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Mapping Nonselectable Genes of *Escherichia coli* by Using Transposon Tn*10*: Location of a Gene Affecting Pyruvate Oxidase

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Mutants of *Escherichia coli* K-12 deficient in pyruvate oxidase were isolated by screening for the production of $^{14}CO_2$ from [1-^{14}C]pyruvate by the method of Tabor et al. (J. Bacteriol. **128**:485–486, 1976). One of these lesions (designated *poxA*) decreased the pyruvate oxidase activity to 10 to 15% of the normal level but grew well. To map this nonselectable mutation, we isolated strains having transposon Tn*10* inserted into the chromosome close to the *poxA* locus and mapped the transposon. These insertions were isolated by the following procedure: (i) pools of Tn*10* insertions into the chromosomes of two different Hfr strains were prepared by transposition from a λ::Tn*10* vector; (ii) these Tn*10*-carrying strains were then mated with a *poxA* recipient strain, and tetracycline-resistant (Tet^r) recombinants were selected; (iii) the Tet^r recombinants were then screened for $^{14}CO_2$ production from [1-^{14}C]pyruvate. This method was shown to give a >40-fold enrichment of insertions of Tn*10* near the *poxA* gene as compared with transduction. Calculations indicate that a similar enrichment should be expected for other genes. The enrichment is due to the much greater map interval over which strong linkage between selected and unselected markers is found in conjugational crosses as compared with transductional crosses. The use of Hfr conjugative transfer allows isolation of transposon insertions closely linked to a nonselectable gene by scoring hundreds rather than thousands of colonies. Using a Tn*10* insertion >98% cotransduced with the *poxA* locus, we mapped the *poxA* gene on the *E. coli* genetic map. The *poxA* locus is located at 94 min, close to the *psd* locus. The clockwise gene order is *ampA*, *poxA*, *psd*, *purA*. The *poxA* mutation is recessive and appears to be a regulatory gene.

The pyruvate oxidase of *Escherichia coli* is a peripheral membrane enzyme that catalyzes the oxidative decarboxylation of pyruvate to acetic acid plus CO_2 (16). The enzyme has been purified to homogeneity (40) and is a tetramer consisting of four identical polypeptide chains. Thiamine pyrophosphate and flavin adenine dinucleotide are required cofactors of the reaction (16). The activity of pyruvate oxidase increases 20-fold or more in the presence of various lipids or detergents, and these agents are active at very low concentrations (4, 12–14, 36). Pyruvate oxidase has a high-affinity binding site for lipid (36), and this enzyme-lipid interaction seems to increase activity by triggering a conformational change of the protein (36). A similar activation of the enzyme is observed upon controlled proteolysis of pyruvate oxidase (34). This protease-activated form of the oxidase is not further activated by lipid, and proteolysis results in loss of the high-affinity lipid binding site (35). These in vitro experiments indicate that pyruvate oxidase is an excellent enzyme for studying protein-lipid interaction.

Although the enzymological and physical characterization of *E. coli* pyruvate oxidase has been thorough, the physiological function of this enzyme is unclear. The major enzyme catalyzing the conversion of C3 compounds to C2 compounds (pyruvate to acetyl coenzyme A) in *E. coli* is pyruvate dehydrogenase (22, 25, 26). Mutants deficient in pyruvate dehydrogenase (*aceEF*) require an exogenous source of acetate for growth (22, 25, 26). Since the pyruvate oxidase activity of *E. coli* is substantial (similar to that of pyruvate dehydrogenase in crude extracts) and produces acetate, it would seem that a deficiency in pyruvate dehydrogenase should not elicit an acetate requirement. It has been suggested that the pyruvate oxidase activity is too low to produce sufficient acetate to support the growth of *ace* strains (22). However, it also seems possible that the rate of conversion of the free acetate produced by the oxidase to

TABLE 1. Bacterial strains

Strain	Relevant markers	Source (reference)[a]
KL333	HfrC, *leu-40*	CGSC4337
JRG596	F$^+$ Δ(*aroP-aceEF*), *pps*	J. R. Guest (25)[b]
CY265	HfrC, Δ(*aroP-aceEF*)	P1 (JRG596) × KL333
CY314	λ*c*I857 lysogen of CY265	This work
χ478	*thi-1*, *proC32*, *purE42*, *leuB6*, *metE70*, *trpE38*, *lysA23*, *rpsL109*, *lacZ36*, *ara-14*, *xyl-5*, *mtl-1*, *tsx-67*, *azi-6*, *supE45 tonA23*	B. Low
AB2880	*aroD6*	
PA505-1-5	*pps-4*	CGSC4869
KL290	*serC13*, *serS14*	CGSC4341
CB898	*pfl-1*	M. Pascal (38)
PC0950	*purA54*, *serB28*	CGSC5409
JB1	*uxuA1*	CGSC5999
CM8	*uxuB108*	CGSC5671
PC1	*dnaC1*	CGSC5030
AB4141	*valS7*	CGSC4141
H882	*purA*	
T850	*mop-1*	CGSC4812
EH450	*psd-2*, *ampA73*	E. Hawrot (20)
Hfr strains H, 6, KL16, P801, P4X, J4, Ra2	Hfr (Fig. 1)	K. B. Low (27, 28)
KLF17/KL132	F117 *purA*$^+$ *pyrB*$^+$/*pyrB*, *hisG*, *thyA*, *recA*	CGSC4255
KLF18/KL132	F118 *purA*$^+$ *pyrB*$^+$/*pyrB*, *hisG*, *thyA*, *recA*	CGSC4259
YYC7	*poxA1* of CY314	This work
YYC16	*zdh*::Tn*10*, *aroD*$^+$ of AB2880	This work
YYC21	*zdh*::Tn*10* of PA505-1-5	P1 (YYC16) × PA505-1-5
YYC33	*zca*::Tn*10*, *ser*$^+$ of KL290	This work
YYC34	*zca*::Tn*10* of CB898	P1 (YYC33) × CB898
YYC63	*pps-4*, *pfl-1* of CY265	This work
YYC65	*pfl-1* of JRG596	P1 (YYC34) × JRG596
YYC85	*zac*::Tn*10*, *ace*$^+$ of YYC65	This work
YYC88	*zac*::Tn*10* of JRG596	P1 (YYC85) × JRG596
YYC91	*rpsL* of YYC7	
YYC107	*pps-4*, *pfl-1* of YYC7	This work
YYC109	*zje-2*::Tn*10*, *poxA*$^+$ of YYC107	This work
YYC115	*zje-1*::Tn*10*, *poxA*$^+$ of YYC91	This work
YYC170	*zje-1*::Tn*10*, *psd*$^+$ of EH450	P1 (YYC115) × EH450
YYC176	*zje-1*::Tn*10*, EH450	P1 (YYC115) × EH450
YYC177	Δ(*aroP-aceEF*), *poxA*, *zje-1*::Tn*10* of X478	This work
YYC183	YYC107 cured of λ prophage	Heat-pulse curing

