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Development of an Arabinose-Fermenting *Zymomonas mobilis* Strain by Metabolic Pathway Engineering

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The substrate fermentation range of the ethanologenic bacterium *Zymomonas mobilis* was expanded to include the pentose sugar, L-arabinose, which is commonly found in agricultural residues and other lignocellulosic biomass. Five genes, encoding L-arabinose isomerase (*araA*), L-ribulokinase (*araB*), L-ribulose-5-phosphate-4-epimerase (*araD*), transaldolase (*talB*), and transketolase (*tktA*), were isolated from *Escherichia coli* and introduced into *Z. mobilis* under the control of constitutive promoters that permitted their expression even in the presence of glucose. The engineered strain grew on and produced ethanol from L-arabinose as a sole C source at 98% of the maximum theoretical ethanol yield, based on the amount of consumed sugar. This indicates that arabinose was metabolized almost exclusively to ethanol as the sole fermentation product, with little by-product formation. Although no diauxic growth pattern was evident, the microorganism preferentially utilized glucose before arabinose, apparently reflecting the specificity of the indigenous facilitated diffusion transport system. This microorganism may be useful, along with the previously developed xylose-fermenting *Z. mobilis* (M. Zhang, C. Eddy, K. Deanda, M. Finkelstein, and S. Picataggio, Science 267:240–243, 1995), in a mixed culture for efficient fermentation of the predominant hexose and pentose sugars in agricultural residues and other lignocellulosic feedstocks to ethanol.

Ethanol is widely used as a transportation fuel in Brazil, but its current production cost limits its use in the United States as a fuel oxygenate. More than 1 billion gallons of ethanol each year are produced solely from corn and other starch crops to meet current U.S. demand. One approach to meet the projected increase in ethanol demand is the conversion of low-cost, renewable, lignocellulosic biomass. Unlike corn starch, however, lignocellulosic feedstocks are composed predominantly of cellulose, hemicellulose, and lignin and are naturally resistant to chemical and biological conversion. Because the feedstock can represent a significant portion of all process costs, an economical biomass conversion process will depend on the rapid and efficient conversion of the hexose and pentose sugars present in both the cellulose and hemicellulose fractions to ethanol. Whereas microorganisms can efficiently ferment the glucose component in cellulose to ethanol, conversion of the pentose sugars in the hemicellulose fraction, particularly xylose and arabinose, is much more difficult (7). Xylose is the predominant pentose sugar derived from the hemicellulose of most hardwood feedstocks, but arabinose can constitute a significant amount of the pentose sugars derived from various agricultural residues and other herbaceous crops, e.g., switchgrass, that are being considered for use as dedicated energy crops (13). *Escherichia coli* strains capable of fermenting these pentose sugars have been developed by introduction of the genes for ethanol production from *Zymomonas mobilis* (9).

Z. mobilis is a bacterium that has been utilized in the production of alcoholic beverages, such as pulque and palm wines, and is naturally tolerant of high ethanol concentrations. In addition, the spent grains from *Z. mobilis* fermentation have proven to be an effective animal feed, an essential co-product for ethanol produced from corn starch (14). *Z. mobilis* is the only microorganism known in which pyruvate decarboxylase and alcohol dehydrogenase activities are coupled to the Entner-Doudoroff pathway to permit efficient glucose fermentation to ethanol (17). Comparative performance trials have suggested that *Z. mobilis* may become an important fuel ethanol-producing microorganism because of its 5 to 10% higher yield and up to fivefold-higher specific productivity compared with traditional yeast fermentations (10). Unfortunately, its substrate range is restricted to the fermentation of glucose, sucrose, and fructose, and, as such, wild-type strains are unable to ferment the pentose sugars derived from lignocellulosic feedstocks because they lack the essential pentose assimilation and metabolism pathways. To address this need, we have recently developed a xylose-fermenting strain of *Z. mobilis* by introducing genes that encode the xylose isomerase, xylulokinase, transaldolase, and transketolase activities necessary for converting xylose to common intermediates of the glycolytic Entner-Doudoroff pathway (19). This strain offers the potential to increase ethanol yield and lower capital and operating costs through rapid, efficient, and simultaneous fermentation of the glucose and xylose in lignocellulosic feedstocks to ethanol. We report here the development of an arabinose-fermenting strain of *Z. mobilis* that may be useful, along with the xylose-fermenting strain, for conversion of the predominant hexose and pentose sugars in agricultural residues and other lignocellulosic feedstocks.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* DH5α [F⁻ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* λ⁻ *thi-1 gyrA96 relA1*] (GIBCO BRL, Gaithersburg, Md.) was used as a cloning host. *E. coli* B/r was used as the source of the *araBAD* operon (12). *E. coli* UP1098 (CGSC 5289) (F⁻ *thr-11 leu-19 araA202*), LBB323 (CGSC 7251) [F⁻ *araB6* λ⁻ IN(*rrnD-rrnE*)*1*], and LBB324 (CGSC 7252) [F⁻ *araD54* λ⁻ IN(*rrnD-rrnE*)*1*] were obtained from the *E. coli* Genetic Stock Center of Yale University and used as hosts for confirmation of *araA*, *araB*, and *araD* genes, respectively. *Z. mobilis* ATCC 39676 was used as the transformation host for the arabinose assimilation and pentose phosphate pathway genes. *Z. mobilis* ATCC 39676, containing the shuttle vector pZB186 (19), was used as the control strain. *Z. mobilis* strains were cultured in

