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Stabilization of *pet* Operon Plasmids and Ethanol Production in *Escherichia coli* Strains Lacking Lactate Dehydrogenase and Pyruvate Formate-Lyase Activities

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In the last decade, a major goal of research in biofuels has been to metabolically engineer microorganisms to ferment multiple sugars from biomass or agricultural wastes to fuel ethanol. *Escherichia coli* strains genetically engineered to contain the *pet* operon (*Zymomonas mobilis* pyruvate decarboxylase and alcohol dehydrogenase B genes) produce high levels of ethanol. Strains carrying the *pet* operon in plasmid (e.g., *E. coli* B/pLOI297) or in chromosomal (e.g., *E. coli* KO11) sites require antibiotics in the media to maintain genetic stability and high ethanol productivity. To overcome this requirement, we used the conditionally lethal *E. coli* strain FMJ39, which carries mutations for lactate dehydrogenase and pyruvate formate lyase and grows aerobically but is incapable of anaerobic growth unless these mutations are complemented. *E. coli* FBR1 and FBR2 were created by transforming *E. coli* FMJ39 with the *pet* operon plasmids pLOI295 and pLOI297, respectively. Both strains were capable of anaerobic growth and displayed no apparent *pet* plasmid losses after 60 generations in serially transferred (nine times) anaerobic batch cultures. In contrast, similar aerobic cultures rapidly lost plasmids. In high-cell-density batch fermentations, 3.8% (wt/vol) ethanol (strain FBR1) and 4.4% (wt/vol) ethanol (strain FBR2) were made from 10% glucose. Anaerobic, glucose-limited continuous cultures of strain FBR2 grown for 20 days (51 generations; 23 with tetracycline and then 28 after tetracycline removal) showed no loss of antibiotic resistance. Anaerobic, serially transferred batch cultures and high-density fermentations were inoculated with cells taken at 57 generations from the previous continuous culture. Both cultures continued to produce high levels of ethanol in the absence of tetracycline. The genetic stability conferred by selective pressure for *pet*-containing cells without requirement for antibiotics suggests potential commercial suitability for *E. coli* FBR1 and FBR2.

In 1987, L. O. Ingram and his colleagues were the first to report a metabolically engineered bacterium for high levels of alcohol production. The cloned *Zymomonas mobilis* genes for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*) were combined to form the *pet* operon, which was transformed into *Escherichia coli* on a pUC-based plasmid to form pLOI295 (8). The resultant recombinant strain produced more than 3.4% (wt/vol) ethanol from glucose in media containing ampicillin (AMP), with positive selection pressure for the plasmid. *pet* operon plasmids and gene expression were improved by changes in promoters (7), insertion of a tetracycline (TET) gene to form pLOI297 for plasmid selection, and selection of hardier *E. coli* strains (1). Although the last researchers showed that *E. coli* B maintained pLOI297 for 25 generations in the absence of TET, we found rapid plasmid losses after this time, and recently Lawford and Rousseau (9, 10) have shown even more rapid declines in levels of ethanol production. A considerably more stable strain was developed by Ohta et al. (12) by integrating the *pet* operon and chloramphenicol (CHL) resistance gene into the *E. coli* chromosome. The resultant *E. coli* strains did not require CHL in the growth media for retention of the *pet* operon; however, ethanol production levels were much lower, presumably because of reduced *pet* gene copy number. When mutants were selected for resistance to high levels (600 μg/ml) of CHL, high levels of ethanol production were restored, as shown with strain KO11. However, Lawford and Rousseau (9, 10) have found that these mutants also lose the ability to produce high levels of ethanol in the absence of CHL.

