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A Regulatory Gene of Phenylalanine Biosynthesis (*pheR*) in *Salmonella typhimurium*

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4-Fluorophenylalanine-resistant mutants of *Salmonella typhimurium* were isolated in which synthesis of chorismate mutase P-prephenate dehydratase (specified by *pheA*) was highly elevated. Transduction analysis showed that the mutation affecting *pheA* activity was not linked to *pheA*, and conjugation and merodiploid analysis indicated that it was in the 95- to 100-min region of the *Salmonella* chromosome. Evidence is presented for the hypothesis that the mutation responsible for constitutivity of chorismate mutase P-prephenate dehydratase occurred in *pheR*, a gene specifying a cytoplasmic product that affected *pheA*. *pheR* mutants were found to carry a second mutation, *tyrO*. The *tyrO* mutation acts *cis* to cause increased levels of the tyrosine biosynthetic enzymes 3-deoxy-D-arabinoheptulosonate 7-phosphate synthetase (tyr) and prephenate dehydrogenase, but it has no effect on regulation of *pheA*.

Several mechanisms which regulate biosynthesis of tyrosine and phenylalanine have been elucidated by the study of mutants selected for resistance to aromatic amino acid analogues. In *Salmonella*, *tyrO* (12), and *tyrR* (E. G. Gollub and D. B. Sprinson, Fed. Proc., p. 491, 1972; manuscript submitted for publication), mutants were isolated by their resistance to 4-fluorophenylalanine. In *Escherichia coli*, *aroK* (22; analogous to *tyrO* in *Salmonella*) and *tyrR* (2, 18, 32), mutants have been selected by their resistance to 4-aminophenylalanine or 4-fluorophenylalanine. *pheO* mutants resistant to 2- and 4-fluorophenylalanine have also been described in *E. coli* (19). These appear to carry operator constitutive mutations of *pheA* operon in which only chorismate mutase P-prephenate dehydratase was affected. Such mutants have not as yet been found in *Salmonella*. The present report describes the isolation of 4-fluorophenylalanine-resistant mutants of *Salmonella* which have highly derepressed levels of chorismate mutase P-prephenate dehydratase. Tyrosine inhibitable 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthetase (EC 4.1.2.15) and prephenate dehydrogenase (EC 1.3.1.a) were also derepressed (Fig. 1). Genetic and biochemical studies indicated that two separate mutations were present: one was responsible for constitutivity of the tyrosine enzymes, and the other was responsible for derepression of chorismate mutase P-prephenate dehydratase. The mutation affecting the latter enzyme is not a *pheA* operator mutation since it is not linked to *pheA*. Evidence is presented suggesting that this mutation has resulted in alteration of a diffusible product specified by *pheR*. Apparently, only regulation of *pheA* is affected; regulation of *aroG*, the structural gene for DAHP synthesis (phe), is not affected. *pheR* is located in the 95- to 100-min region of the *Salmonella* chromosome.

MATERIALS AND METHODS

Materials. DL-4-Fluorophenylalanine and 4-hydroxyphenylpyruvic acid were purchased from commercial sources. Monocyclohexylammonium enolpyruvate-P was prepared by the method of Clark and Kirby (4); erythrose-4-P was prepared by the method of Ballou and MacDonald (1); barium prephenate was prepared by the method of Dayan and Sprinson (8); and chorismic acid was prepared by the method of Gibson (11).

Media. The minimal medium used was previously described (14). Supplements were added as indicated in tables. Difco nutrient broth and nutrient agar served as complete media for preparation of phage lysates and for transductions. Penassay Broth (Difco) was used to grow donors and recipients for conjugational crosses and episome transfers.

Bacterial strains. *Salmonella* strains were derivatives of *Salmonella typhimurium* LT-2. Hfr strains (with the exception of SC19) and strain *serA13* were kindly supplied by K. E. Sanderson: strain SC19 was

supplied by J. S. Gots; strains *tyrA3*; *pheA20*, and *aroD80* were supplied by Y. Nishioka; and *E. coli* K-12 episome carrying strains were supplied by B. Low. Other strains were isolated or prepared in this laboratory. The strains used and their properties are listed in Table 1. Map positions of relevant loci and direction of chromosome transfer of Hfr strains are shown in Fig. 2.

