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Phenylalanine Biosynthesis in *Escherichia coli* K-12: Mutants Derepressed for Chorismate Mutase P-Prephenate Dehydratase

S. W. K. IM AND J. PITTARD

Department of Microbiology, University of Melbourne, Parkville, Australia

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Mutants were isolated which are derepressed for the synthesis of chorismate mutase P-prephenate dehydratase. No other enzymes involved in the synthesis of phenylalanine are derepressed in these strains. These mutants are able to grow in concentrations of *o*- and *p*-fluorophenylalanine that inhibit the growth of AB3259, the strain from which they were derived. They also excrete phenylalanine. Genetic analysis shows that the mutations causing this derepression are closely linked to the structural gene for this enzyme (cotransduction frequency of 95% or more with *pheA*). The gene in which they occur has been designated *pheO* since this gene has all of the properties predicted for an operator gene controlling the *pheA* structural gene. Finally, the *pheO* mutant alleles have been shown to be dominant in diploids.

In the pathway of phenylalanine biosynthesis in *Escherichia coli*, the rate of synthesis of the multifunctional enzyme chorismate mutase P-prephenate dehydratase, which is responsible for the conversion of chorismate through prephenate to phenylpyruvate, is affected by phenylalanine. Although fully repressed levels are still fairly high (10), the synthesis of this enzyme can be derepressed two- to threefold by starving the cells for phenylalanine. The synthesis of the multifunctional enzyme chorismate mutase T-prephenate dehydrogenase of the tyrosine pathway is repressed by tyrosine to undetectable levels and, in the absence of tyrosine, is derepressed to become the major chorismate mutase enzyme (10). Prephenate dehydratase activity is feedback inhibited by phenylalanine, and prephenate dehydrogenase activity in feedback inhibited by tyrosine. Chorismate mutase T activity is not inhibited by tyrosine (F. Gibson, *personal communication*) and chorismate mutase P activity can only be reduced to 70% activity by phenylalanine (B. Davidson, *personal communication*).

The synthesis of the enzyme DAHP (3-deoxy-D-arabino-heptulosonic acid 7-phosphate) synthetase (phe) which is one of the three isoenzymes which carry out the first reaction of aromatic biosynthesis is also repressed by phenylalanine (4, 16, 18). It is the purpose of this paper to describe mutant strains of *E. coli* in which the synthesis of chorismate mutase P-prephenate dehydratase is derepressed but in which the synthesis of DAHP synthetase (phe) is unaltered.

MATERIALS AND METHODS

Organisms. Strains used in this work are all derivatives of *E. coli* K-12 (Table 1). Strain KLF43/KL253 was obtained from B. Low.

Media and culture methods. Media and culture methods have been described by Adelberg and Burns (1).

Buffers. Sodium phosphate buffers were prepared by the method of Dawson and Elliott (6).

Chemicals. Chemicals were obtained commercially and were not further purified. Reagent grade Selectacel [diethylaminoethyl (DEAE) cellulose] was obtained from Brown Co., Berlin, N.H. D-Erythrose-4-phosphate dimethylacetal dicyclohexylammonium salt (A grade) was obtained from Calbiochem, Los Angeles, Calif. Free erythrose-4-phosphate was prepared by the method of Ballou, Fischer, and MacDonald (3). Chorismic acid was prepared by the method of Edwards and Jackman (8). Barium prephenate was prepared by heating a solution of chorismic acid at 70 C for 1 hr; it was purified by chromatography as for chorismic acid before being converted to the barium salt.

Mating procedures. The conditions under which the mating experiments were carried out were those of Pittard and Wallace (14).

Transduction. Transductions with phage P1 were carried out by the method of Pittard and Wallace (14).