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RM media containing yeast extract (10 g/liter), potassium phosphate, dibasic (2 g/liter), tetracycline (10 μg/ml), and sugar at the designated concentrations. *E. coli* strains were cultured in LB medium (Difco) containing tetracycline (10 μg/ml) or ampicillin (50 μg/ml), as appropriate.

Isolation of the L-arabinose isomerase, L-ribulokinase, and L-ribulose-5-phosphate-4-epimerase genes and fusion to a *Z. mobilis* GAP promoter. The L-arabinose isomerase (*araA*), L-ribulokinase (*araB*), and L-ribulose 5-phosphate 4-epimerase (*araD*) genes were isolated separately from the native *araBAD* operon of *E. coli* B/r (12) by PCR synthesis and were subcloned to form a modified *araBAD* operon devoid of repetitive extragenic palindromic sequences. To express the L-ribulokinase, L-arabinose isomerase, and L-ribulose 5-phosphate 4-epimerase (*araBAD*) genes in *Z. mobilis*, they were precisely fused to a *Z. mobilis* glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter (3) by a PCR-mediated overlap extension technique (8). This approach allowed precise fusion of the GAP promoter containing a ribosome binding site to the translational start codon of the L-ribulokinase gene (*araB*), thus ensuring that the expression of the *araBAD* genes would be directed solely from the GAP promoter. To accomplish this precise fusion, 308 bp of 5′-flanking DNA upstream of the GAP structural gene comprising the GAP promoter and the first 582 bp of the *araB* structural gene were separately synthesized in a PCR using a common linking oligonucleotide primer. The individual DNA fragments were recovered from an agarose gel and combined in a second PCR in which the complementary ends at the 3′ end of the GAP promoter and the 5′ end of the *araB* gene were annealed. The addition of the 5′ GAP and 3′ *araB* primers then allowed the synthesis of a 902-bp DNA fragment comprising a precise fusion of the GAP promoter to the *araB* gene.

The primers used to synthesize the 308-bp DNA fragment comprising the GAP promoter were based on the known DNA sequence of the 5′-flanking region of the GAP gene (3) and included the following:

5′-GGAATTCGCGGCCGCGTTCGATCAACAACCCGAATCC-3′

5′-**CAATTGCAATCGCCAT**GTTTATTCTCCTAACTTATTAAGTAGCTATTATATTCC-3′

A 15-bp DNA sequence comprising restriction sites for the restriction enzymes *Eco*RI and *Not*I (underlined) was incorporated at the 5′ end of the synthesized GAP promoter. A 16-bp DNA sequence (boldface) corresponding to the 5′ end of the *araB* gene was added to the 3′ end of the synthesized GAP promoter. The primers used to synthesize the DNA fragment comprising the first 582 bp of the *araB* gene were based on its known DNA sequence (12) and included the following:

5′-**GTTAGGAGAAACATG**GCGATTGCAATTGGCCTCGATTTTGGC-3′

5′-CGGGCGGGTGGTACCGGAAAG-3′

A 15-bp DNA sequence (boldface) corresponding to the 3′ end of the GAP promoter was added to the 5′ end of the synthesized *araB* gene fragment. Following the second PCR synthesis, the 902-bp PCR fragment was purified by preparative agarose gel electrophoresis and digested with *Eco*RI and *Kpn*I to generate the 891-bp *Eco*RI-*Kpn*I DNA fragment, comprising a precise fusion of the GAP promoter to the *araB* gene.

The 2,679-bp DNA fragment comprising the 3′ end of the *araB* and *araA* genes was obtained by PCR synthesis from the *E. coli* B/r chromosome. The primers used to synthesize this DNA fragment were based on its known DNA sequence (12) and included the following:

5′-CTTTCCGGTACCACCCGCCCG-3′

5′-TAACATGTTGACTCCTTCTCTAGACTTAGCGACGAAATCCGTAATACAC-3′

A 26-bp DNA sequence, comprising the restriction site for *Xba*I, was incorporated at the 3′ end of the *araA* gene. Following PCR synthesis, the 2,679-bp PCR fragment was purified by preparative agarose gel electrophoresis and digested with *Kpn*I and *Xba*I to generate the 2,652-bp *Kpn*I-*Xba*I DNA fragment comprising the 3′ end of the *araB* and *araA* genes.

