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Mutants of *Escherichia coli* Defective in Acid Fermentation

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ABSTRACT

Wild type *E. coli* ferments glucose to a mixture of ethanol and acetic, lactic, formic, and succinic acids. Mutants defective in acid production have now been isolated, including those defective in lactate dehydrogenase (LDH) or with excess alcohol dehydrogenase. These mutations had no phenotype without a *pfl* mutation. Novel mutants affecting acetate metabolism were isolated by insertion of the fusion vector Mudl. These *aceG* mutants cannot grow anaerobically on glucose or aerobically on acetate yet lack the pleiotropic growth defects of previously known *pta/ack* mutants. In some genetic backgrounds acetate negative mutations suppress the growth defects of *adh* mutations. These results are discussed in terms of redox balance.

Index Entries: Anaerobic growth; alcohol dehydrogenase; lactate fermentation; acetic acid, redox balance.

INTRODUCTION

When wild type *Escherichia coli* is grown under anaerobic conditions, sugars are fermented to a variety of products (Fig. 1). There are two major alternative fates for pyruvate. Under acidic conditions pyruvate is largely converted to lactate by the fermentative lactate dehydrogenase (LDH) so consuming one NADH (i.e., two reducing equivalents) per 3C. This balances the production of one NADH per 3C by the glyceraldehyde phosphate dehydrogenase step of glycolysis. The NAD-linked LDH is soluble enzyme which must not be confused with the FAD-linked,

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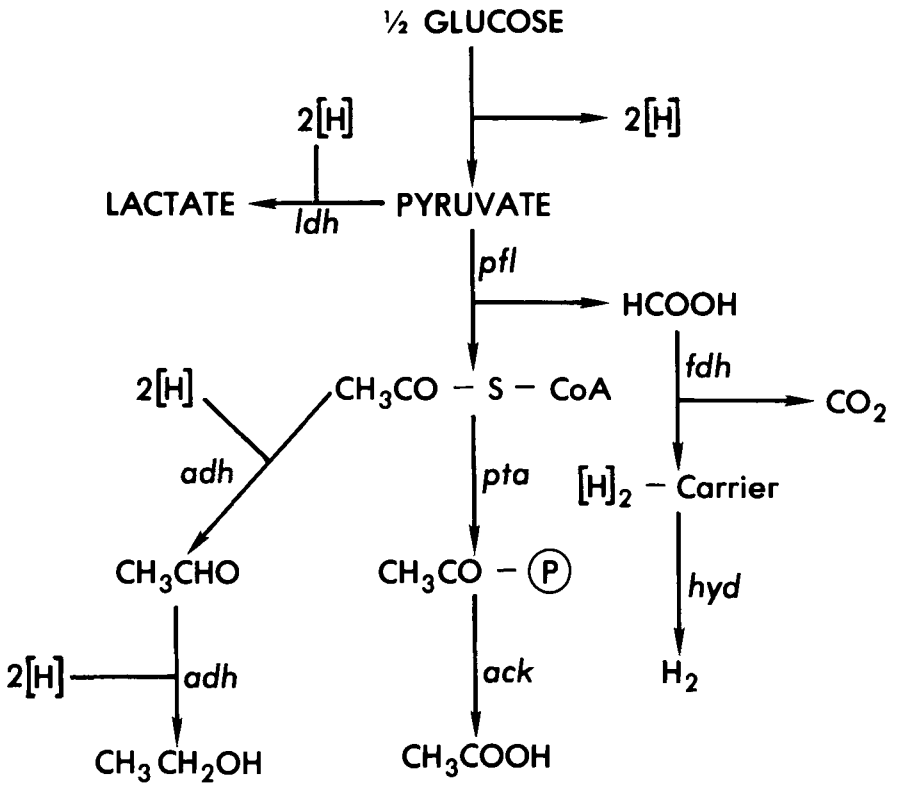

Fig. 1. Fermentation in *Escherichia coli*. The fate of pyruvate is shown together with the flow of reducing equivalents. Genes indicated code for lactate dehydrogenase (*ldh*), alcohol and acetaldehyde dehydrogenase (*adh*), hydrogenase (*hyd*), formate dehydrogenase (*fdh*), pyruvate formate lyase (*pfl*), acetate kinase (*ack*), phosphotransacetylase (*pta*). 2H refers to a pair of reducing equivalents usually in the form of NADH.

membrane-bound, lactate "dehydrogenases" (lactate oxidases) involved in aerobic growth with lactate as carbon source.

