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Engineering of a Xylose Metabolic Pathway in *Corynebacterium glutamicum*

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The aerobic microorganism *Corynebacterium glutamicum* was metabolically engineered to broaden its substrate utilization range to include the pentose sugar xylose, which is commonly found in agricultural residues and other lignocellulosic biomass. We demonstrated the functionality of the corynebacterial *xylB* gene encoding xylulokinase and constructed two recombinant *C. glutamicum* strains capable of utilizing xylose by cloning the *Escherichia coli* gene *xylA* encoding xylose isomerase, either alone (strain CRX1) or in combination with the *E. coli* gene *xylB* (strain CRX2). These genes were provided on a high-copy-number plasmid and were under the control of the constitutive promoter *trc* derived from plasmid pTrc99A. Both recombinant strains were able to grow in mineral medium containing xylose as the sole carbon source, but strain CRX2 grew faster on xylose than strain CRX1. We previously reported the use of oxygen deprivation conditions to arrest cell replication in *C. glutamicum* and divert carbon source utilization towards product production rather than towards vegetative functions (M. Inui, S. Murakami, S. Okino, H. Kawaguchi, A. A. Vertès, and H. Yukawa, J. Mol. Microbiol. Biotechnol. 7:182–196, 2004). Under these conditions, strain CRX2 efficiently consumed xylose and produced predominantly lactic and succinic acids without growth. Moreover, in mineral medium containing a sugar mixture of 5% glucose and 2.5% xylose, oxygen-deprived strain CRX2 cells simultaneously consumed both sugars, demonstrating the absence of diauxic phenomena relative to the new *xylA*-*xylB* construct, albeit glucose-mediated regulation still exerted a measurable influence on xylose consumption kinetics.

Ethanol and most biochemicals are currently typically produced by converting the hexose sugars contained in corn starch or sugarcane syrup. However, these feedstocks are relatively expensive and have a competing value as food. On the other hand, lignocellulosic biomass from agricultural waste represents an abundant and cost-effective renewable energy source that is to date underutilized. This material thus represents a promising candidate as an alternative substrate for the biotechnological production of commodity chemicals. Lignocellulose is composed mainly of cellulose (40 to 50%), hemicellulose (25 to 30%), and lignin (10 to 20%) (54). Lignin is a noncarbohydrate polyphenolic compound. Cellulose hydrolysates comprise glucose and various levels of cellobiose and other glucose oligomers. On the other hand, hemicellulose hydrolysates are more complex mixtures as they include several hexoses (glucose, galactose, and mannose) and pentoses (xylose and arabinose) (53). Despite the fact that typical lignocellulosic carbohydrate fractions are composed primarily of glucose, typical pentose fractions represent a nonnegligible portion of the sugar fraction of lignocellulosic biomass, as they reach the 5 to 20% range for xylose and 1 to 5% for arabinose (1). As a result, the capability to efficiently utilize pentoses is a key attribute of microbial converters for optimizing the economics of lignocellulose-based processes (28). Several fungi and bacteria have been shown to grow aerobically on xylose, but relatively few wild-type strains can utilize xylose as a fermentable substrate (21). While recent extensive research efforts have been made to develop efficient industrial biotechnological schemes for deriving useful products from lignocellulose (52), the bioconversion of xylose remains a limiting step (8, 22, 48). The lack of industrial microbial biocatalysts with improved properties thus constitutes a major bottleneck to the implementation of successful industrial processes using lignocellulosic biomass as their primary feedstock.

The nonmedical corynebacteria are gram-positive bacteria that belong to the *Actinomycetes* subdivision of *Eubacteria*. *Corynebacterium glutamicum* has been widely used for the industrial production of various amino acids and nucleic acids (24, 47). We previously demonstrated that, in mineral medium and under conditions of oxygen deprivation, this aerobic bacterium is essentially under bacteriostasis but maintains its main metabolic capabilities and is thus able to excrete in significant amounts several metabolites, such as lactic, succinic, or acetic acids, while cellular growth is essentially arrested (20). The arrest of cellular replication enables the organism to limit by-product generation and reach higher productivities, since most of the carbon source can be channeled towards product production rather than towards vegetative functions. Combined with the use of a reactor filled to a high density with cells derived from aerobic culture, these features led to a bioprocess with high volumetric productivity. The unique properties of *C. glutamicum* under oxygen deprivation were exemplified by lactic acid and ethanol production (19, 34).

Commonly used substrates in industrial production by *C. glutamicum* include sucrose- and glucose-based media. However, the use of xylose-based media is currently not possible, owing to the inability of *C. glutamicum* to metabolize this sugar (7). As discussed above, xylose utilization is an important trait for an economically feasible production of ethanol and commodity chemicals from lignocellulosic biomass by microbial

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
E. coli		
MG1655	Prototroph	ATCC 47076
JM109	*recA1 endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) e14$^-$ (*mcrA*) *supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*Z*Δ*M15*]	Takara
JM110	*dam dcm supE44 hsdR17 thi leu rpsL1 lacY galK galT ara tonA thr tsx* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*Z*Δ*M15*]	39
DH5α	F′ φ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Takara
C. glutamicum		
R	Wild type	25
CRX1	Cm^r; *C. glutamicum* R (wild type) bearing pCRA810	This work
CRX2	Cm^r; *C. glutamicum* R (wild type) bearing pCRA811	This work
CRX3	Cm^r; *xylB*::Km^r; *C. glutamicum* R *xylB* mutant bearing pCRA810	This work
Plasmids		
pTrc99A	Ap^r; *E. coli* expression vector, source of *trc* promoter	Pharmacia
pCRA801	Ap^r; pTrc99A with a 1.4-kb EcoRI-SmaI PCR fragment containing the *E. coli xylA* gene	This work
pCRA802	Ap^r; pTrc99A with a 1.6-kb EcoRI-SmaI PCR fragment containing the *E. coli xylB* gene	This work
pCRA1	Cm^r; α-*lac* multicloning site; *E. coli-Corynebacterium* sp. shuttle vector derived from pHSG298 and pBL1	26
pCRA810	Cm^r; pCRA1 with a 1.6-kb BglII-BamHI PCR fragment containing the P_{trc}-*xylA* gene construct	This work
pCRA811	Cm^r; pCRA810 with a 1.7-kb FbaI-BamHI DNA fragment containing the P_{trc}-*xylB* gene construct	This work

cells. This limitation was resolved in this study by constructing a recombinant *C. glutamicum* strain that is capable of efficiently and concomitantly metabolizing both glucose and xylose.

MATERIALS AND METHODS

Bacterial strains, media, cultivation conditions, and plasmids. All bacterial strains and plasmids used in this study are listed in Table 1. For genetic manipulations, *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium (39). For aerobic growth conditions, *C. glutamicum* R and recombinant strains CRX1, CRX2, and CRX3 were precultured at 33°C overnight in nutrient-rich medium (A medium) containing (per liter) 2 g urea, 2 g yeast extract, 7 g Casamino Acids, 7 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 6 mg $FeSO_4 \cdot 7H_2O$, 4.2 mg $MnSO_4 \cdot H_2O$, 0.2 mg biotin, and 0.2 mg thiamine, supplemented with 4% (wt/vol) glucose (20). Where appropriate, media were supplemented with antibiotics. The final antibiotic concentrations were as follows: for *E. coli*, 50 μg ml^{-1} of ampicillin and 50 μg ml^{-1} of chloramphenicol, and for *C. glutamicum*, 5 μg ml^{-1} of chloramphenicol and 50 μg ml^{-1} of kanamycin.

