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Expression of the *Escherichia coli pmi* Gene, Encoding Phosphomannose-Isomerase in *Zymomonas mobilis*, Leads to Utilization of Mannose as a Novel Growth Substrate, Which Can Be Used as a Selective Marker†

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Wild-type *Zymomonas mobilis* can utilize only three substrates (sucrose, glucose, and fructose) as sole carbon sources, which are largely converted into ethanol and carbon dioxide. Here, we show that although D-mannose is not used as a growth substrate, it is taken up via the glucose uniport system (glucose facilitator protein) with a V_{max} similar to that of glucose. Moreover, D-mannose was phosphorylated by a side activity of the resident fructokinase to mannose-6-phosphate. Fructokinase was purified to homogeneity from an *frk*-recombinant *Z. mobilis* strain showing a specific activity of 205 ± 25 U of protein mg^{-1} with fructose (K_m, 0.75 ± 0.06 mM) and 17 ± 2 U mg^{-1} (relative activity, 8.5%) with mannose (K_m, 0.65 ± 0.08 mM). However, no phosphomannoseisomerase activity could be detected for *Z. mobilis*, and this appeared to be the reason for the lack of growth on mannose. Therefore, we introduced the *Escherichia coli* gene *pmi* (*manA*) in *Z. mobilis* under the control of a *lacI*^{q}-P*tac* system on a broad-host-range plasmid (pZY507; Cm^r). Subsequently, in *pmi*-recombinant cells of *Z. mobilis*, phosphomannoseisomerase was expressed in a range of from 3 U (without isopropyl-β-D-thiogalactopyranoside [IPTG]) to 20 U mg^{-1} of protein in crude extracts (after IPTG induction). Recombinant cells of different *Z. mobilis* strains utilized mannose (4%) as the sole carbon source with a growth rate of 0.07 h^{-1}, provided that they contained fructokinase activity. When the *frk* gene was additionally expressed from the same vector, fructokinase activities of as much as 9.7 U mg^{-1} and growth rates of as much as 0.25 h^{-1} were detected, compared with 0.34 h^{-1} on fructose for wild-type *Z. mobilis*. Selection for growth on mannose was used to monitor plasmid transfer of pZY507*pmi* from *E. coli* to *Z. mobilis* strains and could replace the previous selection for antibiotic resistance.

The gram-negative bacterium *Zymomonas mobilis* is an efficient ethanol producer with favorable features that are at least equal to those from the more familiar brewer's yeast. However, its substrate spectrum is narrow, since it uses only sucrose, glucose, and fructose as carbon sources (29). Numerous efforts have been undertaken to broaden its substrate or product range (for reviews and references therein, see references 14, 27, and 28). A prerequisite for genetic improvement for utilization of abundant and inexpensive compounds, for example, xylose or mannose from plant hemicellulose, is the uptake of the respective sugars into the cells. *Z. mobilis* uses a uniporter (glucose facilitator protein [GLF]) for glucose transport which does not need metabolic energy for its function (8, 19, 26, 34) and was shown recently to also accept fructose (34). From indirect competition assays, mannose, xylose, galactose, and other sugars were also suggested to be substrates for the GLF transporter (19). Detailed uptake studies for GLF were performed in *glf*-recombinant *Escherichia coli* mutant strains which lacked both transport and phosphorylation of glucose and fructose (19, 34). In further studies of GLF in *E. coli*, we recognized that mannose is also a substrate for GLF and is phosphorylated by the fructokinase (FRK) of *Z. mobilis*. This led us to further investigate whether a mannose catabolic pathway could be engineered in *Z. mobilis* strains by introducing the *pmi* gene encoding phosphomannoseisomerase (PMI) from *E. coli.*

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† Dedicated to the memory of the late Michael Ciriacy.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *E. coli* K-12 strains were grown on Luria-Bertani or on MacConkey agar base indicator plates with 1% sugar supplementation. Defined mineral salts media (*E. coli* mineral salts media [EMM]) (30) were prepared with a 0.5% carbon source. Ampicillin was added at 100 mg liter^{-1}, and chloramphenicol was added at 25 mg liter^{-1}. *Z. mobilis* strains were cultivated anaerobically in complex medium with a 4% carbon source (6), complex medium consisting of (per liter) 1 g of $(NH_4)_2 SO_4$, 1 g of KH_2PO_4, 0.5 g of $MgSO_4$, 10 g of yeast extract, distilled water, and chloramphenicol (100 mg liter^{-1}). Cells were grown at 30°C in 200-ml glass vessels filled with 50 ml of medium. Growth was recorded turbidometrically in a Shimadzu UV-160 spectrophotometer at a wavelength of 600 nm.

