APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1987, p. 2420–2425 Vol. 53, No. 10
0099-2240/87/102420-06$02.00/0
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Genetic Engineering of Ethanol Production in *Escherichia coli*†

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The genes encoding essential enzymes of the fermentative pathway for ethanol production in *Zymomonas mobilis*, an obligately ethanologenic bacterium, were inserted into *Escherichia coli* under the control of a common promoter. Alcohol dehydrogenase II and pyruvate decarboxylase from *Z. mobilis* were expressed at high levels in *E. coli*, resulting in increased cell growth and the production of ethanol as the principal fermentation product from glucose. These results demonstrate that it is possible to change the fermentation products of an organism, such as *E. coli*, by the addition of genes encoding appropriate enzymes which form an alternative system for the regeneration of NAD$^+$.

During glycolysis, cells convert simple sugars, such as glucose, into pyruvic acid, with a net production of ATP and NADH. In the absence of a functioning electron transport system for oxidative phosphorylation, at least 95% of the pyruvic acid is consumed in short pathways which regenerate NAD$^+$, an obligate requirement for continued glycolysis and ATP production. The waste products of these NAD$^+$ regeneration systems are commonly referred to as fermentation products. Under conditions of oxygen insufficiency, mammalian muscle tissues produce lactic acid as the principal fermentation product (12), whereas ethanol is the major fermentation product in the roots of higher plants (11) and in some fish (18). Microorganisms are particularly diverse in the array of fermentation products which are produced by different genera (10). These products include organic acids, such as lactate, acetate, succinate, and butyrate, as well as neutral products, such as ethanol, butanol, acetone, and butanediol. Indeed, the diversity of fermentation products from bacteria has led to their use as a primary determinant in taxonomy (10). The microbial production of these fermentation products forms the basis for our oldest and most economically successful applications of biotechnology and includes dairy products, meats, beverages, and fuels.

End products of fermentation share several fundamental features. They are relatively nontoxic under the conditions in which they are initially produced but become toxic upon accumulation. They are more reduced than pyruvate, their immediate precursors having served as terminal electron acceptors during glycolysis. The diversity of end products and their distribution suggest that few additional constraints have operated during the evolution of such pathways among different organisms.

In the present study, the genes encoding the NADH-oxidizing system of *Zymomonas mobilis* were inserted into *Escherichia coli*. *Z. mobilis* is an obligately fermentative bacterium which lacks a functional system for oxidative phosphorylation (14). This organism, like *Saccharomyces cerevisiae* (20), produces ethanol and carbon dioxide as principal fermentation products. The NADH-oxidizing pathway in this organism consists of two enzymes. Pyruvate decarboxylase (EC 4.1.1.1) catalyzes the nonoxidative decarboxylation of pyruvate to produce acetaldehyde and carbon dioxide. Two alcohol dehydrogenase isozymes (EC 1.1.1.1) are present in this organism and catalyze the reduction of acetaldehyde to ethanol during fermentation, accompanied by the oxidation of NADH to NAD$^+$ (15, 21). Recent studies have described the cloning of the gene coding for pyruvate decarboxylase from *Z. mobilis* (1), its sequence (4, 16), and its expression at high levels in *E. coli* (1, 4). The cloning, characterization, and expression of the *Z. mobilis* alcohol dehydrogenase II gene in *E. coli* have also been completed (5). These two genes from *Z. mobilis* were combined and placed under the control of a single enteric promoter to produce an artificial operon for the production of ethanol, designated the *pet* operon.

MATERIALS AND METHODS

Organisms and growth conditions. *E. coli* TC4 (4) and plasmid-containing derivatives were used in the present study. Plasmids containing the pyruvate decarboxylase gene (pLOI276) and the alcohol dehydrogenase B gene (pLOI284) have been described previously (4, 5). The construction of a plasmid containing both of these genes, pLOI295, is described in this paper. All organisms were grown in Luria broth (13) containing the indicated concentrations of glucose. Growth was measured as A_{550}; 1.0 absorbance unit is equivalent to 0.25 mg of total cell protein per ml.

