) was initially set at 0.05/h until an \( A_{600} \) of 1.5 was reached, after which it was gradually increased to a final value of 0.1 or 0.2/h, depending on the strain and medium employed. On MMG, the final D was set at 0.2/h for each tested strain. On MMX, the final D was set at 0.2/h for strain S12xylAB2 and 0.1/h for strain S12xylIXAD. The latter strain could not be maintained on MMX at \( D = 0.2/h \), which is in agreement with the low growth rate observed in MMX-grown shaker flasks (23, 24). Transcript profiles of \textit{P. putida} S12xylIXAD were almost identical in MMG-grown chemostats at \( D = 0.1/h \) and 0.2/h and hence, transcriptome profiles at \( D = 0.1/h \) and 0.2/h could be safely compared. Cultures were considered to be at steady state when, at least after 5 volume changes, no changes were observed in the carbon source concentration (<50 \( \mu \text{M} \), cell density, and agitation speed.

\textbf{Microarray Analysis—}Transcriptome analyses were performed on steady-state, carbon-limited chemostat cultures (for details, see \textit{supplemental Data S1}). Sampling from steady-state chemostat cultivations, mRNA isolation, and cDNA preparation for transcriptome analysis were performed as described previously (27). The microarrays used were custom-made high-density microarrays based on the genome sequence of \textit{P. putida} S12.\(^4\) The end-labeled cDNA fragments were hybridized to the microarray according to standard manufacturer’s protocols. The hybridized arrays were scanned by ServiceXS (Leiden, The Netherlands) on a high resolution Gene Chip Scanner 3000 7G system with autoloader (Affymetrix) using standard default analysis settings (filter, 570 nm; pixel size, 2.5 \( \mu \text{m} \)). The resulting data were imported into Genespring GX software package version 7.3.1 (Agilent Technologies) using the GC RMA algorithm. After normalization of the data, one-way analysis of variance (\( p < 0.05 \)) was used to select genes that changed significantly between the conditions tested.

\textbf{Construction of Expression Plasmids—}Plasmid pBNNmcst(Km) was constructed as follows. The chloramphenicol (Cm) marker from pBBR1mcst was amplified with primers 1 and 2 (\textit{supplemental Table S2}) as an AvaI/MluI fragment into MluI/\( \text{Km} \) as described previously (27). The microarrays used were custom-made high-density microarrays based on the genome sequence of \textit{P. putida} S12.\(^4\) The end-labeled cDNA fragments were hybridized to the microarray according to standard manufacturer’s protocols. The hybridized arrays were scanned by ServiceXS (Leiden, The Netherlands) on a high resolution Gene Chip Scanner 3000 7G system with autoloader (Affymetrix) using standard default analysis settings (filter, 570 nm; pixel size, 2.5 \( \mu \text{m} \)). The resulting data were imported into Genespring GX software package version 7.3.1 (Agilent Technologies) using the GC RMA algorithm. After normalization of the data, one-way analysis of variance (\( p < 0.05 \)) was used to select genes that changed significantly between the conditions tested.

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strains S12xylAB2. The PCR fragments were subsequently ligated into vector pBNNmcs(t)(Km) using the restriction sites listed in supplemental Table S2. The resulting plasmids were named pBNNmcsA and pBNNmcsABC.D.

The gnd and hexR genes were amplified by PCR using genomic DNA from P. putida S12 as the template and oligonucleotide primers 6–9 (supplemental Table S2). The resulting DNA fragments were ligated into vector pBTmcs (28) using the corresponding restriction sites listed in supplemental Table S2. The resulting plasmids were designated pBTgnd and pBThexR.

Recombinant plasmid pTXylAB_tkt-tal was constructed by cloning the tktA-tal genes from P. putida S12 into plasmid pTXylAB (23). The tktA and tal genes were amplified by PCR using oligonucleotide primers 13–16 (supplemental Table S2). First, tktA was ligated into vector pTXylAB using restriction sites Bsp120I and NotI. Subsequently, tal was ligated into plasmid pTXylAB_tkt using restriction sites NotI and XmaJI, yielding plasmid pTXylAB_tkt-tal.