Isolation of mutants. The conditions under which cells were treated with the mutagen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, were those described by Adelberg, Mandel, and Chen (2). Phenotypic expres-

TABLE 1. *List of strains*

Strain	Sex	Genetic loci relevant to this work[a]						
		aroG	*aroH*	*aroF*	*tyrA*	*pheA*	*aroB*	*pheO*
AB3259	F^-	+	367[b]	363	+	+	+	+
AB3265	F^-	+	367	363	+	+	351	+
AB3313	F^-	+	+	+	4	1	+	+
AT2092	F^-	+	+	+	+	2	+	+
JP171	F^-	+	367	363	+	+	+	351
JP175	F^-	+	367	363	+	+	351	351
JP547	F^-	+	367	363	+	+	+	352
JP548	F^-	+	367	363	+	+	+	353
JP564	F^-	+	+	+	4	+	+	+
JP565	F^-	+	+	+	4	+	+	351
JP566	F^-	+	+	+	4	+	+	352
JP567	F^-	+	+	+	4	+	+	353
KLF43/KL253	F143	+	+	+	+/−	+	+	+

[a] Only genes concerned with aromatic biosynthesis are listed. *aroG*, the structural gene for DAHP synthetase (phe); *aroH*, the structural gene for DAHP synthetase (trp); *aroF*, the structural gene for DAHP synthetase (tyr); *tyrA*, the structural gene for chorismate mutase T-prephenate dehydrogenase; *pheA*, the structural gene for chorismate mutase P-prephenate dehydratase; *aroB*, the structural gene for dehydroquinate synthetase; *pheO*, operator of pheA.

[b] Allele numbers were allocated in these laboratories and the laboratories of E. A. Adelberg and A. L. Taylor.

sion of the mutants was allowed to occur by growth in complete medium for 4 hr after treatment with the mutagen.

Syntrophism tests. The method of Gibson and Jones (9) was used for syntrophism tests, except that 0.01% peptone was omitted from the medium.

Growth of cells and preparation of cell-free extracts. Cells were grown in minimal medium containing all the amino acids necessary for growth. The composition of minimal medium and the concentrations of the amino acids which were added were previously described by Adelberg and Burns (1). When minimal medium was supplemented with the aromatic amino acids, they were added in the following final concentrations: DL-phenylalanine, 5×10^{-4} M; DL-tryptophan, 2×10^{-4} M; L-tyrosine, 2.5×10^{-4} M. Other growth requirements were added in the following final concentrations: shikimic acid, 5×10^{-5} M; *p*-aminobenzoic acid, 10^{-6} M; *p*-hydroxybenzoic acid, 10^{-6} M; and 2,3-dihydroxybenzoic acid, 10^{-6} M. The cells were harvested at mid-exponential phase of growth, washed with chilled 0.9% NaCl, and suspended in 0.1 M sodium phosphate buffer (*p*H 7.0). Cell breakage was achieved by ultrasonic oscillation using a 500-w Ultrasonic Disintegrator (Measuring & Scientific Equipment, Ltd.) at output setting 3 for 40 sec. Cell-free extracts were obtained by centrifugation at $16,000 \times g$ for 15 min.

Column chromatography of enzymes on DEAE cellulose. The procedure for column chromatography of enzymes on DEAE cellulose was essentially that of Wallace and Pittard (18); in this work, however, ethylene diaminetetraacetic acid and mercaptoethanol were not included in the buffer.

Assay of DAHP synthetase. The method of Doy and Brown (7) was used for assaying DAHP synthetase.

Assay of chorismate mutase and prephenate dehydratase. The methods for assay of chorismate mutase and prephenate dehydratase were based on those described by Cotton and Gibson (5). Ethylenediaminetetraacetic acid (0.1 μmole) was included in the incubation mixture. Mercaptoethanol was also included: 1 μmole for the assay of chorismate mutase and 20 μmoles for the assay of prephenate dehydratase.

Assay of transaminase. The method of Silbert, Jorgensen, and Lin (15) using phenylalanine as substrate was used for assay of transaminase.

Protein estimation. Protein was estimated by the method of Lowry et al. (12).

Specific activities. One unit of specific activity is expressed as the number of 0.1 μmole of substrate used or product formed per 20 min per milligram of protein at 37 C.

Curing of F′ males. Acridine orange was used in the curing of F′ males, as described by Hirota (11).

RESULTS

Isolation of mutants derepressed for chorismate mutase P-prephenate dehydratase. To isolate such mutants, a selection procedure was used involving growth inhibition by the phenylalanine analogues, *ortho*- or *para*-fluorophenylalanine (FPA).

On the assumption that FPA inhibits growth by being incorporated into protein instead of phenylalanine and that the extent of this incorporation is dependent on the relative intracellular levels of phenylalanine and the analogue, it would be expected that one class of FPA-resistant mutants should be strains with a greatly increased rate of synthesis of phenylalanine. Such strains might also be expected to excrete phenylalanine and hence to cross-feed phenylalanine auxotrophs.

