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Engineering *Pseudomonas putida* S12 for Efficient Utilization of D-Xylose and L-Arabinose▽

Jean-Paul Meijnen,[1,2,3]* Johannes H. de Winde,[2,3] and Harald J. Ruijssenaars[1,2]

TNO-Quality of Life, Business Unit Food and Biotechnology Innovations, Julianalaan 67, 2628 BC Delft, The Netherlands[1]*; B-Basic, Julianalaan 67, 2628 BC Delft, The Netherlands*[2]*; and Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands*[3]

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The solvent-tolerant bacterium *Pseudomonas putida* S12 was engineered to utilize xylose as a substrate by expressing xylose isomerase (XylA) and xylulokinase (XylB) from *Escherichia coli*. The initial yield on xylose was low (9% [g CDW g substrate^{-1}], where CDW is cell dry weight), and the growth rate was poor (0.01 h^{-1}). The main cause of the low yield was the oxidation of xylose into the dead-end product xylonate by endogenous glucose dehydrogenase (Gcd). Subjecting the XylAB-expressing *P. putida* S12 to laboratory evolution yielded a strain that efficiently utilized xylose (yield, 52% [g CDW g xylose^{-1}]) at a considerably improved growth rate (0.35 h^{-1}). The high yield could be attributed in part to Gcd inactivity, whereas the improved growth rate may be connected to alterations in the primary metabolism. Surprisingly, without any further engineering, the evolved D-xylose-utilizing strain metabolized L-arabinose as efficiently as D-xylose. Furthermore, despite the loss of Gcd activity, the ability to utilize glucose was not affected. Thus, a *P. putida* S12-derived strain was obtained that efficiently utilizes the three main sugars present in lignocellulosic hydrolysate: glucose, xylose, and arabinose. This strain will form the basis for a platform host for the efficient production of biochemicals from renewable feedstock.

The increasing price of oil and imminent shortage of fossil fuels raise the necessity for the development of alternative technologies for the production of petrochemicals. The use of lignocellulosic biomass as feedstock for the chemical industry is a promising alternative that is being studied widely and extensively. Ethanol and other biochemicals are currently produced from glucose by organisms such as *Zymomonas mobilis* and *Saccharomyces cerevisiae*. Although glucose is the primary sugar in lignocellulosic biomass, a considerable fraction consists of xylose and arabinose, which can make up to 25% of the total sugar amount (14). Therefore, expanding the substrate range of whole-cell biocatalysts with these pentose sugars will greatly contribute to the economic feasibility of biochemical production from renewable feedstock.

Several approaches have been used to achieve the utilization of pentose sugars by whole-cell biocatalysts. Expressing xylose isomerase and/or xylulokinase, encoded by, respectively, *xylA* and *xylB*, has proven to be a successful strategy to enable phosphorylative growth on xylose (11, 12, 31). Also, genes encoding xylose reductase and xylitol dehydrogenase have been employed, especially for engineering yeast cells (6). Still, problems like redox imbalance or an incomplete pentose phosphate (PP) pathway have been encountered, hampering efficient xylose utilization (6, 31). In addition to xylose utilization, microorganisms have been engineered to utilize arabinose, e.g., by expressing the AraBAD pathway from *Escherichia coli* (10) or *Lactobacillus plantarum* (30). Ultimately, microorganisms should be engineered to efficiently and concomitantly utilize glucose, xylose, and arabinose to attain cost-effective production of biochemicals.

Our laboratory is developing *Pseudomonas putida* S12 as a platform for the production of chemicals from renewable feedstock via central metabolites as the precursor (17, 18, 26, 29). *P. putida* S12 is exceptionally tolerant to organic solvents (1), which makes this strain an excellent host for the production of chemicals that are generally toxic to other bacterial cells, such as substituted aromatic compounds. For these compounds, the use of mixtures of hexoses and pentoses as substrate may offer a specific advantage, as they are derived from the aromatic amino acids L-phenylalanine and L-tyrosine (17, 18, 26, 29). The key precursors for the aromatic amino acids are phosphoenol pyruvate and erythrose-4-phosphate, which are respectively derived efficiently from hexoses (via the Entner-Doudoroff pathway) and pentoses (via the PP pathway). The aim of this study was to construct a *P. putida* S12 strain that is capable of utilizing glucose, xylose, and arabinose to serve as an optimized host strain for efficient, green production of chemicals from renewable lignocellulose-derived feedstock.

MATERIALS AND METHODS

Culture conditions. The strains and plasmids used in this study are presented in Table 1. The media used were Luria broth (22) and a phosphate-buffered mineral salts medium described previously (8). In the mineral salts medium, 10 mM glucose (MMG), 12 mM xylose (MMX), or 12 mM arabinose (MMA) was used as a sole carbon source unless stated otherwise. Antibiotics were added as required, in the following concentrations: ampicillin, 100 μg ml^{-1} (*E. coli*); gentamicin, 30 μg ml^{-1} for Luria broth or 10 μg ml^{-1} for mineral salts medium; and kanamycin 50 μg ml^{-1}. Shake-flask experiments were performed in Boston bottles containing 20 ml MMG, MMX, or MMA in a horizontally shaking incubator at 30°C for *P. putida* S12 or 37°C for *E. coli*. For *E. coli*, 26.5 mg liter^{-1} thiamine was added to the mineral salts medium.