Another approach of our research program has involved cloning lactate dehydrogenase genes from anaerobic bacteria. A lactate dehydrogenase (*ldh*)-pyruvate formate lyase (*pfl*) double mutant of *E. coli* (11) has been used as the cloning host. While capable of aerobic growth, this mutant strain FMJ39 is incapable of anaerobic growth because of the inability to regenerate oxidized pyridine nucleotides by reduction of pyruvate to lactate. Clones having recombinant plasmids containing an *ldh* gene can be isolated by complementing for anaerobic growth by this strain. We reasoned that an alternative complementation for anaerobic growth would be the *pet* operon-containing plasmids, since expression of the *pdc* and *adh* genes would convert pyruvate to ethanol and regenerate oxidized pyridine nucleotides. If so, the resultant strains should be quite stable anaerobically for ethanol production. These strains should also not require antibiotics in the growth media to maintain positive selective pressure for cells containing the *pet* operon plasmid, because loss of the plasmid would be a conditionally lethal event. In this paper, we show that this is a successful approach to stabilize strains with *pet* operon-containing plasmids and that the resultant strains produce high levels of ethanol.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. Strains *E. coli* B (ATTC 11303) and *E. coli* KO11 and plasmids pLOI295 and pLOI297 were obtained from L. O. Ingram (University of Florida, Gainesville, Fla.). *E. coli* FMJ39 (K-12) [F⁻ *thr-1 leu-6 thi-1 lacY tonA22 straA pfl-1 ldh*) was received from D. P. Clark (Southern Illinois University, Carbondale, Ill.). All strains were grown on

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TABLE 1. Stability of AMP resistance and *pet* operon in *E. coli* FBR1

No. of generations/ days and plating medium	Viable cell count (10^5) under the following growth conditions:			
	Aerobic	Aerobic + AMP	Anaerobic	Anaerobic + AMP
20/3				
LB	2,500	400	200	500
LB + AMP	1.7	7.8	130	130
40/6				
LB	8,200	8,300	600	630
LB + AMP	1.3	5.0	470	690
60/9				
LB	5,400	7,200	730	1,300
LB + AMP	1.0	7.1	730	1,200

[a] Batch cultures were transferred (1% [vol/vol]) on a daily basis with LB broth, with (50 μg/ml) or without AMP added, and were grown under an aerobic (air) or anaerobic (nitrogen) atmosphere.

LB broth (10 g of tryptone, 5 g of yeast extract, 5 g of sodium chloride per liter) supplemented as indicated with AMP (50 to 100 μg/ml), CHL (40 μg/ml), or TET (5 to 15 μg/ml) and arabinose or glucose. Antibiotics were added separately as filter-sterilized solutions, and sugars were added separately as autoclaved or filter-sterilized solutions. For anaerobic growth of *E. coli*, LB broth also contained acetate (1 g/liter) as an additional carbon source, and the culture flasks or tubes were flushed with nitrogen gas and sealed with rubber stoppers.

Genetic procedures. Plasmids were routinely prepared from cells harvested from culture samples (1 to 10 ml) by routine procedures (13). The purity of plasmid preparations was verified by standard agarose gel electrophoresis of undigested and restriction endonuclease (*Eco*RI)-digested plasmids. *E. coli* cells were transformed with plasmid DNAs (5), and recombinants were selected by plating on LB agar media containing AMP (pLOI295) or TET (pLOI297).

High-cell-density fermentations. Minifermentors with automatic pH control were constructed and operated essentially as described by Beall et al. (2). Each 500-ml fleaker culture vessel contained 350 ml of LB broth containing 10% (wt/vol) sugar plus the appropriate antibiotic and was inoculated to an initial optical density at 550 nm (OD_{550}) of 0.5 by using washed cells from a seed culture grown overnight. The culture was incubated at 35°C and sampled (1 to 3 ml) periodically over 96 h of incubation for measurements of OD, ethanol, sugars, organic acids, plasmid isolations, or viable cell counts.

Continuous culture fermentations. The long-term stability of antibiotic resistance and ethanol production were assessed with a glucose-limited continuous culture. The chemostat consisted of a modified 2-liter Multigen fermentor (New Brunswick Scientific, New Brunswick, N.J.) containing 600 ml of LB broth maintained at 35°C and sparged with nitrogen gas. The dilution rate was maintained at 0.1 h^{-1} and the vessel pH was nearly constant (pH 6.2 to 6.4) over the entire run. The added LB medium contained 0.5% glucose and 15 μg of TET per ml and was pumped from a 20-liter reservoir. After 23 generations (7 days), the reservoir was replaced with one containing TET-free medium, and growth was continued for another 28 generations. Culture samples were taken twice daily to monitor OD and to determine cell numbers and for other analyses.

