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Mutants of *Escherichia coli* Deficient in the Fermentative Lactate Dehydrogenase

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Mutants of *Escherichia coli* deficient in the fermentative NAD-linked lactate dehydrogenase (*ldh*) have been isolated. These mutants showed no growth defects under anaerobic conditions unless present together with a defect in pyruvate formate lyase (*pfl*). Double mutants (*pfl ldh*) were unable to grow anaerobically on glucose or other sugars even when supplemented with acetate, whereas *pfl* mutants can do so. The *ldh* mutation was found to map at 30.5 min on the *E. coli* chromosome. The *ldh* mutant FMJ39 showed no detectable lactate dehydrogenase activity and produced no lactic acid from glucose under anaerobic conditions as estimated by in vivo nuclear magnetic resonance measurements. We also found that in wild-type strains the fermentative lactate dehydrogenase was conjointly induced by anaerobic conditions and an acidic pH. Despite previous findings that phosphate concentrations affect the proportion of lactic acid produced during fermentation, we were unable to find any intrinsic effect of phosphate on lactate dehydrogenase activity, apart from the buffering effect of this ion.

When facultative anaerobes such as *Escherichia coli* ferment sugars to pyruvate under anaerobic conditions, there are two major alternative pathways for the formation of terminal fermentation products. The more complex pathway involves splitting the pyruvic acid into acetyl coenzyme A and formic acid by means of pyruvate formate lyase (11, 24). The acetyl coenzyme A is then converted to an approximately equal mixture of ethanol and acetic acid (24, 25). The simpler alternative pathway involves the direct conversion of pyruvate to lactic acid in a single step catalyzed by the fermentative lactate dehydrogenase (LDH) (28).

E. coli contains three LDHs. Two of these are membrane-bound flavoproteins which couple to the respiratory chain and are better described as lactate oxidases (12, 14). These enzymes, one specific for the D isomer and the other specific for the L isomer, are required for aerobic growth on lactate (12, 14, 22). The conversion of pyruvate to lactic acid under anaerobic conditions is catalyzed by a third enzyme (28). This is a soluble, NAD-linked enzyme that is specific for the production of D-lactic acid (27, 28). The fermentative LDH has been purified and is allosterically activated by its substrate, pyruvate (28, 29). The fermentative LDH is found in both aerobically and anaerobically grown cultures (27, 28). In contrast, the accumulation of lactic acid by fermenting *E. coli* occurs only under anaerobic conditions and increases at acidic pH (25, 30). We have further investigated this aspect of fermentation by measuring LDH enzyme activity under various conditions.

Mutants lacking pyruvate formate lyase (*pfl* mutants) cannot grow anaerobically on glucose minimal medium due to their inability to generate acetyl coenzyme A for biosynthesis. However, provision of acetate allows them to grow by lactate fermentation (31). Even in the presence of exogenous acetate *pfl* mutants cannot grow anaerobically on sorbitol or other sugar alcohols, since the excess reducing equivalents relative to glucose cannot be disposed of by lactic acid and the *pfl* mutants cannot make the more highly reduced fermentation product ethanol. We have used these properties of *pfl* mutants to isolate mutants deficient in the fermentative LDH. Such *ldh* mutants were characterized to assess the physiological importance of lactate production.

MATERIALS AND METHODS

Bacterial strains, media, and genetic methods. All bacteria were strains of *E. coli* K-12 except for strain C1, which is the *E. coli* C type strain (Table 1). Rich broth contained (per liter) tryptone (10 g), NaCl (5 g), and yeast extract (1 g). Minimal medium M9 (15) was supplemented with carbon sources at 0.4% (wt/vol) and, where appropriate, with amino acids (50 mg/liter). Solid media contained 1.5% (wt/vol) Difco Bacto-Agar. Anaerobic growth was performed in Oxoid anaerobic jars under an H_2-CO_2 atmosphere generated by Oxoid gas-generating kits. All anaerobic growth media were supplemented with trace elements Fe (50 μM), Se (5 μM), Mo (5 μM), and Mn (5 μM) as previously described (8, 34). Colonies were stained for pyruvate formate lyase by the benzyl viologen procedure (31). Methods for mutagenesis with ethyl methane sulfonate, for transduction with phage P1 *vir*, and for mapping with the Hfr::Tn*10* set (32) have been detailed in recent publications from this laboratory (1, 34).