The alternative route is more complex and starts with the cleavage of pyruvate to acetyl-CoA and formate by pyruvate formate lyase (PFL). The acetyl-CoA may be converted to acetate in two steps by the successive action of phosphotransacetylase (PTA) and acetate kinase (ACK) so generating one ATP per acetyl-CoA. However, this route consumes no NADH and in order to achieve redox balance an equal amount of acetyl-CoA must be converted to ethanol by the associated alcohol and acetaldehyde dehydrogenases that consumes two NADH per acetyl-CoA, but generates no energy. A variety of mutation affecting alcohol and acetaldehyde dehydrogenase have been under investigation in our laboratory over the last few years. (*1–3*). Mutants lacking PTA and ACK have been isolated by Guest (*4*) and cannot grow anaerobically on sugars. However, the known *ack* and *pta* mutants are also incapable of anaerobic respiration using fumarate as electron acceptor suggesting that they may not be in the structural genes for PTA/ACK. Mutants lacking the fermentative

LDH have not been reported before. This work compares the properties of previously known *adh* and *pta/ack* mutants with some new mutants affecting these two fermentation routes as well as mutants deficient in LDH.

Materials and Methods

Bacterial Strains and Media

All bacteria used were strains of *Escherichia coli* K-12 and are listed in Table 1. Minimal medium was medium E (5) supplemented with carbon sources (0.4%, w/v) and, where required, amino acids (50 mg/L) and vitamins (5 mg/L). Tryptone broth contained 10 g tryptone and 5 g NaCl/L. Rich broth was tryptone broth plus yeast extract (1 g/L). For aerobic proton suicide selections, half strength tryptone broth was supplemented with a sugar (0.8%, w/v) and equimolar sodium bromide plus sodium

Table 1
Bacterial Strains

Strain	Relevant characteristics	Source/ref
DC271	*fadR*	(*1,2*)
DC272	*fadR, adhC81*	(*1,2*)
DC511	Acetate negative fusion of MC4100	*See* text
DC512	Acetate negative fusion of MC4100	*See* text
DC864	*adhE2* of MC4100	This work
DC865	*adhE2* of W1485	This work
DC866	*adhE2* of LCB320	This work
DC867	*adhE478* of W1485	This work
DC868	*adhE478* of LCB320	This work
FMJ32	*ldh* of LCB898	*See* text
FMJ33	*ldh* of LCB898	*See* text
FMJ38	*ldh* of LCB898	*See* text
FMJ39	*ldh* of LCB898	*See* text
FMJ112	pfl^+ of FMJ39	*See* text
JRG1078	*facA*	(*4*)
LCB320	*thi-1 thr leu rpsL*	M.C. Pascal
LCB898	*pfl-1* of LCB320	M.C. Pascal
MC4100	DE (*argF-lac*) *araD relA ptsF rbsR rpsL*	M. Casadaban
PRC118	*adhE478* of MC4100	This work
PRC478	*adhE478* of DC272	(*3*)
RMK6	ADH overproducer of DC271	Ana-PS[a]
RMK8	ADH overproducer of DC271	Ana-PS
RMK3	LDH deficient of DC271	Ana-PS
RMK14	LDH deficient of DC271	Ana-PS
RMK24	LDH deficient of DC271	Ana-PS
TA3516	DE (*pta, ack*)	(*10*)
W1485	Wild type	B. Bachmann
WL2	*adhE2* of DC272	(*2*)
WNK120	Proton suicide of MC4100	(*6*)

[a]Ana-PS = Anaerobic proton suicide mutants.