Analytical procedures. Amounts of ethanol and other alcohols were determined by gas liquid chromatography as described previously (6). Amounts of glucose and fermentation acids were determined by high-pressure liquid chromatography with a refractive index detector and Aminex HPX-87H column (Bio-Rad, Richmond, Calif.) run at 65°C and eluted (0.6 ml/min) with 5 mM sulfuric acid. Enzyme activities for pyruvate decarboxylase and alcohol dehydrogenase were measured (3, 4) with cell extracts prepared from French pressure cell-disrupted cell suspensions.

RESULTS AND DISCUSSION

Construction of *E. coli* FBR1. *E. coli* FMJ39 was transformed with pLOI295 DNA, and recombinant clones were screened with plates of LB agar medium containing 100 μg of AMP per ml. Approximately 10 clones were picked and verified for AMP resistance, for ability to grow anaerobically, and for the presence of plasmids with the same electrophoretic mobility as that of pLOI295. One of these clones, strain FBR1, was chosen for further work. This strain was grown aerobically or anaerobically with or without AMP (Table 1). Aerobic growth without AMP resulted in a very rapid loss of AMP-resistant cells within 20 generations. Even with 50 μg of AMP per ml, less than 1% of the cells remained resistant after 20 generations but could be increased by 50-fold or more when levels of 100 μg or more of AMP per ml were used. In contrast, anaerobic growth led to almost all of the cells being AMP resistant, regardless of whether AMP was present in the medium. Plasmids with pLOI295 characteristics could be readily isolated from the cells grown anaerobically without AMP.

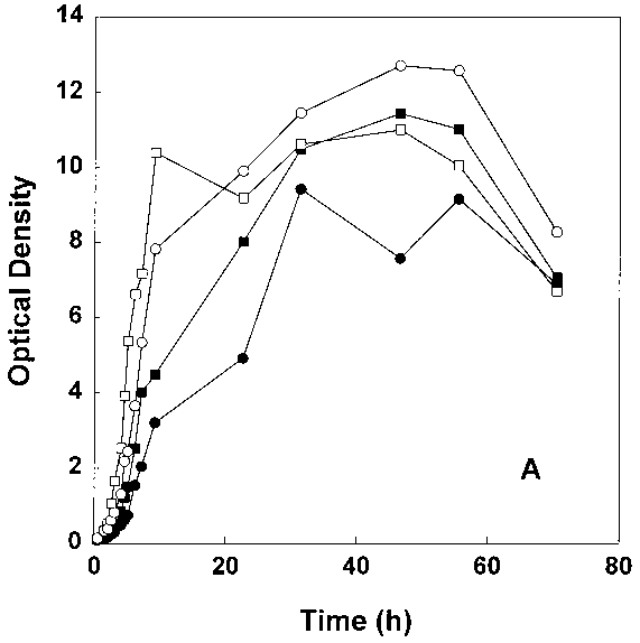
TABLE 2. Ethanol fermentations by *E. coli* KO11 and *E. coli* FBR1[a]

Strain	Sugar (10%)	OD	Generation time (min)	Amt of ethanol (% [wt/vol])	Ethanol yield (g/g)
KO11	Glucose	11.6	69	4.36	0.47
KO11	Arabinose	12.3	83	4.24	0.45
FBR1	Glucose	12.7	74	3.85	0.41
FBR1	Arabinose	9.6	86	3.51	0.37

[a] Fermentations were carried out in fleaker fermentors containing LB broth initially supplemented with 600 μg of CHL (KO11) or 50 μg of AMP (FBR1) per ml.

