

Novel Mutations in the *pheA* Gene of *Escherichia coli* K-12 Which Result in Highly Feedback Inhibition-Resistant Variants of Chorismate Mutase/Prephenate Dehydratase

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Received 2 March 1992/Accepted 11 May 1992

The bifunctional enzyme chorismate mutase/prephenate dehydratase (EC 5.4.99.5/4.2.1.51), which is encoded by the *pheA* gene of *Escherichia coli* K-12, is subject to strong feedback inhibition by L-phenylalanine. Inhibition of the prephenate dehydratase activity is almost complete at concentrations of L-phenylalanine greater than 1 mM. The *pheA* gene was cloned, and the promoter region was modified to enable constitutive expression of the gene on plasmid pJN302. As a preliminary to sequence analysis, a small DNA insertion at codon 338 of the *pheA* gene unexpectedly resulted in a partial loss of prephenate dehydratase feedback inhibition. Four other mutations in the *pheA* gene were identified following nitrous acid treatment of pJN302 and selection of *E. coli* transformants that were resistant to the toxic phenylalanine analog β -2-thienylalanine. Each of the four mutations was located within codons 304 to 310 of the *pheA* gene and generated either a substitution or an in-frame deletion. The mutations led to activation of both enzymatic activities at low phenylalanine concentrations, and three of the resulting enzyme variants displayed almost complete resistance to feedback inhibition of prephenate dehydratase by phenylalanine concentrations up to 200 mM. In all four cases the mutations mapped in a region of the enzyme that has not been implicated previously in feedback inhibition sensitivity of the enzyme.

The common aromatic pathway of *Escherichia coli* K-12 results in the biosynthesis of chorismate, the common precursor of many important aromatic compounds, including the amino acids tyrosine, tryptophan, and phenylalanine (Fig. 1). Phenylalanine is synthesized in three enzymatic steps from chorismate, the first two of which are accomplished by the bifunctional enzyme chorismate mutase/prephenate dehydratase (CMPD) encoded by the *pheA* gene. The final step, a transamination reaction, is carried out by the aromatic and aspartate aminotransferases (15). Phenylalanine biosynthesis is regulated predominantly by control of CMPD through phenylalanine-mediated inhibition of *pheA* transcription (18) and by feedback inhibition of the prephenate dehydratase and chorismate mutase activities of the enzyme (8).

Studies on a number of *E. coli* mutants that were altered in *pheA* regulation initially suggested that transcription of *pheA* was regulated both by operator-mediated repression and by attenuation (17, 20). The presence of an attenuator region analogous to that of other *E. coli* biosynthetic operons was confirmed by nucleotide sequence analysis and transcription studies in vitro (31). However, recent studies have shown that changes in *pheA* regulation that were previously attributed to repressor-operator interactions are due to altered attenuation (12-14). Direct experimental evidence of a *pheA* repressor protein is lacking, although the -10 region of the promoter does overlap a region of dyad symmetry that is typical of *E. coli* operator sequences.

Regulation of CMPD through feedback inhibition is mediated by allosteric binding of phenylalanine, which favors a

shift in the aggregation state of the enzyme from an active dimer to less active tetrameric and octameric species (3). Inhibition is most pronounced upon the prephenate dehydratase activity; almost total inhibition occurs at phenylalanine concentrations of 1 mM (8, 20). In contrast, chorismate mutase activity is maximally inhibited by only 40% (8).

The deregulation of CMPD activity has been a central focus of attempts to overproduce phenylalanine in *E. coli*. This has required both the derepression of *pheA* transcription and the relief of CMPD feedback inhibition. Since the promoter and attenuator elements have been clearly defined, it has been relatively easy to obtain high levels of CMPD expression through replacement of the native regulatory regions with suitable strong promoters and expression of the gene on multicopy plasmids (10, 11, 27). In addition, several workers have described the use of phenylalanine analogs to select *E. coli* mutants in which the CMPD is resistant to feedback inhibition by phenylalanine at concentrations that are close to normal intracellular levels (27, 28). In this report we describe the isolation and genetic characterization of CMPD mutants that exhibit almost total resistance to feedback inhibition at very high phenylalanine concentrations.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. The strains which we used were all derivatives of *E. coli* K-12 (Table 1). Liquid cultures were grown at 37°C in Lenox LB broth (GIBCO/Bethesda Research Laboratories). Plate cultures were grown at 37°C on LB agar or on M9 (24) minimal salts medium containing 1.5% agar and supplemented with 0.2% glucose. Where appropriate, kanamycin was added at a concentration of 40 μ g/ml and ampicillin was added at a concentration of 200 μ g/ml. The plasmids which we used are shown in Table 1.