To remove the repetitive extragenic palindromic sequences between *araA* and *araD* in the native *araBAD* operon, the *araD* gene encoding L-ribulose-5-phosphate-4-epimerase was isolated separately from the *E. coli* B/r chromosome by PCR synthesis and then linked to the 3′ end of *araA* to form a modified *araBAD* operon. The primers used to synthesize the 916-bp DNA fragment comprising the *araD* gene were based on its known DNA sequence (12) and included the following:

5′-CGGATTTCGTCGCTAAGTCTAGAGAAGGAGTCAACATGTTAGAAGATCTC-3′

5′-CCCCCAAGCTTGCGGCCGCGGCCCGTTGTCCGTCGCCAG-3′

A 23-bp DNA sequence comprising a restriction site for *Xba*I was incorporated at the 5′ end of the *araD* gene, and a 19-bp DNA sequence comprising restriction sites for *Hin*dIII and *Not*I was incorporated at the 3′ end of the *araD* gene to facilitate its subsequent subcloning. Following PCR synthesis, the 916-bp PCR fragment was purified by preparative agarose gel electrophoresis and digested with the *Xba*I and *Hin*dIII to generate the 892-bp DNA fragment comprising the *araD* gene, which was ligated to plasmid pUC18 that had been digested with the same restriction enzymes. The ligated DNA was used to transform *E. coli* DH5α, and restriction analyses of the plasmid DNA from ampicillin-resistant transformants confirmed the presence of the expected plasmid, which was designated pUC-*araD*.

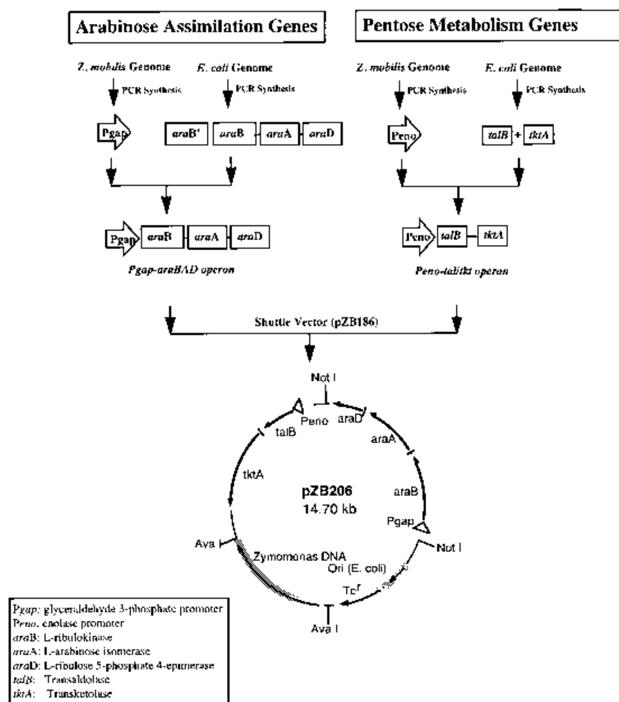

FIG. 1. Cloning strategy for construction of plasmids bearing arabinose assimilation and pentose phosphate pathway genes (see text for details).

To construct a new *araBAD* operon, *araD* was linked to the 3′ end of *araA*. The 2,652-bp *Kpn*I-*Xba*I DNA fragment comprising the 3′ end of the *araB* and the *araA* genes was ligated to pUC-*araD*, which had been digested with restriction enzymes *Kpn*I and *Xba*I. The ligated DNA was used to transform *E. coli* DH5α, and restriction analyses of the plasmid DNA from ampicillin-resistant transformants confirmed the presence of the expected plasmid, which was designated pUC-*araB′AD*. pUC-*araB′AD* contains the partial modified *araBAD* operon.

The plasmid pBRMCS, which was constructed by inserting the *Eco*RI-*Hin*dIII multiple cloning site fragment of pUC18 into the *Eco*RI and *Hin*dIII sites in pBR322, was used to subclone the new P_{gap}-*araBAD* operon. The 3,544-bp *araB′AD* fragment was isolated by preparative agarose gel electrophoresis following digestion of pUC-*araB′AD* with *Kpn*I and *Hin*dIII and ligated to pBRMCS, which had been digested with the same restriction enzymes. The ligated DNA was used to transform *E. coli* DH5α, and restriction analyses of the plasmid DNA from ampicillin-resistant transformants confirmed the presence of the expected plasmid, which was designated pBRMCS-*araB′AD*. The previously obtained 891-bp *Eco*RI-*Kpn*I DNA fragment, comprising a precise fusion of the GAP promoter to the *araB* gene, was then ligated to pBRMCS-*araB′AD*, which had been digested with restriction enzymes *Kpn*I and *Eco*RI. The ligated DNA was used to transform *E. coli* DH5α, and restriction analyses of the plasmid DNA from ampicillin-resistant transformants confirmed the presence of the expected plasmid, which was designated pBR *gap-araBAD*. Digestion of this plasmid with the restriction enzyme *Not*I liberates an approximately 4.4-kb restriction fragment containing the L-arabinose isomerase, L-ribulokinase, and L-ribulose-5-phosphate-4-epimerase genes under the control of the *Z. mobilis* GAP promoter, designated the P_{gap}-*araBAD* operon (Fig. 1).

