Escherichia coli Phenylalanyl-tRNA Synthetase Operon: Characterization of Mutations Isolated on Multicopy Plasmids

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Plasmid pB1 carries the genes for threonyl-tRNA synthetase, phenylalanyl-tRNA synthetase, and translation initiation factor IF3. Strains carrying this plasmid overproduce phenylalanyl-tRNA synthetase about 100-fold. Spontaneous mutant plasmids were obtained which no longer caused the overproduction of the enzyme. Three classes of mutations were found. (i) Deletion mutations were found, some of which had the interesting property of fusing different genes together, e.g., putting phenylalanyl-tRNA synthetase under the control of the threonyl-tRNA synthetase promoter. (ii) Insertion mutations were found; one insertion in particular was studied. This insertion is located in front of the structural gene for phenylalanyl-tRNA synthetase and is shown to interrupt a *cis*-acting regulatory region. (iii) Mutations that showed no major change in DNA structure were found. One of these mutations is apparently purely structural, as it produces a small subunit of phenylalanyl-tRNA synthetase with a reduced molecular weight. This protein is less stable than the wild-type enzyme. These mutations represent useful tools to investigate how the phenylalanyl-tRNA synthetase operon is regulated.

Phenylalanyl-tRNA synthetase is a tetrameric enzyme with two different subunits ($\alpha_2\beta_2$ structure). The genes corresponding to the small subunit (*pheS*) and to the large subunit (*pheT*) have been shown to be adjacent on the *Escherichia coli* chromosome at 38 min (10, 23). A λ transducing phage was isolated which carries, besides *pheS* and *pheT*, the structural genes for threonyl-tRNA synthetase (*thrS*) and for translation initiation factor IF3 (*infC*) (12, 13, 26). The order of the genes is *thrS infC pheS pheT* (23, 25). These four genes are transcribed in the direction from *thrS* to *pheT* (i.e., counter-clockwise on the standard *E. coli* map [2]), and *pheS* and *pheT* are transcribed from the same promoter, located after *infC* (21, 27).

The expression of the *pheS, T* operon is constitutive, as expected, since phenylalanyl-tRNA synthetase is an essential enzyme. However, this synthetase, like several other aminoacyl-tRNA synthetases, is subject to metabolic regulation (20), i.e., its synthesis rate increases with the growth rate of the cell. It has also been shown that withdrawing phenylalanine from a phenylalanine bradytroph provokes an increased synthesis of phenylalanyl-tRNA synthetase (19). The biosynthesis of multimeric protein systems presents a special problem, namely, the stoichiometric synthesis of the various components. The cotranscription of the *pheS, T* operon causes the equimolar synthesis of the mRNA of the two genes; this, however, does not necessar-

ily ensure the equimolar synthesis of the gene products.

One way to investigate the mechanisms regulating the expression of the *pheS, T* operon is to study mutants with an altered expression. This work describes the isolation and characterization of spontaneous mutations directly on plasmids. The fact that the mutations are isolated on plasmids has several advantages: (i) mutations can be isolated that would be lethal on the chromosome; (ii) the analysis of the mutant plasmid DNA makes it easy to classify the mutations as deletions, insertions, or point mutations; (iii) as wild-type *pheS, T* mRNA is strongly expressed from the type of multicopy plasmid employed, significant mRNA measurements can be made even with "down" mutations.

A variety of information has been derived directly from the mutants described here, e.g., relative strength of promoters and physical location of regulatory and structural regions of the *pheS, T* operon. Some of the mutants characterized in this paper are the object of the transcription studies reported in the accompanying paper (22) and provide information about how the operon is regulated.

MATERIALS AND METHODS

Bacterial strains and plasmids. IBPC1365 is F^- and *thi-1 argE3 his-4 proA2 lacY1 galK2 mtl-1 xyl-5 tsx-29 supE44* λ^- (27); IBPC2001 has the same markers as IBPC1365, except it is *recA1* and *rpoB* (27); IBPC1671

has the same markers as IBPC1365, except it is *rpsL*, *recA1 pheS5* (23); IBPC1772 has the same markers as IBPC1365, except that it is *rpsL recA1 pheT354* (23). IBPC4771 is identical to IBPC1365, except that it is *rpsL thrS1029* (27). Plasmid pB1 (23; with revised sizes of the DNA restriction fragments, G. Fayat et al., personal communication) is shown in Fig. 1, together with the structures of the mutant plasmids derived from it. The isolation of plasmids pGC87 and pG6-7 has been described elsewhere (21). They carry, respectively, the *Pst*I2 to *Pst*I3 and *Pst*I2 to *Pst*I4 fragments of pB1 cloned into plasmid pGA39 (1). Plasmid pG6-7 carries the whole structural gene for *pheS*, whereas pGC87 carries only the promoter proximal part. Both plasmids confer resistance to chloramphenicol on the host bacteria.