Ethanol production by *E. coli* FBR1. When grown in aerobic batch cultures with 5% glucose, *E. coli* FMJ39 produced approximately 0.16% ethanol after 48 h of incubation, whereas *E. coli* FBR1 produced approximately 0.9% (wt/vol) ethanol after 24 h and 1.5% after 48 h of incubation. Ethanol production and growth characteristics of *E. coli* FBR1 were then more carefully examined in fleaker fermentors (2) that are routinely used to evaluate various *pet* operon-containing *E. coli* and other species (Table 2). Simultaneously, studies were conducted with *E. coli* KO11, which appears to be the best ethanol-producing strain developed thus far (12). Strain KO11 is a highly selected B strain with the *pet* operon chromosomally integrated apparently near or in the *pfl* gene, having a Tn*10*-induced fumarate reductase mutation to eliminate succinate formation, and possesses resistance to high levels (600 μg/ml) of CHL that concomitantly results in high-level ethanol production. Strain KO11 produced approximately 4.4% ethanol from glucose (Table 2), and time course measurements (Fig. 1) indicated that this level was attained by 50 h. Similar ethanol levels and production rates were found with arabinose (a major sugar found in xylan hydrolysates of biomass) as the substrate. The other major xylan sugar, xylose, was not tested, since parental strain FMJ39 cannot use it. These results compare very favorably with those found by Ingram and colleagues (2, 12). In comparison, the ethanol levels produced by *E. coli* FBR1 were approximately 10% less, and yield was reduced (Table 2). In addition, ethanol productivity rates were slightly lower, and maximal yields were attained at 75 h (Fig. 1). In other experiments, it was found that strain FBR1 produced approximately 1.0% succinate, whereas strain KO11 formed less than 0.1% in batch cultures containing 5% glucose.

Construction of and ethanol production by *E. coli* FBR2. *E. coli* FMJ39 was transformed with pLOI297 to create strain *E. coli* FBR2. When grown either aerobically or anaerobically in serial batch cultures with TET (15 μg/ml), essentially all of the cells were TET resistant. In contrast, when grown without TET in the medium, aerobic serial batch cultures contained less than 0.1% TET-resistant cells after 60 generations, but more than 30% were TET resistant under anaerobic conditions. Although it was unexpected, the apparent high percentage of TET-sensitive anaerobic cells might have been a result of several factors, such as cell death occurring prior to production of TET-inducible proteins and recovery on TET-containing plate media. It also is possible that limited anaerobic growth might



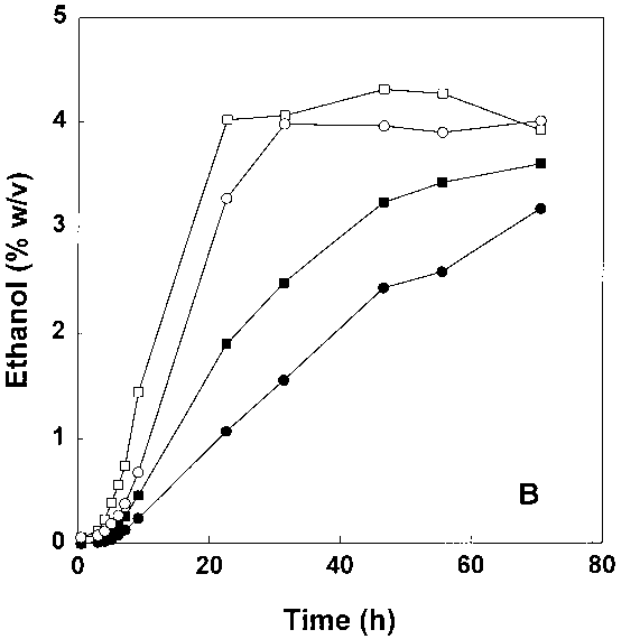


FIG. 1. High-cell-density ethanol fermentations by *E. coli* FBR1 and KO11. Fleaker fermentors were inoculated with strain FBR1 (solid symbols) or strain KO11 (open symbols). The cultures were grown in the presence of air on LB medium containing 8% glucose (□ and ■) or arabinose (○ and ●) and 50 μg of AMP (FBR1) or 600 μg of CHL (KO11) per ml.

