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Fermentation of Biomass-Derived Glucuronic Acid by *pet* Expressing Recombinants of *E. coli* B

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ABSTRACT

The economics of large-scale production of fuel ethanol from biomass and wastes requires the efficient utilization of all the sugars derived from the hydrolysis of the heteropolymeric hemicellulose component of lignocellulosic feedstocks. Glucuronic and 4-*O*-methyl-glucuronic acids are major side chains in xylans of the grasses and hardwoods that have been targeted as potential feedstocks for the production of cellulosic ethanol. The amount of these acids is similar to that of arabinose, which is now being viewed as another potential substrate in the production of biomass-derived ethanol.

This study compared the end-product distribution associated with the fermentation of D-glucose (Glc) and D-glucuronic acid (GlcUA) (as sole carbon and energy sources) by *Escherichia coli* B (ATCC 11303) and two different ethanologenic recombinants—a strain in which *pet* expression was via a multicopy plasmid (pLOI297) and a chromosomally integrated construct, strain KO11. pH-stat batch fermentations were conducted using a modified LB medium with 2% (w/v) Glc or GlcUA with the set-point for pH control at either 6.3 or 7.0. The nontransformed host culture produced only lactic acid from glucose, but fermentation of GlcUA yielded a mixture of ethanol, acetic, and lactic acids, with acetic acid being the predominant end-product. The ethanol yield associated with GlcUA fermentation by both recombinants was similar, but acetic acid was a significant by-product. Increasing the pH from 6.3 to 7.0 increased the rate of glucuronate fermentation, but it also decreased the ethanol mass yield from 0.22 to 0.19 g/g primarily because of an increase in acetic acid production. In all fermentations there was good closure of

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the carbon mass balance, the exception being the recombinant bearing plasmid pLOI297 that produced an unidentified product from GlcUA. The metabolism of GlcUA by this metabolically engineered construct remains unresolved. The results offered insights into metabolic fluxes and the regulation of pyruvate catabolism in the wild-type and engineered strains. End-product distribution for metabolism of glucuronic acid by the nontransformed, wild-type *E. coli* B and recombinant strain KO11 suggests that the enzyme pyruvate-formate lyase is not solely responsible for the production of acetylCoA from pyruvate and that derepressed pyruvate dehydrogenase may play a significant role in the metabolism of GlcUA.

Index Entries: Glucuronic acid; recombinant *E. coli* B; ethanol; pyruvate metabolism; acetic acid derepressed pyruvate dehydrogenase.

INTRODUCTION

Lignocellulosic biomass and wastes are being targeted as an economic alternative to agricultural food crops such as corn, cereal grains, and sugar cane, for the large-scale production of fermentation ethanol for use as an alternative liquid transportation fuel *(1–3)*. Fermentation feedstock costs dominate the economics of fuel ethanol production *(4,5)* and the efficient utilization of nonglucose sugars represents an opportunity to significantly reduce the cost of producing fuel ethanol from lignocellulosic biomass and wastes *(6,7)*.

Woody biomass consists primarily of three polymeric substances, cellulose, hemicellulose, and lignin *(8)*. Cellulose is a homopolymer of glucose and comprises about half of the dry mass; however, it is strongly resistant to depolymerization unless the lignocellulose is pretreated to remove the impediments to enzymic digestion that are caused by lignin and the hemicellulose fraction of biomass *(9–11)*. Unlike cellulose, hemicellulose is a heteropolymer with a structure and composition that is source dependent *(12,13)*. The term hemicellulose was introduced by Schulze in 1891, but it is non-descriptive, and in Europe the preferred term is "wood polyoses" *(12)*. Hemicellulose represents about one-third of the carbohydrate content of hardwood lignocellulosic biomass with the five-carbon sugar, D-xylose, being a major component. Thermochemical depolymerization of hemicellulose using dilute acid is efficient and cost effective *(10,14)*. The hemicellulose of temperate zone hardwoods (*Angiospermae*) such as aspen (poplar), beech, and oak is well conserved with a relatively invariant composition *(8,12,15)*. In chemical terms, hardwood hemicellulose consists of *O*-Acetyl-(4-*O*-methylglucurono)xylans accompanied by small proportions of galactomannan *(16)*. Hardwood (4-*O*-methylglucurono)xylan is completely devoid of arabinose and its presence in hydrolysates probably relates to the hydrolysis of other polysaccharide materials such as the pectic material of the primary cell wall *(12)*. The linear xylan backbone consists of

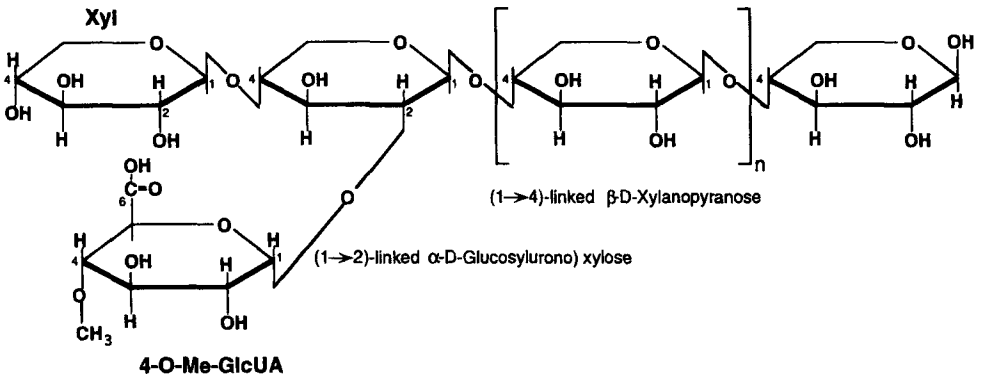

Fig. 1. Chemical structure of hardwood hemicellulose. The linear (1→4) β-D-xylopyranosyl backbone carries occasional substitutions at the C-2 position by 4-*O*-methyl-α-D-glucopyranosiduronic acid as well as randomly distributed acetyl groups (acetylation is not shown). The 4-*O*-methyl-α-D-glucopyranosiduronic acid linkage to xylose is highly resistant to acid hydrolysis and pretreatment of lignocellulosic hardwood biomass yields the disaccharide 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylopyranose.

(1→4)-linked β-D-xylopyranose residues with the 4-*O*-methyl-glucuronic acid attached directly to the C-2 of (on average) approximately every tenth xylose residue (Fig. 1). X-ray analysis has revealed that hardwood xylan has a threefold screw axis with 120° for each xylose residue and a repeat length of 15å (cellulose has a twofold screw axis) *(12)*. The (4-*O*-methylglucurono)xylan is extensively acetylated in a random fashion, with acetyl groups (not shown in Fig. 1) amounting to about 3–5% of the wood substance *(8,17)*. In hardwood hemicellulose, the mole ratio of acetic acid to D-xylose is approx 7:10 (equivalent to a mass ratio of acetic acid to xylose of 0.28:1.0). It has been shown that ester groups play an important role in plant cell wall resistance to enzyme hydrolysis *(18)*.

