Cloning, sequencing, and expression of the P-protein gene (*pheA*) of *Pseudomonas stutzeri* in *Escherichia coli*: implications for evolutionary relationships in phenylalanine biosynthesis

RANDY S. FISCHER,* GENSHI ZHAO and ROY A. JENSEN

Department of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611, USA

(Received 1 October 1990; revised 11 February 1991; accepted 27 February 1991)

The *pheA* gene encoding the bifunctional P-protein (chorismate mutase: prephenate dehydratase) was cloned from *Pseudomonas stutzeri* and sequenced. This is the first gene of phenylalanine biosynthesis to be cloned and sequenced from *Pseudomonas*. The *pheA* gene was expressed in *Escherichia coli*, allowing complementation of an *E. coli pheA* auxotroph. The enzymic and physical properties of the P-protein from a recombinant *E. coli* auxotroph expressing the *pheA* gene were identical to those of the native enzyme from *P. stutzeri*. The nucleotide sequence of the *P. stutzeri pheA* gene was 1095 base pairs in length, predicting a 365-residue protein product with an M_r of 40844. Codon usage in the *P. stutzeri pheA* gene was similar to that of *Pseudomonas aeruginosa* but unusual in that cytosine and guanine were used at nearly equal frequencies in the third codon position. The deduced P-protein product showed sequence homology with peptide sequences of the *E. coli* P-protein, the N-terminal portion of the *E. coli* T-protein (chorismate mutase: prephenate dehydrogenase), and the monofunctional prephenate dehydratases of *Bacillus subtilis* and *Corynebacterium glutamicum*. A narrow range of values (26–35%) for amino acid matches revealed by pairwise alignments of monofunctional and bifunctional proteins possessing activity for prephenate dehydratase suggests that extensive divergence has occurred between even the nearest phylogenetic lineages.

Introduction

Micro-organisms are diverse in the arrangement and variety of enzymes comprising the terminal pathways of L-phenylalanine biosynthesis (Fig. 1). *Bacillus subtilis*, *Corynebacterium glutamicum*, and other Gram-positive bacteria synthesize phenylalanine via phenylpyruvate. The latter is produced from chorismate in consecutive steps catalysed by the enzymes chorismate mutase and PDT (Berry *et al.*, 1987; Fazel & Jensen, 1980; Pierson & Jensen, 1974). In *B. subtilis*, the chorismate mutase reaction may be catalysed by a low-activity species carried on a bifunctional protein (also containing the catalytic site for DAHP synthase, the initial enzymic

step of the pathway which carries out the condensation of phosphoenolpyruvate and erythrose 4-phosphate to form DAHP) or by a monofunctional, high-activity species (CM-F). The relationship of these enzymes has been reviewed by Byng & Jensen (1983). The coexistence of chorismate mutase and DAHP synthase on a bifunctional protein has also been reported for *Brevibacterium flavum* (Shiio & Sugimota, 1979).

Escherichia coli, *Pseudomonas stutzeri*, and many other Gram-negative bacteria synthesize phenylalanine employing the foregoing enzymic steps, but utilize a bifunctional protein (P-protein) to catalyse the overall conversion of chorismate to phenylpyruvate (Ahmad & Jensen, 1986). Other micro-organisms such as *Pseudomonas diminuta* use an entirely different route, the arogenate pathway (Zamir *et al.*, 1985). In this case three monofunctional enzymes, CM-F, prephenate aminotransferase and ADT, catalyse steps mediating the chorismate-to-phenylalanine conversion. *Pseudomonas aeruginosa* exemplifies those organisms having a pathway arrangement whereby dual pathways to phenylalanine coexist. In addition to the P-protein, *P. aeruginosa*

Abbreviations: ADT, arogenate dehydratase; CDT, cyclohexadienyl dehydratase; CM-F, monofunctional chorismate mutase; DAHP, 7-phospho-2-dehydro-3-deoxy-D-arabino-heptonate; PDT, prephenate dehydratase.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M35545.

Fig. 1. Diversity of enzymic routes for the conversion of chorismate to L-phenylalanine. Abbreviations: CHA, chorismate; PPA, prephenate; PPY, phenylpyruvate; PHE, L-phenylalanine; AGN, L-arogenate; CM-F, 'free' chorismate mutase which is not a component of a bifunctional protein; CM-P, chorismate mutase component of the P-protein; PDT, prephenate dehydratase; CDT, cyclohexadienyl dehydratase which can utilize both PPA and AGN as substrate; ADT, arogenate dehydratase; AT denotes an unknown number of aminotransferases having overlapping substrate specificities and able to transaminate PPA or PPY *in vitro*. The CM-P and PDT activities are fused to form the P-protein, represented by the shaded rectangle. The route to PHE via AGN that is catalysed by the substrate-ambiguous CDT is depicted by the dashed line.

has a broad-specificity CDT that can utilize either prephenate or L-arogenate as substrate (Patel *et al.*, 1977). Another type of dual-pathway arrangement is found (unpublished data) in *Zymomonas mobilis*, which expresses two monofunctional substrate-specific dehydratases (ADT and PDT).

In organisms where the dehydratase activity is catalysed by a monofunctional PDT, ADT or CDT, a monofunctional CM-F is inevitably present (Ahmad & Jensen, 1988a). We designate monofunctional chorismate mutase as CM-F to distinguish it from the chorismate mutase components of the bifunctional proteins.

The complexity of phenylalanine biosynthesis in *Pseudomonas* is particularly intriguing since most of the pathway alternatives illustrated in Fig. 1 can be demonstrated in different pseudomonad species. The relative simplicity of phenylalanine biosynthesis in *P. stutzeri*, a close relative of *P. aeruginosa* in rRNA homology group I, has led to its choice for initial investigations aimed at delineation of the evolutionary relationships of genes encoding the monofunctional and bifunctional phenylalanine-pathway enzymes of parallel catalytic function in these Gram-negative organisms.