To investigate the growth performance of *C. glutamicum* under aerobic conditions, both the wild-type strain and the recombinant strains CRX1, CRX2, and CRX3 were harvested by centrifugation (5,000 × *g*, 4°C, 10 min). Cell pellets were subsequently washed twice with mineral medium (BT medium) containing (per liter) 7 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 6 mg $FeSO_4 \cdot 7H_2O$, 4.2 mg $MnSO_4 \cdot H_2O$, 0.2 mg biotin, and 0.2 mg thiamine. After the second wash, cells were resuspended in 100 ml of BT medium containing an appropriate concentration of sugars. The resulting mixture was incubated at 33°C with constant agitation (200 rpm) in a 500-ml flask.

Organic acid production under oxygen deprivation. For organic acid production, both wild-type and recombinant CRX2 cells grown in aerobic-phase cultures were harvested by centrifugation (5,000 × *g*, 4°C, 10 min). Cell pellets were subsequently washed twice with mineral medium (BT medium). Following the second wash, cells were resuspended, concentrated to the appropriate cell concentration in 80 ml of BT medium containing 100 mM sodium bicarbonate, and incubated at 33°C with constant agitation in a lidded 100-ml medium bottle. Organic acid production was started by adding variable amounts of sugar. The pH was monitored using a pH controller (DT-1023; Biott Co. Ltd., Japan) and maintained at pH 7.5 by appropriately supplementing the medium with 2.5 N ammonia.

DNA manipulations. Plasmid DNA was isolated either by the alkaline lysis procedure (39) or by using a HiSpeed plasmid Midi kit (QIAGEN) according to the manufacturer's instructions, modified, when extracting DNA from corynebacteria, by using 4 mg ml^{-1} lysozyme at 37°C for 30 min. Chromosomal DNA was isolated from corynebacteria and *E. coli* following methods previously described (39), modified by using 4 mg ml^{-1} lysozyme at 37°C for 30 min. Restriction endonucleases were purchased from Takara (Osaka, Japan) and used per the manufacturer's instructions. PCR was performed using a GeneAmp PCR system (Applied Biosystems, Foster City, CA) in a total volume of 100 μl with 50 ng of chromosomal DNA, 0.2 mM deoxynucleoside triphosphates, 2% dimethyl sulfoxide in LA *Taq* polymerase buffer with $MgCl_2$, and 4 U of LA *Taq* polymerase (Takara) for 30 cycles at temperatures of 94°C for denaturation (1 min), 55°C for annealing (1 min), and 72°C for extension (2 min). Oligonucleotide primers used in this study are listed in Table 2. The resulting PCR fragments were purified with a QIAquick PCR purification kit (QIAGEN).

Corynebacteria were transformed by electroporation as previously described (50). Transformation of *E. coli* was performed by the $CaCl_2$ procedure (39).

Construction of recombinant plasmids containing xylose metabolism genes. The 1.4-kb *E. coli xylA* gene (41) was amplified using *E. coli* K-12 chromosomal DNA as the template and the oligonucleotide primers primer 1 and primer 2 (Table 2) to generate a DNA fragment with EcoRI and SmaI cohesive ends. The PCR amplicon was subsequently ligated to EcoRI- and SmaI-digested pTrc99A plasmid DNA, yielding plasmid pCRA801 (Table 1). Similarly, the 1.6-kb *E. coli xylB* gene (29) was amplified by PCR using *E. coli* K-12 chromosomal DNA as the template and the oligonucleotide primers primer 3 and primer 4 (Table 2). The resulting PCR product, which was designed to have EcoRI and SmaI cohesive ends, was subsequently ligated to EcoRI- and SmaI-digested pTrc99A DNA, yielding pCRA802 (Table 1).

Plasmid pCRA801 contains the *xylA* gene under the control of the *trc* promoter on a 1.6-kb BglII-BamHI fragment. This fragment was subsequently amplified by PCR using pCRA801 plasmid DNA as the template and the oligonucleotide primers primer 5 and primer 6 (Table 2). The resulting PCR product had BglII and BamHI cohesive ends that were used for its cloning into BglII- and BamHI-digested pCRA1 plasmid DNA, yielding plasmid pCRA810 (Table 1).

Similarly, the pCRA802 1.7-kb FbaI-BamHI DNA fragment containing the *xylB* gene under the control of the *trc* promoter was amplified by PCR using pCRA802 plasmid DNA as the template and the oligonucleotide primers primer 6 and primer 7 (Table 2). The PCR product was designed to have FbaI and BamHI overhangs that were subsequently used to ligate the amplicon to BamHI-circularized pCRA810 plasmid DNA, yielding plasmid pCRA811 (Table 1). The restriction map of plasmid pCRA811 is given in Fig. 1. *C. glutamicum* R was

TABLE 2. Oligonucleotides used in this study

Oligonucleotide primer	Target gene or plasmid	Sequence (5′–3′)[a]	Cohesive end
Primer 1	*E. coli xylA*	CTCTGAATTCACCTGATTATGGAGTTCAAT	EcoRI
Primer 2	*E. coli xylA*	CTCTCCCGGGCATATCGATCGTTCCTTAAA	SmaI
Primer 3	*E. coli xylB*	CTCTGAATTCTTTAAGGAACGATCGATATG	EcoRI
Primer 4	*E. coli xylB*	CTCTCCCGGGTTCAGAATAAATTCATACTA	SmaI
Primer 5	pTrc99A	CTCTAGATCTCCGACATCATAACGGTTCTG	BglII
Primer 6	pTrc99A	CTCTGGATCCCTTCTCTCATCCGCCAAAAC	BamHI
Primer 7	pTrc99A	CTCTTGATCACCGACATCATAACGGTTCTG	FbaI
Primer 8	Native *xylB*	CGCGCAGATCCATGTGATTG	
Primer 9	Native *xylB*	GGTGCGGTTGATCGACAATA	

[a] The restriction site overhangs used in the cloning procedure have been underlined.

transformed by electroporation with either pCRA810 or pCRA811 plasmid DNA. Transformants were selected on the basis of chloramphenicol resistance and subsequently screened for growth on xylose as the sole carbon source. For each plasmid, one of these clones able to metabolize xylose was isolated to purity to yield strains CRX1 and CRX2 (Table 1).

Transposon mutagenesis of the native corynebacterial *xylB* gene. Disruption of the corynebacterial *xylB* gene was achieved via transposon-mediated mutagenesis by electroporation of released Tn*5* transposition complexes (16). EZ::Tn transposon containing the kanamycin resistance gene *tpnA* was mixed with EZ::Tn transposase to generate the transforming transposome according to the manufacturer's instructions (Epicentre, Wisconsin). The resulting transposome was mixed with *C. glutamicum* R cells that were subsequently transiently permeabilized by electroporation. Transposon mutants were recovered on kanamycin-containing agar plates. The presence of the transposome in the DNA fragment coding for the corynebacterial xylulokinase was confirmed by PCR performed using the oligonucleotide primers primer 8 and primer 9 (Table 2) present in the native *xylB* gene. Insertional mutagenesis of *xylB* was also confirmed by xylulokinase activity measurements.

