Microbiology (1997), **143**, 187–195 Printed in Great Britain

The *ldhA* gene encoding the fermentative lactate dehydrogenase of *Escherichia coli*

Pamela K. Bunch, Fairoz Mat-Jan,† Norizan Lee and David P. Clark

Author for correspondence: David P. Clark. Tel: +1 618 453 3737. Fax: +1 618 453 8036. e-mail: clark@micro.siu.edu

Department of Microbiology, Southern Illinois University, Carbondale, IL 62901, USA

Under anaerobic conditions, especially at low pH, *Escherichia coli* converts pyruvate to D-lactate by means of an NADH-linked lactate dehydrogenase (LDH). This LDH is present in substantial basal levels under all conditions but increases approximately 10-fold at low pH. The *ldhA* gene, encoding the fermentative lactate dehydrogenase of *E. coli*, was cloned using λ10E6 of the Kohara collection as the source of DNA. The *ldhA* gene was subcloned on a 2·8 kb *Mlu*I–*Mlu*I fragment into a multicopy vector and the region encompassing the gene was sequenced. The *ldhA* gene of *E. coli* was highly homologous to genes for other D-lactate-specific dehydrogenases but unrelated to those for the L-lactate-specific enzymes. We constructed a disrupted derivative of the *ldhA* gene by inserting a kanamycin resistance cassette into the unique *Kpn*I site within the coding region. When transferred to the chromosome, the *ldhA*::Kan construct abolished the synthesis of the D-LDH completely. When present in high copy number, the *ldhA* gene was greatly overexpressed, suggesting escape from negative regulation. Cells expressing high levels of the D-LDH grew very poorly, especially in minimal medium. This poor growth was largely counteracted by supplementation with high alanine or pyruvate concentrations, suggesting that excess LDH converts the pyruvate pool to lactate, thus creating a shortage of 3-carbon metabolic intermediates. Using an *ldhA–cat* gene fusion construct we isolated mutants which no longer showed pH-dependent regulation of the *ldhA* gene. Some of these appeared to be in the *pta* gene, which encodes phosphotransacetylase, suggesting the possible involvement of acetyl phosphate in *ldhA* regulation.

Keywords: lactate dehydrogenase, fermentation, acetyl phosphate, anaerobic growth

INTRODUCTION

Under anaerobic conditions, *Escherichia 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 (White, 1995). In the absence of such electron acceptors, *E. coli* ferments and generates energy via substrate-level phosphorylation (Clark, 1989; White, 1995). During fermentation, sugars are converted to reduced organic compounds such as ethanol and acetic, lactic, formic and succinic acids (Clark, 1989; Ogino *et al.*, 1980; Stokes, 1949). The soluble NADH-linked lactate dehydrogenase (LDH) is responsible for the formation of lactic acid from pyruvate (Tarmy & Kaplan, 1968a, b). 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.

E. coli actually contains three LDHs. Two of these are membrane-bound flavoproteins which couple to the respiratory chain and are better described as lactate oxidases. These enzymes, one specific for the D-isomer and the other for the L-isomer, are required for aerobic growth on lactate (Haugaard, 1959; Kline & Mahler, 1965). The conversion of pyruvate to lactic acid under anaerobic conditions is catalysed by a third enzyme; this is a soluble NADH-linked enzyme that is specific for the production of D-lactic acid (Tarmy & Kaplan, 1968a). The fermentative LDH has been purified and is allo-

†**Present address:** Immucor Inc., 3130 Gateway Drive, PO Box 5265, Norcross, GA 30071, USA.

Abbreviation: LDH, lactate dehydrogenase.

The GenBank accession number for the nucleotide sequence reported in this paper is U36928.

sterically activated by its substrate, pyruvate (Tarmy & Kaplan, 1968b). The fermentative LDH is induced approximately 10-fold in anaerobically grown cultures at acidic pH (Clark, 1989; Mat-Jan *et al.*, 1989).

Fermentation of sugars to yield lactic acid is widespread. Mammalian muscle generates L-lactic acid when in oxygen debt. A variety of bacteria produce lactic acid during anaerobic growth; in some cases, the L-isomer is made, in others D-lactate. Among the enterobacteria, the D-isomer of lactic acid is produced along with a variety of other products by the mixed-acid fermentation (Clark, 1989). In contrast, many lactobacilli produce solely, or largely, L-lactic acid. Typically, these organisms produce L-lactic acid by means of an L-specific LDH which is activated by fructose bisphosphate, rather than pyruvate (Mayr *et al.*, 1982). However, a few species of *Lactobacillus* (e.g. *L. plantarum*) possess both D- and L-specific fermentative LDHs; these two types of LDH are not at all homologous (Taguchi & Ohta, 1991). We were therefore interested to see if the D-specific LDH of *E. coli* belonged to the same protein family as the D-LDHs of Gram-positive bacteria such as *Lactobacillus*.

We have previously isolated mutants, designated *ldhA*, which are deficient in the fermentative LDH (Mat-Jan *et al.*, 1989). *E. coli* carrying an *ldhA* mutation shows no observable anaerobic growth defects and can still ferment sugars to a variety of other products (Mat-Jan *et al.*, 1989). When an *ldhA* defect is present together with a mutation in *pfl* (pyruvate-formate lyase), anaerobic growth on a wide range of sugars and derivatives is no longer possible. Using this phenotype, the *ldhA* mutation was mapped to 30·5 min on the *E. coli* chromosome (Mat-Jan *et al.*, 1989). By using our mapping data we have been able to identify a lambda from the Kohara collection (Kohara *et al.*, 1987) which carried the *ldhA* gene. Here we describe the cloning and sequencing of the *ldhA* gene of *E. coli* and show that it is indeed highly homologous to the D-specific LDHs of several other organisms. In addition, we have used the cloned *ldhA* gene to initiate studies on its regulation.

