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# Molecular cloning and over-expression of the glyoxylate bypass operon from *Escherichia coli* ML308

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A recombinant plasmid carrying an 11 kb restriction-endonuclease-*Cla*I fragment of genomic DNA from *Escherichia coli* ML308 was constructed. This plasmid complements an *aceA* mutation. The plasmid encodes the structural genes of the glyoxylate bypass operon, namely malate synthase A (*aceB*), isocitrate lyase (*aceA*) and isocitrate dehydrogenase kinase/phosphatase (*aceK*), as judged by overexpression of enzyme activities and transcription/translation experiments *in vitro*. Subcloning confirmed that expression of the *aceK* gene is essential for growth on acetate.

## INTRODUCTION

During growth of micro-organisms on acetate, the glyoxylate bypass is used to generate the precursors necessary for biosynthesis [1]. The competition between the bypass and the tricarboxylic acid cycle at the level of isocitrate is resolved, in *Escherichia coli* and other enteric bacteria, by partial inactivation and phosphorylation of isocitrate dehydrogenase (ICDH, EC 1.1.1.42) [2–5]. The phosphorylation system provides a mechanism whereby flux through the glyoxylate bypass can be controlled very precisely [5,6].

Phosphorylation and dephosphorylation of ICDH are catalysed by a single bifunctional enzyme, ICDH kinase/phosphatase [7,8]. In our laboratory the kinetic and regulatory properties of this enzyme [9], ICDH [8,10] and isocitrate lyase [11] have been extensively studied by using *E. coli* ML308. This has allowed us to identify factors that control the phosphorylation state of ICDH and the flux through the glyoxylate bypass *in vivo* [2,3,12,13].

The genes encoding the enzymes of the glyoxylate bypass, malate synthase A (*aceB*) and isocitrate lyase (*aceA*), and that encoding ICDH kinase/phosphatase (*aceK*) comprise an operon termed the 'glyoxylate bypass operon' [14,15]. This operon is located at 90 min on the *E. coli* chromosomal map and is controlled by the genes *iclR* and *fadR* [14,16,17]. Expression of the operon is de-repressed during growth on acetate [14,16]. The *aceK* gene has been cloned from *E. coli* K12 [18], but no molecular characterization of the glyoxylate bypass operon has been reported. We wished to clone this operon from *E. coli* ML308 to facilitate both isolation of large amounts of the three enzymes and analysis of the control of the expression of the operon, and our results are presented here.

## EXPERIMENTAL

### Materials

Restriction endonucleases were purchased from Bethesda Research Laboratories, Paisley, Renfrewshire, Scotland, U.K. Calf intestinal phosphatase was from Boehringer, Lewes, East Sussex, U.K. The nick-translation kit, *in vitro* transcription/translation kit, L-[$^{35}$S]methionine, [$\alpha$-$^{32}$P]dCTP (sp. radioactivity 3000 Ci/mmol) and T4 DNA ligase were from Amersham International, Amersham, Bucks., U.K. Other materials were as described previously [19].

### Enzyme preparations

ICDH and isocitrate lyase were isolated from *E. coli* ML308 grown on glycerol and acetate respectively [11,19]. Malate synthase A was purified from *E. coli* KAT-1/pEM9 (C. MacKintosh & H. G. Nimmo, unpublished work). ICDH kinase/phosphatase was purified from *E. coli* KAT-1/pEM9 by a modification of the method given in [8].

### Bacterial strains and growth conditions

*E. coli* ML308 (A.T.C.C. 15224) was grown in a mineral-salts solution supplemented with either 20 mM-glycerol or 40 mM-sodium acetate [19,20]. *E. coli* strains LE392 (F$^-$, *hsdR*514, *supE*44, *supF*58, *lacY*1, *galK*2, *galT*22, *metB*1, *trpR*55) and KAT-1 (an *aceA* derivative of LE392 obtained by transposon Tn10 insertion [14]) and plasmid pCTS (see below) were from Dr. W. D. Nunn (University of California, Irvine, CA, U.S.A.). They were grown as described above, except that media were supplemented with L-methionine (50 $\mu$g/ml) and vitamin $B_1$ (1 $\mu$g/ml). For plasmid-bearing strains, the media also contained ampicillin (100 $\mu$g/ml).