bromate (50–150 μ*M*, as indicated). The selective medium was usually adjusted to a mildly acidic pH in order to reduce the level of bromate plus bromide required, as described by Winkelman and Clark (*6*). Anaerobic proton suicide was carried out on M9 minimal agar containing glycerol (0.4%), fumarate (0.2%), proteose peptone (0.1%), and glucose (0.8%). Less than 10 μ*M* each of bromide and bromate was sufficient even though this medium is initially buffered to pH 7.0. Tetrazolium indicator plates contained (per liter): tryptone (10 g), NaCl (5 g), sugar (8 g), triphenyl tetrazolium chloride (0.05 g), and agar (15 g). MacConkey indicator plates contained 1% (w/v) of the appropriate sugar added to Bacto MacConkey Agar Base. The PPPS medium contains the nutrients of MacConkey base medium with the dyes and bile salts omitted, i.e., 17 g of bacto-peptone, 3 g of proteose-peptone, and 5 g of NaCl/L. Solid media contained bacto-agar (1.5% wt/vol). Anaerobic growth was performed in Oxoid anaerobic jars under a H_2/CO_2 atmosphere generated by means of Oxoid Gas Generating Kits. All media used for anaerobic growth were supplemented with trace elements as follows: $FeSO_4$ (50 μM), H_2SeO_3 (5 μM), and $(NH_44)_6Mo_7O_{24}$ (5 μM with respect to Mo). Growth was followed turbidometrically using a Klett-Sumerson colorimeter equipped with a green (540 nm) filter. Aerobic liquid cultures of 10 mL vol, were incubated in 150 mL culture flasks and shaken rapidly in a New Brunswick G76 gyrotory water bath (speed setting 6). Anaerobic liquid cultures were grown without agitation in tubes inside and anaerobic jar. Resazurin indicators were used to ensure anaerobic conditions. Cultures were grown for 3–4 cell doublings before beta-galactosidase assay.

Genetic Manipulations

A set of Tn*10* containing Hfr strains with points of origin scattered around the *E. coli* chromosome was used for mapping. The Tn*10* insertion in each Hfr strain was close to and behind the origin of transfer and thus acted as a proximal marker which could be positively selected. This set of Hfrs was constructed in the laboratory of Dr. B. Wanner, and made available by Dr. B. Bachmann of the Coli Genetic Stock Centre. Each fusion strain was crossed with each Hfr using standard procedures (*7*). A 30-min mating period was used. Exconjugants were selected on rich broth agar containing tetracycline (10 mg/L) to select for transfer of the Tn*10* into the recipient and streptomycin (100 mg/L) to select against the donor. Colonies obtained were checked for the retention or loss of the fusion phage be testing for sensitivity to ampicillin.

Transductions were carried out with P1*vir* essentially as described by Miller (*7*). *Tn10*-containing transductants were selected by addition of tetracycline (10 mg/L) to solid media.

Enzyme Assays

Beta-galactosidase was assayed as previously described (*8*) except that cultures were grown to mid-exponential phase (approx. 5×10^8 cells/ml) before assay in a variety of media, under both aerobic and

anerobic conditions. The units of beta-galactosidase activity are micromoles of *o*-nitrophenylgalactoside hydrolyzed/10^9 cell/h, at 37°C. Alcohol deydrogenase was assayed as before (*1,3*). Acetate kinase and phosphotransacetylase assays have been described (*4*) as has the LDH assay procedure (*8*).

RESULTS

Acetate Related Mutants

We have been previously shown that mutants defective in alcohol dehydrogenase (ADH) cannot grow anaerobically on sugars such as glucose or sugar alcohols such as sorbitol, yet can grow by anaerobic respiration using fumarate or nitrate as an electron acceptor (*3*). The phosphotransacetylase (*pta*) and acetate kinase (*ack*) mutants of Guest (*4*) or Ames (*10*) were tested for growth on a variety of carbon sources both aerobically and anaerobically. As shown in Table 2 (*pta/ack*) mutants are defective in anaerobic fermentation as already known (*4,10*). However, the *pta/ack* mutants were also defective in anaerobic respiration with fumarate, although not with nitrate. This suggested that the *pta/ack* mutations which are closely linked to the menquinone genes required for fumarate respiration (*4,11*) might be regulatory mutations, affecting several aspects of anaerobic growth. We, therefore, isolated new acetate mutants by two approaches. The first approach was the proton suicide technique of Winkelman and Clark (*6*) which selects for the survival of mutants which no longer produce acid. Acid producers are killed by the reaction of protons released into the medium with a bromide/bromate mixture that generates elemental bromine.

Selection of acid nonproducers on glucose medium aerobically gave a variety of mutants (*6*). However, among these were some incapable of aerobic growth on acetate. Mutants defective in ACK or PTA grow poorly

Table 2
Growth Properties of Acetate Mutants[a]

		Aerobic	Anaerobic		
Strain	Mutation	ACE	GLC	GRL/NIT	GRL/FUM
MC4100	Wild type	+ +	+ +	+ +	+ +
DC511	Ace fusion	—	—	+ +	+ +
DC512	Ace fusion	—	—	+ +	+ +
JRG1078	*facA*	—	—	+ +	—
TA3516	DE (*pta, ack*)	—	—	+ +	—
W1485	Wild type	+ +	+ +	+ +	+ +
WNK120	Ace (PS)[b]	—	—	+ +	+ +

[a]ACE = acetate, GLC = glucose, GRL/NIT = glycerol plus nitrate, GRL/FUM = glycerol plus fumarate.

