Mutants of *Escherichia coli* K12 with Defects in Anaerobic Pyruvate Metabolism

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A strain of *Escherichia coli* with a mutation in the *ana* gene was shown to lack acetaldehyde dehydrogenase and alcohol dehydrogenase. The requirement of this strain for an external oxidant to grow anaerobically on glucose shows that the reduction of acetyl-CoA is the principal means of reoxidation of NADH produced during glycolysis in *E. coli*. Further mutants derived from the *ana* strain were shown to be affected in the enzymes involved in the fermentation of pyruvate (pyruvate formate-lyase, phosphotransacetylase, acetate kinase). A gene controlling acetate kinase (*ackB*) activity has been located at 39 min on the chromosomal map. Evidence is presented that anaerobic nitrite reduction with pyruvate involves at least the dehydrogenase subunit of the pyruvate dehydrogenase complex.

INTRODUCTION

When nitrate or nitrite is present during the anaerobic growth of *Escherichia coli*, it is used as electron acceptor for the reoxidation of the NADH produced during glycolysis. The presence of an exogenous electron acceptor, however, is not required for growth, since this organism is able to balance fermentation with endogenous electron acceptors produced by glycolysis. In the scheme of pyruvate metabolism given in Fig. 1, pyruvate formate-lyase (PFL) is thought to be functional in anaerobiosis (Knappe *et al.*, 1972) while the pyruvate dehydrogenase complex (PDH; pyruvate dehydrogenase plus dihydrolipoamide acetyltransferase plus dihydrolipoamide dehydrogenase) is only active in the presence of oxygen (Hansen & Henning, 1966).

We have isolated a mutant of *E. coli* which required nitrate or nitrite as an exogenous electron acceptor for anaerobic growth (Casse *et al.*, 1976). Although the biochemical basis of this mutation (*ana*) was not resolved, its existence made possible the selection of a variety of secondary mutants which were unable to grow on glucose plus nitrite (Nir$^-$). One group (group B) of these Nir$^-$ mutants was able to produce hydrogen from formate but not from pyruvate, thus suggesting a common defect in pyruvate formate-lyase (Abou-Jaoudé *et al.*, 1978). The aim of the work described in this paper was to identify the nature of the original *ana* mutation, to characterize more fully the mutants belonging to group B and to elucidate the role of pyruvate in anaerobic nitrite and nitrate reduction in *E. coli*.

METHODS

Strains. The strains used are listed in Table 1. Nir$^-$ phenotypes of strains LCB26, LCB130, LCB190 and LCB197 are due to mutations in genes distinct from *pfl* or *ack* genes. We will designate as *nirG* the *nir* gene

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Table 1. *Escherichia coli strains used*