DNA techniques. DNA preparation, cloning, restriction analysis, and transformation were according to established methods (13, 22) and according to manufacturers' instructions. Chromosomal DNA from *E. coli* CA 8000 (4) was prepared according to a lysozyme–freeze-thaw method (10). DNA fragments were recovered by excision from agarose gels in Tris (40 mM)-acetate (20 mM) buffer at pH 8.0 and were purified with glassmilk-iodine (GeneClean kit; Dianova). For *Taq* DNA polymerase-dependent cyclic DNA amplification (18), oligonucleotides were used as primers (50 ng each), and chromosomal DNA (0.5 μg) from *E. coli* CA 8000 was used as the template. DNA amplification was done in 50 mM KCl–1.5 mM $MgCl_2$–10 mM Tris-HCl–0.1 mg of gelatin ml^{-1} at pH 8.3 (20°C) with *Taq* DNA polymerase (Boehringer Mannheim) for 30 cycles of 94°C (initially for 7 min; afterward, for 1 min), 55°C (1 min), 70°C (2 min; last cycle, 10 min). The amplified fragments were used without further purification and were subjected to restriction. For the cloning of the *pmi* gene, chromosomal DNA from *E. coli* K-12 CA 8000 was used in conjunction with the PCR primers 5′-TAG GAT CCA CAT TAA AAC AGG GAT TGA TC-3′ and 5′-GCG AAG CTT AGC AAG AGA TGT TAA-3′ including an engineered *Bam*HI site and the indigenous *Hin*dIII site (underlined), respectively, which were based on the

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Size (kb)	Relevant genotype, mode of construction, or feature[a]	Reference or source
E. coli K-12			
CA 8000		Wild-type, prototrophic	4
ZSC112LΔ*ptsHI-crr*		*zfc*::Tn*10* Δ(*ptsHI-crr*) *glk ptsG ptsM fruA*	B. Erni
LR2-177		*galP manA nagE ptsG ptsM fruA ptsI mak*^{o}	1
DH5α		Δ(*lacZYA-argF*)U169 *recA1 hsdR17* φ80d*lacZ*ΔM15	Laboratory stock
S17-1		Mobilization strain	25
Z. mobilis			
ZM1 (ATCC 10988)		Wild-type strain	29
ZM6 (ATCC 29191)		Wild-type strain	29
F^-1959 (DSM3126)		ZM6 FRK deficient	6
CP4 (ATCC 31821)		Wild-type strain	12
Plasmids			
pUCBM20	2.7	*bla*; MCS; *lacZ*α	Boehringer Mannheim
pZY507	10.1	pSUP104-derived vector with *lacI*^{q}-P*tac*; MCS cloned into *tet*; Cm^{r}	34
pBM20*glf*	4.2	pUCBM20 with *glf* as *Bam*HI-*Hin*dIII fragment	34
pBM20*frk*	3.7	pUCBM20 with *frk* as *Kpn*I-*Bam*HI fragment	34
pZY507*glf*	11.6	pZY507 with *glf* gene from *Z. mobilis* CP4	34
pZY507*frk*	11.1	pZY507 with *frk* (ZM6) as *Kpn*I-*Bam*HI fragment	This study
pZY507*glf-frk*	12.6	pZY507*glf* with *frk* gene from *Z. mobilis* ZM6	34
pZY507*pmi*	11.7	pZY507 with *E. coli pmi* as *Bam*HI-*Hin*dIII fragment	This study
pZY507*frk-pmi*	12.7	pZY507*frk* with *pmi* as *Bam*HI-*Hin*dIII fragment	This study

[a] MCS, multiple cloning site; *mak*^{o}, deficient for mannofructokinase activity (1).

DNA sequence of the *E. coli pmi* (*manA*) gene (17), the oligomers being identical or complementary to bp 368 to 391 and 1584 to 1604, respectively, of the published *pmi* sequence.

For cloning and expression in *Z. mobilis*, a low-copy-number vector (pZY507) which confers chloramphenicol resistance and contains a *lacI*^{q}-P*tac* regulatory system (34) was used. The gene *pmi* was cloned by using the unique restriction sites *Bam*HI and *Hin*dIII to result in plasmid pZY507*pmi*. The *frk* gene from *Z. mobilis* had been amplified by PCR earlier (34) and was cloned as a *Bam*HI-plus-*Kpn*I fragment to yield pZY507*frk*. Insertion of the *pmi* gene into vector pZY507*frk* led to the tandem pZY507*frk-pmi* vector as summarized in Table 1.

Transport kinetics of heterologously expressed GLF. *E. coli* mutant strain ZSC112LΔ *ptsHI-crr*, which transports neither glucose, fructose, or mannose nor can phosphorylate them, was used. ZSC112LΔ *ptsHI-crr* carrying the vectors pZY507 (control) and pZY507*glf* was grown in defined mineral salts medium (30) with a 0.5% carbon source and adequate supplementation with thiamin (1 mg liter^{-1}) and chloramphenicol (25 mg liter^{-1}). For induction, isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) was added at an optical density at 600 nm (OD_{600}) of 0.2 to 0.4. Cells were harvested at an OD_{600} of 1.6 to 2.0, cooled on ice, washed twice in ice-cold EMM, and kept on ice at an OD_{600} of 30. In the uptake assay, 50 μl of ^{14}C-labeled substrate was added to 50 μl of cells (both incubated at 5°C), and the mixture was immediately vortexed (all radioisotopes were from Amersham Buchler, Braunschweig, Germany). The reaction was stopped by adding 5 ml of EMM with 8% glycerol at −3°C, and the cells were immediately filtered and washed twice with 5 ml of the stop solution (−3°C). The filters were immediately transferred to scintillation vials and counted after 30 min of incubation time in scintillation fluid. Specific activities of the various labeled substrates were 9.8 to 149 MBq mmol^{-1} (from Amersham Buchler). The internal label was measured as described above. For numerical analysis of the kinetic data, the program Enzfitter (Biosoft, Cambridge) was used. For determination of the substrate specificity of GLF, uptake of 22 mM mannose (11.3 MBq mmol^{-1}) was measured in the absence and presence of a 14-fold excess of unlabeled sugars.

