0273-2289/96/5758—0277$9.00

The Relationship Between Growth Enhancement and *pet* Expression in *Escherichia coli*

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ABSTRACT

The *pet* operon consists of genes coding for enzymes responsible for ethanol production and consists of pyruvate dehydrogenase and alcohol dehydrogenase II from the high-performance ethanologen *Zymomonas mobilis*. This article describes the physiological influence of *pet* expression in *Escherichia coli* B (ATCC 11303) in terms of growth rate and overall concentrations of cell mass and catabolic end products achieved under well-defined cultivation conditions that included constant pH and carbon (energy) limitation. Glucose, mannose, and xylose were used as substrates, because they represent the principal fermentable components of lignocellulosic biomass and because fermentation of these sugars involves different metabolic pathways. Two different types of ethanologenic recombinants were used—a strain in which *pet* expression was via a multicopy plasmid (pLOI297) and a chromosomal integrant, strain KO11. Under the condition of sugar substrate limitation, there was no growth enhancement by *pet* expression with either glucose or mannose. Whereas the host strain produced exclusively lactic acid from glucose and mannose, both recombinants produced mostly ethanol. Both the plasmid-carrying strain and the *pet* integrant exhibited slower growth compared to the host culture with glucose or mannose as fermentation substrate. With mannose, the plasmid recombinant grew appreciably slower than either the host culture or the recombinant KO11. Use of a magnesium-deficient medium produced different results with glucose since substrate and turbidometric measurements proved to be unreliable in terms of estimating overall biomass levels. At pH 6.3, *pet* expression improved overall biomass yield; but at pH 7.0, the cell yields exhibited by the plasmid recombinant and the host strain were the same. *E. coli* B did not grow well on xylose as sole carbon source. With xylose, *pet* expression increased the growth rate, but had no effect on the overall biomass yield. In comparing our observations with the reports of others, it was concluded that the effect of *pet* expression on growth of *E. coli* is dependent on several different biochemical, physiological, genetic, and environmental factors, which largely precludes a statement of generality regarding this phenomenon.

Index Entries: ATP yield; xylose; recombinant *E. coli*; ethanol; bioenergetics; defined medium mannose; growth yield; *pet* plasmid; *E. coli* KO11.

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INTRODUCTION

Recombinant DNA technology provides interesting possibilities for analyzing and studying in vivo behavior of complex systems through the introduction of well-defined perturbations. The essence of metabolic engineering is the introduction of a change in metabolic flux at a metabolic junction (branch point in a pathway). It is important that we understand why and how flux distribution is accomplished in nonlinear metabolic systems in order to predict the total physiological response to metabolic perturbations introduced by genetic manipulations.

The facultative gram-negative bacterium *Escherichia coli* is capable of utilizing a broad spectrum of sugar substrates and is generally regarded as being heterofermentative, producing a mixture of metabolic end products *(1–3)*. In addition to strain specificity, certain physicochemical environmental factors are known to result in various degrees of end-product selectivity, but the nature of cellular control strategies that determine selectivity by regulating flow through competing pathways is poorly understood.Various excreted metabolites can be differentiated by the energy gain (G_{ATP}), and it has been proposed that selectivity is determined by energy demand *(4,5)*. For example, acetate production generates extra energy via acetate kinase, and a correlation has been suggested between acetate production and high growth rates *(5)*. Although knowledge of mechanisms by which cells generate and conserve energy has advanced *(6)*, an understanding of how energy fluxes are regulated in relation to the needs of cell synthesis is largely lacking.

Ingram's recombinant *E. coli (7–11)* represents a model with which to examine the physiological influence of a particular metabolic perturbation designed to linearize terminal carbohydrate metabolism and maximize ethanol yield. Anaerobic metabolism is branched at the level of pyruvate through competition for substrate by several different enzymes, including pyruvate dehydrogenase, pyruvate formate lyase, and lactic acid dehydrogenase *(2)*. Pyruvate decarboxylase activity (coded for by the *pdc* gene) is absent from *E. coli (12)*. Overexpression of *Zymomonas* pyruvate decarboxylase and alcohol dehydrogenase II (collectively called the *pet* operon) *(9)* results in a redirection of metabolism from mixed-acid fermentation to the production of ethanol *(9)*. It is postulated that the high affinity of pyruvate decarboxylase for pyruvate permits this enzyme to compete successfully against the other pyruvate catabolic enzymes, principally lactic acid dehydrogenase (pyruvate reductase *[9]*); however, it is known that a high level of *pdc* expression is critical to ensuring high ethanol selectivity *(9–11)*. Furthermore, other potentially competitive enzymes are regulated both by effector modulation and at the level of transcription through induction or repression *(1)*. For example, respiratory NADH oxidase is induced under aerobic conditions and is a potential competitor for the NADH produced by pyruvate decarboxylase *(13)*. Similarly, the amount and the activity of pyruvate dehydrogenase is subject to regulation *(14,15)*. Hence, flux control at the level of the pyruvate "metabolic intersection" is a complicated matter *(13)* and one that is not entirely understood.