Enzyme assays. Soluble extracts were made by breaking cells in the French press followed by ultracentrifugation to remove membrane fragments. This procedure was previously used to prepare extracts for the assay of alcohol dehydrogenase (5, 6). The NAD-linked LDH was assayed by a slight modification of the method of Tarmy and Kaplan (28). One unit of LDH activity is 1 A_{340} unit per min.

NMR. The nuclear magnetic resonance (NMR) experiments were modeled on the work of Ogino et al. (16, 17), who monitored the synthesis of fermentation products by in vivo NMR scans of whole cultures. Cells were grown in M9 medium (pH 7.2) with 0.1 M glucose as the sole carbon source. When the cell density reached approximately 5×10^8/ml the cells were collected by centrifugation at 7,000 rpm (5,900 × *g*) for 2 min at 5°C. The cell pellets were suspended in M9 buffer and washed twice with the same buffer. The cells were finally suspended in M9 buffer (pH 7.2) containing 0.1 M glucose and anaerobic trace metals at a cell density of 5×10^8/ml. A 0.9-ml sample of cell suspension was placed in

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TABLE 1. Bacterial strains

Strain	Relevant characteristics	Source
C1	*E. coli* C wild type	B. Bachmann
DC271	*mel fadR supR*	D. Clark
		Laboratory collection
DC655	*recE101*::Tn*10* of JRG861a	P1 (JF1096) × JRG861a
DC706	*fnr-1 recE101*::Tn*10 relA1 spoT1*	P1 (DC655) × DC271
FMJ32 through 41	*ldh* derivatives of LCB898	See text
FMJ42	*aroA273*::Tn*10* of W1485	P1 (LCB273) × W1485
FMJ44 and 45	*aroA273*::Tn*10 pfl*$^+$ of FMJ32	P1 (FMJ42) × FMJ32
FMJ46 and 47	*aroA273*::Tn*10 pfl*$^+$ of FMJ34	P1 (FMJ42) × FMJ38
FMJ48 and 49	*aroA273*::Tn*10 pfl*$^+$ of FMJ38	P1 (FMJ42) × FMJ39
FMJ50 and 51	*aroA273*::Tn*10 pfl*$^+$ of FMJ39	P1 (DC706) × FMJ39
FMJ92	*recE101*::Tn*10 ldh* of FMJ39	P1 (DC706) × FMJ39
FMJ93	*recE101*::Tn*10 fnr ldh* of FMJ39	P1 (DC706) × FMJ39
FMJ102	*zda-268*::IS*10* Cam^r IS*10 fnr ldh* of FMJ39	P1 (KF1340) × FMJ39
FMJ105	*zda-268*::IS*10* Cam^r IS*10 fnr ldh*$^+$ of FMJ39	P1 (KF1340) × FMJ39
FMJ106	*recE101*::Tn*10* of LCB898	P1 (FMJ93) × LCB898
FMJ112	*aro*$^+$ of FMJ50	P1 (LCB320) × FMJ50
HB235	F$^-$ *thr-1 ara-14 leuB6 tonA31 tsx-78 trg-2*::Tn*10 eda-50 hisG4 rpsL136 xyl-5 mtl-1 thi-1*	G. Hazelbauer
JP2781	F$^+$ *his29*(Am) *kdpABC5 zch352*::Tn*10 tyrR366*	J. Pittard
JRG861a	*fnr-1*	J. Guest
KF1096	F$^-$ *fnr-1 trpA9761 gal-25 rpsL recE101*::Tn*10*	K. Fouts
KF1340	F$^-$ *lacZ4 gal-44 supE44 endA1 his-317 zda-268*::IS*10* Cam^r IS*10*	K. Fouts
LCB273	F106-3 *aroA273*::Tn*10 lacY gal pyrD trp*::Tn*5 mal thi* Val^r	M. Pascal
LCB320	F$^-$ *thr-1 leu-6 thi-1 lacY tonA22 strA*	M. Pascal
LCB898	*pfl-1* of LCB320	M. Pascal
MC4100	F$^-$ *araD* DE (*argF-lac*) *relA rpsL thiA flbB deoC ptsF*	M. Casadaban
UB1005	*metB nalA rel fru*	U. of Bristol
W1485	F$^-$ wild type	B. Bachmann
W3110	F$^-$ IN (*rrnD-rrnE*)*1*	B. Bachmann

