

Reversion from Erythromycin Dependence in *Escherichia coli*: Strains Altered in Ribosomal Sub-unit Association and Ribosome Assembly

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A mutant of *Escherichia coli* dependent on erythromycin for growth spontaneously gives erythromycin-independent strains with altered or missing ribosomal proteins. Strains with defects in ribosome assembly were sought and obtained from among these revertants. Two organisms in which ribosomal protein L19 is altered and absent respectively have 70S ribosomes whose dissociation into sub-units is particularly sensitive to pressures generated during centrifuging. The mutant that lacks protein L19 also accumulates ribosome precursor particles during exponential growth as do others including mutants that lack proteins S20 or L1. These strains also show unbalanced synthesis of RNA and so will be useful in investigating both the pathways and the regulation of ribosome assembly.

INTRODUCTION

The 70S ribosome of *Escherichia coli* comprises three ribosomal RNAs and 52 different ribosomal proteins. From comparisons with other organisms and organelles, this is about the minimum number of components required by a functioning ribosome. The secondary structures of the ribosomal RNAs are highly conserved across bacterial species. There are also strong homologies in amino acid sequence of the individual ribosomal proteins of different bacteria so that there is, for example, little difficulty in correlating equivalent proteins from *Escherichia coli* and *Bacillus stearothermophilus* (Wittmann, 1986). Although the ribosomal RNAs are increasingly thought to be directly involved in protein synthesis (e.g. Cundliffe, 1986; Moazed & Noller, 1987), the conservation of ribosomal proteins and the numerous studies that have implicated them in the various stages in protein synthesis show that they also have a vital role in ribosome function. However, some mutant strains of *E. coli* lack individual ribosomal proteins. These strains were obtained as organisms that had reverted to independence from dependence for growth on an antibiotic such as kasugamycin or erythromycin. (A review is by Dabbs, 1986.) Some mutants without a ribosomal protein grew slowly while others (e.g. those lacking ribosomal proteins L29 or L30) had no obvious impairment. The existence of these strains shows that all ribosomal proteins are not absolutely required for protein synthesis and emphasizes the flexibility of the ribosome.

Mutants of *E. coli* that lack a ribosomal protein have been used to help locate that protein on the surface of non-mutant ribosomal sub-units (e.g. Dabbs *et al.*, 1981; Stöffler *et al.*, 1984) and to explore the functions in which the protein is involved (e.g. Subramanian & Dabbs, 1980). Little attention has been paid to organisms in which altered ribosome assembly results from absence of a ribosomal protein. This phenotype should be frequent if, as suggested by Ehrenberg *et al.* (1986), a major role of some ribosomal proteins is to maintain a fast kinetic pathway for assembly. A mutant lacking protein L1 oversynthesized protein L11 (Dabbs *et al.*, 1981). This is part of the evidence (reviewed by Lindahl & Zengel, 1986) that translation of mRNA from the L11-L1 operon is inhibited by protein L1; the consequences for ribosome assembly, if any, were not reported. Another revertant to erythromycin independence failed to make protein L24

because of an ochre mutation in the *rplX* gene (Nishi *et al.*, 1985). The 50S ribosomal sub-units of this mutant were as active as those from wild-type organisms in polyphenylalanine synthesis. However, *in vitro*, assembly of 50S sub-units was extremely slow (Herold *et al.*, 1986), in accordance with other evidence that protein L24 is near-essential at an early stage in reconstitution (Nowotny & Nierhaus, 1982).

Mutants lacking ribosomal proteins do not always have properties that might be predicted for them. We (Butler *et al.*, 1980; Butler & Wild, 1984, 1985) studied a strain of *E. coli* (TP28) with a mutation in the *rpmB,G* transcription unit that halved rates of synthesis of proteins L28 and L33. The consequences of this lesion for the synthesis of other ribosomal proteins were widespread: about ten proteins were made at rates up to 75% faster than required to sustain ribosome synthesis and the excess usually degraded. Moreover, this strain accumulated large quantities of '47S' particles. These are unusual precursors to 50S ribosomal sub-units and synthesis proceeded as though they, rather than the sub-units, were the end-product of assembly. Ribosome assembly in strains that lack proteins L28 and/or L33, rather than underproduce them, might then be expected to be even more severely restricted. However, unpublished work (by B. A. Maguire & D. G. Wild) shows that this is not so. Mutant strains AM90 and AM108 lack ribosomal proteins L28, and L28 plus L33, respectively (Dabbs, 1979). Neither shows substantial accumulation of ribosome precursor particles, and rates of synthesis of other ribosomal proteins are less perturbed than in strain TP28. These differences are presently unexplained but, together with the other considerations above, prompted experiments that isolated phenotypic revertants from erythromycin dependence with altered ribosome assembly. The properties of some of these revertant strains are described in this paper.

METHODS

Bacteria and their growth. Experiments used *E. coli* strain A19 (Gesteland, 1966) and derivatives obtained as described in Results. Unless otherwise stated, organisms were grown with shaking at 37 °C in D-broth, which contained, per litre, 10 g tryptone, 10 g yeast extract, 5 g glucose, 7 g K_2HPO_4 , 3 g KH_2PO_4 , 500 mg trisodium citrate. $3H_2O$, and 100 mg $MgSO_4 \cdot 7H_2O$. D-agar was the same medium solidified with 1.5% (w/v) agar. Erythromycin (from Boehringer-Mannheim) was added to media from a filter-sterilized solution (usually 10 mg ml^{-1}) in 50% (v/v) ethanol.

Preparation of extracts and density-gradient centrifugation. Organisms grown in 10–25 ml D-broth to $OD_{450} \sim 0.5$ were harvested, washed with 2–5 ml THMK buffer (10 mM-Tris/HCl, pH 7.4, 10 mM-magnesium acetate, 100 mM-KCl) and broken in a French pressure cell. Portions of extracts (usually 100 μ l) were layered onto 15–30% (w/w) sucrose gradients made in THMKSp buffer (THMK buffer containing 1 mM-spermidine. HCl) and centrifuged at 4 °C usually for 2.5 h at 50000 r.p.m. (237000 g_{av}) in a Spinco SW 55Ti rotor. (Other rotors and conditions of centrifuging are noted in the text.) Gradients were collected through an Isco density-gradient fractionator. For some experiments, [^{14}C]uracil and/or [3H]uracil were added to the growth medium; then, after centrifuging, about 50 three-drop fractions were collected and assayed for radioactivity (Markey & Wild, 1976).

