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Expression of Different Levels of Ethanologenic Enzymes from *Zymomonas mobilis* in Recombinant Strains of *Escherichia coli*†

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The expression of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II in *Escherichia coli* converted this organism from the production of organic acids to the production of ethanol. Ethanol was produced during both anaerobic and aerobic growth. The extent to which these ethanologenic enzymes were expressed correlated with the extent of ethanol production. The replacement of organic acids with ethanol as a metabolic product during aerobic and anaerobic growth resulted in dramatic increases in final cell density, indicating that these acids (and the associated decline in pH) are more damaging than the production of ethanol. Of the plasmids examined, the best plasmid for growth and ethanol production expressed pyruvate decarboxylase and alcohol dehydrogenase II at levels of 6.5 and 2.5 IU/mg of total cell protein, respectively.

Escherichia coli serves as the primary vehicle for the cloning and modification of genes for biotechnology and as one of the most important hosts for the production of recombinant products. We recently demonstrated that it is possible to redirect the central metabolism of this organism by the introduction of genes encoding the required enzymes for fermentative ethanol production from *Zymomonas mobilis*, pyruvate decarboxylase and alcohol dehydrogenase II, under the control of the enteric *lac* promoter (10). The genes encoding the two ethanologenic enzymes, denoted the *pet* operon, were expressed at high levels and dominated carbon flow from pyruvate and NADH oxidation during anaerobic growth. Under these conditions, the flow of pyruvate carbon skeletons was diverted from the production of organic acids to the production of ethanol as the principal fermentation product in *E. coli*.

The ability to change the central metabolism of *E. coli* has several interesting consequences. It demonstrates the potential to develop recombinants of *E. coli* for commercial ethanol production and illustrates the feasibility of drastic changes in metabolic flow for the future development of novel products from *E. coli*. In addition, strains containing the *pet* operon grew to higher cell densities than did the parent organism under anaerobic conditions with glucose (10) and offer the potential for the increased production of recombinant products in *E. coli* while reducing complications associated with acid production.

In this study, we constructed a series of plasmids in which the *pet* operon is expressed under the control of different promoters at various levels in *E. coli*. These plasmids were used to examine the effects of different levels of *pet* operon expression on cell growth and the distribution of fermentation products during the growth of *E. coli* in complex medium containing glucose under both aerobic and anaerobic conditions.

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MATERIALS AND METHODS

Strains and growth conditions. All experiments and strain constructions were conducted with strain TC4 (5) as the host organism. Plasmids pUC18 and pUC19 (22), pLOI204 (4), and pLOI295 (10) have been previously described. The construction and properties of pLOI292, pLOI291, pLOI292, pLOI308, pLOI308-2, pLOI308-5, and pLOI308-10 are described in this paper. Cultures were grown at 37°C in Luria broth (14) containing 50 g of glucose per liter. Cells for enzyme analyses and inocula for fermentation studies were grown in tubes (13 by 100 mm) containing 3 ml of broth at 37°C in a tube rotator. Overnight cultures were diluted 100-fold into fresh medium. Aerobic cultures (50 ml of broth in 250-ml flasks) were shaken in a reciprocating water bath (120 oscillations per min). Anaerobic cultures were grown in stoppered serum bottles (100 ml of broth in 130-ml bottles) with gyratary agitation (150 rpm) in a 37°C incubator. Anaerobic cultures were vented with a 25-gauge needle to allow escape of gaseous fermentation products.

Growth was monitored spectrophotometrically with a Spectronic 70 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) at 550 nm. Disposable culture tubes (10 by 75 mm) were used as cuvettes. One absorbance unit under our conditions contained approximately 0.25 mg of cellular protein per ml.

Genetic techniques. Transformations, plasmid constructions, DNA digestions, and analyses were carried out as previously described (6). Recombinants were selected on solid media (1.5% agar) containing 2 g of glucose per liter and appropriate antibiotics. Recombinants containing functional ethanologenic genes from *Z. mobilis* were identified by their growth as oversized colonies on Luria agar plates containing glucose and were confirmed by their poor growth on Luria agar plates lacking glucose (10) and by the expression of alcohol dehydrogenase on aldehyde indicator medium (6).

Enzyme assays. Cells were disrupted, heat-inactivated, and assayed for pyruvate decarboxylase activity (thermostable) as described previously (5). Cells were prepared and assayed for alcohol dehydrogenase II activity in the direction of ethanol oxidation as described previously (6), except

that cells were washed and disrupted in 30 mM potassium phosphate buffer to which solid ferrous ammonium sulfate (final concentration, 0.5 mM) and sodium ascorbate (10 mM) had been freshly added as described by Neale et al. (16). This modification coupled with the immediate assaying of alcohol dehydrogenase activity without storage resulted in a much higher specific activity than that previously reported (6, 10). Protein was measured with the Folin phenol reagent (13).

