Appl Microbiol Biotechnol (1995) 43:70–75

ORIGINAL PAPER

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Improved strains of recombinant *Escherichia coli* for ethanol production from sugar mixtures

Received: 25 March 1994/Received revision: 26 July 1994/Accepted: 5 August 1994

Abstract Hemicellulose hydrolysates of agricultural residues often contain mixtures of hexose and pentose sugars. Ethanologenic *Escherichia coli* that have been previously investigated preferentially ferment hexose sugars. In some cases, xylose fermentation was slow or incomplete. The purpose of this study was to develop improved ethanologenic *E. coli* strains for the fermentation of pentoses in sugar mixtures. Using fosfomycin as a selective agent, glucose-negative mutants of *E. coli* KO11 (containing chromosomally integrated genes encoding the ethanol pathway from *Zymomonas mobilis*) were isolated that were unable to ferment sugars transported by the phospho*enol*pyruvate-dependent phosphotransferase system. These strains (SL31 and SL142) retained the ability to ferment sugars with independent transport systems such as arabinose and xylose and were used to ferment pentose sugars to ethanol selectively in the presence of high concentrations of glucose. Additional fosfomycin-resistant mutants were isolated that were superior to strain KO11 for ethanol production from hexose and pentose sugars. These hyperproductive strains (SL28 and SL40) retained the ability to metabolize all sugars tested, completed fermentations more rapidly, and achieved higher ethanol yields than the parent. Both SL28 and SL40 produced 60 g l^{-1} ethanol from 120 g l^{-1} xylose in 60 h, 20% more ethanol than KO11 under identical conditions. Further studies illustrated the feasibility of sequential fermentation. A mixture of hexose and pentose sugars was fermented with near theoretical yield by SL40 in the first step followed by a second fermentation in which yeast and glucose were added. Such a two-step approach can combine the attributes of ethanologenic *E. coli* for pentoses with the high ethanol tolerance of conventional yeasts in a single vessel.

Florida Agricultural Station publication number R-03693

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Introduction

Cellulose and hemicellulose are the two most abundant forms of carbohydrate in nature and represent potential feedstocks for the production of chemicals to replace petroleum (Lynd et al. 1991). Hemicellulose represents 25%–50% of the dry weight of agricultural residues (Bertin et al. 1988; Puls 1993) such as corn cobs and stalks, seed hulls and husks, processing pulps (apple, beet, citrus), etc. Unlike cellulose, hemicellulose is readily hydrolyzed at modest temperatures by dilute acids to produce a syrup containing pentose and hexose sugars (Grohmann 1993). Previous studies have reported the genetic engineering of *Escherichia coli* B to produce ethanol from pentoses and hexoses. These recombinant strains contain *Zymomonas mobilis* genes (*pdc*, *adhB*) encoding the ethanol pathway on plasmids (Beall et al. 1991; Ingram et al. 1987) or as in strain KO11, integrated into the chromosome (Ohta et al. 1991). The fermentation of hemicellulose hydrolysates containing mixed sugars by KO11 and related organisms was incomplete or slow in some cases with over 10 g xylose l^{-1} remaining after 48 h (Barbosa et al. 1992; Beall et al. 1992; Lawford and Rousseau 1991, 1993). The sparing of xylose during fermentation is presumed to result from catabolite repression.

Catabolite repression has been studied extensively in *E. coli* and *Salmonella typhimurium* with respect to the ability of glucose to block induction of genes for other substrates via the phospho*enol*pyruvate-dependent

phosphotransferase system (PTS) and cAMP (Lin 1987; Postma et al. 1993; Roseman and Meadow 1990). Fosfomycin (L-*cis*-1,2-epoxypropylphosphonic acid) has been used as a selection agent to isolate PTS mutants that are unable to transport glucose or other PTS sugars (Cordaro et al. 1976). It is a bacteriocidal analog of phospho*enol*pyruvate which inhibits the first step in peptidoglycan biosynthesis (Christensen et al. 1969; Gunetileke and Anwar 1966; Hendlin et al. 1969).