DNA sequencing. All sequencing was performed by the dideoxy chain termination method as previously described (40) with an ABI Prism 3100 genetic analyzer (Applied Biosystems) using a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems). The nucleotide sequences of both strands were determined. DNA sequence data were analyzed with the Genetyx program (Software Development, Tokyo, Japan). Database searches were performed using the BLAST server of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Enzyme assays. Cell extracts obtained from batch experiment samples were used for assaying enzyme activities. Enzyme activities were measured at 340 nm and 30°C in a final volume of 1.0 ml by using a Beckman DU800 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). Cultures were harvested by centrifugation at 5,000 × *g* at 4°C for 10 min. Cell pellets were washed once with extraction buffer (100 mM Tris-HCl [pH 7.5], 20 mM KCl, 20 mM $MgCl_2$, 5 mM $MnSO_4$, 0.1 mM EDTA, and 2 mM dithiothreitol). The resulting cell suspensions were sonicated using an ultrasonic homogenizer (Astrason model XL2020; Misonix) in an ice water bath for three 2-min periods, interrupted by 2-min cooling intervals. Cell debris was removed by centrifugation (20,000 × *g*, 4°C, 30 min). The cell lysates thus produced were subsequently used as crude extracts for enzyme assays. One unit of enzyme activity was defined as the amount of activity necessary to convert 1 μmol of NADH to NAD^+ per min. Protein concentrations were determined using a Bio-Rad protein assay kit.

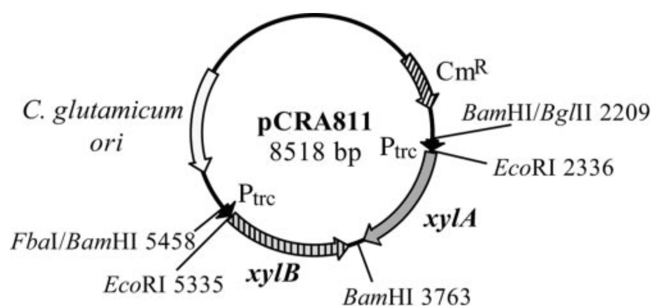

FIG. 1. Restriction map of plasmid pCRA811. Plasmid pCRA811 contains the *E. coli*-derived *xylA* and *xylB* genes cloned in opposite orientations on an EcoRI promoterless cassette. The strong constitutive promoter P_{trc} enables constitutive expression of the two xylose utilization genes, thus circumventing the effect of potential transcriptional regulators. The corynebacterial replication origin is from the rolling circle plasmid pBL1.

Lactate dehydrogenase (LDH) assays were performed as previously described (4). Xylose isomerase activity was determined based on NADH oxidation by sorbitol dehydrogenase as previously described (14). Xylulokinase assays were performed as reported elsewhere (12).

Analytical procedure. Samples were centrifuged (10,000 × *g*, 4°C, 10 min), and the resulting supernatants were analyzed for the presence of sugars and organic acids. Organic acid concentrations were determined by high-performance liquid chromatography using an apparatus (8020; Tosoh Corporation, Tokyo, Japan) equipped with an electric conductivity detector and a TSKgel OApak-A column (Tosoh Corporation, Tokyo, Japan) operating at 40°C with a 0.75 mM H_2SO_4 mobile phase at a flow rate of 1.0 ml min^{-1}. Sugar concentrations were determined by high-performance liquid chromatography using an apparatus (8020; Tosoh Corporation, Tokyo, Japan) equipped with a refractive index detector and a TSKgel Amide-80 column (Tosoh Corporation, Tokyo, Japan) operating at 85°C with an 80% acetonitrile mobile phase at a flow rate of 1.0 ml min^{-1}. Cell mass was determined by measuring the absorbance at 610 nm (A_{610}) using a spectrophotometer (DU800; Beckman Coulter, Inc., CA). An A_{610} of 1 corresponded to 0.39 mg (dry weight) cells ml^{-1}.

Nucleotide sequence accession number. The DDBJ/EMBL/GenBank accession number for the corynebacterial xylulokinase gene (*xylB*) is AB234288.

RESULTS

Xylose metabolism gene in corynebacteria. Most corynebacteria are known not to utilize xylose as a carbon source (7). Unlike in *E. coli*, which is capable of growth on xylose as a sole carbon source, no xylose isomerase-encoding gene is present in any of the corynebacteria sequenced to date. While corynebacteria are unable to catabolize xylose, analysis of the whole genome sequence of *C. glutamicum* R (data not shown) revealed one open reading frame, the deduced product of which shows significant homology to xylulokinases (encoded by *xylB*). A similar xylulokinase is also observed in the genome of *C. glutamicum* ATCC 13032, though its functionality has not been investigated (23, 37). Likewise, we searched the whole-genome sequences of other corynebacteria, including *C. diphtheriae* NCTC 13129 (5), *C. efficiens* YS-314 (33), and *C. jeikeium* K411 (46). While the deduced polypeptide gene encoding xylulokinase in *C. glutamicum* R shares a high level of homology with the putative xylulokinases of *C. glutamicum* ATCC 13032 (99% amino acid identity) and *C. diphtheriae* NCTC 13129 (28% identity), *C. efficiens* YS-314 and *C. jeikeium* K411 do not possess this gene. These observations thus support the view

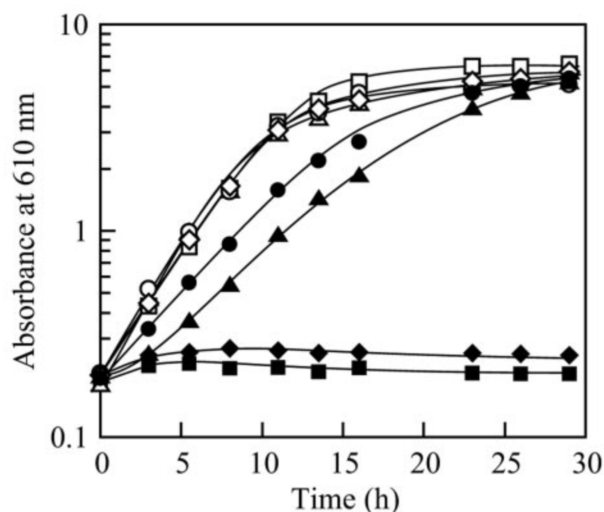

FIG. 2. Comparative aerobic growth of *C. glutamicum* strains in mineral medium containing either glucose (open symbols) or xylose (filled symbols). Wild-type *C. glutamicum* (□ and ■) and recombinant strains CRX1 (△ and ▲), CRX2 (○ and ●), and CRX3 (◇ and ◆) were first grown aerobically to late log phase in A medium (containing 40 g liter^{-1} glucose). These precultures were used to inoculate to an initial A_{610} of 0.2 mineral medium containing either 111 mM (20 g liter^{-1}) of glucose or 133 mM (20 g liter^{-1}) of xylose as the sole carbon source. The reported data represent the averages calculated from triplicate measurements.

that a partially functional pathway for xylose metabolism is present in only some corynebacterial species.

Construction of a xylose-metabolizing strain of *C. glutamicum*. The presence in the *C. glutamicum* R genome of a putative xylulokinase suggests that the introduction and expression of a heterologous gene coding for the enzyme xylose isomerase could be sufficient to enable this strain to convert xylose to common intermediates of the pentose phosphate pathway and thus to grow on media containing xylose as a sole carbon source. However, the possibility that a more efficient xylose-utilizing strain could be generated by the concomitant expression of both *xylA* (xylose isomerase) and *xylB* (xylulokinase) genes could not be ruled out. In order to clarify the functionality of this putative xylulokinase and to develop an engineered *C. glutamicum* strain that is capable of efficient xylose metabolism, two recombinant strains were constructed by cloning the *E. coli xylA* gene either alone or in combination with *E. coli xylB*.

