Journal of Industrial Microbiology & Biotechnology (2002) 29, 221–227

www.nature.com/jim

Fermentation of sugar mixtures using *Escherichia coli* catabolite repression mutants engineered for production of L-lactic acid

BS Dien, NN Nichols and RJ Bothast

Fermentation Biotechnology Research Unit, National Center for Agricultural Utilization Research, USDA[a], Agricultural Research Service, 1815 North University Street, Peoria, IL 61604, USA

Conversion of lignocellulose to lactic acid requires strains capable of fermenting sugar mixtures of glucose and xylose. Recombinant *Escherichia coli* strains were engineered to selectively produce L-lactic acid and then used to ferment sugar mixtures. Three of these strains were catabolite repression mutants (*ptsG*$^-$) that have the ability to simultaneously ferment glucose and xylose. The best results were obtained for *ptsG*$^-$ strain FBR19. FBR19 cultures had a yield of 0.77 (g lactic acid/g added sugar) when used to ferment a 100 g/l total equal mixture of glucose and xylose. The strain also consumed 75% of the xylose. In comparison, the *ptsG*$^+$ strains had yields of 0.47–0.48 g/g and consumed 18–22% of the xylose. FBR19 was subsequently used to ferment a variety of glucose (0–40 g/l) and xylose (40 g/l) mixtures. The lactic acid yields ranged from 0.74 to 1.00 g/g. Further experiments were conducted to discover the mechanism leading to the poor yields for *ptsG*$^+$ strains. Xylose isomerase (XI) activity, a marker for induction of xylose metabolism, was monitored for FBR19 and a *ptsG*$^+$ control during fermentations of a sugar mixture. Crude protein extracts prepared from FBR19 had 10–12 times the specific XI activity of comparable samples from *ptsG*$^+$ strains. Therefore, higher expression of xylose metabolic genes in the *ptsG*$^-$ strain may be responsible for superior conversion of xylose to product compared to the *ptsG*$^+$ fermentations.
Journal of Industrial Microbiology & Biotechnology (2002) **29,** 221–227 doi:10.1038/sj.jim.7000299

Keywords: catabolite repression; lactic acid production; xylose fermentation; *Escherichia coli*

Introduction

Lactate is used by the food, pharmaceutical, and cosmetic industries. Global demand is approximately 130,000 metric tons/year [27] and is projected to grow by 8.5% per year through 2003. Growth in demand will be driven by new industrial uses of lactate as a feedstock for polymers and solvents [20]. Polylactate (PLA) is a high-quality plastic that has applications in the clothing and packaging industries. Cargill Dow Polymers — a partnership of Cargill and Dow Chemical — expects to begin production of PLA in 2002 [20]. The plastic will be marketed as a biodegradable polymer manufactured from renewable resources and is expected to be cost-competitive with specialty plastics [15]. Lactate is also used for the manufacture of ethyl lactate, which is a nontoxic solvent suitable for use as a cleaning agent [11].

Market growth for lactate as an industrial chemical will be very sensitive to its selling price. For example, a less expensive source of lactic acid is needed for PLA to compete in the polystyrene market [27]. Glucose is currently used as the feedstock for production of lactic acid by fermentation [24,32]. Lignocellulosic biomass might offer a less expensive feedstock than glucose for producing lactic acid. Sources of lignocellulosic biomass available for fermentation include agricultural residues, food processing wastes, wood, municipal solid wastes, and wastes from the paper and pulp industry. Available biomass reserves in the US are approximately 200 million tons [3]. However, conversion of lignocellulosic biomass to lactic acid is problematic because sugars prepared from them are a mixture of primarily glucose and xylose. Xylose is a pentose that is not fermented by most lactic acid bacteria, including commercial strains. Lactic acid-producing microorganisms that ferment xylose, including the commercially used fungus *Rhizopus oryzae*, have low productivity and (usually) low yield and selectivity [18,19,26,35]. Most lactic acid bacteria also produce a racemic (D and L) mixture. Optically pure lactic acid is required for manufacturing PLA [27].

We have constructed a series of *Escherichia coli* strains that selectively produce L-lactic acid. *E. coli* engineered for production of lactic acid has several advantages for lactic acid production compared to other microorganisms. First, *E. coli* can be metabolically engineered to produce lactic acid with little production of other fermentation products [7]. Second, as is common for *E. coli*, the strains are capable of utilizing a wide variety of sugars, including xylose. Third, these strains produce an optically pure leugrotary (L) product [13].

The lactic acid-producing strains described here were constructed by transforming *E. coli* strains that are unable to grow fermentatively with a plasmid encoding the L-specific lactic acid dehydrogenase (*ldh*) gene from *Streptococcus bovis* [34]. The strains carry mutations in two genes: pyruvate formate lyase (*pfl*) and lactate dehydrogenase (*ldhA*). The *pfl* mutation prevents the cells from forming acetate, ethanol, and formate, and the *ldhA* mutation prevents formation of D-lactate. Expression of an extrachromosomal *ldh* gene restores the ability of the mutant to grow fermentatively by allowing production of L-lactate. Furthermore, the strains stably maintain the *ldh* encoding plasmid

Correspondence: Dr Bruce S Dien, Fermentation Biotechnology Research Unit, National Center for Agricultural Utilization Research, USDA, Agricultural Research Service, 1815 North University Street, Peoria, IL 61604, USA

[a]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

Received 14 December 2000; accepted 28 June 2002

when cultured anaerobically [13]. These strains have been determined to readily ferment either glucose or xylose, but have not been used to ferment a mixture of these two sugars [13].