Construction of Knock-out Mutants—P. putida S12 knock-out mutants were constructed as described previously (23). Primers used for amplification of the flanking regions of target genes are presented in supplemental Table S2. Gene replacement vectors for edd (6-phosphogluconate dehydratase), eda (2-keto-3-deoxy-6-phosphogluconate aldolase), aceA (isocitrate lyase), gtsABCD (d-glucose ABC-transporter), and hexR (regulatory) genes were constructed in pJQ200SK (29). These vectors were used to delete or interrupt selected genes in wild-type P. putida S12 and P. putida S12xylAB2 by homologous recombination. After confirming deletion of the target gene by PCR and curing the knock-out strain from the antibiotic marker using the cre-loxP system (30, 31), xylAB was introduced into the mutant strains to construct the strains listed in supplemental Table S1.

DNA Techniques—Genomic DNA was isolated using the FastDNA kit (Qiogene). Plasmid DNA was isolated with the QIAprep spin miniprep kit (Qiagen). DNA concentrations were measured with an ND-1000 spectrophotometer (Nanodrop). Agarose-trapped DNA fragments were isolated with the QIAEXII gel extraction kit (Qiagen). PCRs were performed with Accuprime PfX polymerase (Invitrogen) according to the manufacturer’s instructions. Plasmid DNA was introduced into electrocompetent cells using a Gene Pulser electroporation device (Bio-Rad). DNA sequencing reactions were performed by Eurofins MWG Operon (Ebersberg, Germany).

Analytical Methods—Optical densities were measured at 600 nm (A600) using an Ultrospec Cell Density Meter (Amersham Biosciences). An optical density of 1.0 corresponds to a cell dry weight of 0.49 g/liter. Sugars and organic acids were analyzed by ion chromatography (Dionex ICS3000 system) as described previously (23).

NAD⁺ and NADH concentrations were measured using the EnzyChrom™ NAD⁺/NADH Assay Kit (BioAssay Systems) according to the suppliers’ instructions. Samples were taken from mid-log phase cultures and prepared following the manufacturer’s instructions. The assay is based on an alcohol dehydrogenase cycling reaction, in which the tetrazolium dye 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is reduced by NADH in the presence of phenazine methosulfate. The color intensity of the reduced product, measured at 565 nm, is proportional to the NADH/NAD⁺ concentration in the sample. The absorbance was measured in 96-well plates using a TECA.IN Infinite 200 microplate reader.

Enzyme Assays—Cell extracts for enzyme assays were prepared by sonication of 5 ml of concentrated cell suspensions (0.9 g/liter cell dry weight in 100 mm Tris-HCl buffer, pH 7.5) from overnight cultures. Cell debris was removed by centrifugation and supernatants were desalted using PD-10 desalting columns (GE Healthcare) prior to activity assays.

The activity of 6-phosphogluconate dehydrogenase was determined spectrophotometrically by continuously measuring NADH or NADPH formation at 340 nm, using 6-phosphogluconate as substrate. The assays were performed at 30 °C, in a total volume of 1 ml. The assay mixture contained 100 mm Tris-HCl buffer (pH 7.5), 2.0 mm NAD⁺ or NADPH and cell extract. The reaction was started by adding 6-phosphogluconate to the reaction mixture to a final concentration of 1.0 mm.

RESULTS

Rearrangement of Central Carbon Metabolism Facilitates Efficient D-Xylose Utilization—To identify the metabolic changes associated with the improved D-xylose utilizing phenotype of the evolved strain P. putida S12xylAB2, which metabolizes D-xylose via the PP pathway, transcriptomic profiles were determined in steady-state chemostats on D-xylose as the sole carbon source. The nonevolved D-xylose-utilizing strain P. putida S12xylAB2, which metabolizes D-xylose oxidatively via the TCA cycle (24), was employed as a control strain. A thorough comparison of these strains was expected to reveal transcriptional effects specifically associated with (optimized) D-xylose utilization via the D-xylose isomerase/PP pathway. In addition, all transcriptomic profiles from D-xylose-grown cultures were compared with profiles of D-glucose-grown cultures, to identify generic effects associated with growth on D-xylose, or with the evolutionary selection procedure. The key findings from the transcriptome comparisons are summarized below; an overview of all differentially expressed genes (fold-change ≥2) is provided in supplemental Data S1. For a schematic representation of the central carbon metabolism of P. putida S12, please refer to Fig. 1.

Up-regulation of Pentose Phosphate Pathway—P. putida S12xylAB2 converts D-xylose into D-xylulose 5-phosphate (Xu5P) via the introduced D-xylose isomerase pathway. Because Xu5P is further metabolized via the nonoxidative branch of the PP pathway (23), the observed up-regulation of PP pathway genes tktA and tal was expected (Table 1, Fig. 1). However, the extent of up-regulation was rather modest.