Two-dimensional gel electrophoresis of ribosomal proteins. Organisms were grown in 300 ml D-medium to late exponential phase, harvested, washed with TMKSH buffer (10 mM-Tris/HCl, pH 7.4, 15 mM-magnesium acetate, 60 mM-KCl, 7 mM-2-mercaptoethanol) and resuspended in 8 ml of this buffer. An extract, made using a French pressure cell, was clarified (15 min; 25000 r.p.m.; Spinco Type 40 rotor) and the supernatant then centrifuged (2 h; 50000 r.p.m.; Spinco Type 65 rotor). The pellet was resuspended in 5 ml TMASH buffer (20 mM-Tris/HCl, pH 7.4, 10 mM-magnesium acetate, 500 mM- NH_4Cl , 14 mM-2-mercaptoethanol), underlayered with 3 ml TMASH buffer containing 1.1 M-sucrose and centrifuged for 16 h at 40000 r.p.m. (Spinco Type 50 rotor). The pellet of ribosomes was dissolved in 1 ml 8 M-urea containing 1% (v/v) 2-mercaptoethanol. The ribosomal proteins were extracted with acetic acid according to Hardy (1975), precipitated with acetone (Barritault *et al.*, 1976), dried *in vacuo* and dissolved in 500 μ l 8 M-urea, 1% 2-mercaptoethanol. Samples were analysed in the 'standard' two-dimensional electrophoresis system of Kenny *et al.* (1979) without alkylation of proteins. Some samples were also run with SDS/urea gels (Madjar *et al.*, 1979) in the second dimension. Gels were stained for about 2 h with Coomassie Blue R250 (0.1%) in destaining solution (methanol/water/acetic acid, 5:5:1, by vol.).

RESULTS

Isolation of an erythromycin-dependent strain and revertants to independence

The erythromycin-dependent strain 103 was one of several isolated by an antibiotic-underlay method (Dabbs, 1979). For this, *E. coli* strain A19, suspended ($OD_{450} \sim 0.5$) in buffer (50 mM-

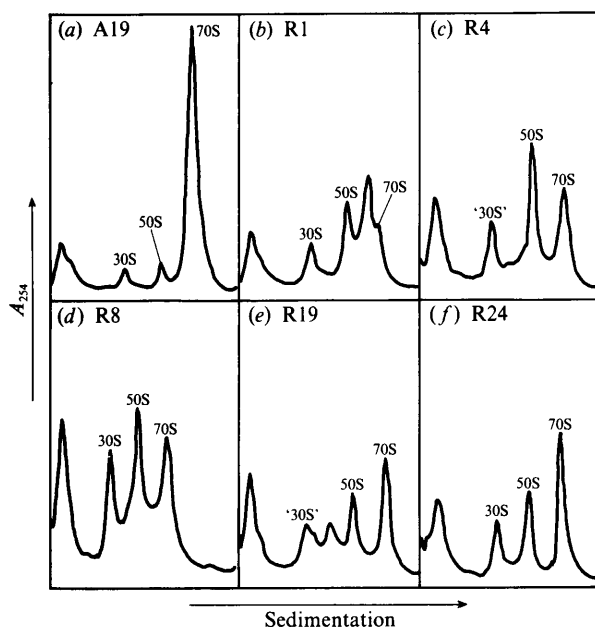


Fig. 1. Sedimentation profiles of ribosomal particles from wild-type and mutant strains after sucrose-density-gradient centrifugation under 'standard' conditions (see Methods). Extracts were made from (a) strain A19; (b) strain R1 (revertant no. 1); (c) strain R4; (d) strain R8; (e) strain R19; (f) strain R24.

Tris, 50 mM-maleic acid; pH brought to 6.2 with 5 M-NaOH) containing 200 μg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) ml^{-1} was incubated for 20 min at 37 °C. Survival was about 50%. Washed organisms were spread on D-agar (~25 ml agar per plate) and incubated for 3 h at 37 °C. The agar was then underlayered with 0.15 ml (5 mg) erythromycin. The antibiotic was allowed to diffuse for 3 d at 4 °C. Plates were then incubated for 2 d at 37 °C and colonies patched with sterile toothpicks onto D-agar without and with 200 μg erythromycin ml^{-1} . About 0.1–0.5% of resistant strains were also dependent on erythromycin for growth. One of these, strain 103, grew exponentially in D-medium containing 50, 100 or 200 μg erythromycin ml^{-1} with generation times at 37 °C of 75, 80 and 100 min respectively but did not grow in the absence of erythromycin. Strain A19 had a generation time of 25 min in D-medium; its growth was severely inhibited by 50 μg erythromycin ml^{-1} .

Strain 103 (but not strain A19) grew on D-agar containing 100 or 200 μg erythromycin ml^{-1} . On D-agar without erythromycin about 0.0005% of organisms spread gave colonies of varied sizes. A selection of these 'revertants' was purified by restreaking and then grown in D-medium to $\text{OD}_{450} \sim 0.5$. Extracts were made in THMKSp buffer and centrifuged through sucrose gradients in this buffer. Under these conditions the sedimentation profile of strain A19 showed native 30S and 50S sub-units to be present in small quantities relative to 70S ribosomes (Fig. 1a); the profile of strain 103 (after growth in D-medium containing 100 μg erythromycin ml^{-1}) was similar but with rather more material in the region of 30S sub-units (e.g. Fig. 3a, b). Most (about 90% of) revertant strains gave profiles similar to that of strain A19. Some did not. For example, revertant strain no. 1 (R1 hereafter) had a profile (Fig. 1b) in which the most prominent component has a sedimentation coefficient of about 60S and lay between the 50S sub-units and the few 70S ribosomes. With strain R4 (Fig. 1c), there were also few 70S ribosomes; the major ribonucleoprotein component was in the approximate position of 50S sub-units and there was also an excess of '30S' material. The profile of strain R8 (Fig. 1d) also showed few 70S ribosomes; most ribonucleoprotein was in the position expected of ribosomal sub-units. Rather similarly, strain R24 (Fig. 1f) had an excess of material in the region of sub-units; so did strain

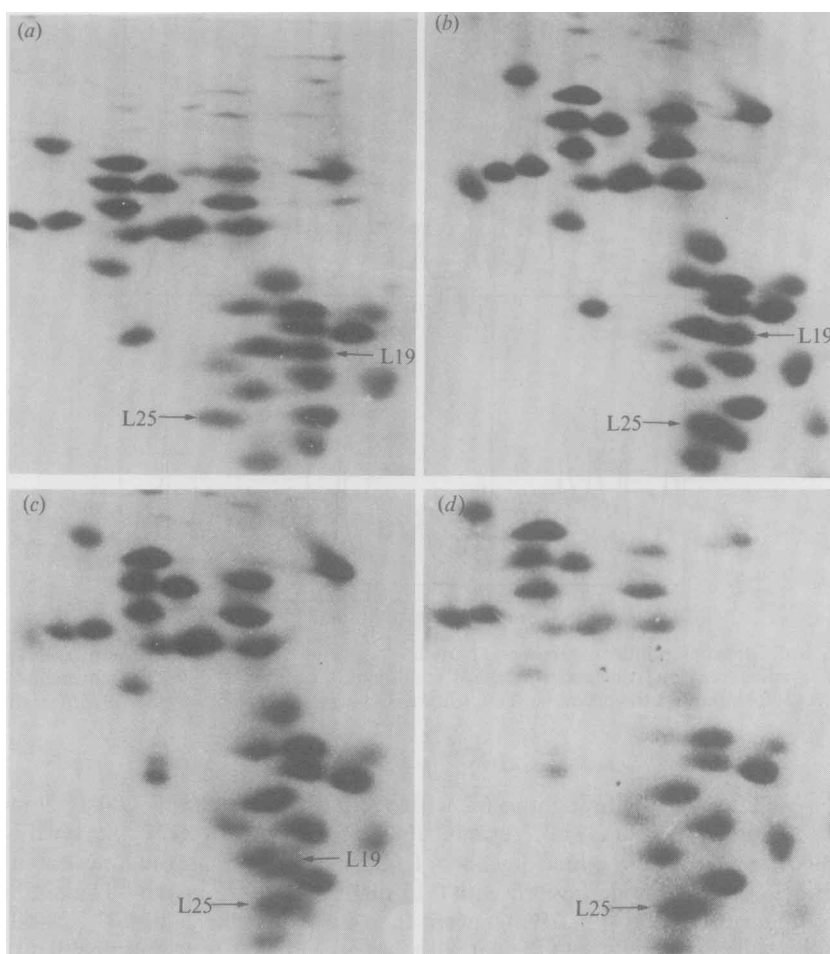


Fig. 2. Two-dimensional gel electrophoresis of ribosomal proteins from wild-type and mutant strains. (a) Strain A19; (b) strain 103; (c) strain R1; (d) strain R8.

