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Escherichia coli Mutants Overproducing Phenylalanyl- and Threonyl-tRNA Synthetase

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The structural genes for threonyl-tRNA synthetase (ThrRS) and phenylalanyl-tRNA synthetase (PheRS) are closely linked on the *Escherichia coli* chromosome. To study whether these enzymes share a common regulatory element, we have investigated their synthesis in mutants which were selected for overproduction of either ThrRS or PheRS. It was found that mutants isolated previously for overproduction of ThrRS as strains resistant to the antibiotic borrelidin (strains Bor Res 3 and Bor Res 15) did not show an elevated level of PheRS. PheRS-overproducing strains were then isolated as revertants of strains with structurally altered enzymes. Strain S1 is a temperature-resistant derivative of a temperature-sensitive PheRS mutant, and strain G118 is a prototrophic derivative of a PheRS mutant which shows phenylalanine auxotrophy as a consequence of an altered K_m of this enzyme for the amino acid. In both kinds of revertants, S1 and G118, the concentration of PheRS and ThrRS was increased by factors of about 2.5 and 1.8, respectively, whereas the level of other aminoacyl-tRNA synthetases was not affected by the mutations. Genetic studies showed that the simultaneous overproduction of PheRS and ThrRS in revertants G118 and S1 is based upon gene amplification, since this property was easily lost after growing the cells in the absence of the selective stimulus, and since this loss could be prevented by the presence of the *recA* allele. By similar criteria, the four- and eightfold overproduction of ThrRS in strains Bor Res 3 and Bor Res 15, respectively, was very stable genetically, indicating that it is caused by a mutational event other than gene amplification. From these results, we conclude that the concomitant increase of PheRS and ThrRS in strains G118 and S1 is an expression of gene duplication and not of a joint regulation of these two aminoacyl-tRNA synthetases. This conclusion is further supported by the result that, in mutant G118 as well as in its parental strain G1, growth in minimal medium lacking phenylalanine led to an additional twofold increase of their PheRS concentration. This increase was restricted to the PheRS, since the level of other aminoacyl-tRNA synthetases, including the ThrRS, stayed unchanged.

Intensive studies on the regulation of formation of aminoacyl-tRNA synthetases in bacteria have revealed the existence of two different modes of control. The first one consists of the change of the rate of formation of these enzymes in answer to certain growth conditions. Thus, for 10 of the 20 aminoacyl-tRNA synthetases it has been shown that shortage of the cognate aminoacyl-tRNA leads to a two- to threefold derepression of the respective aminoacyl-tRNA synthetase (cf. 36). The exogenous supply of the amino acid to the "derepressed" culture, as shown for the phenylalanyl- and isoleucyl-tRNA synthetases (PheRS and IleRS), causes the onset of "repression" again (34). For IleRS, it was demonstrated recently that DNA-dependent in vitro synthesis of this enzyme is markedly stimulated by guanosine-5'-diphosphate-3'-diphosphate (ppGpp) (47). An increased formation rate of this enzyme in vivo elicited by isoleucine deficiency is also accompanied by an increased level of ppGpp (45).

Alteration of the level of aminoacyl-tRNA synthetases as a response to an environmental change may be also brought about by changing the growth rate of the cells not by amino acid limitation but by enriching the medium or changing the carbon source. This kind of control is called metabolic regulation, and it has been shown that several of the 20 aminoacyl-tRNA synthetases react concomitantly, though to a very different degree (31).

The second mode for altering the intracellular level of an aminoacyl-tRNA synthetase is by

specific mutations. Depending on the selection conditions, mutants were obtained which overproduce either a structurally altered seryl-tRNA synthetase (SerRS), glycyl-tRNA synthetase (GlyRS) or leucyl-tRNA synthetase (LeuRS) (9, 17, 27, 41, 44) or the wild-type form of methionyl-tRNA synthetase (MetRS) or threonyl-tRNA synthetase (ThrRS) (8, 39).

One of the amino acid-activating enzymes which has been biochemically and genetically well characterized is PheRS. It consists of two dissimilar subunits, α and β (15, 19), and it has been shown recently that the structural genes for these polypeptides are closely linked (10). Moreover, a specialized transducing λ phage has been isolated which, in addition to the α and β subunits of PheRS (23), also carries the gene for another aminoacyl-tRNA synthetase, namely, the threonine-specific enzyme (22). Therefore, it would be of interest to study the regulatory relationship of these two aminoacyl-tRNA synthetases. PheRS- and ThrRS-overproducing mutants might yield such information. ThrRS-overproducing mutants of *E. coli* are already available (36), so here we describe the isolation and analysis of *E. coli* mutants which overproduce PheRS and provide a further characterization of the ThrRS-overproducing strains.