Lactic acid-producing strains that are catabolite repression mutants were also constructed for this study. These strains should be advantageous for fermentation of sugar mixtures because glucose repression has been eliminated in these strains by modifying the phosphoenolpyruvate-dependent glucose phosphotransferase system (PTS), a multiprotein complex that is involved in glucose repression [30]. When glucose is present, other sugars are prevented from entering the cells (inducer exclusion) and transcription of related metabolic genes is blocked. The catabolite repression mutants used in this study have had the gene (*ptsG*) encoding $IIBC^{Glc}$ disrupted, which prevents IIA^{Glc} from mediating glucose repression. The mutation also disables transport of glucose through PTS. Instead, glucose is transported by galactose permease (*galP*) and phosphorylated by glucose kinase [14]. *E. coli* PTS mutants have been observed to simultaneously use glucose and xylose [17,28].

In this study, fermentations of glucose and xylose sugar mixtures in *E. coli* catabolite repression mutants were compared to the parent strains. The strains were selected on the basis of lactic acid yield and xylose utilization. The superior strains were further characterized for fermentation using media containing glucose and xylose in different ratios. Finally, observed yields were related to the physiological state of the cultures by monitoring xylose isomerase (XI) induction and cell viability during a fermentation of sugar mixtures.

Materials and methods

Bacterial strains, plasmid, growth media, and reagents

E. coli strains and the plasmid used in this study are listed in Table 1. Complex medium ingredients were Difco® products (Difco, Becton Dickinson Microbiology Systems, Sparks, MD). Antibiotics, glycine betaine, glucose, and D-xylose were purchased from Sigma (St. Louis, MO). All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ). All chemicals and enzymes were research or analytical grade. Strains were grown aerobically on LB medium (10 g of tryptone, 5 g of yeast extract, 5 g/l sodium chloride, and 15 g/l Bacto agar for solid medium) supplemented with 4 g/l glucose and 1 g/l sodium acetate. Antibiotic, acetate, and sugars were added separately as sterilized solutions.

Lactic acid-producing strains were routinely cultured in anaerobic medium. For anaerobic growth of *E. coli*, LB broth was prepared without NaCl and supplemented with 4 g/l glucose (unless stated otherwise), 1 g/l acetate as an additional carbon source, 4 g/l sodium carbonate as a buffer, and 0.5 g/l cysteine HCl as a reducing agent. Anaerobic medium was flushed with a 20% CO_2, 80% N_2 gas mixture, and sealed with butyl rubber stoppers. Sugars and acetate were prepared as separate stock solutions and autoclaved under a 100% nitrogen atmosphere. The pH of the buffered medium was 7.

Genetic procedures

Plasmid DNA was isolated from liquid cultures and introduced into cells by chemical transformation [31]. Transformants were selected on LB agar medium containing 100 mg/l ampicillin. Prior to transformation, parental strains were screened on TTP medium [2] for the ability to metabolize xylose.

ND18, a catabolite repression mutant of the nonfermentative *E. coli* B strain ND10, was constructed by phage P1 transduction [25]. First, the *ptsG21* mutation was transduced from *E. coli* AFP308 into *E. coli* WA837, a B strain that is restriction-minus, modification-plus, to create ND14. Then, the mutation was transduced from ND14 into ND10, resulting in strain ND18. Recipients were selected for tetracycline (10 mg/l) resistance encoded on a closely linked Tn*10*. Transductants were blue (*ptsG*$^-$) rather than pale (wild type) on M9 medium containing 0.4% (wt/vol) each lactose and glucose, and 30 mg/l chromogenic substrate X-gal [31].

Batch fermentations of glucose and xylose

Bioreactors with automatic pH control were operated as described previously [1,12]. Production cultures (300 ml) were grown at 35°C in medium containing 10 g/l tryptone and 5 g/l yeast extract supplemented with 80–100 g/l sugar(s) (final concentration), 1 g/l acetate, and 0.1 ml/l antifoam 289 (Sigma). The pH was maintained at 6.7 by addition of a concentrated alkali solution (2.5 N KOH+2.5 N NaOH). The medium and acetate solution were prepared as stock solutions and sterilized using an autoclave. For one experiment, 1 mM glycine betaine (filter-sterilized as a stock solution) was added to the medium. Nitrogen was bubbled through the medium for 30 min followed by a 30-s burst of carbon dioxide to remove oxygen prior to inoculation and to supply carbon dioxide, which is required as a growth factor. The fermentation vessels were inoculated to a concentration of 0.06 mg dry cell mass/ml medium (an optical density of 1.0 at 550 nm was determined to be equal to a concentration of 0.30 mg cell dry weight/ml). Seed cultures used to inoculate the fermentation vessels were also grown in pH-controlled bioreactors for 18 h at 35°C and pH 6.7. The medium used for the seed culture was similar to that used for the fermentation, except seed cultures were supplemented with either 20 g/l glucose or, where stated, xylose. Ethanol, sugars, organic acids, and $OD_{550\ nm}$ were determined periodically with 1.5-ml samples of cultures. Each experiment was run in duplicate, except where indicated. Viable cell numbers were determined by serially diluting each culture and growing the cells aerobically on LB solid medium. Colonies were counted after overnight incubation at 37°C.

Table 1 Bacterial strains and plasmid relevant to this study

Strain or plasmid	Relevant characteristics	Reference
E. coli		
AFP308	*zce726*::Tn*10 ptsG21*	[8]
AFP310	NZN111 *zce726*::Tn*10 ptsG21*	[8]
ND10	B strain; Δ*pfl*::Cm *ldhA*::Kn	[13]
ND14	WA837 *zce726*::Tn*10 ptsG21*	This work
ND18	ND10 *zce726*::Tn*10 ptsG21*	This work
ND19	NZN111 *zce726*::Tn*10 ptsG21*Δ (*frdABCD*)	[28]
NZN111	K12 Δ*pfl*::Cm *ldhA*::Kn	[5]
WA837	$r_B - m_B +$ *gal met*	[33]
FBR12	ND10 (pUCLDH1)	This work
FBR17	AFP310 (pUCLDH1)	This work
FBR18	ND18 (pUCLDH1)	This work
FBR19	ND19 (pUCLDH1)	This work
FBR20	NZN111 (pUCLDH1)	This work
Plasmid		
pUCLDH1	*ldh* from *S. bovis*	[34]