Strain	Sex	Genotype	Source or method of isolation
C600	F^-	*thr-1 leu-6 lacY1 tonA22 thi-1 str*	
P4X	Hfr	*metB1* $(\lambda)^+$	
AB3295	F^-	*pabB3 his-4 ilvC7 argE3 thi-1 xyl-5 strA704*	A. J. Pittard
K63	F^-	*his-1 fadD88 man-1 gal-3 str*	G. Novel
A2T3	F^-	*aceE2 trpE*	J. R. Guest
LCB26	F^-	*thr-1 leu-6 lacY1 tonA22 thi-1 ana-1 str nirE26 facA26*	Abou-Jaoudé *et al.* (1978), Abou-Jaoudé (1979)
LCB49	F^-	*thr-1 lacY1 tonA22 thi-1 aceE2 str*	Transduce C600 with P1 propagated on A2T3. Select Leu^+. Screen for Ace^-
LCB90	Hfr	*thr-1 leu-6 ackB nirG ana-1*	Conjugate P4X with LCB190. Select Lac^+ Met^+. Screen for Ack^-, Nir^- and Hfr
LCB129	F^-	*aroA-2 pfl-1*	M. C. Pascal
LCB130	F^-	*thr-1 leu-6 lacY1 tonA22 thi-1 ana-1 str pfl-130 nir-130*	Abou-Jaoudé *et al.* (1978)
LCB190	F^-	*thr-1 leu-6 lacY1 tonA22 thi-1 ana-1 str ackB nirG*	Abou-Jaoudé *et al.* (1978), Abou-Jaoudé (1979)
LCB197	F^-	*thr-1 leu-6 lacY1 tonA22 thi-1 ana-1 str pfl-197 nirH*	Abou-Jaoudé *et al.* (1978), Abou-Jaoudé (1979)
LCB522	F^-	*dsdA aroC purF argF thi-1 str xyl mtl gal lac*	E. McFall
723-4	F^-	*his-1 fadD88 man-1 ackB nirG ana-1*	Conjugate LCB90 with K63. Select Gal^+ Thr^+ Leu^+. Screen for Ack^-, Nir^-
LCB898	F^-	*thr-1 leu-6 lacY1 tonA22 thi-1 pfl-1 str*	S. Varenne
LCB900	F^-	*thr-1 leu-6 lacY1 tonA22 thi-1 ana-1 str*	F. Casse

affected in strain LCB190 and located at 39 min, and as *nirH* the *nir* gene impaired in strain LCB197 and mapping at 19 min (Abou-Jaoudé, 1979).

Growth conditions. The minimal medium was that described by Kelmers *et al.* (1971) supplemented with 0·02% (w/v) leucine, 0·02% (w/v) threonine and 0·004% (w/v) thiamin. Bacteria were grown with 2% (w/v) glucose at pH 7 and 37 °C, harvested from mid-exponential phase and stored as a cell paste at −90 °C. For anaerobic growth the media were flushed with nitrogen and the pH was kept constant by addition of NaOH through a pH-stat. For studies of anaerobic growth on pyruvate (1%, w/v), the medium was inoculated with 0·01 vol. of a mid-exponential phase culture grown aerobically with glucose. Growth was followed for at least 36 h.

Bacteria used for the determination of nitrate and nitrite reduction were grown as described by Abou-Jaoudé *et al.* (1978). The media contained either glucose (0·2%, w/v) plus KNO_3 (0·1%, w/v) or glycerol (0·2%, w/v) plus KNO_2 (0·01%, w/v).

Measurement of enzyme activities. A suspension of bacteria (5 g) in 15 ml 50 mM-potassium 3-(*N*-morpholino)-propanesulphonate (MOPS) buffer pH 8 (K^+ is essential for the stabilization of phosphotransacetylase) was subjected to sonication (Branson Sonifier type S75) for 75 s at 0 to 10 °C and centrifuged at 30000 *g* for 1 h. The supernatant fraction contained 25 to 30 mg protein ml^{-1} (determined by the Biuret method). For the determination of pyruvate formate-lyase, centrifugation was at 140000 *g* for 90 min (to pellet the pyruvate dehydrogenase complex).

Aldehyde dehydrogenase [acetaldehyde:NAD^+ oxidoreductase (CoA-acylating); EC 1.2.1.10] was assayed as described by Stadtman & Burton (1955) using 0·1 M-potassium phosphate buffer pH 7 (Rudolph *et al.*, 1968). Alcohol dehydrogenase [alcohol:NAD^+ oxidoreductase; EC 1.1.1.1] activity was measured in anaerobic cuvettes under argon (to prevent NADH oxidation). The assay mixture (1 ml) contained 50 µmol MOPS pH 8, 0·4 µmol NAD, 6 µmol dithiothreitol and 0·1 µmol $(NH_4)_2Fe(SO_4)_2$. After preincubation for 30 min the cell extract (0·3 to 1·5 mg protein) was added and NADH absorption was observed for several minutes prior to the addition of 10 µl 1 M-acetaldehyde. D(−)-Lactate dehydrogenase [D-lactate:NAD^+ oxidoreductase; EC 1.1.1.28] was determined according to Tarmy & Kaplan (1968), acetate kinase [ACK; ATP:acetate phosphotransferase; EC 2.7.2.1] was determined according to Rose (1955), and phosphotransacetylase [PTA; acetyl-CoA:orthophosphate acetyltransferase; EC 2.3.1.8] was measured by the coupled spectrophotometric assay described by Blaschkowski *et al.* (1979).