[a] Strains designated CGSC were obtained from the Coli Genetic Stock Center, Yale University, New Haven, Conn., through the kindness of the curator, B. Bachmann.

[b] Strain JRG596 has also been referred to as strain KΔ15 (25, 26). This strain is also deficient in pyruvate oxidase, but the site of this lesion is unknown. We failed to transduce this strain to *pox*$^+$ with P1 phage grown on a *pox*$^+$ strain. It should be noted that this strain was heavily mutagenized during its derivation. We also found that this strain is F$^+$ rather than F$^-$.

acetyl-coenzyme A could limit the utilization of this acetate source.

To approach problems of lipid-protein interaction by genetic means, we isolated mutants of *E. coli* K-12 deficient in pyruvate oxidase. The mutants were isolated by screening for $^{14}CO_2$ production from [1-^{14}C]pyruvate, using the method of Tabor et al. (37). Since these mutants have no selectable phenotype, we developed a method in which the transposon Tn*10* is used to map one class of these mutants, designated *poxA* (for pyruvate oxidase).

MATERIALS AND METHODS

Bacterial strains. All bacteria were derivatives of *E. coli* K-12 and are listed in Table 1. Strains for studying pyruvate oxidase have to be *ace* (lacking pyruvate dehydrogenase [16, 25]). An *ace* mutation was introduced by either of two methods. In the first method, *leu* strains (e.g., KL333) were transduced to *leu*$^+$ with P1 phage grown on strain JRG596 to give the Δ*aceEF* derivatives (e.g., CY265). In the second method, strain YYC85, a strain with a Tn*10* insertion very close (>99% cotransduced) to the *ace* locus, was isolated by transducing strain YYC65 with P1 phage grown on a

pool of Tn*10* insertions into the *E. coli* chromosome (see below and Table 1). Strain YYC88 (Tet^r *ace*) was obtained by transducing strain JRG596 with P1 phage grown on strain YYC85. A P1 stock prepared from strain YYC88 was then used to construct Tet^r *ace* strains (e.g., YYC177).

Construction of *pps* strains was also done by using a closely linked Tn*10* insertion. The *pps* locus maps very close to the *aroD* locus at 37 min of the *E. coli* chromosome (1, 7). Strain AB2880 (*aroD*) was transduced by P1 phage grown on a pool of random Tn*10* insertions into the *E. coli* chromosome to give the Tet^r *aroD*$^+$ strain YYC16. Strain PA505-1-5 (*pps*) was transduced with P1 phage grown on strain YYC16, and Tet^r colonies were scored for *pps* (lack of growth with lactate as sole carbon source). A phage P1 stock grown on strain YYC21 (Tet^r *pps*) was then used to transduce strain CY265 or other strains to give *pps* derivatives (the cotransduction frequency of Tet^r and *pps* was ~67%).

The method for construction of *pfl* strains was analogous to that used for constructing *pps* strains. The *pfl* locus maps very close to the *serC* and *serS* loci, at 20 min of the *E. coli* chromosome (1, 38). Strain KL290 (*serC serS*) was transduced with P1 phage grown on a pool of random Tn*10* insertions into the chromosome to give the Tet^r *serC*$^+$ *serS*$^+$ strain YYC33. Strain CB898 (*pfl*) was transduced with P1 phage grown on strain YYC33, and Tet^r colonies were isolated and screened for the *pfl* phenotype. *pfl* colonies were identified by a soft agar overlay method (30). Soft agar (5 ml) containing 2.5 ml of 1.5% agar, 1.37 ml of 10% sodium pyruvate, 1 ml of 0.5% benzyl viologen, and 0.125 ml of 1 M potassium phosphate buffer (pH 7.0) was poured over the colonies to be screened. *pfl* strains gave white colonies in the soft agar overlay, whereas *pfl*$^+$ colonies were purple. The *zca*::Tn*10* insertion cotransduces ~60% with the *pfl* locus. Strains that had been made *pps* or *pfl* as described above had a Tn*10* insertion near the locus. The Tet^r was deleted by the method of Bochner et al. (5) as modified by Maloy and Nunn (29). These Tn*10*-deleted strains were then used again in crosses, with Tn*10* as the selected marker. In fact, several strains had been through several cycles of insertion and deletion of Tn*10* (e.g., strains YYC63 and YYC107; see Table 1). Strain YYC183 is strain YYC107 cured of its λ prophage by heat-pulse curing (17).

