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Glyoxylate Bypass Operon of *Escherichia coli*: Cloning and Determination of the Functional Map

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In *Escherichia coli*, a single operon encodes the metabolic and regulatory enzymes of the glyoxylate bypass. The metabolic enzymes, isocitrate lyase and malate synthase, are expressed from *aceA* and *aceB*, and the regulatory enzyme, isocitrate dehydrogenase kinase/phosphatase, is expressed from *aceK*. We cloned this operon and determined its functional map by deletion analysis. The order of the genes in this operon is *aceB-aceA-aceK*, with *aceB* proximal to the promoter, consistent with the results of previous experiments using genetic techniques. The promoter was identified by S1 nuclease mapping, and its nucleotide sequence was determined. Isocitrate lyase and malate synthase were readily identified by autoradiography after the products of the operon clone were labeled by the maxicell procedure and then resolved by electrophoresis. In contrast, isocitrate dehydrogenase kinase/phosphatase, expressed from the same plasmid, was undetectable. This observation is consistent with a striking downshift in expression between *aceA* and *aceK*.

In *Escherichia coli*, adaptation to growth on acetate requires the induction of the enzymes of the glyoxylate bypass: isocitrate lyase and malate synthase (12, 13). This bypass is essential for growth on acetate since it prevents the net loss of the acetate carbons as carbon dioxide in the Krebs cycle (Fig. 1). Once induced, the flow of isocitrate through the glyoxylate bypass is regulated, in part, by the phosphorylation of isocitrate dehydrogenase (IDH), the Krebs cycle enzyme which competes with isocitrate lyase (2, 8, 9, 11, 31). During growth on acetate, ca. 70% of the IDH is maintained in the inactive phosphorylated form (4, 16, 18), reducing the activity of this enzyme and thus forcing isocitrate through the bypass (19, 26). The phosphorylation and dephosphorylation of IDH are catalyzed by a bifunctional enzyme, IDH kinase/phosphatase (17), which is encoded by a single gene, *aceK* (15).

In addition to being regulated by the phosphorylation of IDH, the glyoxylate bypass is regulated at the level of gene expression. Isocitrate lyase and malate synthase (expressed from *aceA* and *aceB*, respectively) are induced during growth on acetate and are repressed on most other carbon sources (12). Brice and Kornberg (5) determined that *aceA* and *aceB* mapped close to each other at 90 min on the *E. coli* chromosome. Maloy and Nunn (22) subsequently found that these genes are in the same operon and that *aceB* is upstream from *aceA*. We have demonstrated that *aceK* is also in this operon, downstream of both *aceB* and *aceA* (18). Thus, the organization of the glyoxylate bypass operon is

$$\frac{aceB\ aceA\ aceK}{P}$$

where P indicates the promoter. The expression of the glyoxylate bypass operon is regulated, directly or indirectly, by the products of *iclR* and *fadR* (12, 22, 30).

One of our long-term goals is to characterize the mechanisms which control the glyoxylate bypass operon and the factors which influence the differential expression of its products. Here, we report the cloning of this operon and the determination of its physical and functional maps. We also used the maxicell procedure to confirm our previous observation of a striking downshift in expression between *aceA* and *aceK* (18).

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MATERIALS AND METHODS

Materials. Restriction enzymes, other DNA modification enzymes, *Hin*dIII linkers, and NACS52 Prepac columns were purchased from Bethesda Research Laboratories, Inc. [^{35}S]methionine was obtained from Amersham Corp. All other reagents were the purest grades available.

Bacterial strains and growth media. The *E. coli* strains used in this study and their genotypes are given in Table 1. The minimal acetate medium used was the MOPS (morpholinepropanesulfonic acid)-based medium described by Neidhardt et al. (25), containing 2% sodium acetate and the appropriate amino acids. When indicated, 1% sodium succinate was also included. L broth contained 1% tryptone (Difco Laboratories), 1% NaCl, and 0.5% yeast extract. M63 medium contained 0.4% glucose, 20 mM KH_2PO_4, 40 mM K_2HPO_4, 15 mM $(NH_4)_2SO_4$, 3 μM $FeSO_4$, 1 mM $MgCl_2$, and when indicated, 1% Casamino Acids (Difco). Ampicillin was included at 200 μg/ml for the growth of strains harboring plasmids.

Recombinant DNA techniques. Except when indicated, manipulations of recombinant plasmids were accomplished by standard techniques (23).

Cloning of the glyoxylate bypass operon. Two independent clones of the glyoxylate bypass operon were isolated. One isolate was subcloned from a plasmid carrying the appropriate region of the *E. coli* chromosome as a 20-kilobase (kb) insert. This plasmid, obtained from W. D. Nunn, had been constructed by partially digesting chromosomal DNA with *Sau*3A followed by ligation into the *Bam*HI site of pBR322. (This procedure did not regenerate the *Bam*HI sites.) To locate the glyoxylate bypass operon within this insert, we determined the restriction map of the plasmid and compared it with that of *aceK* (15). This comparison indicated that one of the fragments generated by the digestion of this plasmid with *Hin*dIII carried the operon and most of the vector sequences. The *Hin*dIII digest was ligated with T4 DNA ligase and used to transform strain MM294A to ampicillin