Preparation of cell extracts and enzymatic assays. To prepare cell extracts of *Z. mobilis* CP4 or its derivatives, cells were harvested after growth in complex medium from the late-exponential growth phase (OD_{600} of 1.8) by centrifugation and were washed with Tris-HCl buffer (0.1 M; pH 7.0). After ultrasonic treatment (6 cycles of 30 s at a 40-W output, with cooling in an ethanol-NaCl ice bath; Branson Sonifier), cell debris was removed by centrifugation at 38,000 × *g*, and the supernatant was used as crude extract as described. Enzyme activities were determined in a Shimadzu UV-160 spectrophotometer thermostatted at 25°C. Activities of kinase enzymes were assayed with an NADP-coupled system (9) with auxiliary enzymes (all from Boehringer Mannheim, at 1 U ml^{-1} each) glucose-6-dehydrogenase (glucokinase) plus phosphoglucose isomerase (for FRK) plus PMI (for mannokinase). Unless specified otherwise, the assay mixtures contained in Tris-HCl buffer (0.1 mM; pH 7.4), ATP (final concentration, 1 mM), $MgCl_2$ (10 mM), $NADP^+$ (2.5 mM), and the respective sugars at 5 mM. Protein concentrations were determined by a dye-binding method (3).

Purification of FRK. Cells of *Z. mobilis* CP4/pZY507*frk* were grown anaerobically in 1 liter of complex medium with 2% glucose and chloramphenicol until an OD_{600} of 0.6 was reached. After addition of IPTG (final concentration, 1 mM), growth was prolonged and cells were harvested at an OD_{600} of 2.5 by centrifugation. After washing, they were resuspended in 200 ml of buffer I (20 mM Tris-HCl [pH 7.5], 0.5 mM dithiothreitol). The cells were broken in a prechilled glass-bead Disintegrator-S (BIOmatik GmbH, Rodgau-Hainhausen, Germany) for 5 min at 5,000 rpm. Cell debris was removed by centrifugation, and the supernatant was subjected to an ammonium sulfate precipitation (first step, 0 to 45%; second step, 45 to 70%). The sediment of the latter precipitation was resuspended in buffer I and was concentrated with an Amicon 8200 cell (Amicon Inc., Beverly, Mass.) and a 10YM10 ultrafiltration membrane. This concentrate was passed over an anion exchange column (MonoQ HR10/10; Pharmacia LKB, Freiburg, Germany). Buffer I was used for both column equilibration and elution. Elution was with an increasing NaCl gradient (0 to 500 mM) and with a constant flow rate of 2 ml min^{-1}. FRK activity eluted typically at approximately 175 mM NaCl. Active fractions were pooled and concentrated to a volume of 1 ml with a Centricon 30 (Amicon) centrifuge cell at 1,400 rpm. This solution was applied on a gel filtration column (60 cm in length and 1.6 cm in diameter; total volume, 120 ml) packed with Superdex 200 from Pharmacia LKB. Equilibration and elution were with buffer II (20 mM Tris-HCl [pH 7.5], 0.5 mM dithiothreitol, 150 mM NaCl) at a constant flow rate of 1 ml min^{-1}. FRK eluted at a relative mass corresponding to 60 kDa. Active fractions were collected, and were tested for activity and for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15).

RESULTS

GLF transporter from *Z. mobilis* also accepts D-mannose and D-xylose. In a previous publication, we had shown that expression of the genes *glf* plus *frk* restored growth on fructose of an *E. coli* host which lacked uptake and phosphorylation of fructose (34). This provided evidence that the GLF transporter accepts fructose as an additional substrate. To investigate possible further substrates of GLF, we assayed uptake of mannose and xylose by GLF using the *E. coli* host strain ZSC112LΔ *ptsHI-crr*. This strain lacks all phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) uptake systems because of mutations either in the structural genes or in the general components of the PTS. Moreover, FRK and glucokinase activities are absent (7). Similar to a method developed for measuring sugar uptake in *Saccharomyces cerevisiae* (33), a stopping procedure was employed which combined fast dilution into a buffer solution containing high glucose concentrations, which was followed by rapid washing with the same buffer at low temperature. With cells from one batch for up-

TABLE 2. Uptake of sugars via GLF in the heterologous host *E. coli* ZSC112LΔ*ptsHI-crr*/pZY507*glf*[a]

Substrate	K_m ± SD (mM)	V_{max} at 5°C (nmol min^{-1} mg [dry wt]$^{-1}$)
D-Glucose	4.1 ± 0.5	52
D-Fructose	39 ± 4	64
D-Mannose	8.4 ± 0.6	45
D-Xylose	40 ± 5	115

[a] Cells were grown in mineral salt medium with gluconate (0.5%) as the carbon source as described in Materials and Methods. To determine saturation kinetics, substrates were used at different concentrations (at least six), ranging from 0.5 to 43 mM for glucose, 1 to 43 mM for mannose, and 2 to 345 mM for fructose and xylose. Data are from three independent measurements. To determine V_{max}, cells from the same batch were used for the different substrates.

take assays of glucose, xylose, and mannose in parallel, linear uptake kinetics in the recombinant *E. coli* strains could be measured (Table 2). A Michaelis-Menten-type kinetics was observed for mannose (Fig. 1) and for xylose (data not shown). Interestingly, uptake of D-xylose was more than twice as fast as that of glucose. Rates for D-mannose and D-fructose uptake were about the same as those for D-glucose (Table 2).