Genetic methods. Plasmid DNA was isolated and purified as previously described (4). DNA-modifying enzymes were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and were used according to the recommendations of the supplier. Methods for transformation and selection of recombinants have been described previously (4, 5).

Enzyme assays. Cells were disrupted and assayed for pyruvate decarboxylase (4) and alcohol dehydrogenase (5) as previously described. Enzyme activity is reported as micromoles per milligram of total cell protein per minute.

Analysis of fermentation products. Fermentation products were determined in clarified broth with a Millipore/Waters high-performance liquid chromatograph (Millipore Corp., Bedford, Mass.) equipped with a refractive index monitor and an electronic integrator. Separations were performed on an Aminex HPX-87H column (300 by 7.8 mm) purchased from Bio-Rad Laboratories, Richmond, Calif., at 65°C at a flow rate of 0.25 ml/min (100-μl injection volume). Peaks were identified by using authentic standards. The two peaks

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† Publication 7838 from the Florida Agricultural Experiment Station.

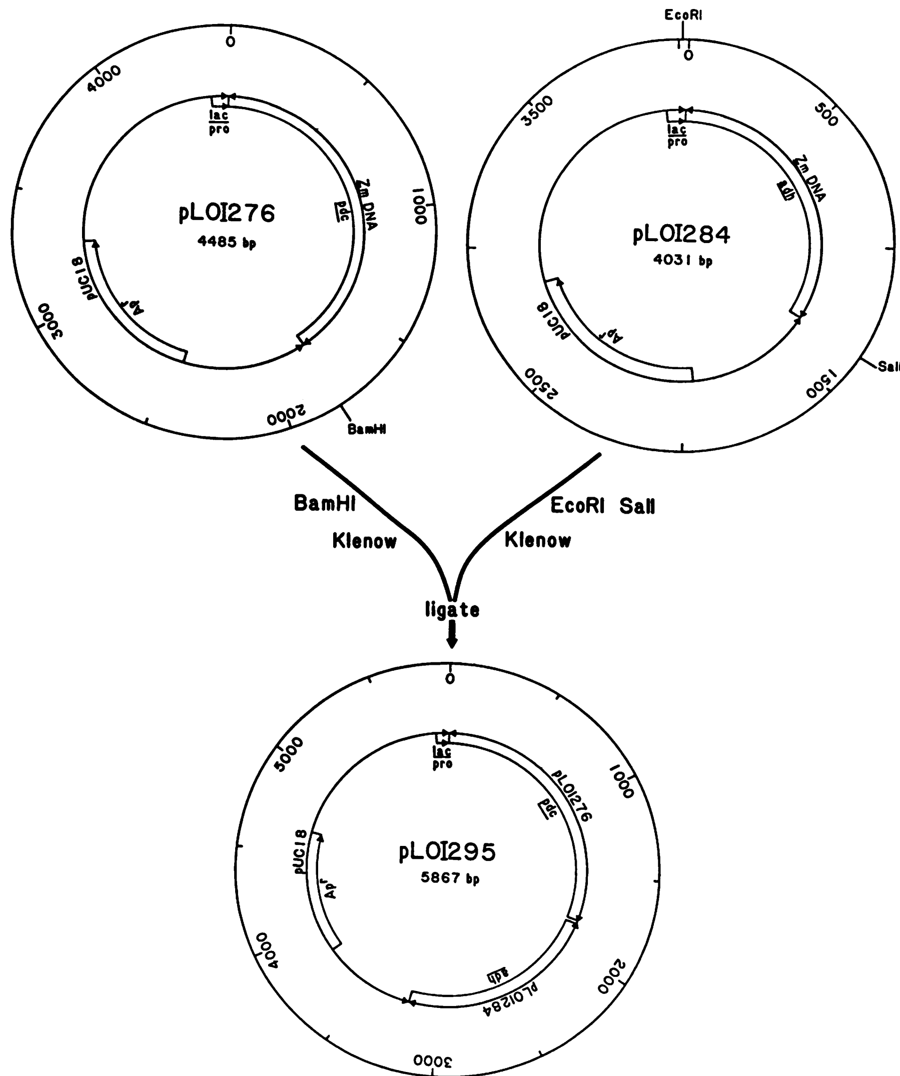

FIG. 1. Construction of pLOI295 containing genes encoding pyruvate decarboxylase and alcohol dehydrogenase II from *Z. mobilis* under the control of the *lac* promoter from *E. coli*. The three plasmids involved in this construction are derivatives of pUC18 and are ampicillin resistant (Ap^r). The *lac* promoter is labeled with a single-ended arrow indicating the direction of transcription. The coding regions for the pyruvate decarboxylase gene (*pdc*), the alcohol dehydrogenase II gene (*adh*), and the sources of DNA fragments (double-ended arrows) are also indicated. The joint presence of these two genes from *Z. mobilis* under the control of the *lac* promoter is referred to as the *pet* operon, so named because of the production of ethanol.