Isolation of mutant strains. Minimal agar plates containing 8 mM DL-4-fluorophenylalanine were inoculated with 10^8 cells of an overnight culture of wild-type *S. typhimurium* strain LT-2 and were incubated for 2 days at 37 C. Several colonies which were surrounded by a zone of background feeding appeared. These were purified on 8 mM fluorophenylalanine and tested for excretion of phenylalanine and tyrosine by spotting the test colony on the surface of minimal and analogue-containing agar plates seeded with tyrosine and phenylalanine auxotrophs.

Transduction. Standard procedures were followed for preparation of donor phage lysates and their assay (16). Transductions were performed with the nonlysogenizing mutant phage, P22-L4 (30), by spreading 0.1 ml of an overnight broth culture of cells and 0.1 ml of a nutrient broth suspension of phage (10^{10}/ml) directly onto the surface of minimal agar plates. Replica plating was carried out as previously described (12).

Conjugation. Crosses were performed by plate mating, and by the membrane filter (Millipore Corp.) technique described by Sanderson (28). For plate mating, donors and recipients were grown to log phase in Penassay Broth. Donor strains were used directly or diluted 1 to 10, and 0.1 ml was spread together with 0.1 ml of recipient cells on the surface of appropriately supplemented agar plates which were incubated at 37 C (usually for 36–48 h). Recombinants were purified at least twice by single-colony isolation on selective media.

Preparation of extracts. Organisms were grown with vigorous shaking at 37 C to late log phase (optical density at 660 nm = 1.0) in 500-ml flasks containing 100 ml of minimal media. Cells were chilled, harvested by centrifugation at 5 C, washed with 0.01 M phosphate buffer (pH 7), and suspended in 2 ml of buffer. Extracts were prepared as described previously (14) and used for enzyme assays.

Assay procedures. Total DAHP-synthetase activity was assayed as described previously (15), except that 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate buffer (pH 7.6) was substituted for tris(hydroxymethyl)aminomethane maleate (9a). Tyrosine and phenylalanine-inhibited isoenzymes were estimated by assaying in the presence of 0.5 mM tyrosine and phenylalanine, respectively, and by calculating decreases in specific activity resulting from tyrosine or phenylalanine inhibition. Prephenate dehydratase and chorismate mutase were determined by the method of Cotton and Gibson (6). Prephenate dehydrogenase was assayed by the method of Dayan and

TABLE 1. *Bacterial strains used in this work*

Strains	Properties
S. typhimurium	
LT2	Wild type
SG12	*tyrO*: operator constitutive mutant for *aroFtyrA* operon; previously designated *fpr-2* (12)
SG300	4-Fluorophenylalanine resistant[a]
SG301	4-Fluorophenylalanine resistant[a]
SG302	Methionine requiring derivative of SG300
SG303	Thymine requiring derivative of SG300
SG304	Tyrosine requiring derivative of SG300
SG305	Proline requiring derivative of SG300
SG350	Derived from SG300
SG351	Lysine requiring derivative of SG350
serA13	Serine auxotroph
tyrA3	Tyrosine auxotroph
pheA20	Phenylalanine auxotroph
aroD80	*aroD* mutant lacking chorismate synthetase[b]
SA464	HfrK1, *serA13*
SA540	HfrK2, *purE8*
SA486	HfrK3, *serA13*
SA534	HfrK4, *serA13*
SA535	HfrK5, *serA13*
SC19	Hfr, *met*$^-$
E. coli	
KLF11/JC1553	F111 episome[c]
KLF16/KL110	F116 episome[c]
KLF43/KL259	F143 episome[c]

[a] Described in Materials and Methods.
[b] Requires phenylalanine and tyrosine for growth (25).
[c] See Fig. 2.