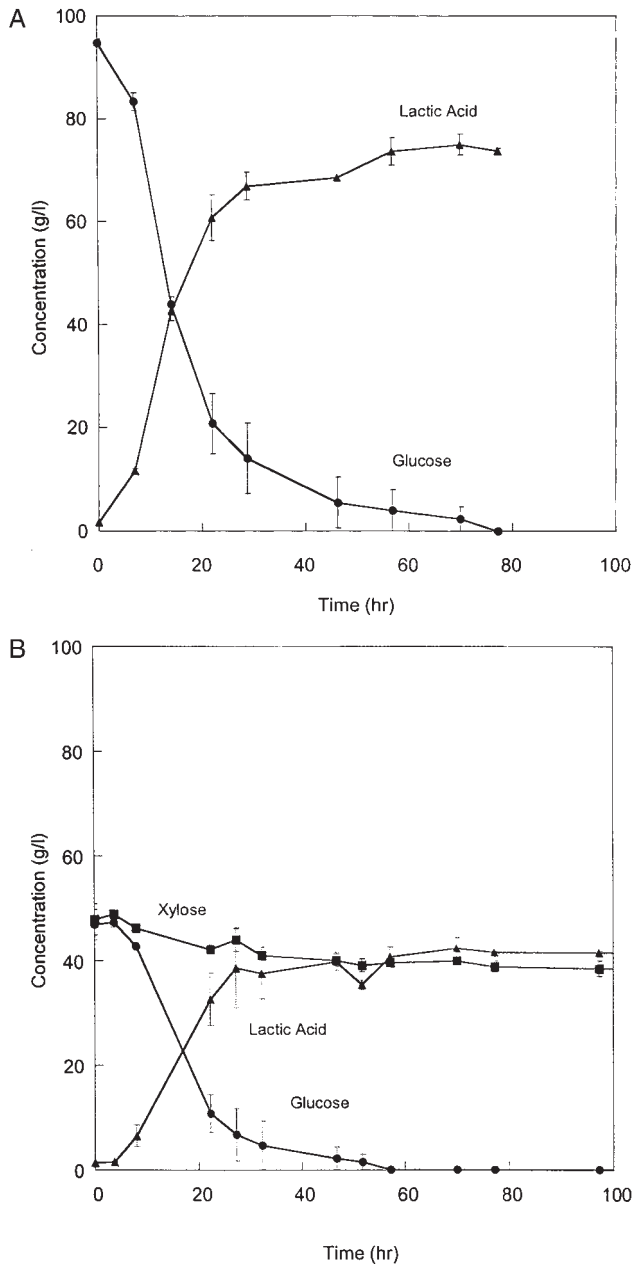

Figure 1 (A) FBR12 was used to convert glucose (100 g/l) to lactic acid. Results shown are the average of duplicate runs. Glucose (circles) and lactic acid (triangles). (B) FBR12 was used to ferment a glucose (50 g/l) and xylose (50 g/l) mixture. Results shown are the average of duplicate runs. Glucose (circles), lactic acid (triangles), and xylose (squares).

XI assay

A cell pellet was recovered from the fermentation broth (10 ml) by centrifuging it at 5000×*g* for 15 m at 4°C. The cells were washed in 1 ml of 50 mM sodium phosphate (pH 7) buffer and stored at −20°C. The pellet was resuspended in 1 ml of sodium phosphate buffer, and cells were broken by agitating the suspension with ziconia/silica beads (diameter 0.1 mm; Biospec, Bartlesville, OK) using a Fastprep FP120 (Savant, Farmingdale, NY) at a speed setting of 4 for 20 s. The resultant mixture was centrifuged at 16,000×*g* and 4°C for 30 min. Protein in the recovered supernatant was measured using the method of Bradford [4]. XI activity was measured at 35°C using a modified coupled enzymatic assay described previously [6]. Briefly, 100 μl of crude protein solution was added to 1 ml of an assay solution containing: xylose (50 mM), MOPs (10 mM, pH 7.2), $MgSO_4$ (10 mM), NADH (0.2 mM), and sorbitol dehydrogenase (1 μg/ml). XI activity was determined by measuring change in absorbance at 340 nm, which corresponds to NADH oxidation, using a Hewlett Packard/Agilent 8453 spectrophotometer (Palo Alto, CA). Prior to the addition of xylose, the background NADH oxidation rate was monitored for each reaction. XI activity was calculated as the difference between this rate and the rate of NADH oxidization following addition of xylose. Each assay was performed in triplicate.

Analytical procedures

Optical densities (1 cm light path) of cultures were monitored at 550 nm. Concentrations of sugars and fermentation-generated organic acids (i.e., lactate and succinate) were determined by high-pressure liquid chromatography (HPLC) using an Aminex HPX-87H column (Bio-Rad, Richmond, CA) and refractive index detector. Samples were run at 65°C and eluted at 0.6 ml/min with 5 mM sulfuric acid.

Calculation of lactic acid yields

Lactic acid yields were calculated from the maximum lactic acid concentration, the total amount of sugar added to fermentation, and the final volume of the fermentation. The total amount of lactic acid produced was calculated by multiplying the maximum lactic acid concentration by the fermentation volume. The lactic acid yield (g/g sugar) was then calculated by dividing total lactic acid by the amount of sugar added to the fermentation.

Results

Construction of lactic acid-producing strains

E. coli strains FBR12, FBR17, FBR18, FBR19, and FBR20 were created by transforming nonfermentative strains ND10, AFP310, ND18, ND19, and NZN111, respectively, with pUCLDH1 (Table 1). This high copy number plasmid carries the *S. bovis ldh* gene [34], which encodes an LDH that is specific for production of L-lactic acid. The ability of transformants to grow fermentatively was verified as described in Materials and Methods (data not shown).