FPA-resistant mutants were isolated in strain AB3259 which contains only a single functional DAHP synthetase isoenzyme, DAHP synthetase (phe), by selecting for growth on minimal medium containing shikimic acid (0.5 mM), tyrosine (0.25 mM), and tryptophan (0.2 mM), and either *o*-FPA (1 mM) or *p*-FPA (10 mM). [This strain, possessing only DAHP synthetase (phe), was used to facilitate the study of the regulation of this enzyme in any mutants that were obtained.] Shikimic acid was added to overcome any inhibitory effect of the analogue on the activity of DAHP synthetase (phe) and tyrosine and tryptophan to prevent the metabolism of the branch point compound, chorismic acid, along these two terminal pathways.

Cells of AB3259 were treated with the mutagen, N-methyl-N-nitro-N′-nitrosoguanidine, as described above and plated to the selective media. The clones that appeared on these plates were purified and tested for their ability to cross-feed phenylalanine auxotrophs. Three strains which were strong cross-feeders (JP171, JP547, and JP548) were chosen for further study.

Repression of chorismate mutase P-prephenate dehydratase. The levels of prephenate dehydratase in cell-free extracts prepared from the three strains, JP171, JP547, and JP548, were approximately 10-fold higher than that of the parent strain AB3259 (Table 2). The presence of the aromatic end-products in the growth medium had no effect on the levels of this enzyme. That is to say repression below levels obtained in minimal medium was not observed even with the parent strain. To confirm that the rate of synthesis of this enzyme can be affected by phenylalanine, *aroB*⁻ derivatives of both the parent and one of the mutant strains, JP171, were prepared. The *aroB* gene codes for the enzyme carrying out the second reaction of the common pathway of aromatic biosynthesis and strains possessing the *aroB351* mutation require tyrosine, phenylalanine, tryptophan, and the aromatic vitamins for growth. To starve the *aroB*⁻ derivatives for phenylalanine, the cells were grown in minimal medium containing excess tyrosine, tryptophan, and the aromatic vitamins but a low level of phenylalanine (0.02 mM). Cells were harvested at 4 hr after growth had ceased owing to depletion of phenylalanine, and cell-free extracts were prepared. When AB3265, the *aroB351* derivative of the parent AB3259, was starved for phenylalanine, a threefold derepression of prephenate dehydratase was detected. This result confirmed that the rate of synthesis of this enzyme was controlled by phenylalanine (Table 3). However, as can be seen in Table 3, there was no further derepression when the *aroB351* derivative of the mutant JP171 was starved for phenylalanine, indicating an alteration in the normal system of repression of prephenate dehydratase in this strain and not simply a change in the rate of synthesis of this particular enzyme.

TABLE 2. *Specific activities of enzymes in parent AB3259 and mutants JP171, JP547, and JP548*

Strain no.	Prephenate dehydratase		DAHP synthetase (phe)	
	Cells grown in minimal medium	Cells grown in minimal medium supplemented with end products[a]	Cells grown in minimal medium	Cells grown in minimal medium supplemented with end products
AB3259	3	3	16	13
JP171	32	35	15	15
JP547	37	34	7	7
JP548	26	31	10	11

[a] End products include phenylalanine, tyrosine, and tryptophan.

Repression of DAHP synthetase (phe). The levels of DAHP synthetase (phe) in these mutants are not derepressed (Table 2). In fact, specific activities obtained in the case of JP547 and JP548 are lower than those obtained in either the parent AB3259 or the mutant JP171.

When the DAHP synthetase (phe) produced by these four strains was examined for its sensitivity to inhibition by phenylalanine, the enzymes produced by JP547 and JP548 were found to be feedback resistant. Although a concentration of 0.1 mM phenylalanine caused 90% inhibition of the enzyme from AB3259 and JP171, the same concentration caused only 5% inhibition of the enzymes from JP547 and JP548. Although this phenomenon has not been studied further, it seems likely that the alteration to feedback re-

TABLE 3. *Enzyme levels in aroB⁻ strains starved for phenylalanine*[a]

Strain no.	Specific activity	
	Prephenate dehydratase	DAHP synthetase (phe)
AB3265 (*aroB351*)	9	20
AB3259 (*aroB*⁺)	3	16
JP175 (*aroB351*)	30	21
JP171 (*aroB*⁺)	31	15

[a] The *aroB*⁺ strains included below were not starved for phenylalanine.

sistance in both these cases has impaired the catalytic activity activity of the enzyme.