METHODS

Bacterial strains, plasmids and growth media. All bacteria were strains of *E. coli* K-12 and are described in Table 1. Plasmid pJOE810 carries tyrosinase genes from *Streptomyces* and allows its host cells to make melanin, provided the growth medium contains tyrosine. Insertion of exogenous DNA into the polylinker inactivates melanin synthesis, and white colonies (instead of black) are obtained (Altenbucher, 1988). Plasmid pUBT1 carries a *chlB*$^+$ gene and is used to directly isolate insertions by selecting for chlorate resistance in a Δ*chlB* host strain (Reiss & Klingmuller, 1987). Plasmid pMAK904 is a low copy number, pSC101-based vector with a *lacZ* blue/white polylinker cloning system and specifying kanamycin resistance. Derivative plasmids are listed in Table 1.

Rich broth contained (per litre): tryptone (10 g), NaCl (5 g) and yeast extract (1 g). Minimal medium M9 (Miller, 1972) was supplemented with carbon sources at 0·4% (w/v) and, where appropriate, amino acids (50 μg ml^{-1}). Solid media contained 1·5% (w/v) Difco Bacto-Agar. Anaerobic growth was performed in Oxoid anaerobic jars under a H_2/CO_2 atmosphere. Anaerobic liquid cultures were grown in milk dilution bottles filled to overflowing before sealing. Anaerobic growth media were supplemented with the trace elements Fe (50 μM), Se (5 μM), Mo (5 μM) and Mn (5 μM).

Preparation of cell-free extracts and LDH assays. Batch cultures (200 ml) were grown to about 10^9 cells ml^{-1} and were harvested by centrifugation at 8000 r.p.m. for 10 min at 4 °C. The supernatant was decanted. After washing the cell pellet in 50 mM KH_2PO_4 buffer pH 7·4, the cells were resuspended in 3·0 ml phosphate buffer and were ruptured by passage of the suspension through a French pressure cell (American Instrument Co.). The high-speed supernatant fraction was obtained by centrifugation at 145000 *g* for 1 h at 4 °C. The cell extract was stored at −20 °C until used for biochemical assays.

Protein content was assayed by the Bio-Rad assay using gamma-globulin (2 mg ml^{-1}) as standard. Samples of 1·0 ml were mixed with 5·0 ml Bio-Rad dye reagent (diluted 1:4 with distilled water). After incubation for 5 min, the absorbance was measured at 595 nm.

LDH was assayed spectrophotometrically using a Perkin-Elmer 552A UV/VIS spectrophotometer with a Fisher Recordall series chart recorder. The assay followed the decrease in absorbance at 340 nm as NADH was oxidized to NAD^+ by pyruvate as catalysed by LDH. The LDH assay mix contained the following: 1 M sodium pyruvate pH 7·5 (30 μl); 50 mM MOPS pH 7·0 (400 μl); 6·4 mM NADH (30 μl); enzyme preparation (10–40 μl); distilled water to bring up the volume to 1·0 ml. One unit of enzyme activity is the amount of enzyme necessary to convert 1 mmol NADH to $NADH^+$ min^{-1} (Mat-Jan *et al.*, 1989; Tarmy & Kaplan, 1968a). Enzyme assays were performed in duplicate and, if within 10% of each other, were averaged. If the discrepancy was greater, another pair of assays was performed.

DNA procedures. Chromosomal DNA was isolated and purified as described by Sato & Miura (1963). Lambda DNA was isolated as described by Sambrook *et al.* (1989). Plasmids were isolated by alkaline lysis followed by ethidium bromide/CsCl density centrifugation (Sambrook *et al.*, 1989). The rapid plasmid isolation technique of Birnboim & Doly (1979) was used to screen plasmid constructs. Ligations using T4 DNA ligase and restriction enzyme digests were performed under conditions recommended by the manufacturers (Bethesda Research Laboratories). DNA fragments were separated by electrophoresis on 0·7% agarose gels in TBE (89 mM Tris, 89 mM boric acid and 0·2 mM EDTA) (Sambrook *et al.*, 1989). DNA restriction fragments and PCR products were purified from agarose gels by Gene Clean (Bio 101). Transformation procedures were essentially as described by Hanahan (1983). Both strands of DNA were completely sequenced using the technique of Sanger *et al.* (1977).

RESULTS

Cloning the *ldhA* gene

Our previous work led to the mapping of the *ldhA* gene at 30·3 min on the *E. coli* genetic map, between the *rac* prophage and an insertion of the IS10-Cam-IS10 element at 30·4 min (Mat-Jan *et al.*, 1989). This suggested that the *ldhA* gene would be carried by λ10E6 and/or λ4E7 of the Kohara collection (Kohara *et al.*, 1987). We did in fact succeed in cloning the complete *ldhA* gene from λ10E6. In retrospect, it became clear that λ4E7 carries