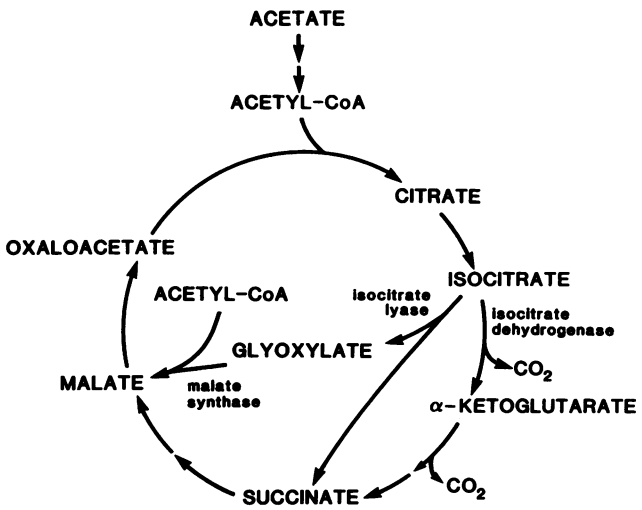

FIG. 1. Krebs cycle and the glyoxylate bypass.

resistance. Plasmid DNA was isolated from the transformants, and the identity of the plasmid was confirmed by restriction mapping. This plasmid is designated pCL8.

Although pCL8 contained the structural genes of the glyoxylate bypass operon (see Results), it contained relatively little DNA upstream of *aceB*. To ensure the isolation of the entire 5′ untranscribed region, we isolated a second clone of this operon. Southern blot analysis indicated that digestion of chromosomal DNA with *Hin*dIII and *Eco*RI would produce a clone of the desired size. Chromosomal DNA was isolated from *E. coli* MM294A, digested with *Hin*dIII and *Eco*RI, and ligated between these sites in pBR322. The ligation mixture was then transformed into strain 5236 (*aceB glc*), and the appropriate clone was identified by its ability to complement the *aceB* mutation, restoring this host to growth on acetate. The identity of this plasmid, designated pCL1000, was confirmed by restriction mapping and by testing its ability to express the enzymatic activities encoded by the glyoxylate bypass operon.

Deletion analysis. To determine the functional map of the glyoxylate bypass operon, a series of nested deletions were generated starting at the 3′ end of the operon. pCL8 was cleaved with *Hin*dIII and then digested with Bal31. Samples were removed at intervals, and the reaction was stopped with EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid)]. After this digestion, the pooled samples were extracted with phenol, and DNA was isolated by ethanol precipitation. The ends were repaired with the Klenow fragment of DNA polymerase I, and *Hin*dIII linkers were attached. The DNA was then cleaved with *Hin*dIII and *Sal*I, purified on a NACS52 Prepac column, and ligated between the *Sal*I and *Hin*dIII sites of pBR322. After transformation into strain MM294A, plasmid DNA was isolated from individual colonies. The extent of each deletion was determined by restriction mapping. The effects of these deletions on the expression of the products of the operon are presented in Fig. 3, 4, and 5.

To delete the promoter of the glyoxylate bypass operon, pCL8 was partially digested with *Bam*HI and then digested to completion with *Eco*RI. The appropriate fragment was purified by electrophoresis and ligated between the *Eco*RI and *Bam*HI sites of pBR322. Plasmid DNA was isolated from individual transformants, and its identity was confirmed by restriction mapping. (This procedure resulted in the deletion of the promoter of the tetracycline resistance gene.) The plasmid generated by this procedure, pCL800, is identical to its parent, pCL8, except for the removal of the insert sequences upstream of the first *Bam*HI site (Fig. 2).

Maxicell labeling. The maxicell technique was used to specifically label the protein products of the recombinant plasmids with [^{35}S]methionine. The method used was similar to that described by Sancar et al. (28), with the following significant modifications. The strain used for these experiments, MM294A, carried a *recA* mutation but not a *uvrA* mutation. Light-activated repair of chromosomal DNA was avoided by covering the cultures with aluminum foil after UV irradiation. Cells surviving the irradiation were killed by the addition of D-cycloserine to 200 μg/ml two h after irradiation. To induce expression from the promoter of the glyoxylate bypass operon, growth and labeling of MM294A, harboring the appropriate plasmid, were performed in minimal acetate medium. To specifically label IDH kinase/phosphatase, MM294A harboring pCL4 (which expresses *aceK*$^+$ from the *tac* promoter) was grown on M63 medium supplemented with Casamino Acids and labeling was then performed in this medium without Casamino Acids.

After the labeling step, samples were subjected to sodium dodecyl sulfate-gel electrophoresis as described by Laemmli (14). The gel was stained with methanol-acetic acid-water (5:1:5) containing 0.1% Coomassie brilliant blue R and destained with 10% acetic acid. The gel was then treated with En^{3}Hance (New England Nuclear Corp.) as recommended by the manufacturer and dried. Autoradiography was performed at −70°C. Molecular weight standards were myosin (205,000), β-galactosidase (116,000), phosphorylase *b* (97,400), bovine serum albumin (66,000), ovalbumin (45,000), and carbonic anhydrase (29,000).

