THE IDENTIFICATION AND CHARACTERIZATION OF PROTEINS SIMILAR TO L7,L12 IN RIBOSOME-FREE EXTRACTS OF ESCHERICHIA COLI

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SUMMARY

Proteins similar to ribosomal proteins L7,L12 have been found in the high-speed supernatant fraction of E. coli. This identification is based on immunochemical characteristics, molecular weight and amino acid composition. However, unlike L7,L12 which has been isolated from the ribosome, the supernatant L7,L12 will not bind to L7,L12 depleted ribosomes. In E. coli B cells, these proteins represent approximately 0.4-1.4% of the total supernatant protein.

INTRODUCTION

The 50S ribosomal subunit proteins L7 and L12 are identical except that in L7 the N-terminal serine is acetylated (1). These two acidic proteins are unique, since there are a total of three moles of L7,L12 per ribosome, while most other ribosomal proteins occur in molar quantities (2, 3). Proteins L7,L12 are required for initiation (4-7), elongation (8-12) and termination (13) of protein synthesis and appear to be necessary for the proper binding of IF2, EFTu, EFG and RF[1] to the 50S subunit (13,14). In addition, it has been shown in vitro that these proteins stimulate the initiation of RNA synthesis by E. coli RNA polymerase (15,16) and stimulate the transcription of the lac operon (17).

It has generally been thought that the pool of ribosomal proteins in the post-ribosomal supernatant of E. coli is very small. This has been based on kinetic experiments which measured the rate of pulse-labeled ribosomal protein incorporation into mature ribosomal particles (18-21). In contrast, however, one study (22) found an apparent pool size of

[1]Abbreviations used - initiation factor 2, IF2; elongation factor Tu, EFTu; elongation factor G , EFG: release factor, RF.

ribosomal proteins as high as 8-14% of the total soluble protein when antibodies to ribosomal proteins were used as the assay. During the course of *in vivo* studies on the biosynthesis of ribosomal proteins L7 and L12 in which antibodies to the proteins were employed, it was noticed that a significant pool of L7,L12-like proteins were present in the post-ribosomal supernatant. This communication describes the identification, quantitation and properties of these proteins from high-speed supernatants of *E. coli*.

MATERIALS AND METHODS

Bacteria and preparation of S-200 extract. *E. coli* B was grown in either minimal salts glucose media or Bacto pennassay broth (Difco) in a rotary shaker at 27°. Cells were harvested at various times by centrifugation, washed twice with minimal salts medium and finally suspended in a small volume of 10 mM Tris acetate pH 7.8, 14 mM magnesium acetate, 60 mM potassium chloride and 6 mM β mercaptoethanol (Buffer A). The cells were disrupted with either a French pressure cell or by sonication. The extract was centrifuged at 30,000 x g for 15 min, and the resulting supernatant (S-30) was further centrifuged at 200,000 x g for 1 hr with or without a 5% sucrose cushion in buffer A. The supernatant (S-200) was carefully withdrawn and centrifuged again at 200,000 x g for 1 hr. The upper 2/3 of the second high-speed centrifugation was retained for analysis.

Immunological techniques. Proteins L7,L12 and [^{3}H] L7L12 were isolated and purified from NH_4Cl washed 70S ribosomes as described previously (1,9,12). Antibodies to purified L7 and L12 were raised in rabbits with the aid of Freund's complete adjuvant. Double diffusion analysis was performed according to Ouchterlony (23). Proteins L7 and L12 were quantitated by a radial-immuno-diffusion technique (24) which consisted of 1% agar double diffusion plates containing 50 mM potassium phosphate pH 7.5 and antisera to L7 or L12. Aliquots of the supernatant and known quantities of L7,L12 were placed in individual wells cut in the agar plates. The plates were developed overnight at room temperature. The diameter of the resulting precipitation rings were measured, and the L7,L12 concentrates of the supernatants were calculated by comparison to standards.

For gel electrophoresis, proteins L7,L12 were purified from the supernatants by immunoprecipitation in 1% Triton X-100, 0.5 M sodium chloride and 50 mM sodium phosphate. The resulting immunoprecipitates were washed three times in this buffer to remove unreacted supernatant and serum proteins. As a control, purified ribosomal L7,L12 was immunoprecipitated under similar conditions. The immunoprecipitate was dissolved in SDS and electrophoresed on a 10% acrylamide slab gel in a system described by Laemmli (25). The protein bands which migrated with a molecular weight of 12,000 were sliced from the gel and subjected to amino acid analysis as described by Stein, *et al.* (26).