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source/reference*
***E. coli* K-12**		
LCB320	*thr-1 leu-6 thi-1 lacY tonA22 rpsL*	Varenne *et al.* (1975)
FMJ115	*recA*::Cam *ldhA39*	Lab. collection
FMJ123	Δ*pfl*::Cam	Lab. collection
FMJ147	Δ*chlB recA srl*::Tn*10 rpsL gyrA* Δ(*argF–lac*)*U169 araD139*	Lab. collection
FMJ149	*recA56 srl*::Tn*10* Δ*pfl*::Cam Δ*ldhA his ilv pro arg*	Lab. collection
FMJ177	pFBP6 in FMJ149	This work
FMJ210	pFBP12 in FMJ115	This work
FMJ212	pFBP15 in FMJ115	This work
FMJ220	pFBP6 in FMJ115	This work
FMJ249	pFBP15 in FMJ149	This work
NZN91	*ack zfa*::Tn*10* of LCB320	Lab. collection
NZN92	Δ(*pta/ack*), *zfa*::Tn*10* of LCB320	Lab. collection
NZN111	Δ*pfl*::Cam *ldhA*::Kan	See text
NZN116	*ldhA*::Tn*10* of LCB320	P1 (SE1752) × LCB320
NZN117	*ldhA*::Kan of LCB320	P1 (NZN111) × LCB320
NZN130	pZAN5 in LCB320	See text
NZN131	Cam^R mutant of NZN130	See text
NZN132	Cam^R mutant of NZN130	See text
LEO13	*recA56 srl*::Tn*10 fadR mel tyrT*	Lab. collection
SE1752	*ldhA*::Tn*10*	L. O. Ingram
SHH300	*zfa*::Tn*10*	Gupta & Clark (1989)
SHH527	*pta zfa*:Tn*10* of LCB320	Lab. collection
Plasmids		
pJOE810	Amp^R melanin screening	Altenbucher (1988)
pMAK904	Kan^R low copy number vector	S. R. Kushner
pSPORT1	Amp^R sequencing vector	Gibco/BRL
pUBT1	Amp^R *chlB*$^+$	Reis & Klingmuller (1987)
pFBP1	1·3 kb *Eco*RI fragment of λ10E6 in pUBT1	This work
pFBP2	12·1 kb *Eco*RI fragment of λ10E6 in pUBT1	This work
pFBP4	6·7 kb *Eco*RI/*Pst*I fragment of λ10E6 in pJOE810	This work
pFBP6	4·4 kb *Bam*HI/*Pst*I fragment of λ10E6 in pJOE810	This work
pFBP7	5·3 kb *Hin*dIII/*Pst*I fragment of λ10E6 in pJOE810	This work
pFBP8	5·0 kb *Kpn*I/*Eco*RI fragment of λ10E6 in pJOE810	This work
pFBP10	3·1 kb *Kpn*I fragment of λ10E6 in pJOE810	This work
pFBP12	4·4 kb *Bam*HI/*Pst*I fragment of pFBP6 in pMAK904	This work
pFBP13	2·7 kb *Kpn*I/*Kpn*I fragment of pFBP6 in pMAK904	This work
pFBP14	1·7 kb *Pst*I/*Kpn*I fragment of pFBP6 in pMAK906	This work
pFBP15	2·8 kb *Mlu*I fragment of pFB6 in pJOE810	This work
pFBP16	Kan of pUC4::KISS in *Kpn*I site of pFBP15	This work
pUC4::KISS	Amp^R with Kan cassette	Pharmacia
pMFH6	Cam of Tn*9* in pUC1318	Y. Y. Chang
pZAN5	Cam from pMFH6 in *Sal*I site of pFBP12	This work

*L. O. Ingram, University of Florida, USA; S. R. Kushner, University of Georgia, USA; Y. Y. Chang, University of Illinois, USA.

approximately half the *ldhA* gene, located next to the left lambda arm.

DNA of λ10E6 was prepared by standard methods and digested with the restriction enzymes *Eco*RI, *Pst*I, *Hin*dIII, *Bam*HI and *Kpn*I to confirm the restriction map of Kohara *et al.* (1987) (data not shown). The chromosomal insert of λ10E6 is 13·4 kb long and consists of two *Eco*RI fragments, of 12·1 and 1·3 kb. Both fragments were ligated into the multiple cloning site of pUBT1, a plasmid designed to allow direct selection of inserted DNA by anaerobic chlorate resistance (Reiss & Klingmuller, 1987). We found that this selection worked poorly, due to the appearance of frequent chromosomal mutations conferring resistance. Only about 10% of the transformants carried pUBT1 with DNA inserts. One isolate of pUBT1 carrying the 1·3 kb *Eco*RI fragment was designated pFBP1, and one with the 12·1 kb *Eco*RI fragment was designated pFBP2. Strain FMJ147 and derivatives carrying pFBP1 and pFBP2 were assayed for

Table 2. LDH activity conferred by plasmids

Host strain	Plasmid	LDH specific activity*	
		Aerobic	Anaerobic
LCB320	None	0·23	0·96
FMJ115 *ldhA39*	None	0·06	0·05
FMJ115 *ldhA39*	pFBP6	101·1	73·8
FMJ115 *ldhA39*	pFBP15	89·7	10·2
FMJ149 Δ*ldh* Δ*pfl*::Cam	None	< 0·06	< 0·04
FMJ149 Δ*ldh* Δ*pfl*::Cam	pFBP6	18·2	8·7
FMJ149 Δ*ldh* Δ*pfl*::Cam	pFBP15	554·8	15·4
NZN116 *ldhA*::Tn*10*	None	0·01	0·01
NZN117 *ldhA*::Kan	None	0·01	0·01

* Specific activity in mmol NADH min^{-1} (mg protein)$^{-1}$. All cultures were grown in rich broth plus 0·4% glucose for 6 h at 37 °C.

LDH activity. pFBP1 had no effect on the level of LDH whereas pFBP2 conferred greatly elevated levels of LDH (data not shown). Throughout the subcloning process, we followed the LDH activity and representative measurements for some plasmids constructed later in this process are shown in Table 2.

DNA of pFBP2 was digested with various restriction enzymes to give several fragments which were ligated into the multiple cloning site of the vector pJOE810 (Altenbucher, 1988). We transformed the ligation mixtures into LEO13 (*recA*) and picked white, ampicillin-resistant colonies. Plasmid DNA was isolated and checked with the appropriate restriction enzymes. Plasmids pFBP4 to pFBP10 (Fig. 1) were constructed by this means. These plasmids were then transformed into strain FMJ149 (Δ*pfl*::*Cam* Δ*ldhA recA*) which has a large chromosomal deletion, including the *ldhA* gene, and hence produces no LDH under any growth conditions. Plasmids pFBP4 (carrying the 6·7 kb *Pst*I–*Eco*RI fragment from pFBP2) and pFBP6 (4·4 kb *Pst*I–*Bam*HI fragment) restored LDH activity to this strain. In contrast, plasmids pFBP7 (5·4 kb *Eco*RI–*Pst*I fragment), pFBP8 (5·0 kb *Kpn*I–*Eco*RI fragment) and pFBP10 (3·1 kb *Kpn*I–*Kpn*I fragment) were negative for LDH. The DNA fragments carried by these plasmids as derived from λ10E6 are shown in Fig. 1. Thus the *ldhA* gene appears to be centred about the *Kpn*I site located at λ10E6 coordinate 16·3 kb.