MATERIALS AND METHODS

Organisms and culture conditions. The *E. coli* strains used in this study are listed in Table 1, together with their genotype and their source or derivation. Gene symbols are adopted from Bachmann et al. (2). The minimal medium used was salt solution P (18), supplemented with 0.2% $(NH_4)_2SO_4$, and 0.4% glucose. Required amino acids and vitamins were added to a concentration of 50 and 5 μg/ml, respectively. For growth of phenylalanine auxotrophs, the concentration of phenylalanine was 200 μg/ml. Rich medium (TGYE) consisted of 1% tryptone (Oxoid Ltd.), 0.5% yeast extract (Oxoid) and 0.2% glucose. Plates contained agar at a concentration of 1.5%.

Bacteriological and genetic techniques. Mutagenesis by ethylmethanesulfonic acid and penicillin enrichment of mutants were carried out by the method of Miller (32). Phenylalanine prototrophic revertants from strain G1 were selected for growth on minimal medium plates at 37°C. Thermoresistant revertants from strain JP1116 were selected for growth on TGYE plates at 40°C. Phage P1*kc* lysates were obtained by the plate lysis technique given by Lennox (28). For transductions, the procedure of Miller (32) was followed. Selection for *aroD*$^+$ recombinants was on minimal medium plates lacking the aromatic amino acids. *pps*$^+$ strains were selected on minimal plates supplemented with 0.8% pyruvate as sole carbon source. The ThrRS-overproducing strains Bor Res 3 and Bor Res 15 have been described (22, 36, 39). For testing the stability of the genetic alterations in these mutants, they were transfered sequentially more than 10 times on TGYE plates without the selective agent borrelidin. Overnight cultures inoculated from the last transfer were grown, and appropriate dilutions were plated on TGYE plates and incubated at 37°C. From each strain, 250 colonies were picked and streaked on minimal medium plates containing a borrelidin concentration

TABLE 1. *E. coli strains used*

Strain	Genotype	Source or derivation (reference)
AS19	Prototrophic	(26)
K-10	Hfr (Cavalli) *rel-1 tonA22* T2^r	(1)
JP1116	HfrH *pheT354*[b] *thi galE-PL5 rel-1*	R. R. B. Russell (43)
NP37	Hfr (Cavalli) *pheS5*[a] *rel-1 tonA22* T2^r	F. C. Neidhardt (13)
MC103	*pheT354*[b] *thi argE3 proA2 aroD6 rpsL*	M. M. Comer (10)
S1	HfrH *pheT354*[b] *sup-950 thi galE-PL5 rel-1*	Spontaneous thermoresistant revertant of JP1116
PA505-1-5	F *pps-4 metA90 argH1 proA44 icl-4 str-9 thi* λ^-	H. L. Kornberg (5) via B. J. Bachmann
G1	F *pheS76*[c] *pps-4 metA90 argH1 proA44 icl-4 str-9 thi* λ^-	Phenylalanine-auxotrophic mutant of PA505-1-5, obtained by EMS mutagenesis and penicillin selection[d]
G118	F *pheS76*[c] *sup-968 pps-4 metA90 argH1 proA44 icl-4 str-9 thi* λ^-	G118 and G124 are spontaneous phenylalanine prototrophic revertants of G1
G124	F *pheS76*[c] *sup-974 pps-4 metA90 argH1 proA44 icl-4 str-9 thi* λ^-	
G118-1	F *pheS76*[c] *sup-968 metA90 argH1 proA44 icl-4 str-9 thi* λ^-	Spontaneous *pps*$^+$ revertant of G118
K-12B	Wild type	(3)
Bor Res 3	Elevated ThrRS level	(8)
Bor Res 15	Elevated ThrRS level	(8)

[a] Temperature-sensitive PheRS mutation in the α subunit.
[b] Temperature-sensitive PheRS mutation in the β subunit.
[c] PheRS mutant with altered K_m for phenylalanine (mutation in the α subunit).
[d] EMS, Ethyl methane sulfonate.

which inhibits the growth of the parental strain (120 μg/ml). Furthermore, from each of the strains three colonies were purified three times by single-colony formation resulting in the repurified strains Bor Res 3 I-III and Bor Res 15 I-III.

Preparation of crude cell extracts. Exponentially growing cells were harvested and washed twice with and resuspended in buffer containing 20 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.5), 30 mM NH_4Cl, 10 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), and 6 mM 2-mercaptoethanol (PheRS buffer). The cells were then disrupted by passage through a French pressure cell at 18,000 lb/in^2. Cell debris was removed by centrifugation at 12,000 × *g* for 30 min. For the formation of immunoprecipitates, the supernatant was further centrifuged for 2 h at 140,000 × *g* at 2°C. The protein contents of the crude extracts were determined by the Folin phenol procedure (29) with bovine serum albumin as the standard. In the case of the ThrRS-overproducing mutants and their parental strain K-12B, crude extract was prepared by using potassium phosphate buffer and sonic oscillation for disruption of the cells as described earlier (45).

Purification of PheRS and its subunits. The PheRS was purified from *E. coli* K-10 as described previously (25). α and β subunits were separated as given by Hennecke and Böck (21). Dilutions of enzyme and subunits were made in 20 mM Tris-hydrochloride (pH 7.5), 6 mM 2-mercaptoethanol, and 0.2 mM EDTA, containing 50 bovine serum albumin at 50 μg/ml (BSA buffer).

