*A MUTANT OF E. COLI WITH AN ALTERED SUPERNATANT FACTOR**

By G. P. Tocchini-Valentini and E. Mattoccia

INTERNATIONAL LABORATORY OF GENETICS AND BIOPHYSICS, NAPLES, ITALY

Communicated by Jean Brachet, July 8, 1968

Conditional lethal mutants[1] offer a powerful tool in the study of cellular processes. The contribution of amber and *ts* mutants to our understanding of bacteriophage T4 and λ physiology has been so decisive that the possibility of applying a similar approach to bacteria has been considered. Several laboratories have isolated *ts* mutants of *Escherichia coli*,[2, 3] and strains with altered aminoacyl-tRNA synthetases have been described.[4]

In order to investigate macromolecular synthesis we have isolated several sets of *E. coli* mutants and have used suicide by tritiated precursors[5] to select for a particular phenotype.

We wish to report biochemical experiments which indicate that mutant G1 from our collection has an alteration in a supernatant factor required to synthesize proteins.

Materials and Methods.—Strains and medium: Our wild type is Hfr C met$^-$ RC^{rel} (from Dr. S. Brenner's collection), and the mutant G1 derived from it is a member of a set of *ts* mutants, blocked in protein synthesis, isolated by one of us (G. P. T.-V.) in Dr. S. Brenner's laboratory (MRC Molecular Biology Unit, Cambridge, England). The mutants were selected by labeling a stock of wild type mutagenized by nitrosoguanidine with tritiated phenylalanine and tritiated tyrosine of high specific activity at 42°C for a 45-min period. The suicide was then carried out according to Person.[5] The surviving fraction was found to be enriched in mutants unable to carry out protein synthesis at 42°C.

The bacteria were always grown in Penassay broth (17 gm/ml Difco Antibiotic Medium).

Cell-free system: (a) *Preparation of s-30:* Wild-type and mutant G1 cells harvested in early log phase were washed and disrupted by grinding with alumina (twice the weight of washed cells). The resulting slurry was extracted with an equivalent weight of buffer containing 0.01 *M* Tris, pH 7.8, 0.01 *M* magnesium acetate, 0.06 *M* KCl, and 0.006 *M* β-mercaptoethanol. Alumina and intact cells were removed by centrifugation at 20,000 × *g* for 30 min. The supernatant fluid was treated with 2 μg/ml of DNase, incubated at 30°C for 45 min, and centrifuged at 20,000 × *g* for 30 min. The supernatant of this centrifugation constituted the s-30.

(b) *Supernatant and ribosomes:* The s-30 was centrifuged at 105,000 × *g* for 3 hr. The upper two thirds of the liquid layer was aspirated and re-centrifuged at 105,000 × *g* for 3 hr. The upper layer resulting from the second centrifugation constitutes the supernatant.

The ribosomal pellet obtained from the first high-speed centrifugation was treated according to Apirion[6] and used for the experiments reported in Table 2, column *A*.

For the experiments reported in Table 2, column *B*, the ribosomes were repeatedly washed according to Lucas-Lenard and Lipmann;[7] the ribosomes prepared in this way are free of the factors Ts and Tu and retain a small amount of the factor G (measured as GTPase).

Preparation of the 40–65% $(NH_4)_2SO_4$ fraction: The 40–65% $(NH_4)_2SO_4$ fraction was prepared according to Nishizuka and Lipmann.[8] Cells were ground with alumina, and the resulting s-30 was treated with a 2% solution of protamine sulfate. The resulting precipitate was collected by centrifugation and extracted with 0.05 *M* K_2HPO_4 containing 0.002 β-mercaptoethanol.

The $(NH_4)_2SO_4$ fractionation was performed on this extract. The fraction precipitating between 40 and 65% saturation was taken up in 10^{-2} *M* Tris-HCl buffer, pH 7.4, containing 0.002 *M* β-mercaptoethanol and dialyzed against the same buffer overnight.

