# Ribosomal Proteins and Ribonucleic Acids of Ribosome Maturation Mutants of *Escherichia coli*†

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ABSTRACT: The rRNA of four ribosomal maturation (*rim*) mutants of *Escherichia coli* and the parental strain were analyzed by polyacrylamide gel electrophoresis. The 43S and 32S particles accumulated by the *rim* mutants at 20° were shown to contain 23S RNA, which indicates that the particles are related to precursors of the 50S subunit. The 43S particles also contained 5S RNA. Precursor 16S RNA could be isolated from 30S subunits of both *rim* mutants and the parental strain when these strains were grown at 20°. Precursor 23S RNA could be isolated from the 50S subunit of the parental strain at 20°. The conversion of precursor RNA to mature RNA is therefore a very late step in ribosomal subunit maturation. Analysis of 50S and 30S ribosomal proteins of three *rim* mutants and the parental strain was performed by two-dimensional polyacrylamide gel electrophoresis. No difference in electrophoretic mobility of any ribosomal protein was detected, which provides evidence that the *rim* mutations do not involve alterations in ribosomal proteins.

Mutants which have blocks in the assembly of ribosomes provide a means of studying the mechanism of ribosome assembly *in vivo*. Guthrie *et al.* (1969a) and Tai *et al.* (1969) have shown that cold-sensitive mutants of *Escherichia coli* or *Salmonella typhimurium* are a rich source of ribosome assembly mutants. Many of the cold-sensitive, ribosome assembly defective mutants reported thus far map in the *str A* region and presumably involve ribosomal proteins (Guthrie *et al.*, 1969b; Nashimoto *et al.*, 1971; Tyler and Ingraham, 1973). The block in ribosome assembly imposed by certain cold-sensitive mutations in the protein S-5 has been well characterized (Nashimoto *et al.*, 1971).

If there are factors which play a role in ribosome maturation *in vivo*, other than ribosomal proteins and RNA, such factors should be revealed by the analysis of ribosome assembly defective mutants. In a previous publication (Bryant and Sypherd, 1974), we presented genetic evidence for the existence of genes which might control assembly factors or enzymes for the 50S subunit in *E. coli*. That evidence consisted of the observation that four ribosome maturation (*rim*) mutants mapped away from *str A* and were recessive to the wild-type allele. In this paper we present a characterization of the ribosomal proteins and RNA of *rim* mutants.

## Materials and Methods

*Strains.* The isolation of cold-sensitive (cld-S) mutants and screening of mutants for defective ribosome assembly by zone sedimentation have been described (Bryant and Sypherd, 1974). The parental strain was AB 1472. Most of the ribosomal protein and RNA analyses were performed on *rim* strains which were ribonuclease I deficient, constructed as described (Bryant and Sypherd, 1974). *Rim* mutants are phenotypically cold sensitive, *i.e.*, have growth rates slower than that of the parental strain at 20°. The doubling time of each organism, in MOPS medium, was: parental strain, AB 1472, 40 min at 37°, 155 min at 20°, *rim A*, 55 min at 37°, 350 min at 20°; *rim B*, 45 min at 37°, 170 min at 20°; *rim C*, 75 min at 37°, 410 min at 20°; and *rim D*, 70 min at 37°, 360 min at 20°. As previously reported (Bryant and Sypherd, 1974), mutants bearing mutations in *rim A*, *rim B*, or *rim D* accumulate 43S particles. Stains bearing mutations in the *rim C* locus accumulate 32S particles. Figure 1 shows the genetic map location of the *rim* mutants studied here. Cells were grown for experimental purposed in MOPS medium, consisting of morpholinopropanesulfonic acid, 20 g/l.; KCl, 2 g/l.; $NH_4Cl$, 2 g/l.; $Na_2SO_4$, 0.05 g/l.; $Na_2HPO_4$, 0.04 g/l.; $MgCl_2$, 0.5 g/l.; glucose, 5 g/l.