S1 nuclease mapping. Strain MM294A was grown to the mid-log phase on minimal acetate medium, and total RNA was isolated by the method of Summers (29). Plasmid pCL1000 was end labeled at the *Bss*HII sites by using T4 polynucleotide kinase and cleaved with *Cla*I, and the appropriate fragment was isolated by gel electrophoresis. S1 nuclease protection analysis was performed by the method of Berk and Sharp (3). The probe (ca. 100,000 cpm) was mixed with 50 μg of RNA and precipitated with ethanol. The sample was dissolved in 50 μl of 80% formamide–40 mM PIPES [piperazine-*N*-*N*′-bis(2-ethanesulfonic acid)] (pH 6.5)–400 mM NaCl–1 mM EDTA and then denatured for 10 min at 75°C. Hybridization was performed at 49°C for 4 h followed by the addition of 250 U of S1 nuclease in 500 μl of 30 mM sodium acetate (pH 4.6)–250 mM NaCl–1 mM $ZnSO_4$–5% glycerol–20 μg of salmon sperm DNA per ml. This reaction mixture was incubated at 37°C for 1 h. The reaction was stopped by the addition of 50 μl of 7.5 M ammonium acetate–100 mM EDTA, and the mixture was extracted with phenol-chloroform (1:1). The protected frag-

TABLE 1. Bacterial strains used

Strain	Genotype	Reference or source
DEK2011	*aceK1 his-4 thi-1 rpsL31 lacBK1 sfiB*	18
MM294A	*endA thi hsdR recA*	D. E. Koshland
5236	*aceB glc ppc*	CGSC[a]
BN1	*aceA zja*::Tn*10 metB galK galT trpR lacY1* or Δ*lacIZY*	W. D. Nunn

[a] CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

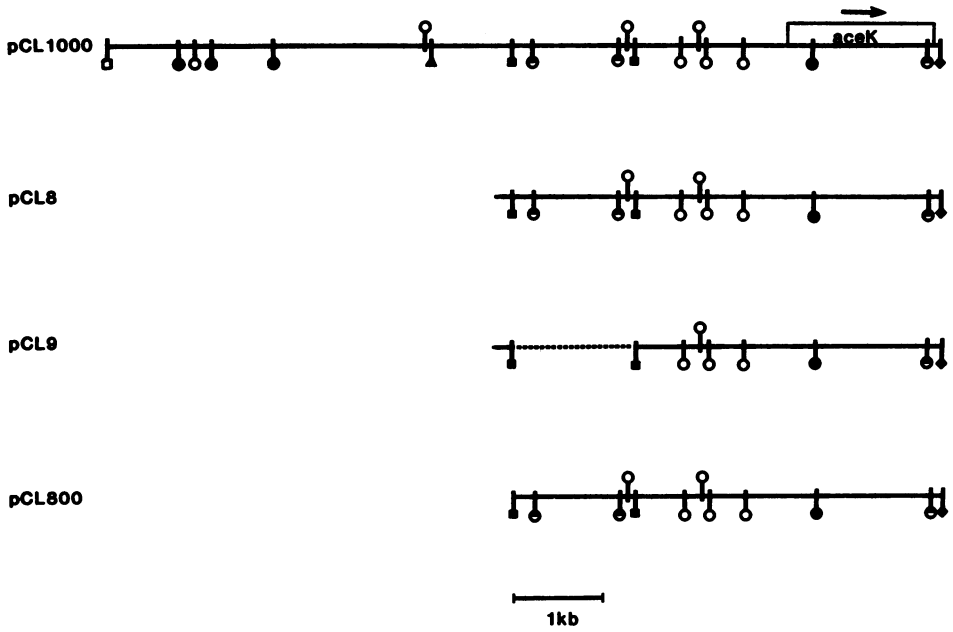

FIG. 2. Restriction maps of the inserts in pCL8 and pCL1000. The location and transcriptional orientation of *aceK* were determined by comparison of its restriction map (17; Klumpp et al., submitted) with those of pCL8 and pCL1000. Plasmids pCL8 and pCL1000 are independent isolates carrying the glyoxylate bypass operon. Plasmid pCL9 was derived from pCL8 by deletion of the *Bam*HI fragment. Deletion of the sequences upstream of the left *Bam*HI site in pCL8 produced pCL800. Restriction sites: □, *Eco*RI; ●, *Ava*I; ○, *Pvu*II; ▲, *Cla*I; ⊖, *Bss*HII; ■, *Bam*HI; ◆, *Hin*dIII.

ments were then isolated by ethanol precipitation. The DNA probe was also subjected to the sequencing reactions of Maxam and Gilbert (24). Samples were then resolved by electrophoresis under denaturing conditions (23).

Enzyme assays. All assays were performed at 37°C. The activity of IDH phosphatase was measured by monitoring the release of [^{32}P]phosphate from [^{32}P]phospho-IDH, as described previously (17). The standard reaction mixture consisted of 25 mM MOPS (pH 7.5), 0.1 μM [^{32}P]phospho-IDH (ca. 30,000 cpm), 1 mM ATP, 5 mM $MgCl_2$, 100 mM NaCl, 2 mM dithiothreitol, 0.5 mM EDTA, 5 mM 3-phosphoglycerate, 1 mM DL-isocitrate, and 2 mg of bovine serum albumin per ml. One unit of IDH phosphatase activity is defined as the amount of enzyme required to achieve 50% dephosphorylation of the phospho-IDH in 1 min.

