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Establishment of Oxidative D-Xylose Metabolism in *Pseudomonas putida* S12[▽]

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The oxidative D-xylose catabolic pathway of *Caulobacter crescentus*, encoded by the *xylXABCD* operon, was expressed in the gram-negative bacterium *Pseudomonas putida* S12. This engineered transformant strain was able to grow on D-xylose as a sole carbon source with a biomass yield of 53% (based on g [dry weight] g D-xylose^{-1}) and a maximum growth rate of 0.21 h^{-1}. Remarkably, most of the genes of the *xylXABCD* operon appeared to be dispensable for growth on D-xylose. Only the *xylD* gene, encoding D-xylonate dehydratase, proved to be essential for establishing an oxidative D-xylose catabolic pathway in *P. putida* S12. The growth performance on D-xylose was, however, greatly improved by coexpression of *xylXA*, encoding 2-keto-3-deoxy-D-xylonate dehydratase and α-ketoglutaric semialdehyde dehydrogenase, respectively. The endogenous periplasmic glucose dehydrogenase (Gcd) of *P. putida* S12 was found to play a key role in efficient oxidative D-xylose utilization. Gcd activity not only contributes to D-xylose oxidation but also prevents the intracellular accumulation of toxic catabolic intermediates which delays or even eliminates growth on D-xylose.

The requirement for renewable alternatives to replace oil-based chemicals and fuels necessitates development of novel technologies. Lignocellulose provides a promising alternative feedstock. However, since the pentose sugar fraction may account for up to 25% of lignocellulosic biomass (12), it is essential that this fraction is utilized efficiently to obtain cost-effective biochemical production. In a previous study, the solvent-tolerant bacterium *Pseudomonas putida* S12, known for its use as a platform host for the production of aromatic compounds (15, 16, 19, 22), was engineered to use D-xylose as a sole carbon source. This was achieved by introducing genes encoding the phosphorylative D-xylose metabolic pathway of *Escherichia coli*, followed by laboratory evolution (14). Prior to evolutionary improvement, extensive oxidation of D-xylose to D-xylonate occurred, resulting in a very low biomass-for-substrate yield as D-xylonate is a metabolic dead-end product in *P. putida*. The evolution approach resulted in elimination of the activity of periplasmic glucose dehydrogenase (Gcd), the enzyme responsible for D-xylose oxidation, which turned out to be a critical step in optimizing phosphorylative D-xylose utilization in *P. putida* S12.

Instead of prevention of endogenous oxidation of D-xylose, this oxidation may be used to our advantage when it is combined with an oxidative D-xylose metabolic pathway, such as the pathways described for several *Pseudomonas* species, *Caulobacter crescentus*, and *Haloarcula marismortui* (7, 11, 18, 20). In these pathways, D-xylonate is dehydrated to 2-keto-3-deoxy-D-xylonate. This intermediate either can be cleaved into pyruvate and glycolaldehyde (7) or is further dehydrated to α-ketoglutaric semialdehyde (α-KGSA). In the final step of the latter pathway, α-KGSA is oxidized to the tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate (18, 20).

In addition to Gcd (PP1444), some of the enzymes required for oxidative D-xylose metabolism are expected to be endogenous in *P. putida* S12. Transport of D-xylonate into the cytoplasm likely occurs through the gluconate transporter (encoded by *gntP* [PP3417]). The enzyme catalyzing the final step of the pathway, α-KGSA dehydrogenase, is also likely to be present (presumably PP1256 and/or PP3602) because of the requirement for metabolism of 4-hydroxyproline (1), a compound that is efficiently utilized by *P. putida* S12. In view of these properties, the most obvious approach for constructing D-xylose-utilizing *P. putida* S12 is reconstruction of a complete oxidative D-xylose metabolic pathway by introducing the parts of such a pathway that complement the endogenous activities. Recently, the genetic information for one such oxidative D-xylose pathway has become available (18), enabling the approach used in the present study, i.e., expression of the oxidative D-xylose metabolic pathway of *C. crescentus* in *P. putida* S12 and investigation of the contribution of endogenous enzyme activities.

MATERIALS AND METHODS

Culture conditions. The strains and plasmids used in this study are shown in Table 1. The media used were Luria broth (17) and a phosphate-buffered mineral salts medium, as described previously (9). In the mineral salts medium, 10 mM D-glucose, 12 mM D-xylose (resulting in MMX), or 12 mM D-xylonate was used as a sole carbon source, unless stated otherwise. Biotin was added to the culture medium to a final concentration of 20 mg liter^{-1} for growth of transformant *P. putida* S12 strains on MMX. D-Xylonate was purchased as a calcium salt (Acros Organics), and before addition to the culture medium, the calcium ions were exchanged for potassium ions by adding an equimolar amount of dibasic potassium phosphate (K_2HPO_4). The insoluble calcium phosphate [$Ca(PO_4)_2$] was precipitated by centrifugation. Antibiotics were added as required at the following concentrations: gentamicin, 10 μg ml^{-1} for mineral salts medium and