Isolation of mutant pB1 plasmids. The mutant plasmids described here all arose spontaneously. Systematic screening was carried out as follows. Independent isolates of IBPC1365 and IBPC2001 carrying pB1 were grown in 3 ml of LB supplemented with ampicillin (50 μ g ml⁻¹) and tetracycline (10 μ g ml⁻¹). After being aged at room temperature for 1 to several weeks, the cultures were diluted and regrown to saturation. After being streaked out on LB plates containing 50 μ g of ampicillin per ml and 10 μ g of tetracycline per ml, single colonies were taken and grown in LB containing ampicillin and tetracycline, and extracts were analyzed by sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis as described previously (15). Plasmid DNA was extracted from non-overproducing mutant strains by the rapid alkaline extraction method (4) and analyzed with restriction enzymes as described previously (23). Plasmid DNA was introduced into cells made competent by treatment with CaCl₂ (23). Strains were cured of plasmids by many generations of growth in the absence of antibiotic selection.

Assay of aminoacyl-tRNA synthetases. IBPC2001 carrying pB1, pB1T, pB1S, or pBR322 was grown in morpholinepropanesulfonic acid (MOPS) medium supplemented with all of the amino acids (20), 50 μ g of ampicillin per ml, and 10 μ g of tetracycline per ml. Samples of exponentially growing cells (absorbance at 650 nm [*A*₆₅₀] = 0.4) were spun down in small polyethylene tubes and frozen at -80°C. Just before being assayed, S30 extracts were made by suspending the cells in 300 μ l of 10 mM Tris-hydrochloride (pH 6.8)–0.1 mM EDTA–10% glycerol–10 mM mercaptoethanol and then sonicating 4 $\times$ 15 s, with 15 s cooling in ice in between. The sonicated extracts were centrifuged for 4 min in a microfuge to give a clear supernatant, which was decanted and assayed immediately for synthetase activity.

Synthetases were assayed in the following buffer: 50 mM Tris-hydrochloride (pH 7.5), 10 mM MgCl₂, 10 mM KCl, 10 mM β -mercaptoethanol, 1 mM ATP, 4 mg of tRNA per ml, and 50 μ M ¹⁴C-labeled threonine or phenylalanine (specific activity, ~20 mCi mmol⁻¹) or ³⁵S-labeled methionine (specific activity, ~50 mCi mmol⁻¹). Incubation was at 37°C in 100 μ l. The S30s were diluted as necessary into the sonication buffer to give a linear response in 15 min (less than 20% maximum tRNA charging capacity). Reactions were quenched with ice-cold 7% trichloroacetic acid containing 0.5% Casamino Acids. Precipitates were collected on Whatman GF/C filters, extensively washed with the trichloroacetic acid containing Casamino Ac-

ids, rinsed with 70% ethanol, and dried. Protein concentration in the S30s was estimated by the Coomassie blue method (6), using bovine serum albumin as a standard.

Labeling of cells with [³⁵S]methionine and immunoprecipitation. IBPC2001 carrying plasmids pB1, pB1T, pB1S, or pBR322 was grown as described for the test for synthetases. At *A*₆₅₀ = 0.4, the cells were spun down, washed, and suspended in the same medium minus methionine. After 10 min of growth to starve for methionine, the cultures were pulse-labeled with [³⁵S]methionine at 40 μ Ci ml⁻¹ (specific activity, 200 Ci mmol⁻¹). After 2 min, unlabeled methionine was added to a final concentration of 1 mM. Samples (1 ml) of the labeled cultures were removed at 2, 10, 30, and 120 min and, when the cultures were saturated, were chilled in ice, collected by centrifugation, washed with 10 mM Tris-hydrochloride (pH 6.8)–1 mM EDTA and frozen at -80°C. The frozen cells were suspended in 10 mM Tris-hydrochloride (pH 6.8)–1.0 mM EDTA, lysed by three cycles of freezing and thawing, treated with 100 μ g of DNase per ml, and suspended in sodium dodecyl sulfate sample buffer (14). Samples were immune precipitated with antisera to phenylalanyl-tRNA synthetase in 10 mM sodium phosphate (pH 7.0)–150 mM NaCl–0.5% Triton X-100–0.1% sodium dodecyl sulfate as described previously (27). The immunoglobulins with bound antigens were separated from the bulk of the radioactive material by absorbing to protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). After being washed the antigen-antibody-protein A complex was disrupted by the addition of sodium dodecyl sulfate sample buffer and then analyzed on 2-mm-thick 10% sodium dodecyl sulfate-polyacrylamide gel. The gel was treated for fluorography (17) and exposed to RP X-Omat film at -70°C.

Construction of λ B1S and λ B1T. The structures of the starting phages λ 512 (14), an *Eco*RI substitution vector, and λ NM540 (5), a *Hind*III insertion vector are shown in Fig. 2. The principle of the cloning is to insert the *Eco*RI-*Hind*III fragment of pB1S and pB1T between the left-hand arm of λ 512, ending with an *Eco*RI site, and the right-hand arm of λ NM540, ending with a *Hind*III site (Fig. 2). Phages λ 512 and λ NM540 were digested, respectively, with *Eco*RI or *Hind*III, and pB1S and pB1T were digested with both enzymes. Each phage DNA (0.6 μ g) was ligated with 0.2 μ g of pB1S or pB1T and used to transfect IBPC2001 made competent with CaCl₂ (23). Several hundred plaques (over four plates) were obtained, which were pooled to produce a low-titer lysate. Phages capable of transducing IBPC4771 (a *thrS* mutant auxotrophic for threonine) to prototrophy were selected by plating the lysate on IBPC4771 and picking lysogens from the center of the plaques. Lysogens capable of growing in the absence of threonine and exhibiting the *imm*21 character of λ NM540 were purified. UV induction of these lysogens gave lysates with titers of 10⁹ PFU ml⁻¹. These lysates were tested for their ability to transduce the thermosensitive *pheS* and *pheT* recombination-deficient (IBPC1671 and IBPC1772) strains to thermoresistance.