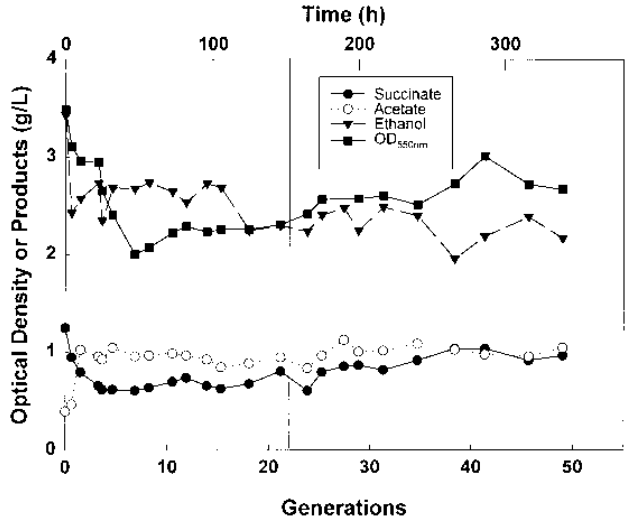


FIG. 2. Growth and product formation during long-term, anaerobic continuous culture fermentation by *E. coli* FBR2. The culture was inoculated (1% [vol/vol]) and grown in batch for 24 h prior to initiation of continuous culture. After 23 generations (vertical bar) with 15 μg of TET per ml in LB medium with 0.5% (wt/vol) glucose, the medium reservoir was replaced with one containing the same medium without TET.

occur after plasmid loss because of residual pyruvate decarboxylase and alcohol dehydrogenase activities coupled with partial utilization of reducing equivalents during succinate formation.

Initial batch culture fermentations with 5% glucose resulted in the production of approximately 1.6% (wt/vol) ethanol after 48 h of incubation by strain FBR2. While *E. coli* FMJ39 possessed less than 0.03 U of alcohol dehydrogenase or pyruvate decarboxylase activities per mg of protein, the levels of these enzymes in *E. coli* FBR2 were 1.7 and 6.2 U/mg, respectively. Ethanol production by *E. coli* FBR2 was determined in high-cell-density fleaker fermentors. For comparison, fermentations were also conducted with an *E. coli* B wild-type strain which had been transformed with pLOI297. The results indicate that *E. coli* FBR2 produced higher levels of ethanol (4.4%), with better yields (0.46 to 0.48 g/g) than those of *E. coli* B (4.1%; 0.41 g/g). In addition, strain FBR2 outperformed strain FBR1 (Table 2).

Continuous culture of *E. coli* FBR2. Lawford and Rousseau (10) showed that the presence of TET and AMP in short-term continuous cultures of *E. coli* B with pLOI297 unexpectedly resulted in loss of ethanol production, implying loss of the plasmid. Using a long-term continuous culture, we examined the stability of strain FBR2, which also contains pLOI297, first in the presence (15 μg/ml) and after removal of TET. During the first 3 to 4 days after inoculation, the microbial population appeared unstable, as reflected in the rapid decline in OD, but after 10 generations the OD remained somewhat constant, with a small increase after TET removal (Fig. 2). Culture stability was also supported by the rather constant levels of acetate, ethanol, and succinate produced. However, a relatively long generation time (ca. 7 h) was maintained, which probably induced some physiological stress. Nevertheless, microscopic examination of the cells revealed only single rods or pairs of rods.

Viable cell counts on LB agar medium alone (total counts) or with 15 μg of TET per ml added (TET-resistant cell counts) were determined. Regardless of whether TET was present, TET-resistant cells were generally 70 to 85% of the total cell counts. On the basis of two samples taken after 35 generations, the cells were plated on media containing only 5 μg of TET per ml, and in both cases the cell counts were approximately 15% higher than the counts on 15-μg/ml TET plates. As noted earlier, these data might suggest that some viability losses occur on the plates until the TET-induced proteins are fully expressed. Overall, TET resistance was maintained at high

TABLE 3. Serial batch culture fermentations by *E. coli* FBR2[a]

Series/transfer	Amt of ethanol (% [wt/vol])		Viable cell counts (10^7)	
	24 h	48 h	LB	LB + AMP
A/1	1.15	2.32		27
A/2	1.12	2.23	29	24
A/3	1.44	3.15	41	41
B/1	2.07	3.30		160
B/2	1.80	2.75	130	130
B/3	2.00	3.05	90	99

[a] Anaerobic LB broth with 10% glucose plus 0.2 M $NaPO_4$ (pH 7.0) inoculated (1% [vol/vol] with cells after 55 generations in continuous culture (series A) or with cells from fresh stock cultures (series B).

levels in the TET-free continuous culture. In addition, the plasmid of appropriate size could be recovered from FBR2 culture samples.