Isocitrate lyase activity was monitored by using the method of Maloy et al. (21). The reaction mixture contained 100 mM potassium phosphate (pH 7.0), 6 mM $MgCl_2$, 4 mM phenylhydrazine, and 12 mM cysteine and was monitored at 324 nm. One unit of isocitrate lyase produced 1 μmol of glyoxylate per min.

Malate synthase activity was measured by monitoring the cleavage of the thioester bond of acetyl coenzyme A at 232 nm. The reaction mixture consisted of 80 mM Tris (pH 8.0), 125 μM acetyl coenzyme A, 10 mM sodium glyoxylate, and 15 mM $MgCl_2$. One unit of malate synthase cleaved 1 μmol of acetyl coenzyme A per min.

Protein concentration was determined by the method of Lowry et al., with bovine serum albumin as a standard (20).

RESULTS

Cloning of the glyoxylate bypass operon. We isolated two independent clones of the glyoxylate bypass operon of *E. coli* (see Materials and Methods). These clones, designated pCL8 and pCL1000, carry the operon within inserts of 5 and 9.5 kb, respectively. Restriction mapping indicated that these inserts are identical (Fig. 2), except that pCL1000 carries an additional 4.5 kb of DNA upstream from *aceB* (see below). Comparison of these restriction maps with that determined from the nucleotide sequence of *aceK* (D. J. Klumpp, D. Plank, C. S. Stueland, T. Chung, and D. C. LaPorte, submitted for publication) indicated that these clones included the intact gene; allowed us to estimate its position and orientation.

The ability of these plasmids to express the products of the glyoxylate bypass operon was tested by enzymatic assay. Expression of the operon clones was induced in mutant strains which were deficient in IDH kinase/phosphatase (DEK2011), isocitrate lyase (BN1), or malate synthase (5236) (Table 2). Both pCL8 and pCL1000 restored the ability of each of these strains to express the deficient enzyme, indicating that the full operon had been cloned. We also found that plasmid pCL8 yielded 10- to 20-fold overproductions of the operon products in wild-type strains (data not shown).

Plasmid pCL8 and the parental plasmid from which it was subcloned complemented the *aceB* mutation of strain 5236, restoring the ability of this strain to grow on a minimal acetate medium. Similarly, the parental plasmid complemented the *aceA* mutation in strain BN1 and the *aceK* mutation in strain DEK2011. In contrast, pCL8 did not complement the *aceA* or *aceK* mutation. The failure of pCL8 to complement these mutations very probably resulted from general growth-inhibitory effects of this plasmid, since pCL8 also inhibited the growth of congenic wild-type strains on acetate. Growth inhibition by this plasmid may result from the overproduction of isocitrate lyase (which normally composes 5 to 10% of the soluble protein during growth on acetate) and malate synthase. Alternatively, the presence of a strong promoter on a multicopy plasmid may be sufficient to inhibit growth on this medium, a possibility which is

TABLE 2. Expression of the glyoxylate bypass operon

Strain[a]	Relevant genotype	Plasmid	Amt (U/mg) of:		
			Malate synthase	Isocitrate lyase	IDH phosphatase
DEK2011	*aceK1*	None			0.00
		pCL1000			1.06
		pCL8			0.85
		pCL9			0.89
		pCL800			0.01
BN1	*aceA*	None		0.0	
		pCL1000		2.0	
		pCL8		2.0	
		pCL9		1.8	
		pCL800		0.0	
5236	*aceB glc*	None	0.0		
		pCL1000	7.3		
		pCL8	8.0		
		pCL9	0.0		
		pCL800	0.0		

[a] The glyoxylate bypass operon was induced in DEK2011 and BN1 by growth to the stationary phase on L broth containing 2% sodium acetate and in 5236 by growth on MOPS minimal medium containing 2% sodium acetate–1% sodium succinate.

consistent with similar results which we have obtained with plasmids which use the *tac* promoter (15).

The products of pCL8 were examined by sodium dodecyl sulfate-gel electrophoresis after in situ labeling by the maxicell procedure (see Materials and Methods). In addition to β-lactamase (molecular weight, 28,300), expressed from the *bla* gene of the vector two protein products were detectable (Fig. 3, lane 2). Comparison of the electrophoretic mobilities of these proteins with that of IDH kinase/phosphatase, expressed from pCL4 (Fig. 3, lane 1), indicated that neither of these bands corresponded to IDH kinase/phosphatase. One of these bands had an apparent molecular weight of 46,000, which is very similar to the apparent molecular

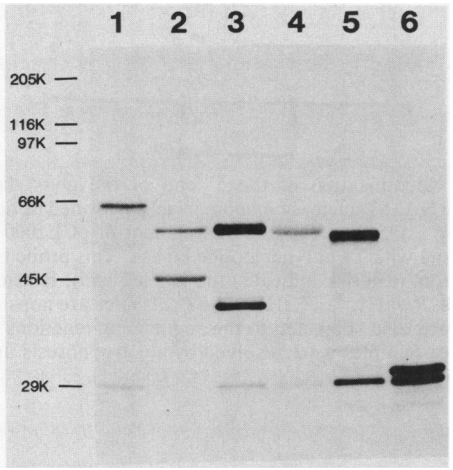

FIG. 3. Identification of the products of pCL8 by maxicell labeling. The maxicell technique was used to specifically label plasmid-encoded proteins with [^{35}S]methionine. The labeled products were resolved by sodium dodecyl sulfate-gel electrophoresis and detected by autoradiography (see the text). Samples were derived from *E. coli* MM294A harboring pCL4 (which expresses *aceK* from the *tac* promoter) (lane 1), pCL8 (lane 2), or the 3′ deletion mutants derived from pCL8 which are indicated by the corresponding numbers in Fig. 4 and 5 (lanes 3 to 6). K, Molecular weight in thousands.