Inhibition of prephenate dehydratase. Table 4 shows the inhibitory effect of phenylalanine and FPA on the activities of prephenate dehydratase in cell-free extracts. All mutant enzymes, except that of JP171, responded in a similar manner to that shown by the parent AB3259. Prephenate dehydratase in cell-free extract prepared from JP171 showed an alteration in feedback inhibition by *o*-FPA. Whether this effect is due to a separate mutation affecting the structural gene of this enzyme has not been established.

TABLE 4. *Per cent inhibition of prephenate dehydratase by phenylalanine and fluorophenylalanine*

Strain	PHE[a] (1 mM)	*o*-FPA (5 mM)	*p*-FPA (5 mM)
AB3259	85	70	66
JP171	88	10	70
JP547	83	76	70
JP548	83	65	66

[a] Abbreviations: PHE, phenylalanine; *o*-FPA, *o*-fluorophenylalanine; *p*-FPA, *p*-fluorophenylalanine.

Chromatography on DEAE cellulose. Since the activities of chorismate mutase P and prephenate dehydratase are associated in the same protein, the former is also expected to be derepressed in mutant strains JP171, JP547, and JP548. No attempt was made to measure the levels of chorismate mutase P in crude extract because of the possible presence of chorismate mutase T of the tyrosine pathway. To demonstrate that chorismate mutase P was derepressed in these mutants, these two enzyme activities, chorismate mutase P and T, were separated by chromatography on DEAE cellulose (19).

The results for extracts prepared from AB3259 and JP171 are summarized in Fig. 1. It can be seen that chorismate mutase P in JP171 was derepressed and that all of the mutase activity in the cell-free extract was accounted for by chorismate mutase P. When cells are grown in the presence of the aromatic amino acids, the contribution by chorismate mutase T is in fact negligible. Total transminase activity for the reaction

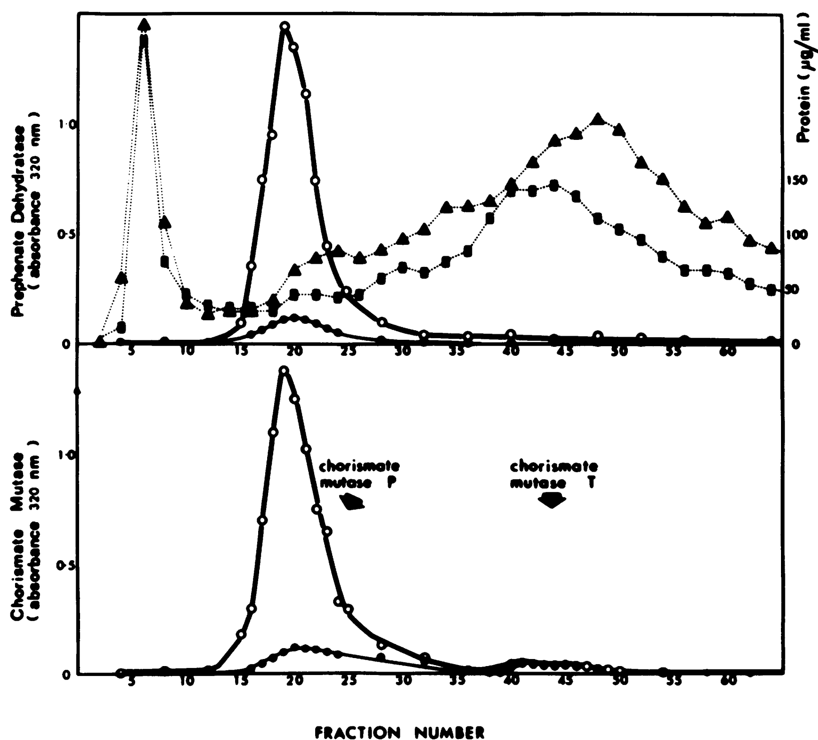

FIG. 1. *Chromatography of cell-free extracts on DEAE cellulose. Symbols:* ●, *enzyme activity in extract prepared from AB3259;* ○, *enzyme activity in extract prepared from JP171;* ■, *protein in extract prepared from AB3259;* ▲, *protein in extract prepared from JP171. Arrow indicates the approximate position where the elution of chorismate mutase T is expected (18). Cell-free extracts were prepared from cells grown in the presence of phenylalanine, tyrosine, and tryptophan.*

phenylpyruvate to phenylalanine was determined in crude cell extracts and in fractions obtained from the column and was found to be unaltered in the mutant JP171.