During the formative stages of research relating to the creation of the *pet* operon, it was observed that recombinants exceeded the growth of the host on solidified complex medium containing fermentable sugar *(7)*, and enlarged colony morphology was a useful distinguishing phenoptypic characteristic of *pet* transformants. Recombinants also were observed to grow to higher cell densities generally in unbuffered broth media *(7)*, and this was interpreted in terms of the

beneficial effect of eliminating the synthesis of the potentially toxic acidic end products and modifying pH during fermentation whereby the pH did not fall as rapidly to a level intolerable for growth. Collectively, these observations led to the concept that *pet* expression has a positive influence on growth yield *(9–11)*. Diaz-Ricci et al. *(16,17)* used well-defined cultivation conditions to explore further the influence of the expression of the *pet* operon on *E. coli*. Using different host cultures (K12 and HB101) from that used by Ingram, batch fermentations were conducted under glucose-limiting conditions, at constant pH. Diaz-Ricci et al. *(16)* confirmed that the growth yield of *pet*-expressing recombinants was improved over that of the wild-type culture. Furthermore, in an investigation of the effect of *pet* expression on changes in the concentration of various intracellular metabolites in *E. coli* using phosphorus-31 nuclear magnetic resonance (NMR), it was concluded that growth enhancement could not be attributable to effects on external pH or ΔpH, but rather to the effect of the switch in pathways from mixed acid to ethanol fermentation *(17)*.

The purpose of this investigation was to examine the physiological influence of the expression of the *pet* operon on the growth of wild-type *E. coli* B (ATCC 11303) under defined environmental conditions. The concept of growth enhancement was examined from both a kinetic and stoichiometric perspective in terms of growth rate and growth yield under carbon (energy) limitation. Two different types of recombinants were used—a strain in which *pet* expression was via a multicopy plasmid (pLOI297) *(10)* and a chromosomally integrated construct, strain KO11 *(18)*. Batch fermentations were conducted under anaerobic conditions with both complex and defined media at constant pH. This investigation employed three different sugars—namely, glucose, mannose, and xylose. In addition to representing the major fermentable components of lignocellulosic biomass, these sugars are not metabolized by the same pathway, and this introduced another variable hitherto unexplored with respect to this phenomenon.

MATERIALS AND METHODS

Organisms

The wild-type, host culture *E. coli* B (ATCC 11303) was obtained from The American Type Culture Collection (Rockland, MD). Recombinant *E. coli* B (ATCC 11303 carrying the *pet* plasmid pLOI297) *(10)* and the chromosomally integrated strain KO11 *(18)* 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 Luria broth (LB) medium supplemented with glycerol (20 mL/dL) and sodium citrate (1.5 g/dL). Inocula were prepared as previously described *(19)* using complex or defined media buffered with 100 m*M* phosphate (pH 7.0). Batch fermentations were inoculated by transferring approx 100 mL of the 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 wt cells/L.

Culture Media

The nutrient-rich, complex culture medium LB consists of 5 g Bacto yeast extract (Difco Laboratories, Detroit, MI), 10 g Bacto Tryptone (Difco), and 5 g NaCl/L

of distilled water. In experiments with glucose, a modified formulation of this medium (designated as "mLB") as described by Grohmann et al. *(20,21)* was also used, and it contained reduced amounts of yeast extract (2.5 g/L) and tryptone (5 g/L), but no NaCl. The defined minimal medium was comprised of 2.25 g NH_4Cl, 0.1 g $MgSO_4$, 0.7 mg $FeCl_3 \cdot 6H_2O$, 2.72 g KH_2PO_4, 3.48 g K_2HPO_4, 5.0 g NaCl, 12 mg $CaCl_2 \cdot 2H_2O$, 9.9 mg $MnCl_2 \cdot 4H_2O$, 0.05 mg thiamine, and 0.21 g citric acid/L of distilled water. All media were sterilized by autoclaving. Stock sugar solutions were autoclaved separately and added at the concentration specified. When the plasmid-bearing strain was used, filter-sterilized antibiotics (final concentration of 40 mg/L ampicillin and 10 mg/L tetracycline) 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). Unless specified otherwise, the pH was controlled at 6.3 by the addition of 4*N* KOH.

Analytical Procedures

Growth was measured turbidometrically at 550 nm (1-cm lightpath), and culture dry wt was measured by microfiltration. Following vacuum filtration, the filters (0.45-μ pore size) were washed and dried to constant weight under an infrared heat lamp. Compositional analyses of culture media and cell-free spent broths were determined by HPLC using an HPX-H column (Bio-Rad, Hercules, CA) as described previously *(19)*. The final concentrations of metabolic end products in spent fermentation broths was not corrected for the dilution caused by the addition of titrant during fermentation.

Determination of Growth Yield and Percent Carbon Recovery

The mass-based growth yield coefficient with respect to carbon (energy) source (i.e., the sugar added to the medium) was calculated by dividing the maximum cell density (g dry wt cells/L) by the concentration of sugar consumed. When complex media were used, the cell yield was corrected for the contribution of the nonsugar nutrients to the total cell mass (cor.$Y_{x/s}$). This value was experimentally determined to be on average 0.41 and 0.25 g dry wt cells/L for LB and mLB media, respectively. Carbon balances (expressed as percent carbon recovery) were calculated as described previously *(19)*. The carbon content of the *E. coli* cell mass was assumed constant at 47.6% carbon *(19)*.