Sprinson (7). Protein was determined by the method of Lowry et al. (21). Specific activity is defined as micromoles of substrate used or product formed per hour per milligram of protein.

Column chromatography. Cell extracts were chromatographed on Whatman DE-52 diethylaminoethyl-cellulose as previously described (9).

RESULTS

Properties of phenylalanine regulatory mutants. Fluorophenylalanine resistant mutants SG300 and SG301 were tyrosine excretors when tested on analogue containing agar plates, but not when tested on minimal agar plates. This phenotype resembled that of previously described *tyrO* mutants (12), but enzyme activities of the new isolates revealed significant differences from *tyrO* strains SG11 and SG12 (12; previously designated *fpr-1* and *fpr-2*). DAHP synthetase (tyr) and prephenate dehydrogenase were derepressed in strains SG300 and SG301 as well as in strain SG12 (Table 2). However, prephenate dehydratase was more than 10-fold derepressed in strains SG300 and SG301, but was normal in strain SG12. Combined chorismate mutase (P plus T) was also derepressed (Table 2); it was only slightly repressed (not shown in Table 2) in strain SG300 by 1 mM tyrosine, which normally repressed chorismate mutase T (9). Furthermore, diethylaminoethyl-cellulose column chromatography of extracts of SG300 showed a very large fraction of prephenate dehydratase activity coincident with chorismate mutase activity. Hence, only chorismate mutase P was derepressed in this mutant. On the other hand, DAHP synthetase (phe) activity (Table 2) was close to wild-type levels. (This enzyme is specified by *aroG*, which is cotransducible with *gal*; A. B. DeLeo and D. B. Sprinson, unpublished data). DAHP synthetase (tyr) and prephenate

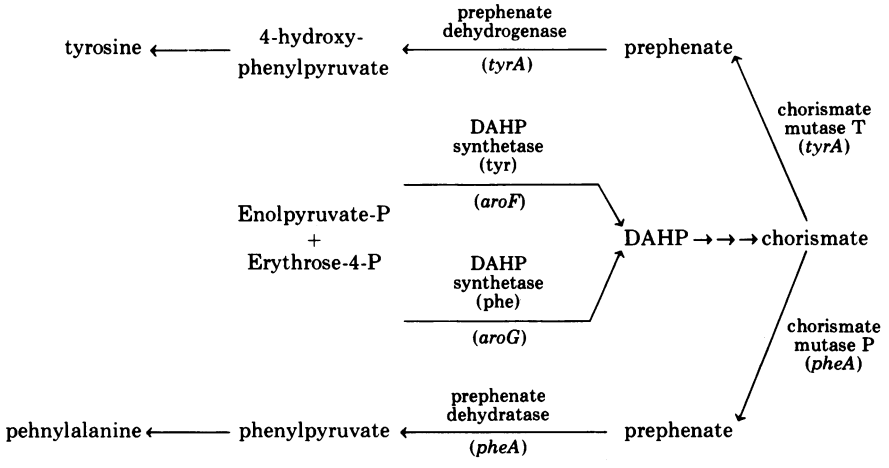

FIG. 1. *Enzymes and structural genes of phenylalanine and tyrosine biosynthetic pathways.*

TABLE 2. *Activities of phenylalanine- and tyrosine-specific enzymes in parent and mutant strains*[a]

Strain	DAHP synthetase[b]		Prephenate dehydratase	Chorismate mutase[c]	Prephenate dehydrogenase
	tyr (*aroF*)	phe (*aroG*)	(*pheA*)	(*tyrA*, *pheA*)	(*tyrA*)
LT2	0.40	1.2	0.27	0.60	0.13
SG12	6.5	1.8	0.22	1.0	5.0
SG300	3.2	0.9	5.7	5.0	1.8
SG301	5.0	1.0	3.8	4.0	2.0

[a] For methods and definitions of units see Materials and Methods. The results are averages of assays performed on at least two independently prepared extracts.