Genetic analysis. Since chorismate mutase P-prephenate dehydratase was the only enzyme to be derepressed in these mutants, transduction experiments were carried out to determine whether the mutations causing this derepression were closely linked to the structural gene to this enzyme, *pheA*. When P1 phage lysates were prepared on JP171, JP547, and JP548 and were used to transduce *pheA* into the *pheA*$^-$ recipient AT2092, over 95% of the *pheA*$^+$ transductants inherited the mutant properties, namely, the ability to cross-feed phenylalanine auxotrophs in syntrophism tests and resistance to FPA. Assay of prephenate dehydratase in cell-free extracts prepared from a number of these FPA-resistant transductants confirmed that they were derepressed for this enzyme. In the control cross using P1 lysate of the parent AB3259 as donor of *pheA*$^+$, none of the transductants showed such properties. These results indicated that the mutations are closely linked to *pheA*, the structural gene for chorismate mutase P-prephenate dehydratase.

Dominance test. Since the mutations causing derepression of chorismate mutase P-prephenate dehydratase in JP171, JP547, and JP548 are closely linked to the structural gene of the derepressed enzyme, *pheA*, and since derepression is restricted to this enzyme alone, these mutations are probably operator constitutive mutations. Henceforth, they are referred to as *pheO* mutations, and their allele numbers are 351, 352, and 353. Merodiploids were constructed to study dominance relationships between constitutive and wild-type (*pheO*$^+$) alleles.

Strain KLF43/KL253 was used as donor of the F143 episome covering the region between *lysA* and *tyrA*. The recipients used were derived from a *tyrA*$^-$ strain (AB3313) into which the *pheO* mutations had been separately introduced. These *tyrA*$^-$ derivatives, JP564, JP565, JP566, and JP567 carried alleles *pheO*$^+$ (from AB3259), *pheO-351* (from JP171), *pheO-352* (from JP547), and *pheO353* (from JP548), respectively. Merodiploids (*tyrA*$^+$/*tyrA*$^-$) were selected in interrupted mating experiments on minimal medium not containing tyrosine. Merodiploidy was confirmed by testing these resulting merodiploids for the ability to transfer *pheA*$^+$ into the *pheA*$^-$ strain AT2092 and by their ability to give rise to *tyrA*$^-$ segregants. The levels of prephenate dehydratase in these merodiploids are shown in Table 5. It can be seen that the presence of the *pheO*$^+$ allele in the *trans* position has no effect on the constitutive synthesis of prephenate dehydratase occurring as a result of the *pheO352*, *pheO352*, and *pheO353* alleles. Partial repression of prephenate dehydratase is observed in these *tyrA*$^-$ strains carrying the O^c alleles when the cells are grown under conditions of repression (Table 5). No such response occurs with the same alleles in the original strains (Table 2). A possible explanation for partially constitutive enzyme synthesis in the *tyrA*$^-$ strain in contrast to constitutive synthesis in the original O^c mutants is that these strains possess different genetic backgrounds. Partially constitutive enzyme synthesis has also been observed in operator mutants of the *trp* operon (17) and of *aroH*, the structural gene for DAHP synthetase (trp) (Camakaris and Pittard, *unpublished data*).