Table 2 Fermentation of glucose and xylose mixtures by FBR12

Added sugars		Lactic acid (g/l)	Lactic acid yield[a] (g/g)	Xylose consumed[b] (%)
Glucose (g/l)	Xylose (g/l)			
0[c]	40	34.6	1.02	100
10	40	36.4±0.9	0.86±0.02	80±4
20	40	25.7±1.1	0.49±0.02	30±1
30	40	31.3±0.5	0.53±0.01	27±2
40	40	36.9±1.5	0.55±0.03	25±0

[a]Gram of lactic acid produced per gram of sugar added to the medium.
[b]Percent gram of xylose fermented per gram of initial xylose; 100% of glucose was consumed in all cases.
[c]Duplicate run not completed.

Table 3 Comparison of lactic acid production from glucose (50 g/l) and xylose (50 g/l) mixtures by recombinant *E. coli* strains

Strain	*ptsG*	Genetic background	Lactic acid (g/l)	Lactic acid yield[a] (g/g)	Xylose consumed[b] (%)	Glucose consumed[b] (%)	Succinic acid[c] (g/l)	Maximum cell concentration (g/l)
FBR17	–	K12	52.3±1.4	0.62±0.01	56±2	100±0	1.0±0.0	1.5±0.2
FBR18	–	B	32.1±3.5	0.35±0.04	54±3	45±2	0.7±0.1	1.2±0.1
FBR19	–	K12	64.3±2.2	0.77±0.03	75±8	100±0	0.0±0.0	1.5±0.0
FBR12	+	B	42.2±0.3	0.47±0.00	18±2	100±0	1.1±0.0	1.4±0.1
FBR20	+	K12	43.4±0.1	0.49±0.00	22±0	100±0	1.1±0.1	1.5±0.0

[a]Gram of lactic acid per gram of total sugar added to fermentation.
[b]Percent gram of xylose or glucose fermented per gram added to fermentation.
[c]No other fermentation products detected other than lactic and succinic acids.

Fermentation of glucose and a glucose/xylose mixture by an E. coli B strain

E. coli strain FBR12 was constructed initially because it is a B strain of *E. coli*, which is more tolerant to lactic acid than K12 strains [13]. The ability of the strain to grow fermentatively and produce lactic acid was confirmed on amended LB medium supplemented with 100 g/l glucose. The sugar was completely converted to lactic acid in 50–70 h (Figure 1A) with an average yield of 0.93±0.04 g lactic acid/g glucose added to the medium; the theoretical yield is 1.0 g/g. The only product detected besides lactate and biomass (3.2±0.4 g/l) was succinic acid (1.4±0.0 g/l). FBR12 was next used to ferment amended LB medium containing a mixture of glucose and xylose (50 g/l of each). The glucose was totally consumed. However, even after 142 h (Figure 1B), only 18±2% of the added xylose had been consumed. Once again, the only products other than lactate were cell biomass (1.4±0.1 g/l) and succinic acid (1.1±0.0 g/l). The final average lactic acid yield from these mixed sugar fermentations was 0.47±0.00 g/g, approximately one half of the yield realized with glucose.

Incomplete xylose utilization was investigated further by using FBR12 to ferment a series of glucose and xylose mixtures. The xylose concentration was kept constant (40 g/l), and glucose concentrations were varied from 0 to 40 g/l (Table 2). Glucose was totally consumed by 21–26 h (data not shown). However, xylose was completely fermented only when glucose was excluded from the media. Even when 10 g/l glucose was added, only 80% of the added xylose was consumed. When more glucose was added, between 25% and 30% of the xylose was consumed.

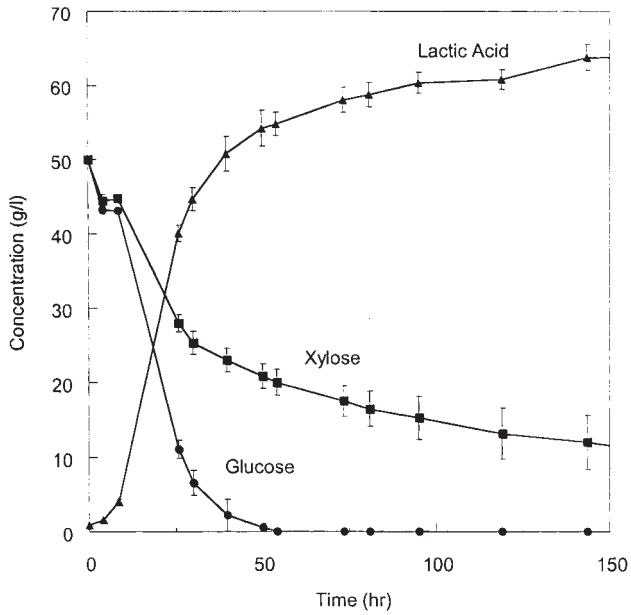

Figure 2 FBR19 was used to ferment a glucose (50 g/l) and xylose (50 g/l) mixture. Results shown are the average of duplicate runs. Glucose (circles), lactic acid (triangles), and xylose (squares).

Fermentation of a glucose/xylose mixture by E. coli catabolite repression mutants

Next, several lactic acid-producing strains were compared for their ability to ferment sugar mixtures (Table 3). A total of three catabolite repression mutants and two parental strains (*ptsG*$^+$ controls) were used to ferment a mixture of glucose and xylose. The *ptsG* mutants had either K12 or B strain origins (Table 1). FBR19 carries an additional mutation (Δ*frdABCD*) that blocks succinate production. All strains, except FBR18, completely utilized the glucose. Though none completely consumed the xylose, the catabolite repression mutants fermented more of the xylose than the control strains. The highest yields were observed for strains FBR17 and FBR19 (Table 3). The consumption pattern observed in this experiment for the *ptsG*$^+$ strains FBR12 and FBR20 (rapid use of glucose with very little consumption of xylose) was virtually identical to that displayed in Figure 1B for FBR12. The *ptsG* mutants FBR17 and FBR19 also consumed the glucose rapidly, but unlike FBR12 and FBR20, they continued to ferment xylose (Table 3, Figure 2).