[b] Both tyr and phe refer to the tyrosine and phenylalanine inhibitable isoenzymes, respectively, of DAHP synthetase.

[c] Comprising chorismate mutase T (specified by *tyrA*) and chorismate mutase P (specified by *pheA*).

dehydrogenase were repressed by growth on tyrosine to the same extent in strains SG300 and 301 as in strain SG12, but prephenate dehydratase was not affected (not shown).

Growth on 1 mM phenylalanine did not repress prephenate dehydratase, chorismate mutase, or DAHP synthetase (phe) in strains SG300 and SG301. However, phenylalanine does control prephenate dehydratase, as shown by the effect of phenylalanine starvation on an *aroD* mutant that requires tyrosine and phenylalanine for growth (Table 3). Strain *aroD80* was grown on minimal medium containing 40 μg of tyrosine/ml and 3 μg of phenylalanine/ml until the latter was exhausted, and the cells were shaken for 3 h longer. Prephenate dehydratase activities were increased 10-fold, whereas DAHP synthetase (phe) remained essentially unchanged (1.5- to 2-fold derepressed).

Transduction of pheA and tyrA with phenylalanine regulatory mutants. To test for cotransducibility of the mutation affecting prephenate dehydratase activity with *pheA*, phage lysates were prepared from both strains SG300 and 301 and used to transduce a *pheA* mutant and a *tyrA* mutant to prototrophy. Transductants were scored for fluorophenylalanine resistance, and their phenylalanine and tyrosine specific enzyme activities were determined. Most of the prototrophic transductants from both crosses were resistant to fluorophenylalanine, but chorismate mutase-prephenate dehydratase was not derepressed. The mutation affecting prephenate dehydratase was, therefore, not linked to *pheA* and was not an operator mutation. However, DAHP synthetase (tyr) and prephenate dehydrogenase activities were 5- to 10-fold higher in these transductants than in the wild type, confirming the presence of a second mutation that was linked to *tyrA* and that affected the function of only *aroF* and *tyrA*. Since strains SG300 and 301 gave identical results, only strain SG300 was analyzed further.

TABLE 3. *Effect of phenylalanine starvation on DAHP synthetase (phe) and prephenate dehydratase in aroD80*

Conditions of growth	DAHP synthetase (phe)	Prephenate dehydratase
Repressed[a]	0.92	0.35
Derepressed[b]	2.1	3.5

[a] Supplemented with 0.35 mM phenylalanine and tyrosine, and cells harvested in late log phase.

[b] Supplemented with 0.35 mM tyrosine and 0.018 mM phenylalanine, and cells harvested 3 h after growth had stopped.

Conjugation of phenylalanine regulatory mutations with Hfr strains. To obtain the approximate location of the new phenylalanine regulatory gene, auxotrophic derivatives of strain SG300 were conjugated with Hfr donors whose points of origin are shown in Fig. 2. Prototrophic recombinants were selected and screened for resistance to fluorophenylalanine. Selection was complicated since recombinants that had incorporated donor chromosome from the region of the *aroFtyrA* cluster became sensitive to fluorophenylalanine even if prephenate dehydratase and chorismate mutase were derepressed. Moreover, recombinants producing repressed levels of prephenate dehydratase in combination with derepressed DAHP synthetase (tyr), and prephenate dehydrogenase were fluorophenylalanine resistant. It was, therefore, necessary to screen recombinants by enzyme analysis as well as by analogue resistance. The results provided only a general location of chromosomal linkage for the regulatory gene. In crosses SC19 × SG305 and SA534 (HfrK4) × SG305 in which selection was made for Pro$^+$ recombinants, and in SA464 (HfrK1) × SG302 in which selection was for Met$^+$, all of the Pro$^+$ and Met$^+$ recombinants tested showed derepressed levels of chorismate mutase-prephenate dehydratase characteristic of the recipient parent strain. On the other hand, crosses SA540 (HfrK2) × SG304, SA486 (HfrK3) × SG302, and SA535 (HfrK5) × SG303 yielded, respectively, some Tyr$^+$, Met$^+$, and Thy$^+$ recombinants that had repressed levels of chorismate mutase-prephenate dehydratase. Hence, the regulatory gene controlling *pheA* activity was located on the chromosome roughly between 70 and 117 min. Closer mapping was attempted by dominance studies with episomal strains.