The binding of L7,L12 to ethanol-NH_4Cl washed 70S ribosomes was determined as described previously (3) except that L7,L12 concentrations were determined by radial-immunodiffusion. Briefly, this consisted of adding supernatant or purified ribosomal L7,L12 to a buffer which contained in a final concentration, 10mM Tris-chloride, pH 7.5, 10mM magnesium chloride and 10 mM ammonium chloride. An aliquot was removed for analysis of the L7,L12 concentration by radial immunodiffusion. Ethanol-NH_4Cl extracted 70S ribosomes were added, and the mixture incubated at 37° for

two minutes followed by centrifugation at 200,000 x g for 1 hr. The supernatant was carefully withdrawn and the binding of L7,L12 was calculated by the disappearance of L7,L12 immunoequivalents from the supernatant upon addition of the ethanol-NH_4Cl washed ribosomes.

Protein concentrations in the supernatant fractions were determined by the method of Lowry, et al. (27).

RESULTS

Identification of L7,L12-like proteins in the post-ribosomal supernatant. In the course of characterizing antibodies to E. coli ribosomal proteins L7,L12, it was noticed that there was a strong cross-reactivity with the post-ribosomal supernatant fraction. The supernatant protein(s) was found to be immunochemically identical to ribosomal protein L7,L12 by double diffusion analysis as demonstrated in Fig. 1 The protein(s) also has the same molecular weight (i.e. 12,000) as L7,L12 as indicated by SDS gel electrophoresis of the solubilized immunoprecipitates (Fig. 2). Furthermore, amino acid analysis of the stained bands (of 12,000 daltons)

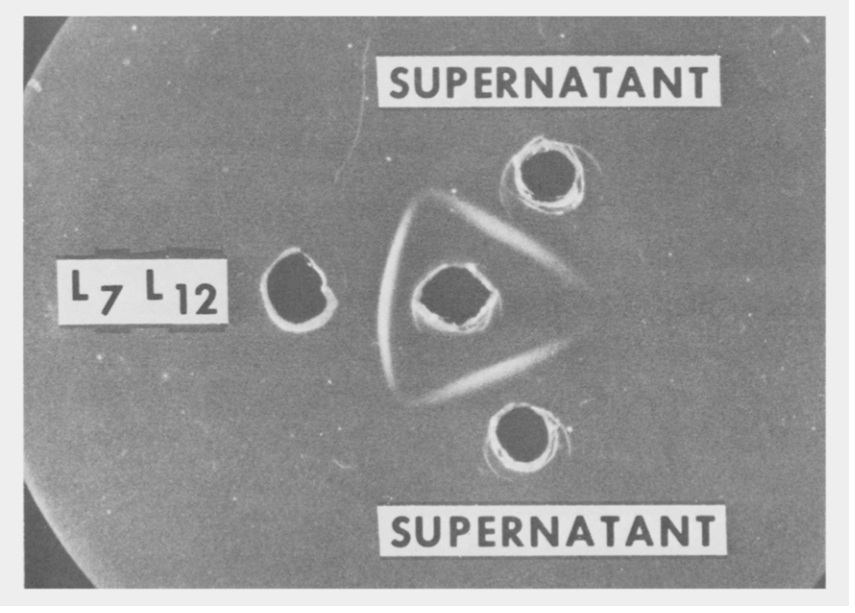

Fig. 1: Ouchterlony double diffusion analysis of L7,L12 from E. coli ribosomes and high-speed supernatants. The center well contains antibody to E. coli ribosomal L7,L12. The peripheral wells contain 2 µg purified L7, L12 or 100 µg of high-speed supernatant protein.

