*Proc. Natl. Acad. Sci. USA*
Vol. 87, pp. 2657–2661, April 1990
Biochemistry

# Structure and probable genetic location of a "ribosome modulation factor" associated with 100S ribosomes in stationary-phase *Escherichia coli* cells

**(growth-dependent control/translation regulation/two-dimensional gel electrophoresis)**

AKIRA WADA*, YUKIKO YAMAZAKI†, NOBUYUKI FUJITA†, AND AKIRA ISHIHAMA†‡

*Department of Physics, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto 606, Japan; and †Department of Molecular Genetics, National Institute of Genetics, Mishima, Shizuoka 411, Japan

*Communicated by Motoo Kimura, January 2, 1990 (received for review November 24, 1989)*

ABSTRACT **The decrease in overall translation activity occurring concomitantly with the transition from the exponential growth phase to the stationary phase of *Escherichia coli* cells was found to be accompanied by the appearance of 100S ribosomes (dimers of 70S ribosome monomers). Analysis of ribosomal proteins by the radical-free and highly reducing method of two-dimensional gel electrophoresis indicated that a protein, designated protein E, was exclusively associated with 100S ribosomes. From the results, we propose that protein E is a "ribosome modulation factor" (RMF), which associates with 70S ribosomes and converts them to a dimeric form. A homology search of the partial amino acid sequence of RMF using the DNA sequence data bases revealed that the *rmf* gene, which encodes RMF, is located next to the *fabA* gene at 21.8 min on the *E. coli* chromosome.**

Two-dimensional gel electrophoresis is a powerful tool for identification of a large number of different proteins and has been applied to the resolution of ribosomal proteins (1, 2) and total bacterial proteins (3, 4). Under commonly used experimental conditions, however, proteins are more or less modified by free radicals and are oxidized during gel electrophoresis. To avoid such artifacts, we developed a radical-free and highly reducing (RFHR) method of two-dimensional polyacrylamide gel electrophoresis (5). Using this RFHR method, we have identified four additional proteins, proteins A, B, C, and D, in *Escherichia coli* ribosomes (5, 6).

Protein D was found in the 30S ribosomal subunits, while proteins A, B, and C were found in the 50S subunits. The gene coding for protein A maps at 37.6 min on the *E. coli* chromosome between *infC* and *rplT*, which encode translation initiation factor 3 and ribosomal protein L20, respectively (7). These three genes may form an operon together with two downstream genes, *pheS* and *pheT*, which code for subunits of phenylalanyl-tRNA synthetase. The gene for protein B is identical to gene X, previously identified as the last promoter-distal gene in the *spc* operon (8). It has been proposed that proteins A and B be designated L35 and L36, respectively, and the genes encoding proteins A and B be designated *rpmI* and *rpmJ*, respectively (7). Partial amino acid sequence data indicated that protein C is a precursor of L31 (A.W., unpublished observation). The gene for protein D was cloned by using a synthetic DNA probe prepared on the basis of the partial amino acid sequence; it mapped at 33 min (A.W. and Y. Komine, unpublished observation), but the physiological role of protein D is not known yet.

Using the RFHR method, we recalculated the copy number of each ribosomal protein, including these four additional proteins, and found that the copy numbers of 10 species of ribosomal proteins, including proteins B (L36), C (pre-L31), and D were less than 0.5, but protein A (L35) was classified into a group of unit copy number ribosomal proteins (A.W., unpublished observation). These results support the concept that ribosomes are heterogeneous as to their protein composition (for example, see ref. 9).

To elucidate the physiological significance of the structural heterogeneity of ribosomes in *E. coli*, we carried out a systematic analysis of the assembly state and protein composition of ribosomes prepared at different growth phases. As described in this report, cell lysates should be prepared without prior washing of the cells in order to preserve the native state of ribosomes under various growth conditions. Taking this precaution, we found that native 100S ribosomes (dimers of 70S ribosomes) exist in stationary-phase cells. Although 70S ribosomes undergo dimerization to 100S particles under high $Mg^{2+}$ concentrations (for a review, see ref. 10), the native 100S ribosomes stay in a dimer state at low $Mg^{2+}$ concentrations. The level of 100S ribosomes increases concomitantly with the transition from exponential growth to the stationary phase. A protein, designated protein E, was found to be exclusively associated with 100S ribosomes. Based on these observations, we propose that 100S ribosomes are a storage form of ribosomes and that protein E is a "ribosome modulation factor" (RMF), which associates with 70S ribosomes and then converts them into a dimeric form. A homology search of the partial amino acid sequence of RMF using the DNA sequence data bases indicated that the gene, *rmf*, encoding this protein is located next to the *fabA* gene at 21.8 min on the *E. coli* chromosome.