Competition with mannose allows screening of other substrates for GLF. On the basis of the measured kinetic constants, mannose was an ideal substrate for competition studies aimed to screen other substrates of the GLF. In a previous study (19), glucose had been used as major transport substrate, which, however, is not well-suited because of its relatively high affinity, at least in comparison to other substrates tested. Mannose, which showed an intermediate K_m of 8 mM, was preferable to fructose, the high K_m (ca. 40 mM) of which would require extremely high additions of the competing substrate. Thus, we used D-mannose at a concentration of 22 mM and applied competing substrates at a 14-fold excess. As a control, the known substrates glucose, fructose, xylose, and mannose were also used. Several other sugars (e.g., D-glucosamine, D-galactose, L-sorbose, and D-arabinose) effectively competed with mannose in the uptake reaction, whereas sucrose, mannitol, or the L-enantiomer of arabinose did not (Table 3).

Mannose is phosphorylated by a side reaction of *Z. mobilis* FRK. When studying expression of the *glf-frk* genes in *E. coli*, we realized that derivatives of strain ZSC112LΔ*ptsHI-crr* concomitantly became mannose positive, whereas those of strain LR2-177 (*manA*) remained mannose negative. Expression of *glf* or *frk* alone, however, did not lead to mannose-positive clones of ZSC112LΔ*ptsHI-crr* (data not shown). We reasoned that after its uptake via GLF, mannose was phosphorylated by FRK to mannose-6-phosphate and then could enter the glycolytic pathways. One difference between ZSC112LΔ*ptsHI-crr* and strain LR2-177 is the lack of the amphibolic, constitutive PMI (gene *pmi* or *manA*) which catalyzes the reversible reaction from mannose-6-phosphate to fructose-6-phosphate (11). We found that heterologously expressed FRK in crude extracts of *E. coli* also phosphorylated mannose with a relative rate of approximately 8 to 10% compared with fructose as the substrate. In the *E. coli* strains we used, no other mannose-phosphorylating activity [neither from the PTS nor from a mannofructokinase (24)], could be detected.

FRK from *Z. mobilis* had been previously enriched and purified. One report stated that mannose was no substrate of FRK (9); in another report, a relative rate for the purified enzyme of less than 0.5% toward mannose was given (23). The reason for this discrepancy with our findings remains unclear. Cell extracts from several *Z. mobilis* CP4 derivatives, whether grown on glucose or fructose, showed relatively low mannose phosphorylation rates between 0.06 and 0.15 U mg^{-1} (Table 4). When they were grown with glucose as the substrate, addition of mannose did not affect growth, whereas mannose was clearly inhibitory under conditions of growth on fructose (Table 5). Therefore, we reasoned that mannose competed for both GLF and FRK in *Z. mobilis.*

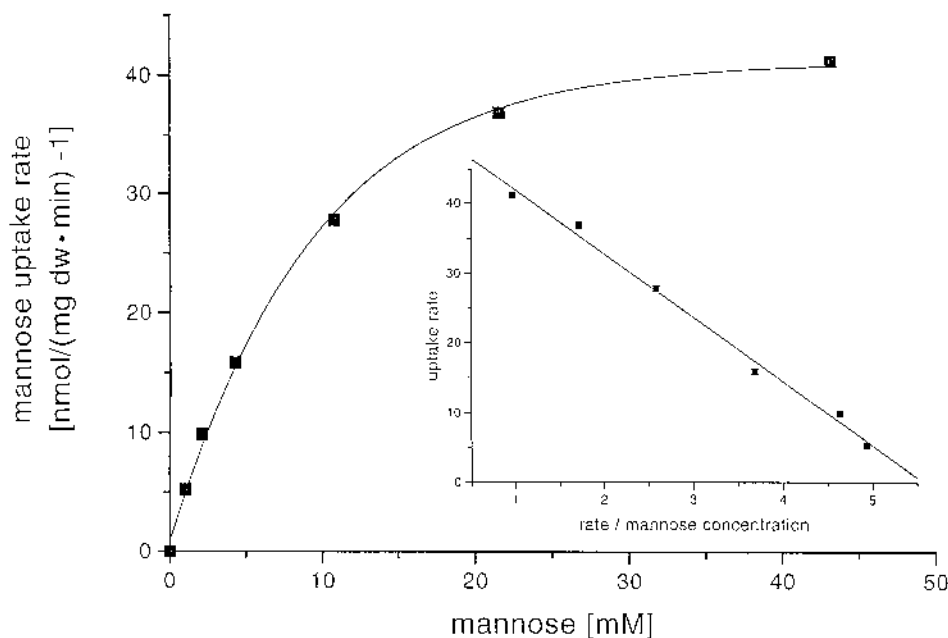

FIG. 1. Determination of K_m and V_{max} values for mannose uptake in recombinant *E. coli* ZSC112LΔ*ptsHI-crr*/pZY507*glf*. ^{14}C-labeled mannose at various concentrations was added to cells at 5°C. Uptake was measured as described in Materials and Methods. In the insert, an Eadie-Hofstee plot of the data is given.