a 5-mm NMR tube with 0.1 ml of D_2O. The suspension was bubbled with argon to remove oxygen. The cell suspension was incubated at 30°C for 4 h, and proton NMR spectra were measured with a Varian VXR-500 spectrometer operating at 500 MHz. The parameters used were as follows: pulse width, 1.5 μs; delay time, 5 s; 300 acquisitions per spectrum. The field was locked on the D_2O, and H_2O was used as a reference peak (4.65 ppm). Proton chemical shifts are reported in parts per million upfield from H_2O. Authentic samples of fermentation products were dissolved in the same M9 buffer to assign the NMR signals. The metabolites shown in the figures are from low to high field, succinate, pyruvate, acetate, lactate, and ethanol. A formate peak (not shown) was also identified at 8.39 ppm.

RESULTS

Regulation of LDH activity by pH and carbon source. Several factors were investigated for their effect upon LDH activity in several wild-type strains of *E. coli* K-12. These factors included the optimum pH for LDH activity in vitro, the pH of the growth medium, the type of buffer used, and the nature of the sugar fermented. The effect of pH on LDH activity in vitro was measured by using several buffers with pK_a values ranging from 5.0 to 10.0 at a final concentration of 50 mM. The source of soluble cell extract was strain W1485 grown anaerobically in rich broth plus glucose. The optimal pH for LDH activity was found to be between pH 6.5 and 7.0 when assayed in the direction of pyruvate reduction. Similar results were found by Tarmy and Kaplan (29) for LDH from *E. coli* B.

The acidity of the growth medium influences lactic acid accumulation by fermenting cells (24, 25, 30). To examine this, three sets of anaerobic cultures were grown in rich broth plus glucose; one set was buffered with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 8.0), another was buffered with TAPS [*N*-(Tris)hydroxymethyl methyl-3-aminopropane sulfonic acid] (pH 9.0), and the other was unbuffered. To buffered cultures, neutral red dye (pK_a 6.7) was added to a concentration of 0.03 g/liter. The buffered cultures were harvested just as the dye turned red, indicating that the medium had just become mildly acidic. One set of unbuffered cultures was harvested after equivalent times of growth (approximately 10 to 12 h), and another was grown for 18 h. The pH of the medium at the end of the growth period varied slightly among strains, but overall the pH of unbuffered cultures was substantially more acidic than for those grown in buffered medium. LDH activity was induced about 6- to 10-fold in more acidic medium in most strains (Table 2). One exception was strain UB1005, a derivative of W1655, which showed little acidification of the medium upon anaerobic growth and low LDH activities.

We also grew anaerobic cultures with various concentrations of phosphate buffer (pH 8.0). The more buffer, the less the acidification of the growth medium, and the less LDH activity was observed (data not shown). Although we tested a variety of cultural conditions, changes in LDH activity were all attributable to the pH, and we were unable to observe an effect of phosphate per se as has been previously suggested (11, 25). We also grew these strains aerobically in the same medium buffered to pH 5.0 with 50 mM citrate, to pH 7.0 with morpholinepropanesulfonic acid, or to pH 9.0 with AMPSO [3(*N*-α,α-dimethyl hydroxyethyl)amino-2-hydroxypropane sulfonic acid]. We found LDH levels of 1.7 to 2.1 U/mg of protein at pH 9.0 (i.e., very similar to the basal anaerobic levels with 200 mM TAPS [pH 9.0]). However, at

TABLE 2. Effect of acidity on LDH activity[a]

Strain	Buffer	(mM)	Initial pH	Final pH	LDH activity
DC271	None (18 h)[b]		6.8	4.7	15.0
	None		6.8	5.7	11.9
	HEPES	(50)	8.0	6.8	4.0
	HEPES	(200)	8.0	7.1	3.6
	TAPS	(50)	9.0	6.7	4.6
	TAPS	(200)	9.0	7.4	1.5
MC4100	None (18 h)		6.8	4.7	15.2
	None		6.8	5.7	17.7
	HEPES	(50)	8.0	6.6	4.9
	HEPES	(200)	8.0	7.2	2.8
	TAPS	(50)	9.0	6.8	4.1
	TAPS	(200)	9.0	7.3	2.8
W1485	None (18 h)		6.8	4.8	18.0
	None		6.8	5.6	16.3
	HEPES	(50)	8.0	6.4	9.7
	HEPES	(200)	8.0	7.3	2.5
	TAPS	(50)	9.0	6.9	4.1
	TAPS	(200)	9.0	7.2	2.0
UB1005	None (18 h)		6.8	7.0	4.1
	None		6.8	6.5	7.5
	HEPES	(50)	8.0	7.4	5.2
	HEPES	(200)	8.0	7.5	2.6
	TAPS	(50)	9.0	7.2	3.5
	TAPS	(200)	9.0	7.1	3.2