Sequence of the *ldhA* gene

The insert of pFBP6 was cut twice by *Mlu*I as shown in Fig. 1. The *Mlu*I–*Mlu*I fragment of 2·8 kb was subcloned into pJOE810 to give pFBP15, which was LDH-positive.

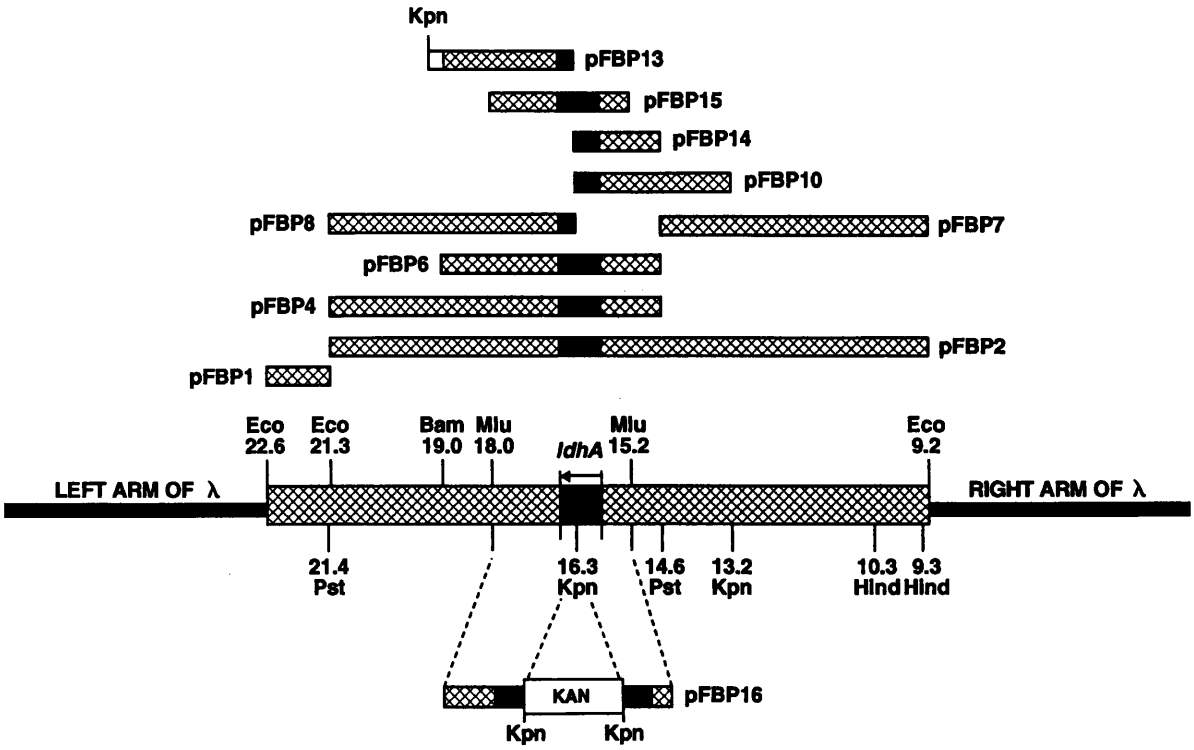

Fig. 1. Plasmids constructed. The inserts in the pFBP series of plasmids are shown relative to λ10E6 of the Kohara collection. Coordinates are given in kb starting from the right-hand end of λ. All plasmids consist of vector plus *E. coli* chromosomal DNA, except pFBP13 (which carries a small fragment from pJOE810 between coordinate 19.0 and the *Kpn*I site) and pFBP16, which carries the KAN cassette from pUC4::KISS inserted into the *Kpn*I site of pFBP15. Eco, *Eco*RI; Hind, *Hin*dIII; Bam, *Bam*HI; Kpn, *Kpn*I; Mlu, *Mlu*I; Pst, *Pst*I. Cross-hatching, *E. coli* chromosomal DNA; grey shading, *ldhA* gene (the arrow shows direction of transcription); black, λDNA; unshaded, DNA from pJOE810 or pUC4::KISS.