R19 (Fig. 1e) although the profile of the latter had an additional major '40S' component. Thus some revertant strains re-acquire the ability to grow without erythromycin at the expense of abnormalities in ribosome metabolism. In these cases at least, 'reversion' is unlikely to involve reversal of the mutation(s) that created the dependence of strain 103 on the antibiotic.

Ribosomal proteins of dependent and revertant strains

Strains A19, 103 and several revertants were grown in D-medium and their ribosomal proteins (obtained as in Methods) examined by two-dimensional gel electrophoresis. The standard system resolved about 48 of the 52 ribosomal proteins; these were identified with the key provided by Madjar *et al.* (1979). Comparison of strains A19 and 103 (Fig. 2a, b) shows that in the latter the mobility of protein L25 was greater in both dimensions. The second dimension of the 'standard' electrophoresis separates partially by molecular mass. When this was replaced by an SDS/urea gel, the mobility of protein L25 from strain 103 was not distinguishable from that from strain A19. Protein L25 from strain 103 has thus gained in positive charge rather than decreased in molecular mass. The altered L25 was also apparent in all revertant strains examined. Some of the latter had additional changes. In strain R1, the mobility of protein L19 was reduced in the first dimension and increased in the second (Fig. 2c); in strain R8, this

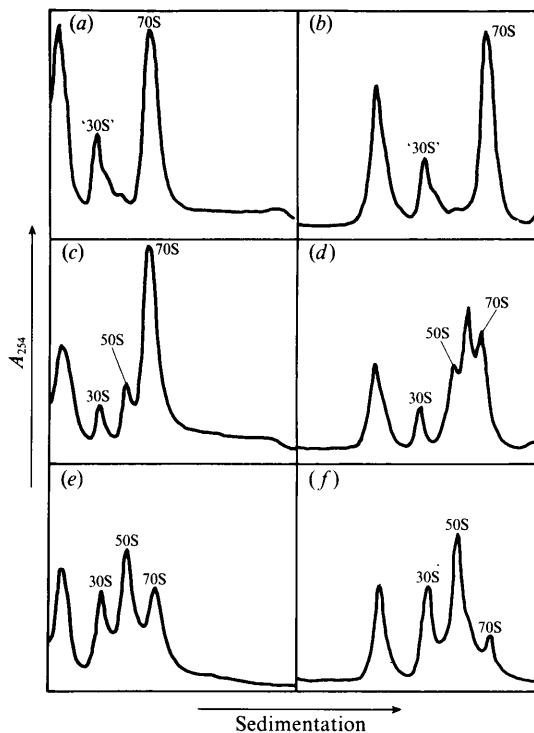


Fig. 3. Effect of pressure on sedimentation profiles. An extract of strain 103 was made in THMKSp buffer containing 7.5% (w/v) sucrose and loaded on (a) a 14 ml 15–30% sucrose gradient; (b) a 10 ml 15–30% sucrose gradient that was then overlaid with 4 ml THMKSp buffer. Gradients (c) and (d) were as (a) and (b) respectively but used an extract from strain R1. Gradients (e) and (f) were as (a) and (b) respectively but used an extract from strain R8. Centrifuging was for 2.5 h at 40000 r.p.m. in a Spinco SW 40Ti rotor.

protein was missing from its usual position and was not detected elsewhere (Fig. 2*d*). The set of proteins from strain R4 lacked protein S20, while strain R24 had no protein L1. Because some proteins are extracted rather variably and/or stain poorly, absences were confirmed by electrophoresis of at least three separate preparations of proteins from each revertant. 'Absence' does not distinguish between failure of synthesis and presence in some grossly altered and undetected form. Not all revertants showed alterations additional to that in protein L25; none was detected in strain R19, in which ribosome assembly was abnormal (Fig. 1*e*), or in strains R10 and R15, which had sedimentation profiles similar to that of strain A19.

Pressure-sensitivity of ribosomes from revertant strains

Revertant R1 grew faster (generation time about 40 min) than other revertants (Fig. 7*a*) but gave a sedimentation profile with a preponderance of '60S' material and few 70S ribosomes (Fig. 1*b*). This suggested that the 70S ribosomes of strain R1 were dissociating during centrifuging at the hydrostatic pressures generated under 'standard' conditions (50000 r.p.m.; 2.5 h). Extracts centrifuged more slowly (20000 r.p.m.) for longer (15 h) gave a profile (not shown) in which 70S ribosomes were now the major component; 70S ribosomes also predominated when 20 mM-Mg²⁺ replaced the 10 mM-Mg²⁺ of THMKSp buffer. Effects of hydrostatic pressure were shown directly. For this, extracts of strains 103 and R1 were each layered on two sucrose gradients made with 14 ml and 10 ml of THMKSp buffer respectively. The 10 ml gradients were then overlaid with 4 ml of buffer. Centrifuging was for 2.5 h at 40000 r.p.m. in the SW 40Ti rotor. Under these conditions the profile of strain R1 (but not strain 103) showed 60S material only at the higher pressure generated by the buffer overlay (Fig. 3). The defect in strain R1 was localized

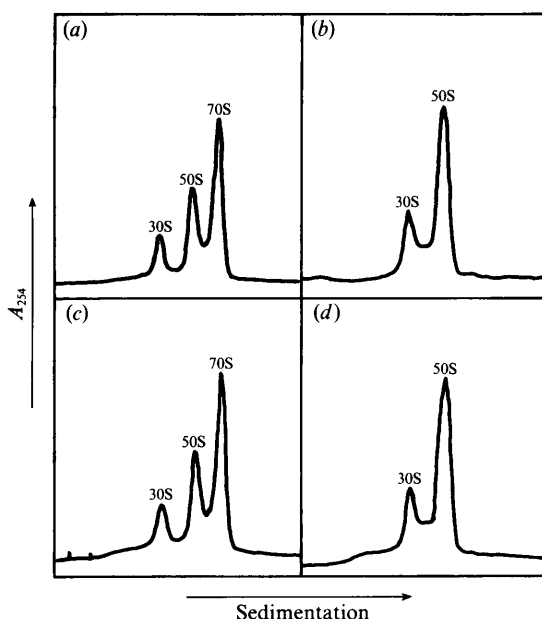


Fig. 4. Re-association of ribosomal sub-units. Extracts of strains A19 and R1, made in TLMKSH buffer (10 mM-Tris/HCl, pH 7.4, 2 mM-magnesium acetate, 100 mM-KCl, 7 mM-2-mercaptoethanol), were centrifuged through sucrose gradients in this buffer and fractions containing 30S or 50S ribosomal sub-units pooled separately. Magnesium acetate (1 M) was added to each pool (final concn 10 mM). Mixtures (total vol. 150 μ l; A_{254} of 50S and 30S sub-units in a ratio of 2:1) were made, diluted with 150 μ l THMKSH buffer (THMK buffer + 7 mM-2-mercaptoethanol) and incubated at 37 °C for 15 min. Each mixture was loaded on a 15–30% sucrose gradient (10 ml) in THMKSH buffer and overlaid with 4 ml of this buffer. Centrifuging was for 4.5 h at 30000 r.p.m. in the SW40 Ti rotor. (a) 50S sub-units from strain A19, 30S sub-units from strain R1; (b) 30S sub-units from strain A19, 50S sub-units from strain R1; (c) 30S and 50S sub-units from strain A19; (d) 30S and 50S sub-units from strain R1.