[a] LDH activity is expressed as units per milligram of protein.
[b] Grown for 18 h. All cultures were grown anaerobically in rich broth medium plus glucose.

pH 5.0 the aerobic cultures gave only a twofold increase (from 3.4 to 3.9 U/mg of protein). Thus acid induction is barely significant during aerobic growth.

Strain W1485 was grown anaerobically for 18 h with several different sugars without buffer or with 50 mM HEPES (pH 8.0) as above. The same general pattern was observed here in that the more acidic the growth medium, the higher the LDH activity. Whereas growth on glucose showed a fivefold acid induction of LDH, sorbitol, fructose, and rhamnose showed less medium acidification and only a two- to threefold induction. Little pH effect was seen with gluconate.

Isolation of LDH mutants. Mutants that lacked the fermentative LDH activity were isolated by using strain LCB898, which is defective in pyruvate formate lyase and therefore unable to grow anaerobically on glucose unless acetate is provided. This strain was grown in rich broth and mutagenized with ethylmethane sulfonate. After overnight growth in rich broth to allow segregation, samples of the mutated cells were plated out on M9-glucose agar and incubated aerobically. Several hundred colonies were picked and screened for anaerobic growth on M9-glucose agar with 0.1% acetate added. Isolated colonies that were unable to grow anaerobically on glucose plus acetate but which grew well aerobically were kept and assayed for LDH activity. Other samples of the mutagenized culture were subjected to anaerobic penicillin selection in M9 with glucose plus acetate. Samples were plated on M9-glucose agar aerobically, and the surviving colonies were screened for anaerobic growth on glucose and acetate, as before. Ten mutants were isolated with various degrees of LDH deficiency, from one that completely lacked detectable LDH activity to others in which LDH levels were reduced by about 90%. These mutants did not grow anaerobically on glucose plus acetate in minimal medium yet grew well on the same medium in air.

TABLE 3. LDH mutants of *E. coli*

Strain	Genotype	LDH activity[a]	Activity ratio[b]
LCB320	Wild type	1.33	
LCB898[c]	*pfl*	1.86	1.00
FMJ32	*pfl ldh-2*	0.06	0.03
FMJ33	*pfl ldh-3*	0.06	0.03
FMJ34	*pfl ldh-4*	0.07	0.04
FMJ35	*pfl ldh-5*	0.09	0.05
FMJ36	*pfl ldh-6*	0.06	0.03
FMJ37	*pfl ldh-7*	0.09	0.05
FMJ38	*pfl ldh-8*	0.16	0.09
FMJ39	*pfl ldh-9*	0.00	0.00
FMJ40	*pfl ldh-10*	0.18	0.10
FMJ41	*pfl ldh-11*	0.18	0.10

[a] LDH activity is given as units per milligram of protein. All strains were grown aerobically up to 200 KU in M9 medium with glucose (40 g/liter), casein amino acid (10 g/liter), and thiamine (5 mg/liter).
[b] Ratio of activity of mutant to activity of LCB898.
[c] Parental strain. All of FMJ32 through FMJ41 carry the *pfl-1* mutation of LCB898.

Four mutants were isolated directly from the mutagenized LCB898 culture; they were designated FMJ38 through FMJ41 (Table 3). Their LDH activity was reduced to about 10% of the wild-type level except for FMJ39, which completely lacked LDH activity. The other six mutants, FMJ32 through FMJ37, were isolated after penicillin selection and possessed from 3 to 5% of parental LDH activity (Table 3). It should be noted that LDH was assayed with aerobic cultures of these mutants because *pfl ldh* double mutants cannot grow anaerobically even in rich broth plus glucose. Values for strain LCB898 and its pfl^+ parent, LCB320, are provided for comparison in Table 3.