The disaccharide that is formed at the branch in the hardwood xylan polymer is a (1→2)-linked (4-*O*-methyl-alpha-D-glucopyranosyluronic acid)-D-xylopyranose. This alpha-(1→2)-glycosidic bond between the 4-*O*-Me-GlcUA and xylose is the most acid stable bond found in woody biomass—being even more resistant to hydrolysis than the β-(1→4)-D-glucosidic bond in cellobiose *(12)*. Hence, this disaccharide is a by-product of hemicellulose acid hydrolysis. It is difficult to quantitate by the usual HPLC analysis (using a HPX-87H column) because it coelutes with several other di- and trimers. Other by-products of dilute-acid pretreatment include substances such as acetic acid, furfural, and lignin-derived phenolics *(19)* that are toxic to ethanologenic micro-organisms *(20–22)*. Recent advances in the area of the bioconversion of biomass hemicellulose to ethanol have been reviewed by McMillan *(23)*.

Considerable research has been directed to the search for organisms capable of high-performance fermentation of biomass prehydrolysates.

This search for xylose-fermenting ethanologenic micro-organisms has produced several alternatives including bacteria, yeasts, and fungi (for review *see* ref. *24*). In addition to natural isolates, several genetically engineered biocatalysts have been constructed for this purpose and prominent among these have been the patented ethanologenic *Escherichia coli* cultures that carry genes for ethanol production, namely pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*) cloned from *Zymomonas mobilis* CP4 *(25–27)*. Although *E. coli* is heterofermentative and produces primarily acid end-products *(28,29)*, it has been metabolically engineered to exhibit a very high degree of ethanol selectivity *(30)*. In the early stages of development, transformation of *E. coli* involved insertion of the ethanol production genes from *Zymomonas* (referred to as the *pet* operon) *(26)* on multicopy plasmids carrying marker genes responsible for resistance to tetracycline and ampicillin *(27,30)*. Although the pioneering work was done with *E. coli* K12 *(31–33)*, a subsequent physiological assessment of growth characteristics of several different potential host cultures of *E. coli* identified the wild-type Luria strain B (ATCC 11303) as a "hardy strain and a suitable host" *(34)* for *pet* transformation using the plasmid designated as pLOI297 *(30)*. For several years, we have been assessing the fermentation performance characteristics of this patented recombinant *E. coli* 11303:pLOI297 using both synthetic lab media *(35–42)* and biomass prehydrolysates prepared by different thermochemical processors from a variety of biomass/waste feedstocks, including both hardwood (aspen) *(43)* and softwood (pine) *(44)*, newsprint *(45)*, spent sulfite liquors *(46)*, and corn crop residues *(47)*.

Plasmid-bearing recombinants suffer from two limitations: firstly, they are inherently less stable than strains in which the foreign genes have been integrated into the host chromosome *(41,48)*, and secondly, high copy number plasmids are known to impose an energetic burden on the host *(49)*, which is often reflected in a reduced growth rate and yield *(40)*. With this in mind, Ingram and his associates engineered chromosomally integrated strains of *E. coli* B ATCC 11303 in which the *Zymomonas pdc* and *adhB* genes were inserted into the pyruvate-formate lyase gene (*pfl*) of the host *(48)*. However, it was discovered that single copy inserts of the *pdc* and *adhB* genes did not result in the same high level of activities of *Zymomonas* enzymes that had been achieved in multicopy plasmid-based recombinants *(48)*. In one series of constructs involving chromosomal integration, the transformation vector also contained the gene for chloramphenicol acetyl transferase (*cat*), which is responsible for conferring resistance to chloramphenicol (Cm). A spontaneous mutant, designated as strain KO11, was selected for resistance to high levels (600 μg/mL) of Cm and has been shown to express high levels of both the *Zymomonas* genes and *cat*. In addition, strain KO11 carries a mutation in its fumarate reductase gene that impairs its ability to produce succinate as a fermentation end-product *(48)*.

In addition to exhibiting a high level of conversion efficiency in laboratory media *(48)*, recombinant KO11 has been shown to ferment

prehydrolysates prepared from pine *(50)*, and agricultural crop residues *(51,52)*. However, claims relating to its long-term stability *(48)* have recently been challenged in a study involving continuous culture *(41)*. Recently conducted comparative surveys of xylose-fermenting ethanologenic micro-organisms have concluded that recombinant *E. coli* strain KO11 is currently one of "the best candidates" for ethanol production from hemicellulosic hydrolysates *(23,53,54)*.

Filtered enzymic hydrolysates of peel and pulp wastes associated with the production of citrus fruit juices are a rich source of fermentable carbohydrates *(55)*. Almost one-third of the total mass of monosaccharides in an enzymic orange peel hydrolysate was shown to be galacturonic acid (GalUA), with the remainder being a mixture of glucose, fructose, galactose, and arabinose. In addition to the expected five-carbon and six-carbon neutral sugars, recombinant *E. coli* KO11 utilized the galacturonic acid. Using a nutrient-rich laboratory medium containing 2% (w/v) D-galacturonic acid, Grohmann et al. *(55)* showed that, at pH 7.0, *E. coli* KO11 produced equimolar amounts of acetic acid and ethanol, with carbon dioxide as the only other detectable fermentation product. Based on the proposed theoretical maximum ethanol yield of 0.237 g/g, the observed ethanol yield of 0.19 g/g *(55)* represents a conversion efficiency of 80%.

One objective of this work was to compare the ability of two high profile *E. coli* ethanologenic recombinants, specifically the plasmid recombinant 11303:pLOI297 and the chromosomal integrated strain KO11, to ferment D-glucuronic acid. Based on a knowledge of the chemical structure of hardwood hemicellulose, it is reasonable to assume that dilute-acid hydrolysis will produce 4-*O*-methyl-glucuronic acid and the C-2 xylose derivative disaccharide 2-*O*-(4-*O*-methyl-α-D-glucuronic acid)-D-xylose; however, in the absence of the commercial availability of either of these substances, our fermentation experiments were based on pure D-glucuronic acid as sole carbon (energy) source. Since end-product distribution has the potential to offer insights into how a substance is metabolized, a second objective of this work involved comparing the anaerobic catabolism of Glc and GlcUA by both the nontransformed wild-type culture and the two different metabolically engineered strains. Because the metabolic engineering was directed specifically toward alterations in pyruvate metabolism, it was hoped that the results of this study would shed light on metabolic fluxes and the regulation of pyruvate metabolism in *E. coli* B.