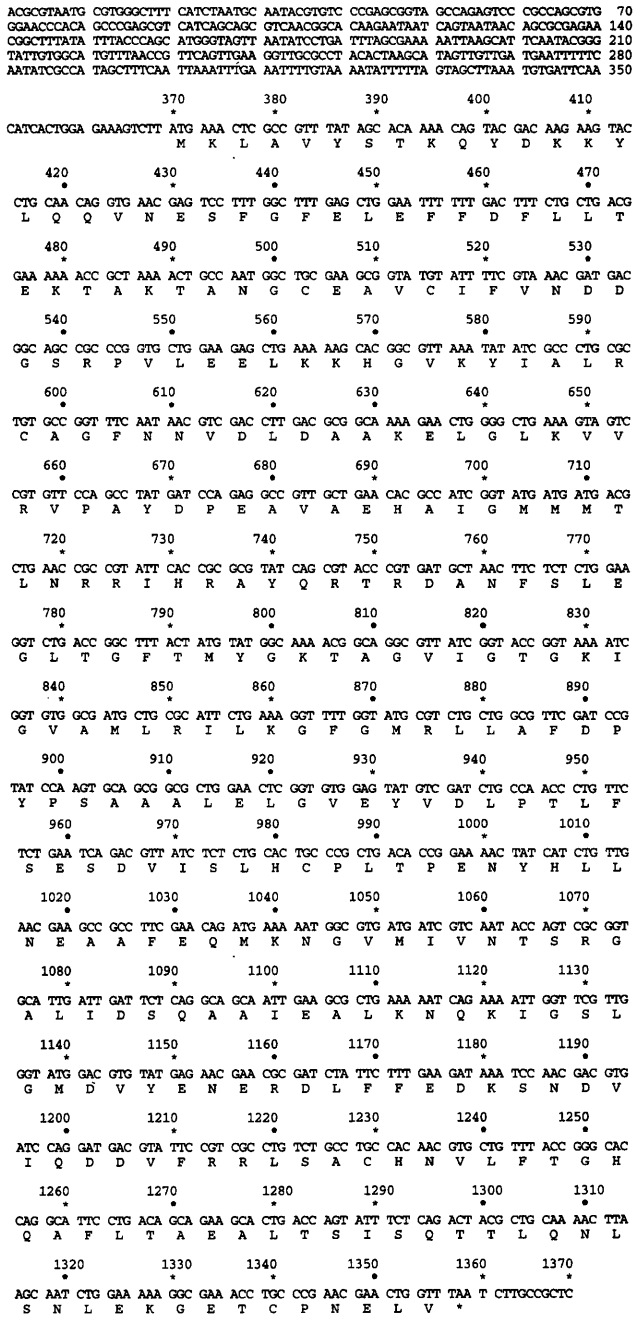

Fig. 2. Sequence of *ldhA*. The sequence of the *MluI–MluI* fragment of 2588 bp which includes the *ldhA* gene is shown. The *ldhA* gene starts at bp 370 and the translated protein sequence is shown beneath the DNA. A putative Shine–Dalgano sequence is seen at coordinates 358–362.

Both strands of the 2·8 kb *MluI–MluI* fragment from pFBP15 were completely sequenced by the dideoxy method. To achieve this the 2·8 kb *MluI–MluI* fragment was cut in two with *KpnI* and the two pieces (1·7 kb and 1·1 kb) were ligated individually into the sequencing plasmid pSPORT1. After sequencing into each fragment as far as possible from both ends using the M13/pUC forward and T7/T3 'backward' sequencing primers, we used new internal primers based on the sequences found. We confirmed the sequence around the *KpnI* site by sequencing across this junction region using pSPORT1 carrying the complete 2·8 kb *MluI–MluI* fragment. The '2·8 kb' fragment was 2588 bp long when sequenced (Fig. 2).

Reading frame analysis indicated only one ORF of appropriate size. This ran from coordinate 370 to 1356 and encoded a protein of 329 amino acids. No other ORF of more than 450 bp (150 aa) was found in the 2588 bp sequence. Evidence that the 329 aa ORF is indeed the *ldhA* gene is of two kinds: protein gels and homology. Firstly, protein gels were run using extracts from an *ldhA* deletion strain with or without a plasmid restoring LDH activity. The plasmid-positive strain showed a protein of just under 40 kDa which was absent in the Δ*ldhA* parental strain (data not shown). [The molecular mass calculated from the sequence of the 329 aa ORF is 36532 Da.] Secondly, the amino acid sequence of the proposed *E. coli* LDH protein showed marked homology with the D-LDHs of *Lactobacillus delbruekii* and *L. plantarum*. Three other hydroxy-acid dehydrogenases showed lesser, but significant, homology (Table 3).

Insertional inactivation of the *ldhA* gene

Plasmid pFBP15, which carries an intact *ldhA* gene, was opened with *KpnI*, which cuts in the middle of the *ldhA* ORF. The kanamycin resistance cassette cut from pUC4::KISS with *KpnI* was ligated into the *KpnI* site of pFBP15, to yield pFBP16 (*ldhA*::Kan). Plasmid pFBP16 did not confer the ability to produce LDH on *ldhA*-negative host cells (data not shown). pFBP16 was transformed into the *rec*$^+$ wild-type strain LCB320, and transformants selected by ampicillin plus kanamycin. Several isolates of LCB320/pFBP16 were cultured for 20 to 30 generations in the presence of kanamycin but without ampicillin. These cultures were diluted and plated for single colonies in rich broth agar plus kanamycin. We found several colonies which were ampicillin-sensitive though kanamycin-resistant, indicating that the *ldhA*::Kan region had inserted into the chromosome by reciprocal crossing over, followed by subsequent loss of the plasmid.

We previously showed that *pfl ldhA* double mutants cannot grow anaerobically on any sugar or sugar alcohol even when supplemented with acetate, in contrast to single *ldhA* mutants, which show no anaerobic growth defect (Mat-Jan *et al.*, 1989). Therefore to confirm that *ldhA*::Kan had replaced the wild-type *ldhA*$^+$ allele, we used P1 to transduce the putative *ldhA*::Kan into strain

Table 3. Homology matrix

Sequence (length, aa)*	Residue identity (%)†						
	1	2	3	4	5	6	7
1. *E. coli* D-LDH (329)	100	33	34	31	29	29	26
2. *L. plantarum* D-LDH (332)		100	48	43	23	23	21
3. *L. delbruekii* D-LDH (332)			100	39	25	24	19
4. *L. casei* D-HICDH (335)				100	21	24	22
5. Cucumber D-glycerate DH (338)					100	21	22
6. *E. coli* D-3PGDH (410)						100	16
7. *E. coli* D-E4PDH (378)							100

* Abbreviations: LDH, lactate dehydrogenase; HICDH, hydroxy isocaproate dehydrogenase; 3PGDH, 3-phosphoglycerate dehydrogenase; E4PDH, erythrose-4-phosphate dehydrogenase.

† Residue identity = (number of identical residues in pairwise alignments divided by length of the shorter sequence) × 100.

FMJ123 *pfl*::Cam, using kanamycin resistance as selective marker. The resulting *pfl*::Cam *ldhA*:Kan double-mutant strains, e.g. NZN111, behaved as expected, confirming that the donors did indeed have *ldhA*::Kan as a chromosomal allele. We also cotransduced the *ldhA*::Kan with characterized Tn*10* insertions to confirm that it mapped at the previously identified *ldhA* locus (data not shown). Finally, the *ldhA*::Kan insert was re-transduced into a fresh isolate of LCB320 to give strain NZN117. When NZN117 cells were grown anaerobically in rich broth plus glucose for 24 h, the LDH activity was barely detectable (Table 2). An *ldhA*::Tn*10* insertion isolated by the laboratory of Dr L. O. Ingram was also transduced into strain LCB320 to give NZN116 and this gave essentially identical results to NZN117 *ldhA*::Kan (Table 2).

