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Molecular Cloning of *pheR* in *Escherichia coli* K-12

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The regulator gene *pheR*, which in *Escherichia coli* controls the expression of *pheA*, the structural gene for chorismate mutase P-prephenate dehydratase, was cloned on to multicopy plasmids directly from the *E. coli* chromosome; this was achieved with the aid of the tetracycline resistance transposon, Tn*10*, that had been inserted very close to the *pheR* gene. Subsequently, *pheR* was subcloned on a 1.1-kilobase-pair fragment on the plasmid vector pBR322; its position on the plasmid was localized by the method of γδ-mediated transpositional inactivation. The *pheR* gene product was identified in maxicells and found to be a protein of subunit molecular weight 19,000, suggesting that the coding segment of the gene is about 500 nucleotide pairs long.

The expression of the structural genes for the enzymes of the aromatic amino acid biosynthetic pathway in *Escherichia coli* K-12 is controlled by the products of three regulator genes, *tyrR* (5), *trpR* (8), and *pheR* (12). The *trpR* product controls the expression of *aroH* and the *trp* operon; *tyrR* is involved in the regulation of expression of eight different transcription units concerned with the biosynthesis or transport of the aromatic amino acids (H. Camakaris and J. Pittard, *in* K. Herrmann and R. L. Somerville, ed., *Amino Acid Biosynthesis and Genetic Regulation*, in press). The *pheR* gene product is involved in the phenylalanine-mediated repression of *pheA*, the structural gene for chorismate mutase P-prephenate dehydratase (EC 5.4.99.5/4.2.1.51), the first enzyme in the terminal pathway of phenylalanine biosynthesis. *pheR* maps at min 93 on the *E. coli* chromosome (12).

The *tyrR* and *trpR* genes have been cloned previously (15, 27; Cornish et al., submitted for publication), and the nucleotide sequence of *trpR* has been determined. The *trpR* gene is 0.38 kilobase pair (kb) long and codes for a small protein of subunit molecular weight (M_r) 12,356 (14) or 10,250 (32). The *tyrR* gene, in contrast, codes for a large protein of M_r 63,000 (Cornish et al., submitted). The difference in the sizes of these two proteins is possibly related to the fact that the *trpR* gene product interacts with only a single effector molecule, L-tryptophan, whereas the *tyrR* product interacts either singly or in combination with each of the three aromatic amino acids. Furthermore, the *tyrR* product, in controlling the expression of eight different transcription units, may recognize a greater variety of operator sequences than does the *trpR* product (Camakaris and Pittard, in press). The expression of both *tyrR* and *trpR* has been shown to be autogenously regulated at the level of transcription (6, 14).

In this report, we describe the cloning of *pheR* on a multicopy plasmid directly from the *E. coli* chromosome and its subsequent localization by subcloning and by transpositional mapping of the cloned gene. The *pheR* gene product was identified in maxicells (29) as a protein of M_r 19,000, similar in size to the *trpR* gene product.

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains used were derivatives of *E. coli* K-12 and are listed in Table 1. The plasmids constructed were derived from the vectors pACYC177 (7) and pBR322 (4) and are described in Fig. 1. γδ insertion derivatives of pMU54 are described in Fig. 2.

Chemicals and media. Most chemicals were commercially obtained and not further purified. Barium prephenate was a gift from G. Hudson. [^{35}S]methionine was from Amersham Co.

Luria agar and Lennox broth were routinely used as nutrient media, and minimal media were prepared from the 56/2 buffer of Monod et al. (24). Cells were grown for enzyme assays in minimal medium supplemented with repressing concentrations of the aromatic end products, containing 10^{-3} M L-tyrosine, 10^{-3} M L-phenylalanine, 5×10^{-4} M L-tryptophan, 10^{-4} M shikimic acid, 5×10^{-6} M *p*-aminobenzoic acid, 5×10^{-6} M *p*-hydroxybenzoic acid, and 7×10^{-5} M 2,3-dihydroxybenzoic acid.

Antibiotics were used at the following concentrations in nutrient media: tetracycline, 15 μg/ml; ampicillin, 50 to 200 μg/ml; and kanamycin, 20 μg/ml.

Isolation of chromosomal DNA. Chromosomal DNA was prepared by the method of Marmur (22) from cells in the early exponential phase.