MATERIALS AND METHODS

Organisms

The wild-type, nontransformed host culture, *Escherichia coli* B (ATCC 11303) was obtained from The American Type Culture Collection (Rockland, MD). Recombinant *Escherichia coli* B (ATCC 11303 carrying the *pet* plasmid

pLOI297) *(30)* and the chromosomally integrated strain KO11 *(48)* were received from L. O. Ingram (University of Florida, Gainesville, FL). Cultures grown from single colony isolates on selective antibiotic-containing agar medium were stored at –10°C in LB medium supplemented with glycerol (20 mL/dL) and sodium citrate (1.5 g/dL). Inocula were prepared using complex or defined media buffered with 100 m*M* phosphate (pH 7.0). Batch fermentations were inoculated by transferring approx 100 mL of an overnight flask culture directly to 1400 mL of medium in the stirred-tank bioreactor. The same sugar was used for preculture and fermentation. The initial cell density was monitored spectrophotometrically to give an OD_{550} in the range 0.1–0.2 corresponding to 30–50 mg dry cell mass (DCM)/L.

Culture Media

The nutrient-rich, complex culture medium Luria broth *(56)* was modified as described by Grohmann et al. *(55,57)* and contained 2.5 g Bacto Yeast Extract (Difco Laboratories, Detroit, MI) and 5 g Bacto Tryptone (Difco) per liter of distilled water. D-Glucuronic acid was obtained from Sigma Chemical (St. Louis, MO). The medium was sterilized by autoclaving. Stock sugar solutions were autoclaved separately and added at the concentration specified. When the *pet* transformed cultures were used, filter-sterilized antibiotics (final concentration of 40 mg/L ampicillin and 10 mg/L tetracycline for pLOI297 and 40 mg/L chloramphenicol for KO11) were added to the autoclaved fermentation media after cooling.

Fermentation Equipment

pH-stat batch fermentations were conducted in a volume of 1500 mL in MultiGen (model F2000) stirred-tank bioreactors fitted with agitation, pH, and temperature control (30°C) (New Brunswick Scientific, Edison, NJ). The pH was controlled either at 6.3 or 7.0 by the addition of 4N KOH.

Analytical Procedures

Growth was measured turbidometrically at 550 nm (1 cm lightpath) and dry cell mass (DCM) was measured by microfiltration as described previously *(40)*. Compositional analyses of culture media and cell-free spent broths were determined by HPLC using a HPX-87H column (Bio-Rad Labs, Richmond, CA) as described previously *(40)*. The concentration of metabolic end-products in spent fermentation broths was not corrected for the dilution caused by the addition of titrant during fermentation.

Determination of Fermentation Parameters

The molar growth yield coefficient with respect to carbon (energy) source was calculated by dividing the maximum cell density (g DCM/L) by the molar concentration of sugar added to the medium. The ethanol yield (Yp/s) was calculated as the final mass concentration of ethanol

divided by the initial sugar concentration. The average volumetric rate of sugar consumption ($_{av}Q_s$; g S/L.h) was determined by dividing the initial concentration of sugar (S) by the total time (post inoculation) required for the complete exhaustion of sugar from the medium. Carbon mass balances (expressed as percent carbon recovery) were calculated as described previously *(41)*. The carbon content of the *E. coli* dry cell mass was assumed constant at 47.6% carbon *(41)*.

RESULTS AND DISCUSSION

Figure 2 compares the growth and fermentation performance of recombinant *E. coli* 11303:pLOI297 in a nutrient-rich complex medium (mLB) with either 2% (w/v) Glc or GlcUA as sole carbon (energy) source. With GlcUA as carbon source, increasing the pH control set-point from 6.3 to 7.0 markedly improved both the sugar utilization rate and the ethanol productivity (Fig. 2), but both the growth yield and the rate of sugar consumption ($_{av}Q_s$) were decreased compared to Glc as substrate (Table 1). Recombinant 11303:pLOI297 exhibits a very high glucose-to-ethanol conversion efficiency (98%), with the ethanol yield being higher at pH 6.3 (0.50 g/g) than 7.0 (0.43 g/g) (Table 1). This observation confirms earlier observations *(30,34,35)*. There was good closure of the carbon mass balance and, at pH 7.0, the lower ethanol yield can be attributed to the formation of lactic acid (Table 2). Whereas Glc is converted primarily to ethanol, GlcUA is converted to acetic acid and ethanol (Table 2). On a weight basis, the ethanol yield (Yp/s/s) was 0.24 and 0.19 g/g at pH 6.3 and 7.0, respectively (Table 1). In the case of GlcUA at pH 6.3, the carbon mass balance did not exhibit closure (Table 1) and an unknown substance was detected in the spent broth from this fermentation. Attempts to positively identify this 'unknown' substance were unsuccessful, although under the conditions of operation of our HPLC system (*see* Materials and Methods), it eluted after acetic acid and before ethanol with a retention time of about 18 min. The only substance that exhibited a similar retention time was methylglyoxal *(29)*. Interestingly, Cooper and Anderson *(58)* have shown that *E. coli* B can synthesize methylglyoxal from the glycolytic intermediate dihydroxyacetone phosphate *(58)*.

The cell mass concentration in Glc and GlcUA fermentations is significantly different (Table 2) with the molar growth yield associated with Glc fermentation being about 1.7 times greater than with GlcUA as substrate (Table 1). The molar growth yield is a reflection of the net gain in energy (ATP) derived by substrate-level phosphorylation reactions associated with anaerobic sugar catabolism. Table 1 shows that the ATP gain (G_{ATP}, mol ATP/mol sugar) is greater with Glc compared to GlcUA as energy source. This bioenergetic parameter can be calculated based on the end-product distribution in combination with a knowledge of the metabolic pathway responsible for the metabolism of each sugar *(41)*. For Glc