[b]WNK120 is an acetate negative mutant of MC4100 by proton suicide.

or not at all on acetate depending on the strain background and we therefore thought these mutants were in *pta* or *ack*. Although some were indeed in *pta/ack* as demonstrated by cotransduction with a nearby Tn*10* insertion, the majority were at another, as yet unidentified locus (*6*). These novel acetate negative strains are exemplified by WNK117, WNK120, and WNK145.

Our second approach was to screen a bank of random insertions of the *lac* fusion vector MudI(Ap^r,*lac*). This bank of random gene fusions has been previously described (*12,13*). Eight gene fusion mutants incapable of aerobic growth on acetate were isolated and most were defective in the glyoxylate cycle. However two mutants were found that behaved very much like *pta/ack* mutants except that their mutations did not cotransduce with the Tn*10* insertion close to *pta/ack*. Moreover they differed from previously isolated *pta/ack* mutants in using fumarate as an anaerobic electron acceptor (Table 2)/ These mutants DC511 and DC512, therefore behave as expected if they had defects in the structural genes for ACK or PTA.

The strains DC511 and DC512 together with the WNK proton suicide isolates therefore represent a novel type of acetate defective mutation. Preliminary mapping experiments indicate a location in the 80–90 min region, far from the known *pta/ack* genes.

Revertants of ADH Mutants

Mutants defective in PFL can grow anaerobically on glucose by fermenting pyruvate to lactate so long as they are provided with small amounts of acetate for biosynthetic purposes. However, neither *adh* nor *pta/ack* mutants (old or new) can grow under such conditions even though they have a functional lactate fermentation pathway. This suggests that it is not the lack of ADH/ACDH or ACK/PTA per se that is fatal, but the redox imbalance caused by production of acetic acid in the absence of ethanol or vice versa. If this is true, then double mutants with defects in both branches of the acetyl-CoA fermentation pathway should be able to grow anaerobically by lactate fermentation, in the same way as *pfl* mutants.

We contructed strains bearing the *adhC81 adhE2* and *adhC81 adhE478* mutations in several genetic backgrounds since preliminary observations had shown that different lines of *E. coli* K12 varied substantially in their anaerobic growth properties. Next, revertants of these *adhC adhE* strains were selected for growth on glucose minimal medium anaerobically. Revertants were purified and checked for anaerobic growth on glucose and aerobic growth on ethanol or acetate. Mutants regaining ADH in full constitutive levels should regain the ability to grow on ethanol in air (Eth$^+$) whereas those which have lost PTA and/or ACK should grow poorly or not at all on acetate aerobically (Ace$^-$). A third possibility consists of leaky mutants in PFL which produce enough acetate for biosynthesis.

We therefore tested for anaerobic growth on sorbitol, which is more reduced than glucose and yields 3 NADH per hexose. PFL defective mutants do not grow anaerobically on sorbitol irrespective of acetate supplemention since lactate fermentation can only dispose of 2 NADH per hexose. As shown in Table 3 all three types of revertant were found although not every parent strain gave each type of revertant. In addition, a fourth type of revertant (Ana^+ in Table 3) was found that was neither Ace^- nor Eth^+, but that could grow anaerobically on sorbitol. These revertants are not understood, but might be partial revertants of any of the other classes.

Anaerobic Proton Suicide

The proton suicide technique was adapted for anaerobic conditions by incorporating glycerol plus fumarate into the selection medium in order to allow the growth of fermentation defective mutants by fumarate respiration. Aerobically, concentrations of up to 150 μM bromide and bromate are required for selection. Anaerobically we found that 2–5 μM each of bromide plus bromate were sufficient. We selected mutants by anaerobic proton suicide and screened them for growth on a variety of carbon sources both aerobically and anaerobically. We found that almost none of these mutants had any observable phenotype. We then assayed about 20 mutants for ADH and LDH. We found several mutants that had substantially reduced levels of LDH and several other with an approximately two-fold increase in ADH. Selected examples are given in Table 4. The majority of the anaerobic proton suicide mutants remain enigmatic. Although we tried various parent strains and variant procedures we were unable to obtain an *ldh* mutant lacking more than about 75% of initial LDH activity by proton suicide.