To observe the LDH activity of the mutants under anaerobic conditions, the growth defect due to the *pfl* mutation was removed. Four of the double mutants, FMJ32, FMJ34, FMJ38, and FMJ39, were transduced to pfl^+ with P1 grown on FMJ42, which carries a Tn*10* insertion in the nearby gene, *aroA*. Tetracycline-resistant transductants were selected, and those gaining the pfl^+ allele grew anaerobically on sorbitol or on glucose without acetate. These transductants were also checked with the *pfl* colony-staining method (see Materials and Methods). The wild-type strain, LCB320, and the pfl^+ transductants of the *ldh* mutants were grown anaerobically in rich broth plus glucose with or without HEPES buffer. Both the pH of the supernatants and the LDH activity of the cells were measured. The data confirmed that these *ldh* mutants are indeed deficient in LDH activity. This was most noticeable in buffered cultures. In particular FMJ50 and FMJ51, two pfl^+ derivatives of FMJ39, were completely lacking LDH activity with or without buffer (Table 4). Culture medium acidification substantially increased the amount of residual LDH activity in most other *ldh* mutants. For example, pfl^+ derivatives of FMJ38 had around 10% of parental LDH levels when buffered but 50% when unbuffered.

Growth properties of LDH mutants on different sugars. All of the LDH mutants and their derivatives were tested for aerobic and anaerobic growth on a variety of sugars in minimal medium. Glucose, fructose, maltose, rhamnose, xylose, sorbitol, and gluconate were used. The *ldh pfl* double mutants FMJ32 to FMJ41 were tested with or without 0.1% acetate added. Results were identical irrespective of the

TABLE 4. Effect of acidity on LDH activities[a] of LDH mutants

Strain[b]	*pfl*	Parent	Buffered[c]		Unbuffered	
			pH	LDH activity	pH	LDH activity
FMJ44	+	FMJ32	7.4	1.9	5.3	6.1
FMJ45	+	FMJ32	7.6	2.2	5.4	7.0
FMJ46	+	FMJ34	7.6	2.0	5.3	6.9
FMJ47	+	FMJ34	7.5	2.4	5.2	7.4
FMJ48	+	FMJ38	7.5	2.3	5.5	12.5
FMJ49	+	FMJ38	7.4	2.8	5.4	9.1
FMJ50	+	FMJ39	7.3	0.0	5.0	0.0
FMJ51	+	FMJ39	7.2	0.0	5.0	0.0
LCB898	−	LCB320	6.9	17.9	5.8	22.7
LCB320	+		6.8	10.4	5.7	15.2

[a] LDH activity is given as units per milligram of protein. All strains were grown anaerobically in rich broth medium plus glucose, aromatic amino acids, and thiamine.
[b] FMJ44 through FMJ51 are *aroA*::Tn*10* *pfl*$^+$ derivatives of the indicated *ldh* mutants.
[c] 50 mM HEPES, initial pH is 8.0.

acetate. Aerobically, all mutants grew on all sugars tested. As expected, the *pfl ldh* double mutants were unable to grow anaerobically on any fermentable sugar even with acetate added. However, they could grow by anaerobic respiration with nitrate or fumarate as an electron acceptor. When the *pfl ldh* double mutants were made *pfl*$^+$ by transduction, all of them could grow anaerobically on any fermentable sugar. To further confirm this, the *aroA*::Tn*10* insertion was removed from FMJ50 by transducing to *aro*$^+$ with P1 phage grown on LCB320 to give strain FMJ112, which only has the *ldh* defect; again no anaerobic growth defects were observed.

NMR analysis of fermentation. Cultures of LCB320 (wild type), LCB898 (*pfl*), FMJ39 (*pfl ldh*), and FMJ112 (*ldh*) were grown in minimal glucose medium to around 5×10^8 cells per ml. The cultures were centrifuged and suspended in minimal medium plus glucose and allowed to ferment anaerobically in an NMR tube at 30°C. Scans were collected at appropriate intervals. Sample scans after 4 h for LCB320 and FMJ112 appear in Fig. 1. Strains deficient in LDH did not make any observable lactic acid, whereas *ldh*$^+$ strains made substantial amounts. Both the *ldh* mutant FMJ112 and the wild type, LCB320, made ethanol and acetate as expected (Fig. 1). Upon prolonged (24 to 36 h) incubation the *ldh* strain FMJ112 accumulated substantial amounts of pyruvic acid, whereas this was not seen in the wild type (data not shown). These data demonstrate that the NAD-linked LDH that is missing in FMJ39 and its derivative FMJ112 is indeed responsible for the synthesis of lactic acid during fermentation.