Media. Rich broth contained (in grams per liter): tryptone, 10; NaCl, 5; and yeast extract, 1 and was supplemented with 10 mM sodium acetate for all Δ*aceEF* strains. Rich agar media were rich broth plus 1.5% agar. Minimal media were medium E (31) or medium M9 (31) supplemented with thiamine (1 mg/liter). The carbon sources used (at 0.4%) were glucose, succinate, lactate, or D-glucuronic acid. L-Amino acids (50 mg/liter), casein hydrolysate (0.1%), nucleic acid bases (25 mg/liter), streptomycin (100 mg/liter), and tetracycline-hydrochloride (10 mg/liter) were added to media as indicated.

The triphenyltetrazolium chloride (TTC)-containing medium was that of Bochner and Savegeau (6) containing 0.5% sodium pyruvate and 0.01% sodium acetate.

Chemicals. Sodium [1-^{14}C]pyruvate was a product of New England Nuclear Corp. *N*-Methyl-*N'*-nitrosonitrosoguanidine and 2,3,5-triphenyltetrazolium chloride monohydrate were products of Aldrich Chemical Co. Sodium ferricyanide was obtained from ICN Pharmaceuticals, Inc.; benzyl viologen and Triton X-100 were obtained from Sigma Chemical Co.; and hyamine was obtained from Packard Instrument Co.

Mutagenesis and screening of mutants. Strain CY314 (a strain lysogenic for λ*c*1857) was grown in medium E plus glucose and acetate until log phase and then mutagenized with *N*-methyl-*N'*-nitroso-nitrosoguanidine at a final concentration of 0.1 mg/ml for 30 min at 33°C (8, 9). The bacteria were washed once, suspended in the same medium, and grown overnight. Over 90% of the organisms were killed by the mutagenesis. The mutagenized cells were diluted and plated on minimal medium E containing glucose and acetate. Single colonies were isolated and screened on microtiter plates as described below.

Screening for pyruvate oxidase mutants was done on 96-well microtiter plates as described by Tabor et al. (37) with some modifications. A colony was inoculated into the well of a microtiter plate containing 0.1 ml of M9 medium (with the pH decreased to 6.0) plus 0.4% glucose and 2 mM acetate. After growing overnight at 33°C, the cultures were inoculated into a second microtiter plate with a 48-prong stamping device. The second microtiter plate contained 0.1 ml of the same medium, except the glucose concentration was growth limiting (0.02%). After these cultures were grown overnight at 33°C (whereupon growth was limited by glucose deficiency), a mixture of 40% glucose and 10% casein hydrolysate was added to each well with the stamping device (transferring ca. 3 μl per well). The plates were incubated at 33°C for 2 h and then shifted to 42°C for 3 h to induce the temperature-sensitive λ prophage and lyse the cells. To each well of these lysates was added 0.1 ml of a mixture of 0.2 M sodium phosphate buffer, pH 6.0, 20 mM $MgCl_2$, 0.2 mM sodium thiamine pyrophosphate, 10 mM [1-^{14}C]-pyruvate sodium salt (6.25×10^{-2} Ci/mol), and 16 mM $Na_3Fe(CN)_6$ (final concentrations are given). The plates were immediately covered with filter paper that had been impregnated with $Ba(OH)_2$ as described by Tabor et al. (37). The plate was incubated at 42°C for 4 to 5 h, and the filter paper cover was replaced by a second $Ba(OH)_2$-treated filter paper. The plate was then incubated at 42°C for 12 to 16 h. Both filter papers were autoradiographed (to give an exposure in the linear range of the film for most wells). A lack of exposure of the film above the position of a microwell on the autoradiogram indicated a possible pyruvate oxidase mutant.

Preparation of pools of random insertions of Tn*10* into the *E. coli* chromosome. Phage λNK370 was plaque purified twice, and a stock with a titer of 2×10^{10} to 3×10^{10}/ml was prepared (8, 9, 24). Pools of random insertions of Tn*10* from λNK370 into the chromosomes of three strains of *E. coli*, CY314, KL16, and YYC63, were prepared essentially as previously described (8, 9). The *E. coli* strains were grown overnight in tryptone broth supplemented with acetate and maltose. Approximately 10^{10} bacteria were infected with λNK370 at a multiplicity of 0.2. After incubation for 45 min, they were plated on rich media supplemented with acetate, 2.5 mM sodium pyrophosphate, and 20 mg of tetracycline-hydrochloride per liter. After 2 to 3 days, individual Tet^r colonies appeared. A total of 2,000 to 4,000 independent Tet^r

colonies of each strain were pooled by washing the colonies from the plates with medium E. The pooled colonies of the random insertions of Tn*10* into strains CY314 and KL16 were used for conjugation experiments as described below. A P1 stock was prepared on the pooled colonies of strain YYC63, which was used for experiments involving the indicator medium.

A pool of random insertion of Tn*10* into the chromosome of the wild-type strain W1485 was prepared as described previously by A. Klages Ulrich of this laboratory (8, 9). P1 phage grown on this pool was used in experiments to isolate strains YYC85 (*zac*::Tn*10*), YYC16 (*zdh*::Tn*10*), and YYC33 (*zca*::Tn*10*) as described above.

Screening *pox* mutants on TTC-containing medium. Screening on TTC-containing medium is reliable only when <100 colonies are present per plate. This behavior upon crowding (6) therefore precluded scoring by replica plating onto TTC-containing medium. Another problem was that the presence of tetracycline interfered with scoring on TTC medium. For these reasons, the screening of *poxA* mutants on TTC medium was usually done by either of the following two methods. In the first method, all colonies (e.g., Tet^r colonies) to be screened were eluted from plates by washing the entire plate with medium E. This mixture of colonies was then diluted and plated on TTC medium to give approximately 100 colonies. The *pox* colonies were recognized as white colonies (in an *ace pps pfl* genetic background) after 2 days of growth (the presence of any pyruvate-utilizing enzyme gives a red colony on this indicator medium). In the second method, the colonies to be screened were gridded onto master plates. Each colony was then inoculated into a well of a 96-well microtiter plate containing 0.1 ml of medium E supplemented with 0.4% glucose and acetate. After growth overnight, the stamping device was inserted into the first microtiter plate and used to inoculate a second microtiter plate containing the same medium. The cultures in the second plate were allowed to grow overnight. The contents of each well were then serially diluted with the stamping device into a series of microtiter plates containing medium E. After proper dilution, the stamping device delivered only a few bacteria per prong onto the TTC medium. This method avoided artifacts of crowding and allowed scoring of ordered arrays of colonies.