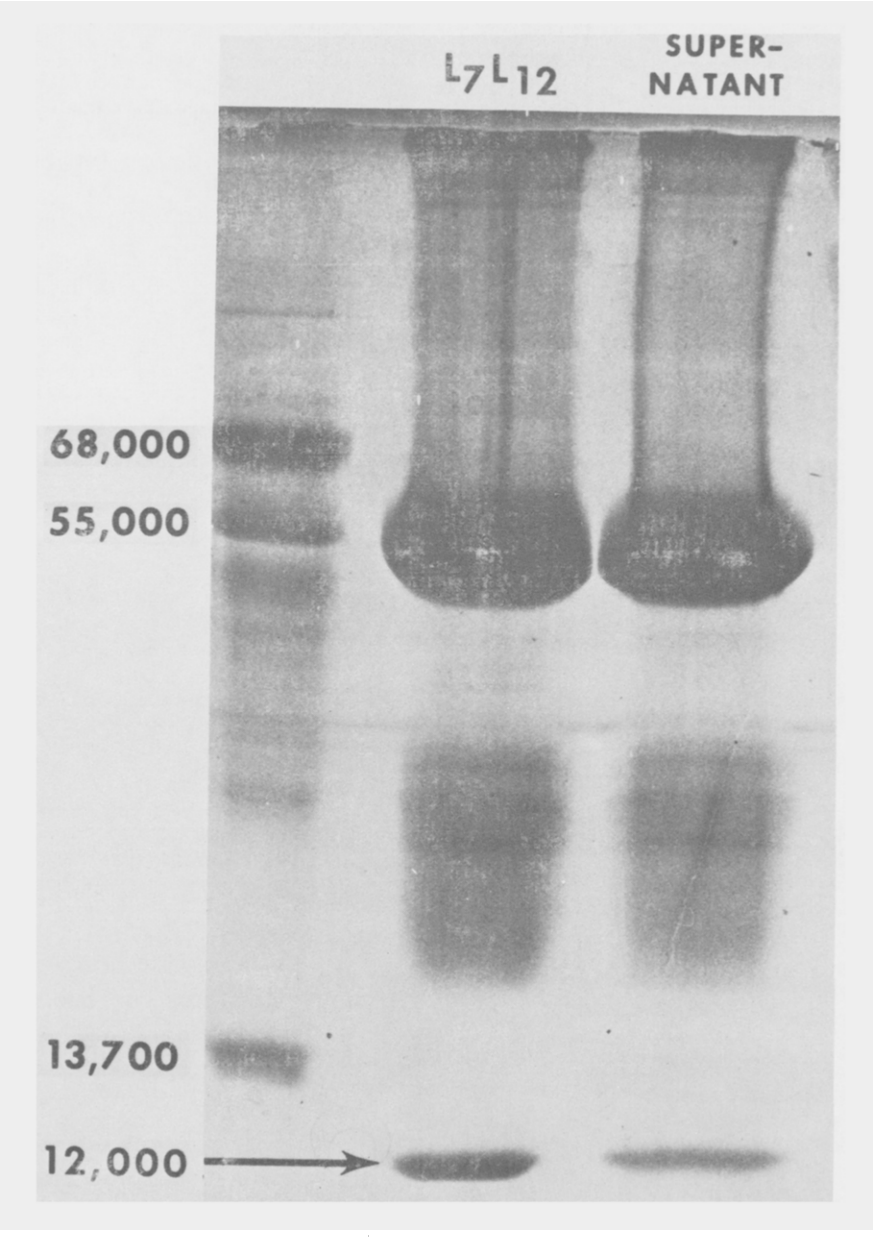

Fig. 2: SDS gel electrophoresis of solubilized immunoprecipitates derived from purified L7,L12 or the high-speed supernatant. Antiserum to L7,L12 was added to purified L7,L12 or to a high-speed supernatant each containing approximately 5 μg of antigenic L7,L12. Immunoprecipitation and gel electrophoresis was performed as described in Methods. The large protein bands in the experimental gels at 50,000 molecular weight and between 20-30,000 molecular weight represent the antibody proteins. Marker proteins (left gel) were bovine serum albumin (68,000) gamma globulin heavy chain (55,000) and RNase (13,700).

derived from the SDS gel (25) revealed essentially the same amino acid composition for the immunoprecipitable material isolated from both the supernatant and ribosomes (Table I). The amino acid compositions of the proteins isolated by immunoprecipitation and SDS gel electrophoresis are comparable to the composition of ribosomal L7 and L12 isolated either by the procedure of Hamel, et al. (9) or of Terhorst, el al. (29) (see Table I). The close identity of the supernatant protein in E. coli ribosomal proteins L7 or L12 appears to be further corroborated by the absence of

TABLE I

AMINO ACID COMPOSITION OF L7,L12 ISOLATED FROM RIBOSOMES AND SUPERNATANT

Amino Acid	Supernatant L7,L12[1]	Ribosomal L7,L12 A[1]	Ribosomal L7,L12 B[2]	Terhorst et al. (29)
	mole %			
Aspartic acid	7.1	7.0	7.3	6.7
Threonine	3.1	3.0	3.0	3.0
Serine	4.4	5.0	4.4	5.1
Glutamic acid	14.7	15.0	15.0	14.5
Proline	2.6	2.5	2.4	1.8
Glycine	19.7	21.0	22.7	23.8
Alanine	19.7	21.0	22.7	23.8
Cysteine	0	0	0	0
Valine	11.6	12.0	13.0	13.3
Methionine	4.0	4.0	4.0	3.6
Isoleucine	4.0	4.0	4.0	3.6
Leucine	7.1	7.0	7.3	6.1
Tyrosine	0	0	0	0
Phenylalanine	2.2	2.0	2.0	1.6
Histidine	0	0	0	0
Lysine	9.8	10.5	11.3	9.8
Arginine	1.7	1.0	1.2	0.9

[1]Isolated by immunoprecipitation and SDS gel electrophoresis.