Strain FBR19 was chosen for further study. The same mixtures of glucose and xylose fermented with FBR12 (Table 2) were repeated with FBR19 (Table 4). Strain FBR19 completely utilized the glucose supplied (0–40 g/l) in all of the fermentations. None of the fermentations completely utilized the added xylose, though when 10 g/l glucose was added, 98% of the xylose was consumed.

Table 4 Fermentation of glucose and xylose mixtures by *ptsG* mutant FBR19

Added sugars		Lactic acid (g/l)	Lactic acid yield[a] (g/g)	Xylose consumed[b] (%)
Glucose (g/l)	Xylose (g/l)			
0	40	32.4±4.2	0.88±0.12	86±8
10	40	44.5±1.4	1.00±0.03	98±1
20	40	47.9±0.3	0.91±0.01	84±4
30	40	46.0±0.8	0.74±0.02	51±2
40	40	57.1±3.0	0.83±0.06	65±14

[a]Gram of lactic acid per gram of total sugar added to fermentation.
[b]Percent gram of xylose fermented per gram of xylose added to fermentation; 100% of glucose was consumed.

When greater than 10 g/l glucose was added, the lactic acid yield and percent xylose consumed were lower. As expected, the maximum lactic acid concentration was realized for the fermentation with the greatest amount of added glucose.

Induction of XI during fermentation of glucose/xylose mixtures

Further fermentation of sugar mixtures with FBR19 (*ptsG*$^-$) and FBR20 (*ptsG*$^+$) was compared to determine what leads to incomplete xylose utilization (Figure 3). Each strain was used to ferment a mixture of glucose (40 g/l) and xylose (40 g/l). Seed cultures were grown on either glucose or xylose. In addition to measuring sugar consumption and lactic acid production, XI activity and viable cell counts were also monitored. XI, which catalyzes the first reaction in xylose metabolism, was used as a convenient indicator of activity of the xylose metabolic regulon.

Lactic acid yields were higher for FBR19 (Figure 3A) than FBR20 (Figure 3B), 0.70±0.02 vs. 0.53±0.01 g lactic acid/g added sugars. This result was expected from the previous experiment (Table 3). The control (*ptsG*$^+$) strain FBR20 fermented all of the glucose and only 25% of the xylose (Figure 3B). Results were similar regardless of whether the FBR20 seed culture was grown on glucose or xylose. The *ptsG*$^-$ strain FBR19 used all of the glucose and 50–55% of the xylose (Figure 3A). While xylose