TABLE 3. Inhibition of mannose uptake via GLF in *E. coli* ZSC112LΔ*ptsHI-crr*/pZY507*glf*[a]

Competitor	Residual D-mannose uptake (%)	Calculated mannose uptake (%)[b]
D-Glucose	9	5
D-Mannose	15	9
D-Xylose	34	32
D-Fructose	35	32
D-Glucosamine	20	
D-Galactose	44	
L-Sorbose	59	
D-Arabinose	67	
N-Acetyl-D-glucosamine	73	
D-Lyxose	75	
D-Gluconate	90	
D-Mannitol	97	
Sucrose	97	
L-Arabinose	100	
D-Mannose (22 mM)	100	

[a] Uptake of ^{14}C-labeled D-mannose (22 mM; 11.3 MBq mmol^{-1}) was measured in the presence or absence of a 14-fold excess (308 mM each) of a potential inhibitor at 5°C as described in Materials and Methods.

[b] Theoretical values which were calculated from the kinetic data obtained in Table 2.

Expression of the *pmi* gene of *E. coli* in *Z. mobilis*. No PMI activity could be detected in crude extracts of wild-type *Z.mobilis* (less than 0.001 U mg^{-1}). However, since mannose was clearly taken up and phosphorylated by *Z. mobilis*, it was interesting to find whether the addition of a functional PMI activity would complete a catabolic pathway for mannose in this bacterium. Using the PCR technique, we amplified and cloned the *E. coli* K-12 *pmi* (*manA*) gene, according to the earlier established sequence (17), from the wild-type strain CA 8000 (4) as a target. The *pmi* gene was inserted in vector pZY507, which allowed expression under a *lacI*^{q}-P*tac* system both in *E. coli* and in *Z. mobilis*. The vector was then mobilized from *E. coli* S17-1 into *Z. mobilis* CP4. Selection was on complex medium with glucose, chloramphenicol, and nalidixic acid to counterselect both parent strains (31). *Z. mobilis* Cm^r exconjugants were purified and tested for the presence of the plasmid DNA and for expression of the *pmi* gene. In cell extracts from CP4/pZY507*pmi*, PMI could be detected at a level of high activity (3.3 ± 0.1 U mg^{-1}) already in the uninduced state; addition of IPTG to the growth medium raised the activity level to 18.5 ± 0.8 U mg^{-1}. These values are several orders of magnitude higher than those for the constitutive PMI activity found for *E. coli* wild-type cells (16). Invariably, the *pmi* recombinant strains of *Z. mobilis* would already grow on complex medium with 4% mannose (growth rate, approximately 0.07 h^{-1}), independently of the presence of IPTG (Table 6). This we take for evidence that all other cellular functions necessary for catabolism of mannose were present in the *pmi*$^+$ strains (Fig. 2).

As an explanation for the relatively low growth rate of 0.07 h^{-1} on mannose, compared with 0.34 h^{-1} for fructose in wild-type CP4 cells, the FRK reaction on mannose could be the rate-limiting step in the novel catabolic pathway. We measured FRK activities after growth of *pmi* strains in glucose, fructose, and mannose (all at 4%). As shown in Table 4, FRK activities were present already with glucose as the growth substrate and were doubled when fructose was the substrate. This was in good agreement with earlier observations (35). Interestingly, with growth on mannose, FRK activities were raised to approximately fourfold compared with growth on glucose (Table 4). The relative mannose-phosphorylating activities in cell extracts of *Z. mobilis* were in the range of 8 to 12% of the activity with fructose as the substrate.

Since FRK appeared to be growth limiting on mannose as the carbon source, we wanted to increase its expression by cloning of the *frk* gene in tandem with the *pmi* gene onto vector pZY507. The PMI activities in cell extracts from CP4/pZY507*frk-pmi* were in the same range (without and with IPTG, 3.0 ± 0.2 and 11.5 ± 0.8 U mg^{-1}, respectively) as those from the pZY507*pmi* strain. Then, FRK activities in the pZY507*frk-pmi* strain could be augmented by the addition of IPTG and reached maximal values of 3.5 U mg^{-1} (Table 4) when the strain was grown on glucose. When it was grown on mannose, FRK activities of as much as 9.7 U mg^{-1} were detected (Table 4). Correspondingly, growth rates on mannose were increased (0.18 h^{-1} without IPTG and 0.25 h^{-1} in the presence of IPTG [Table 6]). Interestingly, these *frk-pmi* strains displayed enormously prolonged lag phases when they were inoculated into fructose media, pointing to a metabolic imbalance as observed earlier with *glf-frk* recombinant strains of *E. coli* grown on fructose (34).

In order to exclude the involvement of mannose-phosphorylating enzymes in *Z. mobilis* other than FRK, an FRK-deficient strain (F$^-$1959 or DSM3126 [6]) was used as a recipient for both pZY507*pmi* and pZY507*frk-pmi*. Although PMI activity was found in strain DSM3126/pZY507*pmi* at levels similar to those of the corresponding derivative of strain CP4 (data not shown), the DSM3126/*pmi*$^+$ strain remained both fructose and mannose negative. However, the *frk*$^+$-*pmi*$^+$ derivatives of strain DSM3126 not only had regained the ability to grow on fructose but invariantly became mannose positive. This underlines that FRK is the only mannose-phosphorylating activity in *Z. mobilis* cells.