Heat inactivation experiments. Crude extracts were incubated at 50°C at a protein concentration of 100 μg/ml. At the times indicated, samples were taken and assayed for residual PheRS activity under standard conditions at 28°C.

Enzyme assay. The activity of PheRS was assayed by aminoacylation of tRNA with L-[^{14}C]phenylalanine (10 μCi/μmol) in the test system described previously (25). For determination of the K_m values for phenylalanine, a blank without extract protein was run for each individual substrate concentration employed. The enzymatic activity of the ThrRS was measured as previously described (45), the final threonine concentration being 0.4 mM, unless otherwise indicated.

In vitro complementation of mutant PheRS activity in crude extract. The procedure described for the reconstitution of active PheRS from its inactive subunits (21) was used with the modifications described recently (10). Before reconstitution, the crude extracts (1 mg of protein per ml) from strains NP37 and JP1116 were preincubated for 5 min at 40°C, and that of strain G1 at 50°C. A 12.5-μg amount of protein of these preparations was then incubated in a total volume of 0.15 ml of BSA buffer for 30 min at 30°C either alone or in combination with 0.5 μg of purified α or 1.25 μg of purified β subunits, respectively. Subsequently, 100 μl of an appropriately concentrated test mixture was added to adjust the reaction mixture to the substrate concentrations used in the standard aminoacylation assay. Incubation was for 10 min at 28°C.

Radioactive labeling of cultures. For radioactive labeling, cultures were grown for at least six generations in 5 ml of glucose minimal medium supplemented with the required growth factors and with a labeling mixture containing 80 μM L-isoleucine, 120 μM L-valine, 160 μM L-leucine, and 10 μCi of L-[4,5-^{3}H]leucine per ml (37). Growth was monitored by measuring the absorbance at 420 nm (A_{420}) of an unlabelled parallel culture. At an A_{420} of 1.5, the radioactively labeled culture was chilled and mixed with 2 ml of an overnight culture of a reference strain grown in the presence of 3 μCi of L-[1-^{14}C]leucine per ml (specific radioactivity 56 μCi/μmol). The cells were sedimented from this mixture by centrifugation at 8,000 × *g* for 10 min. The cell pellet was taken up in 2 ml of PheRS buffer plus 0.5 ml of unlabeled carrier cells (about 125 A_{420} units), and the cells were again collected by centrifugation.

Immunological procedures. The preparation of antisera directed against PheRS from *E. coli*, the immunotitration of PheRS activity with antibodies, and the performance of Ouchterlony immuno-double-diffusion experiments have been described in a recent report (40). Minor modifications of these procedures are specified below, when the respective experiments are described.

Preparation of immunoprecipitates of labeled cultures. The harvested cell pellet was suspended in 0.5 ml of Tris-chloride buffer (10 mM, pH 7.9) containing 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 50 mM KCl, and 0.6 mg of lysozyme per ml (42). After five cycles of freezing and thawing, 1 ml of the following buffer was added: 10 mM Tris-chloride (pH 7.9), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 10 mM KCl, 5% glycerol, 20 μg of deoxyribonuclease I per ml and 10 μg of ribonuclease A per ml (42). After sonic oscillation for 30 s at setting 3 (Branson Sonifier W140), the samples were incubated for 30 min at 24°C. Finally, they were clarified by centrifugation at 30,000 × *g* for 20 min, and the supernatant was adjusted to a final concentration of 50 mM Tris-chloride (pH 7.7), 0.4 M KCl, and 1% Triton X-100. A 0.2-ml amount of a 1:1 mixture of the immunoglobulin G fractions of antisera directed against native wild-type PheRS and against the purified α subunit were added. The precipitate was allowed to be formed at 4°C for at least 15 h. It was collected by centrifugation for 4 min at 12,000 × *g* in plastic vials, washed two times with 50 mM Tris-chloride (pH 7.5), 1 M KCl, and 1% Triton X-100 and once with 50 mM Tris-chloride (pH 7.5)–0.1 M NaCl (42). It was finally taken up in 50 μl of sample buffer containing 50 mM Tris-chloride (pH 6.8), 5% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol, 2 mM EDTA, and 0.001% bromophenol blue, heated in the boiling water bath for 4 min, and subjected to polyacrylamide gel electrophoresis using the gel system of Laemmli (25).

After staining the gels with 0.2% Coomassie brillant blue R-250 in 30% methanol and 10% acetic acid and destaining in the same solution without dye, the bands corresponding to the α and β polypeptides of PheRS were cut out, dried at 60°C, and burnt in a Packard 306 sample oxidizer. Scintillation fluid for ^{3}H was Monophase 40, and for ^{14}C it was Carbosorb II and Permafluor V. Pilot experiments in which the gels were sectioned had shown that, under the precipitation and washing conditions used, the α and β polypeptides of PheRS are the predominantly labeled bands present in the gels (Comer and Böck, unpublished data).

Preparation of the immunoprecipitates of ThrRS

and IleRS was carried out in the same way as described above for the phenylalanine enzyme.