Recombinant DNA techniques. Restriction endonucleases were either purchased from commercial sources and used as recommended or purified as described previously (13). T4 DNA ligase was purchased from Boehringer Mannheim. Miniscreen prep-

TABLE 1. List of *E. coli* K-12 strains

Strain	Genotype[a]	Origin or reference
JP3290	F⁻ *purE trp his argG ilv leu metA* or *metB rpsL recA56 srl-1300*::Tn*10* Δ*lacU169* (λ p *pheA-lac*)	(12)
JP3299	JP3290 *pheR374*	(12)
JP3364	JP3299 Δ(*srl-1300*::Tn*10*) *58*	From JP3299, by method of Bochner et al. (3)
JP3368	JP3290/F117	(12)
JP3370	JP3299/F117	(12)
JP3151	F⁺ *purE trp his argG ilv leu metA* or *metB rpsL* Δ*lacU169* (λ p *pheA-lac*)	(12)
JP3301	JP3151 *pheR372 recA56* Δ(*srl-1300*::Tn*10*) *59*	By P1 *kc* transduction, followed by Tet[s] selection (3)
JP3305	JP3151 *zjd-351*::Tn*10*[b]	By P1 *kc* transduction
CSR603	*thr1 leuB6 proA2 recA1 argE3 thi-1 uvrA6 rpsL31 supE44 gyrA98*	(29)

[a] The nomenclature for genetic symbols follows that of Bachmann and Low (1), and the nomenclature for transpositional insertions follows that of Kleckner et al. (19). Allele numbers are indicated where they are known. Fermentation markers are not described.

[b] The *zjd-351*::Tn*10* insertion is linked 95% in P1 *kc* transductions to the *pheR* gene (12).

arations of plasmid DNA were obtained by the method of Birnboim and Doly (2), whereas large-scale preparations were made by running cleared lysates on a cesium chloride-ethidium bromide gradient (31). Transformations were performed by the method of Kushner (20). Agarose gel electrophoresis and visualization of DNA restriction fragments were by standard procedures (9).

Screening for *pheR*⁺ plasmids in transformations. The strain JP3364, which was routinely used as the recipient in transformation experiments, is a *pheR* strain that carries the *pheA-lac* fusion (11). Colonies of JP3364 were therefore strongly Lac⁺ on lactose indicator media. *pheR*⁺ transformants of JP3364, on the other hand, were only weakly Lac⁺ on these plates, because *lac* gene expression in these strains was repressed in the presence of functional *pheR* gene product. Colonies with *pheR*⁺ plasmids could, therefore, be readily distinguished from the parental strain on eosin methylene blue-lactose plates.

Genetic methods. P1 *kc* transduction and conjugation were performed by methods described previously (25, 26). Tetracycline-sensitive derivatives of strains carrying Tn*10* were selected by the method of Bochner et al. (3).

Maxicell preparations. Proteins encoded by the various plasmids constructed in this study were identified by the modified maxicell technique of Sancar et al. (30).

Enzyme assays. β-Galactosidase activity was assayed by the method of Miller (23) and is expressed in the units defined therein. Prephenate dehydratase was assayed by the method previously described (10) and is expressed in international units. Protein concentrations in cell extracts were determined by the method of Lowry et al. (21).

RESULTS

Primary cloning of *pheR* from the *E. coli* chromosome. Since no satisfactory selection technique exists for the *pheR*⁺ phenotype, the strategy adopted was to clone the neighboring Tet[r] locus from a strain in which the 9.3-kb tetracycline resistance transposon, Tn*10*, was located very near the *pheR* gene (12). Chromosomal DNA was prepared from JP3305 and subjected to a partial digestion with the restriction endonuclease *Pst*I. Tn*10* is not cleaved by *Pst*I (17); this meant that the restriction fragments of interest in the partial digest would be at least 10 kb in length. The DNA fragments were fractionated on a sucrose gradient, and fractions containing fragments greater than 10 kb were pooled; the DNA was then concentrated and ligated into the *Pst*I site of the plasmid vector pACYC177. The ligated DNA was transformed into the *pheR pheA-lac* fusion strain JP3364, and Tet[r] colonies were selected.

The Tet[r] colonies obtained were screened for kanamycin resistance and ampicillin sensitivity; they were also scored for the *pheR*⁺ phenotype on the basis of their fermentation reaction on lactose indicator media. All of the Tet[r] colonies tested were also Kan[r] Amp[s] *pheR*⁺, and the plasmid isolated from one of them, pMU50, was chosen for further mapping studies.