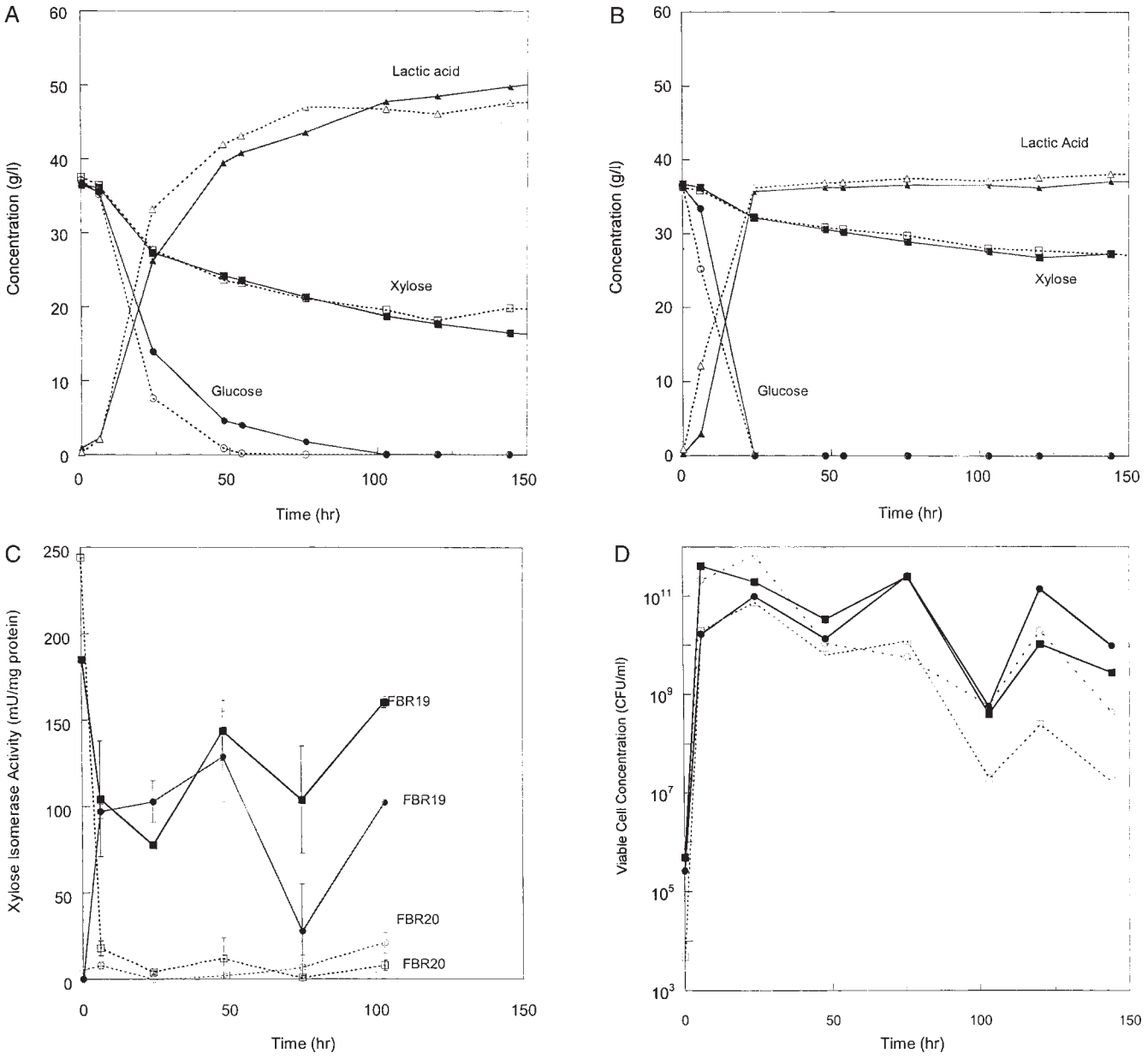

Figure 3 (A) FBR19 *ptsG*$^-$ was used to ferment a glucose (40 g/l) and xylose (40 g/l) mixture. Seed culture was grown on either glucose (dashed lines) or xylose (solid lines). Glucose (circles), lactic acid (triangles), and xylose (squares). (B) FBR20 *ptsG*$^+$ was used to ferment a glucose (40 g/l) and xylose (40 g/l) mixture. Seed culture was grown on either glucose (dashed lines) or xylose (solid lines). Glucose (circles), lactic acid (triangles), and xylose (squares). (C) Measurement of XI activity during mixed sugars fermentations. Fermentations were conducted with FBR19 (solid lines and symbols) or FBR20 (dashed lines and open symbols). The seed culture was grown on either xylose (squares) or glucose (circles). (D) Measurements of viable cell concentrations during mixed sugars fermentations. See (C) for legend.

utilizations in the FBR19 production cultures were similar using glucose- or xylose-grown inoculum, glucose was fermented more rapidly when a glucose-grown inoculum was employed.

Trends in XI activity reflected differences in lactic acid yields. XI activities were higher in FBR19 than FBR20 throughout the fermentation (Figure 3C). When the FBR20 seed was grown on xylose, the inoculum had XI activity of 244 mU/mg protein (0 h; Figure 3C). However, by 6 h, XI activity had decreased to 18 mU/mg. The average XI activities for *ptsG*$^+$ strain FBR20, once glucose was depleted, were 10±8 and 7±7 mU/mg for glucose- and xylose-grown inocula, respectively. In contrast, FBR19 average XI activities, including all but time 0 h, were 92±38 and 118±33, respectively. In the *ptsG*$^+$ strain, XI was not induced even after the glucose was exhausted. In contrast, XI activity was present in the *ptsG* mutant throughout fermentation of the sugar mixtures. Viable cell concentrations remained high (>10^7 cells/ml) throughout the fermentations with both FBR19 and FBR20 (Figure 3D). These results suggest that failure of FBR20 to ferment more xylose cannot be attributed to an absence of viable cells.

An attempt was made to prevent possible osmotic inhibition by adding glycine betaine, which has been observed to partially reverse the effects of osmotic stress for *E. coli* cultures growing in high NaCl concentrations [9,22,29]. In this experiment, medium containing an equal mixture of glucose and xylose (80 g/l total) was supplemented with glycine betaine (1 mM) and fermented with FBR20. Seed cultures were grown on either glucose or xylose. Similar to results observed for fermentations of sugar mixtures without glycine betaine, glucose was rapidly consumed, but only 20–37% of the xylose was consumed (data not shown).

Discussion

Production of lactic acid from lignocellulosic biomass requires a microbe capable of fermenting glucose and xylose. Five *E. coli* strains, engineered to produce L-lactate, were compared for yields on a mixture of glucose (50 g/l) and xylose (50 g/l). Lactate yields were 0.49–0.77 (g lactic acid/g initial sugar) (Table 3). The superior strain (FBR19) is a *ptsG*$^-$ catabolite repression mutant. Both *ptsG*$^+$ strains (FBR12 and FBR20) had much lower yields (0.47 and 0.49 g/g). The difference in yields among the strains can be directly correlated with residual xylose because lactate and cell mass are the only major fermentation products formed by these strains (Table 3). FBR19 fermented 75% of the xylose compared to 18–20% for FBR12 and FBR20. The differences in yields were also reflected in the sugar utilization patterns for the strains. FBR19 and FBR17 (another *ptsG*$^-$ strain) cofermented glucose and xylose, and continued to ferment xylose after glucose was exhausted. FBR12 and FBR20 (both *ptsG*$^+$) quickly fermented the glucose, but showed very little fermentative activity afterwards.

Few studies have been published on conversion of sugar mixtures and lignocellulosic hydrolysates to lactic acid. The scarcity of studies probably reflects the fact that few lactic acid bacteria ferment pentoses and those that do, with very few exceptions, use the heterofermentative lactic acid pathway that yields only 60% lactic acid [21]. It is difficult to directly compare those studies that have been carried out using sugar mixtures because the media and sugar contents vary. The most promising lactic acid yield was reported for *Lactobacillus casei* subsp. *rhamnsous* (ATCC10863), which produced 0.83 (g lactic acid/g added sugar) from a mixture of glucose (15 g/l), xylose (5 g/l), mannose (5 g/l), and galactose (2.5 g/l) [19]. However, the fermentation took 156 h because of slow xylose utilization. When *Lactococcus lactis* IO-1 was used to ferment a glucose (20 g/l) and xylose (20 g/l) mixture, the fermentation was complete in 40 h and the yield was 0.66 g/g [18]. Likewise, *Lactob. pentosus* B-227 was used to ferment municipal solid waste hydrolysate and after 3 days had produced 0.65 g lactic acid/g added sugar [26]. However, it is unclear how much of the residual sugar (20%) was xylose because individual sugar consumption was not monitored. By comparison, FBR19 had among the highest lactic acid yields (0.83 g/g) when fermenting a glucose (40 g/l) and xylose (40 g/l) mixture (Table 4); albeit, the fermentation took 130 h. The lactic acid produced here is also of a higher quality than reported in the other studies because it does not contain significant amounts of acetic acid (Table 3).