RESULTS

Isolation of the mutants. Plasmid pB1, a derivative of pBR322 with an *E. coli Eco*RI to *Hind*III

FIG. 2. Construction of λ B1T and λ B1S. The structure of λ 512 (14) and λ NM540 (5) is indicated with relevant restriction sites. E, *Eco*RI; Hd, *Hind*III; $\square$, *lac* substitution derived from λ plac5 of λ 512; $\square$, *imm*21 substitution of λ NM540; $\square$, deleted DNA. The positions of genes in pB1T and pB1S are as indicated in the legend to Fig. 1. $\square$, pBR322 DNA.

insert (Fig. 1), carries *thrS*, *infC*, *pheS*, and *pheT* (21). Cells carrying this plasmid overproduced phenylalanyl-tRNA synthetase, translation initiation factor IF3 (IF3) and, to a lesser extent, threonyl-tRNA synthetase. Enzyme activity measurements (Table 1) showed that strains carrying pB1 overproduce phenylalanyl-tRNA synthetase on the order of 100-fold and threonyl-tRNA synthetase on the order of 10-fold compared with pBR322-carrying strains. Similar levels of overproduction of the two synthetases have been measured by the ATP-pyrophosphate exchange assay (A. Ducruix, G. Fayat, S. Blanquet, personal communication). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of extracts from pB1-carrying strains shows an overproduction of both subunits of phenylalanyl-tRNA synthetase and also of IF3, but no clearly visible overproduction of threonyl-tRNA synthetase (Fig. 3, lane 2).

The overproduction of proteins caused by the presence of pB1 in the cell might be somehow detrimental to the cell and could explain why spontaneous mutants, which no longer cause phenylalanyl-tRNA synthetase overproduction, are easily isolated. The frequency of these mutations is such that they can be screened for by the systematic polyacrylamide gel electrophoresis of extracts from clones isolated from pB1-carrying strains. In all cases, the loss of protein overproduction was found to be associated with the plasmid. A strain that no longer overproduced phenylalanyl-tRNA synthetase and had been cured of its plasmid gave, upon retransformation with wild-type pB1 DNA, a strain overproducing both phenylalanyl-tRNA synthetase

and IF3. Plasmid DNA extracted from the non-overproducing strain, on introduction into another strain, exhibited the mutant pattern of protein synthesis.

Polyacrylamide gel analysis of several such non-overproducing mutants is shown in Fig. 3. Several of these mutants overproduced neither IF3 nor phenylalanyl-tRNA synthetase, some still overproduced IF3 but not the synthetase, and one still overproduced the synthetase but not IF3. All of the mutants, except pB1TZ, were isolated from pB1 in a single step. As well as being analyzed for protein overproduction in saturated cultures, exponentially growing cells were pulse-labeled with [35 S]methionine, and extracts were examined on denaturing poly-

TABLE 1. Aminoacyl-tRNA synthetase activities in IBPC2001 carrying different plasmids^a

Plasmid	Aminoacyl-tRNA synthetase		
	Thr	Phe	Met
pBR322	7 $\pm$ 3	20 $\pm$ 8	2 $\pm$ 2
pB1	55 $\pm$ 25	1,500 $\pm$ 500	0.8 $\pm$ 0.5
pB1S	100 $\pm$ 20	70 $\pm$ 30	1.1 $\pm$ 0.1
pB1T	110 $\pm$ 60	20 $\pm$ 10	1.2 $\pm$ 0.3
pB1M1	5 $\pm$ 2	1,020 $\pm$ 150	1.6 $\pm$ 0.1
pB1M5	33	19	1.8

^a Activities are expressed in picomoles of tRNA aminoacylated per microgram of extract per 15 min. Error limits indicate the level of reproducibility of extracts made from cultures grown at different times. For pB1 and pBR322, these are the average of at least five experiments. For pB1S, pB1T, and pB1M1, they are the mean of two experiments; for pB1M5, the result of a single experiment is shown.

FIG. 1. Structure of plasmid pB1 and mutants derived from it. The positions of the four genes *thrS* ($\square$), *infC* ($\square$), *pheS* ($\square$), and *pheT* ($\square$) are taken from data in references 23, 24, and 27. The positions of the restriction sites are taken from the revised map of pB1 (G. Fayat et al., personal communication). Deletions are indicated by blocked-in regions. The serrated edges indicate that the exact end of the in vivo deletions is not known.