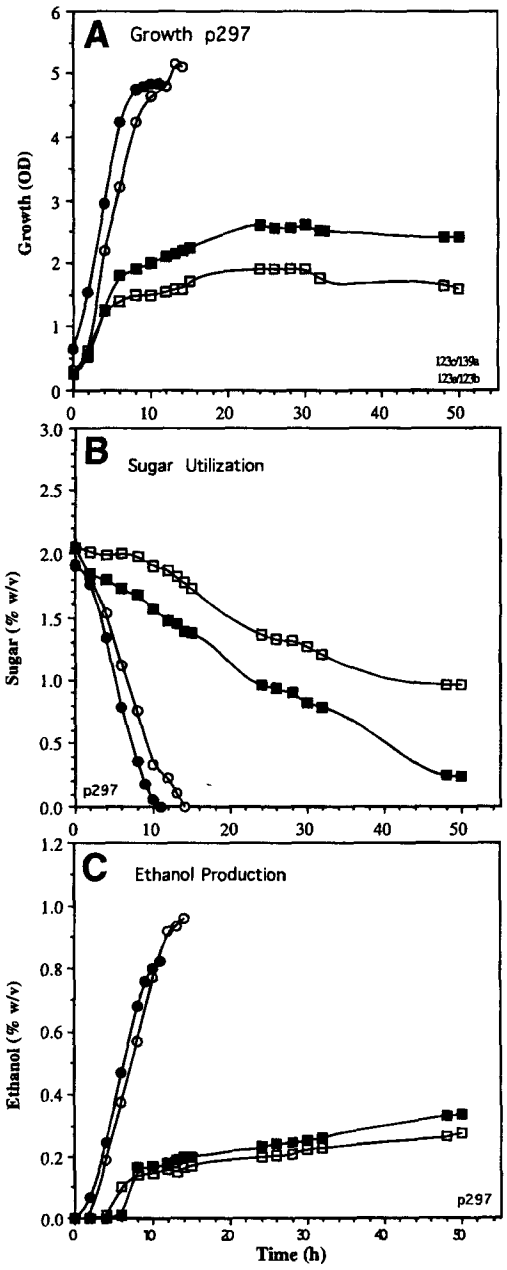

Fig. 2. Comparative growth and fermentation of glucose and glucuronic acid by recombinant *E. coli* B 11303:pLOI297. (**A**) growth, (**B**) sugar utilization, and (**C**) ethanol production. The medium was modified LB (mLB) with either approx 2% (w/v) glucose (Glc) or glucuronic acid (GlcUA) as sole carbon source (*see* Materials and Methods). Experimental data are summarized in Tables 1 and 2. ○ Glc pH 6.3; ● Glc pH 7.0; □ GlcUA pH 6.3; ■ GlcUA pH 7.0.

catabolism, the G_{ATP} per mole of ethanol, lactic acid, and succinate is 1.0 and 1.5 for acetic acid *(59)*. However, because GlcUA is metabolized differently *(60,61)* (*see* more detailed discussion following), the G_{ATP}/mol of ethanol, lactic acid, and succinate is only 0.5 and 1.0 for acetic acid.

Table 1
Summary of Growth and Fermentation Parameters

Culture	Sugar	pH	av Q_s (g S/L.h)	Growth Yield (g DCM/mol S)	G_{ATP} (mol ATP/mol S)	EtOH Yield (g EtOH/g S)	Carbon Recovery (%)
11303							
	Glc	6.3	0.83	11.5	2.00	-	105
		7.0	0.96	12.9	1.90	-	101
	GlcUA	6.3	0.37	8.7	1.59	0.12	114
		7.0	0.87	15.7	1.60	0.08	110
p297							
	Glc	6.3	1.36	16.9	2.01	0.50	113
		7.0	1.74	17.1	1.96	0.43	110
	GlcUA	6.3	0.22	10.9	0.83	0.24	77
		7.0	0.36	9.5	0.89	0.19	73
KO11							
	Glc	6.3	1.11	15.3	2.17	0.50	113
		7.0	1.41	12.3	2.38	0.44	113
	GlcUA	6.3	0.62	9.4	1.42	0.22	102
		7.0	1.45	17.6	1.62	0.19	114

$Y_{x/s}$ = molar yield (g DCM/mol S).

Furthermore, the calculated value of G_{ATP} for GlcUA fermentation by the plasmid-bearing recombinant is made lower by the production of the unidentified metabolic end-product for which an energy yield equivalence can not be assigned.

Acetic acid inhibits *E. coli* growth and fermentation *(62)*. The amount of acetic acid produced, and the sensitivity of *E. coli* to acetic acid inhibition, is known to be both pH and strain dependent *(63)*. In a previous study, we examined the sensitivity of *pet*-plasmid transformed *E. coli* B to acetic acid as a function of pH using different sugar substrates *(37)*. Because the undissociated (protonated) form of acetic acid is responsible for the inhibition, the inhibitory effect of acetic acid is decreased at pH 7.0 because of the lower concentration of the undissociated acid. However, the concentrations of acetic acid produced from GlcUA by the recombinant are well below the inhibitory threshold *(37)*.

Figure 3 compares the growth and fermentation performance of recombinant strain KO11 in mLB medium with either 2% (w/v) Glc or GlcUA as sole carbon (energy) source (note that for purposes of comparison the scales for the plot axes are the same in Figs. 2 and 3). With Glc as substrate, strain KO11 grows slower than the plasmid-bearing recombinant

Table 2
End-Product Distribution Associated with Glucose and Glucuronic Acid Fermentations by Wild-type *E. coli* B and *pet*-transformed Recombinants

		Glc		GlcUA		Cell mass	EtOH	Succ.	Lactic	Acetic	
	pH	g/L	mM	g/L	mM	gDCM/L	mM	mM	mM	mM	(Total)
ATCC 11303											
	6.3	20.8	115.7			1.33	0.0	0.0	219.8 (1.90)	5.9 (0.05)	(1.95)
	7.0	26.9	149.3			1.92	0.4	0.0	266.0 (1.78)	9.6 (0.06)	(1.84)
	6.3			17.8	91.5	0.86	48.0 (0.52)	6.5 (0.07)	42.5 (0.46)	97.3 (1.06)	(2.11)
	7.0			19.1	98.6	1.55	32.1 (0.32)	6.8 (0.07)	32.5 (0.33)	122.4 (1.24)	(1.96)
11303:p297											
	6.3	19.0	105.3			1.78	208.4 (1.98)	3.5 (0.03)	0.0	0.0	(2.01)
	7.0	19.2	106.5			1.82	178.4 (1.68)	7.1 (0.07)	22.1 (0.21)	0.0	(1.96)
	6.3			10.8	55.9	0.61	57.0 (1.02)	2.9 (0.05)	0.0	16.5 (0.30)	(1.37)*
	7.0			18.0	92.8	0.88	74.2 (0.80)	6.8 (0.07)	0.0	41.6 (0.45)	(1.32)*
KO11											
	6.3	18.8	104.4			1.60	203.2 (1.95)	0.4	0.0	11.2 (0.11)	(2.06)
	7.0	22.6	125.7			1.54	216.4 (1.72)	0.0	12.1 (0.10)	35.0 (0.28)	(2.10)
	6.3			21.0	108.0	1.02	99.0 (0.92)	4.6 (0.04)	0.0	102.0 (0.94)	(1.90)
	7.0			17.5	90.0	1.58	73.6 (0.82)	0.0	4.3 (0.05)	106.0 (1.18)	(2.05)

Note: Formic acid was not detected in any of these expts. Medium = modified LB (mLB). Bracketed values represent molar yield of end-products (mole P/mole S).