Also the genes of the oxidative branch of the PP pathway (gnd, zwf-2) were up-regulated. In the oxidative PP pathway, 6-phospho-D-gluconate (6-PG) dehydrogenase (encoded by gnd) catalyzes the oxidative decarboxylation of 6-phospho-D-gluconate to D-ribulose 5-phosphate (Ru5P). Ru5P is subsequently isomerized to D-ribose 5-phosphate (Ri5P) by Ru5P isomerase. Thus, the oxidative PP pathway is not directly involved in Xu5P metabolism, but it does provide Ri5P that, in
addition to Xu5P, is required to maintain nonoxidative PP pathway fluxes (Fig. 1). Although the gnd gene was up-regulated to a relatively limited extent (Table 1), the 6-PG dehydrogenase activity increased from 40 units/g in nonevolved P. putida S12xylAB, to 83 units/g in strain S12xylAB2 (Table 2). Also the gene encoding Ru5P isomerase (rpiA) was up-regulated. It may be noted that Ru5P can also be produced by direct epimerization of Xu5P. However, the associated rpe gene was slightly down-regulated in strain S12xylAB2 (Table 1), which suggested a minor role for this conversion.

Redistribution of 6-PG between PP and ED Pathways—6-Phospho-D-gluconate is the central metabolite of hexose metabolism in Pseudomonads, which is metabolized almost exclusively via the Entner-Doudoroff (ED) pathway (32–33). During growth on D-xylose, however, 6-PG appeared to be the major source of Ri5P in P. putida S12xylAB2 as argued above. This implies that part of the 6-PG pool must be redirected from the ED pathway to the oxidative branch of the PP pathway (see also Fig. 1).

The supply of 6-PG is controlled genetically by zwf-1 and pgl genes, which are part of the eda operon. The demand for 6-PG in the ED pathway, on the other hand, is controlled by edd. A redistribution of the 6-PG pool between the PP and ED pathways may therefore be achieved by tight control of edd expression, whereas maintaining or increasing the zwf-1 and pgl expression levels.

Both eda operon genes and edd were down-regulated during growth on D-xylose (compared with D-glucose), in P. putida S12xylAB2 as well as P. putida S12xylXAD (Table 1). However, the extent to which these genes were down-regulated and, moreover, their relative expression levels, differed considerably between these two D-xylose utilizing strains. The results clearly show that the eda operon genes were up-regulated relative to the edd gene in the evolved strain. Moreover, when transcript levels were compared between D-glucose grown P. putida S12xylAB2 and D-glucose grown P. putida S12pJTmscs (non-D-xylose-utilizing empty vector control), up-regulation of the eda operon genes was observed (Table 1). Thus, the evolutionary

FIGURE 1. Simplified overview of the central carbon metabolism in P. putida S12. Only metabolic conversions relevant for this study are depicted. Black arrows indicate key metabolic conversions involved in the D-xylose metabolism of P. putida S12; gray arrows indicate conversions that are of minor importance for the D-xylose metabolism. Genes are represented in italics. The abbreviations used are: G6P, D-glucose 6-P; F6P, fructose 6-P; GAP, glyceraldehyde 3-P; PEP, phosphoenolpyruvate; Ac-CoA, acetyl-CoA; OGA, 2-ketogluconate; OAA, oxaloacetate; gcd, D-glucose dehydrogenase; zwf, D-glucose-6-P 1-dehydrogenase; pgl, phosphoglucuronolactonase; gnd, 6-phospho-D-gluconate dehydratase; rpiA, D-ribose-5P isomerase; rpe, D-ribulose-5-P epimerase; tktA, transketolase; tal, transaldolase; pgi, phosphoglucoisomerase; edd, 6-phosphogluconate dehydratase; eda, 2-keto-3-deoxy-6-phospho-D-gluconate aldolase; gap-1, glyceraldehyde 3-P dehydrogenase; aceA, isocitrate lyase; glcB, malate synthase; mdh, malate dehydrogenase.
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**TABLE 1**
Differentially expressed genes in D-xylose-utilizing strains of *P. putida* S12