FIG. 3. Proteins synthesized by bacteria carrying pB1 and derived plasmids. Plasmid-carrying strains were grown to saturation in LB medium containing 50 μ g of ampicillin per ml and 10 μ g of tetracycline per ml. Cells were collected by centrifugation and lysed by suspending in sodium dodecyl sulfate sample buffer (15) and heating in a boiling water bath for 4 min. Samples equivalent to 0.1 A_{650} U were analyzed on a 12.5% polyacrylamide gel. Proteins were visualized with Coomassie blue. The positions of the small and large subunits of phenylalanyl-tRNA synthetase (α PheRS and β PheRS, respectively) and translational initiation factor IF3 are indicated.

acrylamide gels. Comparing strains carrying mutant plasmids with a strain carrying pBR322, we found that pulse-labeling sometimes reveals the synthesis of plasmid-coded proteins that are not visible as overproduced proteins when saturated cells are examined. This would suggest that the stability of the gene products has somehow been affected by the mutation. Plasmids which show this phenomenon are pB1T, pB1T2, pB1E4, and pB1S4. To test the complementation properties of the mutant plasmids, we used their DNA to transform *pheS* and *pheT* thermosensitive recombination-deficient (*recA*) strains. The transformed strains were subsequently tested for growth at the restrictive temperature. Table 2 summarizes the properties of a representative sample of the mutants found.

The level of protein overproduction in bacterial strains due to the presence of genes carried on multicopy plasmids is critically dependent on the copy number of the plasmids studied. pB1 is a derivative of pBR322, a relaxed copy number plasmid, and several observations make us think that the mutations, isolated within the *E. coli* DNA insert of pB1, do not affect the copy number of the plasmid. When a wild-type gene was carried by a plasmid, the overproduction was constant, e.g., when seen on denaturing gels, the level of IF3 was constant (Fig. 2).

Extracts of [35 S]methionine-labeled cultures showed similar amounts of pulse-labeled threonyl-tRNA synthetase. We also measured the level of β -lactamase inside plasmid-carrying strains. Samples of exponentially growing cells were washed three times and sonicated, and β -lactamase was measured by the ability of penicilloic acid (the product of the action of β -lactamase on penicillin) to reduce iodine (M. Trudel, M. Graffe, and M. Springer, manuscript in preparation). These experiments (data not shown) suggest that the copy number of pB1 is on the order of one-fifth that of pBR322. The levels of β -lactamase in pB1- and mutated-pB1-carrying strains varied by a maximum of factor two. This again suggests that the pattern of protein overproduction seen with pB1- and mutated-pB1-bearing strains is not a function of plasmid copy number.

Classification of the mutant plasmids. The mutant plasmid DNAs were analyzed with restriction endonucleases. The structure of these plasmids (Fig. 1) permits a classification of the mutations into three categories: (i) large deletions, such as pB1S3, pB1S4, pB1M1, and pB1M5; (ii) insertions, such as pB1S, pB1T2, and pB1T3; (iii) point mutations where no gross effect on the DNA restriction pattern is observed; pB1T and pB1E4 belong in this category. Initially, we describe the properties of these three classes of mutants and subsequently describe experiments with two of the mutants to explore the regulation of the *pheS,T* operon.

(i) Deletions which caused fusions with the *pheS,T* operon. All of the characterized genes carried on pB1 are transcribed in the same direction (21); creating deletions in pB1 thus yields gene or protein fusions. As all four genes are precisely located on a restriction map of pB1 (27), it is possible to identify the fusions by the analysis of mutant plasmid DNA. Several fusions between *thrS* and *pheS,T* were characterized. The deletion of pB1S4 starts within the *thrS* structural gene and ends to the right of *Pst*I3 (Fig. 1). This plasmid expresses *pheT*, as shown by the complementation and pulse-labeling of pB1S4-carrying strains (Table 2). The large subunit was not overproduced alone (at least not at the level of pB1-carrying strains). The pB1S3 plasmid carries a similar deletion, also starting within the *thrS* structural gene and ending to the right of *Pst*I3. This plasmid did not express *pheT* in a pulse, but did weakly complement *pheT* mutants (Table 2), suggesting that the expression of the *pheT* gene has been affected by the deletion. A possible explanation for the weak complementation is that the deletion removes the NH_2 -terminal part of the structural *pheT* gene, creating a protein fusion between *thrS* and *pheT*. This hypothetical hybrid protein

TABLE 2. Properties of pb1 and derived plasmids

Name	Mutation type	Genetic complementation		Overproduced proteins ^a						Pulse-labeled proteins ^b					
		<i>pheS</i>	<i>pheT</i>	ThrRS	IF3	α PheRS	β PheRS	Other	ThrRS	IF3	α PheRS	β PheRS	Other		
pB1	Wild type	+	+	—	+	+	+		$\pm$	+	+	+			
pB1S3	Deletion	—	$\pm$?	—	—	—	—		—	—	—	—			
pB1S4	Deletion	—	+	—	—	—	$\pm$		—	—	—	+			
pB1M1	Deletion	+	+	—	—	+	+		—	—	+	+			
pB1M5	Deletion	$\pm$?	+	—	—	—	—	22 kd	$\pm$	—	—	—	22 kd		
pB1S	Insertion	+	+	—	+	—	—		$\pm$	+	—	—			
pB1T2	Insertion	+	+	—	+	—	—		$\pm$	+	+	—			
pB1TZ	Insertion	ND ^c	ND	—	—	—	—		ND	ND	ND	ND			
pB1T	Point	+	+	—	+	—	—		$\pm$	+	+ ^d	$\pm$			
pB1E4	Point	ND	ND	—	+	—	—		$\pm$	+	$\pm$	—			

^a The protein band can be seen in total extracts of plasmid-carrying cells analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis compared with the cells carrying pBR322.

