

## Defective Regulation of the Phenylalanine Biosynthetic Operon in Mutants of the Phenylalanyl-tRNA Synthetase Operon

SIMON A. BORG-OLIVIER, DAVID TARLINTON,<sup>†</sup> AND KEITH D. BROWN<sup>‡\*</sup>

*School of Biological Sciences, University of Sydney, Sydney, New South Wales 2006, Australia*

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Among mutants of *Escherichia coli* resistant to *p*-fluorophenylalanine (PFP) were some with constitutive expression of the phenylalanine biosynthetic operon (the *pheA* operon). This operon is repressed in the wild type by phenylalanine. The mutation in three of these mutants mapped in the *aroH-aroD* region of the *E. coli* chromosome at 37 min. A plasmid bearing wild-type DNA from this region restored *p*-fluorophenylalanine sensitivity and wild-type repression of the *pheA* operon. Analysis of subclones of this plasmid and comparison of its restriction map with published maps indicated that the mutations affecting regulation of the *pheA* operon lie in the structural genes for phenylalanyl-tRNA synthetase, *pheST*, probably in *pheS*. Thus, the *pheST* operon has a role in the regulation of phenylalanine biosynthesis, the most likely being that wild-type phenylalanyl-tRNA synthetase maintains a sufficient intracellular concentration of Phe-tRNA<sup>Phe</sup> for attenuation of the *pheA* operon in the presence of phenylalanine. A revised gene order for the 37-min region of the chromosome is reported. Read clockwise, the order is *aroD*, *aroH*, *pheT*, and *pheS*.

The phenylalanine operon of *Escherichia coli*, located at 56 min on the genetic map, has a single gene, *pheA*, which encodes the bifunctional enzyme chorismate mutase-prephenate dehydratase (EC 4.2.1.51, EC 5.4.99.5) (1). This protein catalyzes conversions of chorismic acid to prephenic acid and prephenic acid to phenylpyruvic acid, the immediate precursor of phenylalanine (9).

Regulation of the *pheA* operon is not fully understood. Gollub et al. (15) reported the *pheR* gene of *Salmonella typhimurium*, which maps in the 95- to 100-min region of the chromosome of that species, as the repressor gene for *pheA*. An *E. coli* gene which restores the wild phenotype in *S. typhimurium pheR* mutants is carried by the F-prime plasmid F116, derived from the comparable region of the *E. coli* chromosome, 59 to 65 min (15). Gowrishankar and Pittard (16) propose that this gene is nonfunctional in *E. coli* and that a second functional copy, which they named *pheR*, lies at 93 min on the *E. coli* chromosome. They propose that this latter gene encodes the repressor protein of the *pheA* operon. There is no evidence reported, however, that this protein is a repressor in the sense that it binds to an operator locus in the *pheA* operon, thus blocking transcription.

A *cis*-acting *pheA*-linked regulatory region (designated *pheAo*) has been described previously (16, 17). It is proposed that this is an operator, regulated by a phenylalanine-activated repressor encoded by *pheR*. Evidence has not yet been reported, however, that distinguishes between this possibility and the possibility that the *pheR* and *pheAo* loci are elements of the phenylalanine-mediated attenuation system for the *pheA* operon described by Zurawski et al. (37).

While it is our view that from the evidence available, no conclusion can be drawn about the existence of an operator-repressor system of regulation for the *pheA* operon, there is evidence from *in vitro* transcription and nucleotide sequence information (37) that the *phe* operon is regulated by an

attenuation mechanism analogous to that of the tryptophan (20, 35) and histidine (18, 19) operons.

For both the tryptophan and histidine operons, it has been shown that the cognate aminoacyl-tRNA synthetase is involved in their regulation by attenuation (18, 25, 34-36).

In this paper, we report the isolation and characterization of mutants with elevated, constitutive expression of *pheA*. When plasmids containing the wild-type phenylalanyl-tRNA synthetase operon, *pheST*, were introduced into these strains, wild-type control of *pheA* was restored. Thus, we propose that *pheST* regulates the phenylalanine biosynthetic operon, adding further support for an attenuation mechanism for this operon.