RESULTS

Isolation of PheRS-overproducing strains. Two techniques were used for isolating apparent PheRS-overproducing mutants.

(i) For the first approach, we selected thermoresistant revertants of the temperature-sensitive strain JP1116, which contains a mutation in the β subunit of the PheRS (10). A total of 49 independent revertants were isolated on rich medium plates at 40°C and were tested for their specific PheRS activity in crude extracts (Table 2). Three groups could be distinguished: the first group (29 revertants, e.g., strain S38) did not differ in its PheRS activity from strain JP1116; the second one (19 revertants, e.g., strain S2) possessed an enzyme which is more thermostable than that of JP1116 and therefore seems to be changed in the structural gene; the third one, only one strain, namely, S1, showed a threefold increase of activity without an apparent loss of temperature sensitivity. Since the properties of the latter strain are those which would be expected from a presumptive PheRS-overproducing strain, it was first investigated whether immunodiffusion could reveal any immunological difference between the enzymes from strain S1 and the parental strain JP1116; it was found that both enzymes seem to be identical (not shown). To find out whether the threefold increase in PheRS activity of strain S1 is in fact due to an overproduction of this enzyme, three experimental approaches have been followed. First, the PheRS activity of strain S1 and of the controls K-10 (wild type) and JP1116 was titrated with increasing amounts of PheRS antibodies. From the neutralization curves (Fig. 1) it is obvious that, when normalized to 100%, the PheRS activity in strain S1 extracts was inhibited to a lower extent than in extracts from the controls. Under the assumption that the enzymes of strains S1 and K-10 and JP1116 possess the same immunological reactivity this immunotitration experiment indicates a two- to threefold higher PheRS level to be present in strain S1.

TABLE 2. *Specific PheRS activity of JP1116 and of thermoresistant revertants*

Strain	Enzyme activity at:[a]	
	28°C	37°C
K-10 (wild type)	999	1,490
JP1116	554	133
S38	590	55
S2	576	759
S1	1,569	374

[a] Values are expressed as counts per minute per microgram of crude extract protein.

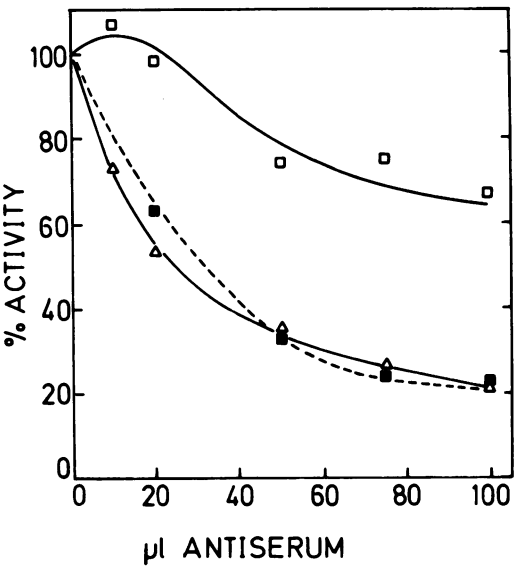

FIG. 1. *Immunotitration of PheRS activity from strains K-10 (△), JP1116 (■), and S1 (□). A 50-µg amount of crude extract of each strain was incubated for 1 h at 0°C with the indicated amounts of diluted antiserum (1:50) raised against wild-type E. coli PheRS. Equal samples were then assayed for residual PheRS activity, and the results were normalized to the value without antiserum.*

In a second experiment, use was made of the fact that the PheRS of JP1116 (and therefore also of S1) dissociates at high temperature and that the α subunits generated during this process can be reacted with purified β polypeptides to deliver active enzyme (10). By the addition of an excess of β, a quantitative determination of α present in crude extracts could be undertaken (Fig. 2). It is evident that saturating amounts of β generated more than fourfold more active enzyme with extracts from strain S1 than with those from JP1116.

In the last experiment, the relative amounts of the radioactive α and β subunits of PheRS, of ThrRS, and of IleRS were measured directly by the technique of Neidhardt and co-workers (37), except that the polypeptides were purified by immunoprecipitation and by one-dimensional polyacrylamide slab gel electrophoresis. The parental strain JP1116 and the revertant S1 were grown under steady-state conditions in medium supplemented with L-[^{3}H]leucine, then mixed with equal amounts of a culture of strain AS19 grown in the presence of L-[^{14}C]leucine. The $^3H/^{14}C$ ratios of radioactivity in the purified α and β polypeptides of PheRS and in ThrRS and IleRS were determined and normalized to the $^3H/^{14}C$ ratio in the total soluble protein. The values in Table 3, therefore, represent the ratios