Growth inhibition by excess LDH

We noticed that strains containing plasmids which carried the *ldhA* gene grew poorly, unless grown in rich media. Fig. 3 shows a representative set of growth curves for strain FMJ115 (*ldhA recA*) and FMJ210, which contains pFBP12, a medium copy number plasmid carrying the *ldhA*$^+$ gene. In minimal medium with glucose as carbon source, the plasmid-bearing strain failed to grow. Addition of Casamino acids allowed some growth, followed by eventual lysis. Supplementation with alanine (200 mg ml^{-1}) allowed almost normal growth of the plasmid-bearing strain. The parental strain FMJ115 grew normally in all three media, although we have only shown its growth on glucose plus alanine, for the sake of clarity.

Supplementation with succinate, aspartate or glutamate had no beneficial effect on the growth of plasmid-carrying strain FMJ210. Strains with high copy number plasmids carrying the *ldhA* gene showed a similar effect, but more severe. In fact, when grown in poor media, such strains often deleted out the *ldhA* gene or occasionally lost the whole plasmid. This resulted in erratic and irreproducible growth curves (not shown).

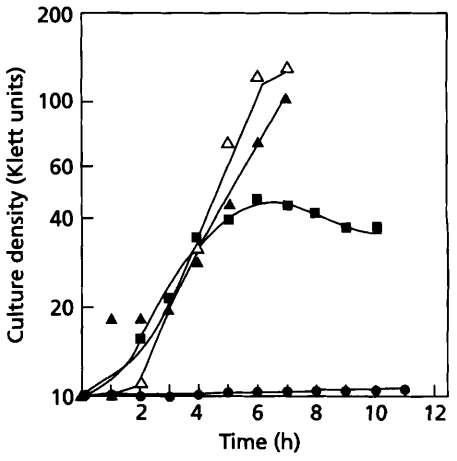

Fig. 3. Growth inhibition by *ldhA* plasmid. Cultures were grown aerobically in M9 minimal medium with 0·4% glucose (●), glucose plus 200 mg alanine ml^{-1} (▲) or glucose plus 0·2% Casamino acids (■). Open symbols are strain FMJ115 (no plasmid); filled symbols are strain FMJ210 carrying pFBP12 *ldhA*$^+$. (Although two early points are somewhat anomalous in this example, in other experiments, cultures of FMJ210 plus alanine gave smooth growth curves.)

Since LDH converts pyruvate to lactate, we believe that excess of this enzyme depletes the pyruvate pool of the cell. Supplementation with the 3-carbon metabolite alanine largely remedies this, whereas 4- or 5-carbon supplements have negligible effect. Addition of pyruvate itself was not as effective as alanine (data not shown), perhaps because high concentrations of pyruvate activate the LDH enzyme hyperbolically (Tarmy & Kaplan, 1968b).

Mutants with elevanted *ldhA* expression

We used the *Sal*I site within the coding region of the *ldhA* gene to insert a chloramphenicol-resistance cartridge. pMFH6 contains the 1·8 kb *Pst*I CamR fragment

Table 4. LDH activity of regulatory mutants

Strain	LDH specific activity*			
	No buffer	HEPES pH 8·0	TAPS pH 8·5	AMPSO pH 9·5
NZN130 (parent)	640	310	250	71
NZN131 (mutant)	1690	850	1050	1050
NZN132 (mutant)	690	780	710	190

* Specific activity in nmol NADH min^{-1} (mg protein)$^{-1}$. All cultures were grown anaerobically in rich broth plus 0·4% glucose for 6 h at 37 °C. Buffers were used at 100 mM.

from Tn9 inserted into pUC1318 (Kay & McPherson, 1987), where it is flanked by two *Sal*I sites. We cut this CamR fragment out with *Sal*I and ligated it into the *Sal*I site in the *ldhA* gene carried on pFBP12 to give pZAN5. In this construct the chloramphenicol acetyltransferase gene (*cat*) retains its own Shine–Dalgarno sequence, but is driven by the *ldhA* promoter. The presence of pZAN5 confers resistance to approximately 25 μg chloramphenicol ml^{-1} on a wild-type host strain, when grown in air.

Mutations in trans-acting regulatory genes affecting *ldhA* should result in an increase in expression of the chromosomal wild-type *ldhA* gene as well as the plasmid-borne *ldhA–cat* fusion. We therefore selected spontaneous mutants of NZN130 (pZAN5 in LCB320) able to grow in the presence of 250–500 μg chloramphenicol ml^{-1} under aerobic conditions. These appeared at a frequency of approximately 10^{-6}. About one in ten of these proved to have elevated LDH levels. Two such mutants, designated NZN131 and NZN132, were assayed for LDH activity, after anaerobic growth with or without buffering (Table 4). In the parent, NZN130, LDH is induced anaerobically and at low pH, as observed previously (Mat-Jan *et al.*, 1989). The mutant strains, NZN131 and NZN132, produced approximately the same levels of LDH, irrespective of the buffer and pH. With moderate buffering, the mutants expressed 3- to 4-fold as much LDH activity as their parent. Under extreme conditions (100 mM AMPSO, initial pH 9·5) where growth is very poor, the *ratio* was increased to 10-fold or more though this is due not so much to increased LDH in the mutants, as to lower parental levels. Aerobically, the mutants showed similar levels of LDH to the parental strain.

The mutants NZN131 and NZN132 grew poorly on acetate in air or with gluconate or glucuronate, anaerobically. These defects are typical of *pta* or *ack* mutants. We cotransduced NZN131 and NZN132 with P1 grown on strain SHH300, which contains a Tn*10* inserted close to the *pta/ack* genes. Approximately 20% of the transductants which received the *zfa*::Tn*10* insert from SHH300 also gained the ability to grow on acetate. This implied that the defect on NZN131 and NZN132 was in the *pta* or *ack* genes.

We therefore assayed LDH levels in well characterized *ack pta* and Δ(*pta ack*) derivatives of strain LCB320. Under standard buffered anaerobic conditions (HEPES pH 8·0) introduction of an *ack* mutation (strain NZN91) had no effect. However, introduction of a *pta* mutation (strain SHH527) or the Δ(*pta ack*) deletion (strain NZN92) resulted in a 3-fold increase in LDH much as in the mutants NZN131 and NZN132 (data not shown).