[2]Direct acid hydrolysis of purified L7,L12.

histidine, cysteine and tyrosine, and the high alanine content, all indicative of these ribosomal proteins. Tryptophan which is also absent from L7 and L12, was not assayed.

Quantitation of supernatant L7,L12-like protein. Having established that the identity of the immunoreactive material in the post-ribosomal supernatant is similar to ribosomal L7,L12, an attempt was made to quantitate the amount of this protein in E. coli supernatants. The concentration of L7,L12 in high-speed supernatants prepared from E. coli harvested at different growth stages was determined by radial-immunodiffusion. Figure 3 shows that cells grown in rich media at all stages of growth contain more of these proteins than similar cells grown in minimal media. Cells in early log phase have about 1% of their supernatant protein as L7,L12 based on this assay. The concentration declines exponentially throughout growth, approaching 0.3-0.4% of the postribosomal supernatant protein at late log and stationary phases.

Biological activity of supernatant L7,L12. Since these proteins have chemical characteristics which are identical to L7,L12, it was of importance to ascertain if they were biologically active. Proteins L7,L12

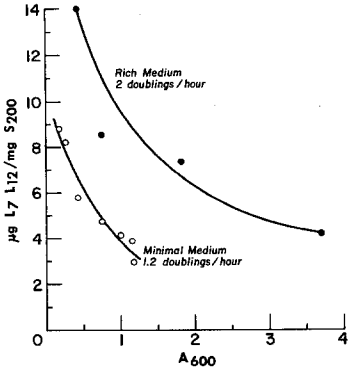

Fig. 3: Quantitation by radial-immunodiffusion of L7,L12 in high-speed supernatants of E. coli B under different growth conditions. Cell growth and analysis were performed as described in Methods.

can be assayed by their ability to restore activity to ribosomes which have had their L7 and L12 removed by extraction with ethanol-NH_4Cl (3,11). The ethanol-NH_4Cl washed ribosomes do not support various reactions involved in protein synthesis (3-14). Ethanol-NH_4Cl washed ribosomes efficiently rebind L7,L12, achieving the normal stoichiometry of almost three L7,L12