| Gene name | Function | S12xyIAB2 vs. D-xylose vs. D-glucose | S12xyIAXD vs. D-xylose vs. D-glucose | S12xyIAB2 vs. S12xyIAXD | S12xyIAB2 vs. S12IPTncs vs. D-glucose |
|-----------|----------|-------------------------------------|--------------------------------------|------------------------|--------------------------------------|
| *edd* operon | | 0.08 | 0.09 | 0.90 | | |
| *gpd* | Glyceraldehyde-3P dehydrogenase | 0.20 | 0.12 | 1.44 | | |
| *gltB* | D-Glucose transporter activator | 0.33 | 0.14 | 2.03 | | |
| *glsABC* | D-Glucose ABC-transporter | 0.28 | 0.14 | 1.84 | | |
| *oprB* | Outer membrane porin | 1.49 | 0.34 | 3.38 | | |
| *hexR* | Transcriptional regulator D-glucose metabolism | 0.33 | 0.18 | 1.68 | | |
| *eda* operon | | 0.33 | 0.07 | 4.15 | 1.63 |
| *zwf* | D-Glucose-6P 1-dehydrogenase | 0.36 | 0.06 | 5.42 | 1.73 |
| *pgl* | D-Glucolactonase | 0.36 | 0.02 | 12.2 | 1.68 |
| *pp* pathway | | 0.10 | 1.60 | | |
| *gnd* | D-Glucose-6P dehydrogenase | 1.07 | 0.62 | 1.80 | | |
| *tkiA* | Transketolase | - | - | 1.24 | | |
| *tal* | Transaldolase | - | - | 1.97 | | |
| *rpe* | D-Ribulose-5P 3-epimerase | 0.89 | - | 0.94 | | |
| *epia* | D-Ribose-5P isomerase | 1.16 | - | 1.20 | | |
| Glycolate shunt | | &nbsp; | &nbsp; | &nbsp; | &nbsp; |
| *aceA* | Isocitrate lyase | 5.62 | 4.80 | 1.10 | | |
| *gltA* | Malate synthase | 2.11 | 1.17 | 1.58 | | |
| Other | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; |
| *crp* | Catabolite repressor protein | 4.08 | 4.21 | 0.95 | | |
| *pqqA* | Coenzyme QQ synthetase protein A | 0.50 | 1.15 | 0.65 | | |
| *pqqB* | Coenzyme QQ synthetase protein B | 0.57 | 1.43 | 0.83 | | |
| *pqqC* | Coenzyme QQ synthetase protein C | 0.69 | 1.74 | 0.39 | | |
| *pqqD* | Coenzyme QQ synthetase protein D | 0.77 | 1.30 | 0.69 | | |
| *pqqE* | Coenzyme QQ synthetase protein E | 0.73 | 1.37 | 0.55 | | |
| *pqqF* | Coenzyme QQ synthetase protein F | 0.76 | 1.36 | 0.51 | | |

*Fold-change in expression level of *P. putida* S12xyIAB2 grown on D-xylose compared with D-glucose. Values below 1 represent down-regulation on D-xylose compared with D-glucose; values above 1 represent up-regulation on D-xylose compared with D-glucose.*

The differences in transcript levels described above clearly hinted at an intrinsically altered *eda* operon expression. Furthermore, the transcript levels of the *edd* operon genes showed a remarkable divergence in D-xylose-grown *P. putida* S12 and *P. putida* S12xyIAB2, in units/g protein. 1 unit represents the amount of enzyme that oxidizes 1 μmol of substrate per min. Values are the average of triplicate measurements ± S.D.

**TABLE 2**
6-Phospho-D-glucanolate dehydrogenase activities of wild-type *P. putida* S12 and evolved D-xylose utilizing *P. putida* S12xyIAB2

Activities were measured in cell extracts of D-glucose, respectively, D-xylose-grown cultures of *P. putida* S12 and *P. putida* S12xyIAB2, in units/g of protein. 1 unit represents the amount of enzyme that oxidizes 1 μmol of substrate per min. Values are the average of triplicate measurements ± S.D.

| Strain and C-source | D-Glucose | D-Xylose |
|---------------------|------------|----------|
|                      | NAD* | NADP* | NAD* | NADP* |
| *P. putida* S12xyIAB2 | 43.1 ± 1.8 | 6.3 ± 0.9 | 39.6 ± 1.5 | 5.8 ± 1.1 |
| *P. putida* S12xyIAB2 | 87.6 ± 6.6 | 12.4 ± 2.2 | 83.4 ± 5.4 | 13.7 ± 2.9 |