Construction of a recombinant plasmid containing arabinose metabolism and pentose phosphate pathway genes and Its transfer into *Z. mobilis*. The pZBET plasmids containing the P_{eno}-*tal*/*tktA* operon comprising the transaldolase (*talB*) and transketolase (*tktA*) genes from *E. coli* cloned precisely under the control of the *Z. mobilis* enolase (ENO) promoter in either clockwise or counterclockwise orientation were previously described (19). To introduce the P_{gap}-*araBAD* operon into these plasmids, the approximately 4.4-kb *Not*I restriction fragment from plasmid pBR *gap-araBAD* was purified by preparative agarose gel electrophoresis and separately ligated to *Not*I-linearized pZBET. The ligated DNA was used to transform *E. coli* DH5α, and restriction analyses of the plasmid DNA from tetracycline-resistant transformants confirmed the presence of the expected plasmids. The plasmid containing the P_{eno}-*talB*/*tktA* operon and the P_{gap}-*araBAD* operon in clockwise orientations was designated pZB200. The plasmid containing the P_{eno}-*talB*/*tktA* operon in clockwise orientation and the P_{gap}-*araBAD* operon in counterclockwise orientation was designated pZB202. The plasmid

containing the P_{eno}-*talB/tktA* operon in counterclockwise orientation and the P_{gap}-*araBAD* operon in clockwise orientation was designated pZB204. The plasmid containing the P_{eno}-*talB/tktA* operon and the P_{gap}-*araBAD* operon in counterclockwise orientations was designated pZB206 (Fig. 1).

Plasmids pZB200, pZB202, pZB204, and pZB206 were separately transformed into *Z. mobilis* ATCC 39676 by electroporation of approximately 10^9 cells per ml with 1.2 to 3.0 μg of DNA in 40 μl of 10% (wt/vol) glycerol at 16 kV/cm, 200Ω, and 25 μF. After electroporation, the cells were allowed to recover at 30°C for 3 to 16 h in a liquid medium comprising 5% (wt/vol) glucose, 10% yeast extract (Difco, Detroit, Mich.), 5% tryptone (Difco), 0.25% ammonium sulfate, 0.02% potassium phosphate, dibasic, and 1 mM magnesium sulfate. Transformants containing pZB200, pZB202, pZB204, and pZB206 were isolated after anaerobic incubation at 30°C for 2 or more days in the same medium containing 1.5% (wt/vol) agar and tetracycline (20 μg/ml) and were subsequently confirmed by restriction analyses of the plasmid DNA from tetracycline-resistant transformants.

Evaluation of arabinose fermentation performance. The comparative fermentation performances of *Z. mobilis*(pZB186) and *Z. mobilis*(pZB206), the latter containing the L-arabinose isomerase, L-ribulokinase, L-ribulose 5-phosphate 4-epimerase, transaldolase, and transketolase genes, was determined in RM medium containing 2.5% (wt/vol) glucose, 2.5% arabinose, or 2.5% arabinose plus 2.5% glucose. The cells were first cultured in RM medium containing 2.5% glucose at 30°C until late log phase and then inoculated into 95 ml of RM medium in a 100-ml bottle to an initial optical density at 600 nm (OD_{600}) of 0.15. Cell growth was monitored as the OD_{600} with a Beckman DU640 spectrophotometer. One OD_{600} unit corresponds to approximately 0.3 mg (dry weight) of cells per ml. The cultures were incubated at 37°C without agitation. Glucose, arabinose, and ethanol were analyzed with a Hewlett-Packard (HP) 1090L high-performance liquid chromatograph equipped with an HP 1047A refractive index detector and a Bio-Rad HPX-87H organic acid analysis column operating at 65°C with a 0.01 N sulfuric acid mobile phase flow rate of 0.6 ml/min. Ethanol yield is based on either the amount of consumed sugar ($Y_{p/s}$) or the total available sugar (Y_p). The maximum theoretical yield is 0.51 g of ethanol per g of glucose or arabinose.