Subcloning of *pheR* from pMU50. pMU50 carries a 17-kb insert into the *Pst*I site of pACYC177. A preliminary restriction map of pMU50 was constructed as an aid to subsequent subcloning procedures and is shown in Fig. 1. The insert contains the 9.3-kb Tn*10* flanked by 2.4- and 5.3-kb segments of chromosomal DNA to its left and right, respectively.

In the subcloning experiments, eosin methylene blue-lactose plates containing the appropriate antibiotic(s) were used for selection after transformations in JP3364, and, as described above, the screening for *pheR*⁺ clones was considerably simplified. The plasmids construct-

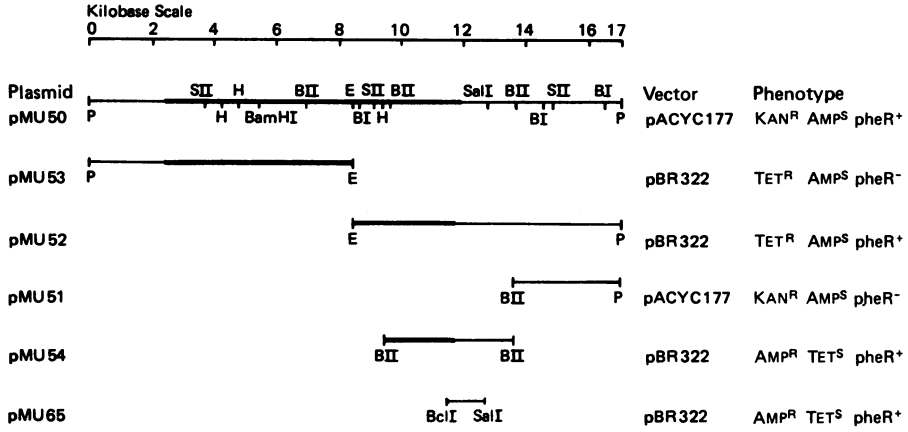

FIG. 1. Physical map of the insert DNA in the plasmid pMU50 and derivatives subcloned from it. The vector into which each of the inserts has been cloned and the corresponding phenotype are given alongside. The heavy line indicates Tn*10* DNA, and the light lines indicate chromosomal DNA. The restriction enzyme cleavage sites in Tn*10* were primarily obtained from the map constructed by Jorgensen et al. (17); the *Sst*II sites were mapped in the course of this study. Abbreviations used for some of the restriction endonucleases: P, *Pst*I; H, *Hin*dIII; E, *Eco*RI; BI, *Bgl*I; BII, *Bgl*II; and SII, *Sst*II.

ed in these experiments, and their phenotypes, are summarized in Fig. 1.

Endonuclease digestion of pMU50 with *Pst*I and *Eco*RI separates the insert DNA into two fragments, one bearing the left 2.4-kb chromosomal segment and the other bearing the right 5.3-kb portion of the chromosome; these were ligated into pBR322 and transformed into JP3364, and *pheR* was shown to reside in the latter fragment (pMU52) (Fig. 1). A subsequent experiment showed that the 4-kb *Bgl*II fragment of pMU54 carried the *pheR* gene, indicating that the gene is located in the 2-kb chromosomal region immediately to the right end of the Tn*10* insertion.

Transpositional mapping of *pheR* in pMU54. The conjugational mobilization of pBR322 and its derivatives by F from *recA* strains is accompanied by the random transposition of the insertion element γδ of F into the pBR322 molecule (16). This has been exploited as a means for the transpositional mapping of the cloned *uvrA*, *uvrB*, and *uvrC* genes (28, 30, 33); we employed this method in a similar manner for the mapping of *pheR*$^+$ on pMU54.

γδ insertions in pMU54 were obtained by mobilization of the plasmid from JP3301 into JP3299, and ampicillin-resistant colonies were selected on eosin methylene blue-lactose plates. Tetracycline was used for contraselection against the donor strain. pMU54 was mobilized at a frequency of 10^{-4} to 10^{-5} per donor cell; approximately 1% of the exconjugants obtained were strongly Lac$^+$, indicating that the plasmids in these strains had undergone insertional inactivation of the *pheR* gene.