Preparation and assay of pyruvate oxidase. Strains of *E. coli* grown to early stationary phase in rich broth supplemented with acetate were harvested and washed once with 0.02 M sodium phosphate buffer (pH 6.0). This same buffer was added to the pellet (2 ml/g [wet weight]), and the suspension was treated at full power of an ultrasonicator fitted with a small probe (50% pulse) for 3 min for each 5 ml of suspension. The suspension was centrifuged at 40,000 × *g* for 1 h, and the supernatant was assayed. All operations were done at 0 to 4°C. The activity of 40,000 × *g* supernatant was stable for at least 1 month if stored at −7°C in the presence of 20% glycerol (4, 34).

Two methods were used to measure pyruvate oxidase activity. Similar results were obtained with either assay to determine the activities of crude extracts of either wild-type or mutant cultures. In the spectrophotometric method, the assay was carried out in 1-ml cuvettes in a Gilford spectrophotometer. Extract (≤0.1 ml) was added to 0.8 ml of an assay mixture consisting of 0.5 ml of 0.2 M sodium phosphate buffer (pH 6.0), 0.05 ml of 0.2 M $MgCl_2$, 0.05 ml of 2 mM sodium thiamine pyrophosphate, and 0.2 ml of 1 M sodium pyruvate. The mixture was incubated at room temperature for 20 min. Then, 0.1 ml of 0.08 M $Na_3Fe(CN)_6$ was added, and the rate of decrease of the absorbance at 450 nm was recorded (4). The enzymatic activity was calculated by using the formula $E_{mM}^{450} = 0.218\ cm^{-1}$ and is expressed as the amount of pyruvate consumed per unit of time, assuming 2 equivalents of ferricyanide are reduced per equivalent of pyruvate decarboxylated (4).

In the radioactive method, the reaction mixture (0.1 ml) consisted of 0.05 ml of 0.2 M sodium phosphate buffer (pH 6.0), 5 μl of 0.2 M $MgCl_2$, 5 μl of 2 mM sodium thiamine pyrophosphate, 5 μl of 1 M sodium [1-^{14}C]pyruvate (6.25×10^{-2} Ci/mol), 10 μl of 0.08 M $Na_3Fe(CN)_6$, and the extract to be assayed. The assay mixture in an Eppendorf vial was placed in a liquid scintillation counting vial as described previously (3). Filter paper (3.5 by 2.5 cm) soaked with 0.15 ml of 1 M Hyamine hydroxide lined the wall of the counting vials to trap the $^{14}CO_2$. After incubating at 33°C for 20 min with shaking, 0.05 ml of 3 N HCl was added to the assay mixture, and incubation was continued for 30 min. After collection, the Eppendorf vials were discarded, 15 ml of toluene scintillation fluid was added to the scintillation vial, and the vials were counted.

Other methods. Protein concentrations were determined by a microbiuret procedure with bovine serum albumin as standard (32). Transductions with P1 *vir* were performed as previously described (11).

Calculations of genetic linkage. Wu (41) has derived the following equation to relate cotransduction frequency with phage P1 to the distance between two markers on the *E. coli* chromosome: $F = (1 - d/L)^3$, where F is the cotransduction frequency, d is the distance between the two markers, and L is the length of the transduced DNA fragment. For 90% cotransduction ($F = 0.9$) and $L = 2.3$ min (the length of DNA packaged by phage P1), $d = 0.07$ min.

An analogous equation relating linkage of unselected and selected markers in prolonged Hfr × F^- conjugational crosses has been derived by de Haan and Verhoef (15, 39): $(1 - \beta) = (1 - \alpha)(1 - e^{-ku})$, where β is the frequency of an unselected marker, α is the incorporation frequency of a given marker, k is the number of breakages per map minute, and u is the distance (in minutes) between the selected and unselected markers. For a linkage of 90% between the selected and unselected markers where α is 0.5 (15, 39) and k is 0.2 (average of the values given in reference 39), $u = 1.1$ min.

The probability of insertion of a transposon into a unique fragment of DNA (that adjacent to the nonselectable marker) was calculated from the following equation of Clarke and Carbon (10): $N = \ln(1 - P)/\ln(1 - f)$, where N is the number of Tet^r recombinants required to detect a transposon ≥90% linked to the nonselectable marker, P is the probability of Tn*10* insertion into a unique DNA sequence, and f is the fraction of the genome into which insertion is required. Since the genome of *E. coli* is 100 min (1, 2), f for ≥90% cotransduction is 0.07/100 = 0.0007 and P = 0.99; N = 6,576. In contrast, in the conjugational

cross, $f = 1.12/100 = 0.0112$. When P is again 0.99, $N = 409$.

This analysis assumes that Tn*10* acts genetically as a point mutation. This assumption appears to be justified (24). However, the assumed random nature of Tn*10* insertion is not accurate (see below).

RESULTS

Characterization of pyruvate oxidase of *E. coli* K-12. The previous work on pyruvate oxidase was on the enzyme from *E. coli* W191-6 (16), a derivative of strain W. Since strain W is genetically cryptic, we used *E. coli* K-12. The properties of pyruvate oxidase from *E. coli* K-12 were found to be very similar to those of pyruvate oxidase from strain W191-6. The activity in the crude extract from the *E. coli* K-12 strain CY314 is dependent on the presence of pyruvate, $MgCl_2$, and thiamine pyrophosphate and is inhibited by the antibody prepared against *E. coli* W191-6 pyruvate oxidase (a gift of R. Kranz and R. Gennis). Detergents such as Triton X-100 increase the activity in the crude extracts somewhat, but the activation is not as dramatic as that of the purified enzyme from strain W191-6 (16). This was attributed to activation by the phospholipids present in the extracts.

