

## Conditionally lethal ribosomal protein mutants: Characterization of a locus required for modification of 50S subunit proteins

(ribosome structure/*Escherichia coli*/localized mutagenesis)

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**ABSTRACT** Mutagenized P1 bacteriophage were used to transduce a marker (*aroE*) adjacent to the cluster of ribosomal protein genes located at 72 min on the *Escherichia coli* chromosome. Linked temperature-sensitive transductants were isolated and characterized. A mutant unable to grow at 44° was found to be defective in protein synthesis both *in vivo* and *in vitro*. At the restrictive temperature mutant cells lost all polyribosomes. Analysis of the ribosomal proteins revealed alterations in at least four 50S subunit proteins. The mutation (called *rimE1*, ribosomal protein modification) mapped between *rpsE* and *aroE*. It is suggested that the *rimE* locus is the structural gene for an activity that modifies a selected number of ribosomal proteins.

Although recent studies *in vitro* have shown that the structural genes for at least 26 ribosomal proteins are clustered between the *trkA* and *trkB* loci on the *Escherichia coli* genetic map (1), inability to select directly for mutationally altered forms of most of the proteins has hampered the analysis of structurally defective ribosomes (2, 3). Antibiotic-resistant strains with altered ribosomal proteins have permitted the identification and mapping of only a limited class of proteins associated with each subunit. Alterations in the structural genes of other proteins may lead to lethality because of pleiotrophic effects on the structure or function of the ribosomes.

With the introduction of localized mutagenesis techniques such as described by Hong and Ames (4), a new approach to the isolation of conditional mutations in the structural genes for ribosomal proteins has become available (5, 6). Transductants of a genetic marker close to a chromosomal region of interest are isolated at a permissive temperature and then screened for linked temperature-sensitive mutations at a restrictive temperature.

We have isolated high-temperature (44°)-sensitive transductants in an *aroE*<sup>-</sup> strain of *E. coli* transduced to prototrophy at 30° by hydroxylamine-treated P1 bacteriophage. In this communication we describe the properties of one high-temperature-sensitive mutation that affects at least four different ribosomal proteins.

### MATERIALS AND METHODS

**Materials.** Reagents and isotopes were obtained from the following sources: radioactive isotopes, Schwarz/Mann; poly(uridylic acid), Miles Laboratory; lysozyme and pancreatic DNase I, Worthington Biochemical Corp.; ethyl methanesulfonate, Eastman Organic Chemicals; streptomycin sulfate and hydroxylamine, Sigma Chemical. Spectinomycin sulfate was the generous gift of The Upjohn Co.

**Bacterial Strains and Media.** The genotypes of the *E. coli* K-12 strains used in this work are listed in Table 1. Nomencla-

ture conforms to that of Demerec *et al.* (9), except that the minus sign is used to indicate a general mutant allele. The gene symbols are those given by Bachmann *et al.* (10) and Champney and Kushner (11). Thus, *strA* becomes *rpsL* and *spcA* becomes *rpsE*.

The complex medium used was Luria broth, which contained 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter, adjusted to pH 7. For solid medium, 2% agar was added. The minimal medium used for plates (M56/2) has been described by Willetts *et al.* (12). Plates used for selection of AroE<sup>+</sup> transductants were supplemented with 0.3% casamino acids that had been treated with Norit to remove aromatic amino acids. Minimal medium for liquid cultures was EC salts (13) supplemented with 0.5% glucose and 0.5% casein hydrolysate.

**P1 Mutagenesis and Transduction.** P1<sup>vir</sup> was prepared by the plate lysate procedure of Willetts *et al.* (12) and mutagenized by a modification of the method of Murgola and Yanofsky (14). P1<sup>vir</sup> (10<sup>11</sup> plaque-forming units/ml) grown on strain JC158 were diluted 1/10 in a solution of 0.45 M hydroxylamine, 2 mM EDTA, and 10 mM CaCl<sub>2</sub>, and incubated for 13 hr at 37°. After centrifugation at 48,000 × *g* for 1 hr, the phage pellet was resuspended in L broth containing 2 mM EDTA and 10 mM CaCl<sub>2</sub>. Debris was removed by centrifugation at 15,000 × *g* for 10 min.