Enzyme assays. *Z. mobilis*(pZB206) was cultured in RM medium containing 2.5% (wt/vol) glucose at 37°C until late log phase. The cells were collected by centrifugation, washed with sonication buffer containing 10 mM Tris-HCl (pH 7.6), 10 mM magnesium chloride, and 1 mM dithiothreitol, and suspended in the same buffer. The cells were ruptured by sonication in an ice bath with a Branson sonifier 450 operated at 20 W and with a 60% pulse. Cell debris was removed by centrifugation (17,530 × *g*, 45 min, 4°C), and the supernatant was recovered. L-Arabinose isomerase activity was measured as described previously (16). L-Ribulokinase activity was measured by a modification of the method described previously (11), using lactate dehydrogenase (Sigma, St. Louis, Mo.) and pyruvate kinase (Sigma), each at 4 U/ml, and purified ATP (Boehringer Mannheim, Indianapolis, Ind.). Transaldolase and transketolase activities were measured as described previously (5). L-Ribulose-5-phosphate-4-epimerase was determined discontinuously as follows. L-Ribulose-5-phosphate was first generated in a reaction mixture containing 50 mM Tris (pH 7.6), 0.33 mM EDTA, 4 mM magnesium chloride, 1 mM ATP, 50 mM L-ribulose, and L-ribulokinase (0.015 μmol of activity purified from the *E. coli araD* mutant per min) for 10 min at 37°C. The reaction was initiated by the addition of cell extract and was terminated by heating at 92°C for 2 min. D-Xylulose-5-phosphate was measured in a 500-μl reaction mixture containing 50 mM Tris HCl (pH 7.6), 10 mM magnesium chloride, 1 mM erythrose-4-phosphate, 1 mM NADP, 0.1 mM thiamine pyrophosphate, 0.2 U of yeast transketolase (Boehringer Mannheim), 10 μg of phosphoglucose isomerase (Boehringer Mannheim), 0.05 μg of glucose-6-phosphate dehydrogenase (Boehringer Mannheim), and 40 μl of L-ribulose-5-phosphate preparation. Reaction rates were determined by comparison with standard D-xylulose-5-phosphate solutions as described previously (6). Protein concentrations were determined with the Bio-Rad protein assay (2), with bovine serum albumin as the standard. Enzyme activities are expressed in micromoles per minute per milligram (units per milligram) of protein.

RESULTS

Isolation and characterization of an arabinose-fermenting *Z. mobilis* strain. Enzymatic analyses of wild-type *Z. mobilis* indicated the lack of L-arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate-4-epimerase, transaldolase, and (sufficient) transketolase activities. This suggested that the introduction and expression of the genes encoding these enzymes were necessary for the completion of a functional metabolic pathway that would permit conversion of L-arabinose to common intermediates of the Entner-Doudoroff pathway and thus enable *Z. mobilis* to grow on L-arabinose and ferment it to ethanol. To construct an arabinose-fermenting *Z. mobilis* strain, the *E. coli araB*, *araA*, and *araD* genes were isolated by PCR synthesis, subcloned to form a modified *araBAD* operon devoid of repetitive extragenic palindromic sequences, and subcloned precisely under the control of a strong, constitutive *Z. mobilis* GAP promoter (3) by PCR-mediated overlap extension (8). The accurate synthesis and construction of a functional *araBAD* operon (Fig. 1) was first confirmed by separate complementation of *E. coli araB*, *araA*, and *araD* mutants. The *araBAD* operon was then subcloned into the chimeric plasmid pZBET, constructed from a 2.7-kb *Z. mobilis* native plasmid, the pACYC184 cloning vector, and the *E. coli* transaldolase (*talB*) and transketolase (*tktA*) genes cloned precisely under the control of the *Z. mobilis* enolase (ENO) promoter (19). The two operons comprising the five arabinose assimilation and pentose phosphate pathway genes in four different orientations were then transformed into *Z. mobilis* ATCC 39676 by electroporation. Transformants were selected on the basis of tetracycline resistance and subsequently screened for growth on arabinose as the sole carbon source. Restriction analyses of the plasmid DNA from tetracycline-resistant transformants confirmed the presence of the expected plasmids. Transformants capable of growth on solid medium containing arabinose as the sole carbon source were obtained from all plasmids except pZB202, in which the P_{gap}-*araBAD* and P_{eno}-*talB/tktA* operons were in a convergent orientation. *Z. mobilis*(pZB206), in which the P_{gap}-*araBAD* and P_{eno}-*talB/tktA* operons are both in a counterclockwise orientation, demonstrated the fastest growth in liquid RM medium containing arabinose as the sole carbon source and was used in all subsequent experiments.

Enzymatic analyses of *Z. mobilis*(pZB206) grown in RM medium containing glucose as the sole carbon source demonstrated the presence of L-arabinose isomerase (7.2 U/mg), L-ribulokinase (2.7 U/mg), L-ribulose-5-phosphate-4-epimerase (0.69 U/mg), transaldolase (2.3 U/mg), and transketolase (0.24 U/mg) activities, thus confirming the expression of all five genes in *Z. mobilis*. These enzymatic activities were largely undetectable in *Z. mobilis*(pZB186), the control strain.