## MATERIALS AND METHODS

**Bacteria.** *E. coli* strain W3110 was grown in medium E (11) containing 2% peptone (Daigo, Tokyo) at 37°C with shaking at 100 cycles per min. At various times, cells were harvested by centrifugation at 4°C and were stored, without being washed, at −20°C until use.

**Ribosomes.** Frozen cells were ground with an approximately equal volume of quartz sand and then extracted with Noll's buffer I (10 mM Tris·HCl at pH 7.6, 10 mM magnesium acetate, 100 mM ammonium acetate, and 6 mM 2-mercaptoethanol) containing DNase I at 2 μg/ml (Sigma) (12). Crude and high salt-washed ribosomes were prepared from the cell extract essentially according to Noll *et al.* (12) with slight modifications, as described (13).

**Ribosomal Proteins.** Ribosomal proteins were prepared from total cell extracts and ribosomes by the acetic acid

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Abbreviations: RMF, ribosome modulation factor; RFHR, radical free and highly reducing.

‡To whom reprint requests should be addressed.

method (14) and, after dialysis three times against 2% acetic acid, were lyophilized and stored at −20°C until use.

**Two-Dimensional Gel Electrophoresis.** The RFHF method of two-dimensional gel electrophoresis (5) was modified as follows: (*i*) 2-mm gels were used instead of the 3-mm gels used for the original procedure, (*ii*) the volume of electrode buffers was reduced to two-thirds of the original volume, and (*iii*) the voltage during sample application and for electrophoresis in the first and second dimensions was increased to 100, 300, and 100 V, respectively.

**Determination of Cysteine Residues.** The number of cysteine residues was determined by the carboxymethylation method (6). In brief, ribosomal proteins were carboxymethylated with iodoacetic acid, and then the number of modified cysteine residues ($x$) was determined by measuring the change in mobility in the first-dimension migration and according to the following equation: $x = -q[(m'/m) - 1]$, where $q$ is the net charge per molecule of the test protein at pH 7.0 and $m$ and $m'$ are the migration distances of the unmodified and modified protein, respectively.

**Amino Acid Sequence.** Total ribosomal proteins were fractionated by two-dimensional gel electrophoresis and then blotted onto polyvinylidene difluoride membrane filters (Millipore) according to the method of Matsudaira (15). The stained protein spots were cut out and were directly subjected to amino acid sequence analysis with an Applied Biosystems sequencer model 470A.

## RESULTS AND DISCUSSION

**Formation of 100S Ribosomes.** Ribosomes from *E. coli* are interconvertible *in vitro* between 70S monomers and 50S and 30S subunits, depending on the concentrations of $Mg^{2+}$ and monovalent cations. It has therefore been difficult to identify the native forms of ribosomes in cells growing under various conditions. An experimental procedure was established to overcome this difficulty. Cells of an *E. coli* culture were harvested by centrifugation at 4°C and were stored, without being washed, at −20°C until use. Washing the cells with fresh buffer affected the state of ribosomes to various extents (data not shown). Cell lysates were immediately centrifuged on sucrose gradients. As shown in Fig. 1, the major species of ribosomes in exponentially growing cells was 70S monomers and, in addition, native 50S and 30S subunits were