DNA techniques. Genomic DNA was isolated by using a DNeasy tissue kit (Qiagen). Plasmid DNA was isolated with a QIAprep spin miniprep kit (Qiagen). DNA concentrations were measured with an ND-1000 spectrophotometer

* Corresponding author. Mailing address: TNO-Quality of Life, Julianalaan 67, 2628 BC Delft, The Netherlands. Phone: (31) 15-2789871. Fax: (31) 15-2782355. E-mail: jean-paul.meijnen@tno.nl.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Source or reference
Strains		
E. coli DH5α	Wild type	Invitrogen
P. putida S12	Wild type	Hartmans et al. (9)
P. putida S12xylAB	*P. putida* S12 containing plasmid pJTxylAB	This study
P. putida S12xylAB_FGH	*P. putida* S12 containing plasmid pJTxylAB_FGH	This study
P. putida S12xylAB2	*P. putida* S12 containing plasmid pJTxylAB, evolved to efficient pentose utilizer	This study
P. putida S12xylAB2c	*P. putida* S12xylAB2 cured from pJTxylAB	This study
P. putida S12Δgcd	*P. putida* S12 glucose dehydrogenase knockout	This study
P. putida S12Δgcd_xylAB	*P. putida* S12 glucose dehydrogenase knockout containing plasmid pJTxylAB	This study
P. putida S12araFGH	*P. putida* S12 containing plasmid pBT*araFGH*	This study
P. putida S12xylAB_araFGH	*P. putida* S12 containing plasmids pJTxylAB and pBT*araFGH*	This study
Plasmids		
pJNTmcs(t)	Ap^r Gm^r; basic expression vector derived from plasmid pJWB1 (28) containing the salicylate-inducible promoter *nagR-nagAa*	Unpublished data
pJTTmcs	Ap^r Gm^r; expression vector containing the constitutive *tac* promoter, derived from pJWB1	Unpublished data
pJTmcs	Ap^r Gm^r; expression vector containing the constitutive *tac* promoter without the *tac* RBS	This study
pBTmcs	Cm^r; expression vector containing the constitutive *tac* promoter without the *tac* RBS	Unpublished data
pJTxylAB	pJTmcs containing the *xylAB* genes from *E. coli* DH5α	This study
pJTxylAB_FGH	pJTmcs containing the *xylAB* and *xylFGH* genes from *E. coli* DH5α	This study
pBTaraFGH	pBTmcs containing the *araFGH* genes from *E. coli* DH5α	This study
pJQ200SK	P15A *ori sacB* RP4 Gm^r(pBluescriptSK); suicide vector	Quandt et al. (20)
pJQgcd::Kana	pJQ200SK containing a Km^r-disrupted copy of the *gcd* gene	This study

[a] Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; *ori*, origin of replication; *sacB*, gene producing levansucrase.

(Nanodrop). Agarose-trapped DNA fragments were isolated with a 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 by using a Gene Pulser electroporation device. DNA sequencing reactions were performed by MWG Biotech AG.

qPCR. The mRNA levels of the glucose dehydrogenase (Gcd) gene *gcd* were analyzed by quantitative PCR (qPCR). Total RNA extractions were performed with an RNeasy kit (Qiagen). qPCR was performed with oligonucleotide primers 9 and 10 (Table 2) using mRNA of mid-log-phase samples of batch cultures with a spectrofluorimetric thermal cycler (iCycler thermal cycler equipped with optical module; Bio-Rad) using IQ Sybr green supermix (Bio-Rad) according to the manufacturer's protocols.

Construction of the expression vector pJTmcs. Expression vector pJTmcs was constructed by using pJTTmcs, formerly named pTac (18) (Table 1), as the backbone. PCR on pJTTmcs was carried out to amplify the plasmid fragment containing the *tac* promoter site, omitting the *tac* ribosomal binding site (RBS). The PCR product was cloned into vector pJTTmcs using restriction sites KpnI and ScaI. The resulting expression vector, pJTmcs, has the same characteristics as pJTTmcs but contains no *tac* RBS.

Construction of recombinant plasmids. *XylAB* was amplified by PCR using genomic DNA from *E. coli* DH5α as the template and oligonucleotide primers 1 and 2 (Table 2). The resulting 2.8-kb DNA fragment was ligated into vector pJTmcs using the restriction sites KpnI and NotI. The resulting plasmid was designated pJTxylAB.