DISCUSSION

A variety of enzymes exist which interconvert lactate and pyruvate. The conversion of L- or D-lactate to pyruvate is catalysed by two separate, isomer-specific, flavoproteins, often called lactate dehydrogenases but better regarded as lactate oxidases. *E. coli* has two such enzymes which are used when lactate is the carbon source and when oxygen or nitrate are available as terminal electron acceptors (Haugaard, 1959; Kline & Mahler, 1965). These flavoprotein oxidases are, in fact, membrane-bound components of the electron transport chain.

Under fermentative conditions, in the absence of oxygen or alternative electron acceptors, pyruvate may be converted to lactate, among other products (Clark, 1989; White, 1995). The reduction of pyruvate is catalysed by soluble NADH-linked fermentative LDHs, not by the FAD-linked enzymes working in reverse (Mat-Jan *et al.*, 1989; Tarmy & Kaplan, 1968a). Some organisms produce L-lactate from pyruvate, whereas others produce D-lactate. Vertebrates and plants produce L-lactate by means of an L-specific fermentative LDH. Prokaryotes usually produce either L-lactate or D-lactate, with a few organisms able to produce both. Thus, in *Lactobacillus plantarum* distinct L- and D-specific LDHs are found in the same cell (Taguchi & Ohta, 1991). Although the L-LDHs from many organisms are all highly homologous, the sequences of the D-LDHs are completely unrelated to them except for the G-X-G-X-X-G motif common to most NAD-linked dehydrogenases (Branden & Tooze, 1991). This motif is part of the $\beta\alpha\beta$-fold which constitutes the NAD-binding domain (Eklund *et al.*, 1981; Wierenga *et al.*, 1986). This region is found at around position 150–160 in the D-LDHs (Branden & Tooze, 1991; Taguchi & Ohta, 1991). However, the corresponding site is located close to the N terminus in the L-LDHs (Branden & Tooze, 1991; Llanos *et al.*, 1992; Taguchi & Ohta, 1991).

E. coli has only a single NADH-linked LDH – a D-specific enzyme (Tarmy & Kaplan, 1968a). We have cloned and sequenced the *ldhA* gene encoding this enzyme. We have found very significant homology between the *E. coli* D-LDH and the D-specific enzyme of *Lactobacillus*. Two D-LDHs from different lactobacilli have been sequenced (Bernard *et al.*, 1991; Taguchi & Ohta, 1991) and showed 48% residue identity with each other. The *E. coli* D-LDH consists of 329 residues and shows approximately 34% identity with the *Lactobacillus* enzymes (333 residues). The D-hydroxyisocaproate dehydrogenase of *L. casei* also belongs to this

homology group, suggesting that the D-hydroxy-acid dehydrogenases form a distinct evolutionary family (Taguchi & Ohta, 1991). This family also includes the D-glycerate dehydrogenase of cucumber, the 3-phospho-D-glycerate dehydrogenase (*serA* product) of *E. coli* and the D-erythrose-4-phosphate dehydrogenase (*pdxB* product) of *E. coli*, which are less closely related (Table 3).

The regulation of the *E. coli* D-LDH is quite distinct from that of *Lactobacillus* LDH (Mat-Jan *et al.*, 1989; Tarmy & Kaplan, 1968b). This is hardly surprising. Lactate is the main fermentation product of the eponymous *Lactobacillus*, and LDH expression is coupled to glycolytic carbon flow (Llanos *et al.*, 1992; Mayr *et al.*, 1982). In *E. coli*, lactate is a subsidiary fermentation product which is produced in the second stage of fermentation, after substantial acidification has already taken place due to the generation of a mixture of acetic acid, formic acid and ethanol. Hence, the *ldhA* gene of *E. coli* is induced about 10-fold by a combination of anaerobiosis and acidity, but not by either condition alone (Clark, 1989; Mat-Jan *et al.*, 1989). As demonstrated here, massive overproduction of LDH under aerobic conditions actually inhibits growth in minimal medium. The suppression of this defect by exogenous alanine or pyruvate argues that excess LDH may deplete the internal pyruvate pools.

We found that the *ldhA* gene was massively overexpressed when present on a multicopy vector, suggesting that it may be under negative control and that the multiple gene copies had titrated out a limited number of repressor molecules. Furthermore, there were significant differences in expression between pFBP6 and pFBP15, which has a shorter upstream region (see Fig. 1). pFBP15 actually expresses D-LDH 10-fold higher in air than anaerobically – the reverse of the normal physiological response – whereas pFBP6 shows little difference between aerobic and anaerobic conditions. Aerobic LDH levels were even greater when pFBP15 was present in strain FMJ149, which has a very large deletion removing *ldhA* and much surrounding material, thus making interpretation hazardous. These observations may well be non-physiological artefacts resulting from the presence of multiple gene copies. We are presently constructing gene fusions to *lacZ*, carrying different portions of the upstream region, in order to examine the regulation of *ldhA* when present in single copy.

Using an *ldhA–cat* gene fusion we isolated mutants whose expression of LDH was increased and had become independent of the pH of the medium. However, these mutations appeared to be in the *pta* gene, which encodes phosphotransacetylase. In contrast, *ack* mutations had no effect, implying that the effect was not merely due to decreased synthesis and excretion of acetic acid. One possible explanation of these results is that anaerobic regulation of LDH depends on the level of acetyl phosphate. In *pta* mutants, no acetyl phosphate is made from acetyl-CoA, and LDH is derepressed. In *ack* mutants, acetyl phosphate can still be made, so no effect is observed. Under low-pH conditions, which serve to induce LDH in wild-type strains, one might expect acetyl phosphate levels to fall as the result of acid hydrolysis. McCleary *et al.* (1993) have suggested that acetyl phosphate might act as a global signal. However, those effects reported so far only occur in mutant backgrounds. Thus, acetyl phosphate affects the phosphate regulon, but only in the absence of the PhoM and PhoR proteins (Lee *et al.*, 1990; Wanner & Wilmes-Riesenberg, 1992) and it affects nitrogen regulation, but only in a GlnL-defective background (Feng *et al.*, 1992). Although our own effect is only of moderate magnitude, it does at least occur in strains wild-type for genes other than *pta* or *ack*.