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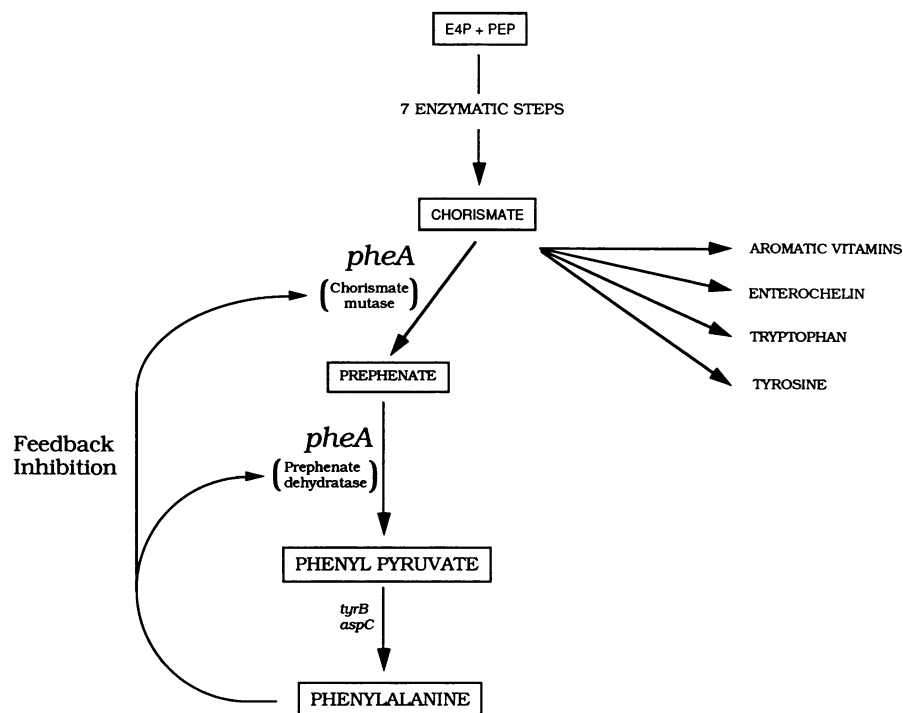


FIG. 1. Role of the *pheA* gene product (CMPD) in the biosynthesis of phenylalanine in *E. coli*. Both enzyme activities are subject to phenylalanine-mediated feedback inhibition. E4P, erythrose 4-phosphate; PEP, phosphoenolpyruvate.

Enzymes and reagents. Restriction endonucleases, polynucleotide kinase, and DNA ligase were purchased from New England Biolabs or from GIBCO/Bethesda Research Laboratories and were used according to the manufacturers' specifications. The Klenow fragment of DNA polymerase I was purchased from Boehringer Mannheim. Chorismate, prephenate, β -2-thienylalanine, and L-phenylalanine were obtained from Sigma Chemical Co. Protein concentrations in cell extracts were determined by the Bradford method (5). Ultrapure agarose and acrylamide, ammonium persulfate, and *N,N,N',N'*-tetramethylethylenediamine for DNA sequencing were obtained from GIBCO/Bethesda Research Laboratories or Sigma. Radioisotopes for DNA sequencing were obtained from Amersham International. All other chemicals were purchased from Sigma or Baker Chemical Co.

Manipulation of plasmid DNA. Bacterial transformation and plasmid DNA isolation were carried out as previously described (21). Restriction analysis and ligations were performed by standard methodology. DNA sequencing was carried out by the dideoxy chain termination method (25) and a Sequenase 1 kit (U.S. Biochemical). Sequences were determined directly by using purified plasmid DNA (2 μ g of plasmid DNA per reaction). Single-stranded plasmid DNA was prepared by a modification of the alkaline denaturation method described in the instructions for the kit. The DNA was denatured in 20 μ l of 0.4 M sodium hydroxide for 5 min at room temperature. The solution was neutralized by adding 8.8 μ l of 5 M ammonium acetate (pH 7.5), and the plasmid was recovered by precipitation in 123 μ l of ethanol. The DNA was washed in 70% ethanol, dried, and suspended in 6 μ l of water. Site-directed mutagenesis was carried out as previously described (30). All oligonucleotides were synthe-

sized with an Applied Biosystems model 380B DNA synthesizer.

Plasmid DNA mutagenesis. Nitrous acid mutagenesis of plasmid pJN302 was carried out with 5 μ g of plasmid DNA in a 200- μ l reaction mixture containing 50 mM sodium acetate (pH 4.6), 88 mM sodium nitrite, and 2 mM spermine. The reaction mixture was incubated at 30°C for a total of 90 min, and samples of 60, 70, and 70 μ l were removed after 30, 60, and 90 min, respectively. Each sample was placed in 30 μ l of 1 M Tris (pH 8.0), and NaCl was added to a final concentration of 200 mM. Plasmid DNA was recovered by precipitation in 2.5 volumes of ethanol, washed in 70% ethanol, and suspended in 10 μ l of water.