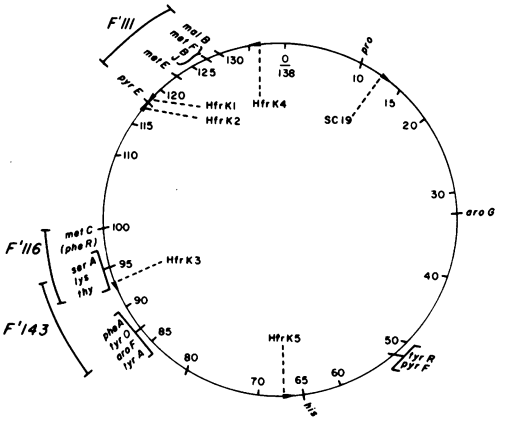

FIG. 2. *Schematic linkage map of Salmonella typhimurium (27).*

Construction and properties of merodiploids from SG300 derivatives and E. coli episomes. Since altered regulation of *pheA* in strain SG300 was not due to a linked operator mutation, it seemed likely that a defective gene product was involved. The wild-type allele of a regulatory gene introduced on an episome should compensate for defective product and show dominance over the mutated chromosomal gene (5, 10, 31). In a merodiploid from strain SG300, chorismate mutase-prephenate dehydratase would, therefore, be repressed.

Merodiploids were constructed from auxotrophic derivatives of strain SG300 and three *E. coli* K-12 F′ strains in which the episomes covered most of the implicated region of the *Salmonella* chromosome (Fig. 2). Prototrophic recombinants were isolated and tested for merodiploidy by successful transfer of episomes to other recipients, and by recovery of segregants which showed the original auxotrophic requirement. Introduction of episome F111 into strain SG302 (*met*$^-$) had no effect on prephenate dehydratase (Table 4). On the other hand, transfer of episome F116 into strain SG303 (*thy*$^-$) resulted in almost complete repression of both prephenate dehydratase and chorismate mutase activities. Furthermore, a *thy*$^-$ segregant reisolated from this merodiploid had derepressed enzyme levels similar to those shown by the original mutant. Episome F116 (which covers approximately the 95- to 100-min region of the *Salmonella* genome), therefore, carried the wild-type allele of a gene, designated *pheR*, which was dominant over the chromosomal mutant allele *pheR*$^-$.

Further evidence for the separate identity of *pheR* and the regulatory mutation affecting the tyrosine operon was provided by a recombinant (SG350) isolated from cross SA535 × SG304 which retained the *pheR* mutation but had a normal *tyr* operon. Episome F116 was transferred into a lysine-requiring derivative of strain SG350 (SG351), and chorismate mutase and prephenate dehydratase were determined in parent and merodiploid strains (Table 4). Again, the presence of F116 episomal genes resulted only in repression of *pheA* gene activity, indicating that the apparent *tyrO* mutation in strain SG300 was not related to *pheA* regulation. Control merodiploids prepared with a *serA*$^-$ strain, and the F116 episome showed that the *E. coli* episome had no effect on prephenate dehydratase levels in *pheR*$^+$ strains.

Effect of F143 in merodiploids. Episome F143, which covers the region between *lys* and *tyrA* and overlaps slightly the F116 episome (Fig. 2), was introduced into strains SG303 and SG304, *thy*$^-$ and *tyrA*$^-$ derivatives, respectively, of strain SG300. Chorismate mutase-prephenate dehydratase was not repressed in the resulting merodiploids (Table 5). Hence, *pheR* is not in the lysine-thymine region of F116, and must be located between 95 and 100 min on the *Salmonella* chromosome. In a merodiploid prepared from F143 and strain SG351, which contained a normal tyrosine operon, prephenate dehydratase was also highly elevated (Table 5), thus lending further support to the conclusion that the mutation affecting the tyrosine operon in strain SG300 was not related to the *pheR* mutation.

