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Molecular Cloning of the Gene (*poxB*) Encoding the Pyruvate Oxidase of *Escherichia coli*, a Lipid-Activated Enzyme

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The pyruvate oxidase structural gene (*poxB*) of *Escherichia coli* was cloned into derivatives of plasmid pBR322. The gene was first cloned into a cosmid vector by selection for the tetracycline resistance determinant of a closely linked Tn*10* insertion (no direct selection for the gene was available). Subsequent subcloning resulted in localization of the gene to a 3.1-kilobase-pair DNA segment. Two of the smaller *poxB* plasmids were shown to cause the overproduction of oxidase activity (by six- to eightfold), and one of these plasmids was shown to encode a protein having the size and antigenic determinants of pyruvate oxidase. Introduction of *poxB* plasmids into strains (*aceEF*) lacking pyruvate dehydrogenase activity relieved the aerobic growth requirement of the strains for exogenous acetate.

Pyruvate oxidase catalyzes the oxidative decarboxylation of pyruvate to acetate and CO_2 (10). The *Escherichia coli* enzyme consists of four identical 60,000-dalton subunits, each of which binds the required cofactors, flavin adenine dinucleotide and thiamine PP_i (10). Although pyruvate oxidase has been extensively characterized biochemically, the role it plays in metabolism is unclear. The most novel aspect of the enzyme is the striking effect of lipids on enzymatic activity. When purified pyruvate oxidase is assayed in the presence of lipids or detergents, a 25-fold increase in the specific activity is observed, accompanied by a 10-fold decrease in the K_m for pyruvate (6, 7, 21). This activation is due to the binding of lipid to the protein (24). A similar extent of activation can be attained by limited proteolysis of the enzyme in the presence of substrate and cofactors, after which the affinity of the protein for lipids and detergents is greatly reduced (20, 21). These properties, coupled with the ease of genetic manipulation in *E. coli*, make pyruvate oxidase an ideal system for studying lipid-protein interactions.

Chang and Cronan (3, 4) have reported the isolation of two classes of mutants deficient in oxidase activity. *poxA* mutants have lesions in a regulatory locus which also affects at least one other pyruvate enzyme (3), whereas *poxB* mutants are lesions in the structural gene (4). These workers have recently reported a *poxB* mutant that contains an oxidase defective in lipid binding (4a). Since the mutant enzyme can be fully activated by limited proteolytic treatment, the mutant enzyme has a normal catalytic site, and thus its inability to function in vivo can be attributed to defective binding of the enzyme to the membrane lipids. To determine the nature of the mutational alteration in this and other *poxB* strains, we wish to determine the amino acid alteration via DNA sequence analysis of the wild-type and mutant oxidase genes. As the first step in this analysis, we report the molecular cloning and physical localization of the pyruvate oxidase gene and demonstrate that the cloned DNA segment encodes pyruvate oxidase.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. All bacterial strains used are derivatives of *E. coli* K-12 (Table 1). Strains YYC162 and YYC187 were provided by Y.-Y. Chang of this laboratory. Strain YYC162 was cured of its λ prophage by heat-pulse curing, and a *thyA* lesion was introduced according to Miller (17). A *recA* derivative of this strain, strain CG3, was isolated by mating the *thyA* strain with the Hfr *recA* strain KL16-99. *recA* derivatives were isolated by screening $thyA^+$ recombinants for sensitivity to UV irradiation.

The complex medium used was rich broth, containing 10 g of tryptone, 5 g of NaCl, and 1 g of yeast extract per liter and was supplemented with 10 mM sodium acetate. The triphenyltetrazolium chloride-containing medium was that of Bochner and Savageau (2), containing 0.5% sodium pyruvate and 0.01% sodium acetate.