eluting before glucose and the later unknown peak eluting at 45.4 to 45.8 min are components of uninoculated medium.

RESULTS

Strain construction. The sizes of the structural genes coding for pyruvate decarboxylase and alcohol dehydrogenase II are 1.7 and 1.1 kilobases, respectively, and these genes encode proteins with molecular weights of 60,000 and 40,100. These genes are each located on derivatives of pUC18 under the control of the *lac* promoter (Fig. 1). The two genes were combined by inserting the promoterless 1.4-kilobase fragment generated by restriction endonucleases *Eco*RI and *Sal*I from pLOI284 (alcohol dehydrogenase) into the *Bam*HI site downstream from the pyruvate decarboxylase gene in pLOI276. These clones were selected for resistance to ampicillin and for the presence and expression of alcohol dehydrogenase activity on a newly developed pararosaniline-ethanol indicator plate which detects the production of aldehydes (5; Fig. 2A). Clones containing the indicated construction, pLOI295, grew poorly on the surface of Luria agar plates (aerobic) in the absence of added glucose but grew to much higher densities than the plasmid-free strain and strains containing pLOI276 or pLOI284 on agar plates containing 2% glucose (Fig. 2B and C). Recombinants containing the *pet* operon were readily detected as larger, more opaque colonies on Luria agar plates (aerobic) containing glucose (Fig. 2D). This difference in colony size and opacity has proven to be a useful marker for the identification of recombinants which contain plasmids expressing both alcohol dehydrogenase and pyruvate decarboxylase genes.

The complete base sequence of pLOI295 is known (4, 5, 22). The open reading frame for the gene coding for pyruvate decarboxylase begins 163 bases downstream from the *lac*