Chorismate mutase-prephenate dehydratase activities in the merodiploids were elevated four- to fivefold over the high constitutive levels present in the parent strain, thus resulting in derepression of over fiftyfold relative to wild type (Table 5). The presence of multiple copies of the episome in the merodiploid is unlikely, since enzyme levels were not excessively elevated in the control diploid F143/*tyrA*$^-$. The unexpectedly high enzyme levels suggest that the mutant repressor may recognize an *E. coli* operator even less effectively than a *Salmonella* operator.

As discussed above, the second mutation that occurred in strain SG300 and that was responsi-

TABLE 4. *Specific activities of prephenate dehydratase and chorismate mutase in merodiploids*[a]

Strain	Mating type	Prephenate dehydratase[b]	Chorismate mutase[b]
SG300	F$^-$	5.7	6.0
SG302	F$^-$	4.2	NT[c]
F111/SG302	F′	4.4	NT
SG303	F$^-$	5.0	6.5
F116/SG303	F′	0.30	0.49
Segregant	F$^-$	3.7	5.1
SG351	F$^-$	3.5	5.1
F116/SG351	F′	0.15	0.35
SerA	F$^-$	0.14	NT
F116/SerA$^-$	F′	0.41	NT
LT-2	F$^-$	0.37	0.61

[a] Methods and definition of specific activity are given in Materials and Methods. Activities are average values from assays of at least two independently prepared cell-free extracts. Merodiploids were grown in minimal medium to avoid growth of segregants. Haploid auxotrophic strains were supplemented with 0.3 mM thymine or required amino acid.

[b] Activities of cells grown on minimal medium. Similar results obtained with cells grown on 0.5 mM phenylalanine.

[c] NT, not tested.

TABLE 5. *Effect of episome F143 on phenylalanine and tyrosine specific enzymes*[a]

Strains	Mating type	Prephenate dehydratase[b]	Chorismate mutase[b]	DAHP synthetase tyr/phe	Prephenate dehydrogenase
SG304	F⁻	4.5	4.5	3.8/1.6	0
F143/SG304	F′	21.7	16.1	4.0/2.1	0.35
SG303	F⁻	6.0	6.5	4.0/1.0	1.0
F143/SG303	F′	23.0	18.1	4.6/2.7	1.6
SG351	F⁻	3.5	5.1	0.15/2.2	0.17
F143/SG351	F′	15.2	10.8	NT[c]	NT[c]
tyrA3	F⁻	0.52	0.60	0.59/0.96	0
F143/tyrA3	F′	1.2	1.2	0.73/1.7	0.24
LT2	F⁻	0.35	0.65	0.40/1.2	0.12

[a] See footnote *a* in Table 4.
[b] See footnote *b* in Table 4.
[c] NT, not tested.

ble for derepressed DAHP synthetase (tyr) and prephenate dehydrogenase was linked to *tyrA*. Since episome F143 carried the tyrosine operon (*tyrO*, *aroF*, *tyrA*), dominance relationships were studied in merodiploids to determine whether the second mutation was in *tyrO*. In F143/SG303 tyrosine operon genes are functional on both the episome and chromosome, whereas in F143/SG304 only the episome carries *tyrA*⁺. Episome F143 had no effect on DAHP synthetase (tyr) in either of the merodiploids, or on prephenate dehydrogenase in F143/SG303 (Table 5). DAHP synthetase (tyr) was derepressed in merodiploid F143/SG304 as in parent strain SG304, whereas prephenate dehydrogenase was present at low wild-type levels. However, both DAHP synthetase (tyr) and prephenate dehydrogenase were derepressed in F143/SG303 as in parent haploid strain SG303. Since the mutant allele was dominant over the wild-type allele and showed *cis* but not *trans* dominance, a *tyrO* mutation was indicated in strain SG300. Both strains F143/SG303 and F143/SG304 excreted large amounts of phenylalanine and were fluorophenylalanine resistant, probably owing to the combined effects of *tyrO* and *pheR* mutations.