*In Expt 123a and 123b an unknown peak on HPLC seems related to p297 metabolism of GlcUA.

(Fig. 3A). This slower growth is likely a result of the much higher level of inhibiting acetic acid (Table 2). The higher acetic acid concentration is reflected in the improved G_{ATP} (Table 1), but the energetic benefit is nullified at the lower pH by the energetic uncoupling effect of acetic acid (*37*). At pH 7.0, the molar growth yield with GlcUA surpasses that achieved with Glc (Table 1) and we have no explanation for this observation. With both recombinants, the ethanol yield from Glc was similar and was lower at pH 7 than at pH 6.3; although the rate of sugar consumption ($_{av}Q_s$) was higher for both recombinants at pH 7.0 (Table 1). With GlcUA as substrate, strain KO11 out performed the plasmid-bearing culture both with respect to growth rate (Fig. 3A) and the rate of sugar utilization (Fig. 3B). Unlike with strain 11303:pLOI297, there was good closure of the carbon balance for GlcUA metabolism by strain KO11 at both pH values (Table 1).

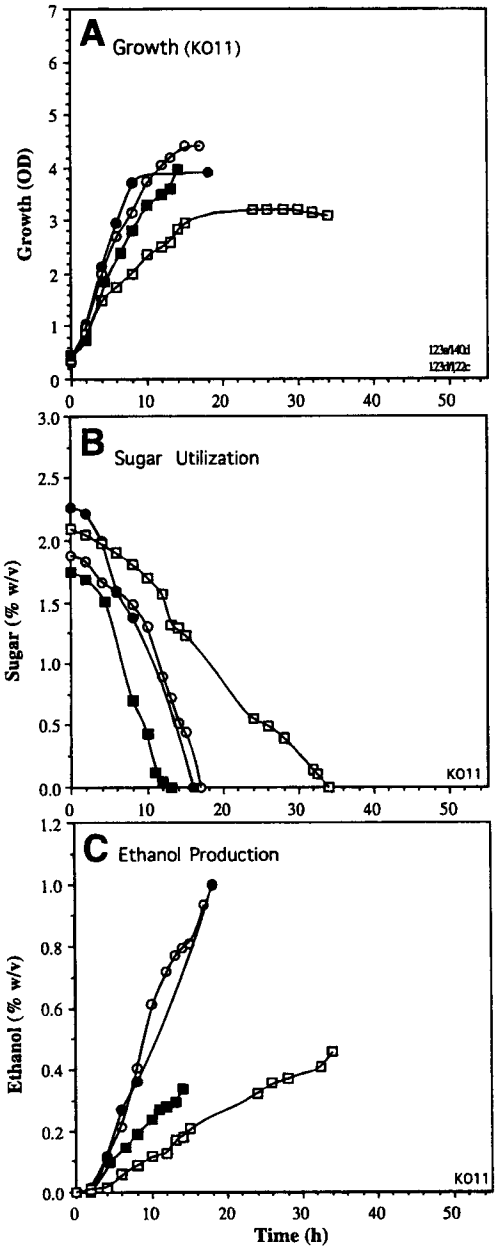

Fig. 3. Comparative growth and fermentation of glucose and glucuronic acid by recombinant *E. coli* B KO11. (**A**) growth, (**B**) sugar utilization, and (**C**) ethanol production. Experimental conditions are as described in legend to Fig. 2. Experimental data are summarized in Tables 1 and 2. ○ Glc pH 6.3; ● Glc pH 7.0; □ GlcUA pH 6.3; ■ GlcUA pH 7.0.

Grohmann et al. *(55,57)* have studied GalUA fermentation by recombinant KO11. Their experimental conditions were similar with respect to substrate concentration (2% w/v) and medium composition (mLB). With the pH controlled at 7.0, the ethanol mass yield from GalUA was 0.19 g/g *(55)*, which is identical to the ethanol yield from GlcUA (Table 1).

Furthermore, the molar yield for ethanol and acetic acid from GalUA was observed to be 0.80 and 0.78, respectively *(55)*, which compares very favorably with the pattern for end-product distribution associated with GlcUA metabolism by KO11 under similar assay conditions, namely 0.82 and 1.18 for ethanol and acetic acid, respectively (Table 2). We observed that at pH 6.3, the distribution with respect to ethanol and acetic acid was equimolar, being 0.92 and 0.94, respectively (Table 2).

The pattern of end-product distribution led Grohmann et al. *(55)* to conclude that GalUA was metabolized according to the following relationship:

$$C_6H_{10}O_7 \rightarrow C_2H_{14}O_2 + C_2H_6O + 2CO_2$$
$$\text{GlcUA} \rightarrow \text{Acetic Acid} + \text{EtOH} + 2CO_2$$

From our observations with strain KO11 under similar conditions (pH 7.0), the same conclusion with respect to GlcUA metabolism would seem appropriate. The fact that this relationship was chemically balanced and that it coincided with their observations was sufficiently satisfying to Grohmann et al. *(55)* for them to suggest that it represented a "novel pattern of galacturonic acid fermentation" and these authors did not speculate regarding the metabolic mechanism responsible for the equimolar amounts of ethanol and acetic acid in recombinant *E. coli* KO11. However, the investigations by Grohmann et al. *(55,57)* were confined to recombinant KO11. In terms of end-product distribution, the results of our comparative study point to a difference in GlcUA metabolism between the two different metabolically engineered constructs that were examined.

In *E. coli*, the uptake and metabolism of both GalUA and GlcUA in terms of the conversion of these uronic acids to pyruvic acid is known to be similar *(64)*. Figure 4 compares the catabolism of Glc and GlcUA. The following relationships represent the metabolic pathways depicted for the catabolism of Glc and GlcUA shown in Fig. 4.

For D-glucose (Glc)

$$\text{Glc} + 2\,\text{NAD}^+ + 2(\text{ADP} + P_i) \rightarrow 2\,\text{Pyruvic acid} + 2(\text{NADH} + H^+) + 2\text{ATP}$$
$$C_6H_{12}O_6 + 2\,\text{NAD} + 2(\text{ADP} + P_i) \rightarrow 2\,(C_3H_4O_3) + 2(\text{NADH} + H^+) + 2\text{ATP}$$

For D-glucuronic acid (GlcUA)

$$\text{GlcUA} + \text{ADP} + P_i \rightarrow 2\,\text{Pyruvic acid} + H_2O + \text{ATP}$$
$$C_6H_{10}O_7 + \text{ADP} + P_i \rightarrow 2(C_3H_4O_3) + H_2O + \text{ATP}$$

Succinic acid is sometimes observed as an end-product in *E. coli* fermentations (Table 2) and the pathway for its production is shown in Fig. 4. In the context of redox balancing, it is important to note that the production of succinic acid from phosphoenolpyruvate requires two pairs of