RESULTS AND DISCUSSION

Under the conditions used in this investigation, the host culture, *E. coli* B (ATCC 11303), and the *pet*-expressing recombinants exhibit the same pattern of growth in LB. Anaerobic growth in LB is characterized by a final cell density of 0.41g dry wt cells/L achieved in about 4–5 h. With the nutritionally leaner mLB medium, the final cell density was only 0.25 g dry wt cells/L (results not shown). Growth in the defined salts medium was entirely dependent on the addition of sugar, since this was the sole source of assimilable carbon. In this study, the mass-based growth yield coefficient with respect to carbon and energy source was used to assess the

physiological effect of *pet* expression on growth quantitatively. Hence, to eliminate the contribution made to cell mass by the nonsugar components of the complex media, the corresponding amounts of biomass produced in the absence of added sugar were routinely subtracted from the values determined for the maximum cell density. Overall growth yield values are limited in their informative value by virtue of the fact that they reflect the aggregate of all effects produced during the entire time period of batch growth. In addition, there exists the potential for growth interference by different metabolic end products. Therefore, in an attempt to minimize possible inhibitory effects, either by ethanol or organic acid end products, the concentration of sugar employed was relatively low at about 20 g/L. Also at this relatively low sugar concentration, carbon-limited growth can be assured using LB *(19,22)*. Under a condition of growth limitation by the carbon (energy) source, the growth yield for carbon can be viewed in bioenergetic terms as an indirect estimate of the net ATP gain (G_{ATP}, mol ATP/mol sugar) *(6,9,23)*. However, the inability to account quantitatively for changes with respect to nongrowth associated energy metabolism (i.e., the amount of ATP required for homeostasis or "maintenance") in an environment of batch culture, limits the generality of interpretation of growth yield determinations with respect to bioenergetics *(19)*. For example, a condition producing an increase in the maintenance energy requirement could result in a decreased overall growth yield without affecting the G_{ATP}. Such an "uncoupling effect" in *E. coli* is known to be produced by acetic acid *(24,25)* and by the maintenance of high-copy-number plasmid vectors *(16)*.

Effect of *pet* Expression with Glucose

Anaerobic batch fermentations were conducted in stirred-tank bioreactors in LB with the pH-controlled at 6.3. Figure 1 shows that under a condition of glucose limitation, the growth rate of the wild-type culture *E. coli* B (ATCC 11303) was faster than that of either the plasmid-carrying strain (11303:pLOI297) or the chromosomally integrated strain KO11. Because the final cell density is, in part, a function of the limiting nutrient and because the sugar concentration was not exactly invariable for different batches, the growth yield provides a means of normalizing the effect of differences in sugar concentration. Figure 1B shows that with LB, the growth yield with respect to glucose (cor.$Y_{x/s}$) for both the wild-type culture and the plasmid recombinant is similar (0.093 and 0.091 g dry wt cells/g glu, respectively) whereas for strain KO11, it is reduced by about 10% (0.083 g dry wt cells/L). A similar observation with respect to the lack of growth enhancement by *pet* expression was made when the nutritionally rich LB medium was replaced with a chemically lean, defined medium (Fig 1B). Table 1 shows stoichiometric data with respect to the distribution of metabolic end products associated with these batch fermentations. The high ethanol selectivity for both recombinant strains shows that *pet* expression clearly functions in the manner consistent with the purpose for which it was designed. Metabolism is redirected by the *Zymomonas* pyruvate decarboxylase and alcohol dehydrogenase from the production of lactic acid as the major end product in the host to ethanol in the recombinants (Table 1). However, our observations with respect to the lack of growth enhancement are in conflict with those of Diaz-Ricci et al. *(16)* who reported that, under similar conditions of glucose limitation in LB medium with pH controlled at 7.0, whereas expression of the *pet* plasmid pLOI295 in *E. coli* HB101 had no effect on growth rate, there was a substantial increase in growth yield, from 0.10 to 0.16g dry wt. cells/L. *E. coli* HB101 is a hybrid of strains