Selection of analog-resistant mutants. Competent cells of strain HW1012 were transformed with 3 μ l of each sample of nitrous acid-treated pJN302 DNA. Transformants were isolated on kanamycin-containing LB plates. All colonies were pooled, washed, and diluted 1:5 in 0.85% saline. Aliquots (100 μ l) were then spread onto minimal plates containing 10 or 20 mM β -2-thienylalanine.

Preparation of cell extracts. A single colony was inoculated into 25 ml of kanamycin-containing LB medium in a 1-liter shake flask, and the flask was incubated overnight. Cells were harvested, washed in 25 ml of 50 mM Tris (pH 8.0) for 5 min, centrifuged again, and resuspended in 1 ml (final volume) of 50 mM Tris (pH 8.0). Lysis was carried out in a French pressure cell at 1,000 lb/in². Debris was removed by centrifugation at 14,000 rpm in a microcentrifuge for 30 min at 4°C.

Assay for prephenate dehydratase activity. The assay for prephenate dehydratase activity, as well as the assay for chorismate mutase activity, was adapted from the assay described previously (8). Prephenate dehydratase activity

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
<i>E. coli</i> K-12 strains		
W3110	Prototroph	S. Primrose
HW1012	<i>pheA7</i>	N. Grinter
HW87	<i>araD139 Δ(ara-leu)7697 Δ(lacIPOZY)74 galU galK hsdR recA56 srl rpsL</i>	W. Tacon
Plasmids		
pBR322		4
pAT153	High-copy-number derivative of pBR322	29
pLG338	Tet ^r Km ^r ; low-copy-number plasmid; derivative of pSC101 ^a	26
pME65	pAT153 carrying the <i>E. coli phe</i> and <i>tyr</i> operons on a 9.6-kb <i>EcoRI</i> fragment	This study
pME149	pAT153 carrying <i>pheA</i> on a 1.7-kb <i>EcoRI</i> -to- <i>Bam</i> HI fragment	This study
pME198	pME149 with synthetic <i>pheA</i> promoter and additional <i>Bam</i> HI site	This study
pJN302	pLG338 carrying <i>pheA</i> on a 1.25-kb <i>EcoRI</i> - <i>Bam</i> HI fragment of pME149	This study
pJN300	Same as pJN302, but with in-frame <i>Bgl</i> III linker at <i>pheA</i> <i>Nco</i> I site; encodes CMPD-M1	This work
pJN302#2	Same as pJN302, but with unidentified spontaneous mutation in <i>pheA</i> ; encodes CMPD-M2	This study
pJN305	Same as pJN302, but with 12-bp deletion of <i>pheA</i> codons 307 to 310; encodes CMPD-M3	This study
pJN306	Same as pJN302, but with A→T transversion in <i>pheA</i> codon 306; encodes CMPD-M4	This study
pJN307	Same as pJN302, but with 6-bp deletion in <i>pheA</i> codons 304 to 306; encodes CMPD-M5	This study
pJN308	Same as pJN302, but with G→T transversion in <i>pheA</i> codon 309; encodes CMPD-M6	This study

^a Tet, tetracycline; Km, kanamycin.

was assayed in 1.25-ml reaction mixtures containing 27 mM Tris (pH 8.0), 1 mM potassium prephenate, and 50 μ l of cell extract. Activity was determined in the absence of phenylalanine and also with phenylalanine added at concentrations of 2, 10, 20, 50, 100, and 200 mM. The concentration of additional phenylalanine derived from cell extracts was at most in the micromolar range since the extracts were diluted approximately 10-fold at lysis and an additional 25-fold in the assay. The reactions were started by adding prephenate, and the preparations were incubated at 37°C for 1 min; then a 0.25-ml sample was removed and mixed with 0.75 ml of NaOH. Absorbance was measured at 320 nm against a water blank. Additional identical samples were removed after 5 and 9 min. The rate of increase in absorbance was calculated and was corrected for any control rate in the absence of extract. Controls containing prephenate but no extract showed no significant increase in absorbance. One unit of prephenate dehydratase activity was defined as the quantity of enzyme that catalyzed the conversion of 1 μ mol of

prephenate to phenylpyruvate in 1 min under our assay conditions.

Assay for chorismate mutase activity. Chorismate mutase activity was assayed in 0.8-ml reaction mixtures containing 1 mM chorismate, 100 mM Tris (pH 7.5), 0.5 mM EDTA, 0.01% bovine serum albumin, and 20 μ l of cell extract. Activity was determined in the absence of phenylalanine and with phenylalanine present at concentrations of 10, 50, and 100 mM. The reactions were started by adding the cell extract, and the reaction mixtures were incubated for 5 min at 37°C; then the reactions were terminated by adding 0.1 ml of 4.5 M HCl. The reaction mixtures were incubated for an additional 10 min at 37°C to convert all of the prephenate to phenylpyruvate, and then 0.1 ml of 12 M NaOH was added and the absorbance was measured at 320 nm. All of the values were corrected for substrate absorbance and for the absorbance change determined for extracts of strain HW1012, which was attributable to the endogenous chorismate mutase activity derived from the *tyrA* gene product, chorismate mutase/prephenate dehydrogenase. One unit was defined as the quantity of enzyme which catalyzed the conversion of 1 μ mol of chorismate to prephenate in 1 min under our assay conditions.