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Reference or source
Strains		
P. putida S12	Wild type	10
P. putida S12xylXABCD	*P. putida* S12 containing plasmid pJTxylXABCD	This study
P. putida S12xylXAD	*P. putida* S12 containing plasmid pJTxylXAD	This study
P. putida S12xylXD	*P. putida* S12 containing plasmid pJTxylXD	This study
P. putida S12xylX	*P. putida* S12 containing plasmid pJTxylX	This study
P. putida S12xylD	*P. putida* S12 containing plasmid pJTxylD	This study
P. putida S12Δgcd	*P. putida* S12 glucose dehydrogenase knockout mutant	14
P. putida S12Δgcd_xylXABCD	*P. putida* S12 glucose dehydrogenase knockout mutant containing plasmid pJTxylXABCD	This study
Caulobacter crescentus	Wild type	ATCC 15252
Plasmids		
pJTmcs	Ap^r Gm^r, expression vector containing the constitutive *tac* promoter, without the *tac* ribosome binding site	14
pJTxylXABCD	pJTmcs containing the *xylXABCD* genes from *C. crescentus*	This study
pJTxylXAD	pJTmcs containing the *xylXAD* genes from *C. crescentus*	This study
pJTxylXD	pJTmcs containing the *xylXD* genes from *C. crescentus*	This study
pJTxylX	pJTmcs containing the *xylX* gene from *C. crescentus*	This study
pJTxylD	pJTmcs containing the *xylD* gene from *C. crescentus*	This study

[a] Ap^r, ampicillin resistance; Gm^r, gentamicin resistance.

30 μg ml^{-1} for Luria broth; and ampicillin, 100 μg ml^{-1} (*E. coli*). Shake flask experiments were performed in Boston shake flasks containing 20 ml mineral salts medium in a horizontally shaking incubator at 30°C.

DNA techniques. Genomic DNA was isolated by using a DNeasy blood and tissue kit (Qiagen). Plasmid DNA was isolated with a QIAprep spin miniprep kit (Qiagen). DNA concentrations were measured with an ND-1000 spectrophotometer (Nanodrop). Agarose-trapped DNA fragments were isolated with a QIAEXII gel extraction kit (Qiagen). PCRs were performed with Accuprime *Pfx* polymerase (Invitrogen) used 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.

Construction of recombinant plasmids. Desired genes were amplified by PCR using genomic DNA from *C. crescentus* as the template and oligonucleotide primers 1 to 7 (Table 2 and Fig. 1). The resulting DNA fragments were ligated in tandem into the vector pJTmcs (14) using the KpnI, XbaI, and XmaJI restriction sites. Transcription of the cloned genes was under the control of the constitutive *tac* promoter. The resulting plasmids are listed in Table 1.

Analytical methods. Optical densities at 600 nm were measured using an Ultrospec cell density meter (Amersham Biosciences). An optical density of 1.0 corresponds to 0.49 g (dry weight) of cells $liter^{-1}$. Sugars and organic acids were analyzed by ion chromatography (Dionex ICS3000 system) as described previously (14).

Activity assays. Cell extracts used for activity assays were prepared by sonication of 5-ml portions of concentrated cell suspensions (0.5 g [dry weight] cells $liter^{-1}$ in 50 mM Tris-HCl buffer, pH 7.5) from mid-log-phase cultures (optical density at 600 nm, 1.0). After centrifugation, supernatants were desalted using PD-10 columns (GE Healthcare) and used for measurement of activity.

The activity of the lower D-xylose metabolic pathway was determined by measuring NADH formation by α-KGSA dehydrogenase using D-xylonate as the substrate. Since α-KGSA dehydrogenase is the final enzyme in the pathway, the α-KGSA dehydrogenase activity represents the overall activity of the three enzymes that catalyze the conversion of D-xylonate to α-ketoglutarate (i.e., the lower-pathway activity).

D-Xylose dehydrogenase (XylB) activity was measured via NADH formation using D-xylose as the substrate. It should be noted that D-xylose dehydrogenase activity cannot be measured independent of the lower-pathway activity since the final reaction in this pathway is also catalyzed by an NAD^+-dependent enzyme, as pointed out above, unless XylB is expressed as a single protein. Accordingly, the total NADH-generating activity is the sum of the true D-xylose dehydrogenase activity and the overall activity of the lower pathway starting from xylonolactone. The lower-pathway activity as measured with D-xylonate as the substrate cannot be subtracted to obtain the true D-xylose dehydrogenase activity, since the effect of the D-xylonolactonase is not accounted for. The D-xylonolactonase activity was not quantified as the chemically unstable substrate was not commercially available. Therefore, the NADH-generating activity with D-xylose as the substrate is referred to below as apparent D-xylose dehydrogenase activity.

For both activities, the assay mixture (total volume, 1 ml) contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 20 mg $liter^{-1}$ biotin, 2 mM NAD^+, and cell extract. The formation of NADH was measured continuously at 340 nm at 30°C after the reaction was started by addition of substrate. For determination of the lower-pathway activity, D-xylonate was added to a final concentration of 0.2 mM. To quantify the apparent D-xylose dehydrogenase activity, D-xylose was added to a final concentration of 2 mM. The substrate concentrations were confirmed to be saturating; i.e., addition of additional substrate did not result in higher activities.