The procedures for transductional crosses were those described by Willetts *et al.* (12). AroE<sup>+</sup> transductants of AB2834 were selected at 30°. When the transductants were just visible to the naked eye, they were replica plated to a casamino acid selective plate that had been warmed to 44°. After 24 hr of further incubation at the respective temperatures, the master and replica plates were compared.

**Cell Growth and Labeling.** Liquid cultures of SK901 or SK911 were grown in Luria broth at 30° in a shaking water bath and shifted at appropriate times to a second water bath maintained at 44°. Growth was followed turbidimetrically with a Klett-Summerson Colorimeter (no. 42 green filter). Samples were removed and spread on Luria agar plates at 30° to determine viable cells per ml. Protein and RNA were labeled by growing the cells in the presence of [<sup>14</sup>C]leucine or [<sup>14</sup>C]uracil.

**Cell Lysis and Gradient Centrifugation.** Cell lysates for sucrose gradient analysis were prepared by the method of Flessel *et al.* (15). Polyribosomes were analyzed by the sedimentation of cell lysates through 15–30% sucrose gradients in R buffer [10 mM Tris-HCl, pH 7.6, 10 mM Mg(OAc)<sub>2</sub>, 50 mM NH<sub>4</sub>Cl]. Centrifugation was performed in an SW 50.1 rotor at 49,000 rpm for 50 min. Ribosomal subunits were separated by centrifugation of cell lysates through 5–20% sucrose gradients in S buffer [R buffer containing only 0.5 mM Mg(OAc)<sub>2</sub>]. The

Table 1. Bacterial strains

Strain no.	Sex	<i>aroE</i>	<i>rpsL</i>	<i>rpsE</i>	<i>rimE</i>	Other	Source
AB2834	F <sup>-</sup>	353	+	+	+	Su <sup>+</sup>	B. Bachmann (7)
JC158	Hfr	+	+	+	+	<i>serA</i> <sup>-</sup>	A. J. Clark
JC5220	F <sup>-</sup>	+	31	+	+	<i>argE3</i> , <i>proA2</i> , <i>his-4</i> , <i>leu-6</i> , <i>thr-1</i>	A. J. Clark
2K405M	F <sup>-</sup>	+	+	+	+	<i>kdpABC5</i> , <i>trkD1</i> , <i>trkA133</i> , Su <sup>-</sup>	W. Epstein
SK577	F <sup>+</sup>	353	—	+	+	SKF2, F' <i>aroE</i> <sup>+</sup> <i>rimE</i> <sup>+</sup> , <i>argD37</i> , <i>proA</i> , <i>thy</i>	Seals <i>et al.</i> (8)
SK901	F <sup>-</sup>	+	+	+	+	Su <sup>+</sup>	AroE <sup>+</sup> transductant of AB2834
SK911	F <sup>-</sup>	+	+	+	1	Su <sup>+</sup>	AroE <sup>+</sup> transductant of AB2834
SK918	F <sup>-</sup>	353	+	—	+	Su <sup>+</sup>	Spc <sup>R</sup> mutant of AB2834
SK922	F <sup>-</sup>	+	+	+	1	Su <sup>+</sup>	AroE <sup>+</sup> transductant of AB2834 with SK911 donor
SK924	F <sup>-</sup>	+	+	+	+	Su <sup>+</sup>	Temperature-resistant revertant of SK922
SK932	F <sup>-</sup>	+	+	—	1	Su <sup>+</sup>	AroE <sup>+</sup> transductant of SK918 with SK911 donor
SK939	F <sup>-</sup>	+	31	—	1	<i>argE3</i> , <i>proA2</i> , <i>his-4</i> , <i>leu-6</i> , <i>thr-1</i>	Spc <sup>R</sup> transductant of JC5220 with SK932 donor
SK949	F <sup>-</sup>	353	+	—	+	<i>trkA133</i> , <i>trkD1</i> , <i>kdpABC5</i>	Seals <i>et al.</i> (8)
SK952	F <sup>-</sup>	+	+	—	1	<i>trkD1</i> , <i>kdpABC5</i> , Su <sup>-</sup>	Spc <sup>R</sup> transductant of 2K405M with SK911 donor
SK1346	F <sup>-</sup>	353	+	+	1	<i>recA56</i> , <i>trkD1</i> , <i>kdpABC5</i>	Derivative of SK949
SK1356	F <sup>+</sup>	353	+	+	1	SKF2, F' <i>aroE</i> <sup>+</sup> , <i>rimE</i> <sup>+</sup>	AroE <sup>+</sup> exconjugant of SK1346 × SK577

gradients were centrifuged for 90 min at 45,000 rpm in an SW 50.1 rotor. Absorbance at 254 nm of gradient fractions was determined.