Analysis of fermentation products. The concentrations of fermentation products in clarified broth were measured by using a high-pressure liquid chromatograph (Beckman Instruments, Inc., Fullerton, Calif.) equipped with a refractive index monitor and an electronic integrator. Separations were carried out at 55°C on an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, Calif.) at a flow rate of 0.5 ml/min (injection volume, 0.1 ml). Peaks were identified by using authentic standards.

RESULTS

Plasmid constructions. Plasmid pLOI295 contains the *Z. mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II under the control of the *lac* promoter (10). This construction is referred to as the *pet* operon (production of ethanol) in this study and was used as the source of ethanologenic genes for the construction of additional plasmids with alternative promoters (Fig. 1). The *Eco*RI-*Sal*I fragment from pLOI295 containing the ethanologenic genes was treated with the Klenow fragment of DNA polymerase to produce blunt ends. This blunt-ended DNA fragment was inserted into the *Sma*I site of pUC19 with the *pdc* gene immediately downstream from the *lac* promoter. The resulting plasmid, denoted pLOI293, contained the *pet* genes flanked by *Bam*HI sites. Plasmids pLOI291 and pLOI292 (opposite orientations) were constructed by inserting the *Bam*HI fragment containing the genes encoding the ethanologenic enzymes into the expression vector pLOI204 (4). The *Bam*HI fragment includes the ribosome-binding site, complete sequences for both genes, and a stem-loop terminator distal to *adhB*. In pLOI292, the two genes are expressed under the control of the *Z. mobilis* promoter contained in the original expression vector. In pLOI291, the two genes are expressed from a cryptic upstream promoter contained in the original expression vector. Although the nature of this cryptic promoter activity has not been investigated, we have observed it previously in the expression of a promoterless chloramphenicol acyltransferase (unpublished data).

Plasmid pLOI308 was constructed to remove the *pet* genes from the control of the *lac* promoter but to retain the upstream *Bam*HI site for the insertion of alternative promoters. Partial digestion of pLOI293 with *Bam*HI and Klenow treatment were used to remove the *Bam*HI site distal to the *adhB* gene. The ethanologenic genes were removed from this plasmid as a promoterless *Bam*HI (immediately proximal to *pdc*)-*Eco*RI (distal to *adhB*) fragment, which was directionally inserted into the *Bam*HI and *Eco*RI sites of pUC18 to produce pLOI308. This plasmid expressed low levels of *adhB* on aldehyde indicator plates but did not exhibit the large-colony phenotype associated with the other functional *pet* plasmids pLOI295, pLOI291, and pLOI292.

Chromosomal DNA from *Z. mobilis* was partially digested with *Sau*3A such that most of the DNA appeared to be less than 4 kilobases long. This unfractionated DNA was used as a source of promoter fragments and was ligated into the dephosphorylated *Bam*HI site of pLOI308. Ampicillin-resistant recombinants with a well-expressed *pet* operon were identified as large colonies on Luria agar plates containing glucose. Three, pLOI308-2, pLOI308-5, and pLOI308-10, were selected for study. The *Z. mobilis* DNA fragments with promoter activity in these plasmids were 6, 2, and 2 kilobases long, respectively. These DNA fragments were not characterized further.

Expression of *Z. mobilis* ethanologenic enzymes in *E. coli*. Table 1 summarizes the activities of pyruvate decarboxylase and alcohol dehydrogenase in overnight cultures of recombinant *E. coli*. The activities of pyruvate decarboxylase ranged from 0.37 IU/mg of cell protein in strain TC4(pLOI291) to 8.23 IU in TC4(pLOI295). Assuming a maximal specific activity of 100 IU for the pure enzyme (5), active pyruvate decarboxylase can be estimated to represent 0.4 and 8.2%, respectively, of the total cellular protein. Since this enzyme was assayed following heat inactivation to eliminate confounding activities, the total amount of enzyme in the cells would be expected to be somewhat higher. Assuming that there is a 20% inactivation by heat treatment (5) and that the soluble proteins represent 70% of the total cellular protein, pyruvate decarboxylase can be calculated to represent approximately 15% of the soluble cellular protein in strain TC4(pLOI295). In terms of pyruvate decarboxylase activity, the recombinant strains of TC4 can be ordered as follows (highest to lowest): pLOI295 > pLOI308-10 > pLOI308-2 > pLOI308-5 > pLOI292 > pLOI291.