RESULTS

Construction of plasmid pJN302. The *pheA* gene was originally isolated on a 9.6-kb *EcoRI* fragment from a genomic library of strain W3110 and was cloned in plasmid pAT153 to give pME65. A 1.7-kb *EcoRI*-to-*Bam*HI fragment containing *pheA* was then subcloned into plasmid pAT153, generating pME149. The nucleotide sequence of *pheA* was determined both in our laboratory and by other workers (9, 18). To relieve transcriptional regulation, the promoter region was replaced by a synthetic DNA fragment that was based on the native sequence but lacked the attenuator region and the dyad symmetry which overlapped the -10 box (Fig. 2). The modified promoter was synthesized as six oligonucleotides which were assembled into a single fragment by sequentially annealing and ligating overlapping oligomers. This fragment was flanked by *EcoRI* and *Hae*II sites, which allowed replacement of the corresponding fragment which encompassed the regulatory region of the wild-type *pheA* gene (18). The synthetic promoter region was cloned into pME149 between the *EcoRI* site upstream from *pheA* and the *Hae*II site in the N-terminal region of the *pheA* coding sequence. To facilitate additional manipulations, a *Bam*HI site was introduced by site-directed mutagenesis 6 bp downstream from the *pheA* translational stop. The resulting plasmid was designated pME198. The modified gene was constitutively expressed and could be isolated on a 1,253-bp *EcoRI*-to-*Bam*HI fragment. This fragment was cloned into vector pLG338, generating plasmid pJN302.

Construction of plasmid pJN300. Plasmid pJN300 was identical to pJN302 except for a four-residue insertion at position 338 in the peptide sequence. The insertion was originally made to facilitate sequencing of the *pheA* gene. The plasmid was cleaved at the unique *Nco*I site. The sticky ends were then filled by using the Klenow fragment of DNA polymerase I prior to insertion of an 8-bp *Bgl*III linker (Fig. 3). The resulting mutation, designated *pheA30*, generated a modified CMPD which unexpectedly exhibited reduced sensitivity of the prephenate dehydratase activity to feedback inhibition by phenylalanine at concentrations up to 20 mM (Fig. 4). This enzyme was designated CMPD-M1.