Methods

Bacterial strains, media and growth. The bacterial strains and plasmids utilized or constructed in this investigation are shown in Table 1. *P. stutzeri* was cultured as described by Carlson *et al.* (1983). *E. coli* strains were grown in M9 (Miller, 1972) minimal medium or LB (Davis *et al.*, 1980a) medium at 37 °C. Amino acids (50 µg ml⁻¹) and thiamin hydrochloride (17 µg ml⁻¹) were added as growth supplements when required. Antibiotics, when appropriate to provide selective

pressure for plasmids, were added to the medium at standard concentrations (Maniatis *et al.*, 1982). X-Gal (5-bromo-4-chloro-3-indoxyl β-D-galactopyranoside) and IPTG (isopropyl β-D-thiogalactopyranoside) were included at concentrations recommended by the supplier (Promega) when recombinants were screened in pGEM-4Z or pGEM-5Z plasmids transformed into JM109 cells. Growth rates were determined by measuring turbidity of exponentially growing cultures at 600 nm in a Perkin-Elmer model 35 spectrophotometer.

DNA manipulation and genetic procedures. Standard molecular biology procedures were performed as described by Maniatis *et al.* (1982) unless otherwise indicated. Bacterial chromosomal DNA was purified by the method of Yuan & Lin (1982). DNA for large-scale plasmid preparations was isolated by a Triton X-100 lysis procedure (Clewett & Helinski, 1969; Kupersztoch & Helinski, 1973) and purified by CsCl/ethidium bromide buoyant density gradient centrifugation (Humphreys *et al.*, 1975). Small-scale plasmid isolation was performed according to the protocol of Davis *et al.* (1980b). *E. coli* strains were transformed by use of a CaCl₂ method (Dagert & Ehrlich, 1979). Natural transformation of *P. stutzeri* JM706 was carried out as described by Carlson *et al.* (1983).

Cloning vectors, reagents, and enzymes for molecular biology. Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs or Bethesda Research Laboratories. Calf intestinal alkaline phosphatase, pHCT9, and the λ DNA packaging kit were obtained from Boehringer Mannheim Biochemicals. X-Gal, IPTG, and the plasmids pGEM-4Z and pGEM-5Zf(+) were purchased from Promega. Each product was used as recommended by the manufacturer.

Preparation and screening of the *P. stutzeri* gene bank. A genomic, randomly generated *Pst*I gene bank was constructed in the cosmid vector pHCT9 by partial methylation of *P. stutzeri* JM300 DNA followed by limit digestion. *P. stutzeri* was chosen for the cloning of the *pheA* gene because: (i) wild-type strain JM300 possesses only the bifunctional P-protein route to phenylalanine; (ii) strain JM706, a Phe⁻ derivative of JM300, demonstrates an absolute growth requirement for phenylalanine, has a low reversion frequency, and is deficient in prephenate dehydratase activity; and (iii) strain JM706 can be transformed to prototrophy with high frequency by natural transformation with homologous chromosomal DNA (Carlson *et al.*, 1983, 1984), regardless of flanking sequences.

The gene library of *P. stutzeri* was prepared as described below to

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
<i>P. stutzeri</i>		
JM300	Prototroph	Carlson <i>et al.</i> (1983).
JM706	<i>pheA</i> mutant defective in prephenate dehydratase activity (Byng <i>et al.</i> , 1983) isolated from NTG mutagenesis of JM300	J. L. Ingraham, Dept. of Microbiology, Univ. of California, Davis
<i>E. coli</i>		
DH1	<i>gyrA96 recA1 relA1 endA1 thi-1 hsdR17 supE44</i>	CGSC* 6040
JP2255	<i>aroF363 pheA361 pheO352 tyrA382 thi strR712 lacY1 xyl-5</i>	Baldwin & Davidson (1981)
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)</i>	Promega
Plasmids		
pHC79	Ap ^R Tc ^R <i>cos</i>	Hohn & Collins (1980)
pGEM-4Z	Ap ^R <i>lacZ</i>	Promega
pGEM-5Zf(+)	Ap ^R <i>lacZ</i>	Promega
pJF958	Phe ⁺ clone from pHC79: <i>Pst</i> I gene bank of JM300	This study
pJF9586	Phe ⁺ 4.35 kb <i>Pst</i> I fragment subcloned from pJF958 into pHC79	This study
pJF1954	Phe ⁺ 1477 bp <i>Sph</i> I/ <i>Ba</i> II fragment subcloned from pJF9586 into pGEM-5Zf(+)	This study
pJF1956	788 bp <i>Sph</i> I/ <i>Eco</i> RI fragment subcloned from pJF1954 into pGEM-4Z	This study
pJF1958	581 bp <i>Sph</i> I/ <i>Eco</i> RV fragment subcloned from pJF1954 into pGEM-5Zf(+)	This study
pJF1960	711 bp <i>Eco</i> RI/ <i>Pst</i> I fragment† subcloned from pJF1954 into pGEM-4Z	This study
pJF1970	896 bp <i>Eco</i> RV/ <i>Ba</i> II fragment subcloned from pJF1954 into pGEM-5Zf(+)	This study
pJF1972	775 bp <i>Sph</i> I/ <i>Pst</i> I fragment subcloned from pJF9586 into pGEM-5Zf(+)	This study

* *Escherichia coli* Genetic Stock Center, Yale University.†The *Pst*I site was that of the multiple cloning region of pGEM-5Zf(+).

ensure that a full-length *pheA* gene would be among the clones. Partially *Pst*I methylated JM300 DNA fragments (size-fractionated to 10–20 kb on preparative agarose) were ligated into *Pst*I-digested pHC79 which had been treated with calf intestinal alkaline phosphatase. Recombinants from the ligation were packaged using a λ DNA packaging kit, employing 2 mM-putrescine to relax size selectivity. Approximately 2600 clones obtained by transfection of *E. coli* strain DH1 with the λ packaging reaction mixture were screened for ability to transform the Phe[−] *P. stutzeri* JM706 to prototrophy. Pools of 90–100 clones were maintained on duplicate master plates of LB/tetracycline medium. For primary screening, small-scale plasmid isolations were prepared from the pooled clones present on each master plate. Transformation of JM706 to prototrophy with plasmid DNA isolated from the pooled clones was used to identify a pool yielding Phe⁺ transformants. The original pool was divided into sub-pools and systematically screened to isolate individual clones. We isolated six clones which could be resolved by restriction analyses to reveal two clones differing only in some low-*M_r* *Pst*I fragments. These clones were characterized as described in Results.