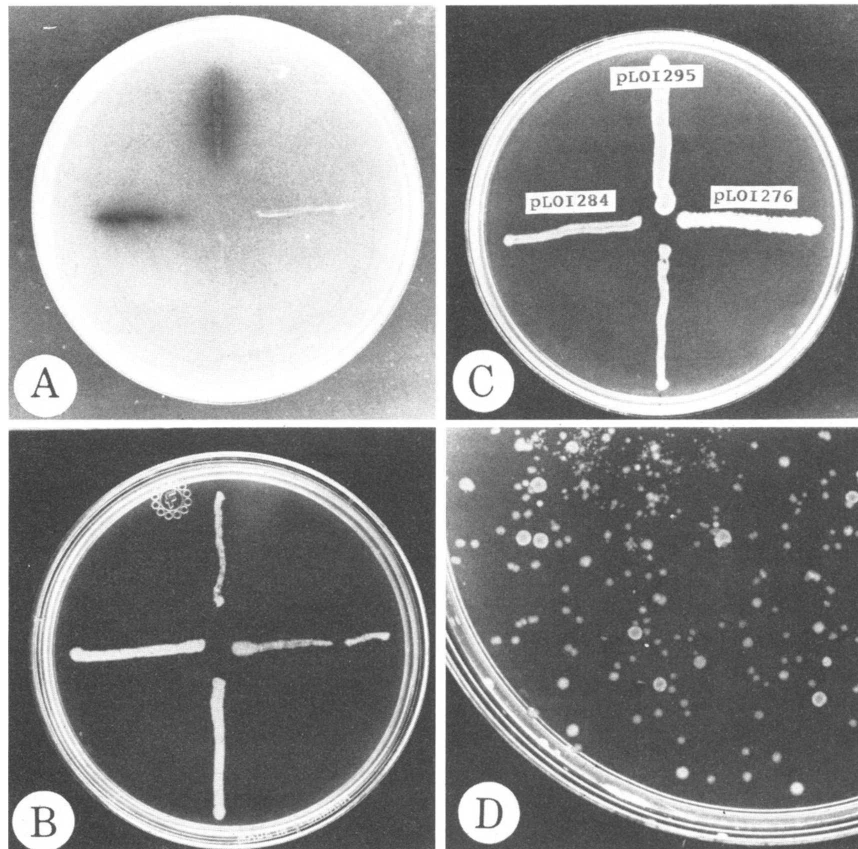

FIG. 2. Comparison of plasmid-free strain TC4 and strain TC4 carrying plasmids encoding alcohol dehydrogenase and pyruvate decarboxylase on solid medium. All plates were incubated under aerobic conditions. Panels A, B, and C show petri plates in which strain TC4 (prototrophic for relevant markers) and plasmid-containing derivatives were streaked in the same orientation for comparison. The lower streak in each of these three panels is plasmid-free strain TC4, the left streak is strain TC4(pLOI276), the right streak is strain TC4(pLOI284), and the upper streak is strain TC4(pLOI295). The plate in panel A was incubated for 2 h at 37°C. The acetaldehyde produced by alcohol dehydrogenase reacted with pararosaniline to produce the diffusible red pigment observed in this alcohol dehydrogenase indicator plate (5). The plates in panels B, C, and D were incubated overnight at 37°C. The plate in panel B contains Luria broth without added glucose. The plates in panels C and D contain Luria broth containing 2% glucose. Panel D is a photograph of transformants which grew in Luria broth with glucose as progeny of a ligation during the construction of pLOI295. The large white colonies (two of which are marked by arrows) were subsequently found to contain plasmid pLOI295.

promoter and ends with two stop codons 85 bases upstream from the open reading frame of the gene coding for alcohol dehydrogenase II. Both genes include sequences which resemble ribosome-binding sites immediately upstream from each open reading frame. The gene encoding alcohol dehydrogenase II contains a single stop codon followed by a palindromic sequence of 13 base pairs which serves as a transcriptional terminator.

Expression of *Z. mobilis* genes in *E. coli*. Both pyruvate decarboxylase and alcohol dehydrogenase II genes were expressed at high levels in *E. coli* under the control of the *lac* promoter singly (pLOI276 and pLOI284, respectively) and jointly (Table 1). Pyruvate decarboxylase is not present in wild-type *E. coli*, but an inducible alcohol dehydrogenase (3) is present at low concentrations. During growth of *E. coli* in the presence of glucose, the specific activities of the *Z. mobilis* enzymes declined by approximately 50%, which is consistent with glucose repression of the *lac* promoter (7). It is interesting that the specific activity of pyruvate decarboxylase, coded for by the proximal gene in the *pet* operon, was threefold higher in pLOI295 than in pLOI276. The specific activity of the product of the alcohol dehydrogenase II gene, the distal gene in the *pet* operon, was expressed in pLOI295 at twice the level in pLOI284.