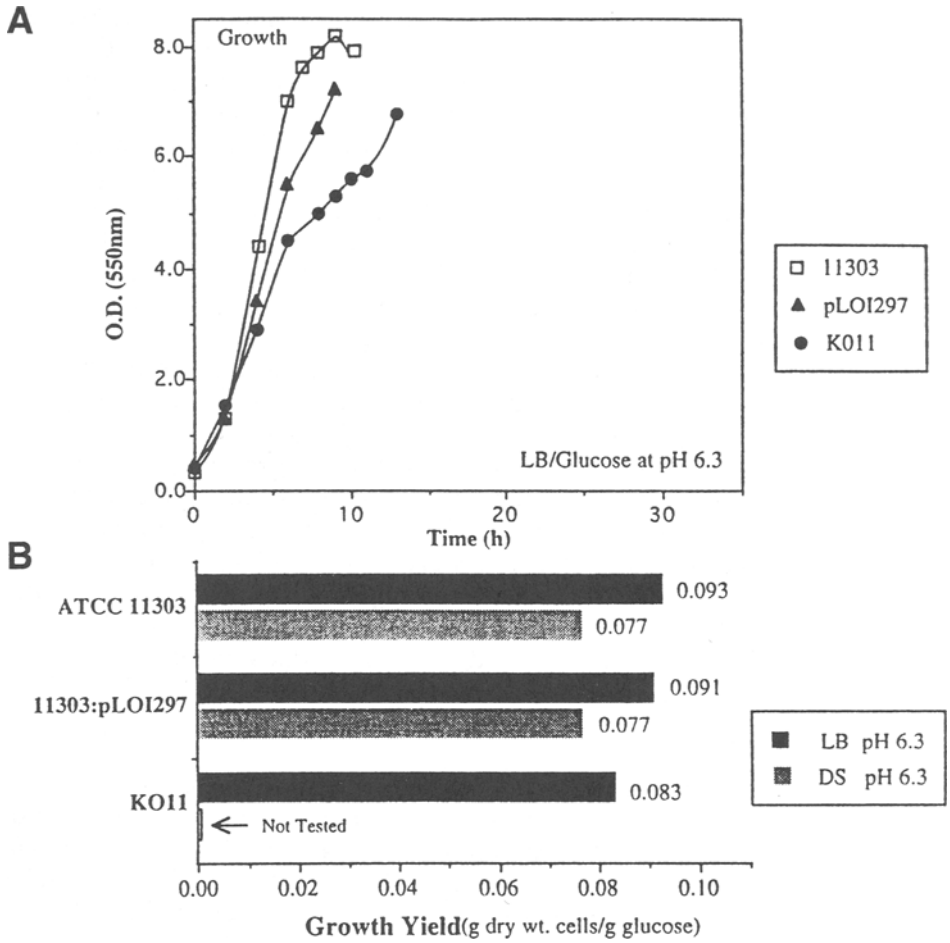

Fig. 1. The effect of *pet* expression on growth of *E. coli* recombinants 11303:pLOI297 and KO11 with glucose as carbon (energy) source. **(A)** Effect on growth rate and **(B)** effect on growth yield coefficient for carbon. The host culture *E. coli* B (ATCC 11303) is included as a control. In the case of the complex media LB, the final biomass concentration was corrected for contribution to growth from nonsugar components of the medium (*see* Materials and Methods).

K12 and Luria strain B *(26)*. In the fermentations reported by Diaz-Ricci et al. *(16)*, *pet* expression affected the end-product distribution, causing a decrease in acetate concentration from 83 to 20 m*M*. Acetic acid is a known byproduct of glucose fermentation by *E. coli*, but the yield of this acid appears to be dependent on both the particular strain and environmental conditions employed *(1–3)*. Furthermore, with *E. coli*, sensitivity to acetic acid toxicity has been shown to be a strain-dependent phenomenon *(27)*. At pH 7.0, 83 m*M* acetate are equivalent to 0.46 m*M* undissociated acid, and whereas this concentration has been shown to have little effect on the growth yield in *E. coli* B *(25)*, it has a pronounced effect of *E. coli* K12 *(28,29)*. In the context of considering the possible effect of acetic acid, it is interesting to note that in another study conducted under similar conditions, expression of the *pet*

Table 1
The Effect of *pet* Expression on Anaerobic Glucose Catabolism
by *E. coli* B (ATCC 11303) and Recombinants 11303:pLOI297 and KO11

Genotype medium	Expt.	pH	Glucose, m*M*	Biomass, g/L	EtOH, m*M*	Lactic, m*M*	Acetic, m*M*	Succ., m*M*	Formic, m*M*	% Carbon recovery
ATCC 11303										
LB	138c	6.3	122	2.46	0	226	0	0	0	106
mLB	128b	6.3	118	1.36	0	183	0	0	0	85
mLB	128d	7.0	117	1.80	20	181	0	0	0	88
DS	112b	6.3	100	1.36	40	125	0	13	0	89
11303:pLOI297										
LB	137a	6.3	116	2.32	224	6	0	8	0	117
mLB	123c	6.3	105	1.78	208	0	0	4	0	113
mLB	139a	7.0	107	1.82	178	22	0	7	0	110
DS	112	6.3	95	1.31	172	0	0	0	0	91
KO11										
LB	139c	6.3	112	2.08	210	11	14	0	11	121
mLB	123e	6.3	104	1.60	203	0	11	0	0	113
mLB	140d	7.0	126	1.54	216	21	35	0	0	113
DS	Not tested									

plasmid pLOI308-10 in a double-deletion mutant of *E. coli* K12 lacking both acetyl phosphotransferase and acetate kinase activity, and thereby genetically blocked in its ability to synthesize acetic acid, did not alter either the anaerobic growth rate or the growth yield with respect to glucose *(30)*. Fortunately, the lack of acetic acid production in our fermentations (Table 1) removed this complication in interpreting the results.