The differences in transcript levels described above clearly hinted at *edd* and *eda*-related causes for the discrepancies between the evolved and nonevolved phenotypes of D-xylose utilizing *P. putida* S12. This was confirmed by the growth behavior of *edd* and *eda* deletion mutants (Table 3). In the nonevolved strain, deletion of *edd* resulted in the inability to utilize D-glucose, D-gluconate, or 2-keto-D-glucanolate. This defect could not be attributed to the associated interruption of the ED pathway, as deletion of *eda* did not show a similar effect. In the absence of a functional ED pathway, 6-PG should be metabolized via oxidative decarboxylation in the PP pathway (Fig. 1). Thus, the effect of the *edd* deletion may be attributed to the inability to produce 2-keto-3-deoxy-6-phospho-D-glucanolate (KDPG; Fig. 1), which is a known inducer of the *edd* and *eda* operons (34, 35). It should be noted that hexose metabolism via the PP pathway requires transcription of additional *edd* and *eda* operon genes, as these encode D-glucose transport (gtsABC, *oprB*), D-glucose phosphorylation (*glk*), and oxidation of Glc-6-P to 6-PG (zwf-1, *pgl*). Apparently, KDPG was essential for induction of the *eda* and/or *edd* operons in the nonevolved strain. The evolved strain, however, retained the ability to utilize hexoses upon deletion of *edd*, showing that the “KDPG effect” was lost, or less stringent, in *P. putida* S12xyIAB2.

An unexpected observation was the inability of both *edd* and *eda* deletion mutants of *P. putida* S12xyIAB2 to utilize D-xylose (Table 3). The deleted genes are clearly not essential for growth on pentoses, as the deletion mutants were able to utilize D-ribose (Table 3). Addition of D-ribose could furthermore relieve the inability to utilize D-xylose for growth.

The up-regulation of the *eda* operon genes relative to *edd*, in addition to the up-regulation of zwf-2, suggested that the supply of 6-PG exceeded the demand of the ED pathway in *P. putida* S12xyIAB2 during growth on D-xylose. The resulting surplus of 6-PG may then be employed to replenish Ri5P via the oxidative PP pathway branch, establishing the metabolic redistribution of 6-PG as described above. The apparently aberrant role of KDPG in *P. putida* S12xyIAB2 indicated that the transcriptional changes of the *edd* and *eda* operons may be the result of a modified transcription control.
**TABLE 3**

**Growth parameters of d-xylose utilizing deletion mutants of evolved and nonevolved *P. putida* S12**

Values represent the biomass-to-substrate (*Y*<sub>ab</sub>) yield in shake-flask cultures, in cmol % (cmol of CDW/cmol of substrate). The numbers in parentheses indicate the time (h) needed to reach the maximum *Y*<sub>ab</sub>. Values are the average of duplicate measurements. The maximum deviation to the average was omitted for clarity, but was always less than 5% of the averaged value.

| Strain<sup>a</sup> | d-Glucose | d-Xylose | d-Gluconate | 2-Keto-d-gluconate | d-Ribose |
|-------------------|-----------|----------|-------------|-------------------|---------|
| S12               | 53 (24)   | NG<sup>a</sup> | 64 (24)     | 58 (24)           | 57 (120) |
| S12ΔgtsABCD       | 51 (24)   | NG       | 61 (24)     | 61 (24)           | 52 (120) |
| S12Δedd           | NG        | NG       | NG          | NG                | 32 (216) |
| S12ΔxylA           | 43 (48)   | NG       | 69 (24)     | 64 (24)           | 53 (192) |
| S12ΔaceA           | 50 (24)   | NG       | 67 (24)     | 61 (24)           | 50 (144) |
| S12ΔxylAB2         | 44 (24)   | 67 (24)  | 61 (24)     | 43 (24)           | 60 (120) |
| S12ΔxylAB2ΔgtsABCD| 37 (48)   | NG       | 67 (48)     | 65 (24)           | 59 (120) |
| S12ΔxylAB2Δedd     | 32 (120)  | NG       | 36 (144)    | 41 (96)           | 35 (336)<sup>b</sup> |
| S12ΔxylAB2ΔaceA    | 36 (120)  | NG       | 37 (144)    | 42 (168)          | 52 (168) |
| S12ΔxylAB2ΔaceA    | 39 (48)   | 20 (72)  | 70 (48)     | 65 (24)           | 60 (120) |

<sup>a</sup> All strains carried the *xylAB* genes from *E. coli* DH5α on plasmid pJT1-xylAB.