Alcohol dehydrogenase activities in the recombinant strains followed the same trend in terms of expression from different plasmids as did pyruvate decarboxylase. The alcohol dehydrogenase activities measured represent a combination of the native enzyme from *E. coli* (3) and the *Z. mobilis* enzyme. The level observed in strain TC4 lacking a plasmid was relatively small in comparison to those observed in strains carrying plasmids with the *Z. mobilis* gene. The activities of the *Z. mobilis* enzyme (corrected for native *E. coli* alcohol dehydrogenase) ranged from 0.13 IU/mg of cell protein for strain TC4(pLOI291) to 9.6 IU in TC4(pLOI295). Assuming the maximal specific activity of the pure enzyme to be 710 IU (average from references 11 and 16), active *Z. mobilis* alcohol dehydrogenase II can be estimated to represent 0.02 and 1.4%, respectively, of the total cellular protein. Assuming that the envelope protein represents 30% of the total cellular protein, alcohol dehydrogenase II can be

TABLE 1. Expression of ethanologenic enzymes from *Z. mobilis* in *E. coli*

Plasmid	Pyruvate decarboxylase		Alcohol dehydrogenase	
	Sp act[a]	% Cell protein[b]	Sp act[a]	% Cell protein[c]
pLOI291	0.37	0.4	0.21	0.02
pLOI292	0.48	0.5	0.30	0.03
pLOI308-2	2.26	2.3	1.54	0.21
pLOI308-5	1.11	1.1	0.76	0.10
pLOI308-10	6.5	6.5	2.51	0.34
pLOI295	8.2	8.2	9.65	1.4
None	0	0	0.08	

[a] Expressed as micromoles of substrate utilized per minute per milligram of total cellular protein.
[b] Calculated assuming a specific activity of 100 for the pure enzyme.
[c] Calculated assuming a specific activity of 710 for the pure enzyme after subtraction of native alcohol dehydrogenase activity.