In nutritional terms, LB is an excessively rich medium, and much leaner complex media have been shown to be compatible with good fermentation performance by ethanologenic recombinant *E. coli (31,32)*. One such formulation, recommended by Grohmann et al. *(20,21)*, was used in this study (designated as "mLB"). However, the mLB medium is known to be deficient in both phosphate and magnesium *(31)*, and whereas the phosphate deficiency is compensated by the volumetric transfer from the phosphate-buffered preculture during inoculation, the concentration of magnesium (estimated at 2 mg/L) remains below the minimal amount required to support a final cell concentration of about 2 g dry wt cells/L. Hence, because growth in mLB medium was not glucose limited, the glucose growth yield coefficient was not an appropriate comparative parameter. Since others have used a variety of environmental conditions, including the lack of pH control and growth that was not limited by the carbon (energy) source, we considered our observations with mLB pertinent for the purpose of comparison. Figure 2 shows the growth of the host culture and the two recombinants in mLB at a nominal glucose concentration of 20 g/L. With the pH controlled at 6.3, turbidometric data indicate an enhancement effect for *pet* expression in the plasmid-bearing culture, but not for the chromosomal integrant (Fig. 2A). However, direct determination of final biomass concentrations revealed that both ethanologenic recombinant cultures surpassed the lactic acid-producing host strain (Table 1). Lactic acid toxicity is pH-dependent, and by changing the pH control set point from 6.3 to 7.0, the final cell density of the host culture was significantly improved (Fig. 2B and Table 1). Furthermore, in a separate experiment with an elevated glucose concentration (80 g/L), but where the mLB medium was replaced with a magnesium and phosphate-fortified LB medium, at pH 6.3, the host culture achieved a final cell density of 3 g dry wt cells/L and a final lactate concentration in excess of 600 m*M* lactic acid (results not shown). Since mLB is magnesium-deficient, we are led to conclude that magnesium may play a role in preventing the negative effect of lactic acid on cell yield. With the pH controlled at 7.0, the optical density (OD) of the host culture surpasses both recombinants (Fig. 2B). However, the final cell densities of the host culture and plasmid-bearing recombinant are similar at 1.8 and 1.82 g dry wt cells/L, respectively (Table 1). Clearly, the ratio of dry cell mass to OD is not the same for the ethanol and acid-producing cultures, and this difference may reside in the light-scattering properties of an ethanologenic culture grown in a magnesium-deficient medium.

In addition to ethanol, recombinant strain KO11 also synthesizes acetic acid (Table 1). Although the growth-inhibiting effects are reduced at the higher pH, the amount produced by strain KO11 at pH 7.0 is equivalent to 1 m*M* undissociated acid, which has been shown to affect the growth of *E. coli* B *(25)*. Collectively, our observations suggest that, under glucose limitation, *pet* expression in *E. coli* B (ATCC 11303) does not enhance growth either with respect to rate or yield. However, in comparing our observations to those of others, it would appear that the effect is dependent on several biochemical, physiological, genetic, and environmental factors, which largely precludes a statement of generality regarding this phenomenon.

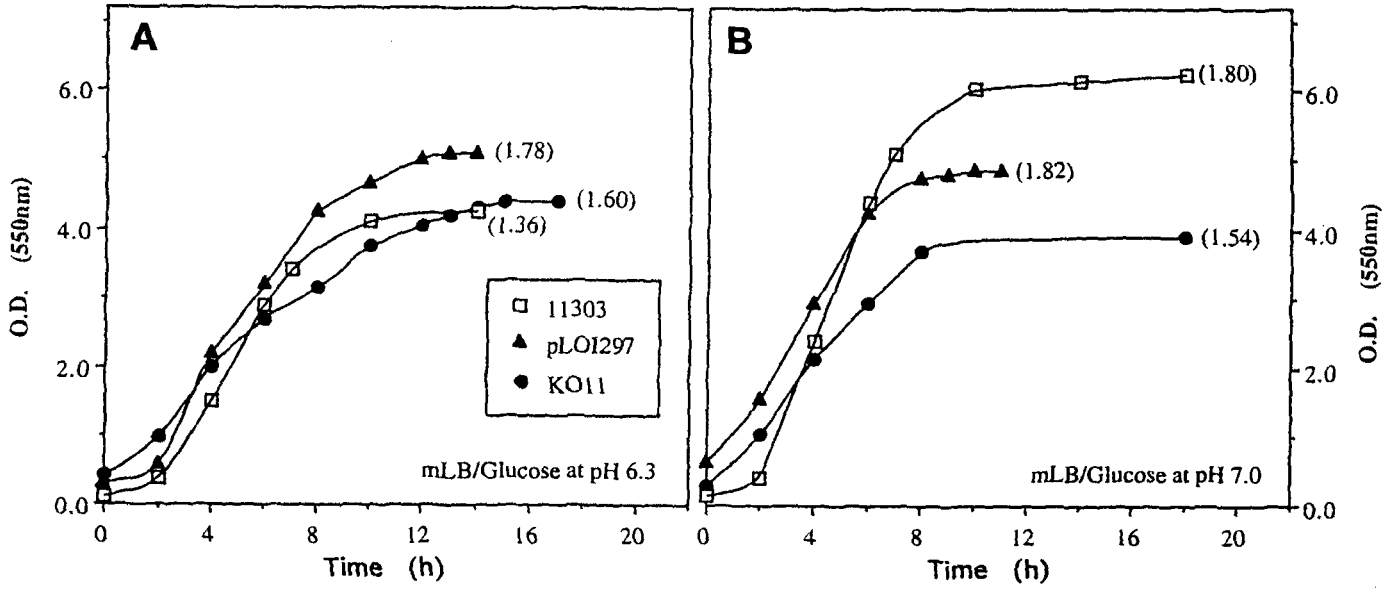

Fig. 2. Anaerobic growth of wild-type *E. coli* B (ATCC 11303) and *pet* recombinants 11303:pLOI297 and KO11 in modified LB medium with glucose at 20 g/L. **(A)** pH controlled at 6.3 and **(B)** pH controlled at 7.0. Maximum biomass and final concentrations of metabolic end products are given in Table 1.