To construct these *C. glutamicum* recombinants, the *E. coli xylA* and *xylB* genes were isolated by PCR and precisely subcloned under the control of the strong constitutive *trc* promoter that is present in vector pCRA1 (26). The PCR product of P_{trc}-*xylA* was cloned into plasmid pCRA1, yielding plasmid pCRA810 (Table 1). Similarly, the PCR product of P_{trc}-*xylB* was cloned into plasmid pCRA810, yielding plasmid pCRA811 (Table 1), where the P_{trc}-*xylA* and P_{trc}-*xylB* are present in divergent orientations. *C. glutamicum* R was transformed by electroporation with either pCRA810 or pCRA811 plasmid DNA. Transformants were selected on the basis of chloramphenicol resistance and subsequently screened for growth on xylose as the sole carbon source. For each plasmid, one of the clones able to metabolize xylose was isolated to purity to yield strains CRX1 and CRX2 (Table 1).

To evaluate the functionality of the native corynebacterial *xylB* gene, a strain in which the native *xylB* gene had been disrupted by Tn*5* transposon mutagenesis was isolated from a *C. glutamicum* mutant library constructed in our laboratory (51). Sequencing of the 1,383-bp *xylB* locus from this mutant confirmed the insertion of the transposon 658 bp downstream of the start codon. Insertional mutagenesis of *xylB* was also confirmed by xylulokinase activity measurements (data not shown). The mutant was subsequently transformed by electroporation with pCRA810 plasmid DNA. Transformants were selected on the basis of chloramphenicol and kanamycin resistance, and a single colony was isolated to purity to yield strain CRX3 (Table 1).

Growth performance of recombinant *C. glutamicum* strains in xylose mineral medium under standard aerobic conditions. To investigate the efficiency of xylose utilization by the recombinant strains CRX1, CRX2, and CRX3, wild-type and transformant strains were grown aerobically in mineral medium containing either glucose (2% [wt/vol]) or xylose (2% [wt/vol]) as a sole carbon source. Strains CRX1 and CRX2 were capable of growth on xylose as a sole carbon source but the wild-type strain was not (Fig. 2). In xylose-containing media, strain CRX2 grew faster than strain CRX1, which expresses only the *E. coli xylA* and the *C. glutamicum xylB* genes, although the specific consumption rates were not significantly different. In contrast, strain CRX3, which harbors only the *E. coli xylA* gene since the native corynebacterial *xylB* gene had been inactivated, hardly grew on xylose. All strains grew on glucose at the same rate (specific growth rate, $\mu = 0.28\ h^{-1}$) but at a much higher rate than on xylose, as exemplified by the observation that the specific growth rate of strain CRX2 was 1.4-fold higher on glucose than on xylose ($\mu = 0.20\ h^{-1}$).

These observations were supported by enzymatic analyses that revealed that xylose isomerase specific activity was observed to occur only in the recombinant strains (Table 3). Likewise, high xylulokinase specific activity was observed with strain CRX2 whereas little activity was observed with the wild-type strain. It is noteworthy that the xylulokinase activity of the corynebacterial *xylB* gene is subject to induction in the presence of xylose, as demonstrated by the ninefold-higher expression of this gene when CRX1 cells were grown on xylose as opposed to glucose. This regulation mechanism is masked in

TABLE 3. Specific activities of xylose isomerase and xylulokinase during aerobic growth in recombinant strains of *C. glutamicum*

Strain	Sp act (U/mg of protein)[c]	
	Xylose isomerase	Xylulokinase
Wild type[a]	ND	0.05
CRX1[a]	0.18	0.05
CRX1 (grown on xylose)[b]	0.26	0.44
CRX2[a]	0.35	12

[a] Cells were grown aerobically in mineral medium containing 222 mM (40 g liter^{-1}) of glucose and harvested in mid-log phase at 6 h.
[b] Cells were grown on 267 mM (40 g liter^{-1}) xylose instead of glucose.
[c] The reported data represent the averages calculated from triplicate measurements. ND, no activity detected.

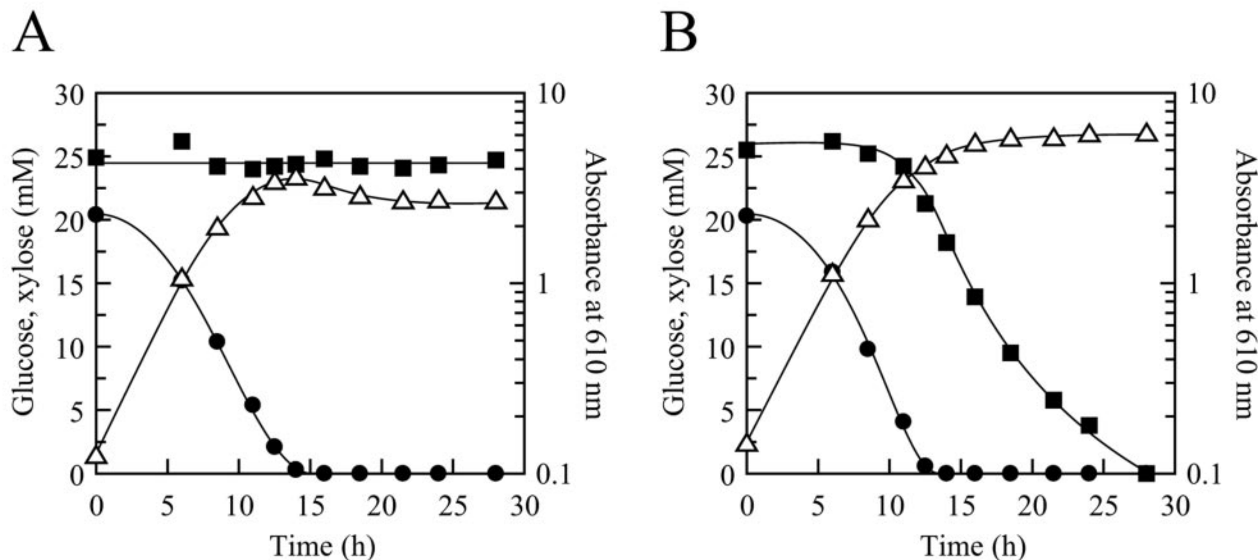

FIG. 3. Comparative aerobic growth of wild-type *C. glutamicum* (A) and recombinant *C. glutamicum* CRX2 (B) in glucose and xylose sugar mixture. Glucose (●), xylose (■), and cell (△) concentrations are shown. Both strains were first grown aerobically to late log phase in A medium (containing 40 g liter^{-1} glucose) with 50 μg ml^{-1} of chloramphenicol (except for the wild type) and then inoculated to an initial A_{610} of 0.2 into mineral medium containing 20 mM (3.6 g liter^{-1}) of glucose and 24 mM (3.6 g liter^{-1}) of xylose. The reported data represent averages calculated from triplicate experiments.

strain CRX2, where both the *E. coli xylA* and *xylB* genes are constitutively overexpressed.

All CRX2 cells tested contained plasmid pCRA811 after at least 12 h of cultivation in the absence of selective pressure.