*RNA Analysis.* The procedure for isolating ribosomal subunits or particles accumulated at 20° (cld-S particles) by *rim* mutants was based on the screening procedure previously described (Bryant and Sypherd, 1974). Cells were differentially labeled with [5-$^3$H]uracil (Schwarz/Mann) for 1 generation at 37° and $H_3{}^{32}PO_4$ (International Chemical and Nuclear Corp.) for 0.3–0.5 generation at 20°. Approximately 20 $\mu$Ci/ml of $H_3{}^{32}PO_4$ and 50 $\mu$Ci/ml of [5-$^3$H]uracil (18 Ci/mmol) were used for 10-ml cultures. A 25-$\mu$l sample was removed from each sucrose gradient fraction for $Cl_3CCOOH$ precipitation. The remaining 0.45-ml fractions were then pooled for the regions of 50S, 43S, and 30S or 30S–32S subunits. (Ethylenedinitrilo)-tetracetic acid, disodium salt (EDTA) at pH 7.6, was added to make a final concentration of 1 mM. Sodium dodecyl sulfate was then added to 1% (w/v) and the preparation was shaken with a Vortex mixture for 5–10 sec. Sodium dodecyl sulfate is effective in dissociating proteins from rRNA (Gilbert, 1963; Girard *et al.*, 1964).

The sodium dodecyl sulfate extracts were subjected to polyacrylamide gel electrophoresis (Bishop *et al.*, 1967). From 0.1 to 1.0 absorbance unit at 260 nm of RNA extract (0.1–0.5 ml) was applied to each gel. The 30S subunit pools were analyzed with 2.8% acrylamide, 0.14% *N,N'*-methylenebisacrylamide ("bis" acrylamide). Composite gels consisting of 3.2% acrylamide and 0.16% "bis" acrylamide in an upper section and 10% acrylamide and 0.5% "bis" acrylamide in a lower section were used for analysis of 50S and 43S pools. Acryl-

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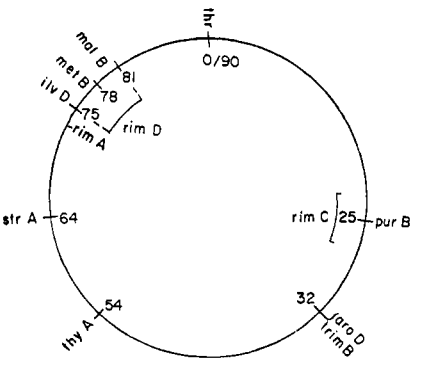


FIGURE 1: Representation of the genetic map of *E. coli*, showing the location of loci involved in the ribosome maturation defect. The genes referred to as *rim* were mapped by conjugation and transduction (Bryant and Sypherd, 1974).

amide and "bis" acrylamide were purchased from Eastman Kodak. Acrylamide was recrystallized from chloroform before use. Gel diameters were 0.9 cm. The 2.8% gels were 10 cm long. The composite gels were 5–6 cm long for the upper section and 10 cm long for the lower section. Gels were run at 10 mA/gel at constant current. Gels were frozen and then sliced into 1-mm fractions with the Mickle Laboratory gel slicer (Gomshall, Surrey, England). The slices were dried on small squares of filter paper and assayed for radioactivity by liquid scintillation spectroscopy. Channel settings were made so that less than 1% crossover of $^{32}P$ into the $^{3}H$ channel was observed. Tritium-labeled marker RNA was run in some gels. Marker RNA was extracted with sodium dodecyl sulfate–EDTA from 50S subunits or from soluble RNA (sRNA) isolated from the parental strain of the *rim* mutants, grown at 37°.

*Ribosomal Protein Analysis.* To provide ribosomal subunits for protein extraction, cultures were grown to late-log phase in 15-l. carboys with aeration at 37°. The medium used was 1% (w/v) tryptone and 0.2% (w/v) yeast extract. Cells were harvested and then broken in the French press at 8000 psi in 10 mM tris(hydroxymethyl)aminomethane–10 mM $MgCl_2$ (pH 7.6 at 20°). Ribosomes were prepared by a modification of the ammonium sulfate precipitation method of Kurland (1966). Separation of subunits was accomplished by sedimentation through a hyberbolic sucrose gradient using the Beckman Ti-15 rotor, as described by Eikenberry *et al.* (1970). Ribosomal proteins were extracted with 2-chloroethanol according to the procedure of Fogel and Sypherd (1968). Proteins were subjected to two-dimensional polyacrylamide gel electrophoresis as described by Kaltschmidt and Wittmann (1970a,b), except that urea was omitted from electrode buffer and a smaller electrophoresis apparatus was used. The pH for the first dimension was 8.6 and for the second dimension, 4.6.

## Results

The *rim* mutants we have studied fall into four genetic loci (Figure 1). The mutants designated *rim A*, *rim B*, and *rim D* accumulate 43S particles when shifted from 37 to 20°, while mutant *rim C* accumulates 32S particles (Figure 2). There are no clearly definable precursors for 30S ribosomes in these mutants, although small amounts of lightly sedimenting material are occasionally seen. The rather broad heterogeneous peak of material around 30S in the *rim C* mutant (Figure 2B) may in fact contain precursors to 30S ribosomes. The parental strain, AB 1472, makes normal 30S and 50S particles at both temperatures (Figure 2C).