Pyrroloquinoline quinone-dependent D-glucose dehydrogenase activity was determined as described by Liu et al. (13) by spectrophotometrically measuring the decrease in absorbance of 2,6-dichlorphenolindophenol (DCPIP) at 600 nm. The assay was performed at 30°C in a 1-ml (total volume) mixture. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 15 mM NH_4Cl, 80 μM

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5′→3′)[a]	Characteristics
1	GCGGGTACCGTGGTTTGTCGGCGGCTTCTAGCA	Start of *xylXABCD* operon from *C. crescentus*, forward primer, KpnI
2	GCGTCTAGATCAGTGGTTGTGGCGGGGCAGCTT	End of *xylXABCD* operon from *C. crescentus*, reverse primer, XbaI
3	GCGGGTACCAGGTCCAGGTCGTGGTTTGTC	Start of *xylX* from *C. crescentus*, forward primer, KpnI
4	GCGTCTAGATTACGACCACGAGTAGGAGGTTTT	End of *xylA* from *C. crescentus*, reverse primer, XbaI
5	GCGTCTAGATGTGGCCGGCCAACCCC	Start of *xylD* from *C. crescentus*, forward primer, XbaI
6	GCGCCTAGGTCAGTGGTTGTGGCGGGG	End of *xylD* from *C. crescentus*, reverse primer, XmaJI
7	GCGTCTAGATTAGAGGAGGCCGCGGCC	End of *xylX* from *C. crescentus*, reverse primer, XbaI

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

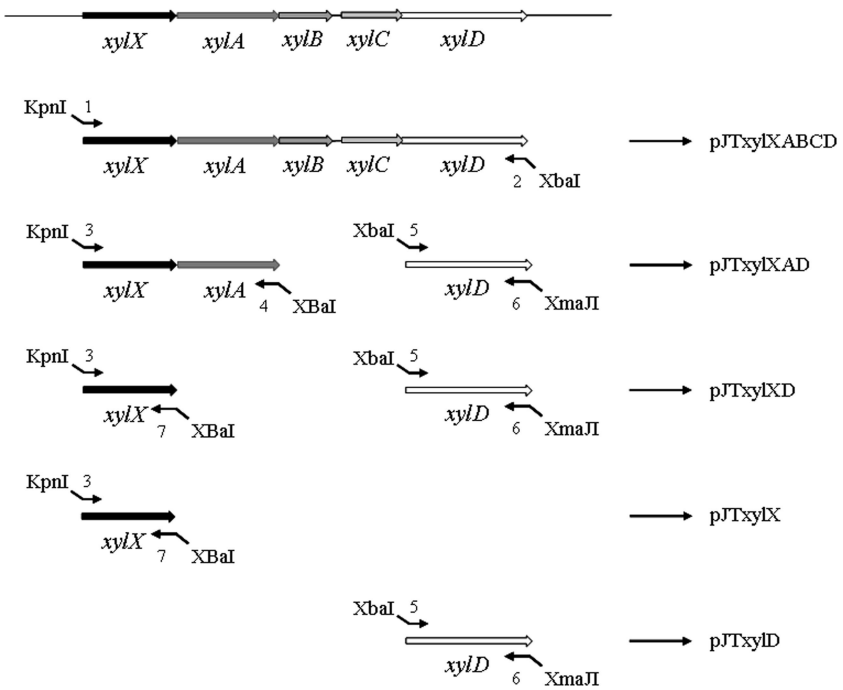

FIG. 1. Construction of expression vectors used in this study. Genes were amplified using the primers indicated by the numbers (see Table 2 for characteristics). Amplified genes were cloned in tandem into the expression vector pJTmcs under transcriptional control of the constitutive *tac* promoter. The resulting plasmids are shown in Table 1.

DCPIP, 1 μM potassium cyanide (KCN), 0.33 mM phenylmethosulfate, and cell extract. The reaction was started by adding D-glucose or D-xylose to a final concentration of 1 mM.

The production of thiobarbituric acid reactive substance was analyzed using the method of Buchanan et al. (5). The reaction mixture (total volume, 250 μl) consisted of 50 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 20 mg liter^{-1} biotin, 2 mM NAD^+, and cell extract. Parallel reactions were initiated by adding 0.2 mM D-xylonate. At set time intervals, one of the parallel reactions was stopped by adding 25 μl 12% trichloroacetic acid (wt/vol). Denatured proteins were removed by centrifugation, and the supernatant was assayed for 2-keto-3-deoxypentonate. The absorbance of the yellow complex was measured spectrophotometrically at 548 nm.

For calculations of enzyme activities the following molar extinction coefficients were used: 6.22 mM^{-1} cm^{-1} for NADH, 19 mM^{-1} cm^{-1} for reduced DCPIP, and 67.8 mM^{-1} cm^{-1} for *tert*-butyl alcohol reactive substance. One unit is defined as the amount of enzyme that oxidizes 1 μmol of substrate per min in the assays described above.

Determination of the pH_{in}. The intracellular pH (pH_{in}) was determined by the method of Breeuwer et al. (4), using 5 (and 6-)-carboxyfluorescein succimidyl ester as the fluorescent probe. D-Glucose-grown cells were harvested and loaded with the fluorescent probe as described previously (4). Determination of the pH_{in} was carried out in 24-well plates using a TECAN Infinite 200 microplate reader. D-Xylose (10 mM), D-glucose (10 mM), or buffer (negative control) was added to the assay mixture to assess the effects of these sugars on the pH_{in}. Calibration curves were prepared using citric acid buffer (50 mM) for pHs ranging from 4 to 6 and Tris-HCl (50 mM) for pH 7 to 10. pH_{in} and extracellular pH were equilibrated by adding valinomycin and nigericin to a final concentration of 1 μM.