Assay of P-protein activities. The methods for preparation of crude extracts from late-exponential cultures of *E. coli* and associated analytical procedures were previously described (Fischer *et al.*, 1986). Chorismate mutase and prephenate dehydratase activities of the

cloned *pheA* gene product were assayed by the method of Cotton & Gibson (1965) as modified by Ahmad & Jensen (1988b), except that substrate concentrations were increased to 2 mM.

Biochemicals and chemicals. Unless indicated otherwise, all biochemicals and chemicals were obtained from Sigma. Barium prephenate was prepared from culture supernatants of a tyrosine auxotroph of *Salmonella typhimurium* (Dayan & Sprinson, 1970) and was converted to the potassium salt with a twofold excess of K₂SO₄ before use. Chorismate was prepared from culture supernatants of *Klebsiella pneumoniae* (Gibson, 1964).

DNA sequencing and analysis of sequence data. Specific restriction fragments were cloned into pGEM-4Z or pGEM-5Zf(+) as shown in Fig. 2. Double-stranded plasmid DNA was sequenced in both directions by methodology using fluorescent chain-terminating dideoxy nucleotides (Prober *et al.*, 1987) at the DNA Sequencing Core Laboratory (DSEQ) of the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida. The DSEQ operates a DNA sequencer (Genesis 2000 DNA Analysis System from DuPont). Primers to the bacteriophage SP6 or T7 RNA polymerase promoters, which flank the multiple cloning site of the pGEM vectors, were used to sequence in from the ends of each pGEM subclone. In order to sequence DNA beyond the range of the SP6 and T7 primers, 17-mer

Fig. 2. Ability of specific restriction-generated fragments of *P. stutzeri* genomic DNA cloned in pHC79, pGEM-4Z or pGEM-5Z to transform or complement P-protein mutants of *P. stutzeri* and *E. coli*. The orientations of pJF958 and pJF9586 relative to the *bla* gene of pHC79 are depicted above. Uppercase letters show restriction sites in the *P. stutzeri* genomic DNA. P, *Pst*I; B, *Bal*I; S, *Sph*I; E, *Eco*RI; R, *Eco*RV; ORF, open reading frame.

oligonucleotides were synthesized to regions upstream of the unsequenced regions at the DNA Synthesis Core Laboratory (DSYN) of ICBR. The DSYN employs an Applied Biosystems 380B synthesizer and a Pharmacia FPLC system for purification, quality control, and analysis of oligonucleotides.

The sequence data were analysed using the Biological Computing Facility (BCF) of ICBR. The BCF operates a MicroVax II, which was accessed through a dial-up modem. The programs and databases from the University of Wisconsin Genetics Computer Group (GCG) package (Devereux *et al.*, 1984), available through the BCF, were used to analyse the DNA and deduced peptide sequences.

Results

Cloning of the *P. stutzeri pheA* gene in *E. coli*

A randomly generated *Pst*I library of wild-type *P. stutzeri* DNA was constructed in *E. coli*, and approximately 2600 recombinants were screened by transformation of *P. stutzeri* JM706. Two plasmids were isolated which transformed JM706 to prototrophy. The presence of the structural gene for the P-protein in DNA sequences carried by these plasmids was confirmed by complementation of *pheA* in *E. coli* JP2255. One plasmid, designated pJF958, was used to localize, subclone and sequence the *P. stutzeri* gene.

The strategy employed for subcloning and characterization of the P-protein gene carried on pJF958 is

illustrated in Fig. 2. The two larger fragments (7.2 and 4.35 kb) resulting from *Pst*I digestion of pJF958 were subcloned into pHC79. The 4.35 kb fragment, but not the 7.2 kb fragment, transformed *P. stutzeri* JM706 to prototrophy. Only one orientation of the 4.35 kb fragment in the *bla* gene of pHC79 was able to complement the P-protein mutation of JP2255, this orientation being the same as that of the original clone, pJF958. Thus, the cloned *pheA* gene appears to be dependent on pHC79 promoters for expression.

One plasmid carrying the 4.35 kb *Pst*I fragment, designated as pJF9586, was utilized for further subcloning. Southern blot hybridization (under moderate conditions of stringency) indicated that the ³²P-labelled 4.35 kb *Pst*I fragment from pJF9586 hybridized with a 4.35 kb fragment of chromosomal DNA of *P. stutzeri* JM300 DNA digested with *Pst*I, whereas no hybridization was detected with *Pst*I-digested chromosomal DNA from wild-type *E. coli* K12 (data not shown).

The restriction sites shown in Fig. 2 were utilized to construct a set of subclones from pJF9586 in pGEM-4Z or pGEM-5Z for nucleotide sequencing. We found that a 1477 bp *Sph*I-BalI fragment cloned into the *Sph*I-EcoRV sites (within the multiple cloning region) of pGEM-5Z complemented the *pheA* mutation in *E. coli* JP2255. Disruption of the *Sph*I-BalI fragment of this plasmid, pJF1954, at the *Eco*RV or *Eco*RI sites (yielding

Table 2. Expression of the *P. stutzeri pheA* gene in *E. coli* JP2255

Plasmid in strain JP2255*	Doubling time (min)	Specific activity† of:		
		Chorismate mutase	Prephenate dehydratase	
			– Tyr	+ Tyr‡
pHC79	66	0.0	0.0	0.0
pJF958	156	3.7	2.5	3.8
pJF9586	84	44.3	33.6	51.2
pGEM-5Z	66	0.0	0.0	0.0
pJF1954	165	4.1	2.8	4.7

* L-Tyrosine was supplemented to the growth medium of all cultures while L-phenylalanine was added only to the control cultures of JP2255(pHC79) and JP2255(pGEM-5Z).

† Specific activity is defined as nmol of phenylpyruvate produced min⁻¹ (mg protein)⁻¹. The values for specific activity are representative of results of one of three independent repetitions.

