Isolation and Mapping of *Escherichia coli* K12 Mutants Defective in Phenylacetate Degradation

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(Received 17 April 1985; revised 5 June 1985)

Mutants of *Escherichia coli* K12 unable to grow on phenylacetate have been isolated and mapped. The mutations were located in the relatively 'silent' region of the *E. coli* K12 chromosome at min 30.4 on the genetic map, with the gene order *rac pac-1 pac-2 trg*.

INTRODUCTION

Reports have appeared recently on the unexpected ability of *Escherichia coli* strains to grow on aromatic acids. The pathways for the catabolism of 4-hydroxyphenylacetate (Cooper & Skinner, 1980) and 3-phenylpropionate (Burlingame & Chapman, 1983) have been described and they are identical to those previously reported for catabolism of these compounds by various soil bacteria (Dagley *et al.*, 1965; Sparnins *et al.*, 1974).

Studies on phenylacetate catabolism in certain *Pseudomonas* sp. implicated 3,4-dihydroxyphenylacetate dioxygenase (Kunita, 1955*a*) and 2,5-dihydroxyphenylacetate dioxygenase (Kunita, 1955*b*) in degradative pathways, but in an extensive study of various pseudomonads grown on phenylacetate neither of these ring-fission dioxygenases could be detected in significant amounts (P. J. Chapman, personal communication). Similarly, no ring-fission reaction involved in phenylacetate catabolism in *E. coli* has yet been found (R. A. Cooper, unpublished observations). It seems, therefore, that there is still uncertainty about the pathway for bacterial phenylacetate catabolism.

Accordingly we have sought to obtain information on phenylacetate catabolism by isolating phenylacetate-negative mutants of *E. coli*. Although the pathway is still unresolved the mutants themselves were of interest since the genes affected were located in a region of the *E. coli* K12 chromosome where very few genes have so far been identified. This report describes the partial characterization of the mutants and their genetic mapping.

METHODS

Bacterial strains and growth conditions. The *E. coli* strains used are listed in Table 1. Bacterial cultures were grown aerobically at 30 °C in minimal medium 63 or Luria broth (Miller, 1972). Individual carbon sources were sterilized separately and added to give the indicated final concentrations. Liquid media were solidified as required by the incorporation of 1.6% (w/v) Oxoid bacteriological agar.

Substrate oxidation. Washed cells were prepared and their oxidative abilities measured as described previously (Cooper & Skinner, 1980).

Isolation of mutants. Cultures were treated in minimal salts medium 63 with ethyl methanesulphonate (EMS) as described by Miller (1972). Survivors were allowed to grow overnight at 30 °C on glucose minimal medium and phenylacetate-negative mutants were isolated after a penicillin enrichment procedure (Miller, 1972).

Metabolism of radioactive phenylacetate. Washed cells from 100 ml exponential phase culture ($OD_{680} \approx 0.55$) were resuspended in 10 ml of minimal medium 63 to give $OD_{680} \approx 5$. A sample (2 ml) was shaken at 30 °C with 1.25 μ Ci (46.3 kBq) phenyl[1- 14 C]acetate and a final phenylacetate concentration of 0.5 mM. Samples (0.2 ml) were removed at timed intervals into 0.8 ml absolute ethanol and heated at 80 °C for 5 min to extract soluble material. Debris was removed by centrifugation and supernatant (0.15 ml) was spotted onto Whatman 3MM

Table 1. *Escherichia coli* K12 strains and plasmids

Genotype		Source*
<i>E. coli</i> K12		
K10	Hfr C <i>thi</i> (?)	F. C. Neidhardt
PA309	F ⁻ <i>thi argHBCE his thr trp rpsL lacY malA xyl ara mtl gal tonA</i>	R. H. Walmsley
HB235	F ⁻ <i>thr leu his eda rpsL lac ara xyl tonA tsx trg-2::Tn10</i>	G. L. Hazelbauer
PLK901	F ⁻ <i>his-51 pro-23 trp-30 thyA lac-28 deoB</i> or <i>deoC rpsL101 AmanA uidA λ⁺ zcj-230::Tn10</i>	P. L. Kuempel
PLK902	As PLK901 but <i>zda-231::Tn10</i> replaces <i>zcj-230::Tn10</i>	P. L. Kuempel
CO160	As K10 but <i>pac-1</i>	EMS mutagenesis
CO161	As CO160 but <i>zda-231::Tn10</i>	P1 (PLK902) × CO160; Tc ^r selection
CO162	As CO160 but <i>srl::Tn10 recA</i>	P1 (<i>srl::Tn10 recA</i>) × CO160; Tc ^r selection
CO164	As K10 but <i>pac-2</i>	EMS mutagenesis
CO165	As CO164 but <i>zda-231::Tn10</i>	P1 (PLK902) × CO164 Tc ^r selection
CO535	As PA309 but <i>his⁺ pac-1</i>	CO160 × PA309; His ⁺ selection
Plasmids		
F ⁺ kit		B. J. Bachmann
pJPB13†		J. P. Bouché
pBS1‡		J. P. Bouché

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† pJPB13 is a derivative of pBR325 with the *Pst*I fragment P41I, position 196–205 kb (Bouché, 1982).