Glucose and xylose consumption during growth. To evaluate whether xylose catabolism mediated by the *E. coli xylA* and *xylB* gene construct driven by the *trc* promoter in a native *C. glutamicum* background is repressed in the presence of glucose, a 100-ml preculture of CRX2 cells was prepared in mineral medium containing 4% (wt/vol) glucose and was used to inoculate 100 ml of mineral medium containing 20 mM (3.6 g liter^{-1}) glucose and 24 mM (3.6 g liter^{-1}) xylose to give a final cell concentration corresponding to an A_{610} of 0.2. In this medium, wild-type *C. glutamicum* R cells ceased to grow upon glucose depletion, while xylose was hardly, if at all, metabolized throughout the incubation period (Fig. 3A). In contrast, strain CRX2 cells consumed both sugars completely, although the maximum specific glucose consumption rate (2.8 mmol h^{-1} g^{-1} [dry weight] cells) was higher than that of xylose (1.5 mmol h^{-1} g^{-1} [dry weight] cells) (Fig. 3B). The specific growth rate of strain CRX2 was comparable to that of the wild type (μ = ~0.30 h^{-1}). Strain CRX2 grew to high cell densities without any apparent diauxic effect (Fig. 3B), though xylose consumption was apparently facilitated once the glucose pool had been depleted. This sequential metabolic shift from glucose to xylose suggests that, along with the constitutive expression of *xylA* and *xylB*, all of the necessary machinery associated with xylose metabolism is present in CRX2 cells, even during glucose metabolism.

Glucose and xylose metabolism under oxygen deprivation. Under conditions of oxygen deprivation, wild-type *C. glutamicum* has been observed to produce organic acids from glucose at high yields in mineral medium (34). Using CRX2 cells incubated under these conditions, we evaluated the productivity of organic acids from xylose by comparing the production of organic acid achieved from xylose to that achieved from glucose (Fig. 4). In both cases, predominantly lactic and succinic acids were produced, with trace amounts of acetic acid. The consumption rate of xylose (33 mmol h^{-1} liter^{-1}) was approximately half that of glucose (57 mmol h^{-1} liter^{-1}). Moreover, the production rates of succinic acid were comparable in both cases, reaching approximately 17 mmol h^{-1} liter^{-1}, whereas the production rate of lactic acid from xylose (29 mmol h^{-1} liter^{-1}) was lower than that from glucose (79 mmol h^{-1} liter^{-1}). The yield of succinic acid was higher from xylose (25%) than from glucose (14%), whereas the yield of lactic acid was lower from xylose (53%) than from glucose (68%). In addition, the xylose consumption rate by CRX2 cells in media containing only xylose and incubated under oxygen deprivation was reduced when glucose was spiked (Fig. 5), reinforcing the view that the chimeric xylose metabolism thus constructed in recombinant *C. glutamicum* remains repressible by glucose.

Effect of carbon source adaptation on xylose metabolism. In order to verify the effects of an eventual carbon source adaptation on xylose metabolism, CRX2 cells grown in mineral medium under standard aerobic conditions were subjected to a switch in carbon source from xylose to glucose and from glucose to xylose (Fig. 6). During aerobic growth on glucose, the specific growth rate attained within 6 h by CRX2 cells precultivated on glucose as the sole carbon source (μ = 0.28 h^{-1}) was reproducibly higher than that attained by CRX2 cells precultivated on xylose as the sole carbon source (μ = 0.20 h^{-1}). Likewise, cells precultured on glucose grew at a faster rate on xylose than cells precultured on xylose. In all experiments where a change in carbon source was implemented, cell growth started without any detectable lag phase. Furthermore, the

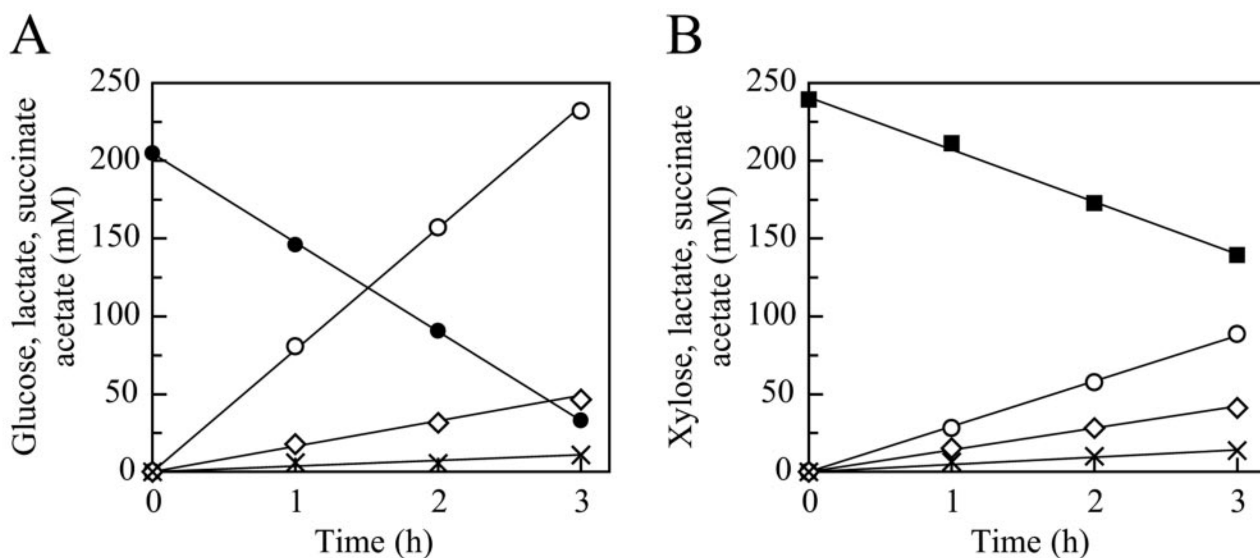

FIG. 4. Organic acid production from either glucose (A) or xylose (B) by CRX2 under oxygen deprivation. The concentrations of glucose (●), xylose (■), lactic acid (○), succinic acid (◇), and acetic acid (×) are shown. CRX2 was first grown aerobically to late log phase in A medium containing 222 mM (40 g liter^{-1}) of glucose with 50 μg ml^{-1} of chloramphenicol and subsequently used to inoculate mineral media containing either 200 mM (36 g liter^{-1}) of glucose or 240 mM (36 g liter^{-1}) of xylose at a final cell concentration of 10 g (dry weight) cells liter^{-1}. The reported data represent averages calculated from triplicate experiments.

activities of xylose isomerase (approximately 0.31 to 0.34 U/mg of protein) and xylulokinase (approximately 11 U/mg of protein) were comparable irrespective of whether or not a carbon source switch had occurred. These different observations suggest that, in recombinant *C. glutamicum* CRX2 cells, all of the enzymes that are required for glucose or xylose metabolism are immediately expressed but other carbon source-dependent

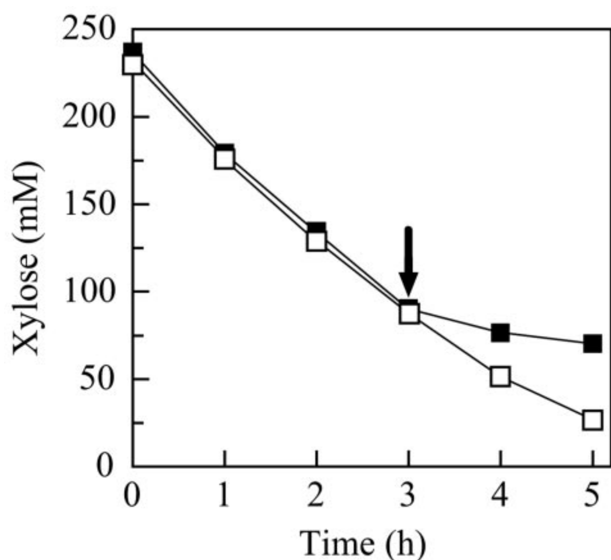

FIG. 5. Effect of glucose on xylose metabolism under oxygen deprivation. CRX2 was first grown aerobically to late log phase in A medium containing 267 mM (40 g liter^{-1}) of xylose with 50 μg ml^{-1} of chloramphenicol and subsequently used to inoculate mineral media containing 240 mM (36 g liter^{-1}) of xylose at a final cell concentration of 10 g (dry weight) cells liter^{-1}. Xylose concentrations with (■) or without (□) the addition of glucose were compared. After 3 h of reactions, 83 mM (15 g liter^{-1}) of glucose was added to the medium. The arrow indicates the time at which the culture was spiked with glucose. The reported data represent the averages calculated from triplicate measurements.