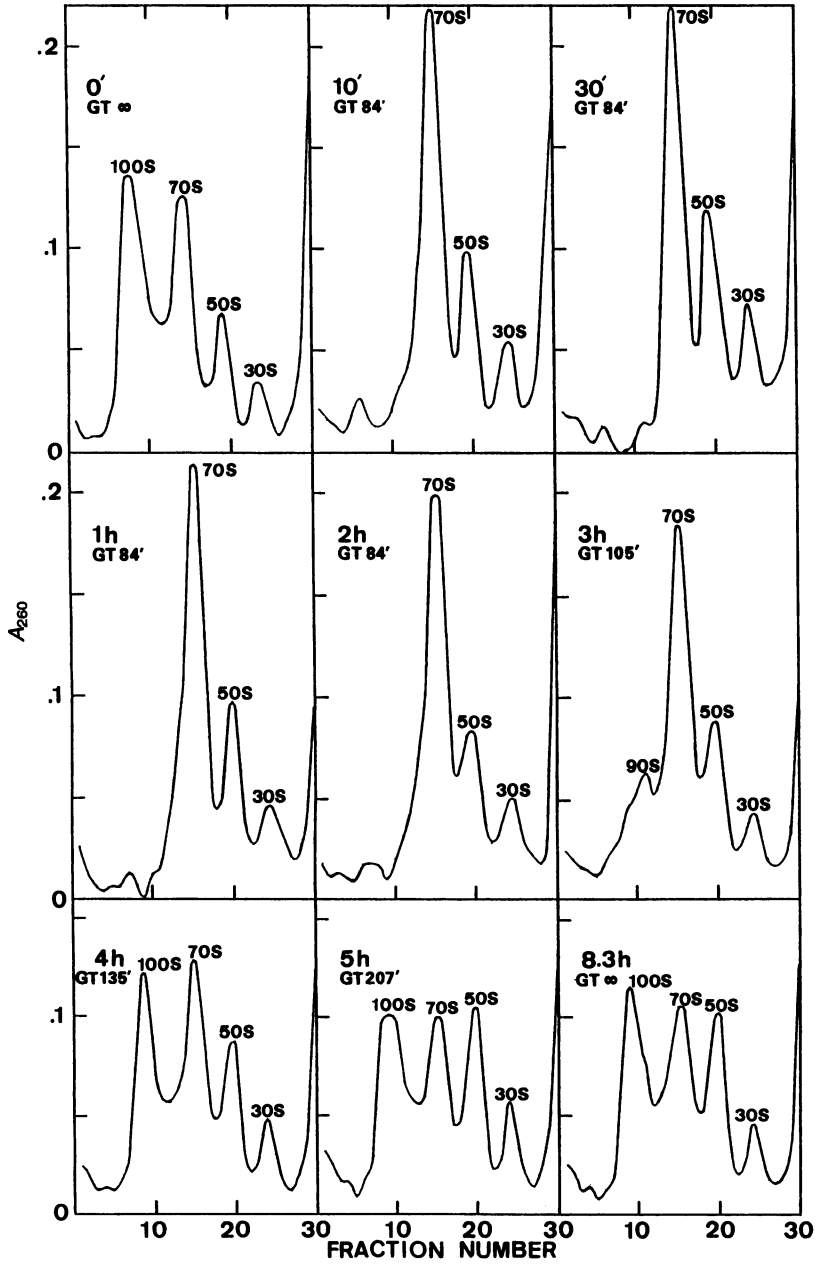


FIG. 1. Sucrose density gradient centrifugation of ribosomes. *E. coli* W3110 was cultured for 24 hr and then transferred into fresh medium. At the indicated times, cells were harvested and lysates were prepared. Samples were layered on 5-ml gradients of 5–20% (wt/vol) sucrose in ribosome buffer (20 mM Tris·HCl at pH 7.6, 15 mM magnesium acetate, and 100 mM ammonium acetate) and centrifuged in a Hitachi RPS50-2 rotor at 40,000 rpm for 80 min at 4°C. The sucrose gradients were fractionated into 34 or 35 tubes (fractions above no. 30 are not shown). GT, generation time estimated from the growth curve at the respective culture time. The generation time at the exponential growth phase was 84 min. The time 0 sample represents the overnight preculture used in this experiment, which was analyzed just before transfer to fresh medium.

detected at about half the level of 70S particles. Concomitant with the transition from the logarithmic phase to the stationary phase, 100S particles (dimers of 70S ribosomes) began to appear. The level of 100S particles reached ≈40% of the total ribosomes and thereafter stayed at a constant level (Fig. 2). Immediately after transfer of the stationary-phase culture to fresh medium, however, 100S particles were rapidly converted into 70S monomers (Fig. 1).

The decrease in the overall level of protein synthesis during the stationary phase was thus found to be accompanied by the conversion of 70S ribosomes into 100S particles. Accordingly, we propose that 100S particles are a storage form of ribosomes.