The suicide vector pJQ200SK (20) was used to construct a gene replacement plasmid for the *gcd* gene as described below. Primers 3 to 6 (Table 2) were used to amplify 1,158 bp of the 5′ end (gcd_1) and 951 bp of the 3′ end (gcd_2) of the *gcd* gene. The kanamycin resistance gene, flanked by *loxP* recombination sites, was amplified by using primers 7 and 8 on pSK-kanalox (unpublished data) as the template. pJQ200SK was digested by using restriction enzymes NotI and BamHI, and gcd_1 and gcd_2 were digested with NotI/XbaI and XbaI/BamHI, respectively. The three resulting fragments were ligated in vector pJQ200SK to yield vector pJQgcd. pJQgcd was linearized with XbaI, and the cohesive ends were dephosphorylated. The *loxP-kanaR-loxP* fragment, digested with XbaI, was cloned into the linearized pJQgcd, yielding pJQgcd::kana. This vector was introduced into wild-type *P. putida* S12, and transformants were selected for kanamycin resistance. Double-crossover mutants were selected for kanamycin resistance (Km^r) and gentamicin sensitivity (Gm^s). The replacement of the native *gcd* by the

TABLE 2. Oligonucleotide primers used in this study

Primer	Target	Sequence (5′ → 3′)[a]	Characteristic
Primer 1	*xylA* from *E. coli*	GCGGCGGGTACCATGCAAGCCTATTTTGACC	KpnI cohesive end
Primer 2	*xylB* from *E. coli*	GCGGCGGCGGCCGCTTACGCCATTAATGGCAG	NotI cohesive end
Primer 3	5′ End of *gcd*	GCGGCGGCCGCTTACTCAGCTAATTTGTAAGCGAT	NotI cohesive end
Primer 4	Positions 1154–1134 in *gcd*	GCGTCTAGACCAACATGTGGTCGATCGCCA	XbaI cohesive end
Primer 5	Positions 1261–1281 in *gcd*	GCGTCTAGAGATCACCCCGGACGGCTCATT	XbaI cohesive end
Primer 6	3′ End of *gcd*	GCGGGATCCATGAGCACTGAAGGTGCGAACC	BamHI cohesive end
Primer 7	5′ End of *loxP*-Km^r-*loxP*	GCTCTAGAATAACTTCGTATAATGTATGCTATAC	XbaI cohesive end
Primer 8	3′ End of *loxP*-Km^r-*loxP*	CGCGCAATTAACCCTCACT	
Primer 9	Positions 2088–2106 in *gcd*	ACGGTAGCAGCAGTACCAC	qPCR primer
Primer 10	Positions 2266–2285 in *gcd*	TACTACCTGATCGCCGGTAT	qPCR primer
Primer 11	Positions 588–608 in *gcd*	AATGCGCCAGGCCTCTTCCAG	Sequencing primer
Primer 12	Positions 397–376 upstream of *gcd*	CGCCACCGTGCATGACAAGAAG	Sequencing primer

[a] The restriction sites used for cloning are underlined.

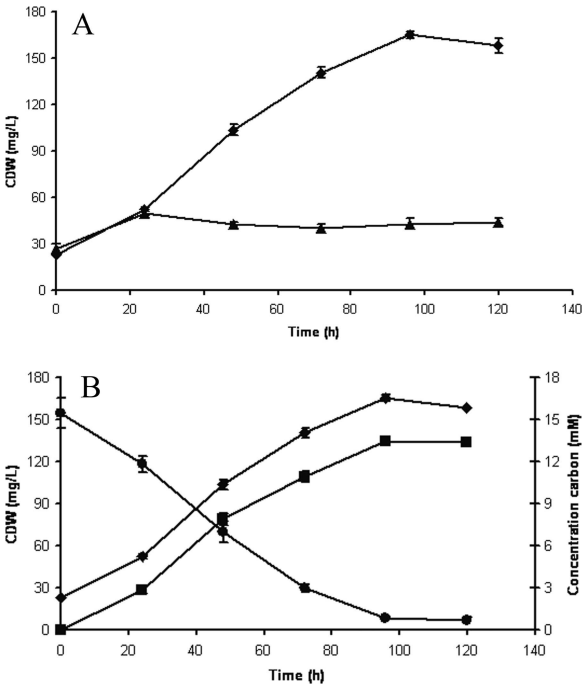

FIG. 1. (A) Growth of wild-type *P. putida* S12 (triangles) and *P. putida* S12xylAB (diamonds) cells in mineral salts medium with xylose as the sole carbon source. Data points are the averages of the results of duplicate measurements. Error bars represent the maximum deviations from the averages. (B) Xylonate (squares) and xylose (circles) concentrations in *P. putida* S12xylAB culture on MMX. *P. putida* S12xylAB cells were grown in mineral salts medium with xylose as the sole carbon source. CDW concentrations are presented by diamonds. Data points are the averages of the results of duplicate measurements. Error bars represent the maximum deviations of the averages. L, liter.