Fermentation of glucose by recombinant strains. Expression of the *pet* operon in *E. coli* resulted in the production of ethanol as the primary fermentation product during anaerobic growth (Fig. 3). The parent strain produced succinate (1.5 mM), lactate (18 mM), and acetate (7 mM) as major fermentation products (Fig. 3A). A small amount of ethanol is typically produced by *E. coli* and was presumed to be present, although it was not observed in the high-performance liquid chromatography profile. An identical fermentation profile was observed in cells containing pLOI284, which carries the alcohol dehydrogenase II gene (Fig. 3C). With pLOI276 carrying the gene coding for pyruvate decarboxylase, an ethanol peak is clearly evident (18 mM), equivalent to one-third of the accumulated fermentation products (Fig. 3D). This higher level of ethanol is presumed to result from the combined activities of the pyruvate decarboxylase from *Z. mobilis* and the native *E. coli* alcohol dehydrogenase. With pLOI295 containing the *pet* operon (both pyruvate decarboxylase and alcohol dehydrogenase II genes from *Z. mobilis*), *E. coli* produced large amounts of

TABLE 1. Specific activities of pyruvate decarboxylase and alcohol dehydrogenase in *E. coli* TC4 and derivatives[a]

Plasmid	Added glucose	Sp act[b] of:	
		Pyruvate decarboxylase	Alcohol dehydrogenase
pLOI295	−	6.3	0.87
	+	3.1	0.33
pLOI248	−	<0.01	0.43
	+	<0.01	0.16
pLOI276	−	2.1	0.03
	+	1.1	0.03
No plasmid	−	<0.01	0.05
	+	<0.01	0.04

[a] Cells were grown at 37°C with shaking in a water bath in Luria broth lacking glucose and in Luria broth containing 2% glucose. An overnight culture was diluted 1:100 into fresh medium (50 ml, 250-ml flask) and harvested by centrifugation at a final cell density of 0.3 at 550 nm (approximately 10^8 cells per ml).

[b] Activities are expressed as micromoles converted per milligram of whole cell protein per minute at 25°C.

ethanol (750 mM; 4.3%, vol/vol), which represented over 95% of the fermentation products (Fig. 3B).

A second approach was also used to construct an ethanol-producing strain of *E. coli*. Recent studies have described cells with constitutive and hyperproducing alcohol dehydrogenase gene mutations (strain DC862 *adhC* and strain DC863 *adhC adhR*, respectively [3a]). Upon transformation of these strains with pLOI276 containing the gene encoding pyruvate decarboxylase, ethanol was also produced as the major fermentation product, thus forming a new hybrid pathway for NADH oxidation.

The high levels of alcohol dehydrogenase and pyruvate decarboxylase produced in cells containing the *pet* operon dominated NADH oxidation in *E. coli* (Fig. 3). Thus, the fermentation of this organism was converted to the equivalent of those of *S. cerevisiae* and *Z. mobilis*. During normal fermentative growth, pyruvate is converted to acetyl coenzyme A by the pyruvate dehydrogenase complex, to oxaloacetate (and on to succinate) by phosphoenolpyruvate carboxylase, to formate and acetyl coenzyme by pyruvate formate lyase, and to lactate by lactate dehydrogenase. This last pathway is the dominant route for the regeneration of NAD^+ in unmodified strains of *E. coli*. However, the K_ms for bacterial lactate dehydrogenases are quite high, ranging from 10 to 1,000 mM (9, 19). The K_m of the pyruvate decarboxylase from *Z. mobilis* is 0.4 mM (2). The abundance of this enzyme, coupled with the lower K_m, effectively diverts the flow of pyruvate from glycolysis into ethanol.