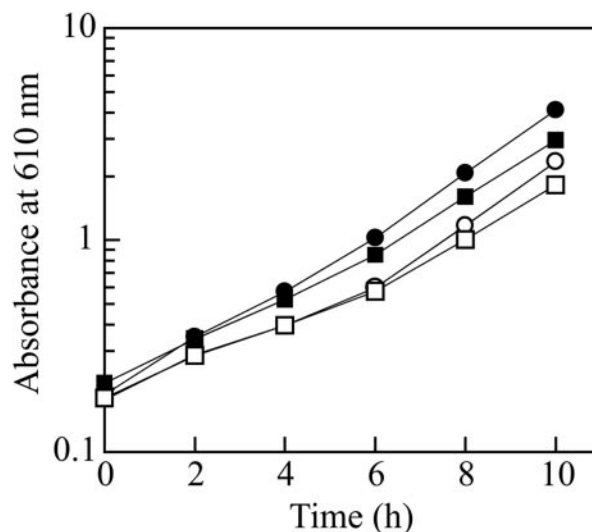

FIG. 6. Comparison of the aerobic growth of recombinant *C. glutamicum* strain CRX2 in mineral medium containing either glucose or xylose. Strain CRX2 was first grown aerobically to late log phase in A medium containing either 222 mM (40 g liter^{-1}) of glucose (filled symbols) or 267 mM (40 g liter^{-1}) of xylose (open symbols) with 50 μg ml^{-1} of chloramphenicol. These precultures were used to inoculate to an initial A_{610} of 0.2 mineral medium containing either 222 mM (40 g liter^{-1}) of glucose (○ and ●) or 267 mM (40 g liter^{-1}) of xylose (□ and ■) as the sole carbon source. The reported data represent the averages calculated from triplicate measurements.

TABLE 4. Effect of carbon source on sugar consumption and organic acid production under oxygen deprivation

Substrate[a]	Aerobic-phase culture[b]	Specific consumption rate[c] (mmol of sugar h^{-1} g^{-1} [dry wt] cells)		Yield (%)[d]		
		Glucose	Xylose	LA	SA	AA
Glucose	Glc	5.7		68	14	3
	Xyl	5.8		64	14	3
Xylose	Glc		3.3	53	25	8
	Xyl		4.6	54	21	6

[a] CRX2 was inoculated into mineral medium containing either 200 mM (36 g $liter^{-1}$) of glucose or 240 mM (36 g $liter^{-1}$) of xylose to give final cell concentrations of 10 g (dry weight) cells $liter^{-1}$.

[b] CRX2 was grown aerobically to late log phase in A medium containing either 222 mM (40 g $liter^{-1}$) of glucose or 267 mM (40 g $liter^{-1}$) of xylose with 50 μg ml^{-1} of chloramphenicol.

[c] Data represent averages calculated from triplicate experiments.

[d] Yields of each organic acid were based on consumed sugar. Theoretical yields of acetic acid (AA), lactic acid (LA), and succinic acid (SA) are 2 mol and 1.67 mol per mol of glucose and xylose, respectively.

TABLE 5. Effect of carbon source on the specific activities of xylose isomerase, xylulokinase, and lactate dehydrogenase of CRX2

Substrate[a]	Aerobic-phase culture[b]	Sp act (U/mg of protein)[c]		
		Xylose isomerase	Xylulokinase	LDH
Glucose	Glc	0.31	10	1.3
	Xyl	0.29	11	0.53
Xylose	Glc	0.30	10	1.0
	Xyl	0.27	11	0.42

[a] Cells were inoculated into mineral medium containing either 200 mM (36 g $liter^{-1}$) of glucose or 240 mM (36 g $liter^{-1}$) of xylose to give final cell concentrations of 10 g (dry weight) cells $liter^{-1}$ and harvested during organic acid production at 2 h.

[b] Cells were grown aerobically to late log phase in A medium containing either 40 g $liter^{-1}$ of glucose or 40 g $liter^{-1}$ of xylose with 50 μg ml^{-1} of chloramphenicol.

[c] The reported data represent the averages calculated from triplicate measurements.

regulatory mechanisms that have observable impacts on the cellular metabolism nevertheless remain.

Similar experiments were conducted under oxygen deprivation conditions to compare the specific sugar consumption rates and organic acid yields. CRX2 cells were grown and incubated with either glucose or xylose (Table 4). Glucose consumption and organic acid production were not significantly influenced by the growth substrate used in the aerobic-phase culture. However, the xylose specific consumption rate of strain CRX2 cells precultured on xylose was 1.4-fold higher than that of strain CRX2 cells precultured on glucose. In addition, the yield of succinic acid is higher during xylose metabolism (approximately 23%) than during glucose metabolism (approximately 14%), whereas the yield of lactic acid is lower from xylose (approximately 54%) than from glucose (approximately 66%). These data promote the view that one or more factors responsible for xylose metabolism may be induced by xylose, even though the *xylA* and *xylB* genes escape any such regulations, as they are expressed from a constitutive promoter. Furthermore, this interpretation is supported by the observation that the xylose isomerase (approximately 0.27 to 0.31 U/mg of protein) and xylulokinase (approximately 10 to 11 U/mg of protein) activities derived from these cultures were comparable (Table 5). In contrast, for CRX2 cells precultured on glucose, LDH activity was lower during xylose metabolism (1.0 U/mg of protein) than during glucose metabolism (1.3 U/mg of protein) (Table 5). Moreover, LDH activity for CRX2 cells precultured on xylose was less than half that attained by CRX2 cells precultured on glucose, whereas LDH activity was lower during xylose metabolism (0.42 U/mg of protein) than during glucose metabolism (0.53 U/mg of protein). Taken together, these various observations reinforce the view that the factors that impact xylose metabolism in CRX2 cells act mainly either at the sugar transport level or downstream of the xylose catabolism mediated by the *xylA-xylB* gene products.