ACKNOWLEDGEMENTS

This research was supported by a grant to D.C. from the Department of Energy, Office of Basic Energy Sciences (contract DE-FG02-88ER13941).

REFERENCES

Altenbucher, J. (1988). A new *E. coli* cloning vector containing a melanin marker for insertion screening. *Nucleic Acids Res* **16**, 8710.

Bernard, N., Ferain, T., Garmyn, D., Hols, P. & Delcour, J. (1991). Cloning of the D-lactate dehydrogenase gene from *Lactobacillus delbruekii* subsp. *bulgaricus* by complementation in *Escherichia coli*. *FEMS Microbiol Lett* **290**, 61–64.

Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**, 1513–1523.

Branden, C. & Tooze, J. (1991). Enzymes that bind nucleotides. In *Introduction to Protein Structure*, pp. 141–159. New York & London: Garland Publishing.

Clark, D. P. (1989). The fermentation pathways of *Escherichia coli*. *FEMS Microbiol Rev* **63**, 223–234.

Eklund, H. H., Samama, P., Wallen, L. & Branden, C.-I. (1981). Structure of a triclinic ternary complex of horse liver alcohol dehydrogenase at 2·9 Å resolution. *J Mol Biol* **146**, 561–587.

Feng, J., Atkinson, M. R., McCleary, W., Stock, J. B., Wanner, B. L. & Ninfa, A. J. (1992). Overproduction of acetate kinase activates the phosphate regulon in the absence of the *phoR* and *phoM* functions in *Escherichia coli*. *J Bacteriol* **174**, 6061–6070.

Gupta, S. & Clark, D. P. (1989). *Escherichia coli* derivatives lacking both alcohol dehydrogenase and phosphotransacetylase grow anaerobically by lactate fermentation. *J Bacteriol* **171**, 3650–3655.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.

Haugaard, N. (1959). D- and L-lactic acid oxidases of *Escherichia coli*. *Biochim Biophys Acta* **31**, 66–77.

Kay, R. & McPherson, J. (1987). Hybrid pUC vectors for addition of new restriction sites to the ends of DNA fragments. *Nucleic Acids Res* **15**, 2778.

Kline, E. S. & Mahler, E. R. (1965). The lactic acid dehydrogenases of *Escherichia coli*. *Ann NY Acad Sci* **119**, 905–917.

Kohara, Y., Akiyama, K. & Isono, K. (1987). The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell* **50**, 495–508.

Le, T.-Y., Makino, K., Shinagawa, H. & Nakata, A. (1990). Overproduction of acetate kinase activates the phosphate regulon

in the absence of the phoR and phoM functions in *Escherichia coli*. *J Bacteriol* **172**, 2245–2249.

Llanos, R. M., Hillier, A. J. & Davidson, B. E. (1992). Cloning, nucleotide sequence, expression and chromosomal location of *ldh*, the gene encoding L-(+)-lactate dehydrogenase, from *Lactococcus lactis*. *J Bacteriol* **174**, 6956–6964.

Mat-Jan, F., Alam, K. Y. & Clark, D. P. (1989). Mutants of *Escherichia coli* deficient in the fermentative lactate dehydrogenase. *J Bacteriol* **171**, 342–348.

Mayr, U., Hansel, R., Deparade, M., Pauly, H. E., Pfleiderer, G. & Tromer, W. E. (1982). Structure–function relationship in the allosteric L-lactate dehydrogenases from *Lactobacillus casei* and *Lactobacillus curvatus*. *Eur J Biochem* **126**, 549–558.

McCleary, W. R., Stock, J. B. & Ninfa, A. J. (1993). Is acetyl-phosphate a global signal in *Escherichia coli*? *J Bacteriol* **175**, 2793–2798.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Ogino, T., Arata, Y. & Fujiwara, S. (1980). Proton correlation nuclear magnetic resonance study of metabolic regulation and pyruvate transport in anaerobic *Escherichia coli* cells. *Biochemistry* **19**, 3684–3691.

Reiss, J. & Klingmuller, W. (1987). Direct selection of recombinant plasmids with chlorate. *FEMS Microbiol Lett* **43**, 201–205.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.

Sato, H. & Miura, K. K. (1963). Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim Biophys Acta* **72**, 619–629.

Stokes, J. L. (1949). Fermentation of glucose by suspensions of *Escherichia coli*. *J Bacteriol* **57**, 147–158.

Taguchi, H. & Ohta, T. (1991). D-Lactate dehydrogenase is a member of the D-isomer specific 2-hydroxyacid dehydrogenase family. *J Biol Chem* **266**, 12588–12594.

Tarmy, E. M. & Kaplan, N. O. (1968a). Chemical characterization of D-lactate dehydrogenase from *Escherichia coli* B. *J Biol Chem* **243**, 2579–2586.

Tarmy, E. M. & Kaplan, N. O. (1968b). Kinetics of *Escherichia coli* B D-lactate dehydrogenase and evidence for pyruvate controlled change in conformation. *J Biol Chem* **243**, 2587–2596.

Varenne, S., Casse, F., Chippaux, M. & Pascal, M.-C. (1975). A mutant of *Escherichia coli* deficient in pyruvate formate lyase. *Mol Gen Genet* **141**, 181–184.

Wanner, B. L. & Wilmes-Riesenberg, M. R. (1992). Involvement of phosphotransacetylase, acetate kinase, and acetyl-phosphate synthesis in control of the phosphate regulon in *Escherichia coli*. *J Bacteriol* **174**, 2124–2130.

Wierenga, R. K., Terpstra, P. & Hol, W. G. J. (1986). Prediction of the occurrence of the ADP-binding $\beta\alpha\beta$-fold in proteins, using an amino acid sequence fingerprint. *J Mol Biol* **187**, 101–107.

White, D. (1995). Fermentations. In *The Physiology and Biochemistry of Prokaryotes*, pp. 272–293. New York: Oxford University Press.

Received 29 April; revised 15 August 1996; accepted 6 September 1996.