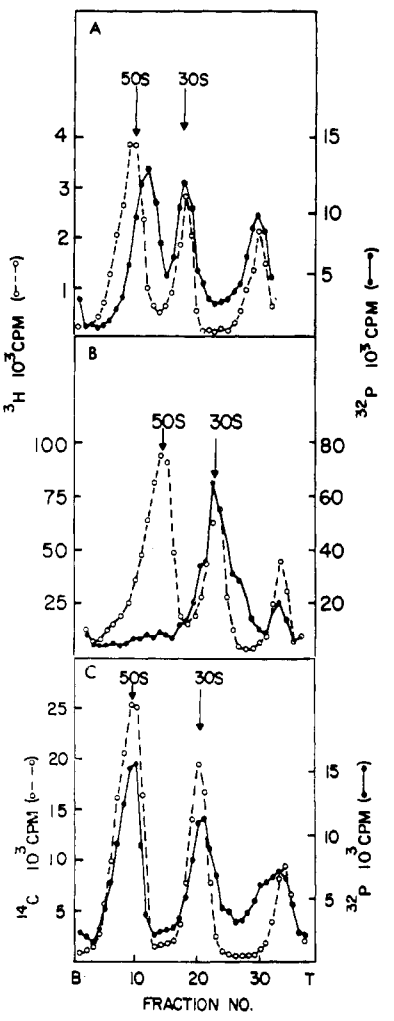


FIGURE 2: Sucrose gradient analysis of extracts from the two types of *rim* mutants. (A) The accumulation at 20° of 43S particles in *rim A* (●—●), which is also characteristic of *rim B* and *rim D*. (B) The accumulation at 20° of 32S particles of *rim C*. The formation of normal 30S and 50S particles at 37° is shown by the incorporation of [$^{3}$H]uracil (O- - - O). (C) The formation of normal 30S and 50S particles at 37 and 20° by the parental strain, AB 1472.

*rRNA Analysis.* The rRNA of the *rim* mutants and the parental strain was analyzed by polyacrylamide gel electrophoresis. The purpose of this analysis was to identify the species of rRNA contained in a cld-S particle. The identity of that RNA would indicate whether the cld-S particle was related to the 50S or to the 30S subunit. Figure 3 shows polyacrylamide gels of RNA extracted from AB 1472, the parental strain. The composite 3.2%/10% gel (Figure 3a) shows 23S and 5S RNA extracted from the 50S subunit. The $^{3}H$ label serves as marker 23S RNA. The $^{32}P$-labeled 23S RNA is in two peaks. One peak is coincident with $^{3}H$-23S RNA. The other peak has a slower electrophoretic mobility. These fast and slow peaks are designated mature (m23S) and precursor (p23S) RNA by analogy to the two types of RNA observed by kinetic analysis (Adesnik and Levinthal, 1969; Hecht and Woese, 1968) and by chemical analysis of pulse-labeled, chloramphenicol-treated and cold-sensitive, ribosome assembly defective cells (Brownlee and Cartwright, 1971; Hayes *et al.*, 1971; Lowry and Dahlberg, 1971; Sogin *et al.*, 1971). These studies and those of M. Sogin (Ph.D. Thesis, University of Illinois, 1972) have demonstrated that molecules of rRNA which migrate more slowly in acrylamide gels—p16 and p23—are larger than the mature species. In these cases