Growth. Shifting the catabolism of glucose to the production of ethanol also affected growth yield and pH drift of the growth medium. Although fermentation products are relatively nontoxic, they may accumulate to toxic levels during fermentation. During anaerobic growth in bottles containing Luria broth containing 10% glucose, the plasmid-free strain and the strain carrying pLOI284 (carrying the gene coding for alcohol dehydrogenase II) achieved a final density of 0.25 mg of cell protein per ml after 48 h, with a final pH of 4.4. The cell density increased by twofold in the strain carrying pLOI276 (carrying the gene coding for pyruvate decarboxylase), with a final pH of 4.5. The final cell density of the strain carrying pLOI295 (*pet* operon) was 2.5 mg/ml, 10-fold higher than that of the control strain. The final pH was 4.7. The similarity in final pH suggests that acidification of the medium may be the primary mode by which the organic acids which are normally produced limit growth. At a density of 2.5 mg of cell protein per ml, magnesium appears to be limiting, and a 1.5-fold-further increase in cell density is readily achieved by the addition of 0.5 mM magnesium sulfate (data not shown). A similar limitation of growth by magnesium in rich medium has also been observed for *S. cerevisiae* (8) and for *Z. mobilis* (17). High cell densities are also achieved during mixed growth conditions with moderate agitation or stirring of culture vessels in which gas exchange is not restricted. Under these conditions, a final pH of 6.3 or above was observed, depending upon the extent of aeration. Strain DC863 (*adhC adhR*; alcohol dehydrogenase hyperproducer) carrying pLOI276 (carrying the gene coding for pyruvate decarboxylase) also grew to high densities, similar to those observed with strain TC4 carrying pLOI295 (carrying the genes coding for alcohol dehydrogenase and pyruvate decarboxylase).

DISCUSSION

The replacement of the native enzymes for the production of fermentation products in *E. coli* with those from *Z. mobilis* without a decrease in growth illustrates that the particular fermentation product produced is relatively innocuous to this organism. Indeed, this represents the replacement of a native pathway, albeit short, with a more useful pathway from an unrelated organism. Many variations of this general approach could be exploited for the production of specific metabolic products from nonconventional substrates or for the transfer of useful pathways from fastidious or less characterized organisms into model organisms in which the full arsenal of genetic and biochemical tools is available.

E. coli is capable of actively metabolizing a wide variety of substrates, including hexoses, pentoses, lactose, etc. Variations of the particular strains constructed in this report may prove to be useful both for the production of ethanol from underutilized sources of biomass, such as hemicellulose (xylose, arabinose, etc.), which represents a major portion of wood and inedible plant parts, and whey (lactose), as well as from other biomass sources. Other organisms with special capabilities, such as extracellular enzymes for the degradation of complex polymers, could be converted to ethanol producers by using pyruvate decarboxylase and alcohol dehydrogenase II genes from *Z. mobilis* under the control of an appropriate promoter. This may be a viable alternative to efforts which are currently under way to genetically engineer the ability of organisms such as *S. cerevisiae* and *Z. mobilis*, which inherently produce ethanol, to utilize new substrates.

The conversion of *E. coli* to ethanolic fermentation may also be of benefit for the production of recombinant products by using *E. coli* expression systems. The maintenance of function in these products may in some cases be related to the pH of the broth during growth in dense culture. The extent of this acidification per unit of cell protein is minimized by the production of ethanol rather than of organic acids. Oxygen transfer is frequently a major limitation during the growth of dense cultures of microorganisms (6), and it is this limitation which results in acid production and pH drift of the growth medium. It is likely that strains containing both functional respiratory chains for oxidative phosphorylation and ethanol production enzymes can be grown to even higher cell densities because of the operation of both sys-