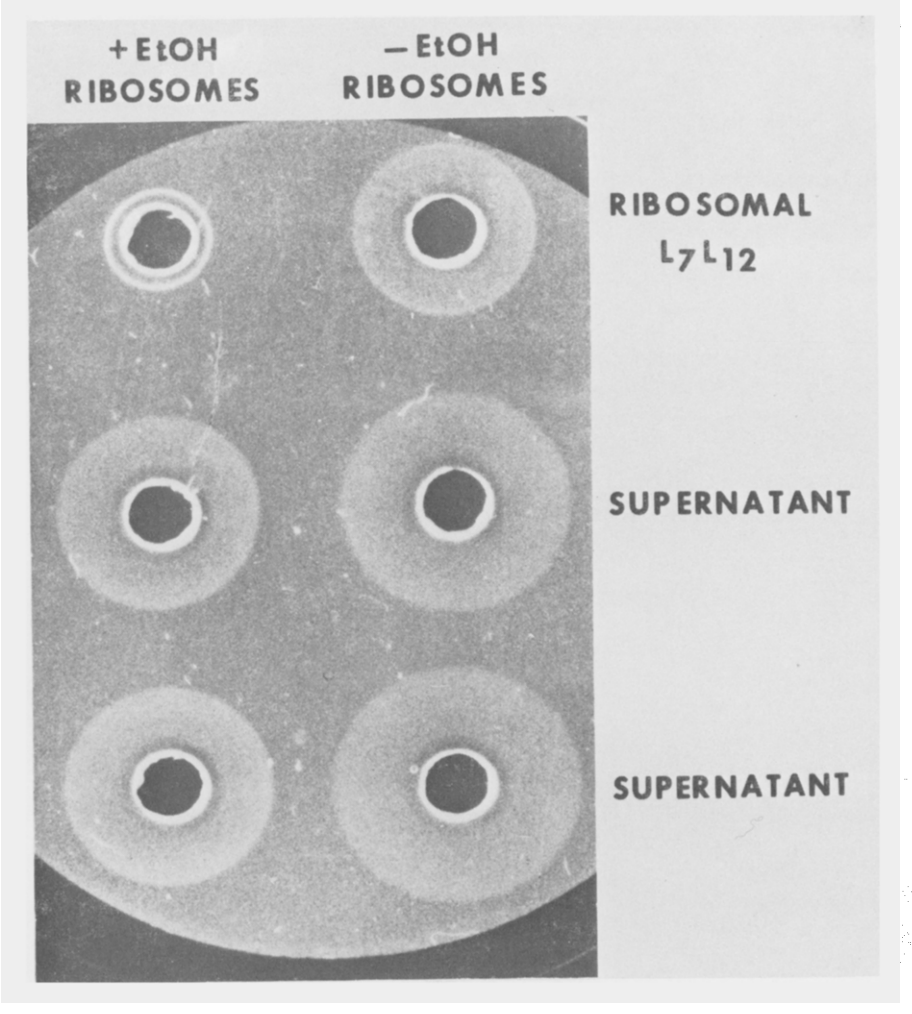

Fig. 4: The binding of ribosomal proteins L7,L12 and supernatant L7,L12 to ethanol-NH_4Cl washed ribosomes. Ethanol-NH_4Cl washed ribosomes (1500 pmoles capable of binding 54 µg of L7,L12) were mixed with either 29 µg of ribosomal L7,L12 or supernatants containing 40-45 µg L7,L12 in a final volume of 0.5 ml of 10 mM each Tris-Cl, pH 7.5, $MgCl_2$ and NH_4Cl. Binding was performed as described in Methods using a radial-immunodiffusion procedure to assay L7,L12.

molecules per ribosome (3). The supernatant L7,L12 immunoequivalents were tested for their ability to bind to ethanol-NH_4Cl washed ribosomes. As seen in Fig. 4, approximately 80% of L7,L12 isolated from ribosomes can rebind to ethanol-NH_4Cl washed ribosomes. In contrast, only about 15% of the supernatant L7,L12-like material binds to these same ribosomes. There does not appear to be an inhibitor of L7,L12 binding in the supernatant, since approximately 70-75% of [^{3}H]-L7,L12 will bind to ethanol-NH_4Cl washed ribosomes in the presence or absence of high-speed supernatant (Table II). This indicates a distinct preference of the ethanol-NH_4Cl washed ribosome for the ribosomal-derived L7,L12, as opposed to the supernatant L7,L12-like proteins, suggesting some differences between the proteins from the two different sources.

TABLE II

Binding of [^{3}H]-labeled Ribosomal L7,L12 in the presence and absence of Supernatant L7,L12

	% [^{3}H] L7,L12 Bound
[^{3}H]-L7,L12	75
[^{3}H]-L7,L12 and supernatant L7,L12	69

Ethanol-NH_4Cl extracted 70S ribosomes (1500 pmoles capable of binding 54 µg L7,L12) were mixed with 29 µg [^{3}H]-labeled L7,L12 in the presence and absence of high-speed supernatant containing 40 µg of L7,L12 as assayed by radial-immunodiffusion in a final volume of 0.5 ml each 10 mM Tris-Cl, pH 7.5, $MgCl_2$ and NH_4Cl. Binding was assayed as described in Methods except that the levels of unbound [^{3}H] L7,L12 were determined by radioactivity instead of radial-immunodiffusion. The two assays gave similar results.

DISCUSSION

This communication provides evidence for the existence of a significant amount of ribosomal proteins similar to L7,L12 in the post-ribosomal supernatant of E. coli. This identification is based on molecular weight, amino acid composition and immunological properties. Our results indicate that 0.5-0.6% of the total soluble protein in supernatants of E. coli at midlog phase of growth cross reacts with antiserum to ribosomal proteins L7,L12. Preliminary results based on gel electrophoresis at pH 6.0 of the supernatant proteins suggest that the major species behaves similar to L7.

The one feature of the supernatant L7,L12 not in common with the ribosomal congener is the apparent lack of biological activity. The lack of binding to ribosomes may be due to subtle difference in the molecule not apparent in the amino acid composition. Alternatively, some protein(s) may be complexed with the supernatant L7,L12 and thus prevent ribosomal binding. If so, the protein(s) would have to be present in close to stoichiometric amounts relative to the supernatant L7,L12, since ribosomal L7,L12 is able to bind in the presence of an S-200 extract.

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