to the 50S ribosomal sub-units. Extracts of strains A19 and R1 were made and centrifuged through gradients in buffer containing 2 mM- Mg^{2+} and in which 70S ribosomes dissociate to sub-units. The latter were isolated and, after increasing the Mg^{2+} concentration to 10 mM, mixed in approximately equimolar proportions. Mixtures were activated (Zamir *et al.*, 1974) at 37 °C for 15 min and then centrifuged (SW 41 rotor; 4.5 h; 30000 r.p.m.) through 10 ml sucrose gradients with an overlayer of 4 ml buffer. When the 50S sub-units were from strain A19, about half the sub-units associated to form 70S ribosomes (Fig. 4a, c), irrespective of the source of the 30S sub-units; when the 50S sub-units were from strain R1, there was no association (Fig. 4b, d).

The sedimentation profiles of strains R4, R8 and R24 obtained under 'standard' conditions (Fig. 1c, d, f) showed few 70S ribosomes. This could result from extensive dissociation to sub-units during centrifuging. However, in contrast to strain R1, the profiles given by strains R4 and R24 did not alter when extracts were centrifuged at low rather than high speed or at different pressures or at 20 mM- Mg^{2+} . Extracts of revertant strain R8 also gave very similar profiles at 10 and 20 mM- Mg^{2+} . However, the profiles of extracts centrifuged at low speed showed rather more 70S ribosomes and, when pressures during centrifuging were increased by an overlayer of buffer, dissociation of 70S ribosomes into ribosomal sub-units (rather than to '60S' material as in strain R1) was virtually complete (Fig. 3e, f).

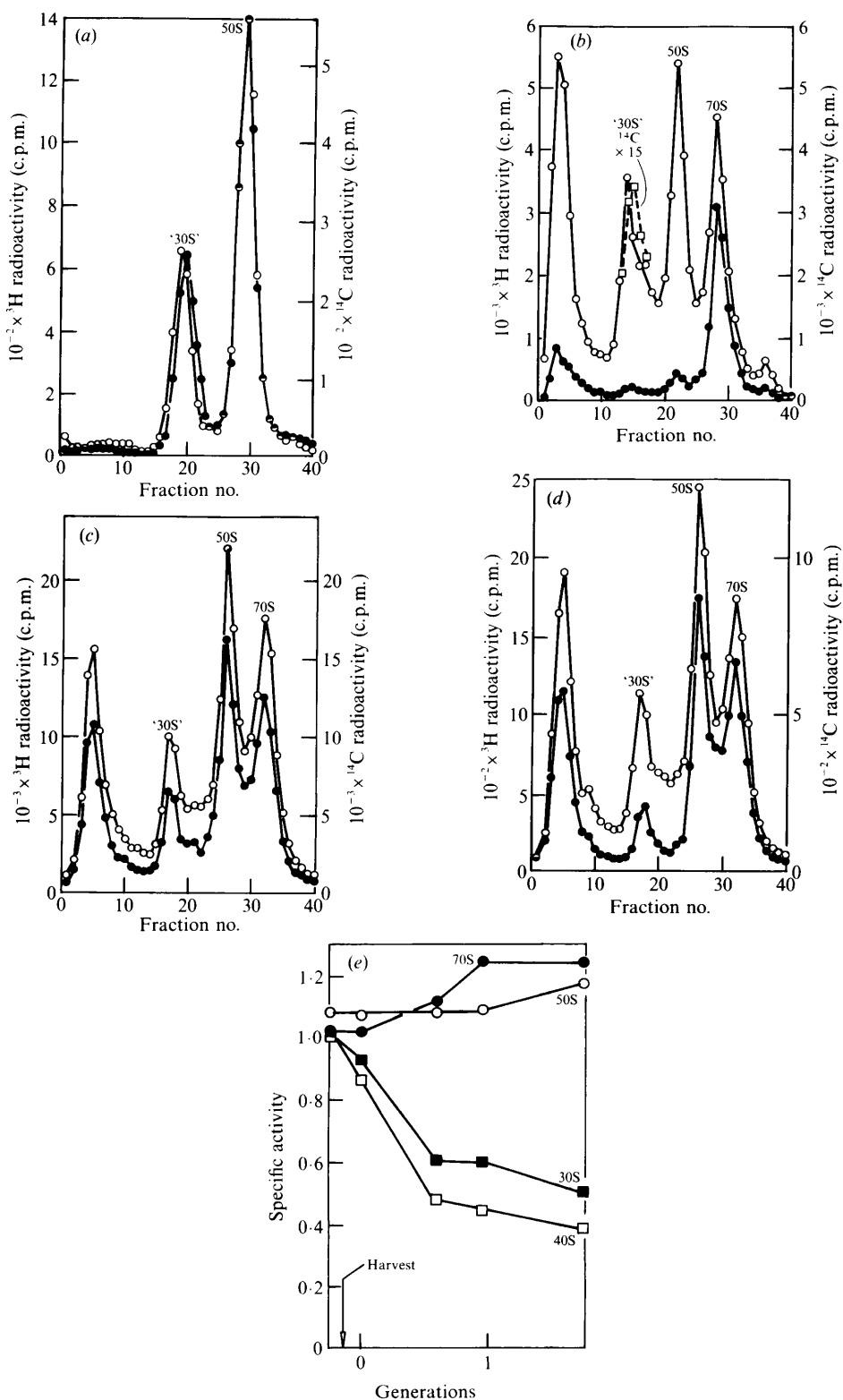
Thus strain R1, with an altered ribosomal protein L19, has a normal complement of 70S ribosomes that are prone to 'dissociate' to 60S material during centrifuging. Strain R8 lacks L19 and has rather few 70S ribosomes. These are also pressure sensitive and possibly more so than those of strain R1.

Ribosome precursor particles in revertant strains

Components that have sedimentation coefficients of 50S or less in profiles or revertant strains could be ribosome precursor particles (as is likely with the '40S' material of strain R19) and/or ribosomal sub-units. Distinction between precursors and sub-units is often not obvious because sedimentation properties can be very similar (Butler *et al.*, 1980). Moreover, in strains that lack ribosomal proteins the sedimentation of the ribosomal sub-units may be altered. These possibilities were examined in two revertant strains, R4 and R8.