**Ribosome Isolation and Two-Dimensional Polyacrylamide Gel Electrophoresis.** Cells were grown at 30° in 1 liter of tryptone broth, and half of the culture was shifted to 44° at an A<sub>600</sub> of 0.15. Cells at an A<sub>600</sub> of 0.5–0.9 were collected by centrifugation, resuspended in R buffer, and lysed by passage through a French pressure cell. Ribosomes were collected from the cell lysates by centrifugation in a Ti50 rotor at 45,000 rpm for 4 hr. The upper half of the supernatant was dialyzed against R buffer and retained at -70° as an S100 fraction for protein synthesis *in vitro*. The ribosome pellet was resuspended in R buffer. Part was retained as low-salt-washed 70S ribosomes for protein synthesis assays, while the remainder of the ribosomes were resedimented through R buffer containing 1 M NH<sub>4</sub>Cl and 5% sucrose. The high-salt-washed ribosomes were kept for gel analysis of total 70S proteins or were separated into subunits by 15 hr of centrifugation through S buffer gradients at 20,000 rpm in an SW 27 rotor. Proteins were extracted from the ribosomes and subunits by the acetic acid method of Hardy *et al.* (16); 1 M LiCl core particles were prepared by the method of Homann and Nierhaus (17). Two-dimensional polyacrylamide gel electrophoresis of the proteins was conducted by the method of Howard and Traut (18).

**Protein Synthesis *In Vitro*.** [<sup>14</sup>C]Polyphenylalanine synthesis with mutant and wild-type ribosomes and S100 proteins was conducted as described by Traub *et al.* (19). Reaction mixtures (150 μl) contained 100 μg of 70S ribosomes and 150 μg of S100 protein. Kinetic experiments were performed by removing 25-μl samples from the incubation mixture at various times.

## RESULTS

**Mutant Isolation.** Since the *aroE* locus is closely linked to the ribosomal protein cluster at 72 min (55–75% cotransduction

frequency), conditional mutants linked to *aroE* might lie in ribosomal protein genes. Accordingly, a P1vir phage lysate of JC158, treated with hydroxylamine as described in *Materials and Methods*, was used to obtain AroE<sup>+</sup> transductants of AB2834. Those transductants obtained at 30° that were unable to grow after replica plating at 44° (high-temperature-sensitive) were picked for further analysis. Five temperature-sensitive mutants were isolated from an initial screening of about 16,000 transductants.

**Characterization of Strain SK911.** One high-temperature-sensitive mutant (SK911) showed no growth on either minimal agar or Luria agar replica plates incubated at 44°. In liquid cultures, growth progressively declined after about one generation at the restrictive temperature, while cell viability was irreversibly lost after 90 min (Fig. 1B). Growth of a temperature-resistant AroE<sup>+</sup> transductant (SK901) was unaffected by the shift to 44° (Fig. 1A). When SK911, grown at 30°, was spread on Luria plates and incubated at 44°, less than 1 in 10<sup>6</sup> survivors were obtained.

**Protein and RNA Synthesis.** The synthesis of protein and RNA *in vitro* in the mutant at permissive and restrictive temperatures was examined. An exponential incorporation of either uracil or leucine was observed at 30°. Upon shifting to 44°, precursor incorporation into protein and RNA progressively declined, in parallel with the cessation of growth as measured by culture turbidity. A complete inhibition of both protein and RNA synthesis was observed after approximately one generation time at 44°. A continuous incorporation of both precursors into protein and RNA was observed in a control strain (SK901) at 44°.