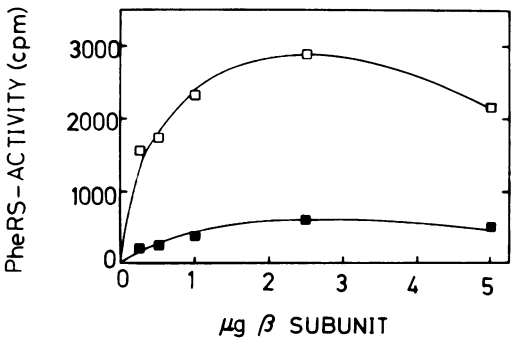

FIG. 2. *In vitro complementation of phenylalanyl-tRNA synthetase. A 5-μg amount of freshly prepared S100 extract protein of strains JP1116 (■) and S1 (□) was incubated for 5 min at 42°C, then mixed with different amounts of purified β subunit of PheRS and further incubated for 30 min at 28°C. The complete mixture was finally assayed for PheRS activity.*

of the individual polypeptides in the total soluble proteins of the strain investigated relative to that of strain AS19. They show that the amounts of α and β subunits increased to an equal extent, about threefold. Surprisingly, in this mutant the amount of ThrRS also increased, though only about twofold. The level of IleRS stayed more or less constant.

(ii) For the second approach to isolating PheRS-overproducing strains, we had at first to isolate a strain which is auxotrophic for phenylalanine because of a mutation in the α or β subunit and then to use such a mutant for selecting prototrophic derivatives. *E. coli* strain PA505-1-5 was mutagenized, and after penicillin treatment a total of 23 independent phenylalanine-auxotrophic mutants were isolated. Crude extracts of these strains were prepared and assayed for PheRS activity. One strain, designated G1, exhibited drastically reduced PheRS activity, whereas the others had normal or slightly reduced PheRS activity in comparison with the parental strain PA505-1-5. The decreased activity of the PheRS of strain G1 was due to an altered K_m for phenylalanine. For wild-type PheRS, an apparent K_m of 6.6 μM was determined, which is comparable to previously published values (14). The PheRS of strain G1 showed a 15-fold higher value, namely, 100 μM. The PheRS of strain G1 was also characterized by a significant thermolability. Incubation of crude extracts from strain G1 at 50°C caused the PheRS activity to decrease with a half-life of first-order reaction of 12 min compared to 60 min for the wild-type enzyme. Nevertheless, strain G1 was able to grow at 42°C, although the growth rate dropped considerably at temperatures above 37°C (not shown).

In analogy to the behavior of the enzyme of several other PheRS mutants (4, 10), this partial temperature sensitivity suggests that the enzyme might be dissociated under this condition. If this is the case, it can be used to localize the defect to either the α or the β subunit by in vitro complementation tests (10, 21). PheRS activity in extracts of strain G1 could be generated after complementation with purified α (Table 4). The PheRS of strain G1 itself was therefore assumed to result from a mutation in the gene for the α subunit. The reconstitution recovery of PheRS activity of G1 extracts was comparably low, but this is probably due to the incomplete dissociation of the enzyme after incubation at 50°C.

The properties of strain G1 made it suitable for the isolation of prototrophic revertants, which then might prove to be PheRS overproducers. For this purpose, a total of 32 independent phenylalanine-prototrophic derivatives from strain G1 were isolated and examined for their PheRS activity in crude extracts. Eight strains were found to have an increased specific enzyme activity as compared to that of mutant G1. As a next step, these strains were screened, by means of a short procedure, for any apparent overproduction of PheRS. Equal amounts of crude ex-

TABLE 3. *Levels of PheRS, ThrRS, and IleRS in strains JP1116 and S1*[a]

Strain	Mean doubling time at 32°C (min)	Synthetase level[b]			
		PheRS		ThrRS	IleRS
		α	β		
JP1116	72	0.94	0.86	0.76	0.67
S1	72	2.56	2.37	1.30	0.72

[a] The cultures were grown at 32°C in minimal medium containing 0.4% glucose, thiamine at 5 μg/ml, and the labeling mixture as outlined in the text.

[b] Values indicate ratio of $^3H/^{14}C$ in synthetase to $^3H/^{14}C$ in total protein. A culture of strain AS19 was used as the ^{14}C reference.

TABLE 4. *Complementation of mutant PheRS with purified wild-type enzyme subunits*

Strain	Enzyme activity in:[a]		
	Mutant extract alone (0.5 μg)	Mutant extract plus α (0.5 μg)	Mutant extract plus β (1.25 μg)
—[b]	—[b]	192	131
NP37	52	1712	192
JP1116	715	1187	5034
G1	78	554	209

[a] Values represent counts per minute of [^{14}C]phenylalanine attached to tRNA measured in the standard aminoacylation assay at 28°C (L-phenylalanine concentration, 20 μM).

[b] —, Without mutant extract.

tract protein of the phenylalanine prototrophs were reacted with two constant amounts of antibodies specific for wild-type PheRS. The result was that the extracts of six strains were inhibited to the same extent as those of mutant G1 or of the wild type, only the extracts of two strains, namely G118 and G124, were far less inhibited (data not shown). This was taken as a first and preliminary piece of evidence that revertants G118 and G124 synthesize more PheRS.

To demonstrate that the revertants overproduce PheRS, three experiments were done to show that these strains produce an enzyme identical to their parent, G1.

(i) Determination of the apparent K_m for L-phenylalanine revealed that the enzymes of strains G1, G118, and G124 exhibited the same values, namely, 100 μM.