Pyruvate formate-lyase [PFL; acetyl-CoA:formate *C*-acetyltransferase; EC 2.3.1.54], which occurs in interconvertible active and inactive forms (Knappe *et al.*, 1972; Knappe, 1978), was measured as follows. The cell extract (2 to 5 mg protein), containing PFL predominantly in the inactive state, was supplemented with 50 µg PFL

activase and subjected to the activation process for 30 min. The procedure described by Knappe *et al.* (1974) was followed but using Tris buffer pH 7·2 and photoreduced 5-deazariboflavin (10 µM) as the reductant instead of the flavodoxin-chloroplast system. PFL activity was subsequently determined by the coupled spectrophotometric assay (Knappe *et al.*, 1974).

Pyruvate dehydrogenase complex activity was measured by the method described by Guest & Creaghan (1973).

All enzyme activities are expressed in terms of µmol substrate transformed min^{-1} (mg $protein)^{-1}$ at 30 °C.

Acetate, D(–)-lactate and glucose in culture media were determined by common enzymic methods (Bergmeyer, 1974).

Measurement of nitrate and nitrite reduction. The formate-nitrate and formate-nitrite reductase activities were determined with whole cells as described previously (Abou-Jaoudé *et al.*, 1979). When pyruvate replaced formate, its concentration was 40 mM.

Genetic techniques. Mating and transduction experiments were performed by the methods of Taylor & Thoman (1964) and Lennox (1955), respectively. The Ack phenotype was tested on solid minimal medium (Davis & Mingioli, 1950) with acetate (0·3%, w/v) as carbon source. The Pab and Fad phenotypes were tested according to Huang & Pittard (1967) and Overath *et al.* (1969), respectively.

RESULTS

The Ana⁻ mutation affects the enzymes of acetyl-CoA reduction

Various properties of the Ana^- strain LCB900 suggested it had a defect in the reoxidation of NADH by the terminal fermentation processes (Chippaux *et al.*, 1974). In *E. coli* K12 the principal hydrogen acceptor during fermentation of glucose is acetyl-CoA (to form ethanol). Indeed, the two enzyme activities concerned with this process, acetaldehyde dehydrogenase and the accompanying alcohol dehydrogenase (Rudolph *et al.*, 1968; Schmitt, 1975), were found (Table 2) to be absent from the extract of strain LCB900. [The other alcohol dehydrogenase of *E. coli* is repressed during growth with glucose (Meyhack *et al.*, 1973).] The D(–)-lactate dehydrogenase activity was similar to that in the parent strain C600 (Table 2).

Mutations affecting the conversion of pyruvate into acetate

The wild-type (C600) and Ana^- (LCB900) strains were both able to grow anaerobically on pyruvate as the sole carbon and energy source with a mean doubling time of 2 to 3 h. Nir^- mutants of group B, which are unable to produce gas from pyruvate, were found to be unable to grow anaerobically with pyruvate. This prompted an investigation of possible defects in the relevant enzymes – pyruvate formate-lyase (PFL), phosphotransacetylase (PTA) and acetate kinase (ACK). The results (Table 3) indicated that strains LCB130 and LCB197 were defective only in PFL; PFL activase was found to be unaffected in these two strains. Strains LCB190 and LCB26 lacked ACK; the latter strain also lacked PTA. During aerobic growth with glucose, ACK-less cells grew slowly and did not accumulate acetate (Table 4). They had also lost the ability to grow aerobically with acetate, a property which was employed in the genetic analysis.