Antibiotics were used at the following concentrations (micrograms per milliliter): sodium ampicillin, 100; tetracycline hydrochloride, 10; and chloramphenicol, 150. Other media and chemicals were those described by Chang and Cronan (4). The *Bam*HI-*Eco*RI adapter was obtained from Amersham Corp., Arlington Heights, Ill. The *Pst*I linker was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

Recombinant DNA techniques. Chromosomal DNA was isolated by the method of Saito and Miura (22). Plasmid DNA was prepared by cesium chloride-ethidium bromide equilibrium centrifugation of chloramphenicol-amplified cultures (16). Rapid minipreparations of plasmid DNA were prepared by alkaline lysis (15). Restriction endonucleases and T4 DNA ligase were obtained from Bethesda Research Laboratories, Gaithersburg, Md., or Boehringer Mannheim, and reactions were carried out under the conditions specified by the suppliers. DNA polymerase I (large fragment) was purchased from Boehringer Mannheim. T4 polynucleotide kinase was obtained from Bethesda Research Laboratories. Phosphorylation of linkers, generation of blunt-ended restriction fragments, and transformations were performed as described by Maniatis et al. (15). Preparation of the cosmid packaging extracts and packaging of DNA were as described by Davis et al. (8), using strains NS433 and NS428.

Preparation of antibody. Homogeneous pyruvate oxidase (20, 21, 24) was kindly provided by R. Gennis. Antibody was raised against pyruvate oxidase by injecting 6-week-old New Zealand White rabbits subcutaneously with 1 mg of pyruvate oxidase, as described by Livingston (14). The rabbits were bled 38 days later. The rabbits were injected again with 0.4

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TABLE 1. Bacterial strains

Strain	Relevant genotype	Source or reference
CY265	HfrC Δ(*aroP-aceEF*)	3
YYC162	*pps-4 pfl-1 poxB1* λ cI857 lysogen of CY265	4
YYC187	*zbi*::Tn*10*, *poxB*$^+$ of YYC162	4
CG1	YYC162 λ$^-$	This work
CG3	*recA1* of CG1	This work
LE392	F$^-$ *hsdR*	L. Enquist
NS428	F$^-$ *recA* (λ *A11 b2 red3 c*I857 *S7*)	N. Sternberg
NS433	F$^-$ *recA* (λ *E4 b2 red3 c*I857 *S7*)	N. Sternberg
CG2	CG1(pCG3)	This work
CG4	CG3(pCG3)	This work
CG5	CG3(pCG4)	This work
CG6	CG3(pCG5)	This work
CG7	CG3(pCG7)	This work
CY377	Δ(*aroP-aceEF*) *zac*::Tn*10*, *leu* of UB1005	This work
CY378	CY377(pCG5)	This work
CY379	Tet^s derivative of CY377	Fusaric acid selection (8)
CY380	CY379(pCG7)	This work

mg of pyruvate oxidase and bled 3 weeks later. The serum was collected, and antibody was purified through the ammonium sulfate step by the method of Livingston (14).

Enzyme analysis. The methods for extract preparation and the spectrophotometric enzyme assay for pyruvate oxidase were described by Chang and Cronan (3). Treatment of extracts with antisera raised against pyruvate oxidase was also described by Chang and Cronan (4). Protein concentrations were determined by a microbiuret procedure with bovine serum albumin as the standard (19).

[^{35}S]methionine labeling and protein gel electrophoresis. Proteins encoded by plasmids were labeled by the maxicell method of Sancar et al. (23) and analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13).

Electrophoresis of DNA and its recovery from gels. Fragments from restriction enzyme digestions were electrophoresed on a 1% agarose gel in Tris-borate buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM sodium EDTA [pH 8.3] [15]), visualized by ethidium bromide staining, and recovered from the gel with DEAE-cellulose paper by the method of Dretzen et al. (9).