Isolation of *pheA* mutants. Plasmid pJN302 DNA was

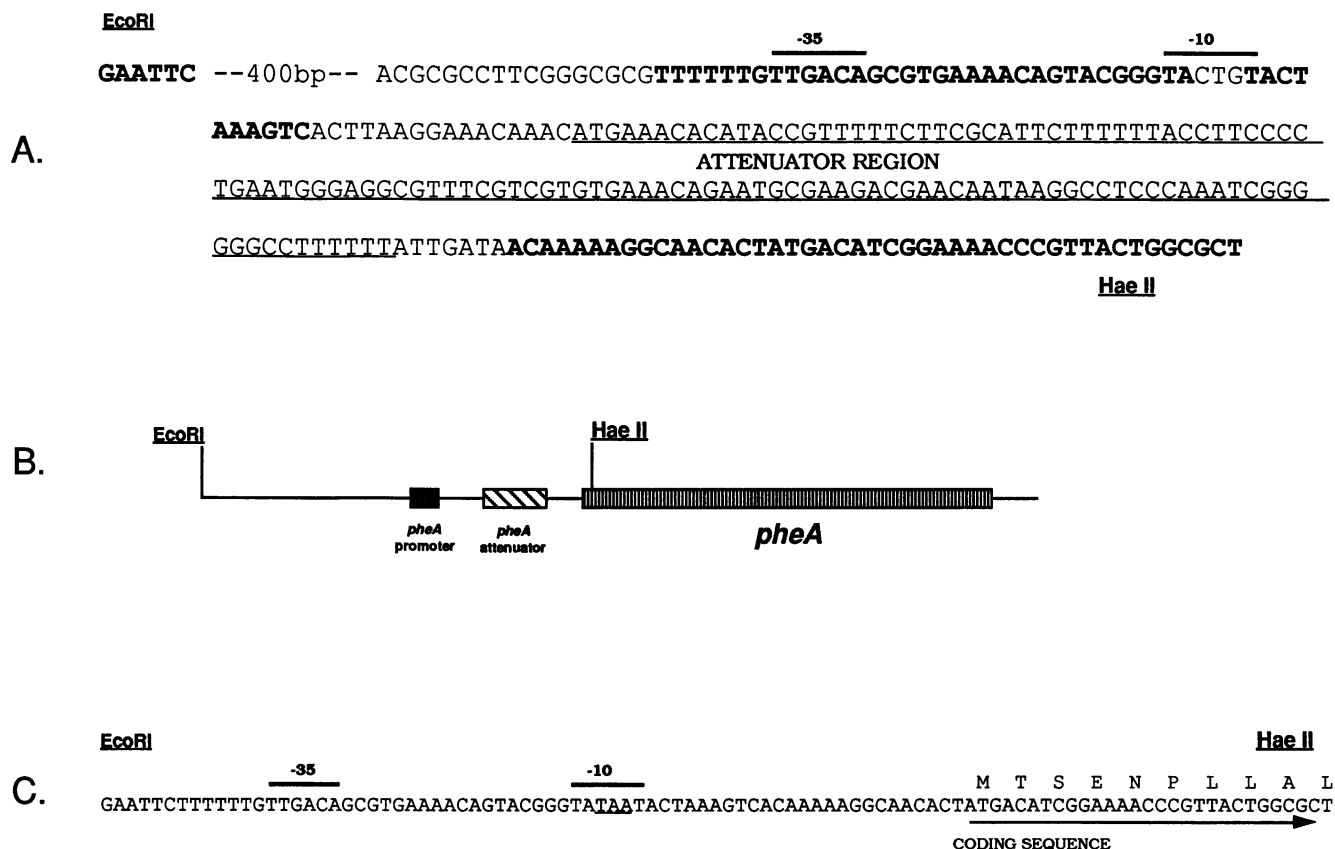


FIG. 2. Basis of the modified *pheA* promoter region. (A) Wild-type regulatory regions within the *EcoRI*-*HaeII* fragment spanning the *pheA* promoter. The positions of the -35 and -10 boxes are indicated. The bases that are retained in the modified promoter are in boldface type. The sequence that is involved in the attenuator region is underlined. (B) Position of the fragment in relation to the *pheA* gene. (C) Sequence of the modified *pheA* promoter region. The altered bases in the -10 region are underlined.

subjected to nitrous acid mutagenesis and was used to transform strain HW1012 as described above. Mutations in the *pheA* gene resulting in feedback inhibition resistance of CMPD were selected on plates containing the toxic phenyl-

alanine analog β -2-thienylalanine at a concentration of 10 or 20 mM. Analog-resistant transformants were isolated at both of the concentrations employed.

More than 1,000 colonies were obtained on the plate

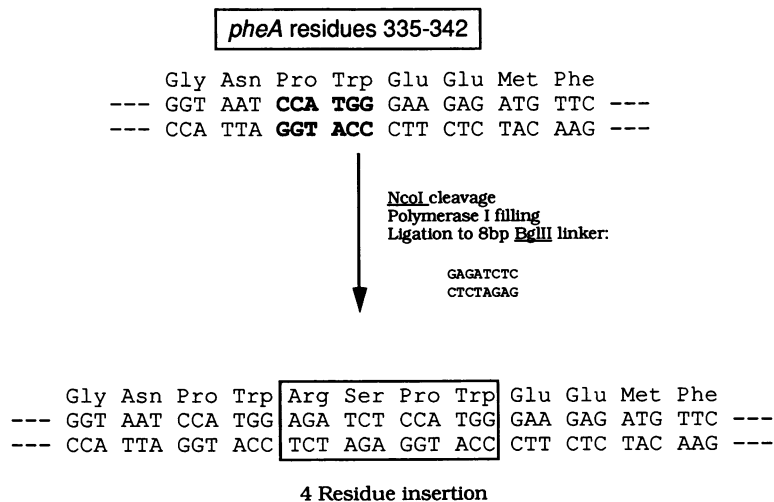


FIG. 3. Mutation of the *pheA* sequence by DNA polymerase I filling the *NcoI* site and insertion of an 8-bp *BglII* linker. The wild-type sequence is shown at the top; the *NcoI* site is in boldface type. The resulting four-residue insertion is enclosed in a box below.