KanaR-disrupted *gcd* was confirmed by colony PCR using primers 3 and 6 and sequence analysis of the disrupted gene. Plasmid pJTNcre (unpublished data) was introduced to cure *P. putida* S12Δgcd from KanaR. This plasmid encodes the Cre recombinase that catalyzes the site-specific recombination at the *loxP* target sites by which the DNA fragment enclosed by the two *loxP* sites is removed, in this case KanaR (23, 25). Vector pJTNcre was removed by overnight culturing in nonselective Luria broth.

Analytical methods. Optical densities were measured at 600 nm using a Biowave cell density meter (WPA Ltd.). An optical density of 1.0 corresponds with a cell dry weight (CDW) of 465 mg liter^{-1}. Sugars and organic acids were analyzed by ion chromatography (Dionex ICS3000 system) using a CarboPac PA20 column with 10 mM NaOH as the eluent for sugars or an IonPac ICE AS6 column with 0.4 mM heptafluorobutyric acid as the eluent for organic acids. D-Xylulose-5-phosphate (Xu5P) was assessed by using a transketolase activity assay.

Enzyme activity assays. Cell extracts for enzyme assays were prepared by sonication of 5 ml of concentrated cell suspensions (0.9 g liter^{-1} CDW in 50 mM Tris-HCl buffer, pH 7.5) from overnight cultures. After centrifugation, supernatants were desalted by using PD-10 desalting columns (GE Healthcare). The resulting cell extracts were used for enzyme assays.

The activity of xylose isomerase (XylA) was determined as described by Gao et al. (5). In the assay, xylose isomerase activity is coupled to NADH consumption via sorbitol dehydrogenase. The assay was performed at 30°C in a total volume of 1 ml. The assay mixture contained 50 mM Tris-HCl buffer (pH 7.5), 10 mM $MgSO_4$, 1 mM triethanolamine, 0.5 U sorbitol dehydrogenase, 0.2 mM NADH, and cell extract. The reaction was started by adding xylose to a final concentration of 50 mM.

The xylulokinase activity was determined as described by Eliasson et al. (3). Xylulokinase activity is coupled to the consumption of NADH in the reduction of pyruvate to lactate by lactate dehydrogenase. The assay was performed at 30°C, in a total volume of 1 ml. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 2.0 mM $MgCl_2$, 2.0 mM ATP, 0.2 mM phosphoenolpyruvate, 10 U pyruvate kinase, 10 U lactate dehydrogenase, 0.2 mM NADH, and cell extract. The reaction was started by adding xylulose to a final concentration of 10 mM.

The transketolase activity was measured to demonstrate D-xylulose-5-phosphate formation from L-arabinose or D-xylose. Transketolase couples D-xylulose-5-phosphate to D-ribulose-5-phosphate to form glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate. The transketolase reaction is coupled to NADH consumption via glyceraldehyde-phosphate dehydrogenase. The assay was performed at 30°C, in a total volume of 1 ml. The reaction mixture contained 216 mM glycylglycine buffer (pH 7.7), 1.7 mM D-ribose-5-phosphate, 0.002% (wt/vol) cocarboxylase, 0.14 mM NADH, 15 mM $MgCl_2$, 2.0 mM ATP, 20 U α-glyceraldehyde-phosphate dehydrogenase–triosephosphate isomerase (based on triosephosphate isomerase units), 0.05 U transketolase, and cell extract. The reaction was started by adding xylose or arabinose to a final concentration of 50 mM.

The pyrroloquinoline quinone (PQQ)-dependent Gcd activity was determined as described by Liu et al. (16). The activity of Gcd was determined spectrophotometrically by measuring the decrease in the absorbance of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm. The assay was performed at 30°C, in a total volume of 1 ml. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 15 mM NH_4Cl, 80 μM DCPIP, 1 μM KCN, 0.33 mM phenazine methosulfate, and cell extract. The reaction was started by adding glucose to a final concentration of 1 mM.

For calculations of enzyme activities, the following molar extinction coefficients were used: 6.22 mM^{-1} cm^{-1} for NADH and 19 mM^{-1} cm^{-1} for reduced DCPIP. One unit is defined as the amount of enzyme that oxidizes 1 μmol of substrate per minute in the coupled assays described above.

RESULTS

Cloning and functional expression of *xylAB* in *P. putida* S12. Genes *xylA* and *xylB*, part of the *xyl* operon of *E. coli*, were cloned into expression vector pJTmcs under the transcriptional control of the constitutive *tac* promoter. The resulting vector, pJTxylAB, was introduced into *P. putida* S12, yielding *P. putida* S12xylAB. The results of enzyme assays confirmed that xylose isomerase and xylulokinase were expressed as functional enzymes. The specific activities were 34 U g^{-1} protein for xylose isomerase and 134 U g^{-1} protein for xylulokinase. The overall activity of XylAB was 22 U g^{-1}.