Genetic mapping. FMJ39 (*pfl ldh*), a derivative of the *pfl* mutant LCB898, lacks the fermentative LDH and hence cannot grow anaerobically on glucose plus acetate. For

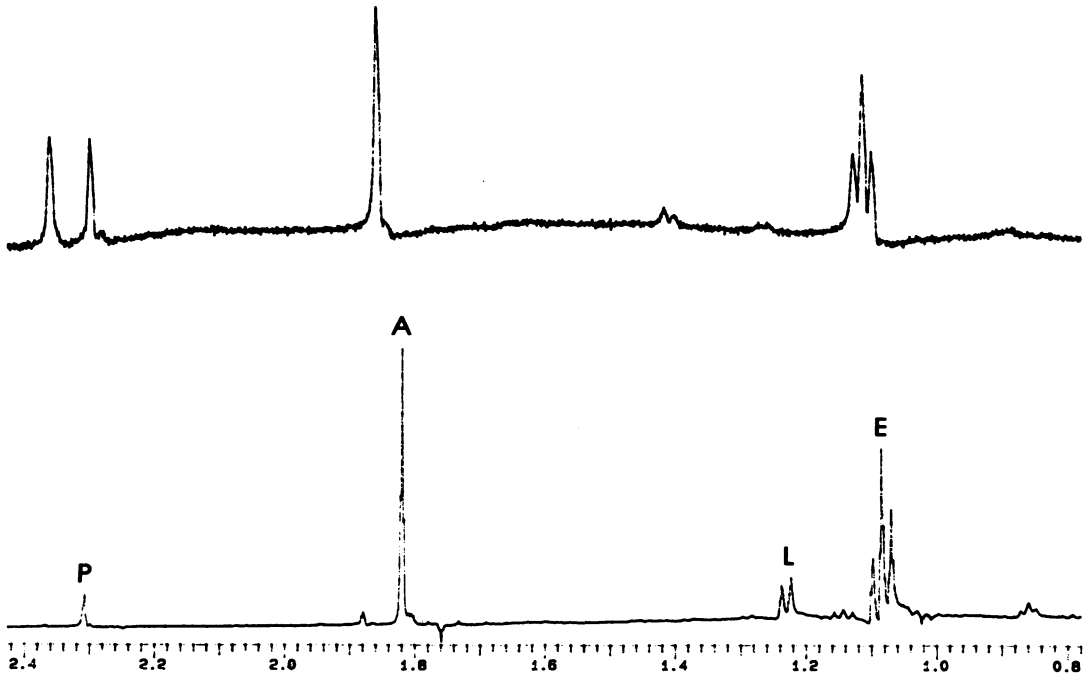

FIG. 1. In vivo NMR of fermentation products. Strain LCB320 (wild type) is shown in the lower panel, and strain FMJ112 (*ldh*) is shown above. Both strains were grown anaerobically in glucose minimal medium. Peaks: A, acetate; E, ethanol; L, lactate; P, pyruvate.

TABLE 5. Three-factor crosses

P1 Donor	Recipient[a]	Selected marker	No. of recombinants											
			Ldh$^+$ Fnr$^+$	Ldh$^+$ Fnr$^-$	Ldh$^-$ Fnr$^+$	Ldh$^-$ Fnr$^-$	Cam^r Ldh$^+$	Cam^r Ldh$^-$	Cam^s Ldh$^+$	Cam^s Ldh$^-$	Tet^r Ldh$^+$	Tet^r Ldh$^-$	Tet^s Ldh$^+$	Tet^s Ldh$^-$
DC706 *recE*::Tn*10 fnr*	FMJ39 *pfl ldh*	Tet	134	76	142	148								
FMJ93 *recE*::Tn*10 fnr ldh*	LCB898 *pfl*	Tet	45	23	19	13								
KF1340 *zda*::Cam^r	FMJ93 *recE*::Tn*10 fnr ldh*	Cam	86	270	17	27								
FMJ92 *recE*::Tn*10 ldh*	FMJ105 *zda*::Cam^r *fnr*	Tet	35	84	10	71								
FMJ106 *recE*::Tn*10*	FMJ102 *zda*::Cam^r *fnr ldh*	Tet	30	91	38	41								
FMJ106 *recE*::Tn*10*	FMJ102 *zda*::Cam^r *fnr ldh*	Tet					18	71	101	10				
FMJ93 *recE*::Tn*10 fnr ldh*	FMJ105 *zda*::Cam^r *fnr*	Tet					75	29	8	88				
KF1340 *zda*::Cam^r	FMJ93 *recE*::Tn*10 fnr ldh*	Cam									218	21	139	22