**Polyribosome Formation.** Both the control (SK901) and mutant (SK911) strains showed normal polysome patterns when the cells were cultured at 30° (Fig. 2A and B). However, no mono- or polyribosomes were detected in SK911 90 min after a temperature shift to 42°, whereas the control exhibited a profile identical to that observed at 30° (Fig. 2C and D). Ex-

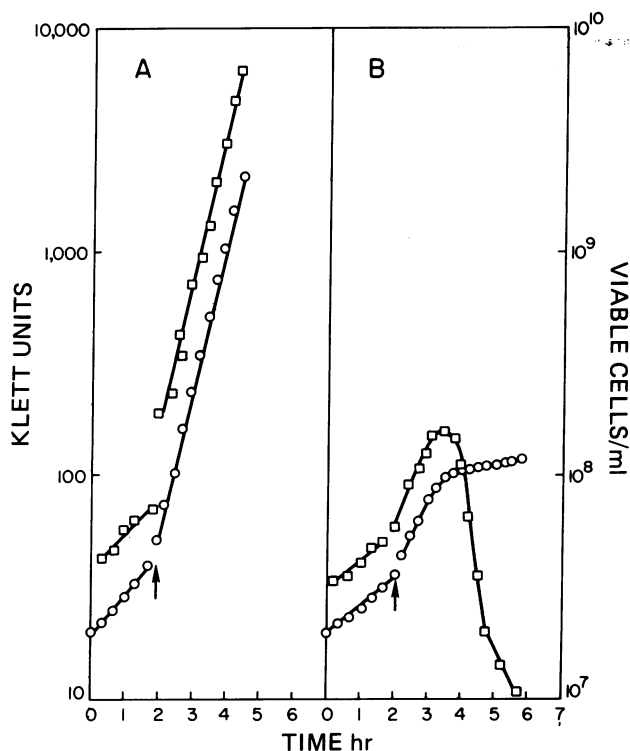


FIG. 1. Growth and viability of SK911 (*rimE1*) and SK901 (*rimE+*). Both strains were grown in Luria broth. Turbidity was determined in a Klett-Summerson Colorimeter with a no. 42 green filter. The arrow indicates the time of the temperature shift. Growing cultures were diluted appropriately with warmed Luria broth to maintain Klett readings below 80. Viable cells were determined on Luria plates at 30°. (A) SK901; (B) SK911. (O) Klett units; (□) viable cells per ml.

amination of ribosomal subunits by sucrose gradient centrifugation of cell extracts prepared from both low- and high-temperature mutant cultures revealed no evidence for accumulation of ribosomal precursor particles. However, increased amounts of low-molecular-weight RNA and protein were clearly evident in gradients, when lysates from the mutant were examined after a shift to 44°.

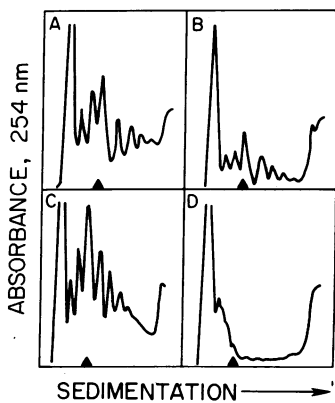


FIG. 2. Polyribosome profiles. Cell lysates from SK901 and SK911 grown continuously at 30° or shifted to 42° were prepared and sedimented through sucrose gradients as described in *Materials and Methods*. The gradients were displaced through an ISCO UV Absorbance Monitor and a continuous recording was made. (A) SK901, 30° polyribosomes; (B) SK911, 30° polyribosomes; (C) SK901, 42° polyribosomes; (D) SK911, 42° polyribosomes. Arrows indicate the 70S monosome peak.