In this paper, we describe the isolation of fosfomycin-resistant mutants of KO11 that have lost the ability to ferment glucose but retain the ability to ferment arabinose and xylose. Additional fosfomycin-resistant mutants were also recovered that were superior to the parent for the fermentation of pentoses and sugar mixtures.

Materials and methods

Bacterial strains

E. coli strain KO11 is a recombinant of *E. coli* ATCC11303 in which the genes for ethanol production from *Z. mobilis* (*pdc*, *adhB*) have been integrated into the chromosome (Ohta et al. 1991). This strain and derivatives were maintained on Luria agar (Sambrook et al. 1989) supplemented with 20 g xylose l^{-1} and 600 mg chloramphenicol l^{-1}.

Preparation of inocula.

To prepare inocula for fermentations, single colonies were transferred to 500-ml flasks containing 200 ml Luria broth with 50 g xylose l^{-1} and incubated at 30°C for 24 h without agitation. Cells were harvested by centrifugation and used to inoculate fermentations at an initial density of 330 mg cells l^{-1} (dry weight).

Fermentation experiments

Fermentations were conducted at 35°C, pH 6.0, in modified 500-ml flasks containing 350 ml Luria broth and sugars as previously described (Beall et al. 1991). Control of pH was provided by the automatic addition of potassium hydroxide (2 M). Glucose, xylose, and complex nutrients were sterilized separately by autoclaving at 121°C for 15 min. Arabinose was sterilized by filtration. Samples were removed at various times and stored at − 20°C until the end of fermentation.

Analyses

Ethanol (g l^{-1}) was determined by gas-liquid chromatography (Dombek and Ingram 1986). Final ethanol yields were corrected for dilution resulting from the addition of base. Yield was computed on the basis of total carbohydrate added to the broth without correction for unmetabolized sugar or the production of cell mass. The theoretical yield of ethanol from both pentose and hexose sugars is 0.51 g ethanol g $sugar^{-1}$. Sugars were analyzed with a Millipore/Waters high-performance liquid chromatograph (Millipore Corp., Bedford, Mass.) using an Aminex HPX-87P column (Bio-Rad Laboratories, Richmond, Calif.) with distilled water (85°C) as the mobile phase. Cell densities were measured at A_{550} on a Spectronic 70 spectrophotometer. All results represent averages from two or more fermentations.

Isolation of fosfomycin resistant mutants

Spontaneous mutants resistant to fosfomycin were isolated in two ways. KO11 was grown to 0.8 A_{550} in Luria broth containing 50 g glucose l^{-1} and harvested by centrifugation at 5000 *g* for 5 min. The cell pellet was washed twice and resuspended in cold 50 mM $CaCl_2$. Approximately 10^8 cells were spread on the surface of Luria agar containing 20 g xylose l^{-1} and 40 mg fosfomycin l^{-1} (Sigma Chemical Company, St. Louis, Mo.). After incubation for 48 h at 30°C, approximately 100 large and 100 small colonies were recovered on each plate. In the second method, cells were spread on Luria agar plates and solid fosfomycin (1–2 mg) was placed in the center of each plate. After overnight incubation at 30°C, small and large colonies were observed within the zone of inhibition.

Utilization of carbohydrate substrates

Mutants were tested for growth on M9 mineral salts agar (Sambrook et al. 1989) containing single carbohydrates (10 g l^{-1}). Growth was scored after 48 h of incubation at 30°C. Xylose, glucose 6-phosphate and glycerol 3-phosphate were sterilized by filtration. Concentrated solutions of other sugars were autoclaved separately. Carbohydrate utilization was also tested using MacConkey agar supplemented with individual sugars (10 g l^{-1}).

