

A Mutant of *Escherichia coli* Defective in Leucyl, Phenylalanyl-tRNA-Protein Transferase*

(regulation of growth/post-translational modification)

R. L. SOFFER AND M. SAVAGE

Department of Molecular Biology, Division of Biological Sciences, Albert Einstein College of Medicine, Bronx, New York 10461

Communicated by Harry Eagle, November 19, 1973

ABSTRACT A mutant of *E. coli* that lacks leucyl, phenylalanyl-tRNA-protein transferase (EC 2.3.2.6) has been isolated. Ability to produce the two activities could be introduced into the mutant from an F' strain whose episome contains genetic material located between 45 and 54 min on the *E. coli* chromosome. When grown into stationary phase and resuspended in minimal medium with glycerol, the mutant exhibited a marked lag before resuming growth. Revertants that did not show this lag were selected and were found to have regained both transfer activities. Extracts of wild-type, mutant, and revertant strains were compared as acceptors for the enzymatic transfer of radioactive phenylalanine. Analysis of the labeled polypeptides by disc gel electrophoresis indicated that certain potential acceptors may be preferentially acylated *in vivo*.

These data provide genetic confirmation that the same enzyme protein catalyzes the transfer of leucine and phenylalanine and suggest that leucyl,phenylalanyl-tRNA-protein transferase is involved in a growth regulatory mechanism.

Aminoacyl-tRNA-protein transferases are soluble enzymes found in bacterial and mammalian cells which catalyze the transfer of certain amino acids from tRNA into peptide linkage with specific NH₂-terminal residues of protein or peptide acceptors (for a recent review see ref. 1). Although they represent an enzymatic mechanism for post-translational modification of the primary structure of special classes of polypeptides, their cellular function is unknown. The *Escherichia coli* enzyme, leucyl,phenylalanyl-tRNA-protein transferase (EC 2.3.2.6) (2) catalyzes the transfer of these amino acids to basic NH₂-terminal amino-acid residues (3-5) and is responsible for the original observation of Kaji, Kaji and Novelli, that ribosome-free extracts could incorporate leucine and phenylalanine into protein (6). *E. coli* offers the possibility of using genetic tools to investigate the role of aminoacyl-tRNA-protein transferases and this report describes the isolation and some characteristics of a mutant which lacks leucyl,phenylalanyl-tRNA-protein transferase.

MATERIALS AND METHODS

Materials. Protosol and Aquasol were from the New England Nuclear Corp. The preparations of [¹⁴C]leucyl-tRNA (0.8 nmol/mg, 262 μCi/μmol), [¹⁴C]phenylalanyl-tRNA (0.4 nmol/mg, 472 μCi/μmol), leucyl,phenylalanyl-tRNA-protein transferase (10 units/mg), and L-phenylalanyl-tRNA synthetase (150 units/mg) were obtained as described elsewhere (4).

* This is paper no. 11 in a series entitled "Enzymatic Modification of Proteins"; the preceding paper in this series is ref. 4.

Bacterial Strains. W4977 was a gift from Dr. Ann Ganesan of Stanford University and is a K12 F⁻ proline auxotroph. An "F' kit" which included 18 strains of *E. coli* and information on their chromosomal and episomal markers was provided by Dr. Barbara Bachman, curator of the *E. coli* Genetic Stock Center, Department of Microbiology, Yale University School of Medicine.

Media. Liquid and solid minimal medium (7) was supplemented with 2 mM L-proline and with 0.5% glycerol. Enriched medium contained 0.8% nutrient broth.

Assays. For screening assays of leucyl,phenylalanyl-tRNA-protein transferase single colonies were grown overnight in 10 ml of enriched medium at 30°. Unfractionated lysates were prepared using EDTA, lysozyme, Brij 58 and DNase (8). Reaction mixtures (75 μl) contained 50 mM Tris·HCl (pH 8.2), 50 mM 2-mercaptoethanol, 0.2 M KCl, 0.3 mg/ml of chloramphenicol, 0.5 mg/ml of α-casein, 0.3 nmol/ml of [¹⁴C]aminoacyl-tRNA and 25 μl of lysate corresponding to approximately 1 × 10⁸ cells. Incubation was for 60 min at 37° and radioactivity insoluble in hot 5% trichloroacetic acid was determined on 50-μl aliquots by the filter paper disc technique (9).

Assays on 105,000 × g supernatant fractions obtained from exponentially growing cells after grinding with alumina (2) were performed similarly, except that the concentration of [¹⁴C]aminoacyl-tRNA was 1.6 nmol/ml and the time of incubation was 10 min.