The pyruvate oxidase activity of *E. coli* K-12 increased during the growth of the bacterial cultures and reached maximum activity during early stationary phase. The activity was unchanged by growth through late stationary phase. No detectable pyruvate oxidase activity was found when strain CY314 was grown under anaerobic conditions with or without sodium nitrate as an electron acceptor.

Isolation and characterization of *pox* mutants. Tabor et al. (37) have reported a method that permits the rapid screening of a large number of bacterial colonies for mutations in pathways producing $^{14}CO_2$. An assay is done on single colonies, each in the well of a microtiter dish, by trapping the $^{14}CO_2$ from each well on a filter paper saturated with barium hydroxide. We utilized this method, with some modifications, as the basis for isolation of pyruvate oxidase mutants (see Materials and Methods). The major modification was that we assayed pyruvate oxidase activity in lysates produced by inducing a phage λ lysogen.

Strain CY314 was mutagenized with *N*-methyl-*N'*-nitroso-nitrosoguanidine, and approximately 1,000 mutagenized colonies were screened by the microtiter plate method. Several mutant candidates were obtained, and they were further characterized by assay of their pyruvate oxidase activities in crude extracts. Most of the candidates had about half of the pyruvate oxidase activity of the parent strain. Only one mutant, YYC7, had a lower activity (about 15% of the parent strain [Table 2]). Thus, we concentrated our effort in characterizing and mapping

TABLE 2. Pyruvate oxidase activities of various strains

Strain	Genotype	Activity (U/mg of protein)[a]	K_m for pyruvate (mM)[b]
Expt 1			
CY314	Wild type	135	14 (33)
YYC7	*poxA1*	16	17 (25)
Expt 2			
CY314	Wild type	113	
YYC7	*poxA1*	16	
CY314 + YYC7[c]		137	
Expt 3			
CY314	Wild type	152	
YYC7	*poxA1*	18	
YYC91	*poxA1*	23	
YYC115	*poxA*$^+$ of YYC91	183	
YYC265	Wild type	141	
YYC63	*pfl pps* of CY265	70	
YYC107	*pfl pps poxA1*	<2	
YYC109	*poxA*$^+$ of YYC107	78	

[a] The spectrophotometric assay was used. One unit of activity equals 1 nmol of pyruvate decarboxylated per min.

[b] The K_m value for pyruvate as determined by spectrophotometric or (values in parentheses) radioactive assay. The differences between the two assays probably reflects a lower extent of activation by endogenous phospholipid in the radioactive assay.

[c] Equal amounts of protein from the two extracts were mixed and assayed.

this mutant, which we designated *poxA* (for pyruvate oxidase).

Although strain YYC7 was deficient in pyruvate oxidase activity, mutant extracts retain significant amounts of oxidase activity (10 to 15% of the normal level). Preliminary experiments indicate that the level of pyruvate oxidase antigen in mutant extracts is similarly decreased. Since the residual activity in the *poxA* strains has a normal Michaelis constant for pyruvate (Table 2), strain YYC7 seems to contain an abnormally low level of a structurally normal pyruvate oxidase. When an extract of strain YYC7 was mixed with an extract of the parent strain, CY314, there was no inhibition of the pyruvate oxidase activity of the latter extract, indicating that no inhibitor was present in the extract of strain YYC7 (Table 2).

Mutant YYC7 grows as well as the parent strain, but at a slightly slower rate. The generation times of strains CY314 and YYC7 were 55 and 95 min, respectively, when grown on rich broth supplemented with acetate at 33°C. On glucose minimal medium supplemented with acetate, strain YYC7 doubled in 115 min, somewhat slower than strain CY314 (100 min). This low growth rate seemed characteristic of the *poxA* lesion, since *poxA* transductants of other strains also grew more slowly than the parental strain.

Insertions of Tn*10* near the *poxA* gene, nonselectable gene. The mapping of the *poxA* gene was complicated by its nonselectable phenotype and by the fact that reliable screening for the enzyme activity required pyruvate dehydrogenase-deficient strains (the assay for the pyruvate decarboxylase component of the dehydrogenase is very similar to that of pyruvate oxidase). We therefore chose to isolate an insertion of the transposon Tn*10* close to the *poxA* gene and map the gene by locating the site of insertion. This is a method put forth by Kleckner and co-workers (24), who also suggested that appropriate insertions could be readily isolated for nonselectable as well as selectable genes. The latter case is clearly true and has been used in a number of laboratories, including our own. However, few nonselectable genes have been mapped by this procedure, owing to the difficulty in isolating closely linked insertions. Calculations (see Materials and Methods) indicate that isolation of such an insertion would require screening of several thousand Tet^r recombinants. Hence, a screening method such as an indicator medium would suffice, whereas a method such as assaying CO_2 evolution of microtiter plate cultures would be most laborious.

We therefore chose to enrich for Tn*10* insertions close to the *poxA* gene by screening Tet^r recombinants of crosses between Tn*10*-carrying *poxA*$^+$ Hfr strains and a *poxA* recipient strain (an F$^-$ phenocopy culture of strain YYC91). The Hfr cultures contain a mixture of several thousand strains, each with a Tn*10* insertion of independent origin. Two Hfr strains having different transfer origins (HfrC and KL16) were used to cover the entire chromosome, and prolonged matings were done. Tet^r recombinants were selected and screened for $^{14}CO_2$ evolution by the microtiter dish assay. Owing to the greater length of DNA transferred in such crosses, Tet^r *pox*$^+$ recombinants were isolated at a much greater frequency than that expected from phage P1 transduction from a pool of Tn*10* insertions.