<sup>b</sup> NG = no growth detected after 240 h. A biomass-to-substrate yield of less than 10 cmol % was regarded as no growth.

<sup>c</sup> Cultivations on d-ribose were prolonged after 240 h because the growth rate on this pentose is intrinsically very low.

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**D-Xylose Import: Involvement of d-Glucose ABC Transporter**—
As discussed above, the *gtsABCD* and *oprB-1* genes were up-regulated in *P. putida* S12ΔxylAB2 during growth on d-xylose, in contrast to other genes of the *edd* operon (Table 1). The up-regulated genes encode the d-glucose ABC transporter and the periplasmic porin OprB-1, which is able to transport d-xylose into the periplasm as previously reported for *Pseudomonas aeruginosa* (36). Whereas growth of *P. putida* S12ΔxylAB2 on d-xylose was only marginally affected by deletion of *gtsABCD* (Table 3), growth on d-xylose was completely eliminated, suggesting that d-xylose was imported via the d-glucose ABC transporter. Moreover, sequence analysis of the *gtsABCD* genes in strain S12ΔxylAB2 revealed two mutations in the gene encoding the sugar binding domain (*gtsA*), resulting in amino acid substitutions T74A and P85L (supplemental Fig. S1). These substitutions may result in improved affinity for d-xylose, but this is subject to further investigation. Overexpressing the mutant d-xylose transporter (*gtsA<sup>B</sup>BCD*) in the nonevolved *P. putida* S12ΔxylAB2 severely affected growth on both d-xylose and d-glucose, suggesting that overexpression of the transporter compromised the general fitness of the strain.

**Up-regulation and Activation of Glyoxylate Shunt**—The genes encoding isocitrate lyase (*aceA*), malate synthase (*gltB*), and malate dehydrogenase (*mdh*) were highly up-regulated in d-xylose-grown *P. putida* S12ΔxylAB2 (Table 1), suggesting an active glyoxylate shunt. In addition, an elevated NADH/NAD<sup>+</sup> ratio was observed in d-xylose-grown cells. This condition typically inhibits the activity of isocitrate dehydrogenase, forcing the isocitrate flux toward the glyoxylate bypass (37). The elevated NADH level likely resulted from the increased oxidative PP pathway activity in *P. putida* S12ΔxylAB2, as the 6-PG dehydrogenase was found to prefer NAD<sup>+</sup> over NADP<sup>+</sup> (Table 2). The importance of the glyoxylate shunt for efficient phosphor-lytic d-xylose metabolism was confirmed by the severely decreased yield and growth rate on d-xylose upon deletion of *aceA* in *P. putida* S12ΔxylAB2 (Table 3).

**Down-regulation of PQQ Biosynthesis**—A number of genes encoding PQQ biosynthetic enzymes were down-regulated in *P. putida* S12ΔxylAB2 (Table 1). PQQ is the cofactor of d-glucose dehydrogenase, which was found to be inactive during the evolutionary selection of strain S12xylAB2. The inactivity of d-glucose dehydrogenase was found to be the major cause of the improved biomass yield of strain S12ΔxylAB2 on d-xylose (23), and some unclarified post-translational effect was proposed to be involved. This unclarified effect may be associated with the down-regulation of PQQ biosynthesis, because binding of PQQ is essential for constituting an active enzyme (38).

**Regulatory Effects Associated with Improved d-Xylose-utilizing Phenotype**—The redistribution of the 6-PG pool between the ED and PP pathways appeared to be a key element of the improved d-xylose-utilizing phenotype of *P. putida* S12ΔxylAB2. In addition, the glyoxylate shunt was shown to play an important role, as well as changes relating to d-xylose import and PQQ biosynthesis. These factors appear to characterize most of the improved d-xylose-utilizing phenotype at the functional level, but do not provide any insight into the regulatory mechanisms behind these changes. Therefore, we specifically mined the transcriptomics dataset for transcriptional changes in, or related to, regulatory genes.