^b The protein band can be seen in autoradiograms of pulse-labeled plasmid-carrying cells analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis compared with cells carrying pBR322. α PheRS and β PheRS are, respectively, the small and large subunits of phenylalanyl-tRNA synthetase corresponding to genes *pheS* and *pheT*. ThrRS is threonyl-tRNA synthetase (gene *thrS*).

^c ND, Not done.

^d The mutant form of α PheRS approximately 1 kd smaller than the wild-type subunit.

would be too unstable to be seen in a pulse-labeling experiment but still sufficiently active to weakly complement *pheT* mutants.

A different type of fusion is shown by the plasmid pB1M5. It caused the overproduction of a 22-kilodalton (kd) protein (Fig. 3, lane 9) which was shown by immune precipitation to carry IF3 antigenic determinants (data not shown). The location of the deletion would strongly suggest a hybrid between *infC* and *pheS* genes. However, antisera against the small subunit of phenylalanyl-tRNA synthetase failed to precipitate the 22-kd protein. This might be expected if the *pheS* part of the hybrid gene is small. However, the size of the pB1M5 deletion and the molecular weight of the overproduced protein do not fit with a hybrid protein made of the NH₂-terminal part of IF3 and the COOH-terminal part of the synthetase small subunit. DNA sequence analysis (G. Fayat, C. Sacerdot, S. Blanquet, personal communication) shows that the distance between the beginning of *infC* and the end of *pheS*

is 2.5 kilobases (kb). The pB1M5 deletion is about 1.3 kb, leaving 1.2 kb for the hybrid gene. A DNA fragment of 1.2 kb should code for a protein of molecular weight greater than 40 kd. As the hybrid protein is of 22 kd, it seems likely that the fusion starting with *infC* is not in the correct reading frame of *pheS*, causing translation of the *pheS* part of the hybrid gene to terminate quickly at a normally out-of-phase termination codon. The weak complementation of *pheS* mutants with pB1M5 would, in this case, be due to some residual expression of *pheS* due to, for instance, an internal translation initiation, giving a protein lacking its NH₂-terminal end.

Plasmid pB1M1 caused the overproduction of phenylalanyl-tRNA synthetase but not IF3. Analysis of the DNA showed a large deletion starting within *thrS* and ending after the *AvaI*3 site. As discussed later, this eliminates the *pheS,T* promoter, so that the expression of phenylalanyl-tRNA synthetase is presumably from the *thrS* promoter. The overproduction by pB1M1 of the two subunits of the synthetase with wild-type molecular weights demonstrates two facts. It shows, firstly, that the intact structural genes of *pheS,T* are located to the right of the *AvaI*3 site and, secondly, that the overproduction of phenylalanyl-tRNA synthetase is not a function of the *pheS,T* promoter. The *thrS* promoter can cause about the same level of phenylalanyl-tRNA synthetase as the *pheS,T* promoter (Table 1), whereas its natural product, threonyl-tRNA synthetase, is only overproduced about 10-fold. This anomaly is still under investigation.

(ii) **Insertion mutants.** Plasmid pB1S carries an insertion of about 1.3 kb located between the *HpaI*5 and *AvaI*3 sites (Fig. 2) to the left of phenylalanyl-tRNA synthetase structural genes. This insertion has been given the designation $\Omega(pheSp::1.3\text{ kb})1001$. Indicative of the fact that this is a regulatory mutant, no synthesis of either *pheS* or *pheT* gene products was detected by pulse-labeling (Table 2 and Fig. 4, lane 11). There was, however, some residual synthesis of phenylalanyl-tRNA synthetase detected by the activity measurements and complementation data (Table 1). As shown later, the insertion interrupts a *cis*-acting regulatory region.

Plasmid pB1T2 carries an insertion of about 0.2 kb between the *PstI*3 and *Bam*HI sites of pB1 (Fig. 2). This fragment contains the end of *pheS* and the start of *pheT*. In a pulse, *pheS* is expressed normally, indicating that the insertion interferes with *pheT* expression only. The insertion is thus located either within the intercistronic region or within *pheT*.