DISCUSSION

Although mutant strains SG300 and 301 were found to be constitutively derepressed for chorismate mutase P-prephenate dehydratase, they did not excrete phenylalanine as might have been expected. However, they excreted tyrosine owing to a second mutant at *tyrO* which derepressed the tyrosine operon and resulted in resistance to 4-fluorophenylalanine (12). Regulation of *pheA* was not affected by the *tyrO* mutation, since *tyrO*⁺ recombinants of strain SG300 showed the same high constitutive levels of chorismate mutase P-prephenate dehydratase, although they were no longer as resistant to fluorophenylalanine as strain SG300. Hence, the *tyrO* mutation was responsible for the selection of these strains on the analogue. Similarly *pheO* mutants in *E. coli* were selected on fluorophenylalanine as doubly mutated strains, the second mutation having resulted in a feedback resistant DAHP synthetase (phe) (19).

Genetic investigation indicated that the regulatory mutation affecting *pheA* was not an operator mutation, and provided evidence for the presence of a regulatory gene *pheR* located at some distance from *pheA*. The presence of the wild-type allele on episome F116 and its absence on F143 established the location of *pheR* at approximately 95 to 100 min on the *Salmonella* chromosome. Precise mapping of its location will depend on availability of a more direct selection procedure than enzyme assay, since *pheR*⁻ mutants cannot be readily selected on fluorophenylalanine. Scarcity of known markers in the region of *pheR* may further contribute to the difficulty of mapping.

Dominance of *pheR*⁺ over *pheR*⁻ suggested that a diffusible, cytoplasmic product was involved in regulating *pheA* activity. The simplest explanation of our results, therefore, would be that *pheR* specifies an aporepressor which with phenylalanine as corepressor acts on the *pheA* operator according to the model of Jacob and Monod (20). It is assumed that phenylalanine, and not charged phenylalanyl transfer ribonucleic acid (tRNA), is the true corepressor for chorismate mutase P-prephenate dehydratase. Although aminoacylated tRNAs apparently act as corepressors for regu-

lating the biosynthesis of several amino acids (3), there is considerable evidence that such derivatives are not involved in regulation of tyrosine (13, 16, 27, 29) and tryptophan (23) biosynthetic pathways. The evidence against function of phenylalanyl tRNA as corepressor is not as clear (24), but it is likely that regulation by phenylalanine is similar to that by tyrosine and tryptophan.

Our evidence indicates that *pheR* does not regulate biosynthesis of DAHP synthetase (phe) which is specified by *aroG*. This enzyme is inhibited but only partially repressed by phenylalanine. It is slightly derepressed by phenylalanine starvation, whereas prephenate dehydratase is 10-fold derepressed under the same conditions. In *E. coli* K-12 it has been postulated (2, 18) that the gene *tyrR* specifies an aporepressor which acts not only with tyrosine as corepressor for regulating tyrosine-specific enzymes, but which under certain conditions also acts with phenylalanine to regulate DAHP synthetase (phe). *AroG* would thus be regulated by *tyrR*, and a separate repressor was predicted for *pheA* (2, 19). However, *pheR* mutants, such as those described in the present report, have not as yet been found in *E. coli*. The limited number of *Salmonella tyrR* mutants so far isolated show an effect only on DAHP synthetase (tyr), prephenate dehydrogenase, and transaminase A (E. G. Gollub and D. B. Sprinson, Fed. Proc., p. 491, 1972). The *pheR* mutants of *Salmonella* provide evidence for a unit of phenylalanine regulation in which structural gene *pheA* is controlled by regulator gene *pheR*.

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