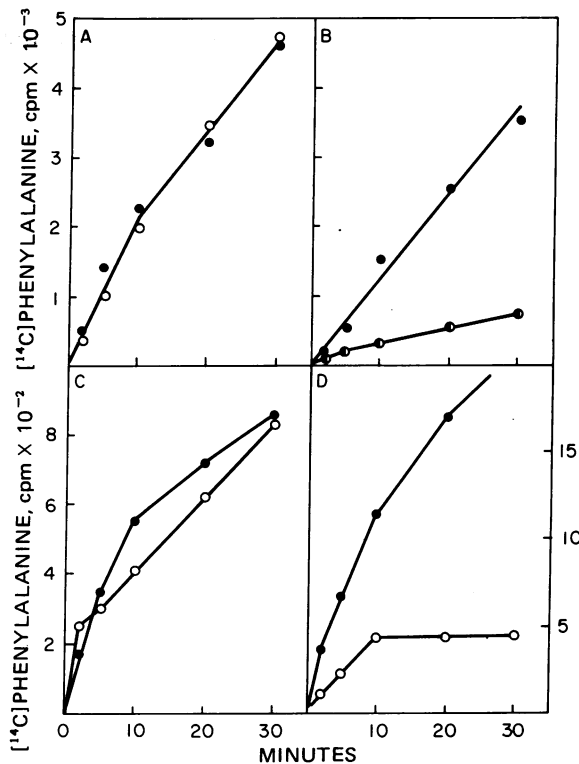


FIG. 3. Kinetics of polyphenylalanine synthesis. Combinations of ribosomes and S100 were incubated at the indicated temperatures and samples removed at various times for measurement of trichloroacetic acid-precipitable [<sup>14</sup>C]phenylalanine. (●) SK901 ribosomes with either SK901 or SK911 S100 proteins; (○) SK911 ribosomes with either SK901 or SK911 S100 proteins; (◐) SK911 ribosomes with SK911 S100 proteins. (A) Ribosomes and S100 proteins isolated and assayed at 30°; (B) ribosomes and S100 proteins isolated at 44°, assayed at 30°; (C) ribosomes and S100 proteins isolated at 30°, assayed at 44°; (D) ribosomes and S100 proteins isolated and assayed at 44°.

**Polyphenylalanine Synthesis.** The ability of ribosomes isolated from SK911 to promote the poly(uridylic acid)-directed synthesis of polyphenylalanine was examined. Ribosomes and S100 proteins from SK911 and SK901 were isolated from cells grown at 30° and from cells that had been shifted to 44°. Ribosomes isolated from either strain grown at 30° showed identical rates of protein synthesis at 30° or 44° using either source of S100 (Fig. 3A and C), although the extent of incorporation was reduced for both systems at 44°.

Ribosomes isolated from cells shifted to 44° showed significant differences in polyphenylalanine synthesis. When assayed at 30°, both mutant and control ribosomes appeared equivalent, if the wild type (SK901), high-temperature S100 was used (Fig. 3B). The S100 fraction from SK911 supported protein synthesis with control ribosomes at 30° (data not shown), but when mutant ribosomes were used, the rate of incorporation was considerably reduced (Fig. 3B). If the same assays were performed at 44°, either S100 supported good protein synthesis with the control ribosomes, but the mutant ribosomes had less than 40% of the control activity with either S100, and were totally inactive after 10 min at 44° (Fig. 3D).

**Analysis of Ribosomal Proteins.** Ribosomal proteins extracted from total 70S ribosomes and from isolated subunits were separated by two-dimensional polyacrylamide gel electrophoresis. Fig. 4A is a photograph of a stained gel of the 70S proteins from SK911 grown at 30°. Although no ribosomal proteins were found to be missing in the mutant, five additional