**Identification of Protein E.** To determine the structural difference between 100S and 70S ribosomes, we carried out two-dimensional gel electrophoresis of ribosomal proteins by using the RFHR method. Using this method, we have identified two additional ribosomal proteins, L35 (protein A) and L36 (protein B), a precursor of L31 (protein C), and an as yet uncharacterized protein, protein D (7). In addition, several marked changes were observed in the protein composition of ribosomes, which depended on the cell growth phase (details will be presented elsewhere). One significant change was the appearance of a new spot, designated protein E, for ribosomes from stationary-phase cells. Fig. 3 shows the gel patterns of total ribosomal proteins from exponentially growing (Fig. 3*a*) and stationary-phase (Fig. 3*b*) cells.

Protein E was only detected in the stationary-phase ribosomes (Fig. 3*b*). The migration position of protein E was, however, close to that of oxidized form of pre-L31 (protein C). For discrimination of the two components, we performed two-dimensional gel analysis under both reducing and oxidizing conditions, and the results are illustrated in Fig. 3*c*. The migration positions of pre-L31 and mature L31 differed markedly between the reducing and oxidizing conditions. Both L31 and pre-L31 migrated more slowly under the reducing conditions than under the oxidizing conditions in the second dimension. The oxidized forms of L31 and pre-L31 contain two pairs of disulfide bonds, while their reduced forms contain four SH residues (ref. 6; A.W., unpublished observation). Previously, these four spots were considered to represent conformers of L31, but our observations, taken together, indicated that they were reduced and oxidized forms of L31 and pre-L31, respectively. In contrast, the migration position of protein E was virtually the same under both the reducing and oxidizing conditions (Fig. 3*c*) and was close to that of the oxidized form of pre-L31. The migration of protein E remained unchanged even when the concentration of the reducing agent was increased 3-fold. This clearly indicates that protein E is not an oxidized form of pre-L31 but is a distinct species of ribosome-associated protein.

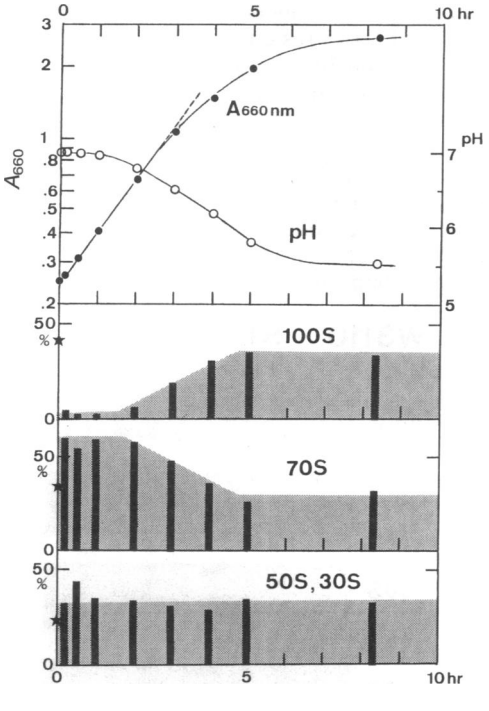


FIG. 2. Levels of 100S ribosomes at various growth phases. The growth curve was determined by measuring the turbidity. Closed circles indicate the turbidity, and open circles indicate the pH of the culture medium. Ribosomes were prepared and analyzed as described in Fig. 1. The levels of 100S and 70S ribosomes and of 50S and 30S ribosomal subunits were determined by measuring the peak area for each particle fractionated by sucrose gradient centrifugation. The stars shown at time 0 represent the preculture levels. The stippling indicates the level of each ribosomal particle.

To confirm this, the number of SH residues in protein E was determined by using the carboxymethylation method (6). Both logarithmic-phase and stationary-phase ribosomes were treated with iodoacetic acid, and the resulting carboxymethylated proteins were analyzed by the RFHR method of two-dimensional gel electrophoresis. The migration positions of both L31 and pre-L31 changed markedly after the modification, while the migration of protein E changed slightly to a position below L30 (Fig. 4). By examining the relative migration patterns of the untreated and carboxymethylated forms and using the equation $x = -q[(m'/m) - 1]$, where $m$ (migration distance in the first-dimension for untreated E), $m'$ (migration distance of carboxymethylated E), and $q$ (net charge per molecule of protein E; $M_r$ = 6000–6500) were estimated to be 27.4 mm, 23.7 mm, and 8.7–9.4, respectively, the number of cysteine residues ($x$) was calculated to be 1.2–1.3, indicating that protein E contains one cysteine residue.