Protein Determinations. The method of Lowry *et al.* (10) was employed using bovine-serum albumin as a standard.

RESULTS

Isolation and Characterization of Mutant and Revertant Strains. W4977 was incubated with 0.5 mg/ml of nitrosoguanidine in Tris·maleate (TM) buffer (11) for 30 min and lysates from 1200 survivors were screened for ability to catalyze the transfer of [¹⁴C]phenylalanine from tRNA to α-casein. The lysate from one strain, MS845, had no detectable activity (<0.3% wild-type) regardless of the growth stage of the cells from which it was prepared and was found to be similarly inactive in the leucine transfer reaction. Preliminary attempts to map the mutation were carried out using 18 F' donors whose episomes comprised almost the entire *E. coli* genome. Equal numbers of exponentially growing donor and mutant cells were mixed and incubated at 37° for 2 hr. The mixtures were plated on minimal agar selecting against the F' donor, and lysates from 60 colonies were assayed for

phenylalanine transfer activity. The only cross which yielded clones containing enzyme activity was that in which strain KLF42/KL253 was used as donor. Lysates from 20 of the 60 colonies in this F-duction possessed phenylalanine transfer activity and these extracts also catalyzed the transfer of leucine. Both activities tended to be lost on subculture, particularly when it was carried out in minimal medium. The episome carried by this donor strain contains genetic material located between genes for serine deaminase (*dsdA*) and fucose utilization (*fuc*) at 45 and 54 min on the *E. coli* chromosome (12). Our data do not distinguish whether it possesses the structural gene for leucyl,phenylalanyl-tRNA-protein transferase or a suppressor active on the original mutation. The latter possibility must be seriously considered, since the episome is known to contain a suppressor (*supN*).

MS845 had the same generation time at 37° as W4977 in enriched medium or in minimal medium supplemented with glycerol. However, when grown into stationary phase it gave a smaller yield and when then resuspended in minimal medium with glycerol it exhibited a pronounced lag before resumption of growth as measured by either absorbance or viable counts. To determine whether the same genetic defect accounted for the growth lag and the absence of leucyl,phenylalanyl-tRNA-protein transferase, revertants with normal growth characteristics were selected by cycling the mutant in minimal medium containing glycerol. Separate revertants were isolated from four mutant clones after 8 cycles. Each of the revertants was found to have regained transfer activities for both leucine and phenylalanine. Representative data on the growth and enzyme activity of W4977, MS845 and one of the revertants, R18, are shown in Fig. 1 and Table 1. Mixing experiments shown in the table ruled out the possibility of an inhibitor accounting for the lack of activity in the mutant.

The mechanism of the growth lag in MS845 has not been delineated. It has been found to last from 3 to 8 hr in minimal medium supplemented with glycerol and to occur regardless of the medium in which the cells are grown into stationary phase. It is absent, however, if the cells are resuspended in enriched medium. During the lag period the rates of synthesis of DNA, RNA, and protein, as determined with pulses of labeled precursors (13), were found to be less than 10% of that for the same number of wild-type cells growing exponentially.

TABLE 1. *Leucyl,phenylalanyl-tRNA-protein transferase activity in soluble extracts from different strains of E. coli*

Extract			Incorporation	
W4977 (mg/ml)	MS845 (mg/ml)	R18 (mg/ml)	Leucine	Phenylalanine (pmol/ml)
0.35	—	—	41	38
0.70	—	—	77	66
—	0.70	—	<1	<1
—	4.60	—	<1	<1
0.70	0.70	—	70	65
—	—	0.17	36	31
—	—	0.34	75	63
—	0.70	0.34	68	64

Supernatant fractions ($105,000 \times g$) were prepared from alumina extracts of cells growing exponentially in mineral medium with glycerol and proline at 37°. Enzymatic transfer of [¹⁴C]amino acid from tRNA to protein was determined after 10-min incubation as described under *Methods*.

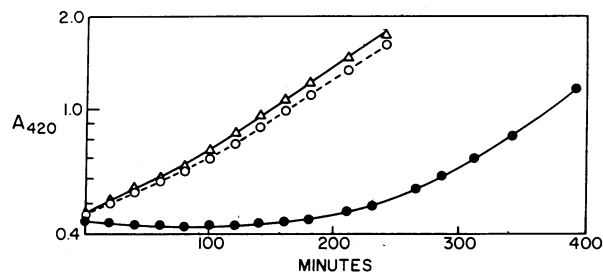


FIG. 1. Growth of stationary phase cells after suspension in fresh medium. Single colonies were grown on a rotary shaker in 50 ml of minimal medium for 72 hr at 37°. The respective optical densities at 420 nm of W4977, MS845, and R18 were 9.0, 3.0, and 9.0. Appropriate aliquots were washed by centrifugation, suspended in 50 ml of fresh medium at time zero and agitated at 37°. W4977 (○); MS845 (●); R18 (△).