### MATERIALS AND METHODS

**Strains.** Strain KB1360 is F<sup>-</sup> *aroD proA argE his4 thi lacZ gal xyl mtl tsx shiA3* (5). Strain KB9195 was derived from KB1360 by mutation and P1 *kc* transduction; it is KB1360 *aroD*<sup>+</sup> *gal*<sup>+</sup> *aroF aroG aroH*. Strains KB8021, KB8026, and KB8090 were derived from KB1360; they are KB1360 *phe(ST)1*, *phe(ST)2*, and *phe(ST)3*, respectively. Strain NP37 (11), obtained from B. Bachmann, is *pheS5 relA1 fhuA22 ompF627 pit-10*.

**Isolation of mutants resistant to PFP.** Colonies of KB1360 were grown in L broth (22) to 5 × 10<sup>8</sup> cells per ml. Washed cells (10<sup>8</sup>) were spread on minimal agar containing non-aromatic growth factors plus shikimic acid (100 µg/ml) and *p*-fluoro-DL-phenylalanine (PFP) (6 mM). After 48 h at 37°C, approximately 50 PFP-resistant colonies appeared per plate.

**Media.** Basal medium was that of Vogel and Bonner (33). It was supplemented when necessary with shikimic acid and amino acids at 100 µg/ml each and thiamine at 10 µg/ml. Complete medium was basal medium with L-tyrosine, L-phenylalanine, and L-tryptophan at 10<sup>-4</sup> M each, L-proline, L-arginine, and L-histidine at 100 µg/ml each, thiamine at 10 µg/ml, shikimic acid at 1 µg/ml, and *p*-aminobenzoic acid, 2,3-dihydroxybenzoic acid, and *p*-hydroxybenzoic acid at 10<sup>-6</sup> M each. Limiting phenylalanine was 8 × 10<sup>-6</sup> M.

**Transformations.** The technique used was that of Cohen et al. (7), except for transformation of the temperature-sensi-

\* Corresponding author.

<sup>†</sup> Present address: Department of Genetics, Stanford University Medical Center, Stanford, CA 94305.

<sup>‡</sup> Present address: DNAX Research Institute, Palo Alto, CA 94304-1104.

TABLE 1. Specific activities of prephenate dehydratase in cell extracts of mutants and merodiploids<sup>a</sup>

Strain and plasmid	Sp act (mU/mg of protein)	
	Phe-rep <sup>b</sup>	Phe-lim <sup>c</sup>
KB1360	6	59
KB8026	79	147
KB8026(F148)	6	NT <sup>d</sup>
KB8026(pAROH13)	12	73
KB8026(pB07)	19	71
KB8026(pB08)	85	NT
KB8026(pB078)	13	NT
KB8026(pB079)	8	60

<sup>a</sup> Cell extracts were prepared and specific activities of prephenate dehydratase were estimated essentially as described previously (6).

<sup>b</sup> Cells were grown in complete aromatic medium and harvested in the exponential phase at  $5 \times 10^8$  cells per ml.

<sup>c</sup> Cells were grown in complete aromatic medium but with limiting phenylalanine ( $8 \times 10^{-6}$  M) and harvested after 3 h in the stationary phase ( $5 \times 10^8$  cells per ml).

<sup>d</sup> NT, Not tested.

tive strain NP37, for which heat shock and phenotypic expression were carried out at 30°C.

## RESULTS

**Isolation of mutants derepressed for chorismate mutase-prephenate dehydratase.** PFP inhibits the growth of *E. coli* (26). Mutants resistant to PFP have been isolated with alterations in phenylalanyl-tRNA synthetase (13), general aromatic amino acid transport (4), feedback inhibition of the first enzyme of the aromatic pathway (3-deoxy-D-arabinoheptulosonic acid 7-phosphate [DAHP] synthetase) (15), and regulation of the phenylalanine operon (15, 17). We searched for mutants whose phenylalanine operon was constitutively derepressed by screening PFP-resistant mutants for high, constitutive activity of prephenate dehydratase. KB1360 was the parent in our mutant hunt, as it is *aroD* and is blocked in the third step (dehydroshikimate synthesis) of the aromatic pathway. KB1360 grows well on the intermediate shikimic acid, since it has the *shiA3* allele for efficient shikimate uptake (5). We reasoned that in this strain, only regulatory mutations which cause overproduction of phenylalanine by interfering with regulation of the terminal phenylalanine pathway would be detected. Mutations affecting regulation of the aromatic pathway before *aroD*, e.g., at DAHP synthetase, would not be expressed.