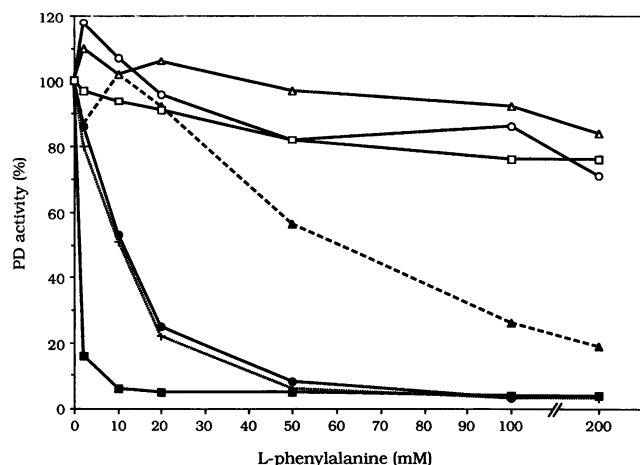


FIG. 4. Feedback inhibition of prephenate dehydratase (PD) activity by L-phenylalanine. The levels of activity retained in the presence of different phenylalanine concentrations are expressed as percentages of the activity in the absence of phenylalanine. Symbols: ■, wild-type CMPD; ●, CMPD-M1; +, CMPD-M2; ○, CMPD-M3; ▲, CMPD-M4; △, CMPD-M5; □, CMPD-M6. Levels of activity were determined in cell extracts of strain HW1012 bearing *pheA* alleles on the plasmids described in the text.

containing 10 mM β -2-thienylalanine. Four of these colonies were characterized and exhibited CMPD activity with feedback inhibition resistance comparable to that of CMPD-M1. Retransformation of strain HW1012 indicated that the activity of each mutant was due to mutation of the plasmid-borne *pheA* gene. The prephenate dehydratase feedback inhibition profile of one isolate (the isolate that contained CMPD-M2) is shown in Fig. 4. The *pheA* allele that encoded CMPD-M2 was designated *pheA31*.

Another four colonies were obtained from the 20 mM β -2-thienylalanine plate. Each of these produced a CMPD with a very high level of resistance to feedback inhibition by phenylalanine. Plasmid DNA was isolated from each of the four resistant colonies and was used to transform fresh cells of strain HW1012. In each case the retransformants exhibited analog resistance and CMPD activity that were identical to the analog resistance and CMPD activity of the original isolate, indicating that the activity was derived from mutation of the plasmid-borne *pheA* gene. The four plasmid isolates were designated pJN305, pJN306, pJN307, and pJN308; these isolates carried the *pheA* alleles *pheA32*, *pheA33*, *pheA34*, and *pheA35* encoding enzymes CMPD-M3, CMPD-M4, CMPD-M5, and CMPD-M6, respectively.

Properties of the mutated CMPD: enzyme assay. Prephenate dehydratase activity was examined in extracts of strain HW1012 bearing each of the four plasmids isolated as described above. In each case the activity was compared with wild-type CMPD, CMPD-M1, and CMPD-M2 activities in the absence of phenylalanine (Table 2). The prephenate dehydratase activities of extracts containing CMPD-M4 and CMPD-M5 were very similar to the wild-type CMPD activity. In contrast, extracts containing CMPD-M3 and CMPD-M6 exhibited reduced prephenate dehydratase activities. This suggests that there was a loss of specific activity by the latter two variants, although differential expression of the alleles could not be ruled out. The activity of wild-type CMPD and the activity of each variant were then examined in the presence of various concentrations of phenylalanine

TABLE 2. Prephenate dehydratase activities in extracts of strain HW1012 bearing *pheA* clones^a

Enzyme	Prephenate dehydratase activity ^b	
	mU/mg of protein	Relative activity
CMPD	72.6	1
CMPD-M1	89.2	1.23
CMPD-M2	86.8	1.20
CMPD-M3	37.8	0.52
CMPD-M4	69.2	0.95
CMPD-M5	71.2	0.99
CMPD-M6	45.4	0.63

^a Enzymes were prepared as described in the text from cultures of strain HW1012 bearing pJN302 or its derivatives.

^b Activity was determined in the absence of phenylalanine.

(Fig. 4). With phenylalanine present at a concentration of 10 mM, the wild-type enzyme exhibited a 95% loss of prephenate dehydratase activity. Conversely, three of the four variants were activated under these conditions. The variants exhibited strong resistance to feedback inhibition at much higher concentrations of phenylalanine, with three of the four exhibiting more than 75% retention of activity in the presence of 100 mM phenylalanine. One variant in particular, CMPD-M5, still retained more than 80% of the prephenate dehydratase activity at the maximum phenylalanine concentration attainable under our assay conditions (200 mM).

Comparable chorismate mutase activity was observed in each of the extracts (data not shown), but differences were also apparent in the chorismate mutase activity profiles of the wild-type and mutant enzymes in the presence of phenylalanine. Each of the mutants exhibited activation in the presence of 10 mM phenylalanine, whereas the wild-type enzyme exhibited 25% inhibition (Fig. 5). At higher phenylalanine concentrations the differences were less pronounced. Although the maximum observed level of inhibition of wild-type chorismate mutase activity by phenylalanine was only 29%, at least one of the mutants, CMPD-M6, appeared to exhibit an increased level of resistance at very high phenylalanine concentrations. Interestingly, at least one other mutant, CMPD-M4, may have possessed increased sensitivity to high concentrations of phenylalanine.