Xylose repression was studied further for the FBR strains to determine why the *ptsG*$^+$ strains metabolized xylose so poorly when fermenting sugar mixtures. There are several possible explanations for the poor xylose utilization: (1) they are unable to ferment xylose; (2) cell death occurs after glucose consumption and before the utilization of xylose induction; and (3) xylose metabolism continues to be repressed when glucose has been exhausted from the medium. Fermentations were conducted using FBR12, FBR19, and FBR20 to determine which of these possibilities were responsible for the poor use of xylose.

The first explanation is unlikely because strains FBR12 and FBR19 were readily able to ferment xylose as a sole sugar source (Tables 2 and 4). Xylose utilization was only problematic when glucose was added to the medium (Tables 2 and 4), nor could the failure of the *ptsG*$^+$ strains to completely ferment glucose and xylose mixtures be attributed to the culture dying before the cells had time to become induced for xylose utilization. When strain FBR20 was used to ferment a mixture of glucose and xylose, cells remained viable long after glucose was exhausted from the medium (Figure 3B and D).

The third possibility is that the *ptsG*$^-$ cultures continued to be repressed for xylose metabolism after exhaustion of glucose. Catabolite repression of non-PTS sugars (e.g., xylose) occurs by a combination of inducer exclusion and transcriptional repression. The proteins required specifically for xylose metabolism are XI (XylA) and xylulose kinase (XylB). The *xylA* and *xylB* genes are regulated as a single operon [23], which is subject to both negative and positive regulation. Induction of the xylose-related genes was monitored, indirectly, by measuring XI activity during fermentations of a glucose and xylose mixture by FBR19 and FBR20. For FBR20, XI activity was low (0–13 mU/mg protein) throughout the fermentation (Figure 3C). In contrast, the measured XI activity for FBR19 on the same sugar mixture was 19-fold higher. Increased XI activity for FBR19 was reflected in increased xylose utilization; FBR19 used two times more xylose from the sugar mixture than did FBR20 (Figure 3A and B). Therefore, little of the xylose present in a glucose/xylose mixture was fermented by FBR20 because the xylose operon was not induced after exhaustion of glucose. However, other factors may be involved because FBR19, which expressed XI, also failed to use all of the xylose.

If FBR12 and FBR20 cultures failed to induce metabolism of xylose even in the absence of glucose, then the glucose transport system is unlikely to be responsible. Possibly, xylose utilization remains repressed as part of an environmental stress response to either lactic acid toxicity or high salt concentrations. Lactic acid toxicity was minimized in the fermentation by controlling pH at 6.7.

Lactic acid toxicity is associated with the protonated form, and at neutral pH, 99.8% of the lactate present is in the anion form. The pH was maintained by adding sodium and potassium hydroxide, which creates an environment of osmotic stress. Once the cells have finished fermenting 40 g/l glucose, enough base has been added to bring the sodium and potassium lactate concentration to 0.5 M. This salt concentration was growth-limiting in prior *E. coli* experiments [16,22,29]. Osmotic stress as a cause of suppressing xylose induction is further supported by our experience with a series of ethanol-producing strains. Ethanol-producing strains were constructed from the same *ldh*$^-$, *pfl*$^-$ strains used in this study. When these strains were used to ferment glucose/pentose sugar mixtures, nearly all of the xylose was fermented [12]. In those fermentations, ethanol instead of lactic acid was produced, and much less base was required to maintain the pH of the medium. However, an attempt to alleviate osmotic stress by supplementing the medium with glycine betaine, a well-studied osmotic protector [9,22,29], failed to improve xylose utilization by FBR12 in cultures containing glucose and xylose. It is possible that adding yeast extract to the media made glycine betaine addition redundant because the former contains choline, which is a precursor of glycine betaine [10].

Acknowledgements

The authors thank Patricia O'Bryan for excellent technical help. Plasmid pUCLDH1 was received from Terence Whitehead, strains AFP308 and AFP310 from Mark Donnelley, and strain NZN111 from David Clark.