RESULTS

Expression of the *C. crescentus* oxidative D-xylose catabolic pathway in *P. putida* S12. The D-xylose operon of *C. crescentus* was cloned into expression vector pJTmcs under the transcriptional control of the constitutive *tac* promoter. The resulting vector, pJTxylXABCD, was introduced into *P. putida* S12, yielding *P. putida* S12xylXABCD. Functional expression of the encoded pathway was confirmed by measuring the apparent D-xylose dehydrogenase activity, as well as the activity of the "lower" pathway. The apparent D-xylose dehydrogenase activity was 896 U g protein^{-1}, whereas the lower-pathway activity was 25.9 U g^{-1}, indicating that both the D-xylose dehydrogenase and the three lower-pathway enzymes were functionally expressed. The two measured activities differed by an order of magnitude, which did not detract from the ability of *P. putida* S12xylXABCD to utilize D-xylose. When inoculated into mineral salts medium with D-xylose as the sole carbon source, *P. putida* S12xylXABCD showed efficient growth (Fig. 2) with a biomass yield (based on g [dry weight] [g D-xylose]$^{-1}$) of 53% and a maximum growth rate of 0.21 h^{-1}.

Partial expression of the *C. crescentus* D-xylose operon results in an oxidative D-xylose metabolic pathway in *P. putida* S12. Previously, various experiments complemented with database searches indicated that *P. putida* S12 contains several, but not all, enzymes required for the conversion of D-xylose into 2-ketoglutarate (Fig. 3). In order to investigate which of the *C. crescentus* enzymes are essential for a functional oxidative D-xylose metabolic pathway in *P. putida* S12, strains that express different parts of the *C. crescentus xylXABCD* operon were constructed. The results demonstrate that only the introduction of D-xylonate dehydratase (XylD) was required to establish growth on D-xylose (Table 3). However, coexpression of the putative 2-keto-3-deoxy-D-xylonate dehydratase (XylX) improved the biomass yield by approximately 10%, while the growth rate was not altered. When XylA, catalyzing the next and final step in the pathway, was also coexpressed, both the biomass yield and the maximum growth rate increased (Table 3).

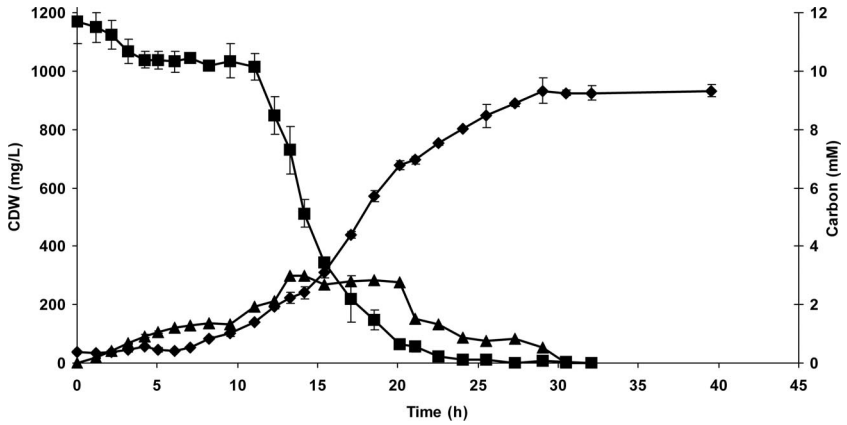

FIG. 2. Growth (◆), D-xylose consumption (■), and D-xylonate production (▲) of *P. putida* S12xylXABCD on mineral salts medium containing D-xylose as the sole carbon source. The symbols indicate the averages for three independently growing cultures. Preculturing was performed on MMX. The error bars indicate the standard deviations. CDW, cell dry weight.

When D-xylose-utilizing *P. putida* S12 strains were inoculated into MMX, only S12xylXAD was able to directly consume all the D-xylose in the culture medium, while the other strains typically required an adaptation period of several days (Table 3). However, when growing cultures on MMX were transferred to fresh D-xylose-containing medium, all strains utilized all of the D-xylose within 50 h after inoculation (Fig. 4). Curing and retransformation of D-xylose-adapted *P. putida* S12xylXABCD did not eliminate the adaptation period after culturing on nonselective medium. Hence, it may be concluded