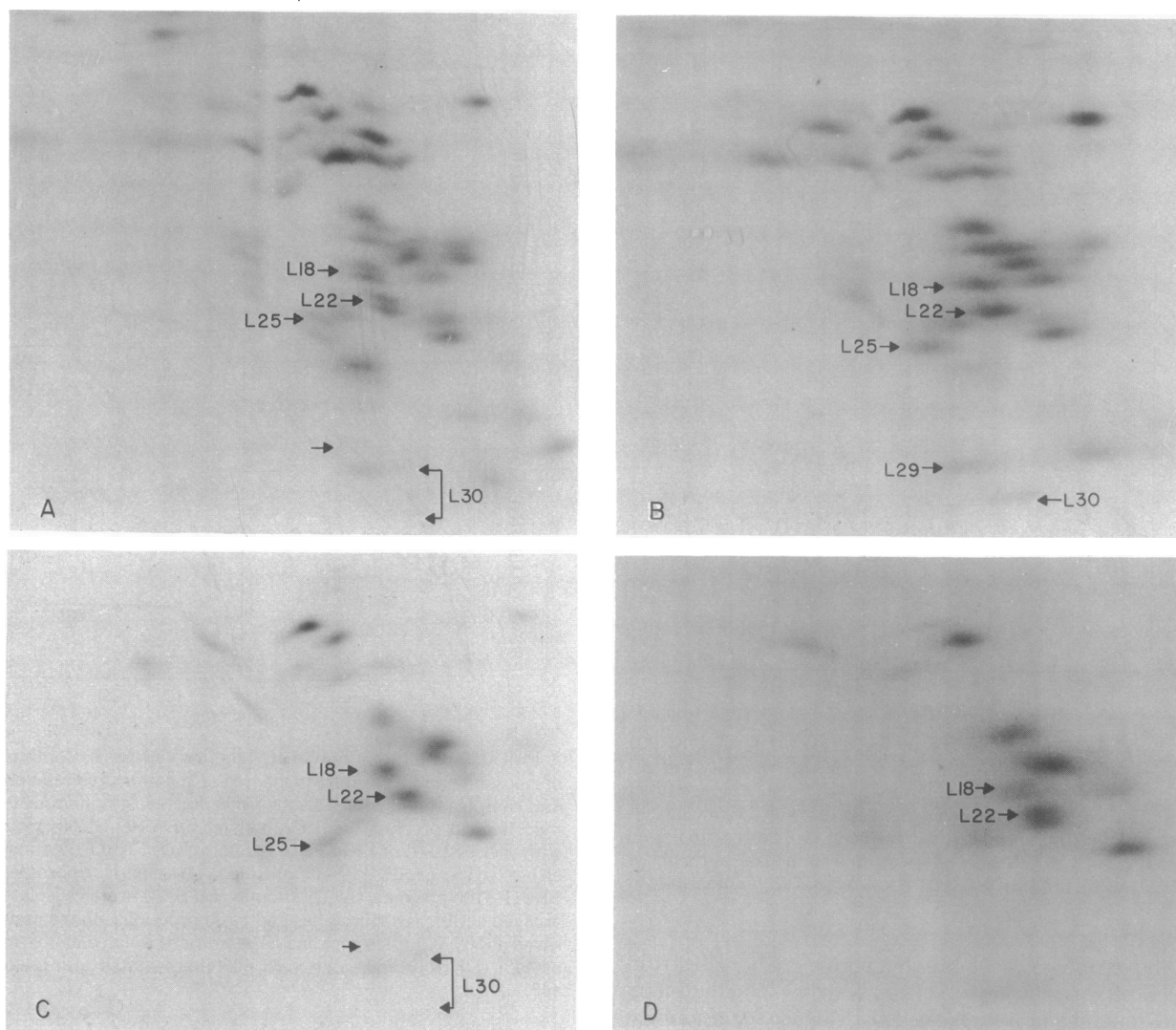


FIG. 4. Two-dimensional polyacrylamide gels of ribosomal proteins from SK911 (*rimE1*) and SK901. (A) 70S ribosomal proteins extracted from SK911 30° ribosomes; (B) 70S ribosomal proteins extracted from SK901 30° ribosomes; (C) 50S ribosomal proteins extracted from SK911 44° 50S subunits; (D) ribosomal proteins remaining after 1 M LiCl extraction of SK911 30° 50S subunits.

proteins were present in the gel (indicated by the arrows in Fig. 4A). These were not present in the parental strain AB2834, in a control strain able to grow at 44° (SK901, Fig. 4B), or in a spontaneously occurring temperature-resistant revertant of SK922 (SK924). Four of the extra proteins may be doublets of the normal ribosomal proteins L18, L22, L25, and L30. An additional protein spot located directly above protein L29 was also present. Without the additional proteins, the 70S gel pattern is typical of *E. coli* K12 strains (20). The four new proteins were also found in the gel of 70S proteins isolated from a strain (SK939) carrying the temperature-sensitive mutation in a different genetic background (Table 1). None of the extra spots was present in a gel of 70S proteins isolated from a partial diploid strain (SK1356, Table 1).

Fig. 4C presents the gel pattern of the 50S ribosomal proteins isolated from SK911 after shift-up to 44°. For the L18, L22, L25, and L30 doublets, the upper spots all increased markedly in stain intensity, compared to the same bands in a gel of 30° proteins (Fig. 4A). In addition, no radioactivity was detected in any of the extra protein bands when <sup>35</sup>S-labeled wild-type proteins were coelectrophoresed in a gel containing SK911 70S proteins. Homann and Nierhaus (17) have shown that treatment of 50S subunits with 1 M LiCl selectively removes certain ri-

bosomal proteins. After this treatment the L18 and L22 doublets remained in the core particles (Fig. 4D), while the L25 and L30 doublets appeared in the split protein fraction (data not shown). No alterations of 30S subunits have been observed.