Plasmid pB1TZ is a double mutant. It was isolated as an IF3 non-overproducer of pB1T,

FIG. 4. Immunoprecipitation with anti-phenylalanyl-tRNA synthetase of IBPC2001 carrying either pB1, pB1T, pB1S, or pB1T2. Exponentially growing cultures were pulse-labeled with [³⁵S]methionine for 2 min and then chased with nonradioactive methionine as described in the text. Samples of the labeled cells, equivalent to 5×10^5 cpm, were immune precipitated with antiserum as described in the text and then analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel (15). After electrophoresis, the gel was treated for fluorography (17) and exposed to R.P. X-Omat film at -70°C for 3 days. We performed the immune precipitation of IBPC2001 pB1, exponentially labeled (lane 1) and then chased for 8 min (lane 2), 28 min (lane 3), 118 min (lane 4), and allowed to saturate (lane 5). Lanes 6 to 10, As for lanes 1 to 5, but with a culture of IBPC2001 pB1T. Lanes 11 and 12, Immune precipitate of exponentially labeled IBPC2001 pB1S and IBPC2001 pB1T2. Lane 13, Total extract of exponentially labeled IBPC2001 pB1 (50,000 cpm).

already a phenylalanyl-tRNA synthetase non-overproducer. An insertion, about 0.6 kb, was located between *Hpa*I4 and *Sac*II2. This could be within the *infC* structural gene or a regulatory region. The insertions, which appear at a fairly high frequency, are apparently stable DNA structures, and no spontaneous revertants were found.

(iii) **Point mutations of the *pheS,T* operon.** Initially classified as a point mutation, pB1T was subsequently shown to be a very small deletion (20 to 30 bp) between *Ava*I3 and *Pst*I3 (Fig. 2). This deletion has been given the designation Δ *pheS1002*. Although genetically a deletion, it is quite different from the large deletions covering several kb of DNA and which were found with much greater frequency. Only one other point mutation, called pB1E4, has been detected. Detailed analysis of this latter plasmid has not yet been undertaken.

The deletion in pB1T (Δ *pheS1002*) is within the *pheS* structural gene, and the *pheS* gene product of this plasmid is slightly smaller than the wild type (approximately 1 kd smaller, corresponding to 25 to 30 bp). As DNA sequence analysis (G. Fayat, C. Sacerdot, S. Blanquet, personal communication) shows that the *pheS* gene ends 216 bp to the right of the *Pst*I3 site, it seems reasonable that the Δ *pheS1002* deletion (located to the left of *Pst*I3) creates an in-phase fusion within *pheS*. (This point is further discussed in the accompanying paper [22].)

Further analysis of the pB1S and pB1T mutations. (i) **Genetic complementation.** Both plasmids complemented thermosensitive mutations in *pheS* and *pheT* (Table 2). However, complementation with multicopy plasmids has not the same significance as classical one-copy complementation with F' episomes of λ transducing phages. To test whether a single copy of the mutations, present on pB1S and pB1T, was sufficient to complement chromosomal mutations in *pheS* and *pheT*, we transferred the mutations to an integration-proficient λ vector. The isolation of λ B1S and λ B1T (Fig. 2) from pB1S and pB1T, respectively, has been described above. Lysogens of these phages with IBPC1671 (*pheS*) and IBPC1772 (*pheT*) thermosensitive recombination-deficient strains were obtained. Both sets of lysogens were thermoresistant. Hence, a single copy (or perhaps two copies if double lysogens were formed) of the mutations complemented *pheS* and *pheT* defects. The fact that the Ω *pheSp1001* mutation is not a null mutation is suggested by the activity measurement of Table 1; strains carrying plasmid pB1S showed a level of phenylalanyl-tRNA synthetase activity somewhat higher than that of a pBR322-bearing control strain. The Δ *pheS1002* mutation is a structural mutation resulting in a

less stable protein (see below) and low phenylalanyl-tRNA synthetase activities in vitro (Table 1). However, within the cell the protein was sufficiently stable to complement a *pheS* mutation.

(ii) **Proteins synthesized by pB1S and pB1T.** Mutations which still allow complementation but not overproduction could be of two conceivable types: those which have a diminished level of expression of the operon (regulatory mutations) and those which show a normal level of expression of an unstable enzyme (structural mutations). To investigate whether the mutations carried by pB1S and pB1T (Ω *pheSp1001* and Δ *pheS1002*, respectively) are structural or regulatory, strains carrying these plasmids were pulse-labeled with [³⁵S]methionine and chased with an excess of nonradioactive methionine for increasing times before lysis. The extracts of the labeled and chased strains were immunoprecipitated with anti-phenylalanyl-tRNA synthetase immune serum, and the immunoprecipitates were analyzed by polyacrylamide gel electrophoresis. The fluorogram of such a gel, made from extracts of pB1-, pB1T-, pB1S-, and pBR322-carrying strains, is shown in Fig. 4. The immunoprecipitate of a pulse from a pB1-carrying strain shows the synthesis of the two wild-type subunits (Fig. 4, lane 1); for a pB1T-carrying strain the pulse shows a somewhat reduced synthesis of an enzyme whose small subunit is smaller than the wild-type one (Fig. 4, lane 6). With a pulse, no more phenylalanyl-tRNA synthetase synthesis was detected with pB1S (Fig. 4, lane 11) than with the control strain carrying pBR322 (Fig. 4, lane 12). For pB1T-carrying strains, the mutated subunit was completely degraded between 30 and 120 min of chase (Fig. 4, lanes 9 and 10). At these same chase times, both subunits of phenylalanyl-tRNA synthetase were detectable with pB1-carrying strains (Fig. 4, lanes 4 and 5). These data show that pB1T synthesized a less stable phenylalanyl-tRNA synthetase, with its small subunit smaller than the wild type, and thus seems to be a typical structural mutant. On the other hand, pB1S synthesized only a low, residual quantity of the synthetase, not detectable by immunoprecipitation, but which must still be sufficient to complement *pheS* and *pheT* mutants. This latter mutant is, as discussed later, a true regulatory mutant. The other minor bands precipitated by the anti-phenylalanyl-tRNA synthetase serum are presumably due to antibodies of different specificities contaminating the antisera, since the same bands, at the same intensities, are precipitated from pBR322- and pB1S-carrying strains (Fig. 4, lanes 11 and 12).