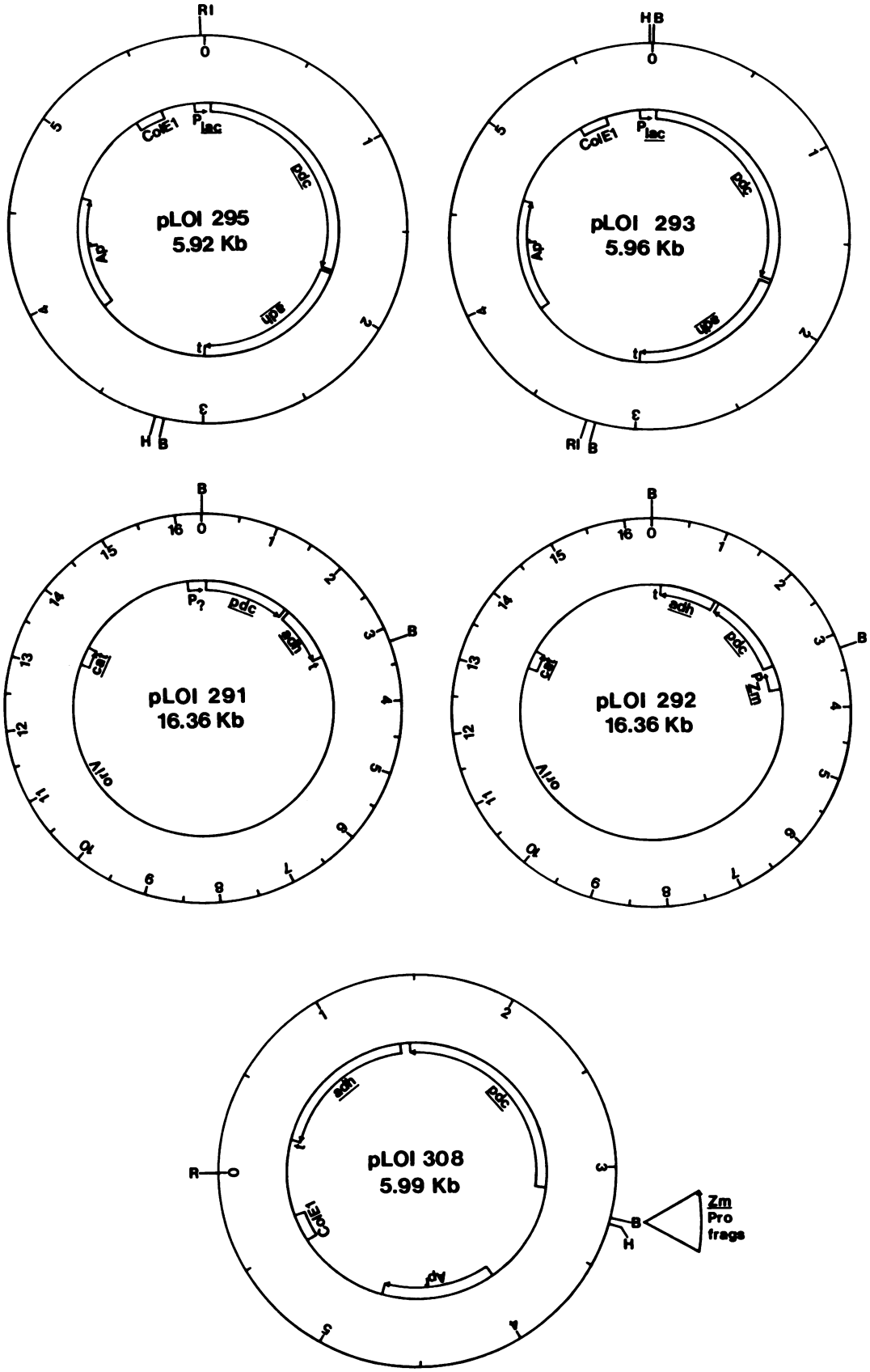

FIG. 1. Plasmids containing the genes encoding pyruvate decarboxylase and alcohol dehydrogenase II from *Z. mobilis*. Fragments of DNA from *Z. mobilis* were inserted into the promoter site of pLOI308 to construct pLOI308-2, pLOI308-5, and pLOI308-10 (not shown). Abbreviations: RI, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; t, terminator; adh, *Z. mobilis* alcohol dehydrogenase II; pdc, *Z. mobilis* pyruvate decarboxylase; cat, chloramphenicol acyltransferase; Ap, β-lactamase; Zm Pro frags, fragments of *Z. mobilis* DNA which exhibit promoter activity; ColE1, replication origin derived from pBR322; oriV, replication origin derived from RSF1010; P_{lac}, *lac* promoter; P_{Zm}, promoter from *Z. mobilis*; $P_?$, cryptic promoter on vector; Kb, kilobases.

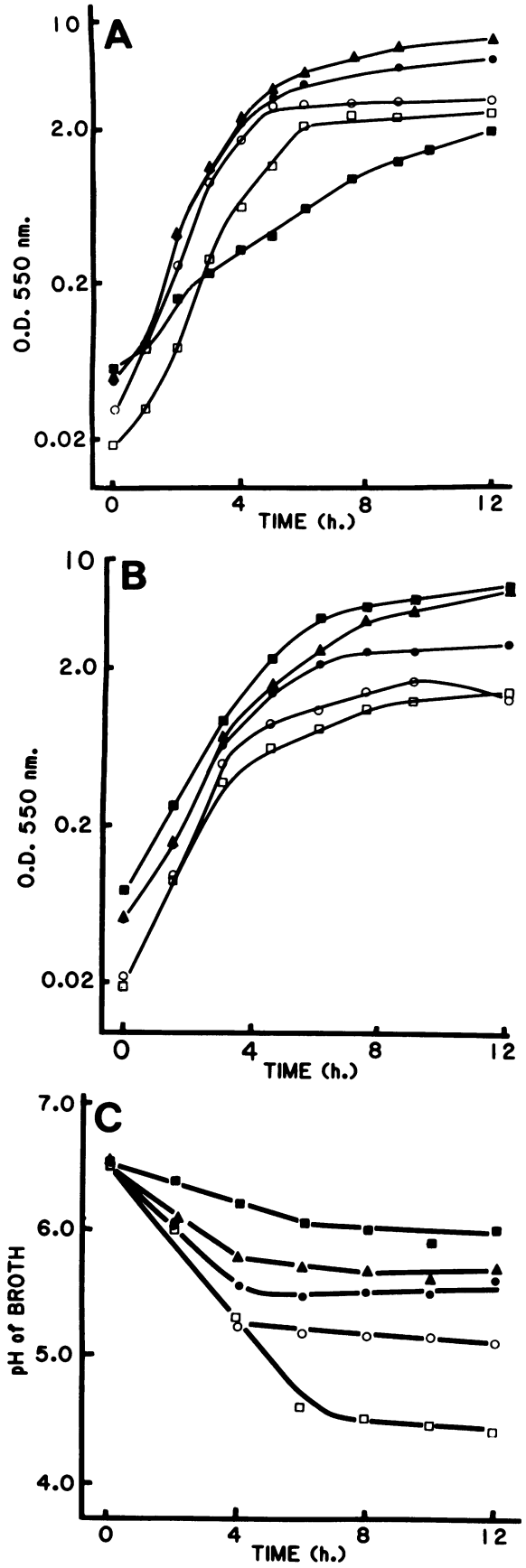

TABLE 2. Maximal generation times, final cell densities, and final pHs of the broth during aerobic and anaerobic growth