‡ L-Tyrosine was added to the reaction mixture to give a final concentration of 0.5 mM.

pJF1956, pJF1958, pJF1960 or pJF1970) resulted in loss of complementation of the *pheA* defect. Neither chorismate mutase nor prephenate dehydratase activities were found in these transformed cells. Natural transformation of *P. stutzeri* JM706 with the restriction fragments shown in Fig. 2 revealed that the mutation responsible for the defective prephenate dehydratase is located within the 581 bp *SphI*–*EcoRV* fragment.

Expression of the *P. stutzeri pheA* gene in *E. coli*

Results of enzymic assays of crude extracts prepared from *E. coli* JP2255 [a mutant which lacks both the P-protein (chorismate mutase:prephenate dehydratase) and T-protein (chorismate mutase:prephenate dehydrogenase) activities] harbouring pJF958, pJF9586 or pJF1954 are summarized in Table 2. Chorismate mutase and prephenate dehydratase activities were demonstrated in cells transformed with each of these plasmids. The allosteric activation of prephenate dehydratase by L-tyrosine is an enzyme character state that typifies most Gram-negative bacteria (Byng *et al.*, 1983), but not enteric bacteria. The enzyme activities given in Table 2 were determined from cultures grown in minimal medium so that the relative abilities of the three plasmids to confer independence of the nutritional requirement for phenylalanine could be estimated. Comparable specific activities were measured in extracts from cultures grown in rich medium (data not shown).

The growth rate of *E. coli* JP2255(pJF958) was still limited by phenylalanine, exhibiting a doubling time of more than 2.5 h. This is consistent with the finding that

specific activities for chorismate mutase and prephenate dehydratase in extracts of JP2255(pJF958) were only about 25% of the values determined for wild-type *E. coli* grown under comparable conditions (data not shown). Subcloning of the 4.35 kb *PstI* fragment from pJF958 to yield JP2255(pJF9586) resulted in elevated expression levels which were about 12-fold greater than those obtained for JP2255(pJF958). The increased growth rate of JP2255(pJF9586) as compared to JP2255(pJF958) probably reflects the elevation in P-protein activities. When the 1477 bp *SphI*–*BalI* fragment was subcloned from pJF9586 into pGEM-5Z to yield pJF1954, the P-protein activities were expressed at much lower levels. These lower P-protein activities are apparently due to vector differences (e.g. such as relative strengths of plasmid-encoded promoters) between pHC79 and pGEM-5Z.

Western blot hybridization, employing antibody prepared to the P-protein from *Acinetobacter calcoaceticus* (Ahmad *et al.*, 1988) (which cross-reacts strongly with the P-protein from *P. stutzeri* but poorly with the P-protein from *E. coli*) was used to demonstrate the presence of protein bands in extracts of wild-type *P. stutzeri* and P-protein clones which have an apparent monomeric M_r of 41 000–42 000 (data not shown).

Sequence of the *pheA* gene

Both strands were sequenced for the gene and surrounding regions except for the 100 bp region upstream from the *SphI* site. The sequence shown in Fig. 3 contains an open reading frame (1095 base pairs) that encodes a protein of 365 amino acids with a calculated M_r of 40 844. Only one open reading frame among the six translation frames of the DNA sequence encoded a protein of sufficient M_r to be the *P. stutzeri* P-protein. This reading frame was in agreement with the orientation of the cloned fragment required for expression in *E. coli*. The sequence GGAG, found 9 bp upstream from the start codon, is presumably the ribosome-binding site (Shine & Dalgarno, 1974, 1975). The reading frame was terminated by the codon TAA. No prokaryotic factor-independent RNA polymerase terminators were detected (Brenzel & Trifonov, 1984).

Discussion

Characteristics of the *P. stutzeri pheA* gene

We have cloned, sequenced, and completed the initial characterization of the *P. stutzeri pheA* gene. This is the first phenylalanine-pathway gene of a pseudomonad species to be cloned and sequenced. Within Superfamily

1	10	30	50	60
61	70	90	110	120
121	130	150	170	180
181	190	210	230	240
241	250	270	290	300
301	310	330	350	360
361	370	390	410	420
421	430	450	470	480
481	490	510	530	540
541	550	570	590	600
601	610	630	650	660
661	670	690	710	720
721	730	750	770	780
781	790	810	830	840
841	850	870	890	900
901	910	930	950	960
961	970	990	1010	1020
1021	1030	1050	1070	1080
1081	1090	1110	1130	1140
1141	1150	1170	1190	1200
1201	1210	1230	1250	1260
1261	1270	1290	1310	1320
1321	1330	1350	1370	1380
1381	1390	1410	1430	1440
1441	1450	1470	1490	1500
1501	1510	1530	1550	1560
1561	1570	1577		

Fig. 3. Nucleotide sequence of the *P. stutzeri pheA* gene and its flanking regions. The one-letter abbreviations for the deduced amino acid sequence are shown below the DNA sequence. The proposed Shine-Dalgarno sequence (Shine & Dalgarno, 1974, 1975) is underlined and labelled S-D.

B. *P. stutzeri* belongs to rRNA homology group I (Palleroni *et al.*, 1973), which includes *P. aeruginosa*, *P. fluorescens*, *P. putida* and *P. syringae*. Group I pseudomonads exhibit considerable diversity for L-phenylalanine biosynthesis (Byng *et al.*, 1983). Although the

pathways for the monofunctional and bifunctional proteins catalysing the chorismate mutase and prephenate dehydratase reactions have been established through detailed enzymological characterization, and extensive evolutionary scenarios have been proposed (Ahmad & Jensen, 1988a), little information about gene organization or molecular-genetic structure is available.

Enzymic activity in transformed cells harbouring the 4.35 kb *PstI* fragment was observed in only one orientation (pJF9586) with respect to the vector pHCT9, indicating that the *pheA* gene was not efficiently expressed from native promoters in *E. coli*. Weak expression of *P. stutzeri* genes in *E. coli* due to inefficient recognition of promoters by *E. coli* RNA polymerase has been suggested (Fujita *et al.*, 1989). Because the nucleotide sequence preceding the initiation codon at position 187 contains an apparent Shine-Dalgarno sequence (Shine & Dalgarno, 1974, 1975) 9 bp upstream at bp 178, the mRNA of the *P. stutzeri* gene is expected to be translated in *E. coli*.