Strain R4. ^3H -labelled 70S ribosomes from strain R4 were isolated from sucrose gradients and mixed with ^{14}C -labelled 70S ribosomes from strain A19. The mixture was dialysed in buffer containing 2 mM- Mg^{2+} and sub-units were displayed by gradient centrifuging. The 30S (but not the 50S) sub-units from strain R4 sedimented rather less far than those of strain A19 (Fig. 5*a*), presumably due to the absence of protein S20. This is an interface protein (synonymous with L26) found associated with either sub-unit; on this evidence, it is better regarded as a small sub-unit protein. ^3H - and ^{14}C -labelled extracts of strains R4 and A19 in THMKSp buffer were also mixed and centrifuged. The '30S' material from strain R4 sedimented less far than the native 30S ribosomes of strain A19 (Fig. 5*b*). However, the following experiment showed that this was not due to differences in sedimentation properties of the 30S ribosomal sub-units of the two strains. Strain R4 was labelled with both [^3H]- and [^{14}C]uracil during growth for three generations. The organisms were collected by centrifuging and resuspended in medium with only [^3H]uracil (at the same specific activity as before resuspension). Growth resumed without lag and there was no loss of ^{14}C radioactivity insoluble at 0 °C in trichloroacetic acid. Extracts made at intervals were centrifuged through sucrose gradients; Fig. 5(*c, d*) shows sedimentation profiles before and 1.55 generations after removal of ^{14}C radioactivity. At the time of resuspension, the profiles of ^3H and ^{14}C material were identical (Fig. 5*c*). The ^3H profile showed no significant change during subsequent growth. However there was a marked loss of ^{14}C radioactivity from 30S material (Fig. 5*d*). Analysis of the redistribution of ^{14}C radioactivity in all samples taken showed that this loss was accompanied by an increase in the normalized specific activity of 70S ribosomes (Fig. 5*e*). The interpretation is that much of the 30S material in strain R4 comprises a precursor(s) to 30S ribosomal sub-units: maturation and combination with 50S sub-units gives 70S ribosomes. There is no large change in ^{14}C radioactivity in native 50S ribosomes since these can exchange with the 50S sub-units of 70S ribosomes. Fig. 5(*e*) also shows that the ^{14}C radioactivity in the minor '40S' component visible in profiles (e.g. Fig. 1*c*) rapidly decays as expected for a precursor particle.

Strain R8. No differences in sedimentation between the 30S, 50S or 70S components of strains R8 and A19 were detected when extracts, made in THMKSp buffer and labelled with [^3H]- and [^{14}C]uracil respectively, were mixed and sedimented through sucrose gradients at low speed (SW 50.1 rotor; 20000 r.p.m.; 15.5 h). However, in TLMK buffer (see legend to Fig. 5), in which 70S ribosomes dissociate to sub-units, the 50S material from strain R8 consistently sedimented rather more slowly than the 50S ribosomal sub-units of strain A19, presumably as a consequence of the lack of protein L19. Strain R8 was grown for three generations with both [^3H]- and [^{14}C]uracil present. As in the comparable experiment with strain R4, organisms were collected and resuspended in medium with only [^3H]uracil added. Growth resumed without lag or loss of acid-insoluble ^{14}C radioactivity. Fig. 6(*a, b*) shows the sedimentation profiles immediately after removal of ^{14}C radioactivity and 1.75 generations later, when the ^3H profile has changed little but the ^{14}C profile shows an increase in the proportion of 70S ribosomes at the expense of both 30S and 50S components. Consideration of the redistribution in all samples taken (Fig. 6*c*) shows that these changes are essentially complete within a generation. This is consistent with the formation of 70S ribosomes from precursors to both ribosomal sub-units but with an excess of sub-units present after maturation is complete.



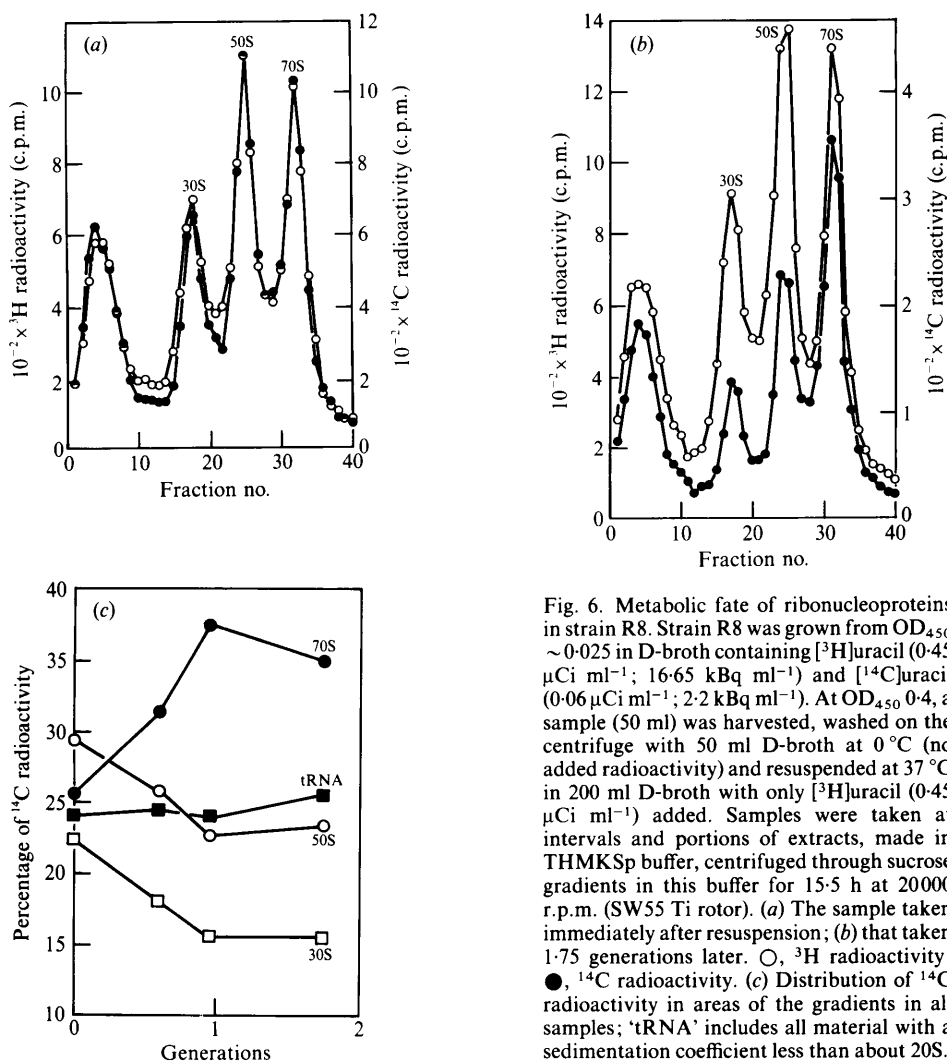


Fig. 6. Metabolic fate of ribonucleoproteins in strain R8. Strain R8 was grown from $\text{OD}_{450} \sim 0.025$ in D-broth containing $[^3\text{H}]\text{juracil}$ ($0.45 \mu\text{Ci ml}^{-1}$; $16.65 \text{ kBq ml}^{-1}$) and $[^{14}\text{C}]\text{juracil}$ ($0.06 \mu\text{Ci ml}^{-1}$; 2.2 kBq ml^{-1}). At $\text{OD}_{450} 0.4$, a sample (50 ml) was harvested, washed on the centrifuge with 50 ml D-broth at 0°C (no added radioactivity) and resuspended at 37°C in 200 ml D-broth with only $[^3\text{H}]\text{juracil}$ ($0.45 \mu\text{Ci ml}^{-1}$) added. Samples were taken at intervals and portions of extracts, made in THMKSp buffer, centrifuged through sucrose gradients in this buffer for 15.5 h at 20000 r.p.m. (SW55 Ti rotor). (a) The sample taken immediately after resuspension; (b) that taken 1.75 generations later. \circ , ^3H radioactivity; \bullet , ^{14}C radioactivity. (c) Distribution of ^{14}C radioactivity in areas of the gradients in all samples; 'tRNA' includes all material with a sedimentation coefficient less than about 20S.