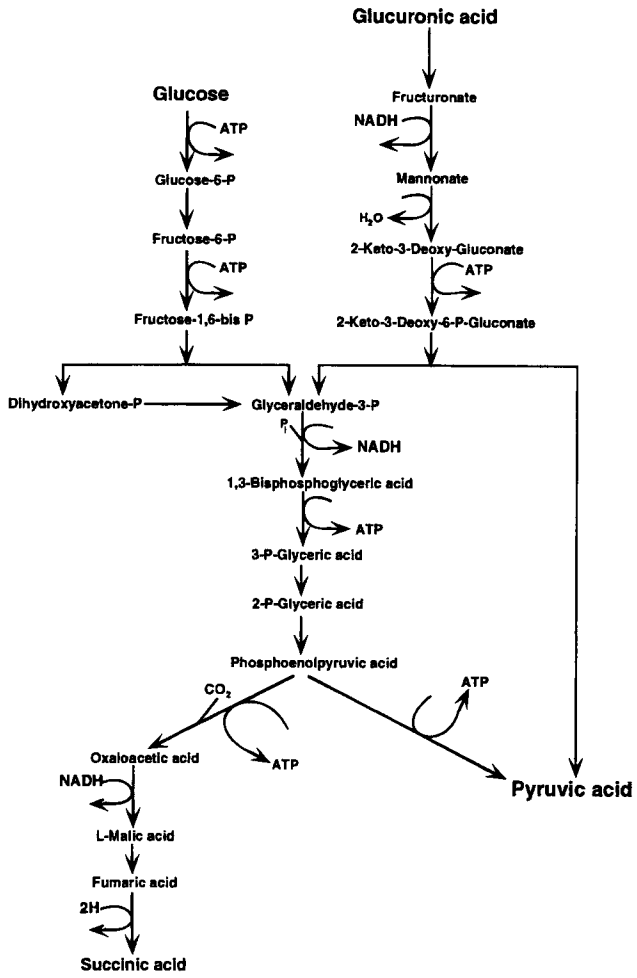

Fig. 4. Metabolic pathways for the partial catabolism of glucose and glucuronic acid by *Escherichia coli* B. The major metabolic pathways for the conversion of glucose and glucuronic acid to pyruvic acid are represented. Phosphoenolpyruvic acid can be converted to succinic acid, but this represents a minor pathway.

reducing equivalents (4H) (Fig. 4). However, the production of succinic acid represents a minor pathway, and furthermore, strain KO11 is supposedly incapable of producing succinate acid by virtue of the engineered interruption of the gene coding for fumarate reductase *(48)*. It is clear from the relationships above that, apart from the participation of different enzymes, the major difference in catabolism of Glc and GlcUA relates to the production of two pairs of reducing equivalents (4H) from Glc, and the production of more energy (ATP) from Glc compared to GlcUA.

Figure 5 illustrates possible pathways for pyruvate metabolism by *E. coli* and includes pathways for both the wild-type, nontransformed, host culture ATCC 11303, and the *pet*-transformed, metabolically engineered, recombinants 11303:pLOI297 and KO11.

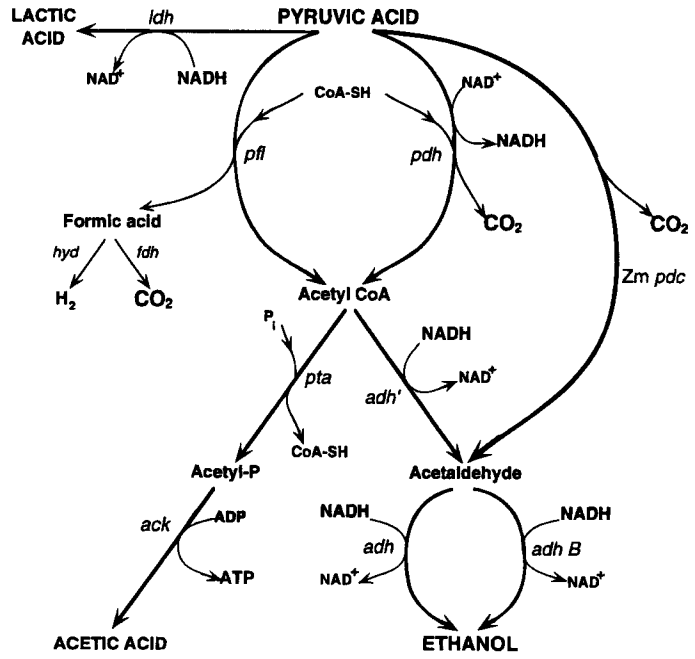

Fig. 5. Pyruvic acid metabolism by *E. coli* B ATCC 11303 and the *pet* recombinants 11303:pLOI297 and strain KO11. *adh* acetaldehyde dehydrogenase; *adh* alcohol dehydrogenase; *adh B* Zm alcohol dehydrogenase; *ack* acetate kinase; *fdh* formate dehydrogenase; *hyd* hydrogenase; *ldh* lactic acid dehydrogenase; pyruvate-formate lyase; *pdh* pyruvate dehydrogenase complex; *pta* phosphotransacetylase; ATP adenosine triphosphate; NADH nicotinamide adenine dinucleotide.

The commonly held view in the literature that deals with the subject of anaerobic glucose metabolism by *E. coli* is that pyruvic acid is reduced to acetyl coenzyme A (acetylCoA) + formic acid by pyruvate-formate lyase (*pfl*) *(65)* (for review *see* ref. *28*) The level of *pfl* is known to be affected by both chemical and physical environmental conditions *(66)*. Lactic acid can also be produced from pyruvic acid by lactic acid dehydrogenase (*ldh*). The metabolic engineering of *E. coli* for ethanol production *(26,27)* was based on this assumed metabolic fate of pyruvate, whereby the high level of expression of the *pet* operon enzymes from *Zymomonas*, namely pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adh B*) resulted in a "redirection of pyruvate metabolism" *(27)* from a mixed acid fermentation to ethanol as the almost exclusive end-product (Fig. 5). If pyruvate were to be metabolized exclusively in this manner, the following end-product distribution, associated with GlcUA dissimilation by recombinant *E. coli*, might be predicted:

$$\begin{array}{llllll} \text{GlcUA} & \rightarrow & \text{Acetic acid} & + \text{Formic acid} & + \text{EtOH} & + CO_2 \\ C_6H_{10}O_7 & \rightarrow & C_2H_4O_2 & + CH_2O_2 & + C_2H_6O & + CO_2 \\ & \rightarrow & C_2H_4O_2 & + C_2H_6O & + 2CO_2 & + H_2 \end{array}$$