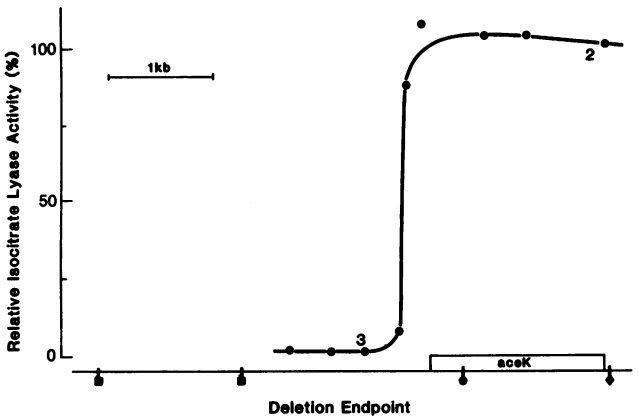

FIG. 4. Identification of the 3′ end of *aceA*. A series of nested deletions were introduced into the 3′ end of the glyoxylate bypass operon within pCL8 (see the text). For each deletion, the left endpoint is at the indicated position and the right endpoint is at the *Hin*dIII site. Expression of the operon was induced by growing strain BN1 (*aceA*) harboring the appropriate plasmid to the stationary phase on L broth plus 2% sodium acetate. Cells were harvested by centrifugation, disrupted by sonication, and then assayed for isocitrate lyase activity, as described in the text. The numbers associated with two of the datum points indicate the clones which were subjected to maxicell analysis and correspond to the lane numbers in Fig. 3. Restriction sites: ■, *Bam*HI; ●, *Ava*I; ◆, *Hin*dIII. The *Bss*HII and *Pvu*II sites are not shown.

weight, 48,000, reported for purified isocitrate lyase (27). The remaining band, with an apparent molecular weight of 61,000, appears to be malate synthase. These assignments were confirmed by deletion mapping (see below). The failure to observe IDH kinase/phosphatase is consistent with our previous observation that the cellular level of isocitrate lyase appears to be 100- to 1,000-fold greater than that of IDH kinase/phosphatase (18).

Mapping the 3′ end of *aceA*. The location and transcriptional orientation of *aceK* had been determined by comparing its restriction map (15; Klumpp et al., submitted) with those of the operon clones (Fig. 2). The 3′ end of *aceA* was identified by examining the effects of serial deletions from the 3′ end of the operon on the activity of isocitrate lyase (Fig. 4). Isocitrate lyase activity was reduced by 90% by a deletion extending 200 base pairs (bp) upstream of *aceK* and was completely eliminated by deletions beyond this point. The 3′ end of *aceA* thus appears to be ca. 200 bp upstream of *aceK*. Deletions beyond this point significantly reduced the size of the *aceA* product (Fig. 3, lane 3), confirming its identity as isocitrate lyase.

We have confirmed the location of the 3′ end of *aceA* while determining the sequence of *aceK* (Klumpp et al., submitted). The reading frame for *aceA* was identified by determining the junction sequence of an in-frame *aceA-lacZ* fusion gene. This open reading frame terminates 184 bp upstream of *aceK*. The deletion which yielded a 90% reduction in isocitrate lyase activity (Fig. 4) extended 14 bp upstream of this termination codon.

Mapping the 3′ end of *aceB*. The 3′ end of *aceB*, the gene encoding malate synthase, was also mapped by deletion analysis (Fig. 5). A deletion extending 1,500 bp upstream from *aceK* reduced malate synthase activity by ca. 50% but had little effect on the size of the *aceB* product (Fig. 3, lane 4). The failure of this deletion to alter the apparent molecular weight of this protein suggests that the deletion endpoint was

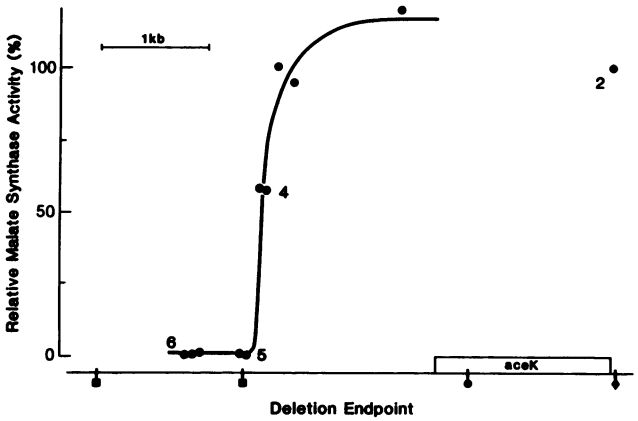

FIG. 5. Identification of the 3′ end of *aceB*. A series of nested deletions were introduced at the 3′ end of the glyoxylate bypass operon within pCL8. For each deletion, the left endpoint is indicated and the right endpoint is at the *Hin*dIII site. Strain 5236 (*aceB glc*), harboring the appropriate plasmid, was grown on MOPS medium containing 2% sodium acetate and 1% sodium succinate to the mid-log phase to induce the expression of the glyoxylate bypass operon. Cells were then harvested, disrupted by sonication, and assayed for malate synthase activity as described in the text. The numbers associated with some datum points indicate plasmids whose products were also characterized by maxicell labeling and correspond to the lane numbers in Fig. 3. Restriction sites: ■, *Bam*HI; ●, *Ava*I; ◆, *Hin*dIII. The *Bss*HII and *Pvu*II sites are not shown.