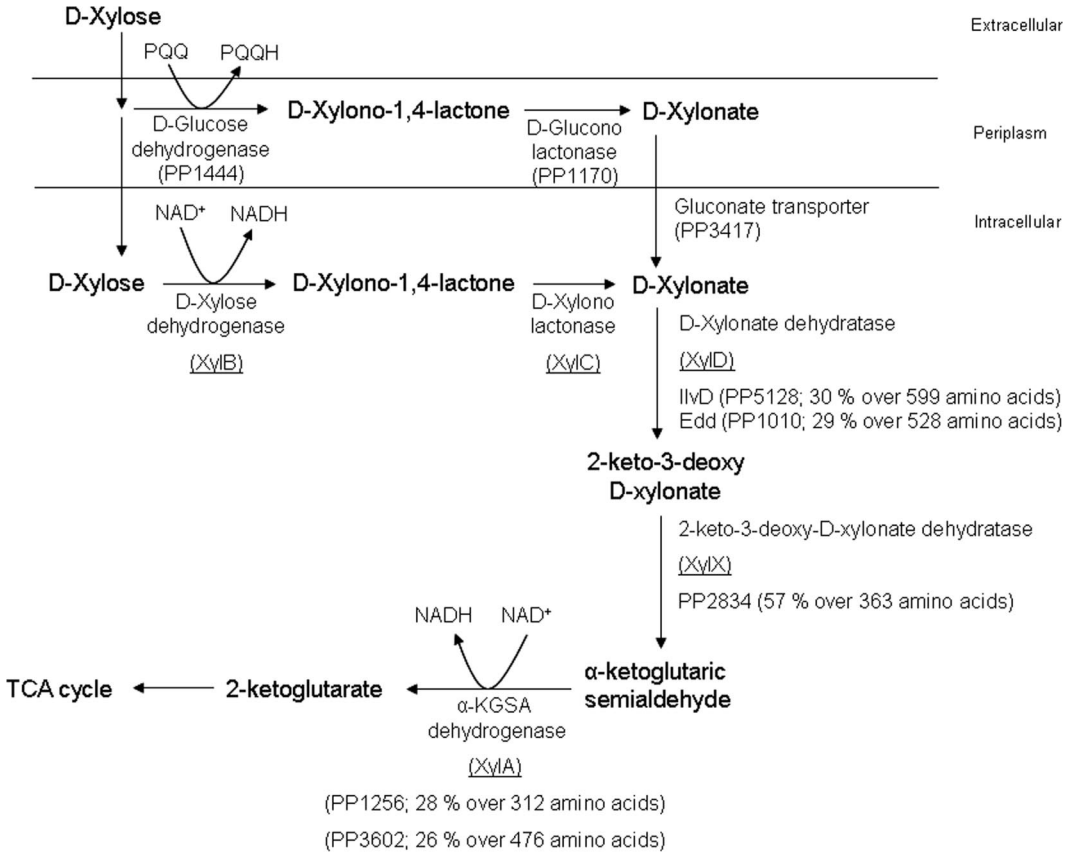

FIG. 3. Presumed native oxidative D-xylose catabolic pathway in *P. putida* S12 in combination with heterologous enzymes from *C. crescentus*. The presence of endogenous enzymes was determined in part from database searches and in part from experimental evidence. Heterologous enzymes are underlined; the other enzymes are thought to be endogenous to *P. putida* S12. The PP numbers indicate orthologues in the *P. putida* KT2440 database (3). The percentages indicate the levels of identity between the endogenous and heterologous enzymes; sequences were aligned with BLAST (2). PQQ, pyrroloquinoline quinone; PQQH, reduced pyrroloquinoline quinone.

TABLE 3. Overview of growth characteristics of oxidative D-xylose-utilizing *P. putida* S12-derived strains

Strain	Growth rate (h^{-1})[a]	Biomass yield (%)[a]	Adaptation time (days)[b]
S12xylXABCD	0.21 ± 0.02	53.2 ± 4.3	7
S12xylXAD	0.21 ± 0.02	56.7 ± 2.0	1.25
S12xylXD	0.12 ± 0.02	52.9 ± 1.0	8
S12xylD	0.11 ± 0.02	43.6 ± 0.8	9
S12xylX	NG[c]	NG	NG

[a] The data are the averages for three independently growing cultures. The biomass yield was calculated on the basis of g (dry weight) g substrate^{-1}.
[b] Adaptation time starting with a preculture on mineral salts medium with 10 mM D-glucose.
[c] NG, no growth was observed.

TABLE 4. Overview of xylose catabolic pathway activities in transformant strains

Strain	Activity on D-xylose (U g^{-1})[a]		Activity on D-xylonate (U g^{-1})[a]	
	DCPIP	NAD^+	DCPIP	NAD^+
S12xylXABCD	11.8 ± 3.0	896 ± 9.2	ND[c]	25.9 ± 3.0
S12xylXAD	10.5 ± 1.2	6.0 ± 1.9[b]	ND	17.8 ± 0.8
S12xylXD	15.9 ± 7.4	4.8 ± 0.5[b]	ND	10.6 ± 0.6
S12xylD	ND	ND	ND	6.8 ± 0.6

[a] One unit was defined as the amount of enzyme that oxidizes 1 μmol of substrate per min. The data are the averages ± standard deviations for three independent cultures growing on MMX.
[b] Endogenous activity on D-xylose.
[c] ND, not determined.

that the ability to efficiently utilize D-xylose is acquired by an adaptational event rather than a mutational event.

Relative contributions of endogenous and heterologous enzyme activities to oxidative D-xylose utilization by engineered *P. putida* S12. As mentioned above, only expression of *xylD* is needed to obtain an oxidative D-xylose catabolic pathway in *P. putida* S12. Active expression of the XylD enzyme was confirmed by performing an adapted version of the classical *tert*-butyl alcohol assay (5, 21), and the specific activity was 6.8 U g^{-1}. To determine the interaction between the endogenous and heterologous activities involved in the oxidative D-xylose catabolism in the engineered *P. putida* S12 strains, D-xylose oxidation and lower-pathway activity assays were performed. As D-xylose can be oxidized by the endogenous periplasmic protein Gcd (14), as well as by the heterologously expressed protein XylB, both activities were determined. The results of the assays are summarized in Table 4. The phenylmethosulfate-mediated reduction of DCPIP confirmed that the pyrroloquinoline quinone-dependent Gcd protein oxidizes D-xylose in the *P. putida* S12 transformant strains tested. As shown in Table 4, the apparent NAD^+-dependent D-xylose dehydrogenase (XylB) activity was over 40-fold higher than the lower-pathway activity in the strain expressing the complete *xylXABCD* operon. Concurrently, the growth rate and the biomass yield were not affected when XylB was not expressed (Table 3). This observation clearly indicated that the heterologous, intracellular D-xylose dehydrogenase plays only a minor role in D-xylose oxidation. It therefore appears that the endogenous periplasmic D-glucose dehydrogenase is the main enzyme for D-xylose oxidation in engineered *P. putida* S12, despite its lower activity as suggested by in vitro assays.