We measured prephenate dehydratase in 50 independent PFP-resistant mutants of KB1360 grown under conditions of *pheA* repression (complete medium). Ten had high specific activities of prephenate dehydratase relative to the parent. The mutations which conferred PFP resistance and high, constitutive prephenate dehydratase synthesis in three of these mutants, KB8021, KB8026, and KB8090, mapped in the *aroD-aroH* region of the chromosome and are the subject of this paper. The specific activities of prephenate dehydratase in KB8021, KB8026, and KB8090 were, on average, 13.7 times higher than in KB1360 (Table 1; only KB8026 and KB1360 shown). When KB1360 was grown on limiting phenylalanine, there was ninefold derepression of prephenate dehydratase relative to cells grown on complete medium (Table 1). When the three *phe* regulatory mutants were grown under these conditions, there was approximately a twofold increase in prephenate dehydratase activity relative to complete medium-grown cells. Moreover, the derepressed activities were twice that in the parent KB1360

(Table 1). Thus, the three regulatory mutants with mutations of the *pheA* operon could be derepressed twofold by phenylalanine limitation but could not be repressed by phenylalanine below the level of the derepressed wild type.

**Mapping the *phe* regulatory locus.** When the F-prime plasmid F148 was introduced by conjugation into mutants KB8026, KB8021, and KB8090, the merodiploids were rendered PFP sensitive and prephenate dehydratase was repressed by phenylalanine (Table 1). Thus, the wild-type phenotype was restored by F148, which carries the 37- to 39- and 42- to 44-min regions of the chromosome (21).

P1 *kc* transduction analysis of the 37-min region indicated that the wild-type *phe* regulatory locus was transduced with *aroD*<sup>+</sup> into the three regulatory mutants at high frequency (69%) (Table 2; only KB8026 shown). To determine the side of *aroD* on which the *phe* regulatory locus lies, the inheritance of a third marker, *aroH*, was examined. The regulatory locus and *aroH* were transferred together as unselected markers at high frequency (91%) when *aroD*<sup>+</sup> transductants were selected (Table 2). Therefore, the regulatory locus and *aroH* lie close together on the same side of *aroD*. *aroH* was transduced with *aroD* at a frequency of 72%, indicating that *aroH* is closer to *aroD* than is the regulatory locus. No published information orients *aroH* with respect to *aroD*. Its current reported position anticlockwise to *aroD* at 37 min on the *E. coli* map (1) is arbitrary. If *aroH* is repositioned on the opposite side of *aroD*, then the position of our *phe* regulatory locus corresponds to the reported position of the phenylalanyl-tRNA synthetase operon (*pheST*) at 38 min (8). We cloned the wild-type *phe* regulatory locus on plasmid pAROH13 and found it to be *pheST* (see below). Complementation of KB9195 and KB8026 showed that this plasmid contains *aroH* but not *aroD*. Therefore, the revised gene order in the 37-min region, reading clockwise, is *aroD*, *aroH*, *pheT*, and *pheS*.

**Cloning the *phe* regulatory locus and identification as *pheST*.** Plasmid pAROH13 (38) contains a 22.6-kilobase (kb) *EcoRI* fragment from F148 with the *aroH* region, inserted in vector pCR1 (10). When transformed with pAROH13, the three regulatory mutants were rendered PFP sensitive and wild-type repression of prephenate dehydratase was restored (Table 1). pAROH13 was mapped with several restriction enzymes (Fig. 1). The restriction map of the *EcoRI* insert in pAROH13 resembles that of the 22-kb insert in plasmid pID1, isolated by Elseviers et al. (12), while the restriction sites within the 9.8-kb *EcoRI-HindIII* region of

TABLE 2. P1 *kc* transduction of the 37- to 38-min region of the *E. coli* chromosome<sup>a</sup>

Genotype of <i>aroD</i> <sup>+</sup> transductants <sup>b</sup>			% Frequency
<i>aroD</i>	<i>aroH</i> <sup>c</sup>	<i>phe</i> regulatory locus <sup>d</sup>	
+	+	+	6
+	+	—	22
+	—	+	63
+	—	—	9

<sup>a</sup> The P1 *kc* transduction method was described previously (3).

<sup>b</sup> Donor strain, KB9195; relevant genotype, *aroD*<sup>+</sup>, *aroH*, wild-type *phe* regulatory locus. Recipient strain, KB8026; relevant genotype, *aroD*, *aroH*<sup>+</sup>, mutant *phe* regulatory locus. Selected marker, *aroD*<sup>+</sup>. Number of transductants examined, 400.