Preparation of phenylalanine-tRNA: *E. coli* B tRNA was charged with radioactive C^{14}-phenylalanine according to Conway.[9]

Standard assay: The reaction mixture contained the following in μmoles/ml: 100 Tris, pH 7.8; 10 magnesium acetate; 50 KCl; 6.0 β-mercaptoethanol; 1.0 ATP; 5.0 PEP; 0.03 GTP; 0.02 C^{14}-phenylalanine, and 20 μg/ml of PEP kinase. The total volume was usually 0.25 ml and for each assay, 0.5–2 mg of s-30 and 20 μg of poly U were used. Samples were incubated at 30°C for 45 min, precipitated with 10% TCA, boiled for 20 min, filtered through millipore, and counted in a gas-flow counter (25% efficiency).

In one instance (Table 3), we measured polyphenylalanine synthesis starting from phenylalanine-tRNA. The incubation mixture was identical to the one used for the standard assay, with the following exceptions: ATP, PEP, PEP kinase, and C^{14}-phenylalanine were omitted and, in the usual total volume of 0.25 ml, 40 μg of C^{14}-phenylalanine-tRNA (3000 cpm) and 20 μμmoles of phenylalanine were added to the already listed components.

Results.—The mutant G1, which we are discussing in this paper, was suspected of having a primary lesion in the protein synthetic machinery due to the rapid cessation of protein synthesis after a shift from 30° to 42°C, and to the fact that RNA synthesis, on the contrary, appeared to proceed normally at both temperatures.

(1) *Nature of the defect:* In order to investigate the biochemical nature of the lesion, poly U-directed synthesis of polyphenylalanine was measured in s-30 prepared both from the wild type and the mutant. The results are reported in Table 1 (expts. 1 and 2).

The activity in the mutant extract is seen to be strikingly low as compared to the wild-type s-30. While these assays were performed at 30°C, the same result was obtained at higher temperatures. Experiments in which equal amounts of the two extracts were mixed showed that the activities were completely additive, indicating that an inhibitor is not responsible for the inactivity of the mutant extract.

In order to find out which part of the machinery required for the synthesis of polyphenylalanine is altered in the extract from G1, supernatant and ribosomes were prepared from the mutant and wild type, and the four possible combinations were examined for the poly U-directed synthesis of polyphenylalanine. Both

TABLE 1. *Polyphenylalanine synthesis by s-30 derived from the wild type and the mutant G1: Effect of the 40–65% $(NH_4)_2SO_4$ fraction.*

Expt. no.	Source of s-30	WT 40–65% $(NH_4)_2SO_4$ fraction	G1 40–65% $(NH_4)_2SO_4$ fraction	Hot TCA-insoluble (cpm/mg protein)
1	WT	−	−	2800
2	G1	−	−	221
3	WT	+	−	2650
4	G1	+	−	1059
5	WT	−	+	2700
6	G1	−	+	235

The conditions of the assay are described in *Materials and Methods*. In experiments 3 and 4, 38 μg of 40–65% $(NH_4)_2SO_4$ fraction prepared from the wild type were present. In experiments 5 and 6, 41 μg of 40–65% $(NH_4)_2SO_4$ fraction prepared from the mutant were present.

supernatant and ribosomes from the wild type appeared to be able to complement the ribosomes and supernatant from the mutant, respectively (Table 2, col. *A*). These results suggest that the extracts from mutant G1 are defective in some supernatant factor, which is bound to the ribosomes to such an extent that it is not removed by a mild washing procedure. In agreement with this interpretation, it was found that after five washes with a buffer containing 1 *M* NH_4Cl,[7] the wild-type ribosomes lost the ability to complement the mutant supernatant (Table 2, col. *B*).

We thus conclude that the activity of a supernatant factor is missing in extracts derived from the mutant.

(2) *Complementation of the defective extract by a fraction prepared from the wild type:* Lipmann[10] and his collaborators have shown that a 40–65 per cent $(NH_4)_2SO_4$ fraction derived from bacterial supernatant contains the factors Tu, Ts, and G, required for the synthesis of polyphenylalanine from phenylalanine-tRNA. We attempted to complement the defective extract prepared from the mutant G1 by using a 40–65 per cent $(NH_4)_2SO_4$ fraction derived from the wild type (results reported in Table 1, expts. 3 and 4). A net stimulation of the extract from the mutant could be detected, but no stimulation of the extract from the wild type could be shown. When the complementing fraction was prepared from the mutant G1, no stimulatory effect could be observed either in the wild-type extract or in the mutant extract (Table 1, expts. 5 and 6).