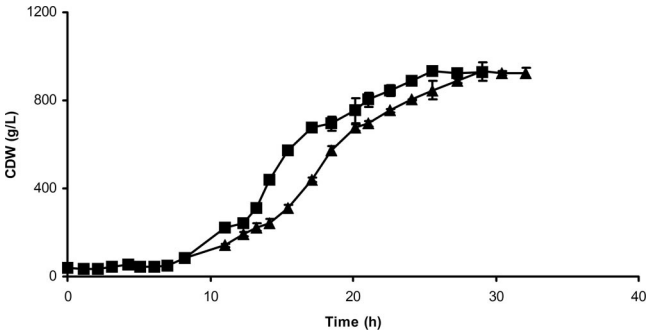

FIG. 4. Growth of S12xylXABCD (◆) and S12xylXAD (■) on mineral salts medium with D-xylose as the sole carbon source. The symbols indicate the averages for three independently growing cultures. Preculturing was performed on MMX. The error bars indicate the standard deviations. CDW, cell dry weight.

In addition to the endogenous D-glucose dehydrogenase, the endogenous α-KGSA dehydrogenases (presumably PP1256 and PP3602) also contribute to the oxidative D-xylose metabolism. The lower-pathway activities of strains S12xylXD and S12xylD, which rely on endogenous semialdehyde dehydrogenase, were about one-half the activity of the strain that coexpresses the *C. crescentus* α-KGSA dehydrogenase (S12xyl XAD). No additional activity was measured with $NADP^+$ as the cofactor, indicating that an endogenous $NADP^+$-dependent semialdehyde dehydrogenase was not present. These observations imply that the endogenous semialdehyde dehydrogenases and the heterologous α-KGSA dehydrogenase contribute equally to D-xylose metabolism. The twofold-higher α-KGSA dehydrogenase activity in strain S12xylXAD than in S12xylXD coincided with a twofold-higher maximum growth rate (Table 3), indicating that the oxidation of α-KGSA is a rate-limiting step in this pathway. Remarkably, it was observed that the lower-pathway activity in D-xylose-grown cells was 2.5-fold higher than the activity in D-glucose-grown cells, indicating that the endogenous genes involved in oxidative D-xylose catabolism are induced or derepressed during growth on D-xylose.

Significance of glucose dehydrogenase for oxidative D-xylose metabolism in engineered *P. putida* S12. The activity assays and the growth characteristics of the *P. putida* S12 constructs expressing partial D-xylose metabolic pathways suggested that the endogenous periplasmic Gcd was the main enzyme responsible for D-xylose oxidation. To confirm this, pJTxylXABCD was introduced into the Gcd knockout strain *P. putida* S12Δgcd. The performance of strain S12Δgcd_xylXABCD on minimal D-xylose medium was significantly poorer than that of strain S12xylXABCD. Growth experiments were not reproducible, and if any growth was observed in MMX, the growth rate was very low (0.03 h^{-1}).

Assuming that D-xylose is transported across the cytoplasmic membrane, an assumption which is supported by previous observations (14), the *gcd* knockout strain must be able to oxidize D-xylose, but only by using intracellularly expressed XylB. Hence, the location of D-xylose oxidation also differs in this strain. The cytoplasmic oxidation combined with the extreme

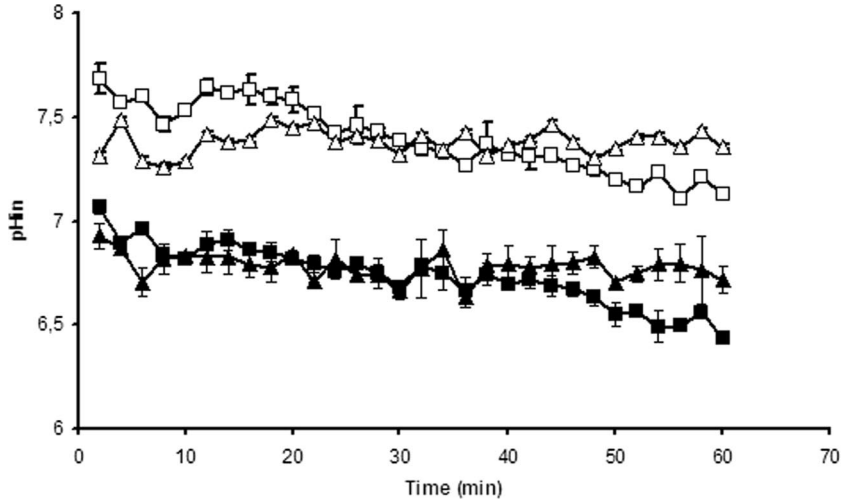

FIG. 5. Determination of the pH_{in} of S12xylXABCD (open symbols) and S12Δgcd_xylXABCD (filled symbols). Glucose-grown cells were loaded with a fluorescent probe. pH_{in} was measured continuously for 60 min after addition of D-xylose (triangles) or D-glucose (squares). The symbols indicate the averages for duplicate measurements. The error bars indicate the maximum deviations of the averages.

difference between XylB activity and lower-pathway activity (Table 4) likely causes intracellular accumulation of D-xylose metabolic intermediates. Considering the very high apparent D-xylose dehydrogenase activity, the most likely intermediates that accumulate are D-xylonate (or rather D-xylonic acid) and D-xylonolactone.