Table 2. *Enzyme activities in extracts of cells grown with glucose*

		Activity [µmol min^{-1} (mg $protein)^{-1}$]		
Strain	Growth condition	Aldehyde dehydrogenase (ALDH)	Alcohol dehydrogenase (ADH)	D(–)-Lactate dehydrogenase (LDH)
C600 (Ana^+)	Aerobic	0·11	0·08	0·45
	Anaerobic	0·5	0·2	1·3
LCB900 (Ana^-)	Aerobic	0·0003	0·001	0·30

Table 3. *Enzyme activities in extracts of cells grown aerobically with glucose*

For *E. coli* K12 (ATCC 10798) the pyruvate formate-lyase activities in extracts from aerobically and strictly anaerobically grown cells (glucose, pH 7) were 0·5 and 1·3, respectively.

	Activity [μmol min^{-1} (mg protein)$^{-1}$]			
Strain	Pyruvate formate-lyase (PFL)	Acetate kinase (ACK)	Phospho-transacetylase (PTA)	Pyruvate dehydrogenase (PDH)
LCB900	0·52	1·6	1·3	0·075
LCB130	<0·02	2·3	1·8	—
LCB197	<0·02	0·9	1·3	—
LCB26	0·36	<0·01	<0·001	0·065
LCB190	0·37	<0·01	1·3	0·081

Table 4. *Aerobic growth of Ana$^-$ mutants on glucose*

The acetate concentration was determined in the medium after growth under vigorous aeration to a cell mass of 3 g wet wt l^{-1}.

Strain	Doubling time (min)	Acetate production (mM)
LCB900 (Ana$^-$)	60	5
LCB130 (Ana$^-$ Pfl$^-$ Nir$^-$)	55	6
LCB197 (Ana$^-$ Pfl$^-$ Nir$^-$)	55	5
LCB26 (Ana$^-$ Ack$^-$ Pta$^-$ Nir$^-$)	110	<0·2
LCB190 (Ana$^-$ Ack$^-$ Nir$^-$)	120	<0·2

Genetic analysis

To determine whether the mutations leading to the loss of PFL in strains LCB130 and LCB197 had affected the *pfl* gene described by Varenne *et al.* (1975), phage P1-mediated transduction experiments were performed using these strains as donors and a *pfl aroA* strain (LCB129) as recipient. All the selected AroA$^+$ recombinants were unable to produce gas from pyruvate indicating that the *pfl* gene was affected in both mutants. On the other hand, strains LCB26 and LCB190 were demonstrated to have an intact *pfl* gene: when used as donors in the transduction experiments they yielded transductants which, like the parent strain LCB900, produced gas from pyruvate.

For genetic mapping of the Ack$^-$ mutation carried by strain LCB190, an Hfr strain was constructed which carried this mutation. For this purpose, the Hfr strain P4X was mated with a λ lysogenic derivative of LCB190; from among the Lac$^+$ Met$^+$ recombinants, a strain having received the F factor and Ack$^-$ character was retained. This Hfr strain, LCB90, was used in subsequent conjugation experiments with strain K63. A strong linkage was observed with *his* (70%) and with *fadD* (62%). A Fad$^-$ Ack$^-$ recombinant (723-4) was used for transduction experiments with strain AB3295 carrying the *pabB* marker. In a cross where 128 Pab$^+$ transductants were selected, Ack$^-$ and Fad$^-$ characters were cotransduced at 4·6 and 6·2%, respectively; moreover, none of the Ack$^-$ transductants were found to be Fad$^-$. In the reciprocal cross where the Ack$^+$ transductants were selected, 5·4% had acquired the Pab$^-$ character while none was Fad$^+$. From these results we conclude that the gene *ackB* affected in strain LCB190 is close to *pabB* but resides on the opposite side to *fadD*, at about 39 min.