Fig. 5 (on facing page). Ribonucleoproteins in strain R4. (a) Strains A19 and R4 were grown from $\text{OD}_{450} \sim 0.025$ to 0.5 in D-broth containing respectively $0.1 \mu\text{Ci}$ (3.7 kBq) $[^{14}\text{C}]\text{juracil ml}^{-1}$ and $1 \mu\text{Ci}$ (37 kBq) $[^3\text{H}]\text{juracil ml}^{-1}$. Extracts made in THMKSp buffer were mixed and centrifuged for 15 h at 21000 r.p.m. through sucrose gradients in this buffer (SW27 rotor). Fractions containing 70S ribosomes were pooled, dialysed for 4 h against TLMK buffer (10 mM-Tris/HCl, 2 mM-magnesium acetate, 100 mM-KCl) and a portion centrifuged (2.5 h; 50000 r.p.m.; SW55 Ti rotor) through a sucrose gradient in this buffer. \circ , ^3H radioactivity; \bullet , ^{14}C radioactivity. (b) Strains A19 and R4 were grown as in (a). Extracts of each were made in THMKSp buffer, mixed and centrifuged (2.5 h; 50000 r.p.m. SW55 Ti rotor) through a sucrose gradient in this buffer. \circ , ^3H radioactivity; \bullet , ^{14}C radioactivity; \square , ^{14}C radioactivity on an expanded scale ($\times 15$). For (c), (d) and (e), strain R4 was grown from $\text{OD}_{450} \sim 0.025$ in D-broth containing $[^3\text{H}]\text{juracil}$ ($0.45 \mu\text{Ci ml}^{-1}$; $16.65 \text{ kBq ml}^{-1}$) and $[^{14}\text{C}]\text{juracil}$ ($0.06 \mu\text{Ci ml}^{-1}$; 2.2 kBq ml^{-1}). At $\text{OD}_{450} 0.4$, a sample (20 ml) was taken for an extract and the remainder (50 ml) harvested and resuspended at 37°C in 200 ml D-broth with only $[^3\text{H}]\text{juracil}$ ($0.45 \mu\text{Ci ml}^{-1}$) added. Samples were taken at intervals and extracts in THMKSp buffer centrifuged (2.5 h; 50000 r.p.m.; SW55 Ti rotor). (c) The sample taken immediately after resuspension; (d) that taken 1.55 generations later. \circ , ^3H radioactivity; \bullet , ^{14}C radioactivity. (e) Specific activity of the components in all the samples, obtained by dividing the ratio ^{14}C radioactivity/ ^3H -radioactivity in the peak fraction corresponding to each component by the corresponding ratio in the peak of tRNA.

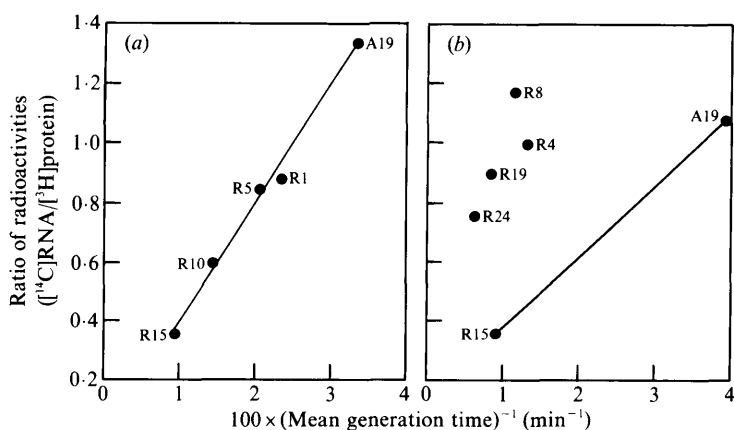


Fig. 7. RNA content of revertant strains. For (a), the strains indicated were inoculated ($\text{OD}_{450} \sim 0.02$) into portions of the same batch of D-broth with $[^3\text{H}]\text{lysine}$ ($2.7 \mu\text{Ci ml}^{-1}$; 99.9 kBq ml^{-1}) and $[^{14}\text{C}]\text{uracil}$ ($0.01 \mu\text{Ci ml}^{-1}$; 0.4 kBq ml^{-1}) added. At $\text{OD}_{450} 0.5$, four samples (0.5 ml) were taken from each culture for the estimation of radioactivities insoluble at 0°C in 5% (w/v) trichloroacetic acid. (b) A second experiment with the strains shown.

RNA content of revertant strains

Several revertants to erythromycin independence thus contain 'extra' ribonucleoprotein either as ribosomal sub-units or ribosome precursor particles or both. This excess could be reflected in a high RNA content of organisms; or, the total RNA content of revertants might be that characteristic of their rates of growth. Experiments that measured RNA/protein ratios of organisms during exponential growth in the same batch of medium containing $[^{14}\text{C}]\text{uracil}$ and $[^3\text{H}]\text{lysine}$ are summarized in Fig. 7. The first comparison (Fig. 7a) is of strains A19, R1, R5, R10 and R15. The latter three strains grew rather slowly but had 'normal' sedimentation profiles very similar to that of A19. All strains have an RNA/protein ratio proportional to their growth rates (expressed as the reciprocal of their generation times). Fig. 7(b) compares strains A19 and R15 with revertant strains R4, R8, R19 and R24. The latter four strains have RNA/protein ratios up to twice those expected from their growth rates. For example, revertant R8 (mean generation time 90 min) has a rather higher RNA content than strain A19 (mean generation time 26 min).