### Assay procedures

Crude cell extracts were prepared and centrifuged as described in [8]. Malate synthase [21] and isocitrate lyase were assayed in the crude supernatants. The lyase was assayed by coupling the formation of glyoxylate with the oxidation of NADH with lactate dehydrogenase [22]. Each cuvette contained, in a final volume of 1 ml, 50 mM-Mops/NaOH, pH 7.3, 5 mM-$MgCl_2$, 5 mM-DL-isocitrate, 0.2 mM-NADH, pig heart lactate dehydrogenase (0.1 mg/ml) and extract. Since ICDH kinase cannot be assayed satisfactorily in crude extracts [8], the enzyme was first partially purified by protamine sulphate

Abbreviation used: ICDH, isocitrate dehydrogenase.

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treatment [8] and fractionation with $(NH_4)_2SO_4$. Proteins that precipitated between 25 and 45% saturation were collected and dissolved in 50 mM-Mops/NaOH (pH 7.3)/1 mM-EDTA/1 mM-dithiothreitol/1 mM-benzamidine hydrochloride. ICDH kinase was assayed by measuring the rate of inactivation of ICDH [8]. Protein was assayed by Bradford's [23] method. Enzymes were assayed at 37 °C and specific activities are quoted per mg of crude supernatant protein.

### Other methods

DNA manipulations and Southern blotting [24] were carried out using methods given in [25]. Bacterial transformation was by the method of Dagert & Ehrlich [26]. Transcription/translation *in vitro* [27] using L-[$^{35}$S]methionine and nick-translation using [$\alpha$-$^{32}$P]dCTP were carried out according to the instructions supplied by Amersham International. Proteins were analysed by SDS/polyacrylamide-gel electrophoresis, a 10% separating gel and a 3% stacking gel [28] being used.

## RESULTS AND DISCUSSION

*E. coli* KAT-1 is an *aceA* mutant and is therefore unable to grow on acetate. No ICDH kinase or ICDH phosphatase activity was detected in this strain in cells grown on glycerol (Table 1). Addition of acetate (40 mM) for periods of up to 18 h elicited only barely detectable levels of activity (results not shown). Plasmid pCTS is a derivative of pBR322 that complements the *aceA* mutation of KAT-1. It contains a 10 kb insert of wild-type *E. coli* K12 DNA encoding the glyoxylate-bypass-operon structural genes *aceA* and *aceB* (W. D. Nunn, personal communication). Strain KAT-1/pCTS overexpresses isocitrate lyase and ICDH kinase some 15-fold relative to LE392 (results not shown), suggesting that pCTS also contains the *aceK* gene.

We prepared a $^{32}$P-labelled probe by nick-translation of pCTS and used it to probe a Southern blot [24] of restriction-endonuclease digests of genomic DNA from *E. coli* K12 and ML308. For both strains, restriction endonuclease *Cla*I digests contained only a single fragment that hybridized with the probe, but digests using endonucleases *Hin*dIII, *Eco*RI, *Bgl*II, *Bam*HI and *Ava*I contained several such fragments (results not shown). We therefore decided to clone this *Cla*I fragment of *E. coli* ML308 DNA in pAT153 [29].

*E. coli* ML308 DNA was digested with *Cla*I and ligated to plasmid pAT153, which had been treated with *Cla*I and calf intestinal phosphatase. A ligation mix containing 50 ng of vector DNA was used to transform *E. coli* KAT-1. When the cells were plated on to L-agar containing ampicillin and tetracycline, 4800 transformants were obtained. It was not possible to determine directly the frequency of recombinants among the transformants by screening for insertional inactivation of the *tet*$^R$ gene of pAT153, as KAT-1 is also tetracycline-resistant by virtue of the Tn*10* insertion in *aceA*. However, a control experiment showed that lengthy phosphatase treatment of the vector had severely reduced the frequency of non-recombinant transformants. Transformants were then replica-plated on to minimal medium containing acetate and ampicillin. Two recombinants able to grow on acetate were obtained. The recombinant plasmids were isolated [30] and shown by restriction-endonuclease analysis to have identical 11 kb inserts in the same orientation (results not shown). One of the recombinant strains, KAT-1/pEM9, was chosen for further analysis, and a partial restriction map of the insert in pEM9 is shown in Fig. 1.