(ii) Measurement of the rate of heat inactivation of the PheRS of strains G118 and G124 yielded exactly the same kinetics as for the enzyme of strain G1 (data not shown).

(iii) Immuno-double-diffusion on agar plates and semiquantitative immunodiffusion experiments showed no antigenic differences between the enzymes of strains G1, G118, and G124 and were in agreement with a four- to eightfold increase in PheRS in G118. A stronger argument for this conclusion is derived from quantitative immunoprecipitation experiments of the α and β subunit protein and subsequent separation of the precipitate by sodium dodecyl sulfate polyacrylamide gel electrophoresis (40). For this purpose, constant amounts of crude extracts of each of the strains PA505-1-5, G1, G118, and G124 which had been grown to the same density in minimal medium (supplemented in the case of strain G1 with 200 μg of phenylalanine per ml) were mixed with three increasing amounts of PheRS antibodies to be sure that the PheRS-antibody complexes were formed under equivalence. The precipitates were analyzed on sodium dodecyl sulfate polyacrylamide gels (Fig. 3). From the intensity of the staining, it is obvious that the revertant strains G118 and G124 contained a substantially higher amount of α and β chains than did the parental strain G1. Scanning of the gels revealed that G124 produced approximately fourfold, and G118 produced fivefold more PheRS. To verify this result, a direct measurement of the absolute amount of enzyme protein was made in the same way as described above for the mutant S1. The results (Table 5) show that both α and β of PheRS in strain G118 were overproduced when compared to the level present in strain G1 or PA505-1-5, the oversynthesis ranging from 2.5- to 3-fold. The level of IleRS stayed constant in all the strains, whereas

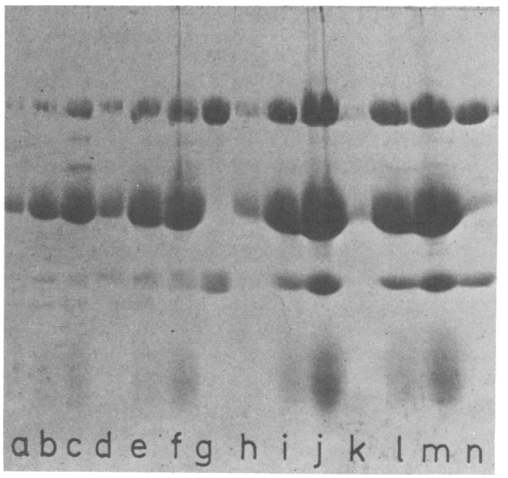

FIG. 3. *Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis of immunoprecipitates formed between three different amounts (0.15 ml, 0.3 ml, and 0.6 ml) of the immunoglobulin G fraction of anti-PheRS serum and 5 mg of crude extract protein of strains PA505-1-5 (a,b,c); G1 (d,e,f); G118 (h,i,j); and G124 (k,l,m). The four main bands are, from top to bottom, β subunit of PheRS, immunoglobulin heavy chains, α subunit of PheRS, and immunoglobulin light chains. Gels g and n show the electrophoresis pattern of 10 μg of purified E. coli PheRS. Strains PA505-1-5, G118, and G124 were grown in minimal medium without phenylalanine; strain G1 was grown in minimal medium supplemented with 200 μg of phenylalanine per ml.*

there was a distinct and (in a second experiment) reproducible increase of ThrRS of about 50%. At the moment, we have no explanation for the finding that the apparent level of the β subunit in all strains seems to be higher than that of α.

ThrRS-overproducing strains. The isolation and some properties of the ThrRS-overproducing strains have already been described (22, 36, 39). Thus, the structurally unaltered ThrRS in strains Bor Res 3 and Bor Res 15 was overproduced four- and eightfold, respectively. In addition, the level of four other aminoacyl-tRNA synthetases, including PheRS, which were measured as control was not altered in those mutants in comparison to the parental strain K-12B (Table 6).

Level of PheRS and ThrRS in the PheRS- and ThrRS-overproducing strains under various growth conditions. For most of the aminoacyl-tRNA synthetases, including PheRS and ThrRS, it is known that growth under limitation of the cognate amino acid or aminoacyl-tRNA leads to a derepression of the respective enzyme (cf. 38; 45). As expected from the reduced growth rate, there was indeed a 1.5- and

TABLE 5. *Levels of PheRS, ThrRS, and IleRS in strains PA505-1-5, G1, and G118*[a]

Strain	Mean doubling time at 37°C (min)	Phenylalanine in medium (μg/ml)	Synthetase level[b]				Sp. act. of PheRS (U/mg of protein)
			PheRS		ThrRS	IleRS	
			α	β			
G1	720		NT	NT	NT	NT	0.0046
	50	200	0.75	0.93	1.36	0.77	0.0033
G118	65		4.64	10.00	2.13	0.77	0.0166
	50	200	1.87	2.88	1.98	0.72	0.0083
PA505-1-5	43		0.74	0.82	1.16	0.90	0.113
	42	200	0.75	1.17	1.38	0.85	0.113

[a] All cultures were grown at 37°C in minimal medium containing 0.4% glucose containing the required amino acids arginine, methionine, and proline each at 50 μg/ml and in the presence of the ^3H labeling mixture as outlined in the text. NT, Not tested. Specific enzyme activities were determined from ribosome-free crude extracts.