The G + C content of the *pheA* gene was 63.5 mol%, which falls within the published range of 60.6–66.3 mol% G + C for the *P. stutzeri* genome (Palleroni, 1984). Codon usage (data not shown) in the *P. stutzeri pheA* gene resembles that in *P. aeruginosa* (West & Iglewski, 1988), showing a marked preference for G or C in the third base in 87.4% of the codons. However, in contrast to all but one *P. aeruginosa* gene, C does not dominate as the nucleotide in the third codon position. Like *phnB* (Essar *et al.*, 1990) [initially named as *trpG* (Crawford & Eberly, 1986) in *P. aeruginosa*], C and G are used at nearly equal frequencies in the *P. stutzeri pheA* gene. It is interesting to note that *phnB* encodes the β subunit of an isoenzyme of anthranilate synthase which functions in pyocyanin synthesis and curiously has been found to resemble the TrpG subunit of the anthranilate synthase of *E. coli* more than the TrpG subunit expressed by *P. aeruginosa*. Whether the codon usage pattern shared by the *P. stutzeri pheA* and *P. aeruginosa phnB* genes portends a relationship of biological significance is unknown. Although the usage of rare codons in the *pheA* gene is low, the tyrosine TAT codon was used equivalent with TAC, and TAA functioned as the termination codon instead of TGA, which is the preferred termination codon in *P. aeruginosa* genes (West & Iglewski, 1988). The effects that these features of codon usage in the *pheA* gene may have on expressivity of mRNA *in vivo* are questions for continuing investigation.

Comparison of the *P. stutzeri* and *E. coli pheA* genes and homology of their predicted gene products

Since the time that the nucleotide sequence of the *E. coli pheA* gene was determined (Hudson & Davidson, 1984),

PS P-PR:	1	MSEADQLKAL	RVRIDSLDER	ILDILISERAR	CAQEVARVKT	ASWPKAEAV	50
EC P-PR:	1	MTSENPLLAL	REKISALDEK	LLALLAERRE	LAVEVGKAKL	LSHRPVRDI	49
PS P-PR:	51	FYRPEREAWV	LKHIMELNKG	.PLDNEEMAR	LFREIMSSCL	ALEQPL....	95
EC P-PR:	50	DRERDL	LERLITLGKA	HHLDAHYITR	LFQLIIEDSV	LTQQALLQQH	95
CG PDT:	1					MSDAPT....	6
PS P-PR:	96	R	VAYLGPEGTF	SQAA....AL	KHFGHSVISK	PMAA	126
EC P-PR:	96	LNKINPHSAR	IAFLGPKGSY	SHLAARQYAA	RHFEQFIESG	C.AK	138
CG PDT:	7	V	VAYLGPAAGT	TEAL....Y	KFADAGVFGD	GEIEQLPAKS	43
BS PDT:	1	MK	VGYLGPAATF	THLAVSSC..	..FQNGAEHV	AYRT	32
PS P-PR:	127	IDEVFREVVA	GAVNFGVVPV	ENSTEGAVNH	TLDSF.LEHD	IVICGEVELR	175
EC P-PR:	139	FADIFNQVET	GQADYAVVPI	ENTSSGAIND	VYDLLQ.HTS	LSIVGEMTLT	187
CG PDT:	44	PQEAVDVRH	GTAQFAVVAI	ENFVDGPVTP	TFDALDQGSN	VQIIAEEELD	93
BS PDT:	33	IPECIDAAVA	GEVDFAFVPL	ENALEGSVNL	TIDYLIHEQP	LPIVGEMTLP	82
PS P-PR:	176	IHHHLVGET	TKTDR..ITR	IYSHAQSLAQ	CRKWLDHAYP	NVERVAVSSN	223
EC P-PR:	188	IDHCLLVSGT	TDLST..INT	VYSHPPFPQQ	CSKFLN.RYP	HWKIEYTEST	234
CG PDT:	94	IAPFSISVRPG	TSLAD..VKT	LATHPVGYQQ	VKNWMATTIP	DAMYLASSN	141
BS PDT:	83	IHQHLLVHPS	RENAWKELDK	IYSHSHAIQA	CHKFLHRHFP	SVPEYANST	132
PS P-PR:	224	ADAARVK..	SEWNS..AAI	AGDMAAQLYG	LSKLAEKIED	RPDNSTRFLI	269
EC P-PR:	235	SAAMEKVAAQ	.AKSPHVAAL	GSEAGGTLYG	LQVLERIEAN	QRQNFTRFVV	282
CG PDT:	142	GAGAQMVA..	EGTAD..AAA	APSRRAELFG	LERLVDDVAD	VRGARTRFVA	187
BS PDT:	133	GAARFVSDH	PELNI..GVI	ANDMAASTYE	LKIVKRDQD	YRDNHTRFVI	180
PS P-PR:	270	IGSQEV..PP	TGD..	DKTSIIIVSMR	..NKPGALHE	LLMPFHSNGI	308
EC P-PR:	283	LARKAINVSD	Q.....VP	AKTLLMATG	..QQAGALVE	ALLVLRNHL	323
CG PDT:	188	VQAQAAVSEP	TGH..	DRTSVIFSLP	..NVPGLSVR	ALNEFGIRGV	228
BS PDT:	181	LSPDENISFE	VNSKLSSR..	PKTTLVMVLP	QDDQSGALHR	VLSAFSWRNL	228
PS P-PR:	309	DLTRIETRPS	RSGKWTVVFF	IDCMGHYQDP	LIKDVLEKID	HEAVALKVLG	358
EC P-PR:	324	IMTRLERPI	HGNPWEEMFY	LDIQANLESA	EMQKALKELG	EITRSMKVLG	373
CG PDT:	229	DLTRIESRPT	RKVFGTYRFH	LDISGHIRDI	PVAEALRALH	LQAEELVFVG	278
BS PDT:	229	NLSKIESRPT	KTGLGHYFFI	IDIEKAFDDV	LIPGAMQELE	ALGCKVRLLG	278
PS P-PR:	359	SYPKAVL...					365
EC P-PR:	374	CYPSENVVVPV	DPT.....				386
CG PDT:	279	SWPSNRRAEDS	TPQTDQLAKL	HKADEWVRAA	SEGKRLN...		315
BS PDT:	279	AYQSYQL...					285