References

1 Beall DS, K Ohta and LO Ingram. 1991. Parametric studies of ethanol production from xylose and other sugars by recombinant *Escherichia coli*. *Biotechnol Bioeng* 38: 296–303.
2 Bochner BR and MA Savageau. 1977. Generalized indicator plate for genetic, metabolic, and taxonomic studies with microorganisms. *Appl Environ Microbiol* 33: 434–444.
3 Bothast RJ, NN Nichols and BS Dien. 1999. Fermentations with new recombinant organisms. *Biotechnol Prog* 15: 867–875.
4 Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 72: 248–254.
5 Bunch PK, F Mat-Jan, N Lee and DP Clark. 1997. The *ldhA* gene encoding the fermentative lactate dehydrogenase of *Escherichia coli*. *Microbiology* 143: 187–195.
6 Callens M, H Kersters-Hilderson, O van Opstal and CK de Bruyne. 1986. Catalytic properties of D-xylose isomerase from *Streptomyces violaceoruber*. *Enzyme Microb Technol* 8: 696–700.
7 Chang DE, HC Jung, JS Rhee and JG Pan. 1999. Homofermentative production of D- or L-lactate in metabolically engineered *Escherichia coli* RR1. *Appl Environ Microbiol* 65: 1384–1389.
8 Chatterjee R, CS Millard, K Champion, DP Clark and MI Donnelly. 2001. Mutation of the *ptsG* gene results in increased production of succinate in fermentation of glucose by *Escherichia coli*. *Appl Environ Microbiol* 67: 148–154.
9 Cosquer A, V Pichereau, JA Pocard, J Minet, M Cormier and T Bernard. 1999. Nanomolar levels of dimethylsulfoniopropionate, dimethylsulfonioacetate, and glycine betaine are sufficient to confer osmoprotection to *Escherichia coli*. *Appl Environ Microbiol* 65: 3304–3311.
10 Csonka LN and W Epstein. 1996. Osmoregulation. In: Neidhardt FC, R Curtiss III, JL Ingraham, ECC Lin, KB Low, B Magasanik, WS Reznikoff, M Riley, M Schaechter and HE Umbarger (Eds), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd edn. ASM Press, Washington, DC, pp. 1210–1223.
11 Datta RS, P Tsai, P Bonsignore, SH Moon and JR Frank. 1995. Technological and economic potential of poly(lactic acid) and lactic acid derivatives. *FEMS Microbiol Rev* 16: 221–231.
12 Dien BS, RB Hespell, H Wyckoff and RJ Bothast. 1998. Fermentation of hexose and pentose sugars using a novel ethanologenic *Escherichia coli* strain. *Enzyme Microb Technol* 23: 366–371.
13 Dien BS, NN Nichols and RJ Bothast. 2001. Recombinant *Escherichia coli* engineered for production of L-lactic acid from hexose and pentose sugars. *J Ind Microbiol Biotechnol* 27: 259–264.
14 Flores N, J Xiao, A Berry, F Bolivar and F Valle. 1996. Pathway engineering for production of aromatic compounds in *Escherichia coli*. *Nat Biotechnol* 14: 620–623.
15 Gerngross TU and SC Slater. 2000. How green are green plastics? *Sci Am* 283: 37–41.
16 Gouesbet G, H Abaibou, LF Wu, MA Mandrand-Berthelot and C Blanco. 1993. Osmotic repression of anaerobic metabolic systems in *Escherichia coli*. *J Bacteriol* 175: 214–221.
17 Hernandez-Montalvo V, F Valle, F Bolivar and G Gosset. 2001. Characterization of sugar mixtures utilization by an *Escherichia coli* mutant devoid of the phosphotransferase system. *Appl Microbiol Biotechnol* 57: 186–191.
18 Ishizaki A, T Ueda, K Tanaka and PF Stanbury. 1992. L-lactate production from xylose employing *Lactococcus lactis* IO-1. *Biotechnol Lett* 14: 599–604.
19 Iyer PV, S Thomas and YY Lee. 2000. High-yield fermentation of pentoses into lactic acid. *Appl Biochem Biotechnol* 84–86: 665–677.
20 Jarvis L. 2001. Lactic acid outlook up as polylactide nears market. *Chem Mark Rep* February 26.
21 Kandler O. 1983. Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuwenhoek* 49: 209–224.
22 Le Rudulier D and L Bouillard. 1983. Glycine betaine, an osmotic effector in *Klebsiella pneumoniae* and other members of the *Enterobacteriaceae*. *Appl Environ Microbiol* 46: 152–159.
23 Lin ECC. 1996. Dissimilatory pathways for sugars, polyols, and carboxylates. In: Neidhardt FC, R Curtis III, JL Ingraham, ECC Lin, KB Low, B Magasanik, WS Reznikoff, M Riley, M Schaechter and HE Umbarger (Eds), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd edn. ASM Press, Washington, DC, pp. 307–342.
24 Litchfield JH. 1996. Microbiological production of lactic acid. *Adv Appl Microbiol* 42: 45–95.
25 Masters M. 1996. Generalized transduction. In: Neidhardt FC, R Curtiss III, JL Ingraham, ECC Lin, KB Low, B Magasanik, WS Reznikoff, M Riley, M Schaechter and HE Umbarger (Eds), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd edn. ASM Press, Washington, DC, pp. 2421–2441.
26 McCaskey TA, SD Zhou, SN Britt and R Strickland. 1994. Bioconversion of municipal solid waste to lactic acid by *Lactobacillus* species. *Appl Biochem Biotechnol* 45/46: 555–568.
27 Mirasol F. 1999. Lactic acid prices falter as competition toughens. *Chem Mark Rep* March 1.
28 Nichols NN, BS Dien and RJ Bothast. 2001. Use of catabolite repression mutants for fermentation of sugar mixtures to ethanol. *Appl Microbiol Biotechnol* 56: 120–125.
29 Perroud B and D Le Rudulier. 1985. Glycine betaine transport in *Escherichia coli*: osmotic modulation. *J Bacteriol* 161: 393–401.
30 Postma PW, JW Lengeler and GR Jacobson. 1996. Phosphoenolpyruvate: carbohydrate phosphotransferase systems. In: Neidhardt FC, R Curtiss III, JL Ingraham, ECC Lin, KB Low, B Magasanik, WS Reznikoff, M Riley, M Schaechter and HE Umbarger (Eds), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd edn. ASM Press, Washington, DC, pp. 1149–1174.
31 Sambrook J and DW Russell. 2001. Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
32 Severson DK. 1998. Lactic acid fermentations. In: Nagodawithana TW and G Reed (Eds), Nutritional Requirements of Commercially Important Microorganisms. Esteekay Associates, Milwaukee, WI.
33 Wood WB. 1966. Host specificity of DNA produced by *Escherichia coli*: bacterial mutations affecting the restriction and modification of DNA. *J Mol Biol* 16: 118–133.
34 Wyckoff HA, J Chow, TR Whitehead and MA Cotta. 1997. Cloning, sequence, and expression of the L-(+) lactate dehydrogenase of *Streptococcus bovis*. *Curr Microbiol* 34: 367–373.
35 Yang CP, L Zhongjing and GT Tsao. 1995. Lactic acid production by pellet-form *Rhizopus oryzae* in a submerged system. *Appl Biochem Biotechnol* 51/52: 57–70.