The accumulation of D-xylonic acid was expected to cause a decrease in the pH_{in}, since the dissociation constant of this compound is lower than the physiological pH. However, no such effect was observed upon addition of D-xylose to prepared cells of strain S12Δgcd_xylXABCD (Fig. 5). In contrast, addition of D-glucose resulted in a greater decrease in pH_{in} for both strain S12Δgcd_xylXABCD and strain S12xylXABCD, which grew equally well on glucose. Therefore, the hypothesis that the poor performance of S12Δgcd_xylXABCD was caused by acidification of the cytoplasm by D-xylonate accumulation was rejected.

D-Xylono-1,4-lactone is the direct product of XylB and has been shown to strongly inhibit the growth of *Pseudomonas fragi* (6). In order to demonstrate that D-xylonolactone is the inhibiting compound, strain S12Δgcd_xylXABCD was inoculated into mineral salts medium with either D-xylonate, D-glucose, or a mixture of D-glucose and D-xylose as the C source. With D-xylonate, the growth characteristics were similar to those of strain S12xylXABCD (not shown), demonstrating that the capacity to metabolize D-xylonate was not affected by deletion of *gcd*. When strain S12Δgcd_xylXABCD was cultivated on a mixture of D-glucose and D-xylose, growth was hampered compared to the growth on D-glucose alone. These results indicate that the accumulation of D-xylonolactone is indeed responsible for the poor performance of S12Δgcd_xylXABCD on D-xylose.

Growth of D-xylose-metabolizing *P. putida* S12 strains on a mixture of sugars. The growth behavior of transformant strains S12xylXABCD and S12xylXAD on a mixture of sugars was investigated. Cells were inoculated into mineral salts medium containing D-glucose and D-xylose as carbon sources. Timed samples were analyzed to determine cell dry weight and sugar content (Fig. 6). The curves obtained demonstrate that both sugars were efficiently, but not simultaneously, consumed. The subsequent utilization of D-glucose, D-gluconate, and 2-keto-D-gluconate and later D-xylose and D-xylonate was reflected in a diauxic shift. D-Xylonate accumulation was not observed

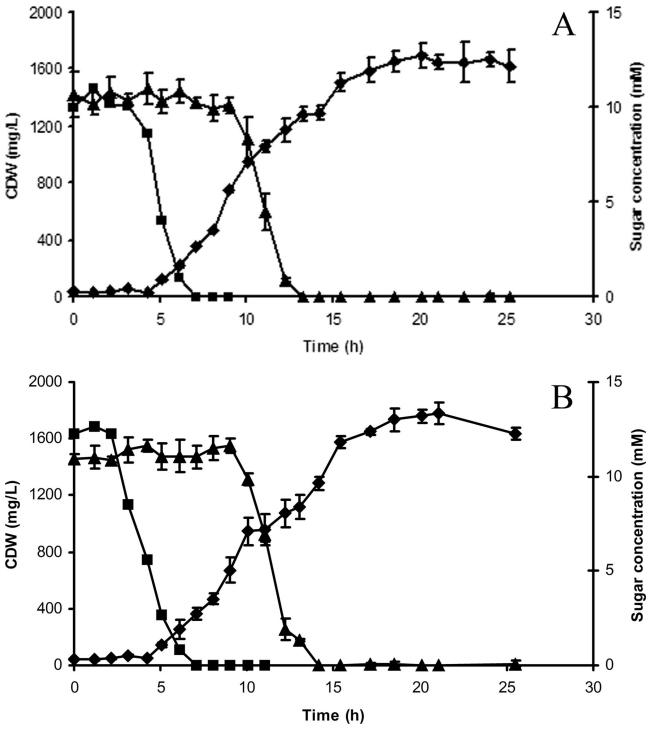

FIG. 6. Growth of and sugar consumption by *P. putida* S12xylXABCD (A) and *P. putida* S12xylXAD (B) growing on a mixture of D-glucose and D-xylose. Preculturing was performed on MMX. ◆, cell dry weight (CDW); ■, D-glucose consumption; ▲, D-xylose consumption. Transformant strains were inoculated into mineral salts medium containing D-glucose and D-xylose as carbon sources. The symbols indicate the averages for three independently growing cultures. The error bars indicate the standard deviations.

until glucose was depleted, which indicates that Gcd had a strong preference for D-glucose over D-xylose (not shown). Since the sugars were oxidized to D-gluconate, 2-keto-D-gluconate, and D-xylonate prior to utilization, growth commenced only after the sugars had been largely depleted (Fig. 2).

DISCUSSION

Efficient D-xylose-utilizing strains derived from *P. putida* S12 were obtained by expressing genes from the D-xylose operon of *C. crescentus*. It was found that introduction of a single gene of this operon, *xylD*, was sufficient to establish a functional oxidative pathway in *P. putida* S12. Database searches identified *P. putida* genes that encoded enzymes similar to those that constitute the pathway from D-xylonate to 2-ketoglutarate in *C. crescentus*. The levels of similarity were, however, rather low (Fig. 3). Apparently, the XylD counterparts in *P. putida* S12 were not similar enough to the *C. crescentus* enzyme to be a functional replacement. Remarkably, no expression of α-KGSA dehydrogenase (XylA) was required for growth on D-xylose, although this protein and its *P. putida* S12 counterparts, PP1256 and PP3602, exhibit even less identity. However, the presence of at least one active α-KGSA dehydrogenase in *P. putida* S12 could be deduced from the ability of this organism to grow on 4-hydroxyproline. The highest level of similarity was found for 2-keto-3-deoxy-D-xylonate dehydratase; XylX and PP2836 are 57% identical. It is therefore assumed that this protein is responsible for the endogenous dehydration of 2-keto-3-deoxy-D-xylonate to α-ketoglutaric semialdehyde in *P. putida* S12.