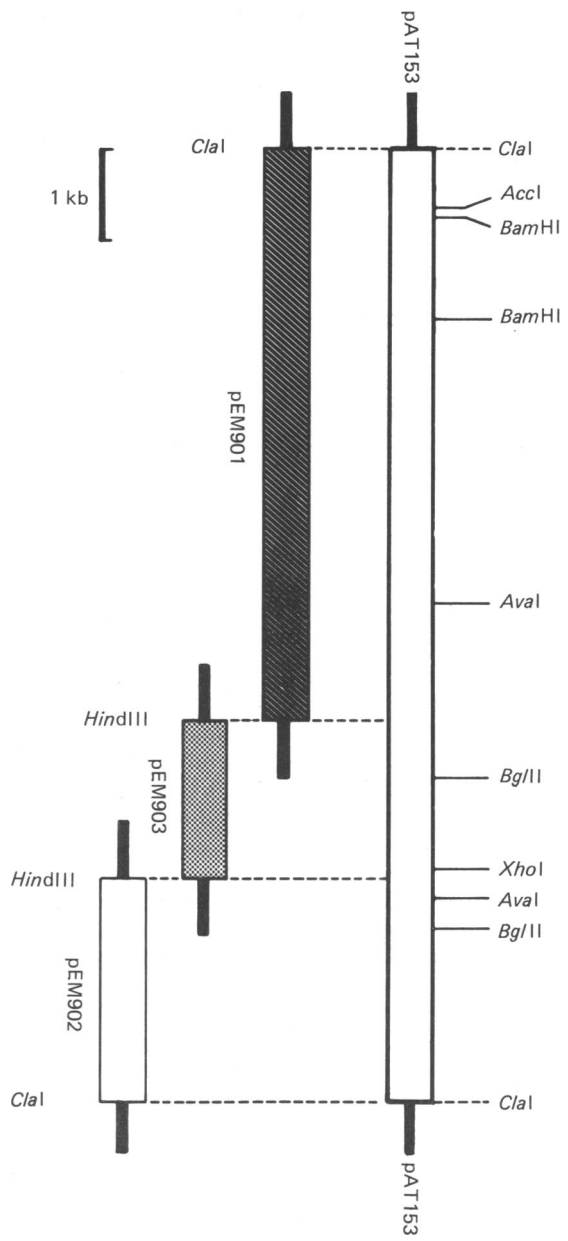


**Fig. 1. Restriction map of the insert in pEM9**

A *Cla*I/*Hin*dIII double digest of pEM9 gave three genomic fragments. These were isolated from a low-melting-point agarose gel and subcloned in pAT153.

The specific activities of isocitrate lyase, malate synthase and ICDH kinase in extracts of *E. coli* ML308, LE392, KAT-1 and KAT-1/pEM9 grown on glycerol or acetate are shown in Table 1. Strain KAT-1/pEM9 overexpresses ICDH kinase 13–18-fold relative to either ML308 or LE392. The overexpression factors for isocitrate lyase and malate synthase are slightly lower. These factors are lower than would be expected from the copy number of pAT153. However, it is evident that, in KAT-1/pEM9 grown on acetate, isocitrate lyase and

**Table 1. Specific activities of enzymes in crude extracts of *E. coli* strains**

Abbreviation: ng, no growth. Values are representative of several experiments.

| | | Specific activity ($\mu$mol·min$^{-1}$·mg$^{-1}$) | | | | Specific activity (nmol·min$^{-1}$·mg$^{-1}$) | |
|---|---|---|---|---|---|---|---|
| | Enzyme... | Isocitrate lyase | | Malate synthase | | ICDH kinase | |
| Strain | Carbon source... | Glycerol | Acetate | Glycerol | Acetate | Glycerol | Acetate |
| ML308 | | 0.04 | 0.67 | 0.12 | 1.26 | 0 | 0.027 |
| LE392 | | 0.24 | 0.81 | 0.48 | 1.39 | 0.010 | 0.036 |
| KAT-1 | | 0 | ng | 0.21 | ng | 0 | ng |
| KAT-1/pEM9 | | 1.49 | 5.44 | 2.70 | 9.14 | 0.080 | 0.490 |