Table 3
Reversion of *adh* Mutants

Strain	Mutation	Background	Revertant type obtained[a]			
			Eth^+	Ace^-	Pfl^-	Ana^+
WL2	*adhE2*	DC271	+	−	−	−
PRC478	*adhE478*	DC271	−	−	−	(+)
DC865	*adhE2*	W1485	+	−	−	+
DC867	*adhE478*	W1485	−	+	+	−
DC866	*adhE2*	LCB320	−	−	−	−
DC868	*adhE478*	LCB320	−	−	−	+
DC864	*adhE2*	MC4100	−	+	−	(+)
PRC118	*adhE478*	MC4100	−	−	−	(+)

[a] + and − indicate whether revertants with the properties indicated were isolated. (+) indicates revertants which grew extremely slowly under anaerobic conditions. The revertant classes are discussed in the text.

Table 4
Mutants from Anaerobic Proton Suicide

Strain	Enzyme level%[a]	
	LDH	ADH
RMK6	100	135
RMK8	100	165
RMK3	14	100
RMK14	13	100
RMK24	34	100

[a]Enzyme levels are given relative to the parental strain DC271. Assays were performed on anaerobic cultures grown in rich broth plus glucose. Twenty other mutants showed negligible changes in either enzyme.

Lactate Dydrogenase Mutants

A mutant defective in pyruvate format lyase, CB898 (*14*) was used as the parental strain. After mutagenesis colonies were replicated onto M9 minimal agar containing glucose plus acetate and one set-incubated aerobically, the other anaerobically. Mutants able to grow aerobically but not anaerobically were assayed for LDH. As Table 5 shows these mutants are completely or almost completely lacking LDH. The *pfl ldh* double mutants were unable to grow anaerobically on any fermentable sugar tested but could grow by anaerobic respiration using nitrate or fumarate as electron acceptors. When the *pfl ldh* mutants were transduced to pfl^+ so giving a strain (FMJ112) with *ldh* as the only defect, no anaerobic growth defects were seen.

DISCUSSION

We have extended our investigation of mutants affecting fermentation (*1–3*) by isolating those with defects in the pathways responsible for acetic and lactic acids. The proton suicide technique devised by our laboratory yields mutants defective in acid production (*6*). We isolated novel acetate defective mutants under aerobic conditions and strains partially defective in LDH using an anaerobic modification of this procedure. Similar novel acetate defective mutants were also found by screening a bank of gene fusions made by insertion of the fusion phage MudI (*12,13*). These novel acetate mutants were unable to grow anaerobically on fermentable sugars, yet could respire anaerobically with fumarate. In contrast, previously described mutants in the *pta/ack* locus at 50 min on the *E. coli* chromosome (*4,10*) were unable to grow anaerobically on sugars or fumarate plus glycerol. Preliminary results suggest a location in the 80–90 min region for the new acetate mutants (data not shown).

Starting with strains carrying defects in the *adhE* locus and lacking both alcohol dehydrogenase and the associated acetaldehyde de-

Table 5
Lactate Dehydrogenase Mutants

Strain	Defect	Anaerobic Growth on[a]			LDH activity %[b]
		GLC	GLC/ACE	SRL	
LCB320	None	+	+	+	100
LCB898	*pfl*	−	+	−	100
FMJ32	*pfl ldh*	−	−	−	3
FMJ33	*pfl ldh*	−	−	−	9
FMJ39	*pfl ldh*	−	−	−	0
FMJ112[c]	*ldh*	+	+	+	0

[a]GLC = glucose; GLC/ACE = glucose plus acetate; SRL = sorbitol.
[b]LDH specific activity is given relative to the parental strain.
[c]FMJ112 is a *pfl*$^+$ derivative of FMJ39.

hydrogenase we isolated revertants capable of growth on glucose anaerobically. The most interesting secondary mutants were not true revertants of *adh*, but had gained an additional defect that resulted in the loss of the ability to grow on acetate aerobically. Such secondary mutants were only isolatable from certain parental strains. In such "revertant" strains, an *adh* mutation preventing ethanol formation is present together with a mutation which presumably prevents interconversion of acetyl-CoA and acetic acid. Nevertheless, these double mutants can ferment glucose, presumably to lactic acid. In striking contrast are those strains that carry only a single defect and cannot grow on sugars anaerobically (*3,4*). This supports the idea that operation of only one branch of the pathway from acetyl-CoA to acetate or ethanol prevents growth as a result of redox imbalance. Production of acetic acid or ethanol in the absence of equal amounts of the other product would result in an incorrect ratio of NADH recycled to NADH produced during glycolysis. However, blocking both branches of this pathway allows redox balanced fermentation by the lactate pathway (Fig. 1).