<sup>c</sup> *aroH* mutant marker (—) was scored by the inability of *aroD*<sup>+</sup> transductants of KB8026 to grow on minimal medium plus phenylalanine and tyrosine (both 1 mM) following receipt of this unselected marker.

<sup>d</sup> The *phe* regulatory locus was scored by sensitivity (+) or resistance (—) to PFP.

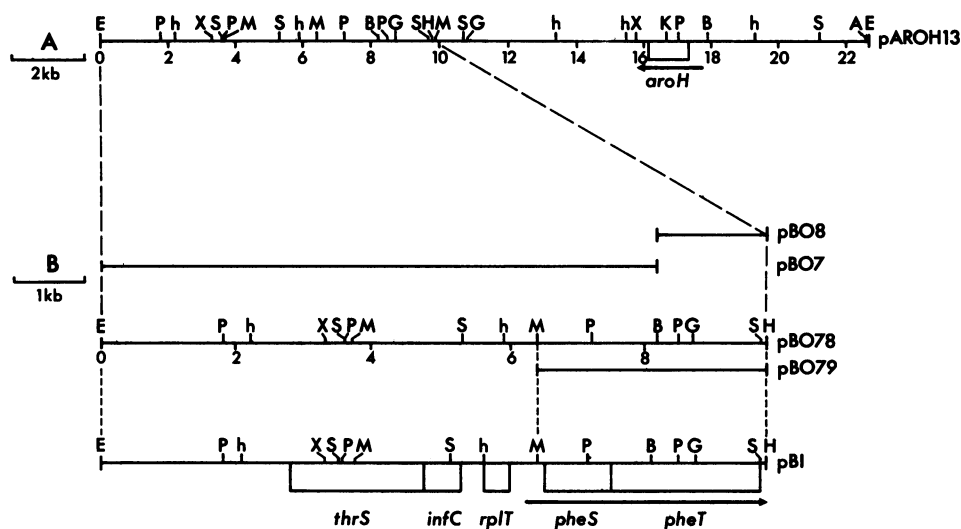


FIG. 1. Restriction maps of the 37-min region. (A) pAROH13 insert. (B) Comparison between pAROH13 DNA and DNA cloned from this region of the chromosome into plasmid pB1 by Plumbridge et al. (29). Symbols: A, *Ava*I; B, *Bam*HI; G, *Bgl*II; E, *Eco*RI; H, *Hind*III; h, *Hpa*I; K, *Kpn*I; P, *Pst*I; M, *Sma*I; S, *Sst*II; X, *Xho*I. Arrows indicate transcription of *pheST* and *aroH*. There are four *Hpa*I sites between the *Hpa*I sites at 2.2 and 5.6 kb in pB078 and pB1. The order of these sites in pB078 was not determined, but fragments obtained by *Hpa*I digestion of this region are similar in size to *Hpa*I fragments from the same region of pB1 (28). Restrictions and gel electrophoresis were performed as described by Maniatis et al. (23).

the pAROH13 insert are similar to those of the 9.8-kb insert in plasmid pB1 isolated by Plumbridge et al. (29) (Fig. 1). Both pB1 and pID1 carry the *pheST* operon (12, 29). pAROH13 complemented the temperature-sensitive *pheS* mutation in strain NP37 (11). When cured of the plasmid, its temperature-sensitive phenotype returned. The *pheST* genes are 6 kb distant from *aroH*. The position of *aroH* in pAROH13 was determined by complementation of the *aroH* mutation in KB9195 by subclones of pAROH13 (data not shown) and from the previously reported nucleotide sequence (38).

A subclone of pAROH13, pB07 (Fig. 1 and 2), made by inserting the 10.1-kb *Sal*I-*Bam*HI fragment into pBR322, partially complemented the regulatory mutations in strains KB8021, KB8026, and KB8090 (as determined by repressibility of prephenate dehydratase [Table 1]). This plasmid contains 8.2 kb of the *E. coli* chromosome. Restriction map comparison between pB07 and the similar region in pB1 indicates that it carries the genes *thrS*, *infC*, *rplT*, and *pheS* and approximately the first third of *pheT*. That pB07 contains the *pheS* gene was confirmed by its ability to complement the temperature-sensitive *pheS* mutation in NP37. pB08, another subclone of pAROH13 which contains the distal portion of *pheT*, had no effect on the levels of prephenate dehydratase in the *phe* regulatory mutants (Table 1; Fig. 1 and 2).