(iii) **Insertion of pB1S interrupts a *cis*-acting sequence.** The insertion of pB1S is situated in

front of the structural genes (discussed later). It is thus possible that it exerts its effect either by interrupting a *trans*-acting regulatory gene or a *cis*-acting sequence. To distinguish these two possibilities, a *cis-trans* test was devised based on the overproduction of phenylalanyl-tRNA synthetase. Two plasmids have been described (21) with parts of the wild-type *pheS,T* operon inserted into a plasmid vector (pGA39 [1]) compatible with pBR322 (and thus with pB1S or pB1T). One plasmid, pGC87, carried the *Pst*I2-*Pst*I3 fragment of pB1 (Fig. 2). This fragment carried the end of *thrS*, *infC*, part of the *pheS* structural gene, and all of the regulatory region of the *pheS,T* operon. It did not complement *pheS* mutants. The second plasmid, pG6-7, carried the *Pst*I2-*Pst*I4 fragment (Fig. 3) and did complement *pheS* mutants without causing the overproduction of the small subunit of phenylalanyl-tRNA synthetase. Both plasmids (conferring resistance to chloramphenicol) were introduced into strains carrying pB1T and pB1S (conferring resistance to ampicillin and tetracycline). The strains were grown in LB containing the three antibiotics, and samples of saturated cultures were analyzed on denaturing polyacrylamide gels (Fig. 5).

Neither the presence of pGC87 nor that of

pG6-7 affected the level of phenylalanyl-tRNA synthetase synthesis from pB1S (Fig. 5, lanes 7 and 8). With pB1T, there was no effect with pGC87 (Fig. 5, lane 4), but the presence of pG6-7 (Fig. 5, lane 5) caused the overproduction of both subunits of the synthetase. The fact that supplying a whole *pheS* gene in *trans* to a *pheS* structural mutation causes overproduction validates the test as a method of detecting *trans*-acting products. The overproduction of phenylalanyl-tRNA synthetase with the two plasmids is less than with pB1. This could be due to the lower copy number of the pG6-7 plasmid (carrying the P15A replicon [8]).

Since no increased synthesis of phenylalanyl-tRNA synthetase was detectable from supplying pB1S with the wild-type region, equivalent to where the insertion is located (carried by pGC87 and pG6-7) in *trans*, we concluded that this insertion exerts its effect in *cis* either by interrupting a *cis*-acting regulatory region or by separating a *cis*-acting regulatory region from the structural genes.

DISCUSSION

Identification of a regulatory region for the *pheS,T* operon. The mutations described here are of such a variety that they give information about many different aspects of phenylalanyl-tRNA synthetase biosynthesis. The most important result is that the pB1S insertion (Ω *pheSp1001*) has permitted the identification of a *cis*-acting regulatory sequence for the *pheS,T* operon. Other examples of *cis*-acting regulatory mutations for aminoacyl-tRNA synthetase have been described, but this is the first where the nature and approximate position of the mutation is known. Based on the isolation of thermoresistant revertants of thermosensitive aminoacyl-tRNA synthetases, three other *cis*-acting mutations, *serO* (9), *leuX* (16), and *valX* (3), closely linked to their corresponding aminoacyl-tRNA synthetase genes, have been defined. These mutations act by producing an increased amount of the thermosensitive enzyme. In addition, mutations conferring resistance to the antibiotic borrelidin cause an overproduction of wild-type threonyl-tRNA synthetase. These mutations, linked to *thrS*, are *cis*-dominant (11). The nature of the mutations is unknown in all cases, but they have been tentatively identified as operator-promoter mutations.

The Ω *pheSp1001* mutation also defines a regulatory region since it reduces considerably the amount of phenylalanyl-tRNA synthetase synthesized. The exact point of the insertion has yet to be determined, but it is situated in the *Hpa*I5-*Ava*I3 fragment (Fig. 1), implying that it is many bp on the 5' side of the structural *pheS,T* genes. Sequence data (G. Fayat, C. Sacerdot, S. Blan-

FIG. 5. *Cis-trans* test for overproduction of phenylalanyl-tRNA synthetase. IBPC2001, carrying the plasmids indicated, was grown to saturation in LB medium containing the appropriate antibiotics: ampicillin ($50 \mu\text{g ml}^{-1}$) and tetracycline ($10 \mu\text{g ml}^{-1}$) for pB1, pB1S, pB1T, and pBR322 and chloramphenicol ($25 \mu\text{g ml}^{-1}$) for pGC87 and pG6-7. Samples were prepared as described in the legend to Fig. 3 and electrophoresed on an 11% sodium dodecyl sulfate-polyacrylamide gel (17). Proteins were stained with Coomassie blue. The positions of the small and large subunits of phenylalanyl-tRNA synthetase (α PheRS and β PheRS, respectively) and chloramphenicol acetylase (CmAc), coded by pGC87 and pG6-7, are indicated.