‡ pBS1 is a derivative of pBR325 with the *Hind*III fragment H36b, position 204–214 kb (Béjar & Bouché, 1983).

chromatography paper. Chromatograms were developed overnight in propan-2-ol/ammonia/water (Smith, 1960) and subjected to autoradiography. Incorporation into cells was measured in a similar system except that the samples (0.2 ml) were filtered on nitrocellulose filters (0.2 µm pore size), washed rapidly on the filter with 5 ml 0.1 M-potassium phosphate buffer pH 7.1 and the filter plus cells transferred to a scintillation vial, containing 4 ml of a dioxan-based scintillant, for counting.

Genetic procedures. Conjugal mating and phage P1 *vir*-mediated transduction were as described by Miller (1972). Cells were transformed by the method of Kushner (1978).

Chemicals. All chemicals used were the best grade commercially available. Phenyl[1-¹⁴C]acetate was from Amersham.

RESULTS AND DISCUSSION

Partial characterization of mutants

The mutants selected for study were affected specifically in phenylacetate catabolism and the gene symbol *pac* was chosen to describe them. On plates, they grew normally with glucose (10 mM), glycerol (20 mM), succinate (15 mM), acetate (30 mM) and 3-phenylpropionate (5 mM) as sole carbon sources.

The ability to catabolize phenylacetate is inducible in *E. coli* K12. Wild-type cells grown in Luria broth or minimal medium with glucose, glycerol or succinate as sole carbon and energy source failed to oxidize phenylacetate. Cells induced by growth on Luria broth supplemented with 5 mM-phenylacetate, or on 5 mM-phenylacetate in minimal medium, readily oxidized phenylacetate.

To seek information on phenylacetate catabolism, the ability of mutant cells induced by growth on Luria broth supplemented with phenylacetate to metabolize phenyl[1-¹⁴C]acetate was monitored.

When mutant CO164 (*pac-2*) was exposed to phenyl[1-¹⁴C]acetate no radioactivity was incorporated into the cells and no radioactive compound other than phenylacetate was seen by chromatography. This suggested that CO164 was affected either in the uptake of phenylacetate

Fig. 1. Location of *pac-1* on the genetic map of *Escherichia coli* K12. An arrow head indicates the unselected marker in a co-transduction experiment. The chromosomal region and the cloned fragments are drawn to the same scale.

or in a regulatory gene controlling phenylacetate catabolism. In contrast, mutant CO160 (*pac-1*) exposed to phenyl[1-¹⁴C]acetate did take up radioactivity and chromatographic analysis of the reaction mixtures showed that 2-hydroxyphenylacetate was produced. Under the experimental conditions employed all the phenylacetate could be converted to 2-hydroxyphenylacetate by this mutant.

To see if 2-hydroxyphenylacetate was an intermediate in phenylacetate degradation, wild-type cells grown on phenylacetate were exposed to 2-hydroxyphenylacetate. No significant oxidation of this compound was detected, so it seems either that 2-hydroxyphenylacetate is not a normal intermediate in phenylacetate catabolism or that it does not enter wild-type cells. However, these experiments showed that the mutants CO160 and CO164 were affected in different genes, so both were mapped to see whether the loci were linked.

Mapping of mutations

To obtain an approximate map position, the defective gene from CO160 was transferred by conjugation into strain PA309. After a 2 h period of mating streptomycin-resistant recombinants that had regained the ability to grow on glucose in the absence of either leucine, arginine or histidine were selected. When the recombinants were tested for growth on phenylacetate the His⁺ recombinants showed the highest proportion of phenylacetate-negatives (about 30%).

One such F⁻ *pac-1* strain, CO535, was then used as recipient in mating experiments with various F prime-carrying strains (Low, 1972) to identify those which suppressed the *pac-1* mutation. F123 from the Hfr B7 carrying the chromosome region min 25–30 and two much shorter F primes from the same Hfr, F618 and F619 (Bitner & Kuempel, 1982), complemented the mutant phenotype indicating that the *pac-1* locus was around min 29–30 on the *E. coli* K12 linkage map.

This region has very few known loci for co-transduction mapping, but a series of transposon insertions in this part of the chromosome have been identified (Binding *et al.*, 1981; Fouts & Barbour, 1982). Two insertions in the *rac* prophage, described by Binding *et al.* (1981), *zcj-230::Tn10* (formerly Tn10₂) and *zda-231::Tn10* (formerly Tn10₄), have been used to help locate *pac-1*.

P1 phage grown on PLK901 (*zcj-230::Tn10*) or PLK902 (*zda-231::Tn10*) were used to transduce mutant CO160 to Pac⁺. When the transductants were tested for tetracycline resistance there was 26% linkage (143/549) for PLK901 and 63% linkage (137/216) for PLK902. In a transduction using P1 grown on PLK902 and CO160 as recipient, with selection for tetracycline resistance, 50% of the transductants (26/52) became Pac⁺. A P1 lysate prepared on CO161 (*pac-1 zda-231::Tn10*) was used to transduce a wild-type strain to tetracycline resistance and 51% (26/51) of the transductants became Pac⁺.