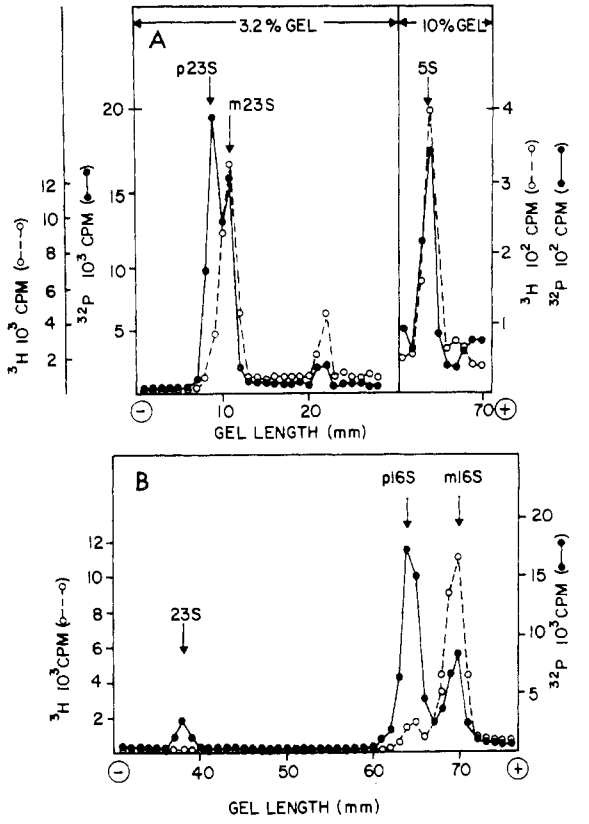


FIGURE 3: Polyacrylamide gel patterns of RNA extracted from ribosomes of AB 1472, parental strain of the *rim* mutants. (A) Composite 3.2%/10% acrylamide gel of RNA extracted from the 50S subunit. (B) 2.8% acrylamide gel of RNA extracted from the 30S subunit.

the processing of the precursor molecules apparently involves the removal of oligonucleotides from the 3′- and 5′-ends of p16 RNA and at least the 5′-end of p23 RNA (M. Sogin, Ph.D. Thesis, University of Illinois, 1972). Regardless of the precise nature of the chemical or physical changes, the available evidence shows that the slower migrating species of rRNA behave kinetically as precursors to the faster forms (Adesnik and Levinthal, 1969; Pace *et al.*, 1970; Hecht and Woese, 1968). Figure 3B shows that p16S RNA, isolated from the 30S subunit, is also synthesized at 20° in the parental strain. The precursor form of 16S RNA was found in all fractions across the 30S peak, suggesting that it is incorporated into a particle very nearly mature. However, it is possible that the p16 RNA resided in a slightly smaller particle that was not well separated from the 30S peak. The accumulation of precursor RNA at 20 probably results from the abruptly slower growth rate imposed by the lower temperature. A second *E. coli* K-12 strain, AB 1360, was also found to accumulate both p16S and p23S RNA at 20°. Since presursor RNA can be found in particles sedimenting at 30 S and 50 S, the conversion of precursor to mature RNA is a very late step in ribosomal subunit maturation.

Figure 4 shows gels of RNA extracted from the precursor particles which were separated on sucrose gradients of the four *rim* mutants. By analogy to the work of others (Adesnik and Levinthal, 1969; Hecht and Woese, 1968), we regard slowly migrating rRNA species to be in the precursor form. The slowly migrating RNA species referred to here as "p23" and "p16" retain their characteristic slower mobility after heat and cooling the RNA preparations. Since the particles from cld-S mutants contain 23S RNA, the particles are either identical or are related to precursors of the 50S subunit. The material analyzed for *rim C* (Figure 4C) was taken from the heavy side of the heterogeneous 30S peak (*e.g.*, fractions 18–20 in Figure 2B). Particles from the lighter side of that peak contain both p16 and m16 RNA.

Figure 4 shows that 5S RNA can be extracted from 43S and 32S particles. Quantitative estimates of the 5S RNA content of the respective 43S particles can be obtained by expressing the radioactivity in 23S and 5S RNA gel peaks as a ratio. We have found significant variation in such 5S:23S RNA