Identification of the *pheA* mutations. To localize the mutations that were responsible for the phenotype of each isolate, specific fragments of plasmids pJN305, pJN306, pJN307, and pJN308 were isolated and substituted for the corresponding fragment of the wild-type *pheA* gene in pJN302. This analysis revealed that in each case the only mutations that were necessary and sufficient for the phenotype mapped to the 208-bp *AlwNI*-to-*NcoI* fragment (Fig. 6). In each case, when this fragment was introduced into the wild-type *pheA* gene, the resulting subclone expressed CMPD with a feedback inhibition resistance profile identical to that of the original isolate.

The nucleotide sequence of the *AlwNI*-to-*NcoI* fragment was determined for each of the four alleles. In addition, the complete nucleotide sequences of the *pheA* genes of pJN305 and pJN307 were determined. In each case the only mutations lay within the region specified by codons 304 to 310 (Fig. 6). In *pheA33* and *pheA35* a single-base mutation led to an amino acid substitution. An A-to-T transversion in *pheA33* replaced Gln-306 with Leu, and a G-to-T transversion in *pheA35* replaced Gly-309 with Cys. In *pheA32* and

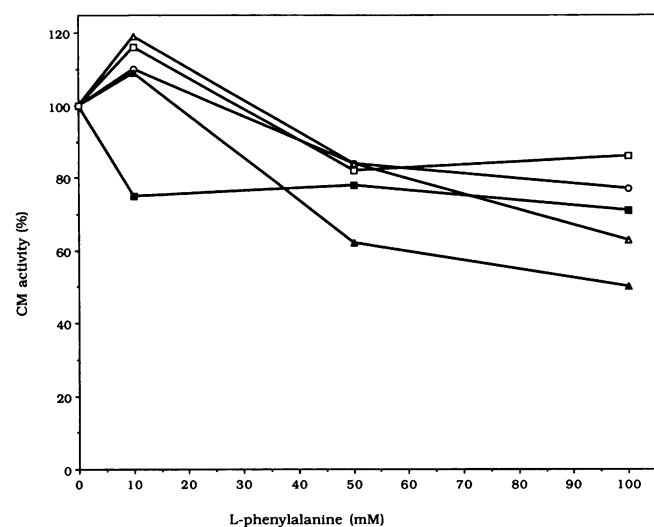


FIG. 5. Feedback inhibition of chorismate mutase (CM) activity by L-phenylalanine. The levels of activity retained in the presence of different phenylalanine concentrations are expressed as percentages of the activity in the absence of phenylalanine. Symbols: ■, wild-type CMPD; ○, CMPD-M3; ▲, CMPD-M4; △, CMPD-M5; □, CMPD-M6.

pheA34, in-frame deletions occurred. A 12-bp deletion excised residues 307 to 310 in *pheA32*, and a 6-bp deletion within codons 304 to 306 of *pheA34* caused the replacement of the residues Thr-Gly and Gln with a single Lys.

DISCUSSION

In this study we constructed a synthetic *pheA* promoter that was derived from the wild type but lacked known and putative regulatory elements. This allowed constitutive expression of the *pheA* gene and was used in the selection of highly feedback inhibition-resistant versions of CMPD. Specific *pheA* mutations which reduce the feedback inhibition sensitivity of CMPD have been described previously by us and by other workers. In each case the region of the mutation has encompassed Trp-338, a residue that was implicated in feedback inhibition sensitivity in early conformational studies on the enzyme (16). Backman and Ramaswamy have described a series of mutations at position 338 that include substitution of the tryptophan residue and truncation of the enzyme at this position (2). Both forms of mutation render CMPD free of feedback inhibition by phenylalanine at a concentration of 1.2 mM.

We have previously described a four-residue insertion at Trp-338 which leads to a partial loss of prephenate dehydratase feedback inhibition (9). In this work we found that this enzyme is essentially free of prephenate dehydratase feedback inhibition by low levels of phenylalanine (2 mM), but is inhibited like the wild-type enzyme at higher phenylalanine concentrations (50 mM). A large number of spontaneous mutants with similar properties were also isolated in this work.

In addition, we identified four separate mutations of the *pheA* gene which encode CMPD activities that exhibit resistance to very high concentrations of phenylalanine. The four mutations vary considerably in the degree of primary sequence perturbation and occur in a common region of CMPD that has not been implicated previously in the feedback