Using this method, we screened about 180 recombinants each from crosses of Hfr strains C and KL16 with a *poxA* recipient (strain YYC91). In the HfrC cross, about 25% of the Tet^r recombinants were *pox*$^+$, whereas no *pox*$^+$ recombinants were found in the cross with strain KL16. From our later experiments in which an indicator plate method was used to screen for *pox*$^+$ (see below), a linkage frequency of 0.6% would have been expected if the experiment had been done by transduction rather than conjugation.

The *pox*$^+$ Tn*10* strains from the conjugational crosses were then screened for those with Tn*10* to *poxA* linkages sufficiently high to be cotransduced. A P1 stock was grown on a pool of these strains. A *poxA* strain was transduced to Tet^r, and the Tet^r transductants were again screened by the microtiter plate method. About 10% of the Tet^r transductants (180 screened) were *pox*$^+$, and several were shown to have a normal level of pyruvate oxidase by the spectrophotometric assay (Table 2). One of these insertions, *zje-1*:Tn*10*, which subsequent transductions showed to be tightly linked (98%) to *poxA*, was used to locate the gene on the genetic map.

Insertions of Tn*10* near the *poxA* gene were later isolated directly by cotransduction. We found that we could score the presence of pyruvate oxidase in individual colonies by using a pyruvate-TTC indicator medium, provided the strains were deficient in all other pyruvate-utilizing enzymes (*ace*, *pps*, *pfl*). Although introduction of the *pfl* mutation into *pox*$^+$ strains decreased pyruvate oxidase levels by about 50%, this did not affect screening for *pox*$^+$ recombinants. We used the indicator medium to screen about 3,600 Tet^r colonies from a transduction of strain YYC107 with a phage P1 stock grown on a pool of Tn*10* insertions into strain YYC63. We isolated 22 presumably independent Tn*10* insertions cotransduced with the *poxA* locus. One of these insertions, *zje-2*::Tn*10*, 98% linked to *poxA*, was used in some mapping experiments. The site of this insertion seems to be identical to that of *zje-1*::Tn*10* (see below).

Mapping of the *poxA* gene. The conjugation

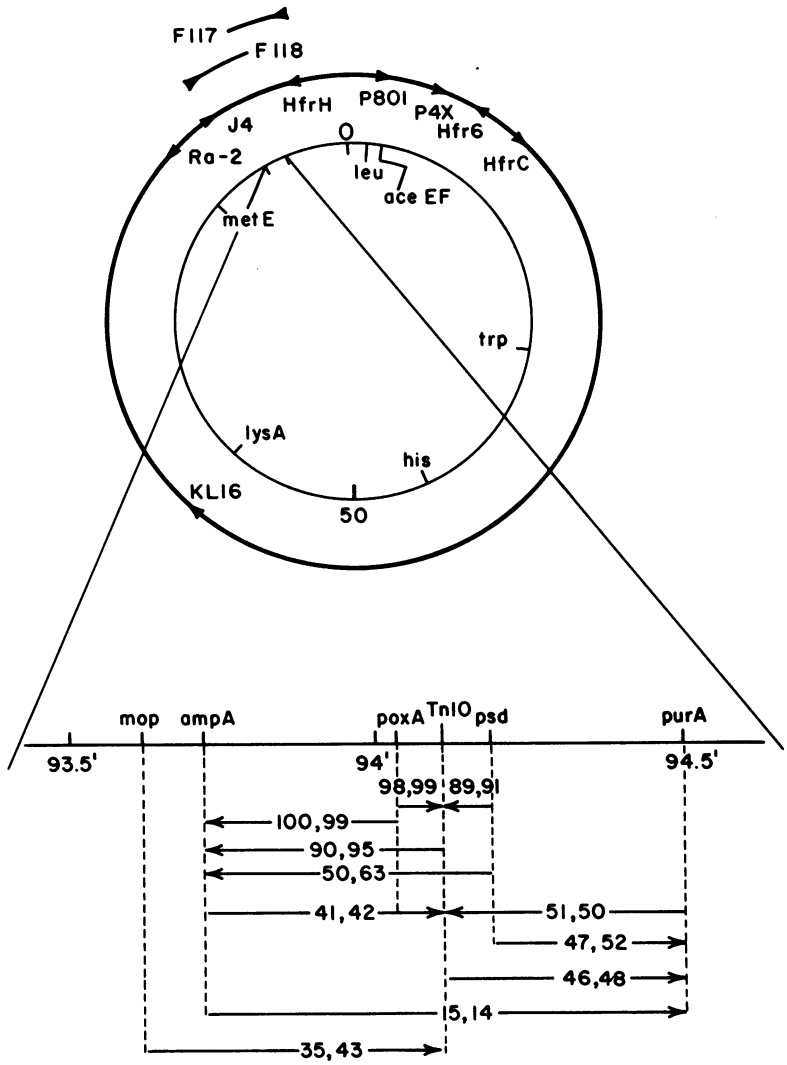

FIG. 1. Relevant portion of the *E. coli* genetic map. The linkages given are percent average cotransduction frequencies taken from experiments similar to those shown in Table 3. The first value is the cotransduction frequency with *zje2*::Tn*10* strain YYC109, and the second value is that with *zje1*::Tn*10* strain YYC115. The map is a modification of that of Bachmann and Low (1).

experiment described above already showed that the *poxA* locus was located between the origins of Hfr strains C and KL16 on the *E. coli* chromosome. Moreover, conjugation between the HfrC strain YYC109 and the F^- strain χ478 (which has markers spaced around the chromosome) showed that the Tn*10* insertion of the donor strain was most closely linked to *leu* (72%). To further delineate the location of *poxA*, a number of Hfr strains (27, 28) in this region were used (Fig. 1). Each Hfr strain was first transduced to Tet^r with phage P1 stock grown on strain YYC109 and then mated with the F^- strain χ478 for a short time (30 min), and Tet^r recombinants were selected. Only the derivatives of Hfr strains P4X, P801, and Ra-2 gave Tet^r recombinants. Identical results were obtained with the Tn*10* insertion of strain YYC115.