Growth condition	Plasmid	Cell density[a] (mg of protein/ml)	Generation time (min)	Final pH[a]
Aerobic	None	0.7	29	4.4
	pLOI291	0.7	46	5.3
	pLOI292	1.3	46	5.1
	pLOI295	1.1	71	5.7
	pLOI308-2	1.7	27	5.5
	pLOI308-5	0.8	30	4.7
	pLOI308-10	2.5	26	5.0
Anaerobic	None	0.3	32	4.4
	pLOI291	0.4	40	4.5
	pLOI292	1.0	48	5.0
	pLOI295	2.1	39	4.7
	pLOI308-2	0.8	42	5.7
	pLOI308-5	0.4	38	4.9
	pLOI308-10	2.2	41	5.2

[a] Measured after 24 h of growth.

calculated to represent 2% of the soluble cellular protein in strain TC4(pLOI295).

Although the trends in activity were similar for both enzymes, the ratios of activity between the two enzymes did not remain constant. These differences may have been due in part to differences in enzyme stability or to differences in the physiological conditions of the cells (extent of growth, pH, cellular damage by fermentation products, etc.).

Growth of recombinant strains containing the ethanologenic enzymes from *Z. mobilis*. The growth of the recombinant strains was examined under both aerobic and anaerobic conditions (Fig. 2). Under aerobic conditions (Fig. 2A and Table 2), strain TC4 grew with a generation time of approximately 30 min during the most rapid phase of growth. Strain TC4 carrying the derivatives of pLOI308 exhibited equivalent maximal rates of growth, with generation times between 26 and 30 min. Strain TC4(pLOI295) grew poorly under these conditions (generation time, 71 min) and was accompanied by partial lysis. Strains TC4(pLOI291) and TC4(pLOI292) are not included in Fig. 2A but grew at intermediate rates, each with a generation time of 46 min.

Under anaerobic conditions (Fig. 2B and Table 2), the generation time for strain TC4 lacking a plasmid was 32 min, considerably shorter than that for the recombinant strains containing the ethanologenic enzymes. All of the recombinants exhibited similar maximal rates of growth, with generation times between 38 and 41 min, except for TC4(pLOI292), which grew somewhat more slowly, with a generation time of 48 min.

All of the recombinants except TC4(pLOI295) grew after 12 h under anaerobic and aerobic growth conditions to cell densities equivalent to or higher than those of strain TC4 lacking a plasmid (Fig. 2A and B). Table 2 summarizes the final cell densities of strain TC4 and the recombinants after 24 h of growth. Under aerobic conditions, strain TC4 con-

FIG. 2. Growth and acid production by strain TC4 and recombinants containing plasmids encoding ethanologenic enzymes. (A) Strains grown under aerobic conditions. (B) Strains grown under anaerobic conditions. (C) Acid production under anaerobic conditions. Symbols: ■, pLOI295; ●, pLOI308-2; ○, pLOI308-5; ▲, pLOI308-10; □, strain TC4 lacking a plasmid. O.D., Optical density.

taining pLOI308-10 reached the highest cell density, followed by TC4 containing pLOI308-2, pLOI292, pLOI295 (with some lysis apparent), and pLOI308-5. This order was not preserved during anaerobic growth. Under anaerobic conditions, the final cell densities of strain TC4 containing pLOI308-10 and pLOI295 were roughly equivalent, followed by those of TC4 containing pLOI292, pLOI308-2, pLOI308-5, and pLOI291.

Figure 3 shows the relationship between the level of pyruvate decarboxylase activity in cells and the final cell density after 24 h of growth. Since the synthesis of pyruvate decarboxylase is coupled to that of alcohol dehydrogenase II in these recombinants, this plot represents the effects of the alternative *Z. mobilis* system for NAD^+ regeneration on final cell density. From these data, it is clear that the expression of the *Z. mobilis* pathway for the production of ethanol increases final cell density under both anaerobic and aerobic conditions. In strain TC4(pLOI308-10), the levels of expression of pyruvate decarboxylase (6.5 IU) and alcohol dehydrogenase II (2.5 IU) were nearly optimal for both anaerobic and aerobic growth. The level of expression of ethanologenic enzymes in strain TC4(pLOI295) appears to be excessive, resulting in diminished cell growth accompanied by partial lysis under aerobic conditions and slightly reduced growth under anaerobic conditions.