After demonstrating that XylAB were functionally expressed, *P. putida* S12xylAB was tested for its ability to utilize xylose as a carbon source. When strain S12xylAB was inoculated into xylose-containing medium, growth was observed. However, the biomass yield was low (9% [g CDW g substrate^{-1}]) (Fig. 1A) compared to the biomass yield on glucose (typically 55%). The growth rate was also much lower on xylose than on glucose (0.01 h^{-1} versus 0.5 h^{-1}). The same characteristics were found for strain S12xylAB_FGH, indicating that xylose transport was not limiting xylose utilization (Table 3). It

TABLE 3. Overview of growth characteristics of pentose-utilizing *P. putida* S12-derived strains[a]

Strain	Xylose		Arabinose	
	Biomass yield (%; g CDW g substrate^{-1})	Maximum growth rate (h^{-1})	Biomass yield (%; g CDW g substrate^{-1})	Maximum growth rate (h^{-1})
S12xylAB	9	0.01	NG	NG
S12xylAB2	52	0.35	52	0.35
S12xylAB2c	NG	NG	NG	NG
S12Δgcd_xylAB	44	0.01	ND	ND
S12xylAB_FGH	10	0.01	NG	NG
S12xylAB_araFGH	14	0.01	13	0.01
S12araFGH	ND	ND	NG	NG

[a] NG, no growth; ND, not determined.

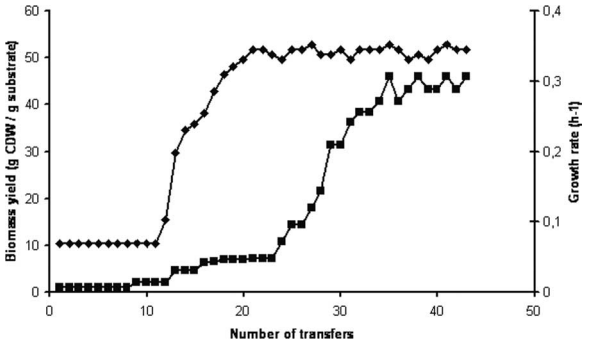

FIG. 2. Laboratory evolution of *P. putida* S12xylAB. Transformant cells were repeatedly transferred into fresh xylose-containing medium in order to optimize biomass yield (diamonds) and growth rate (squares) on MMX.

was observed previously that *P. putida* S12 oxidizes xylose to xylonate, a reaction shown to be catalyzed in other *P. putida* strains by PQQ-dependent Gcd (7). Also in xylose-grown *P. putida* S12xylAB cultures, 81% of the initial amount of xylose was oxidized to xylonate (Fig. 1B), rendering a large part of the xylose unavailable for growth. When the biomass formation in such cultures was related to the amount of xylose that was not oxidized, an apparent yield of 47% (g CDW g substrate^{-1}) was calculated.

Optimizing xylose utilization by laboratory evolution. To optimize the biomass yield and growth rate on xylose, a laboratory evolution approach similar to that described by Kuyper et al. was applied (13). Two parallel cultures were maintained and repeatedly transferred to fresh minimal medium with xylose. Throughout, the best performing of the two parallel cultures was used as inoculum for the next transfer while the other was discarded. Three stages could be discriminated in the evolutionary process (Fig. 2). In the first stage, transformant S12xylAB strains were selected for increased biomass yield. When the culture entered the stationary phase, the culture with the highest biomass yield was selected for further evolution. After approximately 20 transfers, the biomass yield stabilized at 52%, comparable to the yield on glucose (55%). Although the growth rate increased with increasing biomass yield, it stabilized when the maximum yield had been achieved, at only 0.05 h^{-1}. Since this was an order of magnitude lower than the growth rate on glucose, the evolutionary approach was continued to select for a strain with a higher growth rate. Instead of transferring the culture with the highest biomass yield, the faster-growing cultures were selected and reinoculated into fresh medium. After 10 transfers, a strain was obtained that exhibited a significantly higher growth rate of 0.35 h^{-1}. At this stage of the evolutionary procedure, the cultures were found to lyse upon entering the stationary phase. Evolution was therefore prolonged to select for a strain that was less prone to lysis. With every transfer, the susceptibility to lysis decreased, ultimately leading to a nonlysing and efficiently xylose-utilizing *P. putida* S12-derived strain that was designated S12xylAB2.