[a] All recipient strains carried the *pfl-1* mutation to allow scoring of *ldh*.

initial mapping FMJ39 was used as the recipient since it is resistant to streptomycin. This strain was crossed with the Hfr::Tn*10* set of Wanner (32). Exconjugants were selected for resistance to tetracycline and streptomycin and screened for their ability to grow anaerobically on glucose plus acetate or on sorbitol. These crosses indicated that the *ldh* gene was in the 20- to 35-min region of the *E. coli* chromosome (data not shown). Strain FMJ39 was then transduced with P1 phage grown on a set of strains carrying Tn*10* insertions in this region. Positive results were obtained with JP2781 *zcj*::Tn*10* (cotransduction of Tn*10* with *ldh*, 16%), KF1096 *recE*::Tn*10* (48% cotransduction), and HB235 *trg*::Tn*10* (20% cotransduction).

Three-factor crosses were then conducted to order *ldh* with respect to *fnr* (29.4 min), *recE* (29.8 min), and nearby Tn*10* insertions. Transposons inserted in the chromosome within this region were used as selective markers. DC706 has a Tn*10* inserted in the *recE* gene, and KF1340 carries a Tn*10* derivative specifying chloramphenicol resistance at 30.3 min. The presence of *fnr* was scored by anaerobic growth on glycerol plus nitrate, and *ldh* (in a *pfl* background) was screened by anaerobic growth on glucose plus acetate. The first cross, between DC706 (donor) and FMJ39 (recipient), was done to order *ldh* with respect to *fnr* and *recE*. The results (Table 5) showed no outstandingly rare recombinant class, suggesting that *recE*::Tn*10* was between *fnr* and *ldh*. Several backcrosses were performed, and clear double-crossover classes were observed in most cases. Thus the gene order is *fnr recE*::Tn*10 ldh*. Further three-factor crosses ordered *ldh* relative to the chloramphenicol resistance marker of KF1340 (Table 5 and Fig. 2). The cotransduction frequencies were as follows: for Cam and *ldh*, 89%; for *ldh* and *recE*::Tn*10*, 48%; for Cam and *recE*::Tn*10*, 51%; and for *recE*::Tn*10* with *fnr*, 38%. Hence the *ldh* gene maps at 30.3 min on the *E. coli* chromosome (Fig. 2). The *ksgD*::Tn*5* mutation has been mapped to 30.5 min, close to the *zda-1*::Tn*10* insertion. However, *zda-1*::Tn*10* did not cotransduce with *ldh*. The original mapping of *ksgD* (9) contains an ambiguity, and our results suggest that *ksgD* and the linked Tn*10* might be around 29 min, on the other side of *sbcA* from that suggested previously.

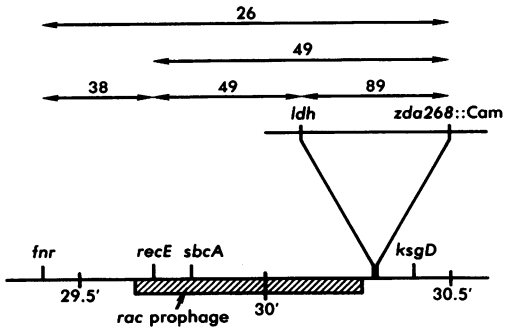

FIG. 2. Map of the 30-min region of the chromosome. The gene orders are from the data in Table 5 and from the map of Bachmann (3). Average cotransduction frequencies obtained during this work are shown above the map.