[b] Values indicate ratio of $^3H/^{14}C$ in synthetase to $^3H/^{14}C$ in total protein. A culture of strain AS19 served as the ^{14}C reference.

TABLE 6. *Specific activity of five aminoacyl-tRNA synthetases in two ThrRS-overproducing mutants and in their parental strain*[a]

Aminoacyl-tRNA synthetase	Sp. act. (U/mg of protein)		
	K-12B	Bor Res 3	Bor Res 15
ThrRS	0.136	0.533	0.985
PheRS	0.069	0.075	0.073
IleRS	0.062	0.060	0.065
LeuRS	0.102	0.094	0.089
SerRS	0.142	0.145	

[a] The cultures were grown at 37°C in glucose minimal medium, harvested at an A_{420} of 1.2, and the aminoacyl-tRNA synthetases were assayed as described in the text.

2-fold increase in PheRS in strains G1 and G118, respectively, when grown in the absence of phenylalanine (Table 5). The smaller increase in PheRS in G1 is readily explained by the fact that G1 had been cultivated in the absence of phenylalanine for less than one generation time whereas G118 grew for three generations without phenylalanine, because we know that the final level of derepression of the PheRS is reached only after growth for about three generations in the absence of phenylalanine (34). The increased production of PheRS in revertant G118 was verified by the technique of direct measurement of the amount of PheRS in phenylalanine-free and phenylalanine-supplemented minimal medium, which also demonstrated a 2.5-fold increase of the PheRS (Table 5). When we performed similar investigations with the PheRS-overproducing strain S1, there was no influence on the content of PheRS, independent of whether the cells were grown in the absence or presence of phenylalanine in minimal medium. Table 7 shows the result of the same analysis for the ThrRS-overproducing mutant Bor Res 3 and its parental strain K-12B. Addition of threonine to the medium did not lead to the repression of ThrRS synthesis in either strain, which is in accordance with previous results (45). On the other hand, a 1.8-fold increase in the enzyme level of strain K-12B occurred when the cells were grown in rich instead of minimal medium, whereas the ThrRS of the overproducing strain Bor Res 3 did not respond to metabolic regulation.

Genetic stability of the mutations leading to overproduction of ThrRS and PheRS. The stability of the mutations leading to overproduction of ThrRS in strains Bor Res 3 and Bor Res 15 was tested as described in Materials and Methods. All 250 colonies picked were found to be resistant to the borrelidin concentration used, meaning that in none of them the mutation causing borrelidin resistance had been lost. A direct measurement of the ThrRS activity in repurified derivatives of the two ThrRS-overproducing strains confirmed this result, since the three repurified strains Bor Res 3 I, Bor Res 3 II, and Bor Res 3 III exhibited a fourfold increase in the ThrRS, and the repurified strains Bor Res 15 I, Bor Res 15 II, and Bor Res 15 III exhibited an eightfold increase in the ThrRS, indicating that the mutations responsible for the ThrRS overproductions were genetically stable. To investigate the stability of mutations leading to PheRS overproduction in strains G118 and S1, they were grown for about 50 generations in nonselective medium (S1 was grown in rich medium at 32°C; G118 was grown in minimal medium supplemented with phenylalanine) and subsequently plated on the same medium. A total of 100 colonies from each strain were then tested for the maintenance of the mutations causing enzyme overproduction. It was found that 5% of the G118 derivatives and 8% of the

TABLE 7. *Specific activity of three aminoacyl-tRNA synthetases in K-12B and Bor Res 3 grown in three different media*

Strain	Medium[a]	Aminoacyl-tRNA synthetase (U/mg of protein)		
		ThrRS	SerRS	LeuRS
K-12B	Minimal	0.145	0.133	0.104
	Minimal + thr[a]	0.149		
	Rich	0.253	0.123	0.089
Bor Res 3	Minimal	0.553	0.142	0.091
	Minimal + thr[a]	0.542		
	Rich	0.526	0.122	0.108

[a] Concentration, 50 μg/ml.

S1 descendents had lost this character, as judged by the acquirement of a phenylalanine prototrophic or a temperature-sensitive phenotype, respectively. Since frequent loss of mutations is characteristic for gene amplification events, and since this has been demonstrated to be a *recA* dependent process (17, 44), we have introduced the *recA1* allele into strains S1 and G118 by standard procedures. Stability tests were performed in the same way as described above, and it was found that the *recA1* derivatives of S1 and G118 were stable.

DISCUSSION

Genetic studies have revealed that the structural genes of the ThrRS and PheRS are closely linked to each other on the *E. coli* chromosome at 37.7 min (22), whereas all other structural genes for aminoacyl-tRNA synthetases mapped so far are scattered around the chromosome (cf. 38). Therefore, it was of interest to study whether the overproduction of one of these two aminoacyl-tRNA synthetases is accompanied by an increase in the level of the other one.