Characterization of *P. putida* S12xylAB2. The increased biomass yield on xylose after laboratory evolution provided a strong indication that xylose oxidation was affected in *P. putida* S12xylAB2. Indeed, no xylonate formation was observed during growth on xylose, and also, glucose was no longer oxidized to its corresponding aldonic acids. This suggested that Gcd had become inactive during the evolutionary procedure, which was confirmed by the results of Gcd assays. Sequence analysis of the *gcd* gene showed no mutations, indicating that the absence of active Gcd was caused at a different level. qPCR was used to determine the *gcd* transcript levels. The mRNA concentration in *P. putida* S12 cells was 7.00 ± 1.1 ng μl^{-1} (average ± standard deviation of the results from three independent exponentially growing cultures); that in *P. putida* S12Δgcd cells was 10.62 ± 2.0 ng μl^{-1}; and that in *P. putida* S12xylAB2 cells was 9.10 ± 0.8 ng μl^{-1}. Surprisingly, the *gcd* mRNA level in S12xylAB2 was increased in comparison to that of the wild-type S12 strain, eliminating impaired transcription of *gcd* as an explanation for the absence of Gcd activity. Also, inefficient translation of *gcd* mRNA is unlikely to have caused the abolishment of Gcd activity as no mutations were found in the *gcd* RBS. Therefore, it is proposed that the inactivation of Gcd results from a yet-unidentified posttranslational event.

In order to investigate whether the improved growth characteristics of the evolved strain on xylose could be attributed to the absence of Gcd activity, the *gcd* gene was disrupted in wild-type *P. putida* S12. The resulting strain, *P. putida* S12Δgcd, was transformed with plasmid pJTxylAB to enable xylose utilization. Like *P. putida* S12xylAB2, strain S12Δgcd_xylAB utilized both glucose and xylose as the sole carbon source without formation of the associated aldonates. Also in strain S12Δgcd_xylAB, in which 107 bp were removed from *gcd*, the *gcd* mRNA level was increased with respect to the level in the wild-type S12 strain (see above). Compared to the yield of the nonevolved strain S12xylAB, the yield of strain S12Δgcd_xylAB was considerably improved (44% [g CDW g xylose^{-1}]), but the growth rate was equally low (0.01 h^{-1}). Thus, the absence of Gcd activity explains only part of the improved xylose utilization. Mutations in the *xylAB* genes of strain S12xylAB2 resulting in a higher xylose conversion rate could be excluded by sequence analysis, which was confirmed by the results of XylAB activity measurements (not shown).

Utilization of arabinose and mixtures of glucose and pentoses by *P. putida* S12xylAB2. The evolved strain S12xylAB2 was able to efficiently utilize xylose, as well as glucose, despite the apparent loss of a key enzyme activity for direct oxidative glucose metabolism. For optimal utilization of lignocellulose-derived feedstock, L-arabinose should also be metabolized in addition to glucose and D-xylose. Although strain S12xylAB2 was not specifically engineered for arabinose utilization, the introduced xylose metabolic enzymes may show nonspecific activity toward L-arabinose, a C4 epimer of D-xylose (19, 21). The ability of strain S12xylAB2 to utilize L-arabinose was therefore assessed by growth in mineral salts medium containing 12 mM arabinose. Surprisingly, L-arabinose was utilized as a carbon source at an efficiency identical to that of growth on xylose, with a biomass yield of 52% (g CDW g arabinose^{-1}) and a maximum growth rate of 0.35 h^{-1}.

Strain S12xylAB2 lost the ability to utilize arabinose when cured from pJTxylAB (Table 3), demonstrating that the *xylAB* genes introduced for xylose consumption were also essential for arabinose consumption. Also, the evolutionary procedure apparently made a key contribution to efficient arabinose consumption

TABLE 4. Key enzyme activities for pentose utilization in cell extracts of *P. putida* S12xylAB2 with xylose or arabinose as substrate[a]

Enzyme	Activity (U g^{-1}) on:	
	Xylose	Arabinose
XylA (pentose isomerase)	34	1.5[b]
XylB ("pentulose" kinase)	134	ND[c]
XylAB (combined isomerase/kinase)	22	16
Putative C4 epimerase[d]	ND	194

[a] ND, not determined.
[b] The activity assay of XylA with L-arabinose was compromised by the low affinity of sorbitol dehydrogenase for L-ribulose.
[c] The activity of XylB was not determined separately as L-ribulose is not commercially available.
[d] The formation of D-xylulose-5-phosphate from L-arabinose was quantified by measuring transketolase activity.

since the nonevolved strain S12xylAB did not utilize arabinose (Table 3). The results of previous work demonstrated that *P. putida* S12 expressing the AraBAD pathway that converts L-arabinose into D-xylulose-5-phosphate did not grow on arabinose unless the high-efficiency arabinose transporter AraFGH was coexpressed (unpublished data). The coexpression of AraFGH and XylAB in wild-type *P. putida* S12 resulted in an arabinose-utilizing strain, whereas the introduction of *araFGH* alone did not establish growth on arabinose (Table 3). These results suggest that the evolutionary procedure improved arabinose transport efficiency. The effect of Gcd having become inactive probably plays a minor role, as wild-type *P. putida* S12 oxidizes arabinose only to a very limited extent (not shown).