Induction of tryptophanase by tryptophan (14) did not occur during this period but was normal during exponential growth. An extract made from the mutant during lag phase was similar to one from exponentially growing wild-type in DNA-dependent RNA polymerase activity (15) and in ability to catalyze the polyuridylylate-directed synthesis of polyphenylalanine (16).

Acceptor Proteins in the Wild-Type, Mutant, and Revertant.

Since MS845 lacks leucyl,phenylalanyl-tRNA-protein transferase activity its acceptor substrates cannot be modified *in vivo* and reflect the total population of potential acceptor molecules in *E. coli*. Leucyl,phenylalanyl-tRNA-protein transferase does not recognize NH₂-terminal leucine or phenylalanine residues (4) so differences in acceptance by mutant and wild-type proteins in the reaction catalyzed by purified enzyme should provide a measure of acceptance by the latter proteins *in vivo*. Therefore soluble proteins and salt-washed ribosomes from the three strains were examined as acceptors for the enzymatic transfer of [¹⁴C]phenylalanine. The average acceptance by mutant soluble proteins determined under conditions of stoichiometric acylation was 0.44 nmol/mg (Table 2). Assuming an average molecular weight of 40,000 for these

TABLE 2. *Acceptance of phenylalanine by soluble and ribosomal proteins from the different strains*

Strain	Acceptance	
	Soluble proteins (nmol/mg)	Ribosomes
W4977	0.31 (0.28–0.35)	0.60 (0.33–1.1)
R18	0.30 (0.20–0.41)	0.68 (0.41–1.1)
MS845	0.44 (0.36–0.50)	0.65 (0.47–1.1)

Acceptor-dependent incorporation of [¹⁴C]phenylalanine into material insoluble in cold 5% trichloroacetic acid and resistant to ribonuclease was measured in the presence of excess transfer enzyme, a phenylalanyl-tRNA-generating system and 0.3 mg/ml of chloramphenicol under conditions previously described for stoichiometric acylation (3). Soluble proteins and ribosomes washed 3 times with 1 M NH₄Cl (18) were prepared from four separate cultures of each strain growing exponentially in minimal medium. The data show the average acceptance with the extreme values in parentheses. Each determination represents a dose-response curve in which the value was derived from a linear relationship between acceptor concentration and phenylalanine incorporation.