Although anaerobic problem suicide gave only partially defective lactate dehydrogenase mutants, we were able to make fully deficient strains by starting with a *pfl* mutant (*14*). Derivatives unable to grow anaerobically even when supplemented with acetate for biosynthetic purposes were found to lack LDH. Removal of the *pfl* defect by transduction yielded strains containing only the *ldh* mutation. As expected, these single mutants had no detectable growth phenotype aerobically.

It is now possible to isolate mutants of *E. coli* with defects in any or all of the various branches of the fermentation scheme (Fig. 1) and the same procedures can be extended to other bacteria. Industrially useful microorganisms may be modified by eliminating or altering one or more fermentation pathway, however, it is necessary to maintain redox balance or the result will merely be a mutant unable to grow anaerobically. For example, *E. coli* cannot be converted to production of alcohol alone

merely by eliminating production of acetate and lactate; it is necessary to reroute the reducing equivalents normally lost as formate. In collaboration with Dr. L.O. Ingram (University of Florida, Gainesville) we have constructed a hybrid organism containing the cloned pyruvate decarboxylase of *Zymomonas* in a fermentation defective *E. coli* host. Pyruvate is all converted to acetaldehyde and no reducing equivalents are lost as formate. The acetaldehyde is then converted to ethanol by the *E. coli* host alcohol dehydrogenase and thus is the only major fermentation product (*15*).

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REFERENCES

1. Clark, D. P., and Cronan, J. E. (1980), *Escherichia coli* mutants with alter control of alcohol dehydrogenase and nitrate reductase. *J. Bacteriol.* **141,** 177–183.
2. Lorowitz, W., and Clark, D. P. (1982), *Escherichia coli* mutants with a temperature-sensitive alcohol dehydrogenase. *J. Bacteriol.* **152,** 935–938.
3. Cunningham, P. R., and Clark, D. P. (1986), The use of suicide substrates to isolate mutants of *Escherichia coli* lacking enzymes of alcohol fermentation. *Mol. Gen. Genet.* **205,** 487–493.
4. Guest, J. R. (1979), Anaerobic growth of *Escherichia coli* K-12 with fumarate as terminal electron acceptor. Genetic studies with menaquinone and fluoroacetate resistant mutants. *J. Gen. Microbiol.* **115,** 259–271.
5. Vogel, H. J., and Bonner, D. M. (1956), Acetylornithinase in *Escherichia coli*. *J. Biol. Chem.* **218,** 97–103.
6. Winkelman, J. W., and Clark, D. P. (1984), Proton suicide: General method for direct selection of sugar transport and fermentation-defective mutants. *J. Bacteriol.* **160,** 687–690.
7. Miller, J. H. (1972), *Experiments in Molecular Genetics,* Cold Spring Harbor Laboratory. Cold Spring Harbor, NY.
8. Tarmy, E. M. and Kaplan, N. O. (1968), Chemical characterization of D-lactate dehydrogenase from *Escherichia coli*. *J. Biol. Chem.* **243,** 2579–2586.
9. Winkelman, J. W., and Clark, D. P. (1986), Anaerobically induced genes of *Escherichia coli*. *J. Bacteriol* **167,** 362–367.
10. LeVine, N. M., Ardeshir, F. and Ames, G. F-L. (1980), Isolation and characterization of acetate kinase and phosphotransacetylase mutants of *Escherichia coli* and *Salmonella typhimurium*. *J. Bacteriol.* **143,** 1081–1085.
11. Bachmann, B. (1983), Linkage map of *Escherichia coli* K12, edition 7. *Microbiol. Rev.* **47,** 180–230.
12. Clark, D. P. (1981), Regulation of fatty acid degradation in *Escherichia coli*: Analysis by operon fusion. *J. Bacteriol.* **148,** 521–526.
13. Clark, D. P. (1984), The number of anaerobically regulated genes in *Escherichia coli*. *FEMS Microbiol. Lett.* **24,** 251–254.

14. Varenne, S., Casse, F., Chippaux, M. and Pascal, M. C. (1975), A mutant of *Escherichia coli* deficient in pyruvate formate lyase. *Molec. Gen. Genet.* **141,** 181–184.
15. Ingram, L. O., Conway, T. Clark, D. P., Sewell, G. S., and Preston, J. F. (1987), Genetic engineering of ethanol production in *Escherichia coli*. *J. Appl. Environ. Microbiol.* In press.