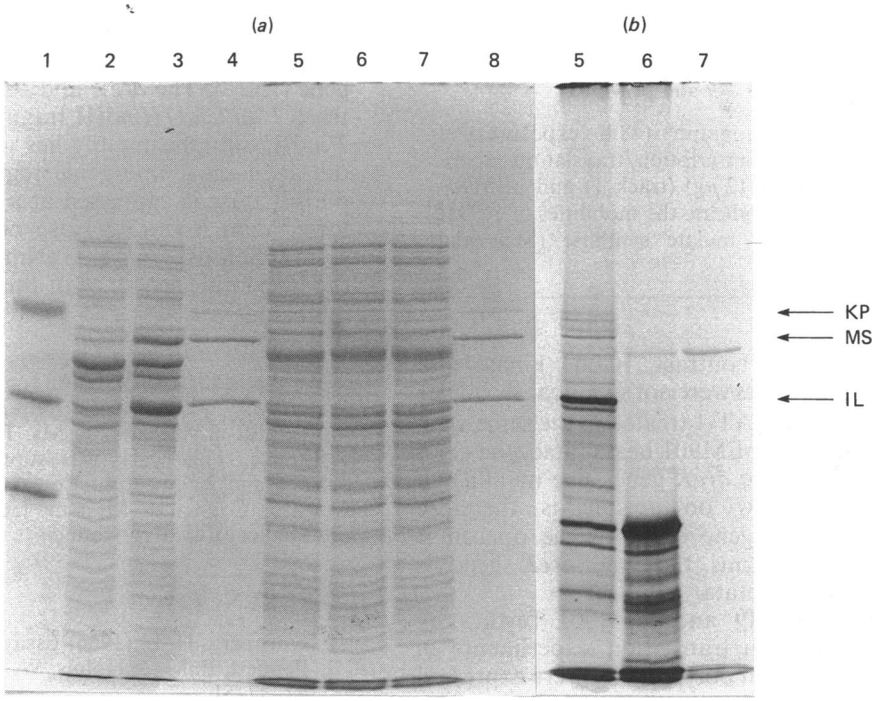


**Fig. 2. Proteins encoded by pEM9**

(*a*) Shows a polyacrylamide gel stained for protein. Track 1, marker proteins bovine serum albumin, *E. coli* ICDH and pig heart lactate dehydrogenase ($M_r$ values 68000, 45000 and 35000 respectively). Tracks 2 and 3, crude supernatants from *E. coli* LE392 (track 2) and KAT-1/pEM9 (track 3); 20 $\mu$g of protein each. Tracks 4 and 8, homogeneous marker proteins: ICDH kinase/phosphatase (1 $\mu$g), malate synthase and isocitrate lyase (2 $\mu$g each). Tracks 5–7, products from transcription/ translation *in vitro* (incubation time 60 min) of pEM9 (1 $\mu$g, track 5), pAT153 (2.5 $\mu$g, track 6) and no DNA (track 7). (*b*) Shows an autoradiograph (48 h exposure ) of tracks 5–7. The arrows indicate the mobilities of ICDH kinase/phosphatase (KP), malate synthase (MS) and isocitrate lyase (IL).

malate synthase together comprise a large part of the total soluble cell protein (see Fig. 2), and this may restrict the degree of overexpression attainable. The results in Table 1 also show that the activities of all three enzymes in each of strains ML308, LE392 and KAT-1/pEM9 are higher during growth on acetate than on glycerol. Expression of the glyoxylate bypass operon is controlled by the gene *iclR*, which is adjacent to the operon itself [14,16]. Since strain KAT-1 is *iclR*$^+$ (see Table 2 in [14]), the results do not test the possibility that pEM9 also carries the *iclR* gene.