Recent studies have located genes *ack*, *pta*, *facA* and *facB* near *purF* (Brown *et al.*, 1977; Guest, 1979). Since strain LCB26, like the *facA* mutants, lacks ACK and PTA activities, transduction experiments were performed with strain LCB26 as donor and strain LCB522

Table 5. *Physiological characteristics of mutants*

	Activity [μmol substrate reduced min^{-1} (mg cells)$^{-1}$]					
	Nitrate reductase		Nitrite reductase		Gas production	
Strain	With pyruvate	With formate	With pyruvate	With formate	With pyruvate	With formate
LCB900 (Ana^-)	0·037	0·135	0·031	0·024	+	+
LCB898 (Pfl^-)	0·027	0·195	0·033	0·022	–	+
LCB49 (Pdh^-)	<0·001	0·225	<0·001	0·106	+	+
LCB130 (Ana^- Pfl^- Nir^-)	0·024	0·057	0·034	0·030	–	+
LCB197 (Ana^- Pfl^- Nir^-)	0·075	0·075	0·013	<0·001	–	+
LCB26 (Ana^- Ack^- Pta^- Nir^-)	0·006	0·084	0·012	0·043	–	+
LCB190 (Ana^- Ack^- Nir^-)	0·001	0·091	0·003	0·011	–	+

(*aroC purF*) as recipient. Among the 200 Pur^+ recombinants selected, 49·5% were Ack^-, while only 6% of the 100 Aro^+ recombinants selected had acquired this phenotype. These results are in agreement with those reported by Guest (1979) for *facA* mutations.

Pyruvate as electron donor for nitrate and nitrite reduction

The Nir^- strains of group B are devoid of NADH-nitrite oxidoreductase activity (Abou-Jaoudé, 1979) but can reduce nitrate and nitrite when using formate as electron donor (Abou-Jaoudé *et al.*, 1978), with the exception of LCB197 (which carries a *nirH* mutation and so is defective in nitrite reduction due to the lack of cytochrome *c*-552). Since these strains which cannot produce formate display a reduced but significant nitrite reduction with glucose (Abou-Jaoudé *et al.*, 1978), intermediate donors other than NADH or formate have been investigated. Using resting cells, nitrite (or nitrate) reduction was observed with the parental strain LCB900 when pyruvate was the electron donor. Although strain LCB898 lacked PFL activity and thus did not produce formate, it could still use pyruvate for nitrite or nitrate reduction (Table 5). A strain carrying a defect in the dehydrogenase subunit of the pyruvate dehydrogenase complex (LCB49) was unable to utilize pyruvate, suggesting that the electron transfer between pyruvate and nitrite (or nitrate) proceeds via the pyruvate dehydrogenase system.

DISCUSSION

During anaerobic growth with glucose at pH 7, *E. coli* K12 strains display a fermentation pattern for energy production which principally follows the equation: glucose → 2 formate + acetate + ethanol (Fig. 1). In this pathway, pyruvate is processed through the pyruvate formate-lyase reaction and regeneration of NAD^+ occurs only subsequently by reduction of one of the two molecules of acetyl-CoA formed. The importance of acetyl-CoA as terminal hydrogen acceptor and its indispensability as long as pyruvate is processed to acetate as an end-product is immediately evident from the growth property of the Ana^- mutant. Despite the fact that it contains D(–)-lactate dehydrogenase, this organism, which lacks acetaldehyde and ethanol dehydrogenases, can only grow when an external oxidant (nitrite or nitrate) is supplied (Casse *et al.*, 1976). However, nitrite or nitrate is no longer required by a further mutant derived from the Ana^- strain, which additionally lacks pyruvate formate-lyase (strain LCB130). This organism, which can grow with glucose provided that acetate is added as the anabolic C_2 source, displays pure lactate fermentation for energy production (data not shown). If nitrate or nitrite is the exogenous electron acceptor, NADH supplies electrons to the nitrate or nitrite reductase terminal enzymes through specific transfer chains.