Fermentation performance of *Z. mobilis* containing the arabinose metabolism and pentose phosphate pathway genes. The results presented in Fig. 2A show that *Z. mobilis*(pZB206) containing the L-arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate-4-epimerase, transaldolase, and transketolase genes demonstrated growth on and ethanol production from L-arabinose as a sole carbon source. The recombinant strain produced ethanol from arabinose at 98% of the maximum theoretical yield based on the amount of sugar consumed in 72 h at 37°C. The *Z. mobilis*(pZB186) control strain neither grew on nor produced any ethanol from arabinose. The overall process yield based on the amount of total available sugar was 84% of the maximum theoretical yield because of residual unfermented arabinose. The near-theoretical consumed sugar yield indicates that, once transported into the cell, arabinose was metabolized almost exclusively to ethanol as the sole fermentation product, with little by-product formation. *Z. mobilis*(pZB206) produced ethanol as rapidly and efficiently as the control strain on 2.5% (wt/vol) glucose and achieved the maximum theoretical ethanol yield in 24 h at 37°C (Fig. 2B). The engineered strain grew slightly more slowly than the control strain but reached the same final cell density.

In the combined presence of glucose and arabinose, and in contrast to the control strain, which fermented only glucose, metabolically engineered *Z. mobilis*(pZB206) fermented both sugars at 99% of the maximum theoretical ethanol yield based on the amount of sugar consumed in 48 h at 37°C (Fig. 2C). The process yield based on total available sugar was only 84% of the maximum theoretical yield due to residual unfermented arabinose. Although no diauxic effect was evident, arabinose

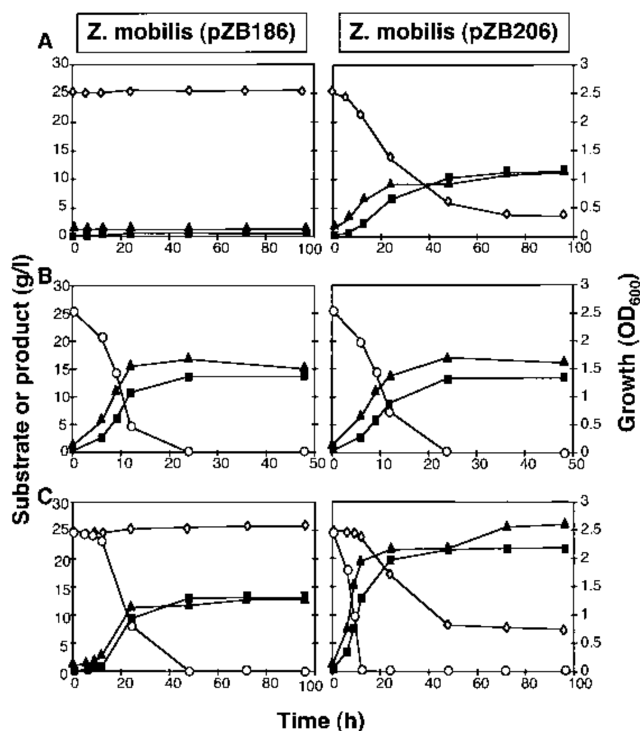

FIG. 2. Comparative fermentation performances of *Z. mobilis*(pZB186) (left panels) and *Z. mobilis*(pZB206) (right panels) in fermentation media containing 2.5% (wt/vol) arabinose (A), 2.5% (wt/vol) glucose (B), or 2.5% (wt/vol) glucose plus 2.5% (wt/vol) arabinose (C). Symbols: ○, glucose; ◇, arabinose; ■, ethanol; ▲, OD_{600}.

was utilized at a considerably slower rate and only after near-complete glucose depletion.

DISCUSSION

The construction of microorganisms capable of rapid and efficient conversion of the D-xylose and L-arabinose commonly found in the hemicellulose fraction of lignocellulosic feedstocks is essential for the development of an economical process for biomass conversion to fuel ethanol. Previously we described the construction of a xylose-fermenting strain of the ethanologenic bacterium *Z. mobilis* that is useful for simultaneous fermentation of the glucose and D-xylose found in these feedstocks (19). We report here the construction of a *Z. mobilis* strain for efficient fermentation of L-arabinose. Five genes that encode the L-arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate-4-epimerase, transaldolase, and transketolase activities necessary for conversion of L-arabinose to common intermediates of the Entner-Doudoroff pathway were simultaneously introduced into *Z. mobilis* under the control of constitutive promoters that would direct their expression even in the presence of glucose. The engineered strain grew on and produced ethanol from arabinose as a sole carbon source at 98% of the maximum theoretical ethanol yield based on the amount of consumed sugar, indicating that arabinose was metabolized almost exclusively to ethanol as the sole fermentation product.