Simultaneous utilization of glucose and xylose under oxygen deprivation. To confirm data attained with growing CRX2 cultures incubated under standard oxygenation conditions, we conducted batch studies under oxygen deprivation by using a synthetic sugar mixture containing 50 g $liter^{-1}$ of pure glucose and 25 g $liter^{-1}$ of pure xylose (Fig. 7) to represent the 2:1 glucose/xylose mass ratio that is typical of hydrolysates of lignocellulosic agricultural residues (1). Wild-type *C. glutamicum* R cells were used as the negative control. The latter cells hardly, if at all, metabolized xylose (Fig. 7A). In contrast, not only did strain CRX2 metabolize glucose and xylose simultaneously, but it also completely consumed xylose (Fig. 7B). The overall rate of sugar consumption for strain CRX2 within the initial 2 hours was comparable to the specific consumption rate of glucose for wild-type cells, based on gram weight (approximately 0.87 g sugar/h/g [dry weight] cells). The xylose consumption rate for strain CRX2 within the initial 3 h doubled once the glucose pool was depleted to reach 1.3 mmol h^{-1} g^{-1} (dry weight) cells (Fig. 7B), thus demonstrating that xylose metabolism under oxygen deprivation remains affected by the presence of glucose.

With both wild-type and recombinant CRX2 strains, the combined yields over the total course of the conversion of lactic, succinic, and acetic acids based on the amounts of sugar consumed was 80% of the theoretical maximum. As expected, following glucose depletion, production of lactic and succinic acids was observed only with strain CRX2 (Fig. 7B). The consequent lactic acid yield from xylose (61%) was comparable to the yield prior to glucose depletion (60%). On the other hand, the succinic acid yield was higher after (19%) than prior to (13%) glucose depletion. Similarly, the combined yields of organic acids after glucose depletion (88%) were higher than those prior to glucose depletion (77%), suggesting that strain CRX2 utilized xylose efficiently as a substrate for organic acid production despite the fact that it utilized xylose at faster kinetics once the glucose pool had been exhausted.

DISCUSSION

We confirmed in this study that wild-type *C. glutamicum* is unable to utilize xylose under both standard aerobic and oxygen deprivation conditions, owing to the lack of xylose isomerase activity (Fig. 2 and 3 and Table 3). On the other hand, wild-type *C. glutamicum* possesses a functional *xylB* gene, as demonstrated in this study by transposon mutagenesis experi-

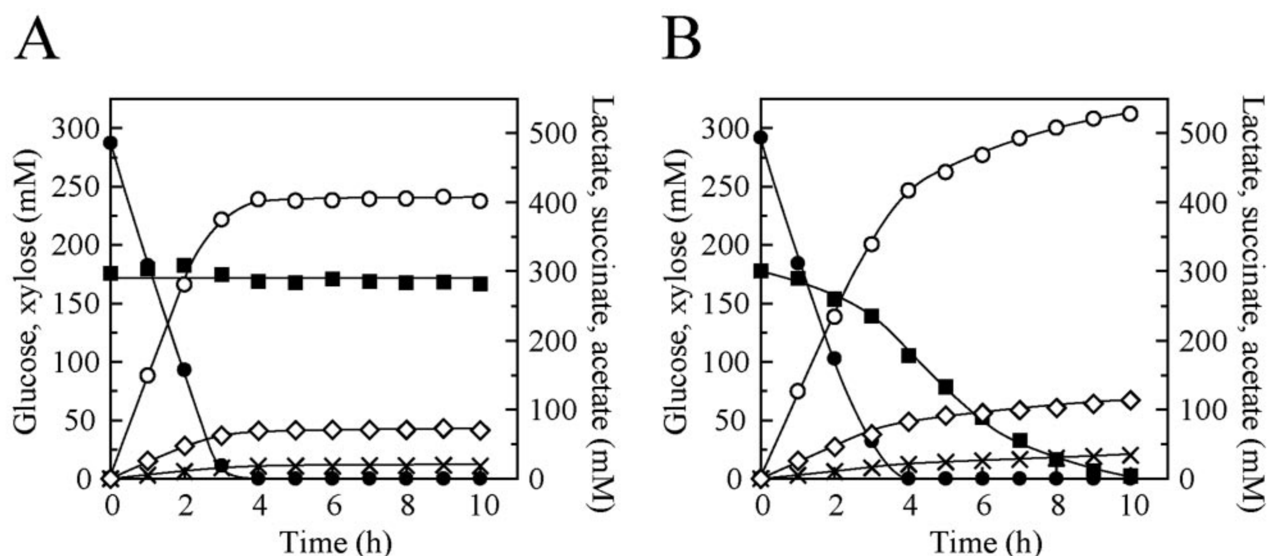

FIG. 7. Organic acid production by the wild-type *C. glutamicum* strain (A) and recombinant *C. glutamicum* CRX2 (B) from a synthetic sugar mixture containing glucose and xylose at a ratio of 2:1 under oxygen deprivation. The concentrations of glucose (●), xylose (■), lactic acid (○), succinic acid (◇), and acetic acid (×) are shown. (Note the different scales at left and right.) Both strains were first grown aerobically to late log phase in A medium containing 222 mM (40 g liter^{-1}) of glucose with 50 μg ml^{-1} of chloramphenicol (except for the wild type) and subsequently used to inoculate mineral medium containing both 278 mM (50 g liter^{-1}) of glucose and 167 mM (25 g liter^{-1}) of xylose in the presence of sodium bicarbonate (200 mM) to give final cell concentrations of 20 g (dry weight) cells liter^{-1}. The pH was maintained at 7.5 with 5 N ammonia. Data represent averages calculated from triplicate experiments.

ments. Moreover, enzymatic assays and physiological studies promoted the view that this native *xylB* gene is inducible over ninefold by xylose (Table 3), suggesting the presence of a xylose-specific regulatory gene. In *E. coli*, the expression levels of both the *xylA* and *xylB* genes are positively regulated by xylose but strictly repressed by glucose under the control of the regulatory protein XylR (15, 43), similar to what is observed with the gram-positive bacteria *Lactococcus lactis*, *Lactobacillus pentosus*, and *Bacillus subtilis* (13, 30). Inspection of the region upstream of the native *xylB* gene in *C. glutamicum* R led to the identification of an open reading frame coding for a putative regulatory protein which shows limited identity to the XylR regulator of the aforementioned strains. A similar genetic organization in a putative operon is also observed for the *C. glutamicum* ATCC 13032 genome.

To expand the catabolic properties of industrial corynebacteria with the objective to make possible the conversion of xylose to a variety of compounds, we cloned in *C. glutamicum* the *E. coli xylA* and *xylB* genes on an episome and under the control of a constitutive promoter (Fig. 1). The resulting transformant, designated CRX2, was able to grow in mineral medium containing xylose as the sole carbon source (Fig. 2). In corynebacteria, only the direct conversion of xylose into xylitol has been reported to occur, albeit at low efficiency (36, 55). Another recombinant organism, designated CRX1, was generated by cloning only the *E. coli xylA* gene. Strain CRX1 grew on xylose at a lower rate than CRX2 (Fig. 2), thus corroborating the view that introduction of both *E. coli xylA* and *xylB* under the control of a constitutive promoter is an efficient strategy to engineer in corynebacteria a functional xylose catabolism pathway that is not subject to catabolite repression, at least at the *xylA-xylB* gene product level.

In addition, we confirmed that, as designed, under aerobic growth conditions strain CRX2 does not exhibit any diauxic growth effect when cultured in media containing both glucose and xylose (Fig. 3B) and that the specific activities of both xylose isomerase and xylulokinase are comparable irrespective of the carbon source used. This property represents a particularly important industrial attribute, as it allows the efficient use of complex sugar mixtures while minimizing the residence time of the reaction broth in the fermentor. Furthermore, the usefulness of strain CRX2 for organic acid production from glucose and xylose mixtures in a 2:1 ratio was demonstrated under oxygen deprivation conditions, with the characteristics that both xylose consumption and organic acid production in this strain are not dramatically negatively affected by the utilization of carbon source mixtures (Fig. 7B).