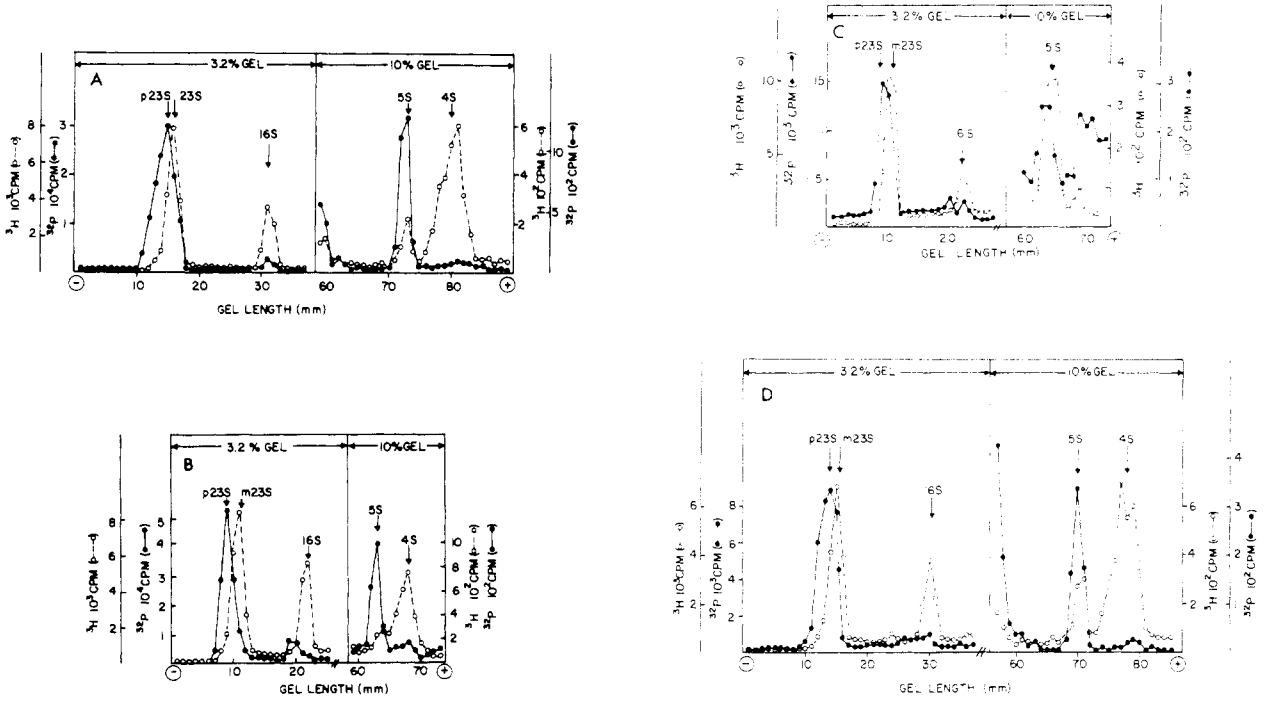


FIGURE 4: Polyacrylamide gel patterns of RNA extracted from the precursor particles of rim mutants. Gels consist of composite 3.2%/10% acrylamide. $^3$H-labeled RNA was added as a marker for the mature species: (A) *rim A*, (B) *rim B*, (C) *rim C*, (D) *rim D*.

ratios for both 43S and 50S subunits from experiment to experiment for the same mutant and for the parental strain. From our data, which are not presented, we conclude that from 50 to 100% of the 5S RNA found in 50S subunits is found in the 43S particles of *rim A*, *rim B*, and *rim D* strains. As has been suggested with regard to the 43S particles in another *E. coli* strain (Monier *et al.*, 1969), it is probable that 43S particles contain the full complement of 5S RNA, but 5S RNA is lost during 43S particle isolation because of weak binding.

Experiments in which *rim* mutants are labeled with radioactive uracil at 20° and then grown at 37° have been performed in order to observe whether the label in cld-S particles "chases" into 50S subunits. The results of these experiments indicate that the particles accumulated at 20° by all four mutants are converted to 50S subunits when the cultures are returned to the permissive temperature. This conversion is complete within 1 hr (or 2/3 generation) after the shift to 37°. Experiments with the *rim A* mutant show that the precursor RNAs are converted to mature forms at the permissive temperature.

*Ribosomal Protein Analysis.* Two-dimensional polyacrylamide gel electrophoresis was used in an attempt to demonstrate whether a ribosomal protein was latered by mutation in any of the mutants. There is a possibility that 50S subunit assembly is dependent upon the 30S subunit (Nashimoto and Nomura, 1970). A mutation in a 30S protein could thereby lead to a block in 50S subunit assembly. Consequently, both 50S and 30S ribosomal proteins were analyzed. Figure 5 shows 50S proteins extracted from the *rim A* mutant and from the parental strain. All of the proteins are visible and are in a pattern similar to that obtained by Kaltschmidt and Wittmann (1970a). The 50S proteins of the *rim A* mutant can be superimposed with those of the parental strain, demonstrating that there is no alteration in electrophoretic mobility of any of the proteins. Figure 6 shows 30S proteins of the *rim A* and of the parental strain. The protein S1 is not reproducibly demonstrable in our analyses. No differences in electrophoretic mobility are apparent. Ribosomal proteins of *rim B* and *rim D* strains were also analyzed. No alterations in electrophoretic mobility were detected for 50S or for 30S proteins. Mutant *rim C* is difficult to grow and, additionally, is a leaky mutant. A more thorough analysis of it is in progress.