either downstream of *aceB* or was near its 3′ end. (Translational readthrough into the vector sequences made very little contribution to the apparent molecular weight of the product of the truncated gene since translational termination codons occur in all three reading frames within 53 bp of the deletion endpoint.) Slightly larger deletions eliminated this activity and reduced the apparent molecular weight of the *aceB* product by ca. 2,000 (Fig. 3, lane 5). Thus, the 3′ end of *aceB* appears to be close to the endpoint of the smaller deletion. Substantially larger deletions resulted in a reduction in the apparent molecular weight of the *aceB* product (Fig. 3, lane 6), confirming the identity of this band as malate synthase.

Identification of the 5′ end of the operon. The approximate location of the promoter of the glyoxylate bypass operon was determined by deletion mapping. Deletion of the internal *Bam*HI fragment from pCL8, generating pCL9 (Fig. 2), eliminated the expression of malate synthase but not that of isocitrate lyase or IDH kinase/phosphatase (Table 2). In contrast, deletion of the sequences upstream of the first *Bam*HI site, yielding pCL800, eliminated the expression of all three products of this operon. These results indicate that the promoter is upstream of the first *Bam*HI site.

The 5′ end of the glyoxylate bypass operon mRNA, expressed from the chromosome, was mapped by S1 nuclease analysis by using the *Cla*I-*Bss*HII fragment of pCL1000, which had been end labeled at the *Bss*HII site. S1 digestion produced a single protected fragment with an electrophoretic mobility corresponding to a point 50 bp upstream of the first *Bam*HI site in the insert (Fig. 6). Sequences immediately upstream of this site exhibit homology with the consensus *E. coli* promoter (Fig. 7). These sequences agree with the consensus (10) at all positions which are conserved by greater than 70%. This agreement includes matches at four of six positions in the −35 region and three of six positions in the −10 region. These two regions are separated by 16 nucleotides, consistent with the consensus separation of 17

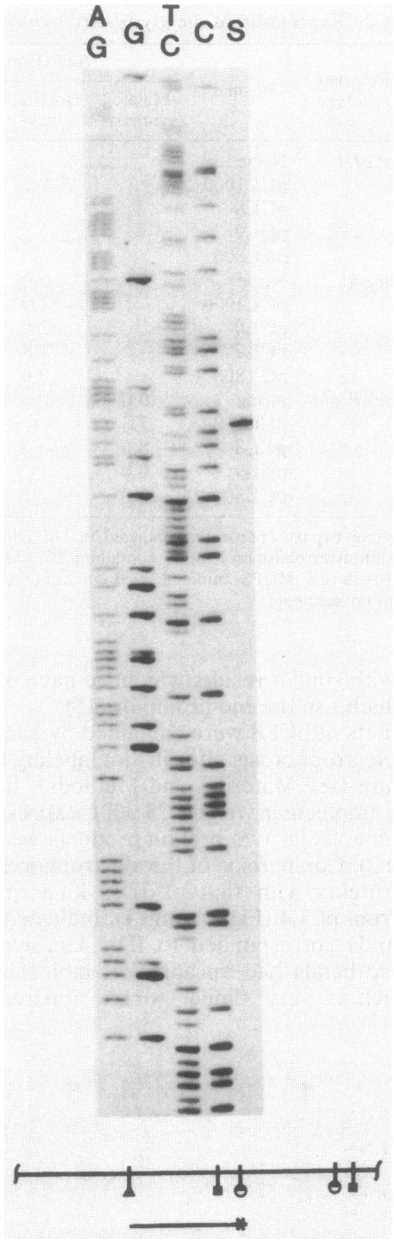

FIG. 6. Identification of the 5′ end of the glyoxylate bypass operon mRNA. S1 nuclease mapping was performed as described in the text by using the *Cla*I-*Bss*HII fragment of pCL1000 labeled at the latter site with T4 polynucleotide kinase. This probe is indicated at the bottom (asterisk indicates the labeled end); restriction sites: ▲, *Cla*I; ■, *Bam*HI; ⊖, *Bss*HII. The *Pvu*II sites are not shown. This fragment was also subjected to the sequencing reactions of Maxam and Gilbert. Samples were resolved by electrophoresis under denaturing conditions.

AAAATGGAAATTGTTTTTGATTTTGCATTTTAAATGAGTAGTCTTAGTTGTGCTGAA

E. coli Consensus: TTGaca←17±1→TAtaaT

FIG. 7. The promoter of the glyoxylate bypass operon. The transcriptional start site is indicated with an asterisk. The consensus *E. coli* promoter is also shown, with bases which exhibit greater than 70% conservation shown in capital letters.