RESULTS

Cloning of the *poxB* gene. Pyruvate oxidase plays only a secondary role in acetate metabolism in *E. coli* (3, 4), and thus even in a *poxB* recipient we had no direct selection for recombinant DNA clones carrying the wild-type oxidase gene. Hence, we cloned this segment of DNA indirectly by selection of the tetracycline resistance (Tet^r) determinant of a Tn*10* insertion closely linked to the *poxB* gene (69% cotransduced by phage P1) (4). The large size of Tn*10* (9.3 kilobases [kb]) plus the 1.6-kb segment needed to encode the *poxB* gene in addition to the large uncertainty in the calculated size (ca. 9 to 15 kb) of the DNA segment between the gene and the transposon (calculated from the P1 cotransduction frequency [1]) precluded cloning into the commonly used plasmid vectors since the resulting Tet^r *poxB*$^+$ recombinant plasmids were very likely to be too large for efficient transformation (15). We therefore used a cosmid cloning system because the in vitro packaging of cosmid clones into phage lambda allows (and selects for) insertion of large (30- to 45-kb) segments of DNA (15). Since all of the available small cosmids were Tet^r, we first inactivated the Tet gene of pHC79, a cosmid derived from pBR322 (12). The cosmid was digested with both *Bam*HI and *Eco*RI and ligated in the presence of a synthetic *Eco*RI-*Bam*HI adapter DNA. The ligation mixture was used to transform strain LE392 to ampicillin resistance (Amp^r), and the resulting colonies were screened for Tet^s. A plasmid, designated pCG1, was isolated which was slightly smaller than pHC79, a result consistent with the 370-base-pair deletion predicted from the base sequence of pBR322. The plasmid also contained a unique *Xho*I site, the result of the base sequence of the inserted adapter.

Segments of chromosomal DNA from strain YYC187 (a *pox*$^+$ strain which carried the *zbi*::Tn*10* insertion) were obtained by partial digestion with *Bam*HI and mixed with the pCG1 cosmid DNA which had been linearized with *Bam*HI. After ligation the DNA was packaged into phage lambda particles in vitro, and the resulting phage suspensions were used to transduce the *poxB1* recipient strain CG1 to Amp^r.

Approximately 2,000 of the resulting ampicillin-resistant colonies were replica plated onto rich plates containing ampicillin and tetracycline. Cultures of seven Tet^r colonies were grown and assayed for pyruvate oxidase activity. One strain (CG2) exhibited a sixfold-higher enzyme level than did the wild-type control strain, whereas the strain lacking the plasmid had no activity (Table 2). The activity of these extracts was specifically inhibited by antibody raised against homogeneous pyruvate oxidase, indicating that the enzymatic activity was that of pyruvate oxidase rather than of another enzyme (the donor DNA was deleted for the pyruvate dehydrogenase [*aceEF*] genes, thus precluding cloning of the *aceE* gene product which has an oxidase-like activity).

Subcloning of the *poxB* gene. The chromosomal DNA insert in the original *poxB* cosmid (pCG3) was large (ca. 40 kb), and the plasmid was unstable, particularly in *rec*$^+$ strains. We therefore reduced the size of the plasmid by partial digestion with *Eco*RI, followed by ligation and transformation of strain CG3 (*poxB1 recA1*). This strain also carried lesions in the *pfl* and *pps* loci, thus allowing scoring of colonies for *poxB* activity on tetrazolium indicator plates (3). After selection for Amp^r, red (*pox*$^+$) colonies were isolated, and the plasmids were extracted from the cells. One such plasmid (pCG4), ca. 30 kb in size, was then digested with both *Bgl*II and *Bam*HI (which make mutually cohesive ends). The double digest was ligated and introduced into strain CG3, and Amp^r *pox*$^+$ colonies were isolated. From one such colony a 10-kb plasmid (pCG5) was isolated and mapped with a number of restriction enzymes. This map (Fig. 1) shows that pCG5 contains 2.7 kb of the pCG1 vector and ca. 7.3 kb of chromosomal DNA. Strains carrying plasmid pCG5 overproduced pyruvate oxidase about eightfold (Table 2), although the extent of overproduction was somewhat variable. The lower levels seen in strain CG3 may be due to the slower growth of *recA* strains.