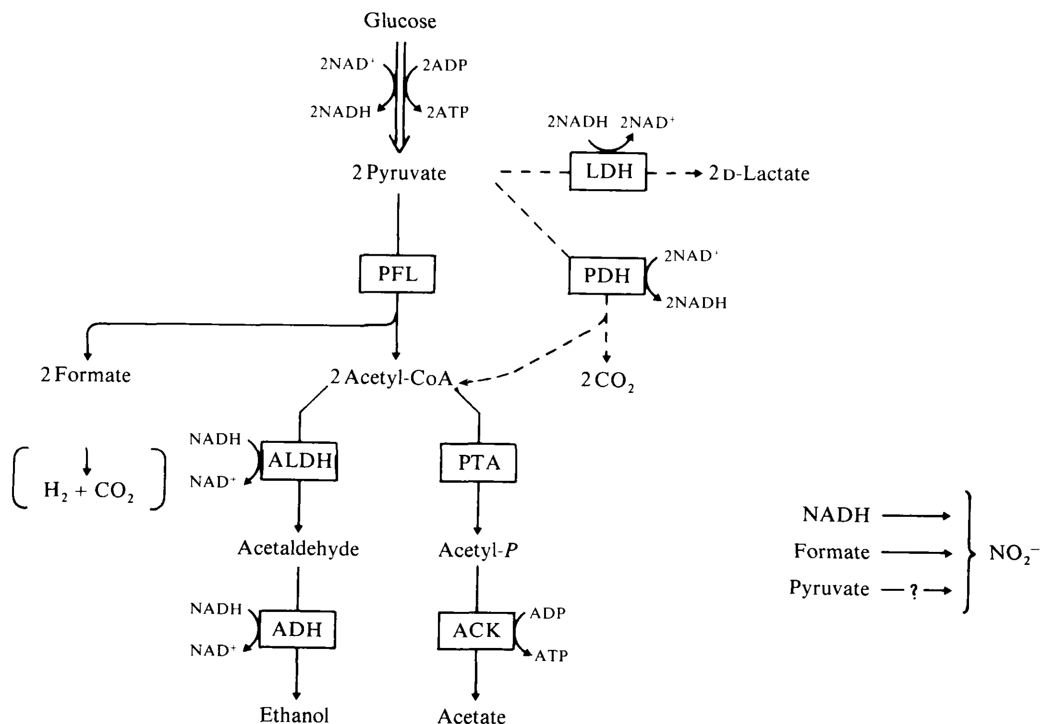

Fig. 1. Glucose fermentation in *Escherichia coli* K12. Enzyme abbreviations: ACK, acetate kinase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; LDH, D-lactate dehydrogenase; PDH, pyruvate dehydrogenase complex; PFL, pyruvate formate-lyase; PTA, phosphotransacetylase.

The simultaneous defect in alcohol and aldehyde dehydrogenases in the Ana^- strain could derive from a mutation in a control gene, from a deletion through adjacent genes or from a polar mutation in an operon; that both enzymes act in sequence could support the existence of a transcription unit. Alternatively, the double defect could be explained by mutations in both structural genes. Since mutants defective in either alcohol or aldehyde dehydrogenase must show the Ana^- phenotype, only the biochemical characterization of recombinants could answer the question. It would be of interest to resume the genetic analyses performed by Casse *et al.* (1976) and to study mutants affected in the structural genes.