Three fragments of the insert in pEM9 were subcloned in pAT153 as shown in Fig. 1. The resulting plasmids, pEM901, pEM902 and pEM903, were used to transform *E. coli* KAT-1, selecting for resistance to ampicillin. The transformants grew on glycerol, but not on acetate, showing that the plasmids could not complement the mutation in KAT-1. However, when KAT-1/pEM901 was grown on glycerol and then challenged with 40 mM-acetate for 2 h, high levels of isocitrate lyase and malate synthase activities were detected. The specific activities of these enzymes in a crude extract were 2.4 and 3.9 $\mu$mol/min per mg of protein respectively, only 2–3-fold lower than the values for KAT-1/pEM9 grown

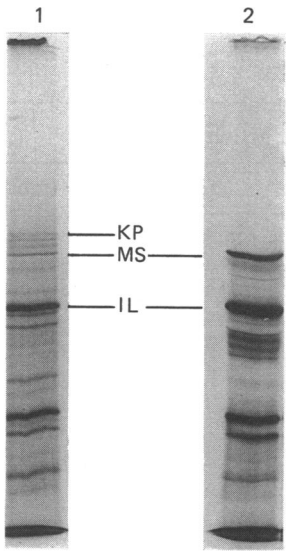


**Fig. 3. Proteins encoded by pEM9 and pEM901**

This shows an autoradiograph (48 h exposure) of products from a separate transcription/translation experiment *in vitro* using pEM9 (2 $\mu$g) (track 1) and pEM901 (2 $\mu$g) (track 2). The bars indicate the mobilities of ICDH kinase/phosphatase (KP), malate synthase (MS) and isocitrate lyase (IL).

on acetate (Table 1). In contrast, ICDH kinase and ICDH phosphatase activities were not significantly higher than in crude extracts of KAT-1 treated in the same way. These results suggest that pEM901 bears intact the *aceA* and *aceB* gene, but not the *aceK* gene. The inability of KAT-1/pEM901 to grow on acetate is therefore expected, given that the gene order in the operon is *aceBAK* [14,15]. It confirms that the *aceK* gene is essential for growth on acetate.

We characterized pEM9 and pEM901 further by carrying out transcription/translation experiments *in vitro* (Figs. 2 and 3). Several polypeptides were expressed from pEM9 and pEM901, but not from pAT153 or in a 'no DNA' control. Since the insert in pEM9 is only some 11 kb, it is likely that several of these bands result from proteolysis or premature termination of transcription or translation. Homogeneous preparations of isocitrate lyase, malate synthase A and ICDH kinase/phosphatase were mixed and run as marker proteins (Fig. 2, tracks 4 and 8). The major polypeptide expressed from both pEM9 and pEM901 co-migrated with isocitrate lyase, and another of the products expressed by both plasmids co-migrated with malate synthase A. pEM9, but not pEM901, directed synthesis of a band which co-migrated with ICDH kinase/phosphatase. This was the least-mobile band observed with either plasmid, and it is therefore very unlikely that it resulted from proteolysis or premature termination. In combination with the activity data (see above), this confirms that pEM9 bears the *aceK* gene but pEM901 does not. The pronounced band seen in both the 'no DNA' control and the other tracks may represent a protein that binds methionine or a methionyl-tRNA very tightly (see the instructions supplied with the transcription/translation kit by Amersham International).

Fig. 2 also shows the polypeptides present in crude supernatants of *E. coli* LE392 and KAT-1/pEM9 grown on acetate (tracks 2 and 3). Both isocitrate lyase and malate synthase are overproduced in the latter strain. The isocitrate lyase from KAT-1/pEM9 is identical with that of ML308 as judged by peptide mapping and *N*-terminal sequence analysis (C. MacKintosh & H. G. Nimmo, unpublished work). Although ICDH kinase/phosphatase is overexpressed in KAT-1/pEM9 (Table 1), it remains a low-abundance protein in this strain.

The work described here has thus allowed us to construct a strain that overexpresses the enzymes encoded by the glyoxylate bypass operon of *E. coli* ML308. Procedures for the purification of isocitrate lyase and malate synthase A from *E. coli* have not been reported, and the overexpression obtained here will greatly facilitate characterization of these two enzymes and of ICDH kinase/phosphatase. Our results are consistent with previous studies on the order of genes in the glyoxylate bypass operon and subcloning of the *aceK* gene [15,18]. The *aceA* and *aceB* genes clearly lie within the 6.7 kb *Cla*I/*Hin*dIII fragment shown in Fig. 1. This *Hin*dIII site presumably lies within the *aceK* gene or in the *aceA–aceK* intergenic region. Since ICDH kinase/phosphatase is expressed at a level some 500–1000-fold lower than isocitrate lyase or malate synthase A [15; C. MacKintosh & H. G. Nimmo, unpublished work], the nucleotide sequence of this region is of considerable interest.

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