These co-transduction frequencies were used to calculate genetic map distances (Wu, 1966), assuming that the effective length of the phage P1 transducing fragment is 2 min. The *pac-1* locus appeared to be 0.4 min from *zda-231::Tn10* (min 30.0) and 0.7 min from *zcj-230::Tn10* (min 29.7) (Bitner & Kuempel, 1981), indicating a location at min 30.4 on the *E. coli* K12 linkage map (Bachmann, 1983) (Fig. 1). No allowance for the size of the inserted Tn10 (9.3 kb) has been made in these calculations.

The proposed location for *pac-1* indicates that it should be close to the *trg* locus at min 31.4. When this was tested, using P1 phage grown on HB235 (*trg-2::Tn10*) and CO160 as recipient, 18% of the tetracycline resistant transductants became *Pac*⁺ (79/447). This suggests that *pac-1* and *trg-2::Tn10* are 0.9 min apart on the genetic map.

It has been reported (Henson *et al.*, 1984) that deletions extending from *trg* towards *rac* can be obtained easily and such deletions cause no detectable alteration of phenotype. We have taken HB235 and selected tetracycline-sensitive colonies by the fusaric acid method (Bochner *et al.*, 1980). About 11% of the tetracycline-sensitive strains obtained were unable to grow on phenylacetate (20/178).

The other *pac* mutant appeared to map in the same region as *pac-1*. Strain CO164 (*pac-2*) was transduced to tetracycline resistance using phage grown on PLK902 and only eight of 52 transductants remained phenylacetate-negative, indicating an 85% linkage between *pac-2* and *zda-231::Tn10*.

To obtain information on the order of the *pac* mutations, crosses were made between pairs of *pac* mutants with the *zda-231::Tn10* of the donor as the selected outside marker. Some *Pac*⁺ progeny were obtained when CO165 (*pac-2 zda-231::Tn10*) was the donor with CO160 (*pac-1*) as the recipient (14/226). In the reverse cross with CO161 (*pac-1 zda-231::Tn10*) as donor and CO164 (*pac-2*) as recipient, no *Pac*⁺ transductants were obtained (0/260).

In these three-factor crosses generation of *Pac*⁺ requires two cross-overs or four cross-overs depending on the positions of the two mutant loci relative to the selected outside marker, *zda-231::Tn10*. When the mutant *pac* locus in the donor is proximal to *zda-231::Tn10*, four cross-overs will be required to produce *Pac*⁺. When it is distal, *Pac*⁺ can arise by two cross-overs. Since four cross-overs will occur significantly less frequently than two cross-overs the cross which produces the highest proportion of *Pac*⁺ progeny will be that in which the *pac* locus of the donor is distal to the *zda-231::Tn10* marker. The results suggest that of the mutants tested the *pac-1* mutation is closest to *zda-231::Tn10*.

The availability of cloned DNA from the region min 30–34 of the *E. coli* K12 chromosome (Béjar & Bouché, 1983) made it possible to seek confirmation of the location of the *pac* genes. To do this two plasmids pBS1 and pJPB13 were transformed into CO162 (*pac-1 recA*) to see if they complemented the mutant phenotype. Growth on phenylacetate was not restored by either plasmid. However, when CO160 (*pac-1 recA*⁺) was transformed with the plasmids and the transformants patched onto phenylacetate plates, large numbers of individual colonies appeared on each patch after 2–3 d. The plasmid-free host strain gave no colonies on phenylacetate even after 10 d. When the plasmids were transformed into CO164 (*pac-2*) and transformants tested on phenylacetate plates a similar phenomenon was seen except that only pBS1 gave rise to phenylacetate-positive colonies in this *recA*⁺ host.

It seems that either the whole or a part of the wild-type *pac-1* gene is present on both plasmids but is either not expressed or does not give a functional protein. In the *recA*⁺ strains repair of the defective gene can occur by recombination between the cloned DNA and the chromosome. Since the only cloned DNA common to both plasmids is that between 204 kb and 205 kb on the physical map of the region (Bouché, 1982) it seems that *pac-1* is located around 204–205 kb. Since *trg* has been located at 247 kb (Bouché *et al.*, 1982), the difference of 42 kb between *pac-1* and *trg* fits well with the map difference of 0.9 min estimated from the cotransduction studies, assuming there are 46 kb per min (Bouché *et al.*, 1982). The precise position of the *pac-2* mutation could not be established, but the apparent presence of the gene on pBS1 and its absence from pJPB13 is in accord with the gene order proposed.

Thus, at least two genes involved in phenylacetate catabolism are located in the region min 30.4 of the *E. coli* K12 linkage map. This region was felt to be devoid of essential genes (Henson *et al.*, 1984) and the suggestion that it may contain metabolically unusual or 'exotic' genes (Bachmann *et al.*, 1976; Bouché *et al.*, 1982) seems to be borne out by the experiments reported here.

We would like to thank B. J. Bachmann, P. L. Kuempel and G. L. Hazelbauer for providing bacterial strains and J. P. Bouché for supplying plasmids. D.C.N.J. was supported by an SERC postgraduate research studentship.

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