quet, personal communication) show that the *pheS* structural gene starts 85 bp to the right of *Ava*I3. However, the functional promoter for *pheS,T* is situated to the left of this site. Springer et al. (25) described an in vivo-deleted derivative of λ p2 (a transducing phage which carries all of the *E. coli* genes for pB1); this phage, λ pp1-16, does not express any of the genes of pB1. However, DNA restriction analysis shows that the deletion is to the left of *Ava*I3, leaving the *Ava*I3 site and all of the DNA to the right of it intact (i.e., the entire structural genes of *pheS* and *pheT* are present). This observation predicts that the natural promoter for *pheS,T* is before the *Ava*I3 site.

Since several of the non-overproducing mutant plasmids still complement mutations in *pheS* and *pheT*, complementation is not a sufficient criterion for the presence of a complete wild-type gene. A better criterion for the wild-type *pheS,T* operon on a plasmid would appear to be the overproduction of phenylalanyl-tRNA synthetase. By employing a *cis-trans* test for overproduction of the synthetase, the insertion, Ω *pheSp1001*, was shown to act in *cis*. Supplying pB1S in *trans* with wild-type DNA of the region, where the insertion is located (via the compatible plasmids pGC87 or pG6-7), did not stimulate an increased synthesis of phenylalanyl-tRNA synthetase. Thus, the insertion is not affecting a *trans*-acting gene but is affecting the *pheS,T* promoter or is situated between the promoter and structural genes. Although the size of this insertion (1.3 kb) is similar to that of many insertion elements (7), we have not identified it.

Only minimal expression of the *pheS,T* genes seems to be necessary for complementation to be observed. This is particularly apparent for pB1S. No phenylalanyl-tRNA synthetase protein synthesis was observed by pulse-labeling of IBPC2001 carrying pB1S, and activity measurements showed only a small enhancement of amino acid charging activity compared with pBR322. However, when transferred to a λ phage, a single copy of the mutated operon still complemented perfectly. The expression of *pheS,T* from the lysogen can only be a fraction of the normal wild-type expression. This could mean that phenylalanyl-tRNA synthetase is normally synthesized in excess.

Structural mutations of the *pheS,T* operon. The pB1T mutation (Δ *pheS1002*) was studied as an example of a purely structural mutation. The 20- to 30-bp deletion within the *pheS* gene produced a smaller protein whose molecular weight is compatible with the size of the deletion. This protein was much less stable than the wild-type subunit (Fig. 4), and this is, presumably, the major reason why phenylalanyl-tRNA synthetase was not overproduced in pB1T-carrying

strains. However, a 2-min pulse-labeling of pB1T with [³⁵S]methionine apparently labeled less phenylalanyl-tRNA synthetase than did a similar pulse with pB1 (cf. Fig. 4, lanes 1 and 6), suggesting that there is a reduction in the synthesis of the mutated enzyme. This might be indicative of some role of the wild-type protein in controlling its own synthesis. This point is considered in detail in the accompanying paper (22). Supplying pB1T with a wild-type *pheS* gene in *trans* restored the ability to overproduce phenylalanyl-tRNA synthetase. Thus, although normally cotranscribed from the single *pheS,T* promoter (21), neither cotranscription nor co-translation is necessary for phenylalanyl-tRNA synthetase overproduction. This is again suggestive of the importance of the wild-type tetrameric enzyme in its own regulation. Finally, we note that pB1M1, deleted for the regulatory regions of *pheS,T* up to, and including, the *Ava*I3 site, still considerably overproduced phenylalanyl-tRNA synthetase, although transcription is from the *thrS* promoter.

In summary, we have described the isolation and preliminary characterization of several mutated plasmids which are shown to be useful in studies of the expression of the *pheS,T* operon. Plasmid pB1M1 localizes the structural genes to the right of *Ava*I3, whereas plasmid pB1S shows that DNA to the left of *Ava*I3 is essential for the normal expression of the *pheS,T* operon. The mutation in plasmid pB1T caused a structural change in the small subunit of phenylalanyl-tRNA synthetase and produced a less stable enzyme. This mutated enzyme is useful for both in vivo experiments to see the effect of an altered protein on phenylalanyl-tRNA synthetase biosynthesis and also, potentially, for in vitro analysis of the biochemistry of the enzyme. Some of the mutants seem to reveal specific features of the *pheS,T* operon, e.g., the weak *pheS* complementation and strong *pheT* expression of pB1M5 could be explained by a translational restart within *pheS*, and the weak *pheT* complementation with pB1S3 could be due to an unstable hybrid protein exhibiting some residual *pheT* activity. The fact that these mutations are isolated on multicopy plasmids makes them easier to analyze and eliminates any problems of essential genes not being susceptible to most types of mutation described here.

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