Results

Isolation of fosfomycin resistant mutants of KO11

Two hundred fosfomycin-resistant colonies were streaked onto Luria agar plates (containing fosfomycin and xylose) for purification. All colonies from the original selection plates contained a mixture of mutant types as shown by differences in colony size. Unexpectedly, approximately 5% of fosfomycin-resistant strains formed larger colonies than the parent. Fosfomycin-resistant mutants were tested on minimal salts agar for their ability to metabolize PTS sugars (glucose, fructose, mannose) and sugars with alternative transport systems (xylose, arabinose, galactose, lactose) (Table 1). Approximately 5% had lost the ability to metabolize glucose and other PTS sugars but retained the ability to metabolize pentoses. Two of these glucose-negative strains were selected for further study, SL31 and SL142. Both formed white to light pink (slight acid production) colonies on MacConkey agar plates containing PTS sugars in contrast to the red (higher acid production) colonies produced by the parent. Glucose-negative mutants retained the ability to metabolize glucose 6-phosphate and glycerol 3-phosphate on minimal agar and produced red colonies on MacConkey agar plates containing sugars with alternative transport systems.

In contrast, 95% of the mutants retained a functional PTS but lost the ability to metabolize both glucose

Table 1 Properties of mutant strains colony size was recorded after 24 h (30 °C) on Luria agar containing chloramphenicol (40 mg l^{-1}) and xylose (20 g l^{-1}): small (+) to large (+ + + +). (*Glu* glucose, *Fru* fructose, *Man* mannose, *Ara* arabinose, *Xyl* xylose, *Gal* galactose, *Lac* lactose, *Glu6P* glucose 6-phosphate, *Gly3P* glycerol 3-phosphate)

Strain	Colony size	Growth on M9 plates with single carbohydrates								
		Glu	Fru	Man	Ara	Xyl	Gal	Lac	Glu6*P*	Gly3*P*
KO11	+ + +	+	+	+	+	+	+	+	+	+
SL31	+ +	−	−	−	+	+	+	+	+	+
SL142	+ +	−	−	−	+	+	+	+	+	+
SL28	+ + + +	+	+	+	+	+	+	+	−	−
SL40	+ + + +	+	+	+	+	+	+	+	−	−
SL26	+ + +	+	+	+	+	+	+	+	−	−
SL119	+ + +	+	+	+	+	+	+	+	−	−
SL137	+ + +	+	+	+	+	+	+	+	−	−
SL150	+ + +	+	+	+	+	+	+	+	−	−

6-phosphate and glycerol 3-phosphate aerobically. The large-colony mutants were in this latter group and retained the ability to metabolize all sugars transported by the PTS. Some of the large-colony mutants were unstable during sequential transfers on solid medium. Two stable clones were retained for further study, SL28 and SL40.

Fermentation of sugar mixtures

Figure 1A shows a comparison of the parent strain (KO11), a glucose-negative mutant (SL31), and a large-colony mutant (SL40) during the fermentation of an equal mixture of glucose, xylose, and arabinose. Surprisingly, ethanol was produced more rapidly by SL40 than by KO11. This was accompanied by less acid production and thus less dilution by base. After correcting for base additions, the ethanol produced by KO11 was 91% of the theoretical yield while that from SL40 approached 100%. Strain SL31 produced less ethanol owing to an inability to transport glucose efficiently. Results obtained with two other mutants, SL28 and SL142, were very similar to those from SL40 and SL31, respectively. In all cases, the pH of the broth began to rise after 36–48 h signalling the exhaustion of sugars and release of ammonia from the catabolism of complex nutrients (Beall et al. 1991). Eighteen PTS-positive fosfomycin-resistant mutants (medium colony size) were also tested. None performed better than KO11. Four are included in Table 1 for comparison.

Table 2 summarizes sugar concentrations after 24 h during a mixed sugar fermentation (glucose, arabinose, and xylose). With SL40, SL28 and KO11, glucose was used preferentially followed by arabinose. Xylose was last with all strains except SL31 and SL142 (glucose-negative). SL31 and SL142 metabolized arabinose followed by xylose with slow utilization of glucose.