The fact that complementation of the xylose-metabolizing pathway allowed *C. glutamicum* to utilize xylose suggests that wild-type *C. glutamicum* possesses a transporter associated with xylose uptake. It has been demonstrated that *C. glutamicum* transports hexose sugars via the phosphotransferase system (25), but the mechanisms of pentose transport in this organism remain unclear. For other gram-positive bacteria, several transport mechanisms responsible for pentose uptake have been reported (44). For instance, a mutant strain of *B. subtilis* has been shown to uptake xylose using the AraE protein, a native H^+ symporter responsible for arabinose uptake, though at an efficiency relatively lower than that measured for arabinose transport (27). Similarly, xylose transport via a low-affinity facilitated-diffusion system was also observed to occur in *L. pentosus* (6). In addition, several recombinant strains of *Saccharomyces cerevisiae* have been demonstrated to uptake xylose by using a nonspecific monosaccharide transport system,

but at an affinity that was nearly 200-fold lower for xylose than for glucose (17). As a result, the possibility that xylose transport in corynebacteria occurs via more than one mechanism, including both specific ATPase-dependent transport systems and low-affinity nonspecific transport systems, cannot be ruled out.

The relative proportion of each organic acid secreted by corynebacteria appears to depend on the carbon source. For example, the yield of succinic acid in strain CRX2 was higher during xylose metabolism than during glucose metabolism (Table 4 and Fig. 7). Similar phenomena were previously observed with *E. coli*, where, during mixed glucose and xylose fermentations, the lactic acid yield decreased when the xylose/glucose ratio increased (9). Xylose is believed to be converted into the two glycolytic intermediates fructose-6-bisphosphate and glyceraldehyde-3-phosphate, leading to lower intracellular fructose-1,6-bisphosphate levels during xylose metabolism than during glucose metabolism. These intermediates are subsequently eventually converted into lactic and succinic acids under anaerobic conditions by the enzymes LDH and succinate dehydrogenase, respectively. LDH is known to be allosterically regulated in lactococci, as it is activated by intracellular fructose-1,6-bisphosphate (49). In addition, lower amounts of *ldh* transcripts on xylose than on glucose have been observed with other genera, for example, *Rhizopus oryzae* (42). All of these previous reports are consistent with the present observation that the LDH activity of strain CRX2 was not only relatively lower when xylose rather than glucose was used as a sole carbon source but also was significantly lower in cells precultured on xylose than in cells precultured on glucose (Table 5). All of these results support the view that both transcriptional and allosteric regulatory mechanisms are responsible at least partially for the metabolic shift from lactic acid secretion to succinic acid secretion during xylose metabolism in strain CRX2. Likewise, the total organic acid yield is higher under xylose metabolism than under glucose metabolism (Fig. 7). This can perhaps be ascribed to the energy balance, by analogy with the xylose uptake mechanism of *E. coli*, which involves an energy-dependent transporter. In *E. coli* cells incubated under anaerobic conditions, the net energy conserved during xylose metabolism is estimated to be 0.67 molecule of ATP per xylose molecule, in other words, less than half of that produced from glucose (~2 ATP molecules/glucose molecule) (45); to compensate, *E. coli* cells utilize relatively more xylose.

The efficient utilization of mixtures of various sugars is critical for attaining the complete conversion of lignocellulosic sugars. The physiological role of carbon catabolite repression mechanisms present in bacteria is generally regarded to be a mechanism that has evolved to ensure sequential carbohydrate utilization (3, 38), with the most energy-efficient carbohydrate being utilized first. While *E. coli* is able to ferment xylose, the utilization of this sugar by this microbe during lignocellulosic hydrolysate fermentation is delayed and is often incomplete (10). Likewise, genetically engineered *Zymomonas mobilis* has been shown to still preferentially utilize glucose during cofermentation of sugar mixtures (31). Current works thus particularly focus on engineering strains that avoid such regulatory systems, as exemplified by the construction of *E. coli* phosphotransferase mutants (32). However, the diminished consumption rate and sequential utilization of sugar mixture still remain to be solved (18).

In the present study, the simultaneous utilization of glucose and xylose was observed only with growth-arrested cells of strain CRX2 incubated under oxygen deprivation, whereas aerobic-growing cells demonstrated sequential consumption of glucose and xylose, suggesting that the inhibitory effects exerted on xylose metabolism are attenuated under oxygen deprivation. On the other hand, when incubated under conditions of oxygen deprivation, xylose-degrading *C. glutamicum* CRX2 cells consumed each substrate at a lower rate when sugar mixtures were used than when either glucose or xylose alone was used (Fig. 7). Thus, xylose metabolism in chimerical *C. glutamicum* seems to remain subject to multifactorial regulations, perhaps acting at the sugar transport level or downstream of the xylose isomerase and xylulokinase nodes in the pentose phosphate pathway. The specific consumption rate of xylose in strain CRX2 remained 1.4-fold higher for cells precultured on xylose than for cells precultured on glucose (Table 4), suggesting that a regulatory mechanism acting at the gene expression level could be involved in xylose metabolism, although *C. glutamicum* is known not to possess the classical catabolite repression system (35). This view is furthermore supported by the observation that *E. coli* cells grown on xylose display higher levels of xylose transporter and pyruvate kinase transcripts than those grown on glucose (15). On the other hand, inducer exclusion in bacteria is a common regulatory phenomenon whereby a carbohydrate inhibits uptake of another carbon source at the enzymatic activity level (3). The present observation that the xylose consumption rate exhibited by strain CRX2 was immediately attenuated upon glucose spiking (Fig. 5) supports the view that a regulatory mechanism at the enzymatic activity level could also be involved in either xylose transport or its metabolism in strain CRX2, even under oxygen deprivation. A detailed study of the pentose phosphate pathway and of xylose transporters and their corresponding genes is necessary to elucidate the molecular basis of this observation. Nevertheless, the present observations that growth-arrested CRX2 cells precultured on glucose consumed xylose immediately (Fig. 4B), as demonstrated by the simultaneous utilization of both glucose and xylose under oxygen deprivation (Fig. 7B), suggest that, under these conditions, the arrest of cellular replication is not predominantly subjected to a regulatory mechanism acting at the gene expression level.

Notably, the overall consumption rates of glucose and xylose by strain CRX2 remained comparable with that of glucose alone under oxygen deprivation (Fig. 7). This observation does not conflict with previous reports regarding the simultaneous consumption of a mixture of glucose and fructose in *C. glutamicum* during aerobic growth. Under this latter growth condition, the rates of consumption of each substrate are lower than with growth on a single substrate alone, but the overall substrate consumption is higher than that of each substrate (11), since hexose and pentose mixtures are catabolized through different pathways (glycolysis and pentose phosphate pathways) and subject to different transport systems.

In conclusion, the results presented here demonstrate the feasibility of introducing into *C. glutamicum* the xylose assimilation pathway genes from *E. coli*. The ability to produce organic acids without growth and without diauxic effect by using complex carbohydrate mixtures is an important attribute for the production of ethanol or of other commodity chemicals

from lignocellulosic biomass (19, 34). The present work expands the range of sugars that can be catabolized by *C. glutamicum* and can be combined with methods that have already been developed for enabling this organism to degrade cellobiose (26) or galactose (2) as a step towards the development of a cost-effective biomass converter.

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