Fig. 4. Alignment of the deduced amino acid sequences of the *P. stutzeri* P-protein, *E. coli* P-protein, *C. glutamicum* PDT, and *B. subtilis* PDT. Gaps have been introduced to maximize alignments with respect to identical and functionally similar amino acids. Identity among amino acid residues of the P-proteins of *P. stutzeri* and *E. coli* is represented by short lines between the sequences. The positions of identical residues among all sequences are designated with asterisks. The locations of amino acids aligning with residues thought to be associated with the enzyme activities of the *E. coli* P-protein are indicated by ■. Numbers to the left and right of the sequences indicate amino acid residue positions. Abbreviations not previously defined: PS, *P. stutzeri*; EC, *E. coli*; CG, *C. glutamicum*; BS, *B. subtilis*; P-PR, P-protein.

no other P-protein gene sequence has been reported. The deduced amino acid sequence encoded by the *P. stutzeri* *pheA* gene is comprised of 365 residues yielding an M_r of 40844. Although slightly smaller, the size of the *P. stutzeri* P-protein falls within the same range as the *E. coli* P-protein, which is reported to consist of 386 amino acid residues having an M_r of 43111 (Hudson & Davidson, 1984). Amino acid identities between the predicted *pheA* gene products of *P. stutzeri* and *E. coli* are shown in Fig. 4. Alignment of the two amino acid sequences revealed that 114 of the 365 residues of the *P. stutzeri* gene product were identical to those of *E. coli*. Significant identity was noted throughout the entire sequence. From the group of three amino acid residues reported to be associated with the activities of the P-protein from *E. coli* (Baldwin & Davidson, 1981; Hudson & Davidson, 1984), the cysteine residue 216 and threonine residue 278 have been conserved in the *P. stutzeri* protein, while the cysteine residue 374 has undergone a change to serine. Interestingly, six residues immediately surrounding the cysteine residue at 374 have been conserved in both proteins.

Alignments of the amino acid sequences of the *P. stutzeri* P-protein, *E. coli* P-protein and *E. coli* T-protein revealed homology in the N-terminal portions of these peptides (data not shown). Of the first 42 residues of the *P. stutzeri* P-protein, 18 and 14 were identical to the *E. coli* P-protein and T-protein, respectively. Similarly, alignments of the *E. coli* P-protein and T-protein peptides showed 18 amino acid matches for this region. The homology between the *E. coli* proteins and the *P. stutzeri* P-protein continues throughout the first 80–90 residues of the *P. stutzeri* gene product but disappears beyond this region. This is consistent with the report by Hudson & Davidson (1984) which demonstrated homology between only the N-terminal portions (chorismate mutase domain) of the two bifunctional *E. coli* proteins.

In *E. coli*, the genes for the P-protein (*pheA*), T-protein (*tyrA*), and DAHP synthase isoenzyme (tyrosine-sensitive) (*aroF*) are organized together in two operons on the chromosome, separated by a bidirectional transcription terminator (Hudson & Davidson, 1984). In addition, unidentified reading frames (URF) were identified at

regions outside of the two operons. The sequences 5' and 3' to the *P. stutzeri pheA* gene were analysed for regions which might possess similarity to the *pheA* and *aroF/tyrA* operons of *E. coli*. After analysis with all six reading frames, no regions were recognized which demonstrated homology to the *aroF* gene product, the *phe* leader peptide, the *tyrA* gene product, or the two URF regions. Experiments to recognize unidentified gene sequences in regions contiguous to the DNA sequenced for this investigation are in progress to reveal potential operons, and to establish gene order and organization.

Evolutionary relationships between enzymes of phenylalanine biosynthesis

In addition to the *E. coli pheA*, two genes encoding monofunctional PDTs have been sequenced from Gram-positive bacteria. The deduced amino acid sequences of the monofunctional PDTs of *Bacillus subtilis* (Trach & Hoch, 1989) and *Corynebacterium glutamicum* (Follettie & Sinskey, 1986) showed strong identity to the C-terminal (residues 90–365) portion of the *P. stutzeri* P-protein. The multiple alignment shown in Fig. 4 demonstrates that the peptide sequences of the *P. stutzeri* P-protein, *E. coli* P-protein, *C. glutamicum* PDT and *B. subtilis* PDT are homologous. In this alignment 34 residues are conserved in all four peptides. Many more identities are seen if the sequences are compared in a pairwise fashion. The results obtained from binary alignments of the *P. stutzeri* peptide sequence with those of the *E. coli* P-protein, *C. glutamicum* PDT, or *B. subtilis* PDT (Table 3) reveal a limited range of identity values (31–35%). Interestingly, the *C. glutamicum* and *B. subtilis* PDTs showed amino acid identities of 26% and 31%, respectively, with the *E. coli* P-protein sequence but only 29% when aligned with each other even though both enzymes are from Gram-positive bacteria, possessing similar allosteric characteristics of being stimulated by remote effectors (hydrophobic amino acids) and feedback inhibited by L-phenylalanine (Ahmad & Jensen, 1986; Berry *et al.*, 1987; Fazel & Jensen, 1980; Pierson & Jensen, 1974).