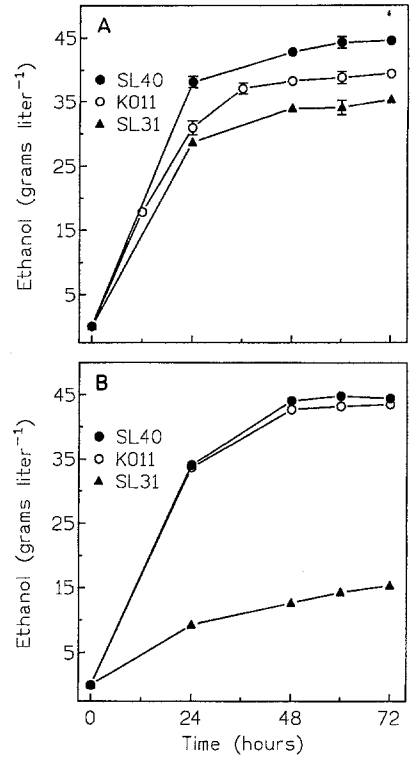

Fig. 1A,B Ethanol production from sugar mixtures. **A** Combination of glucose (30 g l^{-1}), arabinose (30 g l^{-1}), and xylose (30 g l^{-1}). **B** Combination of glucose (60 g l^{-1}) and xylose (30 g l^{-1}). Averages of four fermentations are shown with standard deviations in **A**. Bars are obscured by the data points in some cases. Differences in ethanol levels achieved by these three strains after 48 h were highly significant (95%)

Figure 1B summarizes the fermentation of a mixture of glucose and xylose in which glucose was in excess. Both KO11 and SL40 utilized this combination of sugars very effectively and approached 100% of the theoretical yield. Glucose was consumed at an equal rate by KO11 and SL40 but was metabolized poorly by the glucose-negative mutant, SL31 (Fig. 2). With SL31,

Table 2 Fermentation of sugar mixture containing (30 $g l^{-1}$) each of glucose, arabinose and xylose. Maximal concentration of ethanol (*EtOH*) during the 72-h fermentation. Cell densities (dry weight) at the end of fermentation ranged from 3.0 $g l^{-1}$ to 4.6 $g l^{-1}$. (*Glu* glucose, *Ara* arabinose, *Xyl* xylose)

Strain	Sugar ($g l^{-1}$) after 24 h			Maximum EtOH ($g l^{-1}$)
	Glu	Ara	Xyl	
KO11	0	5	18	41
SL31	27	0	0	34
SL142	26	0	8	35
SL28	0	3	15	44
SL40	0	0	12	45
SL26	0	6	18	39
SL119	0	12	26	28
SL137	0	19	30	23
SL150	0	9	18	39

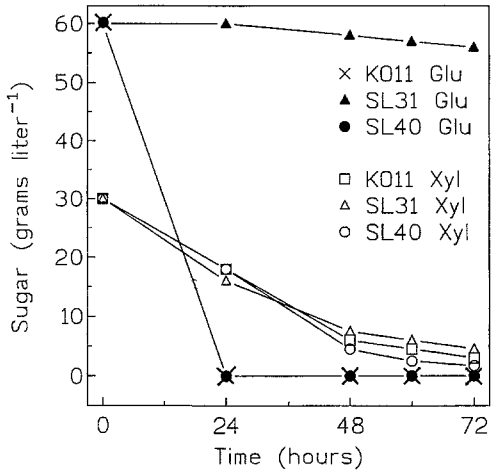

Fig. 2 Sugar utilization during ethanol production from a mixture of glucose (60 $g l^{-1}$) and xylose (30 $g l^{-1}$)