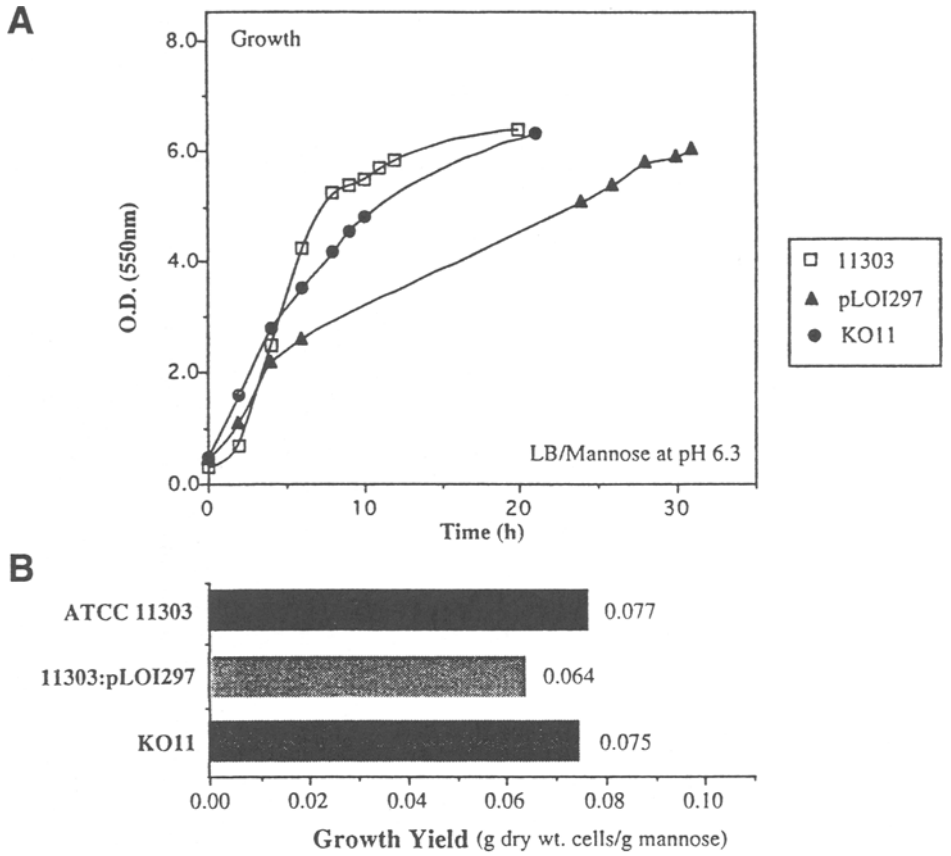

Fig. 3. The effect of *pet* expression on growth of *E. coli* recombinants 11303:pLOI297 and KO11 with mannose as carbon (energy) source. **(A)** Effect on growth rate and **(B)** effect on growth yield coefficient for carbon. The host culture *E. coli* B (ATCC 11303) is included as a control.

Effect of *pet* Expression with Mannose

D-Mannose is an aldohexose and differs from D-glucose only with respect to the configuration of the hydroxyl group at carbon 2. Mannose is a prominent component of the hemicellulose of certain lignocellulosic biomass, for example, softwood *(33)*. Previous studies have examined the physiology of the plasmid-bearing recombinant 11303:pLOI297 with mannose as carbon source *(34)*, and the fermenation performance of both 11303:pLOI297 and KO11 in softwood prehydrolysates has been investigated *(35a)*.

Under a condition of carbon limitation at pH 6.3, the wild-type culture exhibits a slightly reduced growth rate and a marked growth yield relative to glucose as carbon source (Figs. 1 and 3); however, lactic acid is the sole end product of both glucose and mannose catabolism (Tables 1 and 2). The apparent reduction in growth rate may be owing to the short lag that was experienced by the host culture on introduction into the mannose LB medium. The relatively poor growth performance of the plasmid recombinant has been observed previously with mannose as principal carbon source *(34,36)* but no explanation for the reduced growth has

Table 2
The Effect of *pet* Expression on Anaerobic Mannose Catabolism
by *E. coli* B (ATCC 11303) and Recombinants 11303:pLOI297 and KO11