A notable change was observed in the expression of *crp*, encoding the catabolite repression protein Crp (39). The high-level up-regulation of *crp* during growth on d-xylose in *P. putida* S12ΔxylAB2 as well as S12ΔxylXAD (Table 1) clearly illustrated the system-wide derangement provoked by enforcing growth on a non-natural carbon source. In addition, *hexR* was clearly down-regulated in both *P. putida* S12ΔxylAB2 and S12ΔxylXAD during growth on d-xylose, suggesting a generic response associated with growth on d-xylose as observed for *crp*. Down-regulation was less severe, however, in *P. putida* S12ΔxylAB2 (Table 1). The *hexR* gene encodes the key regulator of d-glucose metabolism (34, 35), HexR, through which KDPG exerts its derepressing effect (34, 40). The apparently altered impact of KDPG on transcription of the *eda* and *edd* operons in *P. putida* S12ΔxylAB2 (see above), combined with the relatively mild down-regulation of *hexR* during growth on d-xylose, suggested an important role of HexR (de-)regulation in the improved d-xylose utilizing phenotype.

To obtain more insight into the role of *hexR*, the gene was deleted in the nonevolved d-xylose-utilizing parent of strain S12ΔxylAB2, *P. putida* S12ΔxylAB (23). The growth rate of the resulting strain S12ΔhexR_xylAB was considerably decreased...
on D-xylose (Table 4). However, the biomass yield on D-xylose was improved by nearly a factor 4, to 52.9 cmol % (Table 4), which even exceeded the biomass yield achieved by the gcd knock-out strain P. putida S12Δgcd_xylAB (46.5 cmol %; Table 4). This result strongly suggested that the periplasmic oxidation of D-xylose was affected by deletion of hexR. Production of D-xylanate was indeed absent in D-xylose-grown P. putida S12ΔhexR_xylAB cultures, whereas the introduction of an episomal copy of hexR fully restored D-xylanate formation. It may therefore be concluded that there is a, so far unobserved, connection between HexR and periplasmic sugar oxidation. The nature of this connection appears to be indirect, because an effect of HexR on gcd transcription may be excluded based on previous findings in P. putida KT2440 (35).

Redistribution of 6-PG Pool by Inverse Engineering—HexR deregulation appeared to be an important feature in establishing efficient D-xylose utilization by P. putida S12. However, the dramatically decreased growth rate caused by deleting hexR in the nonevolved strain demonstrated that simply eliminating HexR-controlled repression will not establish efficient growth on D-xylose. This observation confirmed our assumption that tight control of edd transcription by HexR is essential for proper distribution of 6-PG between the ED and PP pathways.

To establish whether the absence of HexR-control could be counteracted by stimulating the flux of 6-PG to the PP pathway, genes encoding both the oxidative and nonoxidative PP pathway branches were overexpressed in the (nonevolved) P. putida S12ΔhexR_xylAB. Overexpression of gnd decreased the time required to fully consume 12 mM D-xylose from 13 to 8 days (Table 4). This supported the notion that Ri5P availability limited the growth rate on D-xylose in the hexR knock-out strain. The overexpression of gnd, however, also resulted in a considerably decreased biomass yield (Table 4). This could likely be attributed to the very high 6-phospho-D-gluconate dehydrogenase activity (3275 ± 291 units/g of protein), which was 37-fold higher than in P. putida S12xylAB2 (Table 2). A correspondingly high flux of 6-PG to Ri5P would obviously lead to extensive loss of carbon via CO₂ formation.

Overexpression of tktA and tal in P. putida S12ΔhexR_xylAB was more effective than gnd overexpression in improving growth on D-xylose: the time required to consume 12 mM D-xylose was reduced to only 4 days, whereas the biomass yield was unchanged or even slightly improved (Table 4). Apparently, the drain on Ri5P caused by overexpression of the nonoxidative PP pathway stimulated replenishment via the oxidative branch without the negative effect on the biomass yield associated with gnd overexpression. When gnd was overexpressed in addition to tktA-tal, the growth performance was heavily affected: 8 days were required to consume 12 mM D-xylose and the biomass yield dropped to 25.9 cmol %.