Further localization of the *poxB* gene. Plasmid pCG5 contained 7.3 kb of chromosomal DNA, whereas only ca. 1.6 kb of DNA was needed to encode pyruvate oxidase. To remove this extraneous DNA, we subcloned various restriction fragments into pBR322. By a process of elimination, the 3.1-kb *Sph*I-*Mlu*I segment of the insert DNA was thought to

TABLE 2. Pyruvate oxidase activity of various strains

Strain	Allele	Plasmid[a]	Pyruvate oxidase activity (U/mg of protein)[b]			Overproduction (fold)
			No IgG[c]	Control IgG	Immune IgG	
Expt 1						
CG1	*poxB1*		5			
CY265	*pox*$^+$		200	143	8	
CG2	*poxB1*	pCG3	1,170	1,185	86	5.9
Expt 2						
CG3	*poxB1*		<1			
CY265	*pox*$^+$		131	144	<1	
CG4	*poxB1*	pCG3	411	388	<1	3.1
CG5	*poxB1*	pCG4	397	333	14	3.0
CG6	*poxB1*	pCG5	1,009	927	57	7.7
CG7	*poxB1*	pCG7	1,238			

[a] Strains CG1 and CG3 harbored the plasmids in experiments 1 and 2, respectively.
[b] One unit of activity equals 1 nmol of pyruvate decarboxylated per min.
[c] IgG, Immunoglobulin G.

carry the complete gene. This was tested by isolation of this fragment from an agarose gel. The fragment was treated with DNA polymerase I to generate blunt ends and ligated to a phosphorylated *Pst*I linker. The ligation mixture was digested with *Pst*I and mixed with *Pst*I-digested pBR322. After ligation and transformation of strain CG3, Tet^r *poxB*$^+$ colonies were isolated. One of these strains carried plasmid pCG7 (ca. 7.5 kb) and also overproduced oxidase activity (Table 2). Plasmids carrying the *Pst*I-*Pst*I fragment in either orientation within pBR322 conferred a *pox*$^+$ phenotype on strain CG3, indicating that the inserted DNA carried the normal *poxB* promoter sequence (data not shown).

The chromosomal insert in pCG7 could be cut into two segments of ca. 1.9 and 1.3 kb with *Sal*I. Since only the larger segment is of sufficient size (1.6 kb) to encode the oxidase, the *poxB* gene had to be either located within the larger segment or lie across the *Sal*I site. The latter possibility was shown to be the case by insertion of an *Xho*I adapter into the *Sal*I site. The insertion of this linker resulted in the loss of *poxB* gene function (data not shown).

Proteins encoded by pCG5. The maxicell technique was used to assay plasmid-coded proteins. Strain CG6, a *recA* strain carrying pCG5, was irradiated with UV light, treated with cycloserine, and labeled with [^{35}S]methionine essentially as described by Sancar et al. (23). The cellular proteins were solubilized and then analyzed by polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate (Fig. 2). Strain CG6 (which carried pCG5) synthesized a protein with a molecular weight identical to that of pyruvate oxidase. Moreover, the labeled protein could be specifically precipitated with antioxidase antibody (Fig. 2), and this precipitation was eliminated by the addition of excess homogeneous oxidase to the antibody-extract mixture (data not shown). Neither the original extracts nor the immunoprecipitates of the strain lacking pCG5 (or of a strain carrying the original cosmid vector [pCG1]) contained the labeled M_r 60,000 protein.