In these experiments, ribosomes were prepared from cultures incubated for 24 hr after inoculation of an overnight preculture into fresh medium. When a stationary-phase culture was transferred to fresh medium, cell growth started immediately without a significant lag period, suggesting that ribosomes in the stationary-phase cells are stored intact and could be utilized immediately for the translation cycle. In agreement with this consideration, 100S ribosomes are converted into 70S ribosomes within 2 min following transfer to fresh medium (data not shown).

**Localization of Protein E.** Stationary-phase ribosomes were fractionated into 100S, 70S, 50S, and 30S particles by sucrose gradient centrifugation. The protein composition was determined for each particle by the RFHR method. As shown in Fig. 5, protein E was found to be associated with only 100S ribosomes and in an ≈1:1 molar ratio (i.e., 1 mol of protein E per 100S particle). We have, however, failed to detect protein E in stationary-phase 70S, 50S, and 30S ribosomes and logarithmic-phase ribosomes. When 100S ribosomes were dissociated into 50S and 30S subunits by decreasing the $Mg^{2+}$ concentration, protein E was recovered in the 50S subunits. It was, however, released from the ribosomes on exposure to 1 M ammonium acetate, supporting the hypothesis that protein E is not an intrinsic component of ribosomes but an accessory factor. Putting these results together, we propose that protein E is a ribosome modulation factor (RMF), which associates with 70S ribosomes and converts them to 100S particles, a storage form of ribosomes during the stationary phase of cell growth.

The present studies were carried out by using *E. coli* strain W3110, but essentially the same results were obtained with strain W3350. In contrast, protein E was not detected in strain Q13, which carries mutations in the *rna* and *pnp* genes, coding for ribonuclease I and polynucleotide phosphorylase, respectively. In agreement with our hypothesis that protein