P1 transduction was used to more finely locate the Tn*10* insertions of strains YYC109 and YYC115. Strains carrying readily scorable markers in this region were transduced with phage P1 stocks grown on strains YYC109 or YYC115, and Tet^r recombinants were selected. Neither Tn*10* insertion cotransduced with *serB* (<0.7%), *dnaC* (<0.5%), *uxuAB* (<1%), *valS*

TABLE 3. Cotransductional mapping

Cross	P1 donor and relevant marker[a]	Recipient	Marker selected	No. of colonies scored	% Cotransduction frequency
1	YYC115 *zje-1*::Tn*10*	PC0950 *purA*	Tet^r	196	48
2	YYC115 *zje-1*::Tn*10*	H882 *purA*	Tet^r	200	49
3	YYC115 *zje-1*::Tn*10*	T850 *mop*	Tet^r	43	42
4	YYC115 *zje-1*::Tn*10*	EH450 *ampA*	Tet^r	96	45
5	YYC115 *zje-1*::Tn*10*	EH450 *psd*	Tet^r	96	95
6	YYC115 *zje-1*::Tn*10*	YYC7 *poxA*	Tet^r	89	95
7	YYC115 *zje-1*::Tn*10*	YYC107 *poxA*	Tet^r	48	100
8	YYC109 *zje-2*::Tn*10*	YYC107 *poxA*	Tet^r	48	98
9	YYC109 *zje-2*::Tn*10*	YYC107 *poxA*	Tet^r	200	98
10	YYC176 *zje-1*::Tn*10 ampA*	YYC183	*ampA*	143	95
11	YYC170 *ampA*	H882 *purA*	*pur*$^+$	144	11
12	EH450 *psd ampA*	YYC183	*ampA*	120	64

[a] The inheritance of the *pox*$^+$ allele of the donor was scored by the $^{14}CO_2$ microtiter dish assay (cross 6), by indicator plates with method 1 (cross 9) or method 2 (crosses 7 and 8). Only a few of the data obtained with strains carrying the *zje-2*::Tn*10* marker are shown. Crosses with these strains were done in parallel with those containing *zje-1*::Tn*10* insertion, and a similar number of transductants were scored. The cotransduction frequencies found for the two insertions to the other markers were essentially indistinguishable (see Fig. 1).

(<0.4%), or *metA* (<3%), but both insertions cotransduced with *purA* at a frequency of about 50% (Table 3).

Further transduction experiments showed cotransduction of both insertions with the *ampA* and *psd* loci (Table 3). The relative cotransduction frequencies suggested that both Tn*10* insertions were located between the *ampA* and *purA* loci, very close to the *psd* locus. A location between *ampA* and *purA* was demonstrated by three-factor transductional crosses (data not shown). Other three-factor crosses were consistent only with the order *ampA*, Tn*10*, *psd*, *purA* (Table 4). The Tn*10* insertions of strains YYC109 and YYC115 were indistinguishable in the crosses performed.

We then mapped the *poxA* locus in relation to the Tn*10* insertion and *psd* locus. Unfortunately, *psd* is a poor selected marker, owing to residual growth at 42°C, and thus we used Tn*10* and *ampA* as the selected markers in these crosses. When Tet^r was the selected marker in a transductional cross with strain YYC176 as the donor and strain YYC183 as the recipient, close linkage to both *poxA* (99%) and *psd* (86%) was found. There was no clear four-crossover class, and thus these data (not shown) suggested the map order *poxA*, Tn*10*, *psd*. Upon scoring the *poxA* markers of the recipient in cross 1 of Table 4, the linkage between *ampA* and *poxA* was 99%, whereas the cotransduction frequencies for both the Tn*10* insertion and the *psd* locus were lower (95 and 63%, respectively). These data are consistent only with the order *ampA*, *poxA*, Tn*10*, *psd*. The large differences in the cotransduction frequency observed between *ampA* and the Tn*10* insertion when the selected marker was shifted from one antibiotic to the other (42% when Tet^r was selected, 95% when *ampA* was selected) has been well documented

TABLE 4. Ordering of *ampA psd* and *zje1*::Tn*10*[a]

Cross	Donor	Recipient	Marker selected	No. of colonies scored	Class	% Frequency
1	YYC176 *zje-1*::Tn*10 psd ampA*	YYC183	*ampA*	143	Tet^r *psd*$^+$	32
					Tet^r *psd*	63
					Tet^s *psd*$^+$	5
					Tet^s *psd*	0
2	YYC115 *zje-1*::Tn*10*	EH450 *psd ampA*	Tet^r	96	*amp*$^+$ *psd*$^+$	42
					amp$^+$ *psd*	3
					ampA psd$^+$	53
					ampA psd	2

[a] In cross 1, the Tet^s *psd* recombinant class is taken as the four-crossover class, indicating the order *ampA*, Tn*10*, *psd*. The lack of a clear four-crossover class in cross 2 is consistent with this order.

with *ampA* (20) as well as other markers (2) and can be explained by the existence of sites within the *E. coli* chromosome that lead to preferential packaging into phage particles (2).