Purification and properties of FRK from overproducing *Z. mobilis* cells. The discrepancy about the extent of mannose phosphorylation via FRK (see references 9 and 23 and this report) led us to study the activity of *Z. mobilis* FRK in more detail. We purified the enzyme from strain CP4/pZY507*frk*,

TABLE 4. Kinase activities of *Z. mobilis* CP4 with different plasmids[a]

Substrate	Vector	IPTG	Activity (U mg^{-1})		
			Glucokinase	FRK	Mannokinase
Glucose	pZY507	−	ND[b]	0.7 ± 0.1	0.06 ± 0.01
	pZY507	+	ND	0.7 ± 0.1	0.06 ± 0.01
	pZY507*pmi*	−	0.77 ± 0.2	0.85 ± 0.1	0.07 ± 0.01
	pZY507*pmi*	+	0.76 ± 0.2	0.83 ± 0.1	0.07 ± 0.01
	pZY507*frk pmi*	−	0.84 ± 0.1	1.31 ± 0.2	0.12 ± 0.01
	pZY507*frk pmi*	+	0.75 ± 0.1	3.53 ± 0.3	0.34 ± 0.02
Fructose	pZY507*pmi*	−	0.86 ± 0.1	1.62 ± 0.1	0.15 ± 0.01
	pZY507*pmi*	+	0.74 ± 0.2	1.49 ± 0.2	0.13 ± 0.01
	pZY507*frk pmi*	−	ND[c]	ND[c]	ND[c]
	pZY507*frk pmi*	+	ND[c]	ND[c]	ND[c]
Mannose	pZY507*pmi*	−	0.79 ± 0.1	3.1 ± 0.2	0.32 ± 0.02
	pZY507*pmi*	+	0.72 ± 0.1	3.2 ± 0.2	0.32 ± 0.02
	pZY507*frk pmi*	−	0.7 ± 0.1	6 ± 0.5	0.51 ± 0.02
	pZY507*frk pmi*	+	0.71 ± 0.1	9.7 ± 0.4	0.85 ± 0.05

[a] Cells were grown in complex media with 4% substrate at 25°C. Kinase activities in cell extracts were determined as described in Materials and Methods. Mean values of two independent determinations are given.

[b] ND, not done.

[c] Because of severe growth retardation (see Table 6), no cells from a comparable growth state could be obtained.

TABLE 5. Influence of increasing mannose concentrations on growth of *Z. mobilis* CP4/pZY507[a]

Mannose concn (mM)	Growth on glucose		Growth on fructose	
	μ (h^{-1})	Y (OD_{600})	μ (h^{-1})	Y (OD_{600})
0	0.38 ± 0.01	2.4 ± 0.3	0.33 ± 0.02	1.6 ± 0.2
27	0.37 ± 0.01	1.9 ± 0.1	0.19 ± 0.02	0.93 ± 0.1
55	0.35 ± 0.02	1.8 ± 0.2	0.13 ± 0.01	0.7 ± 0.1
83	0.35 ± 0.02	1.6 ± 0.1	0.09 ± 0.01	0.52 ± 0.08
111	0.35 ± 0.02	1.6 ± 0.1	0.07 ± 0.02	0.35 ± 0.05

[a] Cells were grown in complex media with 1% carbon source and various amounts of mannose. Growth rate (μ) was recorded by measurement of the OD_{600}. The final growth yields (Y) are also expressed in OD_{600} units. The mean values ± standard deviations of the means of at least two independent determinations are given.

which carried the cloned gene under the control of the *tac* promoter. The enzyme was purified with a two-step ammonium sulfate precipitation, anion-exchange chromatography, and gel filtration (for details, see Materials and Methods). The final protein was homogeneous as judged by Coomassie staining after SDS-PAGE (Fig. 3) and displayed a specific activity with fructose as the substrate of 205 ± 25 U mg^{-1} of protein, comparing favorably with an earlier report (23). By SDS-PAGE, the enzyme showed an apparent molecular weight of 30,000, and on gel filtration a molecular mass of 60 kDa was judged. Thus, the enzyme is likely to be a homodimer. The activity with mannose was 17 ± 2 U mg^{-1} of protein (relative activity of 8.5% compared with that of fructose). K_m values were determined for fructose at 0.75 ± 0.06 mM and for mannose at 0.65 ± 0.08 mM. We conclude that FRK from *Z. mobilis* indeed phosphorylates mannose at a reasonable rate and at an affinity similar to that of fructose.

Use of the *pmi* gene as a selective marker in *Z. mobilis*. Wild-type *Z. mobilis* strains ZM1, CP4, and ZM6 of our laboratory collection did not grow on mannose as a growth substrate, and we did not observe any spontaneous mannose-utilizing clones after several weeks of incubation. Therefore, the mannose-negative phenotype appears to be a stable feature of *Z. mobilis* in agreement with an earlier report (29). Introduction of the *pmi* gene under a *lacI*^{q}/*tac* promoter led to growth on mannose as the sole carbon source in strains ZM1, CP4, and ZM6, whereas the FRK-deficient mutant DSM3126 (6) would grown on mannose only when a plasmid-borne *frk* copy was transferred also (see above). Therefore, FRK appears to be the only major mannose-phosphorylating activity in *Z. mobilis* and the kinase is a prerequisite for growth on this sugar.