Efficient fermentation of L-arabinose to ethanol was thus obtained through a combination of the pentose phosphate and Entner-Doudoroff pathways. With the introduction of an L-arabinose assimilation pathway and the nonoxidative portion of the pentose phosphate pathway into this microorganism, L-arabinose is presumably converted to L-ribulose-5-phosphate and then further metabolized to glyceraldehyde-3-phosphate and fructose-6-phosphate, which effectively couples L-arabinose metabolism to the glycolytic Entner-Doudoroff pathway and consequently to ethanol production (Fig. 3). In the overall fermentation reaction, 3 mol of L-arabinose is converted to 5 mol of ethanol. Neglecting the NAD(P)H balance, the stoichiometry can be shown by the following equation: 3 L-arabinose + $3ADP$ + 3 P_i -------> 5 ethanol + $5CO_2$ + $3ATP$ + $3H_2O$. The theoretical ethanol yield based on this stoichiometry is 0.51 g of ethanol per g of L-arabinose or 1.67 mol of ethanol per mol of L-arabinose. In this new pathway, the net ATP yield from 3 mol of L-arabinose is 2 mol less than that postulated for conventional L-arabinose fermentation through a combination of pentose phosphate and Embden-Meyerhoff-Parnas pathways. Because less substrate is used for biomass formation, ethanol production via L-arabinose fermentation in the engineered *Z. mobilis* strain is more efficient than that in any other known microorganism and equivalent to the efficiency of D-xylose fermentation previously reported for a xylose-fermenting *Z. mobilis* strain (19).

E. coli has been metabolically engineered to produce ethanol from glucose and pentoses by the introduction of ethanol production genes from *Z. mobilis* (9). We demonstrated here another example of the complementary approach of introducing the arabinose assimilation and pentose phosphate pathways into *Z. mobilis.*

In fermentation media that contain glucose as the sole carbon source, *Z. mobilis*(pZB206) produced ethanol as rapidly and efficiently as the control strain. The engineered strain grew slightly more slowly than the control strain but reached the same final cell density. This result probably reflects the surprisingly minor metabolic burden imposed on *Z. mobilis* by the high-level expression of the five additional genes. Although near-theoretical ethanol yields based on consumed sugar were obtained in both arabinose and combined-glucose-and-arabinose media, arabinose was not completely utilized. Incomplete utilization of arabinose could be caused by a decrease in the pH during fermentations without pH control. It was noticed that the final pH decreased to around pH 4.6 in medium containing both glucose and arabinose as compared with pH 4.9 in medium containing arabinose alone. The higher residual arabinose concentration following growth in the presence of both glucose and arabinose as compared with growth on arabinose alone may reflect inhibition of arabinose uptake at low pH. It is not likely that an ethanol concentration of about 20 g/liter caused inhibition of the arabinose assimilation enzymes. In fact, more than 27 g of ethanol per liter was produced in media containing high initial glucose and arabinose concentrations when the pH was controlled at 5.25 (data not shown).

The recombinant strain is stable in the presence of tetracycline or arabinose (data not shown). However, only 40% of the cells retained the ability to ferment arabinose after 20 generations of growth in complex media without selection pressure at 30°C, and they completely lost the ability to ferment arabinose within 7 generations at 37°C. Future work will be directed toward improving stability of the strain by either increasing the plasmid stability or integrating the arabinose fermentation genes into the *Z. mobilis* chromosome.

In the presence of both glucose and arabinose, arabinose was utilized at a considerably slower rate than glucose and only after near-complete glucose depletion. The previously developed xylose-fermenting strain utilized glucose at a faster rate than xylose, but both sugars were utilized simultaneously (19). In *Z. mobilis*, glucose is transported by a low-affinity, high-velocity, energy-independent, glucose-facilitated diffusion (Glf)