Since we were testing the dominance relationship between the constitutive alleles on the chromosome and the wild-type allele on the F′ episome F143, it was necessary to show that the wild-type (*pheO*$^+$) allele on F143 was intact in these strains. A transduction experiment was carried out using P1 phage lysate prepared from the merodiploid F143/JP565 (*pheO351*) as donor of *pheA*$^+$ into a *pheA*$^-$ recipient AT2092. Experiments with the diploid strain JP171 had shown that *pheO351* was cotransducible at frequencies of 95% or more with *pheA*. In this case, however, the expected frequency of the occurrence of *pheA*$^+$, *pheO351* transductants should be reduced to 50% or less, depending on the average number of F′ episomes per cell. The remainder of the *pheA*$^+$ transductants would be expected to be *pheA*$^+$, *pheO*$^+$, having originated from the F′ episome. The selection is further complicated by the fact that *tyrA* is cotransducible with *pheA* (14) and present in a mutated form *cis* to the *pheA*$^+$, *pheO351* alleles. Therefore, a proportion of the *pheA*$^+$, *pheO351* transductants should also be *tyrA*$^-$. The *pheA*$^+$, *pheO*$^+$ transductants, on the other hand, should all be *tyrA*$^+$. The results presented in Table 6 confirm these predictions and also prove that *pheO*$^+$ on F143 is intact and does not have any effect on the *pheO351* allele in the *trans* position.

DISCUSSION

Special problems have to be faced in trying to isolate mutants exhibiting altered repressibility for any of the enzymes involved in the biosynthesis of phenylalanine. These problems arise because, even under repressing conditions, quite high levels of these enzymes are still synthesized by the cells, and the major control mechanism would appear to be that of feedback inhibition. Furthermore, no phenylalanine analogue is

TABLE 5. *Specific activity of prephenate dehydratase in merodiploids*

Strain	Sex	Prephenate dehydratase	
		Cells grown in minimal medium	Cells grown in minimal medium supplemented with end products[a]
JP564	F⁻	3.7	2.5
F143/JP564	F'	8.4	4.4
JP565	F⁻	40.4	25.7
F143/JP565	F'	38.5	31.0
JP566	F⁻	28.0	16.2
F143/JP566	F'	28.3	18.6
F143/JP567	F'	34.3	22.0

[a] End products include phenylalanine, tyrosine, and tryptophan.

TABLE 6. *Test for the presence of intact pheO⁺ allele on F143*

Donor	Recipient	*pheA*⁺ transductants		
		Genotype	No.	Total no. tested
F143/JP565	AT2092	*tyrA⁺ pheA⁺ pheO⁺*	25	
		tyrA⁺ pheA⁺ pheO-351	1	40
		tyrA⁻ pheA⁺ pheO-351	14	
F143/JP564	AT2092	*tyrA⁺ pheA⁺ pheO⁺*	23	40
		tyrA⁻ pheA⁺ pheO⁺	17	

known which can replace phenylalanine in repression but not in feedback inhibition. As a result of this situation, many selection procedures involving analogues of phenylalanine produce only mutants in which the feedback sensitivity of a particular enzyme is altered (Im and Pittard, *unpublished data*). In this publication, we described a method which has allowed us to isolate mutants in which the rate of synthesis of chorismate mutase P-prephenate dehydratase occurs constitutively at elevated rates. Although shikimic acid was added to overcome any effect of the analogues on the first enzyme of the pathway DAHP synthetase (phe), two of the mutants selected were double mutants possessing one mutation creating a feedback-resistant DAHP synthetase (phe) and another causing derepression of chorismate mutase P-prephenate dehydratase. The selection of such double mutants in two strains probably indicates that the rate of utilization of exogenous shikimic acid by these strains limits the rate of synthesis of phenylalanine. Such a conclusion is supported by other data which shows that exogenous shikimic acid is inefficiently used by many strains (Pittard, *unpublished data*). The finding that the specific activity of DAHP synthetase (phe) in these feedback-resistant strains is lower than in the parent AB3259 suggests that the alteration to the enzyme which renders it feedback resistant either alters its stability or its turnover number. We have observed exactly the same effect with feedback-resistant mutants of DAHP synthetase (trp) and DAHP synthetase (tyr) (Pittard and Camakaris, *unpublished data*). The third mutant which is derepressed for an enzyme (chorismate mutase P-prephenate dehydratase), which is also resistant to inhibition by *o*-FPA, is presumably able to convert chorismic acid to phenylalanine more rapidly in the presence of the analogue than strains in which this enzyme is still inhibited by *o*-FPA.

The mutants that have been described in this paper have all the properties predicted for strains in which an operator locus controlling the structural gene *pheA* has been altered. This locus has been found to be very closely linked to the *pheA* gene but no fine structure mapping has yet been carried out.

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