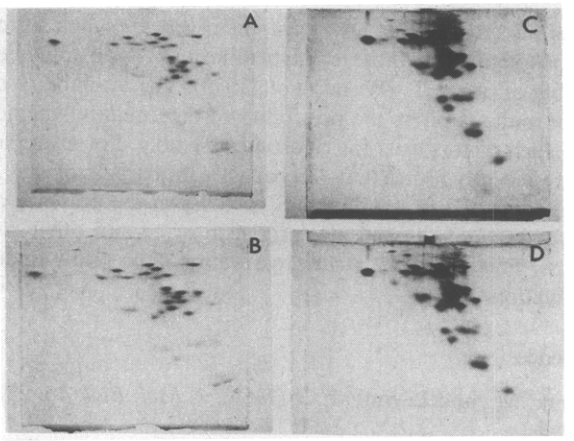


FIGURE 5: Two-dimensional polyacrylamide gel electrophoresis patterns of 50S ribosomal proteins of a *rim A* mutant. (A) *rim A*, (B) parental strain. Gels (A) and (B) were run simultaneously. L31 and L34 are not visible. (C) *rim A*, (D) parental strain. Gels (C) and (D) were run simultaneously.

## Discussion

The rRNA of four mutants of *E. coli* which have conditional blocks in the assembly of ribosomes was analyzed. Three mutants accumulate 43S and one mutant accumulates 32S ribonucleoprotein particles at 20°, but not at 37° The cld-S particles contain 23S RNA in the precursor form, which indicates that the particles are related to precursors of the 50S subunit. At least 50% of the 5S RNA found in the 50S subunit is also present in the 43S particles. The particles probably contain the full complement of 5S RNA, since 5S RNA may be lost from the particles during the course of particle isolation because of weak binding.

Precursor rRNA has been isolated by others from precursor RNP particles, but has not been reported in particles sedimenting at 50 S or 30 S. The demonstration of p23S and p16S RNA in 50S and 30S ribosomes, respectively, synthesized at 20° indicates that maturation of rRNA by removal of oligonucleotides is a very late step in ribosome maturation.

Two-dimensional polyacrylamide gel electrophoresis revealed that the ribosomal proteins from the mutants are indistinguishable in electrophoretic mobility from those of the parent strain. Although many mutations in ribosomal proteins are distinguishable, the failure to demonstrate an alteration in electrophoretic mobility of a protein is not conclusive proof that the protein is not altered by mutation. One would expect that substitution of an amino acid by another amino acid of similar charge would not affect electrophoretic mobility significantly. Also, it is possible that an insertion or deletion mutation could result in a protein with the same charge density as the wild-type protein. This type of altered protein might not be resolved from its wild-type homolog. On the other hand, two-dimensional gel electrophoresis has been quite useful in detecting a variety of mutant ribosomal proteins (Bollen *et al.*, 1973; Deusser *et al.*, 1970; Funatsu *et al.*, 1972; Stöffler *et al.*, 1971).

Failure to find altered ribosomal proteins in these mutants, together with the fact that they map at different loci and quite far from the *str A* locus (Bryant and Sypherd, 1974), suggests that these mutations do not involve the structural proteins of ribosomes. Our present hypothesis is that the alterations in the *rim* mutants involve factors which promote ribosome assembly by posttranscriptional or posttranslational modifications of ribosome components or by affecting the assembly

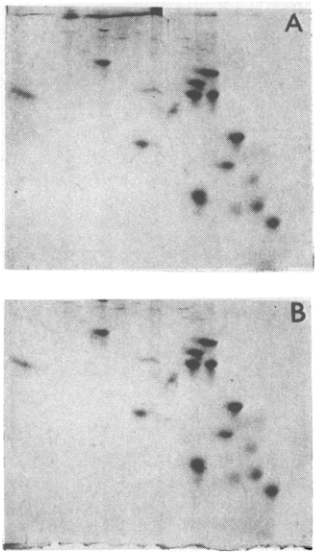


FIGURE 6: Two-dimensional polyacrylamide gel electrophoresis patterns of 30S ribosomal proteins of a *rim A* mutant: (A) *rim A*, (B) parental strain. The gels were run simultaneously.

process directly. In the latter case these genes might control functions similar to those catalytically involved in tail fiber assembly of phage $T_4$ (Wood *et al.*, 1973). The availability of the *rim* mutants offers the possibility of determining whether or not such factors exist for ribosome assembly. For example, we have recently found that 43S particles in extracts of *rim A* can be converted to nominal 50S particles in the presence of crude extracts of the parental organism or certain other *rim* mutants. This apparent *in vitro* maturation is presently under investigation.

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