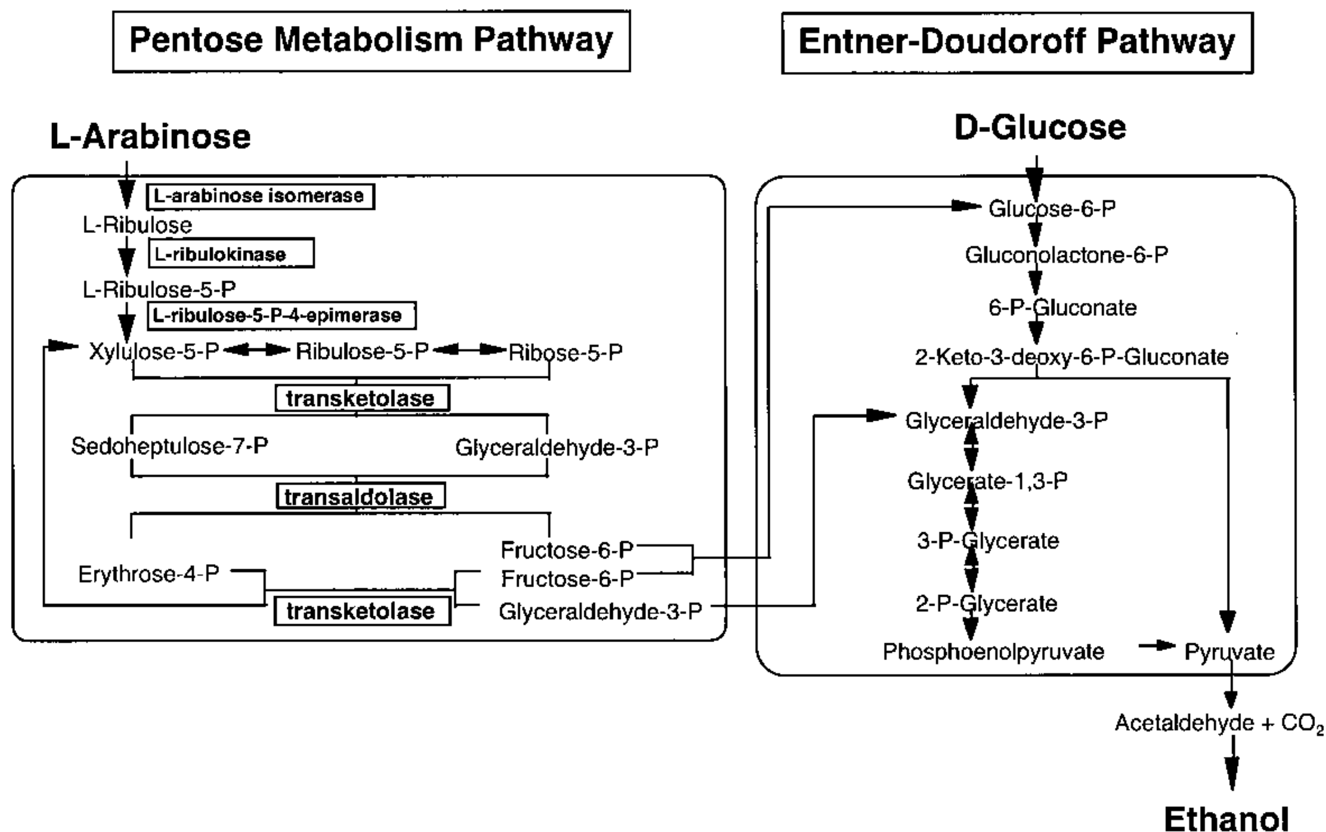

FIG. 3. Proposed metabolic pathway for arabinose fermentation by engineered *Z. mobilis*(pZB206).

transport system (4) that is well suited to the high-sugar plant saps that *Z. mobilis* inhabits (17). The presumptive *glf* gene has been isolated and sequenced (1), and the transport kinetics of its gene product have been characterized in a heterologous system (15). Substrate analog competition experiments have shown that Glf has a very narrow substrate range and is competitively inhibited by closely related analogs, including D-xylose. Recent studies suggest that Glf may be the primary, if not the sole, system required for transport of glucose and fructose (18), the only monosaccharides that *Z. mobilis* naturally ferments. Therefore, it is reasonable to expect that L-arabinose may be transported by this indigenous glucose-facilitated diffusion transport system but susceptible to competitive inhibition at high glucose concentrations. The rapid utilization of arabinose immediately after glucose depletion, as well as the absence of a diauxic growth pattern, suggests that the preferential utilization of glucose before arabinose is probably due to inhibition of arabinose transport by glucose rather than to glucose catabolite repression. Enzymatic assays demonstrated the presence of all five required arabinose assimilation and pentose phosphate pathway enzymes in glucose-grown cells. It is also possible that *Z. mobilis* has adapted to the newly acquired ability to ferment arabinose through fortuitous *glf* mutations that permit pentose sugar transport via the indigenous Glf transport system at rates sufficient to generate enough ATP for growth, maintenance, and efficient conversion to ethanol. Interestingly, we have observed improved arabinose fermentation performance upon sequential transfer of *Z. mobilis*(pZB206) in media that contained L-arabinose as the sole carbon source but have been unable to attribute this improvement to any obvious differences in the levels of arabinose assimilation or pentose phosphate pathway enzyme activities (results not shown).

We expect that, with further improvements in pentose sugar transport, this new arabinose-fermenting *Z. mobilis* strain may be used in a mixed culture with the xylose-fermenting *Z. mobilis* strain for rapid and efficient fermentation of the predominant hexose and pentose sugars in agricultural residues, such as corn fiber, and herbaceous energy crops, such as switchgrass.

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