Of the three amino acid residues reported to be required for activity of the *E. coli* P-protein (Baldwin & Davidson, 1981; Hudson & Davidson, 1984) and designated in Fig. 4, only the threonine at position 278 was conserved in the *P. stutzeri* P-protein, *B. subtilis* PDT, and *C. glutamicum* PDT. It is of interest to note that mutagenesis studies demonstrated that threonine residue 278 was essential for prephenate dehydratase activity (Baldwin & Davidson, 1981). While it appears that the prephenate dehydratases may share a common ancestor, a monophyletic origin of chorismate mutase enzymes is not obvious since the CM-Fs of *B. subtilis*

Table 3. Comparison of binary alignments between various prephenate dehydratase enzymes

Organism and enzyme	Percentage identity/similarity		
	<i>E. coli</i> P-protein	<i>C. glutamicum</i> PDT	<i>B. subtilis</i> PDT
<i>P. stutzeri</i> P-protein	31/57	33/51	35/58
<i>C. glutamicum</i> PDT	26/48		29/55
<i>B. subtilis</i> PDT	31/58	29/55	

(Gray *et al.*, 1990) and *Saccharomyces cerevisiae* (Schmidheini *et al.*, 1989) showed marginal to no similarity to the *P. stutzeri* P-protein, the *E. coli* P-protein, the *E. coli* T-protein, or to each other. The sequence of the gene encoding the bifunctional DAHP synthase:chorismate mutase from *B. subtilis* 168 (Hoch & Nester, 1973; Nasser & Nester, 1967) may provide clues about the evolution of chorismate mutase.

The phylogenetic relationships of *Bacillus*, *Corynebacterium*, *Escherichia* and *Pseudomonas* (rRNA homology group I) are not paralleled by the homology comparisons of the various chorismate mutase and prephenate dehydratase gene products considered in this paper. PDT (*Bacillus* and *Corynebacterium*), P-protein (*P. stutzeri*, *Escherichia*) and T-protein (*Escherichia*) exhibit similar homologies in all combinations of pairwise comparisons. It would appear that the evolutionary time sufficient for maximal divergence has occurred between even the most closely related lineages compared (i.e. *Escherichia* and *Pseudomonas*). The similar level of homology retained for all dehydratase peptides presumably reflects a degree of conservation mandated by constraints linked to catalytic requirements for dehydratase activity. When sequence data are available for more closely related lineages, it will be interesting to see at what phylogenetic depth the molecular tree based upon dehydratase genes parallels the phylogenetic tree based upon 16S rRNA.

This investigation was supported by National Institutes of Health Grant DK38309. This paper is Florida Agricultural Experiment Station Journal no. R-01059.

References

- AHMAD, S. & JENSEN, R. A. (1986). The evolutionary history of two bifunctional proteins that emerged in the purple bacteria. *Trends in Biochemical Sciences* **11**, 108–112.
- AHMAD, S. & JENSEN, R. A. (1988a). New prospects for deducing the evolutionary history of metabolic pathways in prokaryotes: aromatic biosynthesis as a case-in-point. *Origins of Life* **18**, 41–57.
- AHMAD, S. & JENSEN, R. A. (1988b). Phylogenetic distribution of components of the overflow pathway to L-phenylalanine within the enteric lineage of bacteria. *Current Microbiology* **16**, 295–302.