Ethanol production and antibiotic resistance after continuous culture. After approximately 15 days or 55 generations (32 after TET removal), samples were taken from the continuous culture and used to inoculate (1% [vol/vol]) anaerobic batch cultures containing no TET. These cultures produced high levels of ethanol at high rates, and essentially all cells were resistant to AMP, the other antibiotic gene on pLOI297 in strain FBR2. These trends continued even after three serial transfers (Table 3). Simultaneously, cultures were inoculated with cells from fresh batch stock cultures and yielded similar results. To assess maximal ethanol production, pH-controlled anaerobic fleaker fermentors were also inoculated with the same cells from the continuous culture. These fermentors produced approximately 3.6% ethanol, compared with 4.1% with fermentors simultaneously inoculated from fresh stock cultures. These data clearly indicate that high levels of ethanol production and genetic stability can be maintained by *E. coli* FBR2 after prolonged anaerobic growth in the absence of antibiotics with glucose-limited continuous culture which is followed by several batch cultures.

Thus, the requirement for antibiotics, a major economic and environmental drawback to the use of *pet* operon-containing, ethanologenic *E. coli* strains, no longer exists, provided that anaerobic growth conditions are used. It seems that scale-up and pilot plant ethanol production from purified sugars or sugar hydrolysates derived from corn fiber or other biomass would be feasible with *E. coli* FBR1 and FBR2. We are now exploring these possibilities, along with further genetic improvements in these strains, such as introducing mutations to eliminate succinate as a fermentation product.

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REFERENCES

1. **Alterthum, F., and L. O. Ingram.** 1989. Efficient ethanol production from glucose, lactose, and xylose by recombinant *Escherichia coli*. Appl. Environ. Microbiol. **55:**1943–1948.
2. **Beall, D. S., K. Ohta, and L. O. Ingram.** 1991. Parametric studies of ethanol production from xylose and other sugars by recombinant *Escherichia coli*. Biotechnol. Bioeng. **38:**296–303.
3. **Conway, T., Y. A. Osman, J. I. Konnan, E. Hoffman, and L. O. Ingram.** 1987. Promoter and nucleotide sequences of the *Zymomonas mobilis* pyruvate decarboxylase. J. Bacteriol. **169:**949–954.
4. **Conway, T., G. W. Sewell, Y. A. Osman, and L. O. Ingram.** 1987. Cloning and sequencing of the alcohol dehydrogenase II gene from *Zymomonas mobilis*. J. Bacteriol. **169:**2591–2597.
5. **Hanahan, D., J. Jessee, and F. R. Bloom.** 1991. Plasmid transformation of *Escherichia coli* and other bacteria. Methods Enzymol. **204:**63–113.
6. **Hespell, R. B.** Fermentation of xylan, corn fiber, or sugars to acetoin, and butanediol by *Bacillus polymyxa* strains. Curr. Microbiol., in press.
7. **Ingram, L. O., and T. Conway.** 1988. Expression of different levels of ethanologenic enzymes from *Zymomonas mobilis* in recombinant strains of *Escherichia coli*. Appl. Environ. Microbiol. **54:**397–404.
8. **Ingram, L. O., T. Conway, D. P. Clark, G. W. Sewell, and J. F. Preston.** 1987. Genetic engineering of ethanol production in *Escherichia coli*. Appl. Environ. Microbiol. **53:**2420–2425.
9. **Lawford, H. G., and J. D. Rousseau.** 1995. Loss of ethanologenicity in *Escherichia coli* B recombinants pLOI297 and KO11 during growth in the absence of antibiotics. Biotechnol. Lett. **17:**751–756.
10. **Lawford, H. G., and J. D. Rousseau.** 1995. Factors contributing to the loss of ethanologenicity of *E. coli* recombinants pLOI297 and KO11. Appl. Biochem. Biotechnol. **57/58:**277–292.
11. **Mat-Jan, F., K. Y. Alam, and D. P. Clark.** 1989. Mutants of *Escherichia coli* deficient in the fermentative lactate dehydrogenase. J. Bacteriol. **171:**342–348.
12. **Ohta, K. D., D. S. Beall, J. P. Mejia, K. T. Shanmugam, and L. O. Ingram.** 1991. Genetic improvement of *Escherichia coli* for ethanol production: chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl. Environ. Microbiol. **57:**893–900.
13. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.