Genotype medium	Expt.	pH	Mannose, m*M*	Biomass, g/L	EtOH, m*M*	Lactic, m*M*	Acetic, m*M*	Succ., m*M*	Formic, m*M*	% Carbon recovery
ATCC 11303										
Lo	134b	6.3	124	2.12	0	240	0	0	0	109
11303:pLOI297										
LB	052b	6.3	118	1.78	199	5	0	8	0	103
KO11										
LB	137d	6.3	118	2.01	224	4	14	3	1	116

been forthcoming. Figure 3B shows that the growth yield with respect to mannose is similar for both the host culture at strain KO11 being 0.077 and 0.075 g dry wt cells/L, respectively. The growth yield for the plasmid recombinant is 0.064 g dry wt cells/L (Fig. 3B). Measurements of growth yield have previously been used to infer differences in energetics associated with glucose and xylose metabolism by the plasmid-bearing recombinant *(19)*. A similar approach can be used in analyzing the differences in growth yield elicited by glucose and mannose. Assuming that the growth yield with respect to energy (ATP) is constant when either glucose or mannose serves as the energy source for growth, then the reduction in growth yield exhibited under the condition of mannose-limitation suggests that the net gain in ATP is less for mannose than glucose. Carrying a high-copy-number plasmid places an energetic burden on the cell *(16)* and the associated increase in maintenance energy coefficient would reduce the net energy yield, but the issue of why the magnitude of energy required for plasmid maintenance might be different for glucose and mannose remains problematical. In the context of this study, it is important to note that *pet* expression did not enhance growth when mannose was the limiting carbon and energy source (Fig. 3).

Effect of *pet* Expression with Xylose

Because the recombinant cultures under investigation were specifically designed to convert xylose to ethanol efficiently, the majority of previous work with these cultures has involved xylose either as the sole sugar supplement *(35b–37)* or as a component of either synthetic mixtures *(38)* or biomass prehydrolysates *(35a,39)*. Xylose is a poor growth substrate for *E. coli (12)*. Under a condition of carbon limitation at pH 6.3, *pet* expression results in an improvement in both the growth rate (Fig. 4A) and growth yield (Fig. 4B) of *E. coli* B (ATCC 11303) in LB with xylose as the carbon source. With glucose or mannose as fermentation substrates, *pet* expression alters the homofermentative pattern in *E. coli* B from lactic acid to ethanol production. However, with xylose as substrate, *E. coli* B is heterofermentative. In addition to lactic, acetic, and succinic acids, ethanol is synthesized (Table 3). The xylose-based ethanol yield is 0.22 g/g and this observation has been reported previously *(37)*. Hence, with xylose as substrate, *pet* expression improves the xylose-to-ethanol conversion efficiency. The ethanol yield for the plasmid recombinant and KO11 is 0.45 and 0.47 g/g, respectively (Table 3). At pH 6.3, the concentration of the undissociated (inhibitory) form of acetic acid is 1.86 and 0.76 m*M* for the host culture and KO11, respectively (Table 3). The difference in growth rate between these cultures can be explained in terms of the amounts of acetic acid produced (Fig. 4A). The absence of acetic acid production by the plasmid recombinant gives this culture a distinct growth advantage (Fig. 4A). The xylose-based growth yield coefficient in the host culture was 0.055 g dry wt cells/g xylose (Fig. 4B). In the case of the plasmid recombinant, the growth yield of 0.054 g dry wt cells/g xylose has been previously reported *(19)*. The growth yield varies only slightly for the three cultures (Fig. 4B), and under the conditions of assay, this parameter is considered to be unaffected by *pet* expression.

The Concept of Ethanologenic *E. coli* as a Superior Host for Heterologous Expression

Largely because of the vast amount of research that has been done on *E. coli*, especially in terms of molecular biology and genetics, it has been a popular selec-