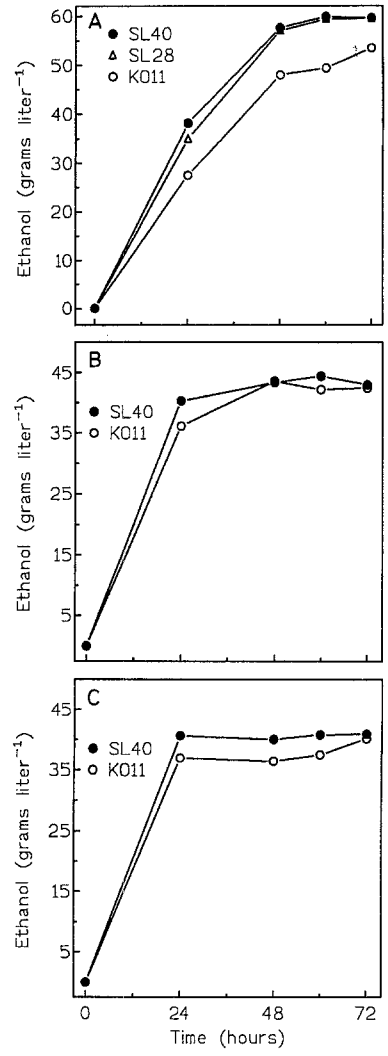

Fig. 3A–C Ethanol production from single sugars. **A** Xylose (120 $g l^{-1}$). **B** Xylose (90 $g l^{-1}$). **C** Glucose (90 $g l^{-1}$)

xylose was metabolized much more rapidly than glucose.

Fermentation of single sugars

SL28 and SL40 were tested further for their ability to ferment single sugars (Fig. 3). Both produced ethanol from 12% xylose more rapidly and at higher concentrations (60 g ethanol l^{-1}) than the parent KO11 (Fig. 3A). SL40 performed consistently better than KO11 (Fig. 3B, C) although differences were small in some cases.

Sequential fermentation

We have explored the possibility of combining ethanologenic *E. coli* and yeast in a sequential fermentation process (Fig. 4). Since strain SL40 produced ethanol from sugar mixtures more effectively than the glucose-negative mutants, this strain was selected for testing. A mixture of glucose, arabinose, and xylose (30 $g l^{-1}$ each) was fermented for 72 h. The total remaining sugar after 72 h was less than 1 $g l^{-1}$. At this time, non-sterile dry glucose was added to a final concentration of 37 $g l^{-1}$ along with 2 $g l^{-1}$ dry commercial yeast (Fermol; IBIS, Charlotte, N.C.). The added glucose was fully converted to ethanol within 24 h indicating that prior growth of *E. coli* was not detrimental to fermentation by yeast. No further additions of nutrients were made and pH control was not provided during the second fermentation step. Glucose added in the yeast-based step was converted to ethanol at approximately 90% of the theoretical yield.