The position and orientation of γδ insertions in the various *pheR* plasmids, and in two others that continued to be *pheR*$^+$, were determined by restriction endonuclease digestions of mini-screen plasmid preparations with *Eco*RI, *Hin*dIII, *Sal*I, *Pst*I, and *Bam*HI. A summary of the results is presented in Fig. 2. Five γδ inser-

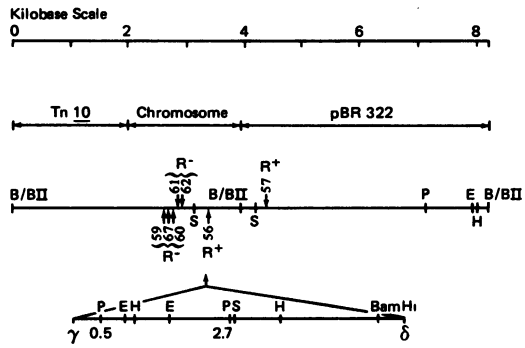

FIG. 2. Location and orientation of γδ insertions in pMU54, a *pheR*$^+$ plasmid carrying a 3.95-kb *Bgl*II insert into the *Bam*HI site of pBR322. Each arrow designates the position of one γδ insertion; the corresponding pMU plasmid number and phenotype with respect to *pheR* (R$^+$ or R$^-$) are marked alongside. Arrows below the line represent γδ insertions in the orientation shown, and those above the line represent insertions in the opposite orientation. The restriction endonuclease cleavage sites in γδ were obtained from Guyer (16); the *Pst*I cleavage sites were mapped in the course of this work. The portions corresponding to Tn*10*, pBR322, and chromosomal DNA are indicated. Abbreviations for the restriction enzyme cleavage sites marked: P, *Pst*I; H, *Hin*dIII; E, *Eco*RI; S, *Sal*I; and B/BII, *Bam*HI-*Bgl*II joined ends.

tions that inactivated *pheR* (in the plasmids pMU59, pMU60, pMU61, pMU62, and pMU67) were clustered in a 0.3-kb region situated 0.2 kb to the left of the *Sal*I site of the chromosome. The γδ insertions in pMU56 and pMU57, the two *pheR*$^+$ plasmids tested, were mapped to the right of the chromosomal *Sal*I site and in the pBR322 vector, respectively.

On the basis of these results, we attempted a further subcloning to determine whether the segment of chromosome between the right end of Tn*10* and the *Sal*I site carried the intact *pheR* gene. The restriction enzyme *Bcl*I is known to cut Tn*10* at a site 70 nucleotides from its end (18) and to generate a 5'-cohesive end identical to that generated by *Bam*HI cleavage. The 1.1-kb *Bcl*I-*Sal*I fragment of pMU52 (prepared from a *dam* mutant strain) was ligated into the *Bam*HI-*Sal*I site of pBR322 to generate pMU65 (Fig. 1); pMU65 was shown to carry the *pheR* gene.

Identification of the *pheR* gene product in maxicells. Maxicell preparations of derivatives of CSR603 carrying some of the plasmids above were made as described by Sancar et al. (30), and plasmid-encoded proteins labeled with [^{35}S]methionine were visualized by fluorography after separation on a 15% polyacrylamide gel with 0.1% sodium dodecyl sulfate (Fig. 3). A

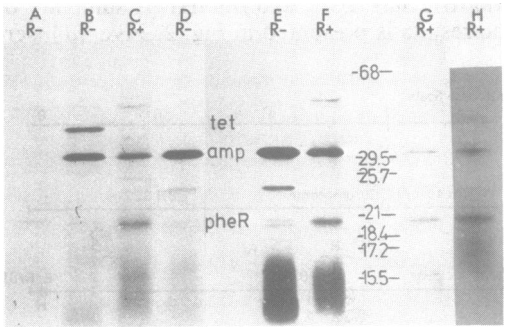

FIG. 3. Fluorograph of [^{35}S]methionine-labeled proteins from maxicell preparations run on 15% sodium dodecyl sulfate-polyacrylamide. Direction of electrophoresis was from above downward. Lane A, CSR603. Lanes B through H, derivatives of CSR603 carrying, respectively, the following plasmids: pBR322, pMU56, pMU60, pMU61, pMU57, pMU54, and pMU65. pMU56 and pMU57 are γδ insertion derivatives of pMU54 that are still *pheR*$^+$; pMU60 and pMU61 are derivatives in which γδ insertions have inactivated *pheR*. The phenotype with respect to *pheR* (R$^+$ or R$^-$) of each plasmid is indicated above the corresponding lane. Lanes A through G were obtained from fluorographs of a single gel. The positions of marker protein bands run on the same gel, with their corresponding molecular masses in kilodaltons, are shown. The 19K *pheR* band and the Amp and Tet protein bands of pBR322 (29) are also marked. The fluorograph of lane H was obtained from a separate gel.