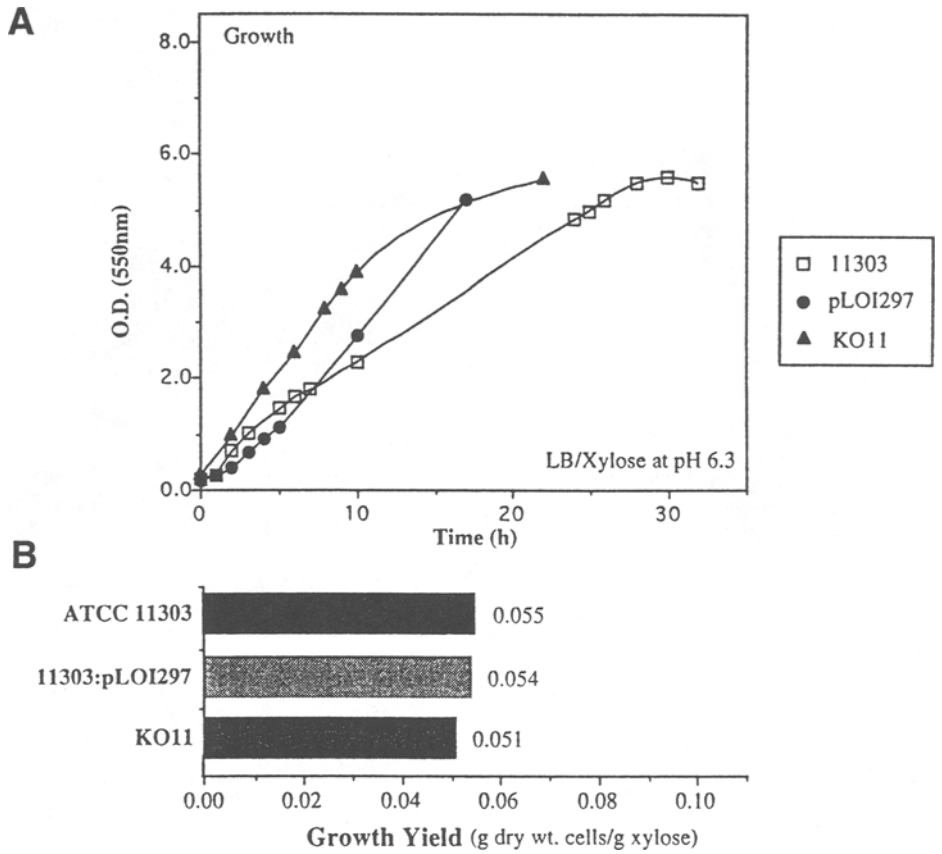

Fig. 4. The effect of *pet* expression on growth of *E. coli* recombinants 11303:pLOI297 and KO11 with xylose as carbon (energy) source. **(A)** Effect on growth rate and **(B)** effect on growth yield coefficient for carbon. The host culture *E. coli* B (ATCC 11303) is included as a control.

tion as host for the expression of heterologous proteins from a variety of origins. To achieve maximum cell yield, recombinants are usually grown under aerobic conditions. In general, aerobic growth of plasmid-bearing cultures is often depressed compared to the host culture, and this has led to studies on optimization of growth of recombinant *E. coli (40–43)*. The excretion of acetic acid (HAc) is cited as the causative agent responsible for both depressed growth yield and protein biosynthesis *(44,45)*. Recombinant cultures are reported to be more HAc-sensitive than wild-type cultures *(46)*.

Based on the claim that *pet* expression in *E. coli* results in growth enhancement, it has been suggested that ethanologenic *E. coli* would be a superior host for the expression of foreign proteins *(9,18)*. However, there are seveal considerations that detract from the general utility of ethanologenic constructs in the biotechnology industry. The inherent instability of plasmid-carrying constructs is a serious consideration *(18,47)*, and the energetic burden imposed by a high-copy-number *pet* plasmid *(16)*, such as pLOI297, not only lessens the potential growth (protein) yield through an increase in energy consumption that is unrelated to growth or

Table 3
The Effect of *pet* Expression on Anaerobic Xylose Catabolism
by *E. coli* B (ATCC 11303) and Recombinants 11303:pLOI297 and KO11

Genotype medium	Expt.	pH	Xylose, m*M*	Biomass, g/L	EtOH, m*M*	Lactic, m*M*	Acetic, m*M*	Succ., m*M*	Formic, m*M*	% Carbon recovery
ATCC 11303										
LB	011c	6.3	137	1.53	98	38	67	25	28	106
11303:pLOI297										
LB	030b	6.3	141	1.54	206	0	0	12	0	108
KO11										
LB	136d	6.3	152	1.58	219	3	26	0	18	109

protein biosynthesis, but also increases the sensitivity to HAc toxicity by reducing the amount of energy available for intracellular pH homeostasis. Under aerobic conditions, expression of the *pet* operon is purported to divert >90% of carbon to ethanol production *(9)*. Successful competition by the *Zymomonas* ethanol production enzymes with the respiratory NADH oxidase for NADH and pyruvate dehydrogenase for pyruvate would result in an energetic debilitation, since the G_{ATP} associated with fermentative metabolism is far less than for aerobic catabolism. As a consequence, it could be anticipated that ethanologenic recombinant *E. coli* would be at an energetic disadvantage under aerobic conditions *(48)*. However, it has been shown that the ethanologenicity of recombinant 11303:pLOI297 is greatly reduced under aerobic conditions *(37,48)*, and this observation challenges the claim regarding the ability of the *pet* enzymes to control and monopolize pyruvate catabolism under aerobic conditions. To overcome the limitations imposed by the potential for genetic instability in plasmid-based *pet* constructs, a number of different chromosomal integrants of *E. coli* B (ATCC 11303) have been developed *(18)*. Furthermore, one of the integrants (strain KO12) has a recA mutation, thereby making it a more suitable host for the production of recombinant proteins *(18)*. It has been suggested that "additional genes can be inserted into plasmids to direct the synthesis of recombinant proteins as valuable coproducts with no expected interference in ethanol productivity" *(18)*. This suggestion has not yet been tested, but if proven tenable, the concept of recombinant protein biosynthesis as a byproduct of fuel ethanol production has interesting economic ramifications. From the perspective of the fuel ethanol industry, it is not known what effect the imposed energy demand of protein production would have on ethanol yield and productivity. Strains KO11 and KO12 were selected on the basis of their resistance to high levels of chloramphenicol (600 μg/mL), and the ultimate utility of these ethanologenic *pet* integrants as hosts for heterologous protein expression is predicated on claims relating to their genetic stability *(47)*. However, at this symposium, we reported the results of our investigation into the genetic stability of Ingram's ethanologenic *E. coli* recombinants, and our observations with continuous culture shed new light on the purported stability of both the plasmid construct 11303:pLOI297 and the chromosomal integrant, strain KO11 *(18)*. There appears to be sufficient evidence to discount the suggested utility of these ethanologenic recombinants as candidate host strains for use by the biotechnology industry.

ACKNOWLEDGMENTS

This research was supported by the Natural Sciences and Engineering Research Council of Canada and The University of Toronto. We are greatful to Lonnie Ingram for the gift of the recombinant *E. coli* cultures.

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