- AHMAD, S., WILSON, A. T. & JENSEN, R. A. (1988). Chorismate mutase:prephenate dehydratase from *Acinetobacter calcoaceticus*. Purification, properties and immunological cross-reactivity. *European Journal of Biochemistry* **176**, 69–79.
- BALDWIN, G. S. & DAVIDSON, B. E. (1981). A kinetic and structural comparison of chorismate mutase/prephenate dehydratase from mutant strains of *Escherichia coli* K-12 defective in the *pheA* gene. *Archives of Biochemistry and Biophysics* **211**, 66–75.
- BERRY, A., AHMAD, S., LISS, A. & JENSEN, R. A. (1987). Enzymological features of aromatic amino acid biosynthesis reflect the phylogeny of mycoplasmas. *Journal of General Microbiology* **133**, 2147–2154.
- BRENDEL, V. & TRIFONOV, E. N. (1984). A computer algorithm for testing potential prokaryotic terminators. *Nucleic Acids Research* **12**, 4411–4427.
- BYNG, G. S. & JENSEN, R. A. (1983). Impact of isozymes upon partitioning of carbon flow and regulation of aromatic biosynthesis in prokaryotes. In *Isozymes*, vol. 8, pp. 115–140. Edited by M. C. Ratazzi, J. G. Scandalios & G. S. Whitt. New York: Alan R. Liss.
- BYNG, G. S., WHITAKER, R. J. & JENSEN, R. A. (1983). Evolution of L-phenylalanine biosynthesis in rRNA homology group I of *Pseudomonas*. *Archives of Microbiology* **136**, 163–168.
- CARLSON, C. A., PIERSON, L. S., ROSEN, J. J. & INGRAHAM, J. L. (1983). *Pseudomonas stutzeri* and related species undergo natural transformation. *Journal of Bacteriology* **153**, 93–99.
- CARLSON, C. A., STEENBERGEN, S. M. & INGRAHAM, J. L. (1984). Natural transformation of *Pseudomonas stutzeri* by plasmids that contain cloned fragments of chromosomal deoxyribonucleic acid. *Archives of Microbiology* **140**, 124–138.
- CLEWELL, D. B. & HELINSKI, D. R. (1969). Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular form. *Proceedings of the National Academy of Sciences of the United States of America* **62**, 1159–1166.
- COTTON, R. G. H. & GIBSON, F. (1965). The biosynthesis of phenylalanine and tyrosine: enzymes converting chorismic acid into prephenic acid and their relationships to prephenate dehydratase and prephenate dehydrogenase. *Biochimica et Biophysica Acta* **100**, 76–88.
- CRAWFORD, I. P. & EBERLY, L. (1986). Structure and regulation of the anthranilate synthase genes in *Pseudomonas aeruginosa*. I. Sequence of *trpG* encoding the glutamine amidotransferase subunit. *Molecular Biology and Evolution* **3**, 436–448.
- DAGERT, M. & EHRLICH, S. D. (1979). Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene* **6**, 23–28.
- DAVIS, R. W., BOTSTEIN, D. & ROTH, J. R. (1980a). In *Advanced Bacterial Genetics*, pp. 201. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- DAVIS, R. W., THOMAS, M., CAMERON, J., ST JOHN, T. P., SCHERER, S. & PADGETT, R. A. (1980b). Rapid DNA isolations for enzymatic and hybridization analysis. *Methods in Enzymology* **65**, 404–411.
- DAYAN, J. & SPRINSON, D. B. (1970). Preparation of prephenic acid. *Methods in Enzymology* **17A**, 559–561.
- DEVEREUX, J., HAEERLI, P. & SMITHIES, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* **12**, 387–395.
- ESSAR, D. W., EBERLY, L., HADERO, A. & CRAWFORD, I. P. (1990). Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. *Journal of Bacteriology* **172**, 884–900.
- FAZEL, A. M. & JENSEN, R. A. (1980). Regulation of prephenate dehydratase in coryneform species of bacteria by L-phenylalanine and by remote effectors. *Archives of Biochemistry and Biophysics* **200**, 165–176.
- FISCHER, R. S., BERRY, A., GAINES, C. G. & JENSEN, R. A. (1986). Comparative action of glyphosate as a trigger of energy drain in eubacteria. *Journal of Bacteriology* **168**, 1147–1154.
- FOLLETTIE, M. T. & SINSKEY, A. J. (1986). Molecular cloning and nucleotide sequence of the *Corynebacterium glutamicum pheA* gene. *Journal of Bacteriology* **167**, 695–702.
- FUJITA, M., TORIGOE, K., NAKADA, T., TSUSAKI, K., KUBOTA, M., SAKAI, S. & TSUJISAKA, Y. (1989). Cloning and nucleotide sequence of the gene (*amyP*) for maltotetraose-forming amylase from *Pseudomonas stutzeri* MO-19. *Journal of Bacteriology* **171**, 1333–1339.
- GIBSON, F. (1964). Chorismic acid: purification and some chemical and physical studies. *Biochemical Journal* **90**, 256–257.
- GRAY, J. V., GOLINELLI-PIMPANEAU, B. & KNOWLES, J. R. (1990). Monofunctional chorismate mutase from *Bacillus subtilis*: purification of the protein, molecular cloning of the gene and overexpression of the gene product in *Escherichia coli*. *Biochemistry* **29**, 376–383.
- HOCH, J. A. & NESTER, E. W. (1973). Gene-enzyme relationships of aromatic acid biosynthesis in *Bacillus subtilis*. *Journal of Bacteriology* **116**, 59–66.
- HOHN, B. & COLLINS, J. (1980). A small cosmid for efficient cloning of large DNA fragments. *Gene* **11**, 291–298.
- HUDSON, G. S. & DAVIDSON, B. E. (1984). Nucleotide sequence and transcription of the phenylalanine and tyrosine operons of *Escherichia coli* K-12. *Journal of Molecular Biology* **180**, 1023–1051.
- HUMPHREYS, G. O., WILLSHAW, G. A. & ANDERSON, E. S. (1975). A simple method for the preparation of large quantities of pure plasmid DNA. *Biochimica et Biophysica Acta* **383**, 457–463.
- KUPERSZTOCH, Y. M. & HELINSKI, D. R. (1973). A catenated DNA molecule as an intermediate in the replication of the resistance transfer factor R6K in *Escherichia coli*. *Biochemical and Biophysical Research Communications* **54**, 1451–1459.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*, pp. 431–435. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- NASSER, D. & NESTER, E. W. (1967). Aromatic amino acid biosynthesis: gene-enzyme relationships in *Bacillus subtilis*. *Journal of Bacteriology* **94**, 1706–1714.
- PALLERONI, N. J. (1984). Gram-negative aerobic rods and cocci. Family I. *Pseudomonadaceae*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 172–173. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- PALLERONI, N. J., KUNISAWA, R., CONTOPULAS, R. & DOUDOROFF, M. (1973). Nucleic acid homologies in the genus *Pseudomonas*. *International Journal of Systematic Bacteriology* **23**, 333–339.
- PATEL, N., PIERSON, D. L. & JENSEN, R. A. (1977). Dual enzymatic routes to L-tyrosine and L-phenylalanine via pretyrosine in *Pseudomonas aeruginosa*. *Journal of Biological Chemistry* **252**, 5839–5846.
- PIERSON, D. L. & JENSEN, R. A. (1974). Metabolic interlock: control of an interconvertible prephenate dehydratase by hydrophobic amino acids in *Bacillus subtilis*. *Journal of Molecular Biology* **90**, 563–579.
- PROBER, J. M., TRAINOR, G. L., DAM, R. J., HOBBS, F. W., ROBERTSON, C. W., ZAGURSKY, R. J., COCUZZA, A. J., JENSEN, M. A. & BAUMEISTER, K. (1987). A system for rapid DNA sequencing with fluorescent chain-terminating dideoxy nucleotides. *Science* **238**, 336–341.
- SCHMIDHEINI, T., SPERISEN, P., PARAVICINI, G., HUTTER, R. & BRAUS, G. (1989). A single point mutation results in a constitutively activated and feedback-resistant chorismate mutase of *Saccharomyces cerevisiae*. *Journal of Bacteriology* **171**, 1245–1253.
- SHIIO, I. & SUGIMOTA, S. (1979). Two components of chorismate mutase in *Brevibacterium flavum*. *Journal of Biochemistry* **86**, 17–25.
- SHINE, J. & DALGARNO, L. (1974). The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. *Proceedings of the National Academy of Sciences of the United States of America* **71**, 1342–1346.
- SHINE, J. & DALGARNO, L. (1975). Determination of cistron specificity in bacterial ribosomes. *Nature, London* **254**, 34–38.
- TRACH, K. & HOCH, J. A. (1989). The *Bacillus subtilis spoOB* stage 0 sporulation operon encodes an essential GTP-binding protein. *Journal of Bacteriology* **171**, 1362–1371.
- WEST, S. E. H. & IGLEWSKI, B. H. (1988). Codon usage in *Pseudomonas aeruginosa*. *Nucleic Acids Research* **16**, 9323–9335.
- YUAN, R. & LIN, Y. (1982). The purification of restriction enzymes and DNA substrates. Their use in DNA sequencing. In *Genetic Engineering Techniques: Recent Developments*, pp. 337–349. Edited by P. C. Huang, T. T. Kuo & R. Wu. New York: Academic Press.
- ZAMIR, L. O., TIBERIO, R., FISKE, M., BERRY, A. & JENSEN, R. A. (1985). Enzymatic and nonenzymatic dehydration reactions of L-arogenate. *Biochemistry* **24**, 1607–1612.