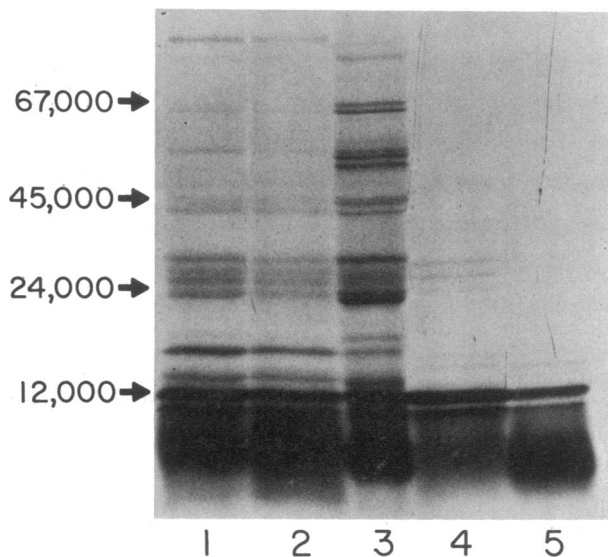


FIG. 2. Autoradiograms of protein acceptors acylated with [^{14}C]phenylalanine and subjected to disc gel electrophoresis. Reaction mixtures (1 ml) were similar to those described under *Methods* except that they contained 2.1 nmol of [^{14}C]phenylalanyl-tRNA, 70 mV of leucyl,phenylalanyl-tRNA-protein transferase and either 2.5 mg of soluble protein or 1.5 mg of ribosomal protein. Under these conditions transfer into ribonuclease-resistant acid-insoluble product was more than 97% dependent upon the added acceptor proteins. After incubation for 30 min at 37° pancreatic ribonuclease (15 μg) was added and incubation continued for 15 min. The samples were precipitated with 1 ml of 10% trichloroacetic acid and the pellets washed successively with 1 ml each of 5% trichloroacetic acid, ether-ethanol (1:1), and ether. The dried precipitates were dissolved in 0.4 ml of 0.06 M Tris·HCl (pH 6.8) containing 2% sodium dodecyl sulfate and 5% 2-mercaptoethanol and boiled for 2 min. Aliquots (8000 cpm) were subjected to electrophoresis in 0.025 M Tris·HCl (pH 8.3), 0.19 M glycine, 0.1% sodium dodecyl sulfate, and 0.1% 2-mercaptoethanol. The procedure was carried out with a slab gel containing a 5–20% linear gradient of acrylamide as described by Maizel (17). The gel was stained for protein with 0.25% Coomassie blue in 9% acetic acid, 50% methanol and then destained with 7% acetic acid, 10% methanol. After drying it was exposed to Kodak medical x-ray film for 35 days. Samples 1, 2, and 3 contained the soluble proteins, respectively, of W4977, R18, and MS845. Ribosomal proteins of W4977 and MS845 were present in samples 4 and 5. The molecular weight markers were bovine albumin, ovalbumin, β -casein, and cytochrome *c*.

proteins, this figure suggests that about 1.8% of them are potential acceptors. The parental and revertant strains had a corresponding average value of 0.31 nmol/mg. If this difference were due solely to acylation occurring *in vivo*, it would suggest that almost 30% of the potential soluble acceptors undergo post-translational modification in the cell. The values on acceptance by ribosomes in all three strains were extremely variable perhaps because subtle differences in their physical structure may be critical in relation to accessibility of appropriate NH_2 -termini.

Labeled acceptors were also analyzed by disc gel electrophoresis. At least 21 potential soluble and three ribosomal acceptors could be detected by inspection of the autoradiograms (Fig. 2). More than 75% of the potential soluble acceptor molecules and more than 97% of the potential ribosomal acceptors migrated with a mobility corresponding to a molec-

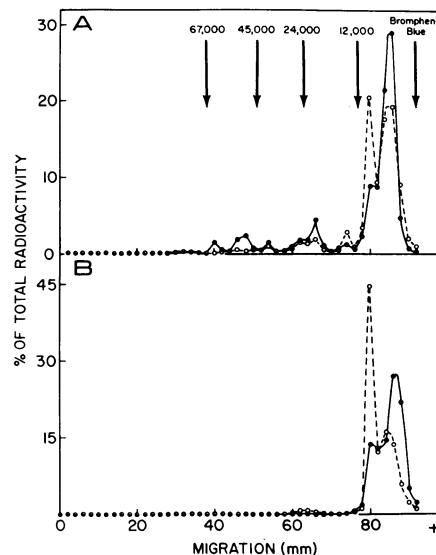


FIG. 3. Quantitative analysis of radioactive acceptor proteins after disc gel electrophoresis. Aliquots of acceptor proteins containing 9000 cpm of [^{14}C]phenylalanine were subjected to electrophoresis on a slab gel as described in Fig. 2. Individual lanes were sliced into 2-mm sections which were incubated for 18 hr at 37° in scintillation vials containing 0.25 ml of Protosol and 0.5 ml of concentrated NH_3 . Aquasol (15 ml) was then added and the samples were chilled at 4° for 48 hr before counting. A, soluble proteins; B, ribosomal proteins. W4977 (O); MS845 (●).

ular weight in the reduced denatured form of less than 12,000 (Fig. 3). The pattern of labeled parental and revertant soluble proteins was almost identical (Fig. 2) and quite different from that of the radioactive proteins of the mutant. In particular, mutant doublets with molecular weights of approximately 64,000, 48,000, and 22,000 were relatively more heavily labeled than the corresponding wild-type proteins. In addition, the most rapidly migrating acceptor(s) (molecular weight <10,000) contained a higher proportion of incorporated radioactivity in the mutant soluble fraction than in the others. These results suggest that several of the potential soluble acceptors may be preferentially acylated *in vivo*.

Ribosomal acceptors migrated identically to the low molecular weight soluble acceptors on gels containing sodium dodecyl sulfate. Different mobilities were observed, however, when they were examined in gels containing 8 M urea at pH 4.5 (not shown). The most prominent labeled acceptor from the wild-type ribosomes possessed a mobility similar to cytochrome *c* on sodium dodecyl sulfate gels as had previously been observed using ribosomes from *E. coli* B (18). In contrast, a smaller polypeptide(s) accounted for most of the total radioactivity in the preparation of mutant ribosomal protein. The latter may, therefore, be a preferred substrate *in vivo*.