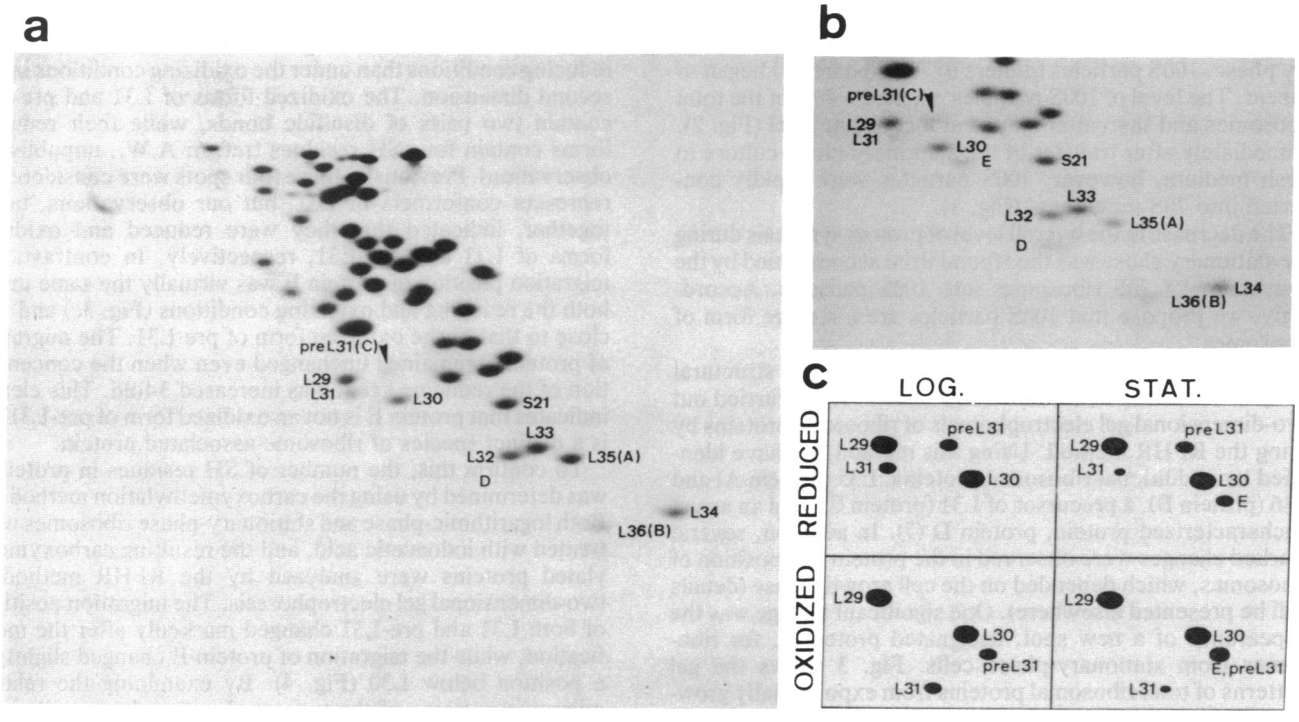


FIG. 3. Two-dimensional gel electrophoresis of ribosomal proteins. (*a*) *E. coli* W3110 cells were harvested during exponential growth (1.5 hr after preculture inoculation). (*b*) Cells were harvested during the stationary phase (24 hr after inoculation). One milligram each of total ribosomal proteins was analyzed by two-dimensional gel electrophoresis by the RFHR method. Gels were stained with 0.1% amido black 10B (Merck). For the stationary-phase ribosomes (*b*), only a part of the gel containing the additional ribosome-associated proteins is shown. (*c*) Total ribosomal proteins from either exponential-growth (Log) or stationary-phase (Stat) cells were subjected to two-dimensional gel electrophoresis under reducing or oxidizing conditions. Areas containing L31, pre-L31, and protein E are illustrated.

E is needed for 100S ribosome formation, a parallelism existed that 100S ribosomes were not found in this strain. The storage form of ribosomes might be resistant to degradation by increased levels of nucleases and/or proteases during the stationary growth phase.

In parallel with the modulation of ribosomes, we also found that RNA polymerase was modulated during the stationary phase of cell growth (M. Ozaki, N.F., and A.I., unpublished observations) and that the modified forms of RNA polymerase are resistant to degradation but the newly synthesized RNA polymerase subunits in the stationary-phase cells are rapidly degraded (reviewed in ref. 16).

**Primary Structure and Genetic Location of Protein E.** To further confirm that protein E is not a degradation product but a distinct ribosome-associated protein, we determined the amino acid sequence of the N-terminal region of protein E. About 1 mg of stationary-phase ribosomes was fractionated by the RFHR method of gel electrophoresis, and then the proteins were blotted onto a polyvinylidene difluoride membrane. The stained spot of protein E was cut out and directly subjected to amino acid sequence analysis with an automated gas-phase sequencer. The amino acid sequence was determined up to residue number 23 and, in addition, residues 25, 27, and 30 were also identified (Fig. 6).

When the partial amino acid sequence of protein E was compared with *E. coli* DNA sequences in DNA data bases [GenBank (release 61), European Molecular Biology Laboratory (release 21), and DNA Data Bank of Japan (release 5)], we found that similar sequences existed downstream of the *fabA* gene, which encodes β-hydroxydecanoylthioester dehydrase (Fig. 6). This putative protein E gene is, however, encoded by the opposite DNA strand from that encoding the

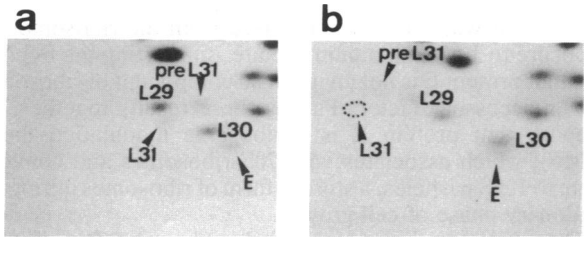


FIG. 4. Gel electrophoresis of carboxymethylated ribosomal proteins. Total ribosomal proteins were carboxymethylated and then subjected to two-dimensional gel electrophoresis by the RFHR method. (*a*) Untreated control ribosomal proteins. (*b*) Carboxymethylated ribosomal proteins.