To examine the effect of the entire *pheST* operon, pB07 and pB08 were cut with *Bam*HI, ligated, and then transformed into KB8026. The fusion of pB07 and pB08 to reconstruct the *pheST* operon results in reformation of an intact tetracycline resistance gene from the two pBR322 vector fragments of the parents. Hence, cells bearing such a plasmid, designated pB078 (Fig. 1 and 2), could be selected for by resistance to tetracycline. Merodiploids of KB8026, KB8021, and KB8090 containing pB078 were PFP sensitive and had Phe-repressible prephenate dehydratase (Table 1).

To test whether our regulatory mutations occur in the *pheST* operon, we constructed plasmid pB079, which con-

tains no chromosomal DNA other than structural genes *pheST*. pB079 was constructed by ligating the 7.4-kb *Sma*I-*Sal*I fragment of pB078 with the 0.5-kb *Eco*RV-*Sal*I fragment of pBR322 (Fig. 2). Restriction mapping (28, 29) and DNA sequence analysis (14, 24) indicate that the *pheST* structural genes are contained within the 3.4-kb *Sma*I-*Hind*III region of pB079 (Fig. 1). pB078 and pB079 were shown to contain *pheS* by complementation of *pheS* in NP37. When the three *phe* regulatory mutants were transformed with pB079, the full wild-type phenotype was restored (Table 1). Thus we conclude that the regulatory mutations in KB8026, KB8021, and KB8090 lie in the *pheST* operon.

## DISCUSSION

We isolated PFP-resistant mutants with altered regulation of the phenylalanine operon. Complementation of these mutants by wild-type phenylalanyl-tRNA synthetase (*pheS*) genes restored wild-type repression of *pheA*. This indicates that wild-type phenylalanyl-tRNA synthetase is required for normal regulation of the phenylalanine operon.

Partial restoration of *phe* regulation in these phenylalanyl-tRNA synthetase mutants by pB07, which contains all of *pheS* but only part of *pheT*, suggests that the mutated gene in the three regulatory mutants is *pheS* (Fig. 2; Table 1). The incompleteness of restoration of wild-type *phe* regulation in pB07 merodiploids may be because *pheS* and *pheT* are cotranscribed on the chromosome (27) and hence are translated on the same mRNA. The mutant  $\alpha$  subunit (the chromosomal *pheS* product) may be better able (owing to proximity) to compete for binding with the normal  $\beta$  subunit (the *pheT* product) than the wild-type  $\alpha$  subunit, whose synthesis is directed by pB07. Hence, *pheST* regulatory mutants bearing pB07 would have a mixed population of phenylalanyl-tRNA synthetase molecules characteristic of wild-type and mutant genes. Alternative explanations are that (i) our mutants are *pheS* with a polar effect on *pheT* or that (ii) they are double *pheST* mutants.