The present studies have also revealed that *E. coli* K12, like *Proteus rettgeri* (Kröger *et al.*, 1974), can grow anaerobically with pyruvate as sole carbon source. Energy is then obtained from pyruvate fermentation to formate and acetate which provides (one) ATP without any redox process. At least one of the enzymes involved in this pathway (pyruvate-formate lyase, phosphotransacetylase or acetate kinase) proved to be affected in the various mutants of the Nir^- group B. That strains LCB26 and LCB190, although they contain pyruvate formate-lyase, are unable to produce hydrogen from pyruvate is expected since any continuous pyruvate conversion requires CoA regeneration from acetyl-CoA and these strains lack both the phosphotransacetylase/acetate kinase (PTA/ACK) and aldehyde dehydrogenase/alcohol dehydrogenase reaction sequences.

The low growth rate and markedly reduced levels of acetate production during aerobic growth of mutants LCB26 and LCB190 indicate that the PTA/ACK reaction sequence also operates during *aerobic* cell growth with glucose. The growth-promoting effect of PTA and ACK (by removing acetyl-CoA and enhancing CoA recycling) could be due to several reasons: several enzymes of the citric acid cycle are subjected to glucose repression (Sanwal,

1970), acetyl-CoA is inhibitory to the pyruvate dehydrogenase complex (Hansen & Henning, 1966) and acetyl-CoA appears to inhibit hexose transport (Morgan & Kornberg, 1969). Mutants impaired in ACK and PTA have previously been described (Brown *et al.*, 1977). A comparison of our strain LCB190 (Ack$^-$) and strain JM591 (Ack$^-$) of these authors revealed that LCB190 is more defective in aerobic growth on acetate or glucose as well as acetate accumulation from glucose. While these differences could possibly be explained by a more severe defect in LCB190 (1% as compared with 5% of wild-type activity), the genetic defects appear to differ since *ack* of JM591 was located at 50 min on the chromosomal map, whereas that of LCB190 resides at 39 min. As a result, we have designated the latter mutation *ackB*. It is so far unclear whether the two *ack* genes code for two distinct ACK enzymes or control a single enzyme. Genetically and biochemically, strain LCB26 resembles the *facA* mutants, i.e. loss of both ACK and PTA activities and cotransduction of the mutation with *purF* (Guest, 1979). We therefore propose the gene affected in LCB26 to be the *facA* gene itself.

Resting cells of *E. coli* grown anaerobically in the presence of nitrate or nitrite are able to reduce these acceptors with pyruvate as an electron donor. One could have supposed that formate was an intermediate in this reaction; however, an *aceE* strain carrying a defect in the dehydrogenase subunit of the pyruvate dehydrogenase complex provided unambiguous evidence that the electrons in fact come from pyruvate via the pyruvate dehydrogenase complex and not from formate via pyruvate formate-lyase and formate dehydrogenase. This would imply that NADH is involved in electron transport between pyruvate and nitrite.

Nevertheless, the involvement of NADH in the electron transfer from pyruvate to nitrite is in contradiction with the fact that strains lacking NADH-nitrite oxidoreductase possess pyruvate-nitrite oxidoreductase activity. This difficulty could be overcome since it has been proposed that the pyruvate dehydrogenase complex of *E. coli* has a site for interaction with an as yet unidentified electron acceptor (Frey *et al.*, 1978). It would be of interest to use a mutant lacking NADH-specific lipoamide dehydrogenase of this complex in order to establish whether or not NADH is involved in the pyruvate–nitrate/nitrite electron transfer.

Anaerobic conditions normally block the pyruvate dehydrogenase complex. This has been ascribed to the inhibitory action of NADH (Hansen & Henning, 1966). The presence of nitrate or nitrite could shift the NADH/NAD$^+$ ratio and thus enable the enzyme to be active.

Although strains LCB26 and LCB190 have normal pyruvate dehydrogenase activity they are unable to reduce nitrate or nitrite with pyruvate. This again can be explained by the inhibitory effect of the accumulated acetyl-CoA.

After the completion of this manuscript, we became aware of the discovery of another mutation, *adh*, which results in the derepression of alcohol dehydrogenase (Clark & Cronan, 1980). This mutation appears to map exceedingly close to the *ana* locus described here.

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