DISCUSSION

In these experiments, mutants dependent on (and resistant to) erythromycin were obtained at a frequency about 0.1% that of resistant organisms. It is difficult to assess whether resistance and dependence in strains such as 103 are conferred by different mutations or a single event. Mutation to streptomycin dependence in *E. coli* is at the same ribosomal protein (*rpsL*) locus that confers resistance. Similarly, kasugamycin dependence and resistance can simultaneously result from an altered ribosomal protein S9 (*rpsI* locus), although in other kasugamycin- (and spectinomycin- and rifampicin-) requiring organisms there are separate mutations for resistance and dependence (Dabbs, 1983). In an erythromycin-dependent strain (Sparling & Blackman, 1973), the resistance mutation was at about 73 min and so probably in *eryA* or *eryB*. (These loci are synonymous with *rplD* and *rplV* respectively, which specify ribosomal proteins L4 and L22: Bachmann, 1983.) The dependence mutation, *mac*, was separate and at about 26 min. *mac* is not near any gene that specifies a ribosomal protein, although, as pointed out by Dabbs (1986), an antibiotic dependence that is suppressed by a series of ribosomal mutations is not itself necessarily ribosomal. Against this background, the status of the altered ribosomal protein L25 in the erythromycin-dependent strain 103 is uncertain. The gene (*rplY*) for L25 is at 47 min; an

alteration could cause the resistance, dependence, or both, or be adventitious and result from the mutagenesis with NTG. Transfer of the alteration to a different genetic background should resolve these questions. The reversions of strain 103 to independence were spontaneous events so that in these cases the additional alterations to ribosomal proteins are very likely to be responsible for the new phenotypes.

Revertant strain R1 has a less basic protein L19 than strains A19 or 103 and also has 70S ribosomes that readily dissociate into ribosomal sub-units during centrifuging. Dissociation would be enhanced if binding of Mg^{2+} to 70S ribosomes were reduced or, more likely, if there were a greater than usual decrease in molar volume when 70S ribosomes dissociate to sub-units (Pande & Wishnia, 1986). Similar but less pronounced effects were noted in some kasugamycin-resistant strains of *E. coli* (Poldermans *et al.*, 1980), whose 70S ribosomes were more prone to dissociate than those from sensitive organisms. Kasugamycin resistance arises from failure to methylate adjacent adenosines near the 3' end of 16S rRNA. This might directly affect sub-unit association if the RNAs of the ribosomal sub-units are in direct contact (Yusupov & Spirin, 1986). Protein L19 has been located at the base of the 50S sub-unit facing the small sub-unit (Stöffler *et al.*, 1984) and is thus at the sub-unit interface. The binding of L19-specific antibodies to 50S ribosomes prevents their association with 30S ribosomes (Noll *et al.*, 1976). Thus altered L19 might affect sub-unit association directly through interaction with proteins (or the RNA) of the smaller sub-unit or indirectly by causing conformational rearrangements in 23S rRNA. Stöffler *et al.* (1984) reported that a strain (AM149) that lacked protein L19 had 70S ribosomes of normal stability. They suggested either that L19 played no essential role in sub-unit association or that the other mutations in strain AM149 compensated for the absence of this protein. In strain R1, an altered L19 does have a marked effect on ribosome stability; here other ribosome mutation(s) present may either fail to influence or even enhance the effect. Transfer of the L19 mutations to other backgrounds should resolve these possibilities.

Similar considerations apply to strain R8, whose 70S ribosomes are also pressure-sensitive and in which protein L19 is either absent or much altered. In addition, strain R8 has defects in ribosome assembly. The experiments suggest that the material in extracts that co-sediments with either 30S or 50S ribosomal sub-units comprises roughly equal proportions of 'genuine' sub-units and precursor particles. A similar situation may exist with strain R24 (lacking protein L1). Strain R8 thus also differs from mutant AM149 (Stöffler *et al.*, 1984), which lacks protein L19 and where there was no evidence for an assembly defect. The genetic background of the various strains may again be responsible for these differences.

Strain R8 grows at about a third the rate of the parent strain A19 yet has a similar RNA content. Indeed, oversynthesis of RNA seems to be diagnostic of revertants to erythromycin independence with altered ribosome assembly. It is found also in strain R24 and in strain R19, whose sedimentation profile shows substantial accumulation of a putative precursor to 50S ribosomal sub-units. Revertant strain R4, which lacks protein S20, accumulates material that, from its sedimentation coefficient and kinetic properties, is a precursor to 30S ribosomal sub-units.

It is difficult to rationalize effects on RNA content and ribosome synthesis in terms of regulatory properties of the ribosomal proteins that are absent or altered. Oversynthesis of RNA is not universal among revertants to erythromycin independence. Nor is it inevitable in revertants that lack ribosomal proteins. Unpublished work (by B. A. Maguire & D. G. Wild) has noted that strains AM90 or AM108 (Dabbs, 1979), without proteins L28, or L28 plus L33 respectively, have virtually normal RNA contents and sedimentation profiles. This contrasts with strain TP28 (see Introduction), which synthesizes L28 and L33 at half the normal rate, oversynthesizes RNA and accumulates a precursor to 50S ribosomes (Butler & Wild, 1984, 1985). Regulation of the L28-L33 transcription unit has not been investigated. Protein S20 (absent from strain R4) regulates its own translation. Similarly, protein L1 (missing from strain R24) is the regulatory protein of the unit that comprises genes for L11 and L1. However, neither protein L19 (whose gene is distal in the 'S16' operon) nor protein L24 has had regulatory properties ascribed to it, although the absence of protein L24 elevates rates of synthesis of about four other ribosomal proteins from scattered transcription units (Dabbs, 1984).

A more general explanation of oversynthesis of RNA can be based on the ribosome feedback regulation model of Nomura *et al.* (1984). This proposes that synthesis of rRNA and tRNA (and perhaps the transcription of ribosomal protein genes) is negatively regulated by products of rRNA operons that are not the rRNAs themselves but may be non-translating ribosomes in the cellular pool. In conformity with this, Takebe *et al.* (1985) found that overproduction of protein S4 not only inhibited translation from its own ('alpha') operon and therefore ribosome assembly but stimulated RNA synthesis. This was seen as a consequence of a decreased number of free ribosomes. Failure to make a ribosomal protein, as found in some revertants from erythromycin dependence, is thus partially equivalent to inhibition of ribosomal protein synthesis. However, strain R8 (and possibly strain R24) accumulates ribosomal sub-units as well as precursor particles so that the situations are not completely comparable. The ribosome feedback model does not specify the molecular mechanism involved in regulation and it is not yet clear to what extent this system is independent of that responsible for 'stringent control'. Here, the synthesis of rRNA and tRNA is shut off during amino acid starvation, most likely by the inhibition of transcription from 'stringent' promoters by the guanosine tetraphosphate (ppGpp) produced by ribosomes in an idling reaction (Lindahl & Zengel, 1986). RNA synthesis in strain TP28 (which over-produces RNA during exponential growth) remains under stringent control. This suggests that mechanisms for stringency and growth-rate control of RNA content are separable (Butler *et al.*, 1985). Even so, strains that have reverted to erythromycin independence and oversynthesize RNA might have ribosomes that produce ppGpp poorly. Alternatively, growth-rate control may be manipulated through some other property of free ribosomes or sub-units that is faulty in these revertants. These possibilities remain to be investigated.