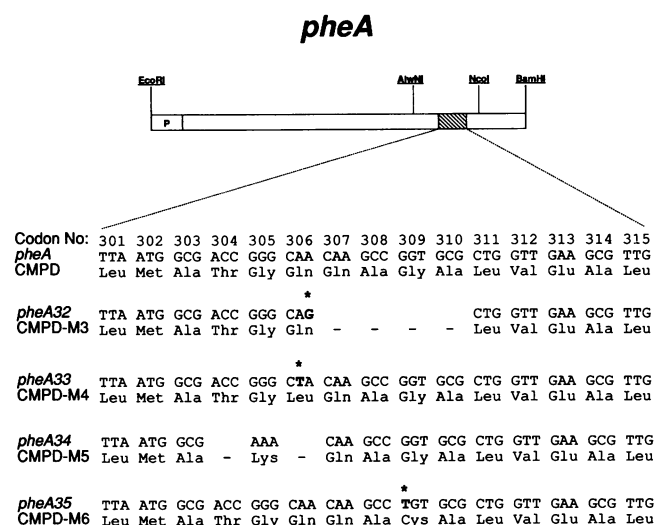


FIG. 6. Mutations in the *pheA* gene giving rise to feedback inhibition-resistant variants of CMPD. The *pheA* gene is shown at the top with the promoter (P) at the left and the mutated region indicated by the cross-hatched box. Alterations to the DNA and amino acid sequence are shown below aligned with the wild-type *pheA* and CMPD sequences. Single-base substitutions are indicated by asterisks and nucleotides in boldface type. The dashes indicate deleted codons.

inhibition sensitivity of the enzyme. Three of the altered enzymes retain more than 70% of the wild-type prephenate dehydratase activity in the presence of 200 mM phenylalanine. These enzymes also exhibit altered chorismate mutase feedback inhibition profiles, although it cannot be determined at the present time whether there are significant differences in chorismate mutase activity when phenylalanine concentrations exceed 10 mM. Certainly, there is clear stimulation of both enzymatic activities at low phenylalanine concentrations. The data suggest that the mutations in CMPD-M4 and CMPD-M5 result in little or no loss of specific activity in the absence of phenylalanine since the total cellular CMPD activity is comparable to that of the wild-type enzyme expressed from an analogous construction.

Previous reports of feedback inhibition-resistant CMPD have not described activity in the presence of phenylalanine concentrations greater than 2 to 5 mM. This level of resistance is sufficient to render the enzyme free of inhibition at normal intracellular phenylalanine concentrations, which in wild-type *E. coli* range from 0.6 to 1.6 mM (6, 22). However, cultures of overproducing strains of *E. coli* readily achieve extracellular phenylalanine concentrations that are greater than 100 mM (19, 27), and we have observed that in such cultures the intracellular phenylalanine concentrations can frequently exceed the extracellular levels (23). Under these conditions, the CMPD step would still be expected to be rate limiting even if a partially feedback inhibition-resistant enzyme, such as the enzyme mutated at Trp-338, were present. In strains that overproduce phenylalanine, the *pheA* gene is frequently overexpressed on high-copy-number plasmids (10, 27), suggesting that there is a need for elevated levels of CMPD synthesis in addition to feedback inhibition resistance to ensure that there are maximum levels of phenylalanine biosynthesis at high phenylalanine concentrations. However, the use of highly expressed genes on multicopy

plasmids can lead to plasmid instability (7) and is not consistent with energy efficiency in amino acid-overproducing strains, since considerable cellular resources are diverted to plasmid and enzyme biosynthesis (1). The retention of almost total prephenate dehydratase activity demonstrated by the CMPD variants at very high phenylalanine concentrations should permit maximum phenylalanine biosynthesis levels to occur with considerably lower levels of CMPD expression.

Inhibition of native prephenate dehydratase activity by phenylalanine has been shown to result from a shift in the aggregation state equilibrium from an active dimer to a less active tetramer or octamer, driven by favored allosteric binding of phenylalanine to the tetrameric form (3). Therefore, feedback inhibition resistance could occur through a reduced ability of the enzyme to adopt aggregation states greater than a dimer or by reduced phenylalanine binding to the tetramer. The clustered nature of the mutations which we observed is perhaps more consistent with alteration of a specific binding site for L-phenylalanine than with perturbation of an oligomerization interface. Since CMPD mutated in the codon 304 to 310 region of the peptide retains full activity at phenylalanine concentrations that are completely inhibitory to CMPD mutated at Trp-338, it is also possible that the mutations relieve feedback inhibition in different ways. Alternatively, the mutations may generate varying degrees of conformational change in a common region in the tertiary structure of the enzyme. Currently, we are purifying the CMPD variants described in this paper to examine the allosteric binding of phenylalanine, the effect upon the aggregation state of the enzymes, and the mechanism of feedback inhibition resistance.

ACKNOWLEDGMENTS

We thank Nigel Grinter and Neil Welker for a critical appraisal of the manuscript and Paul Taylor and Leigh Fozkos for oligonucleotide synthesis. We also thank Stefan Bledig for his assistance in the development of the plasmid-sequencing protocol and Roberta Yoshida and Andrew Pauls for helpful discussions. We are grateful to The NutraSweet Company for providing excellent research facilities for this work.

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