Physiological consequences of overproduction of pyruvate oxidase. *E. coli* strains lacking the genes for pyruvate dehydrogenase require acetate for aerobic growth despite the presence of pyruvate oxidase activity (3, 4). Chang and

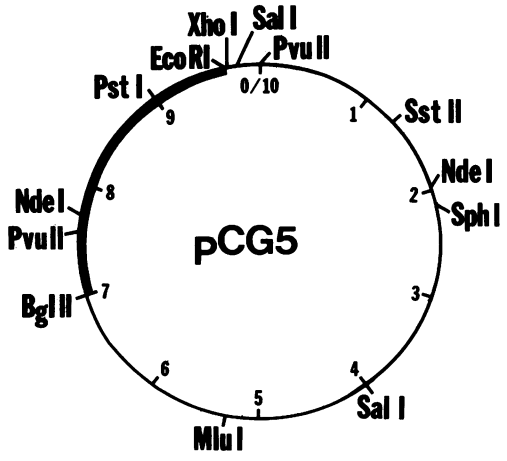

FIG. 1. Restriction enzyme cleavage map of pCG5. The heavy line depicts the DNA segment derived from vector pCG1, whereas the thin line depicts the insert of chromosomal DNA. The construction of pCG5 is described in the text.

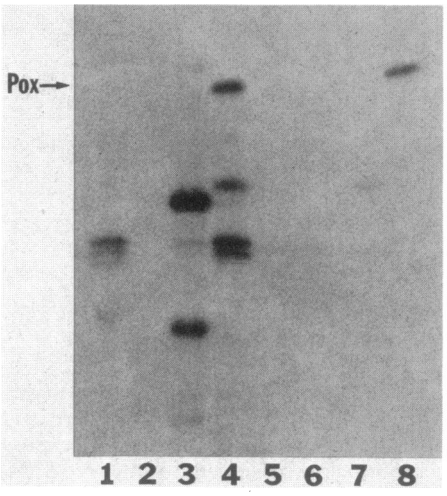

FIG. 2. Autoradiogram of [^{35}S]methionine-labeled maxicells. Lanes 1, strain CG3 carrying pCG1; 2, strain CG3 with no plasmid; 3, strain CG3 carrying pBR328; 4, strain CG3 carrying pCG5. Lanes 5 through 8 are immunoprecipitates of portions of the same extracts run in lanes 1 through 4, respectively. The arrow indicates the migration position of purified pyruvate oxidase. The askew band in lane 8 is due to its location at the edge of the gel.

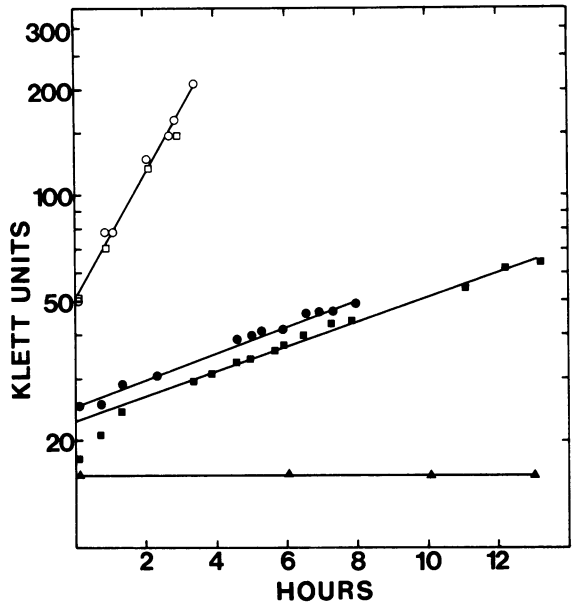

FIG. 3. Growth of strains carrying *poxB* plasmids. The medium used was minimal medium E (25) containing 0.4% sodium succinate and 50 μg each of L-methionine and L-leucine per ml. Potassium acetate (0.2%) was added as indicated. Symbols: ○, strain CY380 plus acetate; ●, strain CY380 without acetate; □, strain CY378 plus acetate; ■, strain CY378 without acetate; and ▲, strain CY377 (or strain CY379) without acetate. The incubation was at 37°C with vigorous shaking. One Klett unit corresponds to ca. 5×10^6 cells per ml.