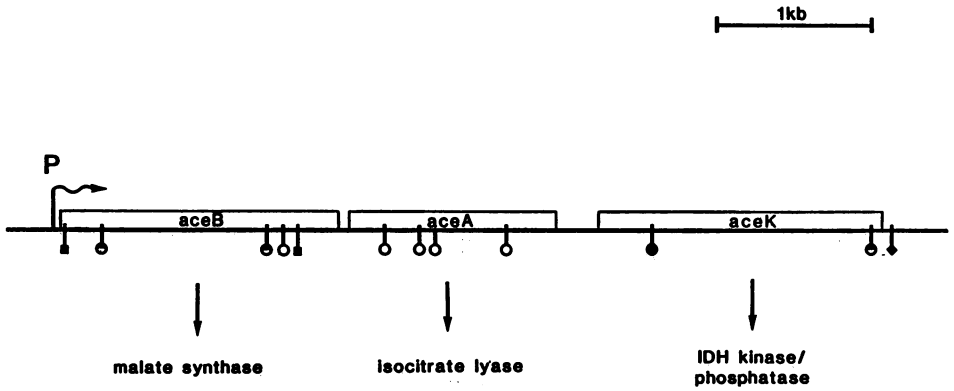

FIG. 8. Map of the glyoxylate bypass operon. The location of *aceK* was determined by comparison of the restriction maps of the operon clones with that predicted from the nucleotide sequence of this gene. The locations of *aceA* and *aceB* were determined from the experiments for which results are shown in Fig. 4 to 6. P indicates the position of the promoter, and the direction of transcription is indicated by an arrow. Restriction sites: ■, *Bam*HI; ◓, *Bss*HII; ○, *Pvu*II; ●, *Ava*I; ◆, *Hin*dIII. The size of the *aceB-aceA* intergenic region and the location of the translational initiation site for *aceB* have not been precisely determined.

± 1 bp. The −10 region begins 6 bp upstream of the transcriptional start site, consistent with the consensus distance of ca. 7 bp. The A+T-rich sequences upstream of the −35 region are also a conserved feature of *E. coli* promoters. Although these sequences exhibit all of the appropriate features, it remains to be rigorously proved that they compose the promoter of the glyoxylate bypass operon.

Attempts to identify the 3′ end of the mRNA by S1 analysis were unsuccessful. Several 3′ ends were identified, the most distal of which occurs near a site corresponding to the *Ava*I site within *aceK*. The apparent absence of mRNA downstream of this site was confirmed by Northern blot (RNA blot) analysis (unpublished observations). The precise significance of these observations remains unclear, but they are likely to be related to the downshift in expression which occurs between *aceA* and *aceK*.

DISCUSSION

In *E. coli*, a single operon encodes the metabolic and regulatory enzymes of the glyoxylate bypass. We cloned this operon and determined its functional map (Fig. 8) as a further step in the characterization of the mechanism(s) of its control and the factors yielding differential expression of its products. The order of the genes determined by deletion mapping is consistent with the results of previous studies which used genetic techniques (18, 22). The approximate sizes of *aceA* and *aceB*, 1.2 and 1.6 kb, respectively, are in good agreement with the apparent molecular weights of isocitrate lyase and malate synthase, 46,000 and 61,000, respectively. The results of S1 nuclease and deletion analysis indicate that the glyoxylate bypass operon is expressed from a single promoter during growth on acetate.

The glyoxylate bypass operon has recently been cloned from *E. coli* ML308 (7). The restriction map reported for the clone is in good agreement with those which we determined for the clones which we isolated. Although the functional map of the ML308 clone was not determined, it seems quite likely that the organization of the operon is similar to that reported here.

Although isocitrate lyase and malate synthase were readily identified after maxicell labeling of cells harboring pCL8, IDH kinase/phosphatase was not detected. This held true even when the gels were overloaded or when the films were subjected to prolonged exposures. This observation is consistent with the conclusion, based on enzymatic activities, that the cellular level of isocitrate lyase appears to be 100- to 1,000-fold greater than that of IDH kinase/phosphatase (18). Thus, there appears to be a striking downshift in expression between *aceA* and *aceK*. This degree of polarity is probably advantageous to the cell since the products of this operon play very different roles: isocitrate lyase and malate synthase are metabolic enzymes, whereas IDH kinase/phosphatase is a regulatory protein.

The degree of polarity observed for the glyoxylate bypass operon is unusual but is not unique to this operon. For example, in *E. coli*, the cellular level of ribosomal protein S21 is 1,000-fold greater than that of DNA primase even though they are expressed from the same operon (6). Similarly, a 10- to 20-fold difference in expression has been reported for the products of the *rxcA* operon of *Rhodopseudomonas capsulata* (1). For both operons, it has been proposed that polarity results, at least in part, from the rapid degradation of the downstream regions of the message (which correspond to the poorly expressed genes) by 3′-specific exonucleases. The upstream sequences appear to be stabilized by stem-loop structures at their 3′ ends. Although the mechanism responsible for the downshift in expression between *aceA* and *aceK* remains to be determined, it is intriguing that potential stem-loop structures have been identified in the *aceA-aceK* intergenic region and within *aceK* (Klumpp et al., submitted). The mechanism responsible for this downshift is under continued study.

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LITERATURE CITED

1. **Belasco, J. G., J. T. Beatty, C. W. Adams, A. von Gabain, and S. N. Cohen.** 1985. Differential expression of the photosynthesis genes of *R. capsulata* results from segmental differences in stability within the polycistronic transcript. Cell **40**:171–181.
2. **Bennett, P. M., and W. H. Holms.** 1975. Reversible inactivation of isocitrate dehydrogenase of *Escherichia coli* during growth on acetate. J. Gen. Microbiol. **87**:37–51.
3. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early

adenovirus mRNAs by gel electrophoresis of S1-digested hybrids. Cell 12:721–732.
4. **Borthwick, A. C., W. H. Holms, and H. G. Nimmo.** 1984. The phosphorylation of *Escherichia coli* isocitrate dehydrogenase in intact cells. Biochem. J. 222:797–804.
5. **Brice, C. B., and H. L. Kornberg.** 1968. Genetic control of isocitrate lyase activity in *Escherichia coli*. J. Bacteriol. 96: 2185–2186.
6. **Burton, Z. F., C. A. Gross, K. K. Watanabe, and R. R. Burgess.** 1983. The operon that encodes the sigma subunit of RNA polymerase also encodes ribosomal protein S21 and DNA primase in *E. coli*. Cell 32:335–349.
7. **el Mansi, E. M., C. MacKintosh, K. Duncan, W. H. Holms, and H. G. Nimmo.** 1987. Molecular cloning and over-expression of the glyoxylate bypass operon from Escherichia coli ML308. Biochem. J. 242:661–665.
8. **Garnak, M., and H. C. Reeves.** 1979. Phosphorylation of isocitrate dehydrogenase of *Escherichia coli*. Science 203:1111–1112.
9. **Garnak, M., and H. C. Reeves.** 1979. Purification and properties of phosphorylated isocitrate dehydrogenase of *Escherichia coli*. J. Biol. Chem. 254:7915–7920.
10. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter sequences. Nucleic Acids Res. 11:2237–2255.
11. **Holms, W. H., and P. M. Bennett.** 1971. Regulation of isocitrate dehydrogenase activity in *Escherichia coli* on adaptation to acetate. J. Gen. Microbiol. 65:57–68.
12. **Kornberg, H. L.** 1966. The role and control of the glyoxylate cycle in *Escherichia coli*. Biochem. J. 99:1–11.
13. **Kornberg, H. L., and N. B. Madsen.** 1951. Synthesis of C_4-dicarboxylic acids from acetate by a "glyoxylate bypass" of the tricarboxylic acid cycle. Biochim. Biophys. Acta 24:651–653.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
15. **LaPorte, D. C., and T. Chung.** 1985. A single gene codes for the kinase and phosphatase which regulate isocitrate dehydrogenase. J. Biol. Chem. 260:15291–15297.
16. **LaPorte, D. C., and D. E. Koshland.** 1983. Phosphorylation of isocitrate dehydrogenase as a demonstration of enhanced sensitivity in covalent regulation. Nature (London) 305:286–290.
17. **LaPorte, D. C., and D. E. Koshland, Jr.** 1982. A protein with kinase and phosphatase activities involved in the regulation of the tricarboxylic acid cycle. Nature (London) 300:458–460.
18. **LaPorte, D. C., P. E. Thorsness, and D. E. Koshland, Jr.** 1985. Compensatory phosphorylation of isocitrate dehydrogenase: a mechanism for adaptation to the intracellular environment. J. Biol. Chem. 260:10563–10568.
19. **LaPorte, D. C., K. Walsh, and D. E. Koshland, Jr.** 1984. The branch point effect: ultrasensitivity and subsensitivity to metabolic control. J. Biol. Chem. 259:14068–14075.
20. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
21. **Maloy, S. R., M. Bohlander, and W. D. Nunn.** 1980. Elevated levels of glyoxylate shunt enzymes in *Escherichia coli* strains constitutive for fatty acid degradation. J. Bacteriol. 143: 720–725.
22. **Maloy, S. R., and W. D. Nunn.** 1982. Genetic regulation of the glyoxylate shunt in *Escherichia coli* K-12. J. Bacteriol. 149: 173–180.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. 65:499–560.
25. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. 119:736–747.
26. **Nimmo, G. A., and H. G. Nimmo.** 1984. The regulatory properties of isocitrate dehydrogenase kinase and isocitrate dehydrogenase phosphatase from *Escherichia coli* ML308 and the roles of these activities in the control of isocitrate dehydrogenase. Eur. J. Biochem. 141:409–414.
27. **Robertson, E. F., and H. C. Reeves.** 1987. Purification and characterization of isocitrate lyase from *Escherichia coli*. Curr. Microbiol. 14:347–353.
28. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-coded proteins. J. Bacteriol. 137: 692–693.
29. **Summers, W. C.** 1970. A simple method for extraction of RNA from *Escherichia coli* utilizing diethyl pyrocarbonate. Anal. Biochem. 35:459–463.
30. **Vinopal, R. T., and D. G. Fraenkel.** 1974. Phenotypic suppression of phosphofructokinase mutations in *Escherichia coli* by constitutive expression of the glyoxylate shunt. J. Bacteriol. 118:1090–1100.
31. **Wang, J. Y. J., and D. E. Koshland, Jr.** 1982. The reversible phosphorylation of isocitrate dehydrogenase of *Salmonella typhimurium*. Arch. Biochem. Biophys. 218:59–67.