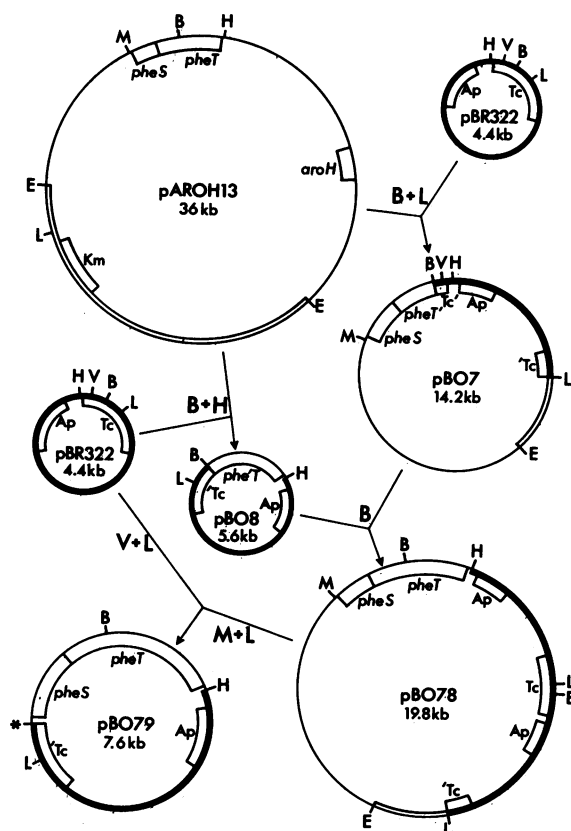


FIG. 2. Cloning strategy used to isolate structural genes for phenylalanyl-tRNA synthetase. Symbols: L, *SalI*; V, *EcoRV*; \*, site of blunt-end ligation of *EcoRV*; and *SmaI*-cleaved DNA; other restriction enzyme symbols are defined in Fig. 1; Ap, Km, and Tc denote genes for resistance to ampicillin, kanamycin, and tetracycline, respectively; other gene symbols are those of Bachmann (1); restriction enzyme symbols adjacent to arrows indicate the enzymes used to cleave parent DNA prior to ligation. Vector DNA is indicated by a thick line for pBR322 (2) and by a double line for pCR1 (10). Ligations were carried out as described by Maniatis et al. (23).

pB079 lacks the *pheST* promoter (Fig. 2), yet it restores *pheS* function in NP37 and *pheST* function in the *phe* regulatory mutants. We presume that expression of *pheST* occurs in the absence of the *pheST* promoter either because of promoter activity in vector DNA 5' to *pheST* (32) or because of the gene dosage effect due to the high copy number of pB079.

The most likely role of *pheST* in *pheA* regulation is that wild-type phenylalanyl-tRNA synthetase function is necessary to maintain sufficient intracellular Phe-tRNA<sup>Phe</sup> for attenuation of the *pheA* operon. We postulate that in our *pheST* mutants, insufficient charging occurs even when phenylalanine concentrations are high. Thus, attenuation is relieved, allowing readthrough transcription of *pheA* and constitutive synthesis of chorismate mutase prephenate dehydratase. Support for this hypothesis comes from studies of the *trp* operon (25, 36), for which it has been shown that the concentration of Trp-tRNA<sup>Trp</sup> mediates attenuation. Mutations in *trpS*, the structural gene for tryptophanyl-tRNA synthetase, decrease transcription termination at the *trp* attenuator and derepress the operon. The *trpS* mutants produce an altered tryptophanyl-tRNA synthetase with re-

duced affinity for tryptophan. An analogous effect of *hisS* mutants on the *his* operon has also been described (18, 34). Our finding that phenylalanyl-tRNA synthetase is essential for wild-type regulation of the *pheA* operon supports the attenuation model for regulation of amino acid biosynthetic operons (19, 20, 35).

Phenylalanine starvation leads to higher *pheA* expression in our *pheST* mutants than in wild-type cells (Table 1). This may be explained if under conditions of Phe starvation in the wild-type strain (KB8526), there remains a low concentration of Phe-tRNA<sup>Phe</sup> that is able to cause some attenuation. In *pheST* mutants, the residual level of Phe-tRNA<sup>Phe</sup> may be further reduced in phenylalanine-starved cells owing to defective phenylalanyl-tRNA synthetase. Hence, the starved cells show greater transcriptional readthrough of *pheA*.

It has recently been shown that the *pheST* operon is regulated by an attenuation mechanism analogous to the attenuation mechanism postulated for the *pheA* operon (14, 31). It is likely that our *pheST* mutants are defective in this regulation as well as in *pheA* regulation, causing depression of the *pheST* operon itself and hence synthesizing more less-active phenylalanyl tRNA synthetase. This possibility, however, has not been investigated.

An alternative explanation for the derepression of *pheA* in our *pheST* mutants is that phenylalanyl-tRNA synthetase is a repressor activated by phenylalanine to bind to a hypothetical operator locus of the *pheA* operon to block *pheA* transcription. An analogy occurs in the *alaS* operon (30), in which the operon product (alanyl-tRNA synthetase), in the presence of alanine, represses transcription of its own gene by binding specifically to a palindromic sequence centered at the transcription start site.

While our rationale for isolation of *phe* regulatory mutants by selecting for PFP resistance did yield such mutants, it does not follow that the overproduction of Phe causes the resistance. Possibly, the alteration of phenylalanyl-tRNA synthetase per se causes resistance, since thereby PFP may not be efficiently activated to give rise to nonfunctional proteins (a major cause of PFP toxicity [26]). Thus our mutants could have altered charging activities for both Phe and PFP. Fangman and Neidhardt (13) indeed showed that their PFP-resistant mutants had a reduced ability to activate PFP.

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