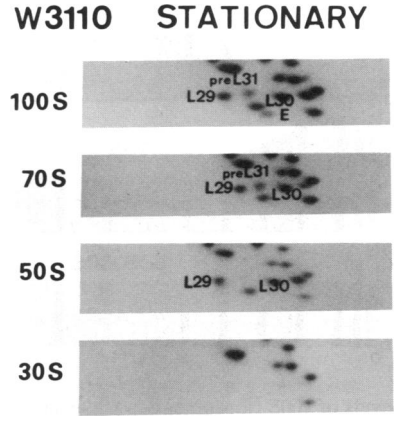


FIG. 5. Gel electrophoresis of ribosomal proteins. A stationary-phase cell extract prepared from an *E. coli* W3110 culture at 24 hr was centrifuged on a 60-ml 10–40% sucrose gradient in ribosome buffer at 40,000 rpm for 80 min in a Hitachi RP42 rotor. 100S (70S dimer) and 70S ribosomes as well as 50S and 30S ribosomal subunits were analyzed by two-dimensional gel electrophoresis by the RFHR method.

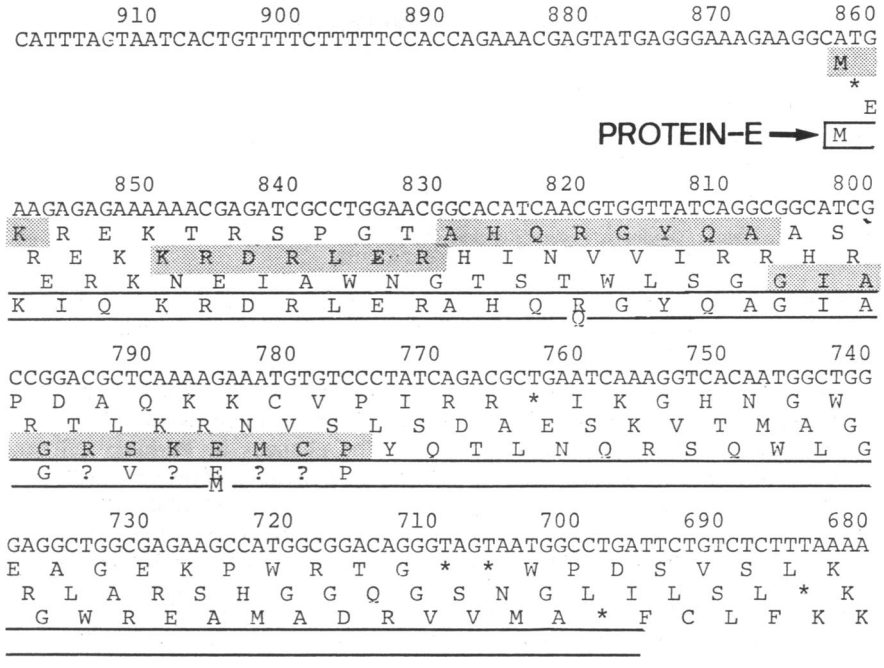


FIG. 6. Amino acid sequence of protein E. The amino acid sequence was determined for the N-terminal region of protein E and compared with *E. coli* DNA sequences in the DNA data bases. The bottom line represents the RMF sequence, whereas the top three lines represent the translation products of the three possible reading frames deduced from the ECFABAA sequence in the DNA data bases. The stippled amino acid residues indicate the sequence homologous to protein E. The stars denote translation termination codons.

*fabA* gene. Thus the *fabA* and *rmf* genes should be transcribed in a convergent direction. The amino acid sequence deduced from the DNA data bases agrees completely with that of protein E except for (*i*) a substitution of guanine for thymine at nucleotide 855, (*ii*) a substitution of guanine for cytosine at nucleotide 853, (*iii*) a deletion of one adenine within the hexaadenine nucleotide sequence between 847 and 852, and (*iv*) insertions of one guanine into two guanine dinucleotide sequences at positions 805–806 and 829–830. The *fabA* sequence was determined using *E. coli* strain CS520 (17), and thus the strain difference could account for the disagreement in the DNA sequence. If the published DNA sequence after amino acid residue 30 is correct, the open reading frame continues up to nucleotide 699, leading to the generation of a polypeptide of 55 amino acid residues with a $M_r$ of 6475, which agrees well with the size of protein E. In agreement with our experimental results, only one cysteine residue was found in this unidentified reading frame. This finding of the location of the *rmf* gene will allow us to study details of the structure and function of RMF and the regulation of the *rmf* gene during phase transitions of cell growth.

This work was supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

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