ThrRS-overproducing mutants were already available (36, 39), and it was shown that the four- and eightfold increase in the concentration of the ThrRS in Bor Res 3 and Bor Res 15, respectively, is restricted to this aminoacyl-tRNA synthetase, since the level of five other activating enzymes, including PheRS, was not changed. These results demonstrate that in the ThrRS-overproducing strains the mutation has affected only the regulation of the intracellular level of the ThrRS and not of the PheRS.

On the other hand, two PheRS-overproducing strains which were isolated by different procedures and in which the level of PheRS was increased by a factor of 2.5 also showed an elevated level of ThrRS, whereas the concentration of other aminoacyl-tRNA synthetases, e.g., of IleRS, was not affected by the mutation. In both mutants, however, the concentration of the ThrRS had only been raised by a factor of 1.8 over the value of the parental strains in comparison to a factor of 2.5 for the PheRS. Similar values for a concomitant increase in PheRS and ThrRS were previously observed in *E. coli* cells carrying on an episome the region of the PheRS and ThrRS genes (22).

The coordinate increase in the level of PheRS and ThrRS in strains S1 and G118 might be due to either a common regulatory element or to the amplification of the chromosomal area in which the respective structural genes are located. Gene duplication as a mechanism for increasing the concentration of an aminoacyl-tRNA synthetase has been already reported for the glycine-specific enzyme (17, 44). Since it is known that gene duplications are easily lost during several cell cycles under growth conditions lacking the selective stimulus, we investigated the stability of the mutations causing the PheRS and ThrRS overproduction in mutants S1 and G118 and found that it decreased between 5 and 10% after about 50 generations of growth. The fact that this loss was prevented in the presence of the *recA1* allele strongly indicates that the increased level of these two enzymes is indeed based on a gene amplification process and is not an indication of a simultaneous regulation. This conclusion is further substantiated by the result that mutations causing the ThrRS overproduction in Bor Res 3 and Bor Res 15, which do not affect the intracellular PheRS concentration, were not lost after several generations of growth in the absence of the selective agent borrelidin. The underlying mutation, therefore, in this case seems not to be a gene amplification event but more probably a mutation in the operator or promoter region of the ThrRS structural gene, since genetic mapping data reveal a close linkage to structural gene mutations (J. Fröhler, J. Thomale, G. Nass, and A. Böck, manuscript in preparation). One possible reason for the preferential isolation of amplification mutants in the case of PheRS could be that each of the structural genes of this enzyme (*pheS* and *pheT*) possesses its own promoter, rendering the selection of concomitant "promoter-up" mutations highly improbable.

It is interesting to note in this connection that the wild-type strain ThrRS is subject to metabolic regulation leading to a derepression of a factor of about 2, whereas in the ThrRS-overproducing mutants the level of ThrRS is not further increased upon growth in rich medium. Possible explanations for this observation could be that either the mutation responsible for overproduction of ThrRS in principal does not

allow the derepression of ThrRS or that the need of an increased ThrRS level in rich medium is already met in these mutants by their four- and eightfold ThrRS concentration, respectively. Thus, our ThrRS-overproducing mutants differ from the also genetically stable MetRS-overproducing strain AB 311, which when grown in rich medium derepressed its MetRS level further, but did not react to a shortage of charged $tRNA^{Met}$ (7). So far, we have not investigated whether the ThrRS of Bor Res 3 or Bor Res 15 is further derepressed when there is an internal deficiency of aminoacylated $tRNA^{Thr}$.

The elevated PheRS concentration in G118 was additionally increased by a factor of about 2 in cells grown in minimal medium lacking phenylalanine. This derepression was restricted to PheRS; the level of other aminoacyl-tRNA synthetases including ThrRS was not affected by the withdrawal of phenylalanine from the medium. A similar increase of PheRS was also observed in the parental strain G1 when grown without exogenous phenylalanine, indicating that two independent regulatory processes contribute to the increased PheRS concentration (about fivefold) in G118 grown in minimal medium without phenylalanine. The stimulus for the derepression of PheRS in phenylalanine-free minimal medium in G1 and G118 is probably the intracellular deficiency of phenylalanine in respect to their PheRS, which possesses lowered affinity for the substrate phenylalanine. Since it is known (6) that exogenous phenylalanine expands its intracellular pool, the PheRS of those mutants is better saturated in phenylalanine-containing medium. Similar regulatory phenomena have been observed in an *E. coli* mutant with an impaired phenylalanine biosynthesis (34).

The result of derepression of only the PheRS in G1 and G118 during growth in the absence of exogenous phenylalanine also supports our conclusion that the formation of the PheRS and ThrRS is regulated independently, although their structural genes are closely linked to each other on the chromosome. In vitro transcription and translation experiments using the specialized transducing phage λ p2 (23) will show whether PheRS and ThrRS also are regulated independently in vitro and might help in the identification of effectors and of the mutations which influence the rate of formation of these enzymes.

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