These results indicate that a *factor* contained in the 40–65 per cent $(NH_4)_2SO_4$ fraction is altered in the mutant G1.

(3) *Properties of the factor:* (a) *Heat inactivation:* The successful outcome of the complementation experiment (Table 1, expts. 3 and 4) provided an *in vitro* assay for the altered factor in the mutant G1 and allowed us to investigate some of its properties. Aliquots of the 40–65 per cent fraction, always prepared from the wild type, were heated for two minutes at various temperatures and tested for the ability to stimulate an s-30 derived from the mutant. Figure 1 shows that at 60°C, about 90 per cent of this activity is heat-resistant. Lipmann[10] and his co-workers could demonstrate that, when heated under the same conditions, one of the factors, Tu, contained in the 40–65 per cent $(NH_4)_2SO_4$ fraction, is considerably more heat-sensitive. It is thus possible to conclude that the factor defective in the extract derived from G1 is not Tu.

(b) *Size:* An aliquot of the 40–65 per cent $(NH_4)_2SO_4$ fraction was run in a sucrose gradient, and the various fractions collected were then assayed for phenyl-

TABLE 2. *Crosses of supernatants and ribosomes from the wild type and the mutant G1.*

Expt. no.	Mixtures	Hot TCA-Insoluble (cpm) *A*	*B*
1	Supn WT + Ribs WT	861	872
2	Supn WT + Ribs G1	772	800
3	Supn G1 + Ribs G1	42	81
4	Supn G1 + Ribs WT	608	37

In each experiment, 0.25 mg of supernatant proteins and 0.36 mg of ribosomes were used. For the experiments reported in column *A*, the ribosomes were prepared according to Apirion;[6] for the experiments reported in column *B*, the ribosomes were treated according to Lucas-Lenard and Lipmann.[7]

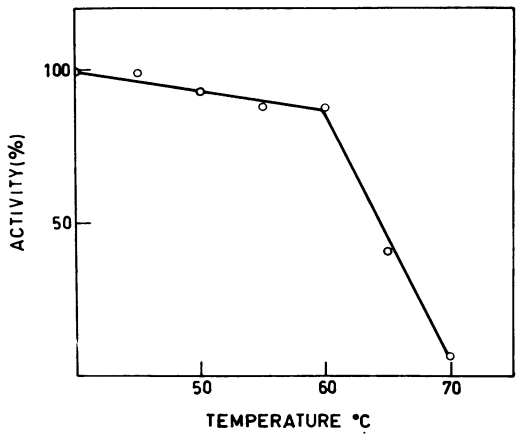

FIG. 1.—Heat stability of the factor. Aliquots of the 40–65% $(NH_4)_2SO_4$ fraction from the wild type containing 38 μg of proteins were heated at the indicated temperatures for 2 min. The ability to stimulate an s-30 derived from the mutant G1 was then measured by the standard assay (see *Materials and Methods*).

alanine-tRNA synthetase and for the ability to stimulate an s-30 derived from the mutant. The factor (Fig. 2) sedimented more slowly then the synthetase and since an $s_{20,w}$ of 8.6 has been reported [11] for the latter enzyme, we could tentatively attribute an $s_{20,w}$ of around 4 to the factor.

(c) *Function:* Our usual assay involved synthesis of polyphenylalanine starting from phenylalanine. Table 3, however, shows that the 40–65 per cent $(NH_4)_2SO_4$ fraction complements the s-30 derived from G1 when C^{14}-phenylalanine-tRNA is added to the assay mixture instead of C^{14}-phenylalanine. It is therefore clear that the defect on which we are focusing does not involve phenylalanine-tRNA synthetase (as was also evident from Fig. 2) or phenylalanine-t-RNA.