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REFERENCES

- BACHMANN, B. J. (1983). Linkage map of *Escherichia coli* K-12, edition 7. *Microbiological Reviews* **47**, 180–230.
- BARRITAU, D., EXPERT-BEZANCON, A., GUERIN, M.-F. & HAYES, D. (1976). The use of acetone precipitation in the isolation of ribosomal proteins. *European Journal of Biochemistry* **63**, 131–135.
- BUTLER, P. D. & WILD, D. G. (1984). Ribosomal protein synthesis by a mutant of *Escherichia coli*. *European Journal of Biochemistry* **144**, 649–654.
- BUTLER, P. D. & WILD, D. G. (1985). The location of a mutation affecting ribosomal protein synthesis by *Escherichia coli*. *Journal of General Microbiology* **131**, 135–144.
- BUTLER, P. D., SIMS, P. F. G. & WILD, D. G. (1980). Intermediates in the assembly of ribosomes by a mutant of *Escherichia coli*. *Biochemical Journal* **190**, 157–170.
- BUTLER, P. D., CATTANEO, E. & WILD, D. G. (1985). Interactions between mutations affecting ribosome synthesis in *Escherichia coli*. *Journal of General Microbiology* **131**, 945–949.
- CUNDLIFFE, E. (1986). Involvement of specific portions of ribosomal RNA in defined ribosomal functions: a study utilizing antibiotics. In *Structure, Function and Genetics of Ribosomes*, pp. 586–604. Edited by B. Hardesty & G. Kramer. Berlin: Springer-Verlag.
- DABBS, E. R. (1979). Selection for *Escherichia coli* mutants with proteins missing from the ribosome. *Journal of Bacteriology* **140**, 734–737.
- DABBS, E. R. (1983). *Escherichia coli* kasugamycin dependence arising from mutation at the *rpsI* locus. *Journal of Bacteriology* **153**, 709–715.
- DABBS, E. R. (1984). Organization and regulation of ribosomal protein genes in bacteria. In *Genetics: New Frontiers* (Proceedings of the International Congress of Genetics, 1983), pp. 35–48. New Delhi: Oxford and IBH Publishing.
- DABBS, E. R. (1986). Mutant studies on the prokaryotic ribosome. In *Structure, Function and Genetics of Ribosomes*, pp. 733–748. Edited by B. Hardesty & G. Kramer. Berlin: Springer-Verlag.
- DABBS, E. R., EHRLICH, R., HASENBANK, R., SCHROETER, B.-H., STÖFFLER-MEILICKE, M. & STÖFFLER, G. (1981). Mutants of *Escherichia coli* lacking ribosomal protein L1. *Journal of Molecular Biology* **149**, 553–578.
- EHRENBERG, M., ANDERSSON, D., BOHMAN, K., JELENC, P., RUUSALA, T. & KURLAND, C. G. (1986). Ribosomal proteins tune rate and accuracy in translation. In *Structure, Function and Genetics of Ribosomes*, pp. 573–585. Edited by B. Hardesty & G. Kramer. Berlin: Springer-Verlag.
- GESTELAND, R. F. (1966). Isolation and characterization of ribonuclease I mutants of *Escherichia coli*. *Journal of Molecular Biology* **16**, 67–84.
- HARDY, S. J. S. (1975). The stoichiometry of the ribosomal proteins of *Escherichia coli*. *Molecular and General Genetics* **14**, 253–274.
- HEROLD, M., NOWOTNY, V., DABBS, E. R. & NIERHAUS, K. H. (1986). Assembly analysis of ribosomes from a mutant lacking the assembly-initiator protein L24: lack of L24 induces temperature sensitivity. *Molecular and General Genetics* **203**, 281–287.
- KENNY, J. W., LAMBERT, J. M. & TRAUT, R. R. (1979). Cross-linking of ribosomes using 2-iminothiolane

- (methyl 4-mercaptobutyrimidate) and identification of cross-linked proteins by diagonal polyacrylamide/sodium dodecyl sulfate gel electrophoresis. *Methods in Enzymology* **59**, 534–550.
- LINDAHL, L. & ZENGEL, J. M. (1986). Ribosomal genes in *Escherichia coli*. *Annual Review of Genetics* **20**, 297–326.
- MADJAR, J.-J., MICHEL, S., COZZONE, A. J. & REBOUD, J.-P. (1979). A method to identify individual proteins in four different two-dimensional gel electrophoresis systems: application to *Escherichia coli* ribosomal proteins. *Analytical Biochemistry* **92**, 174–192.
- MARKEY, F. & WILD, D. G. (1976). An unusual precursor of 50S ribosomes in a mutant of *Escherichia coli*. *Biochemical Journal* **158**, 451–456.
- MOAZED, D. & NOLLER, H. F. (1987). Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature, London* **327**, 389–394.
- NISHI, K., DABBS, E. R. & SCHNIER, J. (1985). DNA sequence and complementation analysis of a mutation in the *rplX* gene from *Escherichia coli* leading to loss of ribosomal protein L24. *Journal of Bacteriology* **163**, 890–894.
- NOLL, M., STÖFFLER, G. & HIGHLAND, J. H. (1976). Dissociation of *Escherichia coli* ribosomes into active sub-units. *Journal of Molecular Biology* **108**, 259–264.
- NOMURA, M., GOURSE, R. & BAUGHMAN, G. (1984). Regulation of the synthesis of ribosomes and ribosomal components. *Annual Review of Biochemistry* **53**, 75–117.
- NOWOTNY, V. & NIERHAUS, K. H. (1982). Initiator proteins for the assembly of the 50S sub-unit from *Escherichia coli* ribosomes. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 7238–7242.
- PANDE, C. & WISHNIA, A. (1986). Pressure dependence of equilibria and kinetics of *Escherichia coli* ribosomal sub-unit association. *Journal of Biological Chemistry* **261**, 6272–6278.
- POLDERMANS, B., BAKKER, H. & VAN KNIPPENBERG, P. H. (1980). Studies on the function of two adjacent *N*⁶,*N*⁶-dimethyladenosines near the 3' end of 16S ribosomal RNA of *Escherichia coli*. IV. The effect of the methyl groups on ribosomal sub-unit interaction. *Nucleic Acids Research* **8**, 143–151.
- SPARLING, P. F. & BLACKMAN, E. (1973). Mutation to erythromycin dependence in *Escherichia coli* K12. *Journal of Bacteriology* **116**, 74–83.
- STÖFFLER, G., NOAH, M., STÖFFLER-MEILICKE, M. & DABBS, E. R. (1984). The localization of protein L19 on the surface of 50S sub-units of *Escherichia coli* aided by the use of mutants lacking protein L19. *Journal of Biological Chemistry* **259**, 4521–4526.
- SUBRAMANIAN, A. R. & DABBS, E. R. (1980). Functional studies on ribosomes lacking protein L1 from mutant *Escherichia coli*. *European Journal of Biochemistry* **112**, 425–430.
- TAKEBE, Y., MIURA, A., BEDWELL, D. M., TAM, M. & NOMURA, M. (1985). Increased expression of ribosomal genes during inhibition of ribosome assembly in *Escherichia coli*. *Journal of Molecular Biology* **184**, 23–30.
- WITTMANN, H. G. (1986). Structure of ribosomes. In *Structure, Function and Genetics of Ribosomes*, pp. 1–27. Edited by B. Hardesty & G. Kramer. Berlin: Springer-Verlag.
- YUSUPOV, M. M. & SPIRIN, A. S. (1986). Are there proteins between the ribosomal sub-units? Hot tritium bombardment experiments. *FEBS Letters* **197**, 229–233.
- ZAMIR, A., MISKIN, R., VOGEL, Z. & ELSON, D. (1974). The inactivation and reactivation of *Escherichia coli* ribosomes. *Methods in Enzymology* **30**, 406–426.