The increased growth of strain TC4(pLOI295) under anaerobic conditions with little apparent lysis in contrast to the poor growth and lysis during growth in rapidly shaken flasks suggested that a highly aerobic environment may be damaging to this construction. Lysis in this recombinant was dramatically reduced and the final cell density was increased during growth in shaken flasks when the speed of oscillation was decreased by one-third (data not shown).

Effects of ethanologenic enzymes on the acidification of broth during growth. Figure 2C shows a plot of the changes in the pH of the broth during anaerobic growth. The pH fell rapidly during the first 6 h of growth of strain TC4 lacking a plasmid but declined more slowly in derivatives containing the ethanologenic enzymes. Acidification during the initial 12 h was reduced to the greatest extent in strain TC4 containing pLOI295, followed by TC4 containing pLOI308-10, pLOI308-2, and pLOI308-5. Data for strains TC4 (pLOI291) and TC4(pLOI292) are not shown but lie below and above those for TC4(pLOI308-5), respectively. Although the recombinants reached a higher final cell density, the pH of the broth from the recombinants grown under both anaerobic and aerobic conditions for 24 h was less acidic than that of the broth from strain TC4 lacking ethanologenic enzymes (Table 2).

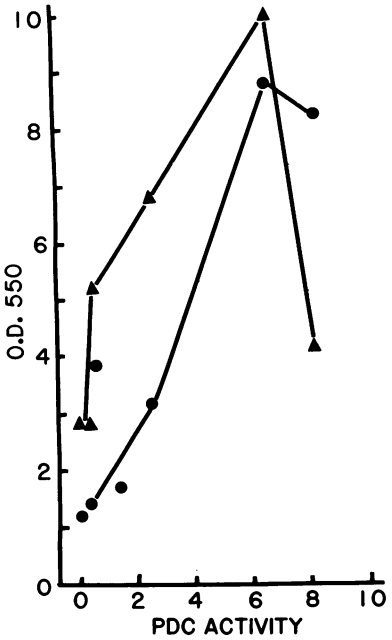

FIG. 3. Relationship between pyruvate decarboxylase (PDC) activity in recombinants and the extent of growth. Cell mass after 24 h of growth is expressed as the optical density at 550 nm (O.D. 550). Symbols: ▲, aerobic growth conditions; ●, anaerobic growth conditions.

The reduced rate and extent of acidification in recombinants accompanied by increased cell growth suggested that the fall in pH was a major factor limiting growth even under highly aerobic conditions. This hypothesis was supported by an 85% increase in the final cell density of strain TC4 (lacking a plasmid) grown in medium supplemented with a 1/10 volume of 1 M sodium phosphate buffer (pH 7.0) (data not shown). Lower levels of buffer addition resulted in intermediate levels of growth.

Effects of ethanologenic enzymes on fermentation products. Table 3 summarizes the analyses of fermentation products made by strain TC4 and the recombinants after 24 h of growth under aerobic and anaerobic conditions. Under aerobic conditions, acetate was the primary fermentation product that accumulated during the growth of strain TC4 lacking a plasmid in rich medium, with no detectable ethanol. The amount of acetate produced was drastically reduced in strains containing the ethanologenic enzymes from *Z. mobilis*, and ethanol appeared as the major fermentation product. Strain TC4 containing pLOI308-10 produced the most ethanol, followed by TC4 containing pLOI295, pLOI308-2, pLOI292, pLOI308-5, and pLOI291. Under these aerobic conditions, small amounts of lactate were also produced (0.6 to 1.2 mM) by all of these strains. Only strain TC4 containing pLOI308-10 accumulated appreciable amounts of succinate, although this product still represented only 1% of the total fermentation products, with 94% being ethanol.

Under anaerobic conditions, lactate was the principal fermentation product that accumulated during 24 h of growth of strain TC4 lacking a plasmid in rich medium containing glucose, with lesser amounts of acetate, succinate, and ethanol being present. Lactate production was dramatically reduced in strains containing the ethanologenic enzymes and was accompanied by the production of substantial quantities of ethanol. Strain TC4(pLOI308-10) produced the largest amount of ethanol, and this product alone represented 97% of the total soluble fermentation products. The trend of ethanol production among the organisms tested was the same as that during aerobic growth. All organisms except strain TC4(pLOI308-10) actually produced less total ethanol after 24 h under anaerobic conditions than under aerobic conditions. It is likely that this lower level of accumulated ethanol was caused by the reduction in total cell mass produced under these anaerobic conditions, thus reducing the volumetric rate of ethanol production.

The extent of ethanol production under anaerobic and aerobic conditions (Table 3) was directly related to the level of expression of the *Z. mobilis* ethanologenic genes (Table 1). Ethanol production appeared to be optimal in strain TC4(pLOI308-10), with a pyruvate decarboxylase activity of 6 IU and an alcohol dehydrogenase II activity of 2.5 IU.