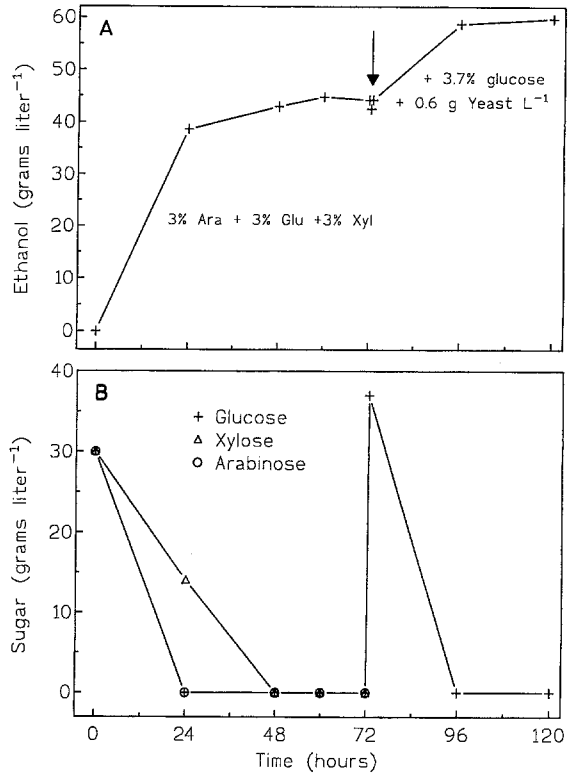

Fig. 4A,B Sequential fermentation of sugar mixtures by *E. coli* strain SL40 and yeast. The first stage of this fermentation contained 30 $g l^{-1}$ each of glucose, arabinose, and xylose and continued for 72 h with pH control. At 72 h, glucose (37 $g l^{-1}$) was added along with dry commercial yeast (2 $g l^{-1}$) and the fermentation continued without pH control. **A** Ethanol production. **B** Sugar utilization

Discussion

We have obtained a series of fosfomycin-resistant mutants of *E. coli* KO11 which may have advantages for commercial ethanol production. Fosfomycin enters enteric bacteria by either of two routes, the *glpT*-encoded permease for L-α-glycerophosphate or the *uhpT*-encoded permease for hexose phosphate (Cordaro et al. 1976; Lin 1970; Kahan et al. 1974). Many of our fosfomycin-resistant strains of KO11 have lost the ability to utilize both sugar phosphates and are presumed to contain mutations in the respective permeases (SL26, SL28, SL40, SL119, SL137, SL150). Double mutants of this type (loss of sugar-phosphate uptake) comprised the most frequently recovered group in previous studies (Venkateswaran and Wu 1972; Wu and Venkateswaran 1974). Surprisingly, strains SL28 and SL40 fermented single sugars and mixtures of sugars more efficiently than the parent strain. These hyperproducers also reached higher final ethanol concentrations (over 60 g ethanol l^{-1} from 12% xylose). Although the nature of the mutation(s) is unknown, SL28 and SL40 appear superior for the complete fermentation of sugar mixtures containing pentoses and hexoses.

Fosfomycin-resistant mutants of KO11 were also isolated that had lost the ability to utilize glucose and other PTS sugars (SL31 and SL142) but retained the ability to ferment pentoses, lactose and sugar phosphates. Strains with this phenotype have been previously reported to contain mutations in *ptsH* or *ptsI* thus eliminating the function of the PTS (Cordaro et al. 1976). Fosfomycin resistance in these mutants was proposed to result from tighter repression of *glpT* and *uhpT*. Our glucose-negative mutants, SL31 and SL142, are presumed to contain an analogous mutation in the PTS genes. Both SL31 and SL142 can be used to ferment pentoses preferentially to ethanol in complex sugar mixtures.

Ethanol tolerance remains a problem with ethanologenic *E. coli* although SL40 and SL28 approached 60 g ethanol l^{-1} from xylose after 48 h. In many cases, higher ethanol concentrations may be desirable to reduce costs associated with fermentation and ethanol purification. One method for solving this problem involves the inclusion of a second fermentation step with yeasts after an initial fermentation of hemicellulose-derived sugars by ethanologenic *E. coli*. Our initial investigation into the feasibility of this approach indicates that yeast are compatible with the beer produced during a bacterial fermentation and rapidly convert added glucose to ethanol. The combination of ethanologenic *E. coli* followed by yeast in a sequential fermentation represents a simple yet powerful approach, which combines the ability to convert pentoses or sugar mixtures efficiently to ethanol with the generation of high product levels in a single vessel. Such an approach may be used to incorporate lignocellulose-derived sugars into existing corn-based or cane-based ethanol plants.

Acknowledgements Sugar analysis were generously provided by Quadrex/Bioenergy, Gainesville, Fla. This research was supported in part by grants from the US Department of Energy, Office of Basic Energy Science (DE-FG05-86ER13574 and the US Department of Agriculture (92-37308-7471 and 58-3620-2-112).

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