Cronan (4) have shown that the normal level of oxidase activity is sufficient to allow only extremely slow aerobic growth of strains lacking pyruvate dehydrogenase. We supposed that the overproduction of oxidase evident in strains carrying plasmids pCG5 and pCG7 might allow more rapid growth. We therefore introduced the plasmids into strains CY377 and CY379. The presence of either pCG5 or pCG7 gave a detectable growth rate (doubling time of ca. 500 min) in the absence of acetate supplementation, although the doubling time was much slower than the rate observed in the presence of acetate (100 min) (Fig. 3). Growth in the absence of acetate supplementation was completely dependent on the presence of a *poxB*$^+$ plasmid (Fig. 3). Strains lacking the plasmids or carrying plasmids in which either the amino-terminal portion or the carboxy-terminal portion of the gene had been deleted (Y.-Y. Chang and J. E. Cronan, Jr., unpublished data) failed to show detectable growth during a 14-h incubation. Derivatives of strain CY378 which became Amp^s due to loss of the plasmid under nonselective conditions also lost the ability to grow rapidly without acetate supplementation. It should be noted that the strain background was important for growth in the absence of acetate. The strain UB1005 background was chosen because this strain grows unusually well on acetate as sole carbon source. In two other Δ(*aceEF*) strains (derivatives of HfrC and Hfr6), introduction of pCG5 or pCG7 failed to allow growth without acetate supplementation.

The growth data indicate that the plasmid-encoded oxidase is active in vivo. To confirm that oxidase activity was not limited in vivo by the supply of another cellular component (e.g., an electron transfer or membrane-binding component), we assayed oxidase activity in vivo by production of $^{14}CO_2$ from [1-^{14}C]pyruvate (4a) and found that similar extents of enzyme overproduction were observed in both the in vitro and in vivo assays (data not shown).

DISCUSSION

We cloned the *poxB* gene by use of a closely linked Tn*10* insertion, a strategy used previously in cloning the *tyrR* locus into phage λ (5). We used a cosmid vector since the large insert size carried by such vectors could accommodate both the Tn*10* element and the very large uncertainties inherent in converting P1 cotransduction frequencies into lengths of DNA (1). Another advantage in using a cosmid vector is that the low (ca. 5) copy number of such plasmids (12) avoids the sensitivity to inducers of Tet gene expression observed with plasmids present in high (>20) copy number (18).

The analysis of the proteins encoded by a subclone of the original cosmid confirms the identification of the *poxB* gene as the structural gene for pyruvate oxidase. We have shown that a protein of the proper size and antigenic determinants is synthesized by this plasmid. Our preliminary DNA sequence analyses (unpublished data) of segments of plasmid pCG7 cloned into phage M13 conclusively demonstrate that the *poxB* gene encodes pyruvate oxidase. We have obtained DNA sequences (from appropriately spaced parts of the cloned segment) that encode two ca. 20-amino-acid-long segments identical to those obtained (M. Recny and L. Hager, personal communication) by amino acid sequencing of the amino and carboxy termini of the intact protein.

The finding that overproduction of the oxidase allows growth of strains lacking pyruvate dehydrogenase in the absence of added acetate confirms the previous indications (4) that the acetate produced by the oxidase is used metabolically. However, growth under these conditions was quite slow (ca. 20% of the acetate-supplemented rate), indicating that acetyl-coenzyme A production limits growth. The level of acetate produced in these cells exceeds by 10-fold the level of acetyl-coenzyme A produced by the pyruvate dehydrogenase (11), and thus the proposal of Chang and Cronan (3, 4) that inefficient conversion of acetate to acetyl-coenzyme A is responsible for the apparent metabolic inertness of pyruvate oxidase is strengthened. The suppression of the *aceEF* phenotype by the *poxB*$^+$ plasmids suggests (in hindsight) that direct selection for the *poxB* clones might have been possible. However, such selection would have depended on a fortuitous choice of the recipient strain and might also have selected plasmids carrying other genes (e.g., pyruvate formate lyase).

The cloning of the gene for pyruvate oxidase makes the study of its interaction with lipid approachable by a variety of in vitro mutagenesis methods. Such mutants will be most useful in understanding the physiological role of the activation of pyruvate oxidase by lipid.

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