TABLE 3. Comparison of fermentation products during aerobic and anaerobic growth

Growth condition	Plasmid	Fermentation product [mM (SD)]			
		Succinate	Lactate	Acetate	Ethanol
Aerobic	None	0.2 (0.1)	0.6 (0.2)	55 (2)	Tr
	pLOI308-2	Tr	1.2 (0.3)	22 (2)	98 (3)
	pLOI308-5	Tr	0.9 (0.2)	43 (3)	15 (2)
	pLOI308-10	4.9 (0.5)	1.0 (0.2)	17 (2)	337 (21)
	pLOI295	Tr	1.1 (0.4)	13 (1)	114 (10)
	pLOI291	Tr	0.6 (0.2)	34 (3)	7 (1)
	pLOI292	Tr	1.3 (0.2)	30 (1.5)	24 (1)
Anaerobic	None	0.9 (0.1)	22 (1)	7 (0.3)	0.4 (0.2)
	pLOI308-2	0.8 (0.1)	7 (0.5)	4 (0.3)	71 (5)
	pLOI308-5	0.3 (0.1)	18 (2)	6 (1)	16 (2)
	pLOI308-10	5.0 (0.4)	10 (1)	1.2 (0.2)	482 (23)
	pLOI295	2.2 (0.20)	6 (1)	3 (0.3)	90 (2)
	pLOI291	1.0 (0.1)	15 (0.5)	7 (0.2)	4 (0.5)
	pLOI292	2.3 (0.2)	9 (0.7)	7.2 (0.3)	21 (1)

DISCUSSION

Derivatives of *E. coli* TC4 containing plasmids which express the ethanologenic enzymes from *Z. mobilis* grew to higher cell densities than did the parent organism lacking a plasmid. The increase in the final cell density, the extent to which ethanol accumulated in the medium, and the reduction in the rate of acidification of the culture broth during growth all correlated with the level of expression of *Z. mobilis* ethanologenic enzymes. Heterologous promoters were used to express the genes in all constructions except pLOI295 (*lac*) to minimize potential problems associated with transcriptional regulation. The level of expression nearest to optimal for growth and ethanol production was provided by pLOI308-10 (6.5 IU of pyruvate decarboxylase and 2.5 IU of alcohol dehydrogenase II per mg of total cellular protein). This level of expression in *E. coli* is considerably higher than that present in *Z. mobilis* CP4 (17), which contains only the ethanol pathway for the regeneration of NAD^+.

The level of expression of the ethanologenic enzymes in *E. coli* was directly related to the replacement of acidic fermentation products by ethanol and to increased cell growth. These results indicate that the production of organic acids as products of glucose metabolism limits the growth of *E. coli* under anaerobic and aerobic conditions.

The level of expression of ethanologenic enzymes appeared to be excessive in strain TC4(pLOI295) (approximately 17% of the soluble cellular protein). This high level of expression was accompanied by partial cell lysis, slower growth, and a reduction in ethanol production under aerobic conditions. These effects were reduced by slower agitation and by growth under anaerobic conditions. The apparent damage and partial lysis that occurred during highly aerobic growth may have been related to the depletion of NADH by a combination of the high levels of *Z. mobilis* alcohol dehydrogenase II and the native NADH oxidase (coupled to the electron transport system).

The production of ethanol as a major product does not appear to adversely affect the growth rate of *E. coli* TC4. Strains containing derivatives of pLOI308 (ColE1 replicon) expressing the *pet* operon and producing ethanol grew as rapidly as did the parent organism under aerobic conditions with glucose and reached higher final cell densities than did the parent organism. Strains containing pLOI291 or pLOI292 with the RSF1010 replicon grew more slowly under aerobic conditions. Since these two constructions expressed lower levels of the ethanologenic enzymes and produced less ethanol than did pLOI308-10, the reasons for the slower growth can be attributed to properties of the vector rather than to the expression of the *pet* operon. Under anaerobic conditions, all recombinant strains grew more slowly than did the parent lacking a plasmid, but the degree of increase in the generation time did not correlate with the level of expression of the ethanologenic enzymes.