DISCUSSION

The mutant described in this report provides a new approach for investigating the function of aminoacyl-tRNA-protein transferases. Our results implicate leucyl,phenylalanyl-tRNA-protein transferase in a growth regulatory process; however, the relationship between the fundamental enzymatic defect in MS845 and its growth phenotype is unclear. The growth lag when suspended in fresh minimal medium containing glycerol appears to be a consequence of an event occurring in sta-

tionary phase. The fact that there is no lag in enriched medium suggests that broth contains a compound essential for growth which the mutant is temporarily unable to produce. One possibility is that the enzyme required to generate this compound is subject to degradation in stationary phase cells and that its catalytic activity can be increased by post-translational acylation with leucine or phenylalanine. In the wild-type acylated enzyme molecules surviving in stationary phase cells might be active enough so that growth in fresh minimal medium could commence almost immediately. In the mutant no such acylated molecules would exist and growth might not occur until sufficient nonacylated enzyme could be made such that the product whose formation it catalyzed was no longer growth-limiting. This hypothesis predicts that the growth lag should be eliminated by addition of the proper compound to the medium. It also predicts that the enzyme responsible for the biosynthesis of this molecule is an acceptor substrate for leucyl,phenylalanyl-tRNA-protein transferase and, therefore, contains an NH₂-terminal arginine or lysine residue; that its activity is diminished in stationary phase to a lower level in the mutant than in the wild-type; that enzymatic acylation of its NH₂-terminal residue increases its catalytic constant; and that it is fractionally acylated in the wild-type *in vivo*.

An understanding of the role played by aminoacyl-tRNA-protein transferases requires identification of their natural substrates. A major obstacle in achieving this objective has been that physiologically important acceptors would be expected to be modified within the cell and could, therefore, not be detected as substrates *in vitro*. The mutant represents a source of acceptors which have not been acylated and a comparison of acceptance by its polypeptides and those of the wild-type has, therefore, provided the first opportunity to investigate which potential acceptors are actually recognized *in vivo*. The data suggest that as much as 30% of the potential soluble acceptors may be modified within the cell and that post-translational acylation with leucine and phenylalanine may account for about 0.5% of the NH₂-termini of bulk

soluble proteins. Since several soluble proteins and at least one ribosomal protein are probably substrates *in vivo*, it is likely that further study of the mutant will reveal considerable pleiotropy in its phenotype.

This work was supported by grants from the National Institutes of Health (GM 11301; AM 12395) and the National Science Foundation (GB 35203). This is Communication no. 310 from the Joan and Lester Avnet Institute of Molecular Biology. R.L.S. is a Faculty Research Associate of the American Cancer Society.

1. Soffer, R. L. (1974) *Advan. Enzymol.* **40**, in press.
2. Leibowitz, M. J. & Soffer, R. L. (1970) *J. Biol. Chem.* **245**, 2066-2073.
3. Leibowitz, M. J. & Soffer, R. L. (1971) *J. Biol. Chem.* **246**, 5207-5212.
4. Soffer, R. L. (1973) *J. Biol. Chem.* **248**, 8424-8428.
5. Momose, K. & Kaji, A. (1966) *J. Biol. Chem.* **241**, 3294-3307.
6. Kaji, A., Kaji, H. & Novelli, G. D. (1965) *J. Biol. Chem.* **240**, 1185-1191.
7. Herzenberg, L. (1959) *Biochim. Biophys. Acta* **31**, 525-538.
8. Godson, G. N. & Sinsheimer, R. L. (1967) *Biochim. Biophys. Acta* **149**, 476-488.
9. Mans, R. J. & Novelli, G. D. (1960) *Biochem. Biophys. Res. Commun.* **3**, 540-543.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275.
11. Adelberg, E. A., Mandel, M. & Chen, G. C. C. (1965) *Biochem. Biophys. Res. Commun.* **18**, 788-795.
12. Taylor, A. L. (1970) *Bacteriol. Rev.* **34**, 155-175.
13. Jacobson, A. & Gillespie, D. (1968) *J. Bacteriol.* **95**, 1030-1039.
14. Bilezekian, J. P., Kaemper, R. O. R. & Magasanik, B. (1967) *J. Mol. Biol.* **27**, 495-506.
15. Burgess, R. B. (1969) *J. Biol. Chem.* **244**, 6168-6176.
16. Nirenberg, M. W. & Matthaei, J. H. (1961) *Proc. Nat. Acad. Sci. USA* **47**, 1588-1602.
17. Maizel, J. V., Jr. (1971) in *Methods in Virology*, eds. Maramorosch, K. & Koprowski, H. (Academic Press, New York), pp. 179-246.
18. Leibowitz, M. J. & Soffer, R. L. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1866-1869.