The involvement of XylA and XylB in L-arabinose metabolism was further confirmed by the results of enzyme measurements (Table 4). NADH consumption was observed when L-arabinose was added as the substrate instead of D-xylose when assaying xylose isomerase and xylulokinase in the cell extract of strain S12xylAB2. In addition, D-xylulose-5-phosphate from L-arabinose could be detected with the transketolase assay (Table 4). Since the product of L-arabinose formed by XylAB is expected to be L-ribulose-5-phosphate, the formation of D-xylulose-5-phosphate suggests the presence of a C4 epimerase activity that remains to be identified. The observation that the nonevolved strain S12xylAB_araFGH also utilizes L-arabinose suggests that the C4 epimerase activity is endogenous to *P. putida* S12 and not the result of the evolutionary procedure.

Finally, the growth of strain S12xylAB2 on mixtures of sugars was investigated. Cells were inoculated into mineral salts medium containing glucose and xylose (MMGX); glucose and arabinose (MMGA); or glucose, xylose, and arabinose (MMGXA). Timed samples were drawn and analyzed for CDW and sugar content. The results show that all sugars in the tested combinations are consumed (Fig. 3). A diauxic shift was observed in all cultures: only after glucose was depleted were the pentoses consumed. When both xylose and arabinose were present in addition to glucose, the pentose sugars were utilized simultaneously when cells were deprived of glucose.

DISCUSSION

A *P. putida* S12 strain was constructed that efficiently utilizes D-xylose, as well as L-arabinose. The expression of xylose isomerase and xylulokinase is essential for the utilization of both pentoses, but the subsequent laboratory evolution is key to the efficiency with which these pentoses are metabolized. The improved yield on xylose attained by the evolved strain could largely be attributed to Gcd having become inactive in the evolved strain, preventing xylose "loss" as a result of oxidation to xylonate. Although targeted disruption of the *gcd* gene in wild-type *P. putida* S12 resulted in an improved biomass yield on xylose, this strategy did not result in an improved growth rate, indicating that other changes occurred in the evolved strain. It may be speculated that mutations occurred that affected the metabolic fluxes through the PP pathway, which is the expected route by which xylose is metabolized. In

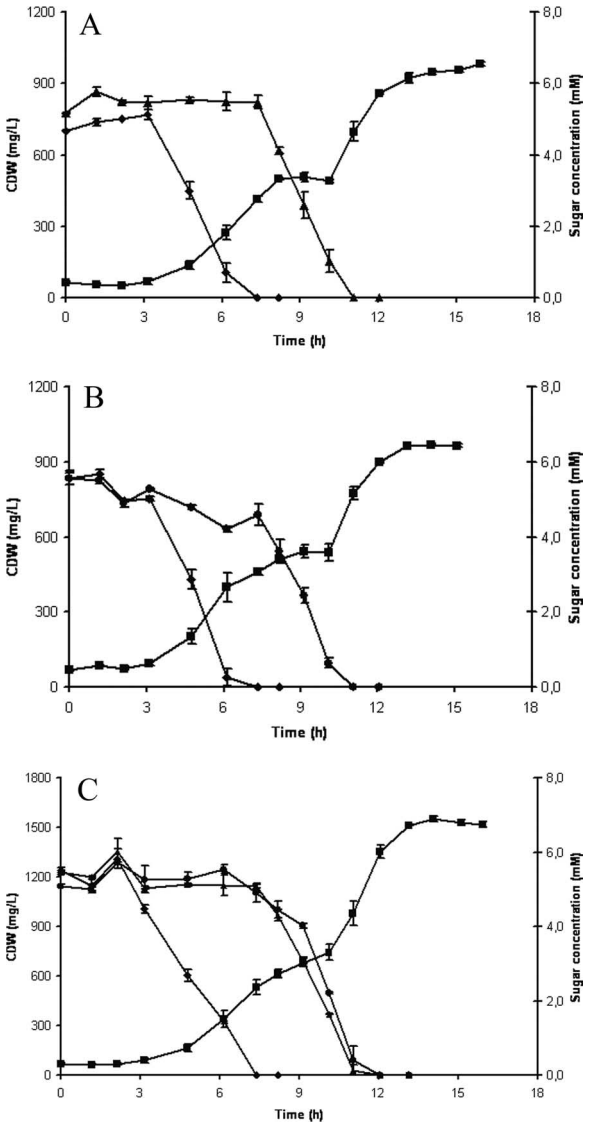

FIG. 3. Growth of *P. putida* S12xylAB2 (CDW is represented by squares) and consumption of glucose (diamonds), xylose (triangles), and arabinose (circles). *P. putida* S12xylAB2 was inoculated into mineral salts medium containing glucose and xylose (A); glucose and arabinose (B); or glucose, xylose, and arabinose (C). Data points are the averages of the results of duplicate measurements. Error bars represent the maximum deviations of the averages. L, liter.