Although *P. putida* S12 requires only XylD to convert D-xylose to 2-ketoglutarate, coexpression of additional enzymes considerably improved the growth performance. Coexpression of XylX resulted in a higher biomass yield, and a significant improvement in both biomass yield and growth rate was achieved when XylA was also coexpressed. It was therefore concluded that α-KGSA dehydrogenase activity is an important bottleneck for optimal D-xylose utilization by *P. putida* S12.

With regard to D-xylose oxidation, it was established that, despite its high apparent activity, the contribution of XylB to overall D-xylose oxidation was negligible and that the major contribution was made by the endogenous periplasmic Gcd. Apparently, D-xylose is oxidized by Gcd rather than transported into the cell, suggesting either that Gcd has a very high affinity for D-xylose or that D-xylose is not very efficiently transported across the cytoplasmic membrane. Indications that support the latter explanation were found in previous research on a *P. putida* S12 construct that utilizes D-xylose via a phosphorylative pathway; the occurrence of growth demonstrated that D-xylose was metabolized intracellularly, but the possibility that D-xylose transport is a growth-limiting factor could not be excluded (14).

In addition to contributing to D-xylose oxidation, Gcd also plays a major role in preventing the intracellular accumulation of toxic D-xylose metabolic products. This became evident when *P. putida* S12 was forced to oxidize D-xylose intracellularly via XylB. This resulted in severely impeded growth that could be attributed to the accumulation of D-xylonolactone. This compound is also produced by Gcd, but in this case the toxic intermediate is present in the periplasm rather than in the cytoplasm, which probably results in less acute toxicity. Furthermore, since no lactonase activities were determined, the possibility that the endogenous periplasmic lactonase activity was higher than the heterologous XylC activity cannot be excluded.

The extremely long lag phase, or adaptation phase, of *P. putida* S12xylXABCD may also be related to D-xylonolactone accumulation. It is thought that induction of a lactonase activity occurs during this adaptation phase. Once the culture is adapted, expression of the lactonase would be induced sufficiently to prevent accumulation of D-xylonolactone, enabling growth on D-xylose and eliminating the adaptation phase upon transfer to fresh D-xylose-containing medium.

A similar explanation may be given for the adaptation phases observed with *P. putida* S12xylXD and S12xylD. The endogenous genes required for a functional D-xylose catabolic pathway in these strains (Fig. 3) apparently require induction or derepression. This may be concluded from the observation that the NAD^+-dependent lower-pathway activity was higher when these strains were grown on D-xylose than when they were grown on D-glucose. Also, the time required for induction likely translates into an adaptation phase. Once the culture is growing, apparently all the required endogenous enzymes are sufficiently expressed, and indeed no adaptation phase is observed upon transfer of the culture to fresh MMX. In *P. putida* S12xylXAD, all genes needed for the conversion of D-xylonate to 2-ketoglutarate are expressed episomally under the control of the constitutive *tac* promoter. Hence, no adaptation phase was observed with this strain.

The growth behavior of transformant strains on a mixture of D-glucose and D-xylose strongly resembles the behavior observed when an optimized phosphorylative D-xylose-catabolizing *P. putida* S12-derived strain is cultivated (14). Both routes show a diauxic shift, in which D-glucose is depleted before D-xylose is utilized. In addition, in both strains growth occurs after depletion of sugars, albeit for different reasons. For the phosphorylative route, indications were found that excess amounts of carbon were converted into glycogen, which is later degraded and used for growth when cells are deprived of sugars. In the oxidative route, sugars are oxidized to the corresponding aldonates prior to uptake and metabolism via central pathways. The diauxic shift may present a new challenge for optimizing the growth characteristics on a mixture of sugars. It was reported previously that a diauxic shift in yeast cells causes lower metabolic fluxes and that many proteins associated with biosynthetic machinery are downregulated as a response to the switch in metabolism (8). These phenomena may result in decreased specific production rates of biochemicals. This issue will be addressed in future research as it is crucial in the development of economically feasible production processes.

In conclusion, a *P. putida* S12-derived strain has been developed that is able to grow efficiently on D-xylose by converting this C_5 sugar into the TCA cycle intermediate 2-ketoglutarate. In this strain, optimal use was made of endogenous enzyme activities, which assisted the introduced heterologous pathway both by contributing to the overall catalytic activity and by preventing intracellular accumulation of toxic intermediates. The oxidative D-xylose metabolic pathway is an alternative to

the phosphorylative pathway, which involves the conversion of D-xylose to the pentose phosphate pathway intermediate D-xylulose-5-phosphate via D-xylulose. The latter pathway may be the pathway of choice when compounds derived from the pentose phosphate pathway are produced. The current strain may be more suited for production of TCA cycle-derived chemicals (e.g., C_4 building blocks), as the supply of 2-ketoglutarate presumably results in greater availability of succinic or fumaric acid. This broadens the range of products derived from renewable D-xylose, thereby contributing to the cost-effective production of biochemicals.

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