DISCUSSION

The molecular background of improved D-xylose utilization by an engineered and evolutionarily selected mutant of P. putida S12 was characterized by a combination of system-wide analysis and inverse engineering. Multiple systemic changes were identified that had apparently accumulated under selective pressure for efficient utilization of this unnatural carbon source. Metabolic redistribution of the 6-PG pool appeared to contribute most to the improved growth rate on D-xylose, increasing the availability of 6-PG for the oxidative PP pathway at the expense of the ED pathway. Thus, supply of Ri5P to the nonoxidative PP pathway was ensured, enabling efficient metabolism of D-xylose via Xu5P. Furthermore, we found indications for improved D-xylose import, via the mutated and up-regulated D-glucose ABC-transporter. Expression levels of the nonoxidative PP pathway genes were only slightly elevated. However, as previously reported for E. coli (41), small transcriptional changes can significantly alter the PP pathway flux. Such an effect was also observed for gnd expression levels and the corresponding 6-PG dehydrogenase activity.

The increased NADH levels, probably resulting from the improved oxidative PP pathway activity, provoked the redirection of the isocitrate flux to the glyoxylate bypass. Historically, the oxidative PP pathway is believed to be associated with NADPH formation. Recent insights, however, show that a preference for NAD⁺, as observed for P. putida S12, is actually quite common (42). The associated metabolic rearrangement bypasses two CO₂ generating steps of the TCA cycle, adding to the already improved biomass yield caused by the inactive periplasmic sugar oxidation pathway (23). Redistribution of the metabolic flux to the glyoxylate shunt through elevated NADH levels was further sustained by up-regulation of the associated genes in P. putida S12xylAB2. In E. coli, these genes are con-
controlled by the cAMP-CRP complex (43), suggesting that the observed up-regulation of crp accounts for this transcriptional effect.

Modified transcriptional control by HexR, the key regulator of hexose metabolism, appeared to underlie many of the observed changes at the metabolic level. In the evolved strain, the eda operon was apparently deregulated, whereas edd appeared to be under regular HexR control. This partial deregulation is highly relevant for the engineered P. putida S12 that is forced to utilize d-xylose, because it has to cope with the unnatural situation that the PP pathway both demands and supplies 6-PG, via Fru-6-P. A high activity of the ED pathway would considerably reduce 6-PG availability for the PP pathway, leading to further reduction of 6-PG levels because supply is controlled by the PP pathway. This kinetic effect will be exacerbated at the transcriptional level under regular HexR control, as KDPG levels (produced by the ED pathway) will be low when 6-PG is scarce, leading to reduced transcription of the genes encoding the ED pathway as well as the oxidative PP pathway. This vicious cycle can be interrupted by deregulating the eda operon while maintaining HexR control on edd transcription. Low KDPG levels will only lead to down-regulation of the ED pathway, securing supply of 6-PG via the PP pathway. This appeared to be the case in P. putida S12xylAB2, resulting in a stable, self-sustaining redistribution of 6-PG between the ED and PP pathways.

Aberrant HexR control in P. putida S12xylAB2 may also be responsible for the divergent regulation of the edd operon genes, resulting in the up-regulation of gtsABCD and oprB1 that were shown to be involved in d-xylose import. Moreover, HexR affected the periplasmic sugar oxidation, probably via transcriptional control of PQQ biosynthesis genes. Down-regulation of PQQ biosynthesis would explain inactivation of the periplasmic sugar oxidation pathway in P. putida S12xylAB2. The exact mechanism of the altered HexR control remains to be clarified, because no mutations were found in the hexR gene itself, or in the promoter regions of the eda and edd operons.

Efficient d-xylose utilization could not be achieved by simple targeted deletion or overexpression of genes that were found to be differentially expressed in P. putida S12xylAB2. Still, these inverse engineering attempts provided valuable insights into the, clearly subtle, metabolic and regulatory changes that were responsible for the optimized d-xylose utilizing phenotype. Although some of the metabolic targets identified in the present study are specific for ED pathway-dependent microorganisms, the generic principles may also be exploited to improve the utilization of non-natural carbon sources by glycolytic microorganisms. Here, the metabolic flux should be controlled at the level of Glc-6-P, rather than 6-PG, as the central node of sugar metabolism. Consequently, phosphoglucose isomerase (Pgi), rather than Edd, may be targeted. Thus, leads for engineering and improving the utilization of non-natural carbon sources were identified that apply to a wide range of industrial microorganisms, and that may contribute to the deployment of renewable, lignocellulosic feed stocks for efficient bioproduction of chemicals and fuels.

Acknowledgments—We thank Hendrik Ballerstedt for performing transcriptomics experiments and Karin Nijkamp for practical assistance. This work was carried out within the research program of the Kuyver Centre for Genomics of Industrial Fermentation, which is part of the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research.

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