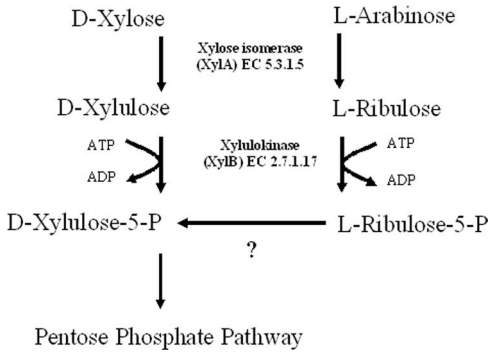

FIG. 4. Utilization of D-xylose and L-arabinose may proceed via (partly) shared pathways. Indications were found that L-arabinose can be converted into D-xylulose-5-phosphate by the combined action of XylAB and a yet-unidentified C4 epimerase (indicated by the question mark).

P. putida, a complete PP pathway is present, but metabolic flux analyses on *Pseudomonas fluorescens* have shown that this pathway mainly serves to replenish biosynthetic intermediates (4). Similarly, modest flux distributions have been demonstrated for *P. putida* S12 (unpublished data). Therefore, the function of the PP pathway may have changed from anabolic to catabolic in the evolved *P. putida* S12xylAB2 strain.

The observation that D-xylose and L-arabinose are consumed with equal efficiency, both in terms of biomass yield and specific growth rate, suggests that both pentoses are consumed via the PP pathway. In addition, the evolved strain appears to have acquired an efficient L-arabinose uptake system, as wild-type *P. putida* S12 required the expression of both XylAB and the AraFGH transporter for arabinose utilization. Although coexpressing a high-affinity xylose transporter (XylFGH from *E. coli*) did not have a significant effect on xylose metabolism in the nonevolved strain (Table 3), the possibility that improved xylose uptake has contributed to more-efficient xylose utilization in strain S12xylAB2 cannot be excluded. Since pentose transporters have been shown to be promiscuous (24), it may be hypothesized that efficient L-arabinose uptake has coevolved with improved D-xylose uptake in strain S12xylAB2.

At this point it is unclear how arabinose is converted into a PP pathway intermediate (Fig. 4). The results of enzyme assays showed that L-arabinose is a substrate for XylAB, and growth on arabinose did not occur without the expression of XylAB. The expected product, L-ribulose-5-phosphate, is not a central pathway intermediate, and a C4 epimerization would be required to form D-xylulose-5-phosphate. Indeed, the formation of D-xylulose-5-phosphate from L-arabinose was suggested by the results of the transketolase assay, but no indications that this strain contains an AraD homologue were found in the *P. putida* S12 genome sequence (unpublished data). Therefore, it is proposed that the endogenous ribulose-5-phosphate-3-epimerase shows nonspecific epimerization activity on L-ribulose-5-phosphate, converting the molecule into D-xylulose-5-phosphate. Further research is ongoing to confirm this hypothesis.

Despite the loss of Gcd activity, glucose was still efficiently used as the sole carbon source by the evolved *P. putida* S12xylAB2. The glucose catabolism in *P. putida* operates through the action of three simultaneous pathways that converge at 6-phosphogluconate. Glucose is preferentially oxidized in the periplasm by Gcd to (2-keto-)gluconate and subsequently phosphorylated in the cytoplasm to yield 6-phosphogluconate (2, 15). Alternatively, glucose is imported by an ABC-transport system, phosphorylated by glucokinase, and oxidized to 6-phosphogluconate (2, 15). Apparently, the evolved xylose-utilizing strain can readily switch to this alternative pathway for glucose oxidation without affecting the yield or growth rate.

The absence of active Gcd may itself provide an explanation for the increased *gcd* transcription levels observed in both the evolved strain and the *gcd* knockout strain. Glucose induces the expression of *gcd* (27), and with active Gcd present, glucose is rapidly oxidized to gluconate and 2-ketogluconate, resulting in a swift downregulation of *gcd*. However, without active Gcd, glucose persists in the medium, resulting in increased levels of *gcd* mRNA. So, with an intact *gcd* gene and associated RBS present in the evolved strain, and apparently even increased transcription levels, the absence of active Gcd must be attributed to some posttranslational effect. The amino acid sequence of Gcd shows that the protein is excreted and that it contains four transmembrane regions (not shown), which is consistent with the periplasmic oxidation of sugars. It raises the possibility that malfunctions appear in the Gcd translocation machinery, leading to faulty localization of the enzyme, improper folding, or inadequate anchoring to the inner membrane. The exact cause of the inactivity of Gcd remains to be investigated.

In conclusion, a *P. putida* S12 strain was obtained that efficiently utilizes the three most-abundant sugars in lignocellulose, glucose, xylose, and arabinose, as sole carbon sources. The applied evolutionary approach proved to be a powerful method to optimize the initial inefficient xylose-utilizing strain. Transcriptome and proteome analyses, as well as metabolic flux analysis, are currently being performed to identify the changes in the metabolism of the evolved xylose-utilizing strain. The insight gained into the molecular background of the efficient pentose utilization will be employed to incorporate this property into optimized substitute-aromate-producing *P. putida* S12-derived strains, thereby contributing to the economical feasibility of the production of such biochemicals from renewable feedstock.

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