Since the expression of one heterologous gene in *Z. mobilis* led to growth on a novel carbon source, it was interesting whether the conjugal transfer of plasmid pZY507*pmi* from an *E. coli* donor to a *Z. mobilis* recipient could be monitored by selection for growth on mannose. Mannose-utilizing exconjugants from a conjugation of *E. coli* S17-1/pZY507*pmi* with the *Z. mobilis* strain ZM1, CP4, or ZM6 appeared on agar plates with 4% mannose after 7 to 10 days of anaerobic incubation, with an apparent rate of 10^{-5} to 10^{-6} per donor cell (data not shown). This was about the same rate as that found when selection was for chloramphenicol resistance (data not shown) and is in the typical range for transfer of RSF1010-derived vectors from *E. coli* donors to *Z. mobilis* (14). All mannose-positive clones were chloramphenicol resistant (100 of 100 randomly picked clones) and showed pZY507*pmi* plasmid DNA, verified by successful retransformation into *E. coli* recipient strains (data not shown). Thus, mannose utilization could be used to monitor plasmid transfer from an *E. coli* donor to a *Z. mobilis* recipient, and no false-positive clones were observed.

DISCUSSION

The present study provides evidence for the transport of mannose via the GLF of *Z. mobilis*. GLF thus accepts not only glucose and fructose (19, 26, 34) but also xylose and mannose as shown in the present study. By measuring the competition for mannose uptake via GLF in recombinant *E. coli*, compounds such as D-galactose, D-glucosamine, L-sorbose, and D-arabinose are likely to be substrates for GLF too. A previous study (19) found that a 100-fold excess of mannose inhibited glucose uptake to as low as 42% residual activity; other competitors were xylose (61%), fructose (74%), galactose (81%), and sucrose (87%). D-arabinose had no inhibiting effect; L-sorbose and glucosamine were not assayed. From our results, we conclude that D-arabinose is an inhibitor of mannose uptake, whereas its L-enantiomer is not. Our results on sucrose argue against an uptake via GLF. Another group (19) used sucrose at 100 mM, which may have contained substantial amounts of free glucose to inhibit [^{14}C]glucose uptake, in particular since glucose was used at the nonsaturating concentration of 1 mM.

Mannose phosphorylation via the *Z. mobilis* FRK (purification factor, 49-fold; K_m for fructose, 0.16 mM) was rejected earlier (9) or was regarded as a minor side activity of less than 0.5% (23). Here, we present evidence that the cloned *frk* gene from strain ZM6 displays mannose-phosphorylating activity shown by growth on mannose, e.g., strain DSM3126/pZY507*frk-pmi* or *E. coli* strains lacking a so-called manno-

TABLE 6. Growth rates on glucose, fructose, or mannose as carbon sources[a]

Strain or vector	IPTG (1 mM)	Mannose		Glucose		Fructose	
		μ (h^{-1})	Y (OD_{600})	μ (h^{-1})	Y (OD_{600})	μ (h^{-1})	Y (OD_{600})
CP4/pZY507	−	ND	ND	0.41 ± 0.01	5.3 ± 0.1	0.34 ± 0.01	2.7 ± 0.2
	+	ND	ND	0.4 ± 0.01	5.2 ± 0.2	0.34 ± 0.01	2.7 ± 0.1
pZY507*pmi*	−	0.07 ± 0.01	1.7 ± 0.1	0.37 ± 0.01	5.3 ± 0.2	0.29 ± 0.01	2.5 ± 0.2
	+	0.07 ± 0.01	1.7 ± 0.2	0.36 ± 0.02	5.4 ± 0.2	0.25 ± 0.02	2.5 ± 0.2
pZY507*frk-pmi*	−	0.18 ± 0.02	4.4 ± 0.3	0.37 ± 0.01	5.5 ± 0.3	0.05 ± 0.01	1.7 ± 0.2
	+	0.25 ± 0.01	3.5 ± 0.3	0.34 ± 0.02	5.4 ± 0.2	0.03 ± 0.01	2.3 ± 0.3

[a] Cells of strain CP4 with the different plasmids (pZY507 as control) were grown in complex media with 4% carbon source and with chloramphenicol. The mean values (± standard deviations) of three independent determinations are given. Y, yield; ND, not detectable; μ, growth rate.