It is interesting to note that the fermentation of glucose to ethanol by *Z. mobilis* results in only a single mole of ATP being produced (net) per mole of glucose consumed via the Entner-Doudoroff pathway (15). Glucose fermentation by the mixed-acid pathway in *E. coli* normally produces 2 to 2.5 mol of ATP per mol of glucose consumed, while homoethanol production (Embden-Meyerhof-Parnas pathway, as in *Saccharomyces cerevisiae*) results in only 2 mol of ATP being produced (9). Thus, the conversion of *E. coli* from being a mixed-acid producer to being an ethanol producer, like *S. cerevisiae*, will reduce the energy yield to the cell by one-third per glucose molecule metabolized. It is tempting to speculate that this less efficient production of ATP is in part responsible for the increase in generation time during anaerobic growth which was not observed with derivatives of pLOI308 during aerobic growth.

Significant amounts of ethanol were produced in recombinants containing the *pet* operon under both aerobic and anaerobic conditions. Although the amounts of ethanol produced were lower under anaerobic conditions, the production of ethanol as a mole fraction of total fermentation products was similar in most cases. The native *E. coli* enzymes involved in pyruvate metabolism are present in concentrations of 0.5 to 1.5 IU/mg of soluble cellular protein (8) and about one-third less when expressed on a total cellular protein basis. Under aerobic conditions in *E. coli*, pyruvate from glycolysis is primarily metabolized by the pyruvate dehydrogenase complex and by lactate dehydrogenase (9), with excess acetyl coenzyme A being converted to acetate. The apparent K_ms for these two enzymes are 0.4 and 7.2 mM, respectively (Table 4). The apparent K_m for *Z. mobilis* pyruvate decarboxylase is equal to (pyruvate dehydrogenase) or lower than (lactate dehydrogenase) those for the two *E. coli* enzymes, thereby facilitating acetaldehyde production. NAD^+ regeneration under aerobic conditions results primarily from biosynthesis and from the NADH

TABLE 4. Comparison of apparent K_m for selected enzymes from *E. coli* and *Z. mobilis*

Organism	Enzyme[a]	K_m of:		Reference(s)
		Pyruvate	NADH	
E. coli	PDH	0.4 mM	176 μM	1, 21
	LDH	7.2 mM	>0.5 mM	20
	PFL	2.0 mM		12, 18
	ALDH		50 μM	19
	NADH-OX		50 μM	8
Z. mobilis	PDC	0.4 mM		2
	ADH II		12 μM	11

[a] PDH, Pyruvate dehydrogenase; LDH, lactate dehydrogenase; PFL, pyruvate formate lyase; ALDH, aldehyde dehydrogenase; NADH-OX, NADH oxidase; PDC, pyruvate decarboxylase; ADH II, alcohol dehydrogenase II.

oxidase (coupled to the electron transport system) with an apparent K_m of 50 μM. The apparent K_m for *Z. mobilis* alcohol dehydrogenase II is over fourfold lower than that for *E. coli* NAD^+ oxidase, allowing the *Z. mobilis* enzyme to compete effectively for endogenous pools of NADH for the reduction of acetaldehyde to ethanol. Thus, the properties of the *Z. mobilis* ethanologenic enzymes and their relatively high levels of expression are well suited for the diversion of carbon flow into ethanol under aerobic conditions.

Under anaerobic conditions in *E. coli*, pyruvate from glycolysis is primarily metabolized by lactate dehydrogenase and pyruvate formate lyase (9). The apparent K_ms for these two enzymes are 18-fold and 5-fold higher, respectively, than that for *Z. mobilis* pyruvate decarboxylase (Table 4). Similarly, the apparent K_ms for the principal enzymes involved in NAD^+ regeneration in *E. coli* are also considerably higher than those for *Z. mobilis* alcohol dehydrogenase II. Thus, the ethanologenic enzymes from *Z. mobilis* are quite competitive for carbon (pyruvate) and reducing potential (NADH) with the normal fermentative enzymes of *E. coli*, allowing the efficient channeling of glycolytic products into ethanol.

The accumulation of organic acids from sugar metabolism is generally regarded as a consequence of fermentation during anaerobic growth. However, appreciable quantities of acetate were produced by the parent strain of *E. coli* (lacking a plasmid) even during rapid agitation under aerobic conditions. Although the time course data are not presented, the production of acetate by strain TC4 is progressive from the earliest stages of growth and is not limited to the later stages, when cell density is high and one could imagine problems in saturating oxygen demands due to limited solubility and mass transfer (7). This production of acetate can be regarded as an overflow of acetyl coenzyme A from glycolysis and the action of pyruvate dehydrogenase in excess of the demands for biosynthesis. This acid production from glucose even under aerobic conditions serves to limit growth in broth and on solid medium, as demonstrated by the increased final cell density in medium supplemented with phosphate buffer. In recombinants expressing the *pet* operon, the ethanologenic enzymes of *Z. mobilis* divert part of the pyruvate from glycolysis to acetaldehyde and reoxidize NADH to produce ethanol, a less damaging product of metabolism.

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