However, this relationship produces an extra 2H and does not balance. From a theoretical perspective, if the recombinants were to produce ethanol through a utilization of the engineered ethanol production pathway from *Zymomonas* (or at least via the *Zymomonas pdc*), there would be a requirement for 2H (NADH). In searching for a possible source of this 2H (NADH), it was postulated that the native NAD-linked pyruvate dehydrogenase (*pdh*) is repressed by glucose, but in a nonrepressive growth environment, *pdh* would operate in competition with *pfl* for the conversion of pyruvate to AcCoA, thereby generating the necessary NADH for either the native or *Zymomonas*-derived alcohol dehydrogenase to convert acetaldehyde to ethanol (Fig. 5). The pyruvate dehydrogenase complex in *E. coli* is known to be subject to catabolite repression by glucose *(67)*, and Langley and Guest *(68)* have observed that with 50 m*M* glucose the levels of pdh were similar in *E. coli* strain H under both aerobic and anaerobic growth conditions. Whereas *pdc* is known to be absent from the nontransformed ATCC 11303 culture *(33)*, nevertheless there remains the potential for ethanol production via the combined action of the native NAD-linked enzymes, acetaldehyde dehydrogenase and alcohol dehydrogenase (Fig. 5). Therefore, it is conceivable that if *pdh* were solely responsible for the formation of AcCoA from pyruvate, equimolar amounts of ethanol and acetic acid could be produced from GlcUA in the recombinants totally independent of the existence (operation) of the engineered *pet* pathway. The balanced fermentation that would result from this proposed action is:

$$C_6H_{10}O_7 \rightarrow \underset{\text{Pyruvic acid}}{2(C_3H_4O_3)} + H_2O$$
$$\rightarrow 2\,\text{AcetylCoA} + 2CO_2 + 2\,(NADH + H^+)$$
$$\rightarrow \underset{\text{Acetic acid}}{C_2H_4O_2} + \underset{\text{EtOH}}{C_2H_6O} + 2CO_2 + 2\,\text{CoA-SH} + 2NAD^+$$

In considering the fermentation of GlcUA, it was noted that the following relationship also balanced:

$$\underset{\text{GlcUA}}{C_6H_{10}O_7} \rightarrow \underset{\text{Acetic acid}}{C_2H_4O_2} + \underset{\text{Lactic acid}}{C_3H_6O_3} + CO_2$$

However, this pattern of end-products is not consistent with the production of acetic acid by either *pfl* and/or *pdh* since both enzymes would generate an extra pair of reducing equivalents either as H_2 or as NADH.

The strategy for metabolically engineering *E. coli* for ethanol production was based on

1. The conversion of pyruvate to AcCoA exclusively by *pfl*, and
2. The absence of *pdc*, and
3. The ability of expressed *Zymomonas pdc* to effectively exclude the competition for pyruvate exerted by NAD-linked lactic acid dehydrogenase (*ldh*) *(26,27)*.

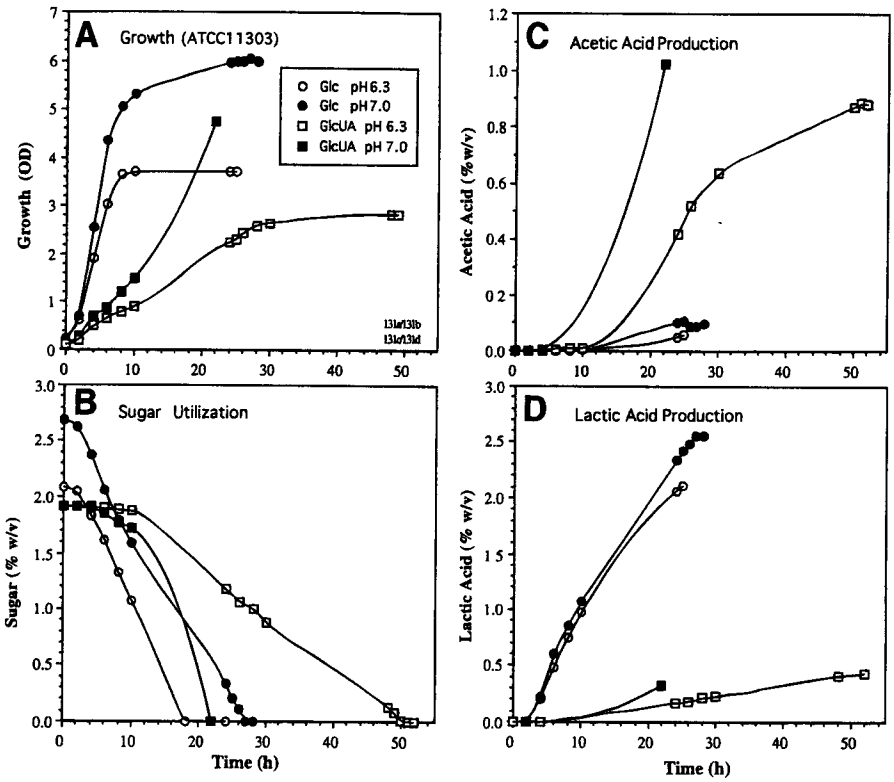

Fig. 6. Comparative growth and fermentation of glucose and glucuronic acid by the nontransformed host culture *E. coli* B ATCC 11303. **(A)** growth, **(B)** sugar utilization, **(C)** acetic acid production, and **(D)** lactic acid production. Experimental conditions are as described in legend to Fig. 2. Experimental data are summarized in Tables 1 and 2. ○ Glc pH 6.3; ● Glc pH 7.0.; □ GlcUA pH 6.3; ■ GlcUA pH 7.0.

However, if glucose acts to repress *pdh*, then under conditions where *pdh* is derepressed, the success of the competition for pyruvate between *pdh*, *pfl*, and *ldh*, will dictate the nature of end-product distribution. If GlcUA did not repress *pdh*, then it seems reasonable to expect that ethanol could be a product of GlcUA catabolism by the wild-type culture. To examine this possibility, the nature of end-product distribution in GlcUA fermentation by the nontransformed culture *E. coli* B ATCC 11303 was investigated. Figure 6 compares the growth and fermentation performance of wild-type *E. coli* B in mLB medium with either 2% (w/v) Glc or GlcUA as sole carbon (energy) source (note that for purposes of comparison the scales for the plot axes are the same as in Figs. 2 and 3).

With Glc as substrate, the higher cell density achieved at pH 7.0 compared to pH 6.3 (Fig. 6A) is a result of, in part, the higher sugar concentration (Fig. 6B and Table 2); however, the growth yield was only slightly higher at pH 7.0 (Table 1). The rate of Glc consumption ($_{av}$ Q_s) was slower at both pH values compared to the recombinant cultures (Table 1), which

may be related to the fact that lactic acid is the sole end-product of Glc catabolism by ATCC 11303 (Fig. 6D and Table 2). The high degree of selectivity for lactic acid associated with Glc fermentation by this culture confirms previous reports in the literature *(31,35,39)*.