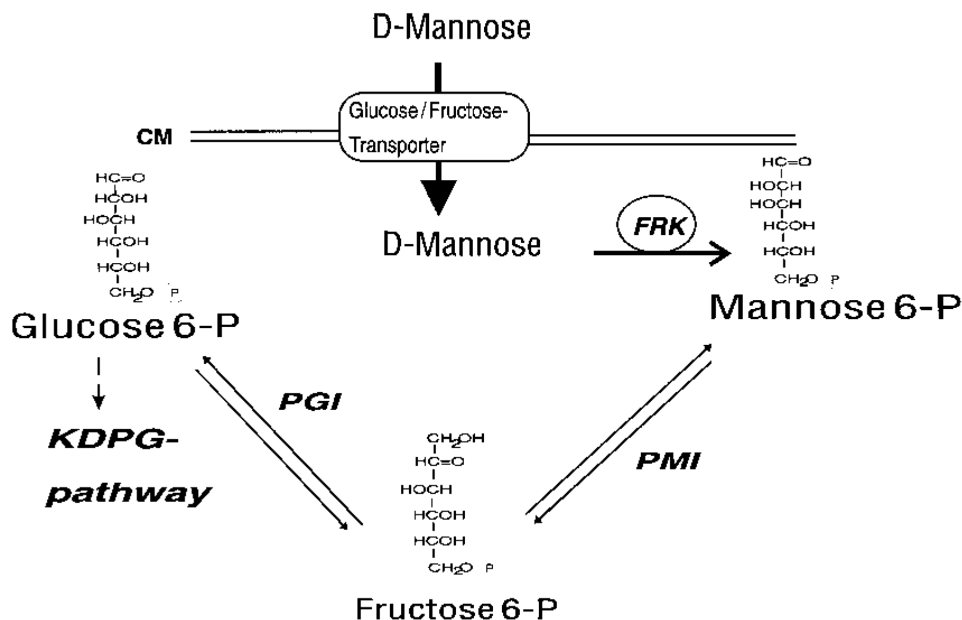

FIG. 2. Model of novel D-mannose catabolic pathway in pmi^{+} recombinant *Z. mobilis* strains. PGI, phosphoglucoseisomerase; KDPG pathway, Entner-Doudoroff pathway; CM, cytoplasmic membrane.

fructokinase activity (mak^{o} [1]). From in vitro measurements with crude extracts both from *Z. mobilis* or from *frk* recombinant *E. coli*, we found relative mannose-phosphorylating activities via FRK ranging from 8% to 12%. This result was corroborated by purification of the FRK, for which a relative rate of 8.5% for mannose phosphorylating activity (compared with that for fructose as the substrate) was observed. Scopes et al. (23) had determined a K_m for fructose of 0.7 mM (at 1 mM ATP), in accordance with our present results. Recently, FRK from *Z. mobilis* has, on an evolutionary tree, been placed into the hexokinase group (including bacterial glucokinases) and apart from the ribokinase group which encompasses other bacterial fructokinases (2, 35). In view of the observed side activity on mannose, this grouping is now correct also in biochemical terms. A mannofructokinase from *Streptococcus mutans* had been found to phosphorylate both sugars, with a K_m for fructose of 0.63 mM and a K_m for D-mannose of 0.37 mM (20), while having similar V_{max}s (11.6 U versus 12.3 U mg^{-1}) when measured with a partially purified enzyme. In this case, fructose phosphorylation was also competitively inhibited by mannose (K_i of 0.1 mM). The authors stated that stereochemical models of α-D-mannopyranose and β-D-fructofuranose share striking topological and spatial similarities, explaining the dual specificity of the enzyme.

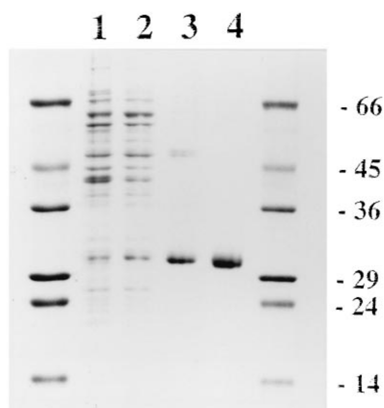

FIG. 3. SDS-PAGE of fructokinase protein purification. Cell extracts (lane 1) of strain CP4/pZY507*frk* were prepared by milling with glass beads in a disintegrator. FRK was purified by ammonium sulfate precipitation (lane 2), anion-exchange chromatography (lane 3), and gel filtration (lane 4). Protein size standards are shown in the left and right lanes.

After expression of the *glf* gene in conjunction with the *frk* gene from *Z. mobilis*, growth on mannose was restored in *E. coli* strains under the prerequisite that PMI activity was present.

This experiment is in full accordance with the fact that mannose-6-phosphate is the product of mannose phosphorylation through FRK. The combination *glf-frk* provided further physiological evidence for mannose being transported via GLF.

After introduction of the *E. coli pmi* gene in *Z. mobilis*, growth on D-mannose occurred with the chromosomally encoded FRK at a rate of 0.07 h^{-1} and could be further increased by a plasmid-borne copy of *frk* to 0.18 and 0.25 h^{-1} (with or without IPTG in the medium), which is already close to the growth rate of wild-type CP4 on fructose (0.34 h^{-1}). In summary, a clear correlation between mannokinase activity and the subsequent growth rate could be observed. The yield on mannose surpassed that observed with fructose. Growth yields on fructose in *Z. mobilis* are well-known to be always lower than those on glucose (5, 32). It is now clear that the functions of both GLF and FRK on the substrates fructose and mannose are similar. Since phosphoglucose isomerase is common to both the fructose and the mannose pathways, it will be interesting to find out why the biomass yield from fructose is lower than that from mannose, and further investigations are necessary to solve these discrepancies.

We showed that the *pmi* marker could be successfully used as selective marker and thus adds to the existing small arsenal of genetic markers for *Z. mobilis* (for a review, see reference 28), which consists at present mainly of antibiotic resistance markers (tetracycline and chloramphenicol). It has been noted

in several laboratories that *Z. mobilis* strains tend to become spontaneously resistant toward tetracycline and/or chloramphenicol, the only two antibiotic markers that are easy to use in *Z. mobilis* (for reviews, see references 21 and 28). *pmi* could substitute for these markers when antibiotics are no longer desired for environmental or process engineering reasons. The spread of antibiotic resistances by interspecific plasmid transfer could also be prevented by the use of a catabolic single-gene marker. Experiments to broaden the applicability of the *pmi* marker in *Z. mobilis* (transposons carrying *pmi* or chromosomal gene disruptions) are under way in our laboratory.

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