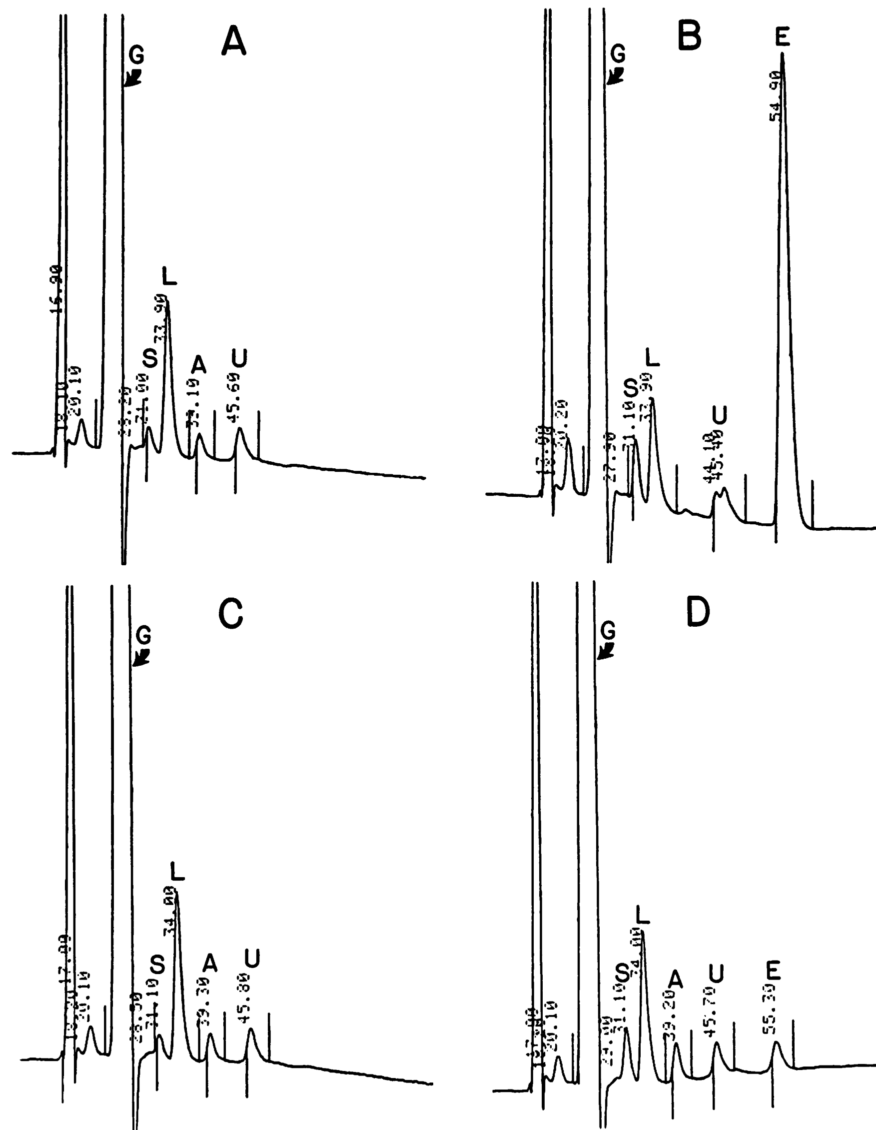

FIG. 3. High-performance liquid chromatography profiles of the fermentation products produced by strain TC4 and derivatives. Cells were grown in Luria broth containing 10% glucose at 30°C with agitation in a gyratory shaker in sealed serum bottles (100 ml of broth per 125-ml bottle, vented with a 25-gauge needle). Overnight cultures were diluted 1:100 and incubated for 48 h. (A) Plasmid-free strain TC4. (B) Strain TC4(pLOI295). (C) Strain TC4(pLOI284). (D) Strain TC4(pLOI276). The peak occurring to the left of the glucose peak includes a complex mixture of medium components. A single unknown peak was observed between the acetate and ethanol peaks in panels A, B, and C, and two unknowns were observed in this region in panel D. Abbreviations: G, glucose; S, succinate; L, lactic acid; A, acetic acid; U, unknown(s); and E, ethanol.

tems during the regeneration of NAD^+ and a reduction in acidic waste products. Such inherent flexibility may result in less stringent process-control requirements, as well as increased yields of recombinant products.

ACKNOWLEDGMENTS

We gratefully acknowledge J. E. Gander, K. T. Shanmugam, and R. A. Jensen for their comments and suggestions during the preparation of this manuscript.

This research was supported in part by the Florida Agricultural Experiment Station and by grants from the Office of Basic Energy Science, Department of Energy (FG05-86ER3574 and AC02-82ER12095), the National Science Foundation (DMB 8204928), and the Alcohol Fuels Program, Department of Agriculture (86-CRCR-12134).

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