Diploidy at the *poxA* locus. Two F' strains, F117 and F118, which carry the chromosomal regions from 93 to 98 and 91 to 95 min of the *E. coli* chromosome, respectively (Fig. 1), were transferred into strain YYC177 (a Δ*aceEF*, *poxA* derivative of χ478). The resulting merodiploid strains and the recipient strain YYC177 were grown in liquid on a medium lacking histidine and thymine to select against the donor strains. Extracts of both merodiploid cultures had the same pyruvate oxidase activity as that of a wild-type strain, whereas the activity of monoploid strain YYC177 was very low. Since 60% of the cells in the merodiploid cultures were able to transfer $purA^+$ to strain H882, most of the cells were merodiploids rather than recombinants. These results indicated that the $poxA^+$ gene is carried by both F117 and F118 and that the *poxA* mutation is recessive to the wild-type allele.

DISCUSSION

Although quite deficient in pyruvate oxidase activity, the *poxA* mutant we isolated does not appear to be a mutant in the structural gene for this enzyme. We have recently isolated a second class of *pox* mutants (designated *poxB*) that preliminary results suggest are lesions in the structural gene for this protein. At this time, we therefore assign a regulatory function to the *poxA* gene. It is of interest that the level of pyruvate oxidase activity was decreased by introduction of the *pfl* mutation (Table 2). It may be that the *pfl* mutant (38) is a lesion in a regulatory gene rather than a structural gene for this complex enzyme. We also noted that pfl^+ *poxA aceEF* strains gave red colonies on the TTC indicator medium, indicating that pyruvate lyase functions in aerobically growing cells, a result in disagreement with a previous report (21).

Kleckner and co-workers (24) have pointed out that nonselectable genes can be readily mapped by isolation of a strain carrying a Tn*10* insertion near the gene of interest. The location of the transposon is mapped, and when the gene is mapped in relation to the transposon (e.g., by enzyme activity or protein profile), the gene can be accurately placed on the genetic map. However, the major difficulty lies in the isolation of a Tn*10* insertion close to the gene. Kleckner and co-workers (24) have implied that a transducing phage lysate of a pool of Tn*10* insertions into a wild-type strain could be used to transduce a strain defective in a nonselectable gene and the resulting Tet^r strains screened for the wild-type allele of the nonselectable gene. This is true, but calculations (see Materials and Methods) indicate that a powerful and facile screening method is needed. Assuming that Tn*10* inserts at random, the probability of a Tn*10* insertion cotransducible with a given gene is very low. For phage P1 transduction of *E. coli*, approximately 6,600 colonies having independent Tn*10* insertions must be screened to isolate a single insertion ≥90% cotransduced with a given gene ($P = 0.99$). Hence, although indicator plates would suffice, screening methods such as the microtiter dish $^{14}CO_2$ evolution assay of Tabor et al. (37) that can readily screen only a few hundred colonies are unfeasible. Since it seems likely that for many of the remaining unexplored genes of *E. coli* only rather laborious and expensive screens will be available, a method more powerful than direct screening of transductional Tn*10* recombinants seems desirable.

The key manipulation of our method is transfer of Tn*10* insertion pools via Hfr mating (rather than transduction) into an F^- strain carrying a scorable allele of the nonselectable marker. During prolonged Hfr × F^- matings, unselected markers are inherited by the recipient strain at a probability depending only on the map distance between the selected and nonselected markers (15, 39). The frequency of inheritance of nonselected markers is quite high. From the data and equations of de Haan and Verhoef (15, 39), markers separated by 1.1 min of the map inherit a ≥90% frequency (see Materials and Methods). Thus, isolation of a Tn*10* insertion close to a given nonselectable gene would require screening only 400 colonies ($P = 0.99$), an enrichment of about 20-fold as compared with a direct screening of transductants. The F^- Tn*10* recombinants that inherit the Hfr allele of the gene of interest can then be pooled, and a transducing phage stock can be grown on this enriched pool and used in a subsequent screening for a highly cotransducible insertion.

A potential difficulty with our method concerns the gradient of chromosome transfer by Hfr strains. Although the gradient of transfer does not affect linkage between selected and nonselected markers separated by distances of <20 min (39), it does affect the efficiency of transfer of the selected marker. Thus, transposon insertions into chromosome segments transferred early will be preferentially found in the population to be screened. In our experiments, a high frequency of linkage was found despite a distance of 20 min between the HfrC origin and the location of the *poxA* gene, but more distal markers have not been tested. One solution to this problem would be the use of more than two Hfr strains; this, however, would increase the number of colonies to be screened. A better

solution would be to alleviate the transfer gradient by immobilization of mating complexes on a membrane filter (19). This procedure would decrease nonrandom transposon transfer to about the level observed in P1 transduction. Phage P1 preferentially transduces those markers close to the origin of chromosome replication (33).

We observed that the frequency of Tn*10* insertions near *poxA* was much higher than that calculated for both the conjugational and transductional cases. However, this was not unexpected, since the calculations assumed Tn*10* insertion to be at random and insertion of this transposon is known to be sequence specific (18, 23). About 90% of Tn*10* insertions are into "hot spots," DNA containing a specific 6-base-pair (GCTNAGC) sequence, whereas the remaining insertions are into DNA having degenerate versions of this sequence (18, 23). The high frequency with which we isolated Tn*10* insertions close to the *poxA* gene and the fact that two independently isolated insertions are genetically indistinguishable indicate that a hot spot for insertion of Tn*10* is located in this area of the chromosome. However, it should be noted that the presence of a hot spot should affect the conjugational and transductional crosses in a similar manner, and thus the relative enrichment of *poxA*-linked Tn*10* insertions predicted by the calculations should be valid. Indeed, a reasonable agreement is seen between the predicted and observed enrichments. Our calculations predict a 15- to 20-fold enrichment, whereas an enrichment of 40-fold was observed. The enrichment should suffice to make this procedure applicable to many nonselectable genes and to highly revertable selectable genes. Its use need not be restricted to Tn*10* and *E. coli* but should also be useful with other transposons and other conjugative bacteria.

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