On the basis of observed ribosomal protein gel patterns, we propose to call this mutation *rimE* (ribosomal protein modification) to indicate the first in perhaps a series of genes that controls alteration or modification of ribosomal proteins necessary for subunit structure. This designation is in accordance with the nomenclature suggested by Bachmann *et al.* (10) and Champney and Kushner (11).

**Genetic Analysis of the *rimE1* Mutation.** The temperature-sensitive phenotype of SK911 was retained by AroE<sup>+</sup> transductants constructed in several different genetic backgrounds [SK922, Su<sup>+</sup>; SK939, multiple auxotrophic; and SK952, Su<sup>-</sup> (Table 1)]. In a partial diploid strain (SK1356), *rimE1* was found to be recessive to *rimE*<sup>+</sup>. Spontaneously occurring temperature-resistant revertants of *rimE1* were isolated at frequencies (10<sup>-7</sup>) suggestive of a point mutation.

In order to localize the *rimE* locus, three factor transductional crosses were carried out with *aroE* and *rpsE* as selected markers and *rimE1* as an unselected marker. The *rimE1* marker was found to be 90% linked to *aroE* and 70% linked to *rpsE* (a more

detailed description of the fine structure mapping will be published elsewhere: V. F. Maples, W. S. Champney, and S. R. Kushner, unpublished data).

### DISCUSSION

Our working hypothesis is that the *rimE1* mutation is an alteration in an enzyme responsible for the modification of at least four 50S ribosomal proteins. Sufficient although incomplete modification of these ribosomal proteins occurs at 30°, allowing for cell viability at this temperature. Presumably a heterogeneous population of ribosomes is formed at the permissive temperature. At 44° the modifying activity is completely inactive, and the cells grow only to the extent permitted by the amount of ribosomes formed at the permissive temperature. The characteristics of the *rimE* mutant described in this paper suggest that ribosome maturation requires some type of processing of the 50S subunit after it has been assembled.

The significant differences in the relative amounts of the doublet pairs suggests that at least in four cases the additional proteins may represent precursors of L18, L22, L25, and L30. Methylation (21), acetylation (22), or proteolytic cleavage of a precursor ribosomal protein are possible candidates for the nature of the *rimE* activity. It is of interest that all four proteins are present in the p<sub>1</sub>50S precursor particle described by Nierhaus *et al.* (23).

Modification of the proteins is presumably critical for the function of the 50S subunit in protein synthesis. Modification may take place after the subunit has been assembled, since no precursor particles have been observed and the additional proteins are isolated from intact 50S and 70S ribosomes using cells grown at either 30° or 44°. The loss of polyribosomes at the high temperature and the accumulation of RNA and protein suggest a degradation of unmodified and nonfunctional ribosomes in the mutant.

The results from the assays of the protein synthesis *in vitro* suggest that ribosomes isolated from cells cultured at 30° have been normally modified to some extent *in vivo* and are capable of active protein synthesis *in vitro* at any assay temperature. (This is consistent with our finding of both forms of the proteins in gels of low temperature ribosomes.) Ribosome modification does not occur *in vivo* at 44°, and ribosomes isolated from these cells can function *in vitro* only to the extent permitted by the amount of low temperature (modified) particles present.

The *rimE1* mutation may be similar to the *sad* (self-assembly deficient) mutants of Guthrie *et al.* (24) and the *rim* mutants of Bryant and Sypherd (25). In these cold-sensitive ribosome assembly mutants, however, an assembly factor or activity is affected, since direct alterations of any of the structural proteins of the particle have not generally been observed. The pleiotropic effects of the *rimE1* mutation on the ribosomal proteins and its location adjacent to the ribosomal protein cluster at 72 min makes this mutation of special interest in studying ribosome function.

In addition, the application of localized mutagenesis should permit a direct genetic analysis of the ribosomal protein gene

cluster through isolation of conditional mutants in many of the structural genes for the ribosomal proteins.

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