The *recE* gene is actually part of a defunct lambdoid prophage, *rac* (2, 26). Strain AB1157, which is deleted for *rac* (2, 26), was therefore assayed for LDH and found to have wild-type levels of LDH activity. We also assayed the C strain of *E. coli*, which was never lysogenized by *rac*. This strain also showed full LDH activity (data not shown). Thus *ldh* is a genuine *E. coli* chromosomal gene and lies outside the *rac* prophage.

DISCUSSION

Under anaerobic conditions, *E. coli* may grow via two alternative energy-generating modes. It will respire if an alternate electron acceptor such as nitrate, fumarate, or trimethylamine oxide is present (11, 13). In the absence of such electron acceptors, *E. coli* ferments and generates energy via substrate-level phosphorylation (11, 24). During fermentation, sugars are converted to reduced organic compounds such as ethanol and acetic, lactic, formic, and succinic acids (16, 17, 25, 30). The soluble NAD-linked LDH is responsible for the formation of lactic acid from pyruvate (27, 28). This reaction consumes one NADH (i.e., two reducing equivalents) per three carbons, thus balancing out the NADH produced in the glyceraldehyde phosphate dehydrogenase step of glycolysis.

Since earlier reports have shown that more lactate is produced in acidic conditions and high phosphate concentrations during fermentation, we studied the effect of these factors on LDH activity in wild-type strains. The use of buffering agents makes the medium less acidic throughout the growth period and reduced LDH activity compared with that in unbuffered cultures (Table 2). Longer incubation led to increased acidity of the growth medium and increased LDH activity. Similar experiments were conducted to investigate the reported effects of phosphate (25). We found that as phosphate concentration increased, there was less acidification of the medium, and LDH activity decreased. We feel that there is no genuine effect of phosphate per se. The buffering properties of phosphate and the pH effect on LDH can fully account for all our observations.

The induction of LDH by low pH was pronounced anaerobically but barely significant aerobically. Thus this enzyme is under dual regulation by acid and anaerobic conditions (Table 2). Similar effects have been shown with formate dehydrogenase (19, 20) and with several anaerobically induced gene fusions isolated previously in our laboratory (34). Slonczewski et al. (23) have directly isolated gene fusions that are induced by external acidification but that do not respond to internal acidification. Thus *E. coli* has some mechanism that specifically detects changes in external pH. Similar pH-dependent gene expression has also been demonstrated in the eukaryote *Aspergillus nidulans* (4).

We have isolated mutants affected in ethanol metabolism (5, 6, 8) and in acid production (7, 33). However, mutants defective in the fermentative LDH have not previously been isolated. To isolate such mutants we mutagenized strain LCB898, which is defective in pyruvate formate lyase (31). Mutants deficient in *pfl* are unable to grow anaerobically on glucose unless acetate is provided for biosynthetic processes, and they cannot grow on sorbitol even if given acetate (31). We isolated several mutants deficient in LDH activity, ranging from some that were completely devoid of LDH to others with their activity reduced to about 10% of the parental level. These doubly mutant *ldh pfl* strains were unable to grow anaerobically on glucose as the carbon source even with acetate supplemented. They were also unable to grow anaerobically on any fermentable sugar tested, but their growth by anaerobic respiration was not affected. The *pfl* defect was then removed to yield strains with only the *ldh* mutation. Such *ldh* strains showed no anaerobic growth defects but still lacked LDH activity. Therefore, the absence of fermentative LDH alone does not prevent fermentative growth.

We mapped the *ldh* mutation of FMJ39 to 30.3 min on the *E. coli* chromosome (3). The gene order for *ldh* is illustrated in Fig. 2 (i.e., *fnr recE ldh* Cam). It seems a curious coincidence that *fnr*, the activator gene for anaerobic respiration (18, 21), and *ldh* should be such close neighbors, except for the fortuitous insertion of foreign (viral) DNA into the *E. coli* K-12 chromosome.

We monitored the production of lactic acid by whole-culture scans of fermenting *E. coli* by NMR. Similar in vivo measurements were previously made by Ogino et al. (16, 17), who demonstrated that fermentation may be observed by this nonintrusive technique which allows the maintenance of anaerobic conditions. The *ldh* mutants did not make lactic acid but still produced ethanol and acetic acid. When incubation was prolonged *ldh* mutants accumulated pyruvate, which was not observed in the wild type.

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