A net stimulation of the synthesis of polylysine directed by poly A was also

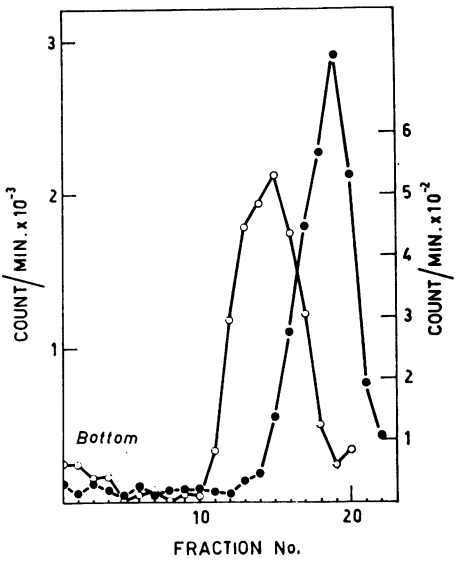

FIG. 2.—Sucrose gradient sedimentation of the factor. 50 μl of the 40–65% $(NH_4)_2SO_4$ fraction from the wild type containing 900 μg of proteins was layered on a 5-ml sucrose gradient (5–20%) and centrifuged at 39,000 rpm for 7 hr. Fractions of 3 drops were collected.

50-μl aliquots of each fraction were assayed for phenylalanine-tRNA synthetase according to Fangman and Neidhardt[14] and for the ability to stimulate an s-30 derived from the mutant G1 by the standard assay (see *Materials and Methods*).

(O—O) Activity of phenylalanine-tRNA synthetase. (●—●) Activity of the factor.

TABLE 3. *Polyphenylalanine synthesis by s-30 derived from the mutant G1, starting from phenylalanine-tRNA: effect of the 40–65% $(NH_4)_2SO_4$ fraction.*

Expt. no.	Source of s-30	WT 40–65% $(NH_4)_2SO_4$ fraction	Hot TCA-insoluble (cpm)
1	G1	−	234
2	G1	+	1554

The conditions of the assay are as described in *Materials and Methods*. In experiment 2, 38 μg of 40–65% $(NH_4)_2SO_4$ fraction prepared from the wild type were present.

TABLE 4. *Polylysine synthesis by s-30 derived from the mutant G1: effect of the 40–65% $(NH_4)_2SO_4$ fraction.*

Expt. no.	Source of s-30	WT 40–65% $(NH_4)_2SO_4$ fraction	Cpm incorporated
1	G1	−	14
2	G1	+	238

The procedures described in *Materials and Methods* for the standard assay of poly U-directed polyphenylalanine synthesis were used with the following substitutions: C^{14}-lysine was used in place of C^{14}-phenylalanine, and poly A in place of poly U. In experiment 2, 38 μg of 40–65% $(NH_4)_2SO_4$ fraction prepared from the wild type were present. The reaction was stopped by the addition of 5% TCA containing 0.25% sodium tungstate as described by Gardner *et al.*[13]

detected when the 40–65 per cent $(NH_4)_2SO_4$ fraction was added to the extract derived from the mutant (Table 4).

On the basis of these results, we conclude that the factor defective in the extract derived from the mutant is not specific for polyphenylalanine synthesis alone.

Discussion.—The isolation of a mutant of *E. coli* defective in a supernatant factor has revealed a new class of mutants deficient in the ability to support protein synthesis.

The possibility of complementing the extract derived from the mutant with fractions purified from the wild type provides an *in vitro* assay and opens new approaches to the study of the factors complementing the ribosomes that are required for amino acid polymerization.

The results of the *in vitro* complementation experiments reported here establish some properties of the factor defective in our mutant; its heat stability and ability to bind tightly to the ribosomes are reminiscent of factor G, described by Lipmann[10] and his collaborators.

In a subsequent paper,[12] evidence will be given suggesting that the factor altered in the mutant G1 corresponds to the G factor.

Summary.—A *ts* mutant of *E. coli* unable to produce proteins at high temperature is described. *In vitro* studies have demonstrated that extracts derived from the mutant are defective in a supernatant factor required to synthesize proteins.

One of us (G. P. T.-V.) would like to acknowledge his debt to S. Brenner for having introduced him to *ts* mutants. Both authors are grateful to F. Graziosi for his stimulating interest in this work.

The following abbreviations are used: tRNA, transfer RNA; Tris, tris(hydroxymethyl)-aminomethane; Ts, Tu, and G are the supernatant factors described by Lipmann;[10] GTP, guanosine 5′-triphosphate; ATP, adenosine 5′-triphosphate; PEP, phosphoenolpyruvate; poly U, polyuridylic acid; poly A, polyadenylic acid; TCA, trichloroacetic acid.

* This work was carried out under contract no. 012-61-12 BIAI from the Association Euratom-C.N.R.-C.N.E.N.

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