comparison of the proteins encoded by pMU54 and four of its γδ insertion derivatives (the structures of which are described in Fig. 2) indicated that the M_r 19,000 (19K) protein is the most likely candidate for being the *pheR* gene product. Thus, pMU56 and pMU57, both of which carry γδ but are still *pheR*$^+$, and pMU54 itself produced prominent bands corresponding to this 19K protein (Fig. 3, lanes C, F, and G). On the other hand, pMU60 and pMU61 (Fig. 3, lanes D and E), plasmids with γδ insertions in opposite orientations that have inactivated *pheR*, showed only faint bands at the same position. The faint 19K band observed in the latter tracks was seen with CSR603 and CSR603(pBR322) as well (Fig. 3, lanes A and B) and therefore probably represents low-level synthesis of a protein of that molecular weight from a chromosomal gene in CSR603. Densitometric analysis showed that the ratio of intensity of the 19K band to that of the 28 to 31K Amp protein band was 0.5 on the *pheR*$^+$ tracks and less than 0.02 on those tracks not carrying the intact *pheR* gene on the plasmids.

The plasmid pMU65 was the smallest that expressed the *pheR*$^+$ phenotype, with a 1.1-kb insert that had inactivated the Tet^r gene on the pBR322 vector. A maxicell preparation of the CSR603 strain with pMU65 demonstrated two proteins encoded by this plasmid; one corresponds to the Amp protein from the vector, and the other is the 19K band, the putative *pheR* gene product (Fig. 3, lane H).

Effect of *pheR*$^+$ plasmids on expression from *pheA*$_p$. The results of β-galactosidase and prephenate dehydratase assays in *pheA-lac* fusion strains with several *pheR* plasmids are presented in Table 2. F117 (an F-prime that carries the 93-min region of the chromosome), pMU50, and pMU65 all conferred a *pheR*$^+$ phenotype in a *pheR* strain. The degree of repression of both β-galactosidase and prephenate dehydratase showed a progressive increase associated with the expected order of increasing *pheR*$^+$ copy

TABLE 2. Enzyme specific activities in *pheA-lac* fusion strains with different *pheR*$^+$ plasmids[a]

Strain	β-Galactosidase (U)	Prephenate dehydratase (mU/mg)
JP3364 (*pheR374*)	1,500	75
JP3290 (*pheR*$^+$)	155	5.0
JP3370 (*pheR374*/F117)[b]	90	3.9
JP3368 (*pheR*$^+$/F117)[b]	75	2.8
JP3364(pMU50)	65	2.5
JP3364(pMU65)	35	1.6

[a] All strains were grown in repressing concentrations of the aromatic end products.

[b] The F-prime F117 carries the *pheR*$^+$ gene (12).

number in the strains; however, this increase was far from coordinate. For example, pMU65 is a small plasmid derived from pBR322 that is expected to exist at about 15 to 20 copies per genome equivalent in the cell; yet the magnitude of repression of *pheA* expression in JP3364(pMU65) was only three- to fourfold higher than that observed in the haploid pheR$^+$ strain, JP3290. Camakaris and Pittard (manuscript in preparation) have made similar observations with regard to the control of *aroF* and *aroG* by the *tyrR* gene and have interpreted them as being consistent with the evidence from operon fusion studies for the autoregulation of *tyrR* (6). Our results suggest that the expression of *pheR* may likewise be subject to autogenous control.

DISCUSSION

We have described the cloning of *pheR* directly from the *E. coli* K-12 chromosome by employing a selection for the transposon Tn*10* inserted very close to the gene. The cloned gene was subsequently mapped by the method of γδ-mediated insertional inactivation and has been subcloned on a 1.1-kb *BclI-SalI* fragment into the pBR322 vector.

The maxicell studies identified the *pheR* gene product as a protein of M_r 19,000. Assuming an average molecular weight of 120 for an amino acid residue, this would suggest that the *pheR* polypeptide is about 160 amino acids long, and that the coding segment of the gene is about 0.5 kb in size. The size of *pheR* corresponds more closely with *trpR* than it does with *tyrR*; this may again reflect the fact that the *pheR* product, like the *trpR* product, is a simple regulatory protein, whereas the regulatory role of the *tyrR* product in the bacterial cell is considerably more complex.

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