With GlcUA as substrate, the nontransformed culture produced acetic acid (Fig. 6C), lactic acid (Fig. 6D), and ethanol (not shown in Fig. 6). In all fermentations with ATCC 11303 (with both sugars) there was good closure of the carbon balance (Table 1). The concentrations of the different end-products associated with GlcUA fermentation are given in Table 2. The molar ratio of the end-products is not the same at pH 6.3 and pH 7.0 (Table 2). At pH 6.3 and 7.0, the yield of ethanol on a weight basis is 0.12 and 0.08 g/g, respectively (Table 1). This yield of ethanol is clearly inferior to that observed with recombinant strains, but the appearance of ethanol supports the proposed pathway for pyruvate metabolism with GlcUA as carbon source.

In the nontransformed culture, if the pyruvate derived from GlcUA were to be converted to ethanol, acetic acid and lactic acid by *pfl* and *ldh*, one could predict the following relationship:

$$\underset{\text{GlcUA}}{C_6H_{10}O_7} \text{ ---------> } \underset{\text{Pyruvic acid}}{2(C_3H_4O_3)} + H_2O$$

$$\text{---------> } \underset{\text{Lactic Acid}}{0.5\,(C_3H_6O_3)} + 1.5\,\text{AcetylCoA} + 1.5\,CH_2O_2$$

$$\text{---------> } \underset{\text{Lactic acid}}{0.5\,(C_3H_6O_3)} + \underset{\text{Acetic acid}}{C_2H_4O_2} + \underset{\text{EtOH}}{0.5\,(C_2H_6O)} + 1.5\,CO_2$$

$$+ 1.5\,H_2$$

However, this relationship produces an extra 2H and does not balance. If the pyruvate derived from GlcUA were to be converted to ethanol, acetic acid and lactic acid by *ldh* and derepressed *pdh*, the fermentation balances as:

$$\underset{\text{GlcUA}}{C_6H_{10}O_7} \rightarrow \underset{\text{Pyruvic acid}}{2(C_3H_4O_3)} + H_2O$$

$$\rightarrow \underset{\text{Lactic acid}}{0.5\,(C_3H_6O_3)} + 1.5\,\text{AcetylCoA} +$$

$$1.5\,CO_2 + 1.5\,(NADH + H^+)$$

$$\rightarrow \underset{\text{Lactic acid}}{0.5\,(C_3H_6O_3)} + \underset{\text{Acetic acid}}{C_2H_4O_2} + \underset{\text{EtOH}}{0.5\,(C_2H_6O)} +$$

$$1.5\,CO_2 + 1.5\,NAD^+$$

This approximates closely the molar ratio of ethanol, acetic acid, and lactic acid that was observed for GlcUA fermentation by ATCC 11303 at pH 6.3 (Table 2). At pH 7.0, there is a shift in favor of acetic acid with the amounts both ethanol and lactic acid decreasing proportionately (Table 2).

To further support our hypothesis concerning a possible role for derepressed *pdh* in GlcUA metabolism, the literature was searched for reports concerning Glc fermentation by *E. coli*. A review of the literature revealed that the end-product distribution in anaerobic mixed acid fermentation by *E. coli* is strain-dependent. Too often generalizations concerning microbial metabolism are made based on the observations with only a single strain or with only a single substrate. Redox balance requires that the molar amount of acetic acid be equal to the sum of ethanol and succinic acid. Beläich and Beläich *(69)* observed that *E. coli* K-12 growing anaerobically in minimal medium at pH 7 produced no lactic acid and equimolar amounts of acetic acid + ethanol and succinic acid with a mass ethanol yield of 0.22 g/g. Varma and Palsson *(70)* observed a similar end-product distribution associated with anaerobic glucose dissimilation by *E. coli* strain W3110 (ATCC 27325) where the ethanol yield was 0.17 g/g. This pattern of end-product distribution is totally consistent with the catabolism of pyruvic acid by *pfl*. However, there are also reports in the literature where the amount of acetic acid produced during anaerobic glucose dissimilation by *E. coli* was not equal to the sum of the amounts of ethanol + succinic acid *(71,72)*. For example, Diaz-Ricci et al. *(72)* have studied the ethanologenicity of *pet* plasmid transformed *E. coli* strain HB101. Strain HB101 is a hybrid of *E. coli* K-12 and *E. coli* B *(73)* and the molar end-product distribution exhibited by the nontransformed culture is:

$$\text{Glucose} \rightarrow 0.86\ \text{EtOH} + 0.11\ \text{Succ} + 0.75\ \text{Acetic acid}$$
$$+0.01\ \text{Lactic acid} + 1.25\ \text{Formic acid}$$

The ethanol yield from glucose exhibited by the nontransformed culture was 0.22 g/g *(72)*. The labile nature of formic acid compromises interpretation based solely on the amount produced; however, pyruvate catabolism by *pfl* would yield an amount of formic acid equal to the sum of ethanol and acetic acid and clearly this is not the case in the work reported by Diaz-Ricci et al. *(72)*. Furthermore, the amount of ethanol + succinic acid is greater than the amount of acetic acid which suggests an additional source of reducing equivalents that would be required to form ethanol from acetylCoA by the combination of acetaldehyde dehydrogenase and alcohol dehydrogenase. It seems reasonable to assume that in this *E. coli* B hybrid strain *pdh* and *pfl* compete for pyruvic acid and that the extra NADH generated by *pdh* activity permits more ethanol to be synthesized. Collectively, these observations in the literature offer indirect support for our conclusion that *pdh* plays a role in GlcUA metabolism in recombinant *E. coli* B.

CONCLUSIONS

From this investigation it is concluded that GlcUA can be fermented by *E. coli* B ATCC 11303 and recombinants 11303:pLOI297 and KO11. Both recombinants produced ethanol from GlcUA in an amount and manner that

was similar to the "novel fermentation" reported by Grohmann et al. (*54,56*) for GalUA with strain KO11. Acetic acid is a significant by-product of GlcUA fermentation by both recombinant cultures. The rate of fermentation is improved by increasing the pH-control set point from 6.3 to 7.0. The production of an unidentified metabolic end-product by the plasmid-bearing recombinant points to a different metabolic pathway operating in this metabolically engineered construct. Finally, our analysis of end-product distribution for metabolism of GlcUA by the non-transformed, wild-type *E. coli* B and recombinant strain KO11 suggests that pyruvate formate lyase is not solely responsible for the production of acetylCoA from pyruvate and that derepressed